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1	Identification of two abundant Aerococcus urinae cell wall-					
2	anchored proteins.					
3						
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34 Abstract

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36 Aerococcus urinae is an emerging pathogen that causes urinary tract infections, 37 bacteremia and infective endocarditis. The mechanisms through which A. urinae 38 cause infection are largely unknown. The aims of this study were to describe the 39 surface proteome of A. urinae and to analyse A. urinae genomes in search for genes 40 encoding surface proteins. Two proteins, denoted Aerococcal surface protein (Asp) 1 41 and 2, were through the use of mass spectrometry based proteomics found to 42 quantitatively dominate the aerococcal surface. The presence of these proteins on the 43 surface was also shown using ELISA with serum from rabbits immunized with the 44 recombinant Asp. These proteins had a signal sequence in the amino-terminal end and 45 a cell wall-sorting region in the carboxy-terminal end, which contained an LPATG-46 motif, a hydrophobic domain and a positively charged tail. Twenty-three additional A. 47 urinae genomes were sequenced using Illumina HiSeq technology. Six different 48 variants of asp genes were found (denoted asp1-6). All isolates had either one or two 49 of these *asp*-genes located in a conserved locus, designated Locus encoding 50 Aerococcal Surface Proteins (LASP). The 25 genomes had in median 13 genes 51 encoding LPXTG-proteins (range 6-24). For other Gram-positive bacteria, cell wall-52 anchored surface proteins with an LPXTG-motif play a key role for virulence. Thus, it 53 will be of great interest to explore the function of the Asp proteins of A. urinae to 54 establish a better understanding of the molecular mechanisms by which A. urinae 55 cause disease. 56 57 58 Key Words: Aerococcus urinae, surface proteome, LPXTG-motif, genes encoding 59 surface proteins.

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67 Introduction

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69 Aerococcus urinae is a Gram-positive coccus that belongs to the phylum of 70 Firmicutes. A. urinae is known to cause urinary tract infection (Christensen et al., 71 1989; Schuur et al., 1997; Senneby et al., 2015), bacteremia (Christensen et al., 1995; 72 Senneby et al., 2016) and infective endocarditis (de Jong et al., 2010; Ebnother et al., 73 2002; Kristensen and Nielsen, 1995; Sunnerhagen et al., 2016), mostly in the elderly 74 male population. Since the introduction of MALDI-TOF MS as a diagnostic tool in 75 clinical microbiology laboratories, A. urinae is more frequently identified in clinical 76 samples, especially in urine cultures, and has attracted attention as an important 77 emerging pathogen (Rasmussen, 2016). A few studies have reported that A. urinae is 78 part of a urinary tract microbiome (Hilt et al., 2014; Pearce et al., 2014). The 79 mechanisms through which A. urinae establishes colonization and causes infection are 80 largely unknown. Only one study has previously targeted the question of A. urinae 81 virulence mechanisms (Shannon et al., 2010), demonstrating that it produces biofilm 82 and is able to induce human platelet activation and aggregation. These are properties 83 of potential importance in the process of establishing infections in the human host 84 (Donlan and Costerton, 2002). 85 At the molecular level, little is known regarding aerococcal virulence factors (Carkaci 86 et al., 2017). For other pathogenic Firmicutes, such as Streptococcus pyogenes and 87 Staphylococcus aureus, cell wall-attached proteins with an LPXTG-motif play key 88 roles for virulence. For instance, the M protein of S. pyogenes is an abundant surface 89 protein, which possesses a broad spectrum of functions, such as the inhibition of 90 phagocytosis and binding of several plasma proteins (Fischetti, 1989; Smeesters et al., 91 2010). The amino (NH₂)-terminal part of the protein displays hypervariability, 92 resulting in antigenic variation (Cunningham, 2000). The LPXTG-proteins share 93 common features, such as a signal sequence in the NH₂-terminal end and three 94 characteristics in the carboxy (COOH)-terminal end; a cell wall-sorting region 95 containing the LPXTG-motif, a hydrophobic membrane-spanning domain and a 96 positively charged tail positioned in the cytoplasm. The LPXTG-motif is recognized 97 by the membrane bound enzyme sortase and is, after cleavage between the threonine 98 and glycine residues, covalently attached to the cell wall (Schneewind and Missiakas,

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.
- 111
- 112

113 Material and Methods

114

115 Bacteria and culturing conditions

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 % CO₂.

