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Pine Oil Cleaner-Resistant *Staphylococcus aureus*: Reduced Susceptibility to Vancomycin and Oxacillin and Involvement of SigB

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Mutants of *Staphylococcus aureus* strain COL resistant to a household pine oil cleaner (POC) were isolated on laboratory media containing POC. *S. aureus* mutants expressing the POC resistance (POC^r) phenotype also demonstrate reduced susceptibility to the cell wall-active antibiotics vancomycin and oxacillin. The POC^r phenotype is reliant on the *S. aureus* alternative transcription factor SigB, since inactivation of *sigB* abolished expression of elevated POC resistance and the reductions in vancomycin and oxacillin susceptibilities. The isolation of suppressor mutants of COL*sigB*::*kan*, which maintain the *sigB*::*kan* allele, indicates that the POC^r phenotype can also be expressed to a lesser degree via a *sigB*-independent mechanism. These results bolster a growing body of reports suggesting that common disinfectants can select for bacteria with reduced susceptibilities to antibiotics. A series of in vitro-selected glycopeptide-intermediate *S. aureus* (GISA) isolates also expressed reductions in POC susceptibility compared to parent strains. Viewed collectively, our evidence suggests that mutations leading to the POC^r phenotype may also be involved with the mechanism that leads to the GISA phenotype.

The cost of infections caused by antibiotic-resistant *Staphylococcus aureus* in the United States has recently been estimated to be between 24 and 36 billion dollars per year (19). Infections with methicillin-resistant *S. aureus* (MRSA) are also associated with higher mortality rates than disease caused by methicillin-susceptible *S. aureus* (27). The glycopeptide antibiotic vancomycin remains the antibiotic of choice for treatment of serious MRSA infections; however, MRSA isolates expressing intermediate levels of vancomycin resistance, i.e., glycopeptide-intermediate *S. aureus* (GISA), have now been isolated from patients (for a review, see references 9 and 12). SigB, an alternative transcription factor of *S. aureus*, is involved with general stress resistance (5) and virulence factor production (2, 6, 15, 24). Inactivation of *sigB* in various strains of MRSA and GISA can also lead to reductions in resistance to oxacillin and vancomycin (30a, 32).

In the laboratory, various disinfectants and antiseptics have been shown to select for bacteria with increased antibiotic resistance (for a review, see reference 17), including MRSA with elevated resistance to antibiotics (1). It is now feared that the continued use of disinfectants in the household will promote the evolution of antibiotic resistance in bacteria (16). Cleaning and disinfecting solutions consisting of pine oil, surfactants, and alcohol are commonly purchased and used in households around the world. It has been previously shown in the laboratory that a pine oil-containing cleaning product can select for *Escherichia coli* mutants with reduced susceptibilities to multiple antibiotics (18).

In an effort to further understand potential links between disinfectant resistance and antibiotic resistance, we have now isolated MRSA mutants expressing elevated resistance to a common household pine oil cleaner (POC). Relationships between POC, oxacillin, and vancomycin resistance and *sigB* were also investigated.

MATERIALS AND METHODS

Bacterial strains, antibiotics, and chemicals. The bacterial strains used in this study are listed in Table 1. COL is a homogenous MRSA strain (28) that has been chosen for genomic sequencing (see http://www.tigr.org/tdb/mdb/mdbinprogress.html). Bacterial strains were maintained on Luria broth agar (LBA) (Difco, Detroit, Mich.), while isolates carrying the *sigB*::*kan* insertion were maintained on LBA supplemented with 100 mg of kanamycin/liter. Unless otherwise noted, chemicals were obtained from Sigma Chemical Co., St. Louis, Mo. The POC (Pine-Sol) was obtained from a local supermarket. Vancomycin, kanamycin, and oxacillin (Bristol Laboratories, Syracuse, N.Y.) were prepared in water, filter sterilized, and stored at −20°C.

