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Characterization of the *penA* and *penR* Genes of *Burkholderia cepacia* 249 Which Encode the Chromosomal Class A Penicillinase and Its LysR-Type Transcriptional Regulator

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***Burkholderia cepacia* is recognized as an important pathogen in the lung infections of patients with cystic fibrosis. An inducible β-lactamase activity has been associated with increased resistance to β-lactam antibiotics in clinical isolates of *B. cepacia*. In this study, we report the revised sequence of the *penA* gene, which encodes the inducible penicillinase of *B. cepacia*, and show that it belongs to the molecular class A β-lactamases and exhibits a high degree of similarity to the chromosomal β-lactamase of *Klebsiella oxytoca*. Analysis of the nucleotide sequence of the DNA region directly upstream of the *penA* coding sequence revealed an open reading frame (*penR*), the transcription of which was oriented opposite to that of *penA* and whose initiation was 130 bp away from that of *penA*. Two potential ribosome-binding sites and two overlapping −10 and −35 promoter sequences were identified in the intercistronic region. The predicted translation product of *penR* was a polypeptide of 301 amino acids with an estimated molecular size of 33.2 kDa. The deduced polypeptide of *penR* showed a high degree of similarity with AmpR-like transcriptional activators of class A and C β-lactamases, with identities of 59 and 58.7% with *Pseudomonas aeruginosa* PAO1 AmpR and *Proteus vulgaris* B317 CumR, respectively. The N-terminal portion of *B. cepacia* PenR was predicted to include a helix-turn-helix motif, which may bind the LysR motif identified in the intercistronic region. Induction of PenA by imipenem was shown to be dependent upon the presence of PenR. Expression of the cloned *B. cepacia penA* and *penR* genes in *Escherichia coli* SNO302 (*ampD*) resulted in a high basal and hyperinducible PenA activity. These results suggest that the regulation of the PenA penicillinase of *B. cepacia* 249 is similar to that observed in other class A and class C β-lactamases that are under the control of a divergently transcribed AmpR-like regulator.**

Over the past decade, *Burkholderia* (*Pseudomonas*) *cepacia* has been recognized as a ubiquitous and opportunistic pathogen of increasing importance, particularly in immunocompromised hosts and cystic fibrosis patients. The treatment of *B. cepacia* infections poses a significant challenge because of its high-level, multiple antibiotic resistance (14). Resistance to β-lactam antibiotics has been attributed to inducible chromosomal β-lactamases in many gram-negative bacteria. Inducible chromosomal class C β-lactamases (cephalosporinases [AmpC]) are found in *Citrobacter freundii*, *Enterobacter cloacae*, *Pseudomonas aeruginosa*, and *Yersinia enterocolitica* (39). A model for the induction mechanism of chromosomal cephalosporinases implicating many regulatory genes is well documented in enterobacteria (40). AmpC induction requires AmpG and is regulated in a negative manner by AmpD. AmpR, a DNA-binding protein of the LysR family (49), activates the transcription of cephalosporinase in the presence of β-lactam inducers such as cefoxitin and acts as a repressor in their absence. Inducible chromosomal class A β-lactamases, regulated by a LysR-type transcriptional regulator, have also been identified in *Serratia marcescens* S6 (36), *E. cloacae* NOR-1 and 1413B (35, 45), *Proteus vulgaris* B317 (10), *Rhodopseudomonas capsulata* sp108 (8), and *Citrobacter diversus* NF85 (21), as well as in gram-positive *Streptomyces cacaoi* KCC SO352 (28, 56).

In *B. cepacia* 249, two different inducible β-lactamases have been identified and characterized biochemically: a cephalosporinase (6) and a penicillinase (43). Southern blot analysis, gel mobility shift assays, and an induction study have shown that the penicillinase is regulated by a DNA fragment located upstream from the *penA* gene encoding this penicillinase (44). However, the sequence of the PenA β-lactamase as published contained large amounts of what appeared to be plasmid vector DNA, and the G+C content was not entirely characteristic of other *B. cepacia* genes (24).

To clarify these issues, we report in this paper the recloning and correct sequence analysis of the *penA* gene encoding the inducible penicillinase of *B. cepacia*, and we confirm that it belongs to the molecular class A β-lactamases. In addition, we describe an open reading frame (ORF) (*penR*) adjacent to the *penA* gene which encodes a polypeptide with high similarity to the AmpR-like transcriptional regulator of the LysR family, and we show that the protein encoded by this gene is both a negative regulator of PenA expression and essential for its induction.

MATERIALS AND METHODS

Bacterial strains and plasmids. The bacterial strains used in this study are listed in Table 1, together with their respective references. *Escherichia coli* SNO3 and SNO302, and plasmid pNU311, were obtained from Staffan Normark (Washington University, St. Louis, Mo.).

