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The *Pseudomonas cepacia* 249 Chromosomal Penicillinase Is a Member of the AmpC Family of Chromosomal β-Lactamases

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***Pseudomonas cepacia* 249 produces an inducible β-lactamase with penicillinase activity. The nucleotide sequence of the *penA* gene, which encodes this β-lactamase, was determined and found to include regions with a significant homology to the *ampC*-encoded β-lactamases of members of the family *Enterobacteriaceae* and *Pseudomonas aeruginosa*. The predicted amino acid sequence of the PenA β-lactamase contained 17 amino acids immediately preceding the putative active-site serine which were highly conserved among the enzymes of the AmpC family. Although the *penA*-coding sequence had a total GC content of 60%, the predicted codon usage was more characteristic of *Escherichia coli ampC*-encoded β-lactamase, with 53% of the codons having G or C in the third position, in contrast to the values for the *P. aeruginosa ampC* (88.5%) or *Pseudomonas cepacia* (88 to 92%) metabolic genes. The inducible expression of *penA* can be regulated by the *E. coli* gene product AmpD. A putative *P. cepacia* AmpR homolog was associated with the positive regulation of both *Enterobacter cloacae ampC* and *P. cepacia penA* expression, as confirmed by gel retardation studies. The *E. cloacae* AmpR did not regulate *penA* expression. Thus, by homology studies, codon usage, and genetic analysis, the *P. cepacia penA* β-lactamase appears to have been acquired from members of the family *Enterobacteriaceae* and belongs to the class C group of β-lactamases.**

Chromosomal β-lactamases are commonly expressed by gram-negative enterobacteria (35, 40). Several species share a similar *ampC* β-lactamase gene which may be expressed constitutively as in *Escherichia coli* or which may be inducible by β-lactam compounds as in *Citrobacter freundii*, *Enterobacter cloacae*, or *Pseudomonas aeruginosa* (24, 34). Common regulatory elements involved in *ampC* expression have been identified in *E. coli*, *C. freundii*, *E. cloacae*, and *P. aeruginosa* (24, 28). These include AmpR, a transcriptional activator (2, 27); AmpD, a negative regulator (20, 22, 43); AmpE, which is postulated to act in signal transduction (15, 26); and *ampG*, a locus involved in activation of *ampC* (19).

Pseudomonas cepacia is a species noted for its exceptional metabolic diversity. It produces an inducible β-lactamase associated with the novel ability to utilize penicillin as a carbon source (3). The induction of *P. cepacia* β-lactamase expression occurs frequently in the clinical setting and is associated with difficulties in the treatment of infections caused by these organisms (8, 13). Biochemical analysis of the penicillinase produced by prototypic strain *P. cepacia* 249 demonstrated a substrate profile and induction kinetics clearly distinct from those of the *ampC*-encoded chromosomal β-lactamases characteristic of members of the family *Enterobacteriaceae* and *P. aeruginosa*, which have cephalosporinase activities (33). Further analysis of the *P. cepacia penA* enzyme as described in this report revealed that the *penA* gene is, nonetheless, very closely related to the *ampC* gene by genetic evidence.

MATERIALS AND METHODS

Bacterial strains and plasmids. The *E. coli* strains used in the present studies were SNO3 (*ampA1 ampC8 pyrB recA rpsL*) (31) and SNO302, an *ampD2* derivative of SNO3 (25) obtained from S. Normark (Washington University, St. Louis, Mo.). Strain JM107 was used for the propagation of the phage M13 (46). *P. cepacia* 249 and the cosmids pTGL70 and pTGL73 containing the *penA* gene cloned from a genomic library of 249 DNA on the vector pLAFR (9) were obtained from T. Lessie (University of Massachusetts, Amherst). The construction of pASP71, which contains a 1.1-kb *Eco*RI-*Hin*dIII fragment from pTGL70, including the *penA* β-lactamase cloned on the vector pMK16 (41), has been described previously in detail (33). The plasmids pNU362, which contains the *E. cloacae* P99 *ampR* gene cloned on pACYC184 (7), and pNU363, which contains the *E. cloacae* P99 *ampC* gene cloned on pNU78 (24), were obtained from S. Normark.

Media and growth conditions. The organisms were grown in LB medium (29). The antibiotics used for selection in *E. coli* were as follows, in micrograms per milliliter: chloramphenicol, 50; kanamycin, 50; and tetracycline, 30. The antibiotics were purchased from Sigma Chemical Co., St. Louis, Mo.

