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Amplification of the Tetracycline Resistance Determinant of pAMα1 in *Enterococcus faecalis* Requires a Site-Specific Recombination Event Involving Relaxase

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The small multicopy plasmid pAMα1 (9.75 kb) encoding tetracycline resistance in *Enterococcus faecalis* is known to generate tandem repeats of a 4.1-kb segment carrying *tet*(L) when cells are grown extensively in the presence of tetracycline. Here we show that the initial (rate-limiting) step involves a site-specific recombination event involving plasmid-encoded relaxase activity acting at two recombination sequences (RS1 and RS2) that flank the *tet* determinant. We also present the complete nucleotide sequence of pAMα1.

Tetracycline resistance in clinical isolates of enterococci is extremely common and is frequently associated with plasmids and/or conjugative transposons (6). pAMα1 is a relatively small (9.75-kb), multicopy tetracycline resistance plasmid originally identified in *Enterococcus faecalis* strain DS5 (10). Although not conjugative, it is readily mobilizable by coresident conjugative elements, and its mobilization has been used indirectly to identify conjugative plasmids devoid of easily selectable markers (11, 12, 28, 29).

When *E. faecalis* cells carrying pAMα1 are grown in the presence of tetracycline, the plasmid accumulates tandem repeats of a 4.1-kb segment of DNA containing the *tet*(L) determinant (9, 40, 41). The phenomenon was found to involve a recombinational event between directly repeated recombination sequences (RSs) that flanked *tet* (41). Three models, not mutually exclusive, were suggested to explain how the process may take place (41); the rate-limiting step involves intra- or intermolecular recombination events between the two RSs. (In the case of an intramolecular event, an uneven crossover between the two RSs within a partially replicated molecule can be easily envisioned and requires only a single crossover, whereas the other models invoke two-step processes.) The generation of tetracycline-sensitive derivatives exhibiting a deletion of the amplifiable segment is explainable by the same mechanism(s). After a single tandem duplication arises, the repeated segments bearing *tet* become available, in their entirety, for further amplification by a RecA-dependent process (43), and growth under selective conditions leads to single molecules with as many as eight repeats (40). The RSs are presumed necessary only for the initial tandem duplication step.

Perkins and Youngman (31) found that pAMα1 represents a composite of two independent replicons, one of which, pAMα1Δ1, corresponds to the amplifiable segment of the plasmid. The other, designated pAMα1Δ2, corresponds to the replicon that remains in *E. faecalis* after loss of tetracycline resistance (i.e., spontaneous deletion of pAMα1Δ1). (It is believed that pAMα1Δ1 is not able to replicate independently in *E. faecalis* but is able to replicate in *Bacillus subtilis* and pAMα1Δ2 is not able to replicate in *B. subtilis*.) Based on its sensitivity to various restriction enzymes, pAMα1Δ1 was found to closely resemble pBC16 and other plasmids in *Bacillus* (1, 2) and pUB110 of *Staphylococcus aureus* (33). Here we report on the nucleotide sequence of pAMα1 and identify the nature of the two RSs. In addition, we show that two mobilization proteins (putative relaxases), one associated with pAMα1Δ1 and one with pAMα1Δ2, participate in site-specific recombinational events related to the first step in amplification.

Determination of the nucleotide sequence of pAMα1. A map of pAMα1 is shown in Fig. 1, while Table 1 provides a list of related open reading frames. All of the determinants have the same orientation, and the presence of genes with near identity to replication and mobilization functions on pBC16 and pS86 (a cryptic plasmid from *E. faecalis* [25]), is clearly evident. The nomenclature utilized here is *repB* and *mobB* (B for *Bacillus*, relating the connection of pAMα1Δ1 to the bacillus plasmid pBC16) and *repE* and *mobE* (E for *Enterococcus*, relating the connection of pAMα1Δ2 to the enterococcal pS86). The two RSs represent segments of 387 bp (Fig. 2), a size that relates well with that previously proposed based on electron microscopy (41) but, interestingly, they exhibit only 57% identity. A core region exhibits identity at 50 out of 56 nucleotides. The RSs overlap the 5′ ends of the adjacent *mob* determinants (Fig. 2). The products of these two determinants are 34% identical and exhibit 58% identity (70% similarity) in their N-terminal 200 amino acids. They both relate to the recently described pMV158 family of relaxases (19). (DNA relaxases are key enzymes in the initiation of DNA transfer via their cleavage at the *nic* site within a specific transfer origin, *oriT*.) Proteins belonging to this family show characteristic amino acid sequence motifs plus one motif that is common to all other relaxase types—that is, a highly conserved pair of histidine residues followed by a stretch of hydrophobic amino acid residues (19, 44) (positions 131 to 139 in MobB and 129 to 136 in MobE). In addition, the DNA sequences of the target *nic* regions are shared among the members of this family. The