Media and reagents. Strains were grown in tryptic soy broth (TSB; Difco Laboratories, Detroit, Mich.), and on tryptic soy agar plates (Difco) containing 50 μg of kanamycin/ml and/or 10 μg of chloramphenicol/ml (Sigma, St. Louis, Mo.) when necessary for plasmid selection. Nitrocefin was from Oxoid (Nepean, Ontario, Canada). Benzylpenicillin, carbenicillin, cefuroxime, cefotaxime, and oxacillin were purchased from Sigma. Imipenem, clavulanic acid, and ceftazidime were gifts from Merck Frosst Canada, Inc. (Pointe-Claire-Dorval, Quebec, Canada), SmithKline Beecham Pharma (Oakville, Ontario, Canada), and Glaxo Canada, Inc. (Toronto, Ontario, Canada), respectively. T4 DNA ligase and the restriction endonucleases were obtained from New England Biolabs (Missis-

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TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Genotype or description	Reference
Strains		
E. coli DH5α	*deoR supE44* Δ(*lacZYA-argFV169*) (ϕ80 d*lacZ*ΔM15) F^- λ^- *hsdR17* (r_K^- m_K^+) *recA1 endA1 gyrA96 thi-1 relA1*	15
E. coli SNO3	*ampA1 ampC8 pyrB recA rpsL*	38
E. coli SNO302	*ampD2*; mutant of SNO3	29
B. cepacia 249	Wild type	43
Plasmids		
pASP72	*penA penR* Km^r; 12.5-kb *Eco*RI fragment from *B. cepacia* 249, cloned into pMK20	44
pNU311	*ampR* Cm^r; 2.3-kb *Sal*I-*Cla*I fragment from *C. freundii* cloned into pACYC184	29
pHUL3-1	2.7-kb *Hin*dIII-*Eco*RI fragment from pASP72 cloned into $pBGS18^+$	This study
pHUL3-2a and -2b	*penA penR* Km^r; 2.8-kb *Hin*dIII fragment from pASP72 cloned into $pBGS18^+$ (2a and 2b represent different orientations)	This study
pHUL3-3	*penA* Δ*penR* Km^r; from pHUL3-2b	This study
$pBGS18^+$	Km^r f1 Ori *lacPOZ*	53

sauga, Ontario, Canada), and ^{35}S-dCTP was supplied by ICN (Mississauga, Ontario, Canada).

Cloning procedure. A restriction map of plasmid pASP72 is shown in Fig. 1. This plasmid provides an inducible penicillinase activity that has been characterized by Prince et al. (43). The 2.7-kb *Hin*dIII-*Eco*RI fragment and the 2.8-kb *Hin*dIII fragment from pASP72 were each cloned into plasmid $pBGS18^+$, and the resulting plasmids were named pHUL3-1 and pHUL3-2a or -2b (depending on orientation), respectively. Plasmid pHUL3-3 ($PenA^+$ $PenR^-$) was constructed from pHUL3-2b as shown in Fig. 1. Restriction enzymes *Sma*I and *Xmn*I were used to delete the 3′ end of the *penR* gene. Plasmid pNU311, which contains the *ampR* gene from *C. freundii* OS60 (Table 1), was used to complement the *penR* deletion of pHUL3-3. Plasmids were transformed in *E. coli* SNO3 (*ampC*) and *E. coli* SNO302 (*ampC ampD*) for induction assays. DNA techniques were performed as described by Sambrook et al. (47). Plasmid DNA was prepared with a DNA preparation kit from Promega (Madison, Wis.).

DNA sequencing and analysis. Selected plasmids with β-lactamase activity were transformed in *E. coli* DH5α for single-stranded DNA production by using the phage M13K07 (57). DNA sequencing was performed by the dideoxynucleotide method (48) with the Deaza Sequencing Kit from Pharmacia Biotech (Baie d'Urfé, Québec, Canada). Both strands of the selected fragment were sequenced. Sequence analysis, alignment, homology study, motif searching, and molecular weight and pI prediction were performed with the Genetics Computer Group sequence analysis software package of the University of Wisconsin. The signal peptide and its cleavage site were identified with the program Signalp (37).

Susceptibility testing. MICs were determined by a twofold microdilution method in TSB with an inoculum of 5×10^5 CFU/ml. MICs were read after 18 h at 37°C and were defined as the lowest antibiotic concentration at which no bacterial growth was detected. The MICs of benzylpenicillin and cefuroxime were measured alone and in combination with lithium clavulanate (2 μg/ml).

Induction assays. Strains of *B. cepacia* 249 and *E. coli* SNO3 and SNO302 containing plasmids were grown in TSB with appropriate plasmid selection (50 μg of kanamycin/ml for $pBGS18^+$, pASP72, pHUL3-2a, and pHUL3-3 and 50 μg of kanamycin/ml plus 10 μg of chloramphenicol/ml for the cotransformed plasmids pHUL3-3 and pNU311), to an optical density at 420 nm of 0.8 U. *B. cepacia* 249 was induced with 2,000 μg of benzylpenicillin/ml. *E. coli* SNO3 and SNO302 containing *penA* plasmids were induced with 0.06 μg of imipenem/ml. After 4 h of induction, cells were washed and resuspended in 50 mM phosphate buffer, pH 7.0. Crude extracts were prepared by incubating the cells with 0.1 mg of lysozyme/ml, followed by disruption with a Virsonic 475 sonicator. Cells were sonicated for 6 cycles of 10 s with pause times of 30 s. The lysates were clarified via centrifugation and were used for β-lactamase assays.

