SCHOOL OF BIOMEDICAL SCIENCES

Molecular Analysis of Genes Encoding Resistance to Cationic Biocides in Staphylococci

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1 LITERATURE REVIEW

1.1 Introduction

Staphylococcus aureus is a nosocomial and opportunistic pathogen once easily treated with antibiotics. *Staphylococcus aureus* harbours a battery of genetic elements including plasmids, bacteriophages, transposons and insertion sequences able to promote transfer and re-assortment of genetic information among staphylococci. It is of no surprise then that *S. aureus* has rapidly acquired antibiotic resistance genes and evolved into multiply resistant strains. Widespread use of antibiotics has promoted this acquisition and dissemination of resistance genes amongst these organisms. Multiply resistant *S. aureus* no longer respond to the usual anti-staphylococcal therapy and currently there are very few treatment options available (Noble *et al.* 1992; Ward *et al.* 2001). There has also been a dramatic increase in the prevalence of hospital-acquired infections caused by strains of *S. aureus* that are not only resistant to numerous antibiotics but also to antiseptics and disinfectants (Gillespie *et al.* 1989; Littlejohn *et al.* 1990; Lyon *et al.* 1982, 1983, 1984; Noguchi *et al.* 1999; Shimizu *et al.* 2002). It is not yet clear whether the acquisition of resistance to antiseptics and disinfectants will increase the threat to patient care or compromise infection control practices.

1.2 Emergence of Antibiotic Resistant *S. aureus*

Before the golden era of antibiotic medicine began some 60 years ago, severe staphylococcal infections were often fatal. With the introduction of benzyl penicillin (Pc) in the early 1940s invasive *S. aureus* infections were treated easily and effectively (Hancock and Knowles 1998; Shanson 1981). Pc resistant strains were rare. However by 1946 it was estimated that 60% of *S. aureus* isolates in United Kingdom (UK) hospitals produced β-lactamase and, as a result, were resistant to benzyl Pc (Lyon and Skurray 1987; Shanson 1981). This prompted the introduction of a succession of antibiotics such as streptomycin (Sm), tetracycline (Tc), chloramphenicol (Cm) and erythromycin (Em) for the treatment of staphylococcal infections but each was countered quickly by the emergence of resistant strains (Hall and Partridge 2001; Lyon and Skurray 1987; Skurray and Firth 1997). Even more

worrying was the fact that many strains that had acquired resistance to these new antibiotics were also retaining their resistance to other antibiotics (Ito and Hiramatsu 1998; Lyon *et al.* 1983; Shanson 1981; Skurray and Firth 1997). The result was the emergence of organisms with a broad spectrum of resistance with a distinct ability to survive and spread in the hospital environment.

1.3 Emergence and Evolution of MRSA

Staphylococcus aureus became a worldwide problem in the 1950s (Cookson and Phillips 1988; Shanson 1981). During the 1960s the introduction of the semi-synthetic β-lactam antibiotics, methicillin (Mc) and oxacillin (Ox), brought about a marked improvement in antimicrobial treatment of *S. aureus* infections (Lyon and Skurray 1987; Pearman 1996; Shanson 1981). However, by the late 1960s to early 1970s strains resistant to Mc and Ox were isolated with an increasing frequency in several countries including Australia (Rountree *et al.* 1968, 1973), Belgium, France, Poland, the UK and the United States of America (USA) (Lyon and Skurray 1987; Mallorqui-Fernandez *et al.* 2004). In general these outbreaks of Mc resistant *S. aureus* (MRSA) were contained by standard cross-infection control procedures (Faoagali *et al.* 1992).

During the late 1970s to early 1980s MRSA was spreading between hospitals and causing a significant number of nosocomial infections (Cookson and Phillips 1990; Gedney and Lacey 1982; Pearman 1996; Skurray *et al.* 1988). Clinicians were so concerned by the apparent ability of these strains to spread within and between hospitals they were termed epidemic MRSA (EMRSA). EMRSAs have subsequently spread to various parts of the world and caused similar outbreaks (Bradley *et al.* 1985; Gillespie *et al.* 1989; Marples and Reith 1992; Pearman 1996; Riley and Rouse 1995; Townsend *et al.* 1984b). Many hospitals introduced patient isolation and barrier-nursing procedures to limit spread of EMRSA between hospitals and patients (Ayliffe *et al.* 1986; Bell 1982; Davies *et al.* 1987; Marples and Reith 1992). A surveillance and control policy was introduced at Royal Perth Hospital (RPH), Western Australia (WA) after an outbreak of EMRSA in 1982 (Riley and Rouse 1995). All incoming patients who had been admitted to a hospital outside of WA in the previous year were screened, and if found to harbor MRSA they were treated in

isolation. This stringent policy is still in place today and has prevented the establishment of EMRSA in this WA hospital (Dailey *et al.* 2005; Pearman 1996; Riley and Rouse 1995).

1.3.1 Typing schemes for MRSA

Episodes of international and intercontinental spread of EMRSA have become increasingly common. Strains of MRSA that have the potential to cause epidemics and affect more than one hospital need to be quickly identified and defined by typing. Typing schemes for MRSA were developed to identify strains that cause epidemics or possess enhanced virulence properties and to monitor their spread within the community and between patients (Abbott and Youngs 1992; Marples and Reith 1992).

1.3.1.1 Bacteriophage typing of MRSA

Bacteriophage typing was previously the standard typing scheme for *S. aureus*. Each isolate was tested for their susceptibility to a bank of 23 staphylococcal bacteriophages of the Basic International Set of Typing Phages and supplementary phages needed for typing MRSA (Richardson *et al.* 1988; Vickery *et al.* 1986). Strains with a similar susceptibility were classified into the same phage group. Although findings by Vickery (1986) indicated that the lysogenicity of MRSA strains isolated after 1976 were distinct from earlier isolates, unfortunately phage typing does not have sufficient discriminatory power for MRSAs appearing post 1980s, as many of the strains displayed similar or identical phage-typing patterns or were non-typable. Alternative typing schemes were thus required to determine how the epidemic strains of MRSA emerging in the 1980s were different from previous strains.

1.3.1.2 Phenotypic and genotypic typing of MRSA

Gedney and Lacey (1982) used a phenotypic scheme to compare Australian EMRSA isolates with MRSA isolates from Europe prior to 1973. The phenotypic properties included antibiotic sensitivity, induction of lysogenic bacteriophage by mitomycin-C,

survival on glass, pigmentation, lipolytic activity and transfer of antibiotic resistance. Gedney and Lacey (1982) concluded that Australian EMRSA were closely related to the earlier European MRSA strains and no property of EMRSA was found to explain the perception that they possessed enhanced ability to spread and survive within hospitals.

An alternative phenotypic and genotypic scheme examined the range and location of resistant determinants and plasmid content of different strains of MRSA. Those isolated in Australian hospitals between 1966 and 1974 were found to be similar to strains isolated in Europe before 1973 (Lacey and Grinsted 1973). These have been referred to as 'classic' MRSA (Lacey and Grinsted 1973). Characteristically the 'classic' strains bore chromosomal resistance to Mc, Em and Sm and carried two resistance plasmids (Coia *et al.* 1988; Townsend *et al.* 1984a). One plasmid was approximately 20-30 kilobase pairs (kb) and encoded production of β-lactamase and resistance to the heavy metals cadmium (Cd), mercury (Hg), phenylmercuric acetate, arsenate and arsenite (Lacey and Grinsted 1973; Townsend *et al.* 1984a, 1985d). The other plasmid was smaller, approximately 4.4 kb in size and encoded Tc resistance (Tc^R) (Townsend *et al.* 1985d). These 'classic' MRSA caused several outbreaks in hospitals worldwide during this time but normal infection control techniques were able to contain these organisms (Faoagali *et al.* 1992).

EMRSAs isolated in the early 1980s differed in their resistance phenotype from the earlier 'classic' strain in that they were resistant to trimethoprim (Tp), Cm, most of the aminoglycoside's and some were also resistant to fusidic acid (Fa) and rifampicin (Rf) (Faoagali *et al.* 1992; Lyon *et al.* 1982). Surprisingly they also exhibited a reduced susceptibility to quaternary ammonium compounds (QAC), ethidium bromide (Eb) and other cationic biocides (Emslie *et al.* 1985b, 1986). EMRSA also differed significantly from the "classic" MRSA in their genotype, these differences are summarised in Table 1.1. Some of the most striking differences were the location of drug resistance determinants and differences in plasmid content (Lyon *et al.* 1982, 1983, 1984; McDonald 1982; Townsend *et al.* 1983b, 1983c, 1985c). Australian EMRSA were found to have chromosomal determinants for penicillinase production and resistance to Mc, Cd, Hg, Tc, Em, Tp, sulphonamides (Su) and low-level resistance to Sm (Gillespie *et al.* 1986; Lyon *et al.* 1982; Townsend *et al.* 1985b,

1985d). The increased plasmid content included the presence of a 2.1 kb cryptic plasmid, a 4.2 kb plasmid encoding Cm^R and a larger plasmid, 27-43 kb, conferring resistance to QAC, gentamicin (Gm), kanamycin (Km), tobramycin (Tm), Tp and in some cases production of a β-lactamase (Brumfitt *et al.* 1985; Coleman *et al.* 1985; Emslie *et al.* 1986; Lyon *et al.* 1982, 1984; Mycock 1985; Skurray *et al.* 1988; Tennent *et al.* 1985; Townsend *et al.* 1983a, 1984d, 1985b, 1985d).

Table 1.1 – Phenotypic and genotypic comparison of 'classic' MRSA and EMRSA

MRSA classification	Chromosomal resistance determinants	Plasmid-borne resistance determinants
'Classic' MRSA	Mc Em Sm	20-30 kb plasmid: β-lactamase production Heavy metal resistance 4.4 kb plasmid: Tc
'Epidemic' MRSA	β-lactamase production Mc Heavy metal resistance Tc Em Tp Su Sm (low-levels)	27-43 kb plasmid Gm Tp QAC β-lactamase production 4.2 kb plasmid Cm 2.1 kb plasmid Cryptic

Many studies have suggested that phenotypic systems have limited typing ability, lacking stability and discrimination (Struelens *et al.* 1992). Genotypic typing also has significant limitations due to problems related to plasmid instability or to poor discrimination among the few conserved plasmids commonly found in MRSA (Cookson *et al.* 1986; Ichiyama *et al.* 1991; Mulligan and Arbeit 1991). The recent developments of molecular typing schemes for MRSA were considered more stable.

1.3.1.3 Molecular typing of MRSA

MRSA strains tend to be similar in many phenotypic and genotypic traits and appear to belong to an evolutionarily restricted subpopulation within the species *S. aureus* (Cookson *et al.* 1986). As a result, discrimination between MRSA strains by classical typing methods has often been difficult. One molecular typing technique is pulsed field gel electrophoresis (PFGE) of restriction fragments, a sensitive method that can detect subtle genetic variations among phylogenetically and epidemiologically related isolates of various strains of MRSA (Cookson and Phillips 1988; O'Brien *et al.* 2006; Struelens *et al.* 1992). However this test alone does not distinguish MRSA strains from one another. A combination of techniques are required to identify EMRSA and separate them from sporadic strains of MRSA (Goh *et al.* 1992; Rossney *et al.* 1994a, 1994b; Tenover *et al.* 1994). Two techniques have arisen as the definitive typing methods for MRSA, multilocus sequence typing (MLST) and SCC*mec* typing.

MLST is a highly discriminatory method of characterising MRSA based upon the combination of seven housekeeping genes sequence types (Enright *et al.* 2000, 2002). Distinct alleles are assigned to the different sequences of the seven housekeeping gene fragments, defining an isolate by its allelic profile or sequence type (ST). Isolates that differ at no more than two alleles are considered to belong to the same clonal complex (cc) and can be members of the same clone.

The second test involves the typing of the Mc^R gene, *mecA*, which is contained within a mobile element known as the *mec* region or staphylococcal cassette chromosome *mec* (SCC*mec*) (Coombs *et al.* 2005). SCC*mec* regions vary between MRSA strains due to insertion and deletion events and the acquisition of various genetic elements (Oliveira *et al.* 2000). Six SCC*mec* types have been identified, differing in their *mec* regulatory region, cassette chromosome recombinases and resistance determinants (Ito *et al.* 2001, 2004).

MLST and SCC*mec* typing can be used to predict ancestral genotypes and patterns of evolutionary descent within groups of related genotypes. The origins of MRSA were poorly understood, initially it was proposed that all MRSAs were descended from a single ancestral *S. aureus* strain (Kreiswirth *et al.* 1993), but more recent studies

using MLST and SCC*mec* typing (Fitzgerald *et al.* 2001; Musser and Kapur 1992) show that MRSAs are very divergent, implying that resistance genes have been transferred between *S. aureus* lineages.

1.3.1.4 Virulence factors of MRSA

Staphylococcus aureus is a well-adapted opportunistic pathogen that is able to move from carriage sites into wounds, the respiratory tract and the urinary tract to cause infection (Cookson and Phillips 1990; Kuroda *et al.* 2001; Wright 2003). *Staphylococcus aureus* is able to maintain its colonist status by producing a variety of proteins that interact with host-cell components. These virulence factors have functions that facilitate attachment, colonisation, cell-cell interactions, immune evasion and tissue damage (Gill *et al.* 2005; Holden *et al.* 2004). Many have suggested that MRSAs are more virulent or invasive than Mc-sensitive *S. aureus* (MSSA), accounting for their increased rates of persistent isolation in the hospital environment. Significant variation in the number of virulence-associated genes carried by EMRSA/MRSA and MSSA strains has been identified through the sequencing of entire genomes (Gill *et al.* 2005; Holden *et al.* 2004; Kuroda *et al.* 2001; Saunders *et al.* 2004). And the presence of some but not all of the virulence-associated genes is thought to be related to the tendency of certain strains to make the transition from benign coloniser to opportunistic pathogen (Peacock *et al.* 2002). However, results based on clinical data indicate that MRSA/EMRSA are no more or less virulent than more susceptible strains (French *et al.* 1990; Peacock *et al.* 1981) and no property has been found to explain their unique ability to spread (Duckworth and Jordens 1990).

1.4 Retention of Resistance Determinants by MRSA

The ability of MRSA and EMRSA to survive and spread within the hospital environment may simply lie in their extensive complement of resistance determinants. Resistant strains survive in a given niche at the expense of sensitive strains of the same species. Chromosomal resistance determinants are generally stably maintained within a cell irrespective of the presence of a selective pressure. Plasmids on the other hand provide an adaptive advantage to the host cell and can be

lost or "cured" from the cell without loss of viability. The host cell may lose the plasmid in the absence of a selective pressure. The Australian EMRSA, also seen in the UK and Canada (Cookson and Phillips 1988; Cookson *et al.* 2003; Richardson *et al.* 1994), carry a range of chromosomal determinants encoding resistance to Mc, Cd, Hg, Em, Sm, Su, Tp and Tc. The cell maintains these chromosomal resistance determinants even in the absence of a selective pressure. The maintenance of EMRSAs plasmids would however indicate the presence of a selective pressure.

1.4.1 Plasmids in EMRSA

EMRSA usually harbour three plasmid families. The smallest of the plasmids is phenotypically cryptic, possessing no known function other than its own maintenance and replication (Skurray *et al.* 1988). There is also a small plasmid 4.2 kb in size that encodes Cm^R. The largest of the plasmids range in size from 27-43 kb and encode resistance to Gm, Km, Tm, Tp, cationic biocides and in some cases produce a β-lactamase (Lyon *et al.* 1984; Skurray *et al.* 1988; Townsend *et al.* 1983c, 1984a, 1984c). A large plasmid pSK1 (28.4 kb), Figure 1.1, was isolated in approximately 50% of all isolates of EMRSA from Australian hospitals at the time, and was therefore considered the reference for this particular family of staphylococcal plasmids (Lyon *et al.* 1983, 1986, 1987; Skurray *et al.* 1988). The plasmids analogous to the pSK1 family include pSK4, pSK23, pSJ24, pWBG53 and pWBG50 (Lyon and Skurray 1987; Townsend *et al.* 1983c, 1984c). Plasmids from the SK1 family were established as belonging to the incompatibility group I (*Inc*I) (Iordanescu *et al.* 1978; Lyon and Skurray 1987; Townsend *et al.* 1985b). The SK1 plasmid family was unstable and needed to be under constant selective pressure for it to be maintained (Cookson *et al.* 1991; Townsend *et al.* 1984a, 1984c). Sequence analysis found members of this family carried several transposons. Transposon Tn*4001* (Skurray *et al.* 1988) and Tn*3851* (Townsend *et al.* 1984d) encode for the bifunctional aminoglycoside resistance gene *aacA-aphD* responsible for linked resistance to Gm, Tm and Km (Skurray *et al.* 1988). Other transposons also belonging to this plasmid family include Tn*4002*, which encodes production of β-lactamase and Tn*4003*, the cointegrated remnant of a dfr*A*-carrying plasmid, encodes Tp^R (Rouch *et al.* 1989; Skurray *et al.* 1988). These transposons account for

the antibiotic resistance profile of the pSK1 family, except resistance to cationic biocides.

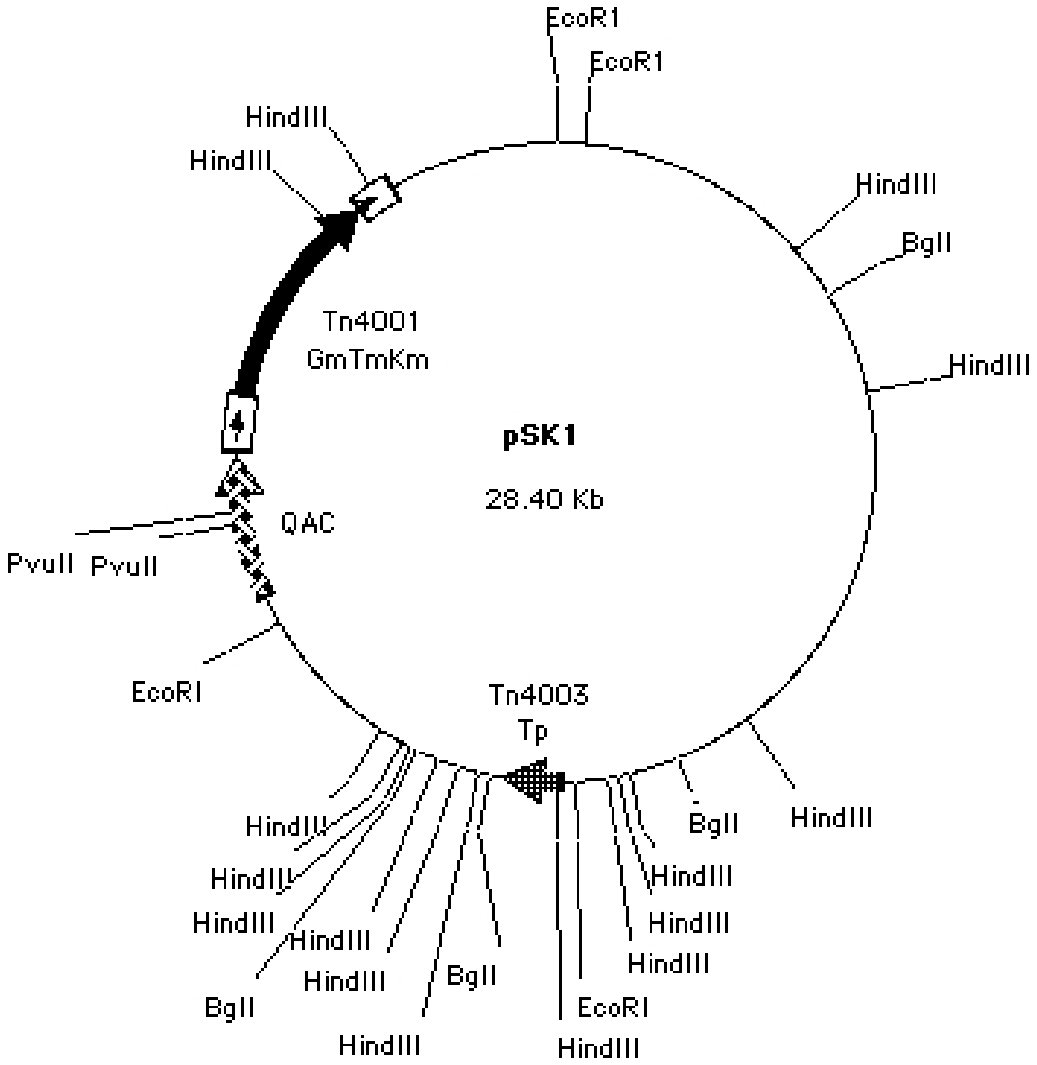


Figure 1.1 - A circular schematic of plasmid pSK1. Arrows depict ORF and transposons. RE sites *Bg*II, *Eco*RI, *Hind*III, and *Pvu*II are indicated. Resistance to Gm, gentamicin; Km, kanamycin; Tm, tobramycin; Tp, trimethoprim; QAC, quaternary ammonium compounds, is indicated.

1.4.1.1 Selective pressures for the retention of the SK1 family of plasmids

Initially the maintenance of the SK1 plasmids by EMRSA was attributed to the determinant for Gm^R and the widespread use of Gm in hospitals during this time (Townsend *et al.* 1983a). However, the transposon carrying the Gm^R determinant, along with Km^R and Tm^R, could transpose into the host chromosome alleviating the

need for EMRSA to retain the plasmid (Rahman *et al.* 1988; Townsend *et al.* 1983b, 1983c). Other antibiotic resistance determinants associated with transposons on plasmid pSK1 were also deemed capable of transferring to the chromosome and therefore the antibiotics Tp, and β-lactams were also considered unlikely to provide a selective pressure for the retention of SK1-like plasmids (Townsend *et al.* 1984c).

Incompatibility studies provided evidence that the presence of a selective pressure was required for the retention of the SK1 family of plasmids. Plasmids from the SK1 family belong to the same *Inc*I as the large 20-30 kb plasmid found in the "classic" MRSA. Many believe that the acquisition of SK1-like plasmids was blocked by the presence of this resident *Inc*I plasmid based on the assumption that EMRSA originated from the same 'clone' as other "classic" MRSA strains (Lacey and Grinsted 1973). Since these two plasmids belong to the same incompatibility group they could not be stably maintained in the same cell. If EMRSA evolved from the "classic" MRSA by acquisition of additional resistance plasmids, then there must have been considerable selective pressure for the preservation of the SK1 plasmids and displacement of the resident *Inc*I plasmid (Townsend *et al.* 1985b). This indicates that the success of EMRSA is directly related to its ability to overcome incompatibility and acquire the pSK1 plasmid. However, more recent analysis of "classic" MRSA and EMRSA using MLST and SCC*mecA* typing indicate that these strains are not clonal as first thought but have arisen by transfer of resistant genes to different strains of MSSA (Fitzgerald *et al.* 2001; Musser and Kapur 1992).

The SK1 plasmids also conferred resistance to QACs and Eb (Tennent *et al.* 1989; Townsend *et al.* 1983b, 1983c). These compounds belong to a large class of antimicrobial compounds referred to as cationic biocides that came under scrutiny as the selective agents (Sivaji *et al.* 1986) that may be responsible for maintenance of the SK1 plasmid family in EMRSA. The prevalence of cationic biocide resistance amongst isolates of MRSA, and particularly those associated with outbreaks, suggested that cationic biocide resistance was an important attribute of clinical isolates of *S. aureus* (Townsend *et al.* 1983a, 1984c).

1.5 Cationic Biocides

Biocide is a general term that includes disinfectants, antiseptics and preservatives (Fraise 2002; Maillard 2005) and is used to describe a chemical agent that inactivates microorganisms (McDonnell and Russell 1999). It does not include antibiotics, which in spite of being biocides in the strictest sense tend to be categorised separately (Maillard 2005). Less is known about the mode of action of biocides than antibiotics. In general biocides have a broader spectrum of activity than antibiotics targeting multiple sites in the cell while antibiotics tend to have specific intracellular targets (McDonnell and Russell 1999). Considerable variation is seen in the level of susceptibility to biocides this is due in part to their chemical structure, and also cell morphology and cellular chemical composition of its target (Denyer and Stewart 1998; Heinzel 1998).

Prominent amongst the biocides are the cationic compounds, remaining to this day the mainstay of routine chemical antisepsis and disinfection. These positively charged molecules are relatively hydrophobic in nature and are believed to bind to the negatively charged cell walls and membranes of bacteria leading to their eventual disruption (Gilbert and Moore 2005).

1.5.1 Range and use of cationic biocides

The range of cationic biocides includes the QACs, diamidines, biguanides, cationic dyes and nuclear stains. Numerous cationic biocides have been used extensively in hospitals for over 40 years (Gilbert and Moore 2005; Rahn *et al.* 1947). They are believed to be an essential part of safe infection control practices and prevention of nosocomial infections (McDonnell and Russell 1999). There has also been an increased trend in recent years to use cationic biocides in the home environment due to an increase in public awareness to hygiene and greater commercial and marketing pressures (Cole *et al.* 2003; Davis *et al.* 2005).

1.5.1.1 QACs

The QACs are considered amphoteric surfactants. Their structure consists of four carbon atoms linked directly to a nitrogen atom through covalent bonds, the general

formula is represented in Figure 1.2 (Fredell 1994; Gilbert and Moore 2005; Merianos 2001; Moore and Payne 2004). The side-groups, R1, R2, R3 and R4, are alkyl or heterocyclic and the nitrogen can be part of a ring system. The nitrogen atom, with the attached side-groups, creates a positively charged cationic group, which is the functional portion of the molecule (Fredell 1994; Merianos 2001). The positively charged nitrogen attaches to negatively charged surfaces (Gilbertand Moore 2005; Östman 1993). For a QAC to have a high antimicrobial activity at least one of the side-groups (designated R in Figure 1.2) must have a chain length in the range of 8 to 18 carbons long (Moore and Payne 2004). The commonly used QAC, cetyl trimethyl ammonium bromide (CTAB), is a mixture of *n*-alkyltrimethyl ammonium bromides where the hydrophobic moiety's (*n*-alkyl groups) are between 8 and 18 carbons long, Table 1.2 (Gilbert and Moore 2005). Another example, benzalkonium chloride (Bc), is a mixture of *n*-alkyldimethylbenzyl ammonium chlorides where the *n*-alkyl groups can be of variable length; the greatest bactericidal activity is associated with the alkyl derivatives of 12 to 14 carbons long, see Table 1.2 (Gilbert and Moore 2005).

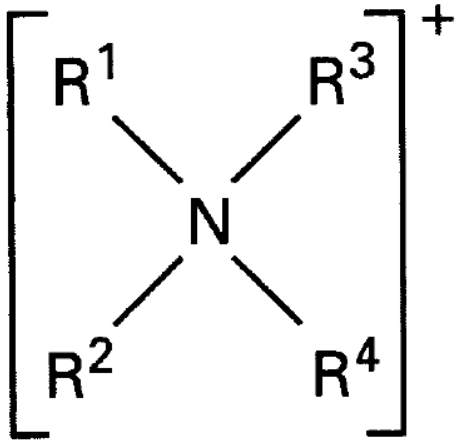
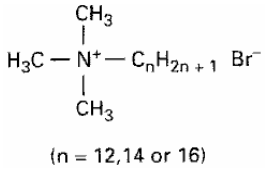
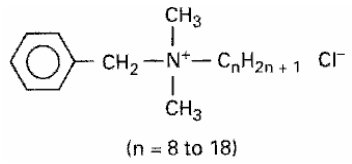
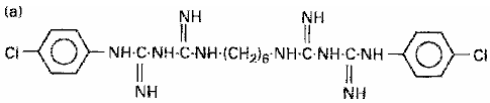
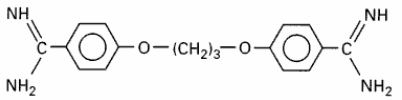
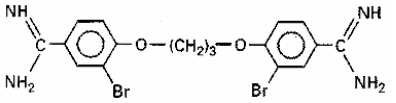
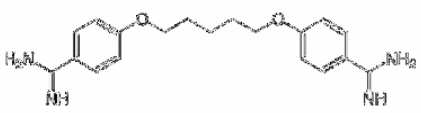
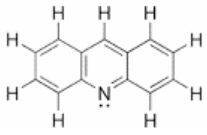
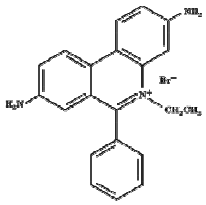


Figure 1.2 – General structure of QACs (Moore and Payne 2004).

Table 1.2 - Chemical structure and use of cationic biocides

Cationic Biocides	Example	Chemical Structure	Function
QACs	CTAB		Disinfectant Antiseptic Preservative Deodorisation Algaecides Fabric softener Antistatic
	Benzalkonium chloride		
Biguanides	Chlorhexidine		Antiseptic Antiplaque agents Preservative Disinfectant
Diamidines	Propamidine		Antiseptic Preservative
	Dibromopropamidine		
	Pentamidine		
Cationic dyes	Acridine		Antiseptic Nuclear stain Fluorescence
	Eb		

QACs have a wide range of applications in the health care market, including applications in hospitals, nursing homes, dental offices and veterinary facilities (Fredell 1994; Merianos 2001). Predominantly the formulations containing CTAB (up to 1%) and Bc (up to 0.1%) are used as antiseptics in the cleansing of wounds and burns, preoperative disinfection of unbroken skin, application to mucous membranes and disinfection of non-critical surfaces (Arugonda 1998; Gilbert and Moore 2005; Maillard 2005). CTAB is also included in several barrier creams and is used in combination with chlorhexidine (Ch) in the household product Savlon (Gardner and Peel 1998). QACs have been recommended for use in food hygiene in hospitals and in food processing industries (Aase *et al.* 2000; Lalla 2004). In addition to having antimicrobial properties QACs are excellent for cleaning hard-surfaces and deodorisation and are also the active ingredients in many algaecides for swimming pools, industrial water reservoirs, and farm ponds (Merianos 2001). The use of QACs as fabric softeners exceeds that of all other applications combined. The positive charge imparts antistatic properties to clothing and fabrics as well as making the fabric easier to dry as the fibre surfaces are now hydrophobic (Cross 1994; Merianos 2001). The compounds are used in hair conditioners, as softeners for textiles and paper products, and as pigment dispersers (Arugonda 1998). QACs were initially developed to be used as disinfectants now formulations containing QACs have found their way into many industries and have myriad applications.

1.5.1.2 Bisbiguanides

The bisbiguanides are symmetrical molecules containing two biguanide (guanylguanidine) groups connected by a methylene bridge and having identical terminal groups (Baker *et al.* 1979). Antimicrobial activity is seen only when the carbon bridge in the compounds is aliphatic and activity is maximal with a bridge length of 6 carbons. Ch is the most renowned member of the bisbiguanide group, and is the most widely used cationic biocide in antiseptic products due to its broad-spectrum activity (Denton 2001; McDonnell and Russell 1999). The structure of Ch is a hexamethylene bridge with para-chlorophenyl terminal groups, Table 1.2 (Baker *et al.* 1979). It is also available as a dihydrochloride, diacetate and gluconate (Denton 2001; Moore and Payne 2004; Russell and Day 1993). Used at concentrations ranging from 0.05-4% in antiseptic products such as hand washes and lotions and

cosmetics, where it is used as a preservative (Gardner and Peel 1998; Maillard 2005). It is commonly used in mouthwashes to reduce dental plaque and reduce gingivitis, and is one of the few recommended bactericides used in eye-drops and contact lens solutions (Maillard 2005; Moore and Payne 2004). Persistent use of Ch has in some cases found to cause skin sensitivity, irritation and hypersensitivity reactions (Maillard 2005).

Other biguanides include alexidine, which differs chemically from Ch in possessing ethylhexyl end groups (McDonnell and Russell 1999; Moore and Payne 2004). Alexidine was found to be more active against *Escherichia coli* and recommended for use as an oral antiseptic and antiplaque compound, and as cleaning agents in the food industry (Moore and Payne 2004).

1.5.1.3 Diamidino compounds

Initially introduced to medicine as a possible substitute for insulin, this group of organic compounds was later found to have useful antimicrobial properties (Moore and Payne 2004). Members of this group include propamidine isethionate (Pi), pentamidine isethionate (Pt) and dibromopropamidine, with typical structures seen in Table 1.2. The two most notable members of this group are Pi and dibromopropamidine; these are usually supplied as isethionates, which is the soluble form. These compounds are found predominantly in a cream emulsion containing 0.15% and used topically on wounds (Moore and Payne 2004).

1.5.1.4 Cationic dyes and nuclear stains

Cationic dyes and nuclear stains are relatively large, flat basic molecules that somewhat resemble a DNA base pair (bp). This group includes the acridines, the triphenylmethane group, and Eb, all of which find applications because of their ability to intercalate with DNA and subsequent antimicrobial activity.

Cationic dyes such as the acridines have traditionally been used as astringents to dry macerated skin around wounds and for their antiseptic properties. The use of acridines as antimicrobial agents for the treatment of infected wounds in the clinical

setting was first introduced in 1917 (Wainwright 2001). The acridines are organic compounds with a heterocyclic structure, Table 1.2. Acridines also have DNA staining/fluorescent properties due to their intercalating abilities. Numerous structurally related derivatives of acridine where developed for use as antimicrobial dyes, including that of acriflavine (Af). Af has been used in the treatment of infected wounds, burns and for skin disinfection. However, prolonged treatment may delay wound healing and hypersensitivity to acridine derivates has been reported and therefore they are now rarely if ever used (Moore and Payne 2004).

The triphenylmethane dyes include crystal violet (Cv), brilliant green and malachite green (Moore and Payne 2004). These were once used as local antiseptics for application to wounds and burns but were also found limited in their effectiveness.

Other cationic dyes are found in association with the staining and fluorescence of DNA including the toxic compound, Eb. The main portion of the molecule is a tricyclic structure with aniline (amino-benzene) groups on either side of a pyridine (six-atom, nitrogen-containing, aromatic ring), Table 1.2. The dibenzopyridine structure is known as a phenanthridine. Because of its chemical structure, it can easily insert into a DNA strand, making it a convenient marker or stain for identifying nucleic acids in electrophoresis gels and its aromatic structure give it fluorescent properties. However it is dangerous to handle by the investigator and is not used as a biocide due to its toxicity.

1.5.2 Mechanism of action of cationic biocides

Despite the prolonged and extensive use of cationic biocides indicated above, there is still a general lack of experimental evidence and understanding surrounding their exact antimicrobial activity.

1.5.2.1 QACs

There have been several studies investigating the antimicrobial activity of QACs and in these investigations the antimicrobial activity varied greatly depending on the type of QAC used, concentration, exposure time and the type of organism being

investigated. The bactericidal spectrum of QACs is narrow. They are primarily active against Gram-positive bacteria, and to lesser extent Gram-negative bacteria, mainly due to the latter's outer membrane acting as a protective barrier (Fredell 1994; Ioannou *et al.* 2007; Langsrud and Sundheim 1997; Moore and Payne 2004). The QACs possess antifungal properties, although they are fungistatic rather than fungicidal (Moore and Payne 2004).

QACs are membrane-active agents with a target site predominantly at the cytoplasmic membrane in bacteria or the plasma membrane in yeasts (McDonnell and Russell 1999). The bacterial cytoplasmic membrane provides the mechanism whereby metabolism is linked to solute transport, flagellar movement and the generation of ATP. Protons are extruded to the exterior of the bacterial cell during metabolism. The combined potential, concentration or osmotic effect of the proton and its electropositivity, is known as the proton motive force (PMF), which drives these ancillary activities (McDonnell and Russell 1999). The QAC CTAB was found to have an effect on the PMF in *S. aureus.* At its bacteriostatic concentration, CTAB caused the discharge of the pH component of the PMF (Johnston *et al.* 2003; McDonnell and Russell 1999). At higher concentrations QACs are also believed to damage the outer membrane of Gram-negative bacteria, thereby promoting their own uptake (McDonnell and Russell 1999). Damage to the cytoplasmic membrane, with resulting changes in cell permeability, is the primary mode of antimicrobial action for QACs (Fredell 1994).

1.5.2.2 Bisbiguanides

Ch has a wide spectrum of antibacterial activity against both Gram-positive and Gram-negative organisms. It also has fungicidal activity and a low order of activity against viruses, but is not sporicidal (Denton 2001; Gardner and Peel 1998; McDonnell and Russell 1999; Moore and Payne 2004; Russell 2002b).

Ch works by being taken up rapidly by the cell, this causes damage to the outer cell layers allowing the agent to then cross the cell wall or outer membrane resulting in an attack on the cytoplasm or inner membrane (El-Moug *et al.* 1985). Leakage of intracellular constituents then follows. The mechanism of action of Ch is

concentration dependant, at low concentrations, up to 200 μg/ml, membrane integrity is affected whereas high concentrations cause congealing of the cytoplasm (Kuyyakanond and Quesnel 1992; McDonnell and Russell 1999; Russell and Day 1993).

Ch has also been claimed to be an inhibitor of ATPase as well as net K^+ uptake in *Enterococcus faecalis* (Harold *et al.* 1969). More recently it has been shown that although Ch collapses the membrane potential, it is membrane disruption rather than ATPase inactivation that is associated with its killing action (Barrett-Bee *et al.* 1994; Kuyyakanond and Quesnel 1992).

1.5.2.3 Diamidino compounds

Both Pi and dibromopropamidine have the highest activity of the diamidino compounds against both Gram-negative and Gram-positive bacteria (Turner 2004). The exact mechanism of action of diamidines is unknown, but as with other cationic-surface active agents they have been shown to inhibit oxygen uptake and induce leakage of amino acids (aa) (Richards *et al.* 1993).

1.5.2.4 Cationic dyes and nuclear stains

The acridine derivatives are slow-acting antiseptics, bacteriostatic against Gram-positive bacteria, less effective against Gram-negative bacteria, and ineffective against spores (Moore and Payne 2004). Eb, though not used as an antiseptic due to its toxicity, is known to have antimicrobial activity towards Gram-positive and Gram-negative bacteria.

The mechanism of action of Eb and acridine dyes works on the nucleic acids in bacteria. The nucleotide base pairs are specifically targeted by the positively charged structure of these cationic dyes and nuclear stains. They intercalate between bp in the double helix, binding to the DNA blocking replication, gene expression and protein synthesis (Wainwright 2001; Wilson *et al.* 1994).

1.6 Resistance to Cationic Biocides by Staphylococci

Compared to that of antibiotic resistance, biocide resistance has not attracted a great deal of interest. Resistance to biocides is unexpected as these compounds have a multitude of target sites upon the cell (McDonnell and Russell 1999; Poole 2002). Resistance generally results from cellular changes to the cell wall or envelope (Denyer and Stewart 1998) or expression of an efflux mechanism (McDonnell and Russell 1999; Poole 2002; Russell 2002b), both preventing cationic biocide accumulation in the cell. The discovery of resistance to cationic biocides in EMRSA strains carrying SK1 plasmids stimulated interest in the range of cationic compounds to which resistance is expressed (Poole 2004). Consequently a range of cationic biocides was investigated as agents that provide selective pressures for EMRSA in hospitals (Emslie *et al.* 1985b; McDonnell and Russell 1999; Townsend *et al.* 1984c).

Investigation of the SK1 family of plasmids found several cationic biocides, including QACs, Eb, acridine yellow (Ay), Pi and Pt that select for strains bearing SK1-like plasmids (Emslie *et al.* 1985b). Interestingly several of these compounds were known to interact with nucleic acids. QACs and diamidinos bind to the phosphodiester backbone of nucleic acids, while Eb and Ay are plate-like molecules that intercalate between the bases of DNA (Russell 1998). With this shared property the compounds were initially referred to as nucleic acid-binding (NAB) compounds and the plasmids that carried resistance determinants to them were thus designated NAB-resistance plasmids (Emslie *et al.* 1985a, 1985b; Littlejohn *et al.* 1992; Mitchell *et al.* 1999; Paulsen *et al.* 1996a). This terminology was later deemed inappropriate as not all the compounds could be classified as NAB, in particular the biguanide Ch. Today's accepted terminology is QAC resistant determinants. This terminology may also seem inappropriate, as it is too narrow in its description of the compounds included in the resistance profiles of QAC-resistant genes.

1.6.1 Resistance to QACs by staphylococci

QAC resistance has been widely recognised in *S. aureus* since 1985 (Al-Masaudi *et al.* 1988; Jones and Midgley 1985; Tennent *et al.* 1989; Townsend *et al.* 1985c). Initially associated with MRSA, QAC resistance has now extended to numerous

staphylococci found in clinical, agricultural and food isolates (Bjorland *et al.* 2001, 2003, 2005; Brooks *et al.* 2002; Heir *et al.* 1999a; Irizarry *et al.* 1996; Leelaporn *et al.* 1994; Littlejohn *et al.* 1990; Nikaido 1998; Noguchi *et al.* 1999; Paulsen *et al.* 1993a, 1996c). Multidrug resistance to QACs has been reported in clinical isolates of *S. aureus* from all over the world including Australia, Europe, Asia, Japan and North America (Alam *et al.* 2003b; Leelaporn *et al.* 1994; Noguchi *et al.* 1999; Townsend *et al.* 1985c; Yamamoto *et al.* 1988). All staphylococci QAC resistant determinants are plasmid borne.

Though resistance to cationic biocides is not a new phenomenon its maintenance by staphylococci, in particular hospital-associated strains, for the past 35 years makes it quite unusual.

1.6.2 Significance of low-level resistance to cationic biocides

One of the remaining cationic biocides still used in the hospital is Ch. Ch is used as a skin antiseptic to prevent cross-infection between staff and patients. Resistance to Ch only confers a low minimum inhibitory concentration (MIC) ranging from 1 to 4 mg/L (Denton 2001), although considered significant, is irrelevant when compared with actual 'in-use' concentrations. Clinically the Ch concentration (in the absence of soil) needs to produce a 99.99% (4 $\log_{10}$ reduction) kill in 10 minutes at 20 °C, which against *S. aureus* has been reported as 25 mg/L (Russell and Day 1993). This concentration is well in excess of the MIC values recorded for resistant *S. aureus*. It has been proposed that the low MICs observed for cationic biocide resistant *S. aureus* reflects the fact that cationic biocide resistant substrates are hydrophobic, and as such these compounds accumulate in the cytoplasm to levels dramatically exceeding the concentration at which they are added to the surrounding environment (Ahmed *et al.* 1994). One study also considered the inactivation by non-ionic surfactants such as Tween 80 and phospholipids when evaluating the activity of cationic biocides such as the QACs and Ch. A neutralising solution consisting of phospholipid (azolectin 0.75%) in 5% Tween 80 was demonstrated to be effective against neutralising high concentrations of Ch (Fitzgerald *et al.* 1989). This would indicate that residual levels of these cationic compounds provide the selective

advantage for the maintenance of the determinants within *S. aureus*, and the low levels of resistance are significant.

Differences in MIC levels between QAC-sensitive and QAC-resistant staphylococcal strains are generally quite small, as little as 2-4 fold. One particular study performed by Irizarry (1996) compared the susceptibility of MRSA and MSSA strains to the cationic biocides, cetylpyridinium chloride (CPC) and Ch, by MIC. On the basis of MICs, MRSA strains were some four times more resistant to Ch and five times more resistant to CPC than the MSSA strains. At 2 μg of CPC/ml concentration in broth, two MRSA strains grew normally with a threefold increase in viable numbers over a 4-hour test period whereas a MSSA strain showed a 97 % decrease in viability. From this the authors concluded that it was reasonable to speculate that the residual amounts of antiseptics and disinfectants found in the hospital environment could contribute to the selection and maintenance of MRSA strains. However their conclusions warrant additional comments. The same experiment performed with a concentration of 4 μg of CPC/ml in broth showed no such pattern. At the higher concentration the viability of the two MRSA and MSSA strains decreased at very similar rates, with one MRSA strain appearing to be more affected than the MSSA strain.

The MIC values for each compound also vary between different batches of media as cationic biocides were found to diffuse poorly in agar or interact with agar constituents (Russell 2002a). Emslie (1986) proposed a more reliable way of comparing MICs between isolates by calculating the ratio of MIC to a compound between the test strain and a sensitive reference strain performed in parallel and in the same batch of media. A ratio of 2-8 was considered a marginal increase in resistance to a particular compound and the test organism was said to possess low-level resistance. At a ratio greater than 8 fold, the test organism was considered to express high-level resistance. Many consider MICs as unsuitable for evaluating cationic biocides and serve only as a preliminary procedure in determining activity (Platt and Bucknall 1988).

Overall there is a general concern that the subtle differences in the cationic susceptibility of antibiotic-resistant strains might result in the selection, maintenance

or evolution of MRSA strains in the hospital. And perhaps there are cationic biocide resistance mechanisms of which we are unaware, that are not detected by a MIC test (Cookson 2005). Fortunately there is very little evidence that exposure of bacterial cultures to subeffective concentrations of cationic biocides leads to the development of resistance in clinical practice (Gilbert and McBain 2003). Cookson (1991) was not able to increase MIC levels of MRSA, EMRSA and MSSA strains to Ch after repeated exposure *in vivo* or serial passage *in vitro*. Also despite extensive use of Ch in dental hygiene, strains of *Streptococcus mutans* have remained sensitive to the biocide (Jarvinen *et al.* 1993).

1.6.3 Resistance to cationic peptides

The rationale behind staphylococci encoding resistance to a range of cationic compounds is debated. The fact that cationic biocide resistance genes are located on plasmids found typically in hospital isolates rather than community isolates, supports the suggestion that resistance to these surface-active compounds is maintained because of selective pressures found in the hospital environment (Leelaporn *et al.* 1994; Tennent *et al.* 1985). Initially it was thought the chronological emergence of QAC-resistance in clinical isolates of *S. aureus* mirrored the introduction and usage of cationic biocides in hospitals, notably Af, the diamidines, QACs (CTAB and Bc) and Ch (Emslie *et al.* 1985b; Paulsen *et al.* 1998). Yet the lack of current widespread use of acridines, the diamidines and QACs in the clinical setting makes this interpretation very unlikely as resistance often results in metabolic penalties for the cell. And if the selective pressures of antiseptic use are withdrawn there is usually a gradual return to sensitivity in the population (Towner 2000). Recent data has also suggested that bacteria are unable to adapt to the additional genetic load of extra resistance genes, returning to their initial physiological fitness in the absence of a selective pressure (Gillespie 2001). Yet the current prevalence of cationic biocide resistance amongst the MRSA strains from around the world indicates there is one or more current resistance(s) that provides a survival advantage to *S. aureus* carrying QAC resistance (Levy and Marshall 2004).

The basis for staphylococci encoding resistance to a range of cationic compounds is unknown. The low levels of resistance may in fact indicate that the resistance is

actually directed against other compounds, prompting attempts to find the original target of these resistance genes. A study performed by Kupferwasser (1999) tested a range of cationic peptides, including antimicrobial peptides, against cationic-resistant *S. aureus.* Antimicrobial cationic peptides are found ubiquitous throughout nature as part of the intrinsic defences of most organisms. These peptides are produced in large quantities at sites of infection and inflammation and can have broad-spectrum antibacterial, antifungal, antiviral, antiprotozoan and antisepsis properties (Friedrich *et al.* 2000; Hancock and Diamond 2000a). A cationic antibacterial peptide is defined as containing a net excess of positively charged residues, approximately 50% hydrophobic residues and a size ranging from 12 to 50 residues (Hancock and Scott 2000b). The exact mechanism of action of cationic peptides varies according to the structure of the peptide (Bush 1997). Their amphipathic structure is known to interact with membranes indicating that this represents their antibacterial target (Wu *et al.* 1999) but have also been found to readily interact with nucleic acids (Zhang and Hancock 2001). They have been hailed as potentially new antibiotic drug candidates to use against multi-resistant bacteria, but have so far been met with limited success (Boman 1995; Mor 2000; Strom *et al.* 2003).

The presence of the plasmid pSK1 was found to mediate resistance against a small cationic peptide, thrombin-induced platelet microbial protein 1 (tPMP-1). tPMP-1 is an 8.5 kDa cationic antimicrobial peptide released from rabbit platelets following thrombin stimulation, produced during endovascular infection (Dhawan *et al.* 1997; Koo *et al.* 2001). It was suggested that the mechanism of resistance worked by modifying the composition of the cytoplasmic membrane so that it was less susceptible to disturbance by tPMP-1 (Bayer *et al*. 2000; Yeaman and Yount 2003). These results indicate that *S. aureus* might have procured the cationic biocide resistance in response to the presence of cationic antimicrobial peptides and further investigation of these naturally occurring antimicrobials was warranted.

Other cationic peptides were subsequently tested against the SK1 family of plasmids, these included human neutrophil defensin 1, protamine, and staphylococcal lantibiotics and nisin (Kupferwasser *et al.* 1999). Also examined was human β-defensin 3 a cationic peptide produced by the skin to protect the body's surface against microbial invasion, an area typically colonised by coagulase-negative

staphylococci and *S. aureus* (Mahendran results not published). There was however, no apparent cross-resistance imparted by strains bearing cationic biocide resistance plasmids to these endogenous cationic antimicrobial peptides that were structurally distinct from tPMP-1 (Kupferwasser *et al.* 1999). Although these cationic peptides gained visible prominence in the area of new antimicrobial agents and though intellectually appealing they have shown limited use in animal models.

1.6.4 Resistance to cationic biocides by other bacteria

Resistance to cationic biocides has also been characterised in various other organisms. Resistance to QACs has been documented in *Pseudomonas aeruginosa* (Stickler and Thomas 1980), *Serratia* species (Moore and Payne 2004), coagulase-negative staphylococci (Heir *et al.* 1995, 1999a; Sidhu *et al.* 2001), *Burkholderia cepacia* (Nagai and Ogase 1990), *Bacillus subtilis*, *E. coli* (Jones and Midgley 1985), *Salmonella* (Randall *et al.* 2001), *Klebsiella aerogenes* (Leelaporn *et al.* 1994), *Providencia*, *Proteus* and *Pseudomonas* genera (Moore and Payne 2004; Russell and Day 1993). This insusceptibility in many of the species listed above is due to the fact that cationic biocides cannot reach their target sites within these cells. Many consider the wide distribution of QAC resistance suggests that cationic biocides are important elements of an organism's potential to survive. Yet it has also been countered that much of this resistance is unstable and not deemed clinically significant (Russell 2000).

1.6.5 QAC resistance genes of staphylococci

There are to date three cationic biocide resistance phenotypes in *S. aureus* that differ markedly in the range of compounds to which they are resistant and the levels of expression of resistance to each compound (Emslie *et al.* 1985a, 1985b; Littlejohn *et al.* 1992; Mitchell *et al.* 1999; Paulsen *et al.* 1996a). These resistance phenotypes have subsequently been associated with three genes, *qacA*, *qacB* and *smr* (formerly *qacC*, also known as *qacD*, and *ebr*). All three genes have been cloned and expressed in *E. coli*, their nucleotide (nt) sequences determined and their protein products analysed.

1.6.5.1 The *qacA/B* gene family

The first gene identified was *qacA* (Littlejohn *et al.* 1990; Tennent *et al.* 1989). This gene was found to confer the broadest resistance phenotype and encoded the highest levels of resistance to all compounds tested, with the exception of CTAB and tetraphenylarsonium chloride (Littlejohn *et al.* 1992). Its resistance includes all four classes of cationic biocides previously described in this review (Leelaporn *et al.* 1994; Littlejohn *et al.* 1990; Mitchell *et al.* 1998; Tennent *et al.* 1989). Restriction mapping and DNA-DNA hybridisation has identified the *qacA* determinant on β-lactamase and heavy-metal resistance plasmids dating back to the 1980s, including pSK57 (Lyon and Skurray 1987), but has more frequently been found on the pSK1 family of multi-resistance plasmids in EMRSA strains in Australia and the United Kingdom since 1980, Table 1.3 (Leelaporn *et al.* 1994; Paulsen *et al.* 1998). Although present predominantly on the pSK1 family of multi-resistance plasmids it may also be present on the chromosome of clinical *S. aureus* strains as a result of integration of cationic biocide resistance genes.

While *qacA* confers resistance to both monovalent and divalent organic cations, the closely related *qacB* determinant confers resistance primarily to monovalent organic cations (Paulsen *et al.* 1998). *qacB* confers similar levels of resistance to QACs and to the cationic dyes compared to *qacA*, yet *qacB* characteristically differs from *qacA* in that it confers very low levels of resistance to the diamidines and no resistance to Ch (Littlejohn *et al.* 1992). The *qacB* gene has been found on large, 20-55 kb, β-lactamase and heavy-metal resistance plasmids such as pSK23 and pSK21 (Leelaporn *et al.* 1994; Littlejohn *et al.* 1992; Lyon and Skurray 1987). Also found on small plasmids, 2-3 kb. The presence of *qacB* on both small plasmids and much larger plasmids is explained by cointegration (Novick 1989).

Molecular comparisons of *qacA* and *qacB* found their nt sequences to differ by 7 nt substitutions (Paulsen *et al.* 1998). Hydrophobicity plot analysis of the sequences predicted a protein comprised of 14 transmembrane segments, Figure 1.3. The structure was confirmed by alkaline phosphatase and β-galactosidase fusions by identifying internal and external motifs (Paulsen *et al.* 1996a). The generation of *qacB* mutants, which conveyed resistance to divalent cations and site-directed

mutagenesis of *qacA* showed that the difference in substrate specificity between QacA and QacB was due to a single aa substitution at position 323 (Paulsen *et al.* 1996b). The presence of an acidic residue, aspartate, in QacA is essential for high levels of resistance to diamidines and biguanides instead of an uncharged residue, alanine, in QacB (Mitchell *et al.* 1998; Paulsen *et al.* 1996b). It is believed that the negative charge of the acidic amino acid at position 323 in transmembrane segment 10 of QacA interacts directly with the positively charged moieties of the divalent cation resulting in increased QacA-mediated resistance of these compounds (Mitchell *et al.* 1998).

It has been postulated that the extensive use of the antimicrobial agent Ch in the hospital environment has resulted in the evolution of *qacA* from *qacB* (Paulsen *et al.* 1998).

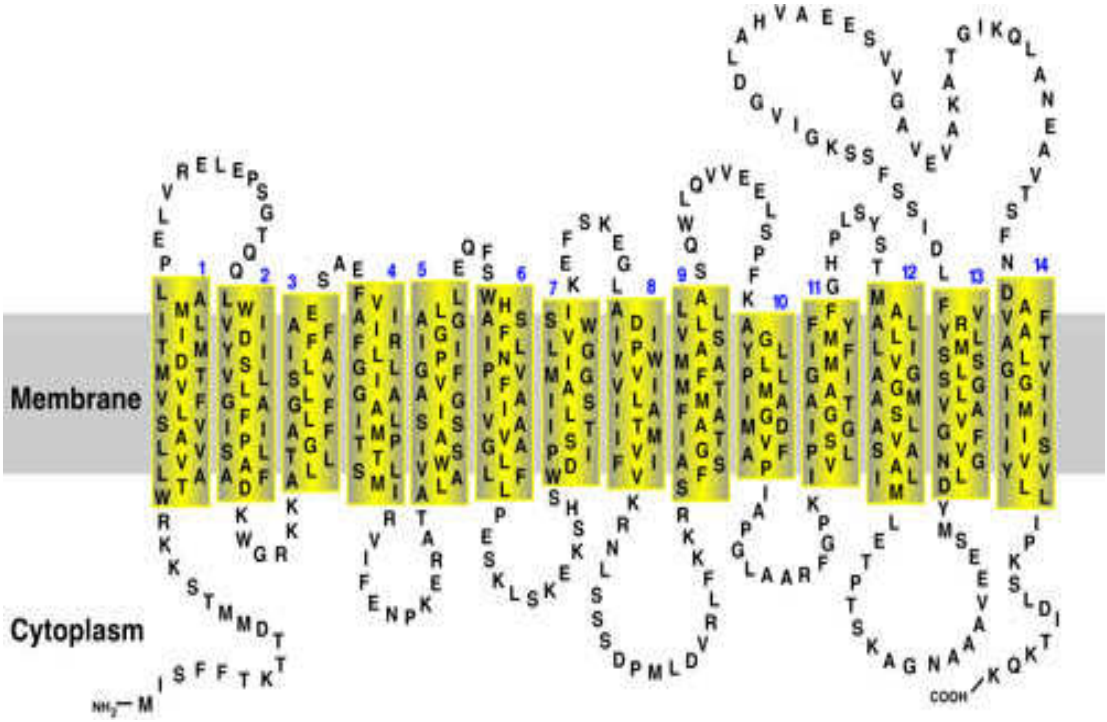


Figure 1.3 – Two-dimensional model of the *S. aureus* protein QacA, transmembrane domains represented in yellow and the cytoplasmic membrane represented in grey (Brown and Skurray 2001).

