ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Apr. 1997, p. 785–790 Vol. 41, No. 4
0066-4804/97/$04.00+0

OXA-15, an Extended-Spectrum Variant of OXA-2 β-Lactamase, Isolated from a *Pseudomonas aeruginosa* Strain

FRANCK DANEL,[1]* LUCINDA M. C. HALL,[1] DENIZ GUR,[2] AND DAVID M. LIVERMORE[1]

Department of Medical Microbiology, St. Bartholomew's and the Royal London School of Medicine and Dentistry, London E1 2AD, United Kingdom,[1] and Section of Infectious Diseases, Department of Internal Medicine, Hacettepe University School of Medicine, 06100 Ankara, Turkey[2]

Received 29 July 1996/Returned for modification 6 November 1996/Accepted 24 January 1997

Pseudomonas aeruginosa **AH, isolated in Ankara, Turkey, was highly resistant to ceftazidime (MIC, 128 μg/ml) and produced a β-lactamase that gave a doublet of bands at pIs 8.7 and 8.9. β-Lactamase production was transferable to *P. aeruginosa* PU21 by conjugation and was determined by a ca. 450-kb plasmid, pMLH54. The transconjugant and *Escherichia coli* transformed with the cloned gene showed increased resistance to ceftazidime (especially) and to cefpirome, ceftazidime, ceftriaxone, moxalactam, and aztreonam, but not to carbapenems. Resistance was not reversed by clavulanic acid or tazobactam. Sequencing revealed that the β-lactamase responsible for this resistance was identical to OXA-2 except that glycine replaced aspartate at position 150. Compared to OXA-2, the new enzyme, named OXA-15, had greater cephalosporinase activity, with increased relative hydrolysis rates for cephaloridine and cephalothin and, most dramatically, for ceftazidime. Cefotaxime and carbapenems remained stable to hydrolysis. Thus, as in the TEM, SHV, and OXA-10 (PSE-2) β-lactamase families, a minor sequence change in OXA-2 gave a major extension of cephalosporinase activity and contingent resistance. The gene encoding the new β-lactamase, bla_{OXA-15}, lay close to the highly conserved 3′ end of an integron and had flanking sequences typical of an integron-associated gene cassette. Restriction mapping and partial sequence data indicated that pMLH54 carries an integron with three putative gene cassettes: bla_{OXA-15} itself, *aadB* [coding aminoglycoside nucleotidyltransferase (2″)-1a], and an uncharacterized cassette.**

Resistance to extended-spectrum cephalosporins in enterobacteria is increasingly associated with extended-spectrum plasmid- or transposon-mediated β-lactamases (ESBLs). Most ESBLs are mutants of molecular class A β-lactamases, specifically, TEM-1, TEM-2, and SHV-1 (2, 13, 21, 29).

Classical TEM and SHV β-lactamases remain uncommon in *Pseudomonas aeruginosa* (4), and only once has an extended-spectrum TEM mutant been reported in the species (31). Nevertheless, *P. aeruginosa* has been a major source of unusual ESBLs. It was the first species found to harbor a plasmidic zinc β-lactamase (47) and the first from which PER-1, a non-TEM, non-SHV class A β-lactamase, was recorded (32, 33). Furthermore, it remains the sole host species for the extended-spectrum mutants of the class D β-lactamase OXA-10 (19), namely, OXA-11, -14, -16, and -17 (8, 10, 11, 14). In the present report, we describe the discovery and characterization of the first extended-spectrum mutant of OXA-2 β-lactamase, also from a *P. aeruginosa* strain.

MATERIALS AND METHODS

Bacterial strains and plasmids. *P. aeruginosa* AH was isolated in April 1992 from a patient in the neurology ward at Hacettepe University Hospital, Ankara, Turkey, and was retained because of its considerable resistance to ceftazidime. Prior to its isolation the patient had received ampicillin plus ciprofloxacin and then ceftazidime plus ciprofloxacin. Reference producers of OXA-2 β-lactamase were *Escherichia coli* K-12 J53-2 with plasmid R46, *P. aeruginosa* Riff 6, and the Riff 6 transconjugant *P. aeruginosa* PU21(pMG40). The following β-lactamases, all from *E. coli* J53-2 and its transconjugants, were used for comparison in isoelectric focusing: AmpC (pI 9.2), SHV-2 (pI 7.6), SHV-3 (pI 7.0), and SHV-5 (pI 8.2). *P. aeruginosa* PU21 *ilv leu* Str^r Rif^r (20) was used as a recipient for transconjugation, and *E. coli* XL1-blue MRF′ was used as a recipient for transformation with the cloning vectors pBC SK+ (Stratagene, La Jolla, Calif.) and pUC19 (48). *E. coli* NCTC 50192 (44) with plasmids of 154, 66, 38, and 7 kb (44) and *P. aeruginosa* PU21 with the 450-kb plasmid pMG2 (20) served as markers in plasmid sizing studies.

Antibiotics. The antimicrobial agents tested were aztreonam and cefepime (Bristol Myers Squibb, Syracuse, N.Y.), cefsulodin (CIBA-GEIGY, Basel, Switzerland), ceftazidime (Glaxo-Wellcome, Greenford, United Kingdom), piperacillin sodium, tazobactam, and tetracycline (Lederle, Gosport, United Kingdom), cephalothin, moxalactam, and tobramycin (Lilly, Basingstoke, United Kingdom), cefoxitin and imipenem (Merck Sharp & Dohme, Hoddesdon, United Kingdom), ceftriaxone (Roche, Welwyn Garden City, United Kingdom), cefotaxime and cefpirome (Roussel, Uxbridge, United Kingdom), amikacin, benzylpenicillin, cephaloridine, chloramphenicol, gentamicin, oxacillin, and rifampin (Sigma, St. Louis, Mo.), ampicillin sodium, carbenicillin disodium, and clavulanate lithium (SmithKline Beecham, Brentford, United Kingdom), and meropenem (Zeneca, Macclesfield, United Kingdom).

Susceptibility tests. MICs were determined on DST agar (Unipath, Basingstoke, United Kingdom) with inocula of 10^4 CFU per spot, as described previously (14).

Plasmid transfer and characterization. Transfer of resistance from *P. aeruginosa* AH to *P. aeruginosa* PU21 was by overnight plate mating of logarithmic-phase cells on drug-free DST agar (Unipath). Transconjugant selection was on the same medium containing ceftazidime at 25 or 50 μg/ml plus rifampin at 100 μg/ml. Presumptive transconjugants were confirmed as nitrocefin-positive auxotrophs.

