SecA2-dependent secretion of autolytic enzymes promotes *Listeria monocytogenes* pathogenesis

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Edited by Roy Curtiss, Washington University, St. Louis, MO, and approved August 21, 2003 (received for review June 13, 2003)

Pathogenic bacteria secrete proteins that promote invasion of host tissues and resistance to immune responses. However, secretion mechanisms that contribute to the enormous morbidity and mortality of Gram-positive bacteria are largely undefined. An auxiliary protein secretion system (SecA2) has recently emerged in *Listeria monocytogenes* and eight other Gram-positive pathogens. Here, a proteomics approach identified seventeen SecA2-dependent secreted and surface proteins of *L. monocytogenes*, the two most abundant of which [the p60 and *N*-acetylmuramidase (NamA) autolysins] hydrolyze bacterial peptidoglycan (PGN) and contribute to host colonization. SecA2-deficient (ΔSecA2) bacteria were rapidly cleared after systemic infection of murine hosts, and in cultured cells showed reduced cell–cell spread. p60 or NamA deficiencies (Δp60 and ΔNamA) caused intermediate reductions in bacterial virulence *in vivo*, yet showed no defect for infection of cultured cells. Restoration of virulence in Δp60 bacteria required full-length p60 with an intact catalytic domain, suggesting that PGN hydrolysis by p60 is crucial for *L. monocytogenes* virulence. Coordinated PGN hydrolysis by p60 and NamA activities is predicted to generate a muramyl glycopeptide, glucosaminylmuramyl dipeptide (GMDP), which is known to modify host inflammatory responses. Thus, SecA2-dependent secretion may promote release of muramyl peptides that subvert host pattern recognition.

A common and conserved strategy for pathogenesis of Gram-negative bacteria is to inject eukaryotic cells with protein effectors that promote or prevent phagocytosis (1), promote growth by avoiding host defense mechanisms (2), and induce or prevent cell death (3–5). The injection of these effector proteins requires a specialized auxiliary protein secretion system known as type III secretion. At least three additional auxiliary secretion systems have also been shown to contribute to the pathogenesis of specific Gram-negative bacteria (6). Yet, despite the demonstrated importance of such secretion systems in Gram-negative pathogens, studies of Gram-positive pathogenesis have largely focused on toxins and superantigens (7, 8). Remarkably little is known about how release of these and other factors is regulated and contributes to Gram-positive pathogenesis.

Recently, an auxiliary SecA paralog (SecA2) was identified in nine Gram-positive bacteria that cause severe or lethal infections of humans. SecA2 is nonessential but nonetheless was required for secretion of a subset of the exported proteomes (secretomes) of *Listeria*, Mycobacteria, and Streptococci (9–12). The *Listeria monocytogenes* SecA2 was identified through its association with bacterial smooth-rough variation, and mutations in *secA2* reduced virulence of *L. monocytogenes* and *Mycobacterium tuberculosis* (11, 12). These findings suggested that SecA2-dependent protein secretion might promote Gram-positive pathogenesis. Here, we identified 17 SecA2-dependent proteins of *L. monocytogenes* and showed that the activity of two abundant autolysins promotes bacterial virulence. We present a mechanism by which SecA2-dependent secretion of these autolysins may facilitate immune subversion.

Methods

Bacterial Manipulations and Proteomics. *L. monocytogenes* strains with in-frame deletions of the indicated genes were generated in the 10403S background by splice-overlap extension (SOE)-PCR and allelic exchange with established methods (13). Each of the strains described here showed growth rates identical to the parental strain in bacteriological media. Bacteria were grown to exponential phase in LB for harvest of supernatant and SDS-extractable cell-surface proteins, as detailed (11). Proteins were separated by 10% SDS/PAGE and stained with colloidal Coomassie blue (Invitrogen). Gel slices containing SecA2-dependent proteins were excised and treated with trypsin for at least 4 h at 37°C. Tryptic peptides were purified on C18 ZipTips and spotted to matrices for analysis on an Applied Biosystems time-of-flight mass spectrometer at the University of California, Berkeley, Howard Hughes Medical Institute Mass Spectrometry Laboratory. Peptide masses were used to fingerprint the corresponding SecA2-dependent proteins in the *L. monocytogenes* genome by using the MS-FIT component of the PROTEINPROSPECTOR software package. This approach unambiguously identified 17 SecA2-dependent supernatant and cell surface-associated proteins. The pPL2 integrational vector (14) was used to complement SecA2 and p60 expression at single copy and in trans in the respective mutant strains. Complementation constructs contained the endogenous regulatory sequences for the respective genes or gene fragments. Transduction with phage U153 was used to engineer Erm-resistant test strains as detailed (11).

