Exposure of vancomycin-sensitive *Staphylococcus aureus* to subinhibitory levels of vancomycin leads to upregulated capsular gene expression

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ABSTRACT

Reduced vancomycin susceptibility in *Staphylococcus aureus* continues to trouble clinical microbiologists and infectious disease specialists. In this study, a vancomycin-susceptible *S. aureus* (VSSA) strain, which was methicillin-resistant (MRSA), was grown with and without subinhibitory levels of vancomycin, and the transcriptional profiles were determined by microarray analysis. Thirty-six genes were upregulated and 42 genes were down-regulated by more than two-fold ($P \leq 0.05$) in the presence of vancomycin. Many of these genes are involved in cell-wall biosynthesis and regulation, but of particular interest was the upregulation of genes in the locus responsible for capsule synthesis. Increased capsule production following exposure of MRSA to low levels of vancomycin could explain treatment failure. This suggests that selected genes of the capsule locus could be used as diagnostic targets for monitoring patients undergoing treatment with vancomycin therapy, as an increase in their expression may indicate progressive development of low-level resistance.

KEY WORDS: Capsule, bacterial.
Gene expression.
Microarray analysis.
Staphylococcus aureus.
Vancomycin.

Introduction

The intensive use of vancomycin in treating methicillin-resistant *Staphylococcus aureus* (MRSA) has led to the emergence of isolates with reduced susceptibility to vancomycin (vancomycin intermediate susceptibility *S. aureus* [VISA]) and heterogeneous vancomycin intermediate susceptibility *S. aureus* (hVISA). However, vancomycin resistance mechanisms, optimal diagnostic approaches and suitable treatment regimens for VISA and hVISA cases are incompletely understood.[1]

It is concluded that patients with the hVISA phenotype are more likely to have high bacterial load infections, initially low vancomycin serum concentrations, and longer in-patient stay,[2] while the hVISA phenotype is associated with longer periods of bacteraemia and greater prevalence of complications.[3]

It is assumed that because of the long clinical vancomycin exposure times required to generate resistance, multiple genes and probably multiple metabolic pathways have been altered.[4] It is possible that such changes may begin to occur during vancomycin therapy for MRSA.

Therefore, this study aims to investigate the effect of sub-minimum inhibitory concentration (MIC) levels of vancomycin on gene expression in a vancomycin-susceptible *S. aureus* (VSSA) strain.

Materials and methods

An EMRSA-15 strain (epidemic MRSA) was identified as VSSA after measuring vancomycin MIC as 0.5 µg/mL. The isolate was grown in tryptic soy broth containing no vancomycin (control) or 0.25 µg/mL vancomycin (test) using three biological and two technical replicates. Aliquots (1.5 mL) were taken from the mid-log-phase culture and transferred immediately into 3 mL RNA Protect reagent (Qiagen) to stabilise the RNA, following the manufacturer's instructions. The QIAamp DNA mini kit (Qiagen) was used to extract DNA from the EMRSA-15 strain, and this was used as control material for the microarrays.

Sample labelling and microarray analysis was performed using the standard protocol of the Bacterial Microarray Group at St. George's, University of London (BµG@S). Briefly, complementary DNA (cDNA) was synthesised from total RNA samples and fluorescently labelled with Cy5 using random primers and SuperScript II reverse transcriptase. Cy3-labelled genomic DNA was prepared using random primers and Klenow DNA polymerase. The Cy3- and Cy5-labelled samples were prepared for each microarray slide, co-purified using MiniElute columns (Qiagen) and co-hybridised to the BµG@S SAv1.1.0 microarray. The array design is available in the BµG@Sbase (Accession Number: A-BUGS-17; http://bugs.sgul.ac.uk/A-BUGS-17) and also ArrayExpress (Accession Number: A-BUGS-17).

The slides were scanned with a Genepix 4000B scanner (Axon instruments, UK). Raw microarray images were analysed using the BlueFuse for Microarrays 3.3 extraction software (BlueGenome, UK). Median normalisation and further statistical analysis was performed in GeneSpring

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Table 1. An ordered list of genes seen to undergo a greater than two-fold upregulation in expression when the VSSA strain was grown in vancomycin, as compared to gene expression in the absence of vancomycin.