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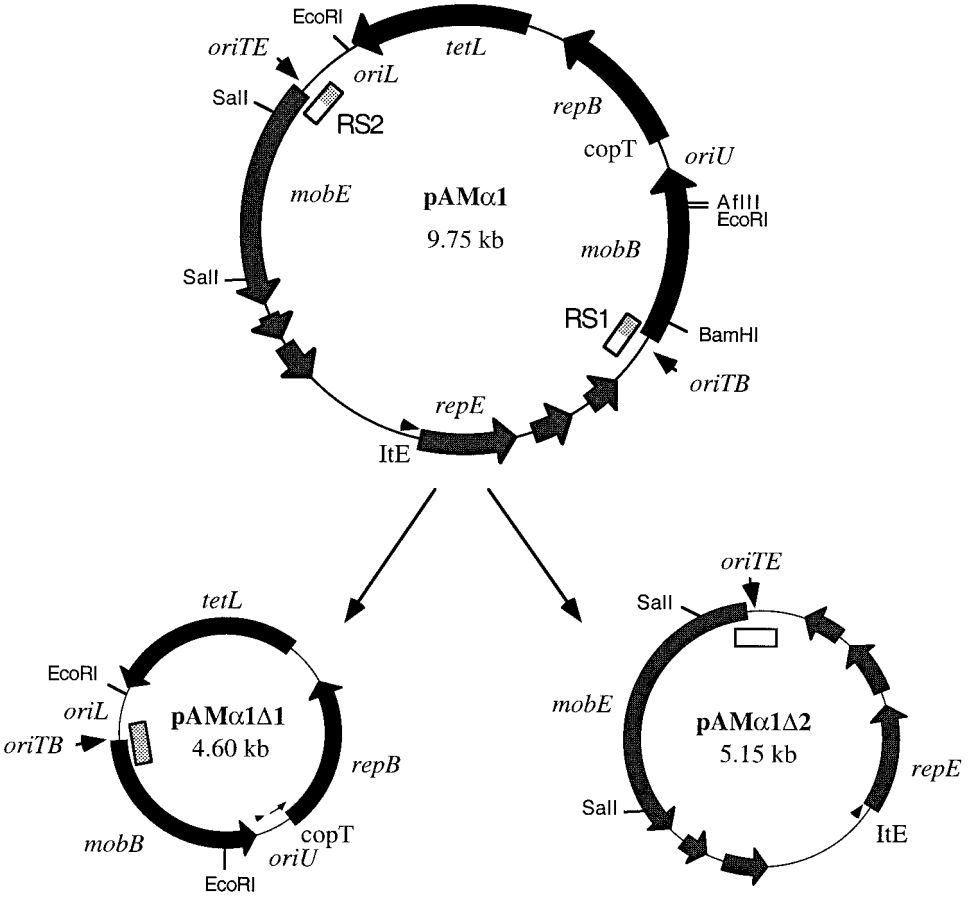

FIG. 1. Structural organization of pAMα1. The positions of the open reading frames and their orientations are indicated with arrows. The cointegrate relationship between pAMα1Δ1 (closely related to pBC16 from *Bacillus cereus*) and pAMα1Δ2 (closely related to pS86 from *E. faecalis*) is illustrated. *oriTB* and *oriTE* are putative *oriT* sites that resemble the pMV158-like *oriT* family and are located within RS1 and RS2, respectively. The recombination in the RSs giving rise to pAMα1 (and in amplified forms [see Fig. 3]) is shown with the differentially shaded rectangles. ItE represents an iteron-repeat region upstream of *repE*; a homologous region in pS86 has been suggested to be involved in replication. *oriU* and *oriL* represent the pAMα1Δ1 double-strand origin and minor origin of replication, respectively, based on homology with pBC16. The positions of the restriction sites used in construction of the pAMα1 mutants and/or for the amplification analyses are indicated.

relaxase MobM of pMV158 (originally from *Streptococcus agalactiae*) has been shown to display the typical properties of DNA relaxases from plasmids of gram-negative bacteria (19, 44). Interestingly, both of the RSs of pAMα1 contain regions resembling the pMV158 *oriT* site and even include the palindrome identified as the pMV158 nicking site (19). Relaxases have been reported previously to facilitate site-specific recombination between two *oriT* sites (3, 17, 24, 27, 37), which raises the question of whether relaxase activity might contribute to the initial tandem duplication step during amplification in the presence of tetracycline. A schematic representation of pAMα1 amplification is shown in Fig. 3.

