

Transposon library screening for identification of genetic loci participating in intrinsic susceptibility and acquired resistance to antistaphylococcal agents

Katy L. Blake† and Alex J. O'Neill*

Antimicrobial Research Centre and School of Molecular and Cellular Biology, University of Leeds, Leeds LS2 9JT, UK

*Corresponding author. Tel: +44-113-343-5600; Fax: +44-113-343-5638; E-mail: a.j.oneill@leeds.ac.uk

†Present address: AmpliPhi Biosciences, Colworth Science Park, Sharnbrook, Bedfordshire, MK44 1LQ, UK.

Received 12 June 2012; returned 18 July 2012; revised 17 August 2012; accepted 22 August 2012

Objectives: To establish an experimental platform in *Staphylococcus aureus* for identifying genetic loci that determine intrinsic antibiotic susceptibility and/or that have the potential to contribute to acquired antibiotic resistance. A near-saturation *S. aureus* transposon (Tn) library was screened for mutants exhibiting altered susceptibility to the antistaphylococcal agents daptomycin, vancomycin and nisin.

Methods: *S. aureus* SH1000 was mutagenized with Tn InsTet^G+2^{Cm} by electroporation of transposomes. Approximately 20500 transposants were screened for increased or reduced susceptibility to the three antistaphylococcal agents and Tn insertion sites were mapped by DNA sequencing in mutants of interest.

Results: Transposants exhibiting hypersusceptibility or reduced susceptibility were identified for all three antibacterial agents; mapping of Tn insertion sites in these mutants identified genetic determinants of intrinsic susceptibility and potential contributors to acquired resistance, respectively. Tn insertions in the *dlt* operon caused cross-hypersusceptibility to vancomycin, daptomycin and nisin. Daptomycin hypersusceptibility was also associated with disruption of genes directing lipoteichoic acid and riboflavin biosynthesis, apparent inactivation of a putative membrane protein encoded by SAOUHSC_00957 and truncation of the cell-division gene *ezrA*. Tn-mediated disruption of the *vraDE*- and SAOUHSC_02953/4-encoded ABC transporters conferred hypersusceptibility to nisin. Reduced susceptibility to both daptomycin and vancomycin was associated with Tn insertions in *rpsU* and upstream of *ycyFG*. Several loci were associated with reduced susceptibility to nisin, including two genes encoding putative glycosyltransferases.

Conclusions: Tn library screening identified both known and novel modulators of antibacterial susceptibility in *S. aureus* and therefore represents a useful approach towards delineating the staphylococcal resistome.

Keywords: *Staphylococcus aureus*, resistome, antibacterial agents

Introduction

Studies in Gram-negative bacteria have revealed that genes across diverse functional categories participate in determining the level of intrinsic susceptibility to antibiotics. Collectively these genetic determinants comprise the intrinsic resistome.¹ The on-going delineation of this resistome is providing fundamental insights, both into antibiotic mode of action and into the bacterial response to inhibition. Moreover, since inhibition of the functions provided by resistome determinants leads to antibacterial hypersusceptibility, this knowledge may have a practical role by identifying targets for co-drug development to potentiate the activity of existing antibiotics.²

In addition to genes that determine intrinsic susceptibility to antibacterial agents, the resistome includes genetic loci that, when subject to mutation or deregulation, can act to further reduce susceptibility to antibacterial agents; such determinants represent potential contributors to acquired antibiotic resistance. Despite the fact that many of these confer only modest reductions in antibacterial susceptibility, the accumulation of mutations in multiple such loci can lead to clinically significant reductions in drug susceptibility, as seen, for example, in intermediate glycopeptide resistance in *Staphylococcus aureus*.³

