

Study of Virulence Factors of *Staphylococcus aureus*

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By

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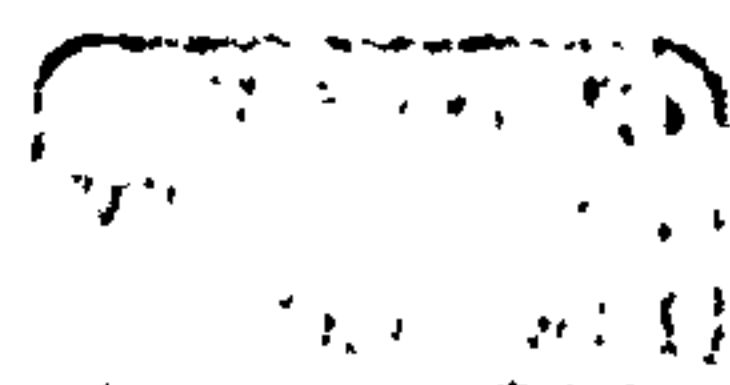


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Abbreviations

amp	ampicillin
BHI	Brain Heart Infusion
CA	community-acquired
ClfA	clumping factor A
cm	chloramphenicol
CoNS	Coagulase-negative staphylococci
Cna	collagen-binding protein
CSPD®	Disodium 3-(4-methoxyspiro {1,2-dioxetane-3,2'-(5'-chloro)tricyclo[3.3.1.1 ^{3,7}]decan}-4-yl) phenyl phosphate
CTAB/NaCl	10% Hexadecyltrimethyl ammonium bromide in 0.7 NaCl
DIG	Digoxigenin
DNA	Deoxyribonucleic Acid
ECM	extracellular matrix
erm	erythromycin
ETA	exfoliative toxin A
ETB	exfoliative toxin B
FnBPA	fibrinogen-binding protein A
HA	hospital-acquired
IE	Infective Endocarditis
IVET	<i>In Vivo</i> Expression Technology
LB	Luria-Bertani
MRSA	Methicillin resistant <i>Staphylococcus aureus</i>
MSCRAMM	microbial surface components recognizing adhesive matrix molecules
PVL	Panton-Valentine Leukocidin
PBS	Phosphate Buffered Saline
PCR	Polymerase chain reaction
PTSAgs	Pyrogenic toxin superantigens
SDS	Sodium dodecyl sulphate
SPE	Streptococcal pyrogenic exotoxins

SSSS	Staphylococcal scalded skin syndrome
STM	Signature-tagged mutagenesis
Tn	Transposon
TNase	Thermostable nuclease
TSST-1	Toxic shock syndrome toxin-1
UV	Ultra violet

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Summary

Staphylococcus aureus is one of the most important pathogens in humans and animals. *S. aureus* is currently the most common cause of infections in hospitalized patients. The bacterium infects diverse tissues and causes a wide spectrum of diseases, suggesting that it possesses a repertoire of distinct molecular mechanisms promoting bacterial survival in disparate *in vivo* environments.

A variety of *in vitro* systems have been developed that simulate certain aspects of the infectious process, enabling the development of screens to study bacterial gene expression and the behaviour of mutant strains in physiological conditions that reflect the situation *in vivo*. *In vitro* assays have been enormously useful and continue to provide much information on the mechanisms of bacterial pathogenesis, but it is obvious that they cannot accurately reproduce all aspects of the host-pathogen interaction. A pathogen may encounter several radically different environments in the host, and it may therefore have very different requirements at various points during infection, particularly in the context of a developing immune response. Consequently, a gene that seems important in *in vitro* studies may not be important *in vivo*, and genes that appear unimportant in an *in vitro* assay may play a critical role during a natural infection.

For these reasons, *in vivo* experimental models are highly desirable. Recently, methods have been developed that enable the identification of genes that are expressed or required for *in vivo* growth. Most of these methods fall into two broad classes. *In vivo* expression technology (IVET) is a promoter-trap strategy designed to identify genes whose expression is induced in a specific environment, typically that encountered in a host. Signature-tagged mutagenesis (STM) uses comparative hybridisation to isolate mutants unable to survive specified environmental conditions and has been used to identify genes critical for survival in the host. Both methods have been used to identify virulence genes in *S. aureus*. The main aim of this project was to find any probable new genes of *S. aureus* that are essential for biofilm formation and infection mouse model by STM.

A library of tagged insertion mutants of *S. aureus* and a series of selected tags in plasmids of *S. aureus* strain RN6390 were used. Most of the experiments with

both the library and selected tags had problems with cross-hybridisation. All the selected tags were therefore sequenced and 33 tags with less than 50% identity were chosen for future experiments. A library of 825 mutants was made with the 33 selected tags. The mutants were arrayed in 25 pools of 33 mutants. Different tests were done to determine that the new library was reliable for a cross-hybridisation free screening. The library was then used in an infection model in mouse and biofilm formation. A total of 12 mutants with significantly reduced signals were sequenced. 7 out of 12 attenuated mutants showed homology to different genes in *S. aureus* and other bacteria. Tetrahydrodipicolinate succinylase homolog, opp-2F, acetoin utilization AcuC protein, phosphate ABC transporter, *dapD*, branched-chain-amino-acid transporter, *pepF*, and *flaR* genes were identified. This work was the first STM screening in a biofilm system, and the *dapD* gene was identified in a biofilm for the first time. 2 out of 12 genes had also been identified in previous STM screens. 5 out of 12 attenuated mutants showed homology to some hypothetical proteins. A hypothetical protein of the same locus was identified in two mutants.

Part I

Introduction

Chapter 1

Introduction

Chapter 1: Introduction

1.1 Microbiology of staphylococci

1.1.1 Morphology

The name *staphylococcus* is derived from the Greek term *staphyle*, meaning “a bunch of grapes”. This name refers to the fact that the cells of these gram-positive cocci grow in a pattern resembling a cluster of grapes. However, staphylococci are spherical cells about 1 μm in diameter arranged in irregular clusters. Single cocci, pairs, tetrads, and chains are also seen in liquid cultures and clinical material. Young cocci stain strongly gram-positive. On ageing, many cells become gram-negative. Staphylococci are non-motile and do not form spores (20, 119).

Staphylococcal species occur worldwide as commensals on the skin of animals and man. They are also found on mucous membranes of the upper respiratory tract and lower urogenital tract and as transients in the digestive tract. They are comparatively stable in the environment. Staphylococcal strains display a selective affinity for particular animal species. Transfer of *S. aureus* strains between animal species and between animals and man is limited (138).

1.1.2 Taxonomy

The genus currently comprises 32 species and 15 serotypes, many of which are found on humans. The species most commonly associated with human disease are *S. aureus* (the most virulent and best-known member of the genus), *S. epidermidis*, *S. saprophyticus*, *S. capitis*, and *S. haemolyticus* (119). *S. aureus* is the only species found in humans that produces the enzyme coagulase, which differentiates it from the other species that are referred to as coagulase-negative staphylococci (20, 119).

1.1.3 Biochemical and metabolic characteristics

Staphylococci are nonmotile, aerobic or facultatively anaerobic, and catalase-positive and grow in a medium containing 10% sodium chloride and at a temperature

ranging from 18°C to 40°C. Relatively simple biochemical tests can be used to differentiate *S. aureus* and the other staphylococci. *S. aureus* has positive reactions for coagulase, heat-stable nuclease, alkaline phosphatase and mannitol fermentation. Differentiation of the coagulase-negative staphylococci is more complex, however, and is not routinely done in many clinical laboratories unless the isolates are demonstrated to be clinically significant (119).

1.1.4 Laboratory identification

Staphylococci grow readily on most bacteriological media under aerobic or microaerophilic conditions. They grow most rapidly at 37°C but form pigment best at room temperature (20-25°C). The most important staphylococcal species is *S. aureus*, which is named for its yellow-pigmented colonies (*aureus* = golden) as the result of the carotenoid pigments that form during their growth. Colonies on solid media are round, smooth, raised, and glistening. *S. aureus* usually forms grey to deep golden yellow colonies. *S. epidermidis* colonies usually are grey to white on primary isolation; many colonies develop pigment only upon prolonged incubation. No pigment is produced anaerobically or in broth. Various degrees of haemolysis are produced by *S. aureus* and occasionally by other species. *Peptostreptococcus* species, which are anaerobic cocci, often resemble staphylococci in morphology (20, 119, 160).

1.2 Epidemiology

Staphylococci are ubiquitous: All persons have coagulase-negative staphylococci on their skin, and transient colonization of moist skin folds with *S. aureus* is common. Colonisation of umbilical stump, skin, and perineal area of neonates with *S. aureus* is common. *S. aureus* and coagulase-negative staphylococci are also found in the oropharynx, gastrointestinal tract, and urogenital tract. Short-term or persistent *S. aureus* carriage in older children and adults is more common in the anterior nasopharynx than in the oropharynx. Approximately 15% of normal healthy adults are persistent nasopharyngeal carriers of *S. aureus*, with a lighter incidence reported for hospitalised patients, medical personnel, persons with eczematous skin diseases, and those who regularly use needles either illicitly or for medical reasons. Although *S.*

aureus rarely causes problems in otherwise healthy people, it is commonly associated with infections of prosthetic devices and surgical wounds, bacteraemia, endocarditis and pneumonia in hospitalised patients. Many of these infections start locally (e.g., skin or catheter infections) then spread to the bloodstream, thus putting patients at risk of developing endocarditis and other metastatic complications (91, 99, 119).

Because staphylococci are found on the skin and in the nasopharynx, shedding of the bacteria is common and is responsible for many hospital-acquired infections. Some characteristics of the staphylococci account for their pathogenicity, which takes many forms. They grow comparatively well under conditions of high osmotic pressure and low moisture, which partially explains why they can grow and survive in nasal secretions (many people carry the bacteria in their noses) and on the skin. This also explains how *S. aureus* can grow in some foods with high osmotic pressure (such as ham and other cured meats) or in low-moisture foods that tend to inhibit the growth of other organisms. The yellow pigment probably confers some protection from the antimicrobial effects of sunlight. Staphylococci are susceptible to high temperatures, as well as to disinfectants and antiseptic solutions. The organisms can survive on dry surfaces for long periods. The organisms can be transferred to a susceptible person either through direct contact or through contact with fomites (e.g., contaminated clothing, bed linens)(119, 160).

Staphylococci are among the most common causes of hospital-acquired infections, including bacteraemia, surgical site infections, pneumonia, and urinary tract infections (50, 51, 121). In hospitals, the areas at highest risks for severe staphylococcal infections are the newborn nursery, intensive care units, operating rooms, and cancer chemotherapy wards. Massive introduction of “epidemic” pathogenic *S. aureus* into these areas may lead to serious clinical disease. Personnel with active *S. aureus* lesions and carriers may have to be excluded from these areas (20), or at least, medical personnel must use proper hand-washing techniques to prevent the transfer of staphylococci from themselves to patients or among patients (119).

S. aureus causes disease through the production of toxin or through the direct invasion and destruction of tissue. The clinical manifestations of some staphylococcal diseases such as impetigo, staphylococcal scalded skin syndrome (SSSS), staphylococcal food poisoning and toxic shock syndrome (TSS) are almost exclusively the result of toxin activity, whereas other diseases result from the proliferation of the organisms, leading to abscess formation and tissue destruction.

1.3.1.2 Beta toxin

β toxin, also called sphingomyelinase C, is a 35 KDa heat-labile protein produced by most strains of *S. aureus*. This enzyme has a specificity for sphingomyelin and lysophosphatidylcholine and is toxic to a variety of cells, including erythrocytes, leukocytes, macrophages, and fibroblasts. It catalyses the hydrolysis of membrane phospholipids in susceptible cells, with lysis proportional to the concentration of sphingomyelin exposed on the cell surface. This is believed to be responsible for the differences in species susceptibility to the toxin. Beta-toxin displays species-dependent activity. Sheep, cow, and goat erythrocytes are most sensitive. Human erythrocytes are intermediate in sensitivity, whereas murine and canine erythrocytes are resistant. The degree of erythrocytes sensitivity depends on membrane sphingomyelin content. The role of β toxin in human disease remains to be proved; however, together with α toxin, it is believed to be responsible for the tissue destruction and abscess formation characteristic of staphylococcal diseases (51, 119).

1.3.1.3 Gamma-toxin

Gamma-toxin is able to lyse human, sheep, and rabbit erythrocytes as well as human lymphoblastic cells. Gamma-toxin, leukocidin, and other bicomponent toxins are a family of proteins encoded by *hlg* and *luk-PV* loci. All of the toxins in this family contain two synergistically acting proteins: one S component (LukS-PV, HlgA, or HlgC) and one F component (LukF-PV or HlgB) designated on the basis of their mobility (slow or fast) in ion-exchange chromatography. The prototype bicomponent toxins are the closely related Panton-Valentine leukocidin ((PVL) and gamma-toxin. The PVL S and F components are LukS-PV and LukF-PV. Gamma-toxin likewise contains S and F components designated HlgA and HlgB, respectively (51). Synergistic function involves sequential binding of the F and S components. The action of gamma-toxin on erythrocytes involves initial binding of HlgB (F) followed by HlgA (S) and subsequent generation of a pore (38, 44).

Studies have shown that the genes for gamma-toxin are transcribed from a single locus, located on a 4.5-kb *ScaI*-digested chromosomal fragment. Extracts from a clone containing this fragment were haemolytic and leukotoxic. The open reading frames, in order, are named *hlgA*, *hlgC*, and *hlgB*, and they correspond to previous identified

gamma-toxin genes. *hlgC* and *hlgB* are transcribed on a single mRNA, while *hlgA* is separately expressed (45).

1.3.1.4 Delta toxin

δ toxin is a 3 KDa polypeptide produced by almost all *S. aureus* strains and the majority of other staphylococci. The toxin has a wide spectrum of cytolytic activity, affecting erythrocytes, other eukaryotic cells, many other mammalian cells, organelles, and spheroplasts and protoplasts, as well as intracellular membrane structures. This relatively non-specific membrane toxicity is consistent with the belief that the toxin acts as a surfactant disrupting cellular membranes by means of a detergent-like action. It has also been reported to be dermonecrotic and lethal in experimental animals when used in high concentration. The activity of delta-toxin is inhibited by phospholipids (36, 119).

δ toxin is a 26-residue long peptide encoded by the *hlg* gene with peak production at the end of the exponential growth phase and can be purified by different procedures (17). The toxin has different affinity for different cells such as neutrophils, monocytes, lymphocytes, and erythrocytes (3). The toxin acts by formation of pores in the membrane, leading to the lysis of erythrocytes and other mammalian cells. At least two variants of δ toxin exist; toxins expressed by human and canine strains of *S. aureus* are only 62% identical and are immunologically distinct (51).

1.3.1.5 Leukocidin

S. aureus can express a toxin that specifically acts on polymorphonuclear leukocytes. Phagocytosis is an important defence against staphylococcal infection so leukocidin should be a virulence factor. Its name arises from the fact that it can kill leukocytes (of which neutrophils are one type). Panton-Valentine Leukocidin (PVL) consists of two protein components, S and F. The S component is like the B part of an A-B toxin in that it binds to G_{M1} gangliosides. However, both have enzymatic activity, involved in phospholipids and phosphatidylinositol metabolism, respectively. Phosphatidylinositol, an important signalling molecule in eukaryotic cells, controls a number of cellular processes. Thus, the action of this two-component toxin appears to alter phospholipid metabolism and cause disruption of normal cellular activities (36,

141). The two components of the PVL bind sequentially to human neutrophils, although most reports indicate that the S component binds first and subsequently forms pores (31).

1.3.1.6 Exfoliative toxins A and B

Two serologically distinct exfoliative toxins have been described, designated exfoliative toxin A (ETA) and exfoliative toxin B (ETB). These toxins cause SSSS, usually seen in new-borns and characterised by intraepidermal separation of layers of the skin at the desmosomes. The illness begins abruptly with a generalised erythema, often near the mouth and spreading over the entire body in the course of a few days. When the skin is lightly rubbed, the epidermal layer wrinkles irreversibly, giving rise to the characteristic positive Nikolsky sign. Later, large flaccid sterile bullae appear that lead to separation through the stratum granulosum layer. The entire sequence from disease onset to recovery is 7 to 10 days. There is no permanent scarring on areas of the skin, and the causative toxins are not themselves lethal to the host (36).

1.3.1.7 Toxic shock syndrome toxin-1

TSS is characterised by fever, hypotension, and rash followed by desquamation and the involvement of multiple organ systems. It is toxin mediated. Toxic shock syndrome toxin-1 (TSST-1), formerly called pyrogenic exotoxin C and enterotoxin F, is an exotoxin secreted during the growth of some strains of *S. aureus* and can reproduce most of the clinical manifestations of TSS in an experimental rabbit model (rash and desquamation are not seen). TSST-1 has not been found in staphylococcal isolates from all patients with TSS, but most of these isolates not producing TSST-1 are reported to produce enterotoxin B. The role of this second toxin in TSS has not been adequately defined. Coagulase-negative staphylococci can also produce TSS (118).

1.3.1.8 Enterotoxins

Staphylococcal enterotoxins (SEs) belong to a large family of staphylococcal and streptococcal pyrogenic exotoxins (PT), sharing common phylogenetic relationships,

structure, function, and sequence homology. The PT family is composed of SEs, TTST-1, and streptococcal pyrogenic exotoxins (SPE). Nine serologically distinct staphylococcal enterotoxins (SEA through SEE and SEG through SEJ) have been identified (14). The enterotoxins are resistant to hydrolysis by gastric and jejunal enzymes and are stable to heating at 100°C for 30 minutes. Thus once a food product has been contaminated with enterotoxin-producing staphylococci and the toxins have been produced, reheating the food will not be protective. Staphylococcal food poisoning is the leading cause of food-borne microbial intoxication worldwide and is usually linked to improper storage of food (76). The SEs are stable in the gastrointestinal tract and indirectly stimulate the emetic reflex centre. Although the vagus nerve is involved, molecular events involved are unclear. There is mounting evidence that mast cell activation occurs and that inflammatory mediators and neuropeptide substance P are released upon SE activity in the gastrointestinal tract and elsewhere (2, 145). *S. aureus* and *S. epidermidis* produce these toxins, with 30% to 50% of all *S. aureus* strains producing an enterotoxin (118).

1.3.2 Staphylococcal enzymes

1.3.2.1 Coagulase

Coagulase production is the principal criterion used in the clinical microbiology laboratory for the identification of *S. aureus*. Although a few strains of *S. aureus* do not produce detectable amounts of coagulase, all strains seem to possess a coagulase gene (*coa*) (163). *S. aureus* strains possess two forms of coagulase: bound (also called clumping factor) and free. Coagulase bound to the staphylococcal cell wall can directly convert fibrinogen to insoluble fibrin and cause the staphylococci to clump. The cell-free coagulase accomplishes the same result by reacting with a globulin plasma factor (coagulase-reacting factor) to form staphylothrombin, a thrombinlike factor. This factor catalyses the conversion of fibrinogen to insoluble fibrin. Coagulase is used as a marker for the virulence of *S. aureus*. The role of coagulase in the pathogenesis of disease is speculative, but coagulase may cause the formation of a fibrin layer around a focal staphylococcal abscess, thus localising the infection and protecting the organisms from phagocytosis. However, coagulase-negative mutants were less virulent than the parental strain in a mouse model of blood-borne

staphylococcal pneumonia (144). This suggests that coagulase may be more important in some types of infections than in others (51, 118).

1.3.2.2 Catalase

All staphylococci produce catalase, which catalyses the conversion of toxic hydrogen peroxide to water and oxygen. Hydrogen peroxide can accumulate during bacterial metabolism or after phagocytosis (118). Different toxic forms of oxygen are produced as inadvertent by-products during the reduction of O_2 to H_2O in respiration. These reactive oxygen intermediates have many deleterious effects on living organisms, ranging from DNA strand damage to peroxidation of membrane lipids. Bacteria have evolved enzymes that destroy toxic oxygen products. The most common enzyme in this category is catalase, which attacks hydrogen peroxide (H_2O_2)(80, 102).

1.3.2.3 Hyaluronidase

Hyaluronidase hydrolyses hyaluronic acids, the acidic mucopolysaccharides that hold together certain cells of the body, particularly cells in the connective tissue. This digesting action is thought to be involved in the tissue blackening of infected wounds and to help the microorganism spread from its initial site of infection. For therapeutic use, hyaluronidase may be mixed with a drug to promote the spread of the drug through a body tissue. More than 90% of *S. aureus* strains produce this enzyme (118, 160).

1.3.2.4 Fibrinolysin

Fibrinolysin, also called staphylokinase, is produced by virtually all *S. aureus* strains and can dissolve fibrin clots. Staphylokinase is distinct from the fibrinolytic enzymes produced by streptococci (36).

1.3.2.5 Lipases

All strains of *S. aureus* and more than 30% of the strains of coagulase-negative staphylococci produce several different lipases. As their names implies, these enzymes hydrolyse lipids, an essential function to ensure the survival of staphylococci in the sebaceous areas of the body. It is believed that these enzymes must be present for staphylococci to invade cutaneous and subcutaneous tissues and for superficial skin infections such as furuncles (boils), and carbuncles to develop. Lipases break fats down into their fatty acid and glycerol components. Each component is then metabolised separately (118, 160).

1.3.2.6 Nuclease

A thermostable nuclease (TNase) is produced by nearly all strains of *S. aureus* and has been used as a diagnostic criterion for this species. TNase hydrolyses single- and double-stranded DNA and RNA at the 5' position of phosphodiester bonds by a calcium-dependent mechanism. The role of this enzyme in the pathogenesis of infection is unknown (51, 118).

1.3.2.7 Penicillinase

More than 90% of staphylococcal isolates were susceptible to penicillin in 1941, the year the antibiotic was first used clinically. However, resistance to penicillin quickly developed, primarily because the organisms could produce penicillinase (beta-lactamase). The enzyme cleaves the β -lactam ring of the penicillin molecule. The widespread distribution of this enzyme was ensured by its presence on transmissible plasmids (118, 160).

1.3.3 Other factors

1.3.3.1 Capsule

A loose-fitting, polysaccharide layer (slime layer) is only occasionally found on staphylococci cultured *in vitro*, but it is believed to be more commonly present *in*

vivo. More than 90% of clinical isolates of *S. aureus* produce capsular polysaccharides. Eleven capsular serotypes have been identified in *S. aureus*, with serotypes 5 and 8 associated with the majority of infections (136). These capsules can also be divided into two distinct groups on the basis of colony morphology. Mucoid-type capsules include the serotype 1 and 2 capsules; strains producing these capsules are heavily encapsulated and are mucoid on solid medium. Microcapsules include the remaining serotype 3 to 11 capsules; strains with these capsules have a thin capsular layer and form non-mucoid colonies on solid medium. Studies showed that the mucoid-type capsules were important antiphagocytic virulence factors that masked C3b deposited on the bacterial cell wall, preventing its recognition by receptors on phagocytic cells. The capsule protects the bacteria from polymorphonuclear leukocytes, as well as by inhibiting the proliferation of mononuclear cells after mitogen exposure. It also facilitates the adherence of bacteria to catheters and other synthetic material (e.g., grafts, shunts, prosthetic valves and joints). This property is particularly important for the survival of relatively avirulent coagulase-negative staphylococci (51, 119). It has been shown that *S. aureus* strains that are defective in microcapsule production are more efficient at inducing experimental infective endocarditis (IE) than their parental strains, suggesting that the microcapsule may obscure important surface-expressed cell wall proteins involved in IE pathogenesis (36).

1.3.3.2 Protein A

The surface of most *S. aureus* strains (but not the coagulase-negative staphylococci) is uniformly coated with protein A. This protein is covalently linked to the peptidoglycan layer and has a unique affinity for binding to the Fc receptor of immunoglobulin (Ig)G₁, IgG₂, and IgG₄, thus effectively preventing the antibody-mediated immune clearance of the organism. The importance of protein A in staphylococcal infections is somewhat unclear. It is possible that, by binding IgG, protein A could interfere with the phagocytosis of opsonized bacteria via receptors on the host cell. Extracellular protein A can also binds antibodies, thereby forming immune complexes with the subsequent consumption of the complement. Recent studies has showed that protein A can mediate bacterial adherence to von Willebrand

Such diseases include cutaneous infections, endocarditis, pneumonia, empyema, osteomyelitis, and septic arthritis. In the presence of a foreign body like a splinter, catheter, shunt, prosthetic valve or joint, significantly fewer staphylococci are necessary to establish disease. Likewise, patients with congenital diseases associated with an impaired chemotactic or phagocytic response, such as Job-Buckley syndrome, Wiskott-Aldrich syndrome, and chronic granulomatous disease, are more susceptible to staphylococcal diseases (51, 119).

1.3 Virulence factors of staphylococci

1.3.1 Staphylococcal toxins

1.3.1.1 Alpha toxin

α toxin (α haemolysin) which is encoded on the bacterial chromosome, is a 33 KDa polypeptide that is produced by most strains of *S. aureus*. The toxin disrupts the smooth muscle in blood vessels and is toxic to many types of cells, including erythrocytes, leukocytes, hepatocytes, platelets, and cultivated cells. It becomes integrated in the hydrophobic regions of host cell membrane, leading to formation of 1- and 2-nm ring-shaped pores (51). The rapid efflux of K^+ and influx of Na^+ , Ca^{2+} , and other small molecules leads to osmotic swelling and cell lysis. The sensitivity to this varies for animal species (rabbit erythrocytes are 1000-fold more sensitive than are human cells) and cell type (e.g., fibroblasts can repair membrane damage more effectively than can erythrocytes). Cells such as rabbit erythrocytes are suspected to have high-affinity binding sites, while other cells such as human erythrocytes have only low-affinity sites (75). α toxin is believed to be an important mediator of tissue damage in staphylococcal disease (119). α toxin is also an important cause of damage to the air-blood barrier in *S. aureus*-induced pneumonia (109). Most *S. aureus* isolates possess *hla*, the structural gene for alpha-toxin, but are variable in its expression or in the amount of toxin expressed. Work with mutants in animal models indicated that strains expressing alpha-toxin are more virulent than isogenic derivatives (51).

factor, an extracellular matrix protein important in normal hemostasis, suggests that protein A may play additional roles in the infectious process (51).

The presence of protein A has been exploited in some serological tests, in which protein A-coated *S. aureus* is used as a non-specific carrier of antibodies directed against other antigens. Additionally, detection of protein A can be used as a specific identification test for *S. aureus* (119).

1.4 Regulation of virulence determinants

S. aureus surface proteins, such as adhesins and protein A, a major surface antigen, are produced early in growth *in vitro* and their production is down-regulated at a later time. Most secreted proteins, such as toxins, haemolysins, and tissue-degrading enzymes, are produced at the end of exponential phase. The production of all of these factors (the virulence response), is controlled primarily by a global regulatory locus *agr* (42), which has been shown in several experimental infection models to be required for virulence (47, 48, 134).

The *agr* locus (66, 70-72) consists of two divergent transcription units using promoters P2 and P3 (figure 1.1)(126). The P2 operon contains four genes, *agrA*, *B*, *C*, and *D*. Genes *agrA* and *C* represent a classic two-component signal transduction pathway, of which *agrC* encodes the signal receptor and *agrA* the response regulator (82, 98, 101). The other two genes, *agrB* and *D*, combine to produce the autoinducer, a small peptide that is the ligand that binds to and activates AgrC (30). *AgrD* encodes the autoinducer propeptide (30) and *AgrB* encodes a protein that is required for processing and possibly for secretion of the autoinducer (104). AgrC is a transmembrane protein that is phosphorylated at a conserved histidine residue in response to the autoinducer *in vitro* (112). The last extracellular loop of AgrC is sufficient for binding of the autoinducer and the subsequent activation by phosphorylation. The primary role of the *agrAC* signaling pathway is to up-regulate both of the major *agr* promoters, P2 and P3, which it does in conjunction with a second transcription factor, SarA (114, 118). It may also up-regulate the coagulase gene (125), but does not directly regulate any of the other known *agr*-dependent genes. The P3 operon specifies a 512 nucleotide transcript, RNA III, that is the effector molecule of the *agr* regulon (70, 71), and happens to encode the 26 amino acid δ -hemolysin which is not involved in regulation. RNA III up-regulates

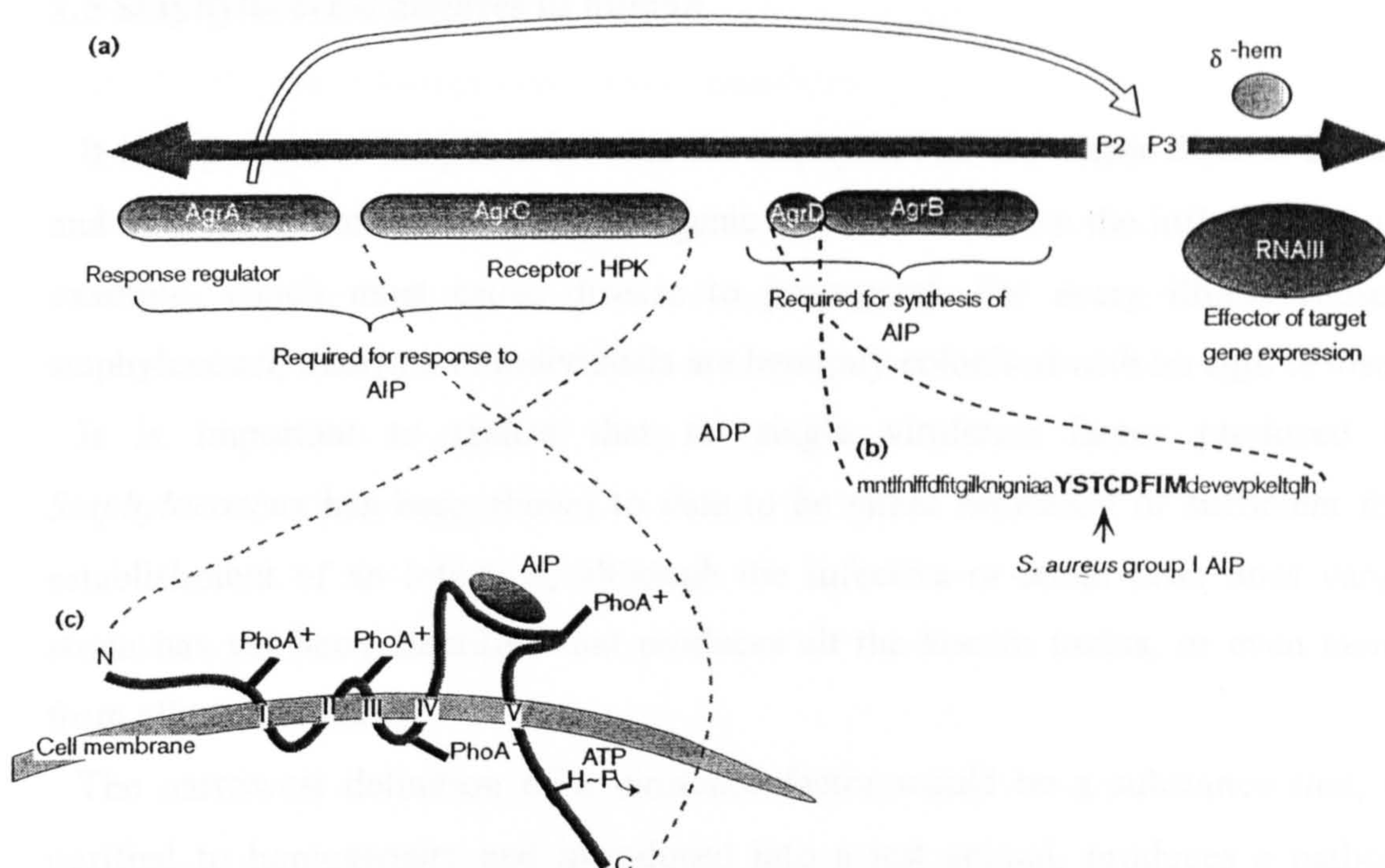


Figure 1.1 The *agr* system. (a) A schematic diagram, showing the two divergent *agr* promoters (P2 and P3) and their transcripts. The P2 operon contains four genes, *agrA*, *B*, *C*, and *D*. Two of these, *agrC* and *agrA*, represent a two-component signal transduction pathway and the other two, *agrB* and *D*, combine to generate a peptide that is the activation ligand for the signal receptor. The function of the signalling pathway is to activate the two *agr* promoters so that there are two-levels of positive feedback (open arrow). The P3 transcript, RNA III, regulates transcription of 30 or more unlinked target genes, including *hla* the α-hemolysin gene, by an unknown mechanism. (b) A blow-up of *AgrD*, showing the amino acid sequence of the autoinducing peptide (AIP) (in bold) that serves as activating ligand for the pathway by binding to *AgrC*. (c) A blow-up of *AgrC*, showing five transmembrane helices, predicted by sequence analysis and confirmed by *phoA* fusions. Deletion analysis suggests that the last extracellular loop is the site of AIP binding, which causes the predicted phosphorylation of a conserved histidine (H) in the carboxy-terminal cytoplasmic domain (126).

transcription of the secretory protein genes and down-regulates transcription of the surface protein genes by an as yet unknown mechanism that probably involves proteins encoded by genes outside of the *agr* locus. RNA III also and independently regulates translation of at least one product α -hemolysin by complementary pairing with the *hla* mRNA leader region, which, in the absence of RNA III, folds into an untranslatable configuration (70, 133).

1.5 Staphylococcal diseases in human

It is important to keep in mind that the staphylococci that cause disease in humans and animals are not inherently pathogenic organisms (unlike the influenza virus, for example, which must cause disease to propagate). For every illness caused by staphylococci, many more individuals are benignly colonised with no sign of disease.

It is important to realise that no single virulence factor produced by a *Staphylococcus* has been shown to date to be either necessary or sufficient for the establishment of an infection, although the infective or lethal dose does vary. No strain has yet been identified that produces all the known toxins, or even members from all toxin families.

The narrowest definition of a virulence factor would be a substance that, when purified to homogeneity and introduced into a test animal, produces a pathogenic effect. Using that definition, most of the virulence factors especially those involved in attachment and probably most of the degradative enzymes (e.g., lipases, proteases, and hyaluronidase) would not be considered true virulence factors. In the broadest sense, any substance that is exported out of the cytoplasm either to the cell surface or into the extracellular environment of the *Staphylococcus* can be thought of as a potential virulence factor. Clearly, those substances that are capable of interacting with the host may affect that host in some deleterious way. This second definition is probably too broad and in many cases it is possible that the production of at least some of these factors may actually assist the host in combating an infection (36).

1.5.1 Bacteraemia and infective endocarditis

S. aureus and coagulase-negative staphylococci (CoNS) are ideal infective endocarditis (IE) pathogens in that they possess a number of virulence factors, which

enable them to establish IE. Acute endocarditis caused by *S. aureus* is a serious disease, with a mortality rate approaching 50%. Although patients with *S. aureus* endocarditis may initially have non-specific influenza-like symptoms, their condition can deteriorate rapidly and include disruption of cardiac output and peripheral evidence of septic embolization. Unless appropriate medical and surgical intervention is instituted immediately, the patient's prognosis is poor. In addition, *S. aureus* continues to be an important cause of both community-acquired (CA) and hospital-acquired (HA) bacteraemia. Commonly associated predisposing medical conditions include diabetes mellitus and renal insufficiency. Most patients with *S. aureus* bacteraemia have an obvious primary focus of infection, usually an intravascular catheter. Overall mortality in patients with *S. aureus* bacteraemia remains high, particularly in the absence of a removable primary focus, older age patients, rapidly fatal comorbid conditions, underlying respiratory or cardiac disease, associated infective endocarditis (IE), and septic shock. Methicillin-resistant *S. aureus* (MRSA) bacteraemia is a growing problem globally (36, 119).

1.5.2 Wound infections

Staphylococcal infections of the skin can be minor or life threatening, depending on the integrity of the skin surface and the invasiveness of the bacterial strain. Staphylococci are the most common species recovered from patients who develop a wound infection after surgery, with *S. aureus* and the CoNS rank first and third among aerobic bacteria associated with wound infections, respectively. In general, staphylococci that cause wound infections come from the flora of the patient or the personnel performing the procedure. For *S. aureus*, studies have shown that the patient's own flora serves as the source of the infecting bacteria in about one-half of cases of surgical infections (36, 51).

1.5.3 Infections of intravascular catheters and vascular devices

Although intravascular catheters and vascular grafts have revolutionised the practice of medicine for the betterment of patients, unfortunately they are associated with a significant incidence of infection. Staphylococci, both *S. aureus* and CoNS, play a pre-eminent role in these infections. Because of their unique environmental niche on

the skin and mucus membranes of humans and their ability to adhere to biomaterials and cause infection, they will undoubtedly continue to cause significant morbidity and mortality as the use of prosthetic medical devices increases. From 20% to 65% of all infections of catheters and shunts are caused by CoNS. The CoNS are particularly well adapted for causing these infections because they can produce a polysaccharide slime that bonds them to catheters and shunts and protects them from antibiotics and inflammatory cells. A persistent bacteraemia is generally observed in patients with infections of shunts and catheters because the organisms have continual access to the blood stream (36, 119).

1.5.4 Skin and soft tissue infections

Staphylococci are inhabitants of the skin and mucous membranes of mammals and birds, where they exist saprophytically. However, factors ranging from tight clothing or scratching to immunosuppression or insertion of a prosthesis alter the host-parasite relationship and cause disease. Colonisation, particularly of the nares by *S. aureus*, leads to hand carriage, and from the hands, the organisms are frequently spread to other areas of the body. Thus, staphylococci often follow a nose to hands to wounds route of infection (172). The variety of enzymes and toxins produced, especially by *S. aureus*, results in a spectrum of disease, part of which may be a direct expression of the toxin damage, but also a result of a variety of pathogenicity factors acting in concert. Purulent skeletal muscle infection (pyomyositis), folliculitis and furuncles, recurrent furunculosis, impetigo, botryomycosis and secondary cutaneous infection in patients with eczema are some staphylococcal infections due to *S. aureus* (36).

1.5.5 Central nervous system

The staphylococci are important causative organisms of cerebrospinal fluid (CSF) shunt infections, acute purulent meningitis, brain abscess associated with trauma, spinal and cranial subdural empyema and epidural abscess. CoNS are the etiologic organisms in most CSF shunt infections. *S. aureus* is the second most common pathogen. The staphylococci typically enter the shunt system from a contaminated wound or from the patient's skin surface at operation.

S. aureus is the causative organism in most cases of staphylococcal meningitis that develop as a complication of a neurosurgical procedure, from a parameningeal focus of infection or a congenital anomaly or during the course of staphylococcal bacteraemia or endocarditis. *S. aureus* is a common causative organism of brain abscesses that develops as a result of cranial trauma, either penetrating wounds of the brain or craniotomy. *S. aureus* is the causative organism in most cases of spinal subdural empyema and in 15 to 25 percent of cases of cranial subdural empyema. CoNS are isolated on rare occasion from cranial subdural empyemas. *S. aureus* is the causative organism in 50 to 60 percent of spinal epidural abscess and in most cranial epidural abscesses that occur as a complication of craniotomy or compound skull fracture (36).

1.5.6 Diseases of the eye

Staphylococcal diseases of the eye include both infections and expression of cell-mediated immunity (CMI) to staphylococcal antigens. The pathogenesis of diseases due to both *S. aureus* and CoNS has been increasingly well-characterised in recent years (36). A model of staphylococcal keratitis in guinea pig has been described (41), and a similar model in rats was used to determine the effects of antibiotics and corticosteroid treatments on the progression of *S. aureus* infection of the cornea (10). Radial incision of the rabbit conjunctiva followed by inoculation of *S. aureus* resulted in purulent conjunctivitis and a model for testing antimicrobial treatments in this disease (16).

1.5.7 Osteomyelitis and other infections of bones and joints

Colonization of the bones and joints by *S. aureus* may occur as a result of either hematogenous infection or it can be a secondary infection resulting from trauma or the extension of disease from an adjacent area (51, 119). During the 1970s, most cases of osteomyelitis were caused by strains of *S. aureus*. During the 1990s, although the incidence of osteomyelitis caused by gram-negative pathogens has increased dramatically, staphylococci are still the most commonly isolated pathogens in most types of osteomyelitis. *S. aureus* is the most frequent single pathogen in adults,

accounting for 50 to 70 percent of cases of osteomyelitis. The estimated frequency of *S. aureus* in pyogenic osteomyelitis in all age groups may reach 80 to 90 percent (36).

S. aureus is the primary cause of septic arthritis in young children and in adults who are receiving intra-articular injections or who have mechanically abnormal joints. Secondary involvement of multiple joints is indicative of hematogenous spread from a localised focus. Staphylococcal arthritis is characterised by a painful, erythematous joint, with purulent material obtained on aspiration. Infection is usually demonstrated in the large joints (e.g., shoulder, knee, hip, and elbow). The prognosis in children is excellent, but in adults, it depends on the nature of the underlying disease as well as the occurrence of any secondary infectious complications (119).

1.5.8 Respiratory tract infections

S. aureus, in distinct contrast to the CoNS, is a significant cause of respiratory tract disease. *S. aureus* respiratory disease can develop after the aspiration of oral secretions or from the hematogenous spread of the organism from a distant site. Aspiration pneumonia is seen primarily in the very young, the aged, and patients with cystic fibrosis, influenza, chronic obstructive pulmonary disease, and bronchoiectasis. Hematogenous pneumonia is common for patients with bacteraemia or endocarditis. While *S. aureus* is not among the most frequent of isolates from patients with sinusitis, bronchitis, or pneumonia, it is isolated from patients with these illnesses. Although *S. aureus* is an uncommon cause of severe community-acquired pneumonia, it is responsible for perhaps 20 to 30 percent of cases of nosocomial pneumonia (36, 51, 119).

1.5.9 Urinary tract infections

Although staphylococci are not commonly thought of as causes of urinary tract infection (UTI), *S. aureus* may cause UTI. The infections range from asymptomatic bacteriuria to infections that are severe and associated with bacteraemia. Given the relative frequency with which the perineal area is colonised with *S. aureus*, it is perhaps surprising that this organism is relatively uncommonly recovered as a cause of UTI (36). *S. saprophyticus* has a predilection for causing UTI in young, sexually

active women and is rarely responsible for infections in other patients. It is also infrequently found as an asymptomatic colonizer of the urinary tract (119).

1.5.10 Toxin-mediated syndromes

Three diseases are associated with specific toxins: (i) toxic shock syndrome (TSS), caused by toxic shock syndrome toxin type 1 (TSST-1), (ii) food poisoning, by staphylococcal enterotoxins; and (iii) staphylococcal scalded skin syndrome (SSSS) by exfoliative toxins. Two other diseases are probably related to staphylococcal toxins: staphylococcal enterocolitis, caused by staphylococcal enterotoxins or δ -toxin; and severe wound infections associated with α -toxin (36).

Staphylococcal toxic shock syndrome (TSS) is an illness that is the result of the protean effects of one or more toxins of *S. aureus* (36). Staphylococcal TSS may manifest in either of two general forms, menstrual or nonmenstrual. Menstrual TSS occurs in women whose vaginal/cervical mucosa are colonised by TSST-1-producing *S. aureus*. Tampon use is a risk factor in menstrual TSS, and a correlation between tampon absorbency and risk of developing TSS has been established. TSS acquired in this way is difficult to reproduce in animals, although some success has been achieved with a simulation of tampon use in rabbits. Attempts have also been made to induce TSS or a TSS-like syndrome by other means in laboratory animals (137), but a definitive model for this disease has not been forthcoming. Nonmenstrual TSS may result from *S. aureus* infection elsewhere in the body. Either staphylococcal enterotoxins (SEs) or TSST-1 may mediate the nonmenstrual form (51). TSST-1 in the bloodstream can trigger a massive release of cytokines that cause shock and death. In animals, TSST-1 makes the animal hypersusceptible to LPS, which may enter the bloodstream regularly in small amounts because of lysis of gram-negative bacteria in the intestine. However, LPS might not even be the most important molecule whose action is potentiated by TSST (141).

