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The *iap* Gene of *Listeria monocytogenes* Is Essential for Cell Viability, and Its Gene Product, p60, Has Bacteriolytic Activity

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Expression of the *iap* gene of *Listeria monocytogenes* in the *L. monocytogenes* rough mutant RIII and in *Bacillus subtilis* DB104 caused the disruption of the cell chains which these two strains normally form under exponential growth conditions. The p60 protein produced by *L. monocytogenes* and *B. subtilis* DB104 also exhibited bacteriolytic activity detected in denaturing polyacrylamide gels containing heat-killed *Micrococcus lysodeikticus*. Purification of the p60 protein led to aggregation of p60 and loss of the cell chain disruption and bacteriolytic activities. A cysteine residue in the C-terminal part of p60 which is conserved in all p60-like proteins from the other *Listeria* species seems to be essential for both activities. The *iap* gene could not be inactivated without a loss of cell viability, indicating that p60 is an essential housekeeping protein for *L. monocytogenes* and probably also for other *Listeria* species. These data suggest that p60 possesses a murein hydrolase activity required for a late step in cell division.

Listeria monocytogenes is a gram-positive facultative intracellular bacterium which can cause severe food-borne infections in humans and animals (16). Virulent strains of *L. monocytogenes* are able to survive and multiply within host macrophages and can invade, replicate, and multiply in nonprofessional phagocytes such as mouse 3T6 fibroblasts (35) and the human colon carcinoma cell line Caco-2 (23). A pathway of entry into epithelial cells is mediated by internalin, an 80-kDa surface protein which has been identified recently (22). After uptake, *listeriae* escape from the phagosome via the action of a cytolytic toxin, listeriolysin, which is an essential virulence factor (23, 35, 43). In the host cell cytoplasm, the bacteria replicate, become coated with actin filaments, and spread intracellularly and from cell to cell by forming a tail of polymerized actin (40, 52, 53). Other *L. monocytogenes* virulence factors with less well-defined roles include a phosphatidylinositol-specific phospholipase C, a metalloprotease, a lecithinase, and ActA, a gene product involved in actin polymerization (12, 13, 31, 38, and 57; for review, see reference 42). The genes for these virulence factors are located in a gene cluster and are positively regulated at the transcriptional level by the regulatory protein PrfA (39).

We have previously described spontaneously occurring rough mutants of *L. monocytogenes* which produce greatly reduced levels of a 60-kDa major extracellular protein termed p60 (34). These mutants show a rough colony morphology and form long chains of cells separated by double septa (34). Rough mutants have reduced virulence in the mouse model of infection (26, 44) and do not efficiently invade mouse 3T6 fibroblasts (34). Upon addition of partially purified p60 to the cell chains, the chains disaggregate, and the resulting single bacteria are invasive (34). These results suggested that p60 may be involved in the invasion of nonprofessional phagocytic cells and, in addition, that p60 may have a role in cell division.

The gene encoding p60 of *L. monocytogenes* EGD has been cloned and sequenced (33). Because of its possible role in invasion, the gene was named *iap* (invasion-associated protein). The *iap* gene encodes a polypeptide of 484 amino acids containing a 27-amino-acid signal sequence which yields a mature polypeptide of 47.5 kDa. Analysis of the deduced amino acid sequence showed that p60 is a basic protein (isoelectric point of 9.3) and contains a domain consisting of 19 Thr-Asn repeats (33). DNA hybridization and immunological studies showed that genes related to *iap* and p60-related proteins were found in all *Listeria* strains tested (9, 9a, 33). The *iap* gene is not regulated by PrfA. Recently, we reported that expression of *iap* is controlled at the posttranscriptional level (32).

In this article, we report the characterization of the cell-chain-shortening activity of the p60 protein. Genetic complementation leading to increased production of p60 in chain-forming *Listeria* and *Bacillus* strains led to shortening of cell chains, and, under certain conditions, p60 had bacteriolytic activity. In addition, we show that *iap* is an essential gene. Our results suggest that p60 is a murein hydrolase which has an essential role in cell division.

MATERIALS AND METHODS

Bacterial strains and plasmids. *L. monocytogenes* serovar 1/2a strain EGD was obtained from S. H. E. Kaufmann, University of Ulm, Ulm, Germany. The *L. monocytogenes* rough mutant RIII (44) is derived from a smooth strain of serovar 1/2a and was obtained from J. Potel, Institute for Medical Microbiology, Medical Academy, Hannover, Ger-

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many. *B. subtilis* DB104 (*his nprE18 nprR2 ΔaprA3*) (30) was obtained from R. Doi, University of California, Davis. This strain carries lesions in the structural genes for extracellular neutral (*nprE*) and serine (*aprA*) proteases. *Escherichia coli* DH5α (*endA1 hsdR17 supE44 thi-1 recA1 gyrA96 relA1*; BRL, Eggenstein, Germany) was used in cloning experiments. Streptococcal plasmid pGB363, which belongs to the pGB301 plasmid family (3), was kindly provided by D. Behnke, Jena, Germany. Plasmid pTV32ts (62) was obtained from P. Youngman. Plasmid pUC18 has been described previously (61). Plasmids constructed for this study are described in detail below.

Media and reagents. *L. monocytogenes* and *B. subtilis* strains were grown in brain heart infusion (BHI) broth (Difco laboratories, Detroit, Mich.) at 37°C. Erythromycin (5 μg/ml) and chloramphenicol (10 μg/ml) were added to broth or agar when required. For electroporation, *B. subtilis* was grown in LBSP medium composed of 1% Bacto Peptone, 0.5% yeast extract, 0.5% NaCl, 8.6% sucrose, and 0.1 M phosphate buffer, pH 7. LBSPG medium was LBSP medium containing 10% glycerol. *E. coli* was cultured in Luria-Bertani medium at 37°C with ampicillin (50 μg/ml) and erythromycin (300 μg/ml) when required. Restriction enzymes and T4 DNA ligase were purchased from Boehringer GmbH, Mannheim, Germany, and used according to the manufacturer's instructions. [γ-^{32}P]ATP (3,000 Ci/mmol) was purchased from Amersham.

DNA isolation and manipulations. The procedures for the isolation of plasmid and chromosomal DNAs from *L. monocytogenes* were as previously described (19, 60). DNA fragments used as probes were purified by electroelution from agarose gels and labeled by random priming (18). Standard protocols were used for recombinant DNA techniques (46).

Southern blot analysis. Chromosomal and plasmid DNAs were digested with appropriate restriction endonucleases, fractionated on a 1% Tris-borate agarose gel (46), and transferred to a nitrocellulose filter by the method of Southern (49). Hybridization and washing of the blots were performed as described earlier (60).