In the present study we sought to define genetic determinants participating in intrinsic susceptibility and acquired antibacterial resistance in the important human pathogen, *S. aureus*. Resistome studies in Gram-negative bacteria have

commonly utilized defined collections of deletion or transposon (Tn) insertion mutants; unfortunately an equivalent, comprehensive set of defined mutants was not available in *S. aureus*. Instead, we undertook screening of a large, random Tn mutagenesis library to identify resistome determinants. We chose three antistaphylococcal agents for this study; the antibiotics daptomycin and vancomycin, and the lantibiotic nisin. Daptomycin and vancomycin were chosen because they are clinically important antibacterial drugs against which acquired resistance in clinical strains usually develops in a step-wise fashion, with multiple genetic loci participating in resistance. Furthermore, a number of genetic loci conferring hypersusceptibility and reduced susceptibility have already been reported for these agents,^{3,4} information that would enable us to judge the effectiveness of our screening approach for identifying resistome determinants. Although nisin is not used clinically, we included it in our study because it exhibits mechanistic overlap with daptomycin and vancomycin; it acts at the cell surface (like both daptomycin and vancomycin), binds lipid II (like vancomycin) and disrupts the staphylococcal membrane (like daptomycin). Furthermore, our current understanding of the molecular basis for staphylococcal resistance to nisin is incomplete.⁵

Materials and methods

S. aureus SH1000⁶ was mutagenized by electroporation of InsTet^{G+2}^{Cm} transposomes⁷ to create a near-saturation library comprising ~20500 transposants (manuscript in preparation). The entire library was screened by growing transposants overnight in 96-well microtitre plates containing Mueller–Hinton (MH) broth supplemented with 50 mg/L Ca²⁺ and spotting 200 nL of these cultures onto selective (containing antibacterial agent) and non-selective MH agar using the Library Copier Tool (V&P Scientific, San Diego, CA, USA). Under these conditions, the MICs for daptomycin, vancomycin and nisin against SH1000 were 1, 2 and 32 mg/L, respectively. The concentrations of antibacterial agents used to identify transposants exhibiting hypersusceptibility or reduced susceptibility were individually optimized for daptomycin (2.5× and 0.5× MIC), vancomycin (1.5× and 0.5× MIC) and nisin (2× and 0.25× MIC) in pilot screens. Briefly, five 96-well microtitre plates containing independent transposants selected at random from the library and a single 96-well microtitre plate containing unmutagenized SH1000 in all wells were spotted onto a series of agar plates containing concentrations of each antibacterial agent ranging from less than to greater than the MIC. Antibacterial concentrations that identified approximately one to four mutants with altered susceptibility per 96-well microtitre plate of transposants, but no mutants for the SH1000 microtitre plate, were chosen for the full screen.

Screening of the entire library identified numerous transposants that appeared to exhibit either hypersusceptibility or reduced susceptibility to one or more of the antibacterial agents. Putative hypersusceptible (HS) transposants were only selected for further study if they completely failed to grow on selective agar; mutants exhibiting only partial attenuation of growth were not investigated. In addition, putative HS transposants were plated onto agar containing ciprofloxacin at 0.5× MIC for SH1000; mutants that were also ciprofloxacin HS were considered growth defective and were not characterized further. To confirm the phenotypes of transposants of interest, all were re-screened twice and then subjected to conventional agar MIC determinations according to CLSI methodology.⁸

Tn insertion sites were mapped by direct sequencing from genomic DNA (Microsynth AG, Balgach, Switzerland), using oligonucleotide primer Tnp_out2 (5'-GCTTGCATGCCTGCAGTCTGACTC).⁷ Genetic loci

associated with altered drug susceptibility were in most cases identified in multiple, independent transposants, thereby providing evidence for an association between phenotype and genotype. For unique Tn insertions, evidence for this association was sought by demonstrating that Φ11 transduction⁹ of the Tn insertion into a clean SH1000 background resulted in co-transfer of the phenotype.

Results and discussion

Transposants exhibiting hypersusceptibility to daptomycin, vancomycin or nisin were identified; mapping of the Tn insertion sites in these mutants revealed determinants of the intrinsic resistome (Table 1). Cross-sensitization to all three antistaphylococcal agents was observed in transposants with Tn-mediated disruption of *dltA* or *dltB*, genes required for D-alanylation of wall teichoic acid (Table 1). Increased bacterial susceptibility to vancomycin and to numerous cationic antimicrobial peptides has previously been associated with *dlt* disruption.¹⁰ No transposants were recovered that exhibited hypersusceptibility to vancomycin without concomitant hypersusceptibility to the other agents.

