The regulation of toxin production in *Staphylococcus aureus*

by

Heather Fairhead, B.Sc.

(University of Nottingham)

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Department of Molecular Biology and Biotechnology,

Firth Court, University of Sheffield.

SUMMARY

Staphylococcus aureus is a major human pathogen causing a wide range of illnesses from the trivial to the life-threatening. *S. aureus* produces many surface-associated and exoproteins, several of which have been implicated in its virulence. Production of these virulence determinants is co-ordinately controlled by several global regulatory elements in a growth phase dependent manner. The best characterised of these regulators are the accessory gene regulator (*agr*) and staphylococcal accessory regulator (*sar*). The *agr* locus comprises a quorum sensing system and encodes a signalling pheromone that autoregulates *agr* in a density dependent manner. Upregulation of *agr* expression leads to production of an mRNA transcript, RNAIII which is the actual effector of virulence gene expression. The RNAIII molecule upregulates several extracellular toxins including haemolysins, toxic shock syndrome toxin 1 (TSST-1) and epidermolytic toxin A (Eta), and down-regulates surface proteins such as protein A and fibronectin binding protein (FnBP) during late exponential growth and stationary phase.

The regulation of toxin production by *S. aureus* is extremely complex and it is not yet understood exactly how this organism responds to environmental stimuli in order to mediate changes in virulence gene expression. In order to determine whether environmental signals are transduced via *agr*, the effect of several stimuli on both *agr* expression and α-haemolysin production was examined using a β-galactosidase reporter gene fusion to the *hld* gene, which is encoded as part of the RNAIII transcript. A number of environmental stimuli were identified which led to changes in *agr* expression. Several of these stimuli resulted in different effects on α-haemolysin activity when compared to RNAIII levels. This suggests the presence of novel regulatory elements involved in the control of Hla production, independently of *agr*.

In order to identify other novel regulators which interact with, or control, *agr*, transposon libraries have been created using Tn*917* and Tn*551*. Two Tn*917* transposon mutants were isolated as deficient in production of β-haemolysin, which is also positively controlled by *agr*. These mutants were found to contain novel transposon insertions in the *agr* locus. Five Tn*551* mutants were isolated which showed pleiotropic effects on virulence determinant levels and did not contain the transposon in previously mapped regulators. The Tn*551* insertions may have therefore occurred in novel regulators of virulence determinant production.

The regulation of toxin production by *S. aureus* in response to environmental stimuli is discussed.

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Abbreviations

BCIP	5-Bromo-4-chloro-3-indolyl phosphate
CHEF	Clamped homogenous electric field (PFGE)
Cm	Chloramphenicol
CDM	Chemically defined medium
DMF	Dimethyl formamide
DMSO	Dimethyl sulphoxide
EDTA	Ethylenediaminetetraacetic acid
EGTA	Ethylene glycol-bis(β-aminoethylether)N,N,N,N'-tetraacetic acid
Em	Erythromycin
FIGE	Field inversion gel electrophoresis
FSB	Frozen storage buffer
GML	Glycerol monolaurate
HL	Homoserine lactone
HU	Haemolysin units
MU	Miller units
4-MU	4-Methyl umbelliferone
MUG	4-Methyl umbelliferone-β-D-galactopyranoside
NBT	Nitroblue tetrazolium
ONPG	*o*-Nitrophenyl β–D-galactopyranoside
ORF	Open reading frame
PEG	Polyethylene glycol
PFGE	Pulsed field gel electrophoresis
PMSF	Phenylmethylsulphonyl fluoride
rpm	Revolutions per minute
Sp	Spectinomycin
SSC	Saline sodium citrate buffer
TAE	Tris/acetic acid/EDTA

TBE	Tris/boric acid/EDTA
TE	Tris/EDTA
TES	Tris/EDTA/sucrose
Tet	Tetracycline
WT	Wild type
X-Gal	5-Bromo-4-chloro-3-indolyl-β-D-galactoside

CHAPTER 1

Introduction

1. INTRODUCTION

1.1 The staphylococci

The genus *Staphylococcus* is currently composed of twenty seven species with seven subspecies (Kloos and Lambe, 1991). Members of the genus are Gram positive cocci, approximately 0.5-1.5 mm in diameter, which occur singly, in pairs, tetrads, short chains of three to four cells and, particularly in the case of *S. aureus*, in irregular grape like clusters. It was this latter form that, in 1883, led Ogston to introduce the name *staphylococcus* (from staphylé which means a bunch of grapes), for a group of micrococci which caused inflammation and suppuration (Kloos and Lambe, 1991).

The staphylococci are non-motile, do not form spores and are commonly catalase positive (Kloos and Lambe, 1991). They are not usually encapsulated but they do have limited capsule formation. Staphylococci are able to ferment, slowly, many carbohydrates producing lactic acid but not gas. Strains of *S. aureus* are alkaline phosphatase positive, urease variable and show no β–galactosidase activity. Most staphylococcal species are facultative anaerobes although apart from two species, *S. saccharolyticus* and *S. aureus* subsp. *anaerobiosis*, their growth is quicker under aerobic conditions (Kloos and Lambe, 1991). Staphylococci are relatively resistant to drying, to heat (they withstand 50 °C for 30 minutes) and to 9 % (w/v) sodium chloride.

The staphylococci are widespread in the environment although they are mainly found living on the skin, skin glands and mucous membranes of mammals and birds. In addition, they are sometimes found in the mouth, blood, intestinal, genito-urinary and upper respiratory tracts of these hosts. The identification of pathogenic staphylococci is most commonly by the ability to produce coagulase and thus clot plasma (Kloos and Lambe, 1991). In particular, only three species are coagulase positive, namely, *S. aureus* which causes acute infections in humans and *S.*

intermedius and *S. hyicus* which are found in animals (Devriese *et al.*, 1978; Hajek, 1976). Some of the remaining coagulase negative staphylococci can also cause disease but are generally considered as opportunistic pathogens causing infection following trauma, pre-disposing diseases, or due to indwelling foreign bodies (Kloos and Lambe, 1991).

Cultured colonies of *S. aureus* are usually large (6-8 mm diameter), smooth, entire, slightly raised and translucent (Kloos and Lambe, 1991). The colonies of most strains are pigmented ranging from creamy yellow to orange.

1.2 *S. aureus* - its effect on man and other animals

Staphylococcal infections cover the complete spectrum from the trivial to the life threatening and are most commonly caused by the coagulase positive species of *S. aureus* (Kloos and Lambe, 1991). These infections include folliculitis, carbuncles, furuncles, cellulitis, impetigo, and post-operative wound infections of various sites. *S. aureus* is also a particular problem following invasive hospital techniques. For example, 20 % of infections involving long term intravascular canulae are caused by *S. aureus*, together with 29 % of all pacemaker infections, usually due to contamination at the time of pacemaker implantation (Bluhm, 1985), with permanent pacemaker infections occurring in more than 6 % of patients (Heimberger and Duma, 1989). The staphylococci cause prosthetic joint infections in up to 1 % of patients undergoing total hip replacement and in up to 1.5 % of patients following knee replacement (Fitzgerald, 1989).

Bacteraemia caused by *S. aureus* is generally the consequence of a local infection with dissemination into the blood stream, but may also be secondary to direct inoculation, i.e. intravenous drug abuse. Septicaemia, or blood poisoning, is the result of the multiplication of micro-organisms in the blood often resulting in

secondary sites of infection. Early reports indicated a 60 % risk of endocarditis with *S. aureus* septicaemia but more recent studies suggest an incidence of 10 % (Waldvogel, 1990).

A particularly serious community-acquired disease, toxic shock syndrome (TSS), has been attributed to infection or colonisation with *S. aureus*. *S. aureus* also causes food poisoning due to ingestion of one, or more, heat-stable, pre-formed toxins resulting in nausea, vomiting, abdominal pains and diarrhoea, 2-6 hours after eating contaminated food (Bergdoll, 1989). The amount of enterotoxin required to cause illness in man is not known but as little as 1 μg of toxin per 100 g of food will induce clinical symptoms (Tranter, 1991).

S. aureus is also a recognised avian pathogen that is responsible for the majority of staphylococcal infections in poultry causing major financial loss (Devriese, 1980).

1.3 Treatment of *S. aureus* infections

The introduction of penicillin for the treatment of severe *S. aureus* infections in the 1940's had a profound effect on morbidity and mortality from this disease. However, by the late 1940's approximately 60 % of hospital-acquired strains were resistant to penicillin (Barber and Rozwadowska-Dowzenko, 1948) with, currently, 80-90 % of community acquired *S. aureus* strains being resistant due to the production of β–lactamase. The staphylococci have proved to be extremely versatile in developing resistance to the commonly used antimicrobial agents although many strains are still susceptible to methicillin and first generation cephalosporins. Resistance to streptomycin emerged soon after its discovery in 1944 (Demerec, 1948) and tetracycline resistance became a major problem during the 1950's. In 1952 erythromycin was introduced, however, in some centres, resistance occurred after only 7-10 days treatment (Haight and Finland, 1952). Shortly following the

introduction into clinical practice of a new β–lactamase resistant penicillin, methicillin, strains resistant to this new drug were reported (Jevons, 1961). Parker and Hewitt (1970) of the Cross-Infection Reference Laboratory in London monitored the frequency of methicillin resistance in *S. aureus* strains and found there was a moderate increase between 1960 and 1963 followed by a stationary phase. In 1968 a second increase began and analysis of the antibiotic resistance pattern of some 100 separate strains showed that, in addition to methicillin resistance, all were β–lactamase producers and were resistant to streptomycin and tetracycline.

Methicillin resistance is chromosomally mediated and results partly from the presence of a novel penicillin binding protein (PBP) 2' which has reduced affinity for methicillin and other anti-staphylococcal β–lactams, thus retaining critical functions necessary for cell survival (Hartman and Tomasz, 1984; Chambers *et al.*, 1985; Boyce, 1989).

Currently, the β–lactamase resistant penicillins are the preferred drugs for all staphylococcal infections caused by penicillin resistant, methicillin susceptible strains. Such agents are bactericidal and, like other penicillins, have a low incidence of adverse reactions. The emergence of methicillin resistant *S. aureus* (MRSA) as a major nosocomial pathogen has led to a significant role for vancomycin in the treatment of serious infections since resistance of *S. aureus* to this drug had not, until recently, been demonstrated in a clinical setting. In 1997 an intermediate level vancomycin resistant strain of *S. aureus* was isolated in a Japanese hospital. It has since been found that up to 20 % of MRSA isolates from hospitals throughout Japan are heterogeneously resistant to vancomycin and that this resistance develops into VRSA upon exposure to vancomycin (Hiramatsu *et al.*, 1997).

1.4 Virulence factors of *S. aureus* - genetics and biochemistry

S. aureus produces more than 25 different extracellular proteins, several of which have been implicated in its virulence (Arvidson, 1983). For example fibrinogen binding protein (a.k.a. clumping factor), encoded by the *clf* gene, is located on the bacterial cell surface anchored by a dipeptide repeat region to an LPXTG motif (Hartford *et al.*, 1997). Some of the genes encoding these extracellular proteins have been mapped on the *S. aureus* chromosome to one of 16 fragments, designated A–P, resulting from a *Sma*I digest of total chromosomal DNA (Pattee *et al.*, 1990). In addition, the genes for some toxins have been found to be located on mobile genetic elements or carried by lysogenic phages (Lindsay *et al.*, submitted; Novick, 1963; Winkler *et al.*, 1965).

1.4.1 Pyrogenic exotoxins

1.4.1.1 TSST-1

Toxic shock syndrome is manifested by the clinical features of fever, profuse diarrhoea, mental confusion and both renal and hepatic impairment (Turnidge and Grayson, 1993). Although TSS was originally described in menstruating females and was associated with a particular type of high-absorbence tampon, the condition has now been associated with a variety of ailments due to strains of *S. aureus* in both sexes, such as abscesses, osteomyelitis, post-surgical infections and following vaginal and caesarean section deliveries. TSS is due to the presence of toxic shock syndrome toxin-1 (TSST-1) which is a potent superantigen. The gene encoding TSST-1, *tst*, was first cloned and sequenced by Kreiswirth *et al.* (1983), and was thought to be found on an accessory genetic element, such as a plasmid, prophage or transposon. However, more recent studies have suggested that the *tst* gene is in fact likely to be carried on a mobile pathogenicity island. Pathogenicity islands range in size from 10-

200 kb and are accessory genetic elements containing one or more pathogenicity genes bordered by directly repeated sequences (Lindsay *et al.*, submitted).

1.4.1.2 Enterotoxins

It has been known for some time that *S. aureus* produces seven serologically distinct enterotoxins which are wholly responsible for the symptoms of food poisoning and whose production is dependent on various environmental factors (Halpin-Dohnalek and Marth, 1989). However, more recently, two new enterotoxins have been identified (Betley *et al.*, 1992; Su and Lee-Wong, 1995). These toxins are produced over a wide range of temperatures (10 °C-44 °C; optimum 35 °C-40 °C and the organism will grow and form toxin in many foods with the exception of those below pH 5 or water activity below 0.86. These enterotoxins are thermostable molecules, inactivated only by prolonged boiling with the amount of inactivation depending not only on temperature but also on composition and pH of the heating medium and purity of the toxin preparations.

The enterotoxins may be considered as "superantigens" since they stimulate production of T-cells and induction of cytokines such as interleukin-1, interferon-γ and tumour necrosis factor (Fast *et al.*, 1989; Parsonnet *et al.*, 1986). Their ability to cause illness may be due to interaction with macrophages causing production of the cytokines mentioned above (Betley *et al.*, 1990).

Of the seven enterotoxins, enterotoxin A (Sea) is encoded by a family of temperate phages (Betley and Mekalanos, 1985) and enterotoxin D (Sed) is encoded within a 27.6 kb plasmid (Bayles and Iandolo, 1989). Enterotoxin B (Seb) and the enterotoxin C's are probably chromosomally encoded (Betley *et al.*, 1990).

1.4.2 Haemolysins

There are four haemolysins of *S. aureus*, α, β, γ, and δ, which are all antigenically distinct.

1.4.2.1 α–haemolysin

α–haemolysin is the 30 kDa protein product of the *hla* gene which lies in *Sma*I fragment A of the *S. aureus* chromosome (Pattee *et al.*, 1990). It binds firmly to target membranes and causes membrane damage resulting in the formation of pores (Bhakdi and Tranum-Jensen, 1991). It shows great variation in its ability to lyse erythrocytes of different animal species. For example, a 400-fold increase in concentration of α–haemolysin is required to lyse human compared to rabbit erythrocytes against which its activity is 100- to 1000-fold greater than in any other species (Bhakdi and Tranum-Jensen, 1991; Möllby, 1983). In fact, clinical isolates from serious illnesses of humans, such as TSS (Clyne *et al.*, 1988) and septicaemia (Christensson and Hedstrom, 1986), have been found not to produce α–toxin. However, this toxin is still considered an important virulence factor in *S. aureus* since human platelets are a highly sensitive target and may be the primary target *in vivo* (Bhakdi *et al.*, 1988). α–toxin also has a powerful action on vascular smooth muscle.

1.4.2.2 β–haemolysin

β–haemolysin is a phospholipase C and is specific for sphingomyelin in cell membranes. Degradation of sphingomyelin in erythrocyte membranes occurs at 37 °C but does not cause lysis of the cell until cooling to 4 °C (Möllby, 1983). It is thus the amount of sphingomyelin in erythrocyte membranes which dictates the sensitivity of erythrocytes from different mammalian species to β–haemolysin. Sheep erythrocytes are very sensitive since 50 % of their erythrocyte membrane

phospholipids consist of sphingomyelin (Rouser *et al.*, 1968). Sheep blood is therefore used to assay the enzyme. This haemolysin needs Mg^{2+} for its activity and is inhibited by chelating agents (Möllby, 1983). β–toxin is encoded by the *hlb* gene which is located in the *Sma*I fragment F of the *S. aureus* chromosome (Pattee *et al.*, 1990).

1.4.2.3 γ-haemolysin

This is the least understood *S. aureus* haemolysin, most probably because it is inhibited by agar (Jackson, 1963) and hence its presence is missed when strains are assayed for haemolysis on blood agar plates. It is also inhibited by lipids (Möllby, 1983). γ-haemolysin is encoded by the *hlg* gene which has been mapped to fragment C of *Sma*I digested chromosomal DNA (Pattee *et al.*, 1990). Although Möllby and Wadstrom (1971) suggested it consisted of a single polypeptide, it has subsequently been reported to consist of two proteins that are expressed from closely linked genes (Taylor and Bernheimer, 1974; Cooney *et al.*, 1988; Clyne *et al.*, 1992). Each component is weakly haemolytic but together they act synergistically against rabbit, sheep and human erythrocytes, although its activity is 4-8 times greater against rabbit cells compared to sheep or human (Möllby, 1983). It has also been reported that one of the components of γ-haemolysin is a protease which acts on the other peptide to give enhanced haemolysis (Clyne *et al.*, 1992).

1.4.2.4 δ-haemolysin

This haemolysin is encoded by the *hld* gene which comprises part of the accessory gene regulator (*agr*) locus which lies in fragment F of *Sma*I digested *S. aureus* chromosomal DNA (Pattee *et al.*, 1990). It comprises a 26-residue peptide that acts, possibly as a surfactant, to affect several cell types, including erythrocytes and

leukocytes, by forming pores in phospholipid bilayers (Mellor *et al.*, 1988). This haemolysin is not species specific.

1.4.3 Fibronectin binding protein (FnBP)

There are two forms of fibronectin, a soluble form secreted into the bloodstream and a cross-linked form which acts as an adhesive protein made by many cell types including fibroblasts and epithelial cells (Ruoslahti, 1988). *S. aureus*, together with many other pathogenic micro-organisms binds fibronectin by a fibronectin binding protein (FnBP) on the surface of the cell wall (Höök *et al.*, 1989). In addition, *S. aureus* produces a free extracellular FnBP (Lindberg *et al.*, 1990). The relative levels of cell wall-associated and free extracellular FnBP is most likely affected by calcium concentration since addition of EGTA (6.25 mM) to a growth medium results in a 6-fold increase in cell wall-associated FnBP and a >40-fold decrease in free extracellular FnBP (Lindberg *et al.*, 1990). It has been postulated that the ability of *S. aureus* to bind to fibronectin represents a mechanism of host-tissue adhesion, i.e. a substrate for bacterial attachment and colonisation (Lindberg *et al.*, 1990). It has been proposed that plasma-fibronectin could serve as an opsonin such that after coating of bacterial cells it mediates recognition and uptake of bacteria by phagocytic cells. It is thus possible that the extracellular free FnBP produced by *S. aureus* could serve to reduce the binding of plasma fibronectin to the bacterial cell itself. (Lindberg *et al.*, 1990).

1.4.4 Coagulase

The coagulase protein is encoded by the *coa* gene, which is located in *Sma*I fragment E, and is considered an important virulence factor of *S. aureus* (Pattee *et al.*, 1990). It is an extracellular protein that coagulates plasma. To assay for coagulase activity *in vitro*, it is necessary to use a plasma that contains a sufficient level of coagulase-

reactive factor and fibrinogen as a substrate (Jeljaszewicz *et al.*, 1983). For *in vitro* assays, the plasma should also contain oxalic acid or citrate to prevent the physiological clotting of fibrinogen (Jeljaszewicz *et al.*, 1983). The coagulase reactive factor of sera reacts with coagulase to generate both esterase and clotting activities in a manner similar to the activation of prothrombin to thrombin. The coagulase actually binds prothrombin to form staphylothrombin (Hemker *et al.*, 1975). Staphylothrombin stimulates the clotting reaction in plasma. Coagulase may deposit fibrin on the surface of staphylococci, possibly altering their ingestion by phagocytic cells, or their destruction within such cells.

1.4.5 Protein A

Between 93 and 99 % of *S. aureus* strains of human origin have been found to contain Protein A as a cell wall protein (Forsgren, 1970). Protein A is the product of the *spa* gene which lies in fragment G of *Sma*I digested *S. aureus* chromosomal DNA (Pattee *et al.*, 1990). It is a 42 kDa protein which, at its COOH-terminal end, is anchored in the cell membrane with its NH_2-terminal immunoglobulin-binding domain extending outside the cell wall (Schneewind *et al.*, 1995). This ability to anchor to the cell wall depends on 35 amino acid residues at the COOH-terminus of protein A, partly comprising an LPXTG motif (Schneewind *et al.*, 1992; Schneewind *et al.*, 1995). *In vivo*, protein A produces several immunological responses, in particular, allergic reactions including anaphylaxis and histamine release (Forsgren *et al.*, 1983). It binds to several immunoglobulins and, through its interaction with immunoglobulin G (IgG) in particular, causes platelet injury (Forsgren *et al.*, 1983; Hawiger *et al.*, 1979). Phagocytosis is inhibited by the presence of protein A and it initiates blood coagulation.

1.4.6 Hydrolases

1.4.6.1 Lipase

Lipases catalyse the hydrolysis or formation of ester bonds of water insoluble substrates, particularly lipids (Götz, 1990). Many strains of staphylococci, including *S. aureus*, produce a true extracellular lipase (or glycerol ester hydrolase) which is active against oils and fats in their emulsified states (Lee and Iandolo, 1986; Shah and Wilson, 1965). Within staphylococcal abscesses there are at least two host-derived bactericidal lipids which aid in bacterial clearance (Engler and Kapral, 1992; Shryock *et al.*, 1992) and it is thought that the staphylococcal lipase inactivates these bactericidal lipids to enhance the survival of staphylococci within abscesses (Kapral *et al.*, 1992). The gene (*geh*) encoding this 76 kDa lipase is 2070 bp and has been mapped to fragment E of *Sma*I digested chromosomal DNA of *S. aureus* strain 8325 (Lee and Iandolo, 1986: Pattee *et al.*, 1990). The activity of the *geh* gene has been shown to be negatively regulated by bacteriophage lysogenization (Rosendal and Bulow, 1965). Bacteriophage L54a interrupts the lipase gene between nucleotides 2608 and 2698 (Lee and Iandolo, 1986).

Ideally lipase activity should be measured using the long chain fatty acid triacylglycerol, triolein. Tributyrin or Tweens have been used as substrates for lipase activity although these are not specific since esterases can also cause their degradation (Farrell *et al.*, 1990; Tyski *et al.*, 1983). Agar containing egg yolk has also been used to assay for lipase activity (Arvidson, 1983) although it has been reported that extracellular enzymes from *S. aureus* produce two types of zones on egg yolk agar, one on the surface, and one sub-surface (Owens, 1974). It has been suggested that, whilst the granular surface zone may be attributed to the action of the staphylococcal lipase, the sub-surface zone of opacity is the result of the hydrolysis of

phosphatidylcholine (Owens, 1974). Thus, it may be best to avoid the use of egg yolk as a definitive, or quantitative, test for lipase activity.

1.4.6.2 Proteases

Most strains of *S. aureus* are proteolytic (Baird-Parker, 1965) producing at least three different types of protease including serine protease, metallo-protease and thiol-protease (Arvidson, 1983). Serine protease (protease I) is produced by at least 67 % of *S. aureus* strains, mainly during post-exponential growth (Björklind and Arvidson, 1977; Björklind and Arvidson, 1978). It is produced as an inactive precursor which is activated by the metallo-protease (Drapeau, 1978). The serine protease specifically cleaves the peptide bonds on the carboxyl terminal side of aspartic or glutamic acid residues (Drapeau *et al.*, 1972). The extracellular metallo-protease of *S. aureus* was identified by Arvidson *et al.* (1972) and has been shown to specifically cleave peptide bonds involving the amino terminal side of hydrophobic residues (Björklind and Jornvall, 1974). Arvidson *et al.* (1973) also demonstrated the existence of the thiol-protease. This protease is only active in the presence of reducing agents and has only been identified in one strain of *S. aureus*. The enzyme is very unstable at 37 °C, losing 50 % of its activity within 3 hours (Arvidson, 1983). All three proteases can be assayed on casein agar plates where each gives a different type of zone of clearing (Arvidson, 1973).

1.4.6.3 Nuclease

In 1956, Cunningham *et al.* identified a very thermostable extracellular DNase produced by *S. aureus* which was shown to require Ca^{2+} for its activity. It has both endonuclease and exonuclease activities on DNA and RNA molecules (Novick, 1993). The gene (*nuc*) encoding this 149 amino acid staphylococcal nuclease, known as nuclease A, has been isolated and sequenced (Shortle, 1983). It was found that

immediately preceding the 149 codons specifying nuclease A, there were nineteen codons encoding the amino terminal end of nuclease B, which is the membrane-bound precursor of nuclease A, found in exponentially growing cells (Davis, *et al.*, 1977; Shortle, 1983).

1.4.6.4 Staphylokinase

Staphylokinase dissolves blood clots by activating the conversion of the proenzyme, plasminogen to the fibrinolytic enzyme, plasmin by cleavage of a single arginine-valine bond (Arvidson, 1983). The gene encoding this enzyme can be located in the bacterial genome or may be carried by at least 15 lysogenic phages (Arvidson, 1983; Winkler *et al.*, 1965).

1.4.7 β–lactamase

Extracellular β–lactamase is responsible for the resistance of *S. aureus* to penicillins and cephalosporins (Barber, 1962). β–lactamases hydrolyse the C-N bond in the β–lactam ring of both these antibiotics. This enzyme is encoded by the *bla* gene which has been mapped to fragment F of *Sma*I digested chromosomal DNA of *S. aureus* (Pattee *et al.*, 1990).

1.4.8 Hyaluronate lyase

Hyaluronic acid is a major glycosaminoglycan, that is the polysaccharide chain in proteoglycans. Proteoglycans consist of units made up of polysaccharides (approximately 95 %) and proteins (approximately 5 %) and bind water and cations thus forming the extracellular medium, or ground substance, of connective tissue (Stryer, 1973). Hyaluronate lyase appears to be produced by 95-100 % of *S. aureus* strains (Arvidson, 1983). The hyaluronate lyase gene (*hysA*) of *S. aureus* 8325-4

shows 35 % amino acid sequence homology with group B streptococcal hyaluronate lyase (Farrell *et al.*, 1995). From work on the group B streptococcal hyaluronate lyase it has been suggested that its role as a virulence factor may lie in its ability to facilitate the invasiveness of the producing organism and to subvert some normal host defence mechanisms (Lin *et al.*, 1994).

1.5 Genetic control of production of virulence determinants

Bacterial survival is competitive, uncertain and, to a large extent, depends on a cell's ability to adapt quickly to environmental changes. For example, the supply of nutrients is often scarce and cells need to be able to respond quickly to take advantage of the appearance of novel nutrients and metabolites by producing transport and metabolic machinery to utilise them. In pathogenic bacteria, including *S. aureus*, several conditions characteristic of a host environment, such as temperature and concentration of various chemicals including iron, calcium etc., will induce the production of toxins and other virulence factors (Bliska *et al.*, 1993) (see section 1.7). Thus, a co-ordinated signalling system is necessary such that environmental stimuli can be sensed by the bacteria and then translated into appropriate gene expression. There are many different modes of genetic regulation involving both repressors and transcriptional activators. A common mechanism of transduction of environmental stimuli to bring about changes in gene expression has been identified in many bacteria and eukaryotes (Kavanaugh and Williams, 1996; Parkinson and Kofoid, 1992). These so-called, two component sensor regulator systems have been found to mediate an adaptive response to many stimuli, including metabolite utilization such as carbon by *Escherichia coli* and *Salmonella typhimurium*, (Island *et al.*, 1992) phosphate by *E. coli* and *Klebsiella pneumoniae* (Lee *et al.*, 1989) and nitrogen by *E. coli* (Miranda-Rios *et al.*, 1987). Other adaptive systems include stress from antibiotics, heavy metals and osmolarity by *E. coli* for example (Comeau *et al.*, 1985). Bacterial interaction with its host leading to production of virulence factors

can also be mediated by two component sensor regulator systems (Parkinson and Kofoid, 1992).

1.5.1 Two component sensor regulator systems

These systems are generally characterised by two protein components, a sensor protein often located in the cytoplasmic membrane and a response regulator protein which is usually located in the cytoplasm (Parkinson, 1993). The sensor monitors environmental stimuli and causes the response regulator to mediate a response, generally causing changes in gene expression (Parkinson, 1993). Sensor proteins commonly contain 5-8 transmembrane segments in their N-terminal domain and these segments are connected to a C-terminal domain that functions as a signal transmitter (Kleerebezem *et al.*, 1997) (Fig. 1.1). The signal sensed by the N-terminal input domain of the sensor protein will cause the C-terminal transmitter domain to transduce the signal to the response regulator. The response regulator detects the incoming signal from the sensor, also at its N-terminal domain, and alters the activity of its C-terminal domain to trigger a response (Parkinson, 1993) (Fig. 1.1). Of the many two component sensor regulators so far characterized, the mechanism of communication has always been found to involve phosphorylation and dephosphorylation reactions (Parkinson and Kofoid, 1992). The sensor transmitter has an autokinase activity and can thus attach phosphoryl groups from ATP to a histidine residue which is located near the N-terminus of the transmitter domain. This phosphohistidine acts as a high energy intermediate and can transfer the phosphoryl group to an aspartate residue in the receiver domain of the response regulator (Sanders *et al.*, 1992) (Fig. 1.1). The receiver catalyses hydrolytic loss of its phosphoryl group, this process occurring from within a few seconds to many minutes (Parkinson, 1993). The output domain of the response regulator, in most cases, has a DNA binding or other regulatory function that provides transcriptional control of one

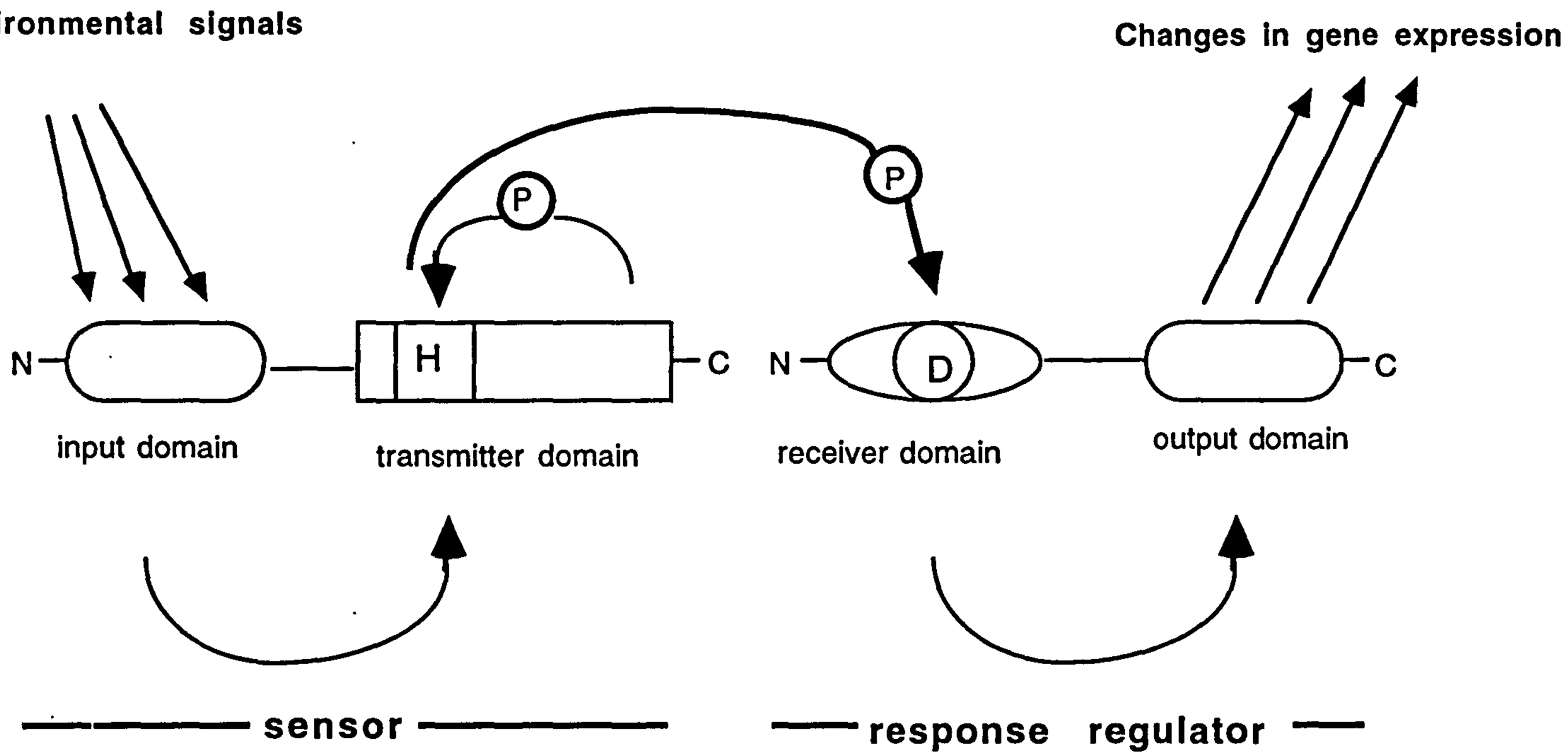


FIGURE 1.1

Model of a two component sensor regulator system (modified from Parkinson and Kofoid, 1992). An environmentally generated signal flows from the input domain to the transmitter domain within the sensor, and from the receiver domain to the output domain within the response regulator, by phosphorylation reactions (arrows labelled P) involving histidine (H) and aspartate (D) residues.

or more target genes. NMR studies have shown that phosphorylation of the receiver causes substantial changes in conformation but it is not yet known how this actually effects the output mechanism of the response regulator (Parkinson, 1993).

1.5.2 The role of two component sensor regulator systems in virulence

Two component sensor regulator systems have been found to have a role in pathogenesis in a number of species. The *bvg* (*Bordetella* virulence gene) regulon of *Bordetella pertussis* and the *pho* regulons of *E. coli* and *S. typhimurium* are examples. The *bvg* locus of *B. pertussis* contains two genes, *bvgS* and *bvgA* which encode the sensor and response regulator of a two component signal transduction system, respectively. The *bvg* regulon co-ordinately regulates expression of several toxins, including pertussis toxin, in response to environmental stimuli such as temperature and the presence of Mg^{2+} (Arico *et al*, 1991) (see section 1.7). The *pho* regulon of *E. coli* and *S. typhimurium* is controlled by the *phoP* and *phoQ* genes, encoding a sensor and regulator protein respectively (Parkinson and Kofoid 1992). In Salmonellae, the *phoP* product regulates the expression of genes encoding factors that protect against the bactericidal activity of macrophage proteins (Miller *et al.*, 1989b). The *phoP* gene is required for the expression of three, and perhaps more, unlinked loci, at least one of which, *pagC* is likely to encode a virulence factor required for intracellular survival in macrophages (Miller *et al.*, 1989b).

1.5.3 Quorum sensing systems

Another mode of global regulation of gene expression has been described in many species. This involves a process of intercellular communication which enables cells to sense population levels. This signalling mechanism uses diffusable signalling molecules (pheromones) to regulate physiological processes in a cell density

(quorum) or growth phase dependent manner (Kleerebezem *et al.*, 1997). Pheromones are substances which are secreted by a cell and received by a second cell in which they cause a specific action (Karlson and Lüscher, 1959). The first identified and best understood quorum sensing system is the bioluminescent (*lux*) phenotype of *Vibrio fischeri*. Here, synthesis of the signalling molecule necessary for induction of the luminescence system, L-homoserine lactone, is mediated by the *luxI* gene (Engebrecht and Silverman, 1984). The protein product of the *luxR* gene binds the L-homoserine lactone and becomes the transcriptional activator of the bioluminescence genes (Engebrecht and Silverman, 1986). Cultures of *V. fischeri* express *lux* genes only weakly during early growth phase with expression being strongly activated in late exponential growth, i.e. in a density dependent manner (Eberhard, 1972).

The phenomenon of quorum sensing has also been identified in several Gram positive bacteria but has not been found to involve an N-acyl homoserine lactone-like signalling molecule (Kleerebezem *et al.*, 1997). Examples of quorum sensing systems in Gram positive bacteria include the competence inducing systems of *Bacillus subtilis* and *Streptococcus pneumoniae*, the virulence response of *S. aureus* and the production of antimicrobial peptides by several different species (Håvarstein *et al.*, 1995; Ji *et al.*, 1995; Magnuson *et al.*, 1994). In Gram positive bacteria, the signalling molecule seems most often to be a post-translationally processed peptide. This peptide pheromone is commonly recognised by the sensor component of a two component signal transduction system. Further, in Gram positive bacteria, the genes encoding the signalling molecule precursor and its processor protein, together with the genes encoding the proteins involved in the two component sensor regulator, are often transcriptionally linked (Kleerebezem *et al.*, 1997). Thus the increase in expression of such a locus is auto-regulatory.

Quorum sensing in *B. subtilis* is utilised in developing genetic competence, whereby cells can take up exogenous DNA, in a cell density dependent manner (Magnuson *et al.*, 1994). Four genes are involved in this regulatory process, *comQ, X, A* and *P*. The *comQ* gene post-translationally modifies the product of the *comX* gene to a 10 amino acid peptide. The cellular response to this peptide is mediated by a two component sensor regulator with *comP* encoding the sensor and the *comA* gene encoding the response regulator (Magnuson *et al.*, 1994).

The signalling pheromone inducing competence in *S. pneumoniae* cells is a 17 amino acid peptide which is processed by the *comB* gene product from a 41 amino acid proform encoded by the *comC* gene (Håvarstein *et al.*, 1995; Zhou *et al.*, 1995). This peptide signal is transduced by the gene products of the *comD* and *E* genes comprising the sensor and regulator of a classical two component sensor regulator system (Håvarstein *et al.*, 1996).

The quorum sensing system of *S. aureus* is discussed in detail in section 1.6.

1.6 Control of virulence determinant production in *S. aureus*

S. aureus has been found to control production of a whole range of virulence determinants by means of several regulatory loci which act either independently or co-ordinately. These global regulators include the accessory gene regulator (*agr*) (Recsei *et al.*, 1986; Morfeldt *et al.*, 1988), the staphylococcal accessory regulator (*sar*) (Cheung *et al.*, 1992), extracellular protein regulator (*xpr*) (Smeltzer *et al.*, 1992; Smeltzer *et al.*, 1993) and *sae* (*S. aureus* exoprotein expression) (Giraudo *et al.*, 1994b). The *S. aureus* virulence response is regulated, at least partly, by a density dependent signalling system that is analogous to the quorum sensing systems of other Gram positive and Gram negative bacteria (see section 1.5.2). In particular, *agr* expression is growth phase dependent occurring at mid to late exponential phase.

Accordingly, those genes which are positively controlled by *agr* are up-regulated at this time and those genes which are negatively regulated by *agr* are down-regulated at mid-late exponential phase (see section 1.6.1.1). The expression of individual transcripts of the *sar* locus is also dependent on growth phase (Bayer *et al.*, 1996). For example, expression of *sarB* occurs during early-mid log phase whilst *sarC* transcription is most active during stationary phase. There is also evidence for co-ordinate transcriptional control of some virulence factors such as protein A and α–haemolysin (Hla) synthesis (Janzon *et al.*, 1986; Vandenesch *et al.*, 1991). Production of α-haemolysin is controlled temporally as well as by *agr* (Vandenesch *et al.*, 1991) (see section 3.1). It has also been demonstrated that environmental conditions, such as pH, temperature, availability of divalent cations and O_2, together with glucose and NaCl concentration, affect the production of virulence determinants under the control of at least one global regulator, *agr*, and also production of α–haemolysin both via and independently of this global regulator (Regassa and Betley, 1993; Regassa *et al.*, 1992; Sheehan *et al., 1992*; Vandenesch *et al.*, 1991).

1.6.1 Regulators of virulence determinant production

1.6.1.1 *agr* (accessory gene regulator)

The accessory gene regulator was the first discovered and is the best understood of all the known global regulators of virulence determinant production in *S. aureus*. It has been shown to up-regulate production of, amongst others, the haemolysins, enterotoxin B, TSST-1, epidermolytic toxin A, staphylokinase, serine protease and acid phosphatase. It also down-regulates coagulase and protein A production. Regulation by *agr* occurs in a growth phase dependent manner, during late exponential-early stationary phase (Björklind and Arvidson, 1980; Recsei *et al.*, 1986). The *agr* locus was identified at almost the same time in two separate

laboratories, although several years were to elapse before either group of workers were to realise that they had identified a global regulatory system.

1.6.1.1.1 The discovery of *agr*

In 1982, Mallonee *et al.* isolated a Tn*551* insertion mutant in what was thought to be the α–haemolysin (*hla*) gene. Transformation of this mutation into an isolate that produced α-, β– and δ-haemolysin, together with a range of other extracellular proteins, showed that the mutation caused loss not only of α–haemolysin but also β– and δ-haemolysin, a 100-fold drop in TSST-1 and a 50-fold drop in staphylokinase (Kreiswirth *et al.*, 1983; Recsei *et al.*, 1986). In contrast, synthesis of the extracellular protein, surface protein A, was elevated 20-fold (Recsei *et al.*, 1986).

It was established by probing wild type and mutant chromosomal DNA with probes originating from both the α–haemolysin and TSST-1 structural genes, that Tn*551* had not inserted into either of these structural genes. These data indicated that the transposon had inactivated a trans-active positive control element. This locus was subsequently designated *agr* for accessory gene regulator (Recsei *et al.*, 1986).

At the same time, a class of pleiotropic mutants were isolated, designated *exp*, which also showed decreased production of α–toxin, serine protease, staphylokinase, nuclease and acid phosphatase and a simultaneous increase in the production of coagulase and protein A (Björklind and Arvidson, 1980). Subsequent analysis of specific exoprotein mRNA in one of these *exp* mutants showed that the regulation of exoprotein gene expression exerted by *exp* was at the level of transcription (Janzon *et al.*, 1986).

It was noted by Morfeldt *et al.* (1988) that the *exp* mutant showed an almost identical phenotype to the *agr* mutant described by Recsei *et al.* (1986). In order to facilitate

further study of this regulatory mechanism and to enable cloning of this element they used Tn*551* to create mutants which were phenotypically identical to the *exp* mutants (Morfeldt *et al.*, 1988). The mutation in one of the clones with an *exp* phenotype (WA205) was phage transduced into strain 8325-4 using phage 80α. More than 90 % of the transductants had the *exp* phenotype, indicating that the Tn*551* insertion was closely linked to, or caused the *exp* phenotype.

A transductant (WA250) was used for Northern blot analysis to demonstrate that transcription of protein A and α–haemolysin was altered by the Tn*551* insertion. Following cloning of the *exp* locus and further Northern blot analysis it was shown that *exp* coded for an RNA transcript of approximately 3.5 kb. It was concluded that *exp* and *agr* are identical since both *exp* and *agr* mutants have a reduced level of this 3.5 kb RNA compared to wild type strains (Morfeldt *et al.*, 1988).

1.6.1.1.2 The *agr* locus

The *agr* locus was first cloned and partially sequenced by Peng *et al.* (1988). A 6.1 kb chromosomal fragment which was cloned was found to contain a 241 codon open reading frame (ORF) which was designated *agrA*. Gene fusion analysis using a promoterless β–lactamase gene showed that there was a weak, but constitutive promoter 5' to the *agrA* gene (Peng *et al.*, 1988). The remainder of the *agr* locus was subsequently sequenced and found to contain six more ORFs, three of which were transcribed in the same direction as *agrA* and three transcribed in the opposite direction (Kornblum *et al.*, 1990; Arvidson *et al.*, 1990) (Fig. 1.2). The ORF upstream of *agrA*, together with the *agrA* gene, were found to be related to a gene encoding a sensor and response regulator of a two component signal transduction system (Kornblum *et al.*, 1990). In addition, *agr* was found to contain two other promoters, P2 and P3 (Morfeldt *et al.*, 1988). P2 initiates a 3 kb transcript, designated RNAII, starting at position 1750 (Fig 1.2) and P3, transcribed in the

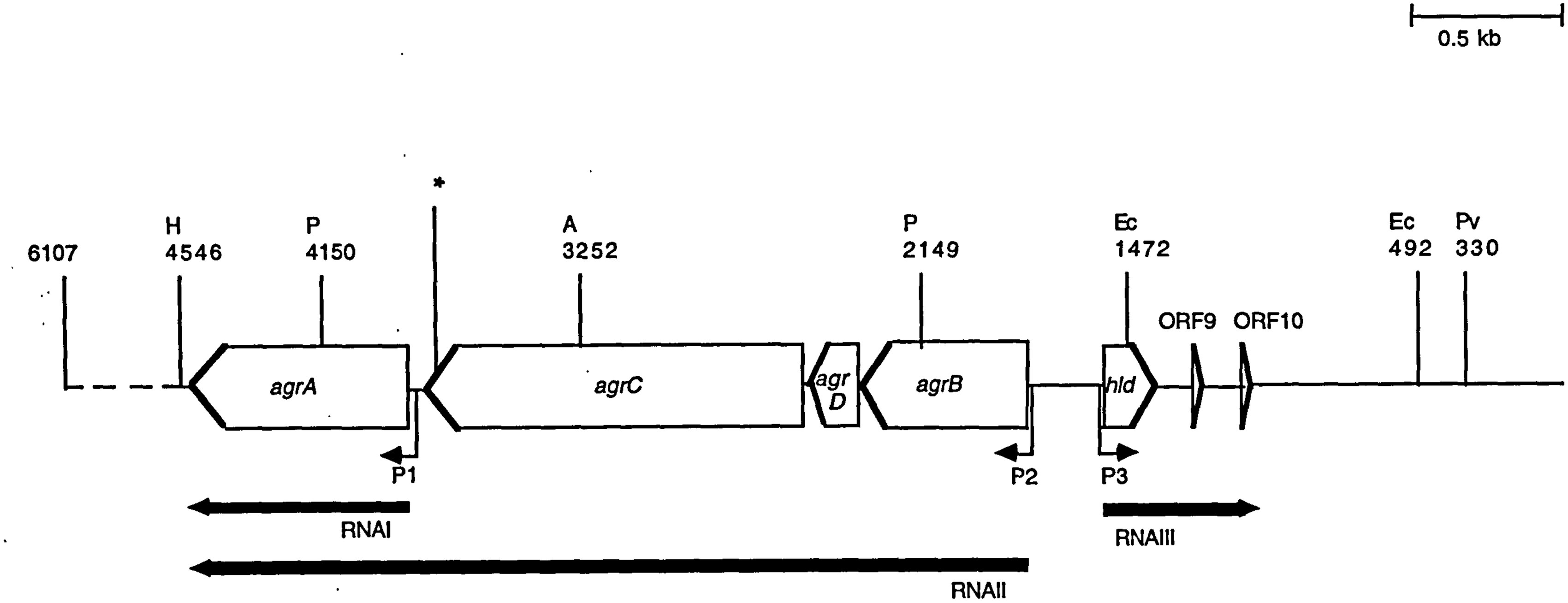


FIGURE 1.2

Physical map and transcription of the *agr* locus (modified from Kornblum *et al.*, 1990). The positions of the three *agr* promoters, P1, P2 and P3 are shown, together with the approximate extent of their transcripts. The *agrC* and *agrA* genes encode a sensor and response regulator, respectively, of a two component signal transduction system. The *agrD* gene product is modified, probably by the product of the *agrB* gene, to give an octapeptide known as RAP (RNAIII activating protein). The *hld* gene encodes δ-haemolysin. The symbol, * marks the approximate insertion site of Tn*551* within strain WA250 (Morfeldt *et al.*, 1988). The restriction sites denoted are: A, *Asp*700; Ec, *Eco*RV; H, *Hinc*II; P, *Pst*I; Pv, *Pvu*II. (The scale is denoted on the top, right).

opposite direction to P2, initiates a transcript of 0.5 kb, designated RNAIII, starting at position 1566 (Fig. 1.2). The 0.5 kb RNAIII transcript is an abundant, long-lived mRNA with a half life of 15 minutes (Arvidson *et al.*, 1989). It was found to contain an ORF of 160 nucleotides encoding a 26 amino acid residue protein identical to the sequence of the *S. aureus* δ-haemolysin (Janzon *et al.*, 1989; Fitton *et al.*, 1980). Approximately 110 nucleotides downstream of the δ-haemolysin (*hld*) gene is a second ORF with a poor ribosome binding site which could ostensibly encode a 21 amino acid peptide. There is also a third ORF contained within the RNAIII transcript comprising 19 codons. However, this latter ORF does not appear to have a ribosome binding site. Thus, it appears that the 3' end of the transcript is untranslated although it has been reported that an insertion in the 3' end of RNAIII reduced *agr* activity but did not affect P2 or P3 transcription (Janzon and Arvidson, 1990). The role of RNAIII as the molecule regulating exoprotein gene expression was largely confirmed by the finding that an insertion in the RNAIII 3' region did not impair δ-haemolysin production but resulted in a typical *agr* phenotype (Arvidson *et al.*, 1989). In addition, it has been shown that a cloned RNAIII defective in δ-haemolysin production can restore the positive and negative effects on virulence gene expression in both an RNAIII deletion mutant and an *agr* null strain (Janzon and Arvidson, 1990; Novick *et al.*, 1993). From these experiments it was not possible to determine whether RNAIII was acting at a transcriptional or post-transcriptional level. This was addressed by transcriptionally fusing promoters from the α–haemolysin (*hla*) and protein A (*spa*) genes to the staphylococcal plasmid pI258 β–lactamase gene (*bla*) and examining expression from these promoters in agr^+ and agr^- strains (Novick *et al.*, 1993). Expression from the *hla* and *spa* promoter in each strain was thus reflected in the levels of β–lactamase activity which, in each case, were found to correlate with the respective levels of the native proteins. Thus, *hla::bla* was expressed during post-exponential growth phase in the agr^+ strain but not in the agr^- strain and the converse for the *spa::bla* fusion. Similar fusions using the *tst* (TSST-1) or *hlb* (β–haemolysin) promoters gave similar results to the *hla::bla* fusion (Novick *et al.*,

1993). It has also been demonstrated that RNAIII affects translation of, at least, α–haemolysin since *hla* mRNA is not translated in the absence of the 5' region of RNAIII (Novick *et al.*, 1993). The mechanism by which RNAIII regulates translation of α–haemolysin has since been deduced. It has been shown that RNAIII binds to the *hla* transcript in a way that exposes its ribosome binding site, thus allowing translation (Morfeldt *et al.*, 1995). This prevents a stem-loop structure being formed within *hla* mRNA which blocks its ribosome binding site in the absence of RNAIII (Morfeldt *et al.*, 1995) (Fig. 1.3). These results confirm that RNAIII has two functions, one as a transcriptional activator and another as an activator of translation (Morfeldt *et al.*, 1995). This was probably the first example of an "antisense RNA" stimulating translation of target mRNA (Morfeldt *et al.*, 1995).

It is now known that the four genes encompassed in the RNAII transcript comprise a quorum sensing signal transduction system (Balaban and Novick, 1995; Ji *et al.*, 1995). This is mediated via an extracellular signalling peptide and two component sensor regulator system which are transcriptionally linked and encoded by the *agrBDCA* operon (Fig. 1.2).

1.6.1.1.2.1 The *agr* two component sensor regulator system

agrC and *agrA* (Fig. 1.2) are homologous to genes encoding the signal sensor and response regulator of a classical two component signal transduction system, respectively (see section 1.5.1). AgrC resembles a histidine phosphokinase signal transducer and acts as a peptide pheromone sensor. Via phosphorylation of AgrA, it stimulates RNAIII production. It has not yet been demonstrated that AgrA binds directly to DNA and it has been suggested that it might rather influence binding of another regulatory component, SarA, to the P2 and P3 promoters of *agr* (Morfeldt *et al.*, 1996a/b) (see section 1.7.2.2) (Fig. 1.4).

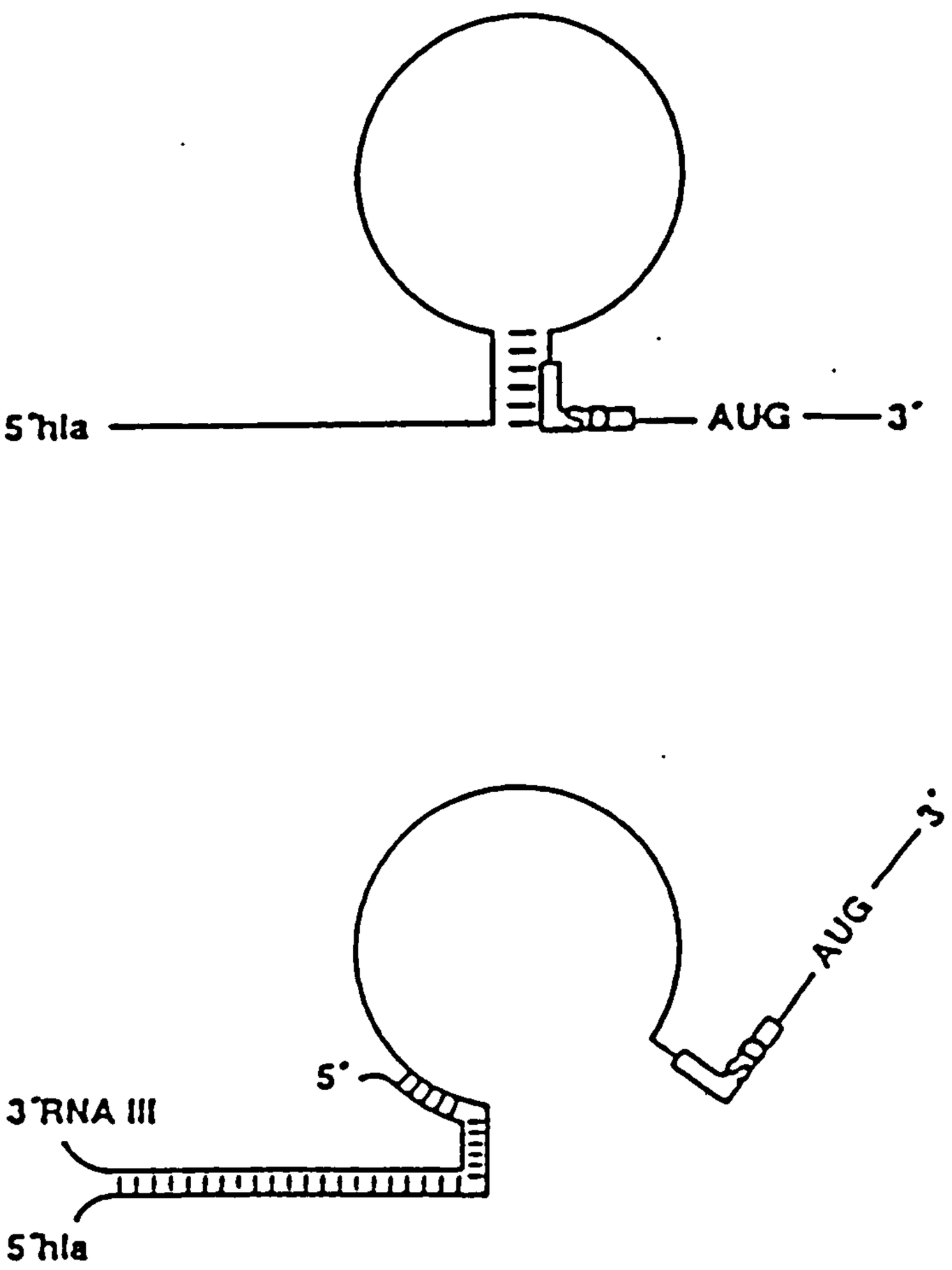


FIGURE 1.3

Model for the translational control of α-haemolysin. The figure shows how the intramolecular base pairing in *hla* mRNA blocks the SD (ribosome binding site), and thereby translation, and how this is released by the interaction with RNAIII (from Morfeldt *et al.*, 1995).

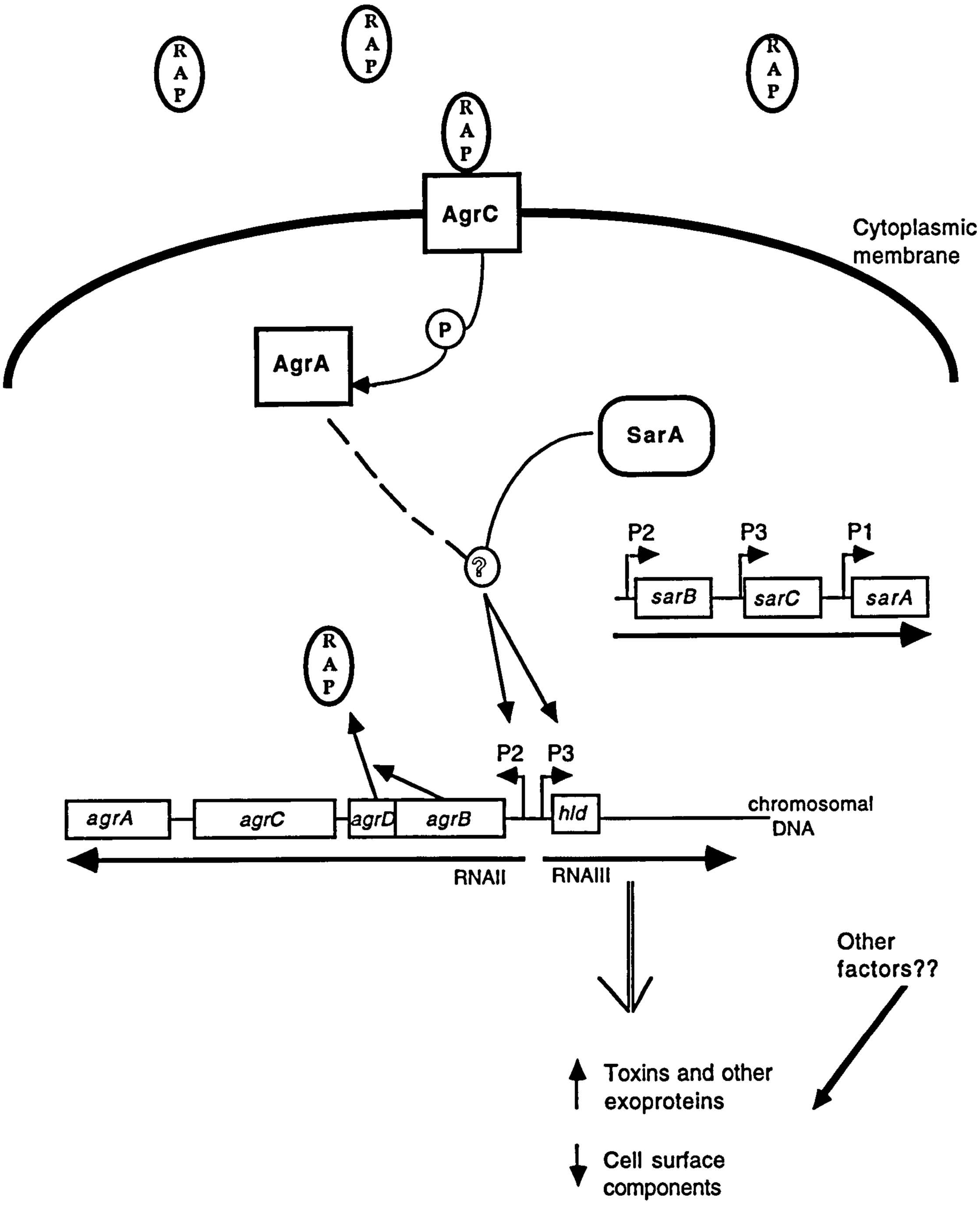


FIGURE 1.4

Model of interaction between the *agr* and *sar* gene products (depicted in bold type) which mediate changes in expression from the P2 and P3 promoters of *agr*. AgrC and AgrA represent the sensor and response regulator, respectively, of a two component signal transduction system. RAP (RNAIII activating protein) is encoded within the *agrD* gene and is modified from a 46 amino acid pre-peptide to an octapeptide, probably by the product of the *agrB* gene. RAP has been proposed as a signalling pheromone that, probably via AgrC, leads to increased transcription from the P2 and P3 promoters of *agr* in a density dependent manner (see section 1.6.1.2.2). The RNAIII transcript has been shown to up- or down-regulate production of some exoproteins, as indicated. (Not to scale).

1.6.1.1.2.2 The *agr* signalling pheromone

Transcription of the *agr* operon from P2 is auto-regulated in a density dependent manner by the gene product of *agrD* (Ji *et al.*, 1995) (Fig. 1.2). *agrD* encodes a 46 amino acid residue pre-peptide which is subsequently processed, probably by the gene product of *agrB*, to give an octapeptide known as RAP (RNAIII activating protein) (Balaban and Novick, 1995; Ji *et al.*, 1995) (Fig. 1.4). RAP accumulates gradually during growth of a culture (Balaban and Novick, 1995) but the response exhibits a sharp concentration dependent activation threshold (Ji *et al.*, 1995). Addition of exogenous RAP to early exponential phase cultures causes immediate activation of transcription from both the P2 and P3 promoters (Balaban and Novick, 1995; Ji *et al.*, 1995).

Interestingly, it has also been demonstrated that a secreted factor produced by one strain of *S. aureus* is able to inhibit *agr* expression by different strains (Balaban and Novick, 1995; Ji *et al.*, 1997). It was found that *S. aureus* strains could be divided into three groups, with members of one group inhibiting *agr* expression by members of the other groups (Ji *et al.*, 1997). Ji *et al.* (1997) confirmed that RAP was also responsible for this strain specific inhibition as well as activating *agr* in the producing strains. In the different strains it was found that RAP varied in length from 7-9 amino acid residues which were again processed from the *agrD* product (Ji *et al.*, 1997).

1.6.1.2 *sar* (staphylococcal accessory regulator)

Another regulator of exoprotein expression in *S. aureus*, designated *sar*, was identified as a Tn*917* (see section 1.7.2) transposon mutant (Cheung *et al.*, 1992). The strain used for transposon mutagenesis was a wild type strain of *S. aureus* isolated from blood, designated DB. Transposition of Tn*917* into the host, DB,

chromosome was found to be high (5 x 10^{-4}, i.e. one Tet^r transposant out of every 2000 cfu). Mutants were selected for a fibrinogen binding protein negative phenotype. One clone, designated 11D2, exhibiting this characteristic was identified out of 17,000 clones screened following enrichment for this phenotype. The mutant phenotype when transduced back into the parental strain DB led to a total lack of δ-haemolysin and a decrease in levels of fibrinogen and fibronectin binding proteins by 73 and 65 % respectively and extracellular protein A by 87 % compared to the parental strain. There was an increase in serine protease and lipase activity and a 64 % increase in α–haemolysin production (Cheung *et al.*, 1992). Southern blot hybridisation using a probe derived from the *agr* locus, showed that the novel locus is distinct from *agr* and was designated *sar* (staphylococcal accessory regulator) (Cheung *et al*, 1992). Subsequently the *sar*::LTV1 mutation was phage transduced into two other strains, one α–haemolysin negative and one haemolytic lab strain (Cheung and Ying, 1994). Similar levels of DNase and β–lactamase were found in the transductants compared to their parental strains, but both transductants showed decreased levels of α-, β– and δ-haemolysin and fibronectin binding protein (FnBP). Both transductants displayed increased protease levels. Northern blot analysis confirmed that the levels of transcripts for both α– and β–haemolysin were decreased throughout the growth cycle indicating that these haemolysins are regulated by *sar* at the transcriptional level (Cheung and Ying, 1994). The regulation of α– and β–haemolysin by the *sar* locus was found to begin at mid-log phase and continue into post-exponential phase in a similar way to that in which *agr* regulates gene transcription (Cheung and Ying, 1994). By Northern blot analyses it was found that RNAIII transcript levels were significantly reduced or absent in *sar* mutants (Cheung and Projan, 1994). Thus the effect of *sar* on α– and β–haemolysin transcription may be mediated via *agr*. Chromosomal DNA flanking the Tn*917*-LTV1 insertion was cloned and sequenced.

1.6.1.2.1 The *sar* locus

The transposon was found to have inserted into an ORF of 372 bp, designated *sarA* (Cheung and Projan, 1994). The *sar* phenotype (reduction in RNAIII levels) could be partially complemented by the provision of an intact copy of *sarA in trans*. This suggested that the levels of RNAIII were related to a functional *sarA* gene possibly by binding of SarA to the promoter region of RNAIII (Cheung and Projan, 1994). Analysis of the SarA amino acid sequence showed that it shared homology with DNA-binding proteins. In fact, *sarA* shares sequence similarity with the *virF* gene of *Shigella flexneri* (Cheung and Projan, 1994). VirF regulates genes involved in bacterial invasion via the control of *virB*, a second positive regulatory gene (Hale, 1991). Thus Cheung and Projan (1994) hypothesised that the similarity between SarA and VirF suggested that SarA may control the expression of some exoproteins, such as α– and β–haemolysins by positively controlling RNAIII levels. The sequence of the entire *sar* locus was found to be 1.2 kb in size. There are three overlapping *sar* transcripts, designated *sarA, sarC* and *sarB* (Bayer *et al.*, 1996; Heinrichs *et al.*, 1996) (Fig. 1.5). These three transcripts have common 3' ends but originate from three distinct promoters, P1, P2 and P3 respectively (Fig 1.5). Of the three promoters, P1 and P2 sequences are similar to those of σ^{70} dependent promoters whilst the P3 promoter is very similar to a *B. subtilis* σ^{B} dependent promoter (Deora *et al.*, 1997) (see section 1.6.2.3). The *sarA* transcript is 0.58 kb and has a single ORF, *sarA*, with a predicted molecular size of 14.7 kDa. The *sarC* transcript is 0.8 kb and *sarB* is 1.15 kb (Bayer *et al.*, 1996). The *sarB* transcript encompasses the *sarA* gene and two upstream ORFs (ORF3 and ORF4) encoding peptides of 39 and 18 amino acids, respectively. All three transcripts are under temporal, growth phase dependent regulation with *sarA* and *sarB* expression at their highest during early exponential phase and *sarC* expression being highest at the end of stationary phase (Bayer *et al.*, 1996). It was suggested that maximum *agr* expression was dependent on an intact *sarB* transcript (Heinrichs *et al.*, 1996) and, in fact, a single copy of the

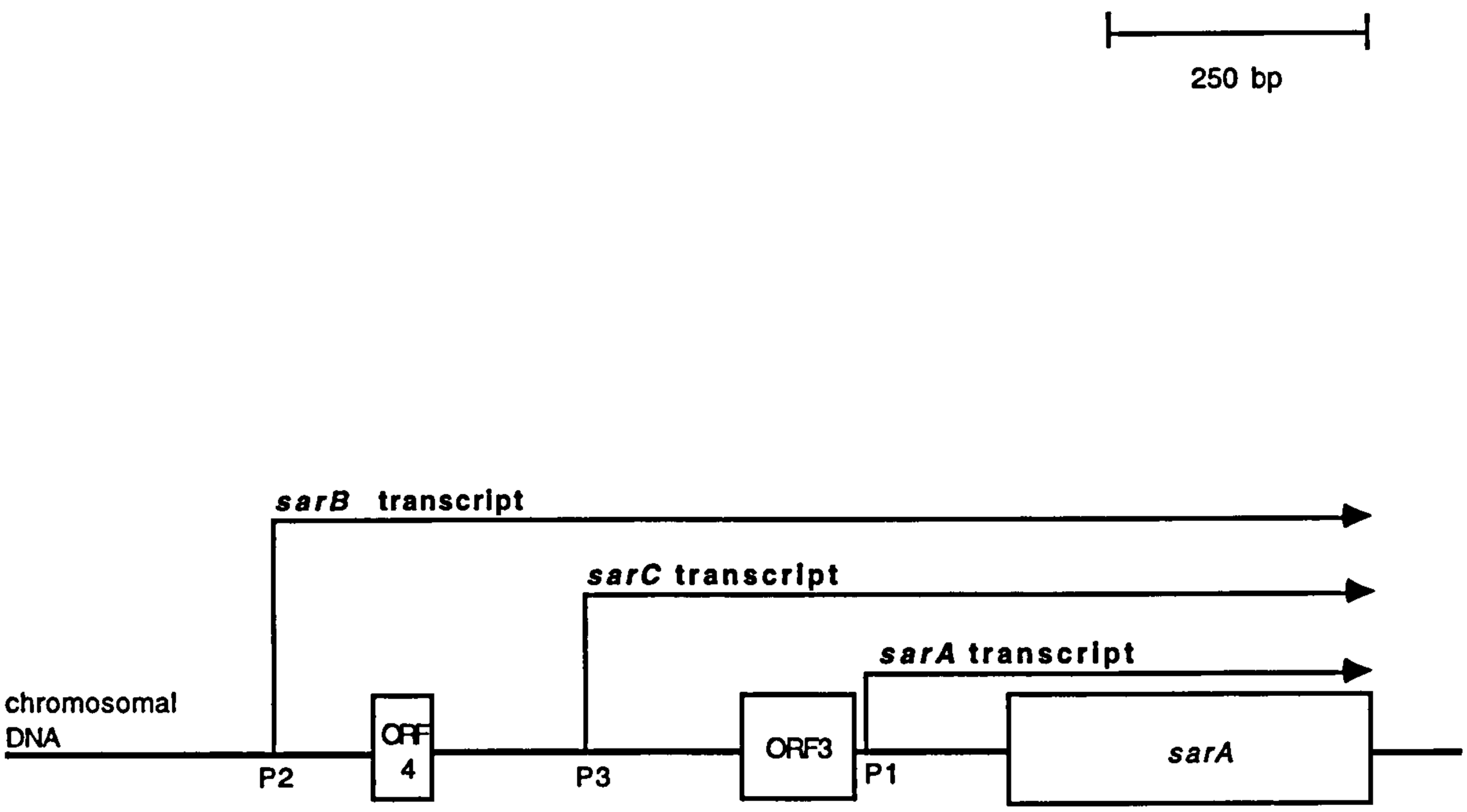


FIGURE 1.5

Transcription of the *sar* locus (modified from Bayer *et al.*, 1996). The map of the *sar* region shows the location of the three *sar* promoters and the approximate extent of their transcripts. (The scale of this diagram is marked on the top, right).

whole *sar* locus was found to be necessary to restore RNAIII levels in an *sar* mutant to those found in wild type strains (Heinrichs *et al.*, 1996). The SarA protein has been found to bind to both the P2 and P3 promoter regions of *agr* (Morfeldt *et al.*, 1996; Cheung *et al.*, 1997). It has also been suggested that the 18 and 39 amino acid peptides encoded by the *sar* locus may play a role with the SarA protein at a physiological level to influence *agr* transcription (Cheung *et al.*, 1997).

It appears that *sar* and *agr* do interact at some level but also act independently *in vivo* since an *sar agr* double mutant shows markedly reduced virulence compared to either single mutant (Booth *et al.*, 1997; Cheung *et al.*, 1994).

1.6.1.2.2 The role of SigB in *sar* transcription

Sigma factors are proteins that bind to core RNA polymerase to form holoenzymes that bind to, and utilise, specific promoters (Moran, 1993). Sigma factors can be divided into two broad groups with the primary sigma factor being necessary for the expression of housekeeping genes whose products are necessary for exponential cell growth (Deora *et al.*, 1997). The second group comprises the alternative sigma factors that control expression of a wide range of genes involved in many cellular functions. The first alternative sigma factor identified in prokaryotes was SigB of *B. subtilis* (Haldenwang and Losick, 1980). SigB controls the expression of many genes in response to several environmental stresses, such as entry into stationary phase, starvation, heat and osmotic shock (Boylan *et al.*, 1993). The *sar* P3 promoter has been shown to be SigB dependent and thus, like other SigB dependent promoters, is most active during stationary phase (Cheung *et al.*, 1997). A putative operon comprising four ORFs has recently been identified in *S. aureus* which showed close similarities to the *sigB* operon of *B. subtilis* (Wu *et al*, 1996). The protein encoded by the putative *sigB* gene of *S. aureus* has since been purified and found to be similar to sigma B of *B. subtilis* and has thus been designated SigSB (*S. aureus* derived SigB

protein) (Deora *et al.*, 1997). It was also demonstrated that SigSB initiated transcription from the *sar* P3 promoter producing a transcript of the expected size (Deora *et al.*, 1997). This confirms the earlier observation of a SigB-like consensus sequence for this promoter. The importance of SigB in the regulation of virulence has not however been established.

1.6.1.3 *sae* (*S. aureus* exoprotein expression)

Another class of pleiotropic mutant defective in the production of several exoproteins has been identified by Tn*551* mutagenesis and designated *sae* (*S. aureus* exoprotein expression) (Giraudo *et al.*, 1994b). The mutant showed decreased β–haemolysis on sheep blood agar plates, reduced DNase activity and a lack of extracellular protein A in comparison to the parental strain. However the parental strain itself was deficient in the production of several exoproteins, so the insertional mutation was transduced into a strain (ISP479-C) which was positive for all characteristics tested, except for enterotoxin A. Following transduction of the mutation into this strain it was found that α– and β–haemolysin levels dropped by 97 and >99.9 % respectively whilst δ–haemolysin remained constant compared to ISP479-C. DNase production decreased by 75 % and the strain was coagulase and extracellular protein A negative. Cell wall protein A, staphylokinase, proteases, lipase and acid phosphatase levels remained unaffected. It was originally thought unlikely that *sae* is a positive regulatory locus but perhaps that the Sae phenotype results from some defect at the level of protein processing (Giraudo *et al.*, 1994b). However, subsequent Northern blot analysis revealed that, in an *sae* mutant, the genes for α– and β–haemolysins and coagulase are not transcribed and the gene for protein A is transcribed at a lower level indicating that the *sae* locus does regulate these genes at a transcriptional level (Giraudo *et al.*, 1997).

An *sae agr* double mutant has been constructed, characterized and, in this strain, the high coagulase levels found in an *agr* mutant are diminished to the near null levels found in an *sae* mutant (Giraudo *et al.*, 1996). Conversely, in this double mutant the low levels of protein A found in the *sae* mutant are raised to the high levels characteristic of an *agr* mutant. Also, β–haemolysin is reduced to an even lower level than that found in either single mutant. These observations suggests that *sae* and *agr* interact, but how and at what level is not yet understood (Giraudo *et al.*, 1997).

