Reduced Susceptibility of *Haemophilus influenzae* to the Peptide Deformylase Inhibitor LBM415 Can Result from Target Protein Overexpression Due to Amplified Chromosomal *def* Gene Copy Number[▽]

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Previous genetic analysis of *Haemophilus influenzae* revealed two mechanisms associated with decreased susceptibility to the novel peptide deformylase inhibitor LBM415: AcrAB-TolC-mediated efflux and Fmt bypass, resulting from mutations in the pump repressor gene *acrR* and in the *fnt* gene, respectively. We have isolated an additional mutant, CDS23 (LBM415 MIC, 64 μ g/ml versus 4 μ g/ml against the parent strain NB65044) that lacks mutations in the *acrR* or *fnt* structural genes or in the gene encoding Def, the intracellular target of LBM415. Western immunoblot analysis, two-dimensional gel electrophoresis, and tryptic digestion combined with mass spectrometric identification showed that the Def protein was highly overexpressed in the mutant strain. Consistent with this, real-time reverse transcription-PCR revealed a significant increase in *def* transcript titer. No mutations were found in the region upstream of *def* that might account for altered expression; however, pulsed-field gel electrophoresis suggested that a genetic rearrangement of the region containing *def* had occurred. Using a combination of PCR, sequencing, and Southern blot analyses, it was determined that the *def* gene had undergone copy number amplification, explaining the high level of target protein expression. Inactivation of the AcrAB-TolC efflux pump in this mutant increased susceptibility 16-fold, highlighting the role of efflux in exacerbating the overall reduced susceptibility resulting from target overexpression.

In recent years, there has been a growing appreciation of the need for new antibiotics targeting novel bacterial functions, to combat the spread of resistance to currently used antibiotics. The result of one effort to develop such a compound is LBM415, a potent low-molecular-weight inhibitor of bacterial peptide deformylase that shows promising antibacterial activities against many drug-resistant bacteria (9, 12, 34).

Two main mechanisms mediating resistance to peptide deformylase inhibitors in bacteria have been previously described. The first is amino acid substitutions within the target protein (Def) (17), and the second is “FMT bypass” (4, 18), which results from mutational loss of methionyl tRNA formyltransferase (Fmt). Loss of the Fmt function reduces or eliminates formylation of the initiating methionyl-tRNA^{fMet}. In many bacteria, the initiation of protein synthesis still occurs in

the absence of the Fmt function (1, 18, 20, 33), but the deformylation step mediated by peptide deformylase is then unnecessary (i.e., the formylation-deformylation cycle is bypassed), leading to insusceptibility to peptide deformylase inhibitors (4, 18). Recently, amino acid substitutions in the Fld component of the folate biosynthesis pathway have been shown to confer resistance to the peptide deformylase inhibitor actinonin in *Salmonella enterica*, presumably as a result of reduced formylation of methionyl-tRNA^{fMet} due to interference with synthesis of the formyl group (22).

Given the potency of LBM415 against the respiratory pathogen *Streptococcus pneumoniae* and other gram-positive bacteria (9), there is interest in potential coverage of the respiratory pathogen *Haemophilus influenzae* by peptide deformylase inhibitors. Although not as active against this organism, LBM415 has a MIC₉₀ (determined for 300 isolates) of 4 to 8 μ g/ml (9). The lower intrinsic susceptibility of *H. influenzae* to LBM415 appears to be the result of AcrAB-TolC-mediated efflux (6).

