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Contributions of *sigB* and *sarA* to distinct multiple antimicrobial resistance mechanisms of *Staphylococcus aureus*

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Abstract

Multiple antimicrobial resistance in *Staphylococcus aureus* can result from mutations leading to reduced susceptibility to Pine oil-based cleaners (PS^{RS}) as well as following growth with the non-steroidal anti-inflammatory salicylate. We now define the contributions of alternative sigma factor (*sigB*) and staphylococcal accessory regulator (*sarA*) to these mechanisms. We conclude that *sarA* plays a more prominent role than *sigB* in overall intrinsic multiple antimicrobial resistance. Both genes have similar effects on intrinsic vancomycin resistance, and the salicylate-inducible mechanism is not *sigB*- or *sarA*-dependent. Furthermore, analyses determined that altered expression of *sigB* and *sarA* is not responsible for the salicylate-inducible mechanism, and *sarA* upregulation is associated with the PS^{RS} phenotype.

Keywords

Staphylococcus aureus; Multiple antimicrobial resistance; Alternative sigma factor; Staphylococcal accessory regulator

1. Introduction

Intrinsic antimicrobial resistance in bacteria is maintained by the normal complement of chromosomal genes and probably involves a number of mechanisms (e.g. drug efflux, target gene upregulation). Mutations can occur that leave these intrinsic antimicrobial mechanisms in the 'on' position, such as that displayed by *Escherichia coli* strains expressing the classic 'multiple antibiotic resistance' (or Mar) phenotype discovered in Stuart B. Levy's laboratory (for review see [1]).

The pathogen *Staphylococcus aureus*, known for its propensity to become clinically resistant to antibiotics via horizontal gene transfer and mutation (for review see [2]), also expresses intrinsic resistance to antimicrobial substances that inhibit a number of unique cellular processes[3–11]. A number of factors are involved in the intrinsic multiple antimicrobial resistance mechanism of *S. aureus*: the multidrug efflux pumps NorA, NorB, MdeA and SepA and other chromosomally encoded efflux pumps [3,11–15]; the *norA* regulator [14,16,17] (also referred to as MgrA or Rat [18,19]); the staphylococcal accessory regulator (SarA) [7]; and the alternative sigma factor SigB [9]. Mutations in the *S. aureus* genes or

operons encoding these factors can result in mutants expressing increased [7,9,10,20–23] or decreased [3,11–13,24,25] intrinsic susceptibility to various antimicrobials.

SigB is essential for the general chemical and physical stress response of the organism [26,27] and SarA alters the expression of a number of staphylococcal virulence factors (for review see [28]). In addition, the *sigB* operon and *sarA* loci appear to control the expression of one another [26,27,29], demonstrating that the control of these two genetic elements is intertwined.

Mutants of *S. aureus* expressing reduced susceptibility to common house cleaners demonstrate increased resistance to multiple antimicrobials [9,30], and growth of *S. aureus* in the presence of the non-steroidal anti-inflammatory salicylate also elicits a multiple antimicrobial resistance mechanism [4–6,8,10,15]. The salicylate-inducible mechanism in *S. aureus* is in part mediated by a reduction in the accumulation of antimicrobials [10].

We now demonstrate unique roles for *sigB* and *sarA* in the expression of the house cleaner-selected and salicylate-induced multiple antimicrobial resistance mechanisms of *S. aureus* and evaluate the individual contributions of these genetic loci to these mechanisms. In addition, we provide evidence for a *sigB*–*sarA*-independent salicylate-inducible multiple antimicrobial resistance mechanism and demonstrate alterations in *sigB* and *sarA* expression following growth in the presence of salicylate as well as upon acquisition of the house-cleaner reduced susceptibility phenotype.

