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Analysis of the Peptidoglycan Hydrolases of *Listeria monocytogenes*: Multiple Enzymes with Multiple Functions

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A b s t r a c t

Listeria monocytogenes is a ubiquitous gram-positive, rod-shaped, widespread in nature, facultative intracellular human and animal pathogen that causes infections collectively termed listeriosis. *L. monocytogenes* EGD encodes a total of 133 surface proteins, the abundance of which, as well as the variety of anchoring systems, probably reflects the ability of this bacterium to survive in diverse environments and to interact with many kinds of eukaryotic cells. The group of surface proteins also includes proteins with murein hydrolase activity-autolysins. To date, five *L. monocytogenes* autolysins have been identified: p60, P45, Ami, MurA and Auto. These enzymes are involved in numerous cellular processes including cell growth, cell wall turnover, peptidoglycan maturation, cell division and separation, formation of flagella, sporulation, chemotaxis and biofilm formation, genetic competence, protein secretion, the lytic action of some antibiotics and pathogenicity. We have recently identified a putative sixth listerial peptidoglycan-degrading enzyme, which has surprisingly been identified as FlaA, a flagellar protein of *L. monocytogenes*.

K e y w o r d s: *Listeria monocytogenes*, virulence, cell wall, peptidoglycan hydrolases

Introduction

Listeria monocytogenes is an important food-borne opportunistic pathogen with high mortality rates, which can range from 20 to 60% in adults, especially in the case of infections of the central nervous system, or from 54 to 90% for neonates (Hof *et al*., 1997). The groups at risk for listeriosis are pregnant women and neonates, the elderly (up to 65 years old) and immunocompromised or debilitated adults with underlying diseases (Vazquez-Boland *et al*., 2001). The bacterium is widespread in nature: waters, soil, rotting parts of plants, animal feces and wastewaters. It has also been isolated from 5% of fecal samples from healthy humans (Farber, 1991) and detected in many food products (Schlech, 2000) and recently has been reported present in various waters, including surface and groundwater in mountainous regions (*e.g*. Gugnani, 1999; Schaffter and Parriaux, 2002).

L. monocytogenes is a model organism in studies on the pathogenesis of intracellular parasites. It is able to penetrate, multiply and propagate in various types of eukaryotic cells and is also able to overcome the three main barriers encountered in the host: the intestinal barrier, the blood-brain barrier and the placenta (Vazquez-Boland *et al*., 2001). Once inside the host, this bacterium has the capacity to invade phagocytic and non-phagocytic cells, to replicate intracellularly, and spread directly from cell to cell, thereby escaping the humoral immune response. Each step of the infection process is dependent upon the production of virulence factors (Portnoy *et al*., 1992): the internalins InlA and InlB for entry, listeriolysin O, two phospholipases C – a phospholipase specific for phosphatidylinositol PlcA and phospholipase C specific for phosphosfatidylcholine PlcB for escape from the primary and secondary vacuoles, protein ActA for intra and intracellular movements and a metaloprotease witch is involved in the processing of PlcB into its mature and active form. The genes coding for 6 of these factors are located in the bacterial chromosome next to each other in a virulence gene cluster locus (pathogenicity island) and are regulated by the transcription activator PrfA (Chakraborty *et al*., 2000; Vazquez-Boland *et al*., 2001). The *inlAB* operon is

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located elsewhere (Lecuit *et al*., 2001). Moreover, such other determinants as protein p60 (Park *et al*., 2000), amidase Ami (Milohanic *et al*., 2001), autolysin Auto (Cabanes *et al*., 2004), catalase, superoxide dismutase, siderophores and protein LmaA are also required for full pathogenic activity of the bacterium (Vazquez--Boland *et al*., 2001).