In *H. influenzae*, increased AcrAB-TolC-mediated efflux (6) and FMT bypass (14) resistance mechanisms have been selected through exposure to LBM415. Each of these mechanisms confers a characteristic phenotype: efflux mutants (associated with amino acid substitutions within the pump repressor AcrR) have decreased susceptibilities to LBM415 and to struc-

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turally unrelated pump substrates (e.g., clindamycin) (6). FMT bypass reduces susceptibility to a variety of peptide deformylase inhibitors, with no decrease in susceptibility to AcrAB-TolC pump substrate antibiotics (14). There is also a pronounced in vitro growth defect associated with mutational loss of *fnt*. We observed a potential third mechanism in an *H. influenzae* mutant isolated by single-step selection upon medium containing LBM415. This mutant had no cross-resistance to AcrAB-TolC pump substrates and did not exhibit any significant growth impairment or morphological changes. Supporting this, no mutations were found in the structural genes encoding AcrR, Fmt, or the target protein Def. In this study, we determined the mechanism of resistance in this mutant.

MATERIALS AND METHODS

Bacterial strains and growth media. *H. influenzae* strain NB65044 is RdKW20 (ATCC 51907) (8). Strain CDS23 was selected by single-step exposure of NB65044 to LBM415 incorporated into chocolate agar at 16 µg/ml. Strain CDS01 is NB65044 with *acrB* insertionally inactivated (6). Strain CDS41 is strain CDS23 with the *acrB* gene insertionally inactivated as previously described (6). Cultures were routinely grown on chocolate agar, supplemented with LBM415 at 16 µg/ml as necessary. Liquid cultures were grown in Haemophilus test medium (HTM; Remel) or supplemented brain heart infusion broth (6).

DNA manipulation. *H. influenzae* genomic DNA was isolated using a Puregene tissue kit (Gentra Systems Inc., Minneapolis, MN) according to the manufacturer's instructions. Oligonucleotides for PCR and sequencing (Table 1) were obtained from Genelink (Hawthorne, NY). PCRs were carried out using the Easystart mix-in-a-tube system (Molecular Bio-Products Inc., San Diego, CA) according to the supplied instructions. Prepared genomic DNA or cells from isolated colonies were used as the template in PCRs, and each reaction mixture contained 2 µl genomic DNA or suspended cells (in distilled H₂O), 2 µl of an 8 µM stock of each primer, 1 µl *Taq* DNA polymerase, and 18 µl distilled H₂O. PCR cycles were as follows: 95°C for 2 min, 25 cycles of 95°C for 30 s and 55°C 30 s, 72°C for 10 min, and a hold at 4°C. Restriction endonucleases and modifying enzymes were used according to the instructions supplied with the enzymes. DNA fragments were purified or isolated following agarose gel electrophoresis, using a QIAquick PCR cleanup or gel extraction kit (QIAGEN Inc., Valencia, CA) as specified in the instructions. Nucleotide sequencing was done by Agencourt Inc. (Beverly, MA). Sequence analysis was done using the Sequencer 4.2 (Genecodes Corporation, Ann Arbor MI) and Vector NTI suite 9 (Invitrogen) software packages.

Southern blotting. *H. influenzae* genomic DNA was restriction digested overnight, separated on 0.7% agarose gels, and transferred to Genescreen charged nylon membrane (NEN Life Science Products, Boston, MA). Transfer of nucleic acid to the membrane, probe labeling, hybridization, and detection were carried out using the ECL direct nucleic acid labeling and detection system (Amersham Life Sciences, Cleveland, OH) according to the low-stringency protocol supplied with the kit. The probe encompassing *def* used in Southern blot experiments was an approximately 700-bp PCR fragment generated using primers DEF HI F1 and DEF HI REV (Table 1).

Isolation of protein extracts for two-dimensional gel electrophoresis and mass spectrometry (MS). *H. influenzae* from overnight chocolate agar plates (Remel) was used to inoculate 100 ml HTM (Remel) to an optical density at 600 nm of 0.04. Cultures were grown at 37°C with shaking to mid-log phase, and the cells were collected by centrifugation. Pellets were washed twice with phosphate-buffered saline and frozen in liquid nitrogen. Frozen pellets were then thawed and resuspended in 1 ml modified Rabbiloud buffer (30 mM Tris [pH 8], 7 M urea, 2 M thiourea, 4% [wt/vol] CHAPS {3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate}, and one complete protease inhibitor tablet [Roche diagnostics] per 50 ml buffer) (16, 25) and sonicated on ice, using a Branson model 250 digital sonifier (amplitude, 25%; three cycles of 0.8 s on and 1.2 s off, 24 seconds total). Insoluble debris was removed by centrifugation at 13,000 × g, and supernatants were stored at -80°C.