Staphylococcal food poisoning, one of the most common foodborne illnesses, is an intoxication rather than an infection. Disease is caused by bacterial toxin present in food rather than from a direct effect of the organisms on the patient. Certain strains of *S. aureus* can also cause enterocolitis, which is manifested clinically by watery diarrhoea, abdominal cramps, and fever. Enterocolitis occurs primarily in patients

who have received broad-spectrum antibiotics, which suppress the normal colonic flora and permit the growth of *S. aureus* (119).

Staphylococcal scalded skin syndrome (SSSS) is characterised by the abrupt onset of a localised perioral erythema (redness and inflammation around the mouth) and formation of the bullae or skin blisters and a potential for widespread peeling. The lesions cover the entire body within 2 days. The disease is predominant in children. Bullous impetigo is a localised form of SSSS. Specific strains of toxin-producing *S. aureus* are associated with the formation of superficial skin blisters. Unlike patients with the disseminated manifestations of SSSS, patients with bullous impetigo have localised blisters that are culture-positive (51, 113, 119).

1.6 Veterinary aspects of staphylococci

The first systematic study of pathogenic staphylococci was done by Rosenbach in 1884. The association of staphylococci with veterinary medicine began with the isolation of the organisms from mastitis in sheep by Nocard in 1887. In 1890 Guillebeau reported that staphylococci were responsible for mastitis in cattle. Thus within six years of the first systematic study of pathogenic staphylococci in 1884, the importance of staphylococci in veterinary medicine was established (48). *S. aureus*, *S. intermedius*, and the coagulase-variable *S. hyicus* are important pathogens of domestic animals. Although coagulase-negative staphylococci are usually of low virulence, some occasionally cause disease in animals (138).

1.6.1 Bovine mastitis

Mastitis is an inflammatory reaction of the mammary gland. The most important mastitis pathogen, worldwide, is *S. aureus*. Staphylococcal mastitis may be subclinical, or clinical. The majority of infections are subclinical. In the clinical form, the disease may be peracute, acute, or chronic. Peracute and gangrenous forms are associated with severe systemic reactions and can be life threatening. The peracute form, which is rare, is caused by *S. aureus*, often shows a gangrenous udder, and is frequently fatal. In gangrenous mastitis the affected quarter, which becomes cold and blue-black, eventually sloughs. Tissue necrosis is attributed to the alpha-toxin which causes contraction and necrosis of smooth muscle in blood vessel walls, impeding

blood flow in the affected quarter. In addition, this toxin causes release of lysosomal enzymes from leukocytes. Usually, staphylococcal mastitis begins with an acute episode, including some or all of the cardinal signs of inflammation in the udder: tumour, rubor, calor, and dolor and functio laesa. Systemic signs of disease are not always seen. If not successfully treated, the acute form becomes chronic, with few clots in the milk, or with no clinical signs; the disease subsequently passes into a subclinical, chronic form. An elevated somatic cell count and isolation of bacteria from the milk can only detect this form of mastitis (66, 138).

The route of infection in staphylococcal mastitis is via the teat, and the milking machine is considered to be the most important predisposing factor. However, staphylococcal mastitis does occur in hand-milked cows, in suckled cows, and during the dry period. The bacteria pass the teat duct into the teat and udder cisterns, and subsequently into the milk ducts. They may subsequently establish in an area of secretory tissue. *S. aureus* and other mastitis pathogens, at 37°C, are able to rise with the milk fat. The phenomenon seems to involve IgA antibodies associated with membranes of fat globules in previously infected animals. This phenomenon may aid to invade upper parts of the milk ducts and udder.

The mechanism of penetration via the teat duct is not fully understood, but it is suggested that the organisms gain entrance to the teat cistern by propulsion and/or by bacterial growth. The staphylococci colonise the tip of the teat, especially in areas of teat lesions or erosions. Colonisation often precedes an intramammary infection but does not always result in mastitis. Staphylococci can colonize the teat duct for a prolonged period of time. In the intact teat, the organisms probably exist as distinct microcolonies in the stratum corneum, as they do in other areas of skin. When contact is made between the staphylococci and milk, then a medium is provided in which the staphylococci can grow into the gland. The nearer the organisms are to the milk in the teat cistern, the greater is the risk for mammary infection to occur. Once the organisms pass the barriers of the teat duct, adherence to epithelial cells is thought (but still not proven) to be the next critical step in pathogenesis. Adherence is believed to involve a combination of cation-bridging, hydrophobic interactions, and bacterial adhesin-specific epithelial surface receptors. It has been shown that the ability of *S. aureus* to bind to epithelial cells of the ductules and alveoli in the bovine mammary gland is an important virulence factor, and that antibodies against whole cells inhibit the adherence.

Once established on the surface, nucleases and phosphatases degrade pus into substances that can be utilised as nutrients for the bacteria. Although several potential virulence determinants have been purified, or partly purified, and tested for toxicity in different experimental systems, their relative importance as virulence determinants is not known (66).

The mouse model of intramammary inoculation has been used extensively in studying this disease and represents a reproducible and cheap simulation of the disease in dairy cattle (26). Various attempts have been made in the past to design vaccines directed against the bacterial surface polysaccharides and proteins (adhesins) and against the toxins of *S. aureus* and to test their efficacy in bovine, mouse, and rabbit models (52, 156). More recently, a live attenuated *S. aureus* strain was used to provide protection in mice when inoculated locally during late pregnancy or early lactation (60).

1.6.2 Mastitis in other species

Staphylococci are capable of causing mastitis in domestic animals other than the cow though these infections are far less well documented than staphylococcal mastitis in the cow. In broad terms the mode of infection and the host defence mechanisms are similar to those that operate in the cow. However, staphylococcal mastitis has been seen in other species such as sheep, goats, pigs, rabbits, dogs, cats and horses (48). In sheep and goats, the mastitis is predominantly of the gangrenous type (66).

1.6.3 Exudative epidermitis

Exudative epidermitis (greasy pig disease) is an acute generalised dermatitis, caused by *S. hyicus*, which involves the entire body surface of pigs 5-35 days of age. It is highly contagious and is characterised by hyperhidrosis, excess sebaceous secretion, exfoliation and exudation but no pruritis. These changes result in a loss of skin function, extreme dehydration and rapid exhaustion, which may terminate in death. Piglets under 3 weeks of age may die within 24 to 48 hours. Morbidity rates range from 20% to 100%, and mortality rates can reach 90% in severely affected litters. *S. hyicus* can be isolated from the vaginal mucosa and skin of healthy sows. The

organisms probably enter the skin of young pigs through minor abrasions such as bite wounds (48, 138).

Prevention of the disease is by scrubbing the sow with a suitable disinfectant prior to entering the farrowing crate. The farrowing crate and pen should be clean and all sharp edges and ridges should be removed (48).

1.6.4 Tick pyaemia of lambs

Tick pyaemia is a prenatal infection of lambs with *S. aureus*, characterised by septicaemia and death or by bacteraemia and the localisation of staphylococci in various tissues. In the bacteraemic form of the disease arthritis or meningitis may first appear during the first 2-4 weeks of life; there may also be suppurative lesions in the skin, muscles, tendon sheaths and viscera especially the heart and kidneys. Tick pyaemia was originally found to co-exist with louping ill, tick-borne fever, lamb dysentery and pulpy kidney disease (48, 138).

The association between the disease and hill-grazing regions of Britain and Ireland, where there are suitable habitats for the tick *Ixodes ricinus*, is strong (138).

The first line in control of tick pyaemia must be to ensure that hygienic conditions exist for lambing and carrying out surgical procedures so that staphylococcal contamination and infection is reduced to a minimum (48).

1.6.5 Staphylococcosis of poultry

As in other animals the staphylococci that cause disease in poultry are inhabitants of the skin of the animals and do not differ biochemically from other skin staphylococci. The lesion traditionally associated with staphylococci in poultry is bumblefoot. This is a localised bulbous lesion of the ball of the foot, which arises from the penetration of a foreign body followed by secondary invasion by *S. aureus*. Lameness is often the first symptom of the condition and the swelling may not be obvious until the planter aspect of the foot is examined. However, if *S. aureus* gains entrance to the circulation of birds, then a septicaemia is produced which if not fatal, leads to arthritis and synovitis (48). *S. aureus* can also cause arthritis and septicaemia in turkeys, and omphalitis in chicks (138).

1.6.6 Pyoderma

Pyoderma is one of the most common skin diseases of dogs, but it is relatively rare in cats. Coagulase-positive *Staphylococcus* is the most commonly isolated organism from pyoderma in dogs. Prior to 1982, the organism was called *S. aureus*, but subsequently it has been identified as *S. intermedius*. It is suggested that changes in sebum production provide a milieu in which *S. intermedius* may multiply (66).

1.6.7 Endometritis

The clinical features of endometritis vary from species to species. Frequently no more is observed in the cow than a slight vaginal discharge and repeated return to service. On the other hand the bitch usually presents as an endometritis which has advanced to pyometritis. The uterus is palpably enlarged, there is a vaginal discharge, anorexia and depression. There are often signs of concomitant renal involvement such as polyuria, nocturia and polydipsia (48).

1.7 Treatment and vaccines

Staphylococci quickly developed drug resistance after penicillin was introduced and today less than 10% of the strains are susceptible to this antibiotic. This resistance is mediated by penicillinase which hydrolyses the β -lactam ring of penicillin. The genetic information encoding the production of this enzyme is carried on transmissible plasmids, which facilitated the rapid dissemination of resistance among staphylococci.

Because of the problems with penicillin-resistant staphylococci, semisynthetic penicillins resistant to β -lactamase hydrolysis (e.g., methicillin, nafcillin, oxacillin, dicloxacillin) were developed. Unfortunately, the staphylococci developed resistance to these antibiotics, as well. *S. aureus* which is resistant to an antibiotic called methicillin is referred to as methicillin-resistant *Staphylococcus aureus* or MRSA. Currently, 30% to 50% of the strains of *S. aureus* and more than 50% of the coagulase-negative staphylococci are resistant to these semisynthetic penicillins. Resistance occurs as the result of acquisition of a gene, *mecA*, that codes for a novel penicillin-binding protein, PBP2'. The penicillins and other β -lactam antibiotics kill

bacteria by their ability to bind to penicillin-binding proteins, which are enzymes responsible for construction of the cell wall peptidoglycan. PBP2' is not bound by penicillins but retains its enzymatic activity.

Staphylococci have demonstrated the remarkable ability to develop resistance to most antibiotics. Until recently, the one antibiotic that has remained uniformly active against staphylococci has been vancomycin, the current antibiotic of choice for treating staphylococci resistant to oxacillin. Unfortunately, isolates of *S. aureus* have been found with decreased susceptibility to vancomycin, and frank resistance to vancomycin has been observed in coagulase-negative staphylococci. The mechanism of this resistance is unknown. However, there is evidence that changes in cell wall synthesis have led to reversible vancomycin binding that effectively prevents vancomycin from disrupting peptidoglycan synthesis (119).

Until the 1970s a variety of whole staphylococcal preparations was used in clinical and veterinary trials as a vaccine. These included cultured, attenuated, fixed or lysed organisms. None showed convincing benefit in patients, animal models or farm animals. Staphylococci were known to produce haemolysins and leukocidins. Toxoids prepared from these were largely unsuccessful as vaccines and were accompanied by many adverse reactions. Some toxoids of veterinary value, such as the alpha toxoid, protect ewes against gangrenous mastitis, did not reduce the overall infection rate.

Polyvalent polysaccharide components of the bacterial wall have given some success in animal models. However, clinical trials of staphylococcal vaccines, including those utilising polysaccharides, were beset with problems with loss of potency of vaccine material and difficulties in interpretation of trial data (48, 116).

Aerosol vaccination with *S. aureus* endotoxins have shown good results in animal models and now await clinical trials. In last few years, scientists have tried DNA vaccination against the clumping factor A (ClfA) and passive immunisation with monoclonal antibodies as new strategies in staphylococcal vaccination (116). The results suggested that DNA immunization against adhesins represents a new and valuable approach to combat *S. aureus* infections (21).

1.8 *In vivo* methods for studying bacterial virulence genes

For more than a century, microbiologists have tried to investigate how bacteria cause disease by measuring phenotypes and attributes of the organism *in vitro*. It has

only recently become evident, however, that some genes essential for pathogenesis are turned on only during infection in the host. Thus, no number of adherence assays, enzyme studies, or electron microscopic examinations of cultured microorganisms would reveal a property that is only expressed *in vivo*.

Much of our knowledge about the virulence determinants of pathogenic bacteria comes from experiments with bacteria grown in culture, but there is currently an increasing interest in what happens to bacterial pathogens in the infected host.

Bacterial pathogenicity is a multifactorial property that is only fully manifested *in vivo*, where environmental conditions differ from those in laboratory cultures. These conditions are not only more complex than those *in vitro* but change as infection proceeds in response to inflammation, tissue breakdown and spread from one site to another. Bacterial behaviour will, therefore, be mediated *in vivo* by changes in the availability of nutrients and substrates and the effect of these changes on the regulation of gene expression. Bacterial pathogens *in vivo* will, consequently, differ from those grown *in vitro*, an effect now confirmed for many pathogenic species. The environment *in vivo* will influence bacterial pathogenicity by controlling bacterial growth rate and population size and by affecting the production of virulence determinants.

The fact that environmental conditions *in vivo* differ from those *in vitro*, and change as infection proceeds, has three implications. First, some putative virulence determinants indicated by experiments *in vitro* may not be produced *in vivo*. Second, some of the virulence determinants that are formed *in vivo* may not be produced *in vitro*. Third, the complement of virulence determinants may change with time and anatomical site. Clearly, the production *in vivo* of any putative virulence determinant has to be confirmed, and bacteria grown *in vivo* must be examined to discover novel virulence determinants. Regarding the third implication, virulence determinants must be identified as infection progresses, together with the host factors and regulatory processes that induce them (151).

In the past few years, the study of bacterial pathogenesis has been advanced by a number of techniques that enable investigators to monitor bacterial gene expression during infection. These techniques allow the investigator to select and screen for genes that are required for infection or that are expressed *in vivo*. In particular, three methodologies have dramatically impacted our understanding of bacterial

pathogenesis. Table 1.1 summaries new methods for studying bacterial pathogens *in vivo* (151).

1.8.1 *In vivo* Expression Technology (IVET)

Defining the nature and function of bacterial gene products expressed during infection is critical to understanding the molecular and genetic mechanisms of pathogenesis. Recent advances in gene fusion technology have resulted in the development of a genetic strategy termed *in vivo* expression technology (IVET) that provides a positive selection for bacterial genes that are induced during host infection (82, 104). The protocol for IVET is shown in figure 1.2 (67).

IVET is a strategy for identifying bacterial genes active during mammalian infection but relatively inactive at other times (104). A subset of such genes encodes virulence factors crucial to the infection process. The novel feature of IVET is its use of infected mammalian tissues directly to induce expression of candidate virulence genes, rather than relying on one's ability to duplicate the *in vivo* milieu in the laboratory. Accordingly, it is a powerful advance over earlier gene-finding techniques (98).

The IVET system has several applications in the area of vaccine and antimicrobial drug development. The technique has been designed for the identification of virulence factors and thus may lead to the discovery of new antigens useful as vaccine components. The IVET system facilitates the isolation of mutations in genes involved in virulence and, therefore, should aid in the construction of live attenuated vaccines (150).

The original IVET selection was performed by creating transcriptional fusions of random fragments of the *Salmonella typhimurium* chromosome with a promoterless *purA* gene and introducing this library onto the chromosome of an *S. typhimurium* $\Delta purA$ strain via homologous recombination at the chromosomal fragment. Because purines are limiting for growth of *S. typhimurium* in the mouse, only those strains expressing *purA* from fused promoters would survive (104).

It should be noted that the integration event resulting from a single crossover does not lead to disruption of the wild-type locus on the chromosome, thereby permitting analysis of genes essential for growth *in vivo*. Bacteria representing the pool of chromosomal fusions were then injected intraperitoneally in mice, and the surviving

Pathogenesis of infections in animals

- Photonic detection in living hosts of pathogens rendered bioluminescent by expression of a bacterial luciferase
- Confocal laser scanning microscopy (CLSM) of histopathological specimens
- Fluorescence-activated cell sorting (FACS)

Detection of gene expression induced *in vivo*

- *In vivo* expression technology (IVET): genes expressed *in vivo* provide promoters for various reporting systems
- Differential fluorescence induction (DFI): promoters of genes expressed *in vivo* drive expression of green fluorescent protein (GFP)
- Differential display of cDNAs from mRNAs of genes expressed *in vivo*
- Labelling with diaminopimelate (DAP), a lysine precursor used by bacteria but not the host
- Differences in antigens revealed by reactions of products of gene libraries with antibodies evoked during infection, and those raised against killed bacteria

Identification of virulence genes

- Signature-tagged mutagenesis (STM): non-recovery from mice after inoculating a pool of tagged insertion mutants indicates mutations in genes required for infection
- Complementation of avirulent strains by gene libraries from virulent strains

Global analysis of potential gene expression *in vivo*

- Whole genome sequencing
- Chip technologies

Measurement of environmental parameters *in vivo*

- Quantitative fluorescence microscopy
- X-ray microanalytical microscopy
- Use of reporter genes

Table 1.1 New methods for studying bacterial pathogens *in vivo* (151)

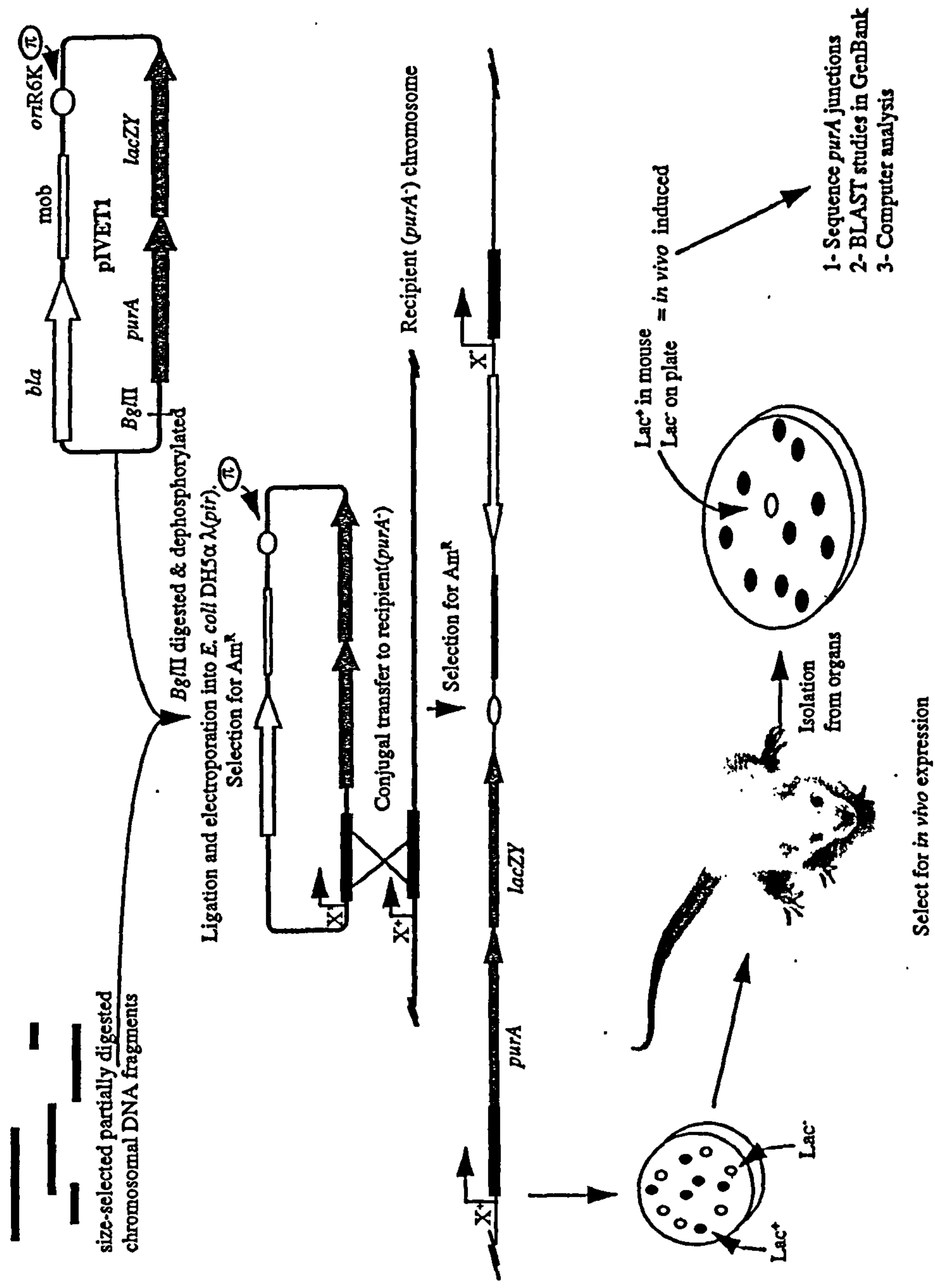


Figure 1.2 IVET is a positive selection method for genes induced specifically in the host (67).

pools were recovered 3 days later and screened on laboratory medium for clones with low promoter activity. Several strains carrying promoters meeting the criteria of *in vivo* expression and *in vitro* inactivity were, on subsequent analysis, found to have severe virulence defects as assayed by oral LD₅₀, thereby validating the ability of IVET to isolate virulence genes (29, 104).

IVET was applied to *S. aureus* using a promoter trap that relies on genetic recombination of the site-specific resolvase of *tnpR* from gamma delta (Tn1000) as a reporter gene expression. A collection of 45 genes were found induced during infection in a murine renal abscess model. Of these, only six had been known previously; 11 others have homology to known non-staphylococcal genes. The known staphylococcal genes included *agrA*, a key locus regulating numerous virulence products; and a glycerol ester hydrolase which may enhance bacterial survival in abscesses. Many of the *ivi* genes identified were not classical virulence factors but rather involved in adaptation to the *in vivo* environment; knowledge of these biochemical pathways is clearly the next phase in IVET systems (98).

To date, there are four variations of IVET, and each relies on the generation of transcriptional fusions of genomic sequences to a reporter gene encoding an enzymatic activity. The variation in the four methods lies in the particular reporter gene utilized. These are (i) auxotrophy complementation-based selections, (ii) antibiotic selections, (iii) dual reporter selections or other types of genes that provide an *in vivo* selection and an *in vitro* screen for promoter activity, and (iv) recombination-based IVET (RIVET) (5).

There are two limitations of IVET selections as follows: (i) *ivi* genes that are transiently expressed or expressed at a low level *in vivo* are difficult or impossible to detect because they either do not produce PurA long enough or do not produce enough of it to allow survival and growth of the strain and (ii) not all *ivi* genes are essential for infectivity. The first of these limitations is of unknown magnitude; however, it is reasonable to predict that only a subset of virulence genes that are transcriptionally silent *in vitro* will be expressed at levels sufficient for survival throughout the course of an infection. The second limitation, applicable to all IVET selections and screens, is often misconstrued, particularly since the advent of signature-tagged mutagenesis (STM), which is a genetic screen used to identify genes that are essential for infectivity (5, 72). In other words, IVET is a selective method that positively selects the bacterial genes that are required for virulence, while STM is

a negative selection method for bacterial mutants that are present in the initial inoculum, but do not survive in the animal. STM is discussed in session 1.8.3.

1.8.2 Differential Fluorescent Induction (DFI)

DFI is a promoter trap in which bacterial promoters drive the expression of the highly sensitive reporter, green fluorescent protein (GFP). The GFP of the jellyfish *Aequorea victoria* is a unique experimental tool that permits monitoring of gene expression and protein localisation in living cells. GFP is stable and, unlike reporter molecules such as *lacZ* or luciferase, it does not require cofactors for its activity. However, with the use of a fluorescence-activated cell sorter (FACS), *in vivo*-expressed fusions are identified by their level of expression compared with growth on laboratory media (70, 167).

1.8.3 Signature-tagged mutagenesis (STM)

Mutagenesis techniques have been used extensively for the identification of genes whose functions are required under certain conditions. One of these techniques that has been used in several pathogens is transposon mutagenesis.

Transposons are the most versatile tools for the genetic analysis of bacteria. Unlike the situation with chemical mutagenesis, transposons produce complete disruption of the mutated gene, resulting in nonleaky phenotypes; moreover, multiple insertions or retranspositions are rare events, and most important, the resulting phenotype is genetically linked to a selectable marker such as resistance to an antibiotic. The spectrum of different types of transposons, their mechanisms of transposition, and their applications in genetic engineering is an interesting subject for molecular biologists (42).

One approach to identifying the traits that contribute to virulence is to make random mutations in the genome of a pathogen using a transposon and screen the transposon-generated mutants for loss of virulence (141).

Transposons are capable of inactivating genes by insertion, and the inactivated genes are readily identified and recovered. A typical strategy is to construct a plasmid carrying the transposon, using a temperature-sensitive vector, e.g., a plasmid that is unable to replicate at an elevated temperature. When this plasmid is introduced into

bacterial cells, and the cells are grown at a temperature permissive for plasmid replication (e.g., 30°C), all the cells will carry the plasmid. If the culture is subsequently shifted to a temperature (e.g., 42°C) at which the plasmid is unable to replicate, the only cells that retain the transposon will be those in which the element has transposed onto the chromosome. It is usual to use a transposon that carries an antibiotic resistance gene (for example Tn5, carrying a gene for kanamycin resistance), so these transpositions can be selected by plating the culture at 42°C on agar containing kanamycin.

This generates a transposon mutant library, with the transposon inserted at a variety of chromosomal positions, and therefore causing a range of mutations. Once the mutants of interest are selected, the gene(s) into which transposon has inserted can be identified. This can be done as described above for deletions and insertions in general (i.e., by looking for a change in size of specific DNA fragments), but the presence of an antibiotic resistance gene on the transposon facilitates a more direct approach. If the total DNA is cut with a restriction enzyme (taking care to select one that does not cut the transposon itself), the collection of fragments can be cloned to produce a gene library. If this gene library is then plated on kanamycin agar, only those clones containing Tn5 will be able to grow. These clones will contain not only Tn5 but also some of the sequence on either side of it; this is the gene that has been inactivated by Tn5 insertion, and it can be proceeded to characterise (39).

On the point of transposon mutagenesis in staphylococci, two transposons have been used successfully in *S. aureus* and *S. epidermidis*:

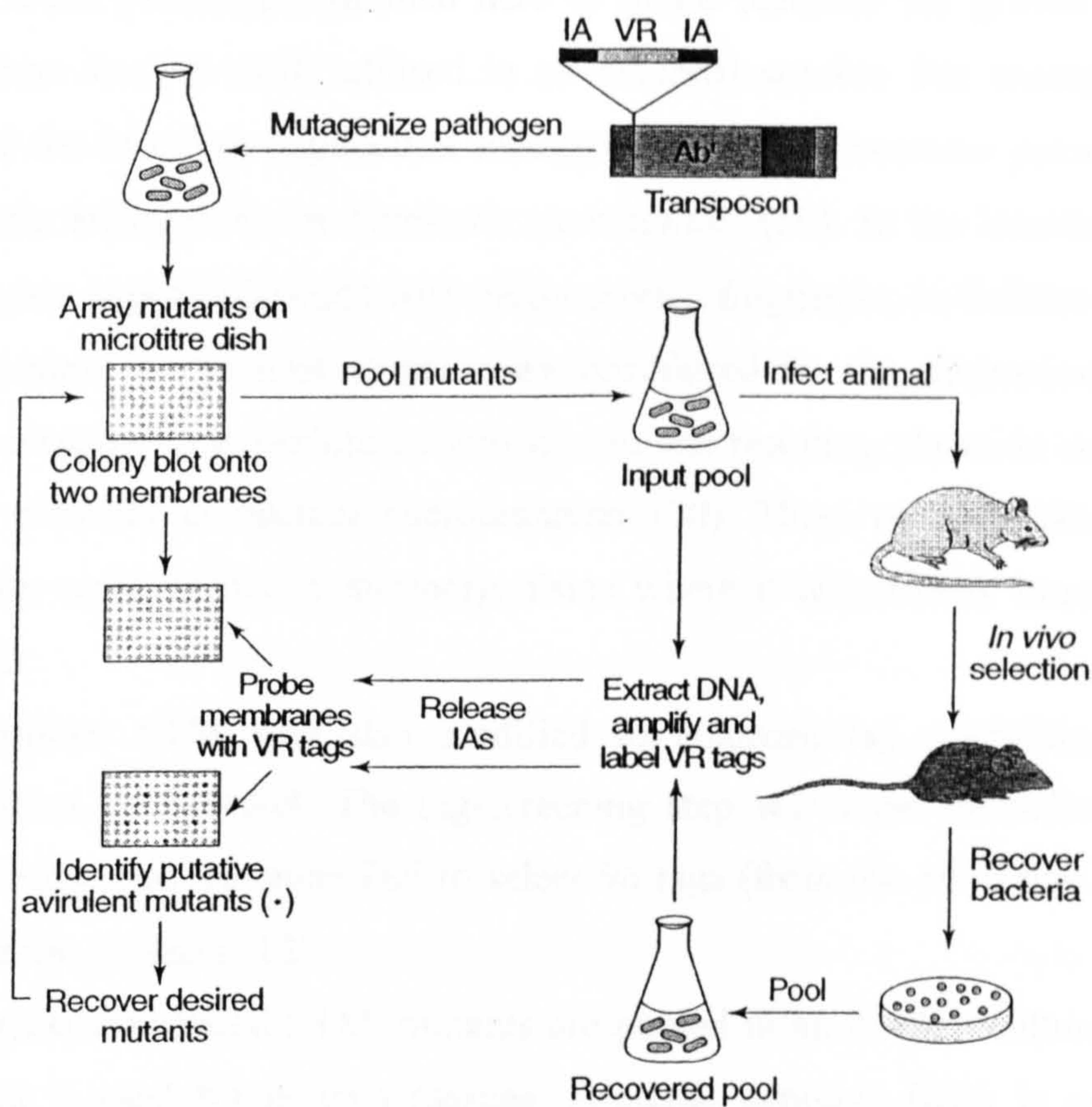
- (1) The closely related class II elements Tn551 and Tn917.
- (2) The closely related conjugative transposons Tn916 and Tn918 (167).

For many applications, mutations in genes of interest can be identified by screening a sufficient number of mutants for a negative phenotype. Such approaches traditionally required individual and labour-intensive analysis of mutants for loss-of-function phenotype. To circumvent this limitation and link mutagenesis techniques with an efficient screening approach a novel technique termed “signature-tagged mutagenesis”, or STM, was developed (71, 72). Although the development of STM was performed for the identification of new bacterial virulence genes the principles of the method can be adapted to the analysis of other organisms and can be extended to the identification of genes whose functions are required for the successful colonisation of certain habitats or the exploitation of specialised metabolic features. A

key feature of STM is the possibility of simultaneous identification of mutants by negative selection. In contrast to approaches where the complementation of mutations can be selected as a positive phenotype, one can identify a mutation resulting in a negative phenotype, such as attenuation of virulence or inability to grow under certain nutritional conditions. A novelty of STM is that such negative selection is not performed by individual testing of mutants from a bank of mutants, but rather by collective screening of pools of mutants in a parallel manner (71).

STM is divided into two steps: firstly, the construction of a library of tagged mutants, and secondly, *in vivo* screening of the library. In the original STM, each insertional mutation carried a different DNA tag, which allowed mutants to be differentiated from each other. The tags were prepared as a pool of oligonucleotides containing a variable central region flanked by conserved sequences. The central region was designed to ensure that the same sequence occurs once in 2×10^{17} molecules (72). Each tag comprised a different central sequence of 40bp flanked by invariant 'arms' of 20bp, which enable the central portions to be co-amplified by the polymerase chain reaction (PCR). Tags were ligated into the pUT mini-Tn5 Km2 transposon and transferred from *E. coli* into the recipient host. Tagged mutant strains are assembled in microtitre dishes, and then combined to form the 'inoculum pool' for infection studies. At an appropriate time after inoculation, bacteria are isolated from the animal and pooled to form the 'recovered pool'. The tags in the recovered pool and the tags in the inoculum pool are separately amplified, labelled and then used to probe filters arrayed with the different tags representing the mutants in the inoculum. Mutants with attenuated virulence are those with tags that give hybridisation signals when probed with tags from the inoculum pool but not when probed with tags from the recovered pool (72). It became quickly apparent that the highly random DNA tags gave cross-hybridisation noise. The original STM tag library was screened to identify pools of 96 tagged mutants giving a high and specific hybridisation signal (92). Figure 1.3 shows the design of STM protocol (130).

When compared to traditional pathogenicity assays, STM minimises the number of animals to be utilised. The crux of STM depends on tagged transposon mutants defective in virulence that cannot be maintained *in vivo*. Attenuated mutants are selected and re-tested to confirm attenuation; disrupted genes are cloned via the transposon marker and identified by DNA sequencing (92). STM is based on the concept of monitoring the frequency of a particular mutant in a population of other



IA, invariant arms; VR, variable region; Ab^r , antibiotic resistance gene

Figure 1.3 A diagrammatic representation of the STM screening process (130).

mutants by marking each mutant with a distinct tag. If a mutant cannot grow in a particular niche, then that mutant's tag will not be detected among the recovered bacteria (110).

A crucial step in STM depends on a high frequency of random transposon insertions into the chromosome. This is not always possible, insertion into an essential gene gives a lethal phenotype (defined here as genes essential for growth *in vitro*) and transposons are not easily utilised in all bacterial species. For example, STM was modified for insertion-duplication mutagenesis in *Streptococcus pneumoniae* (133) and shuttle mutagenesis in *Neisseria meningitidis* (30). In the insertion-duplication mutagenesis, tagged plasmids with chromosomal fragments, to facilitate homologous recombination in the host strain, were introduced by transformation (133). STM shuttle mutagenesis is performed into *E. coli* and resulting plasmids are transformed into the naturally competent microorganism (30). These methods allow the use of transposon mutagenesis in microorganisms where it is normally impossible to use STM (92).

The original STM was also modified to augment tag specificity and reduce hybridisation background. The tag-screening step was done immediately after the initial cloning into the mini-Tn5 to select 96 tags (from the 10^{17}) that gave specific hybridisation signals (112).

In hybridisation-based STM, mutants are pooled in an *in vitro* culture from which an aliquot is used for *in vivo* passage. Bacterial genomic DNA is extracted from bacteria grown *in vitro* and from those recovered after *in vivo* selection. Mutants that gave a hybridisation signal from the *in vitro* pool but not from the *in vivo* pool are detected with respect to persisting *in vivo* or establishing an infection. Chromosomal DNA is used as PCR templates for preparation of radioactive or non-radioactive probes hybridised to colony or DNA dot blots from the arrayed mutant library. Modifications include PCR that can be done directly on bacterial cell lysates (93) and chemiluminescence (92, 112).

Another STM modification is based on PCR, a very powerful technique for DNA detection and amplification, giving a PCR-based STM without hybridisation (93). The initial STM-PCR was built with 12 re-usable and PCR-specific tags eliminating the fastidious tag-screening step. In this method, mutants from the same pool were grown separately, aliquots were pooled and a sample was used as the *in vitro* pool. A second sample from the same pool is used for *in vivo* passage (the *in vivo* pool). The *in vitro*

and *in vivo* pools are used in comparative PCR where one of the tags is used as primer along with a universal primer in the kanamycin resistance gene (92).

In the first application, STM was used in *Salmonella typhimurium* (72) successfully and resulted in the identification of a chromosomal region called *Salmonella* pathogenicity island 2 (SPI2). This encodes a type III secretion system critical for systemic infection (112, 147, 167).

Following the first STM screen in *S. typhimurium* (72, 161), the method has been applied for identification and analysis of virulence factors in a vast variety of gram-positive and gram-negative bacteria including *S. aureus* (33, 112, 146), *Salmonella enterica* serotype Dublin (18), *S. choleraesuis* (96), *Vibrio cholerae* (28, 115), *Yersinia enterocolitica* (40), *Y. pseudotuberculosis* (85, 111), *Proteus mirabilis* (171), *Pseudomonas aeruginosa* (93, 94), *Legionella pneumophila* (49, 132), *Brucella suis* (54), *B. abortus* (77), *B. melitensis* (95), *Listeria monocytogenes* (8), *Escherichia coli* (11, 12, 61, 107), *Neisseria meningitidis* (30, 58, 154), *Streptococcus pneumoniae* (68, 89, 133), *S. agalactiae* (81), *Actinobacillus pleuropneumoniae* (57), *Haemophilus influenzae* (73), *Klebsiella pneumoniae* (106, 153), *Xenorhabdus nematophila* (74), *Burkholderia pseudomallei* (7) and *Pasteurella multocida* (56), and acid fast bacilli like *Mycobacterium tuberculosis* (25, 34). STM has also been used for identification of virulence genes in microorganisms other than bacteria like *Candida glabrata* (32), *Aspergillus fumigatus* (22), *Cryptococcus neoformans* (122), and *Toxoplasma gondii* (86).

Virulence in *S. aureus* has been studied by using STM methodology (33, 112, 146). In the study by Mei et al (112), Tn917 mutants were tested in a murine model of bacteraemia. The majority of loci from 50 mutants that were identified as attenuated were predicted by sequence similarity to be involved in cell surface metabolism (e.g. peptidoglycan cross-linking and transport functions), nutrient biosynthesis, and cellular repair processes, but most of the remainder had no known function. A slightly larger signature-tagged mutant bank was constructed by using the same transposon and tested in models of bacteraemia, abscess and wound formation, and endocarditis (33). This enabled the identification of various genes affecting growth and virulence in specific disease states, as well as 18 that are important in at least three of the infection models. Many of these genes appear to be involved in the same kinds of processes as those identified in the earlier study (112); indeed, seven of the genes

identified by Mei et al (112) were also found by Coulter et al (33). Tables 1.2 and 1.3 summarise some details of the genes recovered in *S. aureus* by STM so far (33, 112).

1.9 Biofilms

Biofilms can be defined simply and broadly as communities of microorganisms that are attached to a surface. It is clear that microorganisms undergo profound changes during their transition from planktonic (free-swimming) organisms to cells that are part of a complex, surface-attached community. Biofilms can comprise a single microbial species or multiple microbial species and can form on a range of biotic and abiotic surfaces. Although mixed-species biofilms predominate in most environments, single-species biofilms exist in a variety of infections and on the surface of medical implants (1, 6, 44, 127). These single-species biofilms are the focus of most current research. *P. aeruginosa* has emerged as the most studied single-species, biofilm-forming gram-negative bacterium, although, among the gram-negative bacteria, *P. fluorescens*, *E. coli*, and *V. cholerae* have also been studied in detail. The gram-positive biofilm-forming bacteria that have been studied include *S. epidermidis*, *S. aureus*, and the enterococci (127).

A number of gram-positive infections, including those caused by *S. epidermidis*, *S. aureus*, and the enterococci, have proven to be particularly difficult to treat with current antibiotic therapies, partly owing to their high-level natural resistance to antimicrobial compounds. Furthermore, these organisms become resistant to the highest deliverable levels of antibiotics when growing in a biofilm (38, 79, 139, 164). The production of an exopolysaccharide matrix, or glycocalyx, is one of the distinguishing characteristics of biofilms. It has been suggested that this matrix, among other functions, prevents the access of antibiotics to the bacterial cells embedded in the community. Either reaction of the compound with, or sorption to, the components of the biofilm can limit the transport of antimicrobial agents to the cells within the biofilm (103). The actual structure of the biofilm matrix will vary greatly depending on the microbial cells present, their physiological status, the nutrients available and prevailing physical conditions (155). It is estimated that more than 60% of nosocomial infections are derived from biofilm-related infections (6, 55, 78). Biofilm bacteria can be up to 1,000-fold more resistant to antibiotic treatment than the same organism grown planktonically, but the mechanisms by which the biofilm-

Animal model	Route of inoculation	Sample site	Number of mutants identified/ number screened	Percentage hit rate	Reference
Mouse bacteraemia	i.p.	Spleen	50/1248	4.01	112
Mouse abscess	s.c.	Skin abscess	108/1520	7.11	33
Mouse wound	s.c.	Wound tissue	127/1520	8.36	33
Mouse bacteraemia	i.v.	Spleen and liver	111/1520	7.30	33

i.p. intraperitoneal; s.c. subcutaneous; and i.v. intravenous

Table 1.2 Summary of published STM screens in *S. aureus*

Classification	Total No.	Blast description (No.)	Reference
Transport / binding proteins	14	nickel-binding periplasmic protein precursor (1)	33
		dipeptide transporter ATP-binding protein (1)	33
		dipeptide transport system permease (4)	33
		oligopeptide permease (1)	112
		oligopeptide ATP-binding protein (C,2; M,1)	33, 112
		di/ tripeptide transporter membrane protein (1)	33
		amino acid carrier protein, sodium/proton dependent alanine transporter (2)	33
Energy metabolism	7	high-affinity proline permease (1)	33
		coenzyme A disulphide reductase (1)	33
		nitric oxide reductase (1)	33
		44 bp 5' of dihydrolipoamide succinyltransferase (E2) (1)	33
		53 bp 5' of oxoglutarate dehydrogenase (E1) of oxoglutarate operon (C,1; M,1)	33, 112
		aconitase (1)	112
		formate c-acetyltransferase, pyruvate formate lyase (1)	33
Amino acid biosynthesis	11	diaminopimelate decarboxylase (C,1; M,1)	33, 112
		51 bp 5' of ykuQ, similar to tetrahydropicolinate succinylase, Lys biosynthesis (1)	33
		tryptophan synthesis:trpA (1)	112
		82 bp 5' of tryptophan synthase (C,1; M,1)	33, 112
		indole-3-glycerol phosphate synthase (1)	33
		tryptophan synthesis: trpD (1)	112
		aspartate semialdehyde dehydrogenase (C,1; M,1)	33, 112
		threonine synthesis (1)	112

Table 1.3 Frequency and distribution of sequenced genes of published STM screens in *S. aureus* (33, 112)

Classification	Total No.	Blast description (No.)	Reference
Translation	5	glutamyl endopeptidase precursor, serine V8 protease (2)	33
		processing protease (1)	33
		peptide methionine sulfoxide reductase (C,1; M,1)	33, 112
Replication	7	DNA polymerase I (1)	33
		ATP-dependent DNA helicase (1)	33
		UV damage repair protein similar to umuC of <i>E. coli</i> (2)	33
		DNA repair recA (1)	112
		3' of asparaginyl tRNA synthetase iin dnaD (1)	33
		DNA mismatch repair (1)	33
Co-factor biosynthesis	1	molybdoprotein guanidinenucleotide biosynthesis protein B (1)	33
Regulatory functions	3	HTH, ARAC family of transcriptional regulators (1)	33
		lac repressor gene (2)	33
Intermediary metabolism	1	77 bp 5' of pyruvate carboxylase (1)	33
Cell envelope	10	ORF-X 3' of femB (4)	33
		ORF similar to femD / ureD (1)	33
		femA (3)	112
		femB (1)	112
		similar to femB (1)	112
Cellular processes	5	prolipoprotein signal peptidase (C,1; M,2)	33, 112
		chaperone for protein degradation (1)	112
		lantibiotic epidermin operon, epiB-like homology (1)	33
Purines, pyrimidines	2	purine operon repressor (1)	33
		purine synthesis (1)	112

Table 1.3 (continued)

Classification	Total No.	Blast description (No.)	Reference
Transposon related functions	2	insertion sequence IS1181, transposase (1)	33
		insertion sequence IS1272, putative transposase (1)	33
Unknown/hypothetical	8	247 bp 3' of accessory regulator (1)	33
		hypothetical protein similar to signal recognition particle (1)	33
		5' of ywhD, unknown function (1)	33
		hypothetical protein, putative histidine phosphokinase (1)	33
		unknown ORF 5' of branched chain amino acid carrier protein (1)	33
		3' of hypothetical protein HI1663, probable hydrolase (1)	33
		3' of hypothetical protein ipa-61d (1)	33
		hypothetical protein 5' of ynxC (1)	33
Low homology	8	similar to protein M type 12 (1)	33
		similar to EF protein (1)	33
		similar to lipase precursor (1)	33
		hypothetical helicase (1)	33
		esterase (1)	33
		putative hydrolase (1)	33
		quinilone resistance protein, efflux pump (1)	33
No database match	51	capsule expression (1)	33
		reference 33(22); reference 112(29)	33, 112

Table 1.3 (continued)

grown bacteria attain this resistance are still a matter of speculation. Mechanisms of resistance that are considered likely include (i) phenotypic changes in bacteria resulting in resistance occurring within the biofilm environment, (ii) inactivation of the antibiotics by extracellular polymers or modifying enzymes, and (iii) nutrient limitation resulting in slowed growth rate (59).

