Machata, S., Hain, T., Rohde, M., Chakraborty, T.
Simultaneous deficiency of both MurA and p60 proteins generates a rough phenotype in Listeria monocytogenes
(2005) Journal of Bacteriology, 187 (24)

JOURNAL OF BACTERIOLOGY, Dec. 2005, p. 8385–8394 Vol. 187, No. 24
0021-9193/05/$08.00+0 doi:10.1128/JB.187.24.8385–8394.2005

Simultaneous Deficiency of both MurA and p60 Proteins Generates a Rough Phenotype in *Listeria monocytogenes*

Silke Machata,[1] Torsten Hain,[1] Manfred Rohde,[2] and Trinad Chakraborty[1]*

Institute of Medical Microbiology, Justus-Liebig-Universität, 35392 Giessen,[1] and Department of Microbial Pathogenesis and Vaccine Research, German Centre for Biotechnology (GBF), 38124 Braunschweig,[2] Germany

Received 24 June 2005/Accepted 23 September 2005

We examined eight spontaneously occurring rough mutants of *Listeria monocytogenes* for their ability to express two previously reported autolysins, p60 and MurA. All mutants lack MurA expression and show strongly reduced levels of extracellular p60. One rough strain harbors a variant of the p60 protein with a partially truncated catalytic domain. In seven cases there were shifts in the localization of p60 to the membrane fraction. Mutations within the *secA2* gene, encoding an auxiliary protein secretion system paralog, were previously shown to be involved in the smooth-rough phenotypic variation seen with *Listeria* strains. An isogenic Δ*secA2* EGDe deletion strain displays a strong pleiotropic reduction of p60 and MurA, in addition to a large number of secreted and surface proteins. However, we observed no apparent SecA2 dysfunction in several of the investigated strains as determined by direct sequencing of the *secA2* gene and complementation of the Δ*secA2* mutant with the respective allele cloned from the rough mutant. To determine the gene products required for the smooth-rough transition, we created mutants lacking the individual *iap* and *murA* genes as well as a Δ*iap* Δ*murA* double mutant. The double mutant displays a rough phenotype and exhibits many of the properties seen with the Δ*secA2* mutant. Our results implicate p60 and MurA as important determinants in controlling the cell shape of *L. monocytogenes*. We also identified homologous MurA and SecA2 proteins in other *Listeria* species. The muramidase in two species, *L. innocua* and *L. welshimeri*, shows activity similar to that of the MurA protein in *L. monocytogenes*.

Listeria monocytogenes is a ubiquitous gram-positive, rod-shaped, nonsporulating, facultative intracellular bacterium that can cause severe food-borne infections in humans and animals. Initial contact of the pathogen with a host cell occurs through surface proteins responsible for the adherence to, invasion of, and interaction with the infected host. A variety of cell wall-associated and secreted proteins are known to be important for pathogenicity. These include well-characterized virulence factors such as the internalins, the actin polymerization-promoting protein ActA, and listeriolysin, all of which enable *L. monocytogenes* to escape intracellular compartmentalization.

Recent studies indicate that cell wall hydrolases, such as the invasion-associated protein (Iap, p60, or Cwh), a cell wall amidase (Ami), and a surface-associated autolysin (Auto), can play a direct role in the pathogenicity of *L. monocytogenes* (3, 22, 27). Cell wall hydrolases are involved in various biological processes including cell division, cell separation, competence for genetic transformation, sporulation, and the lytic action of some antibiotics. Specifically, they hydrolyze distinct components of the bacterial cell wall to enable de novo biosynthesis of the peptidoglycan layer and are classified according to designated sites of hydrolysis (34). Whereas amidases generally cleave the linkage between glycan and peptide, glucosaminidases and muramidases cut the β-1,4 glycosidic bond between *N*-acetylglucosamine and *N*-acetylmuramic acid, respectively. Certain hydrolases, termed autolysins, are able to induce autolysis of bacteria when acting in an unregulated manner. Since these enzymes are potentially lethal, tight control over expression and activity is required. In addition to their function in cell division, murein hydrolases contribute to bacterial susceptibility to certain antibiotics (11) and promote pathogenicity of bacteria (22).

The importance of autolysins in maintaining normal bacterial cell functions is suggested by their widespread occurrence. Diverse studies of gram-positive and gram-negative bacteria demonstrate that deletions of genes encoding murein hydrolases produce defects in cell morphology and cell wall synthesis leading to the generation of long multiseptate filaments (cell chains with cross walls between individual cells). Simultaneous inactivation of several autolysins in *Bacillus subtilis* is concomitant with the formation of longer chains of cells (12, 20, 21, 26). In *Streptococcus pneumoniae*, depletion of PcsB, an essential putative murein hydrolase, elicits the generation of long cell chains (25). Deletion of multiple murein hydrolases in *Escherichia coli* results in the formation of filaments (10).

In *L. monocytogenes*, the 60-kDa secreted autolysin p60 (16) is known to be involved in cell division and the generation of long filaments and is associated with invasion into certain mammalian cell lines. Mutants impaired in the synthesis of p60 were shown to be strongly attenuated in a mouse model of infection (16, 27). The protein was characterized as a murein hydrolase based on homology to a repeat domain of an autolysin in *Enterococcus faecium* (8) and the observation that p60 overexpression induces autolysis in *L. monocytogenes* (35). In various spontaneously occurring phenotypically rough mutants of *L. monocytogenes*, decreased production of extracellular p60 has been observed (14, 16, 35). The p60 hydrolase is encoded by the *iap* gene,

* Corresponding author. Mailing address: Institut für Medizinische Mikrobiologie, Justus-Liebig-Universität, Frankfurter Strasse 107, D-35392 Giessen, Germany. Phone: 49-641 99 41250. Fax: 49-641 99 41259. E-mail: Trinad.Chakraborty@mikrobio.med.uni-giessen.de.

which is not under the control of the transcriptional activator PrfA and whose expression is regulated at the posttranscriptional level (5, 15). Proteins closely related by sequence to p60 have been found in all six *Listeria* species (2).

Another autolysin involved in *L. monocytogenes* cell division is the recently described 66-kDa cell surface protein MurA (NamA) (4, 18). Deletion of the *murA* gene results in chain formation in exponential-growth-phase cultures. This murein hydrolase is important for cell separation and for generalized autolysis in *L. monocytogenes* (4). Both p60 and MurA proteins carry LysM domains, responsible for attachment of the respective proteins to the cell wall, and are secreted out of the bacterial cell in a SecA2-dependent manner. The *secA2* gene has been described as a second *Listeria secA* gene associated with protein secretion and is responsible for the transport of a variety of extracellular proteins in *L. monocytogenes* (18, 19). Secondary SecA homologues have also been described in other gram-positive bacteria such as *Mycobacterium* spp. and *Streptococcus* spp. (19). An *L. monocytogenes secA2* deletion mutant displays a rough phenotype (19) and is defective in the secretion of at least 15 additional cell wall-associated or secreted products in addition to p60/Iap and MurA/NamA. Thus, proteins transported by the SecA2-dependent pathway are assumed to be responsible for generation of long cell chains and rough colony morphology in *L. monocytogenes*.

This study focuses on the determinants for the generation of filamentous rough forms of *L. monocytogenes*. We investigated the protein levels of MurA and p60 in a number of spontaneously occurring rough strains to assess gene products involved in the smooth-rough variation. The impaired synthesis of both proteins in seven examined rough variants suggests that the simultaneous lack of the two autolysins is sufficient to induce filaments in *L. monocytogenes*. We generated mutants with single and multiple deletions of *secA2*, *iap*, and *murA* in the parental EGDe strain that allowed us to study the role of individual genes in the smooth-rough transition. Additionally, we present genetic evidence for the presence of the *secA2* and *murA* genes in nonpathogenic species of *Listeria*.