Selection of *S. aureus* mutants expressing the POC resistance (POC^r) phenotype. Initially, 100-μl aliquots of overnight LB cultures of COL and COL*sigB*::*kan* were spread on LBA containing 1.5 and 0.4% POC (vol/vol), respectively, and incubated at 37°C until surviving POC^r mutant CFU appeared (48 to 72 h). Individual CFU were then passaged three times in LB before being tested for drug resistance levels. Individual POC^r CFU of both *S. aureus* strain COL and COL*sigB*::*kan* surviving on LBA plates prepared with 1.5 and 0.4% POC (vol/vol), respectively, arose at a frequency of 10^{-8}.

Determination of POC, vancomycin, and oxacillin susceptibilities. Resistance levels to POC, vancomycin, and oxacillin were determined by the gradient plate method using LBA, as described previously (23). POC (1% [vol/vol]) was added to the upper LBA layer (0-to-1% [vol/vol] gradient) for all strains examined, except for BB270 and SH108 and their respective GISA mutants, where 0.4% (vol/vol) POC was incorporated into the upper LBA layer (0-to-0.4% [vol/vol] gradient). Vancomycin (1 mg/liter final concentration) or oxacillin (500 mg/liter

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TABLE 1. Strains used in this study

Strain(s)	Relevant background[a]	Reference
COL	MRSA	28
COL*sigB*::*kan*	COL, *sigB*::*kan*	30a
CP170 to CP179	COL, POC^r	This study
CP191 to CP196	CP170*sigB*::*kan*	This study
CP197 to CP199	CP171*sigB*::*kan*	This study
CP182 and CP183	COL*sigB*::*kan,* POC^r	This study
BB568	MRSA, COL	10
$BB568_{V15}$	BB568 GISA	21
BB255	NCTC 8325	10
$BB255_{V3}$	BB255 GISA	21
BB270	MRSA, BB255	10
$BB270_{V15}$	BB270 GISA	21
BB399	MRSA	10
$BB399_{V12}$	BB399 GISA	21
SH108	*atl* mutant	8
$SH108_{V5}$	SH108 GISA	21

[a] Abbreviations: MRSA, methicillin-resistant *S. aureus;* POC^r, pine oil cleaner resistant; GISA, glycopeptide-intermediate *S. aureus.*

final concentration) was added to the upper LBA layer as required, creating 0-to-1-mg/liter and 0-to-500-mg/liter gradients, respectively. Gradient plates were incubated at 37°C for 48 h, and resistance levels were expressed as the distance grown in millimeters on the gradient by each strain investigated (Table 2). Vancomycin resistance population analyses were performed as previously described, using LBA containing increasing concentrations of vancomycin (20, 23). Essentially, *S. aureus* isolates were grown overnight in 3 ml of LB (37°C, 200 rpm) and then serially diluted in fresh LB. Ten-microliter aliquots of the dilutions were then pipetted onto the surface of LBA plates containing increasing concentrations of vancomycin. Plates were incubated for 24 h at 37°C and CFU were scored.

Transduction of *sigB*::*kan* and isolation of chromosomal DNA. Phage 80α lysates of COL*sigB*::*kan* were prepared and used to transduce the *sigB*::*kan* allele into COL mutants expressing the POC^r phenotype. Transductants were selected on LBA supplemented with 100 mg of kanamycin/liter. *S. aureus* chromosomal DNA for PCR analysis was isolated from lysostaphin-treated cells using DNAzol (Molecular Research Center, Cincinnati, Ohio) as recommended by the manufacturer. Chromosomal DNA was precipitated with ethanol, spooled onto a glass rod, and washed two times with 100% ethanol. The spooled washed DNA was then allowed to air dry and resuspended in 50 mM Tris-Cl–EDTA (pH 7.0) buffer (30).

RESULTS

Isolation and characterization of POC^r COL mutants. POC resistance levels were determined initially for POC^r mutants (strains CP170 to CP179) of COL surviving on 1.5% (vol/vol) POC. Distances grown by the POC^r mutants CP170 to CP179 on POC gradients were increased (2.4- to >3.9-fold) relative to the distance grown by the parent strain, COL (Table 2).

