INFECTION AND IMMUNITY, Mar. 1991, p. 1043–1049
0019-9567/91/031043-07$02.00/0

Vol. 59, No. 3

Identification of a New Operon Involved In *Listeria monocytogenes* Virulence: Its First Gene Encodes a Protein Homologous to Bacterial Metalloproteases

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Received 20 August 1990/Accepted 6 December 1990

The region flanking the transposon in a Tn*1545*-induced lecithinase-negative mutant of *Listeria monocytogenes* EGD was cloned and sequenced. The transposon had inserted in ORF D, the open reading frame previously identified downstream from *hlyA*, the gene encoding listeriolysin O. The complete sequence of ORF D from strain EGD has been determined as well as that of two other strains: LO28, a clinical isolate; and LM8, an epidemic strain. ORF D is 1,533 bp long and encodes a protein highly homologous to metalloproteases of bacilli, *Serratia* sp., *Legionella pneumophila*, and *Pseudomonas aeruginosa*. It was renamed *prtA*. Northern RNA blot analysis indicated that *prtA* is the first gene of a 6-kb operon, suggesting that the lecithinase-negative phenotype of the mutant might be due to a polar effect of the transposon insertion.

The gram-positive bacterium *Listeria monocytogenes* is an intracellular pathogen discovered in 1926 during an epidemic among laboratory rabbits and guinea pigs (18) and later shown to be a dangerous pathogen for humans, in particular pregnant women, neonates, and immunocompromised people. It also infects healthy people and has been in recent years the cause of several outbreaks traced to contaminated food and resulting in septicemias, meningitis and meningoencephalitis, and death.

After the studies of Mackaness in 1960 (13), virulence of *L. monocytogenes* was essentially attributed to its capacity to survive and even replicate in professional phagocytes. *L. monocytogenes*, in addition to its capacity to survive intracellularly, is able to cross the intestinal barrier, to spread from cell to cell, to multiply in the liver and spleen (where it creates foci of infection and abscesses), and to cross the endothelial barriers and gain access to the brain and the placenta. Genetic studies to identify the various factors responsible for these different steps recently converged to establish the crucial role of the thiol-activated toxin produced by *L. monocytogenes*. This protein, named listeriolysin O (LLO), allows the escape of the bacterium from the phagosome into the cytosol. The crucial demonstration that LLO is a virulence factor was obtained through a gene complementation experiment: transformation of a transposon-induced nonhemolytic mutant with a plasmid carrying *hlyA*, the gene encoding LLO, led to the concomitant recovery of the hemolytic phenotype and of virulence (8). LLO is, to date, the only bacterial factor identified as an intracellular growth-promoting factor and has even been shown to promote growth of *Bacillus subtilis* in J774 macrophages (3).

LLO was the first factor to be examined not only because clinical strains are hemolytic and all nonhemolytic strains are avirulent in the mouse model but also because the hemolytic phenotype is easily identified on blood agar plates. Another phenotype easily identified on agar plates is the lecithinase phenotype. Secretion of a lecithinase by *L. monocytogenes* was reported for the first time by Seeliger, who detected halos around colonies on egg yolk plates (22). We recently questioned the role of this virulence factor. Purification of the protein was achieved, and four lecithinase-negative mutants were isolated by independent Tn*1545*, Tn*917*, and Tn*917-lac* mutagenesis. These mutants carried a single copy of the transposon inserted at different loci on the chromosome and their virulence, evaluated by their 50% lethal dose, was affected to different extents (20a and our unpublished results).

In this paper, we report the cloning and sequencing of the DNA flanking Tn*1545* in one of the mutants. Unexpectedly, the locus of insertion in the *Listeria* chromosome mapped in ORF D, an open reading frame previously identified downstream from *hlyA*, the gene coding for LLO (15). The wild-type ORF D was cloned and sequenced from three different strains. It encodes a protein homologous to neutral metalloproteases of bacilli, *Legionella pneumophila*, *Pseudomonas aeruginosa*, and *Serratia* sp. and was renamed *prtA*. Northern RNA analysis indicated that this gene is the first gene of a 6-kb operon, suggesting that a polar effect might explain the lecithinase-negative phenotype of the Tn*1545*-induced mutant.