Cloning and sequencing techniques. Recombinant DNA procedures were carried out by standard methods (29). Restriction endonucleases and T4 DNA ligase were purchased from commercial suppliers and were used as recommended by the suppliers. The 1.1-kb DNA fragment containing *penA* was isolated from *Eco*RI digests of pASP71 from low-melting-point agarose gels, cloned into the phage vectors M13mp18 and M13mp19, and used to transfect *E. coli* JM107 (46). Overlapping DNA fragments were cloned, taking advantage of naturally occurring restriction enzyme sites. Single-stranded DNA was prepared (36), and both strands were sequenced by the dideoxynucleotide chain termination method by using a commercially obtained kit (Sequenase II; United States Biochemical, Cleveland, Ohio). dITP was used to prevent GC compression. Analysis

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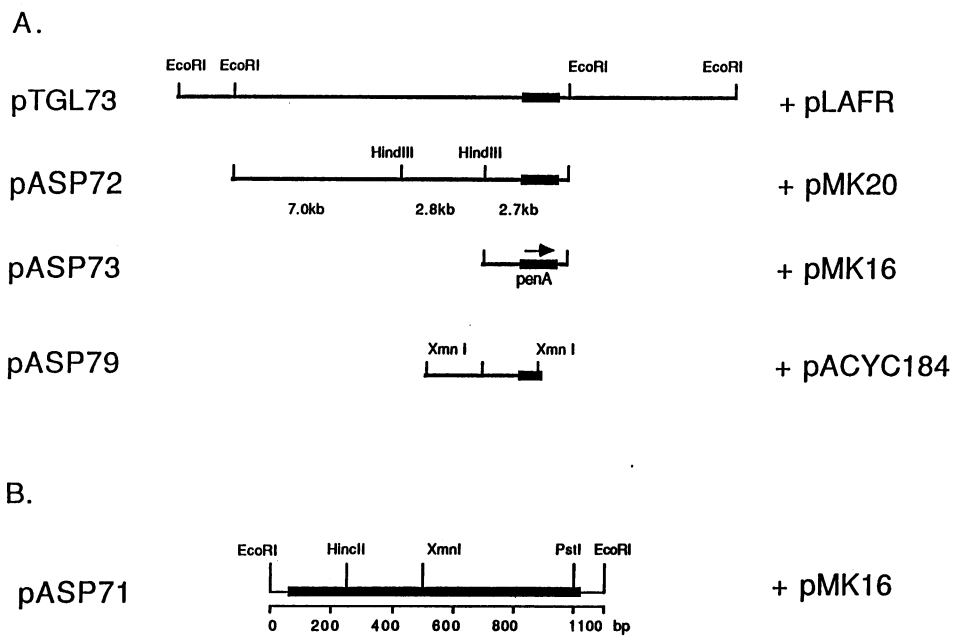

FIG. 1. (A) Schematic maps of the subclones of the cosmid pTGL73. The location of the *penA* gene is marked by the dark solid bar. The subclones of pTGL73, the plasmid vectors, and relevant restriction endonuclease sites are shown. (B) Map of the restriction endonuclease sites used for the sequencing of *penA* from pASP71.

of the DNA sequence was done with the GCG sequence analysis software package (version 6.0) of the University of Wisconsin on a Vax 6310 computer. Homology searches were performed by using the deduced protein sequence against known β-lactamases in the GenBank (release 64.0), EMBL (24.0), Swiss-Prot (release 14), and NBRF-Protein (release 25.0) data bases.

DNA-DNA hybridizations. DNA-DNA hybridizations were performed by the method described by Southern (39) by using high-stringency conditions (10% dextran sulfate, 1.0 M NaCl, 1% sodium dodecyl sulfate [SDS], 100 μg of denatured heterologous DNA per ml) at 65°C for 18 h. High-stringency washes were done with 0.1× SSPE (15 mM NaCl, 1 mM $NaPO_4$, 0.1 mM EDTA [pH 7.4]) and 1% SDS. A *P. cepacia ampR* DNA probe was isolated by eluting the 3.17-kb *Xmn*I DNA fragment from pASP72 from agarose gels. The probe was prepared by the random primer method (29) by using a commercially obtained kit (Boehringer Mannheim, Indianapolis, Ind.), and the probe was labeled with [α-^{32}P]dCTP (New England Nuclear, Boston, Mass.) to a high specific activity. Labeled probe was added to the hybridization mixture at an activity of 10^7 cpm/ml.

Determination of the transcription direction of *penA*. The direction of *penA* transcription was determined by primer extension analysis to map the position of the 5′ end of the RNA transcript (1). The 0.5-kb *Eco*RI-*Xmn*I fragment of pASP71 postulated to include the *penA* promoter sequence was cloned into M13mp18 and M13mp19 in opposite orientations so that they could be used as templates. End-labeled complementary DNAs starting from the primer site were generated with 10 pmol of 5′-end-labeled M13 primer [γ-^{32}P]dATP at 6,000 Ci/mmol (New England Nuclear). The probe DNA was cleaved with appropriate restriction endonucleases and was isolated from a 6% acrylamide gel. The probes (5×10^4 cpm of each probe) were denatured and added to 50 μg of total RNA isolated from JM107/pASP71, JM107, and JM107/M13mp18 in 20 μl of hybridization solution (1 M NaCl, 0.167 M HEPES [*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; pH 7.5], 0.333 mM EDTA [pH 8.0]). This mixture was heated to 65°C for 10 min, and the probes and RNA were allowed to hybridize for 12 h at 30°C. S1 nuclease (300 U) (Boehringer Mannheim) was added, and the mixture was incubated for 30 min at 30°C. The precipitated DNA-RNA hybrids were then electrophoresed on a denaturing acrylamide gel next to ^{32}P-end-labeled molecular weight markers.