Cell Wall Isolation and Zymography. *L. monocytogenes* cell walls were purified and cast in polyacrylamide gels as peptidoglycan (PGN) substrate for digestion by bacterial autolysins as described (15). Surface-associated and secreted proteins isolated from cultures of the indicated *L. monocytogenes* strains grown in LB were electrophoresed into these PGN-embedded gels and incubated for 48 h in renaturation buffer (0.1% Triton X-100/10 mM $CaCl_2$/25 mM Tris·HCL, pH 7.5). *In situ* catabolism of the imbedded cell wall material by the refolded autolysins was visualized as cleared bands in methylene blue-stained gels. To enhance visualization of these cleared bands, the zymograms were scanned and inverted by using PHOTOSHOP software (Adobe Systems, Mountain View, CA).

Mouse and Tissue Culture Infection Assays. Control and Erm-resistant test strains were grown to exponential phase, washed in PBS, and used for i.v. infection of BALB/c mice. The competitive index assay was modified from that described (16), in that numbers of Erm-resistant and sensitive bacteria were quantified by plating organ homogenates (in 0.2% Nonidet P-40 with 0.1 μg/ml Erm to induce resistance) directly on LB agar with or without Erm (1 μg/ml). The observed competitive index for ΔSecA2 vs. 10403S was ≈1 when these strains were cocultured for 2–16 h at 37°C in aerated Luria broth. Bone marrow

This paper was submitted directly (Track II) to the PNAS office.

Abbreviations: PGN, peptidoglycan; BMM, bone marrow macrophage; NamA, *N*-acetylmuramidase; GMDP, glucosaminylmuramyl dipeptide.

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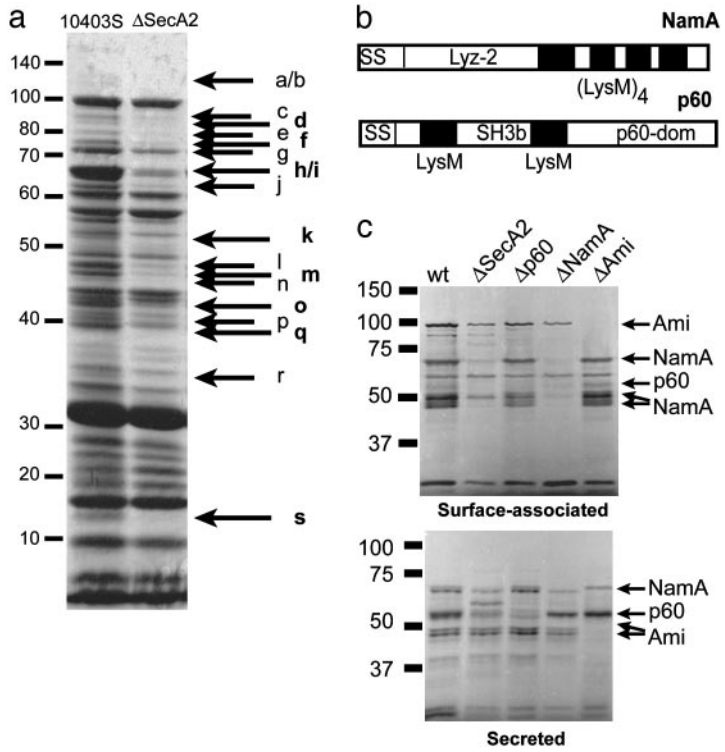

Fig. 1. The SecA2-dependent extracellular proteome of *L. monocytogenes* includes two abundant autolytic enzymes. (*a*) SDS/PAGE of proteins extracted from the surface of log-phase WT and ΔSecA2 bacteria by boiling in SDS sample buffer (2% SDS, 5% 2-mercaptoethanol). Forty-nine protein bands were resolved in WT samples stained with colloidal Coomassie blue, 19 of which were SecA2-dependent and are indicated by arrows. Seventeen of these proteins were identified by mass spectrometry and are listed in Table 1. (*b*) Schematic of the SecA2-dependent NamA and p60 proteins. LysM domains promote adhesion to the bacterial cell wall; Lyz-2 and p60 domains are associated with cleavage of peptidoglycan. (*c*) Zymograms showing bands of autolytic activity present in samples of surface-associated and secreted proteins of the WT and mutant *L. monocytogenes* strains. Only the catalytic activities attributed to p60 and NamA are missing in the samples from the ΔSecA2 strain.

