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Murein-Hydrolyzing Activity of Flagellin FlaA of *Listeria monocytogenes*

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A b s t r a c t

In this preliminary report we show that a 29 kDa surface protein of *Listeria monocytogenes* EGD removed from cells with 4 M LiCl has peptidoglycan (murein) hydrolyzing activity, as revealed by zymographic analysis using *Bacillus subtilis* murein and heat-killed *Micrococcus luteus* cells casted in the gel. Following two-dimensional electrophoresis, the protein was electroblotted to PVDF membrane and its identity (FlaA) was revealed by sequencing. Peptidoglycan hydrolysing activity of FlaA purified by FPLC on Mono-S Sepharose against labelled *Escherichia coli* murein was demonstrated.

K e y w o r d s: *Listeria monocytogenes*, flagellin, FlaA, motility, autolysin

Introduction

Listeria monocytogenes is an ubiquitous gram-positive opportunistic pathogen that causes relatively infrequent but often very serious food-borne infections in humans and animals such as meningitis, meningoencephalitis, septicemia, gastroenteritis as well as abortions (Southwick and Purich, 1996; Hof *et al*., 1997). The infections are particularly severe for newborns and immunocompromised individuals. *L. monocytogenes* has received much attention and an impressive amount of data accumulated in recent years has made this bacterium one of the best characterized intracellular pathogens. Moreover, the data connected with *L. monocytogenes* as regards pathogenesis, immunology, cell biology provide an excellent rational for the use of this microorganism as a carrier for foreign antigens. *L. monocytogenes* elicits a strong cellular immune response following infection and therefore has potential use as a vaccine vector. *L. monocytogenes* induces its own uptake into mammalian cells and then moves within the cells and spreads from one cell to another by virtue of actin-based motility (Cosssart and Kocks, 1994; Cossart and Lecuit, 1998). The majority of genes encoding virulence factors required for intracellular infection, such as ActA, which is necessary for actin-based motility and cell-to-cell spread, are coordinately regulated by the transcriptional activator protein PrfA.

In extracellular environments *L. monocytogenes* can move by means of flagella-based motility. The flagellum is composed of a single protein, called FlaA (Dons, 1992). Previous studies have shown that flagellar motility gene expression in *L. monocytogenes* is regulated by temperature. *L. monocytogenes* strains are highly flagellated and motile at 30°C and below, and are typically not motile at temperatures of 37°C or above, though it has been shown that flagellin expression is maintained at 37°C in some laboratory adapted strains and in approximately 20% of clinical isolates (Way, 2004). FlaA has recently been shown to facilitate, together with the *cheY* and *cheA* gene products, initial contact with epithelial cells and contribute to effective invasion (Dons *et al*., 2004). Several global regulatory factors have been implicated in the control of motility gene expression in *L. monocytogenes*, including PrfA. However, these proteins influence motility gene expression indirectly. Recently, Grundling *et al*. (2004) identified a regulator protein that directly binds to the *flaA* promoter region and proved that this protein, designated as motility gene repressor (MogR), functions as a repressor of motility gene expression and is required for full virulence of *L. monocytogenes*.

In this preliminary report we show that purified FlaA protein functions as a murein hydrolase and exhibits murolytic activity against isolated cell walls of *Bacillus subtilis* and heat-killed *Micrococcus luteus* cells.

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Experimental

Materials and Methods

Bacterial strains and growth conditions. *L. monocytogenes* EGD, serovar 1/2a was from Dr S. Foster (GB). Bacteria were grown in tryptone soy broth (TSB, BioMérieux) at 30°C with mild shaking (120 rpm) to mid-exponential phase of growth. The strain that we have been using in our studies for the past several years is motile at both 30°C and 37°C. *Bacillus subtilis* 168 were grown in Luria-Bertani (LB) medium at 37°C. Growth was monitored at 600 nm in a Novaspec II spectrophotometer (Pharmacia). Lyophilized *Micrococcus luteus* ATCC 4698 cells were purchased from Sigma.

Isolation of cell wall. Bacterial cells were prepared from a culture grown to log phase ($A_{600} = 0.6$). Cells were harvested by centrifugation for 15 min at 8 000 × g, suspended in 50 mm Tris/HCl, pH 7.5 and and five one-minute bursts of ultrasound waves were employed in VCX-600 ultrasonicator (Sonics and Materials, USA) at amplitude 20%. The crude cell wall preparations were sedimented by centrifugation in a Beckman centrifuge (30 min, 100 000 × g at 2°C), suspended in 20 ml of a 1% SDS solution and boiled for 30 min. Crude walls were sedimented by centrifugation in a Beckman centrifuge (30 min, 100 000 × g at 2O°C) and washed five times with water. Radiolabeled murein preps were isolated in the same way after growing *E. coli* W7 or *L. monocytogenes* EGD in the presence of [^{3}H]-A_2pm (1 μCi/ml) to A_{578} 0.4 or 0.6, respectively.

