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Analysis by Using DNA Probes of the OXA-1 β-Lactamase Gene and Its Transposon

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From recombinant clone pTY27, encoding an OXA-1 β-lactamase gene, we performed subcloning experiments to more precisely delimit the gene. We describe the use as probes of six different restriction fragments known from subcloning experiments to be within the structural gene or part of the transposable element, Tn*2603*, flanking the OXA-1 determinant. We showed that the OXA-1 structural gene is slightly related to the OXA-2 determinant and also that sequences within Tn*2603* are common to all the OXA- and PSE-producing strains tested. For epidemiological purposes, we began nucleotide sequencing of the OXA-1 determinant, and from preliminary sequence data we synthesized an oligonucleotide 15 bases in length, corresponding to a sequence within the OXA-1 gene. This oligonucleotide was found to be specific for the OXA-1 determinant, because it hybridized only to bacteria producing that β-lactamase.

β-Lactamases (EC 3.5.2.6.) are responsible for most of the resistance of microorganisms to β-lactam antibiotics such as penicillins and cephalosporins (4). In gram-negative bacteria, most clinically relevant β-lactamases are plasmid encoded. Conjugative plasmids are thought to be responsible for the rapid spread of β-lactam resistance among different species of gram-negative bacteria (23). Another factor believed to be involved in the increase of resistance is the phenomenon of transposition (8), responsible for the spread of β-lactam resistance among different plasmids (23, 26).

The OXA-1 β-lactamase gene, so named because it hydrolyzes best the β-lactam oxacillin, is borne on the resistance plasmid RGN238, first isolated in *Escherichia coli* (5). The OXA-1 β-lactamase gene is carried on a transposon, Tn*2603* (32), which is part of plasmid RGN238. A novel OXA-1 transposon, Tn*2011*, has been described (18) and shows homology with Tn*2603*, at least with regard to restriction sites.

Many β-lactamase genes are on transposons (Table 1). To study the evolutionary relationship of the OXA-1 β-lactamase gene with other plasmid-specified β-lactamases and to develop a molecular probe for OXA-1 detection in epidemiological studies, hybridization experiments were undertaken. By using six different restriction fragment DNA probes known, from subcloning experiments, to be either in the OXA-1 gene or in adjacent parts of Tn*2603*, we showed that the OXA-1 gene has detectable homology with another β-lactamase gene and that other nearby parts of the transposon bearing the OXA-1 gene are intimately related to the sequences of plasmids that mediate unrelated β-lactamase genes. From preliminary DNA sequencing data, we were able to synthesize an oligonucleotide probe that is specific for the OXA-1 β-lactamase gene.

MATERIALS AND METHODS

Strains and plasmids. The plasmids used in this study, along with their host organisms, are described in Table 1. Tomoko Yamamoto kindly gave us a recombinant clone called pTY27 which contains a 2.1-kilobase (kb) *Hin*dIII-*Bam*HI restriction fragment from RGN238 cloned in the vector plasmid pACYC184 (3).

Characterization of a recombinant clone. The recombinant plasmid clone pTY27, resistant to 25 μg of chloramphenicol (Sigma Chemical Co.) per ml and to 25 μg of ampicillin (Sigma) per ml but susceptible to 15 μg of tetracycline (Sigma) per ml, was analyzed by isoelectric focusing (13). Bacteria were grown for 16 h in 20 ml of tryptone-yeast (TY) medium and pelleted at 3,000 × *g* for 10 min at 4°C. The pellet was washed in 5 ml of 10 mM phosphate buffer (pH 7), repelleted and suspended in 5 ml of the same buffer. Intracellular enzymes were released by ultrasonic treatment (13). The resulting crude enzyme preparations were centrifuged at 8,000 × *g* for 20 min at 4°C to pellet the cellular debris. The supernatant fluid was retained and stored at −70°C. A sample (15 μl or less) of the supernatant was applied to an isoelectric-focusing polyacrylamide gel (Ampholine polyacrylamide gel plates; LKB Instruments, Inc.) and electrophoresed as described by Medeiros et al. (17). The bands on the gel were revealed with the chromogenic cephalosporin nitrocefin (Oxoid Ltd.) (20). The pTY27 clone was further characterized by alkaline lysis minipreparation (2), by restriction endonuclease digestion, and by subcloning experiments as discussed in the Results and Discussion sections.