Construction of recombinant plasmids. The 7.0-kb plasmid pGB363-1 was derived from the streptococcal vector pGB363 (3), in which the gene coding for erythromycin resistance was replaced with a 2.2-kb *Hpa*I-*Bam*HI DNA fragment encoding the chloramphenicol resistance gene of plasmid pTV32(Ts) (62). This cloning step led to the deletion of the copy control region of pGB363 (3). Plasmid pGB363-1p60 (9.3 kb) contains a 2.3-kb fragment of chromosomal DNA from *L. monocytogenes* serovar 1/2a EGD encoding the *iap* gene (33) cloned into the *Bam*HI-*Eco*RI polylinker site of pGB363-1. The internal 250-bp *Hin*dIII-*Pst*I fragment of *iap* (33) was cloned into the recently described *E. coli-Listeria* shuttle vector pLSV1 (60) to create plasmid pLSV3.

Transformation of *Listeria* and *Bacillus* strains. *L. monocytogenes* EGD and RIII were transformed by a protoplast transformation method as described previously (60). *B. subtilis* DB104 was transformed with plasmids pGB363-1 and pGB363-1p60 by electroporation with a Gene Pulser apparatus (Bio-Rad Laboratories, Richmond, Calif.). Six hundred milliliters of LBSP medium was inoculated with 6 ml from an overnight culture of *B. subtilis* DB104, and the strain was grown at 37°C to early exponential growth phase. Cells were harvested by centrifugation, and the cell pellet was washed twice with 250 ml of SHMG medium (250 mM sucrose, 1 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid [HEPES]-imidazole, 1 mM magnesium gluconate, 10% glycerin) to remove medium salts. The cell pellet was resuspended in 0.5 ml of SHMG medium (approximately 1.5×10^{10} CFU/ml). Plasmid DNA (500 ng in 5 μl of double-distilled water [ddH_2O]) was added to 400 μl of cell suspension in an Eppendorf tube, mixed, and placed on ice for 20 min. The cell suspension was pipetted into a precooled, sterile electroporation cuvette (0.2-cm interelectrode gap). Electroporation conditions were 1,700 V and 25 μF. After electroporation, cells were suspended in 3.6 ml of LBSPG medium, incubated for 2 to 4 h at 37°C, plated onto Luria-Bertani plates containing chloramphenicol (10 μg/ml), and incubated at 37°C.

Plasmid curing in *L. monocytogenes*. The method described by Bouanchaud et al. (6) was adopted for *L. monocytogenes*. In brief, an overnight culture of the *Listeria* strain carrying the plasmid of interest was diluted 1:150 in BHI broth with ethidium bromide (3 μg/ml) and incubated overnight at 37°C with shaking. The culture was diluted with saline and plated onto BHI agar. Replica plating on BHI plates with and without the antibiotic selective for the plasmid allowed the determination of the rate of plasmid curing in the bacterial population.

Cell chain length determination. Overnight cultures of transformed *L. monocytogenes* RIII or *B. subtilis* DB104 were diluted 1:50 in BHI broth with erythromycin or chloramphenicol and incubated at 37°C with aeration. The optical densities of the cultures were measured with a Klett photometer (Klett Co., New York, N.Y.). At various time points, the lengths of the cell chains were determined under the microscope with an ocular micrometer. Fifty cells were measured per sample. The statistical significance of the mean values was confirmed by a Student *t* test.

Chain disruption assay. The chain disruption activity of p60 was tested with *L. monocytogenes* RIII cell chains from an exponentially growing culture as a substrate. Cell chains were washed with phosphate-buffered saline (PBS) and resuspended to an optical density at 600 nm of 1.0 in PBS containing 10% glycerol and stored at −20°C until use. To test for chain disruption activity, protein samples were diluted serially twofold in PBS containing 2 mM dithiothreitol (DTT), and 10 μl of diluted protein was mixed with 10 μl of RIII cells prepared as described above. Samples were incubated at 37°C for 1 to 3 h, and the degree of chain disruption was quantified by microscopic observation.

Purification of p60 protein. Five liters of BHI broth was filtered through a 10-kDa molecular mass cutoff membrane to remove high-molecular-mass medium components with a Pellicon cassette system tangential-flow ultrafiltration apparatus (Millipore GmbH, Eschborn, Germany). The autoclaved filtrate containing antibiotics (5 μg of erythromycin per ml, 10 μg of chloramphenicol per ml) was inoculated with 50 ml from an overnight culture of *L. monocytogenes* EGD-MR1 (60) carrying high-copy-number plasmid pGB363-1p60 (see above). This strain does not produce listeriolysin and produces high levels of p60 protein due to the presence of *iap* on the multicopy plasmid pGB363-1p60. The culture was incubated at 37°C with aeration until the cells reached stationary growth phase (approximately 10 h). Subsequent steps were performed at 4°C. Cells were removed by centrifugation, and the clear supernatant was concentrated approximately eightfold by tangential-flow ultrafiltration with a 10-kDa molecular mass cutoff membrane. Proteins were precipitated from the retained material by the addition of solid ammonium sulfate to 65% saturation. Precipitated protein was collected by centrifugation (10,000 × *g*, 30 min) and redissolved in 10 ml of 50 mM HEPES buffer, pH 7. This

preparation was filtered through a 0.45-μm-pore-size Millipore filter and desalted with a PD-10 gel filtration column (Pharmacia, Uppsala, Sweden) and 50 mM HEPES, pH 7, as the eluent. The p60 was purified with a fast protein liquid chromatography system (all components from Pharmacia) at room temperature. Aliquots of desalted supernatant proteins were applied to a cation-exchange Mono S HR 5/5 column equilibrated with 50 mM HEPES, pH 7, (buffer A), and eluted with a linear 0 to 0.43 M NaCl gradient in buffer B (50 mM HEPES; 1 M NaCl, pH 7) (20-min gradient; flow rate, 1 ml/min). The p60-containing fractions were pooled, concentrated, and applied (1 ml per run) to a Superose 12 HR 10/30 gel filtration column (Pharmacia) equilibrated with PBS at a flow rate of 0.5 ml/min. Fractions containing p60 were pooled, concentrated, and stored at −20°C. The molecular mass of p60 was estimated by gel filtration with the Superose 12 HR 10/30 column under the conditions described above. Proteins used to calibrate the column were β-amylase (200 kDa), bovine serum albumin (66 kDa), carbonic anhydrase (24 kDa), and cytochrome *c* (12.4 kDa).

During chromatography steps, the protein contents of fractions were monitored by measuring UV A_{278} and chain disruption activity and by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The protein content was determined by the method of Bradford (7), with bovine serum albumin as a standard. Centricon microconcentrators with a 10-kDa molecular mass cutoff (Amicon, Danvers, Mass.) were used to concentrate small volumes.

SDS-PAGE and immunoblotting. SDS-PAGE with 12.5% polyacrylamide gels was used for protein separations as previously described (60). Supernatant proteins were precipitated with trichloroacetic acid (7% [vol/vol]), washed with acetone, and solubilized in Laemmli sample buffer (37). Samples were heated at 95°C for 5 min before electrophoresis. Coomassie brilliant blue R-250 was used for protein staining. For immunoblotting, proteins were transferred from SDS-polyacrylamide gels to nitrocellulose by semidry electrotransfer (36). Nitrocellulose filters were blocked and incubated with anti-p60 serum (33) and horseradish peroxidase-conjguated anti-rabbit immunoglobulins (Dako Laboratories, Hamburg, Germany). The blot was developed with 4-chloro-1-naphthol (0.5 mg/ml) and hydrogen peroxide (0.025%).

