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Molecular Genetic Analysis of Cephalosporinase Production and Its Role in β-Lactam Resistance in Clinical Isolates of *Enterobacter cloacae*

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Two strains of *Enterobacter cloacae* were isolated from a patient before (strain MHN1) and during (strain MHN2) treatment with moxalactam and gentamicin. Strain MHN1 exhibited inducible *ampC* cephalosporinase production. In contrast, strain MHN2 expressed the enzyme constitutively at a 3,000-fold higher level. With the *Escherichia coli ampC* gene as a hybridization probe it was shown that the genomic arrangement of the *ampC* region was the same in both strains. To gain more insight into regulatory phenomena, the *ampC* genes were cloned, and their expression was studied in *E. coli* K-12. The *ampC* gene from MHN1 behaved normally and conferred inducible β-lactam resistance. A regulatory region of at least 800 base pairs involved in controlling repression-induction was located immediately upstream of *ampC*. Surprisingly, when present in *E. coli* the *ampC* gene from MHN2 no longer overproduced the cephalosporinase, and inducible expression was observed. This indicates that in MHN2 stable cephalosporinase overproduction is controlled by another factor which is not linked to the *ampC* gene.

Many members of the family *Enterobacteriaceae* possess a chromosomally encoded cephalosporinase that may contribute to β-lactam resistance. In *Escherichia coli* this enzyme is coded for by the constitutively expressed *ampC* gene (15), which partially overlaps the adjacent fumarate reductase operon, *frdABCD* (10, 26). In the absence of plasmids or transposons, resistance to clinical concentrations of β-lactams is principally due to elevated levels of cephalosporinase production (3). This generally stems from increased transcription of the *ampC* gene; in *E. coli* this is often a consequence of up-promoter mutations (29), although more complicated genetic rearrangements such as gene duplication or transpositional activation have been described (7, 16, 27).

A different situation has been found with some members of the *Enterobacteriaceae*, such as *Enterobacter cloacae*, *Citrobacter freundii*, *Serratia* spp., and *Morganella morganii*. Increased β-lactamase production can be induced in vitro by exposing the bacteria to subinhibitory concentrations of some β-lactam antibiotics; upon their removal, enzyme levels decline. This reversible behavior led to the suggestion that *ampC* expression was regulated by a repressor gene which *E. coli* does not possess. Support for this interpretation was provided by the finding that the *C. freundii* chromosome differs from that of *E. coli*; the *ampC* gene is separated from the *frd* operon by an intergenic sequence of 1,100 base pairs (2). This extra DNA could therefore be involved in regulating transcription of the *ampC* gene or even represent the target of a repressor molecule.

Several mutants of *E. cloacae* have been described which are resistant to β-lactamase-stable cephalosporins. These greatly overproduce the cephalosporinase and are no longer able to turn off *ampC* expression or respond to induction (14, 24, 28, 30, 35). The corresponding mutations would thus appear to define the gene for the putative repressor.

To gain more insight into the regulatory mechanisms and their clinical implications, we have cloned the *ampC* genes from such a mutant of *E. cloacae* and its isogenic parent. Expression of the cloned genes was studied in *E. coli* K-12 and compared with that of both *E. cloacae* strains. As has been reported for *C. freundii*, an additional gene was identified between the *ampC* and *frd* operons and shown to function in both the induction and repression processes. However, the mutation leading to uncontrolled overexpression of the *ampC* gene was not localized in this area, indicating that at least one additional factor is required.

MATERIALS AND METHODS

Bacteria. Two clinical isolates of *E. cloacae* were isolated before (strain MHN1) and during (strain MHN2) treatment with moxalactam and gentamicin. For physiological studies with cloned *ampC* genes, the *E. coli* K-12 strain MI1443 [Δ(*frd-ampC*)*rps*L] was employed.