macrophages (BMM) were cultured from BALB/c mouse femurs and infected on day 7 of culture with WT or mutant bacteria at a multiplicity of 1:10 as described (17). For microscopy, infected BMM were harvested at 6.5 h postinfection and stained with Diff-Quik (Dade Behring, Düdingen, Switzerland). For plaquing, L2 and TIB73 cells were grown to confluency, infected for 1 h, then overlayed with 0.7% agarose in DMEM (Gibco/BRL) containing 10% FCS and 10 μg/ml gentamicin. After 3 days in a 37°C CO_2 incubator, plaques were visualized in the monolayer by addition of neutral red to 0.3%. Animal experiments were approved by the Animal Care and Use Committee at the University of California, Berkeley.

Results

Identification of SecA2-Dependent Proteins. To further examine the role of SecA2 in *L. monocytogenes* virulence, polyacrylamide gel electrophoresis was used to compare the profiles of secreted proteins and surface extracts of exponentially grown ΔSecA2 and WT strains (10403S). Approximately 49 surface-extractable proteins were resolved in preparations from WT bacteria when gels were stained with colloidal Coomassie blue dye (Fig. 1*a*). A similar pattern was seen in parallel preparations from the ΔSecA2 strain, except for 19 proteins that were either undetectable or markedly reduced (arrows in Fig. 1*a*). SecA2 expression was also required for normal secretion of 7 of the ≈25 protein bands resolved from supernatant fractions of *L. monocytogenes* cultures (ref. 11 and data not shown). These results indicated that, under these conditions, less than one-third of the *L. monocytogenes* secretome is SecA2-dependent.

To identify these SecA2-dependent proteins, each affected band was excised from the WT sample and subjected to mass-spectrometry fingerprinting. Peptide masses were compared with the *L. monocytogenes* genome sequence (18) to unambiguously identify 17 SecA2-dependent proteins. Seven of these proteins initiated with N-terminal secretion signal peptides (Table 1), the two most-abundant of which were a surface-associated *N*-acetylmuramidase (referred to here as NamA) and a secreted endopeptidase (p60; ref. 19). The signal peptides of NamA and p60 are 52 or 27 aa, respectively, and each enzyme contains domains to bind (LysM) and digest (Lyz-2 or p60) bacterial PGN (Fig. 1*b*). These observations suggested that autolysins are major targets of SecA2-dependent secretion in *L. monocytogenes*.

To further evaluate the association between SecA2 and the autolytic activities of p60 and NamA, we engineered in-frame deletions in genes coding for NamA, p60, or another abundant (SecA2-independent) autolytic amidase, Ami (15). Zymography was used to evaluate the profile of PGN-digesting activities secreted by each strain (Fig. 1*c*). These zymograms revealed 10–14 distinct bands of PGN-digesting activity in protein extracts from the WT strain (Fig. 1*c*), each of which corresponds to a full-length or proteolytic fragment of an active autolysin. However, the secreted proteins from ΔSecA2 bacteria were missing four autolytic bands. Each of these missing bands was also missing from the Δp60 or ΔNamA strains. Thus, these results confirmed that p60 and NamA digest *L. monocytogenes* cell walls, and that SecA2 promotes extracellular accumulation of these autolysins. Furthermore, the effect of SecA2 on secretion of autolytic activities was specific for p60 and NamA because it did not promote secretion of Ami or other autolytic activities.

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Table 1. SecA2-dependent proteins identified in this study