MATERIALS AND METHODS

Strains, plasmids, and culture media. The *L. monocytogenes* strains used in this study were LO28, a wild-type isolate (29); LM8, an epidemic strain (20); and Bug 13, a Tn*1545*-induced lecithinase-negative mutant of strain EGD (20a). The EGD strain we used is the spontaneous streptomycin-resistant derivative described previously (9). Plasmid pUC18 (32) was used to clone DNA fragments in *Escherichia coli* MC1061 [F$^-$ *araD139* Δ(*ara leu*)*7696* Δ*lacY74 galU galK hsr hsm strA*] (6). *E. coli* strains were grown at 37°C in LB medium (14). For strains containing pUC derivatives, ampicillin was added at a final concentration of 25 μg/ml in liquid medium and 100 μg/ml in solid medium. *L. monocytogenes* strains were grown in brain heart infusion (BHI) broth or agar (Difco Laboratories, Detroit, Mich.) at 37°C with aeration.

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Chemicals and enzymes. Restriction enzymes and ligase were purchased from Amersham (Buckinghampshire, United Kingdom), Boehringer (Mannheim, Federal Republic of Germany), BioLabs (Beverly, Mass.), Gibco BRL (Cergy Pontoise, France), or Genofit (Geneva, Switzerland) and were used as recommended by the manufacturer. [^{35}S]dATP (3,000 Ci/mmol) was purchased from Amersham.

DNA techniques. DNA techniques and plasmid DNA sequencing were performed as previously described (14, 16). Polymerase chain reaction amplification was performed on chromosomal DNA from strain EGD or LM8 by using two pairs of oligonucleotides chosen from the sequence obtained from strain LO28. The amplified fragments were purified by use of a GeneClean kit (Bio 101, La Jolla, Calif.), after electrophoresis on agarose gels, and sequenced as previously described (16). *Listeria* chromosomal DNA was purified by a rapid protocol adapted from the method of Ausubel et al. (1). Briefly, cells from 1.5 ml of overnight culture were washed in 0.1× SSC (1× SSC is 0.15 M sodium chloride–0.015 M sodium citrate), suspended in 60 μl of lysis solution (0.01 M Na_2HPO_4, 20% sucrose, 2.5 mg of lysosyme per ml [freshly added]) and incubated for 1 h at 37°C. Then 507 μl of TE (10 mM Tris HCl, 1 mM EDTA), 30 μl of 10% sodium dodecyl sulfate, and 3 μl of proteinase K (20 mg/ml) were added before a second hour of incubation at 37°C. Cell wall debris, denatured proteins, and polysaccharides were precipitated by adding 100 μl of 5 M sodium chloride and 80 μl of 10% hexadecyltrimethyl ammonium bromide (Sigma, St. Louis, Mo.) in 0.7 M NaCl and incubating for 10 min at 65°C. The mixture was then phenol extracted twice, and DNA was precipitated by adding 0.6 volume of isopropanol and gently inverting the tube. The DNA pellet recovered by brief centrifugation was washed in 70% ethanol, dried, and suspended in 100 μl of TE containing 50 μg of RNase per ml.

RNA techniques. Total cellular RNAs were extracted by a rapid protocol from *L. monocytogenes* LO28 grown at 37°C with aeration in BHI broth (1). Briefly, cells from a 10-ml culture were suspended in 500 μl of lysis buffer (30 mM Tris HCl [pH 7.4], 100 mM sodium chloride, 5 mM EDTA, 1% sodium dodecyl sulfate, 100 μg of proteinase K per ml [freshly added]), frozen on dry ice, thawed, and sonicated three times for 10 s with a microtip sonicator (power setting, about 30 W). The lysate was then incubated for 1 h at 37°C, phenol extracted twice, and chloroform extracted. The aqueous phase was ethanol precipitated overnight at −20°C. The total RNA and DNA pellet was suspended in 100 μl of DNase digestion buffer (40 mM Tris HCl [pH 7.5], 6 mM $MgCl_2$) containing 30 U of RNase-free DNase I (Pharmacia) and incubated for 1 h at 37°C. The mixture was phenol extracted, chloroform extracted, and ethanol precipitated overnight at −20°C. The RNA pellet was finally suspended in 100 μl of diethylpyrocarbonate-treated water and stored at −70°C. Yields (about 10 μg of total RNA per ml of culture) are higher than with the technique previously described (15). Northern blotting was performed as described previously (15), except that we used Immobilon N membranes (Millipore) and the rapid hybridization system of Amersham.

