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Peptide Deformylase in *Staphylococcus aureus*: Resistance to Inhibition Is Mediated by Mutations in the Formyltransferase Gene

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Peptide deformylase, a bacterial enzyme, represents a novel target for antibiotic discovery. Two deformylase homologs, *defA* and *defB*, were identified in *Staphylococcus aureus*. The *defA* homolog, located upstream of the transformylase gene, was identified by genomic analysis and was cloned from chromosomal DNA by PCR. A distinct homolog, *defB*, was cloned from an *S. aureus* genomic library by complementation of the arabinose-dependent phenotype of a P_{BAD}-*def Escherichia coli* strain grown under arabinose-limiting conditions. Overexpression in *E. coli* of *defB*, but not *defA*, correlated to increased deformylase activity and decreased susceptibility to actinonin, a deformylase-specific inhibitor. The *defB* gene could not be disrupted in wild-type *S. aureus*, suggesting that this gene, which encodes a functional deformylase, is essential. In contrast, the *defA* gene could be inactivated; the function of this gene is unknown. Actinonin-resistant mutants grew slowly in vitro and did not show cross-resistance to other classes of antibiotics. When compared to the parent, an actinonin-resistant strain produced an attenuated infection in a murine abscess model, indicating that this strain also has a growth disadvantage in vivo. Sequence analysis of the actinonin-resistant mutants revealed that each harbors a loss-of-function mutation in the *fmt* gene. Susceptibility to actinonin was restored when the wild-type *fmt* gene was introduced into these mutant strains. An *S. aureus* Δ*fmt* strain was also resistant to actinonin, suggesting that a functional deformylase activity is not required in a strain that lacks formyltransferase activity. Accordingly, the *defB* gene could be disrupted in an *fmt* mutant.

In procaryotes and eucaryotes, protein synthesis is initiated with a methionine residue which is removed during protein maturation (13). In bacteria and mitochondria, formyltransferase, the *fmt* gene product, transfers a formyl group to the amino group of the methionine esterified to $tRNA^{fMet}$. Consequently, nascent polypeptides have a formylated methionine at their N termini. In procaryotes the formyl moiety is removed from the growing peptide by peptide deformylase, the product of the *def* gene (1, 5, 13, 21). Although proteins synthesized in mitochondria are formylated, neither a *def* gene nor deformylase activity has been detected in these organelles (17). Searches for sequences homologous to the peptide deformylase among bacterial genomes in publicly available databases reveal the presence of shared open reading frames (ORFs) that encode homologs of *Escherichia coli* transformylase and deformylase proteins, indicating that the corresponding genes are widely distributed among the bacteria (9, 18, 24). It is not possible to construct null mutants of the *def* gene in wild-type *E. coli*, suggesting that the gene is essential for growth (19, 20). On the other hand, it has been shown that deletion of the transformylase-encoding gene, *fmt*, results in impaired growth. In this genetic background, double mutants of *E. coli* that lack both transformylase and deformylase can be constructed; these double mutants have the same impaired growth phenotype as the *fmt* single mutants (11, 19, 26).

Many successful antibiotics inhibit steps of protein synthesis; however, no antimicrobial agent that inhibits protein modification has ever been reported. The widespread occurrence, conservation, and essential nature of deformylase in bacteria, coupled with the absence of this activity in mammalian cells, make it an attractive target for antibacterial drug discovery. Very little is known about deformylase other than the *E. coli* deformylase. Most gram-negative organisms, including *E. coli*, have one chromosomal copy of the *def* gene; however, most gram-positive bacteria have two homologs (9). Redundancy at the genetic or biochemical level can have serious implications for the attractiveness of an enzyme as a drug target, since it provides a relatively facile means of generating resistance. This can be achieved simply through a gene dosage effect or by mutations in which one copy of the gene encodes an enzyme resistant to the antibiotic while the second copy continues to function normally. We have recently identified a potent peptide deformylase inhibitor, actinonin (8). This compound is active against gram-positive and fastidious gram-negative bacteria. The aim of this work was to investigate the suitability of bacterial deformylase as a drug target in *Staphylococcus aureus*. The *S. aureus* deformylase-encoding gene was identified and characterized. In addition, actinonin-resistant mutants were selected and the mechanism of resistance was elucidated.

MATERIALS AND METHODS

Growth conditions and strains. The *S. aureus* and *E. coli* strains and plasmids used in this study are listed in Table 1. Bacterial cultures were incubated at 35°C unless otherwise noted. *E. coli* strains were grown in Luria-Bertani (LB) broth, and *S. aureus* strains were grown in tryptic soy broth (Difco Laboratories, Detroit, Mich.). For antibiotic selection and genetic manipulations, medium was supplemented with 100 μg of ampicillin per ml, 25 μg of kanamycin per ml, 10 μg of chloramphenicol per ml, 10 μg of tetracycline per ml, 5 μg of erythromycin per ml, or 10 μg of gentamicin per ml, as required. Actinonin, antibiotics, and other chemicals were purchased from Sigma (St. Louis, Mo.); linezolid was synthesized in-house. For growth rate determinations, cells were grown in LB broth overnight, diluted to an optical density at 600 nm of 0.04 in fresh medium, and incubated in a rotary shaker. Growth was monitored spectrophotometrically at 600 nm with a DU640 spectrophotometer (Beckman, Fullerton, Calif.).