Code*	MW†	pI‡	Description	Source§	MOWSE score¶
Proteins with signal peptides					
e	81.9	7.05	PBP 2B	SN, SDS	5.8×10^7
h	63.6	9.73	***N*-acetylmuramidase (NamA)**	SN, SDS	2.3×10^7, 6.7×10^{10}
i	62.6	5.3	Pheromone transporter (OppA)	SN, SDS	1.8×10^{12}, 8.8×10^4
k	50.3	9.3	**p60 autolysin**	SN	3.4×10^4
l	46	5.2	Maltose/maltodextrin ABC transporter	SDS	2.4×10^4
p	38.4	5.02	Antigenic lipoprotein (Csa)	SDS	1.5×10^{12}
r	32.7	6.25	Conserved lipoprotein	SDS	4.3×10^9
Proteins without signal peptides (moonlighting proteins)					
a	134.8	8.51	RNA polymerase β' subunit	SN, SDS	4×10^9, 2.5×10^{10}
b	132.6	5.05	RNA polymerase β subunit	SN, SDS	4×10^{13}, 4.5×10^{21}
f	58.2	4.76	***Pyruvate dehydrogenase E2 subunit***	SDS	6.7×10^{10}
g	66.1	4.57	DnaK	SDS	7.8×10^{14}
j	57.4	4.72	***GroEL***	SN, SDS	6.7×10^{10}, 1.2×10^{22}
m	43.3	4.8	EF-Tu	SDS	3.3×10^{19}
n	46.5	4.7	Enolase	SDS	3.3×10^{11}
q	35.3	4.89	***Phosphomannose isomerase***	SDS	2.7×10^4
s	13.1	11.4	Ribosomal protein L19	SDS	551
s	14.4	10.7	Ribosomal protein S9	SDS	249

Signal peptides were predicted using SIGNALP.
*Codes refer to protein bands labeled in Fig. 1*a*.
†Calculated molecular mass (in kDa).
‡Calculated pI.
§Source indicates culture fraction(s) in which the protein was identified by mass spectrometry. SDS, surface extracted; SN, supernatant.
¶Molecular weight search (MOWSE) score from MS-FIT software. Zymography confirmed the effect of SecA2 on release of proteins listed in bold (see Fig. 1*c*). Immunoblotting of culture fractions confirmed that SecA2 expression affected release of those proteins listed in bold italic characters (see Fig. 5).

Requirements for SecA2 and SecA2-Dependent Autolysins in the Infection of Host Tissues *in Vivo*. The relative contributions of SecA2, NamA, and p60 to *L. monocytogenes* virulence *in vivo* were quantified by using a recently developed competitive index assay (16). For these experiments, the mutant bacterial strains were tagged with an erythromycin resistance (Erm^R) marker (Tn917), mixed ≈1:1 with erythromycin-sensitive (Erm^s) WT bacteria (strain 10403S), and used to coinfect BALB/c mice. At the indicated times after infection, the ratios of mutant:WT bacteria were determined by plating tissue homogenates on media with or without Erm. The ≈1:1 recovery of WT and mutant bacteria 6–8 h after infection indicated that ΔSecA2 bacteria seeded spleens and livers of infected mice as well as the WT strain (Fig. 2*a*). After this initial infection, the ratio declined as the ΔSecA2 bacteria were steadily and selectively cleared from mouse livers over a period of 3 days. Conversely, ΔSecA2