Evidence that the initial duplication is RecA independent. Although it was reported a number of years ago that amplification did not occur in a RecA-negative host (43), the methodology utilized may not have resolved the initial step, which

TABLE 1. Open reading frames identified in pAMα1

ORF	Nucleotides (no. of amino acids)	Strand	Gene name	Homology	Similarity (%)	Protein family
1	566–8949 (458)	S-	*tet*(L)	*tet*(L) (pBC16)	100	Sugar transporter (pfam00083)
2	1777–773 (334)	S-	*repB*	ORF alpha (pBC16)	100	Rep (pfam01446)
3	3263–2001 (420)	S-	*mobB*	ORF beta (pBC16)	100	Mob (pfam01076)
4	3921–3697 (74)	S-		ORF3 (pS86)	100	
5	4341–4045 (98)	S-		ORF2 (pS86)	98	
6	5190–4468 (240)	S-	*repE*	*rep* (pS86)	100	Rep (pfam01051)
7	6398–6087 (103)	S-		ORF6 (pS86)	100	
8	6621–6421 (66)	S-		ORF5 (pS86)	100	
9	8389–6710 (559)	S-	*mobE*	*mob* (pS86)	100	Mob (pfam01076)

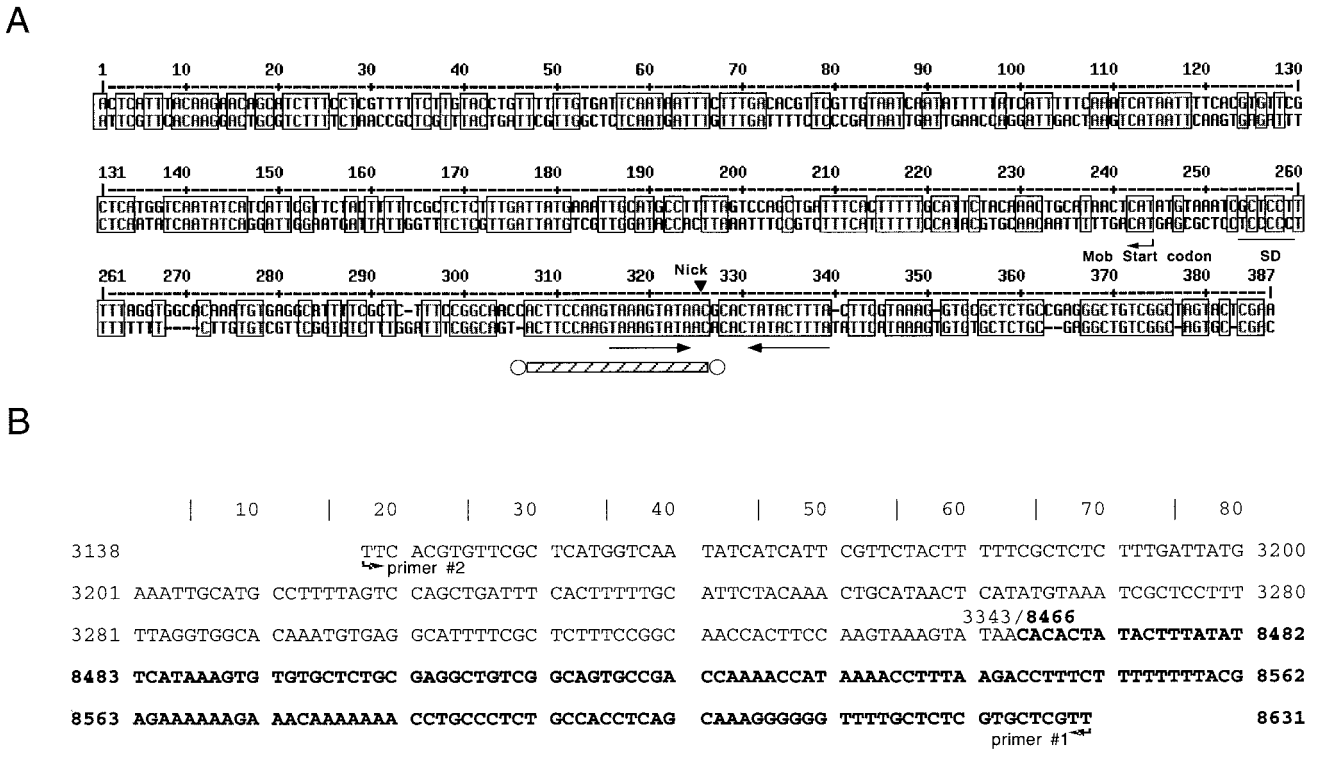

FIG. 2. RS comparisons. (A) Comparison of RS1 (top line) and RS2 (bottom line) sequences. Conserved nucleotides are boxed, and inverted repeat sequences are indicated with arrows. The putative transfer origin nick site is noted with the triangle. The putative Shine-Dalgarno (SD) sequences and the ATG start codons of the corresponding *mob* genes are also shown. The hatched bar indicates the sequence where amplification-related recombination occurs in vivo. (B) Sequence of the junction site of in vivo amplification products, using the 1.5-kb *Eco*RI fragment (see Fig. 3) as template to generate a PCR product from the indicated primers. The probable crossover point is indicated by the switch to bold letters.

might have involved a RecA-independent relaxase activity generating the first tandem duplication. To investigate this point further, the isogenic hosts JH2-2 (RecA$^+$) (22) and UV202 (RecA$^-$) (42), each carrying pAMα1, were grown overnight (0.3% inoculum in 5 ml of Todd-Hewitt broth) in the presence of 5 μg of tetracycline/ml. The cells were then subcultured similarly for a second day in the same concentration of drug. Plasmid DNA isolated (38) from samples taken