A Metaproteomics Approach to Elucidate Host and Pathogen Protein Expression during Catheter-Associated Urinary Tract Infections (CAUTIs)

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Long-term catheterization inevitably leads to a catheter-associated bacteriuria caused by multispecies bacterial biofilms growing on and in the catheters. The overall goal of the presented study was (1) to unravel bacterial community structure and function of such a uropathogenic biofilm and (2) to elucidate the interplay between bacterial virulence and the human immune system within the urine. To this end, a metaproteomics approach combined with *in vitro* proteomics analyses was employed to investigate both, the pro- and eukaryotic protein inventory. Our proteome analyses demonstrated that the biofilm of the investigated catheter is dominated by three bacterial species, that is, *Pseudomonas aeruginosa, Morganella morganii*, and *Bacteroides* sp., and identified iron limitation as one of the major challenges in the bladder environment. *In vitro* proteome analysis of *P. aeruginosa* and *M. morganii* isolated from the biofilm revealed that these opportunistic pathogens are able to overcome iron restriction via the production of siderophores and high expression of corresponding receptors. Notably, a comparison of *in vivo* and *in vitro* protein profiles of *P. aeruginosa* and *M. morganii* also indicated that the bacteria employ different strategies to adapt to the urinary tract. Although *P. aeruginosa* seems to express secreted and surface-exposed proteases to escape the human innate immune system and metabolizes amino acids, *M. morganii* is able to take up sugars and to degrade urea. Most interestingly, a comparison of urine protein profiles of three long-term catheterized patients and three healthy control persons demonstrated the elevated level of proteins associated with neutrophils, macrophages, and the complement system in the patient's urine, which might point to a specific activation of the innate immune system in response to biofilm-associated urinary tract infections. We thus hypothesize that the often asymptomatic nature of catheter-associated urinary tract infections might be based on a fine-tuned balance between the expression of bacterial virulence factors and the human immune system. ***Molecular & Cellular Proteomics 14: 10.1074/mcp.M114.043463, 989–1008, 2015.***

Catheter-associated urinary tract infections (CAUTIs)[1] account for up to 40% of all nosocomial infections and are thus the most prevalent source of hospital-acquired infectious diseases (1, 2). CAUTIs are mostly "asymptomatic" and characterized by less than 10^5 colony-forming units per milliliter urine, which do not cause any signs of infection or symptoms. A symptomatic CAUTI, usually correlated to a number of colony-forming units (CFUs) exceeding the above mentioned threshold, is diagnosed when symptoms commonly associated with urinary tract infections (*e.g.* fever, dysuria, urgency, flank pain, or leukocytosis) occur (3). The risk that CAUTIs become symptomatic increases dramatically during catheter-

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Received, August 10, 2014 and in revised form, January 20, 2015
Published, MCP Papers in Press, February 11, 2015, DOI 10.1074/mcp.M114.043463

Author contributions: C.L. and K.R. designed research; C.L., M.B., and D.C. performed research; C.L., M.B., D.C., and R.J. analyzed data; C.L. and K.R. wrote the paper; A.O. and C.H. mass spectrometry; S.F. provided bioinformatic tools; J.B. provided visualization tool; R.N. provided samples; D.B. provided mass spectrometry know-how; D.H.P., M.J., and D.J. contributed to writing.

[1] The abbreviations used are: AUM, artificial urine medium; BLAST, basic local alignment search tool; UTI, urinary tract infection; CAUTI, catheter-associated urinary tract infection; CLED, cystine lactose electrolyte deficient-agar; CV, coefficient of variation; COG, clusters of orthologous groups; dNTP, deoxynucleoside triphosphates; FDR, false discovery rate; INF, interferon; NGAL, neutrophil gelatinase associated lipocalin; NSAF, normalized spectral abundance factor; NAUC, normalized area under the curve; RPSBLAST, reversed position specific BLAST; PTS, phosphotransferase systems; PRIDE, Proteomics Identifications database; RDP, ribosomal database project; ROS, reactive oxygen species; RT, room temperature; TLR, toll-like receptor.

ization because of the formation of bacterial biofilms on catheter surfaces (4). This explains why the urinary tract of long-term hospitalized patients represents the part of the human body with the highest risk for acquiring sepsis caused by Gram-negative bacteria (5, 6). Long-term catheterization is commonly applied to elderly or disabled persons often for many years (3). Considering the actual demographic development in industrialized nations, problems caused by long-term urinary tract catheterization will certainly increase.

Biofilm formation of bacteria on medical devices, including implants, central venous catheters, and urinary tract catheters has become a worldwide and severe problem (7–9). Surface-associated bacteria, which are embedded in a complex matrix of extracellular polymeric substances (EPS), are highly resistant to antibiotics as well as to the human immune system and therefore hard to eradicate (10–12). Biofilms growing on urinary tract catheters have been demonstrated to often consist of multiple (two to six) species (5). Most frequently *Pseudomonas aeruginosa, Proteus mirabilis*, *Providencia stuartii*, *Morganella morganii*, *Enterococcus faecalis*, and *Klebsiella pneumoniae* have been identified in biofilms of long-term catheterized patients (13, 14).

Until now, the global adaptation mechanisms of uropathogens to their respective habitats, including *P. mirabilis*, *E. faecalis*, and *Escherichia coli*, have been preferentially analyzed by *ex vivo* cultivation in human urine (15–17), by employing murine models (18, 19) or in the human urinary tract (20). The mentioned studies identified the lack of freely available trace metals, especially iron, as a major limiting factor in human (and murine) urine during urinary tract infections (UTIs). For uropathogenic *P. aeruginosa* a strong iron limitation response was observed when cultured as colony biofilm for 6 days on artificial urine medium (AUM) agar (21). Moreover, fimbrial genes, like *mrp* and *fim*, were found highly expressed in uropathogenic *P. mirabilis* and *E. coli* (18), highlighting the importance of bacterial adherence during CAUTIs. Notably, mechanisms required for immune evasion including changes in surface structures (19) and secreted proteases cleaving proteins of the host immune system (20) have been identified in uropathogenic *E. coli* strains. The facultative anaerobic Gram-negative *M. morganii* is a typical secondary invader during multispecies infections. It can be isolated from infected wounds, septicemia, and CAUTIs (22–24). This natural commensal of the human intestinal tract is often regarded as a harmless opportunistic pathogen (25). However, some strains are associated with large nosocomial outbreaks (26). During infection, urease (27) and beta-lactamase (28) are considered to be involved in the maintenance of bacterial fitness in *M. morganii*. Until today, transcriptome or proteome studies targeting global gene expression of *M. morganii* are lacking and its adaptation strategies to the urinary tract environment remain widely unexplored.

Host response to UTIs is achieved by innate and adaptive immunity (reviewed in (29, 30)). Initiated by pathogen recognition via Toll-like receptors, a complex mixture of cytokines, antimicrobial peptides, and proteins is released by infiltrating neutrophils and the urothelium (31–33). Intensely studied protein-effectors are for example, defensins (34), cathelicidin (35), lactoferrin (36), and the tamm-horsfall protein (37). Moreover, the complement system is known to play an important role during urinary tract innate immune response (38) by recognizing bacterial surface structures, followed by an activation of complement cascades. During this process, the central complement compound C3 is activated by C3 convertases, resulting in activated C3a, a chemoattractant for neutrophils. Furthermore, activated C3b covers the pathogen, which subsequently becomes phagocytosed. Finally, the membrane attack complex (C5–C9) forms a pore in the bacterial membrane resulting in cell-lysis (38, 39). The role of the adaptive immunity during UTIs has been discussed controversially. However, it is well-accepted that the humoral (40) and the cellular (41) immunity are involved in the defense against uropathogens. In contrast to the multitude of studies dealing with the human immune response during planktonic UTIs, the impact of catheter-associated UTIs on the innate and adaptive human immunity has not yet been investigated in detail.

The presented study therefore focuses on (1) the comprehensive analysis of structure and functionality of a multispecies catheter biofilm dominated by *P. aeruginosa* and *M. morganii* and (2) the elucidation of the molecular basis of the complex interplay between bacterial virulence of *P. aeruginosa, M. morganii*, and the human immune system in the urinary tract environment. For this purpose, a semiquantitative gel-free metaproteomics approach was employed to obtain a global view on the corresponding *in vivo* protein profiles. Additionally, *in vitro* proteome analyses of *P. aeruginosa* and *M. morganii* grown in artificial urine medium were performed. To our knowledge, here we present the first comprehensive metaproteomics investigation of host–pathogen interactions induced by catheter-associated microbial biofilms. The knowledge gained in our study strongly contributes to a better understanding of *P. aeruginosa* and *M. morganii* urinary tract niche adaptation, bacteria–bacteria and bacteria–host interactions and the corresponding human immune response and will thus also promote the development of novel strategies to fight CAUTIs.

EXPERIMENTAL PROCEDURES

Sample Collection—Suprapubic urinary catheters and urinary drainage bags were collected by the attending urologist after 28 days of use from long-term patients with asymptomatic UTI living in a German nursing home. All samples were completely anonymized. Sampling and further processing have been approved by the ethical review committee of the University Medicine in Greifswald (Internal Registration Number BB 035/13). Immediately after collection, catheters and urine were cooled on dry ice and stored for up to two hours at 4 °C until sample preparation. Catheter tips were cut with a sterile scalpel in 1 cm pieces, which were further processed (see below).