For size estimations, plasmids were extracted by the methods of Kado and Liu (23) or Hansen and Olsen (18) and were electrophoresed at 100 V for 4 h in 0.7% agarose gels at 4°C.

Detection of β-lactamases and their genes. β-Lactamases were characterized by isoelectric focusing (30) of sonic extracts prepared from overnight nutrient agar cultures (14). The enzymes were detected and crudely typed by overlaying the gels with 0.5 mM nitrocefin with or without 0.3 mM cloxacillin. For probing, the total DNA was extracted by the method of Pitcher et al. (36), as modified by Hall et al. (14); briefly, cells from overnight broth cultures were lysed with guanidium thiocyanate, and the proteins were extracted by treatment with a mixture of chloroform and isoamyl alcohol. Total DNA was digested with *Hin*cII and was separated on an agarose gel. By using methods described previously, a Southern blot of the gel was then probed with an oligonucleotide specific for an SHV sequence (28) or with a purified PCR product from bla_{OXA-10} (14).

* Corresponding author. Mailing address: Department of Medical Microbiology, St. Bartholomew's and the Royal London School of Medicine and Dentistry, Turner St., London E1 2AD, United Kingdom. Phone: 44-171-377-7644. Fax: 44-171-247-7669. E-mail: F.H.Danel@mds.qmw.ac.uk.

TABLE 1. MICs for *P. aeruginosa* isolates, transconjugants, and reference strains

Antibiotic	MIC (μg/ml)			
	Isolate AH (OXA-15)[a]	Transconjugants		Recipient PU21
		PU21 (OXA-15)[a]	PU21 (OXA-2)[a]	
Carbenicillin	256	128	128	64
Carbenicillin + clavulanate[b]	128	64	64	64
Carbenicillin + tazobactam[b]	128	128	64	64
Piperacillin	32	32	32	2
Piperacillin + clavulanate[b]	8	8	8	4
Piperacillin + tazobactam[b]	8	8	4	4
Cefepime	8	4	2	2
Cefotaxime	32	16	16	16
Cefpirome	16	16	4	2
Cefsulodin	32	32	4	2
Ceftazidime	128	128	16	4
Ceftazidime + clavulanate[b]	64	64	2	2
Ceftazidime + tazobactam[b]	64	128	2	2
Ceftriaxone	32	32	8	16
Moxalactam	32	32	8	8
Aztreonam	8	8	2	4
Imipenem	4	2	4	1
Meropenem	2	1	1	2
Amikacin	4	4	ND[c]	8
Gentamicin	>128	>128	>128	4
Tobramycin	64	64	ND	1

[a] The OXA-15 enzyme was encoded by plasmid pMLH54; the OXA-2 enzyme was encoded by pMG40.
[b] Inhibitors were used at 4 μg/ml.
[c] ND, not done.

Cloning and sequencing of β-lactamases genes. Total DNA was extracted from broth cultures described as above and was digested to completion with *Hin*dIII, *Eco*RV, or *Eco*RI. The fragments were ligated into the plasmid vector pBC SK+, which encodes resistance to chloramphenicol but not to ampicillin. Trial ligations and transformation to *E. coli* XL1-blue MRF′ were performed by the calcium chloride method (38) with different ratios of DNAs from isolate AH and the pBCSK+ vector, and for each endonuclease, the ratio giving the most recombinants was selected to generate libraries. Subsequently, electroporation at 2,500 V with a capacity of 25 μF and a pulse time of 5 ms was used to transform the ligated plasmids into *E. coli* XL1-blue MRF′ by using an EasyJect apparatus and the protocols of the manufacturer (Phillip Harris, Shenstone, Lichfield, United Kingdom). Isopropyl-β-thiogalactopyranoside and 5-bromo-4-chloro-3-indolyl-β-D-galactoside were added to final concentrations of 5 mM and 100 μg/ml, respectively, and the mixtures were plated on nutrient agar containing ceftazidime at 1 or 5 μg/ml or chloramphenicol at 30 μg/ml, and then the plates were incubated for 12 to 36 h at 37°C. Resistant colonies were subcultured onto fresh plates containing the same antibiotics.

Plasmid DNA was extracted and purified from the recombinants with Qiagen plasmid kits (Diagen; Hybaid, Teddington, United Kingdom). Digestion was undertaken with *Bam*HI, *Eco*RI, *Eco*47III, *Hin*cII, *Nru*I, *Sac*I, *Sph*I, *Sty*I, and *Xho*I, and fragments were subcloned into pBCSK+ or pUC19 by standard methods (38). DNA sequencing was performed by using the universal and reverse universal primers and with a Sequenase, version 2.0, kit, used exactly as advised by the manufacturer (United States Biochemicals, Cleveland, Ohio). Sequencing gels were read with a sonic digitizer and were analyzed with PC/Gene software (Intelligenetics, Mountain View, Calif.). Comparisons with sequences in GenBank (The National Center for Biotechnology Information) by using the alignment method of Altschul et al. (1). Prediction of the secondary structure was done with PC/Gene software.

β-Lactamase purification and assays. A culture of *P. aeruginosa* AH was grown overnight, with shaking, in 0.6 liter of Antibiotic No. 3 Broth (Unipath) at 37°C and was then diluted into 12 liters of fresh, identical medium and incubated for 5 h to yield late-logarithmic-phase cells. The bacteria were harvested at 5,000 × *g* for 15 min at 37°C and washed once in 20 mM Tris H_2SO_4 (pH 7.5) (buffer A). The pellet was resuspended in the same buffer and the suspension was frozen and thawed twice. Residual cells and debris were removed by ultracentrifugation 100,000 × *g* for 45 min at 4°C, and the supernatant was loaded onto a column (2.6 cm in diameter by 40 cm) of DEAE Sephadex A-50 equilibrated with buffer A. The β-lactamase did not bind and eluted with the solvent front. This material was dialyzed against 10 mM piperazine H_2SO_4 (pH 9.5) (buffer B) and was loaded onto a Q-Sepharose high-performance column (1.6 cm in diameter by 10 cm) (Pharmacia LKB, Milton Keynes, United Kingdom) equilibrated with buffer B. Elution was with a gradient of 0 to 0.5 M K_2SO_4 in buffer B. Fractions with β-lactamase activity were collected and subjected to gel filtration on a Sephacryl S-200 high-resolution column (2.6 cm in diameter by 60 cm; Pharmacia) equilibrated with 10 mM phosphate buffer (pH 6.5) (buffer C). Eluant fractions containing β-lactamase were pooled and loaded onto a Q-Sepharose high-performance column (1.6 cm in diameter by 10 cm; Pharmacia) equilibrated with buffer C. Elution was with a linear gradient of 0 to 0.5 M K_2SO_4 in buffer C. The final preparation was stored at −20°C.