β-Lactamase assays. Crude cell extracts containing β-lactamase activity were prepared by isolating the periplasmic contents of 40-ml cultures of bacteria (45) as described previously (33). Penicillinase activity was measured by the starch iodide technique (32) by using a Gilford Response model continuously recording spectrophotometer, and the temperature was controlled at 35°C over 5 to 10 min (6). Reaction rates were shown to be linear for the dilutions of enzyme used. Results are expressed as specific activities representing nanomoles of penicillin hydrolyzed per minute per milligram of protein. Protein content was measured by the method of Bradford (5).

Induction studies were done by adding imipenem (0.06 μg/ml) to the strains grown to the mid-log phase in LB and incubating for another 3 h prior to the preparation of cell extracts, having found imipenem to be a better inducer of *penA* than penicillin, oxacillin, or cefoxitin.

Construction of plasmids containing *penA* and *ampR*. The cosmid pTGL73 (23 kb of *P. cepacia* 249 DNA cloned onto pLAFR [9]), which contained *penA* as well as approximately 10 kb of upstream DNA, was chosen for further study (Fig. 1). A 12.5-kb *Eco*RI fragment from pTGL73 was cloned into the *Eco*RI site of pMK20 (41). This plasmid construction, pASP72, was used to transform the *E. coli* strains SNO3 and SNO302. To isolate a plasmid encoding *penA* alone (pASP73), a 2.8-kb *Hin*dIII-*Eco*RI fragment from pASP72 which contained the ColE1 replicon from pMK20 was eluted from a low-melting-point agarose gel and ligated to a Tc^r

marker from pMK16 (41). The region directly upstream of the *penA*-coding sequence was cloned by isolating a 3.17-kb *Xmn*I DNA fragment from pASP72 and ligating the blunt ends into a *Hin*cII site of pACYC184 to create pASP79. Plasmid DNA was isolated from all of the SNO3 and SNO302 strain constructions and was cleaved with appropriate restriction enzymes to verify that there were no unexpected gene rearrangements or insertions. The effects of these cloned regions of pTGL73, both on *penA* expression and on *ampC* as cloned in pNU363 (24), were determined as described above.

Gel retardation studies. A 240-bp *Eco*RI-*Hin*cII DNA fragment containing the putative *penA* promoter region was isolated from pASP71. This DNA fragment was end-labeled with 50 μCi of [α-^{32}P]dATP (3,000 Ci/mmol) and 2 U of the Klenow fragment of DNA polymerase incubated at 37°C for 1 h. The 240-bp DNA fragment was purified from the reaction mixture by electrophoresis on a 4% acrylamide gel in 1.0× TBE (29) and was extracted from the acrylamide matrix by incubation in 200 μl of 0.3 M sodium acetate at 37°C for 18 h; this was followed by ethanol precipitation. A 200-bp *Apa*I-*Sph*I DNA fragment from pNU363 was isolated and similarly labeled.

Protein extracts were prepared from 250-ml cultures of bacteria grown for 18 h in LB medium under appropriate antibiotic selection conditions. Imipenem (0.06 μg/ml) was added, and the cultures were incubated for another 2 h. The cells were harvested by centrifugation, washed once in 150 mM NaCl, and resuspended in 2.0 ml of 25 mM HEPES [pH 7.5]–5 mM $MgCl_2$–0.1 mM EDTA–5 mM 2-mercaptoethanol–10% glycerol. The cells were sonicated with a Branson sonifier at 40% power, and the cellular debris was removed by centrifugation at 30,600 × *g* for 30 min. The protein concentration was determined by the method of Bradford (5).

The gel mobility shift assay was performed by using the conditions described for AmpR by Lindquist et al. (27), with the following modifications. The labeled DNA (3,000 cpm) was incubated with 1.0 μg of each protein extract in 10 μl of binding assay buffer (25 mM HEPES [pH 7.5], 0.1 mM EDTA, 5 mM dithiothreitol, 10% glycerol, 50 mM KCl) and 1.0 μg of poly(dI-dC) at 25°C for 15 min and was resolved on a 4% nondenaturing polyacrylamide gel in 0.5× TBE (pH 8.5). Nonspecific or specific competitor DNA was added at a concentration of 9 fmol. The gels were fixed in 40% glacial acetic acid and 10% methanol, dried, and exposed to Kodak XAR film with intensifying screens.

Nucleotide sequence accession number. The nucleotide and amino acid sequence data reported here have been assigned the GenBank accession number L02928.

RESULTS

Sequence analysis of the *P. cepacia penA* β-lactamase gene. The analysis of the nucleotide sequence of *penA* revealed a single open reading frame consistent with a 313-amino-acid polypeptide with an expected molecular weight of approximately 33,000, as established previously for the PenA β-lactamase (33) (Fig. 2). This reading frame was preceded by a region with properties consistent with a Shine-Dalgarno sequence, −27 to +1 (30, 38), and was in agreement with the primer extension studies which demonstrated transcription from the plus strand as cloned in M13mp18 (data not shown).

