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Identification of two abundant Aerococcus urinae cell wall-anchored proteins

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1 # Identification of two abundant *Aerococcus urinae* cell wall-

2 # anchored proteins.

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17 Running title: The surface proteome of *A. urinae*

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Abstract

Aerococcus urinae is an emerging pathogen that causes urinary tract infections, bacteremia and infective endocarditis. The mechanisms through which *A. urinae* cause infection are largely unknown. The aims of this study were to describe the surface proteome of *A. urinae* and to analyse *A. urinae* genomes in search for genes encoding surface proteins. Two proteins, denoted Aerococcal surface protein (Asp) 1 and 2, were through the use of mass spectrometry based proteomics found to quantitatively dominate the aerococcal surface. The presence of these proteins on the surface was also shown using ELISA with serum from rabbits immunized with the recombinant Asp. These proteins had a signal sequence in the amino-terminal end and a cell wall-sorting region in the carboxy-terminal end, which contained an LPATG-motif, a hydrophobic domain and a positively charged tail. Twenty-three additional *A. urinae* genomes were sequenced using Illumina HiSeq technology. Six different variants of *asp* genes were found (denoted *asp*1-6). All isolates had either one or two of these *asp*-genes located in a conserved locus, designated Locus encoding Aerococcal Surface Proteins (LASP). The 25 genomes had in median 13 genes encoding LPXTG-proteins (range 6-24). For other Gram-positive bacteria, cell wall-anchored surface proteins with an LPXTG-motif play a key role for virulence. Thus, it will be of great interest to explore the function of the Asp proteins of *A. urinae* to establish a better understanding of the molecular mechanisms by which *A. urinae* cause disease.

Introduction

Aerococcus urinae is a Gram-positive coccus that belongs to the phylum of Firmicutes. *A. urinae* is known to cause urinary tract infection (Christensen et al., 1989; Schuur et al., 1997; Senneby et al., 2015), bacteremia (Christensen et al., 1995; Senneby et al., 2016) and infective endocarditis (de Jong et al., 2010; Ebnother et al., 2002; Kristensen and Nielsen, 1995; Sunnerhagen et al., 2016), mostly in the elderly male population. Since the introduction of MALDI-TOF MS as a diagnostic tool in clinical microbiology laboratories, *A. urinae* is more frequently identified in clinical samples, especially in urine cultures, and has attracted attention as an important emerging pathogen (Rasmussen, 2016). A few studies have reported that *A. urinae* is part of a urinary tract microbiome (Hilt et al., 2014; Pearce et al., 2014). The mechanisms through which *A. urinae* establishes colonization and causes infection are largely unknown. Only one study has previously targeted the question of *A. urinae* virulence mechanisms (Shannon et al., 2010), demonstrating that it produces biofilm and is able to induce human platelet activation and aggregation. These are properties of potential importance in the process of establishing infections in the human host (Donlan and Costerton, 2002).

At the molecular level, little is known regarding aerococcal virulence factors (Carkaci et al., 2017). For other pathogenic Firmicutes, such as *Streptococcus pyogenes* and *Staphylococcus aureus,* cell wall-attached proteins with an LPXTG-motif play key roles for virulence. For instance, the M protein of *S. pyogenes* is an abundant surface protein, which possesses a broad spectrum of functions, such as the inhibition of phagocytosis and binding of several plasma proteins (Fischetti, 1989; Smeesters et al., 2010). The amino (NH_2)-terminal part of the protein displays hypervariability, resulting in antigenic variation (Cunningham, 2000). The LPXTG-proteins share common features, such as a signal sequence in the NH_2-terminal end and three characteristics in the carboxy (COOH)-terminal end; a cell wall-sorting region containing the LPXTG-motif, a hydrophobic membrane-spanning domain and a positively charged tail positioned in the cytoplasm. The LPXTG-motif is recognized by the membrane bound enzyme sortase and is, after cleavage between the threonine and glycine residues, covalently attached to the cell wall (Schneewind and Missiakas,

99   2014). It is at present unclear if *A. urinae* express proteins attached to the cell wall

100   through the LPXTG-motif.

101   Mass spectrometry (MS) based proteomics has previously been shown to be a useful

102   method to detect bacterial surface-associated proteins with protein copies per cell

103   accuracy (Malmstrom et al., 2009) and to determine the surface protein composition

104   of gram-positive bacteria (Kilsgard et al., 2016; Rodriguez-Ortega et al., 2006;

105   Severin et al., 2007). However, a presumption to utilize this technique is the genomic

106   sequence for the analysed bacterial species or strain. Hence, the combination of next

107   generation sequencing and MS-based proteomics constitutes a powerful strategy to

108   search for potential novel virulence factors.

109   The aims with this study were to describe the surface proteome of *A. urinae* and to

110   describe the genes encoding surface proteins.

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Material and Methods

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Bacteria and culturing conditions

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116   The strain ACS-120-V-Col10a (Col10a) was retrieved from the Culture collection of

117   Gothenburg. The AU3 strain was collected at the Clinical Microbiology laboratory,

118   Lund and originated from a blood culture as described previously (Senneby et al.,

119   2012). Forty-six *A. urinae* isolates from blood cultures had been described previously

120   (Senneby et al., 2016) and were designated as AuB followed by a number. *A. urinae*

121   isolates were cultivated in Tryptic Soy Broth with 0.25% glucose (TSBG) for

122   approximately 24 hours at 35° C in 5 % CO_2.

123

Bacterial surface digestion and MS sample preparation

124

125   Surface proteins were released from AU3 and Col10 stationary phase cells in

126   triplicates with a modified protocol as previously described (Rodriguez-Ortega et al.,

127   2006; Severin et al., 2007). The cells (~8 x10^8 colony forming units (CFU)) were

128   washed with 20 mM Tris-HCl, 150 mM NaCl, pH 7.6 (TBS) and resuspended in 1 M

129   d-arabinose, 10 mM $CaCl_2$ in TBS and 5 µg sequencing grade trypsin (Promega) and

130   incubated at 37 °C with 500 rpm shaking for 20 min. The digested mixture was

131   centrifuged with swing-out rotor at 2000 x g for 15 min at 4 °C. ProteaseMAX

132   (Promega) was added to the supernatants (surface fraction) to a final concentration of

133   0.01 % following by heating to 80 °C for denaturation. Cysteine residues were

134   reduced with 25 mM tris(2-carboxyethyl)phosphine (Sigma-Aldrich, TCEP) and

135   alkylated with 25 mM 2-Iodoacetamide (Sigma-Aldrich, IAA). Surface fraction

136   samples were fully digested with 2 µg sequencing grade trypsin (Promega) for 10 h at

137   37 °C and the sample acidified with 0.5 % Trifluoroacetic acid. The digested cells

138   (cellular fraction) were washed with TBS and resuspended in water and homogenized

139   using a Fastprep-96 beadbeater (MPBio) with Lysing Matrix B tubes (MPBio). The

140   cell lysates were denatured with 8 M Urea in 100 mM ammonium bicarbonate (ABC)

141   and then reduced with 25 mM TCEP for 1 h at 37°C, and alkylated with 25 mM

142   iodoacetamide for 45 min before diluting the sample with 100 mM ABC to a final

143   urea concentration below 1.5 M. Proteins were digested by incubation with trypsin

144   (trypsin:protein ratio of 1:100 (w:w)) for 10 h at 37°C. The peptides from both

145   fractions were desalted and cleaned-up with reversed-phase spin columns (Vydac

146   UltraMicroSpin Silica C18 300Å Columns, Harvard Apparatus) according to the

147   manufacturer's instructions.

148

149   **MS data acquisition**

150   Peptide analyses were performed on a Q Exactive Plus mass spectrometer (Thermo

151   Scientific) connected to an EASY-nLC 1000 ultra-high-performance liquid

152   chromatography system (Thermo Scientific). Peptides were separated on an EASY-

153   Spray ES802 columns (Thermo Scientific) using a linear gradient from 3 to 35%

154   acetonitrile in aqueous 0.1% formic acid during 2 h. Data-dependent acquisition mode

155   (DDA) and Data-independent acquisition mode (DIA) instrument settings were

156   identical to as described in (Malmstrom et al., 2016). From the DDA data, spectral

157   libraries were built using the TPP Fraggle workflow (Teleman et al., 2017) using

158   NCBI fasta files GCA_000193205.1_ASM19320 and

159   GCA_001649715.1_ASM164971 respectively concatenated with iRT peptides,

160   contaminants and decoys. The generated spectral libraries were used to extract the

161   DIA data with DIANA v2.0.0 (Teleman et al., 2015) using a 1% peptide false

162   discovery rate.

