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A common system controls the induction of very different genes

The class-A β-lactamase of *Proteus vulgaris* and the enterobacterial class-C β-lactamase

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Among the Enterobacteriaceae, *Proteus vulgaris* is exceptional in the inducible production of a 29-kDa β-lactamase (cefuroximase) with an unusually high activity towards the β-lactamase-stable oximino-cephalosporins (e.g. cefuroxime and cefotaxime). Sequencing of the corresponding gene, *cumA*, showed that the derived CumA β-lactamase belonged to the molecular class A. The structural gene was under the direct control of gene *cumR*, which was transcribed backwards and whose initiation codon was 165 bp away from that of the β-lactamase gene. This resembled the arrangement of structural and regulator genes *ampC* and *ampR* of the 39-kDa molecular-class-C β-lactamase AmpC present in many enterobacteria. Moreover, cloned genes *ampD* and *ampG* for negative modulation and signal transduction of AmpC β-lactamase induction, respectively, were also able to restore constitutively CumA overproducing and non-inducible *P. vulgaris* mutants to the inducible, wild-type phenotype. The results indicate that controls of the induction phenomena are equivalent for the CumA and AmpC β-lactamase. Very different structural genes can thus be under the control of identical systems.

The chromosomal gene *ampC*, encoding a 39-kDa β-lactamase (molecular class C, Jaurin and Grundström, 1981) is present in many species of enterobacteria, including members of the Proteeae (Bergström et al., 1982, 1983). The function of AmpC β-lactamase as a major factor of resistance to β-lactam antibiotics results largely from its inducibility to high concentrations by β-lactam antibiotics. Detailed studies of the AmpC induction mechanism have revealed the essential participation of additional genes, *ampR*, *ampG* and the *ampDE* operon. These encode a transcriptional regulator (Lindberg et al., 1985; Honoré et al., 1986; Lindquist et al., 1989a), a transducer of the external induction signal (Korfmann and Sanders, 1989; Lindquist et al., 1993) and a negative modulator of AmpD (Lindberg et al., 1987) with additional, still undefined functions of *ampE* (Honoré et al., 1989; Lindquist et al., 1989b), respectively.

In *Proteus vulgaris*, the prototype of the indole-positive Proteeae, AmpC β-lactamase is absent and the genes *ampC* and *ampR* are not found in the typical location adjacent to the fumerase-reductase-encoding (*frd*) operon as in AmpC-producing enterobacteria (Cole, 1987). Instead, a chromosomal, inducible β-lactamase is present (Matsubara et al., 1981; Maejima et al., 1987; Cullmann and Seibert, 1986; Aspiotis et al., 1986; Yang and Livermore, 1988; Essig, 1984) which, with its molecular mass of 29 kDa, substrate profile and specific inactivation by clavulanic acid, resembles the molecular class-A plasmid-encoded TEM β-lactamase (Hedges et al., 1974; Sutcliffe, 1978; Fisher et al., 1980) and chromosomal β-lactamases present in a minority of Gram-negative bacteria, such as *Klebsiella pneumoniae* (Arakawa et al., 1986), *K. oxytoca* (Arakawa et al., 1989), *Citrobacter diversus* (Perilli et al., 1991) and *Pseudomonas stutzeri* (Franceschini et al., 1993). The *P. vulgaris* β-lactamase hydrolyses the β-lactamase-stable oximino-cephalosporins (cefuroxime, cefotaxime and congeners) at a high rate and is also effectively induced by β-lactams of this group. Therefore, the designation cefuroximase has been proposed for this enzyme (Yotsuji et al., 1982; Cullmann and Seibert, 1986; Maejima et al., 1987; Essig, 1984).

In the present paper, we report the cloning and nucleotide sequences of the contiguous *P. vulgaris* cefuroximase genes *cumA* and *cumR* encoding a typical class-A β-lactamase CumA and a transcriptional regulator protein CumR, respec-

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Abbreviations. cumA, gene of *Proteus vulgaris* encoding CumA cefuroximase, a β-lactamase hydrolysing cefuroxime and other oximino cephalosporins; *frd*, fumarate reductase gene; *kan*, kanamycin-resistance gene; ORF, open reading frame.

Enzymes. β-Lactamase (EC 3.5.2.6); DNA-ligase (EC 6.5.1.1); RNase A (EC 3.1.27.5); alkaline phosphatase (EC 3.1.3.1); DNA polymerase (EC 2.7.7.7); lysozyme (3.2.1.17); fumarate reductase (EC 1.3.99.1).

Note. The novel nucleotide sequence data published here have been submitted to the EMBL Data Bank and are available under accession number X80128 PVCUMRA.

tively with homology to the class-C β-lactamase regulator AmpR. Moreover, we show that equivalents of the class-C β-lactamase signal transducer and negative modulator genes *ampG* and *ampD* are also present in *P. vulgaris* and can be interchangeably used for inducing expression of the class-C and class-A β-lactamases.

MATERIALS AND METHODS

Bacterial strains and plasmids

P. vulgaris U7, B317 and 1753 were clinical strains obtained from Dr W. Cullmann and Professor W. Opferkuch, Institut für Medizinische Mikrobiologie und Immunologie, Ruhr Universität, Bochum, Germany. The β-lactamase-overproducing mutants B317D and 1753D were isolated from colonies of survivors after plating $1-5\times10^8$ cells of *P. vulgaris* B317 and 1753 on Diagnostic Sensitivity Test Agar (Oxoid; Yang and Livermore, 1988) containing cefotaxime in the range 0.05−0.25 μg ml^{-1}. Mutants B317G and B317R with non-inducible β-lactamase were obtained from B317 bacteria by mutagenesis with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (Adelberg et al., 1965). However, 500 μg *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine ml^{-1} was used. Colonies of the mutagenized cells were replica plated onto agar containing 200 μg ampicillin ml^{-1}, and non-inducible mutants were detected in colonies unable to grow on ampicillin agar. The identity of the original *P. vulgaris* strains and mutants was confirmed with the Api 20E system (bioMérieux).

