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# Inactivation of the *ampD* Gene Causes Semiconstitutive Overproduction of the Inducible *Citrobacter freundii* β-Lactamase

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**In *Citrobacter freundii* and *Enterobacter cloacae*, synthesis of AmpC β-lactamase is inducible by the addition of β-lactams to the growth medium. Spontaneous mutants that constitutively overproduce the enzyme occur at a high frequency. When the *C. freundii ampC* β-lactamase gene is cloned into *Escherichia coli* together with the regulatory gene *ampR*, β-lactamase expression from the clone is inducible. Spontaneous cefotaxime-resistant mutants were selected from an *E. coli* strain carrying the cloned *C. freundii ampC* and *ampR* genes on a plasmid. Virtually all isolates had chromosomal mutations leading to semiconstitutive overproduction of β-lactamase. The mutation *ampD2* in one such mutant was caused by an IS*1* insertion into the hitherto unknown *ampD* gene, located between *nadC* and *aroP* at minute 2.4 on the *E. coli* chromosome. The wild-type *ampD* allele cloned on a plasmid could fully *trans*-complement β-lactamase-overproducing mutants of both *E. coli* and *C. freundii*, restoring the wild-type phenotype of highly inducible enzyme synthesis. This indicates that these *E. coli* and *C. freundii* mutants have their lesions in *ampD*. We hypothesize that induction of β-lactamase synthesis is caused by blocking of the AmpD function by the β-lactam inducer and that this leads directly or indirectly to an AmpR-mediated stimulation of *ampC* expression.**

Most gram-negative enterobacteria and *Pseudomonas* spp. express chromosomally encoded so-called species-specific β-lactamases (33). In *Escherichia coli*, the chromosomal β-lactamase is expressed from the *ampC* gene (5). It has been shown that the β-lactamase genes of other enterobacteria share a large degree of similarity with the *E. coli ampC* gene and that they occupy corresponding chromosomal locations in the different species (2, 12, 13, 15, 16). Thus, these genes are probably derived from a common ancestor. To avoid confusion, we refer to the related structural genes in the different species as *ampC* and to their gene products as AmpC β-lactamases.

In some species, the chromosomal AmpC β-lactamases are constitutively produced, whereas in other species, notably *Citrobacter freundii*, *Enterobacter cloacae*, and *Pseudomonas* spp., expression is inducible by β-lactams (33). In wild-type strains of these species expression is low, but it can be induced more than 100-fold by β-lactams, e.g., 6-aminopenicillanic acid.

The most significant genetic difference between the inducible group, e.g., *C. freundii*, and the group of species producing the enzyme constitutively, e.g., *E. coli*, is the presence of the *ampR* regulatory gene positioned between the *frdD* and *ampC* genes in the inducible group (2, 17).

*E. coli* normally produces its AmpC β-lactamase at a low constitutive level and lacks the *ampR* gene. When the *C. freundii ampR* and *ampC* genes are cloned into *E. coli*, expression from the *C. freundii ampC* gene can be induced, while expression of the *E. coli* AmpC β-lactamase is not affected by this procedure (15). In the absence of the *ampR* gene the basal expression of the *C. freundii* β-lactamase is slightly higher, but enzyme synthesis can no longer be induced.

Clinically, gram-negative bacteria with inducible chromosomal β-lactamases are resistant to most penicillins and cephalosporins. The newer cephalosporins, e.g., cefotaxime, kill members of most of these species at a low concentration. However, resistant mutants of *C. freundii* and *E. cloacae* arise at a frequency of $10^{-5}$ to $10^{-7}$, by mutation to constitutive overproduction of the chromosomal β-lactamase (8, 17, 29). While the relative enzyme level in the wild type can be induced more than 100-fold in 40 min, it can be induced by only about 3-fold in the mutants. We have investigated the inducibility in several such constitutive mutants, and all showed a similar level of residual inducibility (unpublished data). We will therefore refer to such mutants as semiconstitutive in this paper. In the *C. freundii* mutant investigated, the mutation leading to semiconstitutive overproduction of β-lactamase was located outside of the region containing the *ampC* and *ampR* genes (17).