Two-dimensional gel electrophoresis. Two-dimensional electrophoresis of bacterial lysates was carried out according to established procedures (11). For the first-dimension immobilized pH gradient (IPG) strips, 500 to 600 µg of the lysate was diluted to 500 µl with Rabbiloud buffer (7 M urea, 2 M thiourea, 4% CHAPS, 1% dithiothreitol, and 2% Pharmalytes 3 to 10) and loaded onto 24-cm, pH 4 to 7 linear IPG strips (GE Healthcare) by reswelling the strips in sample

TABLE 1. Oligonucleotide primers used for PCR, sequencing, and real-time RT-PCR

Primer	Sequence
Used for PCR and sequencing	
23SF.....	5'-GGTTGGGTGTGTAAGTGATGTG-3'
DEF HI F1.....	5'-CAGAAGGACCCCGTGTGCGAAAG-3'
DEF HI REV.....	5'-CTTTCGACACGGGGTCTTCTG-3'
DF2.....	5'-GCTGTGCTATAATCCCACTAA-3'
DR1.....	5'-ACTTTAGTAACAGGCTCACAGACACC-3'
DEF RACE1.....	5'-ATTCCCGTTTCGCTTCTGAAGCG-3'
DEF RACE3.....	5'-TATCCACCTGAGGTGCAGCTAAACC-3'
FR3.....	5'-GACTGTGCTTCTTCTTTACGTAAGG-3'
Used for real-time RT-PCR	
HIdfST1F.....	5'-GCGATAAACAGAATCAGTTTGTGTTT-3'
HIdfST1R.....	5'-CCCGTTTCGCTTCTGAAG-3'
HIRpsLST1F.....	5'-AACCTTCAAGAGCAGAGTGTGTAT-3'
HIRpsLST1R.....	5'-GCACACCCGGTAAGTCTTTAA-3'
HIAcrBST1F.....	5'-AAAATGATAATTTCGCGTGCTACGG-3'
HIAcrBST1R.....	5'-GAGGTGGATTGATGGCTAACAC-3'
HIdfST1M2 ^a	5'-CCCTGAAATTTTG-3'
HIRpsLST1M2 ^a	5'-ACGACACACCGGATTA-3'
HIAcrBST1M2 ^a	5'-AACGGACTCTGCAACATT-3'

^a TaqMan minor-groove-binder fluorogenic (6-carboxyfluorescein-labeled) probes for real-time RT-PCR.

solution (31). Isoelectric focusing was performed on a Multiphor II apparatus (18-1018-06; GE Healthcare) for approximately 60 kV · h at 20°C, using the following voltage gradient: (i) 3 h at 300 V, (ii) 5 h linear gradient from 300 to 3,500 V, and (iii) continuation at 3,500 V until the target kV · h. After the focusing, IPG strips were equilibrated as described previously (11), with 2% dithiothreitol in the first step and 5% iodoacetamide in the second step. For the second dimension, IPG strips were applied to 23- by 27-cm sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels (12% [wt/vol] total acrylamide [T]; 2.6% [wt/vol] bis-acrylamide [C]), which were run overnight at 100 V and 15°C in a Dodeca electrophoresis chamber (Bio-Rad). Gels were stained with Sypro Ruby (11) or with colloidal Coomassie blue G-250 (2).

