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NOTES

The *lonD* Gene Is Homologous to the *lon* Gene Encoding an ATP-Dependent Protease and Is Essential for the Development of *Myxococcus xanthus*

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***Myxococcus xanthus* contains two genes (*lonV* and *lonD*) homologous to the *Escherichia coli lon* gene for an ATP-dependent protease. We found that the *lonD* gene encodes a 90-kDa protein consisting of 827 amino acid residues. The *lonD* gene product shows 49, 48, and 52% sequence identity to the products of the *M. xanthus lonV*, *E. coli lon*, and *Bacillus brevis lon* genes, respectively. When a *lonD-lacZ* fusion was used, *lonD* was expressed during both vegetative growth and development. However, while *lonD*-disrupted strains were able to grow normally vegetatively, the development of *M. xanthus* was found to be arrested at an early stage in these strains. The mutant strains were able to form neither fruiting bodies nor myxospores.**

Myxococcus xanthus is a unique gram-negative bacterium that undergoes multicellular development involving a cell-cell interaction (for a review, see reference 16). Upon nutrient depletion on a solid surface, cells aggregate to form mounds in which rod-shaped cells are converted into round or ovoid myxospores. The mounds of mature myxospores are called fruiting bodies.

We previously isolated six developmental mutants of *M. xanthus* defective in fruiting body formation (Fru^-) by screening 855 independent kanamycin-resistant (Km^r) clones mutagenized with Tn*V*, a Tn*5* derivative transposon carrying the replication origin of *Escherichia coli* plasmid pSC101 (5). Mutants 221, 328, and 530 were shown to have Tn*V* insertions within a 0.25-kb region of the *M. xanthus* chromosome. Fifteen-kilobase *Sal*I fragments with Tn*V* insertions were cloned from the chromosomal DNAs of these mutants. As described below, the three insertions were located within a single gene that is essential for fruiting body formation. Here, we show that this gene is homologous to the *E. coli lon* gene and is thus designated *lonD*, for the *lon* gene required for development. We have independently identified another gene homologous to the *E. coli lon* gene; this gene has been shown to be essential for the vegetative growth of *M. xanthus* and is thus designated *lonV* (formerly *lon* [19]).

The *lon* gene of *E. coli* encodes protease La (2, 4), an ATP-dependent protease associated with cellular protein degradation. Mutations in the *E. coli lon* gene result in pleiotropic phenotypes, such as increased sensitivity to UV irradiation and SOS-inducing agents, filament formation, mucoidy, and reduced degradation of various abnormal proteins and certain normal proteins.

For cloning the wild-type allele of the *lonD* gene, a genomic library of *M. xanthus* DZF1 was screened by use of the nick-translated 15-kb *Sal*I DNA fragment (5) as a probe. A positive clone, P576, was identified; from it a 13.5-kb *Eco*RI fragment was subcloned into pUC9 (20) to yield pMXL101. For defining the DNA region of the *lonD* locus, 13 new insertion mutations were constructed as described previously (11), and their developmental phenotypes were tested (Fig. 1). First, pMXL101 DNA was digested with various restriction enzymes and ligated with a 1.3-kb DNA fragment containing the Km^r gene of Tn*5* to generate insertion mutations on pMXL101. P1 incompatibility genes were added to the plasmids, which then were introduced into *M. xanthus* DZF1 by P1 transduction (18). The wild-type *lonD* allele of the chromosome was replaced with mutant alleles by double-crossover recombination (18). In each case, replacement-type transductants were selected by screening for Km^r transductants by colony hybridization (15) with vector DNA as a probe. When fruiting body formation was induced on clone fruiting (CF) agar plates (8), of 13 insertion mutants, 4 mutants were normal in fruiting body formation (Fru^+), 8 mutants were defective (Fru^-), and 1 mutant was leaky ($Fru^\pm$), as shown in Fig. 1. In the leaky mutant, fruiting bodies of abnormal morphology were formed at a later stage

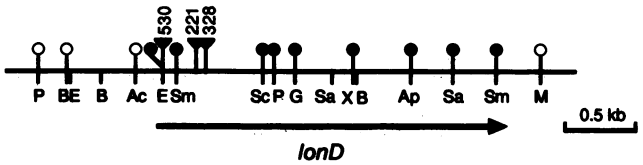

FIG. 1. Restriction map of the area near the *lonD* gene of *M. xanthus*. The locations of insertion mutations at various restriction sites and Tn*V* insertions are indicated. Symbols: ○, Fru^+ mutations; ●, Fru^- mutations (the rightmost Sm site yields a Fru^+ mutation); ▼, Tn*V* insertions with a Fru^- phenotype. The solid arrow below the map denotes the *lonD* coding region and its orientation. Ac, *Acc*III; Ap, *Apa*I; B, *Bam*HI; E, *Eco*47III; G, *Bgl*II; M, *Mlu*I; P, *Pst*I; Sa, *Sac*II; Sc, *Sca*I; Sm, *Sma*I; X, *Xho*I.

