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Expression in *Escherichia coli* and Sequence Analysis of the Listeriolysin O Determinant of *Listeria monocytogenes*

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To evaluate the role of hemolysin production in the virulence of *Listeria monocytogenes*, we have undertaken the analysis of the chromosomal region containing *hlyA*, the gene coding for listeriolysin O. A recombinant cosmid, conferring a hemolytic phenotype to *Escherichia coli*, was shown to express listeriolysin O, by immunoblotting with a specific antiserum against listeriolysin O. The presence of *hlyA* on the cosmid was demonstrated by DNA hybridization with a probe previously shown to contain part of *hlyA*. The complete nucleotide sequence of *hlyA* has been determined. The deduced protein sequence reveals the presence of a putative 25-amino-acid signal sequence: the secreted form of listeriolysin O would have 504 amino acids, in agreement with the molecular weight of purified listeriolysin O (58,000). The protein sequence is highly homologous to those of streptolysin O and pneumolysin. A peptide of 11 amino acids conserved in the three proteins contains the unique cysteine known to be essential for lytic activity. By DNA-DNA hybridization, the listeriolysin O gene was detected in all *L. monocytogenes* strains tested, even in the nonhemolytic type strain. The gene was absent in other species of the genus *Listeria*.

Listeria monocytogenes is a facultative intracellular gram-positive bacterium (34). In the genus *Listeria*, only *L. monocytogenes* and *L. ivanovii* are pathogens for humans and animals. *L. monocytogenes* is increasingly recognized as being responsible for severe infections in both animals and humans. Pregnant women, newborn babies, and immunocompromised patients are especially susceptible to infection.

Among the virulence factors which enable this organism to enter, survive, and grow within cells, including macrophages (27), the SH-activated hemolysin is a serious candidate. The first observation which suggested a correlation between hemolysin and virulence was that all nonhemolytic *Listeria* strains are experimentally nonpathogenic and that all pathogenic strains of *Listeria* produce zones of hemolysis on blood agar plates (3, 20, 33). As hemolysin production is a phenotype easily identified on blood agar plates, genetic studies were undertaken, and transposon mutagenesis was performed to obtain a nonhemolytic (Hly^-) mutant. This mutant was avirulent; insertion of a single copy of the transposon had inactivated the hemolytic phenotype. Spontaneous loss of the transposon led to the recovery of the Hly^+ phenotype and virulence (17). It was further shown that the Hly^- mutant, although phagocytosed at the same rate as that of the hemolytic revertant strain, stayed in the phagolysosome and failed to replicate significantly within the human enterocytelike cell line Caco-2. Electron microscopic study demonstrated that bacteria from the Hly^- mutant remained inside the phagosomes during cellular infection, whereas hemolytic bacteria from *L. monocytogenes* became free in the cytoplasm (16). These data were a strong indication that disruption of vacuole membranes by hemolysin-producing strains of *L. monocytogenes* might be a key mechanism allowing bacteria to escape from phagosomes and to multiply within the cell cytoplasm.

The SH-activated hemolysin (listeriolysin O) has been purified from *L. monocytogenes* culture supernatants. It shares the typical properties of other bacterial sulfhydryl-activated toxins: (i) inhibition by very low amounts of cholesterol, (ii) activation by reducing agents and suppression of the lytic activity by oxidation, and (iii) cross-reactivity with streptolysin O (18). Antiserum raised against the purified protein allowed the demonstration that the Hly^- mutant produced a truncated protein, indicating that the transposon had inserted in the listeriolysin O structural gene. Accordingly, the region of insertion of the transposon was cloned, and sequence analysis showed that it had inserted in an open reading frame (ORF). The deduced sequence of this ORF shared homologies with streptolysin O and pneumolysin. This allowed us to identify the locus of insertion of the transposon in the listeriolysin O gene, called *hlyA* (29). Several questions were still unsolved. First, is the Hly^- phenotype only due to the inactivation of the *hlyA* gene? And is the avirulence due only to this inactivation, or does the mutation affect expression of other genes?

To elucidate the role of listeriolysin O in pathogenicity, and to clarify the conflicting views on the nature and the number of *Listeria* hemolysin(s) (31), we undertook a structural and functional analysis of the chromosomal region carrying *hlyA*. In this paper, we present the complete nucleotide sequence of *hlyA* and an extensive analysis of the deduced protein sequence, especially a comparison with other membrane-damaging thiol-activated hemolysins. In addition, DNA-DNA hybridization studies demonstrate that this sequence is present only in *L. monocytogenes* in the genus *Listeria*.