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TABLE 1. Strains and plasmids used in this work

Strain or plasmid	Relevant characteristics	Source or reference
Strains		
E. coli JM109	[F′ *traD36 proA*$^+$*B*$^+$ *lacI*^{q} Δ(*lacZ*)*M15*] *recA1 endA1 gyr96 hsdR17*(r_K^- m_K^+) *supE44 relA1 thi* Δ(*lac-proAB*)	Promega, Madison, Wis.
E. coli MC1061	F$^-$ *araD139* Δ(*ara leu*)*7697 galE15 galK16* Δ(*lac*)*X74 rpsL hsdR2* (r_K^- m_K^+) *mcrA mcrB1*	Bio-Rad, Hercules, Calif.
E. coli BL21(DE3)(pLysS)	F$^-$ *ompT hsdS*$_B$ (r_B^- m_B^-) *dcm gal* λ(DE3) (pLysS)	Novagen, Madison, Wis.
E. coli VECO2065	MC1061 ΔP_{def}::P_{BAD}; P_{BAD}-regulated *def* gene	This study
E. coli VECO2068	VECO2065 Δ*tolC*	This study
S. aureus ATCC 25923		American Type Culture Collection, Manassas, Va.
S. aureus 1-63	Clinical isolate	H. F. Chambers III (12)
S. aureus NCTC 8325-4		28
S. aureus RN4220		14
S. aureus VSAU6011	ATCC 25923 *fmt* (A108E); actinonin-resistant mutant	This study
S. aureus VSAU6012	ATCC 25923 *fmt* (G117V); actinonin-resistant mutant	This study
S. aureus VSAU6013	ATCC 25923 *fmt* (E157stop); actinonin-resistant mutant	This study
S. aureus VSAU6014	1-63 *fmt* (frameshift, codon 180); actinonin-resistant mutant	This study
S. aureus VSAU7108	RN4220 Δ*defA*::*aac-aph* (pPV158-1)	This study
S. aureus VSAU7136	RN4220 Δ*fmt*::*aac-aph*	This study
Plasmids		
pAW8	*tet* $ori_{E.\ coli}$ $ori_{S.\ aureus}$; shuttle vector	33
pAW9	*tet* $ori_{E.\ coli}$; shuttle vector	33
pBAD/*Myc*-HisB	*bla araC* P_{BAD}	Invitrogen, Carlsbad, Calif.
pBS-SK$^+$	*bla lacZ*α	Stratagene, La Jolla, Calif.
pBS-SK$^-$	*bla lacZ*α	Stratagene, La Jolla, Calif.
pBS-KS$^+$	*bla lacZ*α	Stratagene, La Jolla, Calif.
pBSII-SK$^+$	*bla lacZ*α	Stratagene, La Jolla, Calif.
pBSL99	*kan*	American Type Culture Collection, Manassas, Va.
pBT2	*bla cat repF*(Ts); shuttle vector, temperature sensitive for replication in *S. aureus*	7
pET20b$^+$	*bla* P_{T7}	Promega, Madison, Wis.
pGO1	*aac-aph*	32
pGO514	*aac-aph*	27
pKO3	*sacB cat repA*(Ts)	16
pRDC19	*erm*	F. Arigoni
pT7Blue	*bla lacZ*α	Novagen, Madison, Wis.
pUC19	*bla lacZ*α	New England Biolabs, Beverly, Mass.
pDYD11	pBSII-SK$^+$::*kan* upstream$_{def}$	This study
pDYD12	pBAD/*Myc*-HisB::*def*; P_{BAD}-*def* fusion	This study
pDYD13	pKO3::upstream$_{def}$ *kan araC* ΔP_{def}::P_{BAD}-*def*	This study
pVCRΔtolC	pKO3::Δ*tolC*	This study
pPV16-1	pBS-SK$^-$::*defB*	This study
pPV45-1	pET20b$^+$::*defA*; P_{T7}-*defB* fusion	This study
pPV54-2	pT7Blue::*defA*	This study
pPV58-1	pET20b$^+$::*defB*; P_{T7}-*defA* fusion	This study
pPV46-1	pT7Blue::*repF*(Ts)	This study
pPV72-2	pAW9::*repF*(Ts); shuttle vector, temperature sensitive for replication in *S. aureus*	This study
pPV77-1	*lacG* disruption plasmid derived from pPV72-2	This study
pPV92-3	*gusA* disruption plasmid lacking *S. aureus* homology derived from pPV72-2	This study
pPV120-1	*defB* disruption plasmid derived from pPV72-2	This study
pPV150-1	pT7Blue::P_{defA} Δ*defA fmt*	This study
pPV158-1	pAW8::P_{defA} Δ*defA fmt*; *fmt* complementation	This study
pPV171-1	pBS-KS$^+$::*erm repF*(Ts) shuttle vector, temperature sensitive for replication in *S. aureus*	This study
pPV172-4	pPV46-1::*erm*; shuttle vector, temperature sensitive for replication in *S. aureus*	This study
pPV179-1	pPV171-1::P_{defA} Δ*defA*::*aac-aph fmt*	This study
pPV188-1	pT7Blue::P_{defA} *defA* Δ*fmt*	This study
pPV214-1	pT7Blue::*erm repF*(Ts) P_{defA} *defA* Δ*fmt*::*aac-aph*	This study

Molecular techniques, PCR, and sequence analysis. Molecular techniques, including cloning and DNA purification from *E. coli* and *S. aureus*, were performed by standard protocols (28, 31). Oligonucleotides were synthesized at Operon Technologies (Alameda, Calif.). PCR was performed with Advantage HF Polymerase (Clontech, Palo Alto, Calif.) by using a RoboCycler instrument (Stratagene, La Jolla, Calif.). DNA sequences of cloned or PCR-amplified fragments were determined by using the dideoxy chain termination method (Sequetech, Mountain View, Calif.). Homology searches with BLAST (2) were performed at the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov). Preliminary sequence data were obtained from The Institute for

Genomic Research website (http://www.tigr.org) and from the *S. aureus* Genome Sequencing Project (http://www.genome.ou.edu). Homologs of *E. coli* peptide deformylase retrieved from the public databases were subjected to multiple alignment and phylogenetic analyses by using the GCG software programs (Genetics Computer Group, Madison, Wis.). Plasmids were introduced into *E. coli* and *S. aureus* by electroporation with an Electroporator II apparatus (Bio-Rad, Hercules, Calif.) and by standard procedures. All constructs were confirmed by sequencing or PCR.

