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Supplement

Materials and methods

Bacterial strains and growth conditions

S. pyogenes strain AP1 (strain 40/58 from the WHO Collaborating Centre for Reference and Research on Streptococci, Prague, Czech Republic) is a *covS* truncated clinical isolate of the M1 serotype. The AP1 isogenic mutant BMJ71 has a transposon insertional inactivation of *mga*,[25] a positive gene regulator of several virulence factors, including the two surface proteins M1 and H that are known to bind to several human plasma proteins. An M1 strain isolated from asymptomatic pharyngitis (in the throat) and from necrotic tissue (in the leg) of the same patient[24] was also investigated. The bacteria were grown on agar plates containing 30g/L Todd-Hewitt broth (BD, Sparks, MD, USA) and 15g/L Bacto agar (Saveen Werner AB, Limhamn, Sweden). Single colonies were grown in 30g/L Todd-Hewitt broth (BD) and 6g/L yeast extract (Merck, Darmstadt, Germany) (THY) overnight at 37°C, in 5% CO_2. Five % of the cultures were used to inoculate new tubes containing THY. The bacteria were grown to OD_{620} 0.4 - 0.5 (exponential growth phase), harvested by centrifugation at 3,500×g for 5 min and resuspended in 20mM Tris-HCl(Merck), 150mM NaCl (Sigma-Aldrich, St. Louis, MO, USA), pH 7.6, to a concentration of $2x10^9$ CFU/ml.

Plasma adsorption, glycine elution and sample preparation

Blood plasma was prepared and pooled from 5 healthy individuals as previously described.[24] Plasma was mixed with bacteria ($6.7x10^8$ CFU per ml) and the samples were incubated for 30 minutes at 37°C, allowing plasma proteins to adsorb to the bacterial surface. Bacteria were spun down at 5,000×g for 5 minutes with a swing-out rotor. Supernatants were removed from the resulting pellets, which were resuspended in 20mM Tris-HCl (Merck), 150mM NaCl (Sigma-Aldrich), pH 7.6. This washing procedure was repeated four times. To elute the adsorbed proteins the final pellets were resuspended in 0.1 M glycine (Sigma-Aldrich), pH 2.0, followed by incubation for 10 minutes. Cells were spun down (10,000×g, 5 min) and the supernatants were neutralized to pH 7-8 with 1M Tris (Ultrapure, Saveen Werner AB). Eight M urea (Fluka, Steinheim, Germany) and 0.1M ammonium bicarbonate (ABC) (Sigma-Aldrich) were

added to the supernatants to a final urea concentration of 4M and 0.05 M ABC, before in-solution digestion (see below).

Double digestions

After the washes following plasma adsorption (see above), the cells were washed and resuspended in ice-cold water and transferred to Hydrologix microcentrifuge tubes (Fisher Scientific #229-347201, San Diego, CA, USA) containing 90mg/100μl 0.1 mm Silica beads (Lysing Matrix tubes, Nordic Biolabs #6911100, Täby, Sweden). The cells were lysed with a cell disruptor (Minibeadbeater-96 Cat#1001, Biospec products, Inc., Bartlesville, OK, USA) for 6min. Samples were centrifuged briefly and the supernatants were transferred to new tubes, and 8M urea, and 0.1M ABC were added to a final urea concentration of 4M and 0.05 M ABC. To digest proteins associated with bacterial cell wall fragments, 1μg trypsin (Sequence grade modified trypsin Porcin, Promega, Madison, WI, USA) was added and samples were incubated for 30min at 37°C. Urea (8M) and ABC (0.1M) were added to final concentrations of 6M and 0.075M, respectively. Following incubation for 30minutes on shake at room temperature, the samples were centrifuged and the supernatants were transferred to new tubes and in-solution digestions (see bolow) were performed.

SDS-PAGE

To be able to load the gel with an appropriate amount of protein the concentration was determined using a protein determination kit (Total Protein Kit, Micro Lowry, Peterson's Modification, Sigma) with precipitation. Deviations from standard protocol (B) were that the standard curve was composed of six different concentration of bovine serum albumin (BSA) ranging from 0.02μg/μl to 0.2μg/μl and all volumes were divided by 10.