RESULTS

To examine the role of lecithinase in virulence of *L. monocytogenes*, lecithinase-negative mutants were obtained by transposon mutagenesis with Tn*1545*, Tn*917*, and Tn*917-lac* (20a and our unpublished results). In this study, one of these was further analyzed. It contains a single copy of Tn*1545* inserted in the chromosome and has a higher 50% lethal dose than the parental strain, EGD ($10^{7.6}$ versus $10^{6.2}$).

Cloning and sequence analysis of the chromosome-Tn*1545* junction of a lecithinase-negative mutant: Tn*1545* had inserted in ORF D. Taking advantage of the presence of a kanamycin resistance gene at the left end of Tn*1545*, we cloned, in pUC18, a 8-kb *Hin*dIII fragment containing the junction between the listerial chromosome and the left part of Tn*1545*. Using an oligonucleotide located in the left end of Tn*1545*, we sequenced the transposon-*Listeria* junction. Tn*1545* had inserted 530 bp downstream from the beginning of an ORF previously identified immediately downstream from *hlyA* the structural gene of the LLO and named ORF D. For reasons described below we designated this ORF *prtA*.

Cloning of *prtA* from *L. monocytogenes* EGD, LO28, and LM8 and nucleotide sequence comparison. As most of our genetic work on virulence of *L. monocytogenes* was performed on the clinical strain LO28, we decided to clone and sequence the wild-type *prtA* gene from strain LO28 as well as that from strain EGD. Strains EGD and LO28, respectively, are of the 1/2a and 1/2c serotype. We had previously noticed a restriction fragment length polymorphism in this region, and therefore we also cloned the region from epidemic strain, LM8, of serotype 4b. In this strain, the second *Hin*dIII site located downstream from *hlyA* is absent (10; unpublished results).

We had previously cloned, in pLis3, a 3,454-bp *Bam*HI chromosomal fragment containing the *hlyA* gene and the beginning of *prtA* of strain LO28 (Fig. 1) (15). Using as a probe a 531-bp *Sau*96-A–*Bam*HI fragment internal to *prtA* and purified from pLis3, we cloned, in pUC18, a 1,369-bp *Hin*dIII fragment (pLis11). We then used as a probe a 455-bp *Sph*I fragment purified from pLis11 to clone a 1,591-bp *Dra*I fragment, giving rise to plasmid pLis22, which contained the 3′ end of *prtA*.

For strain EGD, we also used the 455-bp *Sph*I fragment as a probe to clone, in pUC18, a 1,250-bp *Sph*I fragment (pLis33). The 1,369-bp *Hin*dIII fragment harbored by plasmid pLis32 was cloned by using as a probe a 1-kb *Acc*I fragment internal to *prtA* and purified from the plasmid containing the transposon-*Listeria* junction.

Finally, for strain LM8, with a 412-bp *Hin*dIII fragment purified from plasmid pLis3 as a probe, *prtA* was nearly entirely cloned in a 1,781-bp *Hin*dIII fragment (pLis18).

By sequencing on both strands of the DNA the fragments cloned in pLis3, pLis11, and pLis22, we determined the complete nucleotide sequence of *prtA* from strain LO28 (Fig. 2). Inserts of pLis32, pLis33, and pLis18 only partially covered *prtA* of strains EGD and LM8, respectively. To complete the nucleotide sequence of *prtA* of these strains, oligonucleotides were chosen in the sequence of *prtA* region of strain LO28 and used to amplify, by polymerase chain reaction, DNA fragments spanning the 5′ and 3′ ends of the *prtA* genes from strains EGD and LO28, respectively (Fig. 1). These fragments were directly sequenced.

Sequences of the *prtA* genes of strains LO28, EGD, and LM8 are given in Fig. 2. The *prtA* gene, from the ATG start codon arbitrarily chosen as discussed previously (15) up to the TGA stop codon, is 1,533 bp long. The *prtA* nucleotide content is 38% (G+C). Taking strain LO28 as a reference, only 6 differences were detected in the *prtA* sequence of strain EGD versus 79 differences in the case of strain LM8. Most of the changes affect the third position of codons and either are conservative or change an amino acid for a similar one. The mutation responsible for the restriction fragment length polymorphism observed in the *prtA* region corre-