Genetic loci participating in determining the intrinsic level of susceptibility to daptomycin were predominantly associated with structural/functional aspects of the cell envelope (Table 1). Tn-mediated inactivation of the *pgcA* and *gtbB* genes that direct synthesis of diglucosyl-diacylglycerol (Glc₂-DAG), the glycolipid that anchors lipoteichoic acid (LTA) to the staphylococcal membrane (Table 1), led to a daptomycin HS phenotype. This may suggest that Glc₂-DAG-tethered LTA ordinarily acts to limit in some way the action of daptomycin at the cell surface. Alternatively, since *S. aureus* defective in Glc₂-DAG synthesis utilizes DAG to anchor LTA,¹¹ daptomycin hypersusceptibility in these strains might be the result of enhanced daptomycin action in the presence of this substitute LTA anchor molecule. Daptomycin hypersusceptibility also resulted from Tn-mediated 3' truncation of *ezrA*, a gene encoding an essential component of the cell division machinery that acts to regulate FtsZ ring formation. The observation that changes in the divisome can lead to alterations in daptomycin susceptibility fits with the idea that daptomycin preferentially inserts into the staphylococcal membrane at the cell-division septum.¹² A daptomycin HS phenotype was also associated with Tn insertion upstream of SAOUHSC_00957, a gene encoding a putative toxic anion resistance protein with similarity to the TerC tellurium efflux pump in *Escherichia coli*. However, only a single transposant disrupted for SAOUHSC_00957 was recovered from our Tn library, and repeated attempts to transduce the Tn insertion into SH1000 were unsuccessful. Consequently further studies will be required to confirm a role for this gene in daptomycin hypersusceptibility. Disruption of the *ribD/ribE* genes of the riboflavin biosynthesis operon also led to daptomycin hypersusceptibility (Table 1). We note that the *rib* operon may represent a responsive component of the daptomycin intrinsic resistome, since enhanced *rib* expression has been observed in an *rpoB* mutant with reduced daptomycin susceptibility.¹³ However, it is not clear at this stage how altered riboflavin production might impact daptomycin susceptibility.

Although it is well established that reduced MprF-mediated lysinylation of membrane phosphatidylglycerol increases daptomycin susceptibility in staphylococci,¹⁰ null mutants of *mprF* were not among the daptomycin HS transposants identified in

Table 1. Transposants identified in this study exhibiting altered susceptibility to daptomycin (DAP), vancomycin (VAN) or nisin (NIS)

Phenotype/MIC (mg/L)			Locus of Tn integration (number of mutants identified)	Function of locus	Locus previously implicated in antibacterial susceptibility?
DAP	VAN	NIS			
DAP and VAN resistant					
2	3		upstream of <i>yycF</i> (2)	positive regulator of peptidoglycan biosynthesis and turnover ⁴	frameshift mutation in <i>yycG</i> confers reduced DAP susceptibility ⁴
2	3		start codon of <i>rpsU</i> (1)	30S ribosomal protein ¹⁴	point mutation upstream of <i>rpsU</i> confers reduced DAP susceptibility ¹⁴
DAP hypersusceptible					
0.25			upstream of SAOUHSC_00957 (1)	integral membrane protein of TerC family	
0.5			<i>pgcA</i> (1)	synthesis of glycolipid that tethers lipoteichoic acid to the	
0.5			<i>gtaB</i> (1)	cytoplasmic membrane ¹⁶	
0.5			<i>ezrA</i> (1)	negative regulation of <i>ftsZ</i> ring formation ¹⁷	DAP preferentially inserts at the cell division septum ¹²
0.5			<i>rib</i> operon (2)	riboflavin biosynthesis ¹³	enhanced expression of the <i>rib</i> operon reported in an isolate with reduced DAP susceptibility ¹³
DAP, VAN and NIS hypersusceptible					
0.5	0.5	1	<i>dlt</i> operon (3)	D-alanylation of wall teichoic acid ¹⁰	<i>dlt</i> inactivation confers increased susceptibility to antibacterial peptides ¹⁰
NIS resistant					
		128	SAOUHSC_00762 (3)	annotated as putative glycosyltransferase and <i>llm</i> (lipophilic protein affecting lysis rate and methicillin resistance level)	
		128	SAOUHSC_00228 (2)	putative glycosyltransferase	
		128	SAOUHSC_02958 (2)	putative alkaline phosphatase III	
		128	SAOUHSC_02154 (1)	ABC transporter, ATP-binding protein	
NIS hypersusceptible					
		4	SAOUHSC_02953/4 operon (7)	ABC transporter ¹⁶	up-regulated following NIS exposure ¹⁶
		4	<i>nsaS</i> (1)	sensor histidine kinase ⁵	point mutations in <i>nsaS</i> confer NIS resistance ⁵
		4	<i>vraDE</i> operon (2)	ABC transporter ¹⁸	disruption of <i>vraDE</i> confers hypersusceptibility to bacitracin ¹⁸

