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Precise insertion of antibiotic resistance determinants into Tn*21*-like transposons: Nucleotide sequence of the OXA-1 β-lactamase gene

(recombination/hot spot/serine enzyme/site-specific integration)

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ABSTRACT **Several plasmid-encoded β-lactamases are on multiresistance transposable elements. The OXA-1 β-lactamase gene is part of Tn*2603*, which is borne on the R plasmid RGN238. We report here the complete nucleotide sequence of the OXA-1 β-lactamase gene and flanking sequences. The OXA-1 gene shows a >50% sequence divergence from the OXA-2 gene, yet there is significant functional similarity at the peptide level. Analysis of 5′ and 3′ flanking sequences shows that Tn*2603* differs from its probable precursor, Tn*21*, by a precise 1004-base-pair insertion, containing the OXA-1 structural gene, at the target sequence AAAGTT, which is located between the Tn*21* streptomycin/spectinomycin (*aadA*) promoter and its structural gene. A 5- for 6-base repeat of the target sequence is found at the end of the insertion. The same precise insertion and repeat of the target sequence are found for the OXA-2 gene from R46. The 5′ flanking regions of two other genes, the trimethoprim-resistance gene from R388 and the gentamicin resistance (*aadB*) gene from pDGO100, are >98% homologous to the 5′ flanking sequences of the OXA-1, OXA-2, and *aadA* genes until they diverge at the target sequence. From the available sequence data a recombinational hot spot is defined at the nucleotide level 5′ of the *aadA* gene of Tn*21*, and a second potential hot spot is proposed 3′ of this gene.**

In Gram-negative bacteria, β-lactamases (β-lactamhydrolase, EC 3.5.2.6), responsible for most of the resistance to β-lactam antibiotics, are often plasmid mediated (1, 2). Several plasmid-encoded β-lactamases are on transposable elements (2), a factor believed to be responsible for the spread of β-lactam resistance among different plasmids (3). In *Escherichia coli*, the TEM β-lactamases, encoded by Tn*3* and its relatives, are by far the most prevalent type; next in frequency is the OXA-1 type (2, 4). The nucleotide sequences of the plasmid-mediated β-lactamase genes TEM-1 and OXA-2 are known (5, 6).

An increasing number of β-lactamase transposons are found to carry other resistance genes, often including those for resistance to streptomycin/spectinomycin and to sulfonamides. The OXA-1 β-lactamase transposon Tn*2603* (7) is one of these and is part of the resistance plasmid RGN238 (8). We have shown (9) that sequences close to the OXA-1 gene are common to all of the OXA (oxacillin hydrolyzing) (1) and PSE (carbenicillin hydrolyzing) β-lactamase-producing strains tested. In some Tn*21*-related transposons, β-lactamases or other resistance genes are found either 5′ or 3′ to the streptomycin/spectinomycin resistance (*aadA*) gene (Fig. 1). This led to the hypothesis that this gene is flanked by recombinational "hot spots" for the insertion of resistance genes (10, 11).

We report here the complete nucleotide sequence of the OXA-1 β-lactamase gene and flanking sequences from Tn*2603*, and we demonstrate the relationship of the OXA-1 β-lactamase to the other sequenced β-lactamases. The sequence data permit us to precisely locate, and define at the nucleotide level, a recombinational hot spot where several different resistance determinants can insert in the same sequence context between the Tn*21*-encoded *aadA* promoter and the *aadA* structural gene.†

MATERIALS AND METHODS

Strains and Plasmids. The recombinant clone pLQ29, containing a 1.6-kilobase (kb) *Pvu* II/*Hin*dIII fragment from RGN238 that encodes the OXA-1 β-lactamase, has been described (9). The pLQ826 clone was obtained by cloning a 4.0-kb *Bam*HI/*Pst* I restriction fragment from RGN238 into the polylinker of pBGS8 (12). The pLQ826 clone encodes resistance to penicillins and to streptomycin/spectinomycin (Fig. 1). *E. coli* HB101 and JM101 were used as the host strains for plasmid preparation. Strain JM101 was used for M13 cloning using vectors M13mp8, -mp9, -mp18, and -mp19 (Pharmacia).

