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Resistance of *Streptococcus pneumoniae* to Deformylase Inhibitors Is Due to Mutations in *defB*

PETER MARGOLIS, CORINNE HACKBARTH, SARA LOPEZ, MITA MANIAR, WEN WANG, ZHENGYU YUAN, RICHARD WHITE, AND JOAQUIM TRIAS*

Versicor, Inc., Fremont, California 94555

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Resistance to peptide deformylase inhibitors in *Escherichia coli* or *Staphylococcus aureus* is due to inactivation of transformylase activity. Knockout experiments in *Streptococcus pneumoniae* R6x indicate that the transformylase (*fmt*) and deformylase (*defB*) genes are essential and that a *def* paralog (*defA*) is not. Actinonin-resistant mutants of *S. pneumoniae* ATCC 49619 harbor mutations in *defB* but not in *fmt*. Reintroduction of the mutated *defB* gene into wild-type *S. pneumoniae* R6x recreates the resistance phenotype. The altered enzyme displays decreased sensitivity to actinonin.

The formylation-deformylation cycle of the translation-initiating methionine is a characteristic feature of bacterial protein synthesis. Deformylation of the nascent protein is catalyzed by an iron metalloenzyme, peptide deformylase (PDF) (10, 21). The deformylase gene is essential in *Escherichia coli* and *Staphylococcus aureus* (13–15). Novel antibacterials that are PDF inhibitors, such as actinonin, recently have been discovered by screening compound collections and combinatorial libraries (1, 5, 7, 9, 11).

Resistance to PDF inhibitors has been reported in *S. aureus* and *E. coli* (7, 13). The mechanism of resistance is based on the loss of transformylase activity, which renders deformylase nonessential. However, loss of transformylation comes at a cost to the bacteria: *S. aureus fmt* mutants are slow growers, and the virulence of resistant mutants is attenuated (13). In the present work, resistance to this new class of antibiotics is examined in *Streptococcus pneumoniae*.

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MATERIALS AND METHODS

Strains and plasmids used in this study are listed in Table 1. Spontaneous PDF inhibitor-resistant mutants were isolated by plating an exponentially growing culture of *S. pneumoniae* ATCC 49619 on blood agar containing 100 μg of actinonin (Sigma, St. Louis, Mo.) per ml. Growth was determined spectrophotometrically at 600 nm using Mueller-Hinton broth with lysed horse blood. MICs were determined as described elsewhere (5).

E. coli def and *fmt* sequences were used in BLAST searches at NCBI (http://www.ncbi.nlm.nih.gov/blast/blast.cgi) to identify *S. pneumoniae* homologs. The open reading frames (ORFs) were amplified from *S. pneumoniae* R6x by PCR and used in subsequent experiments.

Genes were inactivated in *S. pneumoniae* by insertion-duplication mutagenesis (6). Fragments internal to *defA* (codons 43 to 126), *defB* (codons 77 to 172), or *fmt* (codons 93 to 195) were PCR amplified and cloned into pR326, and transformants were selected as described elsewhere (6, 23). The *defB* allele was replaced in *S. pneumoniae* R6x by using a truncated *defB* fragment (codon 77 through stop) PCR amplified from *S. pneumoniae* ATCC 49619 or the resistant mutants. All constructs were confirmed by PCR and sequencing.

The ability of *def* gene homologs to code for a functional deformylase was tested by complementation of the arabinose-dependent phenotype of *E. coli* VECO2068 with pGEX-5X-3 carrying *def* homologs or with vector alone (see Table 1), as described elsewhere (13). The *E. coli* VECO2068 strain has the chromosomal copy of the essential *def* gene under P_{BAD} control and will grow in the absence of inducer only when an active deformylase is expressed in *trans*.

The *defB* gene (optimized for expression in *E. coli* by 16 silent mutations in the first 48 codons) was cloned into pET20b(+) so as to encode a His-tagged protein. This construct was modified via PCR-mediated site-specific mutagenesis at codon 172 (CAG to aAa) or codon 123 (GCT to GaT). The resulting plasmids were introduced into *E. coli* BL21 for protein expression. Transformants were grown at 37°C in 500 ml of Luria broth supplemented with 100 μg of ampicillin per ml to an optical density at 600 nm of 0.5, at which point IPTG (isopropyl-β-D-thiogalactopyranoside) was added to a concentration of 1 mM. After 3 h of induction, the cells were harvested, resuspended in 35 ml of 10 mM NaCl–20 mM Tris-HCl buffer (pH 8), and then disrupted, in the presence of catalase, by a French press. His-tagged deformylase was purified from the cell lysates by passage over a cobalt affinity column according to the manufacturer's instructions (Clontech, Palo Alto, Calif.).

