The Secretome of *Streptococcus pneumoniae*

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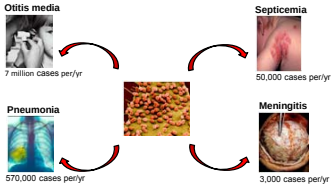
Overview

- LC-ESI-MS/MS approach is applied to identify secreted proteins of *S. pneumoniae* strain D39.
- Known virulence factors (CbpA, PcsB, PcpA) as well as several new proteins were identified.
- Triple autolysin deficient mutant strain was constructed.

Introduction

***S. pneumoniae* Infections**



Streptococcus pneumoniae (S. pneumoniae), a gram-positive pathogen residing in the nasopharynx of up to 40% of the human population is capable of causing life-threatening diseases. Young children, the elderly and immunocompromised patients are particularly predisposed to *S. pneumoniae* infections.[1] Treatment of *pneumococcal* infections has become particularly challenging due to the emergence of antibiotic resistant strains. Since the current preventive vaccines based on capsular polysaccharides have several limitations there is an urgent need to develop alternative pneumococcal vaccines. Development of vaccines based on the surface proteins and secretory proteins (virulence factors) involved in pathogenesis of *S. pneumoniae* is an attractive approach.[2] Despite their significance, a comprehensive characterization of *S. pneumoniae* proteins is still lacking. Recently, using LC-MS we have identified 59 virulence factors associated with another pathogen, *Staphylococcus aureus*.[3] To gain an understanding of the virulence factors and to identify potential vaccine candidates we have employed an LC-MS approach to study the secretome of *S. pneumoniae* strain D39, which is virulent.

Extracellular protein cultures are often contaminated by cytoplasmic proteins.[4] However, to date it is not clear whether these proteins are actively secreted via some unknown transport pathways or released due to cell lysis mediated by autolysins. To address this question, we have developed autolysin deficient mutant strain by removing three major autolysins; cell lysis is expected to be minimal in this strain.

Method

Triple autolysin (LytA, LytC, and CbpD) mutant strain was constructed according to the procedure described by Barendt et al.[5]

S. pneumoniae wild-type (WT) and triple mutant (TM) strains were cultured to an optical density of 0.2 (low OD) and 0.8 (high OD) and secretory proteins were extracted from the nutrient medium by TCA precipitation. Proteins were separated by SCX chromatography with off-line collection of the fractions. These protein fractions were then digested with trypsin and the resulting peptides were analyzed on nanoLC-nanoESI-LTQ-MS/MS. Proteins were identified by MASCOT search.

Results

Secreted proteins identified in *S. pneumoniae*

Protein: PcsB (SPD_2043)
Sequence Coverage: 67.4%

Signal peptide cleavage site ↘ N-Terminal Peptide ↗

MKKKILASLL LSTVMVSQVA VLTTAHA[27] ETT DDKIAAQDNK ISNLTAQQQEAQKQVDQIQEQVSAIQAEQSNLQAE NDRLQAESKKLEGEITELSKNIVSRNQSLEKQARSAQTNGAVTSYINTIVNSKSITEAISRVAAMSEIVSANNKMLEQQ KADKKAISEKQVANNDAINTVIANQQKLADDAQALTTKQAELKAAELSLAAEKATAEGEKASLLEQKAAAEAEARAA AVAEAAYKEKRASQQQSVLASANTNLTAQVQAVSESAAAPVRAKVRPTYSTNASSYPIGECTWGVKTLAPWAGDW GNGAQWATSAAAAGFRTGSTPQVGAIACWNDGGYGHVAVVTAVESTTRIQVSESNYAGNRTIGNHRGWFNPTTTS EGFVTYIYAD

- Peptidoglycan hydrolase, plays a critical role in bacterial cell wall biosynthesis.
- Essential gene of VicRK TCS system, depletion leads to arrest of cell growth, defects in cell wall synthesis and attenuation in virulence. [4]

Protein: Pneumolysin (SPD_1726)
Sequence Coverage: 54.0%

MANKAVNDFILAMNYDKKKLLTHQGESIENRFIKEGNQLPDEFVVIERKKRSLSTNTSDISVTATNDSRLYPGALLVVDE TLLENNPTLLAVDRAPMTYSIDLPGLASSDSFLQVEDPSNSSVRGAVNDLLAKWHQDYGQVNNVPARMQYEKITAHS MEQLKVKFGSDFEKTGNSLDIDFNSVHSGEKQIQIVNFKQIYYTVSVDAVKNPGDVFQDTVTVEDLKQRGISAERPLVYI SSVAYGRQVYLKLETTSKSDEVEAAFEALIKGVKVAPQTEWKQILDNTEVKAVILGGDPSSGARVVTGKVDMVEDLIQE GSRFTADHPGLPISYTTSFLRDNVVATFQNSTDYVETKVTAYRNGDLLLDHSGAYVAQYYITWDELSYDHQGKEVLTPK AWDRNGQDLTAHFTTSIPLKGNVRNLSVKIRECTGLAWEWWRTVYEKTDLPLVRKRTISIWGTTLYPQVEDKVEND

