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Maturation and processing of SpaI, the lipoprotein involved in subtilin immunity in *Bacillus subtilis* ATCC 6633

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Summary

SpaI is a small lipoprotein that provides *Bacillus subtilis* with autoimmunity against the lantibiotic subtilin. We have investigated the maturation of SpaI through the lipoprotein biosynthesis pathway, and analyzed the consequences of maturations in the acylation of the target lipobox in subtilin immunity. Further specificity of lipid acylation of the cysteine within the conserved sequence of the candidate lipobox (LSAC) was studied by site-directed mutagenesis. The mutants LSAA and LSCA blocked lipid attachment to the SpaI protein. Cell-wall stress-sensing *B. subtilis* BSF 2470 was exploited to study the function of each mutant upon heterologous expression. This system allowed the monitoring of β-galactosidase activity to the added subtilin at a sublethal dose. Mutants exhibited 2-fold reduction in β-Gal activity, suggesting their contribution in subtilin autoimmunity.

Introduction

Bacillus subtilis ATCC 6633 produces pentacyclic lantibiotic-subtilin, and protects the lethal action of its own antibiotic through a well-developed immunity system comprising two-component modules, namely the ABC transporter (e.g. LanFEG) and a putative membrane anchored protein (LanI), whose functions have been ascertained by gene disruption and heterologous expression (Klein and Entian 1994; Stein et al. 2005). NisI, a putative homologue of SpaI involved in immunity to nisin, is presumed to bind nisin, thereby preventing its

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penetration and pore-forming activity in the host cell (Stein et al. 2003, 2005), whereas the C-terminal part of NisI is suggested to provide specificity to nisin (Takala and Saris 2006). It has been speculated that the SpaI might exhibit a ''chaperone-like function'' binding to the lantibiotic, preventing its insertion into the cell membrane and/or competing with speculative subtilin-lipid II pore formation (Stein et al. 2005).

Lipid-acylated proteins (lipoproteins) represent about 1–3.5% of the total cellular protein of eubacteria and are involved in several metabolic processes (Tjalsma et al. 1999). Lipoprotein biosynthesis involves diacylglycerylation of specific cysteine residues within the prolipoprotein sequence catalyzed by the enzyme prolipoprotein diacylglyceryl transferase encoded by the *lgt* gene. Subsequently, maturation of the lipidated prelipoproteins by removal of their signal sequences is catalyzed by the signal peptidase type II (Spase II), encoded by the *lsp* gene (Sutcliffe and Russell 1995). Maturation of a target lipoprotein can be easily identified in Lgt- and Lsp-depleted cultures since mutations of these single-copy genes affect global lipoprotein biosynthesis (Antelmann et al. 2001; Babu et al. 2006).

In several lipoprotein biosynthesis studies, it has been observed that non-specific cleavage of the signal peptide is a common phenomenon. Furthermore, secretion of non-lipidated lipoproteins (e.g. NisI) into the medium is reported (Koponen et al. 2004). In a few cases, lipobox-mutagenized lipoproteins were found to be expressed at very low levels due to protein instability, and the function of the mutants could not be analyzed (Kempf et al. 1997). To decipher the contribution of SpaI in subtilin immunity, several studies were undertaken and the response of the target culture was tested by agar well diffusion assay (Klein and Entian 1994; Stein et al. 2005; Heinzmann et al., 2006). Here, we present genetic and molecular evidences for the maturation of SpaI through the lipoprotein-processing pathway, as well as functional analyses of SpaI and its lipobox-mutagenized species using β-galactosidase assay-based cell envelope stress-sensing two-component LiaRS system (Mascher et al. 2004) for studying the immunity function.

Materials and methods

Bacterial strains, plasmids, media and growth conditions

The bacterial strains used in this study were *Escherichia coli* strains DH5α (Stratagene) and BL21 (DE3) (Invitrogen). The lipoprotein biosynthesis defective mutants (MΔL) of *B. subtilis* 168 viz Δ*lgt* and Δ*lsp* (Tjalsma et al. 1999) and *B. subtilis* ATCC 6633, *B. subtilis* BSF2470 (generous gift from Z. Pragai) and its recombinants transformed with P_{xyl}*spa*I, P_{xyl}*spa*I-AA and P_{xyl}*spa*I-CA were used in this study. *E. coli* plasmids pUC19 (New England, Biolab) and pX (Kim et al. 1996) were used for the construction of recombinant plasmids. *B. subtilis* was grown in TY broth (0.8% tryptone, 0.5% yeast extract and 0.5% sodium chloride), Luria-Bertani (LB) medium, DSM, M9, Landy or Spizizen minimal medium supplemented with required mineral salts (Anagnostopoulos and Spizizen 1961) and *E. coli* was grown in LB agar and broth at 37 °C. Chloramphenicol (5 μg ml^{-1}) or erythromycin (1 μg ml^{-1}) or ampicillin (50 μg ml^{-1}) or globomycin (80 μg ml^{-1}, Sankyo Co., Japan) was added to the medium when required.

DNA manipulation and genetic techniques

Chromosomal DNA of *B. subtilis* was isolated by the method of Bernhardt et al. (1978) and transformation of *Bacillus* was performed by the competence transformation method (Anagnostopoulos and Spizizen 1961). Protocols described by Sambrook and Russell (2001) were followed for isolation of plasmid DNA and transformation of *E. coli*. Restriction digestion, ligation and dephosphorylation enzymes were purchased from Promega (Madison, USA) and used according to the manufacturer's instructions.

Oligonucleotide primers and PCR conditions

Disruption of *lsp* gene was verified using primers lsp.F (5′TTAGAACATGGAACTTG GCC3′), Mut.R (5′ATGAAACGCCGAGTTAACGC3′), Mut.F (5′CCACAACTTCCTG TCTGACAGCCCG3′) and lsp. R(5′CTCCTGAGTAGGACAAATCCGCCGC3′). Disruption of *lgt* gene was confirmed by using primers lgt.R (5′GACGTCTACTCCGCG TAC CGCTCC 3′) and Mut.F. A copy of *spa*I gene was amplified by PCR from *B. subtilis* 6633 DNA using primers P.F: (5′GATTGAGG-*ACTAGT*ATGTTGTTTTTG3′) and P.R: (5′ACA*GGATCC*-CTTATTCCTTTTCATTC3′) with *Spe*I and *Bam*HI sites (indicated in italics in forward and reverse primers). PCR was carried out in an Eppendorf Microcycler E apparatus following standard procedure (Sambrook and Russell 2001).

The *spa*I gene was sub-cloned into pUC19 vector and the protocol described in the Quick Change™ site-directed mutagenesis (SDM) kit of Stratagene (USA) was followed for the modification of lipobox.