Extraction of surface-associated enzymes. Cells of *L. monocytogenes* were grown in TSB at 30ºC, harvested by centrifugation, washed in saline and resuspended in an ice-cold solution containing 4 M LiCl and 0.5 mM phenylmethylsulfonylfluoride (PMSF) in 50 mM Tris-HCl buffer, pH 7. The suspension was stirred for 30 min with the use of a magnetic bar in an ice bath, after which the cells were harvested (20 0000 × g, 10 min at 4°C). The supernatant was transferred to dialysis tubing with cutoff value 3 500 (Spectrum) and dialysed for 12 hours at 4°C against 50 mM Tris-HCl, pH 7 containing 100 mM LiCl, with several changes of the buffer. Protein concentrations were determined with protein assay kit (Bio-Rad).

Hydrolysis of labeled *E. coli* cell wall. The reaction mixture contained 10 μl of [^{3}H]-A_2pm-labeled *Escherichia coli* murein, (2 mg per ml) 50 μl sample, 50 μl buffer (10 mM Tris/HCl, pH 7.5) and was incubated for 60 min at 37°C, after which it was placed in ice bath. An equal volume of 1% CTAB solution in water was added. After 20 min. incubation in ice the samples were centrifuged for 15 min at 16 000 × g. 100 μl of the supernatant was taken for counting in liquid scintillation (Beckman) against a control with water instead of studied sample.

Zymographic analysis. Samples containing peptidoglycan hydrolases were analysed by renaturing gel electrophoresis as previously described (Foster, 1992). 12% (w/v) acrylamide gels containing 0.1% (w/v) purified bacterial walls (*Bacillus subtilis* or autoclaved, lyophilized *Micrococcus luteus* ATCC 4698 cells – Sigma). After electrophoresis, the gel was soaked for 48 h with gentle shaking in renaturation buffer (25 mM Tris-HCl, pH 7.5, 1% [vol/vol] Triton X-100) at 37°C with three to five changes of the buffer to remove SDS and allow the protein to be renatured. To obtain better contrast, the gel was rinsed in distilled water and stained with 1% (wt/vol) methylene blue in 0.01% (wt/vol) KOH. After destaining in distilled water, the gel was photographed. If a protein had peptidoglycan-hydrolyzing activity, an unstained clear band appeared in the blue background at the position corresponding to the protein band. The results shown are representative of at least two independent experiments.

Two-dimensional electrophoresis. Samples containing peptidoglycan hydrolases were analysed by two-dimensional electrophoresis as previously described (Copeland, 1982). First dimension electrophoresis was performed in 12% resolving gels and 4.5% stacking gels. Samples were diluted in an equal volume of sample buffer (2%, w/v, SDS, 10%, v/v, glycerol. 5%, v/v, β-mercaptoethanol, 0.002% bromophenol blue, 0.02 M Tris/HCl) and boiled for 3 min at 100°C. After electrophoresis (3 hours, 30 mA), the gels were stained with silver nitrate (Heukeshoven and Dernik, 1988) or with Coomassie blue. For gels stained with silver nitrate 3–10 μg of protein was applied to the wells, compared to 100–200 μg for gels stained with coomassie blue or transferred to nitrocellulose. A molecular mass standard mixture (Amersham Pharmacia Biotech), including α-lactalbumin (14.4 kDa), trypsin inhibitor (20.1 kDa), carbonic anhydrase (30 kDa), ovalbumin (43 kDa), albumin (67 kDa), phosphorylase b (94 kDa), was used in each run. To the stacking gel (4.5%) 0.5 μg ml^{-1} protease SV8 from *S. aureus* was added. The action of protease SV8 was studied to confirm the protein nature of the revealed bands. When the applied sample approached the end of the stacking gel (12.5 mA), electrophoresis was stopped for 30 minut to ensure hydrolysis of all proteins. Subsequently, electrophoresis was continued in 15% resolving gel (25 mA). The gel was stained with Coomassie brilliant blue.

Electroblotting. Following electrophoresis in the first dimension, a slice of the gel containing a putative single protein was purified by electrophoresis in the second dimension. The protein in the gel was transferred to PVDF-type membrane (ProBlott, Perkin-Elmer P/N 4000994), then washed free of salts, buffers, and other interfering substances. The membrane was then directly loaded into automated protein sequencer.