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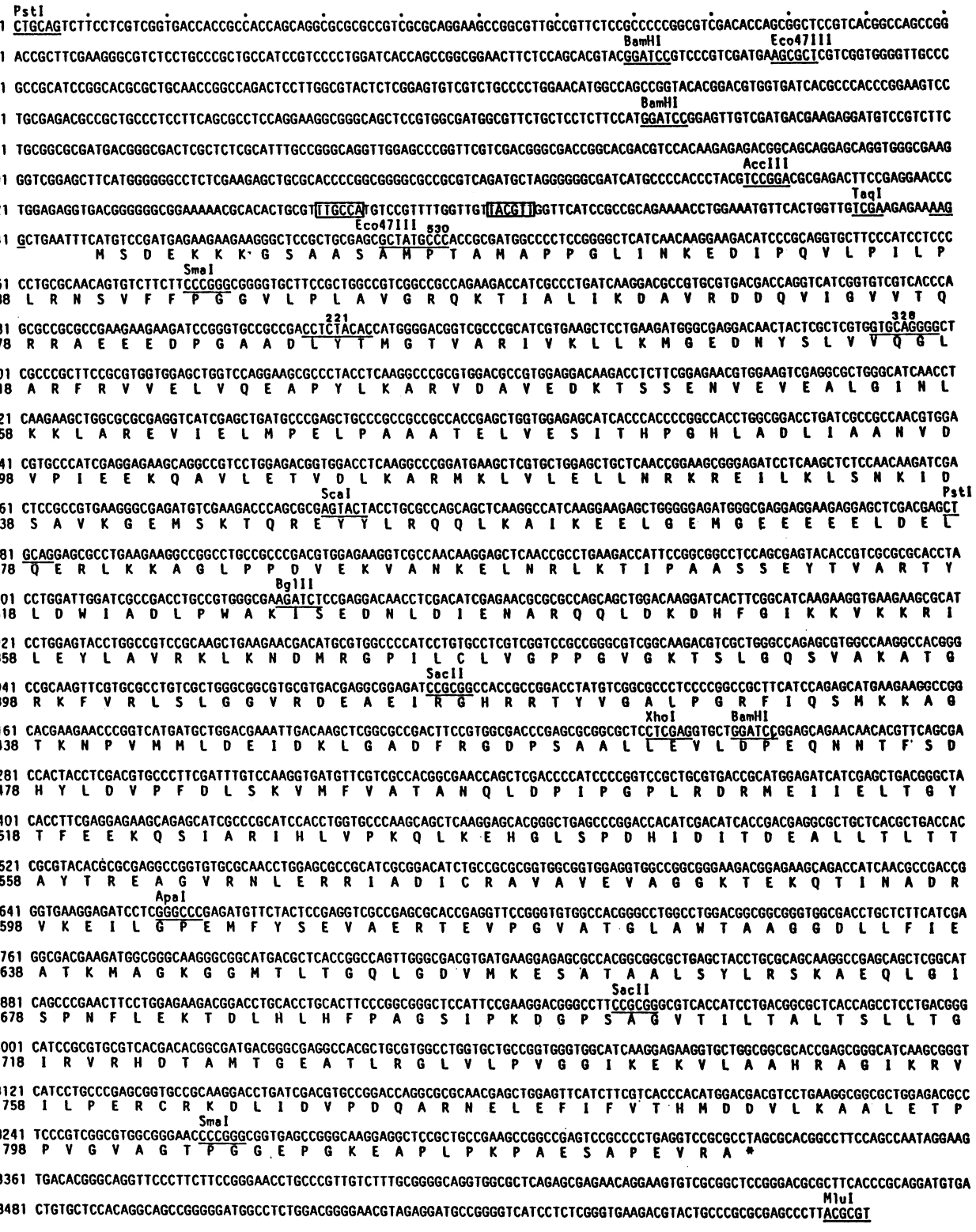

FIG. 2. Nucleotide sequence of the *lonD* gene of *M. xanthus* and its deduced amino acid sequence. Numbers to the left of the top line enumerate the nucleotide bases, and numbers to the left of the bottom line indicate the amino acid residues. Relevant restriction sites are indicated. Duplicated 9-bp sequences at Tn*V* insertion sites are underlined and identified by insertion numbers. Putative promoter sequences are boxed, and a putative Shine-Dalgarno sequence is also underlined.