Table 1.3 – Genes involved in staphylococcal resistance to biocides

Gene	Gene Location	Resistance conferred to
qacA	pSK1 family of multi-resistant plasmids, also β-lactamase and heavy-metal resistant plasmid families	QACs, Ay, Eb, Ch and diamidines
qacB	β-lactamase and heavy-metal resistant plasmid families, also found on small plasmids (< 3 kb)	QACs, diamidines, Ay and Eb
smr/qacC	Large conjugative, multi-resistant plasmids or small rolling circle replicating plasmids (< 3 kb)	Some QACs, Ay and Eb
qacG	pST94, 2.267 kb	Some QACs and Eb
qacH	p2H6, 2.4 kb	Some QACs and Eb
qacJ	pNVH01, 2.65 kb	Some QACs and Eb

1.6.5.2 The *smr* gene family

The third gene to be identified, *smr*, has the narrowest resistance phenotype and encodes the lowest levels of resistance to a number of cationic biocides. The *smr* gene mediates resistance to Ay with reduced levels of resistance to Eb (Littlejohn *et al.* 1992), high levels of resistance to some of the QACs, such as Bc, CPC and CTAB, and low levels of resistance to tetraphenylphosphonium (TPP) (Paulsen *et al.* 1996b) but no resistance to the diamidines or the biguanide, Ch (Littlejohn *et al.* 1992). The *smr* gene is located either on small plasmids < 3 kb such as pSK89 generally found from Australia (Lyon and Skurray 1987), or on large 50 kb conjugative plasmids, such as pSK41 seen in Europe, USA and Japan since the 1970s, see Table 1.3 (Leelaporn *et al.* 1994; Lyon and Skurray 1987). The *smr* gene has also been found duplicated on a Japanese *S. aureus* plasmid, pTZ22, from the study it was concluded that the existence of the duplicated gene conferred double the level of resistance to Eb (Sasatsu *et al.* 1992).

The *smr* gene codes for a much smaller membrane protein than the *qacA/B* gene family (Paulsen *et al.* 1995). The proposed model for the Smr transmembrane protein was deduced by using alkaline phosphatase and β-galactosidase fusions (Paulsen *et al.* 1995). The protein sequences in the Smr family are highly hydrophobic and

studies support the model of four putative membrane domains with short loops in between (Ninio *et al.* 2001; Putman *et al.* 2000). The membrane topology, seen in Figure 1.4, includes four membrane-spanning α-helices that are separated by two periplasmic loops (P1 and P2) and one cytoplasmic loop (C1), with the N-terminus located within the cytoplasm (Paulsen *et al.* 1995). The amino (NH_2) and carboxyl (COOH) termini of this polypeptide are also indicated in Figure 1.4.

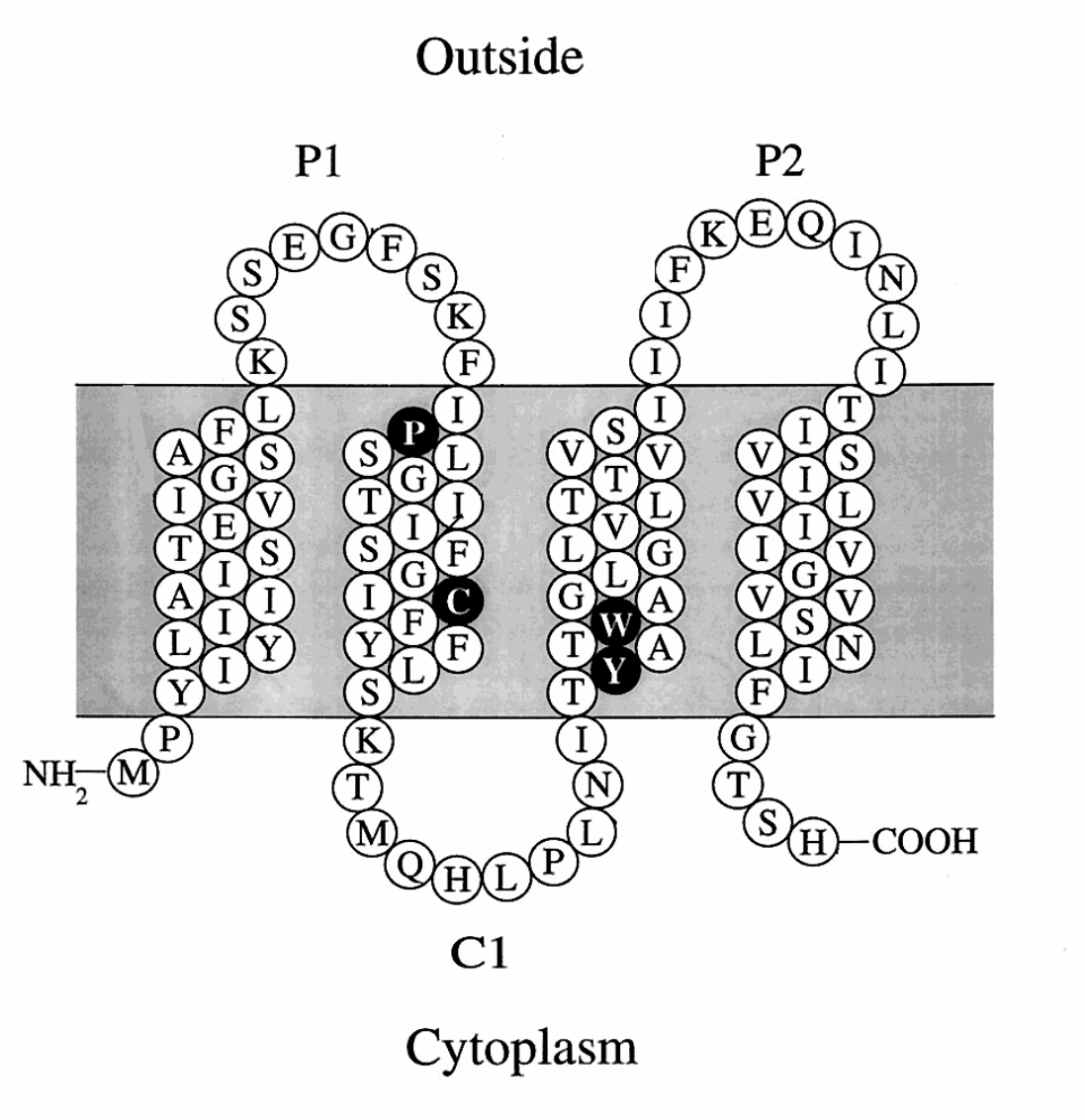


Figure 1.4 – Schematic representation of the QacC polypeptide, showing its proposed transmembrane organization and orientation. The proposed model includes four membrane-spanning alpha-helices that are separated by two periplasmic loops (P1 and P2) and one cytoplasmic loop (C1). The amino (NH2) and carboxyl (COOH) termini of this polypeptide are indicated (Paulsen *et al.* 1995).

The *smr* gene family is composed of the independently identified genes, *qacC, qacC′, qacD, ebr,* and *smr*, sequences of these gene are nearly all identical (Bjorland *et al.* 2001; Grinius *et al.* 1992; Heir *et al.* 1995; Littlejohn *et al.* 1990; Sasatsu *et al.* 1989). The only difference is a single nt substitution found in the *smr* gene reported as *qacC′,* in pST827 (Heir *et al.* 1995) and the *smr* gene in pNVH99 (Bjorland *et al.* 2001). Additional cationic biocide resistant genes conferring low levels of resistance to Eb and several QACS have been recently identified in staphylococci. Identified from the food industry is the *qacG* gene located on the 2.3 kb plasmid pST94, from *Staphylococcus* spp. (Heir *et al.* 1999b). Also identified from staphylococci isolated from the food industry was plasmid p2H6 and sequence analysis revealed another gene with similar homology to *smr,* designated *qacH* (Heir *et al.* 1998) A study of equine staphylococci isolated the *qacJ* gene a novel plasmid-borne gene identified in three staphylococcal species, *S. aureus*, *S. simulans* and *S. intermedius* (Bjorland *et al.* 2003). The newer members of the *smr* family, *qacG, qacH, qacJ,* share 70%, 76%, and 72% identity at the nt level to *smr,* respectively (Bjorland *et al.* 2003; Heir *et al.* 1998, 1999b).

The *smr* gene family is considered highly conserved. Members of the *smr* family generally have several fully conserved residues essential for its function. The highly conserved motif within transmembrane segment (TMS) I contains a glutamic acid residue, Glu-13, which is the only highly conserved positively charged residue within a TMS. This Glu-13 is believed to act as the binding site where lipophilic cations are exchanged for protons (Grinius *et al.* 1992). Glu-13 is extremely sensitive to any substitutions and is proposed to be critical for protein production as well as its activity (Grinius and Goldberg 1994). Site-directed mutagenesis was used to isolate mutants with altered resistance phenotypes and identify the essential residues for function (Paulsen *et al.* 1993a). Those found include Ala-10, Pro-31, Cys-42, Tyr-59 and Trp-62 without which resistance was abolished or greatly reduced (Grinius *et al.* 1992, 1994; Paulsen *et al.* 1993a, 1995).

Not only is the *smr* gene conserved but it has been found that sequences peripheral of the gene are also conserved. Several plasmids carrying the *smr* gene have been sequenced in their entirety uncovering several segments of sequence identity surrounding the resistance gene (Alam *et al.* 2003a). These areas of identity include

direct and indirect repeats, nick site and single strand origin (SSO). These areas have structural features similar to that of cassettes. Cassettes are described as being one of the primary exchange units that can be used to assemble small plasmids such as those that carry *smr* genes (Novick 1989). Cassette exchange is believed to occur by recombination involving short intervening regions, and segments of sequence identity. One study analysed the genomic diversity of the structural unit containing *smr*, which included direct repeat (DR) sequences at both ends of the gene, from clinical *S. aureus* strains detected in a 9-year survey in a Japanese hospital (Alam *et al.* 2003a). As a consequence of this study *smr* gene cassettes were classified into three group types (Alam *et al.* 2003a), illustrated in Figure 1.5. The type 1 cassette was modelled on the genetic organization found in plasmid pSK41, were DRs (that include the SSO) flanked *smr*. In the type 2 cassettes the *rep* gene and a putative replication nick site were found upstream of *smr* and downstream was the SSO and a DR. The third cassette, type 3, was detected in only a single strain and included the insertion sequence IS*431* located between the 3′ end of *smr* and DR. Overall, the cassette structures of the various *smr* plasmids indicate evolution through deletion and insertion of DNA elements.

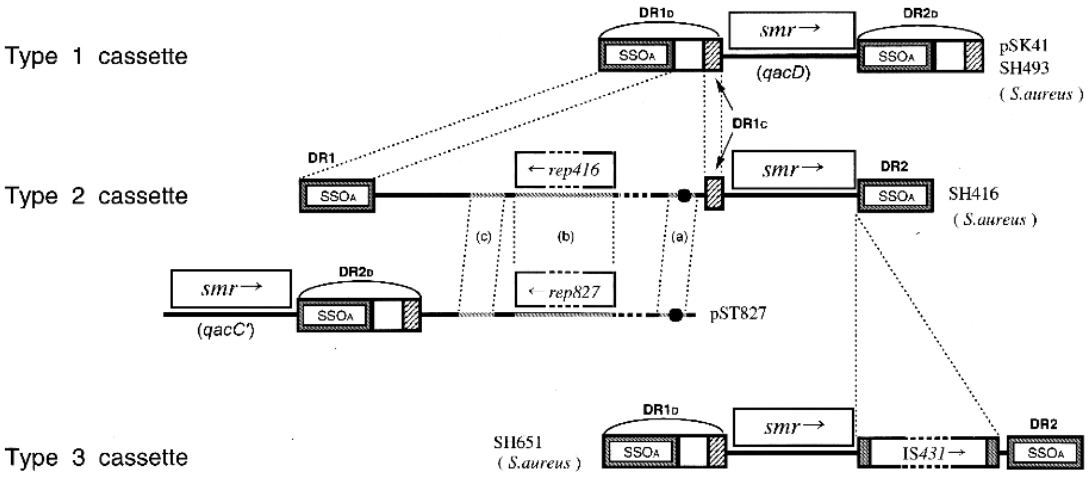


Figure 1.5 – Genomic organization of the three types of *smr* gene cassette (type 1, SH493; type 2, SH416; type 3, SH651) and their relatedness. Horizontal arrows denote directions of ORFs. Solid circles indicate replication nick sites. Hatched portions of the sequence indicated as (a), (b), and (c) represent homologous sequences between the type 2 cassette in SH416 and the downstream region of *smr* in pST827 (Alam *et al.* 2003a).

The potential for QAC resistance genes to be found concomitantly in *S. aureus* was identified in 1% of a distribution study of *qacA/B* and *qacC* genes in 497 European MRSA and MSSA strains (Mayer *et al.* 2001). Up to that time this had only been described in coagulase-negative staphylococci where some 40 % of isolates contain both *qacA* and *smr* genes, with a possible selective advantage in possessing both as opposed to *qacA* or *smr* only (Leelaporn *et al.* 1994).

1.6.6 Mechanism of resistance to cationic biocides

Initial research into the mechanism of resistance to cationic biocides by the three QAC-resistant genes showed they conferred resistance to Eb through efflux of the compound, energised by the transmembrane electrochemical gradient (Jones and Midgley 1985; Littlejohn *et al.* 1990; Rouch *et al.* 1990; Tennent *et al.* 1989). Efflux pumps have the potential to act on a range of chemically dissimilar compounds and have been implicated in both biocide and antibiotic-resistant bacteria (Fraise 2002; Paulsen *et al.* 1996b). Homology of *qac* genes was also found to other energy-dependant transporters, notably the Tc^R transporters (Rouch *et al.* 1990). Efflux pumps are membrane proteins now recognised in all cell types from prokaryotes to superior eukaryotes (Grkovic *et al* 2001; van Bambeke *et al.* 2003). These pumps have been given the names multidrug exporters, multidrug efflux pumps or multidrug transporters because of their ability to handle a wide variety of structurally dissimilar compounds (Grkovic *et al.* 2002; van Bambeke *et al.* 2000).

1.6.7 Regulation of cationic biocide resistance

The discovery of the *qacA* multidrug transporter demonstrating remarkably broad substrate specificity contradicted previously established knowledge of the biochemistry of protein and substrate interactions. However, advances in understanding the basis of multidrug recognition were hindered by the difficultly of performing high-resolution structural analyses of QacA due to the proteins strong association with the cell membrane (Grkovic *et al.* 1998). Analysis of the region immediately upstream from the *S. aureus qacA* gene revealed a putative regulatory gene, *qacR* (Rouch *et al.* 1990). The regulatory protein, QacR, was found to be a

cytoplasmic protein. Structural studies on this cytosolic multidrug binding regulatory protein provided an alternative in defining multidrug binding mechanisms.

Based on homology comparisons, QacR was placed in the TetR family of repressors which regulates expression of the Tc^R gene *tetB* (Grkovic *et al.* 2001; Paulsen *et al.* 1996b). Regulatory proteins from this family share common features associated with a helix-turn-helix (HTH) DNA-binding domains at their N-terminal ends and have highly divergent C-terminals involved in the binding of the inducing compounds (Rouch *et al.* 1990). The site of QacR binding is a large inverted repeat (IR) located immediately adjacent to and downstream from the *qacA* and *qacB* promoters (Grkovic *et al.* 1998). In the absence of *qacR, qacA/B* is expressed constitutively and over expression of *qacR* prevents expression of *qacA.* QacR acts as a transcriptional repressor of *qacA* expression (Paulsen *et al.* 1996b).

The repression of *qacA/B* transcription by QacR has been demonstrated to be overcome by some substrates of the QacA efflux protein (Eb, Cv, rhodamine 6G (R6), Bc, Ch and Pi) and to natural plant antimicrobials (berberine, palmatine and nitidine) (Grkovic *et al.* 1998, 2003). Structural analysis of the binding site of this soluble regulatory protein shows a large hydrophobic area. Binding of these disparate substances has been attributed by Neyfakh (2002) as a combination of hydrophobic effect and electrostatic attraction, rather than by establishing a precise network of hydrogen bonds and other specific interactions characteristic of traditionally studied enzymes and receptors. This suggests that the *qacA-qacR* system provides a general protection system against a broad range of hydrophobic toxic cations, rather than to a specific class of chemicals (Neyfakh 2002).

The antimicrobial pumps that are known to be under regulatory control belong to the *qacA/B* gene family, both of which utilise the PMF. The *smr* family has not been found to be subject to any regulatory controls (Grkovic *et al.* 2002). Other multidrug regulators identified include: BmrR, EmrR and MarR. BmrR is a transcription factor in *B. subtilis* that activates the expression of the multidrug transporter gene Bmr in response to binding a number of hydrophobic cations, many of which are also substrates of the Bmr protein (Markham *et al.* 1996). In *E. coli*, binding of the EmrR repressor to various neutral compounds (Xiong *et al.* 2000) relieves repression of the

EmrAB transporter gene. Also in *E. coli* the intracellular levels of the MarA protein, from the *marRAB* operon, is controlled by MarR, which represses the expression of its own gene and *marA* and *marB* (Grkovic *et al.* 2002). The *mar* locus controls the cells response to multiple toxic substances (Cohen *et al.* 1993a; Ma *et al.* 1993).

1.7 Families of Multidrug Transporters

Multidrug transporters are found in the membrane of all living cells protecting them from numerous structurally dissimilar hydrophobic compounds (Putman *et al.* 2000). It has been estimated that 5-10% of all bacterial genes are involved in transport and a large proportion of these encode efflux pumps (Webber and Piddock 2003). Substrates of multidrug transporters include many anticancer, antifungal and antibacterial drugs, which makes these transporters a serious hindrance in the treatment of cancer and infectious diseases.

Due to their ubiquitous nature multidrug transporters have been classified into several families based on aa similarities. Although the different families share no significant sequence identity, substrate specificity is often shared between them (Paulsen *et al.* 1996b). Of these families several are involved in cationic biocide resistance in both Gram-negative and Gram-positive organisms (Putman *et al.* 2000). The aa sequences of the *qacA*/*B* and *smr* translated proteins were described, compared and placed with the families below.

1.7.1 Major facilitator superfamily (MFS)

The MFS has the ability to catalyse transport of sugars, metabolic intermediates, antibiotics and antiseptics in organisms ranging from prokaryotes to eukaryotes (Marger and Saier 1993). MFS proteins are believed to be closely associated with cellular membranes, which has severely hampered efforts to define the exact molecular mechanisms by which the proteins function (Grkovic *et al.* 2002). However, they are known to confer resistance to cationic compounds by efflux from the cell using energy derived from the PMF (Paulsen *et al.* 1995). The multidrug efflux pump is embedded within the cytoplasmic membrane utilising the transmembrane gradient as a driving force for the efflux of organic cations (Paulsen

et al. 1996a). This prevents the build up of cationic compounds within the cytoplasm and cell death.

Members of the MFS family can be further divided into two separate clusters, those with 14 transmembrane segments (TMS) and those with 12 TMS (Paulsen *et al.* 1996b). The *S. aureus qacA* gene was the first gene encoding a PMF dependent multidrug efflux protein to be described and sequenced (Paulsen *et al.* 1996b). Hydropathy analysis of QacA and QacB proteins identified the structure as having 14 TMS, placing them in the MFS. Other MFS proteins with 14 TMS include Emr (*E. coli*), TetL (*B. subtilis*) and TetK (*S. aureus*) (Paulsen and Skurray 1993b; Xiong *et al.* 2000; Yerushalmi *et al.* 1995). Those with 12 TMS include NorA (*S. aureus*), TetA, TetB (*E. coli*) and Bmr (*B. subtilis*) (Nishino and Yamaguchi 2001; Yoshida *et al.* 1990).

1.7.2 Small multidrug resistance (SMR) family

The first gene to be described in this family was the staphylococcal *smr* gene. Proteins of the SMR family are the smallest multidrug transporters, typically 110 amino acids in length. Due to their small size it was initially unclear if Smr functioned as an independent multidrug transport protein, or if it interacted with other membrane proteins to pump cationic drugs from the cell. An *in vitro* experimental system was set up that was able to demonstrate that the expression of the *smr* gene alone was found to lead to the efflux of TPP and to a net decrease in the uptake of lipophilic cations (Grinius *et al.* 1992). A model for SMR transporter function was proposed by Paulsen (1996c), it was thought that multidrug efflux was catalysed by the first three TMS which are amphipathic with a number of conserved glutamate, serine, tyrosine and tryptophan residues located on the polar faces of these helices. These residues may form part of a transmembrane pathway through which protons and drugs pass (Paulsen *et al.* 1996b). The Smr protein was able to function as a drug pump on its own (Grinius and Goldberg 1994) using the PMF as its driving force.

Comparison of known Smr aa sequences has uncovered over 60 genes encoding Smr-like proteins in several bacteria, and on different plasmids and chromosomal

loci (Grinius and Goldberg 1994; Ninio *et al.* 2001). The *smr* gene product was found to be homologous to a family of small membrane proteins found in *E. coli*, *Pseudomonas aeruginosa, Agrobacterium tumefaciens* and *Proteus vulgaris* (Grinius *et al.* 1992; Nishino and Yamaguchi 2001; Purewal *et al.* 1990). In Gram-negative bacteria three cationic biocide resistance genes have been reported, *ebr, qacE* and *qacEΔ1* (Paulsen *et al.* 1993a; Purewal 1991). One particular member of the Smr family is Ebr, an *E. coli* protein, that has been extensively characterised and found to confer resistance to Eb and methyl viologen (Lewis 1994; Morimyo *et al.* 1992; Purewal 1991). Also showing a high degree of homology to staphylococcal *smr* are *qacE* and *qacE*Δ1 located on an integron found in Gram-negative bacteria (Paulsen *et al.* 1993a). These two genes have identical nt sequences except for the 3′ terminal sequences. It is believed that the *qacE*Δ1 gene represents a disrupted form of *qacE* that has evolved as a result of the insertion of a DNA segment near the 3′ end of the *qacE* gene (Paulsen *et al.* 1993a). Using fluorometric analysis the 115 aa, QacEΔ1 protein, was found to be resistant to Eb by an active export system, energised by PMF (Kazama *et al.* 1998; Littlejohn *et al.* 1990; Paulsen *et al.* 1993a). *qacEΔ1* has also been detected in clinical isolates of *Staphylococcus* and *Enterococcus*. *qacE* has not been detected in any other organism (Bass *et al.* 1999; Kazama *et al.* 1998). Classified by sequence homology the Sug proteins of *E. coli* were also placed in the SMR family (Chung and Saier 2002). The chromosomally located *sugI* locus is understood to be capable of phenotypically suppressing mutations in the molecular chaperone gene *groE* (Paulsen *et al.* 1996c).

The SMR family possesses a number of unique characteristics not observed in other transport systems. Members of this family are widely distributed and are located on plasmids, seen with staphylococcal *smr*, can also be stably maintained on the chromosome, seen with the *ebr* gene of *E. coli* and present on integrons throughout various Gram-negative bacteria. The members of this family are unaffected by inhibitors such as reserpine (Paulsen *et al.* 1993a) and do not display homology with any other known transport systems.

1.7.3 Resistance-nodulation-cell division (RND) family

Transporters of the RND family are usually components of a tripartite efflux system facilitating extrusion of substrates directly into external medium rather than the periplasmic space. This transport systems is only associated with Gram-negative bacteria. Transport across a Gram-negative membrane requires the interaction between the inner-membrane RND transporter, a membrane fusion protein (MFP) and an outer membrane factor (OMF) (Nishino and Yamaguchi 2001; Putman *et al.* 2000). MFP proteins contain a single N-terminal TMS and a large C-terminal periplasmic domain while the secondary structure of RND-type efflux proteins are thought to consist of 12 TMS (Putman *et al.* 2000). The MFP proteins are believed to induce fusion of the inner and outer membrane or form a channel-like structure that spans the periplasmic space.

One particular member of the RND family is the AcrA-AcrB-TolC efflux system of *E. coli*, long known for its resistance to antibiotics and biocides, including dyes (McMurry *et al.* 1998; Moken *et al.* 1997; Nikaido 1996; Okusu *et al.* 1996). The locus was characterised as containing two genes, *acrA* and *acrB*, located on a single operon (Ma *et al.* 1993). AcrA is the MFP while AcrB is a member of the RND family; both are required for drug resistance. Its expression is also regulated to some extent by the repressor AcrR (Ma *et al.* 1993). The normal physiological role of AcrAB is unknown, although it may assist cells in the protection against bile salts in the mammalian intestine (Nikaido 1998; Thanassi *et al.* 1997). A cluster of genes in the genome of *Haemophilus influenzae* has been found homologous to the *acr*AB locus. Disruption to the genes homologous to *acrA* (HI0894) or *acrB* (HI0895) resulted in the loss of resistance to several antibiotics and cationic dyes. Other members of the RND family include the MexA-MexB-OprM pump of *Pseudomonas aeruginosa* and MtrRCDE efflux system of *Neisseria gonorrhoeae* (Putman *et al.* 2000).

1.7.4 ATP-binding cassette (ABC) family

Unlike the MFS, SMR and RND family transporters where efflux is driven by the PMF, transporters of the ABC family utilise ATP as an energy source (Gottesman and Pastan 1993). The best-characterised efflux pump, P-glycoprotein encoded by

human and rodent genes, belongs to this multidrug transporter family. This ATP-dependant efflux pump mediates resistance to many cytotoxic drugs, used for chemotherapy. MsrA of *S. aureus* and LmrA of *Lactococcus lactis* are also members of this family (Bolhuis *et al.* 1994; Gottesman and Pastan 1993).

1.7.5 Multidrug and toxic compound extrusion (MATE) family

A new family of multidrug resistance pumps was recently identified in Gram-negative organisms and is referred to as the MATE family (Kaatz *et al.* 2005). The MATE family is characterised by the presence of 12 putative TMS and by the absence of "signature sequences" specific to the other multidrug transporter superfamilies. The energy source utilised by the MATE family of transporters has been identified as both the proton and sodium ion gradients (He *et al.* 2004; Morita *et al.* 1998). The first MATE pump characterised NorM was found in *Vibrio parahaemolyticus* and subsequent homologues have been identified in other Gram-negative organisms (He *et al.* 2004). The only members of the Gram-positive organisms so far found to have a MATE type pumps are *Clostridium difficile* and very recently in *S. aureus* (Dridi *et al.* 2004; Kaatz *et al.* 2005). The *S. aureus* MATE transporter was designated MepA and confers resistance to several cationic biocides. MepA is part of an operon, *mepRAB*, where MepB does not contribute to the function of MepA and MepR is a repressor of MepA (Kaatz *et al.* 2005; McAleese *et al.* 2005). MepR was stated as having strong homology with MarR-type regulatory proteins.

1.8 Consequences of QAC Resistance/Clinical relevance

1.8.1 Antibiotic and biocide cross-resistance in *S. aureus*

Several studies have suggested that as a consequence of the widespread use of biocides, selection of antibiotic-resistance strains in hospitals has occurred, a mechanism known as cross-resistance. Cross-resistance involves resistance to a number of different members of a group of chemically related agents, which are affected alike by the same resistance mechanism (Towner 2000). Cross-resistance can also be seen occasionally between unrelated antibiotics. This is different to multiple drug resistance, which involves resistance to several unrelated antibiotics by

different resistance mechanisms. If this proposal for cross-resistance is correct then there is also the same potential to arise in domestic environments were biocides have developed popularity (Bloomfield 2002; Russell 2000).

Cell wall changes may play a role in the observed cross-resistance between biocides and antibiotics, probably by reducing permeability (Pearce *et al.* 1999). These are changes occurring in the microbes that result in resistance to both biocides and antibiotics, which is a major cause for concern. Of equal significance is the possibility of genetic linkage between genes for biocide resistance and those for antibiotic resistance (Akimitsu *et al.* 1999). A molecular study performed by Sidhu (2001) demonstrated that some food-related staphylococci harboured both the QAC and the β-lactamase resistance genes on identical plasmids suggesting genetic linkage between the resistance determinants for the disinfectant, Bc and antibiotic, Pc, (Sidhu *et al.* 2001).

1.9 Significance of this Thesis

Microorganisms can adapt rapidly to a variety of environmental, physical and chemical conditions, and it is therefore not surprising that resistance to extensively used antiseptics and disinfectants have been found. The *qacA/B/smr* multidrug efflux pumps represent a growing clinical threat with the presence of one of these single systems in a cell increasing its resistance to a broad range of structurally dissimilar chemotherapeutic agents. The presence of *qacA/B/smr* genes on multi-resistance plasmids poses a control challenge to both the food-processing industries and to clinical settings of hospitals. Also the prevalence of QAC resistance in not only staphylococci but also other organisms, including enterococci, indicates that gene transfer both between different genera and different species within a genus is occurring at a high frequency.

The role of resistance to cationic compounds by staphylococci is unknown. It is also unknown what pressures led to the predominance of the pSK1 family of plasmids over the formerly prevalent β-lactamase/heavy-metal resistance plasmids in ‘clasic’ MRSA. If resistance is directed against cationic biocides in hospitals then these compounds are selecting MRSA and their use should be reviewed to prevent

colonisation and infection of patients. If resistance is targeting human innate defence mechanisms provided by cationic antimicrobial peptides then this may represent a significant shift in the virulence of these pathogens so that not only are they resistant to antimicrobial chemotherapy but also more resistant to innate defence mechanisms of the human body. Perhaps there are other more significant substrates for these multidrug transporters that have not yet been identified. It is essential to endeavour to understand how bacteria respond to antimicrobial pressures in various environmental niches and the reasons why lower resistance levels are encountered.

It is not known whether substrate exposure selects for clinical strains carrying cationic biocide resistance or if reduced biocide susceptibility then allows *S. aureus* to persist in the environment allowing patients who then enter the area to be exposed to the risk of nosocomial infection. Nor is it known whether exposure to cationic biocides selects for antibiotic resistant *S. aureus*. Certainly *qac* genes are predominantly found in antibiotic resistant rather than sensitive staphylococci. Cationic biocides at 'in-use' concentrations are unlikely to be a problem in relation to antibiotic resistance. Residual levels of cationic biocides have not been studied intensively and limited evidence to date suggests that it does not select for antibiotic-resistant bacteria. The clinical relevance of these compounds thus remains uncertain and further research is needed to clarify.

The number of multidrug transporters encoded in each genome is so large that their role in cellular physiology has remained uncertain. The study of multidrug transporters has presented a number of open questions concerning their evolution and biological role. Structural studies of multidrug transporter regulators suggest they possess large hydrophobic binding sites that use simple principles of substrate recognition to explain their broad substrate specificity.

An emerging theme among *S. aureus* strains is that extensive variation exists in gene content, this is particular poignant with respect to the gene content of their plasmids. Plasmids have the ability to carry, gather and disseminate genes that confer a wide range of properties on the bacteria that carry them, while imposing minimal burdens to their host (Towner 2000). In general these properties are not essential for the survival of the bacteria under normal conditions but which offer a survival advantage

in a particular environmental niche. The investigation of these mechanisms of virulence and pathogenesis has led to the sequencing of whole plasmid genomes. The genomes of several large and small *S. aureus* plasmids have been sequenced. They include the 46.4 kb multi-resistance conjugative plasmid pSK41 (Berg *et al.* 1998), the Fa resistant plasmid pUB110 (O'Brien, 2004 unpublished), and smaller plasmids pC194 (Horinouchi and Weisblum 1982a), pE194 (Horinouchi and Weisblum 1982b), pNVH01 (Bjorland *et al.* 2003), and pNVH99 (Bjorland *et al.* 2001).

The genes encoding resistance to QACs have been located on a range of plasmids. These plasmids range in size from large conjugative plasmids to small non-conjugative plasmids (Paulsen *et al.* 1998). One unusual cationic biocide-resistant plasmid is pWBG1773. Plasmid pWBG1773 is approximately 2.88 kb in size; it encodes resistance to Ay and the QACs, CTAB and Bc but differs from other staphylococcal plasmids carrying cationic biocide resistant determinants in that it is sensitive to Eb (Emslie *et al.* 1986). Investigation of plasmid pWBG1773 will increase knowledge and understanding of the molecular mechanisms behind QAC-resistance and in particular this unique plasmid. This study is designed to provide new information about cationic biocide resistance genes in staphylococci.

2 MATERIALS

2.1 Bacterial Strains and Plasmids

Nomenclature and significant properties of bacterial strains and plasmids are summarised in Table 2.1 and Table 2.2, respectively.

Table 2.1 – Bacterial strains and their significant properties

Strain	Characteristics	Source/Reference
Staphylococcus aureus		
RN4220	NCTC8325, restriction-deficient, plasmid-free host strain	Dr. Jostein Bjorland, Norwegian School of Veterinary Science, Norway
RN450 or WBG248	NCTC8325-4, plasmid free and non-lysogenic for ∅11, ∅12 and ∅13. Sensitive control strain for antimicrobial sensitivity testing.	(Novick 1991; Townsend *et al.* 1983c)
WBG541	RN450 mutated to chromosomal Fa^R and Rf^R. Recipient strain for staphylococcal plasmid-mediated conjugation.	(Townsend *et al.* 1983c, 1985e)
WBG1320	RN450 carrying plasmid pWBG53. Carries *qacA* and is used as a resistant control strain for antibacterial sensitivity testing.	(Emslie *et al.* 1985a; Townsend *et al.* 1984d)
WBG1876	WBG541 lysogenised with phage J. Recipient strain for staphylococcal phage-mediated conjugation.	(Townsend *et al.* 1984d, 1985e)

Table 2.1 cont.

Strain	Characteristics	Source/Reference
Staphylococcus aureus		
WBG1979	RN450 with plasmid pWBG72. Carries *qacC* and is used as a resistant control strain for antibacterial sensitivity testing.	(Emslie *et al.* 1985a)
WBG4364	Transcipient strain carrying plasmid pWBG1773. Source of plasmid pWBG1773 for cloning and test strain for antibacterial sensitivity testing.	(Emslie *et al.* 1986)
WBG4483	Strain constructed to carry a range of staphylococcal plasmids varying in size and phenotypic properties. Carries plasmids pWBG615, pWBG115, pWBG3 and pE194.	(Townsend *et al.* 1985e)
BMS11	RN4220 carrying plasmid pL150	This study
BMS12	RN4220 carrying plasmid pCL52.2	This study
Escherichia coli		
DH5α	Highly transformable strain of *E. coli* used for DNA manipulation.	Bethesda Research Laboratories
BMS02	DH5α carrying plasmid pBluescript®SK+ (pSK+).	Stratagene
BMS03	DH5α carrying plasmid pL150.	Dr Frances O'Brien, Curtin University, Western Australia
BMS04	DH5α carrying plasmid pCL52.2.	(Sau *et al.* 1997)
BMS05	DH5α carrying plasmid pDrive.	QIAGEN
DH5αPRO	Recombinant deficient host strain for protein expression.	BD Biosciences

Table 2.1 cont.

Strain	Characteristics	Source/Reference
Escherichia coli		
BMS07	DH5α carrying plasmid pPROTetE233.	This study
BL21(DE3)	Expression host with the T7 RNA polymerase gene under *lac*UV5 promoter control.	Novagen
BMS09	BL21(DE3) carrying plasmid pDrive.	This study

Fa = Fusidic acid; Rf = rifampicin; ^{R} = resistant; ∅ = phage.

Table 2.2 - Plasmids and their significant properties

Plasmid	Properties	Reference/Supplier
pCL52.2	Shuttle vector for transfer of plasmids between *E. coli* and *S. aureus*; 8.119 kb; Sp^R, Tc^R.	(Sau *et al.* 1997)
pDrive	Cloning vector; 3.85 kb; Amp^R, Km^R.	QIAGEN
pE194	3.5 kb; Em^R.	(Horinouchi and Weisblum 1982b)
pL150	Shuttle vector for transfer of plasmids between *E. coli* and *S. aureus*; 5.4 kb; Cm^R.	(Lee and Iandolo 1986, O'Brien *et al.* 2002)
pPROTetE233	Protein expression vector; 2.2 kb; Cm^R.	BD Biosciences
pSK+	Cloning vector; 2.96 kb; AmpR.	Stratagene

Table 2.2 cont.

Plasmid	Properties	Reference/Supplier
pWBG3	Plasmid molecular weight marker; 4.4 kb.	(Townsend *et al.* 1986)
pWBG53	Source of *qacA* for comparison of resistance phenotypes; 27 kb; Gm^R, Km^R, Tp^R, QAC^R, Eb^R, Pi^R.	(Emslie *et al.* 1985a; Townsend *et al.* 1984d)
pWBG72	Source of *qacC* for comparison of resistance phenotypes; 43 kb; QAC^R, Eb^R.	(Emslie *et al.* 1985a)
pWBG115	Plasmid molecular weight marker; 22.4 kb.	(Townsend *et al.* 1984d)
pWBG615	Plasmid molecular weight marker; 40.3 kb.	(Townsend *et al.* 1986)
pWBG1773	2.88 kb; QAC^R.	(Emslie *et al.* 1986)

Amp = ampicillin; Cm = chloramphenicol; Eb = ethidium bromide; Em = erythromycin; Gm = gentamicin; Km = kanamycin; Nm = neomycin; Pi = propamidine isethionate; QAC = quaternary ammonium compounds; Sp = spectinomycin; Tc = tetracycline; Tp = trimethoprim; ^{R} = resistant.

2.2 Media

Brand names and suppliers of the reagents and chemicals used to make media are provided in Appendix 7.1 and 7.2. All growth and storage media were made using distilled water (dH_2O), autoclaved at 121 °C for 15 min and stored at room temperature prior to use.

B2 broth

Casein hydrolysate	100 g
Glucose	5.0 g
K_2PO_4	1.0 g
NaCl	25 g
Yeast extract	25 g
dH_2O	1 L

Brain heart infusion agar (BHIA)

Bacteriological agar	15 g
Brain heart infusion powder	37 g
dH_2O	1 L

The ingredients were mixed together in dH_2O and heated until dissolved.

Brain heart infusion broth (BHIB)

Brain heart infusion powder	37 g
dH_2O	1 L

The powder was added to dH_2O and heated until dissolved.

40% Glycerol

BHIB	60 ml
Glycerol	40 ml

The solution was stored at 4 °C.

Mueller-Hinton agar (MHA)

MHA powder	38.0 g
dH_2O	1 L

The powder was added to dH_2O and heated until dissolved.

NYE agar

Casein hydrolysate	10 g
NaCl	5.0 g
Yeast extract	5.0 g
dH_2O	1 L

The ingredients were added to dH_2O and heated until dissolved.

Phosphate buffered saline (PBS)

KCl	0.2 g
KH_2PO_4	0.2 g
Na_2HPO_4	1.15 g
NaCl	8.0 g

The ingredients were dissolved in dH_2O, the pH was adjusted to 7.4 with 10 M NaOH and the volume made up to 1 L with dH_2O.

Saline

NaCl	8.5 g
dH_2O	1 L

Trypticase soy broth (TSB)

TSB powder	30 g
dH_2O	1 L

The powder was added to dH_2O and heated until dissolved.

Trypticase soy agar (TSA)

TSB powder	30 g
Bacteriological agar	15 g
dH_2O	1 L

The ingredients were mixed together in dH_2O and heated until dissolved.

2.3 Reagent Solutions and Buffers

The chemicals and reagents used to make solutions and buffers were obtained from various suppliers, these are summarised in Appendix 7.1 and their contact details in Appendix 7.2.

All reagent solutions and buffers were prepared using analytical grade chemicals. Unless otherwise stated reagent solutions and buffers were made using “high-pure” water (hpH_2O). The water was purified by a Milli-Q Ultrapure Water System (Millipore, Australia) to a conductivity of <18.2 MΩ-cm. The pH was adjusted as necessary. All solutions were stored at room temperature unless otherwise indicated.

2.3.1 Isolation and purification of plasmid DNA from staphylococci

E buffer (40X)

Ethylenediamine tetra acetic acid (EDTA)	23.38 g
Tris(hydroxymethyl)aminomethane (Trizma®)	193.76 g

The solution was adjusted to pH 8.0 with glacial acetic acid, made up to a final volume of 1 L in hpH_2O and autoclaved at 121 °C for 15 min. The stock solution was diluted 1:40 in hpH_2O to obtain a 1X working solution of 40 mM EDTA and 2 mM Trizma®.

Lysostaphin (150 µg/ml)

Lysostaphin	1.5 mg

The lysostaphin was dissolved in 10 ml hpH_2O at room temperature, dispensed into 1 ml aliquots and stored at -20 °C.

NE buffer

NaCl	146.1 g
EDTA	2.922 g

The pH was adjusted to 8.0 using 10 M NaOH before being made up to 1 L with hpH_2O. The solution was autoclaved at 121 °C for 15 min.

Ribonuclease (RNase)

RNase	10 mg/ml

The enzyme was dissolved in 1X E buffer, steamed for 20 min to destroy any deoxyribonuclease (DNase) and stored at 4 °C.

Sarkosyl lysing solution

CTAB	5.0 g
Sarkosyl	5.0 ml

CTAB was dissolved in hpH_2O, sarkosyl added and volume adjusted to 1 L with hpH_2O.

TE buffer

EDTA	0.292 g
Trizma®	1.211 g

The solution was adjusted to pH 8.0 with HCl, made up to 1 L with hpH_2O and autoclaved at 121 °C for 15 min.

2.3.2 Isolation and purification of plasmid DNA from *E. coli*

Alkaline lysis solution I (ALSI)

EDTA	2.923 g
Glucose	9.008 g
Trizma®	15.138 g

The pH was adjusted to 8.0 with HCl before the solution was made up to 1 L with hpH_2O. ALSI was autoclaved at 121 °C for 15 min then stored at 4 °C.

Alkaline lysis solution II (ALSII)

NaOH	8 g/L
Sodium dodecyl sulphate (SDS)	10 g/L

ALSII was prepared fresh to the required volume.

Alkaline lysis solution III (ALSIII)

Glacial acetic acid	11.5 ml
Potassium acetate (5 M)	60.0 ml
hpH_2O	28.5 ml

The resulting solution was 3 M with respect to potassium and 5 M with respect to acetate. ALSIII was stored at 4 °C.

10 M Ammonium acetate

Ammonium acetate	770 g
hpH_2O	1 L

The solution was sterilised by filtration through a 0.45 μm membrane filter.

Lysozyme

Lysozyme	10 mg/ml

Lysozyme was dissolved in 10 mM Tris-HCl (pH 8.0) and used immediately.

NaCl-PEG

Polyethylene glycol (PEG), MW 8000	13 g
1.6 M NaCl	100 ml

The solution was sterilised by filtration through a 0.22 μm membrane filter.

Phenol

Phenol was equilibrated to a pH >7.8 by the method of Sambrook and Russell (2001). Crystallised phenol was placed in a sealed container and melted in a 68 °C

water bath. In a fume hood an equal volume of 0.5 M Tris-HCl (pH 8.0) was added to the melted phenol. The mixture was stirred on a magnetic stirrer for 15 min. The two phases were then allowed to separate and the upper aqueous layer removed. An equal volume of 0.1 M Tris-HCl (pH 8.0) was again added to the remaining liquid, stirred and allowed to settle before again removing the top aqueous phase. This was repeated three times or until the pH of the phenolic phase was greater than 7.8. Equilibrated phenol was stored in 0.1 M Tris-HCl (pH 8.0) in a foil wrapped bottle at 4 °C for one month.

Phenol/chloroform

Chloroform	1 volume
Phenol (equilibrated to a pH >7.8)	1 volume

STE buffer

NaCl	5.844 g
EDTA	0.293 g
Trizma®	1.211 g

The pH was adjusted to 8.0 before being made up to 1 L in hpH_2O. The buffer was autoclaved at 121 °C for 15 min then stored at 4 °C.

TE buffer

EDTA	2.923 g
Trizma®	12.11 g

The powders were dissolved in hpH_2O and the pH adjusted to 8.0 with HCl before being made up to 1 L with hpH_2O. The solution was autoclaved at 121 °C for 15 min.

1 M Tris-HCl buffer

Trizma®	121.1 g

The Trizma® powder was dissolved in hpH_2O, pH adjusted to 8.0 with HCl and made up to a final volume of 1 L with hpH_2O. The final solution was autoclaved at 121 °C for 15 min. This 1 M stock solution was diluted 1:10 and 1:100 with hpH_2O to make 0.1 M and 10 mM Tris-HCl buffers, respectively.

2.3.3 Isolation of chromosomal DNA from staphylococci

Lysis buffer

EDTA	29.225 g
NaCl	8.766 g
Trizma®	12.11 g

The ingredients were dissolved in hpH_2O, pH was adjusted to 7.5 with HCl and made up to a final volume of 1 L with hpH_2O. The final solution was autoclaved at 121 °C for 15 min.

Lysostaphin (400 μg/ml)

Lysostaphin	15 mg
hpH_2O	37.5 ml

The powder was allowed to reach room temperature, dissolved in hpH_2O then stored at -20 °C.

SDS/ethanol

SDS	50 g
Ethanol	500 ml

The solution was made up to 1 L with hpH_2O.

2.3.4 Electrophoresis and visualisation of DNA

TAE buffer (40X)

EDTA	11.69 g
Sodium acetate	65.62 g
Trizma®	193.76 g

The ingredients were dissolved in hpH_2O and pH adjusted to 7.2 with glacial acetic acid before the buffer was made up to 1 L with hpH_2O and autoclaved at 121 °C for 15 min. The 40X stock solution was diluted 1:40 to obtain a 1X working concentration.

Tracking dye

Bromophenol blue	0.5 g
Ficol	20.0 g
E buffer (1X)	100 ml

The ingredients were dissolved in E buffer and stored at 4 °C.

2.4 Oligonucleotide Primers for DNA Sequencing and PCR

2.4.1 Sequencing primers

DNA sequencing of plasmid pWBG1773 was commenced by sequencing each end of the cloned plasmid using the universal M13 sequencing primers (Table 2.3). Additional primers were then designed with MacVector™7.2 or Primer3 (frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) within the known plasmid sequences to extend sequencing through the rest of the plasmid (Table 2.4). All sequencing primers were obtained from Sigma-Proligo and diluted to 2 pmol before being used in sequencing reactions.

Table 2.3 – Universal M13 sequencing primers (O'Brien, F., personal communication)

Primer	Sequence (5′ to 3′)	Size (nt)
M13 Forward	CAGGACGTTGTAAAACGAC	19
M13 Reverse	GGATAACAATTTCACACAGG	20

Table 2.4 – Primers designed to sequence plasmid pWBG1773

Primer	Primer sequence (5′ to 3′)	Size (nt)	Nt position in pWBG1773*
SeqR1	CTGTTACATTTGGCGTCG	18	1025←1042
SeqF1	ATGTAGATGGGCAGTGTC	18	2039→2056
SeqR2	CAAGACAAGAAGAGACTCG	19	514←531
SeqF2	AATGTCGGCATAGCGTGAGC	20	2494→2513
SeqR3	TTTCCCAACAAAAGCCGC	18	2876←2893
SeqF3	TCGGAAGAAGTGGTTGAAC	19	86→105
SeqR4	AATGCGTGTTCTACTCCC	18	2421←2438
SeqF4	AAGCAACTTTTTTATGGG	18	677→694

*Arrow indicates direction of replication by polymerase

2.4.2 PCR primers

All PCR primers were obtained from Sigma-Proligo and diluted to 62.5 pmol before use. PCR primers designed to sub-clone ORFs from plasmid pWBG1773 are listed in Table 2.5. Primers used to screen MRSAs for sequences similar to ORF2 and ORF3 in plasmid pWBG1773 are given in Table 2.6.

Table 2.5– Primers used to sub-clone ORFs from plasmid pWBG1773

Primer	Primer sequence (5′ to 3′)	Size (nt)	Nt position within pWBG1773*
ORF2A-F	AAAGATGTGGATCCGACTGAA	21	1510→1530
ORF2B-F	CGTTAAAAATGGCTGGGTACA	21	1724→1744
ORF2C-F	GGGTACAACCTGGGTACAA	20	1801→1821
ORF2D-F	GCTTCGCCAGACCATACATT	20	1873→1892
ORF2E-F	TTTTTGAGCCATTTTATGCAA	21	2005→2025
ORF2-R1	AAAGGGGTATTTTGAAGAAACA	22	2182←2203
ORF2-R2	AGCAAAACCAGTTGCGAAAT	20	2061←2081
ORF3-F1	AAGCAACTGTTATACCTGGGACA	23	2819→2842
ORF3-R1	TTTTTGTGCTCGAAACCAGA	20	586←605

*Arrow indicates direction of replication

Table 2.6 - Primers used to screen MRSAs for ORF2 and ORF3 sequences using PCR analysis

Primer	Primer Sequence (5′ to 3′)	Size (nt)	Nt position*
SCRN-ORF2F	GGGGTACAACCTGGGTACAA	20	1769→1788
SCRN-ORF2R	AGCAAAACCAGTTGCGAAAT	20	2061←2081
SCRN-ORF3F	TCGGAAGAAGTGGTTGAACTTT	22	87→108
SCRN-ORF3R	TCTAATTCTGTTACAGTAAATCCATCA	27	401←427

*Arrow indicates direction of replication

2.5 Construction of Plasmids and Strains

Several plasmids and strains were constructed for analysis of ORFs of plasmid pWBG1773. Sequences incorporating potential ORFs of plasmid pWBG1773 were amplified using PCR and sub-cloned into *E .coli* vectors and *E. coli*/*S. aureus* shuttle vectors. Table 2.7 lists the forward and reverse primers used for PCR amplification of potential ORFs. The construction of these plasmids and strains are summarised in Table 2.8.

Table 2.7 – Primer pairs used to amplify potential ORFs of plasmid pWBG1773

Potential ORFs	Forward primer	Reverse primer	Size (nt)	Nt position*
ORF2A	ORF2A-F	ORF2-R1	693	1510→2203
ORF2B.1	ORF2B-F	ORF2-R1	479	1724→2203
ORF2B.2	ORF2B-F	ORF2-R2	357	1724→2081
ORF2C	ORF2C-F	ORF2-R1	402	1801→2203
ORF2D	ORF2D-F	ORF2-R1	331	1873→2203
ORF2E	ORF2E-F	ORF2-R1	198	2005→2203
ORF3	ORF3-F1	ORF3-R1	702	2819→605
ORF2A&3	ORF2A-F	ORF3-R	2012	1510→605
ORF2B&3	ORF2B-F	ORF3-R	1798	1724→605
ORF2C&3	ORF2C-F	ORF3-R	1721	1801→605
ORF2D&3	ORF2D-F	ORF3-R	1648	1873→605
ORF2E&3	ORFE-F	ORF3-R	1516	2005→605

*Arrow indicates direction of replication

Table 2.8 – Strains designed for the characterisation of potential ORFs of plasmid pWBG1773 in *E. coli.*

Strain	Plasmid	Vector::potential ORF	Size (bp)	Host strain
BMS101	pBMS101	pSK+::pWBG1773	5876	DH5α
BMS102	pBMS102	pDrive::ORF2A	4543	DH5α
BMS103	pBMS103	pDrive::ORF2B.1	4329	DH5α
BMS104	pBMS104	pDrive::ORF2B.2	4207	DH5α
BMS105	pBMS105	pDrive::ORF2C	4252	DH5α
BMS106	pBMS106	pDrive::ORF2D	4181	DH5α
BMS107	pBMS107	pDrive::ORF2E	4048	DH5α
BMS108	pBMS108	pDrive::ORF3	4552	DH5α
BMS110	pBMS110	pDrive::ORF2A&3	5862	DH5α
BMS111	pBMS111	pDrive::ORF2B&3	5648	DH5α
BMS112	pBMS112	pDrive::ORF2C&3	5571	DH5α
BMS113	pBMS113	pDrive::ORF2D&3	5498	DH5α
BMS114	pBMS114	pDrive::ORF2E&3	5366	DH5α
BMS115	pBMS115	pL150::ORF2A	6093	DH5α
BMS116	pBMS116	pL150::ORF2B.1	5879	DH5α
BMS117	pBMS117	pL150::ORF2D	5731	DH5α
BMS118	pBMS118	pL150::ORF3	6100	DH5α
BMS119	pBMS119	pL150::ORF2A&3	7412	DH5α
BMS120	pBMS120	pL150::ORF2D&3	7048	DH5α
BMS121	pBMS121	pL150::ORF2E&3	6916	DH5α
BMS122	pBMS122	pCL52.2::ORF3	8821	DH5α

Expression of several of the constructed plasmids was analysed in staphylococci after transferring them to strain RN4220. The construction of these strains is summarised in Table 2.9.

Table 2.9 - Staphylococcal strains carrying shuttle vector with potential ORFs of plasmid pWBG1773

Strain	Plasmid	Vector::potential ORF	Host Strain
BMS132	pBMS115	pL150::ORF2A	RN4220
BMS133	pBMS116	pL150::ORF2B.1	RN4220
BMS134	pBMS117	pL150::ORF2D	RN4220
BMS135	pBMS118	pL150::ORF3	RN4220
BMS136	pBMS119	pL150::ORF2A&3	RN4220
BMS137	pBMS120	pL150::ORF2D&3	RN4220
BMS138	pBMS121	pL150::ORF2E&3	RN4220
BMS139	pBMS122	pCL52.2::ORF3	RN4220

Strains of *E. coli* BL21(DE3) with a gene for T7 RNA polymerase under control of a lacUV5 promoter were constructed carrying the ORF2 sequences inserted into plasmid pDrive (Table 2.10).

Table 2.10– BL21(DE3) strains

Strain	Plasmid	Vector::potential ORF	Host Strain
BMS140	pBMS102	pDrive::ORF2A	BL21(DE3)
BMS141	pBMS103	pDrive::ORF2B.1	BL21(DE3)
BMS142	pBMS104	pDrive::ORF2B.2	BL21(DE3)
BMS143	pBMS105	pDrive::ORF2C	BL21(DE3)
BMS144	pBMS106	pDrive::ORF2D	BL21(DE3)
BMS145	pBMS107	pDrive::ORF2E	BL21(DE3)
BMS146	pBMS108	pDrive::ORF3	BL21(DE3)

Several strains were constructed carrying two plasmids so that interaction between potential ORFs inserted into each plasmid could be analysed (Table 2.11).

Table 2.11 – Strains of *E. coli* DH5α constructed to carry two plasmids

Strain	Plasmids	Potential ORFs	Host Strain
BMS123	pBMS102 & pBMS122	ORF2A ORF3	DH5α
BMS124	pBMS103 & pBMS122	ORF2B.1 ORF3	DH5α
BMS125	pBMS104 & pBMS122	ORF2B.2 ORF3	DH5α
BMS126	pBMS105 & pBMS122	ORF2C ORF3	DH5α
BMS127	pBMS106 & pBMS122	ORF 2D ORF 3	DH5α
BMS128	pBMS107 & pBMS122	ORF 2E ORF 3	DH5α
BMS129	pBMS115 & pBMS122	ORF 2A ORF 3	DH5α
BMS130	pBMS116 & pBMS122	ORF 2B.1 ORF 3	DH5α
BMS131	pBMS117 & pBMS122	ORF 2D ORF 3	DH5α

An *E. coli* recombinant strain BMS160 was constructed by inserting an ORF2 sequence, ORF2B.1 into the expression vector pPROTetE233 (Table 2.12).

Table 2.12 - Strain constructed for protein expression analysis

Strain	Plasmid	Vector::potential ORF	Host Strain
BMS160	pBMS126	pPROTetE233:: ORF2B.1 (excised from pBMS103 with *Bam*HI and *Hind*III)	DH5αPRO

2.6 Antimicrobial Agents

2.6.1 Stock solutions

Stock concentrations of antibacterial compounds used in sensitivity testing are listed in Table 2.13. All antimicrobial stock solutions were stored at -20 °C.

Table 2.13 - Antimicrobial stock solutions

Antibacterial	Stock Concentration	Solvent
Ay	10 mg/ml	dH_2O
Af	10 mg/ml	dH_2O
Amp	100 mg/ml	dH_2O
Bc	10 mg/ml	dH_2O
Cd acetate	5.4 mg/ml	dH_2O
Cm	10 mg/ml	Ethanol
Ch	1 mg/ml	dH_2O
Cv	10 mg/ml	Ethanol
CTAB	10 mg/ml	dH_2O
4, 6-diamidino-2-phenylindole (DAPI)	1 mg/ml	dH_2O
Eb	60 mg/ml	dH_2O
Mercuric chloride	10.86 mg/ml	dH_2O
Pt	10 mg/ml	dH_2O
Phenyl mercuric acid	1.7 mg/ml	dH_2O
Pi	20 mg/ml for discs 10 mg/ml for plates	dH_2O
Quinaldine Red (Qr)	10 mg/ml	Ethanol
R6	10 mg/ml	Ethanol
Safranin O (SO)	10 mg/ml	dH_2O
Sp	50 mg/ml	dH_2O
Tc	1 mg/ml	dH_2O

2.6.2 Antimicrobial content of commercial discs for susceptibility testing

All antibiotic discs listed in Table 2.14 were supplied by Oxoid.

Table 2.14 - Content of commercial antibiotic sensitivity discs

Antibiotic Discs	Disc Content
Cm	30 μg
Ciprofloxacin	5 μg
Em	15 μg
Fa	5 μg
Gm	10 μg
Km	30 μg
Lincomycin	2 μg
Mupirocin	5 μg
Novobiocin	5 μg
Ox	1 μg
Pc	2 μg
Rf	5 μg
Sp	25 μg
Sm	25 μg
Sulphamethoxazole	300 μg
Tc	10 μg
Tp	5 μg
Vancomycin	30 μg

2.6.3 Antimicrobial content of discs prepared for susceptibility testing

Table 2.15 – Antimicrobial disc concentrations

Antimicrobial Agent	Concentration of Stock Solution	Volume of Stock Solution per Disc
Cd acetate	5.4 mg/ml	10 µl
Eb	60 mg/ml	10 µl
Mercuric chloride	10.86 mg/ml	10 µl
Phenyl mercuric acid	1.7 mg/ml	20 µl
Pi	20 mg/ml	10 µl

2.6.4 Replica plates for susceptibility testing and MIC determination

MHA was used as the base medium for all routine susceptibility testing as recommended by Clinical and Laboratory Standards Institute (CLSI), formerly the National Committee for Clinical Laboratory Standards (NCCLS) (CLSI 2006a). Two ml of antimicrobial solution was added to 18 ml of molten MHA that had been allowed to equilibrate at 50 °C in a water bath. The antimicrobial solution and agar were mixed thoroughly and poured into 85 mm sterile Petri dishes and allowed to set. The plates were inverted and dried at 37 °C for 15 min, wrapped in plastic and stored at 4 °C until required. The concentrations of antibiotic and antimicrobial plates are listed in Tables 2.16 and 2.17, respectively.