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MS data analysis

Data was processed with custom R-scripts using the tidyverse package collection together with broom package for statistical functions. For the quantification of proteins, all integrated peptide ion intensities extracted from the MS2 spectra was summed up by protein and then divided by protein length. Peptides matching more than one protein were not included in the analysis. Protein quantification data was normalized based on the sample total intensity. Proteins from Col10a and AU3 were classified into orthologous pairs using ProteinOrtho.pl v5.16b (Lechner et al., 2011) and protein domains predicted using standalone InterProScan v5.11-50 (Jones et al., 2014).

Genome sequencing

Bacteria were pelleted through centrifugation and stored at -70° C until shipment on dry ice to GATC Biotech (Konstanz, Germany). Library preparation was performed using an optimized protocol and standard Illumina adapter sequences were used. Sequencing was performed with Illumina HiSeq 2500 (Illumina, Inc., San Diego, US) with paired-end reads, 2 x 125 bp. The genomes of AU3 (ASM164971v1, GenBank assembly accession GCA_001649715.1) and ACS-120-V-Col10a (ASM19320v1, GenBank assembly accession GCA_00193205.1) had been previously published. For assembly of the genomes, SPAdes 3.9 with the careful-mode on, was used through services on the Center for Genomic Epidemiology's website (Nurk et al., 2013). The computations were performed on resources provided by the Swedish National Infrastructure for Computing (SNIC) at Uppsala Multidisciplinary Center for Advanced Computational Science (UPPMAX). Assembly quality was evaluated using QUAST (Gurevich et al., 2013). Sample contamination was evaluated with Kraken (cross-species) (Wood and Salzberg, 2014) and by mapping the raw sequence data to a reference genome using BWA MEM (Li and Durbin, 2009) and evaluating the presence of minority bases with samtools mpileup (within-species) (Li et al., 2009). In cases with within-species contamination the contaminant gene were eliminated by coverage analysis, i.e. genes with low coverage were excluded. The BLAST software (Zhang et al., 2000) was used for searches in the genomes. Sequence alignment was performed with Clustal Omega. The maximum likelihood method was used for building phylogenetic tress through the use of the MEGA software (version 7.0.26).

200 Annotation of the genomes was performed with RASTtk on the PATRIC platform

201 (Wattam et al., 2017). SignalP 4.1 was used to locate signal peptide cleavage sites

202 (Petersen et al., 2011). Hydrophobic plots were performed using services on the

203 Expasy website (Kyte and Doolittle, 1982). The ClustalW multiple sequence

204 alignment was used to produce identity scores (%) for the amino acid sequences with

205 the following parameters: similarity matrix: gonnet, open gap penalty 10, extend gap

206 penalty 0.1-0.2, gap distance 4, delay divergent 30%. The signal sequences and the N-

207 terminal end (starting with the LPXTG-motive) were deleted prior to analysis.

208 Searches were also performed in 26 *A. urinae* genomes belonging to Bioproject

209 PRJNA315093, that were accessible on the NCBI webpage. The bacterial isolates

210 originated from the female urinary tract.

211

212 **Expression cloning and protein production**

213 The MacVector software (v. 14.5.3) was used to analyse the bacterial genome for

214 suitable primer sites for amplification of *asp* genes. Primers were ordered from

215 Eurofins and can be found in Table 1. Chromosomal DNA from AU3 was extracted

216 using the innuPRerp Bacterial DNA System (Analytik Jena AG). PCR products were

217 inserted into the pGEX-6P-1 expression vector. One Shot TOP10 *E. coli* (Thermo

218 Fisher Scientific) were transformed and used for plasmid amplification. Plasmids

219 were extracted using Qiagen Plasmid Plus kits. The plasmids were sent to GATC

220 GmbH for sequencing to ensure that the sequence was correct. BL21 cells with pLysS

221 were then transformed according to the manufacturer's protocol, with selection for

222 pGEX-6-p-1 (100 μg/ ml ampicillin) and for pLysS (32 μg/ml chloramphenicol).

223

224 For protein expression, an overnight *E. coli* culture carrying the construct was diluted

225 1:25 in fresh TSBG supplemented with antibiotics and grown for 3 h. IPTG (1 mM)

226 was then added and incubation continued for 3 h at 37°C. The bacteria were pelleted

227 and the supernatant discarded. Pellet was dissolved in BugBuster (Merck Millipore)

228 suspended in 20 mM TRIS (pH 7.4) supplemented with 1:100 (v/v) benzonase. After

229 10 minutes of incubation at room temperature bacterial cell debris was collected by

230 centrifugation and the supernatant was loaded onto an equilibrated GST-column,

231 washed, and finally eluted (elution buffer). The sequence of the affinity-purified

232 protein was confirmed by mass-spectrometry at the SciBlu core facility at Lund

233 University.

234

Antiserum generation and ELISA

To generate antiserum against the affinity-purified recombinant proteins, the proteins
were sent to Biogenes GmbH for immunization. To assess binding of antibodies to the
surface proteins an ELISA was performed. Nunc MaxiSorp (ThermoFisher Scientific)
96-well plates were coated with either a suspension of bacteria (AU3, Col10a or *A.
viridans*), or a solution of Asp-proteins in coating buffer (1.69 g Na_2CO_3 and 2.94 g
$NaHCO_3$ in 1 L H_2O). As a control, bacteria with reduced surface protein expression
were generated by incubating a bacterial solution with trypsin (0.0004% w/v) at 37° C
for 20 minutes after which the reaction was stopped by adding trypsin inhibitor at a
molar ratio of 8:1. The trypsinated bacteria and control bacteria (where trypsin
inhibitor was added before the trypsin) were then pelleted by centrifugation, washed
(PBS), and finally resuspended in coating buffer. The bacteria were then heat-killed at
$85^°$C for 30 minutes before adding the samples to the Nunc plate for coating over
night at $4^°$C. The plates were washed (PBST), rabbit serum in different dilutions
added, followed by further washes and addition of protein G coupled HRP (1:3000).
As an HRP substrate, ABTS dissolved 1:20 in a substrate solution (21 g citric acid
monohydrate and 17.8 g Na_2HPO_4 2 H_2O in 1 L H_2O, pH 4.5) was used in
combination with H_2O_2; incubated in room temperature for 15 minutes. The plates
were then analyzed with the iMark Microplate reader (Bio-Rad) at 415 nm.

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Sequence similarity network (SNN) of LPXTG-containing ORFs.

In total, 106 285 ORFs from the 25 *A. urinae* genomes were analyzed with standalone
InterProScan v5.11-50 (Jones et al., 2014) and included the following analyses: Pfam,
PANTHER, Gene3D, CDD, TIGRFAM, ProSitePatterns, SUPERFAMILY, Hamap,
ProSiteProfiles, Coils, SMART, MobiDBLite, PRINTS, PIRSF, SignalP, SFLD and
ProDom. LPXTG-motifs were predicted with InterPro signature accessions PS50847
and/or TIGR01167 and/or PF00746 and sequences identified through this procedure
were analyzed by all-by-all BLAST comparisons using blastp (v2.7.1+ with default
settings). The blast result was filtered with the following thresholds: *E*-value $< 1 \times 10^{-10}$, >50 % sequence identity and >75 alignment length.
The SNN was visualized using Cytoscape (v3.5.1) (Shannon et al., 2003), where each
node represents a protein/ORF and an edge or line between the nodes denotes a

268 similarity relationship between the proteins. The "organic" layout was used whereby

269 nodes are clustered more tightly if they are more highly interconnected and all edges

270 were bundled.

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272

273 # Results

274

275 **Quantitative profiling of *A. urinae* surface proteomes.**

276 To search for putative novel surface-associated proteins, we released surface exposed

277 proteins from hypotonically swelled *A. urinae* cells of the strains Col10a and AU3

278 with a short trypsin digestion step. This produced a surface protein fraction and a cell

279 protein fraction. This method has previously been used for releasing surface exposed

280 proteins from cells of other gram-positive bacteria (Rodriguez-Ortega et al., 2006;

281 Solis et al., 2010). The trypsinated cells (the cell fraction) were homogenized and

282 used as control samples hypothetically containing mostly intracellular and membrane

283 proteins. Both surface and cell fractions were then prepared for MS analysis. All

284 derived peptide samples (n = 12) were analysed using data-independent-acquisition

285 (DIA) followed by targeted data extraction (SWATH-MS) (Fig. 1A). In this analysis

286 we quantified a total of 1118 and 1168 proteins of which 765 and 682 proteins were

287 detected in the surface fractions from strains Col10a and AU3 respectively (Fig. 1B).