1.6.1.4 *xpr* (Extracellular protein regulator)

In 1992 Smeltzer *et al.* described a Tn*551* chromosomal insertion in a *S. aureus* strain which caused an almost complete abolition of expression of extracellular lipase. It was subsequently found that this insertion affected the expression of several exoproteins at the mRNA level with a marked reduction in transcript levels of enterotoxin B, lipase, α– and δ-haemolysin, while mRNA levels for coagulase and protein A were elevated (Hart *et al.*, 1993). The phenotype of this mutant was found to be similar to that of an *agr* mutant. However, genetic and physical mapping showed that insertion of Tn*551* was distinct from the *agr* locus and that this genetic element was in fact a new regulatory locus, designated *xpr* (extracellular protein regulator) (Smeltzer *et al.*, 1993). Due to the reduction in mRNA encoding the δ-haemolysin, thus affecting not only δ–haemolysin but also expression of RNAIII, it was concluded that *xpr* and *agr* are interactive at the genetic level (Hart *et al.*, 1993).

1.6.1.5 Other putative regulatory loci of *S. aureus*

A further putative regulatory locus has been identified by screening a Tn*551* transposon insertion library for fibrinogen binding protein negative mutants (Cheung *et al.*, 1995). A mutant was found to show increased secretion of α-, β– and δ-

haemolysin compared to the parental strain and a decrease in fibrinogen binding capacity and nil levels of cell wall protein A. This pattern was also observed when the mutation was transduced back into its parental strain. Following pulsed field gel electrophoresis the mutation was localised to *Sma*I fragment B of the *S. aureus* chromosome and phenotypic analysis showed that this locus was distinct to the other regulatory loci, *agr, sar* and *xpr*, described above (Cheung *et al.*, 1995). It has been suggested that this locus represents a genetic element that is involved in the expression of potential cell wall virulence determinants, such as protein A (Cheung *et al.*, 1995).

A putative regulatory locus that affects autolysis has also been identified in *S. aureus* (Brunskill and Bayles, 1996). In this work, two genes have been identified, *lytS* and *lytR*, which share homology with genes encoding the sensor and response regulator proteins of a two component signal transduction system (see section 1.5.1). A mutation in the *lytS* gene results in an increased rate of autolysis of cells compared to a parental strain with a functional *lytS* gene.

1.7 The role of environmental stimuli in virulence determinant gene expression

The interaction between a pathogen and its host during the disease process involves microbial survival and multiplication in the face of the rapid and vigorous defence of the host immune system. It is thus important that a bacterium can sense the often subtle environmental signals that indicate that it has entered a host. Such parameters include low iron concentration, temperature, osmolarity, pH, oxygen concentration and the concentrations of other divalent cations such as Mg^{2+} and Ca^{2+} (Mekalanos, 1992) and are discussed below.

1.7.1 Iron

Iron is essential for the growth of virtually all living cells. *In vivo*, intracellular iron is plentiful however extracellular iron is bound to specific iron binding proteins such as transferrin, lactoferrin etc. and thus is not readily available. Therefore, for pathogenic bacteria to survive and multiply in host tissues, they must possess a mechanism for scavenging iron, often by the production of siderophores (Neilands, 1981a). These are low molecular weight chelators and form what is known as a "high affinity" iron transport system thus allowing bacteria to grow in environments where there is <1 μM of free iron (Neilands, 1981b). The best studied siderophore is enterobactin (a.k.a. enterochelin), a complex phenolic derivative which, under conditions of iron deprivation, is produced by *E. coli, Klebsiella pneumoniae, S. typhimurium* and some species of *Shigella* (O'Brien and Gibson, 1970; Pollack and Neilands, 1970; Rogers, 1973; Rogers *et al.*, 1977; Perry and San Clemente, 1979). A novel hydroxamate-type siderophore was found in some clinical isolates of *E. coli* in 1979 and was later confirmed to be aerobactin (Warner *et al.*, 1981; Williams, 1979).

Another mechanism for iron acquisition by bacteria is the production of haemolysins to release iron from intracellular haem and haemoglobin present in erythrocytes (Litwin and Calderwood, 1993). However, not all pathogens which produce haemolysins are able to utilise the iron from haem since it is quickly complexed by haptoglobin in the host as a defence mechanism.

The molecular basis of transcriptional regulation by iron has been most studied in *E. coli* in which co-ordinate regulation of gene expression by iron relies on the product of a single gene, *fur* (ferric uptake regulator). The protein product of the *fur* gene functions as a co-repressor with ferrous iron via interaction with the promoter region of Fur-regulated genes, including aerobactin. Using a transcriptional fusion between *lacZ* and the promoter of the aerobactin gene it was demonstrated that aerobactin

expression is repressed by iron and that this regulation occurs at the transcriptional level (Bindereif and Neilands, 1985). It has been shown that production of shiga toxin by *S. dysenteriae* is repressed in the presence of excess iron and conversely is enhanced under conditions of iron deficiency (Litwin and Calderwood, 1993; Van Heyningen and Gladstone, 1953). In *S. aureus*, it has been found that deficiency of iron up-regulates activity of the accessory gene regulator and production of α–haemolysin (P. F. Chan, personal communication).

1.7.2 Temperature

Temperature is an obvious environmental cue to signal that an organism has entered a host. The transition from usually low ambient temperature to mammalian or avian body temperature causes marked changes in virulence determinant production in several organisms. For example, in *B. pertussis* the *bvgA* and *S* genes encoding a two component regulator system (see section 1.5.1) undergo thermoregulation (Melton and Weiss, 1989). The products of these two genes co-ordinately regulate production of several virulence determinants.

In *S. flexneri*, a change from 30 °C to 37 °C causes the organism to become fully virulent and invasive (Maurelli *et al.*, 1984). Virulence gene expression is mediated in response to increased temperature, by de-repression of a set of genes and steady state expression of these genes at 37 °C (Maurelli *et al.*, 1992). The *virR* gene of Shigella is allelic to *hns* which encodes a histone-like protein that binds to DNA, increases its thermal stability and strongly inhibits initiation of transcription (Maurelli *et al.*, 1992; Spassky *et al.*, 1984). H-NS is believed to mediate changes in DNA supercoiling, by a mechanism which is not yet understood, and thus repress gene transcription (Maurelli *et al.*, 1992). Therefore the ability of Shigella to withstand changes in temperature and co-ordinate a regulatory response could be achieved by

the temperature regulation of *virR* expression. It is possible that this model may be applicable to other temperature sensitive systems in other organisms.

1.7.3 Osmolarity

Osmolarity has also been noted as an environmental signal affecting virulence gene expression in several different pathogens. For example, production of enterotoxin, Yst by *Yersinia enterocolitica* can be induced when osmolarity is increased to that found in an intestinal environment (Alvydas *et al.*, 1994). In *Listeria monocytogenes* cultures, production of a haemolysin is stimulated in the presence of 2.5 % (w/v) NaCl (Dallmier and Martin, 1990). Some organisms are extremely sensitive to osmotic levels. In particular, expression of cholera toxin by *V. cholerae* is optimal at 66 mM NaCl but expression is inhibited at <60 mM or > 250 mM NaCl (Gardel and Mekalanos, 1994). In *S. aureus* high salt concentrations (1.2 M NaCl) resulted in a 16-fold decrease in production of enterotoxin C (Regassa and Betley, 1993). From work done with *E. coli, S. typhimurium* and *S. aureus* it appears that one mechanism of control exerted by osmolarity may be the effect on DNA supercoiling (Higgins *et al.*, 1988; Sheehan *et al.*, 1992). In *S. aureus*, NaCl (0.7 M) causes DNA to become more negatively supercoiled which has been shown to cause a decrease in production of epidermolytic toxin A (Eta) (Sheehan *et al.*, 1992). It is well established that promoter activity is influenced by the level of DNA supercoiling. Higgins *et al.*, (1988), have shown that an increase in extracellular osmolarity increases *in vivo* DNA supercoiling, this seems to be responsible for induction of at least one genetic locus, *proU*, in *E. coli* and *S. typhimurium*. *proU* plays a role in adaptation to growth at high osmolarity (Higgins *et al.*, 1988). The *proU* locus encodes a glycine-betaine transport system which not only balances external osmolarity, but also protects intracellular proteins against denaturation by high ionic strength.

1.7.4 pH

The ability of pathogens to survive the changes in pH during passage through the host digestive tract can be an important factor in their ability to cause disease. Indeed, changes in pH can be interpreted as a signal of exactly where the pathogen is. For example, *Vibrio cholerae* needs to pass through the low pH of the stomach, penetrate the mucus gel layer of the small intestine where pH is more alkaline and then grow and produce cholera toxin. Cholera toxin is optimally produced at pH 8.0 (Gardel and Mekalanos, 1994). Expression of genes in other organisms can also be affected by pH, including the *pagC* gene of *S. tyhphimurium*, which is controlled by the *phoPQ* regulon, is necessary for survival in macrophages and is increased in media of low pH (Miller *et al.*, 1989a).

1.7.5 Anaerobiosis

Changes in oxygen levels can indicate that an organism is entering a host. For example, an anaerobic micro-environment at the site of infection is an environmental signal which can induce an invasive phenotype (Dorman, 1994). It has been shown that growth of *S. typhimurium* under anaerobic conditions leads to increased invasion in tissue culture cell models (Ernst *et al.*, 1990; Francis *et al.*, 1992). In addition, some enteric promoters are sensitive to both osmolarity and anaerobiosis which suggests there might be a collaborative response to those environmental stimuli which indicate that up-regulation of genes necessary for an invasive phenotype is required (Dorman, 1994; Ní Bhriain *et al.*, 1989).

It has been suggested that anaerobiosis can affect the level of supercoiling in chromosomal DNA and it is possible that these changes can play a role in modulating gene expression in response to anaerobic growth (Yamomoto and Droffner, 1985; Higgins *et al.*, 1988).

1.7.6 Divalent cations

1.7.6.1 Ca^{2+}

The level of available calcium has been increasingly implicated in a number of bacterial functions, including differentiation, chemotaxis, heat shock and pathogenicity (Norris *et al.*, 1996). For example, in *Yersinia* species a virulence associated phenomenon known as the low-Ca^{2+} response (*lcr*$^{+}$) confers on the bacterium a dependence on Ca^{2+} for growth at 37 °C and expression of some virulence determinants of *Y. pestis* (Straley and Bowmer, 1986). In addition, the threshold of haemolysin induction in *Actinobacillus pleuropneumoniae* serotype 1 is approximately 700 μM free Ca^{2+} which is the concentration found in blood serum (Frey and Nicolet, 1988). Calcium and magnesium ions have been shown to repress transcription from the promoters of the cell wall protein gene operon of *Bacillus brevis* (Adachi *et al.*, 1991).

In *E. coli*, DnaK, the equivalent of the eukaryotic heat shock protein 70, is induced by stress and has an autophosphorylation activity that is stimulated 10-fold by calcium *in vitro* (Norris *et al.*, 1996). It has been shown *in vivo* that phosphorylation of DnaK increases with temperature (Sherman and Goldberg, 1993). Although the calcium dependence of this phosphorylation is not known, it has been suggested that calcium levels may serve as part of an intracellular thermometer (Norris *et al.*, 1996). A region of DnaK has been shown to have 60 % homology to a classic calmodulin binding site (Stevenson and Calderwood, 1990). Calmodulin is the most common small acidic Ca^{2+} binding protein found in eukaryotic cells and via which calcium regulates a number of cellular processes. Calmodulin has been shown to activate enzymes involved in eukaryotic cellular regulation (Swan *et al.*, 1987). Although the role of calcium binding proteins in bacteria is not well understood, calmodulin-like

activity has also been reported in *B. subtilis*, *E. coli* and *Streptomyces erythraeus* (Fry *et al.*, 1991; Iwasa *et al.*, 1981; Swan *et al.*, 1987).

1.7.6.2 $\underline{Mg^{2+}}$

Several studies have been carried out to determine the effects of magnesium concentration on toxin production in pathogens, in particular TSST-1 in *S. aureus*. Mills *et al.* (1986) found that increasing the concentration of Mg^{2+} from 40 μM to 400 μM in a CDM caused a reduction in TSST-1 production, but a concomitant increase in growth. Similarly, the rate of TSST-1 production was greater in the presence of 20 μM than 1.3 mM. Similar experiments using higher concentrations of Mg^{2+} (25 μM-1.5 mM) showed that Mg^{2+} concentration did not affect production of TSST-1 (Schlievert, 1985). Both these experiments were performed using batch cultures which makes it difficult to measure the effects of changing a single component of a growth medium. However, using a chemostat continuous culture, Taylor and Holland (1989b) showed that, in a Mg^{2+} limited medium, the rate of TSST-1 production is reduced.

1.8 Rationale for this project

It can be seen that there are many factors involved in the response of *S. aureus* to its environment and the consequential regulation of production of virulence determinants. The ways in which these environmental signals cause changes in gene expression is only partially understood. In addition, it remains unclear how, and at what level, the regulators which have been identified to date interact to effect an appropriate response to a changing environment. For this reason it was considered necessary to examine a wide range of environmental stimuli in order to identify those conditions which affect virulence gene expression via the major global regulator, *agr*. It also seems likely that there are other regulators involved in this complex response

system of *S. aureus*. Thus, the work described in this thesis addresses this hypothesis in the attempt to further clarify the mechanism of control of pathogenicity by this organism.

1.9 Reporter gene fusions as tools for analysing gene expression

Reporter gene fusions work by inserting promoterless genes encoding a measurable gene product downstream of a promoter of interest. Thus, when the promoter is transcriptionally active, the reporter gene is transcribed and the resulting gene product can be assayed to give a quantitative measure of promoter activity. Fusions can be made such that the reporter is either transcriptionally or translationally fused to the gene of interest. The use of gene fusions now constitutes a well established method for the study of gene expression and characterization of transcriptional control signals (Silhavy and Beckwith, 1985). Reporter gene fusions have been constructed in a wide variety of bacteria including Bacillus species, *L. monocytogenes*, Mycobacteria and, of course, *S. aureus* (Gordon *et al.*, 1994; Park *et al.*, 1992; Wang *et al.*, 1987; Zuber and Losick, 1983). There are several different reporter gene systems in use.

1.9.1 Classes of reporter genes

Gene fusions using the *lacZ* gene, which encodes β–galactosidase, have been used since the early 1970's (Silhavy and Beckwith, 1985). The *lac* system is probably the most widely used reporter gene system, partly because the *lac* operon has been one of the most intensively studied genetic systems, so that the genetic and biochemical aspects of this system are well understood (Silhavy and Beckwith, 1985). In addition there are several substrate systems which are available. The *lacZ* reporter gene system can be used in both transcriptional and translational fusion studies thus providing a method for distinguishing transcriptional from post-transcriptional control.

Another reporter gene system in wide use employs luciferase encoded by the *lux* operon. Activity is measured by bioluminescence. The advantage of this reporter gene system is that very low levels of luminescence can be detected, for example, transduction of Salmonella with the *luxAB* genes (which encode luciferase) has demonstrated that after one hour as few as 10-100 cells can be detected (Stewart *et al.*, 1989). Also the direct measurement of *in vivo* function without disruption of the cell and loss of viability is a major advantage. However, this system cannot easily be used under anaerobic conditions, interpretation of the intensity of light emission can be complicated and changes in intensity of *in vivo* bioluminescence is dependent of the level of luciferase and is substrates (Meighen, 1991). *lux* gene fusions have been used to detect and measure the strength of promoters under temporal or other regulation during sporulation and germination of Bacillus (Stewart *et al.*, 1989) and the osmotic regulation of *E. coli* (Park *et al.*, 1989). In *S. aureus*, a *lux* reporter gene fusion to the *tst* promoter has been used to study the expression of TSST-1 (Timmins *et al.*, 1995).

β–lactamase has been used as an indicator/reporter for analysing gene expression and its regulation in *S. aureus*. The staphylococcal β–lactamase gene (*blaZ*) has been used to create transcriptional and translational fusions, and in fact transcriptional fusions were used to elucidate the activities of the three promoters of the *agr* locus (Kornblum *et al.*, 1990). The advantage of using β–lactamase as a reporter gene system is that assays are rapid and straightforward and yet it has been shown that the system is sensitive enough to detect activity corresponding to one molecule of enzyme per 10^3 cells (Novick, 1962). Also a variety of colorimetric assays for β–lactamase activity exist (Sykes and Matthew, 1979).

Various other reporter genes have been used to a lesser extent in bacteria including *gus* and *cat.*

CHAPTER 2

Materials and Methods

2 MATERIALS AND METHODS

Components of all the following media and buffers are given in appendix A.

2.1 Media

S. aureus was grown in several media, including two chemically defined media and Tryptic Soy Broth (TSB). However, except where stated otherwise in the following text, Brain Heart Infusion (BHI) was routinely used. Unless stated otherwise, Agar No. 1 [Oxoid] (1 % w/v) was used to make agar plates. Luria-Bertani broth or agar was used for the growth of *E. coli*.

2.2 Antibiotics/inhibitory agent

Antibiotic solutions used in this study are listed in Table 2.1. The stock solutions were filter sterilised where necessary, stored at -20 °C and added to media as appropriate once the media cooled to below 60 °C. Concentrations of antibiotics/ inhibitory agent used for selection were as in Table 2.1, unless stated otherwise in the text.

2.3 Chemicals and enzymes

In general, all chemicals and enzymes were of analytical grade and were obtained from either Sigma, Fisons or BDH, unless otherwise stated. X-Gal (5-bromo-4-chloro-3-indolyl-β–D-galactopyranoside) (Northumbria Biologicals Limited (NBL)) was dissolved in dimethylformamide (DMF) to 20 mg/ml and stored at -20 °C. All restriction enzymes, T4 ligase and buffers for restriction and ligation were purchased from Bethesda Research Laboratories (BRL), NBL or Promega. RNase A (DNase-

Antibiotic/Inhibitory agent	Stock solution	Working conc.
Ampicillin (Amp)	50 mg/ml in H_2O	50 µg/ml
Cadmium (Cd)	$CdCl_2$ (25 mM) in H_2O	0.25 mM
Chloramphenicol (Cm)	30 mg/ml in 95 % Ethanol	5 µg/ml
Erythromycin (Em)	10 mg/ml in 50 % Ethanol	5 µg/ml
Spectinomycin (Sp)	100 mg/ml in H_2O	100 µg/ml

TABLE 2.1

Antibiotics/inhibitory agent used in this study.

free) was purchased from Sigma, dissolved in distilled water to 10 mg/ml, and stored at -20 °C. Lysozyme solutions were prepared fresh on the day of use. Lysostaphin (Applied Microbiology Inc.) was dissolved in 20 mM sodium acetate to 5 mg/ml. Pronase E (Sigma) was dissolved in TES to 10 mg/ml, predigested for 30 minutes at 37 °C, and stored at -20 °C. Phenol was purchased pre-equilibrated from NBL. The constituents of all the buffers and solutions used in this work are described in Appendix A.

2.4 Bacterial strains, plasmids and phages

Bacterial strains and plasmids used in this study are listed in Tables 2.2 and 2.3, respectively. Two bacteriophages (ϕ11 and ϕ85) were used. Both are *S. aureus* specific, temperate, transducing phages of serological group B (Novick, 1991), requiring Ca^{2+} ions for maintenance. Both phages have a genome of approximately 45 kb in size (Novick, 1991).

2.5 Bacterial maintenance, culture and storage conditions

2.5.1 *S. aureus* strains

S. aureus strains were grown on BHI agar plates, containing antibiotics as required. Strains were grown at 37 °C unless otherwise stated. Plate cultures were stored at 4 °C for up to 2 weeks. Long-term storage of strains was at -20 °C in TSB containing 20 % (v/v) glycerol. Liquid cultures were normally inoculated with a colony from an overnight plate into 250 ml flasks containing 100 ml of culture medium and were aerated by shaking on a rotary shaker at 250 rpm.

Strain	Relevant characteristics	Source
S. aureus:		
NCTC 8325	General laboratory strain	Novick, 1990
NCTC 8325-4	Strain 8325 cured of ϕ11, ϕ12 and ϕ13 prophages	Novick, 1990
RN4220	Restriction minus, modification plus strain based on 8325-4	Novick, 1990
WA250	8325-4 containing Tn*551* within the *agr* locus	Morfeldt *et al.*, 1988
SH100	RN4220 containing a chromosomally integrated copy of pHF23 to give *hld::lacZ* fusion and Em^r	This study
SH101	8325-4 containing a chromosomally integrated fragment of genomic DNA from SH100, containing pHF23 to give *hld::lacZ* fusion and Em^r	This study
SH106	RN4220 containing a chromosomally integrated copy of pHF25 to give *hld::lacZ* fusion and Cm^r	This study
SH107	SH100 containing a chromosomally integrated switching casette, pEr::Sp to change Em^r to Sp^r	This study
E. coli:		
XLOLR	Δ(*mcr*A)183, Δ(*mcrCB-hsdSMR-mrr*)173, *endA*1, *thi*1, *recA*1, *gyrA*96, *relA*1, *lac*[F',*proAB*, *lacI*^{q}ZΔM15, Tn*10*, (Tet^r)] λ^r, su^- (non-suppressing)	
DH5α	F-(ϕ80Δ*lac*ZM15), *endA*1, *thi*1, *recA*1, *gyrA*96, *relA*1, *deoR*, *supE*44, *hsdR*17 (r_k-M_k^+) Δ(*lacZYA-argF*) U169	
XL2-Blue MRF'	Δ(*mcrA*)183, Δ(*mcrCB-hsdSMR-mrr*)173, *endA*1, *supE*44, *thi*1, *recA*1, *gyrA*96, *relA*1, *lac* [F', *proAB*, *lacI*^{q}ZΔM15, Tn*10* (Tet^r), Cm^r]	Stratagene

TABLE 2.2

Bacterial strains used in this study.

Plasmids	Characteristics	Source
pUBS1	pUC18 (see below) with multiple cloning site from pBluescript II KS (+/-) phagemid	Foster, 1995
pAZ106	Suicide vector for use in Gram +ve bacteria, promoterless *lacZ* with *spoVG* rbs, *bla* and *erm*	Kemp *et al.*, 1991
pEr::Sp	pIC56 with Er^r::Sp^r	Steinmetz and Richter, 1994
pEr:Cm	pIC56 with Er^r::Cm^r	Steinmetz and Richter, 1994
pTKlac	Suicide vector for use in Gram +ve bacteria, promoterless *lacZ* with *spoVG* rbs, *bla* and *cat*	Kenney and Moran, 1991
pUC18	*E. coli* plasmid cloning vector, *bla*	Vieira and Messing, 1982
pRN3208	Tn*551* delivery vector, Cd^r, *cat, erm*	Novick, 1991
pEX0	pSP64 containing 770 bp fragment of Tn*551* plus 3.8 kb of *S. aureus* flanking chromosomal DNA	Morfeldt *et al.*, 1988
pEX7	pSP65 containing a 2.1 kb *BglII*/*Pst*I fragment containing the *hld* gene of the *agr* locus	Janzon and Arvidson, 1990
pLTV1	Tn*917* delivery vector, *tet, erm, bla*	Camilli *et al.*, 1990
pLTV3	Tn*917* delivery vector, *tet, erm, phe*	Camilli *et al.*, 1990
pHF21	677 bp *PstI*/*Eco*RV fragment of *agr* in *PstI*/*Eco*RV digested pUBS1	This study
pHF23	*XbaI*/*Eco*RV 701 bp fragment from pHF21 in *XbaI*/*Sma*I digested pAZ106	This study
pHF24	701 bp *Bam*HI/*Hin*dIII fragment of *agr* from pHF21 in *Bam*HI/*Hin*dIII digested pTKlac	This study
pHF25	1.8 kb *Asp700*/*Eco*RV fragment of *agr* (from pEX0) in *Sma*I digested pTKlac	This study
pHF26	As pHF25, except insert in opposite orientation	This study
pHF30	1.2 kb *Eco*RI digested PCR product comprising the 3' end of Tn*917* and flanking chromosomal DNA from strain 10.1 in *Eco*RI digested pUBS1	This study
pHF31	1.5 kb *Eco*RI digested PCR product comprising the 3' end of Tn*917* and flanking chromosomal DNA from strain 5B.1 in *Eco*RI digested pUBS1	This study

TABLE 2.3

Plasmids used in this study.

2.5.2 *E. coli* strains

E. coli was grown on LB agar plates, containing antibiotics if necessary to ensure the maintenance of plasmid bearing strains. Plate cultures were stored at 4 °C for 2 weeks before re-streaking onto fresh agar plates. Liquid cultures were prepared in a similar manner to *S. aureus* except that the strains were grown in LB broth. All *E. coli* cultures were incubated at 37 °C. For long-term storage, *E. coli* strains and their plasmid-bearing derivatives were stored in LB broth plus 20 % (v/v) glycerol at -20 °C and/or -70 °C.

2.5.3 Plasmid and chromosomal DNA

Purified plasmid or chromosomal DNA was stored in TE buffer at 4 °C (short-term storage) or -20 °C (long-term storage).

2.6 Phage techniques

2.6.1 Preparation of *S. aureus* phage lysates (Lindsay, pers. comm.)

S. aureus donor strains were inoculated into TSB and incubated overnight at 37 °C with shaking (250 rpm). This overnight culture was used to inoculate (OD_{600} 0.05) 25 ml fresh TSB which was then incubated at 37 °C with shaking (250 rpm) for three hours until mid-log phase. Culture (3 ml) was centrifuged (10,000 rpm, 5 min, RT) and resuspended in fresh TSB (5 ml). Phage buffer (5 ml) and approximately 30 µl of stock lysate (ϕ11 or ϕ85 propagated on 8325-4) was added. A ratio of cells:phage of approximately 20:1 is required. The mixture was incubated at RT for 10 minutes and then transferred to a slow shaker (approximately 50 rpm) at 30 °C for 2-3 hours. If the cells had not lysed by this time they were left overnight at 25 °C without shaking.

The resulting phage lysate contained approximately 10^7-10^{10} pfu/ml. The lysate was filter sterilised and stored at 4 °C.

2.6.2 Phage transduction (Mani *et al.*, 1993)

S. aureus 8325/4 (or SH106, as required) was inoculated into LK broth (50 ml) and incubated overnight at 37 °C with shaking (250 rpm). The culture was then centrifuged (10,000 rpm, 10 min, RT) and resuspended in LK broth (2.5 ml). Aliquots (0.5 ml) were mixed with LK broth (1 ml) containing 10 mM $CaCl_2$ and phage lysate (section 2.6.1) (0.5 ml) was added. The mixture was incubated for 25 minutes at 37 °C without shaking and then for 15 minutes at 25 °C with shaking (250 rpm). Ice-cold Na-citrate (1 ml, 0.02 M) was added and the mixture was centrifuged (10,000 rpm, 10 min, 4°C). The pellet was resuspended in ice-cold Na-citrate (1 ml, 0.02 M) and kept on ice for 2 hours. The cells were then spread onto LK agar plates in 100 µl aliquots and incubated at 37 °C for 2-3 hours until a sheen of growth could be seen. The plates were then overlaid with LK top agar (5 ml) containing 30 µg/ml erythromycin and incubated for up to 2 days at 37 °C. Any resulting colonies were putative phage transductants.

2.7 Transformation

2.7.1 Preparation of competent *E. coli* cells (Hanahan *et al.*, 1991)

A scoop of cells from a fresh overnight plate was inoculated into SOB medium (1 ml) and mixed by vortexing. Cells were inoculated into pre-warmed (37 °C) SOB medium (100 ml) in a 1 l flask and incubated at 37 °C with shaking (150 rpm) until cell density reached OD_{600} 0.5. The culture was chilled on ice for 15 minutes and cells pelleted by centrifugation (3,000 rpm, 15 min, 4 °C). The supernatant was removed and the pellet was resuspended in frozen storage buffer (FSB, 33 ml) by

vortexing. The cells were held on ice for 10-15 minutes and centrifuged and drained as before. The pellet was resuspended in 1/12.5 of the original volume of FSB (8 ml) and DMSO (3.5 %) was added and the tube swirled for 5-10 seconds. The cells were stored on ice for 5 minutes, a second equal volume of DMSO was added, followed by further storage on ice for 10-15 minutes. Aliquots of cells (220 µl) were transferred to microfuge tubes and flash frozen using ethanol/dry ice. The tubes were either used immediately or stored at -70 °C.

2.7.2 Transformation of *E. coli* strains DH5α or XLOLR

A competent cell suspension (Hanahan *et al.*, 1991) (200 µl) was transferred to a pre-chilled microcentrifuge tube and mixed with DNA (approximately 50 ng) in a volume of 20 µl. After 40 minutes storage on ice, the tube was transferred to 42 °C for 90 seconds, and then immediately chilled on ice for 2 minutes. SOC (0.8 ml) was added to the cells, which were then incubated for 45 minutes at 37 °C to allow the expression of plasmid encoded antibiotic resistance markers. Dilutions of the transformed cells were plated on to LB agar containing ampicillin (50 µg/ml) and then incubated overnight at 37 °C until colonies were visible.

2.7.3 Transformation of *E. coli* XL2-Blue MRF' ultracompetent cells (Stratagene)

Ultracompetent cells were thawed on ice and gently mixed by hand. Cells were aliquoted (100 µl) into pre-chilled 20 ml sterile universals and β–mercaptoethanol (Stratagene) (1.7 µl) added to give a final concentration of 25 mM. The contents of the tube were mixed gently and held on ice for 10 minutes, swirling gently every 2 minutes. DNA (50 ng) was added to the cells and swirled gently and the tubes were held on ice for 30 minutes. The tubes were heat shocked in a water bath at 42 °C for 30 seconds and then held on ice for 2 minutes. Preheated (42 °C) NZY broth (0.9 ml)

was added to the tubes and they were then incubated at 37 °C for 1 hour with shaking at 250 rpm. Transformed cells (200 µl aliquots) were plated onto LB agar containing ampicillin (50 µg/ml) and incubated overnight at 37 °C.

2.7.4 Electroporation of *S. aureus* (Schenk and Ladagga, 1992)

2.7.4.1 Preparation of electro-competent cells

Cells to be transformed (*S. aureus* strain RN4220) were grown overnight in B2 broth at 37 °C with shaking (250 rpm). An aliquot (1 ml) was used to inoculate 25 ml of fresh B2 broth and incubated at 37 °C, with shaking, until OD_{600} of 0.5 was reached. The culture was transferred to a sterile tube and centrifuged (5,000 rpm, 10 min, RT). The pellet was washed 3 times by resuspension and centrifugation (5,000 rpm, 10 min, RT) with an equal volume (25 ml) of sterile H_2O at RT, once in a fifth volume (5 ml) of sterile glycerol (10 % v/v) and once in a one tenth volume (2.5 ml) of sterile glycerol (10 % v/v). Following final resuspension in 2.5 ml of sterile glycerol (10 % v/v), the cells were incubated at RT for 15 minutes without shaking. The cells were then harvested (5,000 rpm, 10 min, RT) and resuspended in glycerol (0.8 ml, 10 % v/v). Cells (70 µl) were then removed to a sterile microfuge tube containing dried DNA (1 µg) and mixed, or removed to a sterile microfuge tube, flash frozen and stored at -70 °C until required.

2.7.4.2 Electroporation protocol

For electroporation, 60 µl of each 70 µl aliquot with DNA was removed to a sterile electroporation cuvette (0.2 cm) and electroporated using a Gene Pulser (BioRad) at 20 °C, 100 ohms resistance, 25 mF capacitance and 2.5 kV. Fresh B2 broth (1 ml) was added to the cuvette, mixed and transferred to a sterile universal. Erythromycin (0.05 µg/ml) was added, where appropriate, and cells were then incubated for 1 hour

at 37 °C to allow induction of antibiotic resistance. The cells were then plated in 100 µl aliquots onto TSB agar containing appropriate antibiotics and incubated overnight at 37 °C.

2.7.5 Transformation of *S. aureus* by protoplasting (Novick, 1991)

S. aureus strain RN4220 was inoculated into 2X BHI broth (50 ml) in an acid-washed Ehrlenmeyer flask (250 ml) and grown to mid-log phase (OD_{600} ~2.5). The culture was then centrifuged (5,000 rpm, 10 min, RT), resuspended in 2X BHI (50 ml) and then centrifuged (5,000 rpm, 10 min, RT). The pellet was then resuspended in sterile SMM (10 ml). Lysostaphin was added (50 µg/ml) and the mixture was incubated for 15-20 minutes at 37 °C without shaking. The protoplasts were centrifuged (1,200 rpm, 7 min, RT) and resuspended in sterile SMM (5 ml). DNA (10 µg in 0.25 ml TE) was added to 2X SMM (0.25 ml) in a sterile universal and then the protoplasts (0.5 ml) were added. PEG 8000 (1.5 ml, 40 % w/v) was added immediately and mixed by gentle inversion. After 2 minutes, SMMP (5 ml) was added and protoplasts were collected by centrifugation (1,200 rpm, 7 min, RT). The pellet was resuspended in SMMP (1 ml) containing chloramphenicol (0.15 µg/ml), where appropriate, and incubated at 32 °C with gentle shaking (100 rpm) for 2-3 hours. Aliquots (100 µl) were then plated onto DM3 agar containing appropriate antibiotics and incubated for up to 5 days at 37 °C.

2.8 DNA purification techniques

2.8.1 Small scale plasmid preparation from *E. coli*

Plasmid-bearing cells were grown overnight at 37 °C in 15 ml LB containing ampicillin (50 µg/ml), inoculated from a single colony. The culture was then centrifuged (3,000 rpm, 10 min, RT) and the cells were resuspended in 100 µl of

plasmid preparation solution I in a microcentrifuge tube. Lysozyme (5 µl, 50 mg/ml in 10 mM Tris-HCl [pH 8]) was added, mixed briefly by vortexing and incubated at RT for 5 minutes. Plasmid preparation solution II (200 µl) was added, mixed by gentle inversion 5 times and incubated at RT for 5 minutes. Ice-cold plasmid preparation solution III (150 µl) was added and mixed thoroughly by inversion 5 times to ensure dispersal of solution III through the lysate. Following storage on ice for 10 minutes, the white precipitated debris was pelleted by centrifugation (10,000 rpm, 5 min, 4 °C), and the supernatant transferred to a fresh tube. The plasmid DNA was then precipitated by the addition of 100 % (v/v) ethanol (950 µl) and storage on ice for 2 minutes. The plasmid DNA was then pelleted by centrifugation (10,000 rpm, 10 min, 4 °C). The supernatant was aspirated off and the pellet air dried and resuspended in TE (100 µl) containing RNase A (10 mg/ml). The plasmid DNA was incubated for 20 minutes at 37 °C and then cleaned using a Gene Clean kit (Anachem) (see 2.8.2).

2.8.2 Gene Cleaning of plasmid DNA (Anachem)

To plasmid DNA in a microfuge tube, NaI (3X volume, 6 M) was added, followed by 10 µl glass milk suspension from the Gene Clean kit (Anachem). For purifying DNA from agarose gels, the agarose was dissolved in 3 volumes of the NaI stock solution (6 M) at 45-55 °C prior to addition of glass milk. The tube was stored on ice for 5 minutes, mixing at 1 minute intervals and then centrifuged (10,000 rpm, 5 sec, RT). The supernatant was removed and the pellet washed 3 times in ice cold New Wash (Anachem) (0.5 ml), centrifuging between each wash (10,000 rpm, 5 sec, RT). After the final wash, TE was added (1-2X volume of glass milk used) and the tube heated at 45-55 °C for 3 minutes. The glass milk was pelleted (10,000 rpm, 30 sec, RT) and the supernatant, containing purified plasmid DNA, was transferred to a clean tube.

2.8.3 Large scale plasmid preparation from *E. coli* (or *S. aureus*)

Plasmid-containing cells were grown overnight at 37 °C, with shaking (250 rpm) in LB broth (or BHI for *S. aureus*) (500 ml) containing appropriate antibiotic and were harvested by centrifugation (10,000 rpm, 2 min, RT). The pelleted cells were resuspended in plasmid preparation solution I (18 ml). Freshly prepared lysozyme (0.4 ml, 50 mg/ml), (and lysostaphin (180 µl, 5 mg/ml) for *S. aureus*) was added and mixed briefly by vortexing. The preparation was incubated at RT for 10 minutes for *E. coli* (or at 37°C for 30 minutes for *S. aureus*). Freshly prepared plasmid preparation solution II (40 ml) was added, mixed thoroughly by gentle inversion 5 times and then incubated at RT for 5 minutes. Plasmid preparation solution III (20 ml) was added and the contents mixed by shaking several times. Following storage on ice for 10 minutes, the white precipitated debris was pelleted by centrifugation (4,000 rpm, 15 min, 4 °C). The supernatant was then filtered into a fresh tube through 4 layers of cheesecloth. The plasmid DNA was precipitated by the addition of isopropanol (0.6 volume), and stored at RT for 10 minutes. The plasmid DNA was pelleted by centrifugation (5,000 rpm, 15 min, RT). The pellet was then rinsed with 70 % (v/v) ethanol (5 ml) at RT and, following removal of ethanol, air dried and dissolved in 1 ml TE (pH 8.0) and split between 3 minifuge tubes. The plasmid DNA was then purified by precipitation with polyethylene glycol (PEG).

2.8.4 Polyethylene Glycol (PEG) purification of DNA

To plasmid DNA in a volume of 0.3 ml TE, half volume of ice cold LiCl (150 µl, 10 M) was added. The samples were mixed well and, after centrifugation (10,000 rpm, 10 min, 4 °C), supernatants were transferred to fresh microcentrifuge tubes. An equal volume of isopropanol was added and the DNA pelleted (10,000 rpm, 10 min, RT). The supernatant was discarded and the pellets washed with 70 % (v/v) ethanol at RT. The pellets were air dried and subsequently combined into one microfuge tube,

dissolved in TE (0.5 ml) containing DNase-free RNase (10 mg/ml). The tube was then stored at RT for 30 minutes. NaCl (1.6 M) containing 13 % (w/v) PEG 8000 (0.5 ml) was added and the solution mixed well. The plasmid DNA was recovered by centrifugation (12,000 rpm, 5 min, 4 °C). The supernatant was aspirated and the pellet dissolved in TE (pH 8.0) (0.4 ml). The solution was extracted once with phenol, once with phenol/chloroform and once with chloroform (see section 2.8.5).

2.8.5 Phenol/chloroform purification of DNA

To DNA in TE, an equal volume of phenol was added and, after mixing by vortexing, was centrifuged (10,000 rpm, 10 min, RT). The mixture separated into an upper layer of aqueous DNA and a lower layer of phenol. The aqueous layer, containing plasmid DNA, was removed to a clean microfuge tube and one half volume of phenol and one half volume of chloroform/isoamylalcohol (24:1) was added. After mixing by vortexing and centrifugation (12,000 rpm, 5 min, RT) the upper aqueous layer was again removed to a new microcentrifuge tube containing an equal volume of chloroform/isoamylalcohol and mixed gently. After centrifugation (12,000 rpm, 2 min, RT) the top aqueous layer was removed to a fresh tube and the DNA precipitated with ethanol (see 2.8.6).

2.8.6 Ethanol precipitation of DNA

DNA was precipitated by the addition of 0.1 volume of 3 M sodium acetate (adjusted to pH 4.6 with acetic acid), and 2-3 volumes of ice-cold absolute ethanol. The precipitated DNA was pelleted by centrifugation (10,000 rpm, 15 min, 4 °C), and the pellet was washed with ice-cold ethanol (70 % v/v). The supernatant was removed, the pellet air dried and then dissolved in TE.

2.8.7 Chromosomal DNA preparation

Chromosomal DNA was isolated and purified using a QIAGEN 100/G kit according to the manufacturer's instructions, as follows. The *S. aureus* strain of interest was inoculated into 10 ml of TSB from a single colony and incubated overnight at 37 °C with shaking (250 rpm). The cells were pelleted (3,000 rpm, 10 min, RT) and resuspended in 3.5 ml of buffer 1 (QIAGEN) containing 200 µg/ml RNase A. After vigorous vortexing, lysostaphin (30 µl, 5 mg/ml) and pronase (100 µl, 20 mg/ml) were added and incubated for 30 minutes at 37 °C. To the clear suspension, buffer 2 (QIAGEN) (1.2 ml) was added and briefly vortexed. The tube was then incubated at 50 °C for 30 minutes.

The cell lysate was applied to a QIAGEN 100/G tip equilibrated with buffer QBT (QIAGEN) (4 ml). Once the flow had stopped the tip was washed twice with buffer QC (QIAGEN) (7.5 ml). The genomic DNA was eluted into a sterile 50 ml tube by adding 5 ml of buffer QF (QIAGEN).

Genomic DNA was precipitated by adding 3.5 ml of isopropanol and inverting the tube 20 times and then pelleted (5,000 rpm, 10 min, RT) and washed with 70 % (v/v) ice-cold ethanol. The supernatant was discarded and the pellet dried in a vacuum desiccator. The DNA was dissolved in 250 µl TE.

2.9 *In vitro* DNA manipulation techniques

2.9.1 DNA ligation

All ligations were incubated at 14 °C overnight except for blunt-end ligations which were incubated at 4 °C for 48 hours.

2.9.1.1 Standard DNA ligation

To linearised plasmid vector DNA in TE buffer, DNA insert was added to a ratio of 1:3. Sterile distilled H_2O was added up to 8 µl and then T4 DNA ligase buffer (1 µl, 10X) and 3 Weiss units (1 µl) of T4 DNA ligase were added.

2.9.1.2 Self-ligation of Tn*917* and flanking chromosomal DNA

To 250 µl of digested DNA (1 µg) in TE buffer, 30 µl of 10X ligase buffer and 3 Weiss units (1 µl) of T4 DNA ligase were added. The solution was then made up to 300 µl with sterile distilled water and incubated overnight at 14 °C.

2.9.2 Agarose gel electrophoresis

DNA fragments were separated by horizontal gel electrophoresis using a Horizon 11-14 gel electrophoresis tank (Gibco BRL). An agarose gel (0.8 % w/v in 100 ml TBE) containing 0.2 mg/ml ethidium bromide, was submerged in TBE buffer (500 ml). DNA samples were mixed with one tenth their volume of 10X DNA loading buffer and loaded into wells in the gel. The gel was run at 100 V for approximately 1 hour (the exact time depending on the sizes of the fragments to be separated), and visualised by means of a U.V. transilluminator (2UV, UVP). DNA fragment sizes were estimated using 1 µg λ DNA digested with either *Hin*dIII or double digested with *Hin*dIII/*Eco*RI, as size standards (Table 2.4). The sizes of known fragments (kilobase pairs) were plotted on a logarithmic scale against mobility of fragments (mm) on a linear scale. A best fit curve was drawn by eye and this curve was used to determine the size (in kb) of the DNA fragments in the experimental tracks.

Fragment sizes of digested λ (bp)	
*Hin*dIII	*Hin*dIII/*Eco*RI
23130	21226
9416	5148
6557	4973
4361	4268
2322	3530
2027	2027
564	1904
125	1584
	1375
	947
	831
	564
	125

TABLE 2.4

Sizes of resulting fragments following digestion of λ DNA with *Hin*dIII or *Hin*dIII/*Eco*RI.

2.9.3 Pulsed field gel electrophoresis (Pattee *et al.*, 1990)

2.9.3.1 Preparation of samples

The *S. aureus* strain of interest was inoculated into 10 ml of TSB from a single colony and incubated overnight at 37 °C with shaking (250 rpm). The culture was used to inoculate (OD_{600} 0.05) fresh TSB which was then incubated at 37 °C with shaking (250 rpm) until the OD_{600} reached 0.8. Chloramphenicol (180 µg/ml) was then added and the culture incubated for a further 10 minutes. Cells (2 x 10^8 cfu - approximately 0.5 ml at OD_{600} 1.0 (see section 3.2.3) per plug) were pelleted (10,000 rpm, 3 min, RT) and resuspended, in one half of the total volume of plugs to be made, of cell suspension buffer. The cell suspension was equilibrated to 50 °C, and lysostaphin was added to a final concentration of 100 µg/ml. An equal volume of 2 % (w/v) CleanCut agarose (Sigma) in sterile H_2O (equilibrated to 50 °C) was immediately added and mixed. The cell suspension was then aliquoted into plug moulds and allowed to solidify at 4 °C for 15 minutes. The set plugs were transferred to 50 ml tubes containing lysis buffer (5 ml) and incubated for 1 hour at 37 °C without shaking. The lysis buffer was removed and the plugs were rinsed with wash buffer (25 ml) and incubated at 50 °C overnight without shaking in Proteinase K Reaction Buffer (5 ml). The plugs were subsequently washed 4 times in 50 ml of wash buffer for 30 minutes at RT with gentle shaking. In the second and third washes PMSF (phenylmethylsulphonyl fluoride) was added to 1 mM. Plugs were either stored in wash buffer at 4 °C, or processed.

2.9.3.2 Restriction digestion of DNA *in situ*

The plugs were washed in 50 ml TE for 30 minutes and transferred to individual microcentrifuge tubes containing 1 ml of appropriate 1X restriction enzyme buffer. After incubation for 1 hour with gentle shaking the buffer was removed and 0.3 ml of

fresh 1X restriction buffer added. 50 units of *Sma*I were added and the plugs were incubated overnight at 30 °C. After digestion the buffer was removed and the plugs were washed in 1 ml of wash buffer for 30 minutes with gentle shaking. The plugs were loaded into either, the wells of Clamped Homogenous Electric Field (**CHEF**), or Field Inversion Gel Electrophoresis (**FIGE**) gels.

2.9.3.3 Separation of DNA by PFGE

DNA fragments were separated by using a CHEF-DR II system (BioRad) and a FIGE Mapper system (BioRad). A 1.0 % (w/v) agarose CHEF or FIGE gel was submerged in 0.5X TBE electrophoresis buffer. The CHEF gel was run at 6 V cm^{-1} for approximately 22 hours at 14 °C with 10-50 second switching times. The FIGE gel was run at RT for 16 hours. The forward and reverse voltages were 180 and 120 respectively. The switch time ramps were 0.1-0.4 seconds and were linear in shape. All gels were washed in 0.5 mg/ml ethidium bromide in water to stain the DNA and visualised by means of a UV transilluminator (2UV, UVP). The sizes of DNA fragments were estimated using a λ ladder (BioRad, 50-1000 kb), *Saccharomyces cerevisiae* DNA size standards (BioRad, 240-2200 kb) and DNA size standards of 8-48 kb (BioRad) (Table 2.5). The sizes (kilobase pairs) of known fragments were plotted on a logarithmic scale against mobility (mm) of fragments on a linear scale. A best fit curve was drawn by eye and this curve was used to determine the size (in kb) of the DNA fragments in the experimental tracks.

2.9.4 Gel photography

A permanent record of agarose gels was obtained by photographing the ethidium bromide stained gels illuminated with incident UV light. A Kodak 203 red-orange filter and Polaroid 667 (ASA 3000) film were used.

Size standards (kb)		
Saccharomyces cerevisiae chromosomal fragments	Lambda ladder	8-48 kb size standards
2200	970.0	48.5
1600	921.5	38.4
1125	873.0	35.5
1020	824.5	29.9
945	776.0	24.8
825	727.5	22.6
785	679.0	19.4
750	630.5	17.1
680	582.0	15.6
610	533.5	12.2
565	485.0	10.1
450	436.5	8.6
365	388.0	8.3
285	339.5	
225	291.0	
	242.5	
	194.0	
	145.4	
	97.0	
	48.5	

TABLE 2.5

The sizes of standards used as size markers for pulsed field gel electrophoresis.

2.10 Quantification of DNA (Sambrook *et al.*, 1989)

Spectroscopic measurements at 260 nm were used to determine the concentration of DNA solutions. An absorbance of 1 corresponds to approximately 50 μg/ml for double stranded DNA. The concentration of DNA could be estimated independently by agarose gel electrophoresis, comparing the intensity of the ethidium bromide stained bands to bands containing known amounts of DNA.

2.11 DNA hybridisation techniques

2.11.1 Labelling of DNA probes with Digoxigenin

The commercially available Digoxigenin (DIG) DNA labelling and detection kit from Boehringer Mannheim was used to generate DNA probes for hybridisation by a random priming labelling method. The DNA template (approximately 3 μg in 10 μl TE) was heat denatured in a boiling water bath for 10 minutes and immediately cooled in dry ice/ethanol for 30 seconds. To this denatured DNA, 2 μl each of 10X concentrated hexanucleotide mixture and 10X dNTP labelling mixture (comprising dATP, 1mmol/l; dCTP, 1 mmol/l; dGTP, 1 mmol/l; Dig-dUTP, 0.35 mmol/l) (both supplied with the kit) were added. Klenow enzyme (1 μl of 2 U/μl stock), labelling grade, was added, and the volume of the reaction was made up to 20 μl with distilled water. The reaction was incubated overnight at 37 °C, and then the labelled DNA was purified using a Gene Clean kit (Anachem) (see section 2.7.1.1) and resuspended in 50 μl TE.

Just prior to use, the probe was denatured by placing the tube in a boiling water bath for 10 minutes followed by immediate chilling on ice.

2.11.2 Southern blotting

Agarose gel electrophoresis was performed as described in section 2.9.2, and the gel was photographed prior to Southern blotting analysis. For all of the hybridisations described below, unless otherwise stated, Amersham Hybond-N^+ extra (positively charged nylon membrane) was used. Prior to use, the membrane was cut to the same dimensions as the gel to be blotted.

2.11.2.1 Transfer of DNA from an agarose gel - using a vacuum blotter (Appligene)

Appropriately sized nylon membrane was placed on the vacuum blotter as per the manufacturer's instructions (Appligene). The membrane was wetted with distilled water and the gel placed on top and the vacuum blotter switched on to give a vacuum of approximately 50 mBar. Sufficient HCl (0.25 M) to cover the surface of the gel was added and left for 10 minutes. Excess HCl which remained on the surface of the gel was removed and denaturing solution was then added to the surface of the gel and left for 10 minutes. Following removal of any denaturing solution which remained on the surface of the gel, neutralising solution was added to the top of the gel and left for 10 minutes. Any neutralising solution which remained on the gel was removed and replaced by 10X SSC and the gel left for 30 minutes. The vacuum was then turned off, the gel discarded and the membrane was stored (DNA-side up) on clean filter paper to air dry for approximately 15-30 minutes. The DNA was either, fixed to the membrane by baking in an oven at 80 °C for 2 hours, or fixed to the membrane by U.V. crosslinking (Amersham) (700 mJ, 15 sec).

2.11.2.2 Colony blotting

Colonies for screening were patched in duplicate onto LB agar plates containing

ampicillin and grown overnight at 37 °C. One plate was then stored at 4 °C as a stock plate and the other was used for colony blotting.

Filter paper (Whatman 3MM) was cut into four pieces, each approximately 1 foot square, and placed on individual flat plastic trays. Each of the four pieces was saturated with either, SDS (10 % w/v), denaturing solution, neutralising solution, or 2X SSC and any excess liquid removed. Round nitrocellulose (RN) filters (Whatman) were laid, briefly, on top of each agar plate so that each colony adhered to the RN filter. The RN filters were then removed from each plate and placed, colony side up, on top of the SDS impregnated filter paper and left for 3 minutes. Each RN filter was then removed and laid, briefly (10 sec), colony side up, on a clean paper towel to dry away any remaining SDS. Each RN filter was then laid onto the filter paper containing denaturing solution and left for 5 minutes. Following drying as described above, the RN filters were laid on the filter paper containing neutralising solution and left for 5 minutes. Following drying as described, each RN filter was then laid on the 2X SSC-impregnated filter paper and left for 5 minutes. The RN filters were then dried on paper towels for at least 30 minutes at RT and then baked at 80 °C, *in vacuo*, for 2 hours to fix the DNA to the membrane. The impregnated square sheets of filter paper were discarded.

The RN filters were then floated on the surface of 2X SSC until wetted from beneath and then submerged for 5 minutes. These filters were then transferred to a container of prewashing solution (200 ml of 5X SSC, SDS (0.5 % w/v) and EDTA (1 mM)), covered and incubated at 50 °C for 30 minutes. Any bacterial debris remaining on the surface of a filter was then removed gently by scraping with wet paper towels and the filters were processed as for a standard Southern blot (see section 2.11.3).

2.11.3 Prehybridisation and hybridisation

Membranes to be probed with the DIG-labelled DNA were prehybridised for 1 hour at 68 °C in prehybridisation solution (20 ml per 100 cm^2 of membrane) in a hybridisation oven (Stuart Scientific). The membranes were then hybridised with the labelled probe overnight at 68 °C. The concentration of the probe used was 5-25 ng/ml, diluted in prehybridisation fluid. Unbound probe was removed by washing the membranes twice in 2X SSC containing SDS (0.1 % w/v) for 5 minutes at RT and then washing twice in SSC (0.1 %) containing SDS (0.1 % w/v) for 15 minutes at 68 °C in a hybridisation oven (Stuart Scientific).

2.11.4 Detection of DIG-labelled DNA

The hybridised and washed membranes were equilibrated with buffer 1 for 1 minute, and then blocked for 30 minutes with gentle rocking in buffer 2. The membranes were then transferred to buffer 1 containing a 1:5,000 dilution of Anti-DIG-alkaline phosphatase antibody (Boehringer Mannheim DNA labelling and detection kit) and incubated for 30 minutes with gentle rocking. The membranes were then washed twice with buffer 1 for 15 minutes with gentle rocking and then equilibrated in buffer 3. The membranes were transferred to buffer 3 (15 ml) containing 66 µl of nitroblue tetrazolium (NBT) (50 µg/ml in 70 % (v/v) dimethylformamide) and 33 of µl bromo-4-chloro-3-indolyl-phosphate (BCIP) (50 µg/ml in water) and incubated in the dark, without shaking, until a purple precipitate formed at the labelled DNA. The reaction was stopped by washing the filters in buffer 4.

2.12 Assays

2.12.1 β–Galactosidase assay

2.12.1.1 Using 4-methylumbelliferone-β-D-galactopyranoside (MUG) as the substrate (Youngman, 1990)

Samples (0.5 ml) of cultures of interest were pelleted (10,000 rpm, 5 min, RT) in a microcentrifuge tube. The supernatant was discarded and the pellet resuspended in AB buffer (0.5 ml) containing DNase (100 ng/ml) and lysostaphin (25 μg/ml). An aliquot (50 μl) was removed from each sample and added to 5 μl of MUG solution (0.4 mg/ml in dimethyl sulphoxide). After incubation for 100 minutes at RT, an aliquot of the sample (sufficient to achieve a measurable fluorescence) was added to Na_2CO_3 (2.5 ml, 0.2 M) in a fluorimeter cuvette. A calibration curve of the fluorescent product, 4-MU (4-methyl umbelliferone, 1 mM) was created with final concentrations of 0, 10, 20, 50, 100, 150, and 200 nM in Na_2CO_3. Fluorescence was measured using a fluorimeter (Hoefer DynaQuant 200). For each sample the concentration of 4-MU was related to β–galactosidase activity, using the following equation.

$$\frac{\text{pmol MUG}}{V \times T \times OD_{600}} = \text{MUG units of β–galactosidase activity}$$

where V = volume of culture used (ml), T = time of assay (100 min) and OD_{600} reflects the cell density at the time the sample was removed from the culture. 1 MUG unit of β–Galactosidase activity is defined as the activity that hydrolyses 1 pmol of MUG per min per ml per OD_{600} to 4-MU.

2.12.1.2 Using *o*-nitrophenyl β–D-galactopyranoside (ONPG) as the substrate

Cells were grown to the required OD_{600} and 1 ml samples were transferred to microcentrifuge tubes and immediately placed on ice. A suitable aliquot (starting with 100 μl) of each sample was transferred to a test tube and made up to 1 ml in 'Z'

buffer. Chloroform (100 µl) and SDS (40 µl, 0.1 % w/v) were added to lyse the cells and the samples vortexed for 10 seconds. The tubes were transferred to a water bath at 28 °C for 5 minutes. The reaction was started by adding freshly prepared ONPG (200 µl of 4 mg/ml in 'Z' buffer) and the tubes were shaken for a few seconds. The tubes were held at 28 °C for 15 minutes and the reaction was stopped by the addition of Na_2CO_3 (0.5 ml, 1 M). Approximately 1.2 ml of each reaction mix was transferred to a fresh microfuge tube and centrifuged (10,000 rpm, 1 min, RT) to remove cell debris. The supernatant (1 ml) from each sample was decanted to a 1 ml spectrophotometric cuvette and the OD_{420} determined. An OD_{420} reading of 0.6-0.9 was required and assays were repeated as necessary, varying the amount of cell culture used. Units of β–galactosidase activity were calculated in Miller units (Miller, 1972) and are proportional to the increase in *o*-nitrophenol per minute per bacterium.

$$\frac{OD_{420} \times 1000}{V \times T \times OD_{600}} = \text{Miller units (MU)}$$

where V = volume of culture used (in ml), T = time of the reaction (in minutes) and the OD_{600} reflects the cell density at the time the sample was removed from the culture.

2.12.2 α–haemolysin assay

Cells were grown to the required OD_{600} and 1 ml of culture was removed to a sterile microfuge tube and centrifuged (7,000 rpm, 2 min, RT). The supernatant was transferred to a fresh tube and either assayed immediately or stored at -20 °C. A suitable aliquot of the supernatant was transferred to a fresh microfuge tube (the amount assayed depended on the activity expected but generally the assay was started with a 100 µl sample). Each sample was made up to 1 ml with HA buffer and mixed by gentle inversion. Defibrinated rabbit blood (TCS Biologicals) (20 µl) was added,

mixed gently and the samples were incubated for 15 minutes at 37 °C. The tubes were then centrifuged (7,000 rpm, 30 sec, RT) and the supernatant was transferred to 1 ml spectrophotometric cuvettes. The OD_{543} of each sample was read against a blank of HA buffer containing 20 µl rabbit blood, treated as above. An OD_{543} of 0.5-0.7 was optimum and the amount of sample used was adjusted, if required, to achieve this. Haemolysin units (HU) were calculated using the following equation:

$$\frac{OD_{543} \times 1000}{V \times T \times OD_{600}} = \text{Haemolysin units (HU)}$$

where V = volume of culture used (ml), T = length of assay (min) and OD_{600} reflects the cell density at the time the sample was removed from the culture.

2.13 Transposon mutagenesis

2.13.1 Transposon mutagenesis using Tn*551*

Following transformation of the strain to be mutagenised (SH106) with plasmid (pRN3208), the strain was grown overnight at 30 °C in BHI (100 ml) containing Cm and $CdCl_2$. This overnight culture was used to inoculate BHI (100 ml) containing Cm and $CdCl_2$, to OD_{600} 0.05 and grown, at 30 °C, until the OD_{600} reached 1.5 with shaking at 250 rpm. An aliquot of the culture (3 ml) was removed, centrifuged (3,000 rpm, 10 min, RT) and resuspended in pre-warmed (43 °C) TSB (100 ml) containing erythromycin. This culture was maintained at 43 °C, with shaking at 250 rpm, and grown until the OD_{600} reached 0.4-2.0. An aliquot (3 ml) was then removed to fresh pre-warmed (43 °C) TSB (100 ml) containing erythromycin and grown at 43 °C, with shaking, until the OD_{600} reached 1.5. The culture was then centrifuged (8,000 rpm, 10 min, RT), resuspended in 4 ml of BHI containing glycerol (10 % v/v) and flash frozen in ethanol-dry ice in 0.5 ml aliquots and stored at -20 °C until required. One

sample was immediately defrosted and used to determine the efficiency of transposition and purity of the library (see section 4.2.2).

2.14 Cloning of chromosomal DNA flanking a transposon

2.14.1 Cloning chromosomal DNA flanking Tn*551*

This procedure is described in section 4.2.8.

2.14.2 Cloning chromosomal DNA flanking Tn*917*

Chromosomal DNA (2-5 μg) of each transposon mutant was digested in a total volume of 20 μl with either *Eco*RI or *Xba*I, overnight at 37 °C. The digested DNA was cleaned by phenol/chloroform extraction and resuspended in TE (250 μl). The DNA was then self-ligated (see section 2.9.1.2) overnight and, following ethanol precipitation, resuspended in TE (20 μl). Circularised DNA was transformed into *E. coli* XLOLR or DH5α, selecting for ampicillin resistance. Only the fragment of DNA containing pLTV1 can be transformed and maintained in *E. coli* so that any ampicillin resistant colonies should contain recircularised Tn*917* plus some flanking chromosomal DNA.

2.15 Polymerase Chain Reaction (PCR)

PCR reactions were carried out in an Eppendorf 5330 Mastercycler using the following constituents in PCR reaction tubes.

Template DNA	1 μg
Primer (Forward)	100 pmoles
Primer (Reverse)	100 pmoles

Reaction buffer (10X)	10 µl
$MgCl_2$	3 mM
dNTPs (1 mM stock)	10 µl
Taq polymerase (2.5U)	1 µl
Distilled H_2O	to 100 µl

Samples were equilibrated to 95 °C for 3 minutes to ensure DNA had melted and then the PCR reaction was performed using the cycle; heating to 95 °C for 1 minute to melt the DNA, cooling to 55 °C for 1 minute to anneal primers and template DNA and then heating to 72 °C for 2 minutes to allow extension of DNA. This cycle was repeated 30 times before the samples were cooled to, and then held at, 12 °C until required.

2.16 Automated sequencing

The sequencing reactions were performed using the Taq DyeDeoxy Terminator cycle sequencing kit supplied by Applied Biosystems. Plasmid DNA for sequencing was prepared by the large scale plasmid preparation method. A polymerase chain reaction (PCR) was carried out using 8 µl of the Terminator ready reaction mix, 1 µg template DNA and 3.2 pmol sequencing primer, and the volume made up to 20 µl with distilled H_2O.

The PCR reactions were performed in an Eppendorf 5330 Mastercycler. This was preheated to 96 °C, and then, once the tubes were inserted, a 25 cycle program was started. Each cycle consisted of a rapid thermal ramp to 96 °C which was held for 30 seconds, a rapid thermal ramp to 50 °C for 15 seconds, and finally a rapid thermal ramp to 60 °C for 4 minutes. At the end of the cycles the tubes were ramped to 4 °C and held at that temperature until required.

The reaction products were precipitated by transferring the reaction to a clean tube containing 2 µl sodium acetate (3 M pH 4.6) and 50 µl 95 % (v/v) ethanol. The reaction products were pelleted by centrifugation (14,000 rpm, 30 min, RT), washed with 70 % (v/v) ethanol and dried in a vacuum dessicator. Samples were loaded onto an Applied Biosystems 373A DNA sequencer according to the manufacturer's instructions.

2.17 Sequence analysis

Automated sequence data from the ABI 373A DNA sequencer was edited and analysed using the FASTA program. The database library was accessed via the Genetics Computer Group at the Daresbury laboratory, Warrington, England.

CHAPTER 3

Analysis of the role of environmental and physiological factors in the regulation of *agr* expression and activity

3.1 Introduction

Expression of virulence determinants in *S. aureus* is affected by many environmental conditions but how these stimuli are transduced is at present unknown. pH and glucose have been shown to affect *agr* expression (Regassa *et al.*, 1992) but it is unclear whether this occurs directly or via other components. α–haemolysin is a virulence determinant whose expression is positively regulated by *agr*, via RNAIII, but it is also subject to control in other, as yet unidentified, ways. For example, it has been shown that α–haemolysin production is controlled temporally, occurring at the post-exponential phase of growth in batch culture (Vandenesch *et al.*, 1991). Expression of α–haemolysin usually occurs two hours after RNAIII production, which becomes evident during mid-exponential growth. Experiments extending the exponential growth phase following induction of RNAIII demonstrated that α–haemolysin expression does not necessarily occur two hours after induction of RNAIII but, rather, at the beginning of post-exponential phase (which was some 4.5 hours later) (Vandenesch *et al.*, 1991). Regulation of virulence determinant expression is thus likely to be a complex response to multiple overlapping stimuli. In order to understand more of the regulatory hierarchy it is essential to know which stimuli are transduced via *agr*.

Other workers have measured the effect of environmental conditions on *agr* expression using Northern blots to measure RNA levels. Whilst this method is useful, it is complex, time consuming and can only measure RNA levels at a precise time point. A more simple assay was therefore needed so that the effects of multiple environmental and physiological conditions on *agr* expression could be examined. This has been achieved by the creation of a chromosomal transcriptional reporter gene fusion between *hld*, which measures expression of RNAIII from the P3 promoter (see Fig. 1.2), and *lacZ*.

The variety of reporter gene fusions available and the way in which they work is discussed in section 1.9 but, briefly, transcriptional reporter gene fusions work by

inserting promoterless genes encoding a measurable gene product downstream of a promoter of interest. This means that, when the promoter is transcriptionally active, the reporter gene is transcribed and the resulting gene product can be assayed to give a quantitative measure of promoter activity. Of the many reporter gene systems available, the *lac* system is probably the most widely used and, as mentioned, is the system used in this work.

A variety of directed reporter gene fusions have been developed in *S. aureus* in other laboratories but have mostly been plasmid based. For example, as detailed in section 1.9, the staphylococcal β–lactamase gene (*blaZ*) has been used to create plasmid based transcriptional fusions to each of the three promoters of the *agr* locus in order to study their expression (Kornblum *et al.*, 1990). The luciferase reporter system which was used in *S. aureus* to study the expression of TSST-1 was also plasmid based (Timmins *et al.*, 1995). Regulation of the epidermolytic toxin A (*eta*) gene of *S. aureus* has been examined by simultaneous use of two plasmid based fusions (Sheehan *et al.*, 1992). These two fusions used the luciferase (*luxAB*) genes of *Vibrio fischeri* or the catechol 2,3-dioxygenase (*xylE*) gene from *Pseudomonas putida* as the reporter. Although the *eta-luxAB* fusion provided a measure of real-time changes in promoter activity it was not usable into stationary phase, during which time the *eta-xylE* fusion became necessary (Sheehan *et al.*, 1992). Plasmid fusions suffer from several drawbacks, for example strains need to be maintained and grown in media containing appropriate antibiotics to ensure the plasmid is not lost. In addition, titration of regulatory elements can occur. A chromosomal reporter gene fusion was used in *S. aureus* to determine the expression of the major autolysin gene of *S. aureus, atl* (Foster, 1995). This involved the use of a suicide vector, pAZ106 (Kemp *et al.*, 1991) (Fig. 3.1) which, lacking a replicon functional in *S. aureus*, can only confer antibiotic marker resistance by means of integration into the chromosome. This method avoided problems associated with multi-copy plasmids, but, in this case, resulted in insertional inactivation of the *atl* gene.

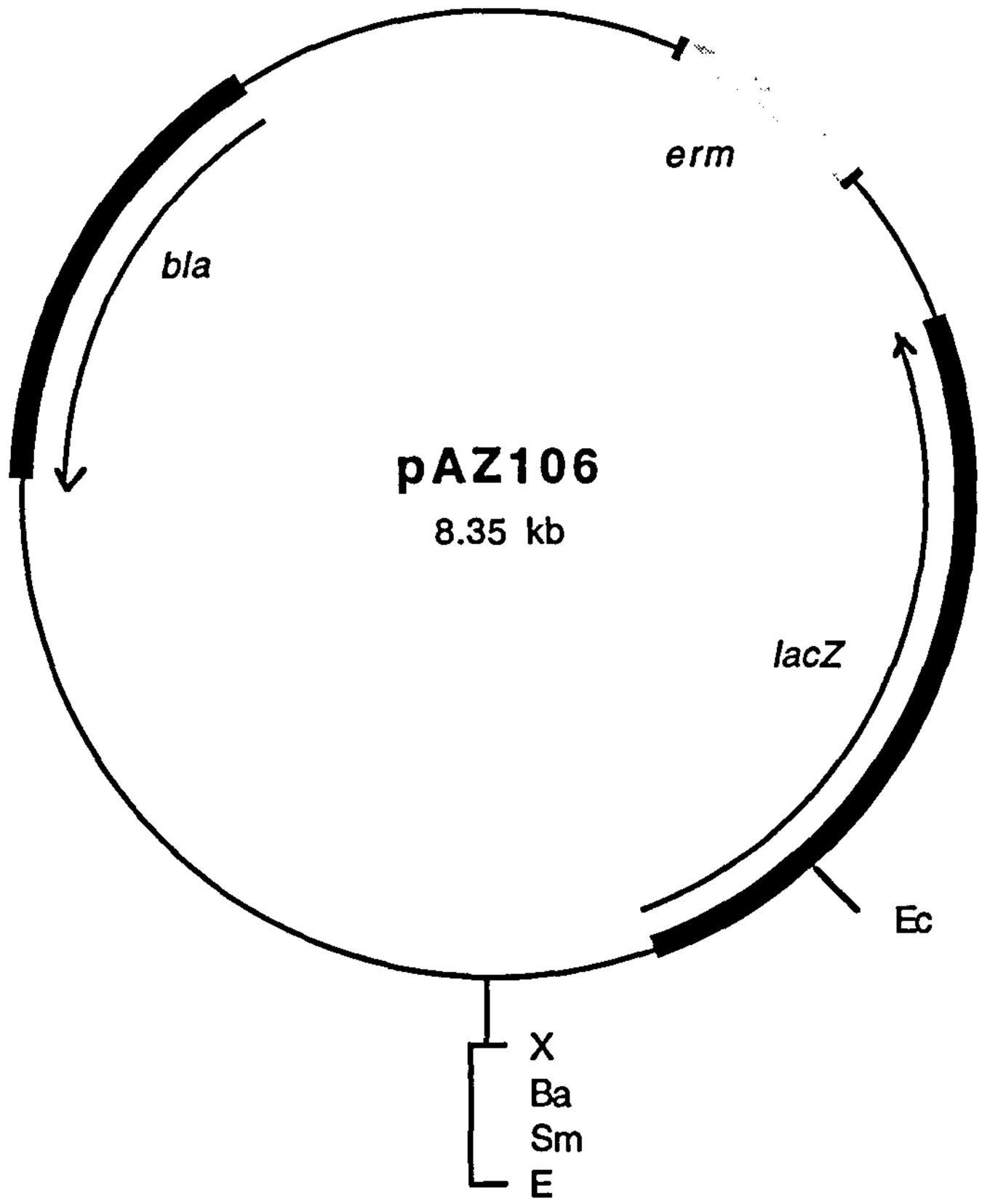


FIGURE 3.1

Map of pAZ106 (Kemp *et al.*, 1991). This plasmid contains an ampicillin resistance gene (*bla*), an Em resistance gene (*erm*) and a promoterless *lacZ* gene. The restriction sites denoted are : Ba, *Bam*HI; E, *Eco*RI; Ec, *Eco*RV; Sm, *Sma*I; X, *Xba*I.

The reporter fusion created in this work has also been produced using the *lacZ* gene of pAZ106 (Fig. 3.1) but without causing insertional inactivation of any part of *agr*. This method provided a stable construct and allowed a convenient assay for expression of RNAIII by monitoring β–galactosidase levels. Simultaneous measurement of α–haemolysin activity has allowed the identification of conditions which not only affect *agr* expression but also the expression of a virulence determinant under the positive transcriptional control of *agr*.

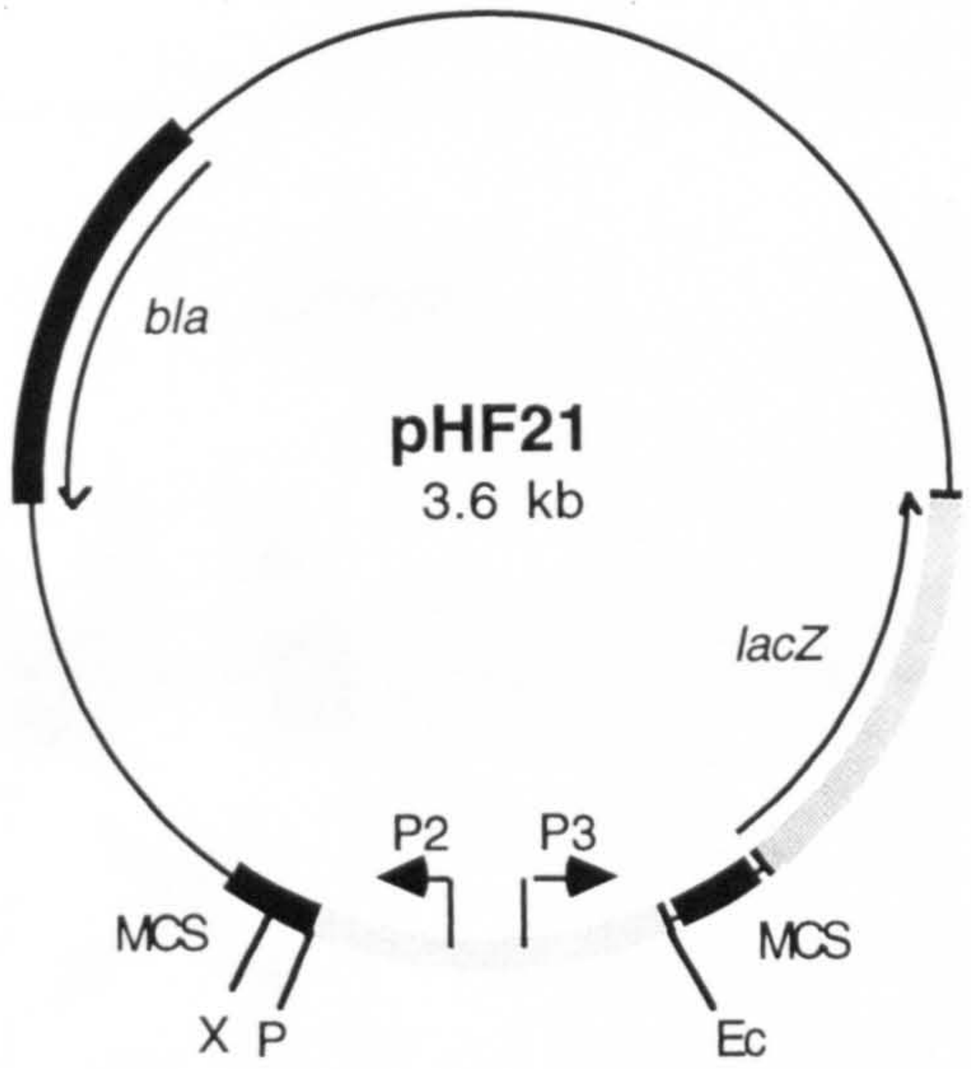
3.2 Results

3.2.1 Construction and verification of *hld::lacZ* fusion

The first step in creating the *hld::lacZ* transcriptional fusion was to isolate a 677 bp *PstI/Eco*RV fragment spanning the P3 promoter of the *agr* locus of *S. aureus* (see Fig. 1.2). This fragment was introduced into the similarly digested, dephosphorylated pUBS1 (Foster, 1995), to give appropriate flanking restriction sites for further subcloning. This insert was isolated from this construct (pHF21) (Fig. 3.2) as a 701 bp *XbaI/Eco*RV fragment and then subcloned into the suicide vector pAZ106 (Fig. 3.1) (digested with *XbaI/Sma*I) upstream of the promoterless *lacZ* gene in the orientation such that a transcriptional fusion between *hld* and *lacZ* was created (pHF23) (Fig. 3.2). pAZ106 has no functional replicon in *S. aureus* and so, following transformation with pHF23, erythromycin (Em) resistant clones occurred only as a result of plasmid integration by a single crossover between the 677 bp fragment of *agr* in the plasmid and homologous chromosomal DNA (Fig. 3.3). Hence a transcriptional *lacZ* fusion was created whereby the *lacZ* reporter gene is under the control of the P3 promoter of *agr*, in addition to an intact copy of *agr* (Fig. 3.3). *S. aureus* RN4220 was used as the host strain as it is able to accept foreign DNA. RN4220 containing integrated pHF23, designated SH100, had blue colonies when grown on BHI-erythromycin plates containing X-Gal, indicating β–galactosidase activity, whereas the parental strain was white. Total genomic DNA from SH100 was prepared and electroporated (Schenk and Ladagga, 1992) (1 μg) into *S. aureus* strain 8325-4. An Em resistant clone was recovered and called SH101. Transformation with naked DNA resulted in a double crossover event allowing stable Em resistance to be maintained.