MATERIALS AND METHODS

Bacterial strains and growth conditions. Bacterial strains, plasmids, and primers are listed in Table 1. Cultivation of *L. monocytogenes* strains was performed at 37°C in brain heart infusion (BHI) broth or on BHI agar plates (Difco). *E. coli* strains were grown in Luria-Bertani (LB) broth at 37°C. Media were supplemented with erythromycin to a final concentration of 300 or 5 μg/ml for growth of *E. coli* or *L. monocytogenes*, respectively. Chloramphenicol was used at a concentration of 8 μg/ml on agar plates or 5 μg/ml in broth for *Listeria* and at 25 μg/ml (plates and broth) for *E. coli*, when needed.

Genetic manipulations. Chromosomal DNA from *L. monocytogenes* was isolated as previously described (28). DNA fragments and PCR products used for cloning were created with the Expand High Fidelity PCR system (Roche) and purified with the QIAquick PCR purification kit (QIAGEN). All constructs were transformed into the *E. coli* INVαF′ strain for plasmid propagation. Plasmid DNA was isolated from *E. coli* and purified with the GFX Micro Plasmid Prep kit (GE Healthcare) as recommended by the manufacturer. For construction of deletion mutants, PCR products and correct insertion into chromosomal DNA of *L. monocytogenes* were verified via automated DNA sequencing.

To construct the deletion mutants, upstream and downstream sequences flanking the open reading frame of interest were amplified by PCR using *L. monocytogenes* EGDe chromosomal DNA as template. These PCR products were subsequently digested with appropriate enzymes and ligated to form a ΔORF insert to knock out the gene by homologous recombination. The insert was amplified by PCR, cloned into the plasmid pAUL-A, and screened for the presence of the insert using the M13for-M13rev primer pair. DNA of pAUL-A containing the insert was introduced into *L. monocytogenes*, and plasmid integration and excision were performed as described by Schaferkordt and Chakraborty (32). The following primer-pair combinations were used to generate suitable inserts to knock out the indicated genes: SecA2F1-SecA2R2 and SecA2F3-SecA2R4 for Δ*secA2* and iap1-iap2 and iap3-iap4 for Δ*iap*. The *L. monocytogenes* strain EGDe Δ*murA* (4) was used to create the Δ*murA* Δ*iap* double mutant by knocking out the *iap* gene using similar techniques.

The Δ*secA2* complement strain was constructed using the site-specific phage integration vector pPL2 (17). Oligonucleotides SecA2F1 and SecA2R2 were used to amplify the *secA2* locus from *L. monocytogenes* genomic DNA for creation of the Δ*secA2-C* strain and from SLCC7503 genomic DNA for generation of Δ*secA2-C*-SLCC7503. This PCR product was cloned into pPL2 (pPL2-*secA2*), and the plasmid was introduced into the *L. monocytogenes* Δ*secA2* strain. Complementation was verified by examining the expression of two SecA2-dependent proteins, NamA and p60 (18), and the strains were subjected to further phenotypic analysis as described in Results.

Construction, expression, and purification of GST-Iap/p60 fusion protein. The coding region of *iap* (excluding the signal peptide) was amplified by PCR using the Iap-GSTfor/Iap-GSTrev primer pair and *Listeria monocytogenes* EGDe as template. The product was digested with BamHI-EcoRI and inserted into the corresponding sites of the glutathione *S*-transferase (GST) gene fusion vector pGEX6P-1 (GE Healthcare). GST-Iap/p60 fusion protein was overexpressed in *E. coli* BL21 transformed with the pGEX6P-1-*iap* construct and purified using the glutathione Sepharose 4B Batch/Column method (GE Healthcare). PreScission protease (GE Healthcare) was used to remove the GST tag from the recombinant p60 protein, and purity was assessed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The eluted protein was stored at −20°C after addition of glycerol to 10%.

Complementation of Δ*murA*, Δ*iap*, and Δ*murA* Δ*iap* strains by treatment with recombinant proteins. The wild-type *L. monocytogenes* EGDe and deletion strains were grown to early exponential phase at 37°C in BHI, and the cells were harvested by centrifugation at 6,200 × *g* for 3 min at 22°C. Cell pellets were resuspended in phosphate-buffered saline containing 1.44 μg/ml MurA and 3.2 μg/ml p60 protein and incubated for 1 h at 37°C with gentle shaking. Excess recombinant protein was removed by exchanging the supernatant with 0.85% NaCl, and the cells were subsequently prepared for light microscopy as described below.

Isolation of proteins from *L. monocytogenes*. The subcellular fractionation procedure was as described by Monk and colleagues (13, 23) with slight modifications. Briefly, 50-ml cultures of *L. monocytogenes* were grown in BHI at 37°C to log phase (optical density at 600 nm [OD_{600}], ≈1.0), and the cells were subsequently harvested by centrifugation at 6,200 × *g* for 15 min at 4°C. Culture supernatant proteins were precipitated on ice overnight by treatment with 10% trichloroacetic acid, pelleted by centrifugation at 4°C, washed with ice-cold acetone, resuspended in 0.2 ml 1 M Tris-HCl (pH 8.8), and stored at −20°C. For subcellular protein fractions, the cell pellet was resuspended in 1 ml wash buffer (10 mM Tris-HCl, pH 6.9, 10 mM $MgCl_2$) containing 500 mM sucrose (SWB) and washed twice to remove traces of the supernatant. Protoplasts were generated by incubating the cells at 37°C for 2 h in 0.1 ml SWB containing 10 mg/ml lysozyme (Merck), 2,500 U/ml mutanolysin (Sigma), and 1 mM phenylmethylsulfonyl fluoride (Sigma). The cell wall fraction was obtained by centrifugation (6,200 × *g* for 15 min at 4°C) and transfer of the supernatant to a fresh tube. Protoplasts were washed in 1 ml SWB, resuspended in 0.2 ml protoplast lysis buffer (PLB; 100 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 100 mM NaCl, 362 U benzonase nuclease [Sigma]), frozen at −20°C, and lysed via three freeze-thaw cycles. The cytoplasmic fraction was obtained by centrifugation (16,000 × *g* for 10 min at 4°C) and transfer of the supernatant to a fresh tube. The resulting pellet containing the cell membrane fraction was washed in 1 ml PLB and resuspended in 0.1 ml TE buffer (10 mM Tris-HCl, pH 8, 1 mM EDTA). The amount of protein in the various subcellular extracts was determined using a Bradford assay (Bio-Rad), and the fractions were stored at −20°C.

Immunodetection and comparative analysis of proteins. Samples containing 10 μg protein were separated using 12.5% SDS-PAGE, transferred to nitrocellulose membranes, and probed with mouse monoclonal antibodies against p60 (Fup60) or MurA (monoclonal antibody αp70128G4) (provided by Jessica Schaumburg, GBF, Braunschweig). Detection was performed using horseradish peroxidase (Santa Cruz Biotechnology)-coupled anti-mouse antibodies and the ECL kit (GE Healthcare) per the manufacturer's instructions. For comparative analysis, relative protein concentrations were derived by scanning (Epson Stylus Color 600) and quantifying both Coomassie blue-stained SDS-polyacrylamide gels and immunoblots. Relative protein expression was estimated by extrapolating the relative protein concentration upon normalization of expression to the