288 Essentially all surface fraction proteins (99.8 %) were also detected in the cellular

289 fractions (Fig. 1C-D), but with large differences of abundances between fractions.

290 The majority of proteins in the surface fraction were much less abundant in the

291 cellular fraction (Fig. 2A). We defined surface associated proteins based on two

292 criteria; 10-fold enriched in the surface associated fractions and a Hochberg adjusted

293 p-value < 0.01 compared to the cellular fraction (Col10a, n= 24; AU3, n = 30) (Fig.

294 2A and B). The stringent cut-off values for the surface associated protein groups were

295 selected to distinctly separate proteins identified in the two fractions. The defined

296 surface associated proteins included the most abundant proteins in the surface fraction

297 (Fig. 2C) and 70% of these proteins had a predicted signal peptide domain as

298 determined by InterProScan analysis (Fig. 2D). Of the proteins identified in the

299 surface fraction but not defined as surface associated, most were ribosomal proteins,

300   metabolic enzymes and transcriptional regulators and only ~9 % had a predicted

301   signal peptide (Fig. 2D).

302   Next we compared the surface associated protein orthology between the two strains as

303   defined by the bidirectional best-hit (Lechner et al., 2011). Of the total 54 surface

304   associated proteins from both strains, 17 pairs were determined to be orthologous by

305   the Proteinortho software (Lechner et al., 2011) (Fig. 2E). Based on this analysis we

306   identified two orthologous protein pairs with predicted LPXTG-sequences and signal

307   peptide protein domains that were abundantly produced on the surface (Fig. 2E, red

308   nodes with dotted edges). The proteins were denoted Aerococcal surface protein

309   (Asp) 1 and 2 and the corresponding genes were accordingly named (*asp1* and *asp2).*

310   The Asp1 was the most abundant protein on the surface, with a relative abundance of

311   approximately 73 % and 26 % of the total proteins on the surface (for Col10a and

312   AU3 respectively). No proteins similar to the Asp proteins were found in the InterPro

313   database that includes high-level structure-based classifications and sub-family

314   classifications. Furthermore, InterPro analysis of motifs and/or domains only

315   identified LPXTG and signal peptide sequences. Additionally, four other surface

316   associated proteins were classified as LPXTG-anchored. However, these proteins

317   lacked an orthologous counterpart or the orthologous counterpart did not harbour a

318   predicated LPXTG sequence (see Fig. 2E). The total number of predicted LPXTG

319   proteins in the genomes was 8 for AU3 and 10 for Col10a (not shown). In summary,

320   the quantitative profiling of *A. urinae* surface proteomes revealed the that less than

321   half all theoretical LPXTG proteins are expressed during the experimental conditions

322   used and that both surfaces are dominated by an orthologous pair of proteins (Asp1).

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324

325   **Genomic and protein sequence analysis of Aerococcal surface protein 1 and 2.**

326   Based on the outcome from the MS-analysis, we continued with acquiring 23

327   additional novel whole genome sequences of *A. urinae* isolates to investigate the

328   presence of genes encoding Asp proteins and also other LPXTG-containing proteins.

329   The genomes of the AU3 and the Col10a isolates were included in the analysis. Thus,

330   25 *A. urinae* genomes were studied. Variants of the *asp*-genes were found in all

331   isolates sequenced. The genes were denoted *asp1-6,* based on a phylogenetic analysis

332   (Figure 3), and the corresponding gene products were denoted Asp1-6. The *asp*-genes

333   were located adjacent to each other in a chromosomal locus of the aerococcal

334   genome, in this study designated as Locus encoding Aerococcal Surface Proteins

335   (LASP). In all isolates it was constituted by, in a 5' – 3' order, a pyruvate carboxylase

336   gene, a gene designated as a "hypothetical cytosolic protein", the asp-gene(s) and a

337   16S rRNA methyltransferase gene. The LASP is schematically depicted in Figure 4.

338   The primary structure of Asp1 contained 284-298 amino acids and Asp2 contained

339   307-382 amino acids. The Asp3 contained 376-407 amino acids, all Asp4 had 425

340   amino acids and Asp5 and Asp6 had 483 amino acids. The comparison of the amino

341   acid sequences for Asp1-6 is presented in Table 2 with identity scores (%). The amino

342   acid sequence of the LPXTG-motif was LPATG in all isolates. The aerococcal

343   isolates had combinations of several different *asp*-genes in their LASP. Four variants

344   were found, designated $LASP_{1-4}$. In $LASP_1$ only the *asp1* was present, $LASP_2$ had

345   both *asp1* and *asp2*, $LASP_3$ had *asp3* and *asp6* whereas $LASP_4$ contained *asp4* and

346   *asp5*.

347   The 26 *A. urinae* genomes in the bio project PRJNA315093 also included *asp* genes

348   and these isolates had either $LASP_1$, $LASP_2$ or $LASP_4$.

349

350   **Expression cloning and immunological assays**

351   The predicted mature forms of Asp1 and Asp2 were recombinantly expressed in *E.*

352   *coli* and were used for production of two polyclonal antisera in rabbits. ELISA

353   demonstrated that the post-immune antisera from both rabbits reacted with Asp1 and

354   Asp2, whereas there was no reaction between the pre-immune sera (data not shown).

355   To confirm that Asp1 and Asp2 were present on the surface of *A. urinae*, ELISA

356   using anti-Asp1 and anti-Asp2 antisera against whole bacteria immobilized in the

357   wells was performed. This showed that both the antisera reacted with *A. urinae* AU3

358   and Col10a but not with the control *A. viridans* that lacked *asp* homologs in the

359   genome (Figure 5A and data not shown). The signal was reduced when the bacteria

360   were treated with trypsin, further indicating that the proteins are located on the

361   surface (Figure 5B).

362

363   **Genome-wide associations of LPXTG-containing proteins in *A. urinae***

364   To expand the information on other potential surface anchored proteins in addition to

365   the Asp-proteins we analysed all ORFs (n=106 285) from the 25 genomes for LPXTG

366   motifs. From this analysis we identified 289 ORFs that contained LPXTG-motifs. In

367   order to cluster these ORFs into groups we compared the translated sequences of the

368 289 ORFs using all-by-all BLAST comparisons, which in turn yielded 83 521

369 comparisons. After applying a BLAST filter as previously described (Mashiyama et

370 al., 2014), 9 647 sequence comparisons remained and these were selected for

371 sequence similarity network (SNN) visualization (Figure 6A). Eleven apparent

372 clusters were identified and these clusters were numbered and colored (Figure 6B).

373 The functional domains that were predicted in the analysis are presented in Figure 6C.

374 Our analysis of the 25 genomes revealed that these isolates had in median 13 genes

375 encoding LPXTG-proteins (range 6-24). Predicted domains of these proteins included

376 the G5 domain, which is widely found in Gram-positive bacteria such as streptococcal

377 species (Lin et al., 2012) and has been proposed to be involved in biofilm formation

378 of *S. aureus* (Bateman et al., 2005). Also, collagen binding domains and domains with

379 collagen triple helix repeats were predicted. Furthermore, Mucin-binding protein

380 domains, which play a role in adhesion of Lactobacillus species to mucin (Chatterjee

381 et al., 2018) and domains with Rib/alpha-like repeats were predicted. Proteins with

382 Rib and alpha repeats can be found in surface proteins of group B *Streptococcus*

383 (Wastfelt et al., 1996).