Bacterial peptidoglycan hydrolases

The cell wall of gram-positive bacteria is host to a wide variety of molecules and serves a multitude of functions, most of which are critical to the viability of the cell. The major structural component of all types of bacterial walls is murein (peptidoglycan), which is composed of glycan chains in which alternating *N*-acetylglucosamine (Glc*N*Ac) and *N*-acetylmuramic acid are β-1,4 bound, cross-linked *via* peptide bridges. Peptide side chains, which are attached to the carboxyl group of muramic acid residues, are primarily composed of five amino acids (L-Ala-D-Glu-X-D-Ala-D-Ala, where X stands either for L-Lys or diaminopimelic acid). The general structure organization of the glycan chains is relatively constant, though certain modifications are known (Navarre and Schneewind, 1999). Other polymers that are associated with murein in

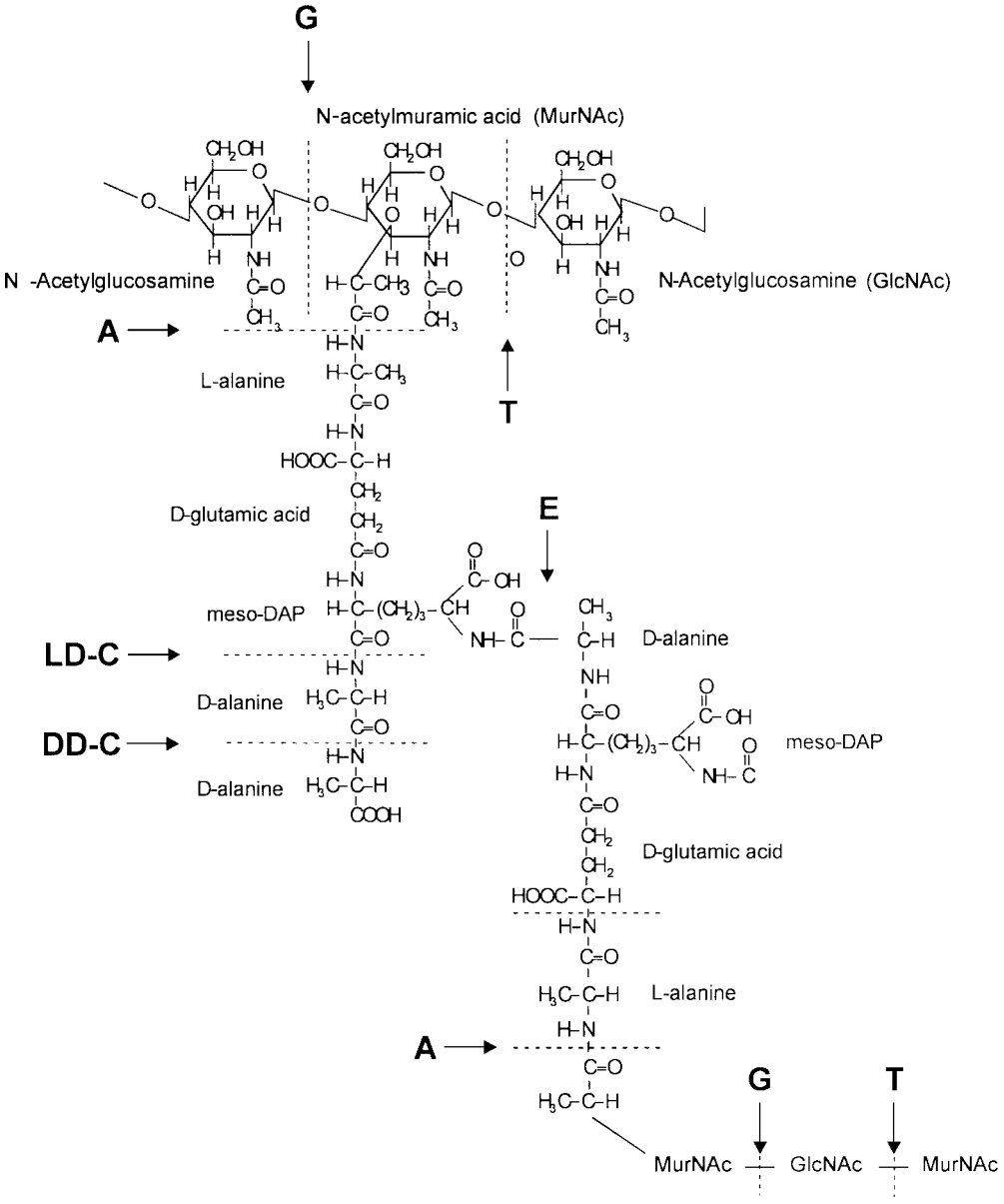

Fig.1. Structure of the disaccharide-pentapeptide monomer of *L. monocytogenes* murein.
An example of each type of bond attacked by glucosaminidase (G), muramidase (T), amidase (A), endopeptidase (E), LD-carboxypeptidase (LD-C) and DD- carboxypeptidase (DD-C).

