Biochem. J. (1990) 272, 627–631 (Printed in Great Britain)

Cloning, sequencing and analysis of the structural gene and regulatory region of the *Pseudomonas aeruginosa* chromosomal *ampC* β-lactamase

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The chromosomal gene from *Pseudomonas aeruginosa* encoding β-lactamase has been cloned, and the sequence determined and compared with corresponding sequences of β-lactamases from members of the enterobacteriaceae. Upstream of the β-lactamase gene is an open reading frame which we postulate encodes a regulatory protein, AmpR. We identified a helix–turn–helix region in AmpR and a putative AmpR-binding site.

INTRODUCTION

Resistance to β-lactam antibiotics in many Gram-negative bacteria, including *Enterobacter cloacae*, *Citrobacter freundii* and *Pseudomonas aeruginosa* is mediated by class I β-lactamases (Piddock & Wise, 1985; Rolinson, 1989) encoded by the chromosomal *ampC* gene. The enzyme is expressed at a low level, but is inducible in response to β-lactam antibiotics (Lindberg *et al.*, 1985; Sabath *et al.*, 1965). These bacteria mutate with high frequency to resistance to most third-generation cephalosporin antibiotics, which is due to high-level constitutive over-expression of the *ampC* gene (Seeberg *et al.*, 1983; Gootz *et al.*, 1984; Curtis *et al.*, 1986).

The structural gene, *ampC*, as well as several of the genes involved in its regulation in *E. cloacae* and *C. freundii*, have been cloned and sequenced (Honoré *et al.*, 1986, 1989; Lindberg & Normark, 1986; Galleni *et al.*, 1988; Lindquist *et al.*, 1989*a*,*b*), and much of the mechanism for control of expression has been elucidated (Honoré *et al.*, 1989; Lindquist *et al.*, 1989*a*,*b*). In contrast, very little is known about the mechanism of regulation of the chromosomal β-lactamase of *Pseudomonas aeruginosa*. Here we describe the cloning of a DNA fragment containing both the structural gene and the regulatory gene from *Pseudomonas aeruginosa* PAO1, which we will refer to as '*ampC*' and '*ampR*' by analogy with their counterparts in the Enterobacteriaceae. We have determined the nucleotide sequence of *ampC* and have shown that the derived amino acid sequence corresponds well with published amino acid data (Knott-Hunziker *et al.*, 1982; Emanuel *et al.*, 1985). The data show sequence similarity, both at the DNA and at the amino acid level, to the corresponding *E. cloacae* and *C. freundii* genes. We have also sequenced the *N*-terminal portion of *ampR*, which shows striking similarity to the *E. cloacae* and *C. freundii ampR* gene and includes the helix–turn–helix DNA-binding motif predicted to be present at the *N*-terminus of bacterial activator proteins (Henikoff *et al.*, 1988).

MATERIALS AND METHODS

The *Pseudomonas aeruginosa* PAO1 gene bank was kindly provided by Dr. K. Piers and Dr. R. E. W. Hancock (University of British Columbia, Vancouver, Canada); this consisted of endonuclease-*Eco*RI fragments from *P. aeruginosa* strain PAO1 DNA cloned in the cosmid pLAFR1 (Friedman *et al.*, 1982) and maintained in the *E. coli* host LE392. Plasmid pAA121 (Kelsall *et al.*, 1985) and a derivative which contains the pUC9 polylinker, pAA121U9, were used as vectors in subcloning experiments in *E. coli* M182 (Casadaban & Cohen, 1979). Antibiotics were used at final concentrations of 100 $\mu g \cdot ml^{-1}$ (ampicillin) and 25 $\mu g \cdot ml^{-1}$ (tetracycline) for *E. coli* strains and 100 $\mu g \cdot ml^{-1}$ (tetracycline) for *P. aeruginosa*. Bacteriophage M13mp18 and M13mp19 were used in *E. coli* strain JM101 to make single-stranded DNA for sequencing.

