

## Model System To Evaluate the Effect of *ampD* Mutations on AmpC-Mediated $\beta$ -Lactam Resistance

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Mutations within the structural gene of *ampD* can lead to AmpC overproduction and increases in  $\beta$ -lactam MICs in organisms with an inducible *ampC*. However, identification of mutations alone cannot predict the impact that those mutations have on AmpD function. Therefore, a model system was designed to determine the effect of *ampD* mutations on ceftazidime MICs using an AmpD<sup>-</sup> mutant *Escherichia coli* strain which produced an inducible plasmid-encoded AmpC. *ampD* genes were amplified by PCR from strains of *E. coli*, *Citrobacter freundii*, and *Pseudomonas aeruginosa*. Also, carboxy-terminal truncations of *C. freundii ampD* genes were constructed representing deletions of 10, 21, or 25 codons. Amplified *ampD* products were cloned into pACYC184 containing inducible *bla*<sub>ACT-1</sub>-*ampR*. Plasmids were transformed into *E. coli* strains JRG582 (AmpD<sup>-</sup>) and K-12 259 (AmpD<sup>+</sup>). The strains were evaluated for a derepressed phenotype using ceftazidime MICs. Some mutated *ampD* genes, including the *ampD* gene of a derepressed *C. freundii* isolate, resulted in substantial decreases in ceftazidime MICs (from >256  $\mu$ g/ml to 12 to 24  $\mu$ g/ml) for the AmpD<sup>-</sup> strain, indicating no role for these mutations in derepressed phenotypes. However, *ampD* truncation products and *ampD* from a partially derepressed *P. aeruginosa* strain resulted in ceftazidime MICs of >256  $\mu$ g/ml, indicating a role for these gene modifications in derepressed phenotypes. The use of this model system indicated that alternative mechanisms were involved in the derepressed phenotype observed in strains of *C. freundii* and *P. aeruginosa*. The alternative mechanism involved in the derepressed phenotype of the *C. freundii* isolate was downregulation of *ampD* transcription.

The overproduction of AmpC  $\beta$ -lactamase by gram-negative organisms results in resistance to most  $\beta$ -lactam antibiotics with the exception of cefepime, cefpirome, and the carbapenems (40). The regulation of *ampC* gene expression can differ between genera of gram-negative organisms. For example, the production of the AmpC  $\beta$ -lactamase in *Enterobacter* spp., *Citrobacter freundii*, *Serratia marcescens*, *Morganella morganii*, and *Pseudomonas aeruginosa* is inducible, and the gene is encoded on the chromosome (4, 12, 13, 17, 33). However, gene expression of the chromosomally encoded AmpC of *Escherichia coli* is regulated by promoter and attenuator mechanisms and is not inducible (13). Furthermore, *ampC* genes from different genetic origins including *C. freundii* and *M. morganii* have been found on plasmids (2, 40). The movement of these genes onto plasmids increased the prevalence of this resistance mechanism by dissemination of the gene into gram-negative organisms which normally do not carry genes encoding AmpC, such as *Salmonella* spp. and *Klebsiella pneumoniae* (2, 3). These plasmid-mediated genes have also been found in *E. coli* (31). Of the more than 20 plasmid-mediated AmpC  $\beta$ -lactamases, five are inducible including DHA-1, DHA-2, ACT-1, CMY-13, and CFE-1 (2, 5, 9, 27, 28).

The induction of AmpC  $\beta$ -lactamase is controlled by the activity of three proteins: AmpG, AmpD, and AmpR. AmpG is a cytoplasmic membrane-bound permease which allows entry of cell wall degradation products. These products, 1,6-anhy-

dromuropeptides, are cleaved by AmpD, an *N*-acetylmuramyl-L-alanine amidase, into 1,6-anhydromuramic acid and peptide (16, 42). The peptide is processed into tripeptide, which is reused by the enzymes of the cell wall recycling pathway, ultimately resulting in the formation of the cell wall precursor, UDP-MurNAc-pentapeptide. AmpR is a transcriptional regulator of *ampC* expression, and binding of 1,6-anhydromuropeptide to AmpR results in induction of *ampC* gene expression (15). Conversely, when AmpR is bound by the cell wall precursor, UDP-MurNAc pentapeptide, *ampC* is repressed.

In a wild-type (WT) cell, AmpC production is expressed at constitutively low levels due to the binding of UDP-MurNAc-pentapeptide to AmpR. Mutations associated with AmpR and AmpD can result in AmpC overproduction, which has been termed derepression (1, 12, 19, 22, 25, 39). Phenotypically, derepressed mutants can be resistant to expanded-spectrum cephalosporins, which is attributed to the overproduction of AmpC (37). The mechanism most associated with constitutive overproduction of AmpC (full derepression) is amino acid substitutions within AmpD (12, 39). It has been suggested that these mutations interfere with the ability of AmpD to cleave the substrate (1,6-anhydromuropeptide), leading to an increase in the cytoplasmic pool of 1,6-anhydromuropeptide compared to UDP-MurNAc-pentapeptide, resulting in AmpC overproduction (39). In addition to the role of AmpD in AmpC production, it is an important enzyme in the cell wall recycling pathway (16). Therefore, *ampD* genes are found in many gram-negative organisms including *K. pneumoniae*, *E. coli*, and *Salmonella* spp (8, 36, 41). Furthermore, these organisms do not normally carry an inducible *ampC* gene and mutations within the *ampD* structural gene which could contribute