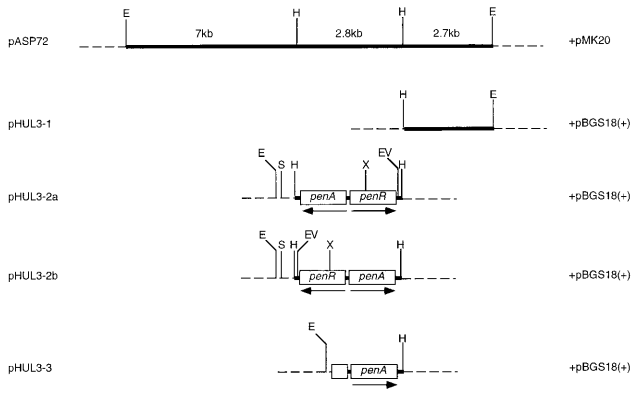

FIG. 1. Construction of plasmids pHUL3-1, pHUL3-2a and -2b, and pHUL3-3 from pASP72. Thick and dashed lines, insert and vector, respectively. The *penA* and *penR* genes are boxed, and the orientation of transcription is specified by an arrow. Abbreviations: E, *Eco*RI; EV, *Eco*RV; H, *Hin*dIII; S, *Sma*I; X, *Xmn*I.

β-Lactamase assays. β-Lactamase activity was determined spectrophotometrically following the hydrolysis of nitrocefin. Assays were performed in 50 mM phosphate buffer, pH 7.0, at 30°C on a Varian Cary-1 spectrophotometer. Specific activities were expressed as micromoles of nitrocefin hydrolyzed per minute per milligram of protein, by using a Δε of 16,000 M^{-1} cm^{-1} for nitrocefin at 486 nm. Protein concentrations of clear lysates were determined by using a Bio-Rad protein assay reagent, with bovine serum albumin (Pierce, Rockford, Ill.) as a standard.

Nucleotide sequence accession number. The nucleotide sequence data reported here appear in the GenBank database under accession no. U85041.

RESULTS

Subcloning of the gene conferring β-lactamase activity. The recombinant plasmids are shown in Fig. 1. The two subclones, pHUL3-2a and pHUL3-2b, which contain the 2.8-kb *Hin*dIII fragment from pASP72 express β-lactamase activity in *E. coli* SNO3. No β-lactamase activity was detected in *E. coli* SNO3 containing plasmid pHUL3-1. Sequencing of the 2.8-kb *Hin*dIII fragment revealed two long ORFs separated by an intercistronic region of 130 nucleotides in which two putative −10 and −35 regions of bacterial promoters were identified (ATAATT and TTTACG for *penR*; GATAGT or GCTGTC and TTGTCG for *penA*) (Fig. 2). Two potential ribosome-binding sites were identified, one 9 nucleotides upstream from the *penA* gene (GAGA) and one 8 nucleotides upstream from the *penR* gene (AAGA on the complementary strand). The first ORF encodes a 303-amino-acid polypeptide (PenA) with a predicted molecular size of 31.7 kDa. A search using the program Signalp (37) showed the presence of a signal peptide with a putative cleavage site at position 28 of the N-terminal region. For the resulting protein of 276 amino acid residues, a molecular size of 29.1 kDa can be calculated. This agrees with the molecular size of 31 kDa observed in lysates of the original strain, *B. cepacia* 249 and in *E. coli* SNO3/pHUL3-2a by using nitrocefin to detect proteins with β-lactamase activity on a nitrocellulose membrane. Mature PenA is calculated to have a pI of 8.8, indicating that the protein has a basic character. In induced cells of *B. cepacia* 249, a second, more intense band of β-lactamase activity was observed to migrate with an apparent

