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Cloning and Nucleotide Sequence of the *Myxococcus xanthus lon* Gene: Indispensability of *lon* for Vegetative Growth

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The *lon* gene of *Escherichia coli* is known to encode protease La, an ATP-dependent protease associated with cellular protein degradation. A *lon* gene homolog from *Myxococcus xanthus*, a soil bacterium which differentiates to form fruiting bodies upon nutrient starvation, was cloned and characterized by use of the *lon* gene of *E. coli* as a probe. The nucleotide sequence of the *M. xanthus lon* gene was determined. It contains an open reading frame that encodes a 92-kDa protein consisting of 817 amino acid residues. The deduced amino acid sequence of the *M. xanthus lon* gene product showed 60 and 56% identity with those of the *E. coli* and *Bacillus brevis lon* gene products, respectively. Analysis of an *M. xanthus* strain carrying a *lon-lacZ* operon fusion suggested that the *lon* gene is similarly expressed during vegetative growth and development in *M. xanthus*. In contrast to that of *E. coli*, the *M. xanthus lon* gene was shown to be essential for cell growth, since a null mutant could not be isolated.

Myxococcus xanthus is a unique gram-negative bacterium which lives in soil and has a complex life cycle (for a review, see reference 27). Upon nutritional starvation on a solid surface at a high cell density, cells undergo a developmental cycle involving cell-cell interactions. Cells move toward aggregation centers by gliding and form raised mounds. Sporulation starts within the mounds, where a portion of the rod-shaped cells differentiate into spherical or ovoid myxospores. Myxospores are resistant to heat, UV irradiation, desiccation, and sonication. In *M. xanthus*, the mounds of myxospores are referred to as fruiting bodies.

The *lon* gene (also known as the *capR* or *deg* gene) of *Escherichia coli* encodes the ATP-dependent protease La (6, 9). This enzyme is a serine protease with an essential ATPase activity (37) and seems to be a heat shock protein (25). *lon* mutations in *E. coli* result in pleiotropic phenotypes such as increased sensitivity to UV irradiation (10, 16) and SOS-inducing agents (35), abnormal cell division (filament formation [16]), mucoid formation (12, 22), defective lysogeny of phages λ and P1 (32, 36), and reduced degradation of various abnormal proteins (13) and certain normal proteins. Various observations indicate that protease La catalyzes rate-limiting steps in the degradation of some regulatory proteins such as SulA and RcsA (23, 34). The phenotypes of its mutants are the result of the failure of rapid degradation of such regulatory proteins. The importance of rapid, energy-dependent proteolysis in cellular regulation was recently reviewed (11). The *lon* gene of *E. coli* has been cloned and sequenced (2, 8). The *lon* gene of *Bacillus brevis* was also cloned and characterized (18).

To reveal a possible role of the *lon* gene during development in *M. xanthus*, we tried to clone and characterize it. Since dramatic changes in the patterns of protein synthesis during the development of *M. xanthus* have been reported (17), the *lon* gene may play an important role in the regulation of development. Recently, Plamann et al. (26) showed that production of a 27-kDa trypsin-like protease is necessary for development in *M. xanthus*. In this article, the cloning and sequencing of a *lon* gene homolog from *M. xanthus* are described. In addition, it is shown that the *lon* gene is essential for vegetative growth of *M. xanthus*.

MATERIALS AND METHODS

Bacteria, phages, and plasmids. The bacterial strains, phages, and plasmids used in this study are listed in Table 1.

Growth conditions. Cultures of *M. xanthus* were grown in Casitone-yeast extract (CYE) medium at 30°C. The agar medium contained 1.5% agar. Development was induced on clone fruiting (CF) agar as described previously (14, 17). Cells were grown to late log phase in CYE broth, sedimented, and resuspended in TM buffer (10 mM Tris-HCl (pH 7.6), 8 mM $MgSO_4$) at a density of 10^{10} cells per ml. Aliquots (10 μl) of cell suspension were spotted on CF plates. Kanamycin sulfate (50 μg/ml) was added for selection of kanamycin-resistant (Km^r) *M. xanthus* cells.