Recombinant DNA Techniques. Plasmid DNA used in the experiments was isolated by the cleared lysate method (13) followed by banding in CsCl/ethidium bromide gradients. The purified plasmid was digested by a series of restriction enzymes using conditions either according to Maniatis *et al.* (13) or to the manufacturer's recommendations. Restriction fragments for subcloning in M13 vectors were isolated from low melting point agarose or 6% polyacrylamide gels as described (9).

DNA Sequencing. The OXA-1 β-lactamase gene was sequenced by the dideoxynucleotide chain-termination procedure (14). Fragments of the 1.6-kb *Pvu* II/*Hin*dIII restriction fragment (Fig. 1) were subcloned in M13 derivatives M13mp8, -mp9, -mp18, and -mp19 in either a directed fashion or, alternatively, in shotgun experiments using restriction endonucleases *Sau*3AI, *Taq* I, *Hpa* II, or *Rsa* I. In addition to the 17-nucleotide universal primer (New England Biolabs) used in the M13 sequencing system, two 15-mer oligonucleotides were synthesized using phosphoramidite chemistry, and they were used as internal primers to complete the sequence on both strands. Computer analysis of the data was performed using the software package of the University of Wisconsin Genetics Computer Group (15) and the program ANALYSEQ (16).

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Abbreviation: ORF, open reading frame.

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†The sequence reported in this paper is being deposited in the EMBL/GenBank data base (Bolt, Beranek, and Newman Laboratories, Cambridge, MA, and Eur. Mol. Biol. Lab., Heidelberg) (accession no. J02967).

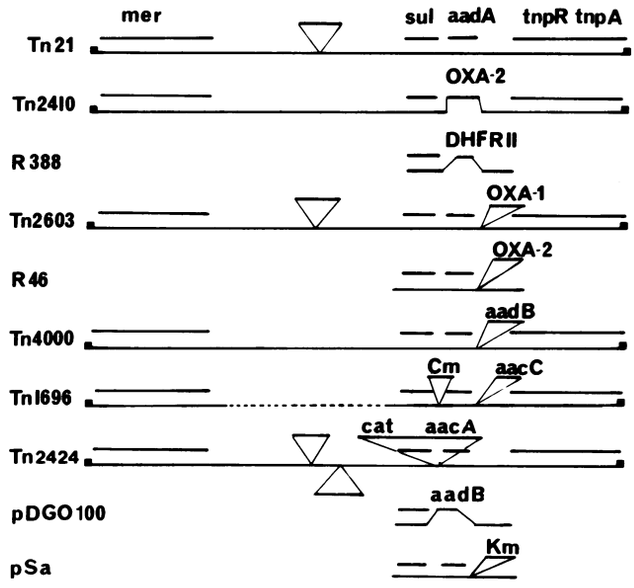

FIG. 1. Schematic structures of Tn*21*-related transposons. Tn*21* harbors genes encoding resistance to mercuric chloride (*mer*), sulfonamide (*sul*), and streptomycin/spectinomycin (*aadA*) along with a transposase and a resolvase (*tnpA* and *tnpR*). The *mer* gene is at the "left" end of Tn*21*; the direction of transcription of *aadA* is from right to left. Resistance determinants either replace the *aadA* gene or insert in 5′ (right) or in 3′ (left) of this gene and not elsewhere, showing that the *aadA* gene is flanked by recombinational hot spots. Mapping data indicate that R46, R388, and pSa contain only part of Tn*21* as shown. Although pDGO100 encodes resistance to mercury (28), only the sequenced region is shown. The lines without gene names indicate the corresponding genes of Tn*21*. Cm, chloramphenicol; Km, kanamycin.

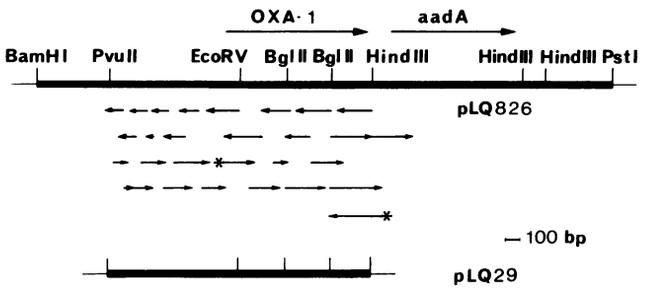

FIG. 2. Structure of pLQ826 and pLQ29 clones and sequencing strategy. Vector and insert segments are depicted by thin and thick lines, respectively. Arrows indicate the direction and extent of the dideoxy sequencing reactions. Arrows with an asterisk show sequences obtained using synthetic primers.