In-gel trypsin digestion. Excised spots were in-gel digested with modified porcine trypsin (V5111; Promega, Madison, WI) as described previously (2), using a microtiter plate format (CB080; Proxeon, Odense, Denmark). Spots were finally eluted with 5% formic acid and tryptic hydrolysates collected in a second microtiter plate. For matrix-assisted laser desorption/ionization (MALDI)-MS and -tandem mass spectrometry (-MSMS) analysis, the tryptic peptides were purified on ZipTips (Millipore Corporation, Bedford, MA), using a Tecan Genesis ProTeam 150 system (Tecan, Maennedorf, Switzerland). After being washed twice with 5 µl 80% acetonitrile-0.1% trifluoroacetic acid (TFA), the tips were equilibrated twice with 5 µl 0.1% TFA and the hydrolysate was applied; after being washed four times with 5 µl 0.1% TFA, peptides were directly eluted onto ABI 4700 MALDI targets (100-well plates) with 2 µl of a solution of α-cyano-4-hydroxycinnamic acid matrix (5 mg/ml in 50% acetonitrile-0.1% TFA containing 2 mM NH₄H₂PO₄) applied to the back ends of the ZipTips.

MALDI-MS and -MSMS. MALDI spots were analyzed using an Applied Biosystems 4700 proteomics analyzer (ABI, Framingham, MA) in automated, combined MS and MSMS mode. Both MS and MSMS data were acquired with an Nd:YAG laser with a 200-Hz repetition rate; 2,000 shots were accumulated for each spectrum in MS mode and 4,000 shots for each of up to five precursor ions in MSMS mode. For MSMS, the five most intense precursor ions with signal/noise ratios of >25 were selected after exclusion of common background signals. MSMS mode was operated with 1 keV, and products of metastable decomposition at an elevated laser power were detected. MS data were acquired with close external calibration and MSMS data using the default instrument calibration. Database searches were performed using the Mascot search engine integrated in GPS Explorer 2.0 (part of the ABI 4700 proteomics analyzer).

RNA isolation, microarray analysis, and real-time RT-PCR. *H. influenzae* NB65044 and CDS23 were grown in 50 ml HTM at 37°C with shaking to an optical density at 600 nm of approximately 0.5. Ten milliliters of culture was removed and mixed with 20 ml RNA Protect reagent (QIAGEN Inc., Valencia, CA), incubated at room temperature for 10 min, and centrifuged for 10 minutes to collect the cells. RNA was isolated from the cell pellets by using a Purescript tissue kit (Gentra Systems Inc., Minneapolis, MN) according to the supplied

TABLE 2. Contribution of AcrAB-TolC-mediated efflux to susceptibility to LBM415 in wild-type and Def-overexpressing *H. influenzae* isolates

Strain	Relevant characteristic(s)	MIC ($\mu\text{g/ml}$) ^a for:		
		LBM415	CLD	TET
NB65044	Parent	4	8	0.5
CDS01	<i>acrB</i>	0.25	0.5	0.5
CDS23	Def overexpressed	64	4	0.5
CDS40	CDS23 <i>acrB</i>	4	0.5	0.5

^a CLD, clindamycin (AcrAB-TolC pump substrate); TET, tetracycline (AcrAB-TolC pump nonsubstrate).

