The application of molecular techniques to investigate the production of sodium channel blocking toxins by heterotrophic marine bacteria

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Presented for the degree of Doctor of Philosophy in

The Division of Infection and Immunity,
Institute of Biomedical Life Sciences,
Faculty of Science,
The University of Glasgow

March 1998

Unless otherwise stated, this thesis is the original work of the author

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Acknowledgements

This work was supported by a NERC CASE studentship, cosponsored by the FRS (formerly SOAEFD) Marine Laboratory, Aberdeen.

The mini-Mu delivery vector and donor strain, J100, were kindly gifted by Dr. J. Östling and Professor S. Kjelleberg. The pUT transposon delivery vectors, and the *E. coli* donor strains with which they were used, were kindly supplied by Dr. V. de Lorenzo and Dr. K.N. Timmis.

The post-column oxidation HPLC analysis contained in this thesis was carried out with the assistance of Dr. J.M. Franco and Miss M.I. Reyero at the Instituto Español de Oceanografía, Vigo, Spain. I thank them and their colleagues for their assistance and generous hospitality during my visit to their institute.

The staff of the FRS Marine Laboratory in Aberdeen performed a number of assays and analysis for SCB toxins during the course of this work. I thank Georgina Hold, Fiona Mackintosh and Stuart Forsyth for the numerous mouse neuroblastoma SCB assays which they performed on my samples and Stephen O'Neill for the preliminary evaluation of the ELISA method for detecting PSP toxins in bacterial cultures. I also thank Margaret Donald and Iain Riddoch for our discussions regarding HPLC analysis of PSP toxins.

This thesis would not have been possible without the patient supervision of Dr. T.H. Birkbeck and Dr. S. Gallacher. I therefore thank them both for their expert advice during my research and thesis preparation. I also thank my fellow students, especially Georgina Hold and Angela Griffen, for their empathy and support.

However, the people whom I consider to have assisted me most in the execution of the research and preparation of this thesis are my wife, Linda Mairi Hosie, my sons, Daniel Iain Hosie and Gregor Thomas Hosie. These three people have assisted me during this work to an extent which they and others may never really appreciate, not least by supplying motivation, support, understanding and, of course, a welcome distraction from scientific research. For this and much more I thank them.

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ABBREVIATIONS

AMP	= adenosine monophosphate
Amp	= ampicillin
Amp^r	= ampicillin resistant
AOAC	= Association of Official Analytical Chemists
AOx	= acidic oxidation
ASW	= artificial sea water
ATP	= adenosine triphosphate
BCIP	= 5-bromo-4-chloro-3-indoyl phosphate
BLAST	= basic local alignment search tool
BLASTN	= basic local alignment search tool for nucleotide sequences
BLASTX	= basic local alignment search tool with translation to protein sequence
CFU	= colony forming units
CI	= confidence interval(s)
Cm	= chloramphenicol
Cm^r	= chloramphenicol resistant
CSPD®	= disodium 3-(4-methoxyspiro{1,2-dioxetane-3,2'-(5'-chloro)tricyclo [3.3.1.1^{3,7}]decan}-4-yl)phenyl phosphate
dc	= decarbamoyl
dH_2O	= distilled water
DIG	= digoxigenin
do	= deoxydecarbamoyl
ELISA	= enzyme linked immunosorbent assay
EST	= estrogen sulfotransferase
FST	= flavanol sulfotransferase
GC-MS	= gas chromatography-mass spectrometry
GTX	= gonyautoxin
hns	= histone-like nucleoid structuring protein gene
H-NS	= histone-like nucleoid structuring protein (H1)
HPLC	= high performance liquid chromatography
HSST	= hydroxy-steroid sulfotransferase
Km	= kanamycin
Km^r	= kanamycin resistant
LC-MS	= liquid chromatography/mass spectrometry
Nal	= nalidixic acid
Nal^r	= nalidixic acid resistant
NBT	= nitroblue tetrazolium
NST	= *N*-sulfotransferase

ODS = octadecyl silane
ONPG = *O*-nitrophenyl-*β*-D-galactosidase
ORF = open reading frame
Ox = alkaline oxidation
p = probability
PAPS = adenosine 3'-phosphate 5'-phosphosulphate
PCR = polymerase chain reaction
PSP = paralytic shellfish poisoning or paralytic shellfish poison
PST = phenol sulfotransferase
RBS = ribosome binding site
RIA = radioimmunoassay
RP = reverse phase
SCB = sodium channel blocking
Sm = streptomycin
Sm^r = streptomycin resistant
sp. = species
SPE = solid phase extraction
ssp = *Shewanella* sp. serine protease gene
Ssp = *Shewanella* sp. serine protease
SSP-PCR = single specific primer PCR
ST = sulfotransferase
STX = saxitoxin
Tc = tetracycline
Tc^r = tetracycline resistant
Tn = transposon
TTX = tetrodotoxin
wt = wild type
X-gal = 5-bromo-4-chloro-3-indolyl-*β*-*D*-galactopyranoside

SUMMARY

Historically the production of tetrodotoxin has been attributed to Puffer fish and the production of paralytic shellfish poisons to dinoflagellates and fresh water cyanobacterial species, especially at times of monospecific blooms. In the last decade, however, a broad spectrum of bacterial species have been reported to produce tetrodotoxin. Similarly, heterotrophic bacteria have been reported to produce paralytic shellfish poisons in culture, although little is known about the biosynthetic pathways of these toxins. In this study the production of sodium channel blocking toxins by heterotrophic marine bacteria was investigated by transposon mutagenesis and other molecular techniques.

The suitability of several bacterial isolates for mutagenesis with a variety of transposons was evaluated by phenotype characterisation with regard to ß-galactosidase activity and spontaneous antibiotic resistance, resulting in the choice of three strains (*Alteromonas tetraodonis* GFC, and sea water isolates A912 and A862, the latter being a putative *Shewanella* sp.) for mutagenesis with three different transposon constructs.

Mutagenesis of *A. tetraodonis* GFC with miniMu(tet^r) was hindered by a high frequency of spontaneous resistance of the *Escherichia coli* donor strain to particular antibiotics; the use of this transposon construct was not pursued. However, pUT::miniTn5 transposon vectors were successfully employed for mutagenesis of strains A912 and A862. A912::miniTn5Km*lacZ*I exconjugants were recovered at frequencies of between $2{\cdot}49\times10^{-6}$ and $1{\cdot}31\times10^{-4}$ per recipient cell and A862::miniTn5Cm*lacZ* exconjugants at frequencies of $2{\cdot}25\times10^{-6}$ and $3{\cdot}73\times10^{-9}$, the latter with an adapted pUT::miniTn5Cm construct to enable promoter activity of mutagenised genes to be monitored by *lacZ* reporter gene expression. Banks of A912 and A862 exconjugants were isolated containing 1,505 and 5,328 exconjugants respectively and further exconjugants may be readily isolated. ß-galactosidase assays and Southern blot analysis confirmed that transposon insertion appeared to be a random event, therefore, the probability of mutating a gene of interest is maximised.

The present methods employed for the detection of sodium channel blocking toxins were not suitable for processing the large number of bacterial samples to be screened for loss of toxin production. A chemical fluorescence assay (Bates and Rapoport, 1975) was adapted for assaying bacterial cultures for paralytic shellfish poisons. However, only strain A862 contained a compound with fluorescent properties indicative of PSP toxins. Although post-column oxidation HPLC analysis indicated that strain A862 may produce neoSTX, $GTX_{1/4}$, B2, C2, C3 and C4 toxins, pre-column oxidation high performance liquid chromatography analysis of this strain showed that the fluorescence detected by the chemical fluorescence assay was not caused by any of these toxins.

Although the compound detected by the chemical fluorescence assay has not been fully characterised, this assay was used in conjunction with a mouse neuroblastoma cell sodium channel blocking assay to screen almost 1,000 A862::miniTn5Cm*lacZ* exconjugants for decreased SCB toxin production. Seven exconjugants were identified which had a significantly lower fluorescence than A862 wt. Inverse polymerase chain reaction was used successfully to amplify the DNA sequence adjacent to the transposon insertion in five of these exconjugants. Subsequent sequence analysis assigned putative functions to two genes located near the insertion site of different exconjugants.

The significance of these two genes and their products to SCB toxin production is unclear. It is possible that the serine protease (Ssp), encoded by one of the genes, could be involved in the post-translational modification of another protein involved in either the post-translational modification of a further protein required for toxin biosynthesis, or alternatively, it could increase the pool of biosynthetic precursors by degrading peptides which are precursors of toxin biosynthesis.

The product of the second gene, a H-NS homologue, is possibly involved in the regulation of expression of other genes involved in SCB toxin biosynthesis as H-NS is known to affect transcription of a number of genes. This is the first description of a H-NS in marine bacteria of the family Vibrionaceae, and therefore further extends the range of organisms known to produce H-NS homologues, which until recently were only described in enterobacteria.

Single specific primer PCR using degenerate primers designed to consensus sequences of known sulfotransferase enzymes, was used in an attempt to amplify from strain A862, genes encoding potential sulfotransferase enzymes involved in PSP biosynthesis. Although SSP-PCR products were obtained, none contained significant homology to known sulfotransferase genes. However, this negative result does not indicate that these enzymes are not present in this organism. If partial amino acid sequences become available for sulfotransferases or other enzymes involved in SCB biosynthesis, the SSP-PCR described here may be used to identify the genes which encode these enzymes.

INTRODUCTION

The sodium channel blocking (SCB) toxins are a group of low molecular weight, non-protein neurotoxins. There are two structurally distinct families of SCB toxins, tetrodotoxin (TTX) and its derivatives and the paralytic shellfish poisons (PSPs). TTX and the PSPs share the same mechanism of action, i.e. they selectively block the voltage-sensitive sodium channels of neurons hence preventing an influx of sodium ions required to initiate an action potential which is necessary for neuromuscular transmission (Levin, 1991; Lipkind & Fozzard, 1994). The SCB toxins have proven to be useful tools in neurobiology research (Kao, 1966; Lipkind & Fozzard, 1994), although it is their status as a public health hazard which makes them worthy of study in their own right. A comparison of the potency of these toxins with other well-known toxic substances (see Table 1) reveals that they are among the most poisonous substances known to man.

The symptoms of poisoning by TTX and PSP are identical, as expected for toxins with the same mechanism of action. Upon ingestion, a tingling sensation develops at the lips and mouth and, shortly after, tingling spreads to the fingers and arms, followed by a numbness in the neck and limbs. As the poisoning progresses other symptoms include loss of co-ordination, headache, nausea and vomiting; ultimately, paralysis occurs, possibly leading to a coma. Deaths caused by SCB toxins are usually attributed to respiratory failure (Mosher *et al.*, 1964; Prince, 1988; Tambyah *et al.*, 1994).

Strict regulatory controls are in place to prevent SCB toxins from reaching the consumer. In Japan there is a potential risk of TTX poisoning following the consumption of toxic puffer fish. However legislation is in place to ensure that chefs who prepare puffer fish are trained to a high standard thereby ensuring that the food they serve contains little toxin (Prince, 1988). In countries where there is a risk of paralytic shellfish poisoning (PSP) the shellfish harvests are monitored for toxicity and, when toxin levels reach a level which is regarded unsafe, the sale of shellfish from that area is prohibited (Van Egmond *et al.*, 1993). The latter of these regulatory measures leads to significant economic losses each year for the shellfish industry (Shumway, 1990).

The occurrence of PSP is believed to be increasing in frequency, magnitude and geographical distribution. Although some of the perceived escalation may be due to more intensive shellfish harvesting and monitoring, there are other factors which may be contributing, including increased human exploitation and pollution of the marine environment (Anderson, 1989). In the light of increased shellfish toxicity, the economic implications for the shellfish industry, and the potential public health risk it is important that we gain a fuller understanding of the production of SCB toxins in the environment. If the mechanisms which are involved in the production of SCB

toxins by marine organisms can be identified it may be possible to predict or control the onset of toxic events and therefore minimise their impact.

TOXIN	MINIMUM LETHAL DOSE (μg/kg)*
TTX (pH 7.8)	10
TTX (pH 8.8)	27
Chiriquitoxin	10
Nor-TTX	40 to 125
4-*epi*-TTX	25
Anhydro-TTX	500
STX (pH 7.25)	10
STX (pH 8.25)	16.7
Neo-STX	10
dc-STX	50
GTX II	50
GTX III	25
Botulinum toxin	0.00003
Tetanus toxin	0.0001
Diphtheria toxin	0.3
Sodium cyanide	10,000

* Minimum lethal dose in mice by intraperitoneal administration.

Table 1. Relative toxicity of TTX, STX, their derivatives and other toxic substances. (Adapted from Mosher *et al.*, 1964 and Kao, 1986)

TETRODOTOXIN

Historical background

Tetrodotoxin (TTX) is most commonly associated with the Japanese delicacy Fugu. Indeed, the name tetrodotoxin is derived from Tetraodontiformes, the order to which puffer fish, or fugu, belong. Trace amounts of TTX present in fugu cause a tingling sensation in the extremities and a feeling of euphoria and warmth. It is these properties that apparently make this dish appealing to many diners (Prince, 1988) and the preparation of fugu in Japan is carefully controlled, only being done by licensed

cooks. This preparation includes the removal of organs where TTX is known to accumulate, namely the ovaries, liver and intestines and thorough washing of the remaining flesh. There are still, however, reports of puffer fish poisoning (Laobhripatr *et al.*, 1990; Tambyah *et al.*, 1994) and ensuing deaths.

The toxicity of puffer fish has been known to man since early civilisation and figures on the tomb of the Egyptian pharaoh Ti, of the Vth dynasty (2500 BC), have been identified as the puffer fish *Tetraodon lineatus*. Thus, it is believed that the early Egyptians were aware of the poisonous properties of puffer fish (Mosher *et al.*, 1964).

The historical voyager Captain James Cook, during his second voyage around the world in 1774, obtained first-hand experience of puffer fish poisoning. In his journals he described the symptoms he suffered, his good fortune at having only tasted the fish and the belated warnings of the natives who had supplied the fish, that it was not for consumption (Mosher *et al.*, 1964).

In addition to the Middle and Far East, toxic puffer fish have been reported in the Mexican state of Baja California and in the coastal waters of the USA as far north as New England (Mosher *et al.*, 1964).

The first comprehensive study of the pharmacology of TTX was published in 1879 by Takahashi & Inoko, who described the hypotension and respiratory depression associated with TTX poisoning and noted that the heart continued to beat after respiration had stopped (Fuhrman, 1986).

It was originally thought that puffer fish were the only source of TTX and that puffer fish themselves synthesised this toxin, perhaps as a defence against predatation. However, Mosher *et al.* (1964) reported that tarichatoxin was identical to tetrodotoxin. The toxin known as tarichatoxin was isolated from the eggs of various species of Western American newts of the genus *Taricha*. The toxin is present in adult newts as well as in eggs. Physical and physiological comparisons of TTX and tarichatoxin proved beyond doubt that they are indeed identical (Mosher *et al.*, 1964).

Subsequently, TTX has been isolated from other species of fish, frogs, gastropods, octopuses, crabs, starfish and flat worms (Fuhrman, 1986; Ali *et al.*, 1990). The presence of TTX in such diverse species was perplexing to workers, especially as other closely related species do not harbour TTX. Indeed, within some species there are variations in the toxicity of different isolates (Laobhripatr *et al.*, 1990), especially depending on the season and geographical location of sampling.

Biological origin of TTX

The observations of Matsui *et al.* (1985) that non-toxic puffer fish became toxic when kept in close contact with toxic puffer fish led these workers to postulate that

symbiotic bacteria were responsible for the production of TTX in puffer fish. The same group had earlier reported that feeding cultured, non-toxic puffer fish on ovaries taken from toxic puffer fish caused the fish to become toxic. However, this did not occur when the fish were exposed to pure TTX (Matsui *et al.*, 1981).

A bacterial origin of TTX could explain why TTX is found in such a diverse range of species and why there are differences in toxicity levels between closely related species and within the same species under different conditions.

There are two possible mechanisms by which bacteria could be responsible for the accumulation of TTX in higher organisms. Either bacteria surviving in a symbiotic relationship within the higher organisms produce TTX, or TTX-producing bacteria are taken up through the food chain. Workers therefore examined bacteria isolated from TTX-containing organisms or their food source for the ability to produce TTX. In 1986, Noguchi *et al.* reported the isolation of a *Vibrio* sp. from the intestines of the Xanthid crab. This isolate was shown by high performance liquid chromatography (HPLC) analysis and gas chromatography mass spectrometry (GC-MS) to produce TTX in culture (Noguchi *et al.*, 1986). Yasumoto *et al.* (1986) reported the isolation of TTX-producing bacteria from a red calcareous alga (*Jania* sp.) which serves as part of the diet of herbivorous fish.

The report of Yotsu *et al.* (1987) of the isolation of TTX-producing bacteria from the skin of puffer fish supports the hypothesis of Matsui *et al.* (1985) that symbiotic bacteria are involved in puffer fish toxicity. Bacteria have also been observed in the cytoplasm of liver cells in toxic puffer fish. The presence of these bacteria does not appear to be detrimental to these cells. It is proposed that the bacteria and liver cells of the puffer fish are in symbiosis and that this symbiotic relationship enhances the production of TTX (Kodama *et al.*, 1995).

A broad spectrum of bacterial species have been reported to produce TTX. These include isolates from a number of marine organisms and established laboratory strains. A summary of bacterial species reported to produce TTX is found in Table 2.

Kogure *et al.* (1988) reported that TTX accumulated in marine sediments, and Do *et al.* (1990, 1991) reported the isolation of a number of TTX-producing bacteria, including actinomycetes, from marine sediments. Marine sediments may, therefore, be an important source of TTX in the marine environment. The report that sinking particles in the water column also contain TTX and other SCB toxins suggests that these too may play an important role in the toxification of marine organisms (Hamasaki *et al.*, 1994).

SPECIES	SOURCE	REFERENCE
Vibrio fischeri	Xanthid crab	Noguchi *et al.* (1986)
Shewanella alga	Red calcareous alga (*Jania* sp.)	Yasumoto *et al.* (1986)
Alteromonas tetraodonis	Skin of fugu	Yotsu *et al.* (1987)
V. alginolyticus *V. damsela* *Staphylococcus* sp.	Starfish	Narita *et al.* (1987)
V. alginolyticus	Fugu intestines	Noguchi *et al.* (1987)
V. alginolyticus *V. anguillarum* *V. costicola* *V. harveyi* *V. parahaemolyticus* *Photobacterium phosphoreum* *Aeromonas hydrophila* *Aeromonas salmonicida* *Plesimonas shigelloides*	Culture collections	Simidu *et al.* (1987)
Bacillus sp. *Pseudomonas* sp. *Vibrio* sp.	Blue-ringed octopus	Hwang *et al.* (1989)
V. alginolyticus	Arrowworms	Thuessen & Kogure (1989)
Bacillus sp. *Micrococcus* sp. *Acinetobacter* sp. *Alteromonas* sp. *Moraxella* sp. *Vibrio* sp.	Marine sediment	Do *et al.* (1990)
Streptomyces sp.	Marine sediments	Do *et al.* (1991)
?	Sinking particles	Hamasaki *et al.* (1994)

Table 2. Bacterial species reported to produce tetrodotoxin or its derivatives.

Structure of TTX and its derivatives

The isolation of crystalline TTX was first achieved by Yokoo in 1950. This facilitated the determination of the structure of TTX, which was presented in 1964 by four different research groups (Fuhrman, 1986). At least 8 toxic derivatives of TTX have subsequently been reported (Kao, 1986; Yasumoto & Murata, 1993). The structures of TTX and its derivatives are shown in Figure 1.

The empirical formula of TTX is $C_{11}H_{17}N_3O_3$. Crystalline TTX is insoluble in water but is very soluble in dilute acid. TTX is reasonably stable in dilute acid solution but rapidly decomposes at pHs above 8. Crystalline TTX is indefinitely stable. At physiological pHs the guanidinium group is protonated and positively charged (Kao, 1966; Mosher, 1986).

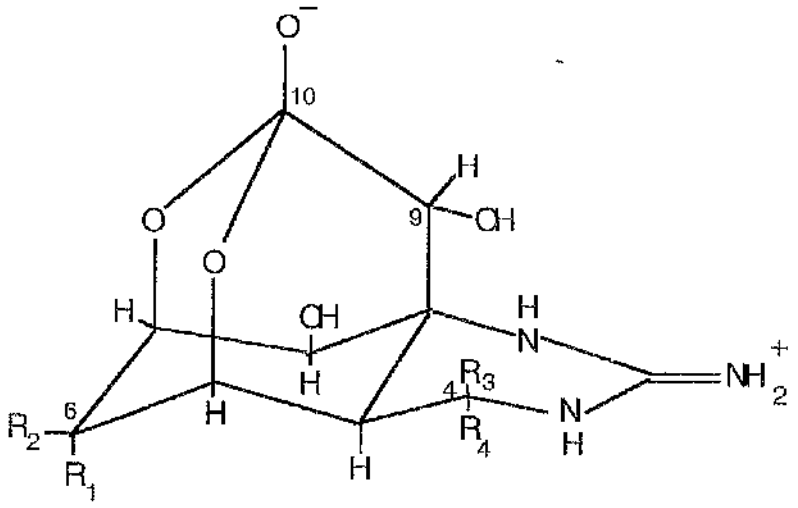
The structural differences between TTX derivatives (Figure 1) and differences in their toxicity (Table 1) have indicated the structural moieties involved in toxin activity. Present evidence suggests the hydroxyls at C9 and C10, together with the guanidinium moiety, are involved in the binding of TTX to sodium channels. It is proposed that the guanidinium moiety forms salt bridges with three carboxyls of the sodium channel, while the hydroxyls at C9 and C10 interact with a fourth carboxyl of the sodium channel (Kao, 1986; Lipkind & Fozzard, 1994).

The elucidation of the biosynthetic pathway of TTX has been hampered by the low amount of toxin produced by bacteria. The biosynthesis is thought however, to involve arginine and a C5 unit derived from either amino acids, isoprenoids, shikimates or branched sugars (Yasumoto & Murata, 1993).

Regulation of TTX production

Gallacher & Birkbeck (1993) investigated the production of SCB by four strains of marine bacteria (*Alteromonas tetraodonis* GFC, *Shewanella alga* OK-1, *Vibrio alginolyticus* NCIMB 1903 and *Vibrio anguillarum* NCIMB 829) reported to produce TTX (Simidu *et al.*, 1987, 1990). TTX was detected in culture supernatants only after these bacteria had entered the stationary phase of growth. This indicates that TTX is a product of secondary metabolism and apparently not required for the active growth of these organisms.

Further investigation of TTX production revealed that the quantity of TTX produced per *A. tetraodonis* cell in sea water was eighty times that produced in marine broth. Therefore TTX production by *A. tetraodonis* is enhanced under nutrient-limited conditions. The addition of phosphate to the growth medium greatly inhibited the production of TTX by *A. tetraodonis*. This inhibition still occurred if phosphate was added late in the growth phase, i.e. up to two hours before TTX was



	R_1	R_2	R_3	R_4
(a) TTX	OH	CH_2OH	H	OH
(b) 4-epi TTX	OH	CH_2OH	OH	H
(c) 6-epi TTX	CH_2OH	OH	H	OH
(d) 11-deoxy TTX	OH	CH_3	H	OH
(e) 11-oxo TTX	OH	CHO	H	OH
(f) 11-nor-TTX-6(r)-ol	H	OH	H	OH
(g) 11-nor-TTX-6(s)-ol	OH	H	H	OH
(h) Chiriquitoxin	OH	$CH(OH)CH(NH_2)COOH$	H	OH

(i) Anhydro-TTX

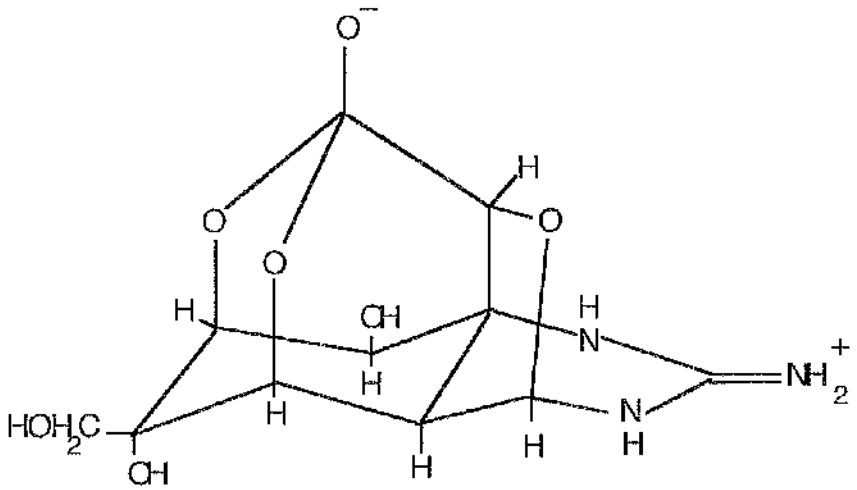


Figure 1. Structure of TTX & its derivatives (adapted from Kao, 1986 and Yasumoto & Murata, 1993)

normally produced. The production of TTX by *A. tetraodonis* is therefore induced when phosphate concentrations are low and the cells are in the stationary phase of growth (Gallacher & Birkbeck, 1993).

PARALYTIC SHELLFISH POISONING

Historical background

Reports of paralysis caused by the consumption of shellfish date back several centuries. One of the earliest detailed accounts is found in the writings of Captain George Vancouver. He reported that during exploratory voyages in 1793 off the coast of British Columbia, a crew member died and others became ill after eating toxic mussels. The symptoms described in this report are typical of paralytic shellfish poisoning (PSP) (Kao, 1966).

Outbreaks of PSP are sporadic and unpredictable. Before regular monitoring of shellfish toxicity levels was introduced, the first indication of a problem was usually a report of human poisoning. It was following an outbreak of PSP in 1927 along the central Californian coast, that the cause of shellfish toxicity was established. Sommer *et al.* (1937) suspected that dinoflagellates, upon which the mussels were feeding, were the source of the poison. A series of experiments proved this link. Acidic extracts of the dinoflagellates and of mussels were reported to kill mice in exactly the same manner. In addition, if non-poisonous mussels were allowed to feed on toxic dinoflagellates, the mussels became poisonous. Sommer *et al.* reported that toxic and non-toxic shellfish have no observable characteristics which could be used for differentiation. The dinoflagellates these workers observed in their studies were identified as *Gonyaulax catenella* (now known as *Alexandrium catenella*) (Sommer & Meyer, 1937; Sommer *et al.*, 1937).

Outbreaks of PSP are most commonly associated with blooms of toxic dinoflagellates. Such blooms, or red tides as they are also known, are dense monospecific populations of dinoflagellates, caused by a rapid growth of the species involved. Diverse environmental factors are involved in the onset of dinoflagellate blooms (Iwasaki, 1989). During the past 20 years there has been a change in the frequency of dinoflagellate blooms and their geographical distribution (Anderson, 1989; Iwasaki, 1989; Sinderman, 1990; Levin, 1991). This increase may be a result of changing environmental conditions, such as water temperature, and increased coastal pollution caused by increased fish farming and nutrient runoff from the land

(Iwasaki, 1989). The presence of an algal bloom is not necessarily indicative of a public health risk as blooms of non-toxic dinoflagellates also occur.

To prevent outbreaks of PSP, toxicity levels of shellfish intended for human consumption are carefully monitored. The mouse bioassay developed by Sommer & Meyer (1937) is still used internationally to estimate toxin levels despite its limitations. The EEC have established a tolerance level of 80µg of STX equivalent per 100g of shellfish flesh (Council of European Communities, 1991). When toxicity is found to exceed this level the sale of shellfish is prohibited. Whilst having the desired effect of minimising cases of human poisoning, this has serious economic implications for the shellfish industry.

Biological origin of PSP Toxins

The major causative toxin of PSP, saxitoxin (STX), was first isolated in 1957 by Schantz *et al.* from mussels, clams, and cultured *A. catenella* (Schantz, 1986). Subsequently, more than twenty toxic derivatives of STX have been described (Oshima, 1995a).

Three morphologically distinct genera of dinoflagellates are reported to produce PSP toxins; *Alexandrium* spp. (formerly known as *Gonyaulax* or *Protogonyaulax* spp.), *Gymnodinium* spp., and *Pyrodinium* spp.. Saxitoxin and its derivatives are also produced by the cyanobacterium *Aphanizomenon flos-aquae* (Ikawa, 1985) and have been detected in a macro-alga, *Jania* sp. (Yasumoto *et al.*, 1983). The PSP toxins thought to be produced by dinoflagellates, cyanobacteria, or macro-algae may be taken up through the food chain and accumulate in shellfish or other marine organisms such as lobsters or xanthid crabs (Shumway, 1995).

A number of observations, however, have challenged the theory that dinoflagellates are the primary producers of STX and its derivatives. Many species closely related to toxic species of dinoflagellate do not produce toxin. Indeed, within known toxic species, variations in toxicity exist between isolates from different geographical locations and the season of sampling (Kodama & Ogata, 1988; Shimizu, 1990b). It has been reported that the toxin content of a single isolate differs depending on growth phase and culture conditions. It has also been reported that subclones of *Alexandrium tamarense* derived from a single clonal cell differ in toxicity. It was, therefore, suggested that PSP toxin production was not a hereditary characteristic (Kodama & Ogata, 1988).

Further questions regarding the source of PSP toxins were raised during the monitoring of the abundance of dinoflagellates and the toxicity of shellfish in Ofunato Bay, Japan (Kodama & Ogata, 1988). The following observations were reported:-

(i) The toxicity of scallops correlated with the abundance of *A. tamarense* but the association of *A. catenella* with shellfish toxicity was not clear.
(ii) The toxicity of shellfish increased to a maximum when the population of *A. tamarense* was decreasing.
(iii) The ratio of toxicity level to cell number of *A. tamarense* varied significantly.
(iv) The scallops showed a repeated rise and fall in toxicity during the period when dinoflagellates were not present.

These observations led workers to investigate alternative sources of PSP toxins. The marine ecosystem is very complex and symbiotic relationships between organisms are common. Romalde *et al.* (1990) suggest that during an algal bloom a "phycosphere" may exist which could influence microbial activity in the surrounding water. Factors involved include competition for nutrients, selective antimicrobial activity and selective stimulation of certain bacteria by algal extracellular products. These factors combine to decrease bacterial diversity during algal blooms (Romalde *et al.*, 1990).

Silva (1982) reported the presence of intracellular bacteria in *A. tamarense* and hypothesised that these bacteria have a role in the production of PSP in this dinoflagellate. Silva suggested that the bacteria could either be the sole producers of PSP toxins or collaborate with the dinoflagellate cell in toxin production. Support for this hypothesis came when Kodama *et al.* (1988, 1989) isolated bacteria from *A. tamarense* grown in the presence of antibiotics. No bacteria were isolated from the culture medium suggesting a possible intracellular location of this isolate. The toxicity of the dinoflagellate was correlated with the number of bacterial colonies derived from *A. tamarense*. No bacteria were recovered from non-toxic dinoflagellate cultures.

The bacterium, identified as a *Moraxella* sp. (Kodama *et al.*, 1990b), was shown to produce STX in culture. However, the amount of toxin produced by this bacterium was very low. In addition, the toxin detected was STX whereas the main toxins reported in *A. tamarense* are gonyautoxins (GTXs). Further studies showed that growth of *Moraxella* sp. under nutrient deprivation conditions increased the amount of toxin produced. Significantly, the toxin profile was found to be comparable to that of *A. tamarense* (i.e. mainly GTXs were produced) under these conditions (Kodama *et al.*, 1990b).

It therefore appears that bacteria may be responsible for PSP toxin production in *A. tamarense*. The dinoflagellate, however, may play a role in the enhancement of toxin production. Differences in the bacterial content of dinoflagellate cells could explain differences in toxicity between different isolates of the same species.

It has been reported however that the toxin profile in *A. catenella* and *A. tamarense* is a hereditary characteristic. F1 progeny of mating parents with different

toxin profiles inherit the toxin profile of one parent in a mendelian manner. It is suggested, therefore, that the genes for PSP toxin synthesis are coded for by a nuclear gene in these algae (Sako *et al.*, 1992, 1995). Doubt still remains, therefore, regarding the relative importance of bacteria and dinoflagellates in the production of PSP toxins.

It is suggested that PSP toxin-producing bacteria are a possible source of such toxins during times of high shellfish toxicity in the absence of dinoflagellates. Kodama *et al.* (1990a) reported that PSP toxins are present in particles of a similar size fraction to bacteria in the sea water of Ofunato Bay, Japan, during times when bivalve toxicity increased in the absence of toxic dinoflagellates. PSP toxins were also detected in particles of similar size separated from cell-free culture medium from *A. tamarense* cultures.

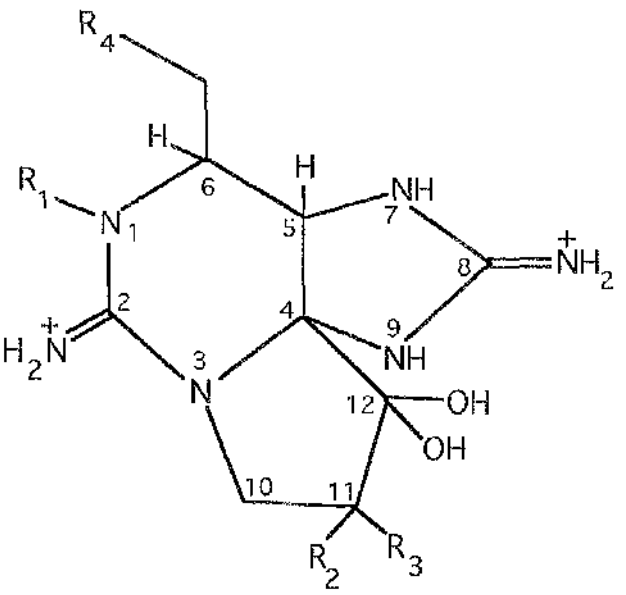
The same group reported that during a bloom of *A. catenella* in Tanabe Bay, Japan, particles which were smaller than dinoflagellates contained a considerable amount of PSP toxins. No significant toxicity was detected in naturally occurring *A. catenella* cells. The particles which contained PSP toxins, although smaller than dinoflagellates, were larger than bacteria. These particles could be small ciliates or flagellates, but such organisms were not observed in high numbers at the time of sampling. However, many detritus and silt particles were observed in water samples. The particles could, therefore, be detritus or silt particles with attached bacteria producing the toxins detected (Sakamoto *et al.*, 1992).

Gallacher and Birkbeck (1995) reported the isolation of SCB toxin-producing bacteria from seawater samples from Ardtoe, Scotland; 36.8% of bacterial isolates taken from this source over a one year period, were demonstrated to produce SCB toxin in culture. The peak incidence of bacteria able to produce SCB toxins, in June 1990, coincided with a major outbreak of PSP. It is possible, therefore, that some of these bacterial species have a role in PSP toxin production in these waters.

Structure of PSP toxins

STX, the most toxic of the PSP toxins, was first isolated from the Alaska butter clam (*Saxidomus giganteus*), California mussels and cultures of *A. catenella* (Schantz, 1986). The empirical formula of STX is $C_{10}H_{17}N_7O_4$. It is a diacidic base, which readily forms a double salt with hydrochloric acid. Purified STX is a white hygroscopic solid which is very soluble in water, partly soluble in methanol and ethanol, but insoluble in most non-polar solvents (Schantz, 1986).

Crystallisation of STX was difficult due to the highly polar nature of the molecule. Schantz *et al.* succeeded in crystallising a p-bromobenzene sulfonic acid derivative of STX and by X-ray crystallography determined its structure. This



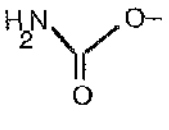
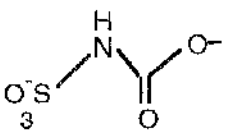
R_1	R_2	R_3	Carbamate Toxins	N- Sulfocarbamoyl Toxins	Decarbamoyl Toxins	Deoxydecarbamoyl Toxins
H	H	H	STX	B1	dc-STX	do-STX
OH	H	H	Neo-STX	B2	dc-Neo-STX	-
OH	H	OSO_3^-	GTX 1	C3	dc-GTX 1	-
H	H	OSO_3^-	GTX 2	C1	dc-GTX 2	do-GTX 2
H	OSO_3^-	H	GTX 3	C2	dc-GTX 3	do-GTX 3
OH	OSO_3^-	H	GTX 4	C4	dc-GTX 4	-
		R_4			HO-	H-

Figure 2. Structure of STX & its derivatives (Adapted from Oshima *et al.*, 1993 and Cembella *et al.*, 1994)

structure was confirmed by Bordner *et al.* by X-ray analysis of saxitoxin ethyl hemiketal (Schantz, 1986).

More than twenty derivatives of STX have been detected in shellfish and dinoflagellates. The structures of STX and its derivatives are shown in Figure 2. The different STX derivatives vary in toxicity. The carbamate toxins are the most toxic, the sulfocarbamoyl toxins the least toxic and the decarbamoyl derivatives have intermediate toxicity (Levin, 1991).

Studies on the toxicity of STX at differing pHs has shown that the 7,8,9-guanidinium group is the active moiety and this corresponds to the guanidinium moiety in TTX. Experiments comparing the toxicity of neosaxitoxin (neo-STX) at different pHs with toxicity of STX at these same pHs suggest the 1,2,3-guanidinium group has a role in the interaction of this molecule with the sodium channel. The two hydroxyls at C12 have also been shown to be important in STX activity (Kao, 1986).

It is proposed that, analogous with TTX binding to the sodium channel, the 7,8,9-guanidinium forms salt bridges with three carboxyls and the two hydroxyls at C12 interact with a fourth carboxyl. In addition, the 1,2,3-guanidinium of STX interacts with a fifth carboxyl on the sodium channel (Lipkind & Fozzard, 1994).

Transformation of PSP toxins

The relative amount of the different STX derivatives detected in shellfish and dinoflagellates varies between different isolates, particularly between isolates from different geographical locations. In dinoflagellates, the N-sulfocarbamoyl toxins often predominate, whereas the proportion of highly potent carbamate derivatives is typically higher in shellfish. The toxin profiles in shellfish do not necessarily reflect those of the dinoflagellates associated with their toxicity. These differences can be explained by transformation of PSP toxins in the shellfish (Cembella *et al.*, 1994).

Shimizu & Yoshioka (1981) reported that the incubation of PSP toxins with scallop tissue homogenates resulted in an increase in the amount of STX and a decrease in gonyautoxins (GTXs) & neoSTX. This change in toxin profile suggests PSP toxins undergo a transformation with STX being the final product. The conversion involves the reduction of N-oxide and O-sulphate groups. An identical conversion can be induced chemically with zinc dust and weak acid (Shimizu & Yoshioka, 1981). Kotaki *et al.* reported that two isolates of bacteria (*Vibrio* sp. & *Alteromonas* sp.) transformed GTXs to STX. Unidentified bacteria from quite distinct environments also carried out this reaction. It is suggested, therefore, that a wide spectrum of bacteria may possess toxin transformation activity (Kotaki *et al.*, 1985). Protein-free extracts of mussel tissue were reported to induce this reduction indicating a non-enzymatic transformation (Oshima, 1995b).

N-sulfocarbamoyl toxins are readily hydrolysed by weak acids to carbamate toxins. Such a reaction could be catalysed in biological systems by N-sulfatases (Shimizu, 1993). Two species of clam (*Mactra chinensis* and *Peronidia venulosa*) were reported to contain an enzyme which catalysed the hydrolysis of N-sulfocarbamoyl toxins to their decarbamoyl derivatives. Carbamate toxins are also hydrolysed by the enzyme of *M. chinensis*, although the enzyme of *P. venulosa* does not hydrolyse this group of toxins (Oshima, 1995b).

Incubation of GTX 2 and GTX 3 with crude enzyme extracts of *A. tamarense* resulted in transformation of these toxins to GTX 1 and GTX 4, indicating the presence of an oxidase in this dinoflagellate. No such activity was observed in *Gymnodinium catenatum* extracts (Oshima, 1995b).

C1 and C2 toxins were formed when GTX 2 and GTX 3 toxins were incubated with adenosine 3'-phosphate 5'-phosphosulphate (PAPS) and *G. catenatum* extracts. This indicated the presence of an N-sulfotransferase in this species. This enzymatic activity was reported to be present in extracts of toxic and non-toxic *G. catenatum* but not in extracts of *A. tamarense*, although this strain produced a high proportion of C2 (Oshima, 1995b). A N-sulfotransferase enzyme has been partially purified from *G. catenatum*. This enzyme transferred sulphate groups from PAPS to N-21 of STX and $GTX_{2/3}$ and produced B1 and $C_{1/2}$ toxins respectively. No transformation of neoSTX and $GTX_{1/4}$ was observed . The enzyme has a molecular weight of about 59,000 and required PAPS as a source of sulphate (Yoshida *et al.*, 1996).

Biosynthesis of PSP Toxins

The biosynthesis of STX derivatives has been investigated by feeding experiments using labelled precursors in *Alexandrium tamarense* and *Aphanizomenon flos-aquae* (Shimizu, 1986; Shimizu *et al.*, 1990a, 1990b). The precursors of the toxins have been revealed to be three molecules of arginine, a molecule of acetate and S-adenosylmethionine. In the decarbamoyl toxins only two arginine molecules are required. A biosynthetic pathway for STX derivatives has been proposed by Shimizu (1993) (see Figure 3). There are at least nine steps in the pathway to form carbamate and N-sulfocarbamoyl toxins, and at least six in the pathway for decarbamoyl toxin synthesis. It is not clear if the formation of sulphated toxins precedes the unsulphated compounds. Similarly, it is not known if N-hydroxy derivatives are the precursors of, or are formed from, STX-type compounds. It is possible that the reactions involved in transformation of PSP toxins are important in the biosynthesis of STX and its derivatives.

Since only the ß-epimers of 11-hydroxy sulphate toxins (C2, C4, GTX 3 and GTX 4) are observed in actively growing dinoflagellate cells, it is proposed that they

are the biosynthetic products. The chemically more stable α-epimers (C1, C3, GTX 2 and GTX 1) are subsequently formed through gradual epimerization (Oshima *et al.*, 1993).

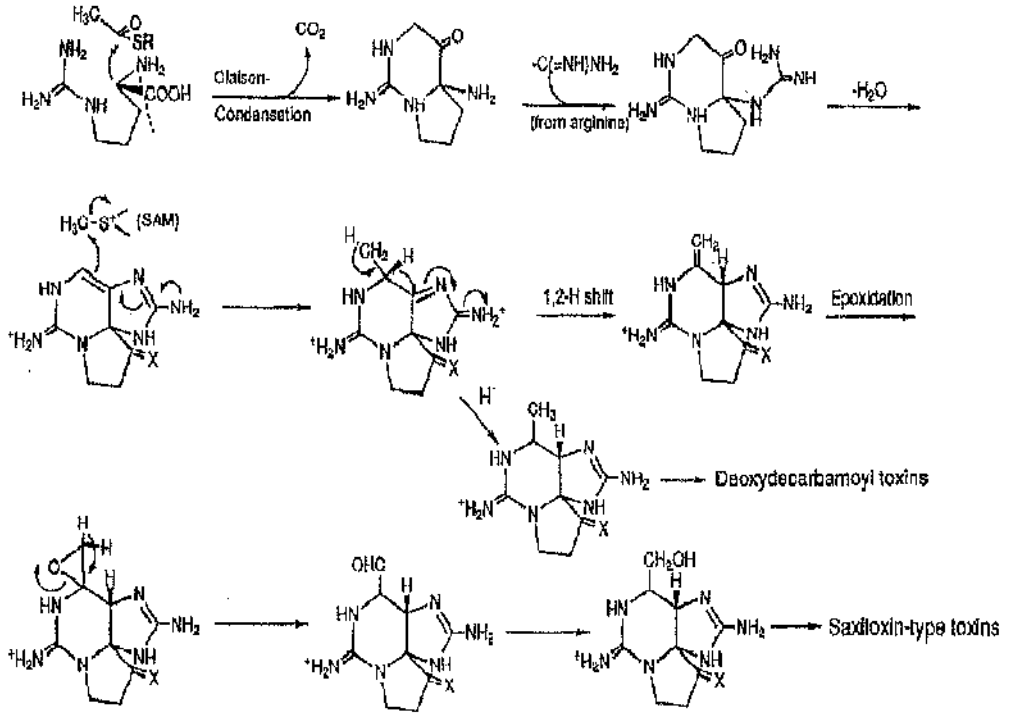


Figure 3. A proposed biosynthetic pathway of saxitoxin derivatives (from Shimizu, 1993)

Regulation of PSP toxin production by bacteria

Kodama & Ogata (1988) reported that *Moraxella* sp. produced PSP toxins at a low level in nutrient-replete medium. Under nutrient limitation conditions, however, the production of PSP toxin was greatly enhanced. The identity of the STX derivatives produced by *Moraxella* sp. was dependent on culture conditions. Only STX was detected during growth in nutrient-rich medium; under nutrient-limiting conditions mainly GTXs were produced (Kodama *et al.*, 1990b).

Doucette and Trick (1995) have suggested that this *Moraxella* sp. would be better characterised as belonging to the *Alteromonas/Pseudomonas* group. This same

group studied PSP toxin production by this bacterium on media containing different nutrient compositions. They reported the detection of PSP toxins in bacterial cells irrespective of its nutritional status. The toxin profile and total toxin content varied with phosphorus limitation depending on which carbon source was provided. Phosphorus-limited cells supplied with α-ketoglutarate contained three times as much toxin as cells grown in nutrient-replete medium. In contrast, when this bacterial strain was grown on succinate or malate under phosphorus limitation there was a decrease in toxin content compared to nutrient-replete cultures. Phosphorus limitation therefore appears to involved in the regulation of PSP production by this strain, but other factors are also involved.

Regulation of PSP toxin production in dinoflagellate culture

PSP toxins are produced in dinoflagellate culture at different rates depending on the stage of growth. In batch culture the toxin content per dinoflagellate cell has been consistently reported to be high in the exponential phase and decreasing as the cells enter stationary phase (Boyer *et al.*, 1985, 1987; Boczar *et al.*, 1988; Anderson *et al.*, 1990b). Growth of *Alexandrium* sp. and *G. catenatum* at sub-optimal temperatures results in an increase in toxin content as the growth rate decreases (Boyer *et al.*, 1985; Ogata *et al.*, 1987, 1989). A similar increase in toxin content was observed when growth rate was decreased by decreasing light intensity. The increase was not however as marked as the increase observed when the growth rate was controlled by temperature. This suggests that photosynthesis is required for toxin production (Ogata *et al.*, 1987, 1989).

Changes in salinity have been reported to affect toxin content, with increased salinity causing increased toxin production (White, 1978). Anderson *et al.* (1990b), however, found no difference in toxin content with varying salinity. Therefore, the effect of high salinity on toxicity is still open to question.

The limitation of different nutrients during growth has different effects on toxin production. Iron limitation for example, has little influence on toxin production (Boyer *et al.*, 1985). In cultures where nitrogen is limited, this limitation has little effect on the initial toxin production during exponential phase, however, during the stationary phase the toxin content continues to decline beyond the levels found in nutrient-replete cultures (Boyer *et al.*, 1985, 1987; Anderson *et al.*, 1990b).

Phosphorus limitation has the most significant effect on toxin production. Boyer *et al.* (1987) reported toxin content of phosphorus-deficient *A. catenella* and *A. tamarense* cells increased three to four times over that observed in nutrient-replete cultures (Boyer *et al.*, 1985, 1987). Phosphorus limitation was also reported to enhance PSP toxin production in batch and semi-continuous cultures of *Alexandrium*

fundyense (Anderson *et al.*, 1990b). In contrast, however, Flynn *et al.* (1994, 1995) reported that growth of *A. minutum* in phosphorus-limited cultures caused a decrease in the rate of toxin production compared with toxin production in nutrient-replete cultures. The toxin content of *A. minutum* cells declined during nitrogen limited conditions. However, if cells are phosphorus limited, then nitrogen stressed, there is no decline in the level of toxin per cell. This observation implicates phosphorus in the regulation of toxin production in *A. minutum*. The variable response of the different *Alexandrium* spp. to phosphorus limitation could reflect species-specific variation associated with differences in toxin composition, or differences in experiment design.

It was originally thought that the toxin profile (i.e. the relative abundance of each STX derivative observed) of a dinoflagellate culture was a fixed genetic trait. The changes in the total toxicity of a culture were regarded as the result of a corresponding decrease or increase in all of the derivatives produced, with no change in the proportion of the different derivatives (Boyer *et al.*, 1985, 1987). However, Boczar *et al.* (1988) reported variations in toxin profile as *A. tamarense* and *A. catenella* cultures entered the stationary phase of growth. It was later reported that the toxin profile of *A. fundyense* varied with growth rate in nitrogen- and phosphorus-limited semi-continuous cultures (Anderson *et al.*, 1990a).

It therefore appears that toxin composition is a variable characteristic of the dinoflagellate which reflects adaptations to nutritional and environmental conditions. It is of note, however, that these changes are in the relative abundance of the different toxins; the range of STX derivatives produced does not vary (Anderson *et al.*, 1990a).

Toxin profile is a function of the expression of enzymes involved in toxin biosynthesis, and the presence or absence of certain toxins provides evidence for genetic differences between and within populations. It has been suggested that toxin profiles are a valid biochemical marker to discriminate between strains of dinoflagellate species. Such a comparison must, however, be under carefully controlled experimental conditions to prevent misinterpretation due to variations caused by growth phase and nutrient availability (Anderson *et al.*, 1990a; Oshima *et al.*, 1993).

The possible role of intracellular bacteria in the production of PSP toxins in dinoflagellate culture cannot be ignored. The response of PSP toxin-producing bacteria to the external environment and to the physiological condition of the host dinoflagellate must be considered when interpreting observations regarding PSP toxin production in dinoflagellate culture (Anderson *et al.*, 1990a).

SCB TOXINS AND SECONDARY METABOLITES

A secondary metabolite has been defined as a naturally produced substance which does not play an explicit role in the internal economy of the organism that produces it (Stone & Williams, 1992). The SCB toxins produced by bacteria, and possibly by dinoflagellates, fit this definition. Within a number of species of bacteria some strains produce SCB toxins while others do not (Do *et al.*, 1990; Gallacher & Birkbeck, 1992). The growth and survival of the non-producing strains does not appear to be hindered by the absence of these toxins. TTX and PSP toxins have been reported to be produced at elevated levels under nutrient-limiting conditions (Kodama *et al.*, 1990b; Gallacher & Birkbeck, 1993; Doucette & Trick, 1995). The toxins are, therefore, apparently not involved in the active growth of the producing strains.

The secondary metabolites are a diverse group of molecules in terms of their chemical structure and biological activity. It is unclear why micro-organisms produce secondary metabolites but in view of their diversity it is unlikely that secondary metabolism has a single universal function. Instead, the individual metabolites probably have a specific function in the producing organism but for the majority of secondary metabolites this function remains unknown (Vining, 1990)

The biosynthetic pathways of secondary metabolites are complex. For example, antibiotic production typically consists of approximately ten to thirty steps catalysed by a corresponding number of different gene products (Hopwood, 1988). Such complex pathways are expensive in terms of energy expenditure and the proportion of the producing organisms genome dedicated to biosynthesis of the secondary metabolite (Maplestone *et al.*, 1992). It is unlikely, therefore, that the capacity to produce the secondary metabolite would be retained if it had no beneficial function and conferred no selectional advantage on the producing organism.

The formation of secondary metabolites is subject to general physiological controls that respond to environmental factors. Under nutrient-replete conditions micro-organisms either do not make secondary metabolites or do so below their potential. The genetic information for the process is fully expressed only when growth is restricted, and the nature of growth limitation usually has a strong influence on the level of expression. The induction of secondary metabolism only when growth is slowed or arrested indicates that the function of the metabolite is of benefit to the organism when it can no longer compete in its environment by exhibiting its full growth potential (Vining, 1990).

It has been proposed that the production of a secondary metabolite increases an organism's fitness for survival by acting as a chemical defence mechanism (Maplestone *et al.*, 1992). The production of secondary metabolites which are active against the physiology of higher organisms could act as a deterrent of microbial

predatation (Vining, 1990). It is tempting to assume that bacteria produce SCB toxins as a defence strategy. The symbiotic relationship that exists between SCB toxin-producing bacteria and higher organisms such as puffer fish and octopuses lends credence to this proposal. Higher organisms clearly use the toxins produced by bacteria as a self defence mechanism (Thuesen & Kogure, 1989).

Alternatively, the primary role of SCB toxin production by bacteria may be a mechanism to treat excess amino acids which accumulate in the cell when primary metabolism is limited (Shimizu, 1993).

The enzymes involved in the biosynthesis of secondary metabolites are specific for the metabolite produced. Some of these enzymes are reported to have a broad substrate specificity, which gives rise to families of closely related compounds. Studies on the organisation of genes encoding the biosynthetic enzymes of secondary metabolites have revealed that most are located in clusters. Indeed, in 1988 Hopwood stated "There are currently no proven exceptions to the generalisation that genes controlling successive steps in antibiotic biosynthesis are clustered." These clusters are located on the bacterial chromosome or on plasmids. The clusters of genes involved in secondary metabolism have been reported to contain not only the genes coding for the biosynthetic enzymes but also genes involved in regulation and, in the case of antibiotics, in conferring resistance to the secondary metabolite on the producing organism (Martin & Liras, 1989; Maplestone *et al.*, 1992; Stone & Williams, 1992).

It is known that within some clusters for secondary metabolites, the biosynthetic genes are ordered in a sequence corresponding to their place in the pathway. Ordering of structural genes allows each enzyme formed by coupled transcription-translation to act on the product of the previous enzyme. In this way an enzyme need not find its substrate by random collision (Martin & Liras, 1989).

SULFOTRANSFERASE ENZYMES

N-Sulfotransferase involved in PSP biosynthesis

One of the enzymes thought to be involved in the biosynthesis of PSP toxins is a *N*-sulfotransferase (NST). The enzyme, which catalyses the transfer of a sulfonate (SO_3) group from adenosine 3'-phosphate 5'-phosphosulphate (PAPS) to the NH_2 group of carbamate PSP toxins producing the corresponding *N*-sulfocarbamoyl toxins, was first detected in extracts of toxic *Gymnodinium catenatum*. The NST is also reported to be present in non-toxic isolates of *G. catenatum* (Oshima, 1995a).

Yoshida *et al.* (1996) have purified a NST from a toxic *G. catenatum* isolate. The enzyme has an apparent molecular weight (M_r) of 59,000 and uses PAPS as a

sulphate donor. The enzyme did not catalyse the transfer of sulfonate to the N-hydroxy PSP toxins (e.g. neoSTX and $GTX_{1/4}$) but did convert STX and $GTX_{2/3}$ to B1 and $C_{1/2}$ respectively. The NST activity was optimal at pH 6 and 25°C.

Eukaryotic sulfotransferases

Sulfotransferases (STs) constitute a superfamily of enzymes which are responsible for the sulfonation of a variety of compounds. In plants, STs are responsible for the sulfonation of flavanols. In mammals, STs catalyse sulphate conjugation of hormones, neurotransmitters, xenobiotic compounds, polysaccharides and peptides. The enzymes which catalyse the sulfonation of hydrophobic compounds (e.g. phenols and steroids) and the enzymes which catalyse sulfonation of hydrophilic macromolecules (e.g. polysaccharides and proteins) belong to two distinct groups. STs which catalyse sulfonation of hydrophobic compounds are located in the cytosol, whereas membrane bound STs are responsible for the sulfonation of polysaccharides and proteins (Weinshilboum *et al.*, 1997; Yamazoe *et al.*, 1994; Rikke & Roy, 1996).

The cytosolic STs are predominately dimers (both homo- and heterodimers) with monomer M_r values that vary from 30 to 36 kDa. However, catalytically active monomers are present in plants and some mammals (Weinshilboum *et al.*, 1997). PAPS acts as a sulphate donor for these STs and the major acceptor groups are aromatic or aliphatic hydroxyls. However, the STs are also capable of conjugating other structural groups, including amines, N-oxides, and hydroxylamines (Falany, 1997a).

Weinshilboum *et al.* (1997) have grouped eukaryotic cytosolic ST cDNAs into three families depending on their amino acid homology (45% homology within families). Mammals express two of the ST families, the phenol STs (PSTs) and the hydroxy-steroid STs (HSSTs). Plants express the third family of STs, the flavanol STs (FSTs). The PST family consists of two subfamilies with 60% amino acid homology within each of the sub-families. The first of these subfamilies (PSTs) preferentially catalyses the sulphate conjugation of simple planar phenols, and the second (ESTs) catalyses the sulfonation of estrogens with very low K_m values.

Human tissues are presently known to express at least five cytosolic ST enzymes. Three are PSTs, one is an EST and the other a HSST. These enzymes differ with regard to their substrate specificity, inhibitor specificity, thermal stability and regulation (i.e. their levels of activity are regulated independently (Weinshilboum *et al.*, 1997).

A comparison of the amino acid sequences of cytosolic STs has revealed four conserved regions. One of these regions, located at the carboxyl terminus, has been identified as a possible PAPS binding site as it is similar to the glycine-rich phosphate

binding loop (P-loop; GxxxxGK) present in some ATP- and GTP-binding proteins (Komatsu *et al.*, 1994). Site directed mutagenesis involving the substitution of an invariant arginine in this region again indicated a role in the binding of PAPS (Marsolais & Varin, 1995). The conserved amino acid sequence of this region is RKGxxGDWKxxFT (Weinshilboum *et al.*, 1997). Rat N-heparan sulphate sulfotransferase possesses a similar consensus sequence (GxxGxxK) at the N-terminus of the molecule. This ST is quite different from cytosolic STs with regard to size (88 KDa) and enzymatic activity (the N-sulfation of a mucopolysaccharide). The presence of a GxxGxxK motif therefore supports the hypothesis that this region is involved in PAPS binding (Komatsu *et al.*, 1994).

Bacterial Sulfotransferases

Bacteria which possess arylsulphate STs have been isolated from human and rat intestinal flora. However, the substrate specificities of the arylsulphate STs produced by these bacteria differ from the STs of mammals. Rather than using PAPS as a sulphate donor, the bacterial STs transfer sulphate groups from phenolic sulphate esters to phenolic compounds (Kim *et al.*, 1986; Kim *et al.*, 1992). It is unlikely, therefore, that bacterial arylsulphate STs are related to the NST reported to convert the carbamate PSP toxins to the corresponding *N*-sulfocarbamoyl toxins as N-ST reportedly uses PAPS as a sulphate donor (Oshima, 1995a; Yoshida *et al.*, 1996).

The NodH protein of *Rhizobium meliloti* is a ST involved in the transfer of the sulfuryl group from PAPS to lipooligosaccharide precursors. These Nod factors are O-sulphated on the carbon 6 of the reducing terminal N-acetyl-D-glucosamine residue. This sulfation determines the host range of *Rhizobium meliloti* infection and nodulation. The overall homology between NodH and mammalian cytosolic STs is poor although there are conserved motifs on a domain spanning 120 amino acids. This domain may contain the PAPS binding site (Roche *et al.*, 1991).

In bacteria, PAPS-specific sulfotransferases are involved in the biosynthesis of cysteine by the assimilatory reduction of sulphate. These STs, also known as PAPS reductase, are encoded by *cysH*. PAPS reductase catalyses the transfer of the sulfonyl moiety of PAPS to a thiol acceptor. In *E. coli*, thioredoxin serves as the thiol acceptor. The eventual fate of thioredoxin-S-SO_3^{-1} is not known, but free sulphite is an eventual product. Sulphite reductase further reduces sulphite to sulphide, which is then used in the conversion of *O*-acetylserine to cysteine (Kredich, 1987). The *E. coli* *cysH* gene encodes a polypeptide of M_r 27,927, consisting of 244 amino acids. The gene product was isolated as a homodimer which exhibited PAPS reductase activity (Krone *et al.*, 1991).

METHODS USED FOR THE DETECTION OF SCB TOXINS

The requirement for shellfish harvests to be tested for the presence of PSP toxins has resulted in a great deal of research into methods of SCB toxin detection. This research has given rise to a diverse array of techniques for the detection and analysis of these toxins. Which of the techniques is used at any one time depends largely on what information is required. Two groups of methods are available, assays which detect the toxicity of a sample and methods of analysis which determine which toxins are responsible for the toxicity. Definitions of "assay" and "analysis" have been supplied by Sullivan (1993). An assay is described as "a method that produces a single response from the collective responses of all the individual components". An analysis is "a method that attempts to differentiate all the various components and quantify them individually". An assay for SCB toxins is only suitable if the total toxicity of a sample is the desired information (e.g. as part of a shellfish monitoring programme). An analysis would be the method of choice if a comparison of toxin profiles of different samples is required.

Bioassays for SCB toxins

The mouse bioassay, first described by Sommer & Meyer (1937), is still the only procedure officially recognised by the Association of Official Analytical Chemists (AOAC) for the detection of PSP toxins (AOAC, 1990). The assay involves injecting mice, of a uniform weight (approximately 20 g), with acid extracts of shellfish tissue. The time taken for the animal to die is taken as a measure of the amount of toxin present. One mouse unit is defined as the amount of injected toxin which would kill a 20 g mouse in 15 minutes (AOAC, 1990; Levin, 1991).

Despite being the most widely used technique for PSP detection, the mouse bioassay has a number of drawbacks. These drawbacks are as follows (Sullivan, 1993):-

(i) The variability of results is reported to be ±20%.
(ii) The bioassay is only quantitative between death times of 5 to 7 minutes therefore dilution and retesting may be required.
(iii) Sensitivity is low (detection limit 40 µg STX per 100 g shellfish flesh).
(iv) A high salt content in samples will suppress toxic effects.
(v) The assay requires the sacrifice of a large number of mice, an issue which is becoming increasingly unacceptable to society.

The house fly has been used as an alternative to mice in the bioassay. Movement of the flies is scored at ten minutes post injection and is used as an

indicator of sample toxicity (Ross *et al.*, 1985). The housefly bioassay has not been widely adopted however, probably due to the inconvenience of working with such a small organism (Sullivan, 1993).

Tissue culture assays for SCB toxins

Competitive displacement assay

The toxicity of the different TTX and STX derivatives is directly proportional to the degree with which they bind to sodium channels. This activity has been utilised in a competitive displacement assay. The assay measures the displacement of radioactive STX from crude rat brain preparations (Davio & Fontelo, 1984; Vieytes *et al.*, 1993) The assay is extremely sensitive and quite specific as it measures only compounds which bind to sodium channels (Sullivan, 1993). The major disadvantage of this assay is the source of the sodium channels. Vieytes *et al.* (1993) reported using between 15 and 40 rats for each preparation.

Sodium channel blocking assays

A tissue culture assay was developed by Kogure *et al.* (1988) which utilised the protective action of SCB toxins on mouse neuroblastoma cells incubated in the presence of ouabain and veratridine. Ouabain is a specific inhibitor of Na^+/K^+ ATPase, which is the energy source for cellular exclusion of sodium ions. Veratridine stimulates an influx of sodium ions into nerve cells. Mouse neuroblastoma cells ordinarily swell and die when incubated with ouabain and veratridine. However, in the presence of SCB toxins they are protected. The level of protection afforded by a sample is a measure of the amount of SCB toxin present. In the original assay Kogure *et al.* (1988) estimated cell viability by direct microscopic observation.

The mouse neuroblastoma assay has been made more quantitative by the incorporation of dyes which stain the cells. Cells affected by ouabain and veratridine lose the ability to adhere to plastic surfaces. Jellet *et al.* (1992) exploited this by staining cells with crystal violet. Non-adherent cells are washed away leaving behind only the cells protected by SCB toxins. The amount of dye present can be determined using a microtitre plate reader. In a separate adaptation of the original assay Gallacher & Birkbeck (1992) stained the viable cells with the vital dye neutral red. Again, the amount of dye present after washing was measured with a microtitre plate reader, giving a measure of the SCB activity of a sample.

Immunoassays for PSP toxins

The first immunoassay for PSP toxins, a haemagglutination assay (Johnson & Mulberry, 1966) was found to have insufficient sensitivity for practical use (Sullivan, 1993). More sensitive radio-immunoassays (RIA) (Carlson, 1984) and enzyme linked immunosorbent assays (ELISA) (Chu & Fan, 1985; Cembella *et al.*, 1990; Usleber *et al.*, 1991, 1994; Chu & Huang, 1992) have been developed using antibodies raised in rabbits following conjugation of STX or neoSTX to a protein carrier (e.g. bovine serum albumin or keyhole limpet haemocyanin). The major disadvantage of the immunoassays developed so far is the antibodies cross-react to a variable extent with the different PSP toxins. The results are therefore greatly influenced by the toxin profile of the sample. This is a particular problem when the antibodies cross-react strongly with a derivative with low toxicity or cross-react poorly with derivatives with high toxicity. The application of immunoassays for PSP toxin detection will be limited until antibodies which cross-react with all the STX derivatives are obtained. This work is however hindered by the limited supply of the different PSP toxins (Sullivan, 1993; Van Egmond *et al.*, 1993).

Chemical assays for PSP toxins

Colorimetric assays for the detection of PSP toxins have been reported which involve the reaction of a sample with picric acid (McFarren *et al.*, 1958) or the Folin-Ciocalteau phenol reagent (Mosley *et al.*, 1985). However, interfering compounds also react with these compounds and the sensitivity is low (Van Egmond *et al.*, 1993).

A more useful chemical assay is the fluorescence assay first described by Bates & Rapoport (1975). Previous workers had reported the degradation of STX by alkaline oxidation with hydrogen peroxide (see Figure 4) (Wong *et al.*, 1971). One of the oxidation products (8-amino-6-hydroxymethyl-2-iminopurine-3(2H)-propionic acid) is converted to a fluorescent pyrimidopurine on the addition of acid. Bates & Rapoport (1975) reported a linear relationship between STX concentration and fluorescence following alkaline oxidation and acidification. The assay has been used to detect PSP toxins in shellfish extracts (Bates & Rapoport, 1975; Shoptaugh *et al.*, 1981).

Investigation of the alkaline oxidation products of the different PSP toxins revealed that they do not fluoresce with equal intensity. Buckley *et al.* (1978) observed that N-hydroxylated toxins (i.e. neoSTX, $GTX_{1/4}$, B2, etc.) fluoresce only poorly following alkaline oxidation with hydrogen peroxide. The fluorescence assay

is therefore not suitable for detecting this group of toxins without further development.

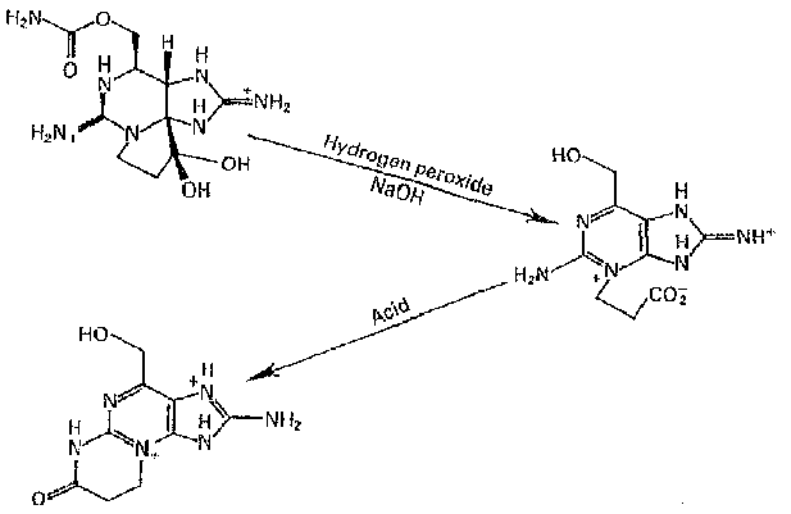


Figure 4. Alkaline oxidation of saxitoxin. (Adapted from Sullivan, 1993)

Chemical analysis of PSP toxins

Thin layer chromatography (Buckley *et al.*, 1976), capillary electrophoresis (Thibault *et al.*, 1991; Buzy *et al.*, 1994) and mass spectrometry (Quilliam *et al.*, 1989, 1993) techniques for the detection of PSP toxins have been described. However, the most commonly employed technique for the analysis of PSP toxins is high performance liquid chromatography (HPLC). PSP toxins neither absorb UV or fluoresce, therefore for detection they must first be converted to fluorescent derivatives (Luckas, 1992). The alkaline oxidation reaction which is the basis of the fluorescence assay (see Figure 4) is the derivatisation method of choice. This oxidation reaction can either be carried out prior to chromatographic separation of the oxidation products (pre-column oxidation), or following separation of the PSP toxins (post-column oxidation).

Post-column oxidation HPLC analysis

Buckley *et al.* (1978) were the first to describe the separation of PSP toxins by liquid chromatography with detection by post-column oxidation and fluorescence. However

poor efficiency of separation limited the application of this method (Sullivan, 1993). More efficient separation was achieved using a polystyrene divinylbenzene resin column (PRP-1; Hamilton) and gradient elution with phosphate buffer solution, containing heptane and hexanesulphonic acid as ion-pair reagents (Sullivan & Iwaoka, 1983; Sullivan & Wekell, 1984). In addition, the detection of the PSP toxins was improved by using periodic acid as an oxidising agent. The alkaline oxidation of PSP toxins with peroxide was not suitable for the derivatisation of the N-hydroxylated toxins. However, alkaline oxidation with periodate did result in the formation of fluorescent derivatives of these toxins (Sullivan & Iwaoka, 1983). This method has been successfully used for PSP detection in shellfish (Jonas-Davies *et al.*, 1984; Sullivan *et al.*, 1985) and dinoflagellates (Boyer *et al.*, 1986; Boczar *et al.*, 1988). The utility of the method is limited by poor separation of the N-sulphocarbamoyl toxins (C1-4) and incomplete separation of carbamate and decarbamoyl toxins (Luckas, 1992).

The methods of Franco & Fernández-Vila (1993) and Oshima (1995b) overcame the problem of poor separation of PSP toxins. Both these methods employ reversed phase chromatography with three separate isocratic elution systems. These different isocratic systems separate three groups of PSP toxins, categorised by their basicity. The N-sulphocarbamoyl toxins (C1-4) belong to one group; $GTX_{1/4}$, $GTX_{2/3}$, B1 and B2 belong to a second group; STX, neoSTX and dcSTX belong to the third group. Both of these methods use post-column periodate oxidation and fluorescence detection.

Alternative post-column oxidation methods, using an electrochemical reactor (Lawrence & Wong, 1995) and a manganese dioxide solid-phase reactor (Lawrence & Wong, 1996), have been evaluated. Analysis of shellfish and plankton extracts gave results comparable to those obtained by post-column periodate oxidation. These new methods have the advantage of requiring less equipment and daily maintenance than established post-column oxidation methods.

Pre-column oxidation HPLC analysis

Pre-column oxidation of PSP toxins was first described by Lawrence *et al.* (1991). The PSP toxins were oxidised under basic conditions with either hydrogen peroxide or periodic acid and the oxidation products separated by reverse-phase HPLC. The oxidation products were eluted from a C18 column with an acetonitrile gradient. In accordance with the work of Buckley *et al.* (1978), the N-hydroxylated toxins did not form fluorescent products following peroxide oxidation. However the periodate oxidation products of these toxins were fluorescent. The non-N-hydroxylated toxins formed highly fluorescent derivatives with both periodate and peroxide oxidation. The UV absorption and fluorescence emission maxima of the oxidation products are

shown in Table 3. The yield of fluorescent N-hydroxylated derivatives was increased by the addition of ammonium formate to the periodate oxidation reaction (Lawrence & Ménard, 1991). Automation of the pre-column oxidation reaction has been reported, therefore improving precision and sample throughput (Janecek *et al.*, 1993). A comparison of this method with the mouse bioassay for the detection of PSP in lobster hepatopancreas revealed a reasonable correlation, although the results for HPLC analysis were consistently higher (Lawrence *et al.*, 1995). The oxidation products of a number of PSP toxins have been characterised by liquid chromatography/mass spectrometry (LC-MS) (Quilliam *et al.*, 1993).

Compound	UV Absorption max.	Fluorescence emission max.
STX	335 nm	386 nm
Neo STX	333 nm	392 nm
B-1	333 nm	391 nm
B-2	331 nm	385 nm
C-2	335 nm	393 nm

Table 3. Absorption & fluorescence emission maxima of the periodate oxidation products of PSP toxins. (From Lawrence *et al.*, 1991)

The major disadvantage of pre-column oxidation HPLC analysis is the formation of a number of different products by the oxidation of some of the toxins, therefore, decreasing sensitivity and complicating identification. In addition, different PSP toxins give rise to identical oxidation products, therefore further complicating toxin identification. The failure of the N-hydroxylated toxins to form fluorescent products by peroxide oxidation can be used to differentiate these toxins from non-N-hydroxylated toxins which yield the same oxidation products. Cation exchange solid phase extraction (SPE) cleanup or strong anion exchange SPE cleanup will remove N- sulfocarbamoyl toxins from a sample. A comparison of treated and untreated samples will therefore allow accurate identification of the toxins present (Lawrence & Ménard, 1991; Lawrence *et al.*, 1995).

An alternative pre-column oxidation HPLC method has been described by Flynn & Flynn (1996). The periodate oxidation used in this method is performed at 5°C in order to minimise the formation of multiple oxidation products.

TRANSPOSON MUTAGENESIS

Transposons are distinct DNA segments which have the unique capacity to move (transpose) to new sites within the genome of their host organisms. The transposition process is independent of the classical homologous recombination (*rec*) system of the organism. Moreover, the insertion of a transposable element into a new genomic site does not require extensive DNA homology between the ends of the element and its target site.

Transposons differ in size and genetic complexity but in most cases the structural organisation follows the same basic pattern. At either end of the transposons are short inverted repeat sequences. Between these inverted repeats are the genes required for transposition and also additional genes unrelated to transposition functions, such as antibiotic resistance, heavy metal resistance or pathogenicity determinant genes (Berg *et al.*, 1989; De Bruijn & Rossbach, 1994).

Shortly after transposons were discovered it was realised that they could be extremely efficient tools in bacterial genetics. The use of transposons to generate mutants has several advantages over classical mutagenesis techniques such as chemical mutagenesis. The advantages are as follows (De Bruijn & Rossbach, 1994):-

(i) The insertion of a transposon into a gene generally results in a null mutation i.e. its complete inactivation.

(ii) The mutation caused by the insertion is relatively stable due to the low frequency of precise excision.

(iii) If a transposon integrates into an operon, it usually exerts a strong polar effect on the genes located downstream of its insertion site. This allows operon structure to be determined.

(iv) Screening for mutants is simplified as transposons carry a selectable phenotypic marker such as antibiotic resistance.

(v) The integration of a transposon introduces genetic markers that allow mapping of the mutated gene and further genetic manipulations.

(vi) Transposons can be used to introduce genes in such a way that they come under the control of an active promoter near the site of integration. Such gene fusions can report on promoter activity and yield information regarding the regulation of gene expression.

To generate transposon insertion mutations in the genome of bacteria, the transposon must be introduced into its new host, and cells in which transposition has occurred must be identified. The transposon, located on either phage or plasmid suicide vectors, is introduced into the bacteria by transformation, transduction or

conjugation. The suicide vectors are so called because they cannot replicate stably or integrate into the genome of the recipient cell. Selection for the phenotypic marker carried by the transposon therefore identifies cells in which the element has transposed from the vector into the genome (De Bruijn & Rossbach, 1994).

Reporter gene fusions

Some transposons have been engineered to facilitate the formation of reporter gene fusions. These transposons have no transcriptional terminators within their terminal sequences. Therefore, if the transposon inserts in the appropriate orientation downstream of a promoter, promoterless genes within the transposon come under the control of that promoter (De Lorenzo & Timmis, 1994).

Reporter gene fusions are useful in studying the regulation of gene expression. This is particularly useful when the product of the gene of interest is difficult to assay. Reporter gene fusions are also useful in the discovery of genes which respond to particular environmental signals, such as DNA damage, heat shock or phosphate starvation (Berg *et al.*, 1989).

There are two types of reporter gene fusions which can be generated using transposon mutagenesis. Type I fusions monitor only transcription because the reporter gene lacks a promoter but contains its own translation initiation signals. Type II fusions monitor translation as well as transcription, because the reporter gene lacks sequences needed to initiate both transcription and translation. Transposons that form type I fusions need only insert into the target gene in the correct orientation for reporter gene expression; transposons that form type II fusions need to insert in both the correct orientation and the correct reading frame for expression. The reporter protein made by a type I fusion consists only of amino acids encoded by the gene carried by the transposon. In contrast, the protein formed in type II fusions is a hybrid protein containing a carboxy-terminal region encoded by the transposons reporter gene and a N-terminal region of variable length encoded by a fragment of the mutated gene (Berg *et al.*, 1989).