The correct insertional event was confirmed by Southern blot analysis (Fig. 3.4). Chromosomal DNA from 8325-4 and SH101 was digested with *Eco*RV and resulting fragments were probed with a digoxygenin labelled 677 kb *Eco*RV/*Pst*I fragment of *agr*,

(a)



(b)

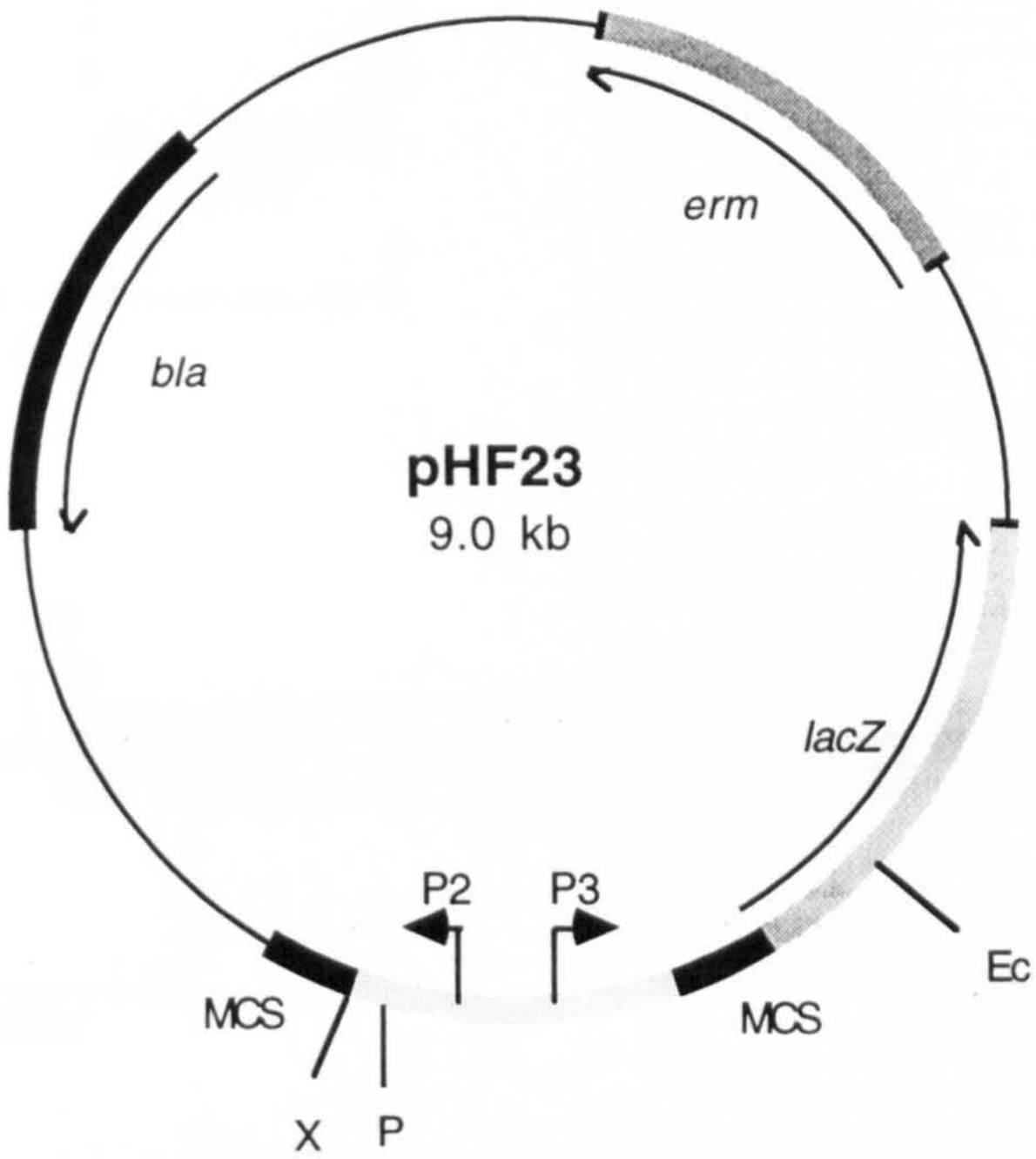


FIGURE 3.2

(a) pHF21 comprising pUBS1 (Foster, 1995) containing the 677 bp *Pst*I/*Eco*RV fragment of *agr*, spanning P3 promoter, within the multiple cloning site (MCS).
(b) pHF23 consisting of *Xba*I/*Sma*I digested pAZ106 (Kemp *et al.*, 1991) containing the 701 bp *Xba*I/*Eco*RV fragment isolated from pHF21.
The restriction enzymes denoted are: Ec, *Eco*RV; P, *Pst*I; X, *Xba*I

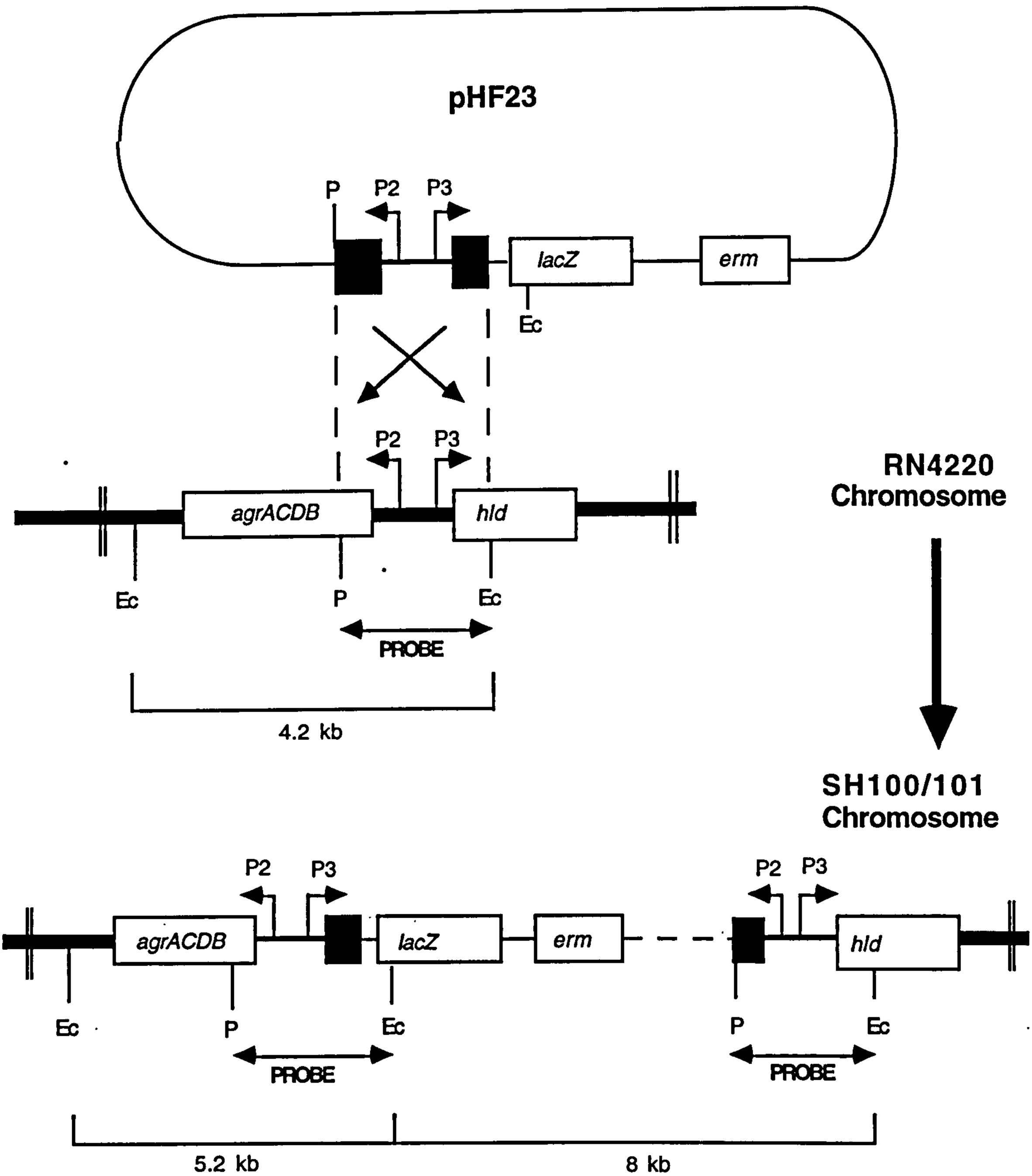


FIGURE 3.3

Construction of the *hld::lacZ* transcriptional reporter gene fusion strain, SH101. The extent of the *agr* locus is indicated by the symbol, ||. The hybridising bands indicated resulted from digesting chromosomal DNA of strains 8325-4 (same restriction pattern as RN4220) and SH101 with *Eco*RV and correspond to bands on the Southern blot (see Fig. 3.4). The extent of the probe (double headed arrow), comprising the digoxygenin labelled 677 bp *PstI/Eco*RV fragment of *agr* spanning the P3 promoter, is shown. The restriction sites denoted are Ec, *Eco*RV and P, *Pst*I. (This figure is not to scale).

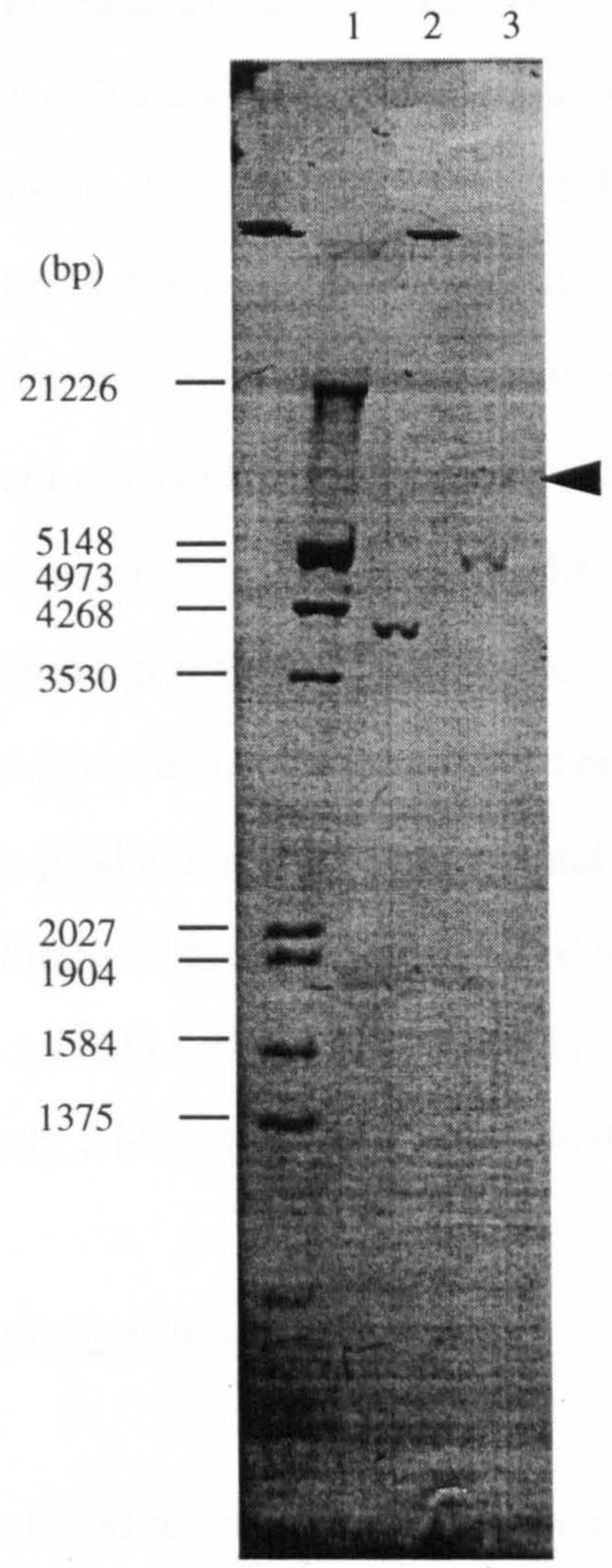


FIGURE 3.4

Southern blot to confirm the construction of strain SH101. Lane 1 shows *Eco*RI/*Hin*dIII digested λ marker, with the sizes of the fragments (bp) indicated on the left. Lanes 2 and 3 show hybridising fragments of *Eco*RV digested chromosomal DNA of strains 8325-4 and SH101, respectively, probed with the digoxygenin labelled 677 bp *Eco*RV/*Pst*I fragment of *agr*. (The arrow on the right indicates a band in lane 3 which was apparent on the original Southern blot but has not reproduced well on the scanned image shown).

spanning the P3 promoter (see Fig. 1.2). One band of approximately 4.2 kb of *Eco*RV digested chromosomal DNA of 8325-4 hybridised with the probe (Fig. 3.4), comprising the number of bases between the *Eco*RV sites at positions 1474 and 5646 of *agr* (Fig. 1.2). As predicted, two fragments of approximately 5.2 and 8 kb of EcoRV digested chromosomal DNA from strain SH101 hybridised with the probe, representing the additional 9 kb of pAZ106 plus the insert (Fig. 3.4). The derivation of these fragments is shown in Fig. 3.3. The integration is stable and does not need to be maintained by the presence of antibiotics. In addition, it is important to note that an intact copy of RNAIII remains in strain SH101 and that this strain grows identically to the wild type, producing α–haemolysin at a comparable level to its parent, approximately 150 haemolysin units (HU) (see section 2.12.2) following overnight growth at 37 °C. In order to investigate *agr* expression, via the *hld::lacZ* reporter gene fusion, under different environmental and physiological conditions, β–galactosidase production was measured and calculated in Miller units (MU) (see section 2.12.1). Strain 8325-4, which was used as a control in all the following experiments, was not blue on X-Gal plates and produced negligible amounts of β–galactosidase (approximately 2 MU after overnight growth).

3.2.2 Development of a standard experimental procedure

The level of expression of *hld::lacZ* was found to be very dependent on the growth conditions of the culture prior to inoculation into the experimental flask (100 ml BHI in a 250 ml flask). For example, inoculating cultures from plates >1 day old led to reduced β–galactosidase production, falling from an average of 100 MU to 35 MU in cultures inoculated from plates 2 or 3 days old. Similarly, inoculating from plates into BHI and growing overnight often resulted in lower levels of β–galactosidase (~100 MU) compared to cultures inoculated from a broth (~140 MU). Smaller volume cultures (25 ml) grown in 250 ml flasks shaking at 250 rpm also produced less β–galactosidase, possibly due to an aeration effect (see section 3.3). The most consistent expression of β–galactosidase was found to be obtained by inoculating BHI (100 ml) from an overnight plate, growing

the culture to exponential phase (OD_{600} of 1-2) (see Fig. 3.5) and then inoculating test and control flasks (250 ml) containing BHI (100 ml) to an OD_{600} of 0.05. Since it appeared that increasing the length of exponential growth encouraged maximal *hld::lacZ* expression these standard conditions were strictly adhered to. Each experiment was repeated between 3-6 times and all the values given below represent the mean obtained for each sample for the number of experiments stated.

3.2.3 Viable counts

Viable counts were taken of cells grown in 1X and 0.25X BHI to ensure that the final OD_{600} showed a relatively constant relationship to final cell numbers even when growth conditions were altered. There was found to be a good correlation between final cell numbers and OD_{600} following overnight growth in BHI at 1X and 0.25X concentrations, reaching 3.2×10^9 cfu/ml in 1X at an OD_{600} of 10 and 1.2×10^9 cfu/ml at an OD_{600} of 3.5 in 0.25X. This is equivalent to 3.2×10^8 cfu/ml or 3.4×10^8 cfu/ml at an OD_{600} of 1.0 in 1X or 0.25X BHI, respectively.

3.2.4 *hld::lacZ* expression during growth

Production of β-galactosidase by SH101 reflects *hld::lacZ* expression and hence RNAIII production and was monitored during growth using ONPG as the substrate (Fig. 3.5) (see section 2.12.1.2). The expression of *hld::lacZ* was very low during exponential phase (<10 MU) but began to rise sharply 5 hours into growth at an OD_{600} of 5.2 which corresponds to the transition between exponential and stationary phase. Thereafter, β-galactosidase levels continued to rise rapidly for 6-8 hours to approximately 160 MU before falling slightly and stabilising at 140 MU. It was possible that the apparent low levels of *hld::lacZ* transcription during exponential phase were due to the insensitivity of the assay and so the more sensitive substrate MUG was used (Youngman, 1990) (see section 2.12.1.1). Using this method, expression became apparent up to 30 minutes

earlier than with ONPG but the expression profile was unchanged (results not shown). Typically, 18 hours was chosen as the standard time for comparative samples to be taken in later experiments. α–haemolysin activity became apparent approximately 2 hours after the onset of *hld::lacZ* expression and increased sharply giving a final activity of approximately 150 haemolysin units (HU) (Fig. 3.5).

3.2.5 Effect of growth medium

Strain SH101 was initially grown in BHI, Tryptic Soy Broth (TSB) and a Chemically Defined Medium (CDM) (Hussein *et al.*, 1991) to evaluate the effect of different media on final OD_{600} and *hld::lacZ* expression. Cultures grown in BHI produced the highest levels of *hld::lacZ* expression, reaching an average of 144 MU after 18 hours, whilst β-galactosidase levels in cultures grown in TSB or CDM reached an average of 85 MU or <10 MU, respectively. α–haemolysin levels were similarly reduced in the different media. A similar trend in the final OD_{600} of cultures was observed with growth in BHI reaching an average OD_{600} of 10.0, in TSB an OD_{600} of 7.0 and in CDM an OD_{600} of 3.5. These values represent the average of 3 independent experiments and varied by less than 11 %.

To give an indication as to whether expression of *hld::lacZ* was related to cell density, the effect of medium concentration was examined. Decreasing the concentration of BHI to 0.5X and 0.25X normal strength (37 g/l) resulted in a marked reduction in β-galactosidase levels to an average of 25 MU and 8 MU, respectively compared to an average of 138 MU in the control cultures (Fig. 3.6). The rate of growth was unaffected but the final OD_{600} was lower in 0.5X (OD_{600} of 5.5) and 0.25X (OD_{600} of 3.2) compared to 1X BHI (OD_{600} of 9.3). α–haemolysin activity was also affected, falling from an average of 154 HU in the control cultures to an average of 62 HU in 0.5X BHI (Fig. 3.6). There was no detectable activity in 0.25X BHI (Fig. 3.6). These values represent an average of 5 independent experiments and varied by less than 15 %.

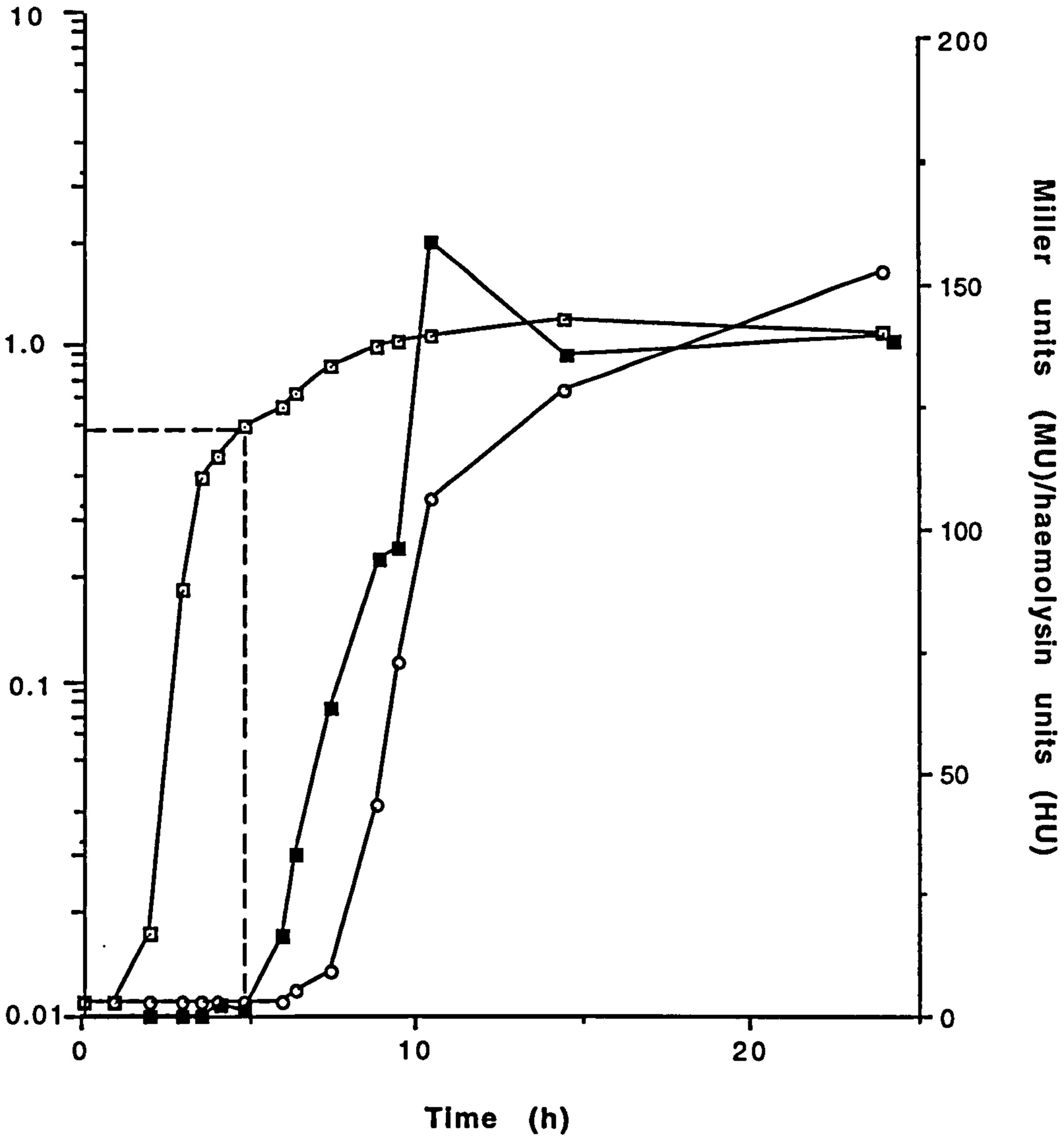


FIGURE 3.5

Expression of *hld::lacZ* and α–haemolysin activity during growth. Strain SH101 was grown in BHI at 37 °C with shaking at 250 rpm. Growth, as absorbance at 600 nm (□), *hld::lacZ* expression as β-galactosidase activity (■ Miller units) and α-haemolysin activity (○ haemolysin units) were measured as described in Chapter 2. The dotted lines indicate the time during growth at which the β-galactosidase level started to rise.

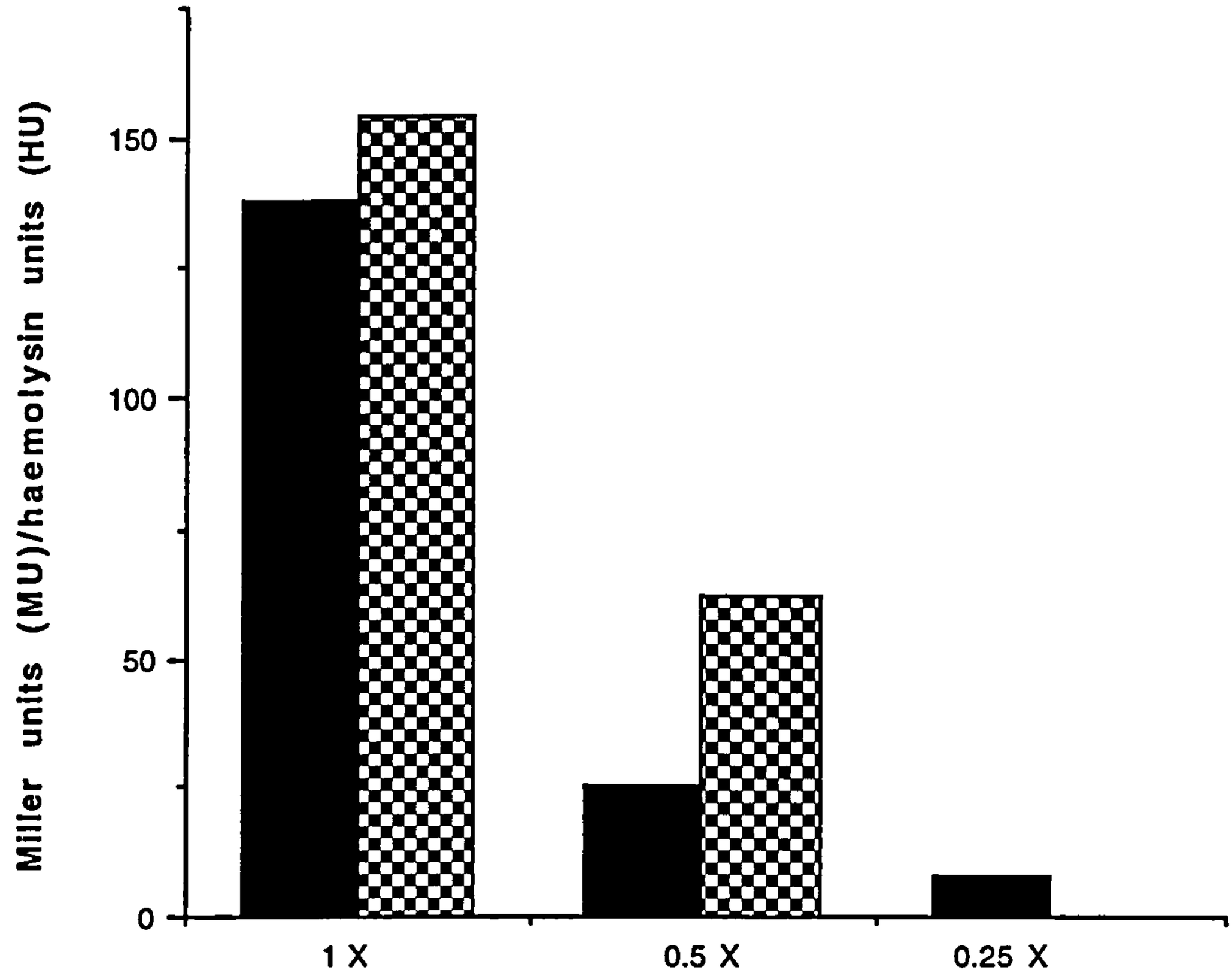


FIGURE 3.6

Effect of medium concentration on *hld::lacZ* expression and α–haemolysin activity. Strain SH101 was grown in 1X, 0.5X or 0.25X BHI and β-galactosidase activity (■ Miller units) or α-haemolysin activity (■ haemolysin units) were measured. The value for each sample represents the mean of 5 independent experiments which varied by <15 %.

Addition of dried skimmed milk (0.1, 1 or 10 % w/v) or bovine serum albumin (0.5 % w/v) to CDM did not lead to an increase in overnight growth yield. β–galactosidase and α–haemolysin production were similarly unaffected.

Subsequently, an alternative CDM (Holland *et al.*, 1994) was tried and, although growth was good, reaching an average OD_{600} of 13 compared to 9.6 in BHI during 3 independent experiments, β–galactosidase levels were an average of 27-fold less than in BHI (5 MU vs 135 MU).

3.2.6 Effect of homoserine or homoserine lactone

Homoserine lactone (HL) is a density dependent signalling compound found in some Gram negatives (see section 1.5.3). To see whether the presence of HL in CDM (Hussein *et al.*, 1991) might activate *hld::lacZ* expression, two variants of HL (L or DL) (1 mM) were added and cells grown overnight. The final level of growth was slightly improved reaching an OD_{600} of 4.1 or 5.1 in L- or DL-HL respectively but no β–galactosidase activity could be measured.

In addition L-homoserine or homoserine lactone (L- or DL-) (1 mm) was added to cells grown in BHI during exponential phase (OD_{600} of 2.0) to determine whether early induction of *hld::lacZ* would occur. The rate of growth was not affected and there was no early induction of *hld::lacZ* expression.

3.2.7 Effect of osmolarity

The effect of the osmolarity of the growth medium on *hld::lacZ* expression was determined by adding NaCl or sucrose (up to 2 M) to BHI. As NaCl concentration increased above the 0.08 M level present in BHI, so β–galactosidase levels rose giving an average 4- to 5-fold increase in 0.5 or 1 M NaCl, respectively, compared to an average of

129 MU in the control samples (Fig. 3.7). Surprisingly, compared with the control (143 HU), α–haemolysin levels decreased to 29 HU and 2 HU in 0.5 M and 1 M NaCl respectively (Fig. 3.7). The lack of α–haemolysin activity was not due to a direct effect of salt on the protein as addition of equivalent concentrations of NaCl to a sample of known haemolytic activity did not inhibit haemolysis. At concentrations of NaCl above 1 M *hld::lacZ* expression sharply declined to 25 MU in 1.5 M NaCl and 2 MU in 2 M NaCl. α–haemolysin activity could not be detected at either concentration of NaCl. The final OD_{600} of the cultures was reduced from 10.0 in BHI to 8.3, 7.1, 6.9 or 5.8 in 0.5, 1, 1.5 or 2 M NaCl, respectively. These values represent an average of 6 independent experiments and varied by less than 25 %.

Supplementing BHI with 0.5 M and 1 M sucrose caused a marked reduction in β–galactosidase levels which, with 1 M sucrose, fell to an average of 9 MU compared to the average of 129 MU obtained in the BHI control (Fig. 3.7). Growth of SH101 was also affected, growing to an average OD_{600} of 6.9 or 5.3 in 0.5 or 1 M sucrose compared to an average OD_{600} of 10.2 in the control. The bacteria proved unable to grow in sucrose concentrations at, or above, 1.5 M. No α–haemolysin activity could be detected, even at concentrations as low as 0.02 M sucrose which did not inhibit β–galactosidase production (Fig. 3.7). These values, similarly, represent an average of 6 independent experiments that varied by less than 12 %.

3.2.8 Effect of glucose

The addition of 0.25 % (w/v) glucose to BHI led to an increase in β–galactosidase levels from an average of 143 MU in the BHI control (which contains 0.25 % w/v glucose) to an average of 206 MU (Fig. 3.8). At 0.5 % or 1 % (w/v) glucose, levels were markedly reduced to an average of 53 MU or 10 MU respectively. Growth was inhibited as glucose concentration increased, typically falling from an OD_{600} of 10.0 in the control to an OD_{600} of 8.4, 7.2 or 6.4 in 0.25, 0.5 or 1 % (w/v) glucose. α–haemolysin activity was

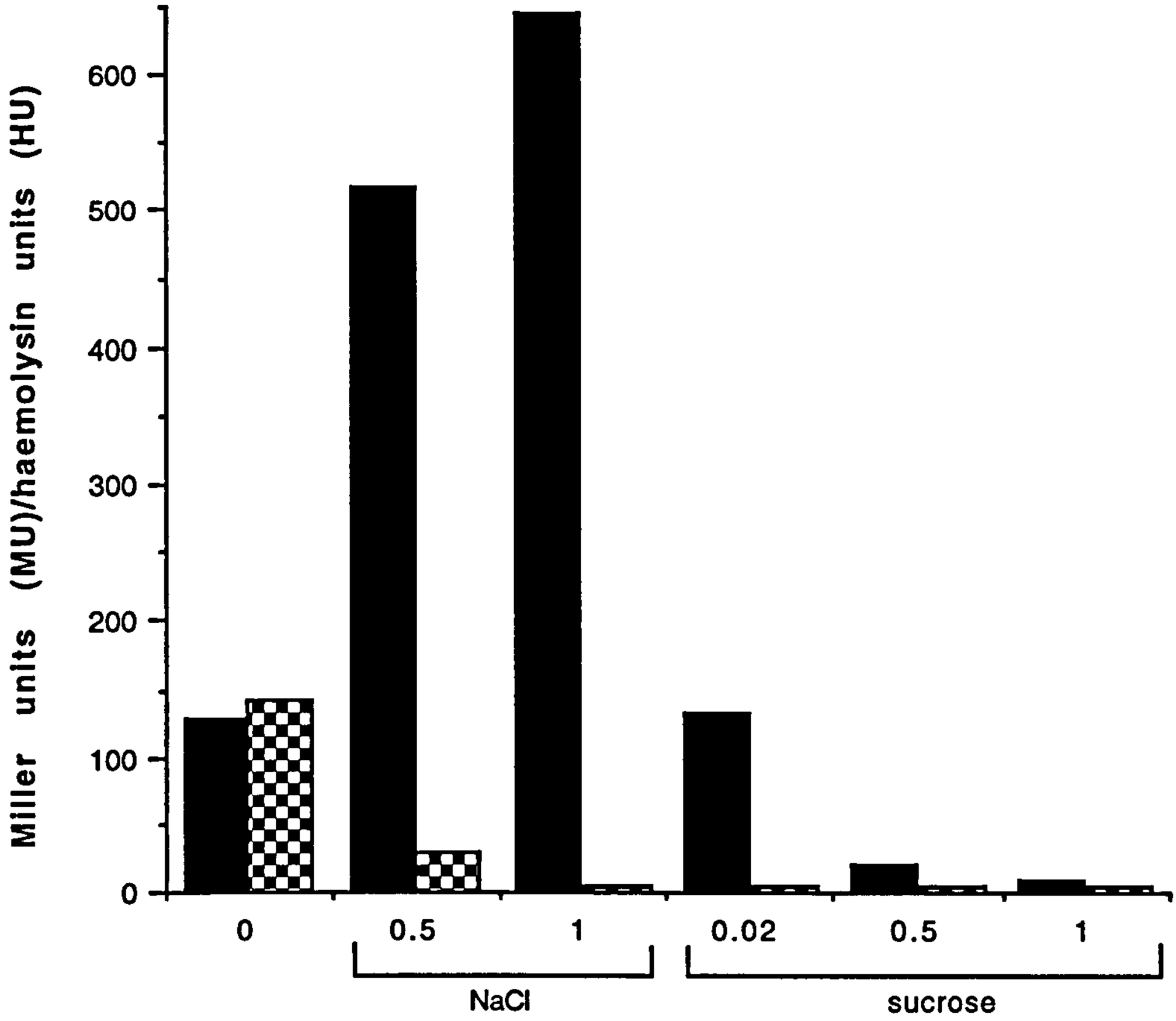


FIGURE 3.7

Effect of NaCl and sucrose on *hld::lacZ* expression and α–haemolysin activity. Strain SH101 was grown in BHI containing NaCl or sucrose, where stated. β-galactosidase activity (■ Miller units) or α-haemolysin activity (◘ haemolysin units) were measured. The value for each sample represents the mean of 6 independent experiments which varied by <25 % (sucrose) or <12 % (NaCl).

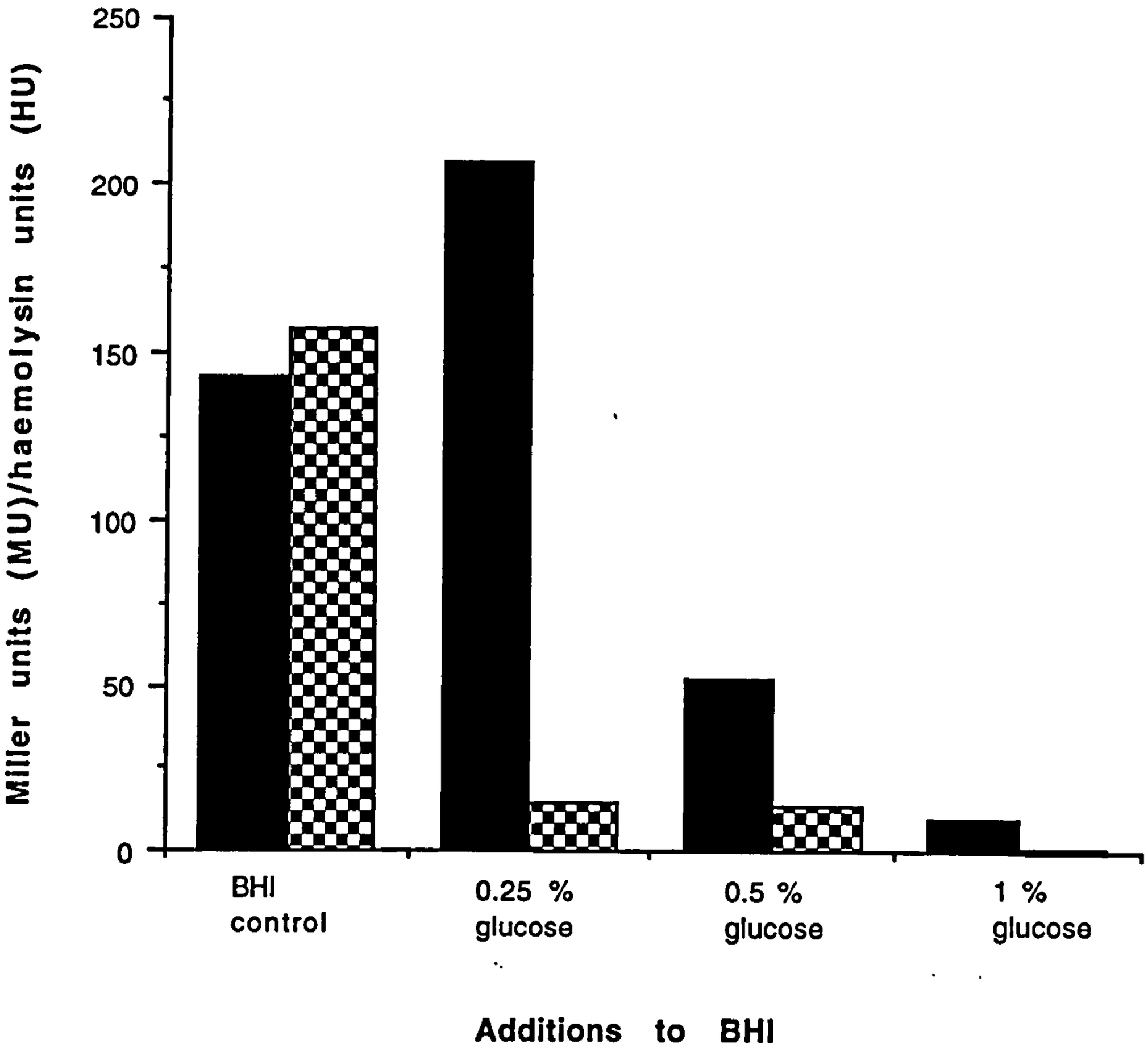


FIGURE 3.8

Effect of glucose on *hld::lacZ* expression and α–haemolysin activity. Strain SH101 was grown in BHI containing glucose (0.25 %, 0.5 % or 1 % w/v) and β-galactosidase activity (■ Miller units) or α-haemolysin activity (⊡ haemolysin units) were measured. The value for each sample represents the mean of 4 independent experiments which varied by <17 %.

reduced to an average of 14 HU, 13 HU or 0 HU at each respective concentration of glucose, compared to an average of 157 HU in the control samples (Fig. 3.8). These values represent an average of 4 independent experiments and varied by less than 17 %.

3.2.9 Effect of calcium and magnesium levels

Cultures grown in BHI supplemented with calcium chloride (5 mM) or magnesium chloride (up to 20 mM) did not have a significant effect either on β–galactosidase activity, α–haemolysin levels or final OD_{600}. However, whilst the addition of 0.5 mM ethylene glycol-bis(β–aminoethylether)N,N,N',N'-tetraacetic acid (EGTA) to create a calcium-restricted environment (Chambers, 1993) did not affect cell growth, there was a 5-fold increase in *hld::lacZ* activity (Fig. 3.9). This effect was reflected more markedly in α–haemolysin levels where there was an increase of up to 18-fold in the calcium restricted medium compared to the BHI control. Supplementing BHI containing EGTA with 5 mM calcium chloride restored both β–galactosidase and α–haemolysin activities to approximately those found in BHI alone. The addition of 20 mM $MgCl_2$ to media already containing EGTA also led to a reduction in β–galactosidase or α–haemolysin levels, to approximately 585 MU or 1153 HU, respectively, compared to 740 MU or 2415 HU in cultures grown in BHI containing only EGTA (Fig. 3.9). These values represent an average of 3 independent experiments and varied by less than 15 %.

The above experiments were also carried out substituting 0.5 mM of ethylenediaminetetraacetic acid (EDTA) (Chambers, 1993) for EGTA. A similar trend was observed with β–galactosidase and α–haemolysin levels, rising up to 5-fold and 10-fold, respectively whilst the final OD_{600} decreased to an average of 6.2 from 9.3 upon addition of EDTA. However, addition of either $MgCl_2$ (20 mM) or $CaCl_2$ (5 mM) restored final growth levels to normal. β–galactosidase activity also returned to control levels, as did α–haemolysin production in the presence of additional $CaCl_2$. Adding $MgCl_2$ to EDTA- treated cultures resulted in α–haemolysin levels only 2-fold above that

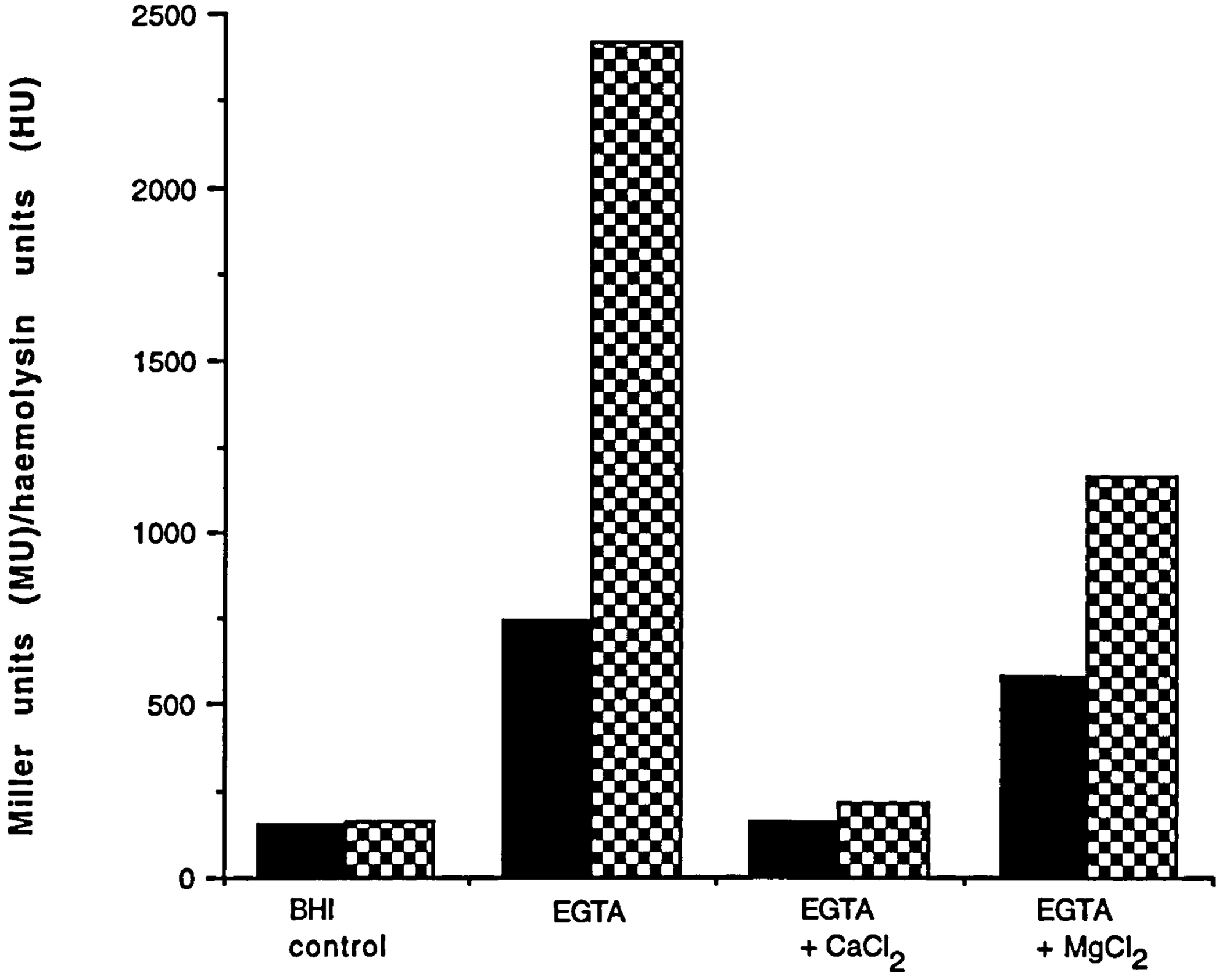


FIGURE 3.9

Effect of availability of divalent cations on *hld::lacZ* expression and α–haemolysin activity. Strain SH101 was grown in BHI containing EGTA (0.5 mM) or EGTA ± $CaCl_2$ (5 mM) or $MgCl_2$ (20 mM and β-galactosidase activity (■ Miller units) or α-haemolysin activity (■ haemolysin units) were measured. The value for each sample represents the mean of 3 independent experiments which varied by <15 %.

produced in BHI alone which showed an average of 155 HU. These values represent an average of 3 independent experiments and varied by less than 20 %.

3.2.10 Effect of temperature

Optimum *hld::lacZ* expression occurred at 42 °C and was approximately twice that of the control which reached an average of 139 MU at 37 °C (Fig. 3.10). Cells grown at 30 °C exhibited an average of only 50 MU. The final growth level was relatively unaffected at 30 °C (OD_{600} of 8.9) but was repressed at 42 °C (OD_{600} of 6.7). The most marked effect of temperature was the dramatic increase in α-haemolysin production at 42 °C, at which temperature levels of this toxin were up to 8-fold higher than the average of 150 HU obtained at 37 °C (Fig. 3.10). These values represent an average of 4 independent experiments and varied by less than 7 %.

3.2.11 Effect of heat shock

SH101 was grown to exponential phase (OD_{600} of 1.0) at 37 °C, heat shocked for 5 or 10 minutes at 47 °C or 53 °C and then returned to 37 °C for approximately 18 hours. This did not result in a significant alteration to the kinetics of growth or the final OD_{600} reached. Levels of β–galactosidase production were not markedly altered whilst α haemolysin activity was affected after heat shocking at 53 °C, falling to an average of 100 HU compared to an average of 148 HU found in the control. Assaying samples at 30, 60 and 90 minutes immediately following heat shock showed that early expression of *hld::lacZ* was not induced. These values represent an average of 3 independent experiments and varied by less than 22 %.

3.2.12 Effect of alcohols

Growth in the presence of isopropanol (1 or 2 % v/v) in BHI resulted in a decrease in β-

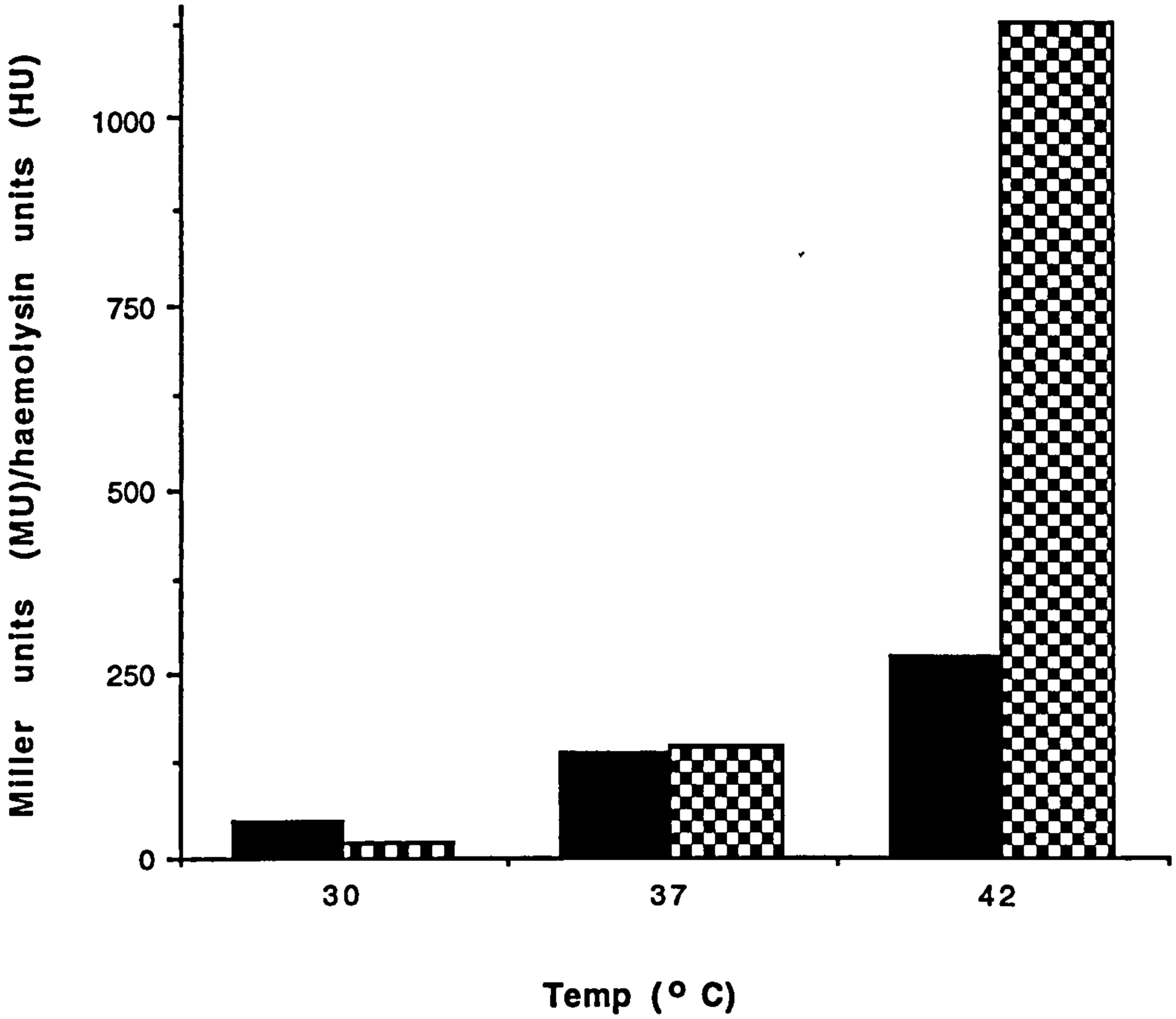


FIGURE 3.10

Effect of temperature on *hld::lacZ* expression and α–haemolysin activity. Strain SH101 was grown in BHI at 30 °C, 37 °C or 42 °C and β-galactosidase activity (■ Miller units) or α-haemolysin activity (■ haemolysin units) were measured. The value for each sample represents the mean of 4 independent experiments which varied by <7 %.

galactosidase levels to an average of 48 MU or 20 MU, and α-haemolysin levels to 36 HU or 2 HU, compared to the BHI control which showed an average of 150 MU or 157 HU (Fig. 3.11). The addition of ethanol (1 or 2 % v/v) enhanced *hld::lacZ* expression to an average of 321 MU or 300 MU, respectively and altered α–haemolysin activity to an average of 273 HU or 31 HU at each respective concentration (Fig. 3.11). Final OD_{600} was 10.0 in the control, 6.8 or 5.9 in 1 or 2 % (v/v) ethanol and 9 or 8.9 in 1 or 2 % (v/v) isopropanol respectively. These values represent an average of 5 independent experiments and varied by less than 21 %.

3.2.13 Effect of glycerol monolaurate (GML)

Cells grown in BHI containing GML (up to 0.2 mg/ml) did not show any marked change in β–galactosidase activity, or final OD_{600} (8.8), compared with cells grown in BHI alone which showed an average of 143 MU (Fig. 3.12). However, there was no α–haemolysin activity in the presence of 0.2 mg/ml GML (Fig. 3.12). This was not due to a direct effect of GML on α–haemolysin activity since adding GML (0.2 mg/ml) to a control sample of known haemolytic activity did not lead to any decrease in haemolysin units. These values represent an average of 3 independent experiments and varied by less than 8 %.

3.2.14 Effect of sodium citrate

When SH101 was grown in BHI supplemented with 0.1 to 0.25 % (w/v) sodium citrate there was a dramatic reduction in β–galactosidase levels from an average of 132 MU obtained in BHI alone. Addition of 0.1 % or 0.25 % sodium citrate resulted in a 5-fold or 20-fold reduction in β–galactosidase activity respectively (Fig. 3.13). There was a corresponding loss of α–haemolysin activity (4-fold at 0.1% and 25-fold at 0.25 %) (Fig. 3.13) but final cell density remained relatively unaffected, reaching an average OD_{600} of 9.4 or 8.2 in 0.1 or 0.25 % sodium citrate compared to an OD_{600} of 9.8 in BHI alone.

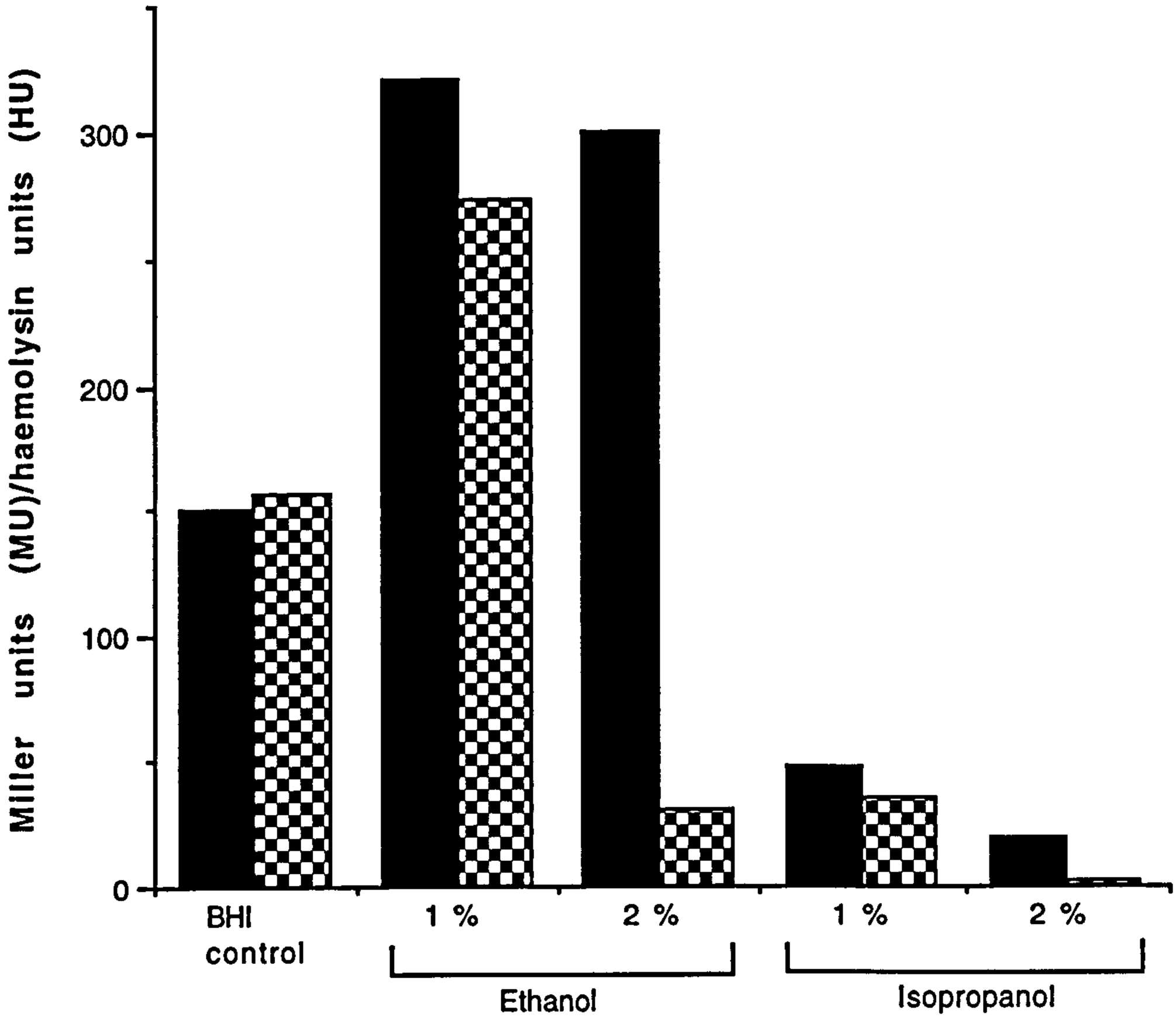


FIGURE 3.11

Effect of isopropanol or ethanol on *hld::lacZ* expression and α–haemolysin activity. Strain SH101 was grown in BHI containing isopropanol (1 % or 2 % v/v) or ethanol (1 % or 2 % v/v) and β-galactosidase activity (■ Miller units) or α-haemolysin activity (◪ haemolysin units) were measured. The value for each sample represents the mean of 5 independent experiments which varied by <21 %.

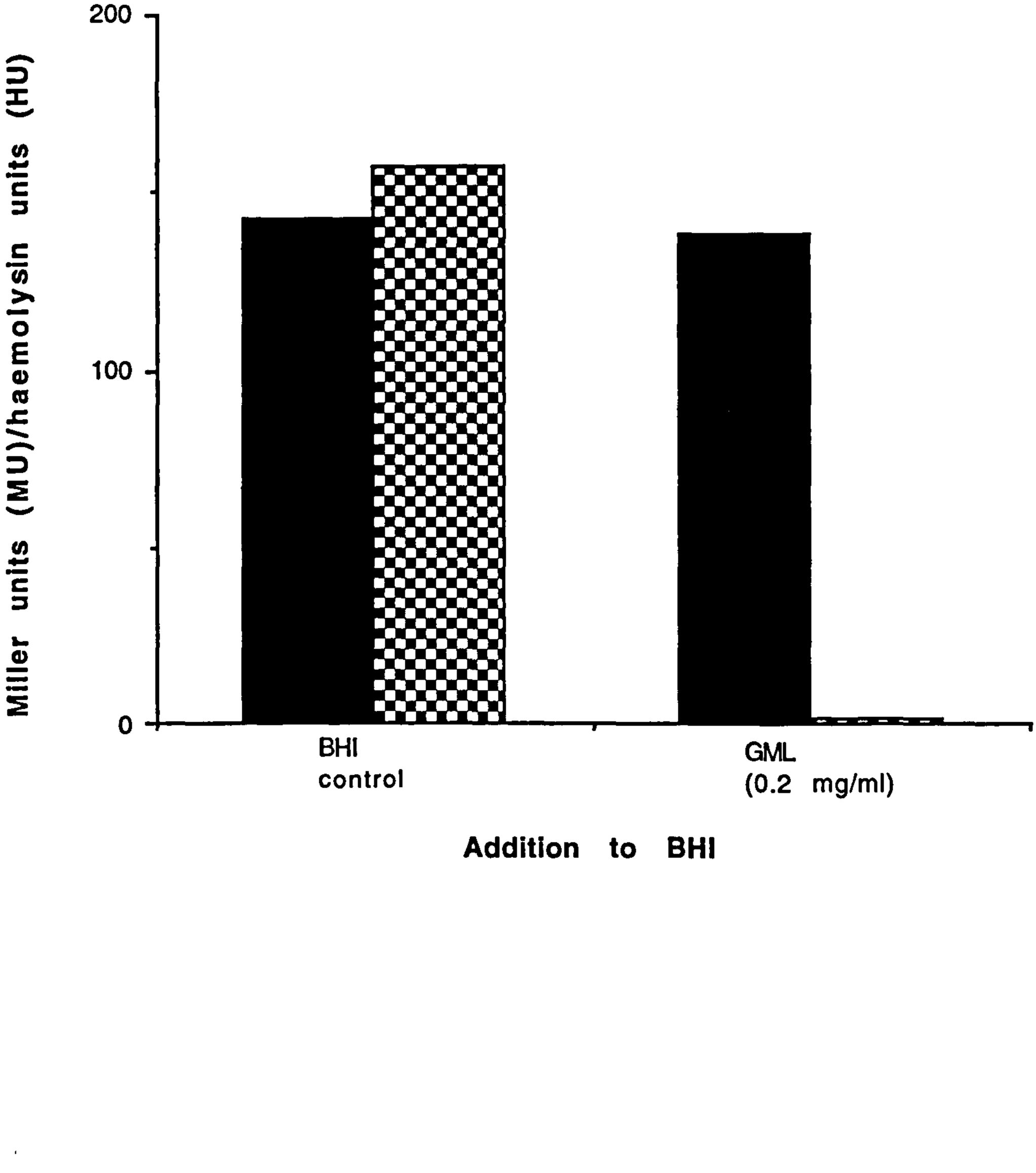


FIGURE 3.12

Effect of glycerol monolaurate (GML) on *hld::lacZ* expression and α–haemolysin activity. Strain SH101 was grown in BHI containing GML (0.2 mg/ml) and β-galactosidase activity (■ Miller units) or α-haemolysin activity (▣ haemolysin units) were measured. The value for each sample represents the mean of 3 independent experiments which varied by <8 %.

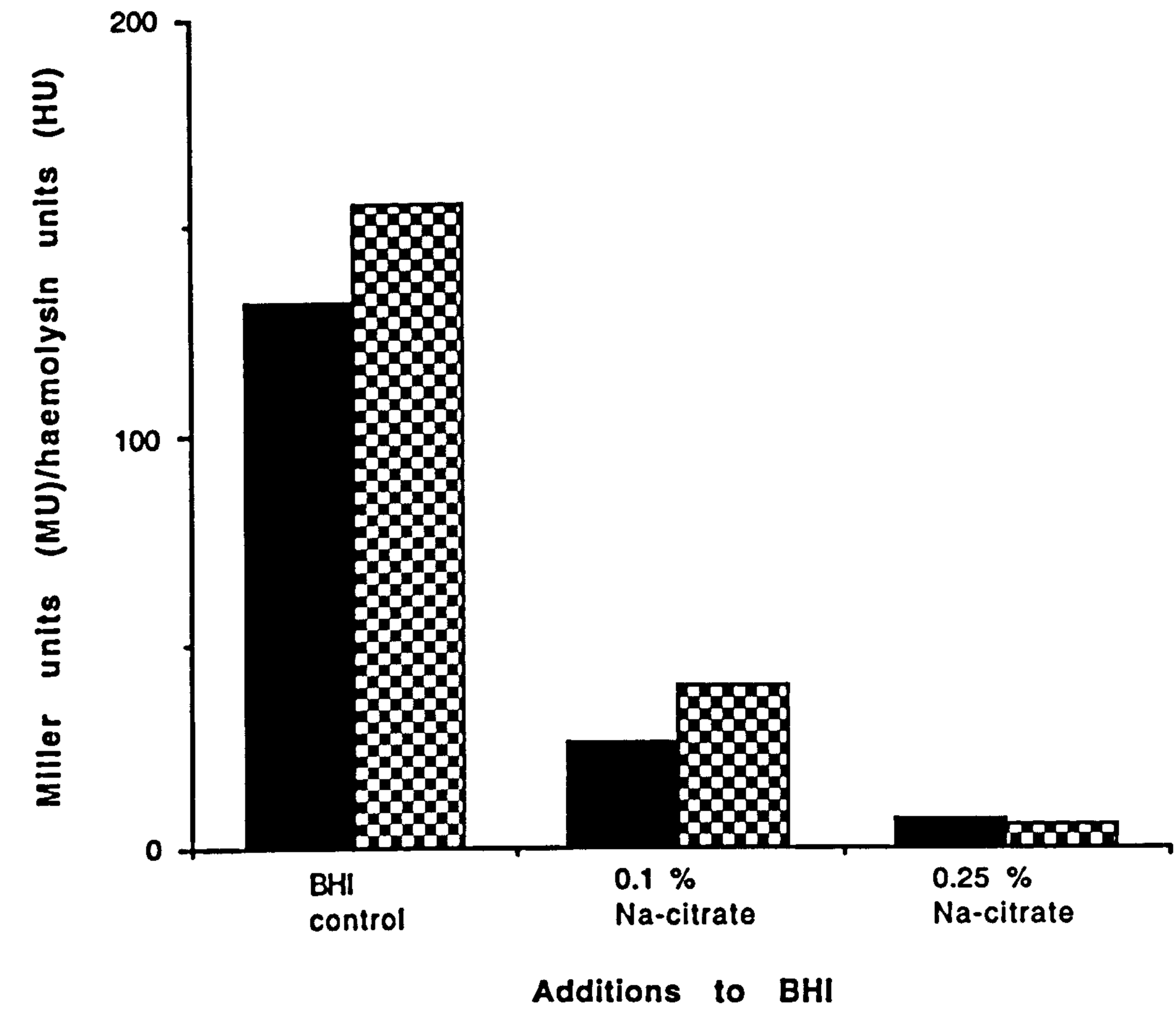


FIGURE 3.13

Effect of sodium citrate on *hld::lacZ* expression and α-haemolysin activity. Strain SH101 was grown in BHI containing either 0.1 % or 0.25 % (w/v) sodium citrate and β-galactosidase activity (■ Miller units) or α-haemolysin activity (◘ haemolysin units) were measured. The value for each sample represents the mean of 3 independent experiments which varied by <6 %.

These values represent an average of 3 independent experiments and varied by less than 6 %.

3.2.15 Effect of oxygen availability

Cultures were grown anaerobically in an anaerobic jar (BBL GasPak System) without shaking. Anaerobic growth resulted in a 15-fold or 20-fold reduction in *hld::lacZ* expression or α-haemolysin activity, respectively when compared to aerobically grown cells which showed an average of 141 MU and 149 HU (Fig. 3.14). Final OD_{600} was severely reduced reaching an average of only 3.2 after 48 hours of anaerobic growth compared to an average OD_{600} of 9.2 after 18 hours of aerobic growth. These values represent an average of 6 independent experiments and varied by less than 33 %.

3.2.16 Effect of sodium bicarbonate

Growing cells in BHI with either 0.2 % or 0.4 % $NaHCO_3$ (w/v) did not result in any change in β-galactosidase levels (Fig. 3.15). OD_{600} was slightly affected reaching an average of 8.9 in the control and 8.2 or 7.5 in the presence of 0.2 % or 0.4 % (w/v) $NaHCO_3$ respectively. α-haemolysin levels were most affected rising >2-fold in both concentrations of sodium bicarbonate from an average of 151 HU obtained in BHI alone (Fig. 3.15). These values represent an average of 4 independent experiments and varied by less than 15 %.

3.2.17 Effect of altering multiple environmental conditions

High levels of *hld::lacZ* expression have been observed under conditions of reduced Ca^{2+}, 0.5 or 1 M NaCl or at increased temperature, up to 42 °C. The effects on *hld::lacZ* expression of altering combinations of two of these environmental conditions was studied. Growth of SH101 in the presence of 0.5 mM EGTA at 42 °C did not

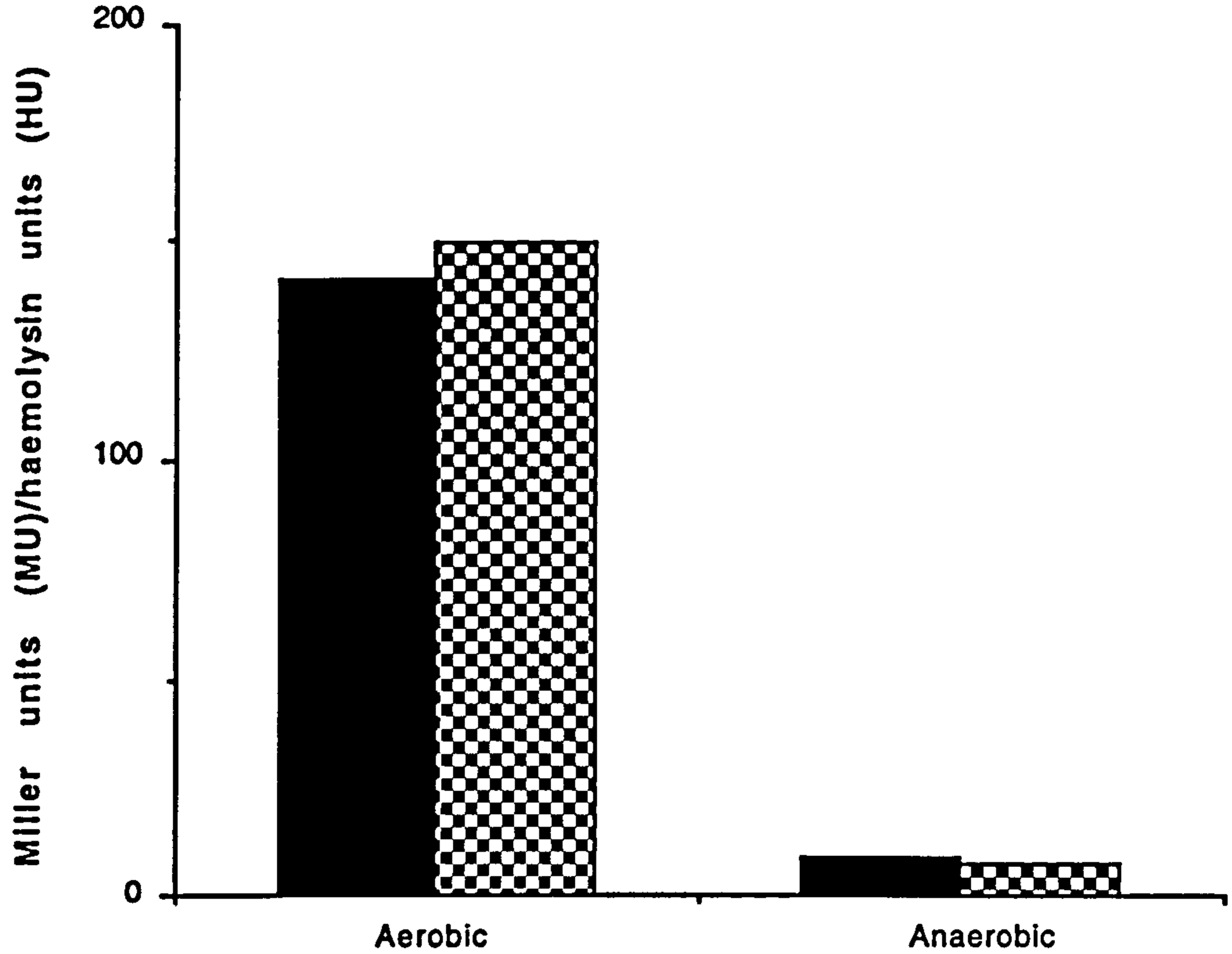


FIGURE 3.14

Effect of oxygen availability on *hld::lacZ* expression and α–haemolysin activity. Strain SH101 was grown in BHI aerobically or anaerobically and β-galactosidase activity (■ Miller units) or α-haemolysin activity (⊡ haemolysin units) were measured. The value for each sample represents the mean of 6 independent experiments which varied by <33 %.