Protein Extraction from Catheter Biofilm—Small pieces of the catheter tip (two times 1 cm) were transferred to 1 ml of urea-containing

buffer (11 M urea, 3 M thiourea, 70 mM dithiothreitol (DTT), 4% (w/v) 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS)). The sample was incubated on ice for 5 min, mixed vigorously for 30 s and then incubated on ice for 10 min; those steps were repeated twice. Sonication was performed for 30 s and repeated five times to dissolve and lyse the cells using a Sonicator (Type UW 2070, Bandelin Electronics, Berlin, Germany). Subsequently, the catheter pieces were removed and the remaining cell lysate was mixed with ice-cold acetone (1:7, v/v). After centrifugation (14,000 × *g*, 12 °C, 40 min) the resulting protein pellet was dried and solubilized in urea-containing buffer supplemented with 1% SDS. Total protein concentration was determined according to Bradford (42) employing the Coomassie PlusTM Protein Assay (Thermo Fisher Scientific Inc.). The absorbance was measured at 595 nm. The protein concentration was calculated using a bovine serum albumin (BSA) standard (Thermo Fisher Scientific Inc.). Metaproteomics analysis was performed in three technical replicates, that is, the protein extract from the catheter biofilm was divided into three subsamples.

Protein Extraction from Urine—Urine samples (200 ml) from three urinary drainage bags from catheterized patients or collected from three healthy control persons were centrifuged for 20 min at 4 °C at 6000 × *g* and the resulting supernatants were mixed with 20% (w/v) trichloroacetic acid and kept overnight at 4 °C. Subsequently, samples were centrifuged for 40 min at 14,000 × *g* at 4 °C, the resulting protein pellets were washed twice with 70% EtOH and thereafter solubilized in a urea-containing buffer with 1% SDS. Protein concentrations were determined as described above. Metaproteomics analyses were performed in three technical replicates, that is, the protein extracts from urine were divided into three subsamples.

Protein Extraction from P. aeruginosa and M. morganii Planktonic Cultures and In Vitro Biofilms—All *in vitro* proteomics analyses were performed with protein extracts of three biological replicates. Planktonic cultures of the *P. aeruginosa* and *M. morganii* isolates (see below) were grown in artificial urine medium (AUM) based on the recipe of Brooks and Keevil (43) with slight modifications (uric acid 0.333 g/l, Na_2SO_4 1.4 g/l). To this end, 100 ml media were inoculated to an $OD_{600\ nm}$ of 0.01 with freshly grown AUM overnight cultures and subsequently incubated at 180 rpm at 37 °C. Cells were harvested in the early stationary growth phase by centrifugation at 6,000 × *g* for 10 min at 4 °C. Proteins were extracted from the resulting cell pellet as described above. The corresponding supernatants were filtered through a 0.2 μm pore filter and concentrated by a vivaspin 20 concentrator (3 kDa MWCO, GE Healthcare) followed by protein precipitation with ice-cold acetone (1:7, v/v) for the investigation of the secretome.

Biofilms were grown on silicone catheters, which were placed into planktonic AUM cultures, inoculated to an $OD_{600\ nm}$ of 0.01 and cultivated for 24 h at 50 rpm at 37 °C. Before harvesting the biofilm, the catheter was washed three times with ¼ ringer solution. Protein extraction from biofilm-grown *P. aeruginosa* and *M. morganii* was performed as described above.

One-Dimensional SDS-PAGE and Tryptic Digestion—The extracted proteins (30–40 μg of protein per biological or technical replicate) were separated on a 12% SDS-polyacrylamide gel (44) and stained overnight with Colloidal Coomassie Brilliant Blue G-250 as described previously (45). Gel blocks were excised from the gel and proteins were digested with trypsin as follows: the excised gel pieces were destained using 50% (v/v) methanol in 100 mM NH_4HCO_3. Proteins were reduced in 50 mM NH_4HCO_3 by using 10 mM DTT for 30 min at 60 °C and carbamidomethylated/alkylated in 50 mM NH_4HCO_3 containing 50 mM iodoacetamide for 60 min in the dark at RT. Subsequently, gel pieces were dehydrated using 100% ACN and allowed to dry. Modified trypsin (sequencing grade, Promega, Fitchburg, WI) was added to a final ratio of 1:10 (trypsin/sample) in 50 mM Tris/HCl, pH 7.5, and the sample incubated at 37 °C overnight. Peptides were iteratively extracted from the gel by a six-step procedure, using ACN, 1% (v/v) formic acid in H_2O, acetonitrile, 10% (v/v) formic acid and two times acetonitrile. Peptide-containing supernatants were pooled and completely dried using a Speedvac concentrator (Eppendorf AG, Hamburg, Germany). Samples were subsequently resolved in buffer A (5% (v/v) ACN, 0.1% (v/v) formic acid) and desalted using ZipTips (C18, Merck Millipore, Billerica, MA). Finally, peptides were again vacuum-dried and stored at −20 °C.

Mass Spectrometric Analyses—The tryptic digest was applied to a reversed phase (RP) chromatographic system (Easy-nLC II operated in "one column setup" (Thermo Fisher Scientific, Waltham, MA)) equipped with a self-packed RP C18 separation column (100-μm i.d. × 200 mm length) as published by Teeling *et al.*(46). Peptides were loaded and desalted on the column followed by elution from the column by a binary gradient of buffers A (0.1% (v/v) acetic acid) and B (99.9% (v/v) ACN, 0.1% (v/v) acetic acid) over a time of 100 min at 300 nl/min. For the analysis of peptide mixtures derived from gel blocks of one gel lane (corresponding to one sample) injection volumes were kept constant. The chromatographic system was coupled on-line to an LTQ-Orbitrap-Velos mass spectrometer (Thermo Fisher Scientific, Bremen, Germany) equipped with a nanoelectrospray ion source. Samples were measured in data-dependent manner with repeated cycles of overview scans in the Orbitrap (r = 30,000) with the lock-mass option enabled, followed by MS/MS acquisition of the 20 most intensive precursor ions in the linear ion trap. Dynamic exclusion was enabled.

Database Assembly and Data Analysis—Phylogenetic information obtained by 16S rRNA gene sequencing was used for the construction of a sample-specific virtual metagenome database designated as "catheterDB" as described by Okuda *et al.* (47). This database contained 342.642 nonredundant protein entries, that is, all available proteins from *Homo sapiens, P. aeruginosa, M. morganii*, and *Bacteroides* sp. extracted from the NCBInr protein database (version 18.01.13). A second database used for protein assignments from urine samples was designated as "urineDB" and contained 46,415 entries, that is, reference proteomes of *H. sapiens, P. aeruginosa, M. morganii, E. faecalis, E. coli, P. mirabilis*, and *Bacteroides fragilis* (Uniprot, version 17.10.14). Because we were in particular interested in human proteins identified in the urine, the additional microbial entries (compared with "catheterDB") were thought to minimize false positive assignments to human proteins.

Proteome discoverer™ software (version 1.4, Thermo Fisher Scientific Inc., Waltham, MA) was used to validate MS/MS-based peptide and protein identifications. Sequest HT database searches were performed with raw files as MudPit experiments. The following search parameters were used: enzyme type, trypsin (KR); peptide tolerance, 10 ppm; tolerance for fragment ions, 0.6 Da; b- and y-ion series; variable modification, methionine (15.99 Da) and carbamidomethylation (57 Da); a maximum of three modifications per peptide was allowed. The percolator node was used to filter peptide identifications based on a fixed false discovery rate (FDR) of 1% depending on a target-decoy approach. Peptide identifications were accepted when they could be established on Sequest: deltaCn scores of greater than 0.05 and XCorr scores of greater 2.2, 3.3, and 3.75 (XCorr filter) for doubly, triply, and quadruply charged peptides. Protein identifications were based on at least two identified distinct peptides per protein. Moreover, only proteins were taken into account that were at least identified in two out of three technical or biological replicates (replicate filter). After application of the filters (XCorr and replicate filter) the initial FDR of 1% was reduced to 0.0001%. Proteins that contained identical peptides and could not be differentiated based on MS/MS analysis alone were grouped together.

Quantification was based on integration of the area under the curve (AUC) from MS1 spectrum peaks performed by the "Precursor Ions Area Detector" node integrated in the proteome discoverer software. Proteome discoverer identification and quantification results (*.msf) were imported into Scaffold (version 4.4, Proteome Software Inc., Portland, OR) and protein quantities were determined as the average of the three highest peptide intensity values for a protein (48). If only two peptides could be quantified, the average of the two highest peptide intensity values for a protein was calculated. Identical distinct peptides identified and quantified from different gel blocks or retention times within a sample were considered. Normalization of these data was achieved by using the following equation:

$$(\mathrm{NAUC})_k = \frac{\mathrm{AUC}_k}{\sum_{i=1}^{N} \mathrm{AUC}_i}$$

NAUC is the normalized intensity value (AUC_k) for a protein divided by the sum of all intensity values per sample (AUC_i). AUC_k is the average of the three highest peptide intensity values for a protein.

