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Molecular Characterization of a Deletion-Prone Region of Plasmid pAE1 of *Alcaligenes eutrophus* H1

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A 93-kb region (D region) of plasmid pAE1 of *Alcaligenes eutrophus* H1 has been found to have a high rate of spontaneous deletion. In this study, we constructed a restriction endonuclease map and carried out limited sequencing of an approximately 100-kb region from pAE1 which includes the D region (the deleted region) in order to detect and characterize repetitive sequences. Two types of repetitive sequences, the R1 and R2 sequences, were observed to flank the D region; within the D region are three copies of insertion element ISAE*1*. The R1 and R2 sequences are arranged in direct and inverted orientations, respectively. Molecular analysis of the end product of the deletion is consistent with the hypothesis that the loss of the D-region DNA is the result of recombination between two copies of the R1 sequence. The R1 sequence encodes a 415-amino-acid protein which exhibits substantial sequence similarity to the lambda integrase family of site-specific recombinases. Its genetic function remains to be determined.

Alcaligenes eutrophus is a gram-negative facultative chemolithoautotrophic (autotrophic) bacterium which can oxidize hydrogen gas for carbon dioxide fixation. Large plasmids (350 to 450 kb) carrying the structural genes for the two hydrogenases, the membrane-bound particulate hydrogenase (*hoxP*) and the NAD^+-reducing soluble hydrogenase (*hoxS*), have been determined to be present in several *A. eutrophus* strains (7, 13, 22). The plasmid from strain H16, pHG1, has been reported to carry genes necessary for carbon dioxide fixation (*cfx* genes). The *cfx* genes are adjacent to the *hox* gene clusters and have a duplicated copy situated on the chromosome (11). An estimated total of 100 kb of pHG1 DNA is required to encode genes involved in the autotrophic growth of *A. eutrophus* H16.

Similar to pHG1, the large plasmid of *A. eutrophus* H1, pAE1, carries the genetic determinants of autotrophic growth (4, 14, 22). Several variants of pAE1 that were smaller in size were observed in $HoxP^-$ mutants generated by Tn*5* mutagenesis, e.g., strain WW1-12. A similar deletion of at least 50 kb of pAE1 DNA has also been observed in strain H1-4, which is a mitomycin-generated $HoxS^-$ derivative of strain H1, as well as other Tn*5*-carrying strains without an obvious defect in autotrophic growth (4, 22). This frequently found deleted region is designated the D region of pAE1 (4). The aim of this work was to determine the extent and physical organization of the D region.

Construction of the restriction endonuclease map of the D region. Southern hybridization analysis has indicated that the D region contains three copies of insertion element ISAE*1* (14). Therefore, the ISAE*1*-containing DNA fragments from pAE1 were used as probes to screen an *A. eutrophus* genomic library constructed by cloning partially *Sau*3A-digested DNA of strain H1 into the *Bam*HI site of Charon 40 DNA (6). The restriction endonuclease map of an approximately 100-kb pAE1 DNA was established by aligning the overlapping clones obtained by DNA walking (Fig. 1A). Most of the probes selected for DNA walking were unique; however, each of the p1 and p18 fragments, which contained no internal *Eco*RI recognition site (Fig. 1), hybridized with two *Eco*RI fragments of strain H1 genomic DNA under a hybridization condition that would allow less than 10% mismatching (Fig. 2). p1 and p18 could produce no hybridization signal with the DNA of a plasmid-cured strain, H1-6 (22), indicating that the redundant hybridization bands for either p1 or p18 were from pAE1. Interestingly, the patterns of hybridization with p1 and p18 differed between DNA of strain H1 and DNA of two plasmid deletion strains, H1-4 and WW1-12 (Fig. 2). A 12.5-kb *Bam*HI hybridization band, which contained two DNA fragments of similar sizes, of strain H1 was missing from the genomic DNA of strain H1-4. Instead, an 8.5-kb *Bam*HI band of strain H1-4 DNA hybridized with p1 and p18. A previous comparison of the *Bam*HI-digested pattern of pAE1 with that of the plasmid of strain H1-4, pAE1-4, revealed that pAE1-4 loses at least three *Bam*HI fragments, i.e., fragments of 15, 12.5, and 10.8 kb, and contains an 8.5-kb fragment (4). The size of this 8.5-kb *Bam*HI fragment of pAE1-4 matched that of the p1- and p18-hybridizing *Bam*HI fragment of strain H1-4. This result implied that the 8.5-kb *Bam*HI DNA of pAE1-4 resulted from the joining of the two partially deleted 12.5-kb *Bam*HI DNA fragments of pAE1.