TABLE 2. POC, vancomycin, and oxacillin gradient plate results[a]

Strain	Parent	Km^r	Growth (mm) on gradient[b]		
			0 → 1% (vol/vol)[c] POC	0 → 1 mg/liter Van	0 → 500 mg/liter Oxa
COL		−	23	34	42
COL*sigB*::*kan*	COL	+	0 ↓	27 ↓	5 ↓
CP170	COL	−	55 ↑	44 ↑	42 −
CP171	COL	−	59 ↑	40 ↑	60 ↑
CP172	COL	−	63 ↑	59 ↑	63 ↑
CP173	COL	−	83 ↑	39 ↑	65 ↑
CP174	COL	−	53 ↑	34 −	63 ↑
CP175	COL	−	>90 ↑	37 ↑	71 ↑
CP176	COL	−	>90 ↑	40 ↑	73 ↑
CP177	COL	−	>90 ↑	42 ↑	73 ↑
CP178	COL	−	>90 ↑	57 ↑	73 ↑
CP179	COL	−	>90 ↑	42 ↑	74 ↑
CP191*sigB*::*kan*	CP170	+	0 ↓	29 ↓	40 ↓
CP192*sigB*::*kan*	CP170	+	0 ↓	29 ↓	27 ↓
CP193*sigB*::*kan*	CP170	+	0 ↓	29 ↓	24 ↓
CP194*sigB*::*kan*	CP170	+	0 ↓	29 ↓	19 ↓
CP195*sigB*::*kan*	CP170	+	5 ↓	34 ↓	30 ↓
CP196*sigB*::*kan*	CP170	+	0 ↓	34 ↓	27 ↓
CP197*sigB*::*kan*	CP171	+	0 ↓	25 ↓	0 ↓
CP198*sigB*::*kan*	CP171	+	0 ↓	19 ↓	0 ↓
CP199*sigB*::*kan*	CP171	+	0 ↓	20 ↓	0 ↓
CP182	COL*sigB*::*kan*	+	35 ↑	32 ↑	14 ↑
CP183	COL*sigB*::*kan*	+	33 ↑	27 −	14 ↑
BB568		ND	7	ND	ND
$BB568_{V15}$	BB568	ND	25 ↑	ND	ND
BB255		ND	14	ND	ND
$BB255_{V3}$	BB255	ND	24 ↑	ND	ND
BB270[c]		ND	17	ND	ND
$BB270_{V15}$[c]	BB270	ND	25 ↑	ND	ND
BB399		ND	7	ND	ND
$BB399_{V12}$	BB399	ND	28 ↑	ND	ND
SH108[c]		ND	22	ND	ND
$SH108_{V5}$[c]	SH108	ND	31 ↑	ND	ND

[a] Abbreviations: Km^r, kanamycin resistant; POC, pine oil cleaner; Van, vancomycin; Oxa, oxacillin; ND, not determined.

[b] Numbers represent millimeters of growth on 90-mm drug gradient plates. Arrows indicate increases (↑) or decreases (↓) in resistance levels expressed by mutant strains compared to relevant parent strains. The "−" indicates no difference in distances grown by mutant and parent strain on drug gradient plates.

[c] A gradient of 0 to 0.4% POC was used for BB270, $BB270_{V15}$, SH108, and $SH108_{V5}$.