Table 2.16 – Replica plates for susceptibility testing

Antibiotic	Volume of stock solution per 100 ml of agar	Final concentration in replica plate
Amp	100 µl	100 µg/ml
Cm for:		
pL150 in *S. aureus*	60 µl	6 µg/ml
pPROTetE233 in *E. coli*	340 µl	34 µg/ml
Sp	100 µl	50 µg/ml
Tc	300 µl	3 µg/ml

Table 2.17 – Replica plates for MIC determination

Antimicrobial Agent	**Range of antimicrobial concentrations in replica plates (increments in concentrations)**	
	E. coli	***S. aureus***
Ay	10-400 μg/ml (10 μg/ml)	0.5-256 μg/ml (doubling dilutions)
Af	2.5-80 μg/ml (doubling dilutions)	0.5-32 μg/ml (doubling dilutions)
Bc	20-400 μg/ml (20 μg/ml)	1-10 μg/ml (1 μg/ml)
CTAB	40-200 μg/ml (10 μg/ml)	1-20 μg/ml (1 μg/ml)
Ch	0.025-10 μg/ml (0.025 μg/ml)	0.025-1 μg/ml (0.025 μg/ml)
Cv	0.25-256 μg/ml (doubling dilutions)	0.05-10 μg/ml (0.05 μg/ml)
DAPI	0.25-16 μg/ml (doubling dilutions)	0.025-1 μg/ml (0.025 μg/ml)
Eb	10-200 μg/ml (10 μg/ml)	0.5-40 μg/ml (1 μg/ml)
Pt	20-400 μg/ml (20 μg/ml)	1.5625-100 μg/ml (doubling dilutions)
Pi	10-400 μg/ml (20 μg/ml)	1.5625-100 μg/ml (doubling dilutions)
Qr	100-800 μg/ml (50 μg/ml)	0.5-40 μg/ml (doubling dilutions)
R6	100-800 μg/ml (100 μg/ml)	0.025-40 μg/ml (0.1 μg/ml)
SO	0.39-400 μg/ml (doubling dilutions)	0.5-32 μg/ml (doubling dilutions)

2.6.5 Turbidity standard for antimicrobial susceptibility testing

McFarland Turbidity Standard (0.5)

Barium chloride (0.048 M)	0.5 ml
Sulphuric acid (1% v/v = 0.18 M)	99.5 ml

2.7 Forced Induction and Expression of ORF2 sequences

2.7.1 Expression of ORF2 sequences in pDrive

Cathode buffer

Glycine	14.4 g
SDS	1.5 g

The ingredients were dissolved in hpH_2O, the pH was adjusted to 8.3 with powdered Trizma® and the volume made up to 1 L with hpH_2O.

Multiple Surfactant Solution (MSS)

ASB-14	0.1 g
Carrier ampholytes	25 µl
CHAPS	0.1 g
200 mM Tributyl phosphine (TBP)	50 µl
Thiourea	0.760 g
Trizma®	24.2 mg
Urea	1.5 g

The ingredients were dissolved and made up to 5 ml with hpH_2O and stored at -80 °C.

Equilibration solution

40% acrylamide/PDA stock	1.4 ml
Glycerol	4.0 ml
SDS	0.4 g
200 mM TBP	0.5 ml
5X Tris-HCl	4.0 ml
Urea	7.2 g

Standard Sample Solubilisation Solution (S4)

Carrier ampholytes	25 µl
CHAPS	0.2 g
200 µM TBP	50 µl
Trizma®	24.2 mg
Urea	2.4 g

The ingredients were dissolved and made up to 5 ml with hpH_2O and stored at -80 °C.

40mM Tris

Trizma®	0.242 g
hpH_2O	50 ml

Tris-HCl (5X)

Trizma®	90.8 g

The powder was dissolved in hpH_2O and pH was adjusted to 8.8 with HCl. The solution was then made up to a final volume of 400 ml.

2.7.2 Forced expression of ORF2B.1 in plasmid pPROTet

40% acrylamide/PDA stock

40% acrylamide	100 ml
piperazine diacrylamide (PDA)	1 g

The solution was covered in foil and stored at 4 °C.

Coomassie blue stain

Coomassie blue	0.1 g
Glacial acetic acid	10 ml
Methanol	50 ml

The solution was made up to a total volume of 100 ml with hpH_2O.

Running buffer

Glycine	14.4 g
SDS	1.0 g
Trizma®	3.03 g

The solution was made up to 1 L with hpH_2O.

1X SDS-PAGE sample loading buffer

Bromophenol blue	0.5 g
Dithiothreitol (DTT)	0.123 g
EDTA	0.003 g
Glycerol	1 ml
SDS	0.2 g
Trizma®	0.012 g

The ingredients were dissolved in hpH_2O, pH adjusted to 8.0 with HCl and the solution was made up to 10 ml with hpH_2O.

4X Separating gel stock buffer

SDS	4.037 g
Trizma®	18.165 g

The ingredients were dissolved in hpH_2O, pH was adjusted to 8.8 with HCl and the solution made up to a final volume of 100 ml with hpH_2O.

Separating gel (12.5%)

40% acrylamide/PDA stock	3.125 ml
4X Separating gel stock buffer	2.5 ml
Sodium thiosulphate (STS) solution	2 μl
hpH_2O	4.375 ml

The solution was degassed for 20 min then the following was added:

10% Ammonium persulfate (APS)	50 μl
TEMED	3.25 μl

The solution was loaded into a mini gel (7 cm x 7 cm) to a level 1 cm below the well former. Once loaded it was overlayed with butanol and allowed to set for 40 min.

Solubilisation solution

ASB-14	100 mg
Carrier ampholytes	25 µl
CHAPS	100 mg
200 mM TBP	50 µl
Thiourea	760 mg
Trizma®	24.2 mg
Urea	1.5 g

The ingredients were dissolved in hpH_2O, made up to a final volume of 5 ml with hpH_2O and stored at -80 °C.

4X Stacking gel stock buffer

SDS	4.037 g
Trizma®	6.055 g

The ingredients were dissolved in hpH_2O, pH was adjusted to 6.8 with HCl and the solution made up to a final volume of 100 ml with hpH_2O.

Stacking gel (4.5%)

40% acrylamide/PDA stock	3.125 ml
10% APS	50 µl
4X Stacking gel stock buffer	2.5 ml
TEMED	3.25 µl
hpH_2O	4.375 ml

The butanol was removed from the separating gel and rinsed with water. The stacking gel was added to the top of the separating gel, a comb inserted and left to set for 30 minutes.

3 METHODS

3.1 Storage and Recovery of Cultures

Stock cultures of bacterial strains were stored in 40% glycerol at -80 °C. Cultures were recovered from storage by inoculating the stock into BHIB and incubating overnight at 37 °C with shaking.

3.2 DNA Isolation

3.2.1 Isolation of plasmid DNA from staphylococci

The technique for isolating plasmid DNA from staphylococci was based on the CTAB method of Townsend *et al* (1985a).

Ten ml of overnight culture grown in BHIB was transferred into a 10 ml Oakridge tube and spun at 3,000 g for 10 min. The supernatant was discarded and the pellet resuspended in 900 µl of NE buffer and 100 µl of lysostaphin (150 µg/ml). The mixture was incubated in a 37 °C water bath for 30 min. Two ml of sarkosyl lysing solution was added at 37 °C. The tubes were placed in a 60 °C water bath for 10 min then spun at 15,000 g for 15 min at 30 °C. The supernatant was decanted into another 10 ml Oakridge tube containing 5 ml of hpH_2O and centrifuged at 3,000 g for 5 min. The supernatant was discarded and the pellet resuspended in 600 µl of 1X E buffer and 300 µl of NE buffer. Twenty µl of RNase (10 mg/ml) was added and mixed by gentle rolling before being incubated for 30 min in a 37 °C water bath. Seven hundred and fifty µl of chloroform was added and the tube was gently rocked 100 times or for 10 min to remove protein material. Tubes were spun at 3,000 g for 20 min and the top aqueous phase removed and placed in a microfuge tube. Seven hundred and fifty µl of isopropanol was added and the solutions mixed by inversion. The tube was spun at 13,500 g speed for 5 min, the supernatant discarded, the pellet dried at 35 °C for 40-50 min and then redissolved in 30 µl of TE buffer. The plasmid DNA was stored at -20 °C.

Further purification of plasmid DNA was required for cloning and sequencing reactions. Plasmid DNA was purified by precipitation with 0.3 M sodium acetate and 2.5 volumes of ethanol. The precipitate was centrifuged at 13,500 g for 5 min and the supernatant discarded. The pellet was washed in 70% ethanol and re-pelleted by centrifugation at 13,500 g for 5 min. The purified DNA pellet was then air-dried at room temperature and resuspended in half its original volume in hpH_2O. The purified plasmid DNA samples were then stored at -20 °C until required.

3.2.2 Isolation of plasmid DNA from *E. coli*

3.2.2.1 Maxi-preparation of *E. coli* plasmid DNA

Prior to cloning or sequencing from *E. coli*, plasmid DNA was prepared using a large-scale method followed by precipitation with PEG (Sambrook and Russell 2001).

Thirty ml of TSB containing an appropriate antibiotic was inoculated with a single colony and incubated overnight at 37 °C with shaking. This overnight culture was then used to inoculate 500 ml of BHIB pre-warmed to 37 °C and containing the appropriate antibiotic in a 1 L flask. The culture was incubated overnight at 37 °C with vigorous shaking. The bacterial cells were harvested by centrifugation at 2,700 g for 15 min at 4 °C. The supernatant was discarded and the bacterial pellet resuspended in 200 ml of ice-cold STE buffer. Bacterial cells were collected by centrifugation at 2,700 g for 15 min at 4 °C and then resuspended in 18 ml of ALSI. Two ml of freshly prepared lysozyme (10 mg/ml) and 40 ml of ALSII were added and the contents were mixed by inverting several times and incubated for 5-10 min at room temperature. Twenty ml of ice-cold ALSIII was added and the contents mixed gently and thoroughly by swirling the bottle several times. The bottle was placed on ice for 10 min. The bacterial lysate was centrifuged at 20,000 g for 30 min at 4 °C. Without disturbing the pellet the clear supernatant was decanted into a graduated cylinder and the volume measured. This was placed into a fresh centrifuge bottle together with 0.6 volumes of isopropanol. The contents were mixed and stored at room temperature for 10 min. Precipitated nucleic acids were recovered by centrifugation at 12,000 g for 15 min at room temperature and washed with 70%

ethanol at room temperature. The open bottle was inverted onto a pad of paper towels for 5 min at room temperature to allow all ethanol to drain. The damp pellet was dissolved in 3 ml of TE buffer (pH 8.0).

The crude plasmid DNA was further purified by precipitation with PEG. The plasmid preparation was transferred to a 40 ml Oakridge tube and chilled on ice. Three ml of an ice-cold solution of 5 M LiCl was added to the crude plasmid preparation, mixed well, and centrifuged at 12,000 g for 10 min at 4 °C. The supernatant was transferred to a fresh 40 ml Oakridge tube, an equal volume of isopropanol was added, mixed and the precipitated nucleic acids recovered by centrifugation at 12,000 g for 10 min at room temperature. The pellet was washed with 70% ethanol at room temperature before being dissolved in 500 µl of TE containing 20 µg/ml RNase. The solution was placed in a microfuge tube and stored for 30 min at room temperature. Five hundred µl of NaCl-PEG was added to this solution, mixed well and centrifuged at 12,000 g for 5 min at 4 °C. The supernatant was removed and the pellet dissolved in 400 µl of TE buffer then extracted once with an equal volume of phenol/chloroform then by an equal volume of chloroform. The aqueous phase was transferred to a fresh microfuge tube and 10 M ammonium acetate added to a final concentration of 2.5 M, along with 2 volumes of ethanol. The tube was incubated for 10 min at room temperature and centrifuged at 12,000 g for 5 min at 4 °C. The pellet of plasmid DNA was rinsed with 200 µl of 70% ethanol at 4 °C, vortexed briefly, and then centrifuged at 12,000 g for 2 min at 4 °C. Ethanol was removed by aspiration and the open tube stored on the bench for 10-20 min to allow the ethanol to evaporate. The damp pellet was dissolved in 500 µl of TE and stored at -20 °C.

3.2.2.2 Mini-preparation of *E. coli* plasmid DNA

A small-scale preparation of plasmid DNA was used to screen *E. coli* clones for recombinant plasmids carrying an insert of foreign DNA (Sambrook and Russell 2001).

TSB containing the appropriate antibiotic was inoculated with a single colony of transformed bacteria and incubated overnight at 37 °C with shaking. The overnight

culture was poured into a 1.5 ml microfuge tube and centrifuged at 13,500 g for 30 s. The supernatant was removed by aspiration and the bacterial pellet was resuspended in 100 µl of ice-cold ALSI with vigorous vortexing. Two hundred µl of freshly prepared ALSII was added to the bacterial suspension and mixed thoroughly by inverting the tube rapidly five times. The tube was placed on ice and 150 µl of ice-cold ALSIII was added and again mixed by inverting the tube several times. The tube was left on ice for 3-5 min before centrifugation at 13,500 g for 5 min. The supernatant was transferred to a fresh tube and the nucleic acids were precipitated from the supernatant by adding 2 volumes of ethanol at room temperature. The solution was vortexed and allowed to stand for 2 min at room temperature. The precipitated nucleic acids were collected by centrifugation at 13,500 g for 5 min and washed with 70% alcohol. All ethanol was carefully removed by gentle aspiration and the tube left open at room temperature until no fluid was visible. The nucleic acids were dissolved in 50 µl of TE containing 20 µg/ml RNase and stored at -20 °C.

3.2.2.3 Purification of plasmid DNA from mini-preparations

Plasmid DNA extracted by the mini-preparation method was further purified for ligation or sequencing reactions. Mini-preparations of plasmid DNA were purified by adding an equal volume of phenol/chloroform, vortexed for 10 s and centrifuged at 13,500 g for 2 min. The top aqueous phase was removed and placed in a clean microfuge tube to which was added 1.0 M sodium acetate to achieve a final concentration of 0.3 M followed by 2.5 volumes of ethanol. The precipitated plasmid DNA was centrifuged at 13,500 g, supernatant removed and all traces of ethanol evaporated by air-drying. Once dry the pellet was dissolved in 15 µl of hpH_2O and stored at 4 °C.

3.2.3 Isolation of chromosomal DNA from staphylococci

The protocol for isolating chromosomal DNA from staphylococci was supplied by Dr B. Bergi-Bachi (O'Brien, F., personal communication).

Strains were inoculated into BHIB and incubated overnight at 37 °C with shaking. Cells were harvested by centrifugation at 15,000 g for 1 min at 4 °C. The supernatant

was discarded, the pellet resuspended in 500 µl of lysis buffer and the cell suspension transferred to a 1.5 ml microfuge tube. One hundred and twenty five µl of lysostaphin (400 µg/ml) was added to the cell suspension and incubated at 37 °C for 30 min. One hundred µl of SDS/ethanol was added and the tube vortexed for 10 s before being left at room temperature for a further 5 min. Four hundred µl of phenol/chloroform was added to the tube and again vortexed for 10 s. The tube was spun at 15,000 g for 15 min at 25 °C. Two separate phases became visible and 500 µl of the upper phase was added to a fresh microfuge tube and overlayed with 1 ml of 100% ethanol at room temperature. The DNA was spooled out using a glass pipette and washed twice in ethanol and allowed to dry in air for approximately 1 min. Finally the DNA was dissolved in 300 µl TE buffer containing 2 µl of RNase (10 mg/ml). The DNA was placed on a shaker overnight at room temperature then stored at -20 °C.

3.3 RE Digestion

3.3.1 RE digestion of plasmid DNA

All RE reagents were added together on ice and comprised 1 µl of RE, 5 µl of staphylococcal plasmid DNA or 2 µl of plasmid DNA from *E. coli*, 2 µl of 10X RE buffer and the volume made up to 20 µl with hpH_2O. The solution was gently mixed and spun at 13,500 g for 1 min. The digest was then incubated in a 37 °C water bath for a minimum of 3 h. The reaction was stopped by incubation at 60 °C for 15 min.

3.3.2 RE digestion for cloning reactions

The cloning vector was converted into linear molecules by digestion with a suitable RE. The DNA used as the source of the cloned insert was digested with the same RE. Table 3.1 provides an example of the digestion reactions used to clone plasmid pWBG1773 into vector pSK+.

Table 3.1 - *Bam*HI digestion of vector pSK+ and plasmid pWBG1773

Reagent	**Vector pSK+**	**Plasmid pWBG1773**
DNA	15 µl	37.5 µl
hpH_2O	115 µl	90 µl
Bam*HI*	15 µl	15 µl
Buffer 10X	5 µl	7.5 µl
Total	**150 µl**	**150 µl**

3.4 Electrophoresis and Visualisation of DNA

3.4.1 Plasmid DNA

Agarose gels for electrophoresis of plasmid DNA were prepared by adding 0.6 g of molecular biology grade agarose to 100 ml of TAE buffer. The agarose was melted and poured into a 15 x 15 cm mould. Once the gel had set sample wells were loaded with 10 µl of plasmid DNA pre-mixed with 3 µl of tracking dye. Electrophoresis was carried out in a sub-cell system for 16 h at 22 V using a Bio-Rad PowerPac (300).

3.4.2 RE digests of plasmid DNA

RE digests of plasmid DNA were separated on 1.0% molecular grade agarose gels (15 x 15 cm). Sample wells were loaded with 10 µl of plasmid DNA pre-mixed with 3 µl of tracking dye. Electrophoresis was carried out in a sub-cell system for 16 h at 22 V using a Bio-Rad PowerPac (Model 300).

3.4.3 PCR products

An agarose gel was used to separate and visualise PCR products to estimate their size. A 2.0% gel was prepared with molecular biology grade agarose and sample wells loaded with 5 µl of the PCR product pre-mixed with 1.5 µl of tracking dye. A PCR molecular weight marker (1 kb DNA ladder, Invitrogen), that contains DNA fragments that range in size from 500 bp to 12 kb (see Appendix 7.3, for complete

size and range of fragments of the ladder), was also loaded into one of the wells in the gel. Electrophoresis was conducted in a sub-cell system for 90 min at 75 V.

3.4.4 Visualisation of DNA

After electrophoresis all gels were immersed for 30 min in 600 ml of hpH_2O containing 300 µl of Eb stock solution (1 mg/ml). Stained DNA was visualised with a UV transilluminator and photographed using Fuji instant black and white film, or with a Fluor-S™ MultImager (Bio-Rad). Gels were scanned and captured as digitised images.

3.5 Cloning Reactions

3.5.1 Alkaline phosphatase treatment of linear vector DNA

Vector DNA was converted into linear molecules using an appropriate RE. The 5′ end of linear vector DNA was dephosphorylated with alkaline phosphatase to prevent self-ligation of vector molecules. Two µl of calf intestinal alkaline phosphatase (CIAP; 1 U/µl), 10 µl of 10X CIAP buffer and 38 µl of hpH_2O were added directly to 50 µl of plasmid DNA digested with appropriate RE(s). The reagents were mixed gently and incubated for 30 min at 37 °C. An additional 2 µl of CIAP was added to the reaction followed by incubation at 37 °C for 30 min. The reaction was stopped by adding 2 µl of 0.5 M EDTA and incubating at 65 °C for 20 min.

3.5.2 Ligation of linear vector and DNA fragments

Linear vector DNA treated with alkaline phosphatase and linear DNA fragments for cloning were cleaned to remove all reagents by following the protocol for purification of *E. coli* plasmid DNA from mini-preparations. Prior to ligation both DNA preparations were electrophoresed at 70 V for 30 min in the same 1% agarose gel, stained with Eb and visualised. This provided a visual estimate of the comparative quantities of linear DNA molecules. Linear DNA fragments for cloning and linear vector DNA were mixed approximately in the ratio 1:1. The DNA mixture

was incubated at 60 °C for 10 min then placed on ice and 10X ligase buffer and ligase (Invitrogen, Australia) were added. The tubes were gently mixed and placed at 4 °C overnight. Appropriate controls were also set up in parallel. An example of a ligation mixture for cloning plasmid pWBG1773 in vector pSK+ is provided in Table 3.2.

Table 3.2 – Ligation mixture for cloning plasmid pWBG1773 in vector pSK+

Reagents	Test	Vector uncut control	Plasmid + ligase control	Vector + ligase control	Vector control
pSK+, cut with *Bam*H1 and dephosphorylated	8.5 µl	-	-	2 µl	2 µl
pWBG1773, cut with *Bam*H1	8.5 µl	-	2 µl	-	-
Ligase (1 U/µl)	1 µl	-	1 µl	1 µl	-
Ligase buffer 10X	2 µl	-	2 µl	2 µl	-
pSK+, uncut	-	2 µl	-	-	-
hpH_2O	-	18 µl	15 µl	15 µl	18 µl
Total	**20 µl**	**20 µl**	**20 µl**	**20 µl**	**20 µl**

3.5.3 Preparation of competent *E. coli* cells

The appropriate strain of *E. coli* was inoculated into TSB and incubated overnight at 37 °C with shaking. A 2% inoculum of the overnight TSB culture was then added to 40 ml of BHIB and incubated at 37 °C with shaking for 2.5-3.0 h. The optical density (OD) of the BHIB culture was monitored at a wavelength of 600 nm and when the OD reached a value between 0.4-0.6 the culture was placed on ice for 10 min. The cells were harvested at 6,000 g for 5 min at 4 °C then resuspended in 20 ml of ice-cold 0.1 M calcium chloride ($CaCl_2$), placed on ice for 1 h and centrifuged again at 6,000 g for 5 min at 4 °C. Finally the cell pellet was resuspended in 2 ml of ice-cold 0.1 M $CaCl_2$. The competent cells were used within 2 h of preparation.

3.5.4 Transformation of *E. coli* by heat shock

Fifty µl of freshly prepared competent *E. coli* cells and 2 µl of a ligation mix were combined in a microfuge tube and placed on ice for 20 min. The tubes were transferred to a 42 °C heat block and heat shocked for exactly 2 min then placed on ice for a further 2 min. Nine hundred and fifty µl of BHIB was added to each tube and incubated for 90 min at 37 °C with shaking. One hundred µl of the culture was lawn inoculated onto TSA selection plates containing Amp (100 µg/ml), X-gal (20 µg/ml) and IPTG (20 µg/ml) and incubated at 37 °C overnight.

White colonies were picked and subcultured onto fresh TSA selection plates and incubated overnight at 37 °C. Plasmid DNA was extracted from pure white colonies using the mini-preparation method for *E. coli* plasmid DNA, digested with the same RE used for linearising the cloning vector and visualised on an agarose gel to identify the desired recombinant plasmid.

3.6 DNA Sequencing

All sequencing reactions were performed at the West Australian Genome Resource Centre. Sequencing was performed with an ABI Prism 3730 48 capillary sequencer (ABI Biosystems) using the BigDye Terminator V3 kit. The thermal cycler conditions were 50 three-step cycles at 96 °C for 10 sec, 50 °C for 5 sec and 60 °C for 4 min.

3.6.1 Analysis of plasmid pWBG1773 sequence

The raw sequence data was exported from the ABI analyser into EditView™1.0.1, AssemblyLIGN™1.0.9, MacVector™7.2 (Accelrys, UK), VectorNTI®10.0 and ClustalX™1.8.3 (Thompson *et al.* 1997) software packages. Sequence assembly and alignments were carried out using EditView™1.0.1, AssemblyLIGN™1.0.9 and ClustalX™1.8.3. Analysis of the sequence for RE sites and maps was performed using MacVector™7.2 and VectorNTI®10.0. Nt sequences of plasmid pWBG1773 were compared with sequences in other plasmids and chromosomes by using an

online basic local alignment search tool (BLAST) at the National Centre for Biotechnology Information (NCBI) server (Altschul *et al.* 1990).

3.6.2 Sequence comparisons using DNA Strider

The DNA Strider 1.4 program was used to compare pairs of nt or protein sequences as a matrix, producing a dot plot. Dot plots detect similarities or differences between two sequences and can also detect internal structures such as repeated segments and IRs by comparing a sequence to itself. Identical nucleotides in each sequence are marked by a dot and if this identity covers a large area of sequence it is seen as an unbroken diagonal line. Where the diagonal line is broken, this indicates a difference between the two sequences. If there are gaps in identity between sequences the diagonal line will appear displaced either vertically or horizontally. Repeat regions can be found when a self matrix is performed and appear as diagonal lines stacked either vertically or horizontally and displaced by a distance equal to the length of the repeat unit. The Tandem Repeat Finder program (Benson 1999; tandem.bu.edu/trf/trf.html) was also used to identify secondary structure features such as repeat regions.

3.6.2.1 G+C% composition

The G+C% composition for plasmid pWBG1773 was calculated using the MacVector™7.2 nucleic acid property profile tool. The algorithm for calculating the percentages involved sampling every third base in a window of 50 codons, counting the number of G and C codons and plotting their combined percentages on a graph. This was repeated for the three different reading frames.

3.6.2.2 Open-reading frame (ORF) prediction and annotation

Open-reading frames (ORFs) within the nt sequence of plasmid pWBG1773 were predicted using the MacVector™7.2 ORF analysis tool. Using the start codons ATG, GTG and TTG and the stop codons TAA, TAG and TGA the program searched the six possible frames of the entire sequence of plasmid pWBG1773. ORF searches were performed looking for peptides with a minimum length of 80 aa. Predicted

ORFs were listed from largest to smallest in size and annotated according to their position, beginning with ORF1.

3.6.2.3 ORF analysis

Using the NCBI database, BLAST searches were performed on the nt (blastn), translated nt (blastx and tblastx) and protein (blastp) sequences for all predicted ORFs. The molecular mass of translated aa sequences was calculated using the aa composition function in MacVector™7.2. Protein and secondary structure analysis was also carried out on ORFs using the Protein Analysis Toolbox function in MacVector™7.2 looking at hydrophilicity, surface probability, flexibility and antigenic index. Predicted transmembrane domains were identified using the TMpred algorithm (www.ch.embnet.org/software/TMPRED_form.html). The SignalP 3.0 server (http://www.cbs.dtu.dk/services/SignalP/) was used to detect the presence of any potential signal peptide cleavage sites in the ORFs aa sequences.

3.7 Antimicrobial Susceptibility Testing

Antimicrobial susceptibility towards a range of antibiotics and heavy metals was performed by disk diffusion. Cationic biocides were found unsuitable for use in the disk diffusion method so antimicrobial susceptibility to them was determined using the minimum inhibitory concentration method.

3.7.1 Disk diffusion susceptibility testing

This method was performed as described by the Clinical and Laboratory Standards Institute (CLSI) (CLSI 2006b). Overnight BHIB cultures were diluted 1:1000 in saline or PBS and used to lawn inoculate MHA plates. Antibiotic discs were placed aseptically onto the MHA plates and incubated at 37 °C for 24 h. The diameters of the inhibition zones were measured to the nearest mm. The strains were interpreted as either sensitive or resistant to a particular antimicrobial agent by comparison to known sensitive and resistant controls. RN4220 and WBG1876 were used as plasmid free sensitive controls and, WBG1979 (*smr*) and WBG1320 (*qacA*) as positive controls.

3.7.2 Minimum inhibitory concentrations (MICs) and relative MIC values

Minimum inhibitory concentrations (MICs) of antimicrobials were determined by the agar dilution technique according to CLSI guidelines (CLSI 2006a). Serial dilutions of antimicrobial compounds (Table 2.17) were added to molten MHA, plates were poured and then dried at 37 °C for 20 min. Test and control strains were grown overnight in BHIB at 37 °C, diluted to a 0.5 McFarland standard then diluted 1:10 in sterile saline or PBS to obtain an inoculum concentration of 10^7 cfu/ml. The inoculum was added onto test plates by a multipoint replicator that delivers approximately 10^4 cfu/spot or 3 µl was inoculated directly onto test plates. The same suspensions were also replicated onto a MHA plate. Plates were incubated at 37 °C for 24 h and only spots with comparative confluent growth to that seen on the MHA plate were recorded as positive. The lowest concentration of antimicrobial compound recorded as negative was taken as the MIC. When testing strains of *S. aureus* RN4220 and WBG1876 were used as plasmid-free sensitive controls and WBG1979 (*smr*) and WBG1320 (*qacA*) were used as resistant controls. Likewise strains of *E. coli* were tested with DH5α as the plasmid-free sensitive control and BMS02, BMS03, BMS04 and BMS05 as vector controls.

Relative MIC values were calculated as the ratio of the MIC of the test strain to the sensitive reference strain.

3.8 PCR Amplification

PCR reagents were thawed and kept on ice at all times. Each 25 µl master mix contained 2.5 pmol/µl of both forward and reverse primers, 2.0 µl of plasmid DNA as template, 2.5 µl of 10X PCR buffer (Promega) and, at their final concentrations, 200 µM dNTPs (Fisher Biotech), 5 mM $MgCl_2$ and 0.25 U/µl of Taq DNA polymerase (5 U/µl, Promega). The final reaction volume was made up to 25 µl with hpH_2O. Table 3.3 provides a summary of the volume and concentration of each component in the PCR mixture.

Table 3.3 – Volume and concentration of reagents used in PCR amplification

Reagent	Volumes for 25µl of reaction mixture	Final concentration
hpH_2O	9.75 µl	-
Buffer 10X	2.50 µl	1X
25 mM $MgCl_2$	5.00 µl	5 mM
Taq polymerase (5 U/µl)	1.25 µl	0.25 U/µl
2 mM dNTPs	2.50 µl	200 µM
Forward primer (62.5 pmol)	1.00 µl	2.5 pmol/µl
Reverse primer (62.5 pmol)	1.00 µl	2.5 pmol/µl
Plasmid DNA template	2.00 µl	

PCR amplification was performed on an iCycler™ 96-well Reaction Module (Bio-Rad, U.S.A.). The protocol for the PCR cycling program is outlined in Table 3.4. The annealing temperature (T_m) of a PCR reaction depended directly on the length and composition of the primers and was adjusted according to the manufacturer's recommendations. All products were stored at 4 °C until required.

Table 3.4 – Thermal cycler settings for PCR amplification

Cycles	Temperature	Time	No. of Cycles
Initial denaturation	95 °C	4 min	1
3-step cycling:			
Denaturation	95 °C	1 min	
Annealing	50-65* °C	1 min	25
Extension	72 °C	1 min	
Final Extension	72 °C	1 min	1
Hold	4 °C	∞	1

*Varied according to the T_M of the primer as recommended by the manufacturer

To generate products larger than 900 bp the method was altered to reduce the stringency by increasing the $MgCl_2$ concentration from 1 mM to 4 mM and adjusting the annealing temperature from 60°C to 55°C.

All PCR products were separated by electrophoresis on agarose gels. The molecular weight of each product was estimated by comparison to a molecular weight ladder included in the electrophoresis.

3.9 Cloning Individual ORFs of Plasmid pWBG1773

3.9.1 Primer design and PCR amplification of ORFs of plasmid pWBG1773

PCR primers were designed using the Primer3 program (Rozen and Skaletsky 2000). All primers were supplied by Sigma-Proligo. The sequences of the PCR primers used in this project and their targets are provided in Tables 2.5 and 2.6. Purified *Bam*HI digested plasmid pWBG1773 DNA was used as the template in PCR amplifications unless otherwise stated.

3.9.2 Cloning PCR products into plasmid pDrive

Prior to cloning all PCR products were purified using Ultraclean™ PCR Purification kit from MOBIO Laboratories (USA). Purified PCR products were stored at -20 °C. The QIAGEN® PCR cloning kit was used to clone PCR products. Taq DNA polymerase generates a single adenine (A) overhang at the end of each PCR product. Linear pDrive cloning vector has a uracil (U) overhang at each end and was hybridised to the PCR products. Ligations were carried out according to the manufacturer's protocol. Ligation mixes were used to transform competent DH5α *E. coli* which was plated onto TSA plates containing Amp (100 μg/ml) and incubated overnight at 37 °C. Plasmid DNA was extracted from colonies using the mini-preparation method for *E. coli* plasmid DNA and visualised on an agarose gel to identify the correct recombinant plasmid.

3.9.3 Verification of the sub-clones

Taq DNA polymerase does not have a proof reading function so all inserts ligated into pDrive were sequenced to ensure they were identical to the original sequence in plasmid pWBG1773. M13 primers were utilised for sequencing.

3.9.4 Sub-cloning of plasmid pWBG1773 ORFs into *E. coli/S. aureus* shuttle vectors

The potential ORF2 and ORF3 sequences were sub-cloned into the *E. coli/S. aureus* shuttle vectors pL150 and pCL52.2 (Table 2.8). This was achieved by cutting out the sequenced insert from the *Eco*RI sites of pDrive, extracting the *Eco*RI fragment from an agarose gel, ligating into pL150 and/or pCL52.2 and transforming into chemically competent DH5α.

The inserts of potential ORF2 and ORF3 sequences were removed from an appropriate recombinant pDrive by *Eco*R1 digestion. A 1% agarose gel was prepared with three well formers taped together to produce one large well. Eight µl of tracking dye was added to 150 µl of digested pDrive and loaded into the single well. Electrophoresis was carried out in a sub-cell system for 90 min at 75 V. The gel was stained for 30 min in 600 ml of hpH_2O containing 300 µl of 1 mg/ml Eb stock solution. The DNA band was visualised over a UV transilluminator and excised from the gel. The volume of agarose was determined by weighing the excised band (1 g = 1 ml). The BRESAclean™ DNA Purification kit (Bresatec, Australia) was used to extract and purify the fragment from the agarose gel following the manufacturer's protocol. DNA was visualised by electrophoresis to confirm the extraction process. Purified DNA was stored at -20 °C.

Ligation mixes were prepared containing the extracted insert and the shuttle vector digested.with *Eco*RI. The ligation mix was transformed into freshly prepared competent *E. coli* DH5α. Transformants carrying ligated pL150 or pCL52.2 were selected on TSA plates containing Amp (100 µg/ml) or Sp (50 µg/ml), respectively. The desired recombinant plasmids were identified by restriction digestion with *Eco*R1 and detection of inserts having the correct size by agarose gel electrophoresis.

3.9.5 Generation of host cells carrying ORF2 and ORF3 sequences

Sequential transformation was performed to generate host cells carrying copies of potential ORF2 and ORF3 sequences each carried on separate vectors (Table 2.11). Each of the potential ORF2 sequences, ORF2A, ORF2B.1 and ORF2D were ligated

into plasmid pL150 to form plasmids pBMS115, pBMS116 and pBMS117, respectively. A copy of ORF3 was ligated into plasmid pCL52.2 to form plasmid pBMS122. Plasmids pL150 and pCL52.2 belong to different incompatibility groups and hence can replicate independently in the same host cell and they also carry different antibiotic resistance markers so each can be selected independently. BMS122, an *E. coli* DH5α strain carrying plasmid pBMS122, was made chemically competent and transformed with plasmid pBMS115, pBMS116 or pBMS117. Transformants were selected on TSA plates containing 50 μg/ml Sp and 10 μg/ml Cm. Colonies were subjected to mini-preparations of *E. coli* plasmid DNA and visualised by electrophoresis to ensure both recombinant plasmids were present in the host.

3.9.6 Electroporation of shuttle vector constructs into *S. aureus*

Although using the *E. coli* host is very convenient for gene cloning and analysis, it was considered important to analyse the ORFs of plasmid pWBG1773 in their native host *S. aureus*. Shuttle vectors pCL52.2 and pL150 carrying ORFs of plasmid pWBG1773 were transformed from *E. coli* into *S. aureus* by electroporation (Table 2.9).

3.9.6.1 Preparation of electrocompetent *S. aureus* cells

Electrocompetent staphylococcal cells were prepared as previously described (Schenk and Laddaga 1992). Cells were inoculated into 5 ml of B2 broth and incubated overnight at 35 °C with shaking. One ml of overnight culture was added to 25 ml of fresh B2 broth and incubated at 35 °C with shaking until the OD_{600} reached 0.4 to 0.7. Cells were pelleted by centrifugation at 8,000 g for 10 min at room temperature. Cells were washed three times with 20 ml of hpH_2O and centrifuged as before. After washing, the cells were resuspended in 120 μl of 10% glycerol, transferred to a 1.5 ml microfuge tube and harvested at 13,500 rpm for 5 min. The supernatant was removed and the pellet was resuspended in 60 μl of 10% glycerol and centrifuged at 13,500 rpm for 5 min. The cells were once again resuspended in 60 μl of 10% glycerol then incubated at room temperature for 15 min. The cells were

pelleted and resuspended in 800 µl of 10% glycerol. The competent cells were used within 1 h of preparation or stored as 50 µl aliquots at -80 °C.

3.9.6.2 Transformation of *S. aureus* by electroporation

Electroporation was carried out as previously described (Augustin and Gotz 1990). Fifty µl of competent cells was mixed with 2 µl of DNA at room temperature. The mix was then added to a 0.2 cm electroporation cuvette (Bio-Rad) and pulsed once with a GenePulser® (Bio-Rad) set to 200 Ω, 25 mF and 2.5 kV. The electroporated cells were immediately transferred to 950 µl of B2 broth pre-warmed to 37 °C and placed in a 37 °C water bath for 60 min. One hundred µl of the electroporated cell suspension was then plated out onto NYE agar containing the appropriate antimicrobial agent for selection. Plates were incubated overnight at 35 °C.

Transformed cells were analysed for their plasmid content to check they carried a plasmid of the appropriate size. Those found with the correct plasmid size were checked for their antimicrobial susceptibility.

3.10 Induction of Cationic Biocide Resistance

A variety of compounds were tested for their ability to induce increased resistance to cationic biocides in strains carrying plasmid pWBG1773. Compounds tested were Cm, Tc, sodium salicylate (Sal) and CTAB.

3.10.1 Induction of cationic biocide resistance with Cm, Tc and Sal

A modification of a method for induction was used (Cohen *et al.* 1993b). BMS101 carries plasmid pWBG1773 inserted into pSK+ and was used as the test strain and *E. coli* DH5α and BMS02 were used as plasmid free and vector controls, respectively. Strains were grown in BHIB overnight at 37 °C with shaking. The overnight broths were diluted 1:10 in fresh BHIB and incubated at 37 °C for 2-2.5 h. The broths were again subcultured 1:10 into fresh BHIB containing the inducing agent. Cm and Tc were added to give a final concentration of 2 µg/ml and 5 µg/ml, respectively (Hächler *et al.* 1991) and Sal was used at 5 mM (Cohen *et al.* 1993b).

The cultures were incubated for 1 h at 37 °C with shaking then diluted 1:10 in PBS. Three μl was then inoculated onto MHA selection plates containing CTAB ranging from 40-240 μg/ml in increments of 20 μg/ml. Plates were incubated overnight at 37 °C.

3.10.2 Induction of cationic biocide resistance with CTAB

S. aureus strain WBG4364 carrying plasmid pWBG1773 was tested for induction of cationic biocide resistance with CTAB. RN4220 and WBG1876 were used as plasmid-free sensitive controls and WBG1979 (*smr*) and WBG1320 (*qacA*) as positive controls. Induction was performed as above with the following modifications. The concentration of CTAB used for induction (1 μg/ml) was approximately 10-fold less than the MIC of WBG4364 to CTAB. Induced cultures were inoculated onto CTAB plates ranging from 2.5-30 μg/ml in increments of 2.5 μg/ml.

3.11 Forced Induction and Expression of ORF2 sequences

The role of the ORF2 gene product in the expression of resistance to cationic biocides was examined by placing ORF2 sequences under the control of inducible promoters.

3.11.1 Induction of cationic biocide resistance by ORF2 sequences cloned in plasmid pDrive

Each of the ORF2 sequences, ORF2A, ORF2B.1, ORF2C, ORF2D and ORF2E, was ligated into pDrive to form plasmids pBMS102, pBMS103, pBMS105, pBMS106 and pBMS107, respectively (Table 2.8). DNA sequence analysis was used to check that each insert of a potential ORF2 sequence was in the correct orientation and downstream from the T7 promoter of pDrive. The recombinant plasmids were transformed into the chemically competent *E. coli* host BL21(DE3) which contains the T7 RNA polymerase gene under the control of the *lacUV5* gene. The presence of IPTG (ranging from 0.1 to 10 mM) in CTAB replica plates was used to promote the synthesis of T7 RNA polymerase, which binds to the T7 promoter in pDrive and

induces expression of the target gene downstream of the promoter. All *E. coli* strains of BL21(DE3) carrying recombinant plasmids (Table 2.10) and control strain BMS09 were grown in BHIB overnight at 37 °C with shaking. The overnight broths were diluted 1:10 in fresh BHIB and incubated at 37 °C for 2-2.5 h. The broths were subcultured 1:100 into fresh BHIB. Three µl of the diluted culture was then inoculated onto MHA replica plates containing IPTG and CTAB (ranging from 40-240 µg/ml in increments of 20 µg/ml). Plates were incubated overnight at 37 °C.

3.11.2 Expression of ORF2A sequence in plasmid pDrive

Bioinformatic analysis of the ORF2 sequence indicates that the protein product may have a hydrophobic C-terminus anchored within the cell membrane. An extraction protocol for hydrophobic proteins was performed on strain BMS140, carrying ORF2A ligated into vector pDrive downstream of the T7 promoter (pBMS102). Strain BMS09 carrying the vector pDrive with no insert was used as a negative control. The protein extracts were compared by two-dimensional SDS-PAGE analysis.

3.11.2.1 Hydrophobic protein extraction protocol

Two hundred and fifty ml of BHIB containing 1 mM IPTG was inoculated and incubated overnight at 37 °C with shaking. The culture was harvested at 4,100 rpm for 15 min at 4 °C, the supernatant was discarded and the pellet was weighed. The pellet was resuspended in 10 ml of 40 mM Tris and 1 ml of protease inhibitor cocktail for every 4 g of pellet. The cell suspension was sonicated four times at 20 s intervals, resting on ice between each sonication and finally vortexed to ensure all large particles had dissipated. One hundred and fifty U of DNase and RNase was added, incubated at room temperature for 20 min and spun at 12,000 g for 8 min at 15 °C. The supernatant was discarded and the pellet resuspended in 1 ml of S4. The solution was vortexed, sonicated four times at 20 s intervals and vortexed again. The sonicated solution was incubated with 150 U each of DNase and RNase for 20 min at room temperature and spun at 12,000 g for 8 min at 15 °C. The supernatant was discarded and the remaining pellet was resuspended in 500 µl of MSS, vortexed, sonicated and vortexed again. The sonicated solution was spun at 12,000 g for 8 min

at 15 °C and the supernatant and stored at -80 °C. This extract contains highly hydrophobic proteins including membrane proteins.

Spectrophotometric (Bradford Assay) and two-dimensional SDS-PAGE analysis was performed on hydrophobic protein extract from BMS140 and BMS09.

3.11.2.2 Bradford assay

The Bradford Assay was performed using the Bio-Rad Quick Start™ Bradford Protein Assay and Bovine Gamma Globulin (BGG) Standard Set (Catalogue No. 500-02090). The method involves the addition of an acidic dye to protein solutions where a differential colour change of a dye occurs in response to various concentrations of protein. Spectrophotometric measurements are taken at a wavelength of 595 nm. Comparison to a standard set plotted on a graph provides a relative measurement of protein concentration.

3.11.2.3 Two-dimensional SDS-PAGE analysis

Two-dimensional SDS-PAGE technology was used to separate and analyse the hydrophobic content between the test strain carrying *orf2* and the control strain. By comparing the two-dimensional SDS-PAGE gels it can be determined if there is any differential expression which will hopefully lead to the identification of the ORF2 protein.

Depending on the size of the IPG strip and the stain being used the amount of protein loaded varied according to the manufacturer's instructions. The recommended amount of protein extract was loaded onto the IPG strip, pH 4-7, (ReadyStrip™ IPG Strip, Bio-Rad) for isoelectric focusing. Once the strip had been loaded it was left to rehydrate for 20 h.

Isoelectric focusing was performed using a Multiphor II Electrophoresis System (Pharmacia Biotech) using the following steps (Table 3.5) for a total of 30 h at 20 °C.

Table 3.5 – Isoelectric focusing parameters

Phase	Time (h)	Constant	Limits
Phase 1	2.5 h	300 V	1mA/5W
Phase 2	2.5 h	1000 V	1mA/5W
Phase 3	25 h	3000 V	1mA/5W

IPG strips were gently rocked in equilibration solution for 20 min. Strips were laid onto the surface of the slab gel and then embedded in a 1% molten agarose solution. Gels were run in cathode buffer at a constant 5 mA per gel for 2 h, then 20 mA per gel for 5 h. The bacterial proteins were detected by staining with coomassie blue for 24 h then placed in 1% acetic acid solution to enhance protein bands and remove background stain.

3.11.3 Expression of cationic biocide resistance by ORF2B.1 cloned in plasmid pPROTet

The sequence encoding potential ORF2B.1 was ligated into the expression vector pPROTet. This placed ORF2B.1 under control of $P_{Ltet0\text{-}1}$, a strong promoter so that expression of ORF2B.1 could be forced in the presence of anhydrotetracycline (anhTc).

3.11.3.1 Cloning ORF2B.1 sequence into the PROTet™6xHN protein expression system

The ORF2B.1 insert of plasmid pBMS103 was excised with *Bam*HI and *Hind*III and purified from an agarose gel. The insert was then re-ligated into the expression vector pPROTetE233 that had been linearised with *Bam*HI and *Hind*III. This plasmid pBMS126 was constructed in *E. coli* DH5α. DNA sequencing was used to confirm that ORF2B.1 was now located downstream from $P_{Ltet0\text{-}1}$ and in the correct reading frame of the start codon in vector pPROTetE233. Plasmid pBMS126 was transformed into competent *E. coli* DH5αPRO and selected on TSA plates containing 35 μg/ml Cm to produce the strain BMS160.

3.11.3.2 Induction of cationic biocide resistance with anhTc

MICs were carried out on BMS160, carrying plasmid pBMS126, in the presence of anhTc to determine if cationic biocide resistance was conferred by the ORF2B.1 sequence. Strain BMS07 was used as a negative control. The induction experiment was carried out as described for the induction of ORF2 sequences in plasmid pDrive. The diluted culture was inoculated onto MHA replica plates containing anhTc and CTAB (ranging from 40-240 μg/ml in increments of 20 μg/ml). The CTAB replica plates contained anhTc to a final concentration of 0.1 ng/ml. Plates were incubated overnight at 37 °C.

3.11.4 Protein expression of ORF2B.1 sequence in plasmid pPROTet

Total protein extracts induced with anhTc were analysed by SDS-PAGE to determine the optimal conditions for induction of gene expression. This was done initially to detect a protein induced from the cloned ORF2B.1 sequence. The insertion site within the vector pPROTet also provides an additional sequence that incorporates a 6xHN tag into ORF2B.1, which assists in the isolation of the protein.

3.11.4.1 SDS-PAGE analysis of protein induction

The expression of ORF2B.1, under the control of $P_{Ltet0\text{-}1}$, was optimised by varying the concentration of the inducer anhTc and the duration of incubation time. The method was taken from the PROtet™6xHN Bacterial Expression System User Manual (PT3161-1). Five ml of TSA containing 35 μg/ml Cm was inoculated with BMS160 and incubated at 37 °C overnight with shaking. Two ml of overnight culture was added to 100 ml of fresh TSA containing Cm and allowed to shake at 37 °C until it reached a reading of 0.5 at OD_{600}. This culture was then used to start five 10 ml cultures each induced with a different concentration of anhTc from 0-100 ng/ml. The cultures were incubated at 37 °C with shaking and 1 ml samples removed at time points 0, 2, 4, and 6 h. At each time point the cells were standardised to an OD_{600} of 0.5 by diluting in TSB then pelleted and stored at -20 °C.

Each induced sample was resuspended in 100 µl of 1X SDS-PAGE loading buffer, boiled for 5 min and spun for 10 min at 13,500 g. Ten µl of the supernatant was loaded into a mini (7 cm x 7 cm) SDS-PAGE gel comprised of 12.5% separating gel and a 4.5% stacking gel. The gel was placed in a tank containing running buffer and run at 150 V until the dye front reached the bottom. The gel was then stained in coomassie blue stain overnight, destained in 1% acetic acid solution and the induction expression of the protein observed by comparing uninduced and induced samples.

3.11.4.2 Extracting the 6xHN-tagged protein

Extraction of the 6xHN-tagged protein was carried as stated by the TALON® Purification Kit (Catalogue No.635606) manufacturer's protocol for standard sample preparation to isolate proteins and batch/gravity-flow column purification. One L of BHIB containing 35 µg/ml Cm was inoculated with a 20 ml overnight culture of BMS160 or BMS07. The culture was incubated for 2 h at 37 °C with shaking before being induced with anhTc at its predetermined optimal concentration and incubation time. The induced culture was divided into four 250 ml centrifuge tubes and harvested at 6,000 rpm for 10 min. The sample was prepared according to the manufacturer's instructions.

A Bradford assay was performed on the eluted proteins to determine their concentration. The protein samples of BMS160 and BMS07 were analysed by two-dimensional SDS-PAGE to determine if an extra protein was present in the test strain and not in the control.

Prior to loading the IPG strip for two-dimensional analysis of the protein any NaCl remaining in the eluted 6xHN-tagged protein was removed. This was achieved by using a 20-30% trichloroethanoic acid (TCA) to precipitate the protein out of solution. The protein was then pelleted at 16,000 g for 20 min at 4 °C. The pellet was washed 4 times with chilled acetone, spun at 10,000 g for 10 min at 4 °C and then air dried at 37 °C for 30 min to remove any remaining acetone. The pellet was stored at -20 °C prior to rehydration in solubilisation solution.

3.12 The Mechanism of Resistance to Cationic Biocides

3.12.1 Effect of CCCP on resistance to cationic biocides

CCCP is an uncoupler of oxidative phosphorylation, making the membrane permeable to H^+ ions and causing the collapse of the proton motive force (PMF) (Lambert and Le Pecq 1984). CCCPs ability to disable the PMF of a cell can be utilised to demonstrate inhibition of multidrug transporters dependant on this form of energy for efflux (Mitchell *et al.* 1999). CCCP was prepared as a solution in 1 mg/ml ethanol and then diluted to 0.1 mg/ml in 10 mM NaOH as described previously (Lambert and Le Pecq 1984). CCCP was used to determine if the multidrug transporter system of plasmid pWBG1773 was dependent on the PMF in *E. coli* and *S. aureus* host strains. Ten μM CCCP was used to test *E. coli* and, after some experiments to determine an appropriate concentration, 5 μM was used for testing *S. aureus* strains. Strains RN4220 and WBG1876 were used as plasmid-free sensitive controls and WBG1979 (*smr*) and WBG1320 (*qacA*) as positive controls. MICs to CTAB were measured by replica plating to duplicate sets of MHA plates containing CTAB with and without CCCP. MICs for *E. coli* strains were measured over the range 40-200 μg/ml, in increments of 20 μg/ml and *S. aureus* strains over the range 2.5-20 μg/ml in increments of 2.5 μg/ml. Overnight cultures were diluted to 0.5 McFarland in PBS and further diluted 1:10 to obtain the correct inoculum. The diluted strains were inoculated onto the plates by replica plating. The plates were incubated overnight at 37 °C.

3.13 Screening MRSAs for the Presence of ORF2 and ORF3 sequences

Preliminary experiments were performed to determine if the genes ORF2 and ORF3 were unique to plasmid pWBG1773 or are found in other MRSA strains from different locations. One hundred MRSA strains from all around the world were chosen to determine if they possessed sequences homologous to ORF2 and ORF3. Several MRSAs strains were chosen from each geographic location (Table 4.14). Strains were not selected on the basis of their resistance to cationic biocides.

Lyophilised strains of MRSA were recovered in BHIB and checked for purity on BHIA plates. All strains were stored in 40% glycerol at -80 °C.

3.13.1 Screening MRSAs for resistance to representative cationic biocides

MRSAs were tested for their MIC to four cationic biocides, each representative of a different class of compound. Eb is a cationic dye, Ch is a biguanide, Pi is a diamidino compound and CTAB is a QAC. Each MIC determination was performed over a range of dilutions with small increments in concentrations (Table 3.6). RN4220 and WBG1876 were included as plasmid free sensitive controls and WBG1979 (*qacC*), WBG1320 (*qacA*) and WBG4364 as resistant controls. Each MRSA strain was then categorised into one of six classes (Table 3.7).

Table 3.6 – Replica plates for determining MICs of MRSAs to cationic biocides

Compound	Range of concentrations in replica plates	Increments in concentration
Eb	10-100 μg/ml	10 μg/ml
Ch	0.25-1 μg/ml	0.25 μg/ml
Pi	10-100 μg/ml	10 μg/ml
CTAB	2-20 μg/ml	2 μg/ml

Table 3.7 – Phenotypic classification of MRSA based on MICs to cationic biocides

Cationic Compound	1	2	3	4	5	6
	MIC to cationic compounds (μg/ml)					
Eb	≥ 100	≥ 100	20-80	≤ 5	≤ 5	≤ 5
Ch	1.00	≤ 0.25	≤ 0.25	≤ 0.25	≤ 0.25	≤ 0.25
Pi	≥ 100	80	20-60	≤ 10	10-50	≤ 10
CTAB	≥ 20	6-10	≥ 20	≥ 20	10-20	≤ 4

3.13.2 Screening for ORF2 and ORF3 sequences in MRSA using PCR analysis

MRSA strains were examined for the presence of sequences homologous to ORF2 and ORF3. Gram-positive plasmid DNA isolation and purification was performed on the MRSA isolates. PCR was used to amplify internal regions of ORF2 and ORF3. The primers for the PCR are listed in Table 2.6. Chromosomal DNA from WBG541 and plasmid DNA from WBG4483 were used as negative controls, plasmid DNA from WBG4364 as the positive control and hpH_2O as a reagent control.

3.13.2.1 Plasmid analysis of MRSAs

All strains of MRSA producing an appropriate PCR product with ORF2 or ORF3 primers were analysed for their plasmid content using the CTAB method for plasmid isolation and visualised after agarose gel electrophoresis.

3.13.3 Sequence analysis of PCR products from MRSAs

A sample of PCR products amplified from MRSA strains with primers for ORF2 and ORF3 were sequenced. PCR products were purified using the Ultraclean™ PCR Purification kit from MOBIO Laboratories (USA) and then sequenced (using either the forward or reverse primer used in the initial PCR amplification. The resulting sequences were compared to plasmid pWBG1773 nt sequence using Clustal X™1.8.3.

4 RESULTS

4.1 Cloning and Sequencing of Plasmid pWBG1773

Plasmid pWBG1773 was isolated from staphylococcal strain WBG4364 using the CTAB method (Townsend *et al.* 1985a). The extracted plasmid DNA was electrophoresed on a 0.6% agarose gel and, based on its mobility, determined to be approximately 2.9 kb in size. RE analysis was performed on plasmid pWBG1773 using the enzymes *Bam*HI, *Cla*I, *Eco*RI and *Hind*III. Single sites were found for the RE's *Bam*HI and *Cla*I. No RE sites were identified for *Eco*RI and *Hind*III. Purified plasmid pWBG1773 was cut with *Bam*H1 and ligated into the cloning vector pSK+ to generate the plasmid pBMS101 (Figure 4.1).

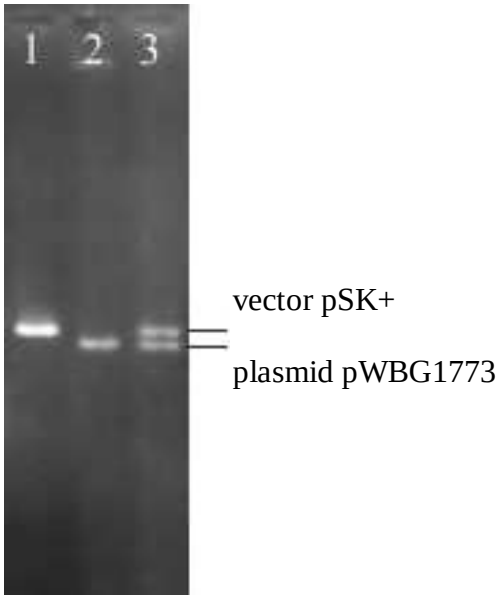


Figure 4.1 – *Bam*H1 digests of vector pSK+, plasmid pWBG1773 and cloned plasmid pWBG1773. Lane 1: vector pSK+ digested with *Bam*HI, lane 2: plasmid pWBG1773 digested with *Bam*HI, lane 3: plasmid pBMS101 digested with *Bam*HI.

Plasmid pBMS101 was isolated from *E. coli* BMS101 by the maxi-preparation method and purified by precipitation with PEG prior to sequencing. The pWBG1773 insert of pBMS101 was sequenced starting at each end of the *Bam*HI site using universal M13 forward and reverse primers (Table 2.3). Primers were designed complementary to the region approximately 50-100 bases from the 3′ end of the previous sequence and the PCR sequencing reaction continued. The complete

sequence of the pWBG1773 insert of plasmid pBMS101 was determined by the "primer walking" technique.

The entire sequence of the plasmid pWBG1773 insert of pBMS101 was determined in both directions. The forward and reverse sequence of the plasmid was obtained using a total of 8 primers (Table 2.4) and combined and aligned by AssemblyLIGN™1.0.9. The position of the overlapping sequences and their respective primers is depicted in Figure 4.2. The sequence file produced from each primer is provided in Appendix 7.4. The assembled sequence of the plasmid pWBG1773 insert was 2,916 bp with 100% concurrence of the sequence in forward and reverse directions.

