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Analysis of the Murein of a *Listeria monocytogenes* EGD Mutant Lacking Functional Penicillin Binding Protein 5 (PBP5)

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Abstract

Cells of a mutant of *Listeria monocytogenes* lacking functional PBP5, an enzyme with DD-carboxypeptidase activity, make thicker cells walls. In this study we show that the muropeptide profile of the mutant, obtained after HPLC analysis of a muramidase digest of cell wall murein, differs from that for the wild type strain. The main differences embrace strongly reduced disaccharide-tripeptide content, strongly increased amounts of pentapeptide-containing muropeptides and a shift in profile from less cross-linked muropeptides (monomers, dimers) towards more highly cross-linked ones.

K e y w o r d s: *Listeria monocytogenes*, penicillin binding protein (PBP), murein, HPLC

The murein of *Listeria monocytogenes* is of the A1g type, with direct cross-links between D-alanine and meso-diaminopimelic acid (Schleifer and Kandler, 1972), though cross-links between two meso-diaminopimelic acid residues in neighbouring peptide side chains, resembling the situation in *Escherichia coli*, have also been observed (Glauner, 1988; Kłoszewska *et al*., this laboratory, unpublished).

Like in other bacteria, the final stages of murein biosynthesis in *L. monoc*ytogenes are catalyzed by the so-called penicillin-binding proteins (PBPs), which are involved in transpeptidation and transglycosylation reactions (Ghuysen, 1991; van Heijenoort, 2001). During transpeptidation the terminal D-alanine is removed from the pentapeptide of the precursor (donor) with subsequent formation of a bond between D-alanine in position 4 of the precursor (or *meso*-diaminopimelic acid in position 3) and *meso*-diaminopimelic acid in position 3 of a second peptide chain (acceptor) (Höltje, 1996). The enzymes catalyzing murein polymerization reactions belong to the class A or B group of high molecular weight (hmw) PBPs. The low molecular weight (lmw) PBPs, on the other hand, as a rule are not directly involved in the biosynthesis of murein, but affect the final structure of the macromolecule, since they determine the length of the peptide side chains. Their activity thus decides how many peptides will serve as acceptors in transpeptidation reactions and consequently, the extent of murein cross-linking. Most of low molecular weight PBPs are DD-carboxypeptidases (Jamin *et al*., 1995; Ghuysen, 1997), though some of these proteins may have DD-endopeptidase activity, hydrolyzing peptide bonds in peptide bridges between adjacent sugar chains, formed as a result of earlier transpeptidation events (Goffin and Ghuysen, 1998).

In *L. monocytogenes* 5 penicillin binding proteins have been identified, though analysis of the genome sequence of this bacterium shows that other putative PBPs may be synthesized. Four of these proteins, PBP1, 2, 3 and 4, belong to the hmw class of PBPs, whereas PBP5 is a lmw penicillin binding protein (Gutkind *et al*., 1989; Vicente *et al*., 1990; 1990a; Korsak *et al.*, 2002). So far, the enzymatic properties of only hmw PBP4, which has transglycosylase and transpeptidase activities (Zawadzka-Skomiał *et al.*, submitted) and the lmw PBP5 (Korsak *et al*., 2005) have been determined. In the latter case, PBP5 has been found to be a DD-carboxypeptidase, and the preferred substrates for the enzyme *in vivo* are low molecular fragments of murein, that is monomeric muropeptides that are formed in the course of the metabolic turnover of the murein sacculus (that is in parallel processes of the degradation and synthesis *de novo* of the macromolecule) (Korsak *et al.*, 2005). A mutant of *L. monocytogenes* lacking PBP5 was found to produce

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a wall with greater thickness than the wild-type strain (Korsak *et al.*, 2005). In view of this finding, the objective of the current study was to determine whether the absence of the DD-carboxypeptidase activity of PBP5 results in changes in the maturation of the cell wall of *L. monocytogenes.*

L. monocytogenes EGD and the mutant strain DA01 lacking functional PBP5 were grown at 37°C in Tryptic Soy Broth (TSB) medium with shaking at 200 rpm. Cell cultures were cooled down and harvested by centrifugation (7 000 × g, 10 min, 4°C). The pellet was resuspended in 50 mM Tris/HCl buffer, pH 7.5. After adding 10 µg DNAse ml^{-1} and phenylmethanelsulfonyl fluoride (1 mM), the cells were broken in the ultrasonicator (Sonics and Materials, type VCX 600). Unbroken cells were removed by centrifugation (7 000 × g, 10 min, 4°C). Hot 8% (v/v 1 : 1) sodium dodecyl sulfate (SDS) was added to broken material, which was then boiled for 30 min. The resulting insoluble wall preparation was then washed with warm distilled water (60°C) at least five times until free of SDS. Covalently attached protein was removed by treatment with 200 µg pronase ml^{-1} for 1.5 h at 60°C. The walls were then recovered by centrifugation (35 000 × g, 30 min, 4°C), washed once in distilled water, and suspended in trichloroacetic acid (final concentration 5%). The mixture was incubated at 4°C for 24 h. The insoluble material was collected by centrifugation (35 000 × g, 30 min, 4°C), and washed five times with cold distilled water. After final centrifugation the pellet was resuspended in 5 ml distilled water and kept frozen at –20°C.