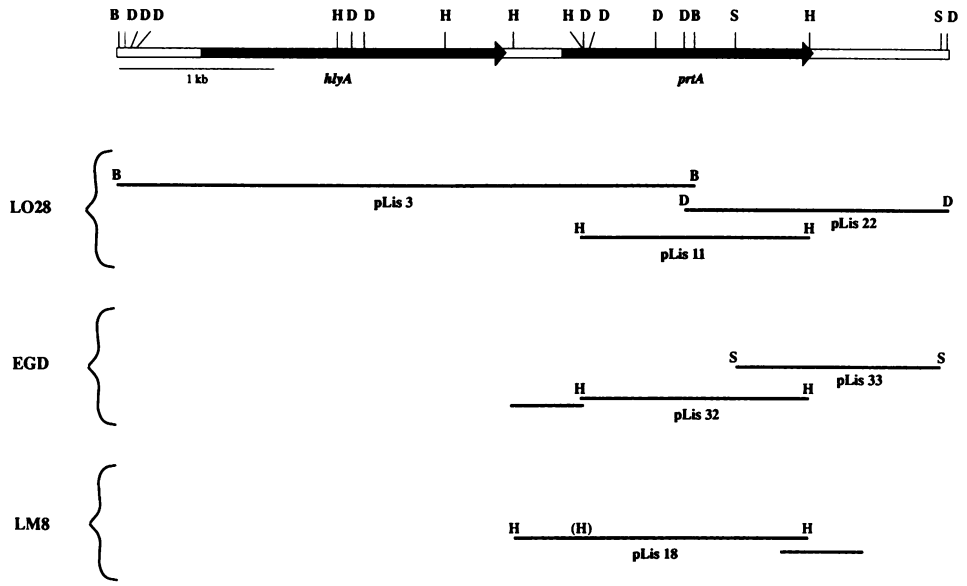

FIG. 1. Schematic drawing and partial restriction map of the *prtA* gene region from strain LO28. The *hlyA* and *prtA* genes are represented by arrows. Restriction sites: H, *Hin*dIII; B, *Bam*HI, S, *Sph*I, D, *Dra*I. Overlapping restriction DNA fragments from strains LO28, EGD, and LM8, cloned in pUC18 and spanning the *prtA* gene, are represented by thick lines. The names of the pUC derivatives containing these fragments are indicated below the lines. Fragments obtained by polymerase chain reaction amplification for direct sequencing of the 5′ and 3′ parts of the *prtA* gene from strain EGD and LM8 are reported with no restriction site indication.

sponds to a G-to-A transition in the *Hin*dIII site in position 139 in LM8.

***prtA* encodes a protein homologous to zinc metalloproteases of the "thermolysin family."** The polypeptide encoded by *prtA* would be 510 amino acids long and have a calculated molecular weight of 57,375. The N-terminal part of the encoded protein has all the characteristics of a signal sequence of a gram-positive bacterium (30). A polypeptide homology search (12) in the NBRF data base revealed substantial similarity between the *prtA*-encoded protein and the amino acid sequences of metalloproteases from *Bacillus stearothermophilus* (26), *Bacillus thermoproteolyticus* (27), *Bacillus cereus* (23), *B. subtilis* (31), and *Bacillus amyloliquefaciens* (28). The prototype of this family of structurally related neutral proteases is thermolysin, the protease produced by *B. thermoproteolyticus*. Interestingly, two other proteins produced by pathogenic gram-negative bacteria, the major secreted protein (MSP) of *L. pneumophila* (4) and the elastase of *P. aeruginosa* (2), also belong to this family.

In all cases where the gene has been cloned and sequenced, a discrepancy between the gene size and the mature protein length was observed, indicating that these proteases are processed from a proprotease into an active form (Fig. 3B). On the basis of protein sequence comparison, the N-terminal amino acid of the *Listeria* mature protease would be located around position 204 (Fig. 2). The mature form of the protease would have a molecular weight of 34,522. Similarities between the putative *Listeria* protease and the other proteases are spread along the whole sequence and are stronger when the *prtA*-encoded protein is compared with proteases from gram-positive bacteria than when it is compared with proteases from gram-negative bacteria. In Fig. 3A, sequences of the putative mature form of the *Listeria* protease (positions 204 to 510) and mature forms of *Bacillus* proteases were aligned (21). Percentages of identity calculated on the basis of this alignment are 35% with *B. cereus*, 40% with *B. thermoproteolyticus* and *B. stearothermophilus*, and 47% with *B. subtilis* and *B. amyloliquefaciens*. For *L. pneumophila*, *P. aeruginosa*, or *Serratia* sp., the percentage of identity is around 25 to 30%.

