CELL WALL-DEFICIENCY IN *STAPHYLOCOCCUS AUREUS* AND ITS ROLE IN ANTIBIOTIC RESISTANCE

SUBMITTED FOR THE DEGREE OF
DOCTOR OF MEDICINE
IN THE SCHOOL OF CLINICAL MEDICAL SCIENCES,
UNIVERSITY OF NEWCASTLE

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MARCH 2007

ABSTRACT.

Cell Wall-Deficiency in *Staphylococcus aureus* and its Role in Antibiotic Resistance.
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Cell wall-deficient bacteria (CWDB) induced from *Staphylococcus aureus* ATCC 9144 (Oxford strain) were generated on medium with elevated osmolality in the presence of sublethal levels of penicillin G. On removal of antibiotic pressure the cell wall-competent (CWC) revertants along with these CWDB exhibited high-level penicillin and methicillin resistance, which was stable in the revertants. The revertants looked visually different, had an altered Gram stain and growth rate. Their matrix-assisted laser desorption/ionisation time-of-flight (MALDI-TOF) 'fingerprint' was also altered and they were more resistant to lysis by lysostaphin in comparison to the wild-type. Reversed-phase high-performance liquid chromatography (RP-HPLC) showed that the revertants' cell walls had shorter glycan chains and more pentaglycine cross-bridges.

A rapid, reproducible method using liquid media was established using the same medium and sublethal levels of penicillin G. The revertants produced using this method had the same characteristics as those cells produced from the original method. The high-level resistance seen in the revertants was homogenous and confirmed to be due to the transient CWD state, along with not being strain-specific. Transmission electron microscopy showed that the CWD cells and the revertant cells, when grown in penicillin, had a very disordered cell wall with areas where the cell wall appeared absent and were indistinguishable.

The revertant cells were *mecA*-negative, β-lactamase-negative and did not contain any mutations in the coding regions of *pbp* genes. The CWD cells and revertant cells, when grown in penicillin, were resistant to lysis by lysostaphin but were very sensitive to lysis with Triton X-100. These data indicate that the resistant cells are not dependent upon an intact cell wall for osmotic stability and they are able to switch readily to this mode of growth in the presence of penicillin G.

ABBREVIATIONS.

ACN: acetonitrile

ATCC: American type culture collection

BDMA: N-benzyldimethylamine

BHI: brain heart infusion

BSAC: British Society for Antimicrobial Chemotherapy

$CaCl_2$: calcium chloride

cfu: colony forming units

CTAB: cetyl trimethyl ammonium bromide

CWC: cell wall-competent

CWD: cell wall-deficient

CWDB: cell wall-deficient bacteria

DDSA: dodecenyl succinic anhydride

DNA: deoxyribonucleic acid

DNase: deoxyribonuclease

EDTA: ethylene diamine tetra acetic acid

GNC: Gram-negative cocci

HPLC: high-performance liquid chromatography

IPTG: isopropyl-β-D-thiogalactopyranoside

kg: kilogram

KH_2PO_4: potassium dihydrogen phosphate

L: litre

LB: Luria-Bertani

LiCl: lithium chloride

mM: millimolar

M: molar

MALDI-TOF: matrix-assisted laser desorption/ionisation time-of-flight

mg: milligram

$MgSO_4$: magnesium sulphate

MIC: minimum inhibitory concentration

ml: millilitre

mOsm: milliosmole

MQ: milliQ

MRSA: methicillin resistant *Staphylococcus aureus*

MSSA: methicillin sensitive *S. aureus*

NaCl: sodium chloride

NAG: *N*-acetylglucosamine

NAM: *N*–acetylmuramic acid

NaOH: sodium hydroxide

ng: nanogram

nm: nanometre

NCTC: National collection of type cultures

PBP: penicillin-binding protein

PCR: polymerase chain reaction

RNase: ribonuclease

RP-HPLC: reversed-phase high-performance liquid chromatography

rpm: revolutions per minute

SDS: sodium dodecyl sulphate

SEM: standard error of the mean

TE: 10 mM Tris.Cl (pH 7.50; 1 mM EDTA)

TEM: transmission electron microscopy

TFA: trifluoroacetic acid

Tris-HCl: Tris (hydroxymethyl) aminomethane hydrochloride

v/v: volume/volume

w/v: weight/volume

XGAL: 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside

ACKNOWLEDGEMENTS.

I would like to thank Katie Elmer for establishing the cell wall-deficient line CS1/19 that my initial work was based on, along with establishing a CWD medium and designing PBP primers. I would also like to thank Fiona Nattress for her work on the antibiotic profiles and β-lactamase status, Bill Simon for his MALDI-TOF and HPLC expertise and Christine Richardson for her electron microscopy expertise. I am grateful to Peter Cook who set the whole project up originally and for his encouragement.

Especially however I would like to thank Tony Fawcett for his supervision and encouragement throughout the research work and writing up, without which I would never have completed this MD, along with allowing me laboratory space at the University of Durham in the first place. I would also like to thank all the other workers in labs 4 and 5 who constantly helped me with my queries, especially Paul, Adrian, Johan, John and Steve.

Finally I would like to thank Mark for his support over the last five years and understanding when I have chosen to spend time working on my MD rather than with him.

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STATEMENT.

This thesis submitted for the degree of Doctor of Medicine entitled: Cell wall-deficiency in *Staphylococcus aureus* and its role in antibiotic resistance is based upon work conducted by the author during the period between 2002 and 2007.

All work recorded in this thesis is original unless otherwise acknowledged in the text or by references.

None of the work has been submitted for another degree in this or any other University.

Signed _____ Date 30/3/07

1. INTRODUCTION.

1.1: CELL WALL-DEFICIENT BACTERIA (CWDB).

Cell wall-deficient bacteria (CWDB) are bacteria that have lost all or part of their cell wall and are able to multiply (Butler and Blakey 1975). Previously CWDB were termed L-forms or spheroplasts, these terms are felt to be synonymous with CWDB, a more inclusive term derived by Louis Dienes. CWDB are pleomorphic variants of bacteria that have atypical colonies, they may require hypertonicity and be highly fastidious in their growth requirements. They may or may not revert to the classical stage (Mattman 2000). Stable CWDB are those that do not revert to a cell wall-competent (CWC) form or do so only with great difficulty, whilst unstable CWDB are those that easily revert to a CWC form (Butler and Blakey 1975). CWDB can occur spontaneously or be induced in the laboratory by various methods. It is thought that probably all known bacterial species can be induced to be cell wall-deficient (CWD) (Domingue and Woody 1997).

Mycoplasmas, a group of organisms known to cause disease for example *M. pneumoniae* causes pneumonia in humans, show similarities with CWDB. They are pleomorphic, lack a cell wall and are therefore resistant to penicillins, cephalosporins and other antibiotics that act on the cell wall. Many of these bacteria are able to pass through 0.45 μm filters that are used to remove bacteria from solutions due to their small size, with an average diameter of 0.2 to 0.8 μm. They divide by binary fission, grow slowly with a generation time from 1 to 6 hours, form small colonies that have a 'fried-egg' appearance and require exogenous sterols from the addition of animal serum to the growth medium to grow (Murray, Drew et al. 1990).

This has lead to the suggestion that mycoplasmas are stable CWDB where the parent form has not been identified. This would mean there was a survival advantage of the CWD form over the parent form leading to a stable CWD form (Butler and Blakey 1975). In fact mycoplasmas are no longer able to synthesise a cell wall or its components, and this is one essential difference between mycoplasmas and CWDB (Mattman 2000).

There is the assumption that all CWDB originated at some point from a CWC bacteria, the so-called parent form. Although in most cases this is easily demonstrated if the CWDB are derived from CWC bacteria in the laboratory, or are unstable and therefore can be reverted back to a CWC form and then identified. However if the mycoplasmas are accepted as CWDB the question remains which CWC bacteria have

they originated from. The mycoplasmas include the *Acholeplasma* species which are sterol-nonrequiring mycoplasmas. In 1982 Neimark and London showed that the seven *Acholeplasma* species they studied were phylogenetically related to streptococci suggesting they evolved from streptococci but the six fermentative *Mycoplasmas* species studied were not related, suggesting that mycoplasmas are derived from various bacteria (Neimark and London 1982).

Historically the first description of CWDB probably was in 1895 by Pfeiffer when a non-rigid bacterial form of *Vibrio chlorae* was observed (Butler and Blakey 1975). In 1935 Klieneberger described non-rigid bacterial forms that she termed L-forms (named after the Lister Institute where Klieneberger worked) and described them as resembling pleuropneumonia-like organisms (PPLO) which were later known as mycoplasmas. Klieneberger suggested a symbiosis between these L-forms and *Streptobacillus moniliformis* which were observed growing together (Klieneberger 1935). Dienes soon after showed that the L-forms Klieneberger was describing originated from *S. moniliformis* by returning them to the parent form and therefore were not a new species (Dienes 1939). He also isolated L-forms from many different bacterial species and showed that these L-forms could multiply in the absence of a rigid cell wall (Dienes and Weinberger 1951).

These L-forms are typically detected by their colonial morphology on solid media with a 'fried-egg' appearance, being very different to the parent form, and reverting back to the parent form for identification. They always stain Gram-negative, regardless of the species and the colonies of CWDB tend to be indistinguishable from one another being small with a 'fried-egg' appearance regardless of the species whilst the colonies of the parent form are characteristic of the species (Hamburger 1968). They are often observed to be very pleomorphic and noted to be osmotically fragile due to the damage to the rigid outer wall. They are able to pass through bacterial filters that would retain the parent form not due to being small in size but due to their non-rigid form (Clasener 1972). It was recognized that the non-rigid form of these L-forms was due to a damaged cell wall with all or part of it absent. Other terms have also been used such as spheroplast and protoplast to describe the degree of damage to the cell wall with spheroplast retaining some cell wall and protoplasts retaining none. Recently these historical terms have been largely superseded with the term cell wall-deficient bacteria (Mattman 2000).

Initially CWDB were thought to occur due to adverse conditions in the environment, i.e. due to an environmental pressure, and that the change seen was due to damage of the cell wall because of the environment and was not a genetic change. Also it was thought that when the CWDB reverted back to the 'classical' or CWC form that it was the same as the parent, with a normal cell wall (Butler and Blakey 1975). Although this still seems largely to be true it is now thought that induction of CWDB probably also occurs spontaneously *in vivo* and antibiotics can cause selection of this sub-population (Domingue and Woody 1997). Certainly it is accepted that many bacteria, such as *Neisseria gonorrhoeae*, *Bacteroides fragilis*, *Haemophilus influenzae*, *Nocardia asteroides*, and various species of *Bacillus*, can spontaneously transform to CWDB (Madoff 1986). Beaman and Bourgeois in their work with *Nocardia* species showed stable changes in the revertant of *N. asteroides* following a transient CWD form in comparison to the parent form, noting that these changes were more likely to occur the longer the bacteria were CWD and suggested these changes probably represented mutational events (Bourgeois and Beaman 1974; Bourgeois and Beaman 1976).

The most common method used to induce cell wall-deficiency is to create an environmental pressure with a cell wall-active antibiotic such as penicillin, this method was used by Dienes to isolate CWDB from *H. influenzae* and *Bacteroides* in the 1940s (Dienes 1947; Dienes 1948). Other inducing agents have been used including amino acids such as glycine, peptidases and cell wall-active enzymes such as lysostaphin. In the right conditions the CWDB are then able to survive and proliferate, and it has been shown that they can proliferate indefinitely in the presence of the inducing agent in a suitable medium (Madoff, Burke et al. 1967). It has been shown that anaerobic conditions help with the induction of CWDB but also help in the reversion (Butler and Blakey 1975).

CWDB are often osmotically fragile and require osmotic protection as loss or damage to the cell wall usually results in lysis and death otherwise. Therefore many of the media used to induce CWDB have increased osmolarity in comparison to standard media, but this is not universal and some CWDB do not require this (Clasener 1972). The addition of salts, such as sodium chloride or potassium chloride, and carbohydrates, such as sucrose, are used to increase the osmolarity of media. Usually CWDB have similar nutritional requirements to their parent form (Madoff 1986) and enriched media allow successful growth, such as the addition of animal

sera or blood lysates and yeast extracts (Domingue and Woody 1997). The addition of magnesium to media also aids growth and increases the yield of CWDB (Brorson, Lundholm et al. 1973). Initially CWDB following induction grow poorly, giving low yields and requiring long incubation periods (Dienes 1967). However with time the CWDB are able to grow on media of normal osmolarity (McGee, Wittler et al. 1971) and many of the properties of older CWDB are different form those recently induced (Dienes 1967). However the growth requirements are different between families of bacteria and even between different strains of the same bacteria (Makino 1983).

Clinically specific growth requirements for CWDB that are not universal make the growth of CWDB from clinical samples difficult. Along with the long incubation periods often required due to the slow growth of CWDB and multiple transfers which make contamination an issue. The optimal conditions needed to grow CWDB means if there are present in clinical samples they may well be missed in the laboratory. However if they are isolated from clinical samples, their significance still needs to be established.

1.1.1: CWDB and disease:

To demonstrate CWDB *in vivo* is difficult due to some of the issues discussed above. There is also often the need of an inducing agent to be present in the media if the CWDB are unstable, but this means there is a possibility that any CWDB grown have actually been induced *in vitro*. However if CWDB are demonstrated *in vivo* the next question is whether they are clinically significant.

Three methods to demonstrate CWDB *in vivo* are suggested by Hamburger in his review in 1968 (Hamburger 1968) which still hold true. They are:

1. Direct demonstration by microscopic methods
2. Growth of CWDB on antibiotic-free hypertonic media
3. Indirect demonstration by making quantitative cultures simultaneously using ordinary isotonic and hypertonic media. If significantly larger numbers of colonies of the CWC form grow on the hypertonic media the assumption is that these bacteria were CWD *in vivo*.

Unfortunately a lot of the published work suggesting growth of CWDB from clinical samples have not followed the above and have used antibiotics or other inducing agents in the media and therefore have to be discounted. Most of the work

that show CWDB are pathogenic and cause disease comes from work with animals, and mainly in the laboratory (Higuchi 1969; Johnson, Wirostko et al. 1993). There are only a few studies or cases that suggest CWDB cause disease in humans, one example is where a clinical burn wound infection caused by a CWD *S. aureus* is discussed by Jiang et al. Cultures of excised material grew small granular colonies typical of CWDB on supplemented media but not on blood agar, with reversion to reveal *S. aureus* (Jiang, Chen et al. 1994). Though there is a lot of work that make strong arguments that CWDB probably do cause disease in humans (Brogan 1977; Goksu, Ataoglu et al. 1996) .

The other issue is whether it is the CWDB that are causing disease or their revertants in the CWC form. To a certain extent both of these possibilities mean that CWDB are involved in disease, as the latter depends on the presence of CWDB, often as a mechanism to allow bacterial persistence in the presence of antibiotics and then subsequent reoccurrence of the infection in the CWC form once the antibiotic pressure is over.

However it has been shown that CWDB are pathogenic, Scheibel and Assandri in 1959 demonstrated that CWD forms of bacteria that produce true exotoxins such as *Clostridium tetani* can still kill (Scheibel and Assandri 1959).

Alderman and Freedman in 1963 inoculated *E. coli* CWDB into the medulla of rabbits' kidneys and then examined the kidneys four or five days later. They recovered significant numbers of the parent form of *E. coli* with some of the kidneys displaying histological evidence of acute pyelonephritis (Alderman and Freedman 1963). Guze and Kalmanson inoculated enterococci intravenously into rats and then treated some of the rats with penicillin for three weeks. The kidneys were then ground in isotonic and hypertonic media and quantitative cultures made showing equal numbers in the untreated rats but growth only on hypertonic media in the rats treated with penicillin (Guze and Kalmanson 1963).

Some of the first work demonstrating CWDB from humans was by Charache. She demonstrated CWDB from blood cultures where the usual laboratory cultures had been negative in 21 patients with septicaemia (Charache 1970).

There has also been a lot of work studying the role of CWDB in urinary tract and renal disease, this is partly due to the recognition that the kidney is hyperosmolar therefore could potentially harbour CWDB. Brogan in 1976 showed that the osmolality required for the survival of coliform CWDB is around 1,100 mOsm/kg

(Brogan 1976) and this is the osmolality found in the renal medulla and in highly concentrated urine (Barker 1963). Brogan over an 18 month period examined all the blood cultures submitted for examination in the standard way but also examined them using Victoria Medium, her own formulated hypertonic medium for isolation of CWDB. Victoria medium consisted of brain heart infusion medium supplemented with sucrose, yeast, magnesium sulphate and heparin; and an osmolality of 1160 mOsm/kg. Cultures from the blood of four patients grew CWDB clinically diagnosed with septicaemia secondary to a urinary tract infection using hypertonic medium where the standard blood and urine cultures were negative. Cultures from the blood and urine of 12 patients were both positive where the cultures were negative for CWDB. These two groups of patients were compared and a history of relapsing urinary tract infection was only found in the first four patients (Brogan 1977).

Beaman and Beaman in their work with *Nocardia* clearly demonstrated that CWDB originating from *N. otitidiscaviarum* are pathogenic in mice with Koch's postulates fulfilled. They also demonstrated CWDB identified as *N. asteroides*, when no CWC forms were demonstrated, in a case of central nervous system nocardiosis (Beaman and Beaman 1994).

CWDB have also been associated with numerous disease processes that are not thought to be due to infective diseases including Crohn's disease, sarcoidosis and even cancer (Chiodini, Van Kruiningen et al. 1984; Macomber 1990; Almenoff, Johnson et al. 1996). However none of these studies are conclusive, being unable to fulfil Koch's postulates. Therefore the main evidence for CWDB causing disease is in known infective diseases as discussed above.

1.1.2: CWDB and antibiotic resistance:

CWDB are resistant to cell wall antibiotics as would be expected as the site of action is no longer present. Singh et al demonstrate that penicillin G, along with other cell wall-active antibiotics, is inactive against the CWD *Staphylococcus aureus* but antibiotics with other modes of action are active against the CWD *S. aureus* (Singh, Petersen et al. 2003). Huber showed that induction of CWD *Enterobacter cloacae* using a hypertonic medium supplemented with ticarcillin resulted in resistant mutants, in comparison to *E. cloacae* grown on standard media. These resistant mutants had high-levels of β-lactamase, persistent resistance and an antibiotic resistance pattern

suggesting *AmpC* derepressed *Enterobacter*. The *ampC* gene is regulated by an *ampR* and an *ampD* region with mutations in the *ampD* region resulting in 'derepressed' mutants that produce high-levels of β-lactamase with resistance to most β-lactam antibiotics (Huber 2002).

However there seems to be little in the literature about what happens if the CWDB return to a cell wall-competent (CWC) state and their antibiotic sensitivity profile then. Beaman and Bourgeois showed that the revertants of CWD *N. asteroides* were morphologically different with altered cell walls in comparison to the original parent form, however there were no alteration in the antibiotic sensitivity profile for cell wall-active enzymes such as penicillin (Beaman, Bourgeois et al. 1981). Similarly Landman showed that the revertants of CWD *Salmonella* species and *Escherichia coli* had the same antibiotic profile as their parent forms (Landman 1968).

1.2: *STAPHYLOCOCCUS AUREUS.*

Staphylococcus aureus is a member of the Micrococcaceae family and appears as clusters of Gram-positive cocci on microscopical examination. *S. aureus* is distinguished from other staphylococci species by the gold pigmentation of colonies, from which it derives its name, and positive results of coagulase, mannitol-fermentation and deoxyribonuclease tests (Lowy 1998).

S. aureus is an organism of great importance clinically and was first described by A. Ogston a surgeon who examined microscopically the pus from a man's leg and described round organisms (Ogston 1882). Since, it has been recognized as causing multiple serious infections other than soft tissue infections including bacteraemia, endocarditis, osteomyelitis and pneumonia. All these have significant morbidity and mortality rates and occur frequently with the mortality from bacteraemia ranging from 11 to 43%, and endocarditis due to *S. aureus* accounting for 25 to 35% of cases with a mortality rate of 20 to 44% (Lowy 1998). However *S. aureus* also colonises the nose and pharynx of around 15% of humans persistently without causing any disease (Murray, Drew et al. 1990).

1.2.1: Cell wall structure and cell wall-active enzymes of *S. aureus*:

The cell wall of *S. aureus* is typical for that of Gram-positive bacteria, being homogeneous and 20 – 40 nm thick (Giesbrecht, Kersten et al. 1998). The major component of the cell wall is peptidoglycan which consists of a disaccharide backbone formed by alternating β-1-4-*N*-acetylglucosamine and *N*-acetylmuramic acid (fig. 1). The average chain length is around 10 disaccharides (Sidow, Johannsen et al. 1990). Tetrapeptides consisting of L-alanine, D-glutamine, L-lysine and D-alanine are attached to the *N*-acetylmuramic acid forming stem peptides. These stem peptides are cross-linked to the stem peptides of another glycan chain by a pentaglycine group in about 90% of cases (Labischinski 1992). In staphylococcal peptidoglycan this pentaglycine is a characteristic feature and connects the ε-amino group of the L-lysine of one stem peptide to the D-alanine of the other one. The stem peptides that are not cross-linked have an extra D-alanine which is cleaved during cross-linking (Giesbrecht, Kersten et al. 1998).

The peptidoglycan complex can be disrupted by selective enzymes and harsh acids. Human polymorphonuclear leukocytes, monocytes and macrophages, tears and saliva contain an enzyme called lysozyme or muramidase, first described by Fleming (Fleming 1922), which can hydrolyse *N*-acetyl muramic 1-4 *N*-acetylglucosamine linkages thus lysing and killing organisms. Lysozyme is a specific enzyme encoded in our genes and in most vertebrates (Kaplan and Tenenbaum 1982).

Gram-positive bacteria produce a thick, multi-layered wall in which peptidoglycan is incorporated on the inner surface and then moves outwards through the wall allowing cell expansion and is shed in small fragments into the media (Popham and Young 2003).

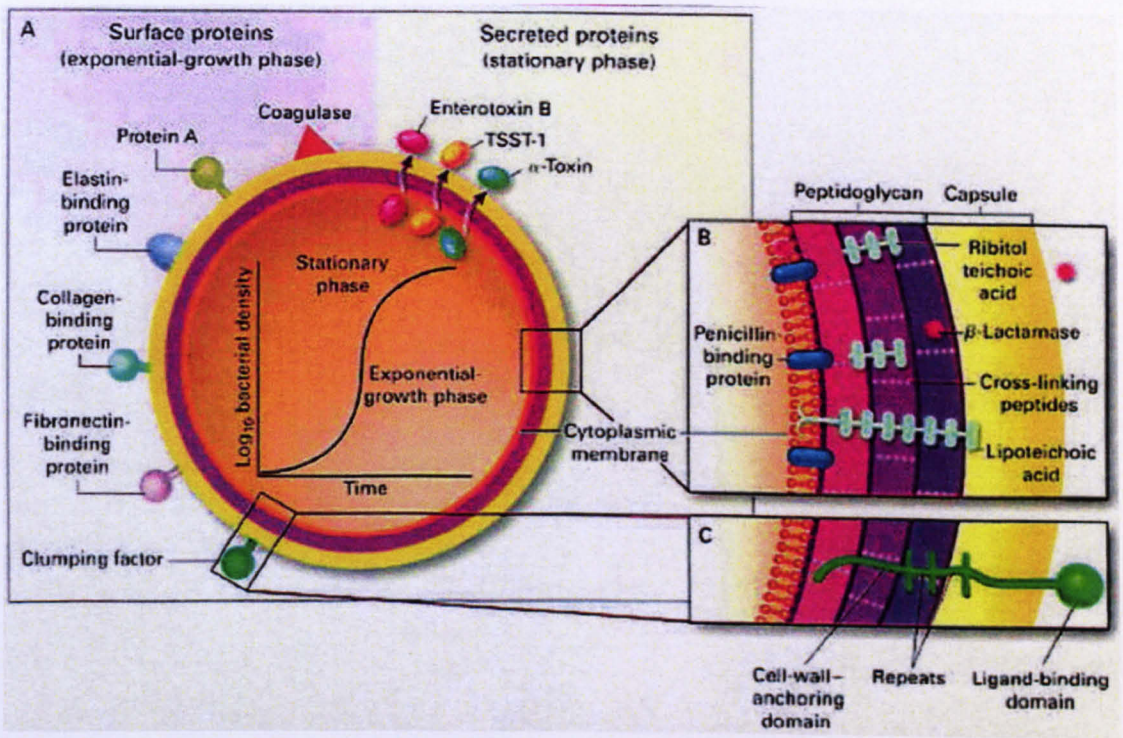
In the synthesis of the cell wall there are several stages, the last being the cross-linking of the polysaccharide strands making up the peptidoglycan structure. Specific enzymes (e.g. transpeptidases and carboxypeptidases) are required to catalyze this final step. These regulatory proteins are also called penicillin-binding proteins (PBPs) because they are bound by β-lactam antibiotics including penicillin, and are situated in the cell membrane. When bacteria are exposed to penicillin and the antibiotic binds to the PBPs in the cell membrane autolytic enzymes are released that degrade the preformed cell wall and cell wall synthesis is also inhibited resulting in bacterial cell death (Navarre and Schneewind 1999).

In *S. aureus* there are four PBPs, named simply PBP 1, 2, 3 and 4. PBP 4 is the only non-essential PBP with the rest being essential for the growth of *S. aureus*. A mutant lacking PBP 4 was found to be slightly penicillin susceptible but to grow normally in the absence of antibiotics, indicating that this PBP is dispensable (Curtis, Hayes et al. 1980). PBP 4 was initially the most studied with the conclusion that it is a transpeptidase involved in the secondary cross-linking of peptidoglycan (Wyke, Ward et al. 1981). PBP 1 is the most important PBP in terms of survival when exposed to β-lactams (Giesbrecht, Kersten et al. 1998). This is supported by the low cross-linking rate in PBP 4-defective *S. aureus* mutants (Henze, Sidow et al. 1993). PBP 2 is the major peptidoglycan transpeptidase and PBP 3 is a septation-associated transpeptidase (Georgopapadakou, Dix et al. 1986).

These roles for the four PBPs are confirmed by Okonogi et al who used cloxacillin, ceftizoxime, cephalexin and cefoxitin to selectively inhibit PBP 1, 2, 3 and 4 respectively. They showed that PBP 1 and 3 play regulatory roles in septum formation, while PBP 2 is involved in peptidoglycan elongation and PBP 4 is involved in additional cross-linking (Okonogi, Noji et al. 1995). This is consistent with *S. aureus* mutants where PBP 2 was inhibited leading to altered cell shape and lack of growth (Pinho, Filipe et al. 2001).

A further cell wall enzyme is autolysin (Navarre and Schneewind 1999). Autolysins are also important in the growth process being involved in enlargement of peptidoglycan and peptidoglycan turnover, along with being involved in remodelling functions, cell division and separation. Though, another presumed important role for autolysin is that of cell suicide, hence the name (Koch 2001).

Figure 1: Structure of *S. aureus* (from Lowy 1998).



1.2.2: *S. aureus* and cell wall-deficiency:

Cell wall-deficient (CWD) *S. aureus* have been induced and grown using various methods since the 1950s. Using a β-lactam antibiotic such as penicillin in sublethal doses to prevent cell wall synthesis has been the most common method of induction (Dienes and Sharp 1956). However other methods not using an antibiotic have been described such as using the cell wall enzyme lysostaphin (Watanakunakorn, Goldberg et al. 1969). Mattman et al showed that CWD *S. aureus* do not require hypertonic media for induction and suggested they probably occur spontaneously. The CWD form also retains the ability to produce coagulase and in the presence of blood about 2% of the CWD forms are found intracellularly (Mattman, Tunstall et al. 1961).

As far as clinical disease due to CWD *S. aureus* there is very little in the literature. One of the best reports is by Jiang et al where a clinical burn wound infection caused by a CWD *S. aureus* is discussed. Cultures of the exudates of the burn wound infection grew *S. aureus*, which was successfully treated leading to negative wound cultures. However the infection returned and again grew *S. aureus*, but a more resistant strain being only sensitive to phosphonomycin and cefmetazon, which spread rapidly leading to systemic symptoms but negative blood cultures. The infection was again successfully treated, with daily soakings with 1:5000 benzalkonium bromide solution and wet dressings with rifampicin solution, leading to negative wound cultures but due to the previous recurrence the residual wounds were excised. Cultures of the excised material were negative on blood agar medium plates but on medium supplemented with 40 g/L sodium chloride to allow the growth of cell wall-deficient bacteria (CWDB) small granular colonies were seen typical of CWDB. The Gram stain was mixed and after two transfers on blood agar medium these CWDB reverted to reveal the typical colonies of *S. aureus*, which were identified as *S. aureus* biochemically. Electron microscopy of the excised tissue also revealed small spherical bacteria with absent walls and loose nucleoids, which were described as CWDB by the authors (Jiang, Chen et al. 1994). This is an important case as it positively demonstrates that CWDB were present and not induced in the laboratory as negative cultures were demonstrated on normal laboratory media and growth only detected on the medium supporting CWDB. The CWDB were then reverted to a cell wall-competent (CWC) state and shown to be *S. aureus*.

Reports of CWD *S. aureus* causing clinical disease go back to the 60s but are infrequent. Conner et al in 1968 studied CWDB from urinary-tract infections and found one case due to *S. aureus*, however there was no control for the growth to exclude induction of the CWDB. Instead of plating the culture on the usual media alongside one that allows the growth of CWDB the authors only did the latter. However the media used for CWDB did not contain anything that would necessarily induce CWD state such as an antibiotic, so the probability that the growth of the CWDB reflected a clinical infection seems likely (Conner, Coleman et al. 1968). Newsom in 1970 reported an empyema due to a CWD *S. aureus* with no growth on normal bacteriological media (Newsom 1970).

Many of the studies can be criticized for inducing CWDB in the laboratories as opposed to showing them to be present in the culture and hence *in vivo*. Bruns and Brown, 1970 is an example of this where the sputum cultures of patients with cystic fibrosis were cultured routinely and in a serum-salt broth medium with methicillin for CWDB. With the addition of methicillin to the serum-salt broth medium the probability is that the *S. aureus* being grown on the routine plates is being induced to a CWD form in the broth. This is confirmed by the fact that in nearly all the cases where CWDB were grown a parent *S. aureus* was grown also. However there were eight cases where nothing was grown routinely but CWDB were seen on the serum-salt broth medium, this is much more convincing evidence of CWDB *in vivo*. The paper discusses five of these eight cases, all of which came from the most severe group of patients with cystic fibrosis, where CWDB were grown in the broth but the routine cultures were negative. In all five cases the patients were taking oxacillin at the time of the cultures, and following the course of oxacillin on reculturing both the parent and CWD forms were grown. This suggests the CWDB induction occurred *in vivo* during the antibiotic treatment, with the CWD form reverting to a CWC i.e. parent form on removal of the antibiotic pressure of oxacillin. Then on reculturing the parent form was grown with the CWD form, with the latter most likely again being induced from the parent form *in vitro* due to the antibiotic pressure of methicillin in the broth. However whether any CWDB were grown without a CWC form when the patient was not on antibiotics is not obvious from the paper (Bruns and Brown 1970).

Calderon et al in 1971 grew CWDB from patients with meningitis including *S. aureus* CWD forms. They took blood for culture routinely and for CWDB around 3 days into the disease, with the medium for CWDB containing penicillin. Again this

raises concerns about the possibility of CWD induction *in vitro*. However they grew CWD forms of *S. aureus* in 7 patients with no growth on the routine plates, this was out of 75 patients, with another 14 with CWDB growth of different organisms with no growth routinely. This again suggests the presence of CWDB *in vivo*, as for CWD induction to occur *in vitro* growth of the parent form would be expected on the routine culture. The possibility of contamination was considered by the authors but discarded as there were 75 patients in a control group where no growth routinely or on the CWD medium, also control plates alongside the CWD plates were used throughout. Most if not all patients were on antibiotics at the time of the cultures as the samples were taken around 3 days into the disease. So again it is likely that the authors were looking at CWD induction *in vivo* due to the antibiotic pressure of the patients' medication. Therefore how much the CWDB are involved in the pathogenesis of meningitis is debateable and the authors recognise this, discussing whether the CWDB could explain the continued activity of an infectious process in spite of antibiotic therapy and why occasionally a chronic state occurs (Calderon, Albuerne et al. 1971).

Although there is limited literature demonstrating clinical disease due to CWD *S. aureus*, recently Michailova et al have successfully infected rats with a stable CWD *S. aureus* and shown that the CWD cells replicate and persist in the lungs of the rats in comparison to the *S. aureus* parental form. This suggests that CWD *S. aureus* may be involved in the pathogenesis of chronic and latent lung infections (Michailova, Kussovsky et al. 2007).

1.2.3: *S. aureus* and antibiotic resistance:

Microbial resistance to antibiotics can be inherent (natural) or acquired. Inherent resistance is where the bacterium is inherently resistant to an antibiotic for example by lacking the target site for the antibiotic e.g. CWDB lack the target site for β-lactam antibiotics. Acquired resistance is due to changes in the bacterial genome either by vertical evolution where mutation and selection occurs or horizontal evolution where exchange of genes between strains and species occurs. Vertical evolution occurs when a spontaneous mutation in the bacterial chromosome leads to resistance and due to natural selection this resistant mutant grows in the presence of the antibiotic whilst the wild-type population is killed. The mutation rate for most bacterial genes is approximately 10^{-8}, therefore a doubling of 10^8 cells can lead to a

mutant being present. Horizontal evolution is the acquisition of genes for resistance from another organism via a genetic exchange, for example via plasmids.

Penicillin resistance in *S. aureus* is very common with the majority of *S. aureus* now resistant to penicillin with less than 5% of isolates remaining sensitive to penicillin (Lowy 1998). The most common mechanism for penicillin resistance in *S. aureus* is the acquisition of β-lactamase, an enzyme that cleaves the β-lactam ring of penicillin (McDougal and Thornsberry 1986). However resistance also occurs because of other mechanisms. Alteration of the target site on the enzyme to which penicillin binds, PBPs for example, due to a point mutation in the *pbp 2* gene (Hackbarth, Kocagoz et al. 1995) can occur. Overproduction of PBP 4 due to mutations in the noncoding *pbp 4* gene promoter region also leads to penicillin resistance (Henze and Berger-Bachi 1995). Finally the horizontal acquisition of a penicillin resistant penicillin-binding protein, PBP 2a leads to resistance, with this also conferring methicillin resistance (Archer, Niemeyer et al. 1994).

There are four PBPs present in *S. aureus* which are required in the assembly of cell wall peptidoglycan. The addition of PBP 2a, which has a low affinity for β-lactam antibiotics, leads to resistance to β-lactam antibiotics as PBP 2a takes over the functions of the other PBPs when they are inhibited by the antibiotics (Pinho, de Lencastre et al. 1998).

Methicillin resistance in *S. aureus* is due to *mecA* gene in nearly all cases which codes for PBP 2a. The *mecA* gene is located on the *Staphylococcus* casette chromosome *mec* (SCC*mec*) which is also seen in coagulase-negative staphylococci, suggesting the possible horizontal transfer at some point, possibly with the *mecA* gene originating from *S. sciuri* (Berger-Bachi and Rohrer 2002).

However methicillin resistant strains that do not produce PBP 2a have also been identified. Berger-Bachi et al selected out methicillin resistance in *S. aureus* by serial passage in increasing concentrations of methicillin which led to homogeneous resistance, a reduced growth rate and changes in the affinity for β-lactam antibiotics of PBPs 2 and 4. They noted there was no genetic relationship between the structural gene for PBP 2a and the PBPs from their mutant strains suggesting that PBP 2a probably did not evolve by a series of point mutations within *pbp 2* (Berger-Bachi, Strassle et al. 1989). In the same year Tomasz et al showed borderline level methicillin resistance in *S. aureus* from clinical isolates lacking the *pbp 2a* gene with homogeneous resistance with decreased activity of PBPs 1 and 2 but increased

activity of PBP 4 (Tomasz, Drugeon et al. 1989). Hackbarth et al in 1995 then took the mutants selected in the *in vitro* experiment by Berger-Bachi et al and the clinical isolate of Tomasz et al, and sequenced their *pbp* genes. This showed that point mutations present in *pbp 2*, leading to reduced binding of PBP 2 and more rapid release of the drug, was causing the low level resistance seen in these isolates (Hackbarth, Kocagoz et al. 1995).

1.3: PREVIOUS WORK.

In previous work carried out by Elmer it had been established that increasing sublethal doses of penicillin in supplemented media with a high osmotic potential could induce cell wall-deficiency in the wild-type *Staphylococcus aureus* Oxford strain ATCC 9144 (Fuller et al. 2005), which is penicillin sensitive (Heatley 1944). These cell wall-deficient (CWD) variants had small, pale colonies, having lost the golden-yellow pigmentation typical of *S. aureus*, and were umbonate in profile with a 'fried-egg' appearance. Gram staining showed that the cells had a diffuse edge, did not show a typical staphylococcal cell arrangement and were Gram-negative. These cells were unstable cell wall-deficient bacteria (CWDB) that reverted to a cell wall-competent (CWC) phenotype on removal of the penicillin, but could be maintained as CWD variants by continuing to grow them on the supplemented media in the presence of penicillin. These CWD cells were resistant to penicillin as would be predicted due to the removal of the site of action of penicillin i.e. the cell wall. However on reversion back to a CWC state, by growing the CWD cells in the absence of penicillin, this penicillin resistance was maintained at a high-level and some altered sensitivities to the non-cell wall-active antibiotics were also noted (Nattress, unpublished see Appendix 1).

This was a surprising result as although it would be expected that the CWDB would be resistant to penicillin, as the target site of penicillin i.e. the cell wall had been removed, on reversion to a CWC state it would be expected that the revertants would return to being penicillin sensitive. This is especially so as the literature suggests this is the case as discussed above (Landman 1968; Beaman, Bourgeois et al. 1981).

1.4: AIMS.

The aims of this work were as follows:

1. To establish whether the altered antibiotic profile seen in the revertants, in comparison to the wild-type, is stable.
2. To establish if there are any cell wall differences between the revertant and the wild-type, determining whether the revertant does have a 'normal' cell wall or not.
3. To establish what role the cell wall-deficient form of *Staphylococcus aureus* plays in acquisition of antibiotic resistance.
4. To study the mechanism of penicillin resistance seen in the revertants, by comparing the revertants to the wild-type, especially focusing on cell wall structure and sequencing the penicillin-binding proteins.
5. Eliminate the known mechanisms of penicillin resistance, such as presence of β-lactamase and PBP 2a.

2. MATERIALS AND METHODS.

2.1: BIOCHEMICAL METHODS.

2.1.1: Bacterial strains:

Wild-type: the Oxford strain of *Staphylococcus aureus* ATCC 9144 (NCTC 6571) was used throughout as the wild-type strain, as it is antibiotic naive (Heatley 1944) and hence is highly susceptible to penicillin. The only exception was the D line which was produced from *S. aureus* strain ATCC 25923 (NCTC 12981), which is again penicillin sensitive (Andrews 2005). Both strains are suggested by the British Society for Antimicrobial Chemotherapy (BSAC) as controls in susceptibility testing for *S. aureus* (Andrews 2005).

CS1/19: this cell wall-deficient (CWD) line was mutated from the wild-type Oxford strain, by transferring on CWD solid medium with increasing concentrations of sublethal penicillin in previous work (Fuller et al. 2005).

RS1/19: this revertant line was obtained from CS1/19 as described in Ch.3.

CL1/19 and CL3/19: these CWD lines were mutated from the wild-type Oxford strain, by transferring on CWD liquid medium with increasing concentrations of sublethal penicillin as described in Ch. 5.

RL1/19 and RL3/19: these revertant lines were obtained from CL1/19 and CL3/19 as described in Ch.5.

CD1/19: this CWD line was mutated from the wild-type ATCC 25923 strain, by transferring on CWD liquid medium with increasing concentrations of sublethal penicillin as described in Ch. 5.

RD1/19: this revertant line was obtained from CD1/19 as described in Ch.5.

2.1.2: Growth media and conditions: bacteria were grown using the following media depending on the strain and experiment. Liquid media were incubated at 37 °C on a

shaker at approximately 200 rpm. Solid media were as per the liquid media with the addition of 1% (w/v) agar and were also incubated at 37 °C.

LB: Luria-Bertani medium; 1% (w/v) bactotryptone, 1% (w/v) NaCl, 0.5% (w/v) bacto-yeast extract at pH 7.5.

BHI: brain heart infusion medium (Oxoid, Basingstoke, United Kingdom).

CWD: cell wall-deficient medium; BHI medium supplemented with 5% (w/v) sucrose, 0.5% (w/v) yeast and 0.2% (w/v) $MgSO_4$ as established in previous work (Fuller et al. 2005). After autoclaving, and once the mixture had cooled to around 50 °C, 10% (v/v) sterile horse serum was added aseptically.