Standard recombinant DNA techniques as described in Maniatis *et al.* (1982) were used throughout. The oligonucleotide probe mixture was purchased from Alta Bioscience of the University of Birmingham and was end-labelled with [γ-^{32}P]ATP and used to probe bacterial colonies immobilized on nitrocellulose filters (Hybond-C; Amersham International). All filter hybridization and Southern blotting was performed according to methods described by Hames & Higgins (1985).

For sequencing, single-stranded DNA was prepared and sequenced using the dideoxy-chain-termination method (Sanger *et al.*, 1980). Sequence ambiguities resulting from the formation of secondary structures were resolved using Deaza ^{T7}Sequencing mixes (Pharmacia) containing 7-deaza-dGTP.

Analysis of the sequence data was assisted by the use of University of Wisconsin sequence analysis software (version 6.2) (Devereux *et al.*, 1984); database searches and sequence alignments were made with the ISIS software package (Akrigg *et al.*, 1988) SWEEP program using the Lipman & Pearson (1985) algorithm. Databases used were GenBank 60, EMBL 19 and OWL 9.

RESULTS AND DISCUSSION

Cloning and sequencing of the putative *P. aeruginosa ampC* gene

A 22 bp oligonucleotide probe was derived from the published amino acid sequence of the active site of the *P. aeruginosa ampC* β-lactamase (Knott-Hunziker *et al.*, 1982). The probe was designed to contain all the alternative coding sequences resulting from the redundancy of the genetic code. A library of *Eco*RI

Abbreviation used: ORF, open reading frame.
The nucleotide sequence data reported will appear in the EMBL, GenBank and DDBJ Nucleotide Sequence Databases under the accession number X54719.

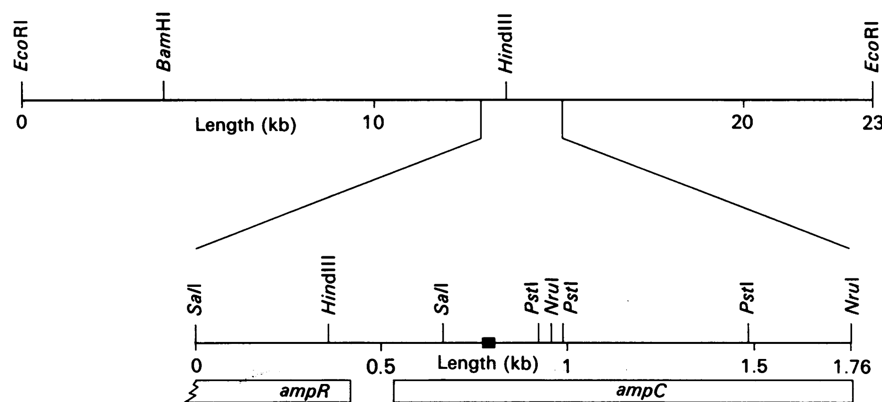

Fig. 1. Restriction map of 23 kb *Eco*RI fragment from *P. aeruginosa* PAO1

The line in the upper half of the Figure represents a cloned *Eco*RI fragment from a *P. aeruginosa* PAO1 library identified by hybridization with an oligonucleotide probe derived from the *P. aeruginosa β*-lactamase amino acid sequence. Below is an enlarged map of the 1.76 kb *Sal*I–*Nru*I fragment, showing restriction sites used in sequencing. The location of the two ORFs, which are similar to *ampR* and *ampC* from *E. cloacae*, is shown below. The location of the oligonucleotide sequence used in hybridization experiments is marked with a filled box.

fragments of *P. aeruginosa* PAO1 DNA in the cosmid pLAFR1 was screened using the radiolabelled oligonucleotide probe. Restriction digests showed that all positive clones contained a 23 kb *Eco*RI fragment of DNA (Fig. 1). Southern-blot hybridization of a *Hin*dIII–*Pst*I restriction digest of the 23 kb fragment identified a 560 bp *Pst*I–*Hin*dIII fragment with which the probe hybridized. This 560 bp fragment was cloned into the M13mp18 and M13mp19 vectors and sequenced; it contained a sequence corresponding to one of the oligonucleotides in the probe mixture. Further analysis showed that the DNA sequence corresponded to the published amino acid sequence flanking the sequence used to derive the probe. Having thus confirmed that the candidate contained sequences consistent with the published amino acid data, the sequence was extended in both directions, sequencing 1.76 kb, on both strands, from the *Sal*I to the *Nru*I sites shown in Fig. 1.

