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DNA Sequence Differences of *ampD* Mutants of *Citrobacter freundii*

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Three groups of mutants with increased levels of β-lactamase synthesis were selected from *Citrobacter freundii* 382010 by β-lactam antibiotics at concentrations just above the MIC. Uninduced cultures of the hyperinducible group had 3- to 5-fold more β-lactamase activity than the parent strain, with one mutant (termed type b) expressing 19 times the activity of the parent strain; the partially derepressed group had a relative 55-fold increase, while fully derepressed strains exhibited a 460-fold increase. Upon induction by growth in the presence of cefoxitin (32 μg/ml) for 2 h, the hyperinducible and derepressed groups had similar relative β-lactamase activities of 650 and 725, respectively. Induction of β-lactamase activity from partially derepressed mutants resulted in a relative activity of only 240. The *ampD* gene including its promoter region was amplified from the parent strain and the mutant strains by PCR. The sequence of *ampD* from the parent strain showed only three nucleotide changes from a previously published sequence, none of which resulted in a change to the deduced amino acid sequence. Hyperinducible mutant strains of type a had an amino acid change of either a tryptophan in codon 95 to an arginine (Trp-95→Arg) (three mutants) or Ala-158→Asp (one mutant). The hyperinducible type b strain had the change Tyr-102→Asp. The derepressed strains had the following changes: Val-33→Gly (one mutant), Asp-164→Glu (one mutant), and Trp-95→termination codon (two mutants). We infer that the amino acid changes in the hyperinducible mutants result in altered AmpD activity, whereas, in contrast, they lead to an inactive protein in derepressed mutants. No nucleotide differences were found in the *ampD* gene from partially derepressed strains.

Several genes are involved in the synthesis of the chromosomally encoded Bush (3) group 1 β-lactamase by *Citrobacter freundii* and *Enterobacter cloacae*. *ampC* is the structural gene for the β-lactamase (2). *ampR* is a regulatory gene that can both repress and, in the presence of a β-lactam inducer, activate transcription of *ampC* (11); the absence of this gene in *Escherichia coli* accounts for the noninducibility of its chromosomal β-lactamase (13). The effects of *ampR* have been studied by cloning the *C. freundii ampC* and *ampR* genes into a strain of *E. coli* lacking a native *ampC* gene (11). The $ampC^+$ $ampR^+$ strain could be induced to synthesize β-lactamase by β-lactam antibiotics, but an $ampC^+$ *ampR* mutant strain was noninducible, although it produced about twice the basal level of β-lactamase as the wild type (11). An *ampR* mutant that constitutively hyperproduces β-lactamase was selected by nitrosoguanidine mutagenesis (1).

Most natural β-lactam-resistant, constitutive β-lactamase producers of *C. freundii* and *E. cloacae* are believed to have mutations in the *ampD* gene (13), although only a relatively small number have been sequenced (Table 1). The AmpD protein is thought be a novel *N*-acetylmuramyl-L-alanine amidase which hydrolyzes the muropeptide 1,6-anhydro *N*-acetylmuramyl-L-alanyl-D-glutamyl-*meso*-diaminopimelic acid, which is believed to be the true inducer (or autoinducer) of β-lactamase synthesis (5, 7). Normark et al. (13) have defined two classes of *ampD* mutants: hyperinducible, in which there is at most a small increase in the level of β-lactamase synthesis in the absence of inducer but in which induction occurs at lower concentrations of a β-lactam inducer than those for wild-type strains; and semiconstitutive, with a 20- to 30-fold elevation in the level of uninduced β-lactamase synthesis and a further 2- to 3-fold increase in the presence of a β-lactam inducer.

A number of other genes, including *ampE* (10, 12), *ampG* (9), and the gene encoding penicillin-binding protein 2 (14), have been reported to be necessary for the induction of β-lactamase synthesis, but the mutations in them have not been reported to result in increased levels of β-lactamase production.