Samples containing muropeptides were digested with Cellosyl (Hoechst AG) as previously reported (Glauner, 1988). Soluble muropeptides were reduced by using sodium borohydride (Glauner *et al*., 1988). The reaction was stopped after 30 min by lowering the pH to 3.5 with phosphoric acid. The samples were analysed by HPLC according to the method of Glauner (1988) on a Hypersil ODS column (250 mm × 4 mm, particle size 3 mm diameter; Teknochroma). Elution buffers were 50 mM sodium phosphate containing 0.8 g sodium azide in 1 liter, pH 4.35 (A) and 15% methanol in 75 mM sodium phosphate, pH 4.95 (B). Elution conditions were 7 min isocratic elution in buffer A, 115 of linear gradient to 100% buffer B and 28 min of isocratic elution in buffer B. The flow rate was 0.5 ml/min and the column temperature was 55°C. Eluted compounds were detected by monitoring A_{205}.

The high performance liquid chromatography (HPLC) technique allows detailed analysis of the so-called muropeptide composition of murein, resulting in a specific pattern, depending on differences in number of sugar residues and number and type of amino acids In the individual muropeptides obtained after digestion of murein with a murolytic enzyme, such as the Cellosyl employed by us. This method allows the demonstrating of changes in the structure of murein, which frequently reflect altered biosynthetic processes, caused by the absence or overproduction of enzymes involved in these pathways, or by environmental factors, *e.g.* presence of certain amino acids or antibiotics in the growth medium (Glauner *et al*., 1988). We applied this technique for the analysis of the murein of the *L. monocytogenes* mutant lacking PBP5, compared to that of the wild type strain.

In an earlier study of *L. monocytogenes* murein five major muropeptides, accounting for approximately 63% of the analyzed murein sample were identified (Kłoszewska *et al*., this laboratory, unpublished). Muropeptides 1 and 2 are monomers – disaccharide-tripeptides, differing in the presence of an acetyl group on the glucosamine moiety of muropeptide 1 (Fig. 1A, peaks 1 and 2). Muropepties 3, 4 and 5 are dimers. Muropeptide 3 is a tri-tetrapeptide, in which the crosslink is between D-alanine and *meso*-diaminopimelic, with both glucosamine residues of the muropeptide being N-acetylated. The structure of muropeptide 4 is similar to that of muropeptide 3, the only difference being that one glucosamine residue is substituted with an acetyl group. In turn, muropeptide 5 is similar to muropeptides 3 and 4, but no glucosamine is acetylated (Fig. 1A, peaks 3, 4 and 5). No tetra monomers or tetra-tetra dimers, at least in significant amounts, were detected (Kłoszewska, 2003).

The muropeptide profile of mutant DA01 is strikingly different from that obtained in the case of the wild type *L. monocytogenes* strain (Fig. 1B). All the muropeptides identified in a murein digest of the wild type murein are absent but at a retention of approx. 25 min two new peaks appear that when compared with the characteristic and well identified muropeptides of a *dacA* mutant of *Bacillus subtilis* (lacking the gene coding for the major DD-carboxypeptidase synthesized by vegetative cells, which have a similar murein structure to that of *L. monocytogenes*); (Atrih *et al*., 1999) may correspond to monomers with disaccharide-pentapeptide structure. The muropeptides that appear in the mutant profile with retention time between 55 and 70 minutes appear to be different forms of a dimer – a bis-disaccharide penta-tetra peptide, even though this has yet to be conclusively demonstrated. On the other hand, the muropeptides with retention time over 75 minutes correspond to trimers and higher oligomers.

Even though the molecular identification of the muropeptides obtained on muramidase digestion of the murein of the mutant lacking functional PBP5 activity is lacking, certain conclusions can be drawn with