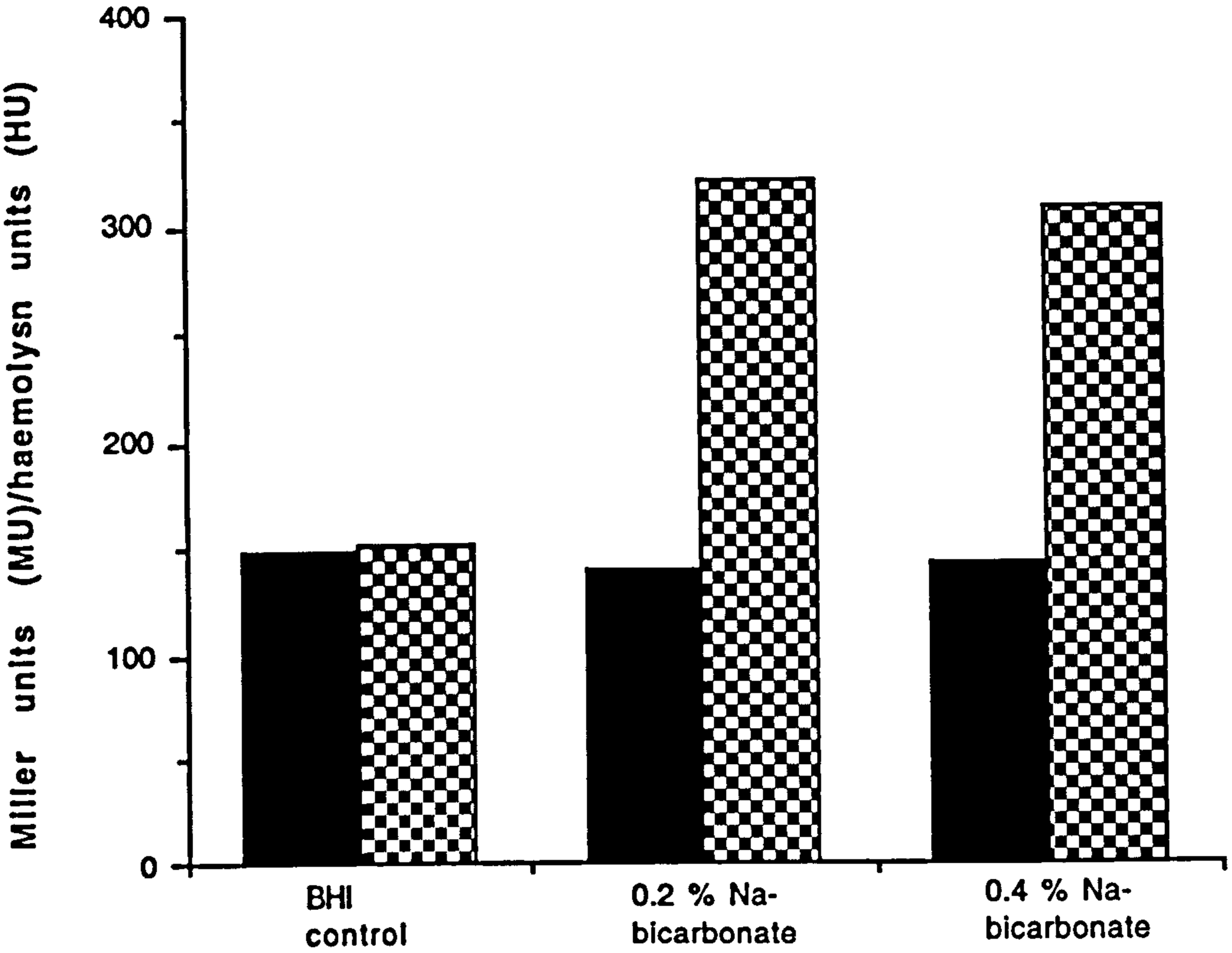


FIGURE 3.15

Effect of sodium bicarbonate ($NaHCO_3$) on *hld::lacZ* expression and α–haemolysin activity. Strain SH101 was grown in BHI containing either 0.2 % or 0.4 % (w/v) $NaHCO_3$ and β-galactosidase activity (■ Miller units) or α-haemolysin activity (◪ haemolysin units) were measured. The value for each sample represents the mean of 4 independent experiments which varied by <15 %.

lead to a significant increase or decrease in β–galactosidase levels above that obtained in the presence of 0.5 mM EGTA alone. Similarly, combining the addition of NaCl (0.5 M or 1 M) and EGTA did not lead to an increase in β–galactosidase production over and above that obtained in either EGTA or NaCl alone. There was an approximately 4-fold increase in cultures grown with combined NaCl and EGTA compared with the control, which reflects the increase in β–galactosidase levels found in BHI containing either NaCl or EGTA. Interestingly, it was found that the presence of EGTA in BHI somewhat counteracted the inhibitory effect of additional NaCl (0.5 M) on α–haemolysin production, with levels being restored to approximately 80% of those obtained in BHI with EGTA alone (see Fig. 3.9).

3.2.18 Effect of addition of spent culture medium

RAP (RNAIII activating protein) has been identified as an octapeptide produced by the *agrD* gene and is the signalling compound of a quorum sensing system (Balaban and Novick, 1995; Ji *et al.*, 1995; Ji *et al.*, 1997) (see section 1.6.1.1.2.2). RAP-containing spent culture medium was prepared by growing strain 8325-4 overnight in TSB at 37 °C, with shaking at 250 rpm. Spent medium and an equal volume of sterile TSB were then freeze-dried and each resuspended to a 10X concentration in sterile H_2O. Adding RAP-containing concentrate to an early exponential culture of SH101 (OD_{600} of 0.8) led to a slight, but consistent, increase in β–galactosidase activity, two hours post-addition (OD_{600} of 5.5) (Fig. 3.16). After three hours there was typically a 6-fold increase in β–galactosidase compared with the background levels (3 MU) present in a control sample with no additions or a sample with 10X sterile TSB added. This experiment was repeated 4 times and the values fluctuated by less than 7 %. The kinetics of growth of the cultures with spent medium added remained identical to cultures grown with sterile medium added.

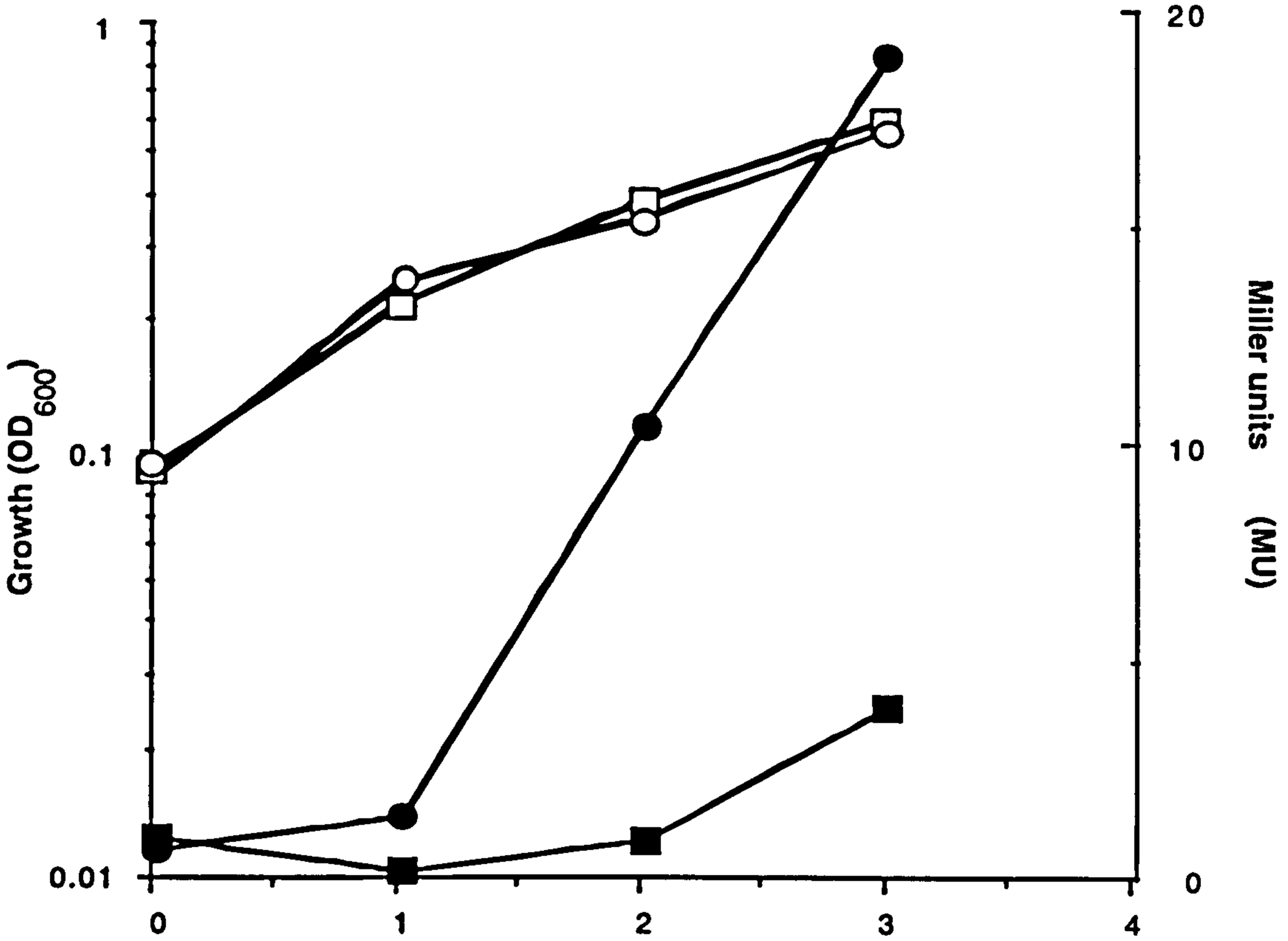


FIGURE 3.16

Effect of spent culture medium on *hld::lacZ* expression. Strain SH101 was grown in BHI until the OD_{600} reached 0.8. (a) Spent culture medium (10X), prepared as described in section 3.2.18, or (b) 10X sterile TSB was added at T=0. Growth, as absorbance at 600 nm (OD_{600} (a) ○; (b) □) and *hld::lacZ* expression as β-galactosidase activity (Miller units (a) ●; (b) ■) were measured at 1, 2 and 3 h post-addition as described in Chapter 2.

3.3 Discussion

The creation of a strain (SH101) containing a chromosomal *hld::lacZ* transcriptional fusion has allowed the convenient assay of *hld::lacZ* expression and hence RNAIII production. Construction of the fusion in single copy in the chromosome avoids problems due to titration of regulatory elements possibly associated with the use of multicopy plasmid based reporter systems. The fusion is stably maintained and does not require constant drug selection. In addition, an intact copy of *hld* remains in the chromosome to prevent the pleiotropic effects associated with an *agr* mutation. Using the reporter system, the effects of a wide range of environmental and physiological conditions on *hld::lacZ* expression have been examined. The *hla* gene (which encodes α–haemolysin) is positively regulated by RNAIII as well as other factors (Vandenesch *et al.*, 1991) and so by the study of α–haemolysin activity it has been possible to investigate the relationship between *hld::lacZ* expression and α–haemolysin levels.

The production of RNAIII has previously been shown to be growth phase regulated by the use of reporter fusion plasmids and transcript analysis (Kornblum *et al.*, 1990; Vandenesch *et al.*, 1991). In SH101 both *hld::lacZ* expression and α–haemolysin activity appear as the cells enter post-exponential phase. This is slightly later than that previously noted, when RNAIII expression is initiated during late exponential phase (Janzon *et al.*, 1989; Vandenesch *et al.*, 1991). α–haemolysin production did not occur until 2 hours after RNAIII transcription, which is consistent with previous observations (Vandenesch *et al.*, 1991). Use of the more sensitive, fluorogenic substrate MUG in the *hld::lacZ* fusion assays did not significantly alter the kinetics of β–galactosidase expression. Overnight growth (18 h) provided a convenient time point for sample collection. The high levels of both α–haemolysin and β–galactosidase in the overnight cultures may not be due to continued expression *per se* but more likely the stability of the respective proteins.

It has been proposed that expression of RNAIII is controlled as part of a density dependent signal transduction pathway via an extracellular octapeptide called RAP (Balaban and Novick, 1995; Ji *et al.*, 1995; Ji *et al.*, 1997). Thus, it would be expected that *hld::lacZ* levels would show a correlation with culture density. The growth media results shown support this hypothesis, especially using dilutions of BHI. Half- and quarter-strength BHI affected the final average OD_{600} of cultures (5.5 and 3.6, respectively) but did not adversely affect the growth rate (results not shown). Significantly lower *hld::lacZ* expression was apparent in both 0.5X (18 %) and 0.25X BHI (6 %) compared with the 1X control. In addition, different types of media have been shown to result in reduced final cell density with a concomitant fall in *hld::lacZ* expression, although this could be due to the different media constituents. Cells grown in TSB or a CDM (Hussein *et al.*, 1991) reach a lower OD_{600} than those grown in BHI and as final cell density is decreased so *hld::lacZ* expression falls.

Attempts were made to identify components which might have led to increased growth and *hld::lacZ* expression in CDM (Hussein *et al.*, 1991). In particular, various solutions containing protein (milk or bovine serum albumin) were supplemented since CDM contains only amino acids whereas other media which support good growth contain proteins and peptides. However, cell growth, *hld::lacZ* expression and α–haemolysin levels were not increased. Similarly, adding L- or DL-homoserine lactone (HL), a density-dependent signalling molecule of some Gram negative bacteria (see section 1.5.3), did not activate *hld::lacZ* expression. L-HL is the natural configuration of homoserine lactone, although DL-HL has also been shown to be active. Interestingly, later experiments on the effect of environmental conditions on *hld::lacZ* expression revealed a number of situations where *hld::lacZ* expression is deregulated from culture density. Addition of EGTA or 1 M NaCl enhanced *hld::lacZ* expression 5-fold. Conversely, 0.5 % (w/v) glucose, 0.25 % (w/v) sodium citrate and the CDM of Holland *et al.* (1994) reduced *hld::lacZ* expression 3-, 20- and 27-fold respectively whilst having no significant effect on final cell density. It has been shown that *S. aureus* grown in defined

media containing 6, 12 or 18 amino acids show comparable growth to cells grown in BHI, but haemolytic activity is negligible (Taylor and Holland, 1989a). Similarly, TSST-1 production is negligible in a 6 or 12 amino acid defined medium and only reaches 50 % that found in BHI in an 18 amino acid defined medium (Taylor and Holland, 1989a). Thus regulation of RNAIII, and control of production of some virulence determinants, is not a simple system but involves the transduction of many signals, including cell density.

S. aureus is an extremely osmotolerant organism, a trait which undoubtedly contributes to its ability to grow in many foods (Troller, 1986). In media containing 1 M NaCl *S. aureus* was found to grow well, with growth still considerable at 2 M NaCl. High salt concentrations (up to 1 M) increased *hld::lacZ* expression, although α–haemolysin levels were markedly decreased. This latter observation has recently been confirmed by showing that growing *S. aureus* in high NaCl (up to 1.2 M) results in repression of *hla* (Ohlsen *et al.*, 1997). Regassa *et al.* (1992) have similarly noted that addition of NaCl to media, at a level as low as 0.05 M, slightly enhances RNAIII expression. Production of two of the enterotoxins (SEB and SEC), whose expression is positively regulated by *agr*, is also adversely affected by high salt concentrations (Genigeorgis and Sadler, 1966; Regassa and Betley, 1993). This additional regulation of SEC is believed to occur at the level of transcription or mRNA stability (Regassa and Betley, 1993). The effect of NaCl concentrations (2-16 % w/v) on SEB production has also been examined in parallel with pH (Genigeorgis and Sadler, 1966). At all pH levels examined, SEB production remained highest at the lowest NaCl concentrations. Production of epidermolytic toxin A (Eta), like α–haemolysin, is positively regulated by *agr*, and *eta* expression decreases dramatically as NaCl concentrations increase from 0 M to 1 M (Sheehan *et al.*, 1992). Thus, regulation of several genes which require RNAIII for high level expression show a common response to high NaCl levels in a manner independent of RNAIII. It has been suggested that the *agr*-independent transcriptional regulation of *eta* may be mediated by salt induced changes in DNA topology (Sheehan *et al.*, 1992). Other *agr*-independent

regulators of virulence determinant production are apparent as the temporal regulation of *hla* during growth occurs irrespective of RNAIII (Vandenesch *et al.*, 1991).

The NaCl-enhanced expression of *hld::lacZ* and reduction in α–haemolysin activity cannot be accounted for by simply an effect of osmolarity, since 0.5 M sucrose strongly inhibits *hld::lacZ* expression as well as α-haemolysin activity. Similarly, α–haemolysin activity is undetectable even at 20 mM sucrose, at which concentration *hld::lacZ* expression is unaffected. This suggests that NaCl and, to an extent, sucrose are mediating their effect on α–haemolysin activity in an *agr*-independent manner. It should be noted, however, that *S. aureus* is able to utilise sucrose (Bergey, 1957) and thus the glucose constituent of sucrose is probably playing a part in the repression of α-haemolysin production and, to some extent, *hld::lacZ* expression (see below). This is particularly so since 0.5 M sucrose is equivalent to 9% glucose which is far greater than the concentration which has been shown, in this work, to inhibit both *hld::lacZ* expression and α-haemolysin production. Interestingly, *eta* expression is also greatly inhibited by the addition of sucrose to the growth medium (Sheehan *et al.*, 1992). Conversely, NaCl (0.5 M) has been shown to stimulate secretion of a haemolysin of *L. monocytogenes*, listeriolysin O (LLO) (Dallmier and Martin, 1990). LLO production is also controlled by a global regulator, *prfA*.

Experiments with sodium bicarbonate provide further evidence that α–haemolysin production is not controlled by *agr* alone. Addition of either 0.2 % or 0.4 % (w/v) $NaHCO_3$ to BHI does not alter *hld::lacZ* expression but causes a 2-3 fold increase in α–haemolysin activity. Bicarbonate is found *in vivo*, where it is in equilibrium with CO_2 and contributes to the buffering of extracellular fluids, at concentrations ranging from 15-40 mM (Dawson and Segal, 1975). In these experiments $NaHCO_3$ concentration was either 24 or 48 mM, which is similar to that found *in vivo* and thus mimics the host environment. Bicarbonate has also been found to affect production of three co-ordinately regulated toxins in *Bacillus anthracis* (protective antigen, edema factor and lethal factor)

at the level of transcription (Sirard *et al.*, 1994). The effect of bicarbonate is unlikely to be one of altered pH since *B. anthracis* cultures grown in the presence or absence of $NaHCO_3$ reached approximately the same final pH (Sirard *et al.*, 1994).

The effects of glucose on growth and gene expression in bacteria is well documented. Glucose, as a rapidly metabolizable carbon source, results in a co-ordinated change of metabolic functions in many bacteria. This is achieved by changing the activity of several proteins which leads to the differential expression of operons encoding metabolic enzymes by a process known as carbon catabolite repression (Wagner *et al.*, 1995). However, in *S. aureus* the effect of glucose is different from the catabolite repression seen in *E. coli* where it can be overcome by the addition of cyclic AMP (Ullmann and Danchin, 1983). The glucose repression of RNAIII synthesis under conditions of non-maintained pH, previously noted by Regassa *et al.* (1992), has been confirmed in this study at concentrations of 0.75 % (w/v) and above. α–haemolysin activity was severely repressed at all tested concentrations of glucose. However, under conditions of maintained pH (6.5), glucose has no effect on RNAIII levels although *hla* and *sec* expression and SEB production are greatly reduced (Jarvis *et al.*, 1975; Regassa *et al.*, 1992). Expression of toxic shock syndrome toxin-1 (TSST-1), also positively regulated by *agr*, is similarly affected (Chan and Foster, submitted; Taylor and Holland, 1988).

Glycerol monolaurate (GML) has also been shown to inhibit extracellular toxin production but not RNAIII expression (Holland *et al.*, 1994; Projan *et al.*, 1994; Schlievert *et al.*, 1992). Glycerol monolaurate is a naturally occurring surfactant commonly used in the food and cosmetic industries as an emulsifier. In this study, *hld::lacZ* expression was found to be unaltered by 0.2 mg/ml glycerol monolaurate but α–haemolysin activity was totally absent. Similarly, it has been reported that in a batch culture pulsed with 0.15 mg/ml GML just prior to onset of TSST-1 production, growth rate is reduced whilst TSST-1 levels are significantly delayed and reduced (Holland *et al.*, 1994). It has been reported that this reduction in TSST-1 production occurs only when

growth of *S. aureus* is affected (Holland *et al.*, 1994). GML has previously been shown to inhibit the production of β–lactamase, TSST-1 and α–haemolysin at a transcriptional level by interfering with signal transduction (Schlievert *et al.*, 1992; Projan *et al.*, 1994). In addition, production of protein A in an *agr* mutant, in which it is normally overproduced, is also inhibited in the presence of GML (Projan *et al.*, 1994). However, inhibition of production of these extracellular factors is not achieved by interfering with *agr* activation but probably other signal transduction pathways involved in their expression (Projan *et al.*, 1994). It has been suggested that a target in cell growth may be involved which in turn influences toxin production (Holland *et al.*, 1994). This provides further evidence that, in addition to α–haemolysin production, TSST-1 may also require a post-exponential phase activation signal additional to *agr*.

Of the many toxins produced by *S. aureus*, toxic shock syndrome toxin-1 (TSST-1) can be fatal and is a problem particularly in women where it is largely associated with the use of some types of tampon. The effect of divalent cations, in particular the presence of Mg^{2+} in tampons, has been well studied. Use of chemostat continuous culture to study the specific effects of Mg^{2+} concentration in a defined medium showed that Mg^{2+} limited conditions led to reduced production of TSST-1 (Taylor and Holland, 1989b). Toxin production increases approximately 23-fold with increased Mg^{2+} concentrations up to 0.4 mM (Sarafian and Morse, 1987). In this work it has been shown that addition of Mg^{2+} (0.2, 2 and 20 mM) to BHI did not significantly alter *hld::lacZ* expression or α–haemolysin activity. However, removing Mg^{2+} (0.5 mM EDTA) led to a 4-5-fold increase in *hld::lacZ* expression and a 10-fold increase in haemolysin units. The effect on β–galactosidase production was reversed when Mg^{2+} (20 mM) was present in addition to EDTA and α–haemolysin levels showed only a 2-fold increase. Thus, our studies suggest that the effect of Mg^{2+} on *tst* noted by other workers is probably specific and occurs independently of *agr*.

Addition of the calcium chelator EGTA results in a 5-fold increase in *hld::lacZ* expression. This response is due mainly to Ca^{2+} as addition of excess Ca^{2+} in the presence of EGTA, reverses the effect. α–haemolysin activity is also enhanced up to 18-fold by a lack of Ca^{2+}. The level of available Ca^{2+} may well be important in enabling *S. aureus* to inhabit certain environments, particularly the airways of cystic fibrosis (CF) sufferers (Kilbourn, 1984). Whilst *S. aureus* is infrequently found in the lower respiratory tract of healthy subjects, more than one-third of CF sufferers harbour this organism in their lower airways (Schwab *et al.*, 1993). The mucus of these patients contains unusually high levels of calcium and it has been shown that the presence of Ca^{2+} enhances adhesion of the bacteria to mucin, a component of mucus (Kilbourn, 1984; Thomas *et al.*, 1993). Calcium has also been shown to play a role in the production of fibronectin binding protein (FnBP), an important virulence factor which is negatively regulated by *agr* (Kornblum *et al.*, 1990; Lindberg *et al.*, 1990). In the presence of calcium, free extracellular FnBP is increased whereas this is suppressed when EGTA (6.25 mM) is added to the medium to chelate free Ca^{2+}. In addition, the presence of EGTA leads to a sharp increase in cell wall-associated FnBP. The results obtained during this work suggest that these changes in FnBP production in response to Ca^{2+} availability are probably not mediated directly by *agr*.

There are several examples in other organisms where changes in extracellular Ca^{2+} concentration have been shown to affect gene expression. For example, in *Streptococcus pneumoniae*, 0.15 mM Ca^{2+} is required for optimal growth but 1 mM triggers a differentiative state (Trombe *et al.*, 1992). In contrast to the situation in *S. aureus*, the presence of free calcium in growth media has been shown to positively regulate gene expression in some serotypes of *Actinobacillus (Haemophilus) pleuropneumoniae* resulting in increased levels of haemolysin (Frey and Nicolet, 1988). In the presence of EGTA, a Ca^{2+} chelator, an almost complete loss of haemolytic activity is observed whereas free calcium concentrations above 3 mM cause a dramatic increase in activity.

Production of siderophores and haemolysins by *S. aureus*, as mechanisms for acquiring iron, is frequently regulated by iron availability, with increased expression occurring when iron is limited (Litwin and Calderwood, 1993; Stoebner and Payne, 1988; Trivier and Courcol, 1996; Waalwijk *et al.*, 1983). In particular, this work has shown that addition of some chelators (EGTA and EDTA) does enhance both *hld::lacZ* expression and α–haemolysin production. Iron-deprivation has also been shown to stimulate LLO secretion (Cowart and Foster, 1991). Citrate in particular chelates minerals, with a high equilibrium constant for iron. Citrate is naturally present in plant foods and is widely used as an antimicrobial agent in foods (Doores, 1993). The inhibitory effect of organic acids, such as citric acid, on microbial growth is attributed to damage caused by the hydrogen ion liberated inside the cell following permeation of the acid in its undissociated form. Acidification of the cytoplasm uncouples both substrate transport and oxidative phosphorylation from the electron transport system (Freese *et al.*, 1973). In the Gram positive organism, *L. monocytogenes*, Listeriolysin O secretion is reportedly stimulated in the presence of citrate (4 % w/v optimum) (Kouassi and Shelef, 1995). This was thought to be possibly due to increased permeability of the cell membrane in the presence of the salt, thus facilitating the release of the haemolysin into the extracellular medium. Growth of *S. aureus* was found to be completely inhibited by 1 % (w/v) Na-citrate and, although growth at 0.1 % (w/v) and 0. 25 % (w/v) is relatively unaffected, there was very little detectable *agr* P3 promoter activity or α–haemolysin production at 0.25 % (w/v).

Temperature regulation of virulence determinant production is common amongst a number of bacterial pathogens as it allows for toxin production only when associated with the mammalian host (Datta and Kothary, 1993; Prugnola *et al.*, 1995). Consistent with this was the ~3-fold increase in *hld::lacZ* expression observed at 37 °C compared to 30 °C. More striking was the 8-fold increase in α–haemolysin activity at 42 °C compared to 37 °C, whereas *hld::lacZ* expression was only 2-fold higher. The finding that α–haemolysin levels are higher at 42 °C has recently been supported by experiments using

an *hla::lacZ* fusion to measure *hla* expression at 37 °C and 42 °C (Ohlsen *et al.*, 1997). In addition, Ohlsen *et al.* (1997) showed that increased temperature altered the temporal expression of *hla* from post-exponential to mid-exponential. This again suggests there are additional factors involved in the regulation of α–haemolysin activity independent of RNAIII. A similar pattern occurs with TSST-1 production which is significantly higher at 37 °C than 30 °C and 2-fold higher at 40 °C than 37 °C (Schlievert and Blomster, 1983). Global regulators of toxin production in some other organisms are similarly affected. For example, transcription of virulence genes controlled by the *bvg* regulon of *Bordetella pertussis* is repressed at 25 °C and increases as temperatures rise to 37 °C (Prugnola *et al.*, 1995). The secretion of listeriolysin O is similarly affected by temperature, showing a 64-fold increase as temperature rises from 26 °C to 37 °C (Datta and Kothary, 1993).

Most bacteria show a dose-dependent inhibition of growth in ethanol over the range 1-10 % (v/v) (Ingram and Buttke, 1984). Growth of *S. aureus* was reduced in the presence of both 1 and 2 % (v/v) ethanol. The presence of alcohols, in particular ethanol, tends to increase membrane rigidity and it is the ability of cells to maintain the fluidity and integrity of membranes in this environment which leads to ethanol tolerance (Couto *et al.*, 1996). Cell membrane alterations affecting possible signal transduction mechanisms may account for the increase in *hld::lacZ* expression and α–haemolysin activity in 1 % (v/v) ethanol grown cells. However, isopropanol reduced *hld::lacZ* expression approximately 3-fold and α–haemolysin activity >4-fold at 1 % (v/v), whilst having no marked effect on the final OD_{600}.

Oxygen availability is likely to be an important stimulus *in vivo*. It has been shown that *S. aureus* grown in batch culture, in flasks, has a reduced growth rate when the ratio of flask size to culture volume is <20:1 (Taylor and Holland, 1988; Taylor and Holland, 1989b). Growing strain SH101 in a flask to media volume of 10:1 led to reduced β–galactosidase activity compared to cells grown in a respective ratio of 2.5:1. Anaerobic

growth led to a very much reduced final OD_{600}, and almost no *hld::lacZ* expression or α–haemolysin activity. It has recently been confirmed that expression of the *hla* gene itself is completely repressed under anaerobic conditions (Ohlsen *et al.*, 1997). Due to the effect on growth it is difficult to determine if the lack of RNAIII is an effect of oxygen availability directly, or indirectly via cell density dependence of RNAIII expression. However, microaerophilic growth led to a large increase in both *hld::lacZ* expression and α–haemolysin activity whilst OD_{600} remained very low (P.F. Chan, pers. comm.). Similarly, decreased oxygen availability has been shown to lead to increased TSST-1 production but decreased cell yield (Sarafian and Morse, 1987) whilst anaerobic conditions have been shown to lead to a reduction in TSST-1 activity of between 4- and 32-fold, depending on media and growth conditions (Sarafian and Morse, 1987; Schlievert and Blomster, 1983; Taylor and Holland, 1989b).

This study has revealed that various environmental factors, such as high temperature (42 °C), EGTA and 1 M NaCl, each lead to increased *hld::lacZ* expression. If these treatments are mediating their effects via independent mechanisms then an additive or even synergistic response to combinations of factors may be expected. No additive effects were seen with *hld::lacZ* expression by this combined approach, but EGTA was able to reverse the inhibitory effect of 1 M NaCl on α–haemolysin activity. Thus, there is some evidence of crosstalk between the regulatory mechanisms which control toxin biosynthesis.

Production of virulence determinants by *S. aureus* is co-ordinately regulated in order that the organism may efficiently respond and adapt to changes in its internal and external environment. Central to this process is the *agr* locus which in itself is a complex regulatory element implicitly required for virulence. Part of the signal transduction process which elicits toxin production is a cell density dependent quorum sensing mechanism (Ji *et al.*, 1995) (see section 1.6.1.1.2.2). The availability of the fusion strain produced in this work and the development of a suitable assay have allowed confirmation

of the existence of such a system in *S. aureus.* Although adding 'spent' media to cultures in early exponential phase caused only a slight increase in Miller units, this response occurred prior to when activity normally would have been expected and the findings were consistently reproducible. The cell density dependent signalling compound of some Gram negatives, homoserine lactone (see section 1.5.3) did not elicit an early increase in *hld::lacZ* expression when added to an exponentially growing culture. Similarly, homoserine (which lacks the fatty acid chain of HL) had no effect. In *E. coli* the non-acylated form of homoserine lactone is thought to be an intracellular starvation signal that induces expression of the *rpoS* stationary phase specific sigma factor (Fuqua *et al.*, 1996).

Multiple environmental signals are being constantly monitored by the cells and responded to by both *agr* dependent and independent mechanisms. This work has revealed a number of the stimuli important in the control of toxin production. Of course, it is not possible to reproduce, *in vitro*, the environment that occurs *in vivo* since this is a complex and dynamic situation where many factors are interacting and changing, often rapidly. Some conditions that may reflect those found *in vivo*, including temperature, the availability of some cations or oxygen, or levels of sodium bicarbonate, have been examined in this work and found to affect *hld::lacZ* expression or α-haemolysin production. However, it is not possible to extrapolate from the data obtained during these *in vitro* studies since the likelihood is that the effects in the body will be somewhat different. Thus, the conclusions that may be drawn from the data presented in this work must be treated with some caution before making parallels between the *in vivo* and *in vitro* behaviour of *S. aureus* and the way in which it regulates toxin production. Nevertheless, it is important to examine the way in which this organism deals with the many stresses it encounters when it infects a host, since the information obtained from this study can be used to predict possible targets for inhibiting toxin production, particularly in foods where levels of glucose or osmotic compounds, or temperature, can be carefully controlled. In particular, the results obtained in this work, and the work of

others, with glycerol monolaurate or sodium citrate may have implications for the food industry.

It is likely that, although several global virulence determinant regulators have already been identified, such as *agr*, *sar* and *sae* (Cheung *et al.*, 1992; Giraudo *et al.*, 1994b; Kornblum *et al.*, 1990), there are many signal response mechanisms, possibly specific to individual stimuli, still to be defined. These response mechanisms form part of a multilayered regulatory hierarchy with *agr* at its heart. *agr* itself probably does not respond to all the environmental signals directly, but is a conduit via which the response of other components is transduced. Fine control of expression of individual or subsets of virulence determinants is likely to be mediated independently of *agr*. Also more global regulatory mechanisms, including DNA topology (Sheehan *et al.*, 1992) or alternative sigma factors (Wu *et al.*, 1996), are likely to input into the ability of *S. aureus* to so deftly inhabit such a range of niches.

CHAPTER 4

Isolation and characterisation of virulence determinant production mutants using Tn*551*

4.1 Introduction

It is well known that *agr* is a major regulator of toxin production in *S. aureus*. However, it has been shown in this work and the work of others, that mechanisms other than *agr* are also involved in the control of production of at least some of the virulence factors (Cheung *et al.*, 1992; Giraudo *et al.*, 1994b; Sheehan *et al.*, 1992; Vandenesch *et al.*, 1991). In particular, several environmental conditions have been found to de-regulate production of some virulence factors from *agr*. Notably, high NaCl concentrations (0.5 and 1 M) led to a large increase in *hld::lacZ* expression but α–haemolysin activity was not detected (see section 3.2.7). Conversely, unavailability of divalent cations due to addition of EGTA or EDTA (0.5 mM) and growth in microaerophilic conditions (P.F. Chan, pers. comm.) have led to a marked increase in α–haemolysin activity compared to a proportionally much smaller increase in *hld::lacZ* expression (see section 3.2.9). In addition, a novel global regulator, staphylococcal accessory regulator (encoded by the *sar* locus) has been identified (Cheung *et al.*, 1992). The protein product of the *sarA* gene, SarA, is thought to up-regulate expression from the P2 and P3 promoters of *agr*, possibly by interacting with AgrA (see section 1.6.1.2.1) (Cheung *et al.*, 1997; Morfeldt *et al.*, 1996b). The *hld::lacZ* fusion reporter strain created during this work (see section 3.2.1) allows an easy assay for expression from the P3 promoter of *agr* with transcription from this promoter being linked, via *hld*, to production of β–galactosidase. Thus, strains bearing mutations in genes encoding components which affect *hld::lacZ* expression would show altered β–galactosidase activity. In order to identify regulators of *agr* transcription, a transposon library has been created in an *hld::lacZ* reporter strain.

Transposons are a valuable and common tool for creating insertional inactivation mutants and as the site of mutation is tagged by the insertion of the transposon it can be relatively easily identified. This technique has been used to characterise many loci

both in *S. aureus* and other organisms (Cheung *et al.*, 1992; Giraudo *et al.*, 1994b; Hart *et al.*, 1993; Recsei *et al.*, 1986).

Transposons are DNA fragments which are inherently mobile and can generally insert at random into plasmids or bacterial chromosomes independently of a host cell recombination system. Such transposable elements were first detected in maize, and their behaviour characterised, by McClintock (1948, 1951) before the structure of DNA was known. There is now good evidence that transposons can be found in all organisms. When transposons move into new genomic locations they may, or may not, significantly alter neighbouring gene expression and have profound mutagenic effects (Singer and Berg, 1991). For example, insertion of a mobile segment into a new genomic site is mutagenic if a coding or critical regulatory segment is disrupted and gene transcription is prevented. Transposons frequently include their own regulatory regions and, in such cases, expression of genes in the neighbourhood of the insertion site may be influenced in complex ways, including the imposition of new regulatory modes. Since transposons carry out initiation of RNA synthesis they sometimes activate previously dormant genes.

Transposons have been exploited for many mutagenesis studies and specific delivery vectors have been created. For example, a streptococcal transposon, Tn*917* (see chapter 5) has been used in *S. aureus* to identify the regulatory locus, *sar*. An engineered Tn*917* has been produced (Camilli *et al.*, 1990) which has several features that make it the method of choice for creating transposon mutants (see chapter 5). However, this vector, pLTV1 (see Fig. 5.1) could not be used to create *hld::lacZ* expression mutants since it contains a *lacZ* gene. As the basis of the proposed screen depends on altered *hld::lacZ* expression this could be compromised. Also, recombination could occur between the two *lacZ* copies. Another transposon widely used in *S. aureus*, and in fact used to create *hld::lacZ* expression mutants, is Tn*551*. Tn*551* has facilitated the identification of several *S. aureus* global regulators, namely

agr, *xpr* and *sae*, all of which have pleiotropic effects on virulence characteristics (see chapter 1). It has also been used extensively as a mutagenic tool for chromosome and plasmid mapping in *S. aureus* (Murphy, 1988; Pattee *et al.*, 1977).

Tn*551* is a 5.3 kb, staphylococcal Tn*3*-like transposon that is found on the penicillinase plasmid, pI258. This transposon specifies constitutive resistance to the macrolide-lincosamide-streptogramin B (MLS) antibiotics. The Tn*3* family of transposons, which also includes Tn*917*, contain homologous short terminal inverted repeats, (in the case of Tn*551* about 40 bp) and generate a 5 bp duplication of the target DNA during transposition (Khan and Novick, 1980; Perkins and Youngman, 1984). Tn*551* has an almost identical restriction pattern to Tn*917* (see Fig. 5.1) but despite their similarities, Tn*551* preferentially transposes into the chromosome (Pattee *et al.*, 1977) whilst Tn*917* transposes to either chromosomal or plasmid sites with a preference for some plasmids (Weaver and Clewell, 1987).

The delivery vector used in this work to introduce Tn*551* into bacterial cells was plasmid pRN3208 (Fig. 4.1), which is derived from pI258 (Kornblum *et al.*, 1986). pI258 carries the gene for resistance to cadmium (Cd) and a β–lactamase gene (*bla*) and, within Tn*551*, there is an erythromycin (Em) resistance gene (*erm*). pRN3208 is temperature-sensitive so that it can only replicate up to a temperature of approximately 30 °C. Thus, by raising the growth temperature to 43 °C it is possible to select only for those bacteria which contain Tn*551* within their chromosome (Kornblum *et al.*, 1986) since resistance to Cd, which is carried on pRN3208, will be lost, whereas Em resistance, which is carried within Tn*551*, will remain.

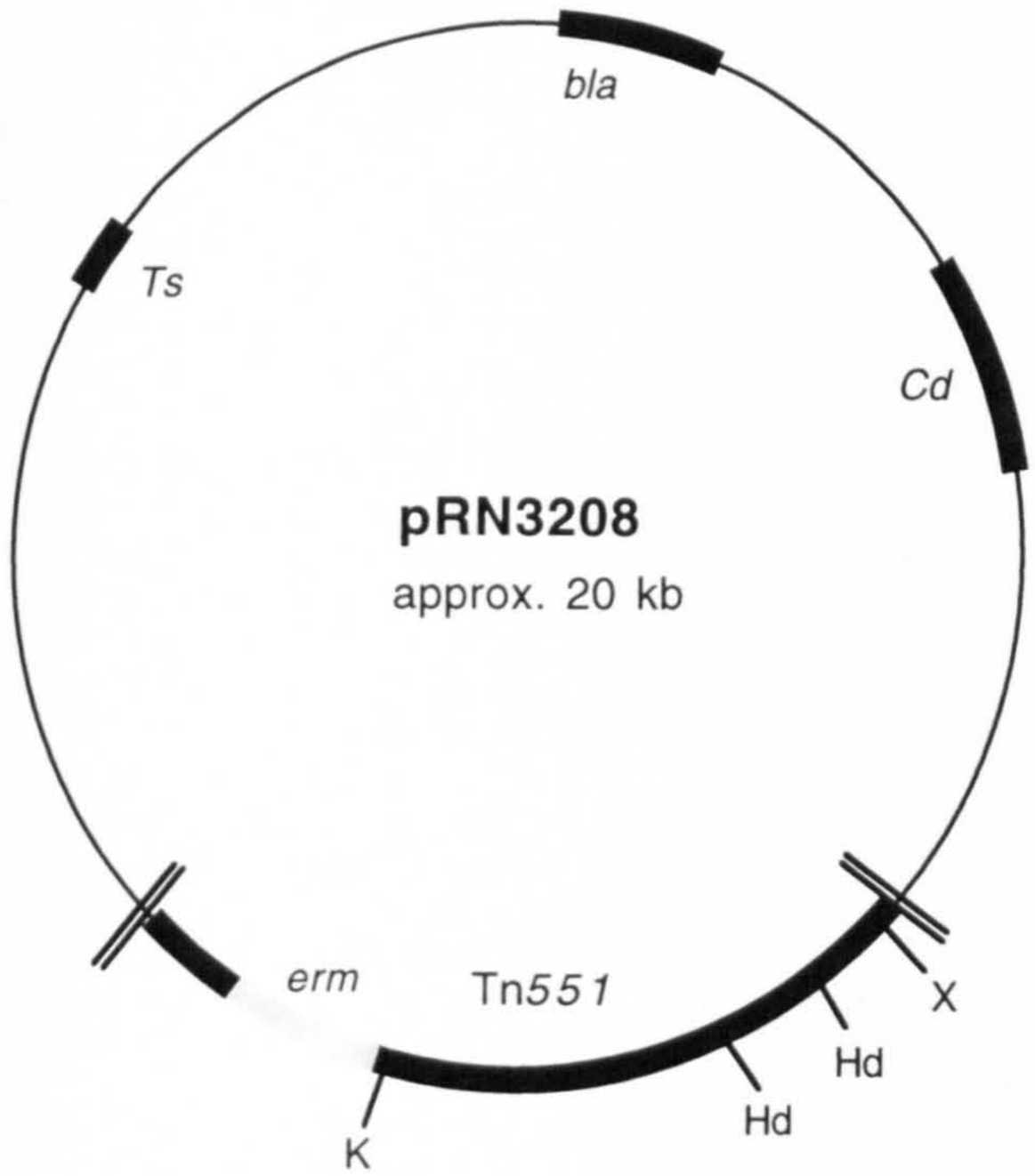


FIGURE 4.1

Approximate map of pRN3208. This plasmid contains an ampicillin resistance gene (*bla*), a cadmium resistance gene (*Cd*) and a temperature sensitive replicon (*Ts*). The extent of Tn*551*, which contains an Em resistance gene (*erm*), is indicated by the symbol, ||. The restriction sites denoted are: Hd, *Hin*dIII; K, *Kpn*I; X, *Xba*I.

4.2 Results

4.2.1 Development of strains for transposon mutagenesis

As mentioned in section 4.1, Tn*551* contains an Em resistance gene (Fig. 4.1). In order to avoid recombination between this Em resistance gene and that contained in the chromosome of the existing *hld::lacZ* reporter strain, SH101 (see section 3.2.1), it was necessary to identify either an alternative transposon or construct a new *hld::lacZ* reporter strain with an alternative resistance marker so that there would be a different selectable marker on the transposon compared to the *hld::lacZ* reporter strain. Since all transposons suitable for use in *S. aureus* contain an Em resistance gene, it was necessary to create a new *hld::lacZ* reporter strain with a novel resistance marker. Two approaches were adopted to create such a construct, although the method described in section 4.2.1.2 was ultimately used to make Tn*551* insertion mutants.

4.2.1.1 Switching of SH100 from Em resistance to spectinomycin (Sp) resistance

Strain SH100 comprises RN4220 (an *S. aureus* strain which accepts foreign DNA) containing pHF23 integrated into the chromosome, to form an *hld::lacZ* fusion (see chapter 3). In order to change the resistance of this strain from Em, two different switching vectors were used. These vectors contain either an Sp resistance gene (pEr::Sp) (Fig. 4.2) or a chloramphenicol (Cm) resistance gene (pEr::Cm) within an Em resistance gene (Steinmetz and Richter, 1994). Thus the Em resistance gene is present in both of these plasmids but is insertionally inactivated by the new respective resistance marker gene (Fig. 4.2). The principle behind this is that, upon introduction of either pEr::Sp or pEr::Cm into strain SH100, the fragments of the Em resistance gene at either end of the Cm or Sp gene would, by a double crossover event, replace the chromosomal Em resistance gene of SH100 resulting in loss of Em resistance and

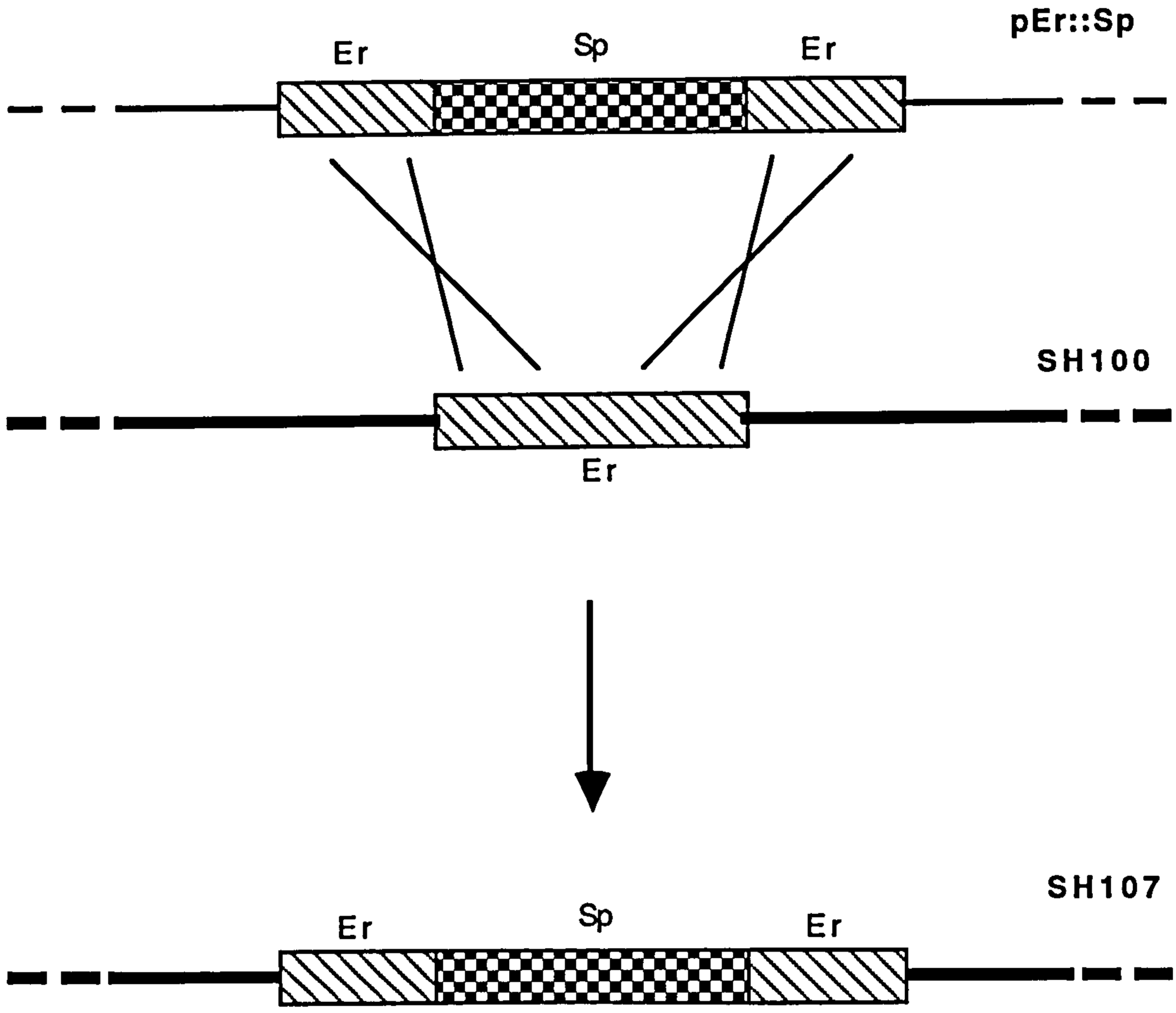


FIGURE 4.2

Schematic diagram to show the alteration of antibiotic resistance associated with a chromosomal insertion (modified from Steinmetz and Richter, 1994). Strain SH100, containing an Em resistance gene (Er) inserted into the chromosome, was transformed with linearised switching vector, pEr::Sp. Plasmid pEr::Sp contains an Em resistance gene disrupted by a gene conferring resistance to Sp. Due to a double crossover event, the Em resistance of strain SH100 was converted to Sp resistance, to give strain SH107.

concomitant gain of Cm or Sp resistance (Fig. 4.2). Accordingly, electroporation (Schenk and Ladagga, 1992) with linearised (by *Eco*RI digestion) pEr::Sp or pEr::Cm (1 μg) into *S. aureus* strain SH100 was attempted. Transformants were selected on agar containing the appropriate antibiotic (Cm or Sp) and subsequently tested to ensure they had lost Em resistance. This would indicate that the double crossover event had occurred between the inactivated Em resistance gene of the switching plasmid and the Em resistance gene in strain SH100. Following four such attempts, a transformant was isolated which was Sp resistant and Em sensitive (strain SH107). No Cm resistant/Em sensitive transformants were isolated. Strain SH107 was blue on agar plates in the presence of X-Gal, indicating that the *hld::lacZ* fusion was intact. This strain was not characterised further due to the success of the approach described below.

4.2.1.2 Creation of an *hld::lacZ* fusion using pTKlac

As an alternative to the above approach, attempts were made to create a new *hld::lacZ* fusion using the suicide vector, pTKlac (Kenney and Moran, 1991) (Fig. 4.3) which carries a Cm resistance marker. The 677 bp *PstI/Eco*RV fragment spanning the P3 promoter of *agr* (see Fig. 1.2) used to create strain SH101 (see chapter 3) was isolated as a 701 bp *Bam*HI/*Hin*dIII fragment from pHF21 (see Fig. 3.2). This 701 bp fragment was ligated into similarly cut pTKlac and transformed into *E. coli* XLOLR. Putative recombinant plasmids were digested with *PstI/Eco*RV, to confirm the presence of the 677 bp fragment. The correct construct, pHF24 (Fig. 4.4), was transformed by electroporation into *S. aureus* RN4220. However, after two attempts no recombinants resistant to Cm were recovered. Two attempts at transformation (Novick, 1991) by protoplasting RN4220 were also unsuccessful. Four further attempts by electroporation (Schenk and Laddaga, 1992) also failed to yield any recombinants. It has been reported that the efficiency of recombination depends on the length of homologous DNA in the suicide vector construct (Perego,

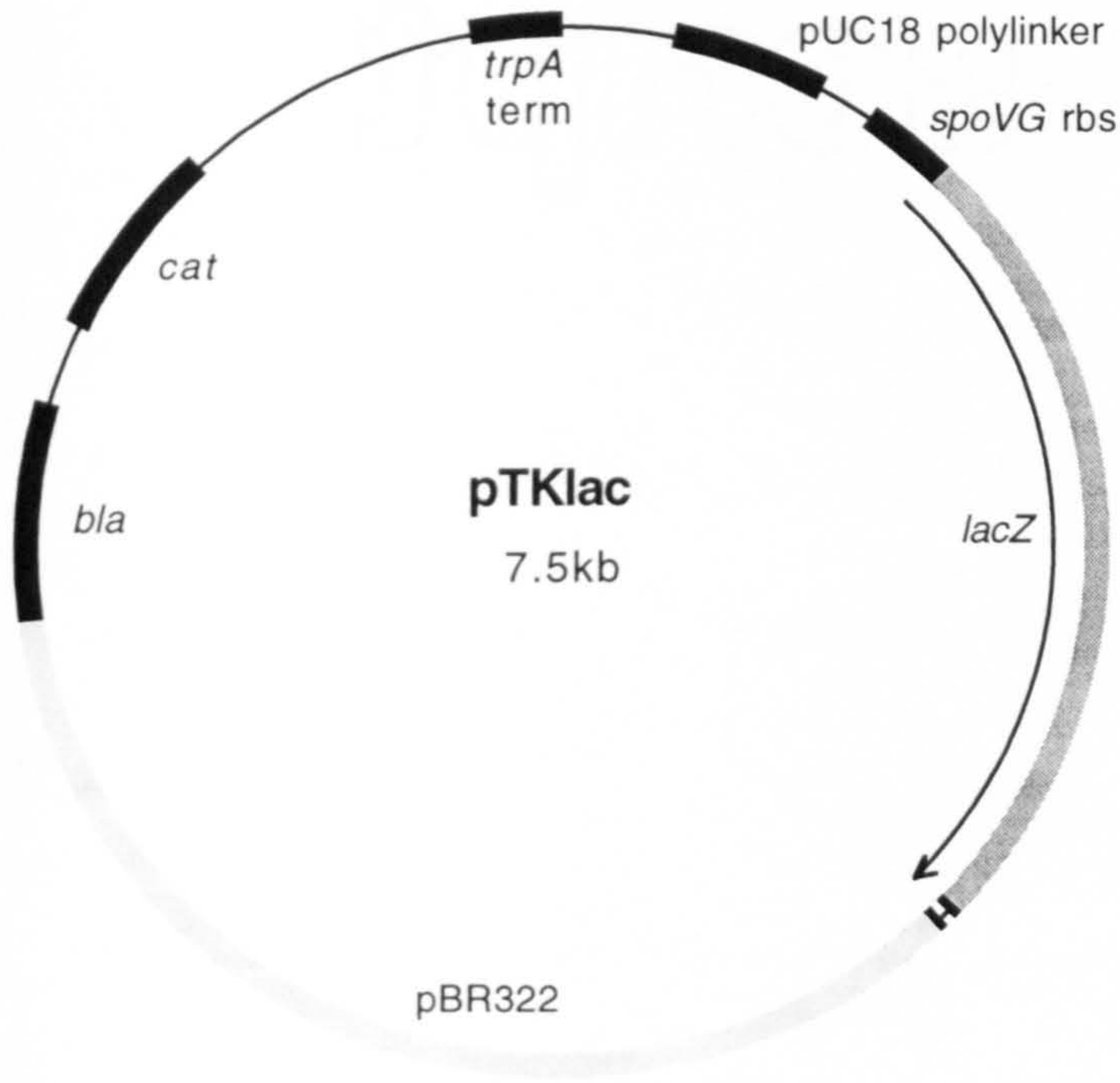


FIGURE 4.3

Map of the suicide vector, pTKlac (modified from Kenney and Moran, 1991). This plasmid contains an ampicillin resistance gene (*bla*), a Cm resistance gene (*cat*) and a promoterless *lacZ* gene, with a *spoVG* ribosome binding site (rbs), encoding β-galactosidase. Plasmid pBR322 backbone is also contained within pTKlac, together with the multiple cloning site from pUC18 and the *trpA* terminator from *E. coli*.

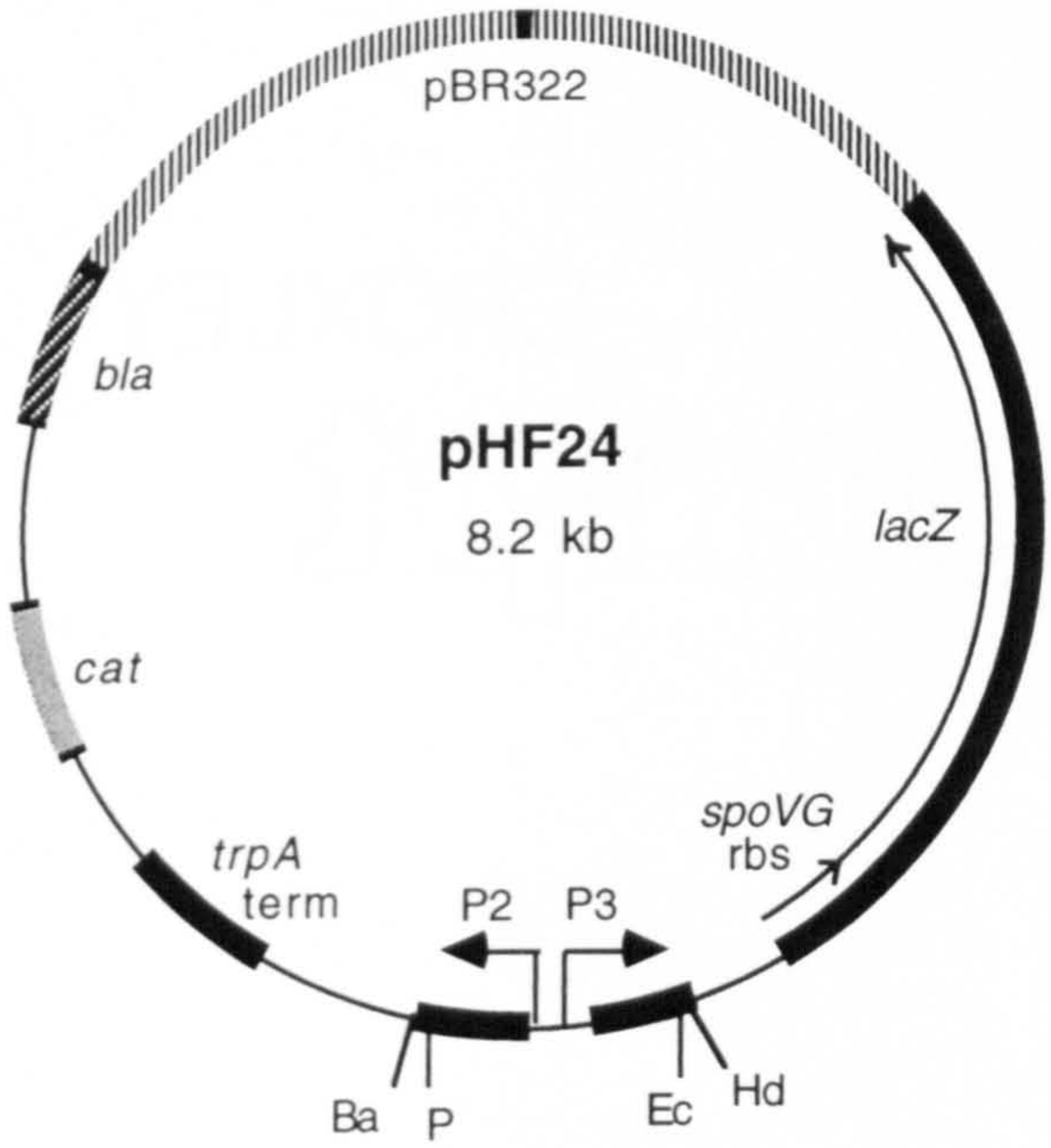


FIGURE 4.4

Map of pHF24. A 701 bp *Bam*HI/*Hin*dIII fragment of *agr* spanning the P3 promoter was isolated from pHF21 (see Fig. 3.1) and ligated into similarly digested pTKlac. The restriction sites denoted are: Ba, *Bam*HI; Ec, *Eco*RV; Hd, *Hin*dIII; P, *Pst*I.

1993), so that a plasmid containing a longer fragment of *agr* would probably stand more chance of recombination.

Plasmid pEXO (Morfeldt *et al.*, 1988) contains an insert of 4.5 kb which includes 3.8 kb of the *agr* locus (Fig. 4.5). A 1.8 kb *Eco*RV/*Asp*700 (see Fig. 4.5) fragment of the *agr* locus was excised from pEXO and purified. The identity of the 1.8 kb band was confirmed by *Pst*I digestion which results in two fragments of 1101 bp and 677 bp (Fig. 4.5). The 1.8 kb fragment (0.25 μg) was ligated into *Bam*HI/*Hin*dIII digested, end-filled, dephosphorylated pTKlac (0.1 μg) and transformed into *E. coli* XLOLR. Appropriate restriction analysis of recombinant plasmids revealed that no plasmids containing the insert has been obtained. Despite repetition and the screening of 85 putative clones, the correct construct was not obtained.

As an alternative, pTKlac was digested with *Sma*I and, following ligation with the 1.8 kb *Eco*RV/*Asp*700 pEXO fragment, was transformed into *E. coli* XLOLR. Ten putative clones were screened by plasmid minipreps and restriction analysis. One of these, pHF25, contained the insert in the correct orientation to give an *hld::lacZ* fusion (Fig. 4.6) and one, pHF26, contained the insert in the incorrect orientation (Fig. 4.6). This was confirmed by restriction analysis, digesting both these plasmids (and pTKlac) with *Sal*I/*Eco*RI to release the 1.8 kb fragment, and with *Pst*I to release the 683 bp fragment from pHF25 (Fig. 4.7). The *Pst*I digest indicated that the 677 bp *Pst*I/ex *Eco*RV fragment spanning the P3 promoter was inserted just 6 bp upstream of the *Pst*I site of pTKlac and thus in the correct orientation for transcription from the P3 promoter through *hld* and the *lacZ* gene of pTKlac (Fig. 4.6). In pHF26, the fragment was in the opposite orientation so would not produce an *hld::lacZ* fusion (Fig. 4.6). Following plasmid preparation (500 ml of culture) pHF25 (2 μg) was transformed into RN4220 by electroporation (Schenk and Ladagga, 1992). Two transformants were selected which were chloramphenicol resistant and blue on plates in the

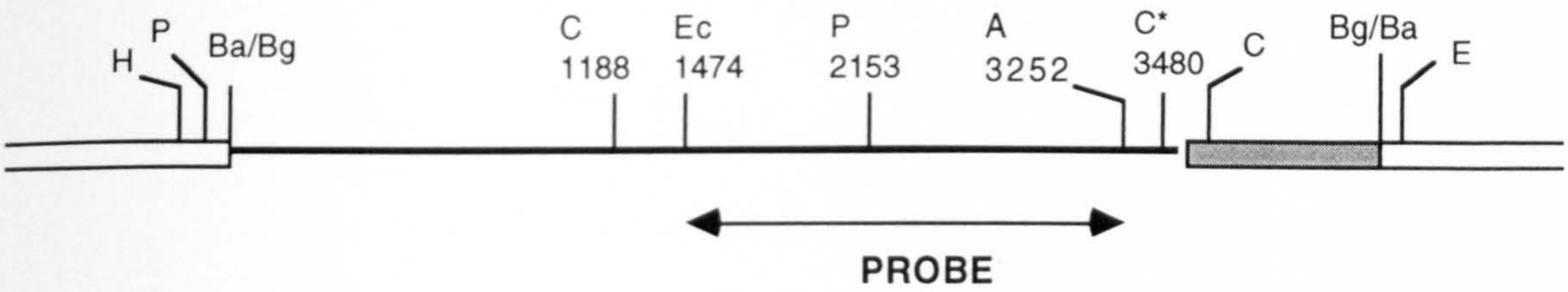
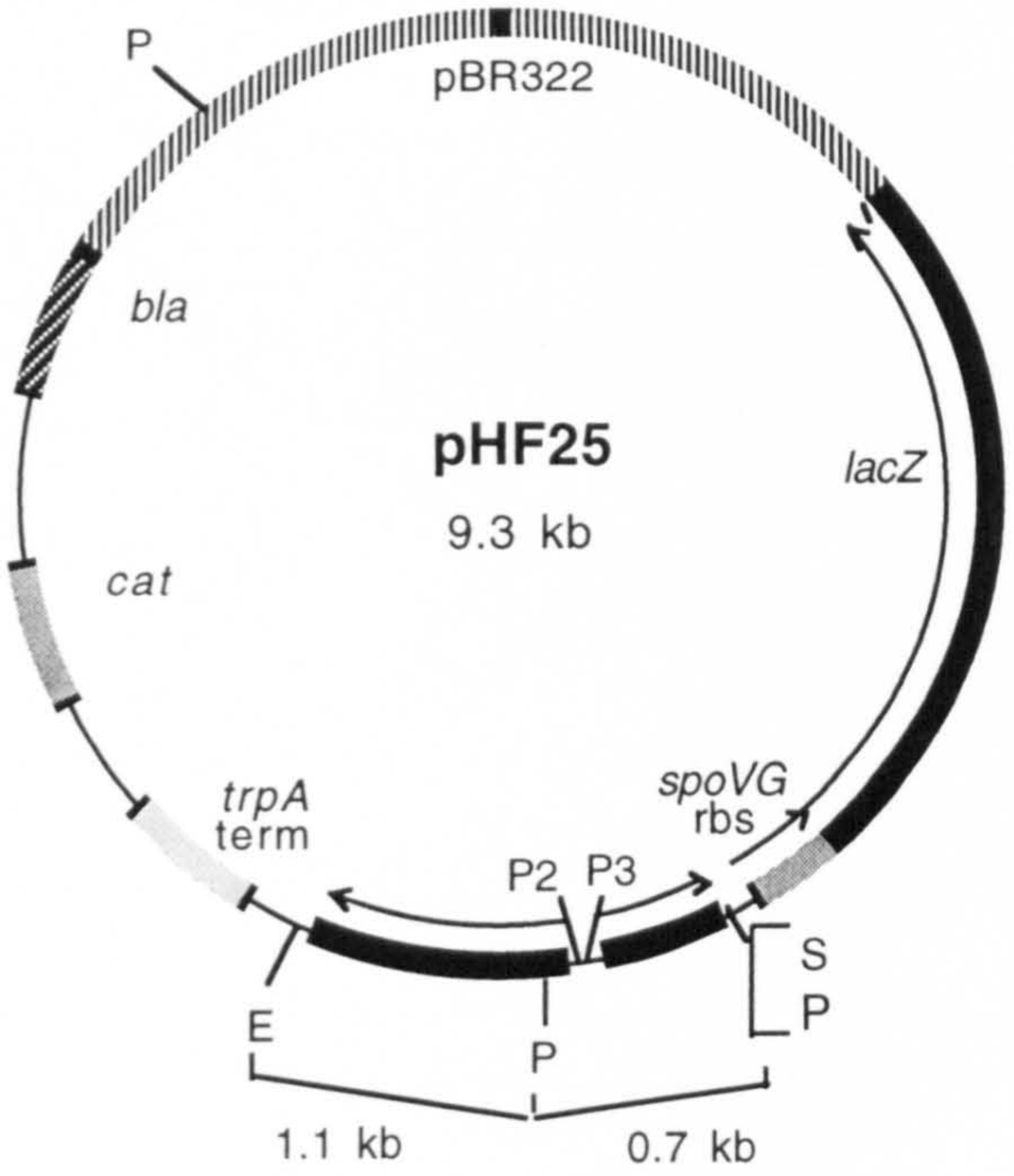


FIGURE 4.5

Partial map of pEXO (Morfeldt *et al.*, 1988). The single line represents 3.8 kb of chromosomal DNA including 3.5 kb of *agr*; the filled bar represents 770 bp of Tn*551*; the open bars represent pSP64. The double headed arrow indicates the origin of the 1.8 kb *Asp*700/*Eco*RV probe used during this work. Restriction sites denoted are: A, *Asp*700; Ba, *Bam*HI, Bg, *Bgl*II; C, *Cla*I; E, *Eco*RI; Ec, *Eco*RV; Hd, *Hin*dIII; P, *Pst*I; C*, a *Cla*I site which is methylated in *E. coli*.



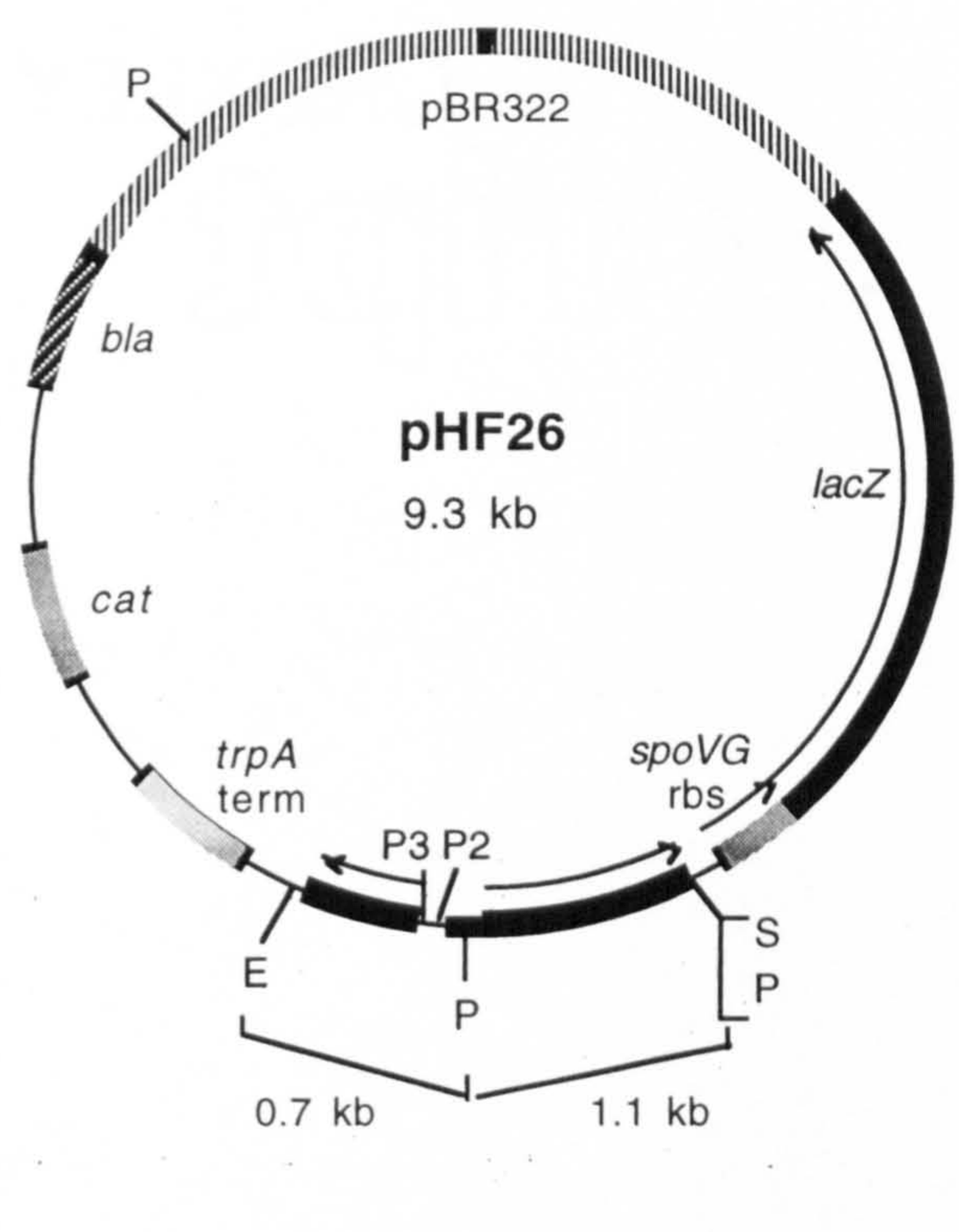


FIGURE 4.6

Maps of pHF25 and pHF26. A 1.8 kb *Eco*RV/*Asp*700 fragment of *agr* spanning the P3 promoter was isolated from pEXO (see Fig. 4.5) and ligated into *Sma*I digested pTKlac (see Fig. 4.3). Insertion of the fragment in the correct orientation (pHF25) and incorrect orientation (pHF26) was confirmed by digestion with *Eco*RI/*Sal*I to release the 1.8 kb fragment and *Pst*I, to release the 689 bp fragment spanning the P3 promoter from pHF25. Restriction sites denoted are: E, *Eco*RI; P, *Pst*I; S, *Sal*I.

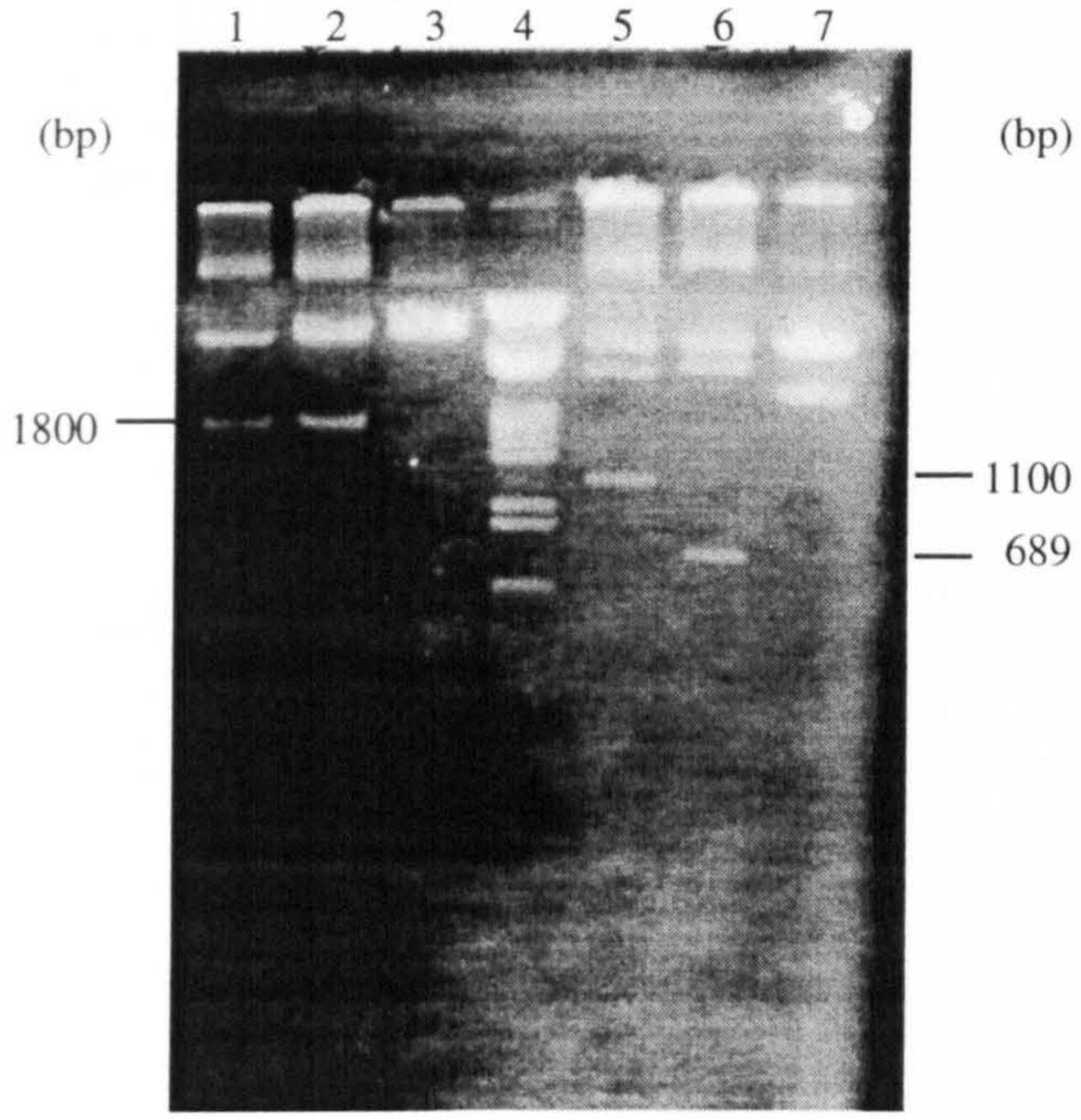


FIGURE 4.7

Agarose gel to confirm the orientation of the 1.8 kb *Eco*RV/*Asp*700 fragment of *agr* isolated from pEXO (see Fig. 4.5) in pHF25 and pHF26. Lanes 1-3 show pHF26, pHF25 and pTKlac (see Fig. 4.3), respectively, digested with *Sal*I/*Eco*RI to confirm the presence of the pEXO fragment (where applicable). The 1800 bp fragment is indicated on the left. Lane 4 is *Hin*dIII/*Eco*RI digested λ (sizes of fragments are shown in Table 2.4). Lanes 5-7 show pHF26, pHF25 and pTKlac digested with *Pst*I to release the 689 bp or 1100 bp fragment (indicated on the right) from pHF25 or pHF26, respectively.

presence of X-Gal. Five attempts were made to transform chromosomal DNA from SH106 into the wild type strain, 8325-4. However, this did not prove possible and strain SH106 was used as the starting strain for transposon mutagenesis. Correct integration of pHF25 into RN4220 was confirmed by Southern blotting with *Pst*I digested chromosomal DNA from two transformants and RN4220 probed with a digoxygenin labelled 1.8 kb *Eco*RV/*Asp*700 fragment isolated from pEXO (Fig. 4.8). Two bands of *Pst*I digested chromosomal DNA from RN4220 hybridised with the probe since there is a *Pst*I site within the region of *agr* covered by the 1.8 kb probe (see Fig. 4.5). Clones 1 and 2 have each of the above bands but also have two additional bands of 689 bp and approximately 3.5 kb indicating the correct insertion. Fig. 4.9 shows how these bands are derived.

