

The Very Large Amplifiable Element AUD2 from *Streptomyces lividans* 66 Has Insertion Sequence-Like Repeats at Its Ends

CHRISTA EICHENSEER AND JOSEF ALTENBUCHNER*

Institut für Industrielle Genetik, Universität Stuttgart, D-70569 Stuttgart, Federal Republic of Germany

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In a spontaneous, chloramphenicol-sensitive (Cm^s), arginine-auxotrophic (Arg^-) mutant of *Streptomyces lividans* 1326, two amplified DNA sequences were found. One of them was the well-characterized 5.7-kb ADS1 sequence, amplified to about 300 copies per chromosome. The second one was a 92-kb sequence called ADS2. ADS2 encoding the previously isolated mercury resistance genes of *S. lividans* was amplified to around 20 copies per chromosome. The complete ADS2 sequence was isolated from a genomic library of the mutant *S. lividans* 1326.32, constructed in the phage vector λ EMBL4. In addition, the DNA sequences flanking the corresponding amplifiable element called AUD2 in the wild-type strain were isolated by using another genomic library prepared from *S. lividans* 1326 DNA. Analysis of the ends of AUD2 revealed the presence of an 846-bp sequence on both sides repeated in the same orientation. Each of the direct repeats ended with 18-bp inverted repeated sequences. This insertion sequence-like structure was confirmed by the DNA sequence determined from the amplified copy of the direct repeats which demonstrated a high degree of similarity of 65% identity in nucleic acid sequence to IS112 from *Streptomyces albus*. The recombination event leading to the amplification of AUD2 occurred within these direct repeats, as shown by DNA sequence analysis. The amplification of AUD2 was correlated with a deletion on one side of the flanking chromosomal region beginning very near or in the amplified DNA. Strains of *S. lividans* like TK20 and TK21 which are mercury sensitive have completely lost AUD2 together with flanking chromosomal DNA on one or both sides.

Chromosomal DNA rearrangements at high frequencies are responsible for the genetic instability observed in the genus *Streptomyces* (for reviews, see references 5, 13, 14, and 22). Deletions of more than 10% of the chromosome lead to an irreversible loss of certain species-specific traits. Sometimes the deletions are accompanied by DNA amplifications. Specific DNA sequences or variable sequences from a certain chromosomal region are amplified by up to several hundred copies per chromosome. According to Hütter and Eckhardt (13), there are two classes of amplifiable elements (amplifiable units of DNA [AUDs] [7]). Elements belonging to class II are flanked by long repeated sequences in the same orientation (direct repeats [DRs]) of 800 to 2,200 bp. The amplified DNA sequences (ADSs) consist of tandemly repeated units of one copy of the DR and one copy of the sequence flanked by the DRs. Class I amplifications arise within the same chromosomal region but differ in size and endpoints. Only a few, if any, directly or inverted repeated base pairs are found at the ends of the sequence used for amplification. Both types of amplifications are accompanied by deletions on one side of the amplified DNA. The deletions always occur on the same side of the amplified DNA. So far, it is unknown whether elements of the two classes amplify by the same mechanism, which enzymes and DNA structures are involved in the amplification process, and how it is correlated to the deletions.

Streptomyces lividans is especially suitable for studying amplification processes. This species segregates chloramphenicol-sensitive (Cm^s) mutants at an average frequency of 0.5% and in a second step arginine-auxotrophic mutants (Arg^-) at a frequency of about 25% that of germinating spores (1). The mutants have extensive chromosomal deletions, and more than

90% of the double mutants show the amplification of a 5.7-kb sequence to several hundred copies per chromosome. This well-characterized amplifiable sequence called AUD1 is a class II element.

Some of the *S. lividans* double mutants show further amplifications in addition to ADS1 (3). An interesting amplification was observed in *S. lividans* 1326.32, a Cm^s Arg^- mutant of *S. lividans* 1326. The DNA sequence, called ADS2 (and the corresponding nonamplified sequence AUD2), was amplified to about 20 copies per chromosome and is one of the largest amplifications described for *Streptomyces* species. It was shown that the mercury resistance genes cloned from *S. lividans* 1326.32 were encoded by ADS2 (23). Therefore, it is one of the rare amplifiable DNAs on which functional genes were detected. A further interesting aspect was the correlation between AUD2 and the endogenous *S. lividans* plasmid SLP3. This plasmid could be demonstrated only by its pock-forming activity on strains without SLP3 but was never isolated (12). Curing of this pock-forming activity by protoplast formation and regeneration of the mycelium led to the simultaneous loss of mercury resistance (23).

