Preliminary analysis of the genetic basis for vancomycin resistance in Staphylococcus aureus strain Mu50

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Glycopeptides, such as vancomycin, are frequently the antibiotics of choice for treatment of infections caused by the now common methicillin-resistant Staphylococcus aureus (MRSA). Incidences of vancomycin resistance in S. aureus (VRSA) have been increasing worldwide for the last 5 years. Complex mechanisms producing changes in cell wall content and composition generate the VRSA phenotype, but the genetic basis of these changes has not yet been determined. To facilitate the genetic investigation, entire genome sequences of the archetypal VRSA (Mu50), and vancomycin-susceptible MRSA strains N315, EMRSA 16 and COL were compared. The in silico analysis revealed several loss-of-function mutations in Mu50, affecting important cell wall biosynthesis and intermediary metabolism genes, not previously reported. The new findings provide further evidence for the hypothesis that vancomycin resistance in Mu50 is due to fundamental changes, important to metabolic pathways that impinge on peptidoglycan biosynthesis. These observations will inform targeted experiments aimed at a complete understanding of the mechanism(s) of vancomycin resistance in S. aureus Mu50 and other VRSA strains.

Background

Staphylococcus aureus has long been recognized as a major cause of hospital-acquired infection. Over the last decade, methicillin-resistant S. aureus (MRSA) strains have become endemic in hospitals worldwide, including in the UK. Accordingly, the frequency of MRSA isolation has increased dramatically. Glycopeptides, such as vancomycin, are often the therapeutic drugs of choice for serious MRSA infections. However, failures of vancomycin therapy against S. aureus, due to the emergence of strains that are significantly less susceptible to vancomycin [vancomycin-resistant S. aureus (VRSA)], are now well established. The first clinical VRSA, Mu50, was isolated in Japan in 1997. The VRSA phenotype of Mu50 is the result of changes to the cell wall structure. In comparison with MRSA strains, Mu50 and Mu50-like strains have a thickened cell wall, release more cell wall material into the culture medium and have increased rates of autolysis. The fine structure of the Mu50 cell wall is similar to that of an MRSA strain such as N315, except that the Mu50 peptidoglycan chains show significantly less cross-linking, and an increased content of pentapeptide chains. The amount of glutamine-non-amidated muropeptide subunits (i.e. those containing D-glutamate rather than D-glutamine) in the Mu50 cell wall increases, and it has been proposed that the reason for decreased cross-linking is that non-amidated muropeptides are poorer substrates for transpeptidases than amidated ones, although this is yet to be confirmed. It is believed that the reason such changes in cell wall thickness and cross-linking result in vancomycin resistance is that the modified cell wall binds more vancomycin, due to the increased amount of terminal D-alanyl-D-alanine dipeptide. To understand the mechanism of vancomycin resistance in VRSA strains, it is obviously necessary first to understand how these microbes synthesize peptidoglycan.

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Production of UDP-N-acetyl glucosamine and UDP-N-acetyl muramic acid, the committed steps of peptidoglycan synthesis

The cardinal component of peptidoglycan synthesis is the high-energy sugar compound, UDP-N-acetyl glucosamine (UDP-GlcNAc). Glucosamine (GlcNH$_2$) is present in complex growth media, and can be imported via specific transport systems and converted to GlcNH$_2$-6-phosphate (GlcNH$_2$-6-P). Alternatively, GlcNH$_2$-6-P can be manufactured from fructose-6-P. GlcNH$_2$-6-P is then modified to GlcNH$_2$-1-P and acetylated and energized to UDP-GlcNAc. UDP-N-acetyl muramic acid (UDP-MurNAc) is made by a two-step modification of UDP-GlcNAc via an intermediate, UDP-GlcNAc-enolpyruvyl transferase; MurB, UDP-MurNAc dehydrogenase.

Figure 1. The production of murein monomers in S. aureus. The figure illustrates two pathways for the production of GlcNH$_2$-6-P. One is through the phosphotransferase-dependent import of GlcNH$_2$, the other is through the amidation of fructose-6-P. Enzyme abbreviations are: GPI, glucose-6-P isomerase; GlmS, glucosamine synthase; GlmM, Gln-6-P mutase; MurA, UDP-GlcNAc-enolpyruvyl transferase; MurB, UDP-MurNAc dehydrogenase.