2. Materials and methods

2.1. Bacterial strains, strain maintenance, chemicals and gradient plate technique

Strain S6C used in this study was previously described by O’Leary et al. [7], and SH1000 (a kind gift of Glenn Kaatz, Wayne State University School of Medicine) is an *rsbU*⁺ derivative of strain 8325-4 [31]. Pine-Sol®-reduced susceptibility (PS^{RS}) strain JO18 was selected by inoculating the surface of a Difco™ Luria broth (LB) base (Becton Dickinson and Company, Sparks, MD) agar (LBA) plate containing 0.9% of Pine-Sol® with 100 µL of an overnight LB S6C culture. PS^{RS} S6C mutants appeared at a mutation frequency of 10^{−7} and JO18 was passaged through drugfree media multiple times before any experiments were performed. All experiments were performed at 37 °C and liquid cultures were maintained at 200 rpm and initiated with 1% (v/v) inocula from overnight LB cultures (18 h). All parent strain working stocks were maintained on LBA at 4 °C or stored following growth in LB and glycerol addition (20%, v/v final concentration) at −20 °C or −80 °C. *sigB::tet*, *sarA::kan* and *sigB::tet/sarA::kan* transductant working stocks were maintained on LBA containing 50 mg/L kanamycin, 10 mg/L tetracycline or both antibiotics and stored following overnight growth in LB containing the appropriate selective antibiotics. *Staphylococcus aureus* strains PC161 (8325-4, *rsbU*[−] *sarA::lacZ*) and MC100 (8325-4, *rsbU*[−] *sigB::lacZ*) (kind gifts of Simon Foster, University of Sheffield UK) harbour an intact chromosomal copy of *sigB* and *sarA*, as well as a truncated version of either *sigB* or *sarA* fused to *lacZ* [26,32]. PC161 and MC100 were maintained on LBA containing 20 mg/L ampicillin and *lacZ* expression was confirmed by subculture to LBA containing 100 µg/mL of X-gal (Sigma, St. Louis, MO).

Stocks of ciprofloxacin (Bayer Corporation, Morristown, NJ), vancomycin, ethidium bromide and sodium salicylate (Sigma) were made in ddH₂O, whilst tetracycline stocks (Sigma) were made in 100% ethanol and all stocks were stored at −20 °C until required. Pine-Sol® (PS) (Clorox Company, Oakland, CA) and Orange Clean® Super Concentrate (OC) (Orange Glo International Inc., Littleton, CO) were stored in their original containers at 25 °C. Single lots of each cleaner formulation were used for all experiments.

Gradient plates were prepared with LBA and inoculated with overnight LB cultures as previously described [7], except that plates in this study were prepared with and without 2 mM salicylate addition into both LBA slanted layers. Inoculated plates (in triplicate) were incubated and read following 48 h incubation. The point at which confluent bacterial growth halted was reported as millimetres grown on the gradient (Table 1).

2.2. Construction and confirmation of *sigB::tet*, *sarA::kan* and *sigB::tet/sarA::kan* transductants

S6C and PS^{RS} mutant JO18 were transduced with phage 80α populations grown on *S. aureus* strains containing *sigB::tet* (PC400; [32]) or *sarA::kan* (S6C*sarA::kan* [7]) constructs. Single and double transductants of S6C and JO18 were selected on LBA containing either kanamycin and/or tetracycline.