Below, we describe the cloning of this unusually large ADS, the characterization of the sequences flanking the element in the wild type, and the rearrangements observed after amplification of AUD2 and the loss of mercury resistance.

Cloning and characterization of ADS2 DNA. A genomic library was constructed from total DNA of *S. lividans* 1326.32 (for the phages, plasmids, and bacterial strains used, see Table 1). The DNA was partially digested with the restriction enzyme *Sau3AI*, with fragments in the range of 14 to 18 kb isolated from a low-melting-point agarose gel (21), ligated to the purified arms of λ EMBL4, and packaged *in vitro* (10). The vector was cleaved earlier with *Bam*HI and also with *Sal*I to remove the replacement fragment of the vector. To identify ADS2 DNA in the genomic library, plaques obtained on *Escherichia coli* NM538 were hybridized with genomic DNA of

* Corresponding author. Mailing address: Institut für Industrielle Genetik, Universität Stuttgart, Allmandring 31, D-70569 Stuttgart, Federal Republic of Germany. Phone: 49 (711) 685-6972. Fax: 49 (711) 685-6973.

TABLE 1. Bacterial strains, plasmids, and phages used in this study

Strain, plasmid, or phage	Relevant characteristics	Reference
<i>S. lividans</i> strains		
1326	Wild type, Mer ^r ; plasmid SLP2, SLP3	12
1326.32	Cm ^s Arg ⁻ Mer ^r ; ND ^a	23
TK19	Wild type, Mer ^r ; plasmid SLP3	12
TK20	Wild type, Mer ^s ; plasmid SLP2	12
TK21	Wild type, Mer ^s	12
TK64	<i>pro-2 str-6</i> Mer ^s	12
<i>E. coli</i> strains		
JM109	<i>recA1 endA1 supE44 relA1 hsdR17 gyrA96 thi Δ(lac-pro)</i> ; F' [<i>traD36 proAB⁺ lacI^q lacZΔM15</i>]	26
NM538	<i>supF hsdR trpR lacY</i>	8
Plasmids		
pIC19H	<i>E. coli</i> cloning vector; <i>bla lacZα</i>	15
pJOE756	5.7-kb <i>Bam</i> HI fragment (ADS1) inserted into pBR325	2
pJOE1364	1-kb <i>Sac</i> I fragment from λRU10 inserted into pIC19H	This work
pJOE1389	1.3-kb <i>Eco</i> RI- <i>Bgl</i> II fragment from λRU26.2 inserted into pIC19H	This work
pJOE1402	3.3-kb <i>Eco</i> RI- <i>Bgl</i> II fragment from λRU1 inserted into pIC19H	This work
pJOE1416	5-kb <i>Bam</i> HI fragment from λRU23.4 inserted into pIC19H	This work
pJOE1440	4-kb <i>Bam</i> HI fragment from λRU2 inserted into pIC19H	This work
pJOE1457	5.5-kb <i>Bgl</i> II- <i>Bam</i> HI fragment from λRU30.2 inserted into pIC19H	This work
pJOE1458	1.73-kb <i>Bgl</i> II fragment from λRU29.4 containing the left end of AUD2 inserted into pIC19H	This work
pJOE1459	1.69-kb <i>Eco</i> RI- <i>Bgl</i> II fragment from λRU23.4 containing the amplified DR of ADS2 inserted into pIC19H	This work
pJOE1460	1.45-kb <i>Eco</i> RI- <i>Bam</i> HI fragment from λRU30.2 containing the right end of AUD2 inserted into pIC19H	This work
pJOE1610	948-bp <i>Eco</i> RI- <i>Bam</i> HI fragment from pJOE459 inserted into pIC19H	This work
pJOE1611	400-bp <i>Hind</i> III fragment from pJOE460 inserted into pIC19H	This work
pJOE1612	440-bp <i>Sal</i> I- <i>Eco</i> RV fragment from pJOE458 inserted into pIC19H	This work
pJOE1801	1.1-kb <i>Eco</i> RI fragment from λRU29.4 inserted into pIC19H	This work
Bacteriophages		
λEMBL4	Replacement vector	8
λRU1, λRU2, λRU4, λRU5, λRU7, λRU9, λRU20, λRU21, λRU23.4, λRU26.2, λRU26.5	λEMBL4 containing 14–18-kb <i>Sau</i> 3A fragments from <i>S. lividans</i> 1326.32	This work
λRU29.1, λRU29.4, λRU30.2	λEMBL4 containing 14–18-kb <i>Sau</i> 3A fragments from <i>S. lividans</i> 1326	This work