Isolation and labeling of DNA fragments. Restriction fragments were isolated by two methods, depending on the size of the fragment to be isolated. For the smaller fragments (smaller than 1 kb), the digested plasmid was electrophoresed on a 6% polyacrylamide gel for 16 h at a constant current (15 mA). Fragments of interest were sliced out of the gel and eluted by the method of Maxam and Gilbert (14). For the larger fragments, the digested plasmid was electrophoresed in 0.5% low-melting-point agarose (Bethesda Research Laboratories, Inc.) with 5 μg of ethidium bromide (Sigma) per ml at 1.2 V/cm for 16 h. The bands of interest, revealed under UV illumination, were sliced out the gel and suspended in 400 μl of 10 mM Tris–1 mM EDTA (pH 8) buffer. The gel and the buffer solution were heated at 65°C for 5 min and after cooling were extracted twice with equilibrated phenol and once with a 1:1 mixture of phenol-chloroform before final extraction with ether. The DNA was concentrated by several *n*-butanol extractions and finally ethanol

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TABLE 1. Plasmid-determined β-lactamases and their transposons

R factor	β-Lactamase type	Transposon	Organism	Reference
pBR322	TEM-1	Tn*3*[a]	*Escherichia coli*	28
R6K	TEM-1	Tn*3*	*Escherichia coli*	15
RP4	TEM-2	Tn*1*	*Escherichia coli*	15
RGN238	OXA-1	Tn*2603*	*Escherichia coli*	32
R1767	OXA-2	Tn*2410*	*Salmonella typhimurium*	10
R57b	OXA-3	—[b]	*Klebsiella pneumoniae*	23
p453	SHV-1	+[c]	*Escherichia coli*	19
R997	HMS-1	—	*Proteus mirabilis*	23
RPL11	PSE-1	Tn*1401*	*Pseudomonas aeruginosa*	17
R151	PSE-2	Tn*1404*	*Pseudomonas aeruginosa*	21
RMS149	PSE-3	—	*Pseudomonas aeruginosa*	23
pMG19	PSE-4	Tn*2521*[d]	*Pseudomonas aeruginosa*	27
pTY27	OXA-1		*Escherichia coli*	This work
pLQ27	bla^{-}[e]		*Escherichia coli*	This work
pLQ28	bla^{-}		*Escherichia coli*	This work
pLQ29	OXA-1		*Escherichia coli*	This work

[a] Only part of Tn*3*.
[b] —, Unknown.
[c] Unnamed.
[d] Encodes PSE-4, but it is not certain that it is part of pMG19.
[e] bla^{-}, No β-lactamase produced.

precipitated. All fragments were labeled by nick translation (24) using [α-^{32}P]dATP (50 Ci/mmol; New England Nuclear Corp.) and a nick translation kit (Bethesda Research Laboratories). The radioactive fragment was separated from the nonincorporated nucleotides by ethanol precipitation. The methodology of colony hybridization and of hybridization and washing conditions were the same as described elsewhere (M. Ouellette, J. J. Rossi, R. Bazin, and P. H. Roy, submitted for publication).

Synthesis, purification, and labeling of the OXA-1 oligonucleotide. Part of the OXA-1 β-lactamase gene was sequenced by the dideoxy method (25). From the sequence data available, we synthesized a pentadecanucleotide by using phosphoramidite chemistry (11) on a Systec Microsyn 1460 synthesizer. The oligonucleotide was then purified on a 20% polyacrylamide gel in the presence of 7 M urea (14). To monitor the homogeneity of the oligonucleotide, the gel was treated with a fresh solution of 0.05% Stains-All (Sigma) in 45% formamide-hydrochloride (pH 7.2). The color was permitted to develop for 2 h in the dark. The gel was washed for 30 min in the dark with 2% glycerol and then washed overnight in a 2% glycerol solution. The oligonucleotide, free of 5′-phosphate, was labeled with T4 polynucleotide kinase (Boehringer Mannheim Biochemicals) and [γ-^{32}P]ATP at 37°C for 30 min. Hybridization and washing conditions for the OXA-1 oligonucleotide were the same as those described for other oligonucleotide probes (Ouellette et al., submitted), except that the two final washes were done at 45°C.