2.1.3: Staphytect test: the latex slide agglutination test Staphytect Plus (Oxoid, Basingstoke, United Kingdom) was used to identify coagulase-positive *S. aureus*. The Staphytect test identifies whether an organism is *S. aureus* or not by testing for the presence of clumping factor, protein A or certain capsular polysaccharides, if one or more of these are present agglutination occurs and the test is positive. Staphytect Plus uses blue latex particles coated with porcine fibrinogen and rabbit immunoglobulin G including specific polyclonal antibodies raised against capsular polysaccharides of *S. aureus*. When the test reagent is mixed on a card at room temperature with colonies of *S. aureus*, rapid agglutination occurs through the reaction between fibrinogen and clumping factor, Fc portion of IgG and Protein A, or specific IgG and capsular polysaccharide. This is compared to control reagent and a positive result being the presence of agglutination within 20 seconds indicating *S. aureus*.

2.1.4: Gram staining: Gram staining uses dyes to stain a bacterial cell so it stands out from its background. It differentiates between Gram-positive and Gram-negative bacteria due to their differing cell wall structure. The specimen is mounted and fixed on a slide before it is stained. The reagents used are crystal violet, the primary stain; Lugol iodine, the mordant; ethanol, the decolouriser and safranin, the counter stain. Gram-positive bacteria will incorporate little or no counter stain and will remain blue-violent in colour from the crystal violet whilst Gram-negative bacteria take on the pink colour of the counter stain.

From solid media: a couple of colonies were mixed with a few drops of milliQ (MQ, de-ionised and distilled water) on a glass slide. The smear was allowed to air dry and then the organisms heat-fixed by passing it through a flame several times. Crystal violet was added to the dried smear for one minute, washed off with MQ and Lugol iodine added for one minute, which was blotted off. A solution of 95% ethanol was agitated over the stain for 30 seconds and washed off with MQ, and finally a counter-stain of safranin was added for 5 seconds and again washed off with MQ. A mixture of air-drying and blotting dried the Gram stain.

From liquid media: a 500 µl aliquot of the culture was centrifuged at 13,000 rpm for 5 min and washed in 500 µl MQ once, to remove the media allowing a clearer Gram stain. The pellet was re-suspended fully in 100 µl MQ, of this 10 µl was added to 10 µl MQ and mixed on a slide. The Gram stain then proceeded as above.

2.1.5: Antibiotic sensitivity testing:

2.1.5.1: E-tests: E-tests were performed by following the BSAC method (Andrews 2001) with modifications. Penicillin E-test strips (AB Biodisk, Sweden) were used with isosensitest (Oxoid, Basingstoke, United Kingdom) or CWD solid media as per experiment. Oxacillin E-test strips (AB Biodisk, Sweden) were used to test methicillin sensitivity using CWD solid medium. The overnight culture of the strain to be tested was diluted with sterile MQ to 0.5 McFarland standard (bioMérieux, Basingstoke, United Kingdom) and an aliquot of 100 µl spread three ways on the plate with a sterile cotton wool bud. A 1.0 McFarland standard was used for the CWD samples to achieve the lawn of growth, i.e. with the colonies merging. The plate was then air dried for 30 min, the strip added and the plate incubated at 37 °C with the results read at 48 h.

2.1.5.2: Penicillin MIC estimation in liquid media: the penicillin MIC estimation in liquid media followed the method by Holt and Brown (Hawkey 1989) with modifications. A 64 mg/L concentration of penicillin G in sterile MQ was made up and 5 ml added to 5 ml of liquid media, the medium as per individual experiment. The 10 ml solution was mixed and 5 ml transferred to a fresh 5 ml of this liquid medium, and this step was repeated until a doubling dilution range of penicillin concentrations

were obtained, from 32 mg/L to 0.03125 mg/L. Diluted aliquots of 75 µl x 10^5 cfu/ml of overnight culture of the strain being tested were added to each of the different penicillin concentrations, along with a 5 ml culture with no penicillin added as a control for growth. These were incubated at 37 °C on a shaker set at approximately 200 rpm. A control for sterility, i.e. 5 ml of liquid media with no inoculum was also incubated. The lowest penicillin concentration without visible growth was considered to be the MIC.

For strains with suspected higher MICs a concentration of 256 mg/L of Penicillin G was started with to obtain a range of 128 mg/L to 8 mg/L.

2.1.6: Growth curves: a single colony of the strain being tested was inoculated from a plate and incubated overnight in liquid media, as required in the individual experiment, at 37 °C on a shaker set at approximately 200 rpm. An aliquot of 500 µl was then transferred to a 50 ml liquid media, to give a 1 in 100 dilution, at time zero. At specific times one ml of culture was taken aseptically and the absorbance read using a disposable cuvette in a visible spectrophotometer, wavelength being set at 600 nm. The spectrophotometer was zeroed with the medium being used in the experiment prior to the reading. The final results then being plotted to obtain a growth curve.

2.1.7: Matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry: the preparation of samples for matrix-assisted laser desorption/ionisation time-of-flight (MALDI-TOF) analysis followed the method by Smole et al (Smole, King et al. 2002) with minor modifications. A single colony of the strain being tested was incubated overnight in liquid BHI medium. The culture was spun at 10,000 g for 5 min and washed three times in MQ. The pellet was re-suspended in 50% acetonitrile (ACN)/0.1% trifluoroacetic acid (TFA) solution and sonicated for a minute using a sonic water bath. An equal volume of 1 mg/ml lysozyme solution, in MQ, was added and this was sonicated for approximately 40 min. Sinapinic acid matrix was prepared as a 10 mg/ml solution in 50% ACN/0.1% TFA. The sample was then spotted onto the MALDI plate with an equal amount of sinnapic acid. The plate was air dried to allow co-crystalisation before being read by the mass spectrometry machine.

2.1.8: Lysostaphin susceptibility: a single colony of the strain being tested was incubated overnight in liquid media, the medium as per individual experiment. **Exponential phase cells:** the overnight culture was used to inoculate 50 ml of liquid media at a 1 in 100 dilution and further incubated until the exponential phase was reached, as judged by an absorbance reading and the previously established growth curves of the relevant strain. The culture was centrifuged at 13,000 rpm for 5 min and washed three times in 20 mM KH_2PO_4 buffer (pH 7.5), and after the final wash re-suspended in the same buffer. The absorbance reading at wavelength 620 nm, using disposable cuvettes and a visible spectrophotometer, was adjusted to approximately 0.25. The spectrophotometer was temperature controlled at 37 °C. One unit of lysostaphin was then added, the cuvette shaken and returned to the spectrophotometer for continuous reading. The data was plotted as a percentage of the initial $A_{620\,nm}$. **Stationary phase cells:** entirely the same process as described above was repeated, with the cells being taken from the initial overnight culture.

2.1.9: Autolysis with Triton X-100: autolysis with Triton X-100 followed the method by Fournier and Hooper (Fournier and Hooper 2000) with minor modifications. Cells were grown in CWD liquid media in triplicate to exponential phase, OD_{600} of 0.6 to 0.8, using the same overnight culture for innoculum. The cells were pelleted by centrifugation and re-suspended in the same volume containing 50 mM Tris (hydroxymethyl) aminomethane hydrochloride (Tris-HCl) (pH 7.5) and 0.1% Triton X-100. The cells were then incubated at 30 °C with shaking, and the changes in OD_{580} were measured. The results were normalized to OD_{580} at time zero (OD_0) i.e. percent lysis at time t = [(OD_0 –OD at time t)/OD_0] x 100 prior to being plotted. All the results shown are means of at least three independent determinations.

2.1.10: Preparation of peptidoglycan: the preparation of peptidoglycan followed the method by de Jonge et al (de Jonge, Chang et al. 1992) with some modifications. Briefly, a 900 ml culture of cells grown in BHI liquid media to an absorbance of approximately 1.0, at wavelength 600 nm, were then rapidly cooled in an ice/water bath and centrifuged at 10,000 g for 20 min at 4 °C. The pellet was washed once in a small volume of MQ and re-centrifuged at 5,000 g for 10 min. The pellets were re-suspended in a small amount of MQ and SDS added to a final concentration of 4%.

This suspension was then boiled for 30 min and centrifuged for 10 min at 30,000 g and 4 °C. The pellet was washed three times in MQ and following re-suspension in 100 mM Tris-HCl (pH 7.5) α-amylase (100 µl/ml) was added. Prior to the addition of α-amylase, small molecular weight protein inhibitors were removed by putting the α-amylase through a Micro Bio-Spin column (Bio-Rad), which uses size exclusion chromatography to separate proteins 6 kD from small sample volumes. The final suspension was incubated at 37 °C on a shaker set at approximately 165 rpm for two hours. Then 20 mM $MgSO_4$, RNase (50 µg/ml) and DNase (10 µg/ml) were added and the solution returned to 37 °C overnight.

The suspension was treated with trypsin (100 µg/ml) in the presences of 10 mM $CaCl_2$ at 37 °C for 4 hours. The enzymes were inactivated by boiling for 15 min in a final concentration of 1% SDS and the walls were collected by centrifugation at 30,000 g, 4 °C for 10 min and being washed in MQ twice. The walls were then washed sequentially in 8 M lithium chloride (LiCl), 100 mM ethylene diamine tetra acetic acid (EDTA) (pH 8.0), MQ twice and acetone. Each spin was 30,000 g, 4 °C and 10 min. The walls were re-suspended in 5 ml MQ and transferred to specimen tubes that were pre-weighed. They were flash frozen prior to lyophilisation and kept at – 20 °C.

2.1.11: Preparation of muropeptide: the preparation of muropeptide from peptidoglycan followed the methods by de Jonge et al (de Jonge, Chang et al. 1992) and Glauner (Glauner 1988). Peptidoglycan (1 mg/ml) was digested in 12.5 mM sodium phosphate buffer (pH 5.5) with 0.02% sodium azide and 5 µg/ml lysostaphin or mutanolysin (as per individual experiment) at 37 °C for 16 hours. This solution was boiled for 5 min and centrifuged for 5 min at 13,000 rpm. The supernatant was discarded and replaced with 0.5 M sodium borate (pH 9.0), and then reduced by the addition of 1-2 mg of sodium borohydride for 30 min. Orthophosphoric acid (85%) was added until the pH reduced to approximately 2.0. Preparations were stored at – 20 °C.

2.1.12: Reversed-phase high-performance liquid chromatography: the muropeptide preparations were fractionated by reversed-phase high-performance liquid chromatography (RP-HPLC). Buffers were degassed prior to use each day by passing through a filter. Various methods using different buffering systems were tried before a

protocol giving the best separation data was established as described in the results. A C18 column was used, aliquots of 100 µl were injected each time and the wavelength UV 206 nm was measured. After an initial wash through of 5 min a gradient from 0% to 50% was run over 20 min before a rapid increase to 100% over 2 min with 5 min at 100% before a reduction down to 0% again over 2 min. The column was washed between different preparations and between runs a blank run with MQ injected through the inject port was ran to ensure all the material had been removed from the column.

2.1.13: Population analysis: the distribution of penicillin resistance in cell populations was determined by spotting 10 µl aliquots of the culture being tested, in a range of dilutions, onto duplicate CWD plates containing a range of penicillin concentrations. The plates were incubated for 48 h before the colonies were enumerated, with an average between the duplicate plates being taken.

2.2: GENETIC PROCEDURES.

2.2.1: Preparation of chromosomal deoxyribonucleic acid: total chromosomal deoxyribonucleic acid (DNA) was prepared using the method by Palomares et al (Palomares, Torres et al. 2003) with modifications. Cells were grown in 5 ml of LB liquid media overnight and pelleted. The pellet was re-suspended in 1.8 ml of TE buffer (Tris 10 mM, EDTA 1 mM, pH 8.0) and 200 µl (1 µg/µl) of lysostaphin added along with 10 µl (50 mg/ml) of mutanolysin, and incubated at 37 °C for one hour.

Then 300 µl of 10% sodium dodecyl sulphate (SDS) and 50 µl of proteinase K (10 mg/ml) were added and the suspension incubated at 65 °C in a waterbath for one hour. Afterwards 500 µl of 5 M sodium chloride (NaCl) and 500 µl of 10% Cetyl Trimethyl Ammonium Bromide (CTAB)/0.7 M NaCl were added after being heated in a waterbath at 65 °C for 10 min to aid release of DNA due to increased solubility. A chloroform/phenol extraction was then performed by adding equal volumes of chloroform and phenol, shaking the mixture, spinning at 4000 rpm for 3 min and pipetting the upper aqueous phase, above the white protein layer, off with this process being repeated twice more. Chloroform was then added alone to remove the phenol,

the mixture shaken and spun with the upper aqueous layer removed. Then 500 µl isopropranol was added, DNA seen precipating out on tipping gently and this was spun at 13,000 rpm for 10 min. The pellet and 70% ethanol were mixed gently and spun 13,000 rpm for 10 min with the supernatant pipetted off carefully. The pellet was then air dried and 100 µl TE buffer added and placed at 4 °C overnight.

2.2.2: Polymerase chain reaction: polymerase chain reactions (PCR) of the penicillin-binding proteins (PBP) 1, 2, 3 and 4 were carried out using KOD polymerase (Novagen), a proof reading enzyme. Each reaction was set up with 5 µl of 10x KOD buffer, 5 µl 2 mM KOD dNTP, 4 µl 25 mM KOD $MgSO_4$, 1 µl KOD, 1 µl of 20 pmoles F and R primers along with approximately 100 ng of chromosomal deoxyribonucleic acid (DNA) made to 50 µl with treated water (to remove any RNases). Four reactions were with the wild-type Oxford strain DNA, and four with the revertant RS1/19 strain DNA, one of each being with the forward and reverse primers for each PBP as discussed in Ch. 4 along with a negative control where no DNA was added. The reactions were set up on ice with the KOD added last straight from the freezer and PCR tips used throughout, and a few drops of mineral oil added on top of the mixture before being moved to the preheated PCR machine. The PCR machine (Robocycler) was set for one cycle of 5 min at 95 °C, 30 cycles of 45 sec 95 °C, 45 sec 50 °C and 5 min 72 °C, finishing with one cycle of 10 min at 72 °C.

Following the PCR reaction an agarose minigel was run with Lambda DNA digested with *Hind*III as a marker to check the size of PCR products present.

Cleaning of PCR products: a QIA quick PCR purification kit (QIAGEN) was used to remove unincorporated nucleotides and PCR primers from the PCR products, and the cleaned PCR product was eluted in 50 µl of buffer EB. An agarose minigel was run to check these cleaned products prior to A-tailing.

A-tailing of PCR products: A-tails were added to purified PCR products using *Taq* polymerase (Bioline). An aliquot of 22.5 µl PCR product was mixed with 3 µl of 10x *Taq* buffer, 1 µl *Taq* 50 mM Mg SO_4, 1 µl 10 mM dATP, 1 µl *Taq* and made up to 30 µl with sterile MQ. The reactions were then transferred to the preheated PCR machine (Perkin Elmer) for 10 min at 72 °C. As previously the mixtures were made up on ice with *Taq* being added last straight from the freezer, PCR tips being used throughout

and the mixtures were mixed by a brief spin of 10 sec up to 6000 rpm and then pipetting up and down.

TOPO ligation: the PCR products were ligated into the vector pCR TOPO TA (Invitrogen) by adding 1 µl salt solution (1.2 M NaCl and 0.06 M $MgCl_2$) and 1 µl TOPO vector both provided by the kit to 4 µl PCR product. The mixture was spun for 30 sec to a maximum 5000 rpm and mixed by pipetting up and down and left at room temperature for 30 min.

Cloning of PCR products: the ligation mixtures were transformed into *Escherichia coli* XL1-Blue (Stratagene) by adding them to competent *E. coli*-XL1 cells. An aliquot of 5 µl of each ligation reaction was added to 40 µl *E. coli*-XL1 cells and mixed by flicking the bottom of tube. An aliquot of 1 µl of a positive control was added to 40 µl *E. coli*-XL1 cells and a 40 µl aliquot of *E. coli*-XL1 cells alone was used as a negative control. Tubes were placed on ice for 30 min prior to heat shock treatment for 30 sec at 42 °C and back to ice for two minutes. Following this, 900 µl of LB liquid medium then added to each tube and mixed by flicking the bottom of tube and the tubes were incubated at 37 °C for one hour. The tubes were spun for 30 sec to a maximum of 12,000 rpm and the supernatant poured out and discarded. Of the approximately 100 µl left this was re-suspended with a pipette and then spread with a glass slide, flamed with 70% ethanol between each use, on to LB plates supplemented with 0.01% (w/v) ampicillin, 0.01% (w/v) Isopropyl-β-D-thiogalactopyranoside (IPTG) and 0.01% (w/v) 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (XGAL). The plates were then incubated at 37 °C overnight. To estimate the rate of transformation, 10 µl of the positive control was spread on one plate and the rest, ie 90 µl spread on another. A white colony for each transformant was transferred to 5 ml LB liquid medium with 5 µl ampicillin (final concentration of 50 µg/ml) and incubated overnight in the usual way for analysis of the recombinant clones.

Minipreparation DNA purification: DNA was prepared for sequencing from the overnight liquid cultures using the Wizard Plus SV Miniprep DNA purification system (Promega). Following the PCR purification an agarose minigel was run, with Lambda DNA digested with *Hind*III as a marker, to quantify the amounts and size of plasmid/DNA (PCR products) present prior to the next step.

Restriction enzyme digest: 5 µl of recombinant plasmid (approximately 1 µg DNA) was mixed with 2 µl 10x buffer H (Roche), 1 µl restriction enzyme (approximately 1

Unit) and made up to 20 µl with MQ. Restriction enzyme used being *Eco*RI and buffer H used as works 100% with *Eco*RI GAATTC. *Eco*RI was added last straight from freezer to stop degradation that would occur if warmed before being added to mixture. Mixed with pipette and incubated at 37 °C for one hour. An agarose minigel was run again with Lambda DNA digested with *Hind*III as a marker to check the size of PCR products present, to see if PBPs present in plasmid before sequencing.

2.2.3: Agarose gels: 1 x TE buffer supplemented with 0.8% (w/v) agarose. Once the mixture had cooled to around 50 °C, 3.75 x 10^{-5}% (v/v) ethidium bromide was added.

2.2.4: Running gels: agarose minigels were used with 10 µl wells. An 1 µl aliquot of orange dye was added to each of the products being run on the gel so the products could be identified under UV light following the run. For each gel Lambda DNA digested with *Hind*III was added to one well as a marker to check the size of the PCR products present. A current of approximately 175 V was run through the gel until the dye was approximately three-quarters the way across the gel, usually 30 min into the run and then the gel was viewed under UV light with a photograph being taken.

2.3: ELECTRON MICROSCOPY.

To prepare samples for transmission electron microscopy the method by Yoshida et al (Yoshida, Kuwahara-Arai et al. 2003) was followed with modifications. Cultures were grown to exponential phase in cell wall-deficient (CWD) liquid medium supplemented with penicillin as required. Cells were collected by centrifugation at 5000 g for 10 min at 4 °C, the supernatant discarded and the cells fixed in 0.1 M sodium phosphate buffer pH 7.4 and 2% glutaraldehyde. After two hours on a revolving shaker, the fixative was removed and 0.1 M sodium phosphate pH 7.4 buffer added to the cells and left overnight at 4 °C. The buffer was then removed and the cells post-fixed in 1% osmium tetroxide and left at 4 °C for two hours.

The cells were then dehydrated by removing the osmium tetroxide, and being left in a graded series of ethanol and propylene oxide. Initially the cells were left in 70% ethanol for 5 min three times, 95% ethanol for 5 min three times and then 100%

ethanol for 10 min three times. The cells were transferred to 100% propylene oxide for 10 min twice and the bacterial cells were then embedded in Araldite CY212. An araldite mix of 2.5 ml araldite CY212, 2.5 ml of the hardener dodecenyl succinic anhydride (DDSA) and 100 µl of the accelerator N-Benzyldimethylamine (BDMA) was made up. The cells were infiltrated with resin at 45 °C with equal amounts of propylene oxide and the araldite mix for 30 min, then the araldite mix alone for 30 min. The cells were then embedded in a plastic mould, covered with fresh araldite left for 12 h at 45 °C and then 24 h at 60 °C. The samples were then sectioned and thin sections (70-90 nm) were stained with uranyl acetate and lead citrate before examination using a Philips 400T microscope operating at 100kV.

The samples from the 1st method on examination had holes in many of the cells. This was felt to be due to lack of penetration of the fixative so the experiment was repeated with minor adaptations. The culture was spun at 13,000 rpm for 1 min, 4% paraldehyde and 2.5% glutaraldehyde in sodium cucodylate buffer 0.1M added, and the mixture pipetted up and down to break the pellet up and allow fixature to all the cells. This was left for 30 min and then respun at 8000 rpm for 1 min and fixative readded. However the two CWD lines produced better micrographs with the 1st method so the samples from the 1st method were used for these two lines and the samples from the 2nd methods used for the wild-type and revertant lines.

3. CHARACTERISATION OF REVERTANTS OF CS1/19.

3.1: INTRODUCTION.

In previous work carried out by Elmer it had been established that increasing sublethal doses of penicillin in supplemented media with a high osmotic potential could induce cell wall-deficiency in the wild-type *Staphylococcus aureus* Oxford strain ATCC 9144 (Fuller et al. 2005). This strain is known to be penicillin sensitive (Heatley 1944). Cell wall-deficient (CWD) variants were generated by inoculating the Oxford strain onto CWD solid medium containing a range of concentrations of penicillin G, and incubating aerobically at 37 °C for up to 72 h. Colonies on the plate with the highest concentration of antibiotic that supported growth were small, had lost the golden-yellow pigmentation typical of *S. aureus* and were umbonate in profile. Gram staining showed that the cells had a diffuse edge, did not show a typical staphylococcal cell arrangement and were Gram-negative. Cells growing at this penicillin concentration were transferred to fresh CWD solid medium containing further incremental increases in the antibiotic concentration and hence CWD variants were maintained. A penicillin G concentration of 19.2 mg/L was reached eventually and this CWD line was named CS1/19. These CWD cells were resistant to penicillin as would be predicted due to the removal of the site of action of penicillin i.e. the cell wall. However on reversion back to a cell wall-competent (CWC) phenotype, by growing the CWD cells in the absence of penicillin, this penicillin resistance was maintained at a high-level. Altered sensitivities to some non-cell wall-active antibiotics were also noted in these revertants (Nattress, unpublished see Appendix 1).

Penicillin resistance in *S. aureus* can be due to an altered target site due to a point mutation in the *pbp 2* gene (Hackbarth, Kocagoz et al. 1995), the acquisition of β-lactamase, an enzyme that cleaves the β-lactam ring of penicillin (McDougal and Thornsberry 1986), overproduction of penicillin-binding protein (PBP) 4 due to mutations in the noncoding *pbp 4* gene promoter region (Henze and Berger-Bachi 1995), or horizontal acquisition of a penicillin resistant penicillin-binding protein, PBP 2a, (Archer, Niemeyer et al. 1994). There are four PBPs present in *S. aureus* which are required in the assembly of cell wall peptidoglycan. The addition of PBP 2a, which has a low affinity for β-lactam antibiotics, leads to resistance to β-lactam antibiotics as PBP 2a takes over the functions of the other PBPs when they are inhibited by the antibiotics (Pinho, de Lencastre et al. 1998).

One hypothesis for the cause of the stable high-level of penicillin resistance seen in the revertants, in comparison to the wild-type, is an altered cell wall structure. An altered cell wall structure could be due to a single mutation, due to an altered protein sequence being encoded that is involved in cell wall structure, leading to resistance to the cell wall-active antibiotics. Also by default some altered sensitivity to the non-cell wall-active antibiotics could occur due to the altered cell wall, for example because of altered cell wall permeability. Alternatively an altered cell wall structure could be due to altered PBPs rather than a single mutation or a combination of both. In continuation of the work of Elmer, my aim was to study these revertant cells in more detail, to understand a potential mechanism for the penicillin resistance.

The work presented in this chapter begins by repeating the reversion of CS1/19 to a CWC phenotype by growth on media without antibiotic selection prior to characterising these revertant cells. Hence all the work described in the following results chapters was performed by the author.

3.2: REVERSION OF CELL WALL-DEFICIENT (CWD) TO CELL WALL-COMPETENT (CWC) PHENOTYPE.

CS1/19 was inoculated onto cell wall-deficient (CWD) (minus penicillin) agar plates for single colonies. Overnight growth at 37 °C demonstrated pale yellow colonies of varying sizes. Three single colonies were sub-cultured for further single colonies on CWD agar and incubated overnight at 37 °C. This process was repeated a further eight times for a total of ten sub-cultures on CWD agar. This was to ensure complete reversion as the generation time for *Staphylococcus aureus* is around 30 min therefore over 24 h and 10 sub-cultures there are around 480 generations, hence the revertant cells will have plenty of opportunity to get back to their normal mode of growth after 10 sub-cultures. These three isogenic revertant lines were named RS1/19/10_1, RS1/19/10_2 and RS1/19/10_3; where the number ten represented the number of sub-cultures and hence changed accordingly on further sub-culturing. Antibiotic sensitivity testing was then carried out on these revertant lines (Nattress, unpublished see Appendix 2). These results confirmed that the revertant lines maintained the high-level resistance to penicillin seen in CS1/19, but also showed altered sensitivities to other antibiotics including non-cell wall-active antibiotics.

3.3: PHENOTYPIC CHARACTERISATION OF REVERTANTS.

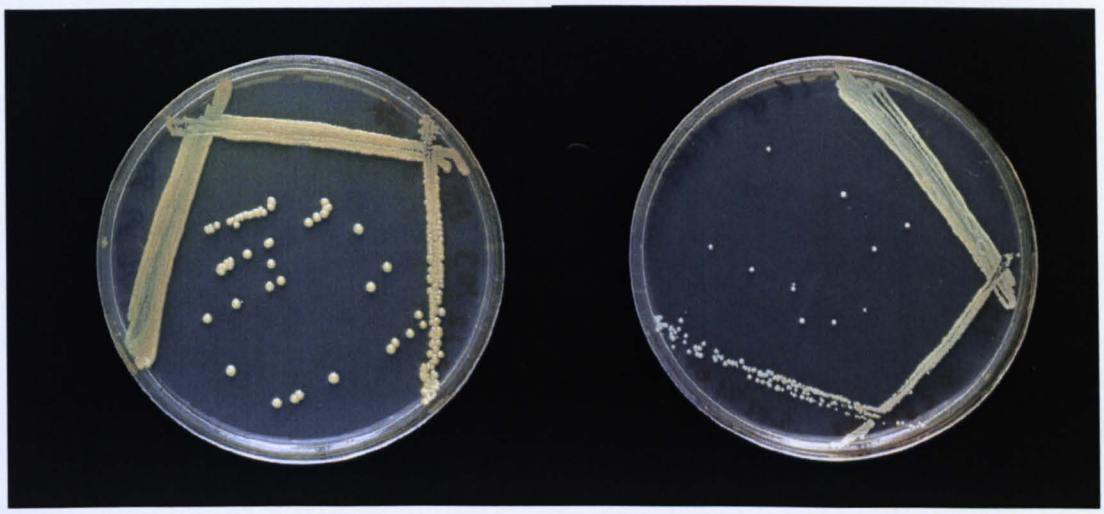
The three isogenic revertant cell lines RS1/19/10_{1-3} all gave positive latex agglutination Staphytect tests confirming them to be *Staphylococcus aureus*. Both the wild-type Oxford and revertant RS1/19 strains were β-lactamase-negative (Nattress, unpublished see Appendix 2). ß-lactamase is an enzyme that cleaves the β-lactam ring of penicillin therefore protecting the bacteria from the antimicrobial activity of the antibiotic.

S. aureus when grown on solid media show typically golden-yellow colonies. This golden colour is due to staphyloxanthine, a carotenoid pigment (Wieland, Feil et al. 1994). However the revertants RS1/19/10_{1-3} appeared paler with smaller colonies in comparison to the wild-type Oxford strain, which showed the typical golden colonies (fig. 3.3.1). In keeping with the colour difference seen on solid media, following centrifugation of cells grown in liquid media the pellet of revertant cells appeared pale yellow almost white in colour, in comparison to the wild-type cells which appeared golden yellow in colour.

S. aureus when Gram stained is Gram-positive due to the presence of the cell wall and typically show a 'bunches of grapes' appearance. The cell wall-deficient, CS1/19 was shown in previous work to stain as Gram-negative (Fuller et al. 2005). As expected the revertants RS1/19/10_{1-3} stained Gram-positive and were almost indistinguishable to the wild-type cells, though appeared to be less clumped (fig. 3.3.2).

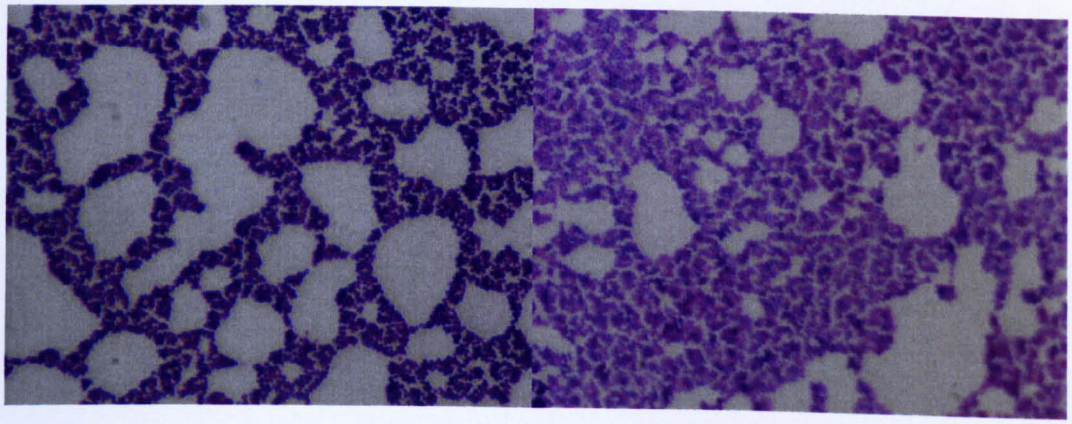
The penicillin minimum inhibitory concentrations (MICs) were measured for the wild-type Oxford and revertant RS1/19 strains in cell wall-deficient (CWD) liquid medium. For the Oxford strain the penicillin MIC was 0.125 mg/L and for RS1/19 strain the penicillin MIC was 32 mg/L, the CWD CS1/19 strain having been grown in 19.2 mg/L of penicillin G. This data was consistent with the previous antibiotic testing as shown in appendix 1 and confirms a stable, high-level of penicillin resistance in the revertant strain.

Figure 3.3.1: Wild-type Oxford and revertant RS1/19 strains.



Wild-type Oxford (on the left) and revertant RS1/19 strains grown on LB solid medium showing the difference in colour between the typical golden-yellow colonies of the wild-type strain and the much paler colonies of the revertant strain.

Figure 3.3.2: Gram stains of wild-type Oxford and revertant RS1/19 strains.



Gram stains of the wild-type Oxford (on the left) and revertant RS1/19 strains showing little distinction between the two, except the revertant cells appear less clumped.

3.4: GROWTH IN LIQUID MEDIA.

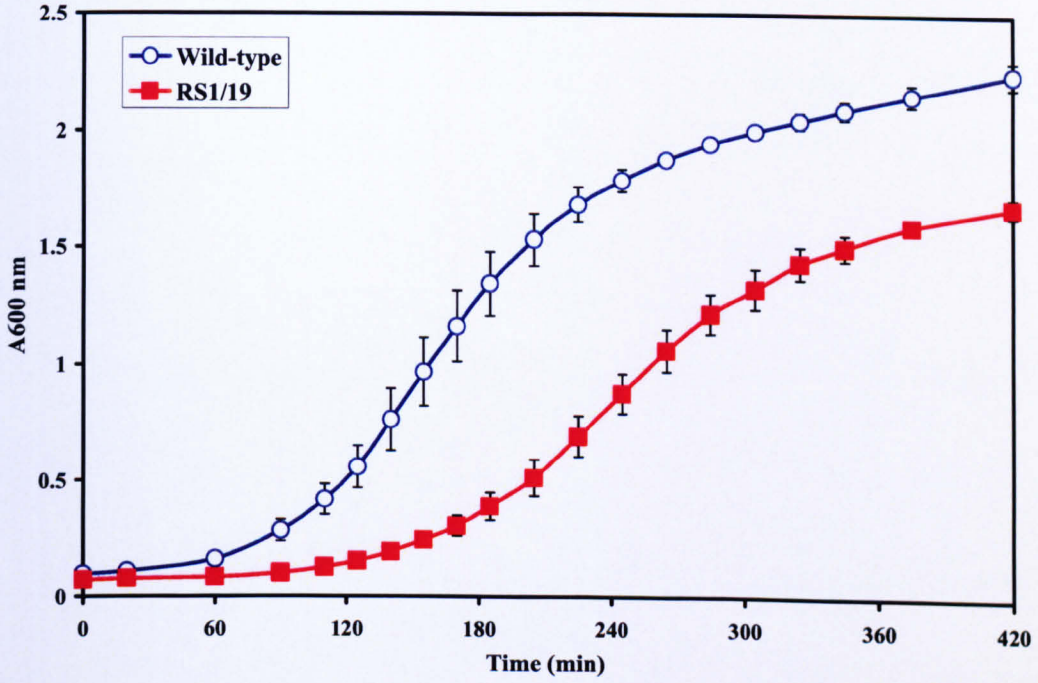
The growth of the wild-type Oxford and the revertant RS1/19 strains were followed by measuring absorbance readings at time periods after inoculation in BHI liquid medium. Growth was monitored for each of the three isogenic lines $RS1/19/10_1$, $RS1/19/10_2$ and $RS1/19/10_3$ and three biological replicates of the Oxford strain. Standard errors were calculated for both strains and the results plotted (figs. 3.4.1 and 3.4.2).

The exponential phase growth of the wild-type Oxford and revertant RS1/19 strains were plotted separately and from these the lag phase times and mean generation times were obtained. For the wild-type the lag phase time was 82 min and mean generation time was 46 min in comparison to the revertant strain which had a lag phase time of 149 min and mean generation time of 66 min respectively. By 24 hours the wild-type had reached an absorbance reading at 600 nm of 2.424 and for the revertant strain 2.370. Therefore the wild-type and revertant strains have the same saturation densities.

Therefore in conclusion the revertant strain had a longer lag phase and slower mean generation time in comparison to the wild-type strain.

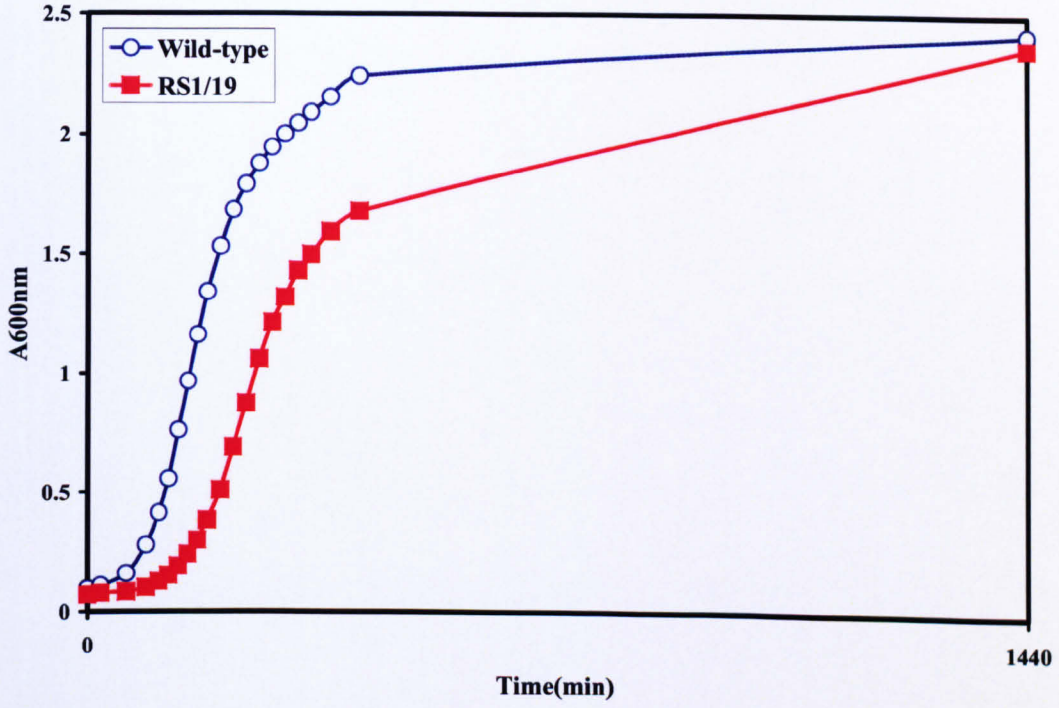
The three isogenic revertant cell lines $RS1/19/10_{1-3}$ proved to be indistinguishable from each other as far as growth on solid and liquid media, Gram staining and growth rates and are henceforth referred to as RS1/19.

Figure 3.4.1: Growth curves of wild-type Oxford and revertant RS1/19 strains.



Growth curves of the wild-type Oxford and revertant RS1/19 strains grown in BHI liquid medium at 37 °C showing that the revertant strain has a longer lag phase, slower mean generation time and lower saturation density in comparison to the wild-type strain. Values are the mean of three experiments and the bars represent the standard error of the mean (SEM).

Figure 3.4.2: Growth curves of wild-type Oxford and revertant RS1/19 strains including 24 hour data.



Growth curves of the wild-type Oxford and revertant RS1/19 strains grown in BHI liquid medium at 37 °C including the 24 hour data and showing that the wild-type and revertant strains reached the same saturation densities. Values are the mean of three experiments.

3.5: MATRIX-ASSISTED LASER DESORPTION/IONISATION TIME-OF-FLIGHT MASS SPECTROMETRY.

Matrix-assisted laser desorption/ionisation time-of-flight (MALDI-TOF) mass spectrometry can be used to obtain structural information as well as molecular weight data from whole cells. The cells from a colony are emulsified in a chemical matrix, added to a sample slide and dried. The sample is then transferred to the mass spectrometer where a laser beam vaporizes the matrix and ionizes large molecular weight (500 – 10,000) fragments from the cell surface into the vapour phase. These ions are extracted into a drift tube, where they separate and are sequentially delivered to a detector in order of their mass:charge (m:z) ratio. Negative and positive ions are produced and either can be analysed. This technique examines the chemistry of the intact bacterial cell surface, yielding spectra consisting of a series of peaks from 500 to 10,000, which represent the mass:charge (m:z) ratios. Each peak corresponds to a molecular fragment released from the cell surface during laser desorption (Edwards-Jones, Claydon et al. 2000).

MALDI-TOF mass spectrometry produces reproducible bacterial fingerprints, for different bacterial strains and has been used to identify and discriminate different *Staphylococcus aureus* strains. Its use to identify methicillin resistant *S. aureus* (MRSA) has been looked into with some peaks unique to MRSA, some unique to methicillin sensitive *S. aureus* (MSSA) and many unique to individual strains. MSSA strains produced a small number of peaks (37 – 67) and MRSA strains produced more (82 – 209), allowing ready visual discrimination (Edwards-Jones, Claydon et al. 2000). Due to its more rapid method of identification compared to traditional microbiological methods it has been suggested MALDI-TOF should be used, for example, to track nosocomial outbreaks of MRSA (Bernardo, Pakulat et al. 2002).

Although spectrometric methods have been described for the identification of microorganisms for many years, use on whole bacterial cells has only been described more recently (Claydon, Davey et al. 1996) and mostly using Gram-negative bacteria. Gram-positive bacteria are more technically challenging with regard to acquiring reproducible, high-quality spectral data (Claydon, Davey et al. 1996). This is thought to be due to the thicker, multi-layered peptidoglycan found in Gram-positive cells and

that physical disruption by laser ablation and ionisation of the sample during the MALDI process is not sufficient to ionize a number of individual proteins either attached to or protected by the cell wall.

Smole et al however showed that using the enzyme lysozyme to cleave the bacterial cell wall prior to the MALDI process led to good quality reproducible data (Smole, King et al. 2002). Lysozyme specifically hydrolyses the β-1, 4 linkages between the peptidoglycan disaccharide subunits *N*-acetylglucosamine (NAG) and *N*–acetylmuramic acid (NAM), and lysozyme treatment of Gram-positive bacteria including *S. aureus* was shown to be successful in increasing the complexity and spectral range (2-14 kDa) to a level similar to that achieved with Gram-negative bacteria (Smole, King et al. 2002).

Therefore any difference in the MALDI-TOF fingerprint of the revertant RS1/19 strain in comparison to the wild-type Oxford strain would confirm that the revertant had different characteristics compared to the wild-type, following the transient cell wall-deficient state, and could even be considered a new strain in comparison. A different fingerprint would confirm that there is a structural difference in the revertant cell in comparison to the wild-type and this structural difference could be within the cell wall and if investigated may lead to an understanding of the mechanism of the altered antibiotic sensitivity profile seen in the revertant RS1/19 strain.