different bacteria are teichoic acids, lipoteichoic acids, polysaccharides and numerous proteins. There are five major mechanisms for displaying protein at the surface of gram-positive bacteria (Popowska and Markiewicz, 2004a). Each mechanism is characterized by specific structural features that can be identified in the sequence of the proteins and are involved in their specific properties. The major types of surface protein are: LPXTG proteins-covalently linked to the cell wall, non-covalent interactions with the cell wall; choline binding proteins, GW proteins, membrane anchored proteins and the other group lipoproteins.

Different kinds of bounds in murein are cleaved by autolysins that can be classified as *N*-acetylmuramidases, *N*-acetylglucosaminidases, *N*-acetylmuramyl-L-alanine amidases, endopeptidases, and transglycosylases (Höltje, 1995) (Fig. 1.).

Autolysins of *Listeria monocytogenes*

To date, five *L. monocytogenes* autolysins have been identified: p60 (CwhA, Iap), P45, Ami, MurA and Auto (Table I). Analysis of the *L. monocytogenes* genome reveals the presence of eleven proteins with a peptidoglycan hydrolysis domain, thus six (at least) are still unidentified. The extracellular protein p60 (gene *iap*) possesses a murein hydrolase activity required for a late step in cell division (Wuenscher *et al*., 1993; Pilgrim *et al*., 2003). The p60 protein has also been shown to play a role in virulence but is not regulated by PrfA (Kohler *et al*., 1991). Spontaneously occurring *L. monocytogenes* mutants (RIII mutants) with reduced p60 levels show rough colony morphology and form long chains of 10–20 cells separated by double septa; the cells fail to separate after cell division (Kuhn and Goebel, 1989). RIII mutant cells also show reduced ability to invade non-professional phagocytic 3T6 mouse fibroblasts (Bubert *et al*., 1992) and a recent report suggested that p60 is directly involved in binding intestinal Caco-2 cells (Park *et al*., 2000). The expression of p60 is controlled at the post-transcriptional level (Wuenscher *et al*., 1993). It was initially thought that this protein was essential for cell viability because *iap* mutations were always lethal. However, a viable mutant harboring a transposon inserted within *iap* has been isolated (Wiśniewski and Bielecki, 1999; Pilgrim *et al*., 2003), indicating that other proteins may be able to compensate for the loss of p60 activity. P60 is a modular protein containing two LysM domains, a bacterial Src homology 3 (SH3) domain and a carboxy-terminal NLPC/P60 domain. The LysM domain (40 residues long) is involved in degradation of the bacterial cell wall. This domain might have a general murein-binding function. Bacterial SH3 domains (60–70 residues) are homologous to eukaryotic SH3 domains and were first characterized in p60 proteins from different *Listeria* species but functions of this domain is as yet unknown (Ponting *et al*., 1999). The NLPC/p60 domain is responsible for peptidoglycan lytic activity (Cabanes *et al*., 2002). One of the hypotheses assumed the possibility that p60 and MurA induce altruistic autolysis that contributes to the SecA2-dependent release of cytosolic bacterial proteins. However, the p60 autolysin is not required for secretion of any heterologous *L. monocytogenes* proteins. Thus, these autolysins may have other roles in promoting bacterial pathogenesis (Lenz *et al*., 2003). 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* peptidoglycan. Conversely, MurA shares homology with enzymes that cleave the *N*-acetylmuramide-*N*-acetylglucosamine linkage. SecA2-dependent secretion would be expected to coordinate peptidoglycan 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

Tabela I
Peptidoglycan hydrolases of *Listeria monocytogenes*

Proteins	Gen	MW*	Description	Function
p60	*iap*	50.3	peptidoglycan lytic activity	cell separation and virulence
P45	*spl*	42.7	peptidoglycan lytic activity	?
Ami	*ami*	102.3	amidase	role in motility
Auto	*aut*	64.1	amidase	autolysis and virulence
MurA	*murA*	63.6	muramidase	cell separation and autolysis
FlaA	*flaA*	30.4	peptidoglycan lytic activity	structural component of flagella

MW* – calculated molecular mass (in kDa)

may also process p60-cleaved peptidoglycan to generate GMDP or muramyl-dipeptide (MDP). GMDP and MDP activate host cell signaling through the Nod2 protein (Girardin *et al*., 2003; Inohara *et al*., 2003), a cytosolic leucine-rich-repeat protein of macrophages. Cleavage of the bond between D-iGlu and mDAP by p60 may interfere with proinflammatory signals (Girardin *et al*., 2003; Chamaillard *et al*., 2003).

