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Sequencing and Analysis of Four New *Enterobacter ampD* Alleles

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Sequences of *ampD* genes from wild-type, temperature-sensitive, and stably derepressed mutants of the wild-type strain *Enterobacter cloacae* 029 and the hyperinducible strain *E. cloacae* 1194E were determined and compared with the *ampD* gene of the wild-type strain *E. cloacae* 14. Seventy nucleotide differences were found between the wild-type sequences, resulting in 13 amino acid changes. The deduced amino acid changes do not correspond to published AmpC regulation mutations and expand the number of known mutations leading to altered AmpC β-lactamase expression in members of the family *Enterobacteriaceae*.

The production of the Bush group 1 (1), chromosomally encoded AmpC β-lactamase of gram-negative bacteria such as *Enterobacter cloacae* and *Citrobacter freundii* is normally inducible. Three genes, in addition to *ampC* (the structural gene for the β-lactamase), which are known to participate in the induction process have been identified. These genes are *ampR*, which encodes a transcriptional regulator; *ampD*, which encodes an enzymatic repressor of induction; and *ampG*, which encodes a transmembrane protein which possibly functions as a permease for the induction signal (7, 9, 10). Under noninducing conditions, AmpR acts to suppress AmpC synthesis by about 2.5-fold, whereas it increases AmpC expression by several hundred-fold under inducing conditions. Recent publications by Jacobs et al. (4, 5) and Höltje et al. (2) identify AmpD as an *N*-acetylanhydromuramyl-L-alanine amidase which functions to cleave the tripeptide from *N*-acetylanhydromuramyl tripeptide (aMT). This muropeptide results from cell wall degradation and is believed to pass into the cytoplasm through AmpG (2, 4, 7). Once there, AmpD cleaves the tripeptide in preparation for reuse in the process of cell wall recycling (4, 5). In the absence of AmpD, aMT is not cleaved and builds up in the cytoplasm (4). Under these conditions AmpC becomes constitutively derepressed. It is likely that β-lactam binding to penicillin-binding proteins results in higher levels of aMT because rebuilding of the cell wall is inhibited, while cell wall hydrolases continue to function. aMT seems the likely candidate as the signal which is transported into the cell and which activates AmpR to induce high-level expression of β-lactamase.

Four phenotypes for AmpC β-lactamase expression which are associated with *ampD* have been identified (8). These include wild type (normal induction), hyperinducible (higher basal level of expression of AmpC and high-level induction in the presence of low levels of inducing drug), temperature sensitive (loss of inducibility at nonpermissive temperatures), and stably derepressed (the well-known constitutive high-level production causing clinical concern) (12). Sequences of *ampD* have previously been determined for a few wild-type strains of *Escherichia coli*, as well as wild-type, stably derepressed, and hyperinducible strains of *C. freundii* and *E. cloacae* (3, 6, 11). We determined sequences for a wild-type *E. cloacae* strain, strain 029, the temperature-sensitive and stably derepressed mutants related to that strain, and a hyperinducible mutant with characteristics different from those of the previously sequenced hyperinducible strain.

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Plasmids containing cloned *ampD* genes were used as double-stranded templates for thermal-cycle sequencing. Specific plasmid designations were as follows: pGKS146-3, *ampD* from *E. cloacae* 029 (wild type); pGKS147-3, *ampD* from *E. cloacae* 029-5 (temperature sensitive); pGKS148-3, *ampD* from *E. cloacae* 029M (stably derepressed); and pGKS165-4, *ampD* from *E. cloacae* 1194E (hyperinducible) (8) (Table 1). The plasmids were isolated from *E. coli* hosts by using the Wizard miniprep kit (Promega, Madison, Wis.). Initial primers were obtained from Research Genetics (Huntsville, Ala.). Additional primers were designed from our own sequence data and were purchased from Life Technologies (Gaithersburg, Md.). The sequences of the primers were as follows: D14-13, 5′-AACGGA TGGCTGGTGGACGCG; D14-301, 5′-ATGTATCAGGGG CGCGAGCGGTGC; D14-C324, 5′-GCACCGCTCGCGCCC CTGATA; D14-C543, 5′-CGACGTGGTAAGCATGGCGT GAAACCG; D14-addFor, 5′-GAAAAGCCCACACTGCTG GTGG; and D14-addRev, 5′-CCACCAGCAGTGTGGGCTT TTC. Thermal-cycle sequencing was performed with the Cyclist kit (Stratagene, La Jolla, Calif.) on a Perkin-Elmer TC-1 thermocycler. Amplification products were labeled by direct incorporation of [α-^{35}S]dATP (Redivue; Amersham, Arlington Heights, Ill.). Cycling parameters were as follows: 95°C for 5 min and then 40 cycles of 95°C for 30 s, 53°C for 30 s, and 72°C for 1 min. The products were separated by electrophoresis through 8% PAGE-PLUS (Amresco, Solon, Ohio)