MATERIALS AND METHODS

Bacterial strains. The bacterial strains used in this study are listed in Table 1.

Media. *Escherichia coli* HB101 was grown at 37°C in L-B medium. For plasmid-containing derivatives, ampicillin was

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TABLE 1. Properties of the bacterial strains

Designation	Strain	Genotype	Source or reference
EGD	*L. monocytogenes* serovar 1/2a		Trudeau Institut (17)
LO28	*L. monocytogenes* serovar 1/2c		Hopital Ramon y Cajal collection
HB101	*E. coli*	F *hsdS20*(r_B, m_B) *recA13 ara-14 proA2 lacY1 galK2 rpsL20* (Sm^r) *xyl-5 mtl-1 supE44* λ^-	(7)
TG1	*E. coli*	K-12, Δ(*lac-pro*) *supE thi hsdD5* F′ *traD36 proA*$^+$*B*$^+$ *lacI*^{q} *lacZ* ΔM15	Gibson (Medical Research Council, Cambridge, U.K.)

added at a final concentration of 25 μg/ml in liquid and 100 μg/ml in solid medium. Strain TG1 was routinely plated on minimal medium containing 0.2% glucose and grown at 37°C. Isolated colonies were used to inoculate 2 × YT liquid medium (tryptone [16 g/l] [Difco Laboratories, Detroit, Mich.], yeast extract [10 g/l] [Difco], sodium chloride [5 g/l]) for overnight cultures, which were subsequently diluted for infection with M13 derivatives or for transformation. Brain heart infusion–5% horse blood agar plates were used to detect hemolytic activity.

Chemicals and enzymes. Restriction enzymes and ligase were purchased from Amersham International plc., Boehringer GmbH, Mannheim, Federal Republic of Germany, or Genofit SA, Geneva, Switzerland and were used as prescribed by the manufacturer. Most solutions were filtered through nitrocellulose filters (0.45 μm) and, when possible, kept frozen at −20°C. [^{35}S]dATP (800 Ci/mmol), [^{35}S]methionine (1,000 Ci/mmol), and sequencing kits were purchased from Amersham International. A "cyclone" system kit and M13 replicative forms were purchased from International Biotechnologies, Inc., New Haven, Conn. Alternatively, nucleotides or dideoxynucleotides were purchased from Boehringer.

DNA techniques. Plasmid DNA was purified by ultracentrifugation in cesium chloride gradients (28). Rapid preparation of plasmid DNA was performed by the method of Birnboim and Doly (6). DNA fragments were purified by electroelution in dialysis bags after electrophoresis on thin (0.35-mm) polyacrylamide gels. Generally, 100 μg of plasmid digest was layered in a 10-cm well. Electrophoresis was carried out in Tris-borate buffer (10^{-2} M Tris base, 2.5 × 10^{-3} M EDTA [pH 8.4], 9 × 10^{-2} M H_3BO_3).

Listeria chromosomal DNA was prepared by a method adapted from that of Flamm et al. (15). Overnight cultures (5 ml) of *L. monocytogenes* in brain heart infusion were centrifuged. Pellets were washed in 1 ml of 0.1× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) and suspended in 0.6 ml of lysozyme solution (0.01 M sodium phosphate, 20% sucrose [pH 7], 2.5 mg of lysozyme per ml, freshly added). The mixture was incubated for 1 h at 37°C, and then 5.4 ml of proteinase K solution (10 mM Tris hydrochloride [pH 8], 1 mM EDTA, 1% sodium dodecyl sulfate [SDS], 500 μg of proteinase K per ml, freshly added) was added. After 1 or 2 h at 37°C, the mixture was gently extracted three times with 6 ml of saturated phenol and then successively chloroform and ether extracted. Then, 600 μl of sodium chloride was added, and DNA was ethanol precipitated for at least 2 h at −20°C. DNA was then suspended in 0.5 ml of TE (10^{-2} M Tris hydrochloride [pH 8], 10^{-3} M EDTA) containing RNase (50 μg/ml). Yield was about 10 μg of DNA per ml of overnight culture.

Subcloning in M13-derived vectors. Purified DNA fragments (500 ng) were ligated with linearized replicative forms (500 ng) of M13mp18, M13mp19, and M13mp21 (30) in a total volume of 10 μl. Competent TG1 cells were transformed as described in the Amersham manual (M13 cloning and sequencing handbook). Single-stranded DNA from white plaques was prepared from 7-h cultures, as described by Carter et al. (9), except that the RNase digestion step was omitted.