^a ND, plasmid not determined.

S. lividans 1326.32 labeled with [α -³²P]dCTP by using a random-primed labeling kit (Boehringer GmbH). Hybridization conditions were as described previously (11). Because of the higher copy number of ADS2 in the genomic DNA, recombinant phages containing ADS2 DNA were expected to give stronger signals on the autoradiogram than phages containing nonamplified chromosomal DNA. Four different types of phages were obtained. Of 200 phages tested, a minority (9 phages) gave no signal on the autoradiogram and probably contained only vector DNA (not tested), some gave low-level signals (126 phages) and were supposed to contain single-copy genomic DNA (not tested), and the remaining phages gave strong and very strong signals. Those which gave the very strong signals (33 phages) carried ADS1 sequences, as shown by hybridization with the plasmid pJOE756 containing the 5.7-kb ADS1 fragment, whereas the others with less intense signals were expected to contain ADS2 sequences. From 20 recombinant phages of the less intense type, DNA was purified and mapped with the restriction enzymes *Bam*HI, *Bgl*II, *Eco*RI, *Pae*R7, and *Sac*I. The inserts mapped into two clusters represented by the phages λRU1, λRU4, λRU5, λRU9, λRU20, λRU21, λRU2, and λRU10 (cluster I) and λRU7

(cluster II). Subcloning of a 1-kb *Sac*I fragment from λRU10 into pIC19H (pJOE1364) and plaque hybridization with this plasmid led to the isolation of λRU23.4 and λRU25.1, giving an overlap between cluster I and II on one side. By subcloning of a 3.3-kb *Eco*RI-*Bgl*II fragment from λRU1 (the *Eco*RI site derived from the vector λEMBL4) into pIC19H (pJOE1402) and screening the genomic library with this plasmid, the phage λRU26.2 was obtained. Finally, the phage λRU26.5 was obtained with a 1.3-kb *Eco*RI-*Bgl*II fragment from λRU26.2 (pJOE1389). Now, the two clusters overlapped on both sides and gave a head-to-tail arrangement of the amplified units (Fig. 1).

Cloning the ends of AUD2. To identify the ends of AUD2, total DNA from *S. lividans* 1326 and the mutant 1326.32 was digested with *Bam*HI, separated on an agarose gel, and blotted on nitrocellulose filters (24). The filters were separately hybridized with labeled DNAs of phage λRU7, λRU26.5, λRU1, λRU5, λRU20, λRU21, λRU2, and λRU23.4. Only with phage DNAs from λRU2 and λRU23.4 was a difference in the hybridization patterns of *S. lividans* wild-type and mutant DNAs seen. Therefore, these phages were supposed to contain the junction generated by the amplification of AUD2. A 5-kb