TABLE 1. Bacterial strains, plasmids, and primers

Strain, plasmid, or primer	Genotype or description	Reference or source
Strains		
L. monocytogenes		
EGDe 1/2a	Virulent clinical isolate	9
Δ*murA*	Δ*murA*	4
Δ*iap*	Δ*iap*	This work
Δ*murA* Δ*iap*	Δ*murA* Δ*iap*	This work
Δ*secA2*	Δ*secA2*	This work
Δ*secA2 attB*::*secA2*	Δ*secA2 attB*::*secA2*	
Δ*secA2-C*	Respective allele cloned from wild-type EGDe	This work
Δ*secA2-C-SLCC7503*	Respective allele cloned from rough strain SLCC7503	This work
SLCC[a]		
SLCC7500	R1	H. Hof, Mannheim, Germany
SLCC7501	R3	H. Hof, Mannheim, Germany
SLCC7503	R5	H. Hof, Mannheim, Germany
SLCC7504	50I	H. Hof, Mannheim, Germany
SLCC7506	153IV	H. Hof, Mannheim, Germany
SLCC7508	1/2	H. Hof, Mannheim, Germany
SLCC7509	4d	H. Hof, Mannheim, Germany
SLCC7510	1/2c	H. Hof, Mannheim, Germany
L. monocytogenes	4b, F2365	24
L. innocua	6a, CLIP11262	9
L. welshimeri	6b, SLCC5334	Laboratory strain collection
E. coli		
INVαF′	F′ *endA1 recA1 hsdR17*(r_K m_K^+) *supE44* λ^- *thi-1 gyrA relA1* ϕ80 *lacZ*αΔ*M15* Δ(*lacZYA-argF*) *deoR*$^+$	Invitrogen
BL21	F$^-$ *ompT hsdSB*(r_B^- m_B^-) *gal dcm*(*DE3*)	Invitrogen
Plasmids		
pAUL-A	Temperature sensitive shuttle vector; Em^r	5
pAUL-A-*iap*	Shuttle vector with flanked *iap* regions	This work
pAUL-A-*murA*	Shuttle vector with flanked *murA* regions	4
pAUL-A-*secA2*	Shuttle vector with flanked *secA2* regions	This work
pCR 2.1 TOPO	TA cloning vector for *E. coli*	Invitrogen
pPL2	*L. monocytogenes* site-specific phage integration vector	17
pPL2-*secA2*	Integration vector harboring the *secA2* gene	This work
pGEX6P-1	Ap^r *lacI*^{q} *tac* promoter	GE Healthcare
pGEX6P-1-*iap*	Vector carrying a 1,380-bp PCR fragment of *iap*	This work
Primers		
SecA2F1	5′-CTCCTGAGCTCTTTCAGAAAATCCCAGTA-3′	
SecA2R2	5′-CATCATAATTGCGGCCGCTTATATAACATCCTCCATAC-3′	
SecA2F3	5′-AGACGGCTTAGCGGCCGCCTAAAATACAACTTGCGTC-3′	
SecA2R4	5′-TGTTAGTCGACGACGTACCTTTTGTGGAG-3′	
0583For1	5′-GTATTTGCGGCCGCATTTTATATTGATTAATACTAAG-3′	
0583Rev2	5′-TGAATGACCCCTTCAGTTTACCTCGAGCTTTTG-3′	
Iap1	5′-TCGATCATCATAATTCTGTC-3′	
Iap2NotI	5′-GATAGTTGCTGCGGCCGCATTCATAAAACTCCTCTC-3′	
Iap3NotI	5′-TTTTATGAATGCGGCCGCTTAATAACTTAAAGTAAC-3′	
Iap4	5′-CATCATCTAAGTAAGTTGC-3′	
Iap-GSTfor	5′-ATCGCATCCGCAGGATCCGTAGTAGTCGAAGCTGGT-3′	
Iap-GSTrev	5′-GTTACTTTAAGTGAATTCTTATACGCGACCGAA-3′	
M13for	5′-GTAAAACGACGGCCAGT-3′	
M13rev	5′-CAGGAAACAGCTATGAC-3′	

[a] SLCC, Seeliger's Listeria Culture Collection, Mannheim, Germany.

constitutive expression levels of two or more unrelated proteins and was processed using the Image J software package (29).

Detection of lytic activity in SDS-polyacrylamide gels. Isolation of cell wall and supernatant protein extracts for the activity assay was performed as described by Domann and Zechel (7). Lytic activity was detected using the protocol of de Jonge and colleagues (6). Briefly, proteins are resolved using 12.5% SDS-polyacrylamide gels containing 0.2% (wt/vol) autoclaved and lyophilized *Micrococcus lysodeikticus* ATCC 4698 cells (Sigma). After electrophoresis, proteins are renatured in 25 mM Tris-HCl (pH 7) containing 1% (vol/vol) Triton X-100 until lytic bands are visible. Visualization of bands is enhanced by staining with 1% (wt/vol) methylene blue (Sigma) in 0.01% (wt/vol) KOH and subsequent destaining with distilled water (4).

Light and electron microscopy. For microscopic images of bacterial colonies, the various strains were grown on BHI agar plates at 37°C (supplemented with 8 μg/ml chloramphenicol as needed) and resultant colonies were analyzed at 50× original magnification. For visualization of bacterial cells, overnight cultures grown in BHI broth were washed in phosphate-buffered saline, Gram stained, and fixed onto objective slides for microscopic analysis at 200× or 1,000× original magnification (Axiophot; Zeiss).

For electron microscopy, 10-ml bacterial cultures were grown to early log phase (OD_{600}, ≈0.3) and the cells were fixed by addition of 1.3 ml formaldehyde, incubation for 5 min at 25°C, and then addition of 0.8 ml glutaraldehyde followed by an additional 2 h of incubation on ice. Cells were harvested by centrifugation (6,200 × *g* at 4°C for 15 min), and the pellet was resuspended in 2 ml of 1%

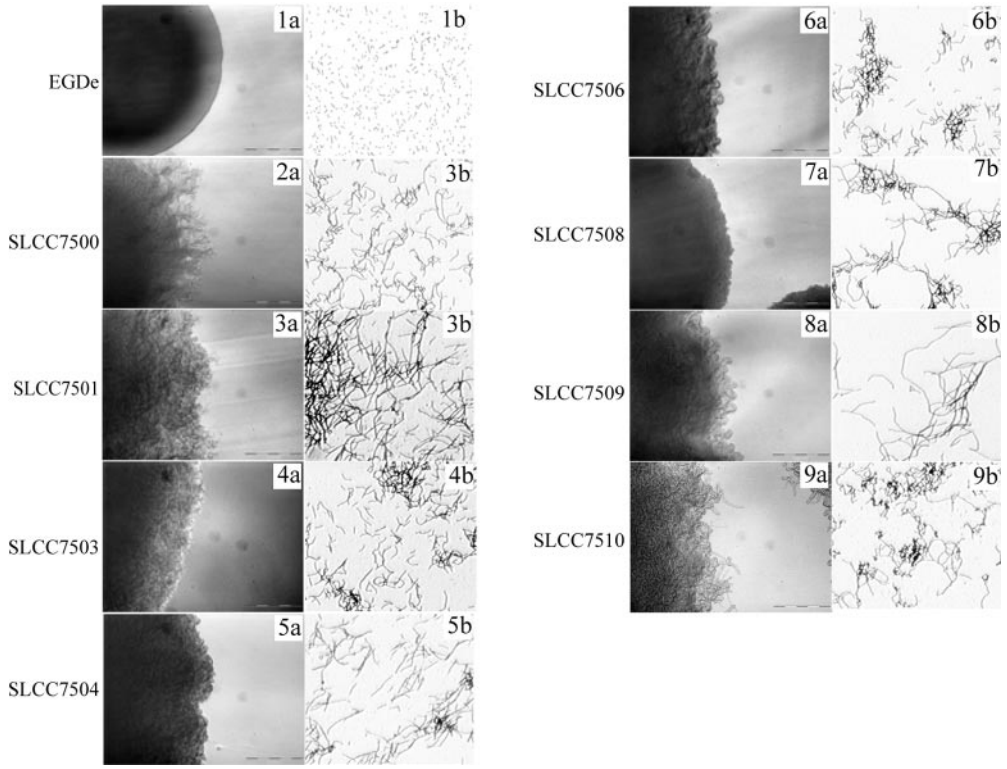

FIG. 1. Microscopic morphology of EGDe and rough isolates. (a) The edges of colonies of the various strains grown on BHI agar plates at 37°C were compared by microscopic analysis (50× original magnification). The wild type showed a smooth outline whereas the R variants have craggy edges. Bars, 500 μm. (b) Images of individual cells of wild-type and R variants are shown (200× original magnification). Bacteria were grown overnight in BHI broth at 37°C followed by Gram staining. All rough isolates exhibit long filaments in contrast to the small individual cells of the wild-type *L. monocytogenes* EGDe.

formaldehyde cacodylate buffer (cacodylate buffer: 0.1 M cacodylate, 0.09 M sucrose, 0.01 M $MgCl_2$, 0.01 M $CaCl_2$, pH 6.9). Samples were embedded in agar, small cubes were cut, and then samples were dehydrated with a graded series of ethanol on ice (10, 30, 50, 70, 90, and 100%) using 15 min for each step. Infiltration was done with LRWhite resin (1 part ethanol/1 part LRWhite, 1 part ethanol/2 parts LRWhite) for 12 h per step. Pure resin was changed three times before the samples were transferred into a gelatin capsule. Polymerization was performed at 50°C for 3 days. Ultrathin sections were cut with a diamond knife, collected onto Formvar-coated copper grids, counterstained with 4% (wt/vol) uranyl acetate for 1 min, washed with distilled water, and air dried. Samples were examined in a Zeiss EM910 transmission electron microscope at an acceleration voltage of 80 kV and calibrated magnifications.