P45 (*spl*), similar to protein p60 (55% similarity and 38% identity) exhibiting peptidoglycan lytic activity (NLPC/p60 domain). This protein was detected in the culture supernatant and at cell surface of *L. monocytogenes* (Schubert *et al*., 2000).

The surface protein Ami (102 kDa) with N-terminal amidase domain and C-terminals eight GW modules that interact with lipoteichoic acids on the bacterial surface (Cabanes *et al*., 2002). The C-terminal amino acid sequence shows homology to the same region of InlB *L. monocytogenes*, while N-terminal domain shows homology to Atl, the major autolysin of *Staphylococcus aureus* (Foster, 1995). Interestingly, the other six of surface protein *L. monocytogenes* containing GW module, like Ami, contain an amidase domain. Ami has a subtle role in motility, observed as a reduction in swarming (McLaughlan and Foster, 1997; McLaughlan and Foster, 1998) and is involved in adhesion to eukaryotic cells (Milohanic *et al*., 2000; 2001). The activity of the pure amidase against *L. monocytogenes* peptidoglycan *in vitro* is only 73% of that against *Bacillus subtilis* vegetative cell walls in spite of the similar primary structure of both substrates. This could reflect the presence of unusual modifications in the peptidoglycan of *L. monocytogenes* (Kamisango *et al*., 1982). However, we have shown that this does not seem to be the case, except for amidation of free diaminopimelic acid residues (Kłoszewska *et al*., in preparation). It is therefore more likely that the activity of the autolytic enzymes of *L. monocytogenes* is very tightly regulated and that their activity is to some extent inhibited even after the death of the cells.

The surface associated autolysin Auto (*aut*) of *L. monocytogenes*, with a predicted molecular mass of 64 kDa, like Ami, containing N-terminal amidase domain and C-terminal cell wall-anchoring domain up of four GW modules was described quite recently (Cabanes *et al*., 2002). The *aut* gene is expressed independently of the virulence gene regulator PrfA and encoding surface protein with an autolytic activity. Although, microscopic analysis of the *aut* mutant did not reveal any defect in cell separation. The *aut* gene is absent from the genome non-pathogenic species *L. innocua*. It is not to be wondered at Auto is required for entry of *L. monocytogenes* into cultured non-phagocytic eukaryotic cells and virulence *in vivo* (Cabanes *et al*., 2004). Reduced virulence of the *aut* mutant after oral and intravenous inoculation suggests that *aut* is critical for several steps of the listeriosis. Cabanes clearly identified Auto as the first *L. monocytogenes* autolysin absent from *L. innocua* directly implicated in virulence process, however, the exact function of Auto remains to be elucidated. The surface location of Auto could lead to the binding to a mammalian receptor directly or through a structure that binds to a eukaryotic receptor like as InlB (Braun *et al*., 2000). Auto as an autolysin could thus appear as a new factor required for a successful infection in controlling the general surface architecture exposed to the host by *L. monocytogenes* and/or the composition of the surface products released by the bacteria, implicating directly autolysins in pathogenicity.

A novel cell wall hydrolase MurA is a 66 kDa cell surface protein which displays two characteristic features: an N-terminal muramidase domain with homology to muramidases from several gram-positive bacterial species and four copies of a cell wall-anchoring LysM repeat motif present within its C-terminal domain (Carroll *et al*., 2003). The deduced molecular mass of MurA is 63,571 Da. The cleavage of a signal peptide would result in mature secreted protein of 57,949 Da. The apparent molecular mass of MurA is larger than would be expected from its primary amino acid sequence, *i.e.*, 66 versus 58 kDa, for the mature protein. Protein database searches showed that the MurA protein of *L. monocytogenes* and *L. innocua* are highly conserved, with 84% identities at the amino acid level. BLAST searches with the amidase_4 motif region of MurA revealed additional ORFs in *L. monocytogenes* EGDe genome (lmo1076 – Auto, lmo1215, lmo1216, lmo2203 and lmo2591) that display homology to this domain. The *murA* gene is preceded by a divergently transcribed putative transcriptional regulator. These cell wall hydrolase is involved in cell separation (mutant *murA*$^{-}$ grew as long chains) and autolysis of *L. monocytogenes* (the deletion mutant is more resistant to both prolonged stationary-phase autolysis and Triton X-100 induced autolysis).