Escherichia coli strains were DH5 α (Gibco BRL) as host for cloning vectors, S17-1 (Simon, 1989) as mobilizing donor for plasmid transconjugation, and SN03/pNU305 (Lindberg et al., 1985) and SN0301/pNU413 (Lindquist et al., 1989b), for expression of AmpR protein.

Plasmid cloning vectors were pK18 (Pridmore, 1987) and pACYC184 (Chang and Cohen, 1978). Derivatives of the mobilizable pKT231 (Bagdasarian et al., 1981) were used for transconjugation in *P. vulgaris*. pMD101 contains cloned *ampR* and *ampC* of *C. freundii* inserted into the *Eco*RI site of pKT321 (Tölg et al., 1993). pMD201 was constructed by inserting the cloned ampDE operon of *E. coli* in an 11-kbp *Eco*RI fragment from pNU413 (Lindquist et al., 1989b) into the *Eco*RI site of pKT231. Plasmid pGKS273-3 ($ampG^+$) was kindly provided by Dr Gisela Korfmann, Bayer AG, Leverkusen, Germany. It contains the cloned gene *ampG* of *E. coli* JRG582 (Lindquist et al., 1993) on an *Eco*RV fragment inserted into the *Eco*RV site of pACYC184 (G. Korfmann, unpublished results). For the construction of pMD301, *ampG* on a 5.5-kbp *Eco*RI fragment from pGKS273-3 (Schmidt, 1991) was inserted into the *Eco*RI site of pKT231. Plasmid pMD401 ($ampR^+$, $ampC^-$) was used for complementation assays with gene *ampR*. It was obtained from pMD101 ($ampR^+$, $ampC^+$) by partial digestion with *Cla*I. The 20.1-kbp plasmid was isolated by excision of the 0.7-kbp fragment between the $ClaI_1$ and $ClaI_2$ sites of gene *ampC* (Bergström et al., 1983), religation and selection of kanamycin-resistant transformants in which the additional $ClaI_3$ site in the *kan* gene of vector pKT231 (Bagdasarian et al., 1981) had remained intact. For the construction of pMD501, *cumR* on a 2.5-kbp *Eco*RI−*Bgl*II fragment from pMDA11 was inserted into the *Eco*RI−*Bgl*IIsite of pKT231.

Plasmid-transfer procedures

Transformation into *E. coli* was performed according to Hanahan (1983). Transconjugation of derivatives of pKT231 from the auxotrophic donor *E. coli* S17-1 into *P. vulgaris* was carried out as described by Tölg et al. (1993).

Culture media

The growth medium for routine purposes was Luria-Bertani broth (Miller, 1972) supplemented with tetracycline (20 μg ml^{-1}), chloramphenicol (20 μg ml^{-1}) and kanamycin (100 μg ml^{-1}), where required. The minimal medium of Grabow and Smit (1967), supplemented with serine and glycine (20 μg ml^{-1}, each), was used for transconjugation into *P. vulgaris*. High yields of β-lactamase for enzyme purification were obtained from *P. vulgaris* grown in Mueller-Hinton medium (E. Merck, Darmstadt; Essig, 1984).

DNA techniques

Chromosomal DNA of *P. vulgaris* B317 was isolated by the method of S. Lindquist and B. I. Marklund as described in Schmidt (1991). Briefly, bacteria were converted to spheroplasts by lysozyme-Tris-EDTA-sucrose treatment and lysed with SDS. The crude lysate was incubated with RNase A. Then, it was extracted repeatedly with a mixture of 2 vol. Tris/HCl-equilibrated phenol and 1 vol. chloroform-2-pentanol (24:1, by vol.), followed by chloroform/2-pentanol extraction. From the aqueous solution, DNA was separated in two successive precipitations with 0.3 M sodium acetate and 2-propanol (0.6 vol.) and ethanol (2.5 vol.), respectively, and washed with ethanol.

Plasmid DNA was prepared with a Qiagen Plasmid Purification Kit (Diagen GmbH).

Two different genomic libraries of *P. vulgaris* DNA were generated. Fragments of partially *Sau*3A-digested DNA were cloned into pK18, linearized with *Bam*HI and dephosphorylated with calf intestine alkaline phosphatase. Alternatively, fragments from completely *Hind*III-digested DNA were separated electrophoretically. Fragments of 5−7 kbp were isolated from the agarose gel with a Glass Max Spin Cartridge Kit (Gibco BRL), and cloned into *Hind*III-cleaved and dephosphorylated pACYC184.

General cloning techniques, digestion with restriction endonucleases, agarose gel electrophoresis of DNA fragments, fragment ligation and other enzymic treatments, and selection procedures were carried out as described by Sambrook et al. (1989).

Polymerase chain reaction (PCR)

The technique described by Saiki et al. (1985, 1988) was used. The reaction mixture (100 μl) contained *P. vulgaris* chromosomal DNA (2 μg), the primers (0.5 μM, each), the deoxyribonucleotide triphosphates (200 μM, each) and Vent DNA Polymerase (4 units) in Vent buffer (New England Biolabs). Samples were submitted to 30 amplification cycles on an automated thermal cycler (New Brunswick), with 1 min denaturation at 94°C, 1.5 min annealing at 45°C and 2 min polymerization at 72°C.