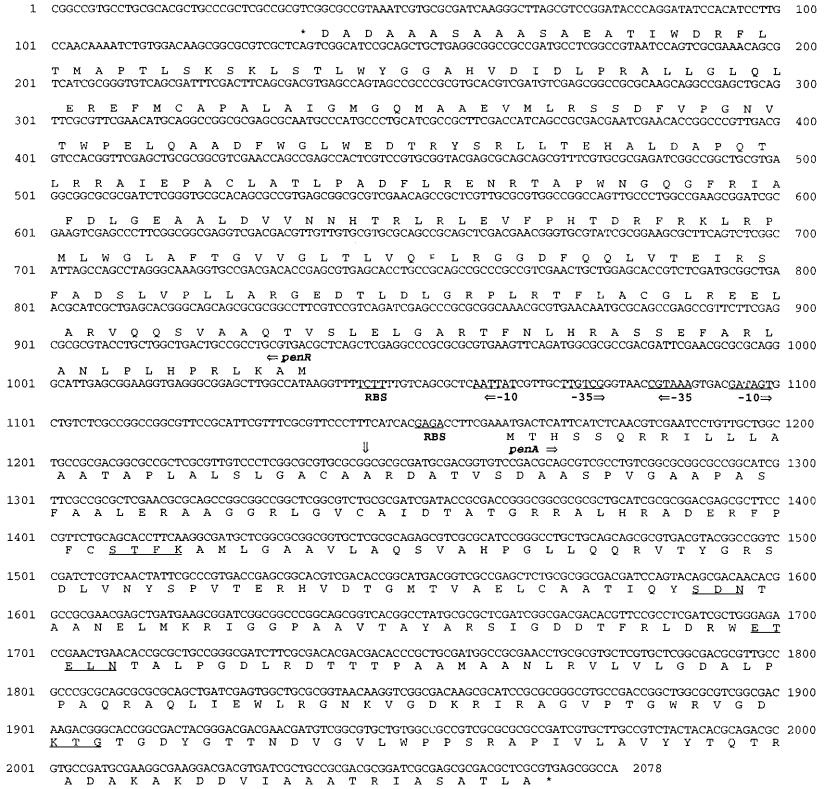

FIG. 2. Nucleotide and deduced amino acid sequences of the *B. cepacia* 249 β-lactamase gene *penA*, its predicted transcriptional regulator gene *penR*, and the intercistronic region. Putative −10 and −35 promoter sequences are indicated. The transcriptional orientation of each gene is specified by an arrow. Potential ribosome-binding sites (RBS) are underlined, as are the conserved sequences of class A β-lactamases (STFK, SDN, ETELN, and KTG). The predicted *penA* signal peptide includes amino acids 1 to 27, and the proposed cleavage site is indicated by a vertical arrow.

molecular size of 39 kDa (data not shown). The comparison of the deduced peptide sequence of PenA with known β-lactamases revealed a relationship to β-lactamases of class A (Table 2) but not with class C β-lactamases (less than 21% identity) (12, 30, 34, 52). The highest degrees of identity were found with the β-lactamases BlaI from *Y. enterocolitica* Y56 (60.3%), K1 from *Klebsiella oxytoca* E23004 (55.1%) and CTX-M-2 of *Salmonella typhimurium* CAS-5 (55.1%). PenA contains the four conserved motifs, ^{70}S*XXK^{73} (where ^{70}S* is the active-site serine), ^{130}SDN^{132}, ^{166}EXXXN^{170}, and ^{234}KTG^{236} (Am-

TABLE 2. Identities among 12 class A β-lactamases

β-Lactamase[a]	% Identity with:											
	PenA Bc	BlaI Ye	K1 Ko	CTX-M-2 St	CTX-M-1 Ei	CdiA Cd	CumA Pv	Sme-1 Sm	NmcA Ec	IMI-1 Ec	BlaL Sc	BlaA Rc
PenA Bc	100											
BlaI Ye[b]	60.3	100										
K1 Ko[c]	55.1	55.5	100									
CTX-M-2 St[d]	55.1	58.3	74.3	100								
CTX-M-1 Ei[e]	54.1	55.5	72.6	79.8	100							
Bla Cd[f]	53.9	53.4	72.5	71.1	70.4	100						
CumA Pv	49.5	55.3	64.7	63.7	63.9	65.1	100					
Sme-1 Sm	46.1	45.9	46.6	44.0	42.8	44.9	43.0	100				
NmcA Ec	46.0	46.7	45.8	45.2	45.1	45.1	44.7	70.3	100			
IMI-1 Ec	45.7	46.4	46.5	44.5	44.4	44.8	44.0	70.3	97.3	100		
BlaL Sc[g]	45.5	39.7	41.4	40.4	40.1	40.1	37.4	38.3	38.1	38.1	100	
BlaA Rc	39.6	34.8	37.6	37.6	37.6	39.1	33.3	29.3	30.0	29.0	34.8	100

[a] β-Lactamase abbreviations are as explained in the legend to Fig. 3 except where specified otherwise.
[b] BlaI from *Y. enterocolitica* Y56 (51).
[c] K1 from *K. oxytoca* E23004 (2).
[d] CTX-M-2 from *S. typhimurium* CAS-5 (5).
[e] CTX-M-1 (MEN-1) from *E. coli* GRI and MEN (4, 5).
[f] Bla (CdiA) from *C. diversus* ULA27 and NF85 (21, 42).
[g] BlaL from *S. cacaoi* KCC SO352 (27).

