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# Novel Locus Required for Expression of High-Level Macrolide-Lincosamide-Streptogramin B Resistance in *Staphylococcus aureus*

P. K. Martin,* Y. Bao, E. Boyer,† K. M. Winterberg,‡ L. McDowell,§ M. B. Schmid,‖ and J. M. Buysse

*Essential Therapeutics, Inc., Mountain View, California 94043*

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**The *yycF1*(Ts) mutation in *Staphylococcus aureus* conferred hypersensitivity to macrolide-lincosamide-streptogramin B ($MLS_B$) antibiotics on strains either containing or lacking *ermB*. The overexpression of the *S. aureus* Ssa protein restored the *yycF1* mutant to wild-type levels of susceptibility. Inactivation of *ssa* in an unmutagenized strain dramatically reduced *ermB*-based resistance. Conditional loss of function or expression of *ssa* in the *yycF1* mutant is proposed to result in the observed hypersensitivity to $MLS_B$ antibiotics.**

One of several phenotypic consequences of the *yycF1*(Ts) mutation in *Staphylococcus aureus* was a hypersensitivity to macrolide-lincosamide-streptogramin B ($MLS_B$) antibiotics (9). This hypersensitivity was returned to wild-type levels when the mutant was cultured under anoxic conditions at neutral pH. Accordingly, when the *ermB*-containing transposon Tn*917lac* was transduced into the original *yycF1*(Ts) mutant (strain NT372), erythromycin-resistant ($Em^r$) transductants could be selected only anaerobically. An isogenic set of strains, SAM1010 [*yycF1*(Ts) Tn*917lac*::*purA571*] and SAM1011 (Tn*917lac*::*purA571*), was constructed by using this strategy (Table 1). Even though both strains were marked with *ermB*, the SAM1010 mutant expressed only an intermediate level of resistance to $MLS_B$-class antibiotics compared to that expressed by SAM1011 (Table 2). In the absence of resistance genes, the NT372 temperature-sensitive mutant was fourfold more sensitive to erythromycin (ERM) than the unmutagenized parental strain (9). Because the *yycF* response regulator was reported to effect changes at the level of transcription (3, 4), it is possible that the conditional underexpression of one or more chromosomally carried genes in the *yycF1*(Ts) mutant of *S. aureus* could account for this observed hypersensitivity.

The NT372 mutant grew very poorly on plating media like Trypticase soy agar (TSA) at 39°C and did not grow at higher temperatures. Thus, by selecting plasmid-based genomic clones that restored high-level $Em^r$ (10 μg/ml) on TSA at this semipermissive growth temperature, it was thought that one or more related genes affecting this partial $MLS_B$ resistance phenotype might be revealed. With a plasmid library (9) constructed from the unmutagenized parental strain (SAM23), a total of 21 $Em^r$ transductants of NT372 were isolated. Under identical selection conditions, over 50,000 $Em^r$ transductants for the isogenic wild type (SAM23) were obtained. Of the 21 $Em^r$ transductants of NT372, 5 could be reselected at 43°C and contained plasmids bearing the original *yycFG* locus (complementing clones). The remaining 16 clones, represented by strain SAM1287 (Table 1), bore equivalently sized genomic inserts (5.2 kb) that conferred high-level ERM resistance (MIC $>$ 512 μg/ml) but did not fully restore the mutant's ability to grow at the higher temperature (43°C).

The inserts of all 16 clones were sequenced and found to be identical. Subcloning and recomplementation of the NT372 mutant (Fig. 1a) correlated the selection of high-level $MLS_B$ resistance to a single open reading frame (ORF), *ssa*. The subclone lacking *ssa*, pMP1025, conferred only very weak growth on solid media at lower ERM concentrations (1 μg/ml), demonstrating that the combination of *ssa* and *ermC* was necessary for the expression of high-level $MLS_B$ resistance in the presence of a *yycF1* chromosomal mutation. To test this apparent correlation between *ssa* expression and the observed antibiotic hypersensitivity, inactivation of the *ssa* locus in an unmutagenized $Em^r$ strain (SAM1011) was attempted.