Tests for bacteriolytic and murein hydrolase activities. Denaturing SDS-polyacrylamide gels (12.5%) containing 0.2% (wt/vol) heat-killed *Micrococcus lysodeikticus* cells (ATCC 4698; Sigma Chemical Co., St. Louis, Mo.) were used to test for bacteriolytic activity, as described by Potvin et al. (45). Protein samples were denatured by heating at 95°C in Laemmli sample buffer before electrophoresis. After electrophoresis, gels were placed in 250 ml of 10 mM Tris, pH 7.6, containing 1 mM DTT (three times for 30 min each, 20°C with agitation). After incubation, the gel was wrapped in plastic wrap and incubated overnight at room temperature. Lytic zones appeared as clear bands within the opaque gel. Gels were photographed on a glass plate above a dark background with illumination from below; thus, areas of lysis appeared as dark bands on a light background in photographs.

For isolation of cell wall material from *L. monocytogenes* EGD, we used a modification of a published method (25). Cells were harvested from 1 liter of stationary-phase culture, washed twice with PBS, and disrupted at 4°C by shaking with glass beads in a Braun homogenizer (three times for 10 min each). Glass beads were removed by low-speed centrifugation, and the supernatant was centrifuged (30,000 × *g*, 1 h, 4°C) to collect crude cell wall material. The crude cell walls were resuspended in 10 ml of ddH_2O, added dropwise with stirring to 10 ml of boiling 8% SDS, and boiled for 15 min. Cell walls were recovered by centrifugation (45,000 × *g*, 15 min, 20°C); the pellet was washed six times with ddH_2O at 20°C and resuspended in 0.01 M Tris-HCl, pH 8.2; and trypsin was added (1 μg/ml, treated with tolylsulfonyl phenylalanyl chloromethyl ketone; Serva) and incubated with gentle stirring at 30°C overnight. Cell walls were recovered by centrifugation, washed once with boiling 1% SDS, washed six times with ddH_2O, resuspended in 2 ml of ddH_2O, and stored at −20°C. To test for cell wall hydrolysis, cell walls were diluted in PBS to an optical density at 600 nm of 0.5, and 200 μl was mixed with the desired protein sample in an Eppendorf tube. After overnight incubation, the samples were assayed for an increase in reducing groups or amino termini by using the ferricyanide reduction assay (24) or 2,4-dinitro-fluorobenzene (24), respectively. The single cysteine of p60 was reversibly blocked with a methanethio (CH_3S) group by using methyl methanethiolsulfonate (MMTS) (Sigma Chemical Co.) as described by Smith et al. (48). Briefly, 2 μg of crude p60 (10 μl) was incubated in 50 mM HEPES–100 mM NaCl, pH 7.8, containing 1 mM MMTS for 30 min at 4°C. After this incubation, the MMTS-treated p60 sample was divided into two parts; DTT (1 mM final concentration) was added to one sample, and buffer without DTT was added to the other. As controls, p60 was incubated under the same conditions without MMTS and RIII cells were incubated with MMTS under the same conditions. These samples were directly tested in the RIII cell chain disruption assay.

Amino acid sequence analysis. Amino acid sequences were analyzed with Genetics Computer Group (University of Wisconsin) sequence analysis software package 7.1 (11).

RESULTS

Increased expression of *iap* causes cell chain shortening. As previously shown, the addition of partially purified p60 protein to *L. monocytogenes* RIII cell chains converts them to single cells (34), suggesting that p60 has a role in normal cell division. We wished to determine whether genetic complementation of *Listeria* and *Bacillus* strains with additional *iap* gene copies introduced on a plasmid also caused shortening of cell chains. A high-copy-number derivative of streptococcal plasmid pGB363 was constructed by deletion of the copy control region to create pGB363-1, and the *iap* gene was cloned into pGB363-1 to create pGB363-1p60. Plasmids pGB363-1 and pGB363-1p60 were transformed into *L. monocytogenes* EGD and RIII and *B. subtilis* DB104. Immunoblot analysis of culture supernatant proteins with anti-p60 serum showed that *iap* was expressed and that increased amounts of p60 were secreted into the culture medium in the strains carrying pGB363-1p60 compared with strains carrying vector pGB363-1 (see Fig. 3A). *L. monocytogenes* RIII and *B. subtilis* DB104 carrying vector pGB363-1 still formed cell chains during exponential growth (Fig. 1C and E) and formed rough colonies on solid media (data not shown). In contrast, cell chains were significantly shorter in *L. monocytogenes* RIII and *B. subtilis* DB104 which carried pGB363-1p60 (Fig. 1D and F), and colonies were smooth in appearance (data not shown). *L. monocytogenes* EGD cell and colony morphologies were not affected by increased p60 production (Fig. 1A and B).

We quantified the chain shortening of the transformed *L. monocytogenes* RIII and *B. subtilis* strains by measuring the