Nucleotide sequence accession numbers. The nucleotide sequence data for *Listeria welshimeri* genes *murA* and *secA2* have been deposited in the EMBL nucleotide sequence database with the accession numbers AM039955 and AM040040, respectively. The nucleotide sequence of *iap* from the rough variant SLCC7506 and the sequences of *secA2* from the strains SLCC6509 and SLCC7510 were submitted to EMBL and assigned the accession numbers AM040043, AM040041, and AM040042, respectively.

RESULTS

Active p60 and MurA are reduced in rough variants of *L. monocytogenes*. We obtained eight clinical isolates (H. Hof, Mannheim, Germany) of *L. monocytogenes* that showed rough colony morphology on agar plates (Fig. 1, panels 2a to 9a) and long filaments in microscopic analysis (Fig. 1, panels 2b to 9b). Wild-type colonies exhibited smooth edges and were built mainly of single, rod-shaped cells (Fig. 1, panels 1a and 1b). In contrast, the rough mutants formed colonies with jagged edges, consisting of long filamentous cells. Rough isolates varied in colony edge formation and in the length of filaments (e.g., compare SLCC7501 to SLCC7506 in Fig. 1). We were interested in whether there are certain gene products responsible for the transition from smooth to rough that are common in these rough isolates. The strains were therefore examined by immunoblot analysis for their ability to express two autolysins, p60 and MurA. Protein extracts were isolated from the supernatant, cell wall, membrane, and cytoplasm of cultures of the wild type and rough mutants of *L. monocytogenes* grown to exponential phase. A Western blot assay was carried out with the monoclonal Fup60 mouse antibody, which is highly specific for the 60-kDa p60 protein. Seven of eight mutants displayed a decreased level of extracellular p60 (from 2% to 30% of wild-type level) while one strain, SLCC7506, showed a moderate level (60%) of a truncated protein in the supernatant (Fig. 2A). Those strains with diminished extracellular p60 exhibited a cellular relocation of the protein, which accumulated in the membrane fraction (3- to 12-fold more than wild-type protein). Most strains also showed increased levels of p60 in the cytoplasm (e.g., SLCC7501 and SLCC7503, with three- and sixfold more protein, respectively). In contrast, no obvious difference from wild type was seen in the cell wall fraction. Western blot assays of the MurA protein were performed using the mouse antibody αp70128G4. The prominent, marked band in Fig. 2B is the MurA protein with a molecular mass of 66 kDa, whereas

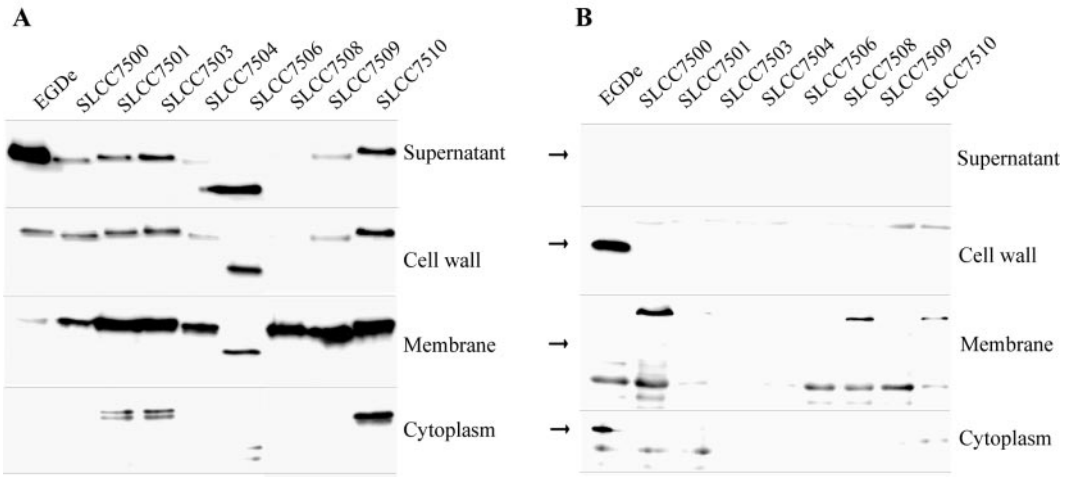

FIG. 2. Immunoblot analysis quantifying MurA and p60 from *L. monocytogenes* EGDe wild type and rough variants. Proteins were isolated from culture supernatant, cell wall, membrane, and cytoplasm. Mouse monoclonal antibody Fup60 was used against p60 (A) and mouse monoclonal antibody αp70128G4 was used against MurA (B) in protein extracts from *L. monocytogenes* EGDe wild type and rough mutants. Arrows indicate MurA protein.

the upper and lower bands in the membrane fraction are from nonspecific antibody binding. For all strains, the MurA protein could not be detected in the membrane fraction, as only nonspecific bands were visible. In wild-type EGDe MurA is mainly located in the cell wall; however, it is not observed in the rough variants. To a lesser extent the protein was also detected in the cytoplasmic fraction of the wild type but not of the rough strains (Fig. 2B).

Truncated p60 lacking most of the C-terminal NlpC/p60 domain is inactive. The *iap* gene of the rough strain SLCC7506, with a truncated p60 having a level of expression comparable to that of wild type, was sequenced to determine the nature of the mutation. The protein carries a number of single, nonsense mutations and has an internal 7-amino-acid deletion (accession number AM040043) (Fig. 3). A stop codon at position 375 truncates the protein by 100 amino acids in the region comprising the NlpC/p60 catalytic domain. Deletion of this region thus appears to inactivate p60, generating the same phenotype as that for mutants that lack p60 expression altogether.

Construction of Δ*iap*, Δ*murA* Δ*iap*, and Δ*secA2* chromosomal deletion mutants and of the Δ*secA2 attB*::*secA2* complementary strain. To determine the gene products required for the smooth-rough transition, we utilized individual mutants of *L. monocytogenes* EGDe lacking the genes for *iap*, *murA*, and *secA2* as well as the Δ*murA* Δ*iap* double mutant. The Δ*iap* mutant was created by standard homologous recombination techniques. The Δ*murA* strain has been described previously (4) and was used for generating the Δ*murA* Δ*iap* double mutant. The lack of the two proteins MurA and p60 in the Δ*murA* Δ*iap* double mutant was confirmed by immunoblot analysis using the *L. monocytogenes*-specific anti-MurA and anti-p60 monoclonal antibodies (Fig. 4). Given our difficulties both in cloning the *iap* gene and with transformation into Δ*iap* strains, complementation of the Δ*iap* and Δ*murA* Δ*iap* mutants was performed by treatment with exogenous recombinant MurA (4) and Iap/p60 protein. Additionally, although a viable complement strain for the Δ*murA* mutant is available (4), treatment with exogenous recombinant MurA produces effective

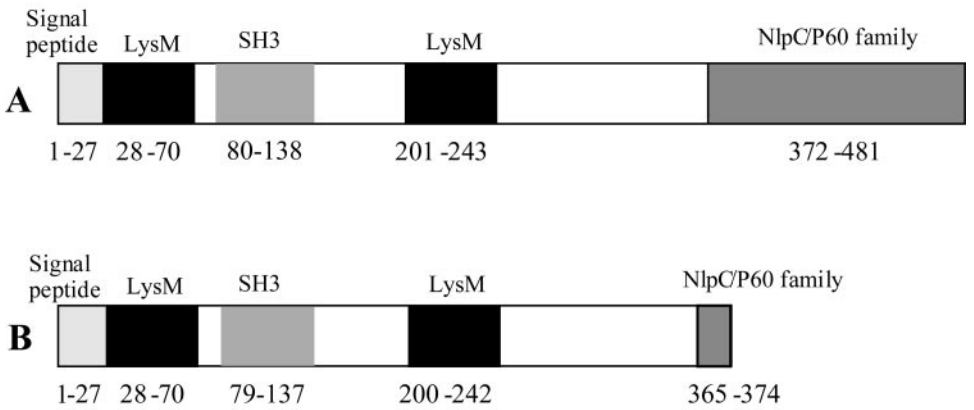

FIG. 3. Nonsense mutations of the truncated p60 in SLCC7506. Diagrams of the precursor protein with its known functional motifs encoded from *L. monocytogenes* EGDe (A) and the R variant SLCC7506 (B).