123

124 Bacterial surface digestion and MS sample preparation

- 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 $\times 10^8$ 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 CaCl₂ 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 $2 \mu g$ sequencing grade trypsin (Promega) for 10 h at 37 °C and the sample acidified with 0.5 % Trifluoroacetic acid. The digested cells 137 138 (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 UltraMicroSpin Silica C18 300Å Columns, Harvard Apparatus) according to the 146 147 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%
- 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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166 **MS data analysis**

Data was processed with custom R-scripts using the tidyverse package collection 167 168 together with broom package for statistical functions. For the quantification of 169 proteins, all integrated peptide ion intensities extracted from the MS2 spectra was 170 summed up by protein and then divided by protein length. Peptides matching more 171 than one protein were not included in the analysis. Protein quantification data was 172 normalized based on the sample total intensity. Proteins from Col10a and AU3 were 173 classified into orthologous pairs using ProteinOrtho.pl v5.16b (Lechner et al., 2011) 174 and protein domains predicted using standalone InterProScan v5.11-50 (Jones et al., 175 2014).

176

177

178 Genome sequencing

179 Bacteria were pelleted through centrifugation and stored at -70° C until shipment on 180 dry ice to GATC Biotech (Konstanz, Germany). Library preparation was performed 181 using an optimized protocol and standard Illumina adapter sequences were used. 182 Sequencing was performed with Illumina HiSeq 2500 (Illumina, Inc., San Diego, US) 183 with paired-end reads, 2 x 125 bp. The genomes of AU3 (ASM164971v1, GenBank 184 assembly accession GCA_001649715.1) and ACS-120-V-Col10a (ASM19320v1, 185 GenBank assembly accession GCA_00193205.1) had been previously published. For 186 assembly of the genomes, SPAdes 3.9 with the careful-mode on, was used through 187 services on the Center for Genomic Epidemiology's website (Nurk et al., 2013). The 188 computations were performed on resources provided by the Swedish National 189 Infrastructure for Computing (SNIC) at Uppsala Multidisciplinary Center for 190 Advanced Computational Science (UPPMAX). Assembly quality was evaluated using 191 QUAST (Gurevich et al., 2013). Sample contamination was evaluated with Kraken 192 (cross-species) (Wood and Salzberg, 2014) and by mapping the raw sequence data to 193 a reference genome using BWA MEM (Li and Durbin, 2009) and evaluating the 194 presence of minority bases with samtools mpileup (within-species) (Li et al., 2009). In 195 cases with within-species contamination the contaminant gene were eliminated by 196 coverage analysis, i.e. genes with low coverage were excluded. The BLAST software 197 (Zhang et al., 2000) was used for searches in the genomes. Sequence alignment was 198 performed with Clustal Omega. The maximum likelihood method was used for 199 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
- 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

The MacVector software (v. 14.5.3) was used to analyse the bacterial genome for suitable primer sites for amplification of *asp* genes. Primers were ordered from Eurofins and can be found in Table 1. Chromosomal DNA from AU3 was extracted using the innuPRerp Bacterial DNA System (Analytik Jena AG). PCR products were inserted into the pGEX-6P-1 expression vector. One Shot TOP10 *E. coli* (Thermo Fisher Scientific) were transformed and used for plasmid amplification. Plasmids 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 and the supernatant discarded. Pellet was dissolved in BugBuster (Merck Millipore) 227 228 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.

235 Antiserum generation and ELISA

236 To generate antiserum against the affinity-purified recombinant proteins, the proteins 237 were sent to Biogenes GmbH for immunization. To assess binding of antibodies to the 238 surface proteins an ELISA was performed. Nunc MaxiSorp (ThermoFisher Scientific) 239 96-well plates were coated with either a suspension of bacteria (AU3, Col10a or A. 240 *viridans*), or a solution of Asp-proteins in coating buffer (1.69 g Na₂CO₃ and 2.94 g 241 NaHCO₃ in 1 L H₂O). As a control, bacteria with reduced surface protein expression 242 were generated by incubating a bacterial solution with trypsin (0.0004% w/v) at 37° C 243 for 20 minutes after which the reaction was stopped by adding trypsin inhibitor at a 244 molar ratio of 8:1. The trypsinated bacteria and control bacteria (where trypsin 245 inhibitor was added before the trypsin) were then pelleted by centrifugation, washed 246 (PBS), and finally resuspended in coating buffer. The bacteria were then heat-killed at 247 85°C for 30 minutes before adding the samples to the Nunc plate for coating over 248 night at 4°C. The plates were washed (PBST), rabbit serum in different dilutions 249 added, followed by further washes and addition of protein G coupled HRP (1:3000). 250 As an HRP substrate, ABTS dissolved 1:20 in a substrate solution (21 g citric acid 251 monohydrate and 17.8 g Na₂HPO₄ 2 H₂O in 1 L H₂O, pH 4.5) was used in combination with H₂O₂; incubated in room temperature for 15 minutes. The plates 252 were then analyzed with the iMark Microplate reader (Bio-Rad) at 415 nm. 253