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385

386 **Discussion**

387

388 In this study, we aimed to describe the surface proteome of *A. urinae* and also to

389 describe aerococcal genes encoding surface proteins. We selected the two strains

390 Col10a and AU3 for the proteomic surface profiling using previously published

391 protocols (Rodriguez-Ortega et al., 2006; Severin et al., 2007). A major issue with

392 studying bacterial surface exposed proteins is contamination of intracellular proteins

393 (Solis et al., 2010). Here we performed a subtraction of the contaminating proteins by

394 comparing the protein abundances between the surface fraction and cellular fraction

395 resulting in 54 surface associated proteins from both strains of which 17 were

396 orthologues. A total of 18 genes encoding potential proteins with an LPXTG-motif

397 were identified in the Col10a and AU3 genomes. However, only two orthologous

398 pairs of them were detected in the surface fractions. These two proteins, denoted Asp1

399 and Asp2 in this study, were among the most abundant proteins on the aerococcal

400 surface. The Asp1 and Asp2 share features with LPXTG-proteins from other bacterial

401   species (for instance streptococcal and staphylococcal species), such as a signal

402   sequence in the NH_2-terminal end, a hydrophobic membrane-spanning domain and a

403   positively charged tail in the COOH-terminal end.

404   In a recent publication by Carkaci *et al.* (Carkaci et al., 2017), the authors described

405   and compared 40 *A. urinae* and eight *A. sanguinicola* genomes of strains isolated

406   from blood, urine and heart valves. In that study, virulence genes encoding LPXTG-

407   containing proteins were not reported. The virulence-associated genes were however

408   identified using a virulence factor database that only included known virulence

409   factors. Thus, aerococcal genes with low similarity to genes in this collection, such as

410   the *asp* genes, were prone to be missed. Prior to our investigation, it was therefore

411   unclear whether all *A. urinae* isolates had genes encoding LPXTG-proteins.

412   Our results show that *A. urinae* genomes of isolates originating from both blood and

413   urine cultures, contain genes encoding LPXTG-proteins and that some of the

414   predicted functional domains of these proteins are of potential importance for

415   virulence. Our results also show that *A. urinae* has a conserved locus, LASP, which

416   comprise one or two of the *asp* genes. The organization of the LASP has a high

417   degree of similarity to the organization of the so-called *mga* regulon of *S. pyogenes*.

418   The genes in the *mga* regulon encode important streptococcal virulence factors (M

419   and M-like proteins and the C5a peptidase (Fischetti, 1989; O'Connor and Cleary,

420   1987)), which are regulated by the transcription factor Mga (Berge et al., 1998;

421   Caparon and Scott, 1987; McIver et al., 1995). Both the aerococcal and the

422   streptococcal loci comprise LPXTG-containing genes, arranged in a conserved

423   fashion, but with a variation between bacterial isolates in the number of genes present

424   and in sequence similarities. The aerococcal protein HCP, with the corresponding

425   gene positioned in the LASP upstream from the *asp* gene(s), was detected in the

426   intracellular fraction in the MS analysis. It is possible that the HCP has a similar

427   regulatory role for the LASP as Mga has for the *mga* regulon of *S. pyogenes*.

428   The function of the aerococcal Asp proteins is unknown. However, LPXTG-proteins

429   in other species are involved in processes such as bacterial adhesion, immune evasion,

430   internalization, iron acquisition and biofilm formation. Since most *A. urinae* isolates

431   are found in urine cultures, it is possible that the Asp proteins act as adhesion

432   molecules to epithelial cells in the human urinary tract or are involved in the process

433   of biofilm formation, which, mentioned previously, has been described as a feature of

434   *A. urinae* (Shannon et al., 2010). A few isolates in this study came from patients

435 diagnosed with infective endocarditis but no common LASP variant was observed for

436 these isolates (data not shown). Conclusion regarding correlation between LASP

437 variants and clinical features could not be made due to the small sample size.

438

439 **Conclusion**

440 In this study, we show for the first time that *A. urinae* has genes encoding surface

441 proteins with an LPXTG-motif and that two of these proteins quantitatively dominate

442 the surface. To establish a better understanding of how *A. urinae* colonize humans and

443 cause disease, it is of great interest to explore the function of the Asp proteins of *A.*

444 *urinae* further.

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469 **References**

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471 Bateman, A., Holden, M.T., Yeats, C., 2005. The G5 domain: a potential N-

472 acetylglucosamine recognition domain involved in biofilm formation.

473 Bioinformatics 21, 1301-1303.

474 Berge, A., Rasmussen, M., Björck, L., 1998. Identification of an insertion sequence

475 located in a region encoding virulence factors of Streptococcus pyogenes. Infect

476 Immun 66, 3449-3453.

477 Caparon, M.G., Scott, J.R., 1987. Identification of a gene that regulates expression

478 of M protein, the major virulence determinant of group A streptococci. Proc Natl

479 Acad Sci U S A 84, 8677-8681.

480 Carkaci, D., Hojholt, K., Nielsen, X.C., Dargis, R., Rasmussen, S., Skovgaard, O.,

481 Fuursted, K., Andersen, P.S., Stegger, M., Christensen, J.J., 2017. Genomic

482 characterization, phylogenetic analysis, and identification of virulence factors in

483 Aerococcus sanguinicola and Aerococcus urinae strains isolated from infection

484 episodes. Microb Pathog.

485 Chatterjee, M., Pushkaran, A.C., Vasudevan, A.K., Menon, K.K.N., Biswas, R.,

486 Mohan, C.G., 2018. Understanding the adhesion mechanism of a mucin binding

487 domain from Lactobacillus fermentum and its role in enteropathogen exclusion.

488 Int J Biol Macromol 110, 598-607.

489 Christensen, J.J., Jensen, I.P., Faerk, J., Kristensen, B., Skov, R., Korner, B., 1995.

490 Bacteremia/septicemia due to Aerococcus-like organisms: report of seventeen

491 cases. Danish ALO Study Group. Clin Infect Dis 21, 943-947.

492 Christensen, J.J., Korner, B., Kjaergaard, H., 1989. Aerococcus-like organism--an

493 unnoticed urinary tract pathogen. APMIS 97, 539-546.

494 Cunningham, M.W., 2000. Pathogenesis of group A streptococcal infections. Clin

495 Microbiol Rev 13, 470-511.

496 de Jong, M.F., Soetekouw, R., ten Kate, R.W., Veenendaal, D., 2010. Aerococcus

497 urinae: severe and fatal bloodstream infections and endocarditis. J Clin Microbiol

498 48, 3445-3447.

499 Donlan, R.M., Costerton, J.W., 2002. Biofilms: survival mechanisms of clinically

500 relevant microorganisms. Clin Microbiol Rev 15, 167-193.

501 Ebnother, C., Altwegg, M., Gottschalk, J., Seebach, J.D., Kronenberg, A., 2002.

502 Aerococcus urinae endocarditis: case report and review of the literature.

503 Infection 30, 310-313.

504 Fischetti, V.A., 1989. Streptococcal M protein: molecular design and biological

505 behavior. Clin Microbiol Rev 2, 285-314.

506 Gurevich, A., Saveliev, V., Vyahhi, N., Tesler, G., 2013. QUAST: quality assessment

507 tool for genome assemblies. Bioinformatics 29, 1072-1075.

508 Hilt, E.E., McKinley, K., Pearce, M.M., Rosenfeld, A.B., Zilliox, M.J., Mueller, E.R.,

509 Brubaker, L., Gai, X., Wolfe, A.J., Schreckenberger, P.C., 2014. Urine is not sterile:

510 use of enhanced urine culture techniques to detect resident bacterial flora in the

511 adult female bladder. J Clin Microbiol 52, 871-876.

512 Jones, P., Binns, D., Chang, H.Y., Fraser, M., Li, W., McAnulla, C., McWilliam, H.,

513 Maslen, J., Mitchell, A., Nuka, G., Pesseat, S., Quinn, A.F., Sangrador-Vegas, A.,

514 Scheremetjew, M., Yong, S.Y., Lopez, R., Hunter, S., 2014. InterProScan 5: genome-

515 scale protein function classification. Bioinformatics 30, 1236-1240.

516 Kilsgard, O., Karlsson, C., Malmstrom, E., Malmstrom, J., 2016. Differential

517 compartmentalization of Streptococcus pyogenes virulence factors and host

518 protein binding properties as a mechanism for host adaptation. Int J Med

519 Microbiol 306, 504-516.

520 Kristensen, B., Nielsen, G., 1995. Endocarditis caused by Aerococcus urinae, a

521 newly recognized pathogen. Eur J Clin Microbiol Infect Dis 14, 49-51.

522 Kyte, J., Doolittle, R.F., 1982. A simple method for displaying the hydropathic

523 character of a protein. J Mol Biol 157, 105-132.