Samples containing urea buffer needed desalting before concentration of the samples. The desalting was done by using Zeba™ Spin Desalting columns, 7K MWCO (Thermo Scientific, Rockford, IL, USA) and supplied desalting procedure protocol. The bacteria concentration used was 6×10^6 CFU per ml plasma. Precasted gels (Criterion™, 12+2 well comb, 45μl, Bio-Rad laboratories Inc., Hercules, CA, USA) were used for the SDS-PAGE, 10μl (max 100μg protein) of sample were mixed with 30μl sample buffer (5% β-

mercapto-ethanol (Merck) in Laemli-buffer (Bio-Rad laboratories Inc.)) and incubated for 5 minutes at 95 °C and cooled on ice and loaded onto the gel. The gel was run at (Criterion™, Bio-Rad laboratories Inc.) 60V until the samples have started to migrate and then the voltage were increased to 160V until the lanes had migrated until about 1cm to the edge of the gel. The gel cassette was removed after the run and the gel was washed gently with deionized water three times 5 minutes and then stained with GelCode® Blue Stain Reagent (Thermo Scientific) for 30-60 minutes. Extensive GelCode® Blue Stain Reagent was washed with deionized water.

In gel digestion

The gel was cut and each lane was fractionated into ten tubes containing 0.1M ammonium bicarbonate (ABC) (Fluka). To destain the GelCode® Blue Stain Reagent from the gel pieces they were put into 50% acetonitrile (ACN) (Fluka), 50mM ABC to shrink and then reswelled with 0.1M ABC, this was repeated until no blue color from the gel pieces could be detected. Then the gel pieces were put in ACN to shrink them until they were white and hard and then completely dried in a speedvac (Savant SPD131DDA Speedvac concentrator, Thermo Scientific).

To reduce the proteins, 20mM dithiothreitol (DTT) (Fluka), 0.1M ABC were added, just enough for swelling the pieces, and the samples were incubated at 55 °C for 30-60 minutes. To alkylate the proteins the residual DTT solution were removed and twice the amount, of 55mM 2-Iodoacetamide (IAA) (AppliChem, Darmstadt, Germany) in 0.1M ABC were added and the samples were incubated at room temperature in the dark for 30-45 minutes. Both the IAA and the DTT solutions were freshly prepared prior the experiment. The gel pieces were then washed by drying the pieces with 50% ACN in 50mM ABC then 100% ACN then the pieces were reswelled with 0.1M ABC and dried again with ACN until they were white and hard and then completely dried in a speedvac.

To digest the proteins, the gel pieces were covered with a minimum volume of 12.5ng/µl trypsin (Sequence grade modified trypsin Porcin, Promega) in 50mM ABC and the pieces were reswelled by trypsin solution on ice for 30 minutes. A small amount of 50mM ABC added before incubating the gel pieces over night at 37 °C. The digestion was stopped by adding 5% Formic acid (J. T. Baker, Deventer, Netherlands) in 50% ACN for 15 minutes repeated twice and the solution was harvested to new tubes. The pieces were then

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reswelled by adding water and then dried completely by addition of ACN, the solution were transferred into the new tubes. To increase the peptide concentration the samples were dried in speedvac and solved in 3% ACN and 0.1% formic acid.

In solution digestion

To reduce the protein sample, Tris (2-carboxyethyl) phosphine (TCEP) (Sigma-Aldrich) were added to a concentration of 5mM and the samples were incubated at 37 °C for 60 minutes. The samples were alkylated by adding 2-Iodoacetamide (IAA) (AppliChem) at a concentration of 10mM and the samples were incubated 30 minutes in the dark at room temperature. Before the digestion the samples were diluted with twice the sample volume in ammonium bicarbonate (ABC) (Sigma-Aldrich) and trypsin (Sequence grade modified trypsin Porcin, Promega) were added to a concentration of 2.5ng/µl and samples were incubates at 37°C over night. Adding formic acid to pH 2-3 stopped the digestion.

