ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, June 1997, p. 1380–1384 Vol. 41, No. 6
0066-4804/97/$04.00+0

Pseudomonas aeruginosa Isolates from Patients with Cystic Fibrosis Have Different β-Lactamase Expression Phenotypes but Are Homogeneous in the *ampC-ampR* Genetic Region

JOAN I. A. CAMPBELL,[1]* OANA CIOFU,[1] AND NIELS HØIBY[1,2]

Institute of Medical Microbiology & Immunology, Panum Institute, University of Copenhagen, 2200 Copenhagen N,[1] and Department of Clinical Microbiology & Danish CF Centre, Department of Pediatrics, Rigshospitalet, Copenhagen,[2] Denmark

Received 16 July 1996/Returned for modification 23 October 1996/Accepted 20 March 1997

Pseudomonas aeruginosa **isolates from 1 of 17 cystic fibrosis patients produced secondary β-lactamase in addition to the *ampC* β-lactamase. Isolates were grouped into three β-lactamase expression phenotypes: (i) β-lactam sensitive, low basal levels and inducible β-lactamase production; (ii) β-lactam resistant, moderate basal levels and hyperinducible β-lactamase production; (iii) β-lactam resistant, high basal levels and constitutive β-lactamase production. Apart from a base substitution in the *ampR-ampC* intergenic region of an isolate with moderate-basal-level and hyperinducible β-lactamase production, sensitive and resistant strains were identical in their *ampC-ampR* genetic regions. Thus, enhanced β-lactamase expression is due to mutations in regulatory proteins other than AmpR.**

The major resistance mechanism in chronic *Pseudomonas aeruginosa* infections in patients with cystic fibrosis (CF) is the production of high levels of β-lactamase. Based on its biochemical properties, the enzyme involved has been implicated to be a group 1 cephalosporinase (classification of Bush et al. [2]) encoded by the *ampC* gene commonly found among members of the family *Enterobacteriaceae* (6). Although the *ampC* gene and its linked regulatory *ampR* gene from *P. aeruginosa* (outlined in Fig. 1) have been cloned and sequenced (11, 12) (refer to GenBank accession numbers X54719 and X67095, respectively), the genetic events underlying the overproduction of the enzyme in the resistant clinical isolates have not been elucidated. Genetic events which can lead to the enhancement of β-lactamase levels include the following: (i) acquisition of a secondary β-lactamase encoded by a plasmid, bacteriophage, or transposon (1, 5), (ii) amplification of the structural gene encoding the chromosomal β-lactamase (13), and (iii) mutations in the regulatory regions and/or regulatory genes which control the expression of the β-lactamase gene (8, 13). In this study, we investigated clinical *P. aeruginosa* isolates from CF patients with chronic infections for evidence of plasmid-mediated β-lactam resistance. In addition, we compared *ampC-ampR*-encoding regions in strains originating from the same patients obtained pretherapy, midtherapy, and posttherapy for possible gene amplifications in *ampC* and for mutations in the *ampC-ampR* promoter regions.

Bacterial strains and plasmids. The *P. aeruginosa* strains were isolated from sputum collected from 17 CF patients regularly attending the Danish CF Center, Rigshospitalet, Copenhagen, Denmark. Sputum collection and subsequent bacterial isolation was during a 2-week course of intensive antipseudomonal therapy in 1989 (6). A total of 400 isolates were screened for the presence of extrachromosomal DNA. Of these, 21 strains isolated on days 1, 7, and 15, representing isolates from nine patients obtained pretherapy, midtherapy, and posttherapy, respectively, were used in the *ampC-ampR* investigations. The reference bacterial strains and plasmids are listed in Table 1.

MIC determinations and measurement of β-lactamase activity. The MICs of different β-lactam antibiotics for the *P. aeruginosa* strains were determined by the agar dilution method or by using E-test strips (Pharmavit A/s, Birkerød, Denmark). Sonicated extracts of the *P. aeruginosa* strains were prepared from cells grown uninduced and induced for β-lactamase production by benzylpenicillin and were assayed for β-lactamase activity spectrophotometrically (6). The β-lactamase preparations were focused in Ampholine PAG plates (pH 3.5 to 9.5; LKB Instruments), and visualization of β-lactamase bands was achieved with a nitrocefin gel overlay (18). When the inhibitors were applied, filter paper soaked in cloxacillin or clavulanate was applied to the focused gel surface for 20 s, before the nitrocefin gel was poured on. β-Lactamase preparations from *P. aeruginosa* 1920E (Rms 149), which has the *ampC* chromosomal β-lactamase and the Rms 149-encoded carbenicillinase PSE-3 (7), were used as reference samples.