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TABLE 1. Primers used in this study

Primer name <sup>a</sup>	Sequence (5' to 3')	Purpose <sup>b</sup>	Nucleotides <sup>c</sup>	GenBank accession no.
<i>E. coli ampD</i>				
ampDUF	CTCTTCAACCCAGCGTTTGC	A	118418–118437	NC_000913
ampDAKR	GCACTGGCAGCTTGATCATCG	A	119360–119340	NC_000913
<i>C. freundii ampD</i>				
CFampDF	CCAGAAGCGCGTACCGTCGG	A	18–37	Z14002
CFampDR	TCATGTCATCTCCTTGTGTGACG	A	717–695	Z14002
XIR1 <sup>d</sup>	CTACTCGGGCCGCAATATTGC	A	635–617	Z14002
XIR2	CTAGGGATCGGTCTTACGCTCG	A	648–630	Z14002
XIR3	CTAGCTAAACCTTGCCCAAGTC	A	680–661	Z14002
<i>P. aeruginosa ampD</i>				
PAampDF	GACGATGCCTTGCTGTTCG	A	436–454	AF082575
PAampDR	GCAGCAATGTGACGAACAGG	A	1422–1403	AF082575
<i>C. freundii</i>				
CFRRNAF <sup>e</sup>	GTTGTGGTTAATAACCGCAGCG	R	436–457	AJ233408
CFRRNAR	GCTTTACGCCAGTAATTCCG	R	556–536	AJ233408
CFampCF	CGAAGCCTATGGCGTGAATC	R	1772–1792	AY125469
CFampCR	CCAATACGCCAGTAGCGAG	R	1906–1888	AY125469
CFampDF	GCATGTTGTTAGACGAGGG	R	152–170	Z14002
CFampDR	CGGCAGGCTGATATTATGC	R	270–252	Z14002

<sup>a</sup> Primer names ending with F represent forward primers; those ending with R represent reverse primers.

<sup>b</sup> Purpose for the primers: A, amplification, cloning, and sequencing; R, real-time RT-PCR.

<sup>c</sup> Nucleotide location of each primer with respect to the GenBank accession number cited, listed 5'–3'.

<sup>d</sup> XIR1, XIR2, and XIR3 primers bind internal to the *ampD* gene and contain an in-frame stop codon.

<sup>e</sup> Primers used to amplify 16S rRNA from *C. freundii* as an endogenous control for real-time RT-PCR experiments.

to a derepressed phenotype would not be detected phenotypically. However, when *E. coli* and *K. pneumoniae* contain an inducible plasmid-mediated AmpC  $\beta$ -lactamase, mutations within the structural gene of *ampD* could lead to increased *ampC* expression from the imported gene, resulting in increased oxyiminocephalosporin MICs (36).

AmpD mutations associated with derepression include point mutations, truncations, and large insertions, primarily disrupting the carboxy terminus of the protein (1, 7, 19, 22, 25, 39). The AmpD protein has two important binding sites: a zinc-binding pocket and a substrate (1,6-anhydromuropeptide) binding site. Nuclear magnetic resonance (NMR) structural analysis of the *Citrobacter freundii* AmpD has identified key amino acids required for AmpD function (11). The amino acids required for zinc binding include an aspartic acid at position 164 and two histidines at positions 34 and 154. Amino acids important for binding substrate are hydrophobic in nature and are concentrated in the amino terminus of the protein. These findings support previous research by Stapleton et al. that mapped point mutations associated with derepression to the aspartic acid residue at position 164 and a valine residue at position 33 (39).

Sequence analysis of *ampD* genes obtained from phenotypically derepressed mutants can identify potential mutations which may affect AmpC production. However, identification of sequence variation alone is not enough to determine the effect that those mutations elicit on AmpC production. Therefore, a model system was developed to identify the role of nucleotide variations identified in different gram-negative organisms in the overproduction of AmpC and resistance to the oxyiminocephalosporin ceftazidime (CAZ). In this model, an inducible *ampC*  $\beta$ -lactamase, *bla*<sub>ACT-1</sub>, was used as the indicator for a

susceptible or resistant ceftazidime phenotype. The inducible ACT-1 system (*bla*<sub>ACT-1</sub> and *ampR*) was cloned into a modified pACYC184 vector in which *ampD* test genes can also be cloned, negating the use of two different vector systems. The *ampD* test genes have nucleotide variations which result in amino acid substitutions. To test the significance of these nucleotide/amino acid variations, the plasmid containing all three genes is cloned into two *E. coli* hosts that differ with respect to the *ampD* gene (i.e., wild type or mutant). If the substitutions identified in the *ampD* sequence do not inhibit the functionality of the *ampD* gene product, the plasmid cloned into the *ampD* mutant host will complement the host gene, resulting in a ceftazidime-susceptible phenotype. If the test gene does not complement the *ampD* mutant phenotype, the resulting ceftazidime phenotype will be resistant. The flexibility of the test was demonstrated by three susceptibility testing methods: agar dilution, Etest, and disk diffusion. The use of this model system suggested that mechanisms other than mutations within the structural gene of AmpD can be responsible for derepressed phenotypes observed in clinical isolates.