1.9.1 Staphylococcal biofilms

Biofilm formation in staphylococci is multifactorial, and the ability to form a biofilm makes the strains much better able to survive in the normally hostile environment of tissue and blood. Numerous studies have implicated a polysaccharide intercellular adhesin (PIA) in biofilm formation in *S. epidermidis*. PIA is a polysaccharide composed of β -1,6-linked *N*-acetylglucosamine with partly deacetylated residues, in which the cells are embedded and protected against the host's immune defence and antibiotic treatment (35, 63). The *ica* genes code for the synthesis of this adhesin (35). Mutations in *ica* operon lead to a pleiotropic phenotype; the cells are biofilm- (35) and haemagglutination-negative, less virulent and less adhesive on hydrophilic surfaces. *ica* expression is modulated by various environmental conditions, appears to be controlled by SigB and can be turned on and off by insertion sequences (IS) elements. Recent studies have shown that *S. aureus*, like *S. epidermidis*, has the *ica* locus, which encodes the functions required for intracellular adhesin. These data suggest that the early stages in biofilm formation may be similar between these two organisms (35, 63). Although it has been shown that *ica* has a role in adhesion for biofilm formation, however, it was not identified in our biofilm experiments.

Bacterial pathogens that are primarily extracellular, such as *S. aureus*, can adhere to components of the extracellular matrix (ECM) of the host to initiate colonization. Adherence is mediated by protein adhesins of the MSCRAMM (microbial surface components recognizing adhesive matrix molecules) family, which in most cases are covalently anchored to the cell wall peptidoglycan (19). Different prototype MSCRAMMs such as fibrinectin-binding protein A (FnBPA), a collagen-binding protein (Cna) and clumping factor A (ClfA) have been recognized in *S. aureus*. Fibrinectin binding is a very common property of *S. aureus* isolates (53).

Proteins have been identified that are also involved in staphylococcal biofilm formation, such as the accumulation-associated protein (AAP), the clumping factor A (ClfA), the staphylococcal surface protein (SSP1), and the biofilm-associated protein (Bap)(37, 63). An *S. aureus* transposon-insertion mutant has been isolated with a significant decrease in attachment to inert surfaces, intercellular adhesion and biofilm formation. The transposon is inserted in the *bap* gene, which encodes the novel cell wall-associated, 2276-amino-acid protein, Bap. The *bap* gene has been found in 5% of the *S. aureus* bovine mastitis isolates, but it was absent in the 75 clinical human *S. aureus* isolates analysed. All staphylococcal isolates harbouring *bap* are highly adherent and strong biofilm producers. In a mouse infection model, Bap is involved in pathogenesis, causing a persistent infection (37).

Biofilm formation is thought to be a two-step process that requires the primary adhesion of bacteria to a substrate surface followed by the formation of multiple cell layers. In other words, at the first stage, the bacterium approaches the surface so closely that motility is slowed. The bacterium may then form a transient association with the surface and/or other microbes previously attached to the surface. This transient association allows it to search for a place to settle down. When the bacterium forms a stable association as a member of a microcolony, it becomes a three-dimensional biofilm structure (43, 166). Studies have shown that teichoic acids, highly charged cell wall polymers, play a key role in the first step of biofilm formation by *S. aureus*. The cell surface of *S. aureus*, as in most bacteria, has a moderately negative net charge at neutral pH, which is probably due to the fact that the teichoic acids contain fewer positively charged D-alanine residues than negatively charged phosphate groups. Nevertheless, *S. aureus* can adhere to hydrophobic or slightly negatively charged surfaces such as polystyrene or glass, respectively. The direct interaction of bacteria and surfaces is dependent on van der Waals forces, which are generally attractive, and interionic forces, which can be either attractive or repulsive. Even if bacteria and surfaces are charged alike, van der waals forces can overcome repulsion and lead to adhesion (64). Figure 1.4 shows the events associated with bacterial adherence to biomaterials (4).

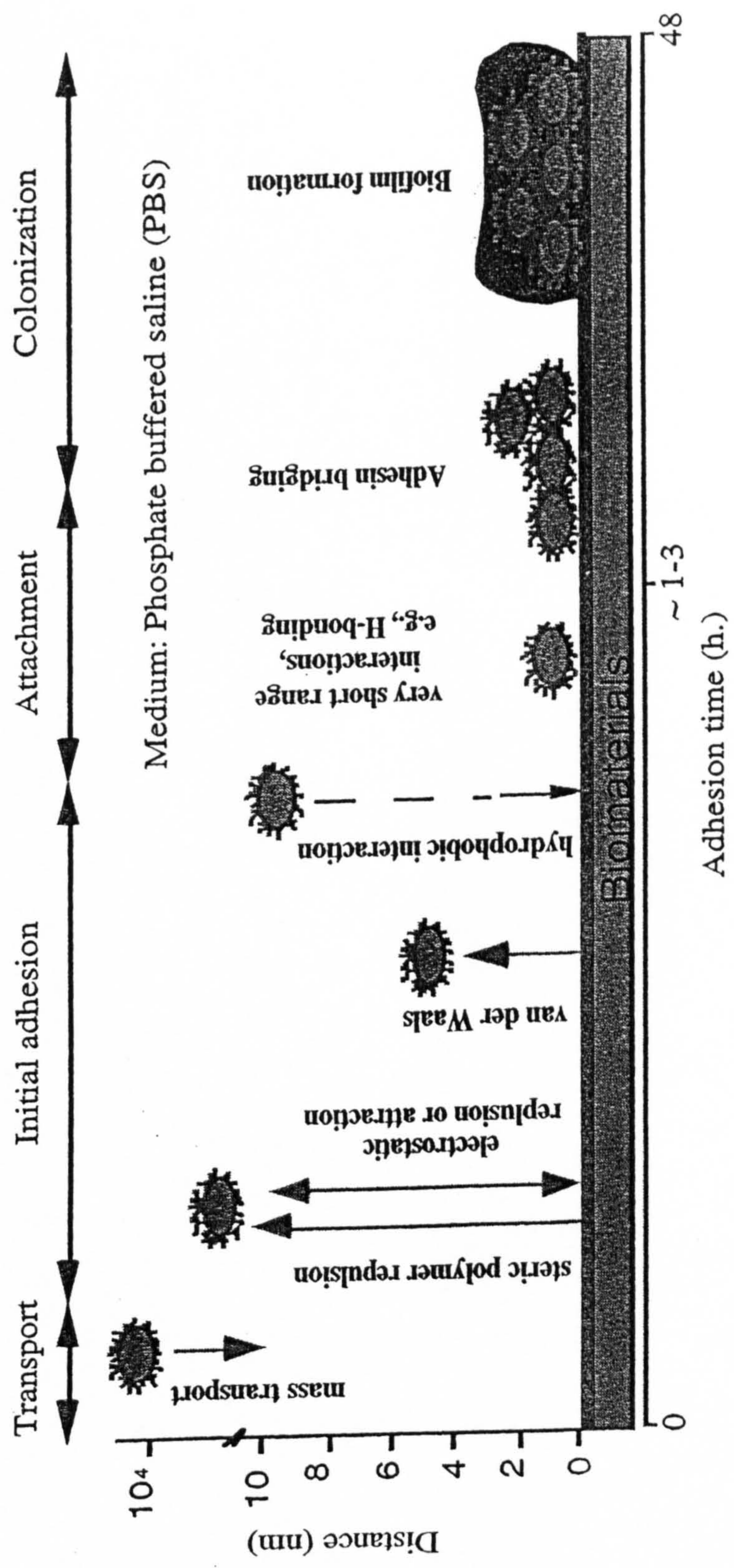


Figure 1.4 Physiochemical forces involved in non-specific bacterial adhesion to biomaterials. The effective distance are based on using phosphate buffered saline as medium (4).

1.10 Aims of thesis project

In recent years, the use of medical implants such as catheters, pacemakers, prosthetic heart valves, and joint replacements has increased dramatically. These devices can become colonised by microorganisms which form a biofilm consisting of a mono- or multilayer of cells embedded within a matrix of extracellular polymeric material. Release of microorganisms from the biofilm may initiate an acute disseminated infection. Implant-associated infections are difficult to resolve, because biofilm microorganisms are resistant both to host defence mechanisms and antibiotic therapy. The majority of implant infections are caused by Gram-positive bacteria, notably staphylococci.

As the STM method has not been used for screening of bacterial virulence genes in biofilm so far, the main aim of this project was to find any probable new genes of *S. aureus* that are essential for biofilm formation by STM. In addition, it may be that biofilm formation and virulence are related, so we attempted to identify genes related in both processes. The general outline of the whole project is shown in figure 1.5.

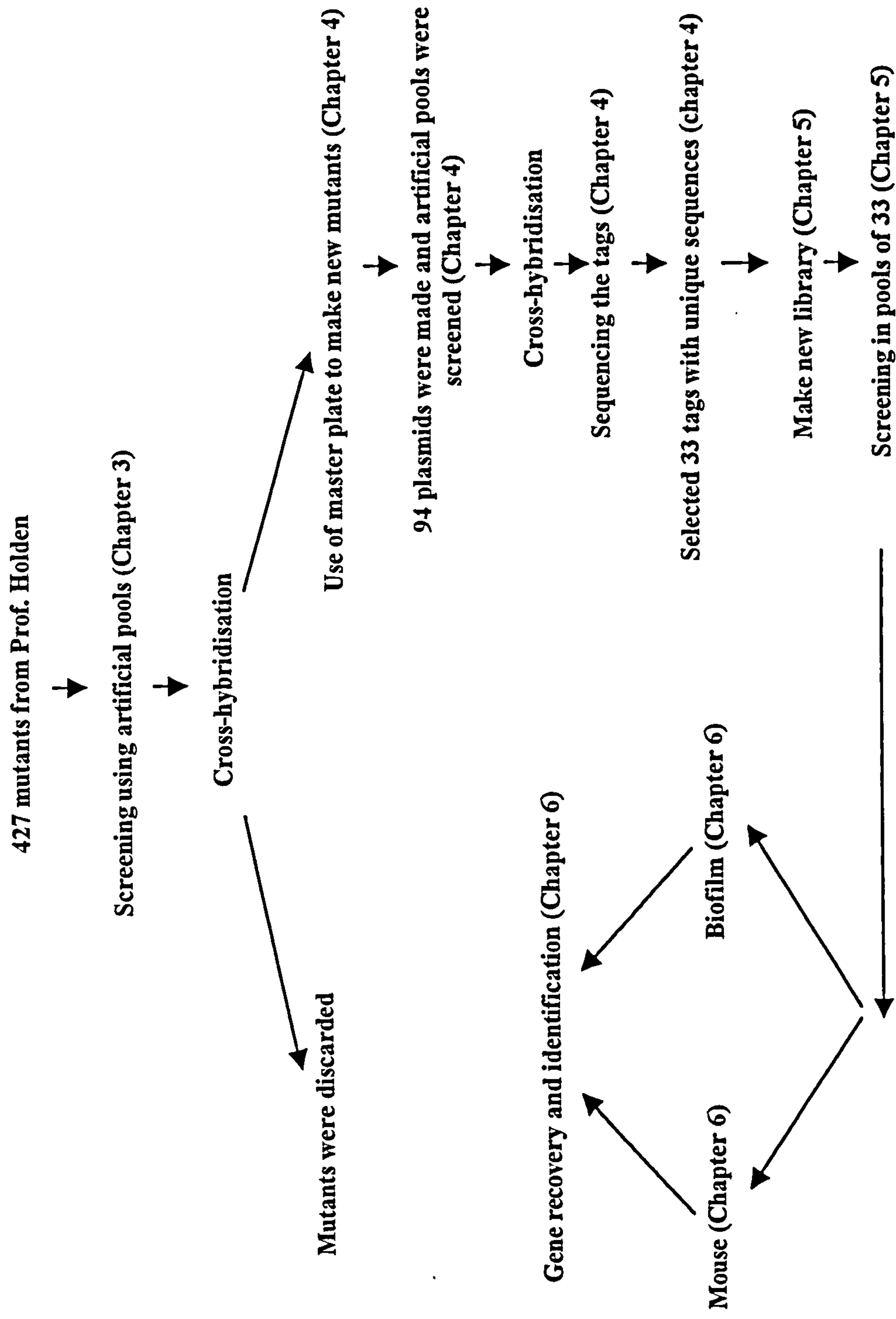


Figure1.5 General outline of the whole project

Chapter 2

General methods

Chapter 2: General methods

General methods are described here. For clarity more specific methods are described in their appropriate chapters.

2.1 Isolation of Genomic DNA from *S. aureus*

Method 1

The bacteria were grown in 5 ml of Brain Heart Infusion (BHI) broth supplemented with erythromycin ($20 \mu\text{g ml}^{-1}$). Erythromycin was added only for growing the mutants. One ml of overnight culture was removed under aseptic conditions and centrifuged in a microcentrifuge tube at 15,000 rpm for 1 minute. The supernatant was discarded and the pellet resuspended in 560 μl of TE buffer. 5 μl of RNase (10 mg/ml) and 5 μl of lysostaphin (10 mg/ml)(Sigma) were added and mixed vigorously for 30 seconds. After 1 h incubation at 37°C , 30 μl of 10% SDS, 5 μl of RNase (10 mg/ml), and 10 μl of proteinase K (10 mg/ml) were added and the incubation continued for an additional hour. Then 100 μl of 5 M NaCl was added followed by 80 μl of prewarmed (65°C) 10% hexadecyltrimethyl ammonium bromide in 0.7M NaCl (CTAB/NaCl), and incubated at 65°C for 10 minutes. An equal volume of chloroform was added and the suspension mixed by vortexing. After centrifugation for 5 minutes at 15,000 rpm, the viscous upper phase was removed and transferred into a new 1.5 ml microcentrifuge tube. The suspension was extracted twice with an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1) and once with chloroform/isoamyl alcohol. The upper aqueous phase was transferred into a new 1.5 ml microcentrifuge tube and the DNA precipitated by adding 500 μl isopropanol. After centrifugation at 15,000 rpm for 10 minutes, the isopropanol was removed and the pellet washed with 1 ml of 70% ethanol. The DNA pellet was air-dried and resuspended in 30 μl of sterile water (108).

Method 2

The bacteria were grown in 5 ml of BHI broth supplemented with erythromycin (20 $\mu\text{g ml}^{-1}$). After overnight incubation at 37°C the cells were harvested by centrifugation (10 min, 3000 g) and resuspended in 5 ml of SET pH 7.5, and then lysozyme to a concentration of 1 mg ml^{-1} was added. After 0.5 h incubation at 37°C, 1/10 volumes of 10% SDS and 0.5 mg ml^{-1} proteinase K were added and incubation continued at 55°C with occasional inversion for 2 h, when 1/3 volumes 5 M NaCl and 1 volume chloroform were added and incubation continued at room temperature for 0.5 h with frequent inversion. The suspension was centrifuged at 4500 g for 15 min and the aqueous phase was transferred to a new tube using a blunt-ended pipette tip. The DNA was precipitated by adding 1 volume of isopropanol and the tube was inverted gently. The DNA was transferred into a microfuge tube, rinsed with 70% ethanol, air-dried and dissolved in a suitable volume of TE buffer (135).

Method 3

Chromosomal DNA from *S. aureus* was isolated using a Qiagen Genomic DNA Kit according to the manufacturer's protocol, except that the bacterial cells were lysed by lysostaphin (Sigma; 200 $\mu\text{g ml}^{-1}$) at 37°C for 30 minutes before DNA extraction.

2.2 Plasmid DNA extraction

Plasmid DNA from *S. aureus* and transformed *E. coli* was isolated using a Qiagen Plasmid Miniprep Kit according to the manufacturer's protocol, except that the staphylococcal cells were lysed by lysostaphin (Sigma; 200 $\mu\text{g ml}^{-1}$) at 37°C for 30 minutes before plasmid purification.

2.3 Gel extraction

Gel extraction of the PCR-amplified products was done using a Qiagen PCR Purification Kit and/or Qiagen QIAEX[®] according to the manufacturer's protocols.

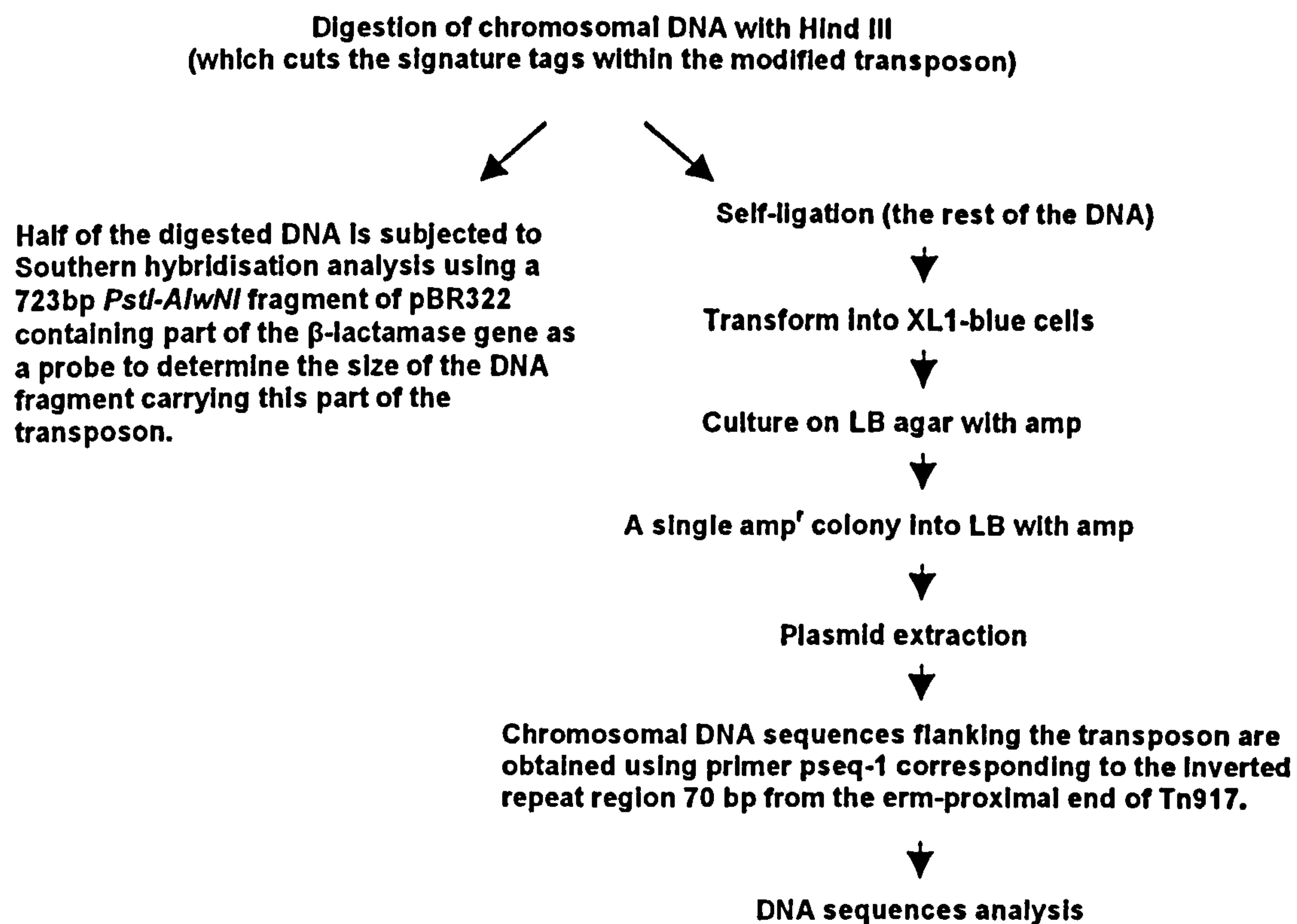


Figure 2.1 Virulence gene identification from mutants with attenuated virulence

a 1 kb ladder (Invitrogen) was used as a marker. The gel was submerged in denaturation solution for 2x 15 min at room temperature. After rinsing with water, the gel was submerged in neutralisation solution at the same conditions. The DNA was blotted to a Hybond N⁺ membrane (Amersham) by capillary transfer, using 20x SSC buffer overnight. The membrane was UV-crosslinked at optimal crosslink (1200 x100μJ/cm²)(Spectro Linker, Spectronics Corporation) without prior washing. The procedure for hybridisation of the membrane was the same as described in section 2.4.

2.5.2 Cloning of attenuated genes

The rest of the digested DNA in section 2.5.1 was resuspended in ligation buffer (Promega) and self-ligated for 12-18 h at 16°C. The ligated products were transformed into *E. coli* XL1-blue, plated onto LB agar containing ampicillin and incubated at 37°C overnight. A single amp^r colony from each transformation was grown in LB broth containing ampicillin. Plasmid DNA was extracted as described in section 2.2. DNA flanking the transposon was sequenced using primer pseq-1 made against the 3' end of the transposon.

2.6 Infection studies

2.6.1 Preparation of bacterial inoculum

To prepare a standard inoculum from *S. aureus* strain RN6390, 0.5 ml of overnight culture was added into 50 ml BHI broth without antibiotics and incubated in a 37°C shaker. The OD₆₀₀ was determined using a spectrophotometer at 15 minute intervals. When the OD ≈ 0.7, 50% sterile glycerol was added and aliquoted into one ml volumes immediately and kept at -80°C. The next day, vials Nos. 1 and 50 were thawed and cultured onto BHI agar plates after making a serial dilution of 10 fold of both. After overnight incubation at 37°C, viable count was done. As there was no significant difference between the viable counts of the two vials, all of the 50 vials were stored as a standard inoculum.

A cryotube containing 1ml of standard inoculum was removed and thawed quickly in 37°C water bath. 900 μl was removed and centrifuged at 13,000 rpm for 3 min. The supernatant was discarded and the pellet was resuspended in 900μl sterile PBS.

Colony forming units (CFU) were counted by carrying out serial 10 fold dilutions of bacterial suspension in PBS. 180µl of PBS was poured in 6 wells of a “U” bottom 96 well plate. 20µl of bacterial suspension was added into the first well and mixed. Then 20µl of this was placed into the next well containing PBS with a new tip. This was continued with a new tip each time until this has been done for a total of 6 times. Then an agar plate was marked into 6 sectors and 3 x 20µl of the lowest dilution was spotted into one of the sectors. The same procedure was done with the remaining dilutions. Another plate was cultured with the same dilutions to give 6 x 20µl spots per dilution. The plates were incubated overnight at 37°C.

The colonies in the sector with more than 40 colonies per spot were counted on the following day. This is the number that is still easy to count (i.e. between 40 and 100 colonies). The number of CFU per ml is achieved with multiplying the number of colonies by the dilution factor (83).

2.6.2 Mouse inoculation

To determine the number of bacteria required for mouse infection, and to see the duration of time required to recover the bacteria from the animals, a series of experiments was done. Different numbers of CFU of *S. aureus* strain RN6390 were injected intraperitoneally into the MF1 hairless mice (female, 6-8 weeks age) and the bacteria were recovered from the mice 24-72 hr after inoculation. Bacteria were not recovered from the mice that received less than 7×10^6 CFU ml⁻¹. The mice which received more than 9×10^6 CFU ml⁻¹ died between 12-18 hr after inoculation with no significant symptoms. We therefore chose a dose of 8×10^6 CFU ml⁻¹ at incubation time of 24 hr.

A series of 25 pools of 33 mutants were used. Each pool contained 33 mutants with individual selected tags (see chapter 5 for details). For mouse inoculation, bacteria from each pool were washed twice with BHI broth by centrifugation at 4000 rpm for 10 min and resuspended in BHI broth. The OD₆₂₀ was determined using a spectrophotometer [OD₆₂₀ of 1.6= 10^9 CFU ml⁻¹] (27). The bacterial suspension was diluted to approximately 8×10^6 CFU ml⁻¹ and added to an equal volume of human serum (Sigma). This mixture (0.2 ml containing approximately 8×10^5 CFU) was injected intraperitoneally into a MF1 hairless mouse (female, 6-8 weeks age). The

number of CFU in the inoculum was verified by viable count after plating a diluted aliquot of the inoculum onto BHI agar. Bacteria were recovered 24 hr after inoculation from spleens of animals as follows.

Half of each spleen was homogenised in 1 ml of sterile PBS in a microfuge tube. Cellular debris was allowed to settle and 1 ml of PBS containing cells still in suspension was removed to a fresh tube and centrifuged for 2 min at 3000 rpm. The supernatant was discarded and the pellet resuspended in 1 ml of sterile PBS. A dilution series was made in sterile PBS and 100 µl of each dilution was plated onto BHI agar containing erm (20 µg ml⁻¹). Bacteria were recovered from plates containing between 1000 and 4000 colonies, and a total of over 10,000 colonies were recovered from each spleen were pooled for DNA extraction (72).

2.7 Biofilm formation

The method for biofilm formation has been adapted from those used for *Candida* species with slight modification (13, 69). Discs of catheter material (surface area, 0.5 cm²) were cut from catheters, and sterilized by UV irradiation for 20-30 minutes each side. Silicone urinary Foley catheters from Vygon, UK were used. The discs were placed in wells of 24-well Nunclon tissue culture plates. Standardized cell suspension (80 µl) was applied to the surface of each disc, and the discs were incubated for 1 h at 37°C. Nonadherent organisms were removed by gentle washing with PBS. The discs were incubated for 24 h at 37°C, submerged in 1 ml of BHI with erythromycin (20 µg ml⁻¹). Discs without cells were incubated in the same way as the control. After biofilm formation, the discs were removed from the medium and gently washed with PBS to remove nonbiofilm cells. Biofilm organisms were washed with 4 ml sterile BHI with erythromycin by vigorous moving of the container. The chromosomal DNA was extracted from the suspension.

2.8 PCR

DNA extracted from single bacterial mutants or pools of mutants was used as template for PCR experiments. The PCR was done in 50 µl reactions containing 75mM Tris-HCl (pH 8.8); 20mM (NH₄)₂SO₄; 2mM MgCl₂; 0.01% Tween 20; 0.2mM each, dATP, dCTP, dGTP, and dTTP; 1.25 U of Taq DNA polymerase (Promega);

400 ng each of primers P12 and P13; and 2.5 µg of target DNA. After initial denaturation of 4 min at 95°C, thermal cycling consisted of 35 cycles of 45 sec at 50°C, 10 sec at 72°C, and 30 sec at 95°C. In some experiments, a Reddy-Load PCR Mix (Advanced Biotechnologies, Surrey, UK) was used at the same conditions.

2.9 Labelling

DNA extracted from single bacterial mutants or pools of mutants were used as templates for PCR to generate labelled probes. The PCR was done in 50 µl reaction volumes and subjected to a [digoxigenin (DIG)-11]-dUTP-labelling PCR using a PCR DIG Probe Synthesis Kit as described by the manufacturer (Boehringer Mannheim) and using primers P12 and P13. After initial denaturation of 4 min at 95°C, thermal cycling consisted of 35 cycles of 45 sec at 50°C, 10 sec at 72°C, and 30 sec at 95°C.

2.10 Ethanol precipitation

PCR products of labelled tags were precipitated with ethanol to concentrate the labelled tags and increase the efficiency of the digestion process in making the probes. 1/10 volume of 3 M sodium acetate pH 5.2 and 2.5 x volume cold absolute ethanol were added and kept on ice for 10 minutes after vortexing. The solution was centrifuged at 13,500 rpm for 15 minutes and the ethanol was discarded. 250 µl cold 70% ethanol was added and centrifuged at 13,500 rpm for 10 minutes. This was done twice. All centrifugation was done at 4°C. The ethanol was discarded and the pellet was air-dried and 20 µl sterile water was added.

2.11 Digestion of the tags

To recover the tags without the arms from the PCR products, 2 µl of the concentrated labelled PCR products of single bacterial mutants or pools of mutants (section 2.10) was digested with *Hind III* enzyme at 37°C overnight. 1 µl (10 U) of *Hind III* enzyme (Life Technologies), 1 µl of buffer, 2 µl of template and 6 µl of water were used in a 10 µl reaction. A total of 30-50 µl reaction was used for each sample to make enough labelled tags as the probes. After overnight incubation, the whole

digestion reaction was run in a 3% agarose gel electrophoresis, and the band of 40 bp was purified using a QIAEX II Kit according to the manufacturer's protocol.

2.12 Gel electrophoresis

Different percentages (0.7%, 1%, and 3%) of agarose gel were used to run the genomic and plasmid DNAs, digestion products of genomic DNAs for Southern blotting, and digestion products of labelled PCR products and probes respectively.

Part II

Results

Chapter 3

Analysis of existing library of mutants

Chapter 3: Analysis of existing library of mutants

The identification of virulence genes and infection mechanisms in bacterial pathogens can lead to new insights for disease-control strategies. Recently, methodologies have been developed that enable genetic approaches to be used for *in vivo* studies of bacterial pathogenesis. One of these new methods is signature-tagged mutagenesis (STM). We were interested to use STM in staphylococcal mouse infection and biofilm. A library of tagged insertion mutants of *S. aureus* strain RN6390 with inserted Tn917 transposon was generously provided by Professor David W. Holden, Imperial College School of Medicine, London, UK.

3.1 Making the genomic DNAs

Genomic DNA from *S. aureus* (as individual and pools of 96 mutants) was isolated by different methods. All protocols described in section 2.1. A typical result is shown in figure 3.1.

3.2 Making the probes

DNA extracted from single bacterial mutants or pools of mutants were used as templates for PCR to generate labelled probes. The PCR was done in 50 µl reactions and subjected to a [digoxigenin (DIG)-11]-dUTP-labelling PCR using a PCR DIG Probe Synthesis Kit as described by the manufacturer (Boehringer Mannheim) and using primers P12 and P13. After an initial denaturation of 4 min at 95°C, thermal cycling consisted of 35 cycles of 45 sec at 50°C, 10 sec at 72°C, and 30 sec at 95°C (see figure 3.2 for results).

The PCR products (approximately 80 bp) were precipitated with ethanol and then digested with *HindIII* (which cuts the signature tags within the modified transposon) at 37°C for 12-18 hr. The digested products (40 bp) were gel purified using a Qiagen PCR Purification Kit and/or Qiagen QIAEX[®] according to the manufacturer's protocols (figure 3.3).

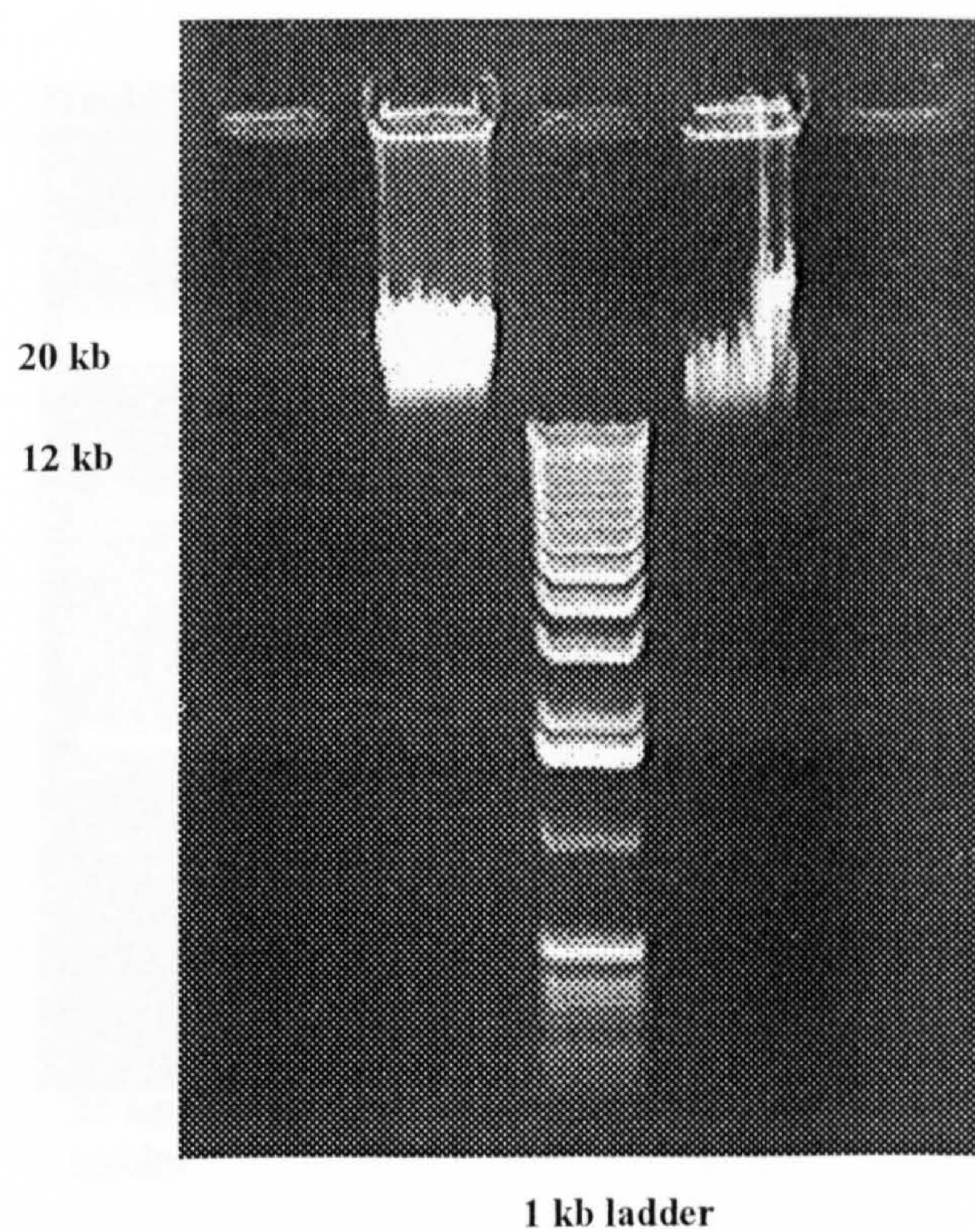


Figure 3.1 Two genomic DNAs from different pools

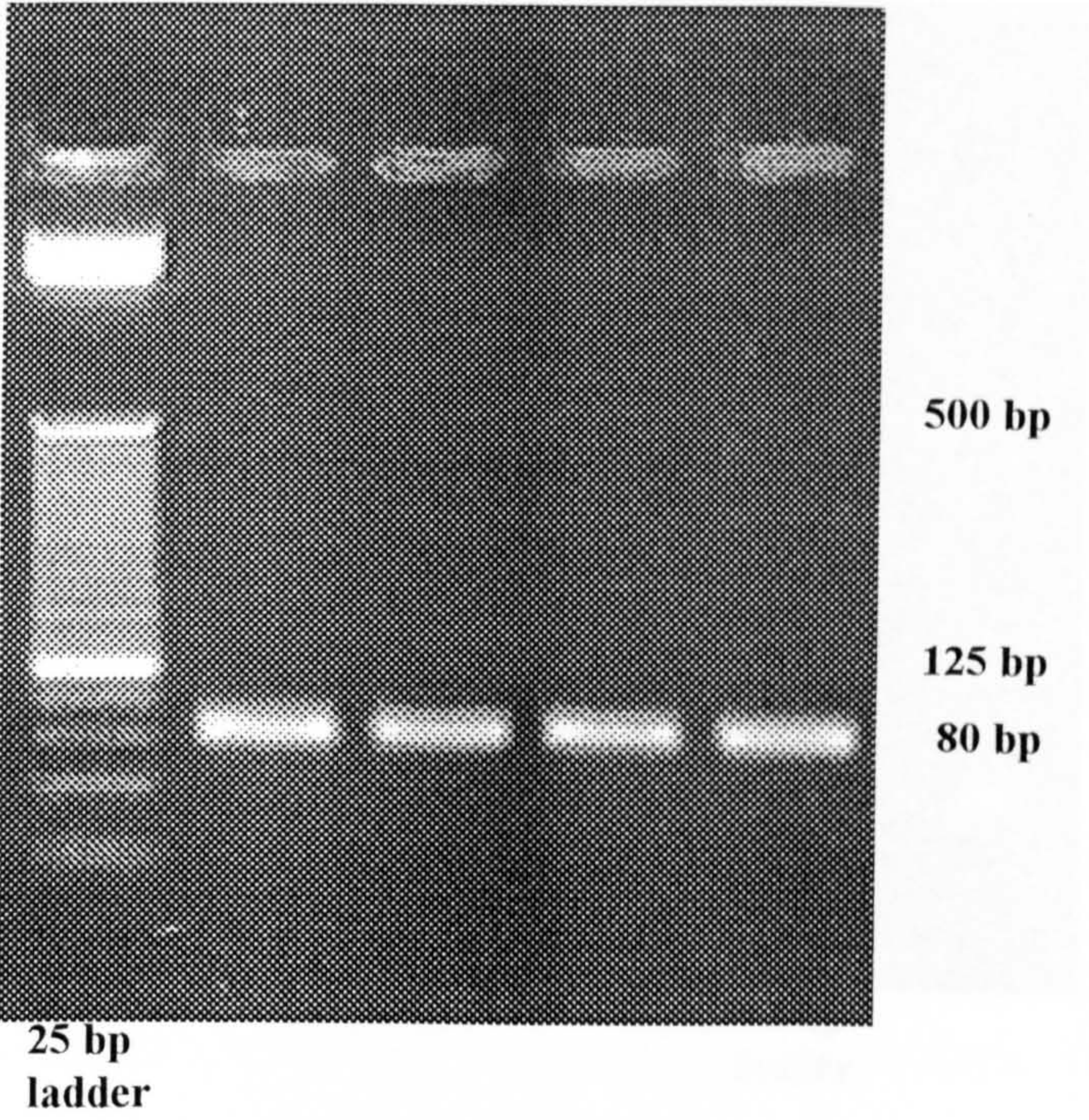


Figure 3.2 PCR products from 4 different genomic DNAs

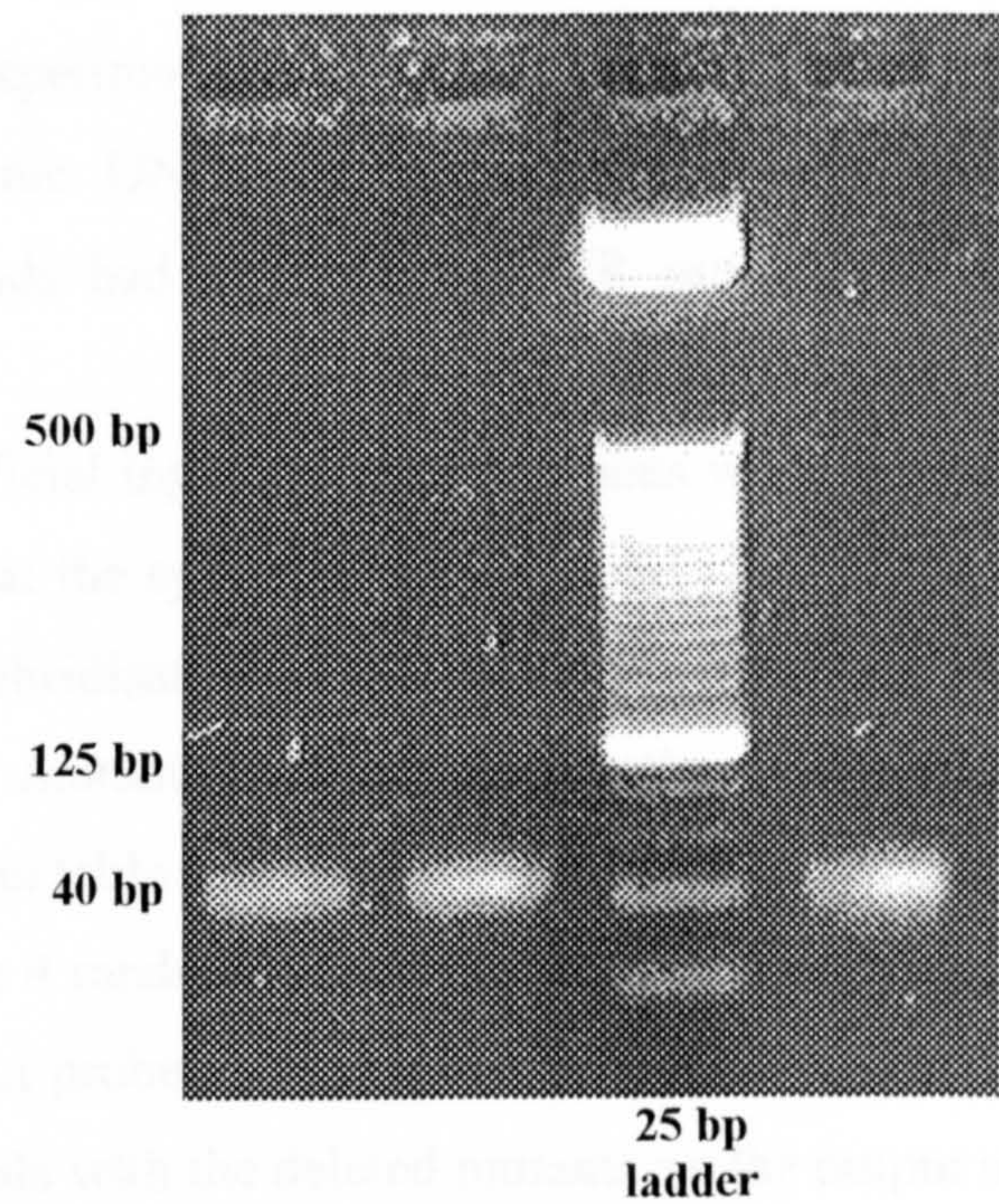


Figure 3.3 Three probes from different genomic DNAs

3.3 Hybridisation experiments

Probes were hybridised to nylon membranes according to the method described in the DIG Probe Synthesis Kit. For dot blot hybridisations, genomic DNAs (0.15-0.2 µg) were transferred onto a Hybond N+ membrane (Amersham).