C18 peptide clean-up

Vydac UltraMicro Spin® Silica C18 300Å Columns (#SUM SS18V, The Nest Group, Inc., Southborough, MA, USA) were placed in tubes and flushed with methanol (Fluka) to wash the column at 100 × g for 1 minute. Buffer A (2 % Acetonitrile and 0.2 % Formic acid) was then added three times following centrifuging at 200 × g for 1 minute. The samples were transferred to the spin column and centrifuged at 200 × g for 1.5 minutes, then reapplied twice before it was discarded. If sample volume was above 450µl the sample adding procedure was repeated until the entire sample had been immobilized. After immobilization the column was washed three times, 200 × g for 1.5 minutes, with buffer A to clean the samples. The columns was placed in new tubes and flushed with Buffer B (50 % Acetonitrile, 0.2 % Formic acid) two times at 100 × g for 1 minute, and a final time at 800 × g for 1 minute to elute the peptides. The samples were placed in the speedvac until completely dry, resuspended in buffer A to desired volume for mass spectrometry, sonicated (Transsonic digitals, Elma, Germany) for 5 minutes, centrifuged briefly and put into MS vials.

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Mass spectrometry analysis

The hybrid Orbitrap-LTQ XL mass spectrometer (Thermo Electron, Bremen, Germany) was coupled online to a split-less Eksigent 2D NanoLC system (Eksigent technologies, Dublin, CA, USA). Peptides were loaded with a constant flow rate of 15 µl/min onto a pre-column (PepMap 100, C18, 5 µm, 0.3 mm x 5 mm, LC Packings, Amsterdam, Netherlands) and subsequently separated on a RP-LC analytical column (10 µm fused silica emitter, 75 µm x 16 cm, PicoTip™ Emitter, New Objective, Inc.Woburn, MA, USA, packed in-house with Reprosil-Pur C18-AQ resin, 3 µm, Dr. Maisch, GmbH) with a flow rate of 300 nl/min. The peptides were eluted with a linear gradient from 95% solvent A (0.1% formic acid in water) and 5% solvent B (0.1% formic acid in acetonitrile) to 35% solvent B over 60 minutes. The mass spectrometer was operated in data-dependent acquisition mode to automatically switch between Orbitrap-MS (from m/z 400 to 2000) and LTQ--MS/MS. Four MS/MS spectra were acquired in the linear ion trap per each FT-MS scan which was acquired at 60,000 FWHM nominal resolution settings using the lock mass option (m/z 445.120025) for internal calibration. The dynamic exclusion list was restricted to 500 entries using a repeat count of two with a repeat duration of 20 seconds and with a maximum retention period of 120 seconds. Precursor ion charge state screening was enabled to select for ions with at least two charges and rejecting ions with undetermined charge state. The normalized collision energy was set to 30%, and one microscan was acquired for each spectrum.

The data analysis was performed as previously described.[28] Briefly, the resulting MS2 data were searched with X!Tandem search engine, version 2009.04.01.1 with the k-score plugin, (1) a common peptide and protein list was generated using the Trans-Proteomic pipeline, version 4.4.0 (2). All searches were performed with full-tryptic cleavage specificity, up to 2 allowed missed cleavages, a precursor mass error of 15 ppm and an error tolerance of 0.5 Da for the fragment ions. Because of the sample preparation cysteine carbamidomethylation was defined as fixed modification in the search parameters. A protein database with sequences for Streptococcus pyogenes (NC_002737 from NCBI) and human (Swiss-Prot, version 57.1 including known splice variants and iso-forms) was used to match the individual spectra to peptide sequences. The database was extended by decoy sequences to validate the resulting peptide-spectrum matches (PSMs) (3). A value of 0.01 for the false-discovery rate (FDR) was

then used to generate the final protein list with ProteinProphet. MS1-based quantification was done using SuperHirn.[29] Features were detected using SuperHirn using a retention time tolerance of 1, MS1 m/z tolerance of 10, MS2 PPM m/z tolerance of 30. Only features with charge 1-5 were included. Any feature for which more than one peptide could be identified at the 1% FDR, hence mapping to more than one protein, were discarded.

