Characterisation of Haemolytic Bacteria Isolated from Turbot, *Scophthalmus maximus* (L.), Larvae

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Declaration

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

Ross Thomson

Dedication

This thesis is dedicated to my fiancée Lynne for her constant love and support.

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Thanks Harry for the opportunity to carry out this research, for the guidance through out this study, and for the time proof reading and correcting this thesis.

Also I would like to thank my Mum and Dad for all the support they have shown thought my studies and Irene for the supply of bacteria free rotifers and algae.

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Abbreviations

Abbreviations

ASW	Autoclaved sea water
bp	Base pair
CFU	Colony forming unit
dH_2O	Distilled water
DNA	Deoxyribonucleic acid
dNTPs	Deoxyribonucleic triphosphates
EDTA	Ethylene diamine tetraacetic acid
g	Grams
hr	hour
KCl	Potassium Chloride
kb	Kilobase pair
kg	Kilograms
L	Litre
LA	Luria-Bertani agar
LB	Luria-Bertani broth
MA	Marine Agar
MB	Marine Broth
mg	Milligram
$MgCl_2$	Magnesium chloride
min	Minute
ml	Millilitre

mm	millimetre
mM	Millimolar
nm	Nanometers
OD^{600}	Optical density at 600nm
PBS	Phosphate Buffered Saline
PCR	Polymerase chain reaction
RFLP	Restriction Fragment Length Polymorphism
rRNA	Ribosomal Ribonucleic Acid
TBE	Tris-Borate EDTA buffer
TCBS	Thiosulphate Citrate Bile-Salts Sucrose
-ve	Negative
+ve	Positive
µg	Micrograms
µM	Micromolar

Abstract

Aquaculture of turbot, *Scophthalmus maximus* (L.), has developed over the last 20 years but there is a major bottleneck in turbot aquaculture due to limited production of juvenile fish. This is due to hatcheries experiencing high mortalities, or 'crashes', during larval rearing, the main cause of which is thought to be haemolytic bacteria introduced with live food organisims.

This thesis describes the characterisation of haemolytic bacteria isolated from turbot larvae and their food, *Artemia*, in order to identify pathogenic bacteria which may be introduced via the *Artemia*. The main method of characterisation was single carbon usage using Biolog GN microplates, but simple biochemical tests, such as Kovac's oxidase, detection of catalase, growth on TCBS medium, and utilisation of sucrose were also used. Selected strains were also tested for possible pathogenicity against turbot larvae. Representitive strains were also identified using partial DNA sequence analysis of 16S rRNA genes, and RFLP analysis of 16S rRNA genes used to group the phenons produced from the biochemical data.

A total of 158 isolates were characterised from both hatcheries and this produced two major phenons. Phenon 1 was comprised of *Vibrio alginolyticus* type bacteria and phenon 2 consisted of *Vibrio splendidus* of both biovars 1 and 2. The RFLP analysis of *V. splendidus* biovar 1 revealed that even though different strains had similar or identical phenotypes their RFLP patterns could be different, and those strains with the same RFLP profile could be phenotypically different. The RFLP patterns of *V. splendidus* biovar 1 and biovar 2 were distinct from each other. Minor strains also detected included *V. tubiashi*, *Marinamonas vaga*, and *Roseobacter gallaeciensis*.

All pathogenic strains belonged to *V. splendidus* biovar 1 group and were found in high concentration in a batch of larvae that suffered high mortality, although they were also present at a low concentration in larval batches with high survival.

The identification of these turbot larvae pathogens allows the development of alternative methods of bacterial control, which would reduce antibiotic usage which tends to select for resistant strains.

Introduction

1.0.0 Introduction

Historically, Man has mostly settled next to water whether the sea or a river, and fish or shellfish would have provided a diet high in protein, vitamins, and fatty acids. Supplemented with vegetables this would have provided early man with a balanced diet. The development of boats and nets allowed early man to catch a wide variety of fish but the increasing efficiency of modern fishing methods and increase in total fishing pressure mean that over the last few years stocks of mature adult fish in the seas have dwindled. Sustainable catches from wild stocks worldwide are estimated to be approximately 100 million tonnes per year (FAO, 1998).

The Chinese, Romans, and Egyptians developed aquaculture more than 2000 years ago. The basis for this early aquaculture involved catching wild juvenile fish and stocking them in ponds or other bodies of water for further growth. The United States of America's system of federal hatcheries for the propagation of *anadromous* fishes (fishes that live and mature in salt water but reproduce in fresh water) was established in the 1870s. Much of the current technology used to reproduce fish in hatcheries has been developed by these federal hatcheries. This has led to the development of modern aquaculture which is now providing a significant contribution to world food supplies, current production exceeding 29 million tonnes per year compared to 10 million tonnes 10 years ago. The bulk of this, approximately 90%, occurs in Asia, but there has been rapid development of salmon aquaculture in Europe as well as North and South America (FAO; 1999). In Europe, Atlantic salmon aquaculture production has grown over the past 30 years to approach 500,000 tonnes per year (FAO; 1999) in Norway, Scotland, Ireland, and Iceland.

1.1 Aquaculture of new species

The success of the Atlantic salmon industry has prompted development of aquaculture of other species. There is substantial production of sea bass and sea bream, mainly in Mediterranean waters, and there is increasing interest in culture of the European turbot (*Scophthalmus maximus*), halibut (*Hippoglossus hippoglosssus*) and cod (*Gadus morhua*) in northern waters (FAO; 1999). The turbot is a species which has been developed for farming in the last 20 years, with much of the initial research carried out in the UK by the White Fish Authority and the Ministry of Agriculture, Fisheries and Food. In recent years the Atlantic coast of northern Spain and southern France, with ideal water temperatures, has seen a rapid commercial development of turbot fisheries and now exceeds 2000 tonnes per year (FAO; 1999). The lack of seawater at a suitable temperature has seen northern Europe focus on rearing juveniles for on growing in southern Europe. The cooling water from electric power stations has been utilised in fish farms in the UK (Golden Sea Produce, Hunterston) and Scandinavia (Paulsen, 1989), although the Hunterston site is no longer operational.

1.2 Taxonomy of Turbot

Turbot is an almost circular flatfish having both its eyes on the left side, allowing the fish to lie 'flat' on the seabed. This, coupled with the turbot's limited ability to change its scaleless skin to the colour of the background, make it a very effective predator. It mainly feeds on small fishes and a variety of bottom dwelling invertebrates such as small crabs, shrimps, and worms (Last, 1979). As the turbot lives on the seabed it is not possible to catch this fish by normal trawling methods,

and the method known as bottom trawling is used. As a result turbot is a high value fish species and this has provided the impetus for the farming of turbot

1.3 Life cycle of farmed turbot

Turbot is farmed onshore in large circular black shallow tanks. The fish normally spawns in mid-summer, however, it is possible to induce spawning by regulating light and temperature conditions to allow the production of fertilised eggs throughout the year (Bye and Htun-Han, 1979; Bromage *et al.*, 1993). Reproduction takes place externally and brood tanks house fish used for sperm and egg production. These tanks are maintained at a low stocking density. i.e. there is a low level of fish per tank with light level and temperature carefully controlled to ensure production of eggs all year round. The fish are persuaded to release eggs and sperm by a type of abdominal massage known as stripping. At any one ovulation a female can release up to $1x10^6$ eggs per kg of body weight. Fertilisation is carried out manually immediately after stripping. After fertilisation, the eggs are rinsed. Hatching occurs 5-6 days after fertilisation at 16°C and is carried out in tanks of filtered seawater to minimise bacterial contamination.

After 3 days the yolk sac reserves have all been utilised and the fish larvae can begin to eat live prey. Rotifers, *Brachionus plicatilis*, are normally used in aquaculture for first feeding (Fernandezreiriz *et al.*, 1993), and this lasts for approximately 15 days before secondary feeding. This consists of the introduction of the live brine shrimp (*Artemia* sp) nauplii along with the rotifers which are given to the larvae for 30 days before being fed on inert foodstuffs. Both the rotifers and *Artemia* must be enriched with fish oils, highly unsaturated fatty acids (HUFA), as fish fed on non-enriched rotifers show abnormal pigmentation (Reitan *et al.*, 1994),

and exhibit slow growth rates and high mortalities which have been attributed to lack of HUFA (Scott and Middleton, 1979). Farming of turbot is not without its problems and very large losses can occur during the stages of hatching to second feeding which accounts for the highest percentage of mortalities during rearing (Nicolas *et al.*, 1989). When the fish are eventually kept in large tanks they are fed on a fishmeal-based pellet. Therefore, they are fed on inert foodstuffs for 6-7 months in order to acclimatise them to this food. They grow from 2g to 100g during this period and are then transferred to on-growing tanks where the turbot spend the next 24 months growing to full adult size. They are then sorted and supplied to market.

1.4 Factors which are important in determining larval survival

1.4.1 Water type

As this is the environment in which they live any changes in water quality, whether bacterial or chemical, can endanger the fish. Rearing systems commonly use "green water " which contains added microalgae (Alderson and Howell, 1973; Nicolas *et al.*, 1989). The microalgae in green water affect the microbiology (Nicolas *et al.*, 1989), nutrition (Scott and Middleton, 1979), behaviour (Naas *et al.*, 1992) and feeding (Reitan *et al.*, 1993) of the larvae, and for turbot rearing improves appetite, initial growth rate and survival of the larvae/fry (Næss *et al.*, 1990; Reitan *et al.*, 1993; Øie and Olsen, 1997). Also with a green water system, the guts of the larvae contain smaller amounts of food and the rotifers which they feed on have a higher energy and protein content (Øie and Olsen, 1997); this may be due to the rotifers feeding on the algae but the short rotifer residence times in the tanks would make the nutritional effect of the algae negligible (Planas and Cunha, 1999). It was also suggested by Støttrup *et al.* (1995) that dead or dying algae would increase the

substrate available for bacteria; therefore, the interaction between algae and bacteria in the larval tanks may be more important than their nutritional value.

1.4.2 **Water quality**

The seawater used for larval culture is one source of bacterial contamination that can de-stabilise larvae production (Douillet and Pickering, 1999). The bacteria in coastal waters are varied (Austin and Allen-Austin, 1985) with numbers in estuaries ranging from 10^5 to 10^7 cells ml^{-1} (Wright *et al.*, 1982; Azam *et al.*, 1983). In some instances microbiological contamination responsible for larvae mortality has been traced to incoming seawater (Loosanoff and Davis, 1963). Disinfection of the seawater used for fish production is an effective means of controlling unwanted microbes (Burrows and Combs, 1968; Bedell 1971) and various methods have been employed, e.g. ultraviolet light, ozone, chlorine, pasteurisation, filtration, and antibiotic prophylaxis. Of these, chlorination (Douillet and Pickering, 1999) and the filtration of seawater are most economical.

1.4.3 **Light**

This is an important factor as the majority of marine fish larvae are visual feeders. Most flatfish hatcheries use light intensities of around 1000-2000 lux (Estévez, 1996). This figure is much higher than the best reported light intensity for turbot larvae of around 50 lux (Darriba, 1997) as light intensities greater than 50 lux were found to result in lower feeding performances and slower growth. Another important factor is the distribution of light within the tank. Naas *et al.* (1996), showed that reflection from the surface on a tank is very important for light distribution in the water body and that black tanks best reproduce natural illumination conditions while

white tanks should be avoided since they would be a perfect wall trap due to phototaxis of the larvae.

1.4.4 **Diet**

Various marine zooplankton are used as food for larval flatfish as suitable artificial diets have not yet been developed. As noted earlier, the live feed of turbot, halibut and other marine fish must contain HUFA (Rainuzzo *et al.*, 1997), of which the most important is the n-3 series, primarily eicosapentaenoic acid (EPA; 20:5(n-3)) and docosahexaenoic acid (DHA; 22:6(n-3)) (Owen *et al.*, 1975; Watanabe, 1982; Sargent *et al.*, 1989). Because of this, the composition of lipids for first feeding larvae should simulate the composition of the yolk (Heming and Buddington, 1988), as an unbalanced diet can be detrimental to the larvae (Planas and Cunha, 1999). The most widely used live feeds for rearing marine fish larvae are rotifers, *Brachionus plicatitis* and *Artemia* (Rainuzzo *et al.*, 1997). These can be enriched by various methods (Watanabe *et al.*, 1983; Frolov *et al.*, 1991; Rainuzzo et *et al.*, 1994; Rainuzzo *et al.*, 1997) to supply the fish with essential lipids. However, it has been noted that high levels of n-3 HUFA frequently lead to poor rearing success (Støttrup, 1992). In Nature, marine fish larvae feed on zooplankton rich in n-3 HUFA, however, in marine fish cultivation it is very likely that the levels of n-3 HUFA within the various lipid classes is unbalanced (Planas and Cunha, 1999). The correct balance is still unknown as research into other fatty acids is currently limited to n-6 fatty acids (Robin, 1995). One particular n-6 fatty acid, arachidonic acid, has been singled out as promoting growth of fish (Cowey *et al.*, 1976; Castell *et al.*, 1994). Arachidonic acid is an important precursor of phosphatidylinositols in fish (Bell *et al.*, 1985b; Lie *et al.*, 1992; Estévez, 1996) and turbot larvae on an arachidonic acid deficient diet showed

high mortality (Bell *et al.*, 1985a). The requirement for n-6 HUFA is low but still unquantified (Estévez, 1996).

Prey size is another important factor in live feed; different strains differ in size and the use of small size rotifers can significantly improve the initial feeding performance of turbot, as also occurs with sea bream larvae at the earlier development stages (Polo *et al.*, 1992; Cunha and Planas, 1995). The diet of the rotifers has little effect on their size (Planas and Estévez, 1989; Øie *et al.*, 1994) which is dependent on the particular strain of rotifer species or strains. An improvement in feeding when small rotifers are used is due to an increase in the number of rotifers eaten rather than digestion rates (Cunha, 1996). Despite the improvements that small rotifers make, only the larger species are commonly used in hatcheries (Planas and Cunha, 1999).

Artemia are also enriched with these n-3 HUFA (Rainuzzo *et al.*, 1997), although the lipid requirements of some fish may be lower during the *Artemia* feeding stage when the necessary lipids for growth have already been supplied during first feeding (Rainuzzo *et al.*, 1997). In general, the enrichment of *Artemia* is less efficient than in rotifers (Léger *et al.*, 1986) and it has also been shown that *Artemia* start to metabolize DHA after enrichment (Danielsen *et al.*, 1995; Evjemo *et al.*, 1997) and so it is important that they are given to the fish immediately after enrichment. The success of the enrichment may be largely dependent on the genetic characteristic of the strain (Dhert *et al.*, 1993). It should also be noted that different strains of *Artemia* metabolize DHA at various rates (Dhert *et al*, 1993). This is important in early first feeding of marine cold water larvae as the *Artemia* can reside in the tank for up to 2 days before being eaten which can have a drastic effect of the nutritional value of the *Artemia*, in particular it's content of DHA(Evjemo *et al*, 1997)

1.4.5 **Bacteria**

It is inevitable that larval fish will encounter microorganisms in commercial aquaculture. Bacteria have adapted to grow on and in various surfaces of fish, such as the fins, gills, and intestinal tract, and potential pathogens can be introduced via various routes including the egg (McFadden, 1969; Bergh *et al.*, 1991), the water (Munro *et al.*, 1993; 1994), and aerosol transfer from neighbourhood sources. The major source of bacterial contamination is usually via the feed (Munro *et al.*, 1993; 1994), as the eggs can be successfully cleared of bacteria by glutaraldehyde disinfection Salvesen and Vadstein, 1995) or antibiotic treatment (Munro *et al.*, 1995), and the water by filtration. Bacterial contamination is of major concern as heavy losses can occur in the larval stages of fish development (Dhert *et al.*, 1993) although it is rare that a particular bacterial species is associated with disease in turbot larvae. It has been noted that opportunistic pathogens rather than obvious marine pathogens are often the major cause of mortalities within hatcheries (Munro *et al.*, 1993, 1995) although primary pathogens can readily infect larvae in laboratory experiments, e.g. *Vibrio anguillarum* (Munro *et al.*, 1995) *Aeromonas hydrophila* (Gatesoupe, 1991) and *A. salmonicida* (Bergh *et al.*, 1997). The *A. hydrophila* reported by Gatesoupe (1990, 1991) was later reclassified as *V. splendidus* and is considered one of the few specific turbot pathogens to date (Gatesoupe *et al.*, 1999). As the bacteria that inhabit the gut normally originate from the feed it would be prudent to minimise this risk, and this can be done in various ways. Antibiotics were used by Shelbourne (1964), who showed that up to 80% of turbot larvae survived to metamorphosis when a mixture of penicillin and streptomycin was routinely used in hatching tanks. Gatesoupe (1982) also showed that antibiotic treatment enhanced survival of turbot larvae. However, within all types of farming the use of antibiotics has led to widespread antibiotic

resistance in the bacterial population. Despite this, fisheries still use antibiotics as a last resort, although they are seeking better ways of controlling unwanted bacteria without endangering the fish. One approach to this is to attempt to stabilise the bacterial flora within the larval fish digestive tract (Ringø and Birkbeck, 1999). Production of a stable gut flora would dramatically decrease the chance for opportunistic pathogens to settle and it may be possible to achieve this by introduction of bacteria that can form a stable flora.

1.5 Development of the gut flora in larval flatfish

The development of the digestive tract of marine fish is important in the development of gut flora. In contrast Wolffish *Anarchichas lupus L.*, fry which do not undergo a long period of endogenous feeding and under go no metamorphosis have a morphologically well developed gut with a distinct intestinal gut flora immediately after hatching (E. Ringø, unpublished data) although it is unknown how the wolf fish gets this flora. However, the gut of most marine larvae is an undifferentiated straight tube (Govoni *et al.*, 1986). This may not provide suitable attachment sites for the bacteria as the larval gut of turbot contains few if any bacteria before feeding begins (Munro *et al.*, 1993, 1994; Ringø *et al.*, 1996; Strøm and Ringø, 1993; Blanch *et al.*, 1997). The gut flora which develops from non-fed marine fish can originate from the resident egg epiflora at the time of hatching (Olafsen, 1994; Hansen and Olafsen, 1989) or the water in the case of milkfish yolk sac larvae ,*Chanos chanos*, (Fernandez *et al*, 1996). However once feeding begins the gut flora mainly originates from the food bacterial flora. This has been shown for many marine fish including black sea bread, *Acanthopagrus schegeli* (Muroga *et al*, 1987), rockfish, *Sebastes* schlegel, (Tanasomwang and Muroga, 1989), turbot, *Scophthalmus*

maximus, (Munro *et al*, 1993, 1994) and Atlantic halibut, *Hippoglossus hippoglossus*, (Bergh *et al*, 1994; Bergh, 1995). Changes also occur at different developmental stages of the fish (Ringø and Birkbeck, 1999). However, some studies have shown that the bacterial flora of the larval gut is not consistent with that of the water or the feed (Campbell and Buswell, 1983; Nicolas *et al.*, 1989). In the larval stage of flatfish species the majority of bacteria present in the gut are *Vibrio* spp. (Munro *et al*, 1993, 1994; Blanch *et al.*, 1997) with *Aeromonas* (Munro *et al.*, 1994, Ringø *et al.*, 1996), *Pseudomonas* (Munro *et al.*, 1994), Acinetobacter and *Moraxella* (Gatesoupe *et al.*, 1997) as well as to a lesser extent Gram-positive bacteria (Ringø *et al.*, 1995, Ringø and Gatesoupe, 1998).

Munro *et al.* (1995) cultured turbot larvae without detectable bacteria for 14 days. These larvae had a mean survival of 71.3%, indicating that bacteria are not essential for survival, nutrition, or development in larval turbot, whereas turbot larvae infected with a pathogenic strain of *V. anguillarum* having a mean survival of 11.6%. Similar survival rates occurred in the presence of several different bacterial species which were present in water and the larval gut at concentrations similar to those found in commercial larval turbot rearing. Therefore, high concentrations of bacteria *per se* are not harmful.

Although specific pathogens have rarely been associated with larval turbot mortalities it has been noted that haemolytic bacteria are more commonly found in batches experiencing high mortality (Nicolas *et al.*, 1989).

Object of Research

Antibiotics are commonly used in aquaculture to limit the number of bacteria during larval rearing. However widespread antibiotic resistance and negative public opinion on the use of antibiotics in farming has forced companies to seek other methods of controlling the bacterial population. Control of the bacterial population is important as opportunistic pathogens can cause high mortalities during larval development. Although *normal* fish pathogens have not been implicated haemolytic bacteria have been associated with significant losses of larvae during the transition to feeding on *Artemia* (Nicolas *et al.*, 1989). Recently Gatesoupe *et al* (1999) identified Vibrio splendidus to be a pathogen associated with high turbot larvae mortalities. Therefore the identification of bacterial pathogens which are identifies from these 'crashes' is essential for the development of alternative methods of controlling the microflora of the larvae. The main point of research was to identify bacteria associated with high mortalities in turbot larval development.

Materials & Methods

Media and solutions

The composition of all solutions and media is shown in Appendix 1.

Culture of Bacteria

All marine bacteria were cultured on marine agar (MA) and incubated at 20°C; *E. coli* was cultured on LB agar and incubated at 37°C.

2.1 Isolation of bacteria from turbot larvae and *Artemia*

This procedure was carried out by the staff of Stolt Sea Farms, Spain and inoculated plates were delivered to Glasgow in a cooled package via a courier service. ten larvae were removed from a larval rearing tank, placed in a small sterile filter and carefully rinsed with sterile PBS. Using sterile forceps the larvae were placed in a sterile glass test tube with 5ml PBS and homogenised with a sterile plunger. Ten-fold serial dilutions were made using PBS and 100µl was spread on MA and TCBS agar. For bacterial sampling from *Artemia* the above procedure was used using 100 *Artemia*.

2.2 Storage of bacteria

Bacterial strains were normally kept on MA plates at 4°C for up to 1 month. For longer term storage at -80°C Protect beads (Technical Service Consultants) were used according to the manufacturer's instructions, except that 100µl of 20% NaCl was added to each vial when storing marine bacteria.

2.3 Extraction of Genomic DNA and PCR amplification of the 16S rRNA gene

Single colonies were picked from marine agar plates and grown overnight in marine broth (Difco). A 1.5ml volume of the culture was centrifuged at 13,000 rpm in a bench top microfuge for 60 sec, the supernatant was removed and the bacteria resuspended in 200µl of Instagene^{TM} Matrix (Bio-Rad). The suspension was incubated for 30 min at 60°C, vortexed, heated at 100°C for 8 min followed by centrifugation for 3 min at 13,000 rpm. Supernatant fluid (5µl) was used for each PCR reaction, with 95µl of the PCR mixture below.

A 10-reaction PCR mixture consisted of

5µl Hotstar Taq (QIAGEN)

100µl 10x PCR buffer (QIAGEN)

50µl 25mM $MgCl_2$ (Final conc. 2.75mM per reaction)

8µl dNTP mix (10mM each)

10µl 27f primer (Oswel) (2.5µM)

10µl 1522r primer (Oswel) (2.5µM)

767µl dH_20

The PCR was carried out on an MJ Research PTC200 Thermal Cycler using the following cycles.

Step 1	95°C for 15 min	
Step 2	94°C for 1min	25 cycles
	55°C for 1.5min	
	72°C for 2min	
Step 3	72°C for 7min	

Products were separated by electrophoresis in 1% agarose (Sigma) containing ethidium bromide (0.5μg/ml), with a 1kB marker (Promega), and bands were visualised by viewing on a UV transilluminator. The amplified 16S rRNA gene was used for direct identification through DNA sequence analysis (Gatesoupe *et al*, 1999) and RFLP analysis (Urakawa *et al,* 1997)

2.4 Cloning and sequencing of 16s rRNA PCR products

2.4.1 Cloning of 16s rRNA PCR products

When multiple bands were obtained in PCR amplification the PCR products were cloned using a Zero Blunt™ TOPO™ PCR Cloning Kit (Invitrogen). The PCR products were blunt-ended using the Expand™ High Fidelity PCR system (Roche Diagnostics); 0.5μl of Expand™ Taq polymerase and 2μl of dNTP mix (10mM each) were added to the PCR product and the mixture incubated at 72°C for 15 min. Electrophoresis of 15μl of the blunt-ended DNA in 1% agarose containing ethidium bromide and visualisation under UV illumination allowed the band of the correct size (1.5kb) to be excised and gel purified using the Quiquick gel purification kit (Qiagen) according to the manufacturer's instructions before cloning. When PCR produced a single band at 1.5kb, the TOPO TA Cloning Kit for Sequencing (Invitrogen) was used. Cloning was carried out according to the manufacturer's instructions with 4μl of PCR product.

2.4.2 Plasmid purification

A single colony of transformed *E. coli* was grown overnight in LB broth containing 50μg ml^{-1} kanamycin (Sigma). Plasmids were purified from 2ml cultures

using an Ultraclean™ Mini Plasmid Prep Kit (Mo Bio Laboratories, Inc. Cat. 12300-100).

2.4.3 Identification of cloned inserts

Inserts were identified by electrophoresis following *Eco*R1 (Roche diagnostics) restriction digestion of the plasmid, digestion being carried out according to the manufacturer's instructions. This produced a band at 1.5kb (the insert) and a band at 3.5kb (TOPO TA sequencing vector) or 4kb (Zero Blunt TOPO vector).

2.4.4 Partial DNA sequence determination of 16S rRNA PCR products

PCR products were purified using the QIAquick™ PCR purification kit (QIAGEN) and DNA concentration was estimated by comparison with a 1kb ladder (Life Technologies). The University of Glasgow Molecular Biology Support Unit (MBSU) carried out the sequence determination. Primers used for sequence determination of PCR products were 27f and 685r (Fig 2.1), and for sequence determination from 16S r RNA genes cloned into plasmid, primers M13f, M13r, and 685r (Fig 2.1) to give forward and reverse sequences for the first 600bp. Sequence analysis was carried out on a Macintosh Performa 630 using the program Seqman2.

27f	5'-AGAGTTTGATCMTGGCTCAG-3'
1522r	5' –AAGGAGGTGATCCANCCRCA- 3'
M13f	5'- GTAAAACGACGGCCAG – 3'
M13r	5'- CAGGAAACAGCTATGAC- 3'
685r	5'- TCTACGCATTTCACYGCTAC-3'

Figure 2.1 Synthetic oligonucleotides used for PCR amplification and sequencing

2.5 RFLP analysis

This was done to identify further strains without the cost of cloning and partial sequencing of the 16S rRNA gene. This was carried out using the method of Urakawa *et al.* (1997) using *Msp*1, *Dde*1, *Rsa*1 (Promega), *Sau3a*1 and *Cfo*1 (Roche Diagnostics) restriction enzymes. For each digest 0.5µl enzyme, 0.2µl BSA solution, 2µl buffer, 10.3µl autoclaved dH_20, and 7µl DNA solution were used. The banding pattern was visualised under UV illumination after electrophoresis of the restriction digest for 90 min in 2.5% agarose gel containing ethidium bromide (0.5µg/ml).

2.6 Characterisation of bacteria isolated from turbot and *Artemia*

2.6.1 Growth on TCBS agar and utilisation of sucrose

TCBS agar (Oxoid) was prepared by the manufacturer's instructions. Samples from single colonies of bacteria from MA plates were streaked onto TCBS agar and incubated overnight at 20°C. Those bacteria which utilised sucrose yielded yellow colonies and sucrose –ve bacteria usually yielded cream or green colonies.