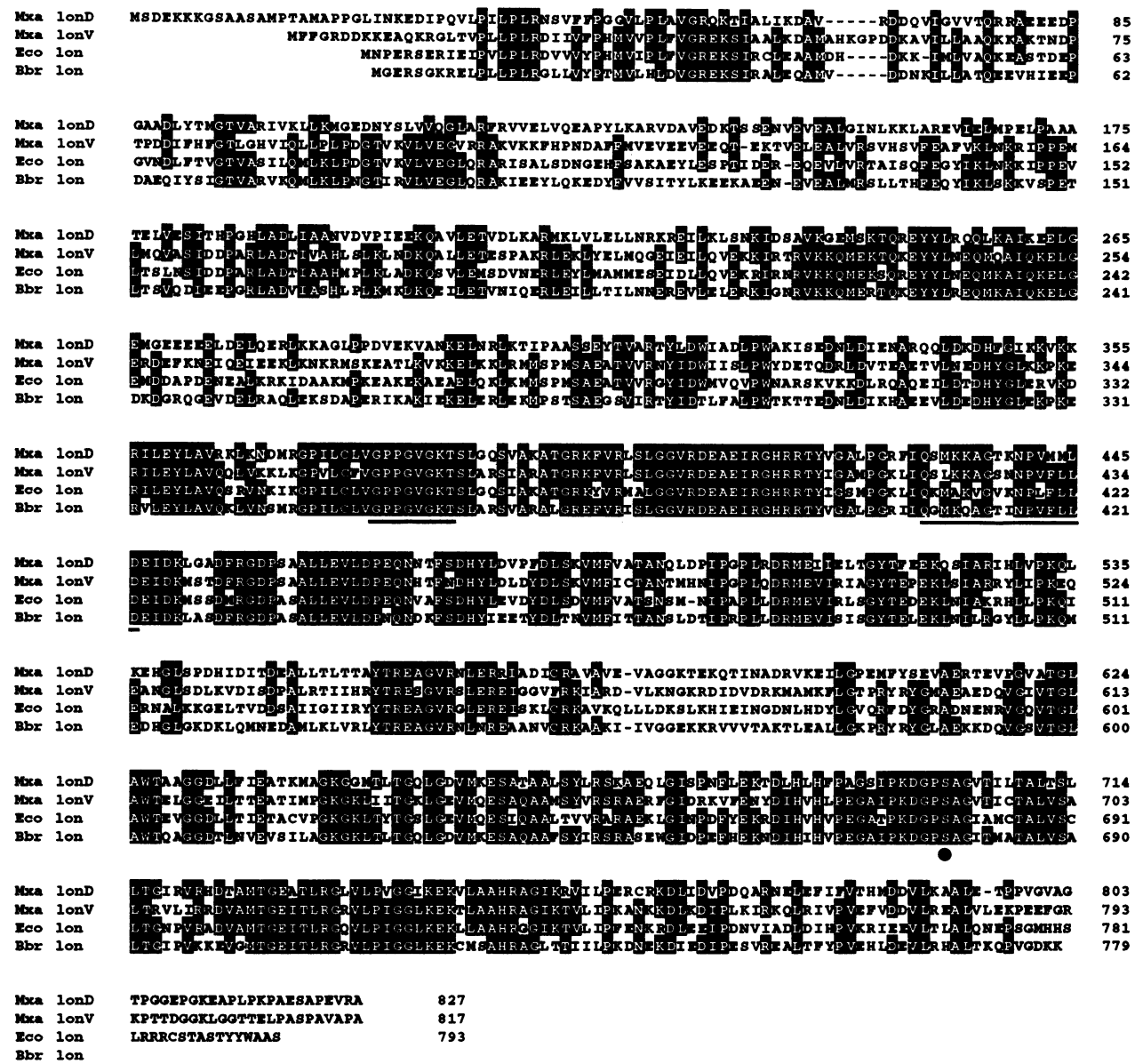

FIG. 3. Alignment of four *lon* amino acid sequences. Mxa lonD, *M. xanthus lonD* gene; Mxa lonV, *M. xanthus lonV* gene; Eco lon, *E. coli lon* gene; Bbr lon, *B. brevis lon* gene. Identical amino acid residues in three or all four sequences are printed in white on black. Putative ATP-binding sequences are underlined, and a serine residue at the putative active site is marked with a circle. *M. xanthus lonV*, *E. coli lon*, and *B. brevis lon* sequences were taken from Tojo et al. (19), Amerik et al. (2), and Ito et al. (10), respectively.