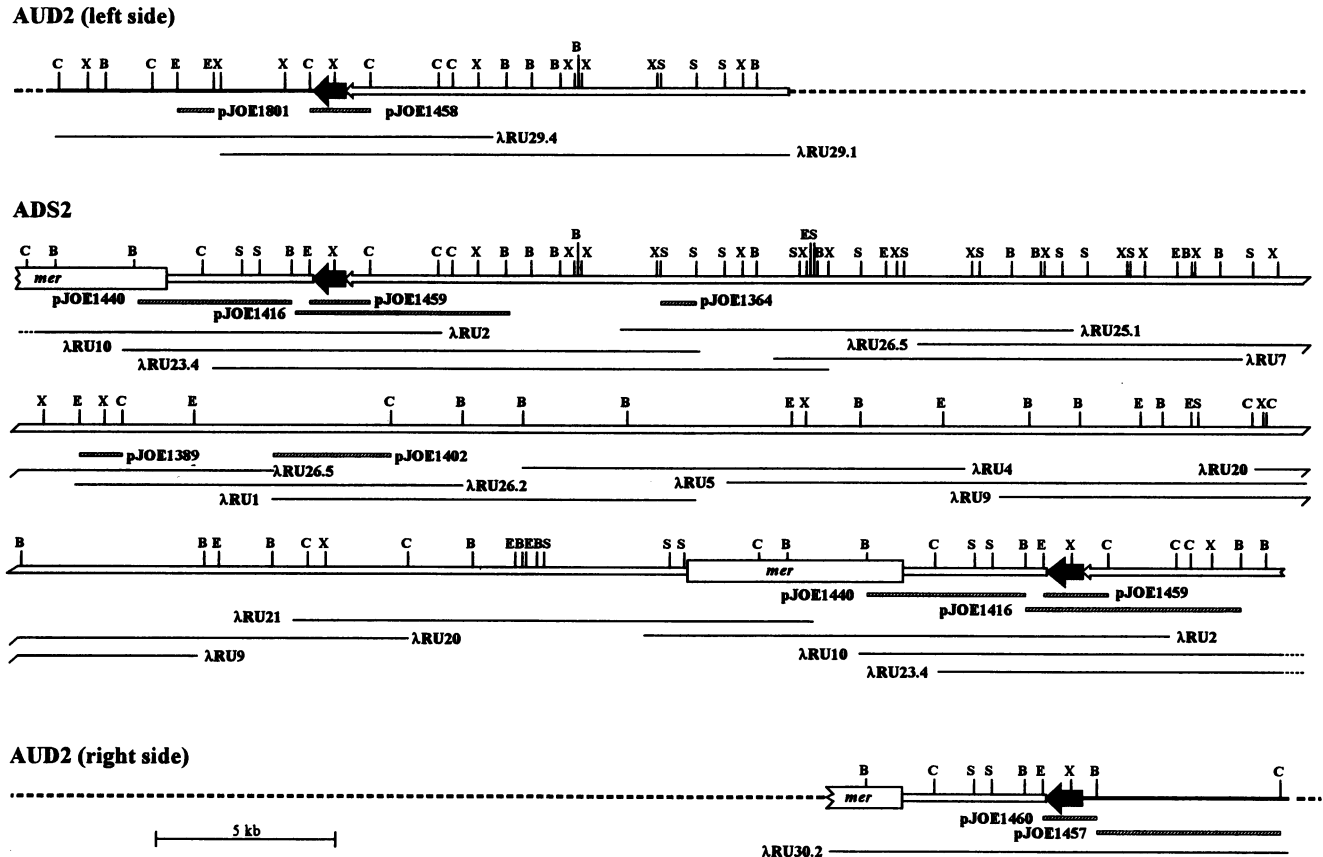


FIG. 1. Restriction map of ADS2 and the left and right ends of AUD2. The ADS2 copy is partly repeated in a head-to-tail arrangement. The 846-bp DRs at the ends of AUD2 and at the junctions of the ADS2 copies (filled arrows), the remaining AUD2 and corresponding ADS2 sequences (open arrows), and the flanking chromosomal DNA (thin bars) are indicated. The positions of the mercury resistance genes (*mer*) encoded by AUD2 and ADS2 are indicated by open rectangles. The sizes and positions of the fragments obtained from the various recombinant λ phages (thin lines) and the fragments subcloned from the phages into the plasmid pIC19H (hatched bars) are depicted below the map. Restriction enzyme abbreviations: B, *Bam*HI; C, *Bgl*II; E, *Eco*RI; S, *Sac*I; X, *Xho*I.