According to the literature (29, 72, 112), the probes should hybridise to the genomic DNAs of the tagged insertion mutants. When we hybridised labelled probes to chromosomal DNA of individual mutants, we were unable to generate a signal. Therefore, a series of experiments was done to compare hybridisation strategies. PCR products of the genomic DNAs and gel extraction of the PCR products were compared. Both methods had signals. The PCR products were chosen for future analysis.

At the next step, artificial input and output probes were hybridised to the different membranes to check that the system worked properly. All of the experiments, except three, showed cross-hybridisation of the tags. We therefore undertook a detailed analysis of the existing mutants to determine whether we could use the library. The results are summarised in table 3.1.

Experiment series 1: 4 random mutants were deleted from a pool of 96 mutants to make an artificial output probe of 92 mutants. The result showed signals from all 96 spots suggesting 4 signals with the deleted mutants on the output membrane. In a real experiment this would suggest no loss of mutants.

Experiment series 2: The experiment in series 1 was done in another way. 4 random mutants (different from the deleted mutants in experiment 1) were deleted from a pool of 94 mutants to make an artificial output probe of 90 mutants. The result showed loss of 2 out of 4 mutants. In other words, 2 out of 4 mutants were not detected.

Experiment series 3: The experiment in series 2 was done in another way. 4 probes were made from the 4 deleted mutants and then each probe was tested with input membrane of 94 mutants separately. The 4 mutants were A6, D4, F9, and H8. The mutants D4 & H8 showed no cross-hybridisation on the output membranes that contained 94 mutants. But mutant A6 had a cross-hybridisation with one mutant (A5), and mutant F9 had cross-hybridisation with 3 mutants (E12, G6, and G7). In other words, the cross-hybridisation results in the previous experiment were confirmed. The

Series	Input Pool	Output Pool	Unexpected cross-hybridisation	Figure
1	96	92	Yes	-
2	94	90	Yes	-
3	94	1 (x4)	Yes/No	-
4	10	1	No	Figure 3.4
5	10	1 (x10)	Yes	Figure 3.5
6	10	8	No	Figure 3.6
7	10	8	Yes	-
8	94	1 (x2)	Yes	-
9	8	6	No	Figure 3.7

Table 3.1 Summary of the results of hybridisation experiments with an existing library of mutants

results in the experiments series 2 & 3 were also confirmed by sequencing the tags (discussed in chapter 4).

As the first three experiments showed there was a problem with the hybridisation, a more detailed set of experiments was done.

Experiment series 4: A probe from one mutant was hybridised to an artificial input of 10 random mutants. As expected there was just one signal on the membrane (Figure 3.4). According to the expected result in this series, we decided to do a series of hybridisation experiments on a pool of 10 random mutants.

Experiment series 5: The experiment in series 4 was repeated ten times with the same mutants, and one mutant was chosen to prepare the probe each time. The results showed that two mutants (Nos. 5 & 9) had cross-hybridisation with each other (Figure 3.5). The details of this series are shown in Table 3.2.

Experiment series 6: This experiment was done on a pool of 10 random mutants. The random selected mutants in this series were different from the 10 mutants in series 5. Mutants Nos. 3 & 9 were deleted to make an artificial output pool with 8 mutants. Probes were made from the 10 mutants as the input and from the 8 mutants as the output pools. Hybridisation experiments were done with both input and output probes. As it is shown in figure 3.6, there were just 8 expected signals on the output membrane. In other words, there were no signals with the 2 deleted mutants in output probe.

Experiment series 7: As we had the expected results in series 6, the same experiment was done on a different series of 10 random mutants. In this experiment, 9 signals were seen instead of 8 expected signals on the output membrane.

Experiment series 8: The probes from the two deleted mutants in series 7 (with cross-hybridisation) were hybridised with input membranes of 94 mutants individually. The 2 deleted mutants were B5 & D2. The results showed that one mutant (D2) had just one expected signal, but the other (B5) had cross-hybridisation. In other words, mutant B5 had 2 signals, but D2 had no cross-hybridisation, both on the membrane of 94 mutants.

Experiment series 9: A pool of 8 random mutants was chosen. Mutants Nos. 6 & 8 were deleted to make an artificial output pool with 6 mutants. Probes were made from the 8 mutants as the input and from the 6 mutants as the output pools. Hybridisation experiments were done with both input and output probes. As expected, there were just 6 expected signals on the output membrane (Figure 3.7).

No. of mutants on each membrane	No. of mutants present in the probe	Result (unexpected signals)	Figure
10	1 (No. 1)	-	-
10	1 (No. 2)	-	-
10	1 (No. 3)	-	-
10	1 (No. 4)	-	-
10	1 (No. 5)	1	Figure 3.5
10	1 (No. 6)	-	-
10	1 (No. 7)	-	-
10	1 (No. 8)	-	-
10	1 (No. 9)	1	Figure 3.5
10	1 (No. 10)	-	-

Table 3.2 The results of hybridisation of 10 different probes with the same mutants

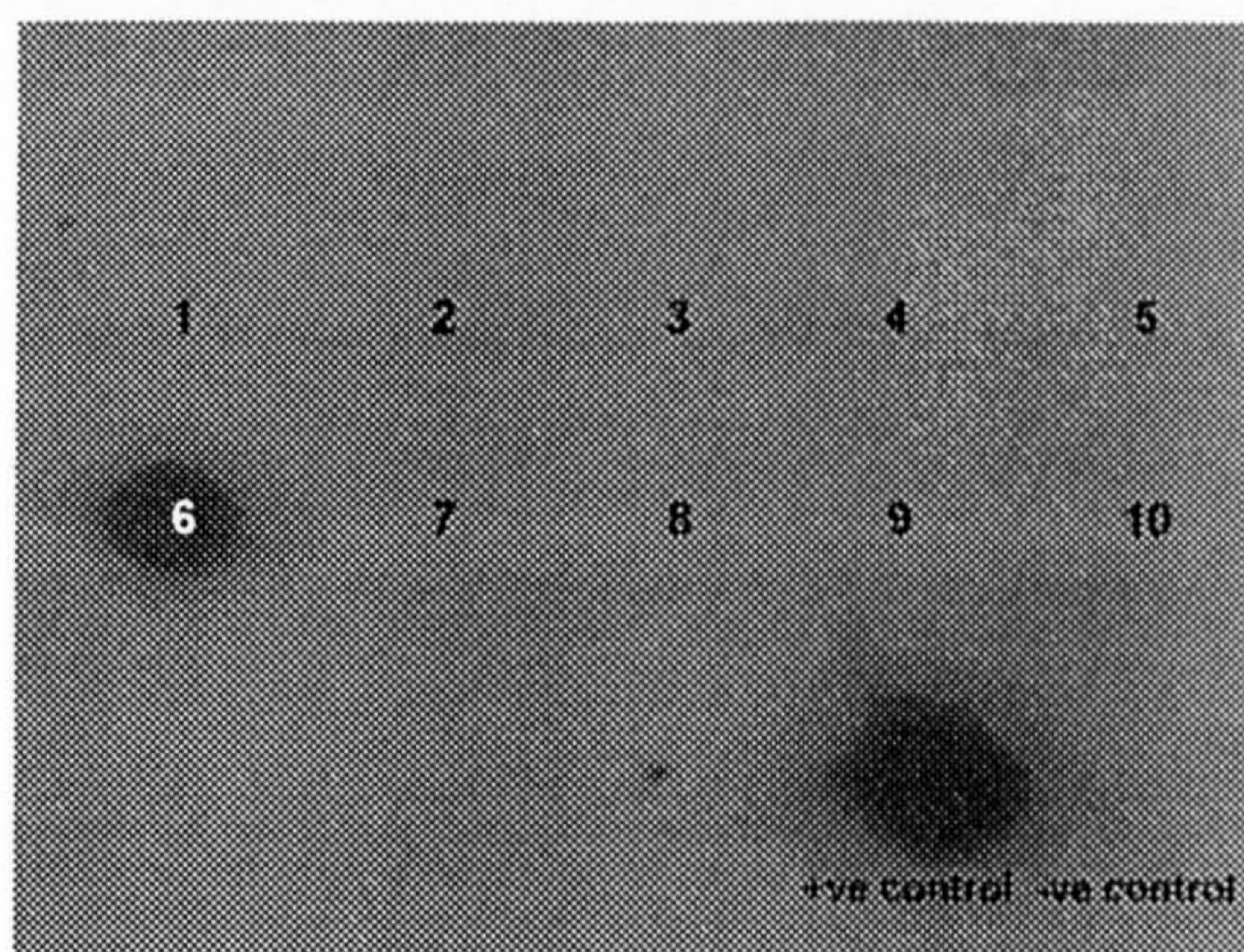


Figure 3.4 Hybridisation of the membrane of 10 mutants with the probe No. 6 that shows expected result.

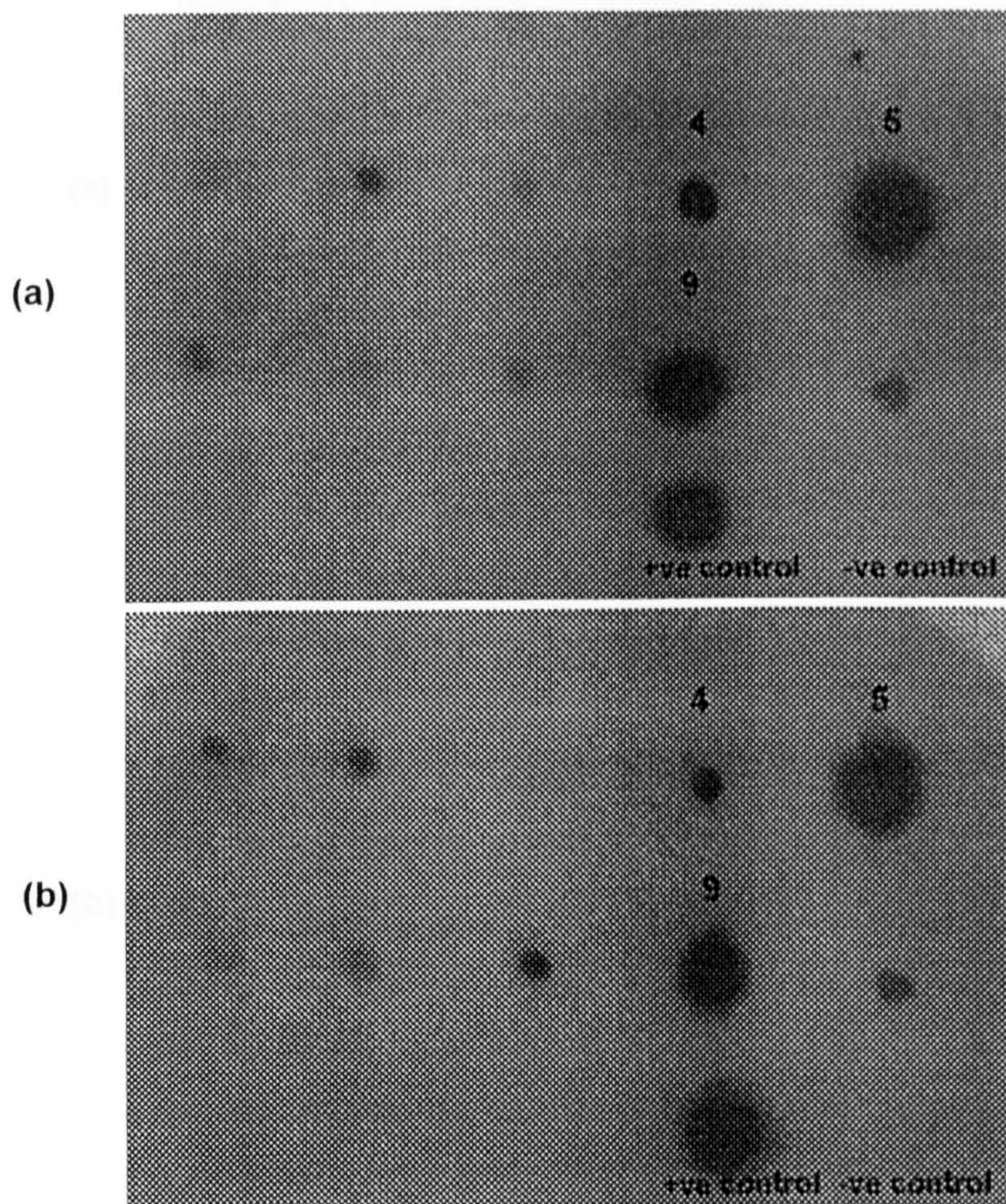


Figure 3.5 Hybridisation of the same membrane of 10 mutants with the probes No. 5 (a) and No. 9 (b). As seen there is also a strong signal (cross-hybridisation) with mutant No. 9 in (a) and with No. 5 in (b). There is also some cross-hybridisation with mutant No. 4 in both membranes.

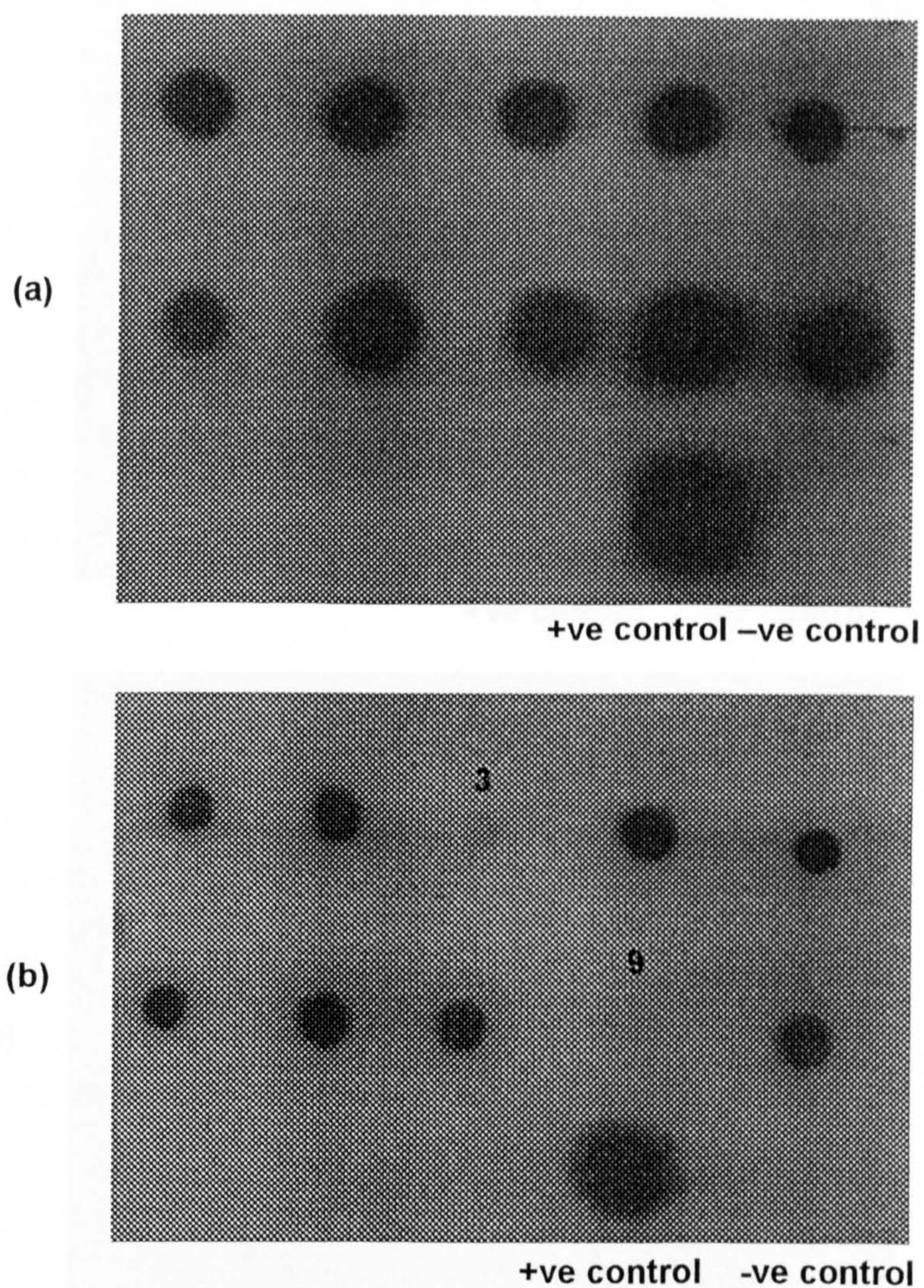


Figure 3.6 Hybridisation of the same membrane with probes of 10 mutants as the input (a) and 8 mutants as the output pools. Mutants Nos. 3 & 9 have been deleted to make an artificial output probe. The experiment showed expected results. There are 10 and 8 signals on the input (a) and output (b) membranes, respectively. The two deleted mutants (Nos. 3 & 9) showed no signals on the output membrane (b).

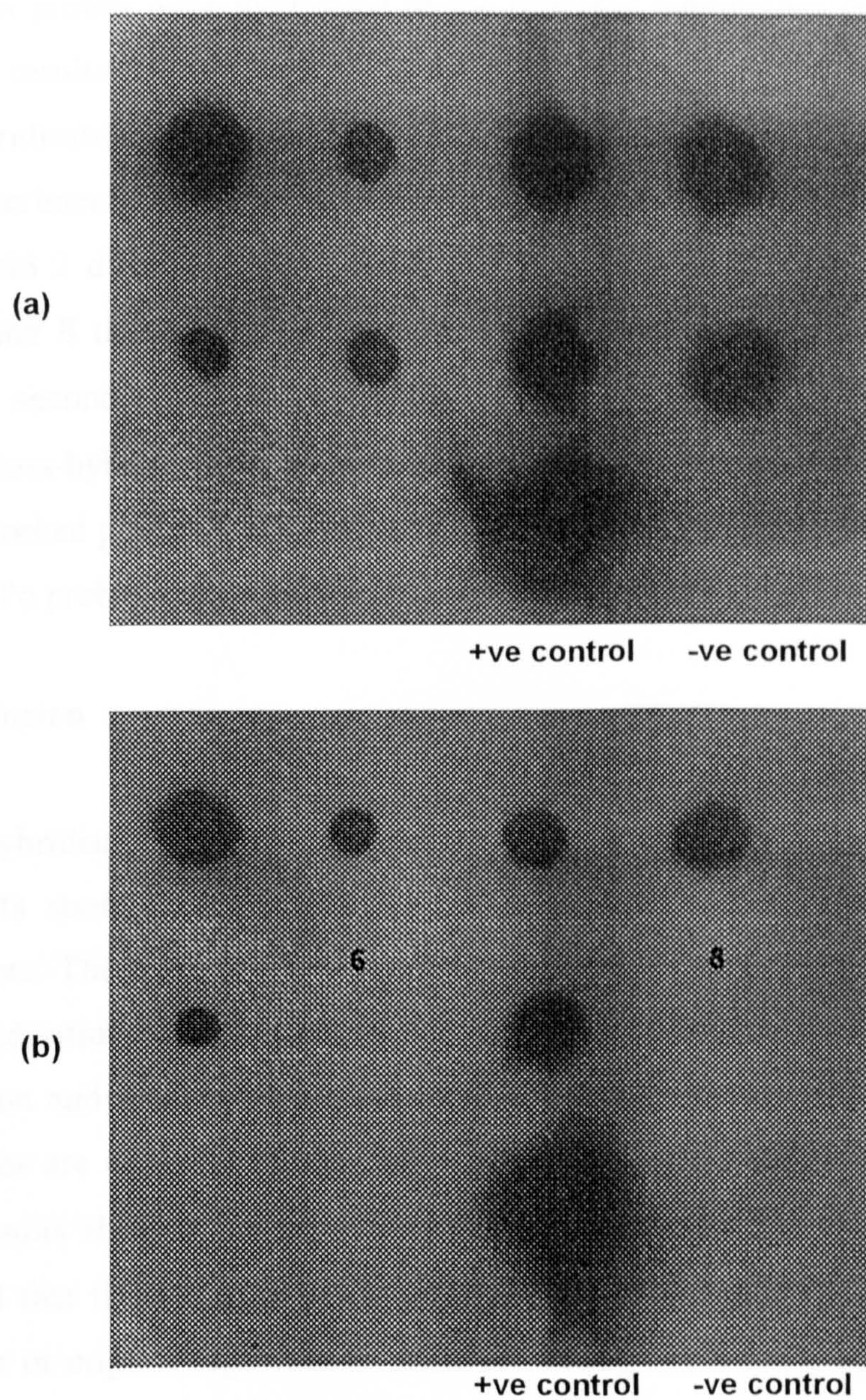


Figure 3.7 Hybridisation of the same membrane with probes of 8 mutants as the input (a) and 6 mutants as the output pools. Mutants Nos. 6 & 8 have been deleted to make an artificial output probe. The experiment showed expected results. There are 8 and 6 signals on the input (a) and output (b) membranes, respectively. The two deleted mutants (Nos. 6 & 8) showed no signals on the output membrane (b).

The details of the experiments on the existing library are shown in table 3.3.

To test the library of mutants and hybridisation process, a series of artificial input and output probes were hybridised to the different membranes (tables 3.1, 3.2, and 3.3). The results showed that all of the experiments, except three, had unexpected cross-hybridisation.

The experiments on the existing library that were done in different scales could be divided into 2 distinct groups. The first group includes series 4, 6, 9, and parts of series 3 and 8 that showed no cross-hybridisation and only expected signals were seen. The second group includes series 1, 2, 5, 7, and a part of series 3 and 8 that showed cross-hybridisation. As we had some expected results, it showed that the STM method worked properly, and the cross-hybridisation in some experiments is probably because of a problem with the library.

3.4 Conclusion

The hybridisation experiments on the existing library took more than one year. The results showed that the library was not reliable for cross-hybridisation free experiments. There are at least 3 possible reasons for this problem, (i) the problem with hybridisation itself, (ii) incomplete removal of the arms of the tags in the process of digestion and making the probes, and (iii) contamination of the tags. The first 2 possibilities are excluded as they are not supported by some series of experiments, and as results showed the method does work in some experiments. It is therefore concluded that the problem was more likely the contamination of the mutants with each other or original tags used to make the library. This could have occurred in the first inoculation from the master plates (which contain all the representative tags in each plate) to the cryotubes.

As the existing library of mutants showed unexpected cross-hybridisation, the whole library was discarded. We next tried to use the selected tags in plasmids of *S. aureus* strain RN6390 for future experiments that will be discussed in details in chapter 4.

Experiment series	No. of mutants on the membrane	No. of mutants present in the probe	Result (unexpected signals)	Deleted mutants
1a*	96	96	-	
		92	4	
1b*	96	96	-	
		92	4	
2	94	94	-	
		90	2	
3a	94	94	-	
		90	2	A6, D4, F9, H8
3b**	94	1 (A6)	1 (A5)	
		1 (D4)	-	
		1 (F9)	3 (E12, G6, G7)	
		1(H8)	-	
4	10	1	-	
5	10	1 (No. 1)	-	
		1 (No. 2)	-	
		1 (No. 3)	-	
		1 (No. 4)	-	
		1 (No. 5)	1	
		1 (No. 6)	-	
		1 (No. 7)	-	
		1 (No. 8)	-	
		1 (No. 9)	1	
		1 (No. 10)	-	
6	10	10	-	
		8	-	Nos. 3 & 9
7a***	10 (pooled)	10	-	
		8	1	B5, D2
7b***	10 (mixed)	10	-	
		8	1	B5, D2
8****	94	94	-	
		1 (B5)	1	
		1 (D2)	-	
9	8	8	-	
		6	-	

Table 3.3 Details of the results of hybridisation experiments with existing library of mutants

*** Two different series were tested.**

**** The same series of mutants in the experiment 3a were tested. Individual probes were made from the mutants which had cross-hybridisation in the experiment 3a.**

***** The same series of 10 mutants were tested twice. In the first experiments, the probes were made from a pool of 10 and 8 mutants as input and output pools respectively, but in the second one, probes were made individually and then mixed up.**

****** The deleted mutants in experiments 7a and 7b were examined with a membrane with larger number of mutants. One of the mutants showed cross-hybridisation with another mutant which was not present in the experiments.**

Chapter 4

Analysis of selected tags in plasmids of strain

RN6390

Chapter 4: Analysis of selected tags in plasmids of strain RN6390

Plasmid pID408 (Figure 4.1) has been constructed for mutagenesis of *S. aureus* strain RN6390 in Professor David W. Holden's laboratory. The $\text{amp}^r/\text{ori}/\text{rop}$ region from plasmid pBR322 allows replication and selection of the plasmid in *Escherichia coli*, while the temperature-sensitive replicon (pE194ts) and the cm^r gene of pTV32ts permit replication and selection in *S. aureus* at 32°C. The DNA signature tags have been incorporated in the *EcoRI* site of transposon Tn917. A series of 96 plasmids carrying different tags has been separately transformed into *S. aureus* strain RN6390. The plate containing 94 of the 96 was sent to us by Professor Holden, and designated the master plate. This was used for all subsequent mutagenesis of *S. aureus*.

A duplicate of the above master plate was generously provided by Professor David W. Holden, Imperial College School of Medicine, London, UK.

4.1 Preparation of plasmid from *S. aureus*

Plasmid DNA from *S. aureus* strain RN6390 which had the selected tags (94 strains) was isolated as described previously in section 2.2. Plasmid DNA was successfully isolated at good concentration (Figure 4.2).

4.2 Hybridisation with probes from *S. aureus* plasmids

Probes were made as described in section 3.2. Probes were hybridised to nylon membranes according to the method described in the DIG Probe Synthesis Kit (Roche Diagnostics GmbH, Germany). For dot blot hybridisations, 0.15-0.2 µg plasmid DNAs were transferred onto a Hybond N+ membrane (Amersham). Artificial input and output probes were hybridised to the different membranes to confirm the system works properly. Cross-hybridisation was seen in the most of the experiments, which were done with the probes from plasmid DNAs.

A series of experiments was done to check whether the selected tags in plasmids of strain RN6390 were suitable for a work of free cross-hybridisation. The hybridisation experiments on the selected tags in plasmids of strain RN6390 took more than one year.

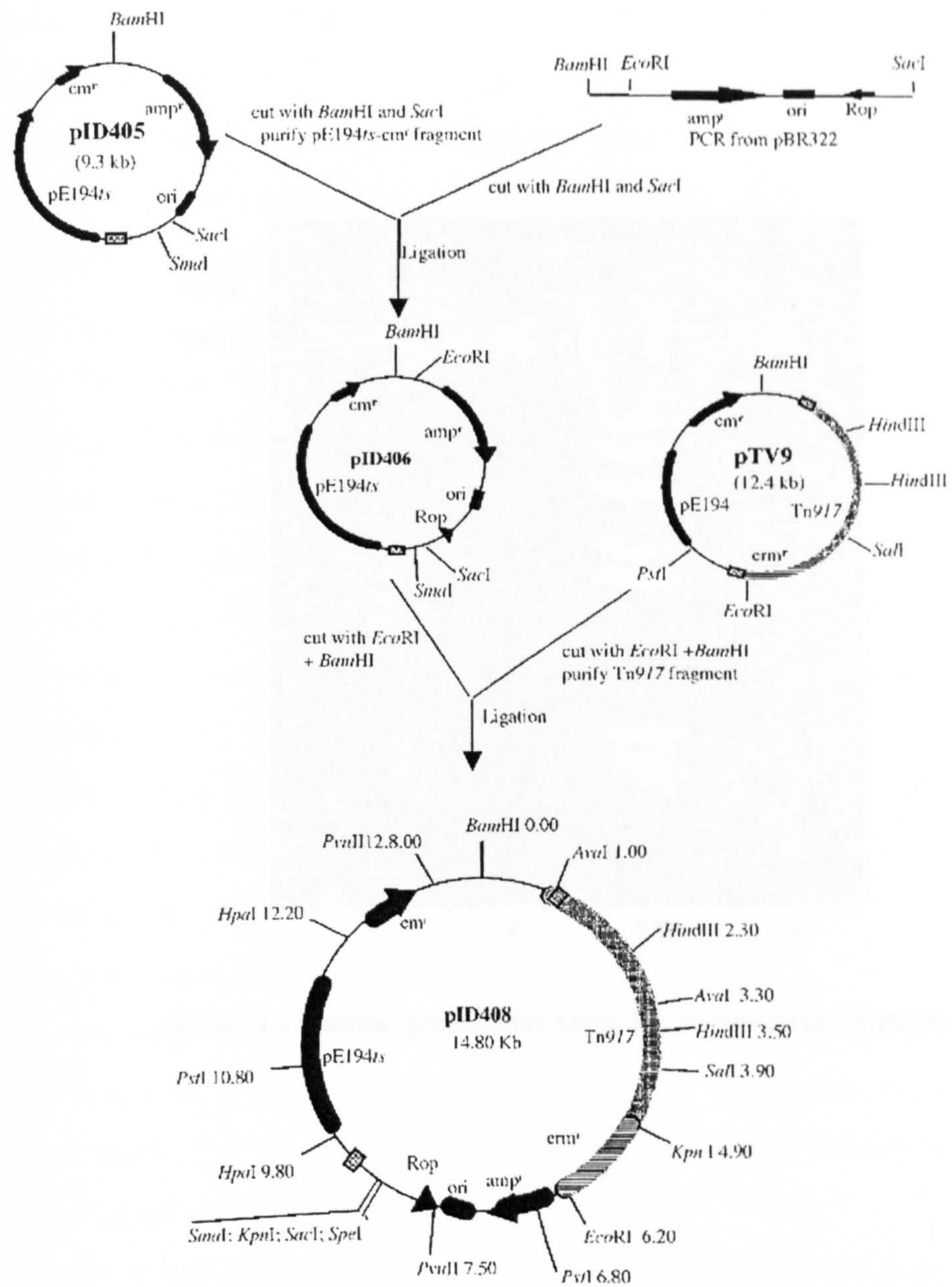


Figure 4.1 Construction of the vector pID408 (by courtesy of Prof. Holden)

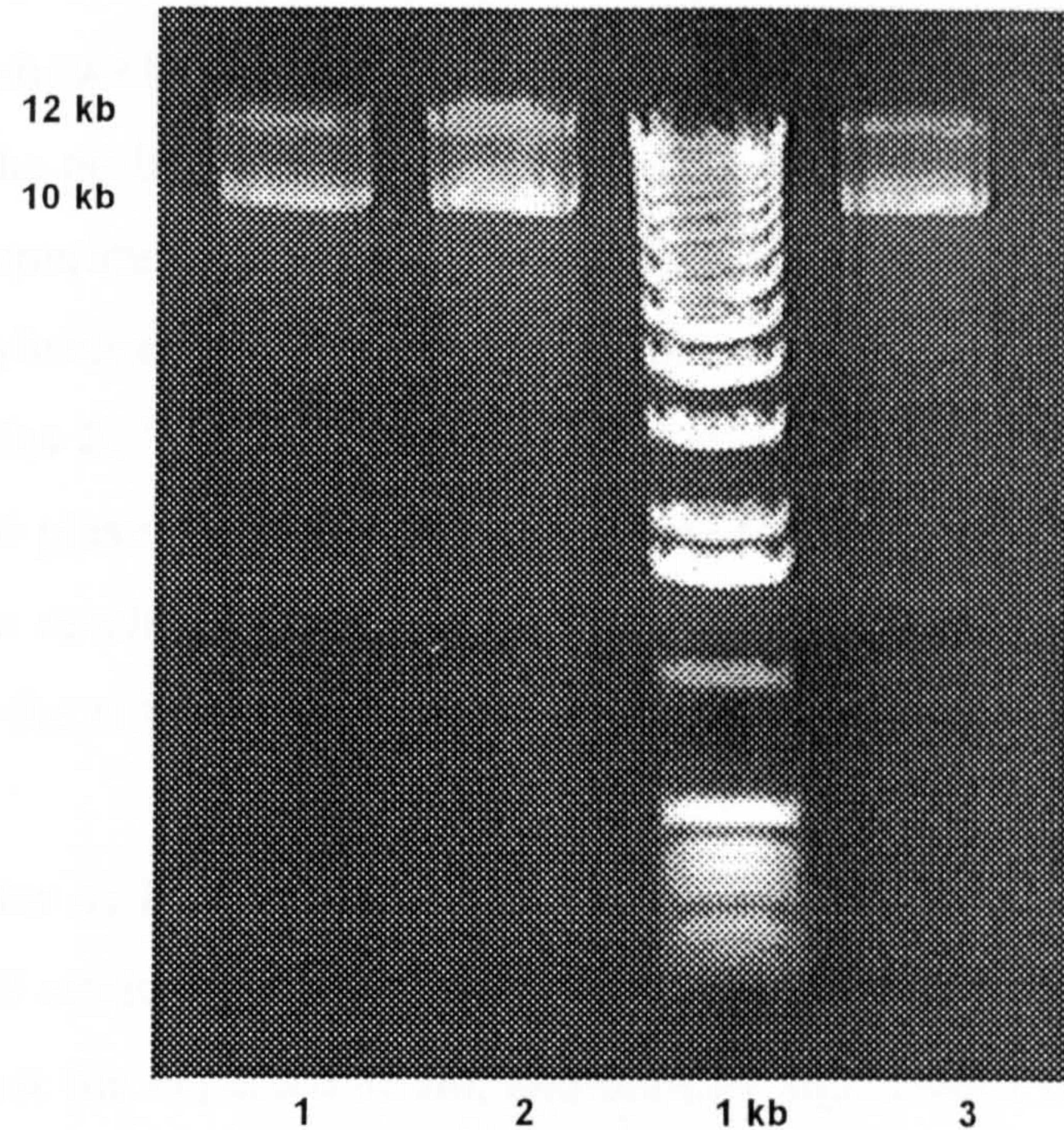


Figure 4.2 Plasmid preparation from 3 *S. aureus* strain RN6390

Experiment series 1: As there was a lot of cross-hybridisation in experiments with genomic DNAs from the existing library, the first two experiments with the selected tags in plasmids of strain RN6390 were done on a small scale. Four strains (A2, A3, A4, and A5) were chosen randomly. Probes were made from strains A2 and A5 and hybridised to the same membranes of the 4 plasmids. Results showed that the probes did not detect just the expected strain. In other words, there were 2 signals instead of one on each membrane.

Experiment series 2: This experiment was repeated 4 times. 4 probes were made, and one different probe was hybridised to a membrane of 94 plasmids each time. Again, the results showed that none of the 4 probes could detect the expected strain on the membranes. The probes which were used in this series were the same 4 plasmids in series 1. This experiment showed that plasmids A2 and A5 had actually more than one signal when hybridised to a large number of plasmids.

Experiment series 3: The experiment series 1 was repeated 4 times with another different series of 4 plasmids. The 4 selected strains for this experiment were A6, A7, A8, and A10. The results showed that none of the 4 probes could detect just the expected strain on the membranes. In other words, there was more than one signal on each membrane.

Experiment series 4: In this series, a probe was made from strain B3. The probe was hybridised to 8 random plasmids including B3. The result showed that the probe could not detect just the expected strain, and another signal with strain B2 was also seen on the membrane (Figure 4.3).

Experiment series 5: In this series, a probe was made from strain E7. The probe was hybridised with 8 random plasmids including E7. The result showed that the probe could detect just the expected strain on the membrane. In other words, there was just one signal on the membrane.

Experiment series 6: In this series, the 2 strains in series 4 and 5 were checked with an artificial pool with a larger number of plasmids to check whether the above results could be confirmed. 2 probes were made from strains B3 and E7, and hybridised to an artificial input of 16 random plasmids including the 2 strains on the same membranes. The results showed that one membrane had just the expected signal (E7), but the probe B3 could not detect just the expected strain, and another signal with strain B2 was also seen on the membrane.

input

output

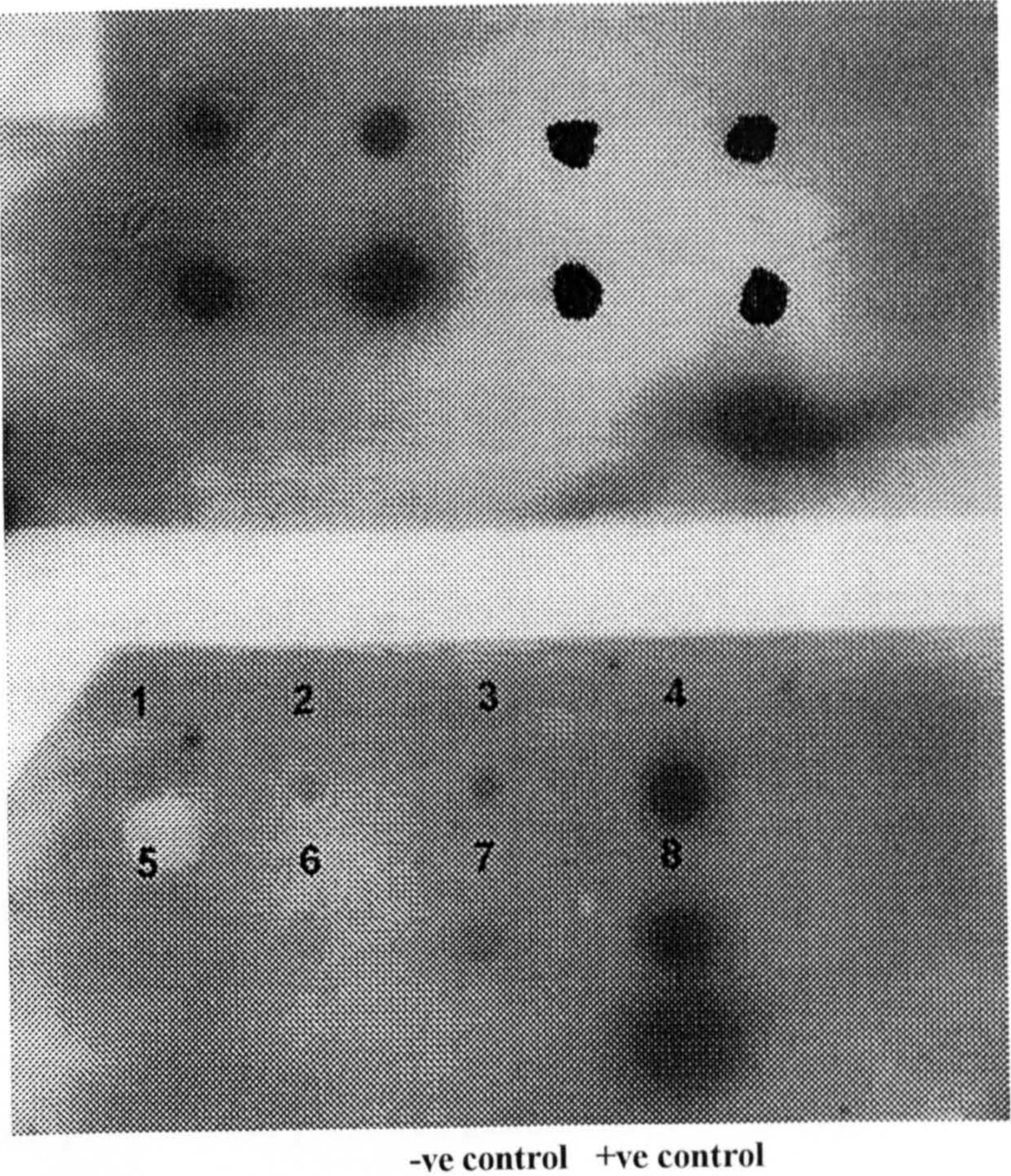


Figure 4.3 Hybridisation of 8 plasmids with a probe from No. 4. There was an additional signal with plasmid No. 8.

Experiment series 7: In this series, the strains B3 and E7 were checked with all of the 93 plasmids. 2 probes of the strains B3 and E7 were hybridised with a pool of 93 plasmids individually. The result showed that the strain E7 had no cross-hybridisation, and only one expected signal was seen on the membrane. However, there were 3 signals on the membrane B3 rather than the one expected signal; the other two were unexpected cross-hybridisations with strains B2 and F7.

The experiments in series 4, 6, and 7 showed that strain B3 could not hybridise free of cross-reactions, whereas the experiments in series 5, 6, and 7 showed that the strain E7 could hybridise clearly.

The results of the experiments with the selected tags in plasmids strains RN6390 are summarised in table 4.1.

4.3 Transformation into *E. coli*

Due to the cross-hybridisation found in the experiments with plasmid DNAs, it was decided to transform all of the plasmid DNAs from *S. aureus* strain RN6390 into suitable competent *E. coli* cells. More DNA was needed to test the tags, and this was not feasible using *S. aureus* strains. 93 out of 94 plasmids were transformed into XL1 blue cells (Stratagene). One sample was not successfully transformed even after several attempts.

4.4 Hybridisation with probes from plasmids generated from *E. coli*

Plasmid DNAs from 93 transformed cells was isolated as described in section 4.1, except using lysostaphin. Probes were made as described in section 3.2 and hybridisation was done as described in section 4.2. Cross-hybridisation was seen in some of the experiments, which were done with the probes from plasmid DNAs from the transformed cells. Therefore, there was a problem with hybridisation of plasmids as isolated from *E. coli*. It was therefore decided to check any possible identity between the tags. Plasmid DNAs of a series of the strains showing cross-hybridisation were therefore sequenced.

Series	No. of mutants on each membrane	No. of mutants present in the probe	No. of unexpected signals
1	4 (A2, A3, A4, A5)	1 (A2, A5)	1
2	94	1 (A2, A3, A4, A5)	2(A5),3(A4),5(A2),7(A3)
3	4 (A6, A7, A8, A10)	1	1(A6),2(A7),1(A8),1(A10)
4	8	1 (B3)	1 (Figure 4.3)
5	8	1 (E7)	-
6	16	1 (B3, E7)	1(B3), -(E7)
7	93	1 (B3, E7)	2(B3), -(E7)

Table 4.1 Summary of the results of hybridisation experiments with the selected tags in plasmids of strain RN6390

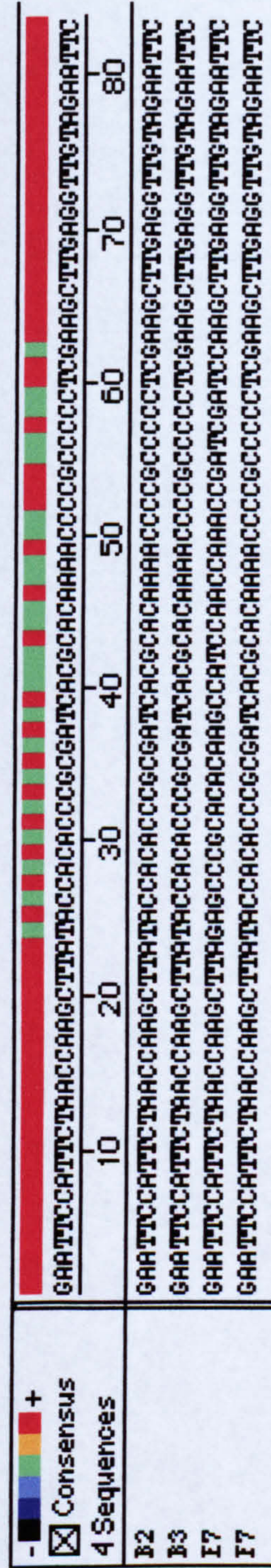
4.5 Sequencing and selecting of non-cross hybridising tags

4 plasmid DNAs from experiments series 4-7 were subjected to sequence analysis. The above experiments showed that strain B3 cross-hybridised with strains B2 and F7, but strain E7 did not cross-hybridise with any of the 93 plasmids. The results showed that the sequences of 3 tags B2, B3, and F7 were exactly the same (Figures 4.4 & 4.5), whereas the sequence of tag E7 was different from the others. The result of sequencing therefore confirmed the hybridisation experiments.