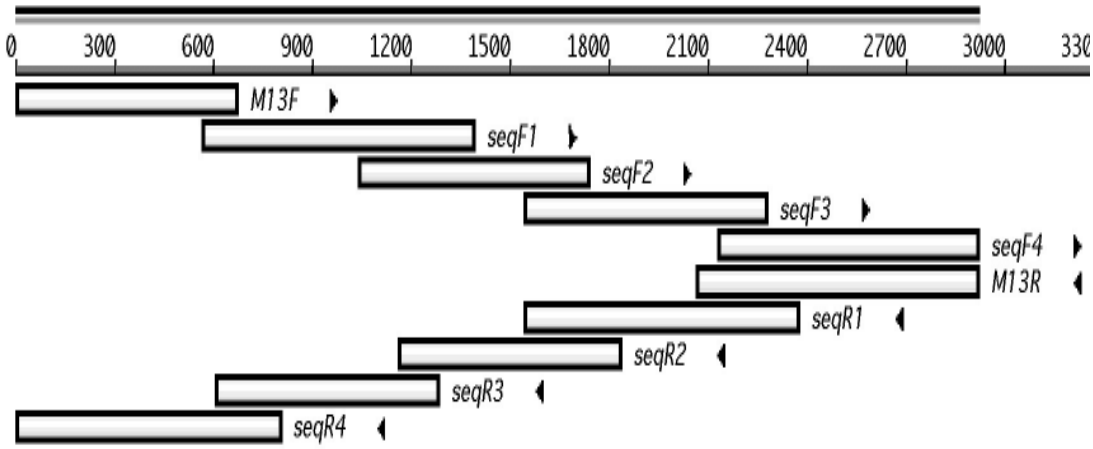


Figure 4.2 - Graphic representation of the sequencing files of plasmid pWBG1773 produced by primer walking. Each sequence is labelled according to the primer used and arrows provide direction of sequencing. The position of each sequence is located relative to the *Bam*HI RE site designated nt position 1. The graphic shows the extent of overlapping sequences in both the forward and reverse directions.

An additional sequencing reaction was performed on uncut purified plasmid DNA of plasmid pWBG1773 isolated from *S. aureus* strain WBG4364 using primer SeqF4. This confirmed that plasmid pWBG1773 was correctly sequenced across the *Bam*HI RE site and that only a single *Bam*HI RE site was present in plasmid pWBG1773. A full list of all RE sites of plasmid pWBG1773 is provided in Appendix 7.5.

4.1.1 Summary of the nt properties of plasmid pWBG1773

Plasmid pWBG1773 consists of 38.05% adenine, 10.73% cytosine, 18.14% guanine and 33.08% thymine. The average G+C content is 28.87%. A G+C plot of the three reading frames was produced using MacVector 7.2 nucleic acid property profile tool (Figure 4.3).

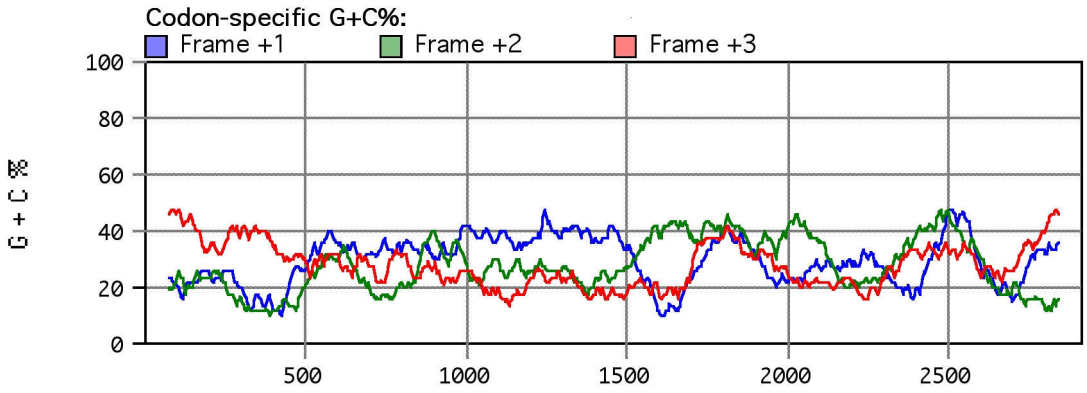


Figure 4.3 – Plot of the percentage G+C content of plasmid pWBG1773. The nt sequence of plasmid pWBG1773 is plotted on the x-axis and the G+C% on the y-axis. Each of the three reading frames, +1, +2, +3, is represented by a different coloured line, blue, green and red, respectively.

4.2 Analysis of the Nt Sequence of Plasmid pWBG1773

4.2.1 Comparison of plasmid pWBG1773 with other sequences in the GenBank database

BLAST searches of GenBank databases were performed on the sequence of plasmid pWBG1773. An overview of homologous sequences in the database aligned to plasmid pWBG1773 is shown in Figure 4.4a. The twenty most significant alignments are listed in Figure 4.4b in descending order of homology. Ten of the most significant alignments are with staphylococcal plasmids and the first six carry a *smr* gene. Plasmid pWBG1773 was found to share greatest homology with plasmid pPI-2, a 2779 bp plasmid carrying *qacC*, isolated from a Japanese *Staphylococcus warneri* (accession number AB125342.1).

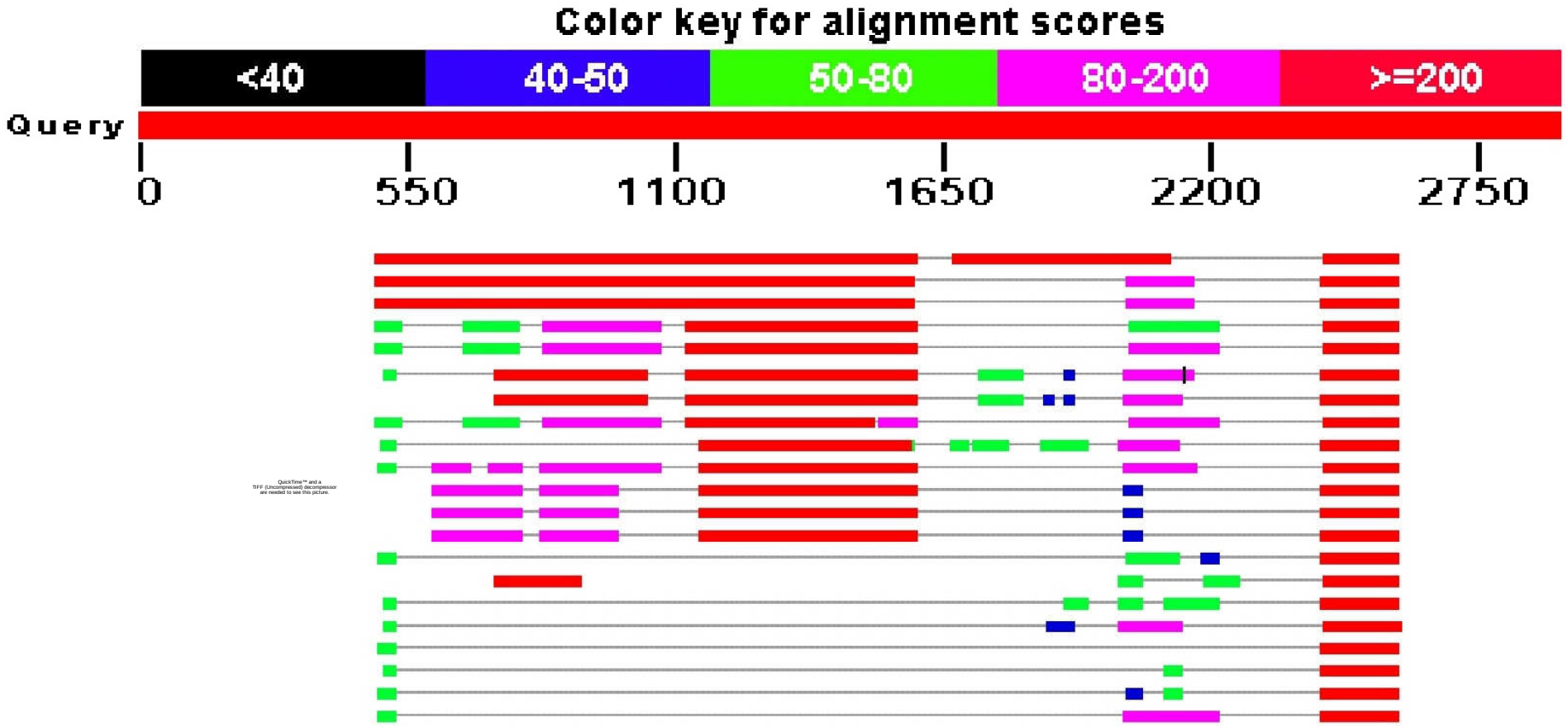


Figure 4.4a – A graphical illustration of the NCBI blastn result for the query sequence of plasmid pWBG1773. The score of each alignment is indicated by one of five different colours, which divides the range of scores into five groups. Multiple alignments on the same database sequence are connected by a striped line.

Accession	Description	E value
AB125342.1	Staphylococcus warneri plasmid pPI-2 DNA, complete sequence	0.0
AM040730.1	Staphylococcus warneri plasmid pSW49	0.0
AM040729.1	Staphylococcus warneri plasmid pSW174	0.0
AY092027.1	Staphylococcus epidermidis CH plasmid pSepCH, complete sequence	3e-179
M37889.1	Staphylococcus aureus replication (rep), control of replication (cop), and resistance protein (QacC) ger	3e-179
AF051917.1	Staphylococcus aureus plasmid pSK41, complete sequence	2e-152
AE017171.1	Staphylococcus aureus plasmid pLW043, complete sequence	2e-152
M33479.1	S.aureus ethidium resistance (ebr) and replication protein (repA) genes, complete cds	2e-137
AP006716.1	Staphylococcus haemolyticus JCSC1435 DNA, complete genome	2e-118
AM399082.1	Staphylococcus chromogenes pLNU9 plasmid	5e-110
U38693.1	Shuttle vector pSEmp18BBXM, complete sequence	2e-103
M91199.1	Staphylococcus xylosus BBXM (pSBBmp18XM) gene, 3'end	2e-103
M91198.1	Staphylococcus xylosus BBM3XM (pSBBM3XM) gene, 3'end	2e-103
AM184102.1	Staphylococcus chromogenes plasmid pLNU4, isolate KNS48	9e-84
AP003088.1	Staphylococcus aureus TY4, ETB plasmid DNA, complete sequence	1e-82
AY917098.1	Staphylococcus aureus strain a53 plasmid pBORa53, complete sequence	2e-81
CP000256.1	Staphylococcus aureus subsp. aureus USA300 plasmid pUSA01, complete sequence	2e-81
AJ512814.1	Staphylococcus aureus pNVH01 plasmid	2e-81
U81980.1	Staphylococcus aureus plasmid pKH4 replication protein Rep (rep) and quaternary ammonium compou	2e-81
AM399083.1	Staphylococcus epidermidis pLNU6 plasmid	8e-81
AM399080.1	Staphylococcus chromogenes pLNU8 plasmid	8e-81

Figure 4.4b – The blastn readout of plasmid pWBG1773 performed using the NCBI databases. Sequences producing significant alignments are listed in descending order with the highest scoring sequences at the top. The expect value (E) is a parameter that describes the number of hits expected by chance when searching a database of a particular size. The lower the E value the higher the homology between the query sequence and the sequences from the NCBI database.

4.2.2 Comparison of plasmid pWBG1773 and plasmid pPI-2 sequences

The complete sequences of plasmids pWBG1773 and pPI-2 were imported into the DNA Strider 1.4 program and plotted as a matrix to detect similarities between the sequences. A sliding window of 11 nt was used to reduce coincidental matches or noise and the stringency was set to 100% match of nucleotides. Diagonal lines seen in Figure 4.5 indicate the areas of greatest homology between these two plasmids. The first region of homology between these two plasmids corresponds to nt positions 490-2122 on plasmid pWBG1773 and nt positions 412-2033 on plasmid pPI-2. This corresponds to a region of pPI-2 comprised of its nick site, *rep* gene and a non-coding sequence. A second region of homology occurs at nt positions 2434-2591 on plasmid pWBG1773 and nt positions 2621-2779 on plasmid pPI-2 corresponding to the latter's SSO. The dot plot identifies regions of plasmid pWBG1773 that correspond to a potential replication gene (approximately nt positions 600-1550), nick site (approximately nt positions 500-570) and SSO (approximately nt positions 2430-2600). Plasmid pWBG1773 does not carry any sequences homologous to the *smr* gene, *qacC*, of pPI-2.

The sequences of plasmids pWBG1773 and pPI-2 were also compared using the Clustal X™1.8.3 alignment program. This result is provided in Appendix 7.6 and provides a detailed examination of the sequence homology between these two plasmids and further supports the DNA Strider 1.4 results.

DNA Strider 1.4 and Clustal X™1.8.3 were also used to compare sequences of plasmid pWBG1773 with five *smr*-carrying plasmids listed in Figure 4.4b. No sequences were detected with homology to known *smr* genes.

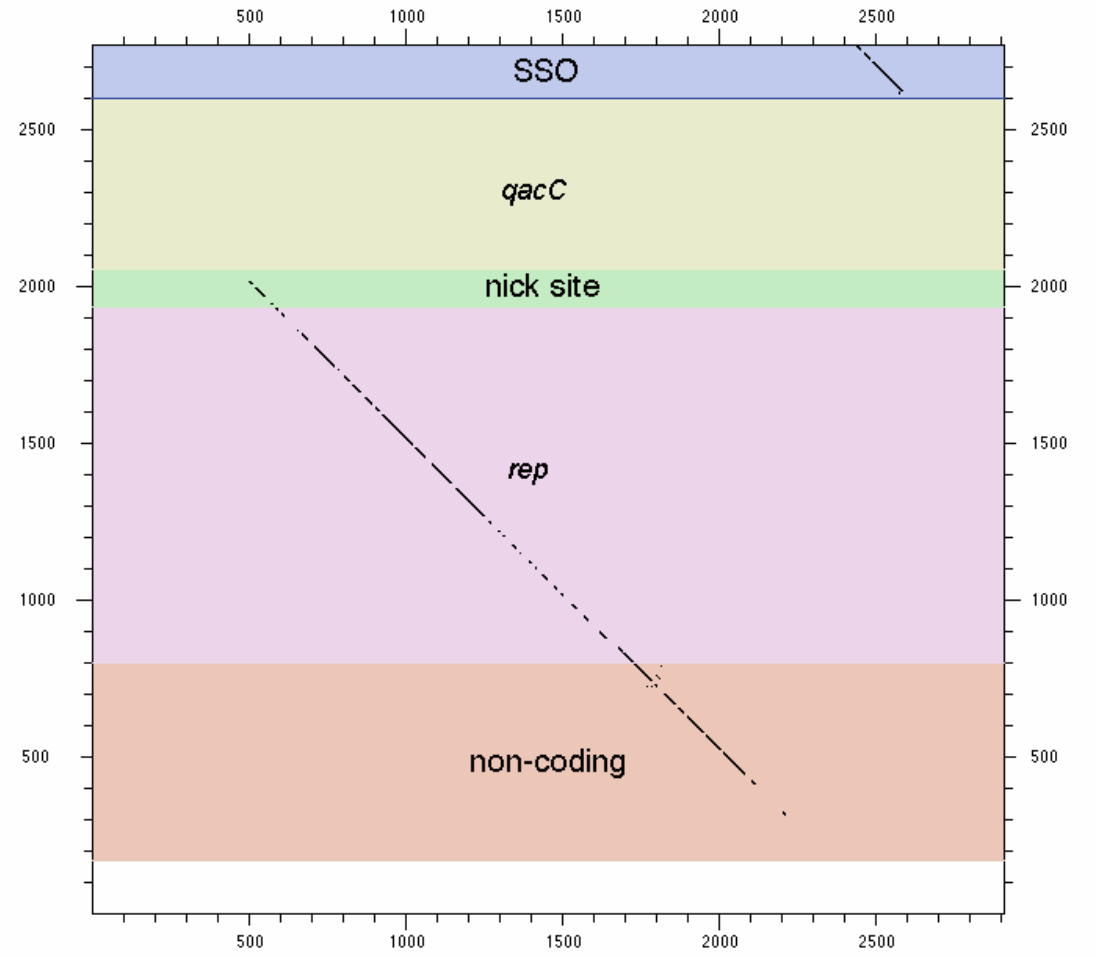


Figure 4.5 – A dot plot comparing the entire nt sequences of plasmids pWBG1773 (x-axis) and pPI-2 (y-axis). Areas of homology between the two plasmids are represented as a diagonal line. Known areas of plasmid pPI-2 (*qacC*, nick site, *rep*, non-coding and SSO) are segmented in different colours and labelled in the centre of each segment.

4.2.3 Coding regions of plasmid pWBG1773

The MacVector™7.2 program was used to analyse the six frames of plasmid pWBG1773 to identify potential ORFs with a minimum aa length of 80, using start codons ATG, GTG or TTG and stop codons TAA, TAG or TGA. Three predicted ORFs were found, all on the plus strand (Figure 4.6). Properties of the three predicted ORFs are summarised in Table 4.1. All three predicted ORFs have ATG start codons, ORF2 and ORF3 have TAA stop codons and ORF1 has a TGA stop codon. All predicted ORFs were submitted to the GenBank database for comparison to other sequences. ORF1 showed sequence similarity to a replication gene, ORF2 was found to have partial identity to a protein of unknown function and ORF3 was found to have homology to a gene involved in regulation (Table 4.1). No other

homology to any other staphylococcal genes was found. These three ORFs comprised 57.4% of the entire plasmid.

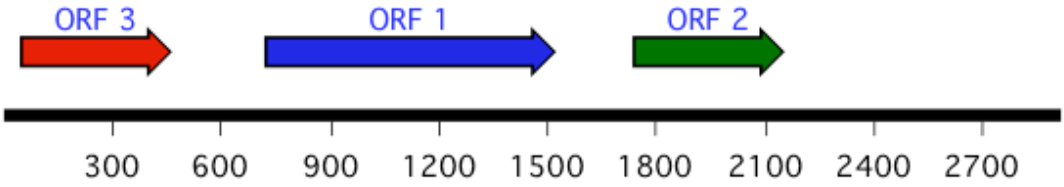


Figure 4.6 - Linear schematic of plasmid pWBG1773 showing the positions of the three ORFs found on the plus strand. Each reading frame is represented by a different colour, +1 blue, +2 green and +3 red.

Table 4.1– Properties of potential ORF products of plasmid pWBG1773 and similarities to proteins in the NCBI database

ORF in plasmid pWBG1773	Position/ coding direction	G+C (%)	Size (aa)	Homologous protein					
				Designation	Possible function	Organism	Match length (aa)	Identity (%)	Accession number/ Reference
ORF1	736⇨1659	29.1	308	Rep protein	replication	*S. warneri*	249/261	95	NP940782
ORF2	1732⇨2163	33.6	144	8.7 kDa protein	unknown	*S. aureus*	26/44	59	CAA24587/ PO386
ORF3	42⇨473	26.3	143	MarR	regulation	*B. cereus*	58/140	41	AAU15466

4.2.3.1 Analysis of pWBG1773 sequence for transcription and translation signals

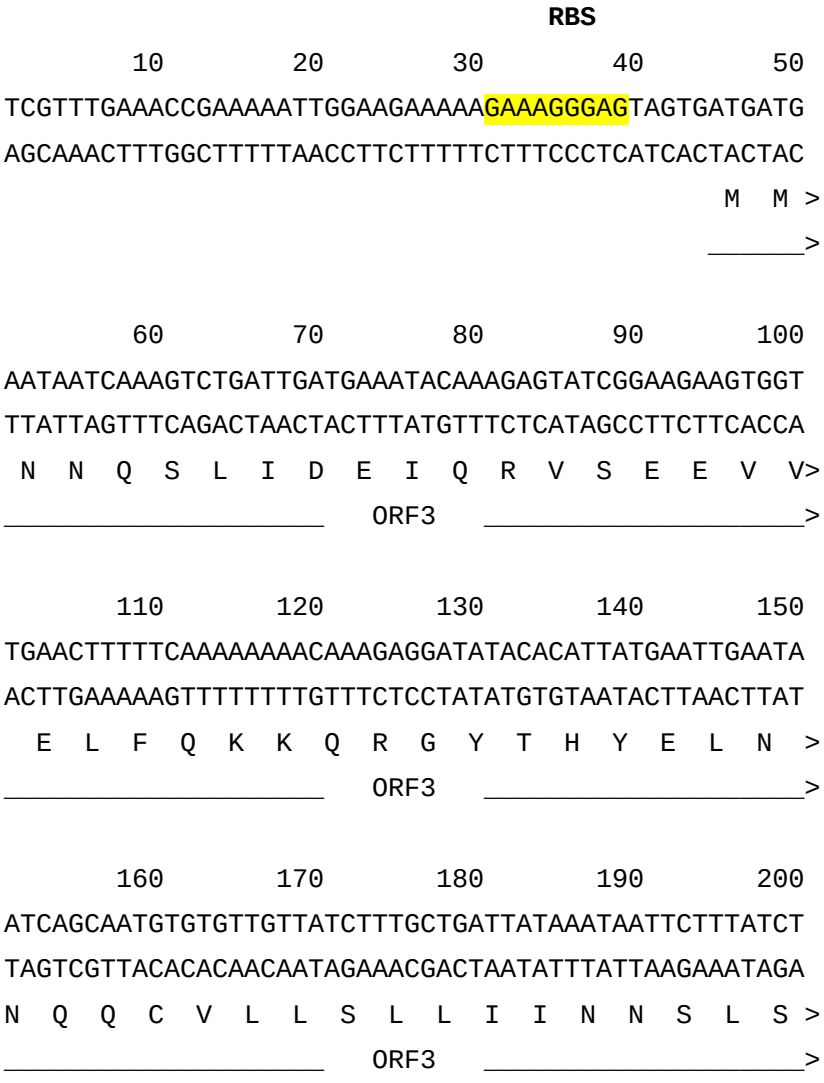
Ribosome binding sites (RBSs) or Shine-Dalgarno sequences typically have a consensus sequence of AGGA or GGAGG and are usually located 4-7 nt 5′ or upstream from a start codon (Davies and Rood 2006; Hawley and McClure 1983; Rao *et al.* 1995). No RBS fitting this description could be located upstream from the putative start codons for ORF1 or ORF2. A potential 5 bp RBS (5′-AGGAG-3′) is located at nt position 669-673 but is 63 bp prior to the predicted start codon for ORF1. Also a potential 4 bp RBS (5′-AGGA-3′) is located 79 bp upstream from the ORF2 start codon at nt position 1650-1653. There are also potential RBS within the sequences of ORF1 and ORF2, both positioned upstream of a potential start codon lying within the ORF. A potential 7 bp RBS (5′-AAGAAGG-3′) is located within ORF1 at nt position 803-809 and is 4 bp upstream of a methionine site at nt position 814-816. ORF2 has a potential 4 bp RBS (5′-AGGA-3′) located at nt position 1983-1986 and is 33 bp upstream of a methionine site at nt position 2020-2022. The ATG start codon of ORF3 is preceded 5 nt upstream by a 9 bp putative RBS (5′-GAAAGGGAG-3′) at nt position 31-39. All the potential RBS sequences in relation to the ORFs are highlighted in Figure 4.7.

The sequences upstream from ORF1, ORF2 and ORF3 were scanned for potential promoter regions. The promoter region consists of two short sequences located -10 and -35 bp upstream from the transcription start site. The sequence at -10 is known as the Pribnow box or -10 signal and has a consensus sequence TA***T with 50% of cases being TATAAT (Graves and Rabinowitz 1986). The sequence at -35 is called the -35 signal and predominantly consists of six nucleotides TTGACA (Graves and Rabinowitz 1986). Two potential RBS were identified for ORF1, the first RBS at nt position 669-673 is preceded by a potential -10 signal (5′-TAAAAC-3′) and a potential -35 signal (5′-TGTATC-3′). The second RBS to be identified for ORF1 at nt position 803-809 is preceded by a potential -10 signal (5′-TAAAAG-3′) but no sequence resembling a -35 signal was identified. ORF2 also has two potential RBS and potential promoter regions were identified upstream of these. Seventeen bp upstream of the first RBS in ORF2 at nt position 1650-1653 is a potential -10 signal (5′-TCAAAT-3′) and 22 bp prior to that is a sequence resembling a -35 signal

(5′-CTGAAC-3′). The second RBS identified for ORF2 located at nt positions 1983-1986 is preceded by a -10 signal (5′-TAAAAT-3′) and a -35 signal (5′-CTTACA-3′). Only ORF3 has a RBS (5′-GAAAGGGAG-3′) within close proximity of the predicted start codon, 5 bp upstream. The sequence 5′-TTTAAT-3′ has five matches to the consensus -10 promoter sequence of Gram-positive bacteria (Graves and Rabinowitz 1986) and was found 99 nt upstream of the start codon of ORF3. The sequence 5′-TGGGACA-3′ has several matches to the consensus -35 promoter sequence and is located 20 nt upstream of this -10 promoter sequence.

4.2.4 The complete annotated sequence of plasmid pWBG1773

The complete annotated sequence of plasmid pWBG1773 is presented in Figure 4.7. The sequence has been submitted to the NCBI public database and can be found under the GenBank accession number EF537646



```
       210       220       230       240       250
CCGATTGAGTTAGCTGATGAAATGAAGATTTCTAAGAGTGCTGTTAGTCA
GGCTAACTCAATCGACTACTTTACTTCTAAAGATTCTCACGACAATCAGT
 P  I  E  L  A  D  E  M  K  I  S  K  S  A  V  S  Q>
_____________________   ORF3   _____________________>

       260       270       280       290       300
GTTATTATTAAAATTAGAAAATGAAGGTTTTGTGATTCGAAAAAAATATA
CAATAATAATTTTAATCTTTTACTTCCAAAACACTAAGCTTTTTTTATAT
  L  L  L  K  L  E  N  E  G  F  V  I  R  K  K  Y  >
_____________________   ORF3   _____________________>
       310       320       330       340       350
AGAATGATAAGCGTTCTGTAACTGTTGAATTAGATGAAAAAGGTAAAATT
TCTTACTATTCGCAAGACATTGACAACTTAATCTACTTTTTCCATTTTAA
K  N  D  K  R  S  V  T  V  E  L  D  E  K  G  K  I >
_____________________   ORF3   _____________________>

       360       370       380       390       400
TATAAAGAAGAAATGAAAGAGTTTGAAAAAGAAATATTAAAGAAATATTA
ATATTTCTTCTTTACTTTCTCAAACTTTTTCTTTATAATTTCTTTATAAT
 Y  K  E  E  M  K  E  F  E  K  E  I  L  K  K  Y  Y>
_____________________   ORF3   _____________________>

       410       420       430       440       450
TGATGGATTTACTGTAACAGAATTAGATAATATGCTTGAATTAATAAAGA
ACTACCTAAATGACATTGTCTTAATCTATTATACGAACTTAATTATTTCT
  D  G  F  T  V  T  E  L  D  N  M  L  E  L  I  K  >
_____________________   ORF3   _____________________>
       460       470       480       490       500
AAAGTAGAGATATTTTATTGTAAAAAGTAGTTTTATTTTAGGTTTTAAAA
TTTCATCTCTATAAAATAACATTTTTCATCAAAATAAAATCCAAAATTTT
K  S  R  D  I  L  L  * >
______   ORF3   _______>

       510       520       530       540       550
ACGAGTTGAAAACGAGTCTCTTCTTGTCTTGATACTATAGGTAACTATTA
TGCTCAACTTTTGCTCAGAGAAGAACAGAACTATGATATCCATTGATAAT

       560       570       580       590       600
CAAGCTACGATAGTTACCTTTAATCTGTTGGTATATCTGGTTTCGAGCAC
GTTCGATGCTATCAATGGAAATTAGACAACCATATAGACCAAAGCTCGTG
```

610 620 630 640 650
AAAAAAAGTTGCTTTTTCGTACCTAGTAATGTATCGTTTAAGGTGTCTAA
TTTTTTTCAACGAAAAAGCATGGATCATTACATAGCAAATTCCACAGATT

RBS

660 670 680 690 700
TCTAAAACTTTACATGAAAGGAGAAAAAGCAACTTTTTTATGGGATTATT
AGATTTTGAAATGTACTTTCCTCTTTTTCGTTGAAAAAATACCCTAATAA

710 720 730 740 750
TTGTATAAAACTAAGTTGTTTTGTTGTGTTATAACATGGATAAGTATACT
AACATATTTTGATTCAACAAAACAACACAATATTGTACCTATTCATATGA
M D K Y T >
_____>

760 770 780 790 800
GAGAAGAAACAAAGAAATCAAGTATTTCAAAAATTTATTAAAAGACATGT
CTCTTCTTTGTTTCTTTAGTTCATAAAGTTTTTAAATAATTTTCTGTACA
E K K Q R N Q V F Q K F I K R H V>
_____ ORF1 _____>

RBS

810 820 830 840 850
AAAAGAAGGGCAAATGGATTTGATAAGGGAGTGCAATACATTCTTGAGTT
TTTTCTTCCCGTTTACCTAAACTATTCCCTCACGTTATGTAAGAACTCAA
K E G Q M D L I R E C N T F L S >
_____ ORF1 _____>

860 870 880 890 900
TTGTGGCAGATAAAACATTAGAGAAACAGAAATTGCATAAATCTAATTTA
AACACCGTCTATTTTGTAATCTCTTTGTCTTTAACGTATTTAGATTAAAT
F V A D K T L E K Q K L H K S N L >
_____ ORF1 _____>

*Cla*I

910 920 930 940 950
TGTAAAAATCGATTTTGTCCTATATGTGCATGGCGAAAAGCGAGAAAAGA
ACATTTTTAGCTAAAACAGGATATACACGTACCGCTTTTCGCTCTTTTCT
C K N R F C P I C A W R K A R K D>
_____ ORF1 _____>

960 970 980 990 1000
TGCGTTAGGTTTATCATTGATGATGCAATATATTAAGCAAAAAGAAGATA
ACGCAATCCAAATAGTAACTACTACGTTATATAATTCGTTTTTCTTCTAT
A L G L S L M M Q Y I K Q K E D >
_____ ORF1 _____>

1010 1020 1030 1040 1050
AACAATTCATCTTTTTAACACTTACGACGCCAAATGTAACAGTTGAGCAT
TTGTTAAGTAGAAAAATTGTGAATGCTGCGGTTTACATTGTCAACTCGTA
K Q F I F L T L T T P N V T V E H >
_____ ORF1 _____>

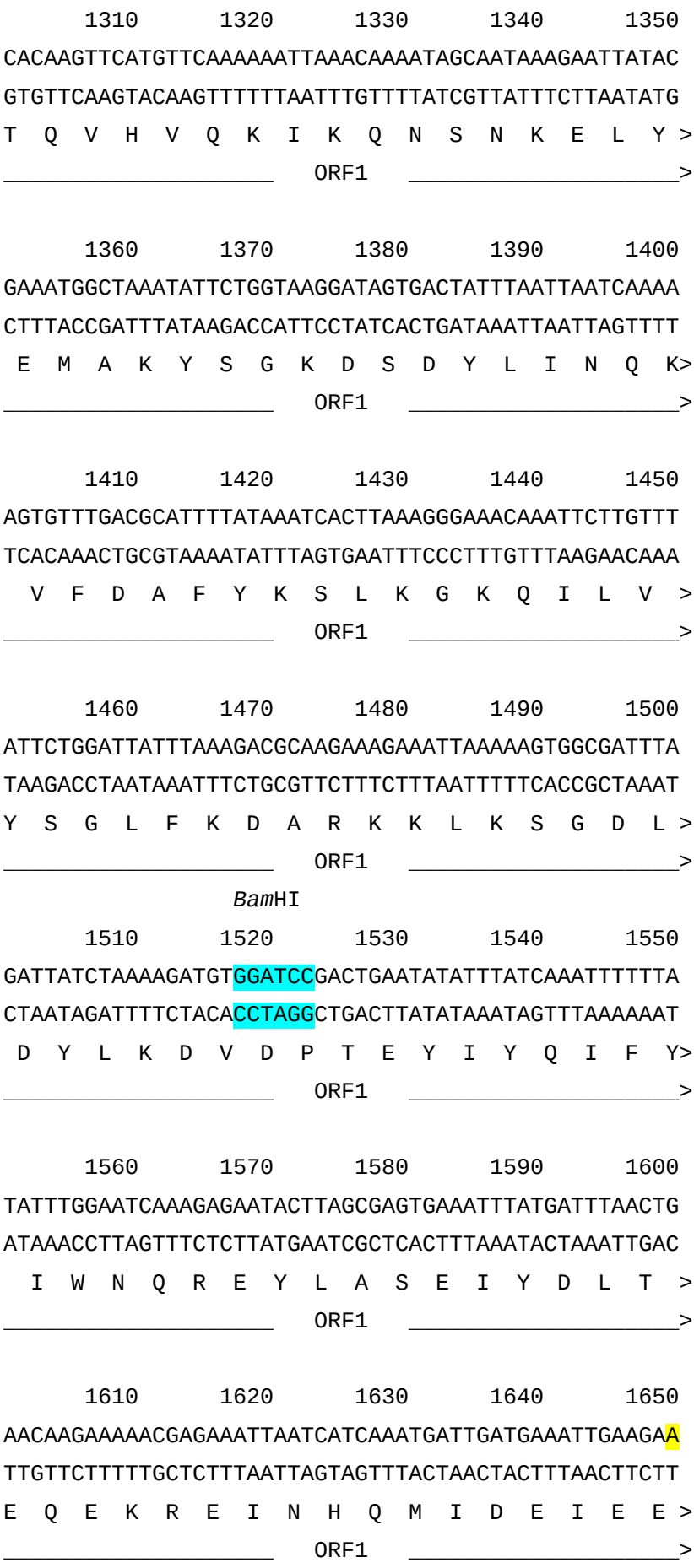
1060 1070 1080 1090 1100
TTGGAAGATGAAATAAAACATTATAATGATTCGTTTAGACGATTAAGTAA
AACCTTCTACTTTATTTTGTAATATTACTAAGCAAATCTGCTAATTCATT
L E D E I K H Y N D S F R R L S N>
_____ ORF1 _____>

1110 1120 1130 1140 1150
TCGTAAACACTTTAAATCTATAGCTAAAGGTTACGTAAGAAAATTAGAAA
AGCATTTGTGAAATTTAGATATCGATTTCCAATGCATTCTTTTAATCTTT
R K H F K S I A K G Y V R K L E >
_____ ORF1 _____>

1160 1170 1180 1190 1200
TCACTTATAACAAAAAACGCGATGATTATAATCCACATTTTCATGTTTTG
AGTGAATATTGTTTTTTGCGCTACTAATATTAGGTGTAAAAGTACAAAAC
I T Y N K K R D D Y N P H F H V L >
_____ ORF1 _____>

1210 1220 1230 1240 1250
ATTGCTGTTAACAAATCGTATTTTACAGACAAACGATATTATATTAGTCA
TAACGACAATTGTTTAGCATAAAATGTCTGTTTGCTATAATATAATCAGT
I A V N K S Y F T D K R Y Y I S Q>
_____ ORF1 _____>

1260 1270 1280 1290 1300
AAAAGAGTGGCTCAATTTATGGCGAGATGTAACGGGGATTTCTGAAATCA
TTTTCTCACCGAGTTAAATACCGCTCTACATTGCCCCTAAAGACTTTAGT
K E W L N L W R D V T G I S E I >
_____ ORF1 _____>


1310 1320 1330 1340 1350
CACAAGTTCATGTTCAAAAAATTAAACAAAATAGCAATAAAGAATTATAC
GTGTTCAAGTACAAGTTTTTTAATTTGTTTTATCGTTATTTCTTAATATG
T Q V H V Q K I K Q N S N K E L Y >
ORF1
1360 1370 1380 1390 1400
GAAATGGCTAAATATTCTGGTAAGGATAGTGACTATTTAATTAATCAAAA
CTTTACCGATTTATAAGACCATTCCTATCACTGATAAATTAATTAGTTTT
E M A K Y S G K D S D Y L I N Q K>
ORF1
1410 1420 1430 1440 1450
AGTGTTTGACGCATTTTATAAATCACTTAAAGGGAAACAAATTCTTGTTT
TCACAAACTGCGTAAAATATTTAGTGAATTTCCCTTTGTTTAAGAACAAA
V F D A F Y K S L K G K Q I L V >
ORF1
1460 1470 1480 1490 1500
ATTCTGGATTATTTAAAGACGCAAGAAAGAAATTAAAAAGTGGCGATTTA
TAAGACCTAATAAATTTCTGCGTTCTTTCTTTAATTTTTCACCGCTAAAT
Y S G L F K D A R K K L K S G D L >
ORF1
BamHI
1510 1520 1530 1540 1550
GATTATCTAAAAGATGTGGATCCGACTGAATATATTTATCAAATTTTTTA
CTAATAGATTTTCTACACCTAGGCTGACTTATATAAATAGTTTAAAAAAT
D Y L K D V D P T E Y I Y Q I F Y>
ORF1
1560 1570 1580 1590 1600
TATTTGGAATCAAAGAGAATACTTAGCGAGTGAAATTTATGATTTAACTG
ATAAACCTTAGTTTCTCTTATGAATCGCTCACTTTAAATACTAAATTGAC
I W N Q R E Y L A S E I Y D L T >
ORF1
1610 1620 1630 1640 1650
AACAAGAAAAACGAGAAATTAATCATCAAATGATTGATGAAATTGAAGAA
TTGTTCTTTTTGCTCTTTAATTAGTAGTTTACTAACTACTTTAACTTCTT
E Q E K R E I N H Q M I D E I E E >
ORF1

RBS

```
      1660      1670      1680      1690      1700
GGAAATTGAAAATAAAATTTGATACTTTTTCTTAAACGTTAGCAAAAAAG
CCTTTAACTTTTATTTTAAACTATGAAAAAGAATTTGCAATCGTTTTTTC
 G  N  * >
_________>

      1710      1720      1730      1740      1750
CTAGCGTTTTTTTGTTGAAAATACGTTAAAAATGGCTGGGTACAAACTGG
GATCGCAAAAAAACAACTTTTATGCAATTTTTACCGACCCATGTTTGACC
                                  M  A  G  Y  K  L  >
                                 _____________________>

      1760      1770      1780      1790      1800
GTACAGACACAATATATAGGGGTACAACCTGGGTACAATCGCGATTTGTA
CATGTCTGTGTTATATATCCCCATGTTGGACCCATGTTAGCGCTAAACAT
G  T  D  T  I  Y  R  G  T  T  W  V  Q  S  R  F  V >
_____________________   ORF2   _____________________>

      1810      1820      1830      1840      1850
GGGGTACAACCTGGGTACAAAATGCAAAAAATGCATACCTTTTTAAAATC
CCCCATGTTGGACCCATGTTTTACGTTTTTTACGTATGGAAAAATTTTAG
 G  V  Q  P  G  Y  K  M  Q  K  M  H  T  F  L  K  S>
_____________________   ORF2   _____________________>

      1860      1870      1880      1890      1900
GCTGTATCCCTTGAAATTTCTGGCTTCGCCAGACCATACATTTTTTAATG
CGACATAGGGAACTTTAAAGACCGAAGCGGTCTGGTATGTAAAAAATTAC
  L  Y  P  L  K  F  L  A  S  P  D  H  T  F  F  N  >
_____________________   ORF2   _____________________>

      1910      1920      1930      1940      1950
TATGCAAATTTCCCTTATGCTCTTACAAAGAATTTAGAGCCAATTTAAAA
ATACGTTTAAAGGGAATACGAGAATGTTTCTTAAATCTCGGTTAAATTTT
V  C  K  F  P  L  C  S  Y  K  E  F  R  A  N  L  K>
_____________________   ORF2   _____________________>
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RBS

```
      1960      1970      1980      1990      2000
TTTGGTGCTTTTTTGAAAGAAATTGTCCTTGCAGGAACTAAAAAAAGTTGA
AAACCACGAAAAAACTTTCTTTAACAGGAACGTCCTTGATTTTTTCAACT
 F  G  A  F  L  K  E  I  V  L  A  G  T  K  K  V  D>
_____________________   ORF2   _____________________>

      2010      2020      2030      2040      2050
TAATTTTTTGAGCCATTTTATGCAAAATTTTAGTTTGCATGTAGATGGGC
ATTAAAAAACTCGGTAAAATACGTTTTAAAATCAAACGTACATCTACCCG
  N  F  L  S  H  F  M  Q  N  F  S  L  H  V  D  G  >
_____________________   ORF2   _____________________>

      2060      2070      2080      2090      2100
AGTGTCTAAAAATTTCGCAACTGGTTTTGCTAAAAATTTGTGATGTGAAA
TCACAGATTTTTAAAGCGTTGACCAAAACGATTTTTAAACACTACACTTT
Q  C  L  K  I  S  Q  L  V  L  L  K  I  C  D  V  K >
_____________________   ORF2   _____________________>

      2110      2120      2130      2140      2150
TCAAAATATATTTATTTGGCTCTCATTTGCGTTTTAAGCGCTTATATAGA
AGTTTTATATAAATAAACCGAGAGTAAACGCAAAATTCGCGAATATATCT
 S  K  Y  I  Y  L  A  L  I  C  V  L  S  A  Y  I  E>
_____________________   ORF2   _____________________>

      2160      2170      2180      2190      2200
ATTTTGGATATAAAACTATGTAAATATTTAGTGTTTCTTCAAAATACCCC
TAAAACCTATATTTTGATACATTTATAAATCACAAAGAAGTTTTATGGGG
  F  W  I  * >
_____________>

      2210      2220      2230      2240      2250
TTTAAAATTAAAAATAAAGGCACTCAAAATTTTTGTAAGGGTTATGATAA
AAATTTTAATTTTTATTTCCGTGAGTTTTAAAAACATTCCCAATACTATT

      2260      2270      2280      2290      2300
AGTTTGTTAAAAGAAAAACTGAAAGTGGATAAATTTATGAGAAAACAGGA
TCAAACAATTTTCTTTTTGACTTTCACCTATTTAAATACTCTTTTGTCCT
```

```
      2310       2320       2330       2340       2350
GTTGTTAAAAAATGAAATTTAGAGAAGCATTAGAGAATTTTTTAACGAAT
CAACAATTTTTTACTTTAAATCTCTTCGTAATCTCTTAAAAAATTGCTTA

      2360       2370       2380       2390       2400
AAGTATGTATATGTTGTGTTGCTGGTACTAGCTATTTATCAAATTTTCAT
TTCATACATATACAACACAACGACCATGATCGATAAATAGTTTAAAAGTA

      2410       2420       2430       2440       2450
GTTGTTTGAATAAAAATTCGGGGAGTAGAACACGCATTTATGCCGAGAAA
CAACAAACTTATTTTTAAGCCCCTCATCTTGTGCGTAAATACGGCTCTTT

      2460       2470       2480       2490       2500
ATTTATTGATGTTGAGAAGAACCCTTAACTAAACTTGAAGACGAATGTCG
TAAATAACTACAACTCTTCTTGGGAATTGATTTGAACTTCTGCTTACAGC

      2510       2520       2530       2540       2550
GCATAGCGTGAGCTATTAAGCCGACCATTCGACAAGTTTTGGGATTGTTA
CGTATCGCACTCGATAATTCGGCTGGTAAGCTGTTCAAAACCCTAACAAT

      2560       2570       2580       2590       2600
AGGGTTCCGAGGCTCAACGTCAATAAAGCAATTGGAATAAATATTAACAG
TCCCAAGGCTCCGAGTTGCAGTTATTTCGTTAACCTTATTTATAATTGTC

      2610       2620       2630       2640       2650
TAAGATTCATTCCAATTGCTTTATTTAAAAAGAAAATGTAGTATATATAT
ATTCTAAGTAAGGTTAACGAAATAAATTTTTCTTTTACATCATATATATA

      2660       2670       2680       2690       2700
TAGTTAATTAAACTAATATATATACTACATGGAGGTTTTATTGTGATTAA
ATCAATTAATTTGATTATATATATGATGTACCTCCAAAATAACACTAATT

      2710       2720       2730       2740       2750
AAGTGAAAAAAAGAAAAGCCCTTTATTATTTAGGATAGGTATAGTACTTT
TTCACTTTTTTTCTTTTCGGGAAATAATAAATCCTATCCATATCATGAAA

      2760       2770       2780       2790       2800
TGATTATTTATGTATTGCTTTGGATAGGAATTATTGTTACTCCTTTTTTA
ACTAATAAATACATAACGAAACCTATCCTTAATAACAATGAGGAAAAAAT
```

```
      2810       2820       2830       2840       2850
CCTGTATCAACGTTTGTAAAAGCAACTGTTATACCTGGGACAATTGTATT
GGACATAGTTGCAAACATTTTCGTTGACAATATGGACCCTGTTAACATAA

      2860       2870       2880       2890       2900
TGCTGAAATACTTTTAATTATTGCTGCGGCTTTTGTTGGGAAAGAGCTTG
ACGACTTTATGAAAATTAATAACGACGCCGAAAACAACCCTTTCTCGAAC