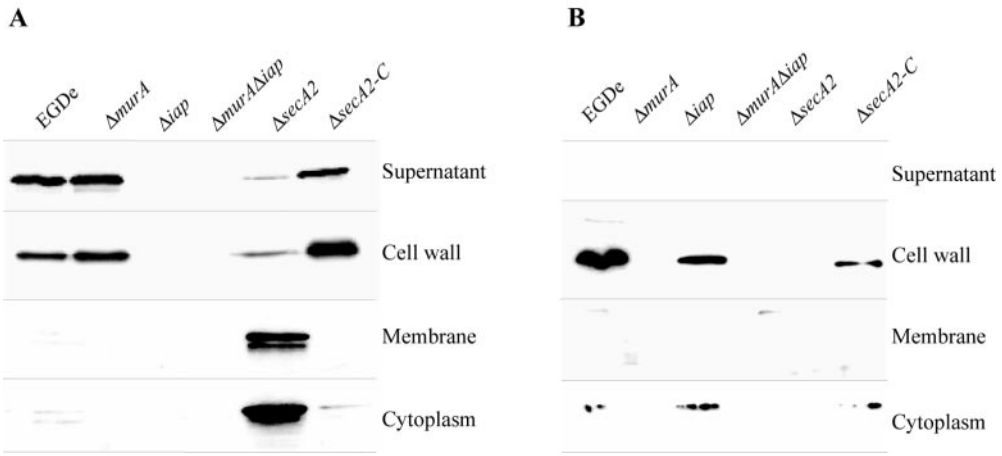

FIG. 4. Immunoblot analysis quantifying MurA and p60 from *L. monocytogenes* EGDe wild type and deletion mutants. Subcellular fractionation and immunoblotting were performed as described in the text. Mouse monoclonal antibody Fup60 was used against p60 in protein extracts from *L. monocytogenes* EGDe wild type and rough mutants (A), and mouse monoclonal antibody αp70128G4 was used against MurA (B).

phenotypic reversion (see below). We also generated an isogenic Δ*secA2* deletion strain, which was independently reported by Lenz and Portnoy (19) and which is known to cause smooth-rough transition in *L. monocytogenes*. As the SecA2 secretion system has been shown to be responsible for the transport of both of the autolysins p60 and MurA, we used the Δ*secA2* strain for phenotypic comparisons. The complementation of the Δ*secA2* mutant was carried out using the *L. monocytogenes* site-specific phage integration vector pPL2 harboring *secA2* (17). Since both MurA and p60 are dependent on the SecA2 transport system, as previously described, the Δ*secA2* deletion strain showed, as expected, a 10-fold-reduced level of the p60 protein in the supernatant and lacked the MurA protein in cell surface extracts. As in the rough mutants, MurA could not be detected in any subcellular fraction of the Δ*secA2* deletion mutant. In contrast, the p60 level was 100-fold increased in the membrane and cytoplasmic fractions of the Δ*secA2* mutant compared to wild type. The Δ*secA2-C* complement strain produced levels of p60 and MurA similar to those of wild-type protein.

Double mutants give rise to a rough phenotype similar to that of the Δ*secA2* strain. We compared the phenotypes of *L. monocytogenes* EGDe and deletion mutants by analysis with light and electron microscopy. The different strains were grown overnight in BHI broth at 37°C to early exponential phase for light microscopy and for electron microscopy. Despite defects in cell division, the growth behavior of the deletion mutants in BHI broth at either 20°C or 37°C did not differ substantially from that of the parental strain (data not shown). The Δ*murA* and Δ*iap* single mutants formed smooth and shiny wild-type-like colonies on BHI agar plates (compare panels 1a, 2a, and 3a in Fig. 5A). However, Δ*murA* Δ*iap* and Δ*secA2* strains lacking both extracellular murein hydrolases displayed rough colony morphology (Fig. 5A, panel 4a; 5B, panel 1a). The deletion of *murA* and *iap* could be complemented by treatment with exogenous recombinant proteins MurA and p60, thus restoring wild-type-like cell morphology (Fig. 5A, panels 2b to 4b and 2c to 4c). For the Δ*secA2* mutant, complement strains were created with the EGDe *secA2* gene (Δ*secA2-C*) as well as with the *secA2* gene of several of the rough variants (Fig. 5B, panels 2 and 3). Microscopic analysis revealed that the double mutant as well as the single *secA2* deletion mutant causes the generation of a rough phenotype with filaments greater than 10 μm in length (Fig. 5C, panels 4a and 5a). Both mutants lack indentations between individual cells but carry septa to separate one cell from another, as verified by transmission electron microscopy (Fig. 5C, panels 4b and 5b). In contrast, deletion of the *iap* gene alone in *L. monocytogenes* resulted in significantly shorter cell chains with an average length of 3 μm (Fig. 5C, panel 3a) and did not cause smooth-to-rough transition as reported earlier (27). Analogous to the double mutant the filamentous phenotype of the Δ*iap* strain showed no distinct partition between individual cells (Fig. 5C, panel 3b). The Δ*murA* mutant, on the other hand, formed long chains only in exponential phase but not in late lag phase, in agreement with the studies by Carroll et al. (4). In late lag phase we observed mostly single cells (data not shown) whereas in early exponential phase the cells formed chains (Fig. 5C, panels 2a and 2b) that, unlike the filaments of the rough strains, showed a clear contraction between each single bacterial cell. Complementation of the *secA2* gene in the Δ*secA2* deletion mutant restored the smooth phenotype and allowed for growth of individual cells.

A secondary *secA* gene and a homologous *murA* gene are present in the apathogenic species *Listeria innocua* and *L. welshimeri*. Through sequence analysis of other *Listeria* strains we identified a secondary *secA* gene in *L. innocua* and *L. welshimeri* (accession number AM040040). Several SecA transport systems have to date been found almost exclusively in pathogenic bacterial strains (19). The presence of active MurA has so far been reported only for *L. monocytogenes* (4). Our analysis revealed that homologous *murA* genes also exist in the chromosomal DNA of *L. innocua* and *L. welshimeri* (accession number AM039955) (Table 2). An alignment of the amino acid sequences showed a high degree of similarity between the MurA proteins of these species. We were therefore interested in whether these MurA homologues show the same activity as the protein in *L. monocytogenes*. To this end, we generated supernatant and cell wall protein extracts from cultures of *L. monocytogenes* EGDe, *L. innocua*, and *L. welshimeri* to assay