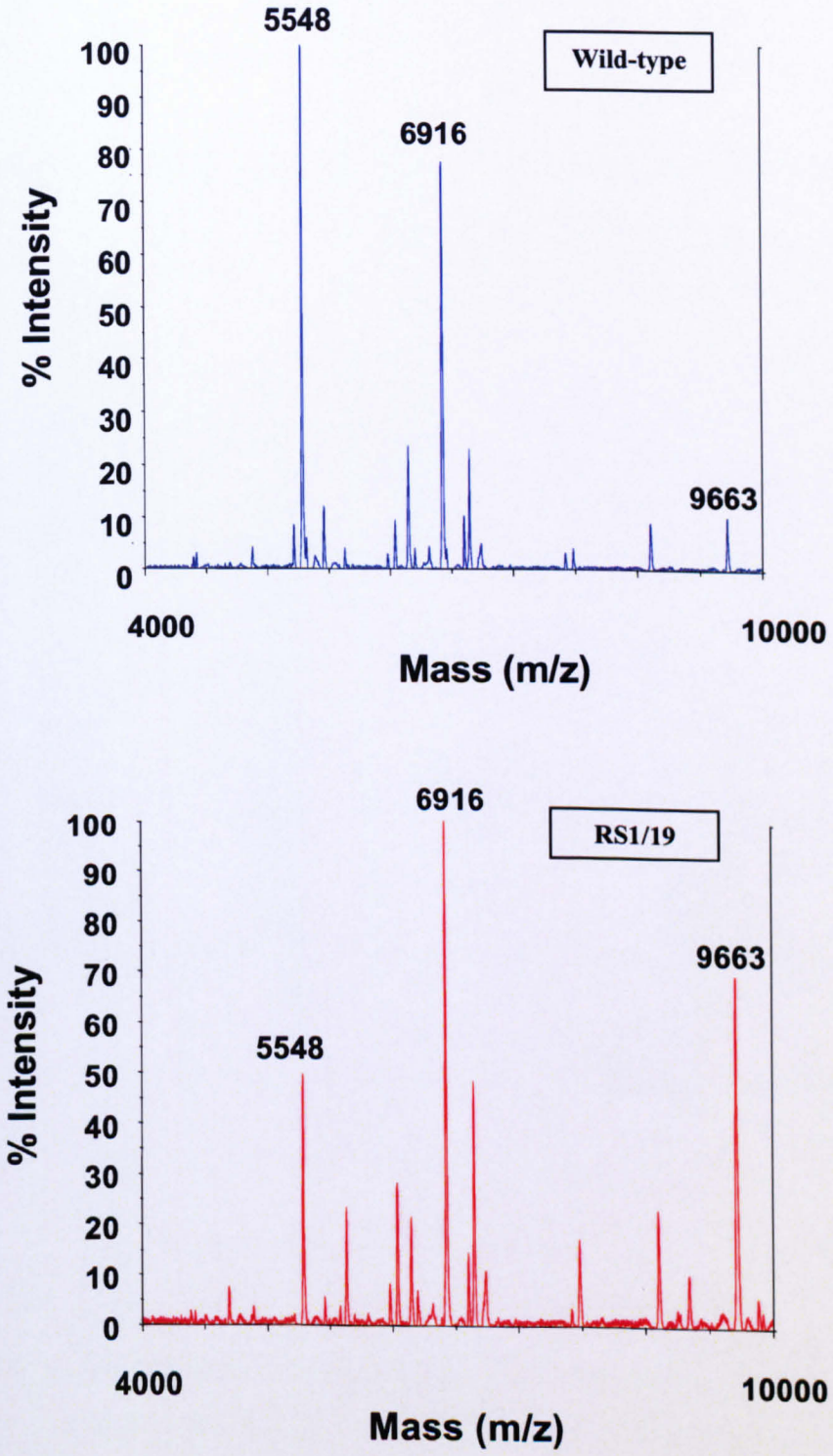
Whole cells were prepared and subjected to MALDI-TOF analysis by the method of Smole et al (Smole, King et al. 2002). Three single colonies of the wild-type and revertant RS1/19 strains were grown overnight in BHI liquid medium. Preparations of these three cultures were made in duplicate and the six subsequent preparations for each strain were spotted on to the MALDI plate twice over. Therefore data was collected to determine variability in terms of the biological material, sample preparation and the MALDI-TOF machine.

There was a clear, reproducible, difference in the MALDI-TOF fingerprint of the wild-type cells and the revertant cells as seen by the different proportions of certain mass ions. The most obvious differences were the peaks of the masses 5548, 6916 and 9663 (fig. 3.5.1). As lysozyme treatment was used for all the samples the lysozyme peaks, 14360 and its double charged mass ion 7180, were used as internal calibration controls. Hence the data was calibrated to the lysozyme peak 14360 with

the heights of the three peaks of the masses 5548, 6916 and 9663 being recorded as a percentage of the lysozyme peak 14360 for each of the 24 results so the data could be compared accurately. The percentages for these three masses, alongside the two lysozyme masses, for the wild-type and revertant strains were plotted with their standard error bars (fig. 3.5.2).

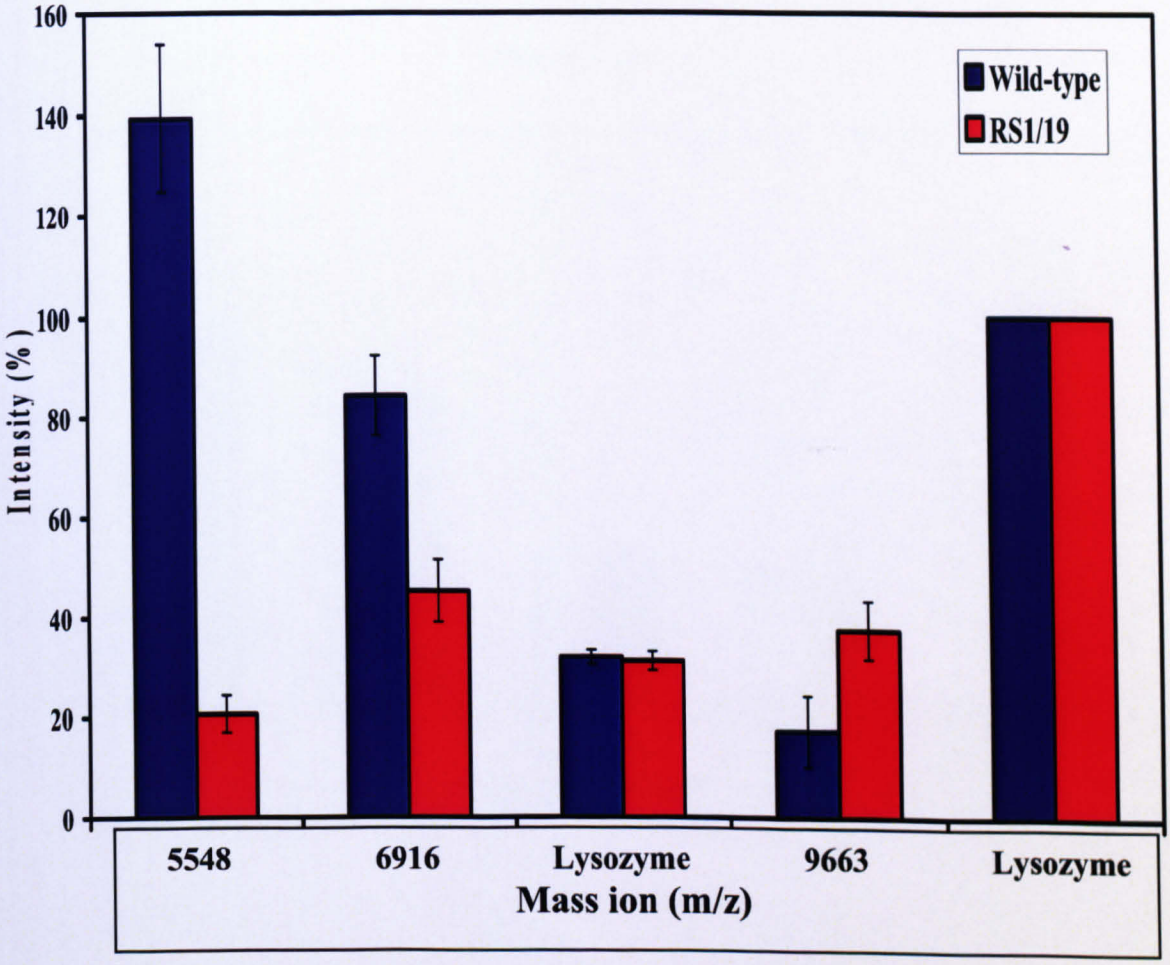
The percentages for the replicates were also compared. The preparations that were spotted in duplicate to test the reproducibility of the MALDI-TOF machine were compared (fig. 3.5.3.1). This shows very good reproducibility. The three cultures for each strain that were prepared in duplicate to test the reproducibility of the sample preparation were also compared (fig. 3.5.3.2). This shows good reproducibility for the mass ion 5548 but less so for the mass ions 6916 and 9663, however still reasonable. This allows comparison for the biological replicates (fig. 3.5.2) and this data shows clearly that the proportions of these three peaks are different between the wild-type and revertant strains consistently with the 5548 peak being larger in the wild-type and the 9663 peak being larger in the revertant strain. This shows there is a fingerprint for the revertant RS1/19 strain and confirms that there are differences between it and the wild-type Oxford strain.

Figure 3.5.1: Typical examples of MALDI-TOF fingerprints obtained for wild-type Oxford and revertant RS1/19 strains.



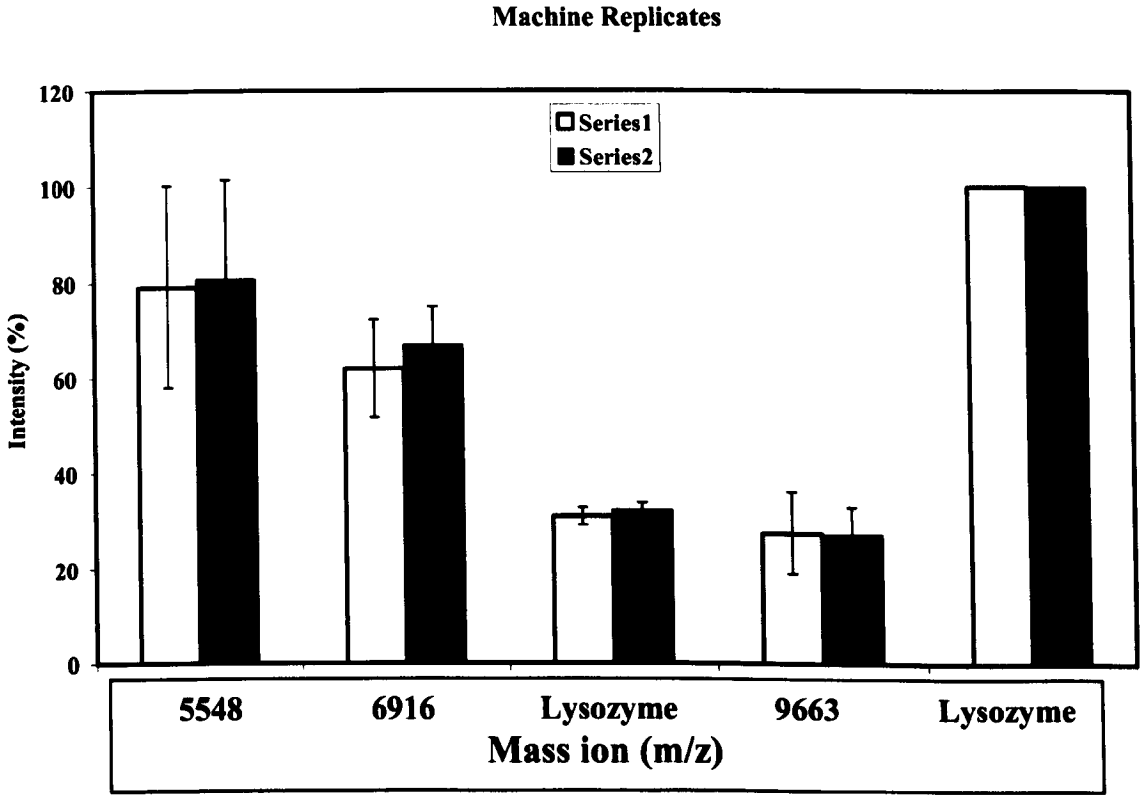
Typical examples of the MALDI-TOF fingerprints obtained for the wild-type Oxford and revertant RS1/19 strains, showing clear differences in the intensities of the peaks for the masses 5548, 6916 and 9663 between the two strains.

Figure 3.5.2: Relative proportions of peaks for the main masses for wild-type Oxford and revertant RS1/19 strains.



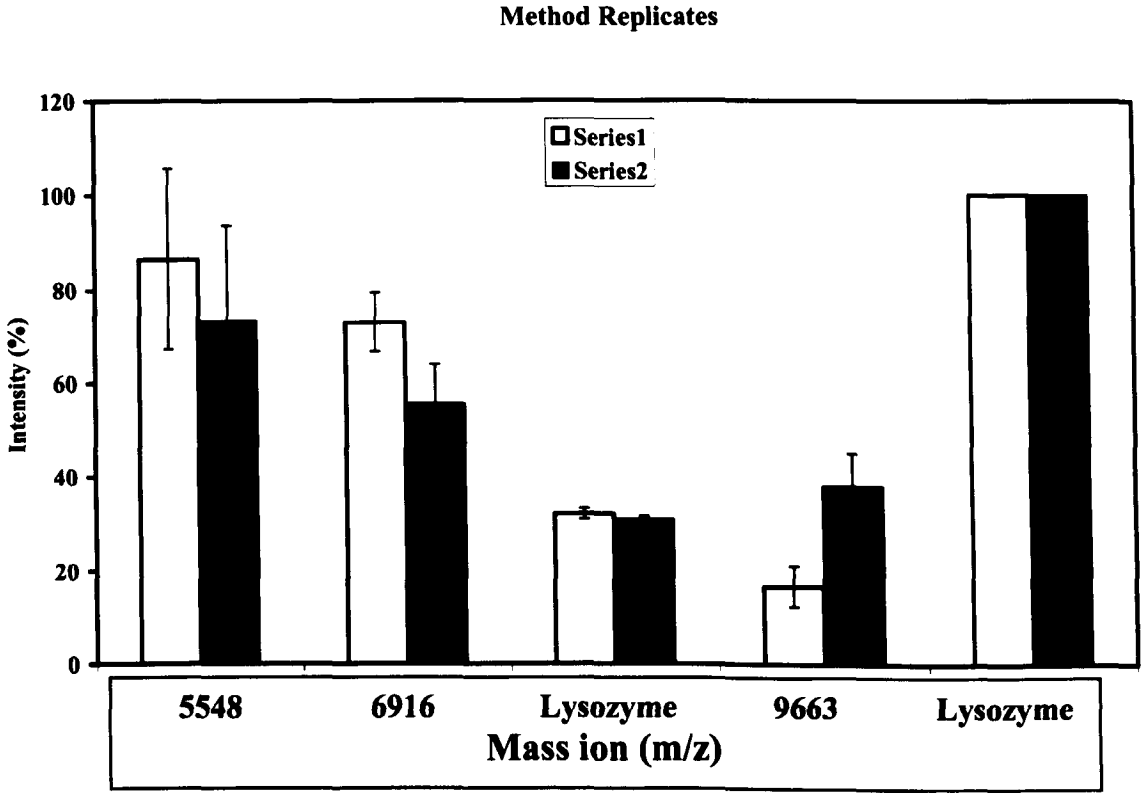
Relative proportions of the peaks for the main masses (± SEM) for the wild-type Oxford and revertant RS1/19 strains. As the masses 7180 and 14360 are the two lysozyme peaks, hence these being more or less identical for the two strains, these were used as internal markers. The heights of the other main peaks were expressed as a percentage of 14360, with the most difference between the two strains seen in the mass 5548.

Figure 3.5.3.1: Relative proportions of peaks for the main masses for the replicates testing the reproducibility of the MALDI-TOF machine.



Relative proportions of the peaks for the main masses (± SEM) for the replicates testing the reproducibility of the MALDI-TOF machine. These show that there is good reproducibility for machine.

Figure 3.5.3.2: Relative proportions of peaks for the main masses for the replicates testing the reproducibility of the method of sample preparation.



Relative proportions of the peaks for the main masses (± SEM) for the replicates testing the reproducibility of the method of the sample preparation. These show reasonable reproducibility for the method.

3.6: LYSOSTAPHIN SUSCEPTIBILITY.

Lysostaphin is an endopeptidase produced by *Staphylococcus staphylolyticus* that cleaves the pentaglycine cross-bridges (fig. 3.6.1, adapted from (Giesbrecht, Kersten et al. 1998) in *S. aureus* peptidoglycan leading to lysis of the cell walls (Giesbrecht, Kersten et al. 1998; Navarre and Schneewind 1999).

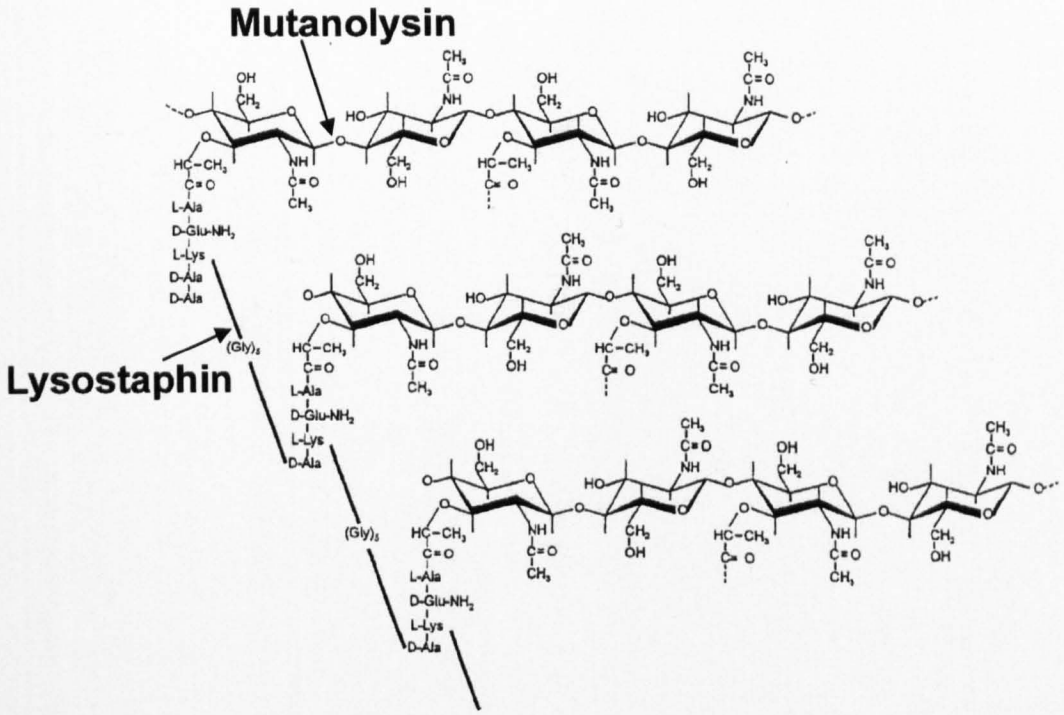
Lysostaphin susceptibility of the wild-type and revertant RS1/19 strains were tested using both exponential and stationary growth phase cells in duplicate using the same culture i.e. technical replicates (figures 3.6.2. and 3.6.3).

These results show that the revertant strain is more resistant to lysostaphin than the wild-type strain during both during the exponential and stationary growth phases, with the exponential phase cells being more susceptible to lysostaphin in both cell types.

For the exponential phase cells 50% lysis occurred after 6.9 min for the wild-type strain and after 14.8 min for the revertant strain, a 2.1-fold difference. For the stationary phase cells 50% lysis occurred after 7.6 and 20.0 min for the wild-type and revertant strains respectively, a 2.6-fold difference.

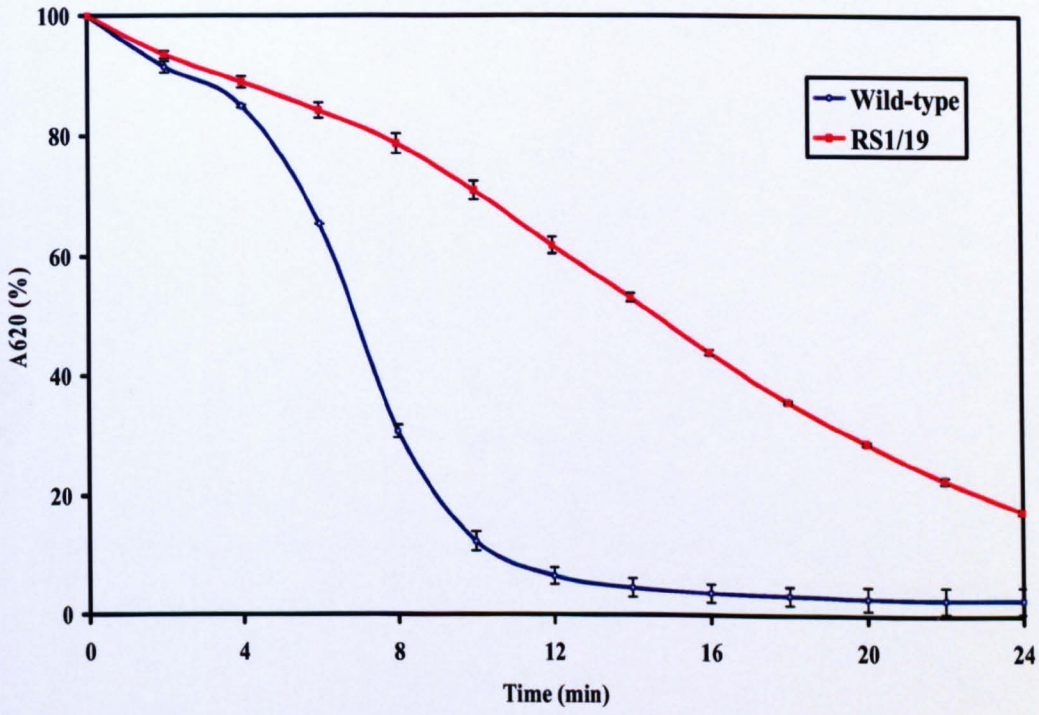
This suggests that the revertant cells do have a different cell wall structure in comparison to the wild-type. This difference in the revertant strain could be a different cross-bridge sequence, more pentaglycine cross-bridges, or due to stearically hindering access of lysostaphin to the target causing the lysostaphin resistance. More pentaglycine cross-bridges could be due to more cell wall in the revertant due to a thicker cell wall or a larger cell, or could be due to more cross-bridges per unit of peptidoglycan. However within *Staphylococcus aureus* cells 90% of the peptidoglycan is cross-linked already (Giesbrecht, Kersten et al. 1998), therefore a thicker cell wall or larger cell is more probable which is consistent with the previous results showing a slower growth rate for the revertant strain and a greater peptidoglycan mass.

Figure 3.6.1: Structure of *S. aureus* peptidoglycan (adapted from (Giesbrecht, Kersten et al. 1998).



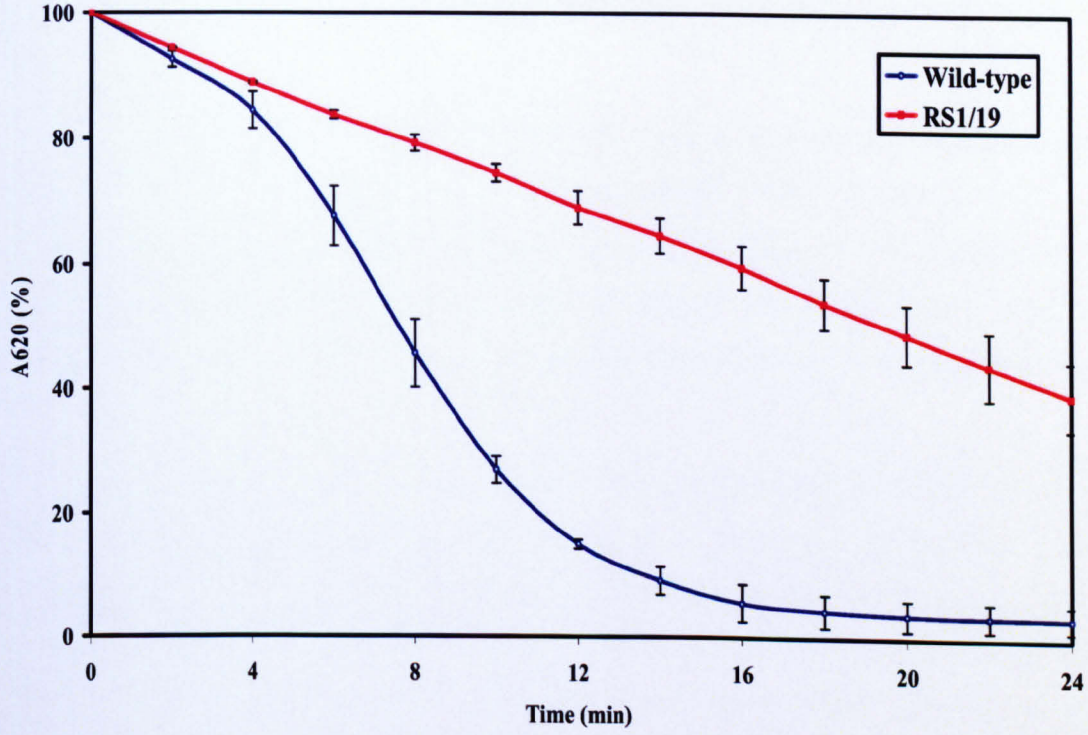
Structure of *S. aureus* peptidoglycan showing positions of cleavage by mutanolysin (cuts glycan chain) and lysostaphin (cuts pentaglycine cross-bridge).

Figure 3.6.2: Lysostaphin susceptibility of wild-type Oxford and revertant RS1/19 strains during exponential growth phase.



Lysostaphin susceptibility of the wild-type Oxford and revertant RS1/19 strains during exponential growth phase (± SEM) at 37 °C, showing the revertant strain is more resistant to lysostaphin in comparison to the wild-type strain. Values are the mean of three experiments.

Figure 3.6.3: Lysostaphin susceptibility of wild-type Oxford and revertant RS1/19 strains during stationary growth phase.



Lysostaphin susceptibility of the wild-type Oxford and revertant RS1/19 strains during stationary growth phase (± SEM), again showing the revertant strain is more resistant to lysostaphin in comparison to the wild-type strain. Values are the mean of three experiments.

3.7: PREPARATION OF PEPTIDOGLYCAN.

Peptidoglycan is the major structural component of the cell wall in *Staphylococcus aureus* and consists of peptide cross-linked glycan chains. Alternating subunits of *N*-acetylmuramic acid and *N*-acetylglucosamine make up the glycan chains and pentapeptide side chains are attached to the *N*-acetylmuramic subunits. The glycan subunits are also cross-linked to the peptide bridges through side chains (Giesbrecht, Kersten et al. 1998).

The hypothesis for the penicillin resistance seen in the revertant RS1/19 strain, and not in the wild-type Oxford strain, is that the antibiotic resistance is due to an altered cell wall structure as discussed in the introduction. Hence to investigate this further peptidoglycan, as the major component of the cell wall, was prepared for each strain to analyse using reversed-phase high-performance liquid chromatography (RP-HPLC).

Preparations of peptidoglycan of the wild-type and revertant RS1/19 strains followed the method by de Jonge et al (de Jonge, Chang et al. 1992) with some modifications. The cultures were grown to absorbance readings at 600 nm of 0.917 for the wild-type strain and 1.012 for the revertant strain. The final masses of the peptidoglycan preparations were for the wild-type strain 0.0924 g and for the revertant strain 0.1639 g, suggesting that the RS1/19 has more peptidoglycan per cell in comparison to the wild-type. The preparation of peptidoglycan from each strain was identical and the starting number of cells for each was roughly the same, as the preparations used cultures with a similar absorbance reading and equal quantities of culture. However, to calculate the difference in peptidoglycan masses more accurately the viable cells were enumerated at several absorbance readings.

Enumeration of wild-type and revertant RS1/19 strains: liquid cultures were grown and using a spectrophotometer set at 600 nm the absorbance was followed for each cell line and at approximately 0.5, 1.0 and 1.5 (i.e. early, mid and late exponential growth phases); serial ten-fold dilutions were made by transferring 1 ml of culture to 9 ml of MQ. Further dilutions were made until 10^{-4}, 10^{-5} and 10^{-6} dilutions were obtained and aliquots of 100 µl from each dilution were then spread

onto LB solid medium. Plates were incubated at 37 °C overnight before the colonies were enumerated.

The results of the colony counts at early, mid and late exponential growth phases are shown in table 3.7. These figures were plotted for each strain and the number of colonies/ml at the OD_{600} at which the peptidoglycan was prepared was determined. For the wild-type strain the OD_{600} was 0.917 this corresponded to 1.3 x 10^9 colonies, and for the revertant strain the OD_{600} was 1.012 this corresponded to 9.7 x 10^8 colonies. Using the relationship between OD_{600} and viable cell counts and the known masses at the end of the preparation a peptidoglycan mass (mg/10^9 colonies) was estimated for each strain. Therefore there were 74.6% of colonies for the revertant strain in comparison to the wild-type strain when the peptidoglycans were prepared. With final masses of peptidoglycan of 0.0924 g or 92.4 mg for the wild-type strain and 0.1639 g or 163.9 mg for the revertant strain before the number of colonies are taken into account, equivalent masses would be 68.9 g per 9.7 x 10^8 colonies for the wild-type strain and 163.9 mg per 9.7 x 10^8 colonies for the revertant strain. Finally these figures are equivalent to 71 mg per 10^9 and 169 mg per 10^9 colonies for the wild-type and revertant strains respectively (table 3.7).

Although this method of enumeration is open to some error it shows the difference seen in the final masses following the peptidoglycan preparation was an underestimate, as the number of colonies at the start of the preparation for RS1/19 was far less than for the wild-type strain. When this is taken into account a 2.4-fold difference is calculated and this indicates that revertant cells have more peptidoglycan and than wild-type cells. Whether this means the revertant RS1/19 has a thicker cell wall in comparison to the wild-type strain due to this increased peptidoglycan mass or whether the cells are larger in size requires further investigation. This however may explain the slower growth rate seen in the revertant strain due to requiring more time to synthesise the increased peptidoglycan.

Table 3.7: Colony count results for early, mid and late exponential growth phases for wild-type Oxford and revertant RS1/19 strains.

Strain	**OD_{600}**	**Colonies/ml**	**Estimated mass ($mg/10^9$ colonies)**
Wild-type	0.515	2.68×10^8	71
	1.089	3.28×10^9	
	1.587	2.016×10^{10}	
RS1/19	0.515	8×10^7	169
	1.089	1.56×10^9	
	1.587	9.6×10^9	

Colony count results for early, mid and late exponential growth phases for the wild-type Oxford and revertant RS1/19 strains, including estimated mass of peptidoglycan in wild-type and revertant strains showing a 2.4-fold difference.

3.8: REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY.

Muropeptides were prepared from peptidoglycan of the wild-type Oxford and revertant RS1/19 strains. The peptidoglycan was digested by lysostaphin or mutanolysin to obtain muropeptides. Lysostaphin is an endopeptidase that is able to cleave the pentaglycine cross-bridges whilst mutanolysin is a muramidase that is able to cleave the glycan chains of the peptidoglycan structure of *Staphylococcus aureus* (fig. 3.6.1). Therefore following lysostaphin digestion the glycan chains are left intact and following mutanolysin digestion the pentaglycine cross-bridges are left. The reduced muropeptides were then fractionated into peptides by reversed-phase high-performance liquid chromatography (RP-HPLC) using a RP18 column. By comparing the RP-HPLC chromatographs of muropeptides following lysostaphin digestion of the wild-type and revertant peptidoglycan any difference in the glycan chains could be analysed and similarly following mutanolysin digestion any difference in the pentaglycine cross-bridges could be analysed.

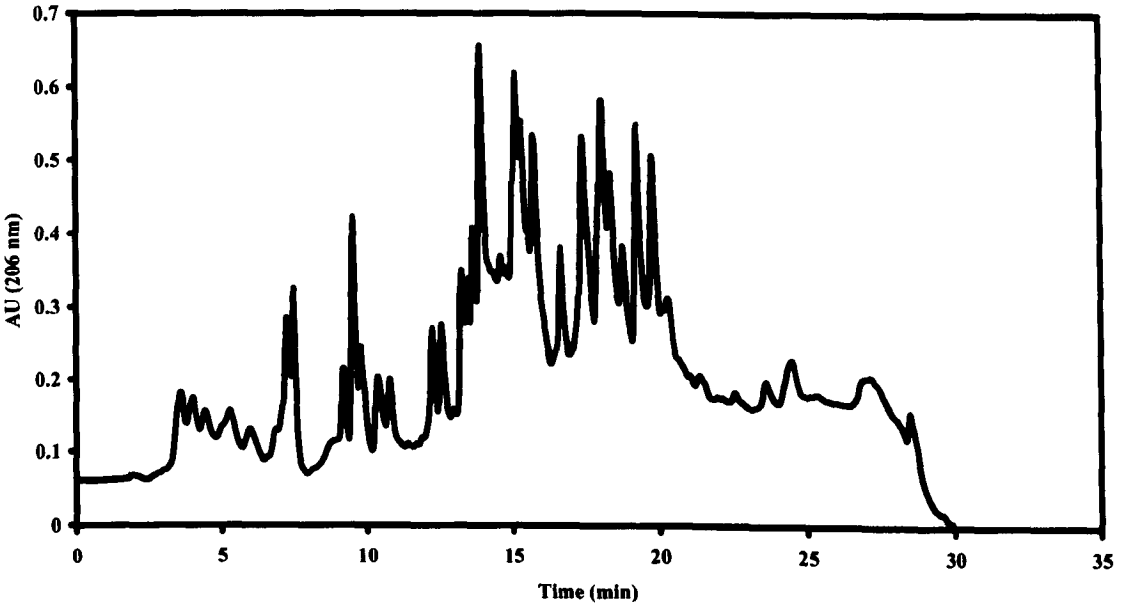
RP-HPLC injects the material to be tested on to a column with buffer running through it. Initially the buffer has a hydrophobic nature (buffer A), for example 5% ACN/ 0.05% TFA, but over time this is replaced by a second buffer (buffer B), for example 95% ACN/ 0.05% TFA, which has a much less hydrophobic nature. Material bound to the column is eluted by running a gradient of 0 to 100% buffer B. As the hydrophobic environment on the column reduces peptides elute from the column and are monitored by a UV detector. For this experiment the fragments with less peptides have low ionic charges and therefore are less hydrophobic and will elute first, whilst the fragments with more peptides are more hydrophobic and will elute later.

Establishing a protocol: initially to test the column a known peptide digest of BSA, digested with trypsin, was chromatographed with a buffering system known to be suitable for analyzing the peptide digest of BSA, 5% ACN/ 0.05% TFA as buffer A and 95% ACN/ 0.05% TFA as buffer B. This gave a result with multiple peaks as expected for a peptide digest of BSA (fig. 3.8.1).

Various previously published buffering systems were compared using the wild-type muropeptide digested by lysostaphin, to establish the most efficient separation. The above buffering system used for the BSA digest was tested in comparison to 5% methanol (pH 2.5) as buffer A and 30% methanol (pH 2.8) as buffer B as established by Glauner (Glauner 1988) (fig. 3.8.2, graphs 1 and 2). Both of these buffering systems failed to separate the peptides fully with a large amount of material eluting together towards the end of the run. To improve the separation of the peptides the methanol gradient was increased to 60% as buffer B and also a longer run time was employed, but again with poor results. Then the solvent gradient used by Roos et al (Roos, Pittenauer et al. 1998) was used, 0% ACN/ 0.05% TFA as buffer A and 17.5% ACN/ 0.035% TFA as buffer B giving good results (fig. 3.8.2, graph 3) with multiple peaks. Figure 3.8.2 shows the three main buffering systems tried, graph 1 shows the system used for the BSA digest with very little resolution of the peptides. Graph 2 shows the buffering system using Glauner's protocol with some resolution of peptides but still with a mass of material at end suggesting that all the peptides were not separated. Graph 3 show the system using Roos' protocol with all the peptides separated with no mass of material at the end. Therefore this buffering system was used to test the wild-type and revertant muropeptides.

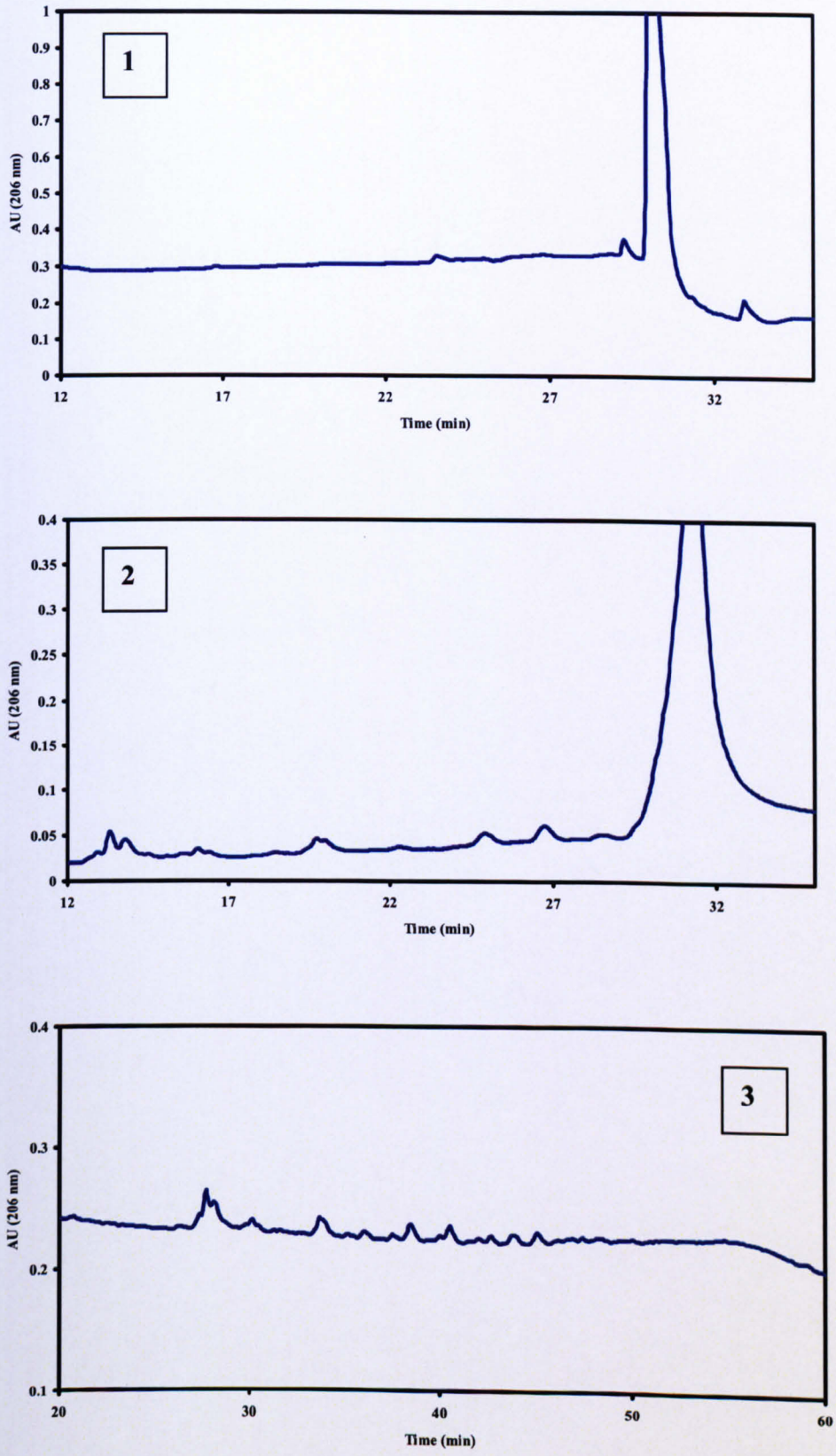
Results: following lysostaphin treatment, the muropeptides of the revertant eluted earlier in comparison to those of the wild-type. The nature of the muropeptides from the revertant cells was therefore less hydrophobic indicating that they contain shorter glycan chains, as lysostaphin cleaves the pentaglycine cross-bridges (fig. 3.8.3). In comparison, following mutanolysin treatment a longer retention time for muropeptides of the revertant was seen; the increased hydrophobicity of these muropeptides indicate that they contain more peptides (fig. 3.8.4). As mutanolysin cuts at the glycan chains this suggests the revertants have a larger number of pentaglycine cross-bridges. This result is consistent with the previous data i.e. greater lysostaphin susceptibility and greater mass of peptidoglycan from equivalent number cells of the revertant RS1/19 strain in comparison to the wild-type Oxford strain. However, these data cannot differentiate whether cell wall of the revertant is thicker or contains more pentaglycine cross-bridges per unit of peptidoglycan.

Figure 3.8.1: RP-HPLC chromatographs of a peptide digest of BSA using a TFA/ACN buffering system.