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium (49) via the PRIDE partner repository with the data set identifier PXD000164. All identified proteins and their respective NAUCs are listed in the supplementary Files S2, S4, S5, and S6. A quantitative comparison of the *in vivo* and the *in vitro* proteome data of *P. aeruginosa* and *M. morganii* proteins was achieved by the separate analysis of proteins identified under both (*in vivo* and *in vitro*) conditions. Quantitative protein data were normalized as described above and used as the basis for fold-change calculation. Proteins detected under *in vivo* conditions were only considered as "highly expressed" if their fold-change in abundance compared with *in vitro* conditions was at least twofold or if they were uniquely identified *in vivo*. Identification of statistical differences in relative protein amounts was performed using *t* test ($p < 0.05$) including adjusted Bonferroni correction and all possible permutations.

Data Processing and Visualization—For metaproteomics data analyses we used the Prophane bioinformatics pipeline (www.prophane.de, (50)). Briefly, corresponding search results were merged using the Scaffold software version 3.6.1 (Proteome Software Inc., Portland, OR). The exported protein reports were submitted to Prophane to screen peptide-sharing proteins for taxonomic or functional similarities. Protein matches pointing to reverse sequences or contaminations were excluded from analyses. Peptide-sharing proteins missing unique peptide identifications were grouped together. The common taxonomic origin of those groups was evaluated considering different taxonomic levels (superkingdom, order, class, family, genus, and species). Groups without common taxonomic origin of all protein members were called "heterogeneous" regarding their taxonomy. Protein functions were transferred by RPSBLAST (51) and HMMER3 (52) alignments, respectively. Using the RPSBLAST algorithm prokaryotic (eukaryotic) protein sequences were aligned *versus* the COG (KOG) database collection (release 22.03.2003) (53). Functional annotation of the first hit (e-value $\leq$ 1E-20) was considered for each protein. Using the HMMER3 algorithm protein sequences were aligned *versus* the TIGRFAMs 15.0 (54) and PFAMs 27.0 (55) collections. Functional annotation of all hits (e-value $\leq$ 1E-5) was considered for each protein. Functional annotation was assigned to each group if respective protein members show the same functional description (proteins without prediction were not considered). If functional prediction varies between protein members group function was named "heterogeneous." In case of multiple TIGRFAMs or PFAMs hits shared by protein group members the hit with the lowest e-value over all proteins were selected. Details regarding the taxonomic and functional assignment of proteins are provided in Suppl. File 1. After computational assignments of protein functions, assignments were carefully checked, completed, and manually curated.

Protein abundances in *in vivo versus in vitro* biofilms were illustrated using treemaps (56). Genes/proteins were functionally classified according TIGRFAMs and visualized in multiple level hierarchically organized regions (*e.g.* energy metabolism) subdivided into subregions (*e.g.* TCA).

DNA Extraction for High-Throughput Sequencing—Total DNA was extracted from the catheter biofilm using the FastDNA Spin Kit for Soil (MP Biomedicals, Santa Ana, CA) (57). Cell disruption was performed employing the Fast Prep® instrument for 40 s with an intensity of 5.5. DNA quality and quantity were determined using a NanoDrop 200 spectrophotometer (Thermo Scientific, Waltham, MA). The V1–2 region of the 16S rRNA gene was amplified using primers based on the 27F and 338R primers as previously described (58) and paired end sequenced on a GAIIx Genome Analyzer (Illumina, Inc., San Diego, CA). Image analysis and base calling were accomplished using the Illumina Pipeline (version 1.7). Sequences were quality filtered, trimmed, collapsed into representative reads and clustered as previously described (58). Phylogenetic assignments were performed manually based on the Ribosomal Database Project (RDP) (http://rdp.cme.msu.edu/) database.

Isolation of Biofilm-Associated Uropathogens—For the isolation of biofilm-inhabiting uropathogens, 0.5 cm of the catheter tips were transferred in 1 ml of ¼ ringer solution, mixed, and incubated for 5 min at RT. After repeating this procedure twice, the resulting bacterial suspension was mixed with 50% (v/v) glycerol (98%) and stored at −80 °C. Dilutions of the glycerol-suspensions were plated on cystine-lactose-electrolyte-deficient (CLED)-agar (Roth, Hamburg, Germany). Single colonies were picked and streaked on the same agar plates. After repeating this procedure four times, cell morphology of the pure cultures was inspected by microscopy and isolates were finally identified by colony PCR as described below. The isolated strains were stored at −80 °C as glycerol stocks for further analysis.

Identification of Isolates by 16S rRNA Gene Sequencing—Genomic DNA of the isolates was extracted using the FastDNA Spin Kit for soil (MP Biomedicals, Santa Ana, CA) following the manufacturer's instructions. 16S rRNA genes were amplified by PCR using the primers Com1f and 1492R (Com1f: CAG CCG CGG TAA TAC and 1492R: AGA AAG GAG GTG ATC CAG CC) (59), which were synthesized by Metabion, Martinsried, Germany. Reaction mixtures contained 1x ThermoPol buffer, deoxynucleoside triphosphates (20 μM of each dNTP), 0.5 μM of each primer, 2.5 U of DNA polymerase (ThermoTaq Polymerase, NEB) and 100 ng genomic DNA. Cycle conditions for the reactions were: initial denaturation at 95 °C (2 min), 28 cycles of 95 °C (20 s), 52 °C (2 min), and 72 °C (2 min) with a final extension at 72 °C (6 min). PCR products were purified by the QIAquick PCR Purification Kit (Qiagen). The DNA sequence of the PCR product was determined using the BigDye® Terminator v1.1 Cycle Sequencing Kit and the 310 genetic analyzer (Applied Biosystems, Waltham, MA) according to the manufacturer's instructions. For sequencing primers 27f and 1492r were used. Identification of the isolates was performed by a BLAST search of the determined 16S rRNA gene fragment sequence against the GenBank database (http://blast.ncbi.nlm.nih.gov/Blast.cgi) (60).

Phenotypic Characterization of Isolates—Biofilm forming capacity of the isolates was tested by a microtiter plate assay according to O'Toole and Kolter (61). Briefly, the bacterial cells were grown in artificial urine medium (AUM) in 96-well microtiter plates (Nunclon, PS) for 24 h at 37 °C. Bacterial growth was monitored by measuring the optical density of the planktonic cultures at 550 nm (Synergy Mx, BioTek, Bad Friedrichshall, Germany). After removing planktonic cells, the remaining biofilms were washed three times with ddH_2O, incubated for 30 min with 0.1% crystal violet and washed again with ddH_2O. Dried biofilms were dissolved in 120 μl DMSO and incubated

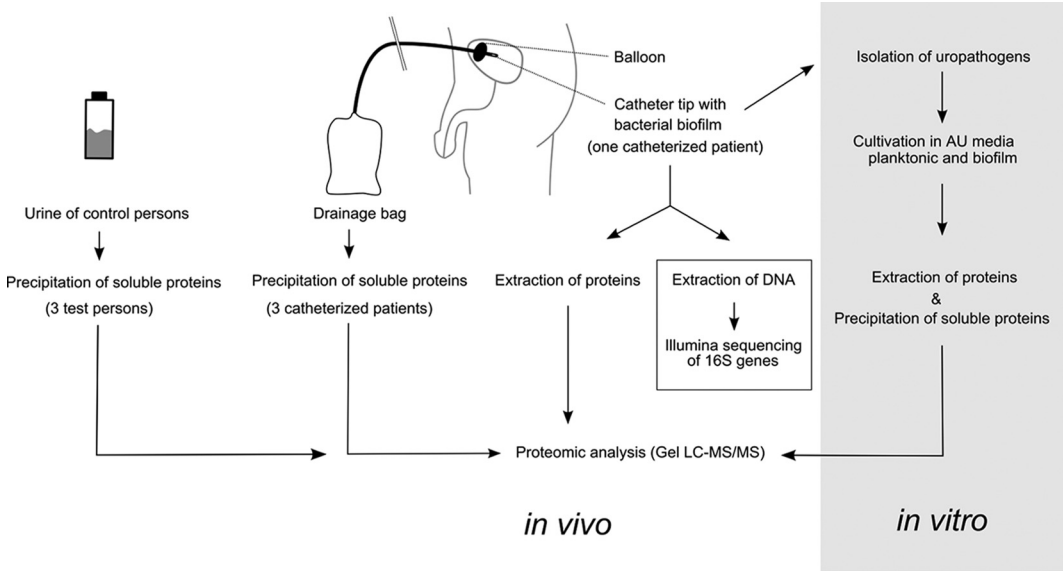

FIG. 1. **Schematic overview of the experimental setup.** A representative multispecies biofilm derived from a long-term catheterized patient was processed together with the corresponding urine for culture-independent metaproteomics analyses. Illumina 16S rDNA sequencing was employed to confirm the phylogenetic composition of the biofilm. Moreover, abundant uropathogens were isolated from the biofilms. The isolates, identified as *P. aeruginosa* and *M. morganii*, were further analyzed by *in vitro* proteome analyses of the extracellular proteins and biofilm-associated cells grown in artificial urine medium (AUM).

for 20 min at RT. Finally, the absorbance at 570 nm was measured and the biofilm-index was calculated (62).

Antibiotic susceptibility of the isolates was tested by a standardized single disk approach (63) with the following antibiotics (μg/disk): nitrofurantoin 100 μg, gentamycin 10 μg, cefalexin 30 μg, sulfamethoxazole/trimethoprim 23.75 μg/1.25 μg, penicillin G 10 μg, trimethoprim 5 μg, tetracycline 30 μg, ciprofloxacin 5 μg, levofloxacin 5 μg, amoxicillin 25 μg, and ampicillin 10 μg (Oxoid Limited, Hampshire, United Kingdom).