#### MATERIALS AND METHODS

**Strains used in this study.** Two *E. coli* strains were used for the cloning experiments: JRG582, an *ampD*-negative mutant ( $\Delta$ *nadC-aroP*) (23), and an *ampD*<sup>+</sup> strain, K-12 259 (GenBank accession number D90770). *ampD* genes were isolated and tested from *E. coli*, *Citrobacter freundii*, and *P. aeruginosa*. A laboratory strain of *E. coli* (HB101) was the source of a wild-type *ampD* gene, and an *E. coli* isolate from a urinary tract infection, CUMC5 (with AmpD amino acid substitutions Val9Ala, Cys143Arg, Lys149Asn, Val176Ala, and Val178Ile), served as the source of mutated *ampD*. The *C. freundii ampD* sources were *C. freundii* strains CF21 (a clinical isolate with a wild-type  $\beta$ -lactam susceptibility pattern) and its single-step mutant selected with cefotaxime, CF21M (derepressed phenotype) (14). Finally, three strains of *P. aeruginosa* were used as

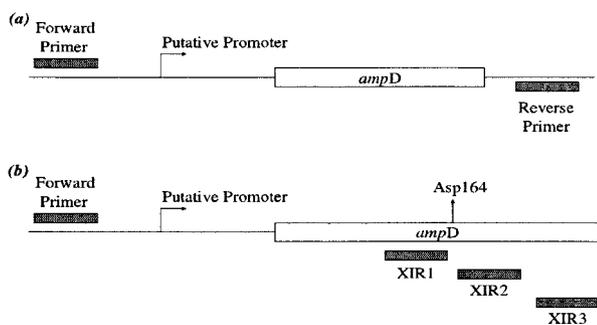


FIG. 1. Primer binding sites for *ampD* amplification and cloning. (a) Specific forward and reverse primers (Table 1) were used to amplify the structural gene, putative promoter, and upstream region of *ampD* test genes used for the generation of clones with *ampD* inserts derived from *E. coli* strains HB101 and CUMC5; *C. freundii* strains CF21 and CF21M; and *P. aeruginosa* strains PAO1, Ps164, and M1. (b) Locations and names of primers used to generate truncated PCR-amplified products of the CF21 *ampD* gene. The PCR was performed using the forward primer CFampDF and XIR1, XIR2, or XIR3, which resulted in truncated products with a loss of 25, 21, or 10 codons from the carboxy terminus, respectively.

source organisms for *ampD*: PAO1, a laboratory control strain; Ps164, a clinical isolate with a wild-type  $\beta$ -lactam susceptibility pattern (34); and Ps164 M1, a single-step mutant selected from Ps164 using cefotaxime which was phenotypically characterized as partially derepressed for AmpC production (10).

**Template preparation.** Template DNA for PCR was prepared from overnight cultures of *E. coli* and *C. freundii* and an 8-hour culture of *P. aeruginosa* using 1.5 ml of culture as previously described (32). A final concentration of 400  $\mu$ g/ml of proteinase K was added to *P. aeruginosa* supernatant prior to lysis to protect the nucleic acid from nuclease degradation.

**PCR of test strains.** *ampD* genes were amplified by PCR as previously described (35) using the proofreading enzymes High Fidelity *Taq* polymerase (Invitrogen) for *E. coli* and *C. freundii* and Precision *Taq* polymerase (Stratagene) for *P. aeruginosa*. The DNA template was generated from strains described above. Primers used for amplification are listed in Table 1. The corresponding binding sites of the primers are depicted in Fig. 1. The amplified product contained the putative promoter region, translational start codon, and structural *ampD* gene. Each forward primer (Table 1) binds 316, 136, and 223 bp upstream of the start codon for *E. coli*, *C. freundii*, and *P. aeruginosa* (respectively) to include the putative promoter for *ampD*. Three internal reverse primers (Table 1) specific to *C. freundii ampD* were used to truncate the gene at codons 160, 165, and 175 and contained a stop codon in frame with the AmpD protein sequence. Amplified *ampD* genes were separated and visualized using a 1.0% agarose gel with ethidium bromide staining.

**Cloning.** The amplified *ampD* genes were extracted from the agarose gel using a SNAP column (Invitrogen), ligated into pCR-TOPO-XL (Invitrogen), and transformed into Top 10 *E. coli* cells (Invitrogen). The *ampD* fragment was subcloned, using the restriction enzymes *Xba*I and *Hind*III, into pACYC184 containing *bla*<sub>ACT-1</sub>-*ampR* (Fig. 2) (36). The resulting plasmid was electroporated into *E. coli* JRG582 ( $\Delta$ *nadC-aroP*) (*ampD* mutant) (23) and K-12 259 (WT *ampD*) as described by Hossain et al. (14).

**Ceftazidime susceptibility assays.** The susceptibility of *ampD* clones to ceftazidime was determined by Etest (AB Biodisk, Sweden) and disk diffusion (30  $\mu$ g) using the manufacturer's instructions and CLSI (formerly NCCLS) criteria (29). The agar dilution methodology assay was performed according to CLSI guidelines to identify the ceftazidime MICs for clones with Etest ceftazidime MICs of  $>256$   $\mu$ g/ml (30).