Nucleotide sequence determination. The dideoxy chain terminator sequencing method was used with the modification of Biggin et al. (5). The technique which generates sequential deletions of the insert, starting at the cloning site, by use of T4 polymerase (11) was used.

Ultrasonic disruption of transformed *E. coli*. Overnight cultures of 500 ml were washed and suspended in a 1/100 volume of phosphate-buffered saline (10 mM sodium phosphate [pH 6.0], 0.15 M sodium chloride) and subjected to ultrasonic disruption at 4°C. The cells were sonicated three times for 10 s each and allowed to cool for 30 s between treatments. The suspension was centrifuged (10,000 × *g*, 15 min, 4°C), and the supernatant was taken for determination of the hemolytic titer. To prepare samples for SDS-polyacrylamide gel electrophoresis and immunoblotting, the protein extract was concentrated by adding 50 μl of a cholesterol solution (10 mg/ml in ethanol) to the supernatant. After centrifugation, the protein-cholesterol pellet was dissolved in 50 μl of 10% SDS solution.

Analysis of plasmid-encoded proteins by SDS-polyacrylamide gel electrophoresis and immunoblotting. SDS-polyacrylamide gradient (7.5 to 25%) gel electrophoresis was performed by the method of Laemmli (24). Volumes of 50 μl of sample buffer (2% [wt/vol] SDS, 10% [vol/vol] glycerol, 5% [vol/vol] 2-mercaptoethanol, 0.001% bromophenol blue in 62.5 mM Tris hydrochloride buffer [pH 6.8]) were added to the samples prepared as described above. Proteins were detected by Coomassie blue staining.

For Western blot (immunoblot) analysis (38), proteins were electrophoretically transferred to nitrocellulose sheets in transfer buffer (25 mM Tris, 200 mM glycine, pH 8.4) containing 20% (vol/vol) methanol. Sheets were incubated for 1 h at room temperature with shaking in TSB (50 mM Tris, 150 mM NaCl solution, pH 8.0) containing 5% (wt/vol) skim milk (Regilait, France Lait, Saint Martin-belle-roche, France) and then incubated for 1 h in anti-listeriolysin O immune serum diluted (1:20) in TSB. Sheets were washed eight times in TSB and then incubated for 1 h in 20 ml of TSB containing 1 μCi (0.37 kBq) of ^{125}I-protein A. Filters were washed six times in TSB supplemented with 0.1% Triton X-100, dried at 80°C, and autoradiographed.

Anti-listeriolysin O serum preparation. Rabbits were immunized by repeated subcutaneous inoculations of purified listeriolysin O (18). The protocol was based on three injections of 75 μg of toxin (in complete Freund adjuvant at days 0, 7, and 14) and one injection of the same dose in incomplete Freund adjuvant (day 21). Blood was harvested 2 weeks after the last injection.

Hemolytic activity titration. The hemolytic activity of the toxin towards sheep erythrocytes was tested as previously described (2). Measurement was made of the absorbance at 541 nm of hemoglobin released from erythrocytes (6 × 10^8

cells per ml) incubated (at 37°C for 45 min) with 1 ml of appropriately diluted toxin in phosphate-buffered saline (pH 6.0) containing 0.1% bovine albumin. The amount of toxin needed to release half the hemoglobin (50% lysis) of the erythrocytes corresponds to 1 hemolytic unit. It is estimated graphically by plotting percent lysis versus toxin volume on a log-probit graph (2).

Southern blot hybridizations. For DNA hybridizations, the purified fragments were labeled by the Multiprime Labeling System of Amersham, *Listeria* DNA was digested by *Hin*dIII, and electrophoresis was carried out in Tris-borate buffer in horizontal 0.7% agarose gels for 18 h at 25 V. DNA was transferred from the agarose to nitrocellulose filters by the method of Southern (28). Prehybridization (1 h) and hybridization with labeled probes (16 h) were carried out at 42°C in 50% formamide–5× SSPE (ref. 28)–10× Denhardt buffer, containing 200 μg of sonicated carrier DNA per ml, in sealed plastic bags. The filters were then washed twice in 1× SSC (ref. 28)–0.1% SDS for 30 min at room temperature and exposed to film at –80°C. Succeeding washings were performed in 0.1× SSC–0.1% SDS at room temperature, and the filters were then reexposed. If necessary, washes were continued in the same buffer at higher temperatures.