Urease production of the isolates was tested in 5 ml urea-containing liquid media (0.1 g/l yeast extract; 9.1 g/l potassium phosphate, monobasic; 9.1 g/l potassium phosphate, dibasic; 20 g/l urea; 0.01 g phenol red), which were inoculated with the test strains (1% (v/v) overnight cultures in LB broth) and incubated at 37 °C for 24 and 48 h at 180 rpm. *P. mirabilis* HI432 was used as positive control (64). After 24 (48) h, the pH-dependent color change (light yellow to dark red) was visually determined as an indicator for urease activity.

Protease activity in cell-free supernatants of the isolates was determined on azocasein (65). Briefly, overnight cultures were centrifuged and the supernatants filtered under sterile conditions. One hundred microliters supernatant and 250 μl substrate solution (2% azocasein, 50 mM Tris/HCl, pH 7.5) were incubated for 3 h at 40 °C. Undigested azocasein was precipitated with 10% trichloroacetic acid and protease activity was quantified by measuring the absorption of the remaining dye at 410 nm. As a positive control *P. aeruginosa* PAO1 (66) was used.

RESULTS

CAUTIs can cause serious problems in long-term patients because of the polymicrobial nature of catheter-associated biofilms and the high bacterial load of up to 5×10^9 cells/cm biofilm. Metaproteomics has been increasingly recognized as a powerful tool to investigate the physiology of complex microbial communities in their natural environment (50, 67–70). Therefore, we have decided to employ such a *state-of-the-art* approach to elucidate the molecular mechanisms underlying pathogen host adaptation and host immune response to bacterial invaders during a multispecies CAUTI of one long-term catheterized patient. An overview on the experimental setup is shown in Fig. 1. A specific patient was selected as his catheter biofilm was found to consist of two major components, *P. aeruginosa* and *M. morganii,* the latter of which can still be considered as poorly characterized; moreover, nothing is known on the interactions between these two opportunistic pathogens during CAUTI. For a further detailed view of bacterial response to the urinary tract the two major bacterial constituents of the biofilm were cultivated in artificial urine medium and their protein profiles were analyzed by *in vitro* proteome analyses (Fig. 1, right panel). Finally, to determine to what extent the biofilm *versus* immune system interaction is reflected by the urine composition of CAUTI patients, the urinary metaproteome of three healthy individuals was compared with that of three CAUTI patients (Fig. 1, left panel).

Metaproteomics Analyses of a Multispecies Catheter-Biofilm and Associated Urine—Spectra recorded for the trypsin-digested proteins extracted from the multispecies biofilm and urine samples were assigned to a total of 1064 and 896 proteins, respectively (Fig. 2, supplemental File S2). Although in the biofilm the number of bacterial proteins (612) clearly exceeded the number of human proteins (452), much fewer microbial proteins could be detected in the urine (509 human proteins *versus* 387 bacterial proteins). However, relative protein quantification indicates a much higher abundance of human proteins on the catheter biofilm (92%) and in the urine (99%) compared with bacterial proteins. The dominance of human

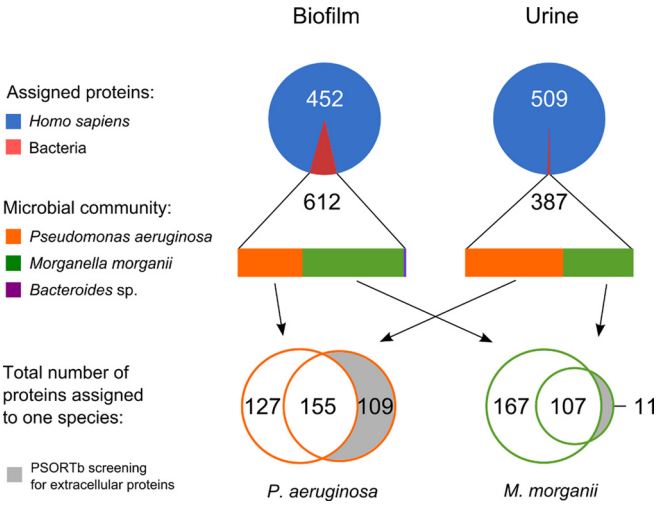

FIG. 2. **Taxonomic distribution of assigned proteins resulting from the metaproteomic analysis of the catheter-associated biofilm and urine of a long-term catheterized patient.** Numbers indicate the assigned proteins per sample (average of three technical replicates), the chart area displays the quantitative distribution (NAUC values) of the assigned proteins to the kingdom or the species level.

proteins is also reflected by a threefold higher median of the NAUC values from human proteins compared with bacterial proteins (supplemental File S3, supplemental Fig. S1).

A Polymicrobial Community Forms the Biofilm—The quantification of the metaproteomics data based on NAUC values revealed that two opportunistic pathogens, that is, *M. morganii* (57%) and *P. aeruginosa* (40%), dominated the biofilm, whereas the obligate anaerobe *Bacteroides* sp. represented only a minor fraction (2.5%) of the biofilm colonizing community (Fig. 2). The observed coefficient of variation (CV) between replicates with regard to the bacterial composition in the biofilm or urine was about 0.1. Relative protein abundance between replicates exhibited a CV of ~0.25. Moreover, 75% of all proteins identified by two distinct peptides were identified in two out of three replicates. The taxonomic composition of the catheter biofilm was confirmed by a 16S rDNA sequencing approach using DNA directly extracted from the catheter biofilm (Fig. 1). Clearly, *M. morganii* and *P. aeruginosa* were confirmed as the predominant biofilm bacterial inhabitants (supplemental File S3, supplemental Tables S1 and S2). The proteins from the urinary sample were assigned to the same taxa found in the biofilm, but the NAUC-based quantification resulted in a predominant occurrence of *P. aeruginosa* proteins (57%), followed by *M. morganii* (41,5%) proteins. Remarkably, only one protein expressed by *Bacteroides* sp. was identified in the urinary proteome (Fig. 2). Notably, a culture-dependent approach gave rise to the isolation and identification of the two dominant biofilm inhabitants *P. aeruginosa* and *M. morganii* (supplemental File S3, supplemental Table S3), which were used for further investigations (Fig. 1, right panel).

Adaptation of P. aeruginosa to the Urinary Tract and the Host Defense—To elucidate the molecular adaptation strategies of *P. aeruginosa* to the infected bladder environment, its catheter-derived proteome profile (282 identified proteins) was compared with the *in vitro* biofilm proteome profile (1332 identified proteins) obtained for the isolated strain grown as biofilm in AUM on a urinary tract silicone catheter (Figs. 1 and 3, supplemental File S4). Interestingly, 28 proteins of *P. aeruginosa* were exclusively detected under *in vivo* conditions on the catheter biofilm (Table I). Moreover, based on our fold-change calculation (described in detail in the experimental procedures), 27 proteins were found to be more abundant in the *in vivo* biofilms compared with the *in vitro* biofilm cultures (Table I, Fig. 3). In the following paragraphs the 55 proteins, which were highly abundant *in vivo*, are described.

Thirteen of these proteins are involved in iron acquisition. Their expression is mostly dependent on the iron-binding Fur regulator (71). Levels of proteins of the pyochelin and the pyoverdine siderophore systems were elevated under *in vivo* conditions, pyoverdine synthase PvdAFH and the corresponding receptors FpvAB were exclusively identified in the catheter biofilms, additionally the pyochelin synthase (PchE) was found to be more abundant *in vivo*. Furthermore, proteins involved in (1) heme/hemin degradation and uptake (HemO, HasR, PhuST), (2) transport of Fe (III) citrate, and (3) uptake of heterologous siderophores (PirA and PfeA receptors) were found to be more abundant under *in vivo* conditions (Table I). Moreover, proteins involved in various functions such as motility and biofilm formation were strongly expressed in the catheter biofilms (Table I): (1) proteins important for biofilm formation/development (*i.e.* the fimbrial proteins PilF, J, N, Q, U, X, and Y1) (68), (2) proteins related to antibiotic resistance and the general stress response (*i.e.* AmpC, Dps, IdpA), and (3) an insulin-cleaving membrane proteinase (IcmP), which has been shown to degrade plasminogen activator, complement convertase and kallikrein (72) and a protease involved in pathogenicity (*i.e.* MucD).

Phenotypic assays were performed to validate biofilm-forming capacity of the catheter isolates. Interestingly, *P. aeruginosa* exhibited a significantly higher (about twofold) biofilm formation than *M. morganii* in AUM (Table II). Both isolated strains were found resistant to all tested antibiotics. Especially, ciprofloxacin and levofloxacin inhibiting the growth of *P. aeruginosa* PAO1 and *P. mirabilis* HI432 were found inactive with the CAUTI isolates (Table II).