The X-ray structure of one of these proteases, thermolysin, has been solved and shown to contain one zinc atom bound to His-142, His-146, and Glu-166. The active site is located around His-231, and two additional sites required for catalysis are located at Glu-143 and Asp-226 (7, 11). The regions from amino acids 346 to 355, 368 to 377, and 428 to 448 of the *Listeria* protease are very similar to the zinc-binding or active site regions of thermolysin (amino acids 138 to 148, 161 to 170, and 222 to 242) (Fig. 3C). On the basis of these similarities, the predicted zinc-binding amino acids of the *prtA*-encoded protein would be His-349, His-353, and Glu-373, and the predicted active site would be His-437. Glu-350 and Asp-432 might also be essential for activity of the protein, by analogy with Glu-143 and Asp-226 of thermolysin. Three zinc ligands and one active site were also predicted for *B. subtilis* (31), *Serratia* sp. (19), *P. aeruginosa* (2), and *L. pneumophila* (4) proteases on the basis of their comparison with thermolysin. The predicted zinc-binding regions of *prtA*-encoded protein are also similar to the zinc-binding regions of these eight other metalloproteases. Recently, sequences of the gp63 metalloproteases produced by the parasitic protozoa *Leshmania chagasi* and *L. major* have been reported (17). They contain the conserved zinc-binding domain, but the overall similarity with bacterial metalloproteases, including the putative *Listeria* protease, is very weak (Fig. 3C).

***prtA* is the first gene of an operon.** To evaluate the role of *prtA* in virulence and determine whether the insertion of Tn*1545* had a polar effect on adjacent genes, we studied the structural and transcriptional organization of the *prtA* gene region. As previously reported, the upstream region encodes LLO and is transcribed as a monocistronic unit (15). The