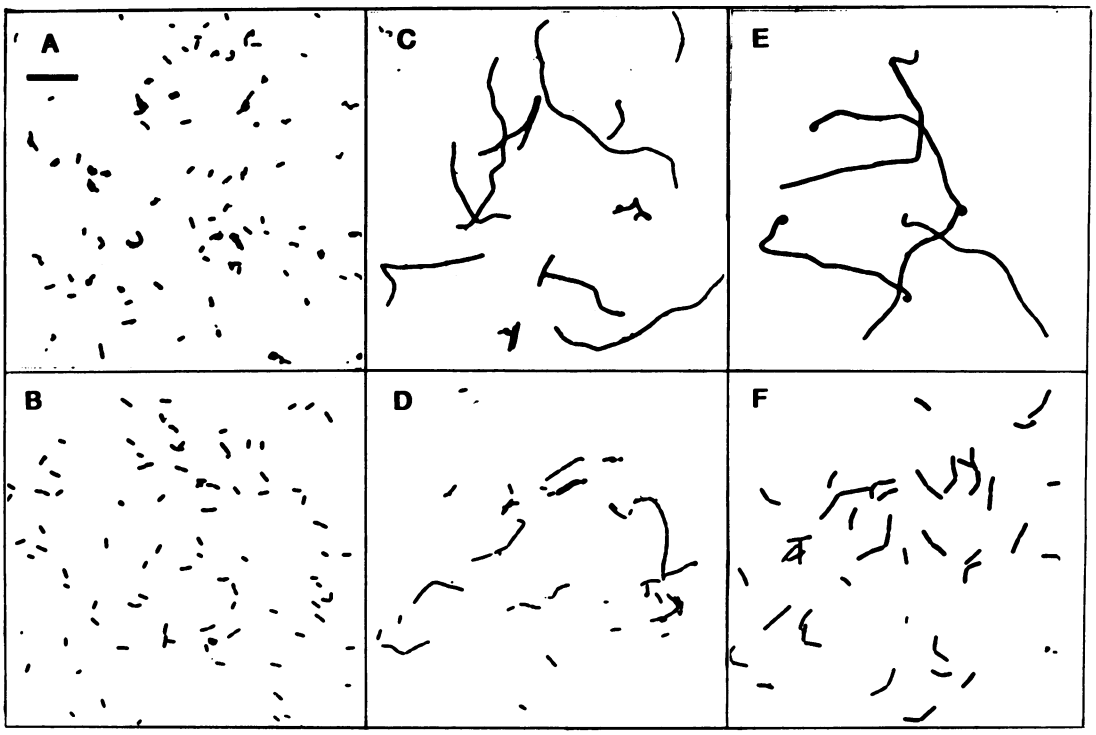

FIG. 1. Photomicrographs of Giemsa-stained cells from exponentially growing broth cultures of strains carrying plasmid vector (pGB363 or pGB363-1) or vector with the cloned *iap* gene (pGB363-1p60). (A) *L. monocytogenes* EGD pGB363-1; (B) *L. monocytogenes* EGD pGB363-1p60; (C) *L. monocytogenes* RIII pGB363; (D) *L. monocytogenes* RIII pGB363-1p60; (E) *B. subtilis* DB104 pGB363-1; (F) *B. subtilis* DB104 pGB363-1p60. Bar, 10 μm.

lengths of cell chains throughout their growth curves. Cell chains of both strains carrying pGB363-1p60 were shorter and more homogeneous than those of the vector-carrying strains at every time point measured; this shortening was most significant during mid-exponential growth, when cell chains were normally longest (Fig. 2). Taken together, these results suggested that p60 possessed an activity which led to more efficient separation of daughter cells during cell division. Interestingly, although the chains of *L. monocytogenes* RIII became shorter, they never became as short as those of wild-type cells. As recently reported, the *iap* gene is not efficiently expressed in *L. monocytogenes* RIII (32); the production of p60 in *L. monocytogenes* RIII carrying plasmid pGB363-1p60, although increased, was still lower than in wild-type *L. monocytogenes* EGD (Fig. 3, lanes 1 and 5). This low level of p60 may not be sufficient for normal cell separation.

Identification of bacteriolytic activity of p60. To gain further insight into p60 activity, we characterized the rough phenotype of *L. monocytogenes* RIII in more detail. The cell chains of mutant RIII were not disrupted by boiling in 4% SDS (data not shown), suggesting that RIII cells are joined by covalent bonds within the murein in the septal region. These SDS-treated RIII cell chains could be converted to single cells by treatment with mutanolysin (a β-1,4-*N*-acetylmuramidase) or by incubation with partially purified p60 (data not shown). These data suggested that p60 may be a type of murein hydrolase. Since murein hydrolases are often bacteriolytic (20, 24, 28), we determined whether p60 possessed bacteriolytic activity by using a gel assay system which has been previously used to identify and characterize bacteriolytic proteins from *Staphylococcus aureus* (50) and *Bacillus* spp. (45). Heat-killed *M. lysodeikticus* cells, which are very sensitive to lysis by murein hydrolases because of a low level of cross-linking in their cell walls, were incorporated into an SDS-polyacrylamide gel. Culture supernatant proteins from *L. monocytogenes* EGD and RIII and *B. subtilis* DB104 carrying pGB363 (vector), pGB363-1 (vector), pGB363p60 (normal-copy-number plasmid with *iap*), or pGB363-1p60 (higher-copy-number plasmid with *iap*) were isolated and analyzed. During incubation of the gel in buffer after electrophoresis, bacteriolysis occurred only at positions corresponding to the p60 protein (Fig. 3B) as shown by parallel immunoblot analysis (Fig. 3A). Lytic activity was higher in the pGB363-1p60-carrying EGD strain than in wild-type EGD, and, in RIII, bacteriolytic activity was detected only in the strains with plasmid-borne *iap* and lytic activity increased with increasing plasmid copy number and p60 production (Fig. 3A and B, lanes 3 to 5). In *B. subtilis* DB104, lytic activity was present only in the strain which carried pGB363-1p60 and produced p60 protein (Fig. 3A and B, lanes 6 and 7). In all strains, several lower-molecular-mass degradation products of p60 also had bacteriolytic activity. p60 also lysed heat-killed *L. monocytogenes* cells in this gel assay system (data not shown). These data suggested that p60 is a type of murein hydrolase.

Purification of p60 protein. In order to test whether p60 had murein hydrolase enzymatic activity, we purified p60 protein from the culture supernatant of listeriolysin-negative mutant *L. monocytogenes* EGD-MR1 (60) carrying plasmid pGB363-1p60, as described in Materials and Methods. This recombinant strain secreted approximately four times more p60 into the culture medium than the wild-type strain, and the amount of other extracellular proteins was reduced (Fig.

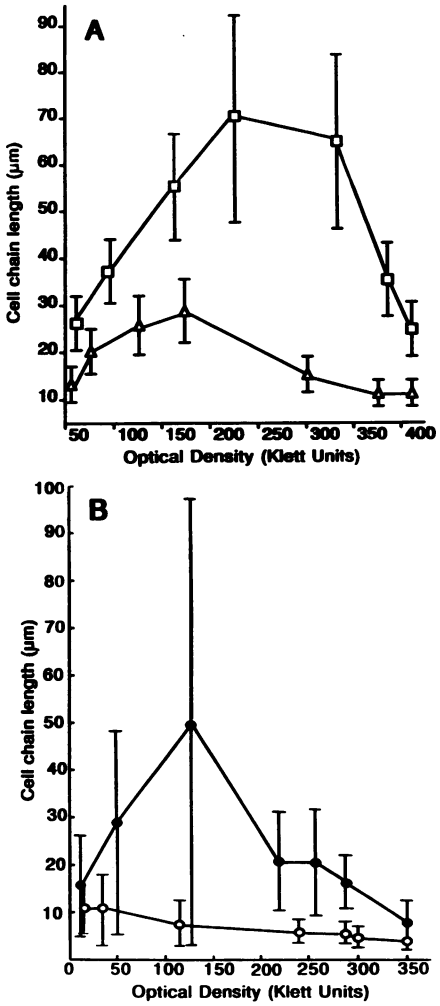

FIG. 2. Quantification of cell chain lengths measured throughout the growth curves of recombinant *L. monocytogenes* RIII and *B. subtilis* DB104 strains. (A) *L. monocytogenes* RIII pGB363 (vector) (□) and *L. monocytogenes* RIII pGB363-1p60 (vector with *iap*) (△). (B) *B. subtilis* DB104 pGB363-1 (vector) (●) and *B. subtilis* DB104 pGB363-1p60 (vector with *iap*) (○). Results at each time point are the average ± the standard deviation determined from 50 randomly chosen cell chains.