In the study described here we compared the β-lactamase inducibility phenotypes and susceptibility patterns of β-lactam-resistant mutants of *C. freundii* with the DNA sequence changes in the *ampD* gene.

MATERIALS AND METHODS

Organisms and antibiotics. *C. freundii* 382010 (inducible for β-lactamase), described by Curtis et al. (4), was used.

The following antibiotic powders of known potency were kindly supplied by the indicated companies: piperacillin, Lederle, Gosport, United Kingdom; cefuroxime and cephaloridine, Glaxo Group Research, Greenford, United Kingdom; cefoxitin, Merck Sharp & Dohme, Hoddesdon, United Kingdom; cefpirome and cefotaxime, Roussel Laboratories Ltd., Wembley Park, United Kingdom; and ampicillin, amoxicillin, and carbenicillin, SmithKline Beecham, Betchworth, United Kingdom.

Reagents. *Taq* DNA polymerase, 10× *Taq* polymerase buffer, and magnesium chloride were supplied by Promega (Promega Corporation, Southampton, England). Nucleotides were obtained from Sigma (Poole, United Kingdom). Sterile distilled water was molecular biology grade (Bio-Rad, Hemel-Hempstead, United Kingdom). Synthetic oligonucleotide primers were derived from the sequence reported by Kopp et al. (8) (GenBank accession number Z14002); primer PSN009 (5′-TTATACGTTCCAGAAGCGCGTCA-3′) is located within the *nadC* gene (positions 78 to 100), and primer PSN004 (5′-TCATGTCAT CTCCTTGTGTGACGA-3′) is located at the 3′ end of the *ampD* gene (positions 451 to 564). Primers were custom made on request by The Advanced Biotechnology Centre (The Charing Cross and Westminster Medical School, London, United Kingdom). The AutoRead sequencing kit and automated laser fluorescent-grade urea were supplied by Pharmacia Biotech (St. Albans, United Kingdom). Hydrolink Long Ranger gel was obtained from Hoefer (Newcastle-under-Lyme, United Kingdom). Microbiological media, brain heart infusion

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TABLE 1. Wild-type and mutant *ampD* genes sequenced previously

Organism	Wild-type strain	Mutant	Phenotype	Mutation	Reference
E. coli	SN03		Wild type		12
		ampD1 (class 1)	Hyperinducible	52-bp frameshift deletion after codon 23	
		ampD2 (class 2)	Derepressed (semi-constitutive)	IS*1* insertion after codon 121	
E. coli	MC4100		Wild type		6
		ampD11	Fully derepressed	T→G transversion in codon 7	
E. cloacae	14		Wild type		8
		ampD02	Fully derepressed	Tandem duplication of TGGCATGC causing frameshift after codon 98	
		ampD05	Hyperinducible	A→G transition in codon 121	
		ampD04	Fully derepressed	AmpD lacking 16 carboxy-terminal residues	

medium (CM255), Diagnostic Sensitivity Test Agar (CM261), and MacConkey agar (CM7), were purchased from Oxoid (Basingstoke, United Kingdom). PADAC was obtained from the Calbiochem Corporation (La Jolla, Calif.). All other reagents were ANALAR grade and were obtained from BDH (Lutterworth, United Kingdom).

Selection of β-lactamase expression mutants. Ampicillin-, carbenicillin-, or cefuroxime-containing MacConkey agar (CM7; Oxoid) plates at concentrations above the MIC (determined on Diagnostic Sensitivity Test Agar [CM261; Oxoid]; see below) for the inducible strain were used to select mixed populations of resistant mutants with different levels of β-lactamase expression. MacConkey agar was chosen to provide the potential for discrimination between resistant mutants with different colonial morphologies and to suppress the growth of contaminants. By use of a microtiter tray assay method (16), large numbers of mutants could be screened and classified quickly on the basis of the rate of PADAC hydrolysis. Resistant mutants were classified as derepressed if the hydrolysis time was less than 2 min, partially derepressed if the hydrolysis time was between 2 and 5 min, and as having a slight increase in β-lactamase activity if the hydrolysis time was between 5 and 15 min (these mutants were subsequently shown to be hyperinducible, and we shall use this term henceforth). Typically, mutants with altered β-lactamase expression occurred at a frequency of 10^{-8} to 10^{-6} depending on the compound used and the mutant phenotype.