E. coli strains were grown in LB medium (28) or 2× YT medium (28) at 37°C. When necessary, antibiotics were added to media at the following concentrations: ampicillin, 100 μg/ml; chloramphenicol, 25 μg/ml; tetracycline, 12.5 μg/ml; and kanamycin, 50 μg/ml.

DNA manipulation and sequencing. Preparation of chromosomal and plasmid DNA, transformation, and other methods of DNA manipulation were as described elsewhere (28). Southern blot analysis was carried out by the method of Southern (31). Colony hybridization analysis was performed as described previously (24). The DNA sequence was determined by the dideoxy-chain termination method (29).

Construction of *lon* deletion and insertion mutants. We attempted to construct *lon* deletion and insertion mutants of *M. xanthus* as described previously (20). We constructed a deletion mutation on a plasmid by digesting pMXL002 completely with *Xho*I to remove all of the *lon* coding sequence and the 2.3-kb upstream region and ligating it with a 1.3-kb *Hin*dIII-*Sma*I DNA fragment containing the Km^r gene of Tn*5*. The plasmid thus constructed was designated pMXL003. To construct insertion mutations on plas-

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

Strain, phage, or plasmid	Genotype or relevant characterization[a]	Source or reference
M. xanthus DZF1	*sglA*	17
E. coli K-12		
JM83	*ara* Δ(*lac-proAB*) *rpsL thi* φ80 *lacZ*Δ*M15*	38
JM109	*recA1 supE44 endA1 hsdR17 gyrA96 relA1 thi* Δ(*lac-proAB*) [F' *traD36 proAB lacI*^{q} *Z*Δ*M15*]	38
SM32	*his pyrD* Δ*lon-100 galE rpsL*	23
Phages		
M13mp18	*lacZ* α fragment	38
M13mp19	*lacZ* α fragment	38
P1 *clr100* Cm	Cm^r, *clr100*	30
Plasmids		
pUC9	Ap^r, *lacZ* α fragment	38
pUC18	Ap^r, *lacZ* α fragment	38
pBR322	Ap^r Tc^r	3
pACYC184	Cm^r Tc^r	5
pHC79	Ap^r Tc^r, λ *cos* site	15
pNT8712[b]	Ap^r pMC871 derivative	This work
pP1inc[c]	Ap^r, P1*inc*	T. Furuichi
pMW4	pBR322 derivative, *E. coli lon*$^+$	19

[a] Cm^r, chloramphenicol resistant; Ap^r, ampicillin resistant; Tc^r, tetracycline resistant.

[b] pNT8712 was constructed by ligating the 2.8-kb *Bam*HI-*Sac*I fragment of pMC871 (4) with the 8.0-kb *Sac*I-*Bam*HI fragment of pMC1403 (4) and then inserting a *Bgl*II linker at the *Sal*I site of the resulting plasmid.

[c] pP1inc was constructed by inserting the 5.4-kb *Eco*RI-*Kpn*I fragment of the P1 phage incompatibility region (30) into the *Eco*RI-*Kpn*I site of pUC19..

mids (pMXL002::KmΩX2, pMXL002::KmΩX3, pMXL002::KmΩX4, pMXL002::KmΩX5, and pMXL002::KmΩX6), pMXL002 DNA was first digested partially with *Xho*I. Next, a DNA fragment cleaved once with *Xho*I was purified by agarose gel electrophoresis and ligated with a Km^r DNA fragment. The sites of Km^r DNA insertions were determined by restriction enzyme analyses. The vector portion of these plasmids was changed to pP1inc to introduce P1 phage incompatibility genes (30). The resulting plasmids were introduced into *M. xanthus* by P1 transduction as described by Shimkets et al. (30).

Construction of a *lon-lacZ* operon fusion. To construct a *lon-lacZ* operon fusion, the 2.3-kb *Sal*I-*Apa*I fragment of pMXL002 DNA was treated with the Klenow fragment of DNA polymerase I and then inserted into the *Sma*I site of pNT8712 in the proper orientation, generating pMXL004. After the P1 incompatibility and Km^r genes were inserted into pMXL004, this plasmid was introduced into *M. xanthus* as described above.