PCR amplification and cloning of *cumR* and *cumA*

Primers constructed according to *P. vulgaris* codon usage (Cole, 1987) were (a) 5′ TCG ATA AGA GCG TAA TAA 3′ (upstream), a consensus sequence of genes *ampR* of *Enterobacter cloacae* (Honoré et al., 1986), *C. freundii* (Lindquist et al., 1998a), *R. capsulata* (Campbell et al., 1989) and *Y.*

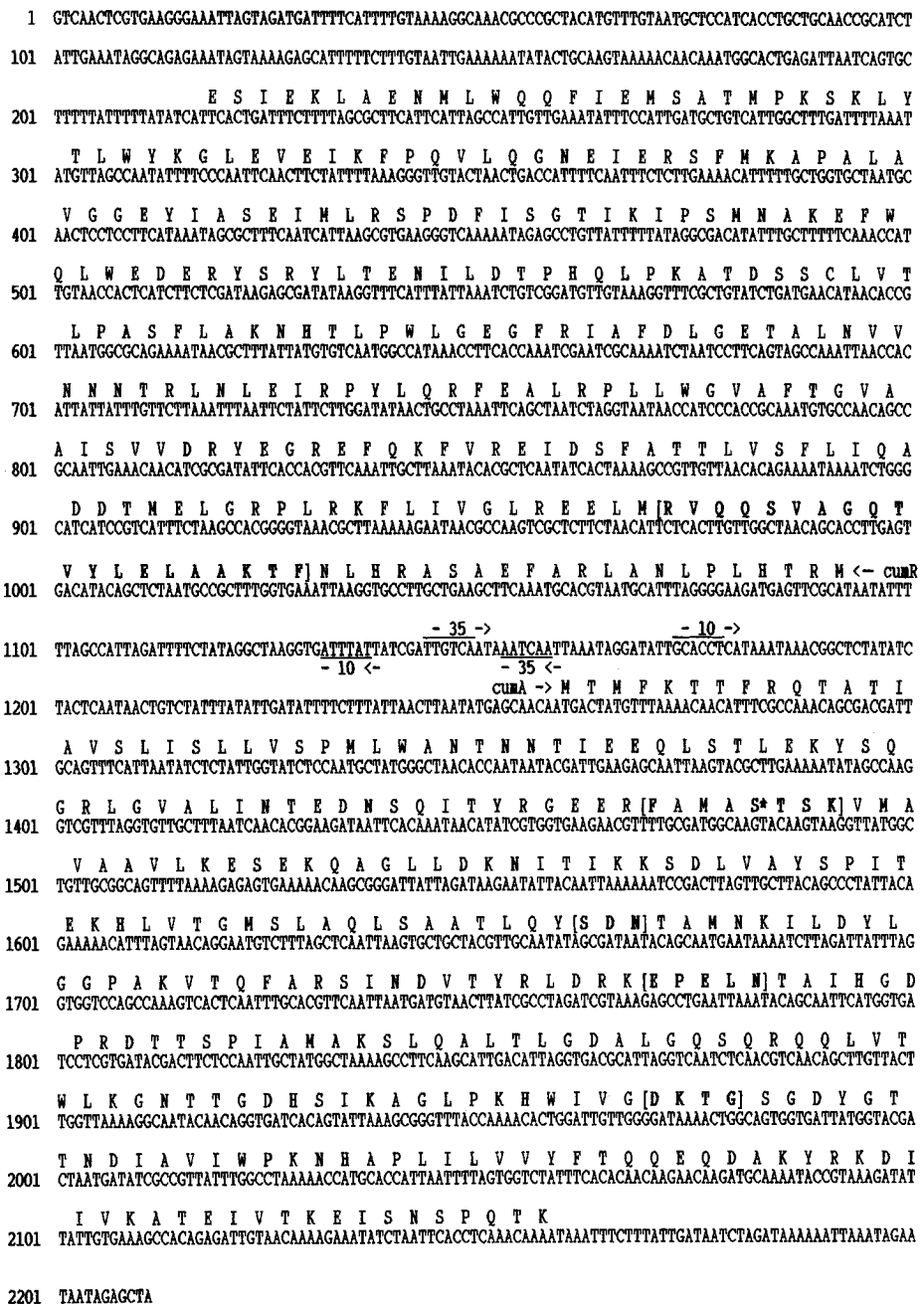

Fig. 1. Nucleotide sequence together with the deduced amino acid sequence in one-letter notation of *P. vulgaris* B317 β-lactamase regulatory and structural genes *cumR* (positions 1093–215) and *cumA* (positions 1259–2161), transcribed in the opposite directions from a 165-bp intercistronic region. The partially overlapping promoters of the two genes are underlined. In the N-terminal part of the protein sequence transcribed from *cumR*, the DNA-binding site is delineated by the segment in brackets derived from nucleotide positions 1030−971. The protein transcript of cumA contains typical structural features of a class-A β-lactamase (shown in brackets), the active site S*-T-S-K (S*, active serine) and its limiting elements S-D-N, E-P-E-L-N and D-K-T-G (Joris et al., 1991).