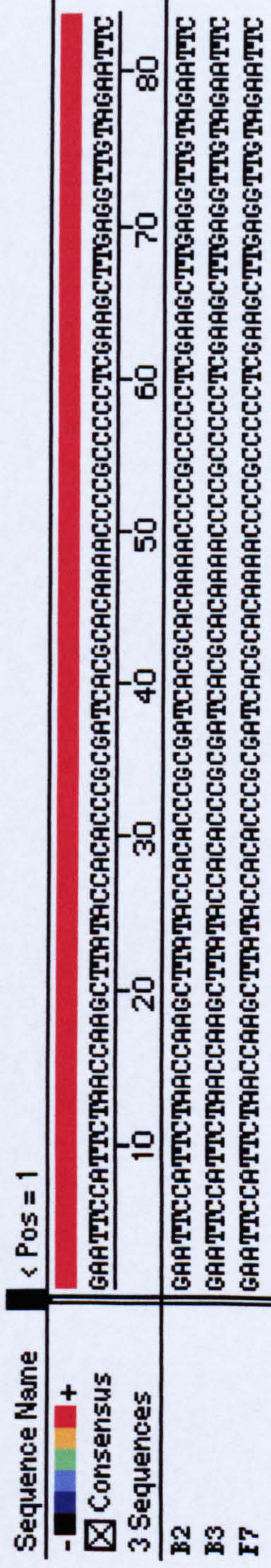
As 3 of 4 plasmids were found to contain similar tags, it was decided that all of the 94 plasmids should be sequenced. 84 out of 94 plasmids were sequenced. The results showed that 51 strains had more than 50% similarity between the tags, and therefore were not suitable for a cross-reaction free hybridisation. The 33 tags with no more than 50% identity were chosen for future experiments (Figures 4.6-4.9). To check whether the system worked properly, a series of 10 random mutants were chosen and artificial input and output probes were hybridised. The results showed no cross-hybridisation. As shown in figure 4.10, there were good signals with all of the 10 mutants in the input membrane (a). In membrane (b), two random mutants (Nos. 2 & 9) were deleted and a probe was made from the remaining 8 mutants. There were still good signals with the 8 mutants, but not with numbers 2 and 9. In the third part of the experiment, 5 mutants (numbers 2, 4, 6, 8, and 9) were deleted and a probe was made from the remaining 5 mutants. There were still good signals with the 5 mutants, but not the deleted ones.

In another experiment, all 33 selected tags were checked. All 33 gave good signals in the output membrane (Figure 4.11)(a). In membrane (b), 4 random tags (Nos. 11, 20, 21, and 29) were deleted and a probe was made from the remainder 29 tags. There were still good signals with the 29 tags, but not the deleted ones.

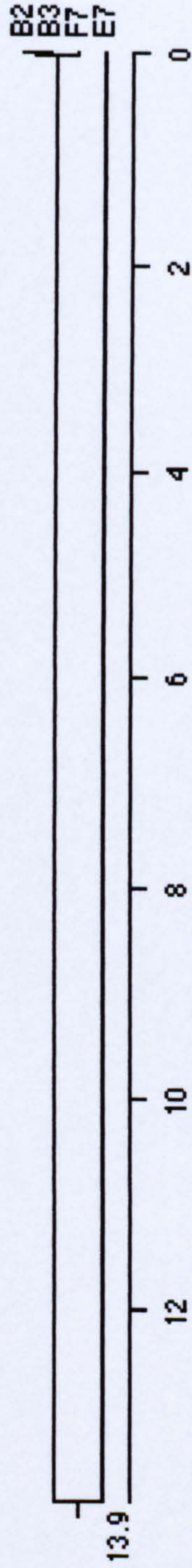
It was therefore concluded that the 33 selected tags had no more than 50% identity and were suitable for cross-hybridisation-free experiments.



(a)



(b)



(c)

Figure 4.4 (a) Alignment of the sequences of the 4 plasmids using the MegAlign program of DNASTAR . See Figure 4.5 for the enzymes sites and tags. (b) Alignment of the sequences of the 3 similar plasmids. (c) Phylogenetic tree of the plasmids in (a). The first 6 bases (gaattc) are the *EcoRI* site. Bases 17-22 (aagctt) are the *Hind III* site which cuts the arms. Bases 23-62 are tags. The tags and different sites are shown in figure 4.5 in colour.

B2	GAATTCATTCTAACC	AAGCTT	ATACCA	CACCCGCGATC	ACGCACAA	ACCCGCCCTCG	AAGCTT	GAGGTTGTAGAATTC
B3	GAATTCATTCTAACC	AAGCTT	ATACCA	CACCCGCGATC	ACGCACAA	ACCCGCCCTCG	AAGCTT	GAGGTTGTAGAATTC
E7	GAATTCATTCTAACC	AAGCTT	AGAGCCG	CACAAAGC	CAATCC	ATCGATCC	AAGCTT	GAGGTTGTAGAATTC
F7	GAATTCATTCTAACC	AAGCTT	ATACCA	CACCCGCGATC	ACGCACAA	ACCCGCCCTCG	AAGCTT	GAGGTTGTAGAATTC

Figure 4.5 Alignment of a part of 4 sequences with the *EcoRI* site in blue, the *Hind III* site in red, similar tags in green, and the only different tag in pink.

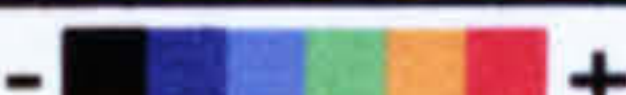

Sequence Name	Pos = 3
-  +	
<input checked="" type="checkbox"/> Consensus	AGAGCGCGCGCGCGAGCTCGCGCGCGCGCTCGCTCCCGCTXX
84 Sequences	10 20 30 40
tag A2	CTAGGCGTGGGAGAGAGAGCGGGTTC TAGTTTG TGAGGG
tag A3	GGCGAGTGAGGGTTGTATTTGGTGGCTGGCGGTGTCGATAT
tag A4	AGTGAGTGAGTGTGCGCGCGCTCGGGAGGGTGTGGAACCG
tag A5	AACAACACACAGACCACCATAGCTCACTCCAGCGCC
tag A6	AACAACACACAGACCACCATAGCTCACTCCAGCGCC
tag A7	ACCGCACACTCTCACCTATCCCTCGAACACACCCCCC
tag A8	CGAGTTCGGGGTGGATGGTTTTGTCTCGCGATAT
tag A9	CGGTGGCGCGGGTGGGGTAGGTGTGGATTTTTGTGTGTG
tag A10	CGAGTTCGGGGTGGATGGTTTTGTCTCGCGATAT
tag A11	ACACCGCCACACACAGCTCCAACTCCACATCG
tag A12	ATAGCAACCGCAATCCAGCCCGACCGCTCCGCCCCAACCC
tag B1	CCCTCCCGACCGACCTCCACACCAATAACTCTCTCAAGAC
tag B2	ATACCACACCCGCGATCACGCACAAAACCCGCCCCCTCG
tag B3	ATACCACACCCGCGATCACGCACAAAACCCGCCCCCTCG
tag B4	AGGGCGCGTTGGCGGTCTCGGTGGTTGGAGATGTGGAGGT
tag B5	CCAACGATAACCCCGCCACCGCAAGCCCTCACACAGCA
tag B6	AGGGTGTAGGTGGATGGTGTGGAGGGCGGGGGTTGGAT
tag B7	GTCGAGGGAGTGCATTTTCGGTGTGGGGGGGTGGCTAGCT
tag B8	GTCGAGGGAGTGCATTTTCGGTGTGGGGGGCTGGCTAGCT
tag B9	CGATCTTG TGGGCTAGGGATCGTTTG TGAGCGTGGTTGGG
tag B10	AGGTAGCGCGGGAGTTGGTTGGCTAGTTATCGTGGGTG
tag B12	CACGAACTAACCATCCCTACACCCACCAATCTCCCAACG
tag C1	ATGGCGCTCTAGGGAGTTAGCGGTGGCGGTGGGGATGTGT
tag C2	AGCTAGGGTGC CGGTGCGGTTGGGTGTAGCGGTGTGT
tag C3	GGAGTGGTGGAGGTAGCTCGGGTGGGGGTGGATATATCG
tag C4	GGAGTGGTGGAGGTAGCTCGGGTGGGGTCTGGATATATCG
tag C7	AACGCACGAGACCGACCCCGCCACTAACGCTCCATCCCT
tag C8	GAGTGGTGGAGGTAGCTCGGGTGGGGTCTGGATATATCG
tag C9	AGAGGGGGCGAGCTAGTGCAGCGCGTTACTGGGTGTTG
tag C11	GGATGGGGCTTGCGGGGGGGGGTTGGTTTGGCTGTGCGG
tag D2	GGAGATATCGCTGGTGTGAGCGCGAGTGAGATGGGGGGAG
tag D3	CGGGCGGTTCGTCGATTTGTGCGGGGTGGTTGCGCGATGT
tag D4	ACAACATAACGCACCATACCCCAACAACCAACGACACCC
tag D5	AGGTGGGGCGCTACGGCGGTACTTTGGGCGCTTAGGTTT
tag D6	AGCGGGTTCGTGCGAGGGGTGTTTGGATGGTGTGTGAG
tag D7	AGCGTGGTTTAGTTAGTTTCGCTGTTGGGCGTTAGTGAGGT
tag D8	AGACACCCCGCCCTAACACAGCTCCACTCTACCTACCC
tag D9	GGAGTGGTGGAGGTAGCTCGGGTGGGGTCTGGATATATCG
tag D12	CGATAACCCAAACCCGCCCTATAGCTCTCCCCCGCACACC

Figure 4.6 Alignments of the 84 tags using the MegAlign program of DNASTAR

tag F1	CTCGATAAATCAACACATCCACGCGCCACCGCCCCCTAT
tag F2	GTGGCTAGCGCGCGTGTGGTGGGGATCGGGGGGGAGCGAT
tag F3	AAACATAACGAGCCCGCCCCACCGACAGCGAGCCATCGCA
tag F4	GGCGCGGGTGGGATGGTTTGGGCGATTTAGTTTGTGATGT
tag F5	GTGGGGGGAGTTAGGGCGCGATTGGGTTGTGTTGTCGGG
tag F6	AACACGCCATATCCCCACTACATCTCTATCACGCCAGCA
tag F7	AGAGCCCGCACACAGCCATCCAACCAAACCGGTCGATCC
tag F8	CTCACCCAGCGCGCAGCAACCGCCAGCCACCTAGCACT
tag F9	AACACGCCATATCCCCACTACATCTCTATCACGCCAGCA
tag F10	ATGGAGAGCGAGAGGGGGAGGTGTCGTGGAGAGCGTGGT
tag F11	GGGGGGAGGTGGGGATGTGTAGTTGGTGATATGTCGTGGG
tag F12	ACCGCAATACCAAGCGCTACCCATCGCGCTAGACCCACCT
tag F1	ACCGCAACCACACCACTCCCCGAGCGCGCCCCCACAG
tag F2	CGATCCACCTCCCTCCATATACCAACCCGATCTAACCACCT
tag F3	CGATCCACCTCCCTCCATATACCAACCCGATCTAACCACCT
tag F4	ACCGCAACCACACCACTCCCCGAGCGCGCCCCCACAG
tag F5	AGGTGGAGCGGGTTAGTGCAGGGTAGCTAGTTTCTTTT
tag F6	CACCTCAGCGTCCCCCGGACAAAGCGAGACAAACCCAT
tag F7	ATACCACACCCGCGATCACGCACAAACCCGCCCCCTCG
tag F8	AGAACTAACCAGCACCAACCACTATCCCAACACCGCC
tag F9	ACCGCAATACCAAGCGCTACCCATCGCGCTAGACCCACCT
tag F10	TTGGCGCGGGGGTATGTTTTGTGCTGGGGAGGGGTCT
tag F11	AGCACAAATCACCATCCACACAGCTCTCGCGCGCTCGCC
tag F12	CTCGAGCGGTAGATGGCGAGGGGGAGAGCTCGGTAGGTGT
tag G1	CAAAAGCGCACACGCCCCCGCAATATACCCCTCACCCGCC
tag G2	CAAAAGCGCACACGCCCCCGCAATATACCCCTCACCCGCC
tag G3	ATCGCCACACACCAACAAACATAATTCTGCTACATCCCACT
tag G5	ACAACACACGCCCCATACATCCAGCCCGCGCACTCACG
tag G6	ACCGCAATACCAAGCGCTACCCATCGCGCTAGACCCACCT
tag G7	ACCGCAATACCAAGCGCTACCCATCGCGCTAGACCCACCT
tag G8	AACCCAAACACATAAACACCCACTCACGCCATAACGCC
tag G9	CTAGCACATACAGCACGATCCCCATACCCCCAAACTCT
tag G11	ATCAAAAGAAAGACACAAAGCCGCTATCCCTCGCGCCCT
tag G12	GGAGAGGGTGTGTGTGTGGGGTCTCTATCTCGTTCTAGTG
tag H1	CTAGGCGTGGGAGAGAGAGCGGGTTCTAGTTTGTGAGGG
tag H2	ACCGCACACCAACCAAGAACTCTCTCGCTCCCTCTCGCTCC
tag H3	ATGTGGTGGTGGAGCGCGATGGTGGGGTGCTGTGGTTT
tag H4	ACATCTAACCCCAAGCAAAACACACCCGCACGACCTATCT
tag H5	TGAGGTAGGGCGATGTGTTTTGGGTTCTGTGATTTCCTGGT
tag H6	TGAGGTAGGGCGAGGTGTTTTGGGTTCTGTGATTTCCTGGT
tag H7	AAACCGCCACAAATCTCTCGACAGCTCTCGCCCCCTAACGCC
tag H8	CCCGCTAGCCACCGAGACCTCCCTAAAGCGCCACCCCTCT
tag H9	ATCCCTAACCCCCCAACTAACGACCAATCTCGACCAAC
tag H10	AGCCAAACAAATATCTACCTATAACACCAAGCTCCCACTCG
tag H11	CTTGGTTTGGAGCTCGGG

Figure 4.6 (continued)

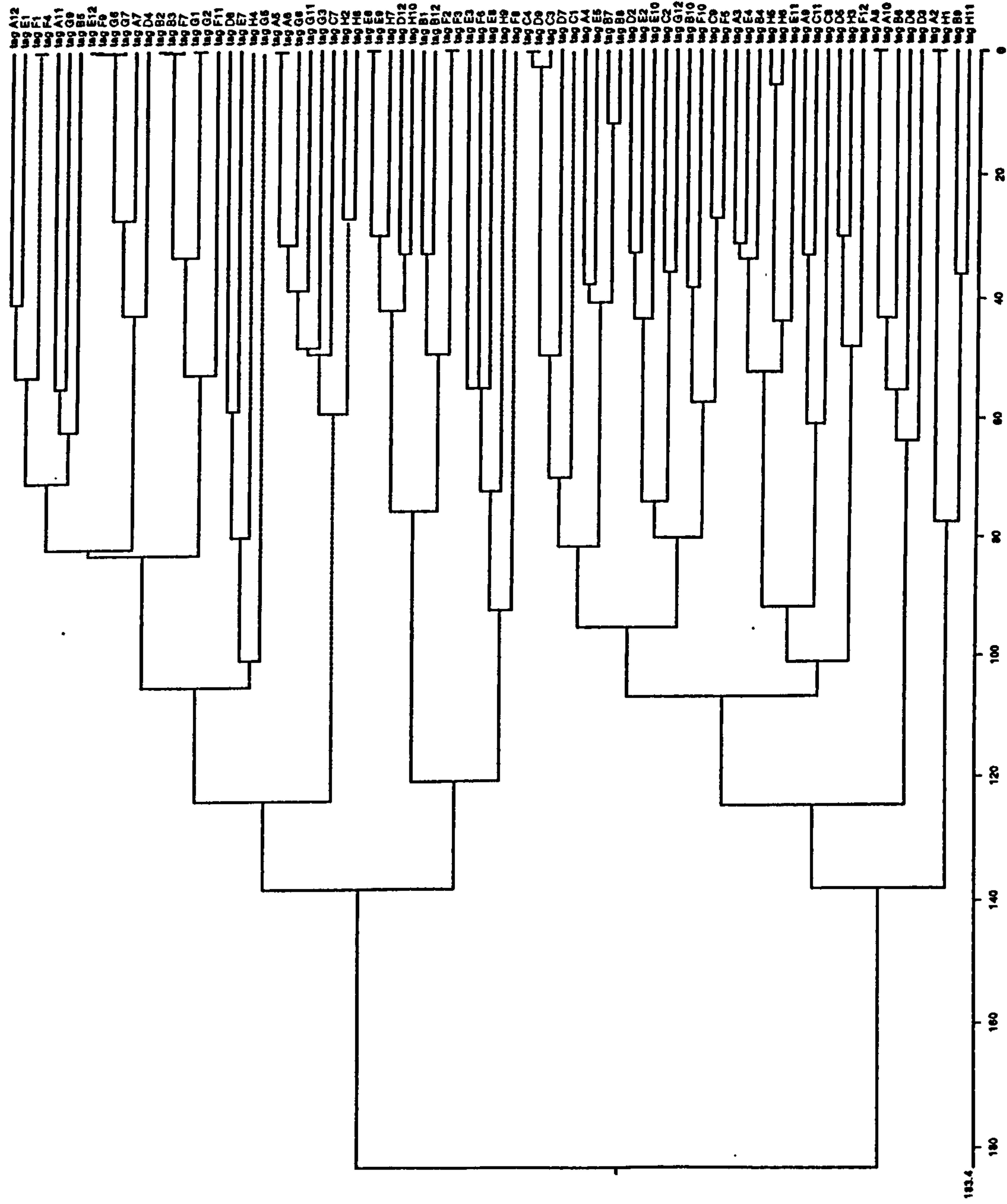


Figure 4.7 Phylogenetic tree of the 84 tags using the MegAlign program of DNASTAR



Sequence Name	< Pos = 1
-  +	
<input checked="" type="checkbox"/> Consensus	AGGG-----GGGCGGGGGAGTGC--GGTGGGGGTGGTTTGTGCGGT-TGAAG
33 Sequences	10 20 30 40 50
A2	CTAG----GC-GTGGGAGAGAGA--GCGGGTTCAGTTTGTGAGG--GAAG
A4	AGTG----AGTGAGTGTGCGCGC--GCT-CGGGAGGGT-GTGGAAAC-CGAAG
A9	CGGT----GGCGCGGGTGGGGT--AGGTGTGGATTTTGTG-TGTGTTGA-AG
A10	CGAG--T---TCGG--GGTGGG--T--GGTTTGTTCCTCGCATATA-AG
B4	CTTGTT-AGAAT--GGAATTCAT--CAACCTCAAGCTTAGGGGGCGC
B6	AGGG-----TGTTAGGTGGATG--GTGCTGGAGGGCGGGGGTTGGATA-AG
B8	GTCG----AGGGAGTGCATTTT--GGTGTTGG--GGCTGGCTAGC-TAA-G
B9	CGAT----CTTGTTGGCTAGGGA--TCGTTGTGAGCGTGGTTGG--GAAG
B10	--AG----GTAGCGCGGGAGTTG--GTTGGCTAGTTATCGTGGGT--GAAG
B11	A--G----GGGTGGGGGGAGAGC--GAT-TGTG-CTGTGGTTCTGGG-TGC-G
C1	AT-----GGCGCTCTAGGGA--GTTAGCGGTGGCGGTCGGGATGTGTAA
C2	AG---CTAGGGTGCGCGTGGG--TTGGGCTGTAGCGGTTG--TGTAAAG
C9	AGAG----GGGGCGAGCTAGTGC--GAG-CGCGTTACT-G-GGTGT-TGAAG
C11	GATG----GGGCTTCGGGGG--G--GGGTTGGTTTGGGCTGT-CG-GG
D2	GGAG----ATATCGCTGGTGTGA--GCGCGAGTGAGATGGGGGA--GAAG
D3	CGGG--C--GGTCGGTCGATTTG--TC--GGGGTGGTTCCGCGCATGTA-AG
D5	AG-G--T--GGG-GCGCTAGGGC--GGTAGTTTGGGCGCTTTAGGT--T-T
D6	AGCG----GGTTCTGTGCGGA--GGGGTGTTGGATGGTGTGTGAGA-AG
D7	AGCG----TGGTTTAGTTAGTTC--GCTGTTGG-GCGTTAGTGAGG-T-AG
D9	GGAG----TGGTGGAGGTAGCTC--GG-GTGGG-GTCTGGATATAT-CGAAG
I2	GTGG--CTAGCGCGCGTGTGGT--GGGATCGGGGGGGAGC--GATTAAG
I4	GGCG--C--GGGTGGGATGGTTT--GGGCGATTAGTTTGTGATGT--A-AG
I5	GTGG--G--GGGAGTTAGGGCGC--GATTGGGTTGTTGTGTCTGGG--A-AG
I10	ATGG----AGAGCGAGAGGGGGA--GGTGTCTGGGAGAGCGTGGTAAG
I11	GGGG----GGAGGTGGGGATGT--TGAGTTGGTGATATGTCGTGG-GA-AG
I5	AGGT----GGAGCGGGTTAGTGC--GAGGTAGCTAGTTCCGCTTT--TTAAG
F10	TTGG----CGCGGGGGGTATGT--TTTGCTGGCTG-GGGAGGGGTCTA-AG
F11	T-----AGCACACATCACCATCCACACAGCTCTCGCGCGCTCGCCAA
F12	CTCG----AGCGGTAGATGGCGA--GG-----GGGAGAGCTCGGTAGGTGT
G12	GGAG----AGGGTGTGT--GTGT--GGGGTCTCTATCTCGTTCTAG-TGAAG
H3	AT-G--T--GG-TGGTGGAGCGC--GATGGTGGGGTGTGTCTGGTTA-AG
H5	TGA-----GGTAGGGCGATGTG--TTTGGGGTTCGTGATTTCTCGGTA-AG
H11	CTTGTTTGGAGCTCGGGAGGCTTGGTTAGAAAGGAATTCACAACTCAAG

Figure 4.8 Alignment of the 33 selected tags using the MegAlign program of DNASTAR

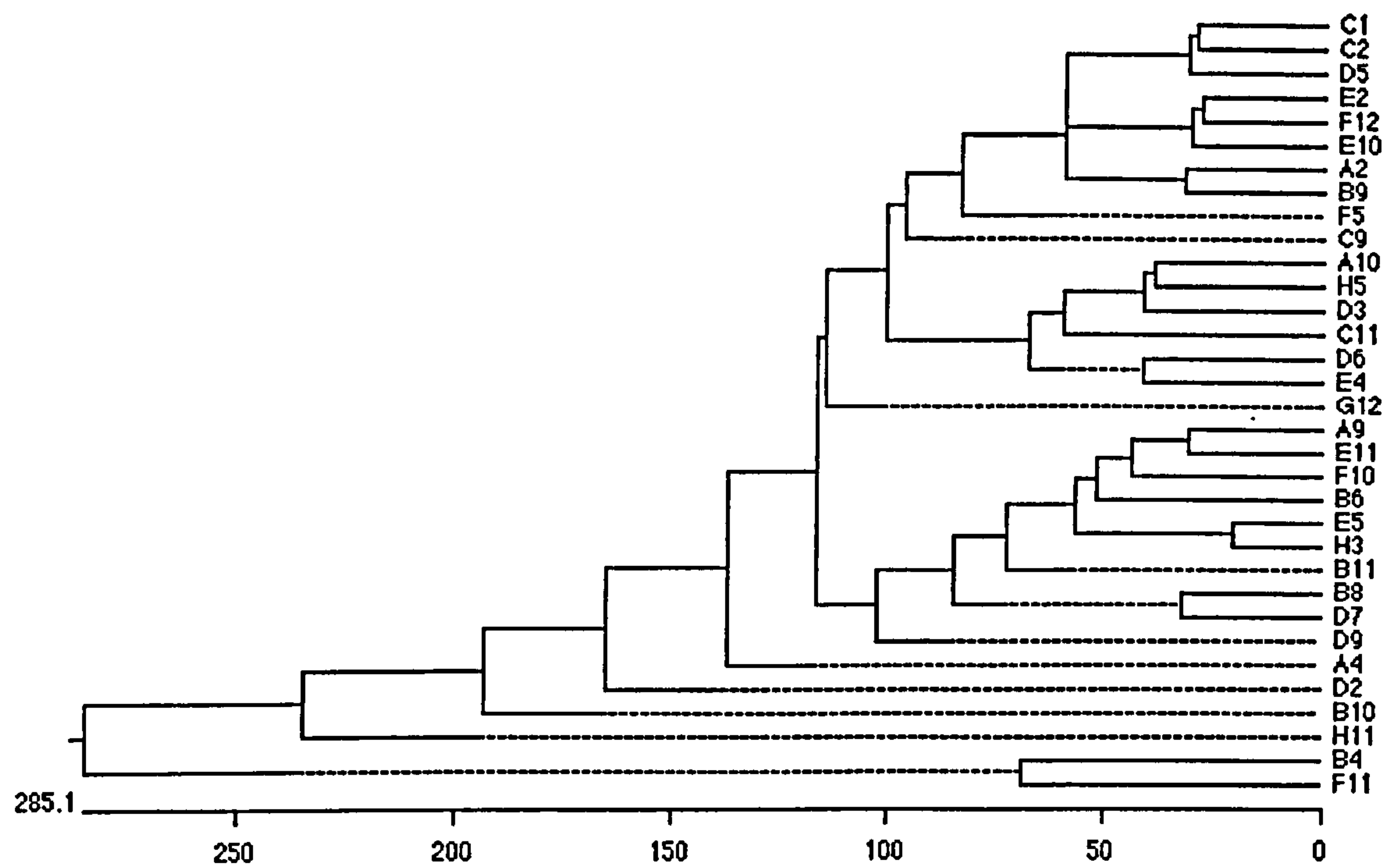


Figure 4.9 Phylogenetic tree of the 33 selected tags using the MegAlign program of DNASTAR

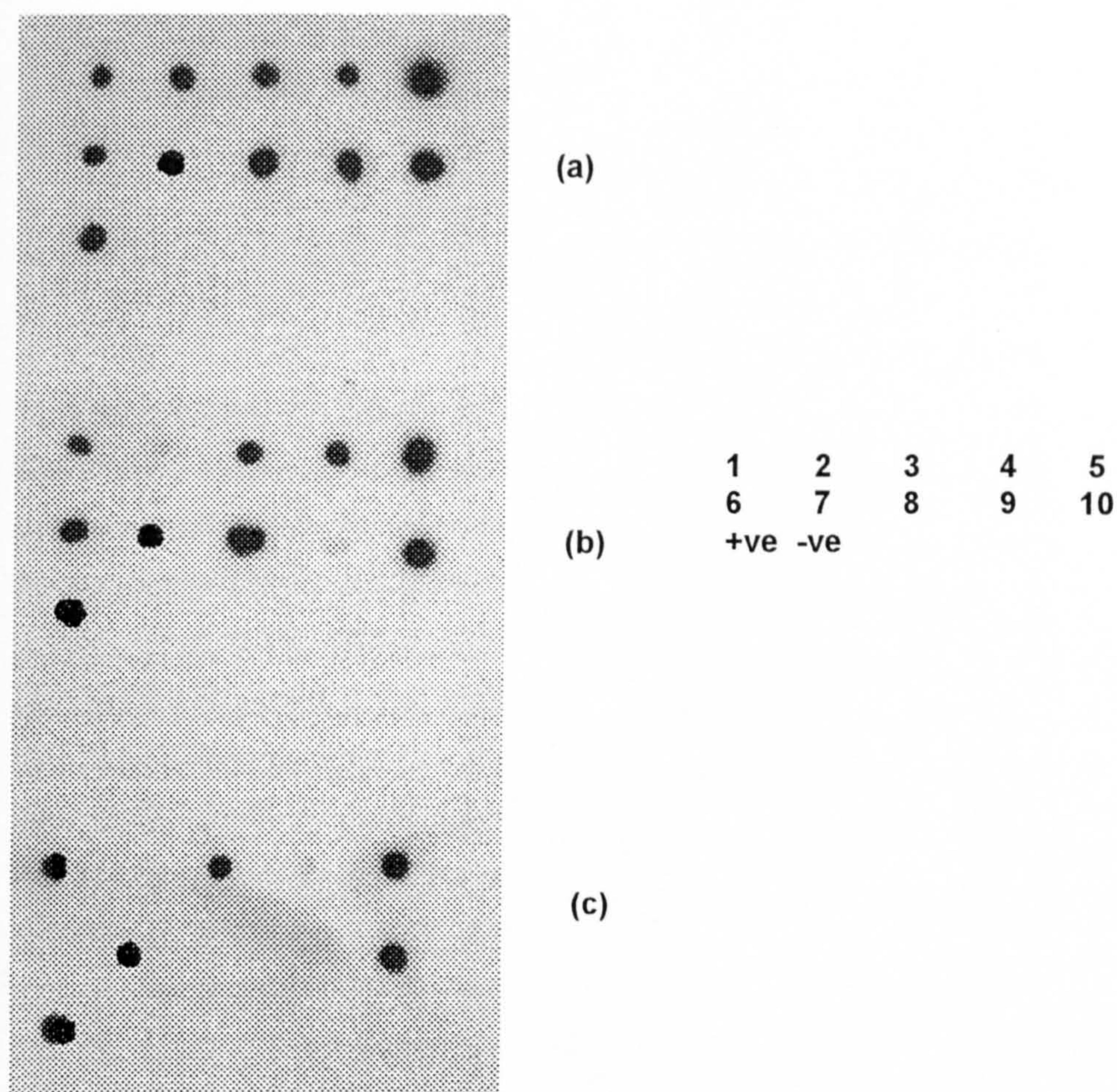


Figure 4.10 Hybridisation of the membranes of 10 random mutants from the 33 selected tags. The membranes were hybridised with the probes from the input pool (a) and the 2 different output pools (b & c). Hybridisation signals from mutants Nos. 2 & 9 in (b), and 2, 4, 6, 8 and 9 (c) are present on the membrane with the input pool but not on the membranes with the output pools.

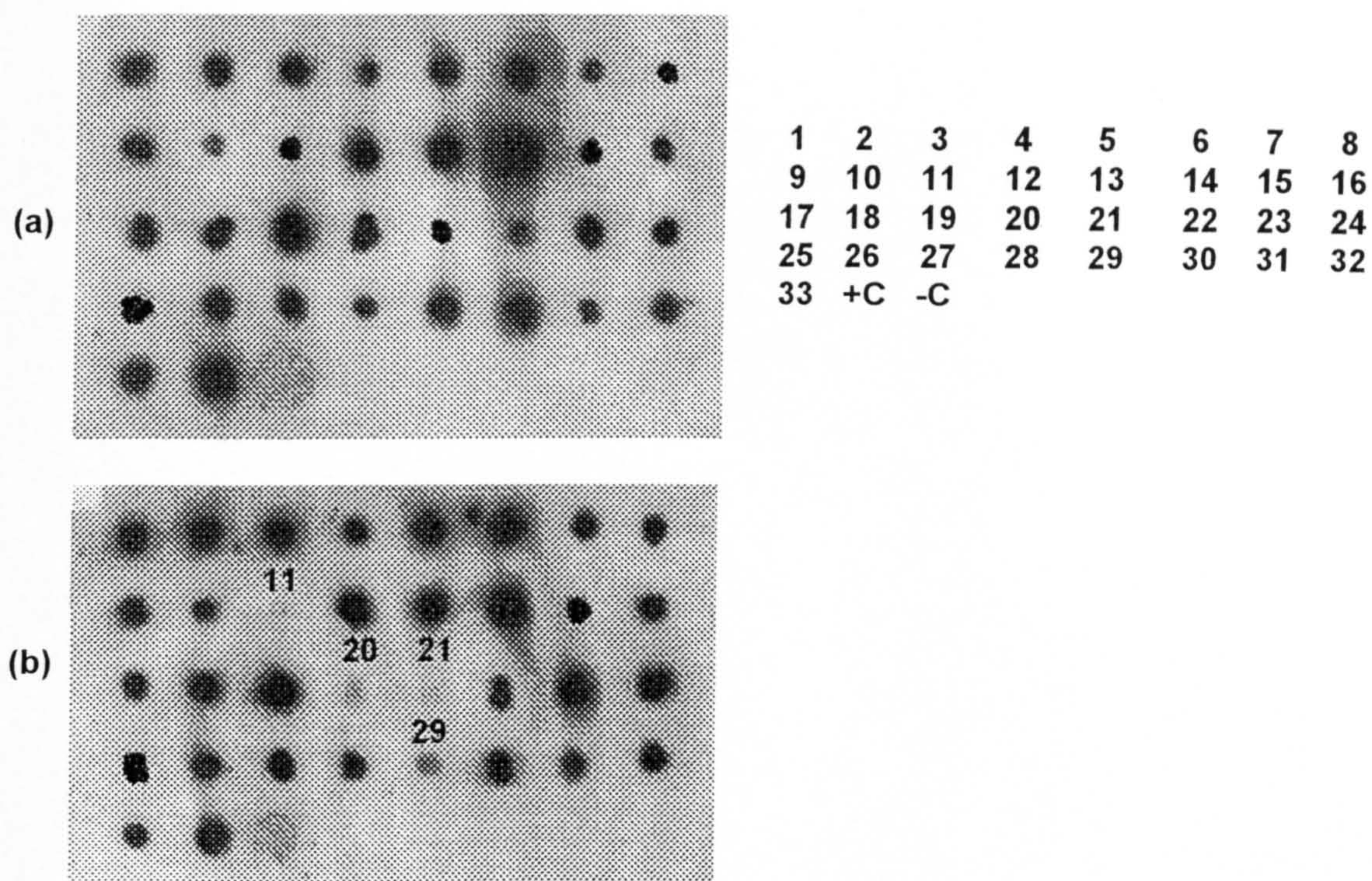


Figure 4.11 Hybridisation of the membranes of the 33 selected tags. The membranes were hybridised with the probes from the input pool (a) and the output pool (b). Hybridisation signals from tags Nos. 11, 20, 21, and 29 are present on the membrane with the input pool but not on the membrane with the output pool.

Chapter 5

Construction of a new library

Chapter 5: Construction of a new library

We were now in a position to use the 33 selected tags to construct a new library. To generate a new library of mutants, 20 μ l of bacterial suspension from each of the selected wells containing 33 non-cross-hybridising tags of the master plate of *S. aureus* strain RN6390 were cultured into the wells of a second microtitre dish containing 180 μ l of BHI and erm (20 μ g ml⁻¹). After overnight incubation at 43°C, bacteria from each well were streaked on BHI agar containing erm and incubated at 43°C overnight to obtain single colonies. Approximately 30-50 different erm^r colonies from each well were transferred onto BHI agar containing cm (20 μ g ml⁻¹) and incubated at 32°C, and onto BHI agar containing erm and incubated at 43°C, to screen for cm^s colonies. The agar plates were incubated overnight. Erm^r cm^s colonies should lack the plasmid and carry a chromosomal insertion of Tn917. Individual erm^r cm^s colonies from each of the 94 wells were transferred into corresponding wells of a third 96-well microtitre dish containing BHI broth and erm. After overnight incubation at 43°C, mutants were transferred to a fourth microtitre dish and grown at 37°C before use as inoculum. Mutant pools were also stored at -80°C in 50% glycerol. A series of 25 pools of 33 mutants were used. Each pool contained 33 mutants with individual selected tags. The total number of mutants made was $33 \times 25 = 825$.

To verify that the constructed mutants lack the plasmid and carry a single chromosomal insertion of Tn917, chromosomal DNA samples from two random individual mutant strains that had been cultured separately were digested with *Hind III* (which cuts the signature tags within the transposon) and then subjected to Southern analysis using a 723bp *PstI-AlwNI* fragment of pBR322 containing part of the β -lactamase gene as a probe to determine the size of the DNA fragment carrying this part of the transposon. The fragment of pBR322 was obtained by PCR using primers P21 and P22. The digested DNA was run in the 1% agarose gel, and a 1 kb ladder (Invitrogen) was used as a marker. The gel was submerged in denaturation solution for 2x 15 min at room temperature. After rinsing with water, the gel was submerged in neutralisation solution at the same conditions. The DNA was blotted to a Hybond N⁺ membrane (Amersham) by capillary transfer, using 20x SSC buffer overnight. The membrane was UV-crosslinked at optimal crosslink (1200

$\times 100 \mu\text{J}/\text{cm}^2$) (Spectro Linker, Spectronics Corporation) without prior washing. The procedure for hybridisation of the membrane was the same as described in section 2.4. As the mutagenesis is random, it is expected that the hybridising fragment in different mutants has a different size. For each of two separately analysed mutants, a single hybridising fragment of different size (6 and 8 kb) was observed (figure 5.1). The different sizes of the DNA fragments therefore confirmed that the mutagenesis was random.

To verify that there was no cross-hybridisation between the mutants of the constructed library, a series of 8 random mutants was chosen and subjected to hybridisation. As shown in figure 5.2, there were good signals with all of the 8 mutants on the input membrane. For output membrane, 2 random mutants (Nos. 6 and 8) were deleted and a probe was made from the remaining 6 mutants. As shown, there were still good signals with the 6 mutants, but not the deleted ones.

5.1 Phenotypic analysis of mutants

To determine that mutagenesis had occurred randomly across the genomic DNA of the mutants of the constructed library, one of the known phenotypic characteristics was traced. Catalase production was chosen for this purpose. Different toxic forms of oxygen are produced as inadvertent by-products during the reduction of O_2 to H_2O in respiration. These reactive oxygen intermediates have many deleterious effects on living organisms, ranging from DNA strand damage to peroxidation of membrane lipids. Bacteria have evolved enzymes that destroy toxic oxygen products. The most common enzyme in this category is catalase, which attacks hydrogen peroxide (H_2O_2) (80, 102). All the mutants of the constructed library were therefore tested individually for catalase production.

5.1.1 Catalase enzyme

All staphylococci produce catalase, which catalyses the conversion of toxic hydrogen peroxide to water and oxygen. Hydrogen peroxide can accumulate during metabolism or after phagocytosis (118). Hydrogen peroxide is produced by bacteria in aerobes and facultatively either by a nonenzymatic reaction of oxygen with reduced

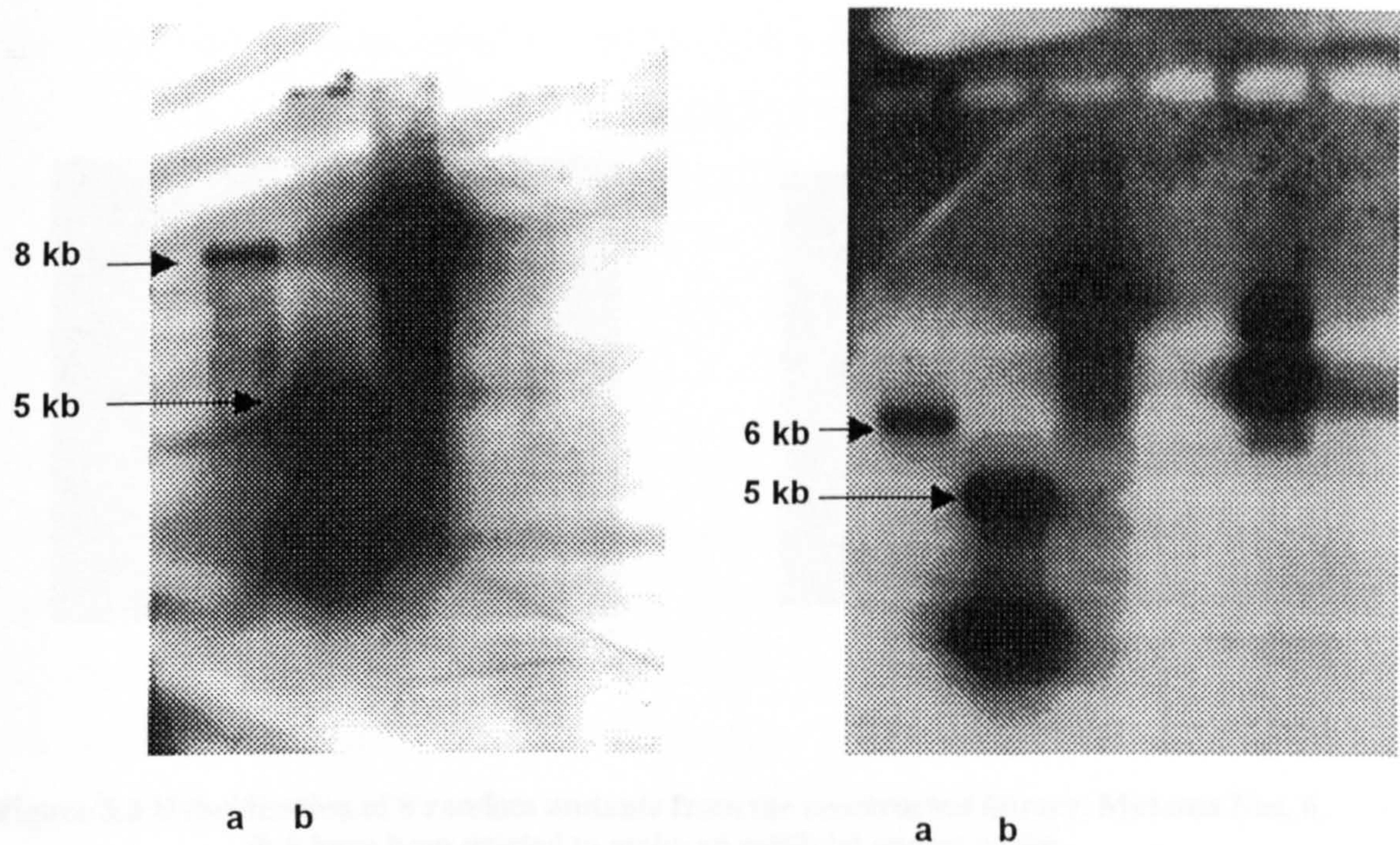


Figure 5.1 Southern hybridisation analysis of Tn917 insertions in the *S. aureus* genome of constructed library of mutants. The lanes (a) are hybridising fragments with different size from individual mutants. Lane (b) is 1 kb marker.