than normal (data not shown). These results indicate that the *lonD* locus is located within the 2.9-kb *Acc*III-*Mlu*I region.

Using some of the insertion mutants described above, we analyzed further other characteristics of the *lonD* mutations. The *lonD* mutants appeared to stop their development at a very early stage; they could not aggregate at all, and no spores were found, even after 7 days on CF agar plates. In contrast, no effect on vegetative growth was noted, and the mutants could form spores when 0.5 M glycerol was added to liquid cultures (16). As noted below, since *lonD* was found to be identical to *bsgA* (7), extracellular complementation with wild-type cells was expected during development (6, 8, 14). However, when the *lonD* mutants were mixed with wild-type cells and examined for complementation on CF agar plates, the *lonD* mutants failed to form spores. This result agrees with the experiment carried out by Kroos and Kaiser to examine extracellular complementation with *bsgA330* (12).

Nucleotide sequence of the *lonD* gene. The nucleotide sequence of the 3,587-bp *Pst*I-*Mlu*I fragment was determined by the dideoxy chain termination method (17) (Fig. 2). The locations of Tn*V* insertions in mutants 221, 328, and 530 were also determined (underlining in Fig. 2). In each Tn*V* insertion, a 9-bp target duplication was found.

Analysis of the nucleotide sequence revealed that there is an open reading frame that shows homology to *E. coli lon*. A putative *lonD* initiation codon was deduced by the coding frame analysis proposed by Hagen and Shimkets (9), on the

basis of the observation that the G+C content at the third codon position is very high within the protein coding region of *M. xanthus* genes. This analysis suggested that the *lonD* gene starts with the ATG codon at position 851. Construction of a *lonD-lacZ* fusion gene by use of the *Eco*47III site at position 884 as described below supported this suggestion. Furthermore, the insertion of 2 nucleotides at the *Taq*I site at position 828 did not affect the expression of the *lonD-lacZ* fusion gene. Thus, we conclude that the *lonD* gene starts with the ATG codon at position 851 and ends with the TAG codon at position 3332. The average G+C contents at positions 1, 2, and 3 of codons in the *lonD* gene were 68, 39, and 94%, respectively. The putative *lonD* initiation codon is preceded by a purine-rich Shine-Dalgarno-like sequence (AAGG). Possible promoter sequences, TTGCCA (positions 769 to 774) and TACGTT (791 to 796), that share homology with the *E. coli* σ^{70} consensus promoter sequence were found in the upstream region.

Amino acid sequence of the *lonD* gene. The proposed *lonD* coding sequence could encode a protein of 827 amino acids with a calculated molecular weight of 90,433 (Fig. 2). When the deduced amino acid sequence of *lonD* was compared with known protein sequences, striking similarities to *E. coli* and *Bacillus brevis lon* genes, with 48 and 52% identity, respectively, were found (2, 10). Furthermore, the proposed amino acid sequence of the *lonD* gene shares 49% identity with that of the *M. xanthus lonV* gene, which was recently identified as a gene essential for vegetative growth by use of the *E. coli lon* gene as a probe (19). Thus, we can conclude that *M. xanthus* bears two *lon*-related genes with different functions: the *M. xanthus lonV* gene is essential for vegetative growth (19), and the *lonD* gene is essential for development.

Figure 3 shows an alignment of four *lon* amino acid sequences. They share highly conserved sequences encompassing almost the entire region. ATP-binding sequences [segment A, $(G/A)X_4(G/A)(H/K/R)X_{0-1}(T/S/K/R/H)$, and segment B, $(H/K/R)X_{5-8}\Phi X\Phi_2(D/E)$, where X and ϕ stand for any amino acid and a hydrophobic amino acid (4), respectively] were found in the *lonD* sequence at the same positions as in the others, and the similarity near these sequences was relatively high for the four sequences (Fig. 3, underlining). Recently, an active-site serine residue in the *E. coli lon* gene was proposed by site-directed mutagenesis (1). This serine residue (indicated with a filled circle) was also conserved in the four sequences (Fig. 3). The similarity in amino acid sequences suggests that the *lonD* product is an ATP-dependent protease. It is noteworthy that the level of similarity of the amino acid sequence of *lonD* to those of the other three *lon* products is rather low in the N-terminal region.