2.6.2 Antibiotic resistance profiles of bacteria

MASTRING-S M11, M14, and M46 (Mast Diagnostics) antibiotic discs were used. An overnight MB culture (150µl) was spread onto each MA plate using a sterile spreader and the plate was allowed to dry before the Mastring was then applied using sterile forceps. The plates were incubated overnight at 20°C. A zone of inhibition of growth round the antibiotic disc indicated that the strain was resistant to that particular antibiotic. No control was used to determine if there were any faulty discs although the three discs used contained two antibiotics twice. Any plate that could not give a accurate reading was re-tested

2.6.3 Single carbon source utilisation using Biolog microplates

This was carried out according to the manufacturer's guidelines except that 15x150mm test tubes were used to record an OD_{600} of between 0.08 and 0.12 using a Corning Colorimeter model 252. Each well was filled with 150µl of suspension before the microplates were incubated for 48 h at 20°C. A change in colour to purple was recorded as +ve, and no colour change was recorded as –ve.

2.6.4 Analysis of Biolog and RFLP data

Data from both the Biolog single carbon usage were clustered using ClustanGraphic4 for Windows (Clustan Limited) and a Viglen Contender C444 computer. Clustering of the data was carried out using the average linkage method. Input of the data used binary code in Microsoft Excel (1, + ve; 0, –ve).

2.7 Effect of bacteria on larval turbot

Fertilised turbot eggs were obtained from Mannin Sea Farms, Derbyhaven, Isle of Man and collected from Glasgow Airport at approximately 1 day post fertilisation.

Day 1: Approximately 1000 fertilised turbot eggs were washed twice using sterile seawater and transferred to a sterile 5L beaker containing 3L of autoclaved seawater containing oxolinic acid, kanamycin, erythromycin (all 10µg/ml), streptomycin (35 µg/ml) and penicillin G (75µg/ml). All antibiotics were obtained from Sigma.

Day 2: The eggs were rinsed in sterile seawater and transferred to new 5L beakers with 3L of fresh autoclaved seawater.

Day 5: The eggs hatched and 50 yolk sac larvae were then transferred using a 50ml pipette to 2L sterile conical flasks containing 1L autoclaved sea water.

Day 8: The larvae were fed and infected at the same time. Depending on the experiment 100µl of a $1x10^9$ ml suspension of unwashed or washed bacteria was added. Bacterial strains that were used were chosen due to their identity and origin. Either 100µl of broth or 100µl of seawater was added to control flasks. Experiment 1 used 4 flasks for controls and 3 flasks per strain, experiment 3 used 8 flasks for controls and 4 flasks per strain. Experiment 2 used 6 flasks for controls and 4 flasks per strain. Rotifers (*Brachionus plicatilis*) rinsed with sterile seawater were added to a final concentration of approximately 1 per ml, together with algae to produce a green-water system within the flask. This system required no further feeding of the fish, reduced the probability of contamination, and removing excess dissolved ammonia (Alderson and Howell, 1973) .

Day 9 – 15: Results of mortality recorded. Dead larvae were not removed from the flasks. Any larvae that had died during the night were decomposed by morning.

The experiment was terminated by adding an overdose of the anaesthetic MS222 to the remaining fish.

Results

Haemolytic bacteria are considered to be detrimental in early larval turbot rearing (Nicolas *et al.*, 1989) and characterisation of these bacteria in the rearing system is of great importance. As it is also generally accepted that the larval gut flora is mainly derived from the live food, haemolytic bacteria were also isolated from *Artemia* rearing systems. Bacteria were isolated from larvae and *Artemia* from two hatcheries, Merexo and Quilmas, in Northern Spain, with 3 sets of samples, one from Merexo (S1) and two from Quilmas (S2, S3). Survival rates are those at day 40.

3.1 Analysis of bacteria isolated from Merexo

The samples S1L1, S1A1, S1L2 and S1A2 were seeded onto marine blood agar by the staff at Merexo and sent to Glasgow by express courier. By the time the plates arrived the bacteria had swarmed over the plate and it was impossible to perform counts. However replicate marine agar plates had been seeded in Spain and counts were provided (Table 1). Of this batch of larvae 43% survived to 40 days.

3.1.1 Antibiotic resistance and basic biochemical profiles of bacteria from the Merexo hatchery.

Antibiotic sensitivity and basic biochemical tests (oxidase, catalase, growth on TCBS, utilisation of sucrose) were used for preliminary grouping. All the bacteria were haemolytic, and oxidase and catalase positive. Therefore, these results were omitted when the data was analysed. (Figure 3.1).

Sample Code	Origin	Bacterial load (cfu /ml)
S1L1	17 day old larvae fed *Artemia* nauplii for 4 days and enriched (DC DHA Selco*) *Artemia* for a further 5 days.	6.9×10^5
S1A1	2 days old *Artemia* were enriched with DC DHA Selco after 24 hours, kept at 4°C for 17 hours, rinsed with sterile water and then fed to larvae. At this point they were sampled.	6×10^3
S1L2	These larvae were sampled from the same tank as S1L1 but 5 hours after they had been fed with the S1A1 *Artemia* sample above.	7×10^4
S1A2	The *Artemia* were 2 days old and were enriched with DC DHA Selco after 24 hours. They were sampled on day 2 after being disinfected with Phylatol, without any period of storage at 4°C.	1.1×10^2

Table 1. Origin, code and concentration of heterotrophic bacteria in samples from Merexo.

*DC DHA Selco is a commercial preparation containing fish oils and highly unsaturated fatty acids fed to *Artemia* to improve nutritional quality

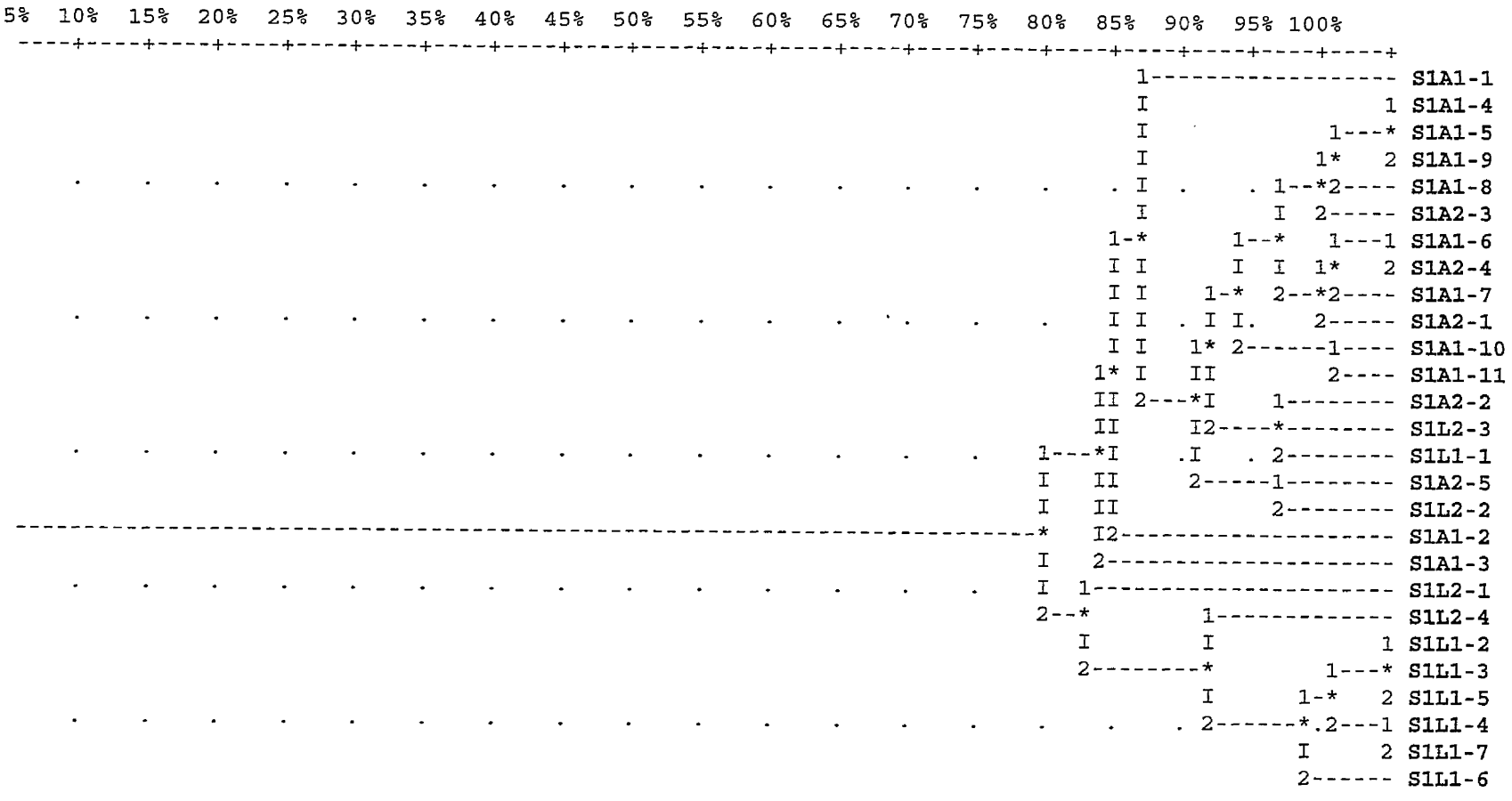


FIGURE 3.1. Dendrogram of 27 Merexo bacterial isolates based on the antibiotic resistance profile and 2 basic biochemical tests.

From the Mastring profiles all 27 isolates were resistant to 15μg tetracycline and 25 μg ampicillin, except for S1L2-1. The only isolates resistant to 100μg tetracycline were S1L1-7, and S1L2-1 to S1L2-4. For streptomycin, 9 isolates were resistant to 10μg (S1A1-3, S1L1-2, S1L1-7, S1L2-2, S1L2 –3). Detailed results are shown in Appendix 3.

3.1.2 **Analysis of Merexo samples using Biolog GN microplates**

Analysis of the above antibiotic sensitivity profiles yielded less identity (i.e. homogeneity) amongst isolates than had been expected. Therefore, samples were tested for their ability to react with 95 organic substrates in the BIOLOG GN characterization system. Results were recorded in binary, by eye due to the incompatibility between the BIOLOG GN plates and the microplate reader (Appendix 4). A number of type strains were included in the analysis and bacterial isolates from turbot larvae that were subsequently identified by partial sequencing (first 600bp) of their 16S rRNA gene. The average linkage method was used for the clustering analysis to produce the dendrogram shown in Figure 3.2.

The isolates formed three major phenons, the first comprising all 16 isolates from *Artemia* cultures, and 3 from larvae (S1L1-1, S1L2-2 and S1L2-3), the second comprised the remaining 6 S1L1 strains, with a further strain, S1L2-4, distinct from both the above phenons. There was good correlation between the dendrograms based on antibiotic sensitivity profiles and Biolog GN analysis.

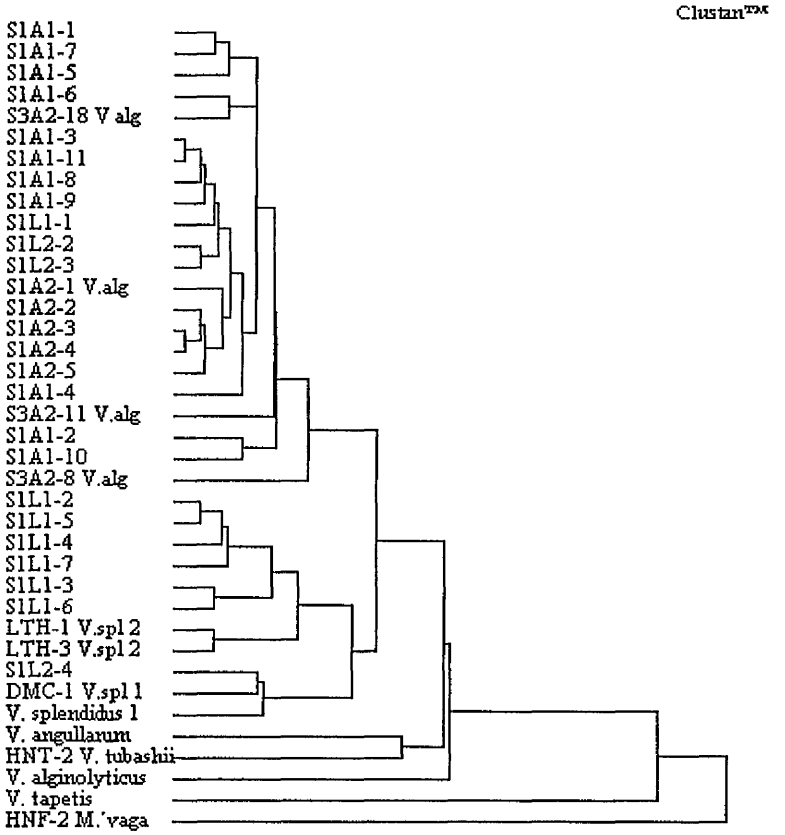


FIGURE 3.2: Biolog GN microplate analysis of 26 Merexo isolates. The dendrogram constructed also used data from type strains and isolates identified on the basis of partial 16S rRNA gene sequences. Reference strains were: *V. splendidus* 1, ATCC 33125; *V. alginolyticus*, ATCC 1339; *V. anguillarum*, ATCC 19264^T; 91079, *V. anguillarum* isolated from vibriosis in juvenile turbot; *V. tapetis*, laboratory strain. V.alg, *V. alginolyticus*; V.spl 1, *V. splendidus* biovar 1; V.spl 2, *V. splendidus* biovar 2.

3.2 Analysis of Bacteria from Quilmas

No haemolytic bacteria could be isolated directly from the *Artemia* cultures from

the hatchery. Bacteria on the plate had swarmed so that an accurate colony count could not be obtained. As before, the hatchery had incubated replicate MA plates from which bacterial concentrations were calculated (Table 2). These larvae had a survival of 42.25% at 40 days.

3.2.1 Antibiotic resistance and basic biochemical profiles of Quilmas bacteria

The same methods were employed as for isolates from Merexo. Detailed results are shown in Appendix 3 and the dendrogram showing the relationships between isolates is shown in Fig. 3.3. Of the 16 samples isolated from Quilmas all were resistant to 10μg tetracycline, and only S2L2-3 was sensitive to 100μg tetracycline. Strains S2L1-1, S2L1-6, and S2L2-1 were sensitive to 2.5μg trimethoprim.

3.2.2 Analysis of Quilmas samples using Biolog GN microplates

The Quilmas S2 samples were analyzed with Biolog GN plates. For reference, the same standard strains used previously were incorporated into the dendrogram (Fig. 3.4). All isolates fell within one phenon that included the standard strains identified as *V. alginolyticus*.

Sample Code	Origin	Bacterial load (cfu /ml)
S2L1	Samples were taken from 21-day-old larvae fed with *Artemia* nauplii for 5 days and enriched *Artemia* for 10 days. The larvae were taken just before the *Artemia* was added to the tank on the last day.	1.5×10^5
S2L2	These larvae were sampled from the same tank as above but 5 hours later.	5×10^5

Table 2. Origin, code and concentration of heterotrophic bacteria in Quilmas bacterial samples.

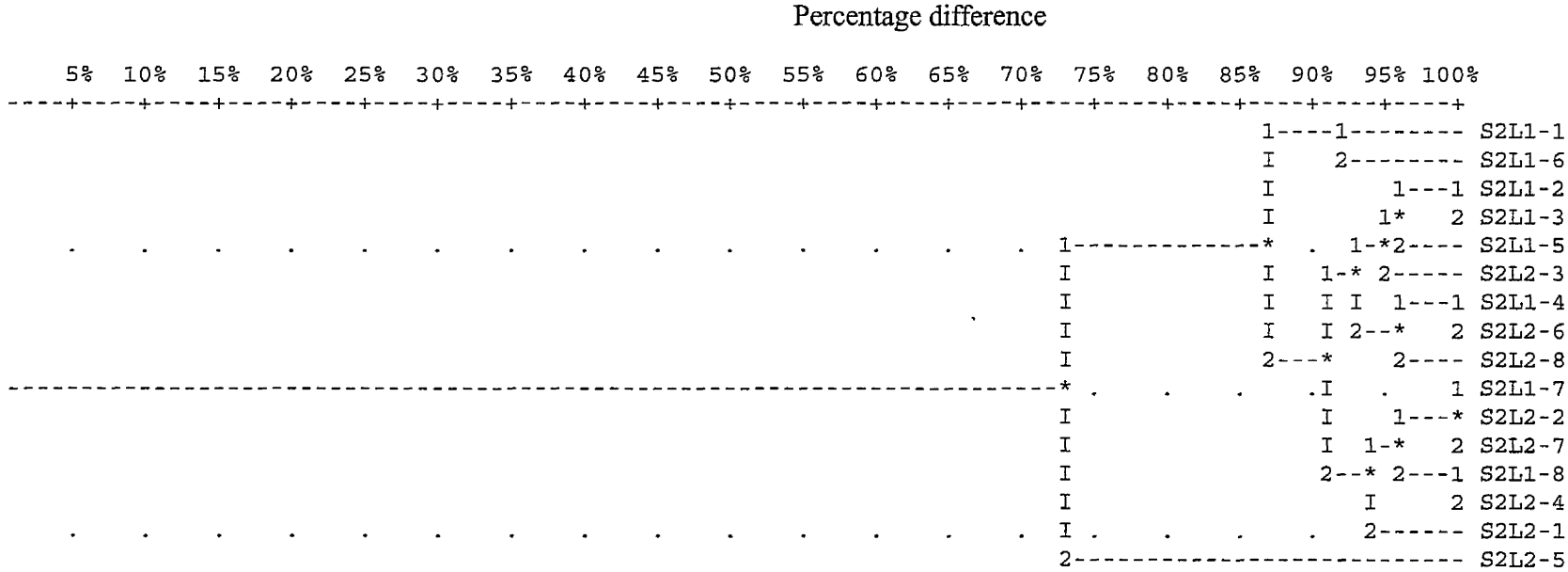


FIGURE 3.3 Dendrogram of 16 Quilmas bacterial isolates based on the antibiotic resistance profile and 2 basic biochemical tests.

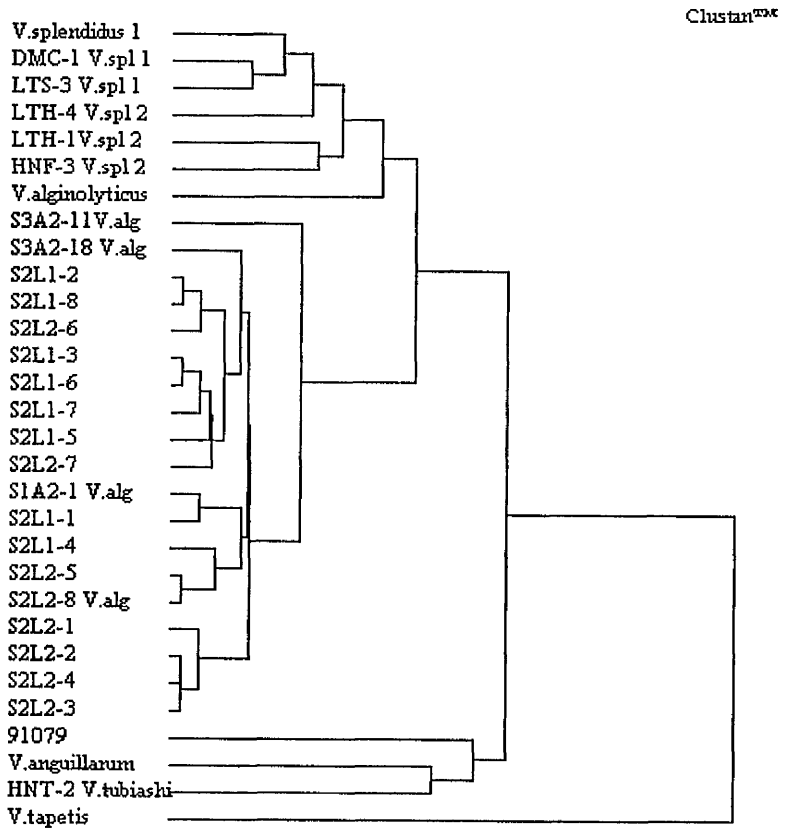


Figure 3.4 Biolog GN microplate analysis of 16 Quilmas isolates. The dendrogram included data from type strains and isolates identified on the basis of partial 16S rRNA gene sequences. Reference strains were: *V. splendidus* 1, ATCC 33125; *V. alginolyticus*, ATCC 1339; *V. anguillarum*, ATCC 19264^T; 91079, *V. anguillarum* isolated from vibriosis in juvenile turbot; *V. tapetis*, laboratory strain. V.alg, *V. alginolyticus*; V.spl 1, *V. splendidus* biovar 1; V.spl 2, *V. splendidus* biovar 2.

3.3 Analysis of a Second Group (S3) of Bacteria isolated from Quilmas

As no haemolytic bacteria were recovered from the first batch of Quilmas *Artemia* isolates a second set of samples was taken from 21-day-old larvae fed *Artemia* nauplii from day 13 and enriched *Artemia* (DC DHA Selco) from day 19. Samples of homogenised larvae and *Artemia* were seeded on MA plates and sent by courier to Glasgow, where colonies were counted (Table 3) and isolates were tested for haemolysis on blood marine agar. These larvae had a survival of 43.2%.

3.3.1 Antibiotic resistance and basic biochemical profiles of S3 isolates

A total of 26 and 32 haemolytic isolates were obtained from larvae (S3L1 and S3L2) and *Artemia* (S3A1 and S3A2), respectively, and these were tested for antibiotic resistance (except for S3A2-23 to S3A2-31), growth on TCBS media, catalase and Kovac's oxidase tests. As all strains were catalase and oxidase positive these parameters were excluded when producing the dendrogram shown in Figure 3.5. At the level of 90% identity 4 phenons were distinguished, and two groups of 7 and 6 isolates, respectively, had identical reactions in the tests applied.

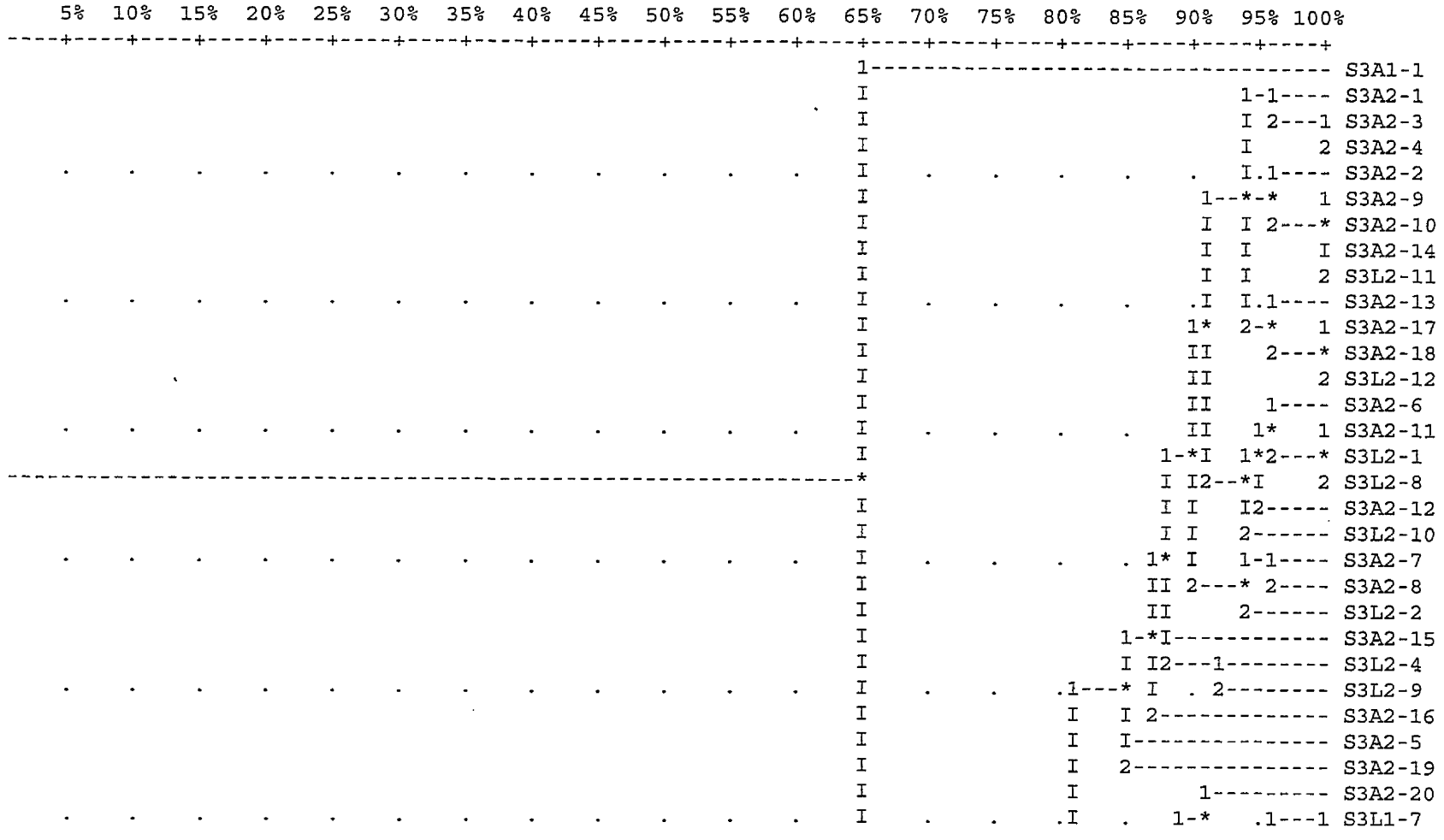
3.3.2 Analysis of S3 Isolates using Biolog GN microplates

All but two of the S3 samples (S3L1-15, which could not be resuscitated and S3A1-1, no biological activity) were analyzed with the Biolog GN system with previously identified strains included to create the dendrogram shown in Figure 3.6. It can be seen that the majority of the *Artemia* isolates and the larval isolates produced independent phenons in both the antibiotic sensitivity and Biolog GN characterization.

Sample Code	Origin	Bacterial load (cfu /ml)
S3L1	Larvae were sampled in the morning prior to feeding.	3.1×10^5
S3A1	Isolates taken from 2-day-old *Artemia* after enrichment and rinsing with sterile fresh water.	2.1×10^3
S3L2	Larvae were taken from the same tank as above but 5 hours later.	7×10^4
S3A2	These *Artemia* were from the same batch as S3A1 but were stored at 4°C for 5 hours.	4×10^3

Table 3. Origin, code and concentration of heterotrophic bacteria in the second set of Quilmas isolates.