Construction of arabinose-dependent P_{BAD}-*def E. coli* strains. The *E. coli def* gene was placed under *araBAD* promoter control by a promoter exchange strategy. First, a kanamycin resistance-encoding gene from pBSL99 was cloned as a *Sac*I-*Xba*I fragment into pBSII-SK$^+$. Next, sequences immediately upstream of P_{def} were amplified from JM109 and were cloned as a *Sac*I-*Asc*I fragment into this plasmid, creating pDYD11. The full-length *E. coli def* gene was cloned as a *Nco*I-*Bgl*II fragment into pBAD/*Myc*-HisB, yielding pDYD12. The *def* suicide vector was constructed by a three-way ligation of (i) the *Ecl*136II-*Nde*I fragment from pDYD11, (ii) the *Nde*I-*Bgl*II *def*-containing fragment from pDYD12, and (iii) the *Sma*I-*Bam*HI-digested cloning vector, pKO3, which contains *sacB* and a temperature-sensitive origin of replication. The resulting clone, pDYD13, was the desired promoter replacement vector. This plasmid was used to replace, in *E. coli* MC1061, the endogenous *def* promoter with the P_{BAD} promoter by conventional allele replacement techniques (16) with the following modifications. All plates were supplemented with kanamycin and arabinose (0.2%; wt/vol) throughout the procedure; clones were passaged twice on counterselection plates that contained sucrose (5%; wt/vol) but that lacked NaCl; and sucrose-resistant recombinants were screened for ampicillin sensitivity, chloramphenicol sensitivity, kanamycin resistance, and arabinose dependence. The resulting P_{BAD}-*def* construct, *E. coli* VECO2065, is arabinose dependent for growth.

In order to introduce a *tolC* deletion mutation into VECO2065, a 2.7-kb fragment containing *tolC* and flanking sequences was amplified from JM109 by PCR and was subcloned into pUC19. A 700-bp internal deletion of the cloned *tolC* gene was created by *Pst*I-*Nsi*I double digestion, followed by ligation of the compatible ends. The fragment that contained the *tolC* deletion allele was subsequently subcloned into pKO3. The resulting plasmid, pVCRΔtolC, was used for allele replacement in *E. coli* VECO2065 as described above. The Δ*tolC E. coli* strain, VECO2068, was identified by screening of sucrose-resistant, ampicillin-sensitive clones on arabinose-supplemented MacConkey agar, which does not support the growth of *tolC* mutants. The presence of Δ*tolC* was confirmed by PCR.

Identification and cloning of *S. aureus def* genes. To complete the sequence of the *S. aureus defA* gene, sequences upstream of the *S. aureus fmt* gene homolog were cloned by inverse PCR (29), taking advantage of the *Bsr*FI restriction site within *fmt*. Briefly, chromosomal DNA from *S. aureus* NCTC 8325-4 was digested with *Bsr*FI, religated, and subjected to PCR amplification with a pair of *fmt*-specific divergently oriented primers. The resulting 1.3-kb fragment was cloned and sequenced. Subsequently, a 0.7-kb fragment, extending from 260 bp upstream of the *defA* start codon to 20 bp downstream of its stop codon, was amplified from *S. aureus* RN4220, cloned into pT7Blue, and sequenced, generating pPV54-2.

An *S. aureus* gene that encoded a functional deformylase was cloned by complementing the arabinose-dependent phenotype of the P_{BAD}-*def E. coli* strain when grown under noninducing conditions. An aliquot of a bacteriophage lambda library of *S. aureus* genomic DNA (Stratagene, La Jolla, Calif.) was phage amplified, and the phagemid was excised according to the manufacturer's instructions. The resulting plasmid pool was used to transform P_{BAD}-*def* strain VECO2065. Recombinants carrying plasmids that complemented for a lack of endogenous deformylase activity were selected for by the ability to grow in the absence of arabinose on LB agar with ampicillin.

Overexpression of *S. aureus def* genes in *E. coli*. The *defA* and *defB* genes were amplified from RN4220 and NCTC 8325-4 such that (i) the ATG start codon was embedded in an *Nde*I restriction site and (ii) the stop codon was followed by an *Xho*I restriction site. The primary PCR products were cloned into pT7Blue and sequenced; there were no differences in the sequences between *def* genes from either strain. The *def* genes were then subcloned, via the flanking *Nde*I and *Xho*I sites, into T7 promoter expression vector pET20b$^+$ to create pPV58-1 and pPV45-1, which carried *defA* and *defB*, respectively. These plasmids were transformed into *E. coli* BL21(DE3)(pLysS). The cells were grown at 30°C in LB broth to an optical density at 600 nm of 0.5, at which point 0.2 mM isopropyl-β-D-thiogalactopyranoside was added. After 4.5 h of induction, the cells were harvested and were resuspended in 20 mM Tris (pH 8)–100 mM KCl–5 mM $NiCl_2$. The cell suspensions were sonicated, and the deformylase enzymatic activity in the cleared lysates was determined by a formate dehydrogenase coupled assay (15). Activity was normalized to the total protein concentration (Protein Assay Kit; Bio-Rad). Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) analysis of the *E. coli* lysates was performed with the ReadyGel system (Bio-Rad).