      2910
TAAGTAAGTATAAAGA
ATTCATTCATATTTCT
```

Figure 4.7 – The complete annotated sequence of *S. aureus* plasmid pWBG1773. Translations of putative ORFs are given below the sequence in the single letter aa code. Direction of the transcription of each ORFs is indicated by arrows at the end of each translation. *Bam*HI and *Cla*I RE sites are highlighted as well as the predicted RBSs.

4.2.5 Secondary structure features of plasmid pWBG1773

Secondary structures such as DRs, IRs, hairpins and palindromic sequences are believed significant in exchange and re-arrangement of DNA sequences, composition of gene cassette structures (Bjorland *et al.* 2003; Murphy 1989) and gene regulation (Manna *et al.* 1998; Ouyang *et al.* 1999). In plasmid pWBG1773 only one set of DRs, DR_1, was located using the Tandem Repeat Finder program (Benson 1999; tandem.bu.edu/trf/trf.html). DR_1 was located within the ORF2 sequence at nt positions 1738-1820 (Figure 4.8, Appendix 7.7).

Although only one set of DRs was identified further analysis of DR_1 found it to be comprised of several internal repeats (Figure 4.8). There are six perfect 7 bp DRs of 5′-GGGTACA-3′ (DR_{1A}), four perfect 9 bp DRs of 5′-CTGGGTACA-3′ (DR_{1B}), two perfect 21 bp DRs of 5′-TAGGGGTACAACTGGGTACAA-3′ (DR_{1C}) and two imperfect 32 bp DRs of 5′-GGGTACAAC(A)CTGGGTACAAT(GA)CA(G)CAA TATATAG-3′ (DR_{1D}). DR_{1A} is found in three pairs. Within each pair the duplicated sequences are only 4 bp apart and each pair is 13-14 bp from the next. BLAST searches of DR_1 found only homology to the *S. warneri* plasmid pPI-2 DNA at nt positions 764-739 and 796-765 and corresponding to areas of non-coding DNA.

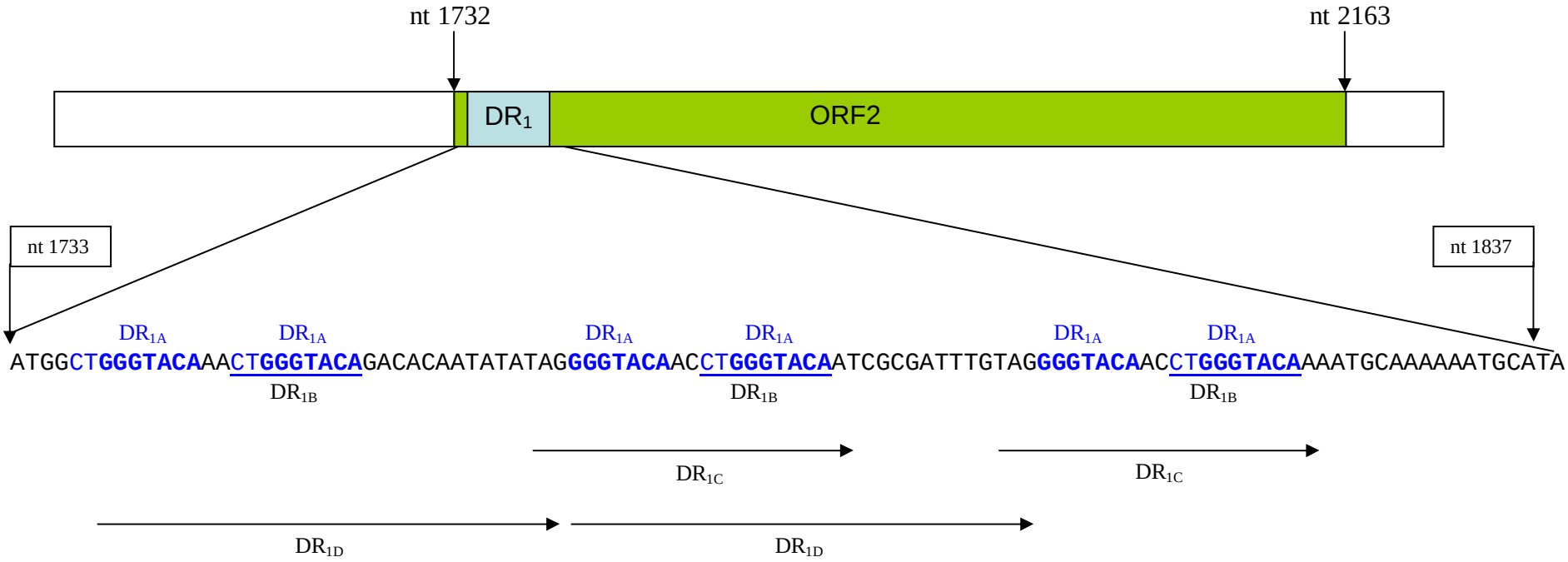


Figure 4.8 – Region of plasmid pWBG1773 containing DRs. Nt coordinates indicate the region of the plasmid pWBG1773 genome. The open arrow indicates the direction of transcription of ORF2. The region of DRs extending from nt positions 1733-1837 is displayed below the figure. DR_{1A} is highlighted in bold blue, DR_{1B} is underlined and, DR_{1C} and DR_{1D}, are indicated by arrows.

Other secondary structures found within the non-coding region of plasmid pWBG1773 included two IRs or palindromes downstream of ORF2. The first, IR_1, is a 54 bp sequence (5′-AATAAAGCAATTGGAATAAATATTAACAGTAAGATTCATTCCAATTGCTTTATT-3′) that forms an imperfect IR located at nt positions 2572-2625 in plasmid pWBG1773 (Figure 4.9). The second IR, IR_2, is a 45-bp sequence (5′-TGTAGTATATATATTAGTTAATTAAACTAATATATATACTACAT-3′) that forms an imperfect IR located 10 bp downstream of IR_1 at nt positions 2636-2680 (Figure 4.9). IR_1, has eight non-complementary nucleotides and IR_2, has a single mismatch. Both IRs were submitted to the NCBI database using the blastn tool. The first 20 bases of IR_1 produced significant homology with a number of staphylococcal plasmid sequences corresponding to an area known as SSO. No significant homology was found for the remainder of IR_1 or the entirety of IR_2 with any other DNA sequence currently in the GenBank database.

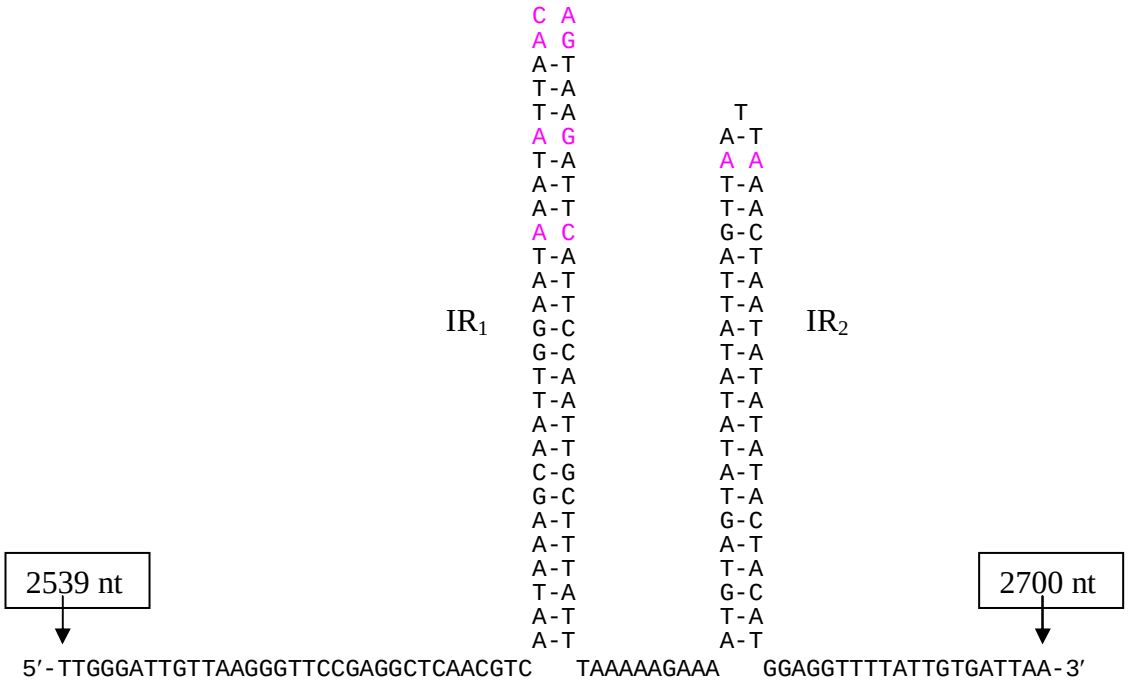


Figure 4.9 – Potential stem or hairpin loop structures of the IRs, IR_1 and IR_2, of plasmid pWBG1773. Non-complementary nucleotides are highlighted in pink.

4.3 Analysis of ORF1

ORF1 extends 924 nt from a potential ATG start codon at nt positions 736-738 to the TGA stop codon at nt positions 1657-1659. It potentially encodes a 308 aa protein with a molecular mass of 37.062 kDa. Comparison of the deduced aa sequence with sequences in the GenBank protein databases revealed that it shared statistically significant similarities with replication proteins from a number of plasmids from a broad range of bacterial genera. The extent of the similarity was assessed by performing pair-wise alignments with the Clustal X™1.8.3 multiple sequence alignment program using the Gonnet series matrix, a gap opening of 10 and a gap extension of 0.20 (Figure 4.10). ORF1 was found to have greatest identity (95%) to the replication protein encoded by the *S. warneri* plasmid pPI-2 (accession number AB125342) and 90% identity with the putative replication initiation proteins, Rep, encoded by the *S. aureus* multi-resistant plasmid pSK41 (accession number AF051917) (Aso *et al.* 2004; Berg *et al.* 1998). Strong homology was also seen with several members of the pC194 family of rolling-circle plasmids such as pSepCH and pSK89 (Figure 4.10).

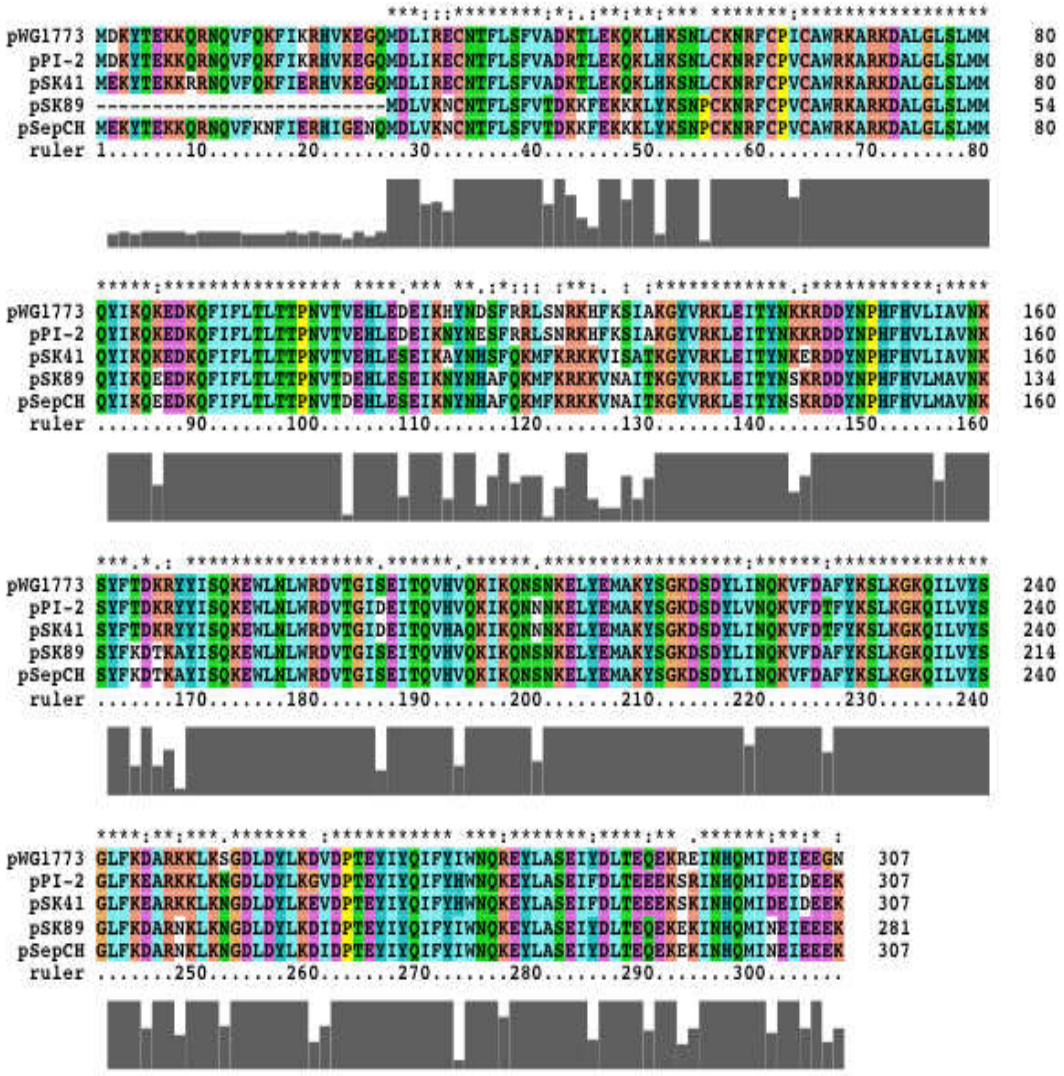


Figure 4.10 – The Clustal X™1.8.3 alignment of the putative plasmid pWBG1773 replication protein (designated here as pWG1773) against homologous replication genes from other staphylococcal plasmids (labelled by their plasmid name). Protein sequences are grouped and coloured as follows: cyan, H Y; blue, M V F I L A W; orange, G; red, K R; pink, C; green, T Q N S; magenta, E D and; yellow, P. A line above the alignment is used to mark strongly conserved positions. Three characters are used: '*' indicates positions which have a single, fully conserved residue, ':' indicates conservation between a group of residues strongly related, and '.' indicates conservation between a group of residues less related. A ruler is displayed below the sequences, starting at 1 for the first residue position. A bar graph at the bottom of the figure represents the extent of homology between all sequences.

Due to its strong homology to staphylococcal replication genes, ORF1 was designated as $rep_{WBG1773}$.

4.3.1 Identification of plasmid pWBG1773 as a rolling circle replication plasmid

Replication by the rolling circle-type mechanism requires three characteristic features: (i) a replication protein, (ii) a double-stranded origin of replication containing a nick site, and (iii) a SSO. The identification of *rep*$_{WBG1773}$ in plasmid pWBG1773 as a potential gene encoding a replication protein for rolling circle replication led to the search for a suitable nick site and SSO.

4.3.1.1 Identification of the double-stranded origin of replication and nick site for plasmid pWBG1773

An analysis of the sequence detected the presence of a double-stranded origin of replication in plasmid pWBG1773. An alignment of the putative double-stranded origin of replication of pWBG1773 with known double-stranded origins was performed using the multiple sequence alignment program Clustal X™1.8.3 (Figure 4.11). Those with the highest homology to plasmid pWBG1773 are all members of the pC194 group of plasmids, including the plasmids pC194, pSK89, pNVH01, pSepCH, and pSK108. All have the conserved nick site sequence CTTGATA found within the double-stranded origin (Seery *et al.* 1993). An exact match of this heptameric motif was detected in plasmid pWBG1773 (Figure 4.11) at nt positions 528-534 and on the same strand of the plasmid encoding the replication gene. The similarity in the sequences extended 11-12 bases 5′ of the nick site.

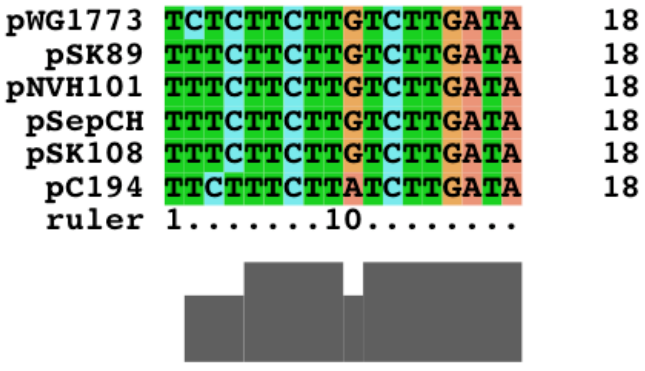


Figure 4.7 –A multiple DNA sequence alignment of a putative nick site within plasmid pWBG1773 aligned to nick sites of other staphylococcal plasmids with rolling-circle replication. A ruler is displayed below the sequences, starting at 1 for the first residue position. A bar graph at the bottom of the figure represents the extent of homology between all sequences.

4.3.1.2 Identification of the SSO of plasmid pWBG1773

Plasmids that replicate with a rolling-circle mechanism commence synthesis of their second strand at a location referred to as SSO, previously known as *palA* (Gros *et al.* 1987; Seery *et al.* 1993). The SSO sequences of other staphylococcal plasmids that replicate via a rolling-circle mechanism were used to identify a potential SSO sequence in plasmid pWBG1773. A search of plasmid pWBG1773 sequence identified a potential SSO located 20 nt down stream of ORF2 on the minus strand at nt positions 2591-2433. This position includes 20 nt of the IR, IR_1, from nt positions 2572-2590. The SSO sequences from other staphylococcal plasmids were aligned with that of the predicted SSO of plasmid pWBG1773 using Clustal X™1.8.3 (Figure 4.12). All SSO sequences downloaded from NCBI were from staphylococcal plasmids carrying a *smr*, *qacC, qacH, qacJ,* or *ebr* gene and all SSO sites were located 5-10 bases downstream of these genes.

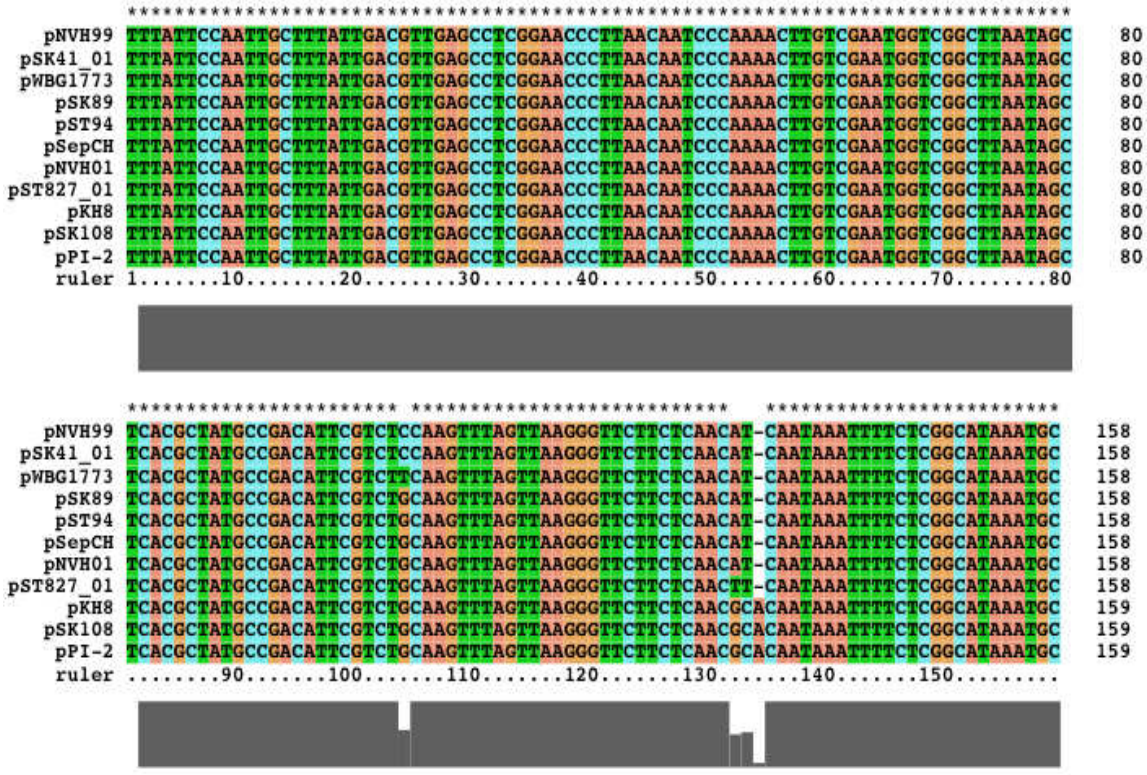


Figure 4.8 – Multiple DNA sequence alignment of a putative SSO within plasmid pWBG1773 to SSOs in other rolling circle plasmids. A line above the alignment is used to mark conserved positions. '*' indicates a position which has a single, fully conserved nt. A ruler is displayed below the sequences, starting at 1 for the first residue position. A bar graph at the bottom of the figure represents the extent of homology between all sequences.

4.4 Analysis of ORF2

ORF2 extends 432 nt from an ATG start codon at nt positions 1732-1734 to the TAA stop codon at nt positions 2161-2163. This encodes a potential 144 aa protein with a molecular mass of approximately 16.543 kDa. There are also three other potential ATG start codons within ORF2. The first occurs directly after the end of DR_1 and is located at nt positions 1822-1824. If this is the coding sequence then ORF2 would be reduced to 341 nt and would encode a protein of 113 aa. The second potential start codon is at nt positions 1831-1833 and, if this is considered the correct start site, it shortens ORF2 to 332 nt in size and would encode a protein of 110 aa. The third ATG start codon is located at nt positions 2020-2022 producing a very short ORF2 of 143 nt in length encoding a protein of 47 aa.

Using the NCBI search tool, blastn, the nt sequence of ORF2 was submitted and compared to DNA sequences within databases at GenBank. ORF2 shared greatest homology to a sequence within plasmid pPI-2 (nt positions 802-412) isolated from *S. warneri* with 96% identity, no gaps and an e value of 0.0. This sequence in pPI-2 was reported as region of non-coding DNA. The original analysis of plasmid pPI-2 sequence was performed using GENETYX-WIN (Software Development) (Aso *et al.* 2004). Another analysis of plasmid pPI-2 nt sequence was performed using the MacVector™7.2 ORF analysis tool. The size of the smallest ORF product previously identified, *qacC′*, was 59 aa and this was used as the minimum aa cut off value when searching plasmid pPI-2 for ORFs. Seven potential ORFs were identified, including the three previously identified genes, *rep*, *qacC* and *qacC′* (Figure 4.13). The new ORFs are labelled W to Z from smallest to largest. The sequences were submitted to the NCBI database to detect homologous sequences using the blastn tool. ORF-X and ORF-Y were associated with plasmid replication genes and ORF-W and ORF-Z had no homology to any known sequences in the GenBank database. ORF-Z of plasmid pPI-2 has significant homology to ORF2 of plasmid pWBG1773. ORF-Z is 426 nt in size and was aligned to ORF2 using the Clustal X™1.8.3 program (Figure 4.14).

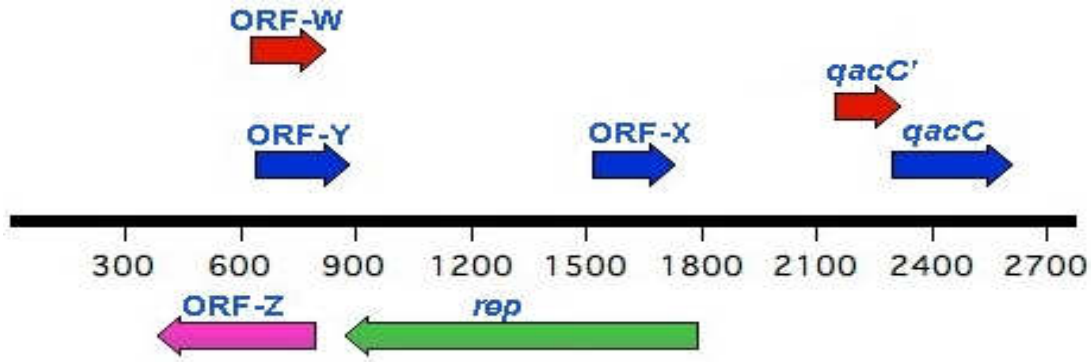


Figure 4.9 – Seven ORFs identified in plasmid pPI-2 using MacVector™7.2 ORF analysis tool with a minimum size of 59 aa. The ORFs *rep*, *qacC* and *qacC′* have been previously identified, and the new ORFs are labelled W to Z from smallest to largest. ORFs positioned above the scale are located on the plus strand (5′-3′), and those ORFs positioned below are on the minus strand (3′-5′). Each reading frame is represented by a different colour, +1 blue, +3 red, -1 pink and -2 light green.

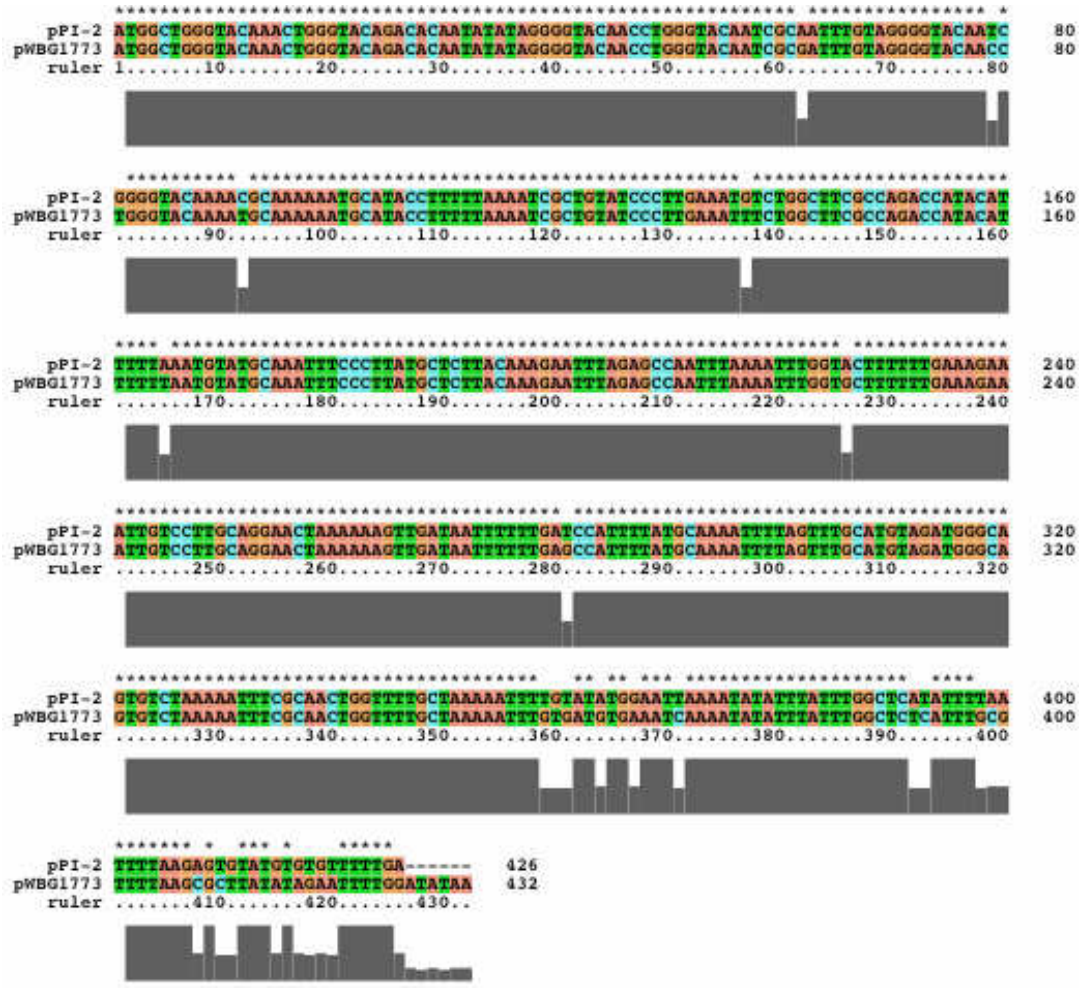


Figure 4.10 – A sequence alignment of ORF-Z of plasmid pPI-2 to ORF2 of plasmid pWBG1773. A ruler is displayed below the sequences, starting at 1 for the first residue position. A bar graph at the bottom of the figure represents the extent of homology between the two sequences.

The blastp search tool was used to search the NCBI database for aa sequences with homology to the translated aa sequence of ORF2. Homology was found to a hypothetical 8.7 kDa protein (reading frame D) encoded by *S. aureus* plasmid pC194 (Accession number P03860) with 59% identity and 70% similarity. Identity only occurred over 44 residues at the C-terminal of ORF2 (aa positions 98-141) and the hypothetical protein product of pC194 (aa positions 37-80) (Figure 4.15). The hypothetical protein product of pC194 has no predicted function (Horinouchi and Weisblum 1982a). No significant homology was found to any other proteins in the NCBI database.

```
pWBG1773    1       MAGYKLGTDTIYRGTTWVQSRFVGVQPGYKMQKMHTFLKSLYPLKFLASP

pC194               --------------------------------------------------

pWBG1773   51       DHTFFNVCKFPLCSYKEFRANLKFGAFLKEIVLAGTKKVDNFLSHFMQNF
                                                                  +NF
pC194       1       ----------TIIKSIMLKCFIILTLYKHYTFVIQILTLLGTIKNHENF

pWBG1773  101       SLHVDGQCLKISQLVLLKICDVKSKYIYLALICVLSAYIEFWI*    144
                    +LHV GQCLK S L LLK   V+ +YIYLA IC L A + F
pC194      48       NLHVTGQCLKKSTLNLLKFLFVELEYIYLAHICFLKASVGF*--     80
```

Figure 4.11 – Diagram indicating areas of similarity between ORF2 of plasmid pWBG1773 and the hypothetical 8.7 kDa protein of plasmid pC194. The region of similarity is indicated in blue, identical amino acids are designated by their respective symbols and amino acids with similar properties are designated by +.

4.4.1 Secondary structure of ORF2

The predicted aa sequence of ORF2 was analysed for the presence of secondary structures that may help identify its function. The Protein Analysis Toolbox function in MacVector™7.2 was used to identify α-helix and β-sheets that make up the secondary structures. The program uses the methods of Chou-Fasman (CF) (Chou and Fasman 1978) and Robson-Garnier (RG) (Garnier *et al.* 1978) and a combination of the two to generate a consensus prediction showing where both methods agree (CfRg), to predict the three conformations, α-helix, β-sheets and turns, from the aa sequences. The three possible conformations are plotted on three separate lines (Figure 4.16). The secondary structures predicted for ORF2 indicate a stronger α-helix conformation rather than β-sheets or turns.

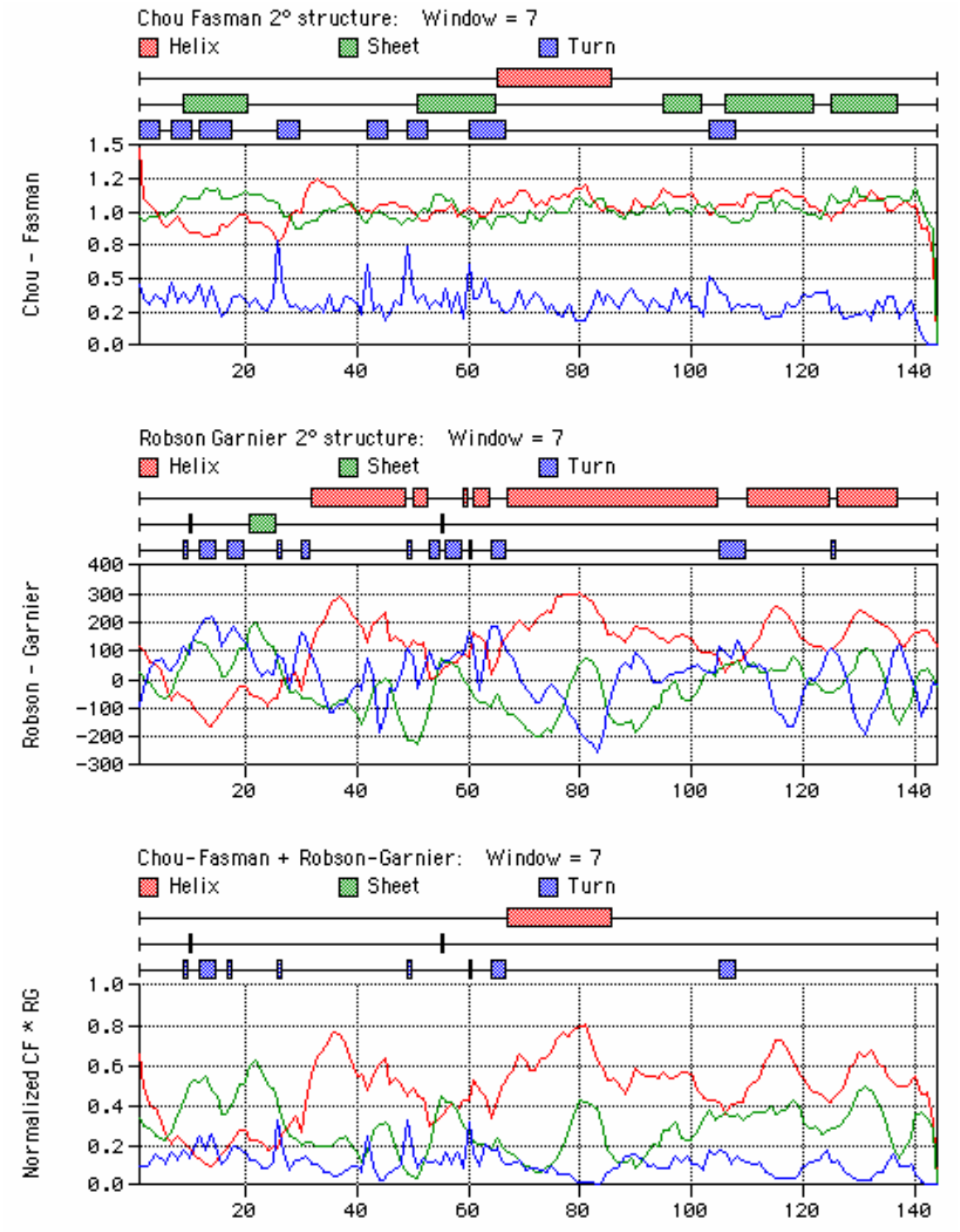


Figure 4.12 - Secondary structure predictions for ORF2 of plasmid pWBG1773 using the CF, RG and combined methods (CfRg) from the Protein Analysis Toolbox function in MacVector™7.2. The potential secondary structure of the protein is plotted using all three methods and the predicted conformation is indicated above the graph. The aa sequence of ORF2 is plotted along the x-axis. A window size of 7 is the number of residues that are grouped together for analysis throughout the length of the sequence.

Another important tool in analysing the secondary structure of a protein is the hydropathy plot. This reveals potential transmembrane spanning regions and is particularly important when predicting membrane bound proteins and trans-membrane proteins that could function as an efflux pump. The 144 aa sequence of ORF2 was submitted to TMpred (www.ch.embnet.org/software/TMPRED_form.html), which predicts membrane-spanning regions and their orientation. The algorithm is based on the statistical analysis of TMbase, a database of naturally occurring transmembrane proteins. The prediction is made using a combination of several weight-matrices for scoring (Hofmann and Stoffel 1993). TMpred predicted a single hydrophobic domain 18 aa in length at aa positions 126-143 at the C-terminus of the ORF2 protein (Figure 4.17). Results also indicate a preference for the transmembrane sequence to orientate inside to outside. This indicates the protein encoded by ORF2 is possibly membrane bound with the N-terminus located internally and the C-terminus inserted into the membrane.

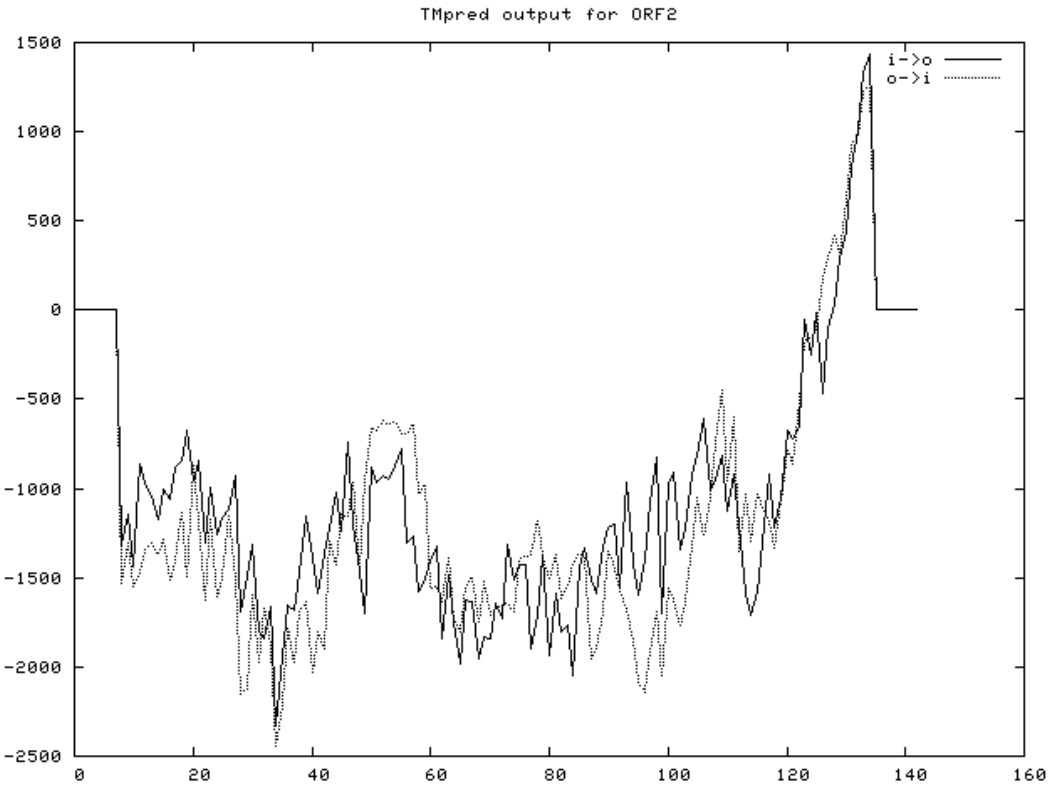


Figure 4.13 – Hydropathy plot of the predicted protein ORF2 using TMpred. The aa residue number is plotted on the x-axis and the hydrophobicity index is plotted on the y-axis. Only scores above 500 are considered significant. The solid line indicates preference of the transmembrane sequence to orientate inside to outside (i - > o) and the broken line indicates a preference for outside to inside (o - > i) orientation.

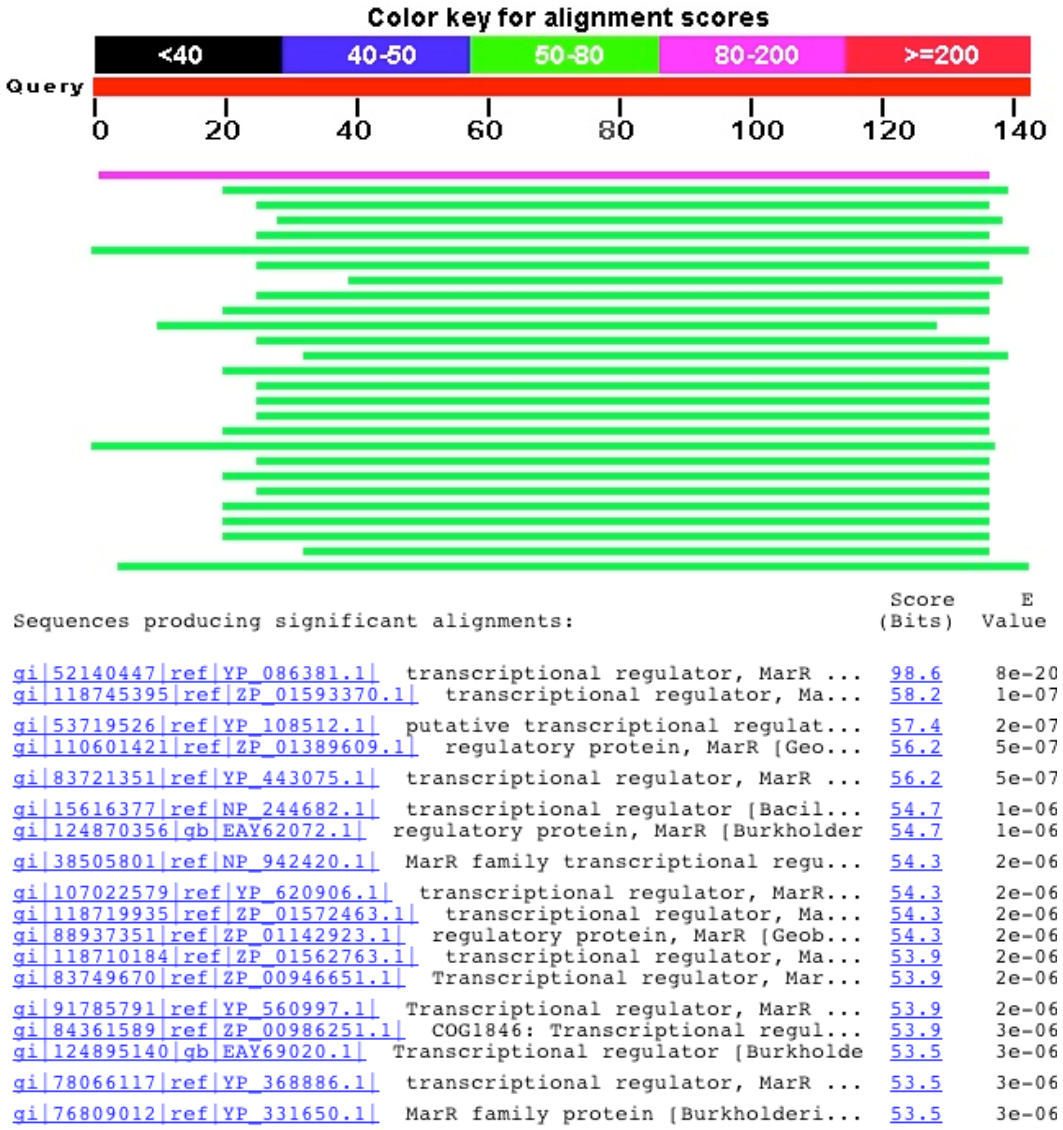
Other transmembrane prediction sites were also used to detect potential transmembrane sequences. These included the Dense Alignment Surface model (DAS) (Cserzo *et al.* 1997; http://www.sbc.su.se/~miklos/DAS/) and TMHMM (Sonnhammer *et al.* 1998; http://www.cbs.dtu.dk/services/TMHMM/) websites. These programs provided similar results to TMpred (Appendix 7.8).

ScanProsite (http://ca.expasy.org/tools/scanprosite/) was used to search for other secondary structures and motifs in the predicted aa sequence of ORF2. No other secondary structures or motifs were found.

The predicted secondary structure for the aa sequence of ORF2 suggested that it may be a secretory protein that contains a signal peptide cleavage site that removes its hydrophobic C-terminus to allow secretion of the hydrophilic N-terminus. The SignalP 3.0 server (http://www.cbs.dtu.dk/services/SignalP/) was used to detect the presence and location of any potential signal peptide cleavage sites. The SignalP 3.0 method incorporates a prediction of cleavage sites and a signal peptide/non-signal peptide prediction based on a combination of several artificial neural networks (NN) and hidden Markov models (HMM). An analysis by NN and HMM trained on signal peptides of Gram-positive bacteria depicted ORF2 as a non-secretory protein with 0.0 probability of containing a signal peptide.

4.5 Analysis of ORF3

ORF3 extends 431 nt from a potential ATG start codon at nt position 42-44 to the TAA stop codon at nt positions 471-473. It encodes a potential 143 aa protein with a molecular mass of approximately 16.765 kDa. The blastp search tool was used to search the GenBank database for sequences with homology to the predicted aa sequence of ORF3 and results revealed 41% identity and 69% similarity along the entire length of MarR, a 143 aa transcriptional regulator (Accession number YP_086381) found on the chromosome of *B. cereus*. The same region of homology was also found in MarR-like sequences from the following organisms *Bacillus anthracis, Bacillus thuringiensis, Methanococcus, Burkholderia mallei, Enterococcus faecium, Streptococcus mutans* and *Clostridium* (Figure 4.18).



Sequences producing significant alignments:		Score (Bits)	E Value
gi|52140447|ref|YP_086381.1|	transcriptional regulator, MarR ...	98.6	8e-20
gi|118745395|ref|ZP_01593370.1|	transcriptional regulator, Ma...	58.2	1e-07
gi|53719526|ref|YP_108512.1|	putative transcriptional regulat...	57.4	2e-07
gi|110601421|ref|ZP_01389609.1|	regulatory protein, MarR [Geo...	56.2	5e-07
gi|83721351|ref|YP_443075.1|	transcriptional regulator, MarR ...	56.2	5e-07
gi|15616377|ref|NP_244682.1|	transcriptional regulator [Bacil...	54.7	1e-06
gi|124870356|gb|EAY62072.1|	regulatory protein, MarR [Burkholder	54.7	1e-06
gi|38505801|ref|NP_942420.1|	MarR family transcriptional regu...	54.3	2e-06
gi|107022579|ref|YP_620906.1|	transcriptional regulator, MarR...	54.3	2e-06
gi|118719935|ref|ZP_01572463.1|	transcriptional regulator, Ma...	54.3	2e-06
gi|88937351|ref|ZP_01142923.1|	regulatory protein, MarR [Geob...	54.3	2e-06
gi|118710184|ref|ZP_01562763.1|	transcriptional regulator, Ma...	53.9	2e-06
gi|83749670|ref|ZP_00946651.1|	Transcriptional regulator, Mar...	53.9	2e-06
gi|91785791|ref|YP_560997.1|	Transcriptional regulator, MarR ...	53.9	2e-06
gi|84361589|ref|ZP_00986251.1|	COG1846: Transcriptional regul...	53.9	3e-06
gi|124895140|gb|EAY69020.1|	Transcriptional regulator [Burkholde	53.5	3e-06
gi|78066117|ref|YP_368886.1|	transcriptional regulator, MarR ...	53.5	3e-06
gi|76809012|ref|YP_331650.1|	MarR family protein [Burkholderi...	53.5	3e-06

Figure 4.14 – An overview of NCBI database sequences aligned to predicted aa sequence of ORF3. The score of each alignment is indicated by one of five different colours, which divides the range of scores into five groups.

No identity was found to any staphylococcal proteins in the NCBI database.

4.5.1 Secondary structure of ORF3

Results from the blastp search of the NCBI database for homologous sequences to the predicated aa sequence of ORF3, indicated a significant degree of similarity to the motif associated with MarR proteins. ORF3 contains a HTH domain extending over 73 aa from location 55-127 (Figure 4.19).

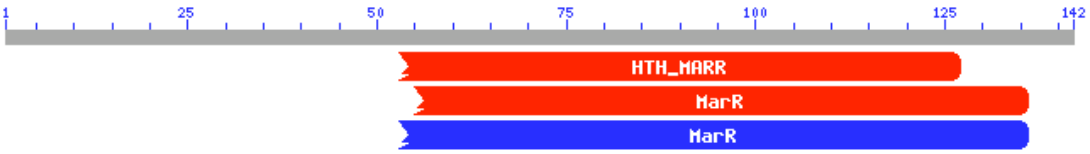


Figure 4.15 – Detection of the putative HTH domain on ORF3, typical of the MarR family of transcriptional regulators (taken from NCBI blastp results). The grey scale indicates the amino acid sequence of ORF3. The red and blue areas indicate areas of homology along ORF3 to conserved HTH MarR motifs.

HTH domains are associated with DNA-binding sites. The conserved sequence associated with the HTH domain was aligned next to other MarR sequences provided in the NCBI database (Figure 4.20).

Using the Protein Analysis Toolbox function in MacVector™7.2 the presence of α-helices and β-sheets that make up the secondary structures were examined. The secondary protein structure predictions of ORF3 (Appendix 7.9) confirmed the results of NCBI, predicting a strong HTH conformation, suggesting that it could represent a DNA binding motif of a regulatory protein.

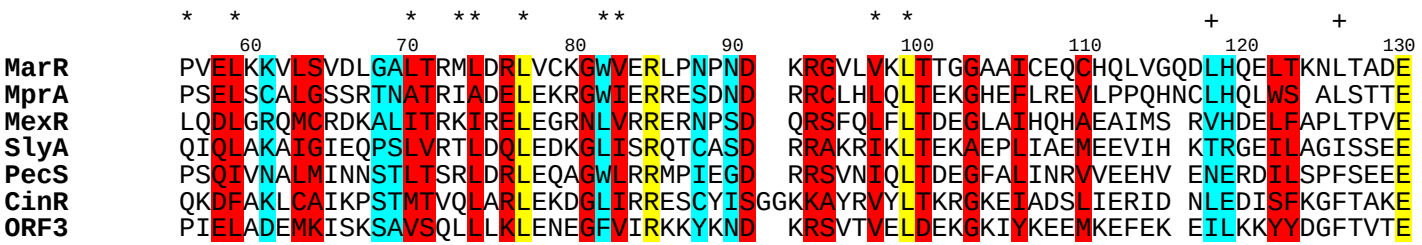


Figure 4.20 – Sequence alignment of ORF3 translated aa with representatives of the MarR family. Numbering is according to the MarR primary sequence. Residues that are identical in all homologs are coloured yellow, highly conserved aa in red and moderately conserved residues in blue. Residues marked with a ‘plus sign’ represent parts of the hydrophobic core of the C-terminal domain of MarR, and those denoted with an ‘asterisk’ form the hydrophobic core of the individual DNA-binding domains.

4.5.2 Detection of a potential 'marbox'

Since ORF3 has strong homology to a family of *marR* genes in other bacteria, the rest of the plasmid sequence was examined for regions homologous to sequences associated with *marR*. One particular consensus sequence of the *mar* regulon is the 'marbox', a defined binding site for a regulatory protein, MarA, in *E. coli*. Using DNA retardation studies and hybrid promoters, a marbox consensus sequence, AYnGCACnnWnnRYYAAAYn, was defined by Martin (1999). In the same study it was also found that the orientation, forward or backward, and spacing of the marbox with respect to potential sites for binding of RNA polymerases was critical for the transcriptional activation by MarA. Using the consensus sequence for the 'marbox', plasmid pWBG1773 was examined for any equivalent sites. A potential 'marbox' was located within ORF2 at nt positions 1961-1942 in the backward orientation (Figure 4.21). The location of this 'marbox' is 60 nt upstream of a potential start codon at nt positions 2020-2022 within ORF2. Putative -10 (TAAAAT) and -35 (TTTTCA) signals are also located near the 'marbox' with an alignment consistent to that documented by Martin (1999).

A.