*Bam*HI fragment from λ RU23.4 was inserted into pIC19H (pJOE1416) and used for plaque hybridization of a genomic library prepared from the *S. lividans* 1326 wild type in λ EMBL4. In this manner, the phages λ RU29.1 and λ RU29.4 containing one end of AUD2 (defined as the left side of AUD2) were obtained. A 4-kb *Bam*HI fragment from λ RU2 was inserted into plasmid pIC19H (pJOE1440) and used to isolate λ RU30.2 from the genomic library of the *S. lividans* 1326 wild type. The insert of this phage contained the right end of AUD2. Comparison of the restriction maps of λ RU29.4, λ RU23.4, and λ RU30.2 allowed a more precise mapping of the borders of AUD2 and the junction in the amplified DNA to a 1.73-kb *Bgl*II fragment in λ RU29.4, a 1.69-kb *Eco*RI-*Bgl*II fragment in λ RU23.4, and a 1.45-kb *Eco*RI-*Bam*HI fragment in λ RU30.2. The fragments were inserted into the vector pIC19H, and the resulting plasmids pJOE1458 (left side of AUD2), pJOE1459 (junction band in ADS2), and pJOE1460 (right side of AUD2) were mapped. The restriction maps displayed in Fig. 2 show a region of about 900 bp identical in all three fragments.

DNA sequence analysis of the ends of the DRs from AUD2 and ADS2. The identities of the restriction maps of pJOE1458, pJOE1459, and pJOE1460 in a region of about 900 bp indicated the presence of DRs. To determine the size of the DRs and their structural features, parts of the plasmids were

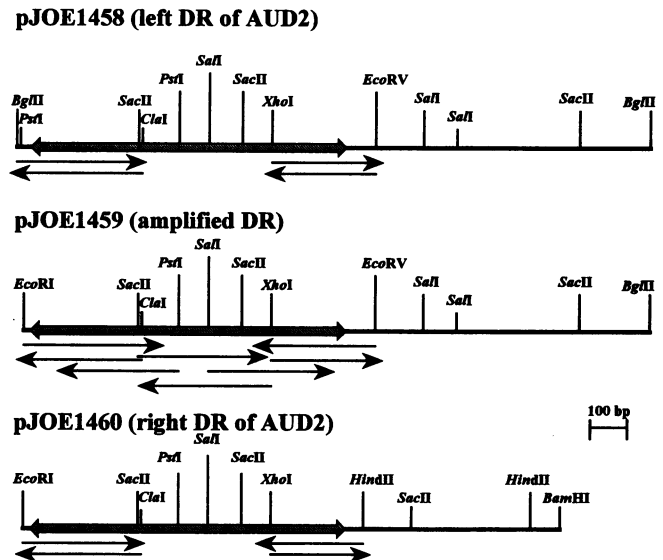


FIG. 2. Restriction map and nucleotide sequencing strategy of the fragments containing the DRs from the left and right sides of AUD2 and the junction in ADS2. The localization of the DRs is indicated on the map by double-headed arrows. The sequenced DNA strands are represented by arrows below the maps.

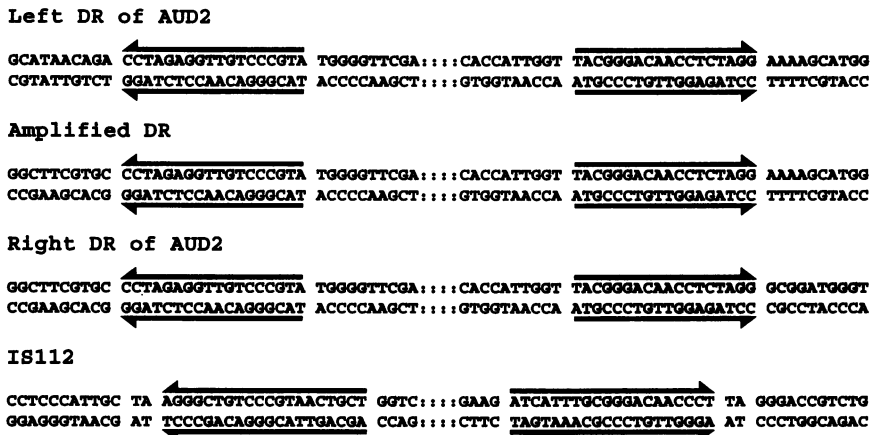


FIG. 3. Nucleotide sequences around the ends of the DRs from the left and right sides of AUD2 and from the junction in ADS2. For comparison, the ends of *IS112* and the target site in the *salR* gene are displayed. The IRs of the elements are indicated by half arrows, and the two base pairs duplicated by insertion of *IS112* into *salR* are separated from the other bases.