DNA methods. All the *P. aeruginosa* test strains were screened for plasmid prevalence by the in-well lysis method (Eckhardt gels), using the horizontal gel modification (16). In order to avoid the masking of some plasmid DNA by chromosomal DNA, the samples were electrophoresed in agarose gels of two different concentrations: 0.6 and 1%. The plasmids borne by the clinical isolates were typed for their incompatibility groups by colony hybridization to the IncN-, IncP-, IncQ-, and IncW-specific probes (4). Transferability of plasmids in the strains with secondary β-lactamases was investigated in triparental mating experiments, in which nalidixic acid- and rifampin-resistant *Pseudomonas fluorescens* R2f was used as the recipient (19). The mobilizable plasmid was an IncQ plasmid (pSKTG) carrying *aadB*, *nptII*, and truncated *cryIVB* genes (19). *P. aeruginosa* strains carrying the *tra*- and *mob*-negative Rms 149 and the *tra*-positive RP4 plasmids served as negative and positive controls, respectively, for the conjugation experiments.

Total DNA preparations, restriction enzyme analyses, blotting of DNA onto nylon membranes, purification of DNA from gels, and DNA ligation were carried out by standard procedures (17). Plasmid DNA preparations from overnight cultures

* Corresponding author. Mailing address: Institute of Medical Microbiology & Immunology, Panum Institute, Bygn. 24.1, University of Copenhagen, Blegdamsvej 3, 2200 Copenhagen N, Denmark. Phone: 45 35 32 78 90. Fax: 45 35 45 64 12.