Identification of the *P. aeruginosa ampC β*-lactamase gene

Fig. 2 shows the nucleotide sequence of 1.76 kb from the *Sal*I to *Nru*I sites identified in Fig. 1. A 1280 bp open reading frame (ORF) was identified which extended from base 554 downstream of the *Sal*I site to a point just short of the second *Nru*I site at bp 1758 (Fig. 1). The nucleotide sequence of this ORF was used to search the GenBank 60 and EMBL 19 databases. The sequence of the *E. cloacae ampC β*-lactamase was identified as showing the most similarity; the *C. freundii* and *E. coli* sequences were also detected. Alignment with the *E. cloacae* sequence was used to locate the translation-initiation codon (Fig. 2). The 33 *N*-terminal amino acid residues of the mature *P. aeruginosa β*-lactamase have been determined (Emanuel *et al.*, 1985), and our nucleotide sequence is in agreement at all but four residues, which occur in the last third of the determined amino acid sequence. These discrepancies may be due to the problems associated with amino acid sequencing over long distances, or alternatively, to strain differences. The sequence data correspond exactly with the published sequence of the tryptic peptide from *P. aeruginosa* containing the active-site serine residue (Knott-Hunziker *et al.*, 1982).

The 1280 bp ORF shows good correlation with the *P. aeruginosa* codon-usage data (West & Iglewski, 1988), having an average codon preference of 1.19 compared with a value of 0.74 using *E. coli* codon-usage data, thus confirming that the clone contains *Pseudomonas* rather than *E. coli* DNA. The derived amino acid sequence for the 1280 bp ORF shows a high percentage identity with the amino acid sequence from *E. cloacae* (44%) and *E. coli* (43%) (Fig. 3). The sequences around the active site show an especially high degree of similarity, as predicted by the amino acid sequences determined directly (Knott-Hunziker *et al.*, 1982). The *Pseudomonas* sequence is longer than either the *E. coli* or the *E. cloacae* sequences, having six additional amino acid residues on the signal sequence and nine more residues at the *C*-terminal end than in the *E. cloacae* sequence.

The conclusion that the 1280 bp ORF codes for the chromosomal *ampC β*-lactamase of *P. aeruginosa* is supported by two pieces of evidence: the sequence data correlate well with the published amino-acid-sequence data and it shows a high degree of similarity at both the DNA and the amino acid level to chromosomal *ampC β*-lactamase from closely related organisms. This was confirmed by mobilizing the 23 kb fragment of DNA in the cosmid pLAFR1 into a non-*β*-lactamase-producing strain of *P. aeruginosa*. The transformed strain turned the chromogenic cephalosporin nitrocefin from yellow to red within 2 min, while a control remained unchanged.

Identification of the putative *P. aeruginosa ampR* regulatory gene

In *E. cloacae* and *C. freundii* a regulatory gene *ampR* is located just upstream of the structural *ampC* gene and is transcribed divergently. We looked for a similar ORF upstream of the *P. aeruginosa ampC* sequence. Analysis of the sequence from bp 1–406 in Fig. 2 reveals an ORF with similarity to the *ampR* sequences from *E. cloacae* and *C. freundii*. The translation-initiation codon has been assigned by aligning the sequence with those of *E. cloacae* and *C. freundii*; interestingly, this is not the usual ATG start sequence, but the rare TTG sequence, which accounts for only 1% of known initiator sequences (Gualerzi & Pon, 1990). Fig. 4 shows that the *P. aeruginosa* sequence shows a high degree of similarity to both the *E. cloacae* and *C. freundii* sequences, suggesting that these proteins have been conserved by evolution. It has been shown that the *E. cloacae* AmpR protein belongs to the LysR family of bacterial activator proteins (Henikoff *et al.*, 1988). Applying the criteria used by Henikoff *et al.* (1990), the *P. aeruginosa* AmpR protein also belongs to this family. In common with the other members of the LysR family, AmpR is predicted to have a helix–turn–helix DNA-binding