4.2.2 Creation of transposon libraries in strain SH106

Three separate transposon libraries were created using the temperature-sensitive plasmid, pRN3208, containing the transposon Tn*551* (Kornblum *et al.*, 1986) (Fig. 4.1). As explained in section 4.1, plasmid pRN3208 has a Cd resistance marker for plasmid selection and an Em resistance marker for the transposon. Thus cells carrying pRN3208 are resistant to Cd but, following insertion of Tn*551* into the chromosome, Cd resistance will be lost and only Em resistance will remain. Creation of transposon libraries is described in detail in chapter 2 but, briefly, strain SH106, containing pRN3208, was grown overnight with shaking at 30 °C in TSB containing Cm and $CdCl_2$. This culture was used to inoculate fresh media containing identical additions which was then grown with shaking at 30 °C until the OD_{600} reached 1.0. An aliquot of this culture (3 ml) was removed, centrifuged (3,000 rpm, 10 min, RT) and resuspended in TSB, containing Em, pre-warmed to 43 °C. The culture was maintained at 43 °C with shaking for approximately 4 hours until an OD_{600} of between 0.4 and 1.0 was obtained. An aliquot of this culture (3 ml) was transferred to

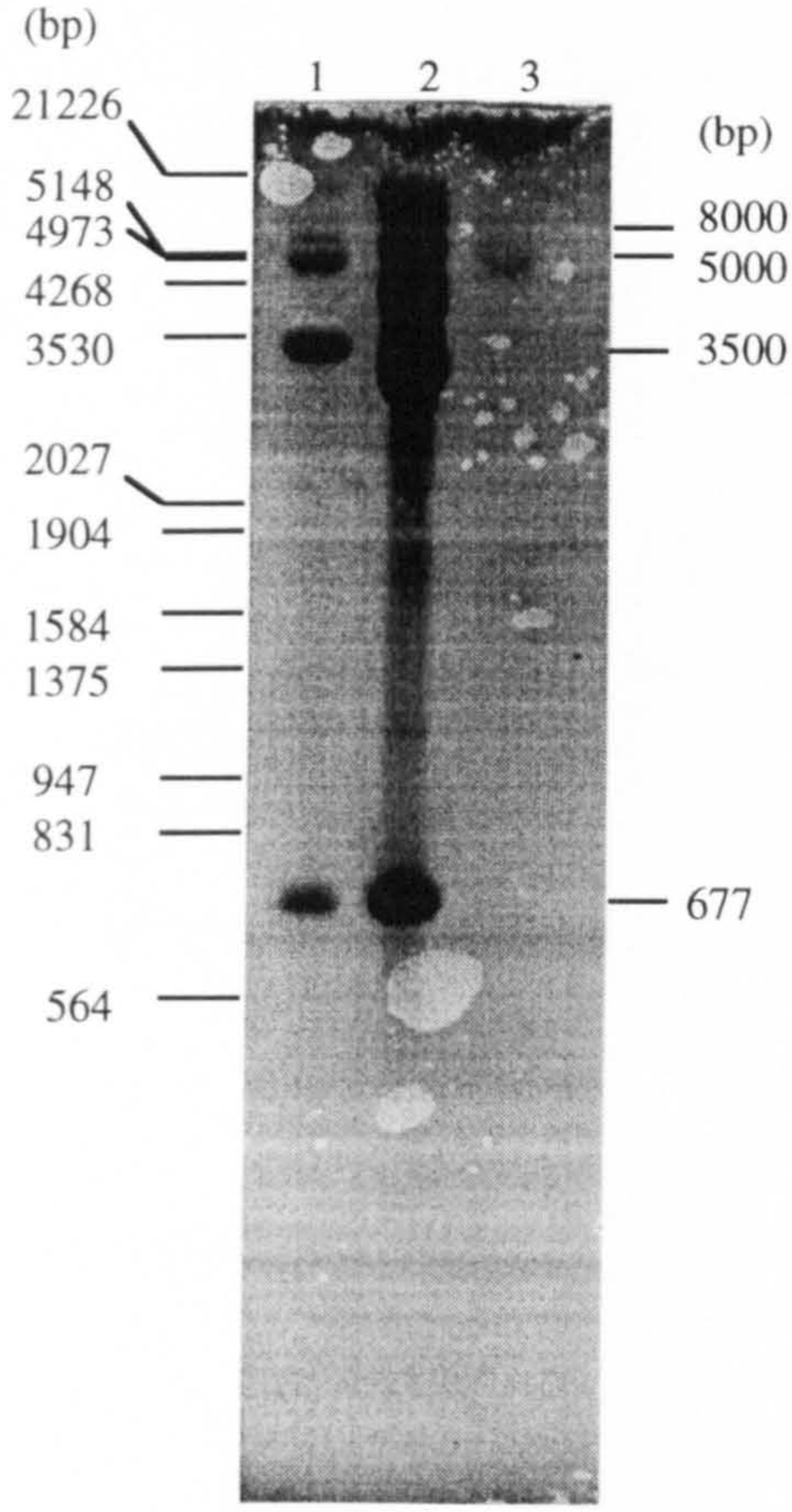


FIGURE 4.8

Southern blot confirming the integration of pHF25 into the chromosome of *S. aureus* RN4220 to give strain SH106. Lanes 1 and 2 show RN4220 containing pHF25 and Lane 3 shows RN4220 digested with *Pst*I, probed with the digoxygenin labelled 1.8 kb *Eco*RV/*Asp*700 fragment isolated from pEXO (see Fig. 4.5). The sizes of *Hin*dIII/*Eco*RI digested λ fragments are indicated on the left (bp). The sizes of hybridising fragments are indicated on the right (bp).

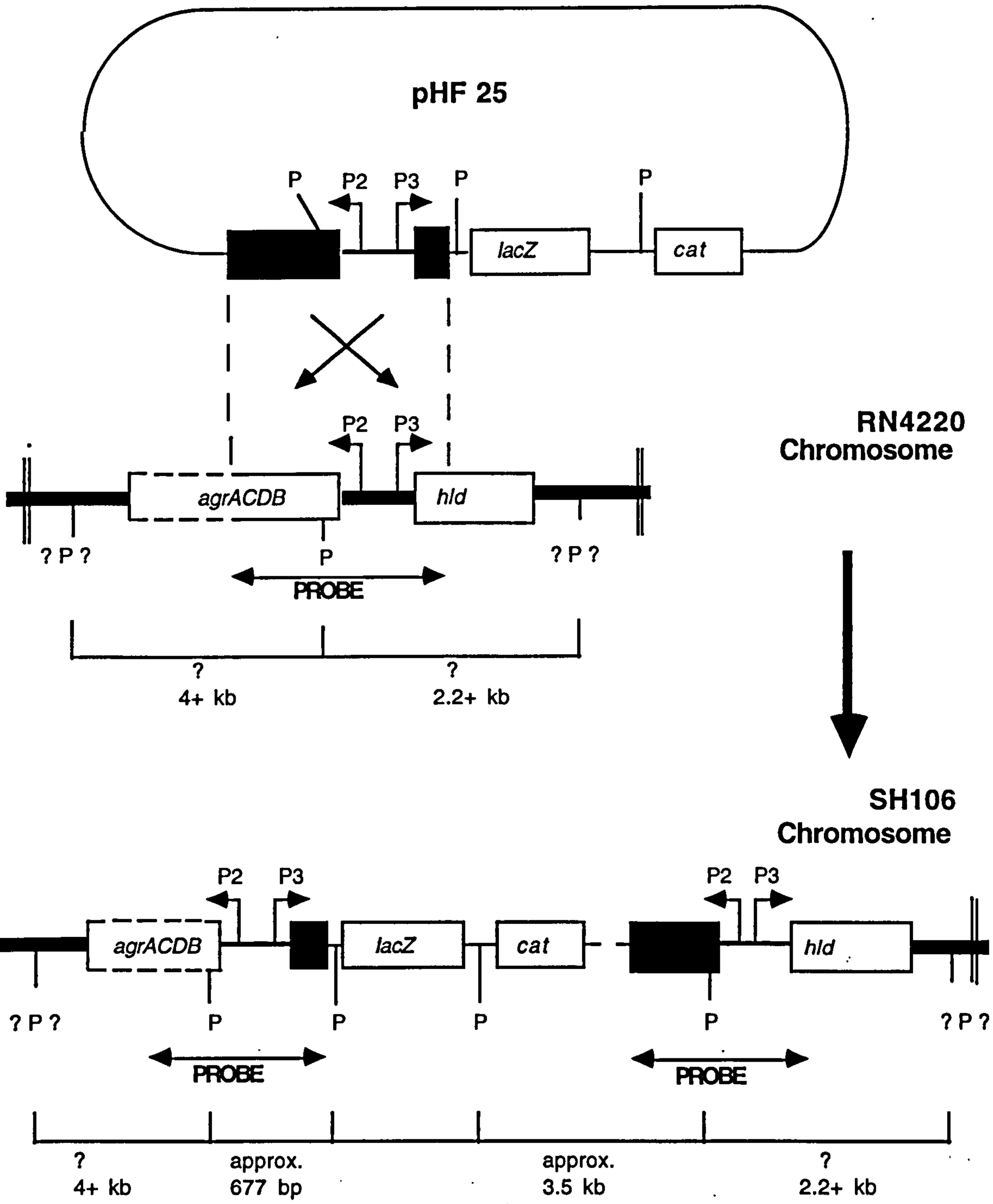


FIGURE 4.9

Construction of the *hld::lacZ* transcriptional reporter gene fusion strain, SH106. The extent of the *agr* locus is indicated by the symbol, ||. The hybridising bands indicated resulted from digesting chromosomal DNA of strains RN4220 and SH106 with *Pst*I (P) and correspond to bands on the Southern blot (see Fig. 4.8). The extent of the 1.8 kb *Eco*RV/*Asp*700 probe (double headed arrow) (see Fig. 4.5) is shown. (This figure is not to scale).

TSB, pre-warmed to 43 °C, containing Em, and grown at 43 °C until the OD_{600} reached 1.5. Cells were centrifuged (8,000 rpm, 10 min, RT), resuspended in TSB/glycerol (10 % v/v) and aliquoted (0.5 ml) into sterile 1.5 ml microfuge tubes. The cells were then flash frozen and stored at -20 °C. One aliquot of each library was immediately defrosted and quantified as shown in Table 4.1. The three libraries contained between 6.5 x 10^6 and 3 x 10^7 colony forming units (cfu) per ml. In order to confirm that the libraries were not contaminated, serial dilutions of each library were spotted (10 µl) onto plates with either no antibiotic or with Cm/Em. In addition, comparison between the number of cfu on plates containing Cm or Cm/$CdCl_2$ revealed that <1 % of the cells were Cd resistant and thus still retained pRN3208.

4.2.3 Isolation of *hld::lacZ* expression mutants

4.2.3.1 Optimisation of mutant selection conditions

Expression of *hld::lacZ* by strain SH106 was measured on plates using the substrate X-Gal. Different concentrations of X-Gal (from 50-150 µl of a 20 mg/ml stock) were either spread directly on to the surface of BHI plates using glass beads or added in an agar overlay (3 ml). Both methods, using 100 µl of 20 mg/ml X-Gal, gave comparable and adequate results so, for ease of experimentation, X-Gal was spread with glass beads and the plates dried prior to plating of bacteria. In order to ensure that the colour of individual colonies would be easily discernible, serial dilutions of cells were plated out to determine an optimum number of colonies per plate. It was apparent that approximately 250 cfu per plate was the maximum that allowed easy differentiation between blue and white colonies.

Library:	cfu/ml		
	Cm/Em	No Ab	Cm/$CdCl_2$
HF1	2.5×10^7	2.6×10^7	9.5×10^4
HF2	3×10^7	3×10^7	5.5×10^4
HF3	6.5×10^6	7×10^6	3×10^4

TABLE 4.1

Determination of the concentration of the transposon libraries (where N = cfu/ml of each library), the purity and efficiency of transposition. (Ab denotes antibiotic).

4.2.3.2 Quantification of the spontaneous *hld::lacZ* mutation rate

In order to determine the likelihood of mutations affecting *hld::lacZ* expression being due to Tn*551*, it was necessary to establish the rate at which spontaneous mutations affecting β–galactosidase production occurred. Thus, approximately 30,000 colonies of strain SH106 (without the transposon) were screened on antibiotic-free BHI plates spread with X-Gal. 36 colonies appeared white and were plated onto BHI with chloramphenicol and X-Gal. Of these, 19 grew to a comparable colony size to SH106, 13 grew very slightly and 3 did not grow at all. On rescreening of the colonies, only one of those able to grow well was consistently white. As *agr* mutants grow well, those colonies which showed diminished growth were deemed unlikely to bear mutations in regulators of *agr* but rather have gross growth defects. It was therefore concluded that 1 in 30,000 cells was naturally white, presumably due to a spontaneous mutation. Strain SH106 was also grown at 43 °C, the temperature used to create the transposon libraries, and it was found that growth at this higher temperature did not lead to an increase in the number of spontaneous white mutants.

4.2.3.3 Isolation of mutants with altered *hld::lacZ* expression

Transposon mutants were selected by plating out approximately 250 colonies per plate on antibiotic-free BHI agar spread with X-Gal. Forty seven mutants (designated A1-A6, B1-B2, C1-C25, D1-D4 and 2/1-2/10) were selected out of approximately 52,000 colonies screened. These mutants were consistently white and grew well on plates in the presence of Cm and Em. The frequency of possible *hld::lacZ* expression mutants in the transposon libraries was therefore deemed to be approximately 1 per 1000 cells.

4.2.4 Phage transduction

4.2.4.1 Transduction into parental strain SH106

In order to determine that the alteration in *hld::lacZ* expression was due to transposon insertion, transposons from all 47 mutants were phage transduced into the parental strain SH106 on two separate occasions using ϕ85. SH106 is blue in the presence of X-Gal due to *hld::lacZ* expression. Phage transduction using lysate from each mutant into SH106 and selection for Em resistant colonies allowed the co-transduction of the transposon and the *hld* expression defect to be assessed. If the transposon insertion had resulted in the alteration in *hld::lacZ* expression then all the transductants should be white on BHI X-Gal plates. Transduction was not achieved with lysate prepared from 8 of the 47 mutants, namely A3, A4, B1, C8, C10, C15, 2/1 and 2/8. However, transductants containing each of the remaining mutations were grown on BHI with X-Gal and Cm/Em, and all colonies remained blue indicating that the transposon had not inserted into a locus affecting *hld::lacZ* expression in any of the mutants. The total number of transductants tested for each mutant is shown in Table 4.2.

4.2.4.2 Transduction into wild type strain 8325-4

Transposon mutations were also transduced into strain 8325-4 and selected as Em resistant colonies. Initially, transductants were inoculated as a single stab onto sheep blood agar plates to measure production of β–haemolysin, and compared visually with 8325-4 (Table 4.2). Several transductants showed variable but reduced levels of β–haemolysin activity, evidenced by approximately a 0-5 mm radius of clearing around the stab compared to approximately 7 mm around strain 8325-4 (Table 4.2). Transductants from five mutants (C2, C4, C5, C11 and C25) showed β–haemolysis reduced to consistently low levels (approximately a 2 mm radius of complete clearing) (Table 4.2). All transductants showed an additional, but faint, area of

Strain	No. of transductants in SH106	No. of transductants in 8325-4	Average (± SD) radius of β-haemolysis (mm)
8325-4			7 ± 1.0
A1	**11**	**31**	**7 ± 1.2**
A2	11	60	4 ± 1.1
A5	**34**	**37**	**3 ± 1.0**
A6	6	43	5 ± 1.1
B2	**22**	**16**	**4 ± 0.7**
C1	31	25	4 ± 0.5
C2	**33**	**22**	**2 ± 0.3**
C4	11	32	2 ± 0.3
C5	**50**	**43**	**2 ± 0.1**
C7	70	56	3.5 ± 0.6
C9	**18**	**44**	**4 ± 1.9**
C11	33	23	2 ± 0.2
C13	**16**	**14**	**5.5 ± 1.3**
C14	6	32	6 ± 0.5
C16	**49**	**28**	**5.5 ± 1.0**
C17	15	37	3 ± 1.5
C20	**12**	**54**	**5 ± 1.0**
C23	14	32	4 ± 1.8
C24	**22**	**35**	**6 ± 1.0**
C25	--	23	2 ± 0.4
2/4	**38**	**45**	**4.5 ± 0.8**
2/5	15	22	3.5 ± 1.5
2/6	**46**	**31**	**7 ± 1.0**
2/7	--	28	7 ± 1.1
2/10	**40**	**25**	**7 ± 2.0**
D1	52	47	5 ± 1.0
D2	**27**	**0**	**---**
D4	141	0	---

TABLE 4.2

Phage transduction of putative regulatory mutants. β-haemolysis was measured using transductants in the 8325-4 background.

clearing up to 3 mm around each colony. The intensity of lysis in this outer halo was greatly reduced compared to the wild type. One transductant of each mutant (suffixed .1) was selected for further study as necessary.

4.2.5 Physical mapping of the transposon insertions

One method for mapping transposon insertions utilises a technique known as pulsed field gel electrophoresis (PFGE) (see section 2.9.3) Chromosomal DNA is first digested with an enzyme which has rarely occurring restriction sites, for example, *Sma*I is used in *S. aureus*. *Sma*I digestion of total chromosomal DNA of *S. aureus* 8325 results in sixteen fragments ranging from 9-670 kb (Pattee *et al.*, 1990) which can be resolved by PFGE. Strains 8325-4 and RN4220 differ from 8325 in that they are cured of three resident prophages, ϕ11, ϕ12 and ϕ13. Prophage ϕ12 is in *Sma*I fragment A (670 kb) whilst both ϕ11 and ϕ13 are in *Sma*I fragment F (205 kb). The PFGE profile of strains 8325-4 and RN4220 thus differs from 8325 in that fragment A is reduced to 630 kb and fragment F decreases to 135 kb so that it appears in the same position as fragment H (Pattee *et al.*, 1990).

Digested chromosomal DNA can be separated by two types of pulsed field gel - clamped homogenous electric field electrophoresis (CHEF) and field inversion gel electrophoresis (FIGE). Under the conditions used in this work, separation of DNA by CHEF results in resolution of fragments ranging from 60-1000 kb and by FIGE from 9-250 kb. This technique allows the separation of such large fragments because, unlike standard gel electrophoresis, the direction of the current is constantly switched. In standard gel electrophoresis, pore size in the agarose matrix alone retards fragments according to their size. In PFGE the switching current means that fragments of DNA passing through the gel have to constantly reorientate themselves. Smaller fragments reorientate themselves much more quickly and progress further through the gel matrix.

In order to physically map the Tn*551* insertion site in the chromosome of each mutant, chromosomal DNA from 12 mutants (A1, A2, A5, B2, C1, C2, C5, C7, C11, C17, 2/4 and 2/8) together with a randomly selected blue transposon mutant (DB33) from a Tn*551* library in SH106 as a positive control, and strain RN4220, was digested with *Sma*I and examined by PFGE (see section 2.9.3). Briefly, the preparation of samples for PFGE involved setting whole cells (2×10^8) of each strain into a plug of agarose and then lysing, washing and digesting etc. *in situ*. Each plug containing the restricted DNA was then inserted into a well of a TBE agarose gel and the fragments separated by clamped homogenous electric field electrophoresis (CHEF). Under the conditions used (see section 2.9.3.3) eleven *Sma*I fragments (from 76-630 kb) from each *S. aureus* strain examined were observed, designated A-K (Fig. 4.10). Since Tn*551* has a high level of homology with Tn*917* (Giraudo *et al.*, 1994b) a digoxygenin labelled 5 kb *Xba*I fragment of Tn*917*, isolated from pLTV3 (Camilli *et al.*, 1990) (see Fig. 5.1) was used to probe a Southern blot of the CHEF gel. Mutant A1 did not show clear evidence of hybridisation with the probe (Fig. 4.11) although it can be seen from the pulsed field gel that strain A1 has a typical *S. aureus* profile when compared with the RN4220 control (Fig. 4.10). This probably indicates that the transposon is in a fragment smaller than those resolved on a CHEF gel. Mutants A2, A5, B2, C1, C2, C5, C11 and 2/4 all contained a transposon within *Sma*I fragment A (630 kb) whilst strains C7 and C17 contained Tn*551* in *Sma*I fragment C (324 kb) (Fig. 4.11). Strain 2/8 did not have an *S. aureus* profile (Fig. 4.10) indicating that this was a contaminant and consequently did not contain Tn*551* (Fig. 4.11).

Chromosomal DNA of strain DB33 (Tn*551* control) showed slight hybridisation with the probe at *Sma*I fragment A but a very strong reaction at the site of *Sma*I fragment G (175 kb) (Fig. 4.11). Subsequent CHEF PFGE revealed that strains B1 and 2/1 were not *S. aureus* as their *Sma*I profiles were not related to the RN4220 control (results not shown). This explains why it was not possible to phage transduce mutations from these strains, or strain 2/8 (see section 4.2.4).

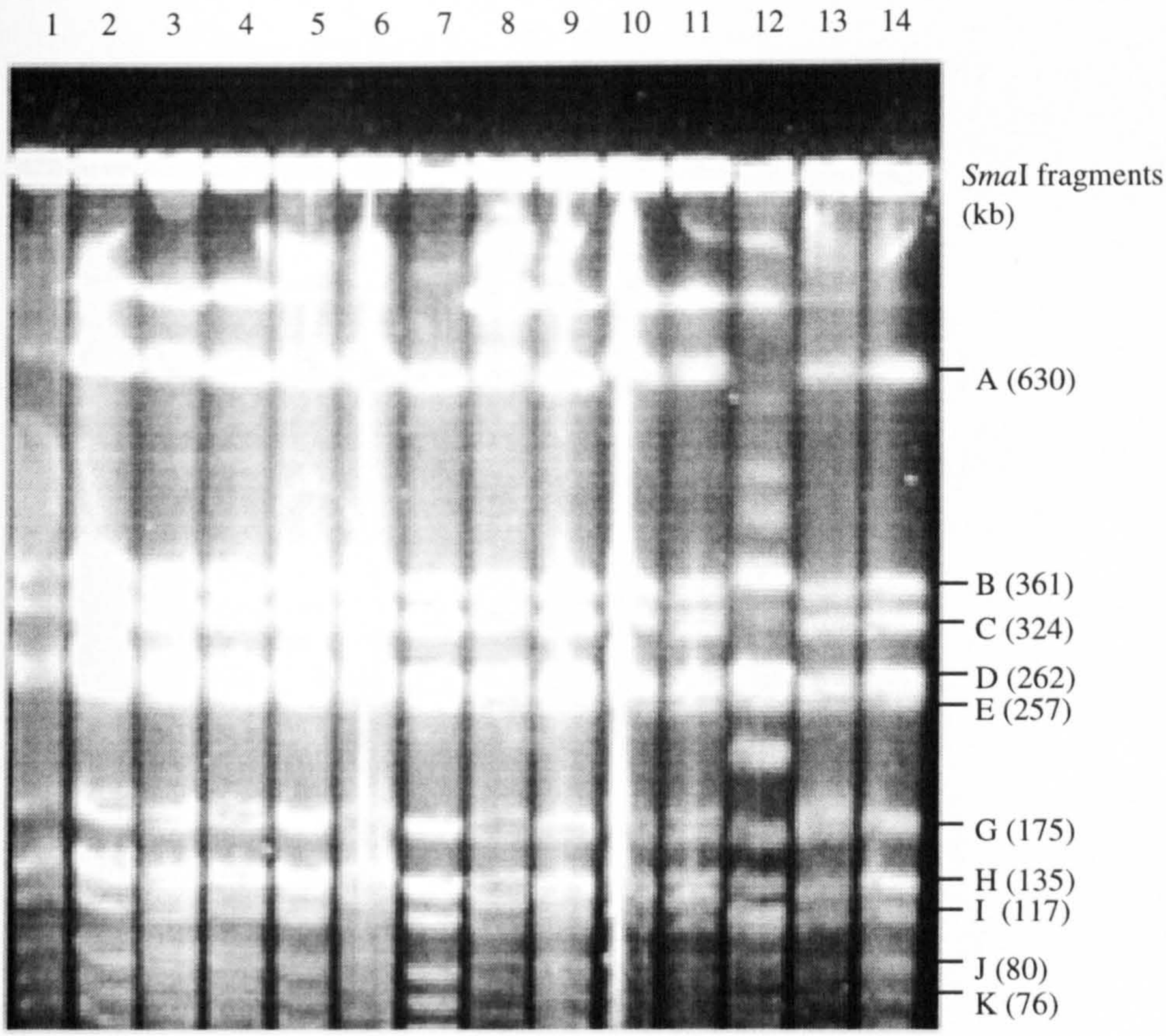


FIGURE 4.10

CHEF pulsed field gel to map Tn*551* insertion sites. Lanes 1 through 14 contain *Sma*I digests of total chromosomal DNA of strains A1, A2, A5, B2, C1, C2, C5, C7, C11, C17, 2/4, 2/8, DB33 and RN4220. The corresponding DNA fragments of RN4220 are shown on the right with their sizes (kb). The gel was calibrated using the standards (not shown) listed in Table 2.5.

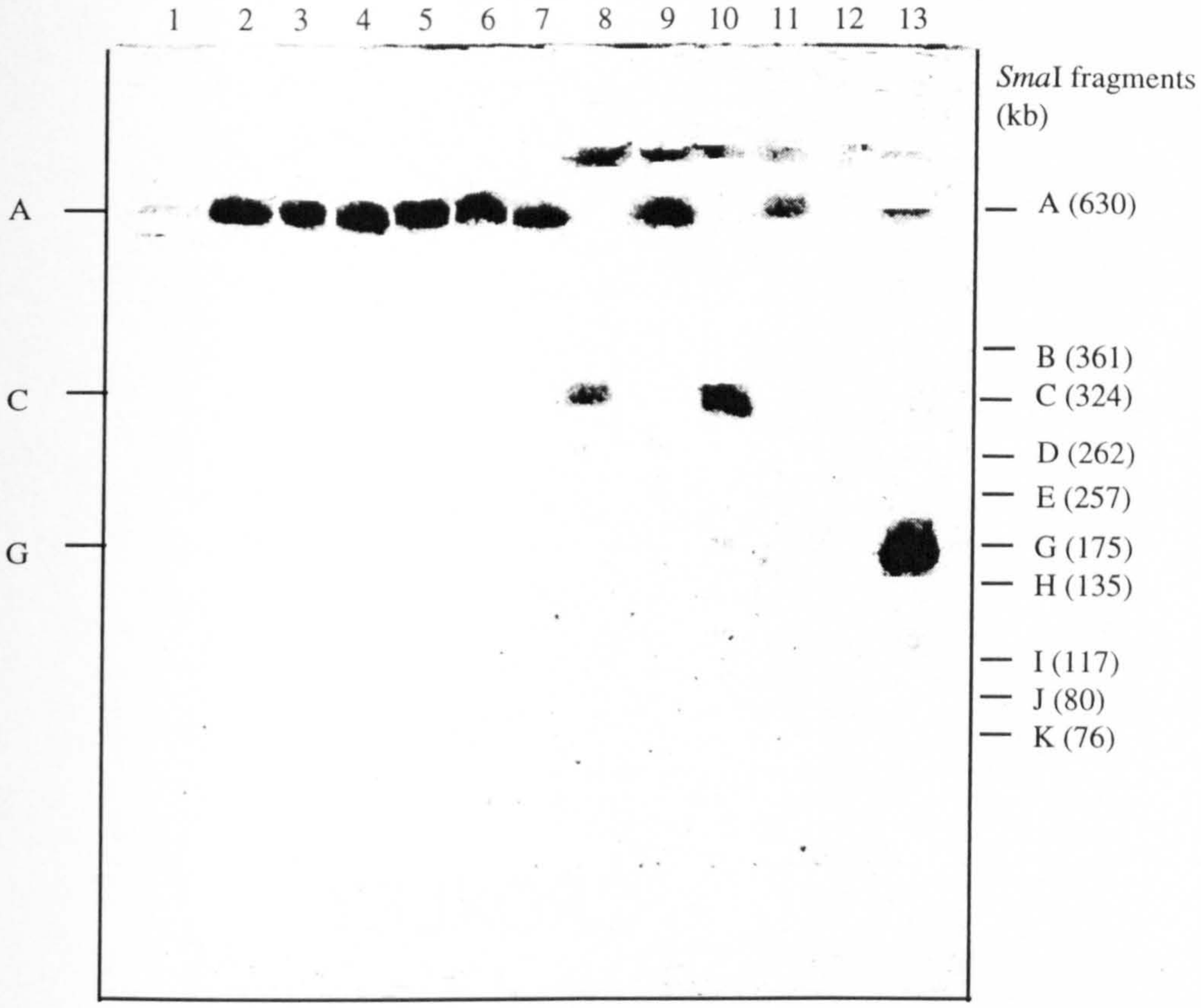


FIGURE 4.11

Southern blot of a CHEF pulsed field gel to map Tn*551* insertions (see Fig. 4.10). Lanes 1 through 13 contain *Sma*I digests of total chromosomal DNA of strains A1, A2, A5, B2, C1, C2, C5, C7, C11, C17, 2/4, 2/8 and DB33 probed with a digoxygenin labelled 5 kb *Xba*I fragment of Tn*917* (see Fig. 5.1). The corresponding DNA fragments of RN4220 are shown on the right with their sizes (kb). Hybridising bands are highlighted on the left.

Each selected transductant (see section 4.2.4.2) of a mutant that contained Tn*551* in *Sma*I fragment A or C was also examined using PFGE. This confirmed that the transposon mutations had transduced to the same fragments in strain 8325-4.

4.2.6 Fine physical mapping of Tn*551* insertions

In order to further map the Tn*551* insertion sites in each mutant, and to determine their relationship with each other, a series of restriction digests and hybridisation studies were carried out. Initially, chromosomal DNA (5 μg) of the *Sma*I fragment A mutants was separated on TBE agarose gels following restriction digestion with *Eco*RI, *Pst*I or *Xba*I. Southern blots of these gels (Fig. 4.12) were probed with a digoxygenin labelled 5 kb *Xba*I fragment of Tn*917* from pLTV1 (Camilli *et al.*, 1990) (see Fig. 5.1). *Xba*I cuts at the same position within Tn*551* so that by using a similarly digested probe only one of the *Xba*I digest fragments covered by the probe will hybridise. From the resulting fragment sizes (Table 4.3) it can be seen that all of the mutants show a different hybridisation pattern indicating that none are siblings. Therefore, the transposon insertions may either be in different loci or at altered positions within the same locus.

Many of the hybridising fragments were fairly large (Table 4.3) so chromosomal DNA (5 μg) of each *Sma*I fragment A mutant, together with *Sma*I fragment C mutants, was digested with *Kpn*I, *Kpn*I/*Eco*RI, *Eco*RI/*Pst*I or *Sal*I to identify smaller fragments suitable for subcloning. Tn*551*, in common with Tn*917*, contains both a *Kpn*I and a *Sal*I restriction site downstream of the Em resistance (*erm*) gene (see Fig. 5.1). Southern blots of these gels (Figs. 4.13-4.16) were probed with a digoxygenin labelled 3 kb *Kpn*I/*Xba*I fragment of Tn*917*-LTV3 (see Fig. 5.1). This probe excludes the Em resistance gene of Tn*551*. Consequently, when chromosomal DNA was digested with *Kpn*I or *Kpn*I/*Eco*RI the probe hybridised to chromosomal DNA flanking only the distal end of Tn*551*, i.e. excluding the Em resistance gene. This

(a)

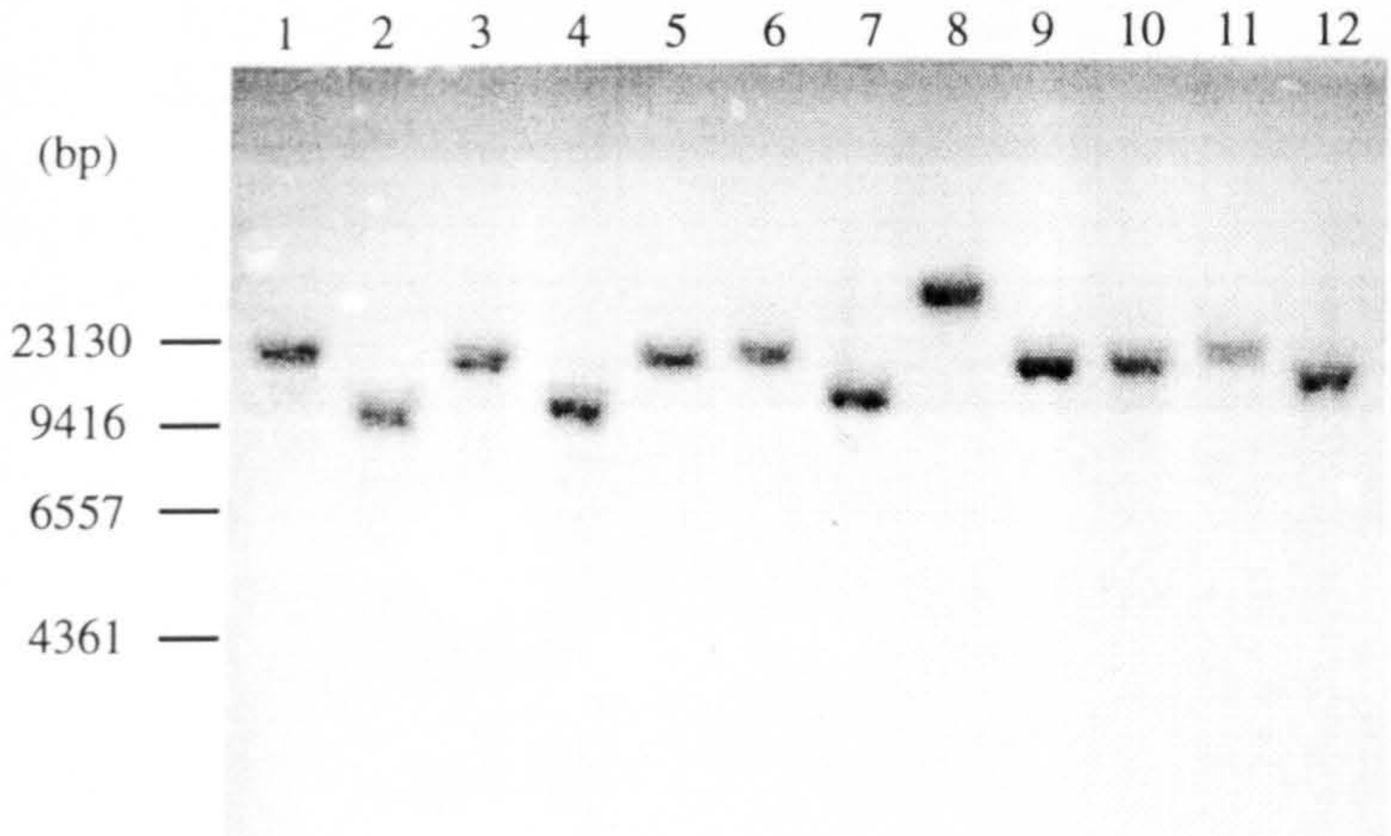
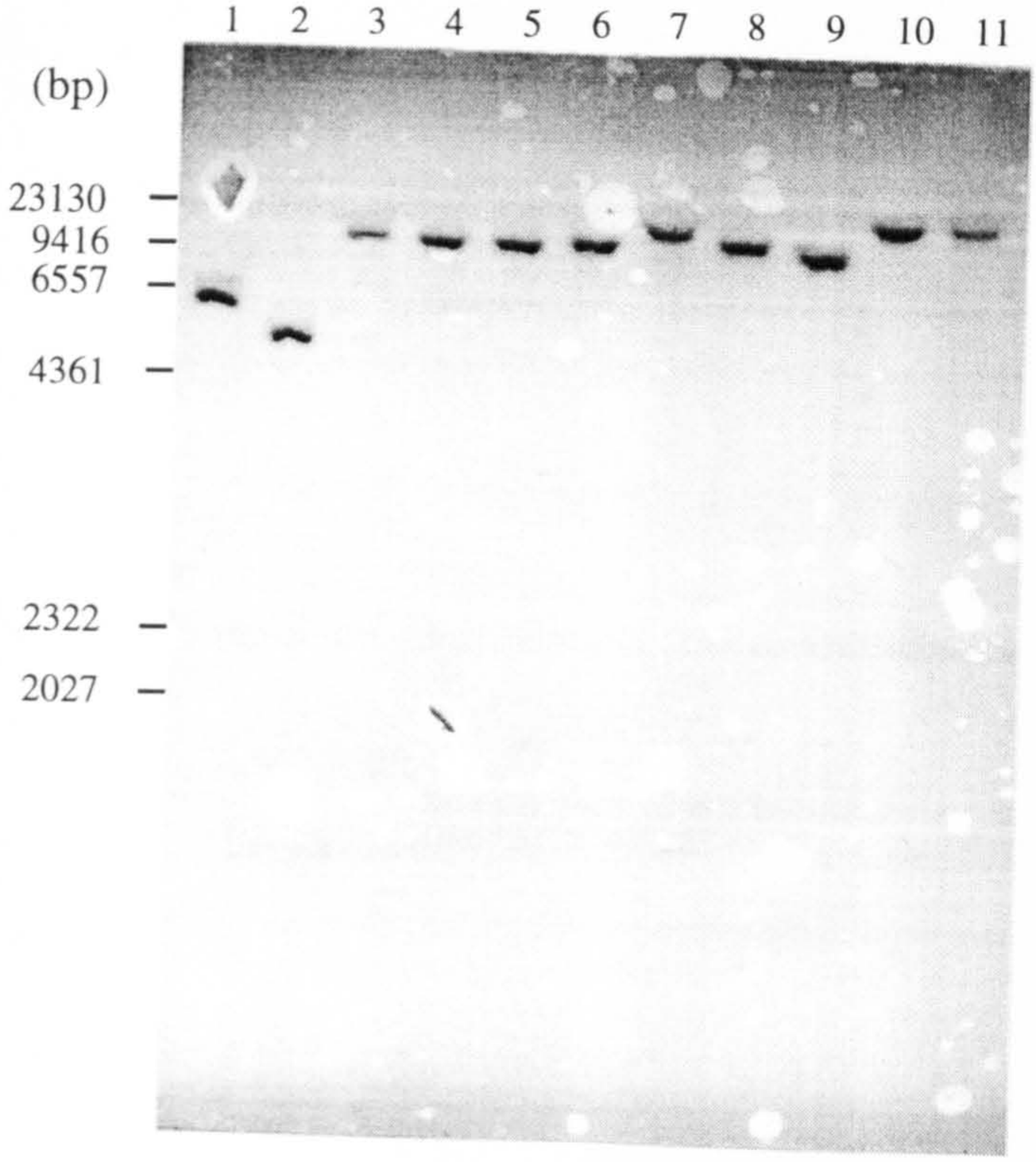


FIGURE 4.12

Southern blots to map Tn*551* insertion sites. **(a)** Lanes 1 through 8 show *Eco*RI digested chromosomal DNA of strains A2.1, A5.1, B2.1, C1.1, C2.1, C5.1, C11.1 and 2/4.1 and lanes 9 through 12 show *Pst*I digested chromosomal DNA of strains A2.1, A5.1, C1.1 and C2.1; **(b,** overleaf) Lanes 1 through 8 show *Xba*I digested chromosomal DNA of strains A2.1, A5.1, B2.1, C1.1, C2.1, C5.1, C11.1 and 2/4.1 and lanes 9 through 11 show *Pst*I digested chromosomal DNA of strains C5.1, C11.1 and 2/4.1. DNA was probed with the digoxygenin labelled 5 kb *Xba*I fragment of Tn*917* (see Fig. 5.1). The sizes of the bands of *Hin*dIII digested λ are shown on the left (bp).

(b)



Mutant	Size of fragment containing Tn*551* (kb)		
	***Eco*RI**	***Pst*I**	***Xba*I**
A2.1	**>23.0**	**21.0**	**6.0**
A5.1	12.0	23.0	5.5
B2.1	**23.0**	**-**	**17.0**
C1.1	12.0	23.0	12.0
C2.1	**23.0**	**20.0**	**12.0**
C5.1	23.0	9.5	12.0
C11.1	**15.0**	**20.0**	**18.0**
2/4.1	>23.0	20.0	12.0

TABLE 4.3

Physical mapping of Tn*551* insertion sites. The sizes of the fragments of chromosomal DNA of each mutant, listed above, which hybridised with the digoxygenin labelled 5 kb *Xba*I fragment of Tn*917* (see Fig. 5.1) are given.

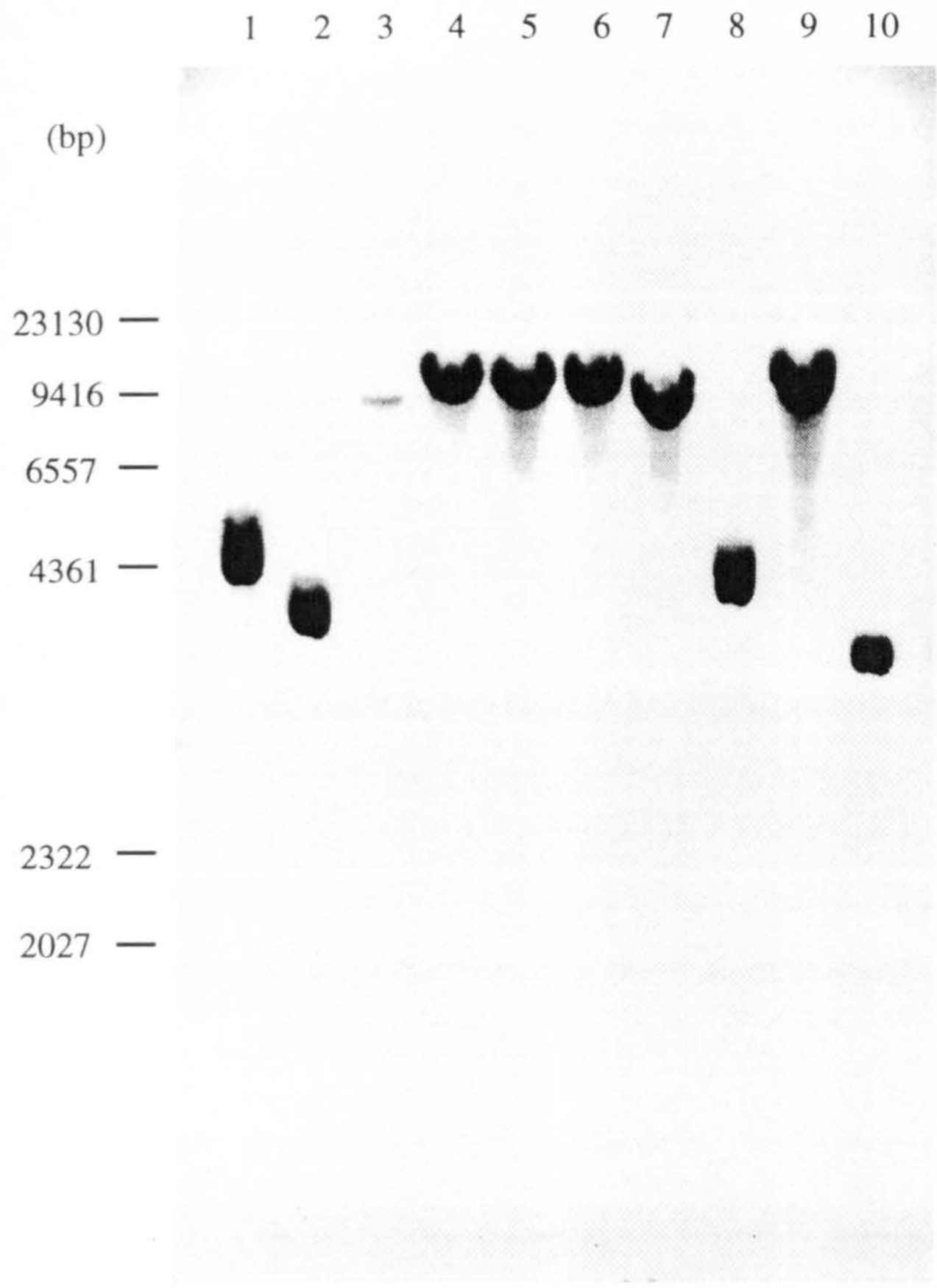


FIGURE 4.13

Southern blot to map Tn*551* insertion sites. Lanes 1 through 10 show strains A2.1, A5.1, B2.1, C1.1, C2.1, C5.1, C7.1, C11.1, C17.1, and 2/4.1 digested with *Kpn*I and probed with the digoxygenin labelled 3 kb *Xba*I/*Kpn*I digested fragment of Tn*917* (see Fig. 5.1). The sizes of the bands of *Hin*dIII digested λ are shown on the left (bp).

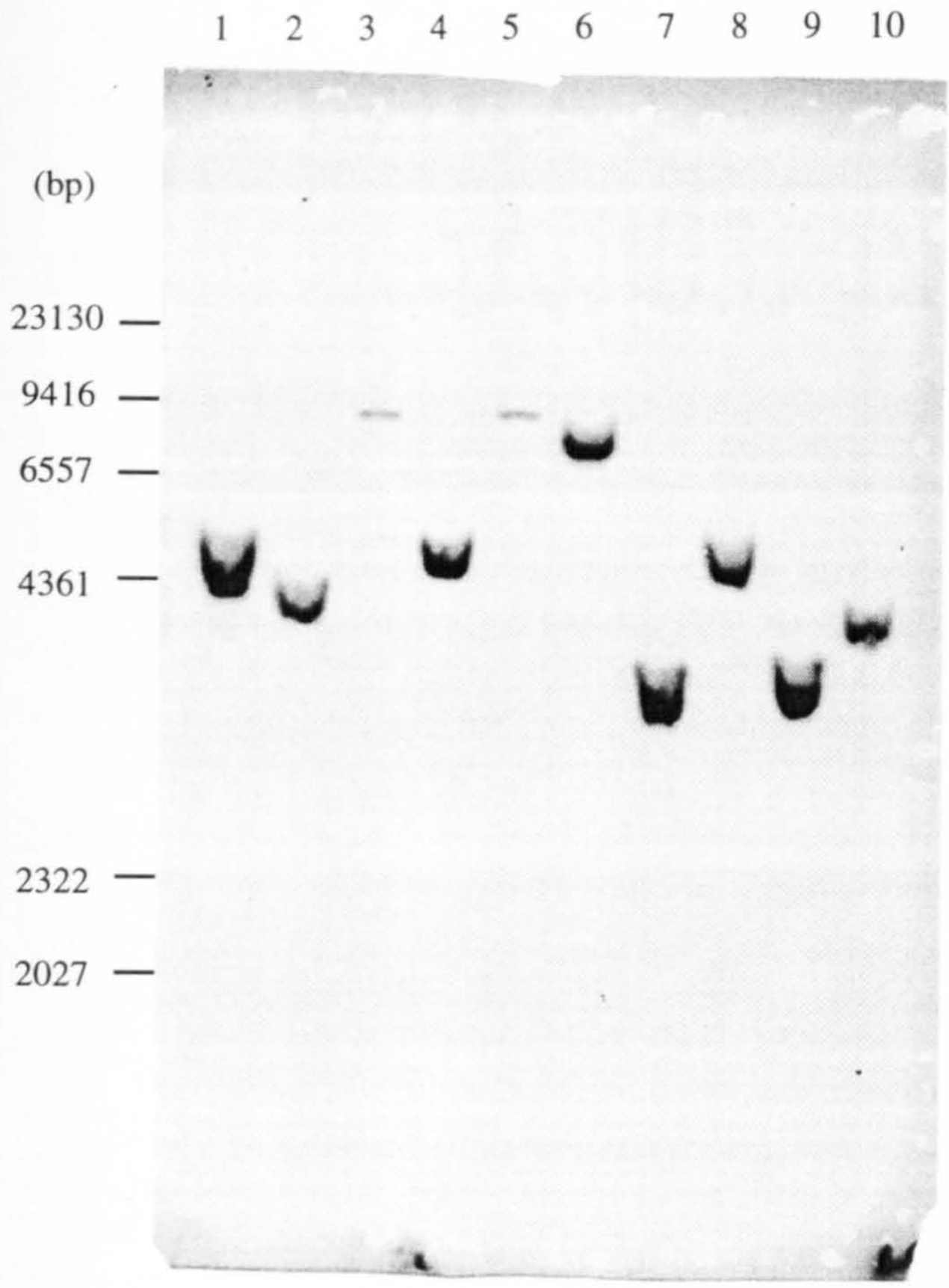


FIGURE 4.14

Southern blot to map Tn*551* insertion sites. Lanes 1 through 10 show strains A2.1, A5.1, B2.1, C1.1, C2.1, C5.1, C7.1, C11.1, C17.1, and 2/4.1 digested with *Kpn*I/*Eco*RI and probed with the digoxygenin labelled 3 kb *Xba*I/*Kpn*I digested fragment of Tn*917* (see Fig. 5.1). The sizes of the bands of *Hin*dIII digested λ are shown on the left (bp).

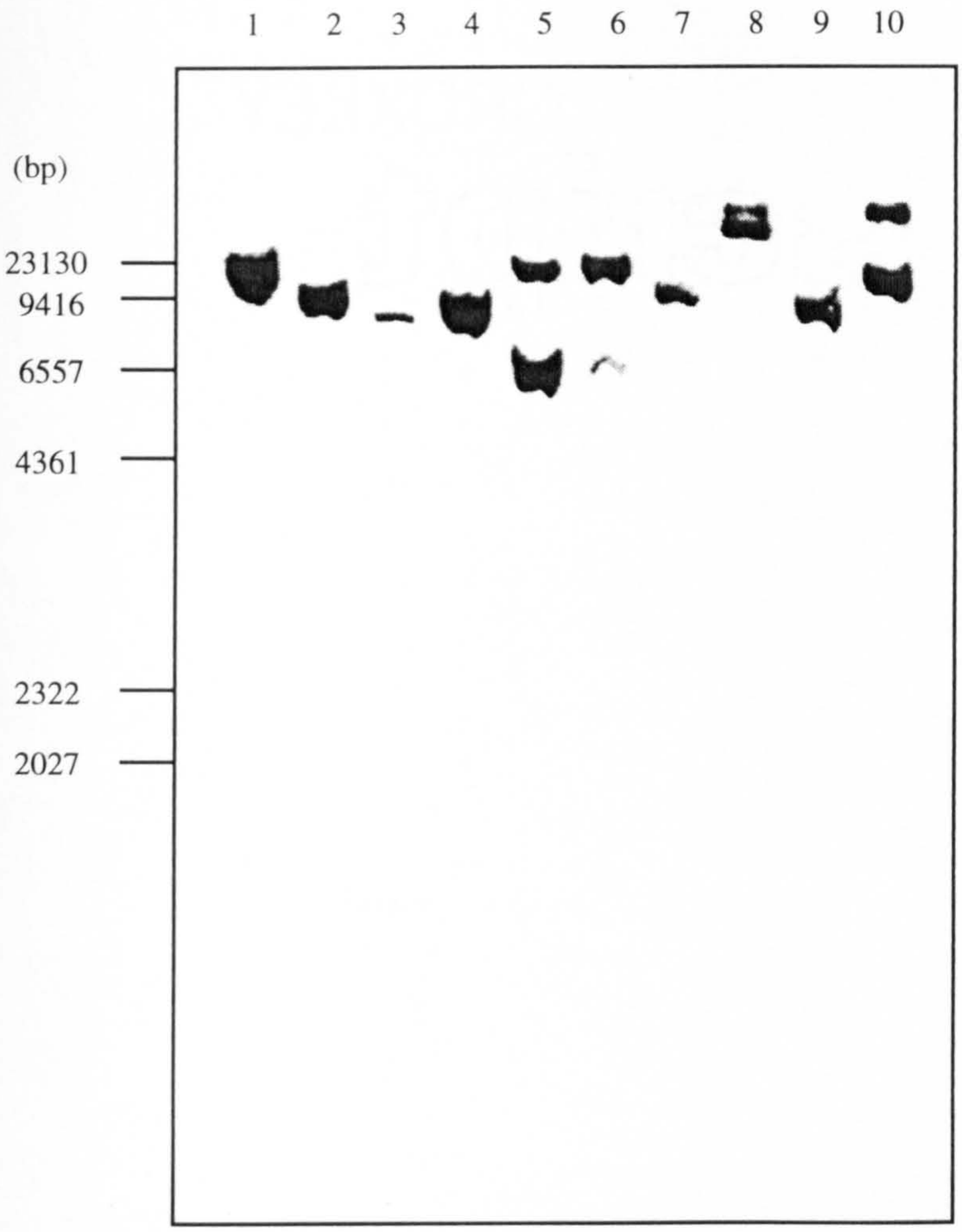


FIGURE 4.15

Southern blot to map Tn*551* insertion sites. Lanes 1 through 10 show strains A2.1, A5.1, B2.1, C1.1, C2.1, C5.1, C7.1, C11.1, C17.1, and 2/4.1 digested with *Eco*RI/*Pst*I and probed with the digoxygenin labelled 3 kb *Xba*I/*Kpn*I digested fragment of Tn*917* (see Fig. 5.1). The sizes of the bands of *Hin*dIII digested λ are shown on the left (bp).

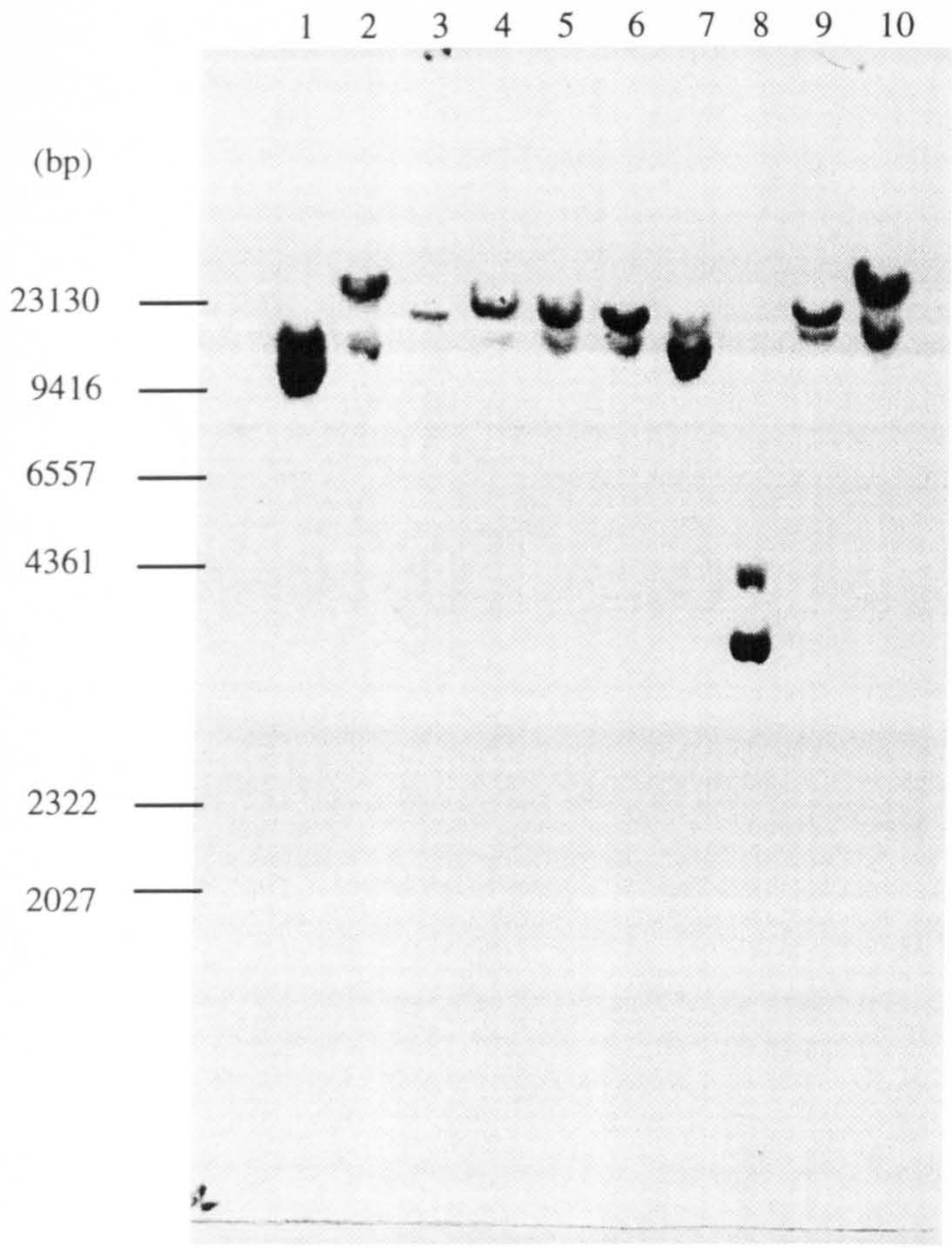


FIGURE 4.16

Southern blot to map Tn*551* insertion sites. Lanes 1 through 10 show strains A2.1, A5.1, B2.1, C1.1, C2.1, C5.1, C7.1, C11.1, C17.1, and 2/4.1 digested with *Sal*I and probed with the digoxygenin labelled 3 kb *Xba*I/*Kpn*I digested fragment of Tn*917* (see Fig. 5.1). The sizes of the bands of *Hin*dIII digested λ are shown on the left (bp).

strategy was adopted since constitutive expression of the Em resistance gene may be toxic to *E. coli* and thus attempting to clone a fragment of DNA containing an Em resistance gene would have been detrimental. In fact, when Morfeldt *et al.* (1988) first identified *exp* (*agr*) by a Tn*551* insertion it proved necessary to clone DNA flanking only the distal end of Tn*551*, excluding the Em resistance gene.

Again all the mutants showed different restriction profiles (Table 4.4). The *Sal*I restriction digest of the chromosomal DNA of each mutant resulted in two bands hybridising with the probe since a *Sal*I site occurs within the region of Tn*551* covered by the probe (Table 4.4). Two fragments of *PstI*/*Eco*RI digested DNA of strains C2.1, C5.1 and C17.1 also hybridised with the probe. However, at least in the case of strains C2.1 and C5.1 it is likely that the larger fragments (23 kb) represent DNA which has digested only with *Eco*RI (see Table 4.3). Strain C17.1 was not previously digested with *Eco*RI or *Pst*I individually so it is impossible to be certain that the extra band is due to partially digested DNA, although this seems likely.

4.2.7 Cloning of transposon insertion sites

In order to clone chromosomal DNA flanking Tn*551* it is necessary to identify a fragment of DNA containing the transposon which is a suitable size (up to 10 kb) to ligate into a plasmid capable of being maintained in *E. coli*. Chromosomal DNA (25 μg) of strains A2.1, A5.1, B2.1, C1.1, C2.1, C5.1, C7.1, C11.1, C17.1, and 2/4.1 was therefore digested with *Kpn*I or *KpnI*/*Eco*RI since these enzymes gave Tn*551*-containing fragments which were 10 kb or less (Table 4.4). In order to confirm the exact sizes of the fragments of chromosomal DNA which contained the transposon, *Kpn*I or *KpnI*/*Eco*RI digested DNA (5 μg) was again separated on TBE agarose gels and Southern blots of each gel (results not shown) were probed as above. Chromosomal DNA (20 μg) from strains A5.1, C2.1, C17.1 and 2/4.1, digested with *Kpn*I and from strains C11.1 and C7.1 digested with *KpnI*/*Eco*RI was separated on a

Strain	Sizes of hybridising fragments (kb)			
	***Kpn*I**	***Kpn*I/*Eco*RI**	***Eco*RI/*Pst*I**	***Sal*I**
A2.1	**4.2**	**4.2**	**12.0**	**16.0 + 9.4**
A5.1	4.0	4.0	9.2	15.0 + >23.0
B2.1	**9.3**	**9.3**	**9.0**	**20.0 + 19.0**
C1.1	9.3	4.4	8.8	17.0 + 20.0
C2.1	**9.3**	**9.3**	**6.0 + 23.0**	**16.0 + 19.0**
C5.1	9.3	7.5	6.5 + 23.0	16.0 + 19.0
C11.1	**7.5**	**3.2**	**15.0**	**12.0 + 18.0**
2/4.1	4.1	4.4	>23.0	3.8 + 4.2
C7.1	**9.3**	**3.4**	**9.2**	**18.0 + 20.0**
C17.1	3.8	3.8	18.0 + >23.0	18.0 + >23.0

TABLE 4.4

Physical mapping of Tn*551* insertion sites. The sizes of the fragments of chromosomal DNA of each mutant, listed above, which hybridised with the digoxygenin labelled 3 kb *Kpn*I/*Xba*I fragment of Tn*917* (see Fig. 5.1) are given.

TAE agarose gel. For each mutant, fragments in the region spanning 2 kb above and below the appropriate size were excised and purified from the gel. Each sample therefore contained many fragments of *Kpn*I or *Kpn*I/*Eco*RI digested chromosomal DNA. The fragments were then ligated into similarly digested, dephosphorylated pUBS1 (Foster, 1995) and transformed into *E. coli* XLOLR by electroporation (Schenk and Laddaga, 1992) or into ultra-competent *E. coli* XL2-Blue MRF' (Stratagene). Putative transformants were then probed, by colony blotting, with a 5 kb *Xba*I fragment of Tn*917*-LTV3 to identify clones containing Tn*551*. However, after probing approximately 100 colonies of each mutant it was not possible to isolate any positive clones.

4.2.8 Phenotypic characterisation of five Tn*551* mutants

The five strains (C2.1, C4.1, C5.1, C11.1 and C25.1) which showed consistently low levels of β–haemolysin activity, along with 8325-4 (WT) and the *agr*⁻ strain, WA250 (Morfeldt *et al.*, 1988) for comparison, were examined for the production of several virulence associated characteristics. Cultures of each strain were grown overnight in BHI and diluted in phosphate buffered saline (PBS) (1 ml) to give an OD_{600} of 0.5. This was assumed to be approximately 10^8 cells per ml (see section 3.2.3). Serial dilutions (from 10^6 to 10^2 cfu/ml) in PBS were spotted (10 µl) onto antibiotic-free plates as appropriate and phenotypic characteristics compared. The results are summarised in Table 4.5.

4.2.8.1 β–haemolysin assay

Strains were plated onto sheep blood agar (see appendix A, section A1.4.1) where strain 8325-4 showed an average 7 mm radius of clearing around single colonies and strain WA250 was β–haemolysin negative (Fig. 4.17). All five mutants showed approximately a 2 mm radius of complete clearing around single colonies. However,

Strain	β-haem.	α-haem.	Protease	Lipase	DNase	Hyaluronate lyase
8325-4	+++	+++	+++	+++	+++	+++
WA250	**-**	**-**	**-/+**	**+++**	**+**	**+**
C2.1	+	++/+++	++	++	++/+++	++
C4.1	**+**	**++/+++**	**+**	**+++**	**+++**	**++**
C5.1	+	++	+	+++	++	++
C11.1	**+**	**++**	**++**	**+++**	**+++**	**++**
C25.1	+	++/+++	++/+++	++	+++	++

TABLE 4.5

Phenotypic characteristics of five Tn*551* mutants compared with strains 8325-4 (WT) and WA250 (*agr*).

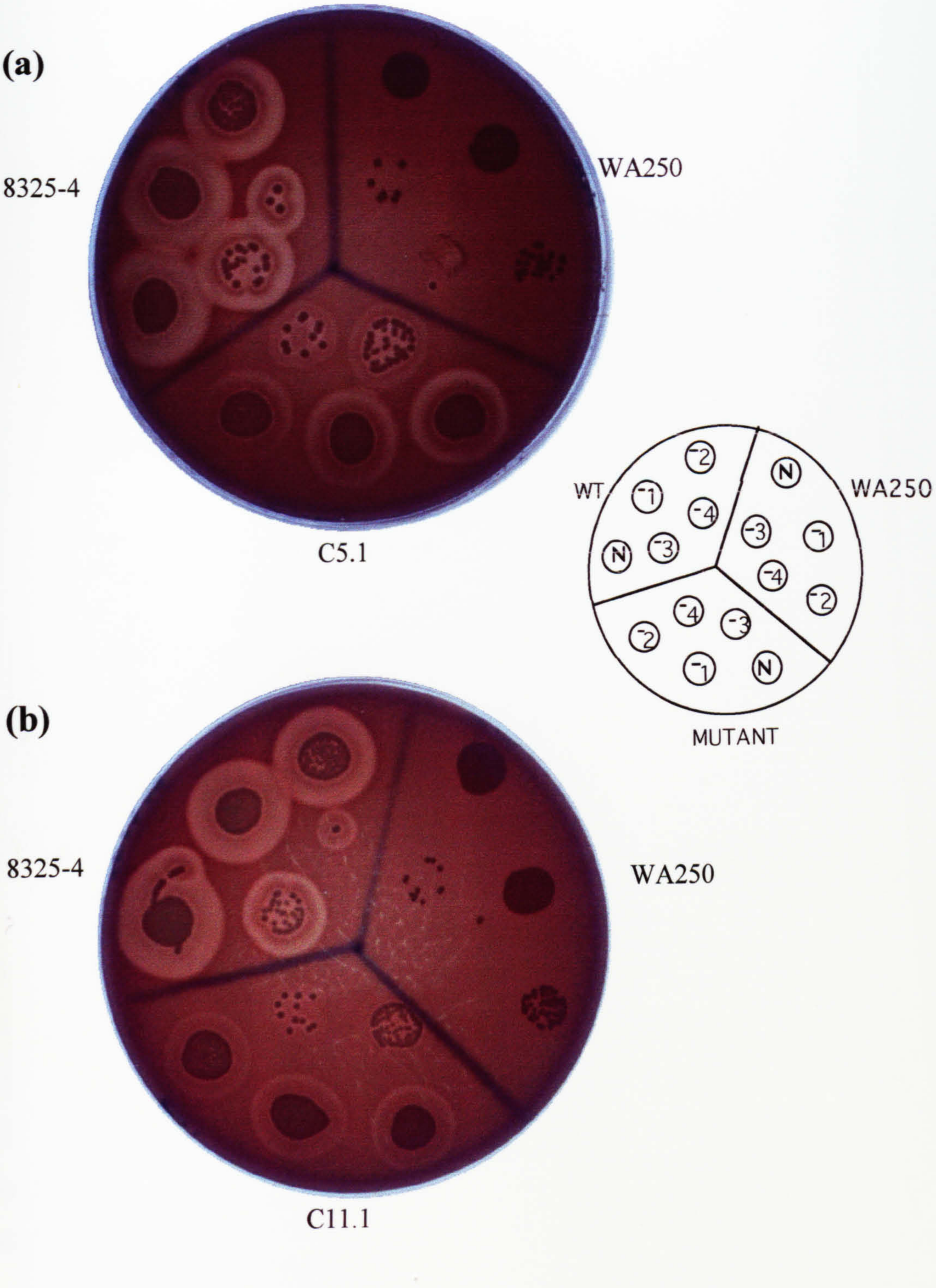


FIGURE 4.17

β-Haemolysin plate assay. Strains 8325-4 (WT), WA250 (*agr)* and strains (a) C5.1 and (b) C11.1 were serially diluted and spotted (10 μl) onto sheep blood agar prior to incubation overnight at 37°C.
Serial dilutions (where N = 10^6 cfu/ml) were plated in the order shown in the key

extending a further 2-3 mm was an area of lysis which was much fainter than that found in 8325-4. Strains C5.1 and C11.1 show the limited range of variation in β–haemolytic activity of these mutants (Fig. 4.17).

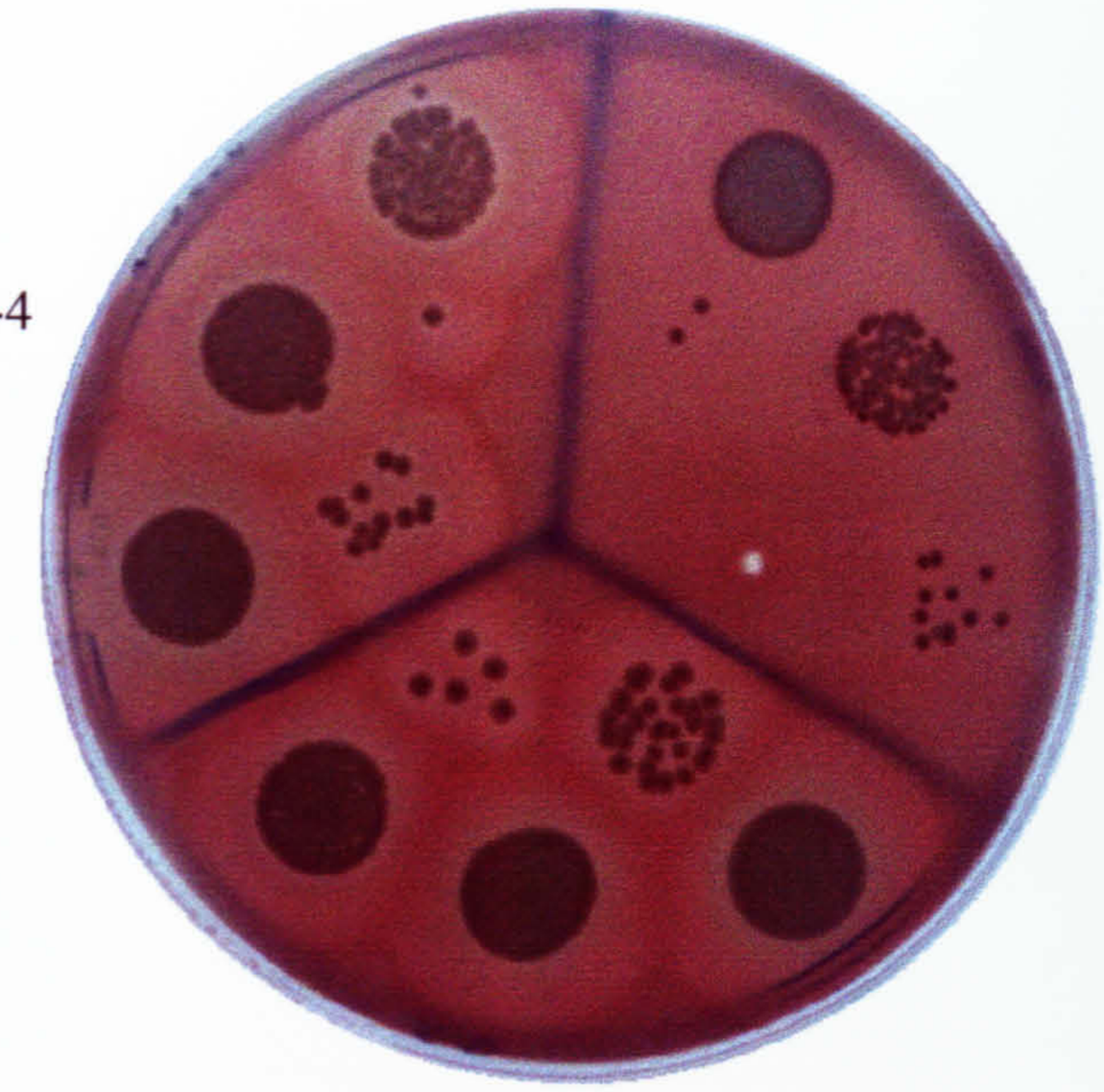
4.2.8.2 α–haemolysin assay

This assay consisted of plating dilutions of cultures on rabbit blood agar (see appendix A, section A1.4.2) and, following growth overnight at 37 °C, measuring zones of clearing around each colony. Strain 8325-4 showed a halo of clearing radiating approximately 1 cm from a colony and WA250 was α–haemolysin negative (Fig. 4.18). Strains C2.1, C4.1 and C25.1 all showed a slight reduction in intensity of clearing which extended approximately 8 mm, 8 mm and 9 mm respectively. Strains C5.1 and C11.1 also showed a reduced radius of clearing which extended approximately 6 mm around single colonies (Fig. 4.18). However, strain C11.1 showed a much less intense halo of clearing (Fig. 4.18).