Deformylase activity was determined by using a deformylase-formate dehydrogenase (FDH) coupled assay (12). Experiments were carried out at room temperature in a buffer containing 10 mM NaCl, 0.2 mg of bovine serum albumin per ml, and 50 mM HEPES (pH 7.2). The reaction was initiated by adding a mixture of 0.5 U of FDH per ml, 1 mM NAD^+, and fMAS at 4 mM (5, 12). Deformylation was followed by monitoring the reduction of NAD due to the oxidation of formate by FDH. For inhibition studies to determine the 50% inhibitory concentration (IC_{50}) values, enzyme was preincubated at different concentrations of actinonin for 10 min prior to the addition of the substrate (5).

RESULTS AND DISCUSSION

Identification of deformylase in *S. pneumoniae*. A BLAST search using the *E. coli def* sequence identified two *S. pneumoniae def* homologs, *defA* and *defB* (Fig. 1). In contrast to many other bacteria (13), neither of these homologs were adjacent to an *fmt* gene. Several pieces of evidence indicate that *defB*, and not *defA*, encodes the *S. pneumoniae* R6x PDF. The predicted DefA protein contains two substitutions (G41C and Q48M) at strictly conserved residues of a key catalytic domain, GXGXAAXQ (Fig. 1). Substitutions at either of the analogous residues of *E. coli* PDF dramatically impair enzyme activity (3, 4, 8, 18, 20). The *S. aureus defA* gene, which also contains two substitutions in this motif (Fig. 1), encodes a protein lacking PDF activity (13).

Despite several attempts, *defB* could not be inactivated by insertion-duplication mutagenesis. However, *defA* or *rafE*, a nonessential gene (23), were readily disrupted. This result im-

* Corresponding author. Mailing address: Versicor, Inc., 34790 Ardentech Ct., Fremont, CA 94555. Phone: (510) 739-3025. Fax: (510) 739-3003. E-mail: jtrias@versicor.com.

TABLE 1. Strains and plasmids used in this work

Strain or plasmid	Relevant characteristics	Source or reference
Strains		
E. coli		
VECO2068	F^- *araD139* Δ*(ara, leu)7697 galE15 galK16* Δ*(lac)X74 rpsL hsdR2* (r_k^- m_k^+) *mcrA mcrB1* Δ*tolC* ΔP_{def}::P_{BAD} = *def*; P_{BAD}-regulated *def* gene	13
BL21(DE3)/pLysS	F^- *ompT* $hsdS_B$ (r_B^- m_B^-) *dcm gal* λ(DE3) (pLysS); expression strain for P_{T7}-regulated gene expression	Novagen, Madison, Wis.
S. pneumoniae		
R6x	Unencapsulated strain for laboratory genetics	24
ATCC 49619		American Type Culture Collection, Manassas, Va.
VSPN6501	ATCC 49619 *defB*(Q172K); actinonin-resistant	This study
VSPN6503	ATCC 49619 *defB*(A123D); actinonin-resistant	This study
VSPN6504	ATCC 49619 *defB*(A123D); actinonin-resistant	This study
VSPN7011	R6x *defA*::pR326*defA; defA* disruption	This study
VSPN7035	R6x $defB^+$::pPV302-7; expresses wild-type *defB*	This study
VSPN7036	R6x *defB*::pPV303-7; expresses *defB* (Q172K)	This study
VSPN7037	R6x *defB*::pPV304-5; expresses *defB* (A123D)	This study
Plasmids		
pET20b(+)	P_{T7}; overexpression vector	Novagen, Madison, Wis.
pET20b*Spn* $defB^{opt}$ (C-His)	pET20b(+) carrying *Spn defB* with optimized codon usage; encodes C-terminally His-tagged PDF (wild type)	This study
pET20b*Spn* $defB^{opt}$ (C-His, Q172K)	pET20b*Spn* $defB^{opt}$ (C-His) mutagenized to introduce Q172K mutation	This study
pET20b*Spn* $defB^{opt}$ (C-His, A123D)	pET20b*Spn* $defB^{opt}$ (C-His) mutagenized to introduce A123D mutation	This study
pGEX-5X-3	P_{tac}; overexpression vector, GST fusion protein	Amersham Pharmacia, Piscataway, N.J.
pGEX*Spn defA*	pGEX-5X-3::*defA*; P_{tac}-*gst-defA* fusion	This study
pGEX*Spn defB*	pGEX-5X-3::*defB*; P_{tac}-*gst-defB* fusion	This study
pR326	*cat; E. coli, S. pneumoniae* shuttle vector	6
pR326*rafE*	pR326::'*rafE*'[a]	23
pR326*defA*	pR326::'*defA*'	This study
pR326*defB*	pR326::'*defB*'	This study
pR326*fmt*	pR326::'*fmt*'	This study
pPV302-7	pR326::'$defB^+$; downstream end of wild-type *defB* ORF	This study
pPV303-7	pR326::'*defB*(Q172K); downstream end of mutated *defB* ORF	This study
pPV304-5	pR326::'*defB*(A123D); downstream end of mutated *defB* ORF	This study