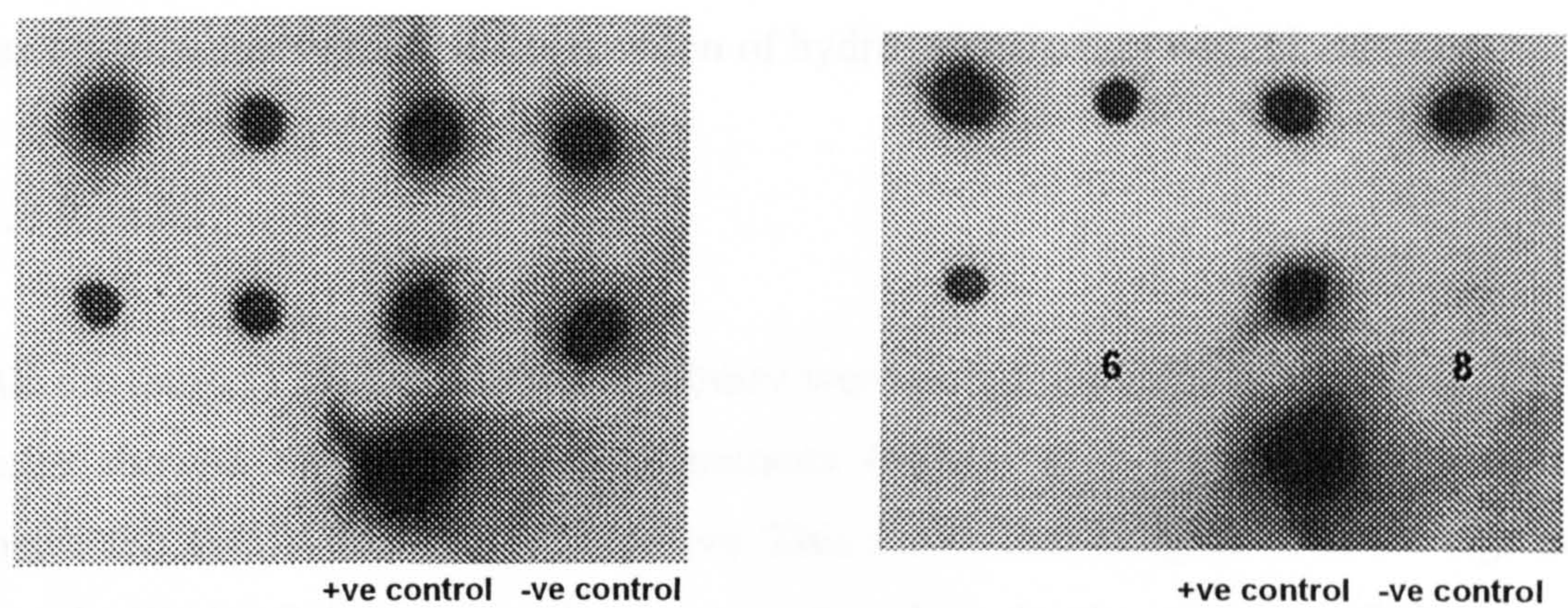


Figure 5.2 Hybridisation of 8 random mutants from the constructed library. Mutants Nos. 6 & 8 have been deleted to make an artificial output probe.

flavoproteins or by enzymatic action on the superoxide radical. Hydrogen peroxide is a highly reactive molecule that can damage cell components.

5.1.2 Catalase test

When a drop of 3% hydrogen peroxide is placed on a culture, bubbles of oxygen gas will appear from the bacteria producing catalase. If no catalase is present, no bubbles will form. The test may be performed on growth transferred to a slide, or to an agar slant. The culture to be tested should be grown on nutrient agar. If the culture is grown on blood agar, and the sample gathered from the blood plate, there may be a false positive reading due to the reaction of hydrogen peroxide with blood.

5.1.3 Results

All the mutants of the constructed library were tested individually for catalase. The results showed that 1 out of 825 mutants (mutant C11 from pool 10) of the constructed library was catalase negative. This shows that integration of the tags into genomic DNA of the mutants has been occurred randomly, as some of the known phenotypic characteristics such as catalase production have been also affected. To test the virulence of the mutant, a mouse was injected intraperitoneally with an overnight culture of the mutant. The overnight culture of the wild type bacteria was also injected into another mouse. The details of the procedure are discussed in section 6.1. The number of recovered bacteria from the mice showed no discernible difference (figure 5.3). It could be concluded that catalase did not affect the growth of bacteria in the mouse. The recovered bacteria from the mouse which had been injected with the mutant showed a negative catalase reaction.

5.1.4 Conclusion

A library of 825 mutants that contained unique tags, with random insertion of transposon Tn917 had been achieved. Thus the STM selection procedure is now working and can be used for mutant selection in the mouse and in biofilms.

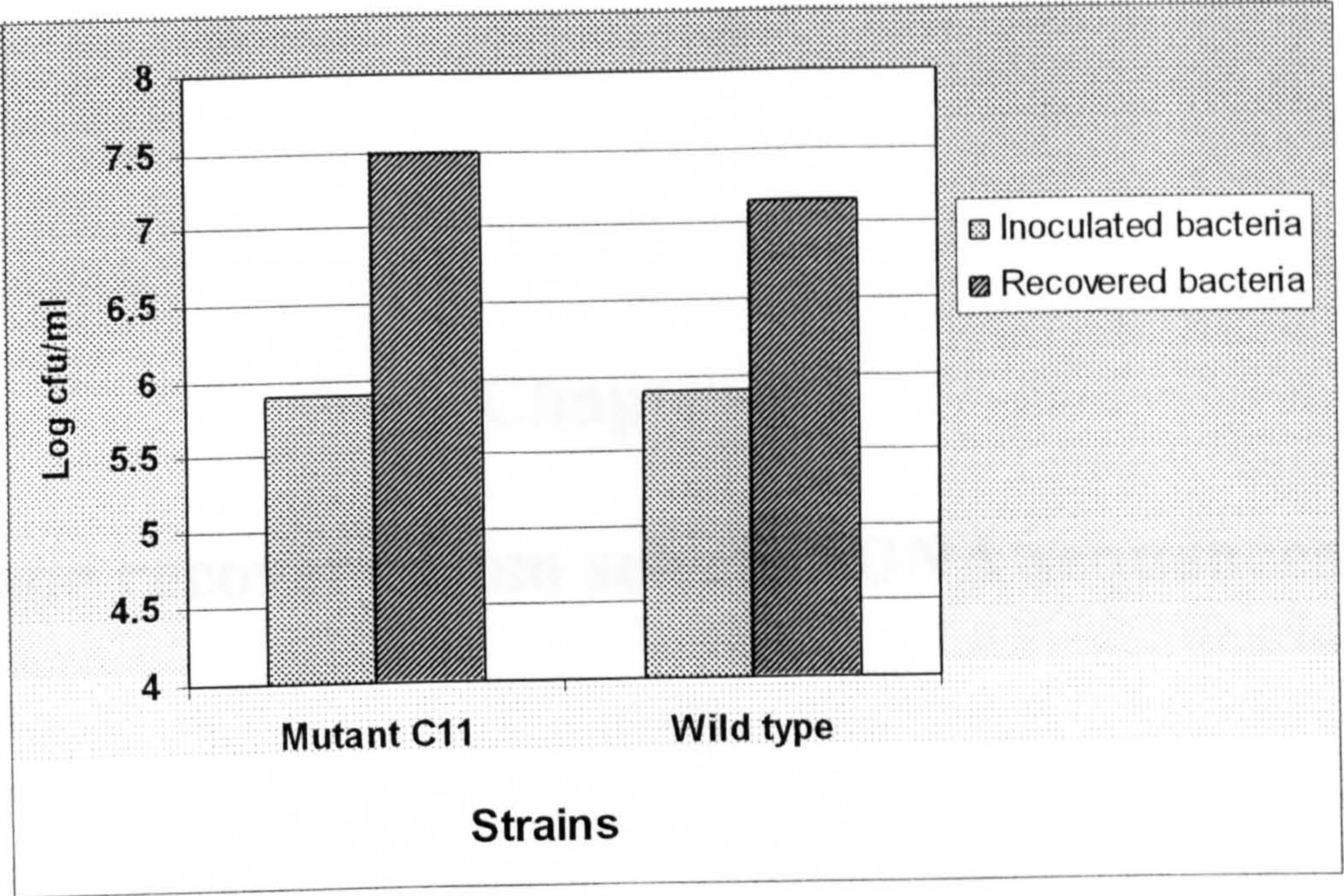


Figure 5.3 Recovery the mutant C11 and wild type strains from mice 24 hr post challenge

Chapter 6

Gene recovery from selected DNA sequences

Chapter 6: Gene recovery from selected DNA sequences

The constructed library was used in two experimental systems: a mouse infection model and in biofilms.

6.1 Mouse infection

A series of 25 pools of 33 mutants was used. Each pool contained 33 mutants with individual selected tags (see chapter 5 for details). The details of procedure for mouse infection and recovery of the bacteria from the mice was discussed in section 2.6.2.

6.1.1 Response to infection

At least one mouse was used for each pool. In some cases, 2-3 mice were used. All mice challenged intraperitoneally with 8×10^5 CFU showed signs of illness (starry coat, hunched appearance, and lethargic) with different severity by 18 hr post-infection. By 24 hr post-infection, all the mice were killed by cervical dislocation. The spleens were removed into 2 ml of sterile PBS in a microfuge tube.

6.2 Biofilm

Discs of catheter material (surface area, 0.5 cm^2) were cut from catheters, and sterilized by UV irradiation for 20-30 minutes each side. The discs were placed in wells of 24-well Nunclon tissue culture plates. A standardized cell suspension (80 μl) was applied to the surface of each disc, and the discs were incubated for 1 h at 37°C . Non-adherent organisms were removed by gentle washing with PBS. To determine the suitable duration for biofilm formation, a series of experiments was done at 24 and 48 h. After 24 h incubation, a layer of bacterial growth was seen on the discs, and the medium was clear. Although it seemed that growth of adherent bacteria was better after 48 h, the growth of nonadherent bacteria was also greater. As a consequence, the medium was cloudy in comparison with the 24 h volumes. Therefore, the 24 h incubation time was chosen for future experiments. At least 5 discs were used for each mutant. The discs were incubated for 24 h at 37°C , submerged in 1 ml of BHI with erythromycin ($20 \mu\text{g ml}^{-1}$). Discs without cells were incubated in the same way as for the control. After biofilm formation, the discs were removed from the medium

and gently washed with PBS to remove non-biofilm cells. Biofilm organisms were washed with 4 ml sterile BHI with erythromycin by vigorous moving of the container. The chromosomal DNA was extracted from the suspension.

6.3 Membranes

After making probes from the extracted genomic DNAs of the output pools from both experiments in mice and biofilm, at least one membrane was used for each pool, and hybridisation was done as described in section 2.4. The result of hybridisation of the 33 mutants of pool 8 from mouse experiment is shown in figure 6.1. Hybridisation signals from mutants A4, A9, B8 and D2 are present on the membrane with the input pool (a) but not on the membrane with the output pool (b).

6.4 Southern blotting and hybridisation

The mutants with attenuated virulence (which had weak or no signals on the output membranes) were subjected to the following procedure for gene recovery. 3-5 µg of *S. aureus* Chromosomal DNA from each mutant with attenuated virulence was completely digested with *HindIII*. Half of the digested DNA was then subjected to Southern hybridisation analysis using a 723bp *PstI-AlwNI* fragment of pBR322 containing part of the β-lactamase gene as a probe to determine the size of the DNA fragment carrying this part of the transposon. The procedure for Southern blotting was described in section 2.5.1. The procedure for hybridisation of the membrane was the same as described in section 2.4.

6.5 Cloning of attenuated genes

The rest of the digested DNA in section 6.4 was resuspended in ligation buffer (Promega) and self-ligated for 12-18 h at 16°C. The ligated products were transformed into *E. coli* XL1-blue, plated onto LB agar containing amp and incubated at 37°C overnight. A single amp^r colony from each transformation was grown in LB broth containing ampicillin. Plasmid DNA was extracted as described in section 2.2. Figure 6.2 shows the Southern hybridisation analysis of 7 mutants from pool 8. As shown in the figure, the size of hybridising fragments in different mutants was different.

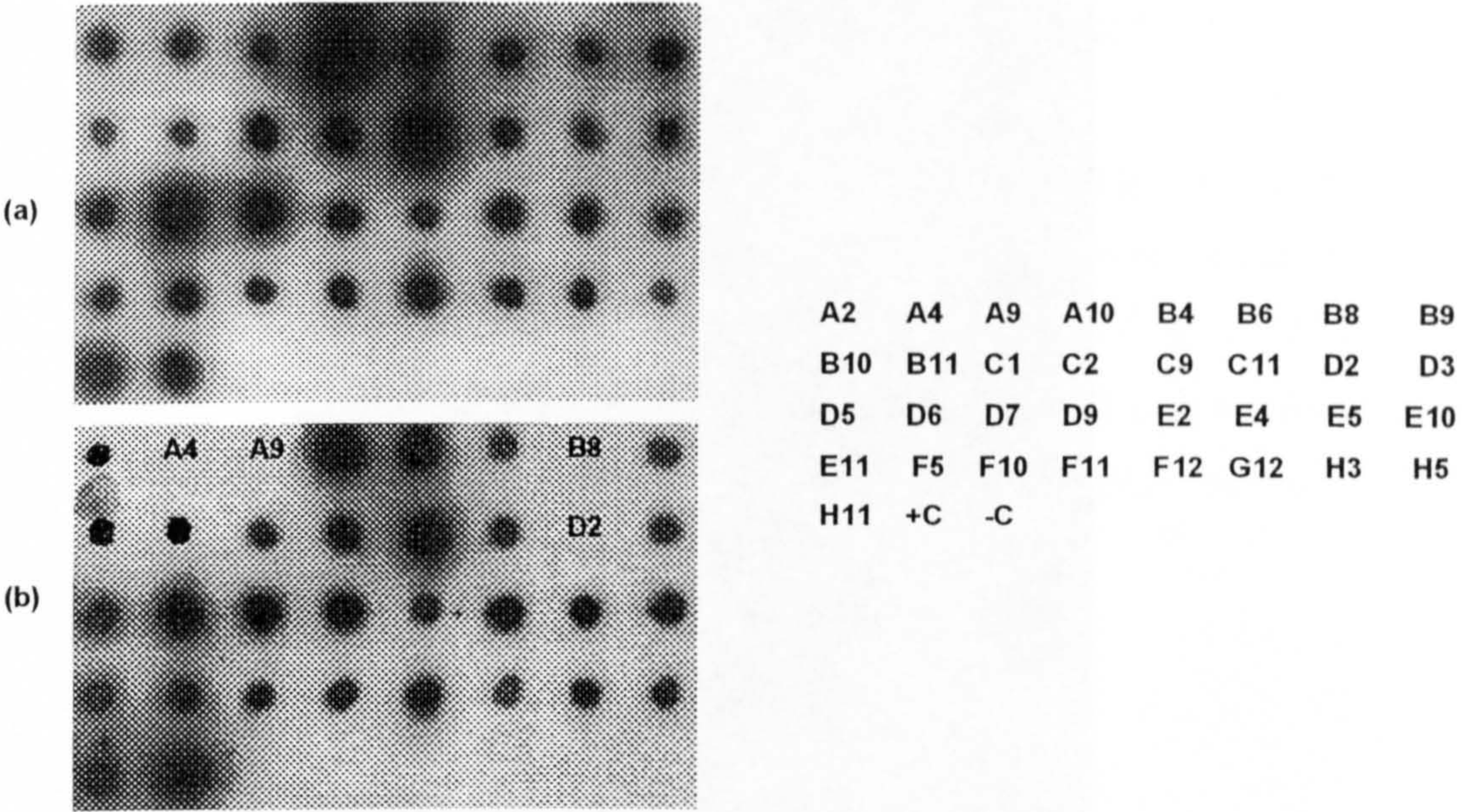


Figure 6.1 Hybridisation of the 33 mutants of pool 8 from mouse experiment. The membranes were hybridised with the probes from the input pool (a) and the output pool (b). Hybridisation signals from mutants A4, A9, B8 and D2 are present on the membrane with the input pool but not on the membrane with the output pool.

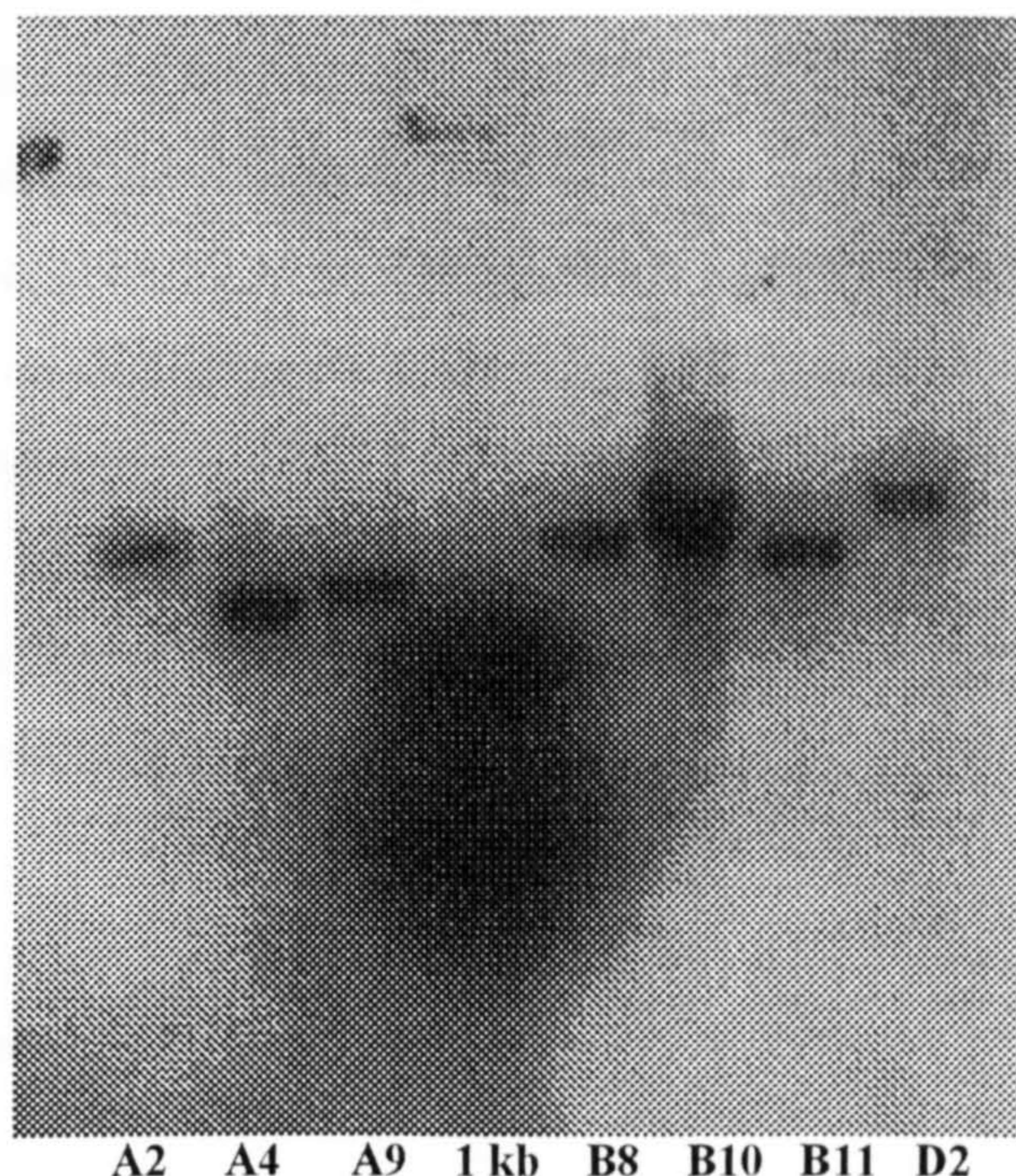


Figure 6.2 Southern hybridisation analysis of 7 mutants from pool 8. The DNAs were digested with *Hind III* and analysed by southern hybridisation using a fragment of the β -lactamase gene of pBR322 as a probe.

6.6 Analysis of the sequences

Chromosomal DNA sequences flanking the transposon in the self-ligated constructed were obtained using primer pseq-1 corresponding to the inverted repeat region 70 bp from the erm-proximal end of Tn917. In total 12 samples with moderately attenuated hybridisation signals were sequenced. The sequencing was done by MBSU DNA Sequencing Service, University of Glasgow, UK. DNA sequences were analysed by searching the TIGR database on complete sequences of different *S. aureus* strains (Mu50, N315, COL, and MW2), BLAST and using DNASTAR software. Mu50 is an MRSA strain with vancomycin resistance isolated in 1997 from the pus of a Japanese male baby with a surgical wound infection that did not respond to vancomycin. N315 is an MRSA strain isolated in 1982 from the pharyngeal smear of a Japanese patient (88). MW2 is a MRSA strain which caused fatal septicaemia and septic arthritis in a 16-month-old girl in North Dakota, USA (9). The results are summarised in tables 6.1 and 6.2.

6.6.1 Mutants from mouse experiments

6.6.1.1 Mutant A4 (pool 8)

The gene from this mutant was identified as encodes a hypothetical protein with a role in the group of hypothetical proteins. The gene had 100%, 100%, and 83.3% identity with SAV2655, SAS090, and MW1322 loci in *S. aureus* strains Mu50, N315, and MW2, respectively. In addition, the gene had 80%-92.9% identity with 5 different loci in each of the 3 strains. Figure 6.3 shows the region surrounding SAV2655 locus in *S. aureus* Mu50, and homology of the recovered gene and its protein. As shown in the figure, the most important genes surrounding the locus SAV2655 are intercellular adhesion protein B, C, and D with unclassified roles.

	Mutant	Biofilm/ Mouse	Locus*	Name of gene	Role
1	A4	Mouse	2655	Hypothetical protein	Hypothetical proteins
2	A9**	Mouse	1394	Hypothetical protein	DNA metabolism
3	A9**	Mouse	1364	Oligopeptide transporter ATP domain (opp-2F)	Protein synthesis
4	B8	Mouse	1722	Acetoin utilization AcuC protein	Energy metabolism
5	B11	Mouse	1362	Hydrolase, putative	Unknown function: general
6	D2	Mouse	1474	Conserved hypothetical protein	Hypothetical proteins
7	F11***	Mouse	1369	<i>PepF</i>	Protein fate
8	G12	Mouse	2190	Conserved hypothetical protein	Hypothetical proteins
9	H3	Mouse	1373	Phosphate ABC transporter	Transport and binding proteins
10	H5	Mouse	1362	Hydrolase, putative	Unknown function: general
11	F10	Biofilm	1382	Tetrahydrodipicolinate succinylase homolog (<i>dapD</i>)	Amino acid biosynthesis
12	F11***	Biofilm	1393	Branched-chain-amino-acid transporter	Hypothetical proteins

* *S. aureus* strain Mu50, ** Pools 8 & 9, respectively; *** Pools 1 & 9, respectively

Table 6.1 The genes recovered from partially attenuated mutants from both mouse and biofilm experiments

	Mutant	Biofilm/Mouse	Locus in <i>S. aureus</i> Mu50	% homology in different <i>S. aureus</i> strains				Homology in <i>S. epidermidis</i> (more than 50%)	Homology in other bacteria (more than 50%, unless mentioned)
				Mu50	N315	COL	MW2		
1	A4	Mouse	2655	100	100		83.3		
2	A9*	Mouse	1394	100	100	100	100	x	<i>B. subtilis</i> <i>O. iheyensis</i>
3	A9*	Mouse	1364	100	100	99.1	98.7	x	
4	B8	Mouse	1722	100	100	99.5	99.2	<i>S. xylosus</i>	<i>B. subtilis</i> (48.8) <i>B. halodurans</i> (48.9)
5	B11	Mouse	1362	100	100	98.0	98.2		
6	D2	Mouse	1474	100	100	100	100	x	<i>B. subtilis</i> (46.4) <i>O. iheyensis</i> (47.3)
7	F11**	Mouse	1369	100	100	99.8	99.8	x	<i>O. iheyensis</i>
8	G12	Mouse	2190	100	100	99.6			
9	H3	Mouse	1373	100	99.7	99.0	99.0	x	<i>B. halodurans</i> <i>O. iheyensis</i>
10	H5	Mouse	1362	100	100	98.0	98.2	x	<i>L. monocytogenes</i> (??)
11	F10	Biofilm	1382	100	100	100	100	x	<i>B. halodurans</i> <i>O. iheyensis</i> <i>E. faecalis</i> Other gram pos. & neg.
12	F11**	Biofilm	1393	100	100	99.7	99.7	x	<i>B. subtilis</i> (37.8) <i>O. iheyensis</i> (34.8)

* Pools 8 & 9, respectively, ** Pools 9 & 1, respectively

Table 6.2 The genes recovered from partially attenuated mutants with their homology in different strains of *S. aureus* and other bacteria

6.6.1.2 Mutant A9 (pool 8)

The gene from this mutant was identified as a hypothetical protein with a role in DNA metabolism. The gene had 100% identity with the four strains of *S. aureus*. The homologous loci for the gene are SAV1394 (strain Mu50), SA1241 (strain N315), SA1445 (strain COL), and MW1229 (strain MW2). The gene also showed some identity with SE0979 in *S. epidermidis*, *yojN* gene in *Bacillus subtilis* 168, and OB0843 loci in *Oceanobacillus iheyensis* HTE831. All of the 3 are nitric-oxide reductase. Nitric-oxide reductase (NOR) is an enzyme involved in the denitrification process, in which nitrate (NO_3^-) or nitrite (NO_2^-) is converted into nitrous oxide (N_2O) via nitric oxide (NO). This enzyme catalyzes the reduction of NO to N_2O ; $2\text{NO} + \text{NADH} + \text{H}^+ \rightarrow \text{N}_2\text{O} + \text{NAD}^+ + \text{H}_2\text{O}$, where NADH is an electron donor to the enzyme. The turnover number of this reaction is over $1,000 \text{ sec}^{-1}$ at 10°C , showing an effective NO diminishing system in cells (148). Although there is no evidence on the specific effect of the enzyme in *S. aureus*, it seems as in other bacteria (24, 143, 162), the above general effect could decrease the side effects of NO on the cells.

Figure 6.4 shows the homology between SAV1394 and *yojN* genes. The homology between the proteins SAV1394 with SE0979, *yojN*, and OB0843 is shown in figures 6.5-6.7. The region view display of the SAV1394, *yojN*, and OB0843 are shown in figure 6.8. On one side, the loci SAV1392 and SAV1393 are a branched chain amino acid carrier protein, and conserved hypothetical protein, respectively. On the other side, there is SAV1395 locus which is a conserved hypothetical protein and has a role in protein fate.

6.6.1.3 Mutant A9 (pool 9)

The gene from this mutant was homologous with SAV1364 (100%), opp-2F (100%), SA1414 (91.7%), and MW1267 (98.7%) loci in *S. aureus* Mu50, N315, COL, and MW2 respectively. The gene had also some homology with SAV1363 locus of *S. aureus* Mu50, and SE1060 locus in *S. epidermidis*. Figure 6.9 shows the protein homology between the recovered gene and SE1060 in *S. epidermidis*. The putative identification of locus SAV1364 is an oligopeptide transporter putative ATPase domain and belongs to the group of transport and binding proteins. This gene has also been identified in another STM screening on *S. aureus* (33). The surrounding

genes of the SAV1364 on the right side are a group of putative oligopeptide transporter genes that belongs to transport and binding proteins and have a role in protein synthesis. On the left side, there are some hypothetical proteins. The family of opps genes encodes the functionally diverse ATP-binding cassette (ABC) transporters proteins. The ATP-binding cassette (ABC) transporter proteins were the largest class which were identified by one of the STM screening on *S. aureus* (33).

There are 2 ABC transporter operons in *S. aureus*, referred to as Opp-1 and Opp-2. The Opp-2 operon is near ORFs encoding the *femA* and *femB* genes that mediate pentaglycine peptidoglycan cross-linking of the *S. aureus* cell wall (152). *S. aureus* is naturally auxotrophic for several essential amino acids, and it is therefore possible that Opp mutants are defective in the import of peptides from which growth-essential amino acids are salvaged. In Gram-negative bacteria, a major function for the Opp system is the salvage of cell wall peptides released from growing peptidoglycan (62). Thus, it may be significant that the Opp-2 operon is in close proximity to the *S. aureus* Fem operon that encodes genes involved in cell wall peptidoglycan biosynthesis. Oligopeptide transporters are also known to play roles in growth regulation, cell competence, adherence to host cells and susceptibility or resistance to host defensins and toxic peptides (90, 129, 131).

6.6.1.4 Mutant B8 (pool 8)

The gene from this mutant was homologous with SAV1722 (100%), *acuC* (100%), SA1785 (99.5%), and MW1678 (99.2%) loci in *S. aureus* Mu50, N315, COL, and MW2 respectively. The gene had also some identity with *acuC* in *S. xylosus*, *B. subtilis* 168 and *B. halodurans* C-125 (figure 6.10). The gene is surrounded by a series of different genes which have some roles in metabolism, regulatory functions, and amino acid biosynthesis.

This gene has been also identified in other bacteria like *B. subtilis* (169), but not in any of the previous STM works on the *S. aureus*. This gene was identified in an output from a mouse infection experiment. *acuC* encodes the enzyme acetoin dehydrogenase and disruption of this gene results in poor growth or sporulation on acetoin in *B. subtilis* (65). The homology between the proteins of the SAV1722 with *acuC* in *S. xylosus*, *B. subtilis*, and *B. halodurans* are shown in figures 6.11-6.13.

6.6.1.5 Mutant B11 (pool 9)

The gene from this mutant was homologous with SAV1362 (100%), SA1209 (100%), SA1412 (98.0%), and MW1265 (98.2%) loci in *S. aureus* Mu50, N315, COL, and MW2 respectively. SAV1362 is a conserved hypothetical protein with unknown function. The surrounding genes of the SAV1362 are the group of oligopeptide transporter putative genes with a role in protein synthesis on one side, and some hypothetical proteins on the other side. SAV1362 and SA1412 encode putative hydrolase enzyme. Homology between the recovered gene and SA1362 is shown in figure 6.14.

6.6.1.6 Mutant D2 (pool 8)

The gene from this mutant was 100% homologous with SAV1474, SA1316, SA1526, and MW1373 loci in *S. aureus* Mu50, N315, COL, and MW2, respectively. The gene had also some identity with SE1174 in *S. epidermidis*, *ypaA* in *B. subtilis* 168 and OB2329 in *O. iheyensis* HTE831 (figures 6.15-6.17). SAV1474 is a conserved hypothetical protein. All of the surrounding genes of SAV1474 locus are hypothetical proteins, except the SAV1473 locus which contains the ferredoxin gene which has a role in energy metabolism. Figure 6.18 shows the region view of SAV1474 in *S. aureus* Mu50, *ypaA* in *B. subtilis* 168, and OB2329 in *O. iheyensis* HTE831, which both have an unclassified role. The homology of the recovered gene with the SAV1474 locus in *S. aureus* Mu50 is shown in figure 6.19.

Work on *ypaA* gene in *B. subtilis* has showed that after inactivation of the gene, the phenotypic pattern obtained showed that this gene controls a system for active flavin transport and, possibly, riboflavin excretion under the conditions of constitutive synthesis (87).

6.6.1.7 Mutant F11 (pool 9)

The gene from this mutant was homologous with SAV1369 (100%), SA1216 (100%), SA1419 (99.8%), and MW1272 (99.8%) loci in *S. aureus* Mu50, N315, COL, and MW2, respectively. The SAV1369 and SA1419 encode oligoendopeptidase F (pepF), and the loci SA1216 and MW1272 are similar to oligoendopeptidase. The

surrounding loci of SAV1369 belong to the group of transport and binding proteins. The gene had also some identity with *S. epidermidis* and *O. iheyensis*. Figure 6.20 shows the homology between the recovered gene (SAV1369 locus in *S. aureus* Mu50) and SE1065 locus in *S. epidermidis*.

PepFs are cytoplasmic endopeptidases that hydrolyze oligopeptides but cannot degrade proteins (140). They are usually named oligopeptidases and constitute a subgroup of the endopeptidase family. Several enzymes of the oligopeptidase family have been found in mammals. A thimet (thiol-dependent metallo) oligopeptidase was purified from various species and tissues. It hydrolyzes peptides which are 6-18 amino acids long. Oligopeptidases are generally characterized by substrate size specificity. It was shown that PepF1 did not cleave peptides containing fewer than 7 or more than 17 amino acids. The substrate size specificity of oligopeptidases is probably related to their role in the cells (170). It seems likely that, in mammals, oligopeptidases participate in the later steps of the degradation of proteins and in the hydrolysis of peptides in the cytoplasm. In addition, they could have a regulatory function on bioactive peptides like bradykinin, enkephalin precursors, luliberin, neurotensin, and angiotensin. The role of oligopeptidases was also investigated in bacteria. OpdA and the dipeptidyl carboxypeptidase of *S. typhimurium* are involved in protein turn-over as revealed by the study of a double negative mutant for both enzymes. In such a mutant, the release of trichloroacetic acid-soluble material due to protein degradation is only 70% of that obtained in the wild strain. These peptidases probably hydrolyze the peptide intermediates formed in the protein breakdown pathway. In *E. coli*, OpdA could play a role in association with the membrane-bound protease IV. The two enzymes are able to degrade *in vitro* prolipoprotein signal peptides. *In vivo*, it is probable that OpdA degrades the products of the initial cleavage of the signal peptide by protease IV that may diffuse into the cytoplasm (123).

Lactococcus lactis has a complex proteolytic system which is essential for its growth in milk. Several oligopeptides have been also purified in lactococci (158, 170). According to the substrate specificities investigated, only two different enzymes are apparently present in *L. lactis*. PepF1 and PepF2 are plasmid- and chromosomally encoded gene products, respectively, of *L. lactis* strain NCD0763. They belong to the M3 family of peptidases that includes the mammalian oligopeptidase 24.15 (140). The gene corresponding to the lactococcal oligopeptidase PepF1 is located on the lactose-proteinase plasmid of *L. lactis* subsp. *cremoris* NCD0763 (120). The gene in *L. lactis*

was found on a 55 kb plasmid, which also carries the genes coding for lactose utilization and for the cell envelope-associated proteinase which are essential for growth in milk. The gene sequence exhibits similarity around the catalytic site with the thimet oligopeptidase and with the bacterial oligopeptidases isolated from *E. coli* and *S. typhimurium* (124). *pepF1* and *pepF2* exhibit 80% identity and encode two proteins which are 84% identical (120).

The *yjbG* gene in *B. subtilis* encodes a protein highly homologous to the PepF1 and PepF2 oligopeptidases of *L. lactis* (84, 117, 120). The oligopeptidase *pepB* in group B streptococci has also showed high similarity to PepF in *L. lactis* (97). Although the homology of the recovered gene and *pepF* and their proteins in other bacteria is strong, there is no evidence on specific function of the gene in *S. aureus*. In other words, it seems the enzyme acts in hydrolysis of oligopeptides like other bacteria.

6.6.1.8 Mutant G12 (pool 9)

The gene from this mutant was homologous with SAV2190 (100%), SA2005 (100%), and SA2196 (99.6%) loci in *S. aureus* Mu50, N315, and COL respectively. Figure 6.21 shows the region view of SAV2190 locus. SAV2190 encodes a conserved hypothetical protein. The surrounding loci of the SAV2190 belong to different groups such as protein fate, cell envelope, metabolism, and amino acid biosynthesis. The homology of the recovered gene from mutant G12 with SAV2190 is shown in figure 6.22.

6.6.1.9 Mutant H3 (pool 9)

The gene from this mutant was homologous with SAV1373 (100%), SA1220 (99.7%), SA1423 (99.0%), and MW1276 (99.0%) in *S. aureus* Mu50, N315, COL, and MW2 respectively. These loci have high homology to SAV1372, SA1219, SA1422, and MW1275 loci in the same strains of *S. aureus* respectively. The putative identification for these loci is phosphate ABC transporter, *pstC* and *pstA* which belong to the group of transport and binding proteins. The gene had also some identity with BH2993 in *B. halodurans* C-125 and OB3135 in *O. iheyensis* HTE831. The region view of the 3 loci is shown in figure 6.23. The main group of surrounding genes is transport and binding proteins. The putative identification for this homology

is also phosphate ABC transporter (permease). The gene had the same identity with different percentage of homology with some other gram positive such as *S. epidermidis* and gram negative bacteria. Figures 6.24-6.26 show the protein homology of recovered gene (SAV1373 in *S. aureus* Mu50) and SE1069 in *S. epidermidis*, BH2993 in *B. halodurans*, and OB3135 in *O. iheyensis* respectively.

ATP binding cassette (ABC) transporters of pathogenic staphylococci are often responsible for antibiotic resistance and for the uptake of essential solutes and are therefore being considered as targets for novel antistaphylococcal drugs. Other ABC transporters are involved in the biosynthesis of a special class of peptide antibiotics, the lantibiotics, and in self-protection of the producer against them (128).

Bacteria can acquire resistance to antibiotics, among other possible mechanisms, by removing the antibiotics from their place of action, often by using drug exporters. In staphylococci, many drug exporters are known, some of which are ABC transporters. MsrA and VgaB are two ABC transporters in staphylococci which confer resistance to erythromycin and streptogramin respectively (128). Many of the proteins involved in cell wall metabolism have been found via their ability to confer resistance to methicillin. Adjacent to the gene for penicillin-binding protein 4 (*pbpD*), an ABC transporter is encoded, which is thought to influence the resistance phenotype by regulatory means and not by serving as an efflux pump (46).

Several ABC transporters have been found in an approach which genetic loci with an impact on growth and survival of *S. aureus* during infection were identified (33). Furthermore, an ABC transporter has been identified as the immunodominant protein during *S. aureus* infections (23). Both findings demonstrate that ABC transporters will play an important role as target molecules in the search for novel antistaphylococcal therapies. Oligopeptide transporters are also known to play key roles in growth regulation, cell competence, adherence to host cells and susceptibility or resistance to host defensins and toxic peptides (33).

6.6.1.10 Mutant H5 (pool 9)

The gene from this mutant was homologous with SAV1362 (100%), SA1209 (100%), SA1412 (98.0%), and MW1265 (98.2%) loci in *S. aureus* Mu50, N315, COL, and MW2 respectively. The surrounding genes of the SAV1362 are the group of oligopeptide transporter putative genes with a role in protein synthesis on one side,

and some hypothetical proteins on the other side. SAV1362 and SA1412 locus is a putative hydrolase. The gene had strong identity with MW1264 as well. The gene had also identity with *flaR* gene in *L. monocytogenes*, and some identity with *S. epidermidis*. FlaR is a histone-like bacterial protein which acts at specific sites to influence DNA topology and, therefore, transcription. *flaR* has different roles in DNA metabolism such as DNA replication, recombination, and repair. *flaR* is the first gene of this class that has been described in gram positive pathogenic bacteria (142). Figure 6.27 shows the homology between recovered gene and its protein (SAV1362 locus in *S. aureus* Mu50) with LMO2669 locus (*flaR*) in *L. monocytogenes*.

6.6.2 Mutants from biofilm experiments

6.6.2.1 Mutant F10 (pool 1)

The gene from this mutant had 100% homology with SAV1382, *dapD*, SA1432, and MW1285 loci in *S. aureus* Mu50, N315, COL, and MW2 respectively. The gene had also some identity with OB1402 in *O. iheyensis* HTE831, EF1133 in *Enterococcus faecalis* V583, BH2669 in *B. halodurans* C-125, and several other gram positive and gram negative bacteria. The SAV1382 locus belongs to the group of amino acid biosynthesis which are also the next 3 genes on the left side, and one on the right side. The other surrounding genes belong to metabolism and cell envelope groups. Figure 6.28 shows the region view of SAV1382 in *S. aureus* Mu50, OB1402 in *O. iheyensis* HTE831, EF1133 in *E. faecalis* V583, and BH2669 in *B. halodurans* C-125. The homology of the recovered gene with SAV1382 locus in *S. aureus* Mu50 is shown in figure 6.29. Protein homology of *dapD* (locus SAV1382 in *S. aureus* Mu50) with SE1077 locus in *S. epidermidis* is shown in figure 6.30.

The recovered gene from this mutant was identified as tetrahydrodipicolinate succinylase homolog. This gene was from one of the biofilm experiments. The *dap* operon contains 6 genes involved in the biosynthesis of lysine. Tetrahydrodipicolinate N-succinyltransferase (*dapD*) is essential in amino acid biosynthesis and catalyzes the succinyl-CoA-dependent acylation of L-2-amino-6-oxopimelate to 2-N-succinyl-6-oxopimelate as part of the succinylase branch of the meso-diaminopimelate/lysine biosynthetic pathway of bacteria, blue-green algae, and plants. This pathway provides meso-diaminopimelate as a building block for cell wall peptidoglycan in most

bacteria, and is regarded as a target pathway for antibacterial agents (15). It has been shown that the enzyme is a sulfhydryl enzyme and involved in diaminopimelate and lysine biosynthesis. It has a pH optimum of 8.2. The enzyme has been identified in another STM screening on *S. aureus* (33), and in other gram positive and gram negative bacteria like *E. coli* (105, 149).

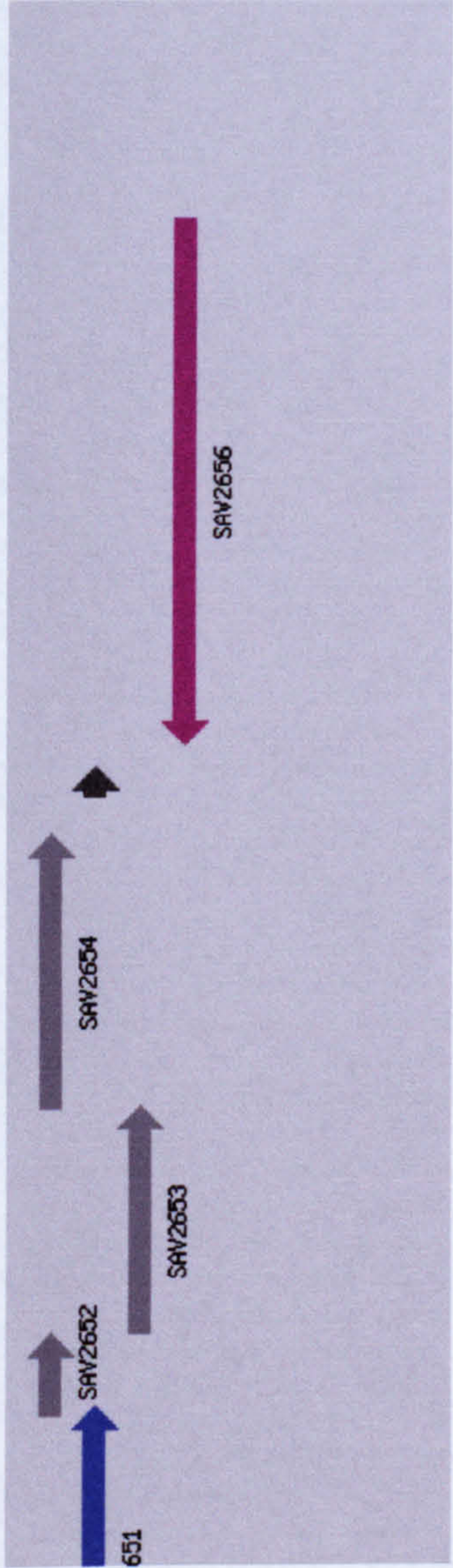
A model system of growth in serum has been shown to be useful for the identification of genes which may contribute to the establishment (surface protein genes) and the persistence (biosynthetic genes) of *S. aureus* in the bloodstream. The serum screen is a simple and complementary approach to both STM and IVET and may allow environmental parameters important in the host to be elucidated. Several genes including *dapA* have been identified in *S. aureus* by using the serum screen (168).

This work is the first that has used the STM method in biofilm systems. This is also the first time that the *dapD* gene has been recovered from a gene screening in biofilm, although it needs more work to confirm the exact role of the gene in biofilm formation. The enzyme has been identified in another STM screening on *S. aureus* (33). This work also showed that the STM method works well, as confirms other findings.