from both the day 1 culture and the day 2 culture was cleaved with *Eco*RI and analyzed by agarose gel electrophoresis. As shown in Fig. 4A, after 1 day the DNA in JH2-2 readily exhibits the presence of a new small (1.5-kb) fragment representing an additional copy of an RS (lane 3), whereas such a band was barely detectable in the case of the UV202 host (lane 1). After the second passage, however, this band could be easily seen (lane 2). *Sal*I digestions further confirmed that the first duplication, and possibly a second repeat, is also obtained in UV202 cells (Fig. 4B, lane 2), while DNA from JH2-2 (lane 4) exhibited a relatively high level of repetition. The two *Sal*I sites in pAMα1 are not within the amplifiable segment; thus, the larger fragments represent increasing lengths due to additional segments containing *tet*. A similar experiment (data not shown) using the RecA$^-$ host and differing only in that the tetracycline concentration was increased from 5 to 10 μg/ml during day 2 resulted in an *Eco*RI pattern in which the 3.05-kb band became approximately twice the intensity of that in the day 1 pattern. Southern hybridization analyses using PCR products representing *mobB* and *mobE* as probes were consistent with the view that only the region representing pAMα1Δ1 (i.e., *tet*) becomes amplified.

As shown in Fig. 4C, PCR products generated from primers designed to flank an RS that was predicted to result from recombination between RS1 and RS2, generating a 1.5-kb *Eco*RI fragment (Fig. 3), resulted in bands of the expected sizes in all cases. The data imply that the first step in amplification is RecA independent. Sequence determination of 16 independently generated amplification products demonstrated the same sequence in each case (Fig. 2B) and indicated that the recombination took place in the 20-bp segment noted in Fig. 2A. This segment contains the sequence resembling the *nic* site for a pMV158-type relaxase, suggesting that such an activity had cleaved and rejoined in the specific *nic* sites in both RS1 and RS2 as part of the duplication process.