524 Lechner, M., Findeiss, S., Steiner, L., Marz, M., Stadler, P.F., Prohaska, S.J., 2011.

525 Proteinortho: detection of (co-)orthologs in large-scale analysis. BMC

526 Bioinformatics 12, 124.

527 Li, H., Durbin, R., 2009. Fast and accurate short read alignment with Burrows-

528 Wheeler transform. Bioinformatics 25, 1754-1760.

529 Li, H., Handsaker, B., Wysoker, A., Fennell, T., Ruan, J., Homer, N., Marth, G.,

530 Abecasis, G., Durbin, R., Genome Project Data Processing, S., 2009. The Sequence

531 Alignment/Map format and SAMtools. Bioinformatics 25, 2078-2079.

532 Lin, I.H., Hsu, M.T., Chang, C.H., 2012. Protein domain repetition is enriched in

533 Streptococcal cell-surface proteins. Genomics 100, 370-379.

534   Malmstrom, E., Kilsgard, O., Hauri, S., Smeds, E., Herwald, H., Malmstrom, L.,

535   Malmstrom, J., 2016. Large-scale inference of protein tissue origin in gram-

536   positive sepsis plasma using quantitative targeted proteomics. Nat Commun 7,

537   10261.

538   Malmstrom, J., Beck, M., Schmidt, A., Lange, V., Deutsch, E.W., Aebersold, R., 2009.

539   Proteome-wide cellular protein concentrations of the human pathogen

540   Leptospira interrogans. Nature 460, 762-765.

541   Mashiyama, S.T., Malabanan, M.M., Akiva, E., Bhosle, R., Branch, M.C., Hillerich, B.,

542   Jagessar, K., Kim, J., Patskovsky, Y., Seidel, R.D., Stead, M., Toro, R., Vetting, M.W.,

543   Almo, S.C., Armstrong, R.N., Babbitt, P.C., 2014. Large-scale determination of

544   sequence, structure, and function relationships in cytosolic glutathione

545   transferases across the biosphere. PLoS Biol 12, e1001843.

546   McIver, K.S., Heath, A.S., Green, B.D., Scott, J.R., 1995. Specific binding of the

547   activator Mga to promoter sequences of the emm and scpA genes in the group A

548   streptococcus. J Bacteriol 177, 6619-6624.

549   Nurk, S., Bankevich, A., Antipov, D., Gurevich, A.A., Korobeynikov, A., Lapidus, A.,

550   Prjibelski, A.D., Pyshkin, A., Sirotkin, A., Sirotkin, Y., Stepanauskas, R., Clingenpeel,

551   S.R., Woyke, T., McLean, J.S., Lasken, R., Tesler, G., Alekseyev, M.A., Pevzner, P.A.,

552   2013. Assembling single-cell genomes and mini-metagenomes from chimeric

553   MDA products. J Comput Biol 20, 714-737.

554   O'Connor, S.P., Cleary, P.P., 1987. In vivo Streptococcus pyogenes C5a peptidase

555   activity: analysis using transposon- and nitrosoguanidine-induced mutants. J

556   Infect Dis 156, 495-504.

557   Pearce, M.M., Hilt, E.E., Rosenfeld, A.B., Zilliox, M.J., Thomas-White, K., Fok, C.,

558   Kliethermes, S., Schreckenberger, P.C., Brubaker, L., Gai, X., Wolfe, A.J., 2014. The

559   female urinary microbiome: a comparison of women with and without urgency

560   urinary incontinence. MBio 5, e01283-01214.

561   Petersen, T.N., Brunak, S., von Heijne, G., Nielsen, H., 2011. SignalP 4.0:

562   discriminating signal peptides from transmembrane regions. Nat Methods 8,

563   785-786.

564   Rasmussen, M., 2016. Aerococcus: an increasingly acknowledged human

565   pathogen. Clin Microbiol Infect 22, 22-27.

566 Rodriguez-Ortega, M.J., Norais, N., Bensi, G., Liberatori, S., Capo, S., Mora, M.,

567 Scarselli, M., Doro, F., Ferrari, G., Garaguso, I., Maggi, T., Neumann, A., Covre, A.,

568 Telford, J.L., Grandi, G., 2006. Characterization and identification of vaccine

569 candidate proteins through analysis of the group A Streptococcus surface

570 proteome. Nat Biotechnol 24, 191-197.

571 Schneewind, O., Missiakas, D., 2014. Sec-secretion and sortase-mediated

572 anchoring of proteins in Gram-positive bacteria. Biochim Biophys Acta 1843,

573 1687-1697.

574 Schuur, P.M., Kasteren, M.E., Sabbe, L., Vos, M.C., Janssens, M.M., Buiting, A.G.,

575 1997. Urinary tract infections with Aerococcus urinae in the south of The

576 Netherlands. Eur J Clin Microbiol Infect Dis 16, 871-875.

577 Senneby, E., Goransson, L., Weiber, S., Rasmussen, M., 2016. A population-based

578 study of aerococcal bacteraemia in the MALDI-TOF MS-era. Eur J Clin Microbiol

579 Infect Dis 35, 755-762.

580 Senneby, E., Petersson, A.C., Rasmussen, M., 2012. Clinical and microbiological

581 features of bacteraemia with Aerococcus urinae. Clin Microbiol Infect 18, 546-

582 550.

583 Senneby, E., Petersson, A.C., Rasmussen, M., 2015. Epidemiology and antibiotic

584 susceptibility of aerococci in urinary cultures. Diagn Microbiol Infect Dis 81, 149-

585 151.

586 Severin, A., Nickbarg, E., Wooters, J., Quazi, S.A., Matsuka, Y.V., Murphy, E.,

587 Moutsatsos, I.K., Zagursky, R.J., Olmsted, S.B., 2007. Proteomic analysis and

588 identification of Streptococcus pyogenes surface-associated proteins. J Bacteriol

589 189, 1514-1522.

590 Shannon, O., Morgelin, M., Rasmussen, M., 2010. Platelet activation and biofilm

591 formation by Aerococcus urinae, an endocarditis-causing pathogen. Infect

592 Immun 78, 4268-4275.

593 Shannon, P., Markiel, A., Ozier, O., Baliga, N.S., Wang, J.T., Ramage, D., Amin, N.,

594 Schwikowski, B., Ideker, T., 2003. Cytoscape: a software environment for

595 integrated models of biomolecular interaction networks. Genome Res 13, 2498-

596 2504.

597 Smeesters, P.R., McMillan, D.J., Sriprakash, K.S., 2010. The streptococcal M

598 protein: a highly versatile molecule. Trends Microbiol 18, 275-282.

599   Solis, N., Larsen, M.R., Cordwell, S.J., 2010. Improved accuracy of cell surface

600   shaving proteomics in Staphylococcus aureus using a false-positive control.

601   Proteomics 10, 2037-2049.

602   Sunnerhagen, T., Nilson, B., Olaison, L., Rasmussen, M., 2016. Clinical and

603   microbiological features of infective endocarditis caused by aerococci. Infection

604   44, 167-173.

605   Teleman, J., Hauri, S., Malmstrom, J., 2017. Improvements in Mass Spectrometry

606   Assay Library Generation for Targeted Proteomics. J Proteome Res 16, 2384-

607   2392.

608   Teleman, J., Rost, H.L., Rosenberger, G., Schmitt, U., Malmstrom, L., Malmstrom, J.,

609   Levander, F., 2015. DIANA--algorithmic improvements for analysis of data-

610   independent acquisition MS data. Bioinformatics 31, 555-562.

611   Wastfelt, M., Stalhammar-Carlemalm, M., Delisse, A.M., Cabezon, T., Lindahl, G.,

612   1996. Identification of a family of streptococcal surface proteins with extremely

613   repetitive structure. J Biol Chem 271, 18892-18897.

614   Wattam, A.R., Davis, J.J., Assaf, R., Boisvert, S., Brettin, T., Bun, C., Conrad, N.,

615   Dietrich, E.M., Disz, T., Gabbard, J.L., Gerdes, S., Henry, C.S., Kenyon, R.W., Machi,

616   D., Mao, C., Nordberg, E.K., Olsen, G.J., Murphy-Olson, D.E., Olson, R., Overbeek, R.,

617   Parrello, B., Pusch, G.D., Shukla, M., Vonstein, V., Warren, A., Xia, F., Yoo, H.,

618   Stevens, R.L., 2017. Improvements to PATRIC, the all-bacterial Bioinformatics

619   Database and Analysis Resource Center. Nucleic Acids Res 45, D535-D542.

620   Wood, D.E., Salzberg, S.L., 2014. Kraken: ultrafast metagenomic sequence

621   classification using exact alignments. Genome Biol 15, R46.