6.6.2.2 Mutant F11 (pool 1)

The gene from this mutant was homologous with SAV1393 (100%), SA1240 (100%), SA1444 (99.7%), and MW1298 (99.7%) loci in *S. aureus* Mu50, N315, COL, and MW2 respectively. The gene had also some identity with branched-chain-amino-acid transporter of *S. aureus*. The homology between the recovered gene and the branched-chain-amino-acid transporter is shown in figure 6.31. The gene had also some identity with *yojO* in *B. subtilis* 168 and OB0842 in *O. iheyensis* HTE831. Figure 6.32 shows the region view of SAV1393 in *S. aureus* Mu50, *yojO* in *B. subtilis* 168, and OB0842 in *O. iheyensis* HTE831.

The recovered gene had some identity with branched-chain-amino-acid transporter of *S. aureus*. The branched-chain-amino-acid transport gene (*brnQ*) encodes a highly hydrophobic polypeptide. The polypeptide has high homology to the Na⁺-dependent branched-chain-amino-acid carriers in several gram negative bacteria, and branched-chain-amino-acid carriers in several gram positive bacteria (165).



mutant A4:	113	ATGATNTTTTGGCAAACCCNTATTTCGTGTCGCCACCCCAACTCGCATTCGCTGTAGAAT	172
SAV2655 :	1	ATGATAATTTTCGC AAAAT-TTATTTTCGTGTCGCCACCCCAACTTGCATTGTTGTAGAAT	59
Mutant A4:	173	TTCATTTTCGAAATTCTCTATGTTGGGGCCCC	203
SAV2655 :	60	TTCTTTTCGAAATTCTTTTGTGGGGCCCC	90
Mutant A4:	1	MIFSQNLFRRPPTTCIVCRISFRNSFCWGPAKILLE	36
		MIFSQNLFRRPPTTCIVCRISFRNSFCWGPAKILLE	
SAV2655 :	1	MIFSQNLFRRPPTTCIVCRISFRNSFCWGPAKILLE	36

Figure 6.3 Region view display of locus SAV2655 (black arrow) in *S. aureus* Mu50 (top). Loci SAV2652, SAV2653, and SAV2654 are intercellular adhesion protein D, B, and C respectively. Homology between the recovered gene from mutant A4 with the same strain (middle). Homology of the protein from the recovered gene in BLAST which indicates a hypothetical protein in the same strain (bottom). As the length of the protein is short, that is actually a peptide.
Note: There are two links for the SAV2655 locus in the GenBank database. One of the genes is larger than the other and is located in the complementary strand.

SAV1394:	6	YKNSDSTVFENDAKALFDLNKNILLKGPTGSGGKTKLAETLSEVVDTPMHQVNCSVDLDTES	65
		YKNSD TVF DAKALF LNKNI LLKGPTGSGGKTKLAETLS V+ PMHQVNCSVDLDTES	
SE0979 :	6	YKNSDQTVFEDAKALFQLNKNILLKGPTGSGGKTKLAETLSNVMKLPMHQVNCSVDLDTES	65
SAV1394:	66	LLGEKTIKTNAEGQQEIVFDGPVIKAMKEGHILYIDEINMAKPETLPVLNGVLDYRRQI	125
		LLGEKTI TN EG QEIVF+DGPVIKAMKEGHILYIDEINMAKPETLP+LNGVLDYRRQ+	
SE0979 :	66	LLGEKTIHTNEEGHQEIVFIDGPVIKAMKEGHILYIDEINMAKPETLPILNGVLDYRRQL	125
SAV1394:	126	TNPYTGEVIKAVPGFENVIAAINEGYVGTLPMNEALKNRFVVIHVDYIDGDILKNVIKEQS	185
		TNPYTGEVIKA PGFNVIAAINEGYVGTLPMNEALKNRF+VI VDYIDGDILK VIKEQS	
SE0979 :	126	TNPYTGEVIKAAPGFENVIAAINEGYVGTLPMNEALKNRFVIEVDYIDGDILKTVIKEQS	185
SAV1394:	186	LLQDDKQIEQIIKFNEDLRTMSKQGQISEEAAASIRALLDCLITVMPVERAIKRTIIDK	245
		LQD++ I+ I+KFNEDLRTM+KQGQISEEAAASIRAL+DL DL TVMP+ERA++RTIIDK	
SE0979 :	186	KLQDEQLIQHIVKFNEDLRTMTKQGQISEEAAASIRALIDLSDLATVMPIERAVQRTIIDK	245
SAV1394:	246	LEDEREQQAIYNAVELNF	263
		LEDEREQQAI NA+ELNF	
SE0979 :	246	LEDEREQQAILNAIELNF	263

Figure 6.5 Homology between the proteins of mutant A9 (SAV1394 locus in *S. aureus* Mu50) and SE0979 locus in *S. epidermidis*

SAV1394:	6	YKNSDSTVENDAKALEFDLNKNILLKGPTGSGKTKLAETLSEVVDTPMHQVNCSVDLDTES	65
YojN	:	Y+ D + DA + KN+LLKGPTGSGKTKLAETLS PMH VNCSVDLD E+	
YojN	: 47	YEADEAILFDAILALAMGKNVLLKGPTGSGKTKLAETLSSYFHKPMHSVNCSVDLDAEA	106
SAV1394:	66	LLGEKTIKTNAEGQQEIVFVDGPVIKAMKEGHILYIDEINMAKPETLPVLNGVLDYRRQI	125
YojN	:	L+G+KTI+ N GQ I FV GPV KAMKEGH LYIDEINMAKPETLP+LNGVLDYR+ +	
YojN	: 107	LVGYKTIE-NQSGQATIEFVSGPVTKAMKEGHFLYIDEINMAKPETLPILNGVLDYRKMM	165
SAV1394:	126	TNPYTGEVIKAVPGFENVIAAINEGYVGTLPMNEALKNRFVVIHVDYIDGDILKNVIKEQS	185
YojN	:	TNP+TGEVI+A GF VIAAINEGYVGT+P+NEALKNRFV+I V YI G++LK V+ QS	
YojN	: 166	TNPFTGEVIRAKSGFGVIAAINEGYVGTVPLEALKNRFVIIDVPYIKGELLKQVLMSSQS	225
SAV1394:	186	LLQDDKQIEQIIKFNEDLRTMSKQGQISEEAAASIRALLDCLITVMPVERAIKRTIIDK	245
YojN	:	L+D+K I++ I + DL + GQ+SEEAASIRAL+D CDL +P RAI+R I++K	
YojN	: 226	ALKDEKLIDREFTLSSDLIVQANNQGVSEEAASIRALIDTCDLAAYIPPRRAIERGIVEK	285
SAV1394:	246	LEDEREQQAIYNAVELNF	263
YojN	:	L+D+RE+ A+ N E F	
YojN	: 286	LDDREKA AVRNI AETLF	303

Figure 6.6 Homology between the proteins of mutant A9 (SAV1394 locus in *S. aureus* Mu50) and *yojN* locus in *B. subtilis*

SAV1394:	6	YKNSDSTVFENDAKALFDLKNKILLKGPTGSGTKLAETLSEVVDTPMHQVNCSDLDTES	65
		Y + + DA + KNILLKGPTG+GGTK AETLS + PM VNCSVDLD ES	
OB0843 :	33	YMPNEELIIDAVTALSMGKNILLKGPTGAGTKKFAETLSLFFQQPMFSVNCSDLDLDAES	92
SAV1394:	66	LLGEKTIKTNAEGQQEIVFDGPVIKAMKEGHILYIDEINMAKPETLPVINGVLDYRRQI	125
		L+GEKT+ + +Q I FV GPV +MK G LYIDEINMAKPETLP++NGVLDYRR I	
OB0843 :	93	LMGEKTLSEYEND-KQTIDFVPGPVTNSMKHGTFLYIDEINMAKPETLPVINGVLDYRRTI	151
SAV1394:	126	TNPYTGEVIKAVPGFNVIAAINEGYVGTLPMEALKNRFVVIHVDYIDGDILKNVIKEQS	185
		TNP+T E+I A F VIAAINEGY+GT+P+NEALKNRF+VI V YI GD L +I +	
OB0843 :	152	TNPFTEIITAEEOFGVIAAINEGYIGTVPLNEALKNRFIVIDVPYIQGDQLNKLITNST	211
SAV1394:	186	LLQDDKQIEQIIKFNEDLRTMSKQGQISEEAASIRALLDCLITVMPVERAIKRITIIDK	245
		L+D+ I + ++DL T +QG++SE+AASIRALLD CDL T +P +RAI R+IIDK	
OB0843 :	212	KLEDETLLNLYVTLSKDLITAVEQQKLSEDAASIRALLDACDLTTFFIPKRAILRSIIDK	271
SAV1394:	246	LEDEREQQAIYNAVELNF	263
		L+++RE+ + N + F	
OB0843 :	272	LDEDREKAFVQNLADTLF	289

Figure 6.7 Homology between the proteins of mutant A9 (SAV1394 locus in *S. aureus* Mu50) and OB0843 locus in *O. iheyensis*

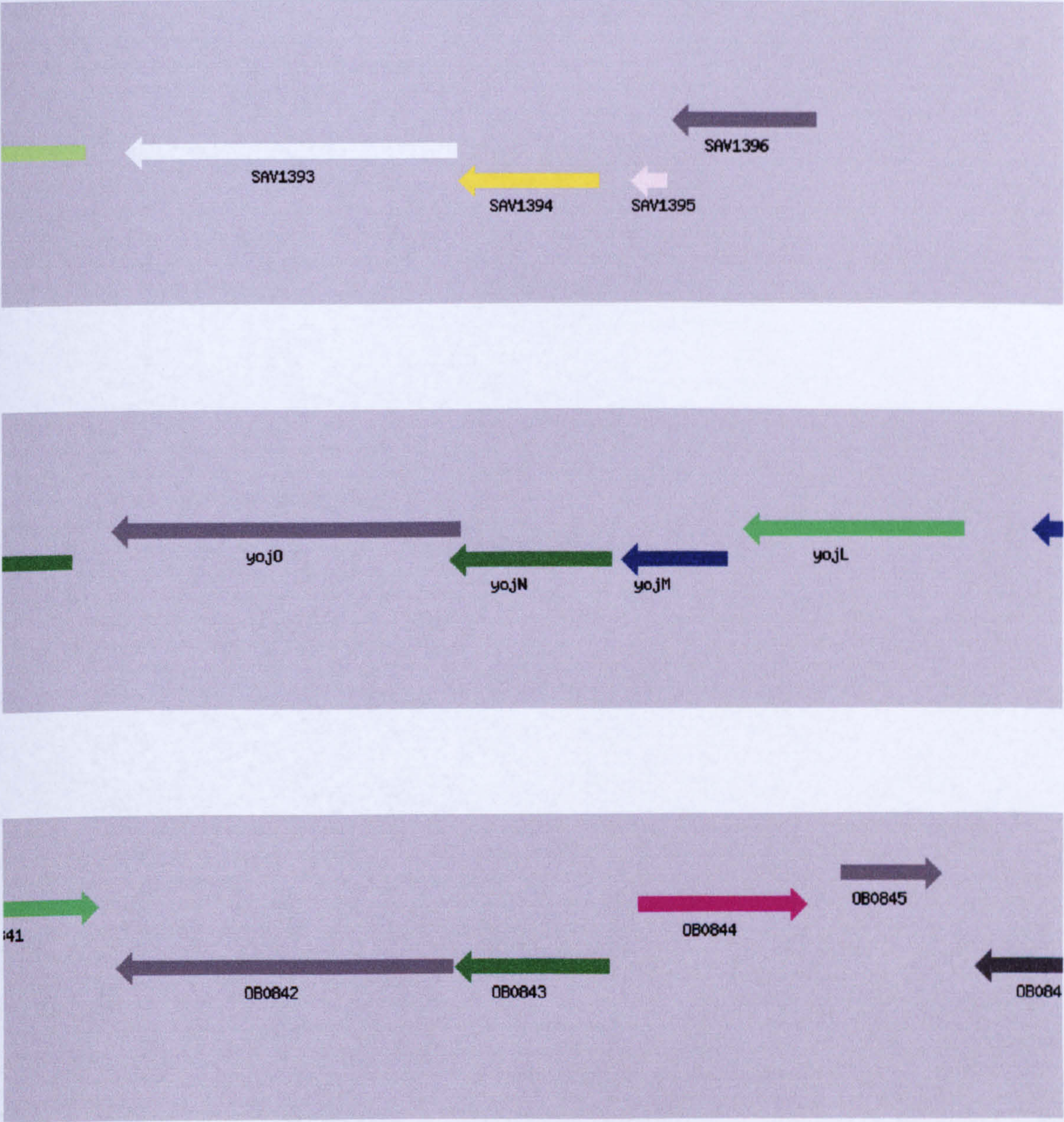


Figure 6.8 Region view display of the SAV1394 locus in *S. aureus* Mu50 (top), *yojN* in *B. subtilis* 168 (middle), and OB0843 in *O. iheyensis* HTE831 (bottom)

SAV1364:	1	MIELKHVTFGYNKKQMVLQDINITIPDGENVGILGESGCGKSTLASLVLGFKPVKGEIY	60
		MI+ HV + Y++KQ VL+DINI+I GE +G+LGESG GKST+ SL+LG KP KG+I	
SE1060 :	1	MIQFDHVDYSYHRKQPVCLKDINISIQRGEKIGVLGESGAGKSTIGSLILGQLKPTKGKIS	60
SAV1364:	61	LSDNAVLTIFQHPLTSENPDWTIETSLKEALYYRGLTDNTAQDQLLLQHLSTFELNAQL	120
		+ VL IFQH SF+ +TIE SL+E L +YR L ++ ++L +L F L+ L	
SE1060 :	61	IDSGKVLPFIQHATESFDRQFTTIEQSLREPLLFYRQLIPQNIKN-IILNYLIEFNLSTD	119
SAV1364:	121	LTKLPSEVSGGQLQRFNVMRSLLAQPRVLCDEITSNLDVIAEQNVINILKAQTITNLNH	180
		+TK P EVSGGQLQR N++RSLLAQP +L+CDEITSNLDV+AEQNVINIL +	
SE1060 :	120	ITKFPQEVSGGQLQRLNIIRSLLAQPDILVCDEITSNLDVMAEQNVINILLNEKNIQNK	179
SAV1364:	181	FIVISHDLSVLQRLVNRIIVLKDGMIVDDFAIEELFNVDHRHPYTKELVQTFSY	233
		IVISHDLSVLQRL NRIIV+KDG IVDDF ++LF+ RHPYTK L+QT+ Y	
SE1060 :	180	LIVISHDLSVLQRLTNRIIVIKDGQIVDDFKSKDLFSHQRHHPYTKLLIQTYEY	232

Figure 6.9 Homology of proteins of recovered gene from M9 (locus SAV1364 in *S. aureus* Mu50) with opp-2F (SE1060 locus) in *S. epidermidis*

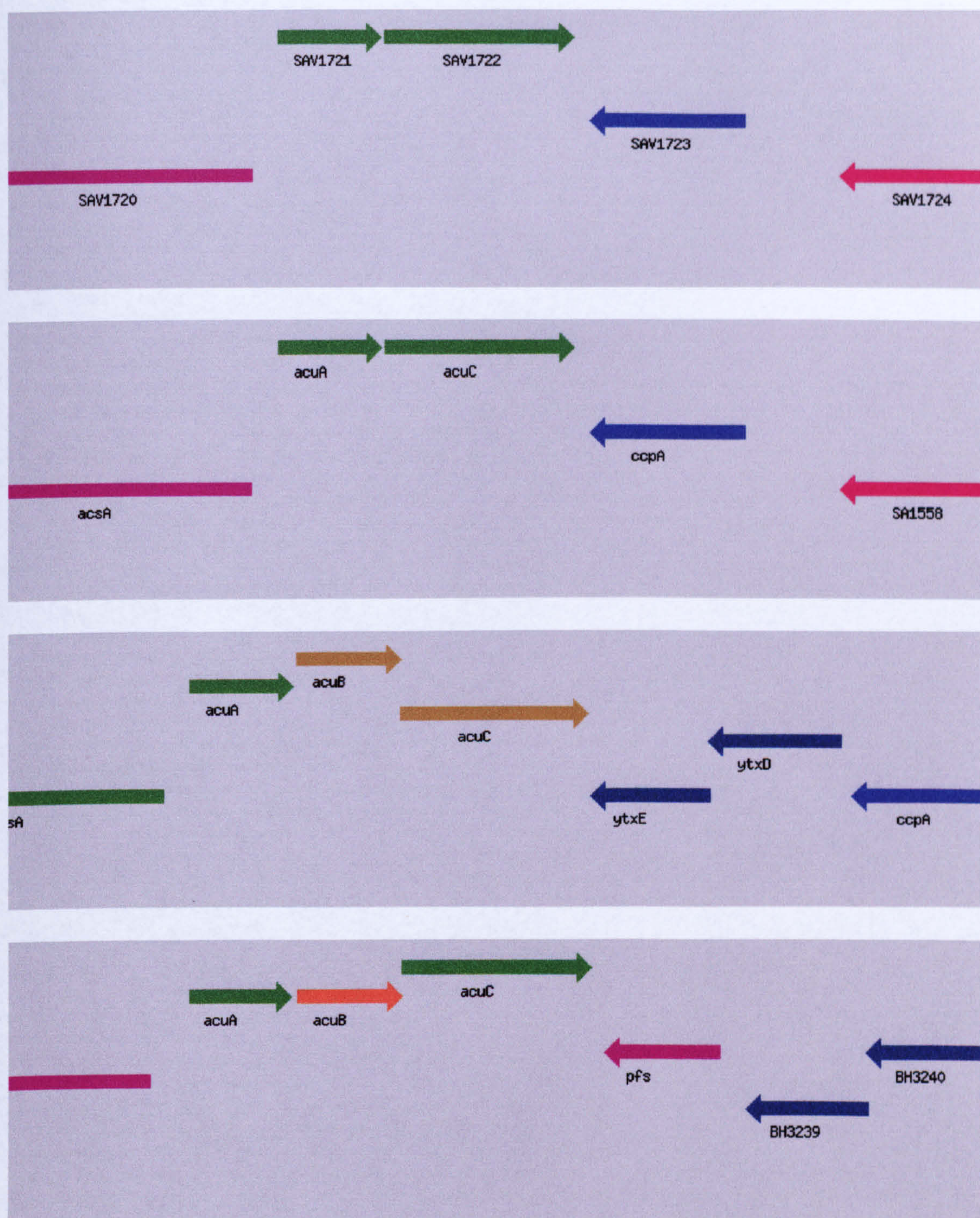


Figure 6.10 Region view display of the SAV1722 locus in *S. aureus* (Mu50), *acuC* (N315), *acuC* in *B. subtilis*, and *acuC* in *B. halodurans* (top to bottom respectively)

SAV1722: 7 KTAYVYSDKLLQYRFHDQHFPNQMRXXXXXXXXXXXXXXXXXSPEQIVQPRIATGDELMLIHK 66
K ++VYS+ L Y+EH HPFN R +IV PR+AT EL LIH
acuC : 3 KASFVYSENQLSYQFHSHTHPFNHQRLRLTYDLLKSMDALDDSEIVAPRVATEAEELRLIHD 62

SAV1722: 67 YDYVEAIKHASHGIISEDEAKKYGLNDEENGQFKMHRHSATIVGGALTADLIMSGKVX 126
+++A+K A +G +S+ A YGL E+ FK+MH +A +VGG LT D +M G
acuC : 63 QAFIDAVKAAAGNGTILSDGVALNYGLGTEDTPIFKNMHEAAALLVGGTLTAVDQVMEGHAE 122

SAV1722: 127 XXXXXXXXXXXXAQPGRASGFCIYNDIAITAQYIAKEYNQRVLIIDTDAHGGDGTQWSFYA 186
G+ASGFC+YND +I +Y+ +++ RVL IDTDAHGGDG QW+FY
acuC : 123 HACHLGGGLHHGFRGKASGFCVYNDSSIAIEYMRRKFGARVLYIDTDAHGGDGVQWAFYE 182

SAV1722: 187 DNHVTTYSIHETGKFLFPGSGHYTERGEDIGYGHTVNVVPLEPYTEDASELECFKLTVEPV 246
++ V T SIHETG++LFPG+G++ E+G+ GYG+++N+PL+ +TED SFL ++ V V
acuC : 183 EDDVCTLSIHETGRYLFPGTGNFNEKGQGEYGYGINIPLDAFTEDDSFELFAYEQAVTNV 242

SAV1722: 247 VKSEKPDIIILSVNGVDIHYRDP LTHLNC TLHSLYEIPYFVKYLADSYTNGKVMFGGGGY 306
VK FKP D+IL+ NG D H+ DPLTHL ++ + IP LA Y G+ I GGGY
acuC : 243 VKFEKPDVILTONGADAHFHDPLTHLCASMRTRYIPQLAHLAHLAHEYCEGRWIAVGGGGY 302

SAV1722: 307 NIWRVVPRAWSHVFLSLIDQPIQS-GYLPLEWINKWKHYSSELLPKRWEDRLNDYTYVPR 365
+IWRVVPRAW+ ++L + DQ ++ G LP W++KW Y+ LP WED + +PR
acuC : 303 DIWRVVPRAWAMIWLEMTDQLQKANGPLPERWLSKWTTYAQHPLPSHWEDENEELPTIPR 362

SAV1722: 366 TKEISEKNKKL 376
EI+EKNKK+
acuC : 363 RDEITEKNKKI 373

Figure 6.12 Homology between the proteins of mutant B8 (SAV1722 locus in *S. aureus* Mu50) and *acuC* locus in *B. halodurans*

SAV1722: 8	TAYVYSDKLLQYRFHQHPFNQMRXXXXXXXXXXXXXXXXXSPQIVQPPRIATGDELMLIHKY 67
+	++YS Y FH +HPFNQ R IV PR+A+ +EL L+H
acuC : 4	SVFIYSPSYQTYMEFHQHPFNQQRVLLTYDLLKKTINAFDDGDIVTPRLASEEELSLVHTD 63
SAV1722: 68	DYVEAIKHASHGIISEDEAKKYGLNDEENGQFKHMRHSATIVGGALTADLIMSGKVXX 127
	DY++A+K A G + +E + YGL E+ F MH ++ +VGG LT AD +MSG+
acuC : 64	DYIQAVKLAGAGKLPAAEGESYGLGTEDTPVFAGMHEAASLLVGGTLTAADWMSGQALH 123
SAV1722: 128	XXXXXXXXXXAQPGRASGFCIYNDIAITAOYIAKEYNQRVLIIDTDAHGGDGTQWSEYAD 187
	GRASGFCIYND A+ QYI K+Y+ RVL IDTDAHGGDG Q++FY +
acuC : 124	AANLGGGLHHGFRGRASGFCIYNDSAVAIQYIQKKYSARVLYIDTDAHGGDGVQFTFYDN 183
SAV1722: 188	NHVTYYSIHETGKELFPGGHYTERGEDIGYGHTVNVPLEPYTEDASFLECFKLTVEPVV 247
	V T SIHETG++LEPG+G E+G GYG++ N+PL+ +TED SFLE ++ V
acuC : 184	PDVCTLSIHETGRYLFPGTGQIQEKSGKGYGSFNIPLDAFTEDDSFLEAYRTAASEVA 243
SAV1722: 248	KSEKPDIIILSVNGVDIHYRDPPLTHLNCTLHSLYEIPYFVKYLADSYTNGKVMFGGGGYN 307
	F+PD+I+S NG D HY DPLTHL+ T++ EIP LA Y GK I GGGY+
acuC : 244	AYFEPDVIISQNGADAHYYDPLTHLSATINIYEIIPRLAHTLAHQYCGGKWIAVGGGGYD 303
SAV1722: 308	IWRVVPRAWSHVELSLIDQPIQSGY-LPLEWINKWKHYSSSELLPKRWEDRLNDYTVVPR 366
	IWRVVPRAW+ ++L + + I G+ +P EWI KW+ LP W D + Y +PR
acuC : 304	IWRVVPRAWARIWLEM--KGIDPGHEIPEWIVKWQKQCPVALPSSWSDPADLYPPIPRK 361
SAV1722: 367	KEISEKN 373
	EI+EKN
acuC : 362	PEITEKN 368

Figure 6.13 Homology between the proteins of mutant B8 (SAV1722 locus in *S. aureus* Mu50) and *acuC* locus in *B. subtilis*

Mutant B11:	101	CAACATGCTAGTAGTGGCTATATTATAGGACCCATCANAAGCNCCCACACACGCAATATT	42	
SAV1362	:	637	CAACATGCTAGTAGTGGCTATATTATAGGACC-ATCAGAAGCATACACACGCAATATT	695
Mutant B11:	41	GAAACTTGATAAAAANAACACATCAATAATAATGCACAAG	1	
SAV1362	:	696	GAAACTTGATAAAATCAAACACATCAATAATAATACACAAG	736

Figure 6.14 (continued)

SAV1474:	1	MQQNKRLITISMLSAIAFVLTFIKEPIPELPPYLTDFSDVPSLLATFTFGPVAGIVVAL	60
		MQQNKRLITISMLSA+AFVLTFIKEP+PF+PPYLTDFSDVP+LLATF	P+AGI+VAL
SE1174 :	1	MQQNKRLITISMLSAVAFVLTFIKEPLPEIIPPYLTDFSDVPTLLATFLLSPIAGIIVAL	60
SAV1474:	61	VKNLLNYLESMGDPVGPEANFLAGASFLLTAYAIYKNKRSTKSLITGLIATIVMTIVLS	120
		+KN+LN+LF++GDPVGP ANFLAG SFLL++Y +Y+ +++ +SLI GLI	TIVMTIVLS
SE1174 :	61	IKNILNLEFNIGDPVGPVANFLAGVSELLSSYYVYRKRKNRSLIYGLITGTIVMTIVLS	120
SAV1474:	121	ILNYFVLLPLYGMIFNLADIANNLKVIIIVSGIIPFNXXXXXXXXXXLLYRRLANFLK	179
		ILNYFVLLPLYGMIFNL D+ NN+K++IVSG+IPFN	LL+RRL + +K
SE1174 :	121	ILNYFVLLPLYGMIFNLGDVLNNVKIVIVSGVIPFNLIKGIISIIIFVLLFRRLRHIK	179

Figure 6.15 Homology of proteins from recovered gene of mutant D2 (SAV1474 in *S. aureus* Mu50) and SE1174 in *S. epidermidis*

SAV1474:	5	KRLITISMLSAIAFVLTFIKEPIPELPPYLTLDFSDDVPSLLATFTFGPVAGIVVALVKNL	64
		K+L+ +SMLS+IAFVL + FP P LP YL +DFSDDVP+++A +GP+AGI V +KN+	
ypaA	: 4	KKLVVVSMLSSIAFVLMLLNFFPGLPDYLNKIDFSDDVPAIIAILIYGPLAGIAVEAIKNV	63
SAV1474:	65	LNYLES---MGDPVGPFFANFLAGASELLTAYAIYKNKRSTKSLITGLIITIVMTIVLSI	121
		L Y+ G PVG ANF+AG F+L ++K S K L L++ T MTI++SI	
ypaA	: 64	LQYIIQGSMAQVPVGVQVANFIAGTLEFILPTAFLEKKLNSAKGLAVSLLLGTAAMTIILMSI	123
SAV1474:	122	LNYSFVLLPLYGMIENLADIANN-LKVIIVSGIIPFNXXXXXXXXXXLLYRRRLANFLKR	180
		LNYS++LP Y + ++++ LK +V+GI+PFN L++ +L ++++	
ypaA	: 124	LNYSVLILPAYTWELHSPALSDSALKTAVVAGILPFENMIKGIVITVVFSLIFIKLKPWIEQ	183

Figure 6.16 Homology of proteins from recovered gene of mutant D2 (SAV1474 in *S. aureus* Mu50) and *ypaA* in *B. subtilis*

SAV1474:	1	MQQNKR-----LITISMLSAIAFVLTFIKFPIPLP-PYLTDFSDVPSLLATFTFGPVA	54
		MQ++KR	
OB2329 :	1	LI I++LS I+ VL FI FP+PFLP PYL +DESDVP+L+A+ F P+A	60
		MOKSKRNNLLKLIILALLSTISLVLEFFINFPLPFLTPYLVDFSDVPALIASLIFSPIA	
SAV1474:	55	GIVVALVKNLLNYLES-MGDPVGPPEFANELAGASFLLTAYAIYKNKRSTKSLITGLIIATI	113
		GIVV KN+L S GDP+G +NF+AG ++ IY + K LITGL+ TI	
OB2329 :	61	GIVVGFKNILYLAVSGSDPIGVLSNFIAGVMLVVPVSMIYHRMKGVKGLITGLVFGTI	120
SAV1474:	114	VMTIVLSILNYFVLLPLYGMIENLADIANNLK-VIIVSGIIPFNXXXXXXXXXXLLYR	172
		VM + +SILNY+++LP YG +++N +K V +++G++PFN L+	
OB2329 :	121	VMAVGMSILNYYLILPAYGWFMGW-EMSNQVKWVSVIAGVLPFNMIKGVFIAVLFIPLEM	179
SAV1474:	173	RLANFLKR	180
		+L +L++	
OB2329 :	180	KLHAWLQQ	187

Figure 6.17 Homology of proteins from recovered gene of mutant D2 (SAV1474 in *S. aureus* Mu50) and OB2329 in *O. iheyensis*

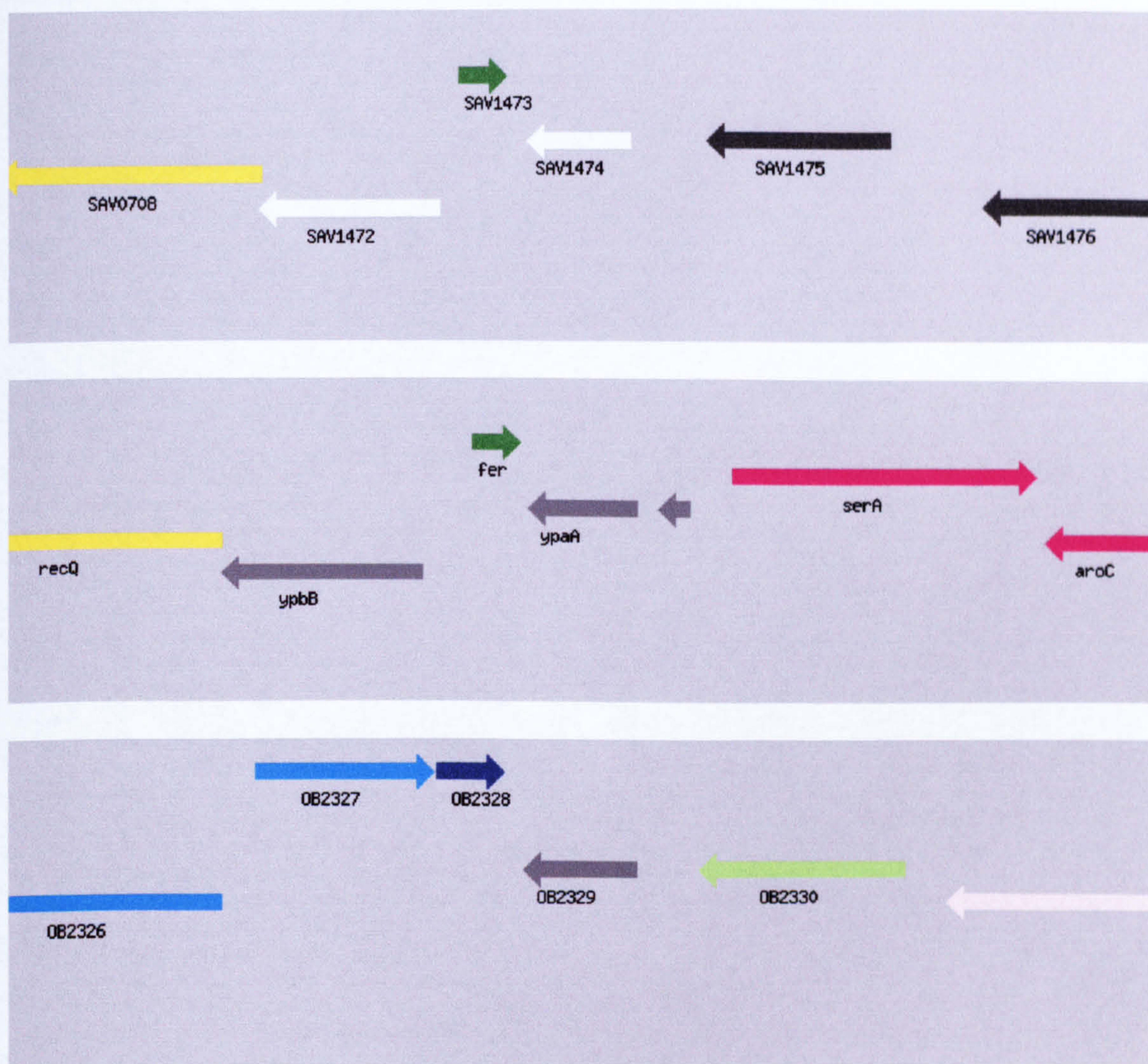


Figure 6.18 Region view display of the SAV1474 locus in *S. aureus* Mu50 (top), *ypaA* in *B. subtilis* 168 (the middle), and OB2329 in *O. ihevensis* HTE831 (bottom).

Mutant D2: 1 taatttgattaaattcttttcaagaaattcgcaagccttctatatagtaaaataataaca 60
|||||
SAV1474 : 210514 taatttgattaaattcttttcaagaaattcgcaagccttctatatagtaaaataataaca 210573

Mutant D2: 61 atagaaataacgataccctttaataantattgaatgggtataatcctgaaacaatgattact 120
|||||
SAV1474 : 210574 atagaaatgacgataccctttaataantattgaatgggtataatcctgatacaatgattact 210633

Mutant D2: 121 ttaagattatttgcgatatcagctaagttaaatatcataccgtacaaaggtaatagaacg 180
|||||
SAV1474 : 210634 ttaagattatttgcgatatcagctaagttaaatatcataccgtacaaaggtaatagaacg 210693

Mutant D2: 181 aaatagttcaaaaataactcaacacgatatgtcataacgattgttgcaatgattaatccagta 240
|||||
SAV1474 : 210694 aaatagttcaaaaataactcaacacgatatgtcataacgattgttgcaatgattaatccagta 210753

Mutant D2: 241 atcaaaagattttgttgaacgtttattttatagatggcgtaagcagttaataagaaaactt 300
|||||
SAV1474 : 210754 atcaaaagattttgttgaacgtttattttatagatggcgtaagcagttaataagaaaactt 210813

Mutant D2: 301 gcgcctgctaaaaagttagcaaatgggtcccaactggatcgccatactaaataagtagttc 360
|||||
SAV1474 : 210814 gcgcctgctaaaaagttagcaaacggcccaactggatcgccatactaaataagtagttc 210873

Figure 6.19 Homology of the recovered gene from mutant D2 and SAV1474 locus in *S. aureus* Mu50

SAV1369: 1 MSQGLPLREDVPVSETWDLVDLFKDDQYYESIDALVQQANQEHHTYATTNLSIEQINTX 60
MS+GLPLRE+VPV ETWDL DLF DQ +Y++++ +VQ + F+HTY LN+IE I
SE1065 : 1 MSEGPLREEVPVKETWDLKDLFTSDQAFYQTLQVQVQMSLDENHTYYQKLNNIETIEKA 60

SAV1369: 61 XXXXXXXXXXXDRLSNYAELRLSVDTSNIEAQVLSAKLSTTYGKIVSQLSFVXXXXXXXXX 120
DRL NY ELRLSVDTSN EAQ ++AKL+TT GK+ LSFV
SE1065 : 61 LDEYERILIEIDRLYNYPELRLSVDTSNEEAQKVNAKLNTTSGKLAGLLSFVDSEILELP 120

SAV1369: 121 XXXXXXXXXXXCPYQHYIKOLIKQKPFQLSASVEQVLATLSPTLNSPYDLYGTTKMLDITF 180
Y H+IKQL +KP+QLSA VE+VLATL+PTL SP++LYGTTK LDI F
SE1065 : 121 DEIISELRSQTKYPHFQIKQLQDRKPYQLSADVEKVLATLTPTLRSPFELYGTTKSLDINF 180

SAV1369: 181 DSEHDGTTYVPVDYATFENDYEDNKDPEFRRKSFKSFSDGIRKYQHTTAATYNNMQVQQEK 240
+SF+++G TYP+DYATFEN+YED+ PEFRRKSF++FSD +R+YQHTTAATYNNMQVQQEK
SE1065 : 181 ESFDYEGVTYPLDYATFENEYEDHPSPEFRRKSFRAFSDALRQYQHTTAATYNNMQVQQEK 240

SAV1369: 241 IEADLRGFESVIDYLLHSQEVTRDMFDRQIDMIMRDLAPVMQYAKLLQRIHGLDNMRFE 300
IEADLRG++SVIDYLL QEVT+DMFDRQID+IM DLAPVMQYAK++QR+H LD MRFE
SE1065 : 241 IEADLRGYDSVIDYLLQDQEVTKDMFDRQIDVIMSDLAPVMQYAKIIRVHNLDKMRFE 300

SAV1369: 301 DLKISVDPDYEPEISIEDSKNYIFGALSVLGDDYTNNMLREAYDQRWIDFAQNKKGKDTGAF 360
DLKIS+DP++EPEISIE+SK YI+GAL VLGDDY ML AYD RWIDFAQNKKGKDTGA+
SE1065 : 301 DLKISIDPNFEEPEISIEESKKYIYGALKVVLGDDYVKMLESAYDYRWIDFAQNKKGKDTGAY 360

SAV1369: 361 CASPYFTHSYVFISWTGKMAEAFVLAHELGHAGHFTLAQKHQPYLESEASMYFVEAPSTM 420
CASPY THSYVFISWTGKMAE FVLAHELGHAGHF LAQ HQ LESEASMYFVEAPSTM
SE1065 : 361 CASPYITHSYVFISWTGKMAETFVLAHELGHAGHFALAQNHNQNLLESEASMYFVEAPSTM 420

SAV1369: 421 NEMLMANYLENTSDNPRFKRWVIGSILSRYYHNMVTHLLEAAYQREVYHKVDQGESLNA 480
NEMLMANYLFN+S+NPRFKRWVIGSILSRYYHNMVTHLLEAAYQREVY +VD GESL A
SE1065 : 421 NEMLMANYLENSSNNPRFKRWVIGSILSRYYHNMVTHLLEAAYQREVYSRVDNGESLTA 480

Figure 6.20 Homology of proteins from the recovered gene from mutant F11 (SAV1369 locus in *S. aureus* Mu50) and SE1065 locus in *S. epidermidis*

SAV1369: 481 PTLNEIMLN VYKQFFGDAVDMTEGAELTWMRQPHYYMGLYSYTSAGLTIGTVVSQIKN 540
P LNEIMLN YK FFGD V+MT+G ELTWMRQPHYYMGLYSYTSAGLTIGTVVSQ IK
SE1065 : 481 PLLNEIMLNTYKAFFGDTVEMTDGVELTWMRQPHYYMGLYSYTSAGLTIGTVVSQCIKK 540

SAV1369: 541 EGQPAVD AWLETLKKGGSVSPVELANIAGVDITTEQPLKSTIQYISDLVDEVEKLTDEIE 600
EGQPAVD WL+TL+ GGS SP+ELA IAGVDITT+ PLK TI YIS+LVDE+E LT +IE
SE1065 : 541 EGQPAVD RWLKT LQAGGSQSPIELAQIAGVDITTDAPLKETINYISNLVDELEVLTYQIE 600

SAV1369: 601 Q 601
+
SE1065 : 601 E 601

Figure 6.20 (continued)

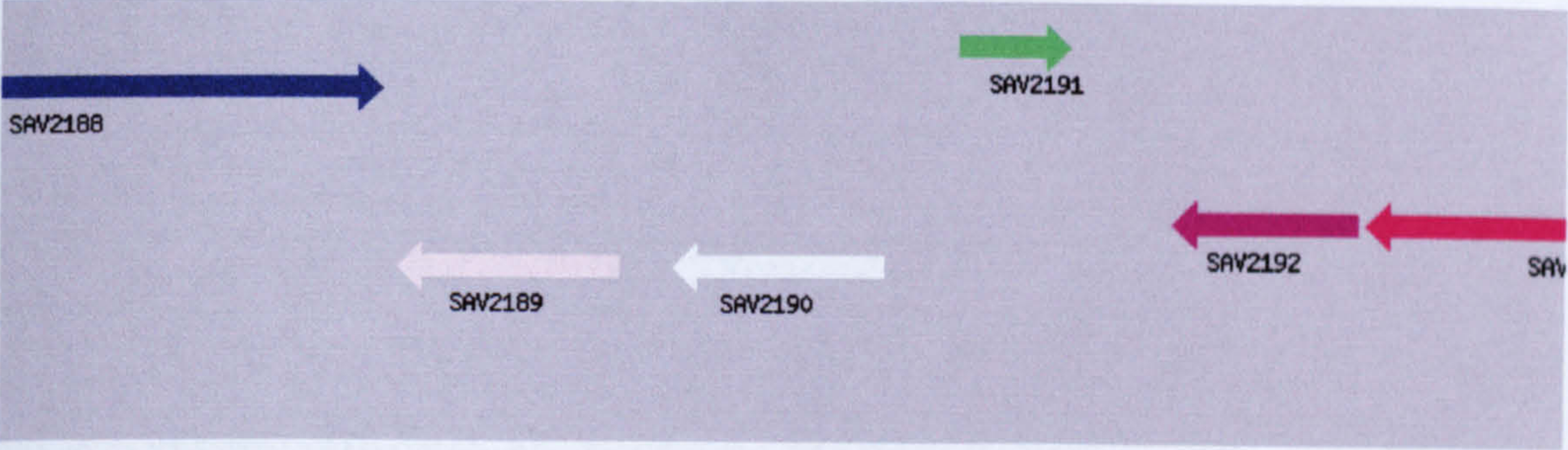


Figure 6.21 Region view display of the SAV2190 locus in *S. aureus* Mu50

Mutant G12:	412	ATGTCAAAGCG-CTATTATTGTTTGGATTTTGATGAAACATATTTTAAACATAATACAAA	470
SAV2190 :	1	ATGTCAAAGCGCTATTATTGTTT-GATTTTGATGAAACATATTTTAAACATAATACAAA	59
Mutant G12:	471	TGAAGGAAGATTTAAGGTCNTTTTAAGAGAAATGGAAAAATTGTTNGGAAAAACCTAAC	530
SAV2190 :	60	TGAAG-AAGATTTAAG-TCATTT-AAGAGAAATGGAAAAA-TTGTT-GGAAAAAC-TAAC	113
Mutant G12:	531	TAATTAATNATNAAAGTGATCACTGCTGTATTGAC	565
SAV2190 :	114	TAAT-AATAATGAAGTGATCACTGCTGTATT-GAC	146

Figure 6.22 Homology of the recovered gene from mutant G12 with SAV2190 locus in *S. aureus* Mu50

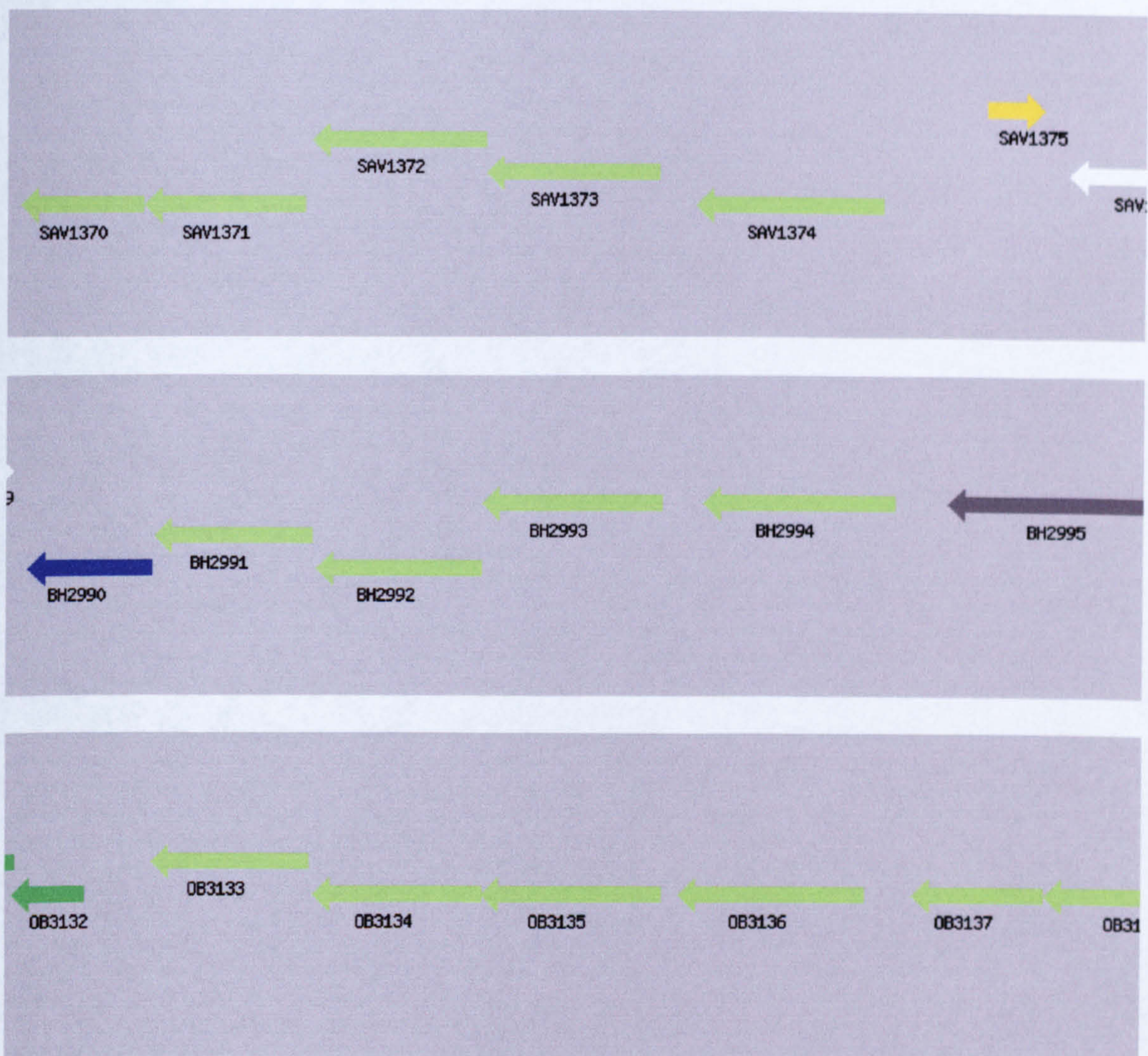


Figure 6.23 Region view display of the SAV1373 locus in *S. aureus* Mu50 (top), BH2993 in *B. halodurans* C-125 (the middle), and OB3135 in *O. iheyensis* HTE831 (bottom). The light green arrows indicate transport and binding proteins group.