By using an *S. aureus* integration plasmid (pMP2376) with a temperature-sensitive origin of replication from pE194*ts* and the *tetK* resistance gene from pT181, the chromosomal copy of this ORF (*ssa*) was inactivated in SAM1011 ($Em^r$) by Campbell-style integration (Fig. 1b). Briefly, exponentially growing cultures containing the integration plasmid were shifted to a temperature restrictive for plasmid replication (39°C) and allowed to grow to saturation at this temperature. Site-specific integration of the disruption plasmid into the *ssa* chromosomal locus (SAM1011s1) was confirmed by genomic PCR and Southern blot analysis (data not shown). The MICs of different $MLS_B$-class antibiotics for the resulting progeny dropped substantially (Table 2), demonstrating that an intact copy of *ssa* was necessary for the full phenotypic expression of resistance in a strain bearing the *ermB* gene (SAM1011).

To demonstrate further that the observed change in susceptibility to $MLS_B$-type antibiotics was a direct result of inacti-

* Corresponding author. Present address: Osel, Inc., 1800 Wyatt Dr., Suite 14, Santa Clara, CA 95054. Phone: (408) 986-0012, ext. 210. Fax: (408) 986-0019. E-mail: pmartin@oselinc.com.

† Present address: Silicon Genetics, Inc., Redwood City, CA 94063.

‡ Present address: University of Wisconsin, Department of Biochemistry, Madison, WI 53706-1544.

§ Present address: Antibacterial Molecular Sciences, Pfizer Global Research and Development, Ann Arbor, MI 48105.

‖ Present address: Affinium Pharmaceuticals, Inc., Toronto, Ontario M5J 1V6, Canada.

TABLE 1. Strains and plasmids

| Strain or plasmid | Relevant genotype or description | Phenotype[a] | Reference |
|---|---|---|---|
| Strains | | | |
| SAM23 | 8325-4 | | 6 |
| NT372 | SAM23 *yycF1* | TS | 9 |
| SAM1010 | SAM23 *yycF1* Tn*917lac*::*purA571* | TS, $Em^r$ | 9 |
| SAM1011 | SAM23 Tn*917lac*::*purA571* | $Em^r$ | 9 |
| SAM1011s1 | SAM1011 *ssa*::pMP2376 | $Tc^r$ | This work |
| SAM1011s1r1 | SAM1011 *ssa*$^+$ | $Em^r$ | This work |
| SAM1287 | NT372 pMP532 | $Em^r$ | This work |
| Plasmids | | | |
| pMP16 | *S. aureus-Escherichia coli* shuttle vector | $Em^r$ $Ap^r$ | 9 |
| pMP532 | pMP16(*ssa nhaC*) | $Em^r$ | This work |
| pMP1025 | pMP16(*nhaC*) | $Em^r$ | This work |
| pMP1029 | pMP16(*ssa*) | $Em^r$ | This work |
| pMP2376 | *ssa* disruption plasmid | *rep*(Ts) $Tc^r$ $Ap^r$ | This work |

[a] TS, temperature sensitive; $Tc^r$ and $Ap^r$, tetracycline and ampicillin resistance, respectively.

vation of the *ssa* ORF, the integration plasmid was allowed to resolve from the chromosome and restore a functional copy of the *ssa* ORF. Single colonies of the integration strain (SAM1011s1) were inoculated into broth (Trypticase soy broth) and cultured in the absence of antibiotic selection at a temperature permissive for plasmid replication (30°C). Serial dilutions of the saturated overnight cultures were plated onto selective medium (TSA with ERM), and numbers of viable cells in these cultures were compared to those observed on nonselective medium (TSA). The number of colonies that had resolved the tandem duplication and had regained phenotypic $Em^r$ were identified at approximately 1:500 CFU/ml, which is in general agreement with our previous observations (9). A subset (eight isolates) of this population was assayed, and all of these isolates had regained full phenotypic $Em^r$ (MIC > 512 μg/ml). Chromosomal DNA was prepared from these eight strains, and a restored copy of the *ssa* ORF at the predicted size was confirmed by PCR for each (eight out of eight).