enterocolitica (Seoane et al., 1992) and (b) 5′ ACC AGC ACC GGT TTT ATC 3′ (down-stream), a consensus sequence of the KTG region of serine β-lactamases. Amplification with chromosomal DNA of *P. vulgaris* B317 and the primers yielded a 1.47-kbp product which was cloned into the *Sma*I site of pK18 to produce pMDA01. Excision of a 1-kbp *Hinc*II fragment from the insert and subcloning into the *Hinc*II site of pK18 yielded pMDA02. The nucleotide sequence of the inserts in both plasmids (Fig. 1) contained two open reading frames (ORF), transcribed in opposite directions from a 165-bp intervening region. The original 1.47-kbp insert contained an incomplete 576-bp ORF1 with the features of a β-lactamase transcriptional regulator (*cumR*) and the 729-bp upstream sequence of a second, incomplete ORF2 with β-lactamase features (*cumA*). The complete ORF1 was detected by colony hybridization. A gene bank of partially *Sau*3A-digested chromosomal DNA of *P. vulgaris* B317 in pK18 was screened in host strain *E. coli* DH5 α, with the 1.47-kbp PCR product as probe. Two recombinant plasmids, pMDA11 and pMDA12, with the complete 879-bp *cumR* gene from start codon ATG at position 1093 to stop codon TGA at position 215 was obtained. For the recovery

of the entire ORF2, fragments of *Hind*III-digested, chromosomal B317 DNA were separated electrophoretically and screened by DNA hybridization with the 1-kbp *Hinc*II fragment from pMDA02 as probe. DNA from a fragment band giving a positive signal on the agarose gel, was eluted, purified, cloned into the *Hind*III site of pACYC184, and transformed into *E. coli* DH5 α. Colonies containing recombinant plasmids were detected by hybridization with the 1-kbp *Hinc*II fragment. A plasmid designated pMDA21 was found which contained the complete 903-bp *cumA* gene from start codon ATG at position 1259 to stop codon TAA at position 2161. For the construction of pMDA31 (cumR$^+$, cumA$^+$), cumR on a 1.6-kbp *Xba*I−*Cla*I fragment from pMDA12 was inserted into the *Xba*I−*Cla*I$_2$ site of pMDA21.

DNA-DNA hybridization

DNA-DNA hybridization was performed with digoxigenin 3′ end-labeled DNA probes. The DIG DNA Labeling and Detection Kit (Boehringer Mannheim) was used as described by the supplier, with slight modifications according to Bertram and Gassen (1991).

Gel-mobility-shift assay

Assays, procedures and preparation of bacterial protein extracts were carried out according to Fried and Crothers (1981) and Lindquist et al. (1989a), with modifications. A 206-bp, digoxigenin 3′ end-labeled DNA segment containing the 165-bp *cumR*−*cumA* intercistronic region of *P. vulgaris* was prepared by PCR using digoxigenin 3′ end labeled oligonucleotides TAGGGGAAGATGAGTTCGCAT and TGTTGTTTTAAACATAGTCATTGTT (positions 1073−1093 and 1279−1255, respectively, of the nucleotide sequence shown in Fig. 1) as primers, and plasmid pMDA31 (*cumR*, *cumA*) as template. Binding mixtures (10 µl) contained 5 µg protein extract, 2 µg poly (dI-dC) (Pharmacia) and 2 pmol labeled DNA segment, in addition to the previously described components.

DNA sequencing

Nucleotide sequences were determined according to Sanger et al. (1977), using M13/pUC universal and reverse oligonucleotides (Boehringer Mannheim) or synthetic oligonucleotides (Eurogentec) as primers, and with denaturation of double-stranded DNA according to Zhang et al. (1988). Sequencing reactions were carried out with the Autoread Sequencing Kit (Pharmacia), with incorporation of fluorescent dATP. The electrophoresis and sequence reading was carried out with an A. L. F. DNA Sequencer (EMBL).

Searches through the nucleic acid (EMBL version 30) and protein (PIR version 32) sequence databases were performed using the procedure of Pearson and Lipman (1988) (FASTA and TFASTA softwares, GCG package).

Isolation and purification of β-lactamase

The β-lactamase overproducing mutant strains *P. vulgaris* B317D and 1753D were grown to late-exponential phase in shake cultures of 10 L Mueller-Hinton medium. The periplasmic β-lactamase was liberated by suspending the harvested bacteria (1 g wet mass ml^{-1}) in an ice-cold solution of Tris/HCl (30 mM, pH 8.0), EDTA (0.2 mM), phenethyl alcohol (1%, by vol.), sucrose (27%, mass/vol.) and lysozyme (8 µg ml^{-1}), followed by three cycles of freezing in methanol/dry ice and thawing at 37°C, and removal of the cell debris from the lysate by centrifugation (Jacobs et al., 1992). From the clear supernatant solution, the β-lactamase was purified in the FPLC system (Pharmacia) by two successive steps of cation-exchange chromatography on carboxymethyl-Sephadex C-50 and Mono S HR 5/5 columns (Pharmacia) in 10 mM potassium phosphate, pH 6.5, and elution with linear 0−1 M NaCl gradients. This was followed by gel filtration on a Sephadex TM 75 column (Pharmacia) in 10 mM potassium phosphate, pH 7.0.

The purity and apparent molecular mass of the β-lactamase were examined by SDS/PAGE. Isoelectric focusing was carried out on ready-made ampholine polyacrylamide gel plates, pH 3.5−9.5, on a Multiphor II system (Pharmacia).

The N-terminal sequence of the purified β-lactamase was determined by Edman degradation with an Applied Biosystems 470-A gas-phase sequenator.

Table 1. Kinetic parameters of hydrolysis of β-lactam substrates by β-lactamase of P. vulgaris B317. n.h., no hydrolysis detected; n.d., not determined.

Substrate	K_m	k_{cat}	k_{cat}/K_m
	µM	s^{-1}	$M^{-1}\,s^{-1}$
Penicillin G	6.0	2.9	4.8×10^5
Nitrocefin	11.6	5.7	4.9×10^5
Cefuroxime	197.0	98.0	5.0×10^5
Cefotaxime	250.0	58.2	2.3×10^5
Oxacillin	n.d.	n.h.	n.d.