[a] Apostrophes indicate the truncation of the gene at the upstream and/or downstream end, as indicated.

plies that *defB* is essential, although the experiment does not exclude the possibility of a polar effect on a downstream gene. A plasmid encoding a GST-DefB fusion protein, but not one encoding GST-DefA, was able to complement the arabinose-dependent phenotype of *E. coli* VECO2068. Expression of the *gst-defA* and *gst-defB* fusions was confirmed by Western blotting (data not shown). Purified GST-DefB was associated with a strong PDF activity (1,300 μmol min^{-1} mg of $protein^{-1}$). Taken together, these results argue that *defB* codes for a true essential deformylase, whereas *defA* is a paralog of unknown but nonessential function or has marginal deformylase activity unable to complement the arabinose-dependent mutant. This is similar to *S. aureus* RN4220, which also harbors two deformylase homologs, only one of which, *defB*, encodes a true PDF (13).

Isolation and characterization of actinonin-resistant mutants. The frequency of resistance in *S. pneumoniae* ATCC 49619 was 10^{-8}, 2 orders of magnitude lower than that obtained with *S. aureus* ATCC 25923 or *S. aureus* 1–63 (13). Three *S. pneumoniae* mutants, VSPN6501, VSPN6503, and VSPN6504, were chosen for further studies. The mutants grew at slower rates, with doubling times approximately 20% longer, when cultured in broth (Table 2). The slow-growth phenotype is less pronounced than in *S. aureus* actinonin-resistant strains derived from *S. aureus* ATCC 25923, where doubling times increase approximately 80% (13). These mutants of *S. pneumoniae* ATCC 49619 showed resistance to PDF inhibitors (Table 2) but were unchanged in susceptibility to penicillin, ampicillin, chloramphenicol, erythromycin, trimethoprim,

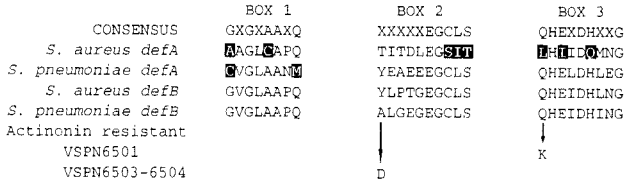

FIG. 1. Alignment of conserved domains of deformylase proteins. Partial sequences of the predicted products of the *defA* and *defB* homologs of *S. aureus* and *S. pneumoniae* are shown aligned with consensus PDF domains (13). Residues that diverge from the consensus are highlighted. Modifications in the deformylase enzyme in the resistant *S. pneumoniae* mutants VSPN6501, VSPN6503, and VSPN6504 are indicated by arrows. The positions of the motifs in the *S. pneumoniae* DefB are as follows: box 1, G69 to Q76; box 2, A123 to S132; and box 3, Q172 to G182. The sequences of the *S. pneumoniae def* homologs from strain R6x have been submitted to GenBank (accession numbers: *defA*, AY014508; *defB*, AY014509). Sequences of the *S. pneumoniae def* and *fmt* homologs from *S. pneumoniae* ATCC 49619 were also submitted to GenBank (accession numbers: *defA*, AY014510; *defB*, AY014511; *fmt*, AY014512).

TABLE 2. Genotypes and susceptibilities to PDF inhibitors, and doubling times of *S. pneumoniae* actinonin-resistant and -susceptible strains[a]

Strain	*defB*	Mean T_2 in min (SD)	MIC (μg/ml)	
			Act	VRC3375[b]
ATCC49619	wt	51 (1.4)	32	16
VSPN6501	Q172K	60 (2.9)	128	256
VSPN6503	A123D	64 (2.6)	64	128
VSPN6504	A123D	66 (2.3)	128	128

[a] wt, wild type; T_2, doubling time; Act, actinonin
[b] PDF inhibitor (D. Chen, C. Hackbarth, Z. J. Ni, W. Wang, C. Wu, D. Young, R. J. White, J. Trias, D. V. Patel, and Z. Yuan, Abstr. 40th Intersci. Conf. Antimicrob. Agents Chemother., abstr. 2175, 2000).

or tetracycline. These results suggest that resistance occurred by a specific mechanism distinct from that for these other antibiotics and further indicates that the slower growth rate did not change the overall susceptibility of the mutant strains. No such differences in susceptibility to PDF inhibitors or doubling time were observed for strain VSPN7011, which carries a disrupted *defA* gene (not shown).