The deduced PenA β-lactamase amino acid sequence contained features expected of a β-lactamase; a short hydrophobic leader sequence (residues 1 to 15) and the S-X-X-K

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   GTATTCGACACATGCCGTGACTCTGCCTGCACGCGCATCTCTATGCACAGATTCGACTAT
 1 ---------+---------+---------+---------+---------+---------+
   GCGCTGTGCAGATCGACGGTACGGCCGTGATTCTGGCGAAGATCTGGTTGAAAGCGTAAT
61 ---------+---------+---------+---------+---------+---------+

                                                          Met - 1
   GCAGCGTATCGGCGTGACCGATTACACCATTCTCGGCACGGTAAAAGGTGCGGAGCTTGA
121 ---------+---------+---------+---------+---------+---------+
     GlnArgIleGlyValThrAspTyrThrIleLeuGlyThrValLysGlyAlaGluLeuGlu - 21
   GCTCGTGCGCTTTACCCATCCGTTTATGGGCTTCGACGTTCCGGCAATCCTCGGCGATCA
181 ---------+---------+---------+---------+---------+---------+
     LeuValArgPheThrHisProPheMetGlyPheAspValProAlaIleLeuGlyAspHis - 41
   CGTTACCCGGATGCCGGTACCGGTGCCGTTCACACCGCGCCTGCCACGGCCCGGACGACT
241 ---------+---------+---------+---------+---------+---------+
     ValThrArgMetProValProValProPheThrProArgLeuProArgProGlyArgLeu - 61
   ATGTGATCGGTCAGAAATACGGCCTGGCAAACCGCTAACCCGGTTGGCCCGGACGGCACT
301 ---------+---------+---------+---------+---------+---------+
     CysAspArgSerGluIleArgProGlyLysProLeuThrArgLeuAlaArgThrAlaLeu - 81
   TATCTGCCGGGCACTTATCCGACGTTGGATGGCGAGAACGTCTTATAGTGACTCTGTCAA
361 ---------+---------+---------+---------+---------+---------+
     IleCysArgAlaLeuIleArgArgTrpMetAlaArgThrSerTyrSerAspSerValAsn - 101
   CTGCCACACTCAACCTATCTCGGCTATCTTTGATTATAAGGATTTGCGATTCGAACCACC
421 ---------+---------+---------+---------+---------+---------+
     CysHisThrGlnProIleSerAlaIlePheAspTyrLysAspLeuArgPheGluProPro - 121
   ATCAAACAGGATTTCGCCTGCTGGGCAAACCAGCGTGGACCGCTTGCTGCAACTCTCTCA
481 ---------+---------+---------+---------+---------+---------+
     SerAsnArgIleSerProAlaGlyGlnThrSerValAspArgLeuLeuGlnLeuSerGln - 141
   GGGCCAGGCGGGTCGAAGGGCAATCAGCTGTTGCCCGCTCTCACTGGTGAAAAGAAAAACCA
541 ---------+---------+---------+---------+---------+---------+
     GlyGlnAlaValGluGlyGlnSerAlaValAlaArgLeuThrGlyGluLysLysAsnHis - 161
   CCCTGGCGCCCAATACGCAAACCGCCTCTCCCCGCGCGCATCGGCAACAACCACCCAGCCAC
601 ---------+---------+---------+---------+---------+---------+
     ProGlyAlaGlnTyrAlaAsnArgLeuSerProArgIleAlaAsnAsnHisProAlaThr - 181
   CCAGCAGACCCTGTTCGAGCTGGGGAGTGGCGCGAAAGAGCGCAACGCAATTAATGTGAG
661 ---------+---------+---------+---------+---------+---------+
                          *
     GlnGlnThrLeuPheGluLeuGlySerGlyAlaLysGluArgAsnAlaIleAsnValSer - 201
   TTACCTCACTGCATTAGGCACCCCAGGCTTTACACTTATGCTTCCGGCTCGTATGTTGTG
721 ---------+---------+---------+---------+---------+---------+
     TyrLeuThrAlaLeuGlyThrProGlyPheThrLeuMetLeuProAlaArgMetLeuCys - 221
   TGGAATTGTGAGCGATAACAATTTCACACAGAAACAGCTATGCCCATCACCAGCGAGGTG
781 ---------+---------+---------+---------+---------+---------+
     GlyIleValSerAspAsnAsnPheThrGlnLysGlnLeuCysProSerProAlaArgCys - 241
   CACTCGTGGCCCAGCGGAGCCTGCAAAACGTGGCCCCTGGTTGGAACCTGGGTTGGTGAT
841 ---------+---------+---------+---------+---------+---------+
     ThrArgGlyProAlaGluProAlaLysArgGlyProTrpLeuGluProGlyLeuValIle - 261
   CAGGAAAGACGGGTTACGCACCGGCAAGCTGTTATCTTCTTTAAGGGGACTTTGTCTGAC
901 ---------+---------+---------+---------+---------+---------+
     ArgLysAspGlyLeuArgThrGlyLysLeuLeuSerSerLeuArgGlyLeuCysLeuThr - 281
   AGTTCTGCGATTTCAGCCAACAGTTCCTTGTTTCTGCCGATTAGCGCTGTCATCCAGCGT
961 ---------+---------+---------+---------+---------+---------+
     ValLeuArgPheGlnProThrValProCysPheCysArgLeuAlaLeuSerSerSerVal - 301
   GGCAATTAGCTCAACAGGCTTGGTCAATTTCTCAAGGTAAGCCTTGAGTTGAGTTTTCAT
1021 ---------+---------+---------+---------+---------+---------+
     AlaIleSerSerThrGlyLeuValAsnPheSerArgEnd                  - 313
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FIG. 2. Nucleotide sequence of the *P. cepacia* 249 chromosomal β-lactamase structural gene *penA* and the deduced amino acid sequence. Numbering of nucleotides is shown on the left, and numbering of the amino acids is shown on the right. Translation starts at position 119 (ATG) and continues until a stop codon at position 1058 (TAA). A bold line indicates a region of sequence similarity to the known active sites of class C β-lactamases. The putative PenA β-lactamase active-site serine is indicated by an asterisk.