Importance of *sigB* in the POC resistance mechanism. Since *sigB* is important in the general stress response of *S. aureus* (5), we determined the effect of *sigB* inactivation on POC resistance expression. Initially, the POC resistance levels of parent strain COL and that of the isogenic *sigB* mutant COL*sigB*::*kan* (Table 1) were analyzed. COL*sigB*::*kan* did not grow on the POC gradient examined, while the parent strain COL grew to a distance of 23 mm (Table 2). Transduction of the *sigB*::*kan* allele into POC^r mutants CP170 and CP171 resulted in kanamycin-resistant strains CP191 to CP196 and CP197 to CP199, respectively (Table 1). Chromosomal preparations of parent strains (COL, CP170, and CP171), COL*sigB*::*kan* (30a), and putative *sigB*::*kan* transductants (CP191 and CP197) were subjected to PCR using an internal *sigB* forward primer (5′-CCT TTGAACGGAAGTTTGAAGC-3′) and a reverse *sigB* primer (5′-TGACACACCATCATTTCTA-3′) (annealing temperature, 55°C) which binds 272 bp downstream of the *sigB* stop codon. In strains COL, CP170, and CP171, a 0.86-kb *sigB* PCR product was generated as expected. In the putative *sigB*::*kan* transductants (CP191 and CP197) and COL*sigB*::*kan*, a 0.86-kb *sigB* amplicon was replaced by a 1.7-kb *sigB*::*kan* PCR product. The 1.7-kb amplicon indicates a *sigB* allele in which a 664-bp *Eco*RV internal *sigB* fragment has been deleted and replaced with a 1.5-kb kanamycin resistance cassette. With the exception of CP195, all CP170 and CP171 *sigB*::*kan* transductants did not grow on the POC gradient examined (Table 2). While transductant CP195 did grow on the POC gradient investigated, it still demonstrated an 11-fold reduction in POC resistance compared to parent strain CP170. This evidence indicates that the loss of *sigB* in a wild-type and POC^r background leads to strains that are highly susceptible to the action of POC.

Two POC^r suppressor mutants of COL*sigB*::*kan* were successfully isolated from LBA plates containing 0.4% (vol/vol) POC. Both mutants CP182 and CP183 retained resistance to kanamycin, and CP182 at least also retained the inactivated *sigB*::*kan* allele as determined by PCR analysis (see above) and the presence of a 1.7-kb amplicon. Both CP182 and CP183 expressed a 33- to >35-fold increase in POC resistance compared to parent strain COL*sigB*::*kan*. The POC resistance levels expressed by CP182 and CP183 were lower than that of *sigB*$^+$ POC^r mutants CP170 to CP179 (Table 2), but they were higher than that of the original strain COL.

Vancomycin and oxacillin susceptibilities in POC^r mutants and isogenic *sigB*::*kan* transductants. Since *sigB* inactivation had such a dramatic effect on POC resistance and *sigB* is also associated with the expression of vancomycin and oxacillin resistance (32; Singh et al., submitted), vancomycin and oxacillin resistance levels were analyzed in the POC^r strains and their isogenic *sigB*::*kan* mutants. The distances grown by the POC^r COL mutants CP170 to CP173 and CP175 to CP179 on a vancomycin gradient were increased (1.1- to 1.7-fold) relative to the distance grown by parent strain COL (Table 2). In contrast, POC^r isolate CP174 grew to the same distance as that grown by parent strain COL on a vancomycin gradient (Table 2). Vancomycin resistance population analyses revealed that on plates containing 0.8 and 1.0 mg of vancomycin/liter, the number of individual CP170 and CP171 CFU surviving was increased by 1.9 and 2.4, and 1.4 and 0.7 log units, respectively, compared to the number of COL CFU surviving on these plates (Fig. 1). Except for the POC^r mutant CP170, the distances grown on an oxacillin gradient by all POC^r isolates were increased (1.4- to 1.8-fold) relative to the distance grown by parent strain COL (Table 2). Besides demonstrating reduced POC resistance, COL*sigB*::*kan*, CP191 to CP196, and CP197 to CP199 also demonstrated elevated vancomycin and oxacillin susceptibilities compared to their respective *sigB*$^+$ parent strains COL, CP170, and CP171 (Table 2). CP171 *sigB*::*kan* transductants (CP197, CP198, and CP199) did not grow at all on the oxacillin gradient examined (Table 2). Vancomycin resistance population analyses also revealed lower numbers of COL*sigB*::*kan*, CP191 (CP170*sigB*::*kan*), and CP197 (CP171*sigB*::*kan*) CFU surviving on increasing vancomycin concentrations compared to their respective parent strains (Fig. 1). At 1.0 mg of vancomycin/liter, the number of CP191 and CP197 CFU surviving was reduced by 1.5 and 1.3 log units, respectively, compared to the number of POC^r parent strain CP170 and CP171 CFU surviving on the same vancomycin concentration (Fig. 1). Oxacillin susceptibility was also reduced 2.8-fold in both POC^r *sigB*::*kan* suppressor mutants CP182 and CP183 compared to parent strain COL*sigB*::*kan*, but only CP182 demonstrated a reduced vancomycin susceptibility (1.2-fold) compared to the parent COL*sigB*::*kan*.