MICs were determined by standard agar susceptibility testing according to CLSI guidelines;⁸ under these conditions, the DAP, VAN and NIS MICs for the parent strain SH1000 were 1, 1 and 16 mg/L, respectively.

our study. Subsequent studies with a strain lacking *mprF* (RN4220 Δ *mprF*) established the reason for this; the absence of this gene did not lead to sufficient retardation of growth at 0.5 \times daptomycin MIC to satisfy the triage criteria of the screen (data not shown). This may also apply to other genetic loci that are known to impact susceptibility to vancomycin and/or daptomycin (e.g. *graRS*), but which were not detected in our screen. Reducing the stringency of the Tn library screen by altering the selecting concentrations of antibacterial agents might allow detection of these genes and other resistome determinants that exert only modest effects on antibacterial susceptibility.

Nisin hypersusceptibility was associated with Tn-mediated inactivation of the nisin susceptibility-associated sensor histidine kinase encoded by *nsaS*⁵ and with disruption of two ABC-type antimicrobial peptide transport systems encoded by SAOUHSC_02953/4 and *vraDE*; all three of these functions have recently been linked to nisin susceptibility (Table 1).

In addition to revealing determinants of the intrinsic staphylococcal resistome to daptomycin, vancomycin and nisin, genes with the potential to participate in reduced susceptibility to these agents were also identified. Transposants uniquely daptomycin- or vancomycin-resistant were not identified, only mutants exhibiting cross-resistance to both agents (Table 1). This points towards an overlap in resistance mechanisms between the two drugs and reflects clinical observations of concomitant reduction in daptomycin and vancomycin susceptibility following vancomycin therapy.¹⁰ In the daptomycin-/vancomycin-resistant transposants, Tn insertions were mapped to the start codon of *rpsU* and to two independent loci in the region upstream of the *yycFG* operon (Table 1). A nucleotide substitution upstream of *rpsU*, which encodes a small ribosomal protein, has previously been detected in a mutant derived *in vitro* exhibiting reduced daptomycin susceptibility,¹⁴ while a frameshift mutation in *yycG* and reduced YycF activity have been reported in an isolate associated with daptomycin treatment failure.⁴ The *yycFGHI* locus is an essential global regulator impacting the expression of numerous genes associated with the cell wall/cell membrane, and has been described as a key effector of daptomycin resistance.⁴ The impact of Tn insertion on *rpsU* and *yycFG*, and the precise role of these loci in reduced susceptibility to daptomycin, requires further study. Nevertheless, the identification of daptomycin-resistant transposants containing Tn insertions at these loci underlines the utility of Tn library screening in *S. aureus* to identify genes with resistance potential relevant to both laboratory and clinical situations.

While relatively few daptomycin-/vancomycin-resistant mutants were recovered in our screen, numerous transposants were identified that exhibited reduced susceptibility to nisin. These included mutants with Tn insertions in SAOUHSC_00228 and SAOUHSC_00762, both of which encode putative glycosyltransferases (Table 1). Disruption of a putative alkaline phosphatase III (SAOUHSC_02958) and truncation of the ATP-binding protein encoded by SAOUHSC_02154 also conferred reduced nisin susceptibility. It is not clear at present how disruption of these loci might impact nisin susceptibility.

In summary, this study has successfully employed Tn mutagenesis and screening for identification of both established and novel modulators of the staphylococcal resistome for daptomycin, vancomycin and nisin. Further work will be required to

establish exactly how the genes identified impact antibacterial susceptibility and to extend this analysis to other classes of antistaphylococcal agents. During the preparation of this manuscript an elegant study was published that employed genome-wide Tn disruption in *S. aureus* to determine antibacterial mode of action.¹⁵ That study, and the work presented here, together reveal transposon mutagenesis as a powerful approach for studying both antibacterial action and resistance in *S. aureus*.

Acknowledgements

We thank Jared Silverman for helpful discussions.