		% Similiarity
PenA	PRIANNHPATQQTLFELGSGAKE	
C.freundii	ADIANNHPVTQQTLFELGSVSKT	77
E.coli	ADIAKKQPVTQQTLFELGSVSKT	69
E.cloacae	ADIAANKPVTPQTLFELGSISKT	65
P.aeruginosa	ASKQBGRRVTPGTLFQIGSVSKT	64

FIG. 3. Comparison of the deduced amino acid sequences of AmpC enzymes from different species and from the *P. cepacia* PenA β-lactamase. The amino acid sequences, represented by single-letter code, immediately preceding the active-site serine are shown.

sequence typical of β-lactam-binding proteins was present, with a serine at residue 190 followed by glycine, alanine, and lysine (17). Comparison of the deduced PenA sequence with other β-lactamases in the GenBank data base revealed striking similarities around this serine with the AmpC (class C) enzymes (Fig. 3). The 17 amino acids immediately upstream of the serine at position 190 were highly similar to those of the active-site regions of the AmpC β-lactamases of *E. cloacae* P99 (11) (65%), *C. freundii* OS60 (23) (77%), and *E. coli* K-12 (16) (69%). The *P. aeruginosa* PAO1 H103 AmpC enzyme (28) had 64% similarity to this region of the PenA β-lactamase.

When compared with the other class C β-lactamases according to the conserved regions described by Joris et al. (17), the first 115 amino acids of the PenA β-lactamase could readily be aligned into the appropriate boxes I to IV. However, the carboxy-terminal portion appeared to have rearrangements. The region which contained the active-site serine (box VII) was present in the PenA β-lactamase sequence at residue 190, which is much closer to the amino terminus than are the β-lactamases with cephalosporinase activity in which this serine is residue 300 (17). Accordingly, the other expected conserved residues (boxes V and VI) actually appeared to be closer to the carboxy terminus. This rearrangement was reflected in the comparison of the *penA* and *ampC* DNA sequences. There were substantial regions of DNA identity in the *P. cepacia* and *E. cloacae* genes, although their locations and orientations within the sequence were not the same (Fig. 4). The DNA sequences corresponding to the active-site regions were found to be highly conserved between the *penA* and the *ampC* enzymes.

The overall GC content of *penA* was 60%, whereas the GC content of reported *P. cepacia* metabolic genes is 67% (18, 47). The predicted codon usage showed that only 53% of the *penA* codons had either G or C in the third or "wobble" position (Table 1), whereas the value was 89 to 92% for the *P. cepacia* metabolic genes. The fraction of *penA* codons predicted to have G or C in the third position was very similar to that of *E. coli* K-12 *ampC* (51%) but was less characteristic of that of *E. cloacae* P99 *ampC* (66.8%) or *P. aeruginosa* H103 *ampC* (88.5%) (Table 1).

Identification of *P. cepacia ampR*. The *penA*-coding sequence was mapped on pTGL73 by Southern hybridization by using pASP71 as a probe, and the properties of the β-lactamase expressed by SNO3 (pTGL73) were demonstrated to be identical to those previously established for PenA (data not shown). AmpR is a transcriptional activator of *ampC* (27). Because *ampR* is immediately upstream of the *ampC*-coding sequence in many members of the family *Enterobacteriaceae* and *P. aeruginosa* (27, 28), the corresponding region was cloned from pASP72 and was tested for AmpR activity (Table 2). This 3.17-kb *Xmn*I DNA fragment (pASP79) had homology to the corresponding *ampR* gene cloned from *E. cloacae* (Fig. 5). As predicted, the gene products encoded by this DNA fragment functioned to positively regulate both the *P. cepacia penA* gene and the *E. cloacae ampC* gene (Table 2). PenA β-lactamase activity was increased three- to fourfold in the presence of the putative *P. cepacia* AmpR. AmpC β-lactamase production was increased threefold in strains containing the plasmid encoding the putative *P. cepacia ampR* homolog. AmpR was necessary but not sufficient for inducibility of *penA* by imipenem, because the sequences present in pASP72 but not in the other plasmid constructions were associated with inducible PenA β-lactamase activity. The *E. cloacae* AmpR did not regulate *penA*.