The similar but not identical hybridization patterns generated by p1 and p18 suggested that p1 and p18 contained homologous DNA sequences. Indeed, p1 and p18 hybridized with each other and with the same recombinant phages, which could be classified into two groups by their restriction endonuclease digestion patterns. The DNA fragments hybridized with either p1 or p18 could be observed in only one of the two groups of the recombinant phages (data not shown). These results are consistent with the established restriction endonuclease map shown in Fig. 1A. The probes taken from DNA upstream and downstream (to the left and the right of p18 and p1, respectively) gave rise to identical hybridization patterns between strains H1 and H1-4, indicating that these probes were outside the D region (data not shown). The p3 fragment, derived from

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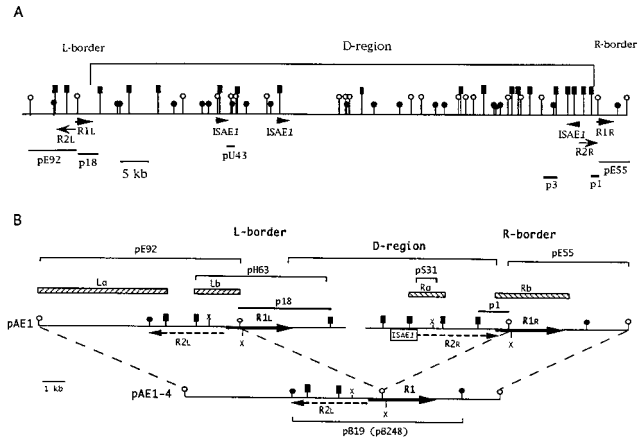

FIG. 1. Restriction endonuclease maps of the D region and its flanking DNA (A) and the L and R borders and the new fusion fragment, pB19 (B). Also shown are some of the cloned fragments used in this study. |, |, |, and X represent the *Bam*HI, *Eco*RI, *Hin*dIII, and *Xho*I recognition sites, respectively. Not all the *Xho*I recognition sites are shown; only the two *Xho*I sites mentioned in the text are indicated. DNA fragments smaller than 300 bp may not be included in the map. The orientations of ISAE*1* are indicated by arrowheads, where the arrowhead points to the R end of the element (14); the orientations of the R1 and R2 sequences are indicated by arrows. The direction of the arrow denotes the 3′ end of the R1 sequence shown in Fig. 3A. ▨ and ▨, the smallest DNA fragments known to hybridize with pS31 and pH63, respectively.

the same 11.5-kb *Eco*RI fragment as p1 was, hybridized only with the DNA of strain H1; thus, it was mapped to the D region (Fig. 1A and 2). In summary, the aforementioned hybridization results suggested that p1 and p18 were situated on the two borders of the D region and that p1 and p18 contained a repetitive sequence. This repetitive sequence was named the R1 sequence. The areas from which p1 and p18 were derived were defined as the R and L borders of the D region, respectively. The two R1 sequences located at the L and R borders were named R1L and R1R, respectively.

Characterization of the R1 sequence. To determine the smallest DNA fragment containing the R1 sequence, pH63 of the L border was hybridized with the R-border DNA. These results are summarized in Fig. 1B, showing that two separate regions (R_a and R_b) of the R-border DNA hybridized with pH63. The 3.5-kb R_b region that overlapped with the p1 fragment most likely contained the R1R sequence. The DNA sequences of the R_b region and the corresponding region of pH63 were determined and compared. The size of the R1 sequence is 3,122 bp if several mismatches at the ends are included (Fig. 3A), whereas it is 3,102 bp if the mismatched sequences are excluded. The first 35 bp of the R1R sequence was identical to a putative repetitive sequence, named the R2 sequence, as described below. The DNA sequence analysis confirms that the R1 sequences of pAE1 are direct repeats.