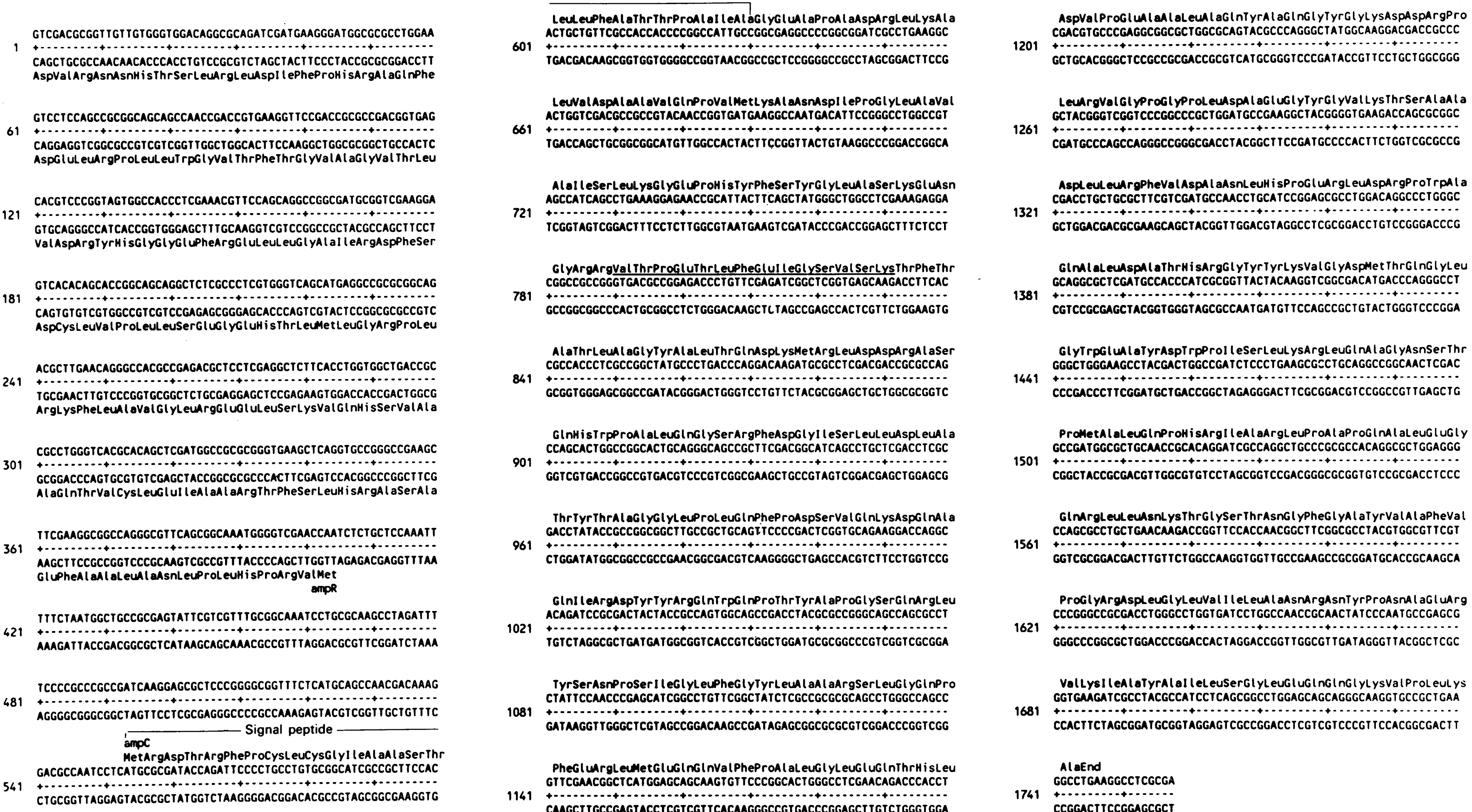

Fig. 2. Nucleotide sequence of the 1.76 kb *SalI–NruI* fragment of *P. aeruginosa* PAO1 DNA