**Sequencing.** Plasmids containing the *ampD* insert were purified for sequencing using a Microcon YM-10 filter column (Millipore Corporation), and PCR-amplified products, including the *ampC* and *ampR* genes of *C. freundii*, were purified using YM-50 filter columns (Millipore Corporation). Sequencing was performed by automated cycle sequencing using an ABI Prism 3100-Avant Genetic analyzer using primers listed in Table 1.

**BLAST analysis.** Mutations and amino acid changes were analyzed by comparing them to wild-type sequence of their respective genera using the BLAST program of the National Center for Biotechnology Information website ([www.ncbi.nlm.nih.gov](http://www.ncbi.nlm.nih.gov)). *E. coli ampD* genes were compared to the *E. coli* strain K-12

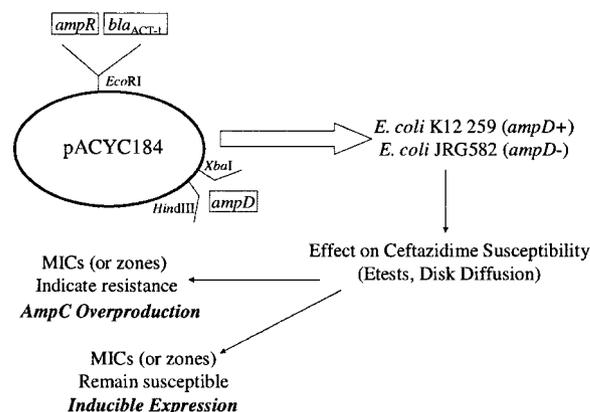


FIG. 2. Development of the *ampD* model system. An inducible *ampC*  $\beta$ -lactamase, *bla*<sub>ACT-1</sub>, is used as the indicator for a susceptible or resistant ceftazidime phenotype. The *ampD* gene to be tested was amplified by PCR and cloned into a moderate-copy-number (12 copies) plasmid, pACYC184, containing the *ampC*  $\beta$ -lactamase gene and its transcriptional regulator gene, *ampR* (35). The amplified *ampD* product contained the entire structural gene as well as upstream sequence to allow gene expression from its native promoter. The plasmid was transformed into two different *E. coli* backgrounds, an *ampD* mutant strain (JRG582) (23) and a WT *ampD*<sup>+</sup> strain (K-12 259). Both of these strains are *E. coli* K-12 derivatives. Following transformation, the ceftazidime MICs were determined. If the test *ampD* gene represented a mutant which results in overproduction of AmpC, the ceftazidime MICs in JRG582 clones were very high ( $>256$   $\mu$ g/ml), representing a failure to complement the *ampD* mutant background of JRG582, but if the test *ampD* gene represented a wild-type gene which would not influence AmpC overproduction, ceftazidime MICs were low ( $\leq 12$   $\mu$ g/ml) for the JRG582 clones, representing a susceptible phenotype. Wild-type AmpD represents a dominant phenotype, so no change in the resistant phenotype was observed when test *ampD* genes were transformed into the AmpD WT strain (K-12 259).

sequence (accession number NC\_000913), *ampD* sequences of *C. freundii* were compared to *C. freundii* strain OS 60 (accession number Z14002), and *ampD* genes from *P. aeruginosa* were compared to the *P. aeruginosa* strain PAO1 (accession number AF082575). Additionally, the *ampC* and *ampR* genes from *C. freundii* CF21M were compared to strain CF21 (accession number AY125469).

**RNA isolation.** Overnight cultures (5 ml) of CF21 and CF21M were diluted 1:20 (100-ml total volume) in Mueller-Hinton broth (Oxoid) and allowed to grow to an optical density at 600 nm of 0.5 at 37°C with shaking at 125 rpm. Cells were centrifuged, and the cell pellet was resuspended in 1 ml of modified Trizol-Max (Invitrogen) solution (36). Protein was removed by treatment with a phenol:chloroform:isoamyl alcohol solution (25:24:1), and the RNA was precipitated with ethanol. To eliminate DNA contamination, 8  $\mu$ g of isolated RNA was treated for 1 hour with 8 units of RQ1 RNase-free DNase (Promega) according to the manufacturer's instructions; however, the 65°C heat inactivation step was omitted to prevent shearing of the RNA.

**Real-time RT-PCR.** DNase-treated RNA isolated from CF21 and CF21M was examined using real-time reverse transcription-PCR (RT-PCR) (43). Each reaction mixture was comprised of 25  $\mu$ l 2 $\times$  QuantiTect SYBR green RT-PCR Master Mix (QIAGEN), 0.5  $\mu$ M of the forward primer and 0.5  $\mu$ M of the reverse primer (Table 1 lists primers used), 0.5  $\mu$ l QuantiTect RT Mix (QIAGEN), and 150 ng of DNase-treated RNA. RNase-free water was added for a total volume of 50  $\mu$ l. An ABI 7000 sequence detection system was programmed for the following parameters: an initial 40-min 50°C reverse transcription step and a 15-min 95°C denaturation and activation step. Next, 40 cycles of PCR were performed: 95°C for 30 s, 50°C for 30 s, and 72°C for 30 s. Data were evaluated using ABI Prism 7000 SDS software with an amplification threshold of 0.4. All RNA samples were evaluated for the presence of DNA template using a reaction mixture for each sample in the absence of reverse transcriptase but in the presence of DNA polymerase. Only samples indicating the absence of DNA were used for analysis. Expression of 16S rRNA was used to normalize the relative expression data for *ampC* and *ampD*. The experiment was performed three times, and the final relative expression of *ampC* and *ampD* was determined by