Owing to the high frequency of mutation, we have hypothesized that the lesion leading to semiconstitutive enzyme overproduction is a loss of function in a gene normally encoding a protein necessary for inducibility (17). If so, this gene should be present also in *E. coli*, since β-lactamase induction is possible after the transfer of the *C. freundii ampC* and *ampR* genes into *E. coli*. To test this possibility, we selected cefotaxime-resistant mutants of *E. coli* harboring plasmids with the *C. freundii ampC* and *ampR* genes. Virtually all mutants investigated were chromosomal. About half of these strains had the same phenotype as the hyperresistant *C. freundii* and *E. cloacae* mutants, i.e., semiconstitutive *ampR*-dependent overproduction of β-lactamase. Here we map the mutated locus, *ampD*, and investigate its role in β-lactamase regulation in *E. coli* and *C. freundii*.

## MATERIALS AND METHODS

**Bacteria.** The bacterial strains used in this study are listed in Table 1, together with their respective source and reference.

**Media and growth conditions.** For routine purpose L broth medium of Bertani (3) was used supplemented with medium E (35), 0.2% glucose, and 1 mg of thiamine per liter. When required, nicotinic acid at 5 mg/liter, quinolinic acid at 30 mg/liter, tetracycline at 10 mg/liter, chloramphenicol at 10

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TABLE 1. Bacterial strains used in this study

| Species (strain) | Relevant genotype | Comments | Reference or source |
|---|---|---|---|
| *Citrobacter freundii* | | | |
| OS60 | *ampR*$^+$ *ampC*$^+$ | Isolate | 17 |
| OS61 | *ampR*$^+$ *ampC*$^+$ *ampD*(?) | Carbenicillin-20$^r$ mutant of OS60 | 17 |
| *Escherichia coli* | | | |
| K-12 | | | |
| SN03 | *ampA1 ampC8 recA* | | 25 |
| SN0302 | *ampD2* | Mutant of SN03 (see text) | This study |
| SN0303 | *ampD* (?) | Mutant of SN03 (see text) | This study |
| SN0305 | *ampD* (?) | Mutant of SN03 (see text) | This study |
| SN0306 | *ampD* (?) | Mutant of SN03 (see text) | This study |
| SN0310 | *ampD* (?) | Mutant of SN03 (see text) | This study |
| HfrC | Hfr | | 18 |
| HfrH | Hfr | | 18 |
| SN1000 | Hfr *ampD2 zad*::Tn*10* | 17% cotransductant between *ampD2* and Tn*10* | This study |
| CSH57a1 | *leu recA*$^+$ | | 23 |
| SN1012 | *ampD2 leu*$^+$ | Cotransductant from SN0302 to CSH57a1 | This study |
| JRG582 | Δ(*nadC-aroP*)2 | Mutant of HfrH | 10 |
| AB1157 | *leu* | | 18 |
| GRB222 | *leu*$^+$ *zab*::Tn*10* | Transduction derivative of AB1157 | G. R. Björk, unpublished |

mg/liter, kanamycin at 50 mg/liter, or β-lactam antibiotics at various concentrations were added. M9 medium (23) containing 0.2% Casamino Acids (Difco Laboratories, Detroit, Mich.), uracil at 50 mg/liter, and thiamine at 1 mg/liter was used in the determination of β-lactamase expression and ampicillin resistance. All bacterial growth was at 37°C.

**Plasmid construction and DNA techniques.** Plasmid pNU305 carries the *C. freundii ampR* and *ampC* genes. This plasmid, its Δ*ampR* derivative pNU307, and the *ampR*-carrying pNU311 have been described previously (Fig. 1) (17). Plasmid pNU344 (Fig. 1) carries the *C. freundii ampR* and *ampC* genes on a pACYC184-derived (6) replicon. It was constructed by ligating the *Sal*I-*Bgl*II fragment of pNU302 carrying *ampR* and *ampC* to the *Sal*I-*Bam*HI backbone of pACYC184. In addition to inducible β-lactamase expression, this plasmid mediates resistance to chloramphenicol.