4.2.8.3 Lipase assay

This assay used egg yolk (10 %) in an overlay (5 ml) on TPEY agar plates (see appendix A, section A1.4.4) to test for lipase activity. Plates were incubated for 48 hours at 37 °C and a positive result was evidenced by a white precipitate around colonies (see section 4.3). Strains WA250 and 8325-4 appeared equally lipase positive, characterised by approximately a 3 mm precipitate around single colonies (Fig. 4.19). Strains C4.1, C5.1 and C11.1 showed lipase activity comparable to the wild type but strains C2.1 and C25.1 showed reduced activity resulting in a precipitate extending approximately 2 mm around single colonies (Fig. 4.19).

(a)



(b)

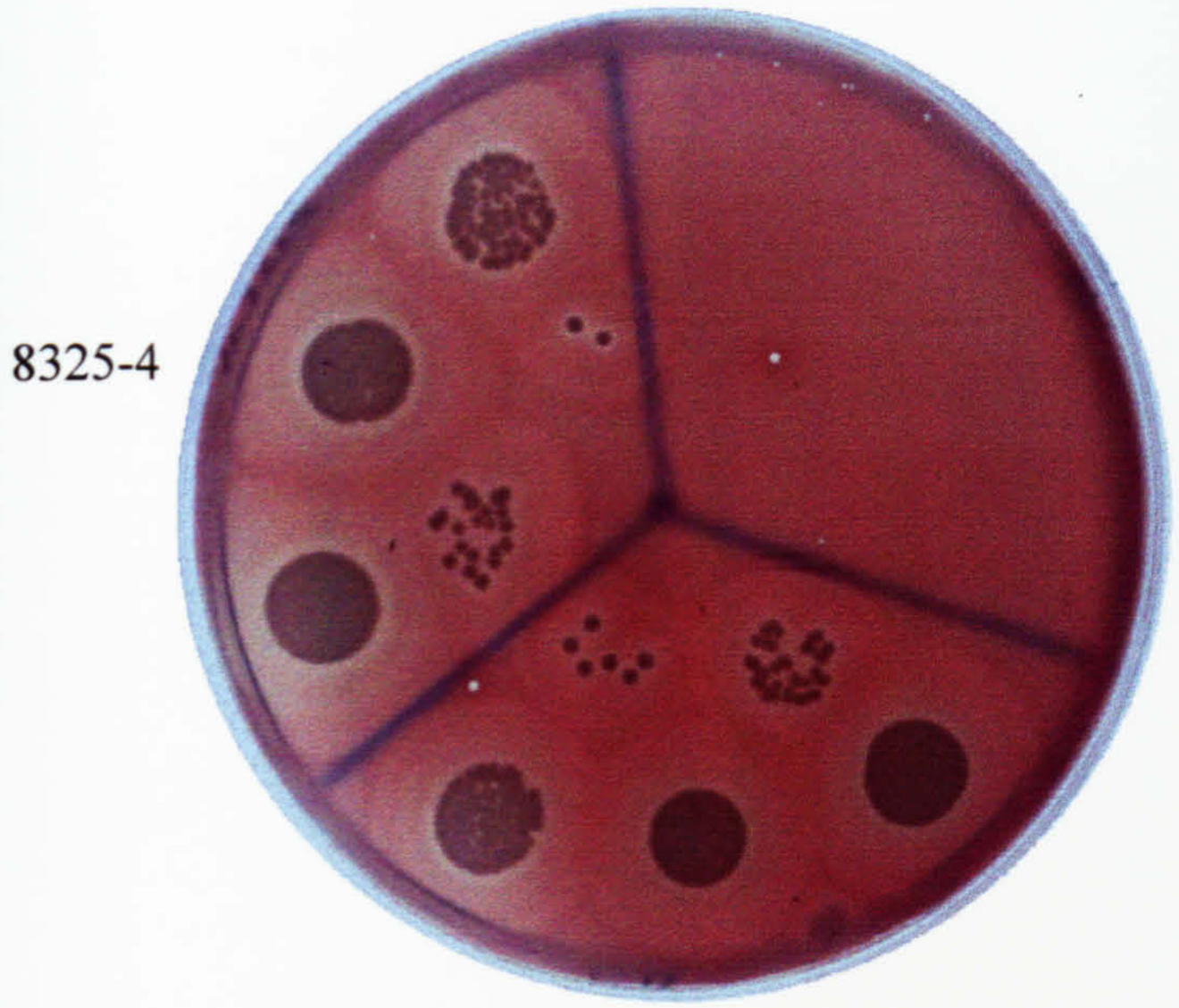


FIGURE 4.18

α-Haemolysin plate assay. Strains C5.1 (a) and C11.1 (b), together with 8325-4 (WT) and strain WA250 (*agr*) were grown for 24 h at 37°C on rabbit blood agar and the zones of α-haemolysis measured and compared. The key for the dilutions plated is as in Fig. 4.17.

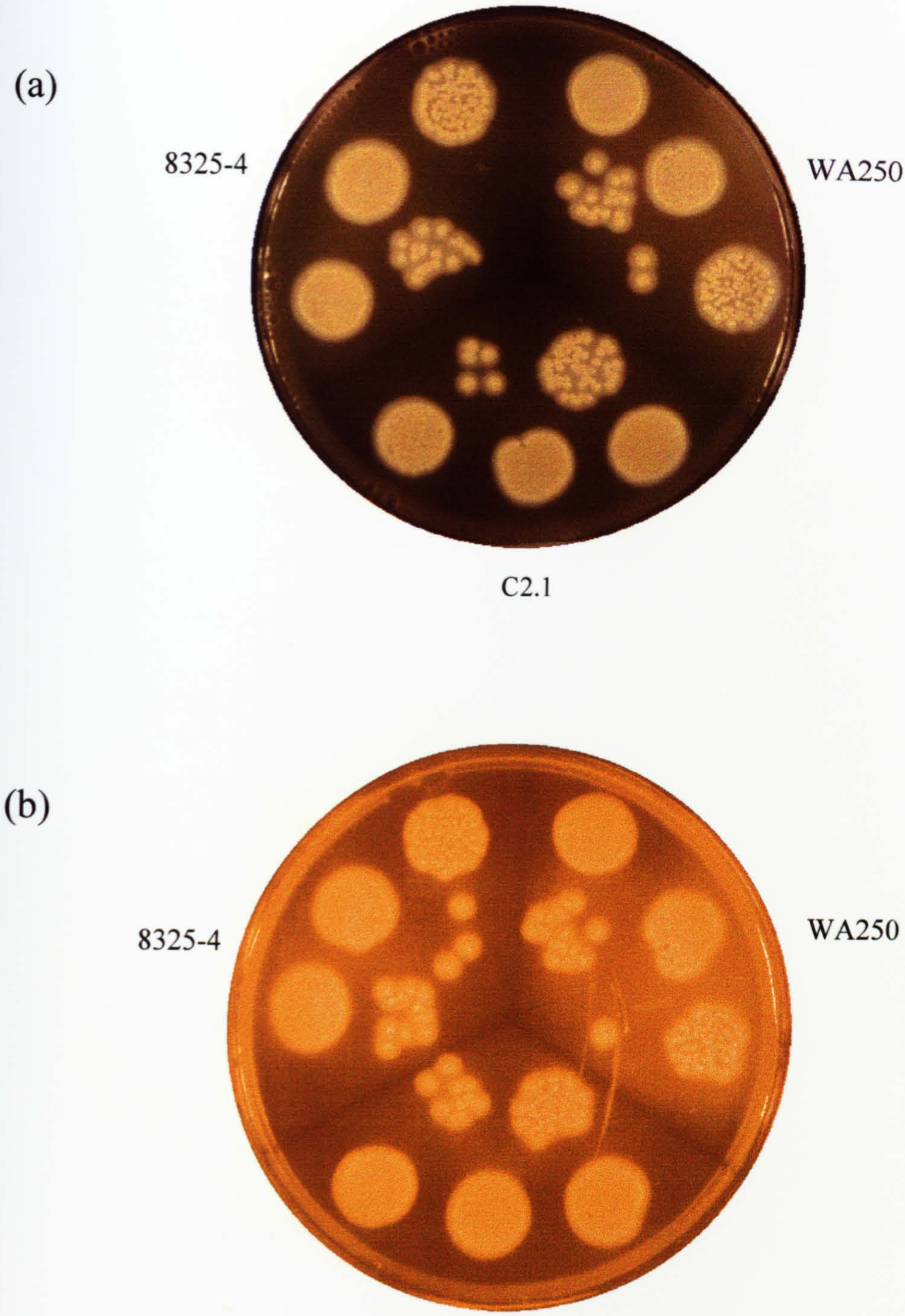


FIGURE 4.19

Lipase plate assay. Strains C2.1 (a) and C25.1 (b) together with 8325-4 (WT) and WA250 (*agr*) were grown for 48 h at 37°C on egg yolk agar (10%). Lipase production is evident as a white zone of precipitation around the colonies. The key for the dilutions plated is as in Fig. 4.17.

4.2.8.4 Protease assay

Strains were plated on BHI agar plates containing dried skimmed milk (1.5 % w/v) (see appendix A, section A1.4.5) for 24 hours at 37 °C and protease activity was evident as a halo of clearing in the opaque background (see section 4.3). All five mutants appeared to have reduced protease activity compared to strain 8325-4 which gave an area of clearing radiating approximately 3 mm around single colonies (Fig. 4.20). A halo of protease activity was apparent in strain WA250 around large clusters of colonies but not around single colonies (Fig. 4.20). Strains C2.1 and C11.1 gave a halo radiating approximately 2 mm, C5.1 showed approximately 1.5 mm of clearing around single colonies (Fig. 4.20) and strain C25.1 approximately 2-3 mm. All mutants showed an area of clearing which was less intense than that observed in the wild type. Strain C4.1 showed the greatest reduction with a very faint halo of clearing extending approximately 1.5 mm from single colonies (Fig. 4.20).

4.2.8.5 DNase assay

Strains were grown overnight at 37 °C on DNase agar plates and then overlaid with 0.5X DNase agar (5 ml) containing methyl green (0.5 mg/ml) (see appendix A, section A1.4.3) and incubated at 37 °C for a further 1-2 hours. DNase positive colonies are characterised by a zone of clearing against the blue/green of the plates (see section 4.3). Strain 8325-4 was DNase positive, evidenced by approximately a 1 cm radius of clearing around colonies and strain WA250 was slightly DNase positive with faint clearing extending approximately 3 mm (Fig. 4.21). Mutants C4.1, C11.1 and C25.1 all showed identical levels of DNase activity to 8325-4. The zone of clearing around colonies of C2.1 tended to appear smaller (approximately 8 mm) and less intense. However, mutant C5.1 had a 5 mm halo of clearing around colonies and this halo was less intense (Fig. 4.21).

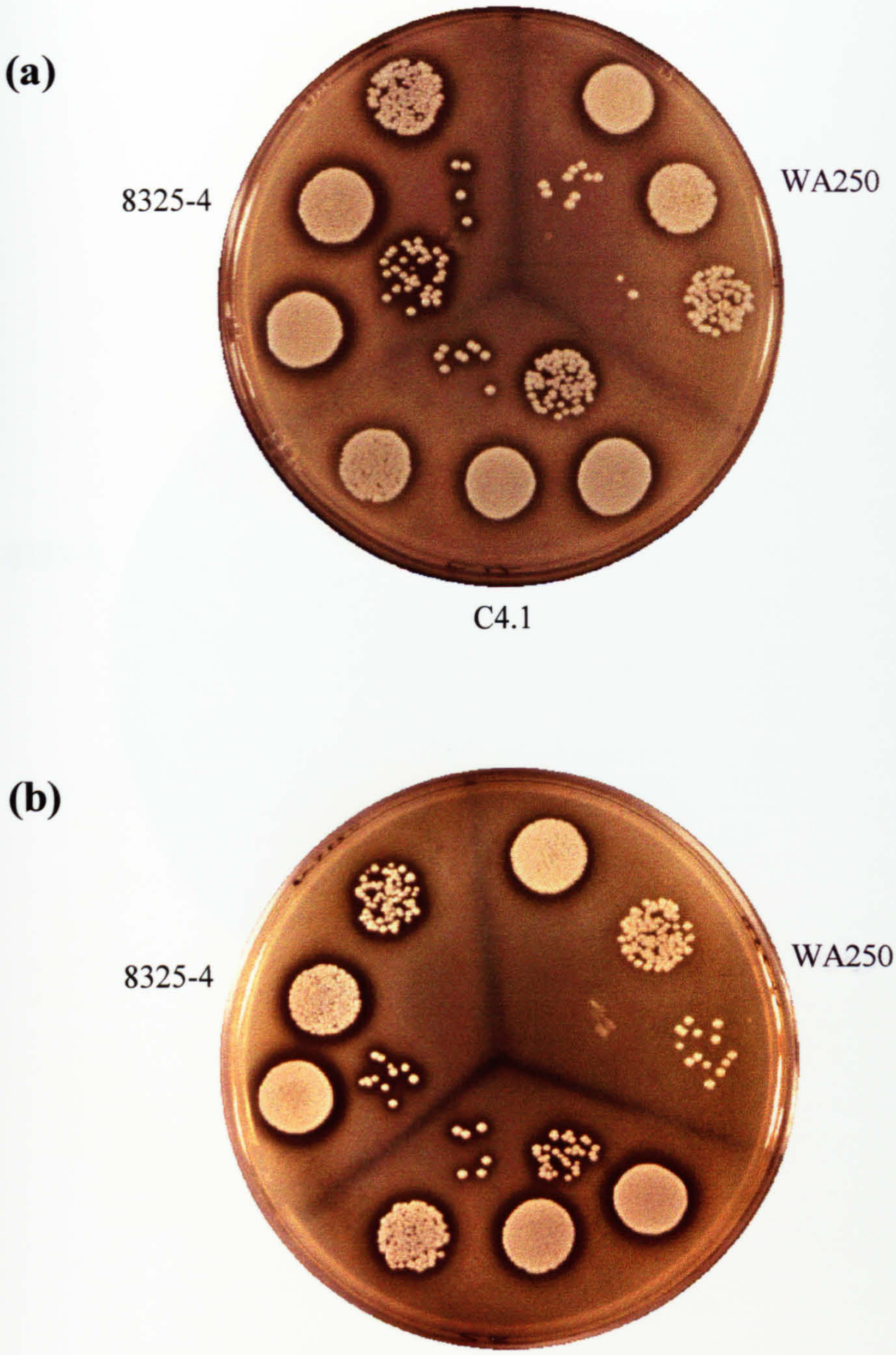


FIGURE 4.20

Protease plate assay. Strains C4.1 (a) and C5.1 (b), together with 8325-4 (WT) and WA250 (*agr*) were grown on BHI agar containing dried skimmed milk (1.5% w/v). Protease production can be seen as a zone of clearing in the opaque background. The key to the dilutions plated is shown in Fig. 4.17.

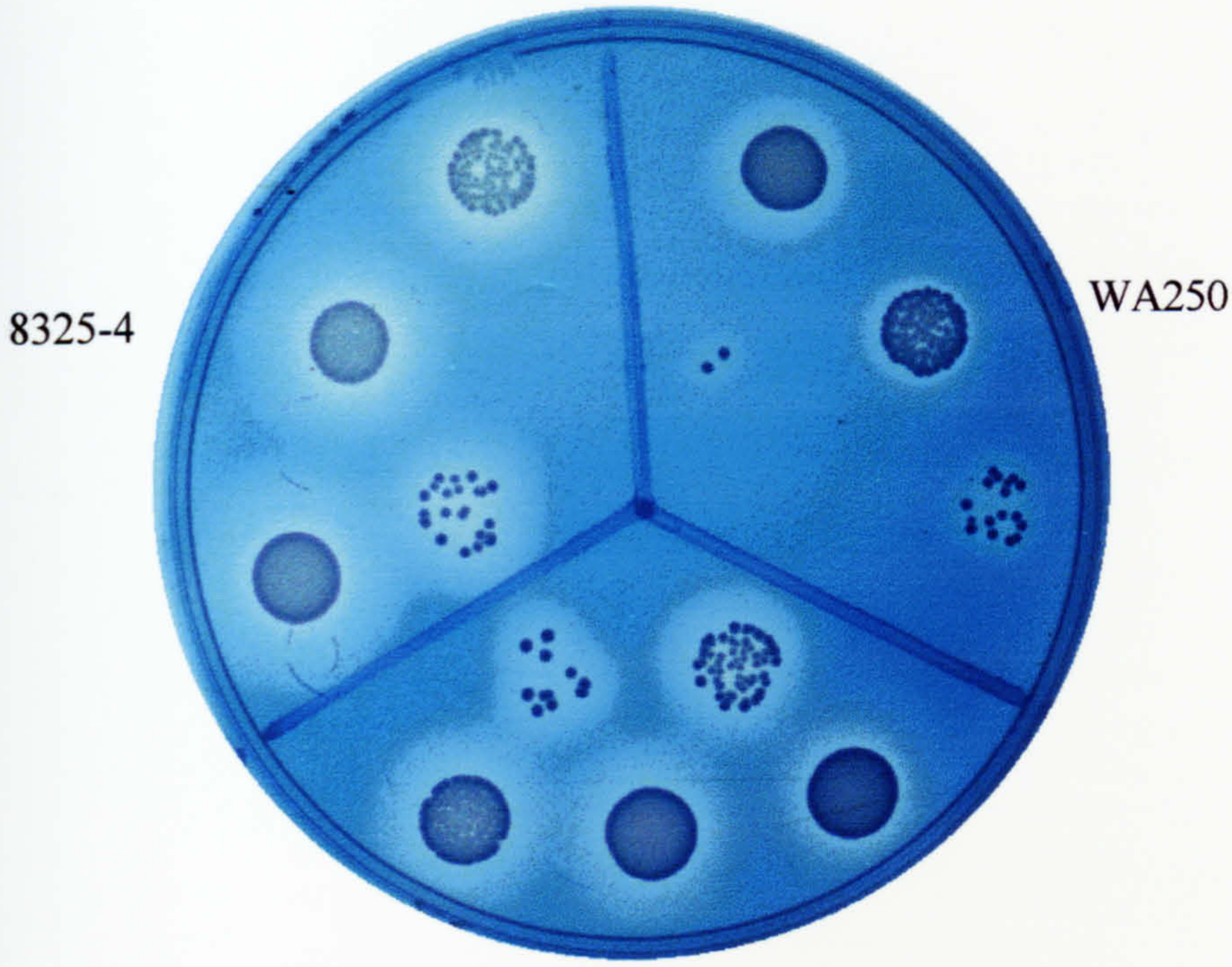


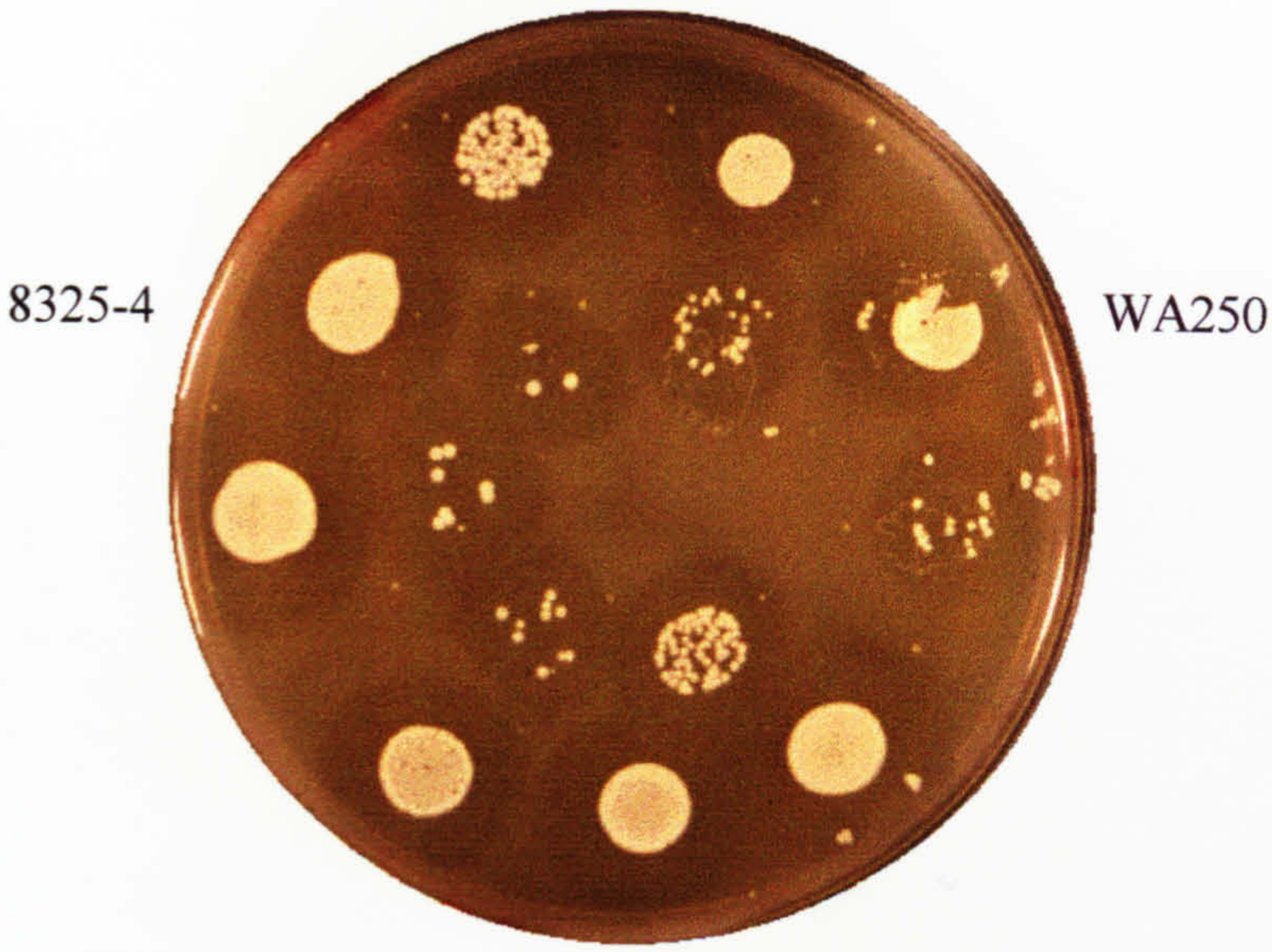
FIGURE 4.21

DNase plate assay. Strains C5.1, 8325-4 (WT) and WA250 (*agr*) were grown for 24 h at 37°C on DNase agar then overlaid with dimethyl green (0.5 mg/ml) in DNase agar (0.5X, 5 ml) and incubated for 1-2 h at 37°C. Zones of clearing were measured to compare production of DNase. Key to dilutions plated is as for Fig. 4.17.

4.2.8.6 Hyaluronate lyase assay

Cells were incubated overnight at 37 °C on BHI agar plates containing BSA (1 % w/v) and sodium hyaluronate (0.04 % w/v) and then flooded with acetic acid (2 M) at room temperature (Farrell *et al.*, 1995; Smith and Willett, 1968) (see appendix A, section A1.4.6). Undegraded hyaluronate precipitates as a conjugate with albumin in the presence of the acetic acid (Farrell *et al.*, 1995) (see section 4.3). Hyaluronate lyase activity was observed as a clear zone against a background precipitate. Zones of clearing around strain 8325-4 extended approximately 7 mm around single colonies and, around strain WA250, approximately 4 mm (Fig. 4.22). All the mutants, including C5.1 and C25.1 showed approximately a 5-6 mm halo of clearing (Fig. 4.22).

(a)



(b)

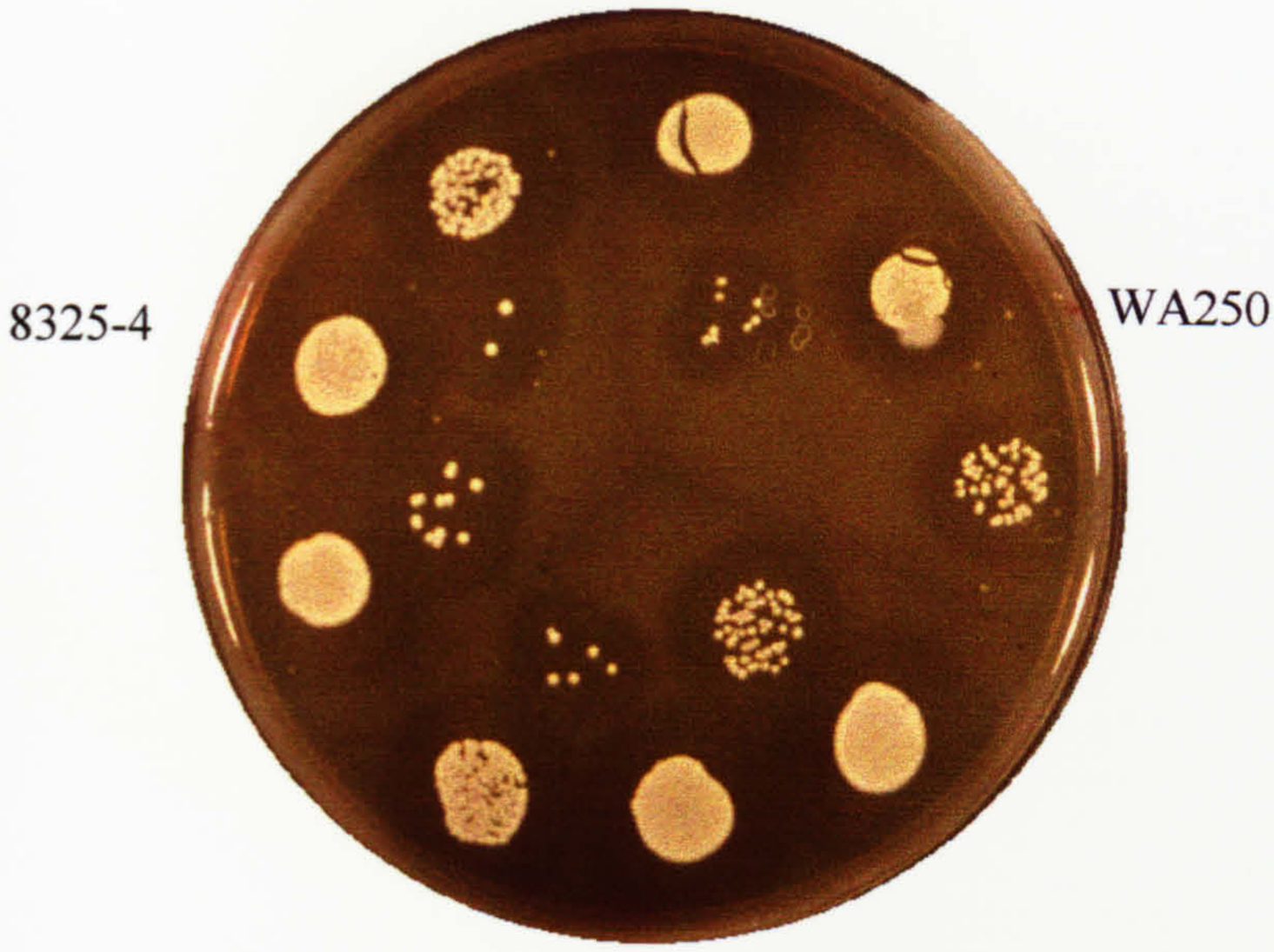


FIGURE 4.22

Hyaluronate lyase plate assay. Strains C5.1 (a) and C25.1 (b) together with 8325-4 (WT) and WA250 (*agr*) were grown for 24 h at 37°C on hyaluronate agar then flooded with 2M acetic acid at RT. Hyaluronate lyase activity is apparent as zones of clearing. The key to dilutions plated is as for Fig. 4.17.

4.3 Discussion

In order to identify possible *agr* regulatory loci, it was proposed to isolate mutants with altered *hld::lacZ* expression. Mutants were created using the staphylococcal transposon Tn*551* (which contains an Em resistance gene) in an *hld::lacZ* reporter strain. Since the *hld::lacZ* reporter strain, SH101 (see chapter 3), contains the Em resistance gene of pAZ106 (Kemp *et al.*, 1991) a new fusion strain with a different selective marker was required. This avoids the likelihood of the Em resistance gene of Tn*551* recombining with the chromosomal Em resistance gene. Thus, the second reporter gene fusion produced in this work, strain SH106, although essentially the same as strain SH101, utilised an alternative suicide vector, pTKlac which carries a Cm resistance gene instead of an Em resistance gene. This fusion is stable, does not need to be maintained with antibiotics and leaves an intact single copy of *agr*. The *lacZ* expression is dependent on the activity of the P3 promoter of *agr*. Thus, *hld::lacZ* expression, measured by β-galactosidase activity, is a reporter of the levels of transcription generated by P3 of the *agr* locus. Tn*551* mutagenesis was thus used to try to isolate mutants with altered *hld::lacZ* expression as evidenced by a change in the blue colour of colonies. Spontaneous mutants arose at a frequency approximately 30 times less than the recovered rate of transposon mutants. Transposon insertion could result in less *hld::lacZ* expression either by insertion in *agr* itself, or perhaps another gene affecting *agr* activity.

Forty seven putative *hld::lacZ* expression mutants were recovered as white colonies on plates containing X-Gal. Phage transduction of these mutations into the parental strain, should have rendered the transductants white also. However, all transductants remained blue. Following phage transduction into the wild type strain, 8325-4, several mutants were identified which showed altered patterns of β–haemolysis. This haemolysin is positively regulated by *agr* and so mutations affecting *agr* activity might be expected to affect levels of β–haemolysin.

Haemolysins are cytolytic toxins which can lyse not only erythrocytes but often other cell types as well (Rowe and Welch, 1994). β–haemolysin production is measured on sheep blood agar plates on which α–haemolysin production is inhibited (Peng *et al.*, 1988). Four mutants (A5.1, C7.1, C17.1 and 2/4.1) showed variable levels of reduction in β–haemolysin activity (approximately 0-5 mm radius of clearing) compared with strain 8325-4 (approximately 7 mm). Five further mutants (C2.1, C4.1, C5.1, C11.1 and C25.1) showed consistently low levels of β–haemolysis (approximately 2 mm radius of clearing).

All these mutants (excluding C4.1 and C25.1) together with a further five mutants (A1.1, A2.1, B2.1, C1.1 and 2/8.1) were examined using CHEF PFGE. Eight mutants were shown to contain the transposon Tn*551* in *Sma*I fragment A and two in fragment C. These initial results suggested that none of the mutants contained the transposon within either the β–haemolysin gene or *agr* since these loci are in *Sma*I fragment F which co-runs with fragment H in 8325-4 and RN4220 (Pattee *et al.*, 1990; Smeltzer *et al.*, 1992)). Similarly, these are not *sar* mutants since the *sar* locus has been mapped to *Sma*I fragment D (Cheung and Ying, 1994).

The location of the transposon within the chromosome of each mutant was mapped more finely by restriction digest to identify whether Tn*551* was inserting into the same locus in each mutant. The restriction data suggested that none of the mutants were identical to each other. Furthermore, following additional characterisation of five mutants (C2.1, C4.1, C5.1, C11.1 and C25.1) it was found that they all showed different profiles of virulence determinant production. This indicates that the transposon in each of these five strains has probably not inserted at different positions within the same gene, but rather that multiple loci may have been affected.

α–haemolysin activity was measured using rabbit erythrocytes as the substrate. This haemolysin forms pores in target cell membranes (see section 1.4.2.1) and is extremely active against rabbit erythrocytes, which is the accepted medium for

quantifying activity. All five mutants showed reduced levels of α–haemolysin compared to wild type strain 8325-4. Strains C2.1 and C4.1 showed similar levels of α–haemolysin but the other three mutants (C5.1, C11.1 and C25.1) were different to each other. None of these mutants contain Tn*551* in the *hla* structural gene since this has been mapped to *Sma*I fragment B (Pattee *et al.*, 1990).

The method employed in this work for assaying lipase activity used a simple plate assay consisting of TPEY agar containing enriched egg yolk (10 % v/v) which gives a cloudy emulsion. Lipase activity is evidenced by a white precipitate around a colony. Only two of the mutants (C2.1 and 25.1) displayed reduced lipase activity compared to the wild type 8325-4. It was also seen that the *agr*⁻ strain WA250 showed no reduction in lipase activity, equalling the levels produced by 8325-4. The lipase activity of *S. aureus* is discussed in section 1.4.6.1. Also as suggested in section 1.4.6.1, other substrates provide more definitive tests for lipase activity than egg-yolk and it has been shown that some strains displaying lipase negative phenotypes on egg-yolk agar are, in fact, lipase positive when tested on the long chain fatty acid triacylglycerol, triolein (Farrell *et al.*, 1990). In addition, a strain of *S. aureus* containing a transposon in *agr,* used by Smeltzer *et al.* (1992), has been reported as showing a decrease in lipase activity compared to an *agr*⁺ strain, using tributyrin as the substrate. This finding does not correlate with the results shown above for the *agr*⁻ strain, WA250. However, it should be noted that the egg yolk assay used in this work has been used as a qualitative rather than quantitative assay and the conflicting result may be due to the choice of substrate.

Two mutants (C2.1 and C5.1) showed reduced DNase activity. The assay used in this work was based on a method described by Langlois *et al.* (1989) and differs from methods used by some other workers in that it uses methyl green to detect the extent of DNase activity (Smith *et al.*, 1969). Methyl green combines solely with DNA which is highly polymerised and thus nuclease activity can be seen as a zone of

clearing in the dye around nuclease-producing colonies. It is possible to use toluidine blue-DNA-agar (Lachica *et al.*, 1970) or acridine orange (40 μg/ml)-DNA-agar which intercalates with the DNA in the agar and fluoresces under UV light with DNase positive colonies evidenced by a non-fluorescent zone. The disadvantage of using acridine orange is that it is toxic and plates can only be viewed under UV light. The nuclease of *S. aureus* is discussed in section 1.4.6.3.

All five mutants showed reduced protease activity with clearing radiating approximately 1.5 mm around single colonies of each mutant compared to approximately 3 mm in strain 8325-4. In addition, the halo of clearing was less intense in all mutants than in strain 8325-4. In this assay, protease activity degrades the casein present in dry skimmed milk changing the milky appearance of the plates to a zone of clearing. The proteases of *S. aureus* are discussed in section 1.4.6.2.

All five mutants also showed reduced hyaluronate lyase activity compared to 8325-4. In this assay the undegraded substrate (hyaluronate), in the presence of 2 M acetic acid, precipitates as a conjugate with the bovine serum albumin leaving a clear zone around those colonies that produce soluble enzymes (hyaluronate lyase) that attack the hyaluronate (Smith and Willett, 1968). Hyaluronate lyase is discussed in section 1.4.8.

As discussed in section 4.1, transposons provide a straightforward way of creating mutants that can be identified by a particular phenotype. Transposon mutagenesis is also a good way of identifying mutants with pleiotropic alterations to virulence characteristics when the transposon is inserted into a regulatory locus. For example, the *agr* locus was originally identified by the insertion of Tn*551* into what was thought to be the α-haemolysin (*hla*) gene (Mallonee *et al.*, 1982) (see section 1.6.1.1.1). Similarly, in 1992 Smeltzer *et al.* described a Tn*551* chromosomal insertion in an *S. aureus* strain which caused an almost complete reduction of

expression of extracellular lipase. It was subsequently found that this insertion, designated *xpr* (extracellular protein regulator) affected the expression of several exoproteins at the mRNA level and that *xpr* and *agr* are probably interactive at the genetic level (Hart *et al.*, 1993) (see section 1.6.1.4).

Tn*551* was also used to identify another class of pleiotropic mutant defective in the production of several exoproteins, this locus was designated *sae* (*S. aureus* exoprotein expression) (Giraudo *et al.*, 1994b) (see section 1.6.1.3).

Transposons can be used to create mutants which can then be selected as displaying a particular phenotype, as in the work described in this thesis where transposon mutants were selected as white colonies with reduced levels of β–haemolysin. Another regulator of exoprotein expression in *S. aureus*, designated *sar* (staphylococcal accessory regulator), has also been identified in this way (Cheung *et al.*, 1992). Here, a streptococcal transposon, Tn*917* was used to create mutants which were initially isolated as fibrinogen binding protein negative and subsequently found to be affected in production of several virulence determinants (see section 1.6.1.2).

In addition, transposons can be used to isolate mutants of a specific phenotype which has been identified by other means which do not allow cloning. An example of this occurred when Björklind and Arvidson (1980) found that protease I negative mutants rapidly accumulated during growth in a chemostat. Further analysis showed that they had identified a class of pleiotropic mutant, originally designated Exp^- and subsequently identified as the same locus as *agr* (see section 1.6.1.1.1).

It is possible to identify novel potentially regulatory loci in bacteria by other means and some of these methods are discussed in Chapter 6.

The mutants described in this thesis are distinct from one another yet share some similarities. More data needs to be acquired before it can be hypothesised that any of the mutants contain Tn*551* within a novel regulatory locus. For example, measuring α– and β–haemolysin in liquid cultures would give a less subjective measure of haemolysin production. In addition production of other virulence factors could also be measured, in particular, β–lactamase, δ–haemolysin and both extracellular and cell bound protein A.

In the meantime, it seems likely that some of these mutants are regulatory mutants since levels of several different virulence determinants are reduced. In addition, at least some of the genes for these virulence determinants are in different *Sma*I fragments to each other and to the fragments in which Tn*551* lies in each mutant. Also, none of the exoproteins examined so far are completely negative, which mirrors the situation found in *sar* mutants, though not *agr* mutants. It may be possible that in some of the mutants, the transposon is in a locus which is resulting in the selection of suppressors in *agr*. In spite of several attempts it has not proved possible to clone chromosomal DNA flanking the Tn*551* insertion sites from any of these mutants. However, following further phenotypic characterisation, this must be the next step on the road to molecular analysis of the novel loci.

CHAPTER 5

Isolation and characterisation of virulence determinant production mutants using Tn*917* insertional mutagenesis

5.1 Introduction

As discussed in chapter 4, transposon mutagenesis provides a good tool for allowing identification of genes involved in global regulation of production of virulence determinants. Previously, Tn*551* was used to create mutants in an *hld::lacZ* fusion strain of RN4220 in order to try to isolate genes directly affecting *agr* expression (see chapter 4).

Another approach is to use the streptococcal transposon, Tn*917* to create random mutations in a population of bacteria and then select for one particular phenotype. Mutants can then be tested for pleiotropic effects on several virulence determinants, such as α–haemolysin, lipase, protease and hyaluronate lyase. This method, selecting for fibrinogen binding protein mutants, was used to identify the *sar* locus of *S. aureus.* A 20.6 kb plasmid, pLTV1, containing Tn*917* has been specifically constructed to carry out efficient insertional mutagenesis and to facilitate the characterisation of disrupted genes (Camilli *et al.*, 1990) (Fig. 5.1). Plasmid pLTV1 carries a highly temperature-sensitive replicon derivative of vector pE194*Ts*, so that a temperature shift (from 30 °C to 43 °C) during growth of cells containing pLTV1 will result in loss of the plasmid and concomitant loss of tetracycline (Tet) resistance. This, then, allows the selection of cells with a transposon inserted in the chromosome, not only by the loss of Tet resistance, but by continued erythromycin (Em) and chloramphenicol (Cm) resistance since the *erm* and *cat* genes respectively are carried within the transposon (Camilli *et al.*, 1990). Another feature of pLTV1 is that it carries a promoterless *lacZ* gene at one end of the transposable element. Thus, provided the transposon inserts into the chromosome downstream of an active promoter and in the correct orientation, a *lacZ* transcriptional reporter gene fusion will be created allowing measurement of promoter activity. A major benefit of using Tn*917*-LTV1 is that it allows rapid cloning of chromosomal DNA flanking the insertion site (Camilli *et al.*, 1990). This transposon also contains a ColE1 replicon and a *bla* gene encoding an ampicillin resistance determinant (Fig. 5.1), both of which

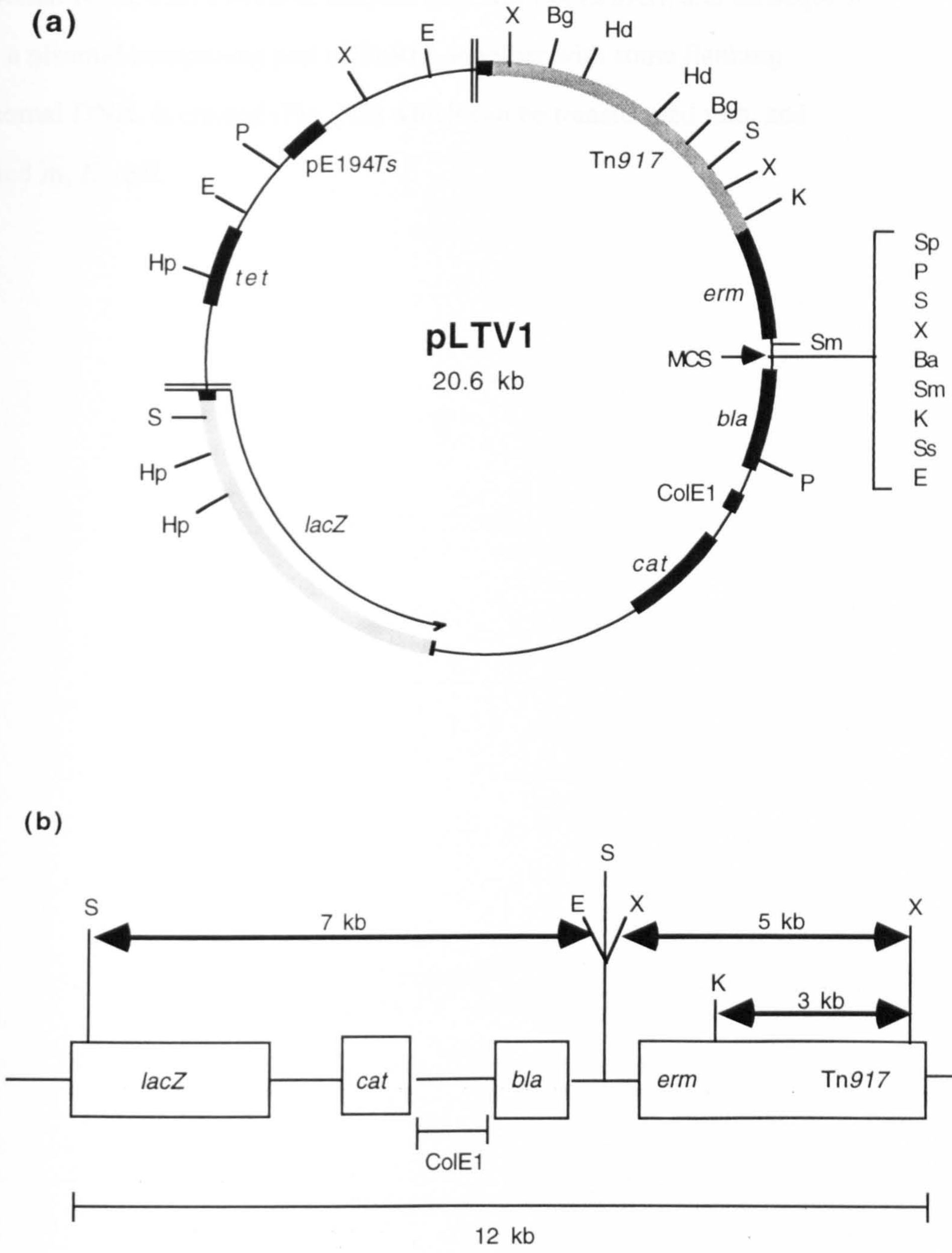


FIGURE 5.1

(a) Map of pLTV1. The multiple cloning site (MCS) contains sites for 9 restriction enzymes (clockwise) Sp, *Sph*I; P, *Pst*I; S, *Sal*I; X, *Xba*I; Ba, *Bam*HI; Sm, *Sma*I; K, *Kpn*I; Ss, *Sst*I; E, *Eco*RI. Other restriction sites indicated are: Bg, *Bgl*II; Hd, *Hin*dIII; Hp, *Hpa*I; Xh, *Xho*I. The extent of the Tn*917* transposable element is indicated by the symbol, ||.
(b) Simplified linear representation of transposable element of pLTV1 indicating fragments used in this work to create probes (double headed arrows).

are functional in *E. coli.* This means that by digestion of transposon mutant chromosomal DNA with a suitable enzyme (e.g. *Xba*I or *Eco*RI), and subsequent ligation, a plasmid comprising part of Tn*917*, together with some flanking chromosomal DNA, is created (Fig. 5.2) which can be transformed into, and maintained in, *E. coli.*

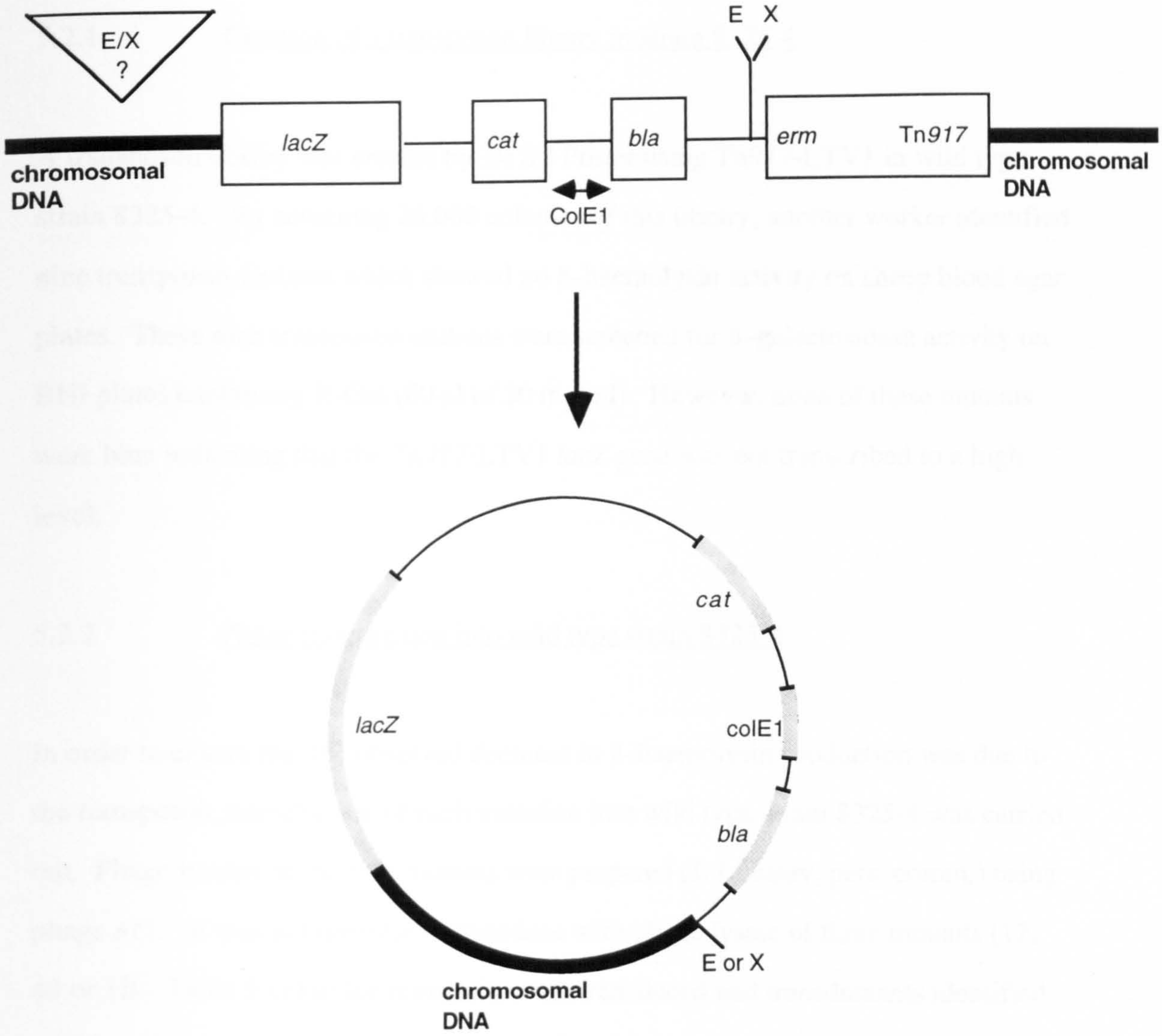


FIGURE 5.2

Method of cloning chromosomal DNA flanking the Tn*917* insertion site. Mutant chromosomal DNA containing the transposon was digested with an enzyme that restricts the DNA within the multiple cloning site of the transposable element, for example *Eco*RI (E) or *Xba*I (X) and somewhere in the chromosomal DNA (heavy line). This generates a DNA fragment containing one end of the transposon plus some flanking chromosomal DNA. Ligation of the ends of this fragment generates a plasmid containing a ColE1 origin of replication and an ampicillin resistance gene (*bla*). This plasmid can be recovered by transformation into *E. coli* and selection for ampicillin resistant colonies.

5.2 Results

5.2.1 Creation of a transposon library in strain 8325-4

A transposon library was created by Dr S J Foster using Tn*917*-LTV1 in wild type strain 8325-4. By screening 26,000 colonies of this library, another worker identified nine transposon mutants which showed no β–haemolysin activity on sheep blood agar plates. These nine transposon mutants were screened for β–galactosidase activity on BHI plates containing X-Gal (80 µl of 20 mg/ml). However, none of these mutants were blue indicating that the Tn*917*-LTV1 *lacZ* gene was not transcribed to a high level.

5.2.2 Phage transduction into wild type strain 8325-4

In order to ensure that the observed decrease in β–haemolysin production was due to the transposon, transduction of each mutation into wild type strain 8325-4 was carried out. Phage lysates of the nine mutants were prepared (J. Lindsay, pers. comm.) using phage ϕ11. It was not possible to transduce with phage lysate of three mutants (12, 43 or 1B - Table 5.1) but the remainder were transduced and transductants identified as Em resistant, indicating that Tn*917*-LTV1 had been transferred. Transductants, together with 8325-4, were plated on to sheep blood agar and production of β–haemolysin was measured and compared (Table 5.1). Transductants of three original mutants (42, 132 and IIA) all showed equal β–haemolysis to 8325-4 (approximately a 7 mm radius of clearing), but the remaining three (10, 5B and 113) showed greatly reduced levels of β–haemolysis (0-2 mm radius of clearing). In fact, 96 %, 97 % and 100 % of 10, 5B and 113 transductants, respectively, were completely non β–haemolytic. These data indicated that, in strains 10, 5B and 113, loss of β–haemolytic activity was associated with the transposon. One transductant of 10, 5B and 113 (suffixed .1) was selected and used for further studies.

Strain/mutant	No. of transductants	Radius of β-haemolysis
8325-4		7 mm
10	**79**	**0-2 mm**
12	-	-
42	**17**	**7 mm**
43	-	-
113	**14**	**0 mm**
132	25	7 mm
IIA	**15**	**7 mm**
1B	-	-
5B	**80**	**0-2 mm**

TABLE 5.1

Number of transductants and average extent of β-haemolysis, using the sheep blood plate assay, following phage transduction into wild type strain, 8325-4 with phage lysate prepared from nine Tn*917* mutants.

5.2.3 Physical mapping of transposon insertions

In order to begin determination of the location of the transposon in the *S. aureus* chromosome in each mutant, pulsed field gel electrophoresis was carried out (see section 4.2.5). Thus, the transposon in 10.1, 5B.1 and 113.1 could be mapped to one of the sixteen fragments resulting from a *Sma*I digest of *S. aureus* chromosomal DNA (see section 4.2.5). Digested chromosomal DNA of each strain was prepared as described in section 2.9.3 and separated using CHEF electrophoresis (Fig. 5.3) which, under the conditions used, resolves approximately eleven *Sma*I fragments ranging from 76-630 kb in 8325-4. The Southern blot of the resulting gel was probed using a digoxygenin labelled 5 kb *Xba*I fragment of Tn*917* from pLTV3 (Camilli *et al.*, 1990) (Fig. 5.4). This fragment is identical to that contained in Tn*917*-LTV1 and comprises the DNA between the multiple cloning site and the distal end of Tn*917* (Fig. 5.1). Digested chromosomal DNA of the *agr*⁻ strain, WA250, and two random blue Tn*917* transposon mutants (BHC1 and BHC2), was also separated on the gel. WA250 contains Tn*551* within *agr* at approximately position 3750 of the published sequence (Morfeldt *et al.*, 1988) (see Fig. 1.2). Tn*551* is 5.3 kb and, since it does not contain a *Sma*I site, does not significantly alter the profile of *Sma*I digested WA250 chromosomal DNA which remains identical to 8325-4 (see section 4.2.5). In addition, Tn*551* has very good homology to Tn*917* and thus hybridises with the Tn*917*-based probe. It has been reported that the *agr* locus is in *Sma*I fragment F of strain 8325 which, in 8325-4 and its derivatives, co-runs with *Sma*I fragment H (Smeltzer *et al.*, 1992; Pattee *et al.*, 1990). This is due to the loss of prophages ϕ11 and ϕ13 in 8325-4 which, in strain 8325, lie in *Sma*I fragment F, and thus their absence reduces the size of fragment F to approximately the 135 kb of fragment H (see section 4.2.5). However, it can be seen from the Southern blot (Fig. 5.4) and the corresponding gel (Fig. 5.3), that WA250 contained Tn*551* within fragment G (175 kb). Tn*917*-LTV1 contains a *Sma*I site (Fig. 5.1) so it would be expected that, following digestion with *Sma*I, the fragment of chromosomal DNA containing this

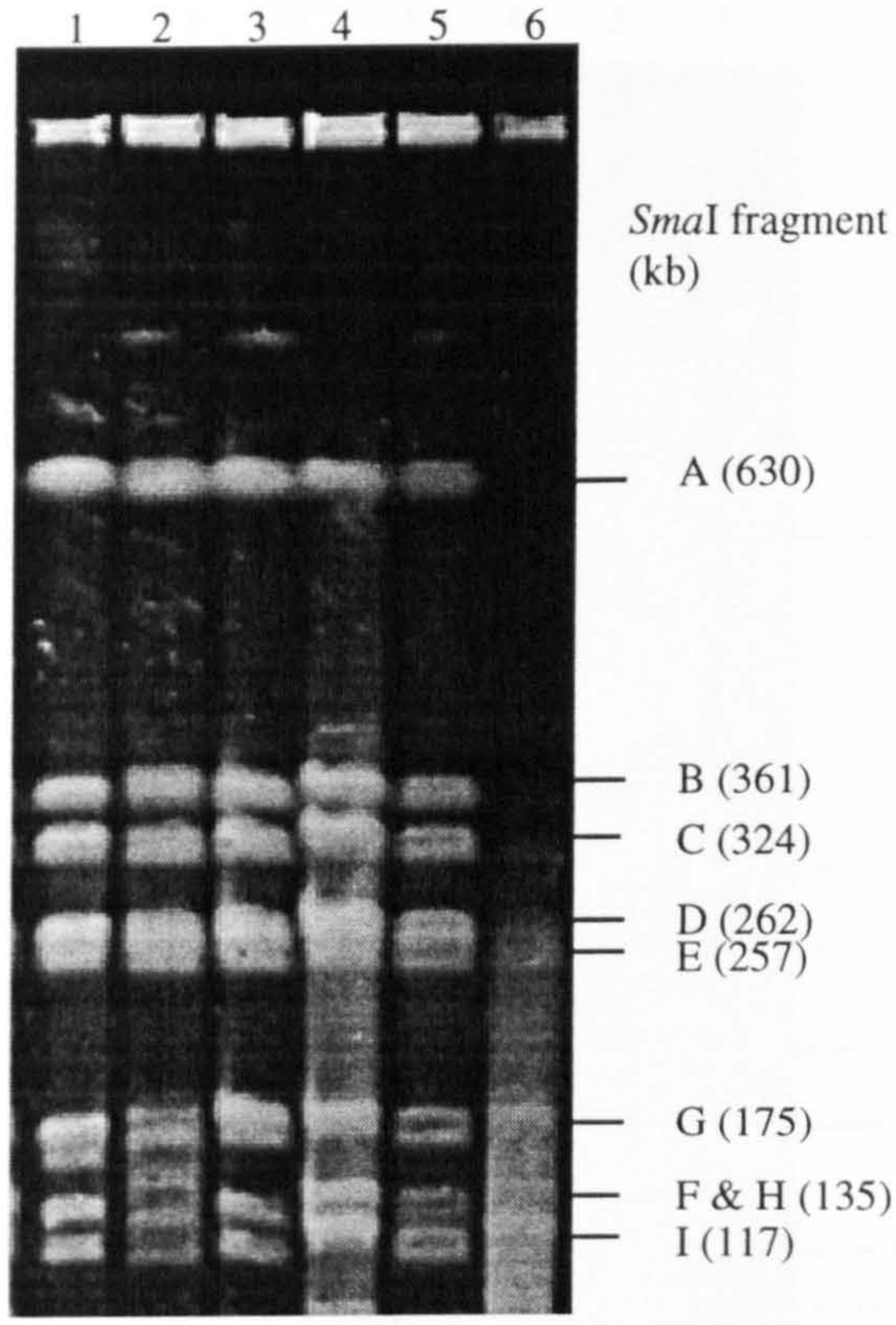


FIGURE 5.3

CHEF pulsed field gel to map Tn*917* insertion sites. Lanes 1 through 6 contain *Sma*I digests of total chromosomal DNA of two controls, BHC1 and BHC2, WA250 (*agr*), 10.1, 5B.1 and 113.1. The corresponding DNA fragments of WA250 are shown on the right with their sizes (kb). The gel was calibrated using the standards (not shown) listed in Table 2.5.

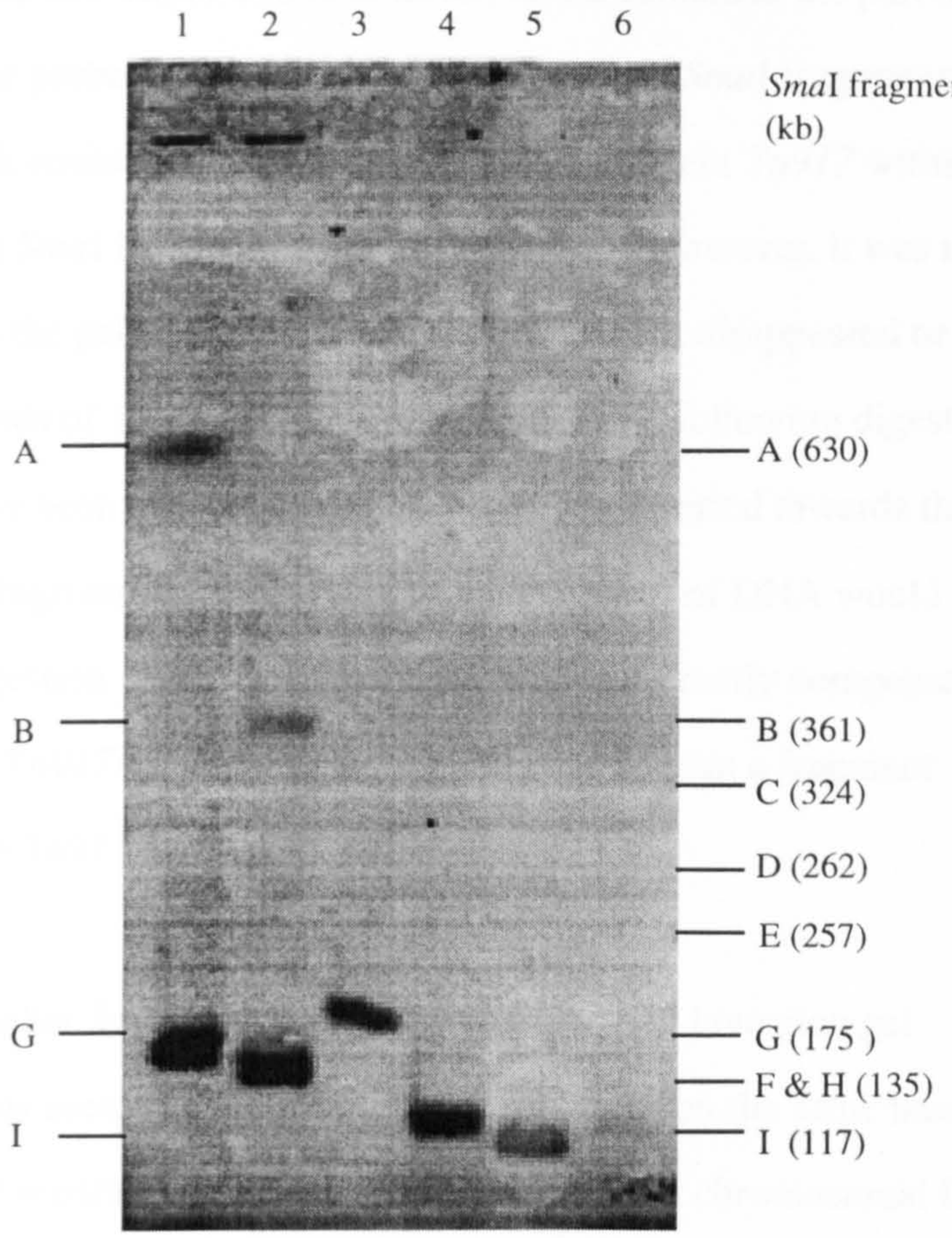


FIGURE 5.4

Southern blot of a CHEF pulsed field gel (see Fig. 5.3) to map Tn*917* insertions. Lanes 1 through 6 contain *Sma*I digests of total chromosomal DNA of two controls, BHC1 and BHC2, WA250 (*agr*), 10.1, 5B.1 and 113.1 probed with a digoxygenin labelled 5 kb *Xba*I fragment of Tn*917* (see Fig. 5.1). The corresponding fragments of WA250 are shown on the right with their sizes (kb). Hybridising bands are highlighted on the left.

transposon would disappear and two smaller, novel bands would appear. The probe comprises the Tn*917* end of Tn*917*-LTV1 (see Fig. 5.1) and thus, only one of these novel fragments of chromosomal DNA will hybridise and be apparent by Southern blotting, representing the region of chromosomal DNA that contains this part of the transposable element. The two Tn*917* controls, BHC1 and 2 contained the part of the transposon covered by the probe in fragments which co-ran with *Sma*I fragments G and H, respectively. Both strains 10.1 and 5B.1 appeared to contain Tn*917* within a fragment that co-ran with *Sma*I fragment I (117 kb) (Fig. 5.4). However, it was not possible to identify, from the gel (Fig. 5.3), either the band which disappeared or the second novel, smaller bands of 10.1 or 5B.1 chromosomal DNA following digestion with *Sma*I. This may have been due to the transposon having inserted towards the end of an existing *Sma*I fragment and thus only a small fragment of DNA would have been cleaved on *Sma*I digestion. Any loss would also have been partly compensated by the addition of part of Tn*917*-LTV1. Strain 113.1 did not contain a fragment which hybridised with the Tn*917* probe (Fig. 5.4).

In order to resolve the smaller *Sma*I fragments more clearly, field inversion gel electrophoresis (FIGE) was used (see section 2.9.3). This works on the same basis as CHEF electrophoresis but separates fragments of 9-250 kb. Total chromosomal DNA of all three strains was digested with *Sma*I and separated on a FIGE gel (Fig. 5.5). From this gel it can be seen that either *Sma*I fragment F or H of both 10.1 and 5B.1 has disappeared (Fig. 5.5). Since fragment F co-runs with fragment H in 8325-4 it is not possible to determine which fragment remains, indicating that the transposon originated in either *Sma*I fragment F or H. The expected appearance of two new fragments can also be confirmed by this gel with *Sma*I digested chromosomal DNA of 10.1 resulting in one novel fragment which co-runs with fragment I (117 kb) and the other between fragments M and N at approximately 20 kb (Fig. 5.5). In strain 5B.1 only one novel *Sma*I fragment can be seen, below fragment I at approximately 110 kb (Fig. 5.5). A Southern blot of this gel, probed as above, confirmed that, in

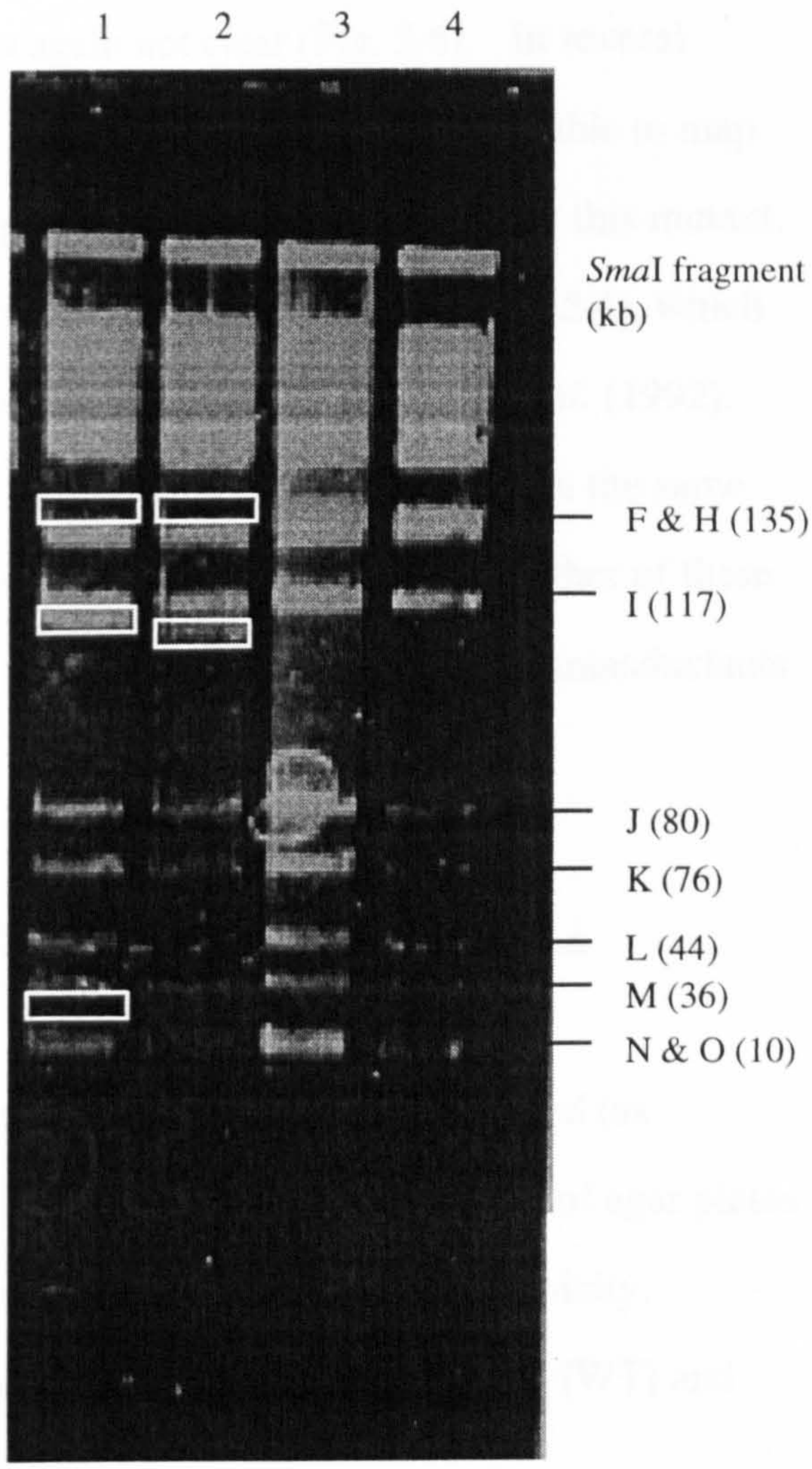


FIGURE 5.5

FIGE pulsed field gel to map Tn*917* insertion sites. Lanes 1 through 4 contain *Sma*I digests of total chromosomal DNA of strains 10.1, 5B.1, 113.1 and 8325-4 (WT). The boxes in lane 1 highlight the missing *Sma*I fragment F or H in strain 10.1 and two novel fragments, compared with 8325-4, of approximately 117 kb and 20 kb. The boxes in lane 2 highlight the missing *Sma*I fragment F or H in strain 5B.1 and one novel fragment of approximately 110 kb. The corresponding DNA fragments of 8325-4 are shown on the right with their sizes (kb). The gel was calibrated using the standards (not shown) listed in Table 2.5.

strain 10.1, the part of the transposon covered by the probe was within a novel fragment that co-ran with *Sma*I fragment I (Fig. 5.6). Strain 5B.1 appeared to contain this part of the transposon in a band which was slightly smaller than *Sma*I fragment I (Fig. 5.6). The result for strain 113.1 was again not clear (Fig. 5.6). In several subsequent Southern blots of FIGE and CHEF gels it did not prove possible to map 113.1 satisfactorily so it was considered prudent not to continue to study this mutant. The transposon in *agr* in strain WA250 mapped to fragment G (see Fig. 5.4), which did not correlate with the previously mentioned findings of Smeltzer *et al.* (1992). However, since the transposon in strains 10.1 or 5B.1 did not originate in the same *Sma*I fragment as Tn*551* in strain WA250, it was thought likely that neither of these mutants contained Tn*917* within the *agr* locus. Accordingly, these two transductants were characterised further.

5.2.4 Phenotypic characterisation of transductants 5B.1 and 10.1

Cultures of strains 10.1 and 5B.1 were serially diluted in PBS and spotted (as described in section 4.2.8), except where indicated, onto different types of agar plates to measure production of various determinants associated with pathogenicity. Activity in the mutant strains was compared visually with strain 8325-4 (WT) and strain WA250 (*agr*$^-$).

5.2.4.1 β–haemolysin assay

This assay is as described in section 4.2.8.1. Strains 10.1, 5B.1 and WA250 were β–haemolysin negative whilst strain 8325-4 showed an approximately 7 mm radius of clearing (Fig. 5.7).

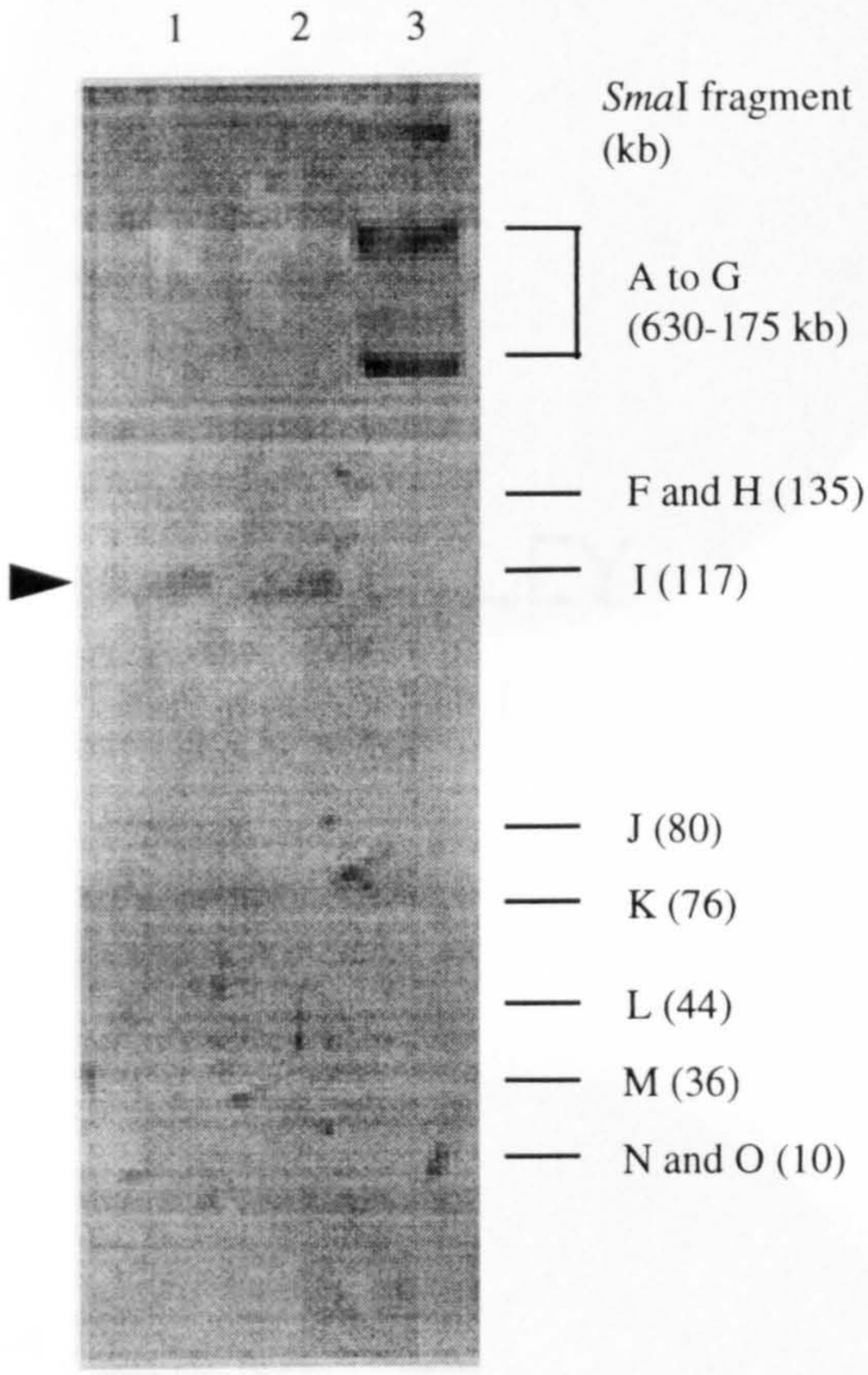
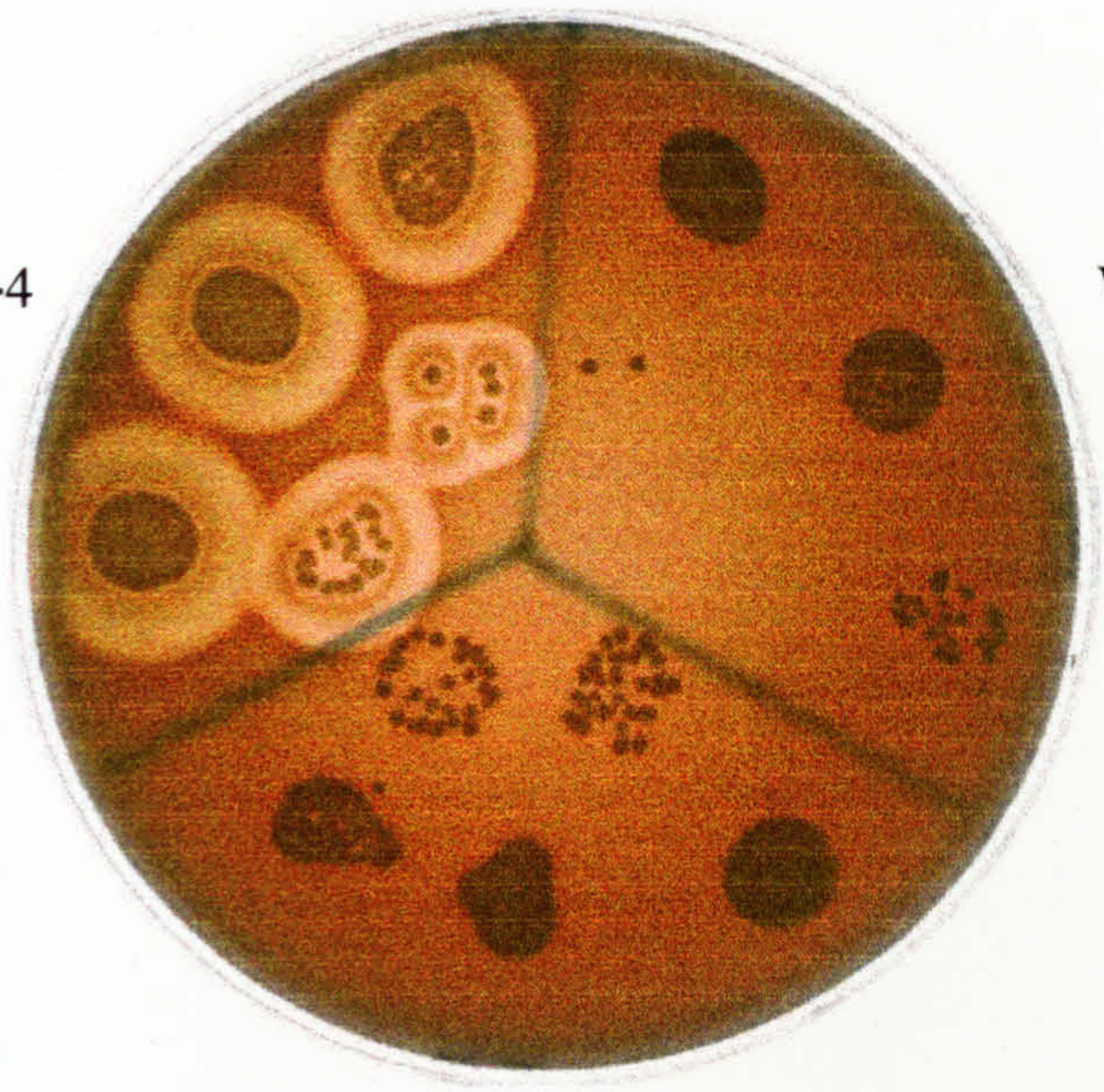


FIGURE 5.6

Southern blot of FIGE pulsed field gel (Fig. 5.5) to map Tn*917* insertion sites. Lanes 1 through 3 contain fragments of *Sma*I digests of total chromosomal DNA of strains 10.1, 5B.1, 113.1 that hybridised with the digoxygenin labelled 5 kb *Xba*I fragment of Tn*917* (see Fig. 5.1). The corresponding DNA fragments of 8325-4 (WT) are shown on the right with their sizes (kb). The arrow on the left indicates the hybridising fragments in lanes 1 and 2.