SAV1373:	1	MTSSTNVKALIEKNINKKGKHN	DKIIPVXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXP	60	
		M S T V+ +I KN KKG +DKI+P+		P	
SE1069 :	1	MASQTKVREMI	AKNNAKKGGLSDKIVPIILAII	SAISILTTIAILFTLLTETITFFTRVP 60	
SAV1373:	61	ITEFLSTTWNPTGSDPKEGIW	ALIIGTLKITVIATIFAVPVGLGAAIYLSEYASDRARR	120	
		++F S TWNPTGS P+EGIW	ALIIGTLKITVIATI AVP+GLGAAIYL+EYASDR+RR		
SE1069 :	61	LSKFFLSGTWNPTGSSPEFGIW	ALIIGTLKITVIATIVAVPIGLGAAIYLN	EYASDRSRR 120	
SAV1373:	121	IIKPILEILAGIPTIVFGFFALT	YVTPVLRSEFISGLGEFNAIS	PGLVVGIMIVPLITSLS 180	
		IIKPILEILAGIPTIVFGFFALT	+VTP+LR+ I LGEFN+ISPG+VVGIMIVP+ITS+S		
SE1069 :	121	IIKPILEILAGIPTIVFGFFALT	FVTPILRNLI	PNLGEFNSISPGIVVGIMIVP	MITSMS 180
SAV1373:	181	EDAMASVPNKIREGAYGLGATK	LEVATKVVLPAATSGIVASIVL	AISRAIGETMIVXXXXX 240	
		EDAM+SVP+KIREGA+GLGATK	EVATKVVLPAATSG+VASIVL	ISRAIGETMIV	
SE1069 :	181	EDAMSSVPDKIREGAFLGATK	FEVATKVVLPAATSGVVASIVL	GISRAIGETMIVSLAA 240	
SAV1373:	241	XXXXXXXXXXXXXX	IQTMTGYIVEIATGDATEGSNI	YYSIYAVGFTLFIFTLIMNLLSQWI 300	
			IQTMTGYIVEIATGDA FGS+I	YYSIYAVGFTLFIFTLIMNLLSQWI	
SE1069 :	241	GSSPTSSLSLTSSIQTMTGYI	VEIATGDAAFGSDIYYSIYAVGFTLFIFTLIMNLLSQWI	300	
SAV1373:	301	SKRFREY	308		
		SKRFREY			
SE1069 :	301	SKRFREY	308		

Figure 6.24 Protein homology for recovered gene from mutant H3 (SAV1373 locus in *S. aureus* Mu50) and SE1069 in *S. epidermidis*

SAV1373:	4	STNVKALIEKNINKGKHN--DKIIPVXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX	61
		+ +V+ +IEKN K N +K+IP	PI
BH2993 :	12	AVDVREMIENRQTKSLANLAEKLI PKCLLVIA TVSILTTIGILYTL LHETIEFFSRIPI	71
SAV1373:	62	TEFLFSTTWNPTGSDPKFGI WALII GTLKITVIATIFAVPVGLGAAIYLSEYASDRARRI	121
		+F T P +P+FG+ L+ GTL TVIA + A+P+GL AI+LSEYASDR RR	
BH2993 :	72	VDFFTGTVLKP LSQLNPEFGVLP LLTGTLISTVIAMVVAIPIGLMTAIFLSEYASDR LRRT	131
SAV1373:	122	IKPILEILAGIPTIVFGFFALT YVTPVLR SFISGLGEFN AISPGLVVGIMIVPLITSLSE	181
		+KP+LE+LAGIPTIV+GFFA T+VTP+LR + GL N +SPG+V+GIMI+P++ SLSE	
BH2993 :	132	LKPMLEVLAGIPTIVYGFFAFTFVTP LLRHVVPGLEATN ILSPGIVMGIMIIPMVASLSE	191
SAV1373:	182	DAMASVPNKIREGAYGLGATKLE VATKVVLPAATSGIVASIVLAISRAIGETMIVXXXXX	241
		DAM+SVPN +REGA LGATKLEV TKVV+PAA SGIVAS VL SRAIGETMIV	
BH2993 :	192	DAMSSVPNAMREGALALGATKLEVTTKVVIPAAISGIVASFVLGFSRAIGETMIVTIASG	251
SAV1373:	242	XXXXXXXXXXXXXIQMTGYIVEIATGDATFGSNIYYSIYAVGFTLFIFTLMNLLSQWIS	301
		+QTMT YIVE+ GDA GS IYYS+YAV TLF+FT +MNL++IS	
BH2993 :	252	SSKNFTFDVTQSMQMTAYIVEVTGGDAPAGSTIYSLYAVAMT L FVTF LMNLLARYIS	311
SAV1373:	302	KRFREEY	308
		+RFREEY	
BH2993 :	312	RRFREEY	318

Figure 6.25 Protein homology for recovered gene from mutant H3 (SAV1373 locus in *S. aureus* Mu50) and BH2993 in *B. halodurans*

SAV1373:	3	SSTNVKALIEKNINKK--GKHNDKIIPVXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXP	60
		++ +V+ +IE N KK + +K++PV	
OB3135 :	9	NTASVRKMIEANKQKKSVSQITEKLVFVFLVLIASISILTTAGIITYTLAESIAFFQRP	68
			P
SAV1373:	61	ITEFLFSTWNPTGSDPKFGIWALIIGTLKITVIATIFAVPVGLGAIYLYSEYASDRARR	120
		I EF T P +P+EG+ L+ GT+ +VIA + A PVGL +A+YLSE+ASD+ RR	
OB3135 :	69	IEFFTGTVLKPLSQNPPEFGVLP LLTGTIISSVIAMLVAGPVGLMSAVYLSEFFASDKVRR	128
SAV1373:	121	IIKPILEILAGIPTIVFGFFALTYYVTPVLRSFISGLGEFN AISPGLVVGIMIVPLITSLS	180
		+KP+LEILAGIPTIV+GFEA T+VTP++R + N +SPG+V+G+MI+P+I SLS	
OB3135 :	129	TVKPLLEILAGIPTIVYGGFFAFTFVTP LIRMVFPVSVEATNILSPGIVMGVMIIPMIASLS	188
SAV1373:	181	EDAMASVPNKIREGAYGLGATKLEVATKVLPAAATSGIVASIVLAISRAIGETMIVXXXX	240
		EDAM+SVPN +REGA LGAT+LE +VV+PAA SGI++S VL ISRAIGETMIV	
OB3135 :	189	EDAMSSVPNSMREGALALGATRLTETFRVVI PAALSGIISFVLGISRAIGETMIVTIAS	248
SAV1373:	241	XXXXXXXXXXXXXXIQMTGYIVEIATGDATFGSNIYYSIYAVGFTLFIFTLIMNLLSQWI	300
		+QTMT YIVE++ G+A GS IYYS+YAV TLF+FTLIMNLL++I	
OB3135 :	249	GSSKNFTFDITQSMQMTAYIVEVSGGEAPAGSTIYYSLYAVALTLEFVFTLIMNLLARYI	308
SAV1373:	301	SKRFREEY	308
		S++FREEY	
OB3135 :	309	SRKFREEY	316

Figure 6.26 Protein homology for recovered gene from mutant H3 (SAV1373 locus in *S. aureus* Mu50) and OB3135 in *O. iheyensis*

SAV1362: 164 ttctagtttacataataatattataggaaagt 195
|||||
LMO2669: 831 ttctagtttacataataatattataggaaagt 800

SAV1362: 312 VFDIDGTLCFDGRLLDQTIIDTLLQLQHDSHELIFASARPIRDL---LPVLPSVFHQHTL 482
+ DIDGTL D + I L+ Q +G +LI AS RP + L H L
LMO2669: 7 ILDIDGTLNDDKQISPETKKALITAEENGVKLLILASGRPRTTGMHVYAEQLEMEKHGGLL 66

SAV1362: 483 IGANGAMISQQSKISVI--KPIHTDTYHHIFKIIQKYELDYIID-DDWNYYAAQLDAENAI 653
+ NGA + + + + + + + + + +E+ +ID +D+ Y N +
LMO2669: 67 VSNGAKVVDCATNEELEFNQALTVEEGKAVLEHMKQFEVKVMIDKEDYMYT-----NDV 120

SAV1362: 654 FERLXPHKLAQCIDVAKY 707
++ P++ + I++ +Y
LMO2669: 121 YDCYVPYR-GEEINIIQY 137

Figure 6.27 Homology between the recovered gene from the mutant H5 (SAV1362 locus in *S. aureus* Mu50) and LMO2669 locus in *L. monocytogenes* which shows 32 identical bases (top). The LMO locus encodes the *flaR* gene. Protein homology between SAV1362 and *flaR* (bottom). The homology of the proteins at the beginning (DIDGTL) may be indicate that the gene is essential for the start of protein synthesis.



Figure 6.28 Region view display of the SAV1382 locus in *S. aureus* Mu50 (top), OB1402 in *O. ihayensis* HTE831 (the second), EF1133 in *Enterococcus faecalis* V583 (the third), and BH2669 in *B. halodurans* C-125 (bottom).

Mutant F10:	653	ATGGTACACCAATTTAACAGCTGAAGAAATTATTCAATATATAAAGTGATGCTAAAAAGTCT	594
SAV1382	:	1	ATGGTACAACATTTAACAGCTGAAGAAATTATTCAATATATAAAGTGATGCTAAAAAGTCT
Mutant F10:	593	ACACCAATAAAAAGTATATTTAAATGGTAATTTTGAAGGCATCACATATCCAGAAAGTTT	534
SAV1382	:	61	ACACCAATAAAAAGTATATTTAAATGGTAATTTTGAAGGCATCACATATCCAGAAAGTTT
Mutant F10:	533	AAAAGTATTTGGTTCAGAACAACTCTAAAGTAATCTTTTGTGAAGCGGATGATTGGAAACCT	474
SAV1382	:	121	AAAAGTATTTGGTTCAGAACAACTCTAAAGTAATCTTTTGTGAAGCGGATGATTGGAAACCT
Mutant F10:	473	TTTTACGAAAGCATATGGTAGTCAATTCGAAGATATAGAAAATTGAAATGGATCGTCGCAAT	414
SAV1382	:	181	TTTTACGAAAGCATATGGTAGTCAATTCGAAGATATAGAAAATTGAAATGGATCGTCGCAAT
Mutant F10:	413	TCTGCTATTCCATTTAAAAGACTTAAACAAATACGAATGCACGAATTGAACCAGTGCGTTT	354
SAV1382	:	241	TCTGCCATTTCCATTTAAAAGACTTAAACAAATACGAATGCACGTATTGAACCAGTGCAATT
Mutant F10:	353	ATTAGAGAACAAAGCCATTATTGAAGATGGTGTGCTGTTATGATGGGCGCAACAATTAAT	294
SAV1382	:	301	ATTAGAGAACAAAGCCATTATTGAAGATGGTGTGCTGTTATGATGGGCGCAACAATTAAT
Mutant F10:	293	ATTGGCGCAGTCGTTGGCGAAGGTACAATGATTGATATGAATGCTACTCTCGGTGGTCGT	234
SAV1382	:	361	ATTGGCGCAGTCGTTGGCGAAGGTACAATGATTGATATGAATGCTACTCTCGGTGGTCGT
Mutant F10:	233	GCTACAACCTGGTAAAAATGTACATGTAGGGGCTGGCGCAGTATTAGCAGGTGTGATTGAA	174
SAV1382	:	421	GCTACAACCTGGTAAAAATGTACATGTAGGGGCTGGCGCAGTATTGGCAGGTGTGATTGAA

Figure 6.29 Homology of the recovered gene from mutant F10 and SAV1382 locus in *S. aureus* Mu50

Mutant F10:	173	CCCCCTAGTGCTTCACCCGGTTATAATCGAGGATGATGTATTAAATCGGTGCAAATGCAGTT	114
SAV1382 :	481	CCCCCTAGTGCATCACCCAGTTATAATCGAGGATGATGTATTAAATCGGTGCAAATGCAGTT	540
Mutant F10:	113	ATTTAGAAAGGTGTACGTGTTGGTAAAGGTGCTATTGTTGCAGCTGGCGGATTGTNACA	54
SAV1382 :	541	ATTTAGAAAGGTGTACGTGTTGGTAAAGGTGCTATTGTTGCAGCTGGCGGATTGTGACA	600
Mutant F10:	53	CAAGATGTACCAGCTGGTGCAGTTGTTGCTGGTACACCTGCAAAGTGATTAA	1
SAV1382 :	601	CAAGATGTACCAGCTGGTGCAGTTGTTGCTGGTACACCTGCAAAGTGATTAA	653

Figure 6.29 (continued)

SAV1382:	1	MVQHLTAEIIQYISDAKKSTPIKVYLNNGFEGITYPESEFKVFGSEQSVIFCEADDWKP	60
		MVQHL+A+EIIQYISDAKKSTP+KVY+NG+FE +T+PESEFKVFGSE SKVIFCEA++WK	
SE1077 :	1	MVQHLSAQEIIQYISDAKKSTPLKVYVNGHFENVTFPESEFKVFGSEHSKVIFCEANEWKQ	60
SAV1382:	61	FYEAYGSQFEDIEIEMDRRNSAIPLKDLTNTNARIEPGAFIREQAIIEDGAVVMMGATIN	120
		FY+ S ++EIEMDRRNSAIPLKDLTNTNARIEPGAFIREQAIIEDGAVVMMGATIN	
SE1077 :	61	FYQQNHSLITEIEIEMDRRNSAIPLKDLTNTNARIEPGAFIREQAIIEDGAVVMMGATIN	120
SAV1382:	121	IGAVVGE GTMIDM NATLGGRATTGKNVHXXXXXXXXXXIEPPSASPVIIEDDVLIGANAV	180
		IGA+VGE GTMIDM NATLGGRATTGKNVH IEPPSASP V+IED+VLIGANAV	
SE1077 :	121	IGAI VGE GTMIDM NATLGGRATTGKNVHV GAGAVLAGVIEPPSASP VIEDNVLIGANAV	180
SAV1382:	181	ILEXXXXXXXXXXXXXXXXXTQDXXXXXXXXXXIKQASEVQDTKKEIVAALRKLND	239
		ILE TQD IKQ SEVQD+K+EIV+ALRKLN+	
SE1077 :	181	ILEGVRVGAGAI VAAGAI VTQDVPAGAVVAGTPAKVIKQTSEVQDSKREIVSALRKLNN	239

Figure 6.30 Protein homology between recovered gene from mutant F10 (SAV1382 locus in *S. aureus* Mu50) and SE1077 locus in *S. epidermidis*

Mutant F11: 24 aaaacacgaaataatttacaagtaataatatTTTancacaaaaataaaagagtctatat 83
 |||||
brn : 2664 aaaacacgaaataatttacaagtaataatatTTTtagcacaaaaataataaga-tctatat 2606

Mutant F11: 84 gaaatatgtgtgacatagcagaaacaaaaacacatgtgtgttttaaatcaaatattatgtcag 143
 |||||
brn : 2605 gaaatatgtgtgacatagcagaaacaaaaacacatgtgtgttttaaatcaaatattatgtcag 2546

Mutant F11: 144 aaaataaaattcttcaaatccactttttaaatagtaactgatgacttttattcacaaattt 203
 |||||
brn : 2545 aaaataaaattcttcaaatccactttttaaatagtaactgatgacttttattcacaaattt 2486

Mutant F11: 204 cataaaactcataatTTTtaaattttataaaagatttaagtagtaannnnnnnaaat 263
 |||||
brn : 2485 cataaaactcataatTTTtaaattttataaaagatttaagtagtaattttttaaat 2426

Mutant F11: 264 ggagagagatgtccaggtaaatgcgcaacaccttcaacgaaaatagcataattgaccataa 323
 |||||
brn : 2425 ggagagagatgtccaggtaaatgcgcaacacc-tcaacgaaaatagcataattgaccataa 2367

Mutant F11: 324 atattatgaattgtttgttcaacatcttcagttatt-ggatcttgactcaaaaaatacatt 382
 |||||
brn : 2366 atattatgaattgtttgttcaacatcttcagttattgggatcttgactc-aaaaatacatt 2308

Figure 6.31 Homology between the recovered gene from mutant F11 and branched-chain-amino-acid transporter of *S. aureus*

Mutant F11: 383 aaatacttcaataccaatttacgtgacatttctacagcttcatacgtatcaataataacc 442
|||||
brn : 2307 aaatacttcaata-caaaatttacgtgacatttctacagcttcatacgtatcaataataacc 2249

Mutant F11: 443 atcttgactataataatgacagacgggttcgccggtctgaaaatacgcattaaaaatcgttg 502
|||||
brn : 2248 atcttgactataataatgacagacgggttcgccggtctgaaaatacgcattaaaaatcgttg 2189

Mutant F11: 503 atgttgatttcgacgcattaatcggtcacttgcaactctaatagcaaca-ccatcac-ga 560
 |||||
 brn : 2188 atgttgatttcgacgcattaatcggtcacaagc-actgtaatggcaacacccatcacgga 2130
 |||||

Mutant F11: 561 ttatcatcttgaggttcaagtgccataatacgtgggcccattcttttcaaaggtgagtaa 620
|| ||||||| ||||||| ||||||| ||||||| ||||||| ||||||| |||||||
brn : 2129 ttgtcatcttgaggttcaa-tgccataatacgtgggcccattcttttcaaaggtgagtaa 2071

Mutant F11: 621 tcataggtaataatttcattaatgataatttgggtgngcatgctcgctgaatcaangca 680
 ||||| ||||| ||||| ||||| ||||| | ||||| ||||| ||||| |||||
 brn : 2070 tcatagtttaataaatttcattaatgataatttgcgttctgcatgctcgctgaatcaaggca 2011

Mutant F11: 681 ttctcactg 689
 . | |||||
 brn : 2010 tcctcactg 2002

Figure 6.31 (continued)

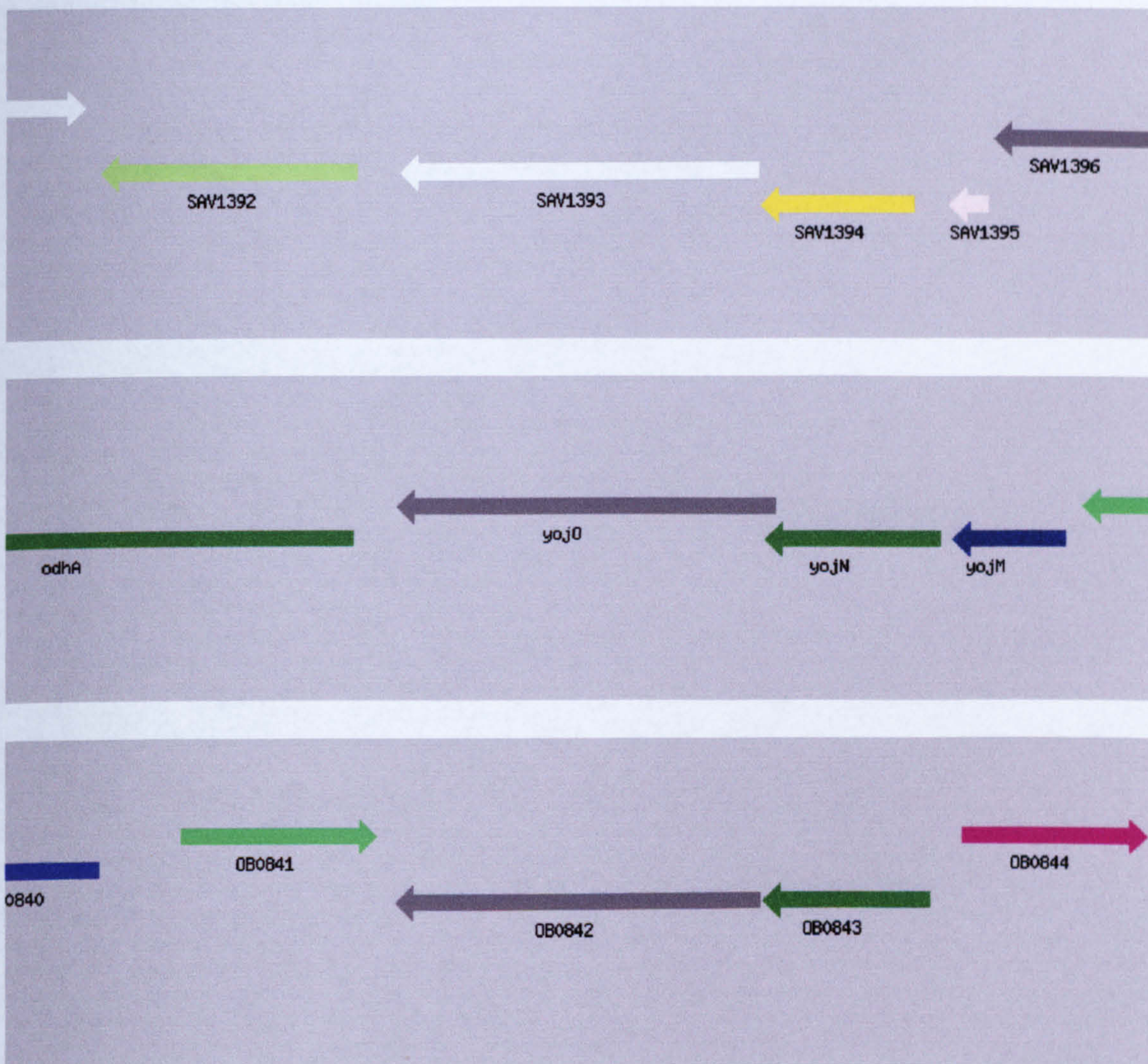


Figure 6.32 Region view display of the SAV1393 locus in *S. aureus* Mu50 (top), *yojO* in *B. subtilis* 168 (the middle), and OB0842 in *O. iheyensis* HTE831 (bottom).
Note: There are some rearrangement of the SAV1394 and SAV1395 loci in the sequence of the whole genome, as the two loci are also seen with different sizes in the opposite direction (see figure 6.28).

Part III

Discussion

Chapter 7

General discussion

Chapter 7: General discussion

S. aureus is a highly versatile pathogen, well adapted to survive in many niches in and on humans. The ability of *S. aureus* to evade the host immune response and cause disease is due to an extensive repertoire of both known and unknown virulence factors. Much of our knowledge about the virulence determinants of pathogenic bacteria comes from experiments with bacteria grown in culture, but there is currently an increasing interest in what happens to bacterial pathogens in the infected host. In the past few years, the study of bacterial pathogenesis has been advanced by a number of techniques that enable investigators to monitor bacterial gene expression during infection. These techniques allow the investigator to select and screen for genes that are required for infection or that are expressed *in vivo*. Two main categories of these techniques are *in vivo* expression technology (IVET) and signature-tagged mutagenesis (STM). Both methods have been used to identify virulence genes in *S. aureus*. The aim of this project was to find any probable new genes of *S. aureus* that is essential for biofilm formation and in a mouse infection model by STM.

IVET has been applied to *S. aureus* using a promoter trap that relies on genetic recombination of the site-specific resolvase of *tnpR* from gamma delta (Tn1000) as a reporter of gene expression. A collection of 45 genes were found induced during infection in a murine renal abscess model. Of these, only six had been known previously; 11 others have homology to known non-staphylococcal genes. The known staphylococcal genes included *agrA*, a key locus regulating numerous virulence products; and a glycerol ester hydrolase which may enhance bacterial survival in abscesses. Many of the *ivi* genes identified were not classical virulence factors but rather involved in adaptation to the *in vivo* environment; knowledge of these biochemical pathways is clearly the next phase in IVET systems (98).

STM is a powerful genetic method, developed by David Holden and his colleagues in 1995, to identify genes in pathogens that are required for growth in an animal. In essence, STM enables one to test a large number of potential mutants simultaneously in one animal, and to identify and recover mutants that fail to colonize host tissues. Factors identified by STM are essential for colonization in the niche tested. This feature is unique to STM among various genomic strategies developed thus far to study pathogens *in vivo*. Other methods, such as IVET, DFI and microarray analysis,

identify genes whose expression is induced in the host; additional experiments are required to determine whether the gene is necessary for colonization of host tissues.

During the past seven years, STM has been successfully applied to a large variety of bacterial pathogens in hosts ranging from cows to amoeba to cultured cells. In most studies, both predicted and unsuspected virulence factors have been identified and include a vast variety of proteins involved in different functions, as well as open reading frames (ORFs) with unknown function or with no homology to other genes in GenBank.

Mutagenesis techniques have been used extensively for the identification of genes whose functions are required under certain conditions. One of these techniques that has been used in several pathogens is transposon mutagenesis. Three limitations of the transposon mutagenesis approach should be kept in mind.

First, transposons carry transcriptional terminators. If a transposon lands in the first gene in an operon, it will eliminate expression not only of that gene but also of downstream genes as well; in other words, transposon insertions are polar. The avirulent phenotype of the mutant could thus be due to loss of expression of either the gene interrupted by the transposon or downstream genes in the operon.

A second limitation is that transposon insertion mutants can be obtained only in genes that are not essential for growth on the selective medium. A transposon insertion in a gene essential for growth will not be isolated because the bacteria will not survive to form a colony. This could be considered an advantage if one assumes that the most interesting virulence genes are not the ones essential for growth in laboratory medium but are genes induced specifically in an animal host (141). The most important disadvantage of transposon mutagenesis is that to screen for loss of virulence would require each mutant to be tested separately. So, we need methods to label individual mutants, and then pools can be screened. In other words, a number of mutants (e.g., 96 from a microtiter dish) can be screened simultaneously.

The third limitation is that transposon mutagenesis is not fully random and may have favoured mutation of certain areas of the chromosome over others (29).

As STM is based on transposon mutagenesis, it has the above limitations. It should be also noted that, depending on the sensitivity of the PCR/hybridisation protocol for detecting such changes in tag populations, STM might not identify mutations causing small or even moderate reduction in survival. This is a particularly important consideration because this category includes mutations in genes that are critical for

causing disease but do not appreciably affect survival of the bacterium in the host. For example, it is the action of cholera toxin that is primarily responsible for the lethality of cholera, but deletion of the cholera toxin genes does not affect the bacterium's ability to colonize the host (159).

In the first step of the project, a library of *S. aureus* tagged insertion mutants was given to us by Professor Holden. A series of artificial input and output pools was chosen and hybridisation experiments were done. Cross-hybridisation was seen in most of the experiments. According to the results of the hybridisation experiments on the existing library, it was concluded that the library was not reliable for a cross-hybridisation free screening. There are at least 3 possible reasons for this problem, (i) the problem with hybridisation itself, (ii) incomplete removal of the arms of the tags in the process of digestion and making the probes, and (iii) contamination of the tags. The first 2 possibilities are excluded as they are not supported by some series of experiments, and as results showed the protocol worked in some experiments. It is therefore concluded that the problem was more likely the contamination of the mutants with each other or original tags used to make the library. This could have occurred in the first inoculation from the master plates (which contain all the representative tags in each plate) to the cryotubes. As the existing library of mutants showed unexpected cross-hybridisation, the whole library was discarded. The details of experiments on the existing library are presented in chapter 3. We next tried to use the selected tags in plasmids of *S. aureus* strain RN6390 for future experiments.

In the second step of the project, the selected tags in plasmids of *S. aureus* RN6390 were used. The plasmids were also given to us by Professor Holden. A series of artificial input and output pools was chosen and hybridisation experiments were done. Cross-hybridisation was seen in most of the experiments. 4 plasmids which had cross-hybridisation were sequenced. The results showed that 3 out of 4 were exactly the same. It was therefore decided that all 94 tags should be sequenced. 84 out of 94 were sequenced. The results showed that 51 out of 84 had more than 50% identity, and were not therefore suitable for a cross-hybridisation free screening. 33 tags with less than 50% identity were chosen for future experiments. The details of the experiments on the selected tags in plasmids of *S. aureus* are presented in chapter 4.

In the third step of the project, we tried to construct a new library of mutants from the 33 selected tags. A library of 825 mutants, divided into 25 pools of 33 individually tagged members was constructed for future experiments. Several random mutants

from the library were chosen and tested in two ways. The results showed that (i) the constructed mutants lacked the plasmid and carry a single chromosomal insertion of Tn917, and (ii) there was no cross-hybridisation between the mutants of the constructed library. In addition, to determine the mutagenesis had occurred randomly across the genomic DNA of the mutants of the constructed library, all the mutants of the constructed library were tested for catalase enzyme production individually. As there was one catalase-negative mutant in the library, this showed that loss of known phenotypes could be detected. In other words, mutagenesis has also occurred in known genes such as catalase gene. The details of the construction of the library of tagged insertion mutants are presented in chapter 5.

In the fourth and last step of the project, the constructed library was used in two experimental systems: mouse infection and biofilm. The mouse infection model has been used before (33, 112, 146), but STM in biofilm experiments has been done for the first time. Totally 25 pools of 33 mutants were screened at least once in each model. The DNA regions flanking the pBR322 side of the transposon insertion points of 12 mutants were cloned as described before in section 6.5. The nucleotide sequences of the flanking regions were determined and subsequently analysed by searching the DNA and protein databases for identical or similar genes (tables 6.1 & 6.2). The details of gene recovery from the attenuated mutants are presented in chapter 6.

To determine the mutagenesis had occurred randomly across the genomic DNA of the mutants of the constructed library, some of the known phenotypic characteristics can be traced. Catalase production was chosen for this purpose. The results showed that 1 out of 825 mutants of the constructed library was catalase negative. This shows that integration of the tags into genomic DNA of the mutants has been occurred randomly, as some of the known phenotypic characteristics such as catalase production have been also affected. The catalase negative and wild type strains were injected intraperitoneally into mice. The number of recovered bacteria from the mice showed no discernible difference (figure 5.3). It could be concluded that catalase does not affect the growth of bacteria in mouse.

The recovered genes in this project can be viewed in different aspects. The first is the homology between these genes and those from the published complete sequences of four strains of *S. aureus*, and other bacterial genomic sequences in GeneBank. In this view, some of the recovered genes have strong homology with the four strains,

and some other gram positive and gram negative bacteria, as well. There are some recovered genes that have homology just with the four strains of *S. aureus*. The third and last group which includes two genes had homology just with 3 out of the four strains. It seems that this could be usual, as there are more than 2500 ORFs in the complete sequence of *S. aureus*, and there are some differences between different strains within a species. On the other hand, bacteria have usually some identical or common genes, and this should be much closer within a definite group such as gram positive and gram negative bacteria. 8 out of 12 genes had some identity with *S. epidermidis*, and the same number had some identity with other bacteria especially gram positive bacteria.

The second point of view could be the kind of recovered genes, and their role in the cells. In addition, the results could be compared with other searches especially those that have done using STM method. 4 out of 12 recovered genes in current study were in the transport and binding proteins group. This maybe represents the frequency and the number of such genes in comparison to the total number of genes in the whole genome. The other possibility, as discussed before, is that transposon mutagenesis is not fully random and may have favoured mutation of certain areas of the chromosome over others (20). As STM has based on transposon mutagenesis, it may show this limitation in some cases.

There were 4 out of 12 sequences identified as conserved hypothetical protein and/or hypothetical protein with unknown function and one with a role in DNA metabolism. All of these sequences were from mice infection experiments. The whole genome of *S. aureus* Mu50 and N315 has 2697 and 2595 open reading frames respectively including 70 putative virulence factors (88). According to the high number of genes in the genome of *S. aureus*, it seems that it is usual to find some of the genes as hypothetical proteins with unknown function in such projects.

One of the conserved hypothetical proteins with locus 1362 has been identified in 2 strains (B11 and H5) of output pools from the mice experiments.

Several sequences showed different homology with *O. iheyensis* HTE831 and *B. subtilis*. *O. iheyensis* is an extremely halotolerant and alkaliphilic gram positive, strictly aerobic, rod-shaped, motile by peritrichous flagella, and spore forming. Phylogenetic placement of the bacterium is comparatively close to that of *B. subtilis* (100). The genome of the bacterium also contains 27 putative phage-associated genes, which are similar to those of several bacteria including *S. aureus* and *B. subtilis* (157).

Media are made selective for staphylococci by including 7.5% (w/v) NaCl. Staphylococci can tolerate unusually high NaCl concentrations, with *S. aureus* capable of growth in media of water activity (a_w) of 0.86, which is equivalent to 3.5 M (20%) NaCl (36). That is not surprising that there is some homology between *S. aureus* and *O. iheyensis* genomes, as high NaCl tolerance is common in both bacteria.

In total 12 (1.45%) samples showed moderately attenuated hybridisation signals in this study. This is less than the number of attenuated mutants in other works by Mei et al (112) and Coulter et al (33) which were 50 (4.01%) and 237 (7.11-8.36% in different infection environments) respectively. In the last work, only 85 out of 237 mutants were sequenced.

Virulence in *S. aureus* has been studied by using STM methodology (33, 112, 146). In the study by Mei et al (112), Tn917 mutants were tested in a murine model of bacteraemia. The majority of loci from 50 mutants that were identified as attenuated were predicted by sequence similarity to be involved in cell surface metabolism (e.g. peptidoglycan cross-linking and transport functions), nutrient biosynthesis, and cellular repair processes, but most of the remainder had no known function. A slightly larger signature-tagged mutant bank was constructed by using the same transposon and tested in models of bacteraemia, abscess and wound formation, and endocarditis (33). This enabled the identification of various genes affecting growth and virulence in specific disease states, as well as 18 that are important in at least three of the infection models. Many of these genes appear to be involved in the same kinds of processes as those identified in the earlier study (112); indeed, several genes identified by Mei et al (112) were also found by Coulter et al (33). As in these earlier studies, both predicted and unsuspected virulence factors, as well as ORFs with unknown function have also been identified in the present study. Two genes identified in this work were also found by Coulter et al (33). This similarity shows: first, our system with the new constructed library worked properly, and second, this maybe strengthen the opinion that transposon mutagenesis (including STM) is not fully random, and may have favoured mutation of certain areas of the chromosome over others.

The assortment of independent mutations recovered in peptide and amino acid transport mechanisms emphasizes the biological significance of these loci on *S. aureus* survival in single or multiple *in vivo* environments.

This work is the first that use the STM method in biofilm systems. This is also the first time that *dapD* gene has been recovered from a gene screening in biofilm, although it needs more work to confirm the exact role of the gene in biofilm formation especially in *S. aureus*. The enzyme has been also identified in a mouse infection model in another STM screening on *S. aureus* (33). In other words, this work also showed that the STM method works well, as confirms other findings. It could also be concluded that there is a link between biofilm formation and infection *in vivo*, as the enzyme has been identified in both systems.

This work could be followed in different ways in future. The more the number of tag insertion mutants in the library are, the more efficient the screening will be. In addition, it is advisable to make defined single mutations to determine and confirm the role of the genes in more details, e.g. defined mutant relating to nitric-oxide reductase and test susceptibility to NO *in vitro*.

Appendix

Materials

Appendix: Materials

Agarose MP (multi purpose agarose):

Roche Diagnostics GmbH, # 1388991

Ampicillin stock solution:

Sigma, # A-9518, 50 mg/ml

Brain heart infusion (BHI) agar:

Oxoid, # CM225, 37 gr/litre with 1.5% agar, autoclaved

Brain heart infusion (BHI) broth:

Oxoid, # CM225, 37gr/litre, autoclaved

Blocking Reagent stock solution:

Roche Diagnostics GmbH, # 1096176, Blocking reagent was dissolved in maleic acid buffer to a final concentration of 10% (w/v) with shaking and heating, and then autoclaved.

Chloramphenicol stock solution:

Sigma, # C-0378, 20mg/ml

Chloroform:

Fisher Scientific, # C/4960/17

CSPD[®] (Disodium 3-(4-methoxyspiro{1,2-dioxetane-3,2'-(5'-chloro)tricyclo[3.3.1.1^{3,7}]decan}-4-yl) phenyl phosphate:

Roche Diagnostics GmbH, # 1755633

CTAB/NaCl (10% hexadecyltrimethyl ammonium bromide in 0.7 M NaCl):

4.09 g NaCl was dissolved in 80 ml distilled water. 10 g CTAB was added and mixed. 20 ml of distilled water was added. The solution is viscous, and should be homogenised by using stirrer and microwave.

Denaturation solution:

0.5 N NaOH, 1.5 M NaCl

Detection buffer:

100 mM Tris-HCl, pH 9.5 (+20°C), 100 mM NaCl

Epicurian Coli[®] XL1-Blue Competent Cells:

Stratagene, # 200249

Erythromycin stock solution:

Sigma, # E-6376, 20 mg/ml

Ethanol:

Bamford Laboratories, # 200-578-6

Glycerol:

Sigma-Aldrich GmbH, # 15523

Hind III:

Life technologies, # 15207-012

Hybond[®] N⁺ Nylon membrane:

Amersham Pharmacia Biotech, # RPN203N

Hydrogen peroxide:

Sigma, # H-1009, 30%

Iso-amyl alcohol:

Fisher Scientific, # A/7080/08

Isopropanol (Iso-propyl alcohol):

BDH Company, # 10224BQ

1 Kb DNA Ladder:

Life Technologies, # 15615-016

25 bp DNA ladder:

Invitrogen, Life technologies, # 10597-011

Luria-Bertani (LB) agar:

Sigma, # L-3522, 25 gr/litre, with 1.5% agar, autoclaved

Luria-Bertani (LB) broth:

Sigma, # L-3522, 25 gr/litre, autoclaved

Lysostaphin:

Sigma, # L-0761; 3M Sodium acetate pH 5.2, 33.3 µl; distilled water 966.7 µl

Lysozyme stock solution:

Sigma, # L-6876

Maleic acid buffer:

Sigma, # M-0375, 0.1M Maleic acid, 0.15 M NaCl, pH 7.5 (+20°C), adjust pH with concentrated or solid NaOH, autoclaved

NaCl:

Sigma-Aldrich GmbH, # 31434

Neutralization solution:

0.5 M Tris-HCl, pH 7.5, 3 M NaCl

N-lauroylsarcosine:

10% (w/v) in sterile H₂O, filtered through a 0.2-0.45 µm membrane

PCR DIG Labelling Mix:

Roche Diagnostics GmbH, # 1585550

Primers:

P1 (5'→3')

CTT CAA TTC CTA TTA TAC

Modification: 5' IRD 700

P2 (5'→3')

CAT ATT TGA ATG TAT TTA G

Modification: 5' IRD 800

P12 (5'→3')

ATT CTA CAA CCT CAA GC

P13 (5'→3')

ATT CCA TTC TAA CCA AGC

P21 (5'→3')

TAA GAC ACG ACT TAT CGC CA

P22 (5'→3')

CGT TGC GCA AAC TAT TAA CT

Pseq-1 (5' →3')

AGA GAG ATG TCA CCG TCA AGT

P009823 (5'→3')

CAA CAT GAC GAA TCC CTC C

P009824 (5'→3')

GGG AAT AAG GGC GAC ACG

Proteinase K stock solution:

Life Technologies, # 25530-015, 20 mg/ml distilled water

RNase:

10 mg/ml, Qiagen

SDS stock solution:

Fisher scientific, # S/P530/53, 10% (w/v) in H₂O, filtered through a 0.2-0.45 µm membrane

SET, pH 7.5:

20% sucrose, 50 mM EDTA, 50 mM Tris Base

SSC stock solution (20x):

3 M NaCl, 0.3 M sodium citrate, pH 7.0 (+20°C), autoclaved

Standard hybridization buffer:

5x SSC, 0.1% N-lauroylsarcosine, 0.02% SDS, 1% Blocking Reagent (from the 10% Blocking Reagent stock solution)

***Taq* DNA polymerase:**

Promega, # M1861

T4 DNA Ligase:

Promega, # M1801

TE buffer:

10 mM Tris-HCl, 1 mM EDTA, pH 8.0 (+20°C)

Trizma[®] Base (Tris[hydroxymethyl]aminomethane):

Sigma, # T-1503

Trizma[®] Hydrochloride (Tris[hydroxymethyl]aminomethane hydrochloride):

Sigma, # T-3253

Tween 20 (Polyoxyethylene-sorbitan monolaurate):

Sigma, # P-1379

Washing buffer:

Add 0.3% (w/v) Tween[®] 20 to Maleic acid buffer.

Washing solution 2x:

2x SSC, 0.1% SDS

Washing solution 0.5x:

0.5x SSC, 0.1% SDS

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