Funding

This work was supported by a research grant from Cubist Pharmaceuticals and by New Investigator Award G0501247 from the Medical Research Council.

Transparency declarations

None to declare.

References

- 1 Alvarez-Ortega C, Wiegand I, Olivares J *et al*. The intrinsic resistome of *Pseudomonas aeruginosa* to β -lactams. *Virulence* 2011; **2**: 144–6.
- 2 Liu A, Tran L, Becket E *et al*. Antibiotic sensitivity profiles determined with an *Escherichia coli* gene knockout collection: generating an antibiotic bar code. *Antimicrob Agents Chemother* 2010; **54**: 1393–403.
- 3 Kato Y, Suzuki T, Ida T *et al*. Genetic changes associated with glycopeptide resistance in *Staphylococcus aureus*: predominance of amino acid substitutions in YvqF/VraSR. *J Antimicrob Chemother* 2010; **65**: 37–45.
- 4 Fischer A, Yang SJ, Bayer AS *et al*. Daptomycin resistance mechanisms in clinically derived *Staphylococcus aureus* strains assessed by a combined transcriptomics and proteomics approach. *J Antimicrob Chemother* 2011; **66**: 1696–711.
- 5 Blake KL, Randall CP, O'Neill AJ. *In vitro* studies indicate a high resistance potential for the lantibiotic nisin in *Staphylococcus aureus* and define a genetic basis for nisin resistance. *Antimicrob Agents Chemother* 2011; **55**: 2362–8.
- 6 O'Neill AJ. *Staphylococcus aureus* SH1000 and 8325-4: comparative genome sequences of key laboratory strains in staphylococcal research. *Lett Appl Microbiol* 2010; **51**: 358–61.
- 7 Bertram R, Kostner M, Muller J *et al*. Integrative elements for *Bacillus subtilis* yielding tetracycline-dependent growth phenotypes. *Nucleic Acids Res* 2005; **33**: e153.
- 8 Clinical and Laboratory Standards Institute. *Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria That Grow Aerobically*, Ninth Edition: Approved Standard M07-A9. CLSI, Wayne, PA, USA, 2012.
- 9 Foster TJ. Molecular genetic analysis of staphylococcal virulence. *Methods Microbiol* 1998; **27**: 433–54.
- 10 Cafiso V, Bertuccio T, Spina D *et al*. Modulating activity of vancomycin and daptomycin on the expression of autolysis cell-wall turnover and membrane charge genes in hVISA and VISA strains. *PLoS ONE* 2012; **7**: e29573.
- 11 Reichmann NT, Grundling A. Location, synthesis and function of glycolipids and polyglycerolphosphate lipoteichoic acid in Gram-positive bacteria of the phylum Firmicutes. *FEMS Microbiol Lett* 2011; **319**: 97–105.

- 12** Baltz RH. Daptomycin: mechanisms of action and resistance, and biosynthetic engineering. *Curr Opin Chem Biol* 2009; **13**: 144–51.
- 13** Cui L, Isii T, Fukuda M et al. An RpoB mutation confers dual heteroresistance to daptomycin and vancomycin in *Staphylococcus aureus*. *Antimicrob Agents Chemother* 2010; **54**: 5222–33.
- 14** Friedman L, Alder JD, Silverman JA. Genetic changes that correlate with reduced susceptibility to daptomycin in *Staphylococcus aureus*. *Antimicrob Agents Chemother* 2006; **50**: 2137–45.
- 15** Wang H, Claveau D, Vaillancourt JP et al. High-frequency transposition for determining antibacterial mode of action. *Nat Chem Biol* 2011; **7**: 720–9.
- 16** Kolar SL, Nagarajan V, Oszmiana A et al. NsaRS is a cell-envelope-stress-sensing two-component system of *Staphylococcus aureus*. *Microbiology* 2011; **157**: 2206–19.
- 17** Yan K, Pearce KH, Payne DJ. A conserved residue at the extreme C-terminus of FtsZ is critical for the FtsA-FtsZ interaction in *Staphylococcus aureus*. *Biochem Biophys Res Comm* 2000; **270**: 387–92.
- 18** Dintner S, Staron A, Berchtold E et al. Coevolution of ABC transporters and two-component regulatory systems as resistance modules against antimicrobial peptides in Firmicutes bacteria. *J Bacteriol* 2011; **193**: 3851–62.