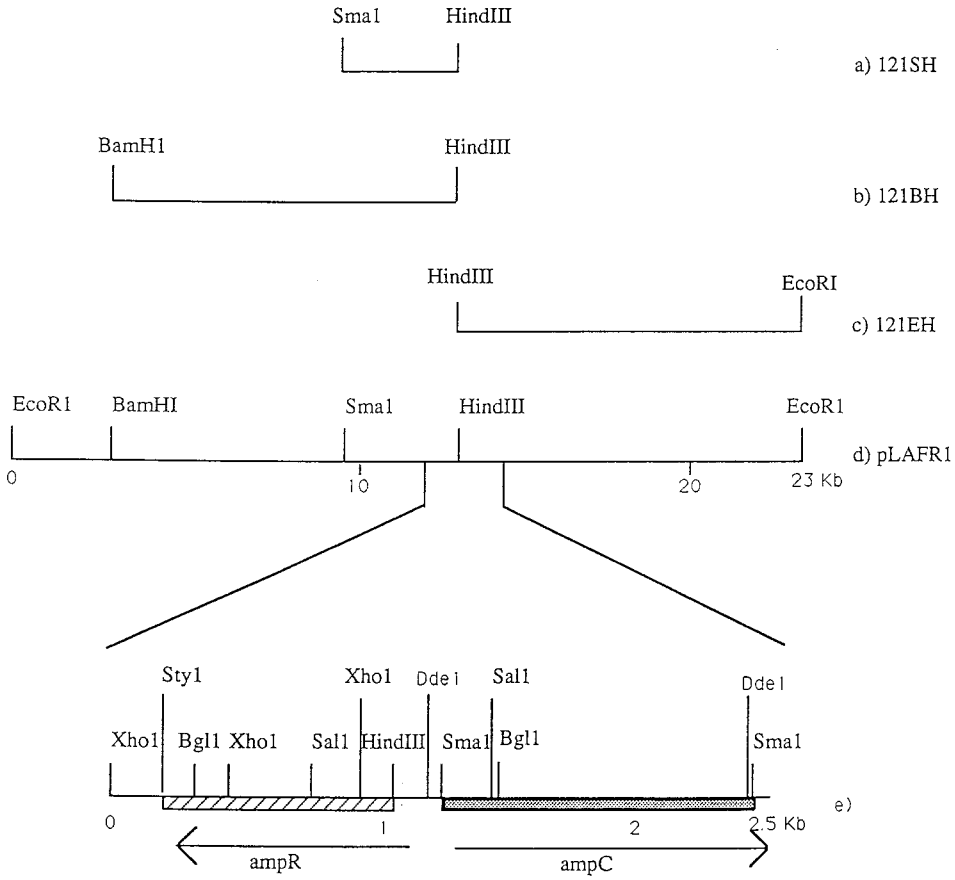

FIG. 1. Restriction enzyme mapping around the *P. aeruginosa ampC* and *ampR* genes in the recombinant plasmid and cosmid clones. The DNA fragments represented in panels a to d correspond to *P. aeruginosa* DNA cloned in plasmids 121SH, 121BH, and 121EH and the cosmid clone pLAFR1 (12), respectively. The arrows in the DNA fragment in panel e represent the *ampR*- and *ampC*-coding regions. The hatched box delineates the *ampR*-specific probe, while the grey box outlines the *ampC*-specific probe.

were made by using Qiagen plasmid preparation kits (Stuers Kebo Laboratories, Copenhagen, Denmark) according to the manufacturer's instructions. A digoxigenin-labelled DNA probe was prepared and used as recommended by the manufacturer (Boehringer Mannheim GmbH).

Secondary plasmid-encoded β-lactamase in the clinical *P. aeruginosa* strains. Fifteen isolates (from 3 patients) of the 400 clinical isolates (from 17 patients) were found to carry extrachromosomal DNA. In line with the enhanced β-lactamase production, most of the plasmid-containing strains were resistant to the β-lactam antibiotics tested (Table 2). When cleared sonicates from the plasmid-containing strains were subjected to isoelectric focusing gels, minor β-lactamase bands focusing at pIs of 7.4 and 7.7, in addition to the major chromosomal β-lactamase band (pI 8.8), were observed in all the isolates hyperproducing β-lactamase (see below). The minor β-lactamase bands in the crude preparations from strains 533, 535, and 569 (patient 2) were completely inhibited by clavulanate but were only partially inhibited by cloxacillin (i.e., the change of the nitrocefin yellow color to pink occurs after 10 to 15 min, as opposed to 3 to 5 min in the absence of the inhibitor). The Rms 149-encoded PSE-3 was also observed to be completely inhibited by clavulanate but only partially inhibited by cloxacillin, suggesting that three of the clinical isolates (from patient 2) might have secondary plasmid-encoded β-lactamase. Transformation of plasmid preparations from the patient 2 strains into competent *Escherichia coli* JM109 gave colonies on Luria broth

TABLE 1. Bacterial strains and plasmids used as controls or standards

Bacterial strain	Plasmids	Phenotype or genotype[a]	Reference or source
P. aeruginosa 1920E	Rms149	Cb^r, Gm^r, Sm^r, Su^r	7
P. aeruginosa PAO1		Wild type	Ackermann[b]
P. aeruginosa ATCC 27853		Wild type Rif^r, Nx^r	19
P. fluorescens R2fN	121BH	Amp^r, nearly all of *ampR*	Busby[c]
	121EH	Amp^r, *ampC*	Busby
	121SH	Amp^r, nearly all of *ampR*	This study
	pULB 2420	repP, Km^r	4
	pULB 2424	repQ, Amp^r, Tc^r	4
	pULB 2426	repW, Tc^r	4
E. coli HB101	pULB 2432	repN, Amp^r, Tc^r	4

[a] Cb^r, Gm^r, Sm^r, Su^r, Rif^r, Nx^r, Amp^r, Km^r, and Tc^r, antibiotic resistance to carbenicillin, gentamicin, spectinomycin, sulfonamide, rifampin, nalidixic acid, ampicillin, kanamycin, and tetracycline, respectively; repP, repQ, repW, and repN replicons specific for plasmid incompatibility groups P, Q, W, and N, respectively.

[b] H.-W. Ackermann, Department of Microbiology, Faculty of Medicine, Université Laval, Laval, Quebec, Canada.

[c] S. J. W. Busby, University of Birmingham, Birmingham, United Kingdom.