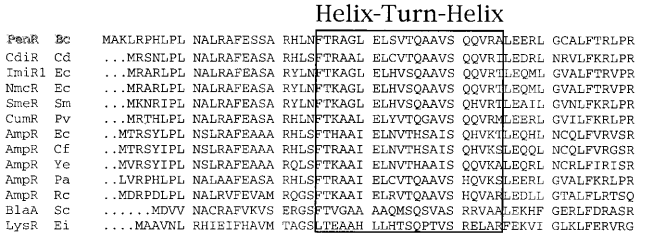

FIG. 3. Alignment of the N-terminal PenR amino acid sequence from *B. cepacia* 249 with those of other LysR-type transcriptional regulators. Abbreviations: Bc, *B. cepacia* 249; Cd, *C. diversus* NF85 (21); Cf, *C. freundii* OS60 (31); Ec, *E. cloacae* 1413B (miR1), MHN1 (AmpR), and NOR-1 (NmcR) (16, 35, 45); Ei, *E. coli* K-12 (55); Pa, *P. aeruginosa* PAO1 (33, 34); Pv, *P. vulgaris* B317 (10); Rc, *R. capsulata* sp108 (8); Sc, *S. cacaoi* KCC SO352 (56); Sm, *S. marcescens* S6 (36); Ye, *Y. enterocolitica* IP97 (52). The conserved helix-turn-helix motif is boxed.

bler's numbering scheme [1]) that are characteristic of class A β-lactamases (Fig. 2) (23).

Sequence analysis of PenR. An ORF encoding a polypeptide of 301 amino acids with a predicted molecular size of 33.2 kDa, which corresponds to those of β-lactamase transcriptional regulators, was found 130 nucleotides upstream from the *penA* gene (Fig. 2). The PenR polypeptide sequence showed high degrees of similarity to β-lactamase transcriptional regulators of the LysR family. The highest degrees of identity were found with *P. aeruginosa* PAO1 AmpR (59%) and *P. vulgaris* B317 CumR (58.7%), and the lowest degree of identity was observed with *S. cacaoi* KCC SO352 BlaA (24.1%) (10, 33, 56). A search for peptide motifs, performed with the Genetics Computer Group program Motifs and the database PROSITE, showed that a helix-turn-helix motif which is usually conserved throughout the LysR family of transcriptional regulators was present in the N-terminal part of the PenR peptide sequence. An alignment of the N-terminal region of the predicted PenR amino acid sequence from *B. cepacia* with those of other LysR-type β-lactamase transcriptional regulators and with that of LysR from *E. coli* K-12, which regulates the transcription of LysA (54, 55), is shown in Fig. 3.

To verify the bacterial origin of the *penA-penR* locus, Southern blot analysis was performed with the 600-bp *Eco*RV-*Xmn*I DNA fragment from pHUL3-2a containing the 3′ end of the *penR* gene as a probe (Fig. 1). Chromosomal DNA from *B. cepacia* 249, *P. aeruginosa* PAO1, and *E. coli* K-12 was digested with restriction enzymes *Hin*dIII, *Eco*RI, and *Pst*I and loaded on a 0.7% agarose gel next to the cloned 2.8-kb *Hin*dIII fragment from pASP72, which contains the *penA-penR* locus from *B. cepacia* 249 and was used as a control. The probe hybridized very strongly to a 2.8-kb *Hin*dIII fragment from the *B. cepacia* chromosomal DNA but not to the chromosomal DNA from *P. aeruginosa* PAO1 or *E. coli* K-12 (data not shown). The *penA* and *penR* genes have G+C contents of 71.4 and 68.7%, respectively, consistent with the *B. cepacia* chromosomal origin of these genes (3).

Genetic organization of the *penA-penR* locus. An alignment of the *B. cepacia* intercistronic region between *penA* and the putative transcriptional regulator *penR* reveals similarities with analogous regions in other bacteria (Fig. 4). On the basis of intercistronic region sequence analysis from *C. freundii* OS60, *E. cloacae* MHN1, *Y. enterocolitica* IP97, *E. cloacae* NOR-1, and *S. marcescens* S6 (16, 31, 35, 36, 52), we identified sequence motifs which appear to be conserved in the PenR-binding region within the *B. cepacia penA-penR* locus. Regions 1 and 2, which correspond to the AmpR DNA-binding region of 38 bp found by Lindquist et al. (31), are indicated. In region 1, we identified a palindromic sequence separated by a T-rich region (GTTTT); region 2 also contains a short palindromic sequence. Partial sequence similarity between these two regions is observed in *B. cepacia*, as in the LysR motif, T-N11-T (13), which occurs around an axis of twofold symmetry in region 1.