To obtain the OXA-2 β-lactamase, 20 ml of an overnight culture of *E. coli* J53-2(R46) in Antibiotic No. 3 Broth was diluted into 0.4 liter of Antibiotic No. 3 Broth (Unipath) and was grown for 5 h. The cells were then harvested, washed, and resuspended in 0.1 M phosphate buffer (pH 7.0) and then frozen and thawed three times. Debris was removed by ultracentrifugation as described above, and the crude enzyme was stored at −20°C.

Hydrolysis assays were by UV spectrophotometry at 37°C in 0.1 M phosphate buffer (pH 7.0). The following wavelengths were used: ampicillin and penicillin G, 235 nm; cephaloridine, 295 nm; cephalothin, 262 nm; cefoxitin, cefotaxime, and ceftazidime, 257 nm; oxacillin, 263 nm; and imipenem and meropenem, 297 nm. The IC_{50} was determined as the inhibitor concentration that reduced the hydrolysis rate of 1 mM penicillin G by 50% under conditions in which the inhibitor and the enzyme were preincubated together at 37°C for 10 min before addition of the substrate.

Nucleotide sequence accession number. The GenBank database accession number for the sequence described in Fig. 3 is U63835.

RESULTS

Susceptibility, β-lactamases, and plasmids of *P. aeruginosa* AH and its transconjugant. Strain AH was more resistant than strain PU21, which was taken as a reference *Pseudomonas* strain, to all β-lactams except cefotaxime and carbapenems, and was also resistant to gentamicin and tobramycin (Table 1). Resistance to ceftazidime was especially striking. Isoelectric focusing of extracts of isolate AH revealed a doublet of β-lactamase bands with apparent pIs of 8.7 and 8.9 (Fig. 1); neither of these activities was inhibited by 0.3 mM cloxacillin. Further β-lactamase bands with pIs below those of the doublet were observed when incubation of the gels was prolonged.

Ceftazidime resistance was transferred to *P. aeruginosa* PU21 at a low frequency (10^{-8} per donor cell) by plate mating, and the transconjugants expressed both the pI 8.7 and 8.9

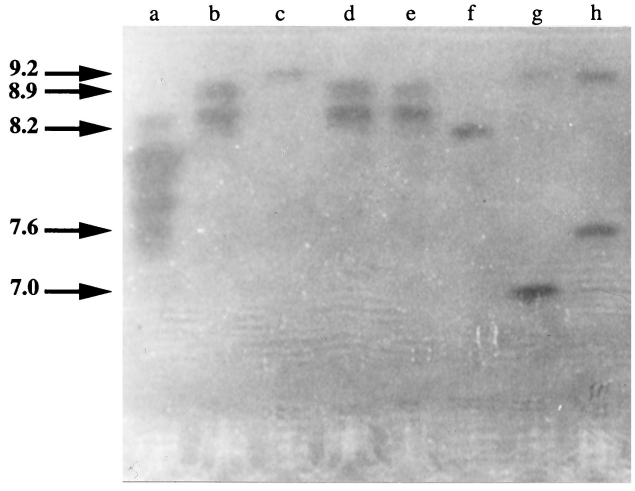

FIG. 1. Isoelectric focusing of β-lactamases from isolate AH, its transconjugants, and β-lactamases used for comparison. Isoelectric focusing of β-lactamases performed on a pH 6.0 to 9.5 gel. Lane a, OXA-2 enzyme from strain PU21(pMG40) with multiple bands between pI 7.6 and 8.2; lane b, pI 8.7 and 8.9 enzyme from isolate AH; lane c, AmpC (pI 9.2) from *E. coli* J53-2; lanes d and e, pI 8.7 and 8.9 enzyme from two different PU21 transconjugants of strain AH; lane f, SHV-5 enzyme (pI 8.2) from *E. coli* J53-2(DPH324); lane g, SHV-3 enzyme (pI 7.0) and the AmpC (pI 9.2) β-lactamases from *E. coli* J53-2(DP192); lane h, SHV-2 enzyme (pI 7.6) and the AmpC (pI 9.2) β-lactamases from *E. coli* J53-2(DPH2).

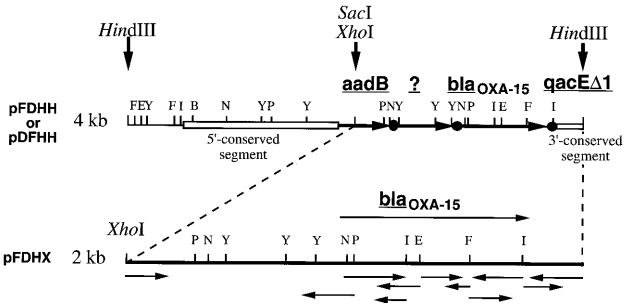

FIG. 2. Restriction map of the 4-kb *Hin*dIII insert fragment in pBC SK+ vector showing the sequences determined. The top line shows the restriction map for the 4-kb *Hin*dIII insert giving ceftazidime resistance. The genes *qacEΔ1*, bla_{OXA-15}, and *aadB* and the 5′ and 3′ conserved segments of the integron are marked. Below is shown in expanded form the 2-kb fragment determining ceftazidime resistance. Restriction sites are indicated as follows: B, *Bam*HI; E, *Eco*RI; F, *Eco*47III; I, *Hin*cII; N, *Nru*I; P, *Sph*I; and Y, *Sty*I. The arrows under the 2-kb fragment represent the sequences determined and their orientations. The 59-base elements at the 3′ ends of cassettes are represented by the closed circles; the question mark indicates the unknown gene cassette.

β-lactamase activities. Strain AH possessed two plasmids with molecular sizes of 4 and 450 kb. The larger of these, designated pMLH54, was acquired by the PU21 transconjugants. The MICs for the transconjugants resembled those for *P. aeruginosa* AH (Table 1) and, compared to that for PU21, were increased by 64-fold for ceftazidime, by 8- to 16-fold for piperacillin, cefpirome, cefsulodin, and moxalactam, but by only 2-fold for carbenicillin, cefepime, ceftriaxone, and aztreonam. The MICs of cefotaxime, imipenem, and meropenem were not increased. Resistances to penicillins and ceftazidime were not reversed by 4 μg of clavulanate or tazobactam per ml.