The following plasmids are all clones of segments of the *nadC-aroP* region of λG78N (10). Plasmid pNU400 was obtained by cloning the *Eco*RI insert of λG78N into pLC339 (32) digested with the same enzyme. The clone was obtained by transformation (21) into *E. coli* JRG582 and selection for tetracycline resistance and the NadC$^+$ phenotype, i.e., growth on nicotinic acid (5 mg/liter) but not on quinolinic acid (30 mg/liter) (34). Subclone pNU401 was constructed in a similar manner, by digesting pNU400 with *Hin*dIII and

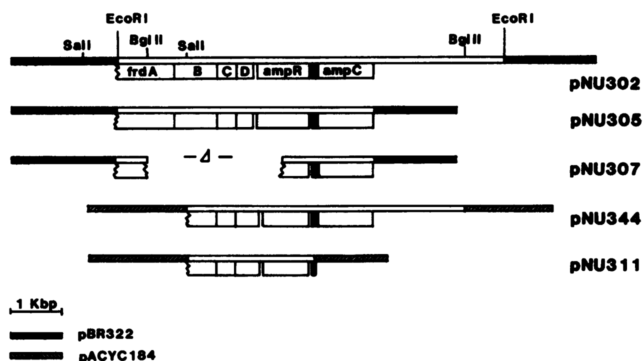


FIG. 1. Restriction maps of the *C. freundii ampR-ampC* region and the subclones used in this paper.

*Bam*HI and ligating this to a digest of pACYC184 treated with the same enzymes. The clone was isolated in *E. coli* JRG582 by chloramphenicol and NadC$^+$ selection. Plasmid pNU403 is an *Sph*I cutback derivative of pNU401, and pNU402 is the complementary clone carrying the thus deleted *Bam*HI$_1$-*Sph*I fragment in vector pACYC184 cut at the corresponding sites. To construct pNU404, an *Ava*I digest of pNU401 was first treated with the large fragment of DNA polymerase I to make the ends blunt (22), following by religation and selection of NadC$^+$, chloramphenicol-resistant clones in JRG582. A member, pNU404, of the smallest class of such clones was further characterized.

A pNU78 derivative corresponding to pNU404 was constructed by the following manipulations. First, a *Bgl*II linker (5′-CCGATCGG-3′) was introduced (22) into the *Eco*RI site of pNU78 (28), giving pNU399. Since the *Eco*RI ends were made blunt by a fill-in reaction, the *Eco*RI site was recreated on either side of the linker. Then pNU399 was digested with *Sac*II and treated with T4 DNA polymerase (22), making the ends flush, followed by digestion with *Bgl*II. Plasmid pNU401 was digested with *Ava*I, the ends were filled in, and then it was digested with *Bam*HI. Plasmid pNU405 was obtained by ligation of these two digests, transformation into *E. coli* JRG582, and selection for NadC$^+$ and tetracycline resistance. This plasmid carries the large *Bam*HI-*Ava*I$_1$ fragment of pNU401 on the *Bgl*II-*Sac*II backbone of pNU399. The construction of pNU406 by recombination of *ampD2* onto pNU405 is described in the Results section.

**Isolation of *E. coli* mutants.** *E. coli* K-12 SN03 was transformed with plasmid pNU305 carrying the *C. freundii ampR* and *ampC* genes. Screw-cap tubes containing 1.5 ml of L broth and tetracycline at 10 mg/liter were inoculated with separate colonies of SN03(pNU305). After overnight growth, the cells (≈$10^9$ CFU) were concentrated to 0.2 ml and spread on L plates supplemented with tetracycline and cefotaxime at 5 to 100 mg/liter. The number of colonies appearing after 36 h of growth was similar in all cultures and showed a threefold decrease going from selection at 5 mg to selection at 50 mg of cefotaxime per liter.

One mutant from each culture was purified, and plasmid DNA (isolated on a small scale) was transformed into SN03 to detect a possible plasmid-mediated increase in resistance. If none of at least 100 transformants was able to grow on 0.5

TABLE 2. Ampicillin resistance and β-lactamase expression of SN03(pNU305) and its mutant derivatives

| Strain | Ampicillin resistance ($IC_{50}$ [mg/liter]) | Relative β-lactamase expression[a] | |
|---|---|---|---|
| | | Noninduced | Induced |
| SN03(pNU305) | 15 | 1.0 | 37.9 |
| SN0301(pNU305) | 300 | 2.3 | 65.2 |
| SN0302(pNU305) | 325 | 37.1 | 101.3 |
| SN0303(pNU305) | 325 | 29.5 | 111.8 |
| SN0304(pNU305) | 300 | 2.1 | 51.7 |
| SN0305(pNU305) | 325 | 39.6 | 139.1 |
| SN0306(pNU305) | 325 | 37.1 | 113.8 |
| SN0307(pNU305) | 300 | 1.4 | 43.8 |
| SN0309(pNU305) | 300 | 1.6 | 47.2 |
| SN0310(pNU305) | 325 | 30.2 | 106.9 |

[a] Relative to SN03(pNU305).

mg of cefotaxime per liter, the mutation was classed as not being plasmid borne.