RESULTS

Identification of the DNA region encoding listeriolysin O. Chromosomal DNA of *L. monocytogenes* L028, digested with *Mbo*I, had been cloned in the *Bam*HI site of the cosmid vector pHC79 (21, 40). After transformation of *E. coli* HB101, hemolytic clones were obtained that were identified as ampicillin-resistant colonies producing a halo of lysis on ampicillin-blood agar plates. Spontaneous deletion of the original clones led to a stable derivative, pCL101. Two deletions were then created between identical restriction sites (*Bam*HI and *Sal*I) on the cosmid pCL101 to give pCL102, which contained an insert of 8.5 kilobases of *L. monocytogenes* DNA (39, 40) (Fig. 1). Hemolytic activity could be detected in the extracts of HB101 harboring pCL101 or pCL102 (7.5 hemolytic units per ml of overnight culture). It was lower than that measured in the *Listeria* supernatants (64 hemolytic units per ml of overnight culture). In addition, listeriolysin O production was detected by Western blot analysis of the extracts, using an antiserum against purified listeriolysin O (data not shown). To localize the gene coding for listeriolysin O in the insert, we took advantage of the recent isolation of a DNA probe shown to contain part of the gene (29), which had been isolated from an Hly$^-$ mutant obtained by transposon mutagenesis with Tn*1545*. This mutant had been shown to produce a truncated protein, indicating that the transposon had inserted in the listeriolysin O gene. A 400-base-pair fragment containing the junction between the transposon and the *Listeria* chromosome had been cloned and sequenced. The results showed that the transposon was inserted in an ORF, namely, the listeriolysin O gene. We used this 400-base-pair DNA fragment carrying part of the listeriolysin O gene as a probe to localize the listeriolysin O gene in pCL102. A 410-base-pair *Hin*dIII fragment of pCL102 hybridized to the probe (Fig. 1). The sequence of this fragment, in the region common to the probe, was identical to the sequence obtained previously. The presence of a stop codon indicated that this fragment presumably contained the end of the listeriolysin O gene. The DNA region spanning upstream from this fragment was then sequenced.

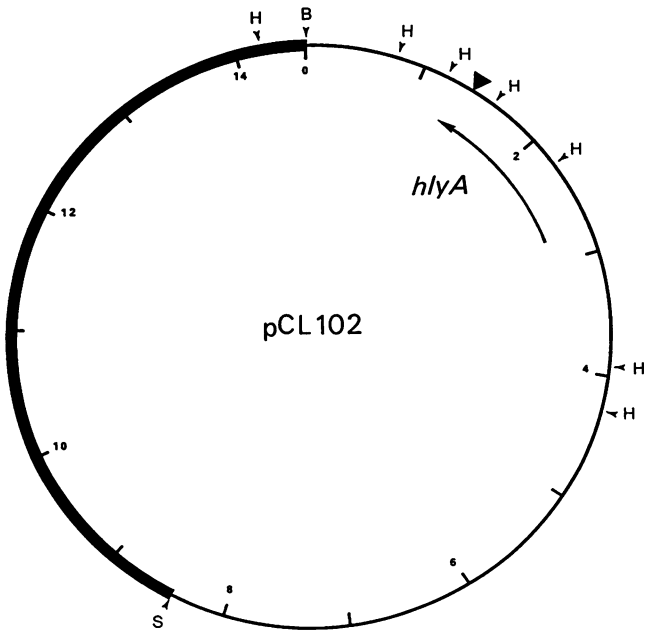

FIG. 1. Physical map of pCL102. Mapping was based on analyses of single and double digestions with the appropriate restriction enzymes. Coordinates in kilobases and the location of the *hlyA* gene are indicated inside the circle. Outside the circle, restriction sites indicated are *Bam*HI (B), *Sal*I (S), and *Hin*dIII (H). Arrowhead corresponds to the insertion of transposon Tn*1545* in the chromosome (17, 29).

Nucleotide sequence of the listeriolysin O gene. The nucleotide sequence of the listeriolysin O gene and its upstream region, completely determined on both strands of the DNA, is presented in Fig. 2. The ORF which ends in the 410-base-pair *Hin*dIII fragment is 1,617 base pairs long; the first ATG in this ORF is located 30 nucleotides downstream from its beginning. It is preceded, 10 nucleotides upstream, by a hexanucleotide (AAGGAG) complementary to the 3′ end of the 16S RNA of *L. monocytogenes* (26) which might be the ribosome binding site (Shine-Dalgarno) for the listeriolysin O gene. This ATG was therefore considered as the putative start codon of the listeriolysin O gene.