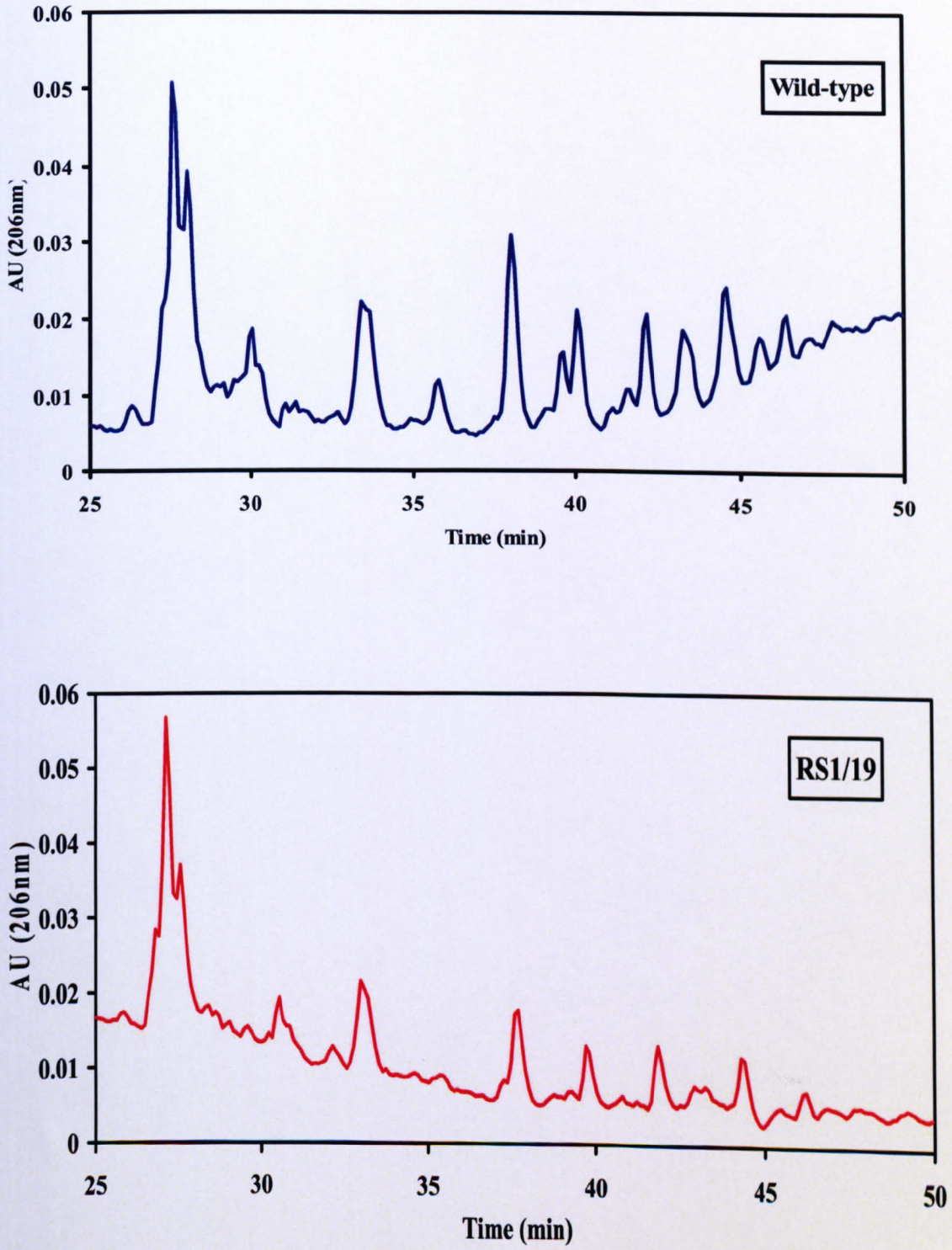
RP-HPLC chromatographs of a peptide digest of BSA using a TFA/ACN buffering system showing the typical peaks expected for this digest, confirming that the column was functioning normally.

Figure 3.8.2: Comparison of different buffering systems.



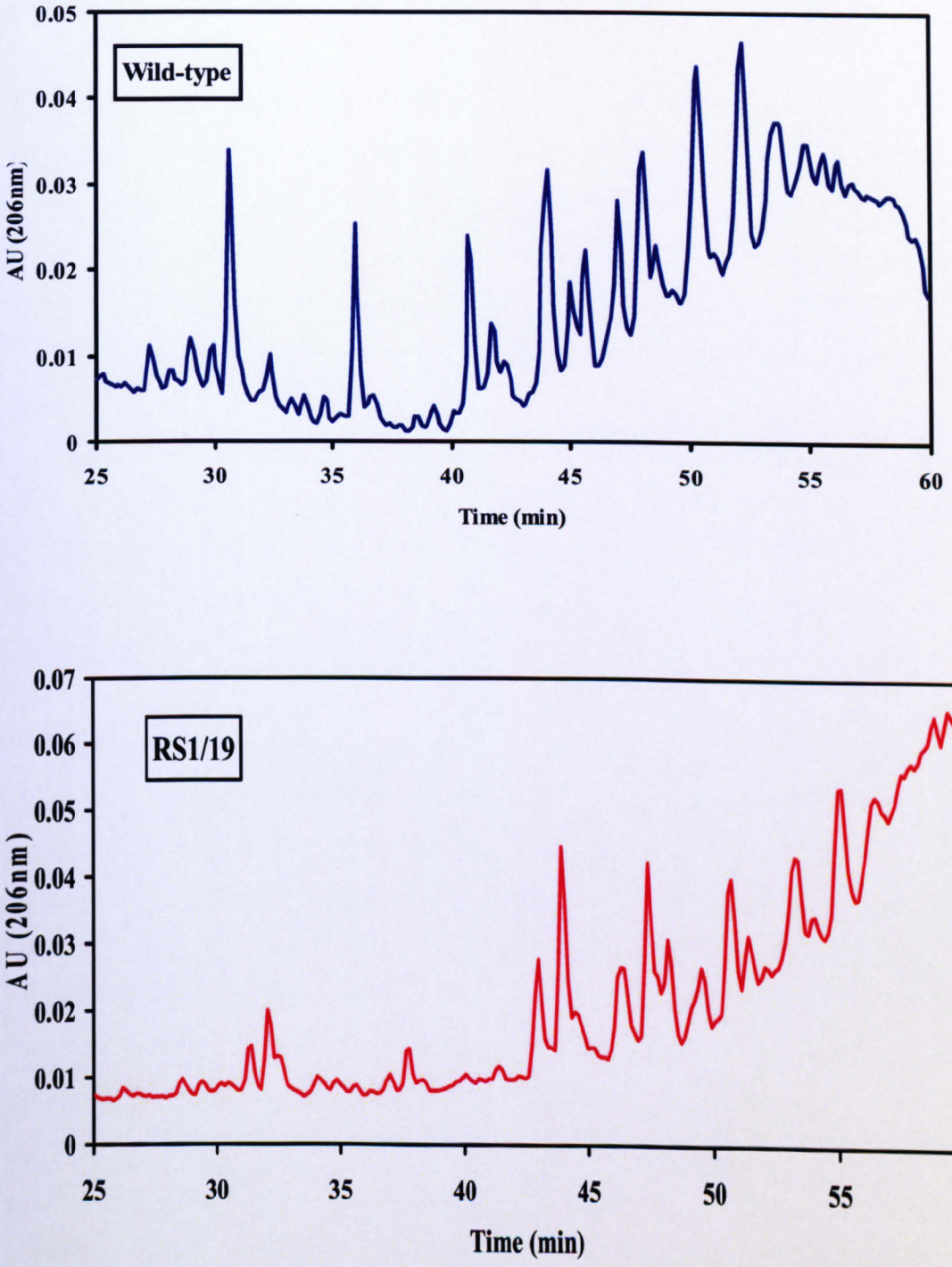
Comparison of different buffering systems. **1:** 5% ACN/ 0.05% TFA to 95% ACN/ 0.05% TFA, **2:** 5% to 30% MeOH and **3:** 0.05% TFA to 17.5% ACN/ 0.035% TFA.

Figure 3.8.3: RP-HPLC chromatographs following lysostaphin treatment of muropeptides of wild-type Oxford and revertant RS1/19 strains.



RP-HPLC chromatographs following lysostaphin treatment of the muropeptides of wild-type Oxford and revertant RS1/19 strains, using a TFA/ACN buffering system.

Figure 3.8.4: RP-HPLC chromatographs following mutanolysin treatment of muropeptides of wild-type Oxford and revertant RS1/19 strains.



RP-HPLC chromatographs following mutanolysin treatment of muropeptides of the wild-type Oxford and revertant RS1/19 strains, using a TFA/ACN buffering system.

3.9: REPRODUCIBILITY OF REVERSION OF CWD TO CWC PHENOTYPE, INCLUDING IMPACT OF MEDIA USED.

The characteristics of the three revertant cell lines RS1/19/10_1, RS1/19/10_2 and RS1/19/10_3 from the cell wall-deficient (CWD) CS1/19 strain were studied with it being noted that these three isogenic lines behaved indistinguishably. The question remained however whether all revertants of CS1/19 behaved in the same way, including if a different media was used for the reversion, this being particularly important to establish whether the media had any effect on the behaviour including resistance seen in the revertants. So not only if the same method of reversion was repeated whether the revertants behaved in the same way, but also if different media was used for the reversion whether the revertants still behaved in the same way. I.e. was it a media-specific observation?

Hence an experiment was designed where reversion of CS1/19 was repeated using CWD solid medium as previously alongside reversion of CS1/19 using LB solid medium. Then the final revertants were compared to each other and the wild-type Oxford strain, using growth curves and lysostaphin susceptibility. These results were also compared to the results of RS1/19.

3.9.1: Repeat reversion of CS1/19 to RS1/19L2 and RS1/19C2: CS1/19 was reverted to a cell-wall competent (CWC) state by transferring on solid media in the absence of penicillin as described previously. However in addition to transferring onto CWD medium single colonies were also transferred onto LB medium, the method otherwise being the same as was described earlier in this chapter. These new revertant cell lines were named RS1/19/10L2 and RS1/19/10C2; where 10 represents the transfer number, 2 represents the 2nd reversion of CS1/19, and L and C represents the type of media the lines were reverted on (L = LB medium and C = CWD medium).

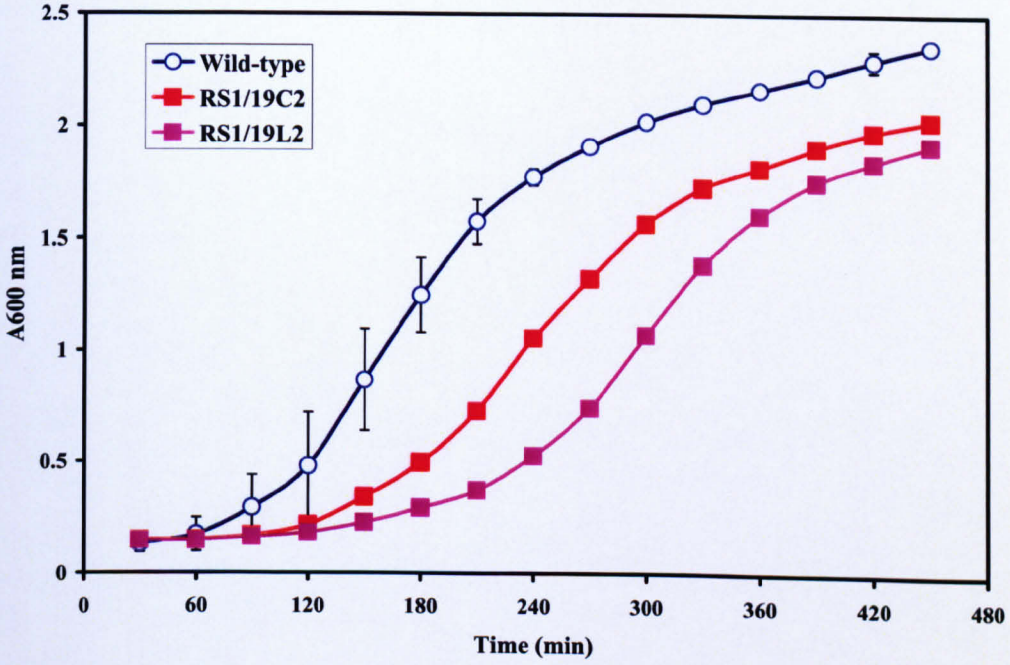
3.9.2: Growth in liquid media: the growth of these new revertant strains was followed in comparison to the wild-type using BHI liquid medium. The growth of the wild-type Oxford strain was followed in duplicate using two different overnight cultures and the standard errors were calculated and the results plotted with the results of the two revertant strains (fig. 3.9.2).

The exponential phase growth of the wild-type Oxford and revertant RS1/19L2 and RS1/19C2 strains were plotted separately and from these the lag phase times were obtained. The linear equations were calculated from these graphs and hence mean generation times were also obtained. For the wild-type the lag phase was 80 min and mean generation time was 49 min, in comparison to RS1/19L2 which was 186 min and 65 min respectively and RS1/19C2 127 min and 66 min respectively. These results also compare to a lag phase of 82 min and mean generation time of 46 min for the wild-type previously, and lag phase of 149 min and mean generation time of 66 min for RS1/19. The saturation density for the wild-type was an absorbance reading at 600 nm of 2.555, for RS1/19C2 2.627 and for RS1/19L2 2.623 at 750 min.

Therefore the method is reproducible as the two results for the wild-type are nearly identical. Also not only do the two revertant lines have near identical mean generation times to each other but also to RS1/19 and again there is a clear difference between the wild-type and the two revertant strains as seen previously with longer lag phases and slower mean generation times seen in the revertant strains. This shows that the media used for reversion does not alter the growth rate of the subsequent revertants. The longer lag phase seen with RS1/19L2 probably is due to this line previously being grown on LB medium which is less rich than BHI medium.

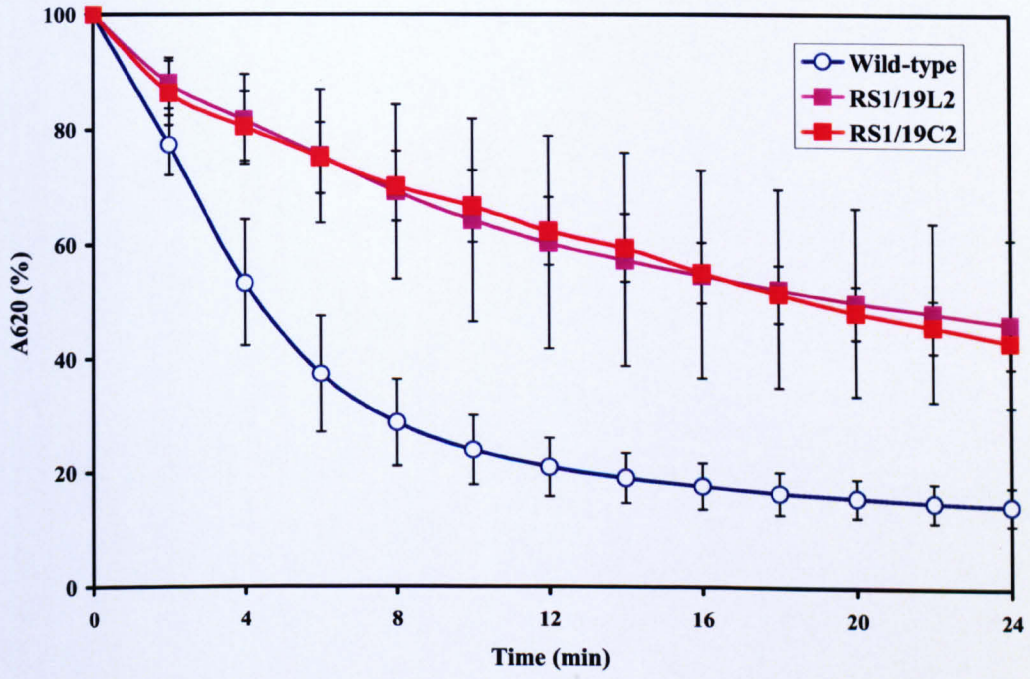
3.9.3: Lysostaphin susceptibility: the lysostaphin susceptibility of the two new revertant strains was tested in comparison to the wild-type. Exponential phase cells were tested for each strain three times, the first with the wild-type Oxford strain and RS1/19L2 grown in LB liquid medium and RS1/19C2 in CWD liquid medium. The experiment was then repeated using BHI liquid medium throughout and each strain tested in duplicate. The results again showed that the revertant strains are more resistant to lysostaphin in comparison to the wild-type strain (fig.3.9.3) with the results of the two revertant strains being indistinguishable. For the wild-type strain 50% lysis occurred after 4.3 min, and after 18.8 min and 20.0 min for the revertant RS1/19C2 and RS1/19L2 strains respectively. This compares to a 50% lysis time of 14.8 min for RS1/19. This again shows that the media used for reversion makes little difference to the lysostaphin susceptibility of the subsequent revertants.

Figure 3.9.2: Growth curves of wild-type Oxford strain and revertant RS1/19C2 and RS1/19L2 lines.



Growth curves of the wild-type Oxford strain (mean and range) and revertant RS1/19C2 and RS1/19L2 lines, grown in BHI liquid medium at 37 °C, showing that the two revertant strains have longer lag phases and slower mean generation times in comparison to the wild-type strain. RS1/19C2C was reverted from CS1/19 on CWD medium and RS1/19L2 on LB medium.

Figure 3.9.3.1: Lysostaphin susceptibility of wild-type Oxford and revertant RS1/19L2 and RS1/19C2 strains.



Lysostaphin susceptibility of the wild-type Oxford and revertant RS1/19L2 and RS1/19C2 strains during exponential growth phase (± SEM) at 37 °C, showing the revertant strains are more resistant to lysostaphin in comparison to the wild-type strain. Values are the mean of three experiments.

3.10: DISCUSSION.

The cell wall-deficient (CWD) line CS1/19 was previously generated (Fuller et al. 2005) from the wild-type *Staphylococcus aureus* Oxford strain ATCC 9144. This was achieved by sub-culturing on CWD solid medium, a supplemented media with a high osmotic potential, with increasing sublethal doses of penicillin G as described in the introduction with the final penicillin G concentration being 19.2 mg/L. The colonies of CS1/19 were small, had lost the yellow pigmentation typical of *S. aureus* and were umbonate in profile. Gram staining showed that the cells had a diffuse edge, did not show a typical staphylococcal cell arrangement and were Gram-negative. This would all be consistent with an absent cell wall. These CWD cells were resistant to penicillin as would be predicted due to the removal of the site of action of penicillin i.e. the cell wall.

CS1/19 was reverted back to a cell wall-competent (CWC) state by growing the CWD cells in the absence of penicillin. This revertant line RS1/19 was also found to be resistant to penicillin. This penicillin resistance was high-level and some altered sensitivities to the non-cell wall-active antibiotics were also noted (Nattress, unpublished see Appendix 1). To study the potential mechanism for this penicillin resistance of the revertants they were studied further.

In comparison to the wild-type Oxford strain RS1/19 was visually paler with smaller colonies, had a longer lag phase and slower mean generation time during growth in liquid media and had an altered matrix-assisted laser desorption/ionisation time-of-flight (MALDI-TOF) 'fingerprint'. The mass of peptidoglycan for RS1/19 was 2.4-fold greater than the wild-type and RS1/19 was more resistant to lysostaphin. After treatment with lysostaphin the peptidoglycan of the revertants on reversed-phase high-performance liquid chromatography (RP-HPLC) analysis showed less hydrophobic peptides suggesting shorter glycan chains, and more hydrophobic peptides after treatment with mutanolysin suggesting a greater number of pentaglycine cross-bridges. This could mean that the revertant has a thicker wall or that there are more pentaglycine cross-bridges per unit of peptidoglycan. On repeating the reversion to a CWC phenotype, all lines had a similar slower growth rate and resistance to lysostaphin suggesting that these characteristics are reproducible for the revertants of CS1/19.

The hypothesis, at the beginning of this work, for the cause of the stable high-level of penicillin resistance seen in the revertants, in comparison to the wild-type, was an altered cell wall structure. The data presented in this chapter shows that the cell wall structure of the revertants is highly altered in comparison to the wild-type strain. Therefore this as a cause for the penicillin resistance seen remains as a possibility, however this requires further investigation as it is possible for an altered penicillin-binding protein to be causing the altered cell wall in the revertant along with the penicillin resistance.

4. CHARACTERISATION OF PENICILLIN-BINDING PROTEINS GENE SEQUENCES OF REVERTANTS OF CS1/19.

4.1: INTRODUCTION.

Following a transient cell wall-deficient (CWD) phenotype, it was shown that the revertant RS1/19 strain has a stable, high-level penicillin resistance phenotype. In comparison to the wild-type *Staphylococcus aureus* Oxford strain ATCC 9144 the revertants were visually paler with smaller colonies, have a longer lag phase and slower mean generation time during growth in liquid media and have an altered matrix-assisted laser desorption/ionisation time-of-flight (MALDI-TOF) 'fingerprint'. Their mass of peptidoglycan was greater than the wild-type and they were more resistant to lysostaphin, an enzyme that acts on the cell wall. After treatment with lysostaphin the muropeptides of the revertants on reversed-phase high-performance liquid chromatography (RP-HPLC) analysis indicated that the peptidoglycan had shorter glycan chains, and after treatment with mutanolysin that it had a greater number of pentaglycine cross-bridges. This suggests a highly altered cell wall structure of the revertants in comparison to the wild-type strain. These altered characteristics may be due to penicillin-binding proteins (PBPs) with altered specificities, which in turn may lead to the penicillin resistance. As discussed previously penicillin resistance in *S. aureus* can be due to an altered target site due to a point mutation in the *pbp 2* gene (Hackbarth, Kocagoz et al. 1995). Also the up- or down-stream sequence of the coding strand may have regulatory properties that affect the expression of genes and a mutation in one of these areas may lead to a change, for example overproduction of PBP 4 due to mutations in the noncoding *pbp 4* gene promoter region (Henze and Berger-Bachi 1995). However in both of these cases the resistance seen was low level and therefore unlikely to explain the resistance seen in the revertant RS1/19 strain (Hackbarth, Kocagoz et al. 1995; Henze and Berger-Bachi 1996).

In the synthesis of peptidoglycan, the major component of the cell wall, there are several stages, the last being the cross-linking of the polysaccharide strands making up the peptidoglycan structure. Specific enzymes (transpeptidases and carboxypeptidases) are required to catalyze this final step. These proteins are situated in the cell membrane and are also called PBPs because they are bound by β-lactam antibiotics including penicillin (Navarre and Schneewind 1999). When bacteria are exposed to penicillin and the antibiotic binds to the PBPs in the cell membrane

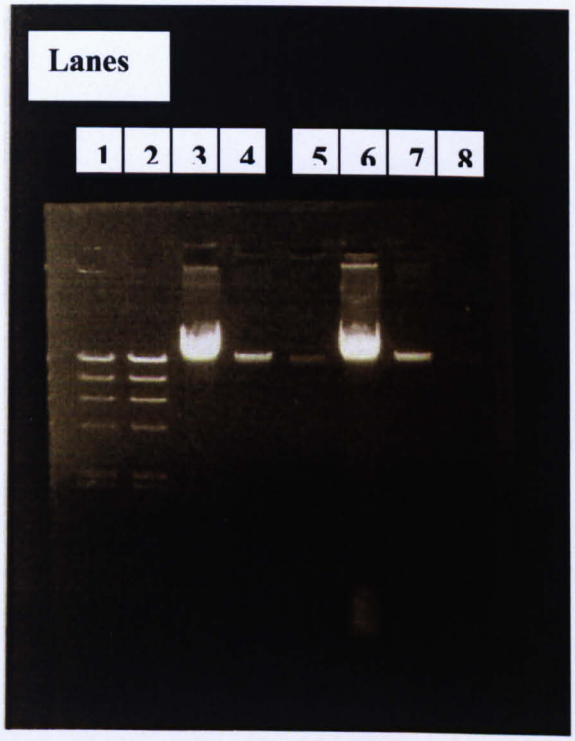
autolytic enzymes are released that degrade the preformed cell wall and cell wall synthesis is also inhibited resulting in bacterial cell death. In *S. aureus* there are four PBPs which are required in the assembly of cell wall peptidoglycan, named simply PBP 1, 2, 3 and 4. PBP 4 is the only non-essential PBP with the rest being essential for the growth of *S. aureus* (Curtis, Hayes et al. 1980). PBP 1 is the most important PBP in terms of survival when exposed to β-lactams (Giesbrecht, Kersten et al. 1998), whilst PBP 4 is a transpeptidase required for secondary cross-linking (Wyke, Ward et al. 1981). PBP 2 is the major peptidoglycan transpeptidase and PBP 3 is a septation-associated transpeptidase (Georgopapadakou, Dix et al. 1986). This was confirmed by Okonogi et al who used cloxacillin, ceftizoxime, cephalexin and cefoxitin to selectively inhibit PBP 1, 2, 3 and 4 respectively. They showed that PBP 1 and 3 play regulatory roles in septum formation, while PBP 2 is involved in peptidoglycan elongation and PBP 4 is involved in additional cross-linking (Okonogi, Noji et al. 1995).

The coding sequence and a portion of up- and down-stream sequence of the four PBPs from RS1/19 alongside those of the wild-type therefore were sequenced to check for any mutations that could explain the altered cell wall and penicillin resistance of RS1/19.

4.2: PREPARATION OF DEOXYRIBONUCLEIC ACID.

Total chromosomal deoxyribonucleic acid (DNA) of the wild-type *Staphylococcus aureus* Oxford ATCC 9144 and revertant RS1/19 strains were prepared using the method by Palomares et al (Palomares, Torres et al. 2003) with modifications. The prepared DNA was fractionated by agarose gel electrophoresis and the concentration estimated by comparison to DNA fragments of known mass (fig. 4.2). This analysis indicated that the concentration was approximately 120 mg ml^{-1} of preparation for both strains.

Figure 4.2: Mini DNA agarose gel showing the DNA preparations for wild-type Oxford and revertant RS1/19 strains.



Mini DNA agarose gel, with 10 µl wells, showing the DNA preparations for the wild-type Oxford and revertant RS1/19 strains. *Hind*III (lanes 1 and 2) was used as a marker at 50% and 100% concentrations, and is shown with the wild-type DNA at 100%, 10% and 1% concentrations (lanes 3,4 and 5) and the RS1/19 DNA at 100%, 10% and 1% concentrations (lanes 6,7 and 8). As the top band of the *Hind*III marker at 50% concentration (lane 1), which represents 120 ng is a similar intensity to the wild-type and RS1/19 DNA bands at 10% concentration (lanes 4 and 7), which is equivalent to 1 µl, 1 µl roughly equates to 120 ng.

4.3: POLYMERASE CHAIN REACTION OF PENICILLIN-BINDING PROTEINS 1, 2, 3 AND 4.

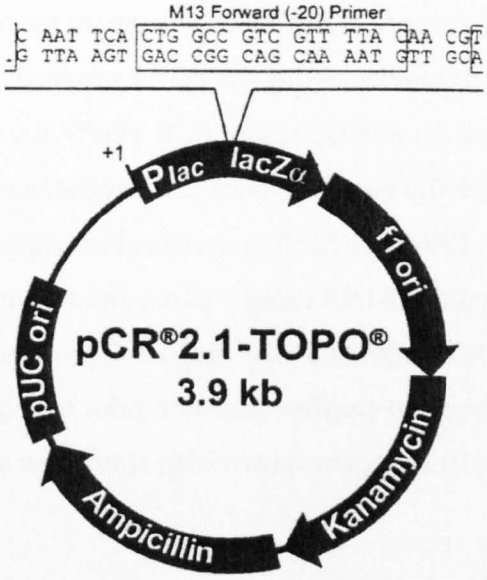
Establishing a protocol: initially to check that polymerase chain reaction (PCR) products were present, *Taq* (Bioline) a non-proof reading DNA polymerase was used. Non-proof reading enzymes give a higher yield but also have a higher error rate so were used to check for PCR products, but proof reading enzymes were then used as they give a lower error rate though a lower yield. A Robocycler screen was performed comparing different anneal temperatures, different $MgSO_4$ quantities and two proof reading enzymes, KOD (Novagen) and VENT (New England Biolabs). Little difference was seen between the anneal temperatures therefore the highest 50 °C was used, however KOD with the highest $MgSO_4$ quantity gave the best product and hence was used.

PCR reactions of the penicillin-binding proteins (PBP) 1, 2, 3 and 4 were carried out using KOD, a polymerase with a proof reading function. Primers for each PBP were designed by Elmer (Elmer, unpublished see appendix 3); however PBP3AS failed to produce any sequence and therefore was redesigned using a primer closer to the coding sequence. The primers used for each PBP are shown in figure 4.3.1. The PCR products were separated from the reaction mixture by QIA quick PCR purification kit (QIAGEN) and an A-tail added to the 3' strand using *Taq* polymerase, before being ligated into the vector pCR TOPO TA (Invitrogen) (see fig. 4.3.2). The ligation mixtures were then transformed into *Escherichia coli* XL1-Blue cells (Stratagene), and positive colonies selected on ampicillin-containing agar plates. DNA was purified from positive colonies using the Wizard Plus SV Miniprep DNA purification system (Promega) followed by restriction enzyme digest using *Eco*RI, GAATTC, to check for the presence and size of the products prior to sending the DNA for sequencing.

Figure 4.3.1: Sense and anti-sense primers used in the PCR of the PBPs.

PBP 1	5′-gatacgcgaggaaagattgc-3′	(sense)
	5′-tttacggcataagaggccag-3′	(anti-sense)
PBP 2	5′-tcgaagtattttggaagag-3′	(sense)
	5′-gtgaatgactgattttacg-3′	(anti-sense)
PBP 3	5′-gtatgattacttgttcggtctc-3′	(sense)
	5′-caaccatgcgctacacaatc-3′	(anti-sense)
PBP 4	5′-gagtaagtttgctcttcg-3′	(sense)
	5′-gtacagaaggcatttcgacg-3′	(anti-sense)

Figure 4.3.2: Map of pCR 2.1-TOPO (from TOPO TA Cloning, Invitrogen).

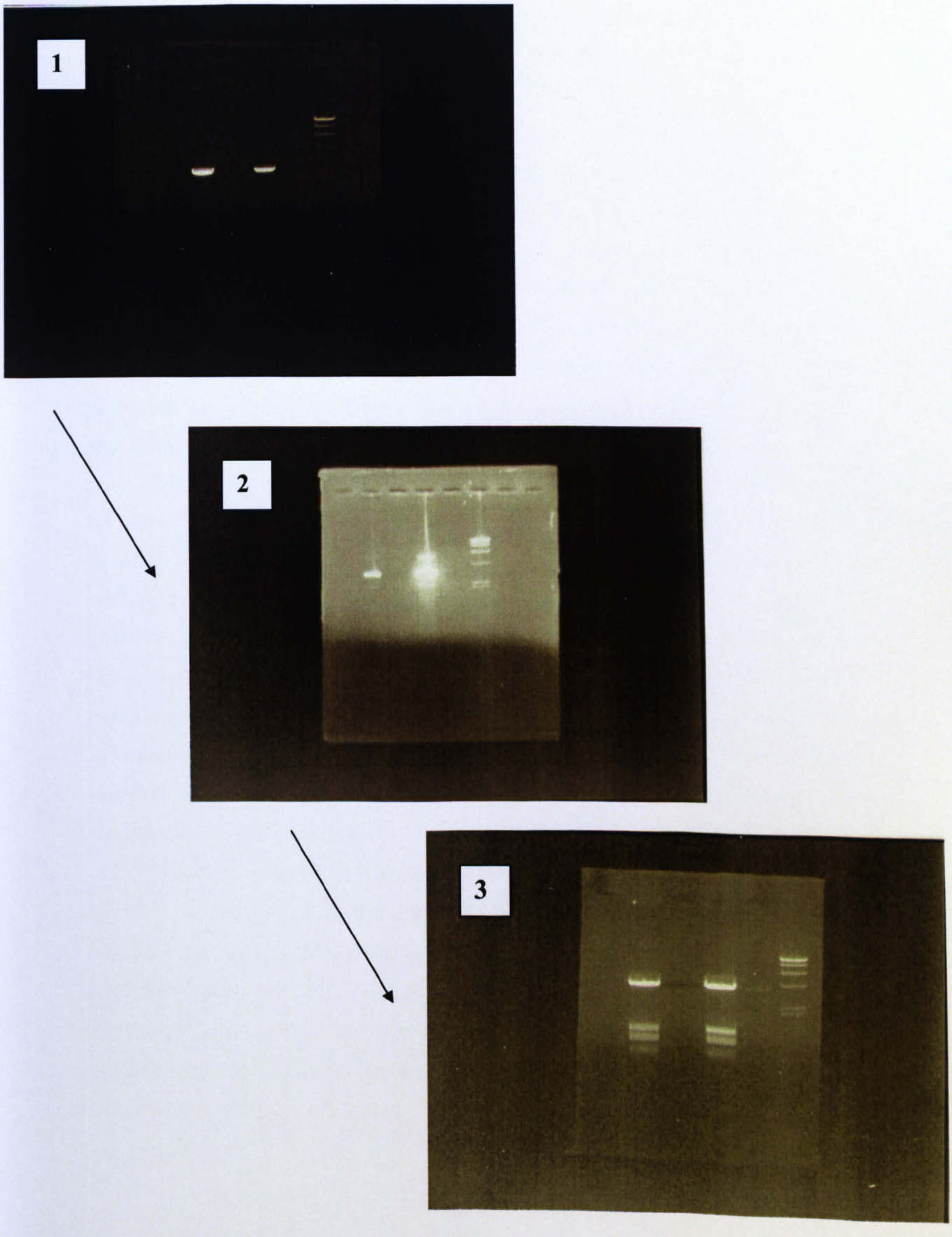


Map of pCR 2.1-TOPO (from TOPO TA Cloning, Invitrogen). The arrow marks the cloning site which is within the P*lac* lacZα fragment with *Eco*RI sites before and after where the PCR product is inserted.

Figure 4.3.3: Mini DNA agarose gels showing the process involved in preparing the gene sequences of penicillin-binding proteins (PBPs) of wild-type Oxford and revertant RS1/19 strains for sequencing, with PBP 2 used as an example.

1: minigel showing the cleaned PCR products following purification using the QIA quick PCR purification kit (QIAGEN). The wild-type (on the left) and the RS1/19 PBP 2 products are shown with *Hind*III as a marker (on the right). The top band of the *Hind*III marker is 120 ng, 5 µl of each product was ran on the gel so 1 µl represents around 60 ng of DNA. **2:** minigel showing the PBP 2 products following A-tailing using *Taq* polymerase, ligation into the vector pCR TOPO TA (Invitrogen), transformation into *Escherichia coli* XL1-Blue (Stratagene) and purification using the Wizard Plus SV Miniprep DNA purification system (Promega). The wild-type (on the left) using 1 µl of product and RS1/19 using 5 µl are shown with *Hind*III as a marker (on the right) using 5 µl. **3:** minigel showing the PBP 2 products following restriction enzyme digestion using *Eco*RI to confirm digestion prior to sequencing. The wild-type (on the left) and RS1/19 are again shown with *Hind*III as a marker (on the right).

Figure 4.3.3:



4.4: SEQUENCING OF PENICILLIN-BINDING PROTEINS 1, 2, 3 AND 4.

The penicillin-binding proteins (PBP) were sequenced initially with the sense and anti-sense primers used for the polymerase chain reaction (PCR) reactions (fig. 4.3.1). Hence the coding and anti-coding strands of the wild-type Oxford and revertant RS1/19 strains were sequenced, giving an internal control. Each sequencing reaction gave around 600 to 700 nucleotides of reliable sequence information. Further sense and anti-sense primers were designed from this initial sequence information to read the next part of the sequence. This process was repeated until all of the PCR product was sequenced on both the sense and antisense strands. Overlapping sequences were formed into contiguous sequences *in silico*. Figure 4.4.1 shows this process for the coding strand of PBP 1 of the wild-type strain. Figure 4.4.2 shows the complete sequence for the coding strand and anti-coding strand (after being reversed and complemented) of PBP 1 of the wild-type strain.

The sequences for the anti-coding strands were reversed and complemented and aligned (using the computer programme DNA Strider) to the coding strands for both the wild-type and the revertant strains to check the results. The coding strands of the wild-type and revertant strains were then aligned to each other to check for any mutations (using DNA Strider). For PBP 1 the revertant coding strand was identical to the wild-type coding strand except the 1681th nucleotide which read T not C, this change was also confirmed by the anti-coding strands, see figure 4.4.3. This point mutation was shown to be a conservative change with CAC of the wild-type strain and CAT of the revertant strain both reading for the amino acid histidine in the protein sequence, see figure 4.4.7.

The three other PBPs were sequenced in the same way and their sequences are shown in figures 4.4.4, 4.4.5 and 4.4.6. The nucleotide sequences were deposited in GenBank and have the following accession numbers: *pbp1*, AY920399; *pbp2*, AY920400; *pbp3*, AY920401 and *pbp4*, AY920402. These PBPs showed no sequence differences between the wild-type and revertant genes. Portions of the up- and down-stream regions of the coding strands of the four PBPs from RS1/19 alongside those of the wild-type were also sequenced to check for any mutations and again no mutations were found.

Figure 4.4.1: Sequencing process of the coding strand of PBP 1 of wild-type Oxford strain showing the coding sequence in capitals and the oligonucleotides used as primers shown in blue. The beginning and the end of the amplified sequence is marked by *Eco*RI sites shown in red. The initial sequence obtained is overlapped with the second sequence obtained from designing the primer shown in bold towards the end of the first sequence. The portion of the first sequence towards the end underlined was then removed and the sequences joined together, and this process repeated until the whole of the penicillin-binding protein had been sequenced. The following four primers were required to complete this process: 5'-aaggcgagtgccaattc-3', 5'-aaagtgggcaaatgacc-3', 5'-ttatgggtgacgcgccgaag-3' and 5'-gtgtcccaccaatcaattag-3'.

gaattcgcccttg**atacgcgaggaaagattgc**agatttagattataaaatagataaa
caatcaagtgaaaacagtgctttacaatctgaaatcaaaaagaattcttcttatgaa
cgcatatacgaaaaggctaagaaacaggggatgagccttgagaacgataatgtaaag
gtagtgcgtagtaATGGCGAAGCAAAAAATTAAAATTAAAAAAAATAAAATAGGGGC
AGTCCTACTTGTTGGTTTATTCGGACTGCTCTTTTTTATATTGGTTTTAAGAATTTC
ATATATCATGATTACTGGACATTCTAATGGTCAAGATTTAGTCATGAAGGCAAATGA
AAAGTATTTAGTTAAGAATGCACAACAACCAGAACGAGGAAAGATATATGATCGTAA
TGGTAAAGTGCTAGCAGAAGATGTAGAAAGATATAAACTTGTTGCAGTAATAGATAA
A**AAGGCGAGTGCCAATTC**TAAAAAACCTAGGCATGTAGTTGATAAAAAAGAGACTGC
*AAGAAATTATCTACAGTCATTAATA

ACTGCAAAGAAATTATCTACAGTCATTAATATGAAGCCAGAGGGAAA
TTGAAAAGAGACTTAGTCAAAAGAAAGCTTTCCAATGAAGCCAGAGGAAATTGAAAA
GAGACTTAGTCAAAAGAAAGCTTTCCAAAATTGAATTTGGGACGCTTGAATTTGGAC
GCAAAGGAACAAATTTAACGTATCAGGACAAATTGAAAATAGAGAAAATGAATTTGC
CTGGTATTTCTTTATTGCCTGAAACAGAACGCTTTTATCCAAATGGCAATTTTGCAT
CACACTTAATTGGTAGAGCTCAGAAAAATCCGGATACTGGTGAACTTAAAGGTGCAC
TTGGAGTTGAAAAGATTTTTGATAGTTATTTAAGTGGATCTAAAGGATCATTGAGAT
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AACGTGGTGATGATGTCCATTTAACAATCGATTCAAATATTCAAGTATTTGTTGAAG
AAGCTTTAGATGGCATGGTTGAAAGATACCAGCCGAAAGATTTATTTGCGGTTGTCA
TGGATGCCAAAACTGGAGAAATTTTAGCATACAGTCAGCGACCAACATTTAATCCTG

AAACTGGTAAAGACTTTGGTAA**AAAGTGGGCAAATGACC**TTTATCAAAACACATACG

AGCCTGGATCAACATTTAAATCATATGGG

AAATCATATGGGTTAGCAGCTGCTATTCAAGAAGGTGCTTTTGATCCTGATA

AGAAATATAAATCTGGACATAGAGATATTATGGGTTCACGTATTTCAGACTGGAATA

GAGTCGGTTGGGGTGAAATCCCAATGTCACTCGGATTTACTTATTCATCTAATACAT

TGATGATGCATTTACAAGATTTAGTTGGTGCAGACAAAATGAAATCTTGGTATGAAC

GATTTGGATTTGGAAAATCAACTAAAGGTATGTTTGATGGAGAAGCACCTGGTCAAA

TTGGATGGAGTAATGAGTTGCAACAAAAAACGTCATCATTTGGTCAATCGACAACAG

TAACACCTGTTCAAATGTTACAAGCGCAATCAGCGTTCTTTAATGATGGTAATATGT

TAAAACCATGGTTTGTGAATAGCGTTGAAAATCCTGTTAGTAAAAGACAATTTTATA

AAGGGCAAAAACAAATCGCAGGCAAACCAATAACAAAAGATACTGCTGAAAAAGTTG

AAAAGCAATTGGATTTAGTTGTGAATAGTAAGAAGAGTCACGCTGCAAACTATCGTA

TTGATGGTTATGAGGTCGAAGGTAAGACTGGTACAGCACAAGTCGCTGCACCTAATG

GTGGTGGATACGTTAAAGGTCCAAACCCATATTTTGTAAGTT**TTATGGGTGACGCGC**

CGAAGAAAAATCCTAAAGTTATTGTATACGCTGGTATGAGCTTGGCACAAAAAAATG

ACCAAGAAGCTTATGAATTAGGTGTTAGTAAAGCG

GAGCTTGGCACAAAAAAATGACCAAGAAGCTTATGAATTAGGTGTTAGTAAA

GCGTTTAAACCAATAATGGAAAATACTTTGAAATATTTAAATGTAGGTAAATCAAAA

GATGACACATCTAATGCAGAGTATAGTAAAGTGCCAGATGTTGAAGGTCAAGACAAA

CAAAAAGCTATTGATAATGTGAGTGCAAAATCATTAGAACCAGTTACTATTGGTTCT

GGCACACAAATAAAAGCACAATCTATAAAAGCAGGGAATAAAGTCTTACCTCATAGT

AAAGTACTGTTATTAACAGATGGAGACTTAACTATGCCTGACATGTCAGGATGGACG

AAAGAAGATGTCATTGCTTTTGAAAACCTAACAAATATTAAAGTAAATTTAAAAGGT

AGCGGTTTT**GTGTCCCACCAATCAATTAG**TAAGGGACAAAAACTTACTGAAAAAGAT

AAAATAGACGTAGAATTTTCATCAGAGAATGTAGACAGCAATTCGACGAATAATTCT

GATTCAAATTC

GACGTAGAATTTTCATCAGAGAATGTAGACAGCAATTCGACGAATAATTCTGATTCA

AATTCAGATGATAAGAAGAAATCTGACAGTAAAACTGACAAGGATAAGTCGGACTAA

caagaatggctacattaaattaggatgtattgtcactatttgtttttactaaagtaa

tgctgtgatagaaaacaacatcttcatttaatctttataaataagtgttgatagaga

attttaaggtagacgaggtcagattaacttgtttgttataggcaaacttttta**ct**

ggcctcttatgccgtaaaaagggc**gaattc**

Figure 4.4.2: Complete DNA sequence of the coding strand and anti-coding strand (after being reversed and complemented) of PBP 1 of wild-type Oxford strain showing the coding sequence in capitals and the oligonucleotides used as primers for the coding strand shown in blue and those for the anti-coding strand in red. The beginning and the end of the sequence are marked by *Eco*RI sites in bold along with the sense and anti-sense primers used in the PCR reaction and initial sequencing. The point mutation is also shown in red. GenBank accession number for the open reading frame is AY920399.