P. aeruginosa proteins found in the urinary proteome but not on the catheter biofilms might be secreted virulence factors, which are involved in host-pathogen interactions (Fig. 2). Only those proteins were considered, which harbored a signal peptide and were also identified in the *in vitro* secretome (supplemental Table S3). This protein group included (Table III): the PvdS regulated protease PrpL, elastase LasB and alkaline protease AprA, the hemolysin-coregulated protein Hcp, the lipase LipA, the chitin binding protein CbpD, a pu-

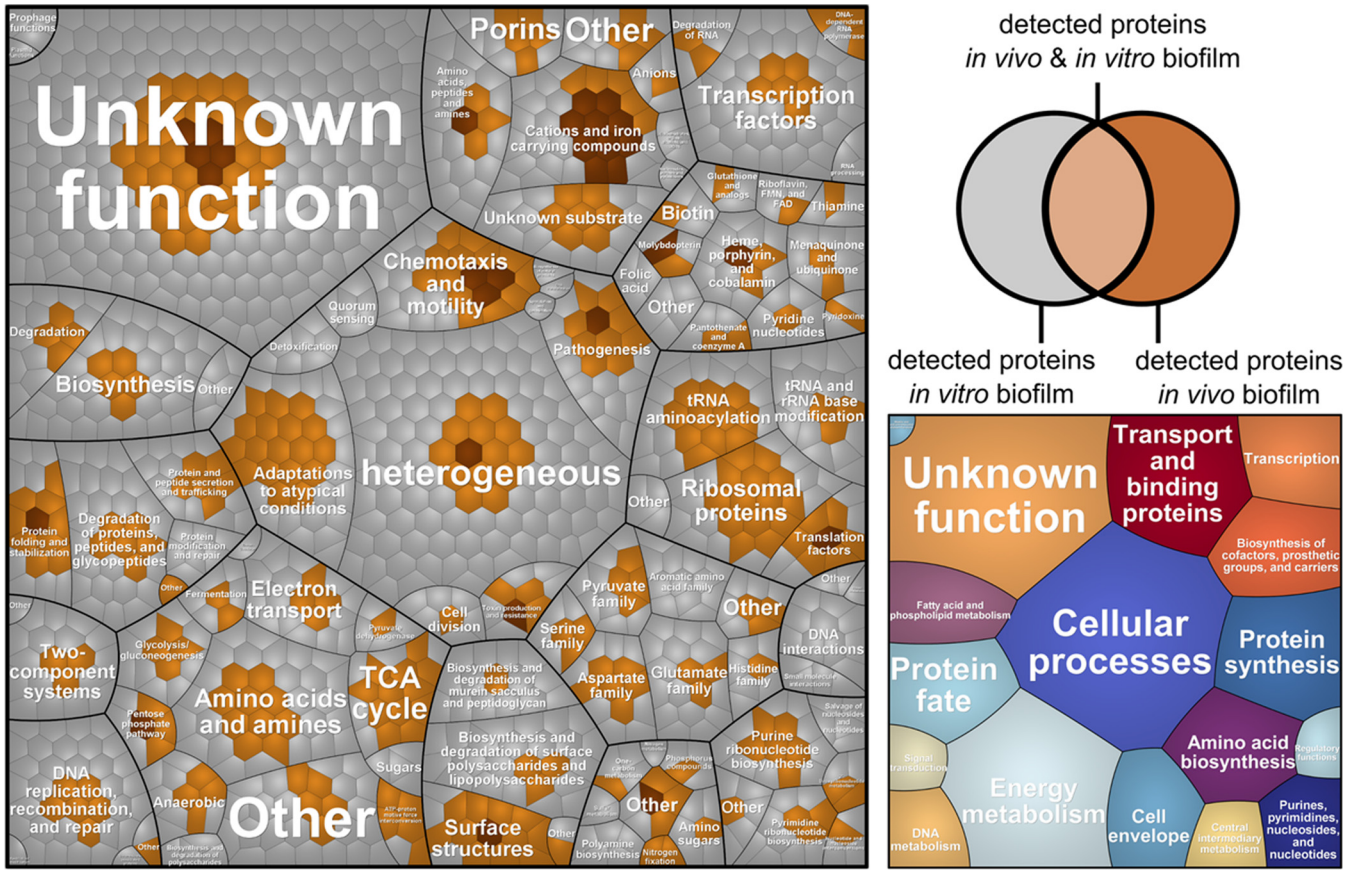

FIG. 3. **Voronoi treemaps (109) comparing protein expression profiles of *P. aeruginosa* grown in catheter biofilms (*in vivo*) or in a catheter model system in AUM (*in vitro*).** Gene functions are based on TIGRFAMS, major TIGRFAMS roles are displayed in the right map and the minor TIGRFAMS roles the left map. Gray proteins were exclusively identified *in vitro*, light brown proteins were identified *in vivo* and *in vitro* and dark brown proteins were exclusively identified *in vivo*.

tative aminopeptidase, and a colicin M-like bacteriocin. These findings were supported by an *in vitro* protease assay, by which a high protease activity was found in cell-free supernatants of the recovered *P. aeruginosa* strain (Table II).

Adaptation of M. morganii to the Urinary Tract and the Host Defense—As described above for *P. aeruginosa*, protein expression profiles of *M. morganii* grown either in mixed catheter-associated biofilms (274 proteins) or monospecies *in vitro* cultured biofilms (1165 proteins) were compared (Figs. 1 and 4, supplemental File S5). Interestingly, 48 proteins were either uniquely detected or highly abundant in the *in vivo* proteome based on the abundance ranking (Table IV, Fig. 4). A major part of the proteins found to be highly expressed *in vivo* is involved in iron and manganese uptake, that is, a ferrous iron transport system (EfeB, EfeO), a manganese uptake system (SitA, SitB), and different siderophore receptors (*i.e.* two TonB-dependent receptors, ferric iron ABC transporter, iron ABC transporter substrate-binding protein, and periplasmic protein p19 involved in high-affinity Fe (II) transport). Moreover, proteins participating in carbohydrate uptake and metabolism appeared to be strongly expressed in the catheter-associated biofilms when compared with the *in vitro* biofilms. Among these proteins were components of phosphotransferase systems (PTS) (*i.e. N*-acetylgalactosamine-specific IIB component, mannose-specific IID component) and various enzymes involved in glycolysis, pentose phosphate pathway, and sugar conversion (*i.e.* D-mannonate oxidoreductase, mannonate dehydratase, 2-dehydro-3-deoxygluconate kinase, enolase, uronate isomerase, 6-phosphofructokinase, NAD-dependent glyceraldehyde-3-phosphate dehydrogenase, galactosamine-6-phosphate isomerase, and gluconokinase). Additionally a β-lactamase was found to be abundant *in vivo*, expression of which was confirmed by our antibiotic susceptibility test revealing that the isolate is multidrug-resistant (Table II). Moreover, proteins involved in general and oxidative stress response were found to be unique or more abundant *in vivo*, that is, a molecular chaperone, a tellurium resistance protein, an iron–sulfur cluster assembly protein, and proteins involved in transport and assembly of lipopolysaccharides.

Notably, urease (UreG, α- and β-unit), an important fitness factor in the urinary tract degrading urea to CO_2 and ammonia (73, 74), was detected in the *in vivo* and *in vitro* proteome of *M. morganii* but not in *P. aeruginosa*. Accordingly, urease activity was only observed for *M. morganii* (Table II).

TABLE I

Proteins of P. aeruginosa exclusively identified or highly expressed in the catheter-biofilm compared to the in vitro biofilm. Proteomic data were compared to published transcriptome analyses (22) that have identified genes, expression of which was induced by either iron-limitation (iron), or surface-associated growth (biofilm). Genes induced under various stress conditions were designated as "core".

Representative GI accession nr.[a]	Protein description	Protein name	PAO1 homolog	ON/FC[b]	Core[c]	Iron[c]	Biofilms[c]
Iron uptake							
15595869	Heme oxygenase	HemO	PA0672	ON	+	+	
313105815	Ferric enterobactin receptor	PirA	PA0931	ON		+	
152989712	L-ornithine N5-oxygenase	PvdA	PA2386	ON	+		
254235400	Pyoverdine synthetase	PvdF	PA2396	ON	+		
152989260	2,4-diaminobutyrate 4-transaminase	PvdH	PA2413	ON	+		
107102212	Ferric enterobactin receptor	PfeA	PA2688	ON		+	
116051427	Heme uptake outer membrane receptor	HasR	PA3408	ON		+	
313109326	Fe(III) dicitrate transport protein	FecA	PA3901	706,7		+	
424938959	Second ferric pyoverdine receptor	FpvB	PA4168	ON	+		
107103807	Heme-transport protein	PhuT	PA4708	ON	+		
254244389	Putative heme degradation protein	PhuS	PA4709	ON	+		
386068373	Dihydroaeruginoic acid synthetase	PchE	-	15,1			
170282658	Ferripyoverdine receptor	FpvA	-	ON			
Biofilm/Chemotaxis							
418593466	Twitching motility protein	PilU	PA0396	2,9			
392981824	Twitching motility protein	PilJ	PA0411	4,5			+
107103325	Type 4 fimbrial biogenesis protein	PilF	PA3805	ON			
319918960	Type 4 fimbrial biogenesis protein	PilX	PA4553	ON			
313107199	Type 4 fimbrial biogenesis protein	PilY1	PA4554	ON			
421156565	Type 4 fimbrial biogenesis protein	PilQ	PA5040	4,7			+
152985916	Type 4 fimbrial biogenesis protein	PilN	PA5043	9,0			+
Defense mechanisms/Pathogenicity							
254239129	Serine protease	MucD	PA0766	2,3			
15596159	Dna-binding stress protein	Dps	PA0962	2,7	+		
15596166	TolQ protein	TolQ	PA0969	7,7			
15598322	Heat-shock protein	IbpA	PA3126	3,7			
313109552	Beta-lactamase	AmpC	PA4110	7,5			+
421170117	Insulin-cleaving metalloproteinase	IcmP	PA4370	4,5		+	
General or unknown functions							
15595620	Hypothetical protein	PasP	PA0423	12,5			
313111914	Putative ClpA/B protease ATP binding subunit	-	PA0459	2,7			
218892989	Pyridoxamine 5′-phosphate oxidase	PdxH	PA1049	2,8			
190613598	Hypothetical protein	-	PA1195	ON			+
15596990	Peptidyl-prolyl cis-trans isomerase B	PpiB	PA1793	2,2	+		
107101462	Hypothetical protein	-	PA2033	ON	+		
15597519	Glyceraldehyde-3-phosphate dehydrogenase	-	PA2323	68,4			
296388958	Hypothetical protein	-	PA2575	9,5			
15598049	Outer membrane lipoprotein	OprI	PA2853	ON			
15598843	Hypothetical protein	-	PA3647	2,7			
308198348	Peptide chain release factor 1	PrfB	PA3701	ON			
116051948	Hypothetical protein	-	PA3911	ON			
15599110	Molybdopterin biosynthetic protein B1	MoaB1	PA3915	ON			
218889787	Hypothetical protein	-	PA3931	3,1			
254242900	Probable ATP-binding component of ABC transporter	-	PA4222	ON	+		
107100081	Hypothetical protein	-	PA4336	11,0			
15599565	Hypothetical protein	-	PA4369	ON			
313107055	Fumarate hydratase	FumC1	PA4470	15,3	+		
15599949	Transcription elongation factor	GreA	PA4755	2,8			
15599987	Hypothetical protein	-	PA4793	2,8			
15600040	Biotin carboxyl carrier protein	AccB	PA4847	ON			
15600128	30S ribosomal protein S6	RpsF	PA4935	3,2			
116053356	Hypothetical protein	-	PA5208	ON			
392983995	Serine hydroxymethyltransferase	GlyA1	PA5415	ON			+
15600668	Hypothetical protein	-	PA5475	ON			+
15600747	ATP synthase F0F1 subunit beta	AtpD	PA5554	2,0			