254 255

256 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,
- 259 PANTHER, Gene3D, CDD, TIGRFAM, ProSitePatterns, SUPERFAMILY, Hamap,
- 260 ProSiteProfiles, Coils, SMART, MobiDBLite, PRINTS, PIRSF, SignalP, SFLD and
- 261 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 < 1×10^{-10}
- 265 ¹⁰, >50 % sequence identity and >75 alignment length.
- 266 The SNN was visualized using Cytoscape (v3.5.1) (Shannon et al., 2003), where each
- 267 node represents a protein/ORF and an edge or line between the nodes denotes a

similarity relationship between the proteins. The "organic" layout was used whereby
nodes are clustered more tightly if they are more highly interconnected and all edges
were bundled.

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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 we quantified a total of 1118 and 1168 proteins of which 765 and 682 proteins were 286 287 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 determined by InterProScan analysis (Fig. 2D). Of the proteins identified in the 298 299 surface fraction but not defined as surface associated, most were ribosomal proteins,

metabolic enzymes and transcriptional regulators and only ~9 % had a predicted
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). 323

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 (LASP). In all isolates it was constituted by, in a 5' - 3' order, a pyruvate carboxylase 335 336 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 LASP₁₋₄. In LASP₁ only the *asp1* was present, LASP₂ had 345 both asp1 and asp2, LASP3 had asp3 and asp6 whereas LASP4 contained asp4 and

346 *asp5*.

The 26 *A. urinae* genomes in the bio project PRJNA315093 also included *asp* genes
and these isolates had either LASP₁, LASP₂ or LASP₄.

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).

384

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 NH₂-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.
445	
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447	
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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

638 SWATH-MS. (b) Average numbers ± standard deviations of quantified proteins per

639 respective strain and fractions using three replicates per condition. (c, d) Number of

640 proteins common between fractions and per respective strain.





643 Figure 2. Proteomic analysis reveals *A. urine* surface associated proteins. (a)

Volcano plots showing Hochberg adjusted -log10 p-values correlated to log2 fold 644 645 changes in surface vs cell fractions of respective strain. Proteins in red represent the 646 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 647 hierarchical clustering of surface associated proteins (red dots in (a)) across individual 648 649 replicates and intensities normalized on total ion current (TIC) normalization (c). 650 Average abundance distribution of surface fraction proteins of respective strain with 651 the surface associated proteins marked with red. (d) The proportion of proteins with a 652 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 653 654 indicated in the plot. (e) Shows the ortholog pairs indicated as dotted lines (edges) 655 between the surface associated proteins (nodes named with GenBank protein 656 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 657 SP: signal peptide and LPXTG: Gram-positive LPXTG cell wall anchor. 658 659



662 **Figure 3.**

- 663 Mid-point rooted maximum likelihood tree visualizing the clustering of the *asp* genes
- 664 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.



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

671 Surface Proteins (LASP).

- 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.
- The distance between genes and length of genes are not according to scale.
- 677



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

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
- of rabbit serum are shown on the x axis and range from 1:400 to no serum at all. B)
- 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.
- 690



692 Figure 6. Sequence similarity network of A. urinae LPXTG proteins.

- A) All A. urinae ORFs (n=106,285) from 25 genomes were analyzed with
- 694 InterProScan to obtain predictions of functional domains. ORFs containing LPXTG
- 695 motifs (n=289) were selected with the criteria of InterPro signature accessions
- 696 PS50847 and/or TIGR01167 and/or PF00746. For sequence similarity analysis the

- 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
- they were detected in.
- 703

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

Table 1. Primers used for expression cloning

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

708 genes were designed with an RBS.

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 %

Table 2.

711 Identity scores (%) for Asp1-6 amino acid sequences.