RESULTS

Nucleotide Sequence of OXA-1 β-Lactamase. In a previous report (9), we showed that recombinant clone pLQ29, consisting of a 1.6-kb *Pvu* II/*Hin*dIII fragment derived from Tn*2603* and cloned into pACYC184, encodes the OXA-1 β-lactamase gene. The purified 1.6-kb *Pvu* II/*Hin*dIII fragment of plasmid pLQ29 was digested independently with various restriction endonucleases and subcloned into the replicative forms of bacteriophage M13mp8, -mp9, -mp18, and -mp19. In each experiment, the inserts of selected clones were sequenced by the chain terminator technique. Further sequencing of the region between the OXA-1 and *aadA* structural genes was done using pLQ826 digested by *Hin*dIII and cloned into M13, and either the universal primer or an oligonucleotide complementary to the DNA sequence encoding amino acids 5–9 of *aadA* (17–19). A partial restriction map of pLQ826 and pLQ29 and the sequencing strategy is shown in Fig. 2. The entire nucleotide sequence obtained consists of 1854 base pairs (bp) and is presented in Fig. 3.

Identification of the OXA-1 β-Lactamase Gene and Regulatory Regions. Computer analysis of the DNA sequence allowed the identification of two open reading frames (ORFs) beginning with an ATG codon and capable of encoding polypeptides at least 150 amino acids long. A first ORF starts with an ATG at position 574–576 (complementary strand in Fig. 3) and extends beyond the *Pvu* II site. The second ORF begins with an ATG codon at nucleotide 786 and terminates with an ochre codon (TAA) at nucleotide 1614 (Fig. 3). A comparison of the deduced polypeptide encoded by this ORF with the sequences of other β-lactamases (5, 6) strongly suggests that this ORF encodes the OXA-1 protein (see below). The OXA-1 pre-β-lactamase gene initiating at nucleotide 786 would encode a protein of 276 amino acids with a predicted size of 30,979 Da. The molecular mass of the deduced polypeptides (28,231 Da) after cleavage of the signal sequence is slightly higher than the 23,300-Da value reported for the mature OXA-1 protein (1). The same is true for the molecular mass of 29,570 Da of the OXA-2-deduced polypeptide (6) compared to the monomer molecular mass of 22,300 Da. Initiation at the ATG codon at position 786 yields an amino-terminal sequence that strongly resembles known prokaryotic signal peptides (20). The three conserved features of such sequences are present. The amino terminus of the signal peptide contains a positively charged amino acid (lysine), which is followed by a long stretch of uncharged predominantly hydrophobic amino acids. A third ORF starts with an ATG codon at position 1729 and corresponds to the published sequence of the *aadA* gene (17–19).

We analyzed the 5′ flanking region of the OXA-1 gene for the presence of transcriptional signals. The gene encoding OXA-1 is preceded by a sequence that closely resembles the consensus sequence of the *E. coli* promoters (Fig. 3). Starting at position 484, the sequence TAAGCT was found, which matches the three most conserved of the six nucleotides found in the ideal TATAAT Pribnow box. Upstream from the proposed Pribnow box, the sequence TGGACA starts at position 461. This sequence matches 5 of the 6 nucleotides found in the consensus *E. coli* −35 box TTGACA. This putative promoter is in a region of almost perfect homology with the 5′ flanking sequence of the Tn*21 aadA* and R388 *DHFRII* genes (see below) and is very similar to the promoters proposed for these two genes (17, 22). Mutations in R388 in this region abolish *DHFR* gene expression (22). No sequences in the vicinity of the proposed ATG codon at position 786 share homology with the canonical Shine-Dalgarno sequence (23). The 3′ flanking region after the stop codon at position 1614 has been examined for the presence of possible transcriptional termination sites. No inverted repeat followed by an oligo(dT) stretch was found. This suggests that the OXA-1 and *aadA* genes may be cotranscribed. It appears that the OXA-1 gene is precisely inserted between the *aadA* promoter of Tn*21* and its structural gene, becoming in effect the first gene of a resistance operon (see below).