β-lactamase assays and determination of enzyme kinetic parameters

Induction of β-lactamase was carried out as described by Lindberg et al. (1985, 1987). β-lactamase activities and values of K_m and k_{cat} were determined by computerized spectrophotometry, using the wavelengths and absorbance variations described by Matagne et al. (1990) for different β-lactam substrates. One unit (U) or milliunit (mU) of β-lactamase hydrolyse 1 µmol or 1 nmol, respectively, of substrate min^{-1} in sodium potassium phosphate (10 mM, pH 7.0). For the β-lactamase inactivator, β-iodopenicillanic acid, the parameter k_2/K of acyl enzyme formation was measured with nitrocefin as reporter substrate (De Meester et al., 1987). The deacylation constant k_3 was determined as k_{cat} at $[S] \gg K_m$.

Specific β-lactam compounds were cefuroxime and cefotaxime (Hoechst), nitrocefin (Glaxo), cephalothin and cefazolin (Eli Lilly), β-iodopenicillanic acid (Pfizer) and 6-aminopenicillanic acid (Beecham). The penicillins were commercial products.

RESULTS

Properties of the β-lactamase of *P. vulgaris* B317D

As estimated by SDS gel electrophoresis, the molecular mass of the enzyme was 29 ± 1 kDa. Its isoelectric pH was 8.0. Table 1 highlights the high activity of the enzyme with oximino-cephalosporins as substrates, a property quite unlike that of class-C β-lactamases. Since the enzyme exhibited no detectable oxacillinase activity and was not inhibited by EDTA, it could be concluded that it belonged either to class

A or a new, original class. The determination of the N-terminal sequence as N T N N T I E E Q L S T did not solve the ambiguity, although an E X Q L S sequence is also found in two class-A β-lactamases, PIT-2 (Barthélémy et al., 1988) and LEN-1 (Arakawa et al., 1986). The B317-enzyme was inactivated rapidly by β-iodopenicillinate in the fashion typical of a class-A β-lactamase (De Meester et al., 1986). The rate and efficiency of acylation were k_2, 0.109 s^{-1} and k_2/K, 3.3×10^5 M^{-1} s^{-1}, with a low rate of deacylation k_3, 2.9×10^{-3} s^{-1}.

Cloning and sequencing of the contiguous β-lactamase transcriptional regulator and structural genes *cumR* and *cumA* of *P. vulgaris*

The chosen cloning technique relied on the observation that structural and transcriptional regulator genes of inducible β-lactamases share common features, not only of sequence homology but also of chromosomal arrangement in different Gram-negative bacteria. Genes *ampR* and *ampC* in *Ent. cloacae* (Honoré et al., 1986), *Citrobacter freundii*, (Lindberg et al., 1985; Lindquist et al., 1989a), *Yersinia enterocolitica* (Seoane et al., 1992) and *Pseudomonas aeruginosa* (Lodge et al., 1990) and also the regulator and structural genes of the class-A β-lactamase in *Rhodopseudomonas capsulata* (Campbell et al., 1989) are equally located in adjacent chromosomal positions. They are also separated by short intercistronic regulatory regions from which they are transcribed in opposite directions.

The cloning of a comparable β-lactamase regulator-structural gene region *cumR*–*cumA* from *P. vulgaris* was carried out by PCR as described in Materials and Methods. The primers were (a) a consensus sequence outside the functionally defined regions of genes *ampR* (Bartowsky and Normark, 1991) of five Gram-negative bacteria, and (b) a consensus sequence of the KTG region common to most serine β-lactamases. Cloned *cumR* in plasmids pMDA11 and pMDA12, and *cumA* in pMDA21, and the contiguous genes *cumR* and *cumA* in pMDA31 were obtained. Fig. 1 shows the nucleotide sequences of the 879-bp *cumR* and 903-bp *cumA* genes, which are transcribed in opposite directions from an intervening 165-bp intercistronic region. The amino acid sequence deduced from *cumR* according to *P. vulgaris* codon usage (Cole, 1987) showed the characteristics of a typical regulator protein with a DNA-binding site in the N-terminal region (Henikoff et al., 1988) and with more than 40% identity to the known *ampR* gene products. The amino acid sequence derived from *cumA* yielded a protein with features of a class-A β-lactamase, such as an active site – F X X S* T X K – (where S* is the active serine) and S D N, E P E L N, and D K T G sequences suitably located approximately 60, 96 and 163 residues after the active site (Joris at al., 1991).

Inducible expression of *P. vulgaris* CumA β-lactamase from cloned genes *cumR* and *cumA* in *E. coli*

When *E. coli* DH5 α was transformed jointly with pMDA11 ($cumR^+$) and pMDA21 ($cumA^+$), or with pMDA31 ($cumR^+$, $cumA^+$), non-induced expression of CumA β-lactamase was low and could not be measured reliably above the background of the AmpC β-lactamase of the host bacterium. However, induction with 6-aminopenicillanic acid (250 μg ml^{-1}) increased the specific activities of the CumA enzyme in the two types of transformants fourfold and 13-fold to 79 mU mg $protein^{-1}$ and 133 mU mg $protein^{-1}$, respectively, and 10-times and 16-times above the control value of the non-inducible *E. coli* host (Lindberg et al., 1985; Honoré et al., 1986). These preliminary results showed that the *P. vulgaris* class-A β-lactamase can be inducibly expressed from cloned *cumR* and *cumA* in *E. coli* DH5 α, although feebly. The interrelation of the induction systems for the class-A and class-C β-lactamases was further studied in the complementation experiments described below.