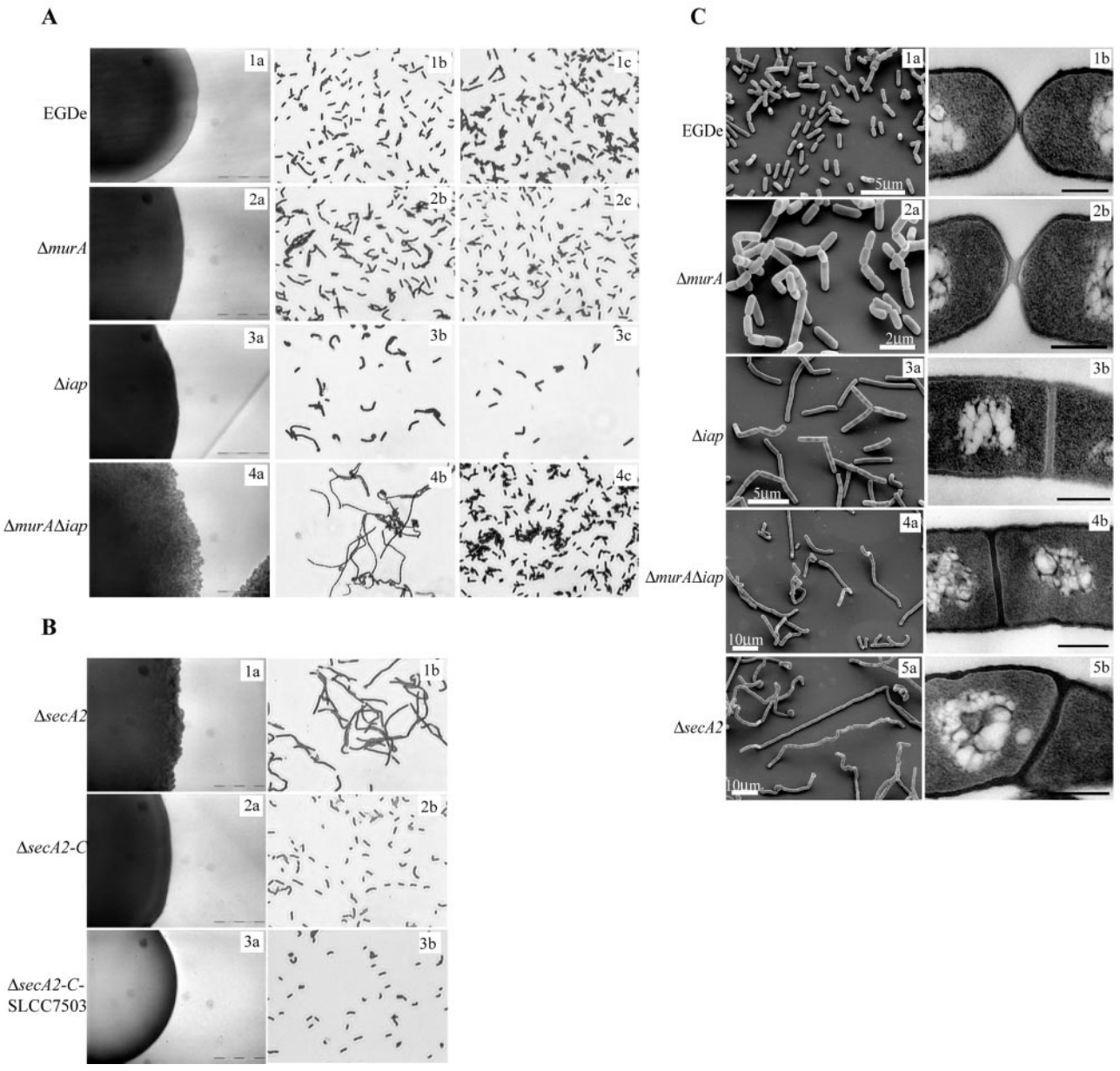

FIG. 5. Microscopic analysis of the cell morphology of *L. monocytogenes* EGDe, deletion mutants, and complemented strains. (A) Images of colony (a) and cell (b) appearance of EGDe and Δ*murA* and Δ*iap* single and double mutant strains are shown. (c) Complementation of Δ*murA*, Δ*iap*, and Δ*murA* Δ*iap* strains by treatment with recombinant proteins MurA and p60. EGDe serves as a control for protein treatment. Pictures in panels b and c were taken from cells in early log phase at 1,000× original magnification. Bars in panel a, 500 μm. (B) Images of colony (a) and cell (b) appearance of the Δ*secA2* strain and its Δ*secA2-C* and Δ*secA2-C*-SLCC7503 complement strains. Bars in panel a, 500 μm. (C) Scanning (a) and transmission electron (b) microscopy of wild type and deletion mutants. For electron microscopy, bacterial cells were harvested at an OD_{600} of ≈0.3 and prepared for further analysis. Bars in panel b, 0.25 μm.

for lytic activity. All strains investigated here showed a lytic band prominent in the fraction associated with cell wall (Fig. 6). Thus, *Listeria* species other than *L. monocytogenes* express a functional, homologous MurA protein.

DISCUSSION

We report the simultaneous reduction in protein level of two autolysins, p60 and MurA, in several rough variants of *L. monocytogenes*. Our study shows that both proteins are in some

TABLE 2. Distribution of genes *murA* and *secA2* in *Listeria* spp.

Organism	% Similarity to *L. monocytogenes* EGDe			
	MurA		SecA2	
	Designation	%	Designation	%
Listeria monocytogenes EGDe	Lmo2691	100	Lmo0583	100
Listeria monocytogenes 4b	Lmof2365_2670	96	Lmo2365_612	99
Listeria innocua	Lin2838	91	Lin0592	97
Listeria welshimeri	Lwe MurA	84	Lwe SecA2	98

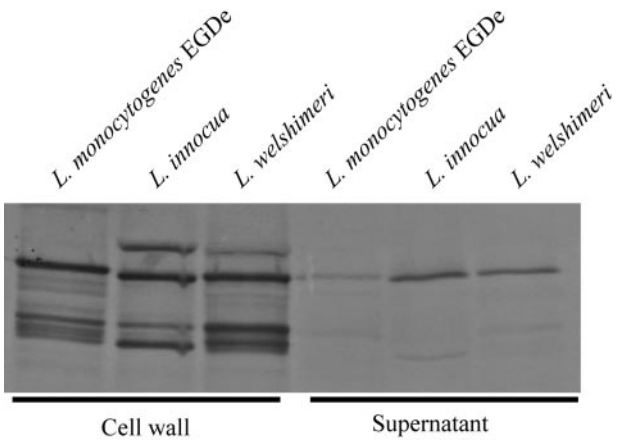

FIG. 6. Activity assay for MurA in *L. monocytogenes* EGDe, *L. innocua*, and *L. welshimeri*. A 12.5% SDS-PAGE assay for cell wall-associated proteins of *L. monocytogenes* EGDe wild type and deletion mutants was run on a gel containing 0.2% lyophilized *Micrococcus lysodeikticus* ATCC 4698 cells. Separated proteins were renatured with 25 mM Tris-HCl (pH 7) containing 1% (vol/vol) Triton X-100, and lytic bands were visualized by staining with 1% (wt/vol) methylene blue (Sigma) in 0.01% (wt/vol) KOH followed by destaining with water.

way either diminished, absent, or impaired in various strains with rough colony morphology. In the case of p60, we were able to detect a relocation of the protein from the culture supernatant to the membrane. This result implies a connection between the necessity for efficient transport of p60 out of the cell and formation of the smooth phenotype, with the rough strains having impaired expression or synthesis of the SecA2 protein. However, sequencing of the *secA2* gene of two of the rough variants (SLCC7509 and SLCC7510) investigated here revealed no evidence of changes either in the promoter region or in the SecA2 protein itself. Since alleles of the *secA2* gene cloned from several rough variants successfully complemented the Δ*secA2* mutant strain, it seems unlikely that a truncation or reduced expression of the SecA2 protein would be responsible for the defect. Nevertheless, defects in other gene products responsible for the proper function of the SecA2 secretion system might be involved. The basis of altered translocation of the p60 proteins in these strains warrants further investigation. Unlike p60, MurA, the second SecA2-dependent hydrolase in *L. monocytogenes*, did not show intracellular accumulation in any of the rough variants or in the Δ*secA2* deletion mutant. As such, it may be that an increased level of intracellular MurA is concomitant with increased degradation.