β-Galactosidase assay during growth and development. For rapid detection of β-galactosidase activity during growth, cells carrying the *lon-lacZ* fusion gene were transferred to CYE agar plates containing 40 μg of 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-Gal) per ml and incubated at 30°C. To quantitate β-galactosidase activity during development (21), fruiting body formation was induced on CF agar, and cells were scraped from the plates and transferred to TM buffer at various times. Extracts prepared by sonication were assayed for their ability to cleave *o*-nitrophenyl-β-D-galactopyranoside (ONPG), and their protein content was measured with the Bio-Rad protein assay reagent. β-Galactosidase specific activity was expressed as nanomoles of *o*-nitrophenol produced per minute per milligram of protein.

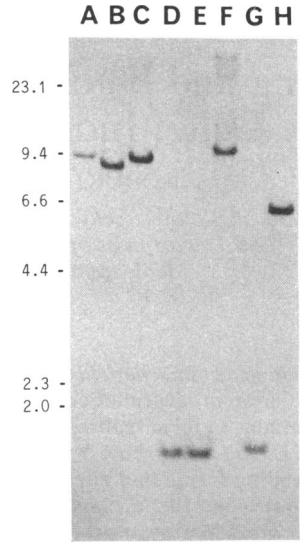

FIG. 1. Southern blot analysis of genomic DNA from *M. xanthus* DZF1. Hybridization was carried out at 37°C in 6× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) containing 50% formamide for 18 h with a nick-translated DNA probe [pMW4(19)] of the *E. coli lon* gene. DNA samples were digested with *Sal*I-*Hin*dIII (lane A), *Bam*HI (lane B), *Sal*I (lane C), *Eco*RI-*Pst*I (lane D), *Pst*I (lane E), *Eco*RI (lane F), *Xho*I (lane G), or *Sma*I (lane H). Numbers at the left represent molecular lengths in kilobases.

Materials. Restriction enzymes were purchased from either Takara Shuzo, Nippon Gene, Toyobo, or Boehringer Mannheim. A 7-DEAZA sequencing kit, a DNA ligation kit, and the Klenow fragment of DNA polymerase I were obtained from Takara Shuzo and used as directed. A nick translation kit was from Nippon Gene. [α-^{32}P]dCTP was from ICN Biomedicals.

Nucleotide sequence accession number. The GenBank accession number of the *M. xanthus lon* gene sequence is D12923.

RESULTS

Cloning of the *lon* gene from *M. xanthus*. Southern blot hybridization was carried out on *M. xanthus* genomic DNA isolated from vegetative cells. The DNA was digested with various restriction enzymes and probed under relaxed conditions with a ^{32}P-labeled DNA fragment containing the C-terminal half of the *E. coli lon* gene (19). For each lane, a single uniquely sized band was detected (Fig. 1), indicating the presence of an *E. coli lon* homolog in *M. xanthus*. For example, the probe hybridized with a 1.5-kb *Pst*I fragment of *M. xanthus* genomic DNA (Fig. 1, lane E).

The *M. xanthus lon* gene was cloned in two steps. First, the 1.5-kb *Pst*I fragment described above was cloned. *Pst*I fragments of *M. xanthus* genomic DNA that migrated at around the 1.5-kb region were purified by preparative agarose gel electrophoresis and inserted into the *Pst*I site of pBR322. Plasmid DNAs from the transformants were isolated and subjected to Southern blot analysis with the same probe. One of the positive clones, designated pMXL001,