Selected reaction monitoring (SRM)

Selected reaction monitoring (SRM) transition assays were constructed by testing the ten most abundant peptide fragments for selected proteotypic peptides identified with high confidence in the LC-MS/MS experiments. Spiked in the RT-peptides (Biognosys AG, Zurich, Switzerland) allowed normalization of the retention time as previously described.[28] The SRM measurements were performed on a TSQ Vantage triple quadrupole mass spectrometer (Thermo Electron, Bremen, Germany) equipped with a nano electrospray ion source (Thermo Electron). Chromatographic separations of peptides were preformed on an Easy-nLC II system (Thermo scientific, San Jose, CA, USA). Peptides were loaded with a constant pressure of 250 Bar onto a pre-column (Thermo Scientific, Easy-Column, 2cm, ID100µm, 5µm C18) and subsequently separated on a RP-LC analytical column (10 µm fused silica emitter, 75 µm x 16 cm, PicoTip™ Emitter, New Objective, Inc.Woburn, MA, USA, packed in-house with Reprosil-Pur C18-AQ resin, 3 µm, Dr. Maisch, GmbH) with a flow rate of 300 nl/min. The peptides were eluted with a linear gradient from 97% solvent A (0.1% formic acid in water) and 3% solvent B (0.1% formic acid in acetonitrile) to 15% solvent B over 3 minutes and then the gradient was increased from 15% to 35% solvent B over 34 minutes. The mass spectrometer was operated in SRM mode, with both Q1 and Q3 settings at unit resolution (FWHM 0.7 Da). A spray voltage of +1700 V was used with a heated ion transfer setting of 270ºC for desolvation. Data were acquired using the Xcalibur software (version 2.1.0). The dwell time was set to 10 ms and the scan width to 0.01 m/z. All collision energies were calculated using the formula: CE = (Parent m/z) x 0.034 + 3.314.

The data analysis was performed as previously described[28] using a 2.5% false discovery rate. The resulting peptide abundances were exported into a database, where protein

abundances were inferred by summing up the abundances for the peptides uniquely mapping to each protein.[29]

Data processing

The initial list of putative interacting proteins was generated by fractionating the proteins prior to LC-MS/MS analysis resulting in high use of instrument time and in complex data outputs. To omit the protein fractionation step and still maintain the same level of sensitivity we turned to SRM. SRM is associated with high sensitivity and specificity enabling quantification of the majority of the target proteins in 1D-LC-SRM-MS.[30] By pooling high, medium and low abundant proteins in separate transitions sets the sample load was optimized for the individual transitions sets. To reduce the number of target proteins certain proteins were removed prior to the SRM analysis. The specific selection of target proteins reflects why there are additional proteins in the plasma abundance graph compared to the plasma adsorption abundance graph. The criteria we used to exclude proteins were as follows. Firstly, all immunoglobulin proteins were excluded since shotgun MS result in many different immunoglobulins due to their variable regions; secondly, proteins unable to generate suitable SRM assays; thirdly, the proteins not detected in the plasma adsorption samples and fourthly proteins with small pXIC ratio between plasma and plasma adsorption.

Equations

$$Enrichment\ ratio = \frac{pXIC_{Plasma\ adsorption}}{pXIC_{Plasma} + pXIC_{Plasma\ adsorption}} \quad (1)$$

Referances

Some references exists in the paper.

1. Craig, R., Beavis, R. C. (2003) A method for reducing the time required to match protein sequences with tandem mass spectra. *Rapid Commun. Mass Spectrom.* 17, 2310-2316
2. Keller, A., Eng, J., Zhang, N., Li, X. J., Aebersold, R. (2005) A uniform proteomics MS/MS analysis platform utilizing open XML file formats. *Mol. Syst. Biol.* 1, 2005.0017
3. Elias, J. E., Gygi, S. P. (2007) Target-decoy search strategy for increased confidence in large-scale protein identifications by mass spectrometry. *Nat Methods.* 4, 207-214