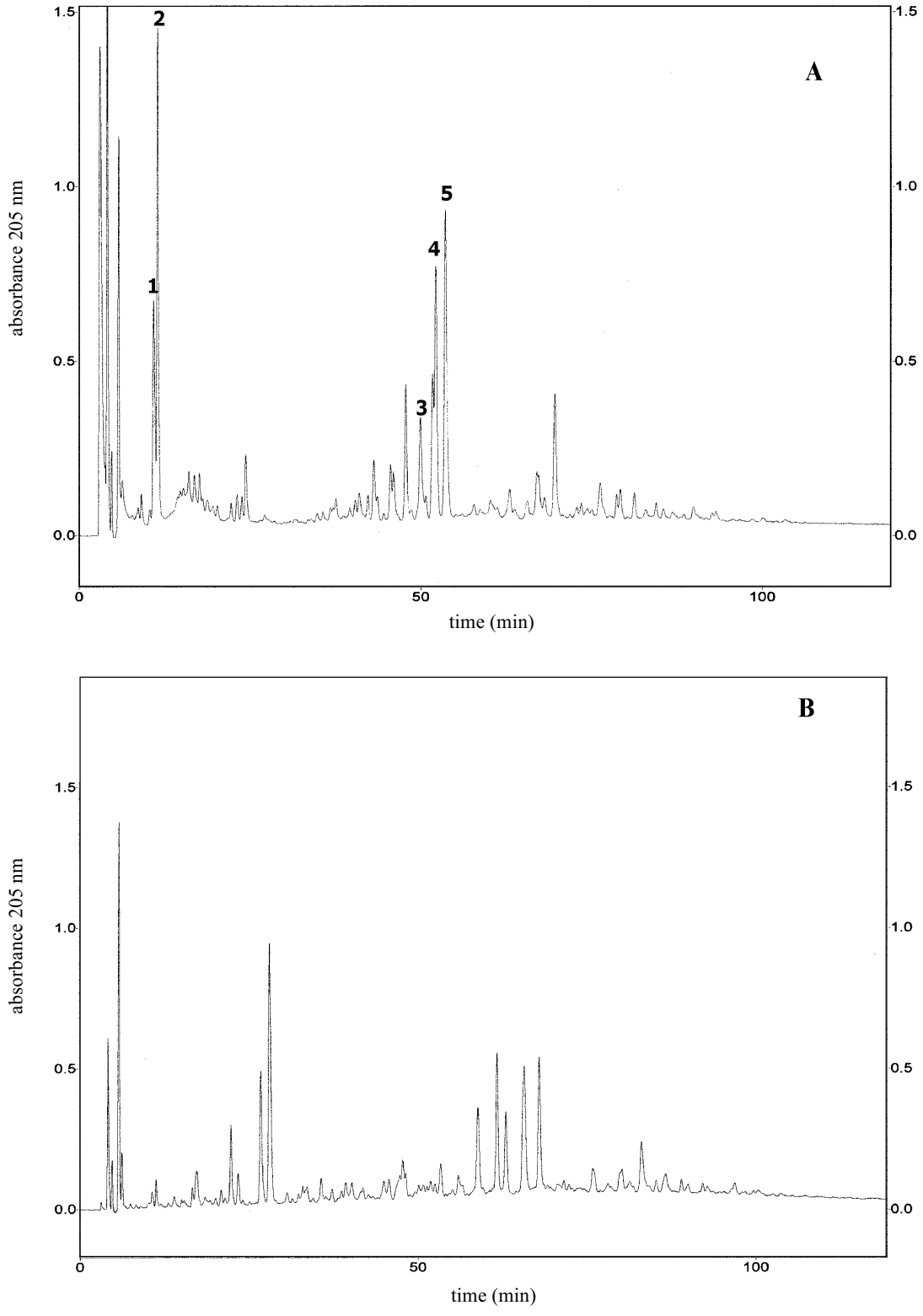

Fig. 1. HPLC muropeptide elution patterns of murein from wild type *Listeria monocytogenes* EGD (A) and *L. monocytogenes* mutant DA01 lacking functional PBP5 (B)

Purified Cellosyl-digested peptidoglycan samples were separated on a Hypersil octadecylsilane column, and the A_{205} of the eluate was monitored: 1, 2 disaccharide-tripeptide monomers; 3, 4, 5 bis-disaccharide tri-tetra peptide dimers.

absolute certainty. Analysis of the muropeptide profile of the mutant murein demonstrates the lack of muropeptides with peptide side chains composed of three amino acids. On the other hand, the appearance of muropeptides, both monomeric and dimeric, carrying pentapeptide side chains is observed. The muropeptide profile shows a dramatic shift towards higher muropeptides (dimers, trimers) which has to translate

to higher overall extent of crosslinking in the murein of the murant. Similar changes have been observed in the murein of a mutant of *B. subtilis* lacking DD-carboxypeptidase activity (Atrih *et al*., 1999). The observed muropeptide profile of mutant DA01 can indicate that PBP5 of *L. monocytogenes*, similar to PBP5 of *B. subtilis*, is a DD-carboxypeptidase that plays a fundamental role in the maturation of the murein of the listerial cell wall. The course of murein turnover in mutant DA01 does not differ from that in the wild type strain, which indicates that PBP5 does not play a role in this process (data not presented).

Of interest is the presence in the murein of wild type *L. monocytogenes* of monomers and dimers carrying tripeptides, which most probably reflects the presence of a protein with LD-carboxypeptidase activity. It thus seems highly probable that besides PBP5, which cleaves off the last D-alanine of the pentapeptide side chain, a penicillin-insensitive protein with LD-carboxypeptidase activity, also participates in determining the length (and role) of the peptide side chains in listerial murein. A protein with such enzymatic activity has been identified in *Escherichia coli*, also a bacterium with 1g type of murein (Ursinus *et al*., 1992).

Further investigations on both the LD-carboxypeptidase of *L. monocytogenes* and the putative enzyme with LD-carboxypeptidase activity, as well as on the putative PBPs of the bacterium, not identified by treating the proteins of the cytoplasmic membrane with radiolabelled b-lactam antibiotic, are required.

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