% Similarity


5% 10% 15% 20% 25% 30% 35% 40% 45% 50% 55% 60% 65% 70% 75% 80% 85% 90% 95% 100%
S3A1-1
S3A2-1
S3A2-3
S3A2-4
S3A2-2
S3A2-9
S3A2-10
S3A2-14
S3L2-11
S3A2-13
S3A2-17
S3A2-18
S3L2-12
S3A2-6
S3A2-11
S3L2-1
S3L2-8
S3A2-12
S3L2-10
S3A2-7
S3A2-8
S3L2-2
S3A2-15
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S3A2-19
S3A2-20
S3L1-7

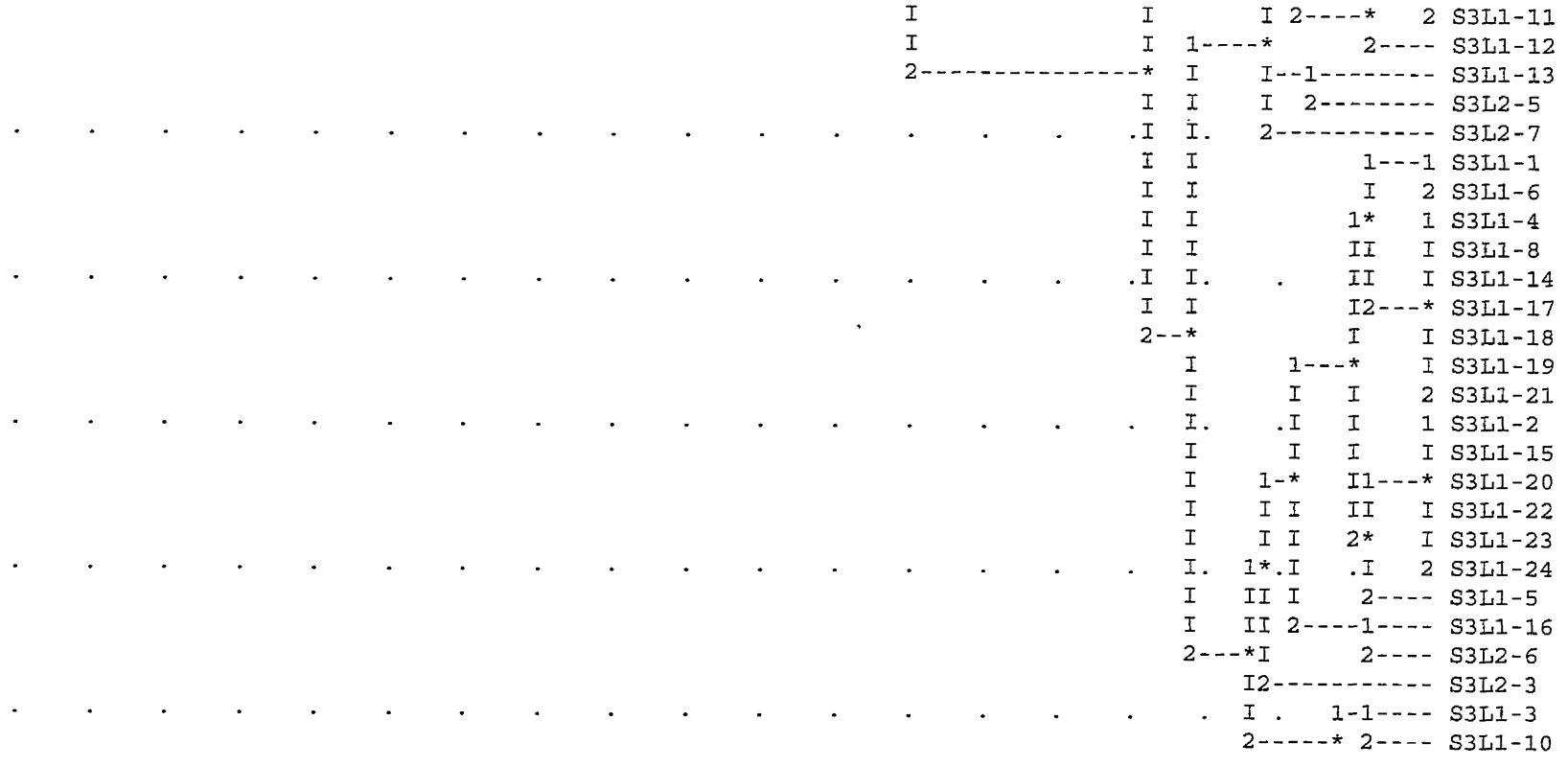
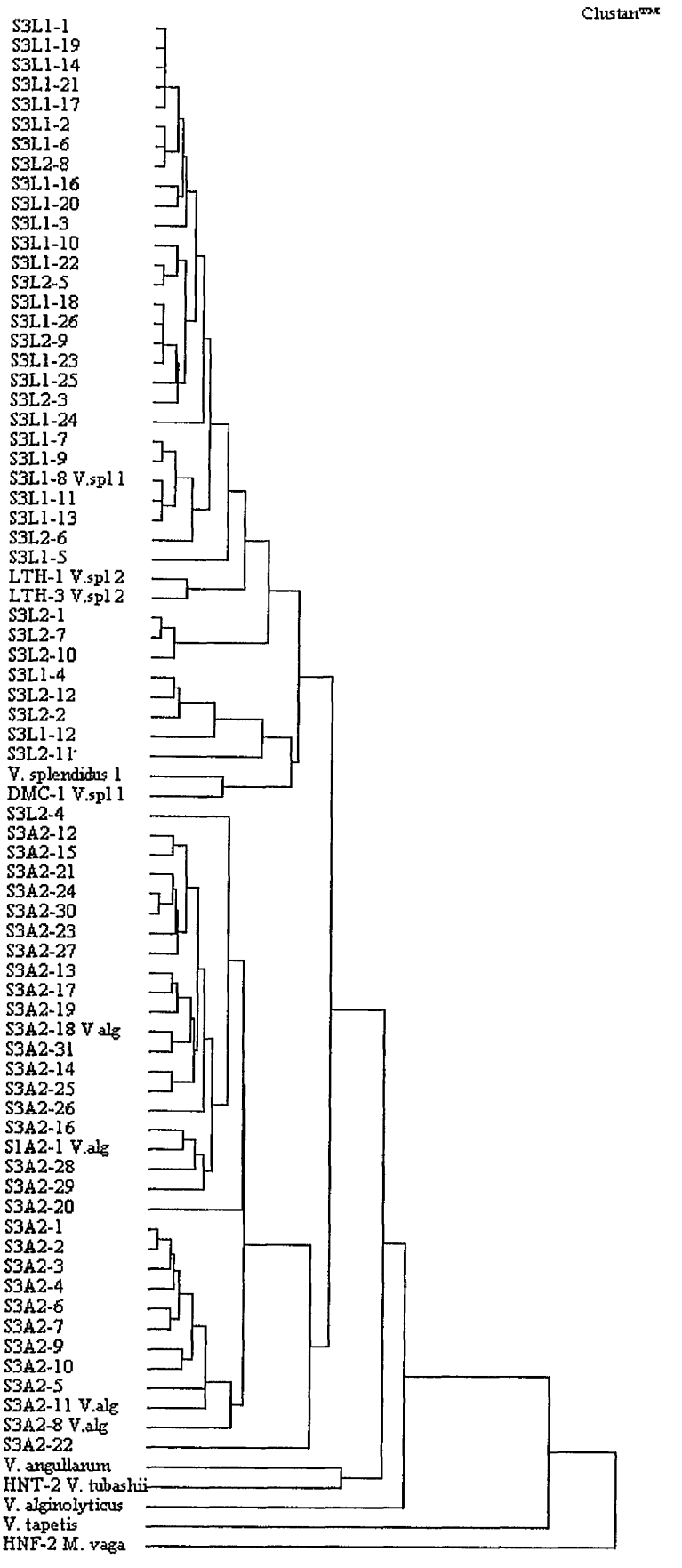


FIGURE 3.5 Dendrogram of Quilmas (S3) bacterial isolates by antibiotic profiling and 2 basic biochemical tests.

Figure 3.6. Biolog GN microplate analysis of 68 Quilmas S3 isolates. The dendrogram included data from type strains and isolates identified on the basis of partial 16S rRNA gene sequences. Reference strains were: *V. splendidus* 1, ATCC 33125; *V. alginolyticus*, ATCC 1339; *V. anguillarum*, ATCC 19264^T; 91079, *V. anguillarum* isolated from vibriosis in juvenile turbot; *V. tapetis*, laboratory strain; V.alg, *V. alginolyticus*; V.spl 1, *V . splendidus* biovar 1; V.spl 2, *V. splendidus* biovar 2.


Clustan™
S3L1-1
S3L1-19
S3L1-14
S3L1-21
S3L1-17
S3L1-2
S3L1-6
S3L2-8
S3L1-16
S3L1-20
S3L1-3
S3L1-10
S3L1-22
S3L2-5
S3L1-18
S3L1-26
S3L2-9
S3L1-23
S3L1-25
S3L2-3
S3L1-24
S3L1-7
S3L1-9
S3L1-8 V.spl 1
S3L1-11
S3L1-13
S3L2-6
S3L1-5
LTH-1 V.spl 2
LTH-3 V.spl 2
S3L2-1
S3L2-7
S3L2-10
S3L1-4
S3L2-12
S3L2-2
S3L1-12
S3L2-11
V. splendidus 1
DMC-1 V.spl 1
S3L2-4
S3A2-12
S3A2-15
S3A2-21
S3A2-24
S3A2-30
S3A2-23
S3A2-27
S3A2-13
S3A2-17
S3A2-19
S3A2-18 V alg
S3A2-31
S3A2-14
S3A2-25
S3A2-26
S3A2-16
S1A2-1 V.alg
S3A2-28
S3A2-29
S3A2-20
S3A2-1
S3A2-2
S3A2-3
S3A2-4
S3A2-6
S3A2-7
S3A2-9
S3A2-10
S3A2-5
S3A2-11 V.alg
S3A2-8 V.alg
S3A2-22
V. angullarum
HNT-2 V. tubashii
V. alginolyticus
V. tapetis
HNF-2 M. vaga

3.4 Analysis of bacteria from dead (D) and live larvae (L)

The bacterial isolates obtained to date were all from healthy larvae but the following strains were isolated from turbot larvae from Merexo that experienced high mortality over a 24-hour period (**D**), and from a parallel batch that fed and grew well with a high survival (**L**). Both batches had been fed the same batch of *Artemia* and were not given any antibiotics during the *Artemia* feeding phase.

The bacteria were seeded on both MA and TCBS, and counts taken when the plates arrived in Glasgow are shown in Table 4.

		Bacterial concentration (cfu/ml)	
Sample	Larval survival %	Marine agar	TCBS agar
Live Larvae (L)	65%	2.42×10^5	1.44×10^5
Dead Larvae (D)	5%	1.3×10^6	4.3×10^5

Table 4. Concentration of bacteria from homogenates of live and dead batches of larvae, isolated on MA and TCBS.

Random isolates were picked from the plates according to their morphology colony and colour, and this was the basis for their initial grouping (Table 5). The organisms were unlike previous isolates in that they did not spread on the plates like *V. alginolyticus*.

3.4.1 Antibiotic resistance and basic biochemical profiles of L and D bacteria

As the larvae had not been treated with antibiotics it was expected that a different profile might be seen to those previously found. Detailed results are shown in Appendix 3

Designation	Origin	Plate Type	Colony morphology	No. of isolates taken	Concentration ($x10^3$ larva $^{-1}$)
LTH	L	TCBS	cream with halo[1]	5	128
LTS	L	TCBS	small round, cream	5	8
LMS	L	MA	small round, cream	4	225
DTY	D	TCBS	round, yellow	5	22
DTC	D	TCBS	cream with halo[1]	5	40
DTR	D	TCBS	round cream	4	390
DMC	D	MA	round cream	7	1300

Table 5 Initial classification and concentration for the isolates obtained from Merexo live and dead batches of larvae.

[1] colony appearance ⊙

All isolates were resistant to ampicillin (10μg), with only the LMS group being sensitive to streptomycin (10μg) and tetracycline (100μg). All but one of the DMC and DTC isolates formed a cluster of organisms that contained no isolates from other groups (Fig 3.7).

3.4.2 **Analysis of Merexo isolates from L and D larvae using Biolog GN microplates**

The results of this analysis are shown in the dendrogram in Figure 3.8, with detailed results shown in Appendix 4. As the isolates were organisms which did not spread on MA it was expected that they would have a different profile from the S1, S2, and S3 strains. The LMS group were not haemolytic but they were included in the analysis as, from the high larval survival, it was thought that they might offer some advantage to the host. All 12 DMC and DTC isolates formed a single phenon which also included strains LTS-3 and LTS-4, and these organisms were closely related to *V. splendidus* biovar 1 (Fig 3.8).

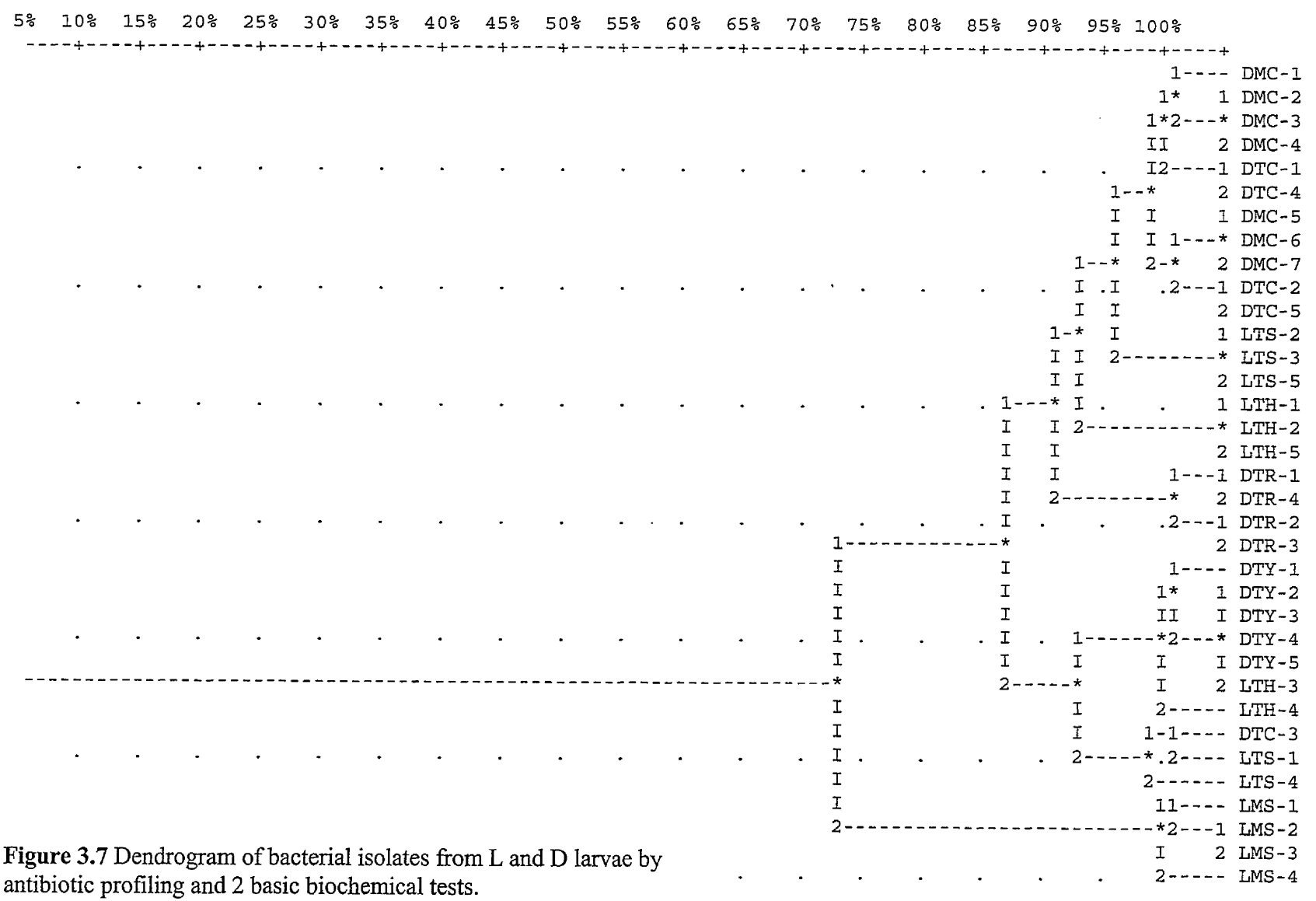


Figure 3.7 Dendrogram of bacterial isolates from L and D larvae by antibiotic profiling and 2 basic biochemical tests.

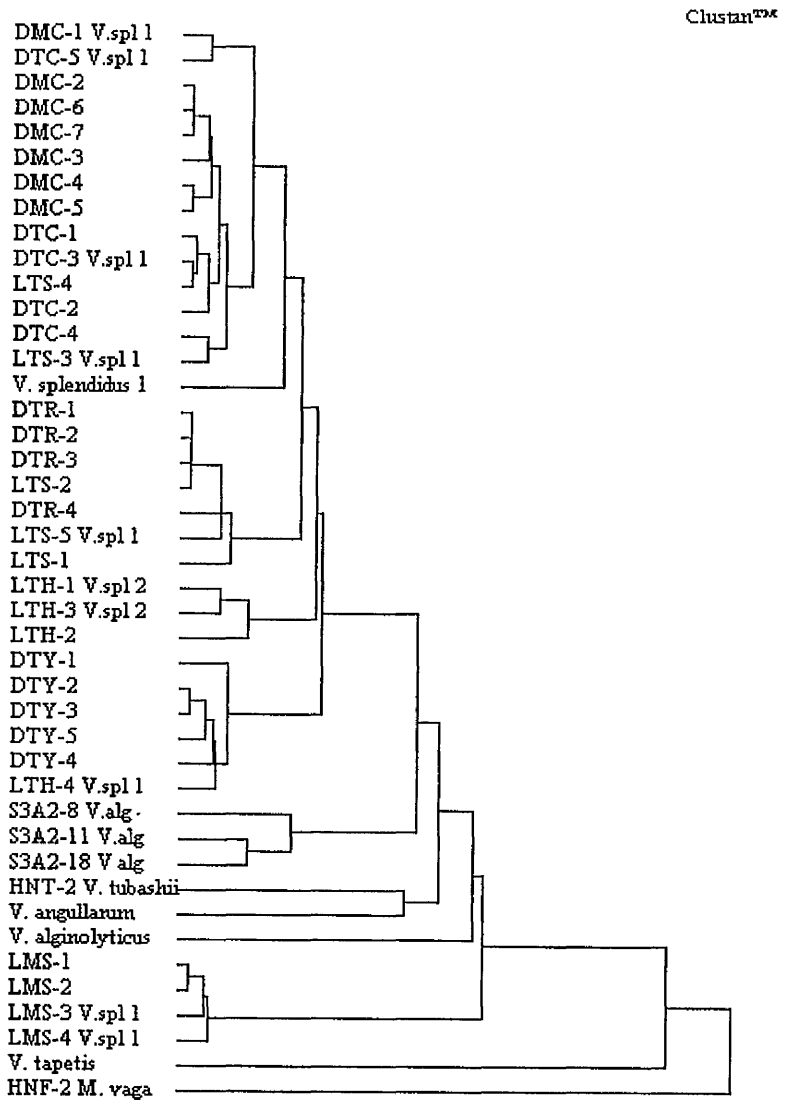


Figure 3.8 Dendrogram constructed using data from Biolog GN microplate analysis of 34 Merexo isolates from L and D larvae. Type strains and isolates identified on the basis of partial 16S rRNA gene sequences are also included. Reference strains were: *V. splendidus* 1, ATCC 33125; *V. alginolyticus*, ATCC 1339; *V. anguillarum*, ATCC 19264^T; 91079, *V. anguillarum* isolated from vibriosis in juvenile turbot; *V. tapetis*, laboratory strain; V.alg, *V. alginolyticus*; V.spl 1, *V. splendidus* biovar 1; V.spl 2, *V. splendidus* biovar 2.

3.5 Analysis of HNT and HNF Merexo samples

A further set of bacterial isolates was obtained following another larval rearing 'crash' in Merexo permitting comparison of bacteria from two larval rearing crashes. These samples were taken only from a batch of larvae that experienced %100 mortality and there was no parallel high surviving group for comparison. From the counts on MA plates the larval homogenates, HNT and HNF, contained 1.83 x 10^8/ml and 1.24 x 10^8/ml bacteria, respectively. Unlike previous samples, these plates contained 3 brown pigmented colonies, one on the HNT and two on the HNF plates. These 3, along with a sample of the other colonies, 8 from HNT and 6 from HNF, were cultured and subjected to the same biochemical tests as before. However, their antibiotic resistance profiles were not recorded.

3.5.1 Characterization of HN isolates using Biolog GN Microplates

The HN samples were analyzed as for the isolates from previous batches and the results used to construct the dendrogram shown in Figure 3.9. However, isolates HNT-9, HNF-1, and HNF-4 did not register biochemical activity on the Biolog GN micoplates. As for previous dendrograms, previously identified species were used as indicators of identity.

3.5.2 Other characteristics of HN isolates

The HN samples were tested on sheep blood MA and TCBS agar media, and tested for catalase and Kovac's oxidase reactions (Table 6). In addition, the brown pigmented isolates HNT-9, HNF-1 and HNF-4 were confirmed as being Gram –ve bacteria.

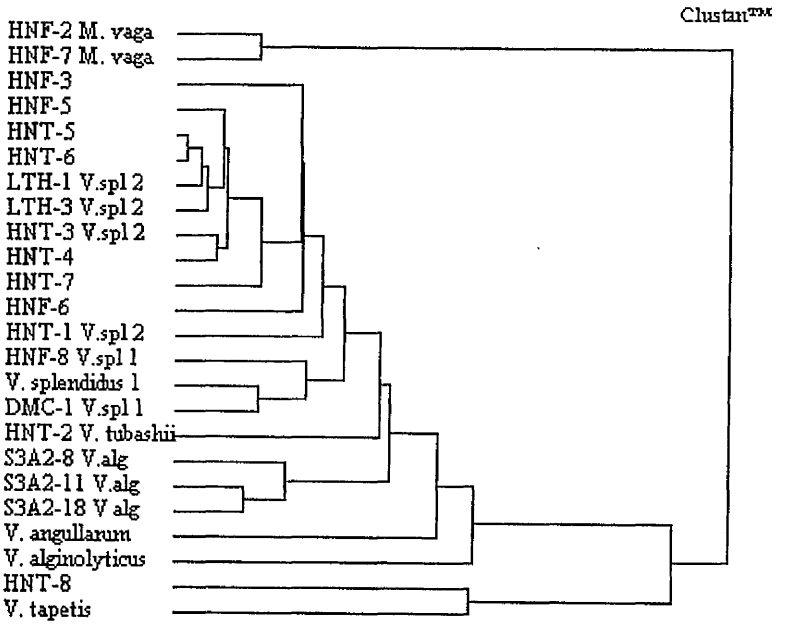


Figure 3.9 Dendrogram showing the relationship between HNF and HNT isolates constructed using data from Biolog GN microplates. Dendrogram also includes data from type strains and isolates identified on the basis of partial 16S rRNA gene sequences. Reference strains were: *V. splendidus* 1, ATCC 33125; *V. alginolyticus*, ATCC 1339; *V. anguillarum*, ATCC 19264^T; 91079, *V. anguillarum* isolated from vibriosis in juvenile turbot; *V. tapetis*, laboratory strain; V.alg, *V. alginolyticus*; V.spl 1, *V. splendidus* biovar 1; V.spl 2, *V. splendidus* biovar 2; M.vaga, *Marinamona vaga*.

Strain	Haemolysis	Growth on TCBS	TCBS sucrose utilization	Catalase	Pigment on MA
HNT-1	-	+	+	+	cream
HNT-2	+	+	+	+	cream
HNT-3	+	+	+	+	cream
HNT-4	+	+	+	+	cream
HNT-5	+	+	+	+	cream
HNT-6	+	+	+	+	cream
HNT-7	+	+	+	+	cream
HNT-8	-	+	+	+	cream
HNT-9	-	-	-	-	brown
HNF-1	-	-	-	+	brown
HNF-2	-	-	-	+	cream
HNF-3	+	+	+	+	cream
HNF-4	-	-	-	+	brown
HNF-5	+	+	+	+	cream
HNF-6	+	+	+	+	cream
HNF-7	-	-	-	+	cream
HNF-8	+	+	-	+	cream

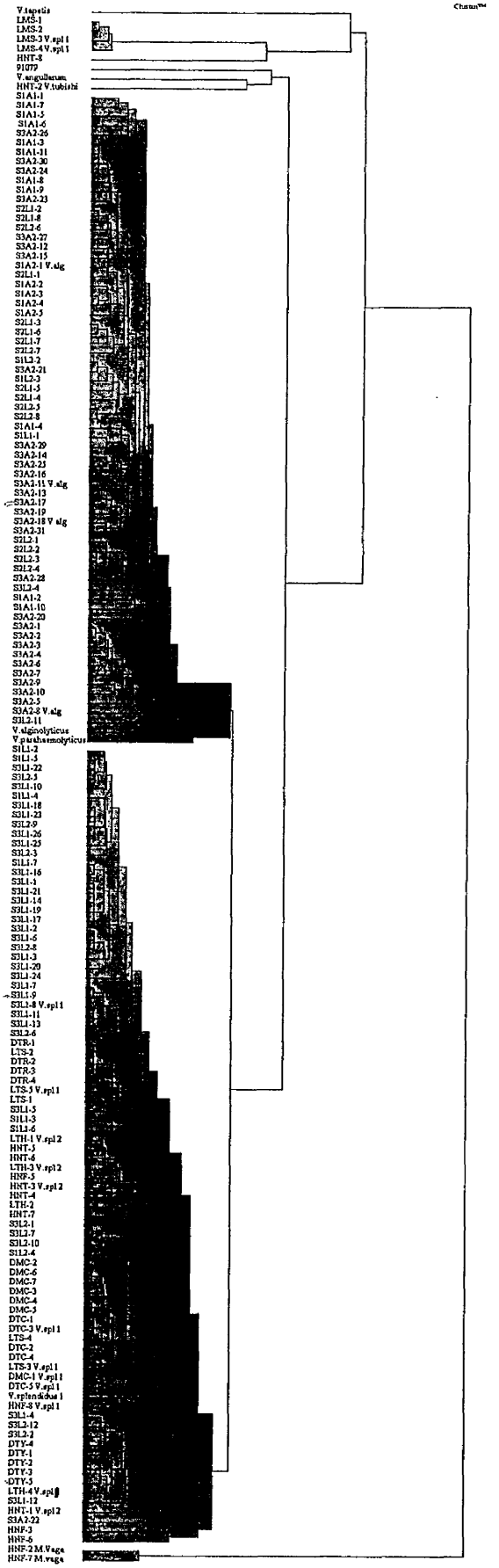
Table 6: Basic characterisation of HN strains.

+ = growth; - = no growth

3.6 Comparison of all isolates using Biolog GN microplates

Using all the Biolog GN data the dendrogram shown in Figure 3.10 was constructed. The highlighted areas show the phenons that were produced. These were chosen arbitrarily as the Clustan program does not show percentage similarity. However, there were clearly two main phenons, a *V. alginolyticus* group and a *V. splendidus* (biovar 1 and 2) group.