Table 1 also presents the MICs for a *P. aeruginosa* PU21 transconjugant with plasmid pMG40 encoding the OXA-2 β-lactamase, to which the present enzyme proved to be related (see below). This transconjugant showed substantially increased resistance to piperacillin compared to PU21, but the MIC of ceftazidime was increased only fourfold and those of carbenicillin, cefepime, cefotaxime, cefpirome, cefsulodin, ceftriaxone, moxalactam, aztreonam, and carbapenem were increased by twofold or less.

Cloning of the β-lactamase gene from strain AH. No hybridization to total cellular DNA from isolate AH was detected with the bla_{SHV} or bla_{OXA-10} probes. To further characterize the β-lactamase from isolate AH, total DNA was digested with the *Hin*dIII, *Eco*RV, or *Eco*RI enzyme, and the fragments were ligated into pBC SK+. The recombinant plasmids were transformed into *E. coli* XL1-blue, and transformants were selected on ceftazidime at 1 or 5 μg/ml. Two resistant colonies were obtained from the *Eco*RV library, 19 were obtained from the *Hin*dIII library, and none was obtained from the *Eco*RI library. The transformants from the *Hin*dIII library contained a 4-kb insert in pBC SK+, and those from the *Eco*RV library had a 16-kb insert that included the 4-kb *Hin*dIII fragment. Two plasmids with the 4-kb *Hin*dIII fragment cloned in opposite orientations were designated pDFHH and pFDHH, respectively. Electrofocusing showed that *E. coli* XL1-blue with pFDHH yielded β-lactamase activities that comigrated with the β-lactamase of isolate AH.

A restriction map was generated for the *Hin*dIII fragment (Fig. 2). Subclones were produced by using restriction enzymes to delete specific fragments from the 4-kb insert. After transformation, these were selected with ceftazidime or chloramphenicol. The β-lactamase gene was localized to a 2-kb section of DNA which, depending on the orientation of the original insert, lay on a *Hin*dIII-*Xho*I (pFDHX) or *Hin*dIII-*Sac*I (pDFSH) fragment. When these fragments were cut with *Eco*RI (Fig. 2) the resulting subclones lacked active β-lactamase, suggesting that an *Eco*RI site lay within the enzyme gene.

Sequence analysis. By further subcloning of pFDHX and pDFSH, a sequence of 1,180 bp was determined and was found to contain an open reading frame of 825 bp which included an *Eco*RI site (Fig. 3). The sequence of this open reading frame was identical to that of bla_{OXA-2} (7) except at position 576, where guanine replaced adenine, giving aspartate instead of glycine at amino acid 150 of the predicted translation product (Fig. 3). This new derivative of OXA-2 β-lactamase is named OXA-15. The encoding gene, bla_{OXA-15}, was organized identically to the OXA-2 cassette in integron In1 from plasmid R46 as described by Stokes and Hall (43), but it differed from the OXA-2 cassette in its flanking sequences. Upstream of bla_{OXA-15} was a sequence with the characteristics of a 59-base element, i.e., an imperfect inverted repeat sequence of the type found at the 3′ end of integron-associated gene cassettes (15). This 59-base element was preceded by a sequence (100 bp) without significant homology to any sequence in the databases examined. Downstream of bla_{OXA-15} lay a sequence identical to the 5′ portion of *qacEΔ1*, which determines resistance to ethidium bromide and quaternary ammonium compounds and which is a part of the 3′ conserved segment of *sulI*-associated integrons (35).

Susceptibility of *E. coli* XL1-blue(pFDHH) producing OXA-15 β-lactamase. The MICs of β-lactams for *E. coli* XL1-blue with or without pFDHH, which encodes the OXA-15 enzyme, and for *E. coli* J53-2 with or without R46, which encodes the OXA-2 enzyme (6, 41), are given in Table 2. The OXA-15 enzyme increased resistance to ampicillin, carbenicillin, piperacillin, cefotaxime, cefpirome, ceftazidime, ceftriaxone, moxalactam, and aztreonam but not cefepime or carbapenems. Resistance to ceftazidime was reduced by twofold by clavulanate at 4 μg/μl and eightfold by tazobactam at 4 μg/ml. Production of the OXA-2 enzyme raised the MICs of penicillins and cefsulodin but not those of any extended-spectrum cephalosporin. The 4-kb insert in pFDHH also determined resistance to gentamicin (Table 2).

Organization of the integron containing bla_{OXA-15}. Sequence analysis of the 1,180-bp segment described above strongly suggested that the bla_{OXA-15} cassette was located in an integron containing the *sulI* gene, adjacent to the 3′ conserved segment. Further investigation of the original 4-kb *Hin*dIII insert revealed a cluster of restriction sites (to the left of the *Sac*I and *Xho*I sites, as depicted in Fig. 2) that exactly matched those found in the highly conserved 5′ segment of *sulI*-associated integrons (16, 42, 43). The 5′ conserved segment would be predicted to end ca. 180 bp to the left of the *Sac*I and *Xho*I sites. A short sequence was determined to the right of the *Xho*I site (Fig. 2) and was identical to a sequence within *aadB* [*ant*(2″)-*1a*], which encodes the ANT(2″)-1a aminoglycoside adenyltransferase and which has previously been found in integrons (3, 26). The occurrence of *aadB* at this position accords with the observation that gentamicin resistance (40) was conferred by plasmids pFDHH and pDFHH, which carry the 4-kb *Hin*dIII fragment, but not by pFDHX, which carries the 2-kb *Xho*I-*Hin*dIII fragment. Extrapolating from the published sequence, *aadB* should start immediately downstream of the 5′ conserved segment and constitute the cassette closest to the 5′ end of the integron. Between *aadB* and bla_{OXA-15} was a ca. 500-bp interval; the 100 bp at the 3′ end of this fell within the 1,180-bp sequence described above and included a typical 59-base element. This 500-bp segment, between *aadB* and