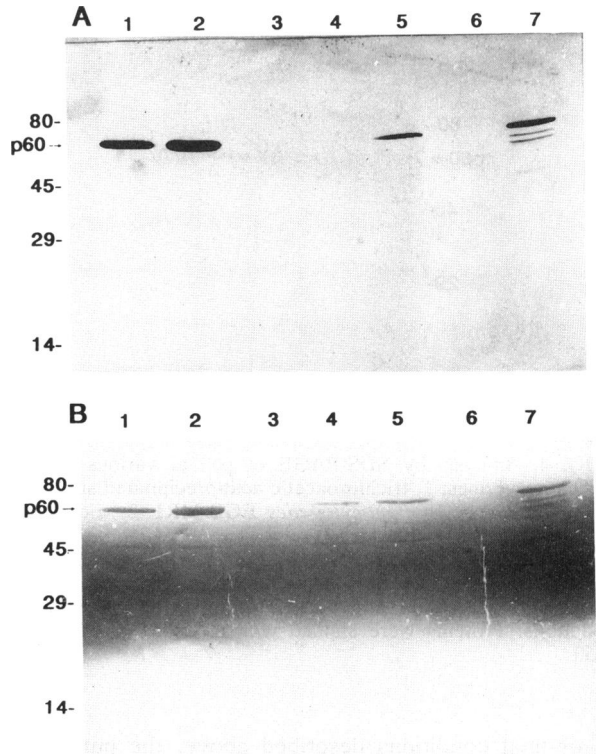

FIG. 3. Anti-p60 immunoblot (A) and bacteriolytic activity gel (B) analyses of supernatant proteins from recombinant *Listeria* and *Bacillus* strains. Lanes: 1, *L. monocytogenes* EGD pGB363-1 (vector); 2, *L. monocytogenes* EGD pGB363-1p60 (high-copy-number plasmid with *iap*); 3, *L. monocytogenes* RIII pGB363 (vector); 4, *L. monocytogenes* RIII pGB363-p60 (normal-copy-number plasmid with *iap*); 5, *L. monocytogenes* RIII pGB363-1p60 (high-copy-number plasmid with *iap*); 6, *B. subtilis* DB104 pGB363-1 (vector); 7, *B. subtilis* DB104 pGB363-1p60 (high-copy-number plasmid with *iap*). The equivalent of 1 ml of culture supernatant was analyzed for all strains. Molecular masses are shown in kilodaltons, and the position of p60 is indicated.

4, lanes 1 and 2). The use of filtered BHI medium greatly reduced medium contaminants, and growth was unaffected. After concentration and ammonium sulfate precipitation of the supernatant, p60 was the major polypeptide present (Fig. 4, lane 3). This preparation was, however, still dark brown because of the presence of low-molecular-mass medium components. p60 has an isoelectric point of 9.3 (34); thus, we performed cation-exchange chromatography as the first high-resolution purification step. Under the conditions chosen, p60 bound to the cation-exchange column but almost all medium components and other listerial proteins did not bind, and p60 was eluted at approximately 0.07 M NaCl in a relatively pure form (Fig. 4, lane 4). Several lower-molecular-mass polypeptides which were coeluted with p60 reacted with anti-p60 antibodies and were probably degradation products of p60. To remove these lower-molecular-mass polypeptides, p60 from this step was further purified by gel filtration and p60-containing fractions were pooled, concentrated, and analyzed by SDS-PAGE, in which p60 appeared as a single polypeptide of 60 kDa (Fig. 4, lane 5). Faint p60 fragments of approximately 40 kDa appeared after prolonged storage of the preparation at −20°C. Approximately 300 μg of purified p60 was isolated from each liter of culture supernatant.

Activity of p60. During purification, we monitored p60-containing fractions for chain disruption activity. Chain-disruption-specific activity increased after ammonium sulfate precipitation, but chain disruption activity was barely detectable after cation-exchange and gel filtration chromatographies (data not shown). To test whether a cofactor or other assay conditions were required for p60 activity, we tested the chain disruption activity of concentrated *L. monocytogenes* EGD-MR1 culture supernatants at 20 and 37°C in the presence of 1 mM DTT; $MgCl_2$, $CaCl_2$, $CuCl_2$, $MnCl_2$, and $ZnCl_2$ (all at 2 and 10 mM); and NaCl (0 to 0.5 M). The chain disruption activity of crude p60 (ammonium sulfate-precipitated supernatant proteins) was optimal in 150 mM NaCl at neutral pH, and DTT clearly stimulated chain disruption activity; the addition of divalent cations had no stimulatory effect (data not shown). However, even under

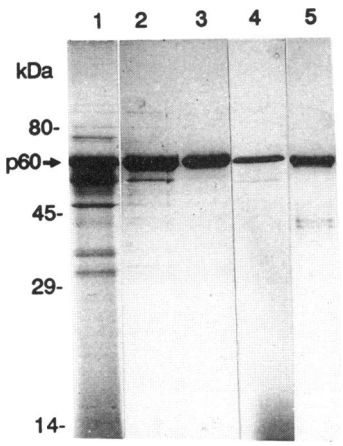

FIG. 4. Analysis by SDS-PAGE of p60 at various stages of purification. Lanes: 1, trichloroacetic acid-precipitated supernatant proteins (1 ml) of *L. monocytogenes* EGD-MR1; 2, trichloracetic acid-precipitated supernatant proteins (1 ml) of *L. monocytogenes* EGD-MR1 pGB363-1p60; 3, ammonium sulfate precipitation (65% saturation); 4, Mono S cation-exchange chromatography; 5, Superose 12 gel filtration chromatography. Molecular masses are shown in kilodaltons. Proteins were stained with Coomassie brilliant blue R-250.

the optimal conditions described above, the purified p60 protein had no detectable activity.

The stimulation of chain disruption activity in crude p60 preparations by DTT suggested that the single cysteine residue in the C-terminal region (position 347 in the mature protein) of p60 may be important for activity. To test this, we reversibly blocked the cysteine residue with MMTS, which blocks the sulfhydryl group with a methanethio (CH_3S) group. This blocking group can be removed by treatment with reducing agents such as DTT (48). Treatment of crude p60 preparations with MMTS almost completely abolished chain disruption activity; however, when the MMTS-treated preparation was unblocked with DTT, chain disruption activity was completely restored (Table 1). These results suggested that the cysteine is important for the chain disruption activity of p60.

We also incubated purified p60 with purified listerial cell walls under the various conditions described above and tested for dissolution of the murein and the appearance of reducing groups (indicative of a glycosidase) or amino groups (indicative of a peptidase or amidase). There was no dissolution of the murein, and no increase in reducing groups or amino groups was detected (data not shown). Purified p60 was, however, bacteriolytic in the SDS-PAGE gel assay system described above (data not shown). These data suggest that the p60 protein indeed has bacteriolytic activity but that the purified p60 was isolated in an inactive form.