**Curing of *E. coli* mutants.** To enrich cells which had spontaneously lost pNU305, we used a modification of the penicillin-enrichment method (23). Bacteria were grown on an optical density at 600 nm of 0.4 in L broth, after which tetracycline (20 mg/liter) was added. After an additional 10 min, D-cycloserine (2 mM) was added (23), and incubation was continued for 2 h at 37°C, killing the tetracycline-resistant bacteria. Subsequently, the cells were washed and grown overnight without selection. The entire procedure was repeated, and plasmidfree clones were identified by their tetracycline sensitivity, which was verified by the absence of plasmid in total DNA preparations.

**Isolation of Tn*10* insertion linked to *ampD2*.** A pool of Tn*10* transposants was constructed from the mutant SN0302 by infection with λ250::Tn*10* as described by Björk and Olsén (4). A P1 lysate was made of this pool and used to infect *E. coli* HfrC(pNU344), selecting for tetracycline-resistant transductants. These were screened for the *ampD2* phenotype by replica plating onto plates with cefotaxime (3 mg/liter). Several potential cotransductants were isolated, and the frequency of cotransduction between *ampD2* and Tn*10* was determined. One of these strains, SN1000, showed a cotransduction frequency of 17% and was chosen for further studies.

**Assay of ampicillin resistance and β-lactamase expression.** Ampicillin resistance was determined on M9CA plates as the $IC_{50}$, i.e., the concentration required to inhibit 50% of the single cells from forming colonies (24). Plates were scored after 36 to 40 h of growth at 37°C. We previously used the 50% lethal dose for the same variable, but the designation $IC_{50}$ is semantically more correct. β-Lactamase activity in sonicated extracts was determined spectrophotometrically (26) at 260 nm with 170 μM cefalexin as the substrate in 0.1 M potassium phosphate buffer, pH 7.0. To prepare extracts, cells were grown logarithmically to an optical density at 420 nm of 0.8 in M9CA medium. A volume of 40 ml taken from this culture was chilled on ice and centrifuged. The cells were washed and suspended in 0.8 ml of phosphate buffer, and 0.2 ml of 50 mM EDTA–500 μg of lysozyme per ml was added. After incubation at 37°C for 30 min, the mixture was freeze-thawed and sonicated on ice by five cycles of sonication (10 s with a Branson sonifier at setting 3 with 30 s of cooling). Usually, 1 to 40 μl of extract was required per ml of substrate solution. Specific activity was expressed as micromoles of substrate hydrolyzed per minute at 22°C per milligram of total protein (20) with bovine serum albumin as the standard. Induction of β-lactamase expression with 6-aminopenicillanic acid (2 g/liter) for 40 min was done as previously described (17).

**Chemicals and enzymes.** All chemicals were of the highest grade commercially available. Ampicillin and 6-aminopenicillanic acid were gifts from Astra Läkemedel, and cefotaxime was kindly provided by Svenska Hoechst AB. Restriction enzymes, DNA ligase, and DNA polymerase were purchased from New England BioLabs Inc. (Beverly, Mass.) or Boehringer GmbH (Mannheim, Federal Republic of Germany).

## RESULTS

**Selection of cefotaxime-resistant *E. coli* mutants carrying cloned *C. freundii ampC* and *ampR* genes.** Plasmid pNU305 carries the *ampC* and *ampR* genes of *C. freundii* OS60 (Fig. 1). When an overnight culture of *E. coli* SN03(pNU305) was plated on L agar containing cefotaxime at 5 to 50 mg/liter, approximately $1 \times 10^{-7}$ to $3 \times 10^{-7}$ bacteria survived and formed colonies. A total of 45 independent mutants were isolated, and plasmid DNA purified from them was transformed into the *E. coli* parent strain SN03. In only one of the mutants could increased cefotaxime resistance be transferred with the plasmid. This mutant was not studied further here.