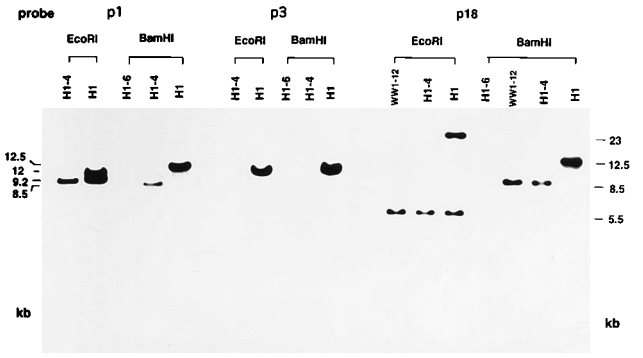

FIG. 2. Southern blots of p1, p3, and p18. [^{32}P]dCTP-labeled probes were hybridized with genomic DNA isolated by a cetyltrimethylammonium bromide extraction procedure (3).

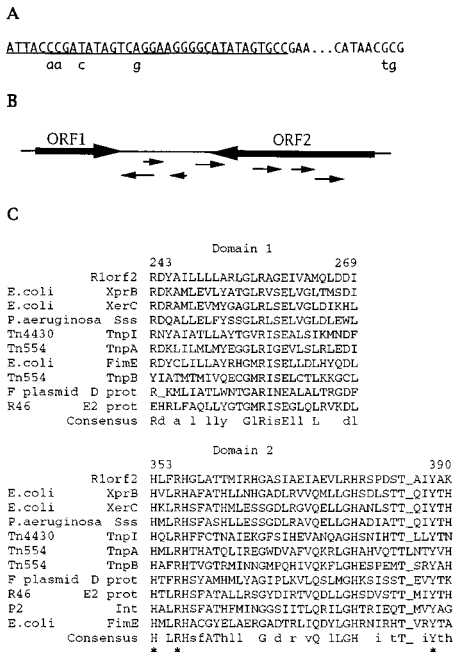

FIG. 3. Sequence analysis of the R1 sequence. (A) Nucleotide sequences of the ends of R1R. The GenBank accession number of a complete R1R sequence is L34580. Lowercase letters indicate the corresponding sequence found in R1L. The underline indicates the portion of the sequence that overlaps with the R2R sequence. (B) Schematic illustration of ORFs. Directions of transcription are indicated by arrows. Small arrows indicate those ORFs shorter than 100 amino acids. (C) Alignment of the predicted protein sequence of ORF2 with those of other site-specific recombinases of the lambda integrase family. Only the two most conserved regions (domains 1 and 2) are shown. Those residues in lowercase type and in uppercase type represent consensus residues present in at least five or eight of the proteins, respectively. Gaps (dashes) were introduced to maximize the homology. Asterisks indicate the absolute conserved residues found in the lambda integrase family (2, 20). The sequences of *E. coli* XprB and *Pseudomonas aeruginosa* Sss are from Lovett and Kolodner (17) and Hofte et al. (10), respectively. The sequence of chromosomally encoded *E. coli cer* site-specific recombinase is from Colloms et al. (5). TnpI is from the *Bacillus thuringiensis* transposon Tn*4430* (18). The TnpA and TnpB are transposition proteins of Tn*554* (19). The D protein (D prot) is a recombinase from the F factor of *E. coli* (16). The sequence of chromosomally encoded FimE recombinase controlling the phase variation of type I fimbriae in *E. coli* is from Klemm (12). The sequence of the integrase of phage P2 is taken from Argos et al. (2). The sequence of E2 protein (E2 prot) of resistance plasmid R46 is from Hall and Vockler (9).

The R1 sequence contained two open reading frames (ORFs) longer than 100 amino acids (Fig. 3B). When the first methionine residue is taken to be the translation initiation codon, ORF1 and ORF2 are predicted to code for 194- and 415-amino-acid polypeptides, respectively. Comparisons of the primary amino acid sequences of the two ORFs with those deposited in the Swiss-Prot and GenBank libraries were made (1). No significant homology existed between the ORF1 product and proteins in the data bank. The ORF2 product displayed significant homology to those proteins which belonged to the lambda integrase family of site-specific recombinases. The ORF2 product showed the highest level of homology (26% amino acid identity and 42% conserved residues) to an *Escherichia coli xprB* gene product over a range of 184 amino