Table I —*continued*

Representative GI accession nr.[a]	Protein description	Protein name	PAO1 homolog	ON/FC[b]	Core[c]	Iron[c]	Biofilms[c]
15600750	ATP synthase F0F1 subunit delta	AtpH	PA5557	2,9			+
30141367	Aminoglycoside acetyltransferase	-	-	3,9			
313107144	Hypothetical protein		-	ON			

[a] Representative GI accession number of *P. aeruginosa* protein groups.

[b] The change (n-fold) was calculated by comparing relative protein amounts of *P. aeruginosa* grown *in vivo* as a biofilm to *P. aeruginosa* grown *in vitro* as a biofilm. Only proteins identified as *in vivo* highly expressed that demonstrated a change of equal to or greater than threefold and exhibiting a statistically significant change ($p \leq 0.05$) are reported. Proteins exclusively detected *in vivo* are indicated by "ON".

[c] Induced expression of the corresponding transcripts during iron-limitation (iron), surface-associated growth (biofilm), or various physiological conditions (core) are indicated (+).

TABLE II

Phenotypic characterization of isolated P. aeruginosa and M. morganii strains. P. mirabilis HI432 and P. aeruginosa PAO1 were used as control strains. (–) no signal, (+) weak signal, (+++) strong signal, (n.a.) not analyzed, (+) resistant or (-) sensitive to the respective antibiotic

	P. aeruginosa Isolate	*M. morganii* Isolate	*P. mirablis* HI432	*P. aeruginosa* PAO1
Urease	–	+	+++	n.a.
Protease	1.33 ± 0.05	0	n.a.	1.39 ± 0.05
Biofilm Index	282 ± 58	141 ± 8	n.a.	107 ± 16
Ciprofloxacin	+	+	-	-
Levofloxacin	+	+	-	-
Tetracycline	+	+	+	+
Amoxillin	+	+	+	+
Ampicillin	+	+	+	+
Penicillin	+	+	+	+
Cephalexin	+	+	+	+
Sulfamethoxazol	+	+	+	+
Nitrofurantion	+	+	+	+
Trimethoprim	+	+	+	+
Gentamicin	+	+	+	+

Only 11 proteins of *M. morganii* were solely present in the urine and not in the catheter biofilm (Fig. 2), of which only one protein, protease III, was considered as a potential secreted virulence factor. However, *M. morganii* protease III does not contain any signal peptide, no protease was found in the *in vitro* secretome (data not shown) and no protease activity was detected in the supernatant of the *M. morganii* isolate (Table II). These observations indicate that the investigated *M. morganii* strain was not capable to secrete proteases into the extracellular matrix of the biofilm or into the urine.

Human Proteins of the Innate Immune System are Abundant on the Bacterial Biofilm—In order to elucidate the human immune response on the basis of the identified proteins to the catheter-associated biofilm (Fig. 1), 452 human proteins were assigned to different functional categories (Fig. 2).

More than 30 of the detected human proteins are directly involved in the human innate immunity including antimicrobial proteins, proteins of the complement system and peptides secreted by epithelial cells or proteins associated to neutrophils or macrophages (Table V, supplemental File S2). Many of these proteins are used by neutrophils to kill bacteria in the phagolysosome in either a reactive oxygen species-(ROS) dependent or ROS-independent manner. Proteins involved in ROS-dependent killing mechanisms are cytochrome b_{245}, neutrophil NADPH oxidase factor 4 and the myeloperoxidase. Interestingly, almost all ROS-independent effector molecules, known to be stored in azurophilic (*i.e.* myeloperoxidase, cathepsin G, proteinase 3, azurocidin, bacterial permeability increasing protein and Hnp-3 defensins) and specific granules (lactoferrin, cathelicidin, lysozyme, cytochrome b_{245}, and neutrophil gelatinase-associated lipocalin) were identified. Besides neutrophil-associated proteins, eosinophil-specific proteins were found, namely eosinophil peroxidase and eosinophil cationic protein (Table V).

Moreover, a significant number of proteins of the complement system, one of the most important components of the innate immune system, were found to be highly abundant in the catheter biofilm, that is,core proteins of the complement system C1–C9, complement inhibitors (H, I, AFD), and activators (D, B).

Human Proteins of the Innate Immune System were also identified in Cell-free Urine—In order to prove whether the high abundance of proteins involved in innate immunity is caused by the presence of the catheter-associated biofilm, the protein profiles of urine of three long-term catheterized patients were compared with the ones of three healthy control persons (Fig. 1, Fig. 5, supplemental File S6). Forty-seven proteins were found to be significantly more abundant or

TABLE III

Potentially secreted P. aeruginosa proteins exclusively identified in the catheter-associated urine when compared to the catheter biofilm proteome. Protein sequences were analyzed by pSORTb and only extracellular and proteins of unknown localization were listed. An identification of the respective protein in the secretome and/or whole cell proteome of the in vitro AUM cultures is indicated by "+"

GI Accession Nr.[a]	Protein Annotation	Protein name	pSORTb	Protein function	Reference	USC[b]	*in vitro* proteomics	
							Secretome	Whole-cell proteome
313111732	Phage tail tube protein FII		Unknown	Phage tail		8.3	+	+
116052217	Pvds-regulated endoprotease	PrpL	Extracellular	Protease (casein, lactoferrin, transferrin, elastin, decorin)	(90)	10.0	+	-
313105877	Colicin M-Like Bacteriocin	PaeM	Unknown	Bacteriocin (inhibits cell wall peptidoglycan biosynthesis)	(93)	4.7	-	+
15598135	Putative aminopeptidase		Extracellular	Peptidase		9.3	+	+
313111732	Phage tail sheath protein FI		Unknown	Phage tail		7.3	+	+
421182152	Chitin-binding protein	CbpD	Extracellular	Pathogenicity	(110)	5.3	+	+
15599752	Type 4 fimbrial biogenesis protein	PilE	Extracellular	Chemotaxis		2.0	-	+
107104369	Hemolysin-coregulated protein	Hcp	Extracellular	Pathogenicity	(111)	2.0	+	-
386066386	Enterochelin esterase		Unknown	Hydrolysis of ferric enterochelin	(112)	8.3	+	-
107103721	Lactonizing lipase	LipA	Extracellular	Lipase activity	(113)	2.0	-	+
107100698	Alkaline metalloproteinase	AprA	Extracellular	Protease (transferrin, complement, cytokines)	(101–104)	2.3	+	-
107103238	Elastase LasB	LasB	Extracellular	Protease (lactoferrin, transferrin, elastin, immunglobulin, collagen, complement)	(101, 103, 105, 106, 114, 115)	2.7	+	-
421169436	Flagellar hook-associated protein	FlgK	Extracellular	Cell motility		1.7	+	+

[a] Representative GI accession number of *P. aeruginosa* protein groups.