622   Zhang, Z., Schwartz, S., Wagner, L., Miller, W., 2000. A greedy algorithm for

623   aligning DNA sequences. J Comput Biol 7, 203-214.

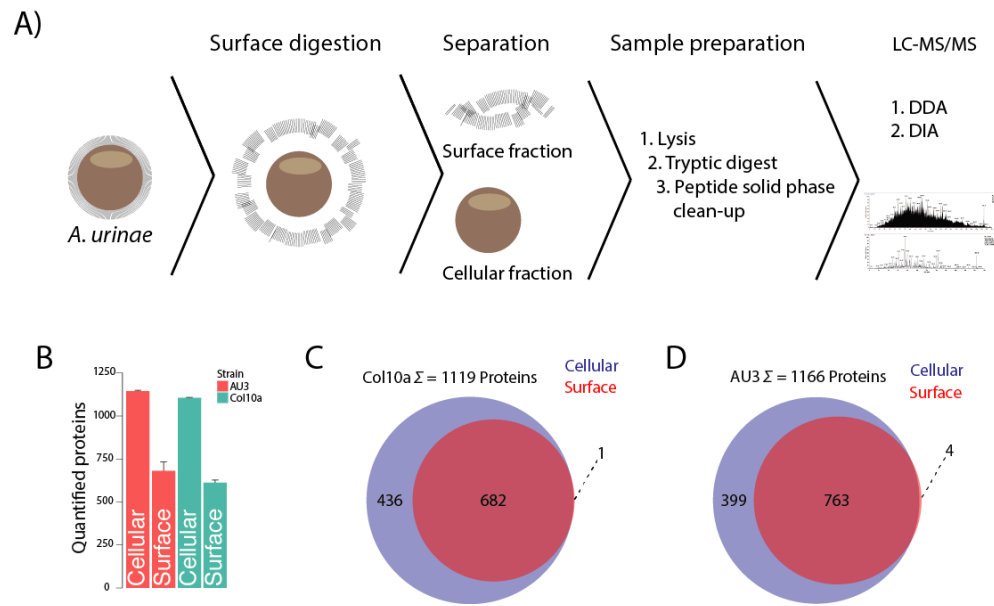624

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Figure 1. Proteomics of *A. urine* cellular and surface fractions. (a) Summary of
the proteomics workflow for *A. urine* cellular and surface fractions. Surface proteins
were released from two different *A. urine* strains using a short incubation with trypsin
and the fractions separated by centrifugation. Following the sample preparation, the
derived peptide samples were analysed with LC-MS/MS using both shotgun- and
SWATH-MS. (b) Average numbers ± standard deviations of quantified proteins per
respective strain and fractions using three replicates per condition. (c, d) Number of
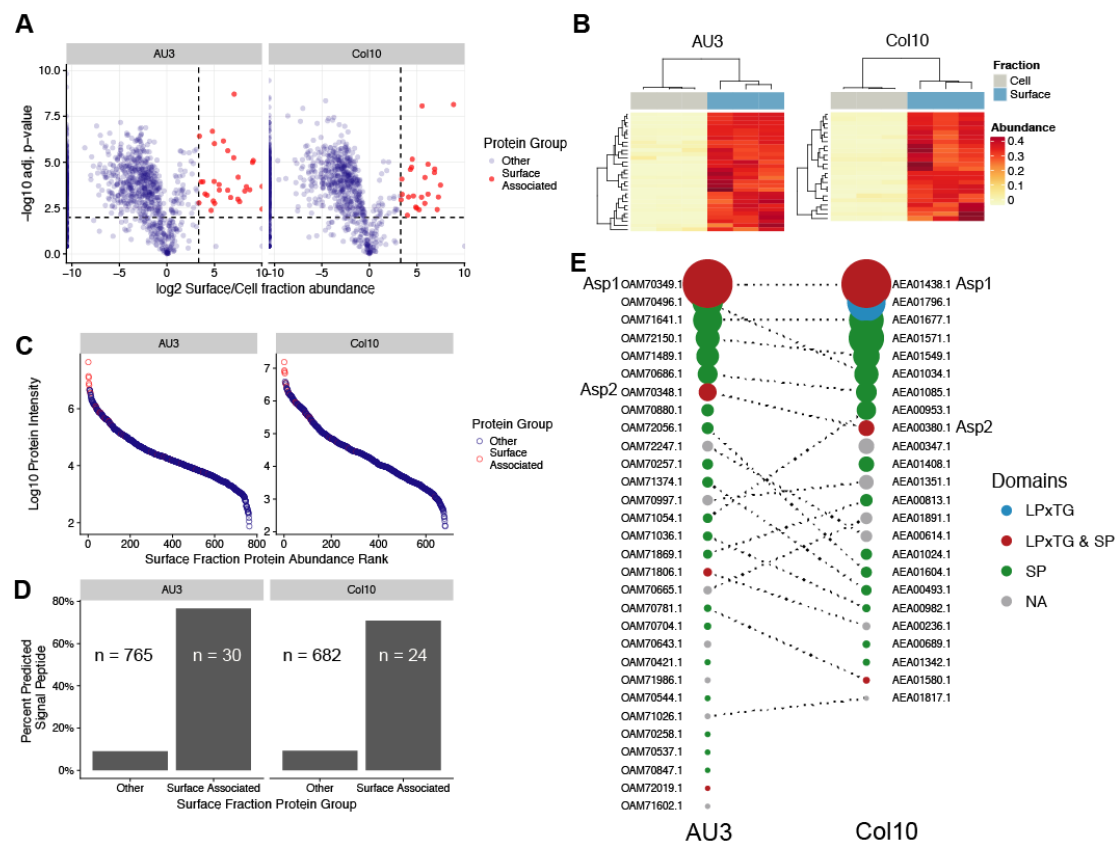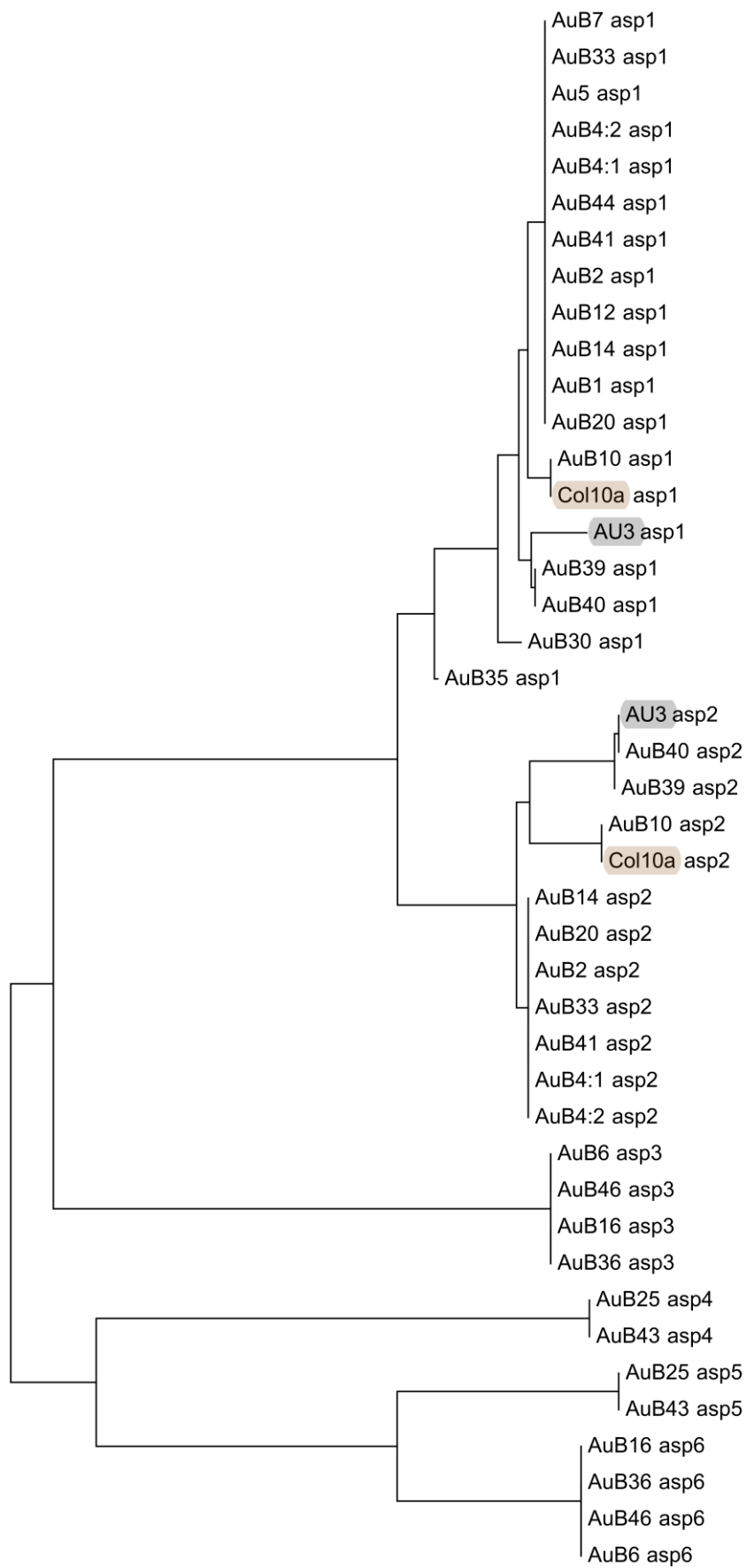proteins common between fractions and per respective strain.