Media and antibiotics. Bacteria were grown in Mueller-Hinton medium for MIC determination, in tryptic soy broth (3.5%, wt/vol) for enzymological studies, and in L broth (25) for the routine subculture of *E. coli* derivatives. The following β-lactam antibiotics were used: ampicillin (Sigma Chemical Co., St. Louis, Mo.), cephalothin and cefamandole (Eli Lilly & Co., Indianapolis, Ind.), cefoxitin (E. Merck AG, Darmstadt, Federal Republic of Germany), and cefotaxime (Roussel UCLAF, France). Nitrocefin (Glaxo Pharmaceuticals Ltd., Greenford, England) and 7-amino cephalosporanic acid (Roussel) were generously provided by the manufacturers. Tetracycline (Sigma) and chloramphenicol (Boehringer GmbH, Mannheim, Federal Republic of Germany) were used at 12.5 and 50 μg/ml, respectively, with plasmid-containing *E. coli* strains.

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TABLE 1. β-Lactamase activity and β-lactam susceptibility of *E. cloacae* MHN1 and MHN2 and of *E. coli* K-12 strain MI1443 carrying *ampC* genes of MHN1 and MHN2

Strain	β-Lactamase activity (mU/mg of protein)		MIC (μg/ml)[a]			
	Uninduced	Induced[b]	AMP	CFT	CFM	CTX
E. cloacae						
MHN1	3	126	1,024	1,024	16	0.25
MHN2	9,284	9,999	8,192	≥8,192	≥2,048	1,024
E. coli K-12						
MI1443	≤1	≤1	1	0.5	0.25	≤0.12
MI1443(pEc1A)	≤1	≤1	1	0.5	0.25	≤0.12
MI1443(pEc1B)	166	334	4	64	2	0.5
MI1443(pEc1C)	32	200	4	32	0.5	≤0.12
MI1443(pEc2A)	≤1	≤1	1	0.5	0.25	≤0.12
MI1443(pEc2B)	160	284	8	64	2	0.5
MI1443(pEc2C)	21	108	4	32	0.5	≤0.12

[a] Drug abbreviations: AMP, ampicillin; CFT, cephalothin; CFM, cefamandole; CTX, cefotaxime.
[b] Cefoxitin (10 μg/ml) was used as the inducer.

Nucleic acid techniques. Chromosomal DNA was prepared by a modified version of the Marmur procedure (22). Restriction endonuclease analysis, agarose gel electrophoresis, Southern transfer, and DNA-DNA hybridization with ^{32}P-labeled probes were performed as previously described (6). Plasmid pNU31 was used as the source of *ampC* or *frd* specific probes (5). Libraries of *Bgl*II restriction fragments from *E. cloacae* strains were obtained by cloning into the positive selection vector lambda L47.1 (20) as recently outlined (12). Recombinant lambda phages carrying the *ampC* gene were identified by the plaque hybridization technique of Benton and Davis (1). The plasmid vector pACYC184 (4) was used for subcloning experiments.

Susceptibility tests. MICs were determined by serial twofold dilution tests with Mueller-Hinton broth and an inoculum of 10^5 CFU/ml. The MIC was defined as the lowest antibiotic concentration that inhibited growth after overnight incubation.

β-Lactamase assays. Microacidimetric assays of β-lactamase activity in cell-free extracts were performed as described previously (17) with cephalothin (33) as the substrate. Isoelectric focusing of cephalosporinase was as described by Matthew et al. (23). Protein concentrations were determined by the method of Lowry et al. (21) with bovine serum albumin as the standard. One unit of enzyme activity was defined as the activity which hydrolyzed 1 μmol of substrate per min. Specific β-lactamase activity was expressed as milliunits per milligram of protein.

RESULTS

Characterization of strains. In *E. cloacae* the production of the chromosomally encoded cephalosporinase is apparently subject to a regulatory network which may influence the exceptionally high level of resistance of this bacterium to the β-lactamase-stable cephalosporins. As a first step toward understanding how this regulation functions, we examined a pair of *E. cloacae* strains isolated from the same patient. These displayed identical biotypes and differed only in the levels of cephalosporinase production. Strain MHN1, isolated before antibiotic therapy, displayed wild-type cephalosporinase activity in that enzyme synthesis responded to induction with cefoxitin (Table 1). In contrast, MHN2, isolated during treatment with moxalactam and gentamicin, constitutively overproduced the cephalosporinase and was resistant to extremely high levels of β-lactam antibiotics. Isoelectric focusing and enzymological studies employing various substrates and inhibitors showed that the enzyme was identical in both strains and that it was overproduced in MHN2 by about 3,000-fold. These findings suggested that the mutation responsible had directly affected a component regulating the expression of the *ampC* gene.