Analysis of the deduced protein sequence. The protein encoded by the ORF starting at the ATG is 529 amino acids long (58.6 kilodaltons). Its amino-terminal sequence presents all the characteristics of signal sequences of gram-positive bacteria (41), as follows. The first residues are hydrophilic and positively charged. They are followed by about 20 hydrophobic residues. The putative cleavage site by the signal peptidase lies probably after lysine 25, as the sequence starting at residue 26 is homologous to the amino-terminal sequence of the SH-dependent hemolysin secreted by *L. ivanovii* (J. Kreft, 4th International Conference on Genetics and Biotechnology of Bacilli, San Diego, Calif., 1987). The signal sequence of listeriolysin O would have a length comparable to the average length of the signal sequences of gram-positive bacteria genes (41). Consequently, the secreted listeriolysin O (without the signal sequence) would contain 504 amino acids and have a molecular mass of 55.8 kilodaltons. This value is in agreement with the molecular weight of the protein purified from *L. monocytogenes* culture supernatants (18). Analysis of the amino acid composition of the protein did not reveal any special feature except the presence of a unique cysteine; this residue is located in the carboxy-terminal part of the sequence, in position 484.

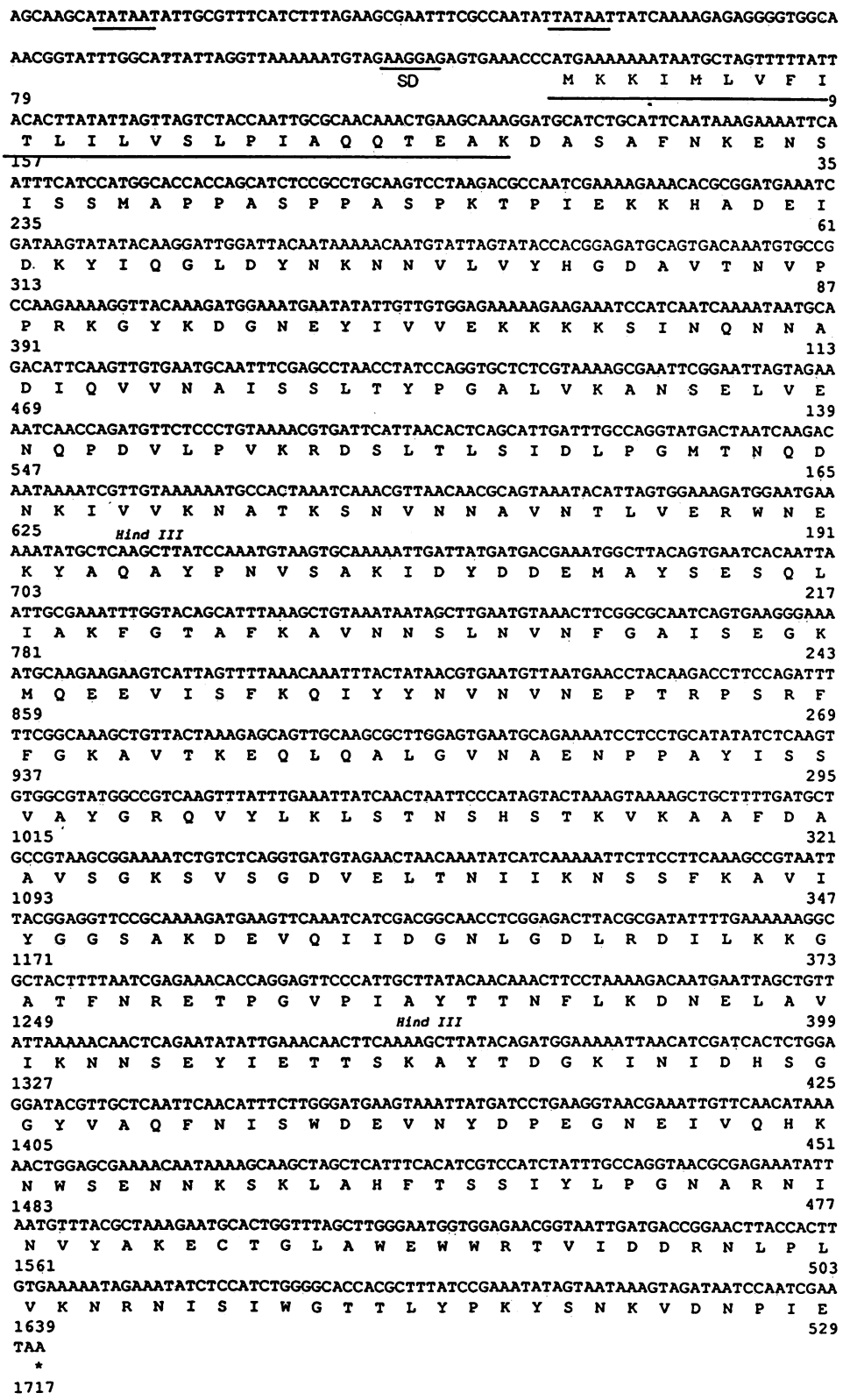

FIG. 2. Nucleotide sequence of the *hlyA* gene and its proximal region (see the text). The *Hin*dIII sites present in the sequenced region are shown over the DNA sequence. The deduced amino acid sequence of the *hlyA* gene, in the single-letter code, is shown. The putative Pribnow boxes and Shine-Dalgarno sequences are underlined (see text), as well as the signal sequence. Coordinates indicated under the sequences at the left or right end of each line correspond respectively to the nucleotide sequence and the deduced peptide sequence.

Comparison of listeriolysin O with streptolysin O and pneumolysin. It is well known that SH-activated hemolysins immunologically cross-react (1). It has been recently shown that streptolysin O from *Streptococcus pyogenes* and pneumolysin from *Streptococcus pneumoniae* share homologies (23). These proteins have identical molecular weight, if one takes into account the secreted form of streptolysin O, the pneumolysin being a nonsecreted protein. They have a unique cysteine located in their carboxy-terminal part, and when one aligns the two sequences at the unique cysteine, the highest homology lies in the region of this unique cysteine. We compared the amino acid sequence of listeriolysin O with the sequences of streptolysin O and pneumolysin. The