RESULTS

Restriction map of pTY27 and subcloning experiments. The OXA-1 β-lactamase protein has a molecular weight of 23,000 (4) which can be accounted for by roughly 230 amino acids. Therefore, the OXA-1 structural gene is approximately 750 to 800 nucleotides in length, allowing for a signal sequence. We made a restriction map of the 2.1-kb fragment to enable us to more precisely localize the gene. Relevant restriction enzyme sites are shown in Fig. 1. Among the restriction

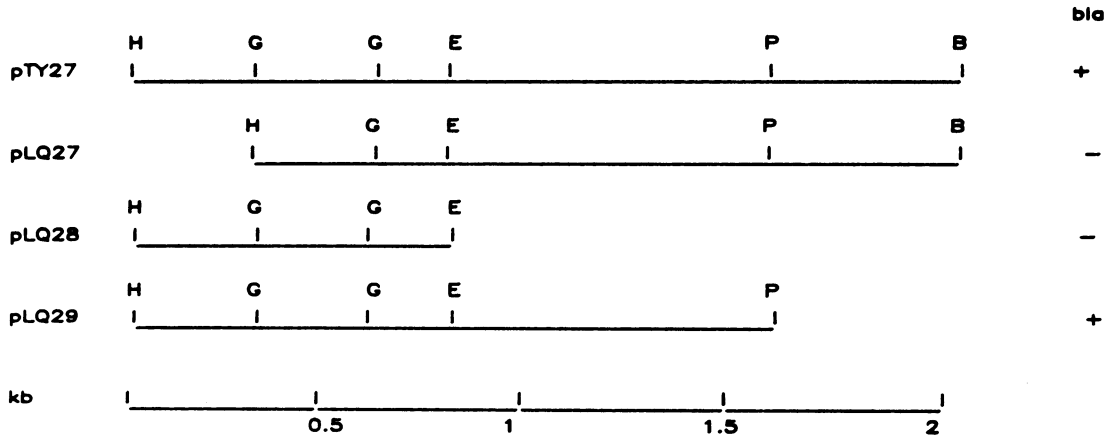

FIG. 1. Restriction mapping and subcloning of pTY27. The pLQ27 derivative was obtained by removing the 0.3-kb *Bgl*II-*Bgl*II fragment from pTY27. pLQ28 was obtained by cloning the *Hin*dIII-*Eco*RV fragment into pACYC184 linearized by the same two enzymes. Finally, pLQ29 was obtained by cloning the *Hin*dIII-*Pvu*II fragment from pTY27 into pACYC184 linearized by *Hin*dIII and *Eco*RV. The capital letters represent restriction sites: H, *Hin*dIII; G, *Bgl*II; E, *Eco*RV; P, *Pvu*II; and B, *Bam*HI. bla indicates the presence of a functional β-lactamase.