Antibiotic susceptibility. The susceptibility of *E. coli* SNO3 containing plasmid pBGS18$^+$ (control) or pHUL3-2a (PenA PenR) to various β-lactam antibiotics is shown in Table 3. *E. coli* SNO3(pHUL3-2a) was highly resistant to benzylpenicillin, carbenicillin, and cefuroxime, an expanded-spectrum cephalosporin. However, this strain was susceptible to imipenem and ceftazidime, a broad-spectrum cephalosporin. The MIC of cephaloridine, a narrow-spectrum cephalosporin, was lower than that of benzylpenicillin. Susceptibility to cefotaxime, a

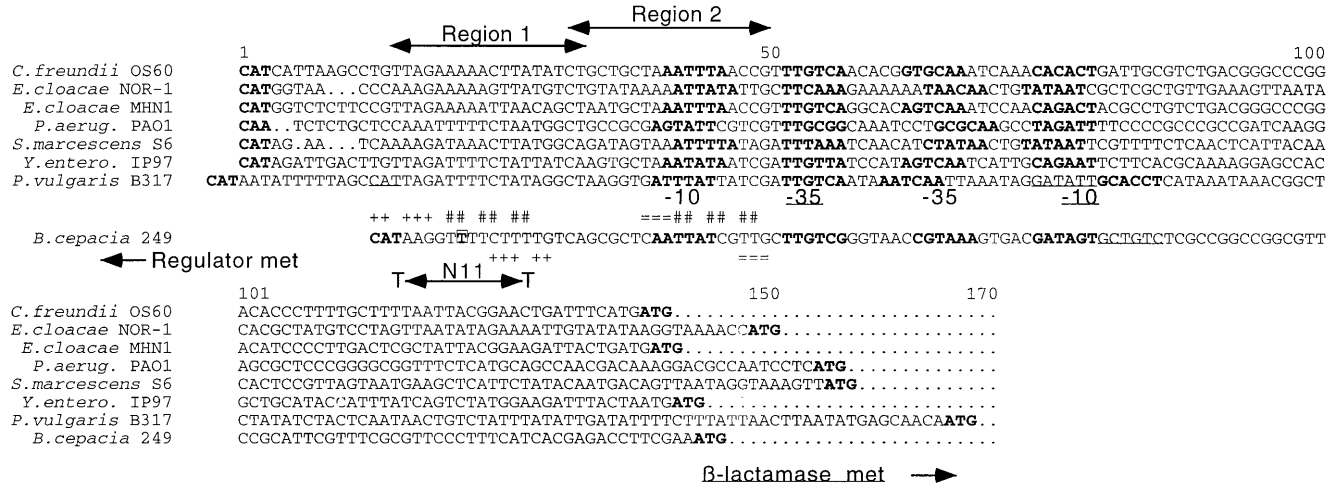

FIG. 4. Alignment of the intercistronic region between the β-lactamase gene and its transcriptional regulator gene from *B. cepacia* with those of seven gram-negative bacteria. Start codons and promoters (−10 and −35) are in bold characters. The β-lactamase promoter is underlined. The conserved regions 1 and 2 of the putative PenR DNA-binding site are indicated. Symbols: + and =, palindromic sequences in regions 1 and 2, respectively; #, sequence homology between both regions. The twofold symmetry axis is indicated by a boxed boldfaced T. The LysR motif T-N11-T is specified. Another potential start codon in *P. vulgaris* and other *−35 sequences of the promoters in P. vulgaris* and *B. cepacia* are underlined.

TABLE 3. MICs for *E. coli* SNO3 strains expressing the *B. cepacia* 249 PenA β-lactamase

β-Lactam	MIC (μg/ml)	
	E. coli SNO3(pBGS18$^+$)	*E. coli* SNO3(pHUL3-2a) (PenA PenR)
Benzylpenicillin	16	256
Benzylpenicillin plus clavulanic acid[a]		16
Carbenicillin	16	128
Oxacillin	256	256
Cephaloridine	4	32
Cefuroxime	4	128
Cefuroxime plus clavulanic acid[a]		4
Cefotaxime	0.06	0.25
Ceftazidime	0.13	0.13
Imipenem	0.5	1

[a] Clavulanic acid at 2 μg/ml.

methoxyimino broad-spectrum cephalosporin, was decreased fourfold compared to the parent. Addition of clavulanate (2 μg/ml) lowered the MICs of benzylpenicillin and cefuroxime to those for the control. Thus, the susceptibility of *E. coli* SNO3(pHUL3-2a) to benzylpenicillin and cefuroxime was restored by the addition of clavulanate.

Induction assays. The expression of the cloned PenA β-lactamase exhibited the expected *penR*-dependent inducibility (Table 4). In the presence of imipenem as an inducer, PenA activity was increased 2.6-fold in SNO3/pHUL3-2a. In SNO3/pHUL3-3, which contains a 3′ deletion of PenR, PenA activity was 7.8-fold higher but was no longer inducible. Although basal PenA activity was 8 times higher in SNO3/pHUL3-2a than in SNO3/pASP72, both constructs exhibited approximately twofold induction. Complementation of pHUL3-3 with pNU311 expressing *C. freundii* AmpR did not restore inducibility. PenA expression was also regulated by AmpD. Basal PenA β-lactamase activity as expressed by pHUL3-2a increased more than 6-fold in a SNO302 (*ampD*) background and 26-fold when expressed from the pASP72 construct; up to 10-fold induction was observed in the presence of imipenem in SNO302/pHUL3-2a. As expected, induction in the SNO302 background was also *penR* dependent. The *penR* deletion in pHUL3-3 did not affect basal β-lactamase activity in the SNO302 background as it did in SNO3. In *B. cepacia* 249, basal and induced β-lactamase activity values were 29.2 and 3,190 μmol of nitrocefin hydrolyzed/min/mg of protein, respectively.