instructions (scaled for the number of cells used). Approximately 80 μg RNA from these preparations was then further purified and size fractionated using RNeasy mini columns (QIAGEN Inc., Valencia, CA), incorporating the on-column DNaseI digestion step according to the supplied protocols. RNA was analyzed as previously described, using custom design Affymetrix microarrays incorporating all open reading frames and intergenic regions derived from the genome sequence of *H. influenzae* strain RdKW20 (6). Primers and probes for real-time reverse transcription (RT)-PCR (Table 1) were designed using Primer Express version 2.0 software (Applied Biosystems, Foster City, CA) and were synthesized by the Applied Biosystems Assays by Design service. The *def* transcript titers were determined by real-time RT-PCR analysis, using an EZ-RT-PCR core reagent kit (Applied Biosystems) based on a one-step RT-PCR for RNA quantitation on an Applied Biosystems PRISM model 7500 sequence detection system. Relative quantitation was done by the comparative cycle threshold method, using both the endogenous internal control *rpsL* (ribosomal protein S12) and *acrB* (encoding a resistance-nodulation-cell division [RND] efflux pump), which were both shown by transcriptional profiling to be invariant in this study (data not shown). Cycle threshold values were calculated using Applied Biosystems sequence detection software (version 1.2.2). For each one-step RT-PCR run, 10 μl (10 ng) of total RNA isolated from either *H. influenzae* NB65044 (the parent strain) or CDS23 was added (based on a preliminary titration experiment) to a reaction mixture prepared on ice containing 1 $\times$ EZ-RT-PCR TaqMan buffer; 3 mM manganese acetate; 300 μM dATP, dCTP, and dGTP; 600 μM dUTP; 0.9 μM of forward and reverse primers (Table 2); 0.25 μM fluorogenic TaqMan-labeled probe (Table 2); and 5 U of *rTth* DNA polymerase in a final volume of 50 μl . Each sample was analyzed in triplicate. The thermocycling conditions were as follows: 60°C for 30 min and 95°C for 5 min, followed by 45 cycles of 95°C for 15 s and 60°C for 1 min. A preliminary experiment was performed to show that both the target (*def*) and the endogenous control (*rpsL* and *acrB*) transcripts were amplified with approximately equal efficiencies. Standard PCRs did not amplify *acrR* and *def* from these RNA samples (1 cycle of 95°C for 3 min and 45 cycles of 95°C for 1 min, 55°C for 1 min, and 72°C for 1 min, followed by a final extension step at 72°C for 10 min, using Accuprime GC-rich DNA polymerase [Invitrogen, Carlsbad, CA]), indicating that there was no significant DNA contamination.

PFGE. Pulsed-field gel electrophoretic (PFGE) analysis of *H. influenzae* strains was conducted using standard methods (24).

Antimicrobial susceptibility testing. Antibiotic MICs were determined by broth microdilution using twofold dilution in HTM (Remel) in accordance with protocols established by the CLSI (Formerly NCCLS) (19). LBM415 (6) was synthesized at Novartis Institutes for Biomedical Research. All other antibiotics were obtained from Sigma (St. Louis, MO).

RESULTS

Strain CDS23 overexpresses peptide deformylase. *H. influenzae* mutant strain CDS23 was selected on chocolate agar plates containing 16 $\mu\text{g/ml}$ LBM415. This strain was significantly less sensitive to LBM415 (MIC, 64 $\mu\text{g/ml}$ versus 4 $\mu\text{g/ml}$ for the parent NB65044). There was no change in susceptibility to structurally unrelated substrates of the AcrAB-TolC efflux pump (e.g., clindamycin), and there was no mutation in the repressor of *acrAB* expression, AcrR (6). Strain CDS23 did not exhibit the pronounced growth deficit on chocolate agar plates

typical of Fmt bypass mutants (14), and no mutation was found in *fnt*, the structural gene encoding methionyl-tRNA^{Met} formyltransferase. Finally, there was no mutation found in *def*, encoding peptide deformylase, indicating that the reduced susceptibility was not the result of amino acid substitutions within the target protein. This suggested that the target, Def, might be overexpressed in the mutant. Supporting this, Western immunoblots using polyclonal antiserum raised against purified *Escherichia coli* Def revealed a dramatic increase in a cross-reactive protein of a size corresponding to that of Def in CDS23, compared to what was found for strain NB65044 (data not shown). To verify the identity of the overexpressed protein as Def, two-dimensional gel electrophoresis of protein extracts from these strains was conducted, revealing large increases in the levels of two protein spots (Fig. 1B). These were excised and subjected to tryptic digestion and mass spectrometry and confirmed as Def. A third spot of a much lower intensity, corresponding to another isoform of Def, was detected in some samples at a more basic position (data not shown). The presence of two major spots confirmed as Def indicated that two distinct forms of peptide deformylase, which were identified by MS not only in the mutant but also in the wild-type sample, were present (Fig. 1A). A characteristic of peptide deformylase enzymes is a propensity for oxidation of an active-site cysteine residue (10, 13, 26). A partially purified sample containing the two forms of Def shown in Fig. 1B was gluC digested and analyzed by liquid chromatography-MSMS, which confirmed the oxidation of C91 (underlined) to cysteic acid (GCLSI⁺PGF RALVPRKE), consistent with these previous observations.