sequenced on an A.L.F. Sequencer (Pharmacia Biotech GmbH) as described previously (17). The sequencing strategy is shown in Fig. 2. According to the sequencing data, the DRs were about 850 bp long. At the ends of the DRs, an 18-bp inverted repeated sequence (IR) was found (Fig. 3). Inside the IRs (about 250 bp was sequenced from each side), the three copies were completely identical. Short IRs are usually present at the ends of insertion sequences (IS). Indeed, a high degree of similarity was found between the IRs of the elements flanking AUD2 and the IRs of *IS112* from *Streptomyces albus* (19). The similarity in nucleotide sequence between these elements continued inside the IRs (as described below).

Another feature of IS elements and transposons is the duplication of short 2- to 11-bp-long sequences on the target DNA generated by the element at the integration site (9). For *IS112*, two base pair duplications were found (19). Such duplications were not present at the ends of the DRs from AUD2. Assuming that the DRs are functional IS elements, it might be that the transposase made blunt ends on the target sequence, like *IS91* (6), or that the duplicated base pairs were lost by later deletions. Another reason might be that the outer ends of the IRs are not part of the element but are generated by the transposase. With the nucleotides TA at positions 3 and 4, the transposase would double 2 bp, like *IS112* (19), or with the nucleotides CTAG at positions 2 to 5, 4 bp of the target DNA would be doubled.

Another interesting aspect was the DNA sequences flanking the DR obtained from the amplified DNA. One side was identical to the nucleotide sequence following the right DR of AUD2, and the other was identical to the sequence following the left DR of AUD2 (Fig. 3). This means that the first recombination leading to the amplification of AUD2 had taken place within the two DRs.

Complete nucleotide sequence of the DR from ADS2. The DNA sequence analysis of the ends of the DRs of AUD2 and ADS2 indicated a close similarity to *IS112* from *S. albus*. To see whether this similarity to *IS112* is present throughout the whole length of the DRs, a 948-bp *EcoRI-EcoRV* fragment from pJOE1459 containing the DR from ADS2 was completely sequenced on both strands. The DNA sequence is shown in Fig. 4. The DR inclusive of the IRs at the ends is 846 bp long. This is slightly smaller than *IS112*, with 883 bp. The element has an overall 65% identity in nucleic acid sequence to *IS112*. An open reading frame with two possible ATG start codons at

nucleotide positions 111 and 132 (Fig. 4) near the inside end of one IR and a stop codon at position 897 near the inside end of the other IR covers nearly the complete element. A codon usage analysis of the open reading frame showed biased codon