```
AYnGCACnnWnnRYYAAAYn
|  |||||||||| |||||||
AAAGCACCAAATTTTAAATT
```

Figure 4.16A- A potential 'marbox' identified in plasmid pWBG1773 as compared to the consensus sequence (top row). Legend: Y = C or T; n = any base; W = A or T; R = A or G; | = matching bases.

B.

3′–**TTAAATTTTAAACCACGAAA**AAACTTTCTTTAACAGGAACGTCCTTGATT**TTTTCA**ACTATTAAAAAACTCGG**TAAAAT**A-5′

Figure 4.21B - Sequence of the potential *mar* regulon promoter showing the relationship of the 'marbox' to the putative -35 and -10 signals (shown in bold). The backwards orientation of the 'marbox' is indicated by the arrow inside the box.

Clustal X™1.8.3 was used to align the potential ‘marbox’ of plasmid pWBG1773 with other known ‘marbox’ sequences including *poxB* (Chang *et al.* 1994), *ribA* (Koh *et al.* 1996, 1999), *acrAB* (Ma *et al.* 1993), *fpr* (Jair *et al.* 1996), *mar* (Alekshun and Levy 1997) and; *fldA* (Zheng *et al.* 1999) (Figure 4.22).

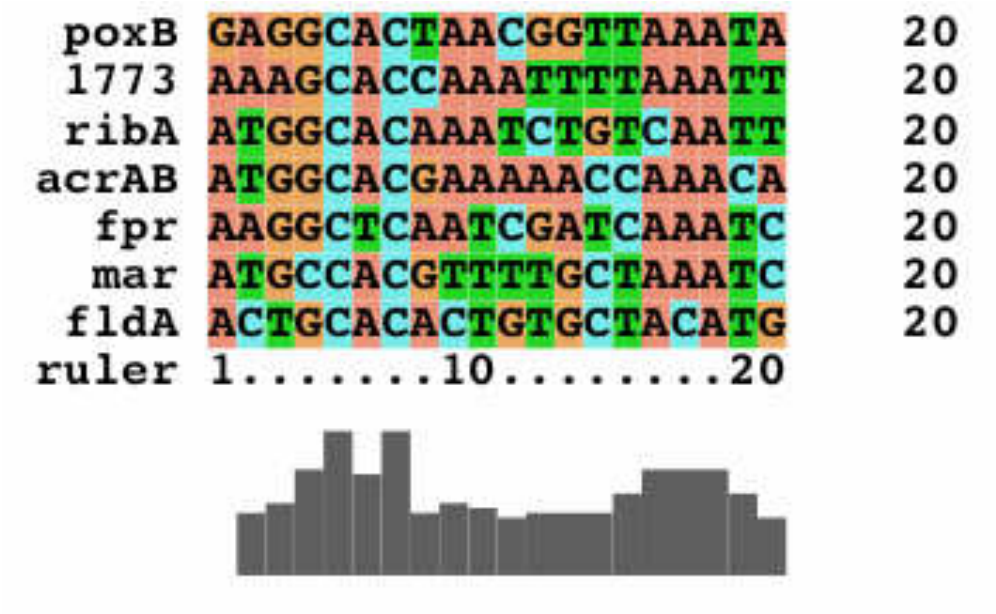


Figure 4.17 – Clustal alignment of ‘marboxes’ from *mar/sox/rob* regulon promoters to the potential ‘marbox’ of plasmid pWBG1773 (labelled 1773 in the alignment). A ruler is displayed below the sequences, starting at 1 for the first residue position. A bar graph at the bottom of the figure represents the extent of homology between all sequences.

The potential ‘marbox’ of plasmid pWBG1773 shares significant homology with the known marbox sequences found in other bacterial species.

Figure 4.23 provides a summary of all the potential significant features from the sequence analysis of plasmid pWBG1773.

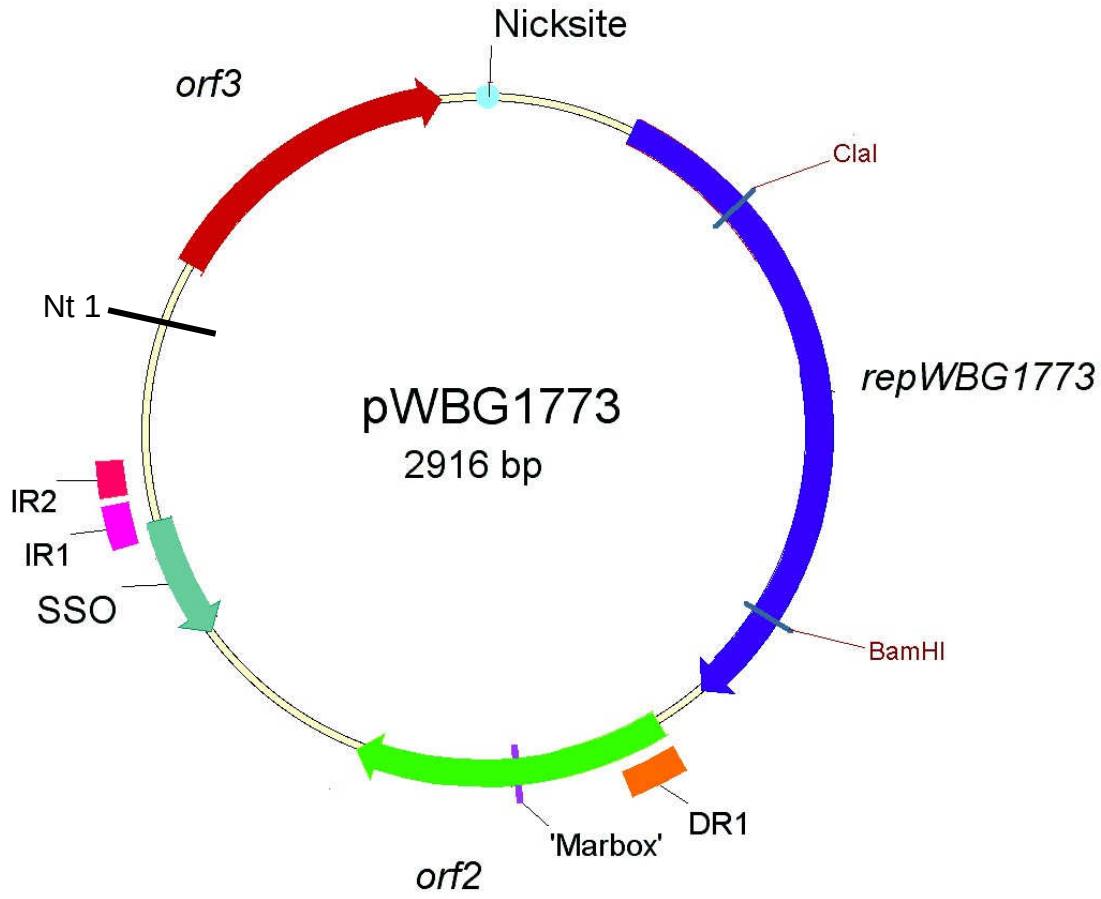


Figure 4.18 - A circular schematic of plasmid pWBG1773. ORFs are depicted in blue, green and red with arrows indicating the direction of transcription. RE sites *Bam*HI and *Cla*I are indicated in red text. The nick site is shown in aqua and the single strand origin (SSO) pale green. The DR_1 is seen in orange and IRs in pink. The position of the first nt is indicated with a black line.

4.6 Comparison of Resistance Phenotype of Plasmid pWBG1773 in *S. aureus* and *E. coli*

4.6.1 Resistance phenotype of plasmid pWBG1773 in staphylococcal host

Disc diffusion testing was used to measure the susceptibility of WBG4364 carrying plasmid pWBG1773 to a range of antimicrobial agents, including 21 antibiotics, 3 heavy metals and 2 cationic biocides (Table 4.2).

Table 4.2 – Disk diffusion results

Antibiotic Discs					
	RN4220	WBG4364 pWBG1773	WBG1979 (*smr*)	WBG1876	WBG1320 (*qacA*)
Amp					
Cm	S	S	S	S	S
Ciprofloxacin	S	S	S	S	S
Em	S	S	S	S	S
Fa	S	R	S	R	S
Gm	S	S	R	S	R
Km	S	S	R	S	R
Lincomycin	S	S	S	S	S
Minocycline	S	S	S	S	S
Mupirocin	S	S	S	S	S
Nm	S	S	R	S	S
Novobiocin	S	S	S	S	S
Ox	S	S	S	S	S
Pc	S	S	S	S	R
Rf	S	R	S	S	S
Sp	S	S	S	S	S
Sm	S	S	S	S	S
Sulphamethoxazole	S	S	S	S	S
Tc	S	S	S	S	S
Tp	S	S	S	S	R
Vancomycin	S	S	S	S	S
Antimicrobial Discs					
	RN4220	WBG4364 pWBG1773	WBG1979 (*smr*)	WBG1876	WBG1320 (*qacA*)
Cd acetate	S	S	S	S	S
Eb	S	S	R	S	R
Mercuric chloride	S	S	S	S	S
Phenyl mercuric acetate	S	S	S	S	S
Pi	S	S	S	S	R

Excluding the chromosomal resistance to Rf and Fa conferred by the host organism, WBG1876, plasmid pWBG1773 did not confer resistance to any of the antimicrobial agents tested by disc diffusion.

Disk diffusion susceptibility was not suitable for testing susceptibility of strains to cationic biocides. No consistent difference in zone diameters could be detected between test and sensitive controls using discs impregnated with various concentrations of CTAB, Qr, Cv, SO, Eb and Pi. Only Eb and Pi produced significant zones diameters. All subsequent testing for susceptibility to cationic biocides was performed via MIC determinations.

The agar dilution method was used to determine MICs to cationic biocides of strains of *S. aureus* carrying plasmid pWBG1773, pWBG72 (*qacC*) and pWBG53 (*qacA*). The range of cationic biocides included Ay, Af, Bc, CTAB, Ch, Cv, DAPI, Eb, Pt, Qr, R6 and SO. The MIC was calculated for each compound and recorded in Table 4.3.

Plasmid pWBG1773 conferred resistance to the cationic biocides Bc, CTAB, Cv, Qr, R6 and SO. Plasmid pWBG1773 did not confer resistance to Af, Ch, DAPI, Eb, Pi and Pt. Plasmid pWBG72 carrying *qacC* conferred resistance to Bc, CTAB, Cv, Eb and R6, and plasmid pWBG53 carrying *qacA* conferred resistance to all cationic biocides tested. The levels of resistance conferred by plasmid pWBG1773 differed from those conferred by plasmid pWBG53. In particular plasmid pWBG1773 confers a lower level of resistance to Cv and a higher level of resistance to Qr (Table 4.3). The results confirm that unlike the other two plasmids, pWBG1773 does not confer any resistance to Eb.

Table 4.3– Comparison of cationic biocide resistance encoded by staphylococcal plasmids pWBG1773, pWBG72 and pWBG53

Strain	Plasmid	MICs to Cationic Biocides[a]												
		QAC		BIG	Diamidine			Dyes						
		CTAB	Bc	Ch	Pt	Pi	DAPI	Eb	Qr	R6	SO	Cv	Af	Ay
RN4220	-	1	1	0.2	20	20	1	2	2	0.2	2	0.05	2	ND
WBG1876	-	1	1	0.2	20	20	1	2	2	0.2	2	0.05	2	ND
WBG4364	pWBG1773	8	4	0.2	20	20	1	2	20	2	8	0.5	2	ND
WBG1979	pWBG72	8	4	0.2	20	20	1	20	2	1	2	0.1	2	ND
WBG1320	pWBG53	6	4	0.5	>300	>300	8	100	5	4	>16	4	40	ND

[a] [a]Compounds tested: QAC, quaternary ammonium compounds (CTAB, cetyltrimethylammonium bromide; Bc, benzalkonium chloride); BIG, biguanidine (Ch, chlorhexidine); Diamidines (Pt, pentamidine isethionate; Pi, propamidine isethionate; DAPI, 4, 6-diamidino-2-phenylindole); Dyes (Eb, ethidium bromide; Qr, quinaldine red; R6, rhodamine 6G; SO, safranin O; Cv, crystal violet; Af, acriflavine). ND, not able to determine MIC. Controls include RN4220 and WBG1876 as plasmid free sensitive controls, WBG1320 (*qacA*) and WBG1979 (*smr*) as positive controls.

Several attempts to demonstrate resistance to Ay, as previously described by Emslie (1986), were not successful. Initial MICs were performed using Ay supplied by ICN but results were inconsistent and little variation was produced between sensitive and resistant controls. ICN did not provide the purity of Ay so another batch of Ay was purchased from Sigma-Aldrich with 90% purity. This Ay could only be dissolved to a maximum solubility of 1 mg/ml, which would not provide high enough concentrations for MIC analysis as previously described. When Ay was dissolved to its maximum solubility in MHA no discernable MIC difference between test and sensitive strains was found.

4.6.2 Resistance phenotype of plasmid pWBG1773 in *E. coli* host

MICs were used to test susceptibility of BMS101, a recombinant *E. coli* carrying plasmid pWBG1773 inserted into vector pSK+ via its *Bam*HI site. The results in Table 4.4 show resistance being expressed to the same cationic biocides as seen in the staphylococcal background (Table 4.3). Individual MICs are higher in the *E. coli* host than in *S. aureus* host but relative MICs are the same or similar (Table 4.5).

Table 4.4- Cationic biocide resistance phenotype of *E. coli* strain BMS101 carrying plasmid pWBG1773 cloned into vector pSK+ (pBMS101).

Strain	Plasmid	MICs to Cationic Biocides[a]												
		QAC		BIG	Diamidine			Dyes						
		CTAB	Bc	Ch	Pt	Pi	DAPI	Eb	Qr	R6	SO	Cv	Af	Ay
DH5α	-	60	20	0.25	40	40	< 1	50	150	400	40	2	20	ND
BMS02	pSK+	60	20	0.25	40	40	< 1	50	150	400	40	2	20	ND
BMS101	pBMS101	180	100	0.25	40	40	< 1	50	300	600	> 200	10	20	ND

[a] [a]Compounds tested: QAC, quaternary ammonium compounds (CTAB, cetyltrimethylammonium bromide; Bc, benzalkonium chloride); BIG, biguanidine (Ch, chlorhexidine); Diamidines (Pt, pentamidine isethionate; Pi, propamidine isethionate; DAPI, 4, 6-diamidino-2-phenylindole); Dyes (Eb, ethidium bromide; Qr, quinaldine red; R6, rhodamine 6G; SO, safranin O; Cv, crystal violet; Af, acriflavine). ND, not able to determine MIC. Controls include: BMS01 as the plasmid-free sensitive control and DH5α carrying the vector pSK+, BMS02, used to rule out the possibility of intrinsic resistance to cationic biocides

Table 4.5 – Relative MICs of *S. aureus* RN4220 and *E. coli* DH5α carrying plasmid pWBG1773

Test Strain	Sensitive Control Strain	Plasmid	Relative MICs to Cationic Biocides[a]											
			QAC		BIG	Diamidine			Dyes					
			CTAB	Bc	Ch	Pt	Pi	DAPI	Eb	Qr	R6	SO	Cv	Af
WBG4364	RN4220	pWBG1773	5	4	1	1	1	1	1	5	10	4	> 5	1
BMS101	DH5α	pBMS101	3	5	1	1	1	1	1	2	1.5	> 5	5	1

[a]Compounds tested: QAC, quaternary ammonium compounds (CTAB, cetyltrimethylammonium bromide; Bc, benzalkonium chloride); BIG, biguanidine (Ch, chlorhexidine); Diamidines (Pt, pentamidine isethionate; Pi, propamidine isethionate; DAPI, 4, 6-diamidino-2-phenylindole); Dyes (Eb, ethidium bromide; Qr, quinaldine red; R6, rhodamine 6G; SO, safranin O; Cv, crystal violet; Af, acriflavine).

4.7 Cloning Individual and Combinations of ORF2 and ORF3

ORF2 and ORF3 of plasmid pWBG1773 were sub-cloned in order to determine their role in conferring resistance to cationic biocides. Each sequence in plasmid pWBG1773 encompassing ORF2 and ORF3 was amplified using specifically designed PCR primers, ligated into *E. coli* vectors and *E. coli/S. aureus* shuttle vectors. Forward and reverse primers were designed to amplify sequence ORF2A. This sequence encompasses a region 5′ to the first start codon of ORF2 so that it would include regulatory signals for transcription. The same forward primer was used to amplify sequence ORF2A&3, which encompasses both ORF2 and ORF3 sequences. Another pair of primers was designed to amplify the ORF3 sequence. All inserts of ORF2A and ORF3 sequences in pDrive were sequenced to confirm they were identical to those in the parent plasmid pWBG1773 (Appendix 7.10). ORF2A and ORF3 sequences were also ligated into *E. coli*/*S. aureus* shuttle vectors, pCL52.2 and pL150. These were transformed into *E. coli* and various constructs were then transformed into *S. aureus* strain RN4220 by electroporation. Recombinant clones carrying ORF2A and ORF3 were tested for their resistance to cationic biocides in *E. coli* (Table 4.6) and *S. aureus* (Table 4.7).

Table 4.6 - MIC values for strains of *E. coli* DH5α carrying ORF2A and/or ORF3 sequences inserted in vector pDrive

Strain	Plasmid	Insert	MICs to Cationic Biocides[a]											
			QAC		BIG	Diamidine			Dyes					
			CTAB	Bc	Ch	Pt	Pi	DAPI	Eb	Qr	R6	SO	Cv	Af
BMS101	pBMS101	pWBG1773	180	100	0.25	40	40	< 1	50	300	600	> 200	10	20
BMS102	pBMS102	ORF2A	60	20	0.25	40	40	< 1	50	150	400	40	2	20
BMS108	pBMS108	ORF3	60	20	0.25	40	40	< 1	50	150	400	40	2	20
BMS110	pBMS110	ORF2A&3	180	100	0.25	40	40	< 1	50	150	400	> 200	10	20
BMS02	pSK+	-	60	20	0.25	40	40	< 1	50	150	400	40	2	20
BMS05	pDrive	-	60	20	0.25	40	40	< 1	50	150	400	40	2	20
DH5α	-	-	60	20	0.25	40	40	< 1	50	150	400	40	2	20

[a]Compounds tested: QAC, quaternary ammonium compounds (CTAB, cetyltrimethylammonium bromide; Bc, benzalkonium chloride); BIG, biguanidine (Ch, chlorhexidine); Diamidines (Pt, pentamidine isethionate; Pi, propamidine isethionate; DAPI, 4, 6-diamidino-2-phenylindole); Dyes (Eb, ethidium bromide; Qr, quinaldine red; R6, rhodamine 6G; SO, safranin O; Cv, crystal violet; Af, acriflavine); Values are shown as μg/ml.

Table 4.7 - MIC values for strains of *S. aureus* RN4220 carrying ORF2A and/or ORF3 sequences inserted into shuttle vectors pL150 or pCL52.2

Strain	Plasmid	Insert	MICs to Cationic Biocides[a]											
			QAC		BIG	Diamidine			Dyes					
			CTAB	Bc	Ch	Pt	Pi	DAPI	Eb	Qr	R6	SO	Cv	Af
WBG4364	pWBG1773	-	10	4	0.2	20	20	1	6	20	2	8	0.5	4
BMS132	pBMS115	pL150::ORF2A	2	1	0.2	20	20	1	6	2	0.2	2	< 0.1	4
BMS139	pBMS122	pCL52.2::ORF3	2	1	0.2	20	20	1	6	2	0.2	2	< 0.1	4
BMS136	pBMS119	pL150::ORF2A&3	10	4	0.2	20	20	1	6	20	2	8	0.5	4
BMS11	pL150	-	2	1	0.2	20	20	1	6	2	0.2	2	< 0.1	4
BMS12	pCL52.2	-	2	1	0.2	20	20	1	6	2	0.2	2	< 0.1	4
RN4220	-	-	2	1	0.2	20	20	1	6	2	0.2	2	< 0.1	4

[a]Compounds tested: QAC, quaternary ammonium compounds (CTAB, cetyltrimethylammonium bromide; Bc, benzalkonium chloride); BIG, biguanidine (Ch, chlorhexidine); Diamidines (Pt, pentamidine isethionate; Pi, propamidine isethionate; DAPI, 4, 6-diamidino-2-phenylindole); Dyes (Eb, ethidium bromide; Qr, quinaldine red; R6, rhodamine 6G; SO, safranin O; Cv, crystal violet; Af, acriflavine); Values are shown as μg/ml.

The *E. coli* strains BMS102 and BMS108 bearing the pDrive constructs carrying ORF2A or ORF3, pBMS102 and pBMS108, respectively, have MICs to cationic biocides identical to that of the sensitive and vector controls (Table 4.6) and were considered sensitive to all the cationic biocides tested.

The ORF2A and ORF3 inserts were orientated downstream of the T7 promoter adjacent to the multi-cloning site of vector pDrive. Constructs were also screened for inserts that were orientated downstream in the same direction from the SP6 promoter. No difference was observed in the MIC values between the strains harbouring plasmids with inserts in either orientation.

Strain BMS110 carries the insert ORF2A&3 and has a MIC to cationic biocides identical to strain BMS101 that contains the entire plasmid pWBG1773 inserted into vector pSK+. This indicated that ORF2 and ORF3 would not express cationic biocide resistance without the other, so both sequences appeared necessary for expression of the resistance phenotype in *E. coli.*

The *S. aureus* strains carrying individual ORF2A, BMS132, or ORF3, BMS139, also produced MIC results identical to that of the vector controls, BMS11 and BMS12, and host control, RN4220 (Table 4.7). Only *S. aureus* strain BMS136, which carries ORF2A&3 expressed resistance to cationic biocides identical to strain WBG4364 carrying plasmid pWBG1773.

4.7.1 Cationic biocide resistance of strains carrying ORF2 and/or ORF3 sequences as a single insert

The exact sequence of ORF2 essential for expression of cationic biocide resistance was examined by reducing the size of ORF2 in the presence of ORF3. Several primers (Table 2.5) were used to produce ORF2 inserts that reduced the size of the ORF2 sequence. The variants of ORF2 were designated ORF2A, ORF2B.1, ORF2B.2, ORF2C, ORF2D and ORF2E (Table 2.7 and Figure 4.24). Primers were also used to amplify the entire region containing ORF2 and ORF3 once again including all potential variants of ORF2 (Figure 4.25). These variants were designated ORF2A&3, ORF2B.1&3, ORF2C&3, ORF2D&3 and ORF2E&3. All

amplicons were ligated into cloning vector pDrive and sequenced to confirm they were identical to those in plasmid pWBG1773 (Appendix 7.10). These inserts were ligated into shuttle vectors, transformed into *E. coli* and *S. aureus* and their MICs to cationic biocides determined (Table 4.8 and 4.9, respectively).

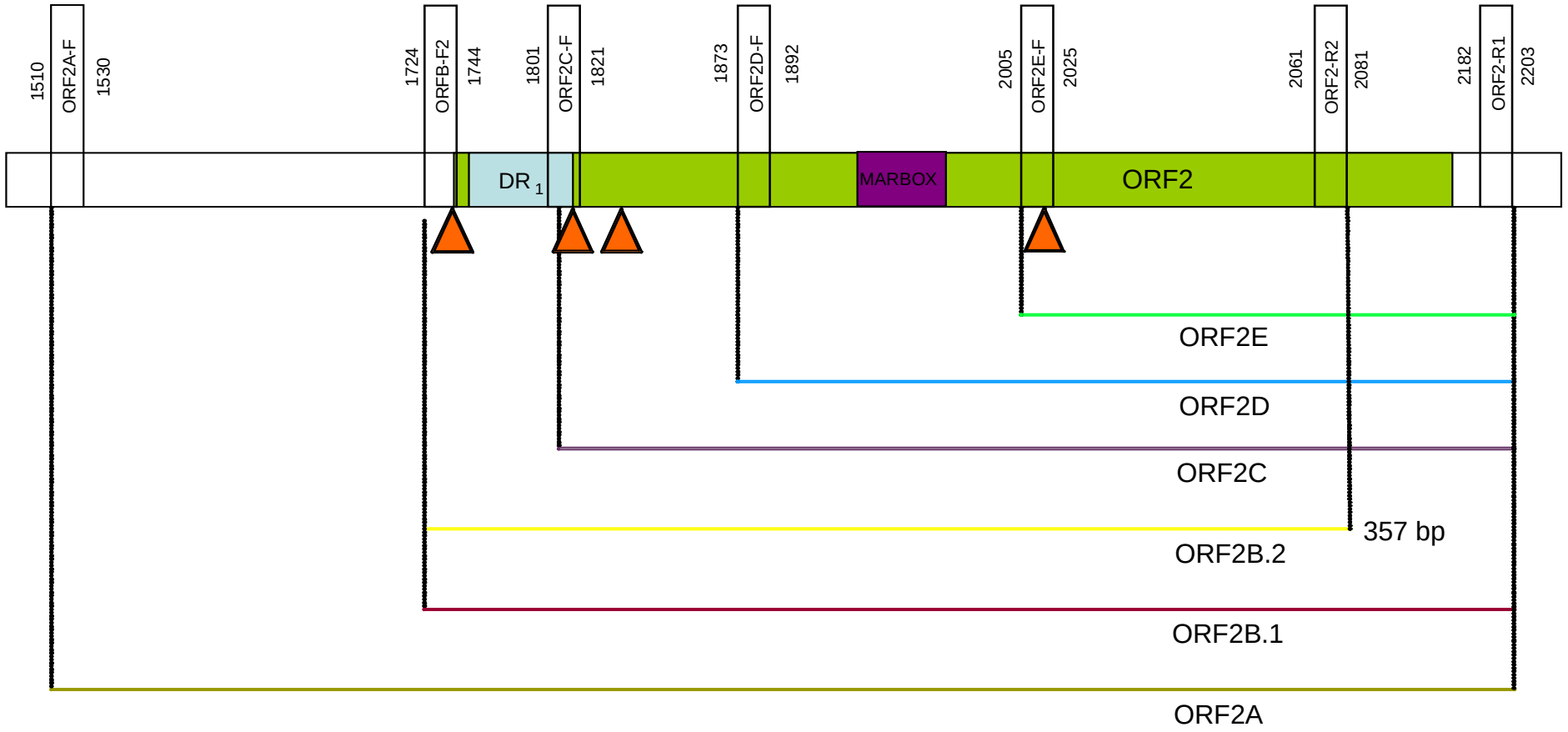


Figure 4.19 – Primer positions are depicted relative to the start and stop codons of ORF2, The ORF is coloured green, the area of DRs is highlighted in blue and the potential 'marbox' indicated in purple. Orange triangles (▲) represent potential ATG start codons. The combination of the PCR products from these primers is extrapolated underneath.

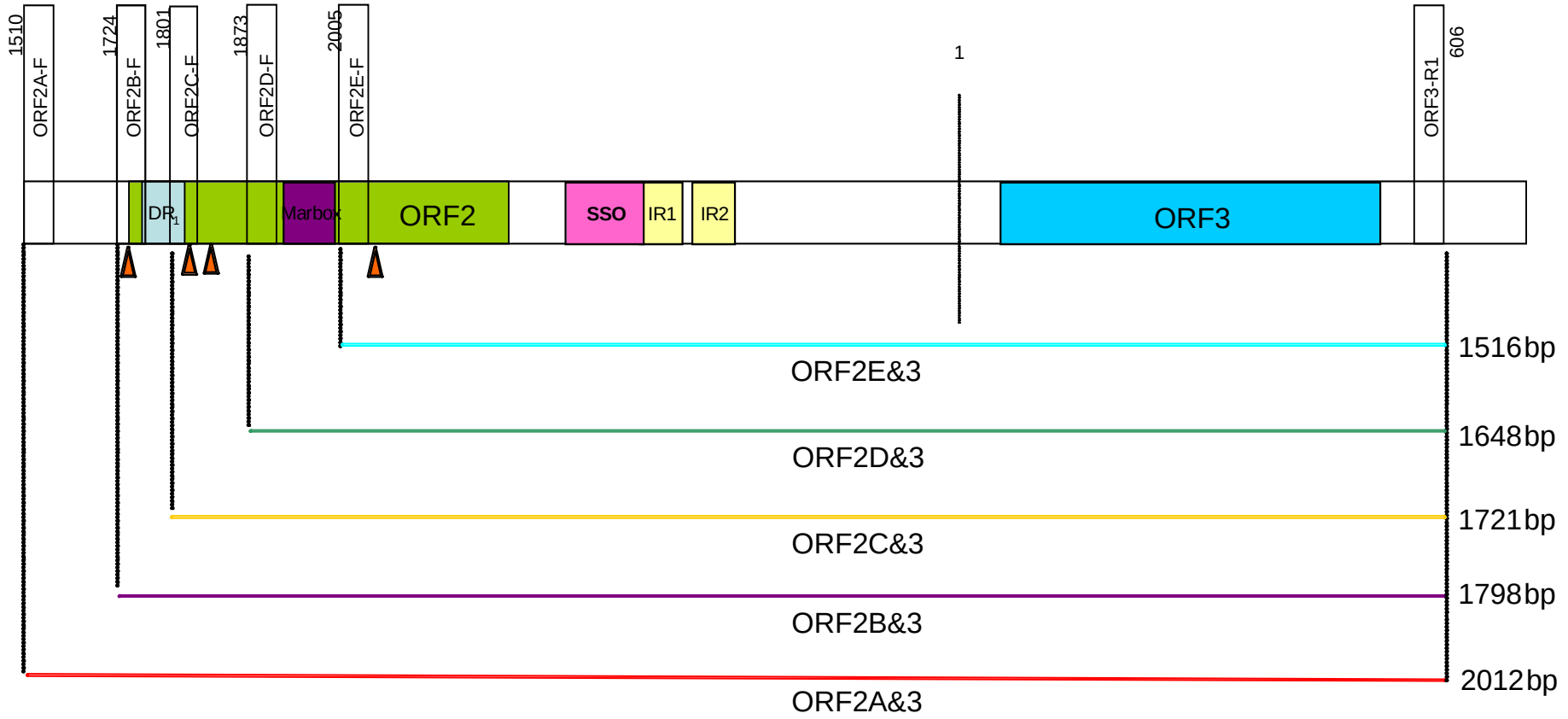


Figure 4.20 – Primer positions are depicted relative to the start codon of ORF2 and stop codon of ORF. The ORF2 is coloured green, ORF3 is blue, the area of DRs is highlighted in light blue and the potential 'marbox' indicated in purple. Orange triangles (▲) represent potential ATG start codons. The combination of the PCR products from these primers is extrapolated underneath.

Table 4.8 - MIC values for strains of *E. coli* DH5α carrying various ORF2 and/or ORF3 sequences inserted in vector pDrive or shuttle vectors pL150 or pCL52.2

Strain	Plasmid	Insert	MICs to Cationic Biocides[a]											
			QAC		BIG	Diamidine			Dyes					
			CTAB	Bc	Ch	Pt	Pi	DAPI	Eb	Qr	R6	SO	Cv	Af
BMS101	pBMS101	pWBG1773	180	100	0.25	40	40	< 1	50	300	600	> 200	10	20
BMS102	pBMS102	ORF2A	60	20	0.25	40	40	< 1	50	150	400	40	2	20
BMS103	pBMS103	ORF2B.1	60	20	0.25	40	40	< 1	50	150	400	40	2	20
BMS104	pBMS104	ORF2B.2	60	20	0.25	40	40	< 1	50	150	400	40	2	20
BMS105	pBMS105	ORF2C	60	20	0.25	40	40	< 1	50	150	400	40	2	20
BMS106	pBMS106	ORF2D	60	20	0.25	40	40	< 1	50	150	400	40	2	20
BMS107	pBMS107	ORF2E	60	20	0.25	40	40	< 1	50	150	400	40	2	20
BMS108	pBMS108	ORF3	60	20	0.25	40	40	< 1	50	150	400	40	2	20
BMS110	pBMS110	ORF2A&3	180	100	0.25	40	40	< 1	50	300	600	> 200	10	20
BMS111	pBMS111	ORF2B&3	180	100	0.25	40	40	< 1	50	300	600	> 200	10	20
BMS112	pBMS112	ORF2C&3	180	100	0.25	40	40	< 1	50	300	600	> 200	10	20
BMS113	pBMS113	ORF2D&3	180	100	0.25	40	40	< 1	50	300	600	> 200	10	20
BMS114	pBMS114	ORF2E&3	60	20	0.25	40	40	< 1	50	150	400	40	2	20
BMS05	pDrive	-	60	20	0.25	40	40	< 1	50	150	400	40	2	20
DH5α	-	-	60	20	0.25	40	40	< 1	50	150	400	40	2	20

[a]Compounds tested: QAC, quaternary ammonium compounds (CTAB, cetyltrimethylammonium bromide; Bc, benzalkonium chloride); BIG, biguanidine (Ch, chlorhexidine); Diamidines (Pt, pentamidine isethionate; Pi, propamidine isethionate; DAPI, 4, 6-diamidino-2-phenylindole); Dyes (Eb, ethidium bromide; Qr, quinaldine red; R6, rhodamine 6G; SO, safranin O; Cv, crystal violet; Af, acriflavine); Values are shown as μg/ml.

Table 4.9 - MIC values for strains of *S. aurues* RN4220 carrying ORF2 and/or ORF3sequences inserted into shuttle vectors pL150 or pCL52.2.

Strain	Plasmid	Genotype	MICs to Cationic Biocides[a]											
			QAC		BIG	Diamidine			Dyes					
			CTAB	Bc	Ch	Pt	Pi	DAPI	Eb	Qr	R6	SO	Cv	Af
WBG4364	pWBG1773	pWBG1773	10	4	0.2	20	20	1	6	20	2	8	0.5	4
BMS132	pBMS115	pL150::ORF2A	2	1	0.2	20	20	1	6	2	0.2	2	< 0.1	4
BMS133	pBMS116	pL150::ORF2B.1	2	1	0.2	20	20	1	6	2	0.2	2	< 0.1	4
BMS134	pBMS117	pL150::ORF2D	2	1	0.2	20	20	1	6	2	0.2	2	< 0.1	4
BMS135	pBMS118	pL150::ORF3	2	1	0.2	20	20	1	6	2	0.2	2	< 0.1	4
BMS136	pBMS119	pL150::ORF2A&3	10	4	0.2	20	20	1	6	20	2	8	0.5	4
BMS137	pBMS120	pL150::ORF2D&3	10	4	0.2	20	20	1	6	20	2	8	0.5	4
BMS138	pBMS121	pL150::ORF2E&3	2	1	0.2	20	20	1	6	2	0.2	2	< 0.1	4
BMS139	pBMS122	pCL52.2::ORF3	2	1	0.2	20	20	1	6	2	0.2	2	< 0.1	4
BMS11	pL150	-	2	1	0.2	20	20	1	6	2	0.2	2	< 0.1	4
BMS12	pCL52.2	-	2	1	0.2	20	20	1	6	2	0.2	2	< 0.1	4
RN4220	-	-	2	1	0.2	20	20	1	6	2	0.2	2	< 0.1	4

[a]Compounds tested: QAC, quaternary ammonium compounds (CTAB, cetyltrimethylammonium bromide; Bc, benzalkonium chloride); BIG, biguanidine (Ch, chlorhexidine); Diamidines (Pt, pentamidine isethionate; Pi, propamidine isethionate; DAPI, 4, 6-diamidino-2-phenylindole); Dyes (Eb, ethidium bromide; Qr, quinaldine red; R6, rhodamine 6G; SO, safranin O; Ay, Acridine yellow; Cv, crystal violet; Af, acriflavine); Values are shown as µg/ml.

All strains of *E. coli* or *S. aureus* carrying any of the ORF2 variants or ORF3 on their own did not express resistance to cationic biocides (Tables 4.8 and 4.9). All strains of *E. coli* or *S. aureus* carrying ORF2 and ORF3 sequences in the same insert did express resistance to cationic biocides except for strains carrying the ORF2E&3 sequence. The latter were sensitive to cationic biocides (Tables 4.8 and 4.9). The ORF2E&3 sequence begins at the 4th potential start codon of ORF2 and ends shortly after the stop codon of ORF3.

4.7.2 Cationic biocide resistance of strains carrying ORF2 and ORF3 sequences on separate plasmids in the same host

Each variant of ORF2 was placed into the same host as ORF3, each carried on separate plasmid vectors. The *E. coli* strain BMS122 carrying ORF3 inserted into vector pCL52.2 was made chemically competent and transformed with the purified plasmid of pDrive or pL150 carrying the various ORF2 inserts. All recombinant clones carrying different combinations of ORF2 with ORF3 were then tested for their resistance to cationic biocides in *E. coli* (Table 4.10).

An attempt was made to electroporate copies of both ORF2 and ORF3 on separate vectors into *S. aureus* RN4220. The *S. aureus* strain, BMS139, carrying the ORF3 insert in vector pCL52.2 (pBMS122) was made chemically competent and electroporated with the purified clone of pL150 carrying the ORF2 insert (pBMS115). All attempts to electroporate both vectors into the RN4220 host were unsuccessful.

Table 4.10 - MIC values for strains of *E. coli* DH5α carrying ORF2 and/or ORF3 sequences inserted into vector pDrive or shuttle vectors pL150 or pCL52.2

Strain	Plasmid(s)	Genotype	MICs to Cationic Biocides[a]											
			QAC		BIG	Diamidine			Dyes					
			CTAB	Bc	Ch	Pt	Pi	DAPI	Eb	Qr	R6	SO	Cv	Af
BMS123	pBMS102 & pBMS122	pDrive::ORF2A & pCL52.2::ORF3	180	100	0.25	40	40	< 1	50	300	600	> 200	10	20
BMS124	pBMS103 & pBMS122	pDrive::ORF2B.1 & pCL52.2::ORF3	180	100	0.25	40	40	< 1	50	300	600	> 200	10	20
BMS125	pBMS104 & pBMS122	pDrive::ORF2B.2 & pCL52.2::ORF3	60	20	0.25	40	40	< 1	50	150	400	40	2	20
BMS126	pBMS105 & pBMS122	pDrive::ORF2C & pCL52.2::ORF3	180	100	0.25	40	40	< 1	50	300	600	> 200	10	20
BMS127	pBMS106 & pBMS122	pDrive::ORF2D & pCL52.2::ORF3	180	100	0.25	40	40	< 1	50	300	600	> 200	10	20
BMS128	pBMS107 & pBMS122	pDrive::ORF2E & pCL52.2::ORF3	60	20	0.25	40	40	< 1	50	150	400	40	2	20
BMS129	pBMS115 & pBMS122	pL150::ORF2A & pCL52.2::ORF3	180	100	0.25	40	40	< 1	50	300	600	> 200	10	20
BMS130	pBMS116 & pBMS122	pL150::ORF2B.1 & pCL52.2::ORF3	180	100	0.25	40	40	< 1	50	300	600	> 200	10	20
BMS131	pBMS117 & pBMS122	pL150::ORF2E & pCL52.2::ORF3	60	20	0.25	40	40	< 1	50	150	400	40	2	20
BMS03	pL150	-	60	20	0.25	40	40	< 1	50	150	400	40	2	20
BMS04	pCL52.2	-	60	20	0.25	40	40	< 1	50	150	400	40	2	20
DH5α	-	-	60	20	0.25	40	40	< 1	50	150	400	40	2	20

[a]Compounds tested: QAC, quaternary ammonium compounds (CTAB, cetyltrimethylammonium bromide; Bc, benzalkonium chloride); BIG, biguanidine (Ch, chlorhexidine); Diamidines (Pt, pentamidine isethionate; Pi, propamidine isethionate; DAPI, 4, 6-diamidino-2-phenylindole); Dyes (Eb, ethidium bromide; Qr, quinaldine red; R6, rhodamine 6G; SO, safranin O; Cv, crystal violet; Af, acriflavine). Values are shown as μg/ml.

In *E. coli* DH5α all ORF2 sequences except two, ORF2B.2 and ORF2E, were able to confer resistance to cationic biocides in the presence of the ORF3 sequence ligated into plasmid pCL52.2 (plasmid pBMS122) (Table 4.10). ORF2B.2 was designed to determine the necessity of the 3′ end of ORF2 by prematurely stopping the sequence 80 bp upstream of the predicted stop codon. ORF2E was designed to incorporate the sequence starting at the 4th start codon and ending at the common stop codon of ORF2.

These results indicate that ORF2D contains the essential sequence of ORF2 and must be accompanied by ORF3 for the expression of cationic biocide resistance.

4.8 Induction of Resistance to Cationic Biocides

ORF3 sequence shares extensive homology with a family of *marR* genes found in other bacteria (Figure 4.18). The product of a *marR* gene is a protein that regulates expression of the *mar* operon, which in turn, regulates expression of resistance to cationic biocides and other antimicrobial agents (Martin and Rosner 1995; Seoane and Levy 1995). The resistance can be induced by several compounds including Tc, Cm and sodium salicylate (Sal) (Cohen *et al.* 1993a, 1993b; Hächler *et al.* 1991). Since ORF3 belongs to the *marR* family of regulatory genes, these same compounds were tested for their ability to induce resistance to the cationic biocide CTAB by plasmid pWBG1773 in *E. coli* strain BMS101. Also CTAB was tested for induction of resistance to itself in *S. aureus* strain WBG4364. No induction of resistance to CTAB was detected in *E. coli* or *S. aureus* (Tables 4.11a and 4.11b, respectively).

Table 4.11a– Induction of CTAB resistance of plasmid pWBG1773 cloned in *E. coli*

E. coli		**MIC to CTAB (μg/ml)**			
Strain	**Plasmid**	**Inducing agent**			
		-	**Tc**	**Cm**	**Sal**
BMS101	pBMS101	180	180	180	180
DH5A	-	60	60	60	60
BMS02	pSK+	60	60	60	60

Table 4.11b – Induction of CTAB resistance of plasmid pWBG1773 in *S. aureus*

S. aureus		**MIC to CTAB (μg/ml)**	
Strain	**Plasmid**	**Inducing agent**	
		-	**CTAB**
RN4220	-	2.5	2.5
WBG1876	-	5	5
WBG4364	pWBG1773	12.5	12.5
WBG1979	pWBG72	15	15
WBG1320	pWBG53	20	20

4.9 Forced Induction and Expression of ORF2

Nt analysis of the ORFs of plasmid pWBG1773 indicate that only ORF2 has the potential to provide the mechanism of resistance to cationic biocides, whereas ORF3 appears to be a regulatory gene. ORF2 was placed under the control of inducible promoters to determine its role in the expression of resistance to cationic biocides independent of ORF3.

4.9.1 Induction of cationic biocide resistance by ORF2 cloned in plasmid pDrive

Plasmid pDrive constructs carrying the various ORF2 inserts ligated downstream of the T7 promoter were transformed into the chemically competent *E. coli* host BL21(DE3). The BL21(DE3) strain contains a T7 RNA polymerase gene under the control of the *lacUV5* gene. The presence of IPTG promotes the synthesis of T7

RNA polymerase, which binds to the T7 promoter in pDrive and induces the expression of the target gene next to the promoter. All of the pDrive constructs listed in Table 2.10 were subjected to induction with IPTG. IPTG was added to the cationic biocide MIC plates at concentrations ranging from 0.1 to 10 mM. The presence of the inducing agent IPTG did not make any difference to the resistance levels expressed by any of these strains as previously recorded in Table 4.8.

4.9.2 Expression of ORF2 in plasmid pDrive

ORF2 may produce a gene product that contains a hydrophobic C-terminus with potential to lodge within the cytoplasmic membrane. An attempt was made to locate this protein within the *E. coli* membrane by analysing the hydrophobic portion of the total protein content. Two-dimensional SDS-PAGE was used to compare the hydrophobic proteins of BMS140 carrying the ORF2 sequence ligated in pDrive (pBMS102) to the vector control strain BMS09, carrying vector pDrive with no insert. Both were induced with IPTG. No variation between the two dimensional SDS-PAGE gels of the test strain carrying the sequence of ORF2 or the control strain was observed (results not shown).

4.9.3 Induction of cationic biocide resistance by ORF2 cloned in plasmid pPROTet

The ORF2B.1 insert from plasmid pBMS102 was excised with REs *Bam*HI and *Hind*III, purified and re-ligated into the expression vector pPROTetE233 to form plasmid pBMS126. This placed the ORF2B.1 sequence under the control of the anhTc-inducible $P_{Ltet0-1}$ promoter in the *E. coli* vector pPROTetE233. The ORF2B.1 insert in plasmid pBMS126 was sequenced to ensure it was ligated in the correct orientation (Appendix 7.11). Attempts were made to place plasmid pBMS126 in the same host as plasmid pBMS122, the pCL52.2 construct carrying the ORF3 sequence. However, all efforts to transform both vectors into the DH5α or DH5αPRO host were unsuccessful. MICs to cationic biocides in the presence of 0.1 ng/ml anhTc were performed on the test strain BMS160, the vector control strain BMS07 and the negative controls DH5α or DH5αPRO. Even under extreme induction no resistance

to the cationic biocides tested was expressed by the ORF2B.1 insert alone (Table 4.12).

Table 4.12 – MICs to cationic biocides of *E. coli* carrying ORF2B.1 inserted in plasmid pPROTetE233 in the presence and absence of anhTc

Strain	Plasmid	MICs (µg/ml)							
		CTAB		Eb		Pi		Ch	
		-	anhTc	-	anhTc	-	anhTc	-	anhTc
BMS160	pBMS126	60	60	50	50	40	40	0.25	0.25
BMS07	pPROTet233	60	60	50	50	40	40	0.25	0.25
DH5αPRO	-	60	60	50	50	40	40	0.25	0.25
DH5α	-	60	60	50	50	40	40	0.25	0.25

CTAB, cetyltrimethylammonium bromide; Ch, chlorhexidine; Pi, propamidine isethionate; Eb, ethidium bromide; anhy, 0.1 ng/ml anydrotetracycline.

4.9.4 Expression of ORF2B.1 in plasmid pPROTet

The pPROTet™ 6xHN Bacterial Expression System was designed to facilitate the expression of a gene and purification of its product. To obtain quantities of the protein product of ORF2 for analysis, the ORF2B.1 sequence was first cloned downstream from a strong RBS and the anhTc-inducible $P_{Ltet0-1}$ promoter in the *E. coli* vector pPROTetE233 to make plasmid pBMS126. The optimal induction conditions for the expression of the protein product of ORF2B.1 were determined from SDS-PAGE analysis (Figure 4.26). These results depicted one band from the total protein sample increasing in intensity with the addition of the inducing agent anhTc over time. This band appeared to peak in concentration after 6 h exposure to 0.1 ng/ml anhTc. These conditions were subsequently used prior to expressing and extracting the 6xHN tagged ORF2B.1 protein.

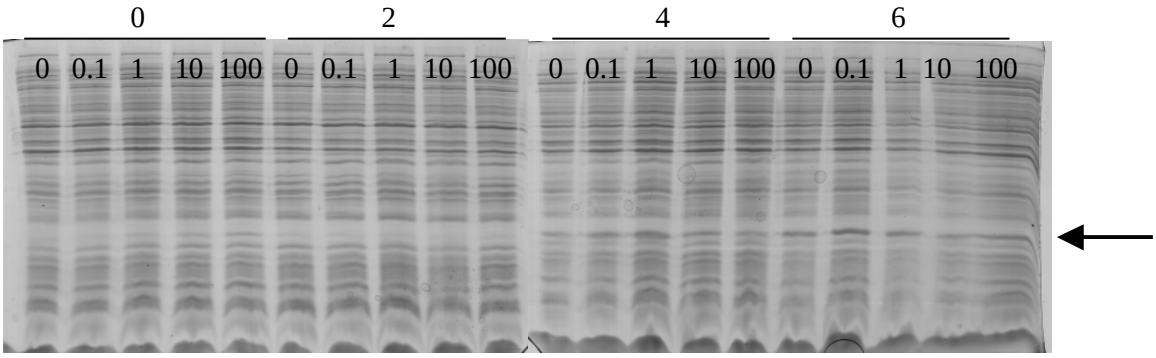


Figure 4.21 - SDS-PAGE patterns of time course expression of pBMS126 (pPROTetE233::ORF2B.1) induced with anhTc. Above the line the numbers correlate to the hours of exposure to anhTc, below the line is the concentration of anhTc (ng/ml). The arrow indicates the only band increasing with intensity.

Using the 6xHN affinity tag incorporated at the C terminus of the protein product of ORF2B.1 would allow purification by CLONTECH's TALON® Purification Kit. Analysis of the extracted 6xHN tagged ORF2B.1 protein by SDS-PAGE revealed a faint band corresponding in size to the band suspected of induction with anhTc identified from the total protein analysis, Figure 4.27. This indicated that the 6xHN tagged protein was strongly induced and extracted to near homogeneity.

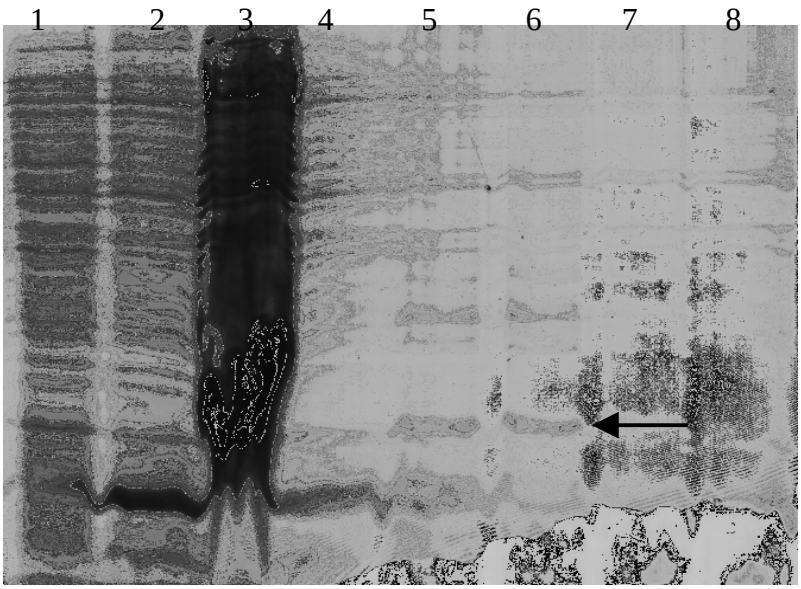


Figure 4.22 – SDS-PAGE analysis of extracted 6xHN tagged ORF2B.1 elution fractions after induction with 0.1 ng/ml anhTc for 6 h. Lane 1, total protein induction control sample; lane 2, total protein; lane 3, clarified sample; lane 4, eluted fraction from vector control strain BMS07; lanes 5-8, eluted fractions from the extraction of BMS160. Arrow indicates the band that corresponds to the induced band in the total protein sample.

Two-dimensional SDS-PAGE analysis was carried out on the elution fractions from both BMS160, carrying pBMS126, and the vector control strain BMS07. There appeared to be no net difference in protein content. The Coomassie blue stained gels contained over 50 potential protein spots with no one spot appearing to increase in concentration with the induction with anhTc (Figure 4.28).

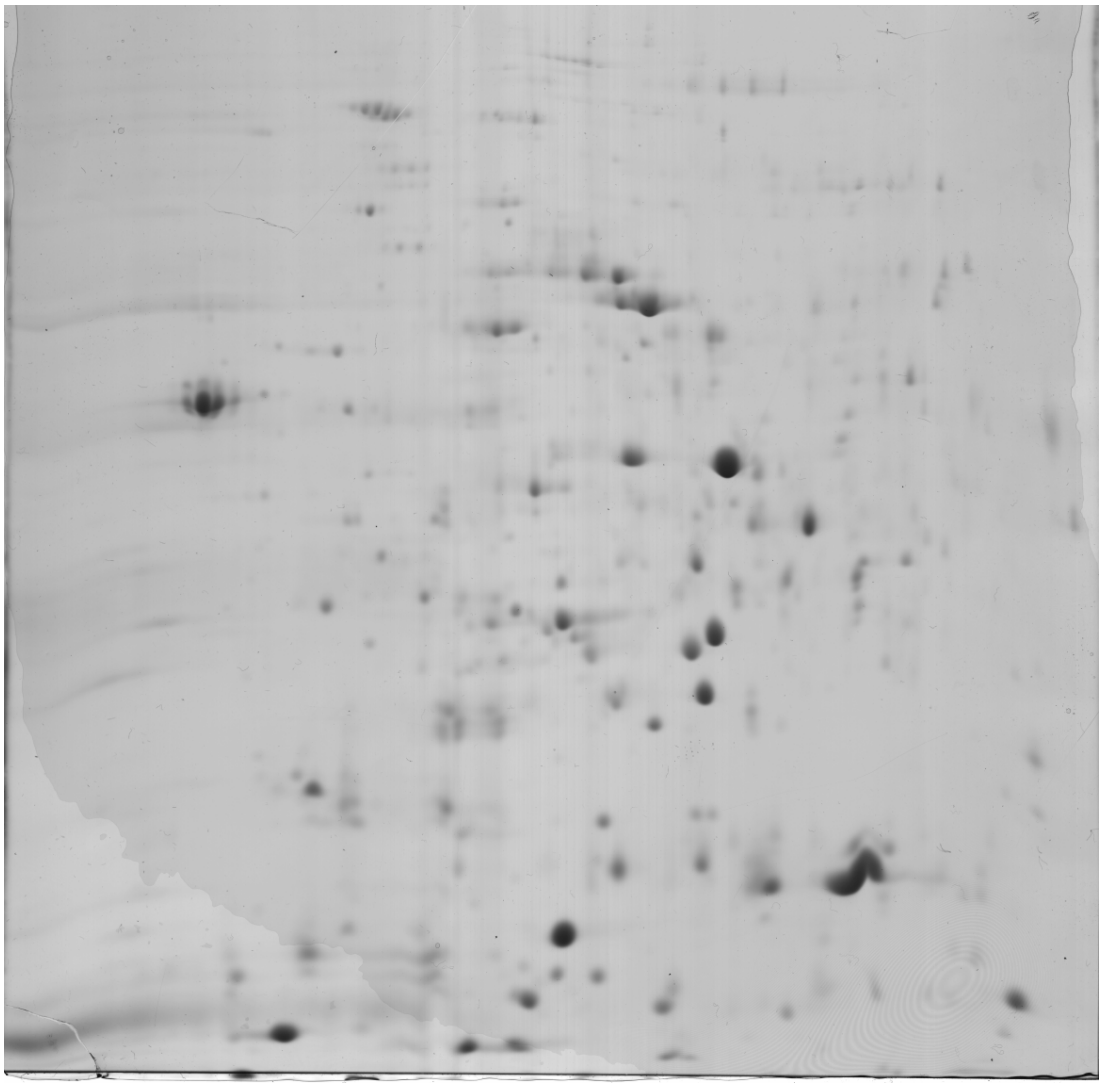


Figure 4.23 – Two-dimensional SDS-PAGE analysis of 6xHN tagged ORF2B.1 extracted sample.

4.10 Mechanism of Resistance to Cationic Biocides

4.10.1 Effect of CCCP on resistance to cationic biocides

Plasmid-borne resistance to cationic biocides involves a membrane-bound pumping mechanism that utilises energy from the cell's PMF (Lambert and Le Pecq 1984).

CCCP interferes with the PMF resulting in a decrease in resistance to cationic biocides. CCCP was tested for its effect on resistance to CTAB expressed by plasmid pWBG1773 in *E. coli* and *S. aureus*. MICs to CTAB were measured in the presence and absence of CCCP. *S. aureus* strains carrying known *smr* and *qacA* genes, WBG1979 and WBG1320, were included as positive controls. The *S. aureus* strains would not grow in the presence of 10 μM CCCP so the concentration of CCCP was reduced to 5 μM which was the highest concentration that allowed growth of colonies. No change was seen in the MICs for all strains carrying pWBG1773 with or without the presence of CCCP in the agar (Table 4.13). Strains WBG1979 and WBG1320 did show a decrease in MICs in the presence of CCCP confirming that this compound does effect the pumping mechanism of the multidrug transporters encoded by *smr* and *qacA* genes (Table 4.13).

Table 4.13 – Effect of CCCP on MICs to CTAB

E. coli			
Strain	**Plasmid**	**MIC to CTAB (μg/ml)**	
		Without CCCP	**With CCCP**
BMS01	-	60	60
BMS02	pSK+	60	60
BMS101	pBMS101 (pSK+::pWBG1773)	180	180
S. aureus			
Strain	**Plasmid**	**MIC to CTAB (μg/ml)**	
		Without CCCP	**With CCCP**
WBG4364	pWBG1773	12.5	12.5
RN4220	-	2.5	2.5
WBG1876	-	2.5	2.5
WBG1979	pWBG72 (*smr*)	15	12.5
WBG1320	pWBG53 (*qacA*)	20	12.5

4.11 Screening MRSAs for the Presence of ORF2 and ORF3

One hundred MRSAs were selected from the *S. aureus* culture collection at Curtin University, School of Biomedical Sciences. The collection contains MRSAs from over 20 locations around the world collected over the last 30 years. Approximately

five isolates were taken from each location. Isolates were not selected on the basis of their susceptibility to cationic biocides. One strain was found to be impure and was discarded from the analysis, leaving 99 MRSAs for screening.

4.11.1 Screening MRSAs for resistance to representative cationic biocides

MICs to a range of cationic biocides were measured for all MRSAs and each was then classified according to its resistance phenotype (Table 3.7). The source and resistance phenotype of each MRSA strain is provided in Table 4.14 and a summary of the distribution of each phenotype is presented in Table 4.15.

Table 4.14 – The phenotype and location of each MRSA isolate

Origin	WBG Strains	Phenotype
Argentina	4428	1
	4429	4
	4430	5
	4431	1
	4432	4
Canada	2891	6
	2892	5
	2893	4
	2894	5
	2896	5
	2899	4
China	8033	5
	8034	1
	8035	1
	8036	1
	8037	4
Copenhagen	7464	4
	7469	6
	7472	4
	7473	4
	7477	6
	7478	4
Denmark	4170	4
	4171	4
	4172	4
	4173	6
	4174	6
Germany	6380	1
	6382	4
	6388	1

Table 4.14 cont.

Origin	WBG Strains	Phenotype
Germany	7704	1
	7706	1
Hong Kong	2909	1
	2914	1
	8447	1
	8452	1
Hungary	7284	6
	7303	6
	7309	5
	7619	4
	7626	4
Ireland	1761	1
	1762	1
	1763	1
	1764	1
	1766	1
Italy	3772	4
	3774	3
	3776	6
	3781	6
	3782	6
Japan	2929	1
	6298	4
	6299	4
	6451	2
	7850	4
Malaysia	1725	6
	2015	1
	7423	1
	7900	3
	8016	1
New Zealand	8145	6
	8146	4
	10031	5
	10035	2
	10036	4
Nigeria	892	4
San Francisco	4369	6
	4371	6
Saudi Arabia	4388	6
Singapore	1990	1
	3751	3
	3850	1
	6316	1
	9031	1

Table 4.14 cont.

Origin	WBG Strains	Phenotype
South Africa	4345	1
	4350	1
	4355	1
	7341	4
	7343	4
Switzerland	10456	5
Switzerland	10457	5
	10460	6
	10461	6
	10462	6
Texas	2133	4
	2137	4
	3770	6
Thailand	4124	3
	4479	2
	4480	2
	4481	4
	4482	6
United Kingdom	1965	1
	2716	1
	4004	1
	7058	1
	7579	4
	10421	6
Controls	1979	3
	4364	4
	4483	1
	RN4220	6

Table 4.15 – Resistance phenotypes of MRSAs and their location

Location	Cationic Biocide Resistance Phenotype						
	1	**2**	**3**	**4**	**5**	**6**	**Total**
Argentina	2	-	-	2	1	-	5
Canada	-	-	-	2	3	1	6
China	3	-	-	1	1	-	5
Copenhagen	-	-	-	4	-	2	6
Denmark	-	-	-	3	-	2	5
Germany	4	-	-	1	-	-	5
Hong Kong	4	-	-	-	-	-	4
Hungary	-	-	-	2	1	2	5
Ireland	5	-	-	-	-	-	5
Italy	-	-	1	1	-	3	5
Japan	1	1	-	3	-	-	5
Malaysia	3	-	1	-	-	1	5
New Zealand	-	1	-	3	1	-	5
Nigeria	-	-	-	1	-	-	1
San Francisco	-	-	-	-	-	2	2
Saudi Arabia	-	-	-	-	-	1	1
Singapore	4	-	1	-	-	-	5
South Africa	3	-	-	2	-	-	5
Switzerland	-	-	-	-	2	3	5
Texas	-	-	-	2	-	1	3
Thailand	-	2	1	1	-	1	5
United Kingdom	4	-	-	1	-	1	6
Total	33	4	4	29	9	20	99

4.11.2 Screening MRSAs for ORF2 and ORF3 sequences using PCR analysis

PCR products were amplified from 20 MRSAs using the PCR primers for ORF2 or ORF3 (Table 2.6 and Figure 4.29). Eighteen MRSAs provided a single PCR product of the correct size using the primers for ORF2. They were WBG4428, WBG8034, WBG8036, WBG4171, WBG4172, WBG6388, WBG7284, WBG1766, WBG7472,

WBG2929, WBG6299, WBG10035, WBG4369, WBG4388, WBG1990, WBG3850, WBG9031 and WBG4482. Nine MRSAs provided a single PCR product of the correct size using the primers for ORF3. These were WBG4428, WBG2894, WBG8033, WBG8036, WBG7472, WBG4171, WBG4172, WBG4482 and WBG7284. Seven MRSAs provided PCR products from both ORF2 and ORF3 primers; these were WBG4428 (Argentina), WBG8036 (China), WBG7472 (Copenhagen), WBG4171 (Denmark), WBG7284 (Denmark), WBG4482 (Thailand) and WBG4172 (Hungary).

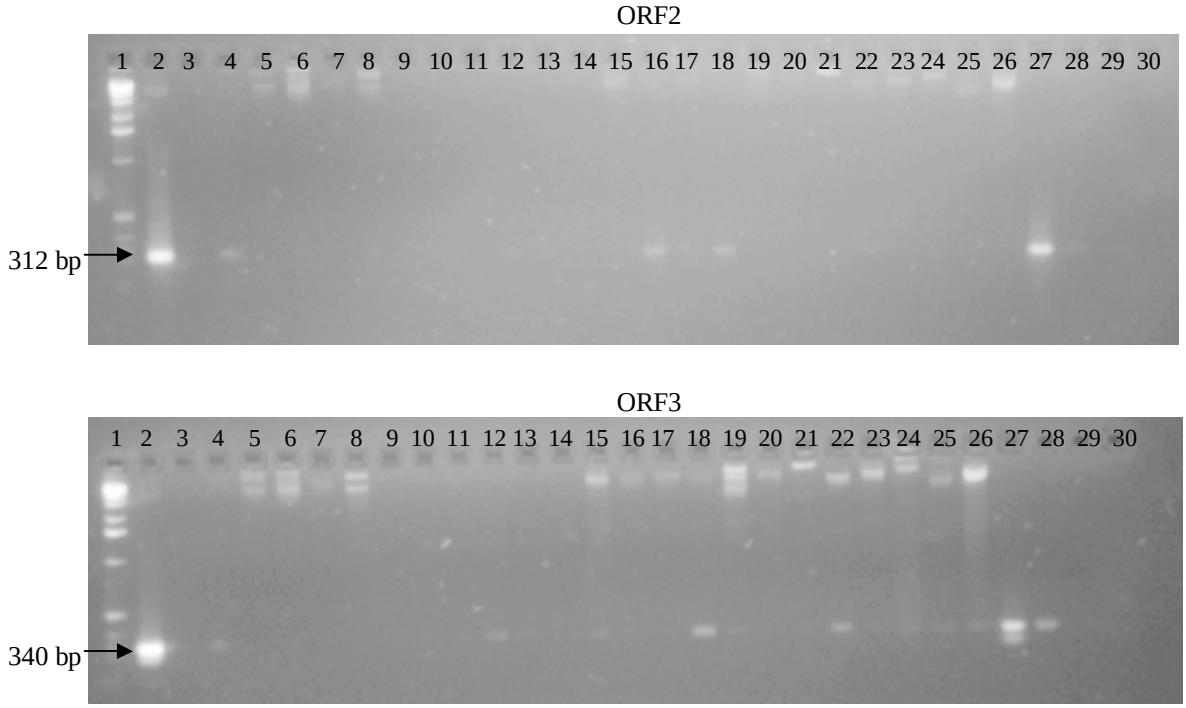


Figure 4.24 – A typical screening PCR gel performed on MRSA isolates, using ORF2 (top) and ORF3 (bottom) primers.

Lane	Sample	Approx. product sizes	
		ORF2	ORF3
1	1-kb DNA Ladder (see Appendix 7.3 for band sizes)		
2	pWBG1773, positive control	312-bp	340-bp
3	WBG541 chromosomal DNA, negative control	-	-
4	WBG4428	312-bp	340-bp
5	WBG4429	-	-
6	WBG4430	-	-
7	WBG4431	-	-
8	WBG4432	-	-
9	WBG2891	-	-
10	WBG2892	-	-
11	WBG2893	-	-
12	WBG2894	-	340-bp
13	WBG2896	-	-
14	WBG2899	-	-
15	WBG8033	-	340-bp
16	WBG8034	312-bp	-
17	WBG8035	-	-
18	WBG8036	312-bp	340-bp
19	WBG8037	-	-
20	WBG7464	-	-
21	WBG7469	-	-
22	WBG7472	-	340-bp
23	WBG7473	-	-
24	WBG7477	-	-
25	WBG7478	-	-
26	WBG4170	-	-
27	WBG4171	312-bp	340-bp, < 300-bp
28	WBG4172	312-bp	340-bp
29	Reagent Control	-	-

The phenotypes of the MRSA strains that provided a PCR product amplified using the primers of ORF2 and/or ORF3 are provided in Table 4.16. Nine of the 20 MRSAs that amplified a PCR product have phenotype 1, which has the broadest and highest resistance profile to the compounds tested. Phenotype 4 closely resembles the resistance profile of plasmid pWBG1773 and 3 out of the 7 strains that amplified PCR products using both ORF2 and ORF3 primers are also classified as phenotype 4. Four of the MRSAs that produced PCR products when amplified with the primers of ORF2 and/or ORF3 were classified as phenotype 6 and are deemed sensitive to the cationic compounds tested.

Table 4.16 – Phenotype classification of MRSAs that provided a PCR product when amplified using the primers for ORF2 and/or ORF3

	Strains that provided a PCR product from ORF sequences			
Phenotype	**ORF2**	**ORF3**	**ORF2 and ORF3**	**Total**
1	WBG8034 WBG6388 WBG1766 WBG2929	WBG1990 WBG3850 WBG9031	WBG8036 WBG4428	9
2	WBG10035	-	-	1
3	-	-	-	0
4	WBG6299	-	WBG4171 WBG4172 WBG7472	4
5	-	WBG8033 WBG2894	-	2
6	-	WBG4369 WBG4388	WBG7284 WBG4482	4
Total	6	7	7	20

There is no correlation between the MRSAs from which PCR products were amplified using the primers of ORF2 and/or ORF3 and their phenotypes.

4.11.2.1 Plasmid profiles of MRSAs

MRSAs from which a PCR product was amplified using primers for ORF2 or ORF3 were analysed for their plasmid content. The plasmid DNA extracts were separated by agarose gel electrophoresis and visualised after staining with Eb (Figure 4.30). There was great variation in the number and sizes of plasmids carried in the MRSAs. Plasmid sizes ranged from < 2.9 kb to > 40.3 kb. There was no consistent pattern of plasmids and some MRSA strains contained no plasmids.

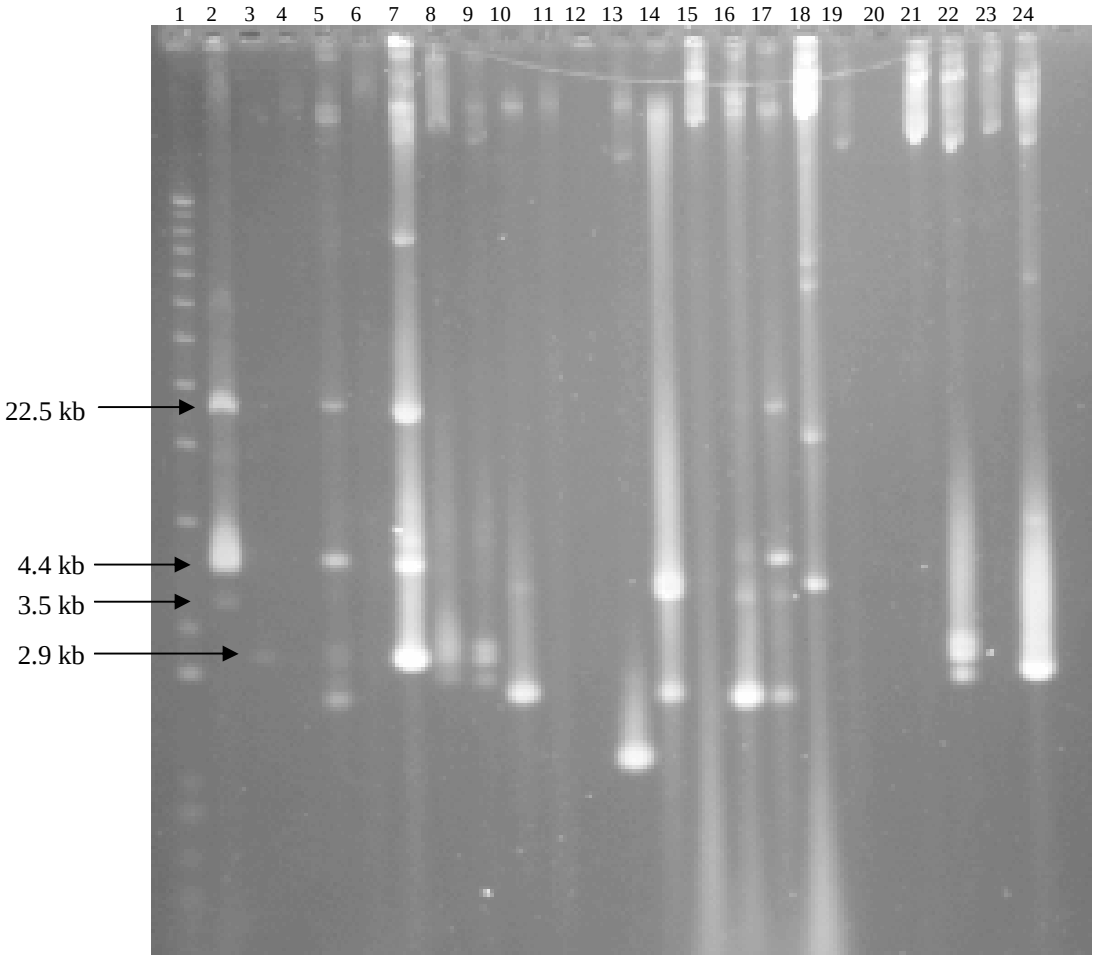


Figure 4.30 – Plasmid profiles of MRSA strains that produced a positive result in the PCR screen (Please note the band corresponding to the 40.3-kb plasmid in lane 2, is very faint in this reproduction of the gel)**.**

Lane	Sample
1	1-kb+ Ladder
2	WBG4483 (pWBG615, pWBG115, pWBG3 and pE194), 40.3-kb, 22.5-kb, 4.4-kb 3.5-kb
3	WBG4364 (pWBG1773), 2.9-kb
4	WBG541 chromosomal DNA
5	WBG4428
6	WBG2894
7	WBG8033
8	WBG8034
9	WBG7472
10	WBG4171
11	WBG4172
12	WBG6388
13	WBG7284
14	WBG1766
15	WBG2929
16	WBG6299
17	WBG10035
18	WBG4369
19	WBG4388
20	WBG1990
21	WBG3850
22	WBG9031
23	WBG4482
24	WBG8036

4.11.3 Homology of ORF2 and ORF3 sequences in MRSAs

Several of the PCR products amplified from MRSAs using ORF2/ORF3 primers were sequenced using the same primers used in the PCR reaction. Three MRSA strains were chosen for comparison to ORF2 and ORF3 sequences, they were: WBG4172 (phenotype 4), WBG4482 (phenotype 6) and WBG8036 (phenotype 1). Extensive sequence homology is evident between the ORF2 and ORF3 sequences of plasmid pWBG1773 and the PCR products obtained from strains WBG4172, WBG4482 and WBG8036 (Figures 4.31 and 4.32, respectively).

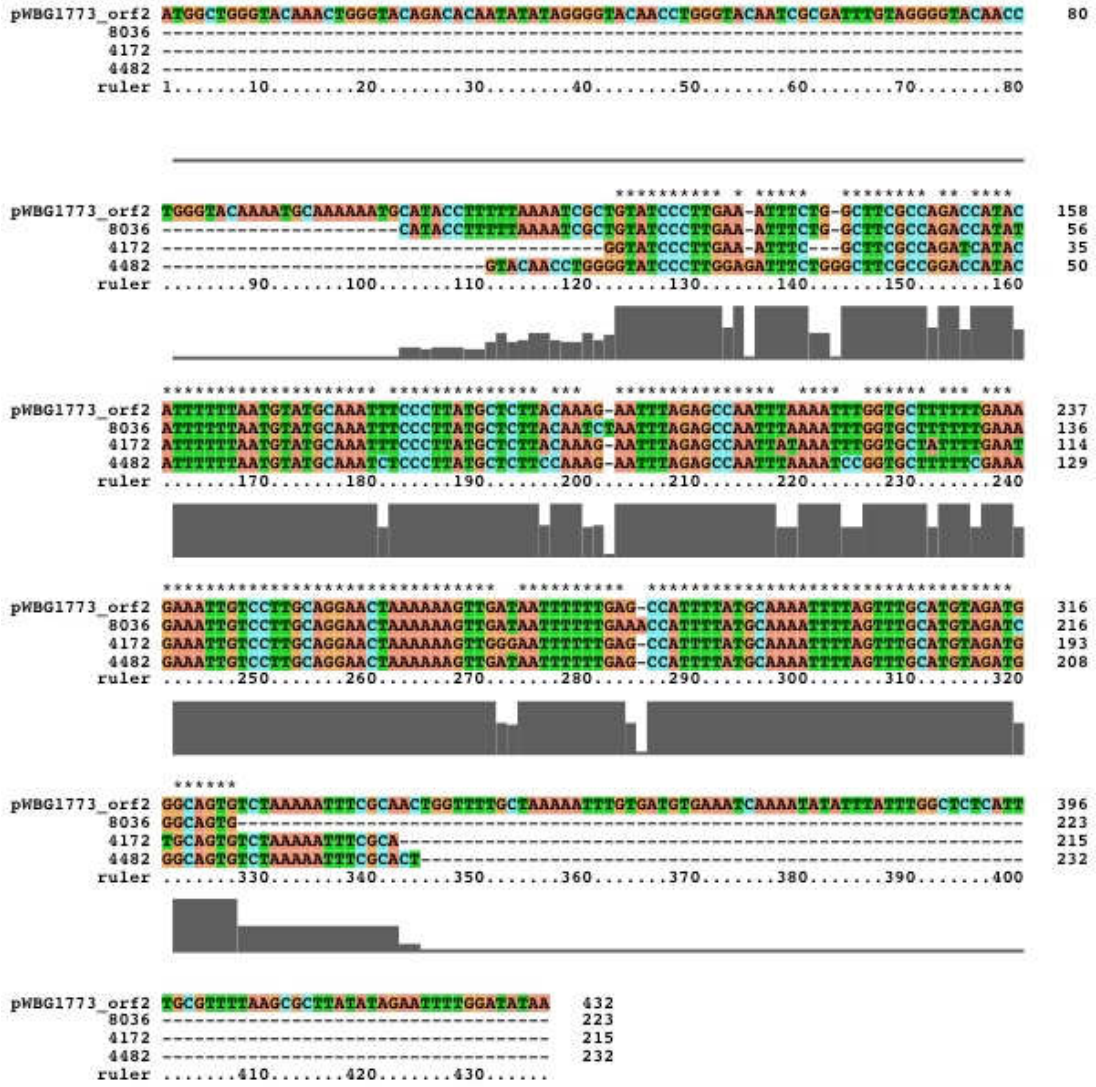


Figure 4.25 - Multiple sequence alignment of pWBG1773 ORF2 with sequenced PCR products from the MRSA screen. A line above the alignment is used to mark conserved positions with '*' indicating positions which have a single, fully conserved nt. DNA sequences are coloured as follows: green, T; blue, C; orange, G and; red, A. A ruler is displayed below the sequences.

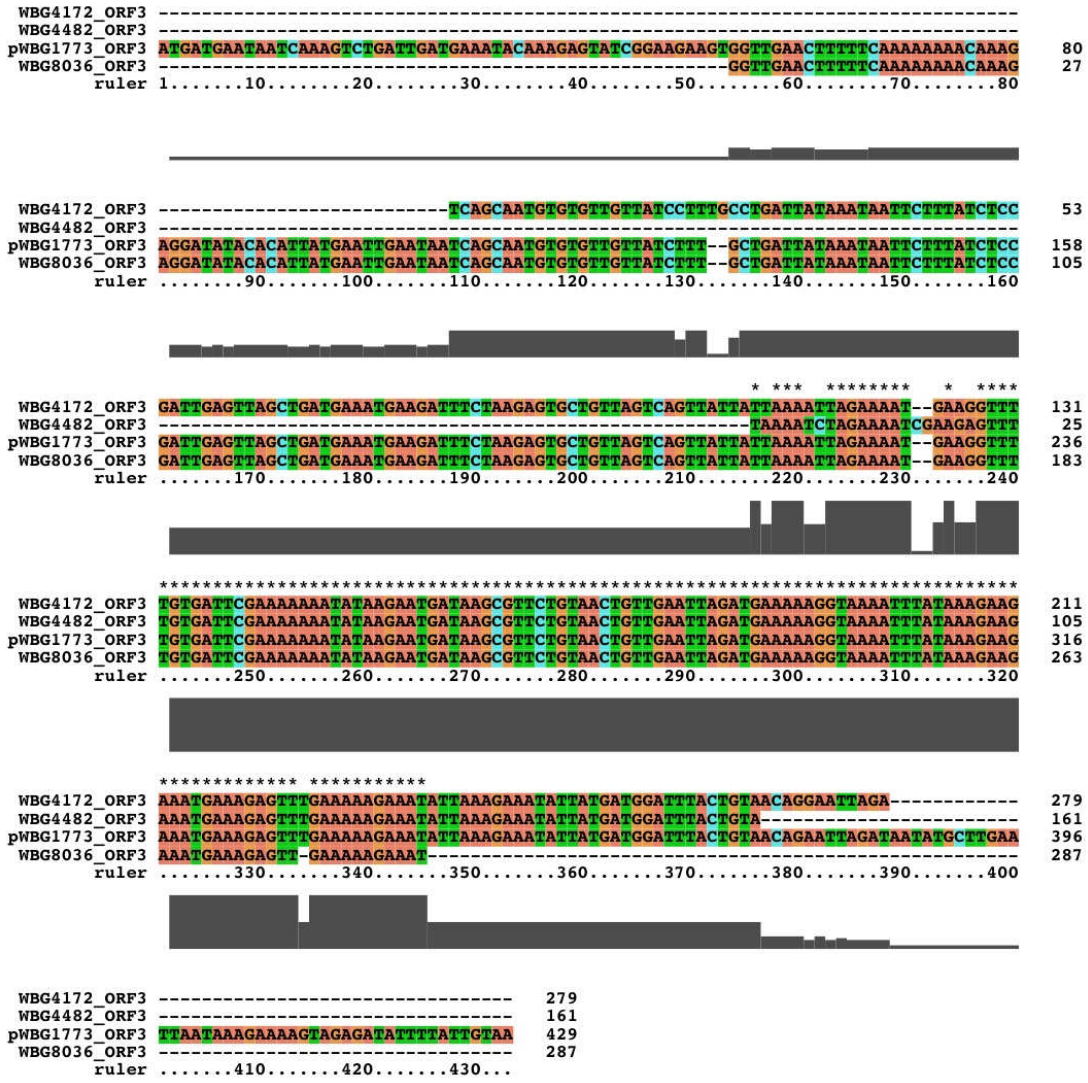


Figure 4.26 - Multiple sequence alignment of pWBG1773 ORF3 with sequenced PCR products from the MRSA screen. A line above the alignment is used to mark conserved positions with '*' indicating positions which have a single, fully conserved nt. DNA sequences are coloured as follows: green, T; blue, C; orange, G and; red, A. A ruler is displayed below the sequences.

5 DISCUSSION

Spread of multiply-resistant *S. aureus* (MRSA) is a worldwide health issue with some strains acquiring resistance to nearly all available antibiotics. MRSA account for a large proportion of nosocomial infections in hospitals and their treatment places a significant burden on patient care and health costs. Cross-infection control of MRSA within hospitals relies partly on the use of biocides in the form of antiseptics and disinfectants. Unlike antibiotics which target specific molecular functions within a bacterial cell, biocides interact with multiple cellular components and so development of resistance to biocides was not expected (Denyer and Stewart 1998; McDonnell and Russell 1999). The discovery of widespread resistance to cationic biocides among MRSA was surprising and led to speculation that this resistance contributed to successful spread and survival of MRSA within hospitals.

Several plasmid-borne genes encode resistance to cationic biocides in staphylococci (Grinius *et al.* 1992; Littlejohn *et al.* 1990, 1992; Mitchell *et al.* 1998; Paulsen *et al.* 1995, 1996a; Tennent *et al.* 1989). Each gene encodes resistance to a different range of cationic compounds. The *qacA* gene has been shown to confer resistance to QACs, dyes, biguanides and diamidino compounds (Tennent *et al.* 1989; Mitchell *et al.* 1998). The *qacB* gene confers resistance to the QACs and dyes but differs from *qacA* as it confers low-level resistance to diamidines and does not confer resistance to biguanides. These two genes have been shown to differ only slightly in their nucleotide sequence. The expression of *qacA* and *qacB* is induced by the presence of cationic biocides that interact with a negative regulator, the product of the *qacR* gene (Grkovic *et al*. 1998). The other resistance determinant for cationic biocides in staphylococci is the *smr* gene. This gene confers resistance to a more limited range of cationic biocides including QACs and nuclear stains such as Eb (Littlejohn *et al.* 1990, 1992). Other resistance determinants for cationic biocides, *qacG*, *qacJ* and *qacH*, have also been found in food and animal isolates of staphylococci (Bjorland *et al.* 2001, 2003; Heir *et al.* 1998, 1999b). These genes share a strong homology to the *smr* gene and are considered members of the same family. The expression of these genes is not under regulatory control.

The QAC genes that provide the mechanism of resistance to cationic biocides encode multi-drug transporters. The *qacA* and *qacB* genes encode membrane proteins with 14 TMS placing them in the MFS family of multi-drug transporters (Paulsen *et al*. 1996a) and the *smr* family of genes encode membrane proteins with 4 TMS that belong to the SMR family of multi-drug transporters (Paulsen *et al*. 1995). Each multi-drug transporter utilises the PMF to pump the cationic biocides out of the cell's cytoplasm and reduce their intracellular concentrations to less inhibitory levels (Grinius and Goldberg 1994; Mitchell *et al.* 1998; Saier *et al.* 1998).

Despite extensive knowledge of the genetic basis and mechanism of cationic biocide resistance in MRSAs and other staphylococci there is still debate over the clinical significance and actual role of this resistance phenotype. More genetic and physiological information is required to clarify the significance and role of resistance to cationic biocides. Emslie (1986) transferred a small plasmid, pWBG1773, from an MRSA isolate that encoded resistance to cationic biocides but differed from other small staphylococcal plasmids in the range of cationic biocides to which it expressed resistance. In particular, plasmid pWBG1773 encoded resistance to CTAB and Ay but did not encode resistance to Eb and diamidines. This plasmid was analysed to determine if its resistance determinants differed from known QAC genes and possibly to shed further light on the significance and role of resistance to cationic biocides.

5.1 Plasmid pWBG1773 is a Small Staphylococcal Plasmid with a Unique Sequence

Plasmid pWBG1773 was cloned into plasmid pSK+ (Figure 4.1) and sequenced with complete agreement in both directions (Figures 4.2 and 4.7). It has a G+C content of 28.87% (Figure 4.3). The G+C content of *S. aureus* is generally 32-36% (Kloos and Schleifer 1986; Kuroda *et al.* 2001) with several staphylococcal plasmids with levels as low as 28.7% (Aso *et al.* 2004). The low G+C content of plasmid pWBG1773 is consistent with prolonged existence in low G+C content hosts, such as staphylococci (Berg *et al.* 1998). The G+C content of the predicted coding region of pWBG1773 is 29.67% and is slightly higher than the overall G+C content. This difference in the G+C content between coding and non-coding DNA is consistent with other genome

sequencing studies (Kuroda *et al.* 2001; Moszer 1998).

The nucleotide sequence of plasmid pWBG1773 shares a high degree of homology with other staphylococcal plasmids. A BLAST search of the GenBank database revealed homology to several staphylococcal plasmids carrying *smr* genes (Figures 4.4a and 4.4b). The ten most significant alignments with plasmid pWBG1773 are all from staphylococci, five are from *S. aureus* and the top six are all *smr*-carrying plasmids. Plasmid pWBG1773 was most related in sequence to a 2779 bp plasmid pPI-2 carrying *qacC* that was isolated from a Japanese strain of *S. warneri* (accession number NC00528). Sequence comparison of plasmid pWBG1773 to pPI-2 using the program DNA Strider 1.4 identified similarities to pPI-2's replication gene, nick site and SSO and also to areas of non-coding DNA in plasmid pPI-2 (Figure 4.5). No identity was shared with respect to the *smr* gene, *qacC*, or the partial duplication of the *smr* gene, *qacC′*, carried by pPI-2. The lack of homology to the *qacC* gene carried by pPI-2 indicates the cationic biocide resistance gene in plasmid pWBG1773 is not related to the *smr* family. Comparison of the nucleotide sequence of plasmid pWBG1773 was extended to other small *smr*-carrying plasmids including pSepCH (Fuentes *et al.* 2005), pSK89 (Littlejohn *et al.* 1990), pSK41 (Berg *et al.* 1998), pLW043 (Weigel *et al.* 2003) and pWBG32 (Grinius *et al.* 1992) (Figure 4.4b). Plasmid pWBG1773 shares a high degree of homology with all these plasmids but has no homology to their *smr* genes.

5.2 Plasmid pWBG1773 has Three ORFs in its Coding Region

Plasmid pWBG1773 was analysed for the presence of ORFs that could encode for proteins of at least 80 aa. Three ORFs were predicted and labelled in order of size, ORF1, ORF2 and ORF3 (Figure 4.6 and Table 4.1). These three ORFs comprised 57.4% of the total genome, which is similar to the total coding region content of other small staphylococcal plasmids such as the *smr*-carrying plasmids pSepCH (64.15%), pSK89 (63.51%), and pKH8 (62.31%) and the Cm resistant plasmid pC194 (64.23%) (Fuentes *et al.* 2005; Horinouchi and Weisblum 1982a; Littlejohn *et al.* 1990).

5.3 Plasmid pWBG1773 Belongs to pC194 Family of Rolling Circle Plasmids

ORF1 of plasmid pWBG1773 was found to have a high degree of homology to a number of replication genes, most of which are found on small plasmids carrying *smr* genes such as plasmids pSK108, pSepCH and pPI-2 (Figure 4.10). All share homology with the replication gene of plasmid pC194, a reference plasmid for small staphylococcal plasmids that replicate by a rolling circle mechanism (Gros *et al.* 1987; Seery *et al.* 1993). The only exception was ORF1 sharing homology to a replication gene on pSK41, a large 46.445 kb conjugative plasmid that encodes resistance to the antibiotics Gm, Tm, Km, and Nm. Plasmid pSK41 also carries a *qac*C resistance gene (Berg *et al.* 1998; Kwong *et al.* 2004) so this homology could be explained if plasmid pSK41 has evolved from integration of a small *smr* plasmid into a larger conjugative plasmid (Berg *et al.* 1998).

The family of plasmids that replicate by a rolling-circle mechanism, represented by plasmid pC194, all possess particular genetic elements. In addition to similar replication genes they share other homologous sequences including a specific nick site and a SSO of replication (Gros *et al.* 1987; Horinouchi and Weisblum 1982a; Seery *et al.* 1993). The nick site provides a sequence in one strand of plasmid DNA that can be cut to trigger the formation of a replication fork for leading strand synthesis of DNA (del Solar *et al.* 1993; Khan 1997; Novick 1989). A potential nick site was located at nt positions 528-534 within a sequence of plasmid pWBG1773 that has extensive homology with similar regions of staphylococcal plasmids belonging to the pC194 group of plasmids (Figure 4.11). Also a site at nt positions 2433-2591 in plasmid pWBG1773 was identified as a potential SSO sequence with overlapping IRs that may provide a signal sequence for the start of plasmid replication (Figure 4.9). The sequence was similar to SSOs of the pC194 family of rolling circle plasmids such as pSK108 (Leelaporn *et al.* 1995), pST827 (Heir *et al.* 1995), pST94 (Heir *et al.* 1999b), pNVH01 (Bjorland *et al.* 2003) and pNVH99 (Bjorland *et al.* 2001) (Figure 4.12).

Since plasmid pWBG1773 possesses all the elements for rolling circle replication and these elements are homologous with those of plasmid pC194 it has been

classified as a member of the pC194 family of plasmids. In light of this classification the ORF1 sequence was designated *rep*$_{WBG1773}$.

5.4 Plasmid pWBG1773 Expresses a Unique Resistance Phenotype to Cationic Biocides in *S. aureus* and *E. coli*

A selection of cationic biocides was chosen for antimicrobial susceptibility testing representing several families of cationic compounds including monovalent and divalent cations. Plasmid pWBG1773 in *S. aureus* expressed resistance to the cationic biocides Bc, CTAB, Cv, Qr, R6 and SO (Table 4.3). There was no detectable resistance to antibiotics or heavy metals (Table 4.2). Emslie (1986) reported that plasmid pWBG1773 conferred resistance to Ay but this could not be verified and may have resulted from use of an impure batch of Ay in the original sensitivity testing.

Phenotypic resistance to cationic biocides has been categorised into three groups based on the MICs and range of cationic biocides to which strains express resistance. The resistance phenotype conferred by plasmid pWBG1773 does not belong to any these groups. Sensitivity to the biguanidine Ch excludes it from the phenotype conferred by the *qacA* gene and sensitivity to all diamidines, including Pi and Pt, excludes it from the phenotypes conferred by *qacA* and *qacB*. Although levels of resistance to several QACs including CTAB and Bc and the cationic dyes Cv, R6 and SO are similar to the phenotype conferred by *smr*, sensitivity to Eb eliminates it as a potential member of this group. Differences in resistance phenotypes have been observed among staphylococcal strains expressing *smr* type proteins, QacG, QacH, QacJ or Smr (Bjorland *et al.* 2001, 2003; Heir *et al.* 1998, 1999b), but never to Eb. Thus the resistance phenotype of plasmid pWBG1773 is unique and confirms its classification into a separate group (Emslie *et al.* 1986).

The resistance phenotype of plasmid pWBG1773 cloned into vector pSK+ in an *E. coli* host mirrors the phenotype observed in the *S. aureus* background. The only difference being the MIC values in *E. coli* were significantly higher than those seen in *S. aureus* (Table 4.4). This may be explained by the presence of an outer membrane in the cell envelope of *E. coli* that may limit the movement of cationic

biocides into the cell's cytoplasm and increase its intrinsic level of resistance (Littlejohn *et al.* 1992; Paulsen *et al.* 1995; Tennent *et al.* 1985). When the levels of resistance in *S. aureus* and *E. coli* are compared using relative MICs, both organisms express resistance to the same range of cationic biocides and with similar relative MICs (Table 4.5). This would indicate that the resistance mechanism of plasmid pWBG1773 is expressed in both hosts and with approximately the same efficiency.

5.5 Determinants of Cationic Biocide Resistance in Plasmid pWBG1773

5.5.1 ORF2 has not previously been associated with resistance to cationic biocides

ORF2 was identified using the ORF analysis tool of MacVector 7.2 at nt positions 1732-2163 and encodes a potential protein of 144 aa in size (Table 4.1). Using the nucleotide blastn search tool ORF2 was found to be homologous to a non-coding region at nt positions 802-412 within plasmid pPI-2. This region was not recognised as a potential coding sequence by the original analysis of pPI-2, which used the GENETYX-WIN (Software Development) to examine the DNA sequence (Aso *et al.* 2004). This particular program identified three ORFs within pPI-2: a *qacC* gene, a partial duplication of the *qacC* gene, *qacC′*, and a *rep* gene. When MacVector™7.2 was used to analyse the pPI-2 sequence for potential reading frames a total of seven potential ORFs were identified, including the three genes previously identified by GENETYX-WIN (Figure 4.13). The ORF-Z sequence in plasmid pPI-2 has 96% homology to the ORF2 sequence identified in plasmid pWBG1773 (Figure 4.14). As the *qacC* gene is already present in pPI-2 it is unknown whether the predicted ORF-Z also plays a role in conferring cationic biocide resistance since the *qacC* gene would mask the role of ORF-Z.

The predicted protein product of ORF2 was found to have similarity to a small region covering 44 aa of a hypothetical protein located on the *S. aureus* rolling circle replication plasmid, pC194 (Accession no. CAA24587.1; Figure 4.15). The role of this hypothetical protein is unknown as neither interruption nor deletion of the gene resulted in loss of plasmid functions such as replication or Cm resistance (Horinouchi and Weisblum 1982a).

An analysis of the possible secondary structures of the protein product of ORF2 suggests that the C-terminal region is hydrophobic and could locate in the cytoplasmic membrane with the hydrophilic N-terminus extending into the cytoplasm (Figures 4.16 and 4.17). Also the structural analysis of the protein product of ORF2 does not reveal any TMS nor bear any resemblance to membrane proteins that form multi-drug transporters.

Although ORF2 and its potential protein product have homology with some sequences within the NCBI database these have not been ascribed a function nor associated with resistance to cationic biocides. This strongly suggests that the cationic biocide resistance encoded by plasmid pWBG1773 is unique and may not depend on a multi-drug transporter.

5.5.2 ORF3 encodes a regulatory protein resembling MarR

ORF3 is located at nt position 42-473 within the sequence of plasmid pWBG1773 and has the potential to encode a 143 aa protein (Table 4.1). ORF3 has a potential 9 bp RBS 5 nt upstream at nt positions 31-39 and potential -10 and -35 promoter sequences 99 nt upstream from the start codon (Figure 4.7). Thus ORF3 has all the genetic elements necessary for transcription and translation into a protein product.