Isolation of actinonin-resistant mutants. Spontaneous actinonin-resistant mutants were isolated by plating approximately 10^7 CFU from an overnight culture of *S. aureus* ATCC 25923 or *S. aureus* 1-63 to each of 10 trypticase soy agar plates (TSA; Difco Laboratories) containing 100 μg of actinonin per ml. The plates were incubated overnight, and colonies that grew were passaged to TSA for further characterization.

Construction of a plasmid bearing Δ*defA fmt*. The construction of an *fmt*-complementing plasmid began with the amplification of Δ*defA fmt* by crossover PCR (16). Specifically, the upstream and the downstream ends of the putative *defA fmt* operon were amplified from *S. aureus* NCTC 8325-4. The products of these two reactions were combined and were used as the template in a secondary PCR with flanking primers. The resulting 2.1-kb PCR product consists of P_{defA} Δ*defA fmt* in which residues 13 to 141 of the 162-residue *defA* ORF have been deleted in frame; the deletion is marked by an *Nhe*I site introduced by PCR. This fragment was cloned into pT7Blue to create pPV150-1 and was subsequently subcloned into pAW8 to generate pPV158-1. Plasmid pPV158-1 carries P_{defA} Δ*defA fmt* on a tetracycline-selectable *E. coli-S. aureus* shuttle vector that bears a gram-positive origin of replication that is compatible with *repF*(Ts)-bearing plasmids.

Construction of temperature-sensitive *E. coli-S. aureus* shuttle vectors. Genetic analysis in *S. aureus* required the construction of *E. coli-S. aureus* shuttle vectors that are temperature sensitive for replication in the gram-positive host. For this purpose, 1.4-kb fragment that contained a temperature-sensitive gram-positive organism origin of replication [*repF*(Ts)] was amplified from pBT2 and was cloned into pT7Blue to give pPV46-1. This insert was subcloned into pAW9, yielding pPV72-2, a tetracycline-selectable shuttle vector. pPV171-1, an erythromycin-selectable shuttle vector, was constructed by subcloning the *erm* gene from pRDC19 and the *repF*(Ts) gene into pBS-KS$^+$. A distinct erythromycin-resistant shuttle vector (pPV172-4) was constructed by subcloning the *erm* gene into pPV46-1. Plasmids pPV72-2, pPV171-1, and pPV172-4 are maintained as episomes when *S. aureus* is grown at 30°C but not at 43°C.

Gene inactivation. To construct a strain that bears a *defA* deletion allele on the chromosome, a *Spe*I-ended *aac-aph* cassette (which provides resistance to gentamicin and which is from pGO514) was inserted into the compatible *Nhe*I site in pPV150-1. A fragment from this plasmid that encompasses Δ*defA*::*aac-aph fmt* was subcloned into shuttle vector pPV171-1. The resulting plasmid, pPV179-1, was transformed at 30°C into *S. aureus* RN4220(pPV158-1) with selection for gentamicin and tetracycline resistance. While maintaining selection with both antibiotics, this strain was grown overnight at 43°C and was then plated at 30°C to obtain isolated colonies. A tetracycline-resistant, gentamicin-resistant, erythromycin-sensitive isolate, *S. aureus* VSAU7108, was selected, and the presence of Δ*defA* on the chromosome was confirmed by PCR.

Construction of a strain that harbors an *fmt* deletion allele also used crossover PCR. A 1.4-kb fragment that encompasses P_{defA} *defA* and the start of the *fmt* ORF was amplified from *S. aureus* NCTC 8325-4; in a separate reaction, a 0.5-kb fragment that spans the downstream end of the *fmt* ORF was amplified from the same source. The two fragments were used as the template in a secondary PCR and were subcloned into pT7Blue, giving plasmid pPV188-1. This plasmid insert consists of P_{defA} *defA* Δ*fmt* in which codons 4 through 164 of the 311 codon *fmt* ORF have been deleted. The deletion is marked by an *Xho*I site, into which a *Sal*I-ended *aac-aph* cassette (from pGO1) was inserted. The 2.4-kb *repF*(Ts) *erm* cassette from pPV172-4 was then introduced via *Hin*dIII. The resulting plasmid, pPV214-1, is an *E. coli-S. aureus* shuttle vector that is temperature sensitive for replication in the gram-positive host and that carries P_{defA} *defA* Δ*fmt*::*aac-aph*. *S. aureus* RN4220 was transformed at 30°C with pPV214-1 with selection for erythromycin resistance. The resulting transformant was passaged first at 43°C (with erythromycin) and was then passaged at 30°C (without antibiotic). Isolates that grew at 30°C were cultured on medium containing actinonin (100 μg/ml) and were rescreened for gentamicin- and actinonin-resistant, erythromycin-sensitive growth. In one such isolate, *S. aureus* VSAU7136, the presence of Δ*fmt*::*aac-aph* on the chromosome was confirmed by PCR.