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Figure 2. Proteomic analysis reveals *A. urine* surface associated proteins. (a)
Volcano plots showing Hochberg adjusted –log10 p-values correlated to log2 fold
changes in surface vs cell fractions of respective strain. Proteins in red represent the
surface associated proteins with a fold-change ≥ 10 (vertical dotted lines) and
adjusted p-value < 0.01 (horizontal dotted lines). (b) Heat map and unsupervised
hierarchical clustering of surface associated proteins (red dots in (a)) across individual
replicates and intensities normalized on total ion current (TIC) normalization (c).
Average abundance distribution of surface fraction proteins of respective strain with
the surface associated proteins marked with red. (d) The proportion of proteins with a
predicted signal peptide of Surface Fraction proteins divided into Surface Associated
and other proteins groups. The number (n) of proteins per protein group and strain are
indicated in the plot. (e) Shows the ortholog pairs indicated as dotted lines (edges)
between the surface associated proteins (nodes named with GenBank protein
accession number) of the two strains. The node size is proportional to relative protein
abundance per strain. Node colour represents predicted presence of protein domains
SP: signal peptide and LPXTG: Gram-positive LPXTG cell wall anchor.

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AuB7 asp1
AuB33 asp1
Au5 asp1
AuB4:2 asp1
AuB4:1 asp1
AuB44 asp1
AuB41 asp1
AuB2 asp1
AuB12 asp1
AuB14 asp1
AuB1 asp1
AuB20 asp1
AuB10 asp1
Col10a asp1
AU3 asp1
AuB39 asp1
AuB40 asp1
AuB30 asp1
AuB35 asp1
AU3 asp2
AuB40 asp2
AuB39 asp2
AuB10 asp2
Col10a asp2
AuB14 asp2
AuB20 asp2
AuB2 asp2
AuB33 asp2
AuB41 asp2
AuB4:1 asp2
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AuB6 asp3
AuB46 asp3
AuB16 asp3
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AuB43 asp4
AuB25 asp5
AuB43 asp5
AuB16 asp6
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AuB6 asp6

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Figure 3.

Mid-point rooted maximum likelihood tree visualizing the clustering of the *asp* genes

in the 25 isolates. The *asp* genes of Col10a (brown) and AU3 (grey) are highlighted in

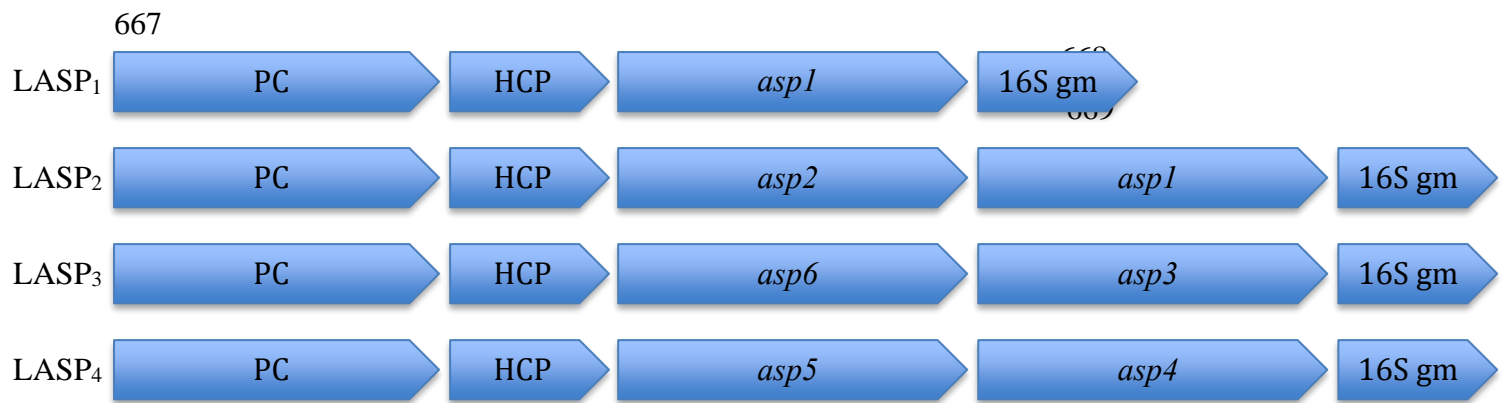the tree. The scale length indicates 0.1 base substitutions per base.

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LASP₁: PC | HCP | *asp1* | 16S gm

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LASP₂: PC | HCP | *asp2* | *asp1* | 16S gm

LASP₃: PC | HCP | *asp6* | *asp3* | 16S gm

LASP₄: PC | HCP | *asp5* | *asp4* | 16S gm

670 **Figure 4. Schematic figure of different types of the Locus encoding Aerococcal**

671 **Surface Proteins (LASP).**

672 The LASP includes three genes enclosing the *asp* gene(s). Four different types of the

673 LASP were identified in the *A. urinae* isolates, denoted by us as LASP₁₋₄.

674 *PC*; Pyruvate carboxylase gene, *HCP*; Hypothetical cytosolic protein gene, *asp;*

675 aerococcal surface protein gene, *16S gm;* 16S rRNA guanine-methyltransferase gene.

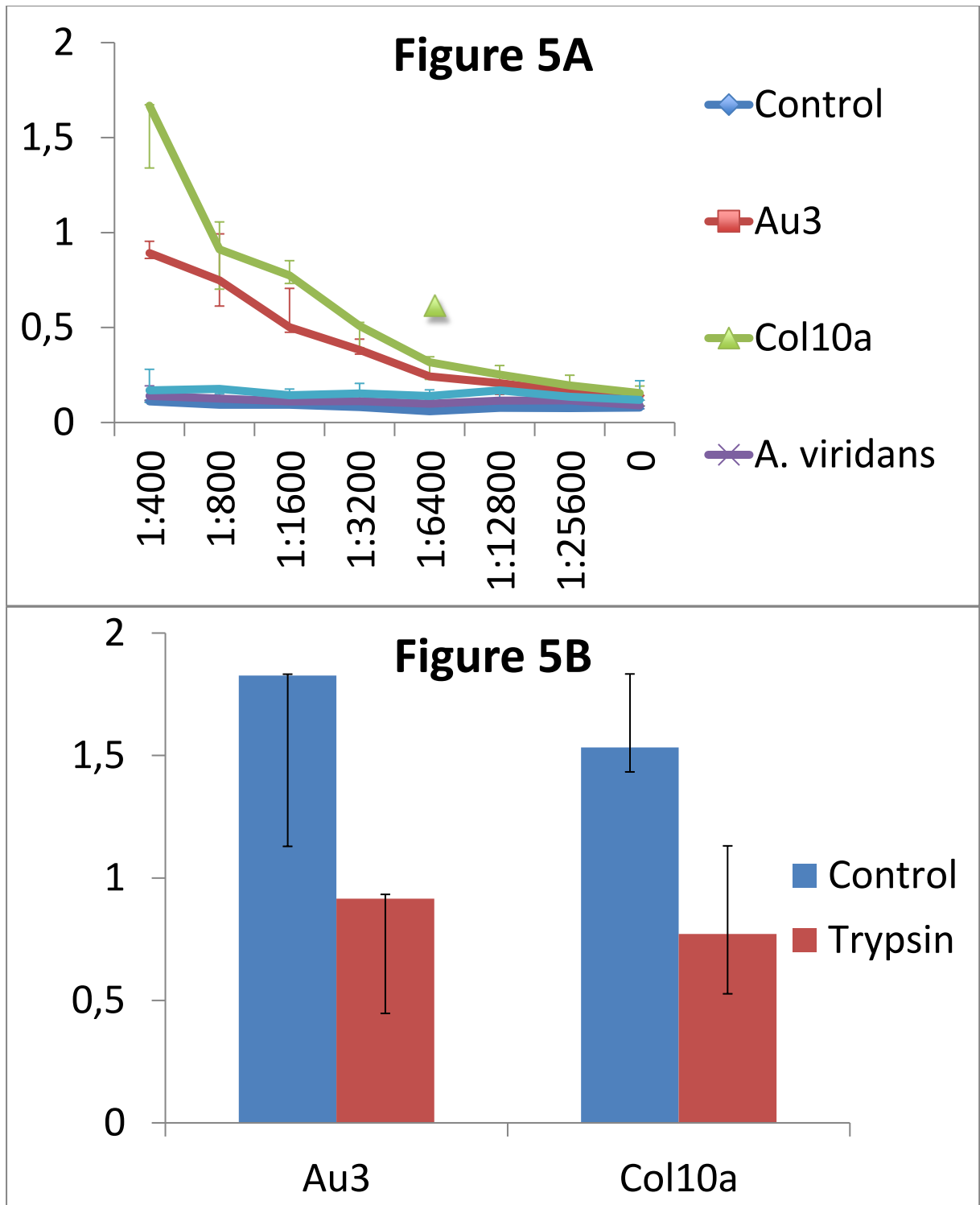676 The distance between genes and length of genes are not according to scale.