MobB or MobE is necessary for the initial duplication. To determine if either of the pAMα1 relaxases (MobB or MobE) was involved in the recombination event, we generated three mutant derivatives with deletions within the related determinants. pAM8501 is a mutant with a 1,264-bp deletion in *mobE*. pAMα1 was cleaved with *Sal*I, and the larger of the two *Sal*I fragments (Fig. 1) was religated and introduced into JH2-2 by electroporation (16). pAM8502 is a mutant with a 789-bp deletion in *mobB*. In this case, pAMα1 was cleaved with *Bam*HI and *Afl*II and filled with DNA polymerase (Klenow), and the larger fragment was religated and introduced into JH2-2 by electroporation. pAM8503 was pAMα1 with mutations in both *mobB* and *mobE* (both of the above mutations). All three

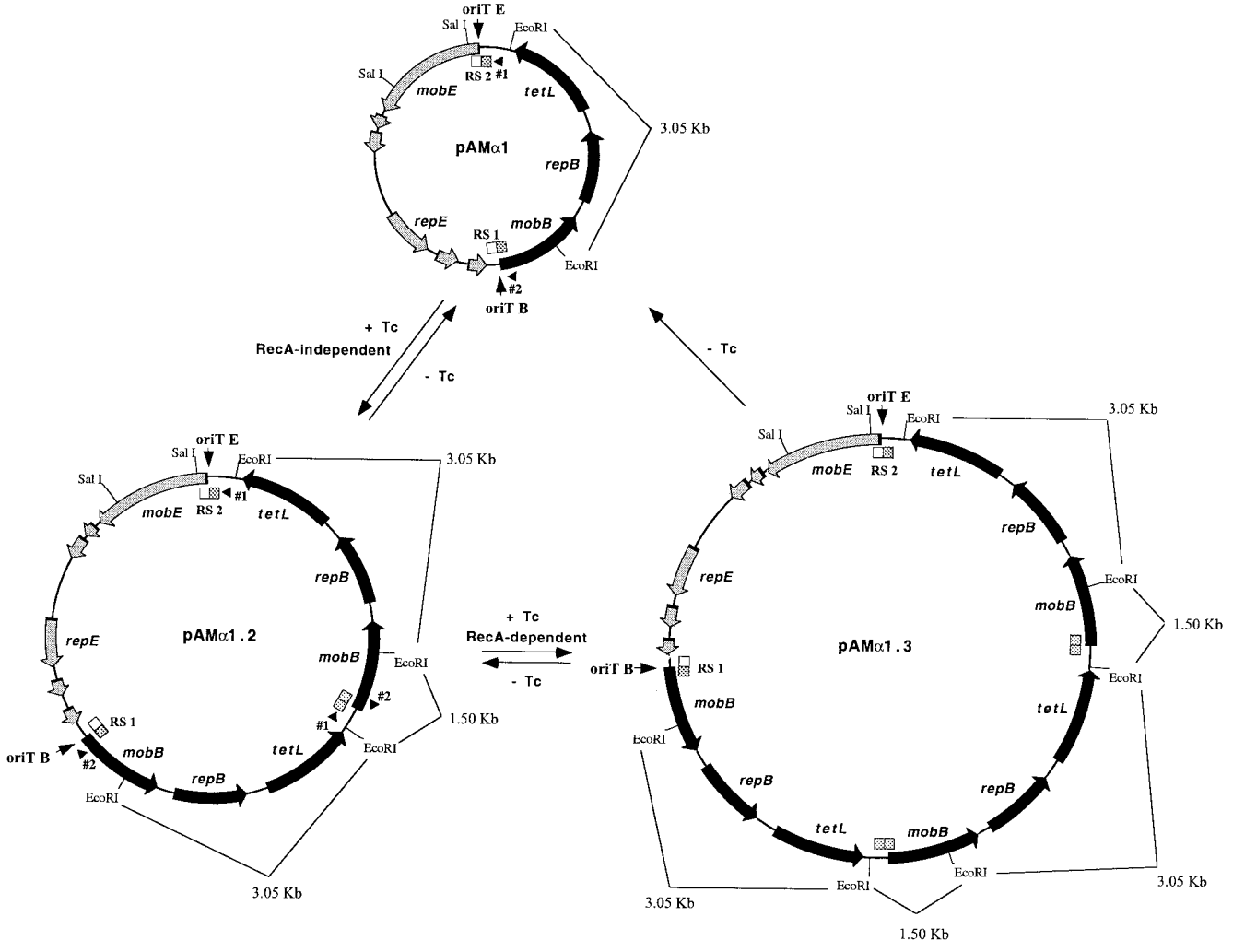

FIG. 3. Schematic representation of pAMα1 tetracycline resistance amplification. The 3.05- and 1.5-kb *Eco*RI segments corresponding to those appearing on the agarose gel analyses shown in Fig. 4 are noted. The locations of the primers used for the PCRs (see Fig. 4) are also indicated.