Table 2. Expression of β-lactamases CumA of *P. vulgaris* (A) and AmpC of *C. freundii* (C) in different strains of *P. vulgaris*, and the *trans*-complementing effect of cloned regulatory genes of AmpC β-lactamase, *ampR*, *ampDE* and *ampG*, and of *P. vulgaris* gene *cumR* on the induction of the cumA enzyme. *P. vulgaris* strains: wild type, U7, B317, 1753; β-lactamase overproducing mutants, B317D, 1753D ($cumD^-$); non-inducible mutants, 317G ($cumG^-$), 317R ($cumR^-$). Induction with 6 μg cefotaxime ml^{-1} (U7, 1753) and 15 μg cefotaxime ml^{-1} (B317). Values are means of triplicate measurements with 0.2 mM cephalothin as substrate.

P. vulgaris strain	Plasmid-borne genes	β-Lactamase, specific activity	
		non-induced	induced
		mU mg $protein^{-1}$	
U7	–	3	40 (A)
U7/pMD101	*ampR*, *ampC*	20	300 (C)
B317	–	8	1900 (A)
B317D	–	2400	2500 (A)
B317D/pMD201	*ampDE*	14	1500 (A)
1753	–	5	1100 (A)
1753D	–	1300	1400 (A)
1753D/pMD201	*ampDE*	10	1100 (A)
B317G	–	2	2 (A)
B317G/pMD101	*ampR*, *ampC*	5	7 (C)
B317G/pMD301	*ampG*	2	1100 (A)
B317R	–	2	1 (A)
B317R/pMD101	*ampR*, *ampC*	4	1200 (C)
B317R/pMD301	*ampG*	2	2 (A)
B317R/pMD401	*ampR*	2	2 (A)
B317R/pMD501	*cumR*	6	1600 (A)

Common functions of genes *ampD* for negative modulation and *ampG* for signal transduction in the induction ofenterobacterial class-C β-lactamase, and *P. vulgaris* class-A β-lactamase

Inducible expression in P. vulgaris *of AmpC β-lactamase from cloned ampR, ampC of* C. freundii

Expression of *C. freundii* AmpC β-lactamase was studied in *P. vulgaris* strain U7, whose own β-lactamase is only poorly inducible (Essig, 1984). *C. freundii* genes *ampR* and *ampC* on plasmid pMD101 (Tölg et al., 1993) were introduced by transconjugation. The transconjugant U7/pMD101 contained an elevated amount of constitutively produced β-lactamase, and the enzyme was induced 115-fold by cefotaxime, a good inducer in *P. vulgaris* (Table 2). The expressed enzyme was identified as an AmpC β-lactamase by its resistance to clavulanic acid, and the typical substrate profile

[Galleni and Frère, 1988; Galleni et al., 1988; see high and very low rates of hydrolysis, respectively, of cephalothin and cefotaxime (data not shown)]. The inducible synthesis of AmpC β-lactamase in *P. vulgaris* indicates that, in this host, analogues of genes *ampD* and *ampG* are present whose functions support the activity of the heterologous genes *ampR* and *ampC*.

Trans-complementation of constitutive overproduction of P. vulgaris *CumA β-lactamase by cloned ampD of* E. coli

In *Ent. cloacae* (Honoré et al., 1989), *C. freundii* and *E. coli* (Lindberg et al., 1987; Lindquist et al., 1989b) mutation in gene *ampD* is known to eliminate the negative modulation of *ampR*-stimulated β-lactamase synthesis. This results in hyperinducibility or constitutive overproduction of AmpC β-lactamase, and thereby confers high β-lactam resistance to the mutant bacteria. In *P. vulgaris*, the isolation of cefotaxime-resistant, β-lactamase-overproducing mutants has also been reported (Yang and Livermore, 1988), suggesting a defect in an *ampD*-like gene in this species.

Cefotaxime-resistant isolates were selected from *P. vulgaris* strains B317 and 1753 as described in Materials and Methods. In the mutants B317D and 1753D, values of non-induced β-lactamase activity were 300-times and 260-times those of the wild type, and minimal inhibitory concentrations of cefotaxime increased 16-fold from 0.12 and 0.06 to 2 and 1 μg ml^{-1}, respectively. However, in contrast to *ampD* mutants of AmpC-synthesizing enterobacteria (Lindberg et al., 1987; Lindquist et al., 1989) and as reported previously (Yang and Livermore, 1988), β-lactamase was not further inducible in the overproducing *P. vulgaris* mutants (Table 2).

Complementation of CumA overproduction in *P. vulgaris* strains B317D and 1753D by *ampD* from *E. coli* was tested by introducing the cloned *E. coli ampDE* operon (Lindquist et al., 1989) on plasmid pMD201 by transconjugation. In transconjugants B317D/pMD201 and 1753D/pMD201, CumA overproduction was eliminated and normal, inducible expression of the enzyme was restored (Table 2). This agrees with the assumption that β-lactamase overproduction in *P. vulgaris* B3127D and 1753D was caused by mutation in a negative modulator gene, tentatively designated *cumD*, and that its function could be replaced by *ampD*.

Trans-complementation of different mutations to the non-inducibility of P. vulgaris *CumA β-lactamase by cloned ampG of* E. coli *or cloned cumR of* P. vulgaris

Non-inducible mutants of *P. vulgaris* B317 and 1753 were obtained by mutagenesis with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine and selection of ampicillin-sensitive clones. Assuming a mechanism of induction in *P. vulgaris* similar to that proposed for AmpC β-lactamase (Lindquist et al., 1993), loss of inducibility could be caused either by a mutational defect in *cumR*, or in the postulated, *ampG*-analogous signal-transducer gene, tentatively named *cumG*. In agreement with this expectation, two types of mutants were distinguished when inducible expression of heterologous AmpC β-lactamase was tested after transconjugation of pMD101(ampR$^+$, ampC$^+$). In mutant transconjugants B317G/pMD101, AmpC β-lactamase also remained non-inducible, suggesting a defect in *cumG*. This notion was supported and functional equivalence of *cumG* and *ampG* was indicated by showing that the inducibility of CumA β-lactamase was restored when plasmid pMD301, containing cloned *ampG* of *E. coli*, was transconjugated into B317G (Table 2).

