Increased Expression of *ampC* in *Pseudomonas aeruginosa* Mutants Selected with Ciprofloxacin[∇]

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Two *Pseudomonas aeruginosa* mutants exhibiting increased expression of *ampC* were selected during exposure to ciprofloxacin. These mutants also exhibited significant increases in *mexCD-oprJ* expression, but further studies failed to show a link between the increased expression of *mexCD-oprJ* and *ampC*. Increased *ampC* expression was not related to mutations within *ampR*, the *ampC-ampR* intergenic region, *ampD*, *ampDh2*, or *ampDh3* or to changes in the levels of expression of these amidase genes. However, *ampD* complementation restored wild-type levels of *ampC* expression and ceftazidime susceptibility, suggesting alternative mechanisms of *ampC* regulation.

The chromosomal cephalosporinase, AmpC, of “wild-type” *Pseudomonas aeruginosa* is produced at a low basal level and can be induced to significantly higher levels in the presence of certain β -lactams (6, 12, 17). Expression of *ampC* is partially controlled by a regulatory factor, AmpR, which represses transcription in wild-type cells. Induction of *ampC* also requires the presence of two other proteins, the AmpG permease and AmpD amidase, and the induction process is intimately linked to the cell wall recycling pathway and increased levels of 1,6-anhydro-*N*-acetylmuramyltripeptides (5). In addition to that of the induction pathway, increased expression of *ampC* can occur through processes of partial or full derepression. Partially derepressed mutants express moderately increased basal levels of *ampC* and retain some degree of inducibility, whereas fully derepressed mutants express high basal levels of *ampC* and lose the induction phenotype. Derepression of *ampC* expression has been associated with mutations within *ampD* (1, 9) and *ampR* (1) and is typically selected for after exposure to β -lactam antibiotics.

In a previous study, ciprofloxacin was used to select two *mexCD-oprJ*-overexpressing efflux mutants, 164M1-94C and 164M1-84C, from *P. aeruginosa* 164M1 that was partially derepressed for *ampC* expression (22). Partially derepressed 164M1 had previously been selected from a clinical isolate, *P. aeruginosa* 164, that was wild type for *ampC* expression. Mutants 164M1-94C and 164M1-84C exhibited significantly higher levels of basal *ampC* expression and AmpC hydrolytic activity than their parental strain, 164M1, and retained their inducibility. These mutants were of interest because they were selected from *P. aeruginosa* 164M1 following exposure to ciprofloxacin rather than a β -lactam. To our knowledge, this is the first report of ciprofloxacin exposure producing mutants with increased expression of *ampC*.

The ciprofloxacin-selected increase in *ampC* expression observed in mutants 164M1-84C and 164M1-94C does not appear to be strictly associated with the overexpression of *mexCD-oprJ*. Two *mexCD-oprJ*-overexpressing mutants, 164-921C and 164-922C, selected from the original clinical isolate, *P. aeruginosa* 164, failed to show a basal increase in *ampC* expression or AmpC hydrolysis activity (Table 1) (22). Similarly, two characterized *mexCD-oprJ*-overexpressing mutants, 922CF and 921OF, selected from a different parent strain, *P. aeruginosa* PAO1, did not exhibit any increase in AmpC activity (Table 1), as determined by a spectrophotometric assay (10).

Although increased *ampC* expression and AmpC hydrolytic activity do not appear to be strictly associated with overexpression of *mexCD-oprJ*, fluoroquinolone exposure is associated with induction of the SOS repair system in bacterial cells and increases in mutational rates (3, 14, 16, 24). Therefore, a fluoroquinolone-induced higher mutational rate may lead to random mutations within genes of the bacterial genome, including *cis*- and/or *trans*-acting factors responsible for regulating *ampC* expression. In an attempt to obtain other ciprofloxacin-selected mutants of *P. aeruginosa* with increases in AmpC hydrolytic activity, *P. aeruginosa* PAO1 was exposed to ciprofloxacin at 2, 4, and 8 $\times$ MIC using an agar-based methodology (20), and 10 ciprofloxacin-resistant mutants were analyzed for basal levels of AmpC hydrolytic activity. All 10 ciprofloxacin-selected mutants had levels of AmpC hydrolysis similar to that of their parental strain, PAO1 (Table 1). These data combined with those from the *mexCD-oprJ*-overexpressing mutants described above suggest that increases in *ampC* expression or AmpC hydrolytic activity after ciprofloxacin exposure are infrequent events.