Sequence Homology Between OXA-1 and Other β-Lactamases. A comparison of the OXA-1 sequence with other β-lactamases reveals some significant regions of homology. The first region of homology corresponds to the putative active site, where the sequence Ser-Xaa-Xaa-Lys is conserved in class A and class C β-lactamases as well as several other penicillin-reactive enzymes (24–26) (Fig. 4*A*). The OXA-1 protein shows greater homology, as defined by amino acid identities, with the OXA-2 protein than with other β-lactamases. From amino acid positions 69–181, 41% of the amino acids are conserved between OXA-1 and OXA-2. The greatest homology is found from amino acid 167–181, where 11 amino acids of 15 are identical (Fig. 4*B*). From the

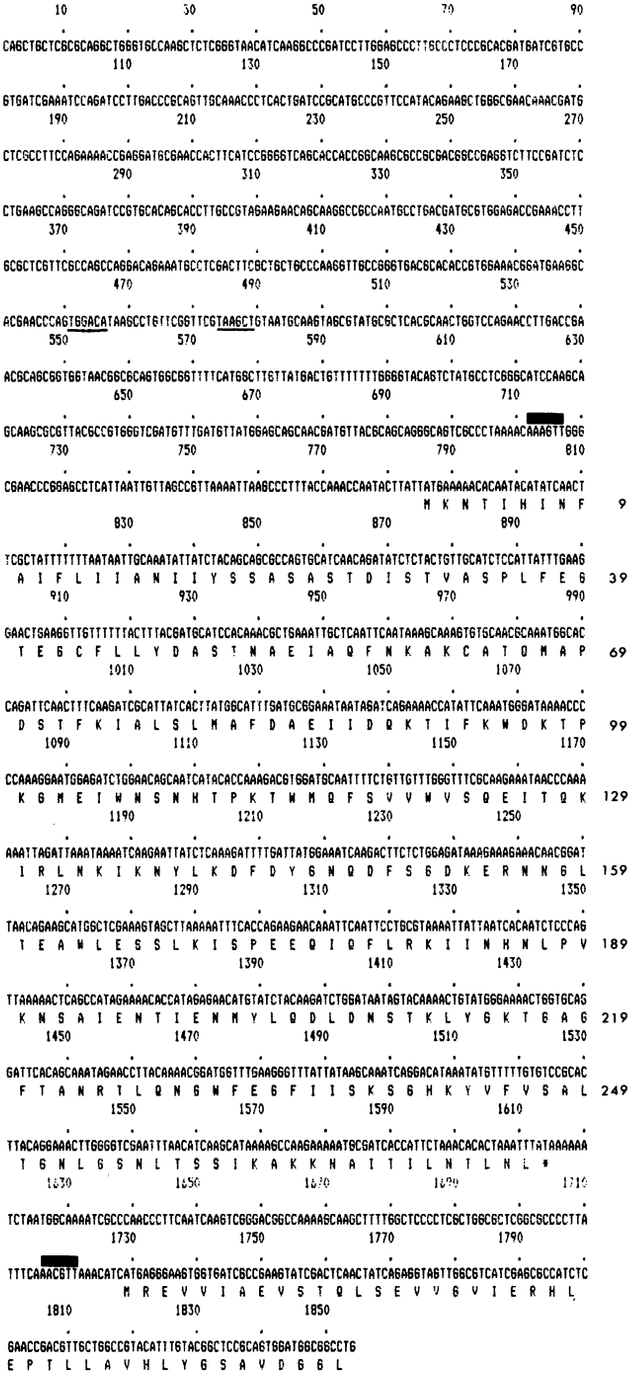

FIG. 3. Nucleotide sequence of the OXA-1 β-lactamase gene. OXA-1 and *aadA* genes start at nucleotide 786 and 1729, respectively. Numbering begins at the *Pvu* II site. Upstream of the OXA-1 gene, the proposed −35 and −10 promoter regions are underlined. The proposed target sequence AAAGTT, which represents the beginning of the OXA-1 insertion (into Tn*21* to generate Tn*2603*) and its 5- for 6-base repeat AACGTT at the end of the insertion, are indicated by boxes. Amino acids are designated by the single-letter code.