mutants were confirmed by restriction analyses and sequencing. Each derivative was introduced into JH2-2 and UV202, and the cells were grown in increasing concentrations of tetracycline. Both single mutants were able to grow in a manner closely resembling that of pAMα1 and exhibited amplification based on the ability to detect a PCR product arising from the 1.5-kb *Eco*RI fragment (Fig. 4D, lanes 3 to 6). However, the double mutant (pAM8503) behaved differently. UV202 or JH2-2 cells harboring pAM8503 were able to grow in the presence of 5 μg of tetracycline/ml but did not give rise to a detectable amplification pattern (data not shown), and a PCR product representative of the expected new (recombinant) RS (i.e., within the 1.5-kb *Eco*RI fragment) could not be detected even after growth in increasing concentrations of tetracycline (Fig. 4D, lanes 7 to 12). The data are consistent with the view that relaxase activity of both MobB and MobE can catalyze site-specific recombination involving the *nic* sites within the two RSs, generating the substrate that can subsequently be utilized for further recombination by the host RecA system.

MobB or MobE is required for conjugative mobilization of pAMα1. As mentioned above, the N-terminal regions of the deduced protein sequences of *mobB* and *mobE* genes in pAMα1 reveal significant homology with corresponding regions of the pMV158 family of relaxases, many of which are encoded in gram-positive plasmids that replicate by rolling-circle replication mechanisms. In several cases, these genes have been shown to be required for conjugative mobilization (14, 30, 34, 36). From this perspective, the plasmid constructions described in the above section were assayed for their ability to be mobilized by pAM307 (pAD1::Tn*917* with wild-type conjugation properties) (8) or a derivative (pAM8130) defective in relaxase (17). (Matings were conducted overnight as described in reference 7.) Table 2 shows that, in the presence of pAM307, mobilization of pAM8503 (deletions in both *mob* genes) was reduced 10-fold compared to that of pAMα1. Derivatives with a deletion of only one or the other of the *mob* genes (i.e., pAM8501 or pAM8502) could be mobilized almost as efficiently as the wild-type pAMα1, suggesting that both Mob proteins were functional. Importantly, when the conjugative pAD1 element was defective in its own relaxase