Figure 2. Production of L-glutamine in S. aureus. L-Glutamine is the key store of ammonia in the bacterial cell, and can only be synthesized in the way illustrated. Enzyme abbreviations are: SDH, succinate dehydrogenase; KDH, 2-ketoglutarate dehydrogenase; GTM, 2-ketoglutarate transaminase (glutamate synthase); GR, glutamate reccemase; GS, glutamine synthetase. GS activity is increased by 2-ketoglutarate and L-glutamate, and inhibited by L-glutamine and GlcNH$_2$-6-P.

The proposed involvement of D-glutamate and D-glutamine in peptidoglycan cross-linking

In S. aureus, the UDP-MurNAc moiety is sequentially loaded with L-alanine, D-glutamate, L-lysine and D-alanyl-D-alanine dipeptide to form UDP-MurNAc-pentapeptide, which transfers to C$_{55}$-undecaprenylphosphate to form lipid I of the cytoplasmic membrane peptidoglycan transporter, releasing UMP in the process (Figure 1). D-Glutamate and D-alanine are formed by specific racemases from L-glutamate and L-alanine, respectively. Three further modifications to MurNAc-pentapeptide are then effected: (i) GlcNAc is added to the C$_{55}$-undecaprenylphosphate using UDP-GlcNAc as the donor—the result is the conversion of lipid I into the lipid II state; (ii) five glycine residues are sequentially added to form a pentaglycine side chain attached to the L-lysine component of the pentapeptide, using glycine-tRNA as the amino acid donor; (iii) the D-glutamate residue is amidated on the carboxyl to D-glutamine. This last reaction uses L-glutamine as the amino group donor, and will only occur if the concentration of L-glutamine in the cell is high enough. This amidation reaction is not essential for transfer of lipid II to the outside of the cell membrane, but may influence subsequent reactions. Once transferred to the outer face of the cytoplasmic membrane, the GlcNAc-MurNAc-pentapeptide-pentaglycine precursors are joined to nascent peptidoglycan chains by transglycosylases, which link the sugar moieties. Transpeptidases then join the pentaglycine side chain of one monomer to the D-alanine residue in the penultimate position of a pentapeptide on a neighbouring polysaccharide chain, releasing the terminal D-alanine.
Mutations in *Staphylococcus aureus* Mu50

The analysis of coding differences between *S. aureus* isolates, Mu50 and N315

The Mu50 cell wall is much thicker and more loosely cross-linked than in vancomycin-susceptible strains, and the content of D-alanyl-D-alanine dipeptide is increased.\(^5,7\) It is likely that this reflects increased murein monomer production, though the activities of the enzymes involved have not been measured. Reduced cross-linking could reflect a lower concentration of L-glutamine in the Mu50 cytosol,\(^6-9\) but there is little specific evidence that it does. Accordingly, it was hoped that an analysis of the differences between the genome sequences of Mu50 and the MRSA strain N315 would point the way to a greater understanding of vancomycin resistance in Mu50 by indicating specific metabolic steps that are altered in Mu50. The paper describing these sequences focused on major differences in multidrug resistance (i.e. the presence of known resistance determinants), virulence and antigenic determinants. It did not directly address the question of vancomycin resistance,\(^15\) so in our study, we have reanalysed the sequences with a view to increasing our understanding of the mechanism of vancomycin resistance in Mu50.

To determine all coding differences between the genomes of Mu50 and N315 (i.e. those that alter protein sequences), the complete sequence flat-files (i.e. the files containing all the information provided by those who determined the sequences) for the Mu50 and N315 genomes were downloaded immediately following publication of the original report.\(^15\) These flat-files were manipulated in Microsoft Word 97 using a specially written macro that eliminates all non-amino acid sequence information to produce a ‘proteome’ for each genome. Each proteome represents a single, uninterrupted amino acid sequence comprising all of the predicted open reading frames (ORFs) joined end to end. In blocks of 30 000 amino acids, the two proteomes were compared using a CLUSTAL_W alignment tool and all differences were noted. The identity of each altered ORF was then determined using standard BLAST analysis.

In this initial analysis, 328 individual differences were noted between the two proteomes. Further sequence analysis (M. B. Avison, unpublished) revealed that many of the differences initially detected reflected errors in the published Mu50 genome sequence. Subsequently, the GenBank flat-files representing Mu50 and N315 have been reanalysed.