Comparison of POC resistance levels of in vitro-selected GISA strains and their respective parent strains. Our results demonstrate a connection between POC resistance, *sigB*, and reduced susceptibility to vancomycin. Therefore, we investigated if in vitro-selected GISA strains representing different genetic lineages (Table 1) also expressed altered POC resistance levels compared to their respective parent strains. The distances grown on POC gradients by all GISA isolates examined ($BB568_{V15}$, $BB255_{V3}$, $BB399_{V12}$, $BB270_{V15}$, and $SH108_{V5}$) were increased relative to the distances grown by each of the respective parent strains (Table 2). This indicates that mutations selected for in vitro that result in the GISA phenotype can also lead to elevated POC resistance.

DISCUSSION

POCs are used in the household for cleaning and disinfection of most surfaces and clothing. The active ingredient of POCs is pine oil, which significantly adds to the ability of POCs to deodorize and disinfect areas and items within a household. The predominant component of pine oil is alpha-terpineol (7), which along with other terpenes has antimicrobial activity (4, 11, 14, 25). We have shown here that mutants of an MRSA strain demonstrating elevated resistance to POC can be isolated on laboratory media containing 1.5% POC (vol/vol) in a single step. We have also determined that the POC^r phenotype leads to reduced oxacillin and vancomycin susceptibility. In vitro-selected *S. aureus* mutants demonstrating elevated resistance to the common household disinfectant triclosan also can express slightly reduced susceptibilities to vancomycin (see Table 3 in reference 31). These results add to a growing body of reports suggesting that common disinfectants can select for bacteria with reduced susceptibilities to antibiotics (16).

The emergence of GISA has become a serious public health threat (9, 12). Some strains of *S. aureus* expressing reduced vancomycin susceptibility have been reported to have vancomycin MICs as low as 1.0 mg/liter (3, 9, 13). It is not until these