The reduced extracellular level of p60 in rough variants of *L. monocytogenes* was previously described (16, 18, 30) and, until the successful generation of an *iap* deletion mutant by Pilgrim et al. (27), was thought to be solely responsible for the rough phenotype (35). Our data agree with the observation of Pilgrim et al. (27) that additional factors, in conjunction with the p60 reduction, are needed. The Δ*iap* deletion mutant generates short chains but does not display the rough colony morphology. Similarly, disruption of the *murA* gene alone does not convert the phenotype from smooth to rough. The Δ*murA* deletion mutant exhibits long chains in exponential phase that fall apart into individual cells when the cultures reach lag phase (4). The filaments of the Δ*murA* mutant are distinct from those of the rough mutants because the chains show a clear constriction between each cell. In contrast, septum formation in strains lacking p60 is visible only via transmission electron microscopy, which shows that constriction of cell poles is inhibited. Thus, MurA seems to be important for the separation of individual cells at a late stage in cell division whereas p60 is involved in cleavage at an earlier phase.

Lenz et al. (18, 19) characterized a rough *L. monocytogenes* strain that resulted from the deletion of a second *Listeria secA* gene associated with protein secretion. They discovered that autolysins p60 and MurA (NamA), as well as various other secreted and surface-associated proteins, are dependent on the SecA2 secretion system. These SecA2-dependent proteins were assumed to be involved in the formation of long cell chains and rough colony morphology in *L. monocytogenes*. By creating the Δ*murA* Δ*iap* double mutant, we were able to identify two specific gene products responsible for the smooth-to-rough phenotype conversion. Mutation of each single gene, however, caused the formation of short (in the case of *iap*) or long (in the case of *murA*) cell chains without rough colony morphology. Only the simultaneous lack of both proteins in Δ*murA* Δ*iap* and Δ*secA2* strains resulted in the smooth-rough transition.

The role of the smooth-rough colony transition in the life cycle of *L. monocytogenes* remains unclear. Diverse rough *Listeria* strains demonstrate decreased pathogenicity by reduced invasion in mouse fibroblast cells (16) and in mouse models (31). In this context, the decreased virulence of rough bacterial strains that generate long filaments could conceivably be the result of an increased size and/or altered shape that inhibits effective adherence and uptake into the eukaryotic cell. Hence, it cannot be concluded that the gene products leading to a rough phenotype have a direct role in bacterial virulence. Rough *Listeria* strains have so far been identified only in the *L. monocytogenes* species. Since the investigated hydrolases and the SecA2 secretion system are also present in *L. innocua* and *L. welshimeri*, the question arises whether it is possible to alter the phenotype of these species from smooth to rough. The role of an additional SecA secretion system for pathogenic strains has been discussed extensively by Lenz and Portnoy (19) and was suggested to play a role in host colonization. The identification of a secondary SecA secretion system in both pathogenic and nonpathogenic *Listeria* strains implies a common role for this system. Genes homologous to p60 and MurA were identified in the genomes of other *Listeria* species also having the SecA2 system. The dependence of both autolysins on the SecA2 system may be linked to the *secA2* gene being conserved in those strains.

Proteins of the NlpC/p60 superfamily exhibit functional diversity and are widely represented in various bacterial lineages (1). The apparently inactive, truncated form of p60 in the rough strain SLCC7506 illustrates the important role of the NlpC/p60 catalytic domain for proper protein function. Although this truncated p60 was expressed in vivo in relatively wild-type amounts, implying stable protein production, the rough phenotype is indicative of a loss in activity. Interestingly, a smaller p60-like protein, p45, with peptidoglycan-hydrolyzing activity is known in *L. monocytogenes* (33). Although a deletion of the *spl* gene encoding p45 has not been described, it would

be worth seeing if such a mutant would exhibit defects in cell division similar to the Δ*iap* mutant.

There are numerous different rough phenotypes that vary in chain length, colony morphology, septum formation, pathogenicity, and motility. *L. monocytogenes* does not generate capsules; thus, a connection between rough colony morphology and variability in capsular material can be excluded. The formation of cell chains in the Δ*iap*, Δ*murA*, Δ*murA* Δ*iap*, and Δ*secA2* mutated EGDe strains highlights the importance of both of the autolysins p60 and MurA for cell division. That additional autolysins other than p60 and MurA might be involved in the transition to a rough phenotype is suggested by the observation that some rough strains do not have a decreased extracellular p60 level (31). In these cases, impaired synthesis or expression of other murein hydrolases may be responsible for the rough phenotypes while strains do not exhibit defects in p60 and MurA expression levels. The necessary participation of more than one cell wall hydrolase likely plays an important role in the generation of a rough phenotype in *L. monocytogenes*. Indeed, a similar situation is seen in *Bacillus* where a combined deficiency of several murein hydrolases increases the length of cell chains (12, 20, 21, 26).

ACKNOWLEDGMENTS

We thank Alexandra Amend for excellent technical assistance and Werner Bouschen (Department of Analytical Chemistry, Justus-Liebig-University) for the use of the dissection microscope. We are grateful to Ulrike Technow for her advice and assistance and indebted to George Silva (Department of Biochemistry, Justus-Liebig-University) for discussions and editing of the manuscript. We also thank Jessica Schaumburg (GBF, Braunschweig, Germany) for providing the MurA monoclonal antibody αp70128G4 and Herbert Hof (Institute of Medical Microbiology and Hygiene, University Hospital Mannheim) for providing the rough variants SLCC7500 to -7510.

This work was supported by a grant (PTJ-BIO/03U213B) made available through the German Ministry of Education and Research (BMBF) through the "Pathogenomik" Network to T.H. and T.C.