TABLE 2. MICs for *ampD* clones in WT and *ampD* mutant *E. coli* background

Plasmid or origin of <i>ampD</i> insert	<i>ampD</i> insert characteristic(s)	K-12 259 <sup>a,k</sup>		JRG582 <sup>b,k</sup>	
		CAZ MIC <sup>c</sup> ( $\mu$ g/ml)	Zone diam <sup>d</sup> (mm)	CAZ MIC <sup>c</sup> ( $\mu$ g/ml)	Zone diam <sup>d</sup> (mm)
<b>Plasmids</b>					
None	No <i>ampD</i> insert	0.25 (S)	30 (S)	0.064 (S)	32 (S)
ACYC184 ( <i>ampR bla</i> <sub>ACT-1</sub> )	No <i>ampD</i> insert	12 (S)	19 (S)	>256 (R)	6 (R)
<b>Origin of <i>ampD</i> insert</b>					
<i>E. coli</i>					
HB101 <sup>e</sup>	WT	16 (I)	19 (S)	24 (I)	18 (S)
CUMC5 <sup>f</sup>	V9A, C143R, K149N, 176A, V178I	16 (I)	19 (S)	12 (S)	19 (S)
<i>C. freundii</i>					
CF21 <sup>g</sup>	R175S	16 (I)	19 (S)	4 (S)	19 (S)
CF21M <sup>h</sup>	R175S	24 (I)	19 (S)	12 (S)	19 (S)
CF21-XIR1	TAG at E160	24 (I)	17 (I)	>256 (R)	6 (R)
CF21-XIR2	TAG at P165	24 (I)	18 (S)	>256 (R)	6 (R)
CF21-XIR3	TAG at R175S	16 (I)	19 (S)	>256 (R)	8 (R)
<i>P. aeruginosa</i>					
PAO1	WT	16 (I)	19 (S)	24 (I)	18 (S)
Ps164 <sup>i</sup>	WT	24 (I)	19 (S)	32 (R)	16 (I)
Ps164 M1 <sup>j</sup>	TAG at Q155	16 (I)	16 (I)	>256 (R)	6 (R)

<sup>a</sup> *E. coli* strain K-12 259: WT *ampD*.

<sup>b</sup> JRG582: *E. coli* strain K-12 derivative lacking the *ampD* gene (23).

<sup>c</sup> Ceftazidime MICs were determined using Etest strips, and susceptibility breakpoints reflect those indicated by CLSI guidelines (29).

<sup>d</sup> Disk diffusion tests for ceftazidime, 30  $\mu$ g.

<sup>e</sup> *E. coli* strain HB101 is a laboratory control strain with wild-type  $\beta$ -lactam susceptibility patterns.

<sup>f</sup> *E. coli* strain CUMC5 is a urine isolate.

<sup>g</sup> *C. freundii* strain CF21 is a clinical isolate which has an inducible chromosomal *ampC* (38).

<sup>h</sup> *C. freundii* strain CF21M is a derepressed (noninducible) mutant derived from CF21 (38).

<sup>i</sup> *P. aeruginosa* strain Ps164 is a clinical isolate susceptible to all  $\beta$ -lactam drugs (10).

<sup>j</sup> *P. aeruginosa* strain Ps164 M1 is a partially derepressed mutant selected from Ps164 using cefotaxime (10).

<sup>k</sup> S, susceptible; I, intermediate; R, resistant.

averaging the results for the respective transcripts. First the cycle thresholds ( $C_T$ ) for each gene were averaged between the three experiments (standard deviation,  $\leq 0.672$ ) and the relative expression was calculated using  $RQ = 2^{-\Delta\Delta C_T}$  (26). Using these parameters, the coefficient of variation for all data used was  $\leq 10\%$ .

## RESULTS

**Susceptibility and phenotypes of *ampD* clones.** The ceftazidime MICs and zones of inhibition for *ampD* clones in *ampD*<sup>+</sup> (K-12 259) and *ampD* mutant (JRG582) genetic backgrounds are listed in Table 2. Disk diffusion was used to evaluate susceptibility for two reasons: (i) this methodology provided an inexpensive alternative to MIC methodologies in establishing a susceptibility phenotype and (ii) these results were used to further evaluate Etest MIC data, especially when an intermediate result was obtained. The recipient strains (JRG582 and K-12 259) with and without the plasmid pACYC184 containing *bla*<sub>ACT-1</sub>-*ampR* were used as controls. When the recipient strains were tested in the absence of plasmid, ceftazidime MICs were 0.064  $\mu$ g/ml for JRG582 (zone size, 32 mm) and 0.25  $\mu$ g/ml for K-12 259 (zone size, 30 mm). Transformation with pACYC184 containing *bla*<sub>ACT-1</sub>-*ampR* into JRG582 and K-12 259 raised the ceftazidime MICs to >256 and 12  $\mu$ g/ml (zones, 6 and 19 mm, respectively), respectively. These data supported the hypothesis that the absence of AmpD (JRG582) correlated with overexpression of *ampC* (*bla*<sub>ACT-1</sub>) and a derepressed phenotype as previously described (35).

