# Dissecting Type III Protein Secretion Mechanism in Enteropathogenic *Escherichia coli* by Dynamic Complexome and Secretome Analysis

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The type III secretion system (T3SS) is a highly specialized bacterial protein secretory pathway. It plays an essential role in the pathogenesis of many Gram-negative bacteria including *Yersinia, Shigella, Salmonella, Bordetella, Pseudomonas*, and Enteropathogenic *E. coli* (EPEC). [1, 2, 3, 4]. T3SS is encoded by a pathogenicity island called the Locus of Enterocyte Effacement (LEE), and injects LEE and non-LEE-encoded effector proteins into the host cell, where these effectors modulate host signaling pathways and immune responses [3,5]. Although a plethora of studies have expanded our knowledge on the structure and function of T3SS and its secreted effectors, the precise mechanisms of type III protein secretion, translocation and their co-ordination remain poorly understood.

In a global approach, cytosolic protein complexes were isolated from wt (in a time-dependent manner) and selected KO mutants (with known effects on T3SS) EPEC cells and fractionated by Native polyacrylamide gel electrophoresis (N-PAGE) or size-exclusion chromatography (SEC). In a targeted approach, selected His-tagged T3SS-related proteins were used as baits for the selective isolation of T3SS-related protein complexes which were further separated by N-PAGE. Protein subunits of the complexes were analyzed by "bottom-up" proteomics using an Easy-nLC coupled via a nanoESI source to a LTQ-Orbitrap MS. Peptides were injected onto an in-house packed 10cm, C18, fused silica emitter column, and eluted into the LTQ-Orbitrap MS using a 175 min linear gradient of increasing ACN concentration. Protein identification was performed by Proteome Discoverer, using both Mascot and Sequest database search algorithms. Scaffold software was used for statistical evaluation of the data. Relative quantitation of the identified proteins was performed by emPAI and iBAQ algorithms [6, 7], using home-made MatLab parsers. The secretome of EPEC was collected in all of the above described conditions, concentrated using TCA protein precipitation, in-solution tryptic digested and analysed by nLC-MS. Protein identification, validation and relative quantitation was performed as described above.

The combination of a global and a targeted approach, with optimized N-PAGE and high accuracy mass spectrometry yielded the reliable identification of more than 1300 unique cytosolic proteins from EPEC cells. This corresponds to ~28% of its total theoretical proteome and ~50% of its predicted cytosolic proteome. The identified proteins represent a wide range of cellular processes as revealed from their GO annotations which is required for a reliable and comprehensive subsequent complexome and protein network analysis. Global complexome analysis determined more than 150 putative cytosolic protein complexes in EPEC including many previously reported complexes in laboratory strains of *E.coli*, providing validation for our approach. Targeted complexome analysis focused on the 54 predicted T3SS-related proteins (LEE and non-LEE encoded). Several T3SS pre-secretion protein complexes and interactions were thus identified in the EPEC cytosol against a background of the house-keeping complexome. More than 15 key cytosolic proteins of EPEC pathogenesis such as EspA, EspB, EspD, CesAB, CesD, Tir, CesT and Map were identified as components of these protein complexes. In an effort to verify these protein complexes and elucidate their precise function during T3S protein secretion, immuno-detection, pull downs and knock-out mutants were used. The time-dependent complexome analysis of EPEC provided significant information about the dynamics of T3S-related chaperone-substrate protein complexes during type III protein secretion. This information was correlated in a time-dependent manner to the orderly secretion of more than 20 specific effectors identified in the extracellular milieu, thus correlating the dynamics of the intracellular T3SS complexome with specific secretion of individual effectors.

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