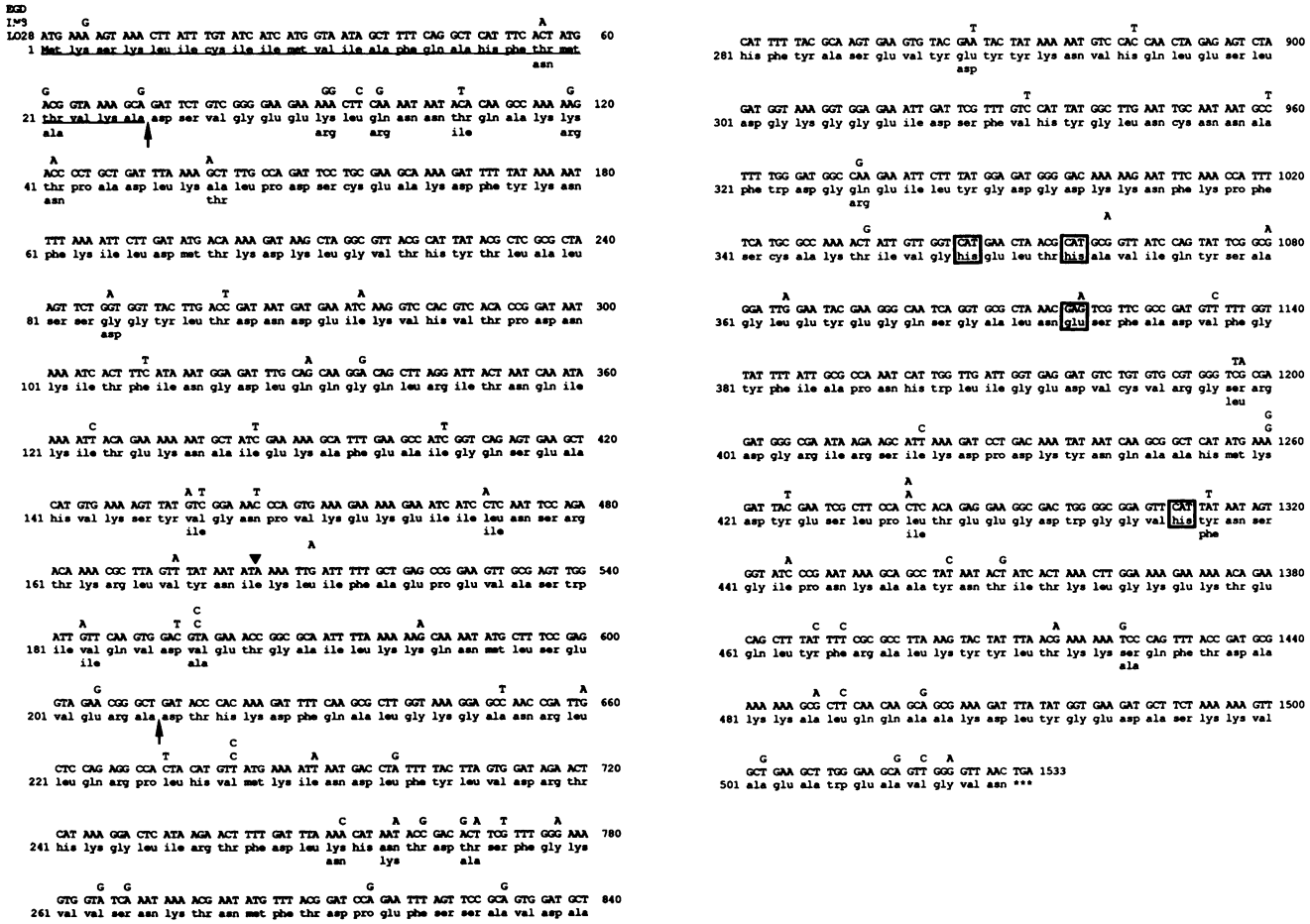

FIG. 2. Complete nucleotide sequence of the *prtA* genes from strains LO28, EGD, and LM8. Only nucleotide changes and amino acid substitutions in the sequences of strains LM8 (serovar 4b) and EGD (serovar 1/2a) compared with the sequence of strain LO28 (serovar 1/2c) are reported. The amino acid sequence deduced from the nucleotide sequence is given under the nucleotide sequence. Nucleotides are numbered on the right side; amino acids are numbered on the left side. The putative signal sequence is underlined. Putative cleavage sites of the signal sequence and of the propeptide (see Results) are indicated by vertical arrows. The insertion point of Tn*1545* is indicated (▼). Amino acids involved in zinc atom-binding and catalytic activities are boxed.

presence of putative ORFs downstream from *prtA* was screened for by use of a program based on codon usage, taking *prtA* as the reference gene (24). One major ORF with a good probability of being a coding sequence was detected. This ORF, in the same orientation as *prtA*, started 199 bp downstream from *prtA* and was not interrupted by a stop codon in the insert of pLis22. In contrast to *hlyA* and ORF U (15), the ORF identified upstream from *hlyA*, palindromic sequences corresponding to putative transcriptional termination signals was not found downstream from *prtA*.

The promoter of *prtA* was previously mapped and shown to be transcribed independently from a promoter located 150 bp upstream from the putative translation initiation codon of *prtA* (15). Northern blot analysis determined the size of the *prtA* transcripts. When total RNAs extracted from bacteria in late-exponential growth were hybridized with a 531-bp *Sau*96-A–*Bam*HI fragment internal to the *prtA* gene, one major band was detected that correspond to transcripts of about 5,700 nucleotides (Fig. 4). This is larger than *prtA*, and the band probably accounts for more than *prtA* and the ORF located immediately downstream from *prtA*.

DISCUSSION

In this paper, we have shown (i) that the locus of insertion of a transposon that resulted in a lecithinase-negative phenotype and impairment of virulence mapped in ORF D, which is located downstream from the LLO gene; (ii) that ORF D, designated herein *prtA*, encodes a protein homologous to bacterial metalloproteases; and (iii) that *prtA* is the first gene of a 6-kb operon. In addition, we sequenced the gene from three different strains and showed that the gene from an epidemic strain has the most distantly related sequence.

prtA was suspected to play a role in virulence on the basis of two pieces of evidence (i) Southern blotting experiments performed with all species of the genus *Listeria* had shown that this region was specific to *L. monocytogenes* even at a low stringency of hybridization (10). (ii) Promoters of *prtA* and *hlyA* share common structural features, suggesting that those two genes might be similarly regulated (15). Transposon Tn*1545*, in the less virulent lecithinase-negative mutant, interrupted *prtA*. This indicates that the *prtA* gene region is involved in virulence. Since *prtA* is the first gene of a 6-kb