To test the association between specific *ampD* mutations

and a derepressed phenotype, *ampD* genes from *E. coli*, *C. freundii*, and *P. aeruginosa* were cloned into pACYC184 containing *bla*<sub>ACT-1</sub>-*ampR* and analyzed for the ability of the cloned *ampD* to complement the JRG582 phenotype as indicated by a reduction of ceftazidime MICs (Table 2; Fig. 2). If the test *ampD* gene represented a mutant which resulted in overproduction of AmpC, the ceftazidime phenotype was resistant for JRG582 clones (by both MIC and zone diameter), representing a failure to complement the *ampD* mutant background of JRG582, but if the test *ampD* gene represented a wild-type gene which would not influence AmpC overproduction, the ceftazidime phenotype was susceptible (both by MICs and by zone diameter) for the JRG582 clones. Wild-type AmpD represented a dominant phenotype, so no change in the susceptibility phenotype was observed when test *ampD* genes were transformed into the AmpD wild-type strain (K-12 259).

**Sequence analysis of *E. coli ampD* and the effects of *ampD* mutations on ceftazidime MICs.** A derepressed phenotype is not normally associated with strains of *E. coli* because the chromosomal *ampC* gene is not inducible. However, when *E. coli* harbors an inducible plasmid-mediated AmpC  $\beta$ -lactamase, it could be considered a derepressed mutant with concomitant increases in  $\beta$ -lactam MICs if AmpD was functionally inactive. To determine if *ampD* mutations were present in clinical isolates of *E. coli*, 31 *E. coli* strains isolated from patients with urinary tract infections were examined by sequencing PCR-generated products of *ampD* and comparing those

sequences to the wild-type *ampD* sequence in *E. coli* K-12 (accession number NC\_000913). Seventy-seven percent (24/31) of the isolates contained mutations within *ampD* which resulted in amino acid substitutions. Five common mutations were observed (Val9Ala, Cys143Arg, Lys149Asn, Val176Ala, and Val178Ile), and the clinical *E. coli* isolates could be grouped as having three (12/24), four (5/24), or all five (7/24) of these mutations. The amino acid substitutions were primarily located in the carboxy terminus with the exception of 13 isolates which also had Val9Ala in the amino terminus. One of the isolates, CUMC5, which contained all five common mutations, was selected for evaluation in the *ampD* model system. *E. coli* HB101 is a wild-type laboratory strain which carries a wild-type *ampD* gene and therefore was used as a control. Transformation of JRG582 with the *ampD* gene of HB101 inserted into the test plasmid resulted in a ceftazidime MIC of 24  $\mu\text{g/ml}$  (zone, 18 mm), and the *ampD* obtained from CUMC5 resulted in a MIC of 12  $\mu\text{g/ml}$  (zone, 19 mm) (Table 2). These data indicated that the amino acid substitutions observed in the CUMC5 *ampD* gene did not influence the production of the indicator, ACT-1.

**Comparison of the *ampD* genes between *C. freundii* *ampD* wild-type and derepressed isolates.** Two native *C. freundii* *ampD* genes from strains *C. freundii* 21 (CF21) and *C. freundii* 21 M (CF21M) were tested in the model. CF21M was a single-step mutant selected from CF21 using cefotaxime and has a cefotaxime MIC of 32  $\mu\text{g/ml}$  compared to 1  $\mu\text{g/ml}$  for the parent, CF21. CF21M is phenotypically derepressed and produces >200 times the amount of *ampC* RNA produced by CF21, which exhibits inducible, wild-type *ampC* production (14). When tested using the *ampD* model system, ceftazidime MICs for JRG582 clones containing the *ampD* genes of CF21 and CF21M were 4 and 12  $\mu\text{g/ml}$  (zones of 19 mm each), respectively. These results were surprising given the derepressed phenotype of CF21M. Sequence analyses of the *ampD* genes from both CF21 and CF21M indicated a mutation resulting in an amino acid substitution at position R175S in both strains. Taken together, these data indicated that the observed mutation did not result in the derepressed phenotype observed for CF21M.

The *ampD* gene cloned from the derepressed mutant, CF21M, did not yield a derepressed phenotype in the model. Therefore, to substantiate the idea that an AmpD-mediated derepressed phenotype could be detected using the *ampD* model system, three PCR-generated mutant *ampD* genes were constructed from *C. freundii* strain CF21. Different internal reverse primers were used (Table 1) and resulted in the loss of 25 (primer XIR1), 21 (XIR2), or 10 (XIR3) codons from the carboxy terminus, respectively. These primers contained stop codons in frame with the amino acid sequence. Clones expressing these constructed *ampD* genes yielded ceftazidime MICs of >256  $\mu\text{g/ml}$  in JRG582 (zones, 6 to 8 mm) and ranged from 16 to 32  $\mu\text{g/ml}$  in K-12 259 (zones, 17 to 20 mm) (Table 2). Strains that exhibited a ceftazidime MIC of >256  $\mu\text{g/ml}$  using Etest were evaluated by agar dilution methodology and found to have a ceftazidime MIC of 128  $\mu\text{g/ml}$ .