Of the remaining 44 mutants, 10, all obtained at 50 mg of cefotaxime per liter, were cured of pNU305 by D-cycloserine enrichment. Loss of the plasmid always resulted in loss of cefotaxime resistance, which was regained upon transformation with pNU305. None of the cured mutant strains differed in any detectable way from the parent in terms of growth rate, colony morphology, nutritional requirements, or resistance to ampicillin and cefotaxime. The 10 mutant strains, cured and retransformed with plasmid pNU305, were assayed for ampicillin resistance and β-lactamase production under inducing and noninducing conditions.

From Table 2 it is apparent that five of them, SN0302, SN0303, SN0305, SN0306, and SN0310, had the phenotype of original *Citrobacter* mutants, i.e., semiconstitutive overproduction of β-lactamase. SN0302 was chosen as a representative of these mutants, and the mutation in this strain was called *ampD2* (allele number corresponds to last digit in strain designation). As previously shown (17), β-lactamase expression from pNU307 (*ampR* $ampC^{+}$; Fig. 1) was low and noninducible in SN03 (Table 3). This effect could be complemented to slightly lower and fully inducible enzyme synthesis by *ampR* on pNU311 (Fig. 1). Expression from pNU307 in the mutants was identical to that in the wild type, whereas with *ampR* in *trans* the overproduction phenotype

TABLE 3. Dependence on *ampR* for ampicillin resistance and β-lactamase expression in SN03 and its mutant derivatives[a]

| Strain | Ampicillin resistance ($IC_{50}$ [mg/liter]) | Relative β-lactamase expression[b] | |
|---|---|---|---|
| | | Noninduced | Induced |
| SN03(pNU307) | 27 | 2.6 | 2.5 |
| SN0302(pNU307) | 27 | 2.5 | 2.6 |
| SN03(pNU307)(pNU311) | 11 | 1.0 | 18.4 |
| SN0302(pNU307)(pNU311) | 150 | 24.1 | 48.6 |
| SN03(pNU311)[c] | <1 | <0.02 | <0.02 |
| SN0302(pNU311)[c] | <1 | <0.02 | <0.02 |

[a] Results with SN0303, SN0305, SN0306, and SN0310 are very similar to those obtained with SN0302.

[b] Relative to SN03(pNU305).

[c] Results do not differ significantly from those obtained with SN03 and SN0302 alone.

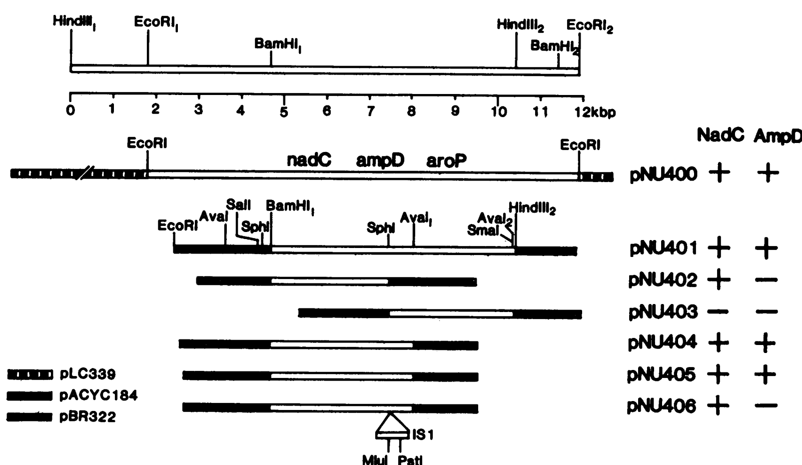


FIG. 2. Restriction map of the *nadC-aroP* region of the *E. coli* chromosome. The subclones used in this study are also shown. Phage λG78N was isolated by Guest and Stephens (10) and carries the 10.1-kbp *Eco*RI fragment which was recloned into pNU401. Plasmids pNU402, pNU403, pNU404, and pNU405 carry DNA derived from λG78N. Plasmid pNU406 carries the *ampD2* mutation of SN0302 (SN1012), transferred by homologous recombination onto pNU405. Plasmid pNU408 (not shown) carries *ampD2* on the 10.5-kbp *Hin*dIII$_{1-2}$ fragment cloned directly from SN0302. The NadC and AmpD phenotypes mediated by the different constructs are also indicated. These were assayed as complementation of JRG582 (pNU305/pNU344) to growth on quinolinic acid (30 mg/liter) and sensitivity to 3 mg of cefotaxime per liter, respectively.

was restored (Table 3). Thus, the effect of *ampD* on β-lactamase synthesis seems to require the presence of an intact *ampR* gene.