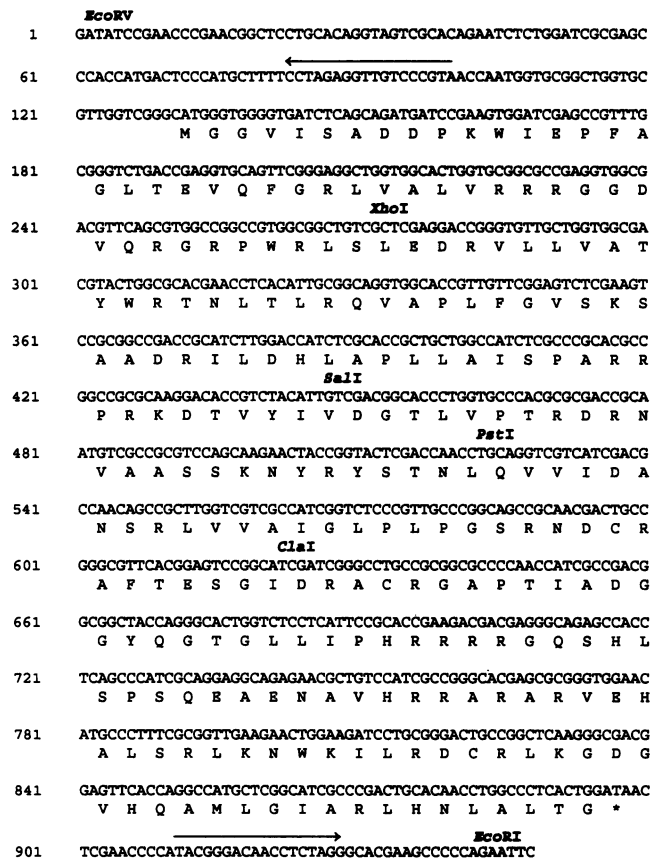


FIG. 4. Nucleotide sequence of the 948-bp *EcoRV-EcoRI* fragment from plasmid pJOE1459 containing the amplified 846-bp DR. The perfect 18-bp IRs are shown by arrows above the sequence, and the potential protein product encoded by the DR is given below the corresponding DNA sequence. Base pairs are numbered at the left. *, stop codon.

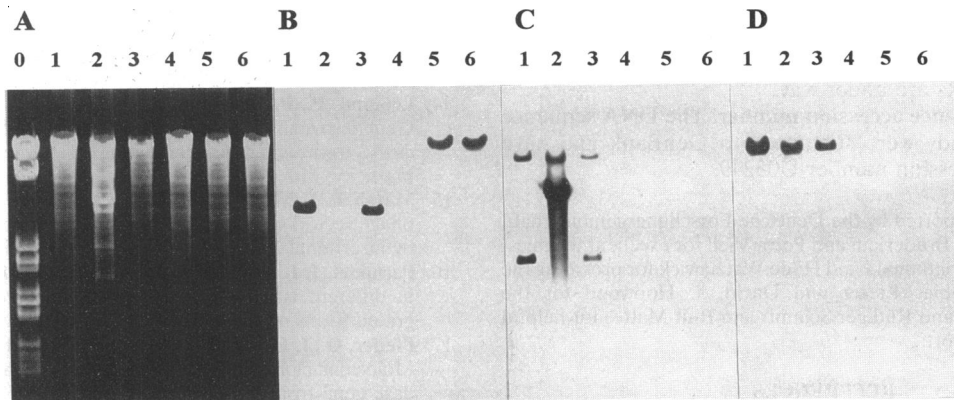


FIG. 5. Agarose gel electrophoresis of genomic DNAs from *S. lividans* 1326 and derivatives digested with *Bgl*II (A) and Southern blots hybridized with the plasmids pJOE1801 (B), pJOE1459 (C), and pJOE1457 (D). Lanes: 0, λ *Bgl*II length standard; 1, *S. lividans* 1326; 2, *S. lividans* 1326.32; 3, *S. lividans* TK19; 4, *S. lividans* TK20; 5, *S. lividans* TK21; 6, *S. lividans* TK64.

usage, with 67% GC at the first position, 49% GC at the second, and 82% GC at the third, typical for *Streptomyces* genes. The predicted amino acid sequence has 62% identity to the putative transposase from IS112. The similarity begins at the second possible start codon. With this start site, the two deduced proteins have nearly identical sizes (255 versus 256 amino acids). Neither of the two possible start codons of the open reading frame is preceded by a sequence fitting the consensus sequence of a *Streptomyces* ribosomal binding site or a promoter sequence (25). For these reasons, the second start site was chosen as the most likely one. The larger size of IS112 was due mainly to an additional 43 bp found in IS112 between the C-terminal end of the presumptive transposase gene and the IR.