TABLE 2. Properties of the *P. aeruginosa* isolates carrying extrachromosomal DNA

Source	Strain no.	β-Lactamase activity (mU)[a]		MIC (μg/ml)			
		Basal	Induced	Aztreonam	Ceftazidime	Piperacillin	Imipenem
Patient 1	247	200	1,290	25	25	50	12.5
	249	180	1,000	200	25	100	6.2
	250	25	730	25	25	25	25
	252	6	580	ND[b]	ND	ND	ND
Patient 2	504	8,870	5,560	100	50	200	3.1
	509	7,800	5,930	100	50	100	3.1
	512	8,800	19,700	ND	ND	ND	ND
	533[c]	420	1,150	50	50	>400	25
	535[c]	40	1,720	>400	400	>400	12.5
	569[c]	5,900	4,200	>400	400	>400	12.5
	614	180	3,060	400	400	>400	12.5
Patient 3	640	110	1,540	200	100	200	12.5
	656	80	5,600	400	400	>400	25
	657	50	770	>400	400	>400	25
	668	260	7,420	>400	400	>400	12.5

[a] One milliunit of β-lactamase is defined as 1 nmol of nitrocefin hydrolyzed per min per mg of protein.
[b] ND, not determined.
[c] These strains contain minor β-lactamase bands focusing at pIs 7.4 and 7.7 which are inhibited by clavulanate.

agar containing 100 μg of ampicillin per ml. A 12-kb plasmid which hybridizes to plasmid DNA of similar size in the *P. aeruginosa* strains from patient 2 was readily recovered from the successful transformants, confirming that the strains from patient 2 had plasmid-encoded β-lactam resistance. Colony blots of the plasmid-containing strains hybridized only to the IncP-specific probe, indicating that the plasmids borne by the strains belong to the IncP incompatibility group of plasmids, which are common among *P. aeruginosa* strains and can be transferable (4). However, in triparental matings in which the *P. aeruginosa* plasmids should provide the transfer function, acquisition of the marked mobilizable plasmid by the *P. fluorescens* recipient was not observed. The lack of the transfer function might explain the low distribution of secondary plasmid-encoded β-lactamases among these *P. aeruginosa* strains.

β-Lactamase phenotype of the selected strains. On the basis of the β-lactamase activity detected with and without penicillin induction, the three reference strains and the *P. aeruginosa* strains from nine different patients could be placed in three phenotypes of β-lactamase expression (Table 3). These are described as (i) low basal levels and moderately inducible β-lactamase production, (ii) moderate basal levels and hyperinducible β-lactamase production, and (iii) high basal levels and constitutive β-lactamase production. The strains with moderate basal levels and hyperinducible β-lactamase production constituted the largest group (16 of 21 strains). Although strain 245 hyperproduces β-lactamase on induction, the low basal enzyme levels might explain its marked antibiotic sensitivity because all the strains with enhanced basal levels of β-lactamase production were resistant to aztreonam, ceftazidime, and piperacillin. However, several of them remained susceptible to imipenem. This may well be due to the fact that for imipenem resistance, other mechanisms such as loss of D2 porin (3) or multidrug efflux mechanisms (10) are more important.

TABLE 3. β-Lactamase expression phenotype and β-lactam MICs for the selected test and reference strains

Phenotype	Source[a]	Isolate	β-Lactamase activity (mU)[b]		MIC (μg/ml)			
			Basal	Induced	Aztreonam	Ceftazidime	Piperacillin	Imipenem
Low basal level, inducible		PAO1	20	850	3.1	0.8	3.1	6.2
		ATCC 57823	15	380	6.2	0.8	3.1	3.1
		1920E	30	240	3.1	1.6	50	3.1
	Patient 4A	176-8	10	250	1.6	1.6	1.6	0.4
	Patient 5A	245	40	3,300	1.6	0.8	6.2	0.8
Moderate basal level, inducible	Patient 4A	237	240	3,420	16[c]	25[c]	25[c]	0.4[c]
	Patient 4B	90	430	6,290	>400	400	400	3.1
	Patient 4C	203	730	6,680	>400	>400	>400	3.1
	Patient 5B	151	180	4,130	ND	ND	ND	ND
	Patient 6A	230-10	250	4,010	25	25	400	25
	Patient 4B	94	590	4,650	>400	400	>400	25
	Patient 7A	235	1,330	8,470	>400	400	200	1.6
	Patient 4B	93	510	4,650	>400	>400	>400	12.5
	Patient 8A	106	120	8,470	>400	100	400	6.2
	Patient 4B	155	170	8,590	200	100	400	6.2
	Patient 9C	248	180	5,870	25	25	50	3.1
	Patient 10A	174	150	5,220	400	200	400	25
	Patient 4B	220	190	4,340	>400	400	400	25
	Patient 11A	238	160	5,540	25	25	50	6.2
	Patient 11B	146	280	6,870	400	200	400	6.2
	Patient 12A	344	180	6,610	25	50	200	12.5
High basal level, constitutive	Patient 4B	176-9	5,260	5,350	>400	200	400	1.6
	Patient 9B	258	8,430	9,150	25	25	50	12.5
	Patient 12B	397	5,580	8,670	>400	>400	>400	25