Determination of MICs. MICs were determined by agar dilution on Diagnostic Sensitivity Test Agar (CM261; Oxoid) with an inoculum of about 10^4 organisms per spot as described previously (16). *E. coli* NCTC 10418 was used as the control strain.

β-Lactamase studies. β-Lactamase synthesis was induced by growing the organisms for 2 h in the presence of cefoxitin (32 μg/ml) as described previously (16). β-Lactamase activity was assessed by measurement of the hydrolysis of nitrocefin (SR112; Oxoid) by ultrasonically disrupted cell suspensions and measurement of the protein concentration as described previously (16). β-Lactamase activities, adjusted for protein content, are given relative to that of the uninduced parent.

Sample preparation for PCR. Wild-type and mutant strains of *C. freundii* were inoculated into 1.5 ml of brain heart infusion broth and were incubated for 20 h at 37°C with shaking. The cells were harvested by centrifugation at 13,000 rpm in an Heraeus Biofuge 13 for 5 min. After decanting the supernatant, the pellet was resuspended in 0.5 ml of sterile distilled water. The cells were lysed by heating at 95°C for 10 min.

Isolation of the *ampD* gene by PCR. The composition of the reaction mixture was as follows: reaction buffer (50 mM KCl, 10 mM Tris-HCl [pH 9.0], 0.1% Triton X-100), 0.2 mM (each) the four deoxynucleoside triphosphates (ATP, CTP, GTP, and TTP), 5 μM (each) the two primers (PSN009 and PSN004), and 1.5 mM $MgCl_2$ in a total volume of 48 μl. A total of 1 μl of sample lysate and 1 U of *Taq* polymerase were added to the reaction mixture, and the samples were mixed by vortexing and were centrifuged briefly before 50 μl of mineral oil was layered onto the surface. Thermal cycling was performed in a Hybaid (Teddington, United Kingdom) Omnigene thermal reactor with the following conditions: 35 cycles of 25 s at 92°C, 2 min at 65°C, and 3 min at 75°C and a final extension step of 5 min at 75°C. Isolation of the *ampD* gene by PCR amplification was performed twice on the parent and mutant strains.

The PCR products were purified by isopropanol precipitation (2 volumes) in the presence of ammonium acetate (4 M, 1 volume) at room temperature for 30 min. DNA was recovered by centrifugation at top speed in a Heraeus Biofuge 13 microcentrifuge (13,000 rpm) for 30 min; this was followed by a 70% ethanol wash. After drying, the pellet was resuspended in sterile distilled water.

Sequencing of the *ampD* gene. The sequencing reactions were performed directly on the purified PCR product by use of the quick annealing method as described in the instructions for the AutoRead Sequencing Kit (Pharmacia Biotech, St. Albans, England). Template DNA (10 μg) and unlabelled primer (PSN009; 25 μM) were first denatured in the presence of NaOH (1 M) at 80°C for 3 min, placed on ice, and then neutralized with HCl (1 M). Labelling of the primer was performed by the addition of 4 U of T7 DNA polymerase and fluorescein-15-dATP labelling mix, with subsequent incubation at 37°C for 10 min. Dideoxy termination reactions were carried out by the addition of the template DNA and the labelled primer to each of four termination mixes and incubation at 37°C for 5 min. The reactions were stopped by the addition of formamide containing dextran blue (5 mg/ml). The DNA sequences of the samples were determined with an automated laser fluorescent DNA sequencer (Pharmacia Biotech). Samples were heat denatured at 80°C for 3 min before loading onto a 6% Hydrolink Long Ranger gel containing 7 M urea and 1.2× Tris-borate-EDTA (TBE). The samples were run at 37 W (1,800 V, 60 mA, 44°C) in 0.6× TBE with a sampling time of 2 s (8-h running time) and a laser power of 3 mW. Sequencing of the coding strand was performed once on two independently generated *ampD* PCR products from each strain.