Organization of the *ampC* region of the *E. cloacae* chromosome. To see whether any gross genetic alterations could account for the difference in cephalosporinase production, chromosomal DNA was isolated from MHN1 and MHN2 and subjected to Southern blot analysis with probes derived from the *E. coli ampC* gene (Fig. 1a). The results of an experiment in which *Eco*RI-digested samples were hybridized with probes specific for the 3′ end of *ampC* (probe A) or the 5′ end plus the adjacent genes *frdCD* (probe B) are

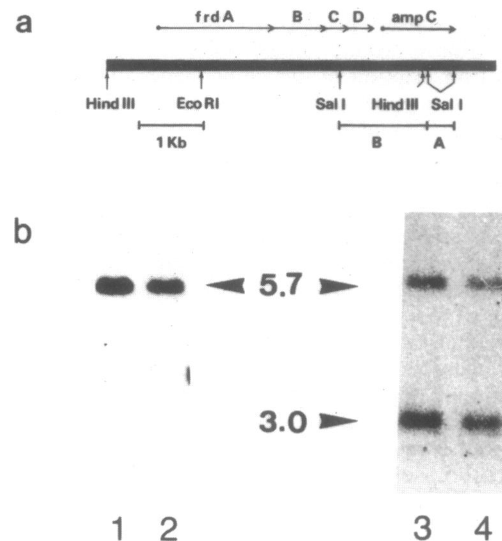

FIG. 1. (a) Partial restriction map of the *frd-ampC* region of the *E. coli* chromosome (5). The locations of the *frdABCD* operon and the *ampC* gene are shown together with the hybridization probes A and B. The scale in kilobase pairs is indicated. (b) Hybridization analysis of the *E. cloacae ampC* region. *Eco*RI-digested DNA from strains MHN1 (lanes 1 and 3) and MHN2 (lanes 2 and 4) was analyzed by Southern blotting with hybridization probes specific for the 3′ end of the *E. coli ampC* gene (lanes 1 and 2) or the 5′ end plus upstream sequences (lanes 3 and 4).

presented in Fig. 1b. A single fragment of 5.7 kilobase pairs (kb) was detected by the former probe, whereas the latter also hybridized to an *Eco*RI fragment of 3 kb. This is in contrast to the situation in *E. coli*, where both probes hybridized with a 6-kb *Eco*RI fragment (36; our unpublished findings). These results suggested that an *Eco*RI site was located between the *frd* operon and the *ampC* gene in *E. cloacae*. When the enzymes *Bgl*II and *Bam*HI were used, no differences in the hybridization patterns for MHN1 and MHN2 were seen, and fragments of 18 and 6 kb, respectively, were detected. Since the same fragments were found in both strains MHN1 and MHN2, the possibility of gene amplification or rearrangement was eliminated.

Cloning of the *E. cloacae ampC* gene. Southern blotting showed that a single *Bgl*I fragment of about 18 kb carried both the *ampC* and *frd* operons. Accordingly, libraries of *Bgl*II fragments from strains MHN1 and MHN2 were prepared in the lambda vector L47.1 (20). Recombinant clones were identified by plaque hybridization (1) with the probes described above (Fig. 1a). Rapid confirmation of the identity of the recombinant phages was obtained by plating them on the *E. coli* K-12 strain MI1443(Δ*ampC*) and applying a drop of nitrocefin to the plaques. Most of those clones exhibiting positive hybridization signals also displayed a positive color reaction indicative of β-lactamase activity. Single isolates derived from strains MHN1 and MHN2 were retained for further analysis and will be referred to as lambda Ecl and lambda Ec2, respectively.

DNA was prepared from the recombinant phages and analyzed with the restriction enzymes used previously, as well as *Apa*I, *Hin*dIII, *Sac*I, *Sma*I, and *Xho*I. For *Eco*RI and *Bam*HI, fragments of the expected size were found which hybridized with *ampC* specific probes. The results of these analyses were used to deduce the restriction map of the *ampC* region of the *E. cloacae* genome (Fig. 2). No restriction site polymorphisms were found, again suggesting that the *ampC* genes of MHN1 and MHN2 are highly similar.