secreted forms of listeriolysin O and streptolysin O are, respectively, 504 and 471 amino acids long, while pneumolysin is 471 amino acids long. The putative signal sequence of listeriolysin (25 amino acids) is shorter than that of streptolysin (33 amino acids). The three sequences can be completely aligned at the unique cysteine, if one introduces two deletions of 1 amino acid for streptolysin O and one deletion of 1 amino acid for pneumolysin (Fig. 3 and Fig. 4). This alignment reveals strong homologies: the percentage of amino acid identity between two sequences compared two by two, in the common 469-residue region, is about 42 to 43%. Interestingly, the signal sequence of listeriolysin O corresponds to the N-terminal part of streptolysin O, the hydrophobic amino acids of listeriolysin O being changed for hydrophilic ones. This is the region of lowest homology (Fig. 4). Homologies between the three proteins are present along the whole sequence, but they are stronger towards the carboxy-terminal end. In particular, around the unique cysteine, an 11-amino-acid peptide is conserved in the three sequences. If one compares the sequences in terms of similarity (and not identity), one observes even stronger homologies, illustrated by the superimposition of the hydrophobicity profiles (data not shown).

Detection of *hlyA* in the different species of the genus *Listeria*. As listeriolysin O is considered to be a major virulence factor (4, 16, 17), it seemed interesting to test whether *hlyA* was present in the different species of the genus *Listeria*. By Southern blot analysis, we looked for the presence of *hlyA* in several *L. monocytogenes* strains and in the other species of the genus, i.e., *L. ivanovii*, *L. seeligeri*, *L. innocua*, *L. welshimeri*, and *L. murrayi* (Table 2). We used as a probe a DNA fragment internal to the gene, the 651-base-pair *Hin*dIII fragment which extends from codon 196 to codon 412. The results with the probe used were unambiguous: *hlyA* was only detected in *L. monocytogenes*, even in a strain which is nonhemolytic on blood agar plates, such as the type strain ATCC 15313.

DISCUSSION

The data presented here are a first step in the elucidation of the structure and regulation of the transcription unit

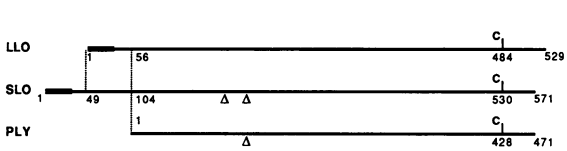

FIG. 3. Schematic comparison of the amino acid sequences of listeriolysin O (LLO), streptolysin O (SLO), and pneumolysin (PLY). Sequences, represented by lines, are aligned on the unique cysteine (C) in the homologous position. Signal sequences are indicated by thick lines. Δ indicates deletions of 1 amino acid, and the numbers correspond to the coordinates in the protein sequence.