Purification of protein FlaA by FPLC. The 4M LiCl extract (20 ml – 1.7 mg) was subjected to affinity chromatography by loading on Mono-S sepharose column (as specified by the manufacturer). Before loading, the column was washed with wash buffer A (40 mM phosphate buffer, pH 6.6). The proteins were eluted with elution buffer B (40 mM phosphate buffer, 1 M NaCl, pH 6.6), flow rate 1 ml/min, 30 min step gradient every 20% NaCl content from 0% to 100%. Each fraction was monitored by SDS-PAGE electrophoresis as previously described and was also assayed for murein-hydrolyzing activity using [^{3}H]-A_2pm-labeled murein from *E. coli.*

Results

Identification of autolytic enzymes associated with the cell surface of *Listeria monocytogenes*. The protein fraction released from the surface of *L. monocytogenes* cells with 4 M LiCl as described under Methods was analysed by electrophoresis on 12% polyacrylamide gel, which was then stained with Coomassie brilliant blue. SDS-PAGE of the 4 M LiCl extract showed more then 20 bands with molecular

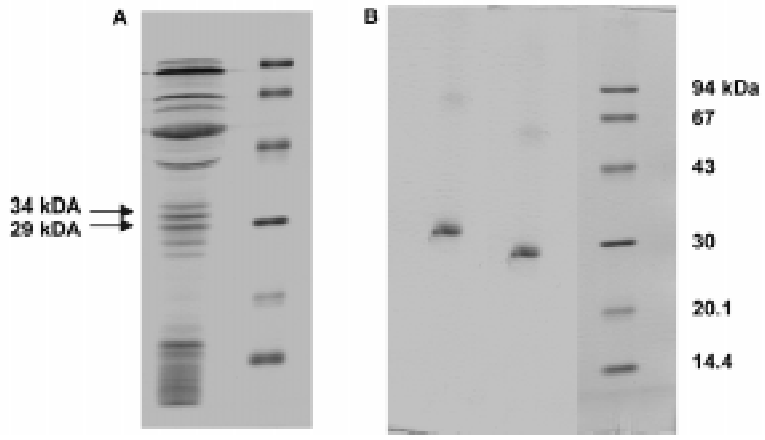

Fig. 1. SDS PAGE analysis of 4M LiCl extract

The protein extracts were analysed on polyacrylamide gel which was then stained with Coomassie brilliant blue. A: First dimension electrophoresis was performed in 12% resolving gels. Lanes: 1, LiCl cell extract; 2, molecular mass standard. The arrows in lanes 1A indicate bands, which correspond to FlaA (~29 kDa), and a ~34 kDa protein which shows homology to phospholipase C. B: Two-dimensional electrophoresis was performed in 15% resolving gels (for details see text).

masses in the 98–12 kDa range (Fig. 1A). An aliquot of the same 4 M LiCl surface extract was subjected to electrophoresis under renaturing conditions on a gel containing either autoclaved *Micrococcus luteus* cells or isolated cell walls of *Bacillus subtilis*. In either case murein hydrolysing activity was observed as zones of clearing against an otherwise opaque background. Highest autolytic activity was observed in the 40 to 104 kDa range and in the 29–34 kDa range, which is in accordance with the results presented by McLaughlan and Foster (1997) for autolytic enzymes released to the growth medium of *L. monocytogenes*. In our study we decided to focus on the presumable low molecular weight molecular weight surface-associated autolytic activities of *L. monocytogenes*. Two-dimensional electrophoresis of the protein bands resolved in the first dimension revealed that seemingly single bands can, in fact, be composed of several proteins with similar molecular mass. However, the bands in the 18 to 36 kDa range corresponded to single proteins. To confirm this, two bands with murein hydrolysing activity, corresponding to proteins of about 29 and 34 kDa, respectively, were cut out of the gel run in the first dimension and subject to electrophoresis in the second dimension. The obtained results proved the presence of single polypeptides, indicating the presence of electrophoretically pure protein fractions (Fig. 1B).

Identification of protein FlaA as an autolysin. Following two-dimensional electrophoresis as described above, the two low molecular weight proteins with murein hydrolysing activity were separately transferred to PVDF membrane for determination of amino acid sequence. The obtained sequences were compared with sequences deposited in GeneBank (NC 003210). The smaller of these proteins was identified as *L. monocytogenes* flagellin FlaA with a calculated molecular mass of 30.4 kDa (Acc. No NP464217, 100% sequence identity) whereas the larger one (~34 kDa) shows strong similarity to phospholipase C PlcA with a calculated molecular mass of 36 kDa (Acc. No NP463732, around 30% primary sequence identity).