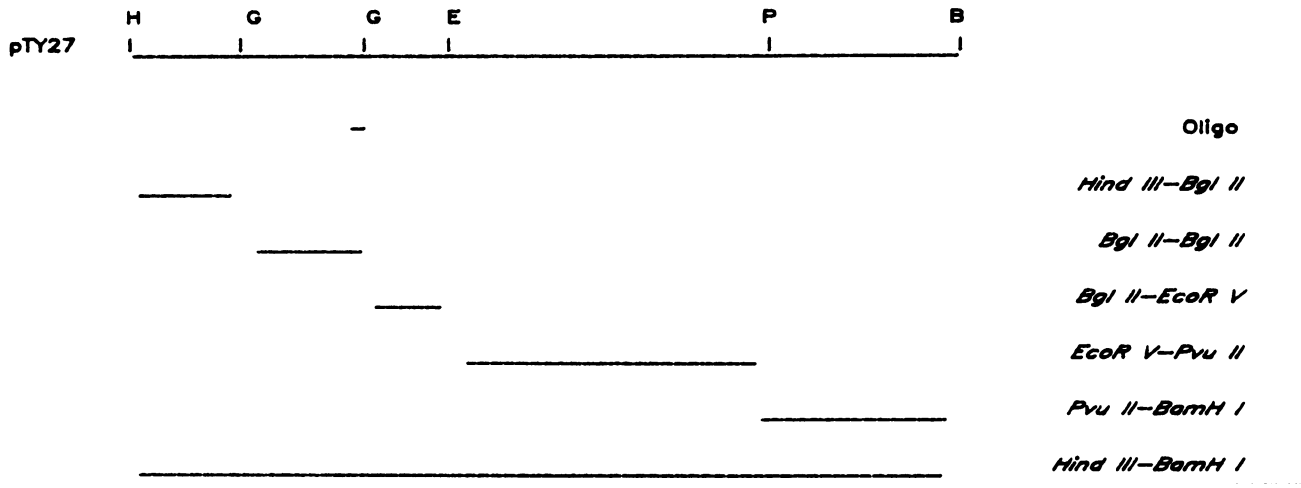

FIG. 2. Probes used in colony hybridization experiments. The oligonucleotide OXA-1, near the *Bgl*II site farthest from the *Hin*dIII site, has the following sequence: 5'-CCAAAGACGTGGATG-3'. The six different restriction fragments used as probes are also shown. H, *Hin*dIII; G, *Bgl*II; E, *Eco*RV; P, *Pvu*II; B, *Bam*HI.

enzymes tested, no sites for the following enzymes were found: *Acc*I, *Bcl*I, *Cla*I, *Hin*cII, *Kpn*I, *Pst*I, *Sma*I, and *Xho*I. As a preliminary subcloning experiment, the 0.3-kb *Bgl*II-*Bgl*II restriction fragment was removed from pTY27. The large fragment generated by that digestion was isolated from a low-melting-point agarose gel and ligated with T4 DNA ligase. The resulting plasmid, named pLQ27 (Fig. 1) was transformed into *E. coli* HB101 and selected on TY chloramphenicol plates. These transformants were replated on ampicillin. The pLQ27 clone lacked β-lactamase activity, because it did not grow on ampicillin plates and did not hydrolyze nitrocefin (20). The *Hin*dIII-*Eco*RV fragment is about 800 nucleotides in length, scarcely long enough to contain the genetic information needed to code for OXA-1. When that fragment was cloned in pACYC184, linearized by the same set of enzymes, the resulting plasmid, named pLQ28 (Fig. 1), lacked β-lactamase activity by both the absence of growth on ampicillin plates and the nitrocefin assay. The *Hin*dIII-*Pvu*II fragment is over 1.6 kb in length and from the results of previous subcloning experiments could be expected to contain all the structural information for the OXA-1 β-lactamase gene. This fragment was cloned in pACYC184 linearized by *Hin*dIII and *Eco*RV (*Pvu*II and *Eco*RV both generate blunt ends), and the resulting pLQ29 clone (Fig. 1) exhibited the β-lactam resistance phenotype by growth on ampicillin plates and by hydrolysis of nitrocefin. The β-lactamase of pLQ29 was analyzed by isoelectric focusing, and the band, after coloration with nitrocefin, migrated at the same distance as the bands of pTY27 and RGN238 (results not shown).

Hybridization with DNA probes. Devising DNA probes for epidemiological studies of β-lactamases will become more and more important as unrelated β-lactamases exhibit isoelectric points (heretofore the standard means of β-lactamase identification) which are very close to each other (Ouellette et al., submitted). For this reason, we took the *Hin*dIII-*Bam*HI restriction fragment and used it as a molecular probe (Fig. 2) to see whether it is a suitable probe for OXA-1 detection. This probe hybridized not only to OXA-1 but also to the genetic material of bacteria harboring plasmids coding for the OXA-2 and -3 and PSE-1, -2, -3, and -4 β-lactamases (Fig. 3). To study more closely whether the structural gene or, alternatively, flanking sequences were responsible for this hybridization, the *Hin*dIII-*Bam*HI fragment was subdivided into five different segments (Fig. 2) which were then used as molecular probes in colony hybridization experiments. The results are shown in Table 2.