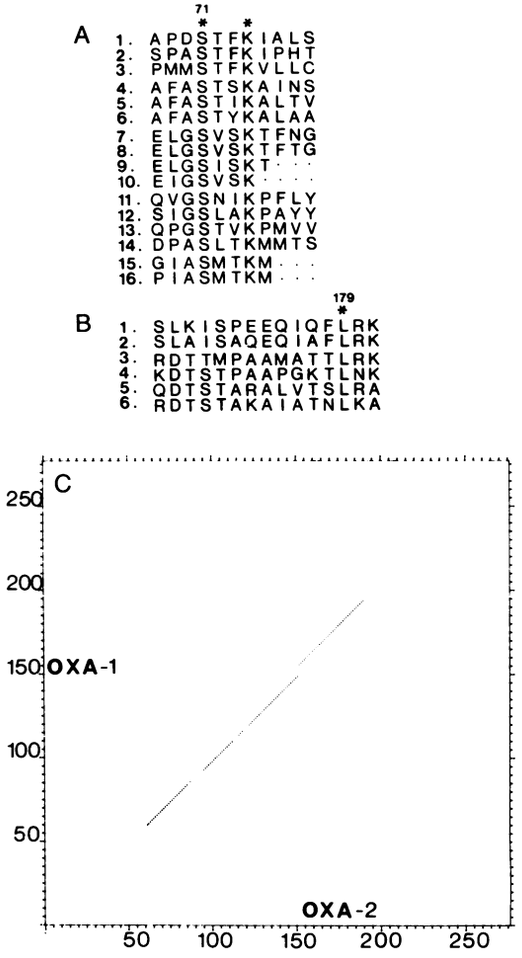

FIG. 4. Sequence homology between OXA-1 and other penicillin-reactive enzymes. A comparison of selected regions of the OXA-1 amino acid sequence (designated by the single-letter code) with other sequences aligned to obtain maximal homology is shown. (*A*) Region of homology corresponding to the active site. The serine and lysine residues, marked by asterisks, are conserved for all penicillin-reactive enzymes shown here. The numbers correspond to the following enzymes: 1, OXA-1; 2, OXA-2; 3, TEM-1; 4, *Staphylococcus aureus* PC1; 5, *Bacillus licheniformis* 749/C; 6, *Bacillus cereus* 569/H β-lactamase I; 7, *Citrobacter freundii ampC*; 8, *E. coli* K-12 *ampC*; 9, *Enterobacter cloacae* P99 *ampC*; 10, *Pseudomonas aeruginosa ampC*; 11, *E. coli* PBP 1A; 12, *E. coli* PBP 1B; 13, *E. coli* PBP3; 14, *E. coli* PBP 5; 15, *Bacillus stearothermophilus* carboxypeptidase; 16, *Bacillus subtilis* carboxypeptidase (adapted from refs. 6 and 24–26). (*B*) Region of homology with the most identities between OXA-1 and OXA-2. A leucine residue, marked by an asterisk, has been conserved through evolution. Numbers are the same as in *A*. (*C*) Functional homology between OXA-1 and OXA-2 expressed as a dot plot. Each dot represents an amino acid similarity score (Dayhoff values) of 330 calculated over a "window" of 30 amino acids.

alignment of sequences shown in Fig. 4*B*, it seems that a leucine residue at position 179 has been conserved throughout evolution in otherwise unrelated β-lactamases. A dot plot graphic, using the values of Dayhoff, which reflect functional similarity of amino acids (27), shows the very high homology between OXA-1 and OXA-2 (Fig. 4*C*).

Recombinational Hot Spot in Tn*21*-like Transposons. By comparing the 5′ flanking sequences of the OXA-1 gene to the GenBank and EMBL data bases‡ and to several recently published antibiotic-resistance gene sequences, we found that several resistance genes can occur in the same sequence context (Fig. 5). First, comparison of our OXA-1 sequence with those of OXA-2 from R46 (6) and of the Tn*21 aadA* gene (17) reveals that both the OXA-1 and OXA-2 structural genes are inserted, in their respective plasmids, between the *aadA* promoter and its structural gene at the target sequence

‡EMBL/GenBank Genetic Sequence Database (1985): GenBank (Bolt, Beranek, and Newman Laboratories, Cambridge, MA), Tape Release 38.0; EMBL (Eur. Mol. Biol. Lab., Heidelberg), Tape Release 7.0.