If the product of a gene is easily detectable by a simple assay it is potentially useful as a reporter gene. The reporter genes which are commonly used to create gene fusions include the ß-galactosidase (*lacZ(YA)*), alkaline phosphatase (*phoA*) and bioluminescence (*luxAB*) genes (De Lorenzo & Timmis, 1994).

Mini-Mu

Bacteriophage Mu is a temperate phage with a 37 kilobase linear dsDNA genome. Mu differs from other temperate phages in that the Mu genome integrates into the host

chromosome irrespective of whether it enters the lytic cycle or lysogenic state. This initial integration event takes place by a transposition mechanism and at a virtually random location in the genome. If the lytic cycle is initiated, Mu DNA is amplified by replicative transposition. For this reason, Mu can be considered to be both a phage and a transposon. Mu is a powerful tool for transposon mutagenesis because of its high transposition frequency and its near-random insertion specificity (Pato, 1989; Groisman, 1991).

Two Mu proteins, MuA and MuB, together with the ends of Mu in their proper orientation, are required for transposition. An enhancer sequence located approximately 1000 base pairs from the left end of Mu is required for high transposition frequencies (Groisman, 1991). The rest of the Mu genome is dispensable with regard to transposition. This has allowed the modification of Mu to decrease its size and to incorporate genes into the transposon to enhance its usefulness for transposon mutagenesis.

Mu dI 1681 is a mini-Mu construct which is a non-viable phage but retains the regions required for transposition. This construct carries a kanamycin resistance gene and a ß-galactosidase gene. Type I reporter gene fusions are formed when Mu dI 1681 integrates into the genome (Berg *et al.*, 1989).

Belas *et al.* (1984) used a tetracycline-resistant derivative of Mu dI 1681 to study the regulation of lateral flagella gene expression in marine *Vibrio* spp. The kanamycin resistance gene of Mu dI 1681 was replaced with a tetracycline resistance gene because the marine strains studied showed considerable natural resistance to kanamycin. The mini-Mu (tet^r) was delivered into the recipient *Vibrio parahaemolyticus* strain using the transducing phage P1. One hundred and twenty four mutants defective in the swarming phenotype were isolated from a bank of 10,000 transductants. The transcriptional fusions produced by this transposon mutagenesis experiment were used to study the expression of the different genes in response to differing growth conditions, namely growth on solid medium and growth in liquid culture.

McCarter & Silverman (1987) used a mini-Mu (tet^r) transposon containing *luxAB*, rather than *lacZ* as the reporter gene, to identify phosphate regulated genes in *Vibrio parahaemolyticus*. The production of a major outer membrane protein (ompP), haemolysin, phosphatase and phospholipase C were shown to be under the control of the *pho* regulon. Loci identified as involved in phosphate regulation were cloned and shown to complement *E. coli pho* regulatory genes.

Transposon mutagenesis using mini-Mu (tet^r) was made simpler by the incorporation of this transposon into a conjugative suicide plasmid. Mini-Mu(tet^r) was placed on the IncP-1 plasmid pRK2013. This plasmid is capable of conjugative transfer into a large range of Gram-negative bacteria. However, this plasmid contains

a ColE1 origin of replication and is, therefore, unable to replicate in most non-enteric bacteria (Ely, 1985). Östling *et al.* (1991) used this system to isolate mutants of *Vibrio* sp. S14 which preferentially expressed the *lacZ* transcriptional fusion gene under starvation conditions.

Although mini-Mu has the advantage of a high frequency of transposition and low specificity of insertion site, it is not an ideal system. The genes required for the transposition of mini-Mu are located on the element itself (Belas *et al.*, 1984). Therefore, these genes are retained after transposition and the loss of the suicide vector. Subsequent transposition events can therefore take place leading to mutants with multiple insertions or genome rearrangements.

Mini-Tn5

Transposon Tn5 is a 5,818 base pair composite transposon, consisting of two inverted repeats of 1,533 base pairs (IS50L & IS50R) flanking a unique central region of 2,750 base pairs. This central region contains an operon consisting of three antibiotic resistance genes. The *nptII* (kanamycin/neomycin resistance), *ble* (bleomycin resistance) and *str* (streptomycin resistance) genes are transcribed from a promoter located at the inside end of IS50L.

IS*50*R encodes a *cis*-acting transposase (TnP) and a *trans*-acting inhibitor (Inh) protein. The Tn5-encoded transposase and associated host factors act upon segments of about 19 base pairs at each end of IS50 that are termed O (outside) and I (inside). These 19 base pair segments are essential for efficient transposition.

Tn5 is capable of transposing at a very high frequency in *E. coli* (10^{-2} to 10^{-3}) by an apparently conservative mechanism. Inserts of Tn5 are bracketed by 9 base pair direct repeats of the target sequence. Tn5 has a low insertional specificity and therefore can insert into a large number of locations in the bacterial genome as well as into multiple positions within single genes, although some hot-spots for insertion have been reported (Berg, 1989; De Bruijn & Rossbach, 1994).

De Lorenzo and co workers have developed a number of mini-Tn5 derivatives for insertion mutagenesis and promoter probing (De Lorenzo *et al.*, 1990; Herrero *et al.*, 1990; De Lorenzo & Timmis, 1994). The mini-Tn5 derivatives contain antibiotic or non-antibiotic (e.g. arsenite or mercury) resistance selection markers. The resistance markers are flanked by the 19 base pair I and O Tn5 ends. Some of the mini-Tn5 derivatives were adapted for the formation of reporter gene fusions and promoter probing. In these derivatives the reporter genes are located between the I and O terminal sequences. The absence of transcriptional terminators within the I and

O sequences allows the expression of the promoterless gene if the mini-Tn5 inserts in the appropriate orientation downstream of an active promoter.

Within many of the mini-Tn5 derivatives there is a unique NotI site which can be used for cloning foreign DNA fragments. These NotI sites can be utilised to insert reporter genes (e.g. *lacZ*) into the derivatives which lack such genes.

An important feature of the mini-Tn5 constructs is that transposase gene (*tnp*) is no longer located on the transposon. Instead *tnp* is located on the delivery vector, pUT. After delivery into the host and transposition the delivery plasmid is lost as it cannot replicate in the host. The transposase is therefore not maintained in the cell after transposition and subsequent transposition events cannot take place.

The pUT plasmid has the origin of transfer, *oriT*, from the RP4 plasmid. This plasmid can therefore be transferred by conjugation into a broad range of bacterial species from donor strains expressing the RP4 conjugative functions (e.g. S17-1*λpir* or SM10*λpir*).

The origin of replication located on pUT is derived from the R6K plasmid. This origin of replication is π protein dependent and the plasmid is therefore only maintained in π protein-producing bacteria, e.g. in *λpir* lysogens of *E. coli*.

PHOSPHATE REGULATION OF GENE EXPRESSION

The environment in which bacteria live is rarely stable. Therefore, bacteria frequently encounter fluctuations in temperature, pH, osmolarity and nutrient availability. In order to optimise their chance of survival and propagate, bacteria must sense any changes in their environment and respond in an appropriate manner. The response invariably involves the differential expression of a number of genes with the induction of some and the repression of others. The group of genes regulated in response to a specific stimulus is known as a regulon. The genes regulated in response to phosphate limitation are said to belong to the *pho* regulon.

E. coli pho Regulon

At least 31 genes belonging to the *E. coli pho* regulon have now been cloned and sequenced (Wanner, 1993). These genes are induced when the cell is starved of phosphate. The level of induction for each gene varies however depending on promoter strength and the nature of other inducers. The induction ratios range from about 3-fold for *psiP* to more than 1000-fold for *phoA* (Wanner, 1987).

The two key proteins involved in the regulation of gene expression in response to phosphate limitation are phoR and phoB. PhoR acts as a sensor and phoB as a response regulator. Both these proteins share homology with the corresponding

proteins in other two-component regulatory systems (Gross *et al.*, 1989). PhoR somehow senses the concentration of phosphate in the environment and passes a signal to phoB, causing PhoB to induce the appropriate response. The signal involves phosphorylation of PhoR and a subsequent phosphorylation of PhoB. PhoR is a histidine protein kinase which autophosphorylates at a histidine residue, receiving its phosphate group from ATP. The phosphorylated phoR then transfers the phosphate group to phoB. Phosphorylated phoB appears to have an inherent autophosphatase activity, therefore reverting back to its non-phosphorylated form (Stock *et al.*, 1989; Russo & Silhavy, 1993). Transcription of *pho* regulon genes requires the phosphorylated form of phoB and controls that regulate the *pho* regulon affect the amount of phosphorylated phoB (Wanner, 1993).

In addition to phoR and phoB, the proteins of the phosphate specific transport (pst) system and phoU are required for the repression, but not activation, of the *pho* regulon (Wanner, 1993).

Phosphate control of the *pho* regulon involves two processes: repression by the pst system, phoU and phoR when phosphate is in excess, and activation by phoR when phosphate is limiting. An extracellular phosphate concentration of 4μM represses *pho* regulon gene expression. This corresponds to the amount of phosphate that would fully saturate the phosphate binding protein pstS (Wanner, 1993).

A model for phosphate transmembrane signal production has been proposed by Wanner (1993). This model involves three important events in phosphate repression: saturation of pstS by environmental phosphate, binding of phosphate bound pstS with pstABC complexes in the membrane and the formation of a "repressor complex" containing phosphate bound pstS, pstABC, phoU and phoR. Such a complex would maintain phoR in its repressor form that would dephosphorylate phoB. Accordingly, phosphate limitation would cause phoR to be released from the complex. This would lead to its conversion to the activated form of phoR (possibly a dimer) that would autophosphorylate and phosphorylate phoB.

The phosphorylated form of phoB is a transcriptional activator of *pho* regulated genes. The promoters of phosphate regulated genes contain one or several copies of an 18-base consensus sequence known as a *pho* box (Wanner, 1993). Phosphorylated phoB promotes transcription by binding to the *pho* box and possibly by direct contact with the σ^{70} subunit of RNA polymerase (Makino *et al.*, 1993).

Pho Regulon of *Vibrio parahaemolyticus*

Induction of the *pho* regulon of the marine bacterium, *V. parahaemolyticus* leads to the production of a major outer membrane protein (ompP), haemolysin, phosphatase and phospholipase C activities (McCarter & Silverman, 1987).

A regulatory locus controlling the expression of phosphate-regulated genes in *V. parahaemolyticus* was reported to complement *E. coli* strains with defects in *phoB, phoR* and *phoU*. Also, the expression of the ompP of *V. parahaemolyticus* responded to phosphate regulation in *E. coli*. The regulation of the *pho* regulon in *V. parahaemolyticus* is therefore analogous to that of *E. coli*. However, differences from the *E. coli* system are evident from the analysis of mutant phenotypes and from the organisation of regulatory loci (McCarter & Silverman, 1987).

Phosphate Regulation of Secondary Metabolites

Secondary metabolism is usually repressed when cells are actively growing and is induced as the cells enter the stationary phase of growth. Stationary phase is entered as one or more of the essential nutrients for growth become limiting. The presence of easily utilisable carbon, nitrogen or phosphate sources is known to inhibit or repress the biosynthesis of some secondary metabolites. High phosphate concentrations (above 1mM) repress the formation of enzymes involved in the biosynthesis of several antibiotics, such as *p*-aminobenzoic acid (PABA) synthase, anhydrotetracycline oxygenase, TDP-glucose-4,6-dehydratase, TDP-mycarose synthase and marocin *O*-methyltransferase (Martin & Liras, 1989; Liras *et al.*, 1990).

PABA synthase is involved in the production of candicidin by *Streptomyces griseus*. A phosphate-regulated promoter has been cloned from the upstream region of the PABA synthase structural gene, *pabS*. This promoter contains 12 nucleotides that are identical to the consensus sequence of 18 nucleotides that make up the *E. coli pho* box. The gene coding for streptomycin phosphotransferase (*sph*) in *S. griseus* is also reported to be regulated by phosphate. The promoter of this gene contains a *pho* box with 13 out of 18 nucleotides identical to the *pho* box of *E. coli* (Liras *et al.*, 1990). The regulation of candicidin and streptomycin biosynthetic pathways, in response to phosphate limitation, is probably controlled in a similar manner to the *E. coli pho* regulon. This may be a general rule for all secondary metabolite biosynthetic pathways regulated by phosphate, including the pathways for SCB toxin production.

SIGMA FACTORS AND GENE EXPRESSION

The transcription of a gene is initiated when the sigma (σ) subunit of RNA polymerase holoenzyme detects the promoter of that gene. In *E. coli* the σ^{70} subunit is the sigma factor involved in the expression of housekeeping genes. However *E. coli* also possesses other sigma factors which are involved in the expression of specific genes at times of environmental stress. For example, σ^{32} mediates the expression of stress proteins in response to heat shock (Yura *et al.*, 1993), and in *B.*

subtilis a cascade of sigma factors are involved in the expression of different genes required for spore formation (Losick & Pero, 1981; Stragier & Losick, 1990).

The different promoters recognised by specific sigma factors are homologous at the -10 and -35 regions. The consensus sequence of these regions is different for the different sigma factors. The production of alternative sigma factors by bacteria allows the co-ordinate regulation of discrete sets of genes in response to environmental stimuli. It is possible that the production of some secondary metabolites requires the presence of one or more alternative sigma factors.

Martin & Liras (1989) reported that most available promoter sequences for antibiotic resistance and biosynthetic genes do not resemble *E. coli* & *B. subtilis* consensus promoter sequences. These promoter sequences may be recognised by different σ subunits of RNA polymerase, which are only present under specific environmental or physiological conditions.

A stationary phase sigma factor, σ^s (σ^{38}), has been identified in *E. coli*. σ^s, encoded by the *rpoS (katF)* gene, controls a regulon of 30 or more genes expressed in response to starvation and during the transition to stationary phase (Kolter *et al.*, 1993; Loewen & Hengge-Aronsis, 1994).

Genes under the control of σ^s may not be regulated directly by this sigma factor; they may alternatively be controlled by the product of a gene which is under the direct control of σ^s. Such a regulatory cascade could explain why no consensus sequence for promoters recognised by σ^s has been determined (Kolter *et al.*, 1993; Loewen & Hengge-Aronsis, 1994).

Although the control of *rpoS* expression is not yet fully understood, it appears that both transcriptional and post-transcriptional controls are involved in the increased expression of *rpoS* during the transition to stationary phase (Kolter *et al.*, 1993; Loewen & Hengge-Aronsis, 1994).

Huisman & Kolter (1994) have reported that homoserine lactone induces the expression of σ^s. Homoserine lactones have previously been reported as extracellular signalling molecules produced during high cell density and involved in the control of bioluminescence and antibiotic production (Horinouchi & Beppu, 1994; Swift *et al.*, 1994). It is possible that homoserine lactones also constitute an intracellular signal that accumulates in starved cells regardless of cell density (Huisman & Kolter, 1994).

It is possible that a stationary phase specific sigma factor is required for the expression of genes involved in the production of SCB toxins in bacteria. This would ensure that these secondary metabolites are only synthesised when active growth has stopped due to nutrient limitation.

POSSIBLE MECHANISM OF SCB TOXIN PRODUCTION BY MARINE BACTERIA

A model of SCB toxin production by marine bacteria has been derived from the literature reviewed above and is presented in Figure 5.

In this model the biosynthetic precursors (e.g. arginine, acetate and S-adenosyl-methionine; Shimizu, 1993) are used as substrates for the enzymes encoded by biosynthetic genes to produce SCB toxins. These biosynthetic genes may (or may not) be clustered on an operon under the control of one or more promoters (Martín & Liras, 1989). The expression of the biosynthetic genes may be regulated in response to one or more environmental stimuli such as limited phosphate availability (Gallacher & Birkbeck, 1993). The environmental stimuli are detected by sensors (e.g. PhoR) and a message (e.g. protein phosphorylation; Stock *et al.*, 1989) transferred to a response regulator (e.g. PhoB) (Gross *et al.*, 1989; Liras *et al.*, 1990; Wanner, 1993). This response regulator regulates the expression of the biosynthetic genes, either directly by enhancing or repressing their expression, or indirectly by regulating the expression of another transcription regulator, which in turn regulates the expression of the biosynthetic genes (Gross *et al.*, 1989; Liras *et al.*, 1990; Wanner, 1993). More than one environmental stimulus may be involved in the regulation of biosynthetic genes and the different environmental stimuli may act individually or in unison. For example, phosphate limitation may not be sufficient to stimulate expression of the biosynthetic genes as another transcription regulator, such as an alternative sigma factor (e.g. RpoS; Loewen & Hengge-Aronsis, 1994), may be required. If the appropriate stimulus (e.g. entry into stationary phase) subsequently induces the production of the other regulator, the expression of the biosynthetic genes will in turn be induced.

The loss of expression of any of the biosynthetic genes involved in PSP production, for example by the insertion of a transposon, will have two affects. Firstly, the production of SCB toxins by the organism will be stopped unless an alternative gene product can substitute for the lost gene. Secondly, if the gene product of the disrupted gene is responsible for catalysing a reaction late in the biosynthetic pathway, biosynthetic intermediates will accumulate in the cell. These intermediates may be toxic derivatives of the SCB toxin normally produced by the organism.

The loss of expression of a gene involved in the regulation of SCB toxin production will either cause an increased production of SCB toxins or inhibit SCB toxin production, depending on whether the regulatory gene usually represses or stimulates the expression of the biosynthetic genes.

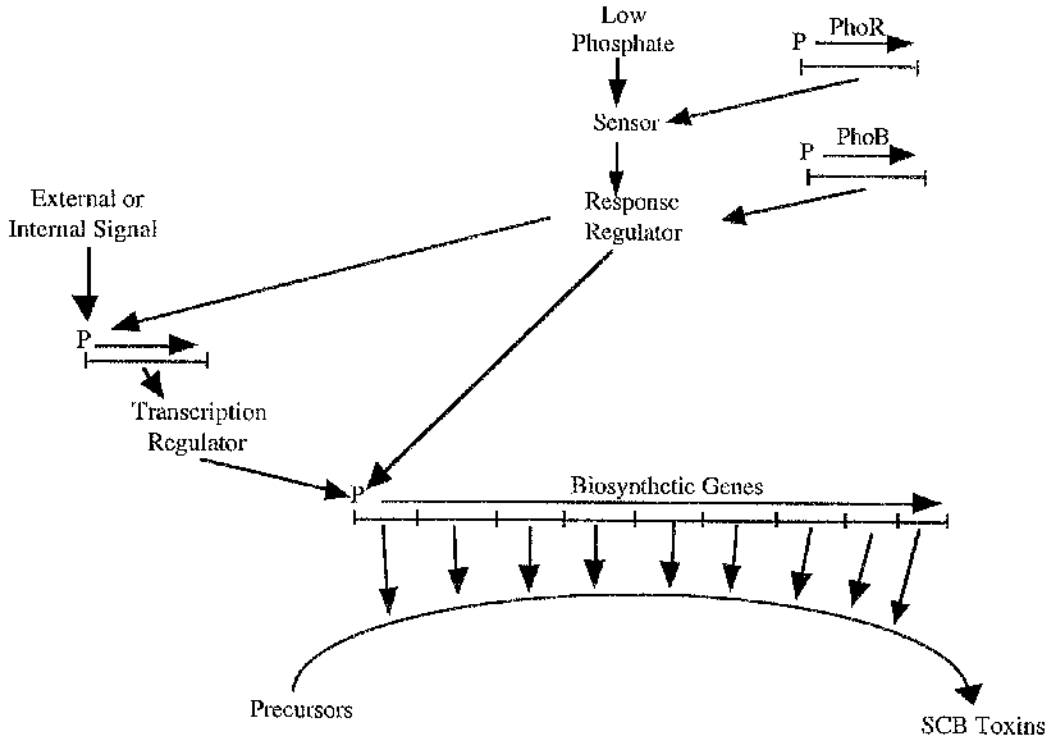


Figure 5. Proposed model of SCB toxin production by marine bacteria.

OBJECT OF RESEARCH

Historically the production of TTX has been attributed to Puffer fish (Mosher *et al*, 1964; Fuhram, 1986) and the production of PSP toxins attributed to dinoflagellates and fresh water cyanobacteria species (Gallacher *et al.*, 1996), especially at times of monospecific blooms. More recently, however, a broad spectrum of bacterial species have been reported to produce TTX in culture (Table 2). Similarly, heterotrophic bacteria isolated from dinoflagellate cultures have been reported to produce PSP in culture (Doucette & Trick, 1995; Gallacher *et al.*, 1997; Kodama *et al.*, 1988). In another study, bacteria isolated from sea water over a 12 month period were screened for their ability to produce SCB toxins using the mouse neuroblastoma assay. One third of the isolates were reported to be capable of producing SCB toxins in culture (Gallacher & Birkbeck, 1995). However, although it is established that various strains of heterotrophic bacteria are capable of producing SCB toxins in culture, almost nothing is known about how these toxins are synthesised by bacteria.

It was therefore the aim of this work to apply transposon mutagenesis and other molecular techniques to identify the genes involved in the production of SCB toxins by heterotrophic marine bacteria so that molecular probes may be constructed to monitor SCB toxin producing bacteria in the environment and to deduce the biosynthetic pathway operating in cyanobacteria and dinoflagellates.

MATERIALS AND METHODS

CHEMICALS AND MEDIA

All chemicals were obtained from Sigma (Sigma-Aldrich Company Ltd., Dorset, U.K.), BDH Laboratory Supplies (Poole, U.K.), or Fisher Scientific (Fisher Scientific UK Ltd., Loughborough, U.K.) unless otherwise stated.

Bacterial culture media were obtained from Difco Laboratory Inc.(Detroit, U.S.A.) or Oxoid (Unipath Ltd., Hampshire, U.K.). The recipes for the culture media used are detailed in Appendix 1.

Nucleic acid modifying enzymes (i.e. restriction enzymes and DNA ligase) were supplied by Life Technologies (Gibco BRL, Paisley, U.K.) with the exception of *Not*I, *Hind*III and *Bam*HI which were supplied by Promega U.K. Ltd. (Southampton, U.K.). All the modifying enzymes were used with the reaction buffer supplied with the enzyme at the temperature recommended by the manufacturer.

The dNTP's for PCR reactions were supplied by Boehringer Mannheim (Boehringer Mannheim UK (Diagnostics & Biochemicals) Ltd., East Sussex, U.K.). DNA oligonucleotides were synthesised by Oswel DNA Service (Southampton, U.K.).

MARINE BACTERIA USED IN THIS STUDY

Ten marine bacterial isolates were considered for transposon (Tn) mutagenesis experiments. The sources of these isolates are summarised in Table 4. Seven of these bacterial strains were isolated from sea water off the Scottish coast and culture supernatants of these strains had previously been shown to possess SCB activity by the mouse neuroblastoma tissue culture assay (Gallacher & Birkbeck, 1995). Strain A862 has been shown by HPLC & ELISA to produce PSP toxins (Gallacher *et al.*, 1996). Strain 4αVS3 was isolated from a dinoflagellate culture (*Alexandrium* sp.). Culture supernatant from this bacterium has tested positive in the mouse neuroblastoma SCB assay (Gallacher *et al.*, 1997). *Alteromonas tetraodonas* GFC was isolated from the skin of a puffer fish, *Fugu poecilonotus*. A culture of this bacterium was shown to contain TTX by HPLC and GC-MS (Yotsu *et al.*, 1987). Strain OK-1 was isolated from the macro-alga *Jania* sp., and demonstrated to produce TTX in culture by HPLC and mass spectrometry analysis (Yasumoto *et al.*, 1986). This isolate has been further characterised and identified as a new species, *Shewanella alga* (Simidu *et al.*, 1990). More recent results have indicated that this isolate also produces PSP toxins in culture (Gallacher *et al.*, in press).

Strain	Species	Source of Isolate	Toxin Group Produced	Reference
OK-1	*Shewanella alga*	Algae (*Jania* sp.)	TTX & PSP	Yasumoto *et al.*, 1986 Gallacher *et al.*, in press
GFC	*Alteromonas tetraodonas*	Skin of puffer fish	TTX	Yotsu *et al.*, 1987
4αVS3	ND	Dinoflagellate	PSP	Gallacher *et al.*, 1996 & Gallacher *et al.*, 1997
A400	ND	Sea water	SCB	Gallacher & Birkbeck, 1995
A862	ND	Sea water	PSP	Gallacher & Birkbeck, 1995 & Gallacher *et al.*, 1996
A903	ND	Sea water	SCB	Gallacher & Birkbeck, 1995
A912	ND	Sea water	SCB	Gallacher & Birkbeck, 1995
A940	ND	Sea water	SCB	Gallacher & Birkbeck, 1995
A1087	ND	Sea water	SCB	Gallacher & Birkbeck, 1995
A1095	ND	Sea water	SCB	Gallacher & Birkbeck, 1995

ND = Not determined SCB = Sodium channel blocking toxins

TTX = Tetrodotoxin PSP = Paralytic shellfish poisons

Table 4. Source of marine bacterial isolates used in this study.

Growth and storage of marine bacteria

Unless otherwise stated all marine bacteria were grown in marine broth or marine agar (Appendix 1) at 20°C for 48 h, the broth cultures being shaken at 175 rpm in a controlled environment incubator shaker (New Brunswick Scientific Co. Ltd, Edison, N.J., U.S.A.).

For short term use the marine isolates were stored on marine agar plates at 4°C but stock cultures were stored in Protect vials (Technical Service Consultants Ltd., Lancashire, U.K.). Prior to inoculation 100µl of sterile 20% NaCl was added to each vial. Following inoculation and mixing the medium was removed from the beads which were then stored at -70°C.

Phenotypic characterisation of marine bacteria

Motility

Marine bacteria were cultured in 2 ml marine broth for 18 h at 20°C, a wet film of each culture was prepared on a microscope slide and motility was observed under a light microscope (×400 magnification).

Oxidase

Whatman filter paper was soaked with 1% tetramethyl-*p*-phenylenediamine. A small portion of growth of the marine bacteria was spotted onto the moist filter paper with a wooden cocktail stick. A blue/purple colour indicated a positive reaction.

Sensitivity to O/129 diagnostic disks for Vibrio sp. identification

Marine bacteria were cultured in 2 ml marine broth for 18 h at 20°C. The cultures were diluted $^1/_{10}$ with ASW (Appendix 1) and 100 µl plated onto marine agar plates. Disks containing 10 µg and 150 µg of O/129 (Oxoid DD10 and DD15) were added and the plates incubated at 20°C for 24 h or until a lawn of growth was observed.

API 20 E identification strips

A large colony of each marine strain was resuspended in 5 ml ASW. API 20 E identification strips (Bio Mérieux SA, France) were inoculated with these bacterial suspensions and incubated at 20°C for 48 h. Only the results for the ONPG, H_2S and gelatinase test wells are presented in Table 8.

Antibiotic sensitivity of marine bacteria used in this study

The marine isolates considered for transposon mutagenesis experiments were used to inoculate 10 ml of marine broth and these cultures were incubated at 20°C for 18 h with shaking at 175 rpm.

To determine the colony forming units (CFU) per ml of culture, the culture was serially diluted in a simple artificial sea water (ASW; Appendix 1) and aliquots of these dilutions were plated onto marine agar plates. Colonies were counted after incubation at 20°C for 48 h.

To determine the spontaneous mutation rate of the marine strains to resistance to particular antibiotics, 100 µl volumes of the undiluted broth cultures were plated onto marine agar containing either chloramphenicol (Cm; 50 µg/ml), kanamycin (Km; 100 µg/ml), streptomycin (Sm; 100 µg/ml) or ampicillin (Amp; 150 µg/ml). The same amount of broth culture (i.e. 100µl) was also plated onto LB20 (Appendix 1) containing tetracycline (15 µg/ml). The plates were incubated at 20°C and the

numbers of colonies present after one week were determined. The spontaneous mutation rate is expressed as the number of resistant colonies on antibiotic containing media per CFU of the original culture used to inoculate the antibiotic plate. Each CFU determination and spontaneous mutation count was set up in triplicate.

TRANSPOSON DELIVERY VECTORS AND STRAINS

The *E. coli* strains and transposon delivery vectors are summarised in Tables 5 & 6, respectively. Diagrams of the structure of pUT::miniTn5Km*lacZ*I, pUJ8 and pUT::miniTn5Cm are shown in Figures 6 & 7. *E. coli* J100, which contains pJO100, was kindly supplied by Staffan Kjelleberg and Jörgen Östling. The *E. coli* strains CC118*λpir*, SM10*λpir* and S17-1*λpir*, the pUT family of plasmids and pUJ8 were kindly supplied by Victor De Lorenzo.

Growth and storage of *E. coli* strains

Unless otherwise stated, all *E. coli* strains were grown in LB broth (Appendix 1) at 37°C for 18 h with shaking at 175 rpm. For solid culture 1·5% agar was added prior to autoclaving.

While in use, the strains were stored on LB plates at 4°C. For longer term storage the strains were stored in Protect vials (Technical Service Consultants Ltd., Lancashire, U.K.). The vials were used as supplied and following inoculation and mixing the medium was removed from the beads which were then stored at -70°C.

The pUT family of plasmids were stored in *E. coli* CC118*λpir* until required for transposon mutagenesis experiments.

CONSTRUCTION OF pUT::miniTn5Cm*lacZ*

Purification of plasmid DNA

Initial attempts to purify pUJ8 and pUT::miniTn5Cm DNA using the alkaline lysis mini-plasmid preparation method (Birnboim & Doly, 1979) were unsuccessful as the resulting DNA was invariably degraded. This problem was overcome by using the Wizard® Miniprep DNA Purification System (Promega U.K. Ltd., Southampton, U.K.). The purification of plasmid DNA was carried out as described by the manufacturer and as summarised below. The composition of the buffers used is given in Appendix 2.

Strain	Genotype
CC118*λpir*	Δ(*ara-leu*) *araD* Δ*lacX*74 *galE galK phoA*20 *thi*-1 *rpsE rpoB argE*(Am) *recA*1 *λpir* phage lysogen
SM10*λpir*	Km^r *thi*-1 *thr leu tonA lacY supE recA*::RP4-2-Tc::Mu *λpir*
S17-1*λpir*	Tp^r Sm^r *recA thi pro hsdR*$^-$*M*$^+$ RP4-2-Tc::Mu::Km Tn7 *λpir*
TG1	*supE hsd*Δ(*lac-proAB*) F'[*traD*36 *proAB*$^+$ *lacZ*ΔM15]
TOP10F'	F'{*lacI*^{q}, Tn10(Tet^r)}*mcrA*Δ(*mrr-hsdRMS-mcrBC*) o80 *lacZ*ΔM15 Δ*lacX*74 *deoR recA*1 *araD*139 Δ(*ara-leu*)7679 *galU galK rpsL*(Str^r) *endA*1 *nupG*
J100	C600 [pJO100]
C600	*supE*44 *hsdR thi*-1 *thr*-1 *leuB*6 *lacY*1 *tonA*21

Table 5. Genotype of *E. coli* strains used for plasmid manipulation and storage.

Transposon Vector	Antibiotic Resistance		Reporter Gene	Reference
	Transposon	Vector		
*pJO100	Tc^r	Km^r Tc^r	*lacZ*	Östling *et al.*, 1991
pUT::miniTn5Km*lacZ*1	Km^r	Amp^r	*lacZ*	De Lorenzo *et al.*, 1990
pUT::miniTn5Sm	Sm^r	Amp^r	none	De Lorenzo *et al.*, 1990
pUT::miniTn5Cm	Cm^r	Amp^r	none	De Lorenzo *et al.*, 1990
pUT::miniTn5Tc	Tc^r	Amp^r	none	De Lorenzo *et al.*, 1990
pUT::miniTn5Cm*lacZ*	Cm^r	Amp^r	*lacZ*	this study

*pJO100 = pRK2013::miniMu(Tet^r)
Tc^r = tetracycline resistance
Km^r = kanamycin resistance
Sm^r = streptomycin resistance
Cm^r = chloramphenicol resistance
Amp^r = ampicillin resistance

Table 6. Summary of transposon vectors used in this study.

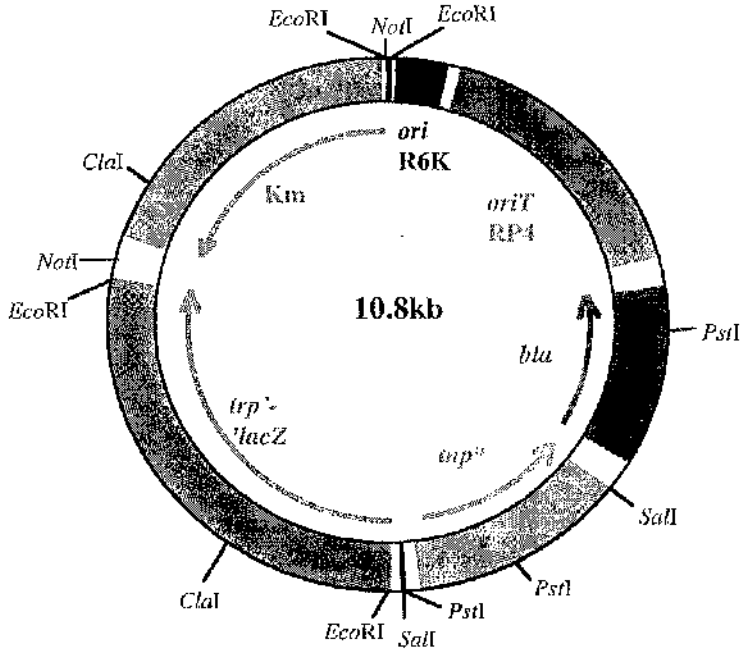


Figure 6. Suicide delivery vector pUT::miniTn5Km*lacZ*I. The transposon region of this vector carries kanamycin resistance (Km) and a promoterless *lacZ* gene (*'trp-'lacZ*). The transposase gene (*tnp**) is located outwith the transposon and is lost following transposition events therefore preventing subsequent transposition taking place. The origin of transfer from RP4 (*oriT*) allows conjugation into a large range of bacterial species. However the origin of replication from R6K allows replication of this plasmid in only the narrow range of bacteria which express the π replication protein.

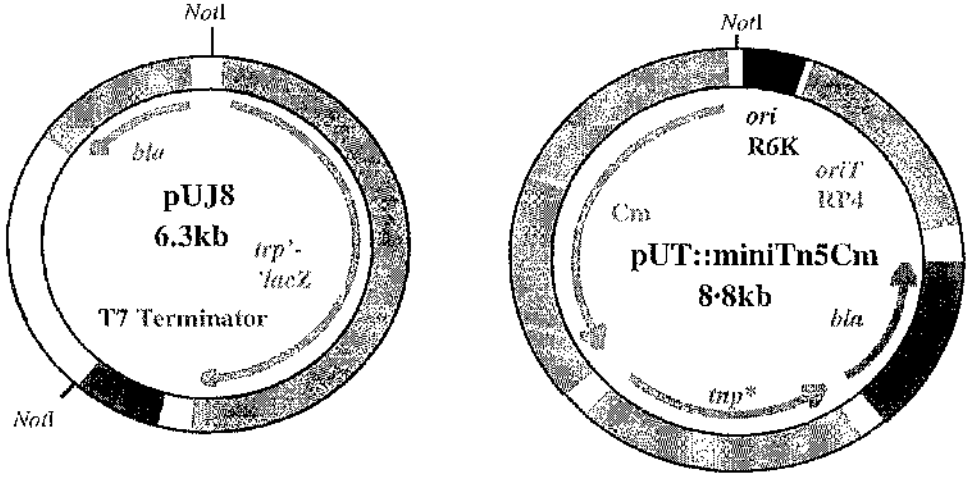


Figure 7. Suicide delivery vector pUT::miniTn5Cm & auxiliary plasmid pUJ8. The suicide vector pUT::miniTn5Cm and auxiliary plasmid pUJ8 were used in the construction of pUT::miniTn5Cm*lacZ*. (See Figure 8)

The cells of an overnight culture (i.e. 3 ml of a 10 ml culture) of *E. coli* CC118*λpir* containing pUJ8 or pUT::miniTn5Cm*lacZ* were recovered by centrifugation (12,000 g for 1 min). The cells were resuspended in 200 µl resuspension buffer and lysed by the addition of 200 µl of cell lysis solution. Once lysis was complete 200 µl of Wizard® neutralisation solution was added to precipitate proteins and genomic DNA. The precipitate was removed by centrifugation at 12,000 g for 10 min. The supernatant was mixed with 1 ml of Wizard® Minipreps DNA Purification Resin and the resulting mix applied to a Wizard® Minicolumn using a 5 ml syringe. The column was washed with 2 ml of Wizard® column wash solution and centrifuged for 2 min to remove excess solution. The plasmid DNA was eluted from the column with 50 µl of sterile distilled water.

Restriction enzyme digestion of pUJ8 and pUT::miniTn5Cm DNA

The plasmid DNA purified by the above procedure were digested with 1 unit of *Not*I (10 units/µl; Promega U.K. Ltd.) and $^1/_9$ volume of 10X React Buffer D (Promega U.K. Ltd.) at 37°C for 4 h.

Gel electrophoresis of DNA

An agarose gel was prepared by adding the appropriate amount of electrophoresis grade agarose (Gibco BRL, Life Technologies, Paisley, U.K.) to 1X TBE (Appendix 2) to give the appropriate concentration of gel (usually 0·7%). The agarose was dissolved in a boiling water bath and, once cooled to 60°C, ethidium bromide was added to a final concentration of 0·5 µg/ml before pouring into the gel cast of a GNA-100 Submarine Electrophoresis System (Pharmacia Biotech, Herts, U.K.).

DNA samples were prepared for electrophoresis by adding $^1/_5$ volume of gel loading buffer (Appendix 2). The samples were then loaded onto an agarose gel and a potential difference of 80 volts applied to the gel in 0·5% TBE buffer, until the first dye front had run an appropriate distance. A 1 kb DNA ladder (Gibco BRL, Life Technologies, Paisley, U.K.) was run alongside the samples to allow the size of DNA fragments to be estimated. To allow the estimation of DNA concentrations, a known amount of a *Hind*III digest of λ DNA (600 ng) was run alongside DNA samples.

Gel purification of DNA fragments

Three bands were observed when the *Not*I digested pUJ8 DNA was separated on a 0·7% agarose gel. The band at 6·3 kb represented pUJ8 which has been cut at only

one of the two *Not*I sites but the other two bands, at 4·1 kb and 2·2 kb, were the products of complete *Not*I digestion. A single band at 8·8 kb was present in the *Not*I-digested pUT::miniTn5Cm sample. This represents linear plasmid DNA digested at the single *Not*I site.

The 4·1 kb fragment from *Not*I digested pUJ8 and the 8·8 kb fragment from *Not*I digested pUT::miniTn5Cm*lacZ* were excised from the gel and the DNA recovered from the agarose using a Sephaglas™ Bandprep Kit (Pharmacia Biotech, Herts, U.K.) as recommended by the manufacturers and described below. The solutions for this procedure are supplied with the kit and their exact composition is not disclosed.

Each agarose gel slice containing the DNA fragments of interest was added to 250 µl gel solubiliser and incubated at 60°C until the agarose had melted. The Sephaglas BP was mixed and 5 µl of this suspension was added to the melted agarose. After incubating at room temperature to allow the DNA to bind, the Sephaglas™ was pelleted by centrifugation (1 min, 12,000 g). The Sephaglas™ was subsequently washed three times with Wash Buffer and, following the final wash, the pellet was allowed to air dry. The DNA fragments were eluted from the Sephaglas™ by the addition of 10 µl Elution Buffer and centrifugation for 1 min at 12,000g.

Ligation of *lacZ* fragment to digested pUT::miniTn5Cm

When the concentration of the gel purified fragments was estimated by agarose gel electrophoresis as described above (see "Gel electrophoresis of DNA") the concentration of the 4·1 kb pUJ8 fragment and the 8·8 kb pUT::miniTn5Cm fragment were found to be comparable.

The 4·1 kb pUJ8 fragment and the 8·8 kb pUT::miniTn5Cm fragment were mixed together in a ratio of 2:1 (i.e. 10µl of the 4·1 kb fragment + 5 µl of the 8·8 kb fragment) and heated to 45°C for 5 min. The mixture of fragments was then chilled on ice. T4 DNA Ligase (1µl of 1 unit/µl stock) and 5X T4 DNA Ligase buffer (2µl) were added and ligation was allowed to proceed at 16°C for 4 h.

Preparation of competent cells for heat shock transformation

The cells of a 50 ml exponential phase culture of *E. coli* CC118*λpir* were collected by centrifugation at 4,000 rpm (3,023 g) for 15 min at 4°C in a Heraeus Sepatech Megafuge 1·0R. The cells were resuspended in 25 ml of ice cold 100 mM $CaCl_2$ and incubated on ice for 30 min, before centrifugation as before and resuspension of the

pellet in 5 ml 100 mM $CaCl_2$. These competent cells were incubated on ice for at least one hour prior to use or were stored at -70°C in 25% (v/v) glycerol.

Transformation of CC118*λpir* with pUT::miniTn5Cm*lacZ*

Various amounts of the pUJ8 + pUTminiTn5Cm ligation mix were added to 100 µl of competent CC118*λpir* cells and these transformation reactions were incubated on ice for 1 h. The cells were then heat shocked for 90 sec at 42°C and chilled on ice. LB broth + 10 mM $MgCl_2$ (500 µl) was added to each transformation reaction and the resulting solutions incubated at 37°C for 1 h. Aliquots of each reaction were plated onto LB plates containing 150 µg/ml ampicillin and 50 µg/ml chloramphenicol.

Confirmation of insertion of the 4·1 kb fragment in pUT::miniTn5Cm

The DNA from selected Amp^r Cm^r transformants was isolated by Wizard® Miniprep DNA Purification as described earlier (see "Purification of plasmid DNA"). These plasmids were digested with a number of restriction enzymes with the appropriate reaction buffer (i.e. *Not*I with React D, *Eco*RI with React 3, *Cla*I with React 1 & *Xba*I with React 2) in single digests or with two different enzymes, at 37°C. The results confirmed the structure of the new construct, pUT::miniTn5Cm*lacZ*, to be that displayed in Figure 8.

PRE-COLUMN OXIDATION HPLC ANALYSIS OF PSP TOXINS

Pre-column HPLC was performed by an adaptation of the method of Lawrence and Menard (1991) and Lawrence *et al.* (1991; 1995; 1996).

Preparation of A862 culture supernatants and cell extracts analysed by pre-column oxidation HPLC analysis

A862 wt and A862 Sm^r were cultured in either 10 ml or 500 ml marine broth for 48 h at 20°C with shaking.

The cells from the A862 cultures were harvested by centrifugation at 3,500 g for 30 min at 4°C in a Sorval RC-5 centrifuge with a GS-3 rotor or a Heraeus Sepatech Megafuge 1·0R. The culture supernatants were decanted and retained for analysis. The cell pellets from 500 ml cultures were resuspended in 5 ml of 0·05 M acetic acid and freeze thawed with 3 cycles of -20°C and room temperature. Insoluble material present in the cell extracts was removed by centrifugation at 3,000g for 15

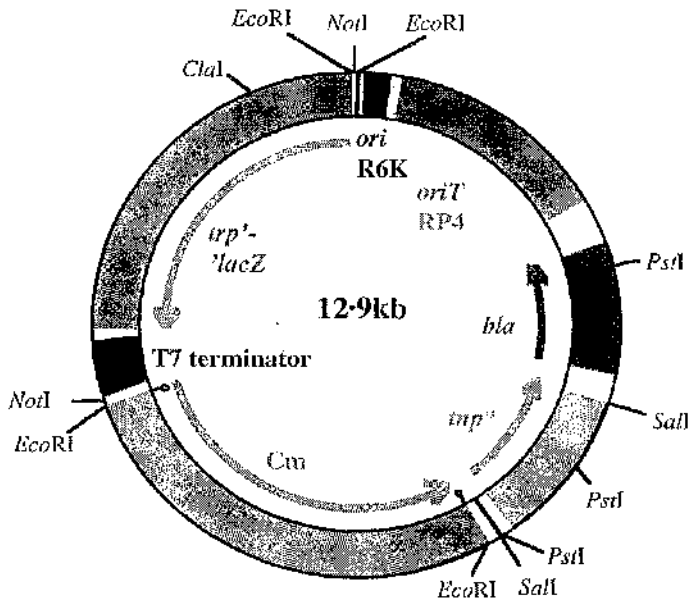


Figure 8. Suicide delivery vector pUT::miniTn5Cm*lacZ*. The plasmid pUT::miniTn5CmlacZ was constructed from the delivery vector pUT::miniTn5Cm and pUJ8 (see Figure 7). The *Not*I fragment of pUJ8 was excised and ligated into the unique *Not*I site of pUT::miniTn5Cm. The transposon carries chloramphenicol resistance (Cm) and a promoterless *lacZ* gene (*'trp-'lacZ*). The transposase gene (*tnp**) is located outwith the transposon and is lost following transposition events, therefore preventing subsequent transposition taking place. The origin of transfer from RP4 (*oriT*) allows conjugation into a large range of bacterial species. However the origin of replication from R6K allows replication of this plasmid in only the narrow range of bacteria which express the π replication protein.

min at 4°C. The culture supernatants and cell extracts were stored at -20°C until required.

Spiking of A862 culture supernatant with C1 or dcSTX toxin

The culture supernatant from a 10 ml marine broth culture (48 h at 20°C) of A862 wt was spiked $^1/_{10}$ with the C1 or dcSTX toxin standards described below (see "PSP toxin standards for pre-column oxidation HPLC analysis").

Acid hydrolysis of A862 culture supernatants

To convert any C toxins present in A862 culture supernatant to their GTX derivatives, acid hydrolysis was performed by the addition of an equal volume of 0·4 M HCl to the samples and incubation in a boiling water bath for 15 min.

PSP toxin standards for pre-column oxidation HPLC analysis

STX, NeoSTX, $GTX_{2/3}$ and $GTX_{1/4}$ toxin standards were supplied by The National Research Council of Canada. These standards were diluted, either individually or in combination, in 0·05 M acetic acid prior to use. The concentration of the undiluted standards were 0·14 mg/ml STX, 0·14 mg/ml neoSTX, 0·12 mg/ml GTX2 + 0·029 mg/ml GTX3 and 0·074 mg/ml GTX1 + 0·032 mg/ml GTX4.

C1 toxin standard was obtained from Molecular Probes Europe BV (Leiden, The Netherlands) and supplied at a concentration of 1 mg/ml.

To obtain a dcSTX standard, 0·014 mg of STX in 7·5 M HCl was incubated in a boiling water bath for 5·5 h (this method was adapted from Laycock *et al.*, 1994).

Pre-column oxidation reactions

Pre-column oxidation was performed with either peroxide or periodate under either alkaline or acidic conditions.

Pre-column peroxide alkaline oxidation reactions were performed by adding 25 µl of 10% (v/v) H_2O_2 and 250 µl of 1 M NaOH to 100 µl of sample. The alkaline oxidation reactions were allowed to proceed at room temperature before acidification with 20 µl of glacial acetic acid.

Periodate oxidant, prepared daily, consisted of 0·03 M periodic acid, 0·3 M Na_2HPO_4 and 0·3 M ammonium formate. Pre-column periodate alkaline oxidation reactions were performed by adding 500 µl of periodate oxidant and 20 µl of NaOH to 100 µl of sample. Oxidation was allowed to proceed at room temperature for 1 min

and 10 µl of glacial acetic acid was added. The acidified periodate oxidation reactions were held at room temperature for 10 min before HPLC analysis.

Acid oxidation reactions were performed as described for alkaline oxidation reactions but the glacial acetic acid was added to the oxidant prior to the addition of sample.

HPLC equipment and mobile phase solutions used for pre-column oxidation HPLC analysis

Pre-column oxidation HPLC analysis was performed using a 3 µm Hypersil ODS column (250 × 4·6 mm i.d.; Capital HPLC Ltd., Broxburn, U.K.) with a 3 µm Hypersil ODS guard column cartridge (HPLC Technology, Cheshire, U.K.). Samples were injected using a syringe loading sample injector (Rheodyne Incorporated model 7125) with a 20 µl sample loop. Two LKB 2150 HPLC pumps and a LKB gradient mixer (part number 2152-400) were used for mobile phase delivery. Peaks were detected using a LKB 2144 Fluorescence Detector with 330 nm excitation and 380 nm emission filters. Data were plotted using a LKB 2210 potentiometric recorder. The apparatus was controlled with a LKB 2152 HPLC Controller.

The mobile phase A was 0·1 M ammonium formate pH 6·0 and mobile phase B consisted of 96% (v/v) 0·1 M ammonium formate pH 6·0 and 4% (v/v) acetonitrile. The mobile phase was delivered at a flow rate of 1 ml/min with a gradient of 0 to 25% mobile phase B from 0 to 15 min, rising to 100% B at 22 min and returning to 0% B at 23 min. The total run time was 35 min.

POST-COLUMN OXIDATION HPLC ANALYSIS OF PSP TOXINS

Post-column HPLC analysis of A862 was performed during a visit to the laboratory of Dr. J.M. Franco in the Instituto Español de Oceanografía (IEO), Vigo, Spain. The method used was as described by Franco and Fernández-Vila (1993).

Preparation of A862 cell extracts and culture supernatants analysed by post-column oxidation HPLC analysis

Strains A862 wt and A862 Sm^r were cultured in 500 ml marine broth for 24 h at 20°C with shaking. The cells from the A862 wt and A862 Sm^r cultures were harvested by centrifugation at 4,000 g (6,000 rpm) for 30 min at 10°C in a Sorval RC-5 centrifuge with a GS-3 rotor. The culture supernatants were decanted and retained for analysis. The cell pellets were resuspended in 5 ml of 0·05 M acetic acid and freeze thawed

with 3 cycles of -20°C and room temperature. Insoluble material in the cell extracts was removed by centrifugation at 3,000 g for 15 min at 4°C. The culture supernatants and cell extracts were stored at -20°C until required.

Immediately prior to analysis by HPLC, the cell extracts were diluted $^1/_2$ with 0·05 M acetic acid and the pH of the culture supernatants was adjusted to between 3 and 4 by the addition of up to 2 drops of glacial acetic acid.

Acid hydrolysis of A862 cell extracts

To convert any C toxins present to their GTX derivatives, acid hydrolysis was performed by the addition of an equal volume of 0·4 M HCl to the samples and incubation in a boiling water bath for 15 min.

PSP toxin standards for post-column oxidation HPLC analysis

The STX, dcSTX and GTX toxin standards were obtained from the National Research Council of Canada. The GTX standard mix was prepared by adding 50 µl of $GTX_{2/3}$ and 250 µl of $GTX_{1/4}$ to 2·7 ml of 0·05 M acetic acid. The final concentrations of the GTX toxins were therefore 0·7275 ng/µl GTX1, 0·2526 ng/µl GTX2, 0·0610 ng/µl GTX3 and 0·3083 ng/µl GTX4.

The qualitative standards used for the third isocratic elution system were an extract of a mixture of *Gymnodinium catenatum* (strain gc19v) plus *Alexandrium minutum* (strain al1v) and an extract of *Alexandrium tamarense* (or *fundyense*) (strain CCMP117). These dinoflagellate cells were extracted with 0·1 M acetic acid following homogenisation with a Teflon pestle and glass homogeniser and treatment with ultrasound (Franco and Fernández-Vila, 1993).

HPLC equipment and solutions used for post-column oxidation HPLC analysis

Separation of PSP toxins was performed using 5 µm LiChrospher 100 RP18 (125 × 4 mm i.d.) with a 5 µm LiChrospher 100 RP18 (4 × 4 mm i.d.) guard column (Hewlett Packard). Samples were injected using a Waters® 717 Plus Autosampler, and a Waters® 510 HPLC pump was used for mobile phase delivery. Post-column reagents were delivered by two Eldex A-30-SW-2 pumps and the post column reaction was performed in a Teflon reaction coil (10 m × 0·5 mm i.d.) at 65°C. Peaks were detected with a Waters® 474 Scanning Fluorescence Detector. Control of the

HPLC apparatus and data capture was performed with a Millennium® Chromatography Manager (Waters®).

The first isocratic eluent was 94% (v/v) 1 mM sodium octansulfanate in 10 mM ammonium phosphate (pH 7·2) and 6 % (v/v) acetonitrile. The second isocratic eluent was 1·5 mM sodium octansulfanate in 10 mM ammonium phosphate (pH 7·0). The third isocratic eluent was 1 mM tetrabutyl ammonium sulphate in 10 mM ammonium phosphate (pH 6·5). The first isocratic eluent was delivered at 1·0 ml/min, and the second and third eluents were delivered at 0·8 ml/min.

Post-column oxidation was performed with 7 mM periodic acid in 50 mM sodium phosphate (pH 9·0). Acidification was performed with 0·5 M acetic acid, the oxidant and acid being delivered to the reaction coil at a flow rate of 0·4 ml/min.

EVALUATION OF A CHEMICAL FLUORESCENCE ASSAY FOR THE DETECTION OF PSP TOXINS

Fluorescence spectrophotometer specifications

A Hitachi F-2000 fluorescence spectrophotometer (Hitachi Scientific Instruments) was used for all fluorescence measurements during the evaluation and application of the chemical fluorescence assay for the detection of PSP toxins. This spectrophotometer was fitted with a 150 W xenon lamp.

Standard fluorescence measurements were performed using the Photometry function with the following instrument settings:-
Intensity data mode; Single wavelength measurement; Excitation wavelength 330 nm; Emission wavelength 380 nm; Initial delay 1 sec; Integration time 1 sec; Response time 0·5 sec; Excitation bandpass width 20 nm; Emission bandpass width 20 nm; Photomultiplier voltage 400 V.

Excitation and emission spectra were obtained using the Wavelength Scan function with the following instrument settings:-
Scan speed 240 nm/min; Response time 0·1 sec; Excitation bandpass width 20 nm; Emission bandpass width 20 nm; Photomultiplier voltage 400 V.

The maximum excitation and emission wavelengths of samples were determined using the pre-scan function on Auto mode. This function determines the optimum excitation and emission wavelength for fluorescence measurements while ignoring peaks of Raman spectrum and high-order scattered radiation.

Preliminary evaluation of the original fluorescence assay

Bates & Rapoport fluorescence assay for PSP toxins

A 2 ml aliquot of the test sample was oxidised by the addition of 2 ml NaOH and 600 µl of 1% H_2O_2. The oxidation reaction was allowed to proceed at room temperature for 40 min before the addition of 200 µl of glacial acetic acid. Simultaneously, a blank reaction was set up by the addition of 2 ml of sample to 2 ml NaOH and 600 µl of dH_2O. The blank reaction was also incubated for 40 min and acidified with 200µl of glacial acetic acid (Bates & Rapoport, 1975). The fluorescence of the oxidised and blank samples were measured in a fluorescence spectrophotometer (Hitachi F-2000) with an excitation wavelength of 330 nm and an emission wavelength of 380 nm. The difference between the fluorescence of the oxidised and blank reactions (i.e. the corrected fluorescence; Ox-B) is regarded as a measure of the PSP content of the sample.

Lawrence peroxide fluorescence assay for PSP toxins

The peroxide pre-column oxidation reaction described by Lawrence *et al.* (1991) was adapted for use in a fluorescence assay for PSP toxins. An 800 µl aliquot of the test sample was added to 2 ml of NaOH and 200 µl of 10% H_2O_2 and oxidation allowed to proceed for 2 min. The reaction was acidified by addition of 160 µl of glacial acetic acid. A blank reaction was set up, simultaneously with the oxidation reaction, by the substitution of dH_2O for the H_2O_2 in the reaction. The fluorescence of the oxidised and blank samples were measured in a fluorescence spectrophotometer (Hitachi F-2000) with an excitation wavelength of 330 nm and an emission wavelength of 380 nm. The difference between the fluorescence of the oxidised and blank reactions (i.e. the corrected fluorescence; Ox-B) is regarded as a measure of the PSP content of the sample.

Lawrence periodate fluorescence assay for PSP toxins

The periodate pre-column oxidation reaction described by Lawrence *et al.* (1991) was adapted for use in a fluorescence assay for PSP toxins. Periodate oxidant, prepared daily, consisted of 0·03 M periodic acid, 0·3 M Na_2HPO_4 and 0·3 M ammonium formate and the pH was adjusted to 8·2 by the addition of NaOH. Samples were assayed for PSP with this reaction by the addition of 4 ml of periodate oxidant to 800 µl of the sample. Oxidation was allowed to proceed at room temperature for 1 min and 80 µl of glacial acetic acid was added. A blank reaction was set up, simultaneously with the oxidation reaction, by the substitution of dH_2O for the periodate oxidant in the reaction. Following acidification the oxidised and blank samples were incubated at room temperature for 10 min and the fluorescence

measured in a fluorescence spectrophotometer (Hitachi F-2000) with an excitation wavelength of 330 nm and an emission wavelength of 380 nm. The difference between the fluorescence of the oxidised and blank reactions (i.e. the corrected fluorescence; Ox-B) is regarded as a measure of the PSP content of the sample.

Flynn periodate fluorescence assay for PSP toxins

The periodate pre-column oxidation reaction described by Flynn & Flynn (1996) was adapted for use in a fluorescence assay for PSP toxins. Periodate oxidant, which was prepared daily, consisted of 0·03 M periodic acid and 0·1 M sodium borate. Samples were assayed for PSP with this reaction by the addition of 2·67 ml of periodate oxidant to 800 µl of the sample. Oxidation was allowed to proceed at room temperature for 10 min and 267 µl of 25% (v/v) acetic acid was added. A blank reaction was set up, simultaneously with the oxidation reaction, by the substitution of 0·05 M sodium borate for the periodate oxidant in the reaction. The fluorescence of the oxidised and blank samples was measured in a fluorescence spectrophotometer (Hitachi F-2000) with an excitation wavelength of 330 nm and an emission wavelength of 380 nm. The difference between the fluorescence of the oxidised and blank reactions (i.e. the corrected fluorescence; Ox-B) is regarded as a measure of the PSP content of the sample.

STX standard curve

A stock solution of 100 µM STX was diluted in acetic acid to give a range of dilutions from 0 to 2·5 µM in 0·05 M acetic acid. The different concentrations of STX were then assayed by the Bates & Rapoport fluorescence assay as described above (see "Bates & Rapoport fluorescence assay for PSP toxins").

Fluorescence of STX following different oxidation reactions

STX was diluted in 0·05 M acetic acid to a concentration of 1 µM and aliquots were assayed by the Lawrence peroxide, Lawrence periodate and Flynn periodate reactions detailed above. The fluorescence of the oxidised and blank samples was measured in a fluorescence spectrophotometer (Hitachi F-2000) with an excitation wavelength of 330 nm and an emission wavelength of 380 nm.

Examination of bacterial cultures with the fluorescence assays

Strain A862 wt, A862 Sm^r, A912 wt and 4αVS3 wt were cultured in 100 ml marine broth at 20°C for 48 h. The cultures and a marine broth control were centrifuged at 10,000 rpm (9,000 g) for 20 min in a Sorval RC-5 centrifuge with a SS-34 rotor. The culture supernatants were decanted and retained for analysis. The cell pellets were resuspended in 10 ml of 0·05 M acetic acid and sonicated with 3 × 20 sec pulses

(Vibra Cell Ultrasonic Processor VCX400 with 3 mm stepped microtip, Jencons Scientific Ltd.). The cell extracts were centrifuged at 3,000g for 15 min at 4°C and the supernatant retained for analysis. The culture supernatants and cell extracts were assayed for PSP toxins by the Bates & Rapoport fluorescence assay described above.

Evaluation of the adapted fluorescence assay

Adapted Bates & Rapoport fluorescence assay for PSP toxins

An 800 µl aliquot of the test sample was oxidised under alkaline conditions by the addition of 1·2 ml dH_2O, 2 ml NaOH and 600 µl of 1% H_2O_2. Simultaneously, a blank reaction was set up by the addition of 800 µl of sample to 2 ml NaOH and 1·8 ml of dH_2O. The alkaline oxidation and blank reactions were allowed to proceed at room temperature for 40 min before the addition of 200 µl of glacial acetic acid. An acid oxidation reaction was also performed by adding 800 µl of sample to 1·2 ml dH_2O, 200 µl glacial acetic acid, 2 ml NaOH and 600 µl of 1% H_2O_2. The fluorescence of the reactions was measured in a fluorescence spectrophotometer (Hitachi F-2000) with an excitation wavelength of 330 nm and an emission wavelength of 380 nm. The difference between the fluorescence of the alkaline oxidation and acid oxidation reactions (i.e. the corrected fluorescence; Ox-AOx) is regarded as a measure of the PSP content of the sample. Latterly, the blank reaction was omitted from the assay as it was superfluous.

Adapted Lawrence peroxide fluorescence assay for PSP toxins

An alkaline oxidation reaction was performed by adding 800 µl of the test sample to 2 ml of NaOH and 200 µl of 10% H_2O_2, and oxidation allowed to proceed for 2 min. A blank reaction was set up simultaneously with the oxidation reaction, by the substitution of dH_2O for the H_2O_2 in the reaction. The alkaline oxidation and blank reactions were acidified by the addition of 160 µl of glacial acetic acid. An acid oxidation reaction was also performed by adding 800 µl of sample to 160 µl glacial acetic acid, 2 ml of NaOH and 200 µl of 10% H_2O_2 .The fluorescence of the oxidised and blank samples was measured in a fluorescence spectrophotometer (Hitachi F-2000) with an excitation wavelength of 330 nm and an emission wavelength of 380 nm. The difference between the fluorescence of the alkaline oxidation and acid oxidation reactions (i.e. the corrected fluorescence; Ox-AOx) is regarded as a measure of the PSP content of the sample. Latterly, the blank reaction was omitted from the assay as it was superfluous.

Examination of STX, and A862 culture supernatant with the adapted fluorescence assays

A862 wt culture supernatant was prepared as described earlier (see "Examination of bacterial cultures with the fluorescence assays") and spiked with STX to give a final concentration of 1 μM STX. This spiked supernatant, together with unspiked A862 supernatant and 1 μM STX in 0·05 M acetic acid controls, were assayed by the adapted fluorescence assays described above.

Examination of bacterial culture supernatants with the adapted fluorescence assays

Strains A862 wt, A912 wt, OK-1 wt and 4αVS3 wt were cultured in 10 ml marine broth for 48 h at 20°C. The cultures were centrifuged at 3,000g for 15 min at 4°C and the culture supernatants retained for analysis. An aliquot of each culture supernatant was spiked with STX to give a final concentration of 1 μM STX. The spiked and unspiked supernatants were assayed by the adapted Lawrence peroxide fluorescence assay as described above.

The effect of marine broth on the adapted fluorescence assay

Standard curves of STX diluted in acetic acid and marine broth

A stock solution of 100 μM STX was diluted in 0·05 M acetic acid or marine broth to give a range of dilutions from 0 to 2·5 μM. The different concentrations of STX were then assayed by the adapted Lawrence peroxide fluorescence assay as described above.

The effect of peptone on the adapted fluorescence assay

STX was diluted in 0·5% peptone (Difco Bacto-peptone 0118), marine broth (Difco 0791), dH_2O or 0·05 M acetic acid to give a final concentration of 1 μM STX. The STX in the different diluents was assayed by the adapted Lawrence peroxide fluorescence assay as described above.

Fluorescence of marine broth following different oxidation reactions

An aliquot of marine broth was oxidised under alkaline and acid conditions as described for the adapted Lawrence peroxide fluorescence assay. Samples were also added to two different blank reactions; an alkaline blank reaction was set up as described for the adapted Lawrence peroxide fluorescence assay and an acid blank reaction was performed by substituting dH_2O for the H_2O_2 in the acid oxidation reaction described for the adapted Lawrence peroxide fluorescence assay. The fluorescence of each of the reactions was measured in a fluorescence spectro-

photometer (Hitachi F-2000) with an excitation wavelength of 330 nm and an emission wavelength of 380 nm.

Fluorescence of strain A862 culture supernatants and cell extracts

Fluorescence of A862 wt and A862 Sm^r following peroxide oxidation

A862 wt and A862 Sm^r were cultured, in triplicate, in 10 ml marine broth for 48 h at 20°C. The cells were pelleted by centrifugation at 3,000g for 30 min at 4°C and the culture supernatants were retained for analysis. Together with three aliquots of marine broth, the culture supernatants were assayed by the adapted Lawrence peroxide fluorescence assay described above. The data obtained for each culture were compared by statistical analysis as described below (see "Statistical Analysis").

Fluorescence of strain A862 at different stages of growth

A862 Sm^r was cultured for 18 h at 20°C in 60 ml marine broth. The cells of this culture were harvested by centrifugation (3,000g for 30 min at 4°C) and resuspended in 15 ml of ASW (see Appendix 1). This cell suspension was used to inoculate 3 × 1 l of marine broth (5 ml of suspension per l). The three 1 l cultures were incubated at 20°C shaking at 175 rpm.

At different times post-inoculation a volume of culture was removed for analysis. The volume of cells removed was varied with growth phase in order to give approximately equal numbers of cells per sample. The total CFU in the samples, determined by diluting the samples in ASW and plating onto marine agar plates, ranged from $7{\cdot}88\times10^{10}$ in the 4 h sample to $5{\cdot}58\times10^{11}$ in the 16 h sample.

The samples of culture were centrifuged for 15 min at approximately 4,000 g in a Sorval RC-5 centrifuge with either a GS-3 or SS-34 rotor. The culture supernatants were retained for analysis. The cell pellets were resuspended in 10 ml of 0·05 M acetic acid and sonicated with 3 × 20 sec pulses (Vibra Cell Ultrasonic Processor VCX400 with 3 mm stepped microtip, Jencons Scientific Ltd.). The cell extracts were centrifuged at 3,000g for 15 min at 4°C and the supernatant retained for analysis.

The culture supernatants and cell extracts were assayed by the adapted Lawrence peroxide fluorescence assay described above.

Further analysis of the fluorescence of strain A862

Emission spectra of A862 wt

Strain A862 wt was cultured in 10 ml marine broth for 48 h at 20°C. The culture was centrifuged at 3,000g for 30 min at 4°C. The culture supernatant was oxidised under

acid and alkaline conditions as described for the adapted Lawrence peroxide assay. The emission spectra of these reactions were determined using the wavelength scan function of a Hitachi F-2000 fluorescence spectrophotometer.

Emission spectra of PSP toxin standards

STX (1 μM), $GTX_{2/3}$ (1 μM) and C1 (40 μM) toxin standards were oxidised by alkaline peroxide fluorescence reaction as described for the adapted Lawrence peroxide fluorescence assay. The emission spectra of these reactions were determined using the wavelength scan function of a Hitachi F-2000 fluorescence spectrophotometer.

Emission spectra of STX in marine broth

STX was diluted to 1 μM in 0·05 M acetic acid or marine broth. The STX in the two different diluents and a marine broth control were oxidised by alkaline peroxide fluorescence reaction as described for the adapted Lawrence peroxide fluorescence assay. The emission spectra of these reactions were determined using the wavelength scan function of a Hitachi F-2000 fluorescence spectrophotometer.

Collection of HPLC fractions

A862 Sm^r was cultured in 10 ml marine broth for 48 h at 20°C. The culture was centrifuged at 3,000g for 30 min at 4°C. The culture supernatant was analysed by pre-column oxidation HPLC analysis with peroxide oxidation (see "Pre-column oxidation HPLC analysis of PSP toxins"). A larger than usual volume of oxidised sample (100 μl) was injected onto the column using a syringe loading sample injector (Rheodyne Incorporated model 7125) with a 200 μl sample loop. During this HPLC analysis 1 ml fractions were collected using a fraction collector (LKB 2212 Helirac). In the same way a mixture of 4·8 μM STX + 3·73 μM $GTX_{2/3}$ were analysed by pre-column oxidation HPLC analysis with peroxide oxidation, 100 μl injection and collection of 1 ml fraction.

Excitation and emission spectra of HPLC fractions

The fluorescence intensity of each HPLC fraction was measured with an excitation wavelength of 330 nm and emission wavelength of 380 nm. The excitation and emission spectra of the HPLC fractions were determined using the wavelength scan function of a Hitachi F-2000 fluorescence spectrophotometer.

Excitation and emission wavelength maxima of PSP toxin standards

The maximum wavelengths of excitation and emission of alkaline peroxide oxidised PSP toxin standards were determined using the pre-scan function of a Hitachi F-2000 fluorescence spectrophotometer.

Statistical analysis

All statistical analysis of data was performed as described by Wardlaw (1985).

The t-test

The found t value was calculated using the equation

$$t = \frac{(\bar{y}_2 - \bar{y}_1)\sqrt{N}}{\sqrt{(s_1^2 + s_2^2)}}$$

where N = the number of observations in each group, $\bar{y}$ = the mean of the observed values, i.e.

$$\bar{y} = \frac{\sum y}{N}$$

and s = the standard deviation, i.e.

$$s = \sqrt{\frac{\sum y^2 - (\sum y)^2 / N}{(N-1)}}.$$

The found t statistic was then compared to the Student t-statistic (tabulated in Appendix A2 of Wardlaw, 1985) for the appropriate degrees of freedom (i.e. n_1+n_2-2). If the calculated t statistic was smaller than tabulated value of t for 5% probability then the difference between the means of the two groups was not considered to be significant. If, however, the found t statistic was equal to or greater than the tabulated value for 5% probability then the difference between the two groups was considered to be significant.

Standard error of the mean

The standard error of the mean (SEM) was calculated by the equation

$$\text{SEM} = s/\sqrt{N}$$

where s, the standard deviation, was calculated by the equation presented above (see "The t-test").

Confidence intervals

Confidence intervals (CI) were calculated using the equation

$$\text{CI} = \bar{y} \pm t(\text{SEM})$$

where t is the tabulated Student t statistic (see Appendix A2 of Wardlaw, 1985) with the degrees of freedom of the sample set (i.e. the number of observations minus 1) and the probability appropriate to the confidence interval. For 95% CI the tabulated value for 5% probability was used in the above equation. Similarly, for 99% CI the tabulated value for 1% probability was used and for 99·9% CI the value for 0·1% probability was used.

Rankit plots

The observations of the data set were arranged in descending rank order. Each of the observations were then assigned a rankit value from a rankit Table (see Appendix A5 of Wardlaw, 1985). A graph was then plotted with the observations against the assigned rankit values and the points of the graph joined to give the observed rankit line. The theoretical rankit line was plotted using with two reference points, i.e. the mean ± the standard deviation ($\bar{y} \pm s$) were plotted against rankit = -1·0 and rankit = +1·0.

TRANSPOSON MUTAGENESIS OF MARINE BACTERIA WHICH PRODUCE SCB TOXINS

Preparation of *E. coli* donor strains

The pUT family of transposon delivery vectors were manipulated and stored in *E. coli* CC118λpir. This strain does not contain the genetic material to facilitate conjugative transfer of these plasmids, therefore, the plasmids must first be transferred into donor strains. This was done by electroporation.

Preparation of electrocompetent cells

E. coli SM10*λpir* and S17-1*λpir* were grown in 1 l of 2YT media (see Appendix 1) until the mid-exponential growth phase was reached. Cells were chilled on ice for 30 min before centrifuging for 20 min at 4,000 g (6,000 rpm) at 4°C in a Sorval RC-5 centrifuge with a GS-3 rotor, and the cell pellet was resuspended in 1 l of ice cold 1mM hepes buffer (pH 7) and centrifuged as before. The cells were washed with 500 ml of ice cold 1mM hepes buffer (pH 7), again centrifuged and the cell pellet resuspended in 20 ml ice cold 1mM hepes (pH 7) + 10 % (v/v) glycerol and centrifuged at 3,000g for 20 min at 4°C. The final cell pellet was resuspended in 3 ml of 10% (v/v) glycerol.

Electroporation of E. coli SM10λpir and E. coli S17-1λpir

Plasmid DNA was purified from *E. coli* CC118*λpir* pUT::miniTn5Cm, CC118*λpir* pUT::miniTn5Cm*lacZ* and CC118*λpir* pUT::miniTn5Km*lacZI* using the Wizard® Miniprep DNA Purification System (Promega U.K. Ltd.) as described earlier (see "Purification of plasmid DNA").

A 5 µl aliquot of each of the purified plasmids was mixed with 50 µl of both types of electrocompetent cells. These plasmid + cell mixes were added to a 2 mm electroporation cuvette (Equibio Ltd., Kent, U.K.) and chilled on ice for one min. The cells were electroporated with one pulse at 25µF, 1·5 kv and 1000 ohms using a Gene Pulser Apparatus and Pulse Controller (Bio-Rad Laboratories Ltd., Hertfordshire, U.K.). SOC medium (1ml) was added to each cuvette and the contents incubated at 37°C for one hour. The electroporated cells were plated onto LB plates containing ampicillin (150 µg/ml) and either chloramphenicol (50 µg/ml) or kanamycin (100 µg/ml), depending on the plasmid electroporated into the cells. The plates were incubated overnight at 37°C.

Isolation of spontaneous antibiotic-resistant mutants of marine bacteria

The wild types of the marine isolates were cultured overnight in 10 ml marine broth at 20°C with shaking and aliquots of the cultures were plated onto marine agar containing either 25 µg/ml nalidixic acid or 200 µg/ml streptomycin. The plates were incubated at 20°C and, after 48 to 72 h, colonies which were present were plated onto fresh agar containing the same concentration of antibiotic. Colonies which grew on the second antibiotic-containing plate were assumed to have mutated to antibiotic resistance.

To confirm the stability of antibiotic resistance the Nal^r and Sm^r mutants were incubated overnight in the absence of antibiotics in marine broth and on marine agar. These cultures were then used to inoculate marine broth and marine agar containing

the appropriate antibiotic at the same concentration as before (i.e. either 25 μg/ml Nal or 200 μg/ml Sm).

Transposon mutagenesis of strain GFC with pJO100

Mutagenesis with the transposon donor strain *E. coli* J100 was first described by Östling *et al.* (1991). The method they describe was adapted for transposon mutagenesis of *Alteromonas tetraodonas* GFC as described below.

Broth cultures of GFC Nal^r and *E. coli* J100 were incubated overnight at 20°C for 24 h and 37°C for 18 h respectively. GFC Nal^r was cultured in 10 ml marine broth and J100 was cultured in 10 ml LB broth containing 50 μg/ml kanamycin and 10 μg/ml tetracycline. The CFU/ml of these two cultures were determined by plating dilutions of the cultures onto either marine agar or LB agar plates.

The cells of the overnight cultures were used in two separate transposon mutagenesis experiments. In the first experiment $2{\cdot}11\times10^7$ colony forming units (CFU) of GFC Nal^r were mixed with $1{\cdot}24\times10^7$ CFU of J100 in 5 ml of 10 mM $MgSO_4$. This gave a donor : recipient ratio of 1:1·7. In the second experiment $6{\cdot}33\times10^7$ CFU of GFC Nal^r were mixed with $1{\cdot}24\times10^7$ CFU of J100 in 5 ml of 10 mM $MgSO_4$. The donor: recipient ratio was therefore 1:5·1.

In each of the two experiments the mixture of GFC Nal^r and *E. coli* J100 cells were filtered onto 0·2 μm nitrocellulose membranes and the membranes laid onto LB15 plates (see Appendix 1). The plates were incubated at 20°C for 20 h to allow conjugation to proceed.

Following incubation, the cells on the conjugation filters were resuspended in 5 ml of 10 mM $MgSO_4$ and plated onto LB20 agar (see Appendix 1) plates containing nalidixic acid (25 μg/ml), tetracycline (15 μg/ml) and X-gal (40 μg/ml). The plates were incubated at 20°C until colonies were visible (i.e. 1 week).

In order to determine the rate of spontaneous mutation of GFC Nal^r and *E. coli* J100 to Tc^r and Nal^r respectively, aliquots of the overnight cultures were plated onto LB20 agar plates containing nalidixic acid (25 μg/ml), or tetracycline (15 μg/ml) and X-gal (40 μg/ml). The plates were incubated at 20°C, or 37°C where appropriate, for one week before the number of colonies were determined.

Transposon mutagenesis of strain A912 with pUT::miniTn5Km*lacZI*

An account of the methods involved in transposon mutagenesis using the pUT::mini-Tn5 derivatives has been published by de Lorenzo and Timmis (1994). The methods

described in this reference have been adapted for mutagenesis of strain A912 as follows.

Broth cultures of A912 Nal^r and *E. coli* S17-1λ*pir*pUT::miniTn5Km*lacZI* were incubated overnight at 20°C for 24 h and 37°C for 18 h respectively. A912 Nal^r was cultured in 10 ml marine broth and *E. coli* S17-1λ*pir*pUT::miniTn5Km*lacZI* was cultured in 10 ml LB broth containing 150 µg/ml ampicillin and 100 µg/ml kanamycin. The CFU/ml of these two cultures were determined by plating dilutions of these cultures onto either marine agar or LB agar plates.