The *def* transcript titer is dramatically increased in CDS23. The large increase in Def protein expression observed in strain CDS23 suggested that the *def* transcript titers might be dramatically increased as well. Microarray experiments revealed an approximately 5-fold increase in *def* transcript titer, with an increase up to 10-fold for the intergenic region upstream of *def* (data not shown). This clearly indicated an increase in *def* titer in the mutant but it did not correlate well with the dramatic increase in protein expression observed. The individual *def* probe signals from the microarrays gave extremely intense signals, suggesting that probe saturation was occurring, limiting the observable change (*n*-fold) that could be obtained from microarrays under normal sample loading conditions. To clar-

FIG. 1. Two-dimensional gel electrophoretic examination of proteins from *H. influenzae* strains. (A) Strain NB65044 (wild type). (B) CDS23 (Def-overexpressing strain). The overexpressed Def protein spots are indicated (arrows). The region of the total gels shown is indicated in panel A (molecular mass and pH ranges).

FIG. 2. Genetic organization of the region containing the *def* gene in *H. influenzae* NB65044 (A) and the additional PCR products A2 (B) and B2 (C) generated by PCRs using the CDS23 genomic DNA template and the primer pairs 23SF-DR1 and DF2-FR3 (D, columns A and B). Isolation and sequencing of bands A1 and B1 (D) confirmed these as the expected products for each of these PCRs, and these are the sole products obtained when the genomic template from NB65044, the parent of CDS23, is used. Lane 1, 1-kb DNA ladder (lowest to highest: 1, 1.5, 2, 3, 4, 5, 6, 8, and 10 kbp; New England Biolabs).

ify this, the same RNA samples were tested by real time RT-PCR experiments, which showed a circa-1,000-fold-higher abundance of *def* transcripts in CDS23 than in the parent strain (946 ± 13.8 - and $1,010 \pm 5.2$ -fold for two biological samples when using *rpsL* as the internal reference and 981.5 ± 10.7 - and $1,015.5 \pm 12.9$ -fold when using *acrB* as the internal reference).

Def overexpression is the result of amplified *def* gene copy number. Since the *def* transcript titer was very high in strain CDS23, the upstream promoter region was sequenced to check for mutations, but none were found between *def* and the upstream 5S rRNA gene (Fig. 2). To verify that CDS23 was indeed derived from NB65044, both strains were analyzed by PFGE. The *ApaI* restriction patterns in these strains differed by one band (Fig. 3C), which was increased in size in CDS23, indicating that there had been a genetic rearrangement in CDS23. Plotting an *ApaI* restriction map for the genome sequence of NB65044 using Vector NTI 9 software revealed that the band of increased size encompassed *def*. In addition, generating *def* by PCR from CDS23 invariably produced the expected product but with additional bands of increased sizes. This phenomenon also occurred when using primer pairs consisting of an internal *def* primer and primers specific for the regions upstream (23S rRNA) or downstream (*fmt*) of *def* (Fig. 2D, bands A2 and B2). This suggested that multiple priming sites for *def*-specific primers existed, consistent with the presence of multiple, adjacent, head-to-tail copies of *def*. Isolation, cloning, and sequencing analyses of bands A2 and B2 confirmed this to be the case, with adjacent copies of *def* separated by the 5S rRNA normally located immediately upstream of *def* (Fig. 2B and C). This indicated that the estimated 30-kb in-