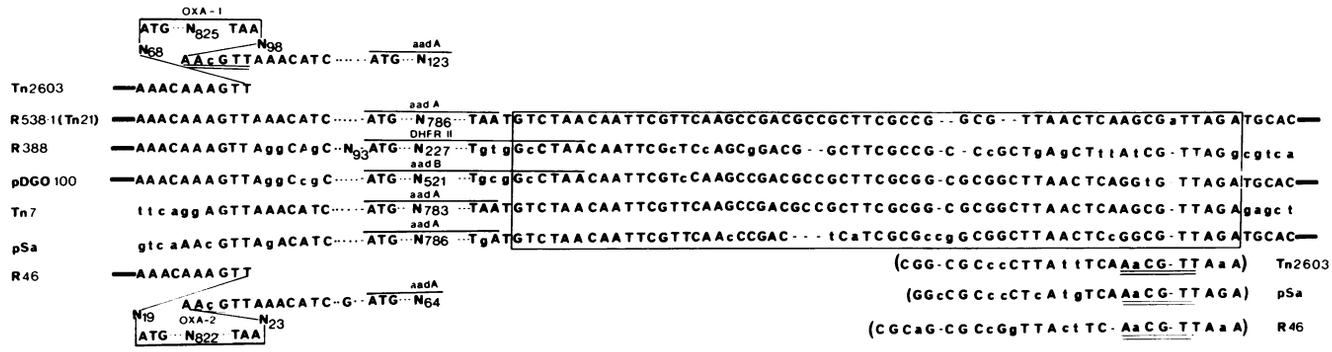

FIG. 5. Recombinational hot spots around the Tn*21*-encoded *aadA* gene. The ATG codon for the *aadA* gene is taken from the N-terminal sequence of the pCN1 aadA protein (19). The OXA-1 and OXA-2 β-lactamase genes insert upstream of the *aadA* gene of Tn*21*, whereas the *DHFRII* and *aadB* genes substitute for it. The sequences 5′ of these determinants are all >98% homologous (represented by a thick line) as far as they have been sequenced (0.1–1.2 kb). The OXA-1 and OXA-2 β-lactamase genes and a kanamycin-resistant determinant in pSa (see text) insert at the sequence AAAGTT. At the end of the OXA-1 and OXA-2 insertions there are near perfect repeats AACGTT (double underlined) of the target sequence. This hot spot is also the point at which genes that substitute for the *aadA* gene diverge from it and where the *aadA* sequences of Tn*7* and Tn*21* converge. The 59-bp element, thought to be involved (11, 28) in recombinational rearrangements in Tn*21* transposons, is boxed. The sequences within parentheses are not colinear with the others but are aligned to show the homology between the distal part of this 59-bp element and the 3′ ends of the OXA-1, OXA-2, and kanamycin-resistance (from pSa) inserts. The initiation codon for *aadB* is an alternative to that of Cameron *et al.* (28).

AAAGTT (see Figs. 3 and 5). In contrast to the 48% homology between the OXA-1 and OXA-2 inserts, the 5′ and 3′ flanking sequences of each (for OXA-1, bases 1–717 and 1722–1854 in Fig. 3) show >98% homology with Tn*21*. Second, the AAAGTT sequence is also at the point where the *aadA* genes from Tn*21*, Tn*7*, and pSa begin their homology. Third, the R388-encoded trimethoprim-resistance gene *DHFRII* (22) and the gentamicin-resistance (*aadB*) gene in pDGO100 (28) have apparently substituted for the *aadA* gene (or vice versa) in their respective plasmids. These sequences are also >98% homologous with the 5′ flanking region of *aadA* up to the sequence AAAGTT. These sequences then diverge and later converge at the common sequence CTAACAATT, which is just 3′ of these three genes. Remarkably, the TAA in this sequence is used by both the *DHFR* and *aadB* genes as their stop codons. The sequence CTAACAATT is at the 5′ end of a 59-bp element (28) (boxed in Fig. 5), after which the Tn*21* and Tn*7* *aadA* sequences diverge. A second copy of this element occurs 5′ of the *aadA* gene in Tn*7* (18) and includes the first 13 nucleotides of the Tn*7* sequence in Fig. 5.