Different amounts of the overnight cultures of A912 Nal^r and *E. coli* S17-1λ*pir* pUT::miniTn5Km*lacZI* were mixed together in 5 ml of 10 mM $MgSO_4$. The CFU of each culture added in the different experiments and the resulting donor:recipient ratios are detailed in Table 13.

The mixture of A912 Nal^r and *E. coli* S17-1λ*pir*pUT::miniTn5Km*lacZI* cells was filtered onto 0·2 µm nitrocellulose membranes and the membranes laid onto LB15 plates containing 10 mM $MgSO_4$. The plates were incubated at 20°C for 18 h to allow conjugation to proceed. Following incubation, the cells on the conjugation filters were resuspended in 5 ml of 10 mM $MgSO_4$ and plated onto marine agar plates containing nalidixic acid (25 µg/ml) and kanamycin (100 µg/ml). The plates were incubated at 20°C until colonies were visible (i.e. 5 days).

In order to determine the rate of spontaneous mutation of A912 Nal^r and *E. coli* S17-1λ*pir*pUT::miniTn5Km*lacZI* to Km^r and Nal^r respectively, aliquots of the overnight cultures were plated onto marine agar plates containing nalidixic acid (25 µg/ml) and kanamycin (100 µg/ml). The plates were incubated at 20°C for one week before the number of colonies was determined.

A total of 1511 Nal^r Km^r exconjugants from the experiment outlined in the final row of Table 13 were picked onto fresh marine agar plates containing nalidixic acid (25 µg/ml) and kanamycin (100 µg/ml) and again incubated at 20°C. The exconjugants were replica plated using a 48 pin replicator, for a total of six passages on marine agar containing nalidixic acid (25 µg/ml) and kanamycin (100 µg/ml). The plates were incubated for approximately 48 h at 20°C between each inoculation onto fresh plates.

Confirmation of sensitivity of *E. coli* donor strains to nalidixic acid

The nalidixic acid sensitivity of the *E. coli* donor strains, S17-1λ*pir*pUT::miniTn5-Cm*lacZ* and SM10λ*pir* pUT::miniTn5Cm*lacZ* was examined by plating the cells of overnight LB broth cultures of these strains onto LB agar and marine agar containing nalidixic acid (25 µg/ml) and chloramphenicol (50 µg/ml). The plates were incubated at 37°C and growth observed after 7 days.

Transposon mutagenesis of strain A862 with pUT::miniTn5Cm*lacZ*

Required adaptations to transposon mutagenesis method

A number of observations required the method described for A912 transposon mutagenesis with pUT::miniTn5Km*lacZ*I to be adapted for mutagenesis of A862 with pUT::miniTn5Cm*lacZ*. The background growth observed when nalidixic acid is used to select against the *E. coli* donor strain makes the isolation of true exconjugants problematic (see "Results" section). Streptomycin resistance was therefore chosen as the selective antibiotic. Unfortunately, *E. coli* S17-1*λpir* is Sm^r and is therefore unsuitable for use as a donor strain. Consequently *E. coli* SM10*λpir* was used to deliver pUT::miniTn5Cm*lacZ* into recipient strains.

During the A912 transposon mutagenesis experiments a mat of bacteria was observed on the nitrocellulose filters indicating that bacterial growth has taken place. Any growth occurring after conjugation could lead to the formation of siblings and potentially increase the number of exconjugants resulting from a single transposition event. The incubation period for conjugation was therefore decreased to 8 h for subsequent transposon mutagenesis experiments.

Initial transposon mutagenesis experiments using A862 Sm^r failed to yield any exconjugants. However, it was also observed that when A862 Sm^r was diluted in 10 mM $MgSO_4$ and plated onto marine agar, no colonies grew on the plates. It was therefore assumed that 10 mM $MgSO_4$ was not a suitable diluent for A862 Sm^r and artificial sea water (ASW; see Appendix 1) was used in its place.

Adapted transposon mutagenesis method

Broth cultures of A862 Sm^r and *E. coli* SM10*λpir*pUT::miniTn5Cm*lacZ* were incubated overnight at 20°C for 24 h and 37°C for 18 h respectively. A862 Sm^r was cultured in 10 ml marine broth and *E. coli* SM10*λpir*pUT::miniTn5Cm*lacZ* was cultured in 10 ml LB broth containing 150 µg/ml ampicillin and 50 µg/ml chloramphenicol. The CFU/ml of these two cultures were determined by plating dilutions of these cultures onto either marine agar or LB agar plates.

Different amounts of the overnight cultures of A862 Sm^r and *E. coli* SM10*λpir* pUT::miniTn5Cm*lacZ* were mixed together in ASW. The CFU of each culture added in the different experiments and the resulting donor : recipient ratios are detailed in Table 14.

The mixture of A862 Sm^r and *E. coli* SM10*λpir*pUT::miniTn5Cm*lacZ* cells were filtered onto 0·2 µm nitrocellulose membranes and the membranes laid onto LB15 plates containing 10 mM $MgSO_4$. The plates were incubated at 20°C for 8 h to allow conjugation to proceed.

Following incubation, the cells on the conjugation filters were resuspended in ASW and plated onto marine agar plates containing streptomycin (200 µg/ml) and chloramphenicol (50 µg/ml). The plates were incubated at 20°C until colonies were visible (approximately 5 days).

To determine the rate of spontaneous mutation of A862 Sm^r to Cm^r and *E. coli* SM10*λpir* pUT::miniTn5Cm*lacZ* to Sm^r, aliquots of the overnight cultures were plated onto marine agar plates containing streptomycin (200 µg/ml) and chloramphenicol (50 µg/ml), and incubated at 20°C for one week before the number of colonies was determined.

A total of 5328 Sm^r Cm^r exconjugants from the experiments outlined in Table 14 were picked onto fresh marine agar plates containing streptomycin (200 µg/ml) and chloramphenicol (50 µg/ml) and again incubated at 20°C. The exconjugants were replica plated using a 48-pin replicator, for a total of six passages on marine agar containing streptomycin (200 µg/ml) and chloramphenicol (50 µg/ml). The plates were incubated for approximately 48 h at 20°C between each inoculation onto fresh plates.

PHENOTYPIC CHARACTERISTICS OF A862::miniTn5Cm*lacZ* EXCONJUGANTS

Ampicillin sensitivity of A912::miniTn5Km*lacZ*1 exconjugants

The 1505 A912 Nal^r Km^r exconjugants which survived the six rounds of replica plating on marine agar containing nalidixic acid (25 µg/ml) and kanamycin (100 µg/ml) were inoculated onto marine agar containing 150 µg/ml ampicillin. The plates were incubated at 20°C and growth recorded after 48 h.

ß-galactosidase activity of selected A862::miniTn5Cm*lacZ* exconjugants & *E. coli* strains used in this study

The ß-galactosidase assay described by Miller (1972) was adapted for use in this study. The strains to be assayed for ß-galactosidase were grown overnight in either marine broth at 20°C or LB broth at 37°C, depending on whether it was a marine isolate or an *E. coli* strain. The cultures were chilled on ice for 20 min before the absorbance of the culture at 600 nm was measured using a Unicam 8625 UV/VIS spectrometer (Unicam Ltd.). Chloroform (50 µl) and 0·1% SDS (50 µl) were added to 75 µl of culture and 675 µl Z buffer (0.06 M $Na_2HPO_4.7H_2O$, 0.04 M $NaH_2PO_4.H_2O$, 0.01 M KCl, 0.001 M $MgSO_4.7H_2O$, 0.05 M ß-mercaptoethanol pH 7.0). The reaction mix was vortexed and allowed to equilibrate to 28°C. ONPG

(150 µl of 4 mg/ml stock solution in 0·1 M phosphate buffer) was added to each reaction and the tubes incubated at 28°C until a yellow colour had developed. The reaction was stopped by the addition of 1 M Na_2CO_3 (375 µl). The cell debris was pelleted by centrifugation (12,000 g for 3 min) and the absorbance measured at 420 nm using the same spectrometer as before.

The units of ß-galactosidase activity were calculated as follows:-

$$\text{Miller Units} = \frac{\text{Absorbance 420 nm}}{\text{t (min)} \times \text{ vol (ml)} \times \text{Absorbance 600 nm}} \times 1000$$

The Miller Unit of ß-galactosidase activity is proportional to the increase in *O*-nitrophenol per bacterium per minute.

Screening of A862::miniTn5Cm*lacZ* exconjugants by the adapted fluorescence assay

Ten replicates of A862 Sm^r, 2 replicates of A862 wt and a total of 886 different A862::miniTn5Cm*lacZ* exconjugants were cultured in 10 ml marine broth for 48 h at 20°C. The cultures were centrifuged at 3,000g for 30 min at 4°C and the supernatants retained for analysis. A portion of each culture supernatant was frozen at -20°C and shipped on ice to FRS Marine Laboratory, Aberdeen, U.K. for analysis by the mouse neuroblastoma sodium channel blocking assay (see below).

Each culture supernatant was assayed by the adapted Lawrence peroxide fluorescence assay as described earlier.

Fifteen exconjugants which gave low corrected fluorescence in the initial screen were selected for further analysis. Six replicate cultures of each of the selected exconjugants, together with marine broth (12 replicates), A862 wt, (6 replicates) and A862 Sm^r (18 replicates) were prepared and assayed by the adapted Lawrence peroxide fluorescence assay as for the initial screen described above.

Pre-column oxidation HPLC analysis of selected exconjugants

Exconjugants of interest were cultured in 10 ml marine broth at -20°C for 48 h. The cells were pelleted by centrifugation at 3,000g for 30 min at 4°C and the culture supernatants were retained for analysis.

The culture supernatants of the exconjugants of interest were analysed by pre-column HPLC analysis as described earlier. The fluorescent properties of the same supernatants were also assayed by the adapted Lawrence peroxide fluorescence assay.

Screening of A862::miniTn5Cm*lacZ* exconjugants by the mouse neuroblastoma cell sodium channel blocking assay

Culture supernatants from A862 wt, A862 Sm^r and A862::miniTn5Cm*lacZ* exconjugants, prepared as described above, were analysed by the mouse neuroblastoma cell SCB tissue culture assay as described by Gallacher & Birkbeck (1992) with the following adaptations:-

(i) Culture supernatants were diluted 10 fold in marine broth prior to analysis.

(ii) Samples were not tested in quadruplicate, but the initial samples were tested in duplicate and latter samples in triplicate.

(iii) The optimum concentrations of veratridine and ouabain were determined at the start of the screening program and kept constant throughout.

(iv) Known amounts of SCB toxins were not included on individual plates, although dose response curves for STX diluted in marine broth were obtained at intervals throughout the screening program (data not shown).

GENOTYPIC ANALYSIS OF A862::miniTn5Cm*lacZ* EXCONJUGANTS

Purification of genomic DNA

Bacterial genomic DNA was isolated by an adaptation of the method described by Johnson (1994), which is derived from the original method of Marmur (1961). The composition of the buffers used in this procedure are detailed in Appendix 2.

A862::miniTn5Cm*lacZ* exconjugants were cultured in 10 ml marine broth for 48 h at 20°C. Approximately half of each culture (5 ml) was centrifuged at 3,000g at 4°C for 15 min. The cells were washed with 5 ml ASW (see Appendix 1) and centrifuged as before. The washed cell pellets were resuspended in 250 µl suspending buffer, and 50 µl of lysozyme (10 mg/ml) and 50 µl of RNaseA (10 mg/ml) added. The cell suspensions were incubated at 37°C for 30 min before the addition of 375 µl of lysing solution and incubation at 50°C for 2 h. The cell lysates were extracted four times with 500 µl phenol:chloroform:isoamyl alcohol (25:24:1) and once with 500 µl chloroform as described by Sambrook *et al.* (1989). The DNA in the final aqueous layers was precipitated by the addition of 25 µl sodium acetate (3M, pH 5·2) and 500 µl 95% (v/v) ethanol. The DNA was spooled with a sealed Pasteur pipette as described by Johnson (1994) and washed with 500 µl of ice cold 70% ethanol. The DNA was air dried and resuspended in 250 µl of sterile distilled water.

Purification of pUT::miniTn5Cm*lacZ* DNA

The Wizard® *Plus* SV Minipreps DNA Purification System (Promega U.K. Ltd., Southampton, U.K.) was used to isolate pUT::miniTn5Cm*lacZ* DNA from *E. coli* CC118*λpir*. The composition of the buffers and solutions used for the purification of plasmid DNA are detailed in Appendix 2 and the method is described below.

E. coli CC118*λpir*pUT::miniTn5Cm*lacZ* was cultured in 10 ml LB broth overnight at 37°C. Approximately half of the culture was centrifuged at 3,000g at 4°C for 15 min. The culture supernatant was removed, the cell pellet resuspended in 250 µl resuspension buffer and the cells lysed by the addition of 250 µl of cell lysis solution; when lysis was complete, 10 µl of alkaline protease solution (supplied by the manufacturers) was added. The lysates were incubated at room temperature for 5 min before the addition of 350 µl of Wizard® *Plus* SV neutralisation solution. The lysate was centrifuged at 12,000 g for 10 min and the precipitate discarded. The supernatant was applied to a Wizard® *Plus* SV Miniprep Spin Column and centrifuged at 12,000 g for 1 min. The column was washed once with 750 µl of Wizard® *Plus* SV column wash solution and once with 250 µl of Wizard® *Plus* SV column wash solution. The plasmid DNA was eluted from the column with 100 µl of nuclease-free water and centrifugation for 1 min at 12,000 g.

Southern blot analysis of exconjugants

Purification of 4·1 kb EcoRI and 1·5 kb SalI fragments

Two different DIG-labelled fragments of pUT::miniTn5Cm*lacZ* were used as probes in Southern blot analysis of exconjugants. The first was a 4·1 kb *Eco*RI fragment which contained the *lacZ* gene, and the other a 1·5 kb *Sal*I fragment which contained the *tnp** gene. These fragments were purified as follows.

The entire yield of a Wizard® *Plus* SV Miniprep purification of pUT::miniTn5-Cm*lacZ* was digested with 1 unit of *Eco*RI with React 3 buffer and the entire yield from a second miniprep was digested with 1 unit of *Sal*I. The digested pUT::mini-Tn5Cm*lacZ* DNA were separated on 0·7% agarose gels as described earlier (see "Gel electrophoresis of DNA"). The agarose containing the 4·1 kb DNA fragment from the *Eco*RI digest and the 1·5 kb DNA fragment from the *Sal*I digest were cut out of the agarose gels and the DNA recovered from the agarose using a Geneclean® III kit (Bio 101, Anachem Ltd., Bedfordshire, U.K.) as recommended by the manufacturer and described below.

Recovery of DNA from agarose with Geneclean® III

All the solutions used for the recovery of DNA from agarose with Geneclean® III (Bio 101, Anachem Ltd., Bedfordshire, U.K.) were supplied by the manufacturer and their exact composition was not disclosed. Approximately $^1/_2$ volume of TBE Modifier® and 4·5 volumes of sodium iodide (NaI) were added to the agarose slice containing the DNA fragment. The agarose was then melted in this solution at 55°C. When the agarose was entirely in solution 5 µl of EZ-glassmilk® was added and incubated at room temperature for 5 min. The EZ-glassmilk® was pelleted by centrifugation at 12,000 g for 10 sec. The EZ-glassmilk® was washed three times with 200 µl of NEW Wash and pelleted as before after each wash. The DNA fragments were eluted of the EZ-glassmilk® with 10 µl of Elution Solution.

Preparation of DIG-labelled DNA probes

The gel purified 4·1 kb *Eco*RI and 1·5 kb *Sal*I fragments were labelled with DIG by random primed DNA labelling using a DIG DNA Labelling and Detection Kit (Boehringer Mannheim UK (Diagnostics & Biochemicals) Ltd., East Sussex, U.K.) as described by the manufacturer. Approximately 200 ng of the 4·1 kb *Eco*RI fragment and approximately 85 ng of the 1·5 kb *Sal*I fragment were denatured in a boiling water bath for 10 min and chilled on ice. Each of the denatured fragments were added to 2 µl Hexanucleotide mixture (10X), 2 µl dNTP labelling mixture (10X) and 1 µl Klenow enzyme (2 units/µl). The labelling reaction was allowed to proceed at 37°C for 20 h, then stopped by the addition of 2 µl EDTA (0·2 M).

Gel electrophoresis of restriction digested genomic DNA

Approximately 1 µg amounts of genomic DNA from selected A862::miniTn5Cm*lacZ* exconjugants and A862 Sm^r were digested with *Cla*I or *Pst*I with the appropriate reaction buffer (React 1 and React 2 buffers respectively) and incubation overnight at 37°C. Control DNA (i.e. approximately 1 ng of pUT::miniTn5Cm*lacZ*) was digested with the same enzyme under identical incubation conditions.

The restriction digests were run separated on 0·5% agarose gels as described earlier (see "Gel electrophoresis of DNA").

Transfer of genomic DNA to nylon membranes by capillary transfer

The 0·5% agarose gel containing DNA of interest was depurinated by incubating in 250 mM HCl for exactly 10 min. The gel was rinsed with distilled water and then incubated in denaturation solution (see Appendix 2) for 30 min. The gel was again rinsed with distilled water and incubated for 30 min in neutralisation solution.

The DNA was transferred to Hybond N+ nylon membrane (Amersham International plc., Buckinghamshire, U.K.) by capillary transfer with 20X SSC (see Appendix 2) as first described by Southern (1975). The assembly used for capillary transfer is shown in Figure 9. The transfer was allowed to proceed for 18 h before the assembly was dismantled. The DNA was cross-linked to the nylon membrane using a UV crosslinker (Spectrolinker XL-1000 UV crosslinker, Spectronics Corporation) and the membrane washed briefly in 2X SSC.

Hybridisation of genomic DNA and DIG-labelled DNA fragments

Nylon membranes containing DNA of interest were prehybridised for at least 2 h with 20 ml of prehybridisation solution (see Appendix 2) at 68°C in a hybridisation oven (Techne Hybridiser HB-1D). The DIG labelled DNA probes were denatured in a boiling water bath for 10 min and chilled on ice. The entire probe was then added to 6 ml of hybridisation solution. (Membranes containing 1 kb DNA ladder were probed with hybridisation solution containing only 5 µl of DIG-labelled 1 kb DNA ladder). The nylon membranes were incubated overnight at 68°C with hybridisation solution containing the appropriate DIG-labelled probe. The membranes were then washed twice for 5 min with 2X wash solution at 68°C and twice for 15 min with 0·1X wash solution at 68°C.

Detection of hybridised DIG-probes

Nylon membranes which had been hybridised with DIG probes and washed with 2X and 0·1X wash solution, were equilibrated in washing buffer (see Appendix 2) for 1 min. The membranes were incubated in blocking solution (see Appendix 2) for 1 h. Anti-Digoxigenin-AP (see Appendix 2) was diluted $^{1}/_{10,000}$ in blocking solution and this antibody was incubated with the membranes for 30 min. Membranes were washed twice for 15 min with washing buffer and then equilibrated with detection buffer for 2 min. A 5 ml solution of 0·25 mM CSPD (Promega U.K. Ltd., Southampton, U.K.) was added to each membrane and incubated at room temperature for 5 min. Excess CSPD was removed and the membranes incubated at 37°C for 15 min. Hyperfilm ECL (Amersham International plc, Buckinghamshire, U.K.) was exposed to the membranes for different lengths of time ranging from 2 min to 1 h. The films were then developed in the dark with Kodak LX24 X-ray developer and fixed with Kodak Unifix (Anachem Ltd., Bedfordshire, U.K.).

Once a suitable exposure was obtained, the membranes were washed with sterile distilled water for 10 min, the membranes were again equilibrated with detection buffer for 2 min and 10 to 20 ml of colorimetric substrate (see Appendix 2) added. The blue/black colour was allowed to develop to the desired intensity before rinsing the membrane in distilled water.

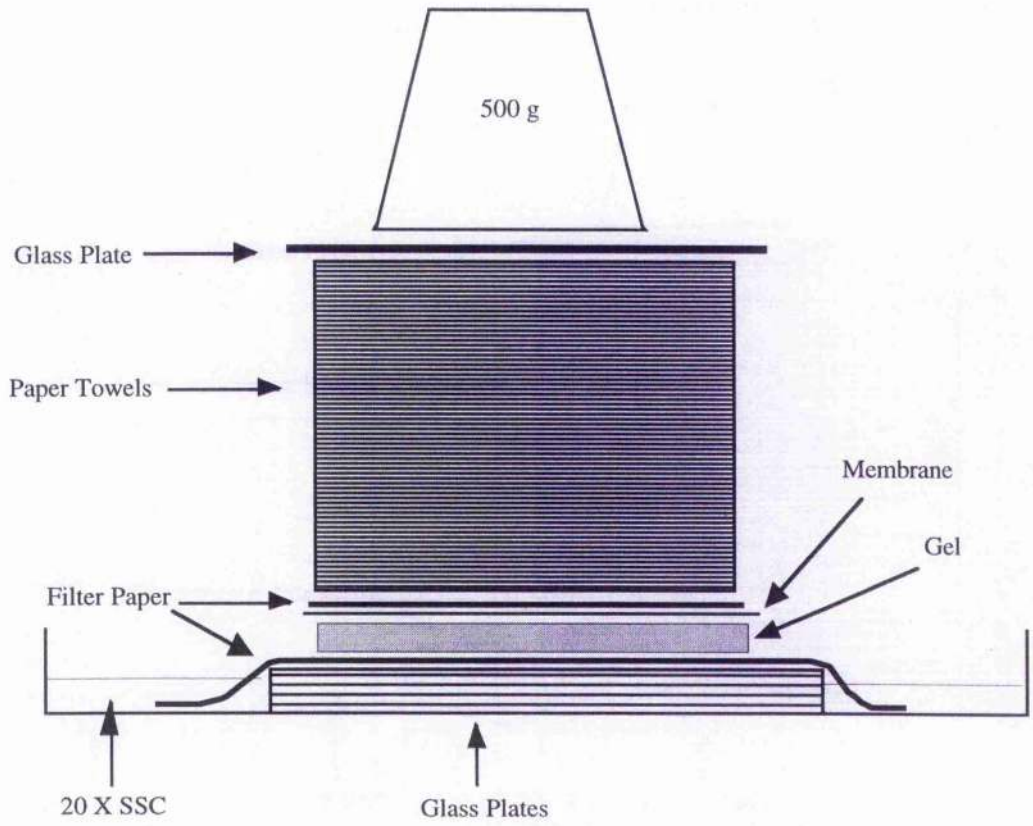


Figure 9. The Southern blot assembly used for capillary transfer of DNA from agarose gels to nylon membranes. Adapted from Crosa *et al.*, 1994.

Inverse PCR amplification of miniTn5Cm*lacZ* flanking sites

Analysis of the DNA sequence of the *E. coli lacZ* gene (accession number V00296) revealed a *Cla*I site at position 836. This restriction enzyme does not cut pUT::mini-Tn5Cm*lacZ* at any other location. It was therefore decided to use this enzyme to digest genomic DNA from exconjugants of interest, circularise the resulting DNA fragments by ligation and to amplify the region of DNA associated with the first 836 nucleotides of the *lacZ* gene by PCR (Triglia *et al.*, 1988). The strategy for this inverse PCR reaction is outlined in Figure 10 and 11. It is clear from these Figures that the DNA associated with appropriate *lacZ* sequence represents a genomic sequence which is adjacent to the miniTn5Cm*lacZ* insertion site.

Design of inverse PCR primers

Inverse PCR primers were selected using the PRIME programme (Wisconsin Package Version 9.0, Genetics Computer Group (GCG), Madison, Wisconsin, USA). A reverse primer which recognised sequences close to the start of the *lacZ* sequence (position 88 to 67) and a forward primer which recognised a sequence (position 730 to 749) close to the *Cla*I site were selected. These primers were named L8867, which has the sequence 5'-GTGCTGCAAGGCGATTAAGTTG-3', and L730749, which has the sequence 5'-GTTCAGATGTGCGGCGAGTT-3'.

Preparation of template DNA

Approximately 5 µg of genomic DNA from exconjugants of interest was digested with 10 units of *Cla*I (Life Technologies). After incubation at 37°C for 4 h, the restriction digests were extracted with phenol:chloroform:isoamyl alcohol (25:24:1) as described by Sambrook *et al.* (1989). The DNA from these digests were further purified using Geneclean® III (Bio 101) as described earlier (see "Recovery of DNA from agarose with Geneclean® III") without the addition of TBE modifier and incubation at 55°C.

The volume of the purified restriction digests was made up to 400 µl with sterile distilled water and 100 µl of 5X T4 DNA ligase buffer and 1 µl of T4 DNA ligase (1 unit/µl; Life Technologies). The ligation reactions were allowed to proceed overnight at 16°C. The ligated DNA was precipitated with 1 ml 95% (v/v) ethanol and 16 µl of 3 M sodium acetate at -70°C for 15 min. The DNA was pelleted by centrifugation at 12,000 g for 15 min. The pellet was washed with 70% (v/v) ethanol and air dried. The DNA was resuspended in 20 µl of sterile distilled water and diluted in sterile distilled water prior to use as template for inverse PCR reactions.

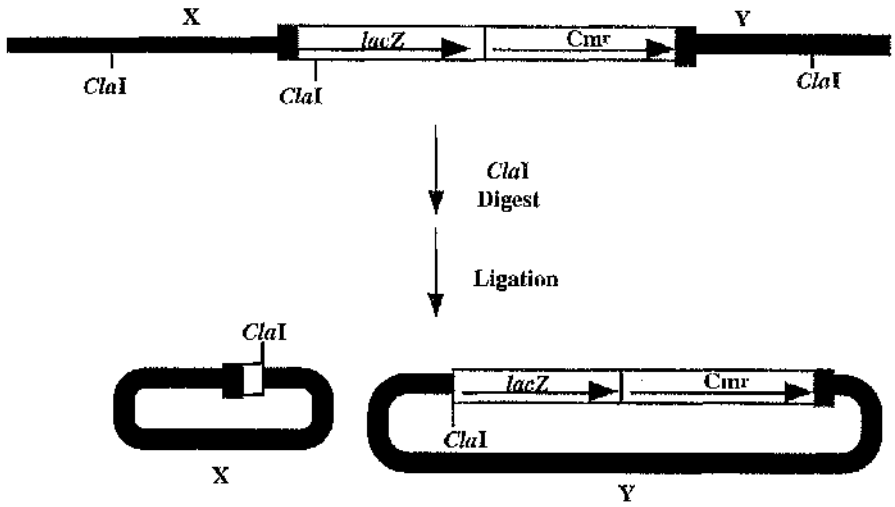


Figure 10. Strategy for the preparation of template DNA for inverse PCR amplification of miniTn5Cm*lacZ* flanking sites. Genomic DNA (represented by the thick black line) of selected exconjugants was digested with the restriction enzyme *Cla*I and ligated in a dilute solution to favour self ligation. The product of this reaction contains many circularised fragments of genomic DNA including the two fragments represented above which contain fragments of the inserted transposon.

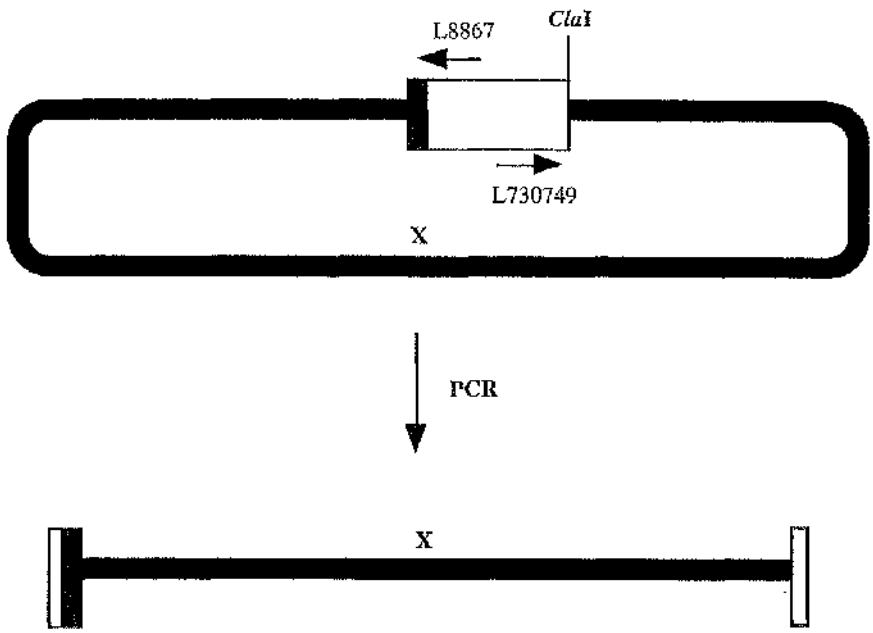


Figure 11. Inverse PCR amplification of miniTn5Cm*lacZ* flanking sites. The template DNA prepared as described in Figure 10 was used to amplify the flanking site x using the above method. Primers L8867 and L730749 recognise sequences of the *lacZ* gene and are orientated so that amplification takes place in the outward direction. The resulting PCR products contain short sequences of miniTn5Cm*lacZ* (represented by the open box) at the end of the DNA of interest.

Inverse PCR reactions

The template DNA prepared from exconjugants of interest was added to the following reaction mix:-

Template DNA	1 µl
dNTP mix (see Appendix 2)	200 µM
Primer L8867	300 nM
Primer L730749	300 nM
Expand™ High Fidelity buffer	1X
dH_2O	to 49·25 µl

Expand™ High Fidelity PCR System enzyme mix (2·6 units) was added to each reaction mix after the initial denaturation cycle of the PCR programme.

The PCR reaction mixes were cycled in a Omn-E Thermal Cycler (Hybaid Ltd., Middlesex, U.K.) using the following programme:-

1X	94°C	2 min
30X	94°C	30 sec
	60°C	30 sec
	68°C	4 min
1X	68°C	7 min

The PCR products were visualised by agarose gel electrophoresis as described earlier (see "Gel electrophoresis of DNA").

Confirmation of the identity of inverse PCR products

An aliquot (15 µl) of the inverse PCR products obtained from exconjugants S5-17 and S9-22 was analysed on an agarose gel (see "Gel electrophoresis of DNA") and the appropriate DNA band excised from the gel and recovered as described earlier (see "Preparation of DIG-labelled DNA probes"). The gel-purified inverse-PCR products were random-primed labelled with DIG as described earlier (see "Recovery of DNA from agarose with Geneclean® III").

The inverse PCR products from exconjugants S5-17 and S9-22, *Cla*I digested pUT::miniTn5Cm*lacZ* DNA, and *Cla*I digested genomic DNA from A862 Sm^r, exconjugant S5-17 and S9-22 were all separated on a 0·5% agarose gel (see Figure 67 and "Gel electrophoresis of DNA"). The gel was transferred to nylon membrane as described earlier (see "Transfer of genomic DNA to nylon membranes by capillary transfer") and the membrane cut into three sections. The two portions of membrane containing the test samples were hybridised with the appropriate DIG-labelled inverse PCR product and the membrane containing 1 kb DNA ladder was probed with DIG-labelled 1 kb DNA ladder (see "Hybridisation of genomic DNA and DIG-labelled

DNA fragments") and the hybridisation was detected as described previously (see "Detection of hybridised DIG-probes").

Cloning of inverse PCR products

The inverse PCR products obtained from exconjugants S5-17, S9-22, S14-4, S16-27 and S19-43 were cloned using the TOPO TA Cloning® Kit (Invitrogen BV, The Netherlands). The inverse PCR products from the exconjugants of interest were prepared as described earlier (see "Inverse PCR reactions"). Immediately following the final extension cycle, 1 unit of *Taq* DNA polymerase (Promega U.K. Ltd.) was added to each reaction and the tubes incubated at 72°C for 10 min. This additional step adds the A overhangs necessary for TA cloning of PCR products. The proof reading property of the Expand™ High Fidelity PCR System enzyme mix removes these overhangs during the standard PCR reactions.

The inverse PCR products (2 µl) were added to the pCR®2·1-TOPO vector (1 µl) in a total volume of 5 µl (i.e. 2 µl sterile water was added) and incubated at room temperature for 5 min. Aliquots (2 µl) of the cloning reactions were added to a vial of One Shot™ TOP10F' cells (Invitrogen BV; see Table 5) and 2 µl of ß-mercapto-ethanol (0·5 M). The cells were incubated on ice for 15 min, then heat shocked at 42°C for 30 sec. SOC (250 µl) medium was added to the transformed cells and incubated at 37°C for 1 hour. The cells were plated onto LB agar containing kanamycin (50 µg/ml) and incubated overnight at 37°C.

Insertion of an insert of the correct size was confirmed by isolation of plasmid DNA by Wizard® *Plus* SV Miniprep DNA Purification System (see "Purification of pUT::miniTn5-Cm*lacZ* DNA") and restriction digest analysis with *Eco*RI.

DNA sequencing of miniTn5Cm*lacZ* flanking sites

The PCR products were sequenced by cycle sequencing with the dye terminator method (Lee *et al.*, 1992) in conjunction with The Molecular Biology Support Unit, Institute of Biomedical and Life Sciences, The University of Glasgow.

Template preparation

Two types of DNA were used as template for the DNA sequencing reactions. Initially the inverse PCR products were purified from an agarose gel (see ""Recovery of DNA from agarose with Geneclean® III") and used as template. Once the inverse PCR products were cloned, the pCR2·1-TOPO DNA containing the inverse PCR products were purified using the Wizard® *Plus* SV Miniprep DNA Purification System as

described earlier (see "Purification of pUT::miniTn5Cm*lacZ* DNA") and used as sequencing template.

Primer design

Three different primers were used to obtain the sequence of inverse PCR products. The primers used for the inverse PCR reaction, L8867 and L730749, were used to obtain the initial sequence. Analysis of the sequences obtained with these two primers revealed a large amount of miniTn5Cm*lacZ* sequence (377 nucleotides) at one end of each PCR product. Another primer was therefore designed which recognised a sequence within this region (nucleotides 276 to 295 of the three sequences shown in Appendix 4). This primer was named AH271290 and has the sequence 5'-AGCTCC-TGCATCAATCGCT G-3'.

Cycle sequencing

The cycle sequencing PCR reaction mix consisted of 8 µl of Terminator Ready Reaction Mix (Perkin-Elmer Corporation, California, USA), 3·2 pmole of primer, template DNA (approximately 60 ng of PCR product or 500 ng of double-stranded plasmid DNA) and sterile distilled water to make a final volume of 20 µl. The reactions were cycled in a Omn-E Thermal Cycler (Hybaid Ltd., Middlesex, U.K.) with 25 cycles of 96°C for 30 sec, 50°C for 15 sec and 60°C for 4 min.

Purification of extension products

The extension products from the cycle-sequencing reactions were purified by the addition of 2 µl of 2 M sodium acetate (pH 4·6) and 50 µl of 95% (v/v) ethanol and incubation on ice for 10 min. The DNA was pelleted by centrifugation at 12,000 g for 15 min, washed with 250 µl of 70% (v/v) ethanol and air dried.

Running the sequencing gel

The ethanol-precipitated extension products were resuspended in 4 µl of gel loading solution (i.e. a 5:1 solution of deionised formamide and 50 mM EDTA). The samples were heated at 90°C for 2 min and placed on ice until loaded onto the sequencing gel. The sequencing gel was a standard 4·5% acrylamide (19:1 acrylamide:bis-acrylamide) gel in an Applied Biosystems 373 DNA Sequencer (Perkin-Elmer Corporation, California, USA).

Sequence analysis of inverse PCR products

The DNA sequences obtained for the inverse PCR products were compared with each other using the FASTA (Pearson & Lipman, 1988), PileUp and Pretty programs of

the GCG package (Wisconsin Package Version 9.0, Genetics Computer Group (GCG), Madison, Wisconsin, USA).

The inverse PCR product DNA sequences were compared with sequences in the SWISS-PROT, GenBank and EMBL databases using FASTA (Pearson & Lipman, 1988; Pearson, 1990), BLASTN (Altschul *et al.*, 1990; Madden *et al.*, 1996; Altschul *et al.*, 1997) and BLASTX (Altschul *et al.*, 1990; Gish & States, 1993; Madden *et al.*, 1996; Altschul *et al.*, 1997).

The DNA sequences were translated in all six frames using the PepData program of the GCG package (Wisconsin Package Version 9.0, Genetics Computer Group (GCG), Madison, Wisconsin, USA). The resulting amino acid sequences were compared to entries in the PROSITE Dictionary of Protein Sites and Patterns using the Motifs search programme (Wisconsin Package Version 9.0, Genetics Computer Group (GCG), Madison, Wisconsin, USA).

Multiple sequence alignments were performed by Mutalin multiple alignment (Corpet, 1988) located at the Internet web page of The Institute of Biology and Chemistry of Proteins, France (http://www.ibcp.fr/multalin.html). The alignments presented in Appendices 4 to 6 were prepared by BOXSHADE (version 3·21).

AMPLIFICATION AND SEQUENCING OF A *hns* GENE FROM THE MARINE BACTERIUM A862

Single specific primer PCR (SSP-PCR) is a method developed to amplify fragments of DNA when only a portion of the sequence is known (Shyamala and Ames, 1989; Novak *et al.*, 1997). This method employs a primer designed to a known DNA sequence and another primer which recognises a sequence of vector DNA. The DNA containing the region of interest is ligated to the vector of choice and this new construct is used as a template for standard PCR reactions.

Primer design

A primer was designed to recognise a sequence downstream from the putative H-NS homologue coding region located on the inverse PCR product of exconjugant S19-43 (see Appendix 4 & 8). This primer, HNS1, has the sequence 5'-GTGGCGGAACT-GTGAGGATA-3' and is complementary to nucleotides 564 to 583 of the inverse PCR product of exconjugant S19-43 (Appendix 4).

Preparation of template DNA

Approximately 1 µg of the A862 Sm^r genomic DNA (purified as described above; see "Purification of genomic DNA") and 0·5 µg of pUC18 DNA (purified by Wizard®

Plus SV Minipreps DNA Purification System; see "Purification of pUT::miniTn5Cm-*lacZ* DNA") were digested with *Hind*III at 37°C for 18 h.

The digested A862 DNA was ligated to digested pUC18 DNA in a 25 µl reaction which included 5 µl of 5X T4 DNA ligase buffer, 1 µl of T4 DNA ligase, 210 ng of A862 genomic DNA and 120 ng of pUC18. Ligation was allowed to proceed at 16°C for 6 h. The ligations were stored at -20°C until required as template for SSP-PCR reactions.

Amplification of a genomic DNA fragment containing a hns *gene*

The ligated A862 DNA and pUC18 were used as template in the following SSP-PCR reaction mix:-

Template DNA	1 µl
dNTP mix (see Appendix 2)	200 µM
Primer HNS1	30 pmol
Primer M13R	20 pmol
Expand™ High Fidelity buffer	1X
dH_2O	to 49·25 µl

Expand™ High Fidelity PCR System enzyme mix (2·6 units) was added to each reaction mix after the initial denaturation cycle of the PCR programme.

The PCR reaction mixes were cycled in a Omn-E Thermal Cycler (Hybaid Ltd., Middlesex, U.K.) using the following programme:-

1X	94°C	2 min
30X	94°C	30 sec
	60°C	30 sec
	68°C	90 sec
1X	68°C	10 min

The SSP-PCR products were visualised by agarose gel electrophoresis as described earlier (see "Gel electrophoresis of DNA").

Cloning and sequencing of SSP-PCR product

The products from the above SSP-PCR reaction were cloned into the pCR®2·1-TOPO vector as described above for the inverse PCR products (see "Cloning of inverse PCR products").

Primer HNS1 was used to sequence the cloned SSP-PCR products as described above (see "DNA sequencing of miniTn5Cm*lacZ* flanking sites"). A second primer, HNS2, was designed to recognise a portion of the sequence obtained using the

HNS1 primer, and this primer was used to obtain further DNA sequence of the SSP-PCR product. The sequence of HNS2 was 5'-GTACGGCCATTTTCTAATTC-3'.

AMPLIFICATION OF POTENTIAL SULFOTRANSFERASE GENES FROM THE MARINE BACTERIUM A862

Comparison of known sulfotransferase sequences

The amino acid sequences of known ST enzymes were retrieved from SWISS-PROT, EMBL and GenBank databases using the StringSearch and Fetch programmes of the GCG package (Wisconsin Package Version 9.0, Genetics Computer Group (GCG), Madison, Wisconsin, USA). Additional accession numbers were obtained from published literature on ST enzymes (Yamazoe *et al.*, 1994; Rikke, & Roy, 1996; Varin *et al.*, 1997; Weinshilboum *et al.*, 1997). The amino acid sequences were compared using Mutalin multiple sequence alignment programme to locate the putative PAPS binding site of each enzyme (Yamazoe *et al.*, 1994; Rikke, & Roy, 1996; Weinshilboum *et al.*, 1997). The sequences of the putative PAPS binding sites were then aligned with Mutalin and BOXSHADE programmes.

SSP-PCR amplification of potential sulfotransferase genes from A862

SSP-PCR primer design

A comparison of the putative PAPS binding site of known ST sequences revealed a conserved motif (RKGxxGDWKxxFT) in the cytosolic ST enzymes of eukaryotes Weinshilboum *et al.*, 1997). Although this motif is not conserved in known bacterial ST enzymes it was decided to use this consensus sequence to design primers for SSP-PCR. The standard codons which encode the amino acids of the conserved motif are listed in Table 7. This information was used to design two degenerate primers which recognise the consensus sequence. The first primer, ST01, has the sequence 5'-GGNGAYTGGAARAAYCAYTTYAC-3' (where N = A, T, G or C, Y = T or C and R = A or G) and recognises the sense strand of the consensus sequence. A second primer, ST03, with the sequence 5'-GTRAARTGRTTYTTCCARTCNCC-3', recognises the complementary strand of the conserved sequence.

Three primers which recognise sequences in pUC18 DNA (accession number L08752) were used in SSP-PCR reactions. The primer L8867, described earlier (see "Design of inverse PCR primers"), recognises a sequence in the *lacZ* gene. Primer M13F, which recognises the M13 forward (-40) primer site of pUC18 has the sequence 5'-GTTTTCCCAGTCACGAC-3'. Primer M13R has the sequence 5'-

CAGGAAACAGCTATGAC-3' and recognises the M13 reverse primer site of pUC18.

Template preparation

Genomic DNA was purified from A862 Sm^r as described earlier (see "Purification of genomic DNA"). Approximately 1 µg of the A862 genomic DNA was digested, in four separate reactions, with different restriction enzymes (*Eco*RI, *Hind*III, *Bam*HI and *Sma*I) at 37°C for 18 h. Four aliquots (approximately 0·5 µg) of pUC18 DNA (purified by Wizard® *Plus* SV Minipreps DNA Purification System; see "Purification of pUT::miniTn5Cm*lacZ* DNA") were digested with the same four restriction enzymes at 37°C for 18 h.

The four different samples of digested A862 DNA were ligated to pUC18 DNA which had been digested with the same restriction enzyme. This ligation reaction was performed in a final volume of 25 µl, which included 5 µl of 5X T4 DNA ligase buffer, 1 µl of T4 DNA ligase, 210 ng of A862 genomic DNA and 120 ng of pUC18. Ligation was allowed to proceed at 16°C for 6 h. The ligations were stored at -20°C until required as template for SSP-PCR reactions.

R Arg	K Lys	G Gly	X_1	X_2	G Gly	D Asp	W Trp	K Lys	X_3	X_4	F Phe	T Thr
cgt	aaa	ggt			ggt	gat	tgg	aaa			ttt	act
cgc	aag	ggc			ggc	gac		aag			ttc	acc
cga		gga			gga							aca
cgg		ggg			ggg							acg
aga												
agg												

Table 7. Possible codons encoding a conserved amino acid sequence of cytosolic sulfotransferases.

SSP-PCR reactions

The ligated A862 DNA and pUC18 were used as template in SSP-PCR reactions using ST01 or ST03 primers (50 pmol) in conjunction with primer M13R (20 pmol) and the reaction conditions described above (see "Amplification of a genomic DNA fragment containing a hns gene"). The SSP-PCR products were visualised by agarose gel electrophoresis as described earlier (see "Gel electrophoresis of DNA").

Nested SSP-PCR reactions

In order to decrease the probability of amplifying non-specific products nested SSP-PCR were performed using primers L8867 (20 pmol) and either ST01 or ST03 (50 pmol) in an initial amplification reaction using the ligated A862 DNA and pUC18 as template and the reaction conditions described earlier (see "Amplification of a genomic DNA fragment containing a hns gene") but with only 15 cycles rather than the usual 30 in the second stage of the reaction. The product of the initial reactions were then used as template in SSP-PCR reactions using primers M13F (20 pmol) and either ST01 or ST03 (50 pmol) and conditions described above.

Cloning and DNA sequencing of SSP-PCR products

The SSP-PCR products were separated on a 0·7% agarose gel (see "Gel electrophoresis of DNA") and the major bands excised with a scalpel. The DNA was recovered from these agarose bands using Geneclean® III (see "Gel purification of DNA fragments"). The gel purified SSP-PCR products were cloned into the pCR®2·1-TOPO cloning vector as described earlier (see "Cloning of inverse PCR products"). The DNA sequences of the SSP-PCR products were obtained by the cycle sequencing and dye terminator method as described earlier (see "DNA sequencing of miniTn5Cm*lacZ* flanking sites"). Four different primers were used in the sequencing of SSP-PCR products, i.e. ST01, ST03, M13F and M13R. Sequence analysis of SSP-PCR DNA sequences was performed as described for the inverse PCR sequence analysis (see "Sequence analysis of inverse PCR products").

RESULTS

EVALUATION OF MARINE BACTERIAL ISOLATES FOR TRANSPOSON MUTAGENESIS

Phenotype of marine bacteria used in this study

A selection of phenotypic characteristics of the strains considered for inclusion in this study were determined (as described in the Materials and Methods) and summarised in Table 8. The results obtained for *Shewanella alga* OK-1 and *Alteromonas tetraodonis* GFC are in agreement with the results of Simidu *et al.* (1990). It was noted, however, that colonies of OK-1 appeared orange when grown on marine agar, a property not described by Simidu *et al.* (1990).

All the strains tested are gram-negative rods and, with the exception of A912, all were motile and oxidase positive. A more comprehensive analysis of the phenotypic characteristics of these strains is required to classify them to genus level. However, it appears from the O129 sensitivity that some of these strains may be *Vibrio* spp.. A preliminary analysis of 16S rRNA sequences has indicated that 4αVS3 may be an *Alteromonas* sp. and the A862 sequence has greatest homology to *Shewanella* spp. For example, accession number D63488 has 95.750% homology and accession number D21225 has 95.618% homology to A862 16S rRNA. (Gallacher, S., Maas, E., Moore, E., Stroempl, C., unpublished data). A phylogenetic tree, constructed with the 16S RNA gene sequence data for strain A862 and a number of selected species (Appendix 3), placed strain A862 on a branch containing known *Shewanella* spp..

Antibiotic sensitivity

Exconjugants which have successfully received a copy of a transposon are selected by the antibiotic resistance encoded by the transposon. The suitability of a bacterial strain for transposon mutagenesis therefore largely depends on its sensitivity to the appropriate antibiotic. In addition, the ideal recipient strain will be sensitive to any antibiotic to which the delivery vector encodes resistance, therefore facilitating the screening of exconjugants for the loss of the delivery vector. If a reporter gene is to be inserted into bacteria of interest the recipient bacteria must lack the phenotype encoded by this gene.

The frequency at which the marine bacteria described above became resistant to selected antibiotics was determined by plating out cells from a broth culture on to marine agar (or LB20 for tetracycline resistance) which contained the antibiotic. The results are summarised in Table 9. Any strain which became resistant to an antibiotic

Strain	Gram Stain	Morph	Pigm	Motil	Oxidase	ONPG	H_2S	Gel	O129 Sensitivity	
									10µg/ml	150µg/ml
OK-1	-ve	rod	orange	+	+	-	+	+	R	R
GFC	-ve	rod	none	+	+	-	-	+	R	R
4αVS3	-ve	rod	none	+	+	+	-	-	R	R
A400	-ve	rod	red/ orange	+	+	-	-	-	R	S
A862	-ve	rod	orange	+	+	-	+	+	R	R
A903	-ve	rod	none	+	+	+	-	+	R	R
A912	-ve	rod	none	-	-	-	-	-	R	S
A940	-ve	rod	none	+	+	-	-	+	R	S
A1087	-ve	rod	orange	+	+	-	+	-	S	S
A1095	-ve	rod	none	+	+	-	-	-	R	S

Morph = morphology
Pigm = pigmentation
Motil = motility
ONPG = ß-galactosidase activity
H_2S = H_2S production
Gel = gelatinase activity
S = sensitive
R = resistant

Table 8. Selected phenotypic characteristics of the marine bacteria used in this study.

at a frequency greater than 1×10^{-6} was considered unsuitable for mutagenesis with transposons carrying a resistance gene to the antibiotic in question.

A number of strains did not grow on LB20 media without the addition of $MgCl_2$ (data not shown). The addition of $MgCl_2$ to this medium would be counter-productive however as it is stated that magnesium ions are antagonists of tetracycline (Sambrook *et al.*, 1989). These strains were, therefore, considered unsuitable for transposon mutagenesis with pJO100 and pUT::miniTn5Tc.

Strain A400 was found to grow significantly slower than the other strains on marine agar. After 72 hours only very small colonies were present. It was decided, therefore, that this strain would only be considered for transposon mutagenesis if the other strains were not suitable.

As it was hoped to use *lacZ* reporter genes to investigate gene expression in mutants of interest, strains which contained ß-galactosidase activity were considered unsuitable for study. As indicated in Table 8, the strains 4αVS3 and A903 both possess active ß-galactosidase and were therefore discounted from any further investigation.

Using the above criteria the following combinations of transposon vector and recipient strains were considered for further study:-

(i) GFC + pJO100

(ii) A912 + pUT::miniTn5Km*lacZI*

(iii) (A862, A912 or A1095) + pUT::miniTn5Sm

(iv) (GFC, A862, A1087 or A1095) + pUT::miniTn5Cm.

Strain	Frequency of Spontaneous Resistance				
	Tet^r	Kan^r	Sm^r	Cm^r	Amp^r
OK-1	~1	$1{\cdot}22\times10^{-5}$	$3{\cdot}89\times10^{-6}$	$3{\cdot}22\times10^{-6}$	~1
GFC	$<7{\cdot}35\times10^{-9}$	~1	$1{\cdot}41\times10^{-6}$	$<2{\cdot}82\times10^{-9}$	$1{\cdot}53\times10^{-7}$
4αVS3	ND	$2{\cdot}41\times10^{-7}$	$3{\cdot}72\times10^{-8}$	$1{\cdot}63\times10^{-8}$	~1
A400	ND	$5{\cdot}13\times10^{-8}$	$<3{\cdot}97\times10^{-7}$	$<3{\cdot}97\times10^{-7}$	$<5{\cdot}13\times10^{-8}$
A862	$9{\cdot}31\times10^{-6}$	$8{\cdot}29\times10^{-6}$	$2{\cdot}75\times10^{-7}$	$<4{\cdot}88\times10^{-9}$	~1
A903	$<4{\cdot}90\times10^{-9}$	$2{\cdot}53\times10^{-5}$	$3{\cdot}47\times10^{-6}$	$<6{\cdot}94\times10^{-9}$	$<5{\cdot}26\times10^{-9}$
A912	ND	$8{\cdot}74\times10^{-9}$	$3{\cdot}30\times10^{-7}$	$8{\cdot}58\times10^{-5}$	$5{\cdot}59\times10^{-7}$
A940	ND	$4{\cdot}80\times10^{-3}$	$6{\cdot}03\times10^{-5}$	$<2{\cdot}51\times10^{-6}$	$<9{\cdot}71\times10^{-6}$
A1087	$3{\cdot}83\times10^{-5}$	$3{\cdot}42\times10^{-6}$	$3{\cdot}50\times10^{-6}$	$<6{\cdot}99\times10^{-9}$	~1
A1095	ND	$2{\cdot}29\times10^{-6}$	$4{\cdot}39\times10^{-8}$	$<4{\cdot}39\times10^{-8}$	$<3{\cdot}92\times10^{-8}$

ND = Not determined

Tet = tetracycline (15μg/ml)

Kan = kanamycin (100μg/ml)

Sm = streptomycin (100μg/ml)

Cm = chloramphenicol (50μg/ml)

Amp = ampicillin (150μg/ml)

Table 9. Frequency of spontaneous antibiotic resistance in marine bacteria.

CONSTRUCTION OF pUT::miniTn5Cm*lacZ*

The transposon vectors pUT::miniTn5Cm, pUT::miniTn5Sm and pUT::miniTn5Tc (obtained from V. De Lorenzo) do not contain a reporter gene which can be used to study gene expression in mutants of interest. The incorporation of a *lacZ* reporter gene is facilitated, however, by the presence of unique *NotI* sites immediately inside the 19 base pair O end of the transposons (see "Mini-Tn5" in the "Introduction"). As pUT::miniTn5Cm is the transposon vector which is suitable for mutagenesis of the

largest number of recipient strains it was decided to concentrate efforts to incorporate a *lacZ* reporter gene into this transposon.

A 4·1 kb *Not*I fragment from pUJ8, which contains a promoterless *lacZ* gene, was successfully ligated into *Not*I digested pUT::miniTn5Cm. Restriction digest analysis confirmed that the orientation of this insert was such that insertion of this transposon downstream from an active promoter would result in expression of the *lacZ* gene. The structure of this new transposon vector, pUT::miniTn5Cm*lacZ*, is shown in Figure 8.

HPLC ANALYSIS OF STRAIN A862

To be able to interpret data from assays for PSP toxin detection it was necessary to determine which PSP derivatives are produced by strain A862. In addition, it was deemed prudent to determine whether the isolation of a spontaneous Sm^R mutant of A862 had affected the toxin profile of this strain. Analysis of toxin profiles was carried out by pre-column oxidation HPLC analysis as described by Lawrence *et al.* (1991; 1995; 1996; Lawrence and Menard, 1991) and post-column oxidation HPLC analysis by the method of Franco and Fernández-Vila (1993).

Pre-column oxidation HPLC analysis of PSP standards

The separation of the oxidation products of the Canadian NRC PSP toxin standards by the method of Lawrence *et al.* (1991; 1995; 1996; Lawrence and Menard, 1991) resulted in well separated peaks (see Figure 13). A comparison of the chromatogram obtained with the hypothetical chromatogram (see Figure 12) and chromatograms of individually analysed toxins (chromatograms not shown) allowed identification of the peaks.

Peak J in the chromatogram of peroxide-oxidised PSP standards (Figure 13a) is the product of STX oxidation. Peaks B, C and H in Figure 12a are the peroxide oxidation products of $GTX_{2/3}$. The presence of peaks F and G in Figure 13a may indicate the presence of dcSTX in the PSP standard mix.

In the chromatogram of periodate-oxidised PSP toxins (Figure 13b) peak J consists of oxidation products of STX and neoSTX. Peaks D and G are also products of neoSTX oxidation. Peaks A, C and H contain oxidation products of $GTX_{1/4}$, although oxidation of $GTX_{2/3}$ is responsible for some of the product forming peak H.

If the PSP standards were diluted in marine broth rather than the usual 0·05M acetic acid, the peak corresponding to peroxide-oxidised STX are greatly increased in

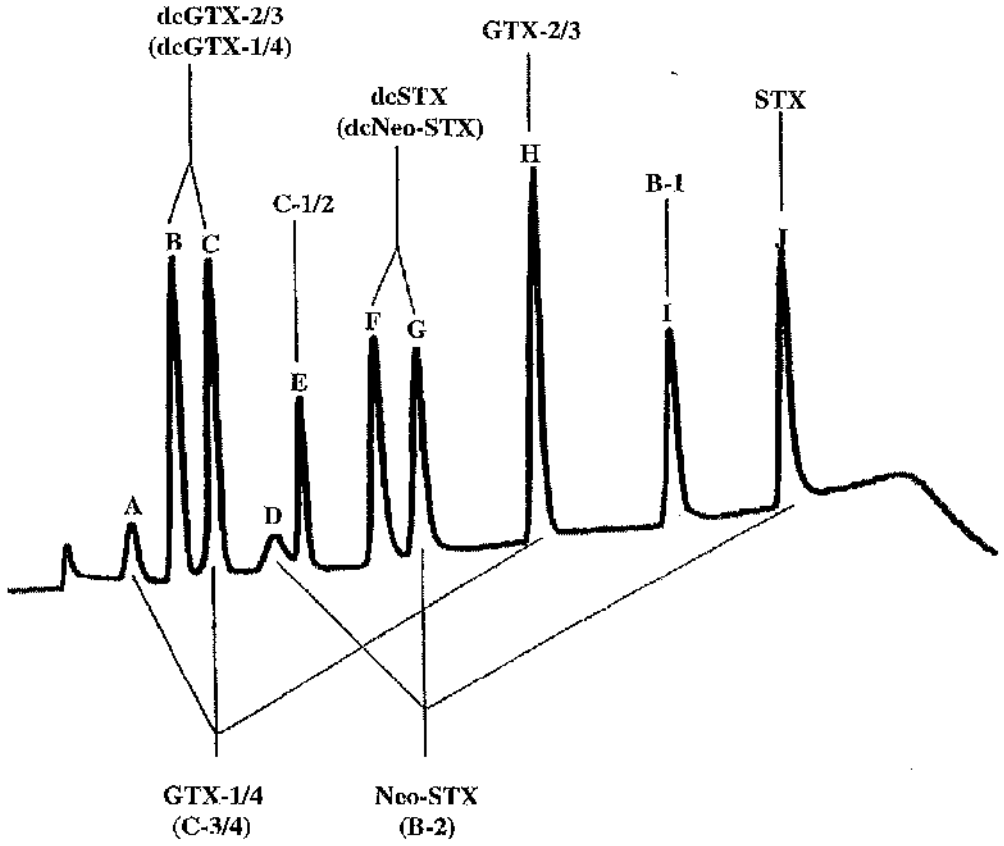


Figure 12. Hypothetical chromatogram for HPLC analysis of the oxidation products of PSP toxins. Adapted from Lawrence *et al.* (1996).

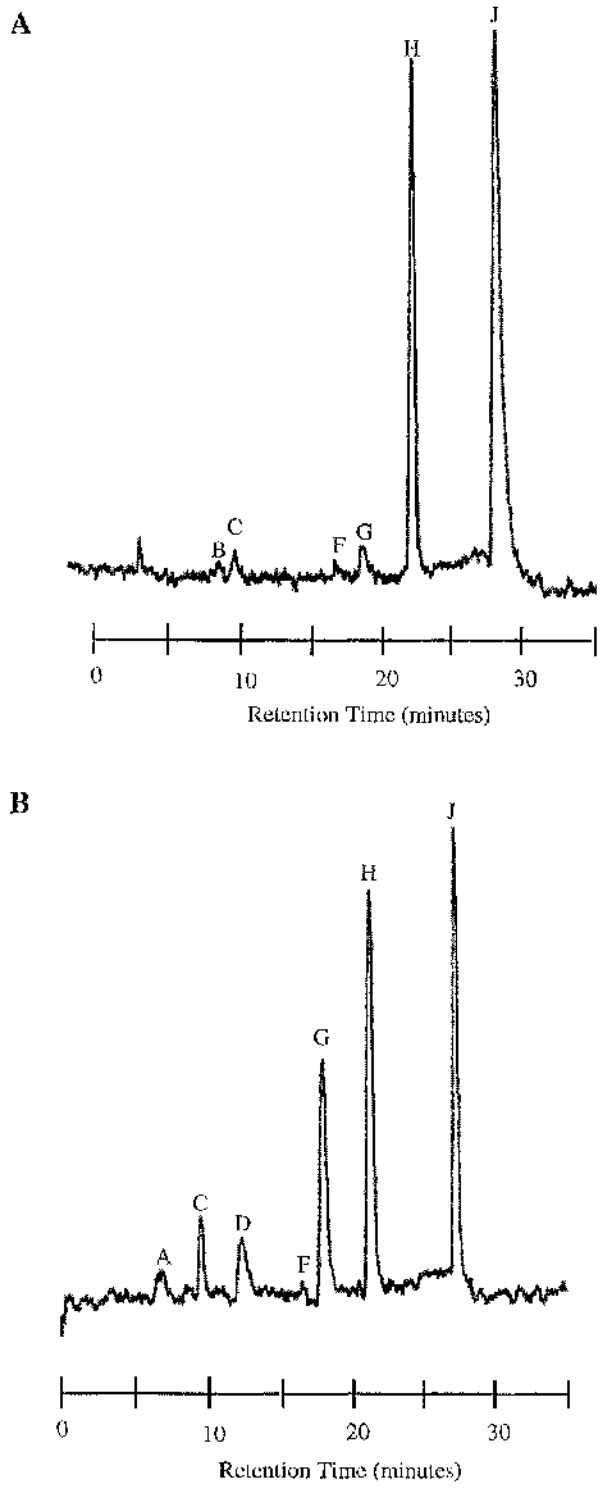


Figure 13. HPLC analysis of pre-column oxidised PSP toxin standards. PSP-1B toxin standards were diluted $^{1}/_{100}$ in 0·05M acetic acid and oxidised by peroxide (**A**) and periodate (**B**) prior to HPLC analysis by the method of Lawrence *et al.* (1991). Peaks labelled A to D, F to H and J correspond to the hypothetical peaks in figure 12.

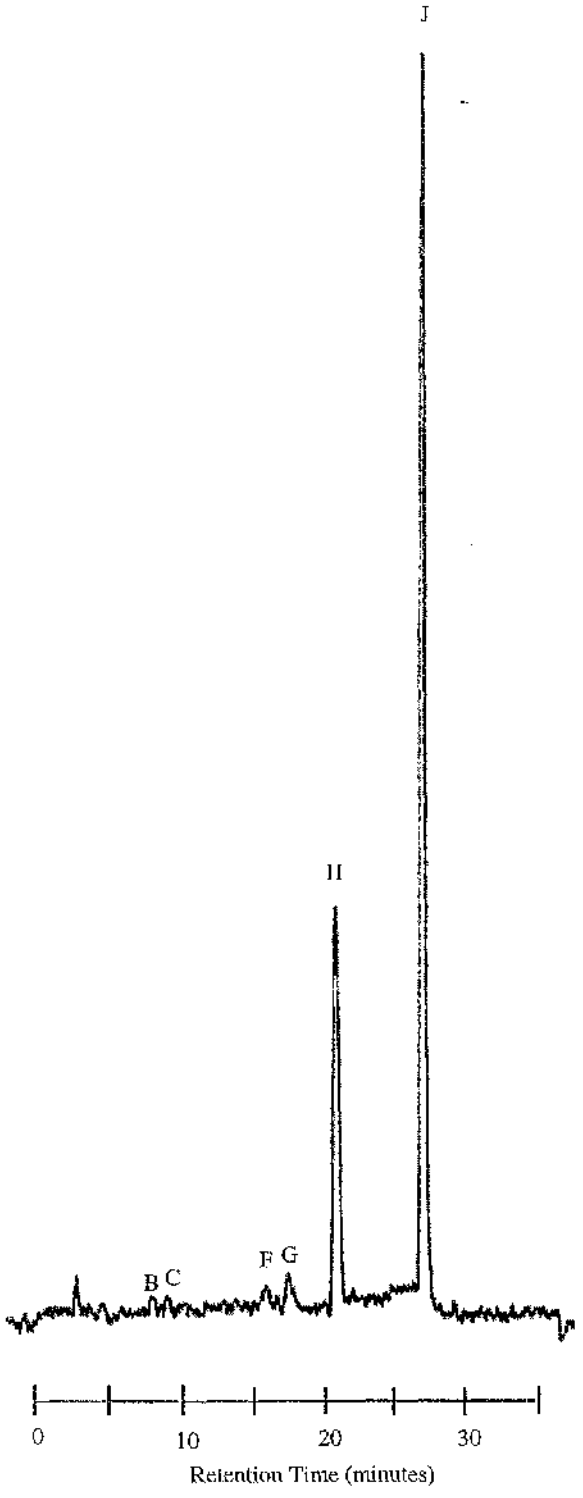


Figure 14. HPLC analysis of pre-column oxidised PSP toxin standards. PSP-1B toxin standards were diluted $^{1}/_{100}$ in marine broth and oxidised by peroxide prior to HPLC analysis by the method of Lawrence *et al.* (1991). Peaks labelled B, C, F, G, H & J correspond to the hypothetical peaks in figure 12.

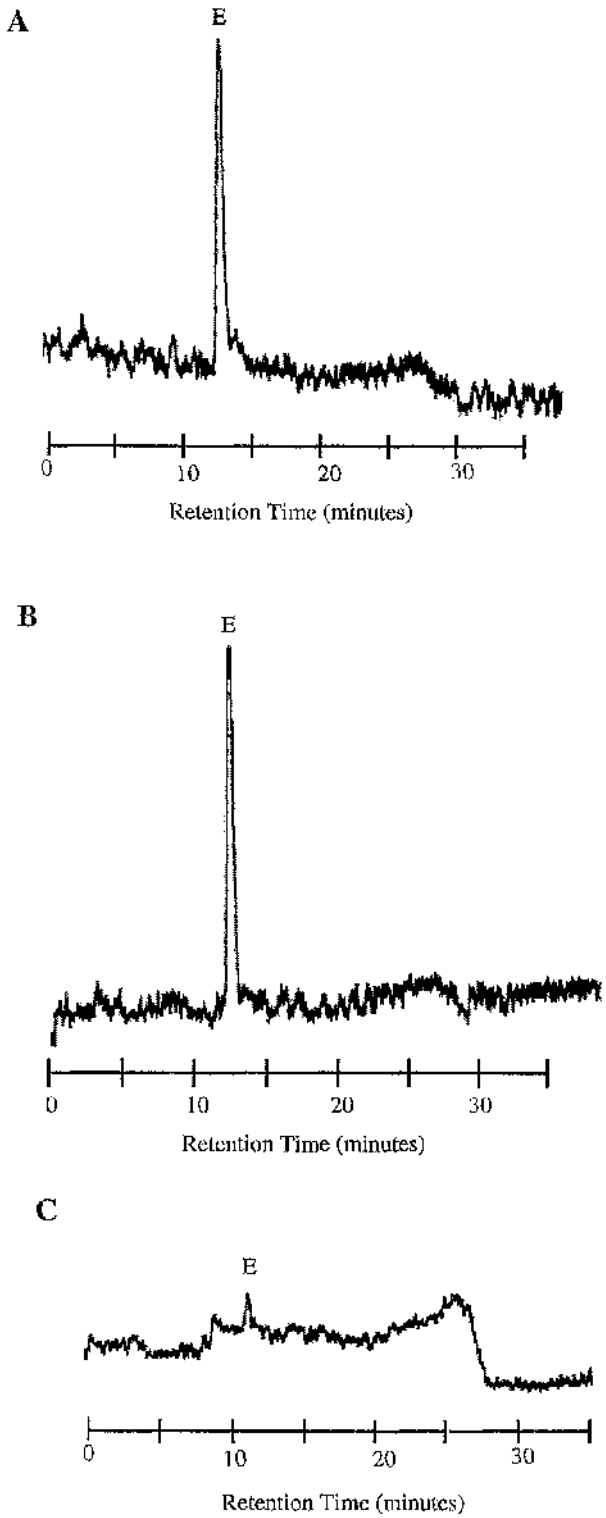


Figure 15. HPLC analysis of pre-column oxidised C1 toxin standard. C1 toxin standard was diluted $^{1}/_{10}$ in 0·05M acetic acid (**A** & **C**) and marine broth (**B**). The diluted toxin was oxidised by peroxide (**A** & **B**) or periodate (**C**) prior to HPLC analysis by the method of Lawrence *et al.* (1991). Peaks labelled E corresponds to the hypothetical peak in figure 12.

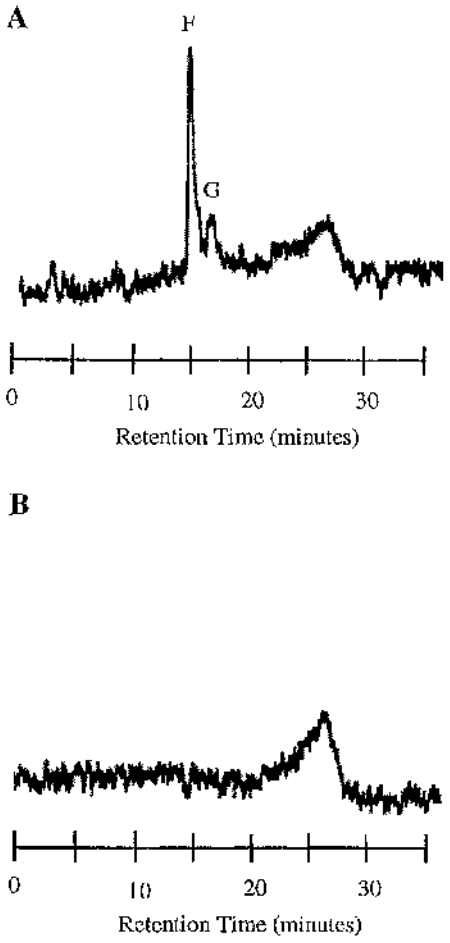


Figure 16. HPLC analysis of pre-column oxidised dcSTX toxin standard. A dcSTX standard was prepared by boiling STX in 7·5M HCl for 5·5 hours. The standard was diluted $^1/_{10}$ in dH_2O and oxidised by peroxide (**A**) or periodate (**B**) prior to HPLC analysis by the method of Lawrence *et al.* (1991). Peaks labelled F and G corresponds to the hypothetical peaks in figure 12.

size (more than twice the height) (see Figure 14). No such increase are observed in periodate oxidised PSP standards.

Pre-column oxidation HPLC analysis of C1 toxin standard gave a peak with a retention time of 13 minutes (see Figure 15). This peak, which corresponds to peak E in the hypothetical chromatogram (Figure 12), was very small in the analysis of periodate oxidised C1 but was present (Figure 15c). There was no significant difference in peak height when the C1 standard was diluted in marine broth rather than 0·05M acetic acid prior to peroxide oxidation (Figure 15a and 15b).

Analysis of pre-column peroxide-oxidised dcSTX revealed two oxidation products (Figure 16a). These two peaks corresponded to peaks F and G in Figure 12 and had retention times of 15 and 16 minutes. No peaks were observed in the analysis of periodate-oxidised dcSTX (Figure 16b).

Pre-column oxidation HPLC analysis of A862 culture supernatants

Pre-column HPLC analysis of culture supernatant from a 10ml marine broth culture of the marine bacteria A862 revealed the presence of an unidentified peak with a retention time of around 16 minutes (Figure 17a). This peak was only present if the supernatant was oxidised by peroxide under alkaline conditions prior to HPLC analysis. This peak was not observed following acidic peroxide oxidation (see Figure 17b) or following oxidation with periodate (chromatogram not shown). HPLC analysis of untreated culture supernatant also failed to reveal a peak corresponding to peak U in Figure 14a (result not shown).

In an attempt to identify the peak present in peroxide-oxidised A862 culture supernatant, the supernatant was spiked with C1 and dcSTX toxin standards (Figure 18 and 19 respectively). Neither peak E, the oxidation product of C1 toxin (and also C2 toxin; see Figure 12), nor peaks F and G, the oxidation products of dcSTX, displayed identical retention times as the unidentified peak (U). This unidentified oxidation product is therefore not derived from $C_{1/2}$ or dcSTX which may be present in A862 culture supernatant.

Hydrolysis of the N-sulfocarbamoyl toxins with 0·2M hydrochloric acid at 100°C for 15 minutes results in the formation of the corresponding carbamate toxins (Franco and Fernández-Vila, 1993). For example, acid hydrolysis of B1 results in the formation of STX and the hydrolysis of C1 results in the formation of GTX 2. The effect of acid hydrolysis on the unidentified peak in A862 culture supernatants was examined by pre-column HPLC analysis of treated and untreated supernatant (see Figure 20). The unidentified peak was present in both acid hydrolysed and untreated samples following peroxide oxidation (Figure 20a and b respectively). A slight increase in peak area was observed in the acid hydrolysed sample.

All the results presented above involved HPLC analysis of the supernatant from strain A862 grown in 10ml of marine broth at 20°C for 48 hours. If, however, the culture volume was increased to 500ml, a different HPLC profile was observed (see Figure 21). A major fluorescent peak was present in both alkaline peroxide and acidic peroxide oxidation reactions. (The result shown in Figure 21 was obtained using A862 Sm^r, however similar results were observed with A862 wt). This peak had a similar retention time to peak U in Figure 17. The peak area was larger in the alkaline-oxidised sample than in the acidic-oxidised sample but the significance of this difference is not known. This peak was also observed in HPLC analysis of untreated and periodate oxidised culture supernatant (chromatograms not shown). The large amount of the fluorescent compound present in 500 ml culture supernatants made it difficult to determine whether there was a second compound present only in alkaline peroxide-oxidised supernatant (e.g. a peak corresponding to peak U in Figure 17).

The retention times of the unidentified peaks in 10 ml A862 culture supernatant and the 500 ml A862 culture supernatant were further compared by pre-column HPLC analysis. The culture supernatant of the 500 ml culture was diluted $^1/_{20}$ in order to give a similar concentration of unidentified peak to that found in the 10 ml A862 supernatant. The A862 10 ml culture supernatant was diluted ($^1/_2$) in order to keep peak heights within the instrument scale. The two different supernatants were analysed individually with both alkaline and acidic pre-column oxidation reactions (see Figure 22a to 22d). A sample of the 10ml A862 culture supernatant was then spiked with A862 culture supernatant from the 500 ml culture. The concentrations of supernatant in this spiked sample were identical to the diluted supernatants which were tested individually. This spiked sample was also tested by both alkaline and acidic pre-column oxidation (Figure 22e and 22f). The unidentified peaks (U) in the two different A862 culture supernatants had identical retention times as only one peak was present in the spiked supernatant sample. However, the fluorescent peak present in the individual analysis of the 10 ml culture supernatant was only present following alkaline peroxide-oxidation reactions. The unidentified peak in the 500 ml culture supernatant was present in both acidic and alkaline peroxide pre-column HPLC analysis.

Pre-column oxidation HPLC analysis of A862 cell extracts

The cells of a 500ml marine broth culture of A862 wt were extracted with 5 ml of 0·05M acetic acid as described in the Materials and Methods section. The cell extracts were subsequently analysed by pre-column oxidation HPLC analysis. The resulting

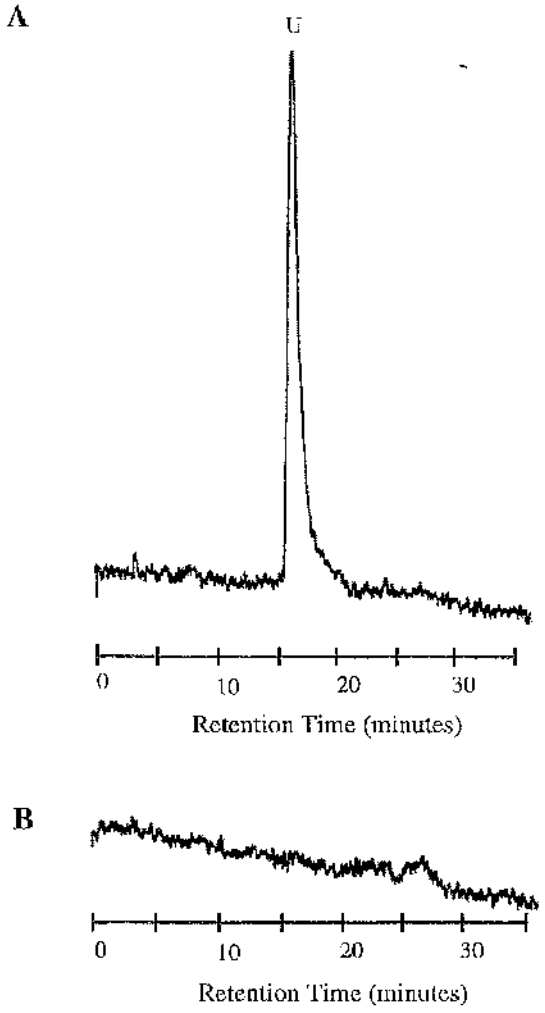


Figure 17. HPLC analysis of pre-column oxidised A862 wt culture supernatant. Culture supernatant from a 10ml culture of A862 wt was oxidised with peroxide in alkaline conditions (**A**) and in acidic conditions (**B**), prior to HPLC analysis by the method of Lawrence *et al.* (1991). The peak labelled U is referred to in the text.