[a] A indicates that the strain was isolated on day 1 of a 2-week course of antipseudomonas therapy. B indicates that the strain was isolated on day 15. C indicates that the strain was isolated on day 7.
[b] See footnote *a* of Table 2 for definition of 1 mU.
[c] MICs were determined by the use of E-test strips (Pharmavit A/s).

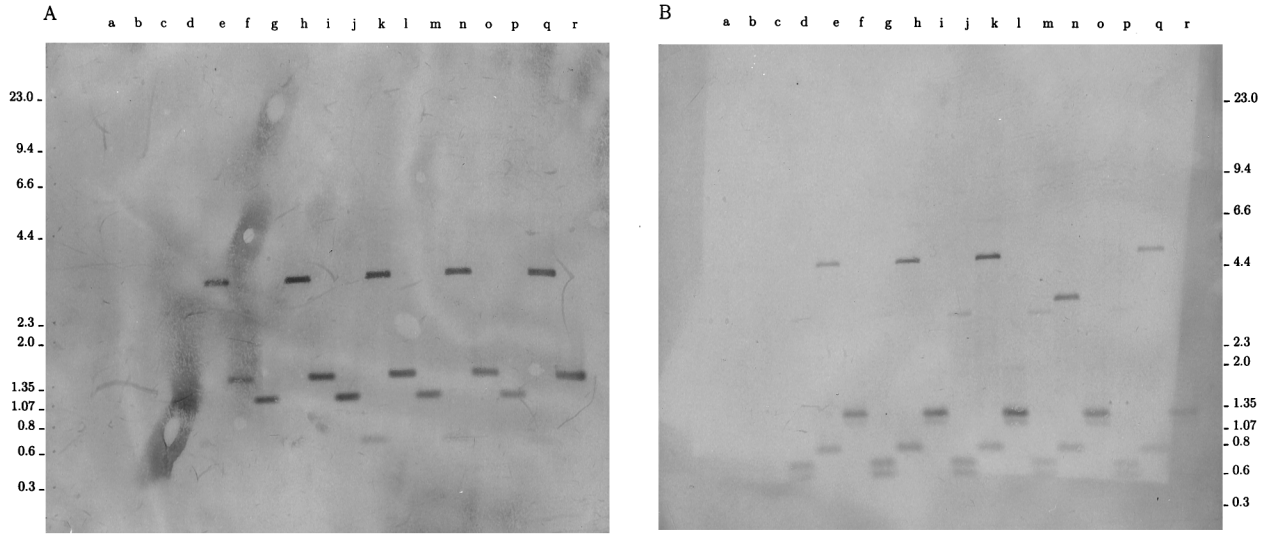

FIG. 2. Comparison of the restriction fragment length polymorphisms around the *ampC*- and *ampR*-coding regions in reference and clinical strains of *P. aeruginosa*. (A) DNA fragments from reference strains *P. fluorescens* R2f; *P. aeruginosa* PAO1, ATCC 27853, and 1920E; and clinical strains *P. aeruginosa* PA 248 (moderate basal level, hyperinducible) and PA 258 (very high basal level, constitutive) were hybridized to the *ampC*-specific probe. Lanes a, b, and c, *P. fluorescens* DNA digested with *Sma*I, *Sal*I, and *Dde*I, respectively; lanes d, e, and f, PA 258 DNA digested with the same respective enzymes; lanes g, h, and i, PA 248 DNA digested with the same respective enzymes; lanes j, k, and l, 1920E DNA digested with the same respective enzymes; lanes m, n, and o, DNA from reference strain ATCC 27853 digested with the same respective enzymes; lanes p, q, and r, DNA from strain PAO1 digested with the same respective enzymes. The migration of standard marker DNA fragments (in kilobases) is indicated on the left. (B) DNA fragments from reference strains *P. fluorescens* R2f; *P. aeruginosa* PAO1, ATCC 27853, and 1920E; and clinical strains *P. aeruginosa* PA 248 and PA 258 were hybridized to the *ampR*-specific probe. Lanes a, b, and c, *P. fluorescens* DNA digested with *Xho*I, *Sal*I, and *Bgl*II, respectively; lanes d, e, and f, PA 258 DNA digested with the same respective enzymes; lanes g, h, and i, PA 248 DNA digested with the same respective enzymes; lanes j, k, and l, 1920E DNA digested with the same respective enzymes; lanes m, n, and o, DNA from clinical strain ATCC 27853 digested with the same respective enzymes; lanes p, q, and r, DNA from strain PAO1 digested with the same respective enzymes. The migration of the standard marker DNA fragments (in kilobases) is indicated on the right.