The biological function of the *ssa* gene in *S. aureus* is not understood. The inactivation of this locus caused no apparent effect upon the viability of the strain in vitro, demonstrating that the expression of the *ssa* gene was not essential. The *ssa* ortholog from *Staphylococcus epidermidis* (8) has been described as encoding a secreted, highly immunogenic protein expressed during the course of infection. The *ssa* ortholog from *S. aureus* described here encoded a predicted hydrophilic polypeptide of 267 amino acids, with a predicted transmembrane region at the N terminus (Fig. 2). At the C-terminal end of this ORF was a predicted block of 67 amino acids, which was found to be highly similar (>50% identity) to the equivalent C-terminal residues of six additional hypothetical proteins in the completed genomic sequence of *S. aureus* strain N315 (7). This apparently conserved motif did not contain the classic gram-positive cell surface anchoring motif, LPXTG (12). The LysM peptidoglycan binding motif (1) was identified in three of these ORFs, suggesting that this group of unassigned proteins may bind to a specific entity on the surface of *S. aureus*, not unlike the choline binding proteins of *Streptococcus pneumoniae* (5).

Aside from those in coagulase-negative staphylococci (8), there do not appear to be structural orthologs of *ssa* in the genomes of other gram-positive bacteria that have been sequenced to date. The conditional loss of function of *ssa* in the *yycF1*(Ts) mutant of *S. aureus* (SAM1010) may explain why the

TABLE 2. Changes in MICs as a result of *ssa* inactivation

| Antibiotic | MIC (μg/ml) for strain: | | |
|---|---|---|---|
| | SAM1010 (Ts) [*yycF1*(Ts) *ermB*] | SAM1011 (*ermB*) | SAM1011s1 (*ssa*::*tetK ermB*) |
| Macrolides (14 and 16 membered) and lincosamides | | | |
| Erythromycin | 16 | >512 | 8 |
| Roxithromycin | 32 | >512 | 4 |
| Josamycin | 8 | >512 | 2 |
| Tylosin tartrate | 4 | >512 | 2 |
| Lincomycin | 1 | >512 | 2 |
| Clindamycin | 0.25 | >512 | 0.25 |
| Representative non-$MLS_B$-type antibiotics | | | |
| Chloramphenicol | 16 | 16 | 8 |
| Phleomycin | 4 | 4 | 4 |
| Streptomycin | 2 | 2 | 2 |
| Vancomycin | 1 | 1 | 1 |
| Methicillin | 1 | 1 | 1 |
| Norfloxacin | 0.5 | 0.5 | 0.5 |