The remaining mutants showed a similar ampicillin resistance, but only a twofold-elevated β-lactamase expression under noninducing conditions. They were therefore excluded from the present study.

**Mapping of *ampD* locus in SN0302 and its complementation with the wild-type allele.** An HfrC derivative, SN1000, with a transposon Tn*10* insertion 17% cotransducible with *ampD2* was isolated (see Materials and Methods). This strategy was chosen since we expected the high frequency of spontaneous mutation to make direct transduction mapping difficult. The location of Tn*10* in SN1000 was determined by interrupted matings (18) after first transducing the insertion into different Hfr strains. Tn*10* was shown to be located between the origins of HfrH and HfrC close to minute 3 on the *E. coli* linkage map (1). F′ KLF4 (F′ 104) originates from HfrH and carries minutes 99 through 6 of the *E. coli* chromosome (19). In an attempt to complement *ampD2* with KLF4, we labeled the F′ with Tn*10* by introducing it into GRB222 (*zab*::Tn*10*), allowing recombinational transfer of Tn*10* onto KLF4. This derivative was then conjugated into SN0302 selecting for tetracycline resistance. From here, KLF4 *zab*::Tn*10* was transferred to SN03(pNU344) and SN0302(pNU344). The F′ complemented *ampD2* in SN0302(pNU344) to give low inducible β-lactamase expression, whereas the phenotype of SN03(pNU344) was not affected (data not shown). These results confirm the mapping data and show that *ampD2* is recessive to the wild-type allele.

By P1 transduction (14), *ampD2* was found to be 40% cotransducible with *leu* and 70% cotransducible with *envA*. No cotransduction was obtained with *proA* or *thr*. This locates the *ampD2* mutation to the *nadC-lpd* region, a region previously characterized by Guest and Stephens (10). *E. coli* JRG582, a Δ(*nadC-aroP*) strain, has the same phenotype as the *ampD2* mutant SN0302, i.e., β-lactamase expression from pNU344 was 80 without and 112 with induction [values relative to those of SN03(pNU344)]. This phenotype could be fully complemented by lysogenization with bacteriophage λG78N (10) carrying the region from *nadC* through *aroP* to the beginning of *aceE* (9). The level of noninduced β-lactamase expression from JRG582(λG78N)(pNU344) was the same as that from SN03(pNU344), and it could be induced 30-fold in 40 min with 6-aminopenicillanic acid (2 g/liter). Thus, phage λG78N carries the wild-type allele of *ampD* or an extragenic suppressor of the *ampD2* mutation. The latter alternative is excluded below.

**Cloning of *ampD* gene from phage λG78N.** Plasmid pNU400 carries the 10,100-base-pair (bp) *Eco*RI insert from phage λG78N. The restriction map of the *Eco*RI fragment has been determined by Guest and co-workers (9) and was confirmed by us (Fig. 2). By various subcloning experiments (Fig. 2 and Materials and Methods) the approximate locations of the *nadC* and *ampD* genes were determined. The *ampD* gene is situated on the *Bam*HI-*Ava*I$_1$ insert on pNU404 and probably spans the *Sph*I site (Fig. 2), since neither pNU402 nor pNU403 complemented the *ampD2* mutation. The *aroP* gene has recently been shown to map between *Ava*I$_1$ and *Hin*dIII$_2$ (Fig. 2) (7), and this gene is therefore distinct from *ampD*.