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Figure 5A

Figure 5B

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682 **Figure 5.**

683 Data shown as median with whiskers showing range, experiments performed in

684 triplicate. A) Binding of serum from rabbits immunized with Asp1 was studied with

685 ELISA. Serum from the same rabbit taken from before the immunization was used as

686   a negative control. The plate was coated with AU3, Col10a, or *A. viridans*. Dilutions

687   of rabbit serum are shown on the x axis and range from 1:400 to no serum at all. B)

688   AU3 and Col10a were treated with trypsin to reduce the amount of Asp1 on the

689   surface. AU3 and Col10a with trypsin inhibitor added were used as controls.
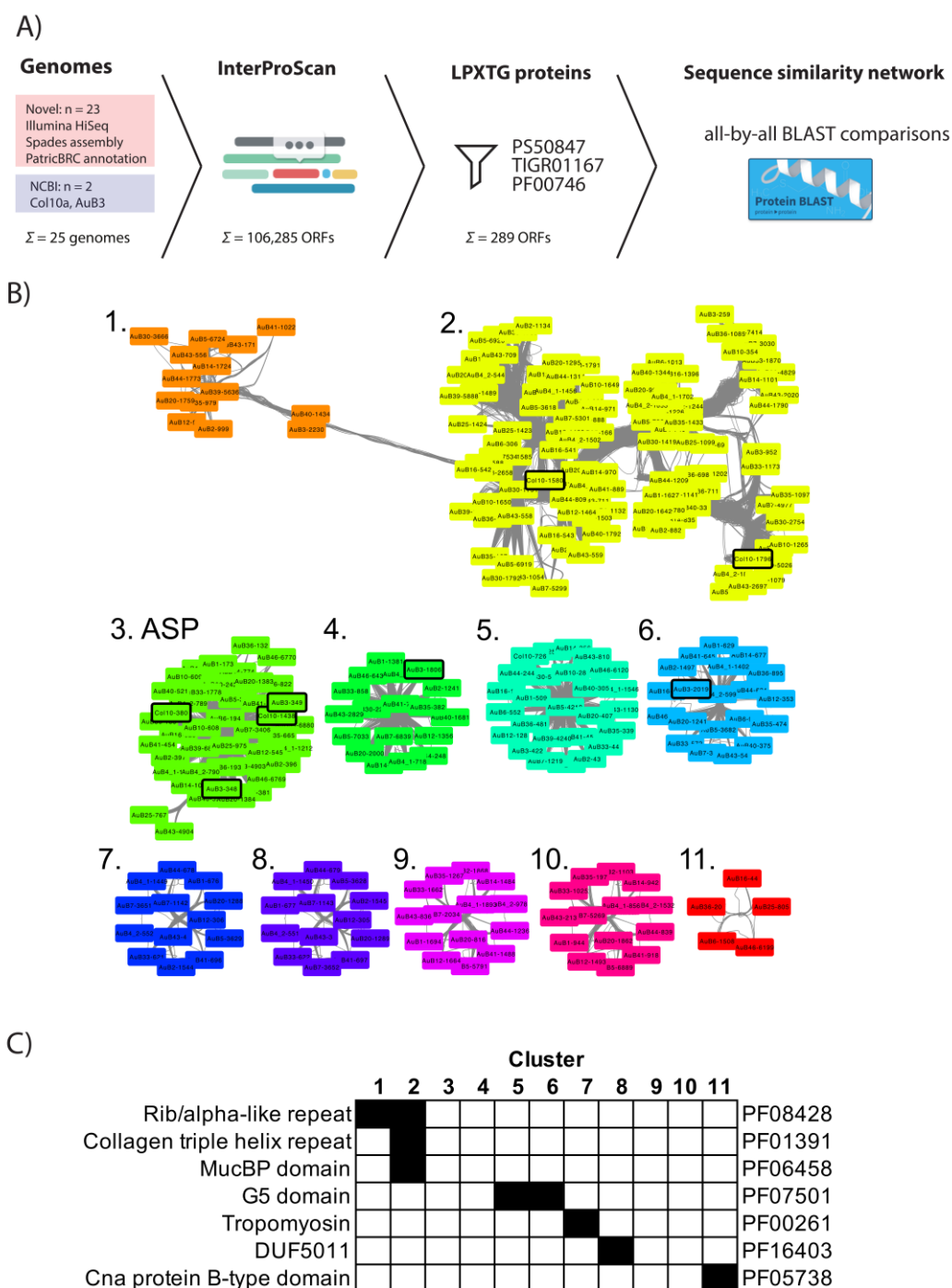
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Figure 6. Sequence similarity network of *A. urinae* LPXTG proteins.

A) All *A. urinae* ORFs (n=106,285) from 25 genomes were analyzed with

InterProScan to obtain predictions of functional domains. ORFs containing LPXTG

motifs (n=289) were selected with the criteria of InterPro signature accessions

PS50847 and/or TIGR01167 and/or PF00746. For sequence similarity analysis the

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697   289 selected ORFs were analyzed all-by-all BLAST comparisons. B) Sequence

698   similarity network of the 289 LPXTG ORFs with 9,647 edges each representing a

699   BLAST hit. Eleven clusters of LPXTG ORFs are colored accordingly. Proteins that

700   were previously defined as being surface associated in Col10a or AU3 were outlined

701   in black. C) Predicted functional domains of the LPXTG-ORFs and in which clusters

702   they were detected in.

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Direction	Gene	
	asp1	*asp2*
Forward	GAGGAATAATAAATGGCAGACGCA TTTGTAACACCAGTA	GAFFAATAATAATAAATGGCAGTT GCTAAAGCTGAAATGTTT
Reverse	AGTAGCTGGTAATTTAGCGTTAGCG TTAGCTTTTTGAGCTTTGC	TAATTTAGCATTAGCTTTTTCAGC TTTAGCTTCTTTATC

706 **Table 1. Primers used for expression cloning**

707 The primers used to construct the pGEX-6p-1 plasmids containing the *asp1* and *asp2*

708 genes were designed with an RBS.

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Asp	1	2	3	4	5	6
1	72-100 %	40-53%	20-21%	12-13%	10-11%	11-12%
2	-	90-100 %	16-17%	14-15%	13%	13%
3	-	-	100 %	16%	14%	15%
4	-	-	-	100 %	12%	13%
5	-	-	-	-	100 %	52%
6	-	-	-	-	-	100 %

710 **Table 2.**
711 Identity scores (%) for Asp1-6 amino acid sequences.
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