TABLE 1. Plasmids and β-lactamase expression phenotypes of *E. cloacae* strains

Plasmid	Source of plasmid-borne genes	Inducibility phenotype	Relative activity[a]	
			Uninduced	Induced
pGKS146-3	029	Wild type	1	39
pGKS147-3	029-5	Temperature sensitive	10	101
pGKS148-3	029M	Stably derepressed	1,362	1,374
pGKS165-4	1194E	Hyperinducible	4	220

[a] Hydrolysis activity against cephalothin, relative to that of the uninduced wild type, which was arbitrarily set at 1.

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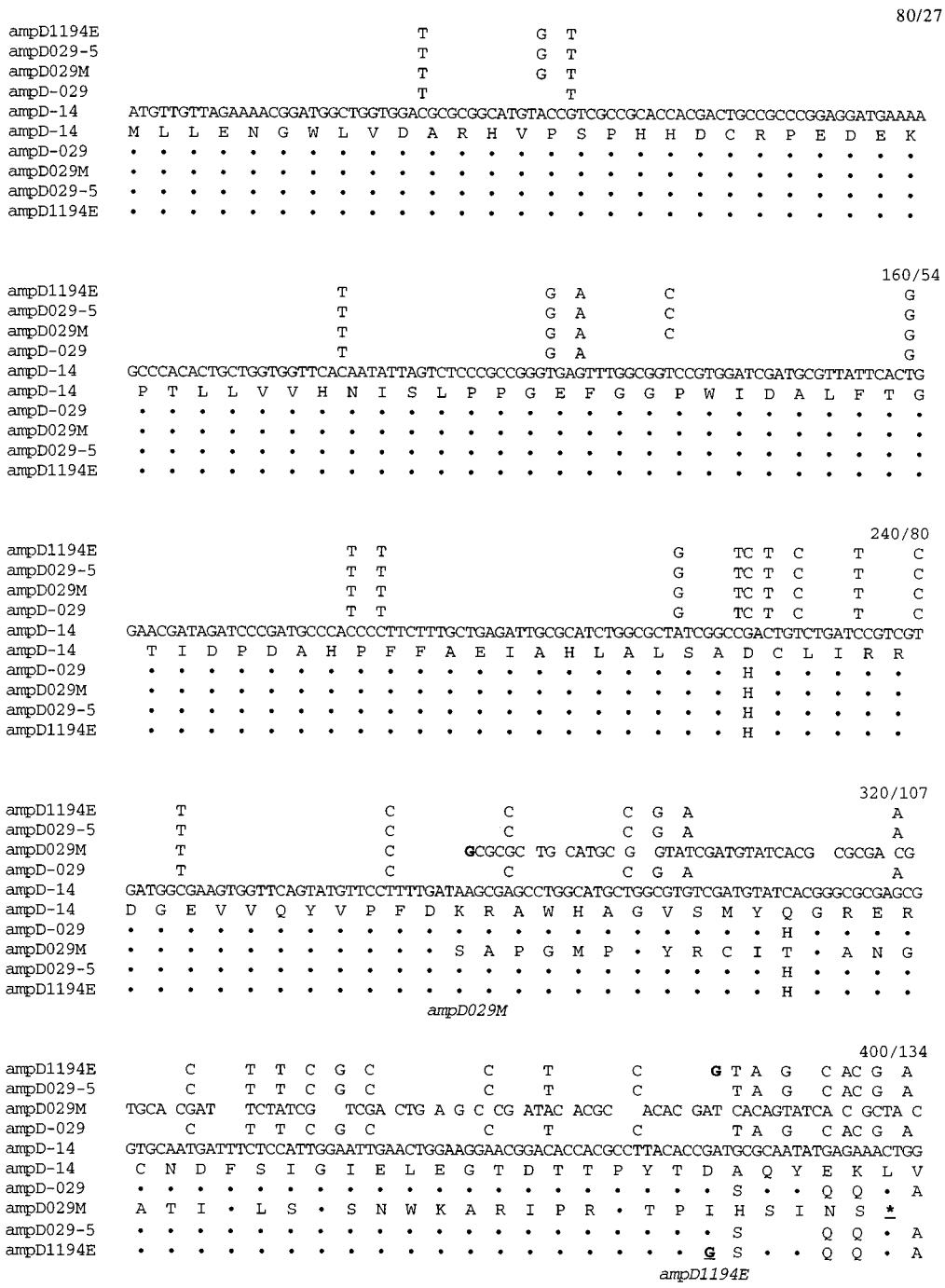