acids (17). The function of XprB is unclear; however, it shows significant homology with several site-specific recombinase proteins (2). The ORF2 product has 32% amino acid identity and 51% conserved residues with the *E. coli* protein XerC in an alignment covering 68 amino acids. The XerC product is required for recombination at the *cer* site of the ColE1 plasmid (5). Figure 3C shows the alignment of the two regions most conserved between the ORF2 amino acid sequence and the sequences of other site-specific recombinases. The conserved carboxy-terminal region (domain 2) of the predicted ORF2 sequence contains the conserved -His-X_2-Arg- residues and an appropriately spaced tyrosyl residue which has been suggested to be the active site of the integrase family of site-specific recombinases (2, 20). The remaining ORFs of the R1 sequence, with coding potential of less than 100 amino acids, were also compared with the data bank. The results revealed no significant homology between the amino acid sequences of these shorter ORFs and those of known transposases. The ends of the R1 sequence and its neighboring DNA contain no typical short inverted or direct repeats found in most prokaryotic transposable elements (8). Thus, despite the fact that the product of ORF2 is homologous to resolvases of several prokaryotic transposons, the R1 sequence is unlikely to be a transposon. The origin of the R1 sequence remains unknown.

DNA homologous (less than 10% mismatches) to the R1 sequence could be detected in the genomes of *A. eutrophus* strains CH34 and H16, whereas such DNA was not detected in *A. eutrophus* ATCC 17700, *Alcaligenes denitrificans* (ATCC 15173 and ATCC 15749), or *Alcaligenes hydrogenophilus* (ATCC 33178) by Southern hybridization (data not shown). There were at least two and three copies of the R1 sequence in the genomes of strains H16 and CH34, respectively. Since ATCC 17700 possesses a large plasmid, the lack of R1-related sequence in ATCC 17700 indicates that the distribution of R1 sequence is not as broad as the presence of large plasmids in various *A. eutrophus* strains.

Evidence for the presence of another inverted repeat, the R2 sequence. The homology between R_a and pH63 could not result from the R1 sequence because R_a did not hybridize with the R1 sequence (data not shown). Thus, it was possible that there was another repetitive sequence present in the L and R borders. An ISAE*1*-sequence-free fragment, pS31 (Fig. 1B), derived from the R_a region was used as a probe to determine the distribution of pS31-homologous sequence in the L border. The result of Southern hybridization indicated the presence of two interrupted homologous regions in L-border DNA (L_a and L_b; the data are summarized in Fig. 1B). The L_b region overlapped with the R1L sequence. Thus, pS31 should contain another repetitive sequence named the R2 sequence. The R2 sequences located at the L (L_b) and R borders were designated R2L and R2R, respectively. Limited sequence comparisons were made to locate the R2 sequence. Results of these comparisons are summarized in Fig. 4. The nucleotide sequence upstream of the R1L sequence of the L_b region (site 3) is complementary to the sequence immediately contiguous to the insertion element ISAE*1* (site 4). One terminus of the R1R sequence (site 2) is complementary to the sequence obtained from DNA near the *Bam*HI site of the L_a region (site 1). Sequences obtained from the termini of the *Hin*dIII fragment located between the L_a and L_b regions (sites 1 and 3) are identical to sequences obtained from the termini of the *Hin*dIII fragment located between the R_b and R_a regions (sites 2 and 4). Hence, the arrangement of the R2 repeat is in an inverted orientation. On the basis of the distribution of homologous sequences, the extent of the R2 sequence is temporally assigned (Fig. 1 and 4A). Such a distribution of R2 sequence

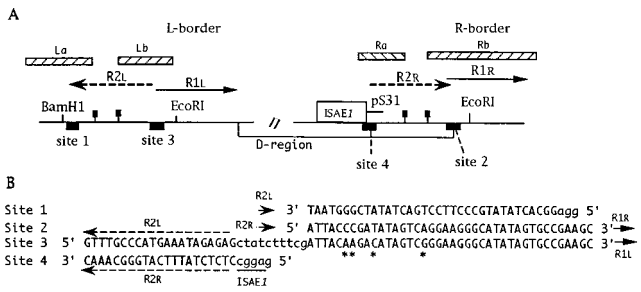

FIG. 4. Comparisons of the nucleotide sequences flanking the R2 sequence. (A) Locations of the sites where sequences shown in panel B were derived. |, *Hin*dIII recognition site. (B) Alignments of nucleotide sequences derived from the four sites shown in panel A. The listed sequences are from the same DNA strand. The sequences of sites 1 and 4 are reversed for easy comparisons, and they are complementary to the sequences derived from sites 2 and 3, respectively. Arrows indicate the directions of repetitive sequences as in Fig. 1. Asterisks indicate the mismatches between R1L and R1R. Lowercase type indicates sequences flanking the R1 and R2 sequences.