RESULTS

Induction of β-lactamase activity. The β-lactamase activities of five resistant mutants from each of the three phenotypic groups (hyperinducible, partially derepressed, and fully derepressed) together with that of the unselected parent were measured under inducing (the presence of cefoxitin) and noninducing conditions. Not surprisingly, the uninduced β-lactamase activity varied between the mutant groups. Strains with the hyperinducible phenotype had 3- to 5-fold more β-lactamase activity than the parent strain, with one mutant (termed type b) expressing 19 times the activity of the parent strain; strains of the partially derepressed phenotype had approximately a relative 55-fold increase, while fully derepressed strains exhibited an approximately 460-fold increase (Table 2). Upon induction strains with the hyperinducible and derepressed phenotypes expressed similar levels of β-lactamase activity (650 and 725 times that of the uninduced parent strain, respectively). Interestingly, induction of β-lactamase activity from partially derepressed mutants resulted in an increase to only 240 times that of the uninduced parent strain (Table 2).

Resistance patterns. The MICs of several β-lactam antibiotics for the wild-type and mutant strains are presented in Table 2. All mutants were more resistant than the parent strain to the compounds tested, apart from the hyperinducible mutants and cefpirome, for which an identical or increased MIC cannot be inferred since end points were not determined. In general, no differences in MICs were found between derepressed and partially derepressed mutants. The hyperinducible mutants were equally resistant to amoxicillin and cephaloridine, which are moderately strong labile inducers of β-lactamase synthesis in the parent strain (12), and to the labile, strong inducer cefoxitin, as were the derepressed mutants. However, the MICs of the labile weak inducers (carbenicillin, piperacillin, cefuroxime, and cefotaxime) were generally between those for the parent strain and those for the derepressed

TABLE 2. Relative level of β-lactamase expression and MICs of β-lactam antibiotics for mutant strains of *C. freundii*

Strain	Phenotype	Relative β-lactamase expression[a]		MIC (μg/ml)							
		Noninduced	Induced	Amoxicillin	Carbenicillin	Piperacillin	Cephaloridine	Cefuroxime	Cefoxitin	Cefotaxime	Cefpirome
Parent	Wild type	1[b]	115	8	1	0.5	8	2	64	0.06	≤0.03
31C8	Hyperinducible a	4	779	512	4	2	256	8	256	0.5	≤0.03
31E3	Hyperinducible a	3	671	>512	2	1	256	8	256	0.5	≤0.03
31A7	Hyperinducible a	5	580	>512	4	2	256	8	256	0.5	≤0.03
CBN3	Hyperinducible a	5	585	>512	4	2	256	8	256	0.5	≤0.03
CBN2	Hyperinducible b	19	573	>512	8	4	256	16	256	1	0.06
31C2	Partially derepressed	48	285	>512	64	16	256	64	256	8	0.25
31E2	Partially derepressed	52	241	>512	64	16	256	64	256	8	0.25
31G9	Partially derepressed	61	242	>512	64	16	256	64	256	8	0.25
31F9	Partially derepressed	52	201	>512	64	16	256	64	256	8	0.25
31F8	Partially derepressed	63	238	>512	64	16	256	64	256	8	0.25
31A12	Derepressed	460	768	>512	32	8	256	64	256	8	0.12
31H11	Derepressed	470	727	>512	64	8	256	64	256	8	0.25
31F11	Derepressed	439	730	>512	64	8	256	64	256	8	0.12
31A4	Derepressed	474	612	>512	16	8	256	64	256	8	0.12
CBN1	Derepressed	451	797	>512	64	8	256	64	256	8	0.12

[a] The level of expression relative to that of the noninduced parent.
[b] A total of 0.1 nkat/mg of protein with nitrocefin as the substrate.

mutants, with that for the type b hyperinducible mutant being twofold different from that for the type a mutant.