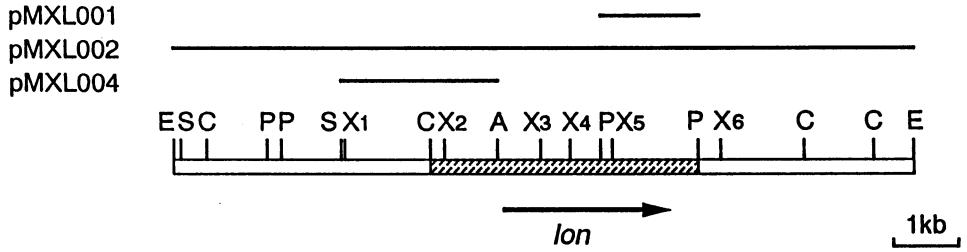

FIG. 2. Restriction map of the 11.1-kb DNA fragment containing the *M. xanthus lon* gene. The sequenced region (Fig. 3) is indicated by the stippled bar. The solid arrow below the map represents the *lon* coding region and its orientation. Solid lines above the map indicate the DNA segments present in various plasmids. A, *Apa*I; C, *Sac*I; E, *Eco*RI; P, *Pst*I; S, *Sal*I; X, *Xho*I. Only the relevant site is shown for *Apa*I. Six *Xho*I sites are numbered from the left.

was partially sequenced and was found to contain a C-terminal region of the *lon* gene (Fig. 2). Second, to obtain the entire region of the *lon* gene, the *M. xanthus* genomic library was screened with the insert of pMXL001 as a probe. The library was constructed on pHC79 as cosmids, and each clone contains a 30- to 40-kb DNA insert (15). Three positive clones, P574, P693, and P726, were isolated, and an 11.1-kb *Eco*RI fragment containing the entire region of the *lon* gene was recloned from P547 into the *Eco*RI site of pACYC184, generating pMXL002. A restriction map of pMXL002 DNA is shown in Fig. 2.

Nucleotide sequence of the *lon* gene in *M. xanthus*. The nucleotide sequence of the *lon* gene in *M. xanthus* was determined by the dideoxy-chain termination method. Figure 3 shows the sequence of the 4,038-bp *Sac*I-*Pst*I fragment (Fig. 2). Each nucleotide was determined in both directions by using overlapping fragments.

Analysis of the sequence revealed a long open reading frame that is initiated by an ATG codon at position 1069 and ends at position 3522 with a TAG stop codon. The initiation codon was postulated by comparing the *M. xanthus* sequence with the *E. coli* and *B. brevis* sequences. The deduced amino acid sequence of the *M. xanthus lon* gene product has 60 and 56% identity with those of the *E. coli* and *B. brevis lon* gene products, respectively. The putative initiation codon is preceded by a purine-rich Shine-Dalgarno-like sequence (AGGTGG). Translation starting at the putative initiation codon is assumed to produce a protein of 817 amino acid residues with a calculated molecular weight of 91,810. Codon usage in the *lon* gene is similar to that of other *M. xanthus* genes so far sequenced (data not shown). As expected from the high G+C content of *M. xanthus* genomic DNA (27), the *M. xanthus* genes have a very high G+C content at the third position of their codons. The average G+C contents at positions 1, 2, and 3 of codons in the *lon* gene were 63, 36, and 93%, respectively.

Failure to isolate a null mutant. To examine the role of the *lon* gene during vegetative growth and development of *M. xanthus*, we attempted to obtain a null mutant of the *lon* gene. For this purpose, we first constructed the desired mutations on cloned copies of the *lon* gene and then tried to replace the chromosomal wild-type *lon* gene with mutant alleles. To construct a deletion mutation, the 5.6-kb DNA segment (*Xho*I sites X_1 to X_6) containing the entire *lon* gene and the 2.3-kb upstream region of PMXL002 was replaced by a 1.3-kb DNA fragment containing the Km^r gene of Tn*5*, generating PMXL003 (Fig. 4). To construct insertion mutations, the same Km^r DNA fragment was inserted into the five different *Xho*I sites of PMXL002, generating PMXL002::KmΩX2, PMXL002::KmΩX3, PMXL002::KmΩX4, PMXL002::KmΩX5, and PMXL002::KmΩX6 (*Xho*I sites are shown in Fig. 2). The *lon* gene was intact in PMXL002::KmΩX2 and PMXL002::KmΩX6, while in the case of PMXL003, PMXL002::KmΩX3, PMXL002::KmΩX4, and PMXL002::KmΩX5, the *lon* gene was deleted or interrupted by the Km^r DNA fragment. The vector portion of these plasmids was changed to pP1inc in order to introduce P1 incompatibility genes.