Analysis of deletions in *S. lividans* 1326.32 and some mercury-sensitive strains. To see whether the amplification of AUD2 is correlated with a deletion on one side of the amplified DNA, DNA fragments were isolated from both sides flanking AUD2 and hybridized to chromosomal DNA of the Cm^s Arg^- mutant *S. lividans* 1326.32. Furthermore, it was interesting to see what happened in the strains *S. lividans* TK20 and TK21 which are sensitive to mercury ions after curing of the plasmid SLP3. Total DNAs of *S. lividans* 1326.32, TK20, and TK21 and (as controls) DNAs of the *S. lividans* 1326 wild type, TK19 (the mercury-resistant progenitor strain to TK21), and TK64 (the Str^r Pro^- derivative of TK20) were digested with *Bgl*II and blotted on nitrocellulose filters after agarose gel electrophoresis (24). The filters were hybridized with the plasmids pJOE1801 containing a 1.1-kb *Eco*RI fragment from outside the left end of AUD2, pJOE1459 with the amplified 846-bp DR of ADS2, and pJOE1457 with a 5.5-kb *Bam*HI-*Bgl*II fragment from outside the right end of AUD2. The results are shown in Fig. 5. The plasmid pJOE1801 hybridized to a 4.2-kb *Bgl*II fragment of the *S. lividans* 1326 DNA as expected on the basis of the restriction map. No hybridization was found with the Cm^s Arg^- mutant, indicating a deletion on the left side of ADS2. No difference in hybridization patterns of the wild type and mutant was seen with pJOE1457 hybridizing to the right side of AUD2. The results were confirmed with the plasmid pJOE1459 which hybridizes to the two AUD2 end fragments (1.73- and 9.3-kb *Bgl*II fragments) in the wild-type DNA. The 9.3-kb fragment from the right side of AUD2 was still present in the mutant, but the 1.73-kb band was replaced by an intensive band of 4.6 kb, the junction band generated by amplification of AUD2. The fact that the 1.73-kb

*Bgl*II fragment ends just 74 bp outside AUD2 means that the deletion ended immediately before or within the amplified DNA.

The mercury-resistant strain TK19 cured for the plasmid SLP2 showed no difference from the parental strain 1326 in hybridization to the three plasmids. The mercury-sensitive strain TK20, cured for SLP3, showed a deletion of AUD2 and both flanking regions, because none of the three plasmids hybridized. The mercury-sensitive strain TK21, independently isolated from TK19 and cured for SLP2 and SLP3, as well as the derivative TK64 showed a large deletion, too. But, in these strains the plasmid pJOE1801 still hybridized. The 4.2-kb *Bgl*II fragment flanking AUD2 on the left side was replaced by a fragment of more than 10 kb. The deletion removing the complete AUD2 and DNA of an unknown size from the right flanking chromosomal region has one of its endpoints within the 4.2-kb *Bgl*II fragment.

An explanation for the simultaneous loss of AUD2 and SLP3 might be that SLP3 is one of the integrative plasmids like pSAM2 or pSLP1 (4, 16) inserted on the right side of AUD2 and the curing in TK20 and that in TK21 were actually large chromosomal deletions removing both elements.

Conclusions. AUD2 shows the typical features of a class II amplifiable element. It has long DRs at its ends, and a deletion occurs on one side when the element is amplified. However, so far the amplification of AUD2 has been observed only once. Twelve more independently isolated Cm^s Arg^- mutants of *S. lividans* 1326 had only an amplification of AUD1 but not of AUD2 (data not shown). For a final association of AUD2 to the class II amplifiable elements, the reproductivity of the amplified structure has to be shown.

Analysis of the DNA flanking the amplified 846-bp DR showed that the first recombination event leading to amplification of AUD2 occurred between the two flanking DRs. The mechanism of amplification might be an unequal crossover recombination or a trapping of the chromosomal replication fork by recombination at the DRs after the replication fork has passed the first DR (27).

AUD2 is the first example of an amplifiable element in streptomycetes with IS element-like sequences at its end. To our knowledge, DNA sequences of DRs of class II elements are available only from AUD1 of *S. lividans* (18). In this case, the 1-kb DRs have no IS element-like structures but encode a putative regulatory protein with similarities to the *lacI* repressor gene.

With an IS-like element at each end, AUD2 resembles composite transposons like Tn9 (20) and might be a large mercury resistance transposon. Experiments to test for transposition of the DRs are under way.

Nucleotide sequence accession number. The DNA sequence data from this study were submitted to GenBank and have been assigned accession number U05249.

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