Chromosomal DNA used for polymerase chain reaction (PCR) was isolated from cell lysates treated with lysostaphin (Sigma). Initially, cells from 20 mL LB overnight cultures were harvested by centrifugation (8000 × *g*, 10 min, 4 °C). The cell pellet was then re-suspended in 3 mL of lysis solution (0.15 M NaCl, 0.1 M EDTA, pH 8.0) and lysostaphin (final concentration 10 mg/L). After 30 min incubation at 37 °C, 0.3 mL of an SDS (5%, w/v)/ethanol (50%, v/v) solution was added and the suspension was vortexed for 10 s. Two millilitres of phenol/chloroform/isoamyl alcohol (25:24:1) (pH 8) was then added, followed by vortexing and centrifugation (10 000 × *g*, 5 min, 4 °C). The aqueous phase was then extracted once with 2 mL of chloroform, and 2 vol. of 100% ethanol (4 °C) was added to precipitate DNA, which was spooled onto a sterile glass rod. While still on the rod the spooled DNA was then washed three times in 100% ethanol and re-suspended from the rod into 1 mL of TE buffer (10 mM Tris-Cl, 1 mM EDTA, pH 8) containing RNase A (Sigma) at a final concentration of 20 mg/L. After 24 h incubation at 25 °C, the RNase was inactivated by addition of 0.2 mL of phenol/chloroform/isoamyl alcohol (pH 8.0) followed by centrifugation as above. The aqueous phase was then extracted with chloroform, centrifuged and the DNA was precipitated by adding sodium acetate (0.3 M final concentration) and 2 vol. of 100% ethanol (4 °C). The chromosomal DNA was then spooled onto a glass rod, washed three times in 100% ethanol and re-suspended in 1 mL of TE buffer and stored at −20 °C.

sarA and *sigB* gene inactivation in single and double transductants was confirmed by PCR using the primers SarA1-1 (5′-TTT CGT TGT TTG CTT CAG TG-3′), SarA1-2 (5′-TCG AGC AAG ATG CAT CAA A-3′) and the primers SigB1-1 (5′-TTG ATT CTT GGC CCA ATT TC-3′) and SigB1-2 (5′-GCG AAA GAG TCG AAA TCA GC-3′), respectively.

2.3. Growth in the presence of salicylate, β-galactosidase assays and real-time PCR

Initially, overnight cultures (18 h) of strains PC161, MC100 and SH1000 were used to inoculate fresh LB media (final optical density of 600 nm (OD_{600nm}) = 0.01), which contained either no addition, or 2 mM or 5 mM salicylate, and OD_{600nm} was recorded over time with a BioMate spectrophotometer (Thermo Spectronic, Rochester, NY). For β-galactosidase assays, cultures of PC161 and MC100 were allowed to grow to mid-exponential phase (OD_{600nm} = 0.7) and then challenged with nothing, or 2 mM or 5 mM salicylate for 1 h before the expression of β-galactosidase in Miller units was determined as previously described [32]. Statistically significant differences in β-galactosidase activity were determined by analysis of variance (ANOVA) analysis using SAS® 9.1 software (SAS Institute Inc., Cary, NC).

RNA for real-time PCR analysis was isolated in triplicate from cultures of PC161, MC100, SH1000, S6C and JO18 grown to an OD_{600nm} = 0.7 before induction with 2 mM salicylate

for 1 h. Following induction, control and induced cultures were harvested ($8000 \times g$, 2 min), re-suspended in 0.2 mL of diethyl pyrocarbonate (DEPC)-treated water and transferred to 2 mL screw-capped tubes containing 1 g of 0.1-mm diameter zirconium/silica beads, 0.6 mL acid-equilibrated phenol:chloroform 25:24 (v/v) (pH 4.7) and 0.5 mL STET buffer (10 mM Tris-Cl, pH 8.0, 0.1 M NaCl, 1 mM EDTA, 5% Triton X-100). Samples were then homogenised in a Mini-BeadbeaterTM (United Laboratory Plastics, St. Louis, MO) at 4200 rpm for 50 s and chilled on ice for 10 min. Cellular debris was removed by centrifugation ($16\,000 \times g$, 10 min) and the aqueous phase was extracted with an equal volume of chloroform and then centrifuged ($16\,000 \times g$, 2 min). RNA was then precipitated by adding isopropyl alcohol and sodium acetate (pH 4.78) (final concentration 50%, v/v and 0.3 M, respectively), incubation at -80°C for 20 min, followed by centrifugation ($16\,000 \times g$, 15 min). Contaminating DNA was removed from purified RNA using DNAfree[®] (Ambion, Austin, TX) and RNA was converted to first strand cDNA using M-MLV SuperScriptTM III Reverse Transcriptase (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Each template cDNA population was then diluted and used for real-time PCR using an iCycler iQTM Real-Time PCR Detection System (Bio-Rad, Carlsbad, CA) and the iQTM SYBR[®] Green Supermix (Bio-Rad). 16S rDNA was used as the reference gene for all experiments. The following primers were used for real-time PCR: SigB2-1 (TTT CAC CTG AGC AAA TTA ACC A); SigB2-2 (TCT TCG TGA TGT GAT TGT CCT T); SarA2-1 (CAT CAG CGA AAA CAA AGA GAA A); SarA2-2 (TTC TTT CAT CAT GCT CAT TAC GTT); 16S-F (TCG TGT CGT GAG ATG TTG); and 16S-R (CTG CCC TTT GTA TTG TCC). Fold change in gene expression was determined using the $2^{-\Delta\Delta\text{CT}}$ method [33].