Gel retardation studies were performed to verify that the putative AmpR homolog encoded by the *P. cepacia* DNA cloned on pASP79 is a DNA-binding protein and to determine whether similar targets are recognized in heterologous species. Extracts from SNO3(pASP79) bound to the 240-bp *penA Eco*RI-*Hin*cII DNA fragment encompassing the DNA immediately upstream of the *penA*-coding sequence, includ-

						% Identity
	penA	483	**CAAA**CAGGA**TTTC**GCCTGCTGGGC	506		71
E.coli	*ampC*	10	**CAAA**TGGGT**TTTC**TACGGCTGGTC	33		
	penA	1027	**AATTGCCACGCTGGATGACAGCGCTA**	1002		69
E.coli	*ampC*	46	**AGTTGTCACGCTGATTGGTATCGTTA**	71		
	penA	638	**ATCGCCAACAACCACCCAGCCACCCAGCAGACCCTGTTCGAGCTGGG**	684		72
E.cloacae	*ampC*	186	**ATCGCGGCGAATAAACCCGTTACGCCTCAGACCCTGTTCGAGCTGGG**	232		
	penA	876	**GGGCCACGTTTTGCAGGCTCCGCTGG**	851		69
E.cloacae	*ampC*	668	**GGTGCGCGTTTCGCCGGGTATGCTGG**	693		
	penA	1131	**GCTGCATGATGATGATGATGTTAAGAGCAGATAAACATGC**	1092		79
E.cloacae	*ampC*	1229	**GCGGCAGGACGATGATGAGGA**.....**AGATGAACACGC**	1261		

FIG. 4. Comparison of the nucleotide sequences of the *P. cepacia* 249 *penA* and *ampC* β-lactamase genes. Five separate regions of the *penA*-coding sequence were found to contain sequences identical to regions within the *E. cloacae* or *E. coli ampC* genes.

TABLE 1. Codon usage in *P. cepacia penA* and *ampC* β-lactamases

Codon	Amino acid	No. of times used[a]				Codon	Amino acid	No. of times used				Codon	Amino acid	No. of times used				Codon	Amino acid	No. of times used			
		Pc	Ec	Ecl	Pa			Pc	Ec	Ecl	Pa			Pc	Ec	Ecl	Pa			Pc	Ec	Ecl	Pa
TTT	Phe	6	7	5		TCT	Ser	5	3	6		TAT	Tyr	2	10	8	7	TGT	Cys	4			
TTC	Phe	7	4	2	13	TCC	Ser	2	3	3	4	TAC	Tyr	3	5	8	11	TGC	Cys	5	2	3	2
TTA	Leu	5	7	1		TCA	Ser	7	2	3		TAA	Ter	1	1	1		TGA	Ter	1			
TTG	Leu	8	5	1	1	TCG	Ser	2	3	3	4	TAG	Ter					TGG	Trp	3	13	11	5
CTT	Leu	6	6	5		CTT	Pro	7	7	4		CAT	His	1	5	4	3	CGT	Arg	6	2	4	
CTC	Leu	7	4	7	11	CCC	Pro	3	7	5	7	CAC	His	4		1	3	CGC	Arg	7	7	5	18
CTA	Leu	3	2	2	1	CCA	Pro	8	6	4	1	CAA	Gln	5	14	2	3	CGA	Arg	6	1		
CTG	Leu	6	10	18	35	CCG	Pro	9	6	14	21	CAG	Gln	6	11	17	22	CGG	Arg	7		1	4
ATT	Ile	6	12	9	2	ACT	Thr	4	4	1		AAT	Asn	4	8	4	2	AGT	Ser	4	3	2	
ATC	Ile	7	9	7	11	ACC	Thr	8	8	9	17	AAC	Asn	6	8	12	7	AGC	Ser	4	6	4	11
ATA	Ile	1	2	1		ACA	Thr	6	9	3	1	AAA	Lys	7	15	12	2	AGA	Arg	1	1	2	1
ATG	Mel	6	7	14	6	ACG	Thr	3	6	10	2	AAG	Lys	3	7	8	13	ACG	Arg	6			3
GTT	Val	6	1	7		GCT	Ala	5	10	5	1	GAT	Asp	6	7	9	5	GGT	Gly	2	10	10	3
GTC	Val	3	5	8	5	GCC	Ala	4	9	17	31	GAC	Asp	6	7	4	19	GGC	Gly	16	11	17	30
GTA	Val	2	6	2	2	GCA	Ala	9	12	6	4	GAA	Glu	5	9	5	5	GGA	Gly	4	3	1	1
GTG	Val	8	10	14	11	GCG	Ala	7	9	20	14	GAG	Glu	4	6	11	10	GGG	Gly	3	5	4	2

[a] Codon usage is given for *P. cepacia penA* (Pc), *E. coli ampC* (Ec), *E. cloacae ampC* (Ecl), and *P. aeruginosa ampC* (Pa).

ing the putative promoter region. Similarly, protein extracts from SNO3(pNU362) shifted the mobility of the *penA* DNA fragment (Fig. 6, lanes C, D, and E). Note, however, that there was no increase in β-lactamase specific activity in strains expressing the *E. cloacae ampR* and *penA*. The expected *P. cepacia* AmpR homolog was able to recognize the appropriate *E. cloacae* target DNA, as evidenced by the mobility shift which occurred in the presence of either specific or nonspecific competitor DNA (Fig. 6, lanes I, J, and K). This interaction correlated with the observed three-