Mutants 164M1-94C and 164M1-84C were further analyzed to determine whether the increased *ampC* transcription resulted from mutational changes within *ampR*, the *ampR-ampC* intergenic region, and/or *ampD*. A DNA template was prepared from the original clinical isolate (164), the partially derepressed parental strain 164M1, and the two ciprofloxacin-selected mutants 164M1-94C, and 164M1-84C, as previously described (13). PCR primers (Table 2) were designed to am-

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TABLE 1. AmpC-mediated hydrolysis

Strain	Phenotype ^a	Hydrolysis rate ^b
Ps 164	WT	0.93
164-921C	CDJ	0.76
164-922C	CDJ	1.20
PAO1	WT	1.11
PAO1-922CF	CDJ	0.76
PAO1-921OF	CDJ	1.24
PAO1-881C	CIP	1.07
PAO1-882C	CIP	1.45
PAO1-941C	CIP	0.99
PAO1-942C	CIP	1.00
PAO1-943C	CIP	1.09
PAO1-944C	CIP	1.52
PAO1-921C	CIP	1.09
PAO1-922C	CIP	1.22
PAO1-84C	CIP	1.17
PAO1-821C	CIP	0.81

^a WT, wild type for *ampC* and *mexCD-oprJ* expression; CDJ, RT-PCR-confirmed overexpression of *mexCD-oprJ*; CIP, ciprofloxacin-selected mutants.

^b Expressed as nanomoles of cephalothin hydrolyzed per minute per milligram of protein.

plify and sequence *ampR*, the *ampR-ampC* intergenic region, and *ampD*, including its putative promoter (8). PCR amplifications were conducted using conditions depicted in an earlier report (23), except that an annealing temperature of 55°C was implemented. Amplicons were sequenced at the Creighton University Molecular Biology Core Facility. No mutations were observed within either *ampR* or the *ampR-ampC* intergenic region. Comparative analysis of *ampD* sequences between strains 164 and 164M1 showed that the partially derepressed phenotype of strain 164M1 was associated with a base transition from C→T at nucleotide 639 (GenBank accession number AF082575), resulting in premature termination of translation (Gln₁₅₅→Stop). Therefore, the AmpD of strain 164M1 has a 34-amino-acid truncation at the carboxy-terminal end causing at least a partial loss of amidase function, as indicated in an *Escherichia coli* model system (18). This same base change was also observed in *ampD* of both mutants,

164M1-94C and 164M1-84C, but no other mutations were identified. Thus, the AmpDs of 164M1 and its ciprofloxacin-selected mutants were identical, suggesting that the further increased basal level of *ampC* expression observed in the mutants was not associated with functional changes in AmpD. These data are not totally unexpected since partial and full derepression of *ampC* has been observed in *P. aeruginosa* isolates that do not exhibit changes in *ampC*, *ampR*, and *ampD* or their promoter regions (1, 2).

Although the truncated AmpD of parent 164M1 appears to be inactive using the recently published *E. coli* model system (18), the caveats of this artificial system do not rule out the possibility that the mutated amidase retains partial activity within its natural *P. aeruginosa* environment. If the AmpDs of the parent 164M1 and mutants 164M1-94C and 164M1-84C retain partial amidase activity, then the increased expression of *ampC* in the ciprofloxacin-selected mutants may involve a decrease in *ampD* expression. This possibility is supported by a recent study demonstrating a link between decreased transcriptional expression of *ampD* and increased transcriptional expression of *ampC* in a mutant of *Citrobacter freundii* (18). Therefore, expression of *ampD* was analyzed in wild-type strain 164, partially derepressed 164M1, and its ciprofloxacin-selected isogenic mutants 164M1-94C and 164M1-84C by real-time reverse transcriptase PCR (RT-PCR) as previously described (22) using the primers listed in Table 2. Expression of the endogenous control gene, *rpsL*, was used to normalize data. Relative quantification was determined by the $2^{-\Delta\Delta CT}$ or delta-delta cycle threshold (C_T) method (11). Levels of expression of *ampD* were similar among all strains (Table 3), indicating that increased *ampC* expression in the ciprofloxacin-selected mutants is not linked to diminished *ampD* expression.