Sequencing of the *ampD* gene from β-lactamase expression mutants. The *ampD* genes including its promoter region were amplified from the parent and the mutant strains by PCR. Sequencing of the PCR products directly without subcloning permitted quick analysis, with the added bonus of masking any bases misincorporated by the *Taq* DNA polymerase, since they would represent only a small proportion of the total population of products. The sequence of the parent (Fig. 1) revealed only three nucleotide positions (position 156, C→T; position 159, T→C; position 399, G→A) at which the nucleotide differed from that in a previously published sequence for *C. freundii* (8); none of these differences resulted in a change to the deduced amino acid sequence. Hyperinducible mutant strains (type a) were characterized by either a change from tryptophan to arginine at codon 95 (three mutants) or a change from alanine to glutamic acid at position 158 (one mutant) (Table 3). The hyperinducible type b strain had a change from tyrosine to aspartic acid at position 102. The derepressed strains had the following changes: valine to glycine at position 33 (one mutant), aspartic acid to glutamic acid at position 164 (one mutant), and tryptophan to the amber termination codon at position 95 (two mutants) (Table 3). No nucleotide differences were found in the *ampD* gene from partially derepressed strains.

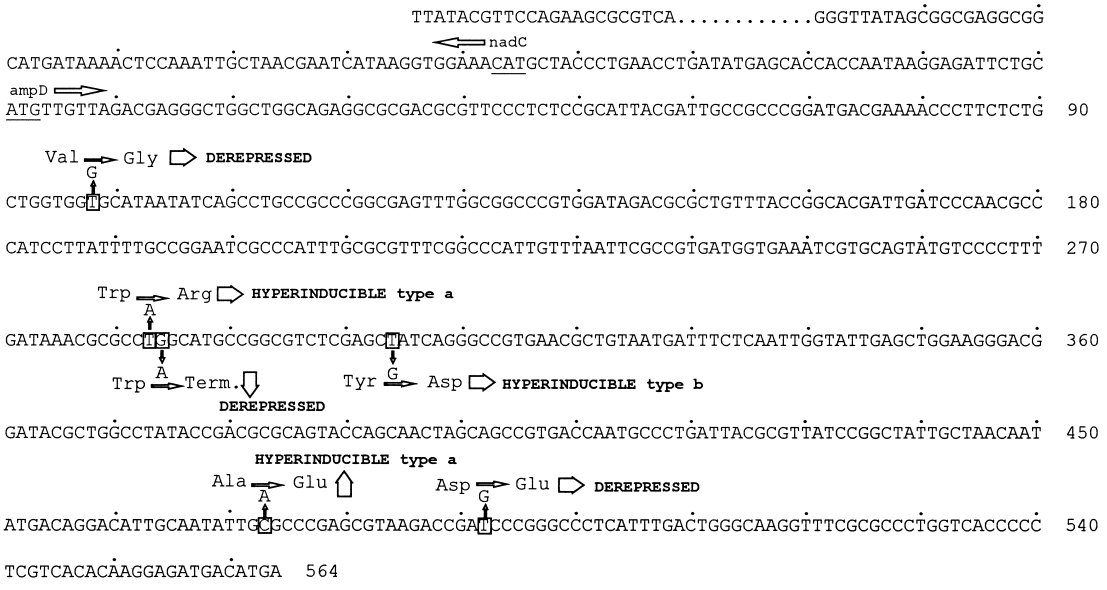

FIG. 1. Nucleotide sequence of *ampD* from *C. freundii* 382010 showing the mutations that occur in β-lactamase expression mutants. Also shown are the upstream *ampD* promoter region and the beginning of the adjacent *nadC* gene.