The only entirely specific fragment probe to the OXA-1-bearing plasmid found was the *Hin*dIII-*Bgl*II fragment. The *Bgl*II-*Bgl*II fragment, essential to OXA-1 expression (Fig. 1, pLQ27) and the *Bgl*II-*Eco*RV fragment, which is, according to subcloning experiments, entirely within the gene, showed cross hybridization with an OXA-2-bearing plasmid (Fig. 3) under the hybridization conditions used (42°C in 6× SSC plus 50% formamide; washing at 65°C in 6× SSC [1 × SSC is 0.15 M NaCl plus 0.015 M sodium citrate]). When the *Eco*RV-*Pvu*II or the *Pvu*II-*Bam*HI fragment was used, cross hybridization was obtained with DNA of strains containing all OXA and PSE plasmid-mediated β-lactamase genes (Table 2). Only the *Hin*dIII-*Bgl*II fragment was found to be specific for the OXA-1 β-lactamase gene, but because at least a small part of that fragment (next to the *Hin*dIII site) must be outside the structural gene (Fig. 1, pLQ28) we were reluctant to use it as a molecular probe, because this region could eventually occur in β-lactamase-positive strains but not necessarily signify that they are OXA-1 producers. For this reason, we began nucleotide sequencing of the OXA-1 gene. From the partial nucleotide sequence thus far obtained, we were able to synthesize an oligonucleotide near the *Bgl*II site farthest from the *Hin*dIII site (Fig. 2). The oligonucleotide is within the OXA-1 gene, because a recombinant clone without the *Bgl*II-*Bgl*II fragment (pLQ27) is β-lactamase negative. The 15-mer probe was found to be specific for the OXA-1 β-lactamase gene (Fig. 3), because it did not hybridize with any other β-lactamase-producing strains. The OXA-1 15-mer could hence serve as a molecular probe for epidemiological studies.

DISCUSSION

A 2.1-kb *Hin*dIII-*Bam*HI restriction fragment from RGN238 was cloned into plasmid vector pACYC184. This insert has all the genetic information required to encode the OXA-1 β-lactamase gene, and its product is identical as indicated by pI determination. To localize the gene more precisely, subcloning experiments were done. We drew

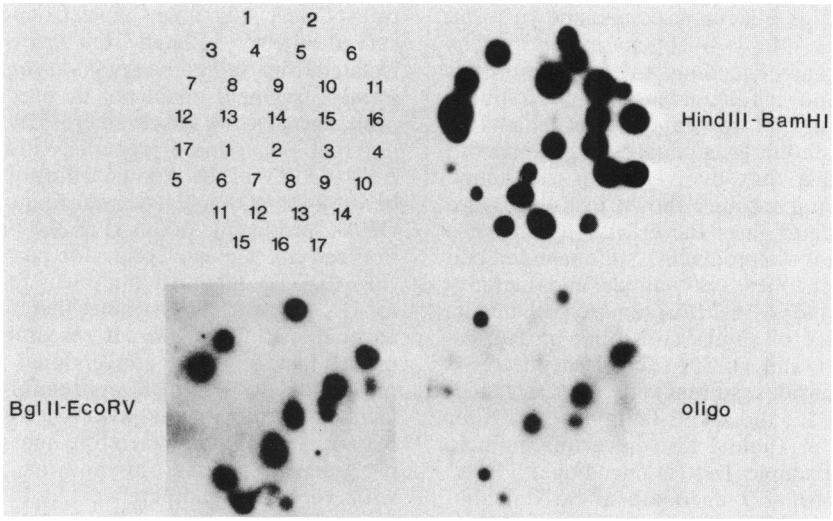

FIG. 3. Colony hybridization autoradiograms. The results obtained with three different probes are shown. The *Hin*dIII-*Bam*HI probe gave many cross hybridizations, the *Bgl*II-*Eco*RV probe cross-reacted only with OXA-2, and the oligonucleotide OXA-1 was entirely specific to the OXA-1 gene. The numbers in the template correspond to bacteria harboring β-lactamases. 1, HB101 (bla−); 2, pTY27; 3, OXA-1; 4, TEM-1; 5, PSE-1; 6, TEM-2; 7, pLQ27; 8, HMS-1; 9, PSE-2; 10, PSE-3; 11, pLQ28; 12, PSE-4; 13, SHV-1; 14, pBR322; 15, pLQ29; 16, OXA-2; 17, OXA-3.

several conclusions from these experiments. First, there are some sequences within the 0.3-kb *Bgl*II-*Bgl*II restriction fragment that are important for OXA-1 expression, because deletion of that fragment (pLQ27) resulted in the loss of β-lactamase activity as shown either by absence of growth on ampicillin plates or by absence of hydrolysis of the chromogenic cephalosporin nitrocefin. Second, the *Hin*dIII-*Eco*RV fragment is about as long as required to encode the OXA-1 structural gene. However, when this fragment was cloned in pACYC184, no expression was obtained. From these two experiments, we concluded that the *Bgl*II-*Eco*RV fragment is entirely within the OXA-1 β-lactamase gene. To further delimit the OXA-1 determinant, we cloned the *Hin*dIII-*Pvu*II fragment. This clone, pLQ29, showed β-lactamase activity. We therefore can decrease the size of the insert to 1.6 kb. pLQ29 seems to have an intact OXA-1 β-lactamase, because the protein comigrated with the β-lactamase of RGN238 in an isoelectric-focusing gel. This result is not surprising, because the OXA-1 gene includes at least part of the *Bgl*II-*Bgl*II fragment and therefore cannot extend to more than about 800 nucleotides after the second *Bgl*II site toward the *Pvu*II site.