TABLE 2. Relative β-lactamase expression from cloned *penA* and *ampC* genes in *E. coli* SNO3 and SNO302

Plasmid(s)	Genotype		Imipenem induction	Sp act of SNO3[a]	MIC (μg/ml)	Imipenem induction	Sp act of SNO302 *ampD2*[a]	MIC (μg/ml)
	penA[b]	*ampR*[c]						
pTGL73	+	+ (PC)	−	7.1	32	−	9.8	32
			+	13.0		+	18.9	
pASP72	+	+ (PC)	−	9.5	32	−	37.7	256
			+	24.9		+	53.8	
pASP73	+	−	−	4.5	8	−	5.2	8
			+	5.2		+	2.8	
pASP73 + pASP79	+	+ (PC)	−	18.2	32	−	32.0	128
			+	14.7		+	31.9	
pASP73 + pNU362	+	+ (ECl)	−	4.4	8	−	3.3	8
			+	4.1		+	2.3	
	ampC[d]	*ampR*						
pNU363	+	−	−	55.2	64	−	79.7	128
			+	59.1		+	71.6	
pNU363 + pASP79	+	+ (PC)	−	172.7	256	−	156.4	512
			+	176.7		+	153.7	
pNU363 + pNU362	+	+ (ECl)	−	132.4		−	556.7	
			+	915.6	4096	+	1,832.9	8,192

[a] The specific activity of the β-lactamase is given as the nanomoles of penicillin hydrolyzed per minute per milligram of protein.
[b] The source of the β-lactamase was *P. cepacia* 249 *penA*.
[c] The source of *ampR* is indicated as *P. cepacia* (PC) or *E. cloacae* (ECl).
[d] The source of *ampC* was *E. cloacae*.

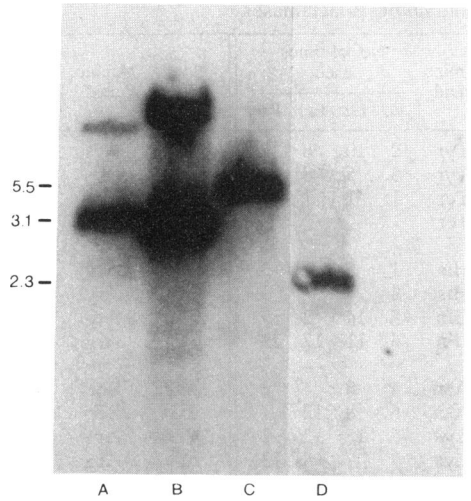

FIG. 5. Southern hybridization with the DNA fragment encoding the putative *ampR* from pASP72 as a probe. Lane A, pTGL73 (original cosmid construction) cleaved with *Xmn*I; lane B, pASP72 (*penA*, putative *ampR* region, and upstream DNA) cleaved with *Xmn*I; lane C, pASP79 (putative *ampR* region) cleaved with *Eco*RI and *Bam*HI; lane D, pNU362 (*E. cloacae ampR*) cleaved with *Cla*I and *Sal*I.

fold increase in the production of the AmpC β-lactamase in strains that also contained pASP79.

Regulation of *penA* by the *E. coli* gene *ampD*. AmpD functions as a negative regulator of *ampC* expression (15, 20, 24, 25) and has been postulated to act in mediating signal transduction (25, 43). The expression of *penA* in *ampD* mutants of *E. coli* was increased (Table 2). There was a 4-fold increase in PenA β-lactamase production in the *ampD2* mutant when *penA* and *ampR* were expressed in the larger plasmid construction (pASP72) and a 1.7-fold increase when the *penA* and *ampR* genes were cloned individually (pASP73 plus pASP79). Other components of the *ampC* regulon appear to be present in pASP72. Expression of the *E. cloacae ampC* gene was regulated as expected by *ampD* when the *E. cloacae* AmpR enzyme was provided; however, when a DNA fragment including the putative *ampR* gene from *P. cepacia* was present, there was no apparent regulation by *ampD*. Although plasmids expressing the putative *P. cepacia ampR* gene were associated with a threefold increase in the production of the *E. cloacae* AmpC enzyme, this effect was independent of *ampD*. The inducibility of *penA* expression was not well preserved in *E. coli* strains containing the cloned *E. cloacae* and *P. cepacia* genes. Inducibility was also lost when the DNA upstream of the putative *ampR* gene was deleted.