Disruption experiments with *S. aureus* required the construction of temperature-sensitive plasmids that bore gene fragments. An internal *defB* fragment (which extended from codons 17 through 139 of 183 total codons) was amplified from *S. aureus* NCTC 8325-4; this fragment was subcloned into pPV72-2 as a *Bam*HI-ended fragment to create pPV120-1. In a separate experiment, the upstream half of the *S. aureus lacG* ORF was cloned into pAW9. The *repF*(Ts) gene was subcloned into this construct via *Hin*dIII, generating plasmid pPV77-1. In a distinct construction, the *E. coli gusA* gene was PCR amplified from *E. coli* JM109 and was subcloned into pPV72-2; the resulting plasmid (pPV92-3) lacks any *S. aureus* DNA homology.

Antibiotic susceptibility testing. MICs were determined by the broth microdilution method with Mueller-Hinton broth (Difco Laboratories) (25). An inoculum of 0.5×10^5 to 1.0×10^5 CFU/ml was used, and the plates were incubated at 35°C for 16 to 20 h. Endpoints were determined by measuring the optical density at 600 nm with a SpectraMax 250 microtiter plate reader (Molecular Devices, Sunnyvale, Calif.). The MIC was the lowest concentration of antibiotic that yielded no visible growth. Experiments were repeated two to five times for each compound. For pPV158-1-containing strains, MICs were determined in the presence of tetracycline in order to maintain the plasmid.

Mouse thigh infection model. A murine abscess model was used to assess the relative virulence of an actinonin-resistant mutant, VSAU6014, compared to that of its parent strain, *S. aureus* 1-63 (12). In this model, an abscess is created in a mouse by using a bacteria-bead suspension (10). The microcarrier beads (Cytodex 1; Sigma) were swollen in phosphate-buffered saline (PBS; 2% [wt/vol]) and were autoclaved prior to use. Log-phase cultures of each strain were grown in brain heart infusion broth (Difco Laboratories), washed, resuspended in PBS, and spectrophotometrically adjusted to the desired inoculum size. Equal volumes

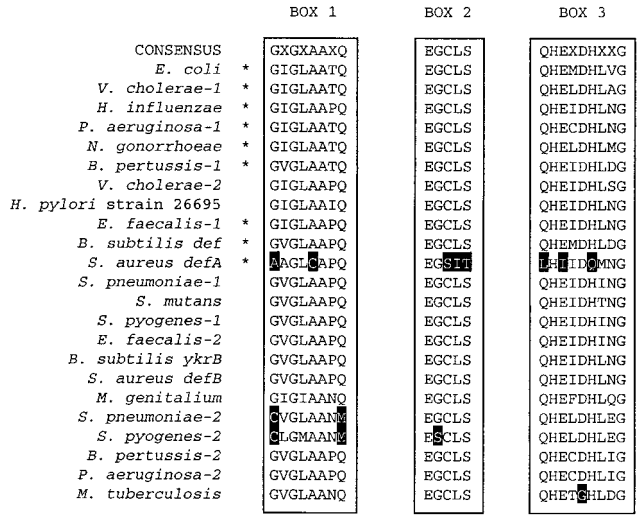

FIG. 1. Alignment of predicted catalytic domains of deformylase. Three conserved motifs that define the catalytic domain of deformylase are shown. The positions of the motifs in the *E. coli* protein are as follows: box 1, G44 to Q51; box 2, E89 to S93; box 3, Q132 to G140. An asterisk next to a bacterial name indicates the presence of an *fmt* homolog downstream of the *def* homolog. Those residues that diverge from the consensus sequence are highlighted.

of the beads and the bacterial culture were mixed together, and infection was initiated by the injection of 0.1 ml of the bacteria-bead mixture into the right thigh of 25-g CD-1 female mice. After 4 days, the mice were killed and their abscessed thighs were removed, weighed, homogenized (Polytron; Brinkman, Westbury, N.Y.) and were quantitatively cultured onto TSA. The results for each group are reported as the mean ± standard deviation of the bacterial titer, expressed as the $\log_{10}$ number of CFU per thigh.

RESULTS

Identification of *S. aureus def* homologs. The ongoing release of microbial genomic sequences has permitted the identification of peptide deformylase homologs from a range of bacteria. By using the sequence of *E. coli* deformylase, BLAST searches of the genomes of pathogenic and nonpathogenic bacteria were performed, and putative peptide deformylase proteins were retrieved and compared (Fig. 1). However, in the case of *S. aureus*, the sequence of a deformylase gene has not been published, nor is the complete genomic sequence of *S. aureus* yet available. To evaluate the utility of deformylase as a target for antibacterial drug discovery, the identification of an *S. aureus def* homolog was necessary.

In other bacteria, the *def* gene is often located immediately upstream of *fmt* (Fig. 1) (18, 20). This conserved gene organization was used to identify an *S. aureus def* homolog, denoted *defA*. An *fmt* locus was identified by BLAST analysis of the existing *S. aureus* genomic sequences; the *fmt* ORF, located near the end of a sequence contig, was preceded by an incomplete ORF predicted to encode the C-terminal end of a deformylase-like protein. Inverse PCR was used to obtain the entire ORF. The derived fragment completed the *defA* ORF and overlapped other existing sequence contigs. The predicted *defA* gene product could be aligned with other peptide deformylases, and its sequence showed the greatest similarity to the sequences of deformylases encoded by genes with adjacent *fmt* loci. However, DefA lacked two of the conserved motifs, EGCLS and HEXXH (Fig. 1), diagnostic of peptide deformylases (9, 18, 24). It appears that DefA is missing consensus cysteine and histidine residues that have been shown in the *E. coli* enzyme to bind to a metal cofactor essential for deformylase activity (3, 4, 22–24, 30). The *defA* gene was therefore considered unlikely to encode a functional peptide deformylase.