TABLE 2. Hybridization of DNA probes derived from pTY27 to β-lactamase genes

β-Lactamase type or R plasmid	Hybridization of probes[a]						
	H-B	H-G	G-G	G-E	E-P	P-B	Oligo-nucleotide
TEM-1	−	−	−	−	−	−	−
TEM-2	−	−	−	−	−	−	−
pBR322	−	−	−	−	−	−	−
OXA-1	+	+	+	+	+	+	+
OXA-2	+	−	+	+	+	+	−
OXA-3	+	−	−	−	+	+	−
SHV-1	−	−	−	−	−	−	−
HMS-1	−	−	−	−	−	−	−
PSE-1	+	−	−	−	+	+	−
PSE-2	+	−	−	−	+	+	−
PSE-3	+	−	−	−	+	+	−
PSE-4	+	−	−	−	+	+	−
pTY27	+	+	+	+	+	+	+
pLQ27	+	+	−	+	+	+	−
PLQ28	+	+	+	+	−	−	+
pLQ29	+	+	+	+	+	−	+

[a] Abbreviations: H-B, *Hin*dIII-*Bam*HI fragment; H-G, *Hin*dIII-*Bgl*II fragment; G-G, *Bgl*II-*Bgl*II fragment; G-E, *Bgl*II-*Eco*RV fragment; E-P, *Eco*RV-*Pvu*II fragment; P-B, *Pvu*II-*Bam*HI fragment.

OXA-1, -2, and -3 were originally set apart from the TEM β-lactamases by the ability of the OXA enzymes to hydrolyze isoxazoyl β-lactam substrates, such as oxacillin and methicillin, although OXA-1 hydrolyzes this substrate about 10 times as rapidly as do OXA-2 and OXA-3 (12). Sykes and Matthew (29) showed that antisera to OXA-1 do not cross-react with OXA-2 or OXA-3. However, Holland and Dale (6) very recently showed that antibodies raised against OXA-2 β-lactamase cross-react with an OXA-3 enzyme but not with OXA-1. From our subcloning experiment, we are sure that the *Bgl*II-*Eco*RV fragment is entirely within the OXA-1 determinant. However, that fragment cross hybridized with an OXA-2-bearing plasmid (Fig. 3), showing that OXA-1 and OXA-2 could have some DNA homology in this region. We have shown (Fig. 3) a slightly overexposed autoradiogram, because the hybridization spot with OXA-2, although always present, was often weak. This latter behavior could reflect only partial homology, but we still always saw a spot associated with OXA-2. This result at first seemed to be in contradiction with the immunological results of Holland and Dale (6). But as they themselves stated, the absence of OXA-1 inhibition by an anti-OXA-2 immunoglobulin G preparation should not be taken as evidence of the absence of homology, as the *Bacillus licheniformis* and *Staphylococcus aureus* β-lactamases do not cross-react (22), despite the fact that they share common sequences (1). Anywhere between 100 and 600 nucleotides in the *Eco*RV-*Pvu*II fragment may be part of the OXA-1 β-lactamase gene, whereas the remaining 200 to 700 nucleotides (and all of the *Pvu*II-*Bam*HI fragment) are outside the gene but still part of Tn*2603*. Cross hybridization of these two fragments with OXA- and PSE-bearing plasmids is very likely due to sequences outside their β-lactamase structural genes.