FIG. 1. Sequences of cloned *ampD* alleles aligned with the sequence of *ampD* from *E. cloacae* 14. Deduced protein sequences are given below the nucleotides. Only nucleotide and amino acid differences from the wild-type *ampD* sequences are given for the other alleles. Numbers appearing above the end of each line represent the last nucleotide/codon of the line. Designations: ampD-14, wild-type strain 14; ampD-029, wild-type strain 029; ampD029M, stably derepressed strain; ampD029-5, temperature sensitive strain; ampD1194E, hyperinducible strain. The nucleotide changes for the *ampD029-5* and *ampD1194E* mutations and the first nucleotide of the *ampD029M* frameshift mutation are indicated in boldface. Amino acid substitutions unique to each mutation are underlined and are in boldface. The name of the mutation appears immediately below the indicated change.

gels containing 7.2% urea. Additional sequence data were obtained from an ABI 373A machine running samples amplified on the GeneAmp System 9600 by using Prism dye terminator label. Both strands were sequenced, and sequences were confirmed by redundant sequencing of all regions with at least three primers. The sequences were assembled, aligned, and analyzed by using the MacVector and AssemblyLign software packages (Eastman Kodak, New York, N.Y.).

The sequences of the four new *ampD* alleles and the previously published sequence from *E. cloacae* 14 (6) are given in Fig. 1. Deduced amino acid sequences are also given in Fig. 1, with the one-letter International Union of Pure and Applied