TABLE 4. Specific activities of *B. cepacia* 249 PenA in *E. coli* SNO3 and SNO302

Strain (plasmid[s] and genotype)	Sp act[a]	
	Noninduced	Induced[b]
SNO3(pHUL3-2a *penA penR*)	56.5 ± 2.8	146 ± 20
SNO3(pASP72 *penA penR*)	7.3 ± 0.8	14.1 ± 0.9
SNO3(pHUL3-3 *penA*)	439 ± 53	490 ± 120
SNO3(pHUL3-3, pNU311 *penA ampR*)	363 ± 51	417 ± 12
SNO302(pHUL3-2a *penA penR*)	331 ± 20	3,360 ± 140
SNO302(pASP72 *penA penR*)	190 ± 22	864 ± 18
SNO302(pHUL3-3 *penA*)	348 ± 8	376 ± 34
SNO302(pHUL3-3, pNU311 *penA ampR*)	487 ± 18	486 ± 22

[a] Values are means of triplicate measurements with 100 μM nitrocefin as substrate and are expressed as micromoles of substrate hydrolyzed per minute per milligram of protein.

[b] Induction was carried out with 0.06 μg of imipenem/ml.

DISCUSSION

B. cepacia is recognized as an important source of infection, particularly in cystic fibrosis patients (14). β-Lactam resistance has been reported frequently in *B. cepacia* and has been associated with the production of an inducible β-lactamase (9). The goal of this study was to characterize the inducible β-lactamase (PenA) of *B. cepacia* 249 and its transcriptional regulator (PenR). It has previously been reported that the purified PenA from *B. cepacia* 249 has penicillinase activity that is not inhibited by clavulanic acid or sulbactam (43). In this report, we demonstrated that the expression of PenA in *E. coli* SNO3 confers high levels of resistance to benzylpenicillin, carbenicillin, and cefuroxime. These data confirm the penicillinase activity of PenA, and they also suggest that this enzyme has a cefuroximase activity similar to that observed for the chromosomally encoded β-lactamases of *P. vulgaris* (10, 41). PenA did not confer resistance to imipenem, and it is thus distinct from the carbapenem-hydrolyzing serine β-lactamases Sme-1 of *S. marcescens* S6, NmcA of *E. cloacae* NOR-1, and IMI-1 of *E. cloacae* 1413B (35, 36, 45). Contrary to the report of Prince et al. (43), we showed that PenA is inhibited by 2 μg of clavulanic acid/ml. Therefore, while PenA had been classified as a group 4 β-lactamase in the functional classification scheme of Bush et al. (7), the current data suggest that PenA is a group 2 enzyme. Assignment of a specific subgroup will require further characterization of the substrate profile of PenA.

Analysis of the deduced amino acid sequence of the *penA* gene product showed that it belongs to the molecular class A β-lactamases (23). The predicted molecular size of 29.1 kDa and the observed value of 31 kDa when it is expressed in *E. coli* SNO302 are also characteristic of class A β-lactamases. Amino acid sequence comparisons revealed that PenA is closely related to *Y. enterocolitica* BlaI, *K. oxytoca* K1, *S. typhimurium* CTX-M-2, *E. coli* CTX-M-1 (MEN-1), and *C. diversus* Bla (CdiA) β-lactamases, with which it has more than 53% identity. The active-site serine penicillin-recognizing enzymes contain seven motifs (1 to 7) of highly conserved residues (22). PenA contains several of these motifs that are known to play critical roles in the catalytic activity of β-lactamases, including box 2 (^{70}S*XXK^{73} with the active-site serine), box 5 (^{166}EXXXN^{170}), box 7 (^{234}KTG^{236}), and the ^{130}SDN^{132} loop (19, 26). In addition, PenA contains many of the residues that are conserved throughout class A β-lactamases: Gly-45 (box 1), Phe-66, Leu-81, Pro-107, Ala-134, Asn-136, Asp-157, Arg-164, Leu-169, Asp-180, Leu-207, and Asp-233. Interestingly, PenA contains 3 cysteine residues at positions 47, 69, and 123, which is uncommon in class A. Cys-69 is common in *K. oxytoca*-related β-lactamases (2, 5, 10, 41, 42, 46), and Cys-123 is also encountered in class A enzymes, such as ROB-1 and a *Y. enterocolitica* enzyme (32, 51). Cys-123 makes a disulfide bridge in enzymes which also possess a cysteine at position 77, such as TEM, SHV, and CARB β-lactamases (1, 11, 20, 50). PenA is further distinguished by the absence of Lys-244, which is replaced with Thr. Lys-244 plays an important role in the enzyme catalysis and inhibition of class A β-lactamases (17, 18, 58) and is also replaced with Thr in many *K. oxytoca*-related β-lactamases (2, 5, 10, 41, 42, 46, 51).