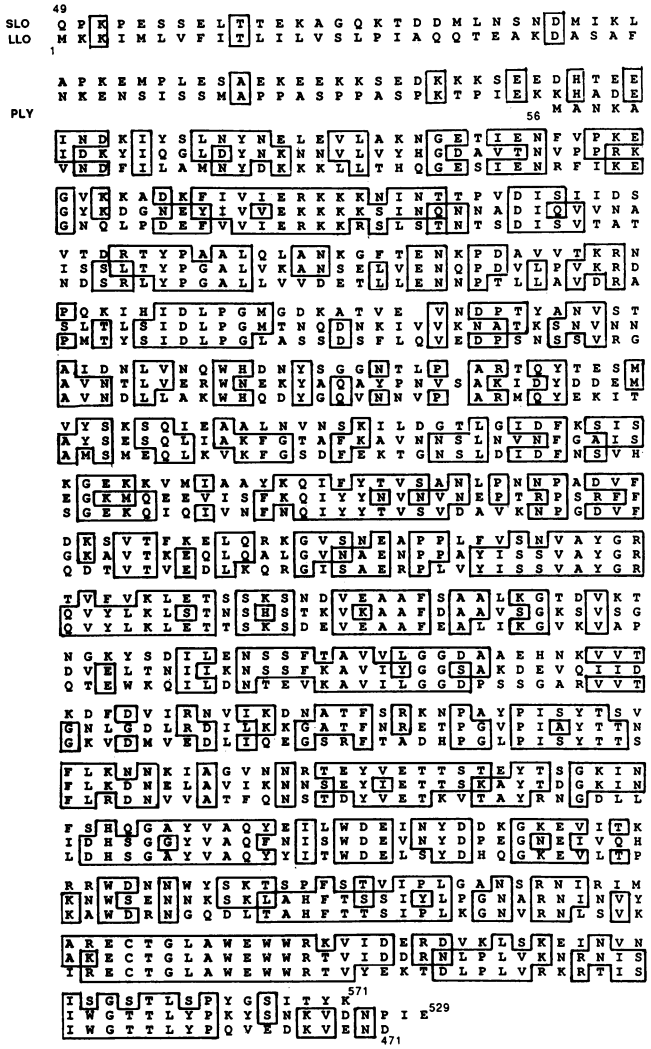

FIG. 4. Alignment of listeriolysin O (LLO), streptolysin O (SLO), and pneumolysin (PLY). Identical amino acids present in two or three sequences have been boxed. Numbers refer to the amino acid coordinates in the sequence.

containing the gene coding for listeriolysin O. This DNA region had been previously shown to play a crucial role in the virulence of *L. monocytogenes* (4, 16, 17). Here, we report the identification and the sequence analysis of *hlyA*, the gene coding for the SH-activated hemolysin of *L. monocytogenes*. This was facilitated by the recent availability of an anti-listeriolysin O antiserum (18) and of a DNA probe containing part of *hlyA* (29). *hlyA* is the first gene of *L. monocytogenes* to be sequenced. It has a GC content of 36%, in agreement with the previously determined GC% of *L. monocytogenes* (37). The exact location of the promoter is unknown: upstream from the ORF, two TATAAT sequences (Pribnow boxes) in positions 9–10 and 52–57 (Fig. 2) are detected, but these sequences are not preceded by a −35 site close to the consensus TTGACA (19). As the hemolysin production is known to be regulated by factors such as iron (10), we looked for sequences similar to the consensus identified as target for iron-binding regulatory proteins (13), but none was detected.

The *hlyA* determinant encodes for a secreted protein of 504 amino acids; its amino acid sequence is homologous to

TABLE 2. Strains tested in the DNA hybridization assays

Strain[a]	Hly phenotype	Hybridization to the 651-base-pair probe
L. monocytogenes		
ATCC 15313 (type strain)	−	+
E.G.D.[b]	+	+
E.G.D. Tn*1545* (18)	−	+
LO28[c]	+	+
CIP 7834	+	+
CIP 7835	+	+
N 62262	+	+
N 3636	+	+
N 2661	+	+
N 74217	+	+
N 8018	+	+
SLCC 5132	+	+
SLCC 5156	+	+
SLCC 3551	+	+
SLCC 4524	+	+
L. ivanovii		
CIP 7842 (type strain)	+	−
SLCC 4121	+	−
L. seeligeri		
CIP 100100 (type strain)	+	−
SLCC 3503	+	−
L. welshimeri		
SLCC 5334 (type strain)	−	−
SLCC 5872	−	−
L. innocua		
SLCC 6463 (type strain)	−	−
CIP 8011	−	−
L. murrayi CIP 76124 (type strain)	−	−

[a] ATCC, American Type Culture Collection, Rockville, Md. CIP, Collection de l'Institut Pasteur. N, Hôpital Necker collection, Paris. SLCC, Special Listeria Culture Collection, H. P. R. Seeliger, University of Würzburg, Würzburg, Federal Republic of Germany, and Institut Pasteur. Other origins are given.