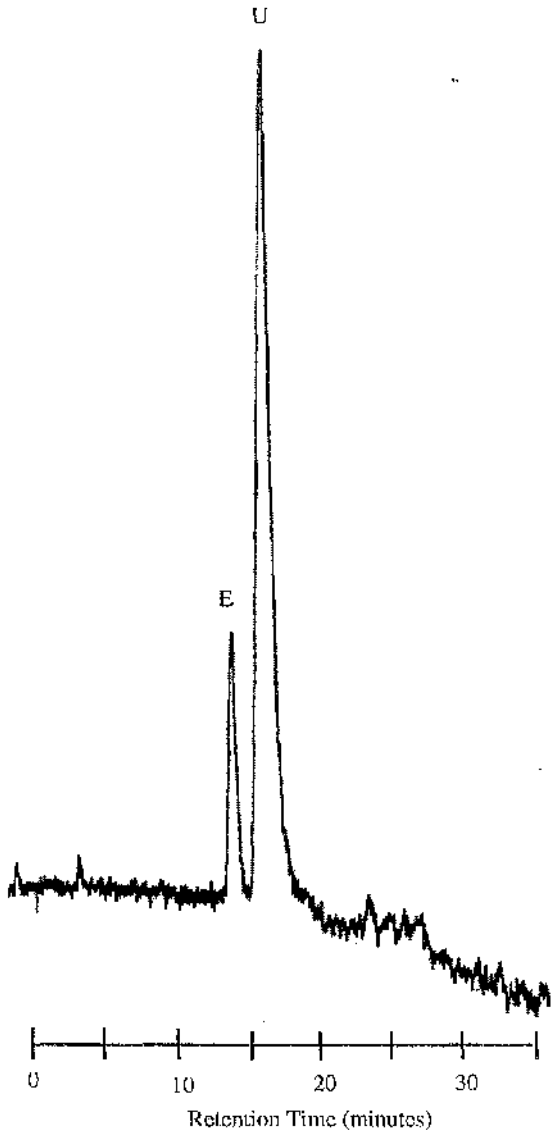


Figure 18. HPLC analysis of pre-column oxidised A862 wt culture supernatant spiked (1/10) with C1 standard. Culture supernatant from a 10ml culture of A862 wt was spiked (1/10) with C1 toxin standard. The spiked culture supernatant was oxidised with peroxide prior to HPLC analysis by the method of Lawrence *et al.* (1991). The peak labelled U is the unidentified peak present in A862 culture supernatant (see figure 17). The peak labelled E corresponds to the hypothetical peak in figure 12.

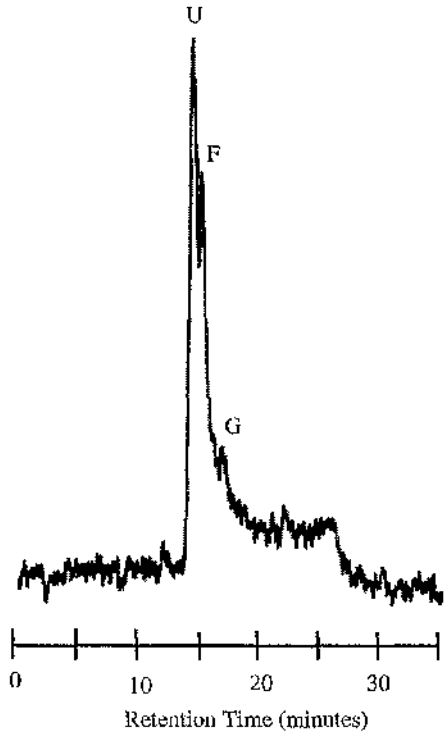


Figure 19. HPLC analysis of pre-column oxidised A862 wt culture supernatant spiked (1/10) with dcSTX standard. Culture supernatant from a 10ml culture of A862 wt was spiked (1/10) with dcSTX toxin standard (prepared by boiling STX in 7·5M HCl). The spiked culture supernatant was oxidised with peroxide prior to HPLC analysis by the method of Lawrence *et al.* (1991). The peak labelled U is the unidentified peak present in A862 culture supernatant (see figure 17). The peaks labelled F and G corresponds to the hypothetical peaks in figure 12.

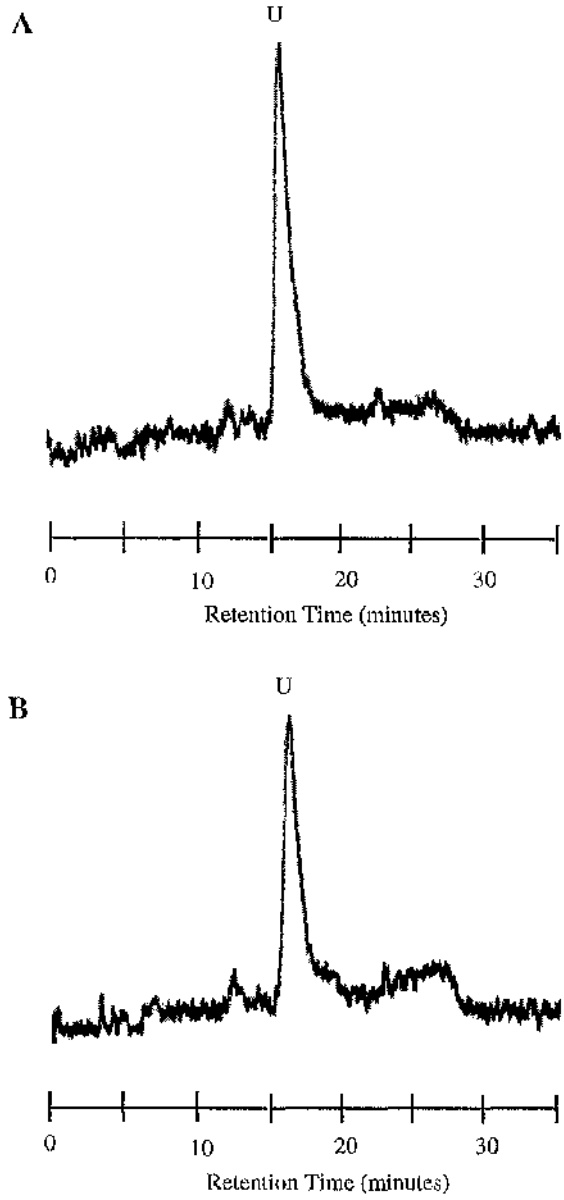


Figure 20. HPLC analysis of acid hydrolysed A862 wt culture supernatant with pre-column oxidation. Culture supernatant from a 10ml culture of A862 wt was acid hydrolysed in 0·2M HCl at 100°C (**A**). Another aliquot of the same supernatant was diluted $^1/_2$ in 0·05M acetic acid (**B**). The treated supernatants were oxidised with peroxide in alkaline conditions prior to HPLC analysis by the method of Lawrence *et al.* (1991).

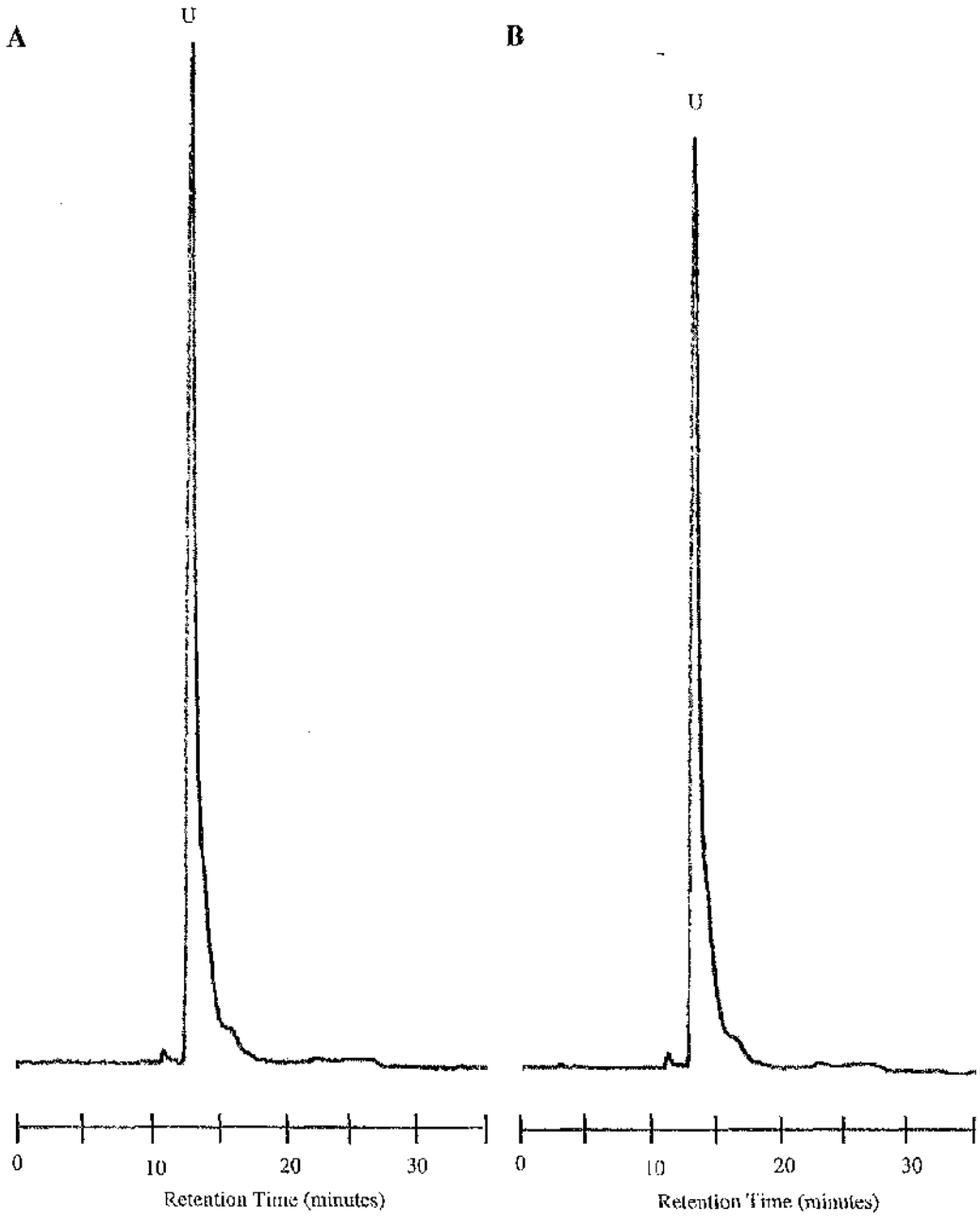


Figure 21. HPLC analysis of pre-column oxidised A862 Sm^r culture supernatant. Culture supernatant from a 500ml culture of A862 wt was oxidised with peroxide in alkaline conditions (**A**) and in acidic conditions (**B**), prior to HPLC analysis by the method of Lawrence *et al.* (1991). The peak labelled U is referred to in the text. The recorder was set 10 fold less sensitive than for the other pre-column HPLC analysis.

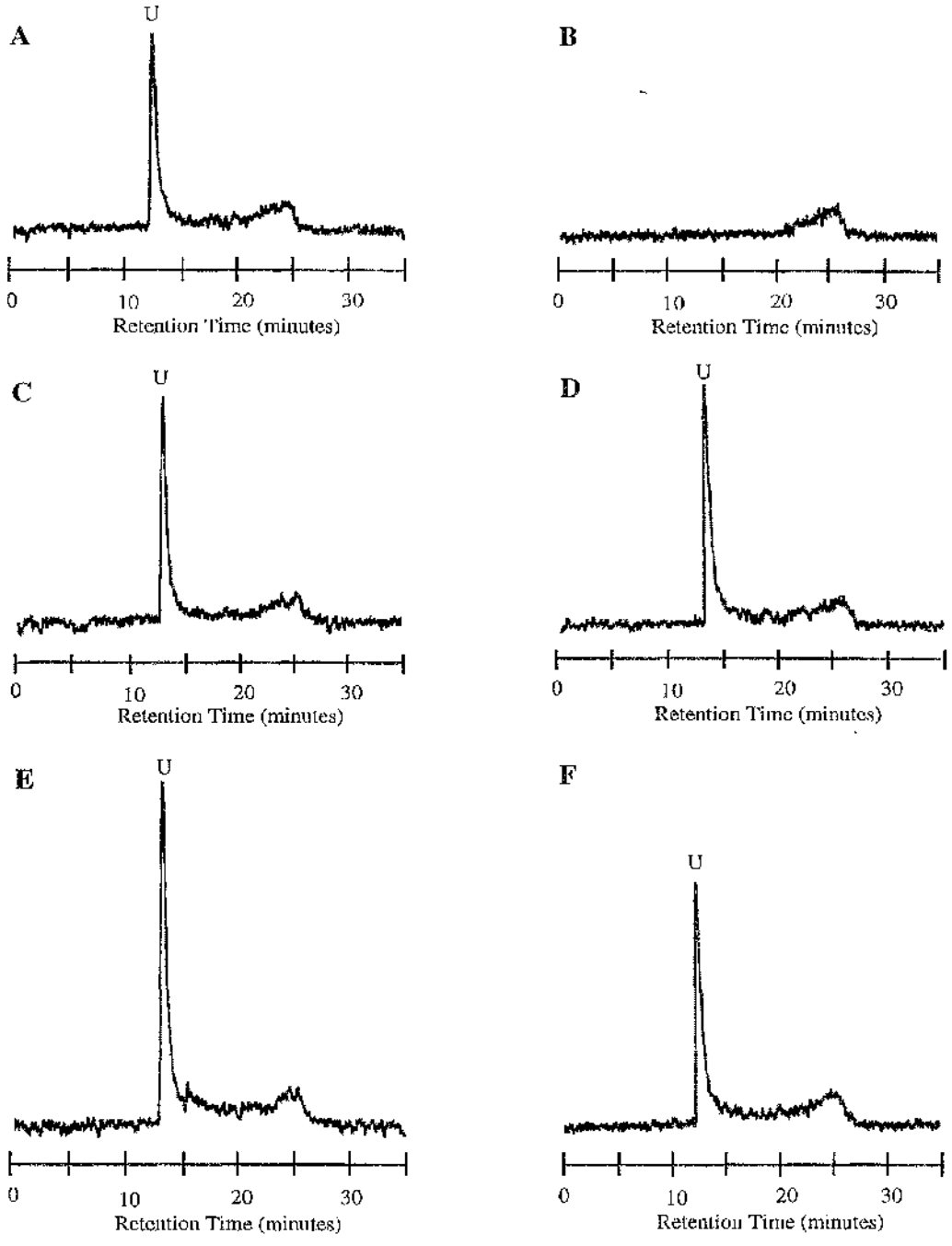


Figure 22. HPLC analysis of pre-column oxidised A862 culture supernatant from 10ml & 500ml cultures. Culture supernatant from a 10ml culture of A862 wt (**A** & **B**) was diluted $^1/_2$, and culture supernatant from a 500ml culture of A862 Sm^r (**C** & **D**) was diluted $^1/_{20}$. A new sample was prepared by spiking the 10ml culture supernatant with the 500ml supernatant to the give a final concentration of each identical to the diluted samples (**E** & **F**). These samples were oxidised with peroxide in alkaline conditions (**A**, **C** & **E**) and in acidic conditions (**B**, **D** & **F**), prior to HPLC analysis by the method of Lawrence *et al.* (1991). The peak labelled U is referred to in the text.

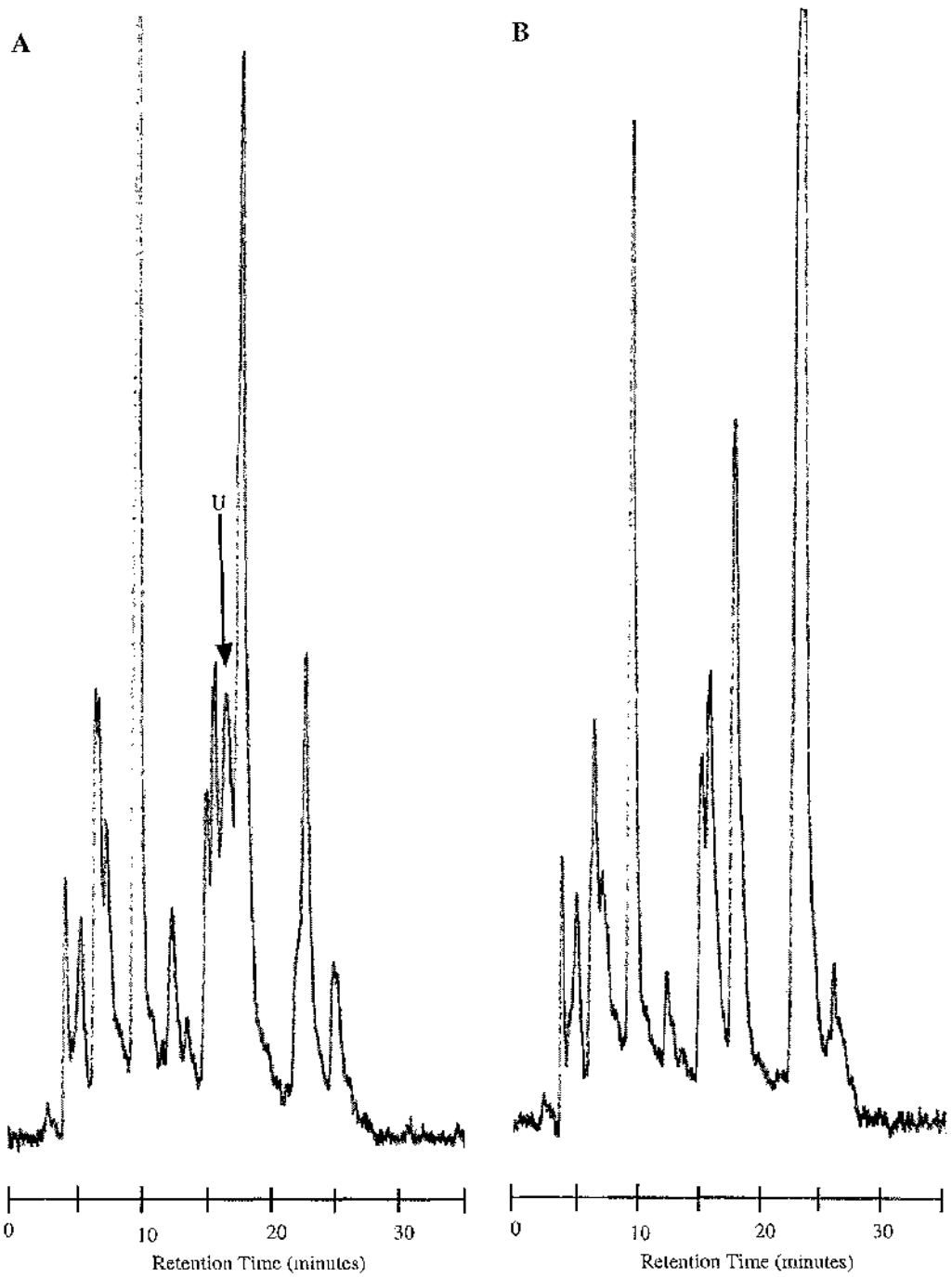


Figure 23. HPLC analysis of pre-column peroxide oxidised A862 wt cell extract. The cells of a 500ml culture of A862 wt were extracted in 0·05M acetic acid. The cell extract was oxidised with peroxide in alkaline conditions (**A**) and in acidic conditions (**B**), prior to HPLC analysis by the method of Lawrence *et al.* (1991). The peak labelled U is referred to in the text.

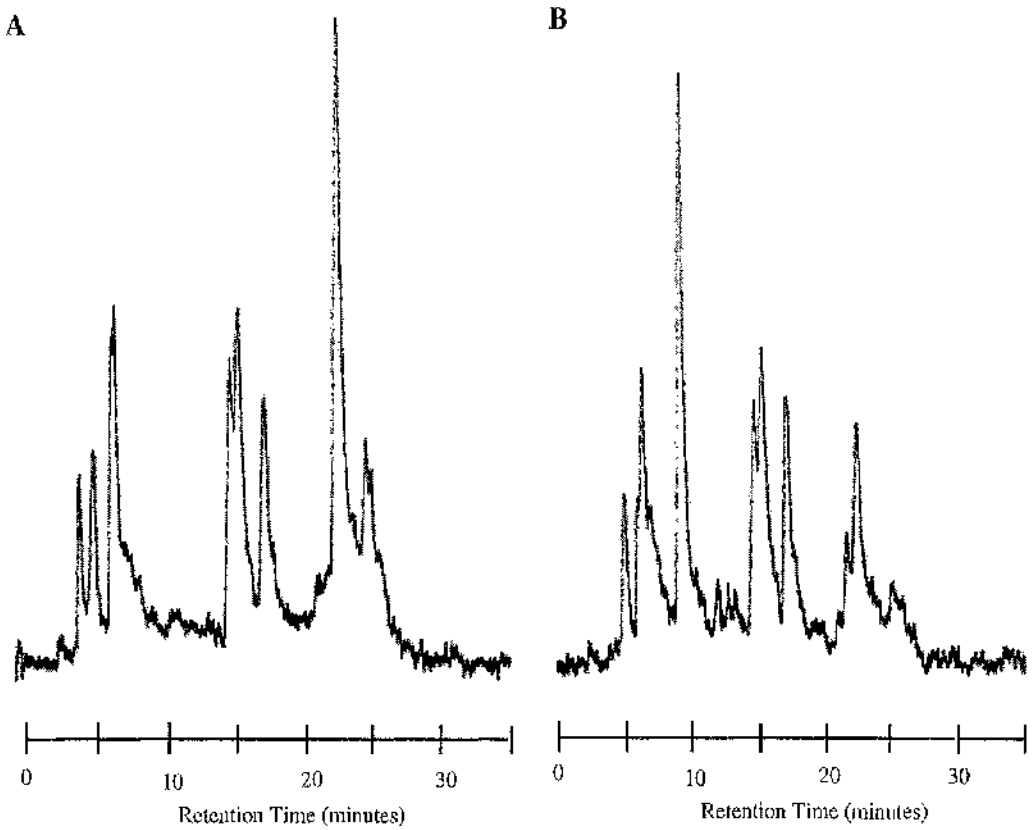


Figure 24. HPLC analysis of pre-column periodate oxidised A862 wt cell extract. The cells of a 500ml culture of A862 wt were extracted in 0·05M acetic acid. The cell extract was oxidised with periodate in alkaline conditions (**A**) and in acidic conditions (**B**), prior to HPLC analysis by the method of Lawrence *et al.* (1991).

chromatograms (see Figure 23 and 24) are difficult to interpret as there are numerous peaks, with many overlapping. However, a close comparison of the alkaline oxidised HPLC analysis (Figure 23a and 24a) and the acidic HPLC analysis (Figure 23b and 24b) revealed that the majority of the peaks were present in both the acidic and alkaline oxidation analysis. Some of these peaks did however display differences in peak height depending on the oxidation reaction conditions used (i.e. acidic or alkaline). Of particular note was the presence of the peak labelled U in the alkaline peroxide oxidised samples. This peak was absent from the acidic peroxide oxidation and both periodate oxidation chromatograms. It is possible that this peak contained the same oxidation product responsible for the peak labelled U in alkaline peroxide-oxidised culture supernatants (Figure 17a).

Post-column oxidation analysis of strain A862

A862 wt and A862 Sm^r were cultured in 500 ml marine broth for 48 hours at 20°C. The cells from these cultures were recovered by centrifugation and the supernatants retained for analysis. The cells were extracted with 0·05M acetic acid as described in the Materials and Methods section. The culture supernatants and cell extracts from both of these cultures were analysed by post-column HPLC analysis using the method of Franco and Fernández-Vila (1993). The cell extracts were analysed by all three isocratic elution systems but the culture supernatants were only analysed by the first two. The cell extracts were tested with and without acid hydrolysis at 100°C for 15 minutes.

First isocratic system

As no neoSTX was available at the time of analysis only STX and dcSTX standards were used in the post-column HPLC analysis using the first isocratic elution (see Figure 25). STX had a retention time of 26 minutes and dcSTX had a retention time of 23 minutes.

None of the fluorescent peaks present in the analysis of A862 cell extracts and supernatants with the first isocratic elution have identical retention times to external STX and dcSTX standards (see Figures 26-28).Two peaks were observed in the analysis of A862 culture supernatant (peak P and Q in Figure 26) with similar retention times to the expected retention time of neoSTX (i.e. 12·5 min; Franco and Fernández-Vila, 1993). Unfortunately as no neoSTX standard was available conclusive statements about the similarity of the retention times of these peaks to that of neoSTX are not possible. However, the shape of peak P is quite different from the small asymmetric peak associated with neoSTX (Franco and Fernández-Vila, 1993). It is therefore unlikely that peak P is neoSTX.

When A862 cell extracts were hydrolysed with acid prior to analysis, a peak (S) was observed in A862 wt and A862 Sm^r cell extracts (Figure 27b and 28b). This peak has a similar retention time to a peak (Q) in A862 wt, and also to the expected retention time of neoSTX. If peak S does indeed correspond to the neoSTX peak, then the presence of this peak only after acid hydrolysis is indicative of B2 toxin (Franco and Fernández-Vila, 1993). However, further HPLC analysis of A862 extracts with neoSTX standards (preferably internal) is required before the identification of peak S as neoSTX can be confirmed.

Second isocratic system

Post-column HPLC analyses of A862 culture supernatants and A862 cell extracts were carried out on two separate occasions. A comparison of the analysis of GTX standards run on the different occasions (see Figure 29a, 30a and 31a) revealed a significant difference in the retention times of each standard. This was the result of an error in mobile phase preparation. Comparison of the chromatograms obtained for the different samples must therefore be carried out with reference to the appropriate GTX standard chromatogram.

Analysis of A862 wt and A862 Sm^r revealed a peak (X) with the same retention time as the GTX 4 standard in cell extracts and culture supernatants (see Figure 29 to 31). Acid hydrolysis of the A862 cell extracts prior to analysis resulted in a decrease in the amount of unretained material (peak W) and a corresponding increase in the height of peak X (Figure 30 and 31). This observation supports the identification of this peak as GTX 4 and may also indicate the presence of C4 in A862 cell extracts.

There is also a peak (Y) in A862 cell extracts which has the same retention time as the GTX 1 standard (Figure 30b and 31b). However, this peak decreases in height if the samples are acid hydrolysed prior to analysis (Figure 30c and 31c). The amount of GTX 1 in a sample will increase following acid hydrolysis if C3 is present. However, GTX 1 itself is stable under the conditions used for this hydrolysis. Therefore, at least a portion of the peak (Y) in the cell extracts consists of a substance other than GTX1. Although it is possible that the remaining peak (Y) after acid hydrolysis consists, at least in part, of GTX 1, this cannot be established by this form of HPLC analysis.

Another peak (Z; Figure 29) present in the culture supernatants of A862 has a similar retention time to GTX 2 and GTX 3. Also there is a minor peak (Z'; Figure 30 and 31) in the A862 cell extracts which may consist of the same material which forms peak Z as it has a very similar retention time. Unfortunately the retention time of these peaks lies between the retention time of the GTX 2 and GTX 3 toxin standards and is therefore probably neither of these toxins.

The HPLC profiles observed for A862 wt and A862 Sm^r differed from one another with regard to the amount of particular fluorescent compounds present in each of the samples. The differences were quantitative rather than qualitative. For example, A862 wt had a larger amount of the compounds forming peaks X and Y in the analysis of culture supernatant (Figure 29b) and cell extracts (Figure 30) than was found in the analysis of A862 Sm^r culture supernatant (Figure 29c) and cell extract (Figure 31). In contrast, however, the analysis of the cell extracts of A862 revealed that the compound forming peak W was more abundant in A862 Sm^r (Figure 31) than in A862 wt (Figure 30). The differences in the HPLC profile of these samples are therefore not likely to be merely the consequence of a difference in the number of cells which formed the two samples.

Third isocratic system

Only the cell extracts of A862 wt and A862 Sm^r were analysed by post-column oxidation HPLC analysis with the third isocratic elution. The qualitative standard used for this analysis was an extract of a mixture of *Gymnodinium catenatum* plus *Alexandrium minutum*. The standards were untreated and acid hydrolysed prior to post-column oxidation HPLC analysis. Acid hydrolysis successfully converted the C toxins to the carbamate derivatives (see Figure 32).

As there was a large peak in the HPLC chromatograms coinciding with the solvent front (i.e. unretained fluorescent compounds) when A862 was analysed by the first and second isocratic systems (Figure 26 to 31), it was not surprising that when the third isocratic system, which includes an ion pair reagent in the elution buffer, was used for A862 cell extract analysis, a number of large fluorescent peaks were observed (Figure 33 and 34). One of these large peaks (J) has the same retention time as C2 toxin. If this peak was C2 however, it would be expected to decrease significantly following acid hydrolysis. No such decrease in the area of peak J was observed indicating that it is possibly not C2, but rather another substance with the same retention time.

A number of peaks (K, L, M and N) present in the analysis of A862 extracts have a similar retention time to C3 and the expected retention time of C4. It is not possible to state with any certainty which, if any, of these peaks correspond to these toxins. Only one of these peaks (peak L in Figure 33) decreases as a result of acid hydrolysis.

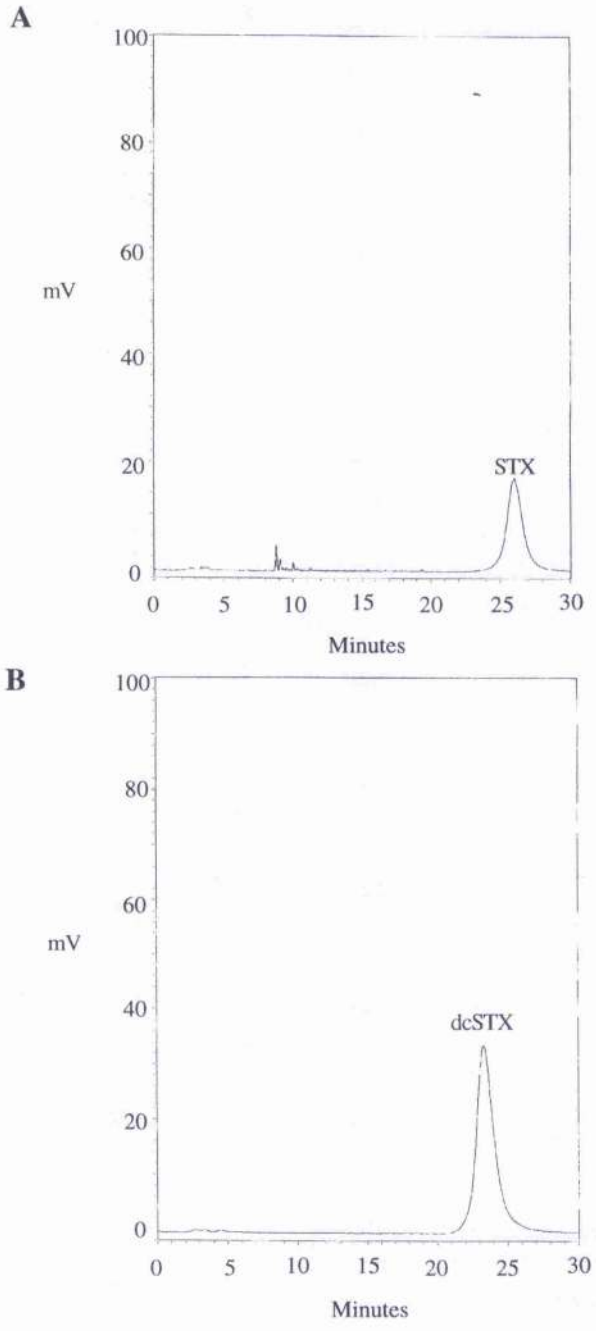


Figure 25. First isocratic system HPLC analysis of STX and dcSTX with post-column oxidation. Qualitative STX and dcSTX standards were analysed by the first isocratic system of Franco & Fernández-Vila (1993).

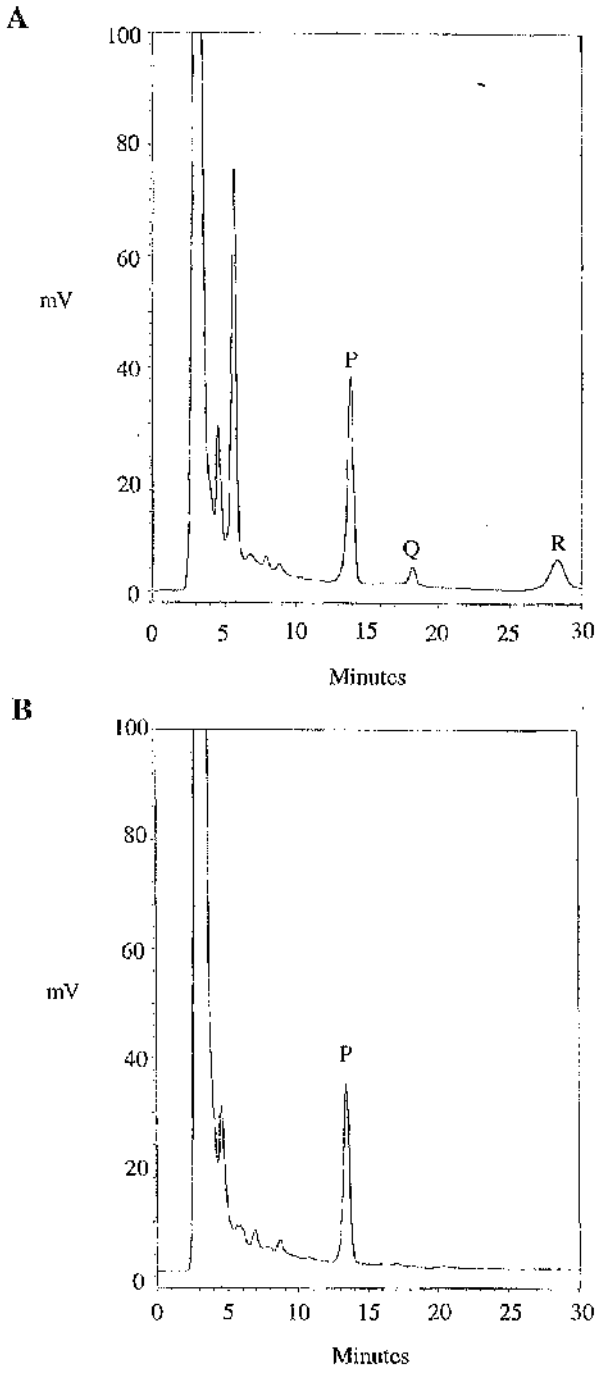


Figure 26. First isocratic system HPLC analysis of A862 culture supernatant with post-column oxidation. A862 wt culture supernatant (**A**) and A862 Sm^r culture supernatant (**B**) were analysed by the first isocratic system of Franco & Fernández-Vila (1993). Peaks P, Q & R are refered to in the text.

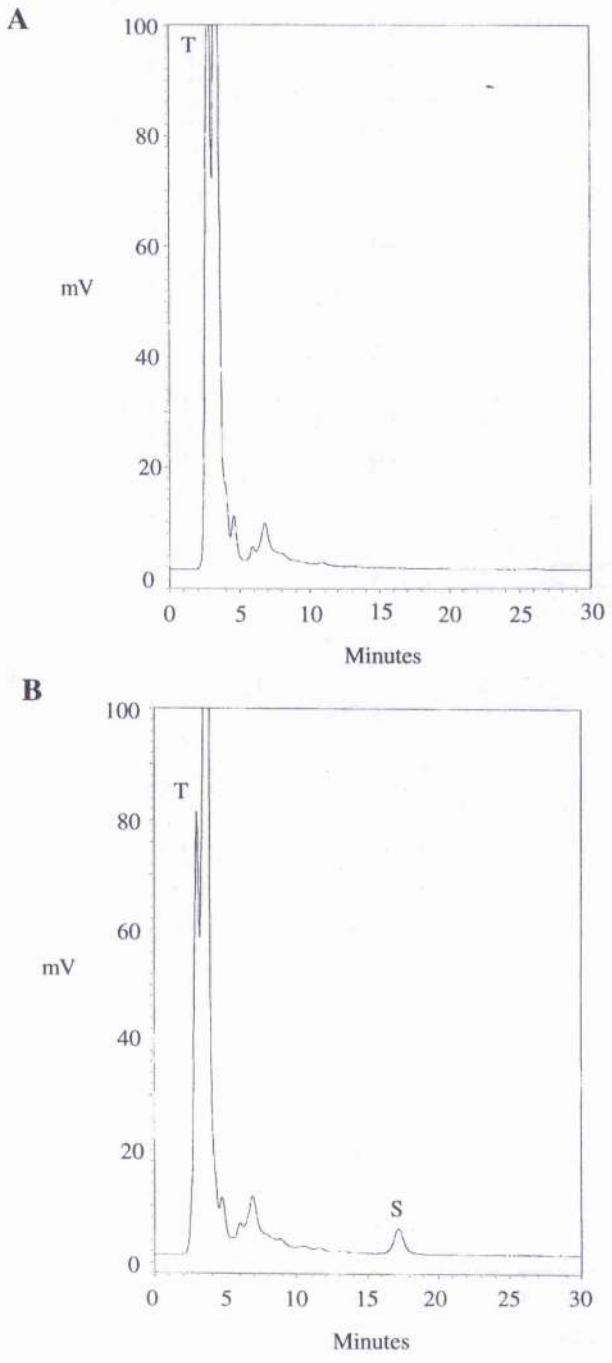


Figure 27. First isocratic system HPLC analysis of A862 wt cell extract with post-column oxidation. A862 wt cell extract was untreated (**A**) and acid hydrolysed (**B**) before analysis by the first isocratic system of Franco & Fernández-Vila (1993). Peaks T & S are refered to in the text.

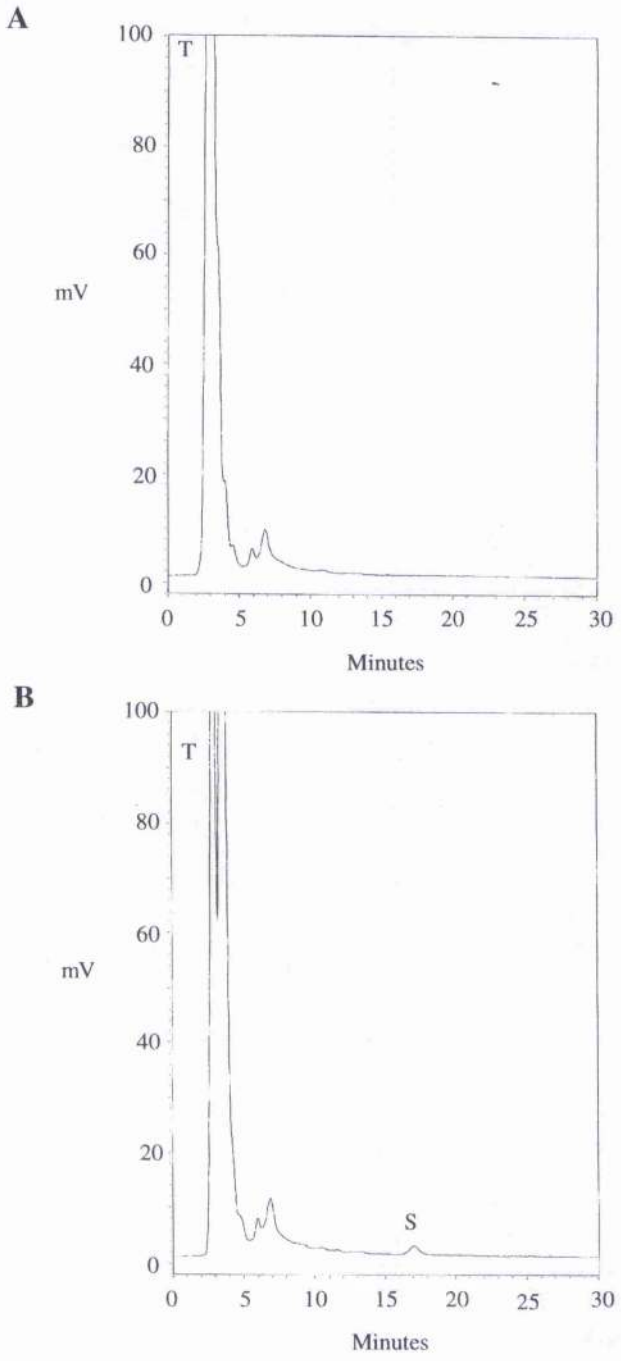


Figure 28. First isocratic system HPLC analysis of A862 Sm^r cell extract with post-column oxidation. A862 Sm^r cell extract was untreated (**A**) and acid hydrolysed (**B**) before analysis by the first isocratic system of Franco & Fernández-Vila (1993). Peaks T & S are refered to in the text.

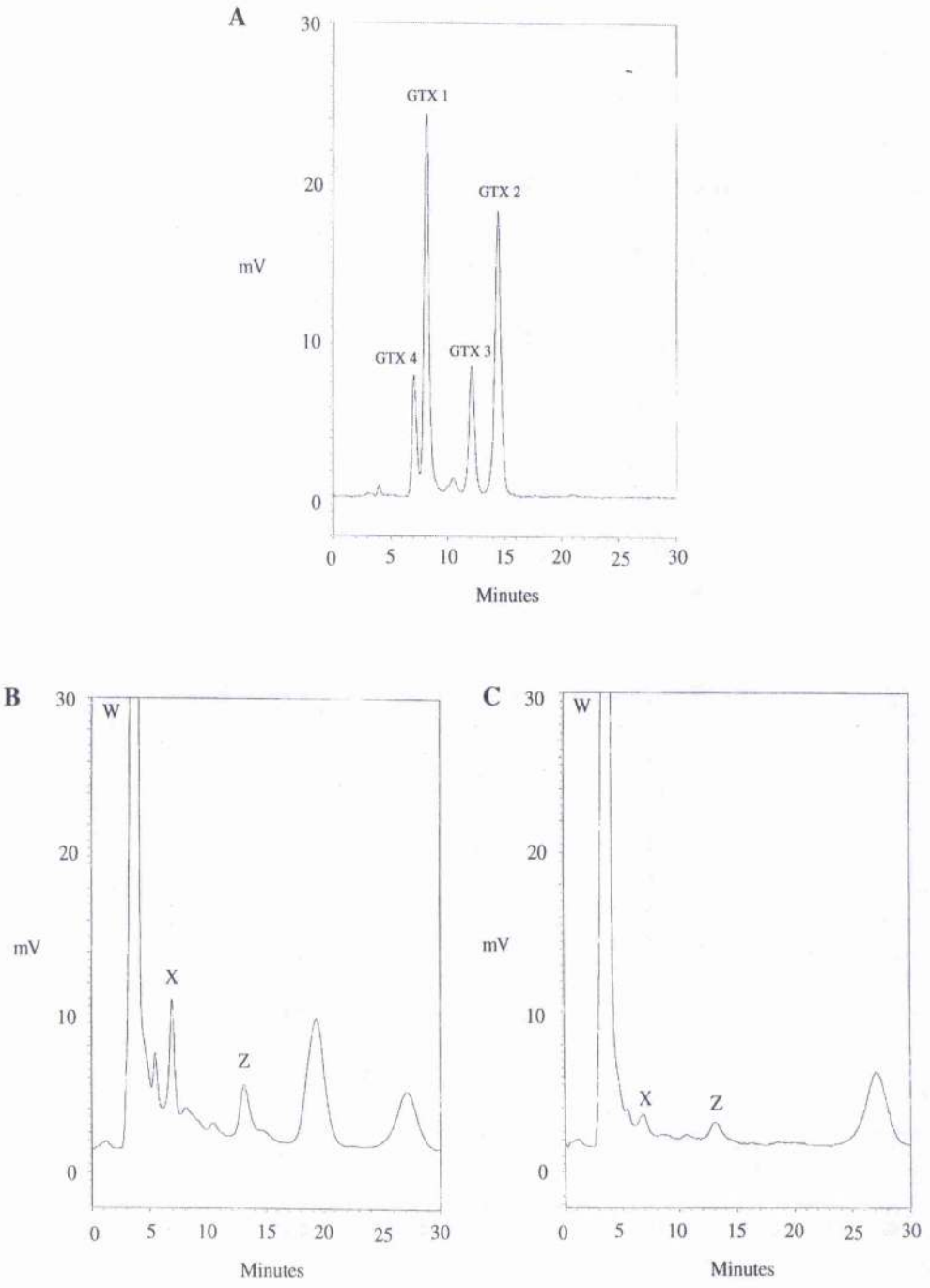


Figure 29. Second isocratic system HPLC analysis of GTX standards and A862 culture supernatant with post-column oxidation. GTX standards (**A**), A862 wt culture supernatant (**B**) and A862 Sm^r culture supernatant (**C**) were analysed by the second isocratic system of Franco & Fernández-Vila (1993). Peaks W, X & Z are refered to in the text.

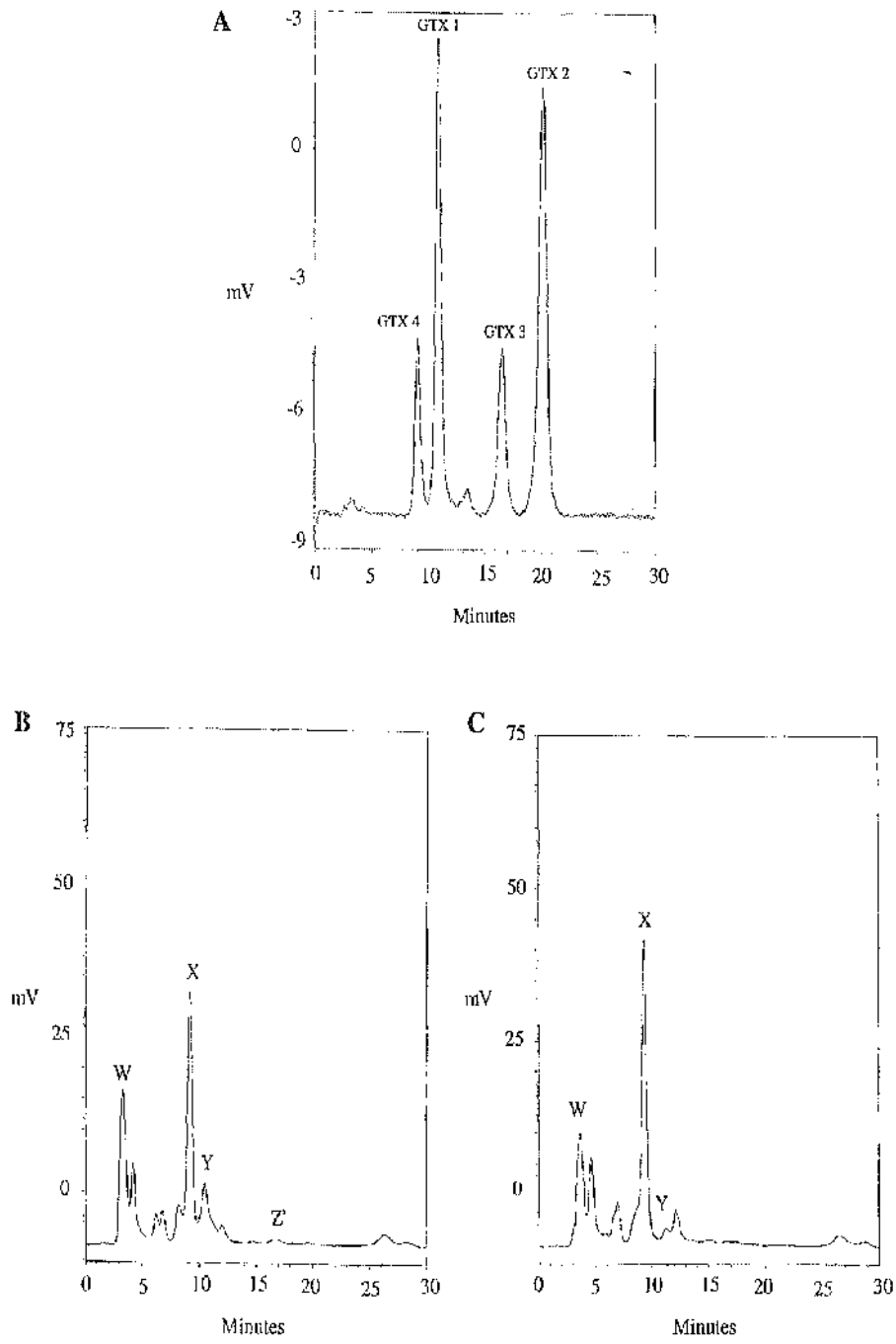


Figure 30. Second isocratic system HPLC analysis of GTX standards and A862 wt cell extract with post-column oxidation. GTX standards (**A**) and A862 wt cell extract (**B** & **C**) were analysed by the second isocratic system of Franco & Fernández-Vila (1993). A862 wt cell extract was untreated (**B**) and acid hydrolysed (**C**) before analysis. Peaks W, X, Y & Z' are refered to in the text.

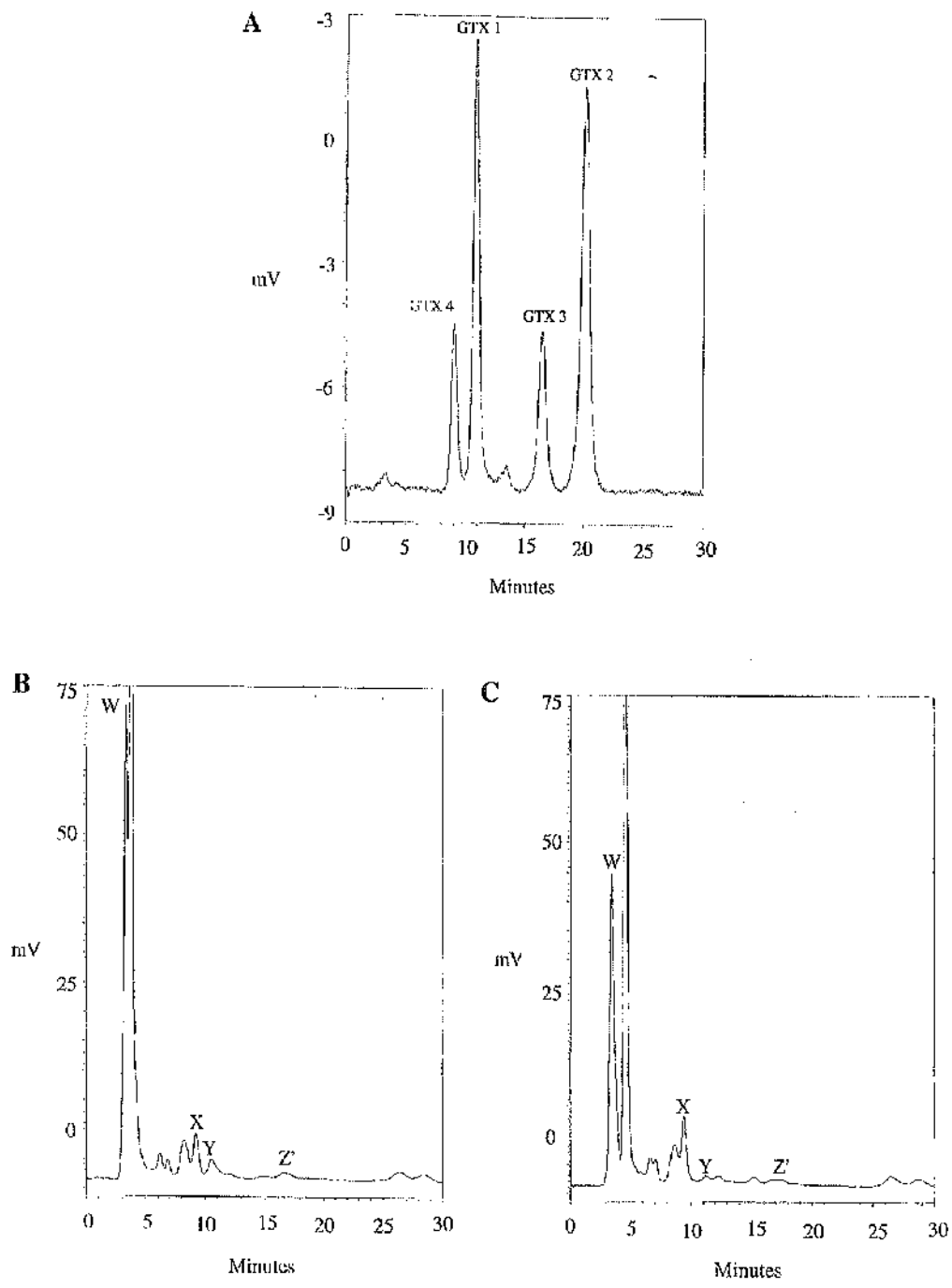


Figure 31. Second isocratic system HPLC analysis of GTX standards and A862 Sm^r cell extract with post-column oxidation. GTX standards (**A**) and A862 Sm^r cell extract (**B** & **C**) were analysed by the second isocratic system of Franco & Fernández-Vila (1993). A862 Sm^r cell extract was untreated (**B**) and acid hydrolysed (**C**) before analysis. Peaks W, X, Y & Z' are refered to in the text.

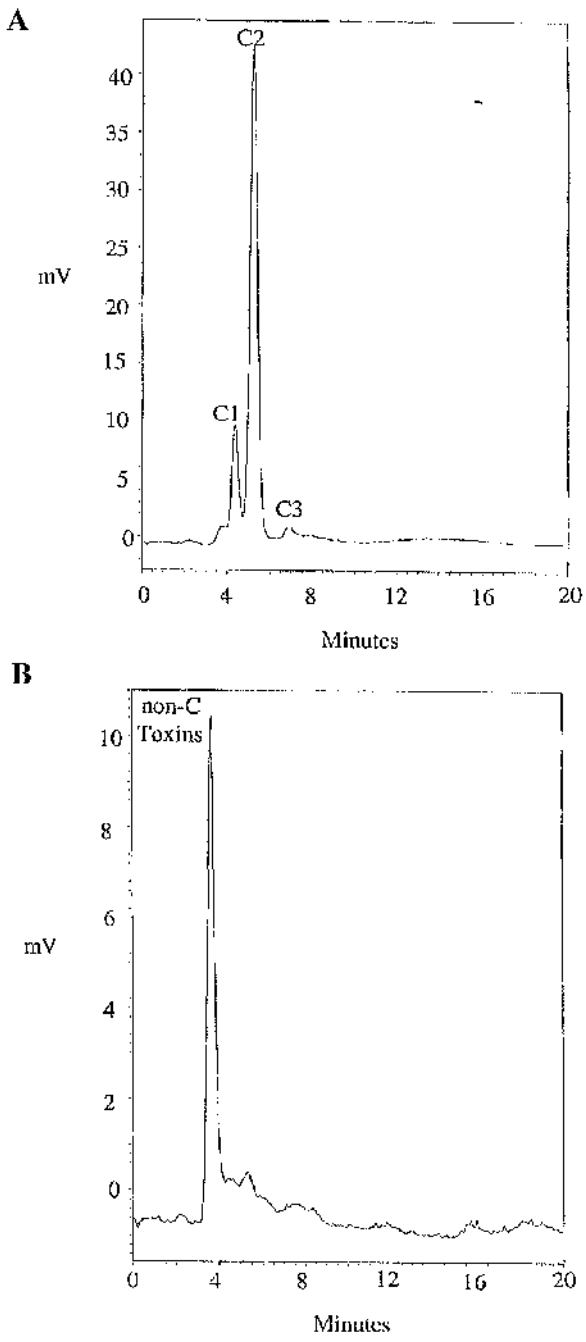


Figure 32. Third isocratic system HPLC analysis of C toxin standards with post-column oxidation. Qualitative C toxin standards (i.e. an extract of a mixture of *Gymnodinium catenatum* plus *Alexandrium minutum*) were untreated (**A**) and acid hydrolysed (**B**) before analysis by the third isocratic system of Franco & Fernández-Vila (1993).

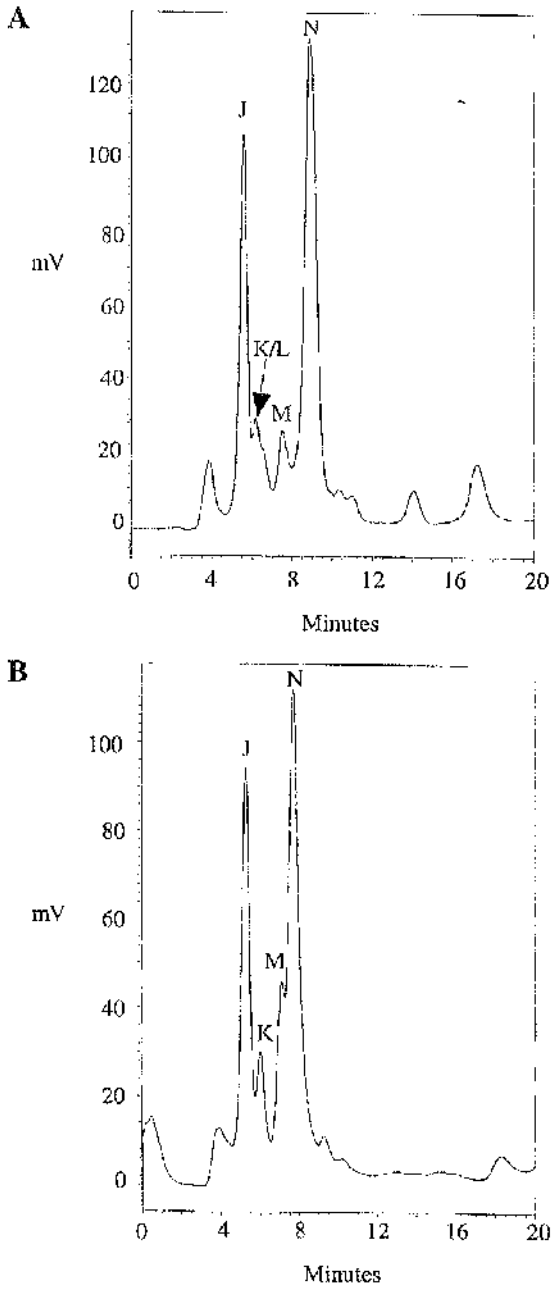


Figure 33. Third isocratic system HPLC analysis of A862 wt cell extract with post-column oxidation. A862 wt cell extract was untreated (**A**) and acid hydrolysed (**B**) before analysis by the third isocratic system of Franco & Fernández-Vila (1993). Peaks J, K, L, M & N are refered to in the text.

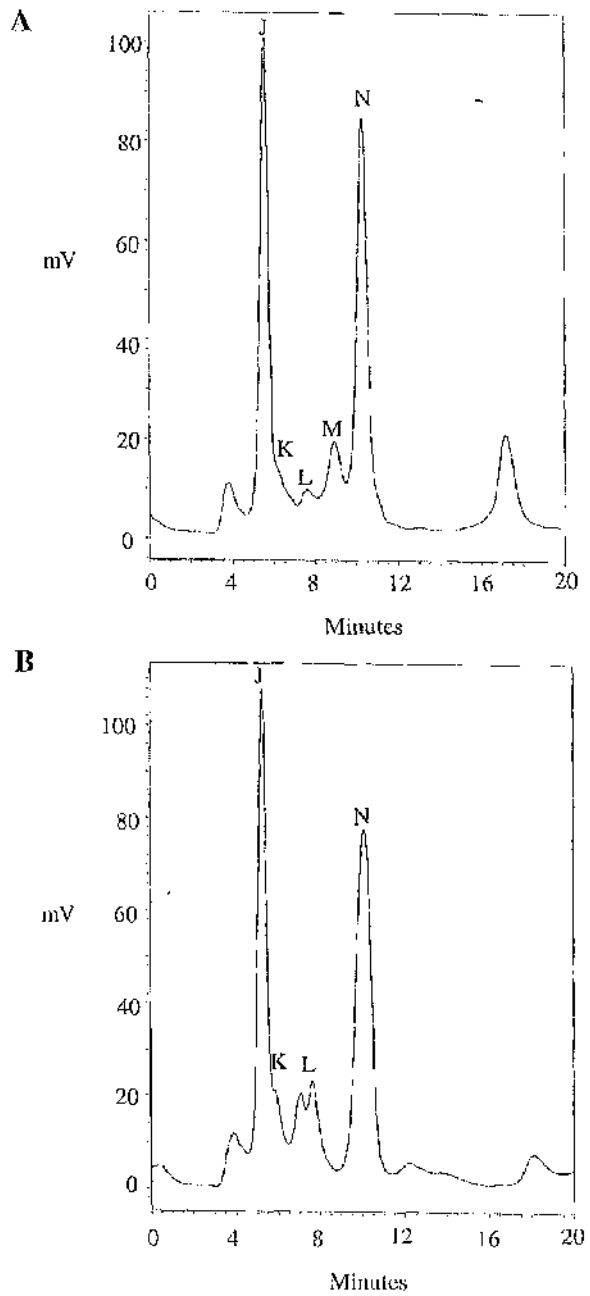


Figure 34. Third isocratic system HPLC analysis of A862 Sm^r cell extract with post-column oxidation. A862 Sm^r cell extract was untreated (**A**) and acid hydrolysed (**B**) before analysis by the third isocratic system of Franco & Fernández-Vila (1993). Peaks J, K, L, M & N are refered to in the text.

EVALUATION OF A CHEMICAL FLUORESCENCE ASSAY FOR THE DETECTION OF PSP TOXINS IN BACTERIA

In order to screen a large number of transposon-induced mutants for the loss of SCB toxin production, a simple, rapid assay must be used. One potential screening method is the chemical fluorescence assay first described by Bates and Rapoport (1975). The basis of this assay is the conversion of PSP toxins to fluorescent derivatives by alkaline oxidation with peroxide, followed by acidification (see Figure 4). The difference between the fluorescence of the oxidised sample and the fluorescence of a blank, unoxidised reaction is correlated to the amount of PSP toxin present in a sample.

Preliminary evaluation of the fluorescence assay

STX was diluted from a 100 μM stock solution to give a range of concentrations of STX from 0 μM to 2·5 μM in 0·05N acetic acid. The solutions (2 ml of each) were oxidised with peroxide as described in the Materials and Methods and a blank reaction also carried out for each. The fluorescence of the oxidised and blank reactions was determined with excitation at 330 nm and emission at 380 nm. The corrected fluorescence (i.e. fluorescence intensity of the peroxide oxidised reaction less the fluorescence intensity of the blank reaction) was plotted against STX concentration (see Figure 35). An excellent correlation was found between STX concentration and fluorescence intensity.

Buckley *et al.* (1978) reported that the different PSP derivatives do not fluoresce with equal intensity following alkaline oxidation with peroxide. In particular N-hydroxylated toxins (e.g. neoSTX, B2, $GTX_{1/4}$, etc.) were only poorly fluorescent following peroxide oxidation. The alkaline oxidation of PSP toxins is the basis of the fluorescence detection mechanism employed for HPLC analysis. The poor fluorescence of the N-hydroxylated PSP toxins has been partially overcome by oxidising with periodic acid rather than peroxide (Sullivan and Iwaoka, 1983; Lawrence and Menard, 1991; Franco and Fernández-Vila, 1993; Oshima, 1995b; Flynn and Flynn, 1996).

The alkaline oxidation reactions which are reported for pre-column oxidation HPLC analysis were evaluated as alternative reactions in the chemical fluorescence assay. STX at a concentration of 1 μM was oxidised in a scaled-up version of the pre-column oxidation reactions adapted from Lawrence *et al.* (1991) and Flynn and Flynn (1996) (see Materials and Methods for details). Blank reactions, consisting of reactions with the oxidant substituted by dH_2O were also performed. The

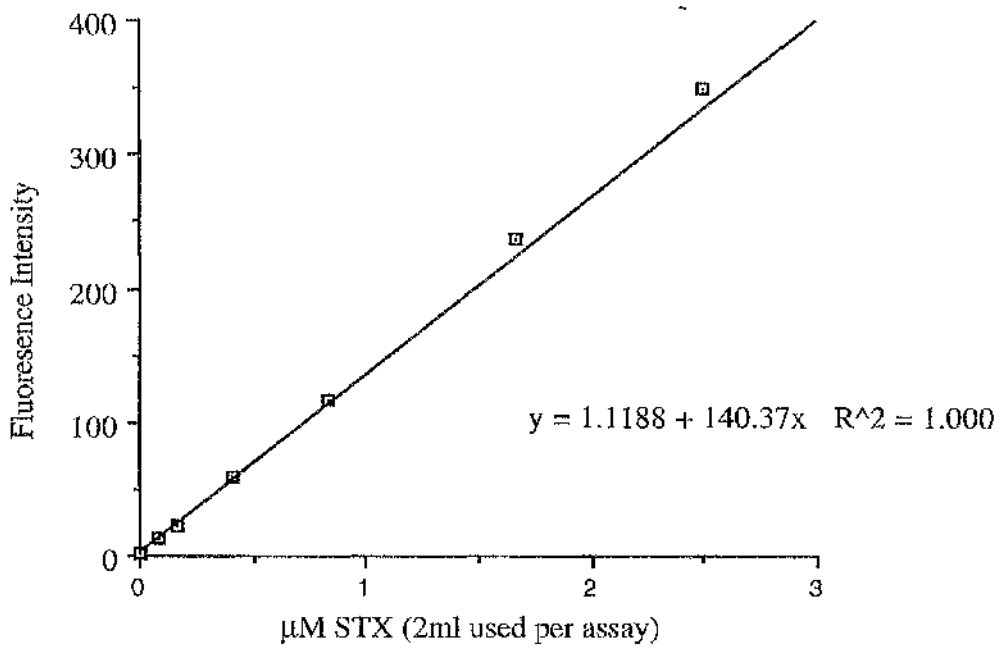


Figure 35. STX fluorescence standard curve following Bates and Rapoport oxidation protocol. A range of dilutions of STX were oxidised by the Bates and Rapoport (1975) oxidation reaction and the fluorescence determined with an excitation wavelength of 330nm and emission wavelength of 380nm. The results were corrected for background fluorescence by the subtraction of fluorescence recorded following a blank oxidation reaction (i.e. peroxide replaced by dH_2O).

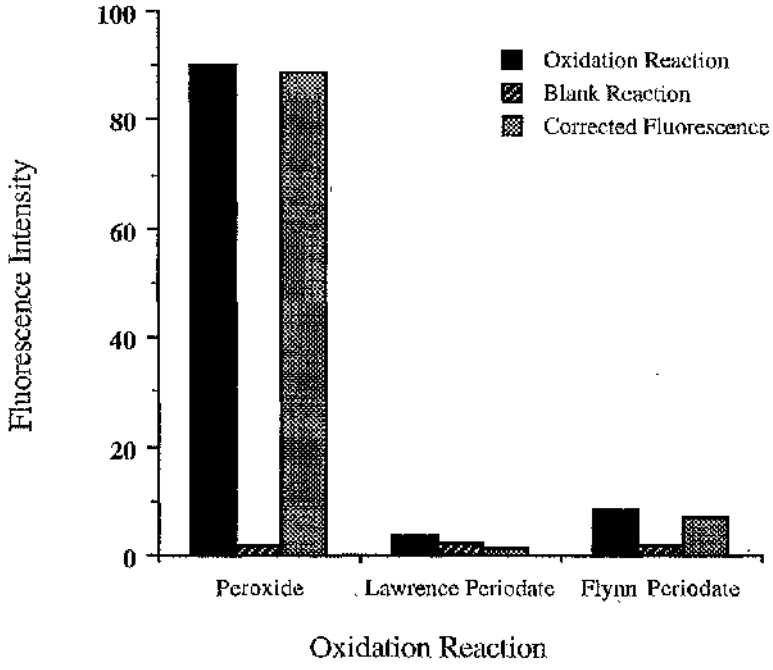


Figure 36. Fluorescence of STX following different oxidation reactions. The three reactions reported for pre-column oxidation prior to HPLC analysis of PSP toxins (Lawrence *et al.*, 1991 and Flynn and Flynn, 1996) were scaled up and used to oxidise 1μM STX. A blank reaction was performed for each type of oxidation by the substitution of dH_2O for the oxidant. Fluorescence was measured with excitation and emission wavelengths of 330nm of 380nm respectively. The corrected fluorescence (i.e. the fluorescence of the blank reaction subtracted from the fluorescence of the oxidised reaction) is a measure of the fluorescence being emitted by STX.

fluorescence of these reaction mixtures was determined (Figure 36) and the corrected fluorescence calculated as before.

A major difference between the oxidation reactions described in the original fluorescence assay (Bates and Rapoport, 1975) and the pre-column oxidation reactions (Lawrence *et al.*, 1991; 1995) is the length of time the reactions are allowed to proceed before acidification. The original assay had an incubation time of 40 minutes but pre-column oxidation is performed for 2 minutes by peroxide or 1 minute by periodate (Lawrence *et al.*, 1991; 1995). To investigate the effect of incubation time on the yield of fluorescent oxidation products, 1μM STX was oxidised by peroxide and periodate by the reactions adapted from Lawrence *et al.* (1991; 1995) and reactions were allowed to proceed for varying lengths of time before the addition of glacial acetic acid. To achieve a true zero time reading (T=0), acetic acid was added to the reaction prior to the addition of sample. The formation of fluorescent oxidation products appeared to be complete within 10 minutes of the addition of sample to peroxide or periodate reactions (Figure 37). The difference between 2 minutes and 10 minutes incubation time was not deemed sufficiently large to merit an increased incubation time.

The original Bates and Rapoport (1975) fluorescence assay was used to assay four bacterial strains (A862 wt, A862 Sm^r, A912 wt and 4αVS3) for the possible presence of PSP toxins (Figure 38). There was very little difference between the fluorescence of oxidised and blank reactions using A912 and 4αVS3 culture supernatants and cell extracts. If the corrected fluorescence is a measure of the PSP content of these samples then the concentration in these samples is below the level of detection of the assay. A862 wt and Sm^r culture supernatants produce a high level of fluorescence. The cell extracts of these strains also contain a higher level of fluorescence than found in the other two strains. As the fluorescence was present in both the blank and peroxide-oxidised reactions indicating that PSP is not responsible for all the fluorescence. However, there is a higher fluorescence intensity in the alkaline oxidation reaction than in the blank reaction, and thus a significant corrected fluorescence. If this corrected fluorescence is due to PSP toxin content then, using the STX standard curve (Figure 35), A862 wt cell extract contains 28 nm STX equivalent, A862 Sm^r cell extract contains 54 nm STX equivalent, A862 wt culture supernatant contains 804 nm STX equivalent and A862 Sm^r culture supernatant contains 800 nm STX equivalent.

The fluorescent properties of A862 cell extracts were further investigated using the adapted pre-column oxidation reactions (adapted from Lawrence *et al.*, 1991) with peroxide and periodate treatment (Figure 39). The corrected fluorescence intensity obtained for the peroxide oxidation reaction (Figure 39a) was comparable with the results obtained by the Bates and Rapoport oxidation reaction (Figure 38).

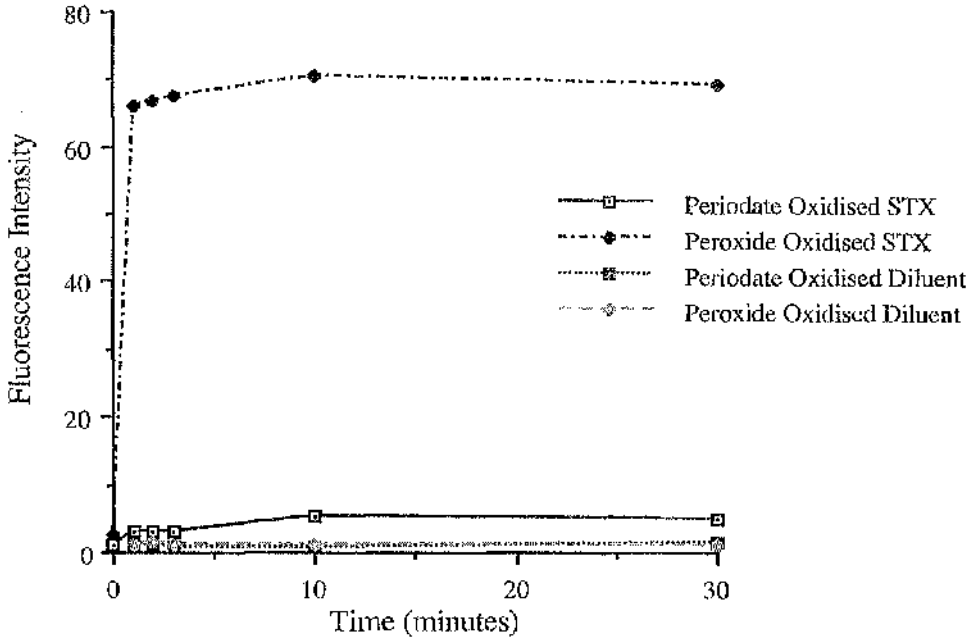


Figure 37. Fluorescence of STX following oxidation reactions with different periods of incubation. STX (1μM) was oxidised by the scaled up peroxide and periodate oxidation reactions of Lawrence *et al.*, (1991). The reactions were allowed to proceed for different periods of time before the addition of glacial acetic acid. (For the t = 0 readings acetic acid was added prior to the addition of sample). Fluorescence was measured with excitation and emission wavelengths of 330nm of 380nm respectively. Diluent (0·05N acetic acid) controls were treated in the same way as the 1μM STX.

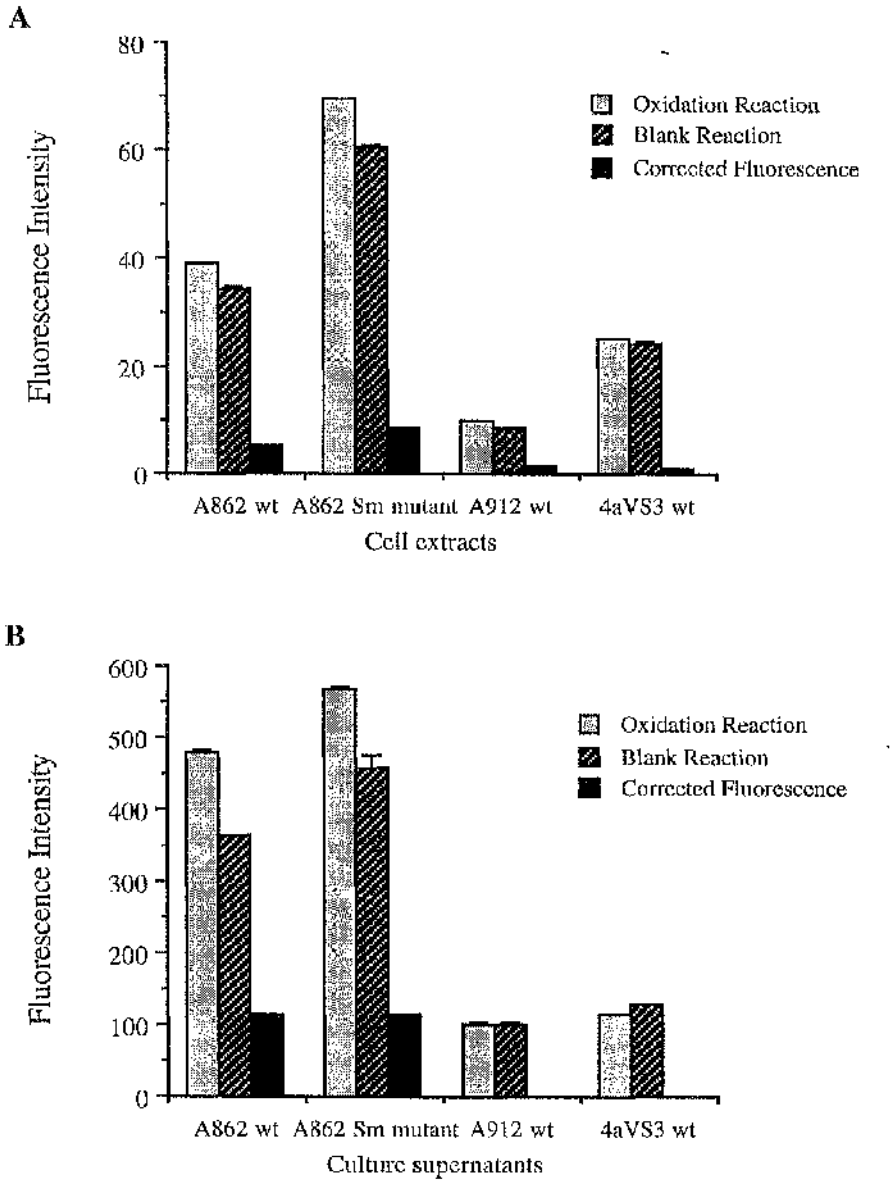


Figure 38. Fluorescence of different marine bacterial strains following Bates and Rapoport oxidation reaction. Cell extracts (**A**) and culture supernatants (**B**) of different bacterial strains were oxidised by the Bates and Rapoport (1975) peroxide oxidation reaction. The blank reaction contained dH_2O in place of peroxide. Fluorescence was measured with excitation and emission wavelengths of 330nm of 380nm respectively. The corrected fluorescence intensity (i.e. the fluorescence intensity of the blank reaction subtracted from the fluorescence intensity of the oxidised reaction) may be a measure of the fluorescence being emitted by PSP toxins in the sample.