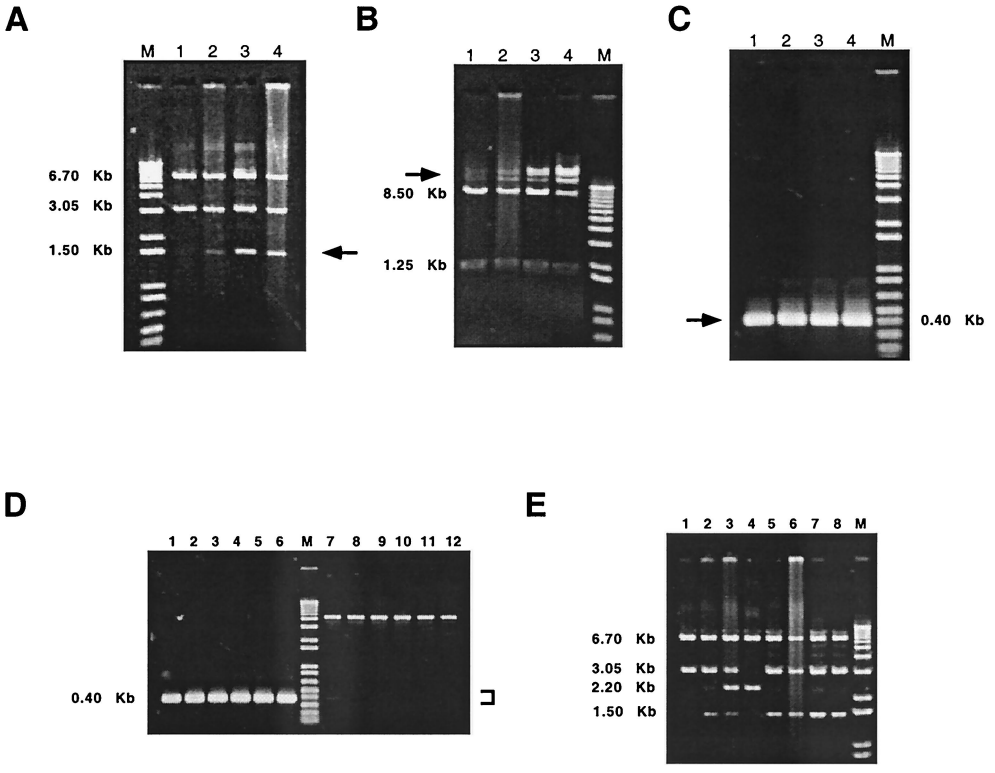

FIG. 4. Analyses of the amplification of pAMα1 and mutant derivatives in the isogenic strains JH2-2 and UV202. (A) *Eco*RI digestions of pAMα1 from UV202 (lanes 1 and 2) and JH2-2 (lanes 3 and 4) cells grown overnight in the presence of 5 μg of tetracycline/ml (lanes 1 and 3) or subcultured for a second day under the same conditions (lanes 2 and 4). The arrow indicates the 1.5-kb band reflecting amplification of *tet*. (B) *Sal*I digestions. Lanes correspond to those in panel A. The new *Sal*I bands indicating tetracycline amplification are noted with an arrow. (C) PCR products indicating in vivo amplification using the above lysates and primers 1 (AACGAGCACGAGAGCAAAACCCC) and 2 (TTCACGTGTTCGCTCATGGTC). (D) PCR products reflecting *tet* amplification or its absence in UV202 cells (lanes 1, 3, 5, 7, and 8) or JH2-2 cells (lanes 2, 4, 6, and 9 to 12). Cells harboring pAMα1 (lanes 1 and 2) or its deletion mutants, pAM8501 (lanes 3 and 4), pAM8502 (lanes 5 and 6), and pAM8503 (lanes 7 to 12), were grown as indicated below. Strains containing pAMα1 or its derivatives pAM8501 or pAM8502 were grown in 5 μg of tetracycline/ml. Strains containing pAM8503 were grown in increasing drug concentrations of 5 μg/ml (lanes 7 and 9), 10 μg/ml (lanes 8 and 10), 15 μg/ml (lane 11), or 20 μg/ml (lane 12). (E) *Eco*RI digestions of pAMα1 from UV202 cells (lanes 1 to 4) or JH2-2 cells (lanes 6 to 8) grown in the presence of increasing concentrations of drug: 5 μg/ml (lanes 1 and 5), 10 μg/ml (lanes 2 and 6), 15 μg/ml (lanes 3 and 7), and 40 μg/ml (lanes 4 and 8). M, the molecular mass marker 1Kb-plus (Invitrogen).

(pAM8130, which is defective in *traX* [17]), no mobilization of pAM8503 was detected, while the mobilization of pAMα1 was not affected. This suggests that pAMα1 relaxases are required for pAMα1 transfer by pAM8130, and pAM307 was utilizing its own relaxase to mobilize pAM8503 (and to a significant degree probably also pAMα1, pAM8501, and pAM8502), unless mobilization involved a transient cointegration event between the two plasmids. Whereas transposition of Tn*917* from pAM307 might be considered a factor in cointegrate formation (8), restriction analyses of the plasmid content of several transconjugants showed the absence of a new Tn*917* copy in the pAMα1 derivative. Thus, if cointegrate formation was occurring in the transfer of pAM8503, it must have involved a different mechanism. We also note here (Table 2) that a pAD1 derivative, pAM8131, with a mutation in *traW*, a *traG*-like determinant (44) required for pAD1 transfer (17), was not able to mobilize pAMα1. Mobilization of small nonconjugative, but mobilizable, plasmids is known to be dependent on a TraG-like function of corresponding coresident conjugative plasmids (44) with one exception, the *Enterobacter cloacae* CloDF13 plasmid (5). This plasmid encodes its own TraG analog.

Appearance of deletions of *repB* in pAMα1. If UV202 cells harboring pAMα1 are subcultured in increasingly higher concentrations of tetracycline (e.g., 5, 10, 15, and 40 μg/ml, sequentially), the appearance of a deletion can be observed. As

TABLE 2. Mobilization frequencies of pAMα1 and its deletion derivatives by pAM307 or its TraX or TraW mutants

Plasmids in donors	Transfer frequency (UV202 to OG1SS)[a]	
	pAD1 derivatives (Er/donor)	pAMα1 derivative (Tc/donor)
pAM307, pAMα1	1	3.5×10^{-5}
pAM307, pAM8501	1	6.0×10^{-6}
pAM307, pAM8502	1	8.5×10^{-6}
pAM307, pAM8503	1	2.1×10^{-6}
pAM8130, pAMα1	$<10^{-8}$	1.5×10^{-5}
pAM8130, pAM8503	$<10^{-8}$	$<10^{-8}$
pAM8131, pAMα1	$<10^{-8}$	$<10^{-8}$
pAM8131, pAM8503	$<10^{-8}$	$<10^{-8}$