Figure 3. Peptidoglycan processing in *S. aureus*. Newly synthesized GlcNAc-MurNAc-pentapeptide-pentaglycine subunits are joined together using transglycosylases (which link the sugar moieties) and incorporated into the existing peptidoglycan network using transpeptidases (which cross-link the pentapeptides using the pentaglycine side chains). The final carboxypeptidase step is only required if the terminal pentapeptide remains uncross-linked. The presence of D-glutamine at position 2 may be required for efficient transpeptidase activity.
corrected. We would suggest that anyone who used the genome sequence data from the original report treats it with extreme caution, downloads the most recent flat-file version available and repeats their analysis. With the corrected sequences, 164 individual differences between the proteomes of Mu50 and N315 were found. Of these, 114 are minor, i.e. single amino acid substitutions, and their effects cannot be predicted, though it cannot be assumed that they are neutral. Accordingly, each product would have to be investigated individually. DNA sequences for the remaining 50 Mu50 ORFs showing more marked differences from those in N315 (deletions, frameshift mutations, truncations, etc.) were obtained. These were then compared with genome sequence data from MRSA COL (obtained from the TIGR website, http://www.tigr.org/tdb/mdb/mdb.html) and EMRSA 16 (obtained from the Sanger centre website, http://www.sanger.ac.uk/Projects/S_aureus/) as additional comparators. In 13 instances, it was the sequence in N315 that was found to differ from those in the other comparators, so these changes could not be linked to the VRSA phenotype. Where the Mu50 genomic copy was found to differ from those in the three MRSA strains (37 instances), many of the changes were located in ‘hypothetical’ ORFs, and genes clearly unrelated to vancomycin resistance (e.g. endotoxin genes and antigenic determinants, etc.). When these are eliminated, a total of 17 loss-of-function mutations (i.e. ORFs harbouring internal frameshifts or large deletions) specific to the Mu50 genome in genes encoding characterized functions were identified (Table). A link between five of these disruptions and the biochemical differences that have been previously noted between Mu50 and MRSA strains is compelling.

### An increased flow of carbon into peptidoglycan biosynthesis in Mu50?

One mutation in Mu50 that stands out is the complete disruption of the *murA* gene, encoding UDP-GlcNAc-enolpyruvyl transferase, which mediates the committed reaction in UDP-MurNAc synthesis (Figure 1). Deletion of *murA* is not a lethal event in *S. aureus* and several other Gram-positive bacteria, because there is redundancy of function (a similar enzyme being encoded by the *murZ* gene). *murZ* is intact in Mu50, so UDP-MurNAc production is presumably assured. The effect of the *murA* mutation on the production of UDP-MurNAc cannot be predicted, but it may alter the dynamics of the GlcNH₂ pool. A second mutation in Mu50 that bears on this is disruption of *mrp* (Table). Insertional inactivation of *fmtB* is known to result in decreased methicillin resistance in MRSA, with no obvious effect on vancomycin susceptibility. The loss of FmtB can be compensated by overexpression of glucosamine mutase (GlnM) and is silent during growth in media containing GlcNAc, though the mutation does not seem to impinge on GlnM activity directly. It is possible, therefore, that the disruption of *mrp* in Mu50 somehow compounds the loss of *murA* to alter the initial stages of peptidoglycan biosynthesis (Figure 1). Given the overproduction of peptidoglycan by Mu50, the overall rate of UDP-MurNAc-pentapeptide production must be somewhat greater than in N315, and it is known that the UDP-MurNAc-pentapeptide pool level is larger in Mu50 than in N315. Hence, if the *mrp* and *murA* knockout mutations are involved, they must result in an increased overall rate of peptido-
glycan synthesis. How this might be achieved is not clear, but specific biochemical experiments can now be targeted at discerning the role of these mutations, if any, in the VRSA phenotype of Mu50.

Two further changes to intermediary metabolic enzymes are notable. The presumed loss of 2-ketoglutarate dehydrogenase (Kdh) and succinate dehydrogenase (Sdh) due to disruption of odhA and sdhB, respectively, would be predicted to disrupt the TCA cycle in Mu50. At first sight, the principal casualty of this would be the loss of succinyl-CoA production, which is essential to the cell. However, in addition to Kdh, S. aureus has a second enzyme that converts 2-ketoglutarate into succinyl-CoA, namely 2-ketoglutarate oxidoreductase. The gene encoding this enzyme is intact in Mu50. Furthermore, S. aureus produces succinyl-CoA synthase, which can convert succinate into succinyl-CoA; the supply of succinate can be maintained from other metabolic pathways.