Fig 3.10 Dendrogram for all hatchery isolates constructed using data from Biolog GN microplates. The dendrogram included data from type strains and isolates identified on the basis of partial 16S rRNA gene sequences are also included. Reference strains were: *V. splendidus* 1, ATCC 33125; *V. alginolyticus*, ATCC 1339; *V. anguillarum*, ATCC 19264^{T}; 91079, *V. anguillarum* isolated from vibriosis in juvenile turbot; *V. tapetis*, laboratory strain; V.alg, *V. alginolyticus*; V.spl 1, *V . splendidus* biovar 1; V.spl 2, *V. splendidus* biovar 2; M.vaga, *Marinamonas vaga.*


V.tapetis
LMS-1
LMS-2
LMS-3 V.spl1
LMS-4 V.spl1
HNT-8
91079
V.anguillarum
HNT-2 V.tubiashi
S1A1-1
S1A1-7
S1A1-5
S1A1-6
S3A2-26
S1A1-3
S1A1-11
S3A2-30
S3A2-24
S1A1-8
S1A1-9
S3A2-23
S2L1-2
S2L1-8
S2L2-6
S3A2-27
S3A2-12
S3A2-15
S1A2-1 V.alg
S2L1-1
S1A2-2
S1A2-3
S1A2-4
S1A2-5
S2L1-3
S2L1-6
S2L1-7
S2L2-7
S1L2-2
S3A2-21
S1L2-3
S2L1-5
S2L1-4
S2L2-5
S2L2-8
S1A1-4
S1L1-1
S3A2-29
S3A2-14
S3A2-25
S3A2-16
S3A2-11 V.alg
S3A2-13
S3A2-17
S3A2-19
S3A2-18 V.alg
S3A2-31
S2L2-1
S2L2-2
S2L2-3
S2L2-4
S3A2-28
S3L2-4
S1A1-2
S1A1-10
S3A2-20
S3A2-1
S3A2-2
S3A2-3
S3A2-4
S3A2-6
S3A2-7
S3A2-9
S3A2-10
S3A2-5
S3A2-8 V.alg
S3L2-11
V.alginolyticus
V.parahaemolyticus
S1L1-2
S1L1-5
S3L1-22
S3L2-1
S3L1-10
S1L1-4
S3L1-18
S3L1-23
S3L2-9
S3L1-26
S3L1-25
S3L2-3
S1L1-7
S3L1-16
S3L1-1
S3L1-21
S3L1-14
S3L1-19
S3L1-17
S3L1-2
S3L1-6
S3L2-8
S3L1-3
S3L1-20
S3L1-24
S3L1-7
S3L1-9
S3L1-8 V.spl1
S3L1-11
S3L1-13
S3L2-6
DTR-1
LTS-2
DTR-2
DTR-3
DTR-4
LTS-5 V.spl1
LTS-1
S3L1-5
S1L1-3
S1L1-6
LTH-1 V.spl2
HNT-3
HNT-6
LTH-3 V.spl2
HNF-5
HNT-3 V.spl2
HNT-4
LTH-2
HNT-7
S3L2-1
S3L2-7
S3L2-10
S1L2-4
DMC-2
DMC-6
DMC-7
DMC-3
DMC-4
DMC-5
DTC-1
DTC-3 V.spl1
LTS-4
DTC-2
DTC-4
LTS-3 V.spl1
DMC-1 V.spl1
DTC-5 V.spl1
V.splendidus 1
HNF-8 V.spl1
S3L1-4
S3L2-12
S3L2-2
DTY-4
DTY-1
DTY-2
DTY-3
DTY-5
LTH-4 V.spl
S3L1-12
HNT-1 V.spl2
S3A2-22
HNF-3
HNF-6
HNF-2 M.vaga
HNF-7 M.vaga

3.7 Molecular analysis of bacterial 16S rRNA genes

3.7.1 Amplification of bacterial 16S rRNA genes

To confirm the identity of representative isolates a section of approximately 1.5kb of the 16S rRNA gene was amplified by PCR using the standard primers 27f and 1525r. This yielded a product of approximately 1.5kb, as expected (Fig. 3.11).

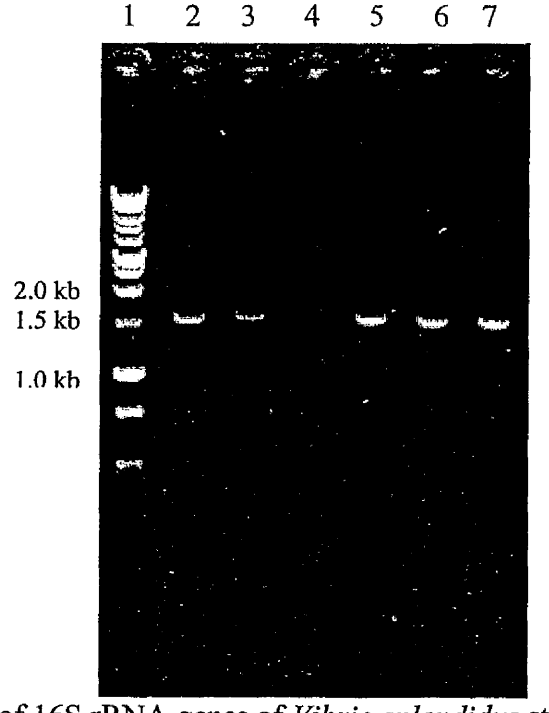


Figure 3.11 PCR amplification of 16S rRNA genes of *Vibrio splendidus* strains. Lane 1, Promega 1kb ladder with selected band sizes; lane 2, HNT-2; lane 3, HNF-1; lane 4, negative control (no DNA); lane 5, DMC-1; lane 6, DTC-5; lane 7, positive control (*E. coli*)

3.7.2 Cloning of PCR products for sequencing

PCR products were cloned into a plasmid to facilitate sequence determination. An *Eco*R1 restriction site on either side of the cloning site allowed the insert to be excised and confirmed by agarose gel electrophoresis to be of approximate 1.5kb in size, with the plasmid, pCR®-4 TOPO® yielding a band at 4kb (Fig. 3.12). Where the 16S rRNA genes

were sequenced from the constructs in pCR®-Blunt II-TOPO, the fragments produced on digestion with *Eco*R1 were an insert of approximately 1500bp, and a plasmid of 3.5kb (data not shown).

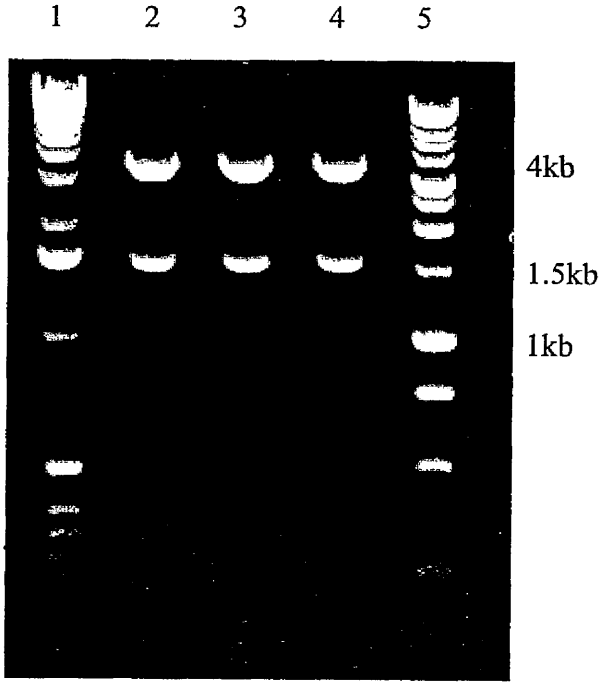


Figure 3.12 Agarose gel of pCR®-4 TOPO® cut with EcoR1. Lane 1, 1kb ladder (Life Technologies); Lanes 2, 3 and 4, cloned 16S rRNA genes of DMC-1, DTC-5, and LMS-3, respectively; Lane 5, 1kb ladder (Promega). 1µl of DNA sample was used in each lane.

3.7.3 Identification of bacterial isolates using partial sequences of the 16S rRNA gene

The 16S rRNA gene within bacteria varies in sequence, from strain to strain, particularly within the first 600bp, making it useful for the identification of bacteria. The identities ascribed to the various strains are shown in Table 7. The determined partial sequences can be found in Appendix 5.

Isolate	Closest 16S rRNA identity (Blast score)
LMS-3	*Vibrio splendidus* biovar 1, ATCC 33125, (1265)
LMS-4	*Vibrio splendidus* biovar 1, ATCC 33125, (1162)
HNT-2	*Vibrio tubiashii*, ATCC 19109^{T}, (1231)
S1A2-1	*Vibrio alginolyticus*, ATCC17749^{T}, (1310)
S3A2-11	*Vibrio alginolyticus*, ATCC17749^{T}, (1310)
S3A2-18	*Vibrio pelagius*, ATCC 25916^{T}, (1203) *
S3A2-8	*Vibrio alginolyticus*, ATCC17749^{T}, (1314)
S1L1-7	*Vibrio splendidus* biovar 1, ATCC 33125, (1221)
S3L1-8	*Vibrio splendidus* biovar 1, ATCC 33125, (1201)
LTS-5	*Vibrio splendidus* biovar 1, ATCC 33125, (1253)
LTH-1	*Vibrio splendidus* biovar 2, ATCC33789, (1314)
LTH-3	*Vibrio splendidus* biovar 2, ATCC33789, (1209)
HNT-3	*Vibrio splendidus* biovar 2, ATCC33789, (1314)
S1L2-4	*Vibrio splendidus* biovar 1, ATCC 33125, (1221)
DTC-3	*Vibrio splendidus* biovar 1, ATCC 33125, (1267)
LTS-3	*Vibrio splendidus* biovar 1, ATCC 33125, (1211)
DMC-1	*Vibrio splendidus* biovar 1, ATCC 33125, (1199)
DTC-5	*Vibrio splendidus* biovar 1, ATCC 33125, (1265)
HNF-8	*Vibrio splendidus* biovar 1, ATCC 33125, (1275)

Table 7. Identification of particular isolates by partial 16s RNA gene sequencing.

*However, from the Ribosome Database Project (RDP) data, S3A2-18 is closest to *Vibrio alginolyticus* str. XII-53 CIP 75.03, with an RDP score of 0.953, *Vibrio pelagius*, ATCC 25916^{T} has an RDP score of 0.930

Table 7 cont.

Isolate	Closest 16S rRNA identity (Blast score)
LTH-4	*Vibrio splendidus* biovar 1, ATCC 33125, (1348)
HNT-1	*Vibrio splendidus* biovar 2, ATCC33789, (1298)
HNF-3	*Vibrio splendidus* biovar 2, ATCC33789, (1302)
HNF-2	*Marinomonas vaga*, ATCC 27119, (973)
HNF-1	*Roseobacter gallaeciensis*, ATCC 700781, (1314)

3.7.4 **RFLP analysis of 16S rRNA genes of bacteria within different phenons**

To provide further evidence that bacteria within the same phenon were of the same species, RFLP analysis of their 16S rRNA gene amplicons was carried out. The specific patterns for each strain are shown in Table 8, with the representative patterns for each enzyme shown in Figure 3.13a – 3.13d.

		Restriction enzyme pattern					
Strain	Identity[1]	*Rsa*I	*Cfo*I	*Dde*I	*Msp*I	*Sau*3AI	Type[2]
LTH-4	V.spl 1	1	1	1	1	1	One
LTS-2		1	1	1	1	1	One
LTS-3	V.spl 1	1	1	1	1	1	One
LTS-5		1	1	1	1	1	One
DTR-1		1	1	1	1	1	One
DTY-1		1	1	1	1	1	One
DTY-2		1	1	1	1	1	One
DTY-3		1	1	1	1	1	One
S3L1-13		1	1	1	1	1	One
DMC-1	V.spl 1	1	1	1	1	2	Two
DMC-2		1	1	1	1	2	Two
DMC-5	V.spl 1	1	1	1	1	2	Two
DTC-1		1	1	1	1	2	Two
DTC-2		1	1	1	1	2	Two
HNF-8	V.spl 1	1	1	1	1	2	Two
LMS-3	V.spl 1	1	1	1	1	2	Two
LMS-4	V.spl 1	1	1	1	1	2	Two
LTS-4		1	1	1	1	2	Two
S1L2-4	V.spl 1	1	1	1	1	2	Two
S3L1-8	V.spl 1	1	3	1	1	1	Three
S3L2-7		1	3	1	1	1	Three
LTH-1	V. spl 2	1	2	1	1	2	Four
HNF-3	V. spl 2	1	2	1	1	2	Four
HNT-3	V. spl 2	1	2	1	1	2	Four
HNT-5		1	2	1	1	2	Four
HNF-2	M. vaga	2	4	2	2	3	Five
HNF-7		2	4	2	2	3	Five

Table 8. RFLP patterns of selected isolates. The restriction patterns are as shown in Figure 3.13.

[1]Isolates identified on the basis of partial 16S rRNA gene sequence.

[2]Designation of RFLP type from specific pattern of the 5 restriction enzymes

V.spl 1 = *Vibrio splendidus* biovar 1

V.spl 2 = *Vibrio splendidus* biovar 2

M.vaga = *Marinomonas vaga*

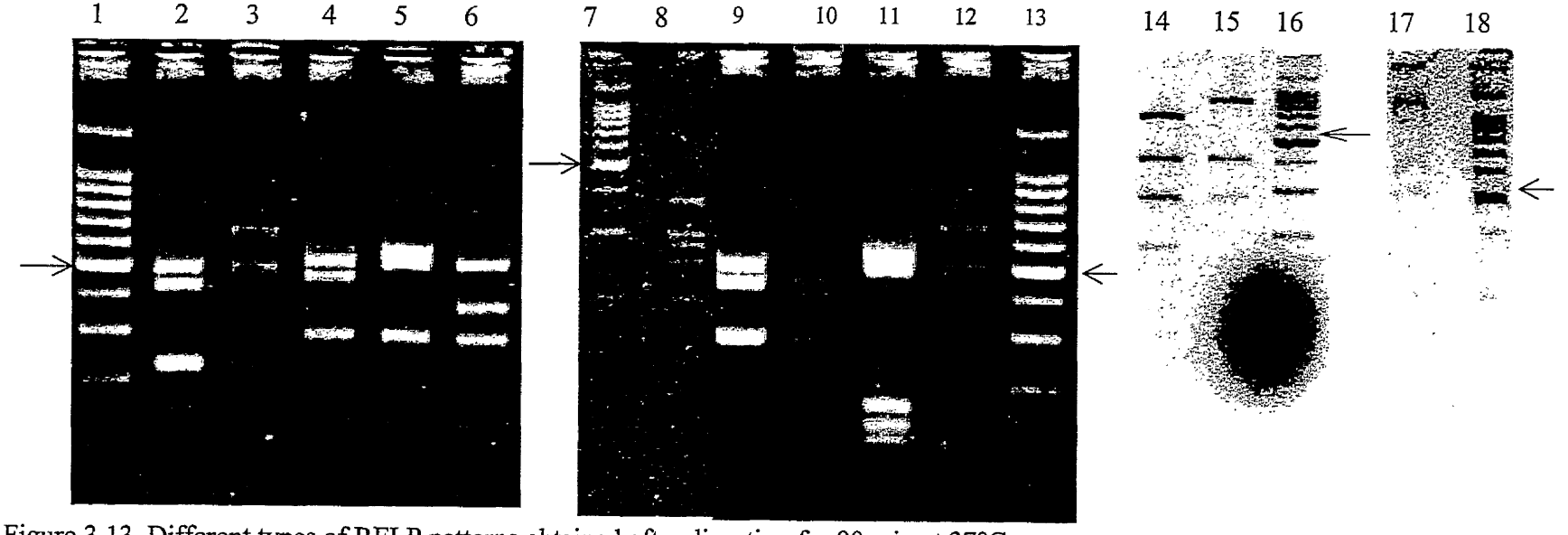


Figure 3.13. Different types of RFLP patterns obtained after digestion for 90 min at 37°C

Lane 1, 7, 13, 14, and 18 100bp ladder (Promega), The brightest band of 500bp is indicated →

Lane 2. Type 1 Digest for *Rsa*I

Lane 3. Type 2 digest for *Rsa*I

Lane 4. Type 1 Digest for *Cfo*I

Lane 5. Type 2 digest for *Cfo*I

Lane 6. Type 3 digest for *Cfo*I

Lane 8. Type 4 digest for *Cfo*I

Lane 9. Type 1 digest for *Dde*I

Lane 10. Type 2 digest for *Dde*I

Lane 11. Type 1 digest for *Msp*I

Lane 12. Type 2 digest for *Msp*I

Lane 15. Type 1 digest for *Sau3A*I

Lane 16. Type 2 digest for *Sau3A*I

Lane 17. Type 3 digest for *Sau3A*I

3.8 Virulence of selected bacteria isolated from turbot larvae

The major part of study was to identify virulent bacteria isolated from the *Artemia* and larval cultures. Bacterial isolates from groups of fish with good survival, and a batch which suffered serious mortality, (section 3.4) provided a good basis for comparison of the virulence of strains from larvae. A total of 23 different challenges were made in four experiments. In each challenge 100µl of a $1x10^9$ ml suspension was used. Bacteria were considered virulent if their P-value (Mann-Whitney test) was <0.05

3.8.1 Challenge experiment one

Because of a poor hatch rate only two strains, DMC-1 and DTC-5, were compared in this experiment (Table 9), which was terminated after 20 hours because of the very high mortality in the challenge flasks. This was possibly due to the high number of eggs in each beaker during the initial antibiotic treatment, as subsequent experiments used lower numbers in each beaker and achieved an increased hatch rate.

Challenge	Mean % survival [1]	SD[1]	SEM[1]	P Value[2]
Control	82.76	17.21	9.94	
DMC-1	16.31	12.43	7.17	0.0518
DTC-5	6.35	11.28	6.51	0.0518

Table 9. Results from first challenge experiment.

[1] mean, standard deviation (SD) and standard error (SEM) for results pooled from all flasks

[2]Mann-Whitney test

3.8.2 Challenge experiment two

After the first experiment using whole cultures, washed cells were used in case extracellular toxins which might kill the larvae were present in the supernatant. In this experiment two samples from the second batch of Quilmas isolates (S3A2-11, S3L2-8) were also included with the L and D larval isolates. Using washed cells, unless otherwise stated, results from the fourteen challenges made in this experiment are shown in Table 10. This experiment lasted for 4 days after infection.

3.8.3 Challenge experiment three

This experiment which was carried out for five days used isolates from L, D and HN larvae for a total of ten challenges. Washed cells of LTS-3, LTH-4, and LMS-1 were tested as in the second experiment. Results are shown in Table 11.

3.8.4 Challenge experiment four

This experiment was originally designed to confirm data already obtained from previous trials but initial heavy moralities reduced the experiment to just four challenges for 4 days post infection. The fish which died were malformed and so only fish which were not disfigured were included in this trial. Results are shown in Table 12.

Challenge	Mean % survival [1]	SD[1]	SEM[1]	P Value[2]
Control	44.6	29.4	13.2	
DTC-2	34.0	13.9	9.8	0.8973
DTC-5 wc[3]	18.0	15.1	13.2	0.2453
DTC-5	55.3	38.3	27.1	0.6056
DTC-5 super[4]	30.7	29.0	20.5	0.6985
DTC-5 heat[5]	32.7	27.3	19.3	0.3017
DTR-2	37.3	20.6	14.6	0.5186
DTY-5	35.3	11.6	8.2	0.5186
LTH-3	34.0	13.9	9.8	1.0000
LTH-4	30.7	21.4	15.1	0.3662
LMS-1	66.0	18.3	12.9	0.5186
LTS-3	13.3	8.1	5.7	0.0528
LTS-4	50.0	44.2	31.3	0.8973
S3A2-11	62.0	20.1	14.2	0.6985
S3L2-8	31.3	17.7	12.5	0.2453

Table 10 Results from the second challenge experiment.

[1] mean, standard deviation (SD) and standard error of the mean(SEM) for results pooled from all flasks.

[2] Mann-Whitney test

[3] Whole cell culture [4] Filtered culture supernatant of DTC-5

[5] Filtered culture supernatant of DTC-5 heated at 100°C for 10 min.

Challenge	Mean % survival [1]	SD[1]	SEM[1]	P Value[2]
Control	31.5	20.7	7.8	
DMC-1	15.0	13.2	7.6	0.3082
DTY-1	18..0	23.2	13.4	0.2027
HNF-1	30.0	35.3	20.4	0.7341
HNF-3	11.0	5.0	2.9	0.1488
HNF-8	9.0	10.5	6.1	0.0508
HNT-2	30.0	30.2	17.4	0.7341
LMS-1	19	8.2	4.8	0.4969
LTH-1	14.0	10.6	6.1	0.1742
LTH-4	19.0	25.6	14.8	0.3502
LTS-3	5.0	6.0	3.5	0.0272

Table 11 Results from the third challenge experiment.

[1] mean, standard deviation (SD) and standard error of the mean (SEM) for results pooled from all flasks

[2] Mann-Whitney test

Challenge	Mean % survival [1]	SD[1]	SEM[1]	P Value[2]
Control	51.0	19.0	8.5	
DMC-1 wc	1.5	3	1.7	0.0142
DMC-1	3.0	4.8	2.7	0.0142
DMC-1 +supernatant[3]	6.0	4.3	2.5	0.0142
LTS-3	14.0	5.9	3.4	0.0142

Table 12 Results from the fourth challenge experiment.

[1] mean, standard deviation (SD) and standard error of the mean (SEM) for results pooled from all flasks

[2] Mann-Whitney test

[5] filtered supernatant of DMC-1 broth culture

3.8.5 **Combined results of all turbot larvae challenge experiments**

Although the experiments were carried out using three flasks for each challenge the pooling together of the results from several experiments shows a truer picture of the virulence of each organism, although this is only for strains used more that once. The results of the pooled P-values are shown in Table 13.

Strain	P-Value[1]	No. Experiments	Virulent[2]
DMC-1 wc[3]	0.0011	2	Yes
DMC-1	0.0033	2	Yes
DMC-1 wc+super[4]	0.0142	1	Yes
DTC-2	0.8973	1	No
DTC-5 wc[3]	0.0067	2	No
DTC-5	0.6056	1	No
DTC-5 super[5]	0.6985	1	No
DTC-5 heat super[6]	0.3017	1	No
DTR-2	0.5186	1	No
DTY-1	0.2027	1	No
DTY-5	0.4404	1	No
HNF-1	0.7341	1	No
HNF-3	0.1488	1	No
HNF-8	0.0508	1	Yes
HNT-2	0.0.7341	1	No
LMS-1	0.8914	2	No
LTH-1	0.1742	1	No
LTH-3	1.000	1	No
LTH-4	0.8645	2	No
LTS-3	0.0001	3	Yes
LTS-4	0.8973	1	No
S3A2-11	0.6985	1	No
S3L2-8	0.2453	1	No

Table 13. Results of pooled larval challenge experiments.

[1] P-Value obtained from a Mann-Whitney test. [2] Virulence based on P-Value <0.05 being significant. [3] Whole culture of cells. [4] Washed cells + filtered culture supernatant. [5] Filtered culture supernatant. [6] Filtered culture supernatant heated at 100°C for 10min

Discussion

4.0 Discussion

The development of larval culture first feeding practices, water and light conditions, and the production of fertilised eggs throughout the year has contributed to an increase in fish production from aquaculture of 300% in the last 10 years (FAO; 1999). The growth of knowledge in aquaculture has led to an increase in the types of fish cultured and these now include European turbot (*Scophthalmus maximus*), halibut (*Hippoglossus hippoglosssus*) and cod (*Gadus morhua*). However, even with the wealth of knowledge that has been acquired in the last 20 years problems still remain for aquaculture of these species but turbot culture is poised to increase substantially once large numbers of juveniles can be produced routinely. The production of a viable inert foodstuff for larvae would be highly desirable for aquaculture, as rotifers and *Artemia* are potential sources of bacterial contamination that can decimate a larval rearing tank. High mortalities in fish aquaculture caused by bacterial contamination usually arise from infection with opportunistic pathogens (Munro *et al.*, 1995). and the purpose of this project was to characterise the haemolytic bacteria associated with commercially reared batches of turbot larvae during transition to feeding on *Artemia*. The identification of these pathogens would help aquaculture in two areas. First, the identification mean that the farm could screen various points of entry for these bacteria in order to find out where they get into the system, and secondly would provide at target for developing alternative methods of controlling bacterial flora in the fish e.g. probiotics.

4.1 Analysis of the bacteria isolated from turbot larvae and *Artemia* from Merexo

The first batch of larvae sampled showed 43% survival to day 40 and suggested that few or no pathogenic bacteria would be found in the bacterial gut flora. The swarming of the bacteria on the plates made it impossible to count the numbers of haemolytic and non-

haemolytic bacterial colonies. The antibiotic resistance profile (Fig. 3.1) divided the bacterial isolates into two phenons at the level of 75% identity, the first phenon consisting of all 16 *Artemia* isolates and 4 larval isolates, while the second phenon contained bacterial isolates from larvae.

The Biolog GN data yielded a dendrogram which showed good correlation with the antibiotic resistance profile. Again, the dendrogram (Fig. 3.2) was split into two major phenons with the same compositions as above. The Biolog GN plates allowed the use of bacteria of known identity to be included for the possible identification of isolates. The first phenon again consisted of mainly *Artemia* isolates and three larval samples (S1L1-1, S1L2-2, S1L2-3) which were also found in this phenon in the antibiotic resistance profile dendrogram. The phenon also contained bacterial isolates S3A2-18, S1A2-1, S3A2-11 which were identified as *V. alginolyticus* from partial DNA sequence of their 16S rRNA genes, and the culture collection type strain *V. alginolyticus* ATCC 1339. Thus, this phenon can be identified tentatively as the *V. alginolyticus* group of bacteria.

The second phenon could be divided into two smaller groups, the first of which contained isolates S1L1-2 through to S1L1-7 and a *V. splendidus* biovar 2 isolate LTH-1. The lower groups within the phenon contained only isolate S1L2-4, and strains LTS-3 and DMC-1 along with strains identified as *V. splendidus* biovar 1 (type strain *V. splendidus* ATCC 33125). Thus, isolates in the second phenon most likely belong to the *V. splendidus* group with the S1L1-2 to S1L1-7 strains being of biovar 2 and S1L2-4 strain being of biovar 1. The lack of *V. alginolyticus* type bacteria in the larval samples could be due to the small sample size as this species has been found in turbot previously (Gatesoupe, 1990; Munro *et al.*, 1993, 1994; Blanch *et al.*, 1997) as well as other species of marine fish e.g. Atlantic halibut, *Hippoglossus hippoglossus*, (Bergh, 1995; Verner-Jeffries, 2000) and rockfish, *Sebastes schlegi*, (Tanasomwang and Muroga, 1989). *Vibrio splendidus* has also been

identified in turbot larvae (Munro *et al.*, 1994; Blanch *et al* 1997) and has been found as a pathogen of turbot larvae (Gatesoupe *et al.*, 1999).

4.2 Analysis of the bacteria isolated from Quilmas turbot larvae

These larvae had a survival of 42.25% at day 40 which is high. Unfortunately, swarming of the bacteria had again covered the plates and comparison of haemolytic to non-haemolytic bacteria was not possible. However, the bacterial counts for these samples were similar to those from Merexo. The swarming was probably due to the plates being wet when inoculated as single colonies were produced when streaking onto fresh MA plates.. The antibiotic resistance profile (Fig. 3.3) also had two phenons (85% similarity) with the lower phenon containing only isolate (S2L2-5). The Biolog GN dendrogram (Fig. 3.4) however included all these isolates in one phenon also containing the strains identified as *V. alginolyticus*. Strain S2L2-5 had the same Biolog GN profile as strain S2L2-8.