Most of the selected clinical isolates produced major β-lactamase bands focusing at pI 8.8, while three strains (strains 90, 203, and 237, from patient 4) had a major band with a pI of 9. Similar to the observations reported by Sanders et al. (18), additional bands at pIs 7.4, 7.7, 8.3, and 8.6 were also observed in all the cleared sonicates from strains in which β-lactamase production was induced as well as in strains with high basal levels of constitutive β-lactamase hyperproduction. However, all the minor β-lactamase bands in these strains were inhibited by cloxacillin but were not affected by clavulanic acid, suggesting that the enzyme with lower pI values in the majority of the isolates is similar to that in the major β-lactamase band with the more basic pI values (8.8 or 9). The β-lactamase bands focusing at the lower pI values may be poorly cleaved derivatives of the major enzyme arising from the extremely high amounts present upon hyperproduction.

Comparison of the *ampC*- and *ampR*-coding regions in the different β-lactamase expression phenotypes. The restriction sites for several enzymes which cut within the regions coding for *ampC* (*Dde*I, *Sal*I, and *Sma*I) and *ampR* (*Bgl*I, *Sal*I, and *Xho*I) as well as in the flanking DNA sequences are indicated in Fig. 1e. The *ampC*-specific DNA probe was generated by PCR amplification of the *Sma*I fragment in the recombinant plasmid 121EH by using flanking primers: CGGTTTCTCAT GCAG and GGGACGAACGCCACG. The PCR conditions were 35 cycles of 94°C for 1 min, 55°C for 2 min, and 72°C for 3 min. The *ampR*-specific probe was derived from a double *Sty*I and *Hin*dIII digestion of plasmid 121SH, generated by ligation of a 6-kb *Sma*I fragment purified from 121BH plasmid DNA.

The *ampC*-specific probe hybridized to the expected 1.36-kb *Dde*I fragment, the 664-bp *Sal*I fragment (straddling the *ampR-ampC* promoter regions), and the 1.11-kb *Sma*I fragment expected from the published *P. aeruginosa ampC* gene sequence in all the strains (Fig. 2A). In addition, a 3-kb *Sal*I fragment comprised of the main part of the *ampC* gene sequences and flanking DNA sequences up to the next rightward *Sal*I site was conserved in all the clinical and reference *P. aeruginosa* strains tested.

In accordance with the restriction enzyme mapping around the *ampR* nucleotide sequence, the *ampR*-specific probe gave strong signals to a 1.05-kb *Bgl*I fragment, a 664-bp *Sal*I fragment (same as the fragment described above [Fig. 2A]), and the 448- and 543-bp *Xho*I fragments (Fig. 2B). Furthermore, the *ampR*-specific probe hybridized weakly to a 950-bp *Bgl*I fragment (containing 100 bp of the DNA between the far left *Bgl*I site within *ampR* and the *Sty*I site), a 2.7-kb *Xho*I fragment (containing 89 bp between the far right *Xho*I fragment and the *Hin*dIII site), and a 5-kb *Sal*I fragment (consisting of the main portion of the *ampR* gene and flanking sequences up to the next leftward *Sal*I site). The only exception was reference strain ATCC 27853, in which the large *Sal*I fragment was shortened to 3.5 kb. Because the observed difference is at a site 2 kb away from the left of the end of the *ampR* gene, we conclude that the *ampR* architecture is also highly conserved in both β-lactam-sensitive and -resistant strains.