- Major Cytotoxin, lacks N-terminal signal peptide.
- Stimulates host cell apoptosis and interferes with host immune responses.[1,2]

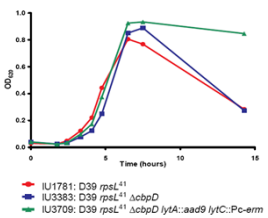
Predicted secretory proteins identified in *S. pneumoniae* WT and TM strains

Protein	Mass Theo.	WT Low OD Seq. Cov.%	WT High OD Seq. Cov.%	TM Low OD Seq. Cov.%	TM High OD Seq. Cov.%
Hypothetical protein (SPD_0010)	48100	0	25.4	36.0	12.6
LysM domain-containing protein(SPD_0104)	17759	23.4	0	65.3	31.1
Choline binding protein F (CbpF)	34279	0	0	0	21.1
D-ala-D-ala carboxypeptidase (SPD_0767)	45220	0	0	0	22.0
Pneumococcal histidine triad protein E (PhtE)	114555	0	8.9	0	10.5
Hypothetical protein(SPD_0913)	35377	26.2	33.3	43.2	64.8
Conserved Hypothetical protein(SPD_0954)	36417	0	20.1	0	0
1,4-beta-N-acetylmuramidase (LytC)	57402	0	19.0	0	0
Transcriptional regulator (SPD_1741)	37543	33.4	39.1	38.8	38.8
LysM domain-containing protein(SPD_1874)	40541	54.5	55.3	59.2	64.5
Hypothetical protein(SPD_1931)	20783	0	62.6	62.6	62.6
Choline binding protein PcpA (PcpA)	75986	0	10.9	15.3	25.3
Carbamate kinase(SPD_1977)	33636	0	16.5	0	0
Choline binding protein A (CbpA)	79049	15.6	6.1	0	8.7
Secreted 45 kDa protein precursor (PcsB)	41672	61.7	67.4	77.3	78.1

- Locate P program predicted 25 secretory proteins.
- Six secretory proteins were identified at low OD and twelve secretory proteins were identified at high OD in WT strain.
- Eight secretory proteins were identified at low OD and twelve secretory proteins were identified at high OD in TM strain.
- For a majority of the secreted proteins, expression (seq. cov%) was highest at high OD
- SPD_0104 was expressed only at low OD in WT strain.
- PcsB was identified as the most abundant secreted protein, this is consistent with our previous finding that there are ~ 5000 copies of PcsB per cell. [4]

Release of cytoplasmic proteins into extracellular medium

Growth curve of triple autolysin deficient mutant strain



Live/dead stain analysis

OD = 0.2	WT (n=172)	TM (n=249)
Live	93.0%	92.7%
Dead	7.0%	7.2%

OD=0.8	WT (n=145)	TM (n=130)
Live	66.2%	94.5%
Dead	33.8%	5.5%

n = number of cells counted

- Deleting *LytA*, *LytC* and *CbpD* in TM strain did not result in any major growth defects
- The growth curves and live/dead stain analysis indicated that removal of three major autolysins reduced cell death in TM strain.
- Several cytoplasmic proteins were identified by MS analysis in WT and TM strains that included abundant proteins such as ribosomal proteins, glycolytic enzymes and elongation factors.

Summary of proteins identified in *S. pneumoniae* WT and TM strains

Category	Predicted	Identified WT Low OD	Identified TM Low OD	Identified WT High OD	Identified TM High OD
Secreted proteins	25	6	8	12	12
Cell wall anchored proteins	13	3	3	3	3
Membrane proteins	435	8	3	6	13
Lipoproteins	47	6	8	11	11
Cytoplasmic proteins	1387	69	61	64	53
Proteins secreted via minor pathways	7	0	0	0	0
Total protein	1914	92	83	96	92

Conclusions

- Using a sensitive nanoLC-nanoESI-MS/MS approach we were able to identify 15 secreted proteins by ≥ 3 peptides. 3 proteins were identified by < 3 peptides. These are probably present in the extracellular medium but in low abundance.
- Seven predicted secretory proteins that were not detected in the present study are probably not secreted under the conditions studied, are present in trace amounts or are bound to the cell surface via non-covalent interactions.
- *S. pneumoniae* secretes fewer proteins and in lower abundance than *Staphylococcus aureus.*
- Release of cytoplasmic proteins in the mutant strain lacking the three major autolysins suggested that other minor autolysins could also play a role in their release. Although a majority of cytoplasmic proteins appear to be released by cell lysis, active secretion of some of the proteins via unknown transport machinery is also possible.

References

1. Jedrzejas, M. J. (2001) Microbiology and Molecular Biology Reviews 65, 187-207.
2. Kadioglu, A., Weiser, J. N., Paton, J. C., Andrew P. W. (2008) Nature Reviews Microbiology 6, 288-301.
3. Ravipaty, S.; Reilly, J. P. (2010) Molecular and Cellular Proteomics 9, 1912.
4. Bendtsen, J. D., Kiemer, L., Fausbøll A., Brunak, S. (2005) BMC Microbiology 5, 58.
5. Barendt, S. M., Sham, L-T., Winkler, M. E. (2009) Journal of Bacteriology 191, 3024-3040.