accounts for the result observed with pH63, which contains R1L and portions of R2L sequences, hybridizing with two regions of the R-border DNA (Fig. 1B). The sequence of site 1 is unrelated to the pS31 sequence; thus, the nature of the L_a region showing homology to pS31 is currently unknown. However, judging from the distribution of the *Hin*dIII site, the L_a region is unlikely to contain an identical copy of the R2 sequence.

Figure 1A summarizes the distributions and orientations of the known repetitive sequences, including the ISAE*1*, R1, and R2 sequences, present in the D region and its flanking DNA. Nucleotide sequence comparison revealed that the R1L and R2L sequences were separated by 10 bp, whereas R1R and R2R overlapped by 35 bp (Fig. 3A and 4B). The other end of the R2R sequence was directly adjacent to ISAE*1*.

Determination of the extent of deletion in strains H1-4 and WW1-12. The results of hybridization between p1 and p18 fragments and DNA of wild-type and deletion strains suggested that the 8.5-kb *Bam*HI DNA of pAE1-4 may result from the fusion of two physically separated 12.5-kb *Bam*HI DNA fragments of pAE1. To determine the breakage-and-fusion site, the 8.5-kb *Bam*HI DNA fragment was cloned from plasmid DNA of strains H1-4 and WW1-12, and the resulting constructs were named pB19 and pB248, respectively. The distributions of *Eco*RI, *Hin*dIII, *Xho*I, and *Sal*I recognition sites within pB19 and pB248 were identical (data not shown). The two *Eco*RI sites outside the cloned pB19 were determined by Southern hybridization (Fig. 2 and data not shown). A comparison of the restriction endonuclease maps indicated that the distribution of restriction endonuclease sites within pB19 and its surrounding DNA matched that of sites within the pE92 and pE55 fragments of the L and R borders, respectively (Fig. 1B). Furthermore, DNA sequence comparison revealed that DNA sequences obtained from each of the two termini of pB19 and pB248 were identical to those near the *Bam*HI sites of pE92 and pE55 (data not shown). DNA sequences of pB19 and pB248 between the two *Xho*I sites shown in Fig. 1B were identical to that of the L border, revealing that the DNA upstream (to the left) of the R1 sequence in pB19 (pB248) was derived from the L border (pE92). The DNA sequence immediately to the right of the R1 sequence of pB19 was identical to that of the R border (pE55). These results were consistent with the theory that pB19 was the fusion (rearranged) fragment that resulted from the deletion of the D region. The deletion end products for pB19 and pB246 were identical on the basis of

restriction endonuclease mapping and partial DNA sequence comparison. Thus, the extents of deletion in strains WW1-12 and H1-4 are identical.

The sequence analysis clearly indicated that the deletion end product observed for pB19 contained a single copy of the R1 sequence flanked by sequences derived from two physically separated pAE1 DNA fragments located on one side of R1R and on one side of R1L. The DNA deleted from strains H1-4 and WW1-12 includes a copy of the R1 sequence and the DNA between the two R1 sequences (the D region). The predicted end product of recombination between two direct repeats of a plasmid was observed in pB19, supporting the possibility that the deletion of the D region is a result of a recombination between the two R1 sequences (21). The length of the R1 sequence indicates that it is sufficient to act as a substrate recognized by the machinery of homologous recombination. However, the R1 sequence encodes a polypeptide whose C-terminal portion shows substantial homology to those of many proteins of the site-specific recombinase family. We cannot determine whether the deletion is a result of homologous or site-specific recombination between the two R1 sequences.