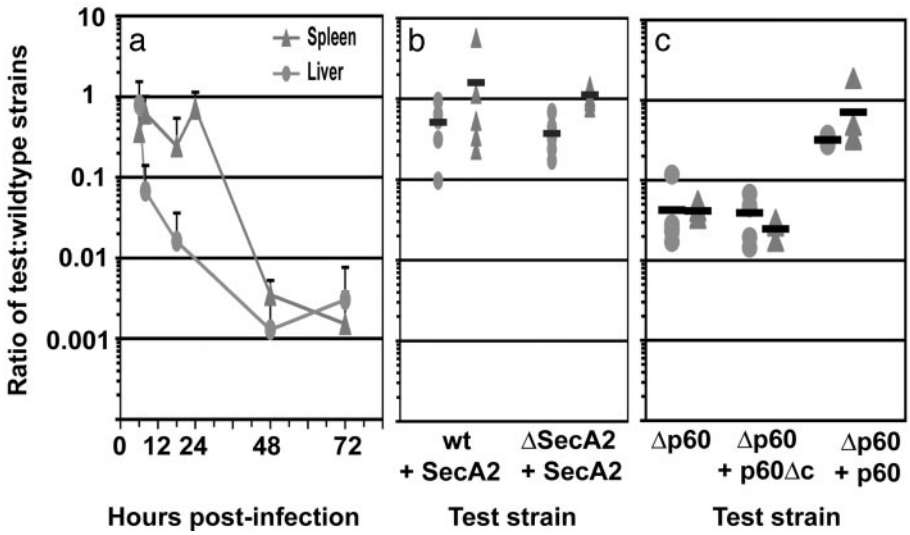

Fig. 2. SecA2 and catalytically active p60 are essential for sustained colonization of mouse tissues by *L. monocytogenes*. (*a*) Kinetic analysis of the virulence defect for the ΔSecA2 strain. Mice were infected with a 1:1 ratio of mutant:WT bacteria. The inability of the marked ΔSecA2 test strain to persist in the host at WT levels is shown by reduced ratios of test:WT strain colonies recovered at later time points after infection. Data are compiled from three experiments comprising at least five mice per time point. Bars indicate SDs. (*b*) Competitive indices for five mice per group demonstrate restored virulence with expression of SecA2 in the ΔSecA2 strain. Expression of additional SecA2 in the parental strain did not affect virulence. Ratios were determined at 72 h postinfection. (*c*) Reduced virulence of *L. monocytogenes* lacking p60. Virulence is restored only by expression of p60 with an intact catalytic domain. Bars in *b* and *c* indicate mean values.

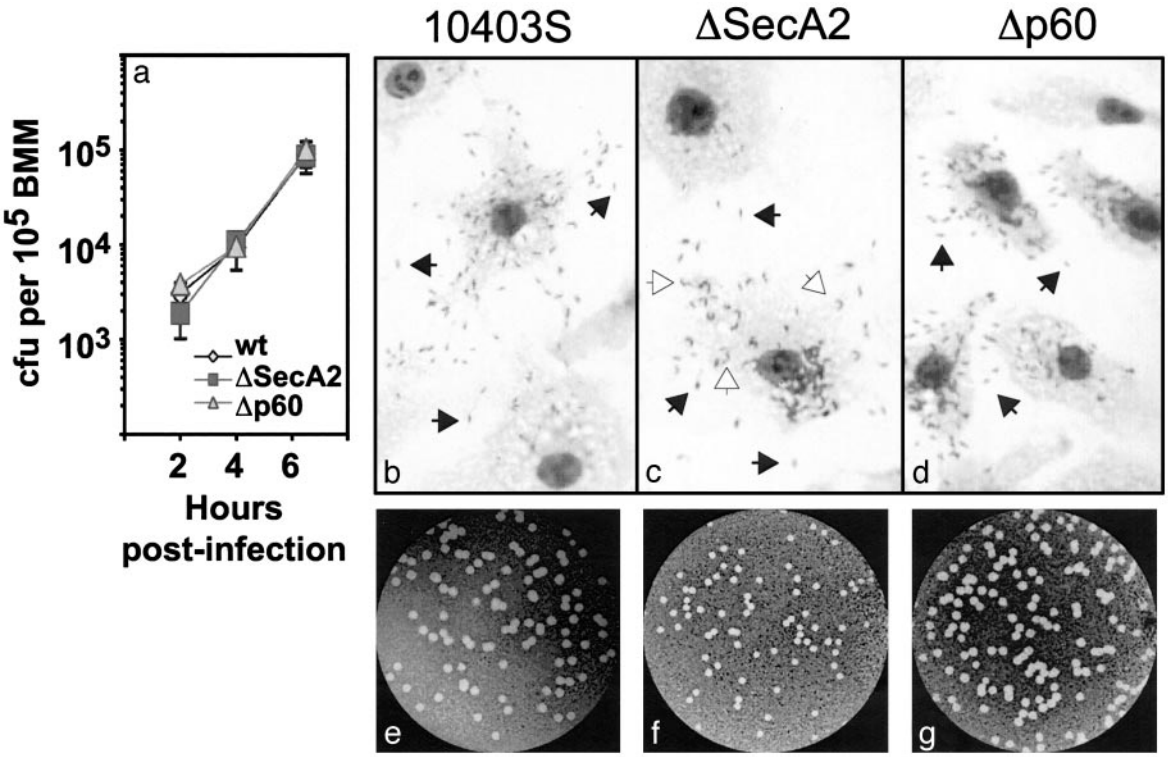

Fig. 3. SecA2 and p60 are not required for intracellular growth of *L. monocytogenes* although SecA2 promotes bacterial cell–cell spread independent of p60. (*a*) Infection and intracellular replication of WT (10403S) and mutant bacteria were measured by plating lysates of primary mouse macrophages at the indicated times after infection. The host cell-impermeable antibiotic gentamycin sulfate was added to kill extracellular bacteria 30 min after infection. The infection efficiency and growth rates (judged by increased colony-forming units over time) were identical for all three strains. (*b–d*) Micrographs of mouse bone marrow macrophages 6.5 h after infection with WT or mutant *L. monocytogenes* strains. Black arrows indicate bacteria-induced pseudopod-like structures. White arrows show doublets of ΔSecA2 bacteria. (*e–g*) Plaques formed in monolayers of TIB73 hepatocytes 72 h after infection with WT or mutant bacteria. The mean diameter of plaques formed by WT and Δp60 bacteria were identical whereas the ΔSecA2 strain showed reduced cell–cell spread as judged by the 32 ± 4% smaller plaque size.