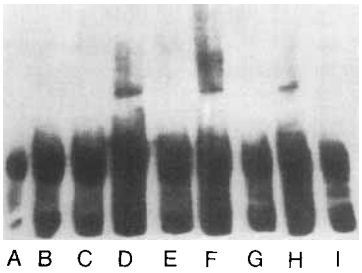

Fig. 2. Selective gel-mobility shift of the *P. vulgaris cumR* and *cumA* intercistronic region by cell extracts containing the CumR regulator protein. The intercistronic sequence in a DIG end-labeled 206-bp PCR product (A, I) was retarded by CumR from wild-type *P. vulgaris* B317 (H) and from pMD501 (cumR$^+$) in *E. coli* S 17−1/pMD501 (D) and in the *cumR*-defective *P. vulgaris* mutant B317R/pMD501 (F). No mobility shift was effected by extracts from mutant *P. vulgaris* 317R (G) and from *E. coli* SN0301/pNU344 (C) and SN03/pNU305 (D) containing AmpR expressed from cloned *ampR* of *C. freundii* (Lindberg et al., 1985; 1987). The position of the retarded fractions of the intercistronic region is indicated by the arrow.

The second type of mutant, B317R, was not reverted to inducibility of the CumA enzyme by pMD301 (ampG$^+$). In contrast, inducible expression of AmpC β-lactamase in transconjugant B317R/pMD101 was unimpaired. This suggested the presence of intact *cumG* and defective *cumR* in B317R. In confirmation of this notion, the inducibility of mutant B317R was restored by introducing cloned *cumR* into pMD501 by transconjugation. Remarkably, however, B317R failed to regain inducibility upon transconjugation of cloned *ampR* of *C. freundii* in plasmid pMD401 (Table 2). These findings indicated a difference in the ability of CumR and AmpR regulator proteins to activate the expression of cumA β-lactamase. An explanation for this difference was apparent from gel-mobility-shift assays testing the binding of *cumR* and *ampR* products to the *cumR−cumA* intercistronic region of *P. vulgaris*. This region was present in a 206-bp, digoxigenin 3′ end-labeled PCR product constructed with the appropriate template and primers. Cellular extracts containing CumR protein were from *P. vulgaris* strains B317 and B317R/pMD501, and from *E. coli* S17-1/pMD501. Preparations with AmpR were obtained from *E. coli* SN03/pNU305 (Lindberg et al., 1985) and SN0301/pNU413 (Lindquist et al., 1989b). Control extracts were from the *cumR*-defective mutant *P. vulgaris* B317R and from AmpR-less *E. coli* S17-1. Gel retardation of the intercistronic region was effected only by extracts from *cumR*-expressing cells but not by extracts containing AmpR and by controls (Fig. 2). This indicated that cumR is a DNA-binding regulatory protein which specifically interacts with the *P. vulgaris cumR−cumA* intercistronic region, whereas AmpR is unable to bind to this region.

Discussion

The chromosomal β-lactamase of *P. vulgaris* B317 described in this study is clearly identified as an enzyme of molecular class-A (Joris et al., 1991) by the protein sequence deduced from that of gene *cumA*. The 42% dC + dG content of *cumR* and *cumA* sequences corresponds to the 40% dC +

dG value in *P. vulgaris* DNA (Falkow et al., 1962), confirming the chromosomal provenance of both genes.

Deduction of the amino acid sequence from the *cumA* gene *according to P. vulgaris* specificity (Cole, 1987) yields a predicted sequence of 300 amino acids. A search using the method of Kyte and Doolittle (1982) shows the presence of a signal peptide with a putative cleavage site at position 29 of the N-terminal region, in agreement with the directly determined N-terminal sequence of the mature protein. For the resulting enzyme with 271 amino acids, a molecular mass of 29750 Da can be calculated. This agrees with the value determined for the isolated β-lactamase. The predicted CumA sequence shows 53, 79, 49 and 49% identity with the known chromosomal class-A β-lactamases of the Gram-negative *K. pneumoniae* (Arakawa et al., 1986), *K. oxytoca* (Arakawa, 1989), *R. capsulata* (Campbell et al., 1989) and to the TEM1 β-lactamase (Sutcliffe, 1978), respectively. There is no sequence similarity to class-C β-lactamases.

The amino acid sequence of the B317 β-lactamase also contains the class-A-specific features of the structural elements 1−4 that limit the active site (Joris et al., 1991).

Conversely, the *P. vulgaris* enzyme differs from many known class-A β-lactamases due to its high content of basic amino acids which is reflected in the high pI value. The structural features related to the efficient hydrolysis of cefuroxime and the cefotaxime group of β-lactamase-stable third-generation cephalosporins remain to be determined. The extended substrate specificity may be due, in part, to the presence of serine in position 237 which is located in box VII of the highly conserved and functionally important regions identified in β-lactam-recognizing active-site-serine enzymes (Joris et al., 1988). This is a change from alanine or glycine in class-A β-lactamases without oximino cephalosporin-hydrolyzing activity. Serine 237 has been found in the extended-spectrum β-lactamases MEN-1 (Barthélémy et al., 1992) and LENZYM (Von Tigerstrom and Boras, 1990). In equivalent positions of cefotaxime-hydrolyzing variants of TEM β-lactamase, serine or threonine have been shown to replace alanine or glycine (Jacoby and Medeiros, 1991).