Gene product	Gene	Change	Functional category
Carbamoyl-phosphate synthase large subunit	*pyrAB*	6.07	Metabolism
Putative ABC transporter, ATP-binding protein	SAR0205	5.62	Transport
Putative aldehyde dehydrogenase	SAR0169	4.48	Metabolism
Capsular polysaccharide synthesis enzyme	*capO*	4.48	Cell envelope
Capsular polysaccharide synthesis enzyme	*capD*	4.15	Cell envelope
Putative extracellular sugar-binding lipoprotein	SAR0206	3.82	Transport
Capsular polysaccharide synthesis enzyme	*capE*	3.81	Cell envelope
Capsular polysaccharide synthesis enzyme	*capH*	3.81	Cell envelope
Capsular polysaccharide synthesis enzyme	*capN*	3.79	Cell envelope
Putative sugar transport system permease	SAR0207	3.69	Transport
Capsular polysaccharide synthesis enzyme	*capE*	3.62	Cell envelope
Putative sugar phosphate transport protein	*uhpT*	3.50	Transport
Capsular polysaccharide synthesis enzyme	*capC*	3.48	Cell envelope
Glycine betaine transporter 1	*opuD1*	3.25	Transport
Proline dehydrogenase	SAR1849	3.24	Unknown
Capsular polysaccharide synthesis enzyme	*capI*	3.22	Cell envelope
3-hydroxy-3-methylglutaryl coenzyme A synthase	*mvaS*	3.08	Metabolism
Conserved hypothetical protein	SAR0558	2.93	Unknown
Capsular polysaccharide synthesis enzyme	*capF*	2.74	Cell envelope
Lipoylsynthase	*lipA*	2.63	Metabolism
Capsular polysaccharide synthesis enzyme	*capL*	2.60	Cell envelope
Cysteine protease precursor	*sspB*	2.54	Cell envelope
Putative succinyl-coa ligase	SAR1222	2.52	Metabolism
Glutamyl endopeptidase precursor	*sspA*	2.51	Cell envelope
Putative pyridine nucleotide-disulphide oxidoreductase	SAR0903	2.41	Unknown
Capsular polysaccharide synthesis enzyme	*cap*	2.40	Cell envelope
60 kda chaperonin (HSP)	*groEL*	2.38	Chaperones
8-amino-7-oxononanoate synthase	*kbl*	2.34	Metabolism
Immunodominant antigen A	*IsaA*	2.33	Cell envelope
Sodium:alanine symporter family protein	SAR1368	2.28	Transport
Putative phosphate transport protein	SAR0674	2.27	Unknown
Aconitate hydratase	*citB*	2.20	Metabolism
Putative NADH ubiquinone/plastoquinone oxidoreductase	SAR0630	2.14	Unknown
DNA polymerase III subunit β	*dnaN*	2.14	Metabolism
Putative exported protein	SAR2295	2.14	Cell envelope
Putative endonuclease	SAR1463	2.06	Metabolism

v7.0 (Agilent Technologies, USA). Differentially expressed genes were identified using a one-way ANOVA with $P<0.05$, and Benjamini and Hochberg false discovery rate correction on selected genes showing more than two-fold change between treated and untreated samples. Fully annotated microarray data have been deposited in BμG@Sbase (Accession Number: E-BUGS- A-BUGS-17; http://bugs.sgul.ac.uk/E-BUGS- A-BUGS-17) and also in ArrayExpress (Accession Number: E-BUGS- A-BUGS-17).

Results and discussion

A total of 36 genes (15 for cell wall [11 capsular genes: *sspA*, *sspB* and *isaA*], six for transport and nine for metabolism) were upregulated by more than two-fold ($P\leq0.05$) in the VSSA strain grown in a subinhibitory level of vancomycin, when compared with the same strain grown in a vancomycin-free medium (Table 1).

Previous studies of VISA have demonstrated the upregulation of cell wall biosynthetic genes.[1,5,6] Current findings regarding capsular gene expression are in agreement with results obtained from McAleese and colleagues who studied gene expression of VSSA and VISA isolates recovered from a patient undergoing extensive vancomycin therapy.[6] These findings are not exclusive to vancomycin, however, as expression of the capsular polysaccharide biosynthesis pathway was upregulated in teicoplanin-resistant *S. aureus* isolates compared to teicoplanin-sensitive strains.[7]

It has been reported that polysaccharide capsule production in *S. aureus* affects immune evasion, changes endothelial binding, alters virulence factor production and protects the bacterium from phagocytic uptake.[1] It has also been reported that over-expression of staphylococcal capsular polysaccharide type 8 protects against *in vitro* opsonophagocytic killing by human neutophils and causes more pronounced infection in mice.[8]

A recent study confirmed that *S. aureus* can evade the innate immune system by changes in gene transcription, including the upregulation of capsular gene expression, which suggests that these genes may represent new targets for therapeutics designed to control *S. aureus* infection.[9]

In this study, *sspA*, which encodes serine protease V8, and *sspB* encoding a cysteine protease, were upregulated. These proteins are part of a cascade pathway controlling autolytic

Table 2. An ordered list of genes seen to undergo a greater than two-fold down-regulation in expression when the VSSA strain was grown in vancomycin, as compared to gene expression in the absence of vancomycin.