Comparison of the antibiotic resistance profiles of bacteria from larval samples from Merexo and Quilmas shows that all strains from Merexo are resistant to 25μg cotrimoxazol, 50μg sulphamethoxazole, 10μg streptomycin (except S1L1-1,S1L2-2, S1L2-3) and all strains from Quilmas are sensitive to 25μg cotrimoxazol, 50μg sulphamethoxazole (except S2L1-4, S2L2-5, S2L2-6, S2L2-8), 10μg streptomycin (except S2L2-5). This is most likely due to different practices at the two hatcheries.

4.3 Analysis of the second group of bacterial isolates from Quilmas turbot larvae and *Artemia*

The total bacterial counts from the second batch of Quilmas larvae and *Artemia* were similar to those of the first batches from Quilmas and Merexo. The antibiotic resistance profile (Fig 3.5) divided the isolates into two large phenons at the level of 80% similarity,

with a single outlying isolate, S3A1-1 (a possible contaminant) The first phenon contained all but one of the *Artemia* strains and eight larval samples (S3L2-1, S3L2-2, S3L2-4, S3L2-8, S3L2-9, S3L2-10, S3L2-11, S3L2-8). There were 3 instances where larval strains had the same resistance profile as *Artemia* isolates, S3L2-11, S3A2-9, S3A2-10 and S3A2-14; S3L2-8 and S3A2-11; S3L2-12, S3A2-17, and S3A2-18 respectively. The second phenon contained only one *Artemia* isolate, S3A2-20, the remainder being from larvae.

The Biolog GN dendrogram (Fig 3.6) divided the *Artemia* and larval isolates into two major phenons, the first of which contained all *Artemia* isolates and only 1 larval isolate, S3L2-4. This "*Artemia*" phenon contained 3 isolates identified as *V. alginolyticus*. Although the *V. alginolyticus* type strain ATCC 1339 is slightly distant from the *Artemia* group in this dendrogram, the Biolog GN profile of this strain had been recorded using the Biolog recorder rather than visually. The lower phenon consists of the samples isolated from larvae and contains isolates identified as *V. splendidus* biovar 1 and biovar 2; it also contains the type strain *V. splendidus* ATCC 33125. This pattern is the same as for bacteria isolated from the Merexo *Artemia* and larvae samples even though the sample size was twice as large.

4.4 General comparison of the characteristics of bacteria isolated from the Merexo and Quilmas hatcheries

When comparing the Biolog GN profiles of all samples together (Fig. 3.10), all but one of the *Artemia* samples, S3A2-22, fell into the *V. alginolyticus* phenon (67 isolates) with 31% of these bacteria isolated from larvae, the majority of which were from the S2 batch of Quilmas larvae. The identity and virulence of S3A2-22 is very important as it lies within the *V. splendidus* phenon in Figure 3.10 and is very close to the *V. alginolyticus* type strain ATCC1339 in Figure 3.6. Both the larval isolates from Merexo and the second set from Quilmas were dominated by *V. splendidus*-type organisms whereas the bacteria isolated from

the first Quilmas batch larvae were all *V. alginolyticus*-type. With a total sample size of 63 from larvae, 42 are *V. splendidus*-type bacteria and 21 were *V. alginolyticus*-type. The total number of *Artemia* isolates is 46 of which only 2% were *V. splendidus*-type and the rest *V. alginolyticus*-type. This suggests that the major haemolytic flora from the *Artemia* is *V. alginolyticus* and the major haemolytic flora of the larvae is *V. splendidus*. However the sample size from *Artemia* is 27% less than the larval sample size. Although the sample size is quite small these results are similar to those of Blanch *et al.* (1997) who found no *V. splendidus* but a high percentage of *V. alginolyticus* in *Artemia* (6 samples, with 200-300 *Artemia* per sample) with the larval flora having both *V. alginolyticus* and *V. splendidus*, although the concentration of *V. alginolyticus* was greater than that of V. splendidus. Had the sample size been larger then there would have been possibility of identifying V. alginolyticus strains present in the larvae

Analysis of the Merexo and Quilmas rotifer bacterial flora might indicate the origin of the larval flora as Munro *et al.* (1994) and Blanch *et al.* (1997) both found *V. alginolyticus* to be present in significant numbers in both rotifers and turbot larvae. It may be that *V. splendidus* is adept at colonizing the gut of turbot larvae but not *Artemia*, and that the organism originates from rotifers. It should be noted that all isolates picked from the original plates, with the exception of the first batch of Quilmas *Artemia* and LMS isolates, were haemolytic.

Although all the S2 Quilmas larva isolates were *V. alginolyticus* the information from this part of the study indicates that the bacterial flora of the gut of these farmed turbot larvae does not, on the whole, originate from the feed at this stage of the feeding process. This partially agrees with findings of Campbell and Buswell (1983), for Dover sole, and Nicolas *et al.* (1989), for turbot. Without samples from the water and rotifers it is impossible to postulate the exact origin of the *V. splendidus*.

4.5 Analysis of the characterisation of bacteria isolated from dead (D) and live (L) turbot larvae

Although no antibiotics were used in the rearing of these larvae the bacteria did not differ significantly in antibiotic resistance profiles from the original bacterial isolates from Merexo larvae. The only isolates which differed significantly from previous isolates were the LMS group which were not haemolytic. Although the bacterial load of the dead larvae was five fold greater than that for live larvae; this in itself may not be significant as previous studies with younger larvae indicate little if any correlation between bacterial concentration in the larval gut and larval survival (Munro *et al.*, 1993). It is probable that the types of bacteria present have more influence in determining larval survival (*Munro et al.*, 1993, 1995). No overtly sucrose-positive colonies were isolated from the '**L**' TCBS plates, but LMS isolates from MA and LTH group were later shown to be sucrose positive. The antibiotic resistance profile (Fig. 3.7) did not split the '**L**' and '**D**' isolates into different groups and the majority of bacteria in these groups had similar antibiotic resistance profiles. The Biolog GN analysis (Fig. 3.8) grouped the isolates together, except for the LMS samples, with bacteria identified as *V. splendidus*, the type strain *V. splendidus* ATCC 33125, and distant from isolates identified as *V. alginolyticus*. The phenon was split into two smaller groups with the DMC, DTC, DTR, and LTS strains close to, or identified as *V. splendidus* biovar 1 with the DTY and LTH strains clustering close to isolates identified as *V. splendidus* biovar 2. The four LMS isolates were not haemolytic and differed significantly from the Biolog patterns of other *V. splendidus* isolates (both biovar 1 and biovar 2) even though 2 out of the 4 isolates were identified as *V. splendidus* biovar 1 by partial DNA sequence of their 16S rRNA genes.

4.6 Analysis of the characterisation of the HN isolates

The concentration of bacteria in these larval samples was significantly greater than in previous samples. Three of the HNT samples, HNT-1, HNT-8 and HNT-9, were not haemolytic and HNT-9 was also brown pigmented on MA with no growth on TCBS. Four of the HNF samples were not haemolytic and all of these did not grow on TCBS, with HNF-1 and HNF-4 producing a brown pigment on MA.

The Biolog GN dendrogram (Fig. 3.9) showed one main phenon comprised of 9 isolates, containing the type strain *V. splendidus* ATCC 33125 and isolates identified as *V. splendidus* biovar 1 and biovar 2. Four other isolates were not included in this phenon. These were HNF-2, HNF-7, HNT-2, and HNT-8. HNF-2 has been given the primary designation (due to a low Blast score) of *Marinomonas vega* (previous not identified in turbot larvae), HNT-2 has been identified as a *Vibrio tubiashii*-type organism by partial DNA sequencing of its 16S rRNA gene whilst it did not prove possible to PCR amplify the 16S rRNA gene of strain HNT-8. As the brown pigmented strains HNT-9, HNF-1 and HNF-4 showed no reaction on Biolog GN HNF-1 was analysed by partial DNA sequencing of the 16S rRNA gene.

The closest match was for *Roseobacter gallaeciensis*, an organism not previously reported in turbot larvae but isolated from larval cultures and collectors of the scallop, *Pecten maximus*, (Ruiz-Pointe *et al.*, 1998). It should be noted that *V. tubiashii* is a well recognised pathogen of bivalve larvae (Tubiash *et al.*, 1965; Hada *et al.*, 1989), and also that *R. gallaeciensis* produces substrates that inhibit bacterial pathogens of the scallop *in vitro*, with possible beneficial effect in larval rearing (Ruiz-Pointe *et al.*, 1999). The three strains HNT-9, HNF-1, HNF-4 had the same colony morphology, were Gram negative, oxidase and catalase positive, except for HNT-9 which was catalase negative.

4.7 The use of RFLP of 16S rRNA genes for identification and analysis of genetic variation among species

The RFLP method of Urakawa *et al.* (1997) has been used for tentative identification of species of bacteria based on the pattern of digestion with their 16S rRNA genes with 5 restriction enzymes. Although originally designed to identify bacteria belong to the family Vibrionaceae the method has been used to identify strains of other bacterial families. Here, isolate HNF-2 was identified as being closely related to *Marinomonas vega* from the DNA sequence of part of its 16S rRNA gene. By RFLP analysis (Fig. 3.13) of the 16S rRNA gene it was possible to identify HNF-7, which had a similar Biolog profile (Fig. 3.10), to the same type of organism as HNF-2.

RFLP analysis also showed that strains with the same Biolog profile, S3L1-8 (identified as *V. splendidus* biovar 1 by partial 16S rRNA gene DNA sequencing) and S3L1-13, can have different restriction digest patterns (Table 8) and that strains which have vastly different Biolog profiles appear to be of the same species. For example, this shows that strains such as LMS-3 and DMC-1 with a similar genotype, can vary greatly in phenotype. The results also show that *V. splendidus* biovar 1 could be distinguished not only by partial 16S rRNA gene sequence determination but also by RFLP analysis of the whole 16S rRNA gene. It also appeared that there may be at least three subclasses, based on 16S RFLP patterns, of *V. splendidus* biovar 1.

4.8 Virulence of bacteria isolated from *Artemia* and turbot larvae

From the results of individual experiments and statistical analysis of the pooled results, the bacterial strains DMC-1, LTS-3, HNF-8 were virulent towards turbot larvae. Bacterial isolate DTC-5 showed virulence towards larvae in initial experiments, however, in subsequent experiments the strain did not appear to be so virulent (although this apparent loss

was not investigated). The organisms used were from fresh overnight cultures grown from Protect beads so loss of virulence through repeated subculture was ruled out. Certain isolates from the **D** larvae, DTY-1, DTY-5, DTR-2, which were minor components of the gut microflora, showed no signs of virulence. However, strain DMC-1, which was representative of the dominant colony morphology on marine agar, was virulent when all challenges were considered (Table 10). Only four of the 17 HN strains were used to challenge turbot larvae; of these strains the two non-vibrios were avirulent, whereas the vibrio isolates, HNF-8, a *V. splendidus* biovar 1, was virulent and HNF-3, a *V. splendidus* biovar 2, possibly virulent. *V. splendidus* biovar 1 strain HNF-8 inflicted heavy mortalities.

Only one *V. alginolyticus* strain was tested, S3A2-11; not only was this non-pathogenic towards the turbot larvae but the percentage survival was greater than in the controls (Table 10). The bacterial isolates, which appeared to be virulent towards turbot larvae all belong to the *V. splendidus* biovars 1. Gatesoupe *et al.* (1999) recently showed that *V. splendidus* biovar 1 is a pathogen of larval turbot but How these bacteria kill the fish is not known although preliminary results using bacteria free culture supernatants concentrated on isolate DTC-5, for which virulence of the whole cell culture or washed cells were not proven. However, the dilution of the filtered culture supernatant would be very high so no definite conclusion can be reached from these experiments.

4.9 General conclusions of research

Vibrio alginolyticus and *V. splendidus* are common marine organisms and the former is well recognised as the dominant organism in the flora of *Artemia* (Olsen *et al.*, 2000). With the possible exception of isolate S3A2-22, *V. splendidus* was not found in any of the *Artemia* samples, whereas the majority of the larval isolates were *V. splendidus*. Only two samples from *Artemia*, S3A2-11 and S3L2-8, were tested for virulence against turbot larvae

and both were non-pathogenic. This does not mean that all of the *V. alginolyticus* strains isolated are non-pathogenic as this is a recognised pathogen of gilt-head sea bream larvae (*Sparus aurata* L.) (Balebona *et al.*, 1998; Colorni *et al.*, 1981; Paperna, 1984). The dominant flora of these larvae were *V. splendidus*, the majority of which were biovar 1. Strains of biovar 1 isolated from larvae were pathogenic. The virulence mechanisms of organisms of this biovar requires further research to determine the difference between the virulent isolates and the non-virulent isolates which are closely related in phenotype. Not all of the *V. splendidus* strains were virulent, as found by Gatesoupe *et al.* (1999). The partial DNA sequence of the DMC-1 16S rRNA gene is very similar to that of the *V. splendidus* isolate described by Gatesoupe *et al.* (1999) from diseased turbot larvae, although the 16S rRNA gene sequence and RFLP pattern cannot indicate that any particular strain is pathogenic. The Biolog GN profile also does not correlate with a strain's virulence, as LTS-4 (non-pathogenic) has a profile very close to that of LTS-3 (pathogenic). The Biolog results of these *V. splendidus* strains does give any particular insight into the virulence mechanisms of the strains as no pattern emerged for pathogenic and non-pathogenic strains. Gatesoupe *et al.* (1999) found that all of the virulent strains were positive for the enzyme valine arylamidase and the non-virulent strains were negative.

The use of partial DNA sequence of 16S rRNA genes was successful in identifying several strains and the majority of those identified in this way had high Blast scores, except for HNF-2. The partial DNA sequence for this particular 16S rRNA gene was of high quality but only matched the type strain *Marinomonas vega* ATCC 27119 in 614bp out of 652bp. It is quite possible that this is a new member of the *Marinomonas* family. HNF-7 has the same RFLP pattern as HNF-2 and is also a member of this family but little information was obtained or unavailable for these strains which have not previously associated with turbot larvae.

A major discovery in this study was the isolation of pathogenic *V. splendidus* bacteria from batches of healthy fish. The strain LTS-3 was highly virulent but was present in low numbers in comparison to those in the **'D'** larvae. The low levels of this organism may be due to the presence of other bacteria in the culture or it may be that these pathogenic strains originated from aerosols from adjacent **'D'** larvae tanks which experienced high mortalities.

5.0 Future work

With the identification of *Vibrio splendidus* as a pathogen of turbot larvae (*Scophthalmus maximus L.*) in this study and by Gatesoupe *et al.* (1999) further work into this organism is required. Determination of the origin of these strains within the hatchery is of great importance as it could indicate how better control measures could be applied. For this purpose it would be useful to develop immunological or nucleic acid probes, or other analytical methods to detect the organisms in rearing tanks and input materials, e.g. food, water and aerosols. The levels of aerosol contamination within the hatchery should be investigated and possible spread of pathogenic *V. splendidus* strains from an infected tank to neighbouring tanks should be monitored such that the danger to other larvae can be determined.

The mode of action of the virulent strains is of great interest and it is not yet known whether they also pose a risk to juvenile and adult fish. If the mode of action is determined it could be the basis for more specific probes which would differentiate virulent from avirulent strains. The survival rate of turbot larvae in the wild is unknown but in aquaculture a consistent survival rate of 40% e.g. S1, S2, S3 would allow considerable expansion of the industry. The very high survival of **'L'** larvae (65%) is of interest given the presence of some pathogenic *V. splendidus* isolates and merits further study to determine whether the reasons were bacterial or due to genetic differences between 'D' and 'L' batches of larvae.

Research into selective breeding would possibly produce a more resistant line of brood stock fish.

A final outcome from this work is that identification of pathogens presumed to have a role in larval rearing success can provide specific targets for development of probiotic bacteria for use in larval rearing. Such probiotics provide a better long term strategy in larval rearing than reliance on antibiotic with their inherent difficulties.

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Appendices

APPENDIX I

Bacterial growth media

Biolog Inoculation Medium

Carageenan (Sigma)	1.5g
NaCl (Fisher Chemicals)	25g
$MgCl_2$.$6H_2O$(AnalaR)	8g
KCl (AnalaR)	0.5g
dH_20	1000ml

Medium was autoclaved at 121°C for 15 min and distributed as required

Luria-Bertani broth + Kanamycin

Tryptone (Oxoid)	10g
Bacto-yeast extract (Difco)	5g
NaCl (Fisher chemicals)	10g
dH_20	1000ml
Kanamycin (Sigma)	1ml of 50mg/ml stock solution

Medium was shaken until dissolved and autoclaved at 121°C for 15min. The Kanamycin was added prior to use.

Luria-Bertani Agar + Kanamycin

This uses the same protocol as Luria-Bertani broth + Kanamycin except that 15g of technical agar No.3 (Oxoid) was added and kanamycin was added prior to pouring into Petri dishes.

Marine Agar

Marine Broth (Difco)	37g
Technical Agar No.3 (Oxoid)	15g
dH_20	1000ml

The medium was autoclaved at 121°C for 15min, cooled to 57°C and 20ml poured into Petri dishes. The medium was stored at 4°C.

Marine Blood Agar

This follows the preparation for MA except that, before pouring, 25ml of defibrinated sheep blood was carefully added per litre and mixed.

Marine Broth:

Marine broth (Difco)	37.4g
dH_20	1000ml

The medium was autoclaved at 121°C for 15min, then distributed as required.

F/2 Medium (Guillard and Ryther, 1962.)

$NaNO_3$	0.075g
$NaH_2PO_4.2H_2O$	0.00565g
Trace elements stock solution (1)	1ml
Vitamin mix stock solution (2)	1ml

Made up to 1000ml with filtered sea water and pH adjusted to 8.0 with 1NaOH or 1M HCl. The solution was autoclaved at 121°C for 15min.

Stock solution (1) Trace elements

EDTA (disodium salt)	4.360g
$FeCl_3.6H_2O$ (Fisher)	3.150g
$CuSO_4.5H_2O$	0.010g
$ZnSO_4.7H_2O$	0.022g
$CoCl_2.4H_2O$ (Fisher)	0.010g
$MnCl_2.4H_2O$ (Fisher)	0.180g
$Na_2MoO_4.2H_2O$	0.006g

Stock solution (2) Vitamin mix

Cyanocobalamin (Vitamin B12)	0.0005g
Thiamine HCl (vitamin B1)	0.1000g
Biotin	0.0005g

Sterile or autoclaved seawater

A container of appropriate size was filled with seawater and was autoclaved at 121°C for 15min.

Appendix 2

Basic Biochemical Tests

Test for Haemolysis

Single colonies of bacteria were picked from MA plates and streaked onto MA plates containing 2.5% defibrinated sheep blood. The plates were incubated overnight at 20°C. Haemolysis was indicated by a clear zone around bacteria colonies.

Kovac's oxidase test

For making 10ml solution

0.1g tetramethly –p- phenyldiamine dihydrochloride

0.01g ascorbic acid

The solution was made up to 10ml in sterile dH_2O and used within 12 h. However, it was possible to prepare a number of universals with the required amounts of dry reagents in advance; 10mls of sterile dH_2O was then added to a universal when required and these were stored in the dark.

Oxidase test

A piece of filter paper was moistened with sterile dH_2O and inoculated with fresh growth of the organism to be tested from a MA plate using a glass or wood applicator. Nichrome wire should not be used as there is a possible risk of false positives. A drop of the oxidase reagent was then added. A deep purple colouration almost immediately indicated a positive reaction. Positive (e.g. *Vibrio anguillarum*) and negative (e.g. *E. coli*) controls were included.

Catalase Activity

A single colony was picked from an MA plate (overnight culture) and smeared onto a filter paper moistened with sterile water. One drop of 30% (w/w) hydrogen peroxide solution was placed onto the smear. Evolution of bubbles of oxygen indicate a positive result.

Growth on TCBS

TCBS (Oxoid) agar plates were prepared according to the manufacturer's instructions (Note: the use of a microwave to dissolve the agar in dH_2O is not advisable as it may not dissolve properly and the plates will not set). Fresh MA cultures of the test organism were spotted on to the prepared plates and incubated at 20°C for up to 48 h. Growth after this time indicated a positive result. A change in colour of the agar from green to yellow (the colony can change colour too) indicates the organism can utilise sucrose.

Appendix 3

Antibiotic resistance and TCBS results of haemolytic bacteria isolated from Merexo and Quilmas

Isolate	Antibiotic, and micrograms/ units per disc																															
	C 25	E 5	FC 10	MT 10	NO 5	PG 1u	S 10	T 25	AP 10	KF 5	CO 25	GM 10	S 10	ST 200	T 25	TS 25	AP 25	NI 50	TC 75	T 100	NA 30	TM 2.5	SMX 50	GM	TCBS	YELLOW
S1A1-1	0	0	1	0	0	0	0	1	1	1	0	0	0	1	1	0	1	0	1	1	0	1	1	0	1	1
S1A1-2	0	0	1	1	0	1	0	1	1	1	0	0	0	0	1	0	1	0	1	1	0	1	1	0	0	0
S1A1-3	1	0	1	1	0	1	0	1	1	1	0	0	1	1	1	0	1	0	1	1	1	1	0	0	1	1
S1A1-4	0	1	1	1	0	1	0	1	1	0	0	0	0	1	1	0	1	0	1	1	1	1	1	0	1	1
S1A1-5	0	1	1	1	0	1	0	1	1	0	0	0	0	1	1	0	1	0	1	1	1	1	1	0	1	1
S1A2-6	0	1	1	1	0	1	0	1	1	1	0	0	0	1	1	0	1	0	1	1	0	1	1	0	1	1
S1A1-7	0	1	1	1	0	1	0	1	1	1	0	0	0	1	1	0	1	1	1	1	0	1	1	0	1	1
S1A1-8	0	1	1	1	0	1	0	1	1	1	0	0	0	1	1	0	1	0	1	1	1	1	1	0	1	1
S1A1-9	0	1	1	1	0	1	0	1	1	0	0	0	0	1	1	0	1	0	1	1	1	1	1	0	1	1
S1A1-10	1	1	1	1	1	1	0	1	1	1	0	0	0	1	1	0	1	0	1	1	1	1	1	0	1	1
S1A1-11	0	1	1	1	1	1	0	1	1	1	0	0	0	1	1	0	1	0	1	1	1	1	1	0	1	1
S1A2-1	0	1	1	1	0	1	0	1	1	1	0	0	0	0	1	0	1	0	1	1	0	1	1	0	1	1
S1A2-2	0	1	1	1	0	1	0	1	1	1	0	0	0	1	1	1	1	0	1	1	0	1	0	0	1	1
S1A2-3	0	1	1	1	0	1	0	1	1	0	0	0	0	1	1	0	1	0	1	1	0	1	1	0	1	1
S1A2-4	0	1	1	1	0	1	0	1	1	1	0	0	0	1	1	0	1	0	1	1	0	1	1	0	1	1
S1A2-5	0	1	1	1	0	1	0	1	1	0	0	0	0	0	1	0	1	0	1	1	0	1	0	0	1	1
S1L1-1	0	1	1	1	0	1	0	1	1	1	0	0	0	1	1	1	1	0	1	0	0	1	1	0	1	1
S1L1-2	0	1	1	1	0	1	1	1	1	0	0	0	1	1	1	1	1	0	1	1	0	1	1	0	1	0
S1L1-3	0	1	1	1	0	1	1	1	1	0	0	0	1	1	1	1	1	0	1	1	0	1	1	0	1	0
S1L1-4	0	1	1	1	1	1	1	1	1	0	0	0	1	1	1	1	1	0	1	1	0	1	1	0	1	0
S1L1-5	0	1	1	1	0	1	1	1	1	0	0	0	1	1	1	1	1	0	1	1	0	1	1	0	1	0
S1L1-6	0	1	1	1	0	1	1	1	1	1	0	0	1	1	1	1	1	0	1	1	0	1	1	0	1	0
S1L1-7	0	1	1	1	1	1	1	1	1	0	0	0	1	1	1	1	1	0	1	1	0	1	1	0	1	0
S1L2-1	1	1	1	1	1	1	1	1	1	1	0	0	1	1	1	1	0	0	0	1	0	1	1	0	0	0
S1L2-2	0	1	1	1	0	1	0	1	1	0	0	0	0	0	1	1	1	0	1	1	0	1	1	0	1	1
S1L2-3	1	1	1	1	0	1	0	1	1	1	0	0	0	1	1	1	1	0	1	1	0	1	1	0	1	1
S1L2-4	1	1	1	1	0	1	1	1	1	0	0	1	1	1	1	1	1	0	1	1	0	1	1	1	0	0
S2L1-1	0	1	1	1	1	1	0	1	1	1	0	0	1	0	1	0	1	0	1	1	0	0	1	0	1	1
S2L1-2	0	1	1	1	0	1	0	1	1	1	0	0	0	1	1	0	1	0	1	1	0	1	0	0	1	1
S2L1-3	0	1	1	1	0	1	0	1	1	1	0	0	0	1	1	0	1	0	1	1	0	1	0	0	1	1
S2L1-4	0	1	1	1	0	1	0	1	1	1	0	0	0	0	1	0	1	0	1	1	0	1	1	0	1	1
S2L1-5	0	1	1	1	0	1	0	1	1	1	0	0	0	0	1	0	1	0	1	1	0	1	0	0	1	1
S2L1-6	0	1	1	1	1	1	0	1	1	1	0	0	0	1	1	0	1	0	1	1	0	1	0	0	1	1
S2L1-7	0	1	1	1	1	1	0	1	1	1	0	0	0	0	1	0	1	0	1	1	0	1	0	0	1	1
S2L1-8	0	1	1	1	1	1	0	1	1	1	0	0	0	1	1	0	1	0	1	1	0	1	0	0	1	1
S2L2-1	0	1	1	1	1	1	0	1	1	1	0	0	0	0	1	0	1	0	1	1	0	0	0	0	1	1
S2L2-2	0	1	1	1	1	1	0	1	1	1	0	0	0	0	1	0	1	0	1	1	0	1	0	0	1	1
S2L2-3	0	1	1	1	0	1	0	1	1	1	0	0	0	1	1	0	1	0	1	0	0	1	0	0	1	1
S2L2-4	0	1	1	1	1	1	0	1	1	1	0	0	0	1	1	0	1	0	1	1	0	1	0	0	1	1
S2L2-5	0	1	1	1	0	1	1	1	1	1	0	0	1	1	1	0	1	0	1	1	1	1	1	0	0	0
S2L2-6	0	1	1	1	0	1	0	1	1	1	0	0	0	0	1	0	1	0	1	1	0	1	1	0	1	1
S2L2-7	0	1	1	1	1	1	0	1	1	1	0	0	0	0	1	0	1	0	1	1	0	1	0	0	1	1
S2L2-8	0	1	1	1	0	1	0	1	1	1	0	0	0	1	1	0	1	0	1	1	0	1	1	0	1	1