The possible existence of a second *S. aureus def* homolog was suggested by the surprising observation that many gram-positive bacteria and some gram-negative bacteria have two *def* homologs (Fig. 1). To identify the *S. aureus* gene(s) that codes for deformylase activity, an *S. aureus* genomic library was screened for clones that were able to complement the arabinose-dependent phenotype of *E. coli* VECO2065 deprived of arabinose. This strain bears a single copy of the *def* gene, located on the chromosome, with expression of this gene under control of the arabinose-regulated P_{BAD} promoter. Growth of *E. coli* VECO2065 is strictly arabinose-dependent in LB broth; cells do not grow in the absence of arabinose. Plasmids isolated by their ability to complement the arabinose-dependent growth phenotype were characterized by sequence, PCR, and restriction site analyses; all of these plasmids shared *S. aureus* sequences that included a *def*-homologous ORF, denoted *defB*. The sequence of the DefB protein conforms to the deformylase consensus sequence, including the presence of the pentapeptide motifs missing from DefA (Fig. 1). The *defB* gene is not adjacent to an *fmt* homolog, and DefB best resembles deformylases encoded by genes that also lack an adjacent *fmt* locus (Fig. 1).

***defB* encodes a functional peptide deformylase.** To assess the deformylase activity of the *S. aureus def* homologs, each gene was overexpressed in *E. coli* via a T7-regulated promoter. SDS-PAGE analysis confirmed the presence of an induced protein of the expected molecular weight in each of the lysates (data not shown). The level of deformylase activity from DefA lysates was comparable to that from the parent strain carrying the vector alone (Table 2). In contrast, overexpression of *defB* resulted in an approximately 1,600-fold increase in the relative amount of specific deformylase activity detected in the *E. coli* lysate (Table 2). Thus, in *S. aureus*, *defB* encodes a functional deformylase.

Whole-cell assays were used to determine whether the *defB* homolog also conferred resistance to the deformylase-specific inhibitor actinonin. Because *E. coli* wild-type strains are naturally resistant to this antibiotic (8), *E. coli* VECO2068, which has a *tolC* mutation, was used. The strain was more resistant to actinonin when it harbored *defB* on a multicopy plasmid than when it carried either *defA* or vector alone (Table 2). *E. coli* VECO2068 additionally bears a P_{BAD}-*def* fusion; the *defA* plasmid was unable to complement the arabinose-dependent phenotype of such a construct. In contrast, when the same strain harbored the plasmid with *defB*, it was able to grow even under noninducing conditions (Table 2).

Inactivation of *S. aureus def* homologs. Inactivation of *defA* was complicated by the potential polarity of *defA* mutations on

TABLE 2. Activity of *S. aureus def* homolog products in *E. coli*[a]

Host *E. coli* strain	Arabinose in medium	Assay	*E. coli* harboring:		
			Vector alone	*defA*	*defB*
BL21(DE3) (pLysS)	NA[b]	Deformylase activity	1	1	1,600
VECO2068	0.2%	MIC (μg/ml) of actinonin	2	2	16
	None	Growth	−[c]	−	+[d]

[a] Enzyme activities were normalized to that of *E. coli* harboring vector alone.
[b] NA, not applicable.
[c] −, no visible growth.
[d] +, growth.

TABLE 3. *defB* gene disruption in *S. aureus*[a]

S. aureus strain	Disruption target	*fmt* genotype	Titer (log_{10} CFU/ml)	
			30°C	43°C
RN4220	*lacG*	*fmt*$^+$	9.8	7.3
RN4220	No homology	*fmt*$^+$	9.5	3.6
RN4220	*defB*	*fmt*$^+$	9.6	3.5
VSAU7136	*defB*	Δ*fmt*	8.5	5.3
VSAU6011	*lacG*	*fmt* mutant	8.7	4.9
VSAU6011	*defB*	*fmt* mutant	8.8	4.3

[a] Viable organisms recovered at permissive (30°C) or restrictive (43°C) temperatures.

fmt. To avoid the effects on *fmt* expression, a plasmid-borne copy of *S. aureus fmt* was supplied in *trans* while *defA* was inactivated. Specifically, plasmid pPV158-1 carries *defA fmt*, from which *defA* codons 13 through 141, of 162 total codons, were removed. Because this deletion is in frame, the episomal copy of *fmt* should still be subject to the same regulation of expression seen in the intact operon. By using an *S. aureus* RN4220 strain carrying this *fmt*-complementing plasmid, the *defA* locus was deleted and replaced by a gene that encoded antibiotic resistance. The *defA* mutant showed no apparent defect in growth.

Inactivation of the *defB* gene was attempted by use of a shuttle vector that harbored a temperature-sensitive gram-positive organism origin of replication. As a control, a strain carrying a temperature-sensitive plasmid that included homology to the nonessential *S. aureus lacG* (phospho-β-galactosidase [6]) was grown at 43°C. Isolates bearing an integrated plasmid were readily recovered (Table 3). Site-specific integration at *lacG* was confirmed by the resulting Lac$^-$ phenotype and by PCR. This integration-disruption assay was also performed with strains carrying either of two other plasmids, one that lacked homology to *S. aureus* and a second that bore a fragment internal to the *defB* ORF. In both cases, growth was rarely observed upon a shift to the restrictive temperature (Table 3). The colonies that did appear at a low frequency at 43°C apparently harbored freely replicating plasmid or ectopically integrated plasmid, as judged by PCR. *S. aureus* strains disrupted in *defB* could not be recovered.