Deletion of the D region occurred spontaneously. A loss of the D region is frequently observed to occur in the Tn*5*-carrying *A. eutrophus* strain H1. Could the process of Tn*5* transposition or selection for kanamycin-resistant colonies induce the observed deletion? Colony hybridization was performed to estimate the frequency of deletion of the D-region DNA. Tn*5* mutagenesis was performed as previously described (4). Thirty Kan^r colonies from each mutagenic treatment and randomly selected *A. eutrophus* H1 colonies were hybridized with pE37 (4, 15) and with pU43 (Fig. 1A). Theoretically, the loss of hybridization signal with pU43, which was derived from the pAE1 DNA flanked by two copies of ISAE*1* arranged in the same orientation, would suggest a loss of either the 12-kb DNA between two ISAE*1* elements, the D region, or the entire pAE1 DNA. Hybridization with pE37, a pAE1 DNA fragment outside the D region and containing part of the plasmid-encoded *hoxP* locus, was used as an indicator of the presence of pAE1. Losses of hybridization signals with both pE37 and pU43 would suggest that the entire pAE1 DNA was lost. By this criterion, approximately 0.15% of the Kan^r colonies lost the large plasmid. This frequency is much lower than the previously reported 1% plasmid curing rate estimated by the phenotype of a complete loss of ability to grow autotrophically (4). Twelve of 1,318 Kan^r colonies (0.91%) from 44 independent Tn*5* treatments hybridized only with pE37 and not with pU43. This frequency was slightly lower than the 1.36% frequency observed in the controlled population (3 of 221 colonies). The difference became less significant if we included three deletion isolates originally excluded because they were not derived from independent Tn*5* treatments. To ascertain the extents of deletion in all the pU43-negative strains, hybridizations with an R1-specific probe and pH63 were performed. All the deletion strains showed hybridization patterns identical to that of strain H1-4 (data not shown). Thus, all of the plasmid deletions in the 18 newly isolated strains occurred between the R1 sequences and none occurred between the two ISAE*1* elements.

The deletion of the D region could be observed at a high frequency in strain H1 prior to exposure to any mutagenic treatment, suggesting that deletion of the D region can occur spontaneously. In contrast to the high frequency of deletion of the D region, none of the 1,541 colonies that we screened had the predicted end product of deletion which would result from a recombination between two ISAE*1* elements arranged in a direct orientation. It remains unclear as to whether this difference in deletion frequency simply reflects the difference in the length of the substrate or the difference in efficiency between homologous and site-specific recombination.

Deletions of portions of the *hox* gene cluster on pHG1 have been reported to occur frequently among Hox mutants derived from strain H16; however, these deletions have not been fully characterized. Two of the four copies of the 1.3-kb insertion element IS*491* are mapped to the *hox* gene cluster. As a result, IS*491* is suspected to be responsible for the deletion observed in pHG1 (7). The phenotypes of the deletion strains derived from strain H1 vary from $HoxP^-$ (strains WW1-11 and WW1-12) and $HoxS^-$ (strain H1-4) to no detectable defect in autotrophic growth (4). The $HoxP^-$ phenotypes of strains WW1-11 and WW1-12 have been determined to result from Tn*5* transpositions into the structural genes of the particulate hydrogenases (15), whereas the HoxS-deficient phenotype of strain H1-4 has not yet been studied. Although the deletion was not characterized for all of the deletion mutants to the nucleotide level, the fact that all of the deletion mutants had hybridization patterns identical to that of an R1-specific probe and pH63 suggested that the nature of the deletion was the same in all isolated deletion mutants derived from strain H1. The isolation of mutants with identical deletions but with different phenotypes concerning the activities of hydrogenase supports our previous conclusion that the D region of pAE1 is unlikely to contain essential genes involved in autotrophic growth (4).

Since no obvious growth alternation, under either heterotrophic or autotrophic conditions, is known to be associated with the loss of the D region, the function of this 93-kb plasmid region remains to be elucidated. Our preliminary data indicated that there was a weak transcription from portions of the D region (23). It has not yet been determined whether the DNA information of the entire D region is conserved in pHG1 or in the plasmids of other *Alcaligenes* species. However, DNA sequences identical to flanking sequences of ISAE*1* are present in pHG1; thus, at least portions of the D region are conserved in pHG1 (14). Consequently, the presence of D-region DNA on the related plasmid pHG1 is quite likely.

Nucleotide sequence accession number. The nucleotide sequence of R1R has been submitted to GenBank and the EMBL data bank with the accession number of L34580.

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