Isolate	Antibiotic, and micrograms/ units per disc																														
	C 25	E 5	FC 10	MT 10	NO 5	PG 1u	S 10	T 25	AP 10	KF 5	CO 25	GM 10	S 10	ST 200	T 25	TS 25	AP 25	NI 50	TC 75	T 100	NA 30	TM 2.5	SMX 50	GM	TCBS	YELLOW
S3A1-1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0
S3A2-1	0	1	1	1	0	1	0	1	1	0	0	0	0	1	1	1	1	0	1	1	1	1	1	0	1	1
S3A2-2	0	1	1	1	1	1	0	1	1	1	0	0	0	1	1	0	1	0	1	1	1	1	1	0	1	1
S3A2-3	0	1	1	1	0	1	0	1	1	0	0	0	0	1	1	0	1	0	1	1	1	1	1	0	1	1
S3A2-4	0	1	1	1	0	1	0	1	1	0	0	0	0	1	1	0	1	0	1	1	1	1	1	0	1	1
S3A2-5	0	1	1	1	0	1	0	0	1	0	0	0	0	1	1	0	1	0	1	1	1	1	0	0	1	1
S3A2-6	0	1	1	1	0	1	0	1	1	1	0	0	0	1	1	0	1	0	1	1	0	0	1	0	1	1
S3A2-7	0	1	1	1	0	1	0	1	1	0	0	0	0	1	1	0	1	0	1	1	0	1	1	0	1	1
S3A2-8	0	1	1	1	1	1	0	1	1	0	0	0	0	1	1	0	1	0	1	1	0	1	1	0	1	1
S3A2-9	0	1	1	1	0	1	0	1	1	1	0	0	0	1	1	0	1	0	1	1	1	1	1	0	1	1
S3A2-10	0	1	1	1	0	1	0	1	1	1	0	0	0	1	1	0	1	0	1	1	1	1	1	0	1	1
S3A2-11	0	1	1	1	0	1	0	1	1	1	0	0	0	1	1	0	1	0	1	1	0	1	1	0	1	1
S3A2-12	0	1	1	1	0	1	0	1	1	1	0	0	0	1	1	0	1	0	1	0	0	1	1	0	1	1
S3A2-13	0	1	1	1	0	1	0	1	1	1	0	0	0	1	1	1	1	1	1	1	1	1	1	0	1	1
S3A2-14	0	1	1	1	0	1	0	1	1	1	0	0	0	1	1	0	1	0	1	1	1	1	1	0	1	1
S3A2-15	0	1	1	1	0	1	0	1	1	0	0	0	0	1	1	1	1	0	1	1	1	0	1	0	1	1
S3A2-16	0	1	1	1	1	1	0	0	1	1	0	0	0	1	1	0	1	0	1	1	0	1	1	0	1	1
S3A2-17	0	1	1	1	0	1	0	1	1	1	0	0	0	1	1	1	1	0	1	1	1	1	1	0	1	1
S3A2-18	0	1	1	1	0	1	0	1	1	1	0	0	0	1	1	1	1	0	1	1	1	1	1	0	1	1
S3A2-19	0	1	1	1	0	1	0	1	1	1	0	0	0	1	1	1	1	1	1	1	1	1	1	1	1	1
S3A2-20	1	1	1	1	0	1	1	1	1	1	0	0	1	1	1	1	1	1	1	1	0	1	1	0	1	1
S3A2-21	0	1	1	1	0	1	0	1	1	1	0	0	0	1	1	1	1	1	1	1	1	1	1	1	1	1
S3A2-22	1	1	1	1	0	1	1	1	1	1	0	0	1	1	1	1	1	1	1	1	0	1	1	0	1	0
S3L1-1	0	1	1	1	0	1	1	1	1	1	0	0	1	0	1	0	1	0	1	1	0	0	1	0	1	1
S3L1-2	0	1	1	1	0	1	1	1	1	1	0	0	1	0	1	1	1	0	1	0	0	0	1	0	1	0
S3L1-3	0	1	1	1	0	1	1	1	1	1	0	0	1	0	1	1	1	0	1	0	0	0	1	0	1	0
S3L1-4	0	1	1	1	0	1	1	1	1	1	0	0	1	1	1	0	1	0	1	1	0	0	1	0	1	1
S3L1-5	0	1	1	1	0	1	1	1	1	0	0	0	1	0	1	0	1	0	1	1	0	0	1	0	1	1
S3L1-6	0	1	1	1	0	1	1	1	1	1	0	0	1	0	1	0	1	0	1	1	0	0	1	0	1	0
S3L1-7	0	1	1	1	0	1	1	1	1	1	0	0	1	1	1	1	1	0	1	1	0	1	1	0	1	1
S3L1-8	0	1	1	1	0	1	1	1	1	1	0	0	1	1	1	0	1	0	1	1	0	0	1	0	1	1
S3L1-9	0	1	1	1	0	1	1	1	1	1	0	0	1	0	1	0	1	0	1	0	1	0	1	0	1	0
S3L1-10	0	1	1	1	0	1	1	1	1	1	0	0	1	0	1	0	1	0	1	0	0	0	1	0	1	1
S3L1-11	0	1	1	1	1	1	1	1	1	1	0	0	1	1	1	0	1	0	1	1	0	0	1	0	1	1
S3L1-12	0	1	1	1	0	1	1	1	1	1	0	0	1	1	1	1	1	0	1	1	0	1	1	0	1	1
S3L1-13	0	1	1	1	1	1	1	1	1	1	0	0	1	1	1	1	1	0	1	1	0	1	1	0	1	0
S3L1-14	0	1	1	1	0	1	1	1	1	1	0	1	1	1	1	1	1	0	1	1	0	1	1	1	0	0
S3L1-15	0	1	1	1	0	1	1	1	1	1	0	0	1	1	1	1	1	0	1	1	0	1	1	0	1	1
S3L1-16	0	1	1	1	0	1	1	1	1	0	0	0	1	1	1	0	1	0	1	1	0	0	1	0	1	0
S3L1-17	0	1	1	1	1	1	1	1	1	0	0	0	1	1	1	0	1	0	1	1	0	0	1	0	1	0
S3L1-18	0	1	1	1	0	1	1	1	1	1	0	0	1	1	1	0	1	0	1	1	0	0	1	0	1	0
S3L1-19	0	1	1	1	0	1	1	1	1	1	0	0	1	1	1	1	1	0	1	1	0	1	1	0	1	1
S3L1-20	0	1	1	1	0	1	1	1	1	1	0	0	1	1	1	1	1	0	1	1	0	1	1	0	1	1
S3L1-21	0	1	1	1	0	1	1	1	1	1	0	0	1	1	1	1	1	0	1	1	0	1	1	0	1	1
S3L1-22	0	1	1	1	0	1	1	1	1	0	0	0	1	1	1	0	1	0	1	1	0	0	1	0	1	0
S3L1-23	0	1	1	1	0	1	1	1	1	1	0	0	1	1	1	1	1	0	1	1	0	1	1	0	1	1
S3L1-24	0	1	1	1	0	1	1	1	1	0	0	0	1	1	1	0	1	0	1	1	0	0	1	0	1	0
S3L1-25	0	1	1	1	0	1	1	1	1	0	0	0	1	1	1	0	1	0	1	1	0	0	1	0	1	0
S3L1-26	0	1	1	1	0	1	1	1	1	0	0	0	1	1	1	0	1	0	1	1	0	0	1	0	1	0

Isolate	Antibiotic, and micrograms/ units per disc																														
	C 25	E 5	FC 10	MT 10	NO 5	PG 1u	S 10	T 25	AP 10	KF 5	CO 25	GM 10	S 10	ST 200	T 25	TS 25	AP 25	NI 50	TC 75	T 100	NA 30	TM 2.5	SMX 50	GM	TCBS	YELLOW
S3L2-1	0	1	1	1	0	1	0	1	1	1	0	0	0	1	1	0	1	0	1	1	0	1	1	0	1	1
S3L2-2	0	1	1	1	0	1	0	1	1	0	0	0	0	1	1	1	1	0	1	1	0	1	1	0	1	1
S3L2-3	0	1	1	1	0	1	1	0	1	0	0	0	1	1	1	0	1	0	1	1	1	0	1	0	1	0
S3L2-4	0	1	1	1	1	1	0	1	1	1	0	0	0	1	1	0	1	0	1	1	1	0	1	0	1	1
S3L2-5	0	1	1	1	0	1	1	1	1	1	0	0	1	1	1	1	1	0	1	1	1	1	1	1	1	1
S3L2-6	0	1	1	1	1	1	1	1	1	0	0	0	1	1	1	0	1	0	1	1	0	0	1	0	1	0
S3L2-7	0	1	1	1	0	1	1	1	1	0	0	0	1	1	1	1	1	0	1	1	1	1	1	0	1	1
S3L2-8	0	1	1	1	0	1	0	1	1	1	0	0	0	1	1	0	1	0	1	1	0	1	1	0	1	1
S3L2-9	0	1	1	1	0	1	1	1	1	1	0	0	0	1	1	0	1	0	1	1	1	0	1	0	1	0
S3L2-10	0	0	1	1	0	1	0	1	1	1	0	0	0	1	1	0	1	0	1	1	0	1	1	0	1	1
S3L2-11	0	1	1	1	0	1	0	1	1	1	0	0	0	1	1	0	1	0	1	1	1	1	1	0	1	1
S3L2-12	0	1	1	1	0	1	0	1	1	1	0	0	0	1	1	1	1	0	1	1	1	1	1	0	1	1
DMC-1	0	1	1	1	0	1	1	1	1	1	0	0	1	1	1	0	1	0	1	1	1	0	1	0	1	0
DMC-2	0	1	1	1	0	1	1	1	1	1	0	0	1	1	1	0	1	0	1	1	1	1	1	0	1	0
DMC-3	0	1	1	1	0	1	1	1	1	1	0	0	1	1	1	0	1	0	1	1	1	1	1	0	1	0
DMC-4	0	1	1	1	0	1	1	1	1	1	0	0	1	1	1	0	1	0	1	1	1	1	1	0	1	0
DMC-5	0	1	1	1	0	1	1	1	1	1	0	0	1	1	1	0	1	0	1	1	0	1	1	0	1	0
DMC-6	0	1	1	1	0	1	1	1	1	1	0	0	1	1	1	0	1	0	1	1	0	1	1	0	1	0
DMC-7	0	1	1	1	0	1	1	1	1	1	0	0	1	1	1	0	1	0	1	1	0	1	1	0	1	0
DTR-1	0	1	1	1	1	1	1	1	1	1	0	0	1	0	1	0	1	0	1	1	1	1	1	0	1	0
DTR-2	0	1	1	1	1	1	1	1	1	1	0	0	1	0	1	0	1	0	1	1	1	0	1	0	1	0
DTR-3	0	1	1	1	1	1	1	1	1	1	0	0	1	0	1	0	1	0	1	1	1	0	1	0	1	0
DTR-4	0	1	1	1	1	1	1	1	1	1	0	0	1	0	1	0	1	0	1	1	1	1	1	0	1	0
DTY-1	0	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	0	1	1	1	1	1
DTY-2	0	1	1	1	0	1	1	1	1	1	0	1	1	1	1	1	1	0	1	1	0	1	1	1	1	1
DTY-3	0	1	1	1	0	1	1	1	1	1	0	1	1	1	1	1	1	0	1	1	0	1	1	1	1	1
DTY-4	0	1	1	1	0	1	1	1	1	1	0	1	1	1	1	1	1	0	1	1	0	1	1	1	1	1
DTY-5	0	1	1	1	0	1	1	1	1	1	0	1	1	1	1	1	1	0	1	1	0	1	1	1	1	1
DTC-1	0	1	1	1	0	1	1	1	1	1	0	0	1	1	1	1	1	0	1	1	1	1	1	0	1	0
DTC-2	0	1	1	1	0	1	1	1	1	1	0	0	1	1	1	1	1	0	1	1	0	1	1	0	1	0
DTC-3	0	1	1	1	0	1	1	1	1	1	0	1	1	1	1	1	1	0	1	1	1	1	1	1	1	0
DTC-4	0	1	1	1	0	1	1	1	1	1	0	0	1	1	1	1	1	0	1	1	1	1	1	0	1	0
DTC-5	0	1	1	1	0	1	1	1	1	1	0	0	1	1	1	1	1	0	1	1	0	1	1	0	1	0

Isolate	Antibiotic, and micrograms/ units per disc																																	
	C	25	E	5	FC	10	MT	10	NO	5	PG	1u	S	10	T	25	AP	10	KF	5	CO	25	GM	10	S	10	ST	200	T	25	TS	25	AP	25	NI	50	TC	75	T	100	NA	30	TM	2.5	SMX	50	GM	TCBS	YELLOW
LMS-1	0	1	1	1	1	1	0	1	0	0	1	1	0	0	0	1	1	0	0	1	0	0	0	1	1	1	0	1	1	1	0	1	1													
LMS-2	0	1	1	1	0	1	0	0	1	1	0	0	0	1	0	0	1	0	0	1	0	0	1	0	1	0	1	1	1	0	1	1													
LMS-3	0	1	1	1	0	1	0	0	1	1	0	0	0	1	0	0	1	0	0	1	0	0	1	0	1	0	1	1	1	0	1	1													
LMS-4	0	1	1	1	0	1	0	0	1	1	0	0	0	1	0	0	1	0	0	1	0	0	1	0	1	0	0	1	1	0	1	1													
LTH-1	0	1	1	1	0	1	1	1	1	1	0	0	1	1	1	0	1	0	1	1	0	0	1	0	1	1	0	0	1	0	1	1													
LTH-2	0	1	1	1	0	1	1	1	1	1	0	0	1	1	1	0	1	0	1	1	0	0	1	0	1	1	0	0	1	0	1	1													
LTH-3	0	1	1	1	0	1	1	1	1	1	0	1	1	1	1	1	1	0	1	1	0	1	1	0	1	1	0	1	1	1	1	1													
LTH-4	0	1	1	1	0	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	0	1	1	0	1	1	0	1	1	1	1	1													
LTH-5	0	1	1	1	0	1	1	1	1	1	0	0	1	1	1	0	1	0	1	1	0	0	1	0	1	1	0	0	1	0	1	1													
LTS-1	0	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1	0	1	1	1	1	1	0	1	1	0	1	1	0	1	1													
LTS-2	0	1	1	1	1	1	1	1	1	1	0	0	1	1	1	1	1	0	1	1	0	1	1	0	1	1	0	1	1	0	1	0													
LTS-3	0	1	1	1	1	1	1	1	1	1	0	0	1	1	1	1	1	0	1	1	0	1	1	0	1	1	0	1	1	0	1	0													
LTS-4	0	1	1	1	0	1	1	1	1	1	0	1	1	1	1	0	1	0	1	1	1	1	1	0	1	1	1	1	1	1	1	0													
LTS-5	0	1	1	1	1	1	1	1	1	1	0	0	1	1	1	1	1	0	1	1	0	1	1	0	1	1	0	1	1	0	1	0													

Origin of strains

S1 = Merexo
S2 = Quilmas
S3 = Qulimas (2nd)
L1 = larvae sample 1
L2 = larvae sample 2
A1 = Artemia sample 1
A2 = Artemia sample 2

Antibiotic resistance
0 = sensitive
1 = resistant

Antibiotics and amounts (microgram/disc)

Codes for antibiotics and bichemical tests

AP : ampicillin
C : chloramphenicol
CO : colistin sulphate
E : erythromycin
FC : fusidic acid
GM : gentamicin
MT : methicillin
NA : naladixic acid
NI : nitofurantoin
NO : novobiocin
PG : penicillin G
S : streptomycin
SMX : sulphamethoxazole
ST : sulphatriad
T : tetracycline
TC : ticarcillin
TM : trimethoprim
TS : cotrimoxazol

TCBS = thiosulphate citrate bilesalts sucrose agar
YELLOW = Utlilsation of sucrose (yellow colony)

Appendix 4 Carbon utilization as recorded using BIOLOG GN microplates

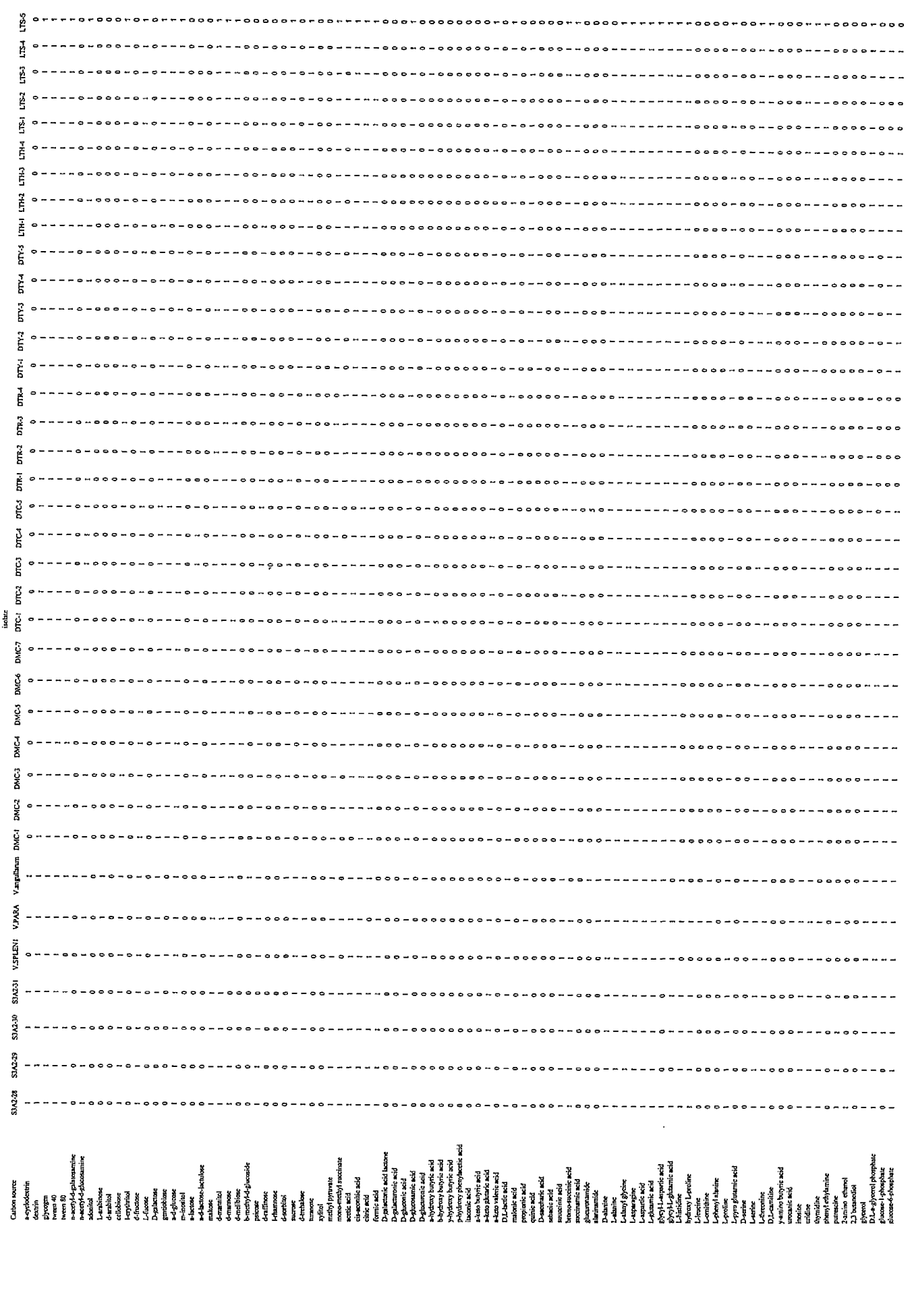
Isolate

Carbon Source	V.tapetis	91079	V.alginolyticus	S1A1-1	S1A1-2	S1A1-3	S1A1-4	S1A1-5	S1A1-6	S1A1-7	S1A1-8	S1A1-9	S1A1-10	S1A1-11	S1A2-1	S1A2-2	S1A2-3	S1A2-4	S1A2-5	S1L1-1	S1L1-2	S1L1-3	S1L1-4	S1L1-5	S1L1-6	S1L1-7	S1L2-2	S1L2-3	S1L2-4	S2L1-1	S2L1-2	S2L1-3	S2L1-4	S2L1-5	S2L1-6	S2L1-7	S2L1-8
a-cyclodextrin	1	0	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	0	0	0	0	1	1	0	1	1	1	1	1	1	1	1
dextrin	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
glycogen	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
tween 40	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
tween 80	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
n-acetyl-D-galatosamine	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
n-acetyl-D-glucosamine	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
adonitol	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
L-arabinose	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
D-arabitol	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
cellobiose	0	0	1	0	1	1	1	0	0	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
I-erythritol	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
D-fructose	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
L-fucose	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
D-galactose	0	1	0	1	1	0	0	0	1	1	0	0	1	0	0	0	0	0	0	0	1	1	1	1	1	1	0	0	1	0	0	0	0	0	0	0	0
gentiobiose	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	1	1	0	0	0	0	1	0	0	0	0	0	0	0	0
a-D-glucose	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
M-inositol	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
a-lactose	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0
a-D-lactose-lactulose	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
maltose	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
D-mannitol	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
D-mannose	1	1	1	0	1	0	0	0	0	0	0	0	1	0	0	0	0	0	0	1	1	1	1	1	1	1	0	0	1	0	0	0	0	0	0	0	0
D-melibiose	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
b-methyl-D-glucoside	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
psicose	0	0	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
D-raffinose	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
L-rhamnose	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
D-sorbitol	0	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
sucrose	0	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	0	0	0	0	1	1	0	1	1	1	1	1	1	1	1
D-trehalose	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
turanose	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
xylitol	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
methyl pyruvate	0	0	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	1	0	1	1	1	1	1	1	1	1	1	1	1	1
mono-methyl succinate	1	0	0	1	1	1	0	1	0	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1	0	1	1	1	1	1	1	1	1
acetic acid	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
cis-aconitic acid	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
citric acid	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
formic acid	0	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	0	0	0	0	1	1	0	1	1	1	1	1	1	1	1
D-galactonic acid lactone	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
D-galacturonic acid	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
D-gluconic acid	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	0	0	1	1	1	1	1	1	1	1	1	1	1	1	1
D-glucosamic acid	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
D-glucuronic acid	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0
a-hydroxy butyric acid	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
b-hydroxy butyric acid	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
y-hydroxy butyric acid	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
p-hydroxy phenylacetic acid	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
itaconic acid	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
a-keto butyric acid	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
a-keto glutaric acid	0	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
a-keto valeric acid	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
D,L-lactic acid	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
malonic acid	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
propionic acid	0	0	0	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
quinic acid	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
D-saccharic acid	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
sebacic acid	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
succinic acid	0	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
bromo-succinic acid	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
succinamic acid	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
glucuronamide	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0
alaninamide	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
D-alanine	0	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
L-alanine	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
L-alanyl glycine	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
L-asparagine	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
L-aspartic acid	0	0	1	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	0	1	1	0	0	1	0	0	0	0
L-glutamic acid	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
glycyl-L-aspartic acid	0	0	1	0	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	1	0	1	1	0	1	1	1	1	0	0	0	1	1	0	0	0
glycyl-L-glutamic acid	0	0	0	0	0	1	0	0	1	0	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1	0
L-histidine	0	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
hydroxy L-proline	0	0	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	0	0	1	0	1	1	0	1	1	1	1	1	1	1	1
L-leucine	0	0	0	0	0	0	0	0	0	0	0	0	1	0	1	1	1	1	1	0	0	0	0	0	0	0	0	0	0	1	0	1	1	1	1	1	0
L-ornithine	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
L-phenyl alanine	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
L-proline	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
L-pyro glutamic acid	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
D-serine	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
L-serine	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
L-threonine	0	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
D,L-carnitine	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0
y-amino butyric acid	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
urocanic acid	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
inosine	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
uridine	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
thymidine	1	0	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
phenyl ethylamine	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
putrescine	0	0	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	0	0	1	0	1	1	0	1	1	1	1	1	1	1	1
2-amino ethanol	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	1	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0
2,3 butanediol	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
glycerol	0	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
D,L-a-glycerol phosphate	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	0	0	0	0	1	1	1	1	1	1	1	1	1	1	1
glucose-1-phosphate	0	1	1	1	0	1	0	1	1	1	0	1	0	1	0	1	1	1	1	0	0	0	0	0	0	0	1	1	1	1	1	0	1	1	0	1	1
glucose-6-phosphate	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	0	0	0	0	1	1	1	1	1	1	1	1	1	1	1

Carbon Source	S1L2-1	S1L2-2	S1L2-3	S1L2-4	S1L2-5	S1L2-6	S1L2-7	S1L2-8	S1L1-1	S1L1-2	S1L1-3	S1L1-4	S1L1-5	S1L1-6	S1L1-7	S1L1-8	S1L1-9	S1L1-10	S1L1-11	S1L1-12	S1L1-13	S1L1-14	S1L1-16	S1L1-17	S1L1-18	S1L1-19	S1L1-20	S1L1-21	S1L1-22	S1L1-23	S1L1-24	S1L1-25	S1L1-26	S1L2-1	S1L2-2	S1L2-3	S1L2-4
a-cyclodextrin	-	-	-	-	-	-	-	-	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	-
dextrin	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
glycogen	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
tween 40	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
tween 80	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
n-acetyl-d-galactosamine	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
n-acetyl-d-glucosamine	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
adonitol	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
L-arabinose	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
d-arabitol	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
cellobiose	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
i-erythritol	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
d-fructose	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
L-fucose	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
D-galactose	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
gentiobiose	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
a-d-glucose	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
m-inositol	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
a-lactose	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
a-d-lactose-lactulose	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
maltose	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
d-mannitol	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
d-mannose	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
b-methyl-d-glucoside	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
palatinose	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
d-raffinose	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
l-rhamnose	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
d-sorbitol	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
sucrose	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
d-trehalose	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
turanose	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
xylitol	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
methyl pyruvate	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
mono-methyl succinate	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
acetic acid	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
cis-aconitic acid	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
citric acid	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
formic acid	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
D-galactonic acid lactone	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
D-galacturonic acid	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
D-gluconic acid	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
D-glucosamic acid	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
D-glucuronic acid	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
a-hydroxy butyric acid	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
b-hydroxy butyric acid	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
y-hydroxy butyric acid	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
p-hydroxy phenylacetic acid	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
itaconic acid	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
a-keto butyric acid	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
a-keto glutaric acid	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
a-keto valeric acid	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
D,L-lactic acid	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
malonic acid	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
propionic acid	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
quinic acid	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
D-saccharic acid	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
sebacic acid	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
succinic acid	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
bromo-succinic acid	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
succinamic acid	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
glucuronamide	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
alaninamide	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
D-alanine	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
L-alanine	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
L-alanyl glycine	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
L-asparagine	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
L-aspartic acid	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
L-glutamic acid	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
glycyl-L-aspartic acid	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
glycyl-L-glutamic acid	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
L-histidine	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
hydroxy L-proline	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
L-leucine	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
L-ornithine	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
L-phenyl alanine	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
L-proline	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
L-pyro glutamic acid	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
D-serine	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
L-serine	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
L-threonine	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
D,L-carnitine	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
g-amino butyric acid	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
urocanic acid	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
inosine	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
uridine	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
thymidine	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
phenyl ethylamine	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
putrescine	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
2-amino ethanol	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
2,3 butanediol	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
glycerol	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
D,L-a-glycerol phosphate	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
glucose-1-phosphate	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
glucose-6-phosphate	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Isolate