**Cloning of *ampD2* mutation from SN0302.** To clone the *ampD2* mutation, we transferred it from SN0302 into the Rec$^+$ strain CSH57a1 by P1-mediated cotransduction with

TABLE 4. Complementation of SN03 and its mutant derivatives with plasmids pNU405 (*ampD*$^+$) and pNU406 (*ampD2*)[a]

| Strain | Ampicillin resistance ($IC_{50}$ [mg/liter]) | Relative β-lactamase expression[b] | |
|---|---|---|---|
| | | Noninduced | Induced |
| SN03(pNU344) | 4 | 1.0 | 7.4 |
| SN03(pNU344)(pNU405) | 4 | 0.8 | 6.2 |
| SN03(pNU344)(pNU406) | 4 | 0.8 | 7.4 |
| SN0302(pNU344) | 400 | 22.9 | 42.0 |
| SN0302(pNU344)(pNU405) | 4 | 0.9 | 12.6 |
| SN0302(pNU344)(pNU406) | 400 | 20.0 | 43.0 |

[a] Results with SN0303, SN0305, SN0306, and SN0310 are very similar to those obtained with SN0302.
[b] Relative to SN03(pNU305).

TABLE 5. Complementation of *C. freundii* OS60 and its β-lactamase-overproducing mutant derivative OS61 with plasmids pNU405 (*ampD*$^+$) and pNU406 (*ampD2*)

| Strain | Ampicillin resistance ($IC_{50}$ [mg/liter]) | Relative β-lactamase expression[a] | |
|---|---|---|---|
| | | Noninduced | Induced |
| OS60 | 10 | 1.0 | 209 |
| OS60(pNU405) | 5 | 1.0 | 67 |
| OS60(pNU406) | 10 | 0.8 | 188 |
| OS61 | 100 | 124.0 | 227 |
| OS61(pNU405) | 5 | 1.4 | 66 |
| OS61(pNU406) | 150 | 151.0 | 217 |

[a] Relative to OS60. [The expression from SN03(pNU305) is 15 relative to OS60.]

*leu*. The resulting *ampD2* strain was called SN1012. To allow recombination of the *ampD2* mutation onto pNU405, we transformed the latter into SN1012. After about 20 generations of growth, plasmid DNA was isolated and transformed into SN1012(pNU344). Among the transformants, cefotaxime (3 mg/liter)-resistant clones appeared at a frequency of 4 $\times 10^{-5}$. These should have picked up the *ampD2* allele from the chromosome by homologous recombination. The plasmid isolated from one of these clones was called pNU406. Plasmid pNU405 (*ampD*$^+$) fully complemented the phenotype of all five mutants back to low-level-inducible β-lactamase production. In contrast, pNU406 (*ampD2*) failed to do so, and expression remained high and semiconstitutive (Table 4). These data show that pNU405 actually carries the *ampD* gene and not some other locus which phenotypically suppresses the *ampD2* mutation, and that, most likely, all five mutants carry their mutations in the *ampD* gene. The *ampD2* locus was also isolated by direct cloning of the 10.5-kb *Hin*dIII$_{1-2}$ fragment from SN0302 (by NadC$^+$ selection in *E. coli* JRG582). Complementation experiments with this clone (pNU409) gave results identical to those obtained with pNU406 (data not shown).

**Characterization of *ampD2* mutation.** By restriction mapping of pNU406 and pNU409 it was observed that they carried an insertion of about 750 bp between *Sph*I and *Ava*I$_1$, suggesting that one end of *ampD* lies between these sites. The presence of the *Pst*I and *Mlu*I sites on the insertion, separated by approximately 250 bp, led us to believe that the insertion is IS*1*. The DNA sequence of the *Pst*I-*Mlu*I fragment was determined (data not shown) and was identical to that published for the corresponding region of IS*1* (27), confirming our assumption.

**Complementation of *C. freundii* β-lactamase-overproducing mutant OS61 with *ampD*.** Plasmids pNU405 (*ampD*$^+$) and pNU406 (*ampD2*) were transformed into *C. freundii* OS60 and its semiconstitutive mutant derivative OS61, selecting for tetracycline resistance. Neither plasmid affected β-lactamase expression in the wild type (Table 5). However, pNU405 fully complemented the semiconstitutive overproducer OS61 to wild-type-level inducible β-lactamase production. This strongly suggests that *C. freundii* OS61 owes its β-lactamase overproduction phenotype to a mutation in the *C. freundii ampD* gene. β-Lactamase expression under noninducing conditions was the same in OS60 and OS60(pNU405), although resistance to ampicillin was significantly higher in OS60 than in OS60(pNU405). Probably, β-lactamase expression in OS60 is induced by ampicillin at concentrations below 5 mg/liter, leading to partial induction on the $IC_{50}$ plates, while this is not the case in the presence of plasmid pNU405.