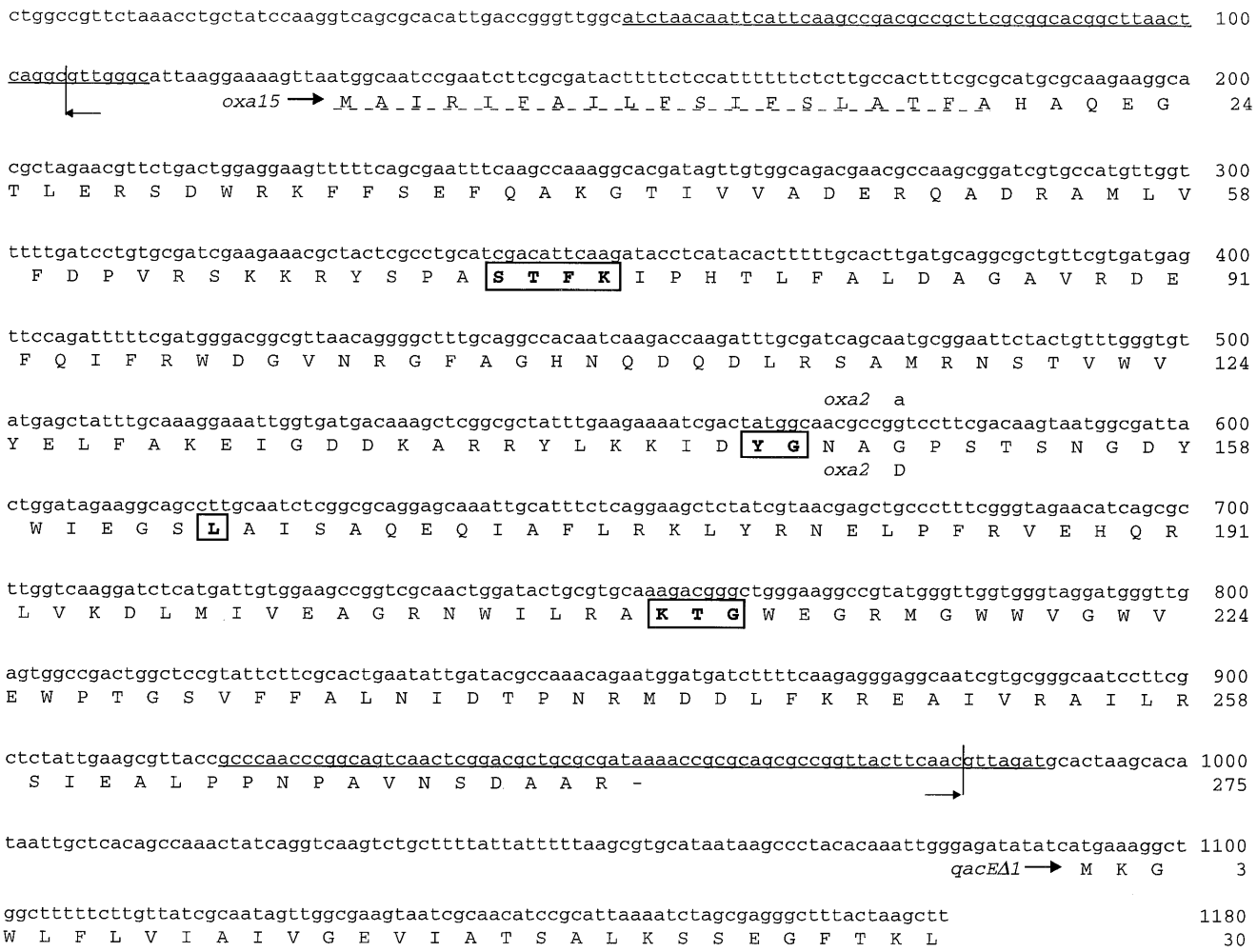

FIG. 3. Nucleotide sequence including the OXA-15 gene from *P. aeruginosa* AH and its deduced amino acid sequence. Amino acid numbering corresponds to that for the full-length precursor protein, as deduced by homology to OXA-2; the amino acid sequence putatively eliminated from the mature protein is indicated by a dashed line. The highly conserved amino acids of class D β-lactamase are given in boxes. The OXA-15 gene cassette lies between the arrows pointing to vertical lines; the single difference from the OXA-2 gene cassette is marked above the sequence for the nucleotide and below the sequence for the amino acid. The 59-base elements are underlined. The nucleotide sequence of *qacEΔ1* and its deduced amino acid sequence are indicated up to its *Hin*dIII site. GenBank accession number U63835 has been assigned to this sequence.

bla_{OXA-15}, represents a putative cassette of unknown function. Overall, the data suggest that bla_{OXA-15} lies in a *sulI*-associated integron (17), as illustrated in Fig. 2.

β-Lactamase purification and hydrolysis assays. Considerable difficulty was experienced in purifying the OXA-15 enzyme, the main problem being that it was not produced in large amounts by the initial isolate or by any of the transconjugants or recombinants. Ultimately, the enzyme was only partially purified, even after three steps of ion-exchange chromatography and one gel filtration, and the yield was insufficient to allow for detailed kinetic study.

As with many class D β-lactamases, biphasic kinetics were observed for several substrates, with the initial hydrolysis rate declining more rapidly than was explicable by substrate depletion, before stabilizing at a steady-state rate. As described by Waley (45), biphasic kinetics was considered measurable only when the decline in reaction velocity between the initial and steady-state rates was substantial (taken as 20% in these studies). For the OXA-15 enzyme, these conditions were met for cephalothin and ceftazidime (Table 3), but smaller effects were seen for the other compounds tested. On the basis of the steady-state rates, the OXA-15 enzyme possessed relatively less activity against penicillins than OXA-2, but was more active against cephaloridine, cephalothin, and, especially, ceftazidime. Neither enzyme hydrolyzed cefotaxime, cefoxitin, imipenem, or meropenem. The IC_{50}s of clavulanic acid for the OXA-2 and OXA-15 β-lactamase were 0.6 and 1.5 μM, respectively.

DISCUSSION

P. aeruginosa AH, from a patient in a neurology ward in a hospital in Ankara, Turkey, was examined because of its ceftazidime resistance. Ceftazidime resistance in *P. aeruginosa* is most often caused by hyperproduction of the chromosomal class C β-lactamase (29) or by increased efflux or impermeability (27). These mechanisms leave the MICs of cefotaxime and ceftriaxone above those of ceftazidime, whereas the MICs for isolate AH showed the opposite pattern.