Isolate
Carbon Source
S1L2-5
S1L2-6
S1L2-7
S1L2-9
S1L2-8
S1L2-10
S1L2-11
S1L2-12
S3A2-1
S3A2-2
S3A2-3
S3A2-4
S3A2-5
S3A2-6
S3A2-7
S3A2-8
S3A2-9
S3A2-10
S3A2-11
S3A2-12
S3A2-13
S3A2-14
S3A2-15
S3A2-16
S3A2-17
S3A2-18
S3A2-19
S3A2-20
S3A2-21
S3A2-22
S3A2-23
S3A2-24
S3A2-25
S3A2-26
S3A2-27
α-cyclodextrin
dextrin
glycogen
tween 40
tween 80
n-acetyl-d-galactosamine
n-acetyl-d-glucosamine
adonitol
l-arabinose
d-arabitol
cellobiose
i-erythritol
d-fructose
l-fucose
d-galactose
gentiobiose
α-d-glucose
m-inositol
α-d-lactose
lactulose-lactulose
maltose
d-mannitol
d-mannose
d-melibiose
b-methyl-d-glucoside
palcose
d-raffinose
l-rhamnose
d-sorbitol
sucrose
d-trehalose
turanose
xylitol
methyl pyruvate
mono-methyl succinate
acetic acid
cis-aconitic acid
citric acid
formic acid
D-galactonic acid lactone
D-galacturonic acid
D-gluconic acid
D-glucosaminic acid
D-glucuronic acid
α-hydroxy butyric acid
b-hydroxy butyric acid
γ-hydroxy butyric acid
p-hydroxy phenylacetic acid
itaconic acid
α-keto butyric acid
α-keto glutaric acid
α-keto valeric acid
D,L-lactic acid
malonic acid
propionic acid
quinic acid
D-saccharic acid
sebacic acid
succinic acid
bromo-succinic acid
succinamic acid
glucuronamide
alaninamide
D-alanine
L-alanine
L-alanyl glycine
L-asparagine
L-aspartic acid
L-glutamic acid
glycyl-L-aspartic acid
glycyl-L-glutamic acid
L-histidine
hydroxy L-proline
L-leucine
L-ornithine
L-phenyl alanine
L-proline
L-pyro glutamic acid
D-serine
L-serine
L-threonine
D,L-carnitine
γ-amino butyric acid
urocanic acid
inosine
uridine
thymidine
phenyl ethylamine
putrescine
2-amino ethanol
2,3-butanediol
glycerol
D,L-α-glycerol phosphate
α-d-glucose-1-phosphate
α-d-glucose-6-phosphate


Carbon source
SJA2-28
SJA2-29
SJA3-30
SJA3-31
V.SPLEN1
V.PARA
V.anguillarum
DMC-1
DMC-2
DMC-3
DMC-4
DMC-5
DMC-6
DMC-7
DTC-1
DTC-2
DTC-3
DTC-4
DTC-5
DTR-1
DTR-2
DTR-3
DTR-4
DTY-1
DTY-2
DTY-3
DTY-4
DTY-5
LTH-1
LTH-2
LTH-3
LTH-4
LTS-1
LTS-2
LTS-3
LTS-4
LTS-5
a-cyclodextrin
dextrin
glycogen
tween 40
tween 80
n-acetyl-d-galactosamine
n-acetyl-d-glucosamine
adonitol
l-arabinose
d-arabitol
cellobiose
i-erythritol
d-fructose
l-fucose
d-galactose
gentiobiose
a-d-glucose
m-inositol
a-lactose
b-d-lactose-lactulose
maltose
d-mannitol
d-mannose
d-melibiose
b-methyl-d-glucoside
psicose
d-raffinose
l-rhamnose
d-sorbitol
sucrose
d-trehalose
turanose
xylitol
methyl pyruvate
mono-methyl succinate
acetic acid
cis-aconitic acid
citric acid
formic acid
d-galactaric acid lactone
d-galacturonic acid
d-gluconic acid
d-glucosaminic acid
d-glucuronic acid
a-hydroxy butyric acid
b-hydroxy butyric acid
g-hydroxy butyric acid
p-hydroxy phenylacetic acid
itaconic acid
a-keto butyric acid
a-keto glutaric acid
a-keto valeric acid
D,L-lactic acid
malonic acid
propionic acid
quinic acid
d-saccharic acid
sebacic acid
succinic acid
bromo-succinic acid
succinamic acid
glucuronamide
alaninamide
D-alanine
L-alanine
L-alanyl glycine
L-asparagine
L-aspartic acid
L-glutamic acid
glycyl-L-aspartic acid
glycyl-L-glutamic acid
L-histidine
hydroxy L-proline
L-leucine
L-ornithine
L-phenyl alanine
L-proline
L-pyro glutamic acid
D-serine
L-serine
L-threonine
D,L-carnitine
γ-amino butyric acid
urocanic acid
inosine
uridine
thymidine
phenyl ethylamine
putrescine
2-amino ethanol
2,3 butanediol
glycerol
D,L-a-glycerol phosphate
glucose-1-phosphate
glucose-6-phosphate

Carbon source	LMS-1	LMS-2	LMS-3	LMS-4	HNF-2	HNT-3	HNF-5	HNF-6	HNF-7	HNF-8	HNT-1	HNT-2	HNT-3	HNT-4	HNT-5	HNT-6	HNT-7	HNT-8
a-cyclodextrin	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
dextrin	-	-	-	-	0	-	-	-	0	-	-	-	-	-	-	0	-	0
glycogen	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
tween 40	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
tween 80	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
n-acetyl-d-galactosamine	-	0	-	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
n-acetyl-d-glucosamine	-	0	-	0	-	0	0	0	0	0	0	0	0	0	0	0	0	0
adonitol	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
l-arabinose	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
d-arabitol	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
cellobiose	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
i-erythritol	-	-	-	-	0	-	-	-	0	-	-	-	-	-	-	0	-	0
d-fructose	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
l-fucose	-	0	-	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
D-galactose	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
gentiobiose	-	-	-	-	0	-	-	-	0	-	-	-	-	-	-	0	-	0
a-d-glucose	-	0	-	0	-	0	0	0	0	0	0	0	0	0	0	0	0	0
m-d-glucose	-	0	-	0	-	0	0	0	0	0	0	0	0	0	0	0	0	0
m-inositol	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
a-d-lactose-lactulose	-	0	-	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
maltose	-	-	-	-	0	-	-	-	0	-	-	-	-	-	-	0	-	0
d-mannitol	-	-	-	-	0	-	-	-	0	-	-	-	-	-	-	0	-	0
d-mannose	-	-	-	-	0	-	-	-	0	-	-	-	-	-	-	0	-	0
d-melibiose	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
b-methyl-d-glucoside	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
psicose	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
d-raffinose	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
l-rhamnose	0	-	0	-	0	0	0	0	0	0	0	0	0	0	0	0	0	0
d-sorbitol	-	-	-	-	0	-	-	-	0	-	-	-	-	-	-	0	-	0
sucrose	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
d-trehalose	-	-	-	-	0	-	-	-	0	-	-	-	-	-	-	0	-	0
turanose	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
xylitol	-	-	-	-	0	-	-	-	0	-	-	-	-	-	-	0	-	0
methyl pyruvate	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
mono-methyl succinate	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
acetic acid	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
cis-aconitic acid	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
citric acid	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
formic acid	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
D-galactonic acid lactone	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
D-galacturonic acid	0	-	0	-	0	0	0	0	-	0	0	0	0	0	0	0	0	0
D-gluconic acid	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
D-glucosaminic acid	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
D-glucuronic acid	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
a-hydroxy butyric acid	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
b-hydroxy butyric acid	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
y-hydroxy butyric acid	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
p-hydroxy phenylacetic acid	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
itaconic acid	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
a-keto butyric acid	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
a-keto glutaric acid	0	-	0	-	0	0	0	0	-	0	0	0	0	0	0	0	0	0
a-keto valeric acid	0	-	0	-	0	0	0	0	-	0	0	0	0	0	0	0	0	0
D,L-lactic acid	-	0	-	0	-	0	0	0	0	0	0	0	0	0	0	0	0	0
malonic acid	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
propionic acid	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
quinic acid	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
D-saccharic acid	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
sebacic acid	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
succinic acid	0	-	0	-	0	0	0	0	-	0	0	0	0	0	0	0	0	0
bromo-succinic acid	-	0	-	0	-	0	0	0	0	0	0	0	0	0	0	0	0	0
succinamic acid	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
glucuronamide	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
alaninamide	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
D-alanine	0	-	0	-	0	0	0	0	-	0	0	0	0	0	0	0	0	0
L-alanine	-	-	-	-	0	-	-	-	0	-	-	-	-	-	-	0	-	0
L-alanyl glycine	-	-	-	-	0	-	-	-	0	-	-	-	-	-	-	0	-	0
L-asparagine	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
L-aspartic acid	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
L-glutamic acid	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
glycyl-L-aspartic acid	-	-	-	-	0	-	-	-	0	-	-	-	-	-	-	0	-	0
glycyl-L-glutamic acid	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
L-histidine	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
hydroxy L-proline	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
L-leucine	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
L-ornithine	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
L-phenyl alanine	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
L-proline	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
L-pyro glutamic acid	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
D-serine	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
L-serine	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
L-threonine	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
D,L-carnitine	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
y-amino butyric acid	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
urocanic acid	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
inosine	-	-	-	-	0	-	-	-	0	-	-	-	-	-	-	0	-	0
uridine	-	-	-	-	0	-	-	-	0	-	-	-	-	-	-	0	-	0
thymidine	-	-	-	-	0	-	-	-	0	-	-	-	-	-	-	0	-	0
phenyl ethylamine	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
putrescine	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
2-amino ethanol	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
2,3 butanediol	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
glycerol	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
D,L-a-glycerol phosphate	0	0	0	0	0	0	-	-	-	-	-	-	-	-	-	-	-	-
glucose-1-phosphate	0	0	0	0	0	0	-	-	-	-	-	-	-	-	-	-	-	-
glucose-6-phosphate	0	0	0	0	0	0	-	-	-	-	-	-	-	-	-	-	-	-

Appendix 5 16S rRNA partial DNA sequences

NAME LMS-3, *Vibrio splendidus* Biovar 1

LENGTH 719 nucleotides

AFFILIATION γ-proteobacteria, *V. splendidus* subgroup

ORIGIN Merexo 'L' group larvae

```
1   ATACGACTCA CTATAGGGCG AATTGAATTT AGCGGCCGCG AATTCGCCCT TAGAGTTTGA
61  TCATGGCTCA GATTGAACGC TGGCGGCAGG CCTAACACAT GCAAGTCGAG CGGAAACGAC
121 ACTAACAATC CTTCGGGTGC GTTAATGGGC GTCGAGCGGC GGACGGGTGA GTAATGCCTA
181 GGAAATTGCC TTGATGTGGG GGATAACCAT TGGAAACGAT GGCTAATACC GCATAATGCC
241 TACGGGCCAA AGAGGGGGAC CTTCGGGCCT CTCGCGTCAA GATATGCCTA GGTGGGATTA
301 GCTAGTTGGT GAGGTAATGG CTCACCAAGG CGACGATCCC TAGCTGGTCT GAGAGGATGA
361 TCAGCCACAC TGGAACTGAG ACACGGTCCA GACTCCTACG GGAGGCAGCA GTGGGGAATA
421 TTGCACAATG GGCGAAAGCC TGATGCAGCC ATGCCGCGTG TATGAAGAAG GCCTTCGGGT
481 TGTAAAGTAC TTTCAGTTGT GAGGAAGGGG GTAGTGTTAA TAGCGCTATC TCTTGACGTT
541 AGCAACAGAA GAAGCACCGG CTAACTCCGT GCCAGCAGCC GCGGTAATAC GGAGGGTGCG
601 AGCGTTAATC GGAATTACTG GGCGTAAAGC GCATGCAGGT GGTTCATTAA GTCAGATGTG
661 AAAGCCCGGG GCTCAACCTC GGAACTGCAT TTGAAACTGG TGAACTAGAG TACTGTAGA
```

NAME LMS-4, *Vibrio splendidus* Biovar 1

LENGTH 644 nucleotides

AFFILIATION γ-proteobacteria, *V. splendidus* subgroup

ORIGIN Merexo 'L' group larvae

```
1   AGAGTTTGAT CATGGCTCAG ATTGAACGCT GGCGGCAGGC CTAACACATG CAAGTCGAGC
61  GGAAACGACA CTAACAATCC TTCGGGTGCG TTAATGGGCG TCGAGCGGCG GACGGGTGAG
121 TAATGCCTAG GAAATTGCCT TGATGTGGGG GATAACCATT GGAAACGATG GCTAATACCG
181 CATAATGCCT ACGGGCCAAA GAGGGGGACC TTCGGGCCTC TCGCGTCAAG ATATGCCTAG
241 GTGGGATTAG CTAGTTGGTG AGGTAATGGC TCACCAAGGC GACGATCCCT AGCTGGTCTG
301 AGAGGATGAT CAGCCACACT GGAACTGAGA CACGGTCCAG ACTCCTACGG GAGGCAGCAG
361 TGGGGAATAT TGCACAATGG GCGAAAGCCT GATGCAGCCA TGCCGCGTGT ATGAAGAAGG
421 CCTTCGGGCT GTAAAGTACT TTCAGTTGTG AGGAAGGGGG TAGTGTTAAT AGCGCTGTCT
481 CTTGACGTTA GCAACAGAAG AAGCACCGGC TAACTCCGTG CCAGCAGCCG CGGTAATACG
541 GAGGGTGCGA GCGTTAATCG GAATTACTGG GCGTAAAGCG CATGCAGGTG GTTCATTTAA
601 GTCAGATGTG AAAGCCCCGG GGCTCAACCT TCGGAACTGC ATTT
```

NAME HNT-2 *Vibrio tubiashii*

LENGTH 651 nucleotides

AFFILIATION γ-proteobacteria, *V. nereis* subgroup

ORIGIN Merexo HN larval samples

```
1   AGAGTTTGAT CATGGCTCAG ATTGAACGCT GGCGGCAGGC CTAACACATG CAAGTCGAGC
61  GGAAACGAGT TAACTGAACC TTCGGGGAAC GTTAACGGCG TCGAGCGGCG GACGGGTGAG
121 TAATGCCTGG GAAATTGCCC TGATGTGGGG GATAACCATT GGAAACGATG GCTAATACCG
181 CATAATAGCT TCGGCTCAAA GAGGGGGACC TTCGGGCCTC TCGCGTCAGG ATATGCCCAG
241 GTGGGATTAG CTAGTTGGTG AGGTAATGGC TCACCAAGGC GACGATCCCT AGCTGGTCTG
301 AGAGGATGAT CAGCCACACT GGAACTGAGA CACGGTCCAG ACTCCTACGGG AGGCAGCAG
361 TGGGGAATAT TGCACAATGG GCGCAAGCCT GATGCAGCCA TGCCGCGTGT ATGAAGAAGG
421 CCTTCGGGTT GTAAAGTACT TTCAGCAGTG AGGAAGGTGG TGTCGTTAAT AGCGGCATCA
481 TTTGACGTTA GCTGCAGAAG AAGCACCGGC TAACTCCGTG CCAGCAGCCG CGGTAATACG
541 GAGGGTGCGA GCGTTAATCG GAATTACTGG GCGTAAAGCG CATGCAGGTG GTTTGTTAAG
601 TCAGATGTGA AAGCCCGGGG CTCAACCTCG GAATTGCATT TGAAACTGGC A
```

NAME S1A2-1, *Vibrio alginolyticus*

LENGTH 673 nucleotides

AFFILIATION γ-proteobacteria, *Vibrio harveyi* subgroup

ORIGIN Merexo *Artemia* samples

```
1   CCCTTAGAGT TTGATCATGG CTCAGATTGA ACGCTGGCGG CAGGCCTAAC ACATGCAAGT
61  CGAGCGGAAA CGAGTTATCT GAACCTTCGG GGAACGATAA CGGCGTCGAG CGGCGGACGG
121 GTGAGTAATG CCTAGGAAAT TGCCCTGATG TGGGGGATAA CCATTGGAAA CGATGGCTAA
181 TACCGCATGA TGCCTACGGG CCAAAGAGGG GGGCCTTCGG GCCTCTCGCG TCAGGATATG
241 CCTAGGTGGG ATTAGCTAGT TGGTGAGGTA AGGGCTCACC AAGGCGACGA TCCCTAGCTG
301 GTCTGAGAGG ATGATCAGCC ACACTGGAAC TGAGACACGG TCCAGACTCC TACGGGAGGC
361 AGCAGTGGGG AATATTGCAC AATGGGCGCA AGCCTGATGC AGCCATGCCG CGTGTGTGAA
421 GAAGGCCTTC GGGTTGTAAA GCACTTTCAG TCGTGAGGAA GGTAGTGTAG TTAATAGCTG
481 CATTATTTGA CGTTAGCGAC AGAAGAAGCA CCGGCTAACT CCGTGCCAGC AGCCGCGGTA
541 ATACGGAGGG TGCGAGCGTT AATCGGAATT ACTGGGCGTA AAGCGCATGC AGGTGGTTTG
601 TTAAGTCAGA TGTGAAAGCC CGGGGCTCAA CCTCGGAATA GCATTTGAAA CTGGCAGACT
661 GAGTACTGTA GA
```

NAME S3A2-11, *Vibrio alginolyticus*

LENGTH 667 nucleotides

AFFILIATION γ-proteobacteria, *Vibrio harveyi* subgroup

ORIGIN Second group of Quilmas *Artemia* samples

```
1   AGAGTTTGAT CATGGCTCAG ATTGAACGCT GGCGGCAGGC CTAACACATG CAAGTCGAGC
61  GGAAACGAGT TATCTGAACC TTCGGGGAAC GATAACGGCG TCGAGCGGCG GACGGGTGAG
121 TAATGCCTAG GAAATTGCCC TGATGTGGGG GATAACCATT GGAAACGATG GCTAATACCG
181 CATGATGCCT ACGGGCCAAA GAGGGGGACC TTCGGGCCTC TCGCGTCAGG ATATGCCTAG
241 GTGGGATTAG CTAGTTGGTG AGGTAAGGGC TCACCAAGGC GACGATCCCT AGCTGGTCTG
301 AGAGGATGAT CAGCCACACT GGAACTGAGA CACGGTCCAG ACTCCTACGG GAGGCAGCAG
361 TGGGGAATAT TGCACAATGG GCGCAAGCCT GATGCAGCCA TGCCGCGTGT GTGAAGAAGG
421 CCTTCGGGTT GTAAAGCACT TTCAGTCGTG AGGAAGGTAG TGTAGTTAAT AGCTGCATTA
481 TTTGACGTTA GCGACAGAAG AAGCACCGGC TAACTCCGTG CCAGCAGCCG CGGTAATACG
541 GAGGGTGCGA GCGTTAATCG GAATTACTGG GCGTAAAGCG CATGCAGGTG GTTTGTTAAG
601 TCAGATGTGA AAGCCCGGGG CTCAACCTCG GAATAGCATT TGAAACTGGC AGACTAGAGT
661 ACTGGGA
```

NAME S3A2-18, *Vibrio alginolyticus*

LENGTH 668 nucleotides

AFFILIATION γ-proteobacteria, *Vibrio harveyi* subgroup

ORIGIN Second group of Quilmas *Artemia* samples

```
1   AGAGTTTGAT CATGGCTCAG ATTGAACGCT GGCGGCAGGC CTAACACATG CAAGTCGAGC
61  GGAAACGAGT TATCTGAACC TTCGGGGAAC GATAACGGCG TCGAGCGGCG GACGGGTGAG
121 TAATGCCTAG GAAATTGCCC TGACGTGGGG GATAACCATT GGAAACGATG GCTAATACCG
181 CATGATGCCT ACGGGCCAAA GAGGGGGACC TTCGGGCCTC TCGCGTCAGG ATATGCCTAG
241 GTGGGATTAG CTAGTTGGTG AGGTAAGGGC TCACCAAGGC GACGATCCCT AGCTGGTCTG
301 AGAGGATGAT CAGCCACACT GGAACTGAGA CACGGTCCAG ACTCCTACGG GAGGCAGCAG
361 TGGGGAATAT TGCACAATGG GCGCAAGCCT GATGCAGCCA TGCCGCGTGT GTGAAGAAGG
421 CCTTCGGGTT GTAAAGCACT TTCAGTCGTG AGGAAGGCGG GTACGTTAAT AGCGTATTCG
481 TTTTGACGTT AGCGACAGAA GAAGCACCGG CTAACTCCGT GCCAGCAGCC GCGGTAATAC
541 GGAGGGTGCG AGCGTTAATC GGAATTACTG GGCGTAAAGC GCATGCAGGT GGTTTGTTAA
601 GTCAGATGTG AAAGCCCGGG GCTCAACCTC GGAATAGCAT TTGAAACTGG CAGACTAGAG
661 TACTGTAG
```

NAME S3A2-8, *Vibrio alginolyticus*

LENGTH 659 nucleotides

AFFILIATION γ-proteobacteria, *Vibrio harveyi* subgroup

ORIGIN Second group of Quilmas *Artemia* samples

```
1   AGAGTTTGAT CATGGCTCAG ATTGAACGCT GGCGGCAGGC CTAACACATG CAAGTCGAGC
61  GGAAACGAGT TATCTGAACC TTCGGGGAAC GATAACGGCG TCGAGCGGCG GACGGGTGAG
121 TAATGCCTAG GAAATTGCCC TGATGTGGGG GATAACCATT GGAAACGATG GCTAATACCG
181 CATGATGCCT ACGGGCCAAA GAGGGGGACC TTCGGGCCTC TCGCGTCAGG ATATGCCTAG
241 GTGGGATTAG CTAGTTGGTG AGGTAAGGGC TCACCAAGGC AACGATCCCT AGCTGGTCTG
301 AGAGGATGAT CAGCCACACT GGAACTGAGA CACGGTCCAG ACTCCTACGG GAGGCAGCAG
361 TGGGGAATAT TGCACAATGG GCGCAAGCCT GATGCAGCCA TGCCGCGTGT ATGAAGAAGG
421 CCTTCGGGTT GTAAAGTACT TTCAGTCGTG AGGAAGGCGG CGTCGTTAAT AGCGGCGTTG
481 TTTGACGTTA GCGACAGAAG AAGCACCGGC TAACTCCGTG CCAGCAGCCG CGGTAATACG
541 GAGGGCGCGA GCGTTAATCG GAATTACTGG GCGTAAAGCG CATGCAGGTG GTTTGTTAAG
601 TCAGATGTGA AAGCCCGGGG CTCAACCTCG GAATAGCATT TGAAACTGGC AGACTAGAG
```

NAME S1L1-7, *Vibrio splendidus* biovar 1

LENGTH 651 nucleotides

AFFILIATION γ-proteobacteria, *V. splendidus* subgroup

ORIGIN Merexo larval samples

```
1   AGAGTTTGAT CATGGCTCAG ATTGAACGCT GGCGGCAGGC CTAACACATG CAAGTCGAGC
61  GGAAACGACA CTAACAATCC TTCGGGTGCG TTAATGGGCG TCGAGCGGCG GACGGGTGAG
121 TAATGCCTGG GAAATTGCCT TGATGTGGGG GATAACCATT GGAAACGATG GCTAATACCG
181 CATAATGCCT ACGGGCCAAA GAGGGGGACC TTTGGGCCTC TCGCGTCAAG ATATGCCTAG
241 GTGGGATTAG CTAGTTGGTG AGGTAATGGC TCACCAAGGC GACGATCCCT AGCTGGTCTG
301 AGAGGATGAT CAGCCACACT GGAACTGAGA CACGGTCCAG ACTCCTACGG GAGGCAGCAG
361 TGGGGAATAT TGCACAATGG GCGAAAGCCT GATGCAGCCA TGCCGCGTGT ATGAAGAAGG
421 CCTTCGGGTT GTAAAGTACT TTCAGTTGTG AGGAAGGGGG TAGTGTTAAT AGCGCTATCT
481 CTTGACGTTA GCAACAGAAG AAGCACCGGC TAACTCCGTG CCAGCAGCCG CGGTAATACG
541 GAGGGTGCGA GCGTTAATCG GAATTACTGG GCGTAAAGCG CATGCAGGTG GTTCATTAAG
601 TCAGATGTGA AAGCCCGGGG CTCAACCTCG GAACTGCATT TGAAACTGGTA
```

NAME S3L1-8, *Vibrio splendidus* biovar 1

LENGTH 656 nucleotides

AFFILIATION γ-proteobacteria, *V. splendidus* subgroup

ORIGIN Second Quilmas larval samples

```
1   AGAGTTTGAT CATGGCTCAG ATTGAACGCT GGCGGCAGGC CTAACACATG CAAGTCGAGC
61  GGAAACGACA TTAACAATCC TTCGGGTGCG TTAATGGGCG TCGAGCGGCG GACGGGTGAG
121 TAATGCCTAG GAAATTGCCT TGATGTGGGG GATAACCATT GGAAACGATG GCTAATACCG
181 CATGATGCCT ACGGGCCAAA GAGGGGGACC TTCGGGCCTC TCGCGTCAAG ATATGCCTAG
241 GTGGGATTAG CTAGTTGGTG AGGTAATGGC TCACCAAGGC GACGATCCCT AGCTGGTCTG
301 AGAGGATGAT CAGCCACACT GGAACTGAGA CACGGTCCAG ACTCCTACGG GAGGCAGCAG
361 TGGGGAATAT TGCACAATGG GCGCAAGCCT GATGCAGCCA TGCCGCGTGT ATGAAGAAGG
421 CCTTCGGGTT GTAAAGTACT TTCAGTTGTG AGGAAGGGGG TAACGTTAAT AGCGCTATCT
481 CTTGACGTTA GCAACAGAAG AAGCACCGGC TAACTCCGTG CCAGCAGCCG CGGTAATACG
541 GAGGGTGCCG AGCGTTAATC GGAAATTACT GGGCGTAAAG CGCATGCAGG TGGTTCATTA
601 AGTCAGATGT GAAAGCCCGG GGCTCAACCT CGGAACTGCA TTTGAAACTG GTGAAC
```

NAME LTS-5 *Vibrio splendidus* biovar 1

LENGTH 666 nucleotides

AFFILIATION γ-proteobacteria, *V. splendidus* subgroup

ORIGIN Merexo '**D**' larval samples

```
1   AGAGTTTGAT CCTGGCTCAG ATTGAACGCT GGCGGCAGGC CTAACACATG CAAGTCGAGC
61  GGAAACGACA CTAACAATCC TTCGGGTGCG TTAATGGGCG TCGAGCGGCG GACGGGTGAG
121 TAATGCCTAG GAAATTGCCT TGATGTGGGG GATAACCATT GGAAACGATG GCTAATACCG
181 CATAATGCCT ACGGGCCAAA GAGGGGGACC TTCGGGCCTC TCGCGTCAAG ATATGCCTAG
241 GTGGGATTAG CTAGTTGGTG AGGTAATGGC TCACCAAGGC GACGATCCCT AGCTGGTCTG
301 AGAGGATGAT CAGCCACACT GGAACTGAGA CACGGTCCAG ACTCCTACGG GAGGCAGCAG
361 TGGGGAATAT TGCACAATGG GCGAAAGCCT GATGCAGCCA TGCCGCGTGT ATGAAGAAGG
421 CCTTCGGGTT GTAAAGTACT TTCAGTTGTG AGGAAGGGGG TAGTGTTAAT AGCGCTATCT
481 CTTGACGTTA GCAACAGAAG AAGCACCGGC TAACTCCGTG CCAGCAGCCG CGGTAATACG
541 GAGGGTGCGA GCGTTAATCG GAATTACTGG GCGTAAAGCG CATGCAGGTG GTTCATTAAG
601 TCAGATGTGA AAGCCCGGGG CTCAACCTCG GAACTGCATT TGAAACTGGT GAACTAGAGT
661 ACTGTA
```