bacteria persisted at near WT levels for at least 18–24 h before their selective clearance from spleens of coinfected animals. The magnitude of the virulence defect at 72 h was similar to that seen by LD_{50} (11), and was fully complemented by reintroducing SecA2 into the ΔSecA2 strain (Fig. 2*b*). Overall, these kinetic analyses revealed that SecA2 is dispensable for initial stages of bacterial entry and replication in mouse tissues, yet plays a crucial role later in the infection.

The NamA autolysin moderately contributed to *L. monocytogenes* persistence in livers of infected mice, with the ΔNamA strain showing an average 7.6 ± 4.5-fold reduction in competitive index ratio at 48 h after infection. The p60 autolysin, however, played a more indispensable role. Recovery of Δp60 bacteria from both livers and spleens was ≈1/50th that of the coinfected WT strain by 48 h postinfection (Fig. 2*c*). This defect was fully complemented by expression of a full-length p60, but not by a truncated p60 that lacked the C-terminal catalytic domain (Fig. 2*c*). These data implicate SecA2-dependent autolysins, particularly p60, as important effectors of *L. monocytogenes* pathogenesis and suggest that p60's contribution involves digestion of *L. monocytogenes* PGN.

Effects of SecA2 and p60 on Infection of Mammalian Cells *in Vitro*. Tissue culture infection assays were next used to evaluate whether SecA2-dependent secretion of p60 or other substrates contributes to cytosolic growth in mammalian cells, as intracellular growth in mammalian cells and bacterial cell–cell spread are crucial stages in *L. monocytogenes* pathogenesis (20). Both p60-deficient and SecA2-deficient bacteria replicated in the cytosol of bone marrow macrophages (BMM) at rates equivalent to those seen for the wt strain (Fig. 3*a*). Infected BMM were also examined microscopically by staining coverslips coated with the infected cells. Infections with WT or Δp60 bacteria were indistinguishable with similar numbers of bacterial cells visible per BMM (Fig. 3 *b* and *d*). These bacteria were distributed similarly throughout the BMM cytosol and in pseudopod-like structures that protruded from the infected cells. The formation of such pseudopods requires ActA-dependent polymerization of host actin filaments and is an intermediate in bacterial cell–cell spread (20). Pseudopods were also observed after infection with ΔSecA2 bacteria. However, ΔSecA2 bacterial cells often appeared as doublets intracellularly, and the frequency of pseudopod formation was visibly reduced as compared with the WT strain (Fig. 3*c*). The relative efficiency of cell–cell spread was more precisely quantified by measuring the diameter of plaques formed on prolonged bacterial infection of murine fibroblast and hepatocyte cell monolayers (Fig. 3 *e–g* and data not shown). Plaque formation by the Δp60 strain was equal to that of the WT parent (Fig. 3 *e* and *g*) whereas plaques formed by the ΔSecA2 strain were 68 ± 4% of WT (Fig. 3*f*). These cell culture infections show that SecA2 and SecA2-dependent autolysins are dispensable for intracellular parasitism *per se*. However, SecA2 expression clearly promotes bacterial cell–cell spread in a manner that is independent of p60. This defect in cell–cell spread may at least partially reflect bacterial chaining and presumably contributes significantly to the reduced virulence of ΔSecA2 *in vivo*. Further study will be needed to determine how the remaining SecA2-dependent proteins contribute to these processes.