A putative novel murein degrading enzyme from *L. monocytogenes*, which would be the sixth identified murein-degrading activity, was recently discovered by us in the course of a zymographic analysis of surface-associated autolysins of the bacterium released using 4 M LiCl (Popowska and Markiewicz, 2004b). To our surprise the enzyme was identified as the previously described flagellar protein FlaA (Dons *et al.*, 1992). The amino acid sequence of the autolysin showed 100% identity with that of FlaA. Our preliminary observations will be followed up in subsequent studies aimed, amongst others, at identifying the bond in murein cleaved by the enzyme.

Conclusions

Autolysins enzymes, defined as murein hydrolases, are ubiquitous among both gram-positive and gram-negative bacteria. However, the exact roles of these enzymes cell growth and division has not yet been fully elucidated. It is obvious that autolysins, which are involved in various biological functions including cell growth, cell wall turnover, peptidoglycan maturation, cell division and separation, formation of flagella, sporulation, chemotaxis and biofilm formation, genetic competence, protein secretion, the lytic action of some antibiotics and pathogenicity, should be under strict spatial and temporal control. Though it is generally considered that the activity of autolysins is regulated post-translation level, examples are known of regulation at the level of transcription. The gene *bvg* product in *Bordetella pertussis* (Tuomanen *et al*., 1990) inhibits the expression of virulence determinants in this bacterium. Avirulent cells easily undergo autolysis, which points to derepression of hydrolase activity by the product of this gene. In *Staphylococcus aureus* identified of *rta* gene, coding a RAT protein, witch appears to be a negative regulator of autolysin genes including *lytM* and *lytN* (Ingavale *et al*., 2003). Sequence analysis indicated that Rat is homologous to the SarA protein families that modulated virulence determinants (Cheung and Zhang, 2002). Mutation in rat resulted in decreased expression of known autolytic regulators *lytRS*, *lrgAB* and *arlRS*. The RAT binds to the *lytRS* and *arlRS* promoters, thus confirming Rat as a DNA-binding protein to these known repressors of autolytic activity. It is known that SarA regulates *lytRS* and *lrgAB* (Fournier *et al*., 2001) but zymographic analysis of the lysates of *rat* and *sarA* mutants suggest that *rat* and *sarA* may act on different target genes in autolysis (Ingavale *et al*., 2003).

It is also known that on the one hand these enzymes make the growth of the cell wall possible, but on the other can lead to the death of a cell in the process of autolysis. Cell wall hydrolases are thought to be involved in autolysis of the bacterial cell, and this phenomenon is usually observed after inhibition of further synthesis of peptidoglycan either nutritionally or by the addition of an antibiotic or treatment with certain non-specific chemicals (Shockman and Höltje, 1994). For these reasons the enzymes are considered very significant in therapy involving the use of antibiotics.

This direct correlation with pathogenicity further reinforces the importance of understanding bacterial autolysis. Recent findings suggest that autolysis is not just an unfavourable side effect of the enzymes that control bacterial cell wall synthesis but that it provides some advantage to the organism that is necessary for its survival (Carroll *et al*., 2003).

The peptidoglycan hydrolase profile of *L. monocytogenes* is complex and consists of multiple bands-autolysins (McLaughlan and Foster, 1997), including members of the P60 family such as p60 (*iap*), P45 (*spl*) and Lmo0394 (Cabanes *et al*., 2002) and members of the amidase protein family such as Ami or Auto and Lmo1215, Lmo1216, Lmo1521, Lmo2203, Lmo2591 as well muramidase MurA. All these enzymes appear to have alternative functions in the organism. The presence of multiple autolysins complicates the process of determining the roles of each autolysin in the organism.

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