3. Results

3.1. Confirmation of PS^{RS} phenotype of strain JO18 and transductant genotypes

Compared with the parent strain S6C, PS^{RS} mutant JO18 demonstrated reduced susceptibility to PS, OC and the cellwall-active antibiotic vancomycin. JO18 and S6C expressed similar resistance levels to ciprofloxacin and, since both strains grew up to >90 mm on the ethidium gradients, possible differences in ethidium resistance were not discerned.

All single and double transductants grew on kanamycin-and/or tetracycline-containing agar, as expected for successful transfer of the *sigB::tet* and *sarA::kan* constructs. Following amplification, chromosomal DNA from S6C produced a 398 bp amplicon with primers SarA1-1 and SarA1-2 and a 355 bp amplicon with SigB1-1 and SigB1-2, as expected. To confirm the inactivation of *sarA* and *sigB* via transduction, we analysed suspected *sigB::tet* and *sarA::kan* constructs in the S6C series of transductants by PCR. Utilising chromosomal DNA from S6C*sigB::tet*, primers SigB1-1 and SigB1-2 produced an amplicon of 2.5 kb, which is consistent with that expected when the tetracycline resistance cassette is present in the *sigB* operon. Amplifying chromosomal DNA from transductant S6C*sarA::kan* with primers SarA1-1 and SarA1-2 yielded a 1.9 kb amplicon, which is consistent with that expected when the kanamycin resistance cassette is present in the *sarA* locus. Amplification of chromosomal DNA from double transductant S6C*sigB::tet/sarA::kan* produced amplicons of 2.5 kb with primers SigB1-1 and SigB1-2 and 1.9 kb with primers SarA1-1 and SarA1-2. These data confirm the presence of appropriate constructs within the transductants produced in this study.

3.2. Gradient plate analysis of multiple antimicrobial resistance levels

In general, drug gradient plate analyses revealed that *sigB::tet*, *sarA::kan* and *sigB::tet/sarA::kan* mutants of S6C and JO18 demonstrated increased susceptibility to multiple antimicrobial drugs (Table 1).

Inactivation of either *sigB* or *sarA* in S6C or JO18 led to increased susceptibility to ciprofloxacin. However, inactivation of *sarA* in the S6C background led to a greater decline in ciprofloxacin resistance compared with *sigB* inactivation. Inactivation of *sarA* or *sigB* in the JO18 background led to similar reductions in ciprofloxacin resistance. Inactivation of both genes in JO18, but not in S6C, led to an additional significant increase in ciprofloxacin susceptibility. In addition, the inactivation of *sigB* and both genes in JO18 led to greater fold decreases in ciprofloxacin resistance (1.5 and 2.1) compared with the inactivation of these genes in S6C (1.1 and 1.5). Regardless of *sigB* and/or *sarA* inactivation, addition of 2 mM salicylate to ciprofloxacin gradients reduced ciprofloxacin susceptibility in all strains (Table 1).