TABLE 3. Summary of the results obtained from sequencing the *ampD* gene of *C. freundii* β-lactamase expression mutants

Strain	Phenotype	Selective agent (concn [μg/ml])	Mutation[a]			
			Nucleotide position	Amino acid codon	Nucleotide change	Amino acid change
CBN3	Hyperinducible type a	Carbenicillin (8)	473	158	C→A	Ala→Glu
31E3	Hyperinducible type a	Carbenicillin (2)	283	95	T→A	Trp→Arg
31A7	Hyperinducible type a	Ampicillin (16)	283	95	T→A	Trp→Arg
31C8	Hyperinducible type a	Ampicillin (64)	283	95	T→A	Trp→Arg
CBN2	Hyperinducible type b	Carbenicillin (8)	304	102	T→G	Tyr→Asp
31C2	Partially derepressed	Ampicillin (64)				
31F8	Partially derepressed	Carbenicillin (2)				
31E2	Partially derepressed	Carbenicillin (2)				
31G9	Partially derepressed	Cefuroxime (8)				
CBN1	Fully derepressed	Carbenicillin (8)	98	33	T→G	Val→Gly
31A12	Fully derepressed	Ampicillin (16)	284	95	G→A	Trp→Term.
31H11	Fully derepressed	Cefuroxime (8)	284	95	G→A	Trp→Term.
31F11	Fully derepressed	Carbenicillin (2)	492	164	T→G	Asp→Glu

[a] No mutations were found among strains of the partially derepressed phenotype.

DISCUSSION

Mutants selected for increased levels of β-lactamase expression have been shown to be of one of three phenotypic types (derepressed, partially derepressed, and hyperinducible) on the basis of their level of β-lactamase expression under noninduced and induced conditions. All were more resistant than the parent strain to β-lactam antibiotics, but, not surprisingly, the hyperinducible group, which had the lowest uninduced β-lactamase activities, was less resistant than the other groups. Despite the approximately 20-fold difference in their uninduced β-lactamase activities, it was not possible to distinguish the derepressed and partially derepressed groups by means of β-lactam MICs.

Characterization of the phenotypes by sequencing the *ampD* gene has located single point mutations in the hyperinducible and the derepressed strains but not in the partially derepressed strains. The finding of mutations in the *ampD* genes from hyperinducible and derepressed strains confirms previous reports that mutations in this gene can give rise to either of these phenotypes, but the locations of the mutations reported here are different from those published previously (6, 8, 12).

In the present study, the strains with hyperinducible phenotypes were divided into two types, types a and b, in recognition of the different levels of uninduced β-lactamase expression. Since uninduced β-lactamase expression was not maximal compared with that by the derepressed strains, it can be presumed that the AmpD proteins in the hyperinducible mutants retained some activity. This increase in the level of β-lactamase expression for hyperinducible mutants, we believe, is a reflection of the reduced ability to process the autoinducer compared with the processing ability of the wild type.

With the model proposed by Jacobs et al. (7), it is possible to put forward a hypothesis explaining the hyperinducible phenotype. If the AmpD protein from hyperinducible mutants were unable to remove the tripeptide from the GlcNAc-anhMurNAc-tripeptide, then under inducing conditions with product inhibition of β-*N*-acetylglucosaminidase, an increased level of activation of AmpR would occur since the additional autoinducer produced would not be inactivated by AmpD. The increase in the uninduced level of β-lactamase expression may be explained by either an excess of GlcNAc-anhMurNAc-tripeptide which is not hydrolyzed by the wild-type AmpD or a reduced ability of the mutant AmpD to process the anhMurNAc-tripeptide. The hyperinducible type b mutant may have a further reduced ability to process the anhMurNAc-tripeptide relative to that of the type a mutants, explaining the difference in the level of uninduced β-lactamase expression in this case. An inability to process one of two possible autoinducers may also explain an interesting property of a hyperinducible mutant reported previously (8): the dominance of the hyperinducible phenotype over the wild-type phenotype in complementation studies.