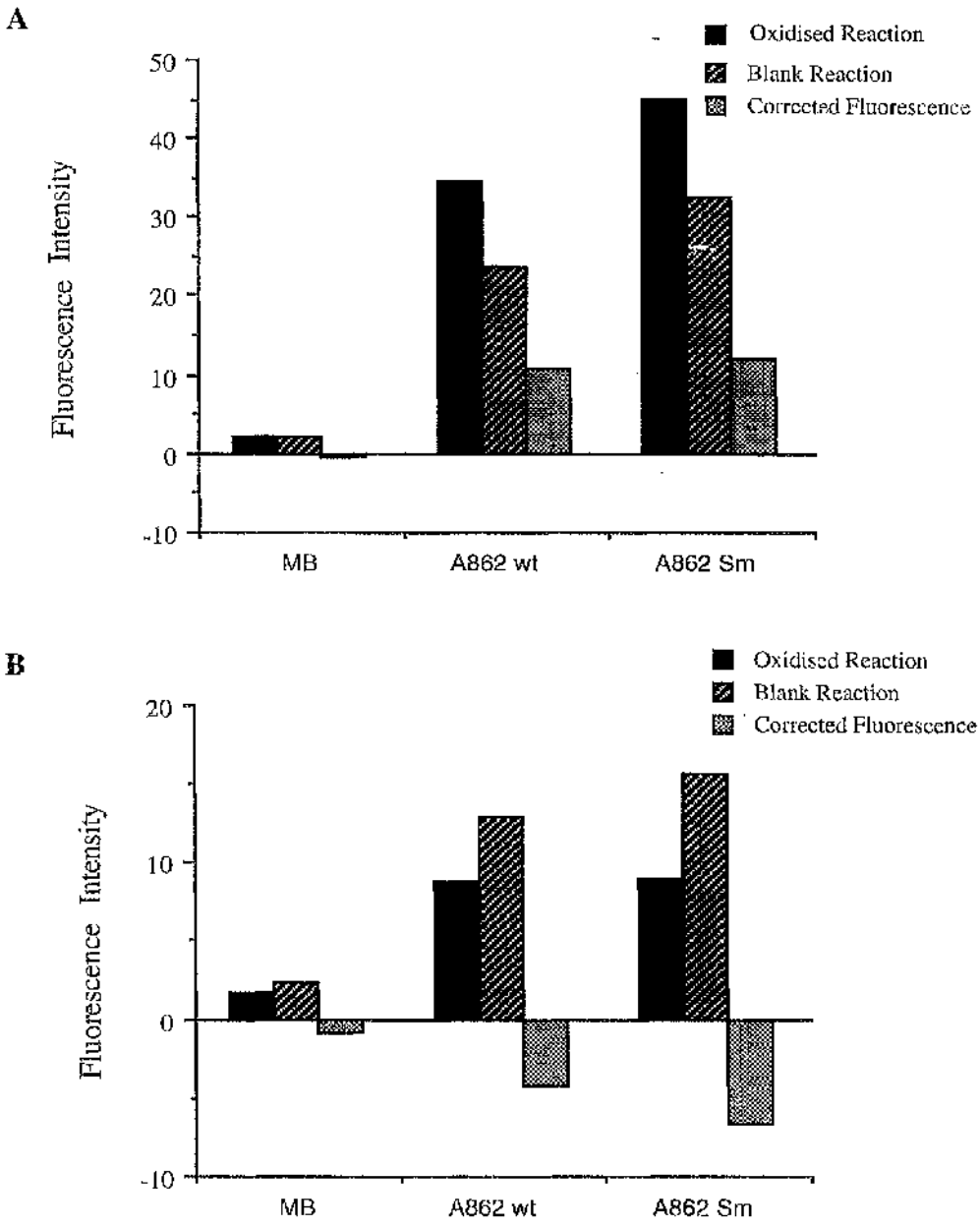


Figure 39. Fluorescence of oxidised A862 cell extracts. Cell extracts from A862 wt and A862 Sm^r cultures, together with a negative control (marine broth; MB) were oxidised by the scaled up peroxide (**A**) and periodate (**B**) oxidation reactions of Lawrence *et al.*, (1991). A blank reaction was also performed for each type of oxidation (i.e. a reaction in which the oxidant is substituted for dH_2O Fluorescence was measured with excitation and emission wavelengths of 330nm of 380nm respectively. The corrected fluorescence intensity is the fluorescence intensity of the blank reaction subtracted from the fluorescence intensity of the oxidised reaction.

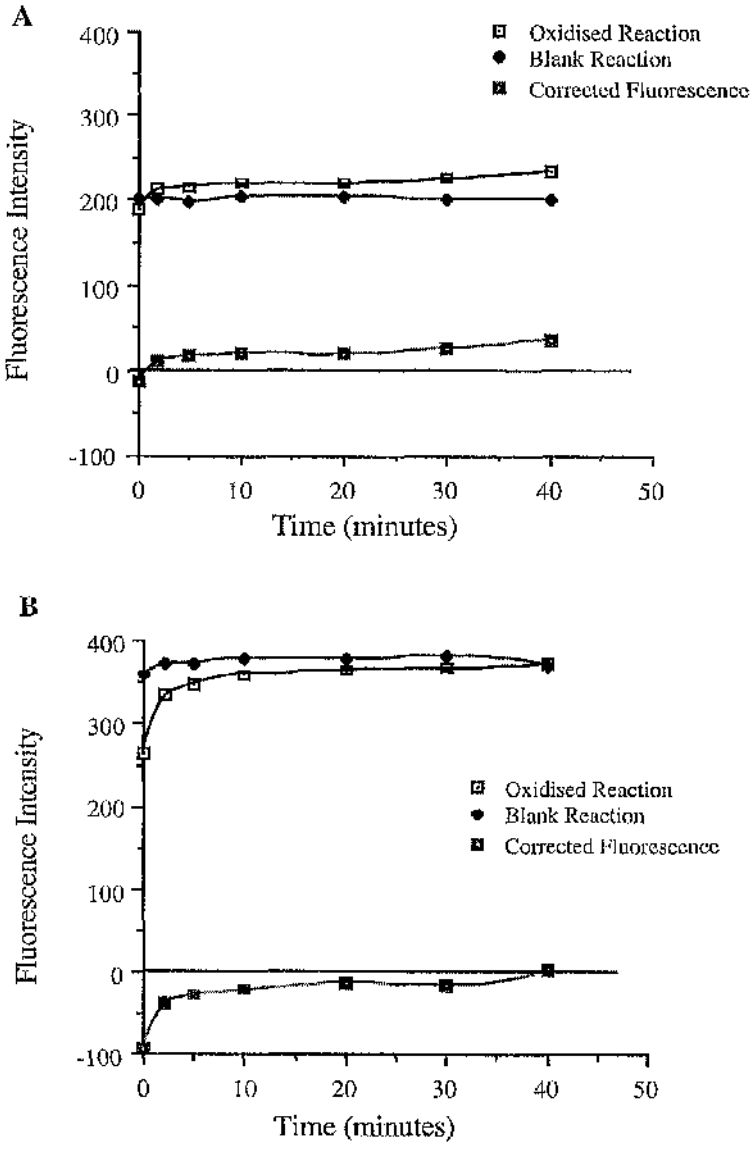


Figure 40. Fluorescence of A862 culture supernatant following peroxide oxidation reactions with different periods of incubation. A862 wt culture supernatant was oxidised by the Bates and Rapoport (1975) peroxide oxidation reaction (**A**) and the scaled up peroxide oxidation reaction of Lawrence *et al.*, (1991) (**B**). The reactions were allowed to proceed for different periods of time before the addition of glacial acetic acid. (For the t = 0 readings acetic acid was added prior to the addition of sample). A blank reaction (i.e. a reaction in which the oxidant is substituted for dH_2O) was also performed for each type of oxidation and for each period of incubation fluorescence was measured with excitation and emission wavelengths of 330nm of 380nm respectively. The corrected fluorescence intensity is the fluorescence intensity of the blank reaction subtracted from the fluorescence intensity of the oxidised reaction.

However, the results obtained following periodate oxidation reactions were unexpected as the corrected fluorescence for each sample was negative indicating higher fluorescence in the blank reaction than in the periodate oxidation reactions. Periodate must therefore be having a negative effect on the fluorescent properties of an unknown compound present in A862 cell extracts.

The effect of oxidation reaction time on the fluorescence of A862 culture supernatants was investigated using the Bates and Rapoport peroxide oxidation reaction and the scaled up Lawrence peroxide oxidation reaction. A series of oxidation reactions and corresponding blank reactions were allowed to proceed for varying lengths of time before acetic acid was added and the fluorescence of the sample determined. To achieve a true zero time reading, acetic acid was added to a reaction prior to the addition of sample. The results for both types of peroxide oxidation reaction are similar (see Figure 40). The change in the fluorescent properties of the supernatant neared completion very rapidly as there was very little difference between the corrected fluorescence at 40 minutes and the corrected fluorescence at 2 minutes. Of particular note however was the result obtained for the T=0 oxidation and blank reactions. If PSP toxins were the only fluorescent compounds in the supernatants which were affected by the oxidation reaction then the fluorescence at T=0 of the oxidation and blank reactions should be identical as PSP toxins are not oxidised under acid conditions (see Figure 37). This was not observed. Rather, the fluorescence of the T=0 oxidised reaction was lower than the fluorescence of the corresponding blank reaction. This observation was most striking with the results obtained using Lawrence peroxide reaction (Figure 40b), although a similar result is observed when the Bates and Rapoport reaction is used (Figure 40a), indicating that peroxide had a negative effect on the fluorescence of an unknown compound in A862 culture supernatants when added under acidic conditions. Any positive influence that alkaline oxidation has on the fluorescence of a sample may be partially masked if acidic oxidation has a subsequent negative affect.

Proposed Adaptation of the Fluorescence Assay

It is evident from the above results that A862 contains fluorescent compounds which are altered in their fluorescence properties when incubated with an oxidising agent. Known PSP toxins are not oxidised under acidic conditions but an unknown compound in the cultures becomes less fluorescent when oxidised under similar conditions. Therefore, if a sample contains PSP toxins and this unknown compound, the fluorescence due to the PSP toxins will be equal to the fluorescence following oxidation under alkaline conditions less any fluorescence of the sample following oxidation under acidic conditions. This can be summarised as follows:-

PSP in sample = (alkaline oxidised fluorescence) - (acid oxidised fluorescence)

It was therefore decided to alter the fluorescence assay replacing the blank reaction with an acidic oxidation reaction (AOx) to reflect more accurately the fluorescence signal in the sample which is not due to PSP toxins. The blank reaction is not a valid measure of this parameter as it does not take into account the effect of peroxide (or periodate) on any other sample component under acid conditions.

Evaluation of the adapted fluorescence assay

To evaluate the proposed adaptation of the fluorescence assay, 1μM STX in 0·05N acetic acid, A862 wt culture supernatant and A862 wt culture supernatant spiked with STX (final STX concentration 1μM) were oxidised by the adapted Bates and Rapoport (1975) and the adapted Lawrence *et al.* (1991) oxidation reactions. Samples were oxidised with peroxide under both alkaline and acidic conditions (Figure 41).

The former calculation for corrected fluorescence (i.e. the fluorescence of the blank reaction subtracted from the fluorescence of the alkaline oxidation reaction; Ox-B) and the new calculation for corrected fluorescence (i.e. the fluorescence of the acidic oxidation reaction subtracted from the fluorescence of the alkaline oxidation reaction; Ox-AOx) yielded the same result for 1μM STX. However, as expected, the former corrected fluorescence gave a much lower value than the new calculation for the A862 culture supernatant. The new method of calculating the corrected fluorescence yielded a positive result for A862 culture supernatant using data from both oxidation reactions, whereas the previous calculation gave a negative value (Figure 41b).

The adapted fluorescence assay was further evaluated by testing culture supernatants from four different strains of marine bacteria, A862, A912, OK-1 and 4αVS3 culture supernatants, which were spiked with STX to give a final concentration of 1 μM. The spiked and unspiked supernatants were assayed by the adapted fluorescence assay (Figure 42). An estimate of the PSP concentration in the samples is shown in Table 10.

Only strain A862 had significant level of PSP in unspiked culture supernatant. The amount of PSP in A912, OK-1 and 4αVS3 culture supernatants was very small and close to, or below the level of detection of this assay. The amount of STX detected in spiked marine broth was higher (1.2 μM) than the expected 1μM, possibly because the STX standard curve was prepared at a different time and with a different batch of marine broth. However, it is still useful for the comparison of the results obtained in this experiment. If the estimated PSP content of the unspiked

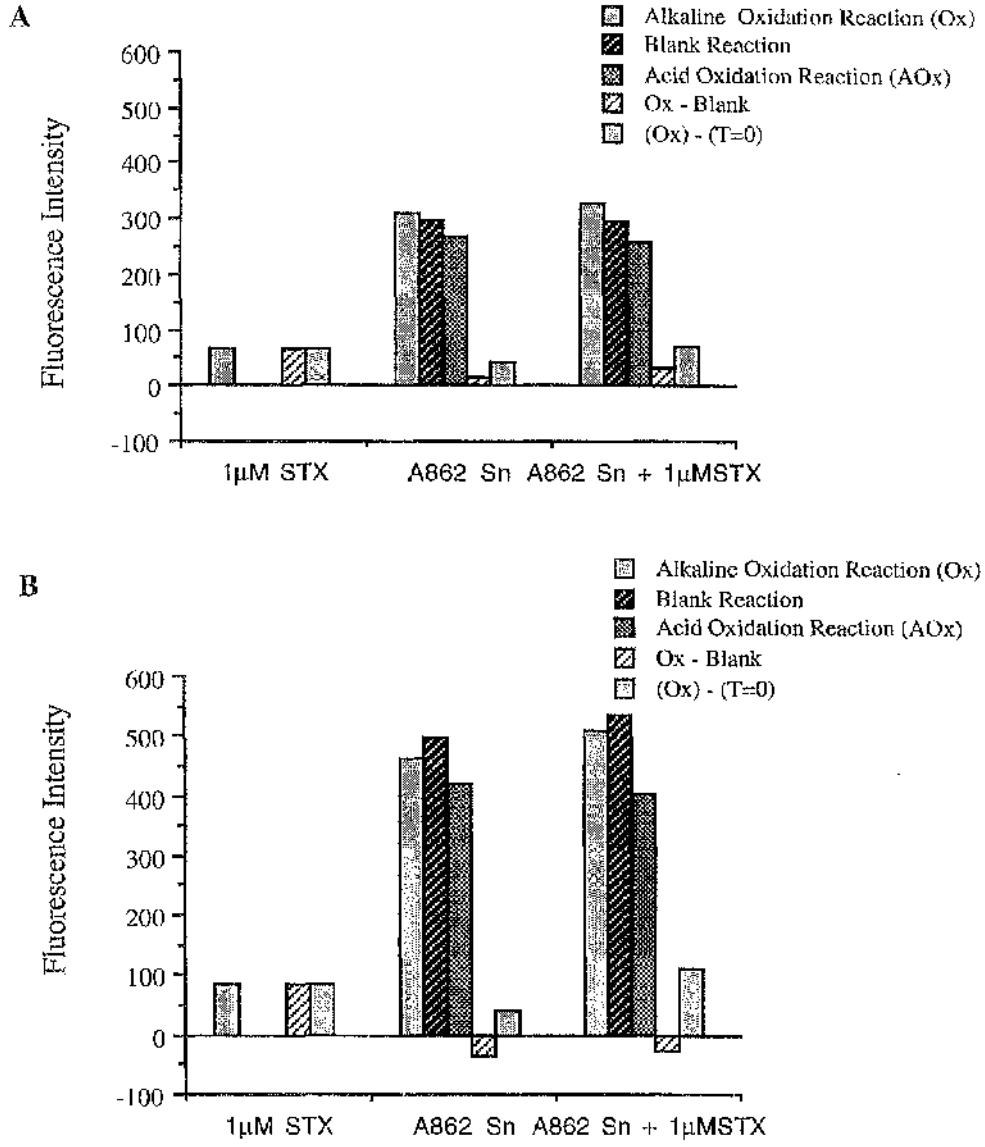


Figure 41. Fluorescence of STX and A862 culture supernatant following the adapted peroxide oxidation reactions. A862 wt culture supernatant, 1µM STX and A862 wt culture supernatant spiked with 1µM STX were oxidised by the peroxide reactions adapted from Bates and Rapoport (1975) (**A**) and Lawrence *et al.* (1991) (**B**). The samples were oxidised with peroxide under both acid and alkaline conditions. In addition a blank reaction containing water instead of peroxide was performed under alkaline conditions. Fluorescence was measured with excitation and emission wavelengths of 330 nm and 380 nm, respectively.

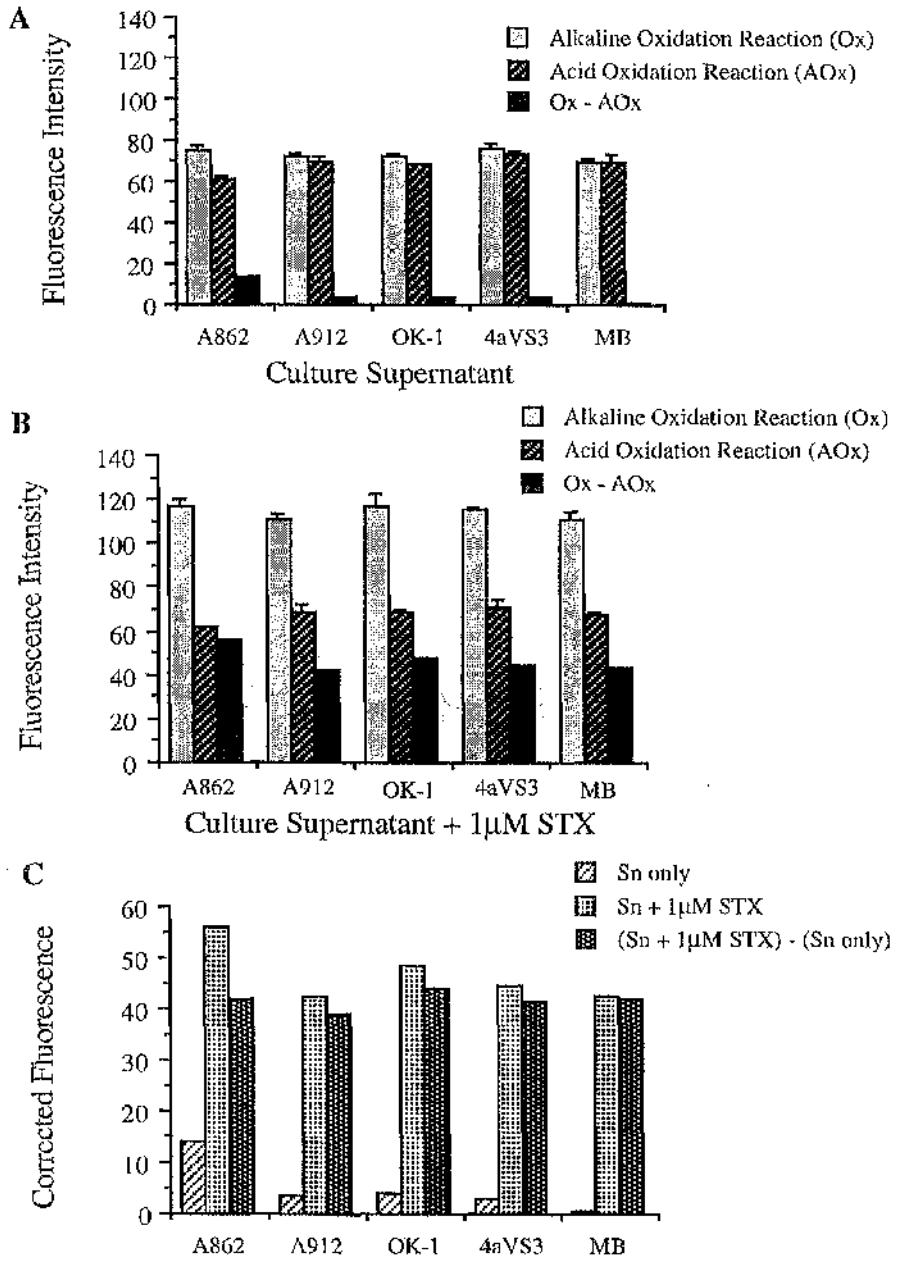


Figure 42. Fluorescence of culture supernatants of different bacterial strains (unspiked and spiked with 1μM STX) following oxidation by the adapted Lawrence peroxide oxidation reaction. Culture superantants from 10ml cultures of a number of bacterial strains and a marine broth (MB) control were spiked with 1μM STX. The spiked supernatants (**A**) and unspiked supernatants (**B**) were oxidised by the adapted peroxide oxidation reactions (adapted from Lawrence *et al.*, 1991) Fluorescence intensity was measured with excitation and emission wavelengths of 330 nm and 380 nm, respectively. The corrected fluorescence intensity is summarised in **C**. The columns represent the mean fluorescence of duplicate oxidation reactions of the same culture supernatant. The error bars indicate the standard error of the mean (SEM).

Bacterial Strain	PSP in sample (STX equivalent)	
	Culture Supernatant	Culture Supernatant + 1μM STX
A862 wt	316nm	1593nm
A912 wt	15nm	1184nm
OK-1	24nm	1363nm
4αVS3	-ve	1253nm
Marine Broth	-ve	1193nm

Table 10. Presumptive PSP content of culture supernatants of different bacterial strains as determined by the adapted fluorescence assay.

culture supernatant is subtracted from the corresponding result for spiked supernatant, the values for each of the culture supernatants (ranging from 1·08 μM for A912 to 1·24 μM for OK-1) are very similar to the value for marine broth (1·17 μM). Therefore, the culture supernatants do not appear to have any affect on the oxidation and subsequent fluorescence of STX apart from what is observed with marine broth.

The effect of marine broth on the fluorescence assay

The corrected fluorescence of A862 culture supernatant spiked with 1μM STX did not equal the sum of the corrected fluorescence of A862 culture supernatant and 1μM STX assayed individually (see Figure 41). Thus, either an unidentified factor influences the fluorescence of STX in the supernatant, or, STX has a negative effect on the fluorescence signal.

This could be due to the marine broth in which A862 was cultured and to investigate this, STX dilutions prepared in 0·05M acetic acid and in marine broth were assayed by the adapted fluorescence assay. Good correlation was found between STX concentration and corrected fluorescence regardless the diluent (Figure 43) although the gradients of the best fit lines for these two sets of results differed significantly.

A major constituent of marine broth is peptone and the effect of peptone on the fluorescence of 1μM STX was therefore examined. No significant difference was found between the corrected fluorescence of STX prepared in acetic acid and STX in distilled water ($t=0.103$; $p>10\%$; Figure 44). but there was a highly significant difference between the corrected fluorescence of STX in acetic acid compared with

STX in marine broth (t=16.575; p<0·1%) and peptone (t=5.080; p<1%). The corrected fluorescence of STX in peptone was also significantly different from the corrected fluorescence of STX in marine broth (t=7.769; p<1%) showing that peptone does have a negative effect on the oxidation or subsequent fluorescence of STX. It is not the only contributing factor as marine broth has a greater affect.

During the course of the above experiments it was observed that marine broth (untreated and oxidised) was itself fluorescent (e.g. see Figure 43b, acid-oxidised reaction). To determine whether the oxidation reactions in the fluorescence assay influenced the fluorescence of marine broth, four samples of marine broth were assayed under acid and alkaline conditions and in the presence and absence of peroxide (Figure 45). There was a highly significant difference between the fluorescence of the alkaline oxidised marine broth and the fluorescence of the alkaline blank treated marine broth (t=5.747; p<1%), but no significant difference between fluorescence intensity of the alkaline oxidised marine broth and the acidic oxidised marine broth (t=2.24; p>5%). Therefore, the oxidation reactions do affect the fluorescence of marine broth. However, if the acidic oxidised sample was used as a control, rather than the blank alkaline oxidation reaction, this effect was not significant.

Fluorescence of strain A862

From results presented earlier (see "Evaluation of adapted fluorescence assay") A862 was the only strain tested which clearly gave positive results with the adapted fluorescence assay. The fluorescence of this strain was therefore examined in more detail in triplicate assays with culture supernatants of A862 wt and A862 Sm^r (Figure 46). The fluorescence intensity was not significantly different (t=1.817; p>10%) following acid and alkaline peroxide treatment of marine broth but was highly significant for strain A862 wt (t=6.645; p<1%) and A862 Sm^r (t=4.365; p<2%). In contrast to earlier experiments (Figure 41) there was no significant difference between the fluorescence intensity of A862 culture supernatants (wt and Sm^r) and marine broth following acidic oxidation (t=2·098; p>5%).

The fluorescence signal of oxidised A862 Sm^r cells and supernatant at different stages of growth was tested with samples removed at different times post-inoculation, cells being extracted with 0·05M acetic acid as described in the Materials and Methods.

Between 0 and 8 hours post-inoculation the culture supernatants displayed fluorescent properties identical to those observed in marine broth (Figure 47a and 48), but by 8 hours post-inoculation another fluorescent compound was detected in

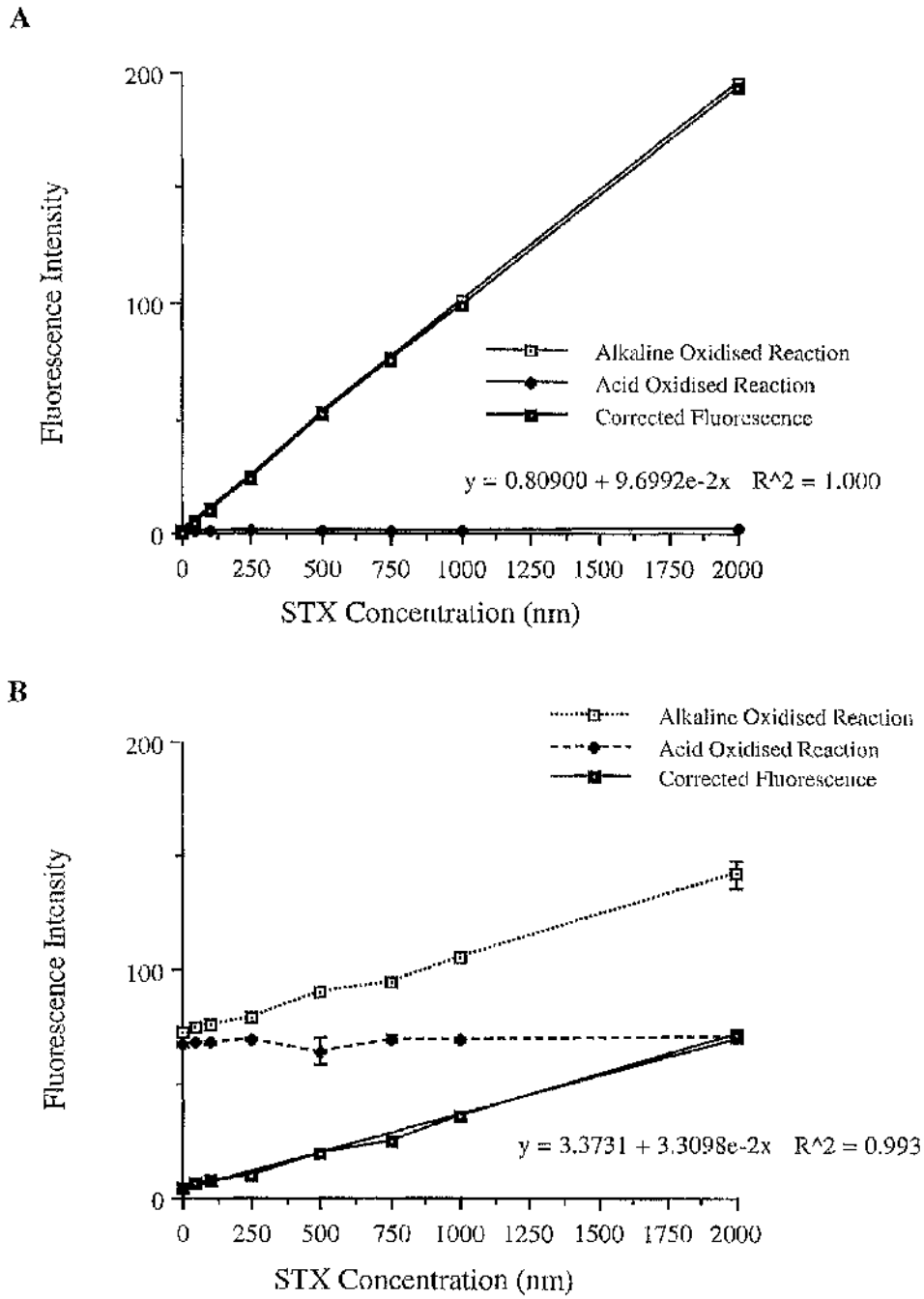


Figure 43. Fluorescence standard curve of STX in different diluents following oxidation by adapted peroxide oxidation reactions. A range of concentrations of STX, diluted in 0·05M acetic acid (**A**) and marine broth (**B**), were oxidised by the adapted peroxide oxidation protocol (adapted from Lawrence *el al.*, 1991). The oxidation was performed under alkaline and acid conditions before fluorescence was measured with an excitation wavelength of 330nm and an emission wavelength of 380nm. The corrected fluorescence (i.e. alkaline oxidation (Ox) - acid oxidation (AOx)) is a measure of the fluorescence emitted by the STX in the sample.

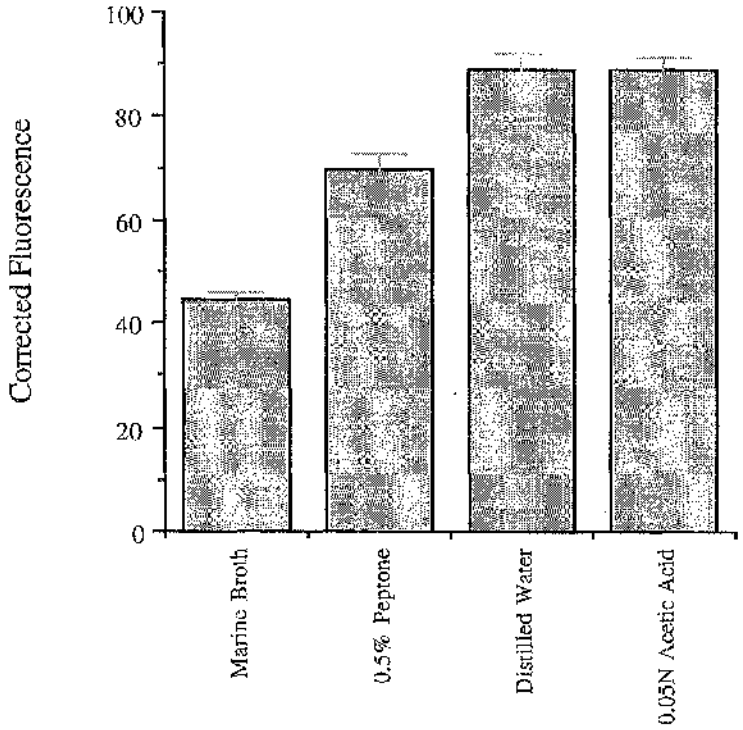


Figure 44. Fluorescence of STX in different diluents following treatment with the adapted Lawrence peroxide oxidation reaction. STX was diluted to 1μM in four different diluents and oxidised by the adapted peroxide oxidation protocol (adapted from Lawrence *et al.*, 1991). The reactions were performed in triplicate under acid and alkaline conditions and fluorescence intensity measured with excitation and emission wavelengths of 330 nm and 380 nm, respectively. The corrected fluorescence (i.e. the fluorescence intensity of the acid oxidation reaction subtracted from the fluorescence intensity of the alkaline oxidation reaction) is a measure of the fluorescence being emitted by oxidised STX. Error bars represent the standard error of the mean (SEM).

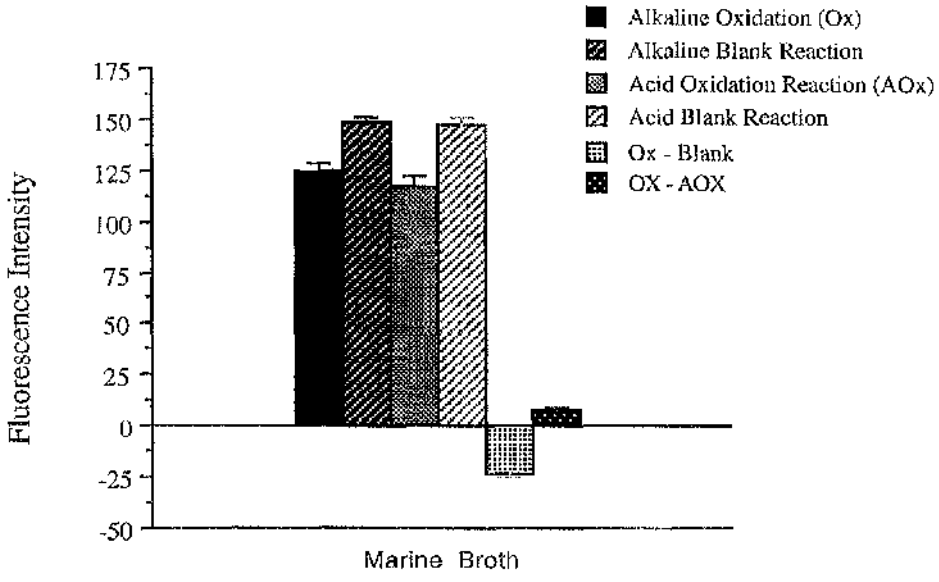


Figure 45. Fluorescence of marine broth following treatment with the adapted Lawrence peroxide oxidation reaction. Four replicate samples of marine broth were oxidised by the adapted peroxide oxidation protocol (adapted from Lawrence *el al.*, 1991). The reactions were performed under acid and alkaline conditions, and in the prescence and absence of peroxide (substituted by dH_2O in blank reactions). Fluorescence intensity was measured with excitation and emission wavelengths of 330 nm and 380 nm, respectively. Two different corrected fluorescence are shown on the graph (i.e. alkaline oxidation - blank and alkaline oxidation - acid oxidation). Error bars represent the standard error of the mean (SEM).

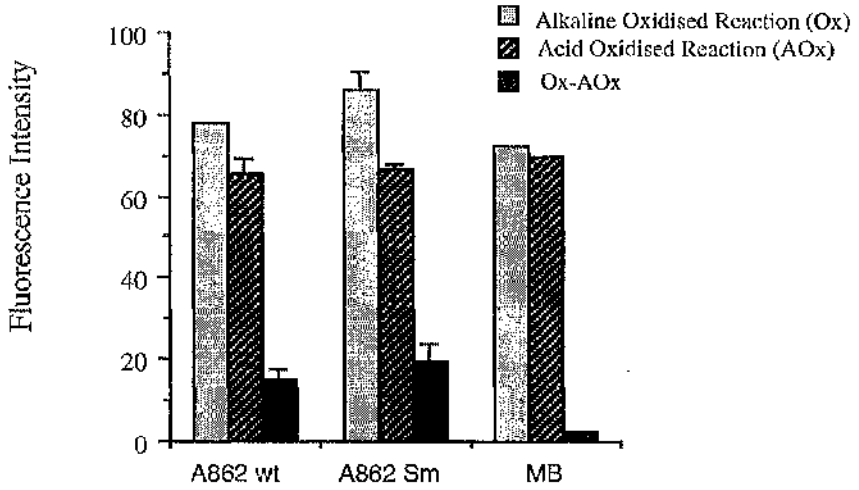


Figure 46. Fluorescence of A862 following oxidation by the adapted Lawrence peroxide oxidation reaction. Supernatant from 24 hour, 10ml cultures of A862 wt and A862 Sm^r, together with a marine broth control, were oxidised by the adapted peroxide oxidation protocol (adapted from Lawrence *et al.*, 1991; The culture supernatants were oxidised by peroxide under acid and alkaline conditions). The columns represent the mean fluorescence measured with excitation and emission wavelengths of 330nm and 380nm, respectively. The error bars indicate the standard error of the mean (SEM). The corrected fluorescence (i.e. alkaline oxidation (Ox) - acid oxidation (AOx)) is a measure of the fluorescence emitted by the STX in the sample.

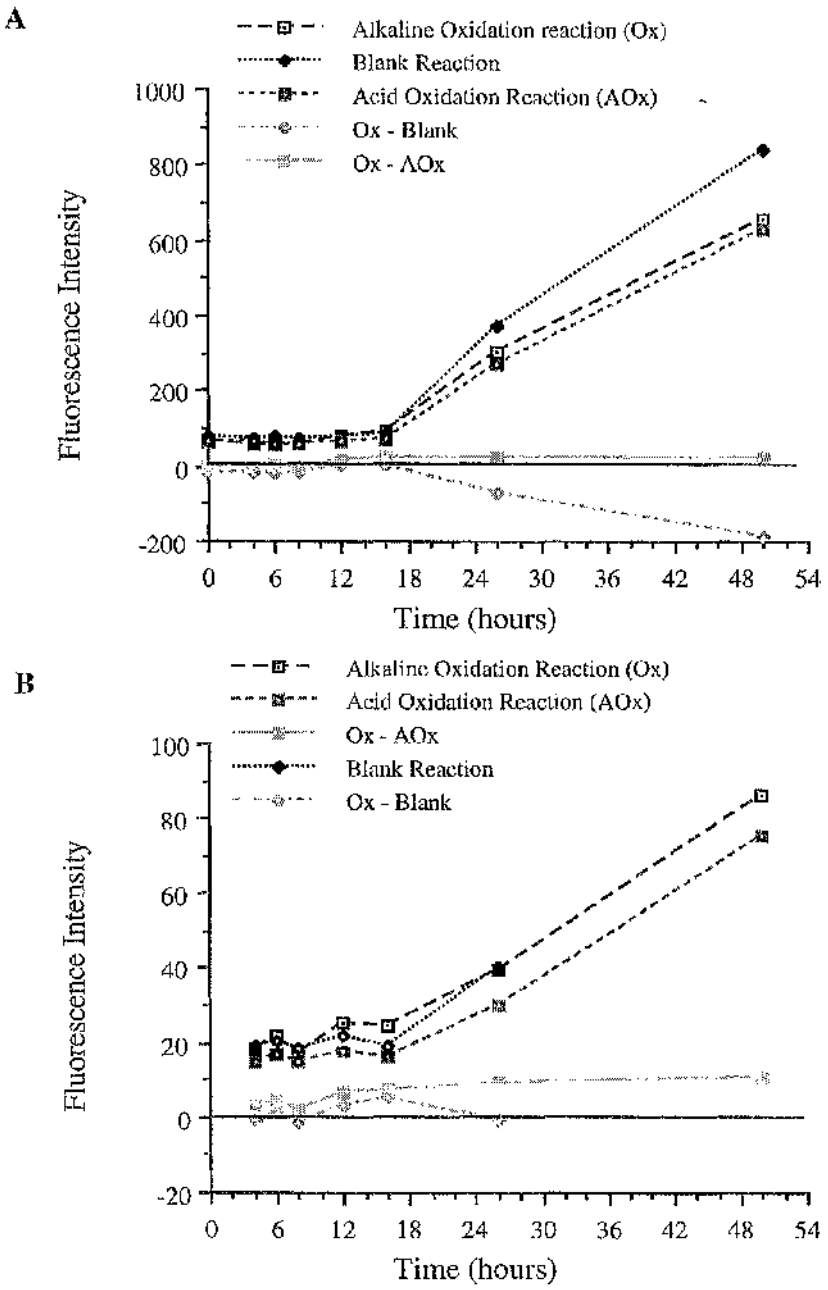


Figure 47. Fluorescence of A862 Sm^r at different stages of growth following oxidation by the adapted Lawrence peroxide reaction. Culture supernatant (**A**) and cell extracts (**B**) from different stages of growth were oxidised by the adapted peroxide oxidation protocol (adapted from Lawrence *et al.*, 1991). The reactions were performed under acid and alkaline conditions, and a blank reaction was performed by substituting water for peroxide in the alkaline reaction. Fluorescence was measured with excitation and emission wavelengths of 330nm and 380nm, respectively. There are two different measures of corrected fluorescence shown on the graphs (i.e. alkaline oxidation - blank and alkaline oxidation - acid oxidation).

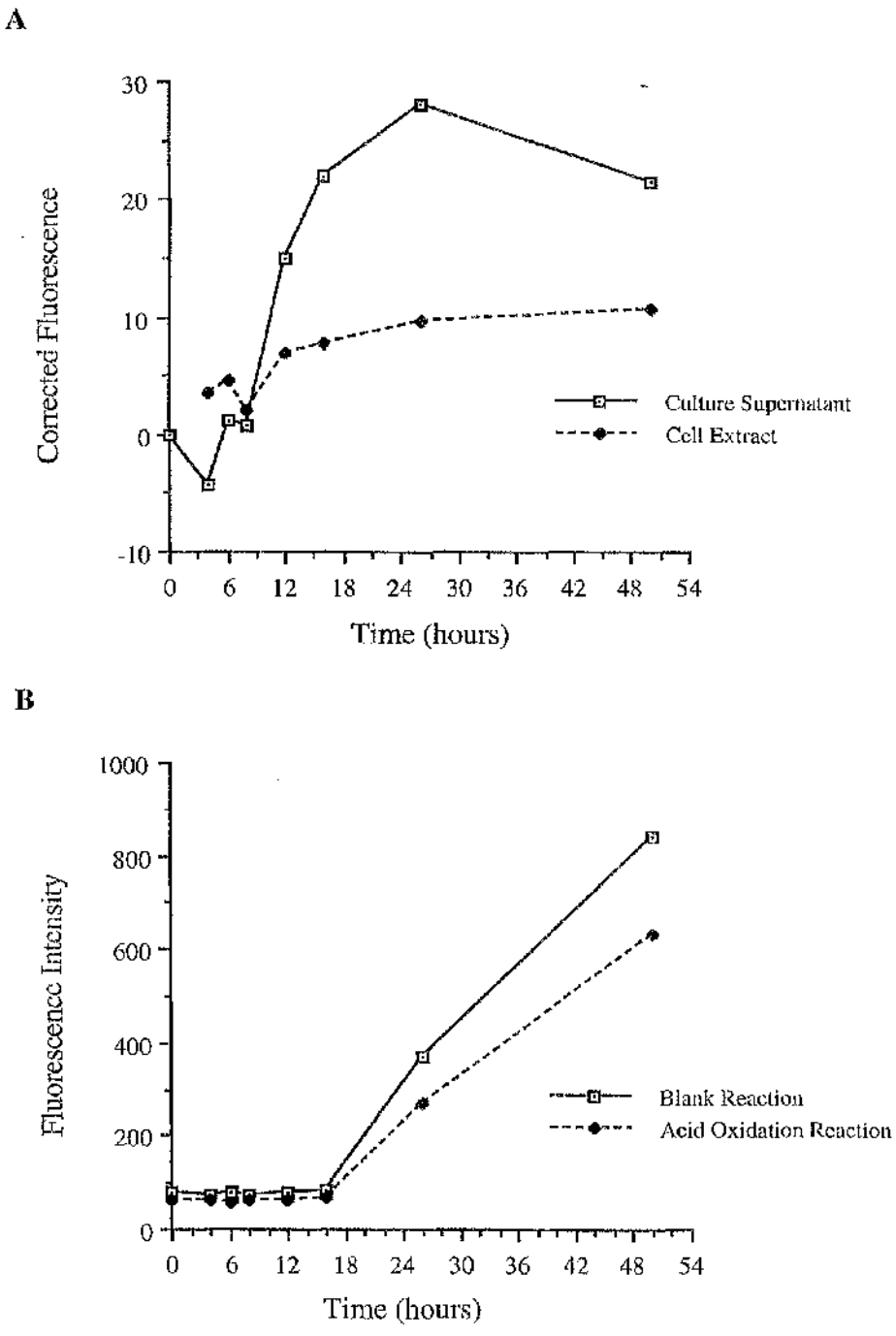


Figure 48. Fluorescence of A862 Sm^r at different stages of growth following oxidation by the adapted Lawrence peroxide oxidation reaction. The fluorescence of A862 Sm^r cell extracts and culture supernatants at different stages of growth was determined as described in the text and Figure 43. The corrected fluorescence (**A**) is the fluorescence intensity of the alkaline oxidation reaction minus the fluorescence intensity of the acid oxidation reaction. The fluorescence of the culture supernatant following treatment by blank and acid oxidation reactions are shown in graph **B.**

the supernatant. This compound was oxidised by peroxide under alkaline conditions, but not under acidic conditions, to yield a fluorescent product (Figure 47a and 48a), properties analogous to those observed for PSP toxins. The amount of this compound in the supernatant, as determined by the corrected fluorescence (Ox-AOx), increased gradually until 16 to 26 hours, before dropping slightly, by 54 hours (Figure 48a). A compound which decreased in fluorescence following acidic peroxide oxidation (as compared to the blank reaction) was detected in the culture supernatant after 16 hours (Figure 48b).

In all the cell extracts tested there was a small difference between the fluorescence of the alkaline oxidised reaction and the fluorescence of the acidic oxidation reaction, possibly indicating the presence of PSP toxins (Figure 47b and 48a). The corrected fluorescence derived from these results was low but increased slightly as growth time increased (there was a possibly erroneous reading at 8 hours). There is a lower fluorescence in acidic oxidised reactions than in the blank reactions for all the cell extracts. The fluorescence of all the reaction types increased from 16 hours, as did the difference between the fluorescence of the blank and acidic oxidised reactions (Figure 47b).

Further analysis of the fluorescence of strain A862

Detailed analysis of the fluorescence assay clearly revealed the presence of fluorescent oxidation products in strain A862, although the identity of these compounds is unknown. PSP toxins may be present but more evidence is required for proof of this.

The emission spectra of alkaline-peroxide oxidised and acidic-peroxide oxidised A862 culture supernatant (10 ml) were obtained by wavelength scanning with an excitation wavelength of 330 nm. The resulting spectra are shown in Figure 49 and those for alkaline peroxide oxidised PSP toxins in Figure 50. Clearly, the emission maximum of A862 culture supernatants (approximately 410 to 420 nm) are different from that of PSP toxins (approximately 390 nm).

To investigate the effect of marine broth on the emission spectrum of STX this was determined in acetic acid and marine broth. The emission spectrum of STX in marine broth was very similar to that obtained for marine broth alone, and very different from the emission spectrum for STX in acetic acid (Figure 51).

It is clear, therefore, that the presence of other fluorescent compounds can mask the true fluorescent properties of PSP in marine broth and, by deduction, possibly also in culture supernatants. To analyse this problem further, 1ml fractions were collected after pre-column peroxide oxidation and HPLC analysis of A862 Sm^r culture supernatant in two separate analyses, and STX + $GTX_{2/3}$ standards. The fluorescent properties of selected fractions were analysed (Table 11 and Figure 52).

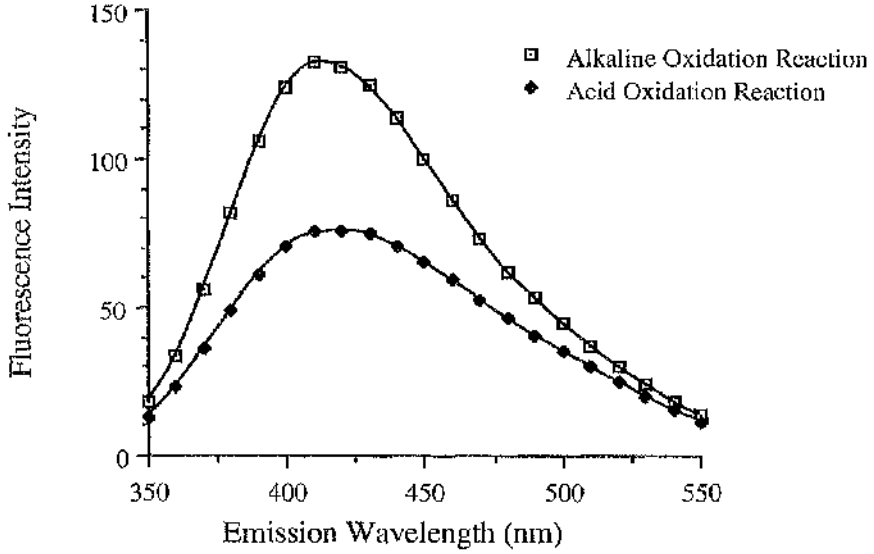


Figure 49. Emission spectra for A862 wt culture supernatant following different oxidation reactions (excitation at 330nm). A862 wt culture supernatant (from a 10ml culture) was oxidised by the acid and alkaline peroxide oxidation reaction (adapted from Lawrence *et al.*, 1991). The emission spectra were determined by wavelength scanning with excitation at 330nm.

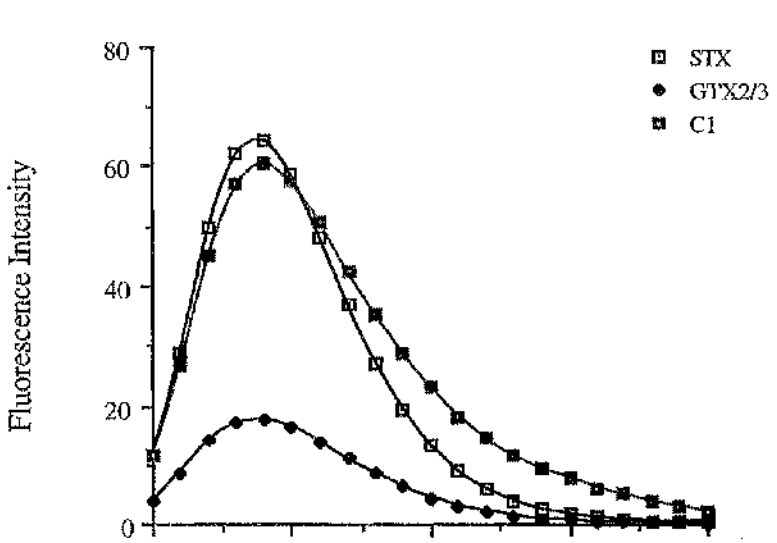


Figure 50. Emission spectra of PSP toxins following alkaline peroxide oxidation (excitation at 330nm). STX, $GTX_{2/3}$ and C1 toxins were oxidised by the alkaline peroxide oxidation reaction (adapted from Lawrence *et al.*, 1991). The emission spectra were determined by wavelength scanning with excitation at 330nm.

Sample	Fraction Number	Fluorescence Intensity (Ex.330nm, Em. 380nm)	Excitation λ max.	Emission λ max.
A862 Sm^r Culture Sn	14	2.451	nd	nd
	15	30.06	316 nm	396 nm
	16	19.18	315 nm	398 nm
	17	12.26	317 nm	397 nm
	18	6.653	nd	nd
A862 Sm^r Culture Sn	13	5.564	nd	nd
	14	27.85	315 nm	396 nm
	15	16.28	315 nm	396 nm
	16	6.208	315 nm	397 nm
STX + $GTX_{2/3}$	21	6.840	nd	nd
	22	16.13	342 nm	387 nm
	26	15.79	341 nm	383 nm
	27	66.55	337 nm	386 nm

Table 11. Fluorescent properties of 1ml fractions collected during pre-column peroxide oxidation HPLC analysis of STX+$GTX_{2/3}$ and A862 Sm^r culture supernatant.

n.d. = not determined.

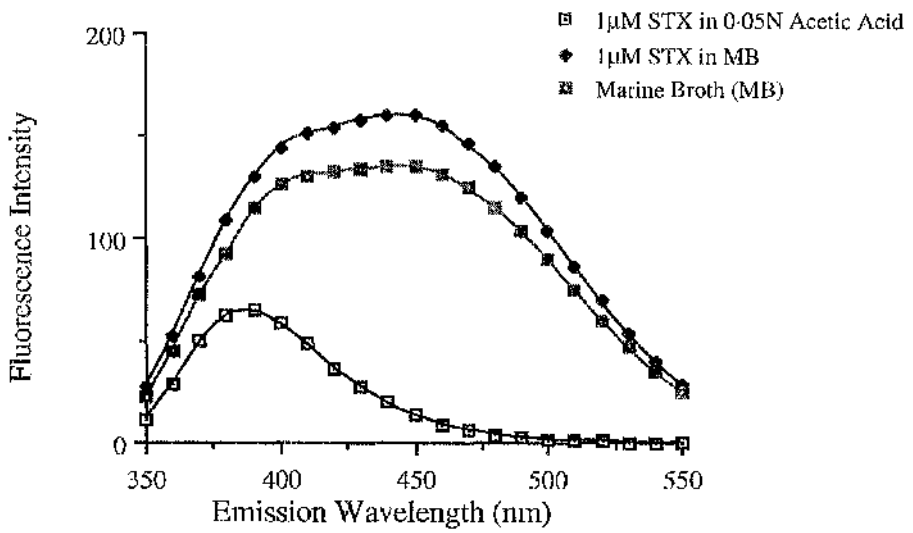


Figure 51. Emission spectra of STX following alkaline peroxide oxidation (excitation at 330nm). STX (1µM) diluted in acetic acid, 1µM STX diluted in marine broth and a marine broth control were oxidised by the alkaline peroxide oxidation reaction (adapted from Lawrence *et al.*, 1991). The emission spectra were determined by wavelength scanning with excitation at 330nm.

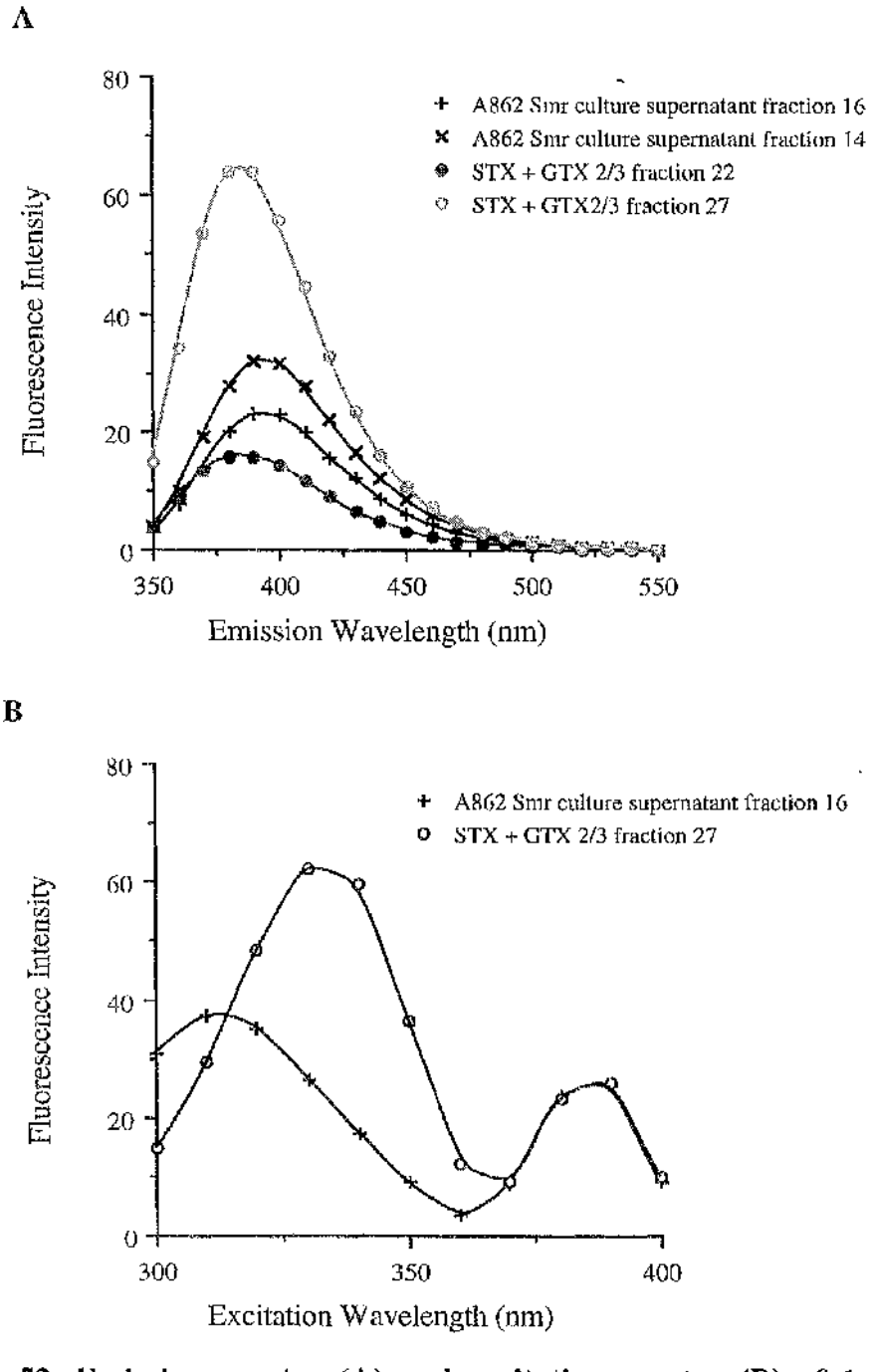


Figure 52. Emission spectra (A) and excitation spectra (B) of 1ml fractions collected during pre-column peroxide oxidation HPLC analysis of STX+GTX$_{2/3}$ and A862 Sm^r culture supernatant. The emission and excitation spectra (**A** and **B** respectively) of fractions collected during HPLC separation of alkaline peroxide oxidised STX+GTX$_{2/3}$ standards and two different separations of alkaline peroxide oxidised A862 culture supernatant were determined by wavelength scan. The emission scan was performed with an excitation of 330nm and the excitation scan performed with an emission of 380nm.

Toxin	Peroxide		Periodate	
	Ex. λ max.	Em. λ max.	Ex. λ max.	Em. λ max.
STX	339 nm	390 nm	342 nm	388 nm
Neo-STX	nd	nd	346 nm	388 nm
$GTX_{2/3}$	341 nm	389 nm	342 nm	391 nm
$GTX_{1/4}$	nd	nd	344 nm	389 nm
C1	335nm	391nm	nd	nd

Table 12. Maximum wavelengths of excitation and emission for fluorescence analysis of PSP toxins. The maximum wavelenths for the fluorescence analysis of PSP toxins were determined by wavelength scan of alkaline peroxide and alkaline periodate oxidation reaction products. The reactions were adapted from Lawrence *et al.*, 1991.

The emission spectra for the A862 culture supernatant fractions (approximately 396 nm) were similar to those for STX and $GTX_{2/3}$ fractions (approximately 386 nm). However, the excitation spectra of fractions from A862 culture supernatant were quite different from those of fractions from HPLC analysis of STX + $GTX_{2/3}$. The excitation maximum of the fractions from A862 culture supernatant was 315 nm and the excitation maximum of the fraction from STX + $GTX_{2/3}$ was 340 nm.

Similar excitation maxima (340 nm ± 6 nm) and emission maxima (390 nm ± 2 nm) were found for all the PSP toxins tested following peroxide and periodate oxidation (Table 12).

TRANSPOSON MUTAGENESIS OF MARINE BACTERIA WHICH PRODUCE SCB TOXINS

Isolation of spontaneous antibiotic resistant mutants of marine bacteria

Several combinations of vector and recipient strains were identified as being likely to yield positive results (see "Evaluation of marine bacterial isolates for transposon mutagenesis") in transposon mutagenesis. One longer term aim of this project was to study the expression of genes of interest using promoter reporters. As not all the transposons considered contain a promoter reporter the list of useful vector/recipient combinations was further reduced to the following:-

(i) GFC + pJO100
(ii) A912 + pUT::miniTn5Km*lacZI*
(iii) (GFC, A862, A1087 or A1095) + pUT::miniTn5Cm*lacZ*.

Before use as recipients for transposon mutagenesis it was necessary to isolate variants of the marine bacteria which could be easily distinguished from the *E. coli* donor strains. Spontaneous mutants of GFC, A912 and A862, which were resistant to nalidixic acid (Nal^r) or streptomycin (Sm^r) were isolated. The antibiotic resistance of the Nal^r and Sm^r derivatives of the three strains was a stable characteristic as the antibiotic resistance remained after growth in broth or solid media containing no antibiotic (data not shown).

Transposon mutagenesis of strain GFC with pJO100

Two separate transposon mutagenesis experiments involving the conjugative transfer of pJO100 from *E. coli* J100 (Östling *et al.*, 1991) to *Alteromonas tetraodonis* GFC were set up simultaneously using $2{\cdot}11\times10^7$ or $6{\cdot}33\times10^7$ CFU of GFC Nal^r mixed with $1{\cdot}24\times10^7$ CFU of *E. coli* J100 (donor : recipient ratios of 1:1·7 and 1:1·5 respectively). Conjugation was allowed to proceed at 20°C for 18 hours. After incubation for one week at 20°C on LB20 agar plates containing nalidixic acid, tetracycline and X-gal, Nal^r Tc^r exconjugants were recovered at frequencies of $3{\cdot}55\times10^{-7}$ and $5{\cdot}64\times10^{-7}$ per recipient cell from the two experiments respectively. Measurement of the spontaneous mutation rate of these strains in the same experiment gave no colonies on the plates inoculated with GFC Nal^r, indicating a spontaneous Tc^r mutation frequency of $<4{\cdot}74\times10^{-9}$. Spontaneous Nal^r of *E. coli* J100 was observed at a frequency of $3{\cdot}79\times10^{-6}$. Therefore spontaneous mutation of *E. coli* J100 to Nal^r occurs at a much higher frequency than the induced Tc^r of GFC Nal^r by transposon mutagenesis with pJO100.

Transposon mutagenesis of strain A912 with pUT::miniTn5Km*lacZI*

Ten transposon mutagenesis experiments were carried out using A912 Nal^r as the recipient strain and *E. coli* S17-1*λpir* containing pUT::miniTn5Km*lacZI* as the donor strain. In each of these experiments different amounts of cells from an 18 hour culture of *E. coli* S17-1*λpir* pUT::miniTn5Km*lacZI* were added to different amounts of cells from a 24 hour culture of A912 Nal^r (see Table 13). Conjugation was allowed to take place, as described in the Materials and Methods, at 20°C for 18 hours before the cells were recovered and plated onto marine agar containing nalidixic acid and kanamycin.

A912 exconjugants which were Nal^r and Km^r were recovered at frequencies of between $2{\cdot}49\times10^{-6}$ and $1{\cdot}31\times10^{-4}$ per recipient cell (see Table 13). The donor : recipient ratio did not appear to have a significant effect on the rate of Nal^r Km^r exconjugant recovery. Increasing the number of cells in the conjugation mix (e.g. from 10^7 to 10^8) had a negative affect on the recovery rate of Nal^r Km^r exconjugants, although the number of exconjugants recovered in any one experiment increased.

A total of 1511 Nal^r Km^r exconjugants from the experiment outlined in the final row of Table 13 were picked onto fresh marine agar plates containing nalidixic acid and kanamycin. The exconjugants were replica plated for a total of six passages on marine agar containing nalidixic acid and kanamycin. The majority (1505) of the exconjugants remained viable after this treatment.

No colonies were observed when the cells of a 24 hour broth culture of A912 Nal^r were plated onto marine agar containing nalidixic acid and kanamycin indicating a spontaneous Km^r mutation rate of A912 Nal^r of less than 1×10^{-8}. However, during the course of the transposon mutagenesis experiments a growth was observed on the selection plates which was different from the formation of true colonies. This background growth consisted of extremely small colonies and was also observed when *E. coli* S17-1*λpir* pUT::miniTn5Km*lacZ*I was plated onto marine agar containing nalidixic acid and kanamycin.

CFU of *E. coli* S17-1*λpir* pUT::miniTn5-Km*lacZ*	CFU of A912 Nal^r	Donor : Recipient Ratio	Exconjugants per Recipient cell
$2{\cdot}6\times10^7$	$4{\cdot}8\times10^7$	1 : 1·8	$1{\cdot}31\times10^{-4}$
$8{\cdot}6\times10^6$	$4{\cdot}8\times10^7$	1 : 5·6	$3{\cdot}95\times10^{-5}$
$2{\cdot}6\times10^7$	$1{\cdot}6\times10^7$	1·6 : 1	$3{\cdot}02\times10^{-4}$
$2{\cdot}6\times10^8$	$4{\cdot}8\times10^8$	1 : 1·8	$7{\cdot}36\times10^{-5}$
$7{\cdot}9\times10^7$	$4{\cdot}8\times10^8$	1 : 6·1	$4{\cdot}85\times10^{-5}$
$2{\cdot}6\times10^8$	$1{\cdot}4\times10^8$	1·8 : 1	$3{\cdot}95\times10^{-5}$
$5{\cdot}2\times10^8$	$9{\cdot}6\times10^8$	1: 1·8	$2{\cdot}39\times10^{-5}$
$2{\cdot}6\times10^8$	$1{\cdot}4\times10^9$	1 : 5·5	$1{\cdot}45\times10^{-5}$
$7{\cdot}9\times10^8$	$4{\cdot}8\times10^8$	1·6 : 1	$2{\cdot}49\times10^{-6}$
$7{\cdot}1\times10^7$	$1{\cdot}4\times10^8$	1 : 2	$3{\cdot}01\times10^{-5}$

Table 13. Frequency of induced Km^r of A912 Nal^r following conjugation with *E. coli* S17-1*λpir* pUT::miniTn5Km*lacZ*.

Transposon mutagenesis of strain A862 with pUT::miniTn5Cm*lacZ*

During preliminary transposon mutagenesis experiments using A862 Nal^r and *E. coli* S17-1 *λpir* pUT::miniTn5Cm*lacZ* the same background growth observed during mutagenesis of A912 Nal^r was observed on marine agar plates containing chloramphenicol and nalidixic acid. The nalidixic acid sensitivity of the *E. coli* donor strains was examined by plating the cells of overnight broth cultures onto LB agar and marine agar containing nalidixic acid and chloramphenicol. One colony was observed on LB + Nal + Cm plates inoculated with *E. coli* S17-1*λpir*pUT:: miniTn5-Cm*lacZ* and no colonies were observed on LB + Nal + Cm plates inoculated with *E. coli* Sm10*λpir*pUT::miniTn5Cm*lacZ*. In contrast, however, marine agar + Nal + Cm plates inoculated with *E. coli* S17-1 *λpir*pUT::miniTn5Cm*lacZ* displayed growth characteristics identical to those observed during the mutagenesis experiments. In addition, marine agar + Nal + Cm plates inoculated with *E. coli* Sm10*λpir*pUT::mini-Tn5Cm*lacZ* showed confluent growth. Therefore, it appears that marine broth has an antagonistic affect on nalidixic acid. Future transposon mutagenesis experiments were performed using the Sm^r derivatives of the marine bacteria. As *E. coli* S17-1*λpir* is Sm^r, it was unsuitable for the proposed experiments, and *E. coli* Sm10*λpir* was used in all subsequent transposon mutagenesis experiments.

Six different transposon mutagenesis experiments were carried out using A862 Sm^r as the recipient strain and *E. coli* Sm10*λpir* containing pUT::miniTn5Cm*lacZ* as the donor strain. In each experiment different amounts of cells from an 18 hour culture of Sm10*λpir* pUT::miniTn5Cm*lacZ* were added to different amounts of cells from a 24 hour culture of A862 Sm^r (see Table 14).

A862 exconjugants which were Sm^r and Cm^r were recovered at frequencies between $2{\cdot}25\times10^{-6}$ and $3{\cdot}73\times10^{-9}$ (Table 14). Experiments in which there were more recipient cells than donor cells in the conjugation mix gave the greatest frequency of induced mutation, with donor : recipient ratios of 1 : 3 or less yielding the highest results.

A total of 5328 Sm^r Cm^r A862 exconjugants were isolated from the six transposon mutagenesis experiments. These exconjugants were replica plated six times on marine agar containing streptomycin and chloramphenicol and only 2803 exconjugants survived this treatment.

No colonies were observed when A862 Sm^r and *E. coli* Sm10*λpir* pUT::mini-Tn5Cm*lacZ* were plated onto marine agar + Sm + Cm; the spontaneous A862 Sm^r Cm^r mutation rate and the spontaneous Sm^r mutation rate of *E. coli* Sm10*λpir* pUT:: miniTn5Cm*lacZ* were less than 2×10^{-9}.

CFU of *E. coli* Sm10*λpir* pUT::miniTn5-Cm*lacZ*	CFU of A862 Sm^r	Donor : Recipient Ratio	Exconjugants per Recipient cell
$5{\cdot}8\times10^{7}$	$2{\cdot}7\times10^{8}$	1 : 3	$3{\cdot}03\times10^{-6}$
$9{\cdot}8\times10^{7}$	$3{\cdot}2\times10^{8}$	1 : 4·6	$3{\cdot}70\times10^{-6}$
$1{\cdot}7\times10^{8}$	$3{\cdot}4\times10^{8}$	1 : 2	$1{\cdot}90\times10^{-7}$
$2{\cdot}8\times10^{8}$	$2{\cdot}9\times10^{8}$	1 : 1	$1{\cdot}25\times10^{-7}$
$3{\cdot}7\times10^{8}$	$1{\cdot}6\times10^{9}$	1 : 4·3	$2{\cdot}25\times10^{-6}$
$5{\cdot}1\times10^{8}$	$1{\cdot}9\times10^{8}$	2·7 : 1	$3{\cdot}73\times10^{-9}$

Table 14. Frequency of induced Cm^r of A862 Sm^r following conjugation with *E. coli* Sm10*λpir* pUT::miniTn5Cm*lacZ*.

PHENOTYPIC CHARACTERISTICS OF A862::miniTn5Cm*lacZ* EXCONJUGANTS

Ampicillin sensitivity of A912::miniTn5Km*lacZ*I exconjugants

The 1505 A912 Nal^r Km^r exconjugants which survived replica plating were plated onto marine agar containing ampicillin. Twelve exconjugants grew on these plates indicating resistance to ampicillin, the result of spontaneous Amp^r mutation or the retention of the delivery plasmid in these exconjugants.

ß-galactosidase activity of random A862::miniTn5Cm*lacZ* exconjugants

Twenty A862::miniTn5Cm*lacZ* exconjugants were selected at random from the 2803 which survived the six replica platings on marine agar + Sm + Cm. These exconjugants and an A862 wt control were cultured in marine broth and the ß-galactosidase activity of the cultures determined. The wild type A862 contained no significant ß-galactosidase activity, but each of the exconjugants tested possessed varying levels of ß-galactosidase activity (see Figure 53).

ß-galactosidase activity of *E. coli* strains used in this study

The ß-galactosidase activity of the plasmid containing *E. coli* strains used in this study was determined as above, except that the *E. coli* cells were grown in LB broth overnight at 37°C. The *E. coli* strains CC118*λpir*, SM10*λpir* and S17-1*λpir* with either no plasmid or pUT::miniTn5Cm, displayed only low levels of ß-galactosidase activity (Figure 54), but, *E. coli* strains containing pUJ8, pUT::miniTn5Cm*lacZ* or pUT::miniTn5Km*lacZ*I contained significant ß-galactosidase activity (Figure 54).

Screening of A862::miniTn5Cm*lacZ* exconjugants by the adapted fluorescence assay

To establish the reproducibility of the adapted fluorescence assay ten replicate 10 ml marine broth cultures of A862 Sm^r and two replicate cultures of A862 wt were inoculated and incubated at 20°C for 48 hours, and the fluorescent properties of the culture supernatants and a marine broth control were determined. A862 Sm^r gave a mean corrected fluorescence of 52·83 (sd = 21·214; N = 10), A862 wt gave a mean corrected fluorescence of 27·07 (sd = 9·815; N = 2) and marine broth had a corrected fluorescence of 10·52 (all are in arbitary units).

The corrected fluorescence data obtained for A862 Sm^r was used to prepare the rankit plots shown in Figure 55. The rankit plot of the unaltered corrected fluorescence data (Figure 55a) did not show gross departure from normality although it differed from that expected from a true normal distribution. Logarithmic transformation of the corrected fluorescence data (Figure 55b) revealed a closer relationship between the theoretical and observed rankit line than was found using the non-transformed data. The corrected fluorescence data is therefore more likely to belong to a log-normal distribution.

Confidence intervals (CI) calculated from the corrected fluorescence data obtained for A862 Sm^r are as follows:-

95% CI = 37·65 to 68·00
99% CI = 31·03 to 74·64
99·9% CI = 20.75 to 84·91.

The CIs obtained from the log corrected fluorescence are :-

95% CI = 38·28 to 64·27
99% CI = 34·26 to 71·97
99·9% CI = 28·76 to 85·73.

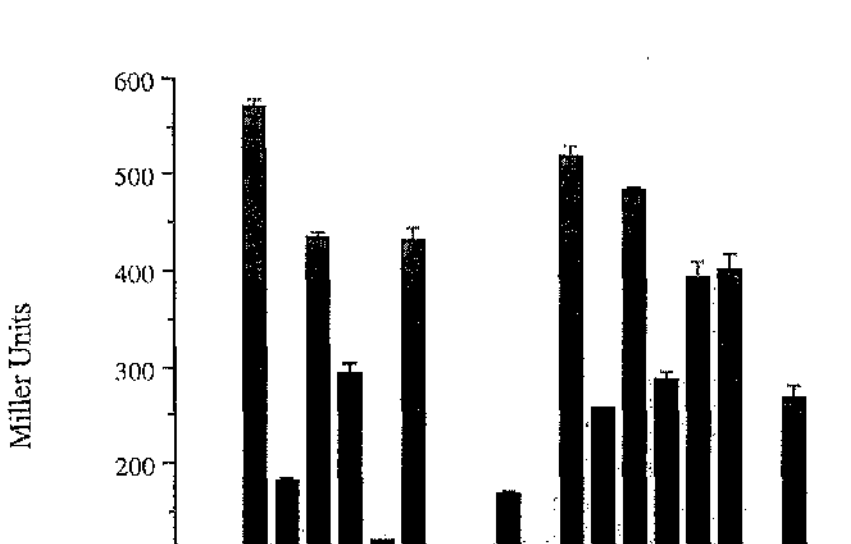


Figure 53. ß-galactosidase activity of A862::miniTn5Cm*lacZ* exconjugants. Twenty A862::miniTn5Cm*lacZ* exconjugants (1-20) were selected at random and, together with wild type A862 (wt), cultured for 24 hours in marine broth. The ß-galactosidase activity of the cells was assayed in duplicate by the method of Miller (1972). Error bars represent the standard error of the mean (SEM).

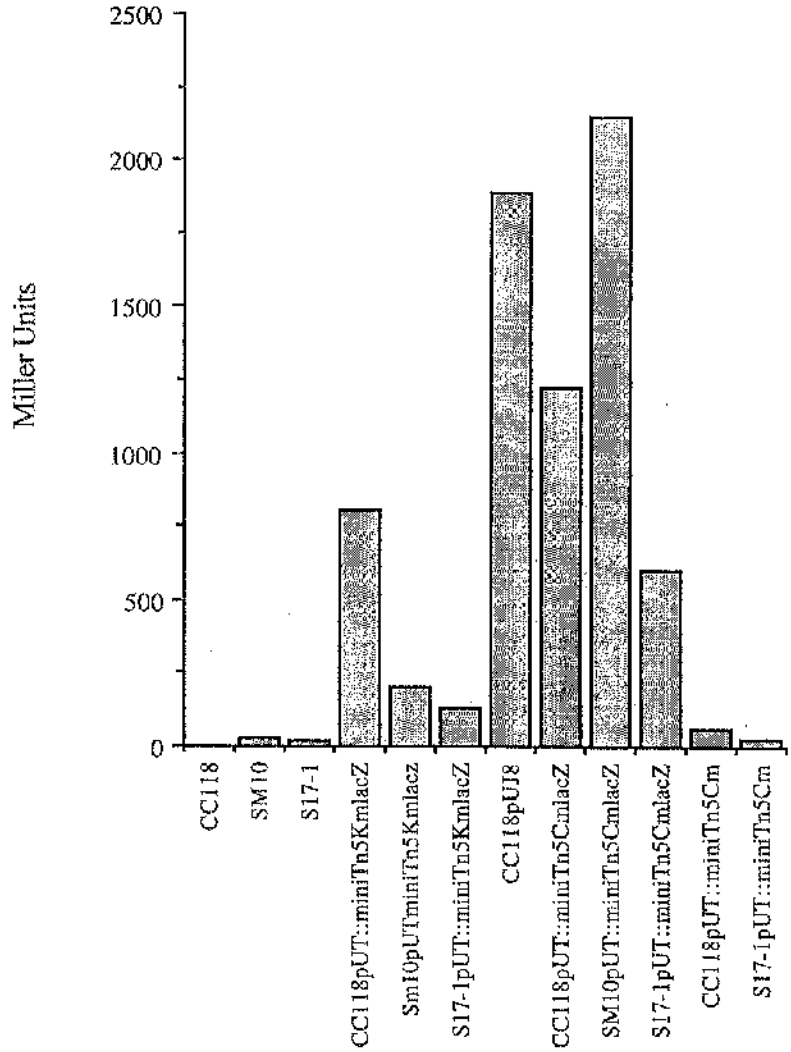


Figure 54. ß-galactosidase activity of *E. coli* donor strains. The *E. coli* strains used for the manipulation of the transposon vectors were cultured overnight in LB broth. The ß-galactosidase activity of the cells was assayed by the method of Miller (1972).