Expression of the *lonD* gene. To investigate the expression of the *lonD* gene, we constructed a fusion of the *lonD* gene with the *lacZ* gene of *E. coli*. The 12th codon of the *lonD* gene was joined in frame to the 8th codon of the *lacZ* gene by insertion of the 666-bp *Eco*47III fragment (positions 221 to 886; Fig. 2) into the *Sma*I site of pMC1403 (3) in the proper orientation. The P1 incompatibility and Km^r genes were added to the resultant plasmid, and the final construct was introduced into *M. xanthus* DZF1 by P1 transduction (18). The *lonD-lacZ* fusion gene was integrated into the homologous region of the *M. xanthus* DZF1 chromosome. The integrated *M. xanthus* strain was merodiploid, containing the *lonD-lacZ* gene under the control of the *lonD* regulatory regions and containing the wild-type *lonD* gene (Fig. 4A). The chromosome structure of the integrated strain was

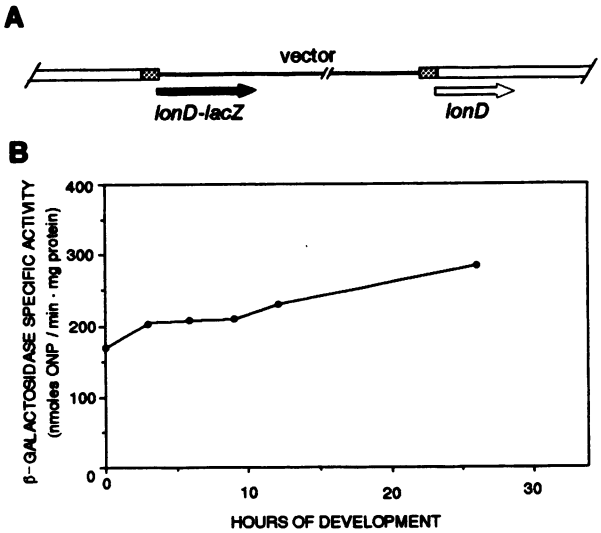

FIG. 4. β-Galactosidase expression during the development of an *M. xanthus* strain carrying a *lonD-lacZ* fusion gene. (A) Chromosome structure of the *M. xanthus* strain carrying the *lonD-lacZ* gene. Unique sequences of the *M. xanthus* chromosome are shown by open bars, and duplicated sequences are shown by stippled bars. A solid line indicates the vector sequence. Arrows indicate *lonD-lacZ* and *lonD* coding sequences. (B) Changes in the specific activity of β-galactosidase during development induced on CF agar plates. ONP, *o*-nitrophenol.

confirmed by Southern blot analysis (data not shown). The integrated strain exhibited normal development on CF agar plates. When the strain carrying the *lonD-lacZ* gene was plated on a Casitone-yeast extract (16) agar plate containing 5-bromo-4-chloro-3-indolyl-β-D-galactoside, the colonies turned green, indicating that the *lonD* gene is expressed during vegetative growth. β-Galactosidase specific activity was measured (13) during development induced on CF agar plates (Fig. 4B). β-Galactosidase activity was present at 170 U/mg of protein at the onset of development and increased gradually during development. These results suggest that the *lonD* gene is expressed during vegetative growth and that its expression increases gradually during development.

On the basis of the present results together with our previous findings (19), we conclude that *M. xanthus* bears at least two essential *lon*-related genes, *lonV* for vegetative growth and *lonD* for development. The *lonV* and *lonD* products may degrade many regulatory proteins during vegetative growth and development. However, the specificities of *lonV* and *lonD* proteases may be different.

Recently, the nucleotide sequence of the *bsgA* gene of *M. xanthus* was determined by Gill et al. (7), and it was found that the *lonD* gene is identical to the *bsgA* gene.

Nucleotide sequence accession number. The nucleotide sequence data shown in Fig. 2 will appear in the EMBL, GenBank, and DDBJ nucleotide sequence data bases under accession number D13204.

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