Transfer experiments revealed that the ceftazidime resistance was associated with a β-lactamase, encoded by a conjugative 450-kb plasmid, with apparent pIs of 8.7 and 8.9. PU21

TABLE 2. MICs for *E. coli* XL1-blue(pFDHH) producing the OXA-15 β-lactamase and reference strains

Antibiotic	MIC (μg/ml)			
	XL1-blue	XL1-blue (OXA-15)[a]	J53-2	J53-2 (OXA-2)[a]
Ampicillin	4	64	4	>512
Carbenicillin	8	128	8	>1,024
Carbenicillin + clavulanate[b]	4	16	4	256
Carbenicillin + tazobactam[b]	8	16	8	256
Piperacillin	0.25	2	1	>128
Piperacillin + clavulanate[b]	0.06	0.25	1	1
Piperacillin + tazobactam[b]	0.12	0.5	1	1
Cefepime	0.12	0.12	0.12	0.12
Cefotaxime	0.06	0.25	0.06	0.06
Cefoxitin	8	16	4	4
Cefpirome	0.03	0.5	0.03	0.06
Cefsulodin	16	32	32	>128
Ceftazidime	0.25	8	0.5	0.25
Ceftazidime + clavulanate[b]	0.12	4	0.12	0.12
Ceftazidime + tazobactam[b]	0.25	1	0.25	0.25
Ceftriaxone	0.06	0.5	0.06	0.06
Moxalactam	0.12	1	0.12	0.25
Aztreonam	0.12	1	0.12	0.06
Gentamicin	0.5	8	0.5	0.5

[a] The OXA-15 enzyme was encoded by plasmid pFDHH; the OXA-2 enzyme was encoded by R46.
[b] Inhibitors were used at 4 μg/ml.

transconjugants with the enzyme showed substantial resistance to ceftazidime which was not reversed by clavulanate or tazobactam and also had increased resistance to penicillins, cefepime, cefpirome, cefsulodin, ceftriaxone, and aztreonam, but not to cefotaxime and carbapenems. Resistance to tobramycin and gentamicin was also transferred. Antibiogram data, isoelectric focusing, and DNA probing gave few positive clues to the identity of the β-lactamase: DNA extracts from producers failed to hybridize with a gene probe for SHV β-lactamases, which are the commonest high-pI ESBLs; the high pI itself argued against an extended-spectrum TEM or an OXA-10 mutant; the lack of greater resistance to cefotaxime than to ceftazidime and the absence of inhibition by cloxacillin argued against the enzyme being an AmpC type β-lactamase.

Accordingly, the β-lactamase was further investigated. Its gene was located, as determined by cloning in *E. coli*, on a 2-kb DNA fragment with an internal *Eco*RI site. Nearly 1.2 kb of the 2-kb fragment was sequenced and was found to contain an open reading frame encoding a class D β-lactamase, now designated OXA-15, that differed from OXA-2 only at residue 150, where glycine replaced aspartate. This substitution accords with the increased pI of OXA-15 (discrete bands at 8.7 and 8.9 compared with multiple bands between 7.6 and 8.2 for OXA-2).

Class D β-lactamases have four highly conserved elements (shown boxed in Fig. 3): namely, element 1 (serine-threonine-phenylalanine-lysine), element 2 (tyrosine-glycine), element 3 (leucine), and element 4 (lysine-threonine-glycine) (22, 37, 39, 46). The mutation distinguishing OXA-15 from OXA-2 lay close to the second highly conserved element (Fig. 2). Secondary structure prediction by the methods of Chou and Fasman (5) and Deleage and Roux (12) and comparison to the model proposed by Zhu et al. (49) for the BlaR signal transducer (involved in induction of class A β-lactamase in *Bacillus licheniformis*) suggest that position 150 is likely to be on a β turn or loop involved in the hydrolysis of β-lactams. Among other class D β-lactamases, aspartate is conserved at this position in OXA-1, -2, -3, -4, -9, and -12, and AmpS, whereas OXA-10, -11, and -14 have asparagine and LCR-1 has glutamate (8, 10, 11, 22, 37, 39, 46). OXA-15 seems unique in not having an acidic or amide side chain at this site. Substitution at this position has not been seen in extended-spectrum mutants of the OXA-10 β-lactamase (8, 10, 11, 14). The ability of OXA-15 β-lactamase to confer resistance to several extended-spectrum cephalosporins correlated with kinetic data: compared to OXA-2, OXA-15 was more active against cephalosporins, although it was less so against penicillins, and showed an especially marked gain in activity against ceftazidime. Cefotaxime escaped hydrolysis by OXA-15; nevertheless, a four-fold increased MIC was observed for the *E. coli* recombinant producing this enzyme. Cephalosporin hydrolysis was, however, complicated by biphasic kinetics, in which the initial rapid hydrolysis rate declined more rapidly than was explicable by substrate depletion and then stabilized at a lower steady-state rate. This behavior was especially apparent for ceftazidime. Various models can explain biphasic hydrolysis (25, 45), including (i) partial unfolding of the acyl enzyme, (ii) progressive accumulation of a stable acyl enzyme complex following release of the 3′ group of cephalosporins, (iii) equilibrium between two forms of free enzyme, or (iv) end product inhibition. However, none of these models can individually explain the behavior of the OXA-2 β-lactamase (24, 25), and more complex or multiple mechanisms may be involved. The OXA-15 β-lactamase was marginally less sensitive to inhibition by clavulanate than OXA-2.

The sequence of bla_{OXA-2} from plasmid R46 constitutes a gene cassette within the integron In1. This cassette structure of bla_{OXA-2} was completely conserved for bla_{OXA-15}, and the ends of the cassette, as defined by homology, are indicated in Fig. 3. The OXA-15 integron is a *sulI*-associated type composed of the conserved 5′ and 3′ segments and three putative cassettes; *aadB*, an unknown gene, and bla_{OXA-15}. *aadB* is often integron linked and was previously associated with the bla_{OXA-2} cassette in an integron in plasmid pBWH301 from *Enterobacter aerogenes* (GenBank database accession number U13880) and in an integron from *Serratia marcescens* (26). Nevertheless, the sequence of cassettes of the bla_{OXA-15} integron differed from that of In1 in R46 or of the integron in pBWH301.