The resultant plasmids were introduced by specialized P1 transduction from *E. coli* into *M. xanthus* DZF1 (30). In Km^r *M. xanthus* transductants, these plasmids were expected to be integrated into the *M. xanthus* chromosome by a single or double homologous crossover, since the vector used does not replicate in *M. xanthus* cells. Single crossover results in merodiploid formation with the wild-type *lon* gene at one copy and the Km^r construct at the other, while double crossover results in gene replacement of the wild-type *lon* gene with the Km^r construct. Km^r transductants were screened by colony hybridization with nick-translated pP1inc DNA as a probe to determine whether each transductant was merodiploid type or replacement type, and the results are shown in Fig. 4. In the case of PMXL002::KmΩX2 and PMXL002::KmΩX6, in which insertions lay outside the *lon* coding sequence, replacement-type transductants were obtained with frequencies of 79 and 70%, respectively, while in the cases of PMXL003, PMXL002::KmΩX3, PMXL002::KmΩX4, and PMXL002::KmΩX5, in which the *lon* gene is deleted or inserted, replacement-type transductants were not found in the colony hybridization. These results strongly suggest that the *lon* gene is essential for vegetative growth of *M. xanthus*.

Expression of the *lon-lacZ* operon fusion. To determine the expression of the *lon* gene during vegetative growth and development in *M. xanthus*, we constructed an operon fusion of the *lon* gene with the *lacZ* gene of *E. coli*. The 2.3-kb *Sal*I-*Apa*I fragment of PMXL002 DNA, which contains the 5′ upstream region of the *lon* gene, was inserted into the *Sma*I site of PNT8712 in the proper orientation, generating PMXL004 (Fig. 2). PMXL004 contained putative *M. xanthus lon* regulatory sequences together with an *E. coli lacZ* coding sequence. This operon fusion plasmid was introduced into *M. xanthus* by P1 transduction after receiving insertions of P1 incompatibility genes and the Km^r gene. In this case, the entire plasmid was integrated into the *M. xanthus* chromosome by single homologous recombination as shown in Fig. 5A. The chromosome structure of the integrated strain was confirmed by Southern blot analysis (data not shown). Integration of PMXL004 into the *M. xanthus* chromosome puts the *lacZ* gene under the control of the *lon* promoter and putative upstream regulatory regions.

FIG. 3. Nucleotide sequence of the *M. xanthus lon* gene and deduced amino acid sequence of its product. Upper numbers at the left in each pair of lines enumerate the nucleotide bases; lower numbers enumerate the amino acid residues. Putative promoter sequences are boxed, and a putative Shine-Dalgarno sequence is underlined. The asterisk indicates the stop codon.