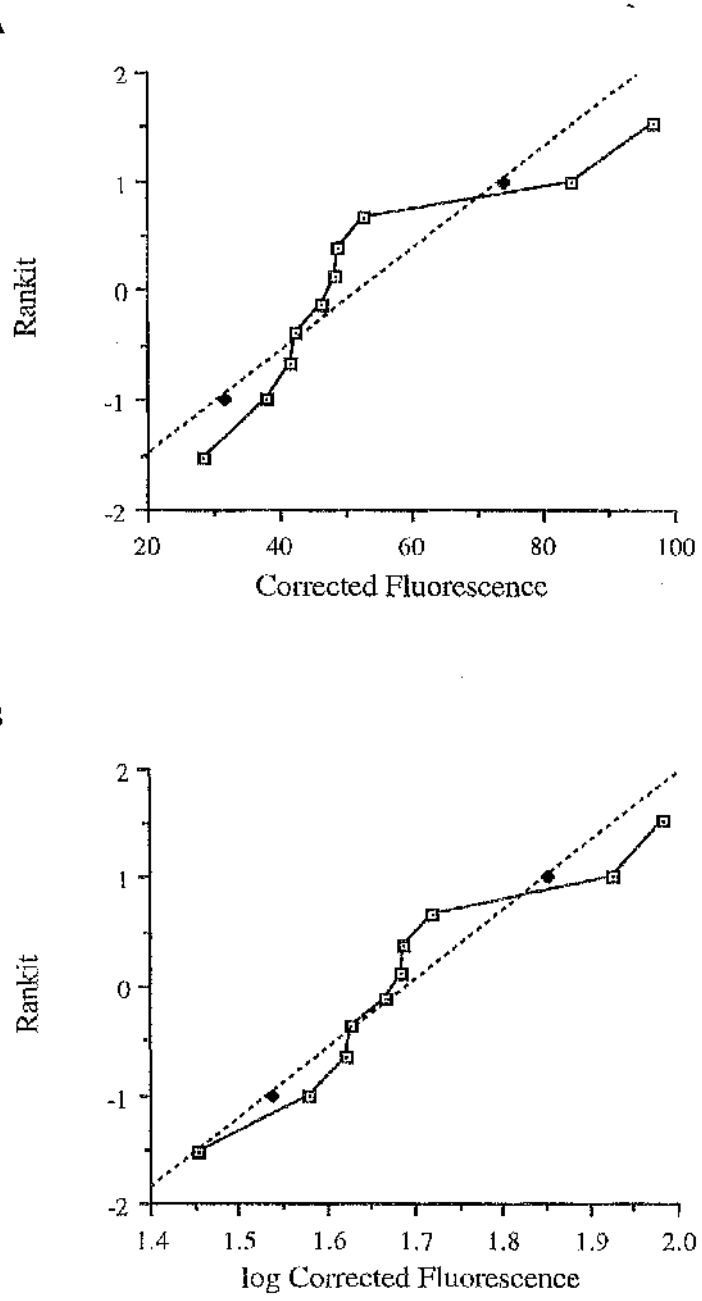


Figure 55. Rankit plot of A862 Sm^r corrected fluorescence. The supernatants from ten 10ml A862 Sm^r cultures were tested by the fluorescence assay. The corrected fluorescence results were used to generate a rankit plot (**A**). The log of the corrected fluorescence results were used to generate another rankit plot (**B**).

These CIs were used to identify exconjugants which gave a corrected fluorescence which lay outside these intervals as differing significantly from the strain from which they were derived (i.e. A862 Sm^r).

Culture supernatants from a total of 886 A862::miniTn5Cm*lacZ* exconjugants were screened for low corrected fluorescence by the adapted fluorescence assay and the number of exconjugants with a corrected fluorescence within each 5 fluorescence unit range is displayed as a histogram in Figure 56. The distribution is slightly asymmetrical with a modal range of corrected fluorescence between 25 to 29·99 units.

Fifteen exconjugants which gave low corrected fluorescence in the initial screening were selected for further analysis. Six replicate cultures of each of the selected exconjugants, together with marine broth (12 replicates), A862 wt, (6 replicates) and A862 Sm^r (18 replicates) were prepared and assayed as before by the adapted fluorescence assay (Figure 57). The corrected fluorescence of A862 Sm^r is higher than the corrected fluorescence of any of the exconjugants. The corrected fluorescence of A862 wt was however similar to the majority of the exconjugants. One exconjugant in particular, S9-22, stood out as having a very low corrected fluorescence, this being similar to that of marine broth. Some of the other exconjugants had a lower corrected fluorescence than A862 wt (i.e. S5-17, S14-3, S14-4, S14-27, S16-27, S19-40, S19-43, S25-18 and S27-37). However, the difference between A862 wt and S14-3 ($t = 1{\cdot}62$; $p>10\%$) and S19-40 ($t = 1{\cdot}51$; $p>10\%$) is not statistically significant.

Pre-column oxidation HPLC analysis of selected exconjugants

Culture supernatants of A862 Sm^r and four of the A862::miniTn5Cm*lacZ* exconjugants which gave significantly lower corrected fluorescence than A862 wt were analysed by pre-column oxidation HPLC analysis. The chromatograms (Figure 58) show a peak of fluorescence in samples from A862 Sm^r and three of the exconjugants. This peak has the same retention time as observed previously (Figure 17) and was absent from all the samples following acidic peroxide oxidation. Exconjugant S9-22 supernant gave no significant fluorescence peak and the chromatogram for this sample was very similar to that obtained for marine broth. The area of the fluorescence peak present in the other three exconjugants was lower than observed in A862 Sm^r. The fluorescent properties of these samples were also assayed by the adapted fluorescent assay and the results (see Table 15) show an excellent correlation with the HPLC chromatograms.

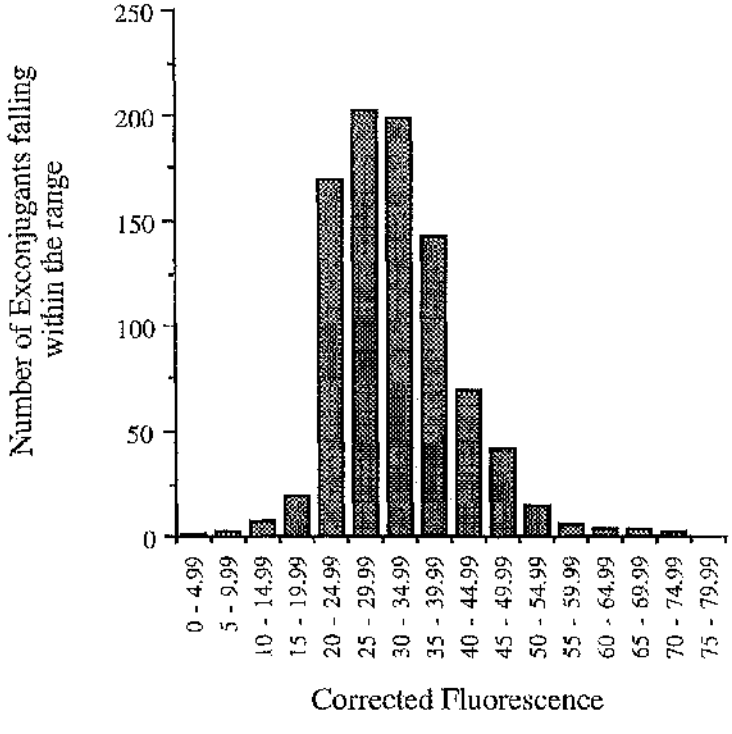


Figure 56. Summary of fluorescence screen results. The culture supernatants of 886 A862::miniTn5Cm*lacZ* exconjugants were tested by the fluorescence assay and the corrected fluorescence (alkaline oxidation fluorescence - acid oxidation fluorescence) calculated. The number of exconjugants yielding results within each 5 fluorescence unit range was determined and is shown here.

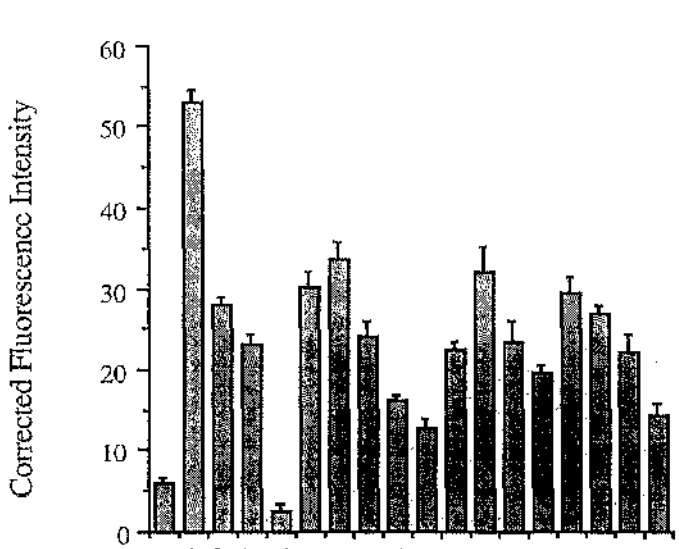


Figure 57. Fluorescence of selected exconjugants. A number of exconjugants which had given low fluorescence in the initial screen with the adapted fluorescence assay were retested using six replicate 10ml cultures grown at 20°C for 48 hours. Six replicates of A862 wt, eighteen replicates of A862 Smr and twelve replicates of marine broth controls were also tested. The fluorescence of the sample following acidic oxidation was subtracted from the fluorescence following alkaline oxidation to give the corrected fluorescence intensity. Error bars represent the standard error of the mean (SEM).

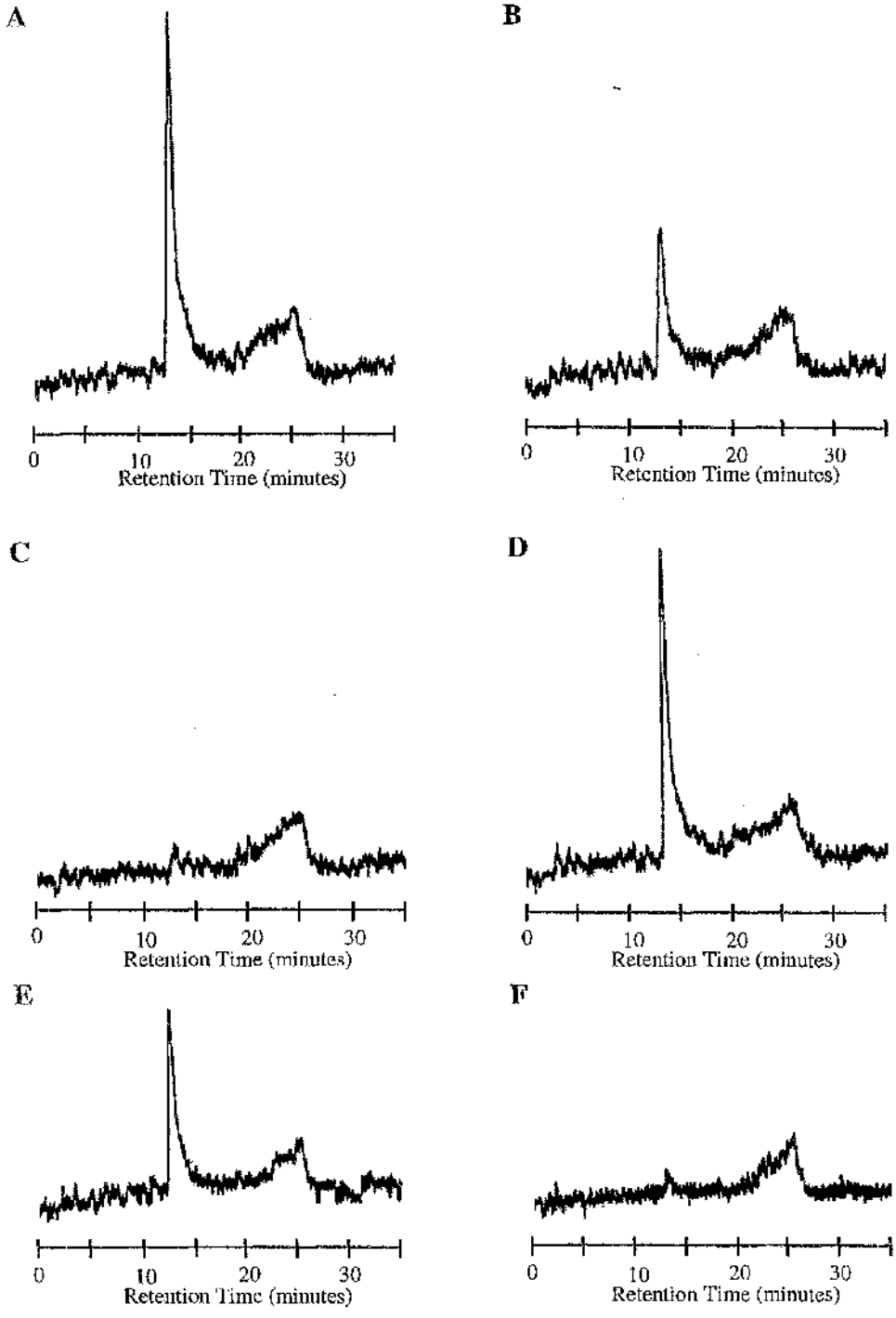


Figure 58. HPLC analysis of pre-column oxidised culture supernatant of selected A862::miniTn5Cm*lacZ* exconjugants. The culture supernatant from 10ml cultures of selected exconjugants was oxidised by peroxide under alkaline conditions prior to HPLC analysis by the method of Lawrence *et al.* (1991).
A = A862 Sm^r, **B** = S5-17, **C** = S9-22, **D** = S16-27, **E** = S19-43 and **F** = Marine Broth.

Sample	Fluorescence Signal (arbitrary units)		
	Alkaline Oxidation Reaction	Acid Oxidation Reaction	Corrected Oxidation Reaction
A862 Sm^r	123·5	67·13	56·37
S5-17	97·82	75·40	22·42
S9-22	79·10	76·48	2·62
S16-27	118·8	78·94	39·86
S19-43	101·6	77·53	24·07
Marine Broth	81·48	82·06	-0·58

Table 15. Fluorescence properties of A862::miniTn5Cm*lacZ* exconjugant samples analysed by pre-column oxidation HPLC determined by the adapted fluorescence assay.

ß-galactosidase activity of selected A862::miniTn5Cm*lacZ* exconjugants

The ß-galactosidase activity of culture supernatants of seven of the A862::miniTn5Cm*lacZ* exconjugants which had significantly lower corrected fluorescence than A862 wt were assayed for ß-galactosidase activity. A positive control consisting of *E. coli* CC118*λpir* pUT::miniTn5Cm*lacZ* was also included. Strain A862 Sm^r and exconjugant S9-22 had no significant ß-galactosidase activity (Figure 59); other exconjugants had variable levels of ß-galactosidase activity but none were as high as found in the control *E. coli* culture.

Screening of A862::miniTn5Cm*lacZ* exconjugants by the mouse neuroblastoma cell sodium channel blocking assay

The culture supernatants of 707 of the 886 exconjugants screened for PSP toxins by the adapted fluorescence assay were also tested by the mouse neuroblastoma cell sodium channel blocking assay. The mouse neuroblastoma cell assay was adapted in order to maximise sample throughput by excluding positive controls on every plate and testing each sample in only two or three wells at a single dilution.

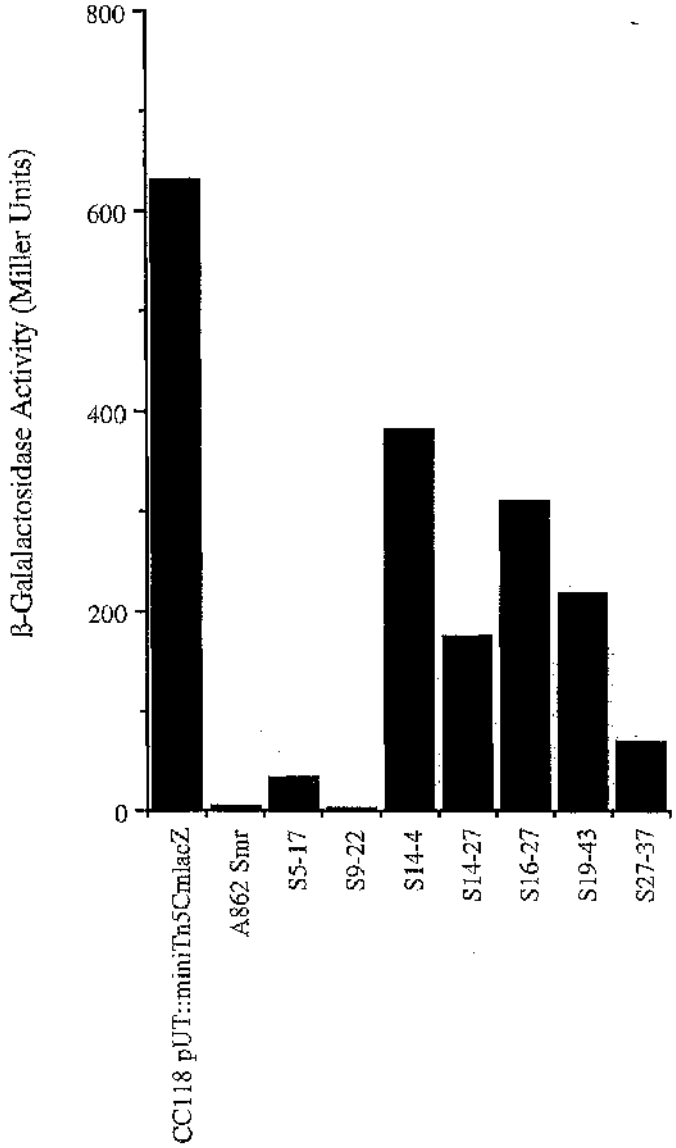


Figure 59. ß-galactosidase activity of selected exconjugants. A number of potentially interesting exconjugants were selected as a result of their low fluorescence in the adapted fluorescence assay. The ß-galactosidase activity of these exconjugants, A862 Sm^r and CC118λ*pir* pUT::miniTn5Cm*lacZ* was determined by the method of Miller (1972) using the cells of an overnight culture (marine broth at 20°C for marine isolates and LB broth at 37°C for *E. coli*).

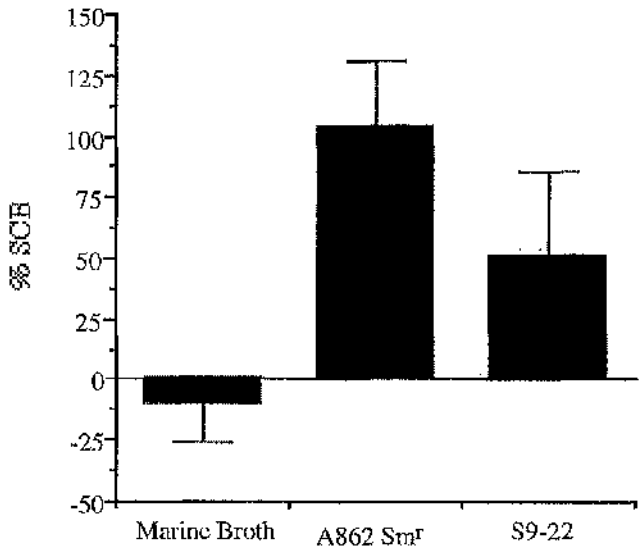


Figure 60. Analysis of A862 Sm^r and exconjugant S9-22 by mouse neuroblastoma SCB tissue culture assay. Marine broth (10 ml) was inoculated with A862 Sm^r and exconjugant S9-22 and the culture supernatant was recovered after incubation at 20°C for 48 hours. Each culture supernatant and an uninoculated marine broth control was split into five replicate samples and these were tested "blind" by the mouse neuroblastoma assay for SCB activity. The % SCB activity (i.e. the percentage protection of cells by the test samples) was calculated as described by Gallacher and Birkbeck (1992) and the mean activity for each supernatant plotted in the graph above. Error bars represent the standard error of the mean (SEM).

The % SCB activity of A862 culture supernatants (Sm^r and exconjugants) detected by this assay was extremely variable. However, there was also considerable variability between the % SCB activity detected when the same culture supernatant was tested on different occasions; indeed, when tested on the same occasion on different 96-well plates (results not shown). It is therefore impossible to give an accurate estimate of the level of SCB toxin present in each sample and comparison of samples on different plates is extremely difficult. However, every exconjugant tested did, on more than one occasion, give a level of SCB activity which could be regarded as positive.

There did not appear to be any correlation between the corrected fluorescence of a sample and its ability to block SCB activity as detected by the mouse neuroblastoma assay (results not shown). To examine this further, five replicate samples of culture supernatant from 10 ml cultures of A862 Sm^r, exconjugant S9-22 and a marine broth control were tested by the mouse neuroblastoma assay. These samples were tested with a batch of exconjugants and their identity was not disclosed to the member of FRS Marine Laboratory staff performing the mouse neuroblastoma assay. The corrected fluorescence of A862 Sm^r, S9-22 and marine broth was 56·85, -4·67 and -5·68 respectively. The mean blocking of SCB activity of the culture supernatants (illustrated in Figure 60) was 103·58% (s.d.= 60·73) for A862 Sm^r, 50·29% (s.d.= 78·19) for S9-22 and -10·34% (s.d.= 36·38) for marine broth. Although there is a high level of variation between the levels obtained for each replicate it is clear that there is a significant level of SCB activity in exconjugant S9-22 despite this exconjugant displaying a negative corrected fluorescence intensity.

GENOTYPIC ANALYSIS OF A862::miniTn5Cm*lacZ* EXCONJUGANTS

Southern blot analysis of selected interesting exconjugants

To confirm that exconjugants had aquired the transposon miniTn5Cm*lacZ*, Southern blot analysis was performed with probes prepared from the carrier plasmid pUT::miniTn5Cm*lacZ*. Therefore, a Southern blot containing *Cla*I digested pUT::miniTn5Cm*lacZ* and *Cla*I digested genomic DNA from A862 Sm^r and seven exconjugants of interest (i.e. S5-17, S9-22, S14-4, S14-27, S16-27, S19-43 and S27-37) was probed with a DIG-labelled 4·1 kb fragment purified from an *Eco*RI digest of pUT::miniTn5Cm*lacZ* (see Figure 61). The probe revealed a different pattern of bands for each sample of digested DNA on the blot. The 4·1kb probe hybridised to control pUT::miniTn5Cm*lacZ* DNA but detected no homologous

sequences in A862 Sm^r genomic DNA. The genomic DNA from each of the exconjugants displayed bands of varying size with homology to the 4·1 kb probe. Exconjugants S9-22, S14-4, S16-27, S19-43 and S27-37 all contained three bands which contain sequence homologous to the 4·1kb probe, but the other exconjugants, S5-17 and S14-27, contained only two.

In order to determine whether the exconjugants of interest had lost the transposon delivery vector, a Southern blot containing *Pst*I digested genomic DNA of the samples of interest was probed with a *tnp** containing probe (see Figure 62). This *tnp** probe was prepared by digesting pUT::miniTn5Cm*lacZ* with *Sal*I and purifying the 1·5 kb fragment which was subsequently labelled with DIG by a random-primed labelling reaction. This DIG-labelled 1·5 kb probe detected the expected bands in *Pst*I digested pUT::miniTn5Cm*lacZ*. Strain A862 Sm^r and exconjugants S5-17 and S9-22 did not possess any homologous sequences to the 1·5 kb probe. The other exconjugants (i.e. S14-4, S14-27, S16-27, S19-43 and S27-37) all contained a number of bands which are recognised by this probe. One of these bands of approximately 800 base pairs, corresponded to the 800 base pair *Pst*I fragment in pUT::miniTn5Cm*lacZ* which contains part of the transposase gene. These five exconjugants therefore appeared to possess a copy of the transposase gene and had probably also retained a full copy of pUT::miniTn5Cm*lacZ*.

Southern blot analysis of selected cured exconjugants

The vector DNA detected in 5 out of 7 interesting exconjugants could either be in the form of a retained plasmid or as a cointegrate (i.e. the pUT::miniTn5Cm*lacZ* DNA incorporated into the genome of the recipient organism). Plasmid inheritance is less stable than cointegrate inheritance, therefore, the exconjugants were grown in media containing no selective antibiotics in an attempt to cure them of any plasmid DNA. After six passages on marine agar the cured exconjugants were plated on marine agar containing chloramphenicol. S14-4 and S14-27 failed to grow on these plates after incubation for one week at 20°C, however all the exconjugants grew in the absence of chloramphenicol. The genomic DNA was prepared from each of these cured exconjugants and analysed by Southern blot analysis (Figure 63 and 64) in the same manner as described above for the uncured interesting exconjugants.

The cured exconjugants S5-17, S9-22, S16-27, S19-43 and S27-37 possessed identical band patterns to those found in the untreated exconjugants (Figure 61 and 62). Southern blot analysis of genomic DNA from cured S14-4 revealed no homology to either the 4·1 kb *Eco*RI probe or the 1·5 kb *Sal*I probe. Fewer bands were detected in the cured S14-27 genomic DNA than were detected in the untreated

S14-27 genomic DNA, although the 800 bp band detected by the *tnp** probe was still present in cured S14-27 (Figure 64).

Southern blot analysis of exconjugants selected at random

Eight A862::miniTn5Cm*lacZ* exconjugants were selected at random from the 886 which were screened by the adapted fluorescence assay. The genomic DNA from these exconjugants was analysed by Southern blot analysis. Hybridisation of *Cla*I digested genomic DNA with the DIG-labelled 4·1 kb *Eco*RI probe revealed a different band pattern for each of the exconjugants (Figure 65). Three out of the eight exconjugants contained three bands and the other five possessed two bands with homology to the 4·1 kb probe. When *Pst*I-digested genomic DNA from the random exconjugants was probed with the 1·5 kb *Sal*I DIG-labelled probe, four of the exconjugants (i.e. the three with 3 bands in Figure 61 plus one other) had an 800 bp band corresponding to the partial *tnp** fragment in pUT::miniTn5Cm*lacZ* (Figure 66).

Inverse PCR amplification of miniTn5Cm*lacZ* flanking sites

Determination of the nucleotide sequence at the region of insertion of miniTn5Cm*lacZ* is possible by inverse PCR (see "Materials and Methods" for details). A DNA fragment immediately adjacent to the miniTn5Cm*lacZ* insertion site in five of the A862::miniTn5Cm*lacZ* exconjugants was amplified succesfully by inverse PCR (see Figure 10 and 11). When the PCR products were analysed by agarose gel electrophoresis (Figure 67) they were of the expected size, as determined from the Southern blot analysis (i.e. the size of the smallest band detected by the 4·1 kb probe (see Figure 61) less a 642 bp fragment of *lacZ* between primer L8867 and L730749).

The successful cloning of the inverse PCR products in the pCR®2.1-TOPO cloning vector was confirmed by restriction digest analysis (result not shown). To confirm the identity of the inverse PCR products, two of these products (from S5-17 and S9-22) were DIG-labelled by random primed labelling reactions and used to probe Southern blots containing A862 Sm^r genomic DNA, exconjugant genomic DNA, pUT::miniTn5Cm*lacZ* DNA and control PCR product (Figure 68). The probes prepared from the PCR products detected the appropriate control PCR product, pUT::miniTn5Cm*lacZ* DNA and a band of the expected size in the exconjugant from which the PCR product was derived. In addition, the S5-17 and S9-22 PCR products each detected a band of different size (1·6 kb and 3·5 kb respectively) in A862 Sm^r genomic DNA. Therefore the PCR products, as expected, contain segments of vector and A862 genomic DNA.

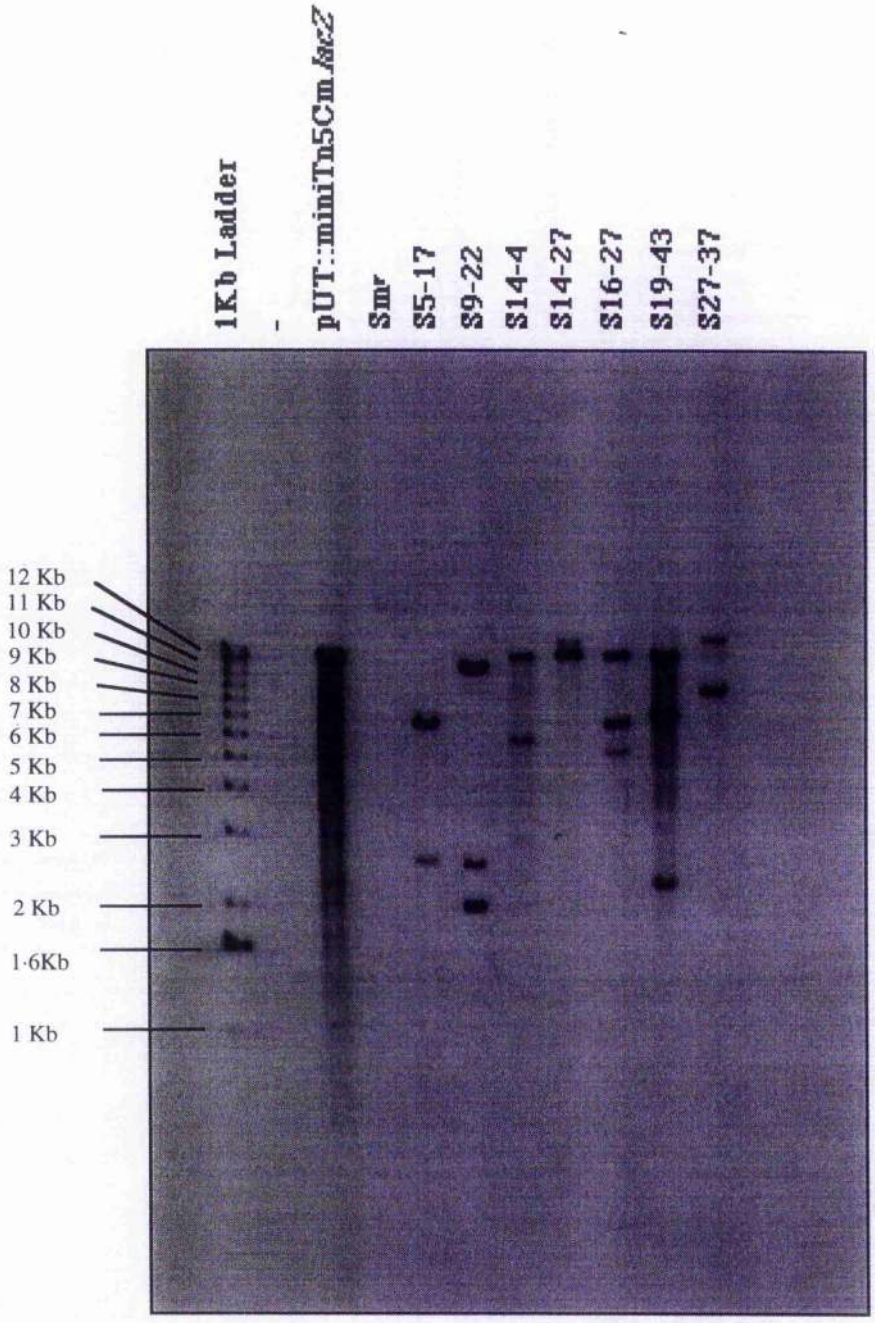


Figure 61. Southern blot analysis of selected interesting exconjugants. A number of potentially interesting exconjugants were selected as a result of their low fluorescence in the adapted fluorescence assay. Exconjugant genomic DNA was digested with *Cla*I, separated on a 0·5% Agarose gel and blotted onto a nylon membrane. The resulting blot was probed with a DIG labelled 4·1kb fragment purified from *Eco*RI digested pUT::miniTn5Cm*lacZ*. The 1kb DNA markers were probed with DIG labelled 1kb DNA ladder.

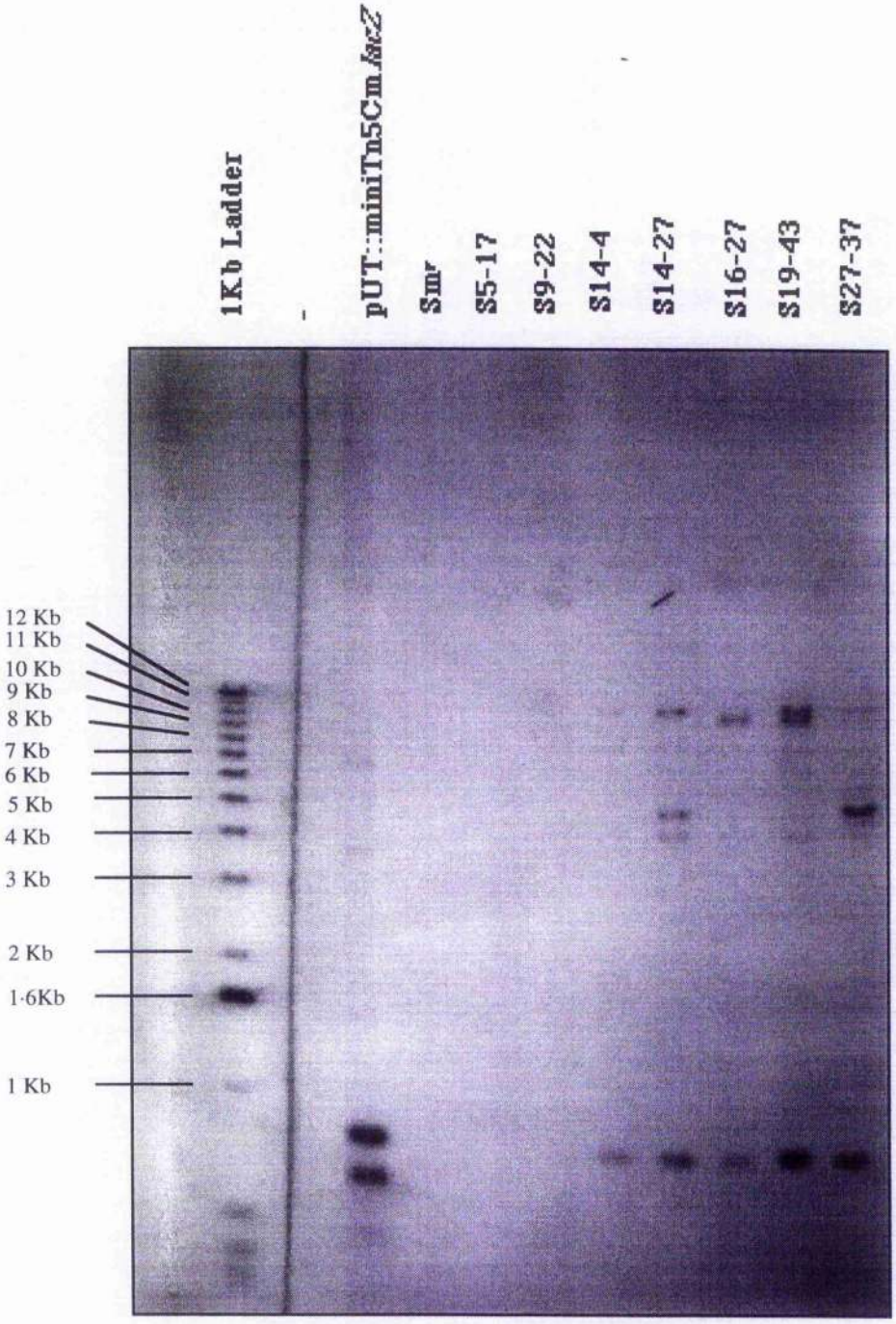


Figure 62. Southern blot analysis of selected interesting exconjugants. A number of potentially interesting exconjugants were selected as a result of their low fluorescence in the adapted fluorescence assay. Exconjugant genomic DNA was digested with *Pst*I, separated on a 0·5% Agarose gel and blotted onto a nylon membrane. The resulting blot was probed with a DIG-labelled 1·5kb fragment purified from *Sal*I digested pUT::miniTn5Cm*lacZ*. The 1kb DNA markers were probed with DIG-labelled 1kb DNA ladder.

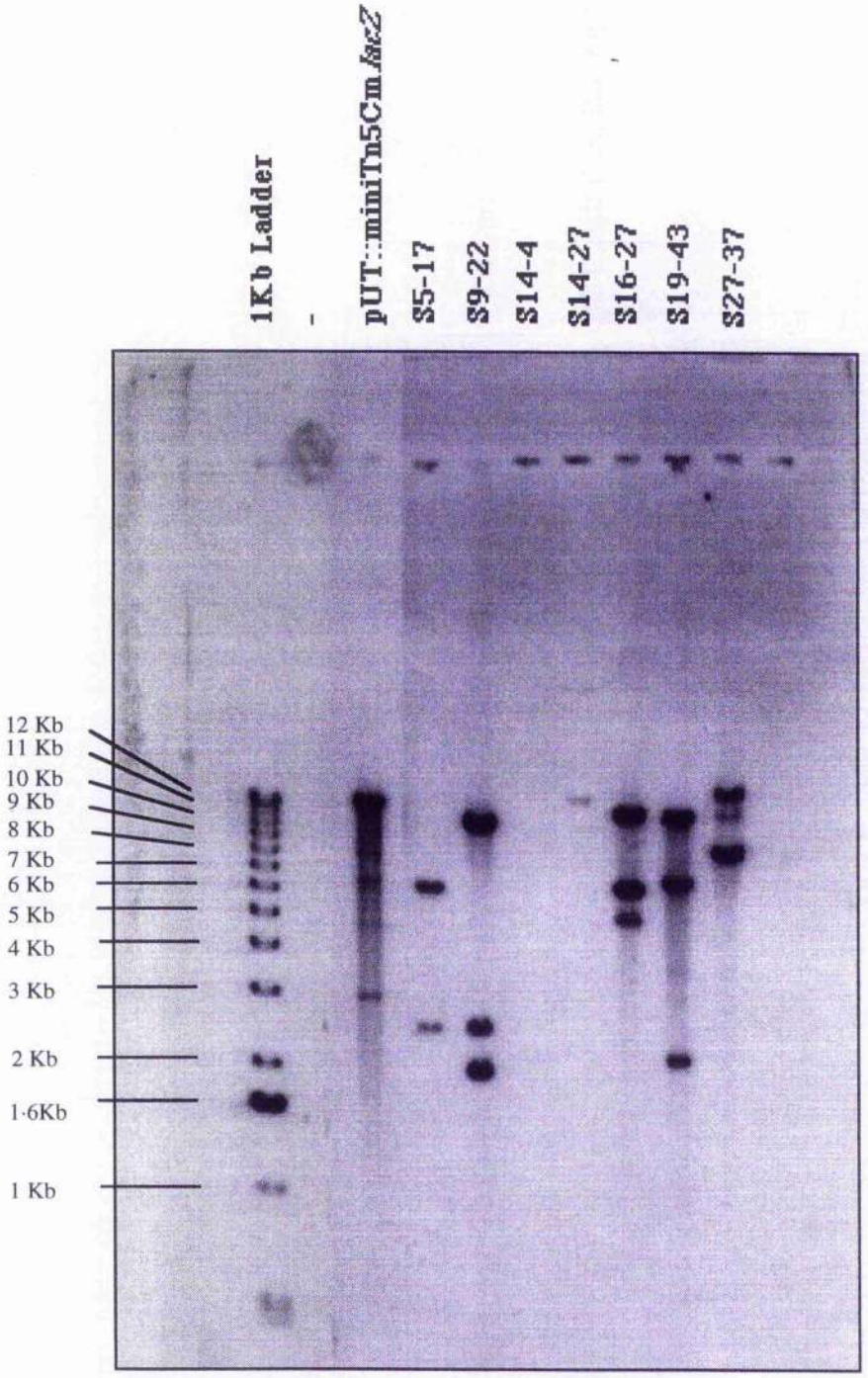


Figure 63. Southern blot analysis of cured exconjugants. Potentially interesting exconjugants were grown in the absence of antibiotics for six passages and genomic DNA isolated from these cured exconjugants. The genomic DNA was digested with *Cla*I, separated on a 0·5% Agarose gel and blotted onto a nylon membrane. The resulting blot was probed with a DIG-labelled 4·1kb fragment purified from *Eco*RI digested pUT::miniTn5Cm*lacZ*. The 1kb DNA markers were probed with DIG-labelled 1kb DNA ladder.

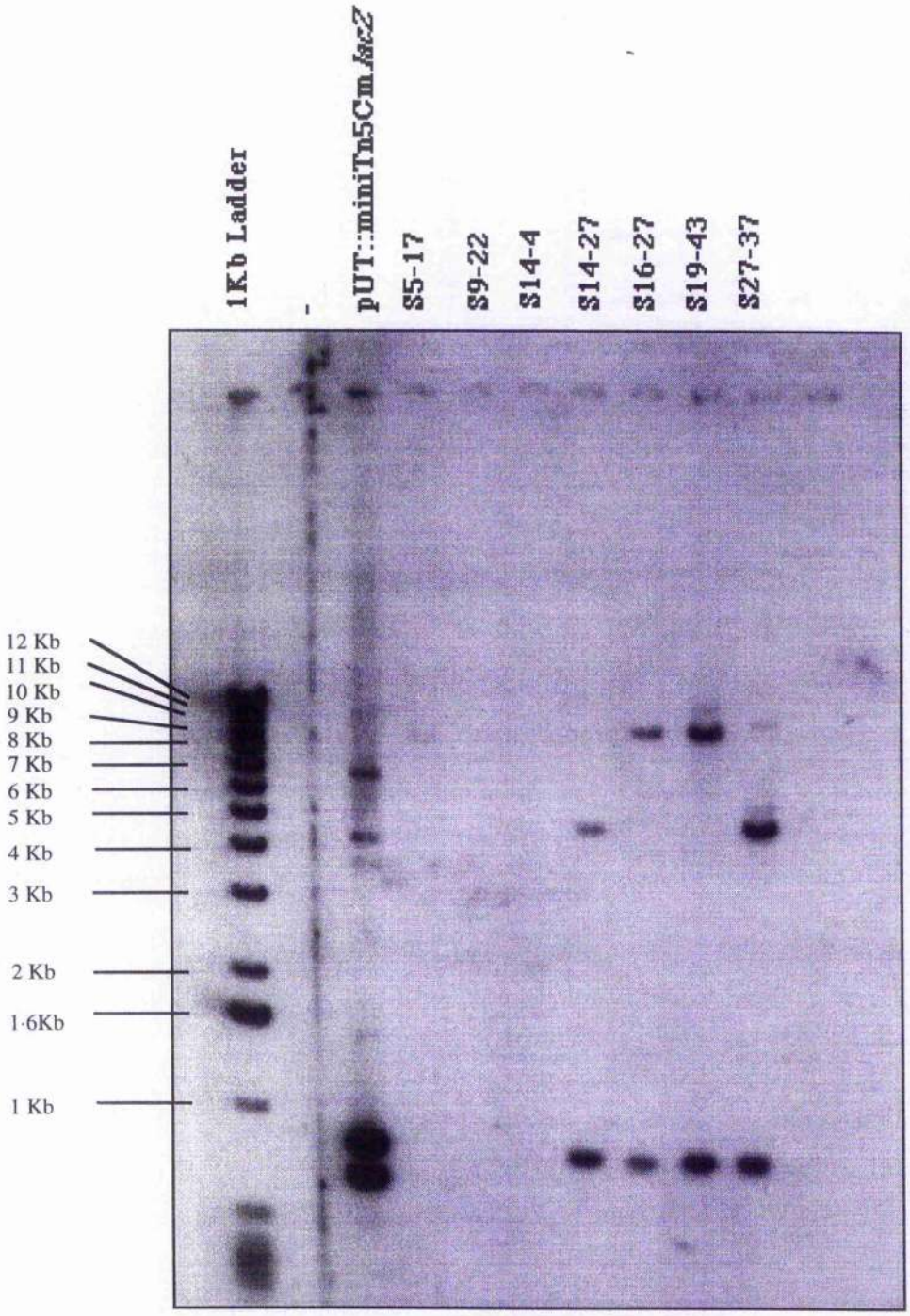


Figure 64. Southern blot analysis of cured exconjugants. Potentially interesting exconjugants were grown in the absence of antibiotics for six passages and genomic DNA isolated from these cured exconjugants. The genomic DNA was digested with *Pst*I, separated on a 0·5% Agarose gel and blotted onto a nylon membrane. The resulting blot was probed with a DIG-labelled 1·5kb fragment purified from *Sal*I digested pUT::miniTn5Cm*lacZ*. The 1kb DNA markers were probed with DIG-labelled 1kb DNA ladder.

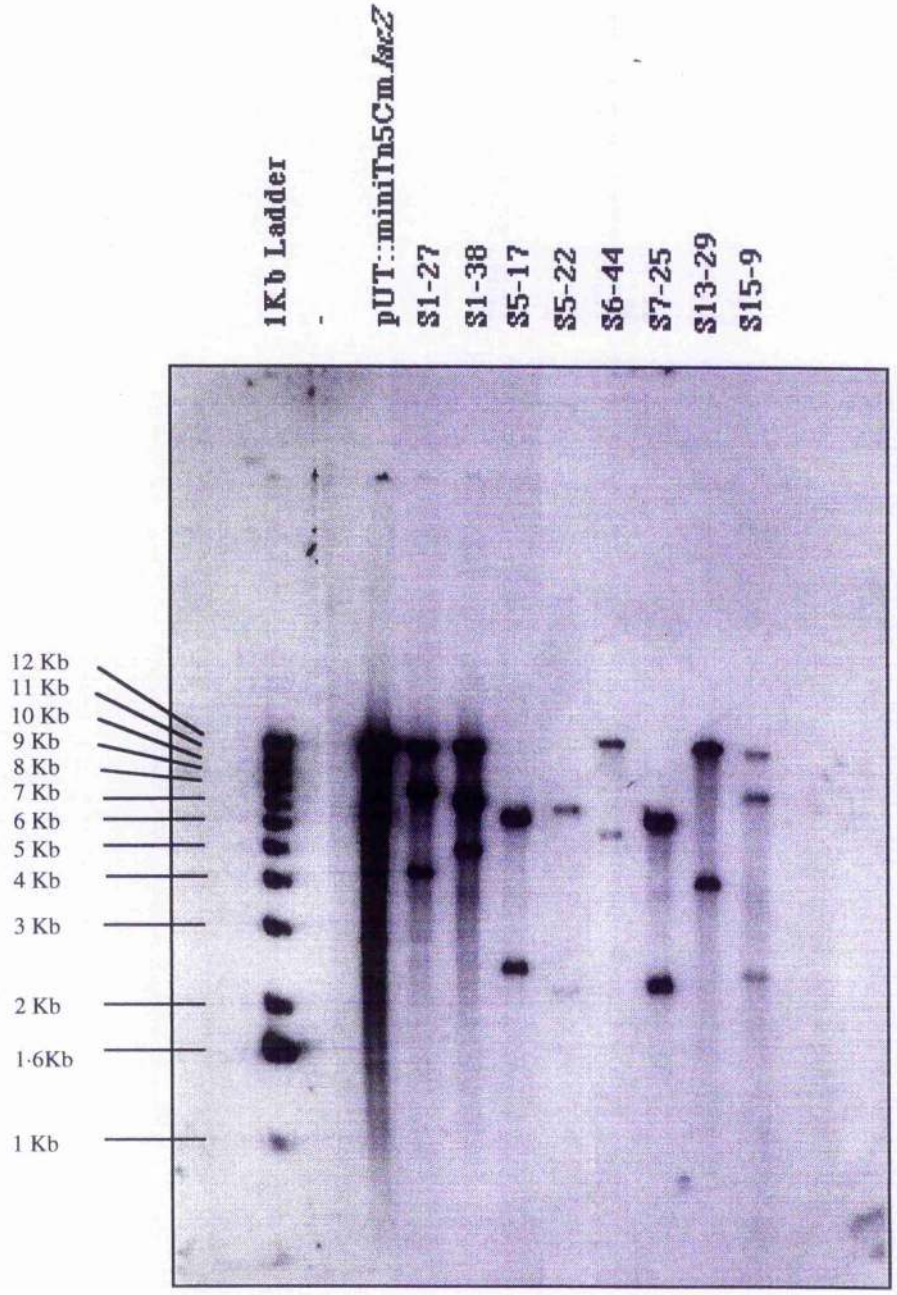


Figure 65. Southern blot analysis of exconjugants selected at random. Genomic DNA was isolated from a number of exconjugants which were selected at random. The genomic DNA was digested with *Cla*I, separated on a 0·5% Agarose gel and blotted onto a nylon membrane. The resulting blot was probed with a DIG-labelled 4·1kb fragment purified from *Eco*RI digested pUT::miniTn5Cm*lacZ*. The 1kb DNA markers were probed with DIG-labelled 1kb DNA ladder.

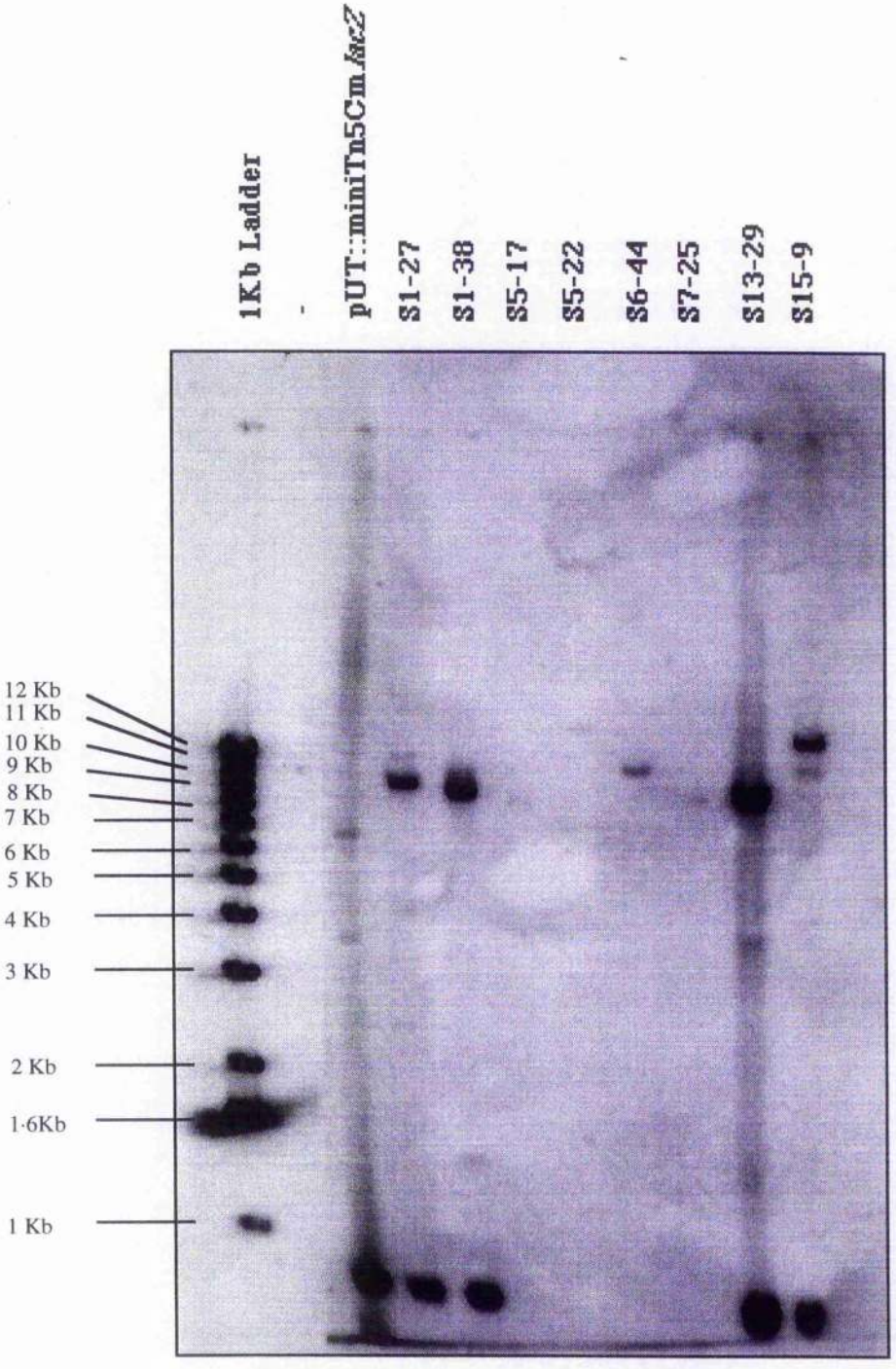


Figure 66. Southern blot analysis of exconjugants selected at random. Genomic DNA was isolated from a number of exconjugants which were selected at random. The genomic DNA was digested with *Pst*I, separated on a 0·5% Agarose gel and blotted onto a nylon membrane. The resulting blot was probed with a DIG-labelled 1·5kb fragment purified from *Sal*I digested pUT::miniTn5Cm*lacZ*. The 1kb DNA markers were probed with DIG-labelled 1kb DNA ladder.

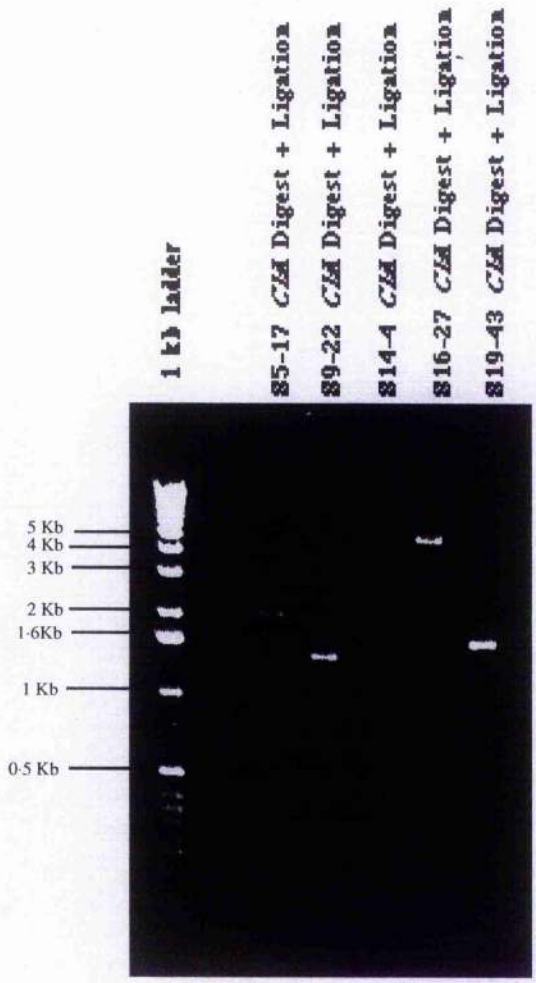


Figure 67. Inverse PCR amplification products containing the flanking sites from selected exconjugants. The inverse PCR products for selected exconjugants were separated on a 0·7% agarose gel.

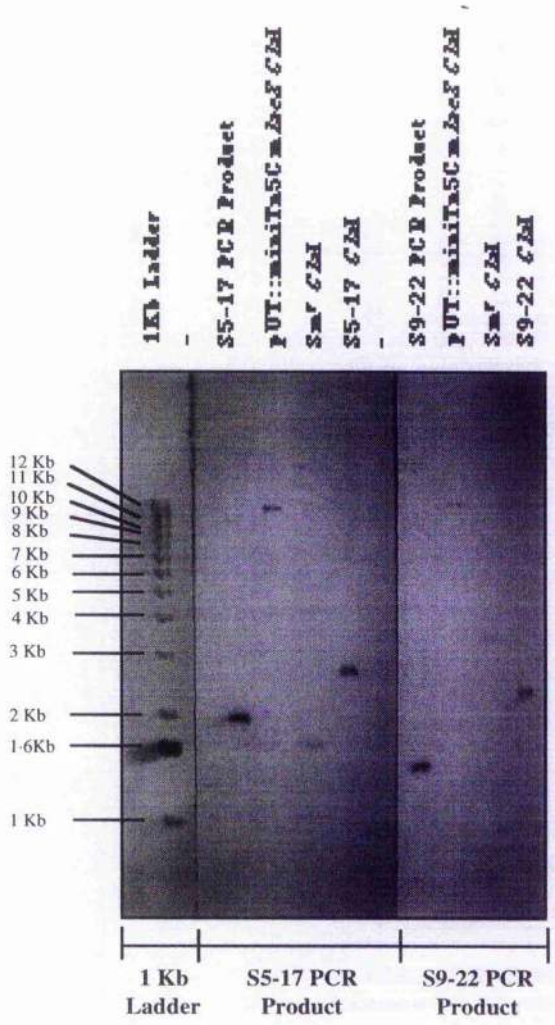


Figure 68. Confirmation of the identity of inverse PCR products from exconjugants S5-17 and S9-22 by Southern blot analysis. The inverse PCR products from exconjugants S5-17 and S9-22, pUT::miniTn5Cm*lacZ* DNA, genomic DNA from A862 Sm^r and genomic DNA from exconjugants S5-17 and S9-22 were separated on a 0·7% agarose gel and Southern blotted onto a nylon membrane. The appropriate DIG-labelled inverse PCR product was used to probe the different sections of this blot as indicated at the bottom of the figure. DIG-labelled 1kb ladder was used to detect the 1kb DNA markers.

DNA sequences of miniTn5Cm*lacZ* flanking sites

The DNA sequences of the inverse PCR products derived from exconjugant S5-17, S9-22 and S19-43 are shown in Appendix 4, the sequences presented for S9-22 and S19-43 spanning the full length of the PCR products. These sequences should not however be considered to be complete as they contain a number of N's, i.e. unresolved nucleotides. The sequence presented for S5-17 is in two parts as the middle or joining sequence has yet to be determined. The underlined portion of the presented sequences of the PCR products contain homology to the expected portion of pUT::miniTn5Cm*lacZ*. That is, the first 377 nucleotides of each of the sequences corresponds to the first 88 nucleotides of the *lacZ* gene (accession number V00296), 270 nucleotides of "spacer" DNA and the 19 bp O end of the miniTn5 construct (Herrero *et al.*, 1990). The last 111 nucleotides of each sequence corresponds to nucleotides 730 to 840 of the *lacZ* gene sequence (accession number V00296).

Sequence analysis of exconjugant S5-17 inverse PCR product

Analysis of the DNA sequence derived from the inverse PCR product of exconjugant S5-17 revealed homology to a number of entries in the sequence databases. A BLASTN search of the S5-17 sequence revealed homology to the gene encoding the *Salmonella enterica* SpaO (surface presentation of antigen) invasion protein. The sequence between nucleotide A848 and A788 of the S5-17 sequence was 68% identical to nucleotides 592 to 652 of the *spaO* gene of a number of *Salmonella enterica* strains (e.g. accession number U29350).

A BLASTX search revealed two regions of amino acid sequence homology between the translated DNA sequence of the S5-17 PCR product and the histone transcription regulator 2 protein (Hir2) of *Saccharomyces cerevisiae* (accession number P32480). The first of these two homologous regions is the translated sequence from nucleotides A784 and A725 of the S5-17 sequence, which is 55% identical (70% similar) to amino acids 138 to 157 of Hir2. The second is the translated sequence from nucleotide A559 to A449, which is 29% identical (54% similar) to amino acids 700 to 736 of Hir2 (see Appendix 5).

A FASTA search revealed homology to a number of DNA sequences in the database, but these were mostly sequences of unknown function or the region homologous to S5-17 was not part of an open reading frame (ORF). One exception to this however was the *melA* gene of *Shewanella colwelliana* (accession number M59289), which encodes an enzyme responsible for the biosynthesis of melanin (Fuqua*et al.*, 1991). The sequence of *melA* between residues 1200 and 1380 is 59% identical to nucleotides A421 and A595 of the DNA sequence of the S5-17 PCR

product. However, a comparison of the amino acid sequence of the MelA protein with the amino acid sequence obtained by translating the S5-17 DNA sequence in all the possible frames, failed to reveal any significant homology.

FASTA also revealed homology between the S5-17 sequence and sequences of *Saccharomyces cerevisiae*. Nucleotides A896 to A660 of the S5-17 sequence are 57% homologous to nucleotides 45450 to 45681 of *S. cerevisiae* chromosome VIII (accession number U00029) and 57% homologous to nucleotides 49950 to 50184 of *S. cerevisiae* chromosome I (accession number L28920).This homology spans a region from approximately 100 nucleotides preceding an ATG start codon (position A799 of the S5-17 sequence) to approximately 140 nucleotides within the ORF, which encode acid phosphatases in *S. cerevisiae* (Arima *et al.*, 1983).

Translation of the S5-17 DNA sequence revealed a possible ORF beginning at nucleotide A799 and reading in the reverse direction. The amino acid sequence of this ORF (shown in Appendix 5) consists of 124 amino acids before encountering a stop codon. A possible ribosome binding site (RBS) is located 5 bp upstream from the ATG start codon. This RBS contains the sequence GAACCA which is similar to the reported consensus sequence for RBS in *E. coli* (TAAGGAGG; Gold, 1988). If however, the second methionine residue of the amino acid sequence is the start codon (i.e. nucleotides A793 to A791) a more likely RBS is located at nucleotides A803 to A799 (i.e. 5 bp upstream of the start codon; TAAAAA). In addition, if the ATG at position A793 is the start codon then the second codon would be AAA. This is one of the two most abundant second codons in *E. coli* mRNAs (Gold, 1988) and therefore supports the allocation of the second ATG as the start codon. The amino acid sequence of this ORF includes the two regions with homology to the Hir2 protein of *S. cerevisiae*.

Sequence analysis of exconjugant S9-22 inverse PCR product

A comparison of the DNA sequence of the S9-22 inverse PCR product with sequences in the databases revealed homology to a number of serine proteases of the subtilase family. For example, BLASTN revealed 56% homology between nucleotides 1120 to 1282 of the S9-22 inverse PCR product and nucleotides 392 to 554 of the pro-hormone convertase 2 (PC2) gene of *Sus scrofa* (pig; accession number X68603). BLASTN also indicated 66% homology between the S9-22 sequence (nucleotides 1061 and 1126) and a *Bacillus* sp. serine protease gene (nucleotides 482 to 547; accession number L29506).

Translation of the S9-22 sequence revealed a possible ORF beginning at the ATG start codon located at position 951 (see Appendix 6). This ORF is not interrupted by any stop codons and ends at the *Cla*I ligation site. This ligation site is a

product of template preparation and is not found in the genome of exconjugant S9-22, therefore this ORF is not interrupted in the genome by the insertion of miniTn5Cm*lacZ*.

A polypurine sequence (GAGA) is located 5 bp upstream of the ATG start codon at position 942 to 945 indicating a possible RBS (Gold, 1988). Sequences with similarity to the -35 (TTGACA) and -10 (TATAAT) consensus sequence of *E. coli* "house-keeping" (σ^{70}) promoters are located at 910 to 915 (TGCACA) and 935 to 940 (TCTGTA) in the S9-22 sequence. The 19 bp gap between these possible -10 and -35 sequences is consistent with the spacing in *E. coli* promoters (15 to 20 bp; Hawley and McClure, 1983).

A MOTIF search of the amino acid sequence of this ORF highlighted an aspartic acid active site of the serine protease subtilase family at amino acid 97 to 99 (i.e. the underlined sequence in Appendix 6).

A comparison of the S9-22 amino acid sequence with the known amino acid sequences of serine proteases of the subtilase family is shown in Appendix 7. This multiple sequence alignment clearly shows that S9-22 contains similarities to members of the subtilisin subfamily (e.g. *Vibrio metschnikovii* alkaline serine protease), thermitase subfamily (e.g. *Thermus aquaticus* aqualysin), proteinase K subfamily (e.g. *Vibrio alginolyticus* Ca-dependent serine protease), kexin subfamily (e.g. *Schizosaccharomyces pombe* dibasic endopeptidase) and pyrolysin subfamily (e.g. *Bacillus* sp. minor extracellular serine protease) of the subtilase serine proteases (Siezen and Leunissen, 1997).

A common feature of many, but not all, serine proteases is an extracellular location and therefore, a signal sequence at the N-terminal end of the unprocessed protein. However, analysis of the S9-22 amino acid sequence failed to reveal a signal sequence which conformed to the typical structure of known bacterial signal sequences (Von Heijne, 1986).

Sequence analysis of exconjugant S19-43 inverse PCR product

A FASTA search of the S19-43 inverse PCR product sequence revealed homology to a number of sequences including *Clostridium difficile* toxin A gene (accession number M30307), *Clostridium botulinum* neurotoxin type F gene (accession number M92906) and *Plasmodium falciparum* major merozoite surface antigen (accession number M19143). However, no significant homology was detected between the amino acid sequence derived from these DNA sequences and the derived amino acid sequence of S19-43.

A BLASTX search revealed 48% identity (72% similarity) between the amino acid sequence encoded by nucleotides 378 to 449 of the S19-43 sequence and 25

amino acids at the carboxyl end of *E. coli* histone like protein H1 (H-NS; accession number X07688). A multiple sequence alignment of the derived amino acid sequence of S19-43 and known bacterial H-NS sequences (see Appendix 8) highlights the similarity of these sequences. It is probable therefore that a putative H-NS gene of A862 has been interrupted by insertion of miniTn5Cm*lacZ*.

DNA SEQUENCE OF A GENE FROM STRAIN A862 WHICH ENCODES A H-NS HOMOLOGUE

The DNA sequence of a single specific primer (SSP)-PCR product from strain A862 which contains a putative *hns* gene is shown in Appendix 9. An ORF, i.e. the putative *hns* gene, is located between the ATG start codon at position 168 and the TAA stop codon at 558. The derived amino acid sequence of this putative H-NS protein is also shown in Appendix 9. Sequence comparisons of the putative H-NS protein from strain A862 and known H-NS-like proteins are shown in Appendix 8 and 10.

AMPLIFICATION OF POTENTIAL SULFOTRANSFERASE GENES FROM THE MARINE BACTERIA A862

Comparison of known sulfotransferase sequences

A comparison of the amino acid sequence of the putative PAPS binding site of eukaryotic cytosolic sulfotransferases (ST) and the amino acid sequence of known bacterial ST revealed some similarity but this homology is not complete (see Appendix 11). The complete RKGxxGDWKxxFT motif reported by Weinshilboum *et al.* (1997) is not found in the bacterial ST. It is not clear however, if this difference is a result of the diversity of the organisms in which these enzymes are found (i.e. humans, plants and eubacteria) or whether it is a result of the different substrate specificity of the enzymes. The only non-cytosolic ST of eukaryotic origin included in the sequence alignment is the rat N-heparan sulphate ST. The amino acid sequence of the putative PAPS binding site of this enzyme also differs from the consensus sequence of cytosolic ST.

SSP-PCR amplification of potential sulfotransferase genes from A862

Single specific primer PCR was performed on four different template preparations using four different primer combinations (see "Materials and Methods"). The

templates were prepared by ligation of genomic DNA from A862 which had been digested with *Eco*RI, *Hind*III, *Bam*HI or *Sma*I to pUC18 DNA which had been digested with the same restriction enzyme. The template DNA were then used in SSP-PCR reactions with one of the two primers designed to recognise the PAPS binding site (i.e. ST01 or ST03) and the M13R primer which recognises the M13 reverse primer site of pUC18. The template DNA was also used in nested SSP-PCR reactions which involved two rounds of PCR amplification. The preliminary amplification was performed with a PAPS binding site primer and primer L8867, which recognises a portion of the *lacZ* gene located on pUC18. The products of the preliminary amplification reactions were then used as template for a SSP-PCR reaction using the appropriate PAPS binding site primer and M13F, a primer which recognises the M13 forward (-40) primer site of pUC18.

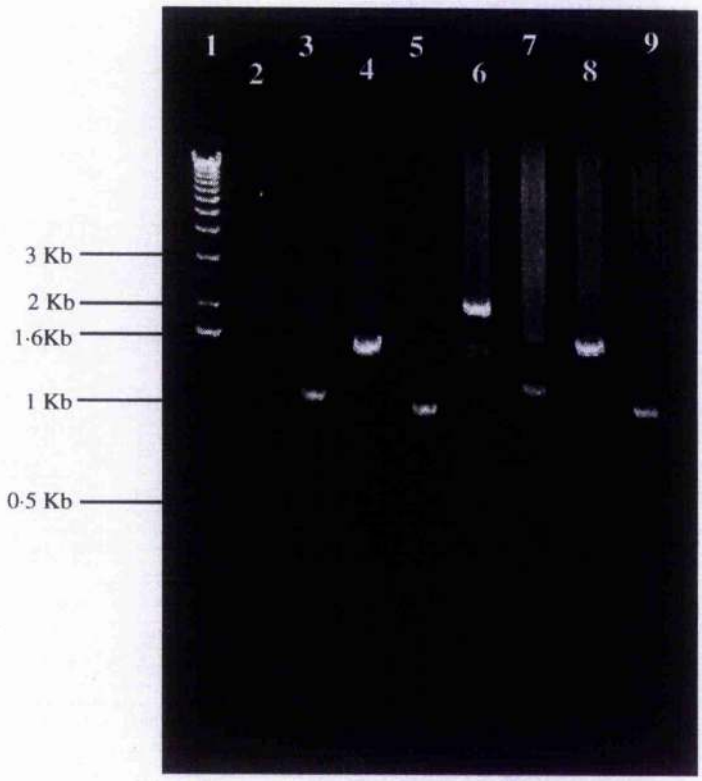
A number of products, of different lengths, were obtained in the different SSP-PCR reactions (see Figure 69 and 70). The major PCR products (i.e. the products with the greatest intensity on an agarose gel) were separated from the other PCR products by gel electrophoresis and Geneclean® purification and the purity of these products determined by gel electrophoresis (Figure 71 and 72). These PCR products were used as templates for automated DNA sequencing or cloned into pCR®2.1-TOPO prior to DNA sequencing (see Appendix 12). The successful cloning of the SSP-PCR products into pCR®2.1-TOPO was confirmed by restriction digest analysis (e.g. see Figure 73).

DNA sequences of SSP-PCR products

Preliminary analysis of the SSP-PCR product sequences (named NST 1 to NST 8 and NST 31 to NST 37) revealed that they all, except one (NST 31), contained the appropriate primer and pUC18 sequences at opposite ends of the product (see Appendix 12). Unfortunately none of the sequences of the SSP-PCR products possessed any significant homology to known sulfotransferases outside the primer site sequence.

Sequence analysis of SSP-PCR products NST 1 and NST 4

Analysis of the SSP-PCR products NST 1 and NST 4 revealed that they were highly homologous and most probably derived from the same genomic sequence. NST 1 is the shorter of these two products and consists of nucleotide 1 to 453 of the sequence shown in Appendix 12. MAPSORT analysis of the NST 4 sequence revealed an *Eco*R1 site at position 454 to 460, therefore supporting the hypothesis that these two products are derived from the same sequence.



Lane 1 = 1 kb DNA ladder.

Lane 2 = A862 Sm^r *Eco*RI + pUC18 *Eco*RI Ligation. ST01/L8867/M13F PCR.

Lane 3 = A862 Sm^r *Hind*III + pUC18 *Hind*III Ligation. ST01/L8867/M13F PCR.

Lane 4 = A862 Sm^r *Bam*HI + pUC18 *Bam*HI Ligation. ST01/L8867/M13F PCR.

Lane 5 = A862 Sm^r *Sma*I + pUC18 *Sma*I Ligation. ST01/L8867/M13F PCR.

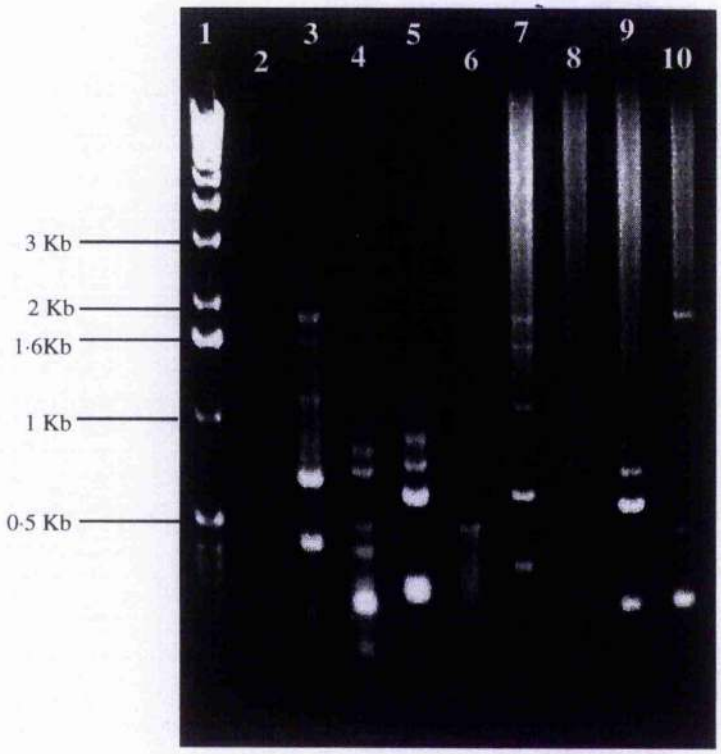
Lane 6 = A862 Sm^r *Eco*RI + pUC18 *Eco*RI Ligation. ST01/M13R PCR.

Lane 7 = A862 Sm^r *Hind*III + pUC18 *Hind*III Ligation. ST01/M13R PCR.

Lane 8 = A862 Sm^r *Bam*HI + pUC18 *Bam*HI Ligation. ST01/M13R PCR.

Lane 9 = A862 Sm^r *Sma*I + pUC18 *Sma*I Ligation. ST01/M13R PCR.

Figure 69. SSP-PCR products using degenerate primer ST01. The products from ST01 SSP-PCR amplification (i.e. using the degenerate primer ST01 in conjunction with pUC18 specific primers) were separated on a 0·7% agarose gel.



Lane 1 = 1 kb DNA ladder

Lane 2 = Empty

Lane 3 = A862 Sm^r *Eco*RI + pUC18 *Eco*RI Ligation. ST03/L8867/M13F PCR.

Lane 4 = A862 Sm^r *Hind*III + pUC18 *Hind*III Ligation. ST03/L8867/M13F PCR.

Lane 5 = A862 Sm^r *Bam*HI + pUC18 *Bam*HI Ligation. ST03/L8867/M13F PCR.

Lane 6 = A862 Sm^r *Sma*I + pUC18 *Sma*I Ligation. ST03/L8867/M13F PCR.

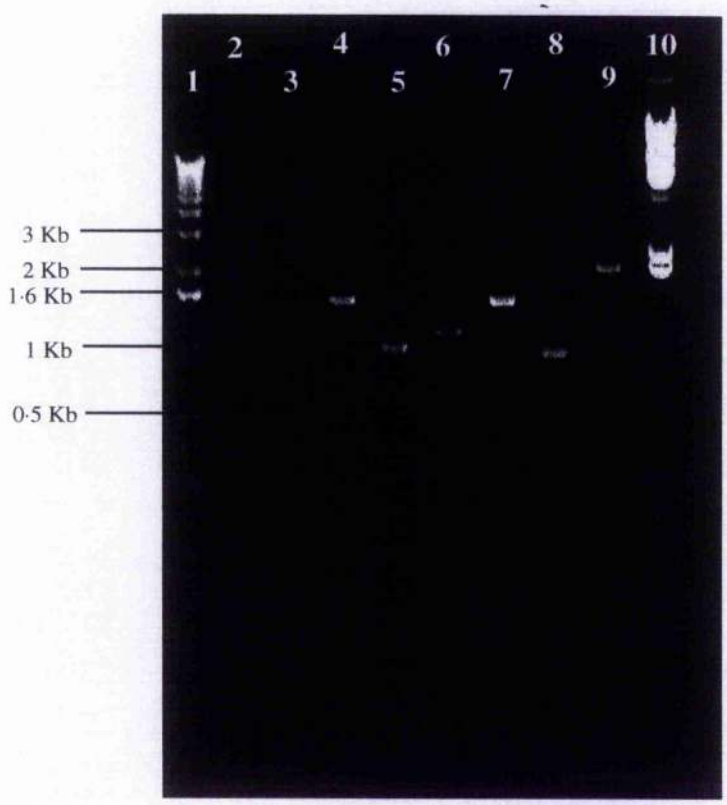
Lane 7 = A862 Sm^r *Eco*RI + pUC18 *Eco*RI Ligation. ST03/M13R PCR.