Gaattcgcccttgatacgcgaggaaagattgcagatttagattataaaatagataaacaatcaagtgaaaacagtgctttacaatctgaaatcaaaaaga**100**
attcttcttatgaacgcatatacgaaaaggctaagaaacaggggatgagccttgagaacgataatgtaaaggtagtgcgtagtaATGGCGAAGCAAAAAA**200**
TTAAAATTAAAAAAAATAAAATAGGGGCAGTCCTACTTGTTGGTTTATTCGGACTGCTCTTTTTTATATTGGTTTTAAGAATTTCATATATCATGATTAC**300**
TGGACATTCTAATGGTCAAGATTTAGTCATGAAGGCAAATGAAAAGTATTTAGTTAA**GAATGCACAACAACCAGAAC**GAGGAAAGATATATGATCGTAAT**400**
GGTAAAGTGCTAGCAGAAGATGTAGAAAGATATAAACTTGTTGCAGTAATAGATAA**AAAGGCGAGTGCCAATTC**TAAAAAACCTAGGCATGTAGTTGATA**500**
AAAAAGAGACTGCAAAGAAATTATCTACAGTCATTAATATGAAGCCAGAGGAAATTGAAAAGAGACTTAGTCAAAAGAAAGCTTTCCAAATTGAATTTGG**600**
ACGCAAAGGAACAAATTTAACGTATCAGGACAAATTGAAAATAGAGAAAATGAATTTGCCTGGTATTTCTTTATTGCCTGAAACAGAACGCTTTTATCCA**700**
AATGGCAATTTTGCATCACACTTAATTGGTAGAGCTCAGAAAAATCCGGATACTGGTGAACTTAAAGGTGCACTTGGAGTTGAAAAGATTTTTGATAGTT**800**
ATTTAAGTGGATCTAAAGGATCATTGAGATATATTCATGATATTTGGGGATATATCGCACCAAATACTAAAAAAGAGAAGCAGCCTAAA**CGTGGTGATGA**900
TGTCCATTTAACAATCGATTCAAATATTCAAGTATTTGTTGAAGAAGCTTTAGATGGCATGGTTGAAAGATACCAGCCGAAAGATTTATTTGCGGTTGTC**1000**
ATGGATGCCAAAACTGGAGAAATTTTAGCATACAGTCAGCGACCAACATTTAATCCTGAAACTGGTAAAGACTTTGGTAA**AAAGTGGGCAAATGACC**TTT**1100**
ATCAAAACACATACGAGCCTGGATCAACATTTAAATCATATGGGTTAGCAGCTGCTATTCAAGAAGGTGCTTTTGATCCTGATAAGAAATATAAATCTGG**1200**
ACATAGAGATATTATGGGTTCACGTATTTCAGACTGGAATAGAGTCGGTTGGGGTGAAATCCCAATGTCACTCGGATTTACTTATTCATCTAATACATTG**1300**
ATGATGCATTTACAAGATTTAGTTGGTGCAGACAAAATGAAATCTTGGTATGAACGATTTGGATTTGGAAAATCAACTAAAGGTATGTTTGATGGAGAAG**1400**

CACCTGGTCAAATTGGATGGAGTAATGAGTTGCAACAAAAAACGTCATCATTTGGTCAATCGACAACAGTAACACCTGTTCAAATGTTACAAGCGCAATC1500

AGCGTTCTTTAATGATGGTAATATGTTAAAACCATGGTTTGTGAATAGCGTTGAAAATCCTGTTAGTAAAAGACAATTTTATAAAGGGCAAAAACAAATC1600

GCAGGCAAACCAATAACAAAAGATACTGCTGAAAAAGTTGAAAAGCAATTGGATTTAGTTGTGAATAGTAAGAAGAGTCACGCTGCAAACTATCGTATTG1700

ATGGTTATGAGGTCGAAGGTAAGACTGGTACAGCACAAGTCGCTGCACCTAATGGTGGTGGATACGTTAAAGGTCCAAACCCATATTTTGTAAGTTTTAT1800

GGGTGACGCGCCGAAGAAAAATCCTAAAGTTATTGTATACGCTGGTATGAGCTTGGCACAAAAAAATGACCAAGAAGCTTATGAATTAGGTGTTAGTAAA1900

GCGTTTAAACCAATAATGGAAAATACTTTGAAATATTTAAATGTAGGTAAATCAAAAGATGACACATCTAATGCAGAGTATAGTAAAGTGCCAGATGTTG2000

AAGGTCAAGACAAACAAAAAGCTATTGATAATGTGAGTGCAAAATCATTAGAACCAGTTACTATTGGTTCTGGCACACAAATAAAAGCACAATCTATAAA2100

AGCAGGGAATAAAGTCTTACCTCATAGTAAAGTACTGTTATTAACAGATGGAGACTTAACTATGCCTGACATGTCAGGATGGACGAAAGAAGATGTCATT2200

GCTTTTGAAAACCTAACAAATATTAAAGTAAATTTAAAAGGTAGCGGTTTTGTGTCCCACCAATCAATTAGTAAGGGACAAAAACTTACTGAAAAAGATA2300

AAATAGACGTAGAATTTTCATCAGAGAATGTAGACAGCAATTCGACGAATAATTCTGATTCAAATTCAGATGATAAGAAGAAATCTGACAGTAAAACTGA2400

CAAGGATAAGTCGGACTAAcaagaatggctacattaaattaggatgtattgtcactatttgtttttactaaagtaatgctgtgatagaaaacaacatctt2500

Catttaatctttataaataagtgttgatagagaattttaaggtagacgaggtcagattaacttgtttgttataggcaaacttttttatactggcctcttat2600

Gccgtaaaaagggcgaa

Figure 4.4.3: Complete DNA sequence of the coding strand and anti-coding strand (after being reversed and complemented) of PBP 1 of revertant RS1/19 strain showing the coding sequence in capitals. The beginning and the end of the sequence are marked by *Eco*RI sites shown in red and the point mutation is also shown in red.

gaattcgcccttgatacgcgaggaaagattgcagatttagattataaaatagataaacaatcaagtgaaaacagtgctttacaatctgaaatcaaaaaga**100**
attcttcttatgaacgcatatacgaaaaggctaagaaacaggggatgagccttgagaacgataatgtaaaggtagtgcgtagtaATGGCGAAGCAAAAAA**200**
TTAAAATTAAAAAAAATAAAATAGGGGCAGTCCTACTTGTTGGTTTATTCGGACTGCTCTTTTTTATATTGGTTTTAAGAATTTCATATATCATGATTAC**300**
TGGACATTCTAATGGTCAAGATTTAGTCATGAAGGCAAATGAAAAGTATTTAGTTAAGAATGCACAACAACCAGAACGAGGAAAGATATATGATCGTAAT**400**
GGTAAAGTGCTAGCAGAAGATGTAGAAAGATATAAACTTGTTGCAGTAATAGATAAAAAGGCGAGTGCCAATTCTAAAAAACCTAGGCATGTAGTTGATA**500**
AAAAAGAGACTGCAAAGAAATTATCTACAGTCATTAATATGAAGCCAGAGGAAATTGAAAAGAGACTTAGTCAAAAGAAAGCTTTCCAAATTGAATTTGG**600**
ACGCAAAGGAACAAATTTAACGTATCAGGACAAATTGAAAATAGAGAAAATGAATTTGCCTGGTATTTCTTTATTGCCTGAAACAGAACGCTTTTATCCA**700**
AATGGCAATTTTGCATCACACTTAATTGGTAGAGCTCAGAAAAATCCGGATACTGGTGAACTTAAAGGTGCACTTGGAGTTGAAAAGATTTTTGATAGTT**800**
ATTTAAGTGGATCTAAAGGATCATTGAGATATATTCATGATATTTGGGGATATATCGCACCAAATACTAAAAAAGAGAAGCAGCCTAAACGTGGTGATGA**900**
TGTCCATTTAACAATCGATTCAAATATTCAAGTATTTGTTGAAGAAGCTTTAGATGGCATGGTTGAAAGATACCAGCCGAAAGATTTATTTGCGGTTGTC**1000**
ATGGATGCCAAAACTGGAGAAATTTTAGCATACAGTCAGCGACCAACATTTAATCCTGAAACTGGTAAAGACTTTGGTAAAAAGTGGGCAAATGACCTTT**1100**
ATCAAAACACATACGAGCCTGGATCAACATTTAAATCATATGGGTTAGCAGCTGCTATTCAAGAAGGTGCTTTTGATCCTGATAAGAAATATAAATCTGG**1200**
ACATAGAGATATTATGGGTTCACGTATTTCAGACTGGAATAGAGTCGGTTGGGGTGAAATCCCAATGTCACTCGGATTTACTTATTCATCTAATACATTG**1300**
ATGATGCATTTACAAGATTTAGTTGGTGCAGACAAAATGAAATCTTGGTATGAACGATTTGGATTTGGAAAATCAACTAAAGGTATGTTTGATGGAGAAG**1400**
CACCTGGTCAAATTGGATGGAGTAATGAGTTGCAACAAAAAACGTCATCATTTGGTCAATCGACAACAGTAACACCTGTTCAAATGTTACAAGCGCAATC**1500**
AGCGTTCTTTAATGATGGTAATATGTTAAAACCATGGTTTGTGAATAGCGTTGAAAATCCTGTTAGTAAAAGACAATTTTATAAAGGGCAAAAACAAATC**1600**
GCAGGCAAACCAATAACAAAAGATACTGCTGAAAAAGTTGAAAAGCAATTGGATTTAGTTGTGAATAGTAAGAAGAGTCA**T**GCTGCAAACTATCGTATTG**1700**

ATGGTTATGAGGTCGAAGGTAAGACTGGTACAGCACAAGTCGCTGCACCTAATGGTGGTGGATACGTTAAAGGTCCAAACCCATATTTTGTAAGTTTTAT**1800**

GGGTGACGCGCCGAAGAAAAATCCTAAAGTTATTGTATACGCTGGTATGAGCTTGGCACAAAAAAATGACCAAGAAGCTTATGAATTAGGTGTTAGTAAA**1900**

GCGTTTAAACCAATAATGGAAAATACTTTGAAATATTTAAATGTAGGTAAATCAAAAGATGACACATCTAATGCAGAGTATAGTAAAGTGCCAGATGTTG**2000**

AAGGTCAAGACAAACAAAAAGCTATTGATAATGTGAGTGCAAAATCATTAGAACCAGTTACTATTGGTTCTGGCACACAAATAAAAGCACAATCTATAAA**2100**

AGCAGGGAATAAAGTCTTACCTCATAGTAAAGTACTGTTATTAACAGATGGAGACTTAACTATGCCTGACATGTCAGGATGGACGAAAGAAGATGTCATT**2200**

GCTTTTGAAAACCTAACAAATATTAAAGTAAATTTAAAAGGTAGCGGTTTTGTGTCCCACCAATCAATTAGTAAGGGACAAAAACTTACTGAAAAAGATA**2300**

AAATAGACGTAGAATTTTCATCAGAGAATGTAGACAGCAATTCGACGAATAATTCTGATTCAAATTCAGATGATAAGAAGAAATCTGACAGTAAAACTGA**2400**

CAAGGATAAGTCGGACTAAcaagaatggctacattaaattaggatgtattgtcactatttgtttttactaaagtaatgctgtgatagaaaacaacatctt**2500**

Catttaatctttataaataagtgttgatagagaattttaaggtagacgaggtcagattaacttgtttgttataggcaaacttttatactggcctcttat**2600**

gccgtaaaaagggc**gaattc**

Figure 4.4.4: Complete DNA sequence of the coding strand and anti-coding strand (after being reversed and complemented) of PBP 2 of wild-type Oxford and revertant RS1/19 strains showing the coding sequence in capitals and the oligonucleotides used as primers for the coding strand shown in blue and those for the anti-coding strand in red, apart from one that is in blue and underlined as it was used both for the coding and anti-coding strand. The beginning and the end of the sequence are marked by *Eco*RI sites in bold along with the sense and anti-sense primers used in the PCR reaction and initial sequencing. GenBank accession number for the open reading frame is AY920400.

gaattcgcccttt**cgaagtattttggaagag**atataaagataatattaaaaagtctataacagttgatgaaatacgaaaaaatggttaccatattcctta**100**
tcagtatcaaccaagattagactatctaaaagcagttgataagttgatattagatgaaagtgaggaccgcgtATGACGGAAAACAAAGGATCTTCTCAGC**200**
CTAAGAAAAACGGTAATAATGGTGGGAAATCCAACTCAAAAAAGAATAGAAATGTGAAGAGAACGATTATTAAGATTATTGGCTTCATGATTATTGCATT**300**
TTTTGTTGTTCTTTTACTAGGTATCTTATTGTTTGCTTATTATGCTTGGAAAGCACCTGCTTTTACCGAAGCTAAATTACAAGATCCGATTCCTGCAAAG**400**
ATATATGACAAGAACGGAGAACTTGTTAAAACATTAGATAATGGCCAAAGACATGAGCATGTAAATTTAAAAGACGTGCCGAAATCAATGAAAGACGCAG**500**
TACTTGCAACTGAAGACAATCGTTTCTACGAACAT**GGCGCACTTGATTATAAACG**TTTATTCGGTGCAATTGGTAAGAACTTGACTGGTGGATTTGGTTC**600**
TGAAGGTGCCTCAACATTAACACAACAAGTTGTTAAAGATGCATTTTTATCACAACATAAATCTATT**GGACGTAAAGCTCAAGAAG**CATACTTATCATAT**700**
CGTTTAGAACAAGAGTATAGTAAAGATGATATCTTCCAAGTATATCTAAACAAAATTTACTATTCTGATGGCGTAACAGGTATTAAAGCTGCTGCTAAGT**800**
ATTACTTTAATAAAGATTTAAAAGATTTAAACTTAGCGGAAGAAGCTTATTTAGCCGGTTTACCTCAGGTTCCAAACAACTATAATATTTATGATCATCC**900**
AAAAGCTGCTGAAGATCGTAAAAACACTGTTTTATACTTAATGCATTATCATAAACGCATTACAGATAAACAGTGGGAAGATGCTAAGAAAATCGATTTA**1000**
AAAGCGAACTTAGTAAATCGTACTGCTGAAGAACGTCAAAACATTGATACAAATCAAGATTCTGAGTATAATTCATACGTTAACTTTGTAAAATCTGAAT**1100**
TAATGAATAATAAAGAATTCAAAGATGAAAATTTAGGTAATGTATTACAAAGTGGTATTAAAATTTATACAAACATGGATAAAGATGTTCAAAAAACATT**1200**
ACAAAATGATGTTGATAATGGTAGCTTCTACAAGAATAAA**GACCAACAAGTTGGTGC**AACGATTCTTGATAGTAAAACTGGTGGTTTAGTTGCTATATCT**1300**

GGTGGACGTGATTTCAAAGACGTCGTTAACAGAAACCAAGCAACAGATCCTCACCCTACTGGTTCATCTTTAAAACCTTTCTTAGCGTATGGACCTGCCA1400

TTGAAAATATGAAATGGGCAACAAACCATGCGATTCAAGATGAATCTTCATATCAAGTTGATGGTTCTACATTTAGAAACTATGATACGAAGAGTCACGG1500

TACTGTATCTATTTATGATGCTTTACGACAAAGTTTCAATATCCCAGCTTTAAAAGCATGGCAATCAGTTAAGCAAAATGCTGGTAACGATGCACCTAAG1600

AAATTCGCTGCCAAACTTGGCTTAAACTACGAAGGCGATATTGGTCCATCTGAAGTACTTGGTGGTTCTGCTTCAGAATTCTCACCAACACAATTAGCAT1700

CAGCATTTGCTGCAATCGCTAACGGTGGTACTTATAACAACGCGCATTCAATTCAAAAAGTAGTTACTCGTGACGGTGAAACAATCGAATACGATCATAC1800

TAGCCATAAAGCGATGAGTGATTACACTGCATACATGTTAGCTGAGATGCTAAAAGGTACATTTAAACCATATGGTTCTGCATATGGCCATGGTGTAGCT1900

GGAGTAAATATGGGTGCTAAGACAGGTACTGGTACTTACGGTGCTGAAACTTATTCACAATATAATTTACCTGATAATGCAGCGAAAGACGTGTGGATTA2000

ACGGCTTTACACCTCAATACACTATGTCAGTGTGGATGGGCTTCAGTAAAGTTAAACAATATGGTGAAAACTCATTTGTGGGACATAGCCAACAAGAATA2100

TCCACAGTTCTTATATGAAAATGTGATGTCAAAAATTTCATCTAGAGATGGCGAAGACTTTAAACGTCCTAGCTCAGTAAGTGGTAGTATCCCATCAATC2200

AATGTTTCTGGTAGTCAAGATAACAACACTACAAATCGTAGTACACACGGTGGTAGTGACACATCAGCAAACAGCAGTGGTACTGCACAATCAAATAACA2300

ATACTAGATCTCAACAATCTAGAAACAGCGGTGGATTAACAGGTATATTCAACTAAtccacacaacataaaatcctcagttataccatatttatggcgta2400

Gctgaggatttttaggttcttcatcttttatggtgggaaggtaaaacttcctgctttttttataagtattcatacgctattgctatattagtagaaacc2500

gtaaaatcagtcattcacaagggcgaattc

Figure 4.4.5: Complete DNA sequence of the coding strand and anti-coding strand (after being reversed and complemented) of PBP 3 of wild-type Oxford and revertant RS1/19 strains showing the coding sequence in capitals and the oligonucleotides used as primers for the coding strand shown in blue and those for the anti-coding strand in red. GenBank accession number for the open reading frame is AY920401.

gatagaaataatttctaaattcatatatgatttaaagtgaaagactttgaatagaggtaggtagttTTGTTAAAAAGACTAAAAGAAAAATCAAATGATG**100**
AAATCGTTCAAAATACAATTAACAAGAGAATTAACTTTATATTTGGTGTGATTGTATTTATTTTTGCAGTACTAGTACTACGTTTAGGTTATTTACAAAT**200**
CGCACAAGGCTCACATTATAAACAAATTATAAAAAATGATGAAAACATTACAGTGAATGAGTCTGTGCCAAGAGGTCGTATTTTAGACAGAAATGGGAAA**300**
GTTTTAGTTGATAATGCTTCTAAAATGGCTATTACATATACTAGGGGTCGAAAAACAACACAATCGGAAATGTTGGATACGGCTGAAAAGTTATCAAAGC**400**
TAATCAAGATGGATACTAAGAAAATTACAGAACGTGATAAGAAAGATTT**CTGGATTCAGTTGCATCC**TAAAAAAGCAAAAGCAATGATGACAAAAGAACA**500**
AGCTATGTTAGCAGATGGAAGTATTAAACAAGATCAATATGATAAACAACTGTTATCGAAAATCGGAAAATCACAATTAGATGAATTGTCTTCTAAAGAT**600**
TTACAAGTTTTAGCTATTTTTCGAGAGATGAATGCAGGAACAGTTTTAGATCCACAAATGATAAAAAATGAAGATGTCAGTGAAAAAGAGTATGCAGCAG**700**
TTTCTCAGCAACTTTCCAAATTACCAGGTGTTAACACGTCTATGGATTGGGATAGAAAATATCCATATGGCGATACTTTAAGAGGTATATTCGGAGATGT**800**
ATCGACACCTGCTGAAGGTATTCCAAAAGAATTGACAGAACATTACTTAT**CCAAAGGATATTCACGC**AATGATCGTGTTGGAAAATCTTACCTAGAATAT**900**
CAATATGAAGATGTATTGCGTGGTAAGAAGAAAGAAATGAAATACACAACGGACAAATCTGGTAAAGTTACATCTTCAGAAGTGTTAAATCCTGGCGCTC**1000**
GCGGTCAAGATTTGAAATTAA**CGATCGATATAGATCTTC**AAAAAGAAGTAGAAGCATTATTAGATAAACAAATTAAGAAGCTTCGCAGTCAAGGTGCCAA**1100**
AGATATGGATAATGCAATGATGGTTGTACAAAATCCTAAAAATGGAGACATTCTTGCGCTTGCCGGAAAGCAGATTAATAAGAGTGGTAAAATGACTGAT**1200**
TATGACATTGGTACGTTTACTTCTCAATTTGCGGTTGGATCTTCTGTAAAAGGTGGAACATTATTAGCTGGTTATCAGAATAAAGCTATCAAAGTTGGAG**1300**
AAACAATGGTCGATGAACCATTACATTTCCAAGGTGGTTTGACAAAACGATCATACTTCAATAAAAACGGGCATGTAACTATTAATGATAAGCAAGCTTT**1400**
GATGCGTTCATCAAACGTATATATGTTTAAAACAGCATTAAAATTAGCGGGAGACCCTTATTATTCTGGTATGGCTTTACCTTCAGACATAA**GTTCACCT**1500
GCCCAAAAGCTAAGAAGAGGATTAAATCAAGTAGGCTTAGGTGTGAAAACAGGGATAGATTTACCAAATGAAACAAGAGGTCAAATCGAACCATTAACAA**1600**
ATAATCCAGGTAATTATCTAGATTTATCAATTGGTCAATAT**GATACCTATACACCATTAC**AATTATCACAATATGTTTCAACTATAGCGAATGATGGTTA**1700**

TAGAATACAGCCACACATTGGATTAACGATTCATGAATCAACTAATAAAGATGAGGTTGGTCCACTCAAGAAGAAAATTAATGGCACTGTCTTGAACAAG**1800**

GTTAATAATACTGAAAAGGAAATCAAACAAATTCAAGAAGGATTCAAAATGGCATTTAATGATAAAGATGGTACTGGATATGTTAGTTTTAAAGATACAG**1900**

TAGTACCTACTGCTGGTAAAACGGGTACCGCTGAAGTGTTCCAAAACGGAGAGCCAAGAGTTAACTCTACT TATATAGGATACGCGCCAATTGATGATC**2000**

CAAAATTAGCGTTTTCAATTGTATATACAAATCAGCCTGTACCACCACCATGGTTAACAGGTGGAGACTTAGGTAGAGATGTAATTAACTACTACTTTAA**2100**

GCAGTTAGGTAAAGATGATAAAAATAAAGACAAAGACAAATAAaatttaacctgacg

Figure 4.4.6: Complete DNA sequence of the coding strand and anti-coding strand (after being reversed and complemented) of PBP 4 of wild-type Oxford and revertant RS1/19 strains showing the coding sequence in capitals and the oligonucleotides used as primers for the coding strand shown in blue and those for the anti-coding strand in red. The beginning and the end of the sequence are marked by *Eco*RI sites in bold along with the sense and anti-sense primers used in the PCR reaction and initial sequencing. GenBank accession number for the open reading frame is AY920402.

gaattcgcccttgagtaagtttgctcttcgtacaaacattataacacctttagctacacacgtaatatatttgcattatttttcaaattaactgcagaaa**100**
actttattttcaacaatttaattaaggtgaagtgaattagcaatgatttccgtaagtaggaatgaccattgcttttagggaatatttaagaattggaaaa**200**
gggaagattaacgctttATGAAAAATTTAATATCTATTATCATCATTTTATTTTTAACATTAAGTATTATGACACCATATGCACAAGCTGCTAACAGTGA**300**
CGTAACCCCTGTACAAGCAGCAAATCAATATGGTTATGCAGGTTTGTCGGCTGCATACGAACCGACGAGTGCTGTTAATGTAAGTCAAACTGGACAATTA**400**
CTGTATCAATACAATATCGATACTAAGTGGAATCCAGCGTCTATGACTAAATTAATGACAATGTACTTAACATTGGAAGCTGTAAATAAGGGGCAGCTTT**500**
CACTTGATGACACTGTCAGAATGACGAACAAAGAATATATTATGTCTACACTACCTGAGTTGAGTAATACGAAACTATATCCTGGACAAGTATGGACAAT**600**
CGCAGACCTATTACAAATTACAGTATCTAATTCTAGTAATGCCGCGGCATTAATTTTAGCTAAGAAGGTATCAAAAAACACGAGCGATTTCGTTGATTTA**700**
ATGAATAACAAAGCTAAAGCTATCGGAATGAAAAATACACATTTCGTCAATCCAACGGGTGCTGAAAATTCAAGATTACGTACATTTGCACCAACAAAGT**800**
ATAAAGACCAAGAACGTACTGTAACGACTGCTAGAGACTATGCCATTTTAGATTTACGCGTGATTAAAGAGACACCTAAAATATTAGACTTTACAAAGCA**900**
GTTAGCACCAACAACGCATGCAGTTACGTATTACACATTCAACTTTTCATTGGAAGGTGCAAAAATGAGTTTGCCGGGTACAGATGGTTTAAAAACTGGA**1000**
TCAAGTGATACAGCAAATTACAACCATACGATTACTACCAAACGAGGTAAATTTAGAATTAATCAAGTTATCATGGGTGCAGGAGACTATAAAAACCTTG**1100**
GCGAGAAGCAACGTAATATGATGGGGAATGCATTAATGGAACGTTCATTTGATCAGTATAAATATGTAAAAATATTGTCTAAAGGTGAGCAAAGGATAAA**1200**
TGGTAAGAAATATTATGTTGAAAATGATCTTTACGATGTTTTACCAAGTGATTTTAGTAAAAAAGATTATAAACTTGTAGTCGAAGATGGTAAAGTACAC**1300**
GCGGACTATCCAAGAGAATTTATTAATAAAGATTATGGACCTCCAACTGTAGAAGTTCATCAGCCAATTATTCAAAAGGCAAATACTGTTGCTAAAAGTA**1400**

TGTGGGCAGAACATCCATTATTCACTATCATTGGTGGTGCATGCCTTGTCGCTGGATTAGCACTTATTGTTCATATGATAATCAATCGTTTATTTAGAAA**1500**

AAGAAAATAAaacatactaaaaacggacaagttgcacattataaagctgcgaaacttgtccgttttatatttatttttataataacctacattaatttatc**1600**

gtttagtatcgatagttctatataatttatttagcatctttaccatataattcttgtttgattttagt**cgtcgaaatgccttctgtac**aagggc**gaattc1700**

Figure 4.4.7: Protein sequence of PBP 1 of revertant RS1/19 strain. The point mutation is shown in red, but is conservative leading to the same protein sequence as the wild-type.

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ATG GCG AAG CAA AAA ATT AAA ATT AAA AAA AAT AAA ATA GGG GCA GTC CTA CTT GTT GGT TTA TTC GGA CTG CTC TTT
M   A   K   Q   K   I   K   I   K   K   N   K   I   G   A   V   L   L   V   G   L   F   G   L   L   F
TTT ATA TTG GTT TTA AGA ATT TCA TAT ATC ATG ATT ACT GGA CAT TCT AAT GGT CAA GAT TTA GTC ATG AAG GCA AAT
F   I   L   V   L   R   I   S   Y   I   M   I   T   G   H   S   N   G   Q   D   L   V   M   K   A   N
GAA AAG TAT TTA GTT AAG AAT GCA CAA CAA CCA GAA CGA GGA AAG ATA TAT GAT CGT AAT GGT AAA GTG CTA GCA GAA
E   K   Y   L   V   K   N   A   Q   Q   P   E   R   G   K   I   Y   D   R   N   G   K   V   L   A   E
GAT GTA GAA AGA TAT AAA CTT GTT GCA GTA ATA GAT AAA AAG GCG AGT GCC AAT TCT AAA AAA CCT AGG CAT GTA GTT
D   V   E   R   Y   K   L   V   A   V   I   D   K   K   A   S   A   N   S   K   K   P   R   H   V   V
GAT AAA AAA GAG ACT GCA AAG AAA TTA TCT ACA GTC ATT AAT ATG AAG CCA GAG GAA ATT GAA AAG AGA CTT AGT CAA
D   K   K   E   T   A   K   K   L   S   T   V   I   N   M   K   P   E   E   I   E   K   R   L   S   Q
AAG AAA GCT TTC CAA ATT GAA TTT GGA CGC AAA GGA ACA AAT TTA ACG TAT CAG GAC AAA TTG AAA ATA GAG AAA ATG
K   K   A   F   Q   I   E   F   G   R   K   G   T   N   L   T   Y   Q   D   K   L   K   I   E   K   M
AAT TTG CCT GGT ATT TCT TTA TTG CCT GAA ACA GAA CGC TTT TAT CCA AAT GGC AAT TTT GCA TCA CAC TTA ATT GGT
N   L   P   G   I   S   L   L   P   E   T   E   P   N   G   N   F   A   S   H   L   I   G   R   A   Q
AGA GCT CAG AAA AAT CCG GAT ACT GGT GAA CTT AAA GGT GCA CTT GGA GTT GAA AAG ATT TTT GAT AGT TAT TTA AGT
R   F   Y   K   N   P   D   T   G   E   L   K   G   A   L   G   V   E   K   I   F   D   S   Y   L   S
GGA TCT AAA GGA TCA TTG AGA TAT ATT CAT GAT ATT TGG GGA TAT ATC GCA CCA AAT ACT AAA AAA GAG AAG CAG CCT
G   S   K   G   S   L   R   Y   I   H   D   I   W   G   Y   I   A   P   N   T   K   K   E   K   Q   P
AAA CGT GGT GAT GAT GTC CAT TTA ACA ATC GAT TCA AAT ATT CAA GTA TTT GTT GAA GAA GCT TTA GAT GGC ATG GTT
K   R   G   D   D   V   H   L   T   I   D   S   N   I   Q   V   F   V   E   E   A   L   D   G   M   V
GAA AGA TAC CAG CCG AAA GAT TTA TTT GCG GTT GTC ATG GAT GCC AAA ACT GGA GAA ATT TTA GCA TAC AGT CAG CGA
E   R   Y   Q   P   K   D   L   F   A   V   V   M   D   A   K   T   G   E   I   L   A   Y   S   Q   R
CCA ACA TTT AAT CCT GAA ACT GGT AAA GAC TTT GGT AAA AAG TGG GCA AAT GAC CTT TAT CAA AAC ACA TAC GAG CCT
P   T   F   N   P   E   T   G   K   D   F   G   K   K   W   A   N   D   L   Y   Q   N   T   Y   E   P
GGA TCA ACA TTT AAA TCA TAT GGG TTA GCA GCT GCT ATT CAA GAA GGT GCT TTT GAT CCT GAT AAG AAA TAT AAA TCT
G   S   T   F   K   S   Y   G   L   A   A   A   I   Q   E   G   A   F   D   P   D   K   K   Y   K   S
GGA CAT AGA GAT ATT ATG GGT TCA CGT ATT TCA GAC TGG AAT AGA GTC GGT TGG GGT GAA ATC CCA ATG TCA CTC GGA
G   H   R   D   I   M   G   S   R   I   S   D   W   N   R   V   G   W   G   E   I   P   M   S   L   G
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TTT ACT TAT TCA TCT AAT ACA TTG ATG ATG CAT TTA CAA GAT TTA GTT GGT GCA GAC AAA ATG AAA TCT TGG TAT GAA
F   T   Y   S   S   N   T   L   M   M   H   L   Q   D   L   V   G   A   D   K   M   K   S   W   Y   E
CGA TTT GGA TTT GGA AAA TCA ACT AAA GGT ATG TTT GAT GGA GAA GCA CCT GGT CAA ATT GGA TGG AGT AAT GAG TTG
R   F   G   F   G   K   S   T   K   G   M   F   D   G   E   A   P   G   Q   I   G   W   S   N   E   L
CAA CAA AAA ACG TCA TCA TTT GGT CAA TCG ACA ACA GTA ACA CCT GTT CAA ATG TTA CAA GCG CAA TCA GCG TTC TTT
Q   Q   K   T   S   S   F   G   Q   S   T   T   V   T   P   V   Q   M   L   Q   A   Q   S   A   F   F
AAT GAT GGT AAT ATG TTA AAA CCA TGG TTT GTG AAT AGC GTT GAA AAT CCT GTT AGT AAA AGA CAA TTT TAT AAA GGG
N   D   G   N   M   L   K   P   W   F   V   N   S   V   E   N   P   V   S   K   R   Q   F   Y   K   G
CAA AAA CAA ATC GCA GGC AAA CCA ATA ACA AAA GAT ACT GCT GAA AAA GTT GAA AAG CAA TTG GAT TTA GTT GTG AAT
Q   K   Q   I   A   G   K   P   I   T   K   D   T   A   E   K   V   E   K   Q   L   D   L   V   V   N
AGT AAG AAG AGT CAT GCT GCA AAC TAT CGT ATT GAT GGT TAT GAG GTC GAA GGT AAG ACT GGT ACA GCA CAA GTC GCT
S   K   K   S   H   A   A   N   Y   R   I   D   G   Y   E   V   E   G   K   T   G   T   A   Q   V   A
GCA CCT AAT GGT GGT GGA TAC GTT AAA GGT CCA AAC CCA TAT TTT GTA AGT TTT ATG GGT GAC GCG CCG AAG AAA AAT
A   P   N   G   G   G   Y   V   K   G   P   N   P   Y   F   V   S   F   M   G   D   A   P   K   K   N
CCT AAA GTT ATT GTA TAC GCT GGT ATG AGC TTG GCA CAA AAA AAT GAC CAA GAA GCT TAT GAA TTA GGT GTT AGT AAA
P   K   V   I   V   Y   A   G   M   S   L   A   Q   K   N   D   Q   E   A   Y   E   L   G   V   S   K
GCG TTT AAA CCA ATA ATG GAA AAT ACT TTG AAA TAT TTA AAT GTA GGT AAA TCA AAA GAT GAC ACA TAT AAT GCA GAG
A   F   K   P   I   M   E   N   T   L   K   Y   L   N   V   G   K   S   K   D   D   T   S   N   A   E
TAT AGT AAA GTG CCA GAT GTT GAA GGT CAA GAC AAA CAA AAA GCT ATT GAT AAT GTG AGT GCA AAA TCA TTA GAA CCA
Y   S   K   V   P   D   V   E   G   Q   D   K   Q   K   A   I   D   N   V   S   A   K   S   L   E   P
GTT ACT ATT GGT TCT GGC ACA CAA ATA AAA GCA CAA TCT ATA AAA GCA GGG AAT AAA GTC TTA CCT CAT AGT AAA GTA
V   T   I   G   S   G   T   Q   I   K   A   Q   S   I   K   A   G   N   K   V   L   P   H   S   K   V
CTG TTA TTA ACA GAT GGA GAC TTA ACT ATG CCT GAC ATG TCA GGA TGG ACG AAA GAA GAT GTC ATT GCT TTT GAA AAC
L   L   L   T   D   G   D   L   T   M   P   D   M   S   G   W   T   K   E   D   V   I   A   F   E   N
CTA ACA AAT ATT AAA GTA AAT TTA AAA GGT AGC GGT TTT GTG TCC CAC CAA TCA ATT AGT AAG GGA CAA AAA CTT ACT
L   T   N   I   K   V   N   L   K   G   S   G   F   V   S   H   Q   S   I   S   K   G   Q   K   L   T
GAA AAA GAT AAA ATA GAC GTA GAA TTT TCA TCA GAG AAT GTA GAC AGC AAT TCG ACG AAT AAT TCT GAT TCA AAT TCA
E   K   D   K   I   D   V   E   F   S   S   E   N   V   D   S   N   S   T   N   N   S   D   S   N   S
GAT GAT AAG AAG AAA TCT GAC AGT AAA ACT GAC AAG GAT AAG TCG GAC TAA
D   D   K   K   K   S   D   S   K   T   D   K   D   K   S   D   *
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4.5: DISCUSSION.

Following a transient cell wall-deficient (CWD) phenotype, it was shown that the revertant RS1/19 strain has a stable, high-level penicillin resistance phenotype and a highly altered cell wall structure. These altered characteristics may be due to penicillin-binding proteins (PBP) with altered specificities, which in turn may lead to the penicillin resistance. Alterations in the coding sequences of PBPs has been shown to impart resistance to β-lactam antibiotics, for example penicillin resistance in *S. aureus* can be due to an altered target site due to a point mutation in the *pbp 2* gene (Hackbarth, Kocagoz et al. 1995).

Therefore the coding sequences of the four PBPs from RS1/19 alongside those of the wild-type were sequenced to check for any mutations that could explain the altered cell wall and penicillin resistance of RS1/19. Apart from the conservative point mutation seen in PBP 1 there were no mutations in the penicillin-binding proteins' coding sequences. Hence an altered PBP is not the mechanism of the penicillin resistance seen in RS1/19.

However the up- or down-stream sequence of the coding strand may also have regulatory properties that affect the expression of genes and a mutation in one of these areas may lead to a change, for example low level β-lactam resistance can be due to overproduction of PBP 4 due to mutations in the noncoding *pbp 4* gene promoter region (Henze and Berger-Bachi 1996). Therefore portions of up- and down-stream sequence of the four PBPs from RS1/19 alongside those of the wild-type were sequenced to check for any mutations and again no mutations were found.

However the position of these operator/promoter regions of the PBPs is not fully known although thought usually to be close to the coding sequence, for example the *prfA-pbpB* operon is adjacent to the *pbp* gene in PBP 2 (Boyle-Vavra, Yin et al. 2003). However in PBP 4 although there is a promoter adjacent to the *pbp* gene there is a further promoter 419 nucleotides upstream in which the mutations were found leading to the low level β-lactam resistance (Henze and Berger-Bachi 1996). Though this can be discounted as a cause of the high-level resistance seen in the revertants it remains a possibility that an altered expression of a PBP with up- or down-regulation of one or more of the PBPs is present and in future work this should be studied.

5. INDUCTION OF CELL WALL-DEFICIENT BACTERIA.

5.1: INTRODUCTION.

The cell wall-deficient (CWD) CS1/19 strain was previously generated from the wild-type *Staphylococcus aureus* Oxford strain ATCC 9144 by sub-culturing on CWD solid medium, a supplemented media with a high osmotic potential, with increasing sublethal doses of penicillin G (Fuller et al. 2005). The final penicillin G concentration being 19.2 mg/L. CS1/19 was then allowed to revert back to a cell wall-competent (CWC) state, by growing the CWD cells in the absence of penicillin, and both the CWD CS1/19 strain and the revertant RS1/19 strain were found to be resistant to penicillin.

However CS1/19 was not 100% Gram-negative on subsequent transfers to CWD solid medium with 19.2 mg/L of penicillin G. This suggested that some of the cells had reverted to a CWC state and hence the line was no longer 100% CWD. In the previous work each time the percentage of Gram-negative cells fell it was returned to a higher level by increasing the penicillin concentration in the medium, hence finally reaching the penicillin concentration of 19.2 mg/L after many transfers. However this no longer occurred above 19.2 mg/L of penicillin, even with the penicillin concentration being increased by 8 times up to 154 mg/L, instead the percentage of Gram-negative cells fell further with more Gram-positive cells suggesting more and more cells were able to revert to a CWC state in the presence of the penicillin. This was probably because CS1/19 had a very high-level of penicillin resistance and doses of penicillin were no longer being reached that required the cells to be Gram-negative to survive.