The regulatory elements of induction of the *P. vulgaris* class-A β-lactamase show a striking similarity to those of the inducible class-C β-lactamases. Typical features of the transcriptional regulatory and structural genes *ampR* and *ampC* of class-C β-lactamase in *Ent. cloacae* (Honoré et al., 1986), *C. freundii* (Lindquist et al., 1989), *Y. enterocolitica* (Seoane et al., 1992) and *Ps. aeruginosa* (Lodge et al., 1990) are close linkage and divergent transcription from an intercistronic sequence carrying overlapping promoters and a binding site for the AmpR regulator protein. The same overall organization of the regulator and structural genes of a chromosomal class-A β-lactamase was first decribed in *R. capsulata* (Campbell, 1989) and is now recognized in detail in the *cumR* and *cumA* genes of *P. vulgaris*.

P. vulgaris-specific deduction of the amino acid sequence from *cumR* yields a regulator protein with 292 amino acids. The AmpR proteins of *C. freundii* (Lindquist et al., 1989a), *Ent. cloacae* (Honoré et al., 1986), *Y. enterocolitica* (Seoanne et al., 1992) and *R. capsulata* (Campbell et al., 1989) are of very similar size. Their sequence identities with CumR are 42%, 43%, 43% and 44%, respectively.

The non-inducible *cumR*-defective *P. vulgaris* mutant B317R could be transcomplemented to inducibility by cloned *cumR* but not by cloned *ampR* from *C. freundii*. An explanation is apparent from a comparison of the promoters and regulator-binding sites in the *ampR*, *ampC* and *cumR*, *cumA*

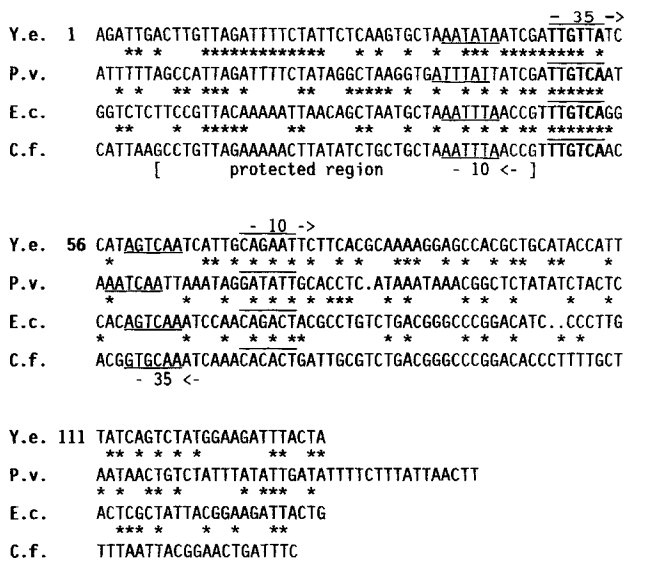

Fig. 3. Comparison of the nucleotide sequences of the intercistronic regions separating the regulatory and structural genes of class-A β-lactamase of *P. vulgaris* [P.v.], and of class-C β-lactamases of *C. freundii* OS60 [C.f.], *Ent. cloacae* [E.c.], and *Y. enterocolitica* [Y.e.]. The −10 and −35 regions of the promoters are shown. The regulator binding site (protected region) of *C. freundii* is indicated. The asteriks denote nucleotides identical with those in the *P. vulgaris* sequence.

intercistronic regions of different Gram-negative species (Fig. 3). In the highly conserved -35 promoter regions of the respective β-lactamase genes there is complete or almost complete identity between *P. vulgaris* and *C. freundii*, *Ent. cloacae* and *Y. enterocolitica*. However, in the regulator protein-binding, protected region described in *C. freundii* (Lindquist et al., 1989), similarity is again much lower, and only 42%, which may be insufficient for the recognition of the *P. vulgaris* binding site by the AmpR of *C. freundii*. This notion was confirmed by our gel-mobility-shift assays, where retardation of the *cumR*−*cumA* intercistronic region was caused only by extracts from cells expressing cumR but not by those containing AmpR.

It remains to provide molecular evidence in *P. vulgaris* for the tentatively assumed presence of genes for signal transduction, *cumG*, and negative modulation, *cumD*, analogues of the regulatory genes *ampG* and *ampD* of class-C β-lactamases. Attempts to locate *cumG* and *cumD* in *P. vulgaris* chromosomal DNA by hybridization with *ampG* and *ampD* probes were unsuccessful, presumably due to *P. vulgaris* DNA sequence specificity (M. Datz, unpublished observation). However, complementation assays have clearly demonstrated activities *of cumG* and *cumD* in *P. vulgaris* and their interchangeability with *ampG* and *ampD* for inducible expression of class-C and class-A β-lactamase. One may thus conclude that the respective gene products are functionally equivalent, if not physically very similar.

These interspecific activities may also reflect more general regulatory functions of genes *ampG* (*cumG*) and *ampD* (*cumD*) in an ubiquitous metabolic pathway of Gram-negative bacteria, and several lines of evidence point to an involvement of these genes in the metabolism of the cell wall peptidoglycan (Tuomanen et al., 1991; Lindquist et al., 1993; Tölg et al., 1993).

The results demonstrate that the induction mechanism of the class-A β-lactamase of *P. vulgaris* utilizes a pathway

which is identical to that observed in the control of synthesis of class-C β-lactamase in Gram-negative bacteria. In Gram-positive bacteria, several examples of a class-A β-lactamase under the direct control of an AmpR-like protein and of similar positionings of this control have already been described (Lampen et al., 1988; Lenzini et al., 1992). The identification of a similar control system in the class-A β-lactamase of the Gram-negative *P. vulgaris* documents the universal application of this regulatory principle in eubacteria.

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