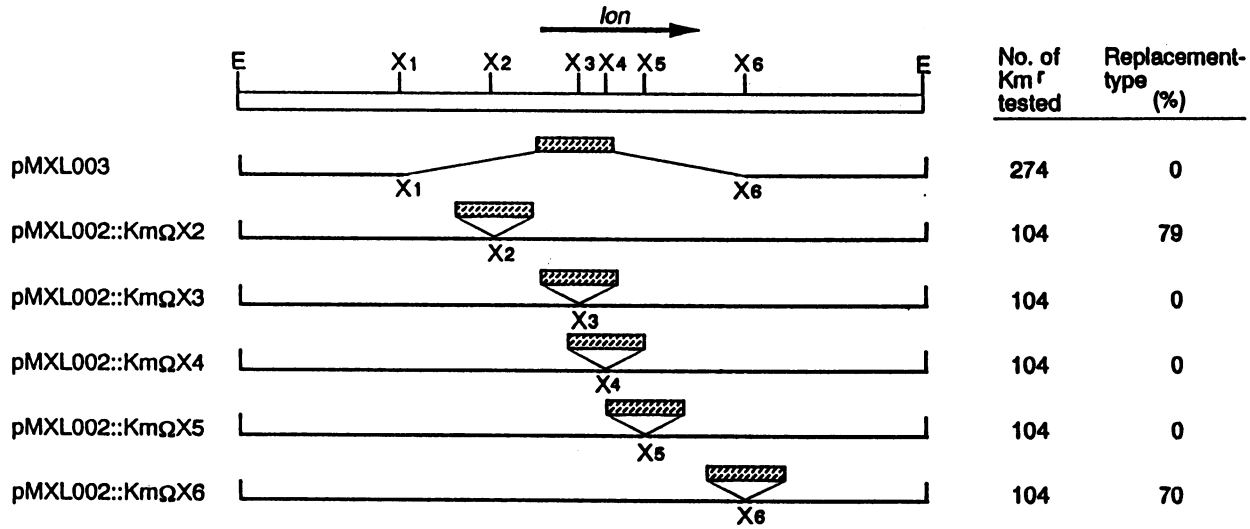

FIG. 4. Frequencies of replacement-type transduction flanking the *M. xanthus lon* gene. At the top, the restriction map of the 11.1-kb *Eco*RI DNA fragment is shown. The arrow represents the *lon* coding sequence. The Km^r DNA fragment is shown by the stippled bar. The positions of the insertions are indicated.

When *M. xanthus* cells carrying the *lon-lacZ* operon fusion were transferred to a CYE agar plate containing X-Gal and incubated at 30°C, blue color induction was observed. Vegetative cells of *M. xanthus* carrying the *lon-lacZ* fusion gene showed β-galactosidase activity of 560 U/mg of protein, indicating that the *lon* gene is expressed during vegetative growth. The strain was subjected to induction of development on CF agar, and the specific activity of β-galactosidase was monitored during development. Development on CF agar of the strain carrying the *lon-lacZ* fusion gene was similar to that of the wild-type strain. As shown in Fig. 5B, the β-galactosidase activity level was 480 U/mg of protein at the onset of development, and the activity increased slightly during development. These results indicate that the *lon* gene is expressed similarly during vegetative growth and development.

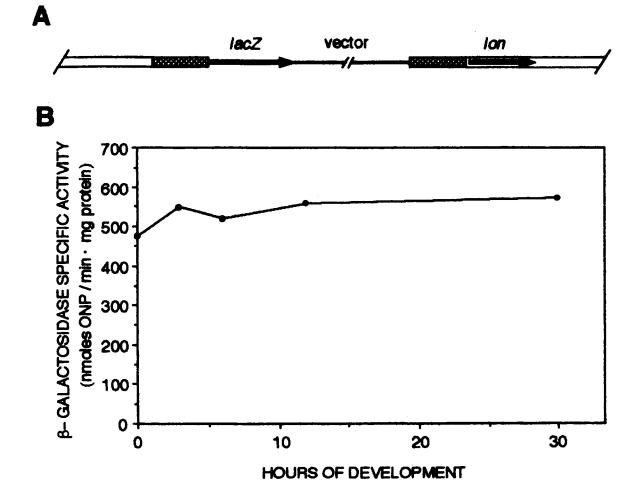

FIG. 5. β-Galactosidase expression during development of an *M. xanthus* strain carrying the *lon-lacZ* operon fusion. (A) Chromosome structure of an *M. xanthus* strain carrying the integrated pMXL004 plasmid. Unique sequences of the *M. xanthus* chromosome are shown by open bars, and duplicated sequences are shown by stippled bars. The solid line indicates the vector sequence of the integrated pMXL004 plasmid. Arrows indicate *lacZ* and *lon* coding sequences. (B) Changes in specific activity of β-galactosidase during development. ONP, *o*-nitrophenol.

DISCUSSION

In the present work, the *lon* gene of *M. xanthus* was cloned and sequenced. The nucleotide sequences of the *lon* genes from *E. coli* (2, 8), a gram-negative bacterium, and *B. brevis* (18), a gram-positive bacterium, have been previously reported. Figure 6 shows an alignment of the deduced amino acid sequences of the three *lon* genes of *M. xanthus*, *E. coli*, and *B. brevis*. The three amino acid sequences are highly conserved throughout most of the gene. The *M. xanthus* sequence shows 60 and 56% identity with those of *E. coli* and *B. brevis*, respectively. The conservation of the three *lon* genes suggests that they play an important role in the physiology of these bacteria. The nucleotide sequences of the three *lon* genes are also conserved within the protein-coding regions (*M. xanthus* and *E. coli*, 60% identity; *M. xanthus* and *B. brevis*, 61% identity). In contrast, the three nucleotide sequences show no significant similarity outside the protein-coding region, suggesting that their regulatory regions evolved diversely. This suggestion is supported by the finding that the *M. xanthus lon-lacZ* operon fusion in PMXL004 was not expressed in *E. coli* cells (data not shown).