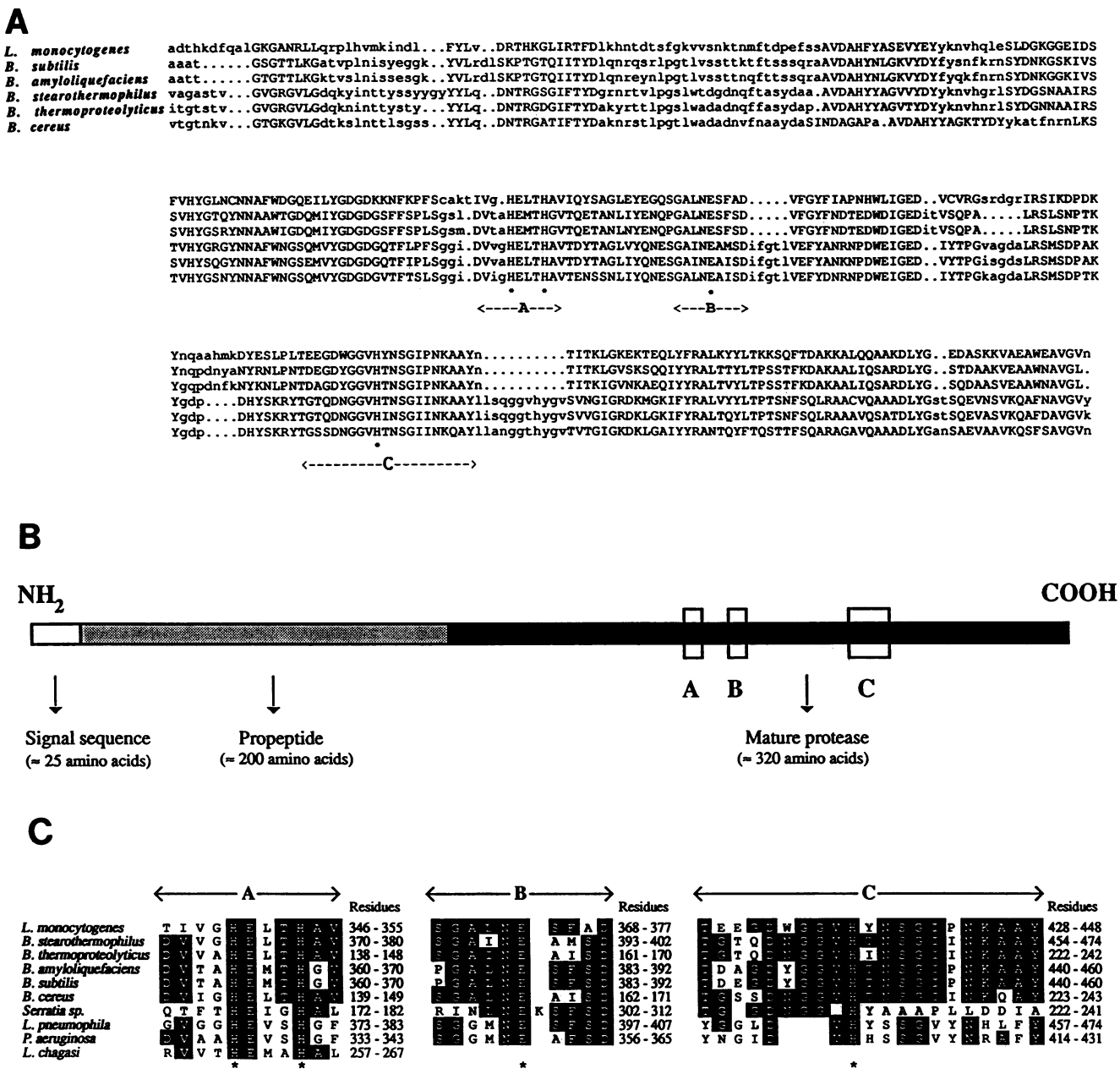

FIG. 3. (A) Comparison of the putative mature form of *Listeria* protease and mature forms of *Bacillus* metalloproteases. Blocks of conserved regions are indicated with uppercase letters as described previously (21). Amino acids involved in zinc atom-binding and catalytic activities are indicated (*). (B) Schematic representation of bacterial metalloprotease structure. A, B, and C correspond to highly conserved regions. (C) Amino acid sequence comparison of three highly conserved domains, A, B, and C, among the bacterial metalloproteases. Identical amino acids are indicated by black boxes.

operon, it is highly probable that insertion of Tn*1545* had at least a partial polar effect and that a lecithinase gene or a gene involved in the lecithinase activity expression is present at some distance downstream from *prtA*. This hypothesis is corroborated by the recent finding of other lecithinase-negative mutations mapping downstream from *prtA* in a gene presenting no homology with other phospholipase C genes (our unpublished results). Whether the *prtA* gene product itself or adjacent genes play a role in lecithinase expression and in virulence remains to be clarified.