[a] Transfer frequencies are expressed as the number of transconjugants per donor cell and represent the average of, at least, two independent experiments. Matings were conducted by using the UV202 host as the donor and OG1SS as the recipient. Er, erythromycin resistance; Tc, tetracycline resistance.

seen in Fig. 4E, the 3.05-kb *Eco*RI band is replaced by a 2.2-kb band. Sequence analysis of this band indicated that a 0.9-kb deletion between two direct repeats (TTTTAAATTCA) had taken place, involving a significant component of *repB* (data not shown). This was a reproducible occurrence and likely related to a phenomenon by which the *repB* promoter is placed closer to the *tet* gene to facilitate an upregulation. Not surprisingly, a similar shift could be seen when pAM8503 (*mobB-mobE* double mutant) was present not only in UV202 but also in JH2-2, again involving loss of the *repB* gene (data not shown). The region containing *repB* would appear to be particularly prone to acquiring a deletion. However, the selection for such derivatives appears much weaker than for amplified molecules, since they become significantly evident only when amplification cannot occur.

Concluding remarks. The ability of bacteria to generate tandem repeats of specific genes as a way of increasing their level of antibiotic resistance is well known; homologous recombination events involving short direct repeats, insertion sequences, and/or transposons are known to be related to such amplification processes (4, 13, 20, 21, 26, 32, 35, 39, 41). However, while it has been previously suggested (15), site-specific recombination events have, to our knowledge, not been reported in such processes. It is now evident that in the case of pAMα1, MobE and/or MobB are important in the first step of the amplification of tetracycline resistance by facilitating a site-specific recombination event between the RS1 and RS2 sequences containing putative *oriT* sites. Further amplification steps occurring via this process might also occur but apparently at a level significantly below that facilitated by the added duplication (i.e., the new 4.6-kb segment, representing an entire unit of pAMα1Δ1) now able to participate in homologous recombination.

While it is conceivable that the initial recombination between RS1 and RS2 might also occur via homologous recombination, the relatively small size (387 bp) and low sequence identity (57%) of these regions would significantly limit such an occurrence. Indeed, in the absence of MobB and MobE (i.e., in the case of the double-mutant pAM8503), growth in the presence of tetracycline in a $RecA^+$ host did not result in amplification. Rather, it gave rise to deletions within *repB* which probably resulted in *tet* being upregulated via its proximity to the *repB* promoter.

Relaxase activities of the Mob proteins play a key role in conjugative mobilization by recognizing an *oriT* site, cleaving at *nic*, covalently attaching to the 5′ nucleotide, and initiating movement of the single strand into the recipient bacterium. Upon completion of the transfer, the reverse reaction of the relaxase (ligation) is believed to recircularize the molecule (23, 44). From an evolutionary perspective, one can easily envision how two coresident plasmids with similar *nic* sites could take advantage of the recombination potential of a related relaxase to generate a cointegrate structure, such as that involving pAMα1Δ1 and pAMα1Δ2 coming together to generate pAMα1. Indeed, an earlier example of this has been reported by Gennaro et al. (18) whereby two small resistance plasmids, pT181 and pE194, in *S. aureus*, could form a cointegrate structure by a RecA-independent, recombination event facilitated by a plasmid-encoded protein designated Pre (for plasmid recombination). The activity involved specific RSs, RSa and RSb, located on the different plasmids and having 24-bp core sequences that were identical. The Pre protein was later found to correspond to a Mob (relaxase) protein based on sequence similarity, and the RSs corresponded to *oriT* sites (44).

Sequence analyses of antibiotic resistance elements continue to reveal the degree to which plasmids, transposons, insertion sequences, and integrons have interacted, as new combinations of determinants evolve and amplify via horizontal transfer through the bacterial world. The growing evidence that proteins relating to the conjugation process (e.g., those encoding relaxase) and related *oriT* sites can participate directly in the recombination process further illustrates the number of genetic tools available to bacteria in their constant effort to survive and evolve.

Nucleotide sequence accession number. The nucleotide sequence of pAMα1 has been assigned the GenBank accession number AF503772.

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