crease in the size of the *def*-containing PFGE restriction fragment (Fig. 3C) likely resulted from the generation of multiple copies of *def* (an estimated 30 to 50 extra copies) between the upstream *rrn* locus and *fmt*. Sequencing revealed the presence of *NdeI* sites between the copies of *def* contained on fragments A2 and B2 (Fig. 2B and C). Therefore, digestion of CDS23 genomic DNA with *NdeI* was predicted to result in the release of a significant amount of an approximately 934-bp fragment containing *def*. Southern blot analysis of *NdeI*-digested genomic DNA isolated from NB65044 and CDS23 showed the presence of a highly emphasized band of the anticipated size hybridizing with a *def* probe (Fig. 3B, lane 2), confirming the presence of multiple copies of *def* separated by this restriction site. Indeed, the appearance of this band is visible in ethidium bromide-stained gels (Fig. 3A, arrow), consistent with a significant expansion of *def* copy number. Extraction of this band and sequence analysis confirmed its identity as the predicted *def*-containing *NdeI* fragment (indicated in Fig. 3). Therefore, Def protein overexpression is mediated by a large increase in chromosomal *def* copy number. The larger *def*-containing *ApaI* band observed by PFGE for the mutant was less intense than other bands (Fig. 3C, lane 2), and there was a faint smear in this region, suggesting that there is a heterogeneous population of *ApaI* bands with different copy numbers of *def*. This in turn suggests that this region is flexible and can expand or contract, a phenomenon previously described for regions containing gene amplifications (7, 27, 28, 32). Consistent with this, PFGE of one CDS23 sample that had been grown in liquid medium containing LBM415 prior to frozen storage showed a larger increase in the size of this band (Fig. 3C, lane 3), possibly the result of maintaining the selective pressure to maintain a

FIG. 3. Genetic analysis of the *def* repeat region of CDS23. Agarose gel (A) and Southern blot (B) analyses of NdeI-digested genomic DNA from strains NB65044 (lane 2) and CDS23 (lane 3). Lane 1, DNA standards. The NdeI fragment encompassing *def* is indicated by the arrow. Panel C shows PFGE analysis of ApaI-digested genomic DNA from strains NB65044 (lane 1) and CDS23 (lane 2). Lane 3 shows the ApaI digest from a CDS23 colony grown in LBM415 prior to being frozen. The *def*-containing fragments are shown (arrowheads). Lane 4, DNA standards. Below is shown the overall genetic arrangement of the *def* repeat region of CDS23, with a large number of copies of *def*, separated by NdeI sites and 5S rRNAs. kb, kilobase pairs; N, NdeI.

higher copy number. Because of this flexibility, a precise determination of *def* copy number was not possible. The presence of multiple adjacent copies of *def* could, however, explain the extreme, 1,000-fold increase in *def* transcript titer determined by real-time RT-PCR (described above) since this arrangement would be expected to generate multiple transcripts, each potentially containing multiple copies of the *def* region. The exact mechanism of this genetic rearrangement, and whether any other changes have also occurred, awaits further examination.

AcrAB-TolC-mediated efflux contributes to the absolute level of susceptibility to LBM415 resulting from target overexpression. Inactivation of the *acrB* gene in strain CDS23 caused a 16-fold increase in susceptibility to LBM415, to a level similar to that of the original parent strain NB65044 (Table 2). Pump loss in this strain gave the expected change in susceptibility to the known pump substrate clindamycin and had no effect on susceptibility to tetracycline, a pump nonsubstrate (Table 2). The 16-fold change in susceptibility to LBM415 is similar to the change (*n*-fold) in susceptibility to LBM415 observed for the parent strain NB65044 upon loss of AcrB (6) (Table 2). Therefore, efflux contributes a constant 16-fold decrease in sensitivity, apparently independent of the level of underlying target-based susceptibility. Strain CDS41 (a Def overexpressor lacking AcrAB) is 16-fold less sensitive to