## DISCUSSION

We have recently shown that mutations leading to semiconstitutive β-lactamase overproduction in *C. freundii* and *E. cloacae* lie outside of the *ampC* and *ampR* genes (16, 17). Here we isolated chromosomal mutants of *E. coli* which similarly affect β-lactamase expression from plasmids carrying the *C. freundii ampC* and *ampR* genes.

The lesion *ampD2* in one of the *E. coli* mutants was mapped to the *nadC-aroP* region at minute 2.4 on the *E. coli* chromosome. The *ampD* gene represents a hitherto unknown gene, distinct from *nadC* and the recently mapped *aroP* (7). The wild-type gene, isolated on a 3.4-kbp *Bam*HI-*Ava*I fragment, complemented the *E. coli* mutants, giving the wild-type phenotype, whereas the corresponding region from the *ampD2* mutant failed to do so. When the *E. coli ampD* clone was introduced into the β-lactamase-overproducing *C. freundii* mutant OS61 this strain reverted to wild-type β-lactamase synthesis and inducibility, whereas no alteration in phenotype could be observed when the *ampD2* clone was introduced. Thus, the basis of the high semiconstitutive β-lactamase expression in *C. freundii* OS61 is most likely a disruption of *ampD*.

Two *trans*-acting components of β-lactamase regulation have been identified. One of these, *ampR*, is present only in species with inducible β-lactamase expression, e.g., *C. freundii* and *E. cloacae*, but not in *E. coli*, which produces its β-lactamase constitutively. The combination of *ampR* from one species with *ampC* from the other yields qualitatively but not quantitatively similar results as homologous combinations (16). This has been interpreted to suggest a direct interaction between AmpR and the *ampC* control region or transcript (16). The other regulatory component, *ampD*, is present in both species with inducible and species with noninducible β-lactamase expression. This gene is fully complementable between all species mentioned, suggesting that its interaction with the rest of the system is very well conserved.

Inactivation of the *ampD* gene with IS*1* gives almost the same result as addition of inducer, suggesting that induction inactivates AmpD, possibly by a direct binding of β-lactam to AmpD. The residual inducibility of *ampD* mutants could be due to a gene product whose functions partially overlap those of AmpD. In analogy, there are certain peptidoglycan-synthesizing enzymes called the penicillin-binding proteins which bind penicillin with concomitant loss of enzymatic function. However, none of the penicillin-binding protein genes, whose chromosomal locations are known (genes for penicillin-binding proteins 1a, 1b, 2, 3, and 5), map in the *ampD* region (36). Since *ampD* is present in *E. coli* and yet does not affect *E. coli* β-lactamase expression (data not shown), it is conceivable that AmpD has some function besides β-lactamase induction. It is possible that β-lactamase induction is mediated by a substrate or product of AmpD action, rather than by direct interaction between the protein and, e.g., AmpR.

Viewing AmpD as a sensor and AmpR as a regulatory effector is reminiscent of the *uhp* system. Synthesis of the hexose monophosphate transporter from the *uhpT* gene is induced by extracellular glucose-6-phosphate, whereas it is insensitive to the intracellular concentration of the compound (30). UhpA is proposed to be an activator of *uhpT* transcription, and UhpR is the transmembrane sensor (31). Here the signal is transmitted by a protein, UhpC, which normally complexes with UhpA, inhibiting it from activating transcription. Binding of glucose-6-phosphate to UhpR on

the outside of the cytoplasmic membrane is proposed to lead to binding of UhpC to the cytoplasmic domain of the sensor, leaving the unblocked UhpA to activate *uhpT* transcription. In the osmoregulation of porin synthesis a similar mechanism is proposed with EnvZ as the sensor and OmpR as the regulator of transcription (11). The mode of transmission of the signal between the two is not yet known.

Whether β-lactamase induction is analogous to these systems remains to be experimentally tested. With the identification and characterization of *ampD* it should be possible to inquire further into the mode of signal transmission from the β-lactam stimulus to the response of increased β-lactamase expression.

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