The antibiotic sensitivity data of CS1/19 and RS1/19 show that the latter is just as resistant to penicillin as the CWD cells (Nattress, unpublished see Appendix 2). As would be expected the CWD cells should be absolutely resistant to penicillin as the target for the antibiotic is no longer present, but the revertants had a similar level of resistance suggesting that even though the cell wall was present it did not alter the resistance to penicillin. Hence perhaps once a pure CWD line is achieved, at 19.2 mg/L for CS1/19, 100% CWD is no longer achievable as sublethal doses of penicillin can no longer be achieved due to the high-level of penicillin resistance of not only in the CWD cells but also their revertants.

In order to have a consistent CWD strain to study the plan was to stabilize this line when 100% Gram-negative by removing the various ingredients that supplement

the CWD medium, finally leaving the basic ingredient i.e. BHI medium. However as achieving a stable 100% Gram-negative CWD strain, i.e. on subsequent transfers the cells remain 100% Gram-negative, did not seem possible this was going to be unlikely. Therefore an alternative was to induce cell wall-deficient bacteria using a rapid and reproducible method, preferably over days rather than weeks required for induction on solid media. So that if necessary a new stock could be generated each time required.

5.2: CELL WALL-DEFICIENT BACTERIA (CWDB) INDUCTION IN LIQUID MEDIA.

To keep as close as possible to the previous method of inducing cell wall-deficient bacteria (CWDB), in the wild-type *Staphylococcus aureus* Oxford strain ATCC 9144, penicillin and cell wall-deficient (CWD) medium were used. However liquid medium was used instead of solid medium for several reasons. The main reason being that the penicillin concentration is more consistent in liquid media, as mixing is easier. Also the culture is within liquid media as opposed to solid media, where some cells in a colony are more exposed to the air and not the media or penicillin, allowing for greater reproducibility. As well as cells in liquid media are metabolically equivalent, rather than cells in a colony that are of different ages and therefore metabolically different. Liquid media was also thought to allow for a quicker method of CWD induction due to more generations occurring on each transfer.

The aim was therefore to transfer wild-type into a range of penicillin concentrations below the MIC and then transfer on the one with the highest percentage of Gram-negative cells, to a range the same and above that penicillin concentration. Then to repeat this process until near 100% Gram-negative cells are achieved.

Method: aliquots of 100 µl of overnight culture of the Oxford strain in CWD liquid medium were transferred to 5 ml CWD liquid medium with a range of penicillin concentrations. The penicillin MIC for the Oxford strain in liquid CWD medium being 0.125 mg/L, so the initial range of penicillin concentrations was 0.01875 to 0.15 mg/L. These were incubated for 48 h at 37 °C on a shaker at 200 rpm and Gram

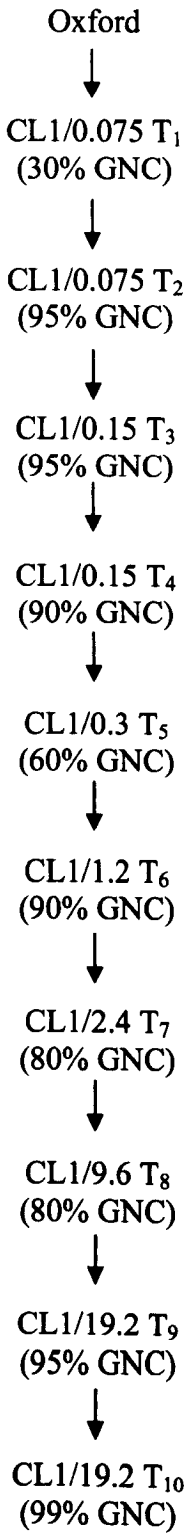
stained. The culture with the highest percentage of Gram-negative cells (taken as an indication of cell wall-deficiency) was used to inoculate CWD medium with a penicillin concentration range between 100% and 400% compared to which the cells were previously grown in. In the case of the 1st transfer the culture which contained the most Gram-negative cells contained 0.075 mg/L, with 30% Gram-negative cells, and this was used to inoculate medium with a range of penicillin concentrations between 0.075 and 0.3 mg/L. At each step the wild-type was transferred to medium without penicillin as a control for growth and for Gram staining, and a tube with medium but no inoculum was used as a control for sterility.

By the 9th transfer the penicillin concentration had reached 19.2 mg/L, and 95% of the cells stained Gram-negative (fig. 5.2). On the 2nd transfer 95% Gram-negative cells had been induced however this dropped on further transfers in the same amount of penicillin, and even dropped on significant increases of penicillin. However between the 9th and 10th transfers with the same amount of penicillin the percentage of Gram-negative cells actually increased further to 99%, and hence these cells were used as the CWD line.

With further transfers at the same penicillin concentration the Gram stain became mixed i.e. both Gram-positive and Gram-negative cells were seen, suggesting some reversion of the CWDB. This observation was the same as what had been seen in the CWD line CS1/19 generated in a similar way but using solid media. This would suggest this CWD line is unstable and will revert back to a cell wall-competent state easily, even in the presence of penicillin. This suggests that the revertants are as resistant as the CWDB and have also acquired the level of penicillin resistance required to grow at that level of penicillin concentration. The above method for CWDB induction was repeated by following the transfers in fig. 5.2, with the same result, i.e. 99% Gram-negativity after 9 transfers and hence was shown to be reproducible.

Aliquots of the 9th transfer were preserved, in 20% glycerol, at – 80 °C after being flash frozen in liquid nitrogen, so that these cells could be recovered and transferred to the 10th transfer to obtain a CWD culture easily and quickly.

Figure 5.2: Transfers from wild-type Oxford strain to CWD CL1/19 strain shown schematically with the Gram stain for each transfer shown in brackets, where 0.075 up to 19.2 represents the penicillin concentration in mg/L, T the transfer number and GNC Gram-negative cocci.



5.3: REVERSION OF CL1/19 TO RL1/19.

The cell wall-deficient (CWD) line of CL1/19 was allowed to revert to a cell wall-competent phenotype, by growing the CWD cells in the absence of penicillin on CWD solid medium using the same method as for the reversion of CS1/19 to RS1/19. This caused the cells to stain Gram-positive as seen previously with RS1/19.

CL1/19 was inoculated onto CWD agar plates for single colonies. Overnight growth at 37 °C demonstrated pale yellow pigment colonies of varying sizes. Three single colonies were sub-cultured for further single colonies on CWD agar and incubated overnight at 37 °C. This process was repeated a further eight times for a total of ten sub-cultures on CWD agar. This revertant line was named RL1/19.

5.4: ROLE OF CELL WALL-DEFICIENT (CWD) STATE IN ACQUISITION OF PENICILLIN RESISTANCE.

This experiment was designed to establish whether the stable antibiotic resistance, seen in cell wall-deficient (CWD) lines and their revertants, is due to the CWD or transient CWD state as opposed to the incremental increases of antibiotic. Berger-Bachi et al, for example showed that methicillin resistance can be selected out in *Staphylococcus aureus* by serial passage in increasing concentrations of methicillin on media without specifically raised osmolality (Berger-Bachi, Strassle et al. 1989).

The above method of CWD induction in liquid media was repeated with a control arm. LB liquid medium was chosen as a control as it does not induce cell wall-deficiency due to the low osmotic potential of LB medium in comparison to the high osmotic potential of CWD medium (Elmer, unpublished). The penicillin MIC for the wild-type Oxford strain was determined in LB liquid medium and found to be 0.0625 mg/L which is half of the penicillin MIC of the Oxford strain in CWD liquid medium. Therefore for the LB liquid medium control arm the penicillin concentrations increased from 0.075 to 9.6 mg/L over 9 transfers.

Population analysis profiles were determined after 9 transfers in each medium with increasing penicillin concentration. Appropriate dilutions of cell suspensions were transferred to a range of plates with different penicillin concentrations using CWD solid medium. Also a second series of population analysis profiles were

performed after a further 5 transfers for each arm in liquid media (CWD for the CWD arm and LB for the LB control arm) without penicillin to allow 'reversion', with the CWD line reverting to a cell wall-competent state. Penicillin E-tests were also performed for completeness for each strain, i.e. the CWD CL3/19 strain and their revertants RL3/19 and their controls LB9 and LB9/2, along with the wild-type Oxford strain for comparison.

The average number of colonies grown per ml on different concentrations of penicillin for CL3/19 and RL3/19 showed the number of colonies only reducing after the penicillin concentration reaches 38.4 mg/L. However for LB9 the number of colonies reduces as soon as the penicillin concentration is increased though some growth is seen hence some penicillin resistance is present, however for LB9/2 most of this resistance is lost. Gram stains for CL3/19 were comparable to the previous experiment of CWD induction in liquid media with increasing percentages of Gram-negative cells seen and Gram-negative cells seen as soon as penicillin was present, however for LB9 no Gram-negative cells were seen with only Gram-positive cells seen. Gram stains for RL3/19 and LB9/2 showed Gram-positive cells only, as expected, throughout. Figure 5.4 shows the figures for the two lines after transfer without penicillin plotted. Table 5.4 shows the results of the E-tests.

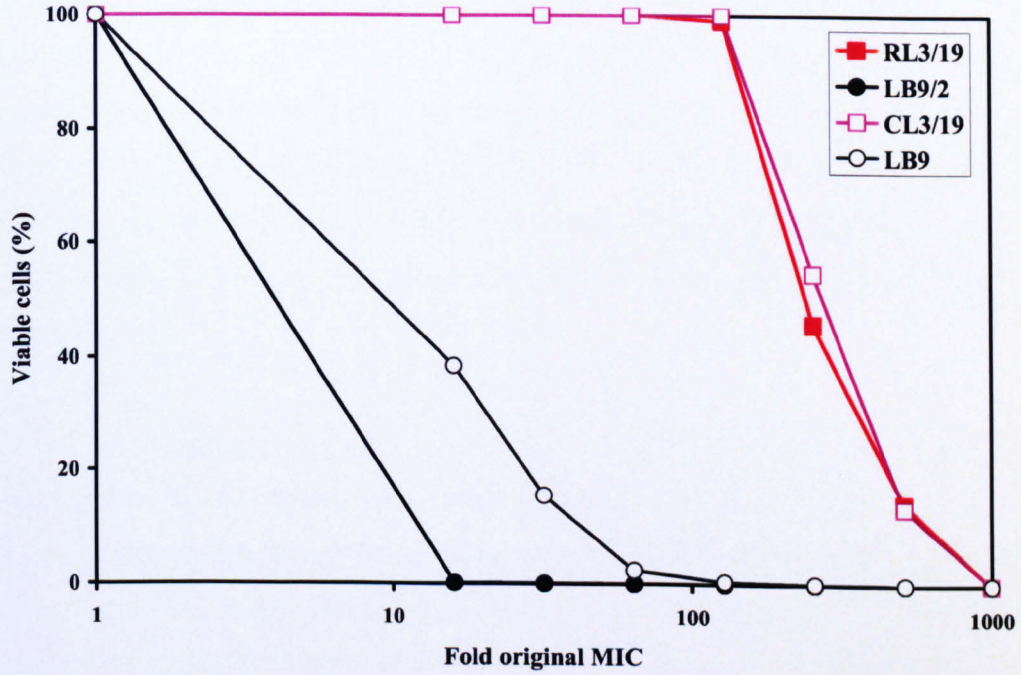
These results demonstrate that even though increasing penicillin concentrations in LB media caused an increase in penicillin resistance, this was much less than that seen in CWD medium and was also quickly lost when the penicillin pressure was removed. In comparison the transient CWD state leads to a high-level of penicillin resistance, MIC >256 mg/L. Hence the transient CWD state is required to obtain this altered antibiotic profile suggesting an alternative mechanism of penicillin resistance in *S. aureus* as a consequence to the transient CWD state.

Table 5.4: Penicillin MICs of CWD line CL3/19 and their revertants RL3/19, along with their controls LB9 and LB9/2, in comparison to wild-type Oxford strain.

MIC test medium	**Wild-type**	**CL3/19**	**LB9**	**RL3/19**	**LB9/2**
CWD	<0.016	192	16	>256	1.5
Isosensitest	<0.016	192	16	>256	0.75

Penicillin MICs (mg/L) determined by E-tests of the CWD line CL3/19 and their revertants RL3/19, along with their controls LB9 and LB9/2, in comparison to the wild-type Oxford strain. This table shows the high-level of resistance to penicillin of the CWD and revertant lines which is not seen in the controls.

Figure 5.4: Population analysis of CWD line CL3/19 and their revertants RL3/19, along with their controls LB9 and LB9/2.



Population analysis of the revertants obtained from the CWD line CL3/19 and their control LB9, showing a homogenous population of penicillin resistance to a high-level in the RL3/19 strain with minimal penicillin resistance in LB9/2. Fold original MIC is shown as a log scale.

5.5: CHARACTERISATION OF REVERTANTS OF CL1/19.

The revertants of CL1/19 and CL3/19 were tested to demonstrate whether they had similar characteristics to those revertants of CS1/19, which were obtained using solid media. Both cell wall-deficient (CWD) lines CL1/19 and CL3/19 were induced using the same method, which used a liquid medium, and were made from the same parental strain. However the reversion of CL1/19 to RL1/19 used the same method as the reversion of CS1/19 to RS1/19, which used a solid medium, whilst the reversion of CL3/19 to RL3/19 used liquid media. Despite this, the experiments used to characterise RL1/19 and RL3/19 gave essentially identical results and hence for ease of reading the results for RL1/19 only are shown. The growth curve of RL1/19 was similar to RS1/19 with a mean generation time of 74 min in comparison to 66 min for RS1/19.

5.5.1: Lysostaphin susceptibility: lysostaphin susceptibility of the wild-type Oxford and revertant RL1/19 strains were tested using exponential growth phase cells. RL1/19 was tested in triplicate to test the reproducibility of the equipment and the Oxford strain was tested once on the same day and a twice more on another day from a different culture. The results show that the revertant strain is more resistant to lysostaphin than the wild-type strain (fig. 5.5.1) with 50% lysis occurring after 3.4 min for the wild-type strain and after 13.2 min for the revertant strain, a 3.9-fold difference. This is very similar to the result obtained for the revertant RS1/19 strain which showed 50% lysis of 14.8 min.

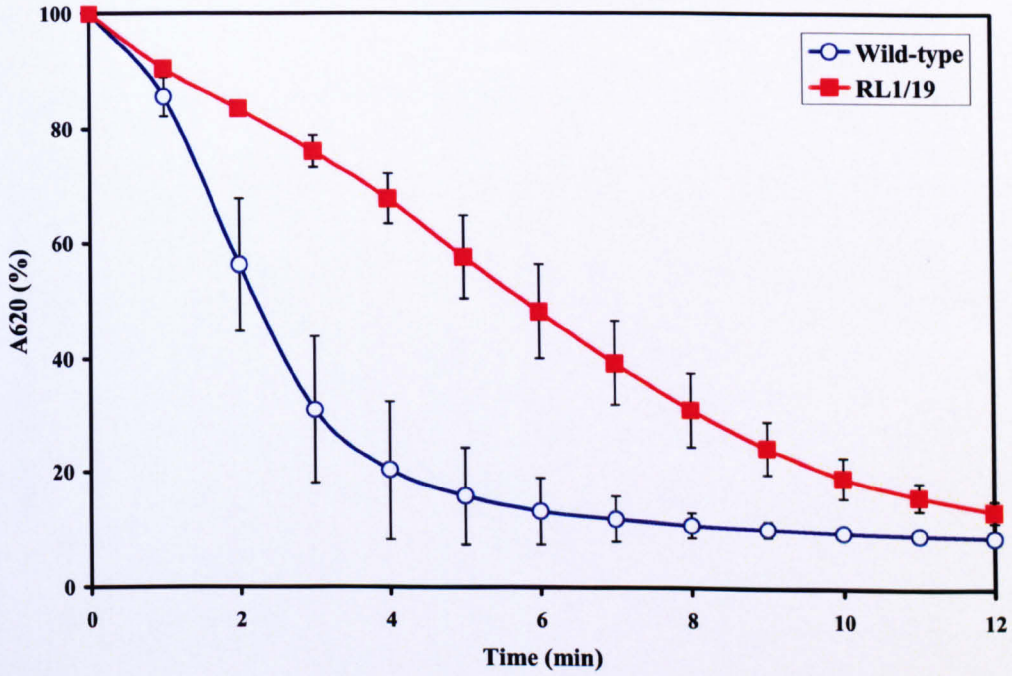
5.5.2: Autolysis with Triton X-100: Triton X-100 is a detergent that induces autolysis of whole cells by releasing the autolysins from the cell membrane leading to cell death (Koch 2001). It has to penetrate the cell wall to do this and therefore if the cell walls are altered in the revertant strain a difference in autolysis by Triton X-100 might be expected. Triton X-100-induced autolysis assays were performed on whole cells of the wild-type Oxford and revertant RL1/19 strains, following the method by Fournier B. and Hooper D.C. (Fournier and Hooper 2000) with minor modifications. Three cultures of each strain were tested i.e. three biological replicates for each strain.

The results show that 50% lysis occurred at 83.0 min for the wild-type strain (fig. 5.5.2), however the revertant strain showed complete resistance to lysis up to

three hours. This does suggest an altered cell wall to the extent that it is impenetrable by Triton X-100. This would be consistent with the earlier findings that suggest the cell walls of the revertant cells are thicker with more pentaglycine cross-bridges. Alternatively the revertant cells could have an absence of autolysin activity due to a single gene mutation as it thought that most of the autolysins of *S. aureus* are the products of a one gene (Foster 1992). However the former seems more likely i.e. an altered cell wall as a by-product of the antibiotic resistance.

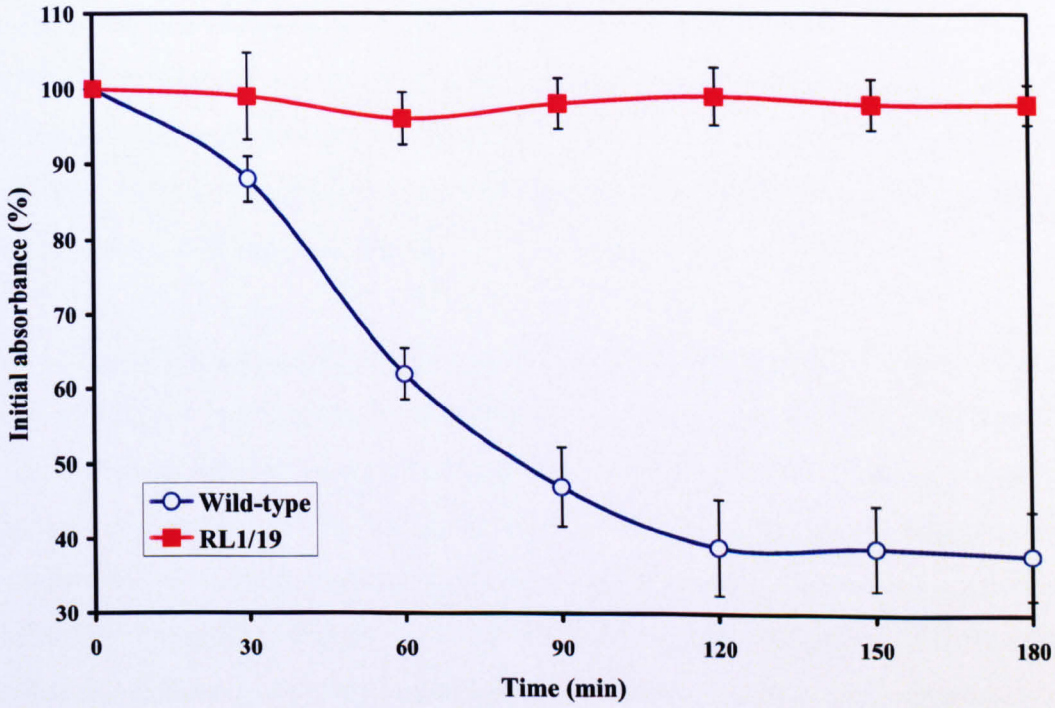
5.5.3: Penicillin MIC estimation in liquid media: this was carried out for RL3/19 to compare to the E-test data. The penicillin MIC for RL3/19 using CWD liquid medium was 128 mg/L which is similar to the E-test result of >256 mg/L, which used CWD solid medium.

Figure 5.5.1: Lysostaphin susceptibility of wild-type Oxford and revertant RL1/19 strains.



Lysostaphin susceptibility of the wild-type Oxford and revertant RL1/19 strains during exponential growth phase at 37 °C, showing the revertant strain is more resistant to lysostaphin in comparison to the wild-type strain. Values are the mean of three experiments and the bars represent the standard error of the mean (SEM).

Figure 5.5.2: Autolysis of whole cells by Triton X-100 of wild-type Oxford and revertant RL1/19 strains.



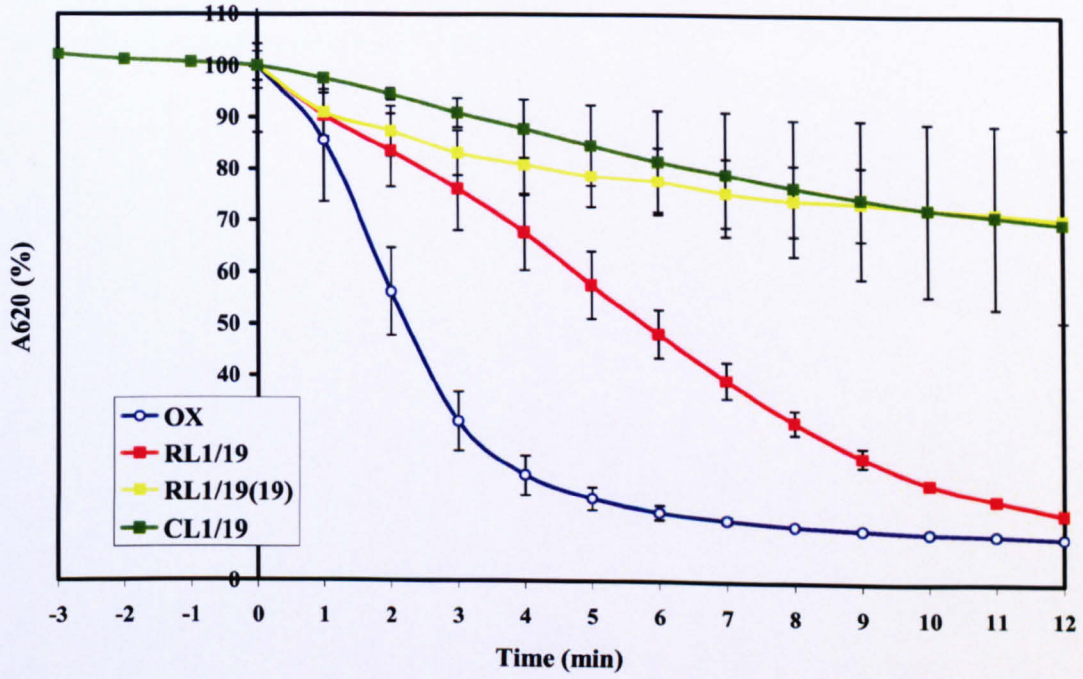
Autolysis of whole cells by Triton X-100 of the wild-type Oxford and revertant RL1/19 strains (± SEM) at 30 °C. The results are expressed as lysis percentages. These show that the revertant strain is resistant to autolysis by Triton X-100, in comparison to the wild-type strain. Values are the mean of three experiments.

5.6: CHARACTERISATION OF CWDB.

For further comparison the cell wall-deficient (CWD) strains CL1/19 and CL3/19 were investigated in the same way as their revertants. To maintain the CWD status, these strains had to be cultured in the penicillin concentration in which they had been induced, i.e. 19.2 mg/L for both, else reversion would occur and the cell wall-deficiency would be lost. Therefore for comparison the revertant was also studied with and without penicillin as part of their culture medium, again 19.2 mg/L being used. The revertant grown in penicillin was referred to as RL1/19(19) or RL3/19(19), with the number in brackets representing the amount of penicillin grown in mg/L to the nearest whole number. Once again the results for CL1/19 and CL3/19 and RL1/19(19) or RL3/19(19) are almost identical therefore for ease of reading the results for RL1/19 only are shown.

5.6.1: Lysostaphin susceptibility: exponential growth phase cells were tested for their susceptibility to lysostaphin. Two cultures of CL1/19 and RL1/19(19) were tested i.e. two biological replicates for each strain. The results for RL1/19 as above are included for ease of comparison (fig. 5.6.1). These show that CL1/19 is almost completely resistant to lysostaphin, especially when the degree of spontaneous lysis occurring before the lysostaphin is added is taken into account. This indicates that these cells either do not have a target for lysostaphin, or that the number of pentaglycine cross-links is so great that the wall integrity is maintained over the period of the assay, or that they do not rely on peptidoglycan for their intactness. Interestingly, there is a difference in the response of RL1/19 to lysostaphin depending on whether the cells are grown in the presence of penicillin or not. RL1/19(19) acts indistinguishably to the CWD line CL1/19. Again this indicates that these cells, when grown in penicillin, either do not have a lysostaphin target, or more pentaglycine cross-links, or that they do not rely on peptidoglycan for their intactness. The latter seems more likely for the CWD cells as they stain Gram negative suggesting an absence of a cell wall, but that the revertant cells when grown in penicillin behave indistinguishably raises the possibility that they also do not rely on a cell wall for its intactness.

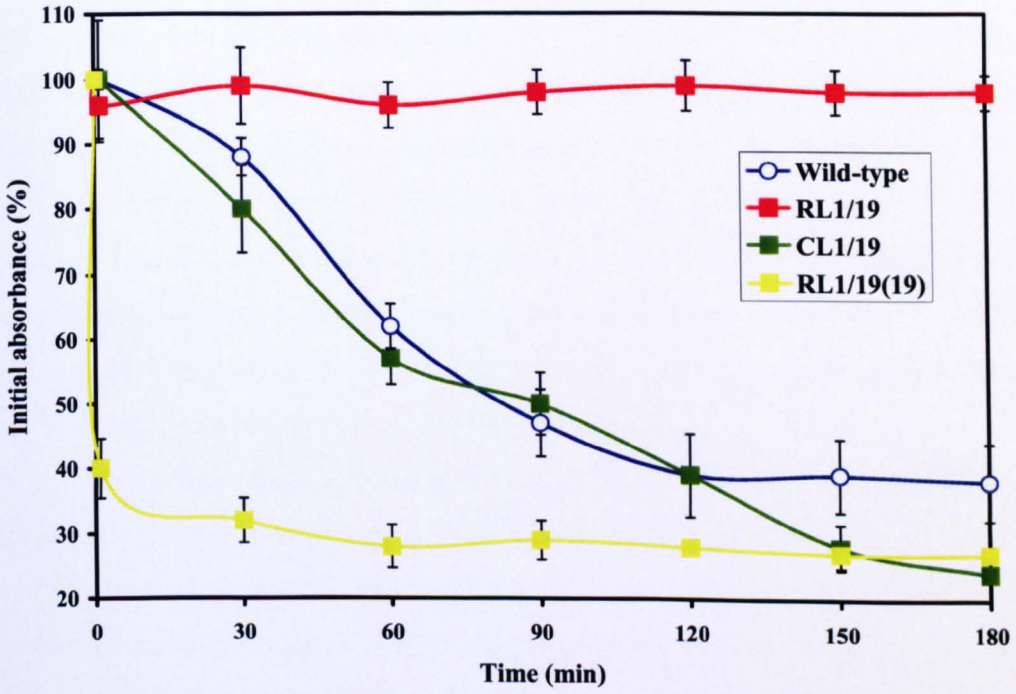
Figure 5.6.1: Lysostaphin susceptibility of wild-type Oxford, revertant RL1/19 and RL1/19(19), and CWD CL1/19 strains during exponential growth phase.



Lysostaphin susceptibility of the wild-type Oxford, revertant RL1/19 and RL1/19(19), the latter being grown in 19.2 mg/L of penicillin G, and CWD CL1/19 strains during exponential growth phase at 37 °C. These results show as previously that the revertant strain is resistant to lysostaphin in comparison to the wild-type strain. They also show that the cell wall-deficient strain is even more resistant to lysostaphin with the revertant strain when grown in penicillin behaving very similarly. Values for the wild-type and RL1/19 are the mean of three experiments and the bars represent the standard error of the mean (SEM). Values for CL1/19 and RL1/19(19) are the mean of two experiments and the bars represent the range.

5.6.2: Autolysis with Triton X-100: this was tested in the same way as previously. The cell wall-deficient CL1/19 strain and revertant RL1/19 strain, both grown in 19.2 mg/L penicillin G, were tested three times using three biological replicates for each strain. The results are shown (fig. 5.6.2) including the data for the wild-type Oxford and revertant RL1/19 strains, the latter being grown without penicillin. These results show that 50% lysis occurred at 90.0 min for the CWD strain (fig. 5.6.2), however RL1/19 grown in penicillin showed rapid lysis, with 50% lysis occurring within the first minute, suggesting increased fragility.

Figure 5.6.2: Autolysis of whole cells by Triton X-100 of wild-type Oxford strain, CWD line CL1/19 with their revertants RL1/19 and RL1/19(19).



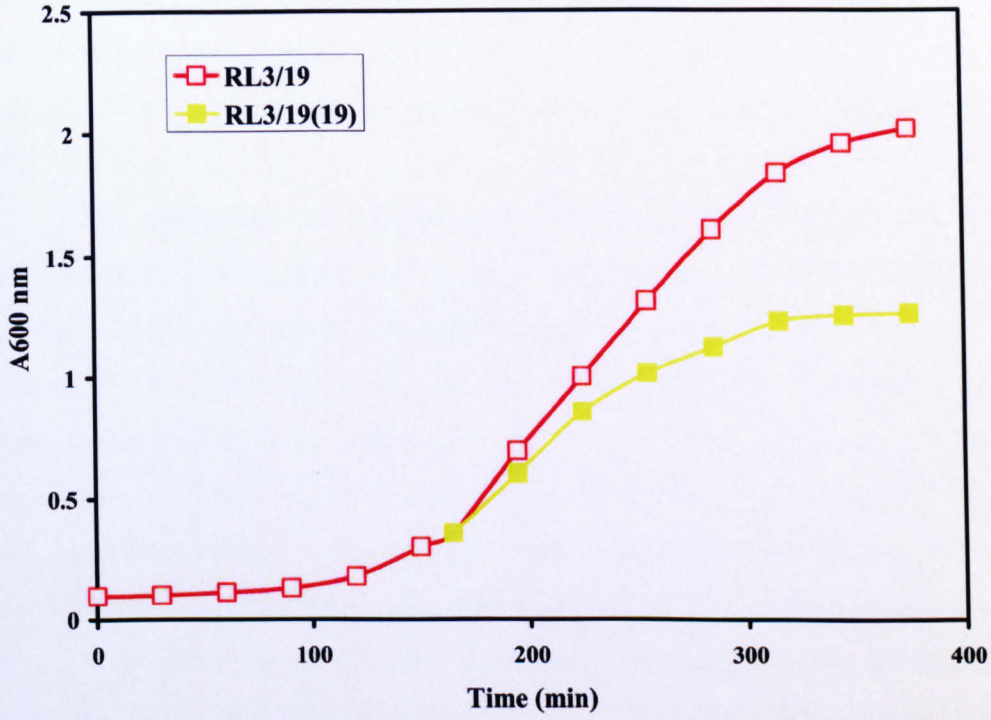
Autolysis of whole cells by Triton X-100 of the wild-type Oxford strain, CWD line CL1/19 with their revertants RL1/19 and RL1/19(19), the latter being grown in 19.2 mg/L of penicillin G, at 30 °C. The results are expressed as lysis percentages. These results show again the almost complete resistance to Triton X-100 of the revertant strain. They also show the almost complete lack of resistance of the cell wall-deficient strain in comparison to the wild-type. Values are the mean of three experiments and the bars represent the standard error of the mean (SEM).

5.7: RATE OF CONVERSION OF REVERTANTS FROM CELL WALL-COMPETENT (CWC) TO CWD STATE.

The characterisation of cell wall-deficient bacteria (CWDB) show that the revertant when grown in penicillin acts like the cell wall-deficient (CWD) line. Lysostaphin susceptibility of the revertant RL1/19 strain when grown in 19.2 mg/L of penicillin was indistinguishable to the CWD CL1/19 strain, which was also in 19.2 mg/L of penicillin. This suggests conversion of the revertant cells from cell wall-competent (CWC) to CWD when grown in penicillin. To examine this further the growth of a culture of RL3/19 in the presence and absence of penicillin in CWD liquid medium was followed. A single culture was grown to early potential growth phase (an A_{600} of 0.3 to 0.4) and this culture was split with half grown in CWD medium and half grown in CWD medium with penicillin at a concentration of 19.2 mg/L added. The growth of the two cultures was followed by absorbance readings until stationary phase cultures were obtained (fig. 5.7).

Gram stains of the cultures at the end of growth i.e. the stationary phase showed 100% Gram-positive cells in RL3/19 without penicillin but mixed Gram-positive and Gram-negative cells in RL3/19(19), i.e. in the presence of penicillin with the number in brackets again representing the amount of penicillin grown in mg/L to the nearest whole number. With RL3/19(19) about 30% of the cells stained Gram-negative and the remaining Gram-positive cells looking pale, misshapen and uniform in size of shape. The growth curve for the revertant RL3/19 after the addition of penicillin follows that of a CWD line, i.e. slower mean generation time and lower saturation density.

Figure 5.7: Growth curves of RL3/19 in CWD liquid medium with half of the culture at early exponential growth phase having penicillin at a concentration of 19.2 mg/L added giving RL3/19(19).



Growth curves of RL3/19 in CWD liquid medium at 37 °C with half of the culture at early exponential growth phase having penicillin at a concentration of 19.2 mg/L added giving RL3/19(19). These results show how the revertant once grown in penicillin has a slower growth rate with a slower mean generation time and lower saturation density.

5.8: CWDB INDUCTION OF ATCC 25923.

So far all the work described has used *Staphylococcus aureus* Oxford strain ATCC 9144 as the wild-type organism, which poses the question whether the same antibiotic resistance is seen in cells that have been through a transient cell wall-deficient (CWD) state but originating from a different parental strain. To test this question the *S. aureus* strain ATCC 25923 was used as the second wild-type *S. aureus* strain, being a widely used fully-sensitive *S. aureus* strain. For example it is recommended as the control organism for *S. aureus* in the British Society for Antimicrobial Chemotherapy (BSAC) guidelines for sensitivity testing (Andrews 2005).

The established liquid culture induction of cell wall-deficiency was used with *S. aureus* ATCC 25923 cells, as it has the same penicillin MIC as the Oxford strain (Andrews 2005). The induction was performed in duplicate. CD1/19T_{10} and CD2/192/19 T_{10}, at the induction, both cultures had >99% Gram-negative cocci on Gram staining. CD1/19 T_{10} was then reverted to a cell wall-competent state by transferring five times on CWD solid medium in the absence of penicillin. Population analyses were then performed on CD1/19 and its revertant RD1/19 along with penicillin and oxacillin E-tests. The results are shown in table 5.8 and figure 5.8.

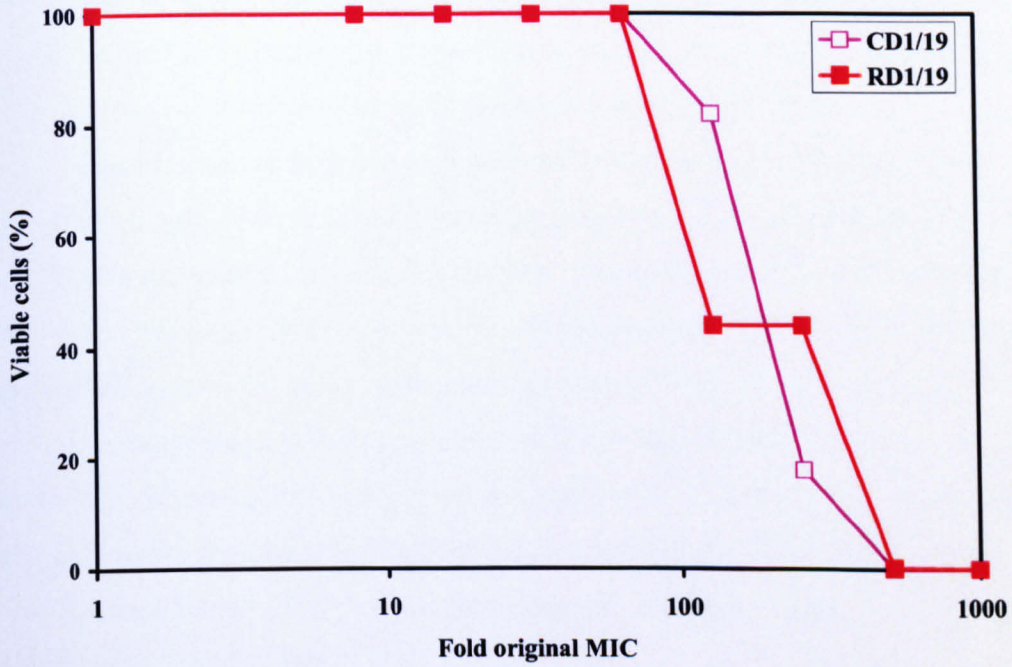
These results show that the altered antibiotic sensitivity seen following a transient CWD state in the wild-type *S. aureus* Oxford strain ATCC 9144 is also seen with the *S. aureus* strain ATCC 25923. The population analyses show there is no drop in the number of colonies until 19.2 mg/L of penicillin both in the CWD CD1/19 and revertant RD1/19 strains. Therefore the same high-level of penicillin resistance with a homogenous population is seen in these strains as was seen when ATCC 9144 was used as the parent strain. Also a high-level resistance to oxacillin is seen, which is used as a marker for methicillin resistance.

Table 5.8: Penicillin and oxacillin MICs of the CWD line CD1/19 and their revertants RD1/19.

MIC (mg/L)	CD1/19	RD1/19
Penicillin	256	128
Oxacillin	>256	>256

Penicillin and oxacillin MICs (mg/L), determined by E-tests on CWD medium, of the CWD line CD1/19 and their revertants RD1/19. This table shows the high-level of resistance to penicillin and oxacillin, therefore methicillin, of the CWD and revertant lines.

Figure 5.8: Population analysis of CWD line CD1/19 and their revertants RD1/19.



Population analysis of the CWD line CD1/19 and their revertants RD1/19, showing a homogenous population of penicillin resistance to a high-level in both strains.

5.9: DISCUSSION.

In this chapter a method for cell wall-deficient (CWD) induction in liquid media is described which was shown to be rapid and reproducible being able to produce cells with the same characteristics as those seen in the cells manipulated on solid media. The new method was then used in an experiment designed to establish the role of the CWD state in the acquisition of penicillin resistance and also the CWD induction of a second *Staphylococcus aureus* strain ATCC 25923.

The experiment designed to establish the role of the CWD state in the acquisition of penicillin resistance was essentially a control for the transient CWD state. It was designed to see whether the penicillin resistance seen in the revertants following a transient CWD state was due to that transient CWD state or due to another mechanism such as the increase in penicillin concentration. It showed that the penicillin resistance seen in the revertants RS1/19 and RL1/19 was homogenous and due to the CWD state, and not the increase in penicillin concentration. The control arm (LB media) showed cells acquired some penicillin resistance whilst exposed to penicillin in the media, but that this resistance was lost when grown in the absence of penicillin for five transfers.

The CWD induction and population analysis was also repeated using ATCC 25923 to demonstrate the reproducibility of the phenomenon of high-level homogenous penicillin resistance following a transient CWD state. This strain was also shown to be methicillin resistant. Therefore a transient CWD state induced using sublethal concentrations of penicillin G on either solid or liquid CWD medium, from the wild-type *S. aureus* strains ATCC 9144 or 25923, produced strains that demonstrated a homogenous high-level of penicillin resistance.

Revertants of CL1/19 that had been grown in the absence of penicillin for five sub-cultures were more resistant to lysostaphin than the wild-type and showed complete resistance to autolysis with Triton X-100. This does suggest an altered cell wall to the extent that it is impenetrable by Triton X-100. This would be consistent with the earlier findings that suggest the cell walls of the revertant cells are thicker with more pentaglycine cross-bridges. However the cell wall-deficient bacteria and the revertants when grown once again in the presence of penicillin were both completely resistant to lysostaphin but showed rapid autolysis with Triton X-100.

When a culture of the revertant line was split, with half having penicillin added, at early exponential phase the latter took on the growth curve of CWD line with a mixed Gram stain at the end with Gram-negative cells seen and atypical Gram-positive cells. This all suggests a possible mechanism for the penicillin resistance of the revertants i.e. on exposure to penicillin the revertants become cell wall-deficient and hence resistant by removing the target of the penicillin.

6. INVESTIGATING CELL WALL STRUCTURE USING ELECTRON MICROSCOPY.

6.1: INTRODUCTION.