Gene product	Gene	Change	Functional category
50S ribosomal protein L33 type 2	*rpmG2*	–1.76	Cell envelope
Threonine dehydratase	*tdcB*	–2.00	Metabolism
Putative transposase	SAR2172	–2.12	Extrachromosomal
Hypothetical protein	SANS086	–2.17	Hypothetic protein
Hypothetical protein	SAR0628	–2.18	Hypothetic protein
Haloacid dehalogenase-like hydrolase	SAR2240	–2.24	Hypothetic protein
Hypothetical protein	SAR2173	–2.39	Unknown
SAC0L2421	*hlgC*	–2.45	Unknown
Cadmium resistant	*cadX*	–2.58	Cell processes
Col1542	C0LB1542	–2.58	Unknown
Serine-aspartate repeat-containing proteins	*sdrD*	–2.60	Cell envelope
Arsenical resistance operon repressor 1	*arsR1*	–2.62	Cell processes
Unknown	MWP006	–2.74	Hypothetic protein
Unknown	8325-2552	–2.75	Unknown
Iron regulated protein	*isdD*	–2.90	Cell envelope
Putative exported protein	SAR0436	–2.97	Cell envelope
Adenylosuccinate lyase	*purB*	–3.07	Metabolism
Putative exported protein	SAR0696	–3.08	Cell envelope
Fosfomycin resistant	*fosB*	–3.34	Cell processes
Col2910	SAC0L2202	–3.37	Unknown
SAC0L2436	SAR2523	–3.48	Unknown
Unknown	SAN1640	–3.60	Unknown
Putative extracellular glutamine-binding protein	SAR1949	–3.99	Transport
Transposase	SAR1827	–4.03	Extrachromosomal
DNA-binding protein HU	*Hup*	–4.04	Cell processes
Isochorismatase family protein	SAR2011	–4.05	Unknown
NADH dehydrogenase subunit	SAV0272	–4.20	Unknown
Col 3629	C0LB3629	–4.21	Unknown
Streptomycin3"-adenylytransferase2	*spc2*	–4.22	Cell processes
Col0068	SAC0L0052	–5.09	Unknown
Recombinase	SAR0689	–5.43	Extrachromosomal
Putative exported protein	SAR2035	–5.43	Cell envelope
Conserved hypothetical protein	SAR0412	–5.69	Unknown
Hypothetical protein	SAV0405	–5.82	Hypothetic protein
Col 2366	C0LB2366	–5.98	Unknown
SAC0L1497	*papS*	–6.06	Unknown
SAC0L0065	SAN0084	–7.19	Unknown
Hypothetical protein	SAV0914	–8.35	Hypothetic protein
Putative haloacid dehydrogenase-like hydrolase	SAR0639	–9.02	Unknown
Putative transposase	SAR0085	–9.08	Extrachromosomal
Unknown	SAN0030	–10.15	Unknown
SAC0L1967	*pcrB*	–11.15	Unknown

activity. It has been confirmed that vancomycin resistance in *S. aureus* will lead to reduced autolytic activity.[10] Therefore, increased expression of *sspA* and *sspB* might have an important role in vancomycin resistance in *S. aureus* through modulation of autolysis.

In the present study, the *isaA* gene encoding a highly immunoreactive antigen was upregulated 2.3-fold. This finding is in accordance with the report that *isaA* is upregulated in highly resistant VISA compared with the parent VISA clinical isolate.[4] The over-expression shown in other genes listed in Table 1 is also in agreement with results obtained from previous studies.[4,6]

A total of 42 genes were down-regulated in the VSSA strain after exposure to vancomycin. The majority (73%) of these genes were extrachromosomal in location or were hypothetical proteins with unknown function. The remainder of the down-regulated genes were involved in cell envelope synthesis (*rpmG2*, *isdD*, *sdrD*, SAR0436, SAR0696, SAR2035). Four are involved in cell process functions, including *cadX* (resistance to cadmium), *arsR1* (resistance to arsenic), *fosB* (resistance to fosfomycin) and *spc2* (resistance to streptomycin) (Table 2).

The increase in cell wall thickness might play a role in resistance mechanisms to different antibiotics, negating the need for their expression, leading to down-regulation. The *tet* gene (responsible for tetracycline resistance) was seen to be down-regulated in a highly resistant VISA strain compared with the parent VISA clinical isolate.[4]

In the current study, the *sdrD* gene, which encodes a serine aspartate-rich motif surface adhesion protein, was down-regulated more than two-fold. A previous study showed a strong association between the presence of the *sdrD* gene and methicillin resistance in *S. aureus* strains,[12] and the *sdrD* gene was down-regulated in *S. aureus* isolates with increasing MICs to vancomycin, and VRSA isolates demonstrate low-level expression of *mecA*.[10] The down-regulation observed in *sdrD* in VSSA exposed to vancomycin in the present study suggests that developing vancomycin resistance in MRSA might affect susceptibility to methicillin.

Avoiding the use of vancomycin to treat MSSA infections, especially deep-seated infections where high serum concentrations may not be achieved, might help to control the increased number of VISA/hVISA strains that are usually associated with delayed therapeutic response and carry an increased risk of complications. Early detection of the development of tolerance/resistance to vancomycin and subsequent selection of alternative therapies is crucial.

Data obtained in the present study suggest that it would be useful to choose selected capsular genes as markers for prediction of the development of VISA/hVISA in MRSA during vancomycin therapy. Further work in this area is warranted and should include a wider assessment of the expression of these key genes in clinical isolates obtained during the course of vancomycin therapy. □

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