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ISID Grant Report

Revealing novels aspects of cell wall synthesis and β-lactam sensitivity in Salmonella enterica *serovar Typhimurium: studies on the interactome of the periplasmic protease Tsp and the outer membrane lipoprotein NlpI*

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Introduction

A rigid shell composed of peptidoglycan (PG) determines shape and contributes to baric and osmotic stress adaptation in many bacteria. β-lactams and other clinically relevant antibiotics target this structure; however, the killing mechanisms of most of these drugs have not been fully elucidated. Recent studies indicate that PG synthesis and degradation are regulated from inside the cell by the divisome and cytoskeletal elements and from outside the cell by proteins in the outer membrane (OM), possibly in response to OM injuries. NlpI is an OM lipoprotein of unknown function in *Salmonella enterica* and other enterics that seems to be activated by a periplasmic protease called Tsp/Prc. This study aimed to identify the interactome of these proteins. Moreover, the role of NlpI in peptidoglycan remodeling was studied under different conditions of peptidoglycan and OM stress.

Materials and Methods

MAll experiments were done with the wild-type (wt) ST strain SR11 and an isogenic *nlpI* mutant obtained by lambda Red-swapping. Intracellular protein crosslinks were obtained at 6 h and 18 h of growth with the membrane permeable, homobifunctional, cleavable reducible crosslinking reagent DSP. Tafter, protein complexes containing NlpI or Tsp were pulled down using protein A resin and antibodies generated from synthetic peptides. Protein complexes were separated through SDS-PAGE after treatment with DTT and the resulting bands were identified by MALDI-TOF MS. This experiment was repeated using a his-tagged NlpI as bait. Growth curves in trypticase soy broth (TSB) or TSB supplemented with bile (3-9%), ethanol (0.5-2.5%), H2O2 (0.75-1.5 μM), sodium deoxycholate (1-5%), polymyxin B (0.1-0.5%), bacitracin (512-2048 μg/ml), lysozyme (750-2500 μg/ml), cephalexin (0.5-2 μg/ml), and cefsulodin (1-5 μg/ml) were recorded to select conditions maximizing differences in the growth kinetics of the wt and the *nlpI* mutant. Cell wall integrity in both strains was tested though comparison of lysis curves in distilled water and of susceptibility patterns to crystal violet and vancomycin of cells stressed for 3, 6 or 24 h to bacitracin, cefsulodin, polymyxin B and lysozyme. The relative level of expression of *nlpI* under these stressing conditions was investigated in the wt through qPCR with the delta-delta Ct method using *rpoB* as endogenous control. PG composition was investigated by means of enzymatic hydrolysis of purified sacculi and HPLC separation of the resulting muropeptides. The subcellular localization of NlpI was investigated by epifluorescence microscopy using a NlpI-GFP fusion cloned into a multicopy plasmid.

Results and Discussion

The *in vivo* interactome of NlpI could not be decipd because this protein was naturally expressed at very low levels. When overexpressed, Tsp was consistently copurified, confirming the anticipated interaction between NlpI and Tsp. Enzymes related to PG synthesis, such as GlmS were also repeatedly co-purified. The *in vivo* interactome of Tsp could not be elucidated

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because the available Tsp antibody was unspecific and also because Tsp purifications were unstable. Compared to SR11, the *nlpI* mutant showed a phenotype of incomplete penetration characterized by formation of elongated cells and filaments likely due to halted septum separation. In agreement with this notion, the growth of the *nlpI* mutant was significantly less inhibited by bacitracin and cefsulodin than that of the wt. The opposite result was obtained for lysozyme. Bacitracin prevents synthesis of new PG through interference with dephophosphorylation of the bactoprenol carrier; hence, cells in which PG synthesis is partially halted have less target molecules and should exhibit lower sensitivity. Cefsulodin specifically inhibits the major peptidoglycan synthase PBP1b, which is known to physically interact with the septal transpeptidase PBP3, and lysozyme could help the nlpI mutant to break down the septae that remain together due to the mutation. Except for polymyxin B, cells of the *nlpI* mutant exposed for 3 h to the four aforementioned stressors lysed at a slower pace than the wt. Wild-type cells stressed for 24 h resumed growth and did not lyse, was mutant cells lysed progressively under the same conditions. These results indicate that the *nlpI* mutation retards lysis and negatively impacts the capacity of the cell wall machinery to repair PG lesions. Indeed, slow lysis is characteristic of cells with incomplete divisomes. Incomplete septae allow entrance to the cell of compounds that do not cross the OM due to their large size. As expected, the *nlpI* mutant, but not the wt, showed phenotypic responses to crystal violet and vancomycin, which are OM-impermeable. Mutant cells were more sensitive to crystal violet than wt cells, confirming that the functional integrity of the cell wall is compromised when NlpI is not present. As to vancomycin, the mutant tolerated well concentrations than inhibited the growth of the wt completely. This suggests that the target for vancomycin is absent in the *nlpI* mutant, perhaps due to transpeptidation alterations. In fact, the PG composition of the wt and the *nlpI* mutant was found to be different, with the latter showing an enrichment of septal precursors. Transcriptomic and western blot analyses showed that *nlpI* expression progresses between 3 h and 24 h of growth and is halted when the stationary phase growth is reached. This pattern changes subtly when the experiments are repeated in presence of bacitracin, cefsulodin, polymyxin B, lysozyme, but no clear trend was observed. Thus, it is unlikely that NlpI is overexpressed in response to envelope stress, as one could anticipate for a sensor of cell wall damage. The subcellular localization of NlpI also changed in a time-dependent manner, with signals accumulating at the cell poles at 3 h of growth and at the center of the cells 16 h later.

Conclusions

Our results confirm that NlpI interacts with Tsp and strongly suggest a role of NlpI in the regulation of septal PG synthesis and remodeling during the very last stages of cell division.

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