Precise examination of the alignment revealed three segments of high conservation (amino acids 215 to 254, 332 to 522, and 591 to 760 in the *M. xanthus* sequence). They have more than 60% identity covering more than 40 amino acids. The first domain contains a cluster of basic amino acids (numbers 223 to 231). An acidic region starts from the C-terminal end of the first domain. The second domain contains ATP-binding sequences (8) consisting of GPPGVG KT and QXXXXXGXXNPVFLLD (Fig. 6). The third domain contains a serine residue at a putative active site (Fig. 6) which was found to be necessary for function in the *E. coli*

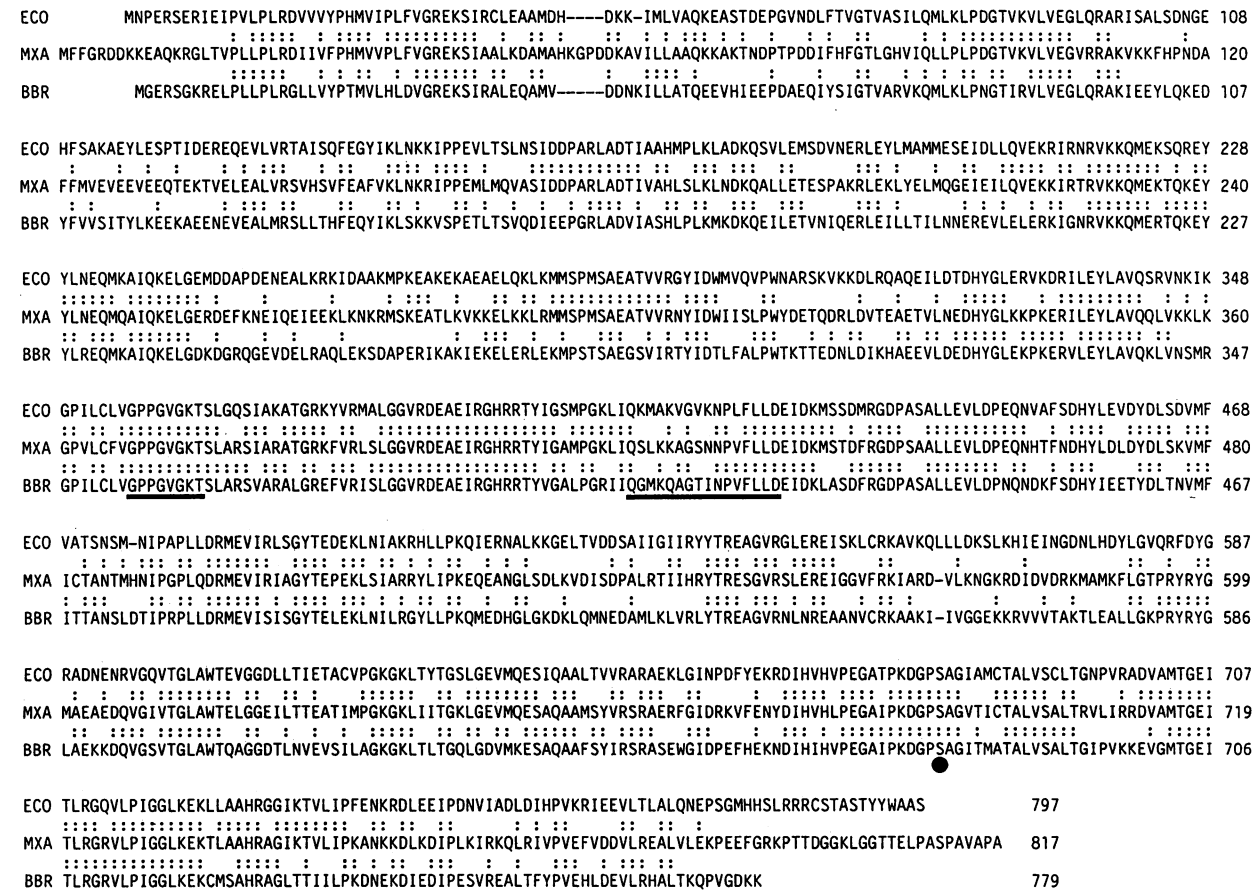

FIG. 6. Alignment of amino acid sequences of three *lon* gene products. ECO, *E. coli*; MXA, *M. xanthus*; BBR, *B. brevis*. Identical amino acid residues (:), a serine residue at the putative active site (●), and putative ATP-binding domains (underlined sequences) are indicated. *E. coli* and *B. brevis* sequences are those determined by Amerik et al. (2) and Ito et al. (18), respectively.

lon gene by site-directed mutagenesis (1). A stretch of seven amino acids containing the active-site serine residue was completely conserved among the three *lon* sequences.

Failure to isolate a *lon* null mutant indicated that the *lon* gene is essential for vegetative growth of *M. xanthus*. This finding is very interesting, since the *lon* gene is not essential in either *E. coli* or *B. brevis*. In both bacteria, *lon* null mutants were isolated (18, 23). Our preliminary data indicated that the *M. xanthus lon* gene can complement the nitrofurantoin-sensitive phenotype of a *lon* mutation of *E. coli*. The *M. xanthus lon* gene was fused in frame to the *lacZ* gene promoter and the α fragment of pUC9 to generate pMXL005; the *E. coli* SM32 (Δ*lon*) strain harboring PMXL 005 was nitrofurantoin resistant in the presence of IPTG (isopropyl-β-D-thiogalactopyranoside), while the same strain harboring pUC9 was nitrofurantoin sensitive (33).