In summary, OXA-15 is the first extended-spectrum derivative of the OXA-2 β-lactamase. Its producer, *P. aeruginosa* AH, was isolated at the same hospital, in the same year, as *P. aeruginosa* isolates with OXA-11, OXA-14, and PER-1 ESBLs (9). It is unclear why this hospital has been such a rich source of unusual ESBLs; nevertheless, it is apparent that at least two class D β-lactamases can gain extended-spectrum activity by mutation. A final, disturbing aspect is that bla_{OXA-15}—like the

TABLE 3. Rates of hydrolysis of 0.5 mM β-lactams at 37°C in 0.1 M phosphate buffer by semipurified OXA-15 and crude OXA-2 β-lactamases

Substrate	Hydrolysis rate (%)	
	OXA-15	OXA-2
Penicillin G	100	100
Ampicillin	44	223
Oxacillin	681	614
Cephaloridine	400	74
Cephalothin	297→148[a]	62
Ceftazidime	65→5[a]	<1
Cefotaxime	<1	<1
Cefoxitin	<1	<1
Imipenem	<1	<1
Meropenem	<1	<1

[a] Biphasic kinetics; the higher rate pertained in the initial phase and the lower rate pertained at steady state.

genes encoding the OXA-1, OXA-2, and OXA-10 enzymes—was on an integron (34, 35), implying that it may readily spread and combine with other resistance determinants.

ACKNOWLEDGMENTS

We are very grateful to G. A. Jacoby, who provided the two strains of *P. aeruginosa* producing of OXA-2 β-lactamase. We thank Merck Sharp & Dohme for financial support in the early part of this study. We are indebted also to the British Council, which sponsored D. Gur's trips to the United Kingdom, and are grateful to B. Duke for excellent technical assistance.