It has been reported that GS activity in Mu50 is higher than in N315. Given that 2-ketoglutarate activates E. coli GS via protein modification, it is possible that 2-ketoglutarate levels are higher in Mu50 than N315 as a result of Kdh inactivation, but this has not been confirmed biochemically. Interestingly, a putative 2-ketoglutarate exporter has also been inactivated in Mu50 (Table). This change would prevent excess 2-ketoglutarate, if present, from being effluxed from the cell, maintaining the larger cytoplasmic 2-ketoglutarate pool. In addition to the increased activity of GS, it has been reported that the activity of glucosamine synthase is increased in Mu50. These findings suggest that Mu50 is geared to direct more carbon into synthesis of GlcNAc, and thence into peptidoglycan, than N315, and the genetic changes revealed here may provide an explanation for the biochemical observations.

It has been proposed that one side-effect of hyperproduction of GlcNAc would be a shrinking of the L-glutamine pool, because it would be used faster to produce glucosamine. This, in turn, would mean that the amount of L-glutamine available for amidation of D-glutamate in cell wall precursors would be reduced. The levels of L-glutamine in Mu50 and N315 have not been determined, however, and an analysis of whether GS is working at full capacity in Mu50 is yet to be performed. The observed disruption of the genes encoding Sdh and Kdh, if confirmed in other VRSA strains, does fit with the hypothesis that perturbation in this branch of intermediary metabolism is important in the VRSA phenotype of Mu50. Following this genetic analysis, biochemical experiments can now be targeted towards determining whether this hypothesis is correct.

**A hypermutable phenotype in Mu50?**

One other interesting finding to emerge from this analysis of the Mu50 genome is that there is a loss-of-function mutation in mutS, a gene encoding a DNA repair protein. It has been shown in several different bacteria that loss of this function leads to a hypermutable phenotype, i.e. the overall rate of spontaneous mutation is 100-fold higher than in a mutS+ strain. Such a phenotype has been associated with the development of resistance to certain antibiotics, where there is a strong survival pressure to generate resistant mutants, even if the hypermutable phenotype of the strain makes it less fit. The perception of loss-of-fitness clearly emerges from our in silico analysis of Mu50. In addition to those already described, there are a number of mutations in genes encoding biosynthetic enzymes and other important proteins (Table), but it is difficult to rationalize a role for these mutations in the VRSA phenotype. It is well documented that Mu50 grows more slowly than N315, which may be explained by the loss of such important functions.

**Conclusions**

The exercise of comparing the coding sequences of Mu50 with those of a number of vancomycin susceptible MRSA isolates has proved to be revealing. It has been hypothesized that the basis of the VRSA phenotype in Mu50 is an increased level of peptidoglycan biosynthesis, resulting in a considerably thicker cell wall. In addition to this, there is a reduced level of glutamate amidation in the cell wall, which may be responsible for reduced cross-linking and the reduced ability of D,D-carboxypeptidases to act on the peptidoglycan network, conserving D-alanyl-D-alanine terminal dipeptides, which bind and sequester vancomycin. Our analysis has identified loss-of-function mutations in genes encoding enzymes responsible, in part, for the committed stage of peptidoglycan biosynthesis and for the manufacture of intermediary metabolites that are precursors of glutamine, and hence glucosamine. These findings will inform future targeted study of the biochemistry of vancomycin resistance in S. aureus.

The analysis is necessarily limited in a number of ways. There may well be other DNA sequence differences in Mu50 that do not cause coding changes but do influence susceptibility to vancomycin, such as mutations in promoter regions or transcription regulatory sites that affect the expression of relevant genes. The analysis of global gene expression in VRSA strains is in progress, although limited analysis of Mu50 has yielded some interesting results. A major limitation is that, of necessity, the genetic analysis only relates to one VRSA strain, Mu50. An investigation to determine whether mutations similar to those found in Mu50 are also present in other clinical VRSA strains is underway.

There is still much to do in our quest to understand how the susceptibility of S. aureus to vancomycin is reduced. Clearly the mechanism is complex, and is likely to confer VRSA with a fitness disadvantage compared with vancomycin-susceptible S. aureus when growing in the absence of
vancomycin. Such is the pressure exerted by vancomycin use, however, that these disadvantages have been tolerated by Mu50. In time, these disadvantages may be rectified and, as a consequence, the prevalence of vancomycin resistance seen in hospitals will rise.

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