The next question that we addressed was whether the mechanism(s) responsible for increased *ampC* expression in the ciprofloxacin-selected mutants would be reversed by the presence of wild-type AmpD. We hypothesized that complementation with wild-type *ampD* would fully restore ceftazidime susceptibility and *ampC* expression in 164M1 to the level of wild-type strain 164. Although we anticipated that *ampD*

TABLE 2. Primers used in this study

Primer	Sequence (5'→3')	Purpose	Product size (bp)	GenBank accession no.
PAmpRF1	CCTTCATCACCGTTGTACG	PCR/sequencing	1,282	AE004827
PAmpRR1	CGCCTCAAACCGTATCAACC	PCR/sequencing		
PAmpRF2	CTGTGTGACTCCTTCGACC	Sequencing		
PAmpDF	GACGATGCCTTGCTGTTCCG	PCR/sequencing	987	AF082575
PAmpDR	GCAGCAATGTGTCAGCAACAGG	PCR/sequencing		
PAmpDh2F	GCTACTGCGCTGATCCTGC	PCR/sequencing	1,153	AE004091
PAmpDh2R	CGAGCCTTTCGTCCAGGTC	PCR/sequencing		
PAmpDh3F	GACCGCTGCGAAAGGCTCTG	PCR/sequencing	1,173	AE004091
PAmpDh3R	GTGCGACGGCATTCATGGC	PCR/sequencing		
PAERUGF	TTACTACAAGGTCGGCGACATGACC	RT-PCR	267	X54719
PAERUGR	GGCATTGGGATAGTTGCGGTTG	RT-PCR		
PAmpDRTF	GGCGTTCTTCCAGAATCGC	RT-PCR	196	AF082575
PAmpDRTR	CCAAGGGAGAAGTCGTTGC	RT-PCR		
PAmpDh2RTF	CATCGTCTCCACTACACCTC	RT-PCR	208	AE004091
PAmpDh2RTR	GATCTCGATGCCGATCGAG	RT-PCR		
PAmpDh3RTF2	CGAGCGCTCGCAGATCAAC	RT-PCR	189	AE004091
PAmpDh3RTR2	GTGGCGTCGTCATACCAGG	RT-PCR		
RpsLF1	GCAACTATCAACCAGCTGGTG	RT-PCR	230	AE004842
RpsLR1	GCTGTGCTCTTGCAGGTTGTG	RT-PCR		

TABLE 3. Ceftazidime susceptibility and transcriptional expression

Strain	Ceftazidime MIC ($\mu\text{g/ml}$)	Expression ^a			
		<i>ampC</i>	<i>ampD</i>	<i>ampDh2</i>	<i>ampDh3</i>
164	2	1.0	1.0	1.0	1.0
164M1	96	99	1.3	2.6	2.1
164M1-94C	48	1,165	1.1	2.6	3.1
164M1-84C	48	1,324	1.2	1.5	1.6
164M1 + p26PAD1 ^b	4	1.1	ND	ND	ND
164M1-94C + p26PAD1	2	0.91	ND	ND	ND
164M1-84C + p26PAD1	2	0.89	ND	ND	ND

^a Transcriptional expression of *ampC*, *ampD*, *ampDh2*, and *ampDh3* as measured by real-time RT-PCR. Values represent the difference (*n*-fold) in gene expression relative to wild-type strain 164. ND, not determined.