TABLE 1. Chain disruption activity of p60 after reversibly blocking the single cysteine of p60 with MMTS

Reaction mix	Chain disruption activity at[a]:	
	1 h	3 h
RIII + DTT + buffer	–	–
RIII + DTT + p60	++	++
RIII + p60 + MMTS	–	±
RIII + p60 + MMTS + DTT	++	++
(RIII + MMTS)[b] + p60	++	++

[a] Symbols: –, no chain disruption; ±, slight chain disruption; +, 25 to 75% chain disruption; ++, more than 75% disruption.

[b] RIII cell chains were pretreated with MMTS, washed, and used as the substrate.

The calculated molecular mass of the mature p60 is 47.5 kDa (33), which is considerably less than the molecular mass of 60 kDa determined by SDS-PAGE. To address this discrepancy, we determined the molecular mass of purified p60 by gel filtration with a calibrated Superose 12 column. The p60 protein was reproducibly eluted from the column at a position corresponding to a molecular mass of 170 kDa (data not shown), suggesting that purified p60 was isolated in an aggregated form, in which each aggregate may contain three to four p60 molecules. These data may also explain why purified p60 was enzymatically inactive.

Disruption of the chromosomal *iap* gene of *L. monocytogenes* EGD. The *E. coli-Listeria* shuttle vector pLSV3, carrying the 250-bp *Hin*dIII-*Pst*I internal fragment of *iap* and an erythromycin resistance gene, does not replicate in the *Listeria* host at the nonpermissive temperature of 42°C because of its gram-positive temperature-sensitive origin of replication [ori(Ts)]. Selection for expression of the erythromycin resistance gene at 42°C should therefore yield clones in which pLSV3 has integrated into the *Listeria* chromosome via homologous recombination between the internal *iap* sequences. We could not isolate any clones under these conditions, although the method has been successfully applied to isolate nonhemolytic mutants of *L. monocytogenes* (60).

Disruption of *iap* was possible, though, after transcomplementation of pLSV3-carrying *L. monocytogenes* clones with plasmid pGB363-1p60 at the permissive temperature of 30°C followed by a temperature shift to 42°C (Fig. 5). Plasmid pGB363-1p60 contained an intact copy of *iap*, which led to increased p60 expression in *L. monocytogenes* (Fig. 3A, lanes 1 and 2). Approximately 50% of the erythromycin- and chloramphenicol-resistant clones which grew at the nonpermissive temperature still contained extrachromosomal pLSV3; this could have been due either to the spontaneous loss of the temperature-sensitive phenotype of plasmid pLSV3 or to integration of pLSV3 into the intact *iap* gene on plasmid pGB363-1p60. The other recombinant clones that expressed both erythromycin and chloramphenicol resistance genes had lost extrachromosomal pLSV3 DNA. In these clones, the chromosomal *iap* gene was disrupted by pLSV3 integration as shown in Fig. 5. This was confirmed by Southern blot analysis, in which a 2.3-kb *Hpa*I DNA fragment containing the entire ori(Ts) and a 440-bp *Hin*dIII internal *iap* fragment were used as probes, thus allowing molecular characterization of the insertion location of pLSV3 in the *Listeria* chromosome (Fig. 6). Total genomic DNA of the transformed and wild-type strains of *L. monocytogenes* EGD and pLSV3 and pGB363-1p60 plasmid DNA were cut with appropriate restriction endonucleases and separated on a 1% agarose gel (Fig. 6A). In the clone carrying pLSV3 in the chromosome, the ori(Ts) probe hybridized with fragments of 2.2 and 1.4 kb in the *Hpa*II digest (Fig. 6B, lane 1) and with a 4.5-kb fragment in the *Hin*cII digest (Fig. 6B, lane 2). This is consistent with the integration of pLSV3 into *iap* in the *L. monocytogenes* chromosome as shown in Fig. 5. Unexpectedly, hybridization also occurred between the ori(Ts) probe and the pGB363-1p60 fragment carrying the chloramphenicol resistance gene from pTV32(Ts) as shown by hybridization of a 2.2-kb *Hin*cII and

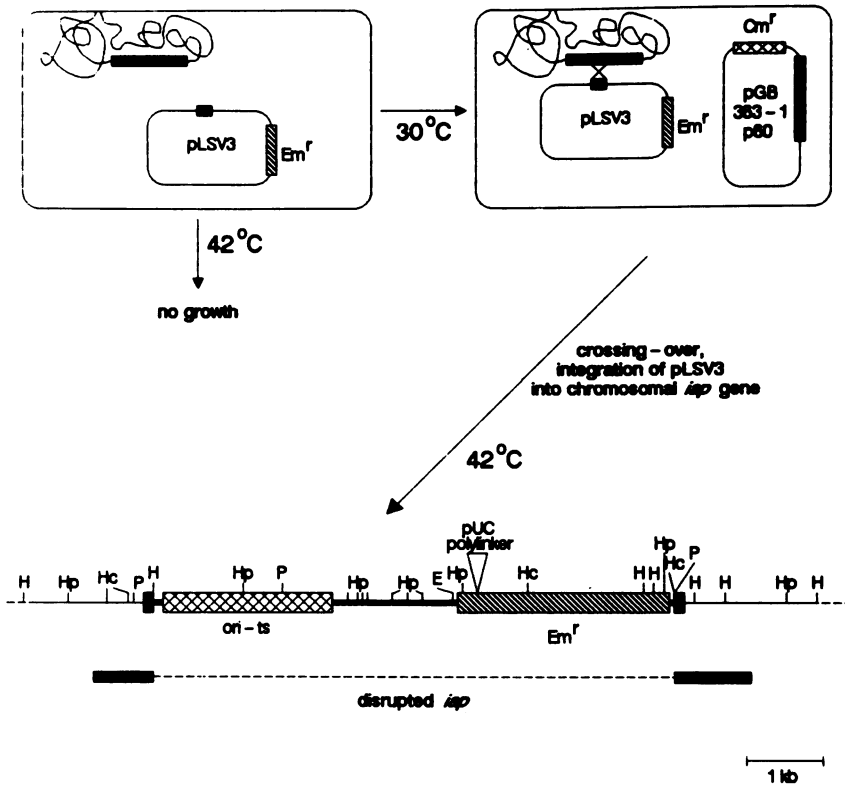

FIG. 5. Transformation of *L. monocytogenes* EGD with plasmids pLSV3 and pGB363-1p60 and disruption of the chromosomal *iap* gene by integration of pLSV3 at 42°C. The bacterial cell and its chromosomal DNA are represented schematically. Solid bars represent *iap* DNA, hatched bars represent the erythromycin resistance gene (Em^r), and cross-hatched bars indicate the chloramphenicol resistance gene (Cm^r) and the ori(Ts). Restriction sites indicated are *Eco*RI (E), *Hin*dIII (H), *Hin*cII (Hc), *Hpa*II (Hp), and *Pst*I (P).