Characterization of actinonin-resistant mutants. Actinonin-resistant mutants arose in *S. aureus* strains at a frequency of $10^{-6.0}$ (for *S. aureus* ATCC 25923) to $10^{-6.3}$ (for clinical isolate 1-63). Three mutant *S. aureus* strains, VSAU6011, VSAU6012, and VSAU6013, were derived from *S. aureus* ATCC 25923; resistant *S. aureus* strain VSAU6014 was isolated from *S. aureus* 1-63. Compared with their parent strains, all of the resistant mutants were highly resistant to actinonin but remained susceptible to other classes of antibiotics, including inhibitors of protein synthesis (Table 4). These mutants were four to eight times more susceptible to kanamycin than their parent strains were. Morphologically, the mutant colonies grew to approximately half the size as their parent strains on agar plates. In accordance with this observation, the in vitro growth rates of the actinonin-resistant mutants were also substantially slower than those of the parent strains (Table 4). The in vitro growth rate of the *S. aureus fmt* mutants was 50% that of the wild type, similar to that found for *fmt* mutants in *P. aeruginosa* and considerably higher than that found in *E. coli*, for which the growth rate of *fmt* mutants was reported to be only 10% of that of the parent strain (11, 26). The relative virulence of *S. aureus* VSAU6014 was assessed in vivo by using a murine abscess model. The difference in bacterial titer between mice infected with the mutant strain ($n = 4$; mean = 4.11 ± 1.47 log_{10} CFU per thigh) and those infected with the parent strain ($n = 4$; mean = 8.04 ± 1.04 log_{10} CFU per thigh) was statistically significant ($P = 0.0047$; Student's unpaired t test). Thus, the ability of the mutant to establish an infection in this model was clearly attenuated. Following infection with *S. aureus* VSAU6014, bacteria recovered from the mice were still uniformly resistant to actinonin.

Mechanism of resistance to actinonin. To ascertain whether actinonin resistance resulted from an altered target, the *defA* and *defB* DNA sequences from the mutant strains were aligned with those from their parent strains. Surprisingly, the sequence comparisons revealed complete identity, even within their upstream promoter domains. In *E. coli*, the ability to knock out *def* depends on the absence of a functional *fmt* gene (19); thus, in the absence of formyltransferase and of formylated proteins, deformylase is no longer essential. We hypothesized that in *S. aureus* resistance to deformylase inhibitors could arise from mutations that altered the *fmt* gene. Indeed, sequence analysis indicated that all four mutants harbored alterations in the *fmt* gene. Two strains had single missense mutations. The other two strains were predicted to encode truncated formyltransferase proteins, one via a nonsense mutation and the other by way of a frameshift (Table 5).

To test whether the *fmt* mutations are the source of the resistance phenotype, the wild-type *S. aureus fmt* gene was supplied in *trans* to the actinonin-resistant mutants. Upon transformation with plasmid pPV158-1 (which harbored *fmt*), the previously resistant mutants were now susceptible to actinonin (Table 5), and their in vitro growth rates were comparable to those of the wild-type parent strains (data not shown). Construction of an *S. aureus fmt* null mutant confirmed the mechanism of actinonin resistance. In this mutant, *fmt* codons 4 through 164, of 311 total codons, were deleted and replaced by a selectable marker. The resulting strain was similar in phenotype to the in vitro-selected mutants: the organism with the Δ*fmt* construct was highly resistant to actinonin and had a decreased growth rate (Table 4); both of these phenotypes were complemented upon the introduction of the *fmt*-bearing plasmid (Table 5).

As noted above, *def* is essential in wild-type *E. coli* but can be mutated or knocked out in an *E. coli fmt* mutant strain. Inactivation of *defB* in the strain that harbored Δ*fmt* confirmed that *defB* is nonessential in an *S. aureus* background that lacks *fmt*. Disruption of *defB* in VSAU6011, which has a point mutation

TABLE 4. Doubling times and antibiotic susceptibilities of actinonin-resistant and -susceptible *S. aureus* strains[a]

S. aureus strain	T_2 (min)	MIC (μg/ml)							
		Act	Cam	Erm	Tet	Clin	Kan	Lin	Van
ATCC 25923	37	16	4	0.5	0.13	0.06	8	2	2
VSAU6011	60	>128	4	0.06	0.13	0.03	1	1	2
VSAU6012	60	>128	4	0.5	0.13	0.13	2	1	2
VSAU6013	ND	>128	4	0.25	0.13	0.06	1	2	1
1-63	34	16	4	0.5	0.13	0.06	2	2	0.5
VSAU6014	62	>128	4	0.13	0.06	0.06	0.5	1	0.5
RN4220	36	16	ND	ND	ND	ND	ND	ND	ND
VSAU7136	63	>128	ND	ND	ND	ND	ND	ND	ND

[a] T_2, doubling time. Antibiotic susceptibilities are to actinonin (Act), chloramphenicol (Cam), erythromycin (Erm), tetracycline (Tet), clindamycin (Clin), kanamycin (Kan), linezolid (Lin), vancomycin (Van). ND, not determined.