Our analysis of the sequence data indicates that *penA* is regulated by *penR*, which is divergently transcribed 130 bp upstream from *penA* and has properties consistent with those of AmpR-like transcriptional regulators of the LysR family. In many gram-negative bacteria, a LysR-type transcriptional reg-

ulator is transcribed divergently from the β-lactamase gene. This has been demonstrated for the class C β-lactamases of *P. aeruginosa* PAO1 (33), *C. freundii* OS60 (31), *E. cloacae* MHN1 (16), and *Y. enterocolitica* IP97 (52), in which AmpR is the transcriptional regulator. Recently, AmpR-like regulators have been found to modulate the expression of class A β-lactamases in *P. vulgaris* B317 (10), *E. cloacae* NOR-1 and 1413B (35, 45), *S. marcescens* S6 (36), *R. capsulata* sp108 (8), *C. diversus* NF85 (21), and *S. cacaoi* KCC SO352 (56). PenR has a deduced molecular size of 33.2 kDa, similar to the sizes of other AmpR-type regulators, and contains the expected helix-turn-helix motif in the N terminus typical of LysR family members. Alignment of the intercistronic region of *B. cepacia* 249 with those of several other class A and class C β-lactamases demonstrated that the −10 and −35 promoter sequences of *penA* and *penR* are both similar and similarly aligned. The general organization of the locus is conserved, particularly in regions 1 and 2, which may serve as the 38-bp protected region, as well as in the palindromic sequences and the LysR motif in region 1 (13). The region of DNA predicted to be protected overlaps the promoter region of *penR*, suggesting that it is autoregulated, as has been demonstrated for *C. freundii ampR* (31).

To verify the activator and repressor functions of PenR, induction assays were carried out on *B. cepacia* 249 and on the cloned *penA* and *penR* genes expressed in *E. coli* SNO3 and SNO302. PenR activates the production of PenA when an inducer is present. Significant differences in PenA activity as expressed in *E. coli* cells by plasmids pHUL3-2a and pASP72 were observed; these may be due to *penA* gene copy number (25, 53) and/or the presence of other regulatory elements in pASP72. Deletion of PenR increases the constitutive PenA expression, showing the repressor function of PenR and its importance in the mechanism of PenA induction. The repressor function of PenR cannot be complemented by the cloned *ampR* gene from *C. freundii*. A similar result was observed with CumR from *P. vulgaris*, which was not complemented by *C. freundii* AmpR (10). The percentage of identity between the intercistronic regions of *B. cepacia* and *P. vulgaris* is higher than those obtained with other bacteria, and there is another possible start codon (ATG) for *cumR*, which is well aligned with that of *penR*. This suggests that transcriptional regulation of *penA* could be more closely related to that of *cumA* from *P. vulgaris*. It is also possible that the helix-turn-helix motif of *C. freundii* AmpR is not sufficiently similar to that of PenR to bind to the intercistronic region. The product of *ampD* is also an important component of the induction of AmpC in the class C type β-lactamases and is a negative regulator of β-lactamase production (40). Basal PenA activity in an *E. coli ampD* mutant, SNO302, was significantly increased and was also hyperinducible, as previously shown (44), suggesting that *penA* is negatively regulated by an AmpD-like protein. The 2.6-fold induction observed in *E. coli* SNO3 containing cloned *penA* is not as high as that which has been determined in the original strain, *B. cepacia* 249 (110-fold); however, the latter also expresses a second inducible β-lactamase of 39 kDa, which contributes to its overall β-lactamase activity. The relative contribution of each of these enzymes to the β-lactamase activity observed in *B. cepacia* has not been determined, but the low level of PenA induction in *E. coli* cells is consistent with that observed in *B. cepacia* 249 when nitrocefin is used to detect β-lactamase activity on a nitrocellulose membrane. These data (not shown) indicated that the 39-kDa β-lactamase is induced to a much higher level than PenA.

The present data clarify earlier studies (44) which suggested that PenA was a member of the AmpC family of β-lactamases. The previous conclusions, based on the observed regulation of PenA by AmpD and its dependence upon an upstream sequence for inducibility, were questioned because of apparent discrepancies in the DNA sequence of *penA* as presented (24). The recloning and sequencing of *penA* as presented in this report allowed us to find an inadvertent cloning error in the earlier work (44). The analysis of the sequence data combined with the current and previously determined genetic data suggests that the *B. cepacia* PenA penicillinase is a class A β-lactamase which is regulated in a manner similar to that observed in other class A and C β-lactamases that are regulated by a divergently transcribed AmpR-like transcriptional regulator.

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