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PGN Hydrolysis by p60 Is Not Essential for Secretion of Heterologous Proteins. Of the SecA2-dependent proteins listed in Table 1, 10 lacked secretion signal sequences and had well-described metabolic or biosynthetic functions in the bacterial cytosol (Table 1). The significance of the SecA2-dependent release of these "moonlighting" proteins by *L. monocytogenes* is not presently clear. However, we were able to confirm that SecA2 expression promotes release of at least three moonlighting proteins: GroEL, phosphomannose isomerase, and the lipoylated E2 subunit of pyruvate dehydrogenase (Fig. 5, which is published as supporting information on the PNAS web site, www.pnas.org.). Thus, it is possible that release of these proteins normally occurs during infection of host animals. Because our data above showed that SecA2-dependent secretion of autolytic enzymes coordinates PGN hydrolyzing activity at the bacterial surface, we considered the possibility that p60's contribution to *L. monocytogenes* virulence was through the release of these or other moonlighting proteins. Bacterial autolysins have previously been associated with protein secretion through the thick bacterial cell wall (21, 22), presumably by generating holes through which flagellar or other structures are assembled. SecA2-dependent autolysins might thus be expected to serve a similar function, or to promote the release of these otherwise cytosolic proteins through induction of bacteriolysis. However, this does not seem to be the case, because the profile and amounts of heterologous proteins secreted by WT and Δp60 *L. monocytogenes* are identical (not shown). These data argue that the contribution of the p60 autolysin to *L. monocytogenes* virulence is not through release of other SecA2-dependent or independent proteins. The Δp60 and ΔSecA2 strains also showed no increase in sensitivity to osmotic stress, treatment with penicillin (a β-lactam antibiotic that interferes with cell wall synthesis), or susceptibility to treatment with an α-defensin (crp4; L.L.L., D.A.P., H. Tanabe, and A. J. Ouellette, unpublished observations). Thus, it is unlikely that SecA2-dependent autolysins are essential for secretion of heterologous proteins or to modify the cell wall in a manner that enhances bacterial resistance to antimicrobial mechanisms.

Discussion

SecA2 is an auxiliary secretory protein that is conserved in several pathogenic Gram-positive bacteria, including *Staphylococcus aureus*, *M. tuberculosis*, *Bacillus anthracis*, and virulent strains of *Streptococcus pneumoniae*. Here we have shown that SecA2 is required for persistent colonization of host tissues by *L. monocytogenes* by using both *in vivo* and *in vitro* assays of bacterial virulence. We furthermore identified 17 proteins that show SecA2-dependent release from WT *L. monocytogenes* during its growth in bacteriological broth. In-frame deletions in the genes coding for the 2 most abundant of these proteins, the p60 and NamA autolysins, led to reduced virulence of *L. monocytogenes*. In the case of the Δp60 strain, the effect on virulence was quite pronounced (≈50×) and could be complemented only by expression of a full-length, catalytically active p60. These data suggest that SecA2-dependent secretion has evolved in part to promote the secretion of autolysins with important contributions to *L. monocytogenes* virulence. Thus, SecA2 may to some extent represent a Gram-positive bacterial equivalent to the specialized secretion pathways (e.g., type I, type II, type III, and type IV) that contribute so importantly to host manipulation by Gram-negative bacterial pathogens.

It remains to be seen how SecA2 identifies target proteins and promotes their secretion by *L. monocytogenes* or other bacteria. Previous work with the *Escherichia coli* SecA showed that this essential protein varies in the affinity of its interaction with different synthetic signal peptides (23), and that signal peptide-independent interactions between SecA and preproteins contribute to activation of SecA ATPase activity (24). Thus, in bacteria with two SecAs, differences between the preprotein-binding domains of SecA1 and SecA2 may confer their respective substrate specificities (11). However, the precise basis for such discrimination is not presently clear, as there does not seem to be any conservation in the signal peptides of SecA2-dependent substrate proteins (Table 1). Furthermore, many of the identified proteins lacked signal peptides and have well-known functions in the bacterial cytosol (Table 1 and Fig. 5). By analogy to a model previously proposed for the LytA autolysin of *Streptococcus pneumoniae* (25), we considered the possibility that p60 and NamA autolysins induce altruistic bacterial autolysis that contributes to the SecA2-dependent release of cytosolic (moonlighting) bacterial proteins. However, as noted above, the p60 autolysin was not required for secretion of any heterologous *L. monocytogenes* proteins (data not shown). More recent data have also cast doubt on the role of LytA in enhancing the release of heterologous *Streptococcus pneumoniae* virulence proteins (26). Thus, these autolysins may have other roles in promoting bacterial pathogenesis.