TABLE 5. Genotypic characterization of actinonin-resistant *S. aureus* mutants[a]

S. aureus strain	*fmt* genotype	MIC (μg/ml) of actinonin in the presence of:	
		No plasmid	pPV158-1
ATCC 25923	Wild type	16	ND[b]
VSAU6011	A108E	>128	4
VSAU6012	G117V	>128	ND
VSAU6013	E157Stop	>128	ND
1-63	Wild type	16	4
VSAU6014	Frameshift[c]	>128	8
RN4220	Wild type	16	2
VSAU7136	Deletion	>128	4

[a] Values for uncomplemented strains are repeated from Table 4. For strains that harbored pPV158-1 (which supplies *fmt* in *trans*), MICs were determined in the presence of 10 μg of tetracycline per ml.
[b] ND, not determined.
[c] A frameshift at codon 180 results in translation termination at codon 184.

in *fmt*, provided further evidence that *defB* is dispensable when *fmt* is inactivated (Table 3). In the case of both of these *fmt* mutants, integration at *defB* by the *defB*-disrupting plasmid was confirmed by PCR analysis.

DISCUSSION

The identification of deformylase homologs in publicly available genomes shows that peptide deformylase is widely distributed in bacteria (Fig. 1). In general, gram-negative organisms have one homolog, while gram-positive bacteria, including *S. aureus*, typically carry two. Our results indicate that only one of the *S. aureus* homologs encodes a true peptide deformylase. As in *E. coli* and related organisms, *defA* is adjacent to *fmt*, apparently in a dicistronic operon. However, the DefA protein sequence lacks the conserved residues of deformylase required for binding to a metal ligand that is essential for deformylase activity (Fig. 1). The significance of this structural difference was confirmed by functional assays. The *defA* gene could not complement the arabinose-dependent growth phenotype of *E. coli* P_{BAD}-*def*. Similarly, *defA* did not provide *E. coli* with increased resistance to actinonin. We cannot exclude the possibility that the lack of complementation or of decreased susceptibility to actinonin was due to failure to express *defA* in *E. coli*. However, overexpression in *E. coli* of the *defA* ORF, under control of a T7 promoter, was not associated with increased deformylase activity, nor is *defA* essential in *S. aureus*, since the gene was easily inactivated even in the presence of an intact *fmt* gene. In contrast, the product of the second *S. aureus* homolog, *defB*, includes the conserved motifs that define the enzyme's metallo-binding site. Consistent with this sequence conservation, DefB has deformylase activity, as demonstrated by complementation by *defB* of the arabinose-dependent growth phenotype of an *E. coli* P_{BAD}-*def* strain. In addition, overexpression in *E. coli* of the *defB* ORF was correlated with a large increase in deformylase activity, while an *E. coli* strain that overexpressed *S. aureus defB* displayed decreased susceptibility to actinonin. In other work that supports this observation, DefB purified from this lysate is strongly inhibited by actinonin (8). In contrast to *defA*, *defB* knockout mutants could not be constructed in wild-type *S. aureus*. However, strains disrupted in *defB* were isolated in a Δ*fmt* background, in which no transformylase activity is expected. If formylation of proteins does not occur, then deformylation becomes a nonessential function. Since inactivation of *defB*, but not of *defA*, is dependent on the Δ*fmt* background, then *defB* encodes a deformylase and *defA* does not. These data are consistent with the observation that *fmt* is epistatic to *def* in *E. coli*. That is, only when the *E. coli* transformylase-encoding gene is inactivated can *def* null alleles be constructed; the resulting double mutant has the same growth phenotype as the *fmt* single mutant (19). Taken together, these results indicate that the product of *defB* is an essential deformylase in *S. aureus*, while *defA* is a nonessential paralog of unknown function.

Actinonin-resistant mutants raised in two distinct *S. aureus* strains were slow growing and did not show cross-resistance to other antibiotics, suggesting a unique mechanism of resistance. Sequence analysis of the deformylase and transformylase homologs showed that these strains carry a mutation in *fmt*. The introduction of a plasmid with wild-type *fmt* restored growth, while the strain simultaneously became susceptible to the deformylase inhibitor actinonin. Phenotypically, these in vitro-isolated mutants are essentially identical to an *S. aureus fmt* deletion mutant. Thus, a mutation in *fmt* is responsible for resistance to actinonin. Presumably, a reduction in transformylase activity shrinks the pool of formylated methionyl-$tRNA^{fMet}$. If unformylated methionyl-$tRNA^{fMet}$ is used instead for the initiation of translation, the resulting proteins will remain unformylated and the requirement for peptide deformylase will be reduced or eliminated. The implication is that a mutation in transformylase renders deformylation, and deformylase, nonessential. In fact, in *S. aureus fmt* mutant strains, *defB* was easily disrupted, in contrast to wild-type *S. aureus*, for which *defB*-disrupted strains could not be recovered.

The viability of the *S. aureus* actinonin-resistant mutants in vitro as well as in vivo is an important factor in predicting whether this kind of resistance will be found in clinical isolates or whether resistant strains will be selected during therapy. In *S. aureus*, *fmt* mutations arose at a high frequency in vitro, but the mutants had a substantially reduced growth rate, indicating that there is a price to pay when transformylase activity is reduced. The virulence of an *fmt* mutant in an abscess model, which mimics a foreign-body infection, was also assessed. In this murine model, bacterial titers for the mutant strain were significantly less than the titers obtained for the parent strain. The *fmt* mutation puts *S. aureus* at a considerable disadvantage in vivo; whether this reduction simply reflects the diminished growth rate or involves other factors is not known. The essentiality of deformylase in *S. aureus* and the attenuated virulence of resistant mutants suggest that deformylase, a bacterium-specific enzyme, is an attractive target for the development of novel antibacterials.

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