The 664-bp *Sal*I fragment is interesting because it contains the *ampR* and *ampC* promoter regions (11). Hence, this *Sal*I fragment from two sensitive strains (*P. aeruginosa* ATCC 27853 and PAO), two clinical strains with moderate basal levels and hyperinducible β-lactamase production phenotype (strains 90 and 248), and two isolates with high basal levels and a constitutive β-lactamase phenotype (strains 258 and 397) were amplified and the nucleotide sequences were determined. DNA amplification was achieved by using the following flanking primers: 5′-GGCGATGTCGACGCGGTTGTTGTGGGT GGACAGGCGCAGATCGATGGAGG-3′ and 5′-GAATGT

CATTGGCCTTCATCACCGGTTGTACGGCGGCGTCGACCAGTGCC-3′. Amplification was done by using 35 cycles of 94°C for 1 min, 55°C for 2.5 min, and 72°C for 3 min. The PCR fragments from clinical strains 90 and 248 were cloned into pUC18 plasmid vector DNA, and the DNA sequence of the inserts was determined in the AP1 Prism 377 automated sequencing system. Direct sequencing of the purified PCR products was carried out for the fragments from *P. aeruginosa* ATCC 27853, PAO1, 258, and 397. The DNA sequence of the *ampR-ampC* intergenic regions from the sensitive strains and all of the resistant clinical strains were identical and differed from the published PAO1 sequence only in the substitution of a G for a C at position −99 (relative to the *ampC* start codon). The only exception was strain 248, which also had a T-to-C substitution at position −96.

Our study suggests that plasmid-mediated β-lactamase may contribute to resistance in only *P. aeruginosa* strains isolated from 1 of 17 CF patients with chronic lung infections. However, IncP plasmids can transfer chromosomal DNA, which then becomes integrated into the chromosome (15). In addition, free bacteriophages have been demonstrated in sputum from patients with CF (14). Hence, there is ample opportunity for the acquisition of extra *ampC* gene copies by conjugation and transduction during the chronic infection. Nevertheless, we found the plasmids to be nonconjugative, and the high degree of conservation around the *ampC* gene from both sensitive and resistant strains suggests that *ampC* sequences have not been amplified.

Mutations in the control regions of the noninducible *ampC* gene of *E. coli* which enhanced the chromosomal β-lactamase expression are known (8). Hence, we investigated the possibility of the existence of changes in nucleotide sequences in the *ampR-ampC* intergenic regions from sensitive and resistant strains. Interestingly, the unusual leucine start codon was maintained in all the strains and the nucleotide sequence in *P. aeruginosa* ATCC 27853, PAO1, and clinical strains 90, 258, and 397 are identical and differ from the published PAO1 sequence in only one base inversion, from a C to a G. This base difference is unlikely to be important for enhanced β-lactamase production because it was found in both the sensitive and the resistant strains. In addition, the substitution should not alter the DNA-DNA hydrogen bonding at the site (c.f. the C-to-T mutation in *E. coli* which produced an up-*ampC* promoter [8]). In moderate-basal-level, hyperinducible strain 248, a T-to-C substitution was also observed. Because strain 248 has the same phenotype as strain 90, which has a promoter sequence indistinguishable from those of the other strains, this T-to-C base change may also be unimportant.

The enhanced expression of *ampC* in *Enterobacter* and *Citrobacter* strains with both hyperinducible and high-level constitutive β-lactamase production has been shown to be due to mutations in another regulatory gene, *ampD* (9), which is not linked to the *ampR* and *ampC* genes. Indeed, a putative *ampD* gene from *P. aeruginosa* PAO1 has been reported (9a). However, the *ampC-ampR* promoter region in *P. aeruginosa* shares no significant sequence similarities with that in enterobacteria, apart from an inverted 38-bp fragment around the *ampR* start codon (12). Experiments involving transposon mutagenesis are in progress to locate and characterize the genes that are expressed in *P. aeruginosa* in response to the presence of β-lactam antibiotics.

We are grateful to S. Busby for kindly providing the recombinant *P. aeruginosa* plasmid clones, Ali Karami for help with DNA sequence analysis, and Pernelle Mathiesen, Lisbeth Andersen, and Jens Saunte for technical assistance.

Clavulanic acid was a gift from Smith Kline & Beecham (Welwyn, Herts, United Kingdom). J.I.A.C. and O.C. were partly supported by a grant from the Danish Health Research Council.

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