^b p26PAD1 represents plasmid pUCP26 containing *ampD* from strain 164.

complementation of mutants 164M1-94C and 164M1-84C would also reduce *ampC* expression and increase ceftazidime susceptibility, we expected the *ampD*-complemented mutants to retain ~9- to 13-fold-higher levels of *ampC* expression since the earlier sequence analysis demonstrated that increased *ampC* expression did not involve mutational changes within *ampD*. In order to test these hypotheses, the *ampD* gene from wild-type strain 164 was amplified by PCR using primers PAampDF1 and PAampDR1 (Table 2) and cloned into the pCR-XL-TOPO (Invitrogen, Carlsbad, CA) vector by using the protocol supplied by the manufacturer. The *ampD* insert was then subcloned into the shuttle vector pUCP26 (21) using the restriction enzymes XbaI and HindIII (Invitrogen). The new plasmid containing wild-type *ampD*, designated p26PAD1, was transformed into competent *E. coli* Top10 cells (Invitrogen). *P. aeruginosa* strains 164M1, 164M1-94C, and 164M1-84C were transformed with p26PAD1 by electroporation according to the methods of Smith and Iglewski (19). The effect of wild-type *ampD* complementation on susceptibility to ceftazidime was evaluated by the Etest according to manufacturer instructions (AB Biodisk North American, Inc., Piscataway, NJ), and the effect on the transcriptional expression of *ampC* was evaluated by real-time RT-PCR using the primers listed in Table 2.

The partially derepressed parent, 164M1, exhibited 99-fold-higher levels of *ampC* transcription than wild-type strain 164 (Table 3). In agreement with previous results (22), expression of *ampC* in the ciprofloxacin-selected mutants 164M1-94C and 164M1-84C was 11- to 13-fold higher than levels in their parental strain 164M1 and ~1,100- to 1,300-fold-higher than the levels in wild-type strain 164. Despite an 11- to 13-fold-higher expression level of *ampC*, ceftazidime MICs were 2-fold lower for 164M1-84C and 164M1-94C than for 164M1 (Table 3). Although this twofold decrease in ceftazidime MICs is not considered significant for susceptibility assays, it is surprising that the increased expression of *ampC* in 164M1-84C and 164M1-94C was not associated with a decrease in ceftazidime susceptibility. One possible explanation is that ceftazidime is a substrate of the constitutively produced MexAB-OprM efflux pump (15), which is downregulated when overexpression of *mexCD-oprJ* occurs (4). Therefore, the slight increase in susceptibility of 164M1-84C and 164M1-94C to ceftazidime may represent an interplay or balance between the decreased efflux of ceftazidime by MexAB-OprM and higher levels of AmpC cephalosporinase.

As hypothesized, *ampD* complementation of strain 164M1 returned both ceftazidime susceptibility and *ampC* expression back to wild-type levels (Table 3). Interestingly, *ampD* complementation of mutants 164M1-94C and 164M1-84C rendered the same effect, with ceftazidime susceptibility and *ampC* expression both returning to wild-type levels (Table 3). Therefore, the unknown mechanism responsible for the increased expression of *ampC* selected for by ciprofloxacin is masked by the presence of a fully functional “wild-type” AmpD.

The data presented in the present study suggest the involvement of additional genes and pathways in the regulation of the *ampC* β -lactamase in *P. aeruginosa*. Homologues of AmpD, namely, AmpDh2 and AmpDh3, have recently been described and were shown to be involved in a stepwise upregulation of *ampC* expression (7). Loss of function or expression of either of these amidases could yield a larger cytoplasmic pool of the AmpR cofactor, 1,6-anhydro-*N*-acetylmuramyltripeptides, resulting in increased *ampC* expression (5). As a result, the *ampD* homologues in the ciprofloxacin-selected mutants were analyzed for changes in sequence and expression compared to the parental strain 164M1 and the wild-type strain 164. *ampDh2* and *ampDh3* were amplified by PCR and sequenced from each strain using gene-flanking primers (Table 2). Expression of both amidase homologues was examined by real-time RT-PCR using the primers listed in Table 2. The *ampDh2* and *ampDh3* sequences were identical among all four strains and expression studies showed similar levels of steady-state transcript (Table 3). Therefore, increased *ampC* expression in 164M1-84C and 164M1-94C was not due to alterations in *ampDh2* and *ampDh3*.

In summary, the two mutants described here offer a perplexing situation wherein exposure to a fluoroquinolone was associated with increased *ampC* expression. Since neither AmpD, AmpDh2, nor AmpDh3 was involved, other components of mureptide recycling or an unidentified pathway in *ampC* regulation that can be masked by a functional AmpD protein may be responsible for the increased expression of *ampC* in these mutants.

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