Lane 8 = A862 Sm^r *Hind*III + pUC18 *Hind*III Ligation. ST03/M13R PCR.

Lane 9 = A862 Sm^r *Bam*HI + pUC18 *Bam*HI Ligation. ST03/M13R PCR.

Lane 10 = A862 Sm^r *Sma*I + pUC18 *Sma*I Ligation. ST03/M13R PCR.

Figure 70. SSP-PCR products using degenerate primer ST03. The products from ST03 SSP-PCR amplification (i.e. using the degenerate primer ST03 in conjunction with pUC18 specific primers) were separated on a 0·7% agarose gel.



Lane 1 = 1 kb DNA ladder

Lane 2 = NST 1 (A862 Sm^r *Eco*RI + pUC18 *Eco*RI Ligation. ST01/L8867/M13F PCR)

Lane 3 = NST 2 (A862 Sm^r *Hind*III + pUC18 *Hind*III Ligation. ST01/L8867/M13F PCR)

Lane 4 = NST 3 (A862 Sm^r *Bam*HI + pUC18 *Bam*HI Ligation. ST01/L8867/M13F PCR)

Lane 5 = NST 4 (A862 Sm^r *Sma*I + pUC18 *Sma*I Ligation. ST01/L8867/M13F PCR)

Lane 6 = NST 5 (A862 Sm^r *Hind*III + pUC18 *Hind*III Ligation. ST01/M13R PCR)

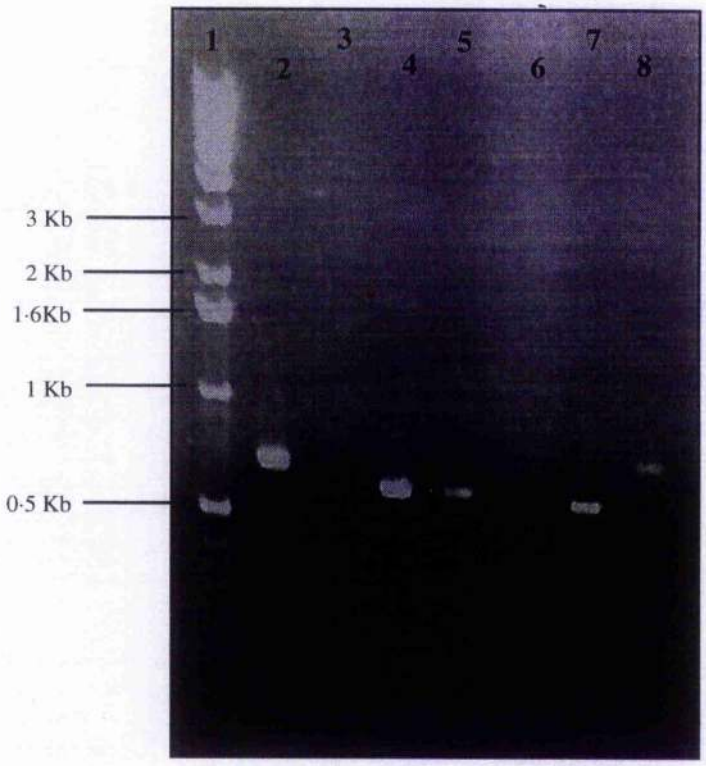
Lane 7 = NST 6 (A862 Sm^r *Bam*HI + pUC18 *Bam*HI Ligation. ST01/M13R PCR)

Lane 8 = NST 7 (A862 Sm^r *Sma*I + pUC18 *Sma*I Ligation. ST01/M13R PCR)

Lane 9 = NST 8 (A862 Sm^r *Eco*RI + pUC18 *Eco*RI Ligation. ST01/M13R PCR)

Lane 10 = λ*Hind*III DNA markers

Figure 71. Gel purified ST01 SSP-PCR products. A number of PCR fragments from the ST01 SSP-PCR reactions were excised from an agarose gel and the DNA recovered utilising a Geneclean III Kit. The gel purified SSP-PCR products were separated on a 0·7% agarose gel.



Lane 1 = 1 kb DNA ladder

Lane 2 = NST31 (A862 Sm^r *Eco*RI + pUC18 *Eco*RI Ligation. ST03/L8867/M13F PCR)

Lane 3 = NST32 (A862 Sm^r *Eco*RI + pUC18 *Eco*RI Ligation. ST03/L8867/M13F PCR)

Lane 4 = NST33 (A862 Sm^r *Bam*HI + pUC18 *Bam*HI Ligation. ST03/L8867/M13F PCR)

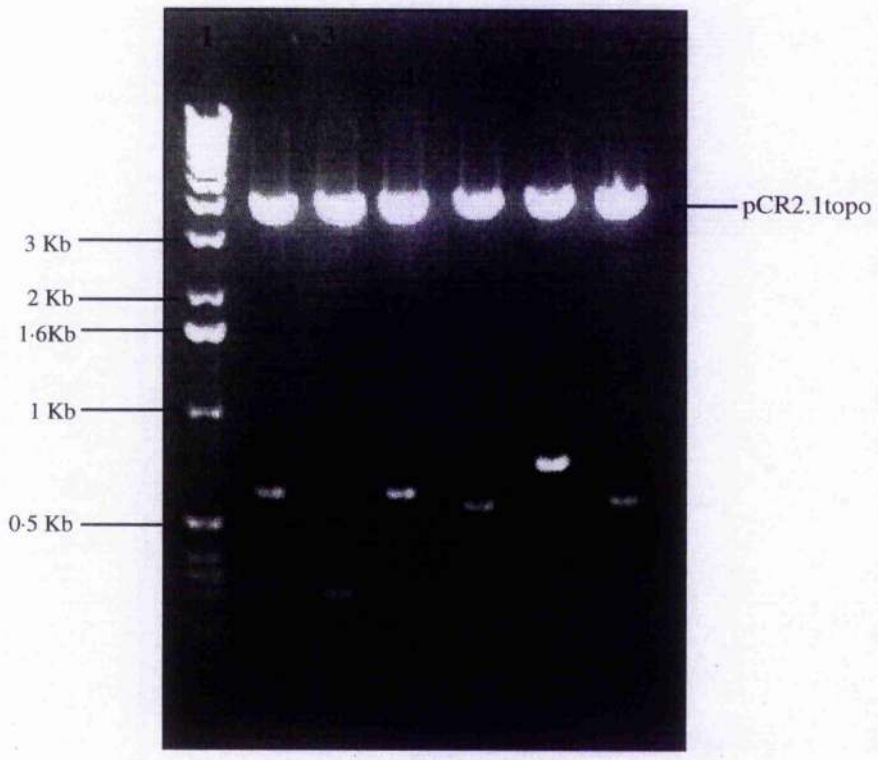
Lane 5 = NST34 (A862 Sm^r *Eco*RI + pUC18 *Eco*RI Ligation. ST03/M13R PCR)

Lane 6 = NST35 (A862 Sm^r *Eco*RI + pUC18 *Eco*RI Ligation. ST03/M13R PCR)

Lane 7 = NST36 (A862 Sm^r *Bam*HI + pUC18 *Bam*HI Ligation. ST03/M13R PCR)

Lane 8 = NST37 (A862 Sm^r *Bam*HI + pUC18 *Bam*HI Ligation. ST03/M13R PCR)

Figure 72. Gel purified ST03 SSP-PCR products. A number of PCR fragments from the ST03 SSP-PCR reactions were excised from an agarose gel and the DNA recovered utilising a Geneclean III Kit. The gel purified SSP-PCR products were separated on a 0·7% agarose gel.



Lane 1 = 1 kb DNA ladder

Lane 2 = pNST31 (pCR®2.1-TOPO + NST31)

Lane 3 = pNST32 (pCR®2.1-TOPO + NST32)

Lane 4 = pNST33 (pCR®2.1-TOPO + NST33)

Lane 5 = pNST36 (pCR®2.1-TOPO + NST36)

Lane 6 = pNST37a (pCR®2.1-TOPO + NST37)

Lane 7 = pNST37b (pCR®2.1-TOPO + NST37)

Figure 73. *Eco*RI restriction digest analysis of cloned ST03 SSP-PCR products. A number of the gel purified ST03 SSP-PCR fragments were incorporated into pCR®2.1-TOPO (Invitrogen). The resulting clones were digested with *Eco*RI and the digests run out on a 0·7% agarose gel. *Eco*RI cuts pCR®2.1-TOPO at either end of the cloning site, therefore releasing any insert DNA.

A comparison of the sequence of NST 1 and NST 4 with sequences in the databases revealed considerable homology (54% over 765 bp from nucleotide 967 to 202) with a sequence of the cyanobacterium *Synechocystis* sp. (accession number D90917; nucleotide 82134 to 82899). This *Synechocystis* sp. sequence encodes a hypothetical protein of unknown function.

Sequence analysis of SSP-PCR products NST 2, NST 3, NST 5 and NST 8

Sequence analysis of the SSP-PCR products NST 2, NST 3 ,NST 5 and NST 8 revealed significant homology between these sequences. It was therefore assumed that these products were derived from the same genomic sequence.

A FASTA search using the consensus sequence derived from these SSP-PCR products revealed homology to hydroxymethylglutaryl-CoA synthase genes from *Mus musculus* and *Sus scrofa* (accession numbers U12790 and U90884 respectively; 62% identity in 96 nucleotides) and staphylococcal protein A gene from *S. aureus* (accession number M18264; 60% identity in 140 nucleotides).

A BLASTN search revealed homology to a genomic sequence of *Synechocystis* sp. (accession number D64005; 63% identity in 141 nucleotides) and the *helA* gene of *Legionella pneumophila* (accession number U49498; 54% identity in 222 nucleotides). HelA is presumed to function with HelC and HelB in the efflux of an unidentified substrate (McClain *et al.*, 1996).

Sequence analysis of SSP-PCR products NST 31 and NST 34

Sequence analysis of the SSP-PCR products NST 31 and NST 34 revealed significant homology between these sequences. It was therefore assumed that these products were derived from the same genomic sequence.

FASTA, BLASTN and BLASTX searches all revealed homology between NST 31 and aspartyl-tRNA synthases (e.g. accession number X53863; 65% identity in 532 nucleotides). Aspartyl-tRNA synthase catalyses the following reaction (Delarue and Moras, 1993) :-

ATP + L-aspartate + tRNA(ASP) → AMP + pyrophosphate + L-aspartyl-tRNA(ASP).

Sequence analysis of SSP-PCR products NST 32 and NST 35

Sequence analysis of the SSP-PCR products NST 32 and NST 35 revealed significant homology between these sequences. It was therefore assumed that these products were derived from the same genomic sequence.

FASTA, BLASTN and BLASTX searches all revealed homology between NST 32 and isopropylmalate dehydratases (synthases) (e.g. accession number X51476; 62% identity in 266 nucleotides). Isopropylmalate dehydratases catalyses the following reaction:-

$$\text{3-isopropylmalate} \rightarrow \text{2-isopropylmalate} + H_2O.$$

The BLASTX search also revealed homology to aconitase hydratase, an enzyme from the tricarboxylic acid cycle that catalyses the reversible isomerisation of citrate and isocitrate (e.g. accession number P49609; 37% identity and 60 % similarity in 40 amino acids). Isopropylmalate dehydratase and aconitase hydratase are highly related enzymes (Hentze and Argos, 1991).

Sequence analysis of SSP-PCR products NST 33, NST 36 and NST 37b

Preliminary sequence anlaysis of the SSP-PCR products NST 33, NST 36 and one of two different NST 37 clones (NST 37b) revealed significant homology between these sequences. It was therefore assumed that these products were derived from the same genomic sequence.

A BLASTN search revealed homology to *Pseudomonas* sp. biphenyl dioxygenase (accession number M86348; 93% identity in 31 nucleotides) and the *Calloselasma rhodostoma* ancrod, a thrombin-like enzyme of the venom of this snake species (accession number L07308; 57% identity in 106 nucleotides).

A BLASTX search revealed homology to acyl carrier protein synthase III (e.g. accession number A64493; 26% identity, 43% similarity in 90 amino acids), proline dipeptidase (e.g. accession number Q11136; 28% identity, 59% similarity in 32 amino acids, 70% identity, 80% similarity in 10 amino acids and 38% identity, 66% similarity in 21 amino acids) and acetyl-CoA-acyltransferase (e.g. accession number P27796; 36% identity, 56% similarity in 44 amino acids).

A FASTA search revealed homology to *Coxiella burnetti* citrate synthase (accession number L33409; 60% identity in 131 nucleotides), a rat lanosterol 14-demethylase pseudogene (accession number D78370; 66% identity in 88 nucleotides)

and caffeoyl-CoA-3-*O*-methyltransferase (accession number A22706; 57% identity in 157 nucleotides).

Sequence analysis of SSP-PCR product NST 37a

BLASTN and BLASTX searches did not reveal any significant homology between the sequence of NST 37b and sequences of known function in the databases. A FASTA search revealed homology to adenylate cyclase genes (e.g. accession number M12057; 68% identity in 68 nucleotides) and a *Drosophila melagaster* gene for furin 1, a serine protease of the subtilase family (accession number L12376; 60% identity in 146 nucleotides). There was no significant homology however between NST 37a and the inverse PCR product of exconjugant S9-22 (a putative serine protease).

DISCUSSION

MARINE BACTERIA USED IN THIS STUDY

Ideally the determination of the molecular mechanisms involved in SCB toxin production would be carried out through studies using the organisms most commonly associated with PSP, the most widespread intoxication associated with SCB toxins. Historically the production of PSP toxins has been attributed to dinoflagellates and cyanobacteria. Although modern techniques are now enabling the molecular biology of these organisms to be studied (e.g. Machabée *et al.*, 1994; Wolk, 1996), such techniques are limited by the complex nature of these organisms. The relative simplicity of the genetics of heterotrophic bacteria make these organisms much more accessible to genetic manipulation and mutation. However, the production of PSP toxins by heterotrophic bacteria is still a controversial subject. Although a number of different techniques (e.g. mouse bioassay, HPLC, mouse neuroblastoma SCB assay; reviewed in Gallacher *et al.*, 1996; and more recently capillary electrophoresis-mass spectrometry; Gallacher *et al.*, 1997) have now been used to detect PSP toxins in bacterial cultures it is still argued by others in the field that conclusive, unambiguous proof is still lacking. Although the quantities of toxin produced are very low, when the accumulated evidence for the production of PSP toxins by heterotrophic bacteria is taken as a whole it seems unlikely that the contrary will prove to be the case. It was therefore considered feasible to use a number of heterotrophic bacteria for this study on the production of SCB toxins.

The heterotrophic marine bacteria used here were selected because they were reported to contain SCB toxins by Yasumoto *et al.* (1986), Yotsu *et al.* (1987), Gallacher & Birkbeck (1995) and Gallacher *et al.* (1997). Although TTX derivatives have historically been associated with puffer fish poisoning, and STX derivatives with paralytic shellfish poisoning, there is now some doubt as to the absolute correlation between the two families of toxins and the intoxications they cause. For example, a recent report has described PSP as the principal toxic component of fresh water puffer fish (Sato *et al.*, 1997). Of particular interest is the finding that at least one bacterial isolate, *Shewanella alga* OK1, appears to produce both TTX and PSP toxin derivatives in culture (Gallacher *et al.*, in preparation). The mechanisms of action of TTX and PSP toxins are identical and, therefore, the symptoms caused by the consumption of these toxins are also indistinguishable. It would not be at all surprising if more cases of puffer fish toxicity are caused, at least in part, by PSP toxins. It is also possible that TTX may contribute to the toxicity of shellfish and paralytic shellfish poisoning. If the genetic apparatus involved in the production of each of these toxin groups can be identified, a clearer picture of their relative importance in sea food intoxication may be established (e.g. by employing probes to detect toxin-producing organisms in the environment). Also, it is not at all clear why

bacteria produce SCB toxins at all. Although TTX and the PSP toxins share the same mode of toxicity, they may not have related roles in their source organisms. A comparison of the molecular mechanisms involved in the production of these two groups of toxins may supply clues as to any relationship between the compounds with regard to their functions.

Several transposons, which carry different antibiotic resistance genes, are available for mutagenesis experiments, and the choice of transposon depends partly on the antibiotic sensitivity of the target organism. For this reason, the antibiotic sensitivity of the marine bacteria considered for use in this study was determined. Any strain with a spontaneous antibiotic resistance rate of greater than 1×10^{-6}, the expected frequency of transposon mutagenesis, was considered unsuitable for use with that antibiotic as the number of false positive colonies would be too high. From Table 9 it is clear that each of the marine isolates was available for use with at least one transposon type as each was sensitive to at least one antibiotic. The miniTn5 derivatives which carry a chloramphenicol resistance marker were deemed the most useful vectors for this study as the majority of the marine isolates were sensitive to this antibiotic and mutated to resistance at only a low rate.

During the course of these experiments it was observed that a number of strains did not grow on LB20 medium without the addition of $MgCl_2$. However, as magnesium ions are reported antagonists of tetracycline (Sambrook *et al,* 1989), these strains were not suitable for mutagenesis with transposons encoding tetracycline resistance.

Four of the ten marine strains tested were completely resistant to ampicillin (Table 9) and because ampicillin resistance is encoded by the suicide vectors used to deliver the transposons into recipient cells it would not be straightforward to demonstrate loss of the suicide vector.

A longer term aim of this project was to investigate the level of expression of the genes involved in SCB production in response to different environmental stimuli. For this purpose transposons carrying a *lacZ* reporter gene were used and it was necessary to incorporate the *lacZ* reporter gene into the pUT::miniTn5Cm transposon. This was facilitated by the auxiliary plasmid pUJ8 and the presence of a unique *Not*I site in pUT::miniTn5Cm. The successful construction of pUT::miniTn5Cm*lacZ* therefore extended the application of *lacZ* promoter reporter technology to strains which display significant Km^r or Tc^r. Unfortunately, two of the strains considered for mutagenesis displayed native ß-galactosidase activity (Table 8) and therefore the *lacZ* reporter genes could not be used with these strains.

Through consideration of all these factors, it was decided that three different combinations of recipient strain and transposon vectors should be used in transposon mutagenesis studies. These were the TTX producing strain *Alteromonas tetraodonis*

GFC with pJO100, strain A912 with pUT::miniTn5Km*lacZI* and strain A862 with pUT::miniTn5Cm*lacZ*.

DETECTION OF PSP TOXINS IN BACTERIAL CULTURE

The successful application of mutagenesis to investigate a specific bacterial characteristic is dependent largely on the ability to detect the desired phenotype. As several thousand exconjugants may have to be screened to identify a mutant with the desired phenotype, the method of detection should preferably be rapid, simple and inexpensive. A number of different methods for the detection of SCB toxins were reviewed earlier (see "Methods used for the detection of SCB toxins"). However, these methods have been chiefly developed for the detection of PSP toxins in shellfish extracts and extracts of dinoflagellates or cyanobacteria. As the levels of toxins detected in cultures of heterotrophic bacteria are significantly lower than those found in shellfish, dinoflagellates and cyanobacteria (Kodama & Ogata, 1988; Gallacher *et al.*, 1997) difficulties arise with regard to assay sensitivity. In addition, most of the techniques routinely employed for detection of PSP toxins in research and monitoring programmes have a low rate of sample turnover. For example, van Egmond *et al.* (1993) state that the mouse bioassay has a rate of analysis of between 10 to 30 samples a day and HPLC has a rate of analysis of 20 samples per day. These techniques are therefore not suitable for screening a large bank of mutants for the loss of PSP production.

One assay which was considered to have a suitable sample analysis rate for preliminary screening for PSP toxicity was the chemical fluorescence assay first described by Bates & Rapoport (1975). It was decided to evaluate this assay for its ability to detect PSP toxins in bacterial cultures. To facilitate the interpretation of the results of fluorescence assays it was necessary to analyse the bacteria of interest by HPLC to identify the toxins present.

Pre-column oxidation HPLC analysis of A862

When the pre-column oxidation HPLC method described by Lawrence & Menard (1991) and Lawrence *et al.* (1991, 1995 and 1996) was evaluated using PSP toxin standards the resulting chromatograms (Figure 13, 15 & 16) equated well with the hypothetical chromatogram of Lawrence *et al.* (1996; see Figure 12). The separation of the different toxins was good and the expected number of oxidation products were observed following oxidation with peroxide and periodate.

However, a surprising result was found when PSP toxin standards were diluted in marine broth prior to pre-column oxidation with peroxide, and HPLC

analysis, in that the peak corresponding to the STX oxidation product was greatly enhanced. As no peak was observed when marine broth was analysed alone, this observation must be due to the influence of marine broth on oxidation of STX. This result was in contrast to the observation when STX in marine broth was oxidised by peroxide and the fluorescence determined by spectrophotometry (Figure 43), where marine broth appeared to have a negative affect on STX oxidation or fluorescence. The enhanced peak area of the STX product, caused by dilution in marine broth prior to peroxide oxidation, is not likely to be a result of any influence marine broth has on the fluorescence of the oxidation product. Rather, the effect is probably on the oxidation of STX to a fluorescent derivative. In addition to this positive influence on oxidation, it is possible that marine broth also has a negative effect of fluorescence. Therefore, the apparent quenching of oxidation or fluorescence observed by fluorescence measurements before separation by HPLC may in fact be a result of two factors; a positive effect on oxidation and a much greater negative effect on fluorescence. As marine broth does not appear to have any significant effect on the oxidation products of the other PSP standards, it does not seem likely that the increased peak for the STX product is a result of abnormal oxidation of the other PSP toxins to yield the oxidation product usually associated with STX.

This observation has important implications for the quantification of PSP toxins by oxidation and fluorescence measurements, with or without HPLC separation. It is important that the PSP standards of known concentration used for calibration of the method are diluted in a solution analogous to that of the sample. In an ideal system internal standards (i.e. spiking of the sample with known amounts of toxin) should be used in preference to external standards. In addition, as different toxins are influenced differentially by some media component(s), accurate quantification can only be assumed if the toxin standard used is identical to the PSP derivative in the sample.

Analysis of A862 culture supernatant from 10 ml marine broth cultures revealed a fluorescent peak which was only present when the sample was oxidised with peroxide under alkaline conditions prior to HPLC analysis (Figure 17). As this peak was not present in unoxidised samples it was thought that this peak may be a PSP toxin. However, as N-hydroxylated PSP toxins are not oxidised by peroxide it is not likely that it belongs to this group of toxins. Also, the peak is not affected by acid hydrolysis (Figure 20) and is therefore probably not an N-sulfocarbamoyl toxin.

The retention time of the unidentified peak was similar to the oxidation products observed for C1 and dcSTX. However, when A862 supernatant was spiked with C1 or dcSTX, the peaks corresponding to the oxidation products of these toxins displayed different retention times from the fluorescent peak in A862 supernatant. It is clear, therefore, that the fluorescent peak in A862 is not formed from the oxidation

of any of the STX derivatives which have so far been analysed by pre-column oxidation HPLC analysis. The oxidation products of a number of PSP toxins have been partly characterised by LC-MS and a number of proposed reaction pathways presented (Quilliam *et al.*, 1993). If the fluorescent peak in A862 is derived from a STX derivative, the structure of the oxidation product of this toxin must be sufficiently different from the known oxidation products to cause a shift in peak retention time.

Recently, cultures of the fresh water cyanobacterium *Lyngbya wollei* have been reported to contain PSP toxins. The HPLC analysis presented for this organism indicated the presence of unknown toxic peaks (Carmichael *et al.*, 1997; Yin *et al.*, 1997), but the publication of the structure of these previously unidentified, putative PSP toxins is still awaited. It is possible, however, that the unidentified peak in A862 culture supernatants is similar to one of these toxins. Alternatively, it may represent a novel PSP toxin, although the possibility remains that it is a compound unrelated to PSP toxins.

Pre-column HPLC analysis of A862 cell extracts and culture supernatant from larger volumes of culture did identify a number of other fluorescent peaks, i.e. in addition to peaks with the same retention time and fluorescent characteristics as peak U in Figure 17 (Figure 21 to 24). However, the fluorescent properties of these peaks in response to different oxidation conditions were not the same as was observed for known STX derivatives (Figure 13 to 16). It is unlikely, therefore, that they represent PSP toxins. It is possible that the large number of peaks present in the cell extracts are masking other peaks which consist of PSP toxins. However, without further sample preparation, including for example, C18 solid phase extraction (SPE), it is difficult to say with any certainty that there are no other putative PSP peaks in the A862 cell extracts.

Therefore, from the pre-column oxidation HPLC analysis of A862 presented here it is not possible to state with any certainty that A862 produces PSP toxins. There is a fluorescent peak in both culture supernatants and cell extracts of A862 which displays the same fluorescence characteristics, with regard to oxidation, as PSP toxins. Further analysis of this fluorescent peak will be discussed later (see "Chemical fluorescence assay for PSP toxins"). However characterisation of the fluorescent oxidation product by mass spectrometry will be required before it is possible to state with any certainty that it is derived from a novel derivative of STX. The contribution, if any, that the parent compound of this fluorescent product may have on A862 toxicity also requires investigation. The parent compound should be purified, for example by liquid chromatography, and assayed for toxicity, preferably by a number of methods, including the mouse bioassay, mouse neuroblastoma SCB assay and receptor binding assays. Further analysis of the purified parent compound,

for example by PSP immunoassays, saxiphilin binding assays, post-column oxidation HPLC and LC-MS, may then confirm the characterisation of this compound as a PSP toxin.

Post-column oxidation HPLC analysis of A862

Fluorescent peaks were observed in A862 culture supernatants and A862 cell extracts when they were analysed by the three isocratic systems (only A862 cell extracts were analysed with the third isocratic elution). However, the identification of these peaks as PSP toxins is not conclusive although A862 culture supernatant may contain neoSTX and GTX 4, and A862 cell extracts may contain B2, GTX 4, GTX 1, C2, C3 and C4. Further, more comprehensive HPLC analysis is required to clarify this. Such analysis must include known standards of all the toxins thought to be present in A862. So that retention times can be more accurately compared, these standards should be internal standards (i.e. spiked samples), rather than the external standards used in this study. However, an identical retention time is not sufficient to identify a fluorescent peak as a PSP toxin. The differences in the chemical characteristics observed for some of the peaks in A862 and the known characteristics of PSP toxins indicate that they are most likely not identical to these toxins. Only one peak in A862 culture supernatants and cell extracts (peak X in Figure 29 to 31) has an identical retention time and a similar response to acid hydrolysis as a PSP toxin standard analysed, i.e. GTX 4.

The post-column and pre-column oxidation HPLC analysis presented here have highlighted a limitation of this technique. HPLC is only suitable for the comparison of the chromatographic characteristics of an unknown substance with those of a known standard. It is a powerful technique for establishing how much of a known substance is present in a given sample. It is not however a suitable technique for the identification of an unknown substance in a sample. It may suggest a possible identification to the analyst (i.e. if the retention time is indistinguishable from a known standard) and indicate what the compound is not (i.e. if the retention time is different from a known standard), but further analysis, for example by mass spectrometry, must be performed to confirm the identity of the substance. Once the identity of an HPLC peak in the given sample is confirmed, it can be assumed, with a reasonable level of certainty, that peaks with the same retention time in similar samples are the same substance. Until there is more definitive proof that heterotrophic bacteria, and indeed the specific species being studied, produce a PSP toxin, HPLC data must be viewed with caution.

In addition, if a fluorescent peak in a HPLC analysis does not match any known standards, it would be premature to conclude that it was not a PSP toxin. It is

almost certain that novel STX derivatives have yet to be identified. This is of particular importance when new species are being analysed, as demonstrated by the recent reports of unidentified toxic peaks in *Lyngbya wollei* (Carmichael *et al.*, 1997; Yin *et al.*, 1997). Although HPLC cannot characterise a substance as being a PSP toxin, it may prove to be a useful tool for the purification of sufficient quantities of the unknown substance for further analysis.

Chemical fluorescence assay for PSP toxins

Preliminary evaluation of the fluorescence assay

The chemical fluorescence assay, first described by Bates & Rapoport (1975), was evaluated for the detection of STX and an excellent correlation was found between the concentration of STX in 0·05 N acetic acid and the corrected fluorescence of the peroxide oxidised sample, at least for the range of concentrations tested (i.e. 0 to 2·5 μM; Figure 35). This observation is in agreement with Bates & Rapoport (1975) who reported a linear relationship between fluorescence and concentrations of STX oxidation product from 1 nM to 10 μM. However, as the different PSP derivatives do not fluoresce with equal intensity following alkaline oxidation with peroxide (Buckley *et al.*, 1978), the oxidation reactions used in HPLC analysis of PSP toxins (Sullivan & Iwaoka, 1983; Lawrence & Menard, 1991; Franco & Fernández-Vila, 1993; Oshima, 1995b; Flynn & Flynn, 1996) were scaled up and evaluated in the chemical fluorescence assay.

The fluorescence of STX following the adapted pre-column peroxide oxidation reaction was found to be 9-fold greater than the fluorescence of STX following the two different pre-column periodate oxidation reactions (Figure 35). This difference, although greater than expected, is in reasonable agreement with the approximate sensitivities for the analysis of STX by pre-column oxidation HPLC with peroxide and periodate oxidation (154 cm/ng & 60 cm/ng respectively; a 2·6 fold difference; Lawrence *et al.*, 1991).

Of the marine isolates which were assayed by the chemical fluorescence assay, only strain A862 displayed a significant difference in the fluorescence intensity of the oxidised and blank reactions (Figure 38 & 39). Therefore, if there were any PSP toxins in the culture supernatants and cell extracts of A912 and 4αVS3, the levels were below the level of detection of this assay. However, it is possible that the predominant toxins in these strains are N-hydroxylated toxins (e.g. neoSTX or $GTX_{1/4}$), but when these strains were assayed by periodate oxidation reactions, no significant corrected fluorescence was observed (data not shown).

The validity of the fluorescence assay as a measure of PSP toxin in strain A862 is equivocal, however, as this strain also displayed a large level of fluorescence in the

unoxidised sample (Figure 38 and 39). This fluorescence cannot be caused by PSP toxins as they are not fluorescent before oxidation under alkaline conditions (Wong *et al.*, 1971). Thus, it was clear that there are other fluorescent compounds in A862 and it was not obvious from the initial evaluation of the fluorescence assay whether the corrected fluorescence was indicative of PSP toxins, or due to an influence of peroxide on an unknown fluorescent compound. Indeed it was apparent from the fluorescence of A862 following periodate oxidation that oxidation has a negative effect on fluorescence. This may lead to an underestimate of the amount of PSP in a sample as any increase in fluorescence resulting from the oxidation of PSP toxins will be offset by the decrease in the fluorescence of the unknown in response to oxidation.

However, while the influence of incubation time on the oxidation and fluorescence of A862 culture supernatants was being evaluated an interesting observation was made. In order to gain a true t=0 fluorescence measurement, the acetic acid, which was usually added after oxidation has taken place, was added to the reaction prior to the addition of sample. Therefore, no oxidation of PSP toxins could proceed as this reaction could only proceed under alkaline conditions (Wong *et al.*, 1971). However, the fluorescence of the t=0 (i.e. acid oxidised or AOx) reactions were found to be lower than the fluorescence of the corresponding blank reaction (Figure 40). It was apparent therefore, that the negative influence which oxidising agents had on the fluorescence of an unknown substance was exerted, at least in part under acidic conditions. However, if A862 culture supernatant was incubated with peroxide in alkaline conditions, the decrease in fluorescence caused by the reaction observed with the t=0 reaction was not as large. It was considered, therefore, that this apparent smaller decrease was in fact the result of a separate positive effect that the oxidising agent has on the fluorescence of another substance, for example PSP toxins, which counteracts, at least partly, the negative effect on the fluorescence of an unknown substance.

The blank reaction used in the original fluorescence assay could not, therefore, be considered a proper control for background fluorescence if a sample contained other fluorescent compounds. In order to take into account any influence that the oxidising agent had on interfering fluorescent substances, it was proposed that the blank reaction was replaced with an acid oxidation reaction. The fluorescence of PSP toxins was not affected by this reaction. Therefore, any change in the fluorescence of a sample resulting from oxidation under acid conditions could be subtracted from the fluorescence of an alkaline oxidation reaction, and the resulting corrected fluorescence taken as a more accurate measure of putative PSP toxin content.

Evaluation of adapted fluorescence assay

When the adapted fluorescence assay, incorporating acid oxidation as a control rather than the blank reaction, was evaluated, the fluorescence of STX was found to retain the linear relationship between concentration and fluorescence (Figure 43). Also, known concentrations of STX could be detected in bacterial culture supernatants (Figure 41 & 42). It was clear from the analysis of spiked supernatants that the original assay (i.e. using the blank reaction as a control) underestimated the amount of STX in the sample, therefore confirming the inadequacy of this reaction as a control and the adapted fluorescence assay as a superior assay for the detection of PSP toxins.

However, as with the original fluorescence assay, only strain A862 displayed a significant difference in fluorescence following alkaline oxidation compared to acid oxidation (Figure 42 & 46), indicating the possible presence of PSP toxins. Therefore, if PSP toxins are present in the supernatants of the other strains, the amount of toxin present is near the limit of detection of this assay.

The effect of marine broth on the fluorescence assay

During the initial evaluation of the adapted fluorescence assay it was noted that the corrected fluorescence of A862 culture supernatant spiked with STX was not equal to the sum of the corrected fluorescence of A862 supernatant and STX assayed separately (Figure 41). It was considered that a component of the culture supernatant inhibited either the oxidation of STX to fluorescent products or the fluorescence of these products. In a subsequent experiment, when STX was added to marine broth and the culture supernatant of four different marine strains, the level of fluorescence in each sample attributed to the added STX was approximately equal (Figure 42). This indicated that the fluorescence of the STX oxidation product was not adversely affected in the supernatants apart from any effect that marine broth may have had on the oxidation of STX or the fluorescence of the oxidation product.

When standard curves of STX corrected-fluorescence were obtained with dilutions of STX in acetic acid and STX in marine broth, it was clear that marine broth indeed had a negative effect on some aspect of STX oxidation and fluorescence, as the standard curve obtained for STX in marine broth displayed a much shallower gradient than the curve obtained for STX in acetic acid (Figure 43). This observation indicates that the sensitivity of the fluorescence assay depends on the matrix of the sample, i.e. the actual detection limit of the fluorescence assay was higher when the PSP was in marine broth rather than in acetic acid. Also, if quantitative estimates of the PSP content of a sample are required, it is important that the standards used for the calibration of the assay are diluted in a matrix as similar to that of the sample as is reasonably possible.

Peptone, a major constituent of marine broth, also inhibited STX fluorescence (Figure 44) but not to the same extent as the inhibition for marine broth, indicating that another constituent of marine broth also contributed to the inhibition.

Other workers have reported that peptone and, more specifically, tyrosine has an inhibitory effect on an assay for lignin peroxidase which involves the oxidation of veratryl alcohol to veratraldehyde. The activity of the lignin peroxidase was inhibited by yeast extract, peptone and L-tyrosine but not by L-phenylalanine, the nonphenolic analogue of L-tyrosine (Ten Have *et al.*, 1997). It is possible that, in an analogous manner, L-tyrosine is the constituent of peptone which contributes to the inhibition of oxidation of PSP toxins by peroxide observed in this study.

Fluorescence of strain A862

Further analysis of the fluorescence properties of A862 confirmed the significant difference between the fluorescence of alkaline peroxide-oxidised supernatants and acid peroxide-oxidised supernatants (Figure 46). Unexpectedly, however, the fluorescence of replicate samples of A862 culture supernatant following acid peroxide oxidation was not significantly different from the fluorescence of acid peroxide oxidised marine broth (Figure 46). This is in contrast to the observation with supernatants from other A862 cultures when fluorescence intensities were at least 3 fold higher than was routinely observed for marine broth (e.g. Figure 42). The major difference between the supernatants was in the growth conditions. It appears that when A862 is cultured in large volumes with good aeration a fluorescent compound is produced which is not present when this strain is cultured in smaller volumes with more limited aeration.

When a culture of A862 was assayed at different stages of growth, it appeared that at least two fluorescent compounds are produced at different stages of growth (Figure 47 & 48). If the corrected fluorescence (Ox-AOx) of the culture supernatants was indicative of PSP toxins, the amount of toxin in the cells remained reasonably constant throughout the growth cycle, but was only detectable in the culture supernatant from mid exponential phase, with the amount of toxin in the medium increasing beyond the stationary phase. These results indicate that the majority of PSP toxin produced by strain A862 is present in the culture supernatant.

During the late exponential or early stationary phase of growth (from 16 hours post inoculation) a different fluorescent factor was detected in culture supernatants and cell extracts. This unidentified substance did not appear to interfere with the fluorescence assay as the corrected fluorescence of these samples remained relatively constant from 18 hours to 54 hours, while the fluorescence of the individual reactions (i.e. non-corrected fluorescence) increased up to 8-fold over the same time period. It is probable that this second unidentified product corresponds to the fluorescent

compound found only when A862 is grown in large volume broth cultures. As small volume cultures were used for screening exconjugants, this compound is not a likely source of interference.

A comparison of the results obtained for strain A862 with the adapted fluorescence assay and the pre-column oxidation HPLC suggests that the corrected fluorescence detected by the assay corresponds to a single peak in the pre-column HPLC chromatograms (i.e. peak U in Figure 17). It was observed that the area of this peak in the HPLC analysis was closely correlated with the corrected fluorescence obtained by the fluorescence assay (e.g. see Figure 57 and Table 15). It is probable that the high level of background fluorescence observed from large volume cultures of A862 also corresponds to peak U in Figure 20. As discussed earlier, these two peaks (i.e. peak U in Figure 17, 57 and 21) have the same retention times but different fluorescent properties so any relationship between these compounds is not apparent.

The excitation and emission maxima for the oxidised PSP standards (Table 12) are comparable to the published maxima for PSP toxins (Table 3; Lawrence *et al.*, 1991; Quilliam *et al.*, 1993). However, when STX was diluted in marine broth its emission spectrum was masked by the emission spectrum of marine broth (Figure 51). It is probable, therefore, that the fluorescence properties of any PSP toxins in A862 culture supernatants are masked by other fluorescent compounds present. Thus, although the emission spectra for A862 culture supernatants (Figure 49) are different from those expected of PSP toxins, it cannot be concluded that PSP toxins are not present.

The emission maximum of HPLC fractions of A862 culture supernatant corresponding to the putative PSP toxin in A862 culture supernatant was approximately 396 nm (Figure 52a and Table 11). This is comparable to the observed emission maxima of the fractions collected during the analysis of STX + $GTX_{2/3}$ (383 to 387 nm; Figure 52a and Table 11) and the reported emission maxima of the oxidation products of PSP toxins (Table 3; Lawrence *et al.*, 1991; Quilliam *et al.*, 1993). The excitation maxima of the A862 supernatant fractions were, however, lower than those of PSP toxins (Figure 52b & Table 11). However, it is possible that there were other fluorescent compounds which co-eluted with the peak of interest (e.g. a trace amount of peak U from Figure 21 may have been present). Such co-eluting compounds could interfere with the fluorescence spectrum of the compound of interest, especially if they have a similar emission maximum to the peak of interest but a lower excitation maximum. Further purification of the peak of interest, for example by HPLC using different chromatographic conditions, is necessary to confirm that the excitation and emission maxima are representative.

Although it is clear that the adapted fluorescence assay can be used to detect PSP toxins in culture supernatants and cell extracts, it is not clear whether the corrected fluorescence observed in cell extracts or culture-supernatants of strain A862 are due to PSP toxins. The corrected fluorescence of strain A862 is correlated with a peak observed in pre-column oxidation HPLC analysis, but the retention time of this peak is not identical to any of the PSP standards tested. Further characterisation of this compound is required before it can be positively identified as a novel PSP toxin. The adapted fluorescence assay can however be used as a screening method for a large number of samples as it is relatively simple and fast to perform. Therefore, it was decided to use this assay as a screening method to identify exconjugants of possible interest. However, as there was doubt as to the identity of factors detected by this assay, a second method (i.e. the mouse neuroblastoma SCB assay) was also used. This allowed a comparison of the two assays and further evaluation of the adapted fluorescence assay. In addition, it was hoped that the characterisation of mutants which had lost the factor detected by the corrected fluorescence assay, would indicate the nature of this compound.

TRANSPOSON MUTAGENESIS OF HETEROTROPHIC MARINE BACTERIA WHICH PRODUCE SCB TOXINS

Mutagenesis of strain GFC with pJO100

Although Nal^r Tc^r exconjugants were isolated by mutagenesis of GFC Nal^r with the mini-Mu suicide delivery vector pJO100, the *E. coli* donor strain, J100, displayed spontaneous Nal^r on LB20 plates at a frequency of $3{\cdot}79\times10^{-6}$ and it is probable that the majority of the Nal^r Tc^r exconjugants had not received a copy of mini-Mu, but were spontaneous Nal^r derivatives of strain J100.

As the high number of expected potential false positives would require many more exconjugants to be screened to find mutants of interest, and because the transposase gene was within the transposon, i.e. mini-Mu(tet^r), and could stimulate subsequent transposition events, the use of pJO100 was not pursued. The alternative transposon vectors available, the pUT::miniTn5 family, have the advantage of increased choice of antibiotic resistance, a transposase gene located outside the transposon and a choice of at least two *E. coli* donor strains (De Lorenzo & Timmis, 1994). These vectors were therefore evaluated for transposon mutagenesis of selected bacterial strains of interest.

Mutagenesis of A912 and A862 with pUT::miniTn5 derivatives

Initial attempts to mutate A912 Nal^r and A862 Nal^r with pUT::miniTn5Km*lacZI* and pUT::miniTn5Cm*lacZ* were hampered by a background growth which was later attributed to resistance of the *E. coli* donor strains (i.e. SM10λ*pir* and S17-1λ*pir*) to nalidixic acid on marine agar. As the donor stains were sensitive to nalidixic acid on LB media, it appears that a constituent of marine agar inhibited the action of nalidixic acid. It is also possible that the spontaneous Nal^r observed for strain J100 on LB20 media (see "Mutagenesis of GFC with pJO100") resulted from a similar antagonistic affect.

Nalidixic acid acts on the DNA gyrase of bacteria which catalyses negative supercoiling of DNA (Hooper & Wolfson, 1989; Neu, 1992). It is possible that high salt concentration in marine broth alters the degree of DNA supercoiling in bacteria and inhibits the interaction of nalidixic acid with DNA gyrase. However, as Sm^r derivatives of the bacteria of interest were readily isolated, it was decided to use streptomycin to select against the donor *E. coli* SM10λ*pir*, which is sensitive to this antibiotic.An alternative donor strain, *E. coli* S17-1λ*pir* is Sm^r and could not be used in this instance.

The problem of antibiotic sensitivity of conjugative donor strains could be circumvented by introducing the transposon vector into the recipient organism by electroporation. This would negate any possibility of false positive exconjugants arising from these strains. Such an approach has recently been reported for mutagenesis of *Pseudomonas fluorescens* with pUT::miniTn5Km (Artiguenave *et al.*, 1997). As many as $7{\cdot}7\times10^5$ *Pseudomonas fluorescens* mutants were recovered per pmole of vector DNA, showing that this is an effective method of mutating strains of interest and an alternative to the conjugation method employed in this study.

Isolation of A912::miniTn5KmlacZI exconjugants

Nal^r Km^r exconjugants of the marine isolate A912 Nal^r, were recovered during ten separate mutagenesis experiments at frequencies between $2{\cdot}49\times10^{-6}$ and $1{\cdot}31\times10^{-4}$ and a bank of 1511 exconjugants isolated. The majority of these exconjugants remained viable after six rounds of replica plating on marine agar containing nalidixic acid and kanamycin. The Nal^r and Km^r of these exconjugants are therefore stable traits and support the belief that they are derived from true conjugation and transposition events. This is supported further by the observation that all but 12 (i.e. less than 1%) of the exconjugants were Amp^r, indicating that the pUT delivery vector had not been retained. If the exconjugants were false positives resulting from spontaneous Nal^r of the *E. coli* donor strain, the plasmid would have been expected to be retained in the presence of the selective antibiotic, kanamycin.

It therefore appears that a bank of 1505 A912 Nal^r Km^r exconjugants had been successfully isolated during transposon mutagenesis experiments and the methodology is available for this number to be readily increased.

Isolation of A862::miniTn5CmlacZ exconjugants

A wide range of exconjugant recovery rates ($2{\cdot}25\times10^{-6}$ to $3{\cdot}73\times10^{-9}$ per recipient cell) was observed during the mutagenesis of A862 Sm^r with Sm10λ*pir* pUT::mini-Tn5Cm*lacZ*. The highest recovery of exconjugants was achieved when there were more recipient cells than donor cells in the conjugation mix, and the frequency of exconjugant recovery only fell to 10^{-9} when there were more donor cells than recipient cells. As no significant effect of varying the donor:recipient ratio was observed in the mutagenesis experiments with strain A912, it appears that this is dependent on the recipient strain. Indeed, the donor:recipient ratio was highlighted by De Lorenzo & Timmis (1994) as a factor which may affect exconjugant recovery, depending on the recipient strain.

The recovery rates of exconjugants observed during mutagenesis of strain A862 and strain A912 were comparable with those reported by other workers for different organisms. For example, Herrero *et al.* (1990) reported exconjugant recovery rates which ranged from 6×10^{-5} to 2×10^{-6} per recipient cell for mutagenesis of *Pseudomonas putida* with a number of pUT::miniTn5 derivatives, Belas *et al.* (1991) reported the isolation of exconjugants at a frequency of about $4{\cdot}5\times10^{-6}$ per recipient cell when *Proteus mirabilis* was mutated with pUT::miniTn5Cm or pUT::miniTn5Sm, and Kolodrubetz & Kraig (1994) recovered spectinomycin-resistant exconjugants at a frequency of approximately 1×10^{-7} per recipient cell when *Actinobacillus actinomycetemcomitans* was mutated by pUT::miniTn5Sp. The recovery of exconjugants at frequencies such as these allows the efficient construction of a large bank of exconjugants which may then be screened for mutants of interest.

In total, 5328 exconjugants were isolated from six separate mutagenesis experiments. Unfortunately, only 2803 (i.e. 53%) of these exconjugants remained viable after six rounds of replica plating on marine agar containing selective antibiotics (i.e. streptomycin and chloramphenicol). It is possible that some of these exconjugants were not derived from true transposition events, but retained copies of the suicide vector, which were subsequently lost as they cannot replicate efficiently in these cells. Alternatively, the transposon may have caused a mutation of a gene which encodes a product which is not essential for the survival of the initial bacterium but is required for the continued survival of its progeny (e.g. if a mutant no longer encodes an enzyme which processes a product of secondary metabolism which is toxic to the cell at high concentrations, the concentration of the metabolite may only become lethal

after several generations). A third possibility is that the transposase gene had been retained, either on a retained copy of the vector or as a result of aberrant transposition. This gene could then allow transposition events to take place after initial isolation of exconjugants, which may lead to lethal mutations. However, a sufficient number of the initial exconjugants did survive the replica plating procedure to proceed with screening for mutants of interest.

ß-galactosidase activity of exconjugants

Insertion of the miniTn5Cm*lacZ* into the A862 genome causes expression of the *lacZ* reporter gene to come under the control of any promoter upstream from the insertion site. A number of exconjugants, selected at random, were assayed for ß-galactosidase activity and each contained a higher activity than was found in A862 wt (Figure 53). The level of ß-galactosidase was variable for the different exconjugants indicating that the level of expression of the *lacZ* gene in each of these exconjugants is different. This indicates that the transposon has inserted into a number of different sites, with the *lacZ* gene coming under the control of promoters of different strength. The *lacZ* reporter gene is therefore active in A862 and analysis of the expression of this gene in an exconjugant of interest will yield information regarding the expression of the inactivated gene.

The ß-galactosidase activity of the *E. coli* donor strains was much higher than expected (see Figure 54). The *lacZ* genes located on pUT::miniTn5Cm*lacZ* and pUT::miniTn5Km*lacZI* do not contain a promoter and therefore expression of this gene is not expected. The observed activity must therefore be a result of residual transcription induced by promoters located elsewhere on the plasmid. As multiple copies of pUT plasmids are present in the cell this residual transcription is sufficient to yield significant ß-galactosidase activity, however when the *lacZ* gene is transferred from the plasmid into the genome residual transcription is expected to be minimal (V. de Lorenzo, personal communication). This is what was observed in A862 exconjugants where some exconjugants had very low ß-galactosidase activity (e.g. exconjugants 1, 2 & 12 in Figure 53), much lower than was observed in the donor strains. It is assumed that these exconjugants have resulted from transposition of miniTn5Cm*lacZ* into a non-expressed region of the genome.

Structure of transposon insertions

Southern blot analysis of selected exconjugants revealed different patterns of hybridisation when the genomic DNA from the exconjugants was digested with *Cla*I and probed with a *lacZ* specific probe (Figure 61 & 65). Transposon insertion had not therefore been restricted to any "hot spot" of insertion, but appears to have been into a unique site in each exconjugant tested. The probability of mutating a gene of

interest is therefore not compromised by the insertion-site specificity of this transposon.

As there is only a single *Cla*I site in the *lacZ* gene, and no other such site is located elsewhere on the transposon, an exconjugant with a simple insertion of miniTn5Cm*lacZ* is expected to yield only two bands when the genomic DNA is digested with *Cla*I and probed with a *lacZ* specific probe (see Figure 74b). However, Southern blot analysis of a number of exconjugants (e.g. S9-22, S16-27 & S1-27; see Figure 61 & 65) had revealed three *Cla*I bands with significant homology to the *lacZ* probe. It is probable therefore that these exconjugants do not contain simple transposon insertions of the structure shown in Figure 74. An alternative to the formation of simple transposon insertion is the formation of cointegrates as shown in Figure 75. Such cointegrates originate from dimers (see Figure 75a) which are formed through recombination of two copies of the suicide vector (Berg, 1989). As the *E. coli* strains used for the manipulation of the delivery vectors are all *recA*$^-$ (see Table 5), it is unlikely that the dimers are formed in these strains. For dimers to be formed in the recipient strain however, more than one conjugative transfer of the plasmid must take place. It would be surprising however if this has occurred in such a large number of exconjugants (at least 50% of those tested by Southern blot analysis).

Further Southern blot analysis confirmed that a large number of exconjugants tested, in particular the majority of those which had three *Cla*I bands with homology to *lacZ*, had retained a copy of the transposase gene, *tnp** (see Figure 62 & 66). This gene is located outwith the boundary of the miniTn5Cm*lacZ* transposon (see Figure 8) and is therefore not retained after simple transposition (Figure 74). The exconjugants which have retained this gene must therefore have retained at least some part of the pUT delivery vector, either in the form of free plasmid or integrated into the genome as a cointegrate (Figure 75). As the *tnp** gene is not lost from the majority of the exconjugants tested, even after repeated growth in the absence of selective antibiotics (Figure 63 & 64), it is probable that the *tnp** gene is retained as part of a cointegrate. Comparison of the theoretical structure of different cointegrates (Figure 75b to 75d) reveals the structure shown in Figure 75b as that most probably formed in the majority of the exconjugants analysed. It is the only structure which would form three bands containing a portion of the *lacZ* gene. The other two cointegrate structures shown contain only a single copy the *lacZ* gene; as there is only a single *Cla*I site on the inserted DNA only two bands would be observed in the Southern blot analysis of exconjugants with such cointegrates.

The formation of cointegrates during transposon mutagenesis still results in the mutation of any gene interrupted by insertion of the transposon and vector DNA.

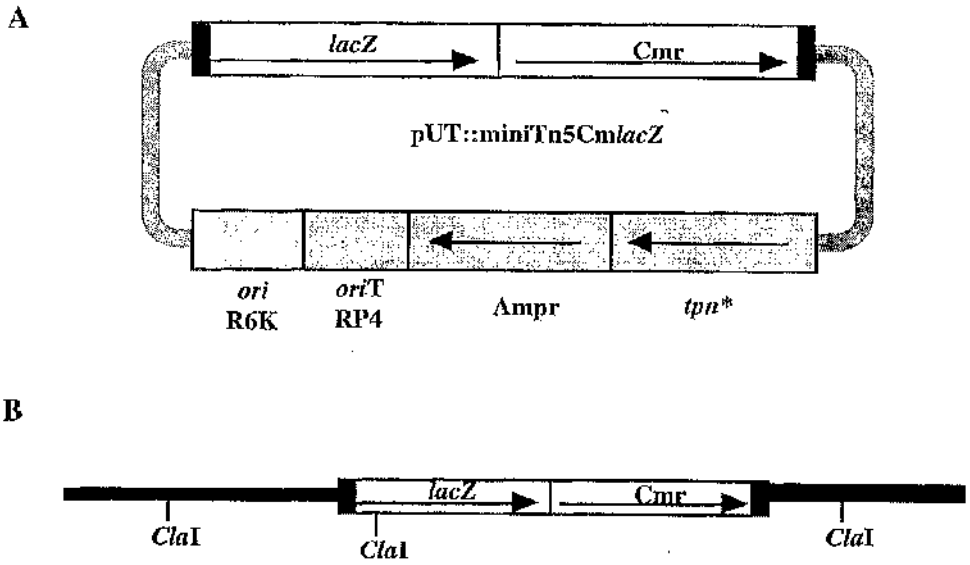


Figure 74. Structure of simple miniTn5CmlacZ insertion. A simple transposition event involving pUT::miniTn5Cm*lacZ* (**A**) will result in an insertion with the structure shown as **B**. The thick black line in **B** represents the recipient genomic DNA.

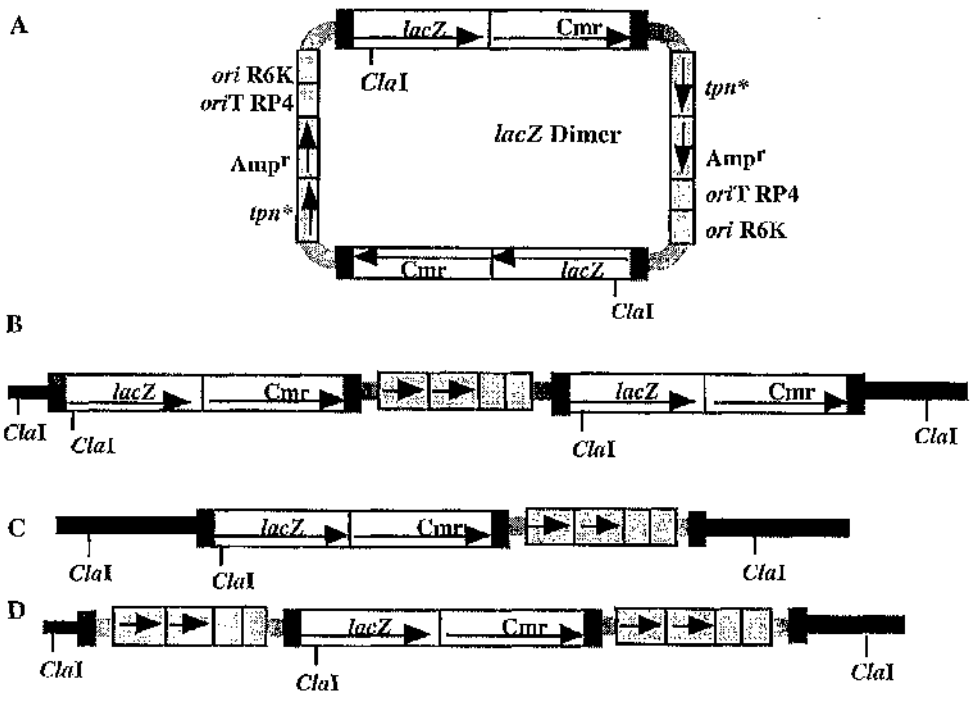


Figure 75. Possible structure of cointegrates. Cointegrates with structures **B**, **C** and **D** may be formed following transposition events involving a dimer of pUT::miniTn5-Cm*lacZ* (**A**). The thick black line in **B**, **C** and **D** represents the recipient genomic DNA.

Therefore, although a large proportion of the exconjugants recovered during the mutagenesis of A862 have cointegrates, the potential use of these exconjugants is not impaired. However, further analysis of these exconjugants must consider the structure of the insertion. This is of particular importance when the ß-galactosidase activity of an exconjugant is used as a measure of the expression of the mutated gene, as more than one copy of the *lacZ* gene, or vector DNA lying between the promoter and the *lacZ* gene, will affect the expression of the reporter gene significantly.

The large number of A862::miniTn5Cm*lacZ* exconjugants containing cointegrates is in contrast to what was observed with A912::miniTn5Km*lacZI* exconjugants. Unlike A862 wt, A912 wt is sensitive to ampicillin, therefore any exconjugants which have retained pUT DNA can be detected by the Amp^r phenotype conferred by the vector. As discussed earlier, less than 1% of the A912::miniTn5Cm-*lacZI* exconjugants were Amp^r, indicating that only a very small number of these exconjugants may contain cointegrates. It is possible that recombination is more common in A862 than in A912. Thus, the formation of dimers, and therefore cointegrates, is more common in this strain.

The frequency of cointegrate formation during the mutagenesis of A862 with pUT::miniTn5Cm*lacZ* may be reduced if the ratio of recipient cells to donor cells is increased and the number of cells in the conjugation mix is decreased, therefore decreasing the probability of any one recipient cell receiving multiple copies of vector DNA, i.e. from more than one donor cell. If cointegrate formation persists, employing a *recA*$^-$ derivative of the recipient strain will minimise the possibility of dimer formation following conjugation. Such *recA*$^-$ derivatives are not easy to construct, however, as this involves the isolation and mutation of the *recA* gene of the recipient strain, followed by replacement of the wild type gene with the mutated gene. Therefore, if cointegrate formation does cause a problem with the characterisation of exconjugants, it may be prudent to consider an alternative recipient strain for further mutagenesis.

SCREENING OF A862::miniTn5Cm*lacZ* EXCONJUGANTS

Screening of exconjugants for loss of PSP toxicity

The adapted chemical fluorescence assay was used for the primary screening of A862::miniTn5Cm*lacZ* exconjugants for PSP toxicity. As discussed earlier, however, (see "Chemical fluorescence assay for PSP toxins"), the fluorescent compound detected by this assay in A862 cultures has not been fully characterised. The fluorescence assay performed well as a screening method in that at least 240 samples per week were assayed by this procedure, a much higher number than could

be processed by alternative methods (e.g. HPLC or the mouse bioassay; van Egmond *et al.*, 1993). Using the assay 7 exconjugants were identified (1·7% of the 886 exconjugants tested) which had a significantly lower corrected fluorescence than A862 wt (Figure 56 & 57). Further analysis of these exconjugants by pre-column oxidation HPLC confirmed that the amount of the fluorescent compound in these exconjugants was lower than in A862 wt & A862 Sm^r, although, with the exception of exconjugant S9-22, it was present (Figure 58). Therefore, the adapted fluorescence assay is a suitable screening method to detect exconjugants which produce a lower quantity of the compound detected by this assay.

In contrast, the mouse neuroblastoma SCB assay did not perform well as a screening method for the loss of SCB toxicity in A862 exconjugants. To increase the number of samples that could be processed, modifications were made to the normal procedure. Even with these modifications, however, the assay was very labour intensive. Although it was possible to process 240 samples in a week, this demanded a large commitment of time. When samples of the same culture supernatant were assayed on the same occasion but on different 96-well plates, considerable variation was observed in the activity detected.

Thus, as known concentrations of a SCB toxin were not included on every plate used for the assay, it proved impossible to compare the level of SCB activity of samples assayed at different times, or even at the same time on the same plate. Therefore, only a qualitative indication of the SCB activity of an exconjugant could be obtained. All of the exconjugants screened by the mouse neuroblastoma assay (i.e. 707) were apparently positive for % SCB activity. However, as it is not known whether strain A862 produces more than one type of SCB toxin (e.g. TTX in addition to STX derivatives), it is possible that any exconjugant with a mutation in a gene essential for the production of one type of SCB toxin, will still give a positive result in this assay. Therefore, it is not clear whether the mouse neuroblastoma SCB assay is a suitable method for screening for the loss of toxin production.

Comparison of results for the same samples assayed by the fluorescence and mouse neuroblastoma assays did not reveal any apparent correlation between the two assays. For example, exconjugant S9-22 consistently gave negative results in the fluorescence assay (Figure 57) and this was confirmed by pre-column oxidation HPLC (Figure 58). However, the mouse neuroblastoma assay revealed that exconjugant S9-22 does exhibit SCB activity (Figure 60). Therefore, it appears that the compound detected by the fluorescence assay is not the only substance in A862 which contributes to SCB toxicity. However, for the reasons discussed above, it is not possible to conclude that this fluorescent compound does not contribute to the SCB toxicity of strain A862.

Alternative assays for screening for PSP toxins in bacterial cultures

The methods employed here to screen exconjugants for loss of SCB toxin production yielded equivocal results and there is still doubt as to what each method is detecting. The substance which yields a fluorescent product upon oxidation in the chemical fluorescence assay is still unidentified and its contribution to SCB activity of the supernatants is not established. In addition, strain A862 may produce both PSP and TTX, as observed in at least one other strain (Gallacher *et al.*, in press). Therefore, it is not clear whether the mouse neuroblastoma SCB assay can differentiate between the wild type and mutants defective in the production of one particular toxin. Also, the mouse neuroblastoma SCB assay is labour intensive and not ideal for screening the large number of samples required to find a suitable number of mutants of interest.

Therefore these methods require further development before more screening for exconjugants is done. Alternatively, another method for the detection of SCB, and in particular PSP toxins, should be adapted or developed for this purpose. Alternative assays which may be considered are the immunoassays for the detection of PSP and the saxiphilin binding assay.

Immunoassays

The different immunoassays which have been developed for the detection of PSP toxins have been reviewed earlier ("Immunoassays for PSP toxins"). The ELISA's which have been developed using polyclonal antibodies raised against STX or neoSTX are rapid techniques which allow a large number of samples to be assayed on microtitre plates (Chu & Fan, 1985; Cembella *et al.*, 1990; Usleber *et al.*, 1991; Chu *et al.*, 1992; Usleber *et al.*, 1994). The use of these methods has been limited, however, as the antibodies against STX and neoSTX cross-react to different extents with the other STX derivatives. Indeed, some of the derivatives are not detected by these antibodies (Sullivan, 1993; Van Egmond *et al.*, 1993). As the toxin profile of the strain from which the majority of exconjugants have been derived (i.e. A862) remains uncertain, the application of the ELISA techniques for the detection of toxins in this organism must first be evaluated. Preliminary results with a commercially available ELISA kit (RIDASCREEN® Saxitoxin, Digen Ltd., Oxford, UK) are ambiguous, perhaps due to the poor cross-reactivity of the antibodies with the toxins present in A862 (S. Gallacher & S. O'Neil unpublished data). It is doubtful that this method will provide an alternative screening method for the detection of PSP toxins in bacterial supernatants.

Saxiphilin binding assay

Saxiphilin is a saxitoxin binding protein which was first detected in various tissues and serum from the North American bullfrog, *Rana catesbeiana* (Mahar *et al.*, 1991). A wider survey of species detected saxiphilin activity in a wide variety of arthropods, fish, amphibians and reptiles (Llewellyn *et al.*, 1997) and, of particular note, in the haemolymph of xanthid crabs (Llewellyn, 1997). Although the saxiphilins from different species have variable specificity for the different STX derivatives (Llewellyn, 1997; Llewellyn *et al.*, 1997), none of the saxiphilins tested bind TTX (Mahar *et al.*, 1991; Llewellyn, 1997).

The ability of saxiphilins to bind STX derivatives but not TTX make them attractive alternatives to the sodium channels used in the receptor binding assays for SCB toxins (Davio & Fontelo, 1984; Vieytes *et al.*, 1993). The receptor binding assay detects SCB toxins in a sample by measuring the amount of ^{3}H-STX displaced from sodium channels of rat brain membrane preparations. As TTX and PSP toxins bind to the same site of sodium channels (Lipkind & Fozzard, 1994), the receptor binding assay cannot differentiate between these two types of toxin. The ability of the saxiphilins to bind STX derivatives was determined by a similar competitive displacement assay to that employed for the receptor binding assay (Mahar *et al.*, 1991; Llewellyn, 1997; Llewellyn *et al.*, 1997). The saxiphilins are therefore suitable alternatives to the sodium channel preparations used in the receptor binding assay and the saxiphilin binding assay has been used successfully to detect PSP toxins in human serum and urine (Gessner *et al.*, 1997) and cell extracts of the cyanobacterium *Lyngbya wollei* (Carmichael *et al.*, 1997).

At present, frog or snake plasma are the source of saxiphilin used in PSP binding assays (Carmichael *et al.*, 1997; Gessner *et al.*, 1997) and the assay is therefore limited by the availability of animals and the ethical constraints on their sacrifice. However, the gene encoding saxiphilin of the bullfrog *Rana catesbeiana* has been cloned and sequenced (Morabito & Moczydlowski, 1994). It should therefore be possible to clone the gene encoding saxiphilin in an expression vector and, if the recombinant protein retains its biological activity, provide a more accessible, economical and ethical source of saxiphilin.

The receptor binding assay has been developed for use on microtitre plates, therefore a reasonable number of samples can be assayed in a short time (Vieytes *et al.*, 1993; Doucette *et al.*, 1997). It should also be possible to adapt the saxiphilin binding assay for use in a microtitre plate format to increase the number of samples which can be assayed.

Therefore, although evaluation of the saxiphilin binding assay for the detection of PSP toxins in cultures of heterotrophic bacteria is required, it is possible that this assay is a suitable alternative screening method for the detection of exconjugants

deficient in toxin production. It has the advantage over the mouse neuroblastoma SCB assay and receptor binding assays that it is specific for PSP toxins and, with some further development, it should be a rapid assay, capable of screening a reasonable number of samples over a relatively short period of time.

ANALYSIS OF SELECTED A862::miniTn5Cm*lacZ* EXCONJUGANTS

Pre-column HPLC analysis of selected exconjugants

The pre-column oxidation HPLC analysis of selected A862::miniTn5Cm*lacZ* exconjugants, which exhibited a lower corrected fluorescence than observed in the non-mutated A862 Sm^r, revealed that the majority of the exconjugants still produced the unidentified peak present in the non-mutated A862 Sm^r (Figure 58). In the analysis of the different exconjugants the area of the peak was directly related to the corrected fluorescence for the same sample (Table 15). Only one exconjugant, S9-22, did not display a detectable level of fluorescence or peak, indicating that only this strain is completely deficient in the production of this compound.

It is possible that a mutation in a gene associated with PSP toxin production could cause a change in the toxin profile of the mutated strain. However, none of the exconjugants analysed by pre-column oxidation HPLC analysis to date have displayed any fluorescent peaks that are not observed in A862 wt (Figure 58 and unpublished data). Therefore, no exconjugant with an altered toxin profile has yet been identified. However, the detection of such a mutant is prejudiced by the screening methods employed in this study, as the adapted mouse neuroblastoma assay was only suitable for the qualitative determination of SCB activity and the chemical fluorescence assay would not allow one to detect changes in the amount of N-hydroxylated STX derivatives present.

ß-galactosidase activity of selected exconjugants

Insertion of the miniTn5Cm*lacZ* transposon into the genome of a recipient bacterium brings the *lacZ* gene under the control of any promoter located upstream from the insertion site. Therefore the level of ß-galactosidase activity of an exconjugant is a measure of the strength and characteristics of the promoter that drives expression of any gene interrupted by the transposon insertion, and genes within the same operon which are downstream from the insertion site.

As exconjugant S9-22 has no significant ß-galactosidase activity (Figure 59) it is probable that the transposon has inserted into a region of the A862 genome which is not expressed. Analysis of the DNA sequence adjacent to the transposon insertion

site of S9-22 DNA confirmed that the transposon has not interrupted any putative open reading frame (see later).

The other exconjugants of interest (i.e. those with consistently low corrected fluorescence by the chemical fluorescence assay) all displayed significant levels of ß-galactosidase activity (Figure 59). However, the interpretation of these results is complicated because the majority of these exconjugants have retained a copy of the delivery vector, probably in the form of cointegrates with two copies of the *lacZ* gene (see below and Figure 61, 62 & 75). Therefore, although the level of ß-galactosidase activity observed in exconjugants S14-4, S14-27, S16-27, S19-43 and S27-37 indicates insertion of the vector DNA downstream from an active promoter, it is possible that promoters located on the vector DNA cause residual expression of the *lacZ* genes. If the vector DNA is indeed retained in the form of a cointegrate, then only one copy of the cointegrate will be present per cell, and the high level of ß-galactosidase activity observed in the *E. coli* control strains, due to multiple copies of the *lacZ* gene, would not be expected in these exconjugants.

It is clear from the Southern blot analysis (Figure 61 & 62) that exconjugant S5-17 has a simple transposon insertion of the type shown in Figure 74. Therefore, the ß-galactosidase activity observed in this exconjugant is indicative of transposon insertion downstream from an active promoter.

Genotypic analysis of selected exconjugants

Southern blot analysis of seven exconjugants revealed that only S5-17 gave the hybridisation band pattern expected for exconjugants with a simple transposon insertion (Figure 61 & 62).

Analysis of exconjugant S9-22 did not reveal significant homology to the *tnp** probe, therefore, the delivery vector has not been retained. However, this exconjugant contained three *Cla*I bands with homology to the *lacZ* gene, a band pattern not consistent with either simple transposon insertion (Figure 74) or the cointegrate structures shown in Figure 75. Without further analysis it is impossible to deduce the structure of the transposon insertion. However, as no copy of *tnp** has been retained, subsequent transposition events cannot take place, so the genotype of exconjugant S9-22 is stable.

In exconjugant S14-4, miniTn5Cm*lacZ* is not retained in the absence of antibiotic (compare Figure 61 & 62), indicating that the transposon insertion is not stable. It is possible that the transposon did not transpose into the recipient genome, but was retained on the vector, which is only maintained by the antibiotic selective pressure. However, the pUT plasmids, which contain the origin of replication from R6K plasmid, require the π protein for replication and this is only encoded by a

narrow range of bacteria, e.g. *λpir* lysogens (De Lorenzo & Timmis, 1994). It is, therefore, unlikely that this plasmid is maintained efficiently in A862. Indeed, if the transposon has not transposed to the genome of A862, it is difficult to explain the change in phenotype observed for exconjugant S14-4 with regard to the fluorescence properties (e.g. see Figure 57). However, as the transposon insertion is unstable, the utility of this exconjugant is limited.

The remaining four exconjugants of interest (i.e. S14-27, S16-27, S19-43 and S27-37) all have a copy of *tnp** (Figure 62) and three *Cla*I fragments with homology to *lacZ*. Therefore, they probably all contain cointegrates with a structure as shown in Figure 75b.

Inverse PCR of insertion sites

The inverse PCR method used in this study successfully amplified the adjacent DNA sequence to the transposon insertion in five exconjugants (Figure 67). The success of the method shows that it could readily be applied to other exconjugants of interest and, using different transposon specific primers, adapted to amplify the DNA sequence adjacent to the opposite end of the transposon.

As inverse PCR products were obtained for exconjugants S16-27 and S19-43, this method is clearly applicable to exconjugants containing cointegrates as well as simple transposon insertions. Theoretically, the inverse PCR method should form two products when applied to exconjugants containing cointegrates with the structure shown in Figure 75b. However, one of these bands, which would be present in all such exconjugants, consists of a full copy of pUT::miniTn5Cm*lacZ*. This plasmid, which is 12·9 kb, is too large to be easily amplified by PCR and would probably not be formed by the inverse PCR method.

The successful amplification of a DNA sequence from S14-4 by inverse PCR indicates that this exconjugant does contain miniTn5Cm*lacZ*. However, it should be noted that the template DNA used for this experiment was prepared from the exconjugant prior to growth in the absence of antibiotics. As the PCR product is only 2 kb, it cannot be formed from an intact copy of pUT::miniTn5Cm*lacZ*, which would form a product of 12·9 kb. However, the DNA sequence obtained from this PCR product (data not shown) indicates that it does not contain the complete sequence of vector DNA present on the other inverse PCR products. This is additional evidence that the transposon insertion in this exconjugant has been abnormal and it was not characterised further.

Southern blot analysis using the inverse PCR products from exconjugants S5-17 and S9-22, confirmed that these PCR products contain DNA with homology to pUT::miniTn5Cm*lacZ*, the appropriate *Cla*I fragment in the exconjugant genomic

DNA and a *Cla*I fragment of a different size in the genomic DNA of A862 Sm^r (Figure 68). Therefore the PCR products contain DNA sequences derived from the vector and from the genome of A862. Also, analysis of the DNA sequences of the inverse PCR products from S5-17, S9-22, S16-27 and S19-43 confirmed that they each contain a region of novel DNA flanked by two regions derived from pUT::mini-Tn5Cm*lacZ* (see Appendix 1). Thus, the structure of the inverse PCR products are as predicted from the strategy outlined in Figures 10 and 11, with the sequence flanked by the vector DNA being that of the genomic DNA adjacent to the transposon insertion site.

DNA sequence of the transposon insertion site of selected exconjugants

Exconjugant S5-17

Analysis of the inverse PCR product of exconjugant S5-17 failed to identify any ORF with extensive homology to genes of known function in the sequence databases. However, a putative ORF was detected, which may encode a protein of 124 amino acids with partial homology to a histone transcription regulator protein (Hir2) of *Saccharomyces cerevisiae*. There are two regions of homology between Hir2 and the putative ORF identified here, but the relevance of this is not clear. The Hir2 protein consists of 875 amino acids (Sherwood *et al.*, 1993) and is therefore much larger than the ORF identified in S5-17. Whereas the two areas of homology between the ORF and Hir2 are separated by 543 amino acids in the Hir2 sequence (accession number P32480) only 55 amino acids separate them in the ORF in S5-17 (see Appendix 2). As the functions of the regions of homology are not known, the importance of the difference in the distance between the two regions is not clear but it does cast doubt on the relevance of the homology of the putative ORF and Hir2.

The putative ORF identified in S5-17 is not interrupted by the insertion of mini-Tn5Cm*lacZ*, neither are any other apparent ORF interrupted by the transposon insertion. Although the potential expression of an ORF is not directly interrupted by this transposon insertion it is still possible that a mutation has occurred if it has inserted into a region involved in the regulation of expression of a gene or it has interrupted an operon. Neither of these possibilities is apparent from the inverse PCR product of exconjugant S5-17 because the PCR product contains only the region of DNA adjacent to one end of the transposon insertion site. If further sequence data is obtained of the area around the insertion site, in particular the region adjacent to the other end of the transposon, it is possible that a gene may be identified which is responsible for the altered phenotype observed in exconjugant S5-17.

Exconjugant S9-22

The inverse PCR product amplified from exconjugant S9-22 contains the N-terminal sequence of a putative serine protease gene. This gene was identified by its homology to sequences in the databases known to encode serine proteases of the subtilase family and also by the subtilase serine protease aspartic acid active site revealed by a MOTIF search. The similarity of this putative serine protease to known serine proteases and its possible role in the production of PSP toxins is discussed later (see "Putative serine protease of the subtilase family").

The putative serine protease gene is located downstream from the transposon insertion site and is not interrupted by the insertion of the transposon. Therefore, the putative serine protease is not directly mutated in exconjugant S9-22. However, it is not clear from the sequence whether insertion of miniTn5Cm*lacZ* upstream from the gene may interfere with its expression by disrupting sequences required for its regulation. Possible -10 & -35 promoter sequences were identified at nucleotides 910 to 915 and 922 to 927 respectively (see "Sequence analysis of exconjugant S9-22 inverse PCR product" and Appendix 2). Even if these sequences do represent a promoter, it is possible that expression of the putative serine protease gene is controlled from another promoter sequence upstream from the transposon insertion site, possibly as part of an operon. If so, the transposon insertion will disrupt the expression of this gene. It is also possible that the transposon insertion has disrupted a sequence upstream from the promoter which is required for binding of a factor required for gene expression.

Therefore, further analysis of exconjugant S9-22 and the sequence of the insertion site is required before it can be established how the expression of the putative serine protease is influenced by insertion of miniTn5Cm*lacZ*. Indeed, it remains to be established that this gene is expressed in A862. This may be achieved by Northern blot analysis of A862 RNA (Lehrach *et al.*, 1977). An S1 nuclease assay (Berk & Sharp, 1977; Weaver & Weissman, 1979) or primer extension assay (Hendrickson & Misra, 1994) could be used to determine the start site and size of the mRNA transcript derived from the DNA sequence of A862 which is interrupted in the exconjugants, therefore revealing the structure of any operon interrupted by miniTn5Cm*lacZ*.