The sequence of ORF3 was found to resemble the *marR*-type gene associated with Gram-positive (*Bacillus, Enterococcus, Streptococcus and Clostridium* sp.) and Gram-negative (*Burkholderia* sp.) organisms (Figure 4.18). This family of transcription regulators is named after *E. coli* MarR (Alekshun *et al.* 1999), a repressor of genes which activate the multiple antibiotic resistance and oxidative stress regulons, and after SlyA from *Salmonella typhimurium* and *E. coli* (Wu *et al.* 2003), a transcription regulator that is required for expression of virulence and survival determinants in the internal environment of a macrophage.

The predicted aa sequence of ORF3 has a high degree of similarity to the MarR protein particularly at its hydrophobic C-terminus (Figure 4.20) that contains the core sequence by which MarR binds to DNA. A secondary structure analysis of ORF3 detected a HTH domain covering approximately 75 residues from aa 55-127 (Figure

4.19). Typically the HTH domain of MarR-like proteins is a DNA-binding, winged HTH (wHTH) domain of about 135 aa and the region spanning aa 61-121 is required for its DNA binding activity (Alekshun *et al.* 2000). The HTH motif present in ORF3 is consistent with a wHTH strongly suggesting that the product of ORF3 is a regulatory protein with its C-terminal region capable of binding to an operator site within a target DNA sequence.

Regulators with the MarR-type HTH are present in bacteria and Archaebacteria, and control a variety of biological functions, including resistance to multiple antibiotics, household disinfectants, organic solvents, oxidative stress agents and regulation of the virulence factor synthesis in pathogens of humans and plants. The crystal structures of *marR*, *mexR* and *slyA* gene products have been determined and show a wHTH DNA-binding core flanked by helices involved in dimerisation (Alekshun *et al.* 2001; Lim *et al.* 2002; Wu *et al.* 2003). Under normal conditions MarR binds to two DRs within the *mar* operator, *marO*, and prevents transcription of the *marRAB* operon (Martin and Rosner 1995). Though DRs were present in plasmid pWBG1773 (Figure 4.8) none matched those related to the DRs of *marO*.

A *marR*-like gene has been previously identified in *S. aureus* within a gene cluster designated *mepRAB* (Kaatz *et al.* 2005, McAleese *et al.* 2005). This chromosomally located operon encodes MepR, a MarR-like transcriptional regulator, MepA, a novel efflux pump of the MATE family, and MepB, a hypothetical protein of unknown function. The MepR protein represses *mepA* expression (Kaatz *et al.* 2005) and auto-regulates expression of its own gene (Kaatz *et al.* 2006). The MepR protein has homology to the MarR-type regulatory proteins due to its putative HTH motif of 21 aa in length and 25% sequence identity and 42% similarity to that of MarR. The majority of proteins showing identity to MarR (MprA, SlyA, HpcR) also have on average an identity of 25% and similarity of 49% to MarR (Seoane and Levy 1995). In comparison, ORF3 has an HTH motif found over 73 aa and sequence identity of 41% and similarity of 69% to that of MarR. An aa sequence alignment of MepR and the potential product of ORF3 results in less than 20% identity between them (results not shown). Thus the potential regulatory protein encoded by ORF3 has even stronger homology to MarR than MepR.

Since ORF3 has strong homology to *marR*, a search was conducted for other sequences associated with the *mar* operon. A potential 'marbox' was identified at nt positions 1942-1961 within ORF2 of plasmid pWBG1773. Only two bases within the 20 nt sequence did not match the defined consensus sequence for the 'marbox' (Figure 4.21A) and this is typical for other 'marbox' sequences from *mar/sox/rob* regulon promoters (Figure 4.22) (Martin *et al.* 1999).

The presence of ORF3 in plasmid pWBG1773 accompanied by a potential 'marbox' is the first report of a MarR-like regulatory system associated with staphylococcal plasmids.

5.5.3 ORF2 and ORF3 are required for expression of resistance to cationic biocides

When ORF2A or ORF3 were sub-cloned as separate sequences into vector pSK+ in *E. coli* there was no expression of resistance to cationic biocides (Table 4.6). If the same individual sequences were placed into *E. coli*/*S. aureus* shuttle vectors and transferred into a staphylococcal host there was still no expression of resistance (Table 4.7). If ORF2A and ORF3 were present in the same sequence as the ORF2A&3 insert of *E. coli* strain BMS110 and *S. aureus* strain BMS136, then resistance to cationic biocides was detected and with the same resistance phenotype as the original plasmid pWBG1773 (Tables 4.6 and 4.7). Thus ORF2A and ORF3 sequences need to be present in the same host for expression of resistance to cationic biocides.

The need for two genes to be present in order to observe a multi-drug resistance phenotype has been previously demonstrated in relation to SMR homologues in *Bacillus subtilus* (Jack *et al.* 2000). These particular SMR homologues are encoded in pairs and are located in a number of distinct operons. There are three gene pairs in *B. subtilus* designated, *ebrA* and *ebrB* (Accession number: Z99113), *yvdR* and *yvdS* (Accession number: Z94093), and *ykkC* and *ykkD* (Accession numbers: AJ002571 and P49856). One member of each protein pair is short (105-106 aa) while the other is longer (111-117 aa). When each gene of the pair was cloned and expressed individually in *E. coli* strain DH5∝, a drug resistance phenotype was not observed.

When both genes of a pair were expressed together in *E. coli* resistance was observed to compounds including cationic dyes, such as Eb, Cv and proflavine, and the biguanide Ch. The study was unable to determine the exact mechanism of resistance but concluded that each protein pair wasn't activating a protein pump, since each protein when synthesised alone had no effect on gene expression. There are obvious strong parallels with the interplay between ORF2 and ORF3 of plasmid pWBG1773 but there is no homology between these sequences and those of the gene pairs in *B. subtilus.*

5.5.4 Defining regions of ORF2 essential for resistance to cationic biocides

The region of plasmid pWBG1773 that contains ORF2 has complex sequence structures including a set of DRs, a potential 'marbox' and 4 start codons (Figure 4.24). A series of sequences were amplified by PCR using primer pairs that bracket different combinations of these sequence structures. The sequence ORF2A included ORF2 and sequences upstream that may carry transcription signals; ORF2B.1 included all start codons and the 'marbox'; ORF2C did not include the first start codon; ORF2D has the 'marbox' and the last start codon; and ORF2E included only the last start codon. Each of these amplicons was cloned into the *E. coli* vector pDrive and the sequence of the insert was verified. Each strain was tested for resistance to cationic biocides and all were found to be sensitive (Table 4.8) Another set of amplicons was produced using the same set of forward primers for ORF2 sequences and a common reverse primer 3' to the stop codon of ORF3. This generated the sequences ORF2A&3, ORF2B.1&3, ORF2C&3, ORF2D&3 and ORF2E&3. Once again these amplicons were ligated with vector pDrive and transformed in *E. coli* DH5α. All insert sequences were verified and MICs to cationic biocides measured. All strains expressed resistance to cationic biocides except for the strain carrying ORF2E&3 (Table 4.10). These results suggest that expression of cationic biocide resistance required the sequence of ORF2D&3 as a minimum.

Confirmation of these results was sought in a staphylococcal host. Variants of ORF2 with and without ORF3 in the same insert were removed from the pDrive vector and re-ligated into *E. coli*/*S. aureus* shuttle vectors pL150 and pCL52.2. These recombinant plasmids were electroporated into *S. aureus* strain RN4220 and MICs to

cationic biocides measured (Table 4.9). Once again ORF2D&3 was shown to be the minimum sequence that encoded resistance to cationic biocides.

ORF2D&3 contains an intervening sequence between ORF2 and ORF3 that includes IRs and SSO of plasmid pWBG1773. Parts of this sequence may be involved in expression of resistance to cationic biocides so a series of *E. coli* strains was constructed each containing a variant of ORF2 inserted into one plasmid and ORF3 inserted into another compatible plasmid. MICs were measured to cationic biocides of strains carrying all possible combinations of ORF2 and ORF3 sequences on separate plasmids. Only strain, BMS128, carrying ORF2E and ORF3 on separate plasmids was sensitive to cationic biocides (Table 4.10). A variation of one of the amplicons, ORF2B.2, was truncated at the 3' end of ORF2 so that it did not include the stop codon at nt position 2163. Strains of *E. coli* carrying this insert did not express resistance to cationic biocides (Table 4.10).

These results define the coding sequence of ORF2 necessary for resistance to cationic biocides as extending over 143 nt starting at the 4th codon of ORF2 at nt position 2020 and finishing at the stop codon at nt position 2163. This sequence would encode a small protein of 47 aa containing a hydrophobic domain at the C-terminus. The DRs of ORF2 were deemed not essential for expression as resistance to cationic biocides was still conferred in their absence. The intervening sequence between ORF2 and ORF3 is also not required. ORF2E contains the start codon at nt positions 2020-2022 but does not express resistance in the presence of ORF3. However ORF2D which has the same start codon as ORF2E does express resistance to cationic biocides. This suggests that the 'marbox' sequence identified in ORF2D but not present in ORF2E is also essential for expression of resistance to cationic biocides.

It is known that 'marbox' sequences are functional in only one orientation at a given site and that the functional orientation is dependent on the distance of the 'marbox' to the binding site of RNA polymerase. A distance of 30 bp brings the regulatory protein at the 'marbox' into correct orientation with RNA polymerase at the promoter site. The regulator protein makes contact with the carboxy-terminal domain

of the α-subunit of RNA polymerase and activates transcription (Martin *et al.* 1999). The potential 'marbox' in ORF2 is located 30 bp upstream of a -10 and -35 promoter sequence to which RNA polymerase can bind (Figure 4.21B). This is similar to the spacing of 'marbox' sequences upstream from the promoters for *fpr* and *zwf* genes in *E. coli* (Jair *et al.* 1996; Martin *et al.* 1999).

Based on these results it is tempting to hypothesise that the product of ORF3 activates resistance to cationic biocides by binding to the 'marbox' sequence and interacting with RNA polymerase at the promoter site to commence transcription of a RNA sequence that includes ORF2E. In support of this there is a RBS 34 bp upstream from the start codon for ORF2E (Figure 4.7) so the RNA transcript can be translated into a protein product. Such an interaction between ORF2 and the product of ORF3 would explain the requirement for both ORF2 and ORF3 sequences for expression of resistance to cationic biocides.

5.5.5 ORF2 variants do not express resistance to cationic biocides in a transcriptional expression vector/host system

One way of confirming that the ORF2 sequences encode a resistance mechanism regulated by a product of ORF3 is to place ORF2 sequences under control of another regulatory system. When the PCR amplicons that contain ORF2 sequences or ORF2 and ORF3 sequences are cloned within vector pDrive, they are located in correct orientation and downstream from a T7 promoter. Each of these recombinant plasmids was transformed into *E. coli* host BL21(DE3) which contains the T7 RNA polymerase gene under the control of the IPTG inducible *lacUV5* gene. The presence of IPTG was used to promote the synthesis of T7 RNA polymerase, which binds to the T7 promoter in pDrive and induces expression of the target gene downstream of the promoter. MICs of all BL21(DE3) strains to cationic biocides were measured in the presence and absence of increasing concentrations of inducing agent IPTG. The MICs were identical to those obtained for the same plasmids in *E. coli* DH5α (Table 4.10) and the presence of IPTG had no effect. These results contradict the prediction that ORF2 sequences encode the resistance mechanism and forced expression by an inducible promoter would provide resistance to cationic biocides independently of ORF3.

The results affirm that *E. coli* host BL21(DE3) carrying ORF2D in vector pDrive must be accompanied by ORF3 either in the same insert or in another compatible plasmid to express resistance to cationic biocides. Thus ORF3 and the 'marbox' sequence present in ORF2D appear to be essential for expression of resistance. There are several possible explanations for the failure of ORF2D to express resistance to cationic biocides in strain BL21(DE3) in the absence of ORF3. The first and most likely explanation is that T7 RNA polymerase is not transcribing the ORF2 sequences inserted into vector pDrive and so expression is still dependent on *E. coli* RNA polymerase, the 'marbox' sequence and the presence of the ORF3 regulatory protein. If the ORF2D sequence is being forcibly transcribed by T7 polymerase but there is no expression of resistance then it implies that translation of the ORF2E sequence is blocked and the role of the regulatory protein produced from ORF3 is to activate translation possibly by binding to the 'marbox' sequence in the RNA transcript of ORF2D. This would be a novel role for a regulatory protein belonging to the MarR family. Another explanation is that the products of ORF2E and ORF3 are components of a signalling pathway in which the 'marbox' is the operator site for another regulatory protein, the ORF2E protein is a membrane receptor for cationic biocides and the ORF3 protein regulates expression of a chromosomal multi-drug transport gene. Under this proposal the ORF2E protein is located in the cytoplasmic membrane and would alter its conformation upon binding with a cationic biocide and, in turn, interact with ORF3 protein so that it changes conformation and activates transcription of a multi-drug transport gene. This would seem an unlikely explanation as no motifs in ORF2 or ORF3 were detected associated with two-component signalling pathways and it would imply plasmid pWBG1773 only carries regulatory genes for expression of resistance to cationic biocides and not the genes for the resistance mechanism itself.

5.5.6 Translation of ORF2B.1 inserted into protein expression vector pProTet

A protein expression system was used in an attempt to force synthesis of a putative protein thought to provide the resistance mechanism to cationic biocides. Vector pPROTet provides an insertion site downstream from a strong promoter inducible with anhTc, a RBS, a start codon and a small sequence that encodes a 6-His tag. A potential coding sequence can be inserted into this site and its corresponding protein

will be synthesised under the control of anhTc. The protein should have a 6-His tag included in its N-terminus that can be used to assist in purification of the protein. At this stage in the analysis of the resistance determinants of plasmid pWBG1773, the sequence ORF2B.1 was thought to be the most likely sequence for providing the resistance mechanism. ORF2B.1 was cut from its location in vector pDrive and ligated into vector pPROTetE233 to construct plasmid pBMS126 in strain BMS160. The DNA sequence of this region was checked to verify that the insert was in the same reading frame as the start codon and its sequence was identical to the original sequence in plasmid pWBG1773. Strain BMS160 was measured for its MIC to CTAB in the presence of increasing concentrations of anhTc. No increase in resistance to CTAB was detected (Table 4.12). Once again this suggested that ORF2B.1 could not express resistance to cationic biocides independently of ORF3. Alternatively the insertion of ORF2B.1 into the pPROTet vector results in a 6-His tag being incorporated into the N-terminus of any protein product and this additional aa sequence tag may interfere with the ability of any ORF2B.1 product to function as a resistance mechanism for cationic biocides. In order to determine if any protein product was being induced by exposure to anhTc, strain BMS160 was checked for production of potential membrane proteins after induction with anhTc. A protein band was detected in SDS-PAGE gels derived from hydrophobic protein fractions that appeared to be induced by the presence of anhTc (Figure 4.26). An attempt was made to detect this protein by two-dimensional SDS-PAGE analysis. Protein fractions from strain BMS160 incubated in the presence and absence of an optimal level of anhTc were passed through columns with high affinity for proteins with a 6-His tag. Proteins were eluted from the columns and fractions analysed by two-dimensional SDS-PAGE. No consistent differences were detected in the pattern of proteins displayed in the two dimensional gel from strains with or without induction by anhTc (Figures 4.27 and 4.28).

Unfortunately this experiment was conducted prior to establishing that ORF2E was the more likely sequence to encode the protein involved in providing the resistance mechanism for cationic biocides. The entire experiment should be repeated with ORF2E as the insert into vector pPROTet. The results obtained with ORF2B.1 inserted into vector pPROTet at least provide confirmation that ORF2B.1 alone, even

under conditions that should lead to strong transcriptional expression of the sequence, is not able to express resistance to cationic biocides.

5.5.7 Resistance determinants of plasmid pWBG1773 are located within cassette structures

The single-stranded replication intermediates produced by rolling circle replication have a high potential for recombination, with evidence suggesting that fragments of functionally related DNA are being shuffled between plasmids (Seery *et al.* 1993). Plasmids that replicate via the rolling circle mechanism are largely composed of cassettes and the SSO is considered part of a cassette structure, which has the ability to be involved in horizontal exchange (Novick 1989). One cassette is considered essential for plasmid function and harbours functions for plasmid replication including the double stranded origin of replication and the *rep* gene (del Solar *et al.* 1993). In plasmid pWBG1773 the replication cassette has been identified in the region at nt positions 500-1610 and includes the double stranded origin of replication and the $rep_{WBG1773}$ gene. Rolling circle plasmids can harbour other coding cassettes (del Solar *et al.* 1993). One particular study of small *smr*-carrying plasmids found specific sequences at the flanking regions of the cationic biocide resistance gene as key indicators of the coding cassette. Cassettes of *smr* genes are bordered predominately by either two sets of DRs including that of the SSO, or by a replication nick site and the SSO (Alam *et al.* 2003a). Analysis of the sequence of plasmid pWBG1773 reveals that apart from the replication cassette it carries another two potential cassettes. One cassette is located between the nick site and SSO on plasmid pWBG1773 and includes ORF3. The second cassette is located between DR_1 and the SSO and includes the 'marbox' and ORF2 (Figure 4.23).

The arrangement of plasmid pWBG1773 into cassettes provides further evidence that this plasmid belongs to the pC194 family of rolling-circle plasmids. This also provides a possible mechanism by which plasmid pWBG1773 has acquired ORF2 and ORF3, either as two individual cassettes or as a single cassette containing both sequences.

5.6 Induction of Resistance to Cationic Biocides

In some cases, exposure to low concentrations of a particular antimicrobial compound can induce resistance to high concentrations of that compound in cells that are otherwise sensitive or resistant to only low levels (Grkovic *et al.* 1998; Horinouchi and Weisblum 1982a, 1982b). In both the *S. aureus qacA-qacR* and *E. coli marRAB* systems expression can be induced by the presence of a range of structurally diverse compounds. The induction of *qacA* expression is correlated with the ability of QacR to dissociate from operator DNA in the presence of several compounds, including substrates of QacA such as Eb, R6, Cv, Ch, pyronin Y and DAPI, the non-substrate methyl green and plant antimicrobials such as berberine, palmatine and nitidine (Grkovic *et al.* 2003). In the *marRAB* system Tc, Cm, Sal and other unrelated compounds bind to MarR causing it to dissociate from an operator site in the operon and induce expression of the *marA* gene (Ariza *et al.* 1994; Cohen *et al.* 1993b; Hächler *et al.* 1991; Seoane and Levy 1995). The resulting gene products activate expression of multi-drug transport genes leading to an increase in resistance.

No induction of CTAB resistance was detected in strains of *E. coli* or *S. aureus* carrying plasmid pWBG1773 (Tables 4.11a and 4.11b) in the presence of sub-inhibitory levels of CTAB, Tc, Cm and Sal. Many more cationic biocides need to be tested for their ability to induce CTAB resistance before any conclusions can be drawn about the role of ORF3 as a regulatory gene. The experimental approach may also have to be modified as induction of resistance to cationic biocides by QacR has proven difficult to demonstrate by measuring MICs alone since the change in MIC can be quite minimal. It may be necessary to purify the protein product of ORF3 and measure binding coefficients of cationic biocides to this protein as has been done for QacR (Grkovic *et al.* 1998, 2003).

5.7 A Proposed Model for Regulation of Cationic Biocide Resistance in Plasmid pWBG1773

The following model is proposed as the most likely explanation for the results of genetic and sequence analysis of the ORF2 and ORF3 sequences (Figure 5.1). In the

model ORF3 encodes a regulatory protein similar to MarR that binds to the ‘marbox’ sequence and activates RNA polymerase bound to a promoter sequence upstream from the ORF2E sequence. The transcript includes the ORF2E sequence, a RBS and a start codon at nt positions 2020-2022. The ORF2E sequence is translated into a 47 aa protein with a hydrophobic C-terminus that allows the protein to insert into the cytoplasmic membrane. The crucial feature of the model is the ORF3 product binding to the ‘marbox’ and activating transcription. This explains the expression of resistance to cationic biocides in recombinant strains carrying ORF2D and ORF3 sequences and loss of resistance when either ORF3 or the ‘marbox’ sequence is absent. The model predicts that a small molecule, as yet unidentified and presumably a cationic biocide, acts as an inducer by binding to the ORF3 regulatory protein and altering its conformation so that it can bind to the ‘marbox’ and activate transcription (Figure 5.1).

The model does not explain the results obtained attempting to force expression of resistance to cationic biocides in the absence of ORF3, particularly when ORF2 sequences were placed under the control of a T7 promoter in vector pDrive and placed in a host which produces T7 polymerase. At least the model provides a rational basis for designing future experiments to clarify the interaction between ORF2 and ORF3 sequences.

In keeping with this model it is proposed that the ORF2E sequence be designated as gene *cbrA* (structural gene for cationic biocide resistance) and ORF3 as *cbrR* (regulatory gene for cationic biocide resistance) (Figure 5.1).

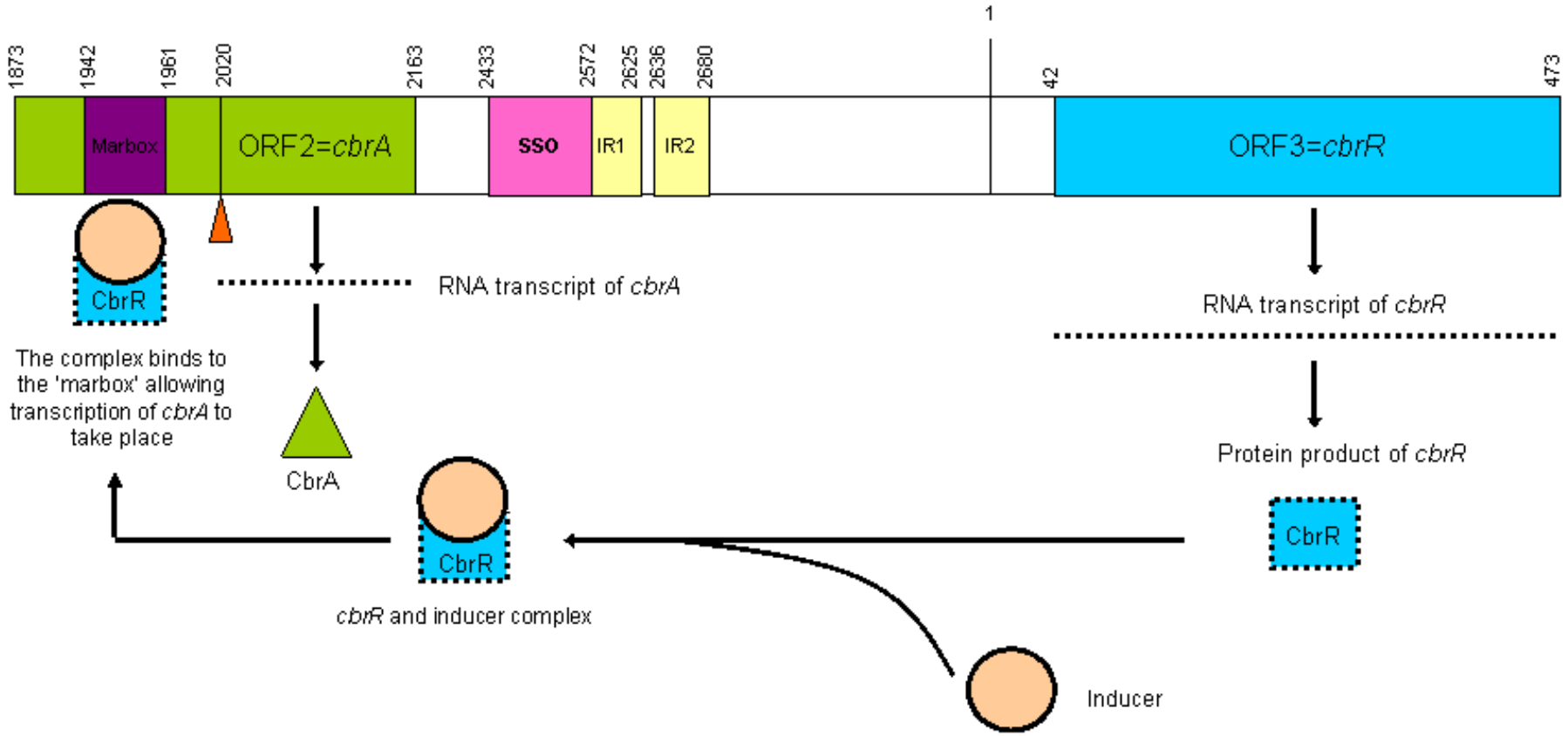


Figure 5.1 – A proposed model for expression of cationic biocide resistance by plasmid pWBG1773.

5.8 Mechanism of Resistance to Cationic Biocides

Multi-drug transport proteins provide the known mechanisms of resistance to cationic biocides in staphylococci. The proteins are located in the cytoplasmic membrane and utilise the PMF as an energy source to drive export of cationic biocides. CCCP and dinitrophenol dissipate the PMF, thereby inhibiting efflux and rendering the organism more susceptible to cationic biocides (Cohen *et al.* 1989; Paulsen *et al.* 1993a). CCCP had no effect on the resistance levels expressed by plasmid pWBG1773 (Table 4.13). This indicates that the mechanism of resistance provided by plasmid pWBG1773 does not utilise the PMF.

An ABC transporter offers another possible mechanism for cationic biocide resistance. The first true bacterial ABC multi-drug transporter was found in *Lactococcus lactis* (Bolhuis *et al.* 1994; Putman *et al.* 2000). The gene encoding this transporter, designated *lmrA*, encodes a 590-aa membrane protein with an N-terminal hydrophobic domain with six putative transmembrane helices and a C-terminal hydrophilic domain, containing the ATP-binding cassette (Putman *et al.* 2000). The sequences of ORF2 and ORF3 do not have any homology to this class of transporter gene nor any motifs associated with an ATP-binding cassette. The multi-drug resistance mechanism encoded by plasmid pWBG1773 maybe unique.

Bacteria continuously adapt to changes in their environment and these responses are largely controlled by two-component signal transduction systems (Henke and Bassler 2004; Stock *et al.* 1989). In essence, these regulatory systems involve the detection of an external stimulus, transmission of that signal across the cell membrane, propagation of the signal through a phosphorelay cascade and, ultimately, the regulation of target gene expression (Cheung and Rood 2006; Parkinson and Kofoid 1992; Stock *et al.* 1989).

One possible explanation for the mechanism of resistance to cationic biocides may be that ORF2 and ORF3 form a two-component signalling system. All two-component signalling pathways consist of a protein kinase that uses ATP to phosphorylate itself at a histidine residue and a response regulator that accepts phosphoryl groups from the kinase at an aspartic acid residue (Parkinson and Kofoid

1992; Stock *et al.* 1989). The length and aa sequence of the sensing domain of histidine kinases are highly variable from one protein to the other, reflecting the variety of signals to which these kinases respond to (Perego *et al.* 1994). Yet the kinase and response regulator proteins of different genera of bacteria share a significant amount of homology (Parkinson and Kofoid 1992). The sensing domain is coupled to a C-terminal cytoplasmic catalytic domain which, consists of a conserved histidine residue and an ATP-binding domain showing high levels of sequence conservation (Henke and Bassler 2004; Parkinson and Kofoid 1992; Stock *et al.* 1989). The response regulator contains a conserved amino-terminal regulatory domain that is phosphorylated on an aspartic acid residue by the histidine kinase to which it is paired. Although the ORF2 sequence has a C-terminus with the potential to be found within the membrane there is no other homology of ORF2 or ORF3 to the two component signalling systems.

5.9 ORF2 and ORF3 Sequences are Widely Distributed in MRSAs

A sample of 99 MRSAs collected from 21 countries around the world during the last 30 years was analysed by PCR for the presence of sequences homologous to the resistance determinants of plasmid pWBG1773. A PCR product indicative of the presence of an ORF2 sequence was detected in 18 of MRSAs and 9 MRSAs possessed sequences homologous to ORF3. Seven of these MRSAs possessed sequences homologous to ORF2 and ORF3 sequences. MRSAs possessing either ORF2 or ORF3 sequences were not confined to a particular period of time nor location. They were distributed over the 30 year time period and13 countries including Argentina, Canada, Germany, Hungary, Ireland, New Zealand, San Francisco, Saudi Arabia, Thailand, China, Denmark, Japan and Singapore. Thus the resistance determinants of plasmid pWBG1773 are not part of an isolated event but must be considered as one of the potential steps in the evolution of different strains of MRSA.

None of the MRSAs carrying sequences homologous to ORF2 or ORF3 have identical plasmid profiles. There was great variation in the number and size of plasmids in these isolates and in some, plasmid DNA bands could not be detected at all (Figure 4.29). This indicates that ORF2 and ORF3 are not confined to small

staphylococcal plasmids and may even be located in the chromosome of some strains. There was no evidence from the sequence of plasmid pWBG1773 that ORF2 or ORF3 were part of a transposable element so until a more detailed analysis of the location these sequences has been conducted there is no explanation for the apparent mobility of ORF2 and ORF3 sequences in MRSAs.

There was no correlation between the resistance phenotype of MRSAs towards cationic biocides and carriage of ORF2 or ORF3 sequences (Table 4.16). Nine MRSAs with the broadest and highest resistance phenotype, phenotype 1, also possessed ORF2 or ORF3 sequences. These MRSAs have a resistance profile indicative of *qacA* that would mask the lower and narrower resistance profile of ORF2 and ORF3. This brings into question why such strains would carry ORF2 or ORF3 sequences. Four other MRSAs possessing ORF2 or ORF3 sequences were completely sensitive to CTAB. This brought into question whether the PCR products were indeed amplicons of ORF2 or ORF3 sequences. Several PCR products were sequenced and shown to have almost identical sequences to those of ORF2 or ORF3 (Figures 4.31, 4.32). These findings taken together with the fact some MRSAs do not carry both ORF2 and ORF3 sequences simultaneously suggest that these genes have another role that does not involve expression of resistance to cationic biocides.

5.10 Clinical Significance of Resistance to Cationic Biocides

Plasmid pWBG1773 expresses levels of resistance to cationic biocides well below the levels used in clinical practice and cross-infection control (Piddock 2006; Russell 2003, 2004). It is therefore difficult to know if the cationic biocide resistance conferred by plasmid pWBG1773 is clinically significant or provides a selective advantage over other organisms to survive and spread in the hospital environment. The ‘in-use’ concentration of cationic biocides such as CTAB, Bc and Ch may exceed that of the MIC for the organism but the actual levels of the cationic biocides on body surfaces may be lower. Cationic biocides are neutralised by the presence of anionic groups of proteins, fatty acids, polysaccharides, soaps and detergents. This may reduce the effective concentration of cationic biocides to a level that can be countered by the resistance mechanism. Alternatively the initial application of the cationic biocide to a body surface may inhibit or kill the microbial flora but as the

concentration of biocide diminishes over time a level is reached at which those bacteria carrying a resistance mechanism to cationic biocides are favoured to re-colonise the surface.

The cationic biocide resistance conferred by plasmid pWBG1773 continues the theme of low-level resistance expressed by all other resistance mechanisms to these compounds with no obvious target for the resistance mechanism. The observation that ORF2 and/or ORF3 sequences may be present in MRSAs that carry other QAC genes or in MRSAs that do not express resistance to cationic biocides brings into question the role of these resistance mechanisms.

5.11 Summary and Conclusion

This study has confirmed that plasmid pWBG1773 confers low-level resistance to QACs and some dyes but does not encode resistance to Eb (Emslie *et al.* 1986). Cloning and sequencing of plasmid pWBG1773 has revealed a unique plasmid of 2,916-bp in length. Plasmid pWBG1773 belongs to the pC194 family of rolling-circle replicating plasmids renowned for their ability to acquire cassettes of resistance genes (Gros *et al.* 1987; Khan *et al.* 1997; Seery *et al.* 1993).

Analysis of the sequence of plasmid pWBG1773 has revealed 3 ORFs. ORF1 shares extensive homology with *rep* genes of other small staphylococcal plasmids belonging to the pC194 family and has been designated $rep_{WBG1773}$. The other two ORFs are required for expression of resistance to cationic biocides. ORF2 was trimmed and it's most likely coding sequence determined to be only 143 nt. This would encode a small protein of 47 aa with a hydrophobic C-terminus that could anchor the protein into the cytoplasmic membrane. The product of ORF3 has all the hallmarks of a MarR regulatory protein and may regulate expression of cationic biocide resistance by binding to a 'marbox' sequence located in ORF2 and activate transcription. As yet no compound has been detected that might interact with ORF3 regulatory protein and act as an inducing agent for cationic biocide resistance. There are no sequences reminiscent of any other QAC gene and no aa sequences capable of forming proteins with TMS that could form part of a multi-drug transport resistance

mechanism. The resistance phenotype, mechanism and genes of plasmid pWBG1773 would appear to be unique.

ORF2 and ORF3 sequences have been detected in other strains of MRSA widely separated by time and place of isolation around the world. This suggests that the appearance of ORF2 and ORF3 sequences in MRSAs is not an isolated event but has contributed to the evolution of different strains of these important nosocomial pathogens.

The role and significance of low-level resistance to cationic biocides in MRSA remains an enigma. The use of cationic biocides may play a role in the selection and survival of MRSAs within hospitals but they do not provide levels of protection able to cope with 'in-use' levels of common antiseptics and disinfectants used in a clinical setting. Others have offered alternative explanations for the existence of resistance mechanisms to cationic biocides including a defence mechanism against cationic peptides encountered in human tissues and plant alkaloids as potential substrates of the resistance mechanism. Staphylococci have been exposed to these antimicrobial compounds for as long as they have colonised animal surfaces so any resistance mechanism to them is likely to have become part of the fabric of chromosomal genes. Plasmid-borne resistance determinants reflect adaptive responses to recent selective pressures. Resistance to cationic biocides is more likely to have emerged in response to exposure to new cationic biocides, particularly in view of their widespread use not only clinically but domestically and industrially.

Unfortunately plasmid pWBG1773 has only added to the enigma. Its resistance phenotype is narrow when compared to other QAC genes, it also confers a very low-level of resistance and its sequences may be present in strains that do not express resistance to cationic biocides at all.

5.12 Future Directions

The model proposed (Figure 5.1) to explain the interaction between ORF2 and ORF3 needs to be verified, particularly in view of the lack of resistance to cationic biocides obtained from cloning ORF2D sequences into vector pDrive under the control of T7

polymerase. RNA extracts should be analysed for transcripts corresponding to ORF2D in the presence and absence of the inducer IPTG for expression of T7 polymerase. If a RNA extract was found corresponding to ORF2D in the absence of ORF3 then this would indicate that translation not transcription was regulated. If however, RNA transcripts can not be found then this indicates there is an inability of the sequence to be transcribed from plasmid pDrive.

Based on the proposed model it is ORF2E that should have been placed in the protein expression vector pPROtet not ORF2B.1. By placing the ORF2E sequence into the correct pPROTet vector and inducing with anhTc to force expression, resistance to cationic biocides should be detected, even in the absence of ORF3. Once correctly inserted into pPROTet the ORF2E 6xHN tagged protein can then be extracted, purified and be used to confirm the aa sequence of the protein product of ORF2E.

One of the key regulatory elements in the proposed model is the binding of the product of ORF3 (CbrR) to the 'marbox' to turn on transcription of ORF2E. To further examine this component of the model a set of primers should be designed that removes the 'marbox' from the ORF2D sequence but includes the promoter region for ORF2E. Once this new insert carrying, ORF2E and its own promoter region, was ligated into plasmid pDrive there should be constitutive expression of cationic biocide resistance in the *E. coli* host DH5α with and without the presence of CbrR.

Preliminary attempts to locate and purify ORF3 have already begun with the ORF3 sequence placed into the protein expression vector pPROTet. The next step would be to induce CbrR expression with anhTc, isolate and purify the protein using the incorporated 6xHN tag, and use the extracted protein to locate the DNA binding site for ORF3. Protein-nucleic acid interactions are commonly studied by several techniques including filter binding, DNA footprinting, methylation interference, and electrophoretic mobility shift assay (EMSA) (Jing *et al.* 2003). Among these methods EMSA is considered the optimum method as it is suitable for detecting small amounts of sequence-specific interaction. EMSA is based upon differences in mobility between the stable protein-nucleic acid complex and free nucleic acids (Jing

et al. 2003; Kass *et al.* 2000; Ruscher *et al.* 2000). This technique would hopefully verify the model and identify the 'marbox' as the binding site of CbrR.

To analyse the 'marbox' and promoter region of ORF2E the sequences upstream of ORF2E that appeared essential for cationic biocide resistance expression (nt positions 1941-2019) should be cloned upstream of a promoterless gene, such as the genes for Cm acetyltransferase (*cat*) or β-lactamase (*bla*). Once in a *S. aureus* or *E. coli* host CAT (Shaw 1975) or BLA (Kuriki 1987) assays can be performed on total soluble protein extracts. Changes in CAT/BLA activity can be detected and directly related to the activity of the promoter. To investigate if particular substrates of plasmid pWBG1773 potentiate the expression of ORF2E, CAT assays could also be performed. Strains of *E. coli* DH5α harboring a plasmid, which contained the ORF3 sequence and the ORF2E 'marbox'/promoter region upstream of the promoterless *cat* gene, could be incubated overnight in sub-MICs of potential inducing compounds and their CAT activity determined (Grkovic *et al.* 1998; Kwong *et al.* 2004). A significant increase in CAT activity would indicate the substrates act as inducers of ORF2E. ORF2E promoter alone would also be tested to see if induction could occur independent of *cbrR*.

Screening for ORF2 and ORF3 sequences should be extended to other strains of staphylococci, both resistant and sensitive to cationic biocides. Locating and analysing these sequences would provide further information on the distribution and maintenance of the sequences of ORF2 and ORF3. More information regarding the presence ORF2 and ORF3 sequences found on cationic biocide sensitive strains would also help to establish the exact sequence of ORF2 and ORF3 essential for cationic biocide resistance.

The strong homology between plasmid pWBG1773 and plasmid pPI-2, particularly with respect to similar the ORF2 sequence, should be further explored. An analysis of the expression of ORF-Z of plasmid pPI-2 would indicate if the potential ORF had any bearing on the cationic biocide resistance conferred by plasmid pPI-2.

These proposed studies should clarify the role of ORF2 and ORF3 sequences in the

expression of resistance to cationic biocides. They may also identify compounds or substrates of the resistance mechanism that can act as inducers and this, in turn, may lead to identifying the targets for this unique resistance mechanism.

The widespread incidence and distribution of resistance to cationic biocides highlights their importance to staphylococci, particularly MRSAs. An understanding of the unique resistance mechanism of plasmid pWBG1773 could solve the enigma that is resistance to cationic biocides.

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7 APPENDICES

Appendix 7.1 – Reagents and their suppliers

Reagent	Supplier
Acridine yellow	ICN/Sigma-Aldrich
Acriflavine	Sigma-Aldrich
40% acrylamide solution	Bio-Rad
Agarose (molecular biology grade)	Promega
Ammonium acetate	Sigma-Aldrich
Ampicillin	Sigma-Aldrich
Ammonium persulfate	Bio-Rad
Antibiotic discs	Oxoid
ASB-14	Sigma-Aldrich
anhydrotetracycline	BD Biosciences
Bacteriological agar	Oxoid
*Bam*HI	Promega
Barium chloride	Sigma-Aldrich
Benzalkonium chloride	Ajax Chemicals
Bradford assay bovine gamma globulin standard set	BIORAD
Brain heart infusion powder	Oxoid
BRESAclean™ DNA Purification kit	Bresatec
Bromophenol blue	TGG
Cadmium acetate	Chem-supply
Calcium chloride	Sigma-Aldrich
Carrier ampholytes	Bio-Rad
Casein hydrolysate	Sigma-Aldrich
CHAPS	Sigma-Aldrich
Chlorhexidine	Fluka
Chloroform	BDH
Calf intestinal alkaline phosphatase	Promega
Calf intestinal alkaline phosphatase 10X reaction buffer	Promega
Cetyl trimethyl ammonium bromide	Ajax Chemicals

Reagent	Supplier
Chloramphenicol	Sigma-Aldrich
*Cla*I	Promega
Coomassie brilliant blue G-250	Bio-Rad
Crystal violet	Sigma-Aldrich
DAPI (4,6-diamidino-2-phenylindole)	Sigma-Aldrich
Disodium phosphate	BDH
Dithiothreitol	Bio-Rad
DNase (RQ1 Rnase free)	Promega
dNTPs, 2mM	Fisher Biotech
dNTP mix (with dTTP), 20 mM	Roche
*Eco*RI	Promega
Ethanol	Merck
Ethidium bromide	Sigma-Aldrich
Ethylenediamine tetra acetic acid	Sigma-Aldrich
Ficol	Sigma-Aldrich
Glacial acetic acid	Sigma-Aldrich
Glucose	Ajax Chemicals
Glycerol	Merck
Glycine	Bio-Rad
Hydrochloric acid	BDH
*Hind*III	Promega
IPG strip, Ready Strip™	Bio-Rad
IPTG	Sigma-Aldrich
Isopropanol	BDH
LiCl	Sigma-Aldrich
Ligase	Invitrogen
Ligase Buffer	Invitrogen
Long Ranger™	Bio-Rad
Lysostaphin	Sigma-Aldrich
Lysozyme	Sigma-Aldrich
Mercuric chloride	Ajax Chemicals
Methanol	Merck

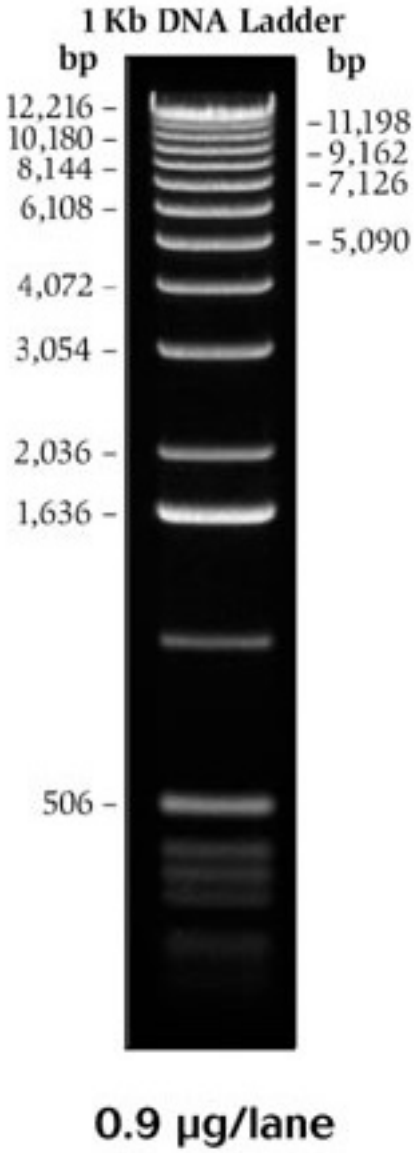
Reagent	Supplier
Magnesium chloride	BDH
Magnesium chloride, 25mM	Promega
Mueller-Hinton agar powder	Gibco Diagnostics
NaCl	Sigma-Aldrich
NaOH	Sigma-Aldrich
PCR Molecular Weight Markers	Invitrogen
Phenol	BDH
Phenyl mercuric acid	Ajax Chemicals
Piperazine diacrylamide	Bio-Rad
Polyethylene glycol (MW 8,000)	Sigma-Aldrich
Potassium acetate	Merck
Potassium chloride	ICN
Potassium dihydrogen phosphate	BDH
Potassium phosphate	BDH
Propamidine isethionate	Sigma-Aldrich
Protein Inhibitor Cocktail	Sigma-Aldrich
Quinaldine Red	Sigma-Aldrich
Rhodamine 6G	Fluka
RNase	Sigma-Aldrich
RNase inhibitor	Promega
Safranin O	Sigma-Aldrich
Sarkosyl	BDH
SequiTherm Excel™ II DNA Sequencing Kit-LC	Epicentre
Sodium acetate	Merck
Sodium dodecyl sulfate	ICN
Spectinomycin	Sigma-Aldrich
Sodium thiosulphate	Bio-Rad
Sucrose	Ajax chemicals
Sulphuric acid	BDH
Taq DNA Polymerase	Promega
TEMED	Bio-Rad

Reagent	Supplier
Tetracycline	Sigma-Aldrich
Thiourea	Merck
Tributyl phosphine reducing agent	Bio-Rad
Triton-X-100	Sigma-Aldrich
Trizma®	Sigma-Aldrich
Trypticase soy broth powder	BBL
Urea	Merck
X-gal	Promega
Xylene cyanol	Sigma-Aldrich
Yeast Extract	Sigma-Aldrich

Appendix 7.2 – Address of suppliers

Company	Address
Ajax Chemicals	Sydney, Australia
BBL	Kansas City, MO64141-2762, USA
BD Biosciences	Mountain View, CA 94043, USA
BDH	Poole, BH15 1TD, England
Bio-Rad	Hercules, CA 94547, USA
Bresatec	Adelaide, Australia
Chem-supply	Gillman, South Australia
Fisher Biotech	Bentley, WA 6102, Australia
Fluka	Riedstr. Steinheim
Gibco Diagnostics	Gaithersburg, MD 20884-9980, USA
ICN	Costa Mesa, CA, USA
Invitrogen	Mulgrave, VIC 4296, Australia
Li-cor	Lincoln, NE 68504, USA
Merck Pty. Ltd.	Kilsyth, VIC 2137, Australia
Novagen	Madison, WI 53719, USA
Oxoid	Basingstoke, England, UK
Promega Corporation	Madison, WI 53711-5399, USA
QIAGEN	Doncaster, VIC 3108, Australia
Roche Diagnostics Australia Ltd.	Castle Hill, NSW 2154, Australia
RPH	Perth, WA 6000, Australia
Sigma-Aldrich	Castle Hill, NSW 2154, Australia
Sigma-Proligo	Lismore, NSW 2480, Australia
Stratagene	Melbourne, VIC 3102, Australia
TGG	London, England

Appendix 7.3 – 1-kb DNA Ladder (Invitrogen)



Appendix 7.4 – Sequence files of plasmid pWBG1773. Each sequence is labelled according to the primer used. Highlighted in blue are the forward primers and in pink the reverse primers, designed from the end of the sequence.

M13F

GGATCCGACTGAATATATTTATCAAATTTTTTATATTTGGAATCAAAGAGAATACTTAGCGAGTGAAA
TTTATGATTTAACTGAACAAGAAAAACGAGAAATTAATCATCAAATGATTGATGAAATTGAAGAAGGA
AATTGAAAATAAAATTTGATACTTTTTCTTAAACGTTAGCAAAAAAGCTAGCGTTTTTTTGTTGAAAA
TACGTTAAAAATGGCTGGGTACAAACTGGGTACAGACACAATATATAGGGGTACAACCTGGGTACAAT
CGCGATTTGTAGGGGTACAACCTGGGTACAAAATGCAAAAAATGCATACCTTTTTAAAATCGCTGTAT
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CAAAGAATTTAGAGCCAATTTAAAATTTGGTGCTTTTTTGAAAGAAATTGTCCTTGCAGGAACTAAAA
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SeqF1

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TGCTTTGGATAGGAATTATTGTTACTCCTTTTTTACCTGTATCAACGTTTGTAAAAGCAACTGTTATA
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AAGTAAGTATAAAGATCG

SeqF2

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AAACAAAGAGGATATACACATTATGAATTGAATAATCAGCAATGTGTGTTGTTATCTTTGCTGATTAT
AAATAATTCTTTATCTCCGATTGAGTTAGCTGATGAAATGAAGATTTCTAAGAGTGCTGTTAGTCAGT
TATTATTAAAATTAGAAAATGAAGGTTTTGTGATTCGAAAAAAATATAAGAATGATAAGCGTTCTGTA
ACTGTTGAATTAGATGAAAAAGGTAAAATTTATAAAGAAGAAATGAAAGAGTTTGAAAAAAGAAATAT
TAAAAGAATATTATGATGGATTTACTGTTACCAGAAATTAGA

SeqF3

GAATAATCAGCAATGTGTGTTGTTATCTTTGCTGATTATAAATAATTCTTTATCTCCGATTGAGTTAG
CTGATGAAATGAAGATTTCTAAGAGTGCTGTTAGTCAGTCTATTATTAAAATTAGAAAATGAAGGTTT
TGTGATTCGAAAAAAATATAAGAATGATAAGCGTTCTGTAACTGTTGAATTAGATGAAAAAGGTAAAA
TTTATAAAGAAGAAATGAAAGAGTTTGAAAAAGAAATATTAAAGAAATATTATGATGGATTTACTGTA
ACAGAATTAGATAATATGCTTGAATTAATAAAGAAAAGTAGAGATATTTTATTGTAAAAAGTAGTTTT
ATTTTAGGTTTTAAAAACGAGTTGAAAACGAGTCTCTTCTTGTCTTGATACTATAGGTAACTATTACA
AGCTACGATAGTTACCTTTAATCTGTTGGTATATCTGGTTTCGAGCACAAAAAAAGTTGCTTTTTCGT
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TATGGGATTATTTTGTATAAAACTAAGTTGTTTTGTTGTGTTATAACATGGATAAGTATACTGAGAAG
AAACAAAGAATCAAGTATTTCAAAAATTTATTAAAAAGACATGTAAAAGAAGGGCCAAATGGATTTGA
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SeqF4

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CATTAGAGAAACAGAAATTGCATAAATCTAATTTATGTAAAAATCGATTTTGTCCTATATGTGCATGG
CGAAAAGCGAGAAAAGATGCGTTAGGTTTATCATTGATGATGCAATATATTAAGCAAAAAGAAGATAA
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ATTATAATGATTCGTTTAGACGATTAAGTAATCGTAAACACTTTAAATCTATAGCTAAAGGTTACGTA
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GAGATGTAACGGGGATTTCTGAAATCACACAAGTTCATGTTCAAAAAATTAAACAAAATAGCAATAAA
GATTATACGAAATGGCTAAATATTCTGGTAAGGATAGTGACTATTTAATTAATCAAAAAGTGGTTTGA
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M13R

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CGATTTTTACATAAATTAGATTTATGCAATTTCTGTTTCTCTAATGTTTTATCTGCCACAAAACTCAA
GAATGTATTGCACTCCCTTATCAAATCCATTTGCCCTTCTTTTACATGTCTTTTAATAAATTTTTGAA
ATACTTGATTTCTTTGTTTCTTCTCAGTATACTTATCCATGTTATAACACAACAAACCAACTTAGTTT
TATACAAAATAATCCCATAAAAAAGTTGCTTTTTCTCCTTTCA

SeqR1

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TCATTTCTTCTTTATAAATTTTACCTTTTTCATCTAATTCAACAGTTACAGAACGCTTATCATTCTTA
TATTTTTTTCGAATCACAAAACCTTCATTTTCTAATTTTAATAATAACTGACTAACAGCACTCTTAGA
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SeqR2

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TCCA

SeqR3

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CATGAAAATTTGATAAATAGCTAGTACCAGCAACACAACATATACATACTTATTCGTTAAAAAATTCT
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TTCTTTTAACAAACTTTATCATAACCCTTACAAAAATTTTGAGTGCCTTTATTTTTAATTTTAAGGGG
TATTTNGAAGAACACTAAATATTTACATAGTTTTTATATCCCAAAATTCTATATAAGCGCTTAAAACG
CAA

SeqR4

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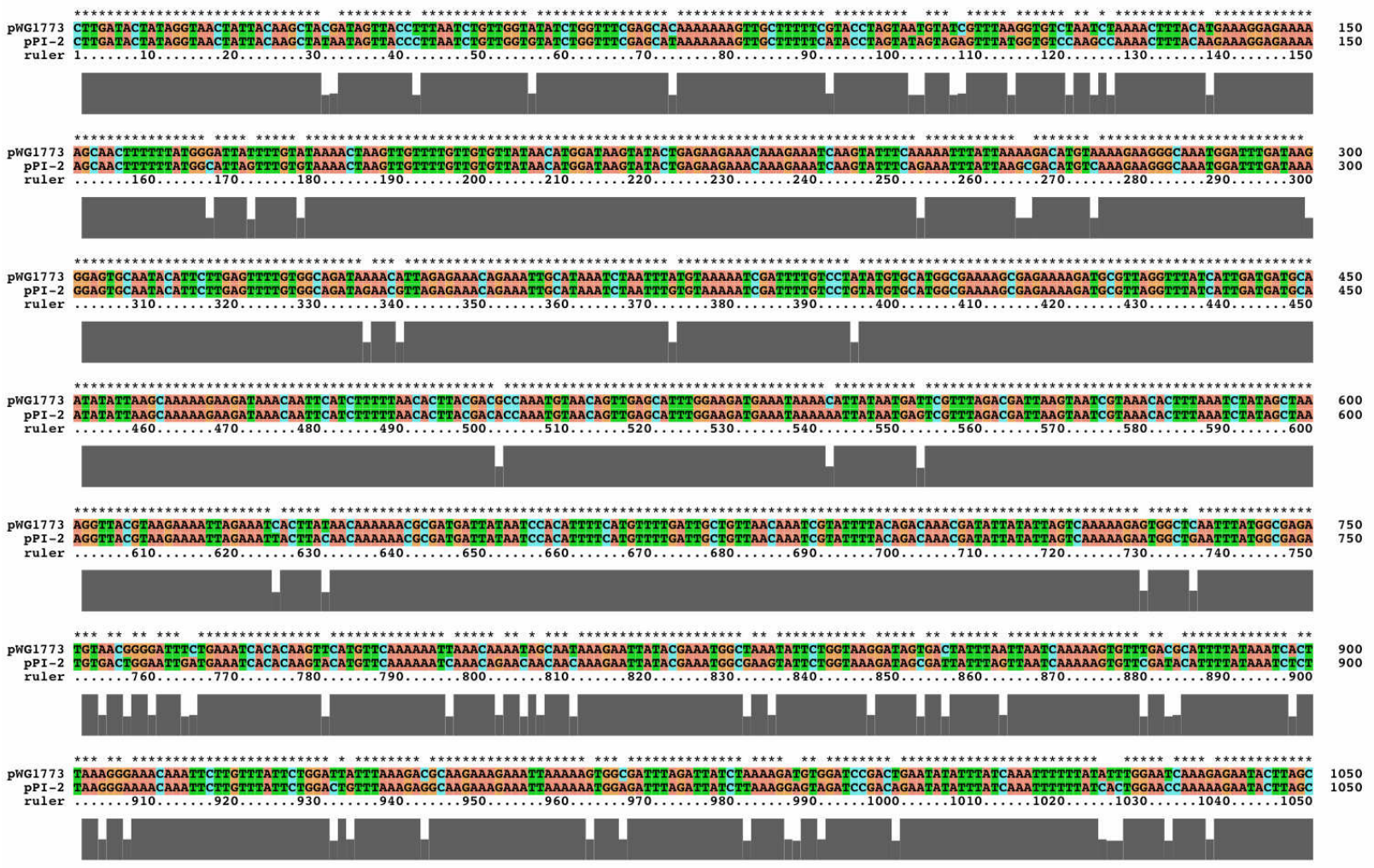
CNCCCCTAAGAAATTCCCCTTGGAATTCCCAAAATNNNAAAAAANTTTGGAAAAA

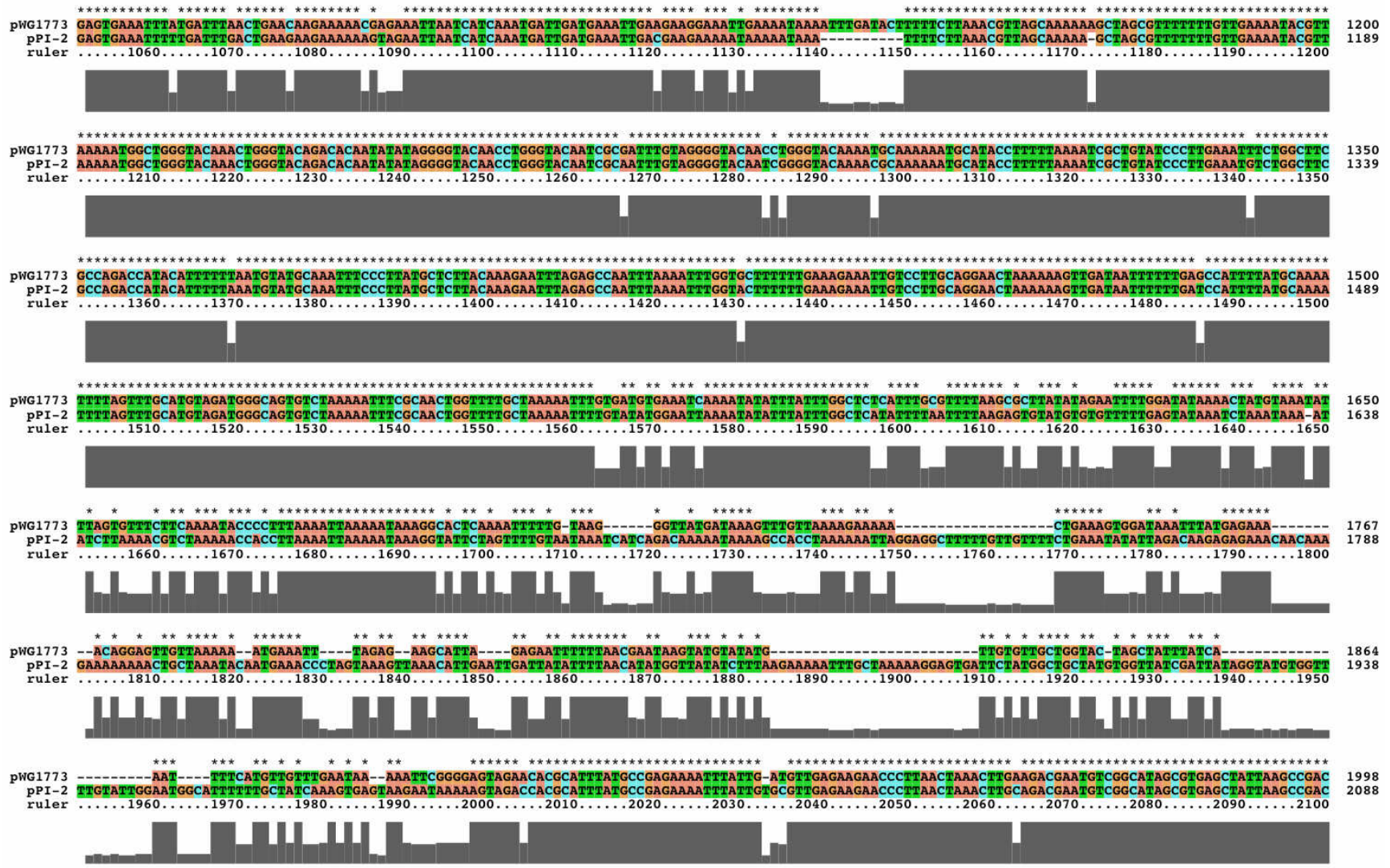
Appendix 7.5 – RE sites and locations for plasmid pWBG1773

Sequence Range: 1 to 2917

Enzyme	#Cuts	Positions
AccI	1	745
AluI	7	213, 554, 1123, 1701, 2382, 2513, 2897
Alw26I	1	521
BamHI	1	1519
Bsp1286I	1	599
BsrBRI	1	1178
BsrSI	2	1752, 2076
Bst71I	1	2861
BstOI	3	1780, 1812, 2836
CfoI	1	2141
ClaI	1	909
Csp45I	1	287
DdeI	4	232, 711, 749, 1573
DpnI	2	1521, 2917
DraI	7	496, 1113, 1464, 1845, 1947, 2204, 2627
Eco47III	1	2140
HaeII	1	2142
HhaI	1	2141
HincII	1	1209
HinfI	5	284, 514, 1078, 1558, 2605
HpaI	1	1209
Hsp92I	1	1027
Hsp92II	9	666, 738, 799, 932, 1195, 1312, 2042, 2402, 2682
MboI	2	1519, 2915
MboII	12	34, 102, 236, 369, 511, 765, 1006, 1066, 1658, 2179, 2479, 2500
NdeII	2	1519, 2915
NheI	1	1701
NruI	1	1792
NsiI	1	1836
RsaI	9	620, 1742, 1753, 1774, 1785, 1806, 1817, 2377, 2746
Sau3AI	2	1519, 2915
ScaI	1	2746
SnaBI	1	1134
SspI	5	385, 396, 1363, 2176, 2593
TaqI	4	287, 593, 909, 2530
VspI	3	441, 1391, 1620
XhoII	1	1519
XmnI	1	106

Appendix 7.6 – Clustal alignment of plasmid pWG1773 vs plasmid pPI-2. A ruler is displayed below the sequences, starting at 1 for the first residue position. A bar graph at the bottom of the figure represents the extent of homology between all sequences.

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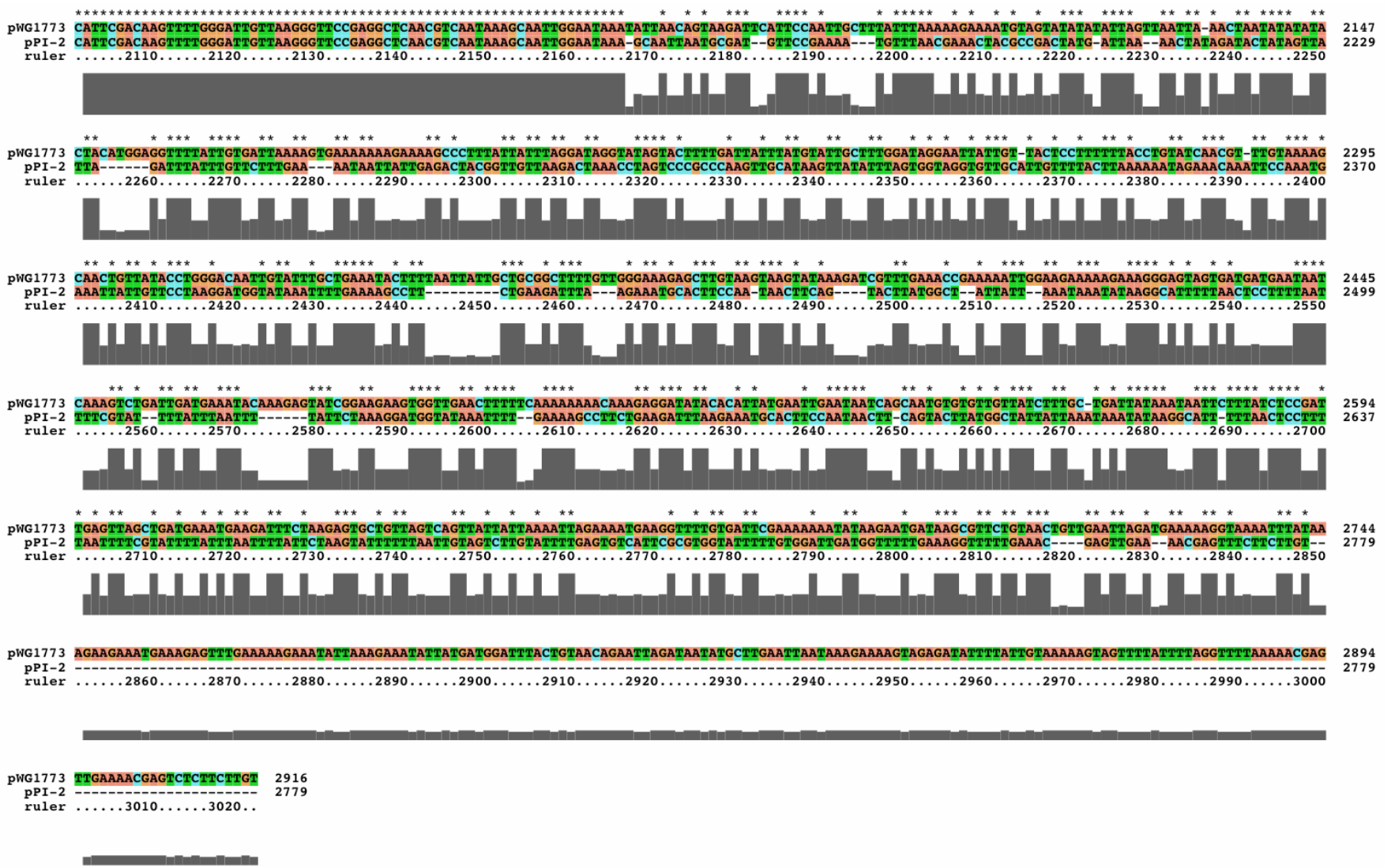
pPI-2 GAGTGAAATTTTTGATTTGACTGAAGAAGAAAAAAGTAGAATTAATCATCAAATGATTGATGAAATTGACGAAGAAAATAAAAATAAA---------TTTTCTTAAACGTTAGCAAAAA-GCTAGCGTTTTTTTGTTGAAAATACGTT 1189
ruler ......1060......1070......1080......1090......1100......1110......1120......1130......1140......1150......1160......1170......1180......1190......1200
pWG1773 AAAAATGGCTGGGTACAAACTGGGTACAGACACAATATATAGGGGTACAACCTGGGTACAATCGCGATTTGTAGGGGTACAACCTGGGTACAAAATGCAAAAAATGCATACCTTTTTAAAATCGCTGTATCCCTTGAAATTTCTGGCTTC 1350
pPI-2 AAAAATGGCTGGGTACAAACTGGGTACAGACACAATATATAGGGGTACAACCTGGGTACAATCGCAATTTGTAGGGGTACAATCGGGGTACAAAACGCAAAAAATGCATACCTTTTTAAAATCGCTGTATCCCTTGAAATGTCTGGCTTC 1339
ruler ......1210......1220......1230......1240......1250......1260......1270......1280......1290......1300......1310......1320......1330......1340......1350
pWG1773 GCCAGACCATACATTTTTTAATGTATGCAAATTTCCCTTATGCTCTTACAAAGAATTTAGAGCCAATTTAAAATTTGGTGCTTTTTTGAAAGAAATTGTCCTTGCAGGAACTAAAAAAGTTGATAATTTTTTGAGCCATTTTATGCAAAA 1500
pPI-2 GCCAGACCATACATTTTTAAATGTATGCAAATTTCCCTTATGCTCTTACAAAGAATTTAGAGCCAATTTAAAATTTGGTACTTTTTTGAAAGAAATTGTCCTTGCAGGAACTAAAAAAGTTGATAATTTTTTGATCCATTTTATGCAAAA 1489
ruler ......1360......1370......1380......1390......1400......1410......1420......1430......1440......1450......1460......1470......1480......1490......1500
pWG1773 TTTTAGTTTGCATGTAGATGGGCAGTGTCTAAAAATTTCGCAACTGGTTTTGCTAAAAATTTGTGATGTGAAATCAAAATATATTTATTTGGCTCTCATTTGCGTTTTAAGCGCTTATATAGAATTTTGGATATAAAACTATGTAAATAT 1650
pPI-2 TTTTAGTTTGCATGTAGATGGGCAGTGTCTAAAAATTTCGCAACTGGTTTTGCTAAAAATTTTGTATATGGAATTAAAATATATTTATTTGGCTCATATTTTAATTTTAAGAGTGTATGTGTGTTTTTGAGTATAAATCTAAATAAA-AT 1638
ruler ......1510......1520......1530......1540......1550......1560......1570......1580......1590......1600......1610......1620......1630......1640......1650
pWG1773 TTAGTGTTTCTTCAAAATACCCCTTTAAAATTAAAAATAAAGGCACTCAAAATTTTTG-TAAG------GGTTATGATAAAGTTTGTTAAAAGAAAAA----------CTGAAAGTGGATAAATTTATGAGAAA------- 1767
pPI-2 ATCTTAAAACGTCTAAAAACCACCTTAAAATTAAAAATAAAGGTATTCTAGTTTTGTAATAAATCATCAGACAAAAATAAAAGCCACCTAAAAAATTAGGAGGCTTTTTGTTGTTTTCTGAAATATATTAGACAAGAGAGAAACAACAAA 1788
ruler ......1660......1670......1680......1690......1700......1710......1720......1730......1740......1750......1760......1770......1780......1790......1800
pWG1773 --ACAGGAGTTGTTAAAAA--ATGAAATT----TAGAG--AAGCATTA----GAGAATTTTTTAACGAATAAGTATGTATATG----------TTGTGTTGCTGGTAC-TAGCTATTTATCA---------- 1864
pPI-2 GAAAAAAAACTGCTAAATACAATGAAACCCTAGTAAAGTTAAACATTGAATTGATTATATTTTAACATATGGTTATATCTTTAAGAAAAATTTGCTAAAAAGGAGTGATTCTATGGCTGCTATGTGGTTATCGATTATAGGTATGTGGTT 1938
ruler ......1810......1820......1830......1840......1850......1860......1870......1880......1890......1900......1910......1920......1930......1940......1950
pWG1773 ---------AAT----TTTCATGTTGTTTGAATAA--AAATTCGGGGAGTAGAACACGCATTTATGCCGAGAAAATTTATTG-ATGTTGAGAAGAACCCTTAACTAAACTTGAAGACGAATGTCGGCATAGCGTGAGCTATTAAGCCGAC 1998
pPI-2 TTGTATTGGAATGGCATTTTTTGCTATCAAAGTGAGTAAGAATAAAAAGTAGACCACGCATTTATGCCGAGAAAATTTATTGTGCGTTGAGAAGAACCCTTAACTAAACTTGCAGACGAATGTCGGCATAGCGTGAGCTATTAAGCCGAC 2088
ruler ......1960......1970......1980......1990......2000......2010......2020......2030......2040......2050......2060......2070......2080......2090......2100


pWG1773 CATTCGACAAGTTTTGGGATTGTTAAGGGTTCCGAGGCTCAACGTCAATAAAGCAATTGGAATAAATATTAACAGTAAGATTCATTCCAATTGCTTTATTTAAAAAGAAAATGTAGTATATATATTAGTTAATTA-AACTAATATATATA 2147
pPI-2 CATTCGACAAGTTTTGGGATTGTTAAGGGTTCCGAGGCTCAACGTCAATAAAGCAATTGGAATAAA-GCAATTAATGCGAT--GTTCCGAAAA---TGTTTAACGAAACTACGCCGACTATG-ATTAA--AACTATAGATACTATAGTTA 2229
ruler ......2110.....2120.....2130.....2140.....2150.....2160.....2170.....2180.....2190.....2200.....2210.....2220.....2230.....2240.....2250
pWG1773 CTACATGGAGGTTTTATTGTGATTAAAAGTGAAAAAAAGAAAAGCCCTTTATTATTTAGGATAGGTATAGTACTTTTGATTATTTATGTATTGCTTTGGATAGGAATTATTGT-TACTCCTTTTTTACCTGTATCAACGT-TTGTAAAAG 2295
pPI-2 TTA------GATTTATTTGTTCTTTGAA---AATAATTATTGAGACTACGGTTGTTAAGACTAAACCTAGTCCCGCCCAAGTTGCATAAGTTATATTTAGTGGTAGGTGTTGCATTGTTTTACTTAAAAAATAGAAACAAATTCCAAATG 2370
ruler ......2260.....2270.....2280.....2290.....2300.....2310.....2320.....2330.....2340.....2350.....2360.....2370.....2380.....2390.....2400
pWG1773 CAACTGTTATACCTGGGACAATTGTATTTGCTGAAATACTTTTAATTATTGCTGCGGCTTTTGTTGGGAAAGAGCTTGTAAGTAAGTATAAAGATCGTTTGAAACCGAAAAATTGGAAGAAAAAGAAAGGGAGTAGTGATGATGAATAAT 2445
pPI-2 AAATTATTGTTCCTAAGGATGGTATAAATTTTGAAAAGCCTT---------CTGAAGATTTA---AGAAATGCACTTCCAA-TAACTTCAG----TACTTATGGCT--ATTATT--AAATAAATATAAGGCATTTTTAACTCCTTTTAAT 2499
ruler ......2410.....2420.....2430.....2440.....2450.....2460.....2470.....2480.....2490.....2500.....2510.....2520.....2530.....2540.....2550
pWG1773 CAAAGTCTGATTGATGAAATACAAAGAGTATCGGAAGAAGTGGTTGAACTTTTTCAAAAAAAACAAAGAGGATATACACATTATGAATTGAATAATCAGCAATGTGTGTTGTTATCTTTGC-TGATTATAAATAATTCTTTATCTCCGAT 2594
pPI-2 TTTCGTAT--TTTATTTAATTT------TATTCTAAAGGATGGTATAAATTTT--GAAAAGCCTTCTGAAGATTTAAGAAATGCACTTCCAATAACTT-CAGTACTTATGGCTATTATTAAATAAATATAAGGCATT-TTTAACTCCTTT 2637
ruler ......2560.....2570.....2580.....2590.....2600.....2610.....2620.....2630.....2640.....2650.....2660.....2670.....2680.....2690.....2700
pWG1773 TGAGTTAGCTGATGAAATGAAGATTTCTAAGAGTGCTGTTAGTCAGTTATTATTAAAATTAGAAAATGAAGGTTTTGTGATTCGAAAAAAATATAAGAATGATAAGCGTTCTGTAACTGTTGAATTAGATGAAAAAGGTAAAATTTATAA 2744
pPI-2 TAATTTTCGTATTTTATTTAATTTTATTCTAAGTATTTTTTAATTGTAGTCTTGTATTTTGAGTGTCATTCGCGTGGTATTTTTGTGGATTGATGGTTTTTGAAAGGTTTTTGAAAC----GAGTTGAA--AACGAGTTTCTTCTTGT-- 2779
ruler ......2710.....2720.....2730.....2740.....2750.....2760.....2770.....2780.....2790.....2800.....2810.....2820.....2830.....2840.....2850
pWG1773 AGAAGAAATGAAAGAGTTTGAAAAAGAAATATTAAAGAAATATTATGATGGATTTACTGTAACAGAATTAGATAATATGCTTGAATTAATAAAGAAAAGTAGAGATATTTTATTGTAAAAAGTAGTTTTATTTTAGGTTTTAAAAACGAG 2894
pPI-2 ---------- 2779
ruler ......2860.....2870.....2880.....2890.....2900.....2910.....2920.....2930.....2940.....2950.....2960.....2970.....2980.....2990.....3000
pWG1773 TTGAAAACGAGTCTCTTCTTGT 2916
pPI-2 ---------- 2779
ruler ......3010.....3020..

Appendix 7.7 – Tandem Repeat Finder Results for plasmid pWBG1773