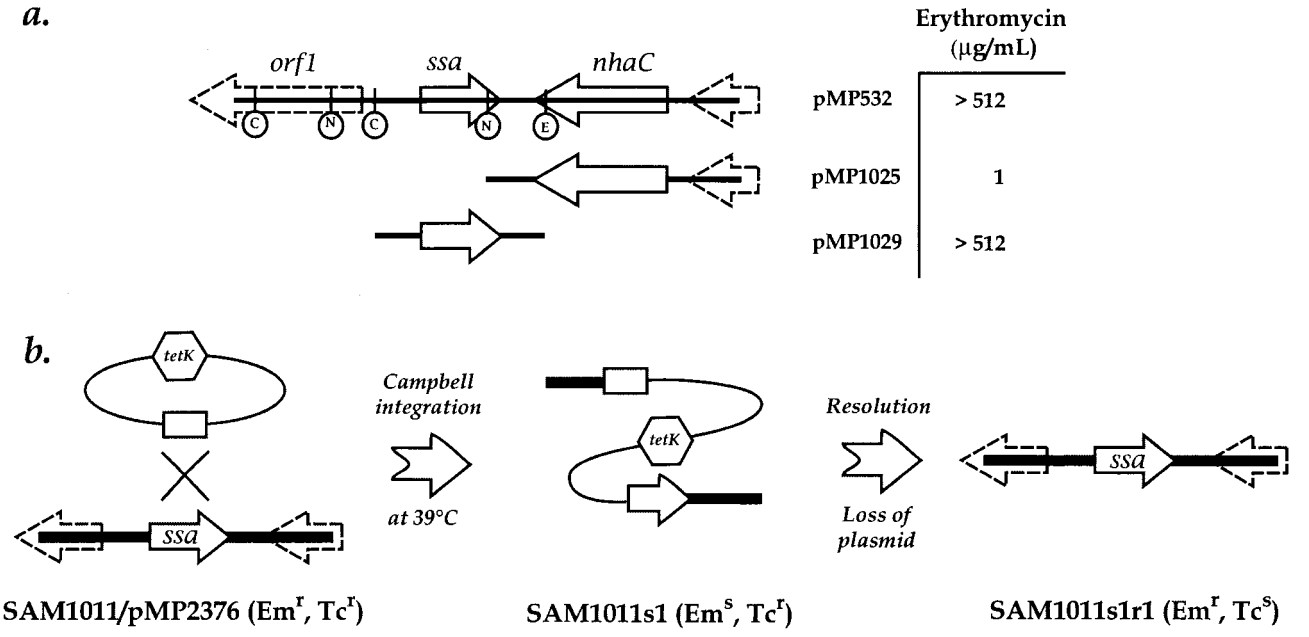


FIG. 1. Mutant complementation and gene inactivation. (a) A partial restriction map of the suppressor clone, pMP532, along with subcloning and mutant recomplementation data are shown. Incomplete ORFs are denoted by dashed arrows. Restriction enzyme abbreviations are as follows: C, *Cla*I; E, *Eco*RI; and N, *Nhe*I. (b) The inactivation of the chromosomal *ssa* locus resulted in a reduction of the MIC of ERM. The resolution of the plasmid integrant and subsequent restoration of an intact copy of *ssa* completely restored $Em^r$.

SAM1010 strain was hypersensitive to $MLS_B$-class antibiotics whereas other gram-positive *yycF* mutants reportedly do not share this defect (3, 4, 10, 11). We have demonstrated that the inactivation of the *ssa* gene in *S. aureus* unexpectedly resulted in the loss of full phenotypic expression of *ermB*-based antibiotic resistance. This is the first report of the expression of a novel uncharacterized cell surface protein as an accessory factor to the manifestation of high-level $MLS_B$ resistance in *S. aureus*. We propose that this is analogous to the identification of the *fem* genes in *S. aureus*, which are chromosomal genes that contribute to the phenotypic expression of methicillin resistance in strains containing the *mec* determinant (2).

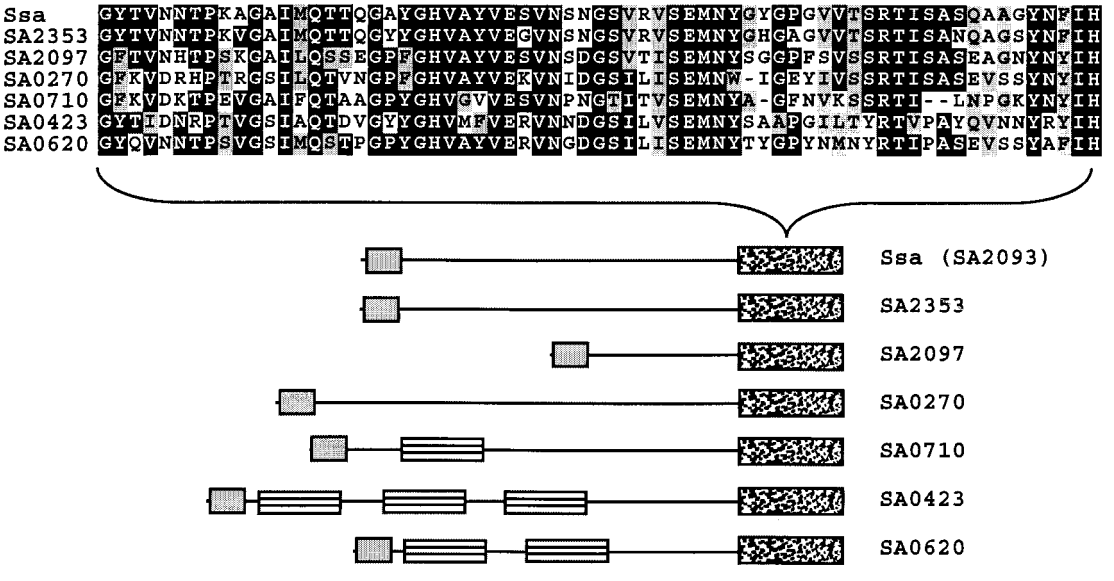


FIG. 2. Alignment of the C-terminal regions of seven *S. aureus* hypothetical proteins. The 67 C-terminal amino acids of each hypothetical ORF show significant identity (>50%). The grey box denotes a predicted transmembrane domain, and the striped box indicates similarity to the LysM domain, common among proteins associated with peptidoglycan processing (1). The mottled box indicates the common C-terminal domain corresponding to the alignment above. Biological functions for these ORFs have not been confirmed; therefore, each ORF is named according to the published genomic data (7) for strain N315 (http://www.bio.nite.go.jp/cgi-bin/dogan/genome_top.cgi?′n315′).

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