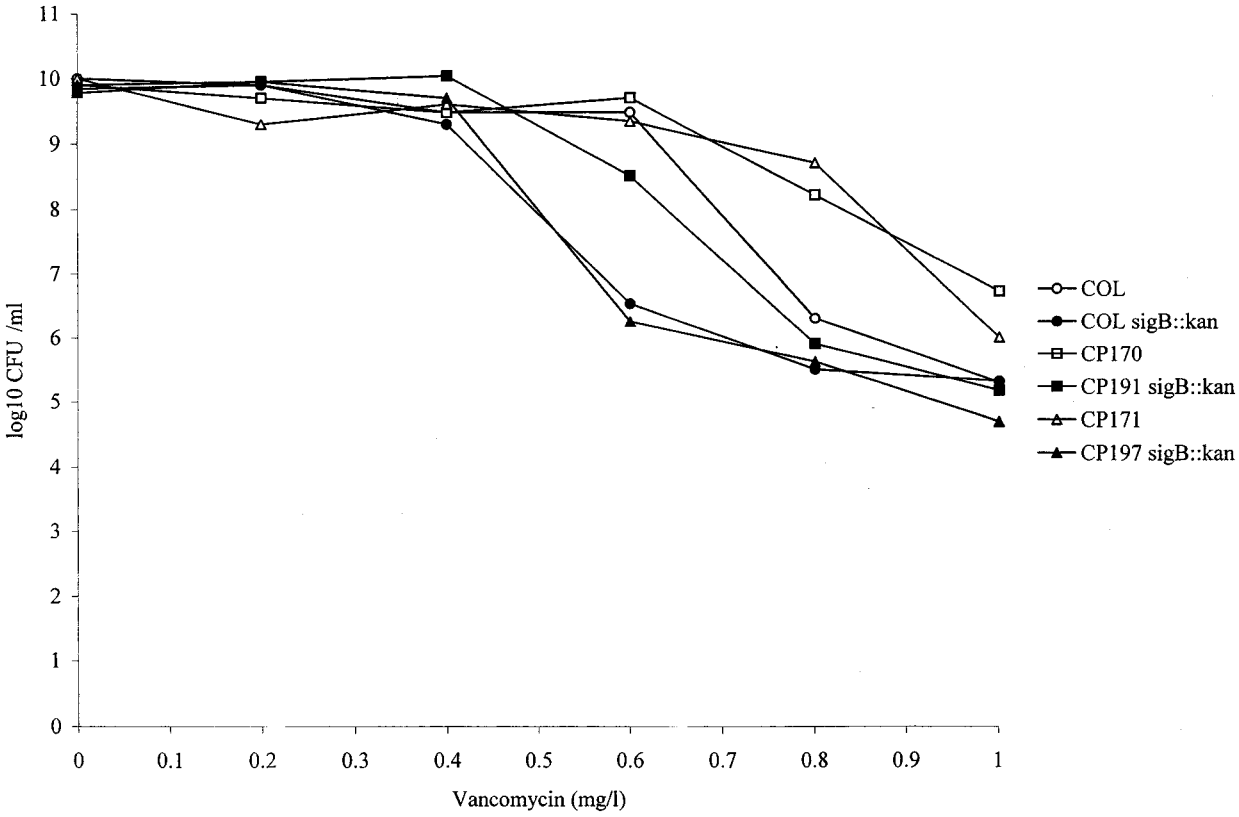

FIG. 1. Vancomycin resistance population analysis for strains COL, COL*sigB*::*kan*, CP170, CP191*sigB*::*kan*, CP171, and CP197*sigB*::*kan*.

strains are screened on media containing vancomycin that their true GISA identity is discovered (for a review, see reference 9). Since low-level vancomycin resistance is relevant, it will be important to determine if POC^r mutants that survive on subclinical vancomycin concentrations also mutate with greater ease to obtain a GISA phenotype.

All in vitro-selected GISA strains examined in our study had increased resistance to POC, even though these isolates had no prior exposure to POC. The increase in vancomycin resistance expressed by the in vitro-selected GISA we have analyzed has been attributed to alterations in cell wall structure and physiology (21). Since POCs are made with pine oil, surfactants, and alcohol, these mixtures probably target the membrane of susceptible microorganisms. Therefore, it is probable that the mutations leading to the POC^r phenotype, similar to mutations leading to the GISA phenotype, also alter the cell wall structure of *S. aureus*.

POC resistance is reliant on *sigB*, since inactivation of this alternative transcription factor led to dramatic reductions in POC resistance and increases in vancomycin and oxacillin susceptibilities. However, the isolation of POC^r suppressor mutants of COL*sigB*::*kan* (CP182 and CP183), which retain the *sigB*::*kan* allele, suggests that a *sigB*-independent pathway can also provide the cell with the means to express the POC^r phenotype. The levels of POC resistance and reductions in vancomycin (when expressed) and oxacillin susceptibilities, however, were lower for POC^r suppressor mutants (CP182 and CP183) compared to *sigB*$^+$ POC^r mutants CP170 to CP179. This indicates that while a suppressor mutation(s) can overcome *sigB* inactivation to allow for the POC^r phenotype, strains with intact *sigB* express greater POC^r-related resistance levels. The *agr* locus is intimately involved with the production of *S. aureus* virulence factors (26). Recently, a report appeared demonstrating that deletion of the *agr* locus leads to reduced vancomycin susceptibility in *S. aureus* (29). Piriz-Duran et al. previously demonstrated that the inactivation of *agr* also leads to reduced oxacillin resistance in *S. aureus* (22). In addition, the *agr* operon is under the control of SigB (2). Therefore, it is possible that mutations leading to the *sigB*-dependent POC^r phenotype involve the *agr* locus.

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