Heteroduplex and restriction mapping data led Schmidt (10) to propose the existence of two recombinational hot spots, flanking the Tn*21* *aadA* gene, at which three types of events can take place: (*i*) insertion at the first hot spot (5′ of *aadA*), (*ii*) insertion at the second hot spot (3′ of *aadA*), and (*iii*) substitution of another resistance determinant for *aadA*, with the ends of the substitution at the two hot spots. Our data and other existing sequence data permit us to precisely locate the first hot spot at the target sequence AAAGTT (Fig. 5). The sequence at the 3′ end of OXA-1 and OXA-2, AACGTT, may have arisen from a repetition of the target site AAAGTT. Sequence data from the substitutions permit us to locate the second hot spot within the 59-bp element, possibly at the sequence CTAACAATT (Figure 5) or, alternatively, at the 3′ end of the 59-bp element, which shows some homology with the first hot spot. Sequence data from instances of gene insertion 3′ of *aadA* such as in Tn*1696* and Tn*2424* (Fig. 1) will be necessary for better definition of this hot spot.

DISCUSSION

A systematic comparison between plasmid mediated β-lactamases, to gain insight into their mode of action, is difficult at present since only 2 of the 25 or so plasmid-specific β-lactamases from Gram-negative bacteria have been sequenced so far. To further study the relatedness of the OXA-1 β-lactamase to other β-lactamases, the sequence of a 1.6-kb *Pvu* II/*Hin*dIII restriction fragment, derived from Tn*2603* and known to encode OXA-1 (9), was done. A comparison of this DNA sequence with that of OXA-2 (6) and TEM-1 (5) shows greater than the overall 48% homology with OXA-2 at nucleotides 988–1036 (33 of 51) and 1278–1352 (43 of 75) but no significant homology with TEM-1 (not shown). However, on comparing the amino acid sequences, regions of homology were apparent. The most recent classification of β-lactamases is based on amino acid sequence homology, assigning the TEM-type β-lactamases to class A and the Zn^{2+}-requiring enzymes to class B (24). Several Gram-negative chromosomal β-lactamases lack significant homology to either class and were assigned to class C (25, 29). Class A and class C β-lactamases as well as penicillin-reactive enzymes have a serine residue at their active site (see Fig. 4*A*). Class A β-lactamases have diverged from a common ancestor (24) and the same is true for class C β-lactamases (25). The only region of homology between OXA-2 and the class A and class C enzymes is adjacent to the active site, and Dale *et al.* (6) have suggested that the homology found in the active-site region may have arisen from convergent evolution. No homology was found between OXA and class B enzymes.

Two regions of homology were found between the OXA-1 β-lactamase and other enzymes. The first one is centered at the active site (Fig. 4*A*) and the second is centered around an oligopeptide (Phe-Leu-Arg-Lys) homologous to OXA-2 and including a leucine residue conserved by class A β-lactamases (Fig. 4*B*). Around these regions OXA-1 exhibits a greater degree of homology with OXA-2 than with any other β-lactamase, and when conservative amino acid substitutions are considered close to identities, the functional homology between OXA-1 and OXA-2 extends from amino acid 60 to 195 (Fig. 4*C*). No significant functional homology, expressed as a dot plot, using the permissive Dayhoff values (27) was found between OXA-1 and TEM-1 or OXA-2 and TEM-1 (data not shown). Since OXA-1 and OXA-2 show homology between themselves but no significant identities with other β-lactamases, we propose that these two enzymes should be assigned to the class D β-lactamases. There are seven different OXA enzymes in nature (30) and it should not be surprising that these enzymes would fall in the class D type, given their enzymatic similarities. For example, the OXA-4 β-lactamase gene hybridized with DNA probes specific for OXA-1 (M.O., unpublished results).