The deduced amino acid sequence of the ORF proposed to encode the chromosomal β-lactamase, *ampC*, is shown, the signal peptide is indicated by a labelled brace, and the active-site sequence (Knott-Hunziker *et al.*, 1982) is underlined. The first 406 bp of the putative regulatory gene, *ampR*, together with the deduced amino acid sequence is also shown. The nucleotide sequence is numbered from the *SalI* site in *ampR*.

```
E.coli              1 MFKTTLCALLITASCSTFAAP...QQINDIVHRTITPLIEQQK 40
                       * *  ****  ***  * *    *    *  *****   *
E.cloacae           1 MMRKSLCCALLLGISCSALATPVSEKQLAEVVANTITPLMKAQS 44
                              **     * *       *    *     * ***
P.aeruginosa  1 MRDTRFPCLCGIAASTLLFATTPAIAGEAPADRLKALVDAAVQPVMKAND 50
                           *  *     *           *     *

E.coli        41 IPGMAVAVIYQGKPYYFTWGYADIAKKQPVTQQTLFELGSVSKTFTGVLG 90
                  ************* * * * ****   *** ******** *********
E.cloacae     45 VPGMAVAVIYQGKPHYYTFGKADIAANKPVTPQTLFELGSISKTFTGVLG 94
                  ** ***    * ***   * *      *** **** ** *****   *
P.aeruginosa  51 IPGLAVAISLKGEPHYFSYGLASKEDGRRVTPETLFEIGSVSKTFTATLA 100
                 *** ***    * * **  * *      **  **** ********   *

E.coli        91 GDAIARGEIKLSDPTTKYWPELTAKQWNGITLLHLATYTAGGLPLQVPDE 140
                 ********* * *  * *** ** *** **  * ****************
E.cloacae     95 GDAIARGEISLDDAVTRYWPQLTGKQWQGIRMLDLATYTAGGLPLQVPDE 144
                 * *      ***    ** * *    **  *************** **
P.aeruginosa 101 GYALTQDKMRLDDRASQHWPALQGSRFDGISLLDLATYTAGGLPLQFPDS 150
                 * *      * *    ** *      ** ** ************** **

E.coli       141 V.KSSSDLLRFYQNWQPAWAPGTQRLYANSSIGLFGALAVKPSGLSFEQA 189
                 *      ********** * *** ***** **************    ***
E.cloacae    145 V.TDNASLLRFYQNWQPQWKPGTTRLYANASIGLFGALAVKPSGMPYEQA 193
                 *  * *     * ***   **   *** * ****** **     * * *
P.aeruginosa 151 VQKDQAQIRDYYRQWQPTYAPGSQRLYSNPSIGLFGYLAARSLGQPFERL 200
                 * *       *  ***  *** **** * ****** **      *  **

E.coli       190 MQTRVFQPLKLNHTWINVPPAEEKNYAWGY.REGKAVHVSPGALDAEAYG 238
                 * ***  **** ******* ***  ***** * **** **** *** ***
E.cloacae    201 MTTRVLKPLKLDHTWINVPKAEEAHYAWGY.RDGKAVRVSPGMLDAQAYG 242
                 *   *   * *  *   ** *  * ** **  *     ** ** ***  **
P.aeruginosa 201 MEQQVFPALGLEQTHLDVPEAALAQYAQGYGKDDRPLRVGPGPLDAEGYG 250
                 *   **  * *  *   ** *     ** **         * ** **** **

E.coli       239 VKSTIEDMARWVQSNLKPLDINEKTLQQGIQLAQSRYWQTGDMYQGLGWE 288
                 **    *** **  *  *         * *** *******  * ********
E.cloacae    243 VKTNVQDMANWVMANMAPENVADASLKQGIALAQSRYWRIGSMYQGLGWE 292
                 ***    *    * **  **       *         *   * * ******
P.aeruginosa 251 VKTSAADLLRFVDANLHPERLDRPWA.QALDATHRGYYKVGDMTQGLGWE 299
                 **    *  *  * ** *         *         *   *** ******

E.coli       289 MLDWPVNPDSIINGSDNKIALAARPVKAITPPTPAVRASWVHKTGATGGF 338
                 ** ***        *** * ***  **    ** * * ********* ****
E.cloacae    293 MLNWPVEANTVVEGSDSKVALAPLPVAEVNPPAPPVKASWVHKTGSTGGF 342
                   **          *    ** *   *    *          ***** **
P.aeruginosa 300 AYDWPISLKRLQAGNSTPMALQPHRIARLPAPQALEGQRLLNKTGSTNGF 349
                   ***          *    **         *          *** * **

E.coli       339 GSYVAFIPEKELGIVMLANKNYPNPARVDAAWQILNALQ* 378
                 ***********  *******  ******* **   ** ***
E.cloacae    343 GSYVAFIPEKQIGIVMLANTSYPNPARVEAAYHILEALQ* 382
                 * **** *    * * ***  ***   **   ** **   *