**Evaluation of *ampD* gene expression in *C. freundii* 21 M (CF21M).** The truncated *ampD* genes verified that the *ampD* model could be used to detect *ampD* mutations which resulted in a derepressed phenotype. Together with the sequence analysis of

the CF21M *ampD* gene, these data confirmed that the amino acid substitution within AmpD of CF21M was not the mechanism responsible for the derepressed phenotype. The *ampR* gene and *ampC-ampR* intergenic region of CF21M were sequenced and evaluated as alternative mechanisms of derepression in this isolate. No mutations were found in these regions compared to the prototype *C. freundii* OS60 (GenBank accession number Z14002). Therefore, *ampD* and *ampC* expression in the clinical isolate CF21 and its mutant CF21M was analyzed using real-time RT-PCR. CF21M exhibited a 403-fold increase ( $C_T$  standard deviation, 0.6) in *ampC* expression and a corresponding 11-fold decrease ( $C_T$  standard deviation, 0.66) in *ampD* expression compared to CF21.

**Evaluation of the *ampD* genes in *P. aeruginosa*.** The plasmid model was further evaluated to determine if *ampD* genes could be tested from an organism other than those grouped as *Enterobacteriaceae* (i.e., *P. aeruginosa*). PAO1 is the prototype strain for *P. aeruginosa* and was used as a control strain for evaluating *P. aeruginosa* mutants. When the WT *ampD* gene from PAO1 was cloned into the plasmid model, the ceftazidime MIC was intermediate (24  $\mu\text{g/ml}$ ) but there was a susceptible zone diameter of 18 mm. However, when the *ampD* gene from a ceftazidime-susceptible clinical isolate of *P. aeruginosa*, Ps164, was cloned into the model, an intermediate-to-resistant phenotype was observed with ceftazidime MICs of 32  $\mu\text{g/ml}$  in JRG582 (intermediate zone size, 16 mm). Insertion of *ampD* from Ps164 M1 resulted in a MIC of >256  $\mu\text{g/ml}$  in JRG582 (zone size, 6 mm). Agar dilution methodology indicated that the ceftazidime MIC of the clone expressing *ampD* of Ps164 M1 in JRG582 was 128  $\mu\text{g/ml}$ . Sequence analysis of the *ampD* genes of the strains tested indicated a wild-type sequence for Ps164 but a truncation of AmpD at amino acid position 155 for Ps164 M1 (D. J. Wolter, N. D. Hanson, and P. D. Lister, Abstr. 44th Intersci. Conf. Antimicrob. Agents Chemother., abstr. C1-955, 2004).

## DISCUSSION

The purpose of this study was to develop a model system to evaluate the effect of sequence variation within *ampD* genes from different genera on AmpC overproduction. Using this model system, alternative mechanisms other than amino acid substitutions were shown to contribute to AmpC derepression. There are several advantages to examining *ampD* sequence variation in this model system. First, the genes corresponding to the inducible  $\beta$ -lactamase phenotype, *bla*<sub>ACT-1</sub>-*ampR*, were cloned into the same plasmid as the *ampD* insert, negating any copy number effect between the genes expressing the  $\beta$ -lactamase and AmpD and eliminating any potential plasmid compatibility issues. Second, JRG582 and K-12 259 are both K-12 *E. coli* derivatives providing a controlled genetic background. Third, the initial effects of *ampD* mutations can be determined inexpensively using disk diffusion compared to more expensive or labor-intensive methods such as Etest or agar dilution methodology. Finally *ampD* genes from different genera can be evaluated for their effects on *ampC* expression in one model system.

A wild-type AmpD encoded by the model system was identified based on decreased ceftazidime MICs (from >256  $\mu\text{g/ml}$  to  $\leq 24$   $\mu\text{g/ml}$ ) or increases in zone size for *ampD* clones in the

*ampD* mutant background (JRG582) with no substantial difference in the K-12 259 background (Table 2). The decrease in ceftazidime MICs indicated a lower constitutive level of *bla*<sub>ACT-1</sub> expression due to complementation of the *ampD* mutant *E. coli* strain JRG582 by the test *ampD* gene in the plasmid. The variation observed for the K-12 259 clones most likely represents fluctuations in Etest results as the zone sizes for all clones indicated a susceptible, or in one case an intermediate, phenotype. Insertion of *E. coli* and *C. freundii* native *ampD* genes as well as the wild-type genes of *P. aeruginosa* PAO1 and Ps164 resulted in decreased ceftazidime MICs in the AmpD<sup>-</sup> *E. coli* strain JRG582 (Table 2). These ceftazidime MICs indicated that *ampD* sequence variations observed for CUMC5 and CF21 and CF21M were associated with a wild-type phenotype. Mutations within AmpD responsible for a derepressed phenotype were identified using the *ampD* model system based on the inability of the test genes to complement the *ampD* mutant phenotype of JRG582 as observed for the CF21 *ampD* truncated clones and strain Ps164 M1 (CAZ MICs remained >256  $\mu$ g/ml [Table 2]).

Although the *ampD* gene product of *E. coli* strain CUMC5 had five amino acid substitutions, the gene encoding that product was capable of complementing an *ampD* mutant phenotype. It is possible that amino acid substitutions that fall outside the highly conserved regions of AmpD do not substantially contribute to the overall function of the enzyme. If these amino acid substitutions contribute to minor changes in the AmpD enzymatic activity, the model system may not be able to differentiate those contributions for at least two reasons: (i) susceptibility data are not sensitive enough to definitively identify small variations in substrate specificity and (ii) the copy number of the plasmid vector can play a role in the overall level of gene expression which may influence the phenotype observed.