Tn*21* harbors genes encoding resistance to mercuric chloride (*mer*), sulfonamide (*sul*), and streptomycin/spectinomycin (*aadA*) along with a transposase, a resolvase, and a modulator of the resolvase (31, 32). In Tn*21*-related transposons, other resistance genes were always found in the vicinity of the *aadA* gene (10, 11, 33), which led to the hypothesis of recombinational hot spots. Two sites into which resistance genes can insert have been found (Fig. 1; ref. 10). The first hot spot, 5′ to the *aadA* structural gene, is where OXA-1 in Tn*2603* (7) and OXA-2 from R46 (6) are precisely inserted at the target sequence AAAGTT and include the sequence AACGTT at the end of the insert. The almost perfect homology (98%) among the flanking sequences as opposed to the considerable divergence (48% homology) between the OXA-1 and OXA-2 inserts strongly suggests that the associations of OXA-1 and OXA-2 with Tn*21*-like sequences are relatively recent events. The kanamycin-resistance gene from pSa (34) is probably a third example of such an insertion. This insertion also ends with AACGTT; although the 5′ end of the kanamycin-resistance gene has not been sequenced, a fragment homologous to a region 5′ of the *aadB* gene hybridizes with pSa (28). Our probes of 5′ flanking sequences, including a *Pvu* II/*Eco*RV fragment (nucleotides 4–869 in Fig. 3), hybridized with strains harboring any of the OXA or PSE β-lactamases tested (9). This suggests that several other β-lactamases can insert precisely 5′ of the *aadA* structural gene. Finally, mapping data suggest that the *aadB* gene in Tn*4000* (10), and the *aacC* gene in Tn*1696* (33), may be inserted at this hot spot (Figure 1).

The second potential hot spot lies 3′ to the *aadA* gene of Tn*21* and is where a chloramphenicol-resistance determinant has been mapped in Tn*1696* (33) and where a 4.1-kb insert encoding amikacin (*aacA*) plus chloramphenicol resistance has been mapped in Tn*2424* (35). Sequence data for these insertions are not yet available. However, a third type of recombinational event involves substitution of the *aadA* gene by OXA-2 in Tn*2410*, by *DHFRII* in R388, and by *aadB* in pDGO100 (refs. 22, 28, and 36; Fig. 1). Which of these three genes substituted for which cannot be established with certitude; however, *aadA-Su*^{r} plasmids are far more widespread than either *DHFR-Su*^{r} or *aadB-Su*^{r} types. Also, these substitutions may in fact be insertions at either hot spot followed by deletion of *aadA*. Sequence data for the latter two examples allow us to approximately localize the second hot spot at a 59-bp element found 3′ of the sequences of the *aadA* genes of Tn*21*, Tn*7*, and pSa and also 3′ of genes that substitute for it (*aadB* and *DHFRII* genes). These sequences converge at the beginning of this element; Tn*7* and R388 diverge from the common Tn*21*-pSa-pDGO100 sequence at the end of this element (Fig. 5).

Recently, Cameron *et al.* (28) published a proposed sequence for the *aadB* determinant from pDGO100. They propose, as Wiedemann *et al.* (11) have done, a role for the 59-bp element in recombinational events around the hot spot. Since Cameron *et al.* state that the coding sequence of *aadB* begins within the 5′ flanking sequence common to several resistance genes (see Fig. 5), they proposed that it is *aadA* that has substituted for *aadB*, and that the 59-bp element played a role in this substitution. This is indeed possible, but it does not explain a role of the 59-bp element, which is 3′ of the *aadA* gene, in subsequent insertions 5′ of the *aadA* gene. However, our finding of good homology (75%) at the 3′ ends of the OXA-1 and OXA-2 and the pSa kanamycin-resistance insertions (sequences in parentheses in Fig. 5), with the distal end of the 59-bp element, suggests that if this region is involved in these insertions, it may be furnished by the insertion rather than by the target.

Alternatively, only the short target sequences themselves may be involved in this recombinational mechanism. While the target site shows no relatedness to transposon termini or to internal resolution sites, the Tn*21* sequence at this site (AAAACAAAGTTA) shows a 9 for 12 homology with the consensus recognition site (AAACCAAGGTTT) of bacterial invertases (37). We speculate that there may be a Tn*21*-encoded enzymatic activity that interacts with the target site.

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