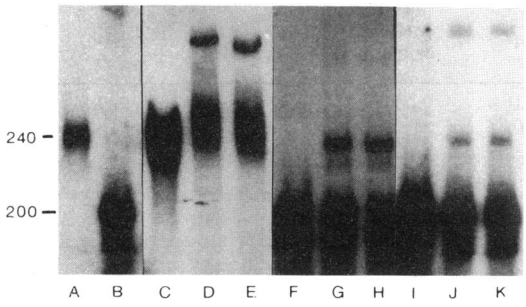

FIG. 6. Gel mobility shift assays demonstrating the interactions of the *P. cepacia* and *E. cloacae* AmpR to their own and heterologous target DNAs. The *P. cepacia* target DNA was a ^{32}P-labeled 240-bp *Eco*RI-*Hin*cII fragment from pASP71 expected to contain the promoter and early coding regions of *penA*; the *E. cloacae* target DNA was a 200-bp *Apa*I-*Sph*I fragment from pNU362 including the *ampR-ampC* intercistronic region. Lane A, control protein extract from SNO3(pACYC184) (plasmid vector) plus pASP71 fragment; lane B, control extract plus pNU362 fragment; lane C, pASP71 fragment alone; lane D, pASP71 fragment plus SNO3(pASP79) extract (*P. cepacia* AmpR); lane E, pASP71 fragment plus SNO3(pNU362) extract (*E. cloacae* AmpR); lane F, pNU362 DNA fragment alone; lane G, pNU362 DNA fragment plus SNO3(pNU362) extract in the presence of 9 fmol of unlabeled specific competitor DNA (200 bp of *Apa*I-*Sph*I fragment); lane H, same as lane G, but with 1 μg of poly(dI-dC) as nonspecific competitor DNA; lane I, pNU362 fragment alone; lane J, pNU362 fragment plus SNO3(pASP79) extract in the presence of specific competitor DNA; lane K, same as lane J, but with 1 μg of poly(dI-dC) as nonspecific competitor DNA.

DISCUSSION

The unusual catabolic capabilities of *P. cepacia* have enabled these organisms to thrive under conditions adverse to most other bacteria. Although originally described as plant pathogens, *P. cepacia* has become an opportunistic human pathogen associated with significant morbidity. In screening studies of clinical isolates and environmental strains of *P. cepacia*, *penA* was found to be highly conserved (3). The inducible production of β-lactamase and the associated ability to metabolize β-lactam compounds are likely to have contributed to the proliferation of *P. cepacia*, particularly in the presence of antimicrobial agents with high in vitro activities, as has been found in a clinical setting (12).

This analysis of the *P. cepacia* 249 penicillinase suggests that the enzyme is the product of a gene that is more closely related to the *ampC* β-lactamases of members of the family *Enterobacteriaceae* (4) than to genes characteristic of either *P. cepacia* or *P. aeruginosa*. Genetic studies have indicated that insertion elements that promote genomic rearrangements have facilitated the ability of *P. cepacia* to acquire and use foreign genes (10). Transposable elements from *P. cepacia* have also been shown to activate foreign or silent genes (37). Analysis of a repeated insertion element RS1100 in *P. cepacia* AC1100, which is capable of growth on 2,4,5-trichlorophenoxyacetic acid, suggested that both the insertion element and the metabolic gene were of foreign origin (14, 42). The PenA β-lactamase in *P. cepacia* appears to be another example of the facility of this species to acquire and maintain foreign genes. In the case of *penA*, not only the structural gene for β-lactamase may have been acquired from other unrelated species but, apparently, some regulatory genes may have been acquired as well.

Although by substrate profile and kinetic parameters the PenA β-lactamase had few of the characteristics of the class C chromosomal cephalosporinases typical of members of the family *Enterobacteriaceae* (33), by DNA homologies and genetic data, the *penA* structural gene belongs to this class of enzymes. Even assuming a common origin, it is not clear how the *P. cepacia* enzyme evolved into a β-lactamase with primarily penicillinase activity as opposed to the cephalosporinase activity expressed by the other AmpC enzymes. The conserved active-site sequences at both the DNA and the protein levels of *penA* and other *ampC* enzymes are striking. The codon usage, which lacks the GC predominance in the third position characteristic of pseudomonads

(44), is more typical of members of the family *Enterobacteriaceae*.

Supporting evidence to classify the *penA* gene as an *ampC*-type enzyme is provided by genetic data: the finding that *penA* expression is regulated by genes encoded by a DNA fragment with homology to *ampR* and that this regulation is *ampD* dependent, as would be predicted by analogous studies of the enterobacterial *ampC* enzymes. AmpR produced by either *E. cloacae* or *P. cepacia* was able to recognize target DNA sequences in *P. cepacia*, as indicated by gel mobility shift studies. However, this physical interaction was not sufficient in an *E. coli* host cell to allow the *E. cloacae* AmpR to activate *penA* expression. The putative *P. cepacia* AmpR was not only able to recognize the heterologous DNA-binding site in *E. cloacae* but was able to positively regulate *E. cloacae ampC* to levels comparable to those achieved by the *E. cloacae* AmpR. Not all of the components of this regulon are equally active in heterologous hosts. The *E. coli* AmpD did not appear to interact successfully when the transcriptional activator and the structural gene were from different species.

Although results of the present studies suggest that *P. cepacia* has acquired a chromosomal β-lactamase from members of the family *Enterobacteriaceae*, it is unclear how this species then evolved the metabolic capabilities to utilize β-lactam compounds as a carbon source (21). Undoubtedly, there are other genes which were not cloned in the present studies which facilitate the 1,000-fold induction of *penA* expression in the presence of penicillin G as a sole carbon source (3). Further analysis of the *P. cepacia* β-lactamase regulatory genes may help to explain whether an entire regulon was imported from a foreign source and whether *penA* expression is at all coordinately regulated with the as yet uncharacterized chromosomal cephalosporinase in *P. cepacia* (3).

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