Only the inactivation of *sarA* in both S6C and JO18 led to increased susceptibility to ethidium on the gradient investigated (Table 1). Interestingly, strains JO18*sarA::kan* and JO18*sigB::tet/sarA::kan* demonstrated greater increased ethidium susceptibility compared with S6C*sarA::kan* and S6C*sigB::tet/sarA::kan*. Furthermore, the addition of 2 mM salicylate to gradients induced elevated ethidium resistance levels in S6C*sarA::kan*, S6C*sigB::tet/sarA::kan*, JO18*sarA::kan* and JO18*sigB::tet/sarA::kan*. Since S6C, S6C*sigB::tet*, JO18 and JO18*sigB::tet* grew to >90 mm on the ethidium gradients investigated, the effects of salicylate induction were not determined.

Inactivation of *sigB* or *sarA* in S6C and JO18 led to similar degrees of increased vancomycin susceptibility (Table 1) and double mutants did not demonstrate significantly increased vancomycin susceptibility compared with the *sigB::tet* or *sarA::kan* transductants of both strains. The addition of 2 mM salicylate to vancomycin gradients significantly induced elevated vancomycin resistance only in strain S6C*sarA::kan*.

The inactivation of *sigB* and/or *sarA* led to reductions in S6C OC and PS resistance levels, and dramatic reductions in JO18 OC and PS resistance levels (Table 1). However, compared with *sigB* inactivation, *sarA* inactivation in S6C and JO18 led to greater reductions in OC and PS resistance levels in S6C. Salicylate addition to gradients induced a significant reduction in OC and PS susceptibility in all strains, except reduced OC susceptibility in strain S6C*sarA::kan* and reduced PS susceptibility in strain JO18*sigB::tet/sarA::kan*. Since JO18 grew to >90 mm on the OC and PS gradients investigated, the potential effects of growth with salicylate were not determined.

3.3. Effect of sodium salicylate on growth and the transcription of *sigB* and *sarA*

Growth of MC100 (Fig. 1), PC161 and SH1000 (data not shown) were inhibited by the addition of 2 mM and 5 mM salicylate. Induction with 2 mM and 5 mM salicylate for 1 h led to an increase in β -galactosidase production in MC100, indicating an increase in *sigB* expression, and a decrease in β -galactosidase production in strain PC161, indicating a decrease in *sarA* expression (Table 2). Real-time PCR confirmed these results in MC100 and PC161 (Table 3). Growth in the presence of salicylate also induced *sigB* transcription in strains SH1000 and S6C, but not as greatly as in MC100 or PC161, and did not have a major effect on *sigB* transcription in JO18 (Table 3). Interestingly, growth in the presence of salicylate induced *sarA* transcription in SH1000 and S6C, but led to a decrease in *sarA* transcription in JO18 (Table 3). Acquisition of the PS^{RS} phenotype in JO18 did not significantly alter *sigB* transcription, but led to a two-fold increase in *sarA* transcription compared with the parent strain S6C (data not shown).

4. Discussion

Compared with the parent strain S6C, PS^{RS} mutant JO18 demonstrated reduced susceptibility to PS, OC and vancomycin, as expected [9,30]. JO18 and S6C expressed

similar levels of susceptibility to ciprofloxacin on gradient plates, suggesting that the PS^{RS} phenotype does involve altered NorA activity, which is an important contributor to intrinsic fluoroquinolone resistance [3].

A number of publications have explored the individual effects of *sigB* or *sarA* on *S. aureus* intrinsic antimicrobial resistance [7,9,20–25,30], but none has investigated the individual contributions of both of these genes to the intrinsic antimicrobial resistance mechanism(s) of *S. aureus*. Using defined isogenic single and double *sigB/sarA* mutants of parent strain S6C and derived PS^{RS} S6C mutant JO18, we have now evaluated the contributions of *sigB* and *sarA* in two intrinsic multiple antibiotic resistance mechanisms.

In general, the overall reductions in resistance to the various antimicrobials investigated caused by inactivation of *sigB* and/or *sarA*, or increases induced by salicylate, differed in S6C compared with the PS^{RS} JO18 background.