P.aeruginosa 350 GAYVAFVPGRDLGLVILANRNYPNAERVKIAYAILSGLEQQGKVPLKA* 399
                 * **** *   ** * *** ****   **   *   **   *
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Fig. 3. Comparison of the derived amino acid sequences of the *P. aeruginosa* PAO1 β-lactamase with *ampC* sequences from *E. cloacae* and *E. coli*

The amino acid sequence of AmpC from *E. cloacae* is from Honoré *et al.* (1986), and the sequence data for *E. coli* are from Jaurin & Grundstrom (1981). The signal sequences are italicized, the active-site sequences (Knott-Hunziker *et al.*, 1982) are underlined, and identical amino acids are marked with an asterisk.

motif near to the *N*-terminus. Fig. 4 shows the location of this motif in the *P. aeruginosa*, *E. cloacae* and *C. freundii* sequences. The very close similarity between the amino acid sequences and the presence of a possible helix–turn–helix DNA-binding motif at the *N*-terminus suggest that this second ORF codes for the *P. aeruginosa* AmpR regulatory protein. On the assumption that this gene product acts to regulate *ampC* transcription by binding to the DNA upstream of the *ampC* gene, we have attempted to align the sequences between the *P. aeruginosa ampR* and *ampC* ORFs with the corresponding regions from *E. cloacae* and *C. freundii*. No alignment giving a high degree of agreement could be found. Fig. 5 shows the best alignment with respect to the *ampR* initiation codons. Lindquist *et al.* (1989*b*) have shown, by DNAase I footprinting, that AmpR from *C. freundii* acts as a transcriptional activator by binding to a 38 bp DNA region in the *ampR–ampC* intercistronic region. This 38 bp sequence is shown above the *E. cloacae* sequence, which has 21/38 bases in common with the *C. freundii* sequence. We were unable to identify any part of the *P. aeruginosa* intercistronic region which directly matched the 38 bp region from *C. freundii*. There is, however, a 38 bp sequence which is significantly similar to the inverted *C. freundii* sequence (Fig. 5). This sequence overlaps the transcription-initiation codon for AmpR. We suggest that this sequence is likely to be the DNA-binding site for the helix–turn–helix identified at the *N*-terminus of AmpR (Fig. 4).

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                                               helix-turn-helix motif
C. freundii     1 MTRSYIPLNSLRAFEAAARHLSFTRAAIELNVTHSAISQHVKSLE 45
                    ***** ****************** ***************** **
E. cloacae      1 MTRSYLPLNSLRAFEAAARHLSFTHAAIELNVTHSAISQHVKTLE 45
                    * *  **** * **** ******* ***** **  * *  ** **
P. aeruginosa   1 MVRPHLPLNALAAFEASARHLSFTRAAIELCVTQAAVSHQVKSLE 45
                    * *   *** * **** ************* **  * *  *****

C. freundii    46 QQLNCQLFVRGSRGLMLTTEGESLLPVLNDSFDRMAGMLDRFATK 90
                    * ********* *********** *********** ********
E. cloacae     46 QHLNCQLFVRVSRGLMLTTEGENLLPVLNDSFDRIAGMLDRFANH 90
                      *   ** *  ****** *** ***** ********* * **
P. aeruginosa  46 ERLGVALFKRLPRGLMLTHEGESLLPVLCDSFDRIAGLLERFEGG 90
                      *   ** *  ****** ********* ***** ** * **

C. freundii    91 QTQEKLKIGVVGTFAIGCLFPLLSDFKRSYPHIDLHISTHNNRVD 135
                    ************* * **  * ** * ******  ********
E. cloacae     91 RAQEKLKIGVVGTFATGVLFSQLEDFRRGYPHIDLQLSTHNNRVD 135
                       *  * ****  * *   ****    * *** *********
P. aeruginosa  91 HYRDVLTVGAVGTFTVGWLLPRLEDFQARHPFIDLRLSTHNNRVD 135
                       *  * ****  * * * ****    * ***  ********
```