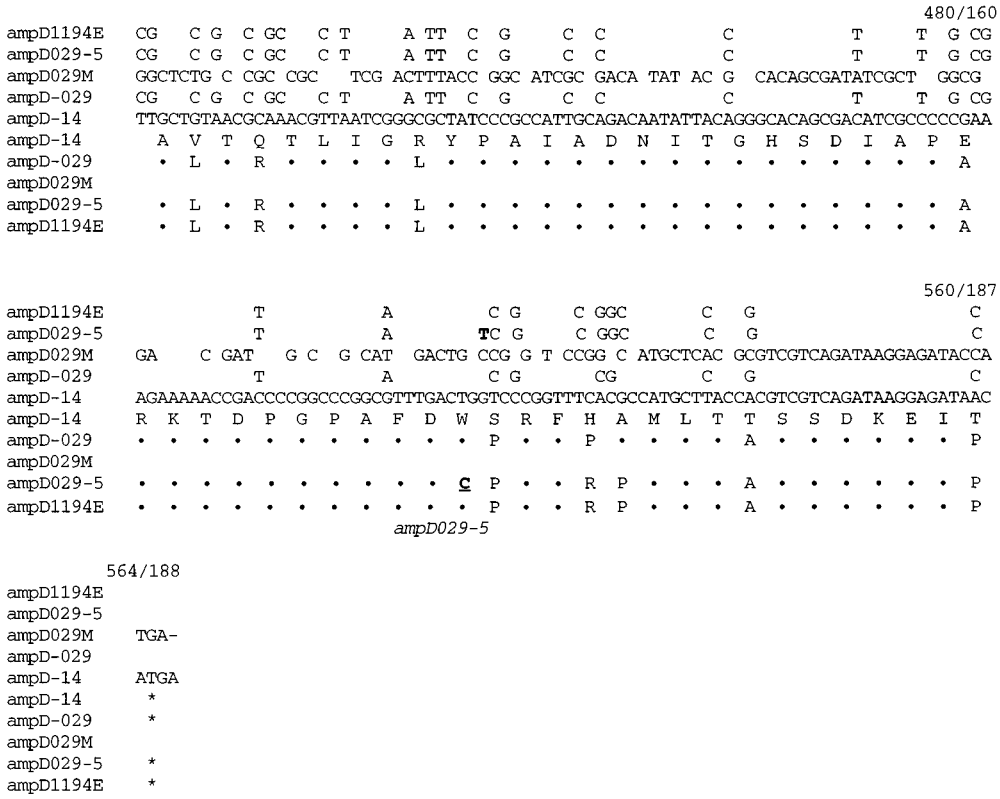

FIG. 1—*Continued.*

Chemistry code for each amino acid appearing under the first nucleotide of its corresponding codon. Alignment of new sequences with the previously published sequence of the *ampD* gene from *E. cloacae* 14 indicated substantial variation between different strains of this species at the nucleotide level (88% identity). This disparity is similar in magnitude (74 to 83% identity) to that found between analogous genes from *C. freundii*, *E. cloacae*, and *E. coli* (6). It is therefore surprising to find this much disparity between alleles in the same species. A total of 39 of the 70 nucleotide differences in the 029 *ampD* sequence correspond to nucleotides in either the published *E. coli ampD* sequence or the *C. freundii ampD* sequence (6). Ten nucleotide differences correspond to *C. freundii*, 11 correspond to *E. coli*, and the remainder ($n = 18$) correspond to nucleotide differences found in both sequences. This may suggest that storage of the clones in *E. coli* hosts has selected for maintenance of sequences preferred by the host. Because the 18 common nucleotides could be interpreted as also belonging to the group preferred by *E. coli*, this would bring the total to 29 of 70 (41%) which converge on the *E. coli* sequence. This would not account for the remaining nucleotides (59%) which are not convergent, and because 40% of the nucleotides could be interpreted as convergent on the *C. freundii* sequence, any such explanation is merely interesting speculation. Previous investigators have noted that the carboxy-terminal region of AmpD is essential for the amidase activity of the mature protein (6) and that loss of this region results in stable derepression of *ampC* (6, 13). A single-base-pair deletion at position 275 (*ampD029M* mutation) leads to amino acid substitutions in 36 of 41 positions (codons 92 to 132) prior to introduction of a stop codon (position 133). Stable derepression of *E. cloacae* 029M may therefore be explained by production of a truncated AmpD protein lacking the 55 carboxy-terminal amino acids. Jacobs et al. (5) searched the on-line protein databases for homology to the *C. freundii* AmpD sequence and found a conserved core region relating this gene with *Bacillus* cell wall hydrolases. The consensus residues of this core region are conserved in three of the four *E. cloacae* AmpD sequences reported here. The frameshift mutation in *ampD* from the stably derepressed mutant occurs in the middle of the coding region for this core region. The stably derepressed phenotype may therefore be alternatively explained as resulting from changes to 6 of the 11 core consensus residues. The *ampD* sequence of the temperature-sensitive mutant contains one unique nucleotide change which results in the replacement of a tryptophan with a cysteine at position 171 (*ampD029-5* mutation). This substitution alone or in combination with one or both of the other amino acid substitutions to the *E. cloacae* 029 wild-type sequence (Arg for Pro at position 175 [Pro-175] and Pro for Ala-176) may be responsible for temperature sensitivity. Both of these additional substitutions are also found in the allele of the hyperinducible mutant. Kopp et al. (6) noted that the characteristics of β-lactamase resistance in the hyperinducible mutant that they sequenced were substantially different from those of *E. cloacae* 1194E (8). These differences in resistance phenotype suggested that hyperinducibility in 1194E might be due to a different *ampD* mutation than the Gly-121 substitution (*ampD05* mutation) found in their strain. Our sequence of *ampD* from 1194E also showed a substitution of glycine for aspartic acid (*ampD1194E* mutation), but the substitution occurred six amino acids downstream (at position 127 rather than position 121) from the position noted by Kopp et al. (6). Recently, Stapleton et al. (13) reported mutations at positions 95, 102, and 158 of the *C. freundii ampD*, yielding two

distinct hyperinducibility phenotypes. None of the *C. freundii* mutations involved a glycine substitution such as that found in the *E. cloacae* protein.

The three mutations found in this work expand the number of known mutations leading to altered Bush group 1 β-lactamase expression. These mutations also support previous findings in that the *ampD029M* mutation demonstrates the essential nature of the carboxy terminus of the mature protein, and the *ampD1194E* mutation is very similar to a previously reported mutation yielding a similar phenotype. Furthermore, the finding of an identical amino acid substitution yielding hyperinducibility at a different position in the *E. cloacae* protein suggests the importance of the region as much as the importance of the Asp-to-Gly change. Finally, the *ampD029-5* mutation is the first which has been related to the temperature-sensitive phenotype.

Nucleotide sequence accession numbers. These sequence data will appear in the EMBL/GenBank nucleotide sequence databases under the accession numbers U40785 for *ampD* from *E. cloacae* 029 (wild type), U40804 for *ampD* from *E. cloacae* 029M (stably derepressed mutant), U40805 for *ampD* from *E. cloacae* 029-5 (temperature-sensitive mutant), and U40806 for *ampD* from *E. cloacae* 1194E (hyperinducible mutant).

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