REFERENCES

1. **Anantharaman, V., and L. Aravind.** 2003. Evolutionary history, structural features and biochemical diversity of the NlpC/P60 superfamily of enzymes. Genome Biol. **4:**R11.
2. **Bubert, A., M. Kuhn, W. Goebel, and S. Kohler.** 1992. Structural and functional properties of the p60 proteins from different *Listeria* species. J. Bacteriol. **174:**8166–8171.
3. **Cabanes, D., O. Dussurget, P. Dehoux, and P. Cossart.** 2004. Auto, a surface associated autolysin of *Listeria monocytogenes* required for entry into eukaryotic cells and virulence. Mol. Microbiol. **51:**1601–1614.
4. **Carroll, S. A., T. Hain, U. Technow, A. Darji, P. Pashalidis, S. W. Joseph, and T. Chakraborty.** 2003. Identification and characterization of a peptidoglycan hydrolase, MurA, of *Listeria monocytogenes*, a muramidase needed for cell separation. J. Bacteriol. **185:**6801–6808.
5. **Chakraborty, T., M. Leimeister-Wachter, E. Domann, M. Hartl, W. Goebel, T. Nichterlein, and S. Notermans.** 1992. Coordinate regulation of virulence genes in *Listeria monocytogenes* requires the product of the *prfA* gene. J. Bacteriol. **174:**568–574.
6. **de Jonge, B. L., H. de Lencastre, and A. Tomasz.** 1991. Suppression of autolysis and cell wall turnover in heterogeneous Tn*551* mutants of a methicillin-resistant *Staphylococcus aureus* strain. J. Bacteriol. **173:**1105–1110.
7. **Domann, E., and S. Zechel.** 1997. Identification and characterization of a novel PrfA-regulated gene in *Listeria moncytogenes* whose product, IrpA, is highly homologous to internalin proteins which contain leucine-rich repeats. Infect. Immun. **65:**101–109.
8. **Furst, P., H. U. Mosch, and M. Solioz.** 1989. A protein of unusual composition from *Enterococcus faecium*. Nucleic Acids Res. **17:**6724.
9. **Glaser, P., L. Frangeul, C. Buchrieser, C. Rusniok, A. Amend, F. Baquero, P. Berche, H. Bloecker, P. Brandt, T. Chakraborty, A. Charbit, F. Chetouani, E. Couve, A. de Daruvar, P. Dehoux, E. Domann, G. Dominguez-Bernal, E. Duchaud, L. Durant, O. Dussurget, K. D. Entian, H. Fsihi, F. Garcia-del Portillo, P. Garrido, L. Gautier, W. Goebel, N. Gomez-Lopez, T. Hain, J. Hauf, D. Jackson, L. M. Jones, U. Kaerst, J. Kreft, M. Kuhn, F. Kunst, G. Kurapkat, E. Madueno, A. Maitournam, J. M. Vicente, E. Ng, H. Nedjari, G. Nordsiek, S. Novella, B. de Pablos, J. C. Perez-Diaz, R. Purcell, B. Remmel, M. Rose, T. Schlueter, N. Simoes, A. Tierrez, J. A. Vazquez-Boland, H. Voss, J. Wehland, and P. Cossart.** 2001. Comparative genomics of *Listeria* species. Science **294:**849–852.
10. **Heidrich, T., A. Ursinus, J. Berger, H. Schwarz, and J.-V. Höltje.** 2002. Effects of multiple deletions of murein hydrolases on viability, septum cleavage, and sensitivity to large toxic molecules in *Escherichia coli*. J. Bacteriol. **184:**6093–6099.
11. **Holtje, J. V., and E. I. Tuomanen.** 1991. The murein hydrolases of *Escherichia coli*: properties, functions and impact on the course of infections in vivo. J. Gen. Microbiol. **137:**441–454.
12. **Ishikawa, S., Y. Hara, R. Ohnishi, and J. Sekiguchi.** 1998. Regulation of a new cell wall hydrolase gene, *cwlF*, which affects cell separation in *Bacillus subtilis*. J. Bacteriol. **180:**2549–2555.
13. **Jonquieres, R., H. Bierne, F. Fiedler, P. Gounon, and P. Cossart.** 1999. Interaction between the protein InlB of *Listeria monocytogenes* and lipoteichoic acid: a novel mechanism of protein association at the surface of gram-positive bacteria. Mol. Microbiol. **34:**902–914.
14. **Kathariou, S., P. Metz, H. Hof, and W. Goebel.** 1987. Tn*916*-induced mutations in the hemolysin determinant affecting virulence of *Listeria monocytogenes*. J. Bacteriol. **169:**1291–1297.
15. **Kohler, S., A. Bubert, M. Vogel, and W. Goebel.** 1991. Expression of the *iap* gene coding for protein p60 of *Listeria monocytogenes* is controlled on the posttranscriptional level. J. Bacteriol. **173:**4668–4674.
16. **Kuhn, M., and W. Goebel.** 1989. Identification of an extracellular protein of *Listeria monocytogenes* possibly involved in intracellular uptake by mammalian cells. Infect. Immun. **57:**55–61.
17. **Lauer, P., M. Y. Chow, M. J. Loessner, D. A. Portnoy, and R. Calendar.** 2002. Construction, characterization, and use of two *Listeria monocytogenes* site-specific phage integration vectors. J. Bacteriol. **184:**4177–4186.
18. **Lenz, L. L., S. Mohammadi, A. Geissler, and D. A. Portnoy.** 2003. SecA2-dependent secretion of autolytic enzymes promotes *Listeria monocytogenes* pathogenesis. Proc. Natl. Acad. Sci. USA **100:**12432–12437.
19. **Lenz, L. L., and D. A. Portnoy.** 2002. Identification of a second *Listeria secA* gene associated with protein secretion and the rough phenotype. Mol. Microbiol. **45:**1043–1056.
20. **Margot, P., M. Pagni, and D. Karamata.** 1999. *Bacillus subtilis* 168 gene *lytF* encodes a gamma-D-glutamate-meso-diaminopimelate muropeptidase expressed by the alternative vegetative sigma factor, sigmaD. Microbiology **145:**57–65.
21. **Margot, P., M. Wahlen, A. Gholamhoseinian, P. Piggot, and D. Karamata.** 1998. The *lytE* gene of *Bacillus subtilis* 168 encodes a cell wall hydrolase. J. Bacteriol. **180:**749–752.
22. **Milohanic, E., R. Jonquieres, P. Cossart, P. Berche, and J. L. Gaillard.** 2001. The autolysin Ami contributes to the adhesion of *Listeria monocytogenes* to eukaryotic cells via its cell wall anchor. Mol. Microbiol. **39:**1212–1224.
23. **Monk, I. R., G. M. Cook, B. C. Monk, and P. J. Bremer.** 2004. Morphotypic conversion in *Listeria monocytogenes* biofilm formation: biological significance of rough colony isolates. Appl. Environ. Microbiol. **70:**6686–6694.
24. **Nelson, K. E., D. E. Fouts, E. F. Mongodin, J. Ravel, R. T. DeBoy, J. F. Kolonay, D. A. Rasko, S. V. Angiuoli, S. R. Gill, I. T. Paulsen, J. Peterson, O. White, W. C. Nelson, W. Nierman, M. J. Beanan, L. M. Brinkac, S. C. Daugherty, R. J. Dodson, A. S. Durkin, R. Madupu, D. H. Haft, J. Selengut, S. Van Aken, H. Khouri, N. Fedorova, H. Forberger, B. Tran, S. Kathariou, L. D. Wonderling, G. A. Uhlich, D. O. Bayles, J. B. Luchansky, and C. M. Fraser.** 2004. Whole genome comparisons of serotype 4b and 1/2a strains of the food-borne pathogen *Listeria monocytogenes* reveal new insights into the core genome components of this species. Nucleic Acids Res. **32:**2386–2395.
25. **Ng, W. L., K. M. Kazmierczak, and M. E. Winkler.** 2004. Defective cell wall synthesis in *Streptococcus pneumoniae* R6 depleted for the essential PcsB putative murein hydrolase or the VicR (YycF) response regulator. Mol. Microbiol. **53:**1161–1175.
26. **Ohnishi, R., S. Ishikawa, and J. Sekiguchi.** 1999. Peptidoglycan hydrolase LytF plays a role in cell separation with CwlF during vegetative growth of *Bacillus subtilis*. J. Bacteriol. **181:**3178–3184.
27. **Pilgrim, S., A. Kolb-Maurer, I. Gentschev, W. Goebel, and M. Kuhn.** 2003. Deletion of the gene encoding p60 in *Listeria monocytogenes* leads to abnormal cell division and loss of actin-based motility. Infect. Immun. **71:**3473–3484.
28. **Pitcher, D. G., N. A. Saunders, and R. J. Owen.** 1989. Rapid extraction of bacterial genomic DNA with guanidium thiocyanate. Lett. Appl. Microbiol. **8:**151–156.
29. **Rasband, W. S.** 1997. ImageJ, v1.34n. National Institutes of Health, Bethesda, Maryland. http://rsb.info.nih.gov/ij/.
30. **Rowan, N. J., J. G. Anderson, and A. A. Candlish.** 2000. Cellular morphology of rough forms of *Listeria monocytogenes* isolated from clinical and food samples. Lett. Appl. Microbiol. **31:**319–322.
31. **Rowan, N. J., A. A. Candlish, A. Bubert, J. G. Anderson, K. Kramer, and J. McLauchlin.** 2000. Virulent rough filaments of *Listeria monocytogenes* from clinical and food samples secreting wild-type levels of cell-free p60 protein. J. Clin. Microbiol. **38:**2643–2648.

32. **Schaferkordt, S., and T. Chakraborty.** 1995. Vector plasmid for insertional mutagenesis and directional cloning in *Listeria* spp. BioTechniques **19:**720–722, 724–725.
33. **Schubert, K., A. M. Bichlmaier, E. Mager, K. Wolff, G. Ruhland, and F. Fiedler.** 2000. P45, an extracellular 45 kDa protein of *Listeria monocytogenes* with similarity to protein p60 and exhibiting peptidoglycan lytic activity. Arch. Microbiol. **173:**21–28.
34. **Tomasz, A.** 1984. Building and breaking of bonds in the cell wall of bacteria—the role for autolysins, p. 3–12. *In* C. Nombela (ed.), Microbial cell wall synthesis and autolysis. Elsevier Science, Amsterdam, The Netherlands.
35. **Wuenscher, M. D., S. Kohler, A. Bubert, U. Gerike, and W. Goebel.** 1993. The *iap* gene of *Listeria monocytogenes* is essential for cell viability, and its gene product, p60, has bacteriolytic activity. J. Bacteriol. **175:**3491–3501.