*E. coli* AmpD shares 88% identity and about 83 to 86% identity with the AmpD proteins of *C. freundii* and *Enterobacter cloacae*, respectively (19). It is interesting, however, that compared to *E. coli*, both *C. freundii* and *E. cloacae* carry an additional four amino acids which represent an SXXL motif at the carboxy end of the protein (19). Previous studies have demonstrated the importance of specific mutations in the carboxy terminus for both function of AmpD and overproduction of AmpC (11, 12, 19, 39). In particular, Kopp et al. have shown the importance of the last 16 amino acids of AmpD from *Enterobacter cloacae* for *ampC* overproduction (19). The current study identified the last 10 amino acids of *C. freundii* AmpD as an essential region for AmpD function as assayed for in the model by elevated ceftazidime MICs as an indication of the overproduction of AmpC (Fig. 1; Table 2). Insertion of PCR-generated *ampD* genes with truncations of the last 10, 21, or 25 codons of the carboxy terminus into the model system indicated a derepressed phenotype (Etest, >256  $\mu$ g/ml). The aspartic acid residue at position 164 and two histidines located at positions 34 and 154 are necessary for zinc-binding and AmpD activity in *C. freundii* (11). However, this study showed that deletion of the last 10 amino acids (leaving Asp164, His34, and His154 intact) results in a MIC of >256  $\mu$ g/ml, indicating their importance for enzymatic activity. The removal of these last 10 amino acids included the removal of the SXXL motif (19). NMR spectroscopy supports these data and suggests that

the last 10 amino acids and the zinc-binding pocket reside in close proximity (11, 24).

CF21M is a phenotypically derepressed mutant selected from the wild-type inducible AmpC parent strain, CF21 (14). The *ampD* sequences for CF21 and CF21M both indicated an amino acid substitution of Arg175Ser. The *ampD* model confirmed that the amino acid substitution observed in the *C. freundii ampD* sequences was not responsible for the derepressed phenotype observed for CF21M. Amino acid substitutions in AmpR have been reported to increase AmpC production (1, 20). Recently an *ampR* mutation resulting in AmpC overproduction was identified in a clinical isolate of *Enterobacter cloacae* (18). However, in the present study no nucleotide sequence variation was observed in *ampR*, *ampC*, or the *ampC-ampR* intergenic region of CF21M. Surprisingly, expression analyses revealed a concomitant 11-fold decrease in *ampD* expression with a 403-fold increase in *ampC* expression in CF21M compared to CF21. The difference between decreased *ampD* expression and amino acid substitutions negating AmpD activity cannot be identified phenotypically. Both mechanisms would result in decreased cleavage of 1,6-anhydromuropeptide and a shift in the cytoplasmic pool concentrations between this muropeptide and UDP-MurNAc-pentapeptide, resulting in overproduction of AmpC. To our knowledge, this is the first report of decreased *ampD* expression coinciding with *ampC* overproduction. The mechanism involved in the decreased *ampD* expression is under investigation.

The *ampD* model showed decreased ceftazidime MICs when *ampD* test genes from PAO1 and Ps164 were evaluated using JRG582 (changes in ceftazidime MICs from >256  $\mu$ g/ml to 24 and 32  $\mu$ g/ml, respectively [Table 2]). These data indicated that the *P. aeruginosa ampD* genes were expressed in *E. coli*. Taken together, the decrease in ceftazidime MICs and sequence data indicating a lack of amino acid substitutions compared to PAO1 suggested that these genes did complement the AmpD mutant background in JRG582. It is possible that the elevated ceftazidime MIC observed for Ps164 was due to less than optimal expression of the *P. aeruginosa ampD* promoter in *E. coli*. Alternatively, *P. aeruginosa* AmpD may not cleave muropeptides within *E. coli* as efficiently as muropeptides produced in *P. aeruginosa* since there is only 65% identity between the two AmpD proteins (21).

The *ampD* gene of *P. aeruginosa* Ps164 M1 contained a stop codon at position 155 and did not complement the *ampD* mutant phenotype of JRG582 (the ceftazidime MICs remained >256  $\mu$ g/ml). However, phenotypically *P. aeruginosa* Ps164 M1 was partially, not fully, derepressed as the sequence and model data would suggest (D. J. Wolter, N. D. Hanson, and P. D. Lister, Abstr. 44th Intersci. Conf. Antimicrob. Agents Chemother., abstr. C1-955, 2004). Taken together, these data indicated that the AmpD of the *P. aeruginosa* strain Ps164 M1 was compensated for by another mechanism within Ps164 M1. The identification of this alternative mechanism is under investigation.

It is clear that AmpD plays a role in the overproduction of AmpC in some clinical isolates (1, 12, 19, 22, 25, 39). But data presented in this study and sequence data presented by others suggest that mechanisms other than mutations within the structural *ampD* gene can be involved in AmpC overproduction (1,

6, 22). The model system presented in this study will be a valuable tool in elucidating these other mechanisms in different genera by eliminating or substantiating the role of amino acid substitutions within AmpD as a mechanism involved in AmpC overproduction leading to a derepressed phenotype.

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