(a)



(b)

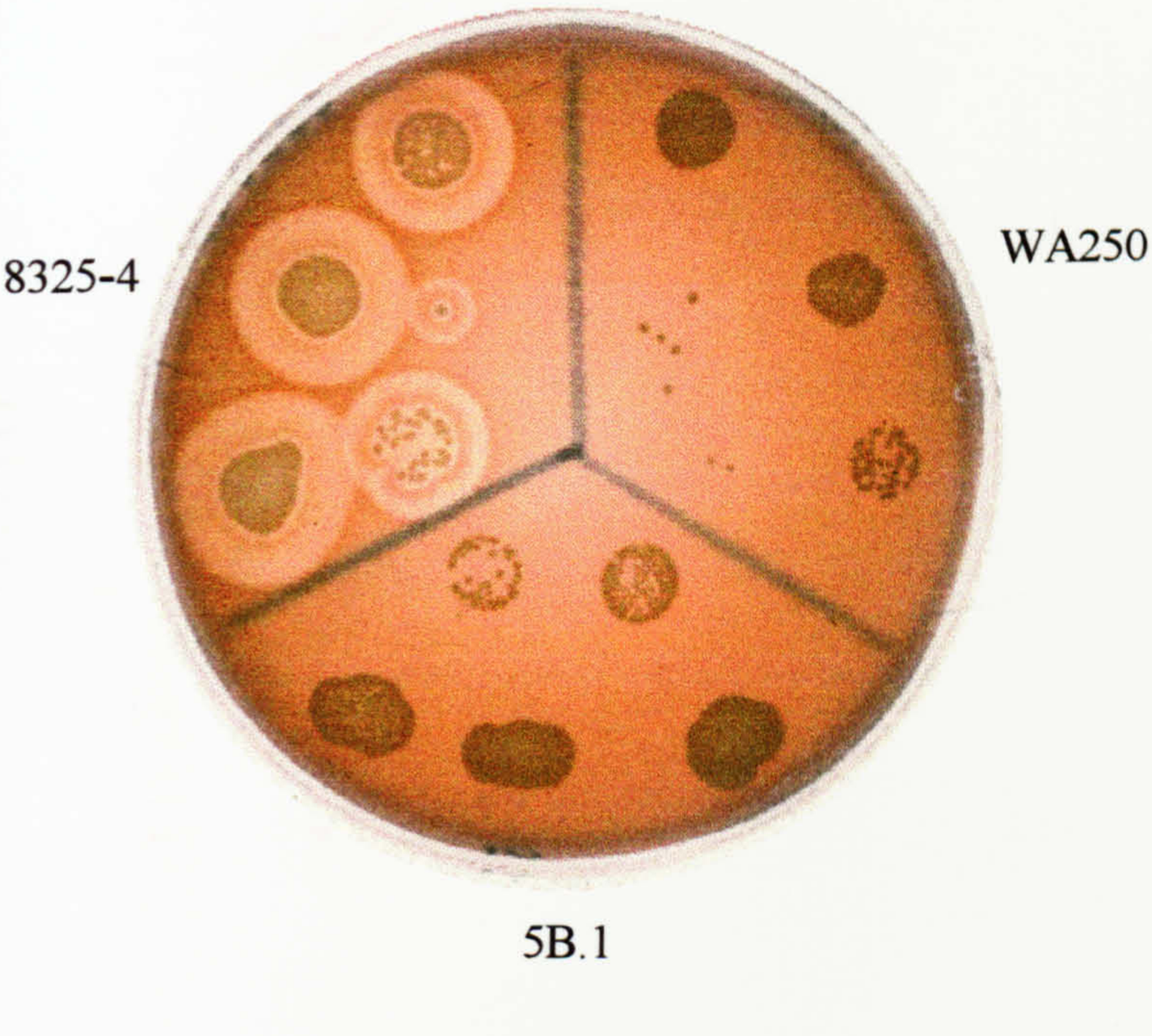


FIGURE 5.7

β-haemolysin plate assay. Strains were serially diluted and spotted onto sheep blood agar prior to overnight incubation at 37°C. (a) Strains 10.1, 8325-4 (WT) and WA250 (*agr*) and (b) Strains 5B.1, 8325-4 (WT) and WA250 (*agr*). Serial dilutions (10 μl) were plated in the order shown in Fig. 4.17.

5.2.4.2 α-haemolysin assay

The basis of this assay is as described in section 4.2.8.2. Strain WA250 was haemolysin negative whilst strain 8325-4 had an approximately 1 cm radius of clearing around colonies (Fig. 5.8a). Strains 10.1 (Fig. 5.8a) and 5B.1 were both α-haemolysin negative.

5.2.4.3 Lipase assay

Following this assay, described in section 4.2.8.3, strain WA250 was lipase negative following 48 hours incubation at 37 °C (Fig. 5.8b) although growth was poor. Strain 8325-4 and both mutant strains were lipase positive showing a white precipitate extending approximately 3 mm from colonies (Fig. 5.8b).

5.2.4.4 DNase assay

The basis of this assay is described in section 4.2.8.5. Both strains 10.1 and 5B.1 (Fig. 5.9a) showed less DNase activity (approximately 4 mm radius of clearing) than 8325-4 (approximately 6 mm radius of clearing) but more than strain WA250 (approximately 2 mm radius of clearing).

5.2.4.5 Protease assay

This assay is described in section 4.2.8.4. Both strains 10.1 and 5B.1 (Fig. 5.9b) showed the same level of protease activity compared to strainWA250, with no clearing evident around single colonies (Fig. 5.9b). Strain 8325-4 showed an approximately 3 mm radius of clearing around colonies (Fig. 5.9b).

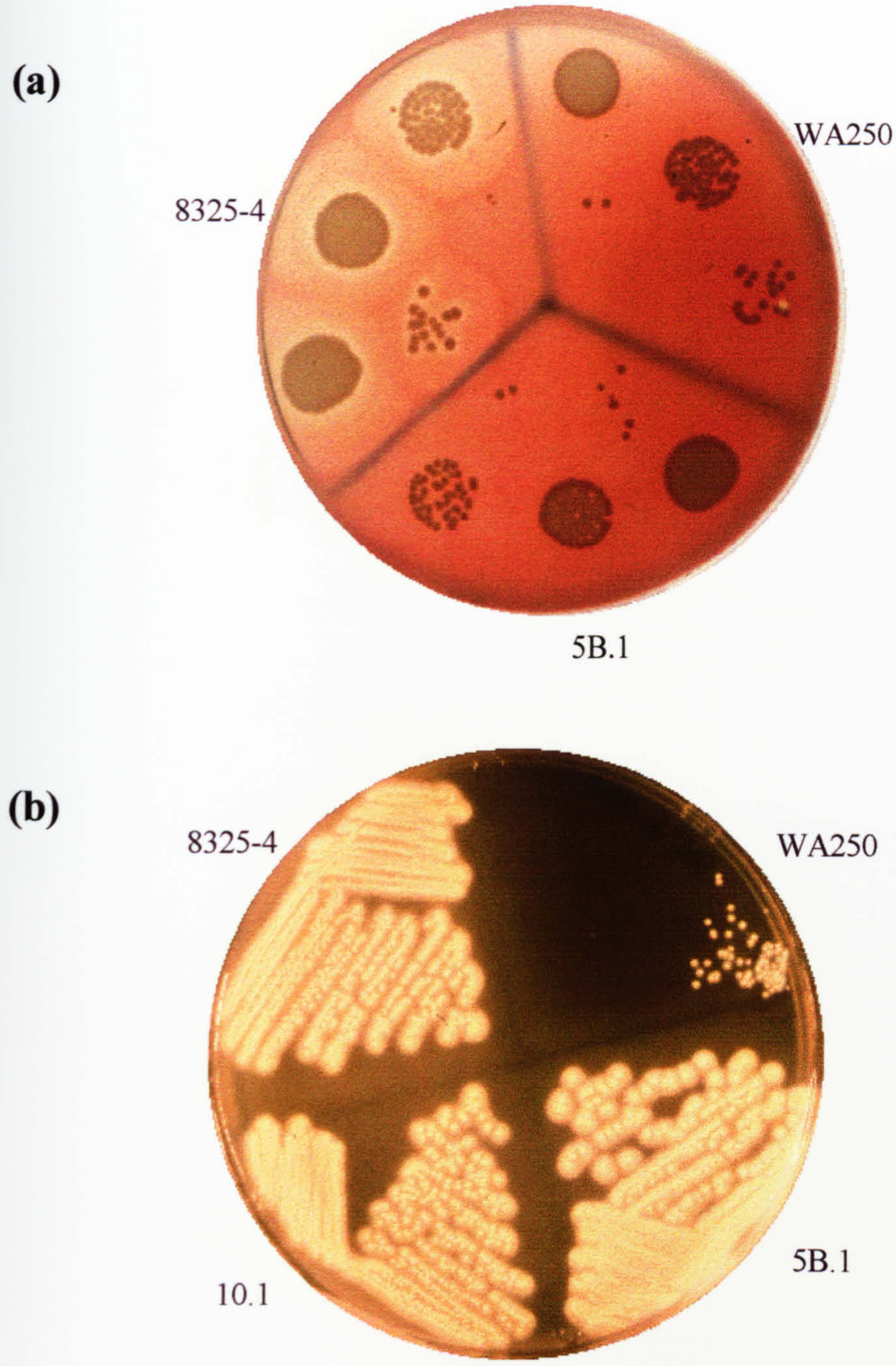


FIGURE 5.8

(a) α-haemolysin plate assay. Strains were serially diluted and spotted onto rabbit blood agar prior to overnight incubation at 37°C. Zones of α–haemolysin activity of strains 10.1, 5B.1, 8325-4 (WT) and WA250 (*agr*) were measured and compared.
(b) Lipase plate assay. Strains 5B.1, 10.1, 8325-4 (WT) and WA250 (*agr*) were streaked onto 10% (w/v) egg yolk agar plates and incubated for 48 h at 37°C. Lipase production is evident as a white zone of precipitation around the colonies.
Key for dilutions is shown in Fig. 4.17.

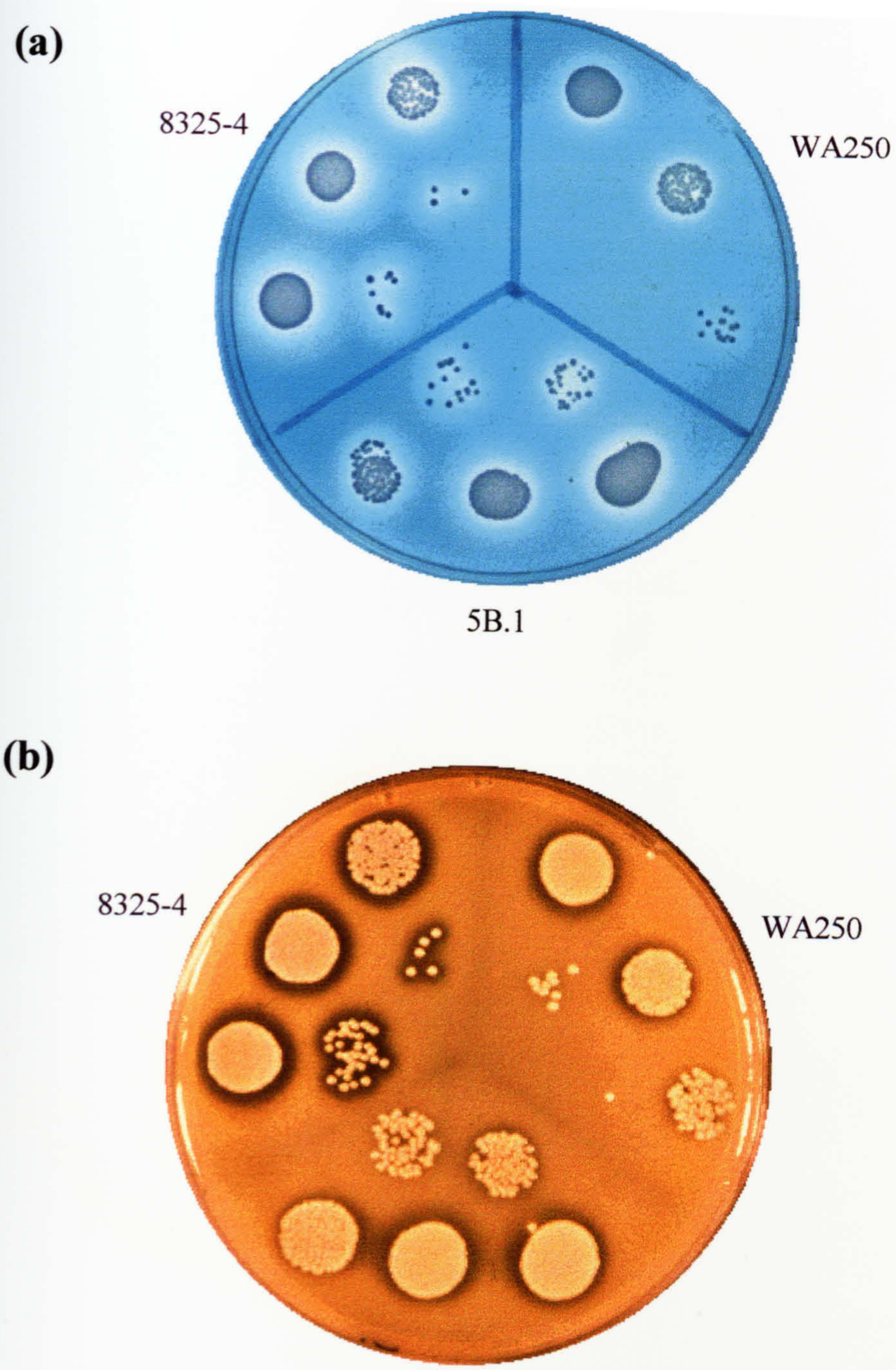


FIGURE 5.9

(a) DNase plate assay. Strains 10.1, 5B.1, 8325-4 (WT) and WA250 (*agr*) were grown for 24 hours at 37°C on DNase agar then overlaid with dimethyl green (0.5 mg/ml) in DNAse agar (0.5X, 5 ml) and incubated for 1-2 h at 37°C. Zones of clearing were measured to compare production of DNase.
(b) Protease plate assay. Strains 5B.1, 10.1, 8325-4 (WT) and WA250 (*agr*) were incubated overnight at 37°C on BHI agar containing 1.5% (w/v) dried skimmed milk. Protease production can be seen as a zone of clearing in the opaque background. Key to dilutions is shown in Fig. 4.17.

5.2.4.6 Hyaluronate lyase assay

This assay is described in section 4.2.8.6. Both mutant strains, 10.1 and 5B.1 showed the same, or slightly greater level of hyaluronate lyase activity compared to WA250 (approximately 4-5 mm radius of clearing around colonies) (Fig. 5.10). Strain 8325-4 showed slightly more activity with an approximately 8 mm radius of clearing (Fig. 5.10).

5.2.5 Identification of the chromosomal sites of Tn*917* insertion

In order to further analyse the putative components involved in the regulation of virulence determinant production, it was important to identify the locus into which the transposon had inserted in each mutant. From the data obtained it was thought likely that neither of these mutants contained Tn*917* within *agr*. In particular, both strains 10.1 and 5B.1 were lipase positive which differed from the phenotype observed by Smeltzer *et al.* (1992) for an *agr* mutant. In addition, the lipase negative phenotype of the *agr*$^-$ strain, WA250 correlated with the findings of Smeltzer *et al.* (1992), although this differed from earlier results where WA250 appeared lipase positive (see section 4.2.8.3). However, as previously suggested, the anomalous behaviour of WA250 was thought to be due to the choice of egg-yolk as the substrate for the lipase assay (see section 4.3). The data obtained from the pulsed field gels also suggested that these were not *agr* mutants since the transposon in *agr* of strain WA250 mapped to *Sma*I fragment G whereas the transposon in each transductant originated in either *Sma*I fragment F or H (see section 5.2.3). In order to determine the exact site at which Tn*917* had inserted in each mutant, chromosomal DNA flanking the transposon insertion needed to be cloned and sequenced. This method is described in section 2.14.2, but briefly, chromosomal DNA (5 μg) of each mutant strain was digested with *Xba*I or *Eco*RI which cut at the multiple cloning site within Tn*917*-LTV1 and somewhere in the chromosome (see Fig. 5.2). Digested DNA was then ligated,

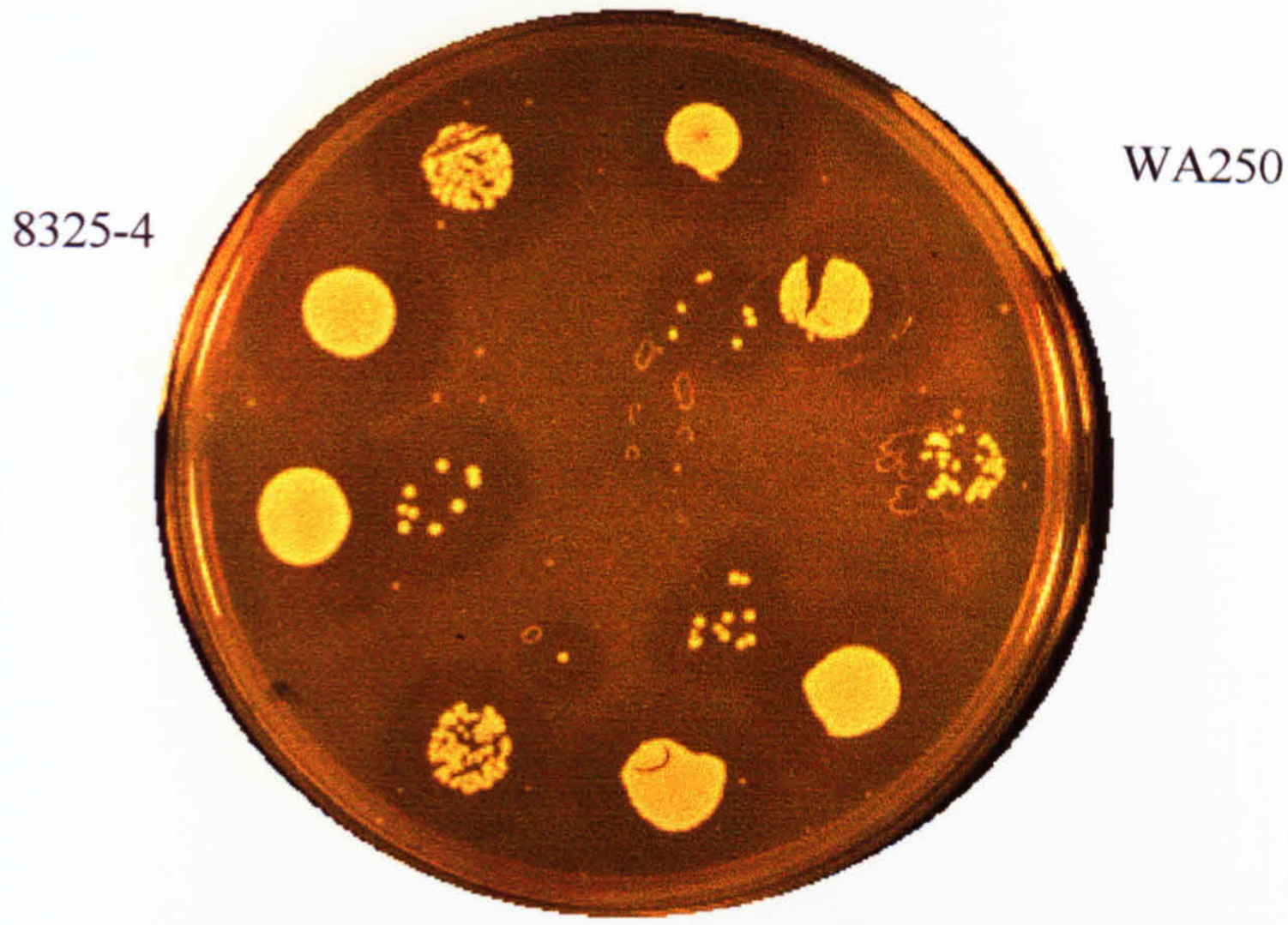


FIGURE 5.10

Hyaluronate lyase plate assay. (a) Strains 10.1, 8325-4 (WT) and WA250 (*agr*) and (b) strains 5B.1, 8325-4 (WT) and WA250 (*agr*) were incubated overnight at 37°C on hyaluronate agar (see Appendix A, section A1.4.6) then flooded with 2 M acetic acid at RT. Zones of clearing in the opaque background were measured after 30 mins to compare production of hyaluronate lyase.
Key to dilutions is shown in Fig. 4.17.

concentrated by ethanol precipitation and used to transform *E. coli* strains XLOLR, DH5α or ultra-competent *E. coli* strain, XL2-Blue MRF' (Stratagene), selecting for ampicillin resistance. This procedure was repeated for each mutant three times, without success, using the various hosts. Plasmids up to 66 kb can be efficiently transformed into DH5 strains of *E. coli*, but transformation frequency declines linearly with increased size (Hanahan *et al.*, 1991). In order to ensure that *Xba*I or *Eco*RI digestion did not result in plasmids which were too large for successful transformation, chromosomal DNA (5 μg) of each mutant was digested singly with *Xba*I, *Eco*RI or additionally, *Sal*I since it was known from the restriction map of pLTV1 that this should result in a transposon fragment of approximately 7 kb. This fragment comprises the 7 kb between the *Sal*I site in the multiple cloning site (MCS) and the *Sal*I site at the end of the *lacZ* gene (see Fig. 5.1). Digested DNA was separated on a TBE agarose gel and a subsequent Southern blot was probed with a digoxygenin labelled 7 kb fragment of *Eco*RI/*Sal*I cut pLTV1 covering the multiple cloning site to the distal end of the *lacZ* gene (see Fig. 5.1). The resulting hybridisation pattern can be seen in Fig. 5.11. The sizes of fragments for each strain which hybridised with the probe and thus contained the transposon are shown in Table 5.2.

It was clear that digesting the mutants with these enzymes was appropriate since the resulting fragments did not exceed approximately 11.5 kb. In addition, these data showed that the mutants were not siblings as the restriction pattern for each strain was different. It can be seen from Fig. 5.11 and Table 5.2 that the hybridising *Sal*I fragment of both transductants was approximately 3 kb instead of the expected 7 kb (see Fig. 5.1). Thus, it was clear that a deletion of part of the 7 kb *Sal*I fragment had occurred. To determine whether this had happened during phage transduction, chromosomal DNA (5 μg) from the original mutants, 10 and 5B was also digested with *Xba*I, *Eco*RI or *Sal*I and a subsequent Southern blot similarly probed. The parental strains were identical to the transductants indicating that the deletion had

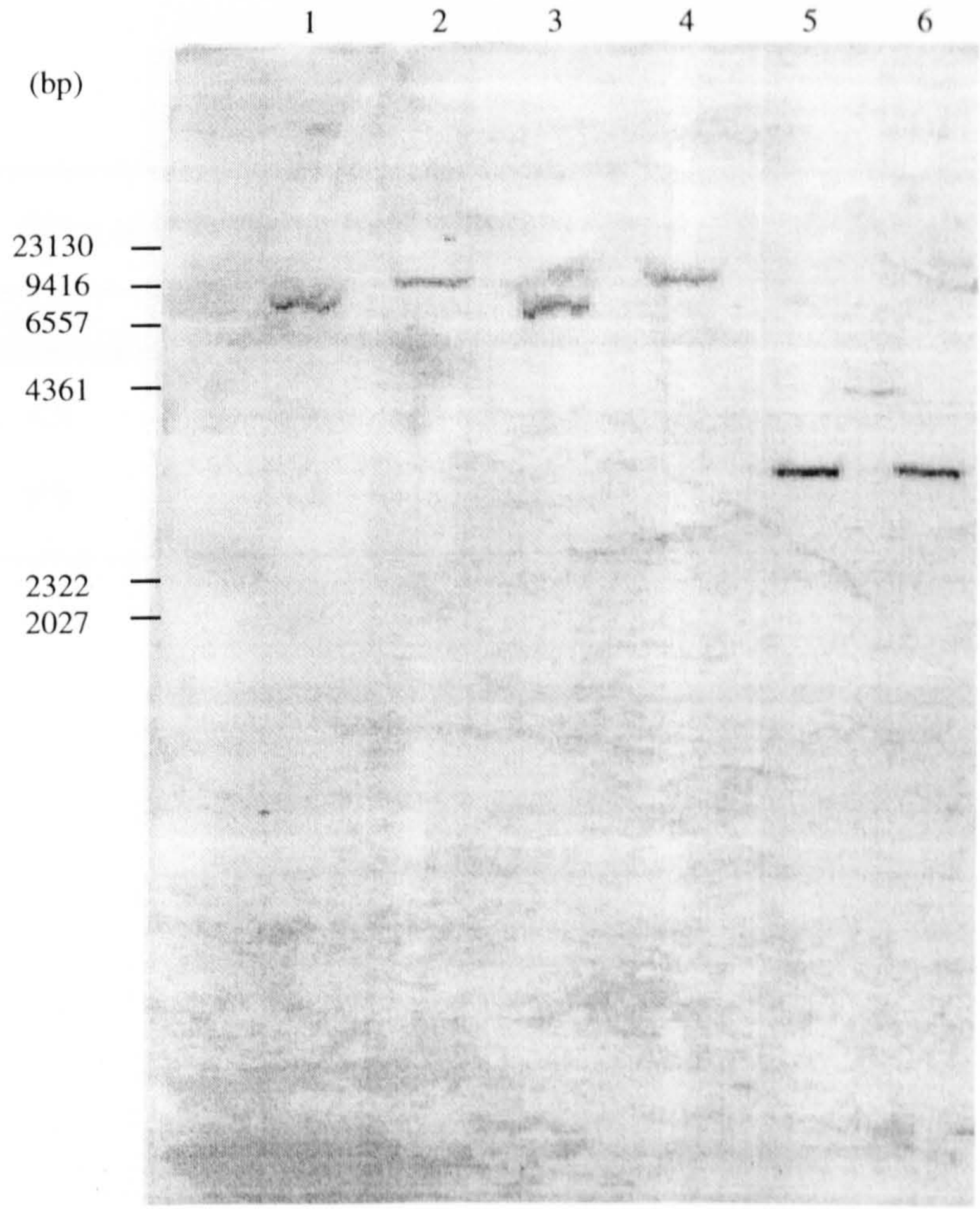


FIGURE 5.11

Southern blot to map Tn*917* chromosomal insertion sites. Chromosomal DNA of strains 10.1 and 5B.1 was digested with *Xba*I (lanes 1 and 2), *Eco*RI, (lanes 3 and 4) or *Sal*I (lanes 5 and 6), and probed with a digoxygenin labelled 7 kb *Eco*RI/*Sal*I fragment of pLTV1 (see Fig. 5.1). The corresponding fragments of *Hin*dIII digested λ are shown on the left with their sizes (bp).

Strain	Sizes of hybridising fragments (kb)		
	*Xba*I	*Eco*RI	*Sal*I
10.1	7.5	7.0	3
5B.1	9.5	11.5	3

TABLE 5.2

Physical mapping of Tn*917* insertion sites. The fragment sizes of *Xba*I, *Eco*RI or *Sal*I digested chromosomal DNA of strains 10.1 or 5B.1 that hybridised with the digoxygenin labelled 7 kb *Eco*RI/*Sal*I fragment of Tn*917* are given.

probably occurred during transposition (results not shown). Thus it was likely that the reason the chromosomal DNA flanking the transposon insertion site could not be rescued directly by cloning was that the ColE1 replicon and/or the *bla* gene, necessary for maintenance and selection in *E. coli*, had been deleted. It was therefore necessary to clone flanking chromosomal DNA in the same way as that attempted for Tn*551* mutants (see section 4.2.7). In order to identify a fragment of chromosomal DNA containing Tn*917* which was a suitable size for cloning into a plasmid, chromosomal DNA (3 μg) of strains 10.1 and 5B.1 was digested with *Xho*I or *Sal*I. Following separation of DNA on a TBE agarose gel, a Southern blot was probed with a digoxygenin labelled 5 kb *Xba*I fragment of pLTV3. Both *Sal*I digested chromosomal DNA of strains 10.1 and 5B.1 resulted in two bands of approximately 9 kb and 2 kb (Fig. 5.12 and Table 5.3). The 2 kb band comprised an internal fragment of Tn*917*-LTV1 (Fig. 5.1), and the other fragment in each strain includes the distal end of Tn*917* and some chromosomal DNA. *Xho*I digested chromosomal DNA of 10.1 resulted in two bands of approximately 7.5 and 8.5 kb and of 5B.1 resulted in two bands which co-resolved at approximately 8 kb (Fig. 5.12).

In order to clone part of the transposon and flanking DNA, chromosomal DNA (25 μg) of both mutants was digested with *Xho*I and separated on a TAE agarose gel. The fragments in the region spanning 2 kb above and below the expected size were excised and purified from the gel. In addition, pUBS1 (5 μg) was digested with *Xho*I, dephosphorylated and then ligated (1 μg) with the gel purified DNA of 10.1 or 5B.1 (5 μg). The ligation mixtures were transformed into ultra-competent *E. coli* strain, XL2-Blue MRF' (Stratagene) and resulting colonies containing plasmids with inserts (>100 for each mutant) were picked onto LB agar plates containing ampicillin (50 μg/ml). Colony blots were performed (see section 2.11.2.2) and probed with the digoxygenin labelled 5 kb *Xba*I fragment of pLTV3. No positives were obtained when compared to the control (pLTV3). Further ligations and transformations did not provide any positive transformants.

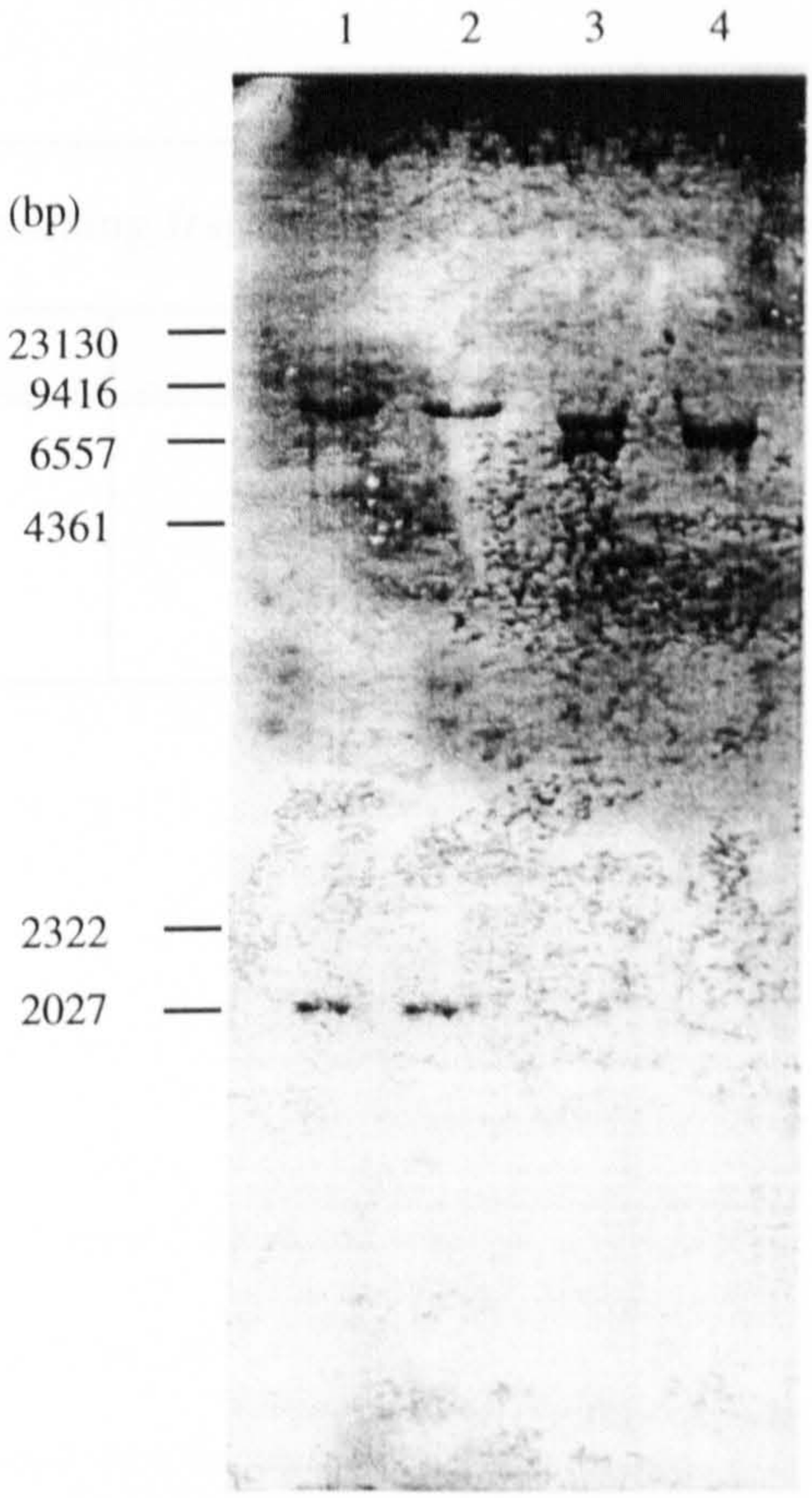


FIGURE 5.12

Southern blot of chromosomal DNA of strains 10.1 (lanes 1 and 3) and 5B.1 (lanes 2 and 4) digested with *Sal*I (lanes 1 and 2) or *Xho*I (lanes 3 and 4), probed with the digoxygenin labelled 5 kb *Xba*I fragment of Tn*917* isolated from pLTV3 (see Fig. 5.1). The corresponding fragments of *Hin*dIII digested λ are shown on the left with their sizes (bp).

Strain	Sizes of hybridising fragments (kb)	
	***Sal*I**	***Xho*I**
10.1	2.0 + 9.0	8.5 + 7.5
5B.1	2.0 + 9.0	8.0 (x 2)

TABLE 5.3

Physical mapping of Tn*917* insertion sites. The sizes of fragments of *Sal*I or *Xho*I digested chromosomal DNA of strains 10.1 or 5B.1 that hybridised with the digoxygenin labelled 5 kb *Xba*I fragment of Tn*917* are given.

Since some of the data obtained in this work was variable it was considered prudent to determine conclusively that the transposon in each mutant was not within *agr* by physical mapping of this area by restriction digest. In particular, the lipase activity in the *agr*⁻ strain, WA250 was variable and thus, although both mutants appeared consistently lipase positive, which suggested that they were not displaying an *agr*⁻ phenotype in this respect (Smeltzer *et al.*, 1992) it was considered that the lipase data may be somewhat unreliable due to the use of egg-yolk as the assay substrate. Also the transposon in both strains 10.1 and 5B.1 originated in *Sma*I fragment F or H. This again was found to be different from the *agr* mutant, WA250 which appeared to contain Tn*551* within *Sma*I fragment G. However, as discussed previously, the *agr* locus is in *Sma*I fragment F in strain 8325 and is in a fragment which co-runs with *Sma*I fragment H in strain 8325-4 (Smeltzer *et al.*, 1992; Pattee *et al.*, 1990).

5.2.6 Fine physical mapping of Tn*917* insertions

To clarify whether Tn*917* had inserted into the *agr* locus in either 10.1 or 5B.1, chromosomal DNA of each mutant and 8325-4 was digested, separated by TBE agarose gel electrophoresis, blotted and probed with a fragment of the *agr* locus. Thus, any change in the size of hybridising fragments in the mutants, compared to 8325-4 (WT), would indicate the presence of additional DNA, almost certainly comprising the transposon. Initially, chromosomal DNA from 10.1, 5B.1, 8325-4 and WA250 was digested with *Pvu*II. This enzyme has restriction sites towards either end of the *agr* locus (positions 332 and 4152) (see Fig. 5.14). A Southern blot of digested DNA was probed with a digoxygenin labelled 1.8 kb *Asp*700/*Eco*RV fragment of *agr* (see Fig. 1.2). One fragment of 3.8 kb of *Pvu*II digested chromosomal DNA of 8325-4 hybridised with the probe, which represented the number of bases between the two *Pvu*II sites of *agr* (Fig. 5.13 and Table 5.4). WA250 contains Tn*551* (within *agr*) which does not contain any *Pvu*II sites, so that the *Pvu*II digestion of strain WA250 chromosomal DNA resulted in hybridisation

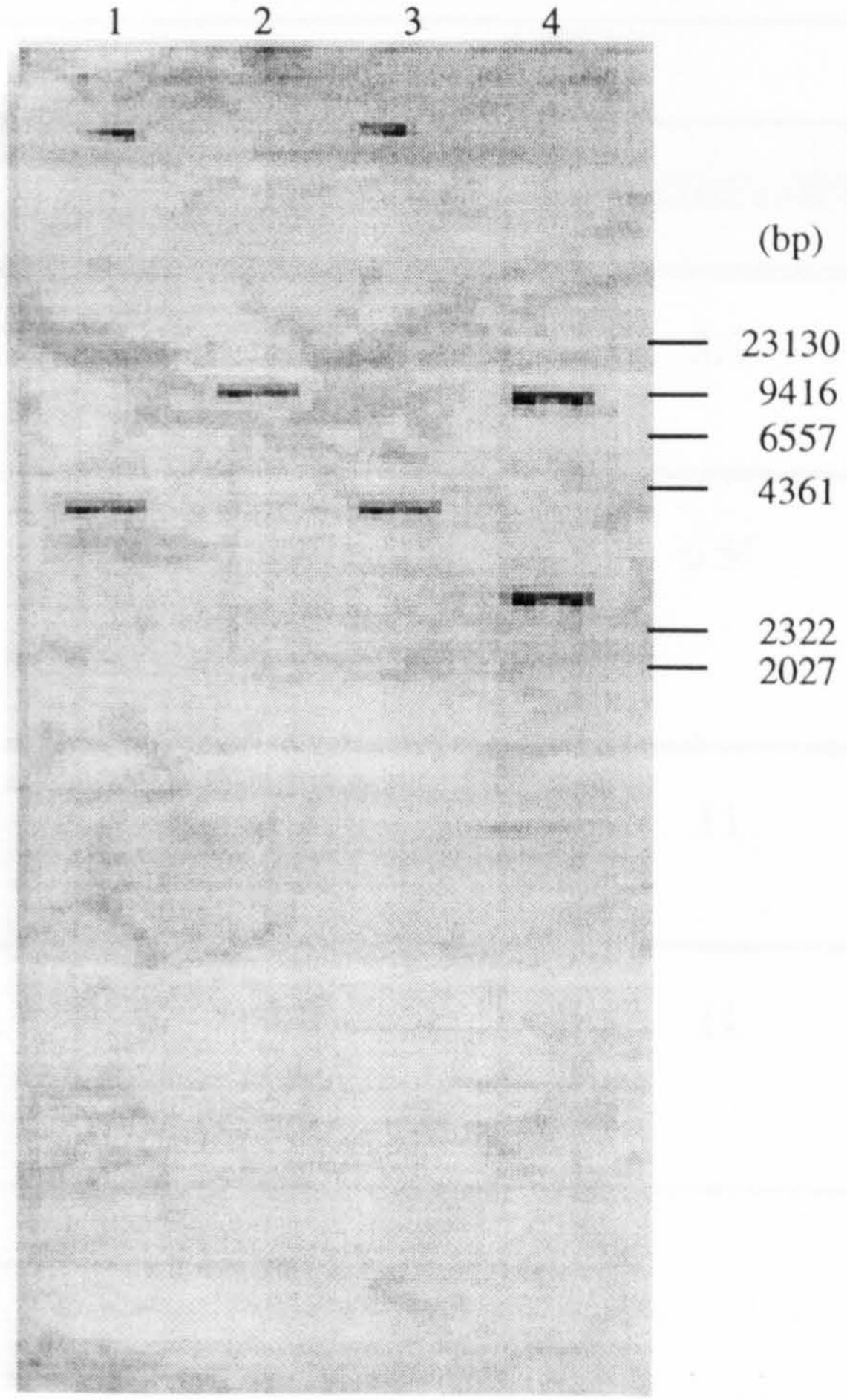


FIGURE 5.13

Southern blot of chromosomal DNA of strains 8325-4 (WT) (lane 1), WA250 (*agr*) (lane 2), 10.1 (lane 3) and 5B.1 (lane 4) digested with *Pvu*II and probed with the digoxygenin labelled 1.8 kb *Asp*700/*Eco*RV fragment of *agr* (see Fig. 1.2). The corresponding fragments of *Hin*dIII digested λ are shown on the right with their sizes (bp).

Strain	Sizes of fragments (kb)		
	*Pvu*II	*Pvu*II/*Pst*I	*Hin*cII/*Eco*RV
8325-4	3.8	1.8 2.0	3.1
WA250	9.1	1.8 9.0† 3.5†	6.5†
10.1	3.8	1.8 2.0	11
5B.1	9.0 2.8	2.0 8.0 0.8	11

† These fragment sizes are anomalous see sections 5.2.6 and 5.3

TABLE 5.4

Sizes of *agr* hybridising fragments. Chromosomal DNA was digested with *Pvu*II, *Pvu*II/*Pst*I or *Hin*cII/*Eco*RV and the sizes of fragments that hybridised with the digoxygenin labelled 1.8 kb *Asp*700/*Eco*RV fragment of *agr* (see Fig. 1.2) were calculated from Southern blots (Figs. 5.13 and 5.15).

with the probe at a single band of approximately 9.1 kb comprising 3.8 kb (*agr*) and 5.3 kb (Tn*551*) (Fig. 5.13). Digested chromosomal DNA of 10.1 gave a hybridising fragment of 3.8 kb (the same as 8325-4) indicating that this transductant possibly did not contain Tn*917* within *agr* (Fig. 5.13). Two fragments of *Pvu*II digested chromosomal DNA of strain 5B.1 hybridised with the probe, one of 9 kb and one of 2.8 kb. This suggested that Tn*917* had inserted in *agr* in strain 5B.1 and also that Tn*917*-LTV1 contains a restriction site for *Pvu*II (Fig. 5.13). This initial analysis could not, however, pinpoint the Tn*917* insertion site. The derivation of fragments following digestion of DNA with *Pvu*II is shown in Fig. 5.14.

To identify more accurately the location of Tn*917* in strain 5B.1, chromosomal DNA of all four strains was digested with *Pvu*II and *Pst*I, which cuts at position 2149 within *agr* (Fig. 5.14). In addition, chromosomal DNA of each strain was digested with *Hinc*II and *Eco*RV (which cut within *agr* at positions 4548 and 1474 respectively, see Fig. 5.14) to further narrow the location of Tn*917* in strain 5B.1 and also to ensure that Tn*917* was not in the remaining 373 bases of *agrA* in strain 10.1. A Southern blot of these digests was probed with the digoxygenin labelled 1.8 kb *Asp*700/*Eco*RV fragment as above. Chromosomal DNA of strain 8325-4, digested with *Pvu*II/*Pst*I, hybridised with the probe at two bands of 1.8 and 2 kb (Fig. 5.15 and Table 5.4). Three fragments of *Pvu*II/*Pst*I digested chromosomal DNA of strain WA250 hybridised with the probe, one of 1.8 kb, one of approximately 9 kb and one of 3.5 kb (Fig 5.15). It seemed likely from these results that the 9 kb band arose from DNA which had been only partially digested and represented the *Pvu*II fragment. There is no *Pst*I site within Tn*551* and it would therefore have been expected that the 3.5 kb band would approximate 7.3 kb (2 kb chromosomal DNA plus 5.3 kb Tn*551*). Digested chromosomal DNA of strain 10.1 hybridised with the probe at the same sized fragments as the wild type (Fig. 5.15) since it has already been shown that Tn*917* does not occur within the *Pvu*II sites of this mutant (Fig. 5.13). Strain 5B.1 contained Tn*917* somewhere between the *Pvu*II sites of *agr* so that, by digesting

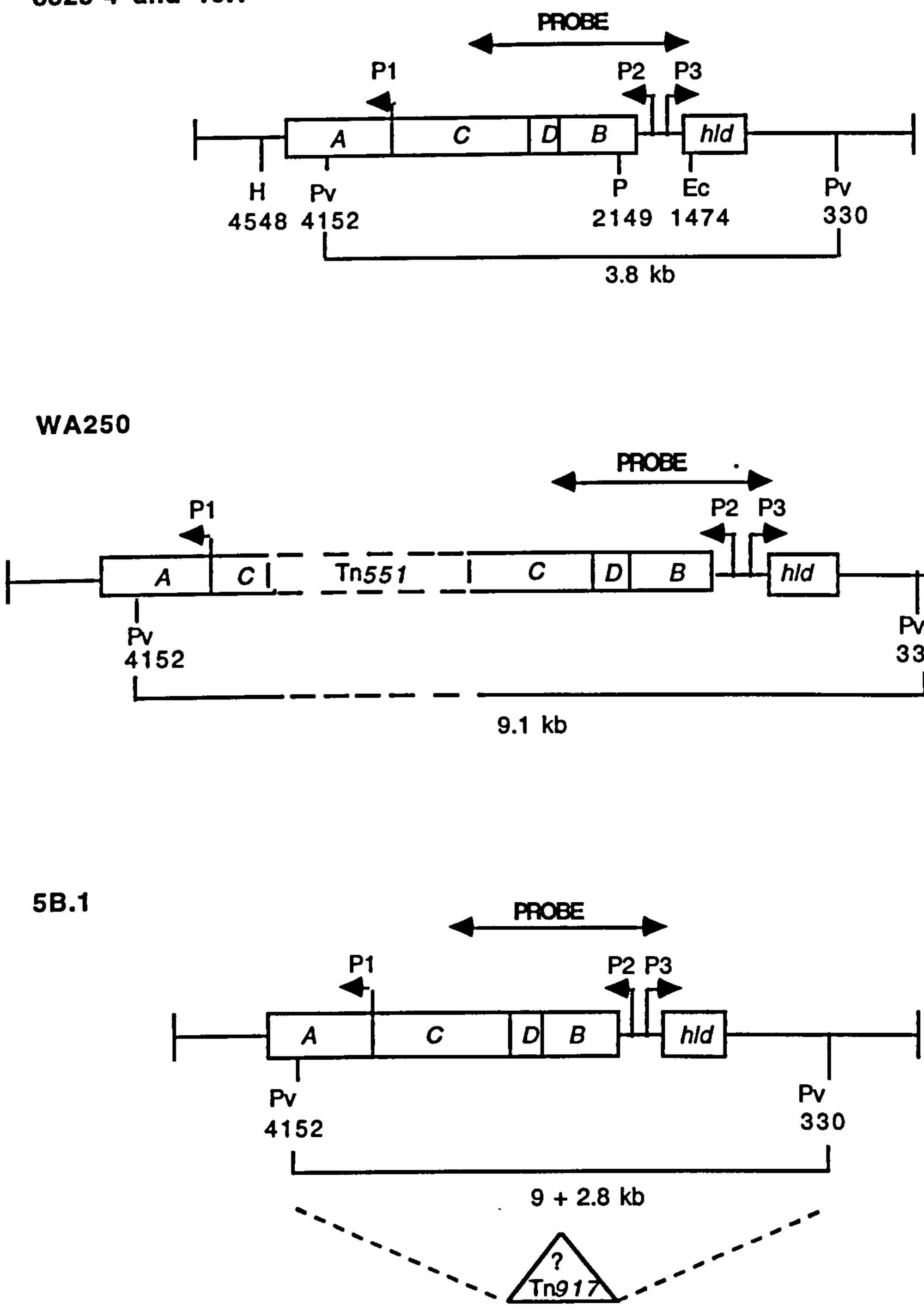


FIGURE 5.14

Physical map of the *agr* region showing the derivation of bands depicted on the Southern blot (Fig. 5.13) of *Pvu*II digested chromosomal DNA of strains 8325-4 (WT), WA250 (*agr*), 10.1 and 5B.1, probed (double headed arrow) with the digoxygenin labelled 1.8 kb *Asp*700/*Eco*RV fragment of *agr* (see Fig. 1.2). The restriction sites denoted are: Ec, *Eco*RV; H, *Hin*cII; P, *Pst*I; Pv, *Pvu*II. (This figure is not to scale).

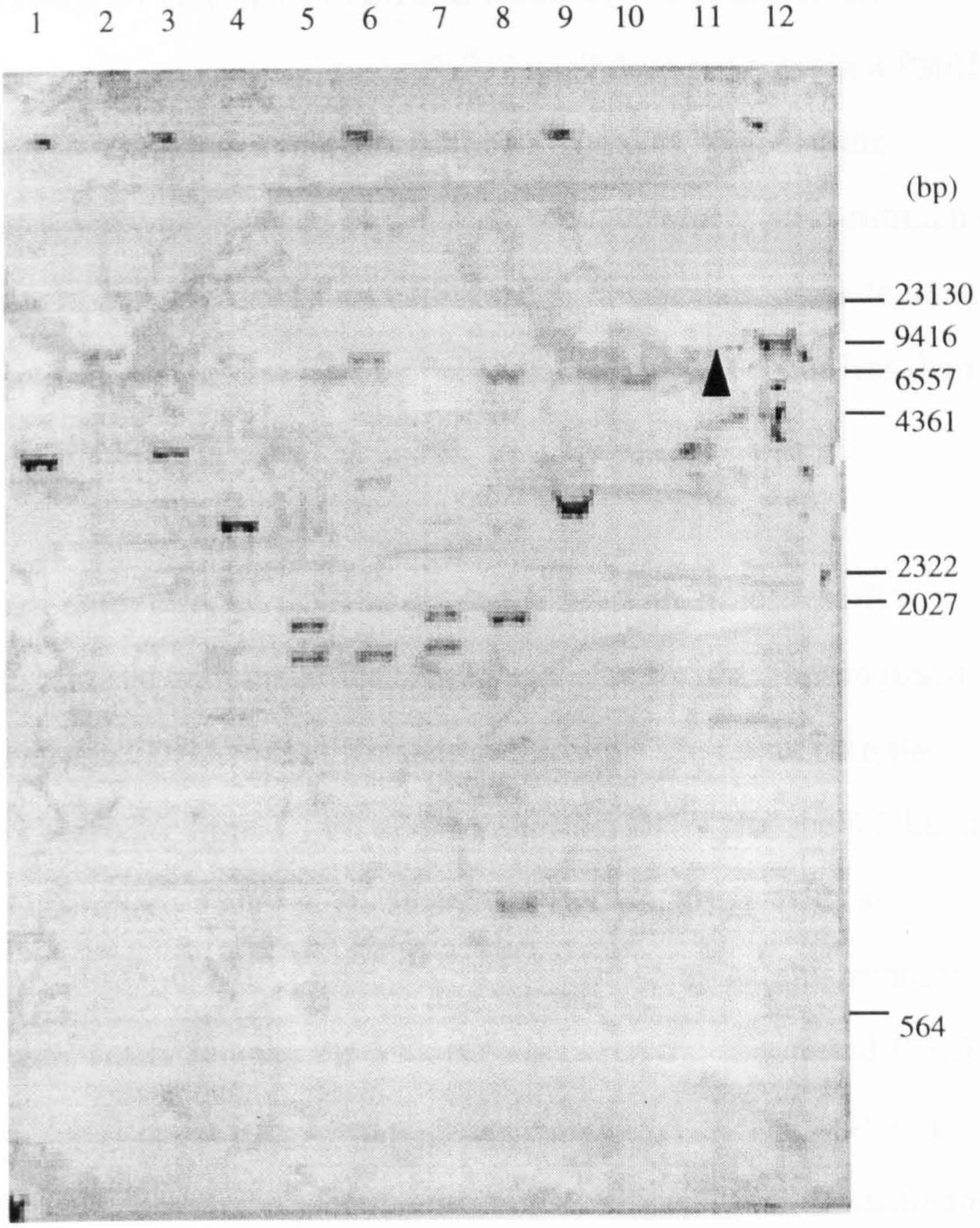


FIGURE 5.15

Southern blot of chromosomal DNA of strains 8325-4 (WT), WA250 (*agr*), 10.1 and 5B.1 digested with *Pvu*II (lanes 1 through 4), *Pvu*II/*Pst*I, (lanes 5 through 8) or *Hin*cII/*Eco*RV (lanes 9 and 12) and probed with the digoxygenin labelled 1.8 kb *Asp*700/*Eco*RV fragment of *agr* (see Fig. 1.2). The corresponding fragments of *Hin*dIII digested λ are shown on the right with their sizes (bp). The arrow in lane 11 indicates a band which was apparent on the original Southern blot but has not reproduced well on the scanned image shown.

simultaneously with *Pst*I, the transposon could be located to one of two fragments of *agr*. Three hybridising bands resulted from this digest, one of approximately, 800 bp, one of 2 kb and the third of approximately 8 kb (Fig. 5.15). It has already been established that Tn*917*-LTV1 contains a *Pvu*II restriction site. This site almost certainly occurs at the *lacZ* proximal end since Tn*917* itself does not contain a *Pvu*II site (Shaw and Clewell, 1985). From this information it could be seen that the transposon had inserted with the *lacZ* end of the transposable element just upstream of the *Pst*I restriction site of *agr* (Fig. 5.16). In addition, the *Pvu*II restriction site must be less than 800 bp into the transposon and, by the same token, must occur less than 800 bp upstream of the *Pst*I site of *agr* (Fig. 5.16).

Following digestion of chromosomal DNA of strain 8325-4 with *Hin*cII/*Eco*RV, a fragment of 3.1 kb hybridised with the probe (Fig. 5.15). The hybridising fragment of strain WA250 chromosomal DNA was again anomalous at 6.5 kb, rather than the expected size of 8.4 kb, arising from 3.1 kb of chromosomal DNA plus the 5.3 kb of Tn*551* (Fig. 5.15). Of similarly digested chromosomal DNA of strain 10.1, a fragment of 11 kb hybridised with the probe (Fig. 5.15) indicating that this strain was also an *agr* mutant with Tn*917* lying in the 396 bases between the *Hin*cII and *Pvu*II sites (Fig. 5.14). Of *Hin*cII/*Eco*RV digested chromosomal DNA of strain 5B.1, a fragment of 11 kb hybridised with the probe, indicating that Tn*917* was downstream of the *Eco*RV site and thus in the 677 bp between the *Pst*I and *Eco*RV restriction sites (Fig. 5.15). The derivation of fragments resulting from digestion of chromosomal DNA with *Hin*cII/*Eco*RV is shown in Figure 5.17.

To locate the transposons exactly within *agr*, the area of the Tn*917* insertion site in each mutant needed to be amplified and sequenced.

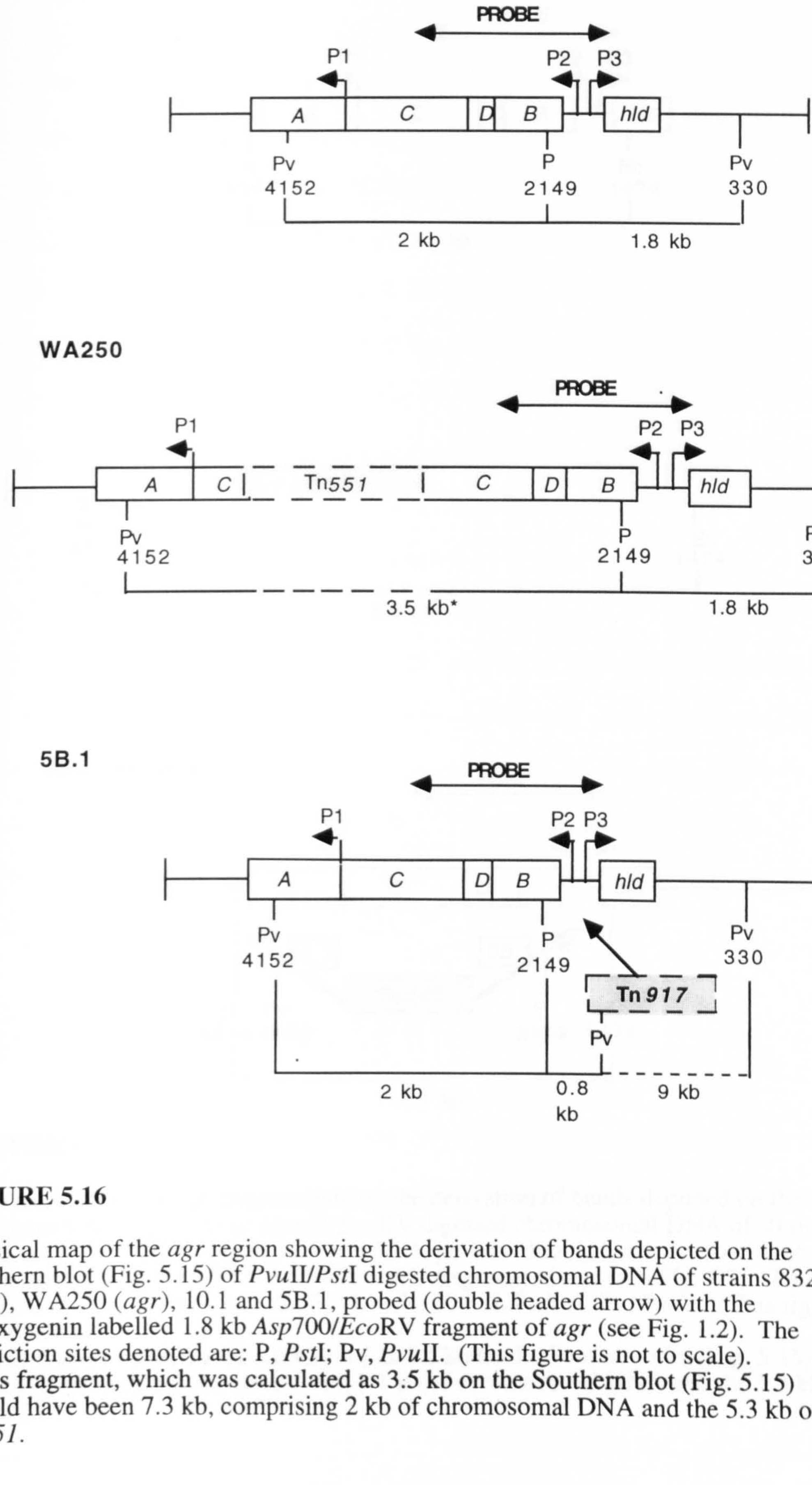


FIGURE 5.16

Physical map of the *agr* region showing the derivation of bands depicted on the Southern blot (Fig. 5.15) of *Pvu*II/*Pst*I digested chromosomal DNA of strains 8325-4 (WT), WA250 (*agr*), 10.1 and 5B.1, probed (double headed arrow) with the digoxygenin labelled 1.8 kb *Asp*700/*Eco*RV fragment of *agr* (see Fig. 1.2). The restriction sites denoted are: P, *Pst*I; Pv, *Pvu*II. (This figure is not to scale).
*This fragment, which was calculated as 3.5 kb on the Southern blot (Fig. 5.15) should have been 7.3 kb, comprising 2 kb of chromosomal DNA and the 5.3 kb of Tn*551*.

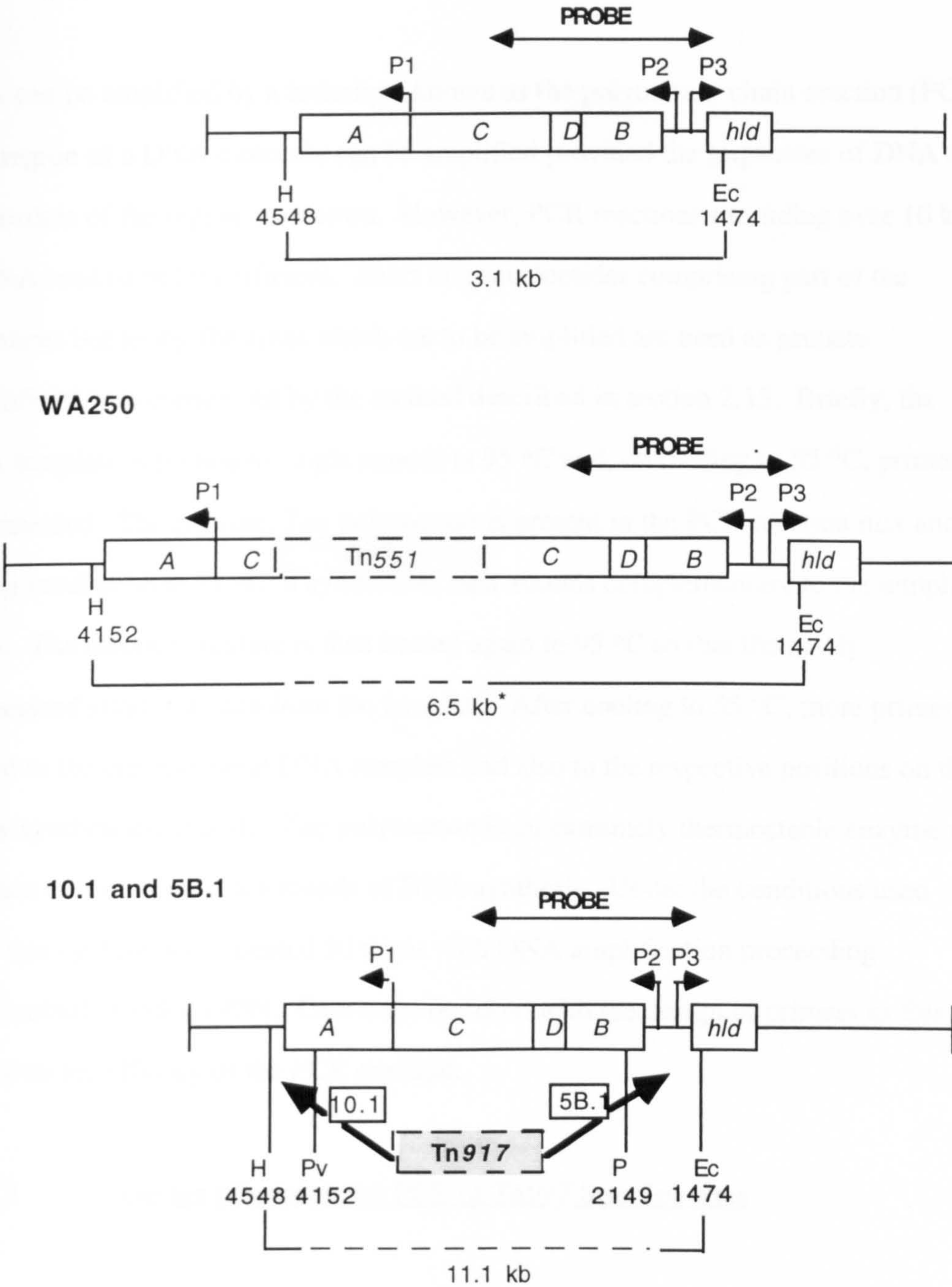


FIGURE 5.17

Physical map of the *agr* region showing the derivation of bands depicted on the Southern blot (Fig. 5.15) of *Hin*cII/*Eco*RV digested chromosomal DNA of strains 8325-4 (WT), WA250 (*agr*), 10.1 and 5B.1, probed (double headed arrow) with the digoxygenin labelled 1.8 kb *Asp*700/*Eco*RV fragment of *agr* (see Fig. 1.2). The restriction sites denoted are: Ec, *Eco*RV; H, *Hin*cII; P, *Pst*I; Pv, *Pvu*II. (This figure is not to scale).
* This fragment, which was calculated as 6.5 kb on the Southern blot (Fig. 5.15), should have been 8.4 kb, comprising 3.1 kb of chromosomal DNA and the 5.3 kb of Tn*551*.

5.2.7 Amplification of chromosomal DNA flanking Tn*917* insertion sites

DNA can be amplified by a technique known as the polymerase chain reaction (PCR). Any region of a DNA molecule can be amplified provided the sequences of DNA at the borders of the region are known. However, PCR reactions extending over 10 kb of DNA tend to be less efficient. Short oligonucleotides comprising part of the sequences bordering the areas which are to be amplified are used as primers. Amplification is carried out by the method described in section 2.15. Briefly, the DNA template is melted to single strands at 95 °C and, on cooling to 55 °C, primers are annealed. The enzyme, *Taq* polymerase is present in the PCR reaction mix and, during incubation at 72 °C, it synthesises new strands complementary to the template DNA. The reaction mixture is then heated again to 95 °C so that the newly synthesised strands detach from the template. After cooling to 55 °C, more primers anneal to the chromosomal DNA template and also to the respective positions on the newly synthesised strands. *Taq* polymerase is an extremely thermostable enzyme and can thus carry out repeated rounds of DNA synthesis. Under the conditions used here, this cycling was repeated 30 times with DNA amplification proceeding exponentially (Saiki, 1990). Care must be taken with the design of primers as this is crucial to the efficacy of the PCR reaction.

5.2.7.1 Design of primers for PCR of Tn*917* insertion sites

At each end of a primer sequence it is preferable to include four sequential bases from the vector into which the PCR products will be cloned, in this case pUBS1. These bases comprise the sequence of pUBS1 just up- or down-stream of an elected restriction sequence (in this case *Eco*RI) within the multiple cloning site. Thus, in the forward direction GCAG and in the reverse direction, TATC (Fig. 5.18) were used. Next in the primer sequence the selected restriction enzyme site (*Eco*RI - sequence

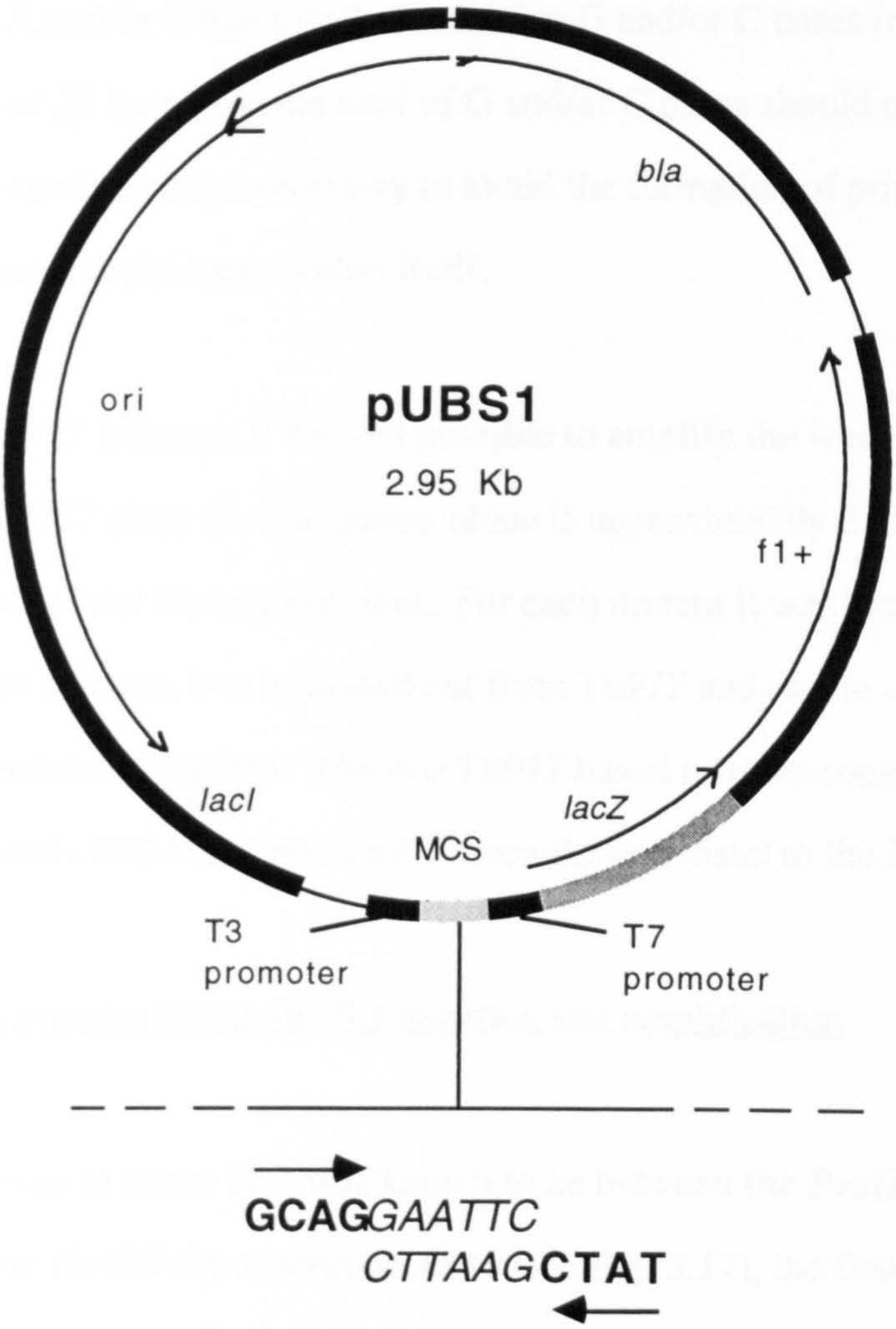


FIGURE 5.18

Partial sequence of the pUBS1 multiple cloning site (MCS) showing the bases used in the creation of PCR primers.

GAATTC) is needed in order to allow the PCR product to be cloned easily into pUBS1. Following the *Eco*RI site, 25 bases of DNA homologous to the chromosomal DNA to be amplified is included in the primer sequence. There should be no more than 3 consecutive A and/or T bases or 2 consecutive G and/or C bases in this complete sequence of 25 bases and the total of G and/or C bases should not exceed 8-10. These last two conditions are necessary to avoid the formation of primer dimers, or the primer forming hairpin loops within itself.

In the case of the Tn*917* mutants, it was not possible to amplify the whole of the region containing Tn*917* since the transposon alone is approximately 8 kb and thus the PCR reaction would not be very efficient. For each mutant it was therefore necessary to use four primers, two to extend out from Tn*917* and two to extend in from flanking chromosomal regions. The two Tn*917* based primers consisted of sequence approximately 800 bases into Tn*917* from the end distal to the *lacZ* gene.