[b] Trudeau Institute.

[c] Ramon y Cajal collection.

that of streptolysin O of *S. pyogenes* and pneumolysin of *S. pneumoniae*, in agreement with the known cross-reactivity of SH-dependent cytolysins (1). The three proteins contain a unique cysteine in a completely conserved undecapeptide. This remarkable feature is to be correlated with the fact that the cysteine is presumed to be essential for binding to cholesterol (32) and consequently for activity. Among SH-activated cytolysins, listeriolysin O is the only one to be produced by an intracellular microorganisms; it also has a distinctive optimum activity pH which is significantly lower than that of all other SH-activated toxins purified so far (18). The cytolytic activity is maximum at pH 5.5 and undetectable at pH 7, whereas the other toxins are mostly active at pH >6.5 to pH 7. It has been proposed that this property might allow *L. monocytogenes* to escape from the acidic phagolysosomal compartment and then replicate in the cytoplasm. Construction of hybrid genes will help in understanding which region is responsible for the low optimum activity pH. It will also be interesting to test whether this optimum pH correlates with the pathogenicity of *L. monocytogenes*.

At the DNA level, the *slo*, *ply*, and *hlyA* genes do not show substantial homology (between 52 and 54% homologies when comparing any two of the three genes). Nevertheless, in the region coding for the conserved undecapeptide, the homology at the DNA level is 73% between *ply* and *hlyA*, 88% between *slo* and *hlyA*, and 82% between *slo* and *ply*. This in agreement with previous results (22); M. Kehoe, using a DNA fragment internal to *slo* as a probe, could not detect homologies between *S. pyogenes* and *L. monocytogenes*. Indeed, the probe used did not correspond to the region of highest homology, and the conditions used could only detect 80% homology. It is highly probable that use of a probe corresponding to the nucleotide sequence coding for the common 11-amino-acid region would have led to different results.

The structural analysis of the chromosomal region containing *hlyA* was undertaken to evaluate the role of listeriolysin in pathogenicity. The first genetic experiments to correlate the hemolytic phenotype with virulence in *L. monocytogenes* were performed by Gaillard et al. (17). Using transposon mutagenesis, they obtained an Hly$^-$ mutant which was avirulent. By cloning and sequence analysis, we showed that the transposon had inserted in *hlyA* (29). This demonstrated that the region containing *hlyA* played a crucial role in virulence. The data presented here show that in the Hly$^-$ mutant the transposon was inserted in codon 481 of *hlyA*, three codons upstream from the cysteine codon, giving rise to a truncated protein devoid of hemolytic activity.

Since increasing evidence argues in favor of listeriolysin O as a major virulence factor (4, 16, 17, 29), we wondered whether *hlyA* was unique to *L. monocytogenes* in the genus *Listeria*. A fragment internal to *hlyA* was used as a probe, and all *L. monocytogenes* strains were positive in the test, even the nonhemolytic type strain. In that latter case, either the gene is present but not expressed or its expression is very low and has not been detected so far. Immunoblotting experiments are in progress to examine for the production of the protein in the strains we tested and in other strains. Preliminary results indicate that all clinical isolates produce listeriolysin O (4). No hybridization was detected in *L. ivanovii*, which is known to have an SH-activated hemolysin which cross-reacts with streptolysin O and listeriolysin O (1, 36). This is probably due to the nature of the probe. The gene was also undetected in *L. seeligeri*, which is hemolytic but in which the hemolysin is uncharacterized. Thus, *hlyA* is specific for *L. monocytogenes*. It is the first species-specific gene reported in the genus *Listeria*; it is specific for a pathogenic organism and could be used to detect its presence in food or other environments. Several techniques have been used to distinguish between pathogenic and nonpathogenic *Listeria* isolates, based either on biochemical reactions (20, 35), the CAMP test (8, 20, 25), or invasion in various cell lines (14, 16). The use of this specific DNA probe should be of great advantage as identification is accurate and rapid. The basis for a colony hybridization assay has been recently published for the genus *Listeria* (12), which should improve the rapidity of the identification of *L. monocytogenes*, especially in dairy and environmental samples.

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