Purification of FlaA by FPLC. The 4M LiCl *L. monocytogenes* cell surface-associated protein

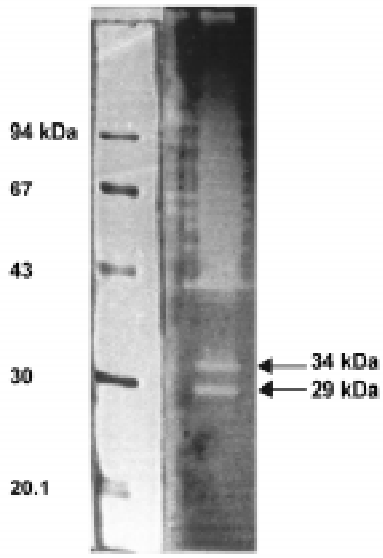

Fig. 2. Zymographic analysis of proteins extracted with 4 M LiCl from *L. monocytogenes* EGD. The rematuring SDS-PAGE was performed as described in Materials and Methods.

The gel contained 0.1% (w/v) purified bacterial walls of *Bacillus subtilis* as the substrate. The molecular masses (in kilodaltons) of standards separated on the same gel are indicated on the right. Autolytic bands are identified on the left.

extract was separated using a Mono-S sepharose column in a FPLC system. Electrophoretic and zymographic analysis revealed murein hydrolysing actitivity in several of the collected fractions (data not presented). This activity was also confirmed in reactions with radiolabeled murein from *E. coli* as a substrate. Protein Fla present in one of the fractions showed 23% of murein-degrading activity compared to calculated total activity loaded onto the column. However, this value most probably does not reflect the actual activity of the protein relative to other autolysins in the extract since the individual enzymes may differ with regard to *E. coli* murein as a substrate (this laboratory, unpublished observations).

Discussion

A cursory analysis of the *L. monocytogenes* genome reveals at least eleven genes thay may code for enzymes with murein-hydrolysing activity, *i.e.* proteins with an N-terminal signal peptide and an enzymatic domain flanked by repeat structures at their N- or C-termini (Joris *et al*., 1992; Cabanes *et al*., 2004). The first of such proteins to be characterized was an autolytic amidase (98 kDa) (McLaughlan and Foster, 1998) which has been shown to function as an adhesin (Milohanic *et al*., 2004) and in the following years several other autolysins have been characterized. The current list, besides the above-mentioned amidase, includes the well described protein p60 (syn. Iap, CwhA) (Hof, 1984), which is implicated in cell division and actin-based motility; the extracellular protein p45 (Schubert *et al*., 2000) with peptidoglycan lytic activity; MurA – a 63.6 kDa *N*-acetylmuramidase which is important for cell separation and generalized autolysis of *L. monocytogenes* (Carroll *et al*., 2003; Lenz *et al*., 2003) and Auto – a 62 kDa protein that has been identified and shown to be necessary for the entry of *L. monocytogenes* into eukaryotic cells (Cabanes *et al*., 2004).

As results from the above, the known *L. monocytogenes* peptidoglycan hydrolysing enzymes are implicated in cell division, autolysis and virulence. In this preliminary report we show that listerial flagellum protein FlaA has peptidoglycan degrading activity and could therefore play an important role in motility of the bacterium. An analysis of the amino acid sequence of flagellin FlaA using the programs BLAST and FASTA did not reveal any significant sequence similarity to other known autolysins. This is not particularly surprising in view of the cellular location and funtions of FlaA. Tandem repeats which are usually found at either the C- or N-termini of peptidoglycan hydrolases serve to bind these molecules to the cell wall (Ghuysen *et al*., 1994). In the case of bacterial flagellin such motifs would apparently be redundant. Similarly, sequences of the catalytic centers strongly differ between autolytic enzymes, depending on the substrate recognized and the bond hydrolyzed.

FlaA has recently been shown to be involved in the initial contact with epithelial cells, thus contributing to effective invasion (Eriksson *et al*., 2004) but no enzymatic activity of the protein has been described. Several bacterial autolysins have been reported to be involved in flagellum-driven motility. Based on the analysis of hypermotile *Bacillus subtilis* strains with increased autolysin level or the impaired motility of amidase-or glucosaminidase-impaired mutants of this bacterium, a strong case for the direct involvement of autolytic murein hydrolases in flagellar assembly has been made (Rashid *et al*., 1993; Lazarevic *et al*., 1994). Direct evidence for the involvement of a lytic transglycosylase in the assembly of the hook complex of the polar flagellum of *Caulobacter crescentus* has recently been presented (Viollier and Shapiro, 2003). A very interesting observation was made for the flagellar protein FlgJ of *Salmonella*, which has been shown to have peptidoglycan hydrolyzing activity and locally digests the murein sacculus to permit assembly of the rod structure pf the flagellum (Nambu *et al*., 1999; Hirano *et al*., 2001). FlaA of *L. monocytogenes* would thus be only the second flagellar protein with peptidoglycan hydrolyzing activity described thus far and the first such case among gram-positive bacteria.

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