Subcloning and location of the *ampC* gene. To locate the *ampC* gene more precisely and to facilitate physiological studies, a series of subclones was produced in the plasmid vector pACYC184 (4) by using restriction endonucleases *Eco*RI and *Bam*HI (Fig. 3). Plasmids pEc1A and pEc1B contain the 3- and 5.7-kb *Eco*RI fragments from lambda Ec1, respectively, whereas pEc1C carries the 6-kb *Bam*HI fragment. A similar set of plasmids (pEc2A, B, and C) was constructed from lambda Ec2. More detailed restriction maps (Fig. 3) of the various plasmids were deduced from double digestion analysis with 10 additional restriction enzymes. When these were compared the *ampC* regions from MHN1 and MHN2 were indistinguishable. By means of Southern blotting and partial DNA sequence studies (data not shown) the *ampC* gene was shown to be separated from the neighboring *frdD* gene by more than 800 base pairs. Sequences around the *Eco*RI 2 and *Sma*I 2 sites (Fig. 2) correspond to those of the *E. coli frdA* and *frdD* genes (5, 10), whereas those around the *Eco*RI 3 site showed no homology to the known sequence of the *E. coli ampC* gene (15). These results suggest that the *ampC-frd* region of *E. cloacae* is organized similarly to that of *C. freundii* (2).

Physiological studies. Expression of the cloned *ampC* genes was studied in the *E. coli* K-12 strain MI1443(Δ *ampC*) and compared with that in *E. cloacae*. As expected from the *ampC* localization studies, β-lactamase activity was detected with plasmids pEc1B and pEc1C carrying the 5.7-kb *Eco*RI and 6-kb *Bam*HI fragments, respectively, but not with pEc1A carrying the 3-kb *Eco*RI fragment (Fig. 2 and 3).

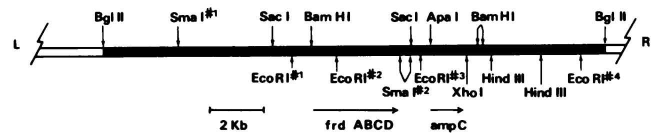

FIG. 2. Restriction map of the *Bgl*II fragment cloned in lambda Ec1. The locations of certain restriction sites are shown, and those sequential sites referred to in the text are identified, e.g., site 1. An additional *Bam*HI site was detected but not localized. The scale in kilobase pairs is shown, and the left and right arms of the lambda vector are represented by L and R, respectively. The approximate locations of the *frd* and *ampC* genes, as deduced from hybridization studies, are shown.

β-Lactam resistance was observed regardless of the origin of the genes (MHN1 or MHN2) or the orientation of the fragments in the plasmids. The MICs of a variety of β-lactam antibiotics obtained with either *E. cloacae* or *E. coli* are presented in Table 1. Major differences in the susceptibility of *E. cloacae* and *E. coli* to ampicillin and cephalothin were observed, as has been reported previously (31, 32). However, a significant difference in the resistance level was observed between pEc1B and pEc1C; pEc1B consistently conferred higher β-lactam resistance. In contrast to the situation in *E. cloacae*, the cloned *ampC* genes from MHN1 and MHN2 displayed identical resistance patterns (Table 1).

Quantitative confirmation of the MIC results was obtained by microacidimetric determination of β-lactamase activity in cell-free extracts from the various strains (Table 1). Again, no significant difference in the levels of enzyme activity was detected for the plasmids pEc1C and pEc2C, whereas enzyme activities in the *E. cloacae* strains from which the genes were originally obtained differed by more than 3,000-fold in the absence of induction. The difference in the β-lactam resistance levels conferred by plasmids pEc1B and pEc1C (or pEc2B and pEc2C) observed in the MIC determinations was also reflected in the amount of enzyme produced (Table 1). This suggested that the DNA sequences situated upstream of or spanning *Eco*RI site 3 (Fig. 2) may be involved in repressing cephalosporinase synthesis. Analytical isoelectric focusing of the *E. cloacae ampC* gene product synthesized either in *E. coli* K-12 or in *E. cloacae* showed the isoelectric point (pI 8.4) to be the same in both cases.