For instance, inactivation of *sarA* in S6C led to a greater increase in ciprofloxacin susceptibility compared with *sigB* inactivation, whilst inactivation of either gene led to similar ciprofloxacin susceptibility increases in JO18. Inactivation of both genes in JO18 led to an additional increase in ciprofloxacin susceptibility. Strains JO18*sarA::kan* and JO18*sigB::tet/sarA::kan* also demonstrated greater increased ethidium susceptibility compared with similar S6C mutants. Perhaps as expected, the reduction in OC and PS resistance caused by *sigB* and/or *sarA* inactivation was also greater in the JO18 background compared with S6C. These findings suggest that mutations leading to the PS^{RS} genotype reconfigure the role of *sigB* and *sarA* in the intrinsic multiple antimicrobial resistance. Only the inactivation of *sarA* both in S6C and JO18 led to increased susceptibility to ethidium, demonstrating a major role for *sarA* in intrinsic ethidium resistance.

Overall, when compared with *sigB* inactivation, inactivation of *sarA* led to greater increases in susceptibility: to ciprofloxacin in S6C; to ethidium and OC in S6C and JO18; and to PS in S6C. Therefore, *sarA* plays a more prominent role than *sigB* in intrinsic multiple antimicrobial resistance expression by *S. aureus*.

Inactivation of *sigB* and *sarA* in S6C and JO18 led to a similar degree of increased vancomycin susceptibility, and inactivation of both genes in S6C and JO18 did not significantly increase vancomycin susceptibility compared with the single mutants. Previously, both *sigB* and *sarA* were demonstrated to play roles in intrinsic and vancomycin-intermediate resistance [9,22,30] and *sigB* exerts control over *sarA* expression [26,29]. We now provide evidence that the activity of these genes with regards to intrinsic vancomycin resistance may not be linked, i.e. *sigB* and *sarA* affect intrinsic vancomycin resistance to a similar degree but perhaps through separate pathways since their subsequent dual inactivation does not lead to a further increase in vancomycin susceptibility. We speculate that *sarA* inactivation may have effects similar to *sigB* inactivation on the vancomycin-intermediate phenotype [22] and experiments are underway to determine this putative association.

Salicylate addition to drug gradient plates resulted in: reduced susceptibility to ciprofloxacin in all strains; reduced susceptibility to ethidium in *sarA::kan* and *sigB::tet/sarA::kan* mutants of S6C and JO18; and reduced susceptibility to OC and PS in all strains with few exceptions (see Section 3). Salicylate addition also significantly induced elevated vancomycin resistance in strain S6C*sarA::kan*, but did not affect vancomycin resistance levels in any other strain. Therefore, the ability of salicylate to induce multiple antimicrobial resistance is not dependent on *sigB* and/or *sarA*, even though both genes are required for the full expression of salicylate-inducible resistance levels. Previously, it was demonstrated that

norA is also not required for salicylate-induced multiple antimicrobial resistance [10], nor does salicylate induce the *norA* promoter [34].

We also show that salicylate reduces the growth of *S. aureus* at a concentration (2 mM) that induces multiple antimicrobial resistance. This reduction in growth rate probably contributes to the overall antimicrobial resistance levels, since slow-growing bacteria are more resistant to antimicrobials than faster growing organisms [35,36]. It is improbable that the reduced growth rate alone explains the salicylate-induced reduction in multiple antimicrobial susceptibility, since salicylate also induces multiple alterations in bacterial transcriptomes and physiology [37–39].