Mutation of *fmt* leads to resistance in *S. aureus* or *E. coli* (7, 13). The *fmt* gene from *S. pneumoniae* ATCC 49619 actinonin-resistant strains was PCR amplified and sequenced. None of the resistant strains carried a mutation in *fmt* or flanking DNA. The inactivation of *fmt* was attempted to assess whether the lack of transformylase activity could provide resistance in *S. pneumoniae* R6x. Despite multiple attempts, the *fmt* gene could not be inactivated. In contrast, the homologous gene can be readily inactivated in strains of *E. coli, Pseudomonas aeruginosa*, or *S. aureus* (13, 14, 19). The inability to inactivate *fmt* suggests that the transformylase gene itself is essential in *S. pneumoniae* R6x, although it is possible that strains disrupted in *fmt* were not obtained because of a polar effect on a downstream gene.

The sequences and flanking DNA of both *defA* and *defB* were PCR amplified and sequenced from *S. pneumoniae* ATCC 49619 and mutant strains. No change was observed among the sequences of the *defA* homolog. However, each of the resistant strains possessed a single missense mutation in *defB* (Fig. 1). For *S. pneumoniae* VSPN6501, a Q172K substitution occurs at a residue that is strongly conserved among PDF proteins (Fig. 1). This position lies immediately upstream of the $_{173}$HEXXH$_{177}$ (*S. pneumoniae* numbering) motif shared by all PDF proteins and characteristic of zinc hydrolases (22). A nonconservative substitution (Q131A) at the equivalent position in the *E. coli* enzyme has been shown to decrease enzyme activity (8). The analogous pair of His residues in the *E. coli* PDF has been shown by genetic and structural studies to bind the metal ion in the catalytic pocket (2–4, 8, 16–18). *S. pneumoniae* VSPN6503 and VSPN6504 have a A123D substitution in the predicted protein. This residue is not conserved among PDF proteins. However, the mutation introduces a charged amino acid five residues upstream of the $_{128}$EGCLS$_{132}$ motif, which has been shown to be involved in binding the metal ion (Fig. 1) (2–4, 7, 8, 16–18). The mutated residue corresponds to a position two residues upstream of an Ile involved in defining a substrate-binding pocket of the *E. coli* enzyme (7). Thus, in all three cases, a mutation in *S. pneumoniae defB* is predicted to cause a substitution close to conserved domains involved in binding the metal ion or the substrate, essential for PDF activity.

TABLE 3. Susceptibilities to actinonin of *S. pneumoniae* R6x strains transformed with wild-type or mutated *defB* and inhibition of purified His-tagged PDF enzymes by actinonin

S. pneumoniae strain	Expressed *defB*	MIC (μg/ml)	Enzyme	IC_{50} (nM)
VSPN7035	*defB*	16	Wild type	53 ± 6
VSPN7036	*defB* (Q172K)	64	Q172K	70 ± 8
VSPN7037	*defB* (A123D)	≥64	A123D	136 ± 16

Mutated *defB* leads to resistance. Genes encoding the wild-type and mutated PDFs were introduced into *S. pneumoniae* R6x (6). Strains expressing either of the two mutated *defB* genes displayed reduced susceptibility to actinonin (Table 3). In addition, the mutated enzymes are indeed less sensitive to inhibition than the wild-type parent PDF (Table 3). These results confirm that, in these mutants, resistance is due to modification of the target rather than the lack of transformylation activity.

Resistance to PDF inhibitors can occur by at least two distinct mechanisms, with different consequences predicted in each case. Resistant mutants of *S. aureus* or *E. coli*, obtained in vitro, lack transformylase activity, bypassing the essentiality of the *def* gene (7, 13). Mutation of *fmt* should lead to cross-resistance to any antibiotic for which PDF is the major target, because inhibition of deformylase would have no consequence for protein synthesis if nascent peptides were not formylated. However, mutation of *fmt* does have consequences for cell growth, as seen in *S. aureus* and *E. coli fmt* mutants. Notably, *S. aureus fmt* mutants have attenuated virulence in abscess or septicemia models, decreasing the chance that such mutants would survive during infection (7, 13). In *S. pneumoniae* R6x, *fmt* cannot be disrupted; instead, resistance to PDF inhibitors derives from modification of the target. In contrast to strains resistant via a lack of transformylation activity, it is possible that these *S. pneumoniae defB* mutants will not be cross-resistant to all PDF inhibitors. More potent inhibitors, or compounds that bind differently to PDF, should be active against these resistant *S. pneumoniae* strains. The essentiality of *fmt* makes PDF an attractive target for the discovery and development of novel antibiotics active against *S. pneumoniae*.

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