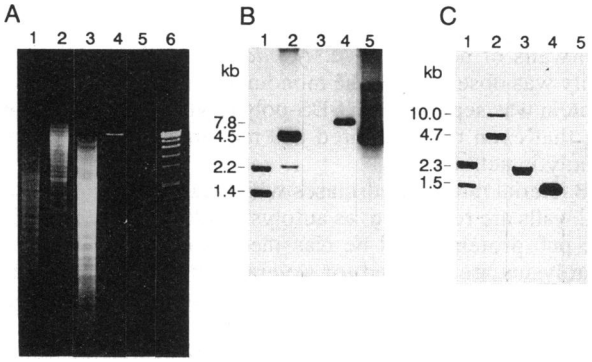

FIG. 6. Analysis of pLSV3 integration into the chromosome of *L. monocytogenes* EGD. (A) Agarose (1%) gel stained with ethidium bromide. (B) Autoradiograph of a Southern blot of the gel shown in panel A, with the 2.3-kb ori(Ts) fragment (double-hatched bars in Fig. 5) as a probe. (C) Autoradiograph of the same Southern blot with the 440-bp *Hin*dIII *iap* fragment as a probe (440-bp *Hin*dIII fragment just 3′ to the integrated Em^r gene in Fig. 5). Shown are the total DNA of *L. monocytogenes* EGD carrying pLSV3 integrated into the chromosome and pGB363-1p60 extrachromosomally, cleaved with *Hpa*II (lane 1) and *Hin*cII (lane 2); the total DNA of wild-type *L. monocytogenes* EGD, cleaved with *Hpa*II (lane 3); plasmid pGB363-1p60 cleaved with *Pst*I (lane 4); plasmid pLSV3 cleaved with *Hin*dIII (lane 5); and DNA standard SPP1 cleaved with *Eco*RI (lane 6) (fragment sizes are indicated in reference 33).

a 7.8-kb *Pst*I fragment of the plasmid with this probe (Fig. 6B, lanes 2 and 4). The model of insertion of pLSV3 into *iap* in Fig. 5 was also confirmed with a probe from within the *iap* gene. The 440-bp *Hin*dIII *iap* probe hybridized with a 2.3-kb *Hpa*II fragment, which was from the intact *iap* gene on the complementing plasmid pGB363-1p60, and with a 1.5-kb *Hpa*II fragment which was from the disrupted chromosomal gene (Fig. 6C, lane 1). Figure 6C, lane 3, shows hybridization of the probe with the 2.3-kb *Hpa*II fragment from the chromosome of wild-type *L. monocytogenes*.

These data indicated that *iap* was an essential gene that could be disrupted on the *Listeria* chromosome only after complementation with extrachromosomal *iap* copies. Plasmid-curing experiments supported this hypothesis. Curing of plasmid pGB363-1p60 could be achieved at a frequency of 3×10^{-2} in the *L. monocytogenes* strain with an intact chromosomal *iap* gene but never in the strain carrying the disrupted *iap* gene under culture conditions selecting for the integrated state of pLSV3 (data not shown).

Protein p60 shows sequence similarity with the repeat domain of an autolysin of *Streptococcus faecalis*. As we have recently reported (9a), the p60 protein of *L. monocytogenes* shares extended sequence homology with a surface protein of *Enterococcus faecium* (21). This homology is restricted to the C-terminal part of both proteins. This region is highly conserved in all p60 proteins of *Listeria* species (Fig. 7) and contains the single cysteine residue which, as described above, seems to be important for the cell chain disruption activity of p60. The biochemical function of this *E. faecium* protein is unknown (21). The recently described autolysin of

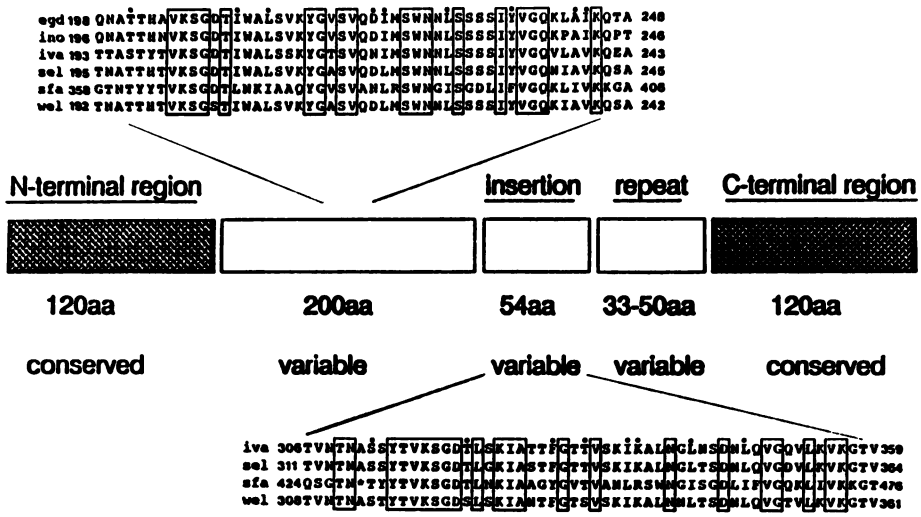

FIG. 7. Amino acid sequence homologies between repeat domains of the *S. faecalis* autolysin protein and portions of various *Listeria* p60 proteins. The p60 proteins are schematically summarized in conserved and variable regions (9a). Identical amino acids are boxed, and positions of homologous amino acid substitutions are marked by dots. The numbers indicate the position of a portion in the corresponding whole protein. The asterisk in the sfa 54-amino-acid insertion indicates a gap inserted in order to maintain the highest degree of homology of the protein sequences. Abbreviations: egd, *L. monocytogenes* serovar 1/2a EGD; ino, *L. innocua* serovar 6b; iva, *L. ivanovii*; sel, *L. seeligeri*; sfa, *S. faecalis*; wel, *L. welshimeri*; aa, amino acids.

S. faecalis contains a repeat region that consists of four tandemly arranged units of about 60 amino acids each (4). As shown in Fig. 7, the sequence of this repeat unit exhibits substantial similarity with part of the 200-amino-acid variable domain of listerial p60. This common sequence is rather conserved in the variable domain of all p60 proteins from the various *Listeria* species. As shown for p60 proteins from *L. ivanovii*, *L. welshimeri*, and *L. seeligeri*, the common sequence occurs twice (Fig. 7) because of a duplication of a region in the variable domain of p60. This duplication is a characteristic feature of p60 proteins within this group of *Listeria* species and does not occur in the p60 proteins from *L. monocytogenes* and *L. innocua*.