Inducible cephalosporinase activity. The β-lactamase levels observed in *E. coli* with pEc1C or pEc2C resembled those of a wild-type *E. cloacae* strain (allowing for copy number effects), so we also examined whether enzyme synthesis could be induced. Although plasmids pEc1B and pEc1C (or pEc2B and pEc2C) both encode the cephalosporinase, only the latter exhibited inducible activity and showed a striking increase in enzyme levels (fivefold or greater) upon cefoxitin addition (Table 1). In *E. cloacae* the level of induction is known to be directly proportional to the inducer concentration (9, 11). Consequently, the cephalosporinase activities were measured in extracts prepared from *E. coli* MI1443(pEc1C) induced with different cefoxitin concentrations (Fig. 4). As has been found for *E. cloacae*, enzyme activity increased in a linear manner. In an attempt to find an inducer which did not cause the bacteriostasis observed with cefoxitin in *E. coli*, we tested the cephalosporin precursor 7-aminocephalosporanic acid but found this to be without effect.

Interestingly, the results of the induction studies with strains harboring plasmid pEc1E or pEc1C (pEc2B or pEc2C) showed that inducible activity correlated with the

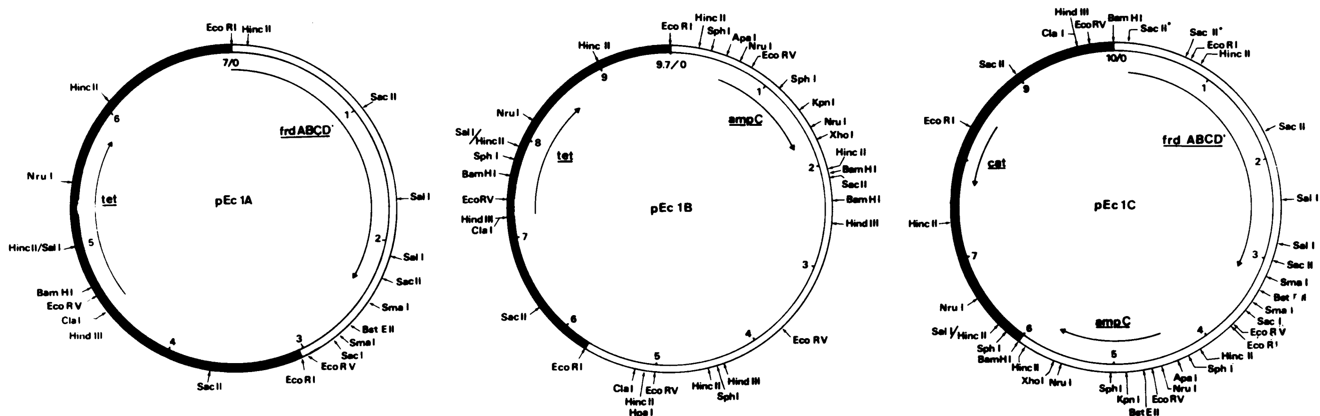

FIG. 3. Detailed restriction maps of recombinant plasmids. The vector portion, pACYC184, of each plasmid is represented by the solid section. The positions of the genes encoding part of fumarate reductase (*frd*), cephalosporinase (*ampC*), tetracycline resistance (*tet*), and chloramphenicol transacetylase (*cat*) are shown. The scale in kilobases is indicated. The precise locations of sites marked with asterisks are uncertain.

presence of the DNA sequence encompassing *Eco*RI site 3. This suggested that the region immediately upstream from *ampC* played a dual role in the induction-repression process. Finally, it should be pointed out that when expressed in *E. coli* K-12 the cloned gene from the deregulated *E. cloacae* mutant MHN2 behaved normally and responded to induction. This strongly suggests that the corresponding mutation is unlinked to the *ampC* gene.