Why is the *lon* gene essential in *M. xanthus*? One possible explanation is that *sulA* expression is constitutive in *M. xanthus*. In *E. coli*, the *sulA* gene is induced by SOS signals, and its expression prevents cell division by inhibiting *ftsZ* gene function (35). Lon protease was shown to degrade the SulA protein rapidly and to restore cell division when the SOS response ends (23). However, further investigation is necessary to reveal the function of the *lon* gene in *M. xanthus*.

In *E. coli*, the *lon* gene is known to be one of the heat shock genes (25), which are induced during the heat shock response and are under control of the *htpR* (*rpoH*) gene. In *B. brevis*, however, the *lon* gene is not a heat shock gene (18). We used the *lon-lacZ* gene to determine whether the *M. xanthus lon* gene is also a heat shock gene. β-Galactosidase activity was not increased when log-phase cells were shifted from 28 to 40°C (33). Hence, it is unlikely that the *lon* gene is a heat shock gene in *M. xanthus*. A potential promoter sequence was found upstream of the *M. xanthus lon* gene. The sequences TTGAGT (positions 782 to 787) and TTCGTT (804 to 809) may correspond to the −35 and −10 sequences, respectively, of the consensus promoter for σ^{70} of *E. coli* RNA polymerase. However, no sequence similar to the heat shock promoter of *E. coli* was found.

Analysis of *lon* gene expression with the *lon-lacZ* operon fusion in *M. xanthus* indicated that the *lon* gene is expressed during vegetative growth at a relatively high level, consistent with the notion that it is indispensable for cell growth. It was also revealed that the *lon* gene is expressed during development at a level similar to the expression level during vegetative growth. We do not know, however, whether the *lon* gene is also necessary for development. The *dsg* gene in *M. xanthus* was shown to be required for both vegetative growth and development. Mutants with mutations in *dsg* were originally isolated as point mutants defective in development. Further investigation found that the *dsg* was necessary for cell viability, since its null mutants could not be isolated (7).

Identification and purification of the *M. xanthus lon* gene product would allow us to answer many unsolved questions. Such projects are now in progress in our laboratory.

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