Fig. 4. Comparison of the *N*-terminal amino acids of AmpR proteins

The deduced amino acid sequence of the *N*-terminal 135 residues of the proposed AmpR protein from *P. aeruginosa* PAO1 is compared with the corresponding sequences from *C. freundii* O S60 (Lindquist *et al.*, 1989*b*) and *E. cloacae* MNH1 (Honoré *et al.*, 1986). Identical amino acids are marked with an asterisk (*). The proposed helix–turn–helix DNA binding motif is shown (Dodd & Egan, 1987).

This work was supported by Cystic Fibrosis Research Trust (grant no. 270). For software support we thank Dr. Martin Horder, University of Birmingham Computing Service, University of Birmingham, and Staff at the Scientific and Engineering Research Council SEQNET Facility, Daresbury, Warrington, Cheshire.

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                             CCTGTTAGAAAAACTTATATCTGCTGCTAAATTTAACC

                             * ********** * * * **  **************                            ampC
                                                              -35              -10        ~~~~>
E. cloacae     AGCTTCGCGTCATGGTCTCTTCCGTTAGAAAAATTAACAGCTAATGCTAAATTTAACCGTTTGTCAGGCACAGTCAAATCCAACAGACTACGCCTGTCTGACGGCCCGGACA
               TCGAAGCGCAGTACCAGAGAAGGCAATCTTTTTAATTGTCGATTACGATTTAAATTGGCAAACAGTCCGTGTCAGTTTAGGTTGTCTGATGCGGACAGACTGCCCGGGCCTGT
                    ampR Met               <~~~~            -10               -35

               *    **    ***** *   *     ***  ***  ** *  **  ******    *   *         *** *  ** * * * ** * ***

P. aeruginosa  ATGGGGTCGAACCAATCTCTGCTCCAAATTTTTCTAATGGCTGCCGCGAGTATTCGTCGTTTGCGGCAAATCCTGCGCAAGCCTAGATTTTCCCCGCCCGCCGATCAAGGAGC
               TACCCCAGCTTGGTTAGAGACGAGGTTTAAAAAGATTACCGACGGCGCTCATAAGCAGCAAACGCCGTTTAGGACGCGTTCGGATCTAAAAGGGGCGGGCGGCTAGTTCCTCG
                     ampR Met
                  ** *  *   *    * * *  ** *********  *

                  GGTTAAATTTAGCAGCAGATATAAGTTTTTCTAACAGG
```

Fig. 5. Alignment of nucleotide sequence from the *ampC–ampR* intercistronic regions from *P. aeruginosa* PAO1 and *E. cloacae*

The nucleotide sequences are arranged showing the best alignment starting with the AmpR initiation codons. The *E. cloacae* MNH1 sequence is from Honoré *et al.* (1986). The sequence of the 38 bp protected region, proposed to be the binding site in *C. freundii* for *ampR* (Lindquist *et al.*, 1989*b*), is shown above the *E. cloacae* sequence, and the corresponding inverted sequence is shown below the *P. aeruginosa* sequence. The translational and transcriptional start points and the −10 and −35 regions from *ampC* and *ampR* are indicated.

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Received 7 September 1990/8 October 1990; accepted 18 October 1990

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