DISCUSSION

The expression and regulation of the *ampC* gene encoding the cephalosporinase of clinical isolates of *E. cloacae* were studied by a gene cloning approach. Strain MHN1 produces low levels of the enzyme, although on induction with cefoxitin cephalosporinase production was amplified up to 40-fold. Upon introduction into *E. coli* K-12 the cloned *ampC* gene from MHN1 behaved in a similar fashion and even displayed the concentration-dependent induction effect (Fig. 4) normally associated with *E. cloacae* (9, 11). Based on these results it can be concluded that the *ampG* gene is regulated by a simple repressor-inducer system as suggested previously (9, 18).

Some support for this interpretation was provided by studies of *E. coli* K-12 harboring the plasmid pEc1C which carries a 6-kb *Bam*HI fragment bearing the *ampC* gene together with all the information necessary for induction and repression. A region involved in repression was identified upstream of *ampC*, since the related plasmid pEc1B, which does not carry this segment, overproduced the cephalosporinase by fivefold. These observations strongly suggest that the intergenic region between the *frd* operon and the *ampC* gene encodes a factor which can negatively regulate *ampC* expression or stimulate its transcription in the presence of β-lactams. Recently, Lindberg et al. reached similar conclusions after studying the expression of the *ampC* gene from *C. freundii* in *E. coli* K-12 (19). Moreover, these workers showed that an additional gene, *ampR*, is situated immediately upstream of *ampC*. The *ampR* gene product is a 31,000-dalton protein which is implicated in the regulation of *ampC* expression. Using the minicell technique, we have detected a similar-sized protein encoded by pEc1C (data not shown) which most likely represents the *ampR* gene product of *E. cloacae*.

An additional level of regulation was uncovered when the *ampC* gene from strain MHN2, a descendant of MHN1 isolated during treatment with the β-lactamase-stable cephalosporin moxalactam, was cloned and analyzed in *E. coli* K-12. *E. cloacae* MHN2 overproduced the cephalosporinase by about 3,000-fold, and its synthesis no longer responded to induction. When present in *E. coli* K-12 the cloned *ampC* gene from strain MHN2 exhibited wild-type behavior, producing low levels of enzyme which could be increased by cefoxitin treatment. These findings indicate that the original mutation leading to cephalosporinase hyperproduction is not closely linked to the *ampC* structural gene or its putative regulatory gene *ampR*. This suggests the involvement of a second factor in the regulation of *ampC* gene expression. Independent support for this interpretation is provided by the studies of Lindberg et al. (19), who found constitutive overexpression of the *C. freundii ampC* gene in *E. coli* to be due to a host chromosomal mutation. It should be pointed

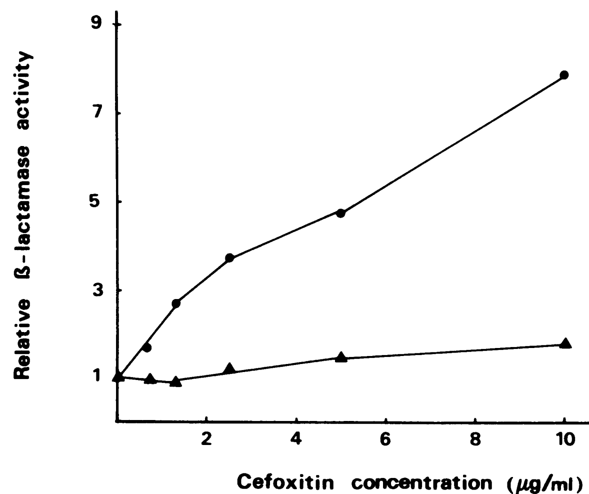

FIG. 4. Induction of β-lactamase activity. Strain MI1443(Δ*ampC*) containing plasmid pEc1B (▲) or pEc1C (●) was treated with various cefoxitin concentrations for 3 h, and the β-lactamase activity in cell-free extracts was measured. Relative activities are expressed as a function of the specific activity in the absence of an inducer.

out that spontaneous mutations affecting the corresponding gene(s) arise at high frequency (10^{-5} to 10^{-6}) both during antibiotic therapy and in the laboratory (8, 18). Furthermore, such mutations, which lead to hyperproduction of the cephalosporinase, are characteristic of bacteria that usually display inducible cephalosporin resistance. This remarkable mutation frequency explains the prevalence of such organisms in hospital infections (14, 34) and undoubtedly highlights the importance of the regulatory network involved.

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