Exconjugant S19-43

Analysis of the inverse PCR product of exconjugant S19-43 revealed a sequence of 72 nucleotides, immediately adjacent to the miniTn5Cm*lacZ* sequence, that encoded an amino acid sequence with homology to the carboxyl end of the *E. coli* H-NS protein (see Appendix 5). SSP-PCR was successfully used to amplify the corresponding sequence from A862 wt and sequence analysis confirmed that the

mutated gene encodes a H-NS homologue. The significance of this finding is discussed below (see "H-NS homologue").

The ß-galactosidase activity observed in exconjugant S19-43 indicates that the transposon has inserted downstream from an active promoter (Figure 59). It is probable, therefore, that the H-NS homologue is expressed in wild-type A862, although this requires confirmation, e.g. by Northern blot analysis.

The carboxyl end of H-NS is required for DNA binding (Shindo *et al.*, 1995; Ueguchi *et al.*, 1996). Therefore, if a truncated form of this protein is expressed in exconjugant S19-43, it is unlikely that the protein will be functional.

PUTATIVE SERINE PROTEASE OF THE SUBTILASE FAMILY

It is clear from the sequence analysis of the transposon insertion site of exconjugant S9-22 that miniTn5Cm*lacZ* has inserted upstream from a putative serine protease gene. Although the identification of strain A862 is incomplete, the 16S rRNA gene sequence obtained from this strain indicates that its closest relatives belong to the genus *Shewanella* (Gallacher, S., Maas, E., Moore, E., Stroempl, C., unpublished data). It is therefore proposed that this gene is named *Shewanella* sp. serine protease (*ssp*), and shall be referred to as such in the remainder of this thesis.

The S9-22 inverse PCR product contains only the first 405 nucleotides of *ssp*, which encode the first 134 amino acids of the putative protease (see Appendices 1, 3 & 4). These 134 amino acids possess significant homology to the N-terminal sequence of known serine proteases of the subtilisin superfamily (Appendix 4). The greatest homology is located around the aspartic acid active site, with the aspartic acid at position 102 being one of the three conserved residues which make up the catalytic triad of subtilases (Rawlings & Barrett, 1994; Siezen & Leunissen, 1997). The other two active sites which are conserved in serine proteases of the subtilisin family are expected to be located further downstream from the sequence contained on the inverse PCR product (Siezen & Leunissen, 1997). Other members of the subtilisin family range in size from 266 to 1775 residues (i.e. the size of the mature enzyme; many members also contain signal peptides; Siezen & Leunissen, 1997). Therefore, a significant portion of the *ssp* gene remains to be identified and sequenced.

The subtilisin superfamily of serine proteases can be divided into subfamilies of more closely related proteases with regard to their sequence homology (Siezen & Leunissen, 1997). The putative serine protease encoded by *ssp* (Ssp) shares homology with members of each of the subfamilies, although the extent of the homology differs. As expected, the region of the Ssp sequence with homology to the majority of the protease sequences analysed was the aspartic acid active site (amino acids 98 to 115).

The subfamilies of serine proteases which share the greatest amino acid homology to the putative serine protease encoded by *ssp* are the kexin and thermitase subfamilies. The region of Ssp which is homologous to the kexin subfamily (amino acids 56 to 115) is different from the region which is homologous to the thermitase subfamily (amino acids 38 to 59 and 89 to 115), although these two regions of homology do overlap. However, the significance of this observation is not apparent. The grouping of the subtilase serine proteases into subfamilies is based upon the similarities of the sequences only within the catalytic domains. An important characteristic of the kexin subfamily is the aspartic acid residue next to the active aspartic acid (position 102 of Ssp) rather than serine or threonine (Rawlings & Barrett, 1994). As Ssp contains threonine at position 103, this indicates that it is probably not a member of the kexin subfamily. As discussed above, the inverse PCR product from S9-22 only contains a small portion of the complete *ssp* gene. When the complete sequence of *ssp* is available, in particular the sequence of the histidine and serine active sites, the relationship between this putative protease and the members of the different subtilase serine protease subfamilies will be more evident.

The subtilisin superfamily of serine proteases, which occur in Archaebacteria, eubacteria, fungi, yeasts and higher eukaryotes, are all putative endoproteases or tripeptidylpeptidases (Rawlings & Barrett, 1994; Siezen & Leunissen, 1997). The subtilases found in bacteria and lower eukaryotes are predominantly extracellular, relatively non-specific enzymes required either for defence or for growth on proteinaceous substrates (Siezen & Leunissen, 1997). However, some of these enzymes, e.g. the members of the lantibiotic peptidase and kexin subfamilies, are specialised enzymes involved in the processing and maturation of proproteins in biosynthetic pathways (Siezen & Leunissen, 1997).

The kexin subfamily of serine proteases consists of a large group of proprotein convertases, which are involved in the activation of peptide hormones, growth factors and viral proteins (Barr, 1991; van de Ven *et al.*, 1993; Siezen & Leunissen, 1997). Although the vast majority of serine proteases of this subfamily are found in eukaryotic organisms (including yeasts, plants, insects, fish and mammals), two prokaryotic kexin serine proteases are known, the *Aeromonas salmonicida* serine protease and the Ca-dependent protease of *Anabaena variabilis* (Siezen & Leunissen, 1997). Interestingly, the kexin subtilases have a high specificity for cleavage after dibasic (Lys-Arg or Arg-Arg) or multiple basic residues (Barr, 1991). As the biosynthetic precursors of PSP toxins include two or three molecules of arginine (Shimizu, 1986; Shimizu *et al.*, 1990a; 1990b), it is possible that a kexin-like protease is involved in the processing of an arginine rich molecule which is the source of the PSP precursors.

The lantibiotic peptidase subfamily consists of specialised enzymes for cleavage of leader peptides from the precursors of lantibiotics, a group of antimicrobial peptides which contain thioether amino acid lanthionine (Jack *et al.*, 1995; Sahl *et al.*, 1995). To date, members of this subfamily have only been found in gram-positive bacteria and are characterised by low sequence similarity with each other and other subtilases (Siezen & Leunissen, 1997). A number of the lantibiotic peptidases are intracellular and therefore do not contain signal peptides (Siezen & Leunissen, 1997).

However, the relevance of the putative serine protease, Ssp, to the production of SCB toxins by strain A862 is not apparent. It is clear from the phenotypic analysis of exconjugant S9-22, that insertion of miniTn5Cm*lacZ* immediately upstream from the *ssp* gene has a negative affect on the formation of a compound detected by the adapted fluorescence assay (Figure 56). However, as the identity of the fluorescent compound detected by this assay has yet to be determined, it is not possible to state with any certainty, the role, if any, which Ssp plays in the biosynthesis of this substance.

It is possible that Ssp is involved in post-translational modification of a protein required for the biosynthesis of the fluorescent compound. However, Ssp does not contain significant homology to the known lantibiotic peptidases, and the amino acid sequence of the aspartic acid active site of Ssp, differs from that of subtilases of the kexin subfamily. Therefore, if Ssp is involved in the specific post-translational modification of a protein, it would represent a new type of subtilase.

Most of the non-specific serine proteases of bacteria are located extracellularly (Siezen & Leunissen, 1997). However, analysis of the amino acid sequence of Ssp (Appendix 3) failed to identify a signal sequence, indicating that, if Ssp is expressed by A862, it is retained within the bacterial cell.

Therefore, it is possible that Ssp is involved in the biosynthesis of PSP toxins, or other SCB toxins, but there is insufficient data available to determine this at present. Completion of *ssp* sequencing and characterisation of the gene product, especially with regard to substrate specificity, may yield an insight into the function of this gene in marine bacteria.

H-NS HOMOLOGUE

H-NS was first identified as an abundant protein of *E. coli* which is involved in compacting DNA (Varshavsky *et al.*, 1977; Spassky *et al.*, 1984). H-NS is a heat stable protein with an approximate molecular mass of 15·6 kDa and it probably exists in solution predominantly as a homodimer (Falconi *et al.*, 1988). H-NS binds to all types of nucleic acid but has greatest affinity for double stranded DNA, with lower affinities for single stranded DNA and RNA (Friedrich *et al.*, 1988). Although the

binding of H-NS to DNA is reasonably non-specific, it does preferentially bind to intrinsically curved DNA (Bracco *et al.*, 1989; Rimsky & Spassky, 1990; Owen-Hughes *et al.*, 1992; Lucht *et al.*, 1994; Tupper *et al.*, 1994; Spurio *et al.*, 1997). In addition to compacting DNA (Spassky *et al.*, 1984), H-NS constrains negative DNA supercoils (Owen-Hughes *et al.*, 1992; Tupper *et al.*, 1994) and induces DNA bending. Thus, it has a significant role in the organisation of the bacterial chromosome (Spurio *et al.*, 1997).

The H-NS homologue described in this thesis (see "DNA sequence of a gene from strain A862 which encodes a H-NS homologue") was initially identified as the mutated gene in exconjugant S19-43. This exconjugant apparently contains a lower level of the compound (i.e. a putative SCB toxin) detected by the adapted fluorescence assay and pre-column oxidation HPLC analysis, than is found in non-mutated A862 (Figure 57 & 58e). However, as the identity of the substance detected by these methods is still in doubt, it is difficult to state how mutation of *hns* may be affecting the production of this compound. It is known that, in other bacterial species, mutation of *hns* leads to an altered level of expression of a large number of genes, including known virulence factors, indicating that H-NS is involved in regulating the expression of these genes (reviewed in Higgins *et al.*, 1990; Ussery *et al.*, 1994; Atlung & Ingmer, 1997). Generally, the mutation of *hns* leads to an increase in the expression of H-NS regulated genes and H-NS is therefore regarded as a repressor of transcription (Williams & Rimsky, 1997). However, the levels of some proteins decrease in *hns* mutants, suggesting that H-NS may also act as an activator of gene expression, although the mechanism by which H-NS affects the production of these proteins is not known (Yamada *et al.*, 1991; Bertin *et al.*, 1990, 1994; Abaibou *et al.*, 1995; Atlung & Ingmer, 1997; Williams & Rimsky, 1997).

It is not yet possible to determine whether H-NS is directly responsible for the regulation of the structural or biosynthetic genes responsible for the production of the unidentified substance detected in A862, or whether H-NS acts on another regulator of these genes or the function of their products. For example, it is known that H-NS is involved in the regulation of the stationary phase sigma factor, σ^S (Yamashino *et al.*, 1995; Barth *et al.*, 1995), a key protein in the regulation of stationary phase gene expression (discussed in the Introduction; see "Sigma factors and gene expression"). The possible involvement of other regulatory components on the production of the compound of interest will only be determined when the nature of this substance is determined and other genes involved in its synthesis are identified. An *hns* mutant, e.g. exconjugant S19-43, may then be used to determine how H-NS, other possible regulators and the biosynthetic genes interact to control the production of this compound in strain A862.

Until recently, H-NS-like proteins had only been described in enterobacteria (i.e. *E. coli*, *Salmonella typhimurium*, *Serratia marscecens*, *Shigella flexneri* and *Proteus vulgaris*) and Southern blot analysis failed to detect *hns* homologues in other bacterial species (e.g. *Aeromonas hydrophila*, *Listeria monocytogenes*, *Bacillus subtilis* and *Clostridium perfringens*; Ussery *et al.*, 1994). However, the complete sequencing of the *Haemophilus influenzae* genome revealed a putative *hns* gene in this organism (Fleischmann *et al.*, 1995) and, more recently, a H-NS homologue (BpH3) has been described in *Bordetella pertussis* (Goyard & Bertin, 1997). The putative *hns* gene described in this thesis is the first *hns* gene described in bacteria isolated from the marine environment and, if the identification of A862 is confirmed, the first report of *hns* in the genus *Shewanella* and the family Vibrionaceae. It is becoming apparent therefore, that H-NS homologues are not only present in enterobacteria, as initial reports suggested (Ussery *et al.*, 1994), but in a wider range of gram-negative bacteria. However, analysis of the complete genome sequence of the gram-positive bacterium, *Bacillus subtilis*, did not reveal a H-NS homologue (Kunst, *et al.*, 1997). It is therefore possible that the distribution of H-NS-like proteins is limited to gram-negative bacteria.

The *hns* homologue present in strain A862 encodes a putative H-NS protein with significant amino acid similarity to known H-NS proteins (Appendix 5 & 12). A comparison of the known H-NS protein sequences confirmed the highly homologous nature of the H-NS proteins from enterobacteria (Ussery *et al.*, 1994), and the more divergent nature of the H-NS like proteins from *Bordetella pertussis*, *Haemophilus influenzae* and putative *Shewanella* sp. (Goyard & Bertin, 1997; Atlung & Ingmer, 1997; this work).

Mutational analysis of *E. coli* H-NS has identified a number of amino acids which are important for the function of this protein (Ueguchi *et al.*, 1996; Williams *et al.*, 1996; Spurio *et al.*, 1997). All but two of these amino acids are conserved or substituted for similar amino acids in the A862 *hns* homologue. The threonine at position 115 and isoleucine at 119 in *E. coli* H-NS are replaced with methionine and phenylalanine respectively in the H-NS of strain A862. The significance of these differences between the H-NS of A862, and the known H-NS sequences is not apparent. However, neither of these residues are conserved in the H-NS homologue, BpH3, in *B. pertussis*, where the equivalent amino acids to threonine 115 and isoleucine 119 of *E. coli* H-NS are replaced with alanine and leucine respectively (Goyard & Bertin, 1997). Although the latter of these two amino acid differences in the *B. pertussis* H-NS is unlikely to have any significant affect on function, these findings do suggest that threonine 115, and possibly isoleucine 119, are not essential for H-NS activity.

AMPLIFICATION OF POTENTIAL SULFOTRANSFERASE GENES FROM HETEROTROPHIC MARINE BACTERIA

The experiments described in this thesis for the amplification of sulfotransferase (ST) genes involved in the biosynthesis of PSP were based on a number of assumptions.

To date, ST activity involved in the conversion of carbamate PSP toxins to the corresponding N-sulfocarbamoyl derivatives has only been described in *Gymnodinium catenatum* (Oshima, 1995a; Yoshida *et al.*, 1996). However, post-column oxidation HPLC analysis of A862 cell extracts using the third isocratic elution revealed a number of peaks which indicated the possible presence of N-sulfocarbamoyl toxins (see Figures 31 to 33). Therefore, although the identity of these peaks has not been confirmed and ST activity has yet to be identified in this strain, it was thought that a sulfotransferase enzyme might be involved in the biosynthesis of these putative C toxins by this bacterium.

The sulphate donor utilised by the ST enzyme to convert the carbamate PSP toxins to N-sulfocarbamoyl derivatives is PAPS (Oshima, 1995a; Yoshida *et al.*, 1996). Other ST enzymes which use PAPS as a sulphate donor have been described and putative PAPS binding sites identified (Yamazoe *et al.*, 1994; Rikke & Roy, 1996; Varin *et al.*, 1997; Weinshilboum *et al.*, 1997). A partial amino acid alignment of the putative PAPS binding site indicated that the RKGxxGDWKxxFT motif described by Weinshilboum *et al.* (1997) is not conserved in all the ST sequences (Appendix 6). It is not clear however, if this difference is a result of the diversity of the organisms in which these enzymes are found (i.e. humans, plants and eubacteria) or whether it is a result of the different substrate specificity of the enzymes. The only non-cytosolic ST of eukaryotic origin included in the sequence alignment is the rat N-heparan sulphate ST. The amino acid sequence of the putative PAPS binding site of this enzyme also differs from the consensus sequence of cytosolic ST. As PSP toxins are of comparable size to the substrates of cytosolic ST, it was decided to use the consensus sequence for the PAPS binding site derived from these sequences to design a primer for PCR amplification. As a number of codons are used to encode for some of the amino acids in the consensus sequence (see Table 7), the primers designed to recognise this sequence contained a number of degenerate nucleotides. This inevitably decreased the specificity of the primer.

Although other conserved sequences have been identified in cytosolic ST enzymes (Weinshilboum *et al.*, 1997), it was considered that using primers designed to one of these regions, in addition to the putative PAPS binding site, would add further variability into the experimental design, thus, decreasing the probability of success. Therefore, a SSP-PCR strategy was employed using the primer designed to recognise the PAPS binding site and another which is specific to vector DNA. This

technique was originally designed for chromosome walking (Shyamala & Ames, 1989) and has also been employed to amplify the sequences adjacent to transposon insertion sites (Novák *et al.*, 1997). Clearly, this method is not as specific as traditional PCR with primers recognising two known sequences but it theoretically may be used to amplify any sequence of DNA if only a short stretch of sequence is known (Shyamala & Ames, 1989).

A number of SSP-PCR products were obtained with each of the two ST specific primers used (Figure 69 & 70). Unsurprisingly, some of the products from the different ligation reactions contained materials of different length but derived from the same genomic sequence (e.g. NST 1 and 4). However, the SSP-PCR reactions were reproducible, yielding the same products each time the reactions were performed (data not shown). These two observations indicate that the ST specific primers are recognising specific genomic sequences. However, sequence analysis of the SSP-PCR products failed to identify any significant homology to known ST sequences, but some of the sequences did contain homology to other genes (e.g. NST31 & NST 34 are homologous to aspartyl-tRNA synthase, NST 32 & NST 35 are homologous to isopropylmalate synthase and NST 1 & 4 are homologous to a hypothetical protein of *Synechocystis* sp.).

It therefore appears that the attempted amplification of sulfotransferase enzymes from strain A862 was unsuccessful. This does not, however, indicate that such enzymes are not present. The primers used for these experiments were degenerate and designed to recognise the PAPS binding site of eukaryotic ST. It is possible that the PAPS binding site of any bacterial ST involved in converting carbamate PSP toxins to the N-sulfocarbamoyl derivatives, varies significantly from the eukaryotic consensus. However, as it has yet to be determined that bacteria possess ST activity specific for PSP toxin conversion, future efforts to identify the genes encoding for the ST enzymes should be performed on organisms which have been shown to possess these enzymes (e.g. *Gymnodinium catenatum*). If ST genes can be identified in these organisms, Southern blot analysis or PCR techniques may be used to identify homologues in cyanobacteria or heterotrophic bacteria involved in PSP production.

A PSP-specific ST has been purified from *Gymnodinium catenatum* (Yoshida *et al.*, 1996). If a portion of the amino acid sequence of this enzyme can be obtained (e.g. by N-terminal protein sequencing) then the SSP-PCR technique described in this thesis may be adapted to isolate the gene which encodes this enzyme. This approach has a greater probability of success than the one used here, as the sequence used for primer design is known and, therefore, the primer will contain greater sequence specificity.

CONCLUSIONS AND FUTURE PROSPECTS

Two different approaches were used in this thesis to investigate the molecular mechanisms involved in the production of SCB toxins by heterotrophic marine bacteria, i.e. transposon mutagenesis and SSP-PCR amplification of genes encoding enzymes which are thought to be involved in PSP biosynthesis.

Established transposon mutagenesis techniques were successfully adapted to generate mutants from two strains of marine bacteria, A862 and A912. Exconjugants were obtained at efficient frequencies and banks containing several thousand exconjugants were isolated. However, the application of this method was hindered by the inadequacy of the present methods for the detection of SCB toxins in a large number of bacterial samples and by the uncertainty regarding the identity of the SCB toxins detected in cultures of the strains used in this study. HPLC analysis (with either pre-column or post-column oxidation) failed to identify fluorescent peaks as specific PSP toxins and further analysis of bacterial cultures by alternative techniques (e.g. mass spectrometry; Quilliam *et al,* 1989; Quilliam *et al,* 1993) is required to determine which SCB toxins are produced by these bacteria.

A chemical fluorescence assay for the detection of PSP toxins (Bates & Rapoport, 1975) was adapted for use with bacterial cultures and successfully used to screen almost 1,000 exconjugants derived from strain A862, for the presence of PSP toxins. However, the specificity of this assay for the detection of PSP toxins is uncertain and the fluorescent product detected in strain A862 remains to be identified. Therefore, the validity of this method as a screen for PSP toxins is questionable.

The other method used to screen exconjugants for SCB toxins was the mouse neuroblastoma SCB assay. However, even after modification of the original method (Gallacher & Birkbeck, 1992) to increase the number of samples which could be processed, this assay was labour intensive and unsuitable for screening a large number of exconjugants. In addition, considerable variation was found with the modified assay, therefore, quantitative determination of SCB activity was difficult and this assay could only provide a qualitative measure of SCB activity of a sample. As some bacterial species may produce both PSP toxins and TTX derivatives (Gallacher *et al.*, in press), mutation of biosynthetic genes for one of these types of toxin may not be detected by this assay, as another SCB toxin may still be produced.

Therefore, although the methods are available for mutation of bacterial strains of interest, further work is required to find a rapid, sensitive and reliable assay which can detect exconjugants which no longer produce SCB toxins. Until such a method becomes available, the application of transposon mutagenesis to determine the biosynthetic genes involved in SCB toxin production by heterotrophic marine bacteria will be limited.

The limitation of the established methods for the detection of SCB toxins in cultures of heterotrophic bacteria is caused, in part, by the low amount of toxin present in these cultures (Kodama *et al.*, 1990b; Gallacher *et al.*, 1996) and the uncertainty regarding the identity of the toxic component (this work). However, the SCB toxic component of cyanobacteria is established to be PSP toxins and these toxins are produced by cyanobacteria in greater quantities than by heterotrophic bacteria (Gallacher *et al.*, 1996). It is probable, therefore, that the methods for the detection of SCB toxins used in this thesis will be more accessible for screening a large number of cyanobacterial cultures. Transposon mutagenesis has been successfully applied to this group of organisms (Borthakur & Haselkorn, 1989; Wolk *et al.*, 1991; Ernst *et al.*, 1992; Cohen *et al.*, 1994). Thus, it is possible that transposon mutagenesis of toxic cyanobacterial species, followed by screening for loss of PSP toxin production, could identify the genes responsible for PSP biosynthesis.

Despite the limitations of the screening methods, the adapted fluorescence assay was used to screen almost 1,000 exconjugants and identified seven exconjugants with a lower corrected fluorescence, and therefore possibly less PSP toxin, than wild type A862. Although some of these exconjugants contain abnormal transposon insertions, characterisation of the transposon insertion site was possible. Inverse PCR and DNA sequencing were successfully applied to a number of exconjugants of interest, and putative functions were assigned to two genes located near the insertion site of different exconjugants. These techniques are readily applicable to other exconjugants of interest which may be identified by further screening of A862::miniTn5Cm*lacZ* exconjugants and can be easily adapted for other transposons and recipient strains.

The possible significance of the putative serine protease (Ssp) and the H-NS homologue of A862 (identified in exconjugants S9-22 and S19-43 respectively) to the production of SCB toxins by this organism is not clear. Both of these exconjugants have a lower corrected fluorescence (as detected by the adapted fluorescence assay) than non-mutated A862. Therefore, if this corrected fluorescence is a measure of PSP toxin content, and thus SCB activity, then these exconjugants contain a mutation which has an adverse effect on SCB toxin production. As discussed in the introduction of this thesis (see "Possible mechanism of SCB toxin production by marine bacteria"), inactivation of either biosynthetic genes or regulatory genes will affect SCB toxin production. H-NS is known to affect the transcription of a number of genes (Higgins *et al.*, 1990; Ussery *et al.*, 1994; Atlung & Ingmer, 1997) and is involved in the post-translational regulation of σ^S (Yamashino *et al.*, 1995; Barth *et al.*, 1995). It is possible therefore that H-NS is involved in the regulation of SCB toxin production by strain A862, and possibly in other heterotrophic bacteria.

Regardless of its relevance to SCB toxin biosynthesis, the discovery of an H-NS homologue in heterotrophic marine bacteria is a significant finding. Until recently it was thought that H-NS-like proteins were only present in enterobacteria (Ussery *et al.*, 1994). However, as genes for H-NS homologues have now been described in *Haemophilus influenzae* , *Bordetella pertussis* and *Shewanella* sp. (Fleischmann *et al.*, 1995; Goyard & Bertin, 1997; this study), it is apparent that H-NS-like proteins are distributed over a wider range of organisms. It is probable therefore that the present understanding of gene regulation in bacteria other than enterobacteria will have to be revised to consider the function of H-NS as a global regulator of gene expression (Williams & Rimsky, 1997) and, in particular, its role in the regulation of virulence factors (Higgins *et al.*, 1990) and gene expression in response to environmental stimuli (Atlung & Ingmer, 1997).

How the putative serine protease Ssp influences SCB biosynthesis is not apparent. It is possible that this serine protease is involved in the post-translational modification of a gene product required for toxin biosynthesis. Although other related serine proteases of the subtilase family (i.e. lantibiotic peptidases and the kexin subfamily of serine proteases) are involved in the specific modification of peptides (Siezen & Leunissen, 1997), the amino acid sequence of Ssp does not indicate that it belongs to either of these subfamilies. The other serine proteases of the subtilase family are relatively non-specific enzymes which are thought to be involved in defence and growth on proteinaceous substrates (Siezen & Leunissen, 1997). If Ssp has a similar function in A862, it is probable that its only role in SCB biosynthesis is to ensure that the biosynthetic precursors are available, by degrading peptides which consist in part of these precursors. However, further analysis of the *ssp* gene and biochemical characterisation of its gene product are required before the function of this protein can be determined.

The attempted amplification of sulfotransferase genes from strain A862 was unsuccessful. However, the primers used for these experiments were degenerate primers designed to recognise sequences which encode the putative PAPS binding site of eukaryotic sulfotransferases. It is not known whether prokaryotes contain similar sulfotransferases with a homologous PAPS binding site. Also, it remains to be determined that heterotrophic bacteria, and specifically A862, possess PSP specific sulfotransferase activity. It may have been more prudent to apply this approach to the organisms in which sulfotransferase activity has been described, i.e. *Gymnodinium catenatum* (Yoshida *et al.*, 1996). However, as the aim of this project was to determine the molecular mechanisms involved in SCB toxin biosynthesis by marine bacteria, it was decided to look directly for these enzymes in this group of organisms. As these initial efforts were unsuccessful, any future attempts to isolate sulfotransferase genes should be carried out in organisms which have been clearly

shown to produce these enzymes. The SSP-PCR approach used in this thesis may be useful in cyanobacteria and dinoflagellates, although modifications to the procedure will be required for the latter group of organisms to take into account their eukaryotic nature (i.e. using cDNA as a PCR template or reverse transcriptase PCR will be required to amplify the sulfotransferase gene without exons). Once PSP-specific sulfotransferases are identified in cyanobacteria or dinoflagellate species, Southern blot analysis or PCR techniques may be applied to identify homologues in heterotrophic marine bacteria.

If biochemical analysis of the biosynthetic pathway of SCB toxins identifies other enzymes involved in this process, the SSP-PCR technique described in this thesis may be adapted to identify the genes which encode these enzymes. The PCR primers may either be designed, as in this thesis, to recognise the conserved motifs in enzymes of the same family, or amino acid sequences derived from purified enzymes. The success of this approach is dependent on designing primers which accurately represent the sequence located on the enzyme of interest.

Over the past few decades, the knowledge of how micro-organisms survive and interact with their environment has increased at a tremendous pace. Molecular techniques have played an integral role in many of these findings and new methods and novel applications are still being described. The work described in this thesis is one of the first reports of how some of these techniques have been applied to determine how bacteria and other micro-organisms produce SCB toxins. Although their role remains to be confirmed, the H-NS homologue and putative serine protease described here are the first genes possibly implicated in SCB toxin biosynthesis. The availability of a growing number of molecular techniques should lead to other genes involved in SCB toxin biosynthesis to be identified. However, this work has again highlighted the requirement for a fast, sensitive and reliable method to detect and analyse SCB toxins. Until such a method is developed, research into this group of toxins will be slow.

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APPENDICES

APPENDIX 1

Composition of media and solutions

The recipes below are for broth cultures. For plates 15 g/l technical agar (agar no. 3; Oxoid L13) was added prior to autoclaving.

Marine Broth

Bacto-Marine Broth 2216	37.4 g/l
(Difco code 0791)	
Boiled for 2 minutes	
Autoclaved 121°C for 15 minutes.	

LB

Tryptone (Oxoid L42)	10 g/l
Yeast Extract (Oxoid L21)	5 g/l
NaCl	10 g/l
Autoclaved 121°C for 15 minutes.	

LB20

Tryptone (Oxoid L42)	10 g/l
Yeast Extract (Oxoid L21)	5 g/l
NaCl	20 g/l
pH 7·8	
Autoclaved 121°C for 15 minutes.	

LB15

Tryptone (Oxoid L42)	10 g/l
Yeast Extract (Oxoid L21)	5 g/l
NaCl	15 g/l
pH 7·6	
Autoclaved 121°C for 15 minutes.	

2YT

Tryptone (Oxoid L42)	16 g/l
Yeast Extract (Oxoid L21)	10 g/l
NaCl	5 g/l
pH 7·0	
Autoclaved 121°C for 15 minutes.	

SOC

Tryptone (Oxoid L42)	20 g/l
Yeast Extract (Oxoid L21)	5 g/l
NaCl	0·5 g/l
KCl (250 mM)	10 ml
pH 7·0	

Autoclaved 121°C for 15 minutes.

After autoclaving add 5ml of 2M $MgCl_2$ & 20 ml of 1M Glucose.

Artificial sea water

NaCl	23.38 g/l	0.4 M
$MgSO_4.7H_2O$	24.65 g/l	0.1 M
KCl	1.49 g/l	0.02 M
$CaCl_2.6H_2O$	4.38 g/l	0.02 M

Autoclaved 121°C for 15 minutes.

APPENDIX 2

Solutions and buffers for use with DNA

TE buffer

Tris-HCl (pH 7·5)	10 mM
EDTA	1 mM

TBE (5X)

Tris Base	54 g/l
Boric Acid	27·5 g/l
EDTA	3·72 g/l

Gel loading buffer

Bromophenol blue	0·25% (w/v)
Xylene cyanol FF	0·25% (w/v)
Glycerol	30% (v/v)
TE	1X

Isolation of bacterial genomic DNA

Cell suspending solution

Tris-HCl (pH 8)	10 mM
EDTA	1 mM
Sucrose	0·35 M

Lysing solution

Tris-HCl (pH 8)	100 mM
NaCl	0·3 M
EDTA	20 mM
SDS	2% (w/v)
Proteinase K	100 µg/ml

(The proteinase K was added just prior to use.)

Wizard® and Wizard® *Plus* SV Miniprep DNA Purification Systems

Resuspension solution

Tris-HCl (pH 7·5)	50 mM
EDTA	10 mM
RNaseA	100 µg/ml

Lysis solution

NaOH	0·2 M
SDS	1% (w/v)

Wizard® Neutralisation solution

Potassium acetate (pH 4·8)	1·32 M

Wizard® Column wash solution

NaCl	200 mM
Tris-HCl (pH 7·5)	20 mM
EDTA	5 mM
Ethanol	55% (v/v)

Wizard® Plus SV Neutralisation solution

Guanidine hydrochloride	4·09 M
Potassium acetate	0·759 M
Glacial acetic acid	2·12 M

Wizard® Plus SV Column wash solution

Potassium acetate	162·8 mM
Tris-HCl (pH 7·5)	27·1 mM
Ethanol	60% (v/v)

Southern blot analysis of DNA

Denaturation solution

NaOH	0·5 N
NaCl	1·5 M

Neutralisation solution

Tris-HCl (pH 7·0)	0·5 M
NaCl	3 M

20X SSC

NaCl	3 M
Sodium citrate	300 mM
pH 7·0	

5X SSC

NaCl	750 mM
Sodium citrate	75 mM
pH 7·0	

2X Wash solution

SSC	2 X
SDS	0·1% (w/v)

0·1X Wash solution

SSC	0·1 X
SDS	0·1% (w/v)

Hybridisation solution

Prehybridisation solution
DIG-labelled probe

Prehybridisation solution

SSC	5 X
N-Lauroyl sarcosine	0·1% (w/v)
SDS	0·02% (w/v)
Blocking solution	1% (w/v)

Washing buffer

Maleic acid	100 mM
NaCl	150 mM
pH 7·5	
Tween 20	0·3% (v/v)

Maleic acid buffer

Maleic acid	100 mM
NaCl	150 mM
pH 7·5	

Blocking solution

Blocking reagent	1% (w/v)
Maleic acid buffer	

Detection buffer

Tris-HCl	100 mM
NaCl	100 mM
pH 9·5	

Anti-Digoxigenin-AP

Anti-Digoxigenin Fab fragments conjugated to alkaline phosphatase	750 units/ml

NBT solution

Nitroblue tetrazolium salt	75 mg/ml
Dimethylformamide	70% (v/v)

BCIP solution

5-bromo-4-chloro-3 indoyl phosphate	50 mg/ml
Dimethylformamide	100% (v/v)

Colorimetric substrate

NBT solution	45 µl
BCIP solution	35 µl
Detection buffer	10 ml

PCR Amplification of DNA

dNTP mix

dATP	10 mM
dTTP	10 mM
dGTP	10 mM
dCTP	10 mM

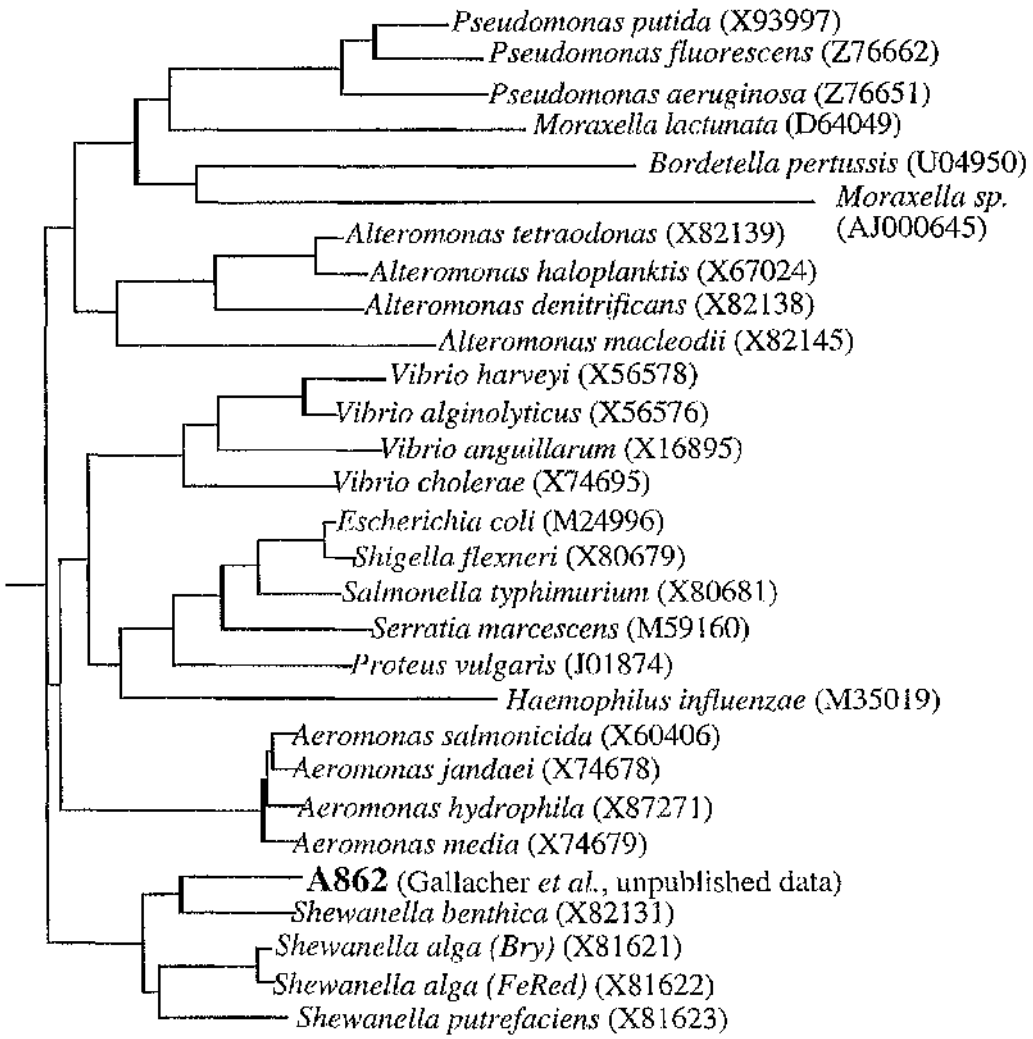
10X Expand™ High Fidelity PCR Buffer

As supplied by Boehringer Mannheim U.K. Ltd; contains 15 mM $MgCl_2$

APPENDIX 3

Phylogenetic tree derived from 16S RNA sequences

The DNA sequence of the 16S RNA genes from a number of species were retreived from the DNA databases using the appropriate accession numbers (shown in brackets in figure below). The 16S RNA sequence for strain A862 was kindly supplied by Gallacher *et al.* (Gallacher, S., Maas, E., Moore, E. and Stroempl, C., unpublished data). These sequences were aligned using CLUSTAL W (version 1·7; Thompson *et al.*, 1994) and this alignment used to construct a phylogenetic tree (unrooted) using the DNAPARS and DRAWGRAM applications of PHYLIP (version 3.5c).



APPENDIX 4

DNA sequence of inverse PCR products containing miniTn5Cm*lacZ* flanking sites.

The underlined portion of the following sequences corresponds to pUT::miniTn5-Cm*lacZ* sequence. The labels L8867 & L730749 indicate the location of the priming sites of these primers.

Exconjugant S5-17

L8867

A1 GTGCTGCAAG GCGATTAAGT TGGGTAACGC CAGGGTTTTC CCAGTCACGA

A51 CGTTGTAAAA CGACGGCCAG TGAATCCGTA ATCATGGTCA TAGCTGTTTC

A101 CTGTGTGATA AAGAAAGTTA AAATGCCGCC AGCGGAACTG GCGGCTGTGG

A151 GATTAACTGC GCGTCGCCGC TTTCATCGGT TGTACAAAAA CTTTCAGTGC

A201 CGCCAGCATT TTCTCTGGCT CATTAATATG TTGCTCGATG ATTTTAACAA

A251 TGGCCGAACC AGAAATCGCG CCCGCAGCTC CTGCATCAAT CGCTGCTTTT

A301 ACCTGATCCG GGGCGGATGC GTCCGGCGTA GAGGATCCCC GGGAATTCGC

A351 GCGGCCGCAC TTGTGTATAA GAGTCAGCTC TAGGGCTATT TATTCACTCA

A401 GCCCAATGGT CATCAACTCT GGAATTAGCA CTTATACAAC TTGTCACCCA

A451 AAATCGCACA GTTGCTATAT TTATCTTCAT TCGGTACGTT GACAATCAAG

A501 GTATTTAAAA ATGAAAAATT ATCAGTTTGA TCTTTTGAGG TTTCCAGATT

A551 TTGTGCGTTC TCACTCCAAC TAGAATCCCC CATCATCCAA ACGTTGTTTT

A601 CATTAATCGA GACAACCACC CTGTACCCCG GTTGGTATCC AGCTAGCTTT

A651 CCTCGTTCAC CATAAATATC AGAGAAAATG TTCCACTGTT TTGCTAGTTG

A701 TTCAACTATA GAAACCATTT CGTAACTCAA GGTGTCGAAG GTATAATGCT

A751 TTAAAAGAAG GACTTCATTA TTTTGGACTA GAAGCGCTTT CATCTTCATT

A801 TTTATGGTTC AGATCATTAT CTAACGTTTG CTTAGTAATT AACCCTTCTT

A851 GCACCTATTC CTAATTAATT GCCACAGCCA AACTGAATTG ACTCAT

B1 NACANATATT CCAAGAGGCT GTTGTCAGTC CATAGTNGAA GAGAACCAAT

B51 TTTGATTGTT CATCTATAGG GCGCTTTATT GGGCTCTTTA TATTTCGTAT

B101 TTAGGATACT CCCAAAGCAA GTGCTAAATC ACTCCGTGTC CCCGAGTATT

B151 TAGGATACTC CCAAAGCAAG TGCTAAATCA CTCCGTGTCC CCGAATTAGT

B201 TTAAAGGGTA ATTACCCATA GCTTTACCAC AGCCTGCTCT TTATATTAGT

B251 TTAAAGGGTA ATTACCCATA GCTTTACCAC AGCCTGCTCT TTATATTAGT

B301 TTAAAGGGTA ATTACCCATA GCTTTACCAC AGCCTGCTCT TTATTTAAAG

B351 CGATAGTGCG ACAGTAAAAT ACATGTTACG TTACATGTAA TATGTCAGAT

B401 ATTGGACTGA ACGTATGGAC CATGCAAAAC TATCCCCACC TGAAAATCGA

B451 TAATTTCACC GCCGAAAGGC GCGGTGCCGC TGGCGACCTG CGTTTCACCC

B501 TGCCATAAAG AAACTGTTAC CCGTAGGTAG TCACGCAACT CGCCGCACAT

L730749

B551 CTGAAC

Exconjugant S9-22

L8867

1 GTGCTGCAAG GCGATTAAGT TGGGTAACGC CAGGGTTTTC CCAGTCACGA

51 CGTTGTAAAA CGACGGCCAG TGAATCCGTA ATCATGGTCA TAGCTGTTTC

101 CTGTGTGATA AAGAAAGTTA AAATGCCGCC AGCGGAACTG GCGGCTGTGG

151 GATTAACTGC GCGTCGCCGC TTTCATCGGT TGTACAAAAA CTTTCAGTGC

201 CGCCAGCATT TTCTCTGGCT CATTAATATG TTGCTCGATG ATTTTAACAA

251 TGGCCGAACC AGAAATCGCG CCCGCAGCTC CTGCATCAAT CGCTGCTTTT

301 ACCTGATCCG GGGCGGATGC GTCCGGCGTA GAGGATCCCC GGGAATTCGC

351 GCGGCCGCAC TTGTGTATAA GAGTCAGCCT CAAGTTACAG CGGTGAAAGA

401 TTGCTGCAAT CAGATAAAAG TCTATTTATT TACCACGATT AAAAAATATT

451 CTTTTCGGTT CTAGGTAGGT ATTTGCTGTT GATATATATG ATTTTTCTTT

501 CAGCTAATGA ATCTTGTGCT AGTAATTACT AATCCAATGT AATTAGCTCT

551 TAAGTGTTAA ATGCTAATTA GGTTCTTTTA AAAAAATGAA GGCTATGACA

601 ACAATCCCTG GGTTAAAAAC TTCCGGAGAT GCATAAGGTG GTTGCAGTAG

651 TCGGTATCTG ATGTGGCTAT GGATGGCACC AAGAGTATTC GAAGTACCGG

701 TTTGAATACA TTTCAAAGGA CTAATAGGAA AACTATTTAT GTTTAAGAAA

751 GCTCACACCA CTAGCCAGCT GCAATTGCTA NCATTGTTGC CGTAATCGTC

801 CGGCTCCAGT TTATGCAAAA AACCCCCTGC AACCCCTGAA ATAATTGAAA

851 AGACCCGCCT TATCNTTGTT TTATAAAGAT AATGCCACCG GCTAGCGAAA

901 CGTTGCTTCT GCACAGCGCT CTAATTACCG GGGCTCTGTA CGAGATATGA

951 ATGCTGACGG TGTTTGATGA TCGCTTTGCA AACGTACTCA ACGGTAAGTT

1001 GGCTCGACTT TCGCTACGCT CAGGATCTGA TGTTGCGCAG GCTATAAAAA

1051 TGATTAGCCG TCACCCCGCG GTCAAATATG CAGAGCCAAA CTATGTAATA

1101 AGAGCTATTG GTACACCTGA TGATCCAGAT TTTGTTTCGT TATGGGCATT

1151 AAACAATACT GGACAAGATG GTGGAACCTC AGATGCCGAT ATTGATGCGT

1201 TAGAAGCTTG GGATATCACT ACGGGTGATG CAAATATTGT CGTTGGTGTT

1251 ATCGACACCG GTGTTGATTA TAACCATCCA GATTTACAAG CTAATATGTG

1301 GGTTAACCCC AATGAGATAG CGGGTAACGG TATAGATGAT GACGGTAATG

1351 GTGTTATCGA TAATTTCACC GCCGAAAGGC GCGGTGCCGC TGGCGACCTG

1401 CGTTTCACCC TGCCATAAAG AAACTGTTAC CCGTAGGTAG TCACGCAACT

1451 CGCCGCACAT CTGAAC

L730749

Exconjugant S19-43

L8867

1 GTGCTGCAAG GCGATTAAGT TGGGTAACGC CAGGGTTTTC CCAGTCACGA

51 CGTTGTAAAA CGACGGCCAG TGAATCCGTA ATCATGGTCA TAGCTGTTTC

101 CTGTGTGATA AAGAAAGTTA AAATGCCGCC AGCGGAACTG GCGGCTGTGG

151 GATTAACTGC GCGTCGCCGC TTTCATCGGT TGTACAAAAA CTTTCAGTGC

201 CGCCAGCATT TTCTCTGGCT CATTAATATG TTGCTCGATG ATTTTAACAA

251 TGGCCGAACC AGAAATCGCG CCCGCAGCTC CTGCATCAAT CGCTGCTTTT

301 ACCTGATCCG GGGCGGATGC GTCCGGCGTA GAGGATCCCC GGGAATTCGC

351 GCGGCCGCAC TTGTGTATAA GAGTCAGGGT CAAGGCCGCA TGCCAACCGT

401 ATTTAAAAAT GAATTAGAAA ATGGCCGTAC TATGGAACAC TTCCTTATCT

451 AAGTTTTCTC TTTGGCCTTT AAGAACCCAG CATTTGCTGG GTTTTTTGTT

501 AATTAACGCT ATATCTACAT TCAAGGTTAC AATTACTACT TAGTATTTAG

551 CATTAAGTTA AGATATCCTC ACAGTTCCGC CACCCCCAAT AACAAACATA

601 AGTACGATAA TGCATAAACA TAAAATAAAA GCCCTTGTCG CCCTACTTAG

651 TGTATTGAGC AGCCATAGCG TAATANCCAN TGAGCAGCTG ACAGATAATA

701 CTCCNTAAAT CACAGCCTTA AGTCATGCTA CCCTGATGTT AGAGCCAGGA

751 GAAATATTGA CTGATGCGAC TTTTNTTAAT TGAAAACAAT CGGAATCAAG

801 TCTNCTTATA AAAAGGACAA TGATATTCCG TCNGGGAGCC TTAGAAAATN

851 AACCTGTCAG GGTCTNATTA TTTACCCCTG GATTTTATTG ACCCCATTCC

901 CTGGAATTTG GCTATTGGAG TNTGGAAGCA AAAGAGTGAA GATAGNCGAT

951 GGTCCCCGGT TNANTGAAAT AAAAGNGNAA TNGGNGCCAA TGCCGGANAA

1001 TGGGCGCGAT CCNATTCNAG AAAAAGGNGT AGTTTAATTN NGTATATCCC

1051 CGACAANGAA CAAGGCTGAA AAATGGATTA ATAATGGNCT TTACCAGGGT

1101 GCAGTCTAGN TGTTTAGATG GNCATATTTN AGAGGACGNG GAGTCAGTTT

1151 ATNTTCTGGC AGATAAAAAA GCGAAACGAT ATAATATACA AAGTTAAAGC

1201 CAATCAATTN ATAGCATTCG ATAANGGTTC ATCAATTCAA GATNACCCGC

1251 AATCATTAAT GGGTAGCATG GCGCTCATTC GACAAACATT CTCAGATGCA

1301 CGTTGGTATA ACAATAATAT AGCCAAAAGT ACTAGTTCAA CTAACAATAT

1351 TGAATTCAAT ATTGCATTAG AANGTNTTTC AAACTTGAAA GAGCAACGTT

1401 TCATCTTTCA AACAAAGAAC TTAAATAATC AATTAAGAGC CGCTAGATTA

1451 TTAAATAAGC AAAAGCTAAA AGGCAGCTTA GTTGGTAATG GACATGAATA

1501 CGCTAGAATT GATGAACTAA AGTCTTACCC CTATAGTTTA GTGTTACCTT

1551 TAAACTATCC TGNTGCACCA ACCATTATCG ATAATTTCAC CGCCGAAAGG

1601 CGCGGTGCCG CTGGCGACCT GCGTTTCACC CTGCCATAAA GAAACTGTTA

1651 CCCGTAGGTA GTCACGCAAC TCGCCGCACA TCTGAAC

L730749

APPENDIX 5

Derived amino acid sequence of a putative open reading frame located on the inverse PCR product of exconjugant S5-17

```
  1  MKMKALLVQN NEVLLLKHYT FDTLSYEMVS IVEQLAKQWN IFSDIYGERG
 51  KLAGYQPGYR VVVSINENNV WMMGDSSWSE NAQNLETSKD QTDNFSFLNT
101  LIVNVPNEDK YSNCAILGDK LYKC*
```

The underlined portion of this sequence contains the regions of homology to *Saccharomyces cerevisiae* histone transcription regulator 2 (Hir2; accession number P32480).

APPENDIX 6

Derived amino acid sequence of a putative open reading frame located on the inverse PCR product of exconjugant S9-22

```
  1  MLTVFDDRFA NVLNGKLARL SLRSGSDVAQ AIKMLSRHPA VKYAEPNYVI
 51  RAIGTPDDPD FVSLWALNNT GQDGGTSDAD IDALEAWDIT TGDANLVVGV
101  IDTGVDYNHP DLQANMWVNP NEIAGNGIDD DGNG
```

The underlined portion of this sequence contains the recognised motif of the aspartic acid active site of the subtilase family of serine proteases (Rawlings & Barrett, 1994).

APPENDIX 7

Multiple sequence alignment of the derived amino acid sequence from the S9-22 inverse PCR product and known serine proteinases

Amino acids in the black boxes are identical to the consensus of the column in which they are located. Amino acids in the grey boxes are similar to the consensus of the column in which they are located.

S9-22 = derived amino acid sequence from nucleotides 951 to 1355 of the DNA sequence of S9-22 inverse PCR product.

avprca = *Anabaena variabilis* calcium dependent protease (accession number X56955; amino acid 132 to 266)

spkrp1 = *Schizosaccharomyces pombe* dibasic endopeptidase (accession number X82435; amino acid 61 to 195)

acpc2 = *Aplysia californica* prohormone convertase 2 (PC2) (accession number X59682; amino acid 108 to 242)

lspc2 = *Lymnaea stagnalis* PC2 (accession number X68850; amino acid 108 to 242)

cepc2 = *Caenorhabditis elegans* PC2 (accession number U04995; amino acid 95 to 229)

hspc2 = *Homo sapiens* PC2 (accession number J05252; amino acid 88 to 222)

hakx2a = *Hydra attenuata* PC2 (accession number M95931; amino acid 123 to 257)

hspc13 = *Homo sapiens* PC1/PC3 (accession number X64810; amino acid 88 to 212)

taaqua = *Thermus aquaticus* aqualysin I (accession number X07734; amino acid 1 to 134)

alapr2 = *Alteromonas* sp. alkaline serine protease II (accession number S68495; amino acid 60 to 194)

vaproa = *Vibrio alginolyticus* calcium-dependent alkaline serine exoprotease A (accession number M25499; amino acid 79 to 213)

alapr1 = *Alteromonas* sp. serine protease (accession number D38600; amino acid 91 to 225)

nahlys = *Natrialba asiatica* halolysin (accession number D10201; amino acid 56 to 190)

tstap = *Thermoactinomyces* sp. thermostable alkaline protease (accession number U31759; amino acid 42 to 176)

bsak1 = *Bacillus* sp. serine proteinase (accession number L29506; amino acid 59 to 193)

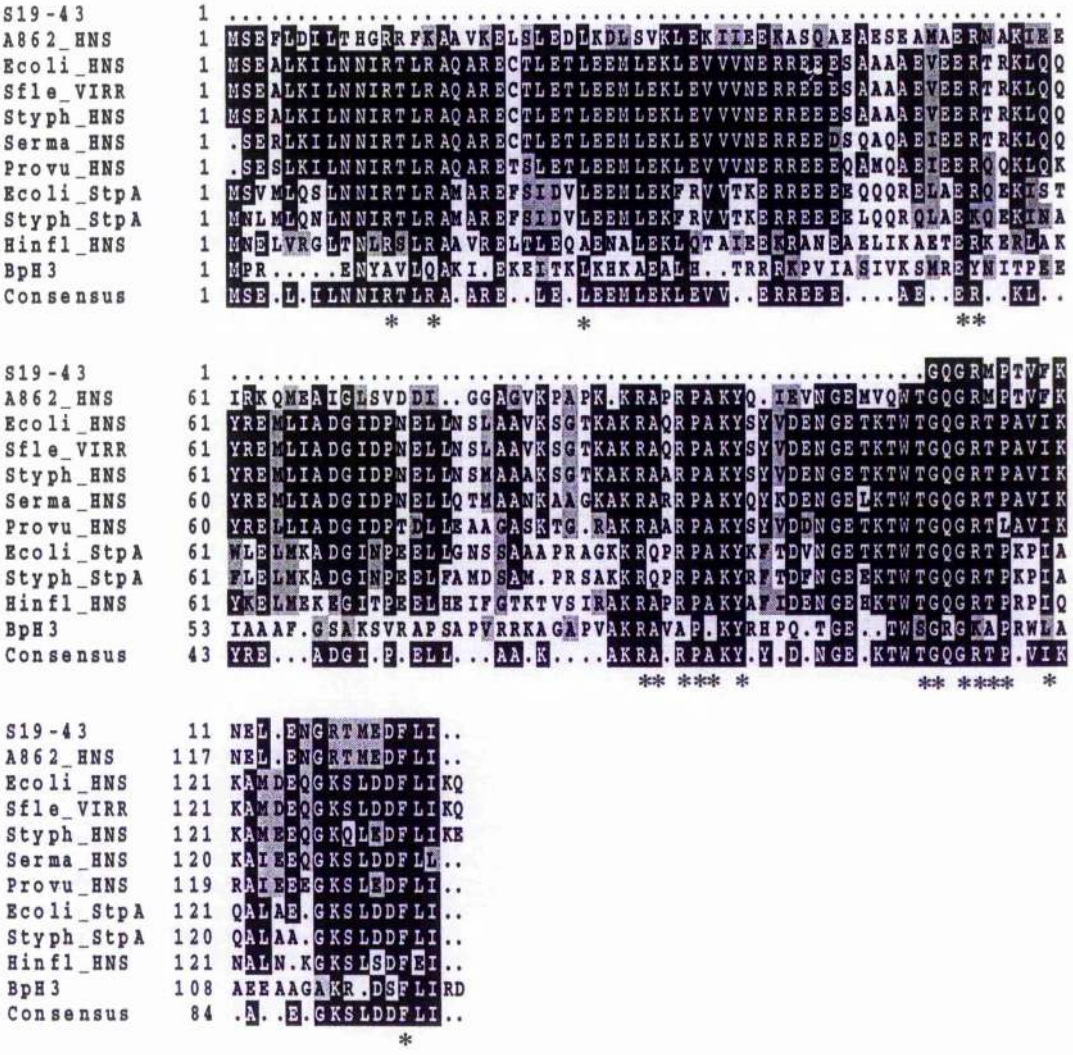
bsvpr = *Bacillus subtilis* minor extracellular serine protease (accession number M76590 amino acid 87 to 221)

asaspa = *Aeromonas salmonicida* serine protease (accession number X67043; amino acid 1 to 134)

vmvapt = *Vibrio metschnikovii* alkaline serine protease (accession number Z28354; amino acid 48 to 182)

APPENDIX 8

Multiple sequence alignment of the derived amino acid sequence from the S19-43 inverse PCR product and known H-NS-like proteins



Amino acids in the black boxes are identical to the consensus of the column in which they are located. Amino acids in the grey boxes are similar to the consensus of the column in which they are located.

Amino acids marked by an asterix have been indicated by mutational analysis as important residues for H-NS function (Ueguchi *et al.*, 1996; Williams *et al.*, 1996; Spurio *et al.*, 1997).

S19-43 = derived amino acid sequence from nucleotides 378 to 449 of the S19-43 inverse PCR product DNA sequence.

A862_HNS = H-NS homologue from strain A862 (see appendix 9)

Ecoli_HNS = *E. coli* histone like DNA binding protein (H-NS; accession number X07688)

Sfle_VIRR = *Shigella flexneri* VirR (analogue of H-NS; accession number X66848)

Styph_HNS = *Salmonella typhimurium* H-NS (accession number M37891)

Serma_HNS = *Serratia marcescens* H-NS (accession number P18955)

Provu_HNS = *Proteus vulgaris* H-NS (accession number P18818)

Ecoli_StpA = *E. coli* StpA (accession number X69210)

Styph_StpA = *Salmonella typhimurium* StpA (accession number AF009363)

Hinfl_HNS = *Haemophilus influenzae* H-NS (accession number U32832)

BpH3 = *Bordetella pertussis* BpH3 (accession number U82566)

APPENDIX 9

DNA and derived amino acid sequence of a H-NS homologue from the marine bacterial strain A862

DNA sequence

```
  1  GATAXTTTTT CATGAATATA ACAAGCCTTT CCCAGTTAAA TGATAATTAA
 51  TCCTTTTTXA TXXTTGATAA AATTAAAATA ATATTTGATC CTTATTGAAA
101  TAGTTTGTAT TATTXACCCA GAATATXAAA TTCTTTATCA ATTTTtttTA
151  ATCGGACTTA ATAATTAATG AGCGAATTTT TAGATATTTT GACCCACGGT
                      →
201  CGTCGTTTTA AAGCTGCTGT AAAAGAGTTG TCTCTTGAAG ACTTGAAAGA
251  CCTTTCAGTA AAGCTAGAAA AAATCATCGA AGAAAAAGCA TCTCAAGCAG
301  AAGCAGAAAG CGAAGCAATG GCTGAGCGTA ATGCTAAAAT TGAAGAAATT
351  CGTAAACAAA TGGAAGCCAT TGGCTTATCA GTTGATGATA TTGGCGGCGC
401  TGGTGTTAAA CCTGCTCCTA AGAAGCGTGC TCCACGTCCT GCTAAATACC
451  AAATCGAAGT TAATGGCGAA ATGGTGCAGT GGACAGGTCA AGGCCGCATG
501  CCAACCGTAT TTAAAAATGA ATTAGAAAAT GCCCGTACTA TGGAAGACTT
551  CCTTATCTAA GTTTTCTCTT TGGCCTTTAA GAACCCAGCA TTTGCTGGGT
                *
601  TKTTTGTTAA TTAACGSTAT ATCTACATTC AAGGTTACAA TTACTACTTA
651  GTATTTAGCA TTAAGTTAAG ATATCCTCAC AGTTCCGCCA CCCCCAATAA
701  CXAACATAAG TACGATAATG CATAAXCATA XAATAXAAGC CCTTGTCGCC
751  CTACTTAGTG TATTGAGCAG CCXXAXCGTX ATAXCC
```

The ORF which encodes the H-NS homologue is underlined in the above sequence. The ATG start codon and the direction of transcription are indicated by the arrow and the stop codon is indicated by the asterix.

Derived amino acid sequence

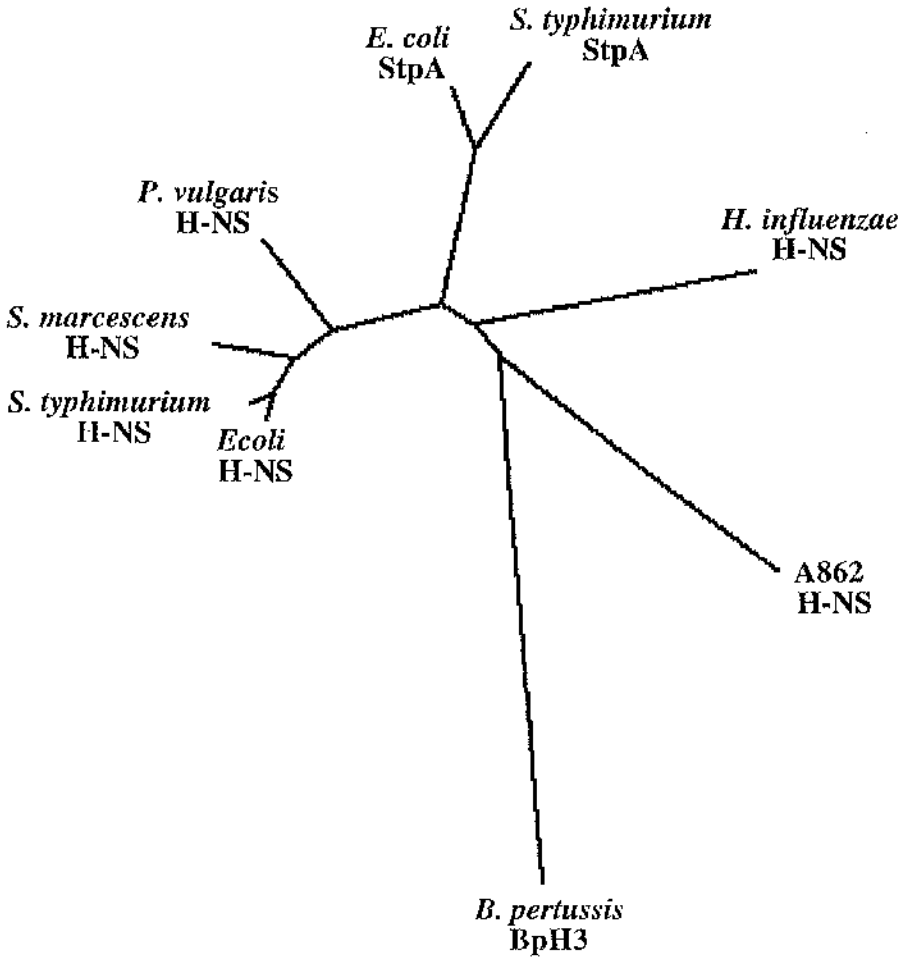
```
  1  MSEFLDILIH GRRFKAAVKE LSLEDLKDLS VKLEKIIEEK ASQAEAESEA
 51  MAERNAKIEE IRKQMEAIGL SVDDIGGAGV KPAPKKRAPR PAKYQIEVNG
101  EMVQWIGQGR MPIVFKNELE NGRTMEDFLI
```

APPENDIX 10

Phylogenetic relationship of H-NS-like proteins

The amino acid sequences of known H-NS like proteins (see Appendix 5 for accession numbers) were aligned using CLUSTAL W (version 1.4; Thompson *et al.*, 1994) and this alignment used to calculate the distances between these proteins using the PROTDIST algorithm of PHYLIP (version 3.5c) and construct a phylogenetic tree (unrooted) using the protein parsimony algorithm (PROTPARS) and DRAWTREE applications of PHYLIP (version 3.5c).

Unrooted phylogenetic tree

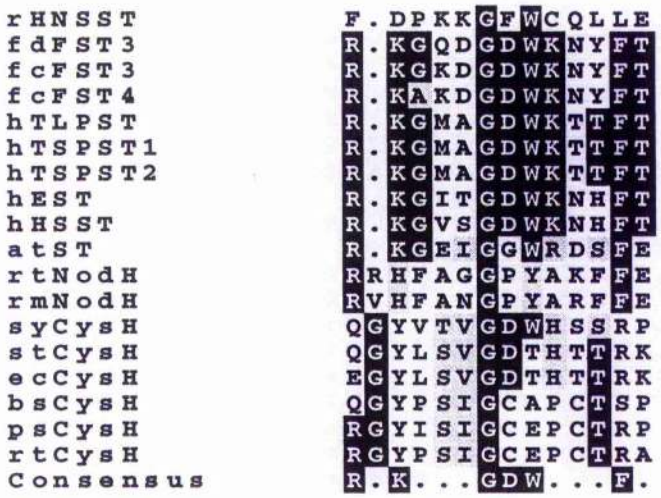


Protein distance matrix of H-NS sequences

		1	2	3	4	5	6	7	8	9
1	*E. coli* H-NS	0.00								
2	*S. typhimurium* H-NS	0.15	0.00							
3	*S. marcescens H-NS*	0.52	0.56	0.00						
4	*P. vulgaris* H-NS	0.71	0.64	0.80	0.00					
5	*E. coli* StpA	1.97	2.08	2.07	1.79	0.00				
6	*S. typhimurium* StpA	2.32	2.41	2.37	2.09	0.48	0.00			
7	*H influenzae* H-NS	2.47	2.64	2.66	2.58	2.43	2.84	0.00		
8	A862 H-NS	3.31	3.27	3.49	3.17	3.29	3.55	3.26	0.00	
9	*B. pertussis* BpH3	6.30	6.37	7.22	7.51	6.30	6.35	7.92	7.55	0.00

APPENDIX 11

Partial sequence alignment of a conserved amino acid domain of sulfotransferase enzymes



Amino acids in the black boxes are identical to the consensus of the column in which they are located. Amino acids in the grey boxes are similar to the consensus of the column in which they are located.

rNHSST = rat N-heparan sulphate ST (Accession no. M92042)
fdFST3 = *Flaveria bidentis* Flavanol 3'-ST (Accession no. U10275)
fcFST3 = *Flaveria chloraefolia* Flavanol 3'-ST (Accession no. M84135)
fcFST4 = *Flaveria chloraefolia* Flavanol 4'-ST (Accession no. M84136)
hTSPST1 = human thermostable phenol ST 1 (Accession no. U09031)
hTSPST2 = human thermostable phenol ST 2 (Accession no. X78282)
hTLPST = human thermolabile phenol ST (Accession no. L19956)
hEST = human estrogen ST (Accession no. U08098)
hHSST = human hydroxysteroid ST (Accession no. X84816)
atST = *Arabidopsis thaliana* ST (Accession no. Z46823)
rtNodH = *Rhizobium tropici* NodH (Accession no. X87608)
rmNodH = *Rhizobium meliloti* NodH (Accession no. M37417)
stCysH = *Salmonella typhimurium* CysH (Accession no. M23007)
ecCysH = *E. coli* CysH (Accession no. M23008)
bsCysH = *Bacillus subtilis* CysH (Accession no. U76751)
syCysH = *Synechococcus* sp. CysH (Accession no. M84476)
psCysH = *Pseudomonas aeruginosa* CysH (Accession no. U95379)
rt CysH = *Rhizobium tropici* CysH (accession no. AJ001223)

APPENDIX 12

DNA sequence of sulfotransferase SSP-PCR products.

The following sequences do not contain the pUC18 DNA sequence which each of the PCR products contain at the end opposite the ST01 or ST03 priming site. The portion of pUC18 DNA varies depending on the restriction enzyme used in template preparation. Each sequence presented ends at the junction between the genomic DNA and the pUC18 DNA. The ST01 or ST03 priming sites are underlined in each sequence.

DNA sequence of the SSP-PCR products NST1 and NST4.

ST01

```
  1  GGGGACTGGA AGAATCACTT CACCACCGTA ACTTAAGTGC TGACGTCCAC
 51  AAGATAGTTC TTTAGGTGGT ATAAGAGACT GAATGTCTTT GTCGAAAATC
101  AGCTCACCTT GCTCGTTAAT GCTGTAAAGC TCGTTAAATT TGTAGCTAGC
151  AAGCCAAGTG TTTAGTGCGT CAAGGTGACC TTTATCGGTT GCACACTTGT
201  TGACAATGAC TTGGTGAGAT TCGCAGTTAC CTTCTAGCTT TTTACCATTA
251  TATTCTGAAG TACCGGTCCA ACCTTTCGCT GTGCGCATTA AAATAACAGG
301  CCAGCGTGGC TTAACAACGT CTTCGCCCGC TCTAGCGCGA GTTTTGAATG
351  TCGTTGATCA TCTCATAAGA AAGTCCATTG CGGCAGTCAT CTGCTCATAA
401  ACATCTTGTT CGAGCTCATC ATCAACAGTG ATTGGGTGGT AACCAAGAAC
451  ACCGAATTCT AAATCAAGTT CTTCCANGGC TCATTCTTCC CATACGAAAT
501  AAGGCCTGAA ATCTTGTTAC CATTAATATG TTCCAAAAGA AGTACTGCAC
551  CGTTGTTCTT TGGGTGAAAC TAAAACGGTT TGCATACCTA AGAAGCGGCA
601  AAAGGACCTG TTTCAGATTC GCCGTCACCA ATTAGGCCAG CGCCAATTAA
651  ATCAGGGTTA TCAAANNTNG GCGCCCCACG CAACTGAAAG TNAGTAACCA
701  AGTTCACCAC CCCNTAGGAT TTGACCTGGT GCTTCCCCGT TCCCNTGAGA
751  AGGGTANCCA TATNGCCCAG AGAACTTTTG GCAAAATATCA GTGATACCCC
801  CTTCGTTTNTA TGGGATGGNG TCAGGATAAA AATGGCTGAG TGAACCTTCC
851  ATGAAGAGGT TCGCTTGTAC TGCAGGGAAA CCGTGGCCAG GGCCAACAAG
901  GTATACAAAC GAGCGGTTAN GNTTAGTAAT CAATCTNTTA ACGTTCGCGT
951  ATACGAAGTT AATTCCC
```

DNA sequence of the SSP-PCR products NST2, NST4, NST5 and NST8.

ST01

```
   1 GGGGATTGGA AGAATCACTT CACCACGGCG CTACCTGCGT TATACGATGG
  51 CCTACAATTC TTAGCGCCAA ACTACGCCAC TGATGGCACA GATATGCTAG
 101 AAATGGGTGA CTACAAGGCT GTTATCAAAC ACTTTCAAAC ATTGCAAAAA
 151 CAGTGGGCAG GTTTTCAGTT TGAATGGCTG CAGGCTTATC AGTTTGCCAA
 201 ATATATATTT TGGTCAAAGC AGGTCGATAA CGTCGATGAA ATACTCAAAG
 251 CTATCAGCAC CAGCTTTCCA GANTCTTTGA CTATACTCAG CATTGAAGTG
 301 GCAAAAGGGC TACTCAAAAA AGGCGATAAT CAACACGCTA AAACTGTGCT
 351 AGAAAACGTC CGTATAGAGG GCGAAGCAAA CCCCAATTGG CATAAGCAAT
 401 TAAGCCTCTA CTACAAGGCC GAAGGTGATG AAAAACAAGC GCAGCAGCAG
 451 TTTAAAACCG CACTAAAACT AGCAGAAAAA ATATCAATTC NATGGCTGGG
 501 AAATTTGGGA ATTAAAGTAA ACTTTCCAGC CCCCCTAACC TTTATGGATA
 551 TAAGGCGCTT ACNCATTATC TCCATCAATC TACGCTTCCA AGTCCNTACT
 601 TAAACCTTTT GTTTACACAA TCTTTGAATA TNACCCNGCC ATACAGGTTT
 651 GGCCACACCA NTAACTTCCA NTGGTGTTGA ANTGAGACAA GACCGNNAAA
 701 GATCANCCCA CCGCCAANGG GTGTTGAATN TCAGTGCCTA CCCCCGTGAA
 751 ATAAGGATAG GTNTCAGCCC TAATGCAGAG GTTAACGCGG TCATTAACAN
 801 GGGACGCAGA CGCCCNACGG TCCCCTCATA AACGGCATCA TACAGCGATG
 851 CATTTGTCTC ATTTTTTACG CTAGCTCGGC GTTGATTTAT TGAATCCACC
 901 AGCACCACAC CATTGAGTAC AGNGACACCA AACAGGGTGA TAAAACCAAT
 951 CGAACTTGGC ACAGAAAGGT AGGTTCCACT GACATATAAG GCAACGACAC
1001 CACCAATGAG TGCTAACGGC ACGTTAGCCA TAATCAATGC GACCTGTTTT
1051 ATCGAGCCAA ACGAGAAGTA CAGCAGCAGC GCAATAAGTG CGCTTGAAAT
1101 GGGGCCCCAC GGGGCTTNTT TAAAGACACC ATCTAANATN CCNGCA
```

DNA sequence of the SSP-PCR product NST31.

The sequence is incomplete as it lacks the ST03 priming site.

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  1  CACCATGGCT TCAGCATCAA CGTTGACTTT ATCAGCACCA AAGAGAATCA
 51  CATCACCAGT TTGTGCACCA CTGCGCTCAA GTAATGCTTT ATCCACATCT
101  TCAGTTAAGA ACTTAAGCAC TGGAGATTGG ATCCCTTCCA TGCCTTTATC
151  AAGATCATTA ACTTTCATCC ATGCTAAGCC TTTAGCACCA TAAATGGTGA
201  CATACTTGGC GTATTCATCA AGTTGCTTAC GCGATAGTTT AGCGCCACCT
251  GGAATGCTAA GCACTGCAAC ACGCCCTTCA GGGTCGTTTG CAGGACCATT
301  AAATACTGCG AACTCGACTT CTTTTACCAG ATCAGCAATG TCAATTAGCT
351  CTAGTGGGTT ACGAAGATCT GGCTTATCAG AGCCATAACG ACGCATCGCT
401  TCAGCAAATG TCATTCTTGG GAACTCACCA AGATCAACAT TTAACAGCTC
451  TTGAAATAGA CCGCGAGTCA TCTCTTCAGT CTTAGCCATC ACTTGCTCAG
501  AAGTCATGAA AGAGGTTTCG ATATCTATTT GGGT
```

DNA sequence of the SSP-PCR product NST32.

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                ST03
  1  GTGAAGTGGT TTTTCCAGTC GCCCAAATTA GNTGCGCCGC AATGTCANTA
 51  GNTTGCAATA CCACTGTTGC ATGGAANGTC GCATCAGCAT CAGTTTTCAA
101  TTCAGCCCAT GCCGCTAAAG CCTGCTGCCA GTTATCGCCT TTAGGTGCAA
151  ATTCACGCCC TTTAAGATAC TCTGCAGTCG TTGCATCAGG CGCTATCATG
201  CCCGCTTTAG CGCCCATCTC AATCGCCATG TTACACACCG TCATGCGCCC
251  TTCCATTGTG AGCGCTTCTA TCGCTTCACC ACAG
```

DNA sequence of SSP-PCR products NST33, NST36 and NST37b.

ST03

1 GTAAGTGGTT TTTCCAGTTG CCAANGGAAT AAATTGTAAT GTGGCGGTGC

51 AGACAAATGA CTGCACTAAC AACTGATGCT GACGAGCACC TTTTAAACCC

101 AAGCTACCAT CTGTCAGTAA CACAGCGACA GCACCTGAAC CACCAGTCAG

151 CGTGGCCAAT GACTGAGCAA AGTTCTGCAT GGTTGGCTCA GCCAGCATTT

201 TGTCGATAGT AATATCAACA ATATGACGAG CTGACTCACA AGAGACCACT

251 AGGCCTGCTT TTATCTGCCC CAGCTCGATA CGGTTGGCAA TATCAAGAAT

301 GCCCGACAGT ACGCCTAAAC AGGCGTTACT TATATCGTAA ATGGCTGTGT

351 CTTTGGAAAC CCCTAATTCA GCAGCGATAC GACATGCTGT TGCGGGCTCA

401 TGTTGATCAC GACAAACACC AGTGTAAACC ACTGCGCCTA AATCGCTGAT

451 CTGAACATCA GTTTCATTGA TTGCCTTATG CGCCGCCGCT AAAGCTCCAT

501 CAGAAAGTCG GTGCCCTTTT GGCCACCAGC GTCTTTCAT

DNA sequence of the SSP-PCR product NST37a.

ST03

1 GTAAAATGGT TTTTCCAGTC GCCCAGTGAG GCCAGTCCTT TTGGCTTTCC

51 TTCGAGTAAA GAAGCTTCCA GTTCAAACAG TCCCTGATAG CAGACGTCAT

101 GGGTAAAATT TAAATTGGTT TGAGCCATAT CATCATGCAA TAAAAAGTGC

151 CCGATCTTAT CGAGCACATA TGCTTCTTCA TTTAGATATA GCAAGCCCAT

201 ACTCAGTCCC ATATCGACTA ATACTCCAAG GGCGTACTCA GACAGTACTG

251 ACTGCTTTGC TAAATCACTT ATGTTGCTAC CTTTTGAGCC GCAATCGGAG

301 ATAAACTGTA AGACATTAAA CTTTAATAGA CAGCGTGCGA CTTGAAAACT

351 TATTGGTGCG AAGGCAATTT TTTGTGCTTC AAACTTGGCA TCAAATGCGC

401 TGATTGATAG AGGAGAGCGA TAGAAAGACA TAAATGAGAC CAATGAGACT

451 GTTACCGAAT TAGTGGAGGC GTATGTTACA TGTTACAGTC TGAACATAGT

501 TTGAACTGGA TCTTGCTTCG CGTTGTTTAA GTAAGCCAAC TTTTCATTTA

551 TTACTTGTTT TGATGTCNTT CCGCTCTCAT TTGTGTTAAN GATGTTTCCA

601 TGCTGGTACC ACTGACCTAC GTTNGGTCGT CCATCAAGCC GAAATTGATG

651 GACAGGCCAA GGCGCCAANA AATCCTTGAA GCGACTTTAC CTTTTAATCC

701 NTTANGGAAN