DISCUSSION

Our data suggest that the major extracellular protein p60 of *L. monocytogenes* is a murein hydrolase which has an essential role in cell division. *L. monocytogenes* rough mutants which synthesize a reduced amount of p60 protein are still viable but form long cell chains with double septa between the individual bacterial cells (34). This morphological phenotype correlates with the amount of p60 secreted into the supernatant of the corresponding mutant. In the exponential growth phase, there is little p60 protein secreted into the supernatant and the *L. monocytogenes* rough mutant RIII forms long cell chains at this time point. More p60 protein accumulates in the supernatant in the stationary growth phase, and a considerable shortening of the bacterial cell chains is observed. The transcomplementation of the RIII mutant with multiple copies of the *iap* gene which encodes the p60 protein also led to significant reductions in the lengths of the RIII cell chains. Interestingly, this complementation caused only a relatively slight increase in the production of the p60 protein in spite of the high *iap* gene dose and the large amount of *iap*-specific mRNA which is synthesized by the complemented strain. This seems to be due to a specific block in the translation of the p60 mRNA in the RIII mutant (32). Expression of *iap* in *B. subtilis* DB104, which normally forms chains in the exponential growth phase, also led to significant reductions in the lengths of cell chains in this organism.

As shown previously, enriched p60-containing fractions are able to disrupt RIII cell chains (34), and our data shown here clearly indicate that this activity can be attributed to the p60 protein. There is, however, a drastic reduction in this activity during purification of the protein. This is most likely due to the tendency of this positively charged protein to form aggregates. The molecular size of the purified protein determined by gel filtration suggests a complex consisting of three to four p60 molecules. This complex has lost not only the chain disruption activity but also the activity to hydrolyze cell walls of heat-killed *M. lysodeikticus*. Bacteriolytic activity was observed for the monomeric form of p60 when the protein was separated on SDS-polyacrylamide gels, suggesting that even the denatured p60 monomer retains this bacteriolytic activity.

Bacterial murein hydrolases which can degrade their own cell walls are referred to as autolysins (24); by this definition, the p60 protein could be classified as a listerial autolysin. Autolysins are required for several important cellular functions, including cell wall growth, turnover and splitting of the septum for cell separation (28). Autolysins may be classified as *N*-acetylmuramidases, *N*-acetylglucosaminidases, *N*-acetylmuramyl-L-alanine amidases, endopeptidases, or transglycosylases (24). Because p60 had lost its chain disruption and bacteriolytic activities after purification, we were unable to determine the nature of its enzymatic activity.

Streptococcus pneumoniae possesses a well-characterized *N*-acetylmuramyl-L-alanine amidase, encoded by the gene *lytA*, which is involved in septum separation and autolysis (47, 54). Deletion of the *lytA* gene results in inefficient daughter cell separation, the formation of short cell chains, and loss of the autolytic phenotype (47). In *S. aureus*, numerous bacteriolytic proteins have been identified (50, 56) and an endo-β-*N*-acetylglucosaminidase has been purified

and implicated in cell separation (51). Autolysin-deficient *B. subtilis* mutants form cell chains (15, 17, 20), and, in *E. coli*, an *N*-acetylmuramyl-L-alanine amidase is involved in septum separation (2, 28, 58). By analogy with these and other similar activities from gram-positive and gram-negative bacteria, and on the basis of experimental evidence presented here, we suggest that the p60 protein possesses, as a basic biochemical function in the listerial cell, a murein hydrolase activity necessary for cell separation.

Autolysis of *L. monocytogenes* has been described previously (55). In that study, the five strains studied began to autolyze immediately at the end of the exponential growth phase. It has also been reported that the rate of cell wall turnover in *L. monocytogenes* is approximately 25% per generation (14). We observed no significant autolysis of *L. monocytogenes* EGD or RIII cultures over a 72-h period when the strains were grown in BHI broth at 37°C (59), suggesting that p60 activity must be tightly regulated in vivo. Regulatory mechanisms of *E. coli* autolysins include activation or inhibition by cofactors or topological restriction of enzyme distribution within the cell wall (28). In *B. subtilis*, in vivo control of autolytic activity is related to the energized membrane (29), and, in some gram-positive organisms, lipoteichoic acids or lipids may regulate autolytic enzymes (27). The mechanism of regulation of p60 autolytic activity will require further study.

Some autolysins are important virulence factors. Inactivation of the *lytA* gene of *S. pneumoniae* (which encodes the *N*-acetylmuramyl-L-alanine amidase) causes a marked reduction in virulence (5). This autolysin is thought to function in pathogenesis by lysing a portion of the invading pneumococci and causing the release of pneumolysin and possibly other toxic substances (5). In *E. coli*, autolysins are important because of their role in the release of highly inflammatory cell wall components (28).

The p60 protein, as a listerial murein hydrolase, could function in the pathogenesis of listeriosis in a similar fashion. Listerial murein causes complement fixation and chemotaxis of human polymorphonuclear leukocytes (1), is a B-cell adjuvant (10), is mitogenic, activates macrophages in vitro, and is a nonspecific immunostimulant (41). The release of listerial cell wall fragments in vivo and/or intracellularly could cause increased inflammation and modulation of the immune response.

The hydrolytic activity of p60 from *L. monocytogenes* on double septa appears to be common to all p60-related proteins of *Listeria* species (8, 9a). There are extended, highly homologous sequences on both termini of these proteins, and the only cysteine residue of p60 is found in the conserved C-terminal region. The data presented indicate that this cysteine is essential for the hydrolytic activity of p60, suggesting that this conserved portion of the p60 protein actively participates in the enzymatic activity or substrate binding.

No extended homology has been detected between the listerial p60 proteins and proteins from other bacterial species. There is, however, an interesting sequence similarity in a stretch of about 50 amino acids which is present in all p60 proteins and in the repeat structure of an autolysin of *S. faecalis*. This repeat domain in the streptococcal autolysin has been suggested to be a possible substrate binding site (4). In the p60 proteins, this common sequence occurs at least once as a rather conserved sequence in an otherwise more variable region (9). In the p60 proteins of the *L. ivanovii-L. welshimeri-L. seeligeri* group, the common sequence occurs twice because of a duplication which also includes this common region (9). Furthermore, we have recently identified an even more extended region of sequence similarity between the C-terminal part of p60 and the C-terminal half of a protein of *E. faecium* which includes the single cysteine (9a, 21). The function of this protein, which has been found in the cell wall fraction, is unknown (21). Interestingly, this protein exhibits some homology to M proteins of group A streptococci and shares with the M protein multiple direct repeats in the DNA and protein sequences (21).

We have recently reported the involvement of p60 of *L. monocytogenes* in the adherence (and invasion) of these bacteria to mouse 3T6 fibroblasts but not to Caco-2 epithelial cells (9a). This activity seems to be rather specific for the p60 protein of *L. monocytogenes* (9a, 34). The most striking difference in the sequences of p60 proteins from the *Listeria* species is the presence of a symmetrically arranged TN repeat domain in the middle part of p60 from *L. monocytogenes* which is virtually absent in the other *Listeria* species. All other listerial p60 proteins and the *E. faecium* protein carry in this region multiple T and N residues which have not become arranged in TN repeats (9a). Taking these data together, we suggest that p60 has evolved in the pathogenic *L. monocytogenes* from an essential housekeeping protein required for cell division into a virulence factor which has become important for the intracellular life cycle of this facultative intracellular microorganism.

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