An increasing number of β-lactamases are known to be encoded by transposons. Many β-lactamase transposons carry other resistance genes encoding, for example, resistance to streptomycin and sulfonamides, and the frequent occurrence of this linkage of resistance determinants in transposons encoding different β-lactamases led Yamamoto et al. (34) to suggest that they evolved from a common ancestor. Many of the transposons shown in Table 1 are multiresistant. Tn*2603* determines the OXA-1 β-lactamase along with resistance to streptomycin, sulfonamide, and mercury (32). Tn*2410* encodes sulfonamide and mercury resistance in addition to the OXA-2 β-lactamase (10), and its map shows a high degree of similarity to that of Tn*2603*. Tn*1401* (17), Tn*1404* (21), and Tn*2521* (27) all encode resistance to some aminoglycosides, sulfonamide, mercury, and, respectively, PSE-1, -2, and -4 β-lactamases. Other transposons not shown in Table 1 also have this multiple resistance pattern, for example Tn*4* (9) encoding a TEM-1 β-lactamase gene, Tn*2101* (7) encoding a carbenicillin-hydrolyzing β-lactamase, Tn*2011* (18) encoding OXA-1, Tn*2610* (34) mediating an enzyme immunologically related to PSE-1 and PSE-4, and, finally, Tn*798* (15) encoding the novel plasmid-mediated β-lactamase AER-1. Tanaka et al. (31) compared multiresistant transposons with transposons encoding mercury resistance alone and mercury, streptomycin, and sulfonamide resistance and suggested that transposons encoding multiple resistance evolved from an ancestral mercury transposon. Very recently, Tanaka et al. (30) demonstrated de novo genesis of a transposon with a structure similar to that of Tn*2603* by recombinational rearrangement between the mercury transposon Tn*21* and the OXA-1 plasmid R753-1. By colony hybridization experiments with probes adjacent to the OXA-1 determinant borne by Tn*2603* (*Hin*dIII-*Bam*HI, *Eco*RV-*Pvu*II, and *Pvu*II-*Bam*HI; Fig. 2), we showed that cross hybridization occurs with strains harboring any of the OXA or PSE β-lactamases listed in Table 1 (Table 2). Interestingly, the OXA-3 and PSE-3 β-lactamases have not yet been reported to be on transposable elements, but from our hybridization experiments it is certainly clear that the bacteria harboring these two β-lactamases have sequences in common with Tn*2603*. R57b (5), which encodes OXA-3, also confers resistance to gentamicin and sulfonamides. Whether these two β-lactamases are on transposons remains to be elucidated. We have cloned a 4-kb *Bam*HI fragment from Tn*2410*, which mediates an OXA-2 β-lactamase (unpublished results), and found that this recombinant clone, transformed into *E. coli* HB101, hybridizes with the *Pvu*II-*Bam*HI probe. The same result was observed for a PSE-1 clone. This shows that at least for these two β-lactamase genes, the fragment adjacent to OXA-1 may also be adjacent for other β-lactamase genes, thus providing an argument for either a common ancestral transposon, such as Tn*2608* or Tn*21* (10, 31), or a common sequence context into which various β-lactamase genes can be inserted.

The experiment was started primarily to construct a DNA probe that could specifically detect the OXA-1 β-lactamase gene among clinical isolates. We have described the use of a restriction fragment for monitoring the presence of TEM β-lactamase genes, but oligonucleotides were necessary to discriminate the closely related TEM-1 and TEM-2 β-lactamase genes (Ouellette et al., submitted). In *E. coli*, the TEM β-lactamases are by far the most prevalent type; next in frequency is the OXA-1 type, followed by SHV-1 (12, 15). Tn*2603* may play a major role in the generation and distribution of R plasmids that carry the OXA-1 gene, because transposons indistinguishable from Tn*2603* have been observed on R455 isolated from *Proteus morganii* and R656a isolated from *Salmonella typhimurium* (30, 33). The *Hin*dIII-*Bam*HI fragment could not be used as an epidemiological probe, because we observed cross hybridization with several different β-lactamase-producing strains (Fig. 3). Even the *Bgl*II-*Eco*RV probe, known from subcloning experiments to be within the OXA-1 gene, was found to cross hybridize with OXA-2-producing strains (Fig. 3). For this reason, we synthesized an oligonucleotide for part of the structural gene (Fig. 2). The oligonucleotide was found to be specific only for OXA-1-producing strains; therefore, it will be useful as an epidemiological tool. If researchers wish to use DNA probes instead of the conventional isoelectric-focusing experiments to perform epidemiological studies on β-lactamases, they might have to use oligonucleotides if cross hybridization occurs when fragment probes are used. Moreover, the fact that there are now many different β-lactamases with very subtle differences in pI (16; Ouellette et al., submitted) might require the use of DNA probes to confirm the identity of β-lactamases whose pIs are such that unambiguous identification by isoelectric focusing is not possible.

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