REFERENCES

1. **Altschul, S. F., W. Gish, W. Miller, E. W. Myers, and D. J. Lipman.** 1990. Basic local alignment search tool. J. Mol. Biol. **215:**403–410.
2. **Bush, K., G. A. Jacoby, and A. A. Medeiros.** 1995. A functional classification scheme for β-lactamases and its correlation with molecular structure. Antimicrob. Agents Chemother. **39:**1211–1233.
3. **Cameron, F. F., D. J. Groot Obbink, V. P. Ackerman, and R. M. Hall.** 1986. Nucleotide sequence of the AAD(2″) aminoglycoside adenyltransferase determinant *aadB*: evolutionary relationship of this region with those surrounding *aadA* in R538-1 and *dhfrII* in R388. Nucleic Acids Res. **14:**8625–8635.
4. **Chen, H. Y., M. Yuan, and D. M. Livermore.** 1995. Mechanisms of resistance to β-lactam antibiotics amongst *Pseudomonas aeruginosa* isolates collected in the UK in 1993. J. Med. Microbiol. **43:**300–309.
5. **Chou, P. Y., and G. D. Fasman.** 1979. Prediction of beta-turns. J. Biophys. **26:**367–373.
6. **Dale, J. W., and J. T. Smith.** 1971. The purification and properties of the β-lactamase specified by the resistance factor R-1818 in *Escherichia coli* and *Proteus mirabilis*. Biochem. J. **123:**493–500.
7. **Dale, J. W., D. Godwin, D. Mossakowska, P. Stephenson, and S. Wall.** 1985. Sequence of the OXA2 β-lactamase: comparison with other penicillin-reactive enzymes. FEBS Lett. **191:**39–44.
8. **Danel, F., L. M. C. Hall, D. Gur, and D. M. Livermore.** 1995. OXA-14, another extended-spectrum variant of OXA-10 (PSE-2) β-lactamase from *Pseudomonas aeruginosa*. Antimicrob. Agents Chemother. **39:**1881–1884.
9. **Danel, F., L. M. C. Hall, D. Gur, H. E. Akalin, and D. M. Livermore.** 1995. Transferable production of PER-1 β-lactamase in *Pseudomonas aeruginosa*. J. Antimicrob. Chemother. **35:**281–294.
10. **Danel, F., L. M. C. Hall, D. Gur, and D. M. Livermore.** 1996. Multiple secondary β-lactamase, including a new OXA-10 mutant, in two Turkish *P. aeruginosa* isolates, abst. T119. *In* Abstracts of the European Congress of Chemotherapy.
11. **Danel, F., L. M. C. Hall, D. Gur, and D. M. Livermore.** 1996. OXA-16: a new OXA-10-related β-lactamase giving ceftazidime resistance in *Pseudomonas aeruginosa* from Turkey, abstr. C027, p. 39. *In* Program and abstracts of the 36th Interscience Conference on Antimicrobial Agents and Chemotherapy. American Society for Microbiology, Washington, D.C.
12. **Deleage, G., and B. Roux.** 1987. An algorithm for protein secondary structure prediction based on class prediction. Prot. Eng. **1:**289–294.
13. **Du Bois, S. K., M. S. Marriott, and S. G. B. Amyes.** 1995. TEM- and SHV-derived extended-spectrum β-lactamases: relationship between selection, structure and function. J. Antimicrob. Chemother. **35:**7–22.
14. **Hall, L. M. C., D. M. Livermore, D. Gur, M. Akova, and H. E. Akalin.** 1993. OXA-11, an extended-spectrum variant of OXA-10 (PSE-2) β-lactamase from *Pseudomonas aeruginosa*. Antimicrob. Agents Chemother. **37:**1637–1644.
15. **Hall, R. M., D. E. Brookes, and H. W. Stokes.** 1991. Site-specific insertion of genes into integrons: role of the 59-base element and determination of the recombination cross-over point. Mol. Microbiol. **5:**1941–1959.
16. **Hall, R. M., H. J. Brown, D. E. Brookes, and H. W. Stokes.** 1994. Integrons found in different locations have identical 5′ ends but variable 3′ ends. J. Bacteriol. **176:**6286–6294.
17. **Hall, R. M., and C. M. Collis.** 1995. Mobile gene cassettes and integrons: capture and spread of genes by site-specific recombination. Mol. Microbiol. **15:**593–600.
18. **Hansen, J. F., and R. H. Olsen.** 1978. Isolation of large bacterial plasmids and characterization of P2 incompatibility group plasmids pMG1 and pMG5. J. Bacteriol. **135:**227–238.
19. **Huovinen, P., S. Huovinen, and G. A. Jacoby.** 1988. Sequence of PSE-2 β-lactamase. Antimicrob. Agents Chemother. **32:**134–136.
20. **Jacoby, G. A.** 1974. Properties of R plasmids determining gentamicin resistance by acetylation in *Pseudomonas aeruginosa*. Antimicrob. Agents Chemother. **6:**239–252.
21. **Jacoby, G. A., and A. A. Medeiros.** 1991. More extended-spectrum β-lactamases. Antimicrob. Agents Chemother. **35:**1697–1704.
22. **Joris, B., P. Ledent, O. Dideberg, E. Fonzé, J. Lamotte-Brasseur, J. A. Kelly, J. M. Ghuysen, and J. M. Frère.** 1991. Comparison of the sequences of class A β-lactamases and the secondary structure elements of penicillin-recognizing proteins. Antimicrob. Agents Chemother. **35:**2294–2301.
23. **Kado, C. I., and S. T. Liu.** 1981. Rapid procedure for detection and isolation of large and small plasmids. J. Bacteriol. **145:**1365–1373.
24. **Ledent, P., X. Raquet, B. Joris, J. Van-Beemen, and J. M. Frère.** 1993. A comparative study of class D β-lactamases. Biochem. J. **292:**555–562.
25. **Ledent, P., and J. M. Frère.** 1993. Substrate-induced inactivation of the OXA2 β-lactamase. Biochem. J. **295:**871–878.
26. **Levesque, C., L. Piche, C. Larose, and P. H. Roy.** 1995. PCR mapping of integrons reveals several novel combinations of resistance genes. Antimicrob. Agents Chemother. **39:**185–191.
27. **Li, X. Z., D. Ma, D. M. Livermore, and H. Nikaido.** 1994. Role of efflux pump(s) in intrinsic resistance of *Pseudomonas aeruginosa*: active efflux as a contributing factor to β-lactam resistance. Antimicrob. Agents Chemother. **38:**1742–1752.
28. **Liu, P. Y. F., D. Gur, L. M. C. Hall, and D. M. Livermore.** 1992. Survey of the prevalence of β-lactamases amongst 1000 gram-negative bacilli isolated consecutively at the Royal London Hospital. J. Antimicrob. Chemother. **30:**429–447.
29. **Livermore, D. M.** 1995. β-Lactamases in laboratory and clinical resistance. Clin. Microbiol. Rev. **8:**557–584.
30. **Matthew, M., A. M. Harris, M. J. Marshall, and G. W. Ross.** 1975. The use of analytical isoelectric focusing for detection and identification of β-lactamases. J. Gen. Microbiol. **88:**169–175.
31. **Mugnier, P., P. Dubroust, I. Casin, G. Arlet, and E. Collatz.** 1996. A TEM-derived extended-spectrum β-lactamase in *Pseudomonas aeruginosa*. Antimicrob. Agents Chemother. **40:**2488–2493.
32. **Nordmann, P., and T. Naas.** 1994. Sequence analysis of PER-1 extended-spectrum β-lactamase from *Pseudomonas aeruginosa* and in comparison with class A β-lactamases. Antimicrob. Agents Chemother. **38:**104–114.
33. **Nordmann, P., E. Ronco, T. Naas, C. Duport, Y. Michel-Briand, and R. Labia.** 1993. Characterisation of a novel extended-spectrum β-lactamase from *Pseudomonas aeruginosa*. Antimicrob. Agents Chemother. **37:**962–969.
34. **Ouellette, M., L. Bissonette, and P. H. Roy.** 1987. Precise insertion of antibiotic resistance determinants into Tn21-like transposons: nucleotide sequence of the OXA-1 β-lactamase gene. Proc. Natl. Acad. Sci. USA **84:**7378–7382.
35. **Paulsen, I. T., T. G. Littlejohn, P. Radstrom, L. Sundstrom, O. Skold, G. Swedberg, and R. A. Skurray.** 1993. The 3′ conserved segment of integrons contains a gene associated with multidrug resistance to antiseptics and disinfectants. Antimicrob. Agents Chemother. **37:**761–768.
36. **Pitcher, D. G., N. A. Sauders, and R. J. Owen.** 1989. Rapid extraction of bacterial genomic DNA with guanidium thiocyanate. Lett. Appl. Microbiol. **8:**151–156.
37. **Rasmussen, B. A., D. Keeney, Y. Yang, and K. Bush.** 1994. Cloning and expression of a cloxacillin-hydrolyzing enzyme and a cephalosporinase from *Aeromonas sobria* AER 14M in *Escherichia coli*: requirement for an *E. coli* chromosomal mutation for efficient expression of the class D enzyme. Antimicrob. Agents Chemother. **38:**2078–2085.
38. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
39. **Sanschagrin, F., F. Couture, and R. C. Levesque.** 1995. Primary structure of OXA-3 and phylogeny of oxacillin-hydrolyzing class D β-lactamases. Antimicrob. Agents Chemother. **39:**887–893.
40. **Shaw, K. J., P. N. Rather, R. S. Hare, and G. H. Miller.** 1993. Molecular genetics of aminoglycoside resistance genes and familial relationships of the aminoglycoside-modifying enzymes. Microbiol. Rev. **57:**138–163.
41. **Smith, J. T.** 1969. R-factor gene expression in gram-negative bacteria. J. Gen. Microbiol. **55:**109–120.
42. **Stokes, H. W., and R. M. Hall.** 1989. A novel family of potentially mobile DNA elements encoding site-specific gene-integration functions: integrons. Mol. Microbiol. **3:**1669–1683.
43. **Stokes, H. W., and R. M. Hall.** 1992. The integron In1 in plasmid R46 includes two copies of the OXA2 gene cassette. Plasmid **28:**225–234.
44. **Vivian, A.** 1994. Plasmid expansion? Microbiol. **140:**213–214.
45. **Waley, S. G.** 1991. The kinetics of substrate-induced inactivation. Biochem. J. **279:**87–94.
46. **Walsh, T. R., L. Hall, A. P. MacGowan, and P. M. Bennett.** 1995. Sequence analysis of two chromosomally mediated inducible β-lactamases from *Aeromonas sobria*, strain 163a, one a class D penicillinase, the other an AmpC cephalosporinase. J. Antimicrob. Chemother. **36:**41–52.
47. **Watanabe, M., S. Iyobe, M. Inoue, and S. Mitsuhashi.** 1991. Transferable imipenem resistance in *Pseudomonas aeruginosa*. Antimicrob. Agents Chemother. **35:**147–151.
48. **Yanisch-Perron, C., J. Vieira, and J. Messing.** 1985. Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. Gene **33:**103–119.
49. **Zhu, Y., S. Englebert, B. Joris, J. M. Ghuysen, T. Kobayashi, and J. O. Lampen.** 1992. Structure, function, and fate of the BlaR signal transducer involved in induction of the β-lactamase in *Bacillus licheniformis*. J. Bacteriol. **174:**6171–6178.