5.2.7.1.1 PCR primers for strain 10.1 insertion site amplification

Because the transposon in strain 10.1 was known to be between the *Pvu*II site at position 4150 and the *Hinc*II site at position 4546 (see Fig. 5.17), the first suitable sequence of DNA upstream or downstream of these sites was used for primers. In the forward direction, upstream of *Pvu*II, primer HF1 (Fig. 5.19) was used (Fig. 5.20) comprising bases 4071 to 4096 of *agr* (Fig. 5.21). In the reverse direction, downstream of *Hinc*II, primer HF2 (Fig. 5.19) was used (Fig. 5.20) comprising bases 4616 to 4592 (Fig. 5.21).

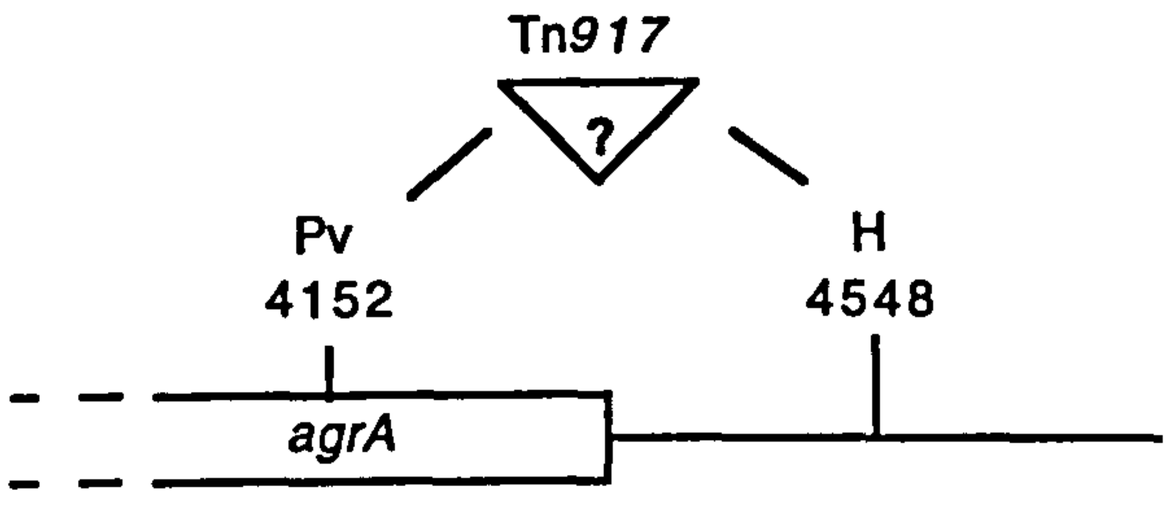
5.2.7.1.2 PCR primers for strain 5B.1 insertion site amplification

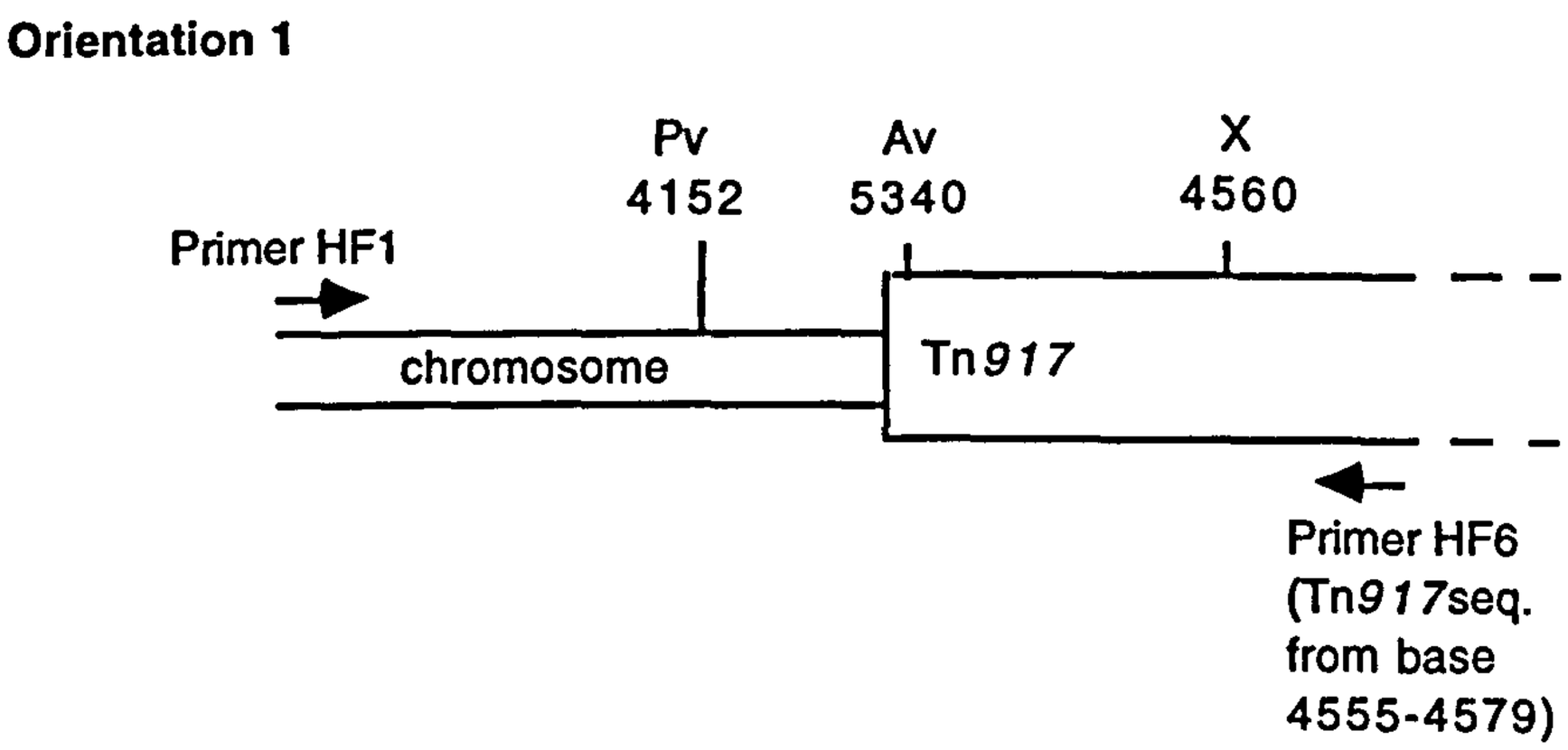
The transposon in this mutant was between the *Eco*RV site at position 1479 and the *Pst*I site at position 2149 (see Fig. 5.17). Consequently, bases 1417 to 1441 (Fig.

PRIMER	SEQUENCE
HF1 - 10.1	GCAGGAATTCGTTACGAGTCACAGTGAACTTACCT
HF2 - 10.1	TATCGAATTCCAGCTATACAGTGCATTTGCTAGTT
HF3 - 5B.1	GCAGGAATTCGAATTTGTTCACTGTGTCGATAATC
HF4 - 5B.1	TATCGAATTCCTCTTTGATGATAAGTGTGATAATG
HF5 - Tn*917*	GCAGGAATTCCAGATCTAGATATTGAGGAACATTT
HF6 - Tn*917*	TATCGAATTCGTCTAGATCTATAACTCCTTGTAAA

FIGURE 5.19

The sequence of primers used to amplify the transposon insertion sites in strains 10.1 (HF1 and HF2) (see Fig. 5.21) and 5B.1 (HF3 and HF4) (see Fig. 5.21). Primers HF5 and HF6 include sequence occurring at the end of Tn*917* distal to the *lacZ* gene.





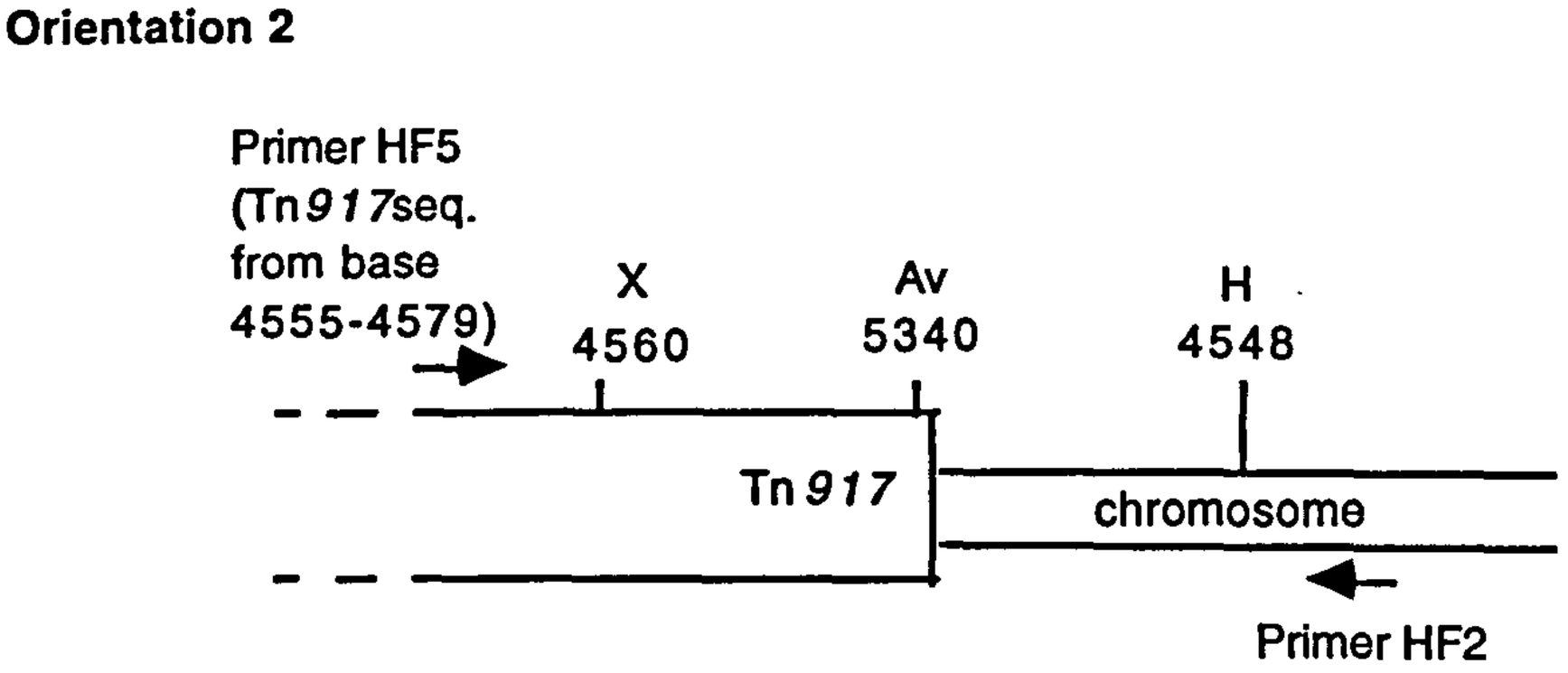


FIGURE 5.20

PCR reactions for the amplification of the transposon insertion site in strain 10.1. The orientation of primers is indicated, allowing for Tn*917* to have inserted in the chromosome in either possible orientation between the *Hin*cII and *Pvu*II restriction sites within the *agr* locus. The restriction sites denoted are: Av, *Ava*I; H, *Hin*cII; Pv, *Pvu*II; X, *Xba*I.

Primer HF3

1401 TCATCTTATT TTTTAGTGAA *TTTGTTCACT GTGTCGATAA TC* CATTTTAC

1451 TAAGTCACCG ATTGTTGAAA TGATATCTTG TGCCATTGAA ATCACTCCTT

Ec

1501 CCTTAATTAA GATAAAAATT CTTAAAATTA AACAACTCAT CAACTATTTT

1551 CCATCACATC TCTGTGATCT AGTTATATTA AAACATGCTA AAAGCATTTA

1601 TTTTCCAATT TTTCTTAACT AGTCGTTTTT TATTCTTAAC TGTAAATTTT

1651 TTTATGTTAA AATATTAAAT ACAAATTACA TTTAACAGTT AAGTATTTAT

1701 TTCCTACAGT TAGGCAATAT AATGATAAAA GATTGTACTA AATCGTATAA

SD ORF-4 (*agrB*)

1751 TGACAGTGAG GAGAGTGGTG TAAAATTGAA TTATTTTGAT AATAAAATTG

1801 ACCAGTTTGC CACGTATCTT CAAAAGAGAA ATAACTTAGA TCATATTCAA

1851 TTTTTGCAAG TACGATTAGG GATGCAGGTC TTAGCTAAAA ATATAGGTAA

1901 ATTAATTGTT ATGTATACTA TTGCCTATAT TTTAAACATT TTTCTGTTTA

1951 CGTTAATTAC GAATTTAACA TTTTATTTAA TAAGAAGACA TGCACATGGT

2001 GCACATGCAC CTTCTTCTTT TTGGTGTTAT GTAGAAAGTA TTATACTATT

2051 TATACTTTTA CCTTTAGTAA TAGTAAATTT TCATATTAAC TTTTTAATTA

2101 TGATTATTTT AACAGTTATT TCTTTAGGTG TAATCTCAGT ATATGCTCCT

P

2151 GCAGCAACTA AAAAGAAGCC CATTCCTGTG CGACTTATTA AACGAAAAAA

Primer HF4

2201 ATATTATGCG ATTATTGTTA GTTTAACCCT TTT*CATTATC ACACTTATCA*

2251 *TCAAAGAG*CC ATTTGCCCAA TTCATTCAAT TAGGCATCAT AATAGAAGCT

FIGURE 5.21

Partial sequence of the *agr* locus indicating the area of DNA used to create PCR primers (see Fig. 5.18) for (a) strain 5B.1 and, overleaf, (b) strain 10.1. Relevant ORFs are indicated. The restriction sites denoted are: Ec, *Eco*RV; H, *Hin*cII; P, *Pst*I; Pv, *Pvu*II.

agrA gene

4001 AACTGATATT AATGGTATCA AATTAGGCAG TGAAATTCGT AAGCATGACC

Primer HF1

4051 CAGTTGGTAA CATTATTTTC *GTTACGAGTC ACAGTGAACT TACCT*ATTTA

4101 ACATTTGTCT ACAAAGTTGC AGCGATGGAT TTTATTTTTA AAGATGATCC

Pv

4151 AGCTGAATTA AGAACTCGAA TTATAGACTG TTTAGAAACT GCACATACAC

4201 GCTTACAATT GTTGTCTAAA GATAATAGCG TTGAAACGAT TGAATTAAAA

4251 CGTGGCAGTA ATTCAGTGTA TGTTCAATAT GATGATATTA TGTTTTTTGA

4301 ATCATCAACA AAATCTCACA GACTCATTGC CCATTTAGAT AACCGTCAAA

4351 TTGAATTTTA TGGTAATTTA AAAGAACTGA GTCAATTAGA TGATCGTTTC

4401 TTTAGATGTC ATAATAGCTT TGTCGTCAAT CGCCATAATA TTGAATCTAT

4451 AGATTCGAAA GAGCGAATTG TCTATTTTAA AAATAAAGAA CACTGCTATG

End of *agrA* gene

H

4501 CATCGGTGAG AAACGTTAAA AAAATATAAT AAGATAATAA AGTCAGTTAA

Primer HF2

4551 CGACGTATTC AATTGTAAAT CTTGTTGGAT TTTAACAAGA TAA*CTAGCAA*

4601 *ATGCACTGTA TAGCTG*GCTT TTTAATTTTA TTAACAAAAT TAAATATGAC

5.21) were used for primer HF3 (Fig. 5.19), upstream of *Eco*RV and bases 2258 to 2235 (Fig. 5.21) were used for primer HF4 (Fig. 5.19), downstream of the *Pst*I site.

5.2.7.1.3 PCR primers internal to Tn*917*

Since the orientation of Tn*917* within the chromosome of each mutant was unknown, two internal primers were designed (Fig. 5.20). Both primers extended out from the same *lacZ* distal end of Tn*917*, thus bases 4555 to 4579 of Tn*917* were used to create primers HF5 and HF6 (Fig. 5.19). These primers were set back approximately 800 bp from the end of Tn*917*.

5.2.7.2 PCR amplification of Tn*917* insertion sites

The PCR reactions were completed as described in section 2.15. Reactions were carried out using primer pairs listed in Table 5.5. It was expected that only one reaction for each mutant would result in a PCR product depending on the orientation of the transposon. An agarose gel of the PCR products revealed that mutant 5B.1 had one band, resulting from the primers HF3 and HF6 (Fig. 5.19), of approximately 1500 bases (Table 5.5). Strain 10.1 had one band resulting from the primers HF2 and HF5 (Fig. 5.19) of 1200 bp (Table 5.5). The PCR products of each mutant were separated on a TAE agarose gel, gel purified and ligated into *Eco*RI digested, dephosphorylated pUBS1. Ligation mixes were transformed into ultra-competent *E. coli* (Stratagene) and transformants selected on LB plates as white, ampicillin resistant colonies. Approximately 100 colonies were obtained from each transformation and, following a mini-prep of two of each, it was confirmed by digestion with *Eco*RI that pUBS1 contained the correct inserts. The plasmids were designated pHF30 and pHF31 for the derivatives from 10.1 and 5B.1 respectively.

Mutant primers	Tn*917* primers	
	HF5	**HF6**
HF1-10.1	-	NP
HF2-10.1	1.2 kb	-
HF3-5B.1	-	1.5 kb
HF4-5B.1	NP	-

TABLE 5.5

Primers used in PCR reactions to amplify chromosomal DNA flanking Tn*917* insertion sites in strains 10.1 and 5B.1. The sizes of PCR products are given where applicable (kb). NP denotes no PCR product obtained.

5.2.8 DNA sequencing of plasmids pHF30 and pHF31

Sequencing of the PCR derived inserts in pHF30 and pHF31 was used to verify the position of Tn*917* within *agr* in each mutant. Sequencing primers consisted of 17 bp oligonucleotides which comprised either the T3 or T7 promoter sequence in pUBS1 (Fig. 5.22). Thus, it was possible to sequence in from either end of the inserted chromosomal and/or transposon DNA since it was not known in which orientation the PCR products had been ligated into pUBS1 (Fig. 5.22). Following these reactions the extension products were purified by ethanol precipitation and pellets dried under vacuum. Automated sequencing was carried out as described in section 2.16.

5.2.8.1 DNA sequence of pHF30

Database analysis (FASTA) of the DNA sequence generated from the T3 primer showed that Tn*917* had inserted after base 4458 of *agr* in strain 10.1 (Fig. 5.23 and Fig. 5.21). The sequence, extending from the T3 promoter region of pUBS1, includes part of the pUBS1 multiple cloning site upstream of the *Eco*RI restriction site, then the *Eco*RI site and the *agr* primer from the PCR reaction, comprising bases 4616 to 4592. The complementary sequence of *agr* then continues from base 4591 to base 4458. The transposon has inserted at base 4458 since, at this point, the sequence of Tn*917* begins from its last base, 5353 (Fig. 5.24).

5.2.8.2 DNA sequence of pHF31

Database analysis (FASTA) of the DNA sequence generated using the T7 primer is shown in Fig. 5.25. The sequence shows that this end of the insert is Tn*917* derived.

The T3 primer generated sequence includes part of the pUBS1 multiple cloning site upstream of the *Eco*RI restriction site, then the *Eco*RI site and the *agr* primer from the

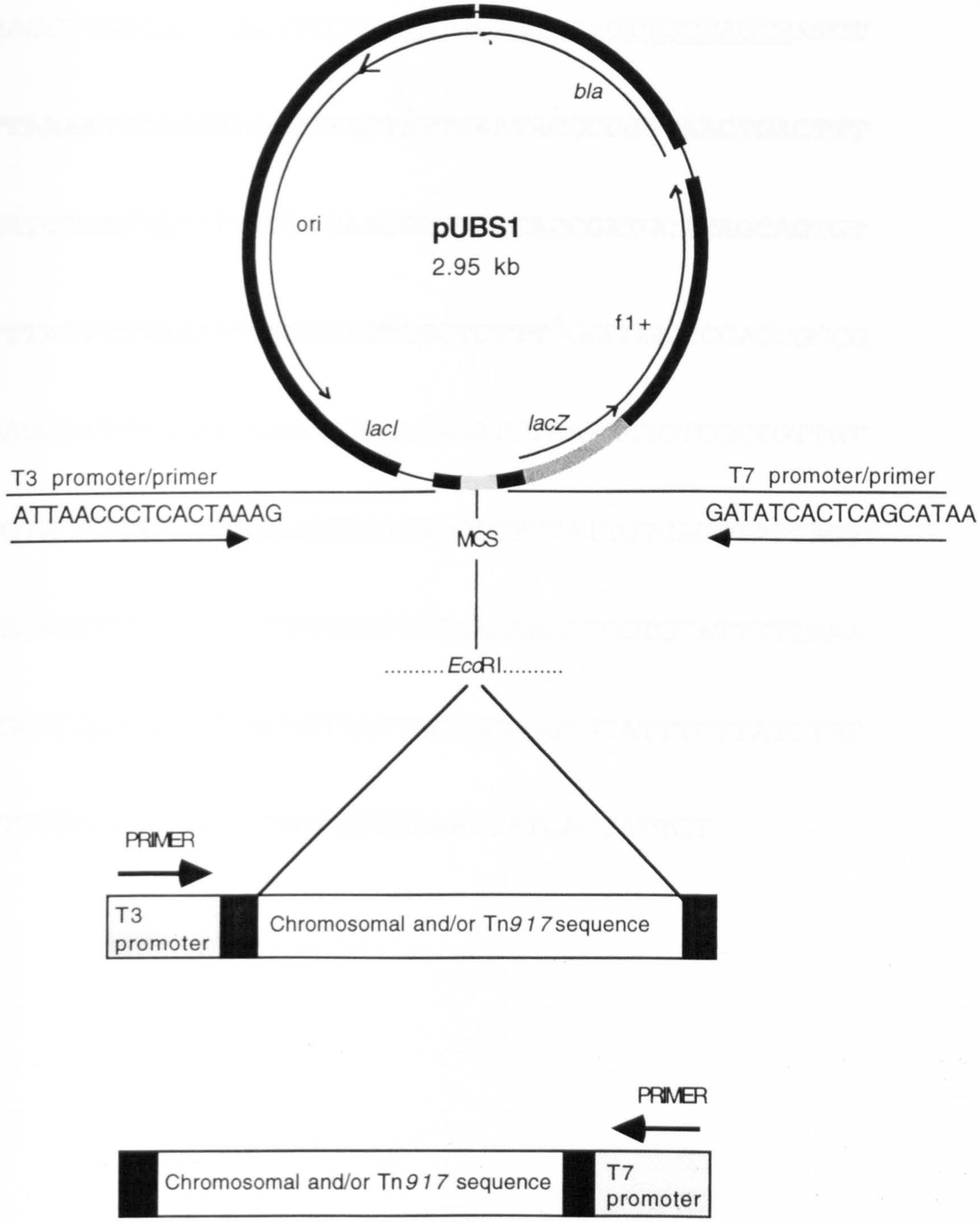


FIGURE 5.22

Position and sequence of the T3 and T7 sequencing primers in pUBS1.

ATAAGCTTGATATCGAATTC<u>CAGCTATACAGTGCAGTTGCTAGTT</u>**ATCT**

TGTTAAAATCCAACAAGATTTACTATTGAATACGCCGTTAACTGACTTT

ATTATCTTATTATATTTTTTTTAACGTTTCTCACCGATGCATAGCAGTGT

TCTTTATTTTTTAAAATAGACAATTCGCTCTTT$^{\Delta}$GGGGGCCCGAGCGCCG

ACGAGGAATTTGTATCGATAAGAAATAGATTTAAAAATGTCGCTGTTAT

TTTGTACATTTAACTTGACGGTGACATCTCTCTATTGTGAGTTATTAGT

GGTACAGTTTTCAACCGTTTTAATTATAAAAAAGTGGTGCATTTTTTAAA

TTGGCACAAACAAGTAACNGTTATTGCAGCAGGTGTATTTCTTATCTAT

GGGTTTAACATGGATTTTATCATTAAAATCATGAGTATTGT

FIGURE 5.23

DNA sequence of pHF30 using the T3 primer of pUBS1.
Key: italics denotes the *MCS* of pUBS1; underlining denotes <u>primer HF2</u>; bold type denotes the **sequence of the *agr* locus** from base 4591 to base 4458; plain text denotes Tn*917* sequence from base 5353. The point of Tn*917* insertion is denoted by the symbol, Δ.

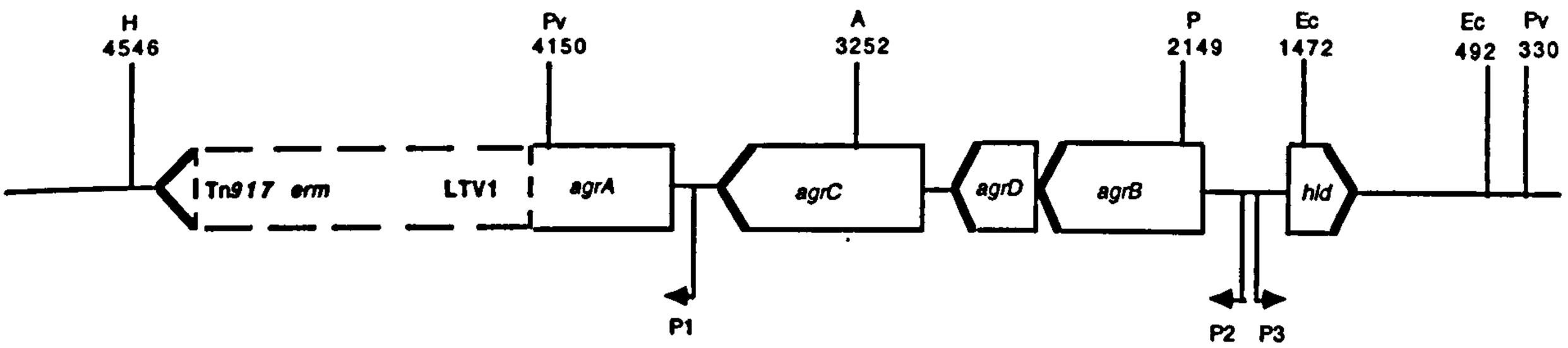


FIGURE 5.24

Approximate insertion site of Tn*917* within the *agr* locus of strain 10.1. The restriction sites denoted are: A, *Asp*700; Ec, *Eco*RV; H, *Hinc*II; P, *Pst*I; Pv, *Pvu*II. (This figure is not to scale).

*CCCGGGCTGCAGGAATTC*CAGATCTAGATATTGAGGAACATTT**CACTGA**

TACAAATGGGTATTCTGATCAGGTGTTTGGAATGACCGCATTACTAGGC

TTTGATTTTGAACCTCGCATCAGAAATATAAAAAAATCACAATTATTTT

CTACAATCACCTTCCTACTACCCTAACTTATCAGAAGATATAAGCGGAA

AAATCAATGTAAAAAATTATTGAAGAAAACTATGATGAAATTAAACGAA

TCGCCTATTCGATTCAAACAGGAAAAGTATCTAGTTCTTTACTATTAGG

AAAGCTAGGCTCATACGCACGTAAGAATAGAGTAGCTCTTGCACTGAGA

GAACTAGGTCGCATTGAAAAGAGCATTTTTATGATAGATTATATTACAG

ATAGTGAGCTACGGCGAAGGATC

FIGURE 5.25

DNA sequence of pHF31 using T7 primer of pUBS1.
Key: italics denotes the *MCS* of pUBS1; underlining denotes primer HF5; bold type denotes the **Tn*917*** sequence from base 4580 to base 4951.

PCR reaction, comprising bases 1417 to 1441 (Fig. 5.26). The *agr* sequence then continues to base 1753. At this point there are 17 bases missing and then the sequence of *agr* resumes from base 1771 to base 1796. At this point, the complement of the sequence of the end of Tn*917* starts, from base 5353 to base 5272. Therefore, Tn*917* has inserted into *agr* following base 1796 (Fig. 5.27 and Fig. 5.21).

*ATAAGCTTGATATCGAATTC*GAATTTGTTCACTGTGTCGATAATC**CATT**

TTACTAAGTCACCGATTGTTGAAATGATATCTTGTGCCATTGAAATCTC

TCCTTCCTTAATTAAGATAAAAATTCTTAAAATTAAACAACTCATCAAC

TATTTTCCATCACATCTCTGTGATCTAGTTATATTAAAACATGCTAAAA

GCATTTATTTTCCAATTTTTCTTAACTAGTCGTTTTTTTATTCTTAACTG

TAAATTTTTTTATGTTAAAATATTAAATACAAATTACATTTAACAGTTA

AGTATTTATTTCCTACAGTTAGGCNATATAATGA*TAAAAGATTGTACT

AAATCGTATAATΔ**GGGGTCCCGAGCGCNTAGGGGAATNTGTATCGATAA**

GGGGTACAAATTCCCACTAAACCAATGTTTCAAGGGCTATTTATTT

FIGURE 5.26

DNA sequence of pHF31 using the T3 primer of pUBS1.
Key: italics denotes the *MCS* of pUBS1; underlining denotes primer HF3; bold type denotes the **sequence of the *agr* locus** from base 1442 to base 1796; plain text denotes Tn*917* sequence from base 5353 to base 5272. The symbol, * indicates the site of a 17 bp deletion in the *agr* sequence. The point of Tn*917* insertion is denoted by the symbol, Δ.

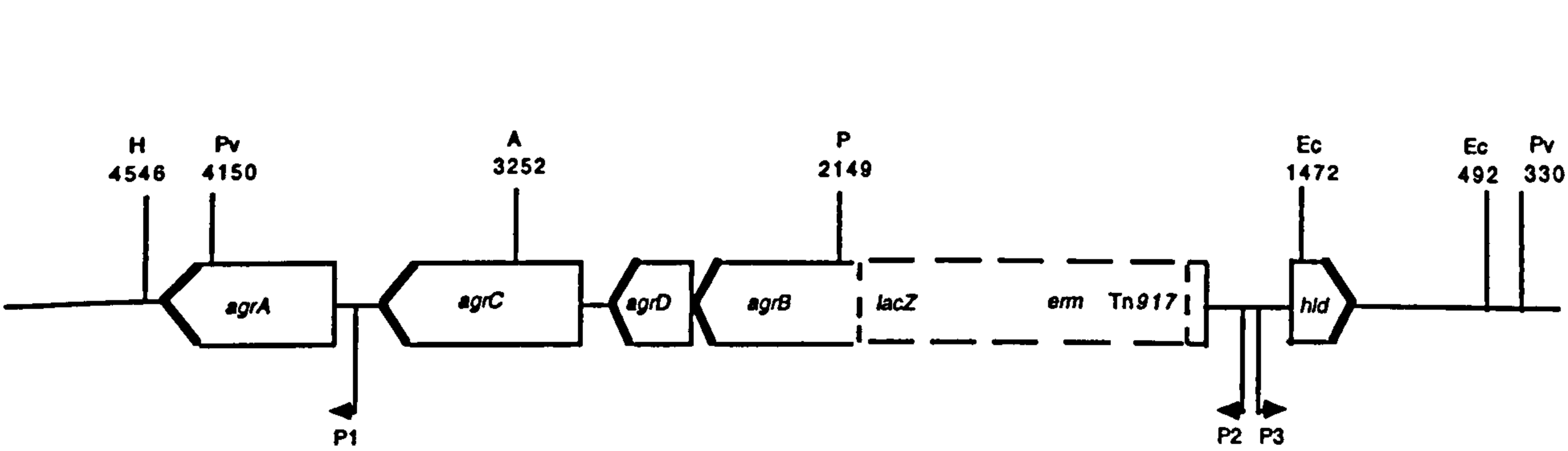


FIGURE 5.27

Approximate insertion site of Tn*917* within the *agr* locus of strain 5B.1. The restriction sites denoted are: A, *Asp*700; Ec, *Eco*RV; H, *Hin*cII; P, *Pst*I; Pv, *Pvu*II. (This figure is not to scale).

5.3 Discussion

A number of putative Tn*917* mutants were isolated on the basis of a reduction in β–haemolysin production. Following phage transduction and pulse field gel electrophoresis, two mutants were chosen for further analysis. Following *Sma*I digestion, the mutations in both these strains (10.1 or 5B.1) mapped to a novel fragment that co-ran with *Sma*I fragment I (117 kb) or a novel fragment of approximately 110 kb, respectively.

Two random controls, BHC1 and BHC2 contained Tn*917* in novel fragments that co-ran with *Sma*I fragments G and H, respectively. Strain WA250 (containing Tn*551* in *agr*) was also examined to identify which fragment contained *agr* and it was found that hybridisation with the Tn*917* probe occurred in *Sma*I fragment G. This was somewhat surprising since *agr* originates in *Sma*I fragment F in strain 8325 which has been reported to co-run with fragment H in 8325-4 (Pattee *et al.*, 1990; Smeltzer *et al.*, 1992). However, repeated PFGE of chromosomal DNA of strain WA250 confirmed that Tn*551*, and thus presumably *agr*, was located in fragment G (results not shown). These results suggested that, either Tn*917* in the newly isolated mutants was not located in *agr*, or that the data acquired for strain WA250 was anomalous.

In addition, the phenotypes of strains 10.1 and 5B.1, being lipase positive, were found to be different to an *agr* mutant examined by Smeltzer *et al.* (1992) who reported that lipase activity in an *agr*⁻ strain was markedly reduced. The *agr* mutant strain, WA250 also appeared, in these particular experiments, to be lipase negative, although this was contrary to the results obtained earlier (see section 4.2.8.3). The production of all other virulence determinants was as for strain WA250 and was typical of an *agr* mutant.

As discussed in section 5.2.5, the transposon used to create these mutants, Tn*917*-

LTV1 was specifically designed for straightforward cloning without the need for an additional intermediate vector. Following digestion with an appropriate enzyme (*Xba*I or *Eco*RI), it should be possible to religate Tn*917*-LTV1 and some flanking chromosomal DNA and transform and maintain stable plasmids in *E. coli*. However, this did not prove possible and it was established, by *Sal*I digestion, that this was due to a deletion of approximately 4 kb from the *lacZ* proximal end of the Tn*917*-LTV1 fragment. This deleted fragment could have contained the ColE1 replicon and thus prevented maintenance in *E. coli*, and probably contained some of the *lacZ* gene, which would explain why the transductants were white in the presence of X-Gal. An alternative method for cloning the chromosomal DNA flanking the transposon insertion was to identify a fragment of chromosomal DNA containing Tn*917* of <10 kb and ligate this fragment into a plasmid suitable for replication in *E. coli*. However, no transformants containing a recombinant plasmid for either mutant were isolated.

To test whether the mutations were actually in *agr*, chromosomal DNA was subjected to Southern blot analysis using an *agr* probe. If the transposons were in *agr* then the restriction profiles would have differed from the wild type strain, 8325-4 and hybridising bands would have increased by the size of Tn*917*-LTV1, i.e. approximately 8 kb. Chromosomal DNA from all four strains strains was initially digested with *Pvu*II which cuts towards each end of *agr* (see Fig. 1.2). Strain 10.1 did not contain Tn*917* between these two restriction sites since the resulting hybridising fragment was 3.8 kb which was the same as for 8325-4. Strain 5B.1 did contain Tn*917* within *agr* resulting in two hybridising fragments of 9 kb and 2.8 kb. Digestion of chromosomal DNA of all four strains with *Pvu*II and *Pst*I, which cuts within the 3.8 kb *Pvu*II fragment, showed that in strain 5B.1, Tn*917* lay approximately 800 bp upstream of the *Pst*I restriction site. The further digestion of 5B.1 chromosomal DNA with *Hin*cII (which cuts 396 bases downstream of *Pvu*II) and *Eco*RV gave confirmation of just how close to the *Pst*I site Tn*917* lay. The

transposon was found to be downstream of the *Eco*RV site and thus somewhere in the 677 bp between the *Eco*RV and *Pst*I restriction sites (see Fig. 5.14). Digestion of strain 10.1 chromosomal DNA using *Hin*cII and *Eco*RV revealed that Tn*917* was located somewhere in these 396 bases. Digestion of chromosomal DNA of 8325-4 with *Hin*cII/*Eco*RV resulted in a hybridising fragment of 3.1 kb representing the number of bases between these two restriction sites within *agr*. The confirmation that both mutants contained Tn917 within the *agr* locus highlighted the fact that a lipase assay using egg-yolk as the substrate must be regarded as somewhat unreliable.

The results for strain WA250 were somewhat anomalous, although digestion with *Pvu*II confirmed the presence of Tn*551* with a 5.3 kb increase in the size of the hybridising fragment compared with strain 8325-4. However, following *Pvu*II/*Pst*I digestion, this extra 5.3 kb did not map to the expected 2 kb fragment downstream of the *Pst*I site of *agr* (see Fig. 5.16). Instead, a hybridising fragment of 3.5 kb was apparent. This could indicate that there is a *Pst*I site within Tn*551*, although in this case there should have been an additional fragment hybridising with the probe of 3.8 kb, comprising the remainder of Tn*551*. In addition, there is very good homology between Tn*551* and Tn*917* (Giraudo *et al.*, 1994b) and Tn*917* does not contain a *Pst*I site (Shaw and Clewell, 1985). Following digestion with *Hin*cII/*Eco*RV a fragment of 8.4 kb of chromosomal DNA of strain WA250 should have hybridised with the probe but, in fact, a fragment of 6.5 kb hybridised with the probe. Since the restriction data acquired for strain WA250 was not as expected and the lipase activity was variable it seemed possible that the strain of WA250 used in some of these experiments had undergone some changes at the genetic level during laboratory use.

Although the orientation of Tn*917* within strain 5B.1 had already been established, PCR reactions for this strain and strain 10.1 were carried out allowing for the transposon to be inserted either way round. As expected, only one reaction for each strain resulted in a PCR product. The PCR products were cloned and sequenced.

In strain 10.1, Tn*917* began immediately prior to base 4458, which is 72 bases before the end of the *agrA* gene. As discussed in section 1.6.1.1.2.1, *agrA* comprises the response regulator of a classical two component signal transduction system. In general, the phosphorylated gene product of a response regulator binds to a promoter and up-regulates target gene transcription. However, it has been suggested that AgrA does not activate transcription from P2 and of RNAIII by binding directly, but rather interacts with SarA to aid RNA polymerase to initiate transcription in some way which is not yet fully understood (Morfeldt *et al.*, 1996b). In any case, absence of AgrA would prevent, by whatever mechanism, the proper functioning of the whole *agr* locus.

In strain 5B.1 it was found that Tn*917* began following base 1796 of *agr*. However, a 17 base gap in the *agr* sequence was identified comprising bases 1754 to 1770, inclusive. This deletion probably arose during insertion of Tn*917* into the original mutant, 10, but it is just possible that these bases were missing in this isolate prior to transposon mutagenesis. If this latter hypothesis was true then, since the 17 bp deletion occurs just 25 bases upstream of the transposon insertion site, it would have been likely that this deletion would co-transduce with the transposon. However, the net effect of either of these mutations would be the same. The missing bases occur within the promoter, P2 and span the purine rich Shine Dalgarno (SD) sequence. Although binding of RNA polymerase is initiated at the -35 region of the promoter, which remained intact, this could not occur because the -10 sequence is also necessary for binding. In addition, the distance between the -35 and -10 sequences is critical. In any case, even if transcription could have occurred, the SD sequence functions as a ribosome binding site so that mRNA could not have been translated to give the AgrB gene product. The transposon in 5B.1 was inserted 20 bases into ORF4 which encodes the *agrB* gene. As discussed in section 1.6.1.1.2.2, the product of *agrB* is possibly involved in processing the gene product of *agrD* from 46 amino acid residues to its final form - the octapeptide signalling molecule, RAP (Balaban and

Novick, 1995; Ji *et al.*, 1995; Ji *et al.*, 1997). Without this signalling molecule, auto-induction of *agr* and RNAIII transcription, and hence some virulence gene expression, can not be activated.

In this work, attempts to identify novel regulatory loci using Tn*917* and screening for clones deficient in β-haemolysin production led only to *agr* mutants. It may be that Tn*917* exhibits a "hot spot" for the *agr* locus although this has not been reported by other workers. It is more likely that a lack of β-haemolysin tends to correlate with an *agr*$^-$ phenotype because *agr* is closely involved in the regulation of production of β-haemolysin. Therefore, screening for β-haemolysin negative clones will tend to result in selection of those clones that are *agr* mutants. It is probably preferable to adopt an alternative virulence determinant as a screen, for example *sar* was identified by screening for a fibrinogen binding protein negative phenotype.

There are many other methods used to identify virulence genes and some of these are discussed in Chapter 6.

CHAPTER 6

General Discussion

6. GENERAL DISCUSSION

The regulation of the production of virulence determinants by *S. aureus* is extremely complex, with the organism producing a wide range of extracellular factors, many of which have a role in pathogenicity. *S. aureus* can grow in a variety of environments, for example in foodstuffs, as well as in humans, and it is therefore important that it can recognise and respond to rapidly changing environments. Obviously, it would not make sense energetically for the bacterium to produce a full range of toxins when it is not in a host environment and it is therefore vital to have a mechanism by which the organism can recognise those signals which indicate that it has entered a host.

A common way in which bacteria sense changes in their environment, and mediate a response by altering gene expression, is via two component sensor regulator systems (Parkinson and Kofoid, 1992; Stock *et al.*, 1989). Such a system is encoded by the major global regulator of *S. aureus, agr*. This global regulator partly comprises a quorum sensing mechanism. The *agr* control mechanism is dependent on cell density which is signalled and sensed via an extracellular factor called RAP. RAP itself is encoded by the *agr* locus and acts to upregulate expression of the whole locus beginning at mid-late exponential growth phase (Balaban and Novick, 1995; Ji *et al.*, 1995). The sensor protein of the two component sensor regulator of *agr*, AgrC, has been proposed to respond to the levels of extracellular RAP and so transduce environmental signals to bring about changes in gene expression via the regulator, AgrA. Many other environmental factors affect expression of both *agr* and other virulence determinants. Some of these signals bring about alterations in virulence determinant gene expression in an *agr*-independent manner.

A key environmental signal that indicates entry into a mammalian host is a change in temperature. Indeed many bacteria, including *B. pertussis* (Melton and Weiss, 1989) and *S. flexneri* (Maurelli *et al.*, 1994) exhibit a virulence phenotype at 37 °C and it has

been shown that *S. aureus* is no exception (see section 3.2.10). In fact, optimum expression of *hld::lacZ* and production of α–haemolysin is obtained at 42 °C with levels at 37 °C greatly exceeding those found at 30 °C.

Many environmental conditions have been examined in this work, of which several led to changes in *hld::lacZ* expression and/or α–haemolysin production. Some of these conditions probably provoke a generalised stress response, i.e. exposure to ethanol or isopropanol, but many correspond to features found in an invasive environment. For example, osmolarity has been found to have a marked effect on the production of several extracellular proteins under the positive control of *agr*. Production of enterotoxins B and C (Genigeorgis and Sadler, 1966; Regassa and Betley, 1993), epidermolytic toxin A (Sheehan *et al.*, 1992) and α–haemolysin (see section 3.2.7) is dramatically reduced in increased osmolarity (NaCl or sucrose). Similarly, *hld::lacZ* expression is decreased in the presence of sucrose but, interestingly, increased in the presence of NaCl indicating that the effect on *agr* is not simply one of high osmolarity. Thus, it appears that production of these virulence factors is de-regulated from *agr* expression in NaCl. As suggested in section 3.3, the inhibitory effect of sucrose is probably also due to the fact that *S. aureus* can ferment sucrose, to give glucose and fructose, as the effects of sucrose are similar to those noted for glucose.

Availability of some divalent cations can have a marked effect on the pathogenesis of many micro-organisms with Mg^{2+} being particularly important in the production of TSST-1 by *S. aureus*, especially associated with some types of tampon (Sarafian and Morse, 1987; Taylor and Holland 1989b). In this work it has been shown that decreased levels of available Mg^{2+} or Ca^{2+} lead to an increase in *hld::lacZ* expression and an even greater increase in α–haemolysin production (see section 3.2.9). Some other conditions which may be indicators of the host environment are iron deprivation and changes in oxygen levels. Many pathogenic bacteria have developed systems for

scavenging iron including production of siderophores and, indeed, in *S. aureus* it has been found that decreased iron availability leads to an increase in both *hld::lacZ* expression and α–haemolysin production (P.F. Chan, pers. comm.). Similarly decreased oxygen availability, but not anaerobic growth, leads to an increase in *hld::lacZ* expression and α–haemolysin production. Growing *S. aureus* in sodium bicarbonate at concentrations equivalent to those found *in vivo* leads to an increase in α–haemolysin production though not *hld::lacZ* expression.

Although the response of *S. aureus* to many environmental signals is mediated via *agr*, the production of some virulence determinants under the control of *agr* can also occur in an *agr*-independent manner, indicating that there is interaction with unidentified factors. In some instances, although α–haemolysin activity shows a similar response to *hld::lacZ* expression following exposure to various environmental stimuli, the response is exaggerated. Conditions which de-regulate α–haemolysin production from *agr* have already been discussed but, in addition, *tst* expression which is positively regulated by *agr*, is also almost abolished in 1 M NaCl (Chan and Foster, submitted). Expression of *tst* is also reduced, like α–haemolysin levels, in the presence of 20 mM sucrose (Chan and Foster, submitted). It is now known that many environmental stresses, including changes in growth phase, osmolarity and temperature, result in changes in bacterial DNA topology, specifically, supercoiling and that the level of supercoiling is important in the control of prokaryotic transcription (Dorman, 1994). The way in which environment influences DNA supercoiling is not yet understood but changes in intracellular ion concentrations may play a role since ions, for example K^+, can alter the topology of DNA directly (Anderson and Bauer, 1978). Another possibility is that the enzymes that effect changes in DNA supercoiling, topoisomerases, are post-translationally modified by phosphorylation catalysed by protein kinases whose activity is environmentally regulated (Dorman, 1994). Thus, it may be that the effect of the changes in the

environment studied in this work is mediated, at least partly, via changes in DNA supercoiling.

The regulation of *agr* itself in response to a changing environment is still not fully understood but since the product of the *agrA* gene comprises the regulatory element of a classical two component sensor regulator system it would seem likely that, in common with other such elements, its function is to influence transcription of target genes, although this has not yet been proven of AgrA. However, it is apparent from the phenotypic data obtained with the Tn*917* mutant, 10.1 (see section 5.2.4), that AgrA is vital for the production of those virulence determinants under the control of *agr*.

The complexity of the system has been demonstrated with the discovery of other global regulators in *S. aureus*. The best characterised of these is *sar*, which has been shown to interact with *agr* to upregulate activity of the P2 promoter of *agr* via SarA (Cheung *et al.*, 1997; Morfeldt *et al.*, 1996b). Whether SarA binds to the P3 promoter has been the subject of conflicting evidence although, at best, SarA binds to a much lesser extent to the P3 promoter (Cheung *et al.*, 1997; Heinrichs *et al.*, 1996; Morfeldt *et al.*, 1996b). However, *sar* expression is unaffected by changes in temperature, increased NaCl levels or deprivation of Ca^{2+}, Mg^{2+} or iron (Chan and Foster, submitted) so it seems unlikely that *sar* levels control changes in *agr* expression, at least under certain environmental conditions. The activity of SarA may be controlled at the post-transcriptional level. It may also be that identified or novel regulatory loci play a part in *agr* control, possibly in response to changes in DNA supercoiling. It has been shown that SarA shows a density dependent effect in the up-regulation of RNAIII with RNAIII production in an *sar* mutant being strongly diminished at late log phase and conversely being increased in the presence of *sar* on a multicopy plasmid (Cheung *et al.*, 1997). RNAII production in an *sar* mutant is much less

affected (Cheung *et al.*, 1997) indicating that P2 promoter activity can occur independently of SarA, although at a lower rate.

It is thus clear that *sar* plays a role in the regulation of *agr*, and it may be that *sar* interacts with the unphosphorylated form of AgrA which is produced constitutively, but at a very low level, from the P1 promoter of *agr*, although it has recently been demonstrated that expression from P1 is higher *in vivo* (Lowe *et al.*, 1998). A low level of expression from the P2 promoter during early growth leads to a gradual accumulation of the signalling molecule RAP and concomitant increase in the level of phosphorylated AgrA (AgrA-P). Expression from the P3 promoter is negligible at this stage, as confirmed by the growth curve of the *hld::lacZ* fusion strain, SH101 (see Fig. 3.5). This is desirable for the cell since, if P3 was up-regulated too early, energy would be wasted producing toxins at an inappropriate time, and surface proteins necessary for colonisation and immune evasion would be down-regulated.

Alternatively, it is possible that unphosphorylated AgrA binds to the *agr* P2 promoter directly or, in conjunction with SarA or another regulator, since expression from P2 is only partially reduced in an *sar* mutant. Although it has not been possible to establish that AgrA binds to P2 or P3, or indeed interacts with SarA (Morfeldt *et al.*, 1996a/b), it is almost certain that AgrA or another factor, or factors, can bind to and up-regulate P2 and/or P3 since it has been shown that *agr* can produce virulence determinants in the absence of *sar*, although final levels are reduced. It may simply be that, in the SarA mutant, the density dependent up-regulation of RNAIII is delayed and similarly toxin production. Since the production of virulence determinants is not apparent until early stationary phase ordinarily, any delay in production could mean that nutrient availability becomes limiting prior to achieving the full potential for toxin production. The observation that α–haemolysin production is delayed some 2-3 hours in an *sar* mutant and reaches only 60 % of wild type levels (P.F. Chan, pers. comm.) correlates with this hypothesis. By culturing an *sar* mutant in a chemostat continuous culture

where an inhibitory lack of nutrients can be avoided it would be possible to determine if finite levels of toxin production in such a strain could equal those reached in a wild type strain.

If AgrA interacts with SarA once phosphorylated, then it would seem likely that expression from the P2 and P3 promoters of *agr* would increase gradually and appear to follow growth. This has been demonstrated not to be the case, with expression from the P3 promoter being negligible until the rapid rise late in exponential growth. This sharp increase occurs independently of any marked increase in *sar* expression. It is likely that it is the level of AgrA-P which controls *agr* P3 promoter activity. The level of AgrA-P is increased as the cell density increases due to the accumulation of RAP and transduction of this signal by AgrC. A proposed model for the way in which all these factors might interact is given in Fig. 6.1.

It seems likely from the Tn*551* mutants isolated during this work that there are probably other regulators yet to be identified. Five Tn*551* mutants have been identified in which the transposon has most likely inserted into a different locus in each mutant and which show varying phenotypic characteristics. Although attempts to clone the chromosomal DNA flanking the Tn*551* insertion site in each mutant proved unsuccessful, this must be the next step. Other methods available to do this include inverse PCR (Silver, 1993). Here, fragments between 300 bp and 3 kb of chromosomal DNA extending from one end of Tn*551*, and including a short sequence of the end of Tn*551*, would need to be excised from a gel and circularised (by self-ligation). Primers comprising each end of the Tn*551* fragment would be designed and the PCR reaction would be carried out, allowing amplification out from each end of Tn*551* into the circularised chromosomal DNA. The linear PCR products could then be DNA sequenced.

Other techniques which are available to identify any novel regulatory loci also

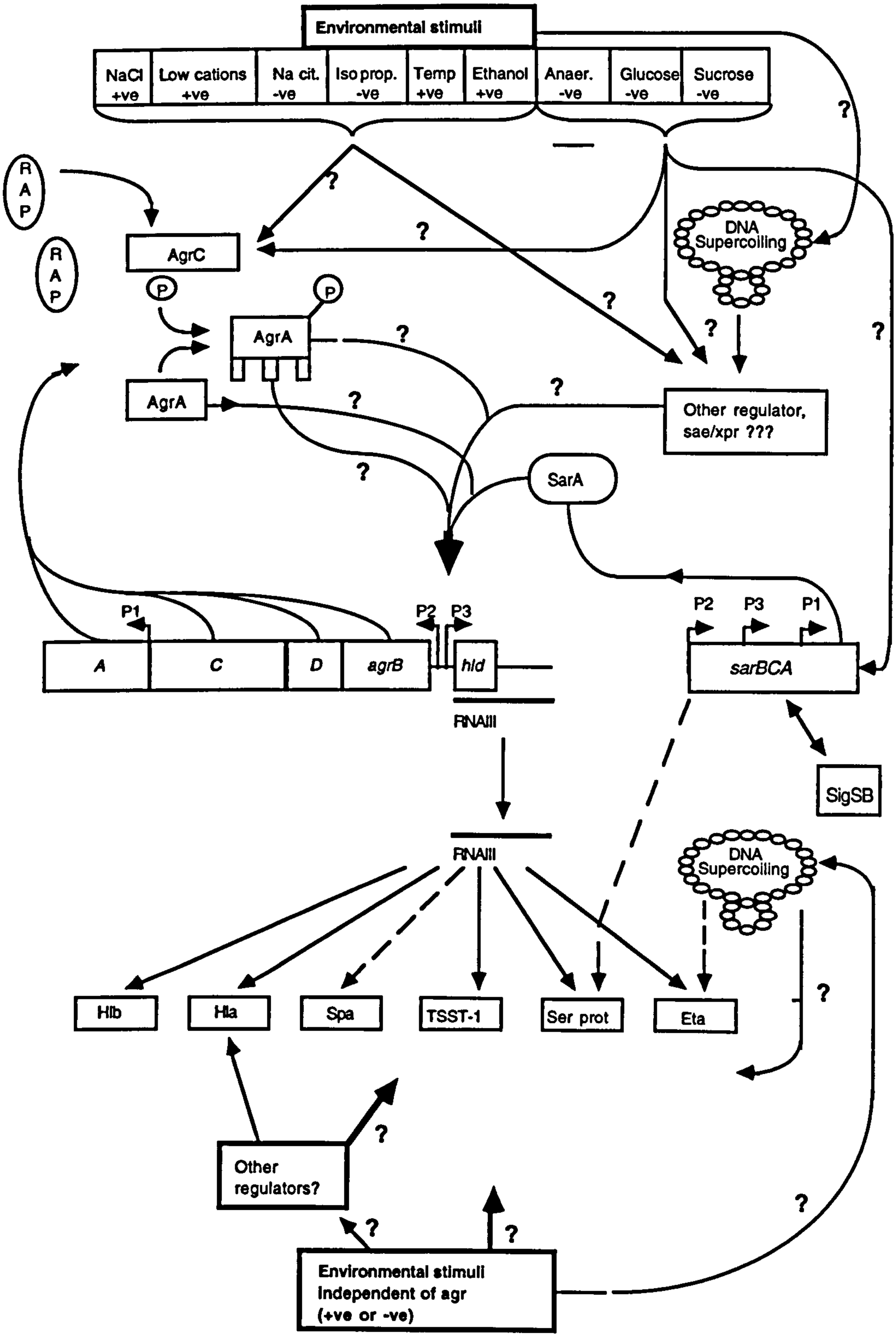


FIGURE 6.1

Proposed model for the control of production of virulence determinants by *agr* and its interaction with other known and putative global regulators. (Dashed lines represent negative regulation). (Not to scale).

include a PCR-based strategy. Such a method was employed by Bayles (1993) who used degenerate sensor-gene specific primers to amplify DNA fragments from *S. aureus*. Two genes were identified, *lytS* and *lytR*, whose deduced amino acid sequences were found to be similar to those of other two component sensor regulator systems (Brunskill and Bayles, 1996). Characterisation of the *lytS* insertional mutant suggested that the gene products control the rate of autolysis in *S. aureus*.

A completely different approach to identifying novel putative regulators is to employ the phenomenon known as "*in vivo* cross-talk". Using this method it is possible to identify novel sensors which can interact with known regulators in strains null in the usual sensor gene for a particular characteristic. By use of this technique two novel *E. coli* genes, *evgS* and *evgA*, have been cloned and sequenced forming both a sensor and regulator (Utsumi *et al.*, 1994). These two genes were found to be structurally and functionally homologous to the two-component sensor regulator genes *bvgA* and *bvgS* which co-ordinately regulate virulence gene expression in *B. pertussis*.

Recently, fifty mutants of *S. aureus* showing reduced virulence were isolated by a method known as signature tagged mutagenesis (Mei *et al.*, 1997). Here, a variety of plasmids are created, incorporating the Tn*917* transposable element, each with a different DNA tag within this element. These tags comprise a variable central sequence flanked by invariant sequences which allow mutants to be differentiated from each other. Following infection of an animal with these various transposon mutants, and subsequent recovery, the tags in the recovered pool and the inoculum pool are separately amplified by PCR using primers based on the invariant flanking sequences. The PCR products are then used to probe the different tags in the inoculum pool. By identifying mutants that hybridise with tags from the inoculum pool but not the recovered pool it is possible to isolate mutants with reduced virulence. Thus genes which are necessary for virulence are tagged since the numbers of bacteria carrying mutations in these genes will be decreased in the infective

population. As has been described earlier, Tn*917* then facilitates easy cloning of these genes in *E. coli.*

A powerful technique which has been used in recent years to identify bacterial genes which are expressed during infection is known as *in vivo* expression technology (IVET) (Mahan *et al.*, 1993). This method has been used to search for genes which allow *S. typhimurium* to cause a typhoid-like disease in mice (Mahan *et al.*, 1993). Purine auxotrophs of *S. typhimurium* cannot infect mice so that, by introducing a promoterless purine biosynthetic gene, *purA* randomly into the chromosome of a *purA*$^-$ strain and infecting an animal, only those bacteria in which the *purA* gene has inserted downstream of a promoter active during infection will be able to survive. Thus *S. typhimurium* chromosomal DNA is partially digested and resulting fragments cloned upstream of a promoterless *purA* gene fused to a promoterless *lacZY* gene to form a plasmid. The plasmids are then introduced into a *purA*$^-$ strain of *S. typhimurium.* Similar to the suicide vectors used in *S. aureus*, described earlier, these plasmids cannot replicate in *S. typhimurium* and so antibiotic resistance (*bla*) will only be conferred to those clones in which the plasmid has integrated into the chromosome. Therefore the bacteria which survive in the mouse can then be harvested and assayed *in vitro* for expression of *lacZ.* In particular, by identifying clones in which *lacZ* is not expressed during *in vitro* growth, it is possible to identify genes which are specifically active during infection.

Using this method in *S. aureus*, six previously identified genes have been found to be expressed *in vivo*, including *agrA* and the lipase gene, *geh* (Lowe *et al.*, 1998). Eleven novel genes showing homology to genes in other organisms have also been found (Lowe *et al.*, 1998).

There is a long way to go before the way in which *agr* interacts with other factors and responds to physiological and environmental stimuli to control expression of

virulence determinants is fully understood. The part the different regulators identified to date play in this complex pattern of gene expression and protein production is still far from clear, and it seems likely that there are yet more novel regulatory components to be discovered. Much work remains in the quest to fully understand how pathogenic bacteria interact with their host to cause infection and disease. Further knowledge in this area will provide vital information which can be used in the struggle to control both *S. aureus* other pathogens.

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Appendix A

A1 MEDIA

A1.1 Growth media

All growth media were made in 1 l volumes in distilled H_2O, unless other stated, and divided into 100 ml aliquots in Ehrlenmeyer flasks (250 ml) prior to autoclaving. Unless stated otherwise in the following text, Agar No. 1 (Oxoid) (1 % w/v) was used to make agar plates.

A1.1.1 Chemically defined media (CDM)

A1.1.1.1 CDM (Hussein *et al.*, 1991)

Component	g/l
Glucose	10
Na_2HPO_4	7
KH_2PO_4	3
$MgSO_4.7H_2O$	0.5
L-aspartic acid	0.15
L-glutamic acid	0.15
L-isoleucine	0.15
L-leucine	0.15
L-proline	0.15
L-threonine	0.15
L-valine	0.15
L-alanine	0.1
L-arginine	0.1
Glycine	0.1
L-histidine	0.1
L-lysine	0.1
L-methionine	0.1
L-phenylalanine	0.1
L-serine	0.1
L-tryptophan	0.1
L-tyrosine	0.1
L-cysteine	0.05
Adenine sulphate	0.02
Guanine HCl	0.02
$CaCl_2.6H_2O$	0.01
$FeNH_4(SO_4)_2.12H_2O$	0.006
$MnSO_4$	0.005
Pyridoxal	0.004
Pyridoxamine diHCl	0.004
D-pantothenic acid	0.002
Riboflavin	0.002
Nicotinic acid	0.002
Thiamin HCl	0.002
Biotin	1×10^{-5}

A1.1.1.2 CDM (Holland *et al.*, 1994)

Component	g/l
L-cystine	0.22
L-glutamic acid	2.22
L-aspartic acid	2.22
L-proline	2.22
L-arginine	0.33
Glycine	2.22
L-lysine HCl	0.56
L-histidine	0.44
L-threonine	2.22
L-serine	2.22
L-alanine	2.22
L-valine	0.44
L-isoleucine	0.56
L-leucine	0.56
L-tyrosine	0.17
L-phenylalanine	0.19
L-tryptophan	0.06
L-methionine	0.17
NH_4Cl	0.51
Nicotinic acid	0.012
Thiamine HCl	6×10^{-4}
MOPS	8.37
Tricine	0.72
$K_2HPO_4.3H_2O$	3.01
K_2SO_4	4.81×10^{-2}
$MgCl_2.6H_2O$	0.27
$FeSO_4.7H_2O$	2.78×10^{-3}
$MnCl_2.4H_2O$	1.58×10^{-5}
NaCl	2.92
H_3BO_3	2.47×10^{-5}
$CaCl_2.2H_2O$	7.35×10^{-5}
$ZnSO_4.7H_2O$	2.88×10^{-6}
$CuSO_4.5H_2O$	2.50×10^{-6}
$(NH_4)_6(Mo)_7O_{24}.4H_2O$	3.71×10^{-6}
$CoCl_2.6H_2O$	7.14×10^{-6}

A1.1.2 Tryptic soy broth (TSB) (Difco)

TSB	30 g/l

A1.1.3 Brain heart infusion broth (BHIB) (Oxoid)

BHI	37 g/l

A1.1.4 Luria-Bertani Broth (LB)

Tryptone (Difco Bacto)	10 g/l
Yeast extract (Difco Bacto)	5 g/l
NaCl	5 g/l

The pH was adjusted to 7.2 using NaOH. Agar (Difco Bacto) (1.5 % w/v) was added for LB agar.

A1.2 Media for transformation

A1.2.1 SOB (Sambrook *et al.*, 1989)

Tryptone (Difco Bacto)	2 % (w/v)
Yeast extract (Difco Bacto)	0.5 % (w/v)
NaCl	10 mM
KCl	2.5 mM

Once autoclaved and cooled, sterile supplements of $MgCl_2$ and $MgSO_4$ were added to 10 mM each (final concentration).

A1.2.2 SOC (Sambrook *et al.*, 1989)

This was prepared by the addition of sterile glucose (10 mM final concentration) to SOB.

A1.2.3 NZY broth (Stratagene)

Casamino acids (Difco Bacto)	10 g
Yeast extract (Difco Bacto)	5 g
NaCl	5 g
	in 1 l

Following autoclaving, the following sterile supplements were added prior to use:

$MgCl_2$ (1 M)	12.5 ml
$MgSO_4$ (1 M)	12.5 ml
Glucose (20 % w/v)	10.0 ml

A1.2.4 Media for electroporation of *S. aureus*

A1.2.4.1 B2 broth

Casamino acids (Difco Bacto)	1 % (w/v)
Yeast extract Difco Bacto)	2.5 % (w/v)
K_2HPO_4	0.1 % (w/v)
Glucose	0.5 % (w/v)
NaCl	2.5 % (w/v)

A1.2.5 Media for protoplasting of *S. aureus*

A1.2.5.1 DM3 agar

Na succinate	67.5 g
Agar (Difco Bacto)	4 g

The pH was adjusted to 7.2 in a total volume of 350 ml of distilled H_2O. The medium was autoclaved, cooled to 65 °C, and the following sterile supplements were added:

Casamino acids (5 % w/v)	50 ml
Glucose (20 % w/v)	12.5 ml

Yeast extract (10 % w/v)	25 ml
KH_2PO_4 (1 M) pH 7.5	50 ml
$MgCl_2$ (1M)	10 ml
Bovine serum albumin (5 % w/v)	5 ml
Chloramphenicol	5 µg/ml

A1.2.5.2 Penassay broth

Penassay broth (Difco)	125 ml
Distilled water	875 ml

A1.3 Media for phage transduction of *S. aureus*

A1.3.1 LK broth

Tryptone (Difco Bacto)	1 % (w/v)
Yeast extract (Difco Bacto)	0.5 % (w/v)
KCl	0.7 % (w/v)

Agar (Difco Bacto) (1.5 % w/v) and Na citrate (0.05 % w/v) were added to make LK agar plates.

Agar (Difco Bacto) (0.7 %) was added to LK broth to make soft agar overlay.

A1.4 Media for virulence determinant assay agar plates

A1.4.1 β–Haemolysin test agar

Blood agar base No.2 (Difco) 39 g/l

Following autoclaving and cooling to 50 °C, defibrinated sheep blood 5 % (v/v) (TCS Biologicals) was added.

A1.4.2 α–haemolysin test agar

Blood agar base No.2 (Difco) 39 g/l
(for top and bottom agar)

Following autoclaving bottom agar was poured. Top agar was cooled to 50 °C, defibrinated rabbit blood (TCS Biologicals) (10 % v/v) was added and used to overlay bottom agar (5 ml per plate).

A1.4.3 DNase test agar

DNA agar (Difco) Bottom agar: 42 g/l

Following incubation for 48 hours at 37 °C, plates were overlaid with 0.5X DNA agar (5 ml per plate) containing methyl green (to a final concentration of 0.5 mg/ml obtained from a stock solution comprising 50 mg/ml in distilled H_2O).

A1.4.4 Lipase test agar

TPEY agar base (Difco) 60 g/l

Once autoclaved, plates were poured and, when set, overlaid (5 ml) with TPEY agar containing Egg Yolk Enrichment [Beta Lab] (10 %).

A1.4.5 Protease test agar

BHI	37 g
Agar No.1 [Oxoid]	10 g
	in 500 ml

Agar was autoclaved and, prior to pouring, molten 2X BHI agar was mixed with autoclaved 3 % (w/v) dried skimmed milk (15 g dried skimmed milk [Boots plc] in 500 ml distilled H_2O) .

A1.4.6 Hyaluronate lyase test agar

BHI	37 g
Bovine serum albumin	1 % (w/v)
Na hyaluronate	0.04 % (w/v)
Agar No. 1 [Oxoid]	10 g

A2 BUFFERS AND STOCK SOLUTIONS

All buffers were prepared in distilled water and stored at room temperature. Solutions for use in microbiological work and *in vitro* DNA manipulations were sterilised by autoclaving.

A2.1 Miscellaneous solutions

A2.1.1 TE (10 X)

Tris-HCl (pH 7.5)	100 mM
Na_2EDTA (pH 8)	10 mM

This solution was diluted 1:10 with distilled water to give TE, and autoclaved.

A2.1.2 Phosphate buffered saline (PBS)

NaCl	8 g
KCl	0.2 g
Na_2HPO_4	1.44 g
KH_2PO_4	0.24 g
Distilled H_2O	1 l

The pH was adjusted to 7.5 using 1 M HCl and the buffer was autoclaved in 100 ml aliquots.

A2.1.3 Phage buffer

$MgSO_4$	1 mM
$CaCl_2$	4 mM
Tris-HCl (pH 7.8)	50 mM
NaCl	5.9 g/l
Gelatin	1 g/l

A2.2 Solutions for agarose gel electrophoresis

Generally, unless stated otherwise in the text, agarose gels were 0.8 % (w/v) agarose (Gibco BRL) in 0.5X TBE. DNA which was to be gel purified was separated on an agarose gel made with 1X TAE.

A2.2.1 TAE (50 X)

Tris	242 g
Glacial acetic acid	57.1 ml
Na_2EDTA (0.5 M pH 8.0)	100 ml
Distilled water	to 1 l

Before use the buffer was diluted 1:50.

A2.2.2 TBE (10 X)

Tris	108 g
Boric acid	55 g
Na_2EDTA (0.5 M pH 8.0)	40 ml
Distilled water	to 1 l

TBE was diluted 1:10 with distilled water before use.

A2.2.3 DNA sample buffer (10 X)

Bromophenol blue	25 mg
Ficoll (Mol. Wt. 400,000)	1.5 g
Distilled water	to 10 ml

A2.3 Solutions for plasmid/chromosomal DNA preparations

A2.3.1 Plasmid preparation Solution I

Solution 1 was prepared in batches of 100 ml and autoclaved.

Glucose	50 mM
Tris-HCl (pH 8.0)	25 mM
EDTA (pH 8.0)	10 mM

A2.3.2 Plasmid preparation Solution II

Solution II was prepared fresh on the day of use.

NaOH	0.2 M
Sodium dodecylsulphate	1 % (w/v)

A2.3.3 Plasmid preparation Solution III

Potassium acetate (5M)	60 ml
Glacial acetic acid	11.5 ml
Distilled water	to 100 ml

A2.3.4 Qiagen buffer B1

EDTA (pH 8.0, 0.5M))	50 mM
Tris-HCl (p H8.0)	50 mM
Tween-20	0.5 % (v/v)

A2.3.5 Qiagen buffer B2

Guanidine HCl	3 M
Tween-20	20 % (v/v)

The pH was adjusted to 5.5 prior to use.

A2.3.6 Qiagen buffer QBT

NaCl	750 mM
MOPS	50 mM
Ethanol	15 % (v/v)
Triton X-100	0.15 % (w/v)

The pH was adjusted to 7.0 prior to use.

A2.3.7 Qiagen buffer QC

NaCl	1 M
MOPS	50 mM
Ethanol	15 % (v/v)

The pH was adjusted to 7.0 prior to use.

A2.3.8 Qiagen buffer QF

NaCl	1.25 M
Tris-HCl (pH 8.5)	50 mM
Ethanol	15 % (w/v)

The pH was adjusted to 8.5 prior to use.

A2.4 Solutions for pulsed field gel eletrophoresis (PFGE)

A2.4.1 Cell suspension buffer

Tris-HCl (pH 7.5)	10 mM
NaCl	20 mM
EDTA	50 mM

A2.4.2 Lysis buffer

Tris-HCl (pH 7.5)	10 mM
NaCl	50 mM
Sodium deoxycholate	0.2 % (w/v)
Sodium lauryl sarcosine	0.5 % (w/v)

A2.4.3 Proteinase K reaction buffer

EDTA (pH 8.0)	100 mM
Sodium deoxycholate	0.2 % (w/v)
Sodium lauryl sarcosine	1.0 % (w/v)
Pronase	1 mg/ml

A2.4.4 Wash buffer

Tris-HCl (pH 8.0)	20 mM
EDTA (pH 8.0)	50 mM

A2.5 Solutions for Southern blots

A2.5.1 SSC (20X)

NaCl	175.3 g
Trisodium citrate.$2H_2O$	88.2 g
Distilled water	to 1 l

The pH of the buffer was adjusted to 7.0 with 1 M NaOH.

A2.5.2 Denaturing solution

NaOH	0.5 M
NaCl	1.5 M

A2.5.3 Neutralising solution

Tris-HCl (pH 7.0)	1 M
NaCl	2 M

A2.5.4 Hybridisation solution

SSC	5X
Blocking reagent (Boehringer Mannheim)	0.5 % (w/v)
N-lauroylsarcosine, Na-salt	0.1 % (w/v)
SDS	0.2% (w/v)

Blocking reagent was dissolved in hybridisation solution by heating at 50-70 °C for one hour.

A2.5.5 Southern buffer 1

Tris-HCl (pH 7.5)	100 mM
NaCl	150 mM

A2.5.6 Southern buffer 2

Southern buffer 1	
Blocking reagent	0.5 % (w/v) in Southern buffer 1

A2.5.7 Southern buffer 3

Tris-HCl (pH 9.5)	100 mM
NaCl	100 mM
$MgCl_2$	50 mM

A2.5.8 Southern buffer 4

Tris-HCl (pH 8.0)	10 mM
EDTA (pH 8.0)	1 mM

A2.5.9 Southern colour solution

NBT (50 µg/ml)	66 µl
BCIP (50 µg/ml)	33 µl
	in 15 ml Southern buffer 3

A2.6 Solutions for assays

A.2.6.1 β–galactosidase assay buffers

A2.6.1.1 AB buffer:

NaCl	100 mM
K_2HPO_4	60 mM
KH_2PO_4	40 mM
Triton X-100	0.1 % (v/v)

A2.6.1.2 'Z' buffer:

$Na_2HPO_4.7H_2O$	16.1 g
$NaH_2PO_4.7H_2O$	5.5 g
KCl	0.75 g
$MgSO_4.7H_2O$	0.246 g
β–Mercaptoethanol	2.7 ml
	in 1 l distilled H_2O

The pH was adjusted to 7.0 and the buffer was stored at 4 °C.

A2.6.2 Haemolysin assay buffer (HA)

NaCl (10 % w/v)	8.5 ml
$CaCl_2$ (1 M)	2 ml
	in 100 ml sterile distilled H_2O

A2.7 Solutions for transformation

A2.7.1 Solutions for transformation by protoplasting of *S. aureus*

A2.7.1.1 SMM

Sucrose	17.1 g
Na maleate	0.23 g
$MgCl_2$	0.4 g
Distilled water	100 ml

A2.7.1.2 SMMP

SMM	100 ml
BHI	7.4 g

A2.7.2 Solutions for transformation of *E. coli*

A.2.7.2.1 Frozen storage buffer (FSB)

KCl	100 mM
$MnCl_2.4H_2O$	45 mM
$CaCl_2.2H_2O$	10 mM
$HACoCl_3$	3 mM
K acetate (1M, pH 7.5)	10 mM

The K acetate was filter sterilised and stored at -20 °C. A 10 mM K acetate/glycerol (10 % v/v) solution was prepared and salts were added as solids. The pH was adjusted to 6.4 using HCl and the solution was sterilised by filtration and stored at 4 °C.