[b] USC (Unique spectral counts), average of three replicates.

uniquely identified in the patient urine, 30 of which are functionally involved in innate and adaptive immunity, among them: (1) key compounds of the complement system (*i.e.* C3, C5, C8, factor H, and factor H related protein 1), (2) antimicrobial peptides and proteins commonly associated with neutrophils, for example, neutrophil collagenase, myeloblastin, bacterial permeability increasing protein, neutrophil defensin 1, cathelicidin, lysozyme C, leukocyte elastase inhibitor, neutrophil gelatinase-associated lipocalin, and histones H3.3 and H2A.1, and 3) iron and calcium binding proteins, that is, lactoferrin, serotransferrin, haptoglobin, hemopexin, hemoglobin, S100-P, EF-hand domain-containing protein D2, and (IV) macrophage-associated proteins that is, chitotriosidase-1, macrophage capping protein, and chitinase-3-like protein, respectively. Interestingly, numerous proteins considered as "acute-phase" proteins expressed in response to microbial infections and other diseases were found to be more abundant in patient urine, that is, fibrinogen, haptoglobin, and serum amyloid A-4 protein (75). Moreover, also proteins considered as "negative acute-phase" proteins were strongly expressed in patient urine, that is, transthyretin and transferrin (75). Lastly, immunoglobulins were found to be more abundant in patient urine.

DISCUSSION

Experimental Design—Until recently, most of the global studies investigating host-pathogen interactions have been employed a murine pathogenicity model system infected with well-characterized laboratory strains (*e.g.* (76–78). For transferring the derived results to human it has to be considered that (1) the murine immune systems differs substantially from the human immune system (79) and (2) pathogenicity and adaptation strategies might vary between laboratory strains and clinical strains isolated from the infection of interest (80). Aiming at a comprehensive and realistic insight into bacterial adaptation mechanisms to the human urinary tract environment and the response of the human host to the long-term colonization of urinary tract catheters, we employed a semi-quantitative *state-of-the-art* metaproteomics approach to investigate a bacterial biofilm directly derived from a catheterized patient. In contrast to metagenome analyses, metatranscriptomics and metaproteomics allow a direct linkage of structure and functionality in microbial communities (68). However, with a metatranscriptomics approach we would probably not detect human mRNA coding for granulocyte-associated proteins because these immune cells originate from the bone marrow and are already differentiated when they reach the site of infection (81, 82). Thus, we would miss mRNA coding for proteins secreted by the urothel as we had only access to catheter biofilms and urine.

Metaproteome coverage (spectra to peptide matching) can be significantly improved if the MS and MS/MS data can be searched against a sample-specific metagenome-based database. However, a recent study demonstrated that virtual

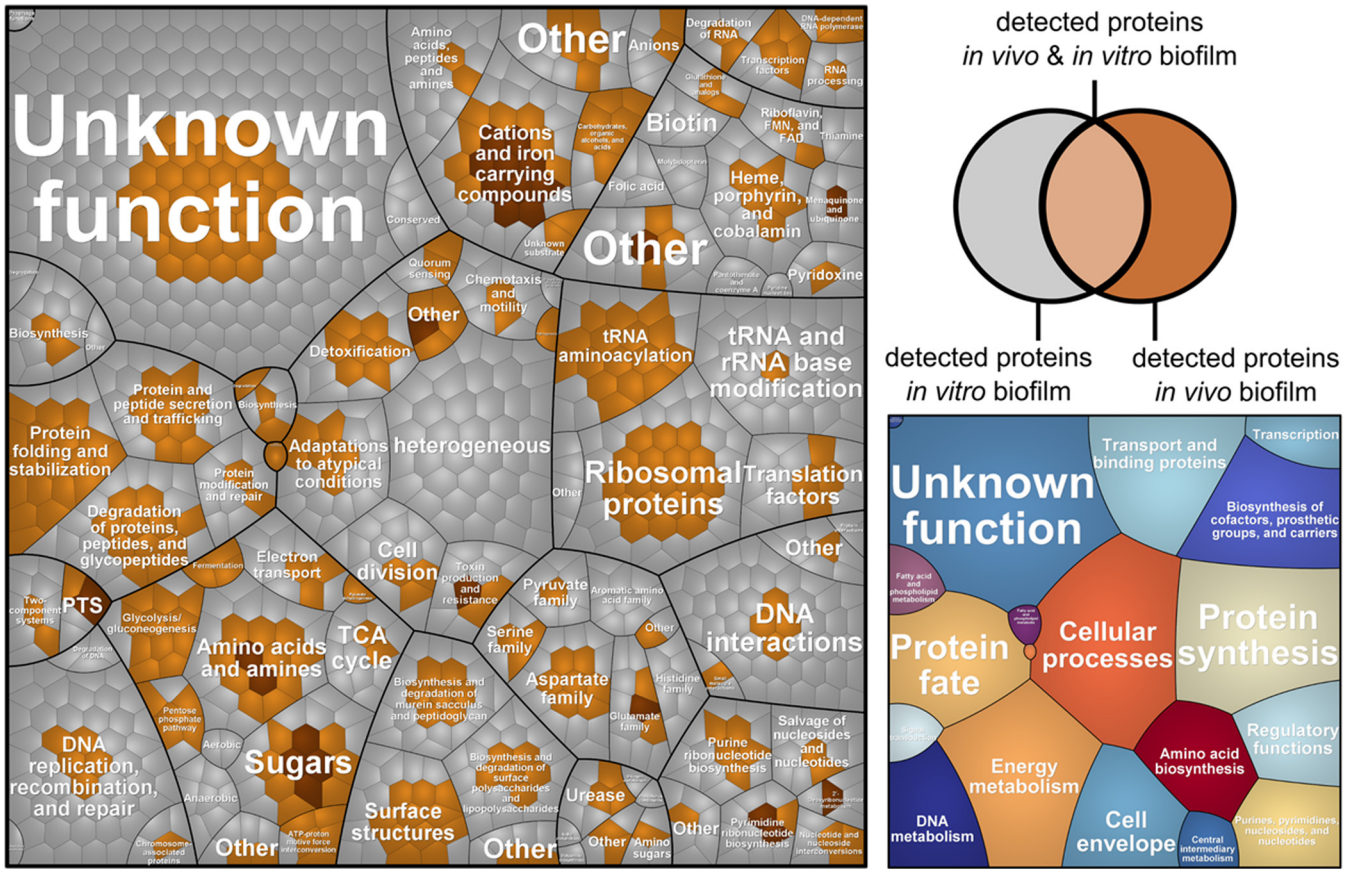

FIG. 4. **Voronoi treemaps (109) comparing protein expression profiles of *M. morganii* grown in catheter biofilms (*in vivo*) or in a catheter model system in AUM (*in vitro*).** Gene functions are based on TIGRFAMS, major TIGRFAMS roles are displayed in the right map and the minor TIGRFAMS roles the left map. Gray proteins were exclusively identified *in vitro*, light brown proteins were identified *in vivo* and *in vitro* and dark brown proteins were exclusively identified *in vivo*.

metagenome databases based on 16S rDNA sequence information can replace a matching metagenome-based database in case the organisms present in the sample have been already sequenced (47, 83). We thus constructed an appropriate database based on the phylogenetic information obtained by 16S rDNA sequencing, which contained all protein sequences of the identified species available in the NCBI database. Nevertheless, as we did not sequence the genomes of the biofilm isolates, we might have missed to identify proteins specific for the strains present in our samples. In contrast to the majority of other metaproteome studies, all identified proteins were exclusively assigned to one of the species present in the biofilm. This is probably because of the specifically designed database, the stringent filter settings, and the limited diversity of the biofilm compared with other environments such as the human intestinal tract or soil.

Even though recent advances in mass spectrometry have turned metaproteomics into a powerful tool for the investigation of complex biological systems, quantification of proteins derived from clinical samples is often still not very accurate and mostly based on NSAFs. Unfortunately, the same applies to our study, in which, albeit we applied more precise NAUC quantification, an accurate comparison of the expression rates of proteins identified from the *in vivo* and *in vitro* samples was hampered by the strongly differing complexity of the two sample types. The catheter metaproteome is certainly much more complex than the proteomes of pure bacterial cultures. The catheter biofilm is composed of at least three bacterial species and contains an enormous amount of human proteins (min. 30,000 proteins), whereas the pure *P. aeruginosa* or *M. morganii* cultures contain a maximum of about 5600 and 3500 proteins, respectively. In addition, bacterial proteins are much less abundant compared with human proteins in the *in vivo* sample (Fig. 2, supplemental File S3, and supplemental Fig. S1). As proteins exclusively identified or highly expressed under *in vitro* conditions, but missing in the *in vivo* data set might simply have escaped mass spectrometric analyses because of the comparably high complexity of the metaproteomics sample, only those proteins were reported that appeared to be exclusively or highly expressed under *in vivo* conditions.