Transmission electron microscopy (TEM) was carried out on the cell wall-deficient (CWD) CL1/19 and revertant RL1/19 strains grown with and without penicillin, along with the wild-type Oxford strain for comparison. This was to visualize the cell wall in the revertant in comparison to the wild-type as the biochemical data obtained suggested a highly altered cell wall. This work suggested shorter glycan chains and more pentaglycine cross-bridges in the peptidoglycan structure making up the cell wall of the revertant in comparison to the wild-type. There was also some suggestion that the cell wall of the revertant may be thicker or alternately the whole cell larger, leading to more peptidoglycan in total. Although this work was done on the revertant RS1/19 strain it was shown that the revertant RL1/19 strain had the same characteristics with lysostaphin resistance etc.. These strains had been obtained in the same way except for liquid media being used instead of solid media for RL1/19.

TEM was also carried out on the CWD strain to visualize the cell wall, to see how much if any cell wall is present and if so what the structure looks like. The experiments of Chapter 5 suggested that CL1/19 was cell wall-independent with no response to lysostaphin, however what this means as far as the cell wall, whether this means alteration in the structure or a total absence, was unknown.

Finally the difference between the revertant strain grown in penicillin and grown without penicillin was compared using EM. The experiments in Chapter 5 had interestingly shown that biochemically at least the revertant line grown in penicillin was behaving like the CWD line suggesting that a possible mechanism for the penicillin resistance was the ability for the revertant to become CWD or cell wall-independent in the face of penicillin.

6.2: RESULTS.

Electron micrographs of the cell lines are shown in figures 6.1 to 6.5. The revertant cell walls, when the revertant line was grown without penicillin, look very similar to wild-type cell walls, but may be thicker and fuzzier around the edge in comparison. The cell wall-deficient (CWD) cells however look very different in

comparison to the wild-type cells with the cells being much larger with very disorganised cell walls, thick in some places but thin or even absent in others. In one field at a 10, 000 magnification around 60 wild-type cells can be seen, however in the same size field at the same magnification around 25 CWD cells can be seen, mainly due to the obvious size difference that can be seen (fig. 6.5). Also there are multiple division planes throughout the cell in comparison to the one seen in the wild-type and revertant grown without penicillin. Most interestingly the revertant grown with penicillin was indistinguishable to the CWD cells. This adds weight to the hypothesis that the revertants' penicillin resistance mechanism is due to the ability to become cell wall-independent in the face of penicillin, with no need for an intact cell wall for osmotic stability. However the cell wall is not entirely absent, suggesting the term cell wall-defective would be more appropriate rather than cell wall-deficient.

Figure 6.1: TEMs of wild-type Oxford strain. **A:** whole cell at 80,000 magnification and **B:** part of the cell wall at 170,000 magnification. Scale bars of 0.1 micron. Both photographs show one division plane.

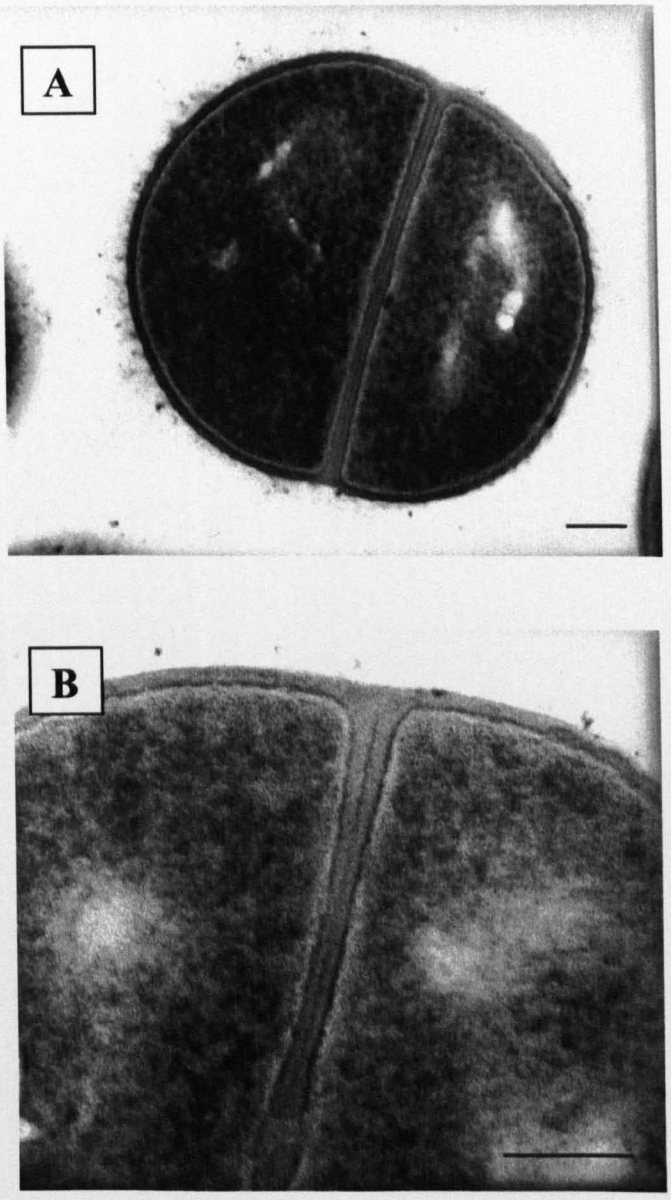


Figure 6.2: TEMs of revertant RL1/19 strain. **A:** whole cell at 80,000 magnification and **B:** part of the cell wall at 170,000 magnification. Scale bars of 0.1 micron. Both photographs show one division plane.

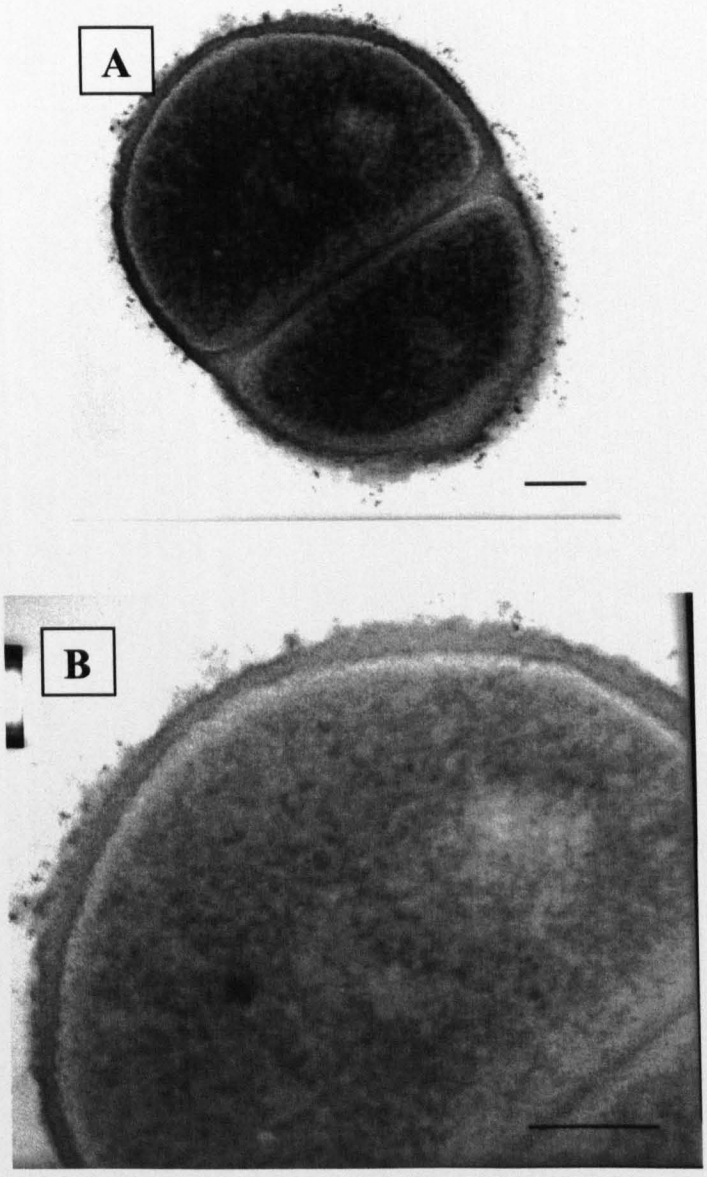
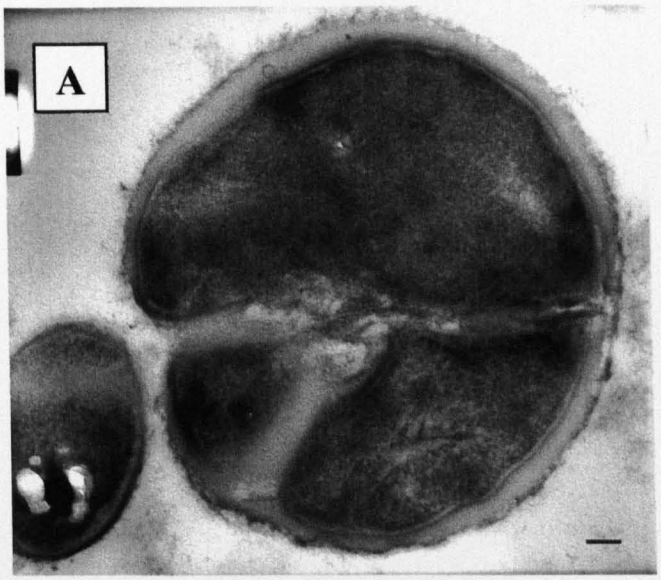


Figure 6.3: TEMs of cell wall-deficient CL1/19 strain. **A:** whole cell at 46,000 magnification and **B:** part of the cell wall at 80,000 magnification. Scale bars of 0.1 micron. Both photographs show multiple division planes.



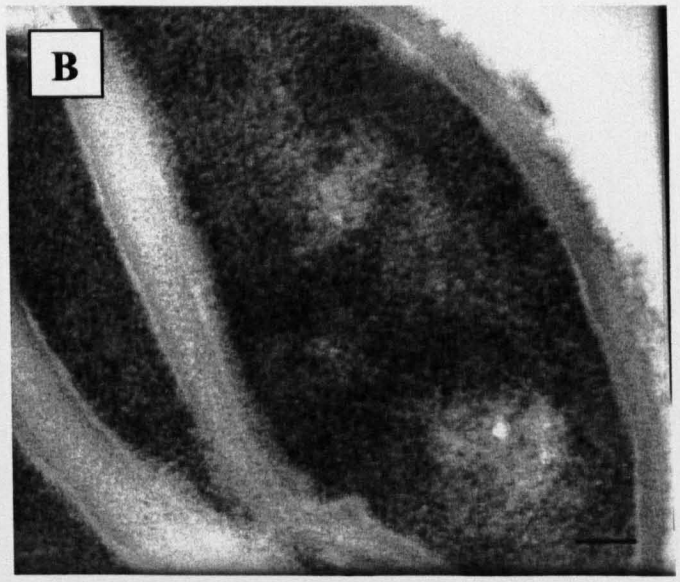


Figure 6.4: TEMs of revertant RL1/19 strain grown in penicillin. **A:** whole cell at 80,000 magnification and **B:** part of the cell wall at 170,000 magnification. Scale bars of 0.1 micron. The whole cell shows multiple division planes.

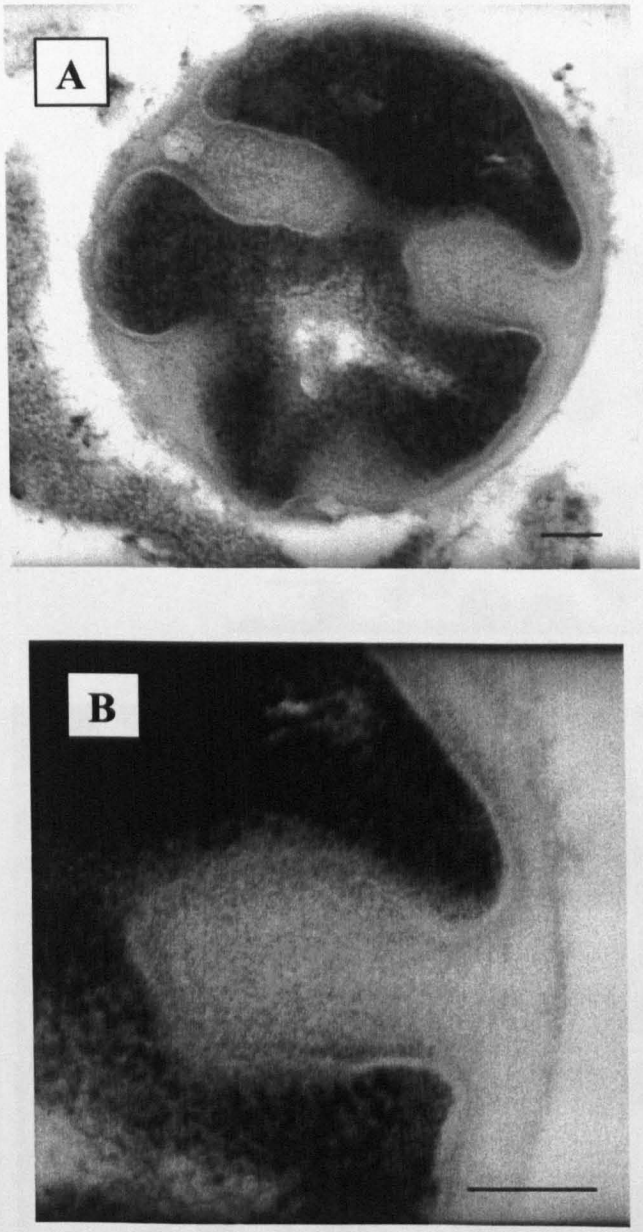
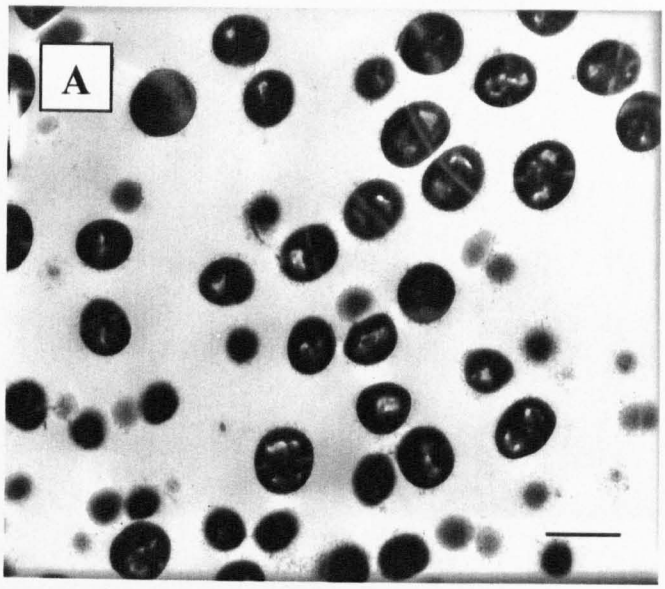
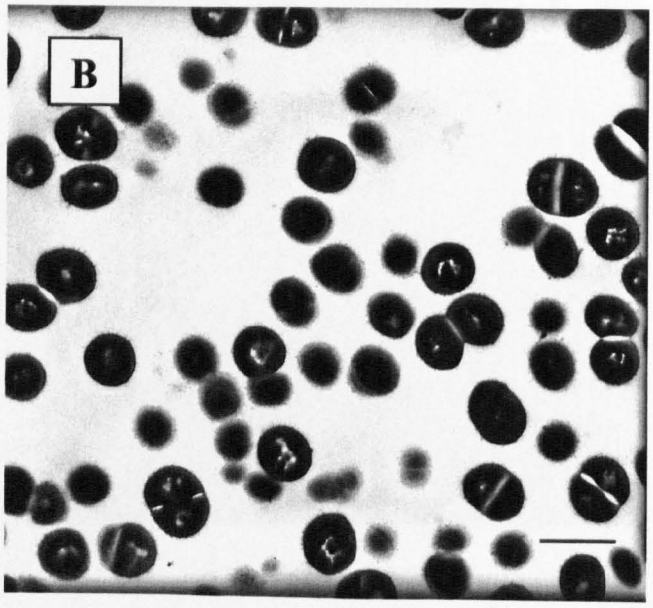
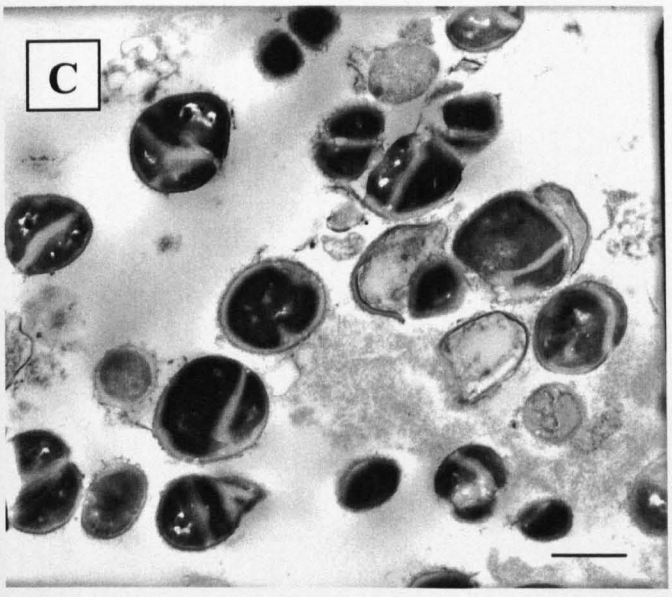
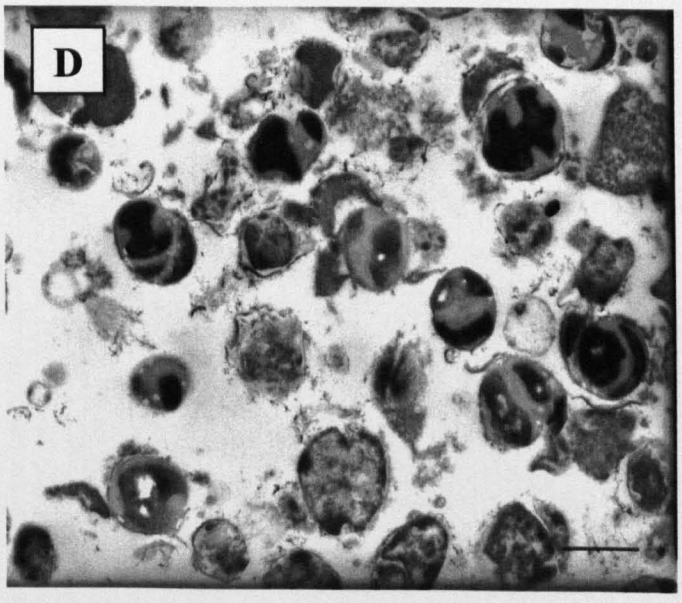


Figure 6.5: Comparison of cells of the different strains at low magnification, power of 10,000. **A:** wild-type Oxford strain, **B:** revertant RL1/19 strain, **C:** cell wall-deficient CL1/19 strain and **D:** revertant RL1/19 strain but grown in penicillin. Scale bars of 1 micron shown.






C


D

6.3: DISCUSSION.

The electron microscopy work carried out gave typical appearances for the Oxford strain that would be expected for *Staphylococcus aureus* cells. The revertant RL1/19 strain showed similar appearances, however the outer edge of the cell wall looked more indistinct and thicker in places in comparison to the Oxford strain. This visual appearance supports the biochemical data that shows that revertant cells contain more peptidoglycan of an altered structure.

However the cell wall-deficient (CWD) CL1/19 strain looked entirely different with a very disordered cell wall, diffuse and thickened but with areas where the cell wall appeared absent. Also there were multiple division planes. Giesbrecht et al showed that staphylococcal cells with multiple complete division planes could be grown using sub-inhibitory levels of penicillin with magnesium chloride, he termed these pseudomulticellular staphylococci due to inhibition of autolytic wall enzymes leading to inhibition of cell separation (Giesbrecht, Kersten et al. 1998). Therefore it seems likely a different mechanism is occurring here with incomplete multiple division planes rather than 'pseudomulticellular' cells. Most interestingly the revertant strain CL1/19 when grown in penicillin gave similar appearances to the CL1/19 strain with a highly disordered cell wall, and again with multiple division planes.

The size of the cells from the CWD strain and revertant strain grown in penicillin were a lot larger than the wild-type and revertant strains grown without penicillin. Also there was a lot more debris around the CWD cells and revertant cells when grown in penicillin, perhaps due to lysis due to the fragility of the cells.

7. DISCUSSION.

In previous work cell wall-deficient bacteria (CWDB) had been generated from the wild-type *Staphylococcus aureus* Oxford strain ATCC 9144 by sub-culturing on a supplemented media with a high osmotic potential with increasing sublethal doses of the cell wall-active antibiotic penicillin G. This cell wall-deficient (CWD) line had been named CS1/19 with a final concentration of penicillin G of 19.2 mg/L and was unstable. The colonies of CS1/19 were small, had lost the golden pigmentation typical of *S. aureus* and were umbonate in profile. Gram staining showed that the cells had a diffuse edge, did not show a typical staphylococcal cell arrangement and were Gram-negative. This would all be consistent with an absent cell wall. These CWD cells were resistant to penicillin as would be predicted due to the removal of the site of action of penicillin i.e. the cell wall. However this resistance persisted on reversion, by subculture in the absence of antibiotic, back to a cell wall-competent (CWC) state.

The current work initially established that the resistance was stable in the revertant line by growing the CWD cells in the absence of penicillin for ten transfers and showing that this revertant line RS1/19 was resistant to penicillin and methicillin to a high-level, with some altered sensitivities to the non-cell wall-active antibiotics also being noted. As discussed resistance to penicillin in CWD bacteria was expected, but if this resistance had been due solely to loss of the antibiotic target susceptibility should have been regained on recovery of the cell wall. On examination of these revertants, RS1/19, they visually looked different with smaller, paler colonies and a more diffuse Gram stain in comparison to the wild-type Oxford strain. They had an altered growth rate with a longer lag phase and slower mean generation time. Their matrix-assisted laser desorption/ionisation time-of-flight 'fingerprint' also was altered, which confirmed that these revertants were a mutant strain and very different to the parent strain i.e. the Oxford strain.

The revertant RS1/19 strain was more resistant to lysis by lysostaphin and had a greater mass of peptidoglycan per cell. Using reversed-phase high-performance liquid chromatography it was shown that the cell walls of RS1/19 were different to the wild-type Oxford strain with shorter glycan chains and more pentaglycine cross-bridges. As lysostaphin cleaves the pentaglycine cross-bridges this is consistent data suggesting either there is a thicker cell wall in the revertants, or the revertant cells are larger, or there are more cross-bridges per unit of peptidoglycan; however even if the

first two are the case the cell wall is still altered with shorter glycan chains. On repeat reversion further revertants had a similar slower growth rate and again were resistant to lysostaphin suggesting reproducibility of these revertants of CS1/19.

Initially the literature on CWDB suggested that on reversion from the CWD form the cells returned to the parent form with a 'normal cell wall' (Butler and Blakey 1975). Studies then suggested this was not the case though this has not been shown in *S. aureus*, however none of the literature suggested that on reversion the cells would have an altered antibiotic profile. Beaman and Bourgeois showed that on reversion of CWD *N. asteroides* the cells were morphologically different with altered cell walls in comparison to the original parent form, however there was no alteration in the antibiotic sensitivity profile (Beaman, Bourgeois et al. 1981). Similarly Landman showed that on reversion of CWD *Salmonella* species and *Escherichia coli* the cells had the same antibiotic profile as their parent forms (Landman 1968).

A method for CWD induction in liquid media, was established, which was shown to be rapid and reproducible being able to produce cells with the same characteristics as those seen in the cells manipulated on solid media. The liquid media method used the same inducing agent, penicillin G and the same CWD medium. The revertants produced via this method were named RL1/19 and had the same characteristics of the revertant RS1/19 strain including the high-level β-lactam resistance.

It was important to establish through a control experiment that this altered antibiotic profile was due to the transient CWD state as this is a potentially novel mechanism for β-lactam resistance in *S. aureus*. Therefore using the CWD induction in liquid media method alongside a control arm, using standard media but the same increasing sublethal doses of penicillin G, it was shown that the penicillin resistance seen in the revertant RS1/19 and RL1/19 strains was due to the transient CWD state, and not the increasing sublethal doses of penicillin. A similar phenomenon has been observed in the acquisition of derepressed β-lactamase mutants in *Enterobacter cloacae*. Huber showed that induction of CWD *Enterobacter cloacae* using a hypertonic medium supplemented with ticarcillin resulted in resistant mutants, in comparison to *E. cloacae* grown on standard media. These resistant mutants had high-levels of β-lactamase, persistent resistance and an antibiotic resistance pattern suggesting *AmpC* derepressed *Enterobacter* (Huber 2002).

The control arm showed some penicillin resistance whilst exposed to penicillin in the media, but lost this resistance when grown in the absence of penicillin for five transfers. This has been shown previously, Berger-Bachi et al selected out methicillin resistance in *S. aureus* by serial passage in increasing concentrations of methicillin which led to homogeneous resistance and a reduced growth rate (Berger-Bachi, Strassle et al. 1989). It was later shown that the low level resistance seen in these isolates was due to point mutations present in the *pbp 2* (Hackbarth, Kocagoz et al. 1995). Therefore it is likely that the resistance seen in the LB line was due to point mutations but these disadvantaged the population and hence were lost quickly.

Therefore the acquisition of high-level, stable resistance was only observed in those cells that had been CWD and not in control cells grown on media that did not allow the formation of CWD bacteria. This control experiment produced the CWD line CL3/19 and their revertants RL3/19, isogenic strains to CL1/19 and RL1/19. The population analysis showed that the penicillin resistance of the revertants was homogenous with resistance present in 100% of the cells.

Decreased microbial susceptibility to penicillin has been widely reported, and a number of resistance mechanisms have been demonstrated. The most common mechanism for penicillin resistance in *S. aureus* is the acquisition of β-lactamase, an enzyme that cleaves the β-lactam ring of penicillin (McDougal and Thornsberry 1986). However resistance also occurs because of other mechanisms such as alteration of the target site on the enzyme to which penicillin binds, for example due to a point mutation in the *pbp 2* gene (Hackbarth, Kocagoz et al. 1995). Overproduction of penicillin-binding protein (PBP) 4 due to mutations in the noncoding *pbp 4* gene promoter region also leads to penicillin resistance (Henze and Berger-Bachi 1996). Finally the horizontal acquisition of a penicillin resistant penicillin-binding protein, PBP 2a leads to resistance, with this also conferring methicillin resistance (Archer, Niemeyer et al. 1994).

Methicillin resistance in *S. aureus* is due to *mecA* gene in nearly all cases which codes for PBP 2a, which has a low affinity for β-lactam antibiotics leading to resistance to β-lactam antibiotics as PBP 2a takes over the functions of the other PBPs when they are inhibited by antibiotics (Pinho, de Lencastre et al. 1998). Borderline methicillin resistance has also been recognized in clinical isolates that do not contain either the *mecA* gene or a β-lactamase but have mutations in the *pbp* genes that cause reduced penicillin-binding (Hackbarth, Kocagoz et al. 1995).

The revertants were shown to be β-lactamase and PBP 2a-negative. There were no mutations in the coding regions of the four PBPs of the revertant line RS1/19 in comparison to the wild-type Oxford strain apart from the conservative point mutation seen in PBP 1. There were also no mutations in the up- and down-stream sequences adjacent to the coding regions of the four PBPs from RS1/19 in comparison to the wild-type Oxford strain. The promoter regions are usually located in these up- and down-stream sequences adjacent to the coding region, for example the *prfA-pbpB* operon is adjacent to the *pbp* gene in PBP 2 (Boyle-Vavra, Yin et al. 2003). However in PBP 4 although there is a promoter adjacent to the *pbp* gene there is a further promoter 419 nucleotides upstream in which the mutation was found causing the low level β-lactam resistance (Henze and Berger-Bachi 1996). Though this can be discounted as a cause of the high-level resistance seen in the revertants it remains a possibility that an altered expression of a PBP with up- or down-regulation of one or more of the PBPs is present and in future work this should be studied further with all the operator/promoter regions of the PBPs sequenced.

Therefore β-lactamase, PBP 2a acquisition or an altered PBP is not the mechanism of the β-lactam resistance seen in RS1/19. Although all the operator/promoter regions have not been sequenced there are no known mutations in these areas that lead to high-level β-lactam resistance. Hence each of the above mechanisms has been eliminated as an explanation for the resistance observed in the CWC cells, confirming that this is a novel mechanism for β-lactam resistance in *S. aureus*.

It was also important to establish that this β-lactam resistance seen in the CWC cells was not strain specific, therefore the CWD induction and population analysis was repeated with a different wild-type *S. aureus* strain to the Oxford strain. The *S. aureus* strain ATCC 25923 was used and this demonstrated the same phenomenon of high-level homogenous β-lactam resistance following a transient CWD state. Therefore a transient CWD state induced using sublethal concentrations of penicillin G and CWD medium, either solid or liquid, from the wild-type *S. aureus* strains ATCC 9144 or 25923 gives a homogenous high-level of β-lactam resistance of unknown mechanism.

To investigate this unknown mechanism of β-lactam resistance the CWC cells were studied further. The revertants of CL1/19 were resistant to lysostaphin in comparison to the wild-type, but showed complete resistance to autolysis with Triton

X-100. This does suggest an altered cell wall to the extent that it is impenetrable by Triton X-100. This would be consistent with the earlier findings that suggest the cell walls of the revertant cells are thicker with more pentaglycine cross-bridges. Alternatively the revertant cells could have an absence of autolysin activity due to a single gene mutation as it thought that most of the autolysins of *S. aureus* are the products of a one gene (Foster 1992). However the former seems more likely i.e. an altered cell wall as a by-product of the antibiotic resistance. However the CWDB and the revertants when grown once again in the presence of penicillin were both completely resistant to lysostaphin but showed rapid autolysis with Triton X-100. It would be predicted that the CWDB would be resistant to lysostaphin as they do not rely on their cell wall for integrity and the increased sensitivity to Triton X-100 is likely to be due to increased access to the membrane. When a culture of the revertant line was split, with half having penicillin added, at early exponential phase the latter took on the growth curve of CWD line with a mixed Gram stain at the end with Gram-negative cells seen and atypical Gram-positive cells. This all suggests a possible mechanism for the penicillin resistance of the revertants i.e. on exposure to penicillin the revertants become cell wall-deficient and hence resistant by removing the target of the penicillin.

Transmission electron microscopic examination of the cells revealed that the outer edge of the revertant cell wall looked more indistinct and thicker in places in comparison to the wild-type cell walls. This visual appearance supports the biochemical data that shows that revertant cells contain more peptidoglycan of an altered structure. The CWD cells however look entirely different in comparison to the wild-type cells with the cells being much larger with a very disordered cell wall, diffuse and thickened but with areas where the cell wall appeared absent. Also there are multiple division planes throughout the cell in comparison to the one division plane seen in the wild-type and revertant cells. Giesbrecht et al showed that staphylococcal cells with multiple complete division planes could be grown using sub-inhibitory levels of penicillin with magnesium chloride, he termed these pseudomulticellular staphylococci due to inhibition of autolytic wall enzymes leading to inhibition of cell separation (Giesbrecht, Kersten et al. 1998). Therefore it seems likely a different mechanism is occurring here with incomplete multiple division planes rather than 'pseudomulticellular' cells.

Most interestingly transmission electron micrographs of the revertant cells when grown with penicillin were indistinguishable to the CWD cells. This adds weight to the hypothesis that the revertant's penicillin resistance mechanism is due to the ability to become cell wall-independent but not cell wall-deficient but more cell wall-defective in the face of penicillin.

In this work it has been shown that a transient cell wall-defective phenotype in *Staphylococcus aureus* leads to an altered antibiotic profile with high-levels of penicillin and methicillin resistance. All known resistance mechanisms were excluded. Uniquely following reversion the integrity of the subsequent cell wall-competent cells does not depend on their reconstituted cell walls if penicillin is reintroduced. The data discussed indicate that the cells have undergone stable genotypic changes that allow them to avoid the action of β-lactam antibiotics by quickly and uniformly dispensing with the need for an intact peptidoglycan sacculus for osmotic stability. Furthermore, cells with a defective cell wall become resistant to other cell wall-active antibiotics and are able to grow and divide. The ability of these cells to survive may be an important bacterial response to attack by cell wall-active agents.

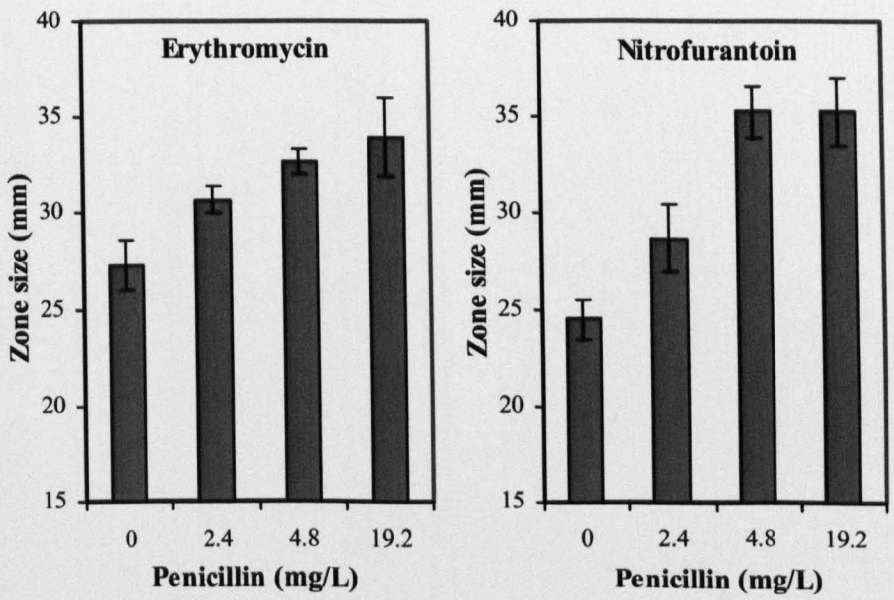
This is a novel mechanism for the development of resistance of great importance. In the future it would be important to establish the cause, probably mutational, behind this ability to become CWD after a transient CWD state so easily. However first it would be useful to study the operator/promoter regions of the PBPs, array data and expression of the PBPs to observe if there is any up- or down-regulation of one or more of the PBPs. Along with establishing that the revertant cells do not have an absence of autolysin activity due to a single gene mutation to explain their resistance to Triton X-100. Also studying the pathogenicity of the reverants especially in the presence of antibiotics, to establish whether they are pathogenic or whether it is a mechanism to persist. Examining any non-PBP 2a methicillin resistant *S. aureus* for these mutations once found would be important to establish if these are present clinically, as the mechanism is an ability to resist all β-lactam antibiotics which would lead to reoccurrence of disease secondary to *S .aureus* and treatment failure. With the high usage of β-lactam antibiotics within medicine if this mechanism of resistance is occurring in vivo due to the antibiotic pressure, the use of β-lactam

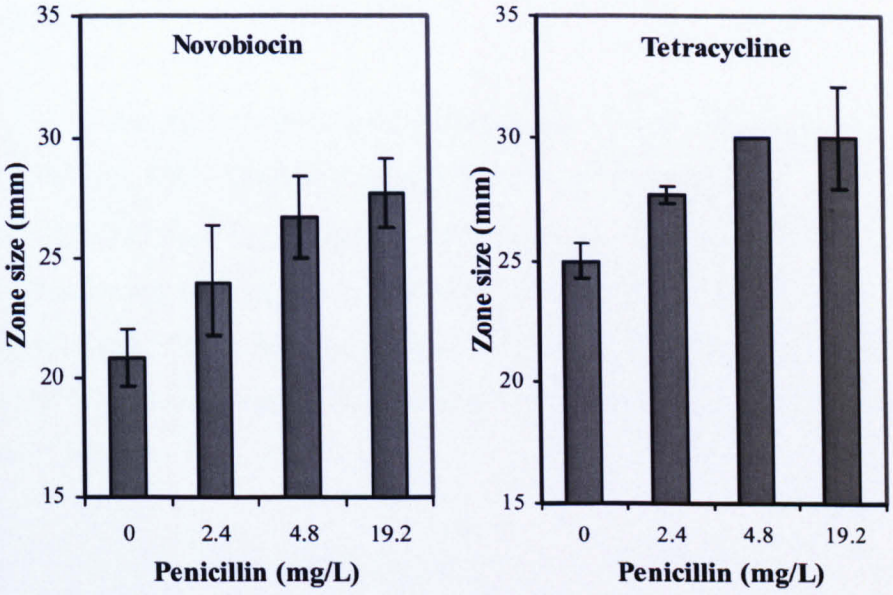
antibiotic as monotherapy may need to be addressed, leading to the need to treat recurrent *S. aureus* infections with a non-β-lactam antibiotic.

Appendix 1.

In previous work by Catherine Elmer had established that increasing sublethal doses of penicillin in supplemented media with a high osmotic potential could induce cell wall-deficiency in the wild-type *Staphylococcus aureus* Oxford strain ATCC 9144. These cell wall-deficient (CWD) variants were generated by inoculating the Oxford strain onto CWD solid medium containing a range of concentrations of penicillin G, with colonies on the plate with the highest concentration of antibiotic being transferred to fresh CWD solid medium containing further incremental increases in the penicillin concentration and hence maintaining these CWD variants. A penicillin G concentration of 19.2 mg/L was reached eventually and this CWD line was named CS1/19.

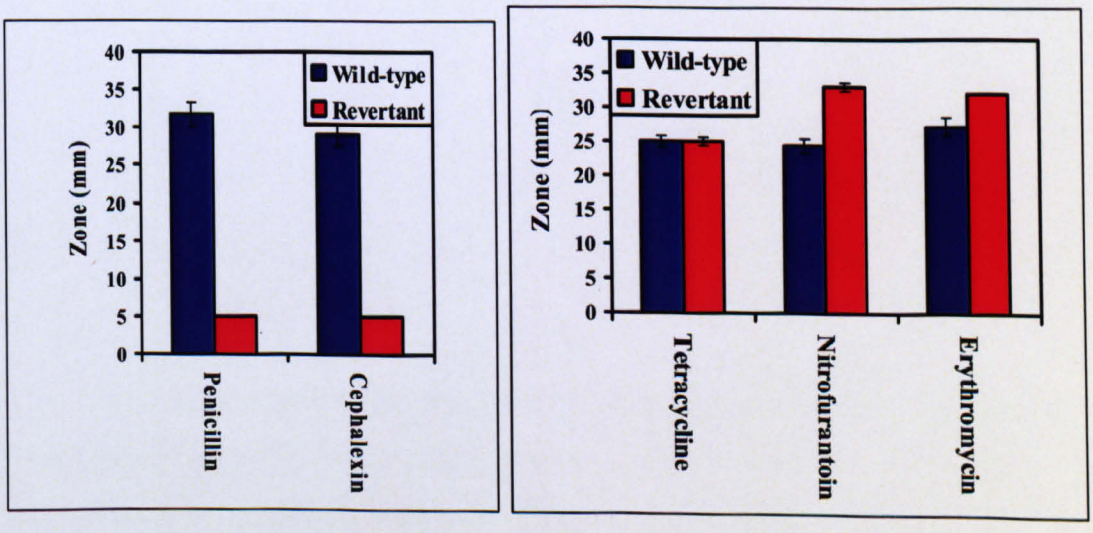
Fiona Nattress performed antibiotic sensitivity tests on this CWD line at several stages as the penicillin concentration was increased, following the British Society for Antimicrobial Chemotherapy (BSAC) guidelines using disc diffusion tests. The results are shown in the following figure with the means shown along with the standard error of the mean (SEM) bars. Disc sizes were 5 mm.





This data shows that the resistance of the CWD line to each of the antibiotics tested increasingly altered as the penicillin concentration used to induce the cell wall-deficiency increased. The antibiotics tested were non-cell wall-active antibiotics.

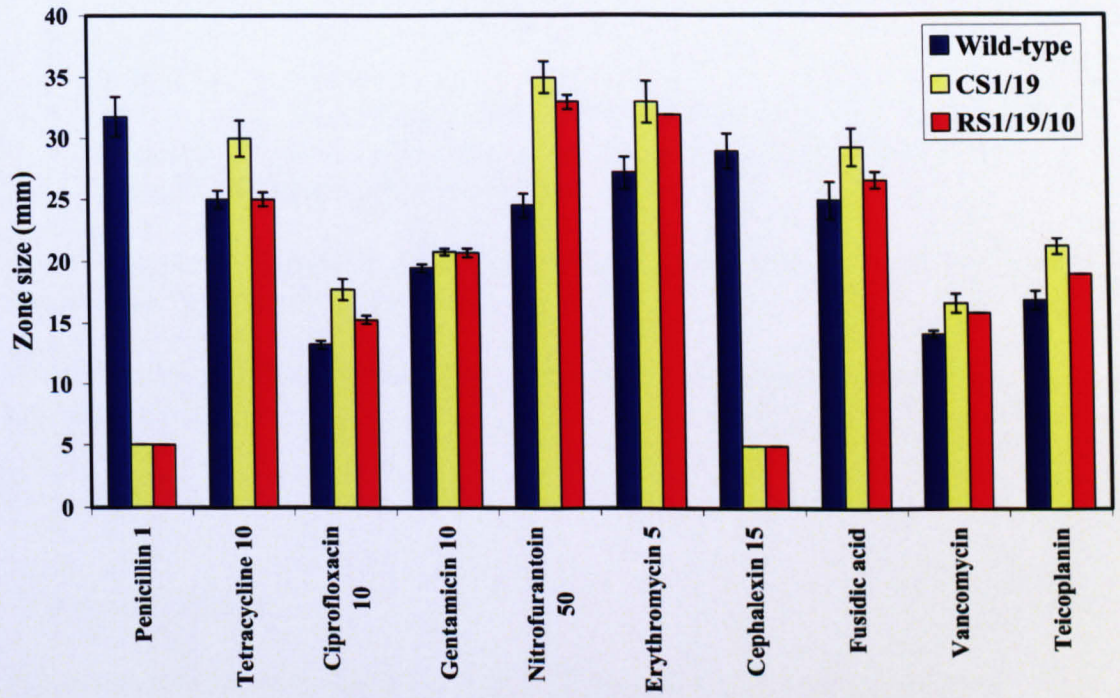
The CWD line CS1/19 was then allowed to revert back to a cell wall-competent phenotype by transferring to fresh CWD solid medium without penicillin. Antibiotic sensitivity tests were then performed in the same way on these revertants.