```
Tandem Repeats Finder Program written by:

Gary Benson
Department of Biomathematical Sciences
Mount Sinai School of Medicine
Version 4.00

Please cite:
G. Benson,
"Tandem repeats finder: a program to analyze DNA sequences"
Nucleic Acid Research(1999)
Vol. 27, No. 2, pp. 573-580.

Sequence: pWG1773
Parameters: 2 7 7 80 10 50 500
Length:  2916

Tables:   1

This is table  1  of  1  ( 1 repeats found )
```

Indices	Period Size	Copy Number	Consensus Size	% Matches	% Indels	Score	A	C	G	T	Entropy (0-2)
1738-1820	32	2.6	32	86	3	105	32	18	28	20	1.96

```
Tables:   1

Alignment explanation


    Indices: 1738--1820  Score: 105
    Period size: 32  Copynumber: 2.6  Consensus size: 32

       1728 AAAAATGGCT

                    *
       1738 GGGTACAAACTGGGTACAGA-CACAATATATAG
          1 GGGTACAACCTGGGTACA-ATCACAATATATAG

                                  * *  * *
       1770 GGGTACAACCTGGGTACAATCGCGATTTGTAG
          1 GGGTACAACCTGGGTACAATCACAATATATAG


       1802 GGGTACAACCTGGGTACAA
          1 GGGTACAACCTGGGTACAA

       1821 AATGCAAAAA

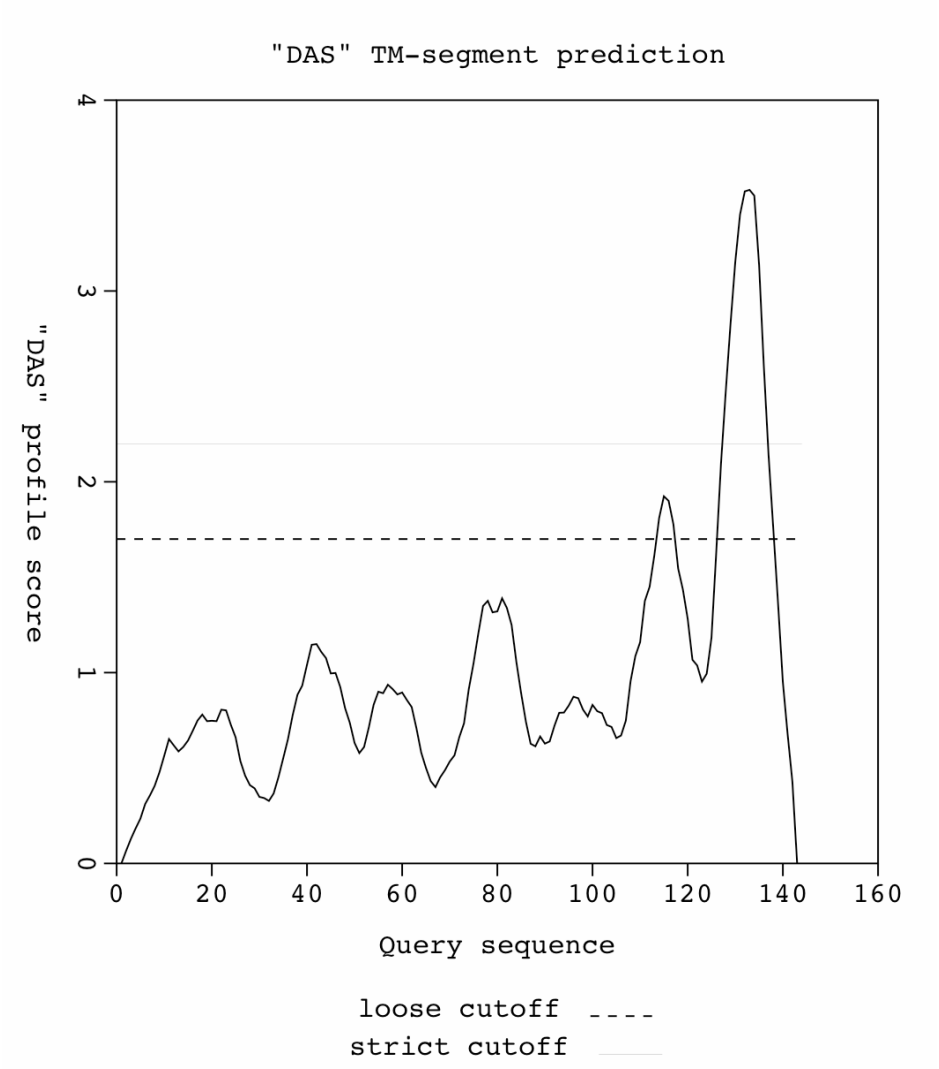

Statistics
Matches: 45,  Mismatches: 5, Indels: 2
        0.87            0.10        0.04

Matches are distributed among these distances:
  31    1  0.02
  32   44  0.98

ACGTcount: A:0.33, C:0.18, G:0.29, T:0.20


Consensus pattern (32 bp):
GGGTACAACCTGGGTACAATCACAATATATAG      Done.
```

Appendix 7.8 – Transmembrane Prediction Results for ORF2


"DAS" TM-segment prediction
"DAS" profile score
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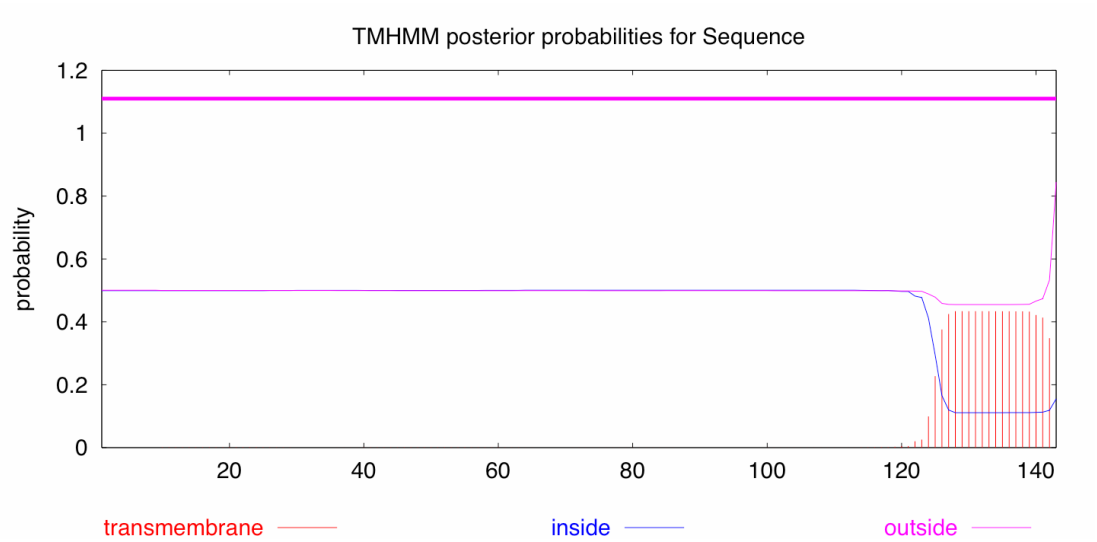
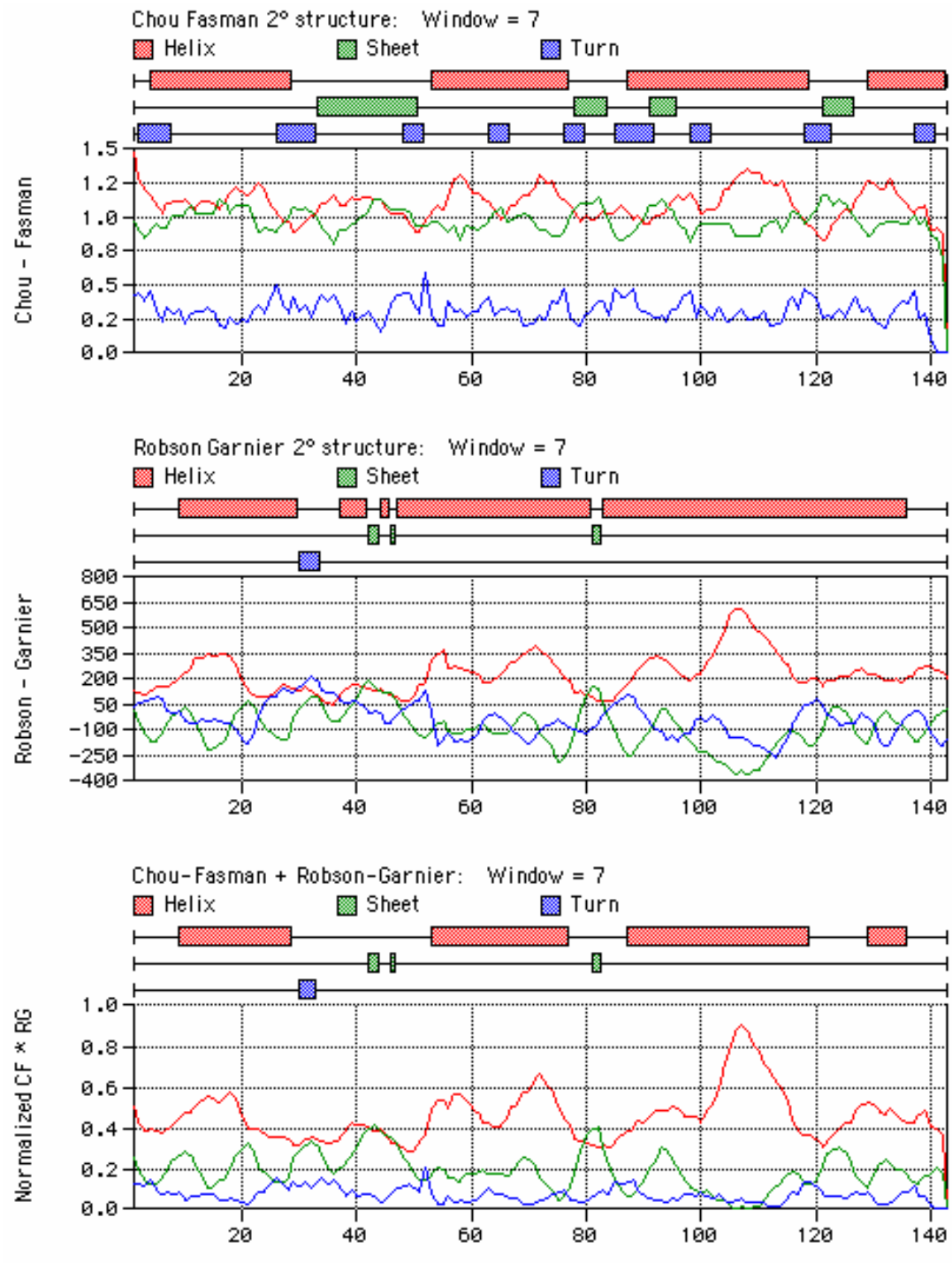
1
2
3
4
0
20
40
60
80
100
120
140
160
Query sequence
loose cutoff ----
strict cutoff


TMHMM posterior probabilities for Sequence
probability
1.2
1
0.8
0.6
0.4
0.2
0
20
40
60
80
100
120
140
transmembrane
inside
outside

Appendix 7.9 – Secondary structure predictions for ORF3 of plasmid pWBG1773 using the Chou-Fasman, Robson-Garnier and combined methods (CfRg) from the Protein Analysis Toolbox function in MacVector™7.2. The potential secondary structure of the protein is plotted using all three methods and the predicted conformation is indicated above the graph. The aa sequence of plasmid pWBG1773 is plotted along the x-axis. A window size of 7 is the number of residues that are grouped together for analysis, throughout the length of the sequence.



Appendix 7.10 - Sequence files of ORF2 and ORF3 inserts in plasmid pDrive. Highlighted in blue are the forward primers and in pink the reverse primers. The sequence highlighted in yellow indicates sequence of plasmid pDrive.

ORF2A

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ORF2B.1

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ORF2B.2

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GAGCTTGGCGTAAT

ORF2C

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CGTGG

ORF2D

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ORF2E

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ORF3

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ORF2A&3

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ORF2B.1&3

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ORF2C&3

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ORF2D&3

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ORF2E&3

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Appendix 7.11 - Sequence file of ORF2B.1 excised from plasmid pDrive by *Hind*III and *Bam*HI digestion and ligated into protein expression vector pPROTetE233 to form plasmid pBMS126. Highlighted in blue is the 5' PROTet Seq primer binding site, in pink the RE sites for *Hind*III and *Bam*HI, and in yellow the pDrive sequence.

```
  5'PROTet Seq Primer      EcoRI          RBS          KpnI
CATCAGCAGGACGCACTGACCGAATTCATTAAAGAGGAGAAAGGTACCCATG GGT

                   6xHN tag
CAT AAT CAT AAT CAT AAT CAT AAT CAT AAR CAC AAC GGT GGA

           EK site                          HindIII      pDrive
GAT GAC GAT GAC AAG GGT GGT CGA CAA GCT TGT CGA CGA ATT

               ORF2B.1
TCA GAT TCG TTA AAA ATGGCTGGGTACAAACTGGGTACAGACACAATATATA
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ATTTATTTGGCTCTCATTTGCGTTTTAAGCGCTTATATAGAATTTTGGATATAAAAC

                                                  pDrive     BamHI
TATGTAAATATTTAGTGTTTCTTCAAAATACCCCTTTATCACGAATTCTGGATCCCT

      pPROTet233
GCAGGCCTCAGGGCCCGATCGATGCGCCCGCTTAAT
```

ABSTRACT

Bacterial resistance to non-antibiotic agents is being increasingly studied. Plasmid-mediated resistance to cationic agents, which are important biocides, has been described in antibiotic-resistant *Staphylococcus aureus*. Multi-resistant *Staphylococcus aureus* (MRSA) are often found to express resistance to a range of cationic biocides including quaternary ammonium compounds (QACs), biguanides, diamidino compounds, cationic dyes and nuclear stains. Three resistance determinants, *qacA, qacB* and *smr* genes, have been identified that confer resistance to cationic biocides in staphylococci. These genes encode multi-drug efflux pumps that remove the cationic biocides from the cytoplasm using a membrane bound pumping mechanism dependent on the cell's proton-motive force (PMF). This prevents the build up of lethal concentrations of cationic compounds within the cytoplasm avoiding cell death.

This research project has focused on the *S. aureus* strain WBG4364, a transcipient strain carrying the cationic biocide resistant plasmid pWBG1773. The plasmid encodes resistance to several QACs, including benzalkonium chloride and CTAB, and cationic dyes rhodamine 6G, crystal violet and safranin O but not to the dye ethidium bromide and therefore differing from other cationic biocide resistant plasmids previously identified in staphylococci (Emslie *et al.* 1986). This unique phenotype was further classified in this study alongside those strains carrying the *qac* gene families, *qacA/B* and *smr*.

Plasmid pWBG1773 was cloned, sequenced and analysed to reveal a unique plasmid of 2,916 bp in length. Plasmid pWBG1773 was placed with the pC194 family of rolling-circle replicating plasmids. This family appear to be largely composed of interchangeable cassette structures.

The plasmid was found to carry three ORFs, designated ORF1, ORF2 and ORF3. ORF1 was homologous to *rep* genes of small staphylococcal plasmids belonging to the pC194 rolling-circle replication family and has been redefined as $rep_{WBG1773}$. ORF2 was found to have no similarity to any proteins of known function in the

GenBank database whereas ORF3 was found to have homology to the *marR* gene, a regulator of the multiple antibiotic resistance (*mar*) operon of Gram-negative organisms. MIC analysis of these ORFs found both ORF2 and ORF3 were essential for expression of resistance to cationic biocides. The exact ORF2 sequence required for resistance to be expressed was reduced to only 141 nt in size. This translated to a 47 aa sequence that contained a hydrophobic C-terminus indicating ORF2 to be a membrane-bound protein. The aa sequence of ORF3 contained a helix-turn-helix motif characteristic of the DNA binding domains of MarR-like proteins. Further analysis of pWBG1773 identified a putative 'marbox', a binding site for the homologous transcriptional activators of *mar*, within the ORF2 sequence. This indicated that ORF3 was binding to the 'marbox' sequence and activating transcription. Induction studies have not been able to ascertain any compounds capable of interacting with the ORF3 regulatory protein resulting in induction of cationic biocide resistance. Each ORF when analysed alone had no effect on the expression of cationic biocide resistance and it is thought that a efflux pump was not involved. This is further corroborated by the CCCP efflux experiments performed in an attempt to determine the mechanism of resistance. The unique ORFs of plasmid pWBG1773 appears to encode a novel cationic biocide resistance phenotype and mechanism.

MRSA strains from all around the world were analysed to determine if they possessed sequences homologous to ORF2 and ORF3. Sequences sharing a high degree of homology to ORF2 and/or ORF3 were detected in several MRSA strains including strains sensitive to all cationic biocides tested. These findings suggest that the appearance of ORF2 and ORF3 sequences in MRSAs was not an isolated event and the fact that some MRSAs do not carry both ORF2 and ORF3 sequences simultaneously indicates that these genes have another role that does not involve expression of resistance to cationic biocides.

Bacteria are noteworthy for their remarkable ability to adapt to changes in their environments and possess an impressive set of tools with which to adjust the blueprint of the cell to this change. The acquisition of a single system that may decrease a potential pathogenic organisms susceptibility to a wide range of cationic biocides,

such as seen in pWBG1773, poses a clinical threat, one that needs to be thoroughly investigated.

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ABBREVIATIONS

A	Adenine
aa	Amino acid
ABC	ATP-binding cassette
anhTc	Anhydrotetracycline
ALSI	Alkaline lysis solution I
ALSII	Alkaline lysis solution II
ALSIII	Alkaline lysis solution III
Amp	Ampicillin
ASB-14	3-(Decyldimethylammonio) propane sulfonate inner salt
ATPase	Adenine triphosphatase
Ay	Acridine yellow
B. cereus	*Bacillus cereus*
B. subtilus	*Bacillus subtilus*
BHIA	Brain heart infusion agar
BHIB	Brain heart infusion broth
BLA	β-lactamase
BLAST	Basic local alignment search tool
blastn	BLAST using nucleotide sequence
blastp	BLAST using protein sequence
blastx	BLAST using translated nucleotide sequence
bp	Base pair(s)
C	Cytosine
$CaCl_2$	Calcium chloride
CAT	chloramphenicol acetyltransferase
cc	Clonal complex
CF	Chou-Fasman
cfu	Colony forming unit
CfRg	The combined Chou-Fasman and Robson-Garnier model
CHAPS	3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate
CIAP	Calf intestinal alkaline phosphatase
CLSI	Clinical and Laboratory Standards Institute

cm	Centimetre
Cm	Chloramphenicol
CPC	cetylpyridinium chloride
CTAB	Cetyltrimethyl ammonium bromide
DAPI	4,6-diamidino-2-phenylindole
DAS	Dense alignment surface
dH_2O	Distilled/deionised water
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
dNTP(s)	Deoxynucleotide triphosphate(s)
DR	Direct repeat(s)
DTT	Dithiothreitol
Eb	Ethidium bromide
E. coli	*Escherichia coli*
EDTA	Ethylenediamine tetra acetic acid
Em	Erythromycin
EMRSA	Epidemic methicillin-resistant *Staphylococcus aureus*
et al.	et alii (and others)
Fa	Fusidic acid
g	Gram(s)
g	Gravity
G	Guanidine
Gm	Gentamicin
h	Hour(s)
HCl	Hydrochloric acid
HMM	Hidden Markov model
hpH_2O	High pure water
HTH	α-helix-turn-α-helix
Inc	Incompatibility
IPG	Immobilised pH gradient
IPTG	isopropylthio-β-d-galactoside
IRD	Infra red
IR	Inverted repeat(s)

K^+	Potassium ions
K_2PO_4	Potassium phosphate
kb	Kilobase pairs
KCl	Potassium chloride
KH_2PO_4	Potassium dihydrogen phosphate
Km	Kanamycin
kV	Kilo volt
L	Litre
m	Meter
M	Moles per litre (molar)
mar	Multiple antibiotic resistance
MATE	Multidrug and toxic compound extrusion
mF	Milli forad (capacitance)
MFP	Membrane fusion protein
MFS	Major facilitator superfamily
mg	Milligram
$MgCl_2$	Magnesium chloride
MHA	Mueller-Hinton agar
MIC	Minimum inhibitory concentration
min	Minute(s)
ml	Millilitre
MLST	Multi-locus sequence typing
mm	Millimeter
mM	Millimolar
MRSA	Methicillin/multiply resistant *Staphylococcus aureus*
MSS	Multiple surfactant solution
MSSA	Methicillin sensitive *Staphylococcus aureus*
MW	Molecular weight
MΩ-cm	ohms/cm (resistivity)
Na_2HPO_4	Disodium phosphate
NAB	Nucleic acid binding
NaCl	Sodium chloride (salt)
NaOH	Sodium hydroxide

NCBI	National Centre for Biotechnology Information
NCCLS	National Committee for Clinical Laboratory Standards
ng	Nanogram(s)
Nm	Neomycin
NN	Neural network
nt	Nucleotide(s)
Nv	Novobiocin
OD	Optical density
OMF	Outer membrane factor
ORF	Open reading frame
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PDA	Piperazine diacrylamide
PEG	Polyethylene glycol
PFGE	Pulsed field gel electrophoresis
pH	Negative logarithm of hydrogen ion activity
pI	Isoelectric point
Pi	Propamidine isethionate
PMF	Proton motive force
pmol	Picomoles
pSK+	pBluescript®SK+
QAC	Quaternary ammonium compound
qac	Quaternary ammonium compound
R	Resistant
®	Registered
RE	Restriction endonuclease
Rf	Rifampicin
RG	Robson-Garnier
RNA	Ribonucleic acid
RNase	Ribonuclease
RND	Resistance-Nodulation-Cell Division
RPH	Royal Perth Hospital
rpm	Revolutions per minute

S4	Standard sample solubilisation solution
s	Second(s)
S. aureus	*Staphylococcus aureus*
SCC*mec*	Staphylococcal cassette chromosome *mec*
SDS	Sodium dodecyl sulfate
Sm	Streptomycin
smr	Small multidrug resistance
SMR	Small multidrug resistance
SSO	Single strand origin
ST	Sequence type(s)
STS	Sodium thiosulphate
TBP	Tributyl phosphine
Tc	Tetracycline
TCA	Trichloroacetic acid
TEMED	N,N,N',N'-tetramethyl ethylenediamine
™	Trademark
T_m	Annealing temperature
TMS	Transmembrane segments
Tn	Transposon
Tp	Trimethoprim
tPMP-1	thrombin-induced platelet microbial protein 1
TPP	tetraphenylphosphonium
Trizma	Tris (hydroxymethyl amino methane)
TSA	Tryptic soy agar
TSB	Trypticase soy broth
U	Units
UK	United Kingdom
USA	United States of America
UV	Ultraviolet
V	Volts
WA	Western Australia
v/v	Volume by volume
X-gal	5-bromo-4-chloro-3-indolyl-β-d-galactopyranoside

/	Per
%	Per cent
μg	Microgram
μl	Microlitre
μM	Micromolar
°C	Degrees Celsius
Ω	Ohm(s)
∅	phage