NAME LTH-1, *Vibrio splendidus* biovar 2

LENGTH 667 nucleotides

AFFILIATION γ-proteobacteria, *V. splendidus* subgroup

ORIGIN Merexo '**L**' larval samples

```
1   GATCATGGCT CAGATTGAAC GCTGGCGGCA GGCCTAACAC ATGCAAGTCG AGCGGAAACG
61  AGTTATCTGA ACCTTCGGGG AACGATAACG GCGTCGAGCG GCGGACGGGT GAGTAATGCC
121 TAGGAAATTG CCTTGATGTG GGGGATAACC ATTGGAAACG ATGGCTAATA CCGCATAATG
181 CCTACGGGCC AAAGAGGGGG ACCTTCGGGC CTCTCGCGTC AAGATATGCC TAGGTGGGAT
241 TAGCTAGTTG GTGAGGTAAT GGCTCACCAA GGCGACGATC CCTAGCTGGT CTGAGAGGAT
301 GATCAGCCAC ACTGGAACTG AGACACGGTC CAGACTCCTA CGGGAGGCAG CAGTGGGGAA
361 TATTGCACAA TGGGCGAAAG CCTGATGCAG CCATGCCGCG TGTATGAAGA AGGCCTTCGG
421 GTTGTAAAGT ACTTTCAGTT GTGAGGAAGG GGGTGTCGTT AATAGCGGCA TCTCTTGACG
481 TTAGCAACAG AAGAAGCACC GGCTAACTCC GTGCCAGCAG CCGCGGTAAT ACGGAGGGTG
541 CGAGCGTTAA TCGGAATTAC TGGGCGTAAA GCGCATGCAG GTGGTTCATT AAGTCAGATG
601 TGAAAGCCCG GGGCTCAACC TCGGAACTGC ATTTGAAACT GGTGAACTAG AGTACTGTAG
661 AGGGGGG
```

NAME LTH-3, *Vibrio splendidus* biovar 2

LENGTH 704 nucleotides

AFFILIATION γ-proteobacteria, *V. splendidus* subgroup

ORIGIN Merexo '**L**' larval samples

```
1   AGAGTTTGAT CATGGCTCAG ATTGAACGCT GGCGGCAGGC CTAACACATG CAAGTCGAGC
61  GGAAACGAGT TATCTGAACC TTCGGGGAAC GATAACGGCG TCGAGCGGCG GACGGGTGAG
121 TAATGCCTAG GAAATTGCCT TGATGTGGGG GATAACCATT GGAAACGATG GCTAATACCG
181 CATAATGCCT ACGGGTCAAA GAGGGGGACC TTCGGGCCTC TCGCGTCAAG ATATGCCTAG
241 GTGGGATTAG CTAGTTGGTG AGGTAATGGC TCACCAAGGC GACGATCCCT AGCTGGTCTG
301 AGAGGATGAT CAGCCACACT GGAACTGAGA CACGGTCCAG ACTCCTACGG GAGGCAGCAG
361 TGGGGAATAT CGCACAATGG GCGAAAGCCT GATGCAGCCA TGCCGCGTGT ATGAAGAAGG
421 CCTTCGGGTT GTAAAGTACT TTCAGTTGTG AGGAAGGGGG TGTCGTTAAT AGCGGCATCT
481 CTTGACGTTA GCAACAGAAG AAGCACCGGC TAACTCCGTG CCAGCAGCCG CGGTAATACG
541 GAGGGTGCGA GCGTTAATCG GAATTACTGG GCGTAAAGCG CATGCAGGTG GTTCATTAAG
601 TCAGATGTGA AAGCCCGGGG CTCGGGTGCG AGCGTTAATC GGAATTACTG GGCGTAAAGC
661 GCATGCAGGT GGTTCATTAA GTCAGATGTG AAAGCCCGGG GCTC
```

NAME HNT-3 *Vibrio splendidus* biovar 2

LENGTH 667 nucleotides

AFFILIATION γ-proteobacteria, *V. splendidus* subgroup

ORIGIN Merexo HN larval samples

```
1   GAGTTTGATC ATGGCTCAGA TTGAACGCTG GCGGCAGGCC TAACACATGC AAGTCGAGCG
61  GAAACGAGTT ATCTGAACCT TCGGGGAACG ATAACGGCGT CGAGCGGCGG ACGGGTGAGT
121 AATGCCTAGG AAATTGCCTT GATGTGGGGG ATAACCATTG GAAACGATGG CTAATACCGC
181 ATAATGCCTA CGGGCCAAAG AGGGGGACCT TCGGGCCTCT CGCGTCAAGA TATGCCTAGG
241 TGGGATTAGC TAGTTGGTGA GGTAATGGCT CACCAAGGCG ACGATCCCTA GCTGGTCTGA
301 GAGGATGATC AGCCACACTG GAACTGAGAC ACGGTCCAGA CTCCTACGGG AGGCAGCAGT
361 GGGGAATATT GCACAATGGG CGAAAGCCTG ATGCAGCCAT GCCGCGTGTA TGAAGAAGGC
421 CTTCGGGTTG TAAAGTACTT TCAGTTGTGA GGAAGGGGGT GTCGTTAATA GCGGCATCTC
481 TTGACGTTAG CAACAGAAGA AGCACCGGCT AACTCCGTGC CAGCAGCCGC GGTAATACGG
541 AGGGTGCGAG CGTTAATCGG AATTACTGGG CGTAAAGCGC ATGCAGGTGG TTCATTAAGT
601 CAGATGTGAA AGCCCGGGGC TCAACCTCGG AACTGCATTT GAAACTGGTG AACTAGAGTA
661 CTGTAGA
```

NAME S1L2-4 *Vibrio splendidus* biovar 1

LENGTH 683 nucleotides

AFFILIATION γ-proteobacteria, *V. splendidus* subgroup

ORIGIN Merexo larval samples

```
1   AGAGTTTGAT CCTGGCTCAG ATTGAACGCT GGCGGCAGGC CCAACACATG CAAGTCGAGC
61  GGAAACGACA CTAACAATCC TTCGGGTGCG TTAATGGGCG TCGAGCGGCG GACGGGTGAG
121 TAATGCCTAG GAAATTGCCT TGATGTGGGG GATAACCATT GGAAACGATG GCTAATACCG
181 CATAATGCCT ACGGGCCAAA GAGGGGGACC TTCGGGCCTC TCGCGTCAAG ATATGCCTAG
241 GTGGGATTAG CTAGTTGGTG AGGTAATGGC TCACCAAGGC GACGATCCCT AGCTGGTCTG
301 AGAGGATGAT CAGCCACACT GGAACTGAGA CACGGTCCAG ACTCCTACGG GAGGCAGCAG
361 TGGGGAATAT TGCACAATGG GCGAAAGCCT GATGCAGCCA TGCCGCGTGT ATGAAGAAGG
421 CCTTCGGGTT GTAAAGTACT TTCAGTTGTG AGGAAGGGGG TAGTGTTAAT AGCGCTATCT
481 CTTGACGTTA GCAACAGAAG AAGCACCGGC TAACTCCGTG CCAGCAGCCG CGGTAATACG
541 GAGGGTGCGA GCGTTAATCG GAATTACTGG GCGTAAAGCG CATGCAGGTG GTTCATTAAG
601 TCAGATGTGA AAGCCCGGGG CTCAACCTCG GAACTGCATT TGAAACTGGT GAACTAGAGT
661 ACTGTAGAGG GGGGTAGAAC TTC
```

NAME DTC-3 *Vibrio splendidus* biovar 1

LENGTH 673 nucleotides

AFFILIATION γ-proteobacteria, *V. splendidus* subgroup

ORIGIN Merexo '**D**' larval samples

```
1   AGAGTTTGAT CATGGCTCAG ATTGAACGCT GGCGGCAGGC CTAACACATG CAAGTCGAGC
61  GGAAACGACA CTAACAATCC TTCGGGTGCG TTAATGGGCG TCGAGCGGCG GACGGGTGAG
121 TAATGCCTAG GAAATTGCCT TGATGTGGGG GATAACCATT GGAAACGATG GCTAATACCG
181 CATAATGCCT ACGGGCCAAA GAGGGGGACC TTCGGGCCTC TCGCGTCAAG ATATGCCTAG
241 GTGGGATTAG CTAGTTGGTG AGGTAATGGC TCACCAAGGC GACGATCCCT AGCTGGTCTG
301 AGAGGATGAT CAGCCACACT GGAACTGAGA CACGGTCCAG ACTCCTACGG GAGGCAGCAG
361 TGGGGAATAT TGTACAATGG GCGAAAGCCT GATGCAGCCA TGCCGCGTGT ATGAAGAAGG
421 CCTTCGGGTT GTAAAGTACT TTCAGTTGTG AGGAAGGGGG TAGTGTTAAT AGCGCTATCT
481 CTTGACGTTA GCAACAGAAG AAGCACCGGC TAACTCCGTGCCAGCAGCCGC GGTAATACG
541 GAGGGTGCGA GCGTTAATCG GAATTACTGG GCGTAAAGCGCATGCAGGTGG TTCATTAAG
601 TCAGATGTGA AAGCCCGGGG CTCAACCTCG GAACTGCATTTGAAACTGGTG AACTAGAGT
661 ACTGTAGAGG GGG
```

NAME LTS-3 *Vibrio splendidus* biovar 1

LENGTH 662 nucleotides

AFFILIATION γ-proteobacteria, *V. splendidus* subgroup

ORIGIN Merexo '**L**' larval samples

```
1   AGAGTTTGATCATGGCTCAGATTGAACGCTGGCGGCAGGCCTAACACATGCAAGTCGAGC
61  GGAAACGACACTAACAATCCTTCGGGTGCGTTAATGGGCGTCGAGCGGCGGACGGGTGAG
121 TAATGCCTAGGAAATGGCCTTGATGTGGGGGATAACCATTGGAAACGATGGCTAATACCG
181 CATAATGCCTACGGGCCAAAGAGGGGGACCTTCGGGCCTCTCGCGTCAAGATATGCCTAG
241 GTGGGATTAGCTAGTTGGTGAGGTAAGTGGCTCACCAAGGCGACGATCCCTAGCTGGTCT
301 GAGAGGATGATCAGCCACACTGGCACTCAGACACGGTCCAGACTCCTACGGGAGGCAGCA
361 GTGGGGAATATTGCACAATGGGCGAAAGCCTGATGCAGCCATGCCGCGTGTATGAAGAAG
421 GCCTTCGGGTTGTAAAGTACTTTCAGTCGTGAGGAAGGGGGTAGTGTTAATAGCGCTATC
481 TCTTGACGTTAGCAACAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATAC
541 GGAGGGTGCGAGCGTTAATCGGAATTACTGGGCGTAAAGCGCATGCAGGTGGTTCATTAA
601 GTCAGATGTGAAAGCCCGGGGCTCAACCTCGGAACTGCATTTGAAACTGGTGAACTAGAG
661 GG
```

NAME DMC-1 *Vibrio splendidus* biovar 1

LENGTH 631 nucleotides

AFFILIATION γ-proteobacteria, *V. splendidus* subgroup

ORIGIN Merexo '**D**' larval samples

```
1   GATTAAACGC TGGCGGCAGG CCTAACACAT GCAAGTCGAG CGGAAACGAC ACTAACAATC
61  CTTCGGGTGC GTTAATGGGC GTCGAGCGGC GGACGGGTGA GTAATGCCTA GGAAATTGCC
121 TTGATGTGGG GGATAACCAT TGGAAACGAT GGCTAATACC GCATAATGCC TACGGGCCAA
181 AGAGGGGGAC CTTCGGGCCT CTCGCGTCAA GATATGCCTA GGTGGGATTA GCTAGTTGGT
241 GAGGTAATGG CTCACCAAGG CGACGATCCC TAGCTGGTCT GAGAGGATGA TCAGCCACAC
301 TGGAACTGAG ACACGGTCCA GACTCCTACG GGAGGCAGCA GTGGGGAATA TTGCACAATG
361 GGCGAAAGCC TGATGCAGCC ATGCCGCGTG TATGAAGAAG GCCTTCGGGT TGTAAAGTAC
421 TTTCAGTTGT GAGGAAGGGG GTAGCGTTAA TAGCGCTATC TCTTGACGTT AGCAACAGAA
481 GAAGCACCGG CTAACTCCGT GCCAGCAGCC GCGGTAATAC GGAGGGTGCG AGCGTTAATC
541 GGAATTACTG GGCGTAAAGC GCATGCAGGT GGTTCATTAA GTCAGATGTG AAAGCCCGGG
601 GCTCAACCTC GGAACTGCAT TTGAAACTGG T
```

NAME DTC-5 *Vibrio splendidus* biovar 1

LENGTH 667 nucleotides

AFFILIATION γ-proteobacteria, *V. splendidus* subgroup

ORIGIN Merexo '**D**' larval samples

```
1   AGAGTTTGAT CATGGCTCAG ATTGAACGCT GGCGGCAGGC CTAACACATG CAAGTCGAGC
61  GGAAACGACA CTAACAATCC TTCGGGTGCG TTAATGGGCG TCGAGCGGCG GACGGGTGAG
121 TAATGCCTAG GAAATTGCCT TGATGTGGGG GATAACCATT GGAAACGATG GCTAATACCG
181 CATAATGCCT ACGGGCCAAA GAGGGGGATC TTCGGGCCTC TCGCGTCAAG ATATGCCTAG
241 GTGGGATTAG CTAGTTGGTG AGGTAATGGC TCACCAAGGC GACGATCCCT AGCTGGTCTG
301 AGAGGATGAT CAGCCACACT GGAACTGAGA CACGGTCCAG ACTCCTACGG GAGGCAGCAG
361 TGGGGAATAT TGCACAATGG GCGAAAGCCT GATGCAGCCA TGCCGCGTGT ATGAAGAAGG
421 CCTTCGGGTT GTAAAGTACT TTCAGTTGTG AGGAAGGGGG TAGTGTTAAT AGCGCTATCT
481 CTTGACGTTA GCAACAGAAG AAGCACCGGC TAACTCCGTG CCAGCAGCCG CGGTAATACG
541 GAGGGTGCGA GCGTTAATCG GAATTACTGG GCGTAAAGCG CATGCAGGTG GTTCATTAAG
601 TCAGATGTGA AAGCCCGGGG CTCAACCTCG GAACTGCATT TGAAACTGGT GAACTAGAGT
661 ACGTAGA
```

NAME HNF-8 *Vibrio splendidus* biovar 1

LENGTH 669 nucleotides

AFFILIATION γ-proteobacteria, *V. splendidus* subgroup

ORIGIN Merexo HN larval samples

```
1   AGAGTTTGAT CATGGCTCAG ATTGAACGCT GGCGGCAGGC CTAACACATG CAAGTCGAGC
61  GGAAACGACA CTAACAATCC TTCGGGTGCG TTAATGGGCG TCGAGCGGCG GACGGGTGAG
121 TAATGCCTAG GAAATTGCCT TGATGTGGGG GATAACCATT GGAAACGATG GCTAATACCG
181 CATAATGCCT ACGGGCCAAA GAGGGGGACC TTCGGGCCTC TCGCGTCAAG ATATGCCTAG
241 GTGGGATTAG CTAGTTGGTG AGGTAATGGC TCACCAAGGC GACGATCCCT AGCTGGTCTG
301 AGAGGATGAT CAGCCACACT GGAACTGAGA CACGGTCCAG ACTCCTACGG GAGGCAGCAG
361 TGGGGAATAT TGCACAATGG GCGAAAGCCT GATGCAGCCA TGCCGCGTGT ATGAAGAAGG
421 CCTTCGGGTT GTAAAGTACT TTCAGTTGTG AGGAAGGGGG TAGCGTTAAT AGCGCTATCT
481 CTTGACGTTA GCAACAGAAG AAGCACCGGC TAACTCCGTG CCAGCAGCCG CGGTAATACG
541 GAGGGTGCGA GCGTTAATCG GAATTACTGG GCGTAAAGCG CATGCAGGTG GTTCATTAAG
601 TCAGATGTGA AAGCCCGGGG CTCAACCTCG GAACTGCATT TGAAACTGGT GAACTAGAGT
661 ACTGTAGAG
```

NAME LTH-4 *Vibrio splendidus* biovar 1

LENGTH 714 nucleotides

AFFILIATION γ-proteobacteria, *V. splendidus* subgroup

ORIGIN Merexo '**L**' larval samples

```
1   CACATGCAAG TCGAGCGGAA ACGACACTAA CAATCCTTCG GGTGCGTTAA TGGGCGTCGA
61  GCGGCGGACG GGTGAGTAAT GCCTAGGAAA TTGCCTTGAT GTGGGGGATA ACCATTGGAA
121 ACGATGGCTA ATACCGCATA ATGCCTACGG GCCAAAGAGG GGGACCTTCG GCCCTCTCGC
181 GTCAAGATAT GCCTAGGTGG GATTAGCTAG TTGGTGAGGT AATGGCTCAC CAAGGCGACG
241 ATCCCTAGCT GGTCTGAGAG GATGATCAGC CACACTGGAA CTGAGACACG GTCCAGACTC
301 CTACGGGAGG CAGCAGTGGG GAATATTGCA CAATGGGCGA AAGCCTGATG CAGCCATGCC
361 GCGTGTATGA AGAAGGCCTT CGGGTTGTAA AGTACTTTCA GTTGTGAGGA AGGGGGTAGC
421 GTTAATAGCG CTATCTCTTG ACGTTAGCAA CAGAAGAAGC ACCGGCTAAC TCCGTGCCAG
481 CAGCCGCGGT AATACGGAGG GTGCGAGCGT TAATCGGAAT TACTGGGCGT AAAGCGCATG
541 CAGGTGGTTC ATTAAGTCAG ATGTGAAAGC CCGGGGCTCA ACCTCGGAAC TGCATTTGAA
601 ACTGGTGAAC TAGAGTACTG TAGAGGGGGG TAGAATTTCA GGTGTAGCGG TGAAATGCGT
661 AGAGATCTGA AGGAATACCA GTGGCGAAGG CGGCCCCCTG GACAGATACT GACA
```

NAME HNT-1 *Vibrio splendidus* biovar 2

LENGTH 664 nucleotides

AFFILIATION γ-proteobacteria, *V. splendidus* subgroup

ORIGIN Merexo HN larval samples

```
1   GAGTTTGATC ATGGCTCAGA TTGAACGCTG GCGGCAGGCC TAACACATGC AAGTCGAGCG
61  GAAACGAGTT ATCTAAACCT TCGGGGAACG ATAACGGCGT CGAGCGGCGG ACGGGTGAGT
121 AATGCCTAGG AAATTGCCTT GATGTGGGGG ATAACCATTG GAAACGATGG CTAATACCGC
181 ATAATGCCTA CGGGCCAAAG AGGGGGACCT TCGGGCCTCT CGCGTCAAGA TATGCCTAGG
241 TGGGATTAGC TAGTTGGTGA GGTAATGGCT CACCAAGGCG ACGATCCCTA GCTGGTCTGA
301 GAGGATGATC AGCCACACTG GAACTGAGAC ACGGTCCAGA CTCCTACGGG AGGCAGCAGT
361 GGGGAATATT GCACAATGGG CGAAAGCCTG ATGCAGCCAT GCCGCGTGTA TGAAGAAGGC
421 CTTCGGGTTG TAAAGTACTT TCAGTTGTGA GGAAGGGGGT GTCGTTAATA GCGGCATCTC
481 TTGACGTTAG CAACAGAAGA AGCACCGGCT AACTCCGTGC CAGCAGCCGC GGTAATACGG
541 AGGGTGCGAG CGTTAATCGG AATTACTGGG CGTAAAGCGC ATGCAGGTGG TTCATTAAGT
601 CAGATGTGAA AGCCCGGGGC TCAACCTCGG AACTGCATTT GAAACTGGTG AACTAGAGTA
661 CTGG
```

NAME HNF-3 *Vibrio splendidus* biovar 2

LENGTH 661 nucleotides

AFFILIATION γ-proteobacteria, *V. splendidus* subgroup

ORIGIN Merexo HN larval samples

```
1   ATCATGGCTC AGATTGAACG CTGGCGGCAG GCCTAACACA TGCAAGTCGA GCGGAAACGA
61  GTTATCTGAA CCTTCGGGGA ACGATAACGG CGTCGAGCGG CGGACGGGTG AGTAATGCCT
121 AGGAAATTGC CTTGATGTGG GGGATAACCA TTGGAAACGA TGGCTAATAC CGCATAATGC
181 CTACGGGCCA AAGAGGGGGA CCTTCGGGCC TCTCGCGTCA AGATATGCCT AGGTGGGATT
241 AGCTAGTTGG TGAGGTAATG GCTCACCAAG GCGACGATCC CTAGCTGGTC TGAGAGGATG
301 ATCAGCCACA CTGGAACTGA GACACGGTCC AGACTCCTAC GGGAGGCAGC AGTGGGGAAT
361 ATTGCACAAT GGGCGAAAGC CTGATGCAGC CATGCCGCGT GTATGAAGAA GGCCTTCGGG
421 TTGTAAAGTA CTTTCAGTTG TGAGGAAGGG GGTGTCGTTA ATAGCGGCAT CTCTTGACGT
481 TAGCAACAGA AGAAGCACCG GCTAACTCCG TGCCAGCAGC CGCGGTAATA CGGAGGGTGC
541 GAGCGTTAAT CGGAATTACT GGGCGTAAAG CGCATGCAGG TGGTTCATTA AGTCAGATGT
601 GAAAGCCCGG GGCTCAACCT CGGAACTGCA TTTGAAACTG GTGAACTAGA GTACTGTAGA
661 G
```

NAME HNF-2, *Marinomonas vaga*

LENGTH 671 nucleotides

AFFILIATION γ-proteobacteria, *Marinomonas* assemblage

ORIGIN Merexo HN larval samples

```
1   ACTCTAGCCT GCCAGTATCG GGTGCAATTC CAAGGTTGAG CCCTGGGCTT TCACATCCGA
61  CTTAACAAAC CACCTACGCG CGCTTTACGC CCAGTAATTC CGATTAACGC TCGCACCCTC
121 TGTATTACCG CGGCTGCTGG CACAGAGTTA GCCGGTGCTT CTTCTGAAGC TAACGTCAAG
181 ATAACTGGAT ATTAGCCAGT CACCCTTCCT CACTTCTGAA AGTGCTTTAC AACCCGAAGG
241 CCTTCTTCAC ACACGCGGCA TGGCTGGATC AGGCTTGCGC CCATTGTCCA ATATTCCCCA
301 CTGCTGCCTC CCGTAGGAGT CTGGGCCGTG TCTCAGTCCC AGTGTGGCTG ATCATCCTCT
361 CAGACCAGCT AAAGATCGTC GCCTTGGTGA GCCTTTACCC CACCAACTAG CTAATCTTAC
421 GCAGGCTCAT CTAATAGCGA AAGGTCCGAA GGTCCCCTCC TTTCCCCCGT AGGGCGTATG
481 CGGTATTAGC ATGCGTTTCC ACATGTTGTC CCCCACTACT AGGCAGATTC CTACGCGTTA
541 CTCACCCGTC CGCCGCTCGT CAGCAAAGAA AGCAAGCTTT CTTCCTGTTA CCGCTCGACT
601 TGCATGTGTT AAGCCTGCCG CCAGCGTTCA ATCTGAGCCA TGATCAAACT CTAAGGGCGA
661 ATTCGCGGCC G
```

NAME HNF-1 *Roseobacter gallaeciensis*

LENGTH 693 nucleotides

AFFILIATION α-proteobacteria, *Rg. algicola* subgroup

ORIGIN Merexo HN larval samples

```
1   GTCGAGCGCA CTCTTCGGAG TGAGCGGCGG ACGGGTTAGT AACGCGTGGG AACGTGCCCT
61  TCTCTAAGGA ATAGCCACTG GAAACXGTGA GTAATACCTT ATACGCCCTT CGGGGGAAAG
121 ATTTATCGGA GAAGGATCGG CCCGCGTTAG ATTAGATAGT TGGTGGGGTA ACGGCCTACC
181 AAGTCTACGA TCTATAGCTG GTTTTAGAGG ATGATCAGCA ACACTGGGAC TGAGACACGG
241 CCCAGACTCC TACGGGAGGC AGCAGTGGGG AATCTTGGAC AATGGGCGCA AGCCTGATCC
301 AGCCATGCCG CGTGAGTGAT GAAGGCCTTA GGGTCGTAAA GCTCTTTCGC CAGAGATGAT
361 AATGACAGTA TCTGGTAAAG AAACCCCGGC TAACTCCGTG CCAGCAGCCG CGGTAATACG
421 GAGGGGGTTA GCGTTGTTCG GAATTACTGG GCGTAAAGCG CACGTAGGCG GATCAGAAAG
481 TTGGGGGTGA AATCCCGGGG CTCAACCCCG GAACTGCCTC CAAAACTCCT GGTCTTGAGT
541 TCGAGAGAGG TGAGTGGAAT TCCGAGTGTA GAGGTGAAAT TCGTAGATAT TCGGAGGAAC
601 ACCAGTGGCG AAGGCGGCTC ACTGGCTCGA TACTGACGCT GAGGTGCGAA AGTGTGGGGA
661 GCAAACAGGA TTAGATACCC CTGGTAGTCC ACC
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Appendix Six

List of Suppliers

BIOLOG	Biolog, Inc., 3938 Trust Way, Hayward, CA 94545 UK agent: Don Whitley Scientific, 14 Otley Road, Shipley, W. Yorks. BD17 7ST
BIORAD	Biorad House, Marylands Avenue, Hemel Hamspstead, Herts
Cambio	34 Newnham Road, Cambridge, CB3 9EY,
Clustan Graphics	Clustan Ltd., 16 Kingsburgh Road, Edinburgh, EH12 6DZ,
Difco	Difco Lab. Ltd., PO Box 14B, Central Avenue, West Molesey
Fisher Scientific	Fisher Scientific UK, Bishop Madow Road, Loughborough, Leicestershire, LE11 5RG
Invitrogen	Invitrogen BV, PO Box 2312, 9704 CH Groningen, The Netherlands,
Life Technologies	Life Technologies Ltd., 3 Fountain Drive, Inchinnan Business Park, Paisley, PA4 9RF
Mast Diagnostics Ltd	Mast International Ltd., Mast House, Derby Road, Bootle, Merseyside, L20 1EA
Oswel DNA Service	Oswell DNA Service, Lab 5005, Medical and Biological Sciences Building, University of Southampton, Boldrewood, Bassett Crescent East, Southampton, SO16 7PX
Promega	Promega, Delta House, Chilworth Research Centre, Southampton, SO16 7NS
Quiagen	Quiagen Ltd., Boundary Court, Gatwick Road, Crawley, RH10 2AX

Roche Diagnostics Ltd	Roche Diagnostics Ltd., Bell Lane, Lewes, East Sussex BN7 1LG,
Sigma-Aldrich Ltd	Sigma-Aldrich Co. Ltd., Fancy Road, Poole, Dorset, BH12 4QH
TSC	Technical Service Consultants Ltd., The Rope Walk, Scofield Street, Heywood, Lancashire, OL10 1DS