This data shows that the penicillin resistance is also seen in the revertants but also the altered sensitivity to some non-cell wall-active antibiotics was maintained.

Appendix 2.

Fiona Nattress performed antibiotic sensitivity tests on the three isogenic lines RS1/19/10_1, RS1/19/10_2 and RS1/19/10_3 along with three wild-type Oxford lines and three CS1/19 lines for comparison, following the British Society for Antimicrobial Chemotherapy (BSAC) guidelines using disc diffusion tests. The results are shown in the following figure with the means of the three lines for the wild-type, CS1/19 and RS1/19/10 shown along with the standard error of the mean (SEM) bars. Disc sizes were 5 mm.



Also Fiona Nattress tested the revertants for β-lactamase activity using the Beta Test kit (Medical Wire and Equipment Co., Bath, United Kingdom). The revertants were negative for β-lactamase activity. Furthermore, incubation of exponentially growing revertants with penicillin G did not activate the antibiotic, confirming the absence of β-lactamase activity and excluding the presence of another antibiotic-inactivating activity.

Appendix 3.

In previous work Catherine Elmer had designed the primers for the polymerase chain reactions of the penicillin-binding proteins (PBP) 1, 2, 3 and 4. Elmer took the available published sequences for each PBP with the surrounding base sequences and identified any differences between the published results. A sense and anti-sense primer was designed for each PBP in the surrounding base sequences of around 20 bases long with reasonably equal CG and AT content. The process for PBP 1 is shown below, along with the primer summary for all the PBPs.

PBP 1:

- Lower case lead in/out sequences
- Upper case coding sequence of gene
- Blue primers
- Red Base which may be different in one or more of the strains
- Positions Base differences within genes numbered with respect to position in "megalign" comparison.

Example sequence chosen for each PBP has the highest consensus with respect to the others in the "megalign" comparison.

Four examples of PBP1 sequenced and published on the net with surrounding base sequences available:

- Mu50
- N315
- MW2
- ATCC83254.

Full gene sequence with additional 228 bases on either end obtained for each.
7 base differences within gene (positions 580, 1536, 1632, 1854, 2077, 2109, 2109, 2193). Change present in one or more of the 4 different sequences.

Gene 2235bp long.
Example MW2.

ttactgtaattgctatgttaagtatttatatgctatctttaaaaatggatgcgtatgatacgcgag
gaaagattgcagatttagattataaaatagataaacaatcaagtgaaaacagtgctttacaat
ctgaaatcaaaaagaattcttcttatgaacgcatatacgaaaaggctaagaaacaggggat
gagccttgagaacgataatgtaaaggtatgtgcgtagta ATG GCG AAG CAA AAA
ATT AAA ATT AAA AAA AAT AAA ATA GGG GCA GTC CTA CTT GTT GGT
TTA TTC GGA CTG CTC TTT TTT ATA TTG GTT TTA AGA ATT TCA TAT

ATC ATG ATT ACT GGA CAT TCT AAT GGT CAA GAT TTA GTC ATG AAG
GCA AAT GAA AAG TAT TTA GTT AAG AAT GCA CAA CAA CCA GAA
CGA GGA AAG ATA TAT GAT CGT AAT GGT AAA GTG CTA GCA GAA
GAT GTA GAA AGA TAT AAA CTT GTT GCA GTA ATA GAT AAA AAG GCG
AGT GCC AAT TCT AAA AAA CCT AGG CAT GTA GTT GAT AAA AAA GAG
ACT GCA AAG AAA TTA TCT ACA GTC ATT AAT ATG AAG CCA GAG
GAA ATT GAA AAG AGA CTT AGT CAA AAG AAA GCT TTC CAA ATT GAA
TTT GGA CGC AAA GGA ACA AAT TTA ACG TAT CAG GAC AAA TTG
AAA ATA GAG AAA ATG AAT TTG CCT GGT ATT TCT TTA TTG CCT GAA
ACA GAA CGC TTT TAT CCA AAT GGC AAT TTT GCA TCA CAC TTA ATT
GGT AGA GCT CAG AAA AAT CCG GAT ACT GGT GAA CTT AAA GGT
GCA CTT GGA GTT GAA AAG ATT TTT GAT AGT TAT TTA AGT GGA TCT
AAA GGA TCA TTG AGA TAT ATT CAT GAT ATT TGG GGA TAT ATC GCA
CCA AAT ACT AAA AAA GAG AAG CAG CCT AAA CGT GGT GAT GAT
GTC CAT TTA ACA ATC GAT TCA AAT ATT CAA GTA TTT GTT GAA GAA
GCT TTA GAT GGC ATG GTT GAA AGA TAC CAG CCG AAA GAT TTA TTT
GCG GTT GTC ATG GAT GCC AAA ACT GGA GAA ATT TTA GCA TAC AGT
CAG CGA CCA ACA TTT AAT CCT GAA ACT GGT AAA GAC TTT GGT AAA
AAG TGG GCA AAT GAC CTT TAT CAA AAC ACA TAC GAG CCT GGA TCA
ACA TTT AAA TCA TAT GGG TTA GCA GCT GCT ATT CAA GAA GGT GCT
TTT GAT CCT GAT AAG AAA TAT AAA TCT GGA CAT AGA GAT ATT ATG
GGT TCA CGT ATT TCA GAC TGG AAT AGA GTC GGT TGG GGT GAA ATC
CCA ATG TCA CTC GGA TTT ACT TAT TCA TCT AAT ACA TTG ATG ATG
CAT TTA CAA GAT TTA GTT GGT GCA GAC AAA ATG AAA TCT TGG TAT
GAA CGA TTT GGA TTT GGA AAA TCA ACT AAA GGT ATG TTT GAT GGA
GAA GCA CCT GGT CAA ATT GGA TGG AGT AAT GAG TTG CAA CAA
AAA ACG TCA TCA TTT GGT CAA TCG ACA ACA GTA ACA CCT GTT CAA
ATG TTA CAA GCG CAA TCA GCG TTC TTT AAT GAT GGT AAT ATG TTA
AAA CCA TGG TTT GTG AAT AGC GTT GAA AAT CCT GTT AGT AAA AGA
CAA TTT TAT AAA GGG CAA AAA CAA ATC GCA GGC AAA CCA ATA
ACA AAA GAT ACT GCT GAA AAA GTT GAA AAG CAA TTG GAT TTA GTT
GTG AAT AGT AAG AAG AGT CAC GCT GCA AAC TAT CGT ATT GAT GGT
TAT GAG GTC GAA GGT AAG ACT GGT ACA GCA CAA GTC GCT GCA
CCT AAT GGT GGT GGA TAC GTT AAA GGT CCA AAC CCA TAT TTT GTA
AGT TTT ATG GGT GAC GCG CCG AAG AAA AAT CCT AAA GTT ATT GTA
TAC GCT GGT ATG AGC TTG GCA CAA AAA AAT GAC CAA GAA GCT TAT
GAA TTA GGT GTT AGT AAA GCG TTT AAA CCA ATA ATG GAA AAT ACT
TTG AAA TAT TTA AAT GTA GGT AAA TCA AAA GAT GAC ACA TCT AAT
GCA GAG TAT AGT AAA GTG CCA GAT GTT GAA GGT CAA GAC AAA
CAA AAA GCT ATT GAT AAT ATG AGT GCA AAA TCA TTA GAA CCA GTT
ACT ATC GGT TCT GGC ACA CAA ATA AAA GCA CAA TCT ATA AAA GCA
GGG AAT AAA GTC TTA CCT CAT AGT AAA GTA CTG TTA TTA ACA GAC
GGA GAC TTA ACT ATG CCT GAC ATG TCA GGA TGG ACG AAA GAA GAT
GTC ATT GCT TTT GAA AAC CTA ACA AAT ATT AAA GTA AAT TTA AAA
GGT AGC GGT TTT GTG TCC CAC CAA TCA ATT AGT AAG GGA CAA AAA
CTT ACT GAA AAA GAT AAA ATA GAC GTA GAA TTT TCA TCA GAG AAT
GTA GAC AGC AAT TCG ACG AAT AAT TCT GAT TCA AAT TCA GAT GAT
AAG AAG AAA TCT GAC AGT AAA ACT GAC AAG GAT AAG TCG GAC
TAAcaagaatggctacattaaataagggtgtattgtcactatttgtttttactaaagtaatgctg

tgatagaaaacaacatcttcatttaatctttataaataagtgttgatagagaattttaaggtaga
cgaggtcagattaacttgtttgttataggcaaacttttatactggcctcttatgccgtaaaaaa
gaaaaattataaattttgattacaactttgtttatt

Sense primer (56-76)

gat acg cga gga aag att gc 20 bases long
(5'-3')

cg content 10 at content 10

Anti-sense primer (2585-2605) (2605-2585)

ga ccg gag aat acg gca ttt 21 bases long ttt acg gca taa gag gcc ag
(3'-5') (5'-3')

cg content 10 at content 11

Primer Summary:

Gene	Primer	Strand	Est. Tm	GC %
PBP 1	gat acg cga gga aag att gc	(sense)	60	50
	ttt acg gca taa gag gcc ag	(anti-sense)	60	50
PBP 2	tcg aag tat ttt gga aga g	(sense)	52	37
	g tga atg act gat ttt acg	(anti-sense)	52	37
PBP 3	gta tga tta ctt gtt cgg tct c	(sense)	62	41
	tc acc aca ttc cgt aca agc	(anti-sense)	60	50
PBP 4	gag taa gtt tgc tct tcg	(sense)	52	44
	gt aca gaa ggc att tcg acg	(anti-sense)	60	50

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ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Dec. 2005, p. 5075–5080 Vol. 49, No. 12
0066-4804/05/$08.00+0 doi:10.1128/AAC.49.12.5075–5080.2005

β-Lactam Resistance in *Staphylococcus aureus* Cells That Do Not Require a Cell Wall for Integrity

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Received 4 May 2005/Returned for modification 13 July 2005/Accepted 18 September 2005

Staphylococcus aureus **ATCC 9144 cells with defective cell walls were generated on a medium with elevated osmolality in the presence of sublethal levels of penicillin G. On removal of antibiotic pressure, the cells exhibited stable penicillin and methicillin resistance. The resistance was homogeneous and its acquisition was enhanced following transient cell wall-defective growth. The resistant cells were *mecA* negative, β-lactamase negative and did not contain any mutations in the coding regions of *pbp* genes. When penicillin was added back to resistant cells, they continued to grow and produced a diffuse cell wall that was resistant to the action by lysostaphin but was very sensitive to lysis with Triton X-100. These data indicate that the resistant cells are not dependent upon an intact cell wall for osmotic stability and they are able to switch readily to this mode of growth in the presence of penicillin G.**

Originally called L-forms, cell wall-defective (CWD) bacteria were first described in *Streptobacillus moniliformis* (11). We now know that many bacteria can become cell wall defective in the presence of various agents, including cell wall-active antibiotics, and can be propagated indefinitely on suitable media. CWD bacteria have been reported under a number of conditions, including burn site infections (10), sarcoidosis (5), and culture-negative febrile episodes in bone marrow transplant patients (15). Indeed, the clinical importance of CWD bacteria may be underestimated, as they do not grow on routine bacteriological media and are resistant to antibiotics that act on the cell wall. Moreover, cell wall-defective variants of *Staphylococcus aureus* were shown to be ingested by rat peritoneal macrophages, without phago-lysosomal fusion and digestion (12), and should therefore be expected to evade the immune system by intracellular refuge.

In the presence of antibiotics, transient cell wall deficiency could also give bacteria time to develop stable antibiotic resistance. This idea is supported by a recent observation that the probability of obtaining β-lactamase-derepressed mutants of *Enterobacter cloacae* rose dramatically when the bacteria were incubated on a medium supporting the growth of CWD bacteria in the presence of ticarcillin (9).

In this paper we discuss an adaptive response of bacteria exposed to antibiotic pressure that has been frequently observed but little understood and its role in facilitating antibiotic resistance. We have demonstrated that transient penicillin-induced loss of the cell wall in *S. aureus* mediates high-level resistance to β-lactam antibiotics and that following recovery of the cell wall, the penicillin resistance is inherited in a stable manner. Furthermore, cells that had previously been cell wall defective had a propensity to lose their cell wall on further penicillin exposure.

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MATERIALS AND METHODS

Bacterial strains and growth. *Staphylococcus aureus* ATCC 9144 was used as a source of cell wall variants because it was β-lactamase negative and susceptible to penicillin (MIC, 0.015 mg/liter in broth dilution tests using CWD medium). *S. aureus* ATCC 25923 was used as an additional control for antibiotic susceptibility testing. Strains were maintained on Luria-Bertani medium (10% [wt/vol] Bacto Tryptone, 5% [wt/vol] Bacto-yeast extract, 10% [wt/vol] NaCl [pH 7.5]). Medium for the induction and growth of cell wall-defective variants (CWD medium) consisted of brain heart infusion broth (Oxoid, Basingstoke, United Kingdom) supplemented with sucrose 5% (wt/vol), yeast extract 0.5% (wt/vol), horse serum 10% (vol/vol), magnesium sulfate 0.2% (wt/vol). Penicillin G was included, at various concentrations, as described. The medium had an osmolality of 597 mosmol/kg as measured by the Advanced Micro-osmometer (model 3MO plus) using Clinitrol 290 as the standard. Where necessary, media were solidified by the addition of agar (1% [wt/vol]). Total cell counts were carried out on blood agar or CWD medium without added antibiotics.

Generation of cell wall-defective bacteria. *S. aureus* ATCC 9144 was streaked onto CWD medium in the presence of various concentrations of penicillin G and incubated at 37°C in air. The plate with the highest concentration of penicillin G that could support growth showed colonies with atypical morphology from which the cells stained gram negative. Cells growing at this penicillin concentration were picked and restreaked upon fresh plates containing further incremental increases in the antibiotic concentration.

CWD bacteria could also be efficiently induced in liquid medium of the same formulation; typically, 5-ml cultures were grown aerobically at 37°C with continuous agitation.

Antibiotic susceptibility testing. Bacteria were grown overnight at 37°C in air on CWD agar, supplemented with penicillin as appropriate. A suspension of each organism that gave semiconfluent growth was used for antibiotic susceptibility testing, which was performed according to the British Society for Antimicrobial Chemotherapy guidelines for disk diffusion, broth dilution, and E-tests (1). For disk-diffusion tests, disks contained either penicillin (1 μg) or oxacillin (1 μg). No differences were observed in zone sizes in the disk-diffusion test, for either *Staphylococcus aureus* ATCC 9144 or ATCC 25923, on CWD medium and on Iso-Sensitest agar.

Population analysis. The distribution of antibiotic resistance in cell populations was determined by spotting undiluted or appropriately diluted aliquots

(10 μl) onto duplicate CWD plates containing various concentrations of penicillin G. Plates were incubated at 37°C for 48 h before the colonies were enumerated.

Electron microscopy. Samples for transmission electron microscopy were prepared from cultures grown in CWD broth supplemented with penicillin as required. The cells were collected by centrifugation and fixed in 0.1 M sodium cacodylate buffer, 4% (vol/vol) paraformaldehyde, 2.5% (vol/vol) glutaraldehyde (pH 7.4). After 2 h cells were postfixed in cacodylate-buffered 1% (wt/vol) osmium tetroxide. After dehydration in a graded series of ethanol and propylene oxide, the bacterial cells were embedded in Araldite CY212. Thin sections (70 to 90 nm) were stained with uranyl acetate and lead citrate before examination using a Philips 400T microscope operating at 100 kV.

Sequencing of *pbp* genes. PCR primers, for each gene, were synthesized from consensus alignments of published *S. aureus* genome sequences. The primers were as follows: pbp1 sense primer, 5′-GATACGCGAGGAAAGATTGC-3′; pbp1 reverse primer, 5′-TTTACGGCATAAGAGGCCAG-3′, which produced a PCR product of 2,596 bp in length, including 172 bp of 5′ untranslated region (UTR) and 189 bp of 3′ UTR; pbp2 sense primer, 5′-TCGAAGTATTTTGGA AGAG-3′; pbp2 reverse primer 5′-GTGAATGACTGATTTTACG-3′, which produced a 2,504-bp product that included 160 bp of 5′ UTR and 163 bp of 3′UTR; pbp3 sense primer, 5′-GTATGATTACTTGTTCGGTCTC-3′; pbp3 reverse primer, 5′-CAACCATGCGCTACACAATC-3′, which produced a 2,228-bp product, including 119 bp of 5′ UTR and 33 bp of 3′ UTR; pbp4 sense primer, 5′-GAGTAAGTTTGCTCTTCG-3′; pbp4 reverse primer, 5′-GTACAG AAGGCATTTCGACG-3′, which produced a 1,679-bp product with 205 bp of 5′ UTR and 178 bp of 3′ UTR.

PCR was carried out for 30 cycles, with each cycle consisting of 94°C for 45 s, 50°C for 45 s, and 72°C for 2 min using KOD polymerase (Novagen). The PCR products were purified using the QIAquick PCR purification system (QIAGEN) and eluted in a volume of 50 μl. A-tails were added to purified PCR products using *Taq* polymerase in the presence of 0.3 mM dATP at 72°C for 10 min. The PCR products were ligated into the vector pCR TOPO TA (Invitrogen) and transformed into *Escherichia coli* XL1-Blue (Stratagene), and recombinant plasmids were selected on plates containing ampicillin and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside.

DNA was prepared for sequencing from overnight liquid cultures using the Wizard Plus SV DNA purification system (Promega) and was sequenced with an Applied Biosystems 373A DNA sequencer using a primer walking approach. Both strands of each gene were completely sequenced, and contiguous sequences were constructed using DNA Strider computer software.

PBP binding assays. Penicillin-binding protein (PBP) binding assays were carried out according to the method of Tonin and Tomasz (14) on purified membrane fractions. Membrane protein concentrations were estimated using the DC protein assay kit (Bio-Rad) using bovine serum albumin as standard. Labeled PBPs were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using a 10% gel and visualized by fluorography. PBPs on the autoradiograph were quantified using NIH Image v1.63 software.

Lysostaphin and Triton X-100 lysis assays. Cells were grown to mid-exponential phase in liquid medium, washed three times in 20 mM potassium phosphate (pH 7.5), and resuspended in the same buffer to an optical density at 620 nm of approximately 0.25. For lysostaphin assays, 1.0 ml of suspended cells was placed into a spectrophotometer at 37°C, and the change in absorbance was recorded continuously following the addition of 1 U of lysostaphin. For Triton X-100 assays, 10 ml of suspended cells was placed in a sterile 25-ml Erlenmeyer flask in a shaking water bath set at 37°C, and Triton X-100 was added to a final concentration of 0.1% (vol/vol). Samples were taken at intervals to monitor the change in absorbance. In both cases values were adjusted to a percentage of the initial absorbance.

Nucleotide sequence accession numbers. The nucleotide sequences determined in the present study have been deposited in GenBank and have the following accession numbers: *pbp1*, AY920399; *pbp2*, AY920400; *pbp3*, AY920401; *pbp4*, AY920402.

RESULTS

Cell wall-defective bacteria grow in the presence of penicillin. CWD variants of *S. aureus* were generated in a medium with elevated osmotic potential (597 mosmol/kg) in the presence of sublethal levels of penicillin G. *S. aureus* ATCC 9144 was incubated aerobically for up to 72 h on media containing a range of concentrations of penicillin G. Approximately 1 in 10,000 of the cells inoculated onto a plate containing half the MIC of antibiotic (0.0075 mg/liter) gave rise to colonies with a cell wall-defective phenotype. The colonies were small, had lost the yellow pigmentation typical of *S. aureus*, and were umbonate in profile. On Gram staining, the cells had indistinct margins, did not show typical staphylococcal cell arrangements, and were gram negative.

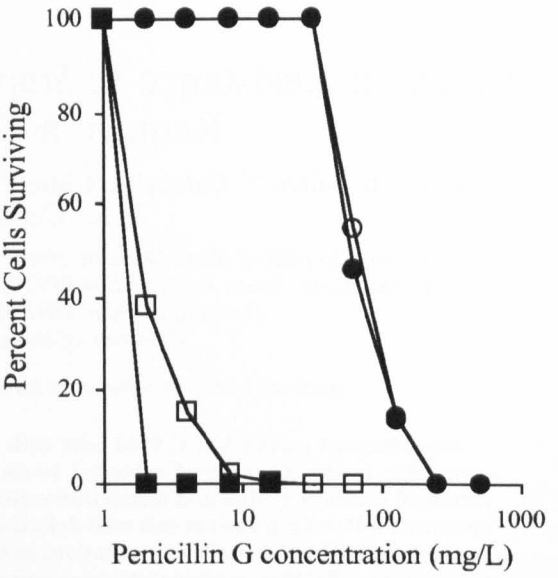

FIG. 1. Population analysis of *S. aureus* cells grown in the presence of penicillin on medium that either induced cell wall-defective variants (CWD medium) or did not (LB medium). CL3/19 (open circles), cells grown in CWD medium in the presence of penicillin G; RL3/19 (closed circles), CL3/19 cells passaged in CWD medium in the absence of penicillin G five times; LB9 (open squares), cells grown in LB medium in the presence of penicillin G; LB9/2 (closed squares), LB9 cells passaged in LB medium in the absence of penicillin G five times.

Cell wall-defective variants could be maintained by subculture on CWD medium containing penicillin. Transfer of the cells to blood agar, LB medium, or CWD medium without addition of penicillin caused them to revert to cell wall-competent (CWC) forms that stained gram positive (data not shown).

Acquired penicillin resistance is stable in CWC cells. The penicillin concentration was increased incrementally in two independent experiments to generate cell wall-defective variant lines on solid medium (CS1/19) and in liquid medium (CL3/19) that were able to grow in the presence of penicillin G at a concentration of 19.2 mg/liter. In order to determine whether the penicillin resistance phenotype of the CWD variants was due solely to loss of the antibiotic target, cells were then allowed to revert to CWC forms prior to antibiotic susceptibility testing by subculturing on CWD medium or LB medium without antibiotic. Following a single passage in the absence of antibiotic, CWC cells (designated RS1/19/1) stained uniformly gram positive. When tested by antibiotic disk diffusion, both CS1/19 and RS1/19/1 strains were resistant to penicillin G and oxacillin, whereas the parental strain, *S. aureus* ATCC 9144, produced large zones of inhibition (zone diameters, 32 mm ± 1.6 mm for penicillin G and 29 mm ± 1.4 mm for oxacillin). In view of the fact that RS1/19/1 might contain a

heterogeneous population, it was then subcultured 10 times on antibiotic-free medium, producing strain RS1/19/10, which was retested with the two β-lactam antibiotics. These cells also stained gram positive and remained completely resistant to the penicillin G- and oxacillin antibiotic-containing disks. Similar results were obtained when either LB or CWD medium was used for antibiotic-free growth. These results showed that the CWC cells had stable resistance to β-lactam antibiotics.

Acquisition of penicillin resistance is enhanced in cell wall-defective bacteria. To determine the extent to which stable high-level penicillin resistance was due to loss of cell wall integrity, *S. aureus* ATCC 9144 cells were exposed to sublethal levels of penicillin G in two different liquid media. One (CWD medium) permitted the cell wall-defective phenotype, and the other (LB medium) did not. Following nine serial passages in increasing concentrations of penicillin G (0.5-fold to 128-fold times the original MIC), the population profiles of the strain grown in CWD medium (CL3/19) and the strain grown in LB medium (LB9) were determined with various concentrations of penicillin G (Fig. 1). In this way the proportions of resistant cells and the maximum level of resistance within each population were determined.

The maximum concentration of penicillin G that LB9 cells were subjected to during the nine serial passages was 9.6 mg/liter. Throughout the experiment, these cells stained uniformly gram positive, indicating that there was no cell wall disruption. Population analysis determined that 0.45% of the cells were able to grow at 9.6 mg/liter of antibiotic, and the maximum concentration at which these resistant cells grew was 38.4 mg/liter.

In contrast, cells grown in CWD medium (CL3/19) showed extensive gram-negative staining early in the experiment, indicating establishment of a cell wall-defective phenotype. The maximum concentration of antibiotic that these cells were subjected to was 19.2 mg/liter, and analysis showed that 100% of the cells in the population were able to grow at that concentration of antibiotic. The maximum concentration of penicillin G that these cells were able to tolerate was 76.8 mg/liter, and this degree of resistance was shown by 13.5% of the population. Clearly, the level of resistance to penicillin and the extent of resistance within the population were dramatically enhanced when cells had defective cell walls.

The stability of penicillin resistance in the two cell populations was tested following a total of five serial passages on the same media in the absence of antibiotic (Fig. 1). The strain that was grown in LB medium (LB9/2) showed a lower resistance to penicillin. Approximately 1 in 10^6 cells in this population was able to grow in the presence of 9.6 mg/liter penicillin G, the maximum concentration at which growth was recorded, showing that resistant cells had been lost from the population in the absence of antibiotic selection pressure. In contrast, 100% of cells in the strain that had been cell wall defective and subsequently recovered cell walls (RL3/19) were resistant to 19.2 mg/liter penicillin G, and the maximum concentration at which growth was recorded was 307.2 mg/liter. The transient loss of cell wall integrity had therefore enabled the cells to develop homogeneous, stable, high-level penicillin resistance. E-tests and broth dilution MIC determinations confirmed that the CWD strain CL3/19 and its CWC derivative (RL3/19) are resistant to high levels of penicillin G (Table 1).

TABLE 1. Penicillin G MICs of strains, determined by E-tests and broth dilution

MIC test/medium	MIC (mg/liter)				
	Wild type (ATCC 9144)	CL3/19	RL3/19	LB9	LB9/2
E-test/CWD	<0.016	192	>256	16	1.5
E-test/Iso-Sensitest	<0.016	192	>256	16	0.75
Broth dilution/CWD	0.016	64	128	ND	ND

[a] ND, not determined.

CWC cells showed no difference in susceptibility to vancomycin, which is a cell wall-active antibiotic with a different target site to that of β-lactams, compared to wild-type cells (MIC, 2 mg/liter). However, following growth of CWC cells in the presence of penicillin and concomitant changes to the cell wall structure, they had a decreased susceptibility to vancomycin (MIC, 512 mg/liter).

CWC cells exhibit *mecA*-negative, β-lactamase-negative methicillin resistance. CWC cells were tested for their susceptibility towards oxacillin, using E-test strips, and were found to have a MIC of 192 mg/liter. In order to establish the cause of this resistance, a series of investigations were undertaken to determine the presence or absence of known resistance mechanisms in these cells.

A duplex PCR using primers designed to amplify the *mecA* gene (3) and the *Staphylococcus*-specific *nuc* gene (4) showed that the *mecA* gene was absent. The cells were also tested with the Beta Test kit (Medical Wire and Equipment Co., Bath, United Kingdom) and were negative for β-lactamase activity. Furthermore, incubation of exponentially growing CWC cells with penicillin G did not inactivate the antibiotic (data not shown), confirming the absence of β-lactamase activity and excluding the presence of another antibiotic-inactivating activity.

Alterations in the coding region of penicillin-binding proteins, notably PBP2, have been implicated in non-PBP2a-mediated methicillin resistance (7). To investigate this possibility, the four *pbp* genes were cloned and sequenced from both wild-type *S. aureus* ATCC 9144 and the penicillin- and methicillin-resistant CWC strain RS3/19. No differences in the DNA sequence of any of the PBP genes were identified between the methicillin-sensitive wild-type strain and the methicillin-resistant CWC derivative. The lack of mutation in the coding and adjacent regions shows that the observed resistance is not due to alterations in the affinity of PBPs for penicillin or in regulatory elements immediately upstream of the genes.

To confirm that the CWC strain did not contain PBPs with reduced affinity for penicillin, 35 μg of membrane proteins from wild-type and CWC strains was incubated with [^{3}H]benzylpenicillin according to the method of Tonin and Tomasz (14). Separation of the membrane proteins by SDS-PAGE and fluorography showed that the CWC strain had increased binding of penicillin to PBP4 (Fig. 2). Densitometric analysis of the penicillin-labeled bands showed that the amount of binding to PBP1, -2, and -3 was similar in wild-type and CWC membrane proteins, whereas binding to PBP4 in the CWC strain was

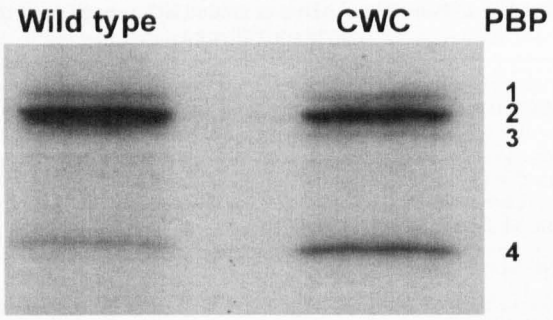

FIG. 2. Binding capacity of PBPs of a penicillin-resistant CWC line and its parent strain for benzylpenicillin. Membrane preparations were exposed to [3H]benzylpenicillin and separated by SDS-PAGE prior to fluorography.

2.75-fold greater than in the wild type. This greatly alters the ratio of penicillin-binding capacity between PBP2 and PBP4 from 4.6 in the wild type to 1.5 in CWC membrane proteins. As no mutations were detected in any *pbp* genes, the increased penicillin binding is likely to be due to an increase in the amount of PBPs in the membrane. This contention is supported by an observation from DNA microarray analysis that *pbp4* mRNA expression is 18-fold higher in CWC cells than in wild-type cells, whereas the other *pbp* genes are expressed at comparable levels in these two strains (T. Fawcett, unpublished data).

CWD variants do not rely upon peptidoglycan for cell integrity. Transmission electron microscopy of ultrathin sections demonstrated that cells grown in the presence of sublethal levels of penicillin G had a disorganized and incomplete cell wall and many cells had more than one division plane. The splitting system (for a review of staphylococcal cell wall morphogenesis, see reference 6) was absent from the cross wall of dividing cells, there was excessive production of wall material, and the margins of cells were indistinct. In contrast, the periphery of each wild-type *S. aureus* cell appeared as a compact structure with cross walls clearly showing the splitting system.

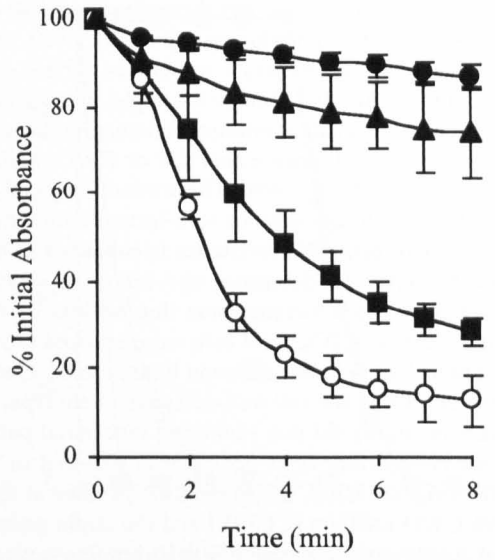

FIG. 4. Lysostaphin lysis of cells. Results shown are for lysostaphin treatment of wild-type cells (open circles), CWD cells (closed circles), CWC cells (closed squares), and CWC cells grown in the presence of penicillin (closed triangles).

On removing the antibiotic pressure, the appearance of CWC forms was indistinguishable from that of wild-type cells, but following growth in the presence of penicillin CWC cells showed major alterations to the cell wall structure. The walls of CWC cells grown in the presence of penicillin appeared similar to CWD cell walls, being thickened and diffuse and with evidence of more than one division plane in a single cell (Fig. 3).

In further experiments to explore the cell surfaces, two lysis methods were performed on bacteria grown in liquid medium: the first employed lysostaphin, a peptidase that cleaves the pentaglycine cross-bridge in staphylococcal peptidoglycan, and the second used the detergent Triton X-100. When wild-type cell suspensions were incubated in the presence of lysostaphin, rapid lysis was observed, which was followed spectrophoto-

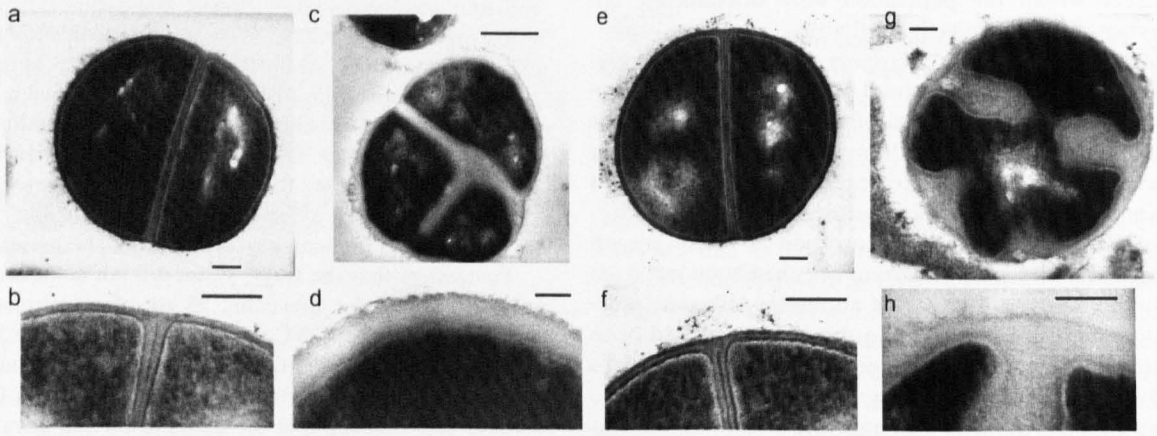

FIG. 3. Transmission electron micrographs of wild-type and cell wall-defective variants of *S. aureus*. (a and b) Wild-type cell wall appeared as an electron-dense structure at the periphery of the cell; nascent cell walls were also visible in dividing cells. (c and d) CWD cells showed a thickened and diffuse cell wall, with no visible splitting system and many cells having greater than one division plane. (e and f) The CWC strain had a cell wall structure visibly indistinguishable from wild-type cells. (g and h) On addition of penicillin to CWC cells, the cell wall became diffuse and cells had greater than one division plane. Bars, 0.1 μm.

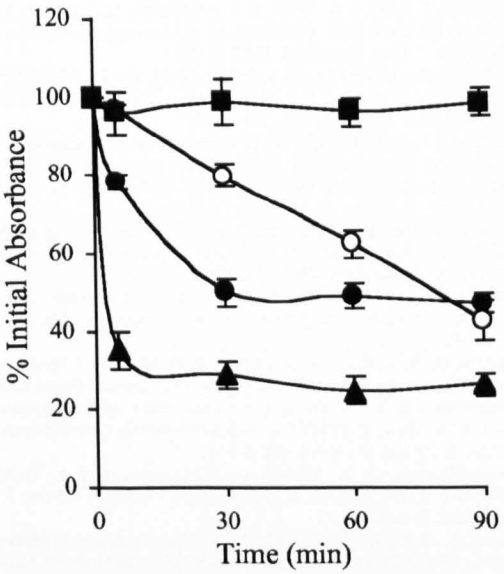

FIG. 5. Triton X-100 lysis of cells. Results shown are for Triton X-100 treatment of wild-type cells (open circles), CWD cells (closed circles), CWC cells (closed squares), and CWC cells grown in the presence of penicillin (closed triangles).

metrically at 620 nm (Fig. 4). CWD cells, however, did not show an increase in the rate of lysis following addition of lysostaphin and are thus not reliant on the presence of peptidoglycan for their intactness. On addition of lysostaphin to the CWC cell line, RL3/19, a decrease in absorbance was observed, demonstrating that these cells have an intact peptidoglycan structure that is integral to their intactness. CWC cells were slightly more resistant to the action of lysostaphin than wild-type cells, suggesting that there may be alterations in the peptidoglycan structure in this line. This change in lysostaphin susceptibility may be due to alteration of the target site for lysostaphin or to an increase in the number of pentaglycine cross-links in the peptidoglycan. Lysostaphin treatment of CWC cells that had been grown in the presence of penicillin caused a slow loss of absorbance with similar kinetics to that shown by CWD cells.

In contrast to lysostaphin treatment, CWD cell suspensions lysed rapidly when incubated with 0.1% Triton X-100, presumably because their membranes were more accessible to the detergent than were those of wild-type cells. CWC cells were resistant to lysis by Triton X-100, indicating that the recovery of a cell wall was impeding access of the detergent to the cell membrane. However, when grown in the presence of penicillin, CWC cells were highly sensitive to lysis by Triton X-100 (Fig. 5).

CWD cells have an altered cell wall appearance, and their resistance to the action of lysostaphin and their increased susceptibility to Triton X-100 show that CWD bacteria do not rely on an intact peptidoglycan structure for their integrity. This phenotype is mimicked only when CWC cells are grown in the presence of penicillin.

DISCUSSION

Cell wall-defective variants of *S. aureus* ATCC 9144 were formed readily on complex media with a high osmolality in the presence of the cell wall-active antibiotic penicillin G. The resulting colonies had an unusual appearance, including loss of the golden pigmentation, and lacked an organized cell wall structure. The CWD bacteria were resistant to β-lactam antibiotics, and this resistance persisted in a stable manner when cells were allowed to regain their cell wall integrity following subculture in the absence of antibiotic. The acquisition of high-level, stable resistance was only observed in those cells that had been cell wall defective and not in control cells grown on media that did not allow the formation of CWD bacteria. A similar phenomenon has been observed in the acquisition of derepressed β-lactamase mutants in *Enterobacter cloacae* (9). Population analysis showed that the resistance was present in 100% of the cells.

Resistance to β-lactam antibiotics in CWD bacteria was expected, but if this resistance had been due solely to loss of the antibiotic target, susceptibility to these antimicrobial agents should have been regained on recovery of the cell wall. However, following growth of CWC cells for many generations in the absence of antibiotics, the cells remained resistant to methicillin as well as penicillin G.

Decreased microbial susceptibility to β-lactam antibiotics has been widely reported, and a number of resistance mechanisms have been demonstrated. In staphylococci the most widely recognized mechanism is the *mecA* gene, which encodes PBP2a, a PBP2 variant with low penicillin-binding affinity. Resistant strains usually also contain an inducible β-lactamase. Borderline methicillin resistance has also been recognized in clinical isolates that do not contain either the *mecA* gene or a β-lactamase (2, 13) but have mutations in the *pbp* genes that cause reduced penicillin binding (7). Each of these mechanisms has been eliminated as an explanation for the resistance we observed in CWC cells. The increase in penicillin binding observed in CWC cells is likely to be due to alterations in the regulation of the gene rather than to mutations in the coding sequence. Overproduction of PBP4 has been previously reported to increase resistance to β-lactams, with the levels of methicillin resistance increasing from two- to sixfold in various strains (8). Though an increase in the level of PBP4 in cells contributes to methicillin resistance in a small way, this alone cannot account for the high-level resistance observed in CWC cells.

Uniquely, we have observed that after reintroduction of penicillin the integrity of CWC cells does not depend on their reconstituted cell walls. The CWC cells have a compact cell wall structure that responds to lysostaphin treatment in a manner similar to wild-type cells, with rapid cell lysis. However, on treatment with penicillin, CWC cell walls appear as diffuse structures, and the cells maintain their integrity in the presence of lysostaphin. CWD and CWC cells, grown in the presence of penicillin, are extremely sensitive to lysis by Triton X-100, likely due to increased access to the membrane because of the diffuse nature of the call wall.

Interestingly, CWC cells are not resistant to other classes of cell wall-active antibiotics unless they are first grown in the presence of penicillin. This suggests that their ability to switch to a mode of growth that does not rely upon the presence of a cell wall for survival may be β-lactam specific. Together, these data indicate that the cells have undergone stable genotypic changes that allow them to avoid the action of β-lactam anti-

biotics by quickly and uniformly dispensing with the need for an intact peptidoglycan sacculus for osmotic stability. Furthermore, cells with a defective cell wall become resistant to other cell wall-active antibiotics and are able to grow and divide. The ability of these cells to survive may be an important bacterial response to attack by cell wall-active agents.

ACKNOWLEDGMENTS

We thank Christine Richardson in the School of Biological and Biomedical Sciences, University of Durham Microscopy Unit, for advice and technical assistance with electron microscopy. The PCR analysis of the *mecA* gene was carried out at the Scottish MRSA Laboratory, Stobhill Hospital, Glasgow, Scotland.

This work was supported by a grant from the University Hospital of North Durham.

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