Since *sigB* and *sarA* are absolutely required for the full expression of intrinsic multiple antimicrobial resistance in *S. aureus* [7,9,30; this study], we wanted to investigate the effects of salicylate and mutations leading to the PS^{RS} phenotype on the expression of these genes. β -Galactosidase assays and real-time PCR with the *rsbU*[−] strains MC100 and PC161 demonstrated that growth in the presence of salicylate increases *sigB* transcription and decreases *sarA* transcription. *rsbU* is required for the activation of *sigB*, and *rsbU*[−] mutants appear to be deficient in *sigB* activity (for review see [27]). These results are in accordance with those of Kupferwasser et al. [40] who reported that salicylate reduced *S. aureus* virulence by upregulating *sigB* and downregulating *sarA* expression in *rsbU*[−] and *rsb*⁺ strains. Growth in the presence of salicylate also induced *sigB* transcription in *rsbU*⁺ strains SH1000 and S6C, once again in accordance with Kupferwasser et al. [40]. However, in our study, salicylate induced *sarA* transcription in SH1000 and S6C, and this result is supported by array data demonstrating that *sigB* expression upregulates *sarA* [27]. This apparent conundrum of the effects of salicylates on *sarA* modulation might be due to differences in salicylate induction concentration (0.3 mM salicylate in Kupferwasser et al. [40] and 2 mM or 5 mM in this study) and the growth phase of bacteria tested (late post-exponential phase in Kupferwasser et al. [40] and exponential phase in this study). To further confuse this issue, salicylate addition does not alter *sigB* expression in the PS^{RS} JO18 background, yet reduces *sarA* expression even though salicylate still induces multiple antimicrobial resistance in this strain. This finding once again suggests that *sigB* and *sarA* activity is altered by mutations leading to the PS^{RS} phenotype.

Since salicylate induces multiple antimicrobial resistance both in the parent strain S6C and the PS^{RS} mutant JO18, we conclude that altered expression of *sigB* (up in S6C, unaltered in JO18) and *sarA* (up in S6C and down in JO18) is probably not absolutely required for the salicylate-inducible multiple antimicrobial resistance mechanism.

We previously speculated that *sigB* was upregulated in a PS^{RS} mutant that had no *sigB* operon mutations yet demonstrated phenotypes attributed to altered *sigB* activity [30]. We now show that the acquisition of mutation(s) leading to the PS^{RS} phenotype does not significantly alter *sigB* transcription, but does lead to a two-fold increase in *sarA* transcription. Therefore, upregulation of *sarA* is associated with expression of the PS^{RS} phenotype.

In conclusion, clearly the mechanism of intrinsic multiple antimicrobial resistance requires functional *sigB* and *sarA* genes, whatever their unique contributions to these mechanisms may be. Furthermore, whilst salicylate alters *sigB* and *sarA* transcription, it does not appear that these alterations are required for the salicylate-inducible multiple antimicrobial resistance mechanism. Further work is required to identify how salicylate affects the staphylococcal cell.

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Fig. 1.
Growth of strain MC100 in the presence of salicylate.

Table 1

Effects of *sigB* and/or *sarA* inactivation on *Staphylococcus aureus* multidrug resistance expression^a

Strain	Drug gradients									
	0→2.0 (mg/L)					0→40 (mg/L)				
	CIP	FD	CIP + Sal	FI	EtBr	FD	EtBr + Sal	FI	VAN	FD
S6C	48 ± 2.5		<90 ^{**}	<1.9	<90		<90	N.D.	30 ± 0.6	
S6C <i>sigB::tet</i>	42 ± 0.6 [*]	1.1	61±0.6 ^{**}	1.5	<90	N.D.	<90	ND	17 ± 0 [*]	1.8
S6C <i>sarA::kan</i>	30 ± 0 [*]	1.6	60±1.5 ^{**}	2.0	50±3.0 [*]	<1.8	<90 ^{**}	<1.8	19 ± 0.6 [*]	1.6
S6C <i>sigB::tet/sarA::kan</i>	32 ± 1.5 [*]	1.5	49±0.6 ^{**}	1.5	50±2.0 [*]	<1.8	<90 ^{**}	<1.8	18 ± 1.5 [*]	1.7
JO18	45 ± 5.0		<90 ^{**}	<2.0	<90		<90	N.D.	39 ± 0.6	
JO18 <i>sigB::tet</i>	30 ± 0.6 [*]	1.5	56±0.6 ^{**}	1.9	<90	N.D.	<90	N.D.	21 ± 1.0 [*]	1.9
JO18 <i>sarA::kan</i>	29 ± 0.6 [*]	1.6	55±1.5 ^{**}	1.9	32±0.6 [*]	<2.8	59±0.6 ^{**}	1.8	20 ± 2.0 [*]	2.0
JO18 <i>sigB::tet/sarA::kan</i>	21 ± 1.7 [*]	2.1	40±2.5 ^{**}	1.9	32±0.6 [*]	<2.8	59±1.2 ^{**}	1.8	17 ± 1.2 [*]	2.3