LBM415 than strain CDS01 (which has wild-type Def levels and lacks AcrB), indicating that *def* overexpression, in the absence of the pump, also results in an approximately 16-fold decrease in susceptibility (Table 2). The 16-fold decrease in susceptibility to the potent compound LBM415 conferred by *def* overexpression in the absence of the pump results in a strain that is still fairly sensitive to LBM415 (CDS41; MIC = 4 μ g/ml) (Table 2). This is an indication of how potent LBM415 is in terms of target inhibition. From this starting point, however, the additional 16-fold decrease in sensitivity engendered by the intact efflux pump provides an absolute level of insusceptibility that is likely more clinically significant (CDS23; MIC = 64 μ g/ml) (Table 2).

DISCUSSION

In developing novel antimicrobials, it is prudent to investigate resistance development in target pathogens as early as possible in order to more fully understand the biology of the intracellular target. These efforts have led to a better understanding of the potential impact of resistance mechanisms, such as Fmt bypass (4, 18) and efflux (6), vis-a-vis peptide deformylase inhibitors. In this study, we have shown that target overexpression resulting from *def* gene copy number amplification on the chromosome can decrease the susceptibility of *H.*

influenzae to LBM415. Gene copy number amplification has been previously shown to mediate beneficial phenotypes or survival under selective pressure, including antibiotic exposure (7, 21, 22, 27–29, 32), and is believed to be a frequently occurring mechanism of genomic evolution (23, 30). Nonetheless, chromosomal gene copy number expansion has not been reported as a bacterial resistance determinant as frequently as might be expected. This may be because initial gene duplication (illegitimate recombination) is a lower-frequency event and/or is limited to those targets where overexpression can be physiologically tolerated. This mechanism is theoretically beneficial in that the copy number can expand and contract, and once the selection pressure is removed, it can quickly be reversed. We have not tested this systematically here, but our PFGE analysis of CDS23 at different times has shown that the band containing the amplification of *def* is variable, suggesting such flexibility (Fig. 3C). It has also been shown that genes located near *rrn* loci are subject to a higher frequency of duplication events (3). Therefore, the location of the *def* gene immediately downstream of one of the 5S rRNAs (Fig. 3) included as part of one of the five *rrn* loci on this small chromosome (8) may predispose this particular gene to amplification under exposure to LBM415 or other peptide deformylase inhibitors. Resistance to the peptide deformylase inhibitor actinonin due to target overexpression resulting from *def* promoter mutation has also been reported previously (5).

RND family efflux pumps have recently been implicated in the development of target-based resistance, for example, in the case of fluoroquinolone resistance development in *Pseudomonas aeruginosa* (15). We have shown here that the absolute decrease in susceptibility to LBM415 mediated by the overexpression of Def in CDS23 is significantly enhanced by the AcrAB-TolC efflux pump, further indicating that modest intrinsic efflux can become very significant when paired with an underlying target-based resistance mechanism. Since the Def-overexpressing mutant is still relatively susceptible to LBM415 in the absence of the AcrAB-TolC pump (LBM415 MIC = 4 µg/ml), the presence of this efflux pump could contribute to the survival (selection) of the Def-overexpressing phenotype and would determine to a significant extent the ultimate resistance level. This underscores the potential benefit of developing peptide deformylase inhibitors that better circumvent the AcrAB-TolC efflux pump.

In conclusion, the addition of this mode of target overexpression and its interplay with AcrAB-TolC efflux to the mechanisms reducing the effectiveness of peptide deformylase inhibitors provides yet another example of the genetic flexibility that bacteria can demonstrate against efforts to develop novel antimicrobial compounds.

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