In this study, we cloned and sequenced the *prtA* gene from three *L. monocytogenes* strains, EGD (serovar 1/2a), LO28 (serovar 1/2c), and LM8 (serovar 4b). Those strains had been classified in two groups on the basis of a restriction fragment length polymorphism in the *prtA* gene region (10; unpublished results). By multilocus enzyme electrophoresis analysis, Piffaretti et al. (20) estimated the genetic distances between 175 *L. monocytogenes* strains and classified them in two distinct groups. In this classification, LO28 and EGD were in the same group; LM8 was in a different group that contained all of the epidemic strains tested (20). Comparison of the three *prtA* sequences is in agreement with a shorter

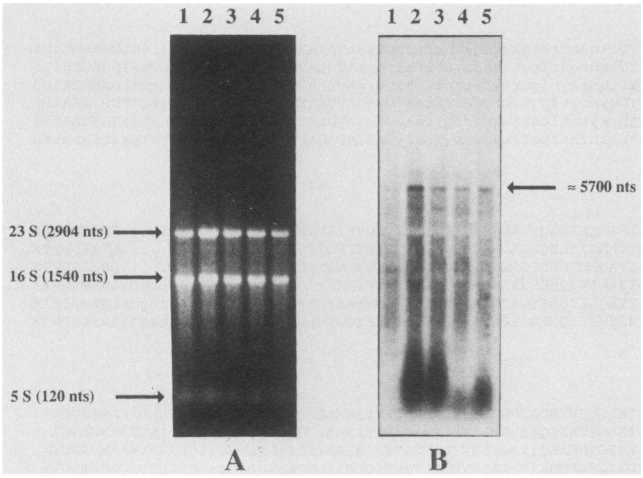

FIG. 4. (A) Agarose gel electrophoresis of 25 μg of total RNA from *L. monocytogenes* LO28. Total RNA was extracted as described in Materials and Methods from cultures in late-exponential growth (A_{600}, 1.7). Samples 1, 2, 3, 4, and 5, respectively, correspond to cultures at 10, 11, 12, 13, and 14 h. rRNAs are indicated by arrows and used as size standards (in nucleotides [nts]). (B) Autoradiogram of Northern blot prepared from the gel shown in panel A and hybridized with a ^{32}P-labeled *prtA* probe. The probe is the 531-bp *Sau*96-A–*Bam*HI fragment internal to *prtA* and purified from pLis3. The approximate size of the major *prtA* transcript is indicated.

genetic distance between EGD and LO28 than between LO28 or EGD and LM8.

On the basis of amino acid sequence comparison, *prtA* was found to encode a protease closely related to metalloproteases produced by *Bacillus* species and, to a lesser extent, to those produced by gram-negative bacteria such as the elastase of *P. aeruginosa*, MSP of *L. pneumophila*, and the protease of *Serratia* sp. The role of elastase in *P. aeruginosa* virulence has not been clearly established. For *L. pneumophila*, it has recently been shown that MSP plays no role in intracellular growth or cell killing (5, 25). Nevertheless, for these two human pathogens, proteolytic activity of the protease is easily detected. This type of activity has not been described in *Listeria* species. In this work, the gene *prtA* was shown to be transcribed, but the proteolytic activity, despite several attempts, was not detected. We were unable so far to find the substrate that would allow detection of the proteolytic activity, to detect a cross-reacting material with antibodies raised against thermolysin or MSP, or to identify conditions in which proteolytic activity could be expressed. In bacilli metalloproteases are synthesized as proenzymes. Cleavage of the amino-terminal part of the molecule releases the active form of the protease. In *Listeria* species, this proteolytic activation might occur in environmental conditions that remain to be discovered.

ACKNOWLEDGMENTS

We thank J. Davies for his constant interest in this work, K. Reich and H. Shuman for help in detecting the activity, and M. Horwitz for the gift of anti-MSP monoclonal antibodies.

This work was supported by the World Health Organization as part of its program for "Transdisease Vaccinology," the EEC (action de stimulation, contrat de jumelage no. STJ2-0319-C), the Institut National de la Santé et de la Recherche Scientifique CRE no. 891003, the Centre National de la Recherche Scientifique U.R.A. 209, and the Pasteur Institute.

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