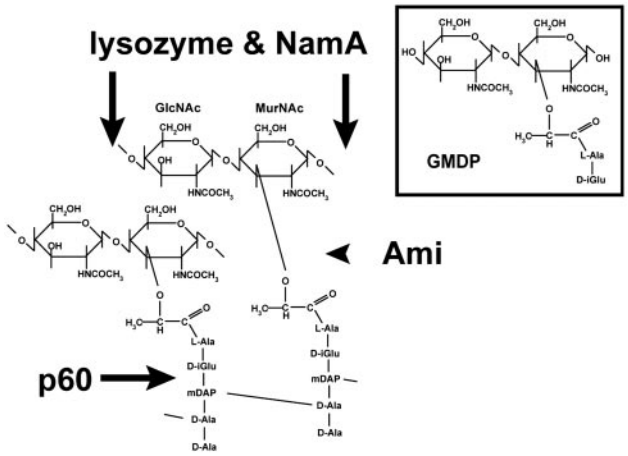

Fig. 4. Structure of *L. monocytogenes* PGN, including predicted cleavage sites for p60, NamA, and other select bacteriolytic enzymes. (*Inset*) The structure of the GMDP glycopeptide predicted to result from cleavage by the combined activities of NamA and p60. GlcNAc, *N*-acetyl-glucosamine; MurNAc, *N*-acetyl muramic acid; iGlu, *iso*-glutamine; mDAP, *meso*-diaminopimelic acid. Note that the GlcNAc-MurNAc bond may also be cleaved by host lysozymes.

It will be important to investigate the regulation of SecA2 and bacterial autolysins under various *in vitro* and *in vivo* conditions. The genes for SecA2 and p60 are linked and divergently transcribed whereas the gene for NamA is found at a separate chromosomal location. We previously reported that ΔSecA2 bacteria form chains in broth culture (11), and recent work by Pilgrim *et al.* showed that mutation of p60 and putative regulatory sequences in the SecA2-p60 intragenic region also cause bacterial chaining (27). However, as shown here, in-frame deletions of NamA or p60 did not cause chaining or other defects *in vitro* or in cultured cells and instead selectively affected virulence in animals. These data suggest that autolysins affect virulence independent of affects on bacterial chaining.

Why then is p60-dependent PGN digestion required for virulence? Based on its similarity to LytF (28), p60 is predicted to digest the peptide bond linking the D-*iso*-glutamine and *meso*-diaminopimelic acid moieties of the peptide side-chain in *L. monocytogenes* PGN. Conversely, NamA shares homology with enzymes that cleave the *N*-acetylmuramide-*N*-acetylglucosamine linkage (Fig. 4). SecA2-dependent secretion would be expected to coordinate PGN digestion by these two autolytic activities, the minimal product of which would be *N*-acetylglucosaminyl-β1-4-*N*-acetylmuramyl-L-alanyl-D-isoglutamine

(GMDP). *In vivo*, host lysozymes and glucosaminidases may also process p60-cleaved PGN to generate GMDP or muramyl-dipeptide (MDP). One might imagine that production and release of these muramyl peptides by *L. monocytogenes* would enhance host resistance to infection because synthetic GMDP and MDP have immunomodulatory activity and use as adjuvants (29). However, there is also accumulating evidence that these muramyl peptides may, under some circumstances, blunt host inflammatory responses. Specifically, GMDP has shown some promise in the treatment of inflammatory immune disorders such as psoriasis and sepsis (30–32). Likewise, GMDP and MDP were recently shown to activate host cell signaling through the Nod2 protein (33, 34), a cytosolic leucine-rich-repeat protein of macrophages. Mutations that disrupt Nod2 activation seem to promote development of Crohn's disease (35, 36) whereas activating mutations induce Blau syndrome (37, 38). Thus, Nod2 activation is clearly important for appropriate regulation of host inflammatory responses, and, in some settings, such activation protects the host from hyperinflammation. Our data suggest that cytosolic *L. monocytogenes* may take advantage of this or other aspects of host pattern recognition by intentionally promoting the release of (G)MDP (Fig. 4). Cleavage of the bond between D-iGlu and mDAP by p60 may also interfere with proinflammatory signals that were recently shown to result from Nod1-dependent detection of PGN fragments containing this dipeptide (39, 40). Future study will be geared toward identifying host responses that are targeted by SecA2-dependent autolytic activity and defining the precise nature of PGN fragments released by these activities. Such study should elucidate more precisely the mechanisms for regulation of host inflammatory responses, as well as mechanisms by which pathogens subvert host pattern recognition to promote their own survival.

We gratefully acknowledge the advice and assistance of A. Falick, D. King, and S. Zhou with mass spectrometry and protein identification. We also thank D. Raulet and M. O'Riordan for helpful comments on the manuscript. This work was supported by U.S. Public Health Service grants to L.L.L. (AI10481) and D.A.P. (AI29619 and AI27655).

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