Strain	Drug gradients									
	0→0.15% (v/v)					0→0.6% (v/v)				
	OC	FD	OC + sal	FI	PS	FD	PS + sal	FI	PS	FD
S6C	26±0.6		38±1.0 ^{**}	1.5	7±0.6		30±0.6 ^{**}	4.3		
S6C <i>sigB::tet</i>	16±1.2 [*]	1.6	26±2.6 ^{**}	1.6	4±0.6 [*]	1.8	30±0.6 ^{**}	7.5		
S6C <i>sarA::kan</i>	8±2.6 [*]	3.3	15±4.1	N.D.	0 [*]	<7	17±0.6 ^{**}	17		
S6C <i>sigB::tet/sarA::kan</i>	5±0.6 [*]	5.2	12±2.5 ^{**}	2.4	0 [*]	<7	17±0.6 ^{**}	17		
JO18	<90		<90	<90			<90	N.D.		
JO18 <i>sigB::tet</i>	15±0.6 [*]	<6	21±1.5 ^{**}	1.4	0 [*]	<90	13±0.6 ^{**}	13		
JO18 <i>sarA::kan</i>	9±1.7 [*]	<10	14±1.5 ^{**}	1.6	0 [*]	<90	6±0.6 ^{**}	6		
JO18 <i>sigB::tet/sarA::kan</i>	8±2.6 [*]	<11.3	14±0.6 ^{**}	1.8	0 [*]	<90	0	N.D.		

CIP, ciprofloxacin; Sal, 2mM salicylate; EtBr, ethidium bromide; VAN, vancomycin; OC, Orange Clean® Super Concentrate; PS, Pine Sol®; FD, fold decrease compared with parent strain; FI, fold increase compared with non-induced strain; N.D., not determined.

^aNumbers represent millimetres grown on 90mm drug gradient plates and standard deviations ($n = 3$).

* Significant decrease compared with parent strain ($P < 0.05$).

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Significant increase compared with non-induced strain ($P < 0.05$).

Table 2

Effects of salicylate on β -galactosidase production by strains MC100 and PC161

Strain	Miller units of β -galactosidase ($\pm$ S.D.)				
	0	2mM Sal.	FI/FD	5mM Sal.	FI/FD
MC100	128.26 $\pm$ 7.12	146.66 $\pm$ 7.13	FI 1.14 *	159.83 $\pm$ 5.84	FI 1.25 *
PC161	29.43 $\pm$ 3.54	25.28 $\pm$ 3.28	FD 1.16 *	25.73 $\pm$ 1.90	FD 1.14 *

Sal., salicylate; FI, fold increase compared with non-induced strain; FD, fold decrease compared with non-induced strain; S.D., standard deviation ($n = 3-9$).

* Significant change ($P < 0.05$).

Table 3

Real-time polymerase chain reaction analysis of *sigB* and *sarA* transcription following growth with salicylate

Strain	Fold change in expression (Livak method $2^{-\Delta\Delta CT}$)	
	<i>sigB</i>	<i>sarA</i>
MC100	3.31	−1.37
PC161	4.38	−1.27
SH1000	1.38	1.21
S6C	1.23	2.97
JO18	1.07	−1.75