To date, DNA sequences have been reported for only two hyperinducible mutants (Table 1); only one of these, in *E. cloacae* 14, resulted from a single point mutation (8). This mutation, an A→G transition in codon 121 (Asp-121→Gly), confers a phenotype different from those conferred by the mutations described in this report. The previously described mutation does not give rise to an increase in the level of uninduced β-lactamase expression compared with that by the wild type, and the cefotaxime MIC remains unchanged. These differences, we believe, can be explained by differences in the levels of uninduced β-lactamase expression by these mutants and the inability of cefotaxime to induce β-lactamase expression. Since cefotaxime is a poor inducer of β-lactamase expression and is labile to its action, cefotaxime MICs will be similar for the wild type and strains with uninduced levels of β-lactamase similar to that of the wild type. Cefotaxime MICs will be higher for strains with increased uninduced levels of β-lactamase, because of the action of the additional β-lactamase on the labile compound. This was seen with the increased cefotaxime MIC for the hyperinducible type a strains and the raised MIC for the hyperinducible type b mutant compared with those for the type a mutants. The increase in the cefoxitin MICs for both the Asp-121→Gly mutant of *E. cloacae* and the hyperinducible mutants reported here is a reflection of cefoxitin's greater ability to induce β-lactamase expression. In the presence of cefoxitin more autoinducer will be produced, which, because of the hyperinducible nature of these mutants, will lead to an elevated level of β-lactamase expression, comparable to the level of expression of the derepressed mutants; hence, the cefoxitin MICs will be identical for hyperinducible and derepressed strains.

Interestingly, a hyperinducible phenotype with a two- to threefold increase in the level β-lactamase expression under noninduced conditions has been reported to result from a 52-bp frameshift deletion after codon 23 (12). It has been suggested that ribosome translational frame shifting may occur

to yield an AmpD protein with a 17-amino-acid deletion (*ampD1*) (8). Here we reported a mutation that is located within this region that gave rise to a derepressed phenotype that was the result of a change from valine to glycine at position 33. Why these differential effects occur is not clear. The presence of a glycine residue, if it is located in a structural element (i.e., α-helix or β-sheet) within this region, may, because of its greater conformational freedom, promote the unfolded rather than the folded conformation(s), leading to a nonfunctional protein. Possibly within the AmpD1 protein a whole structural element may have been removed, the absence of which may be compensated for by internal rearrangement in the tertiary structure, affecting but not eliminating the protein's function. However, such explanations can be offered only tentatively in the absence of the known protein structure.

It is difficult to make generalizations about the positions of the mutations giving rise to the mutant phenotypes. Those giving rise to the derepressed phenotype do appear near the amino and carboxy termini (excluding the Trp to termination codon at position 95), but the significance of this is not known. An Asp-164→Glu change found in a derepressed strain is interesting. Although a minor change structurally, chemically the difference could be very important if the Asp is located within the active site or the regulatory region. The finding of a mutation leading to a hyperinducible type a mutant in this region (Ala-158→Glu) adds weight to the argument that this is an important region. The locations of the mutations in the two other hyperinducible mutants are interesting because our search of the EMBL protein database showed that the Trp-95→Arg mutation lies within and that the Tyr-102→Asp mutation lies close to a small region (18 amino acids, 14 of which were positive matches) of homology with an *N*-acetylmuramyl-L-alanine amidase from a *Bacillus* sp. (15) (data not shown). Again, the significance of this is not known, but it is clear from the search that this is not a common motif among known muramyl amidases (data not shown).

It would appear from the results presented here that point mutations can be found at various locations in the *ampD* gene from hyperinducible and derepressed strains. In the case of derepressed strains it is assumed that the mutations give rise to a nonfunctional protein which by its absence leads to the constant activation of AmpR and, hence, constitutive β-lactamase production. The mutants with hyperinducible phenotypes all have mutations that alter the charge at particular loci, altering the local hydrophobicity profile (data not shown). It is assumed from the raised level of β-lactamase expression exhibited by these mutants that the AmpD protein has altered activity.

Further work is needed to determine the structure of the AmpD protein in the context of the nature of the mutations and their effect on β-lactamase expression. The partially derepressed phenotypes were not caused by mutations within the *ampD* gene, and work is in progress to determine the genetic locus or loci responsible.

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