Iron was Identified as one of the Major Limiting Factors in the Bladder Environment—Proteins involved in iron-acquisition were among the most abundant proteins in the metapro-

TABLE IV
Proteins of M. morganii exclusively identified or highly expressed in the catheter-biofilm compared to the in vitro biofilm

Representative GI accession nr.[a]	Protein description	Protein name	MU9 homolog	ON/FC[b]
Iron and cation uptake				
410084858	Ferric iron ABC transporter, iron-binding protein	-	MU9_1225	2,2
421493824	TonB-dependent receptor	-	MU9_2104	ON
421492020	Manganese ABC transporter, periplasmic-binding protein	SitA	MU9_2451	41,7
410086754	Manganese ABC transporter, ATP-binding protein	SitB	MU9_2452	26,1
421494274	Iron ABC transporter substrate-binding protein	-	MU9_2748	7,5
410086951	Ferrous iron transport peroxidase	EfeB	MU9_2852	ON
421494564	Ferrous iron transport periplasmic protein	EfeO	MU9_2853	ON
410086947	Periplasmic protein p19 involved in high-affinity Fe2+ transport	-	MU9_2856	6,6
421494894	TonB-dependent receptor	-	MU9_2961	ON
Carbohydrate uptake and metabolism				
410086198	2-dehydro-3-deoxygluconate kinase	-	MU9_376	ON
421494175	Uronate isomerase	UxaC	MU9_377	34,2
410086200	D-mannonate oxidoreductase	-	MU9_378	ON
410086201	Mannonate dehydratase	UxuA	MU9_379	ON
410086204	TRAP-type C4-dicarboxylate transport system, periplasmic component	-	MU9_382	15,3
410087600	6-phosphofructokinase [Morganella morganii SC01]	PfkA	MU9_571	2,5
410084920	Enolase	Eno	MU9_1162	4,0
410087699	PTS system, mannose-specific IID component	-	MU9_1934	9,4
410085950	NAD-dependent glyceraldehyde-3-phosphate dehydrogenase	-	MU9_2337	9,8
410088110	PTS system, N-acetylgalactosamine-specific IIB component	-	MU9_3143	ON
410088115	Galactosamine-6-phosphate isomerase	-	MU9_3148	ON
Defense mechanisms/Pathogenicity				
421493572	Molecular chaperone	Skp	MU9_943	7,8
410084895	Tellurium resistance protein	TerD	MU9_1186	2,6
410087933	LPS-assembly lipoprotein	lptE	MU9_1348	2,2
410085873	Iron-sulfur cluster assembly protein	SufD	MU9_2411	ON
40781704	Beta-lactamase, class C	-	MU9_3299	19,0
410087140	LPS transport protein	LptA	MU9_3355	2,2
Amino acids and amines				
410085074	Methylaspartate mutase, E subunit	-	MU9_1022	ON
410085059	Ethanolamine utilization protein	EutQ	MU9_1035	3,2
410085048	Ethanolamine ammonia-lyase heavy chain	-	MU9_1045	4,1
421494057	N-acetylneuraminate lyase	-	MU9_1230	ON
General or unknown functions				
410086339	LSU ribosomal protein L9p	RplI	MU9_205	2,5
410086267	Protein yifE	YifE	MU9_269	3,1
410088675	LSU ribosomal protein L3p	RplC	MU9_310	2,7
410086197	4-Hydroxy-2-oxoglutarate aldolase	-	MU9_375	13,7
410086264	Glutathione S-transferase	-	MU9_450	15,0
410085838	ATP synthase beta chain	AtpD	MU9_474	2,4
410088376	Translation elongation factor Ts	Tsf	MU9_935	2,2
410085053	Acetaldehyde dehydrogenase, ethanolamine utilization cluster	-	MU9_1040	8,4
410087956	Ribonucleotide reductase of class Ib (aerobic), beta subunit	-	MU9_1325	ON
410085589	Outer membrane receptor protein	-	MU9_1681	ON
410087848	SAM-dependent methyltransferase	-	MU9_2098	ON
421493822	Hypothetical protein	-	MU9_2102	ON
410087871	NAD(P) transhydrogenase alpha subunit	-	MU9_2119	19,0
410087905	N-ethylmaleimide reductase	-	MU9_2153	ON
421494395	Alcohol dehydrogenase	-	MU9_2315	43,3
421493969	Pseudouridine-5′-phosphate glycosidase	PsuG	MU9_3155	ON
410088124	Pseudouridine kinase	-	MU9_3156	ON
410088158	Putative ATP-binding protein	YbbA	MU9_3188	3,3

[a] Representative GI accession number of *M. morganii* protein groups.

[b] The change (n-fold) was calculated by comparing relative protein amounts of *M. morganii* grown *in vivo* as a biofilm to *M. morganii* grown *in vitro* as a biofilm. Only proteins identified as *in vivo* highly expressed that demonstrated a change of equal to or greater than threefold and exhibiting a statistically significant change ($p \leq 0.05$) are reported. Proteins exclusively detected *in vivo* are indicated by "ON".

teomics data set (Tables I and IV; Figs. 3 and 4), which indicates that iron is highly limited in the urinary tract environment and thus strongly affects protein expression of the uropathogens. Our findings are in good agreement with earlier studies demonstrating an increase of iron-uptake mechanisms on the transcriptome level when opportunistic patho-

TABLE V

Human proteins identified from the catheter-biofilm and assigned to the innate immune system (complement proteins are not shown). References indicate cells/tissues in which expression of corresponding proteins has been demonstrated

GI accession nr.	Protein description	Function	Azurophil granules of neutrophils	Specific granules of neutrophils	Expression in neutrophils	Expression in epithelial cells	Expression in kidney	Spectral counts (biofilm)
34719	Myeloperoxidase	Oxidative burst	(116)		(117)			48
20664221	Cathepsin G	Protease	(116)		(118) (119, 120)			13
1633225	Proteinase 3 (Myeloblastin)	Protease	(116)		(121) (122)		(123)	4
227250	Azurocidin	Protease	(116)		(124)			10
157830420	Bacterial permeability increasing protein	Antimicrobial peptides	(116)		(125)			8
109156990	Alpha-Defensin-4	Antimicrobial peptides	(116)		(126–128)			3
229858	Defensin HP-3	Antimicrobial peptides	(116)					6
6996021	Cytochrome b_{245}	Oxidative burst		(116)	(129)			6
54607120	Lactoferrin	Iron chelating		(116)	(130)		(131)	75
348041314	Cathelicidin	Antimicrobial peptides		(116)	(132)	(133)	(35)	7
157832581	Lysozyme	Cell wall lysis		(116)	(117)			17
119587431	Neutrophil collagenase	Protease		(116)	(134)			10
300181	Neutrophil gelatinase-associated lipocalin	Binding of bacterial siderophores		(116)	(135)	(136)		13
62898209	Neutrophil NADPH oxidase factor 4	Oxidative burst						6
31183	Eosinophil peroxidase	Oxidative burst			(137)			21
13400006	Eosinophil Cationic Protein (RNase 3)	Cell wall lysis			(137, 138)			6

gens were cultivated in urine (*E. coli*, *P. mirabilis*), in the urinary tract (*E. coli*) or on AUM agar plates (*P. aeruginosa*) (18–21). In an infected bladder environment the availability of iron is even more restricted by competition for iron in multi-species biofilms (84) and the presence of siderophore- and iron-binding proteins such as NGAL and lactoferrin in the urine representing the nutritional immunity (Fig. 5, supplemental File S6) (85).

The expression of *P. aeruginosa* outer membrane receptors for heterologous siderophores (PirA and PfeA) produced by cocolonizers, supports the hypothesis that biofilm inhabitants in the bladder environment strongly compete for iron. These receptors have been described to be involved in ferric enterobactin/enterochelin uptake (86, 87). Our findings suggest that *P. aeruginosa* is able to utilize siderophores produced by *M. morganii* and/or *Bacteroides* sp., which are structurally related to enterobactin (Fig. 6).

As mentioned above, the concentration of freely available iron in the urinary tract is further decreased by lactoferrin, which is secreted by the urothelium and expressed in neutrophils. Lactoferrin has been identified as a biofilm-associated protein and was also found to be highly abundant in the urine of catheterized patients compared with the control persons (supplemental Files S1 and S6). It has been shown that lactoferrin reduces the extracellular free iron concentration to $\sim 10^{-18}$ M (88) and inhibits *P. aeruginosa* biofilm formation (89). In the urine the siderophore pyoverdine and the high-abundant PvdS-regulated lactoferrin degrading protease PrpL were identified, which compensate iron limitation induced by the nutritional immunity of the host (90) (Fig. 6). Moreover, NGAL was found to be expressed in neutrophils that is able to bind ferric-siderophores and thereby interferes with the bacterial siderophore uptake (91). It has been demonstrated that *P. aeruginosa* pyoverdine is able to evade NGAL recognition (92), suggesting that pyoverdine-mediated iron acquisition of *P. aeruginosa* is not hampered by NGAL. Notably, a *P. aeruginosa* colicin M-like bacteriocin was identified in the urine, which has been demonstrated to inhibit peptidoglycan biosynthesis (93). Colicin M-like bacteriocins produced by *Pectobacterium carotovorum* and *E. coli* have been shown to enter the periplasm of potential competitors via TonB-dependent iron-uptake systems (94, 95). These observations suggest, that iron limitation promotes colicin uptake of cocolonizing bacterial species.

The Catheter Biofilm is Characterized by an Oxygen Gradient—It is well known that bacterial biofilms are characterized by a decreasing oxygen gradient from the biofilm-surface to the bottom. The identification of proteins involved in the adaptation to microaerobic conditions indicates that also cells in the catheter-associated biofilm are exposed to oxygen-limiting conditions. These results are supported by several other findings: First, the obligate anaerobe (96) *Bacteroides* sp. was identified in the biofilm (Fig. 2). Second, the *P. aeruginosa* proteins NirS, NosZ, and NarG, which are involved in denitrification and used for energy production under oxygen limitation, were found. They are part of the anaerobic regulon induced by Anr, Dnr, and NarXL (97, 98). Third, proteins of the innate immune system involved in ROS production, that is, myeloperoxidase, NADH peroxidase, and eosinophil peroxidase, were identified from the biofilm (99) (Fig. 5, Table V). Notably, a significant number of proteins associated with neutrophils were found in the biofilm metaproteome. This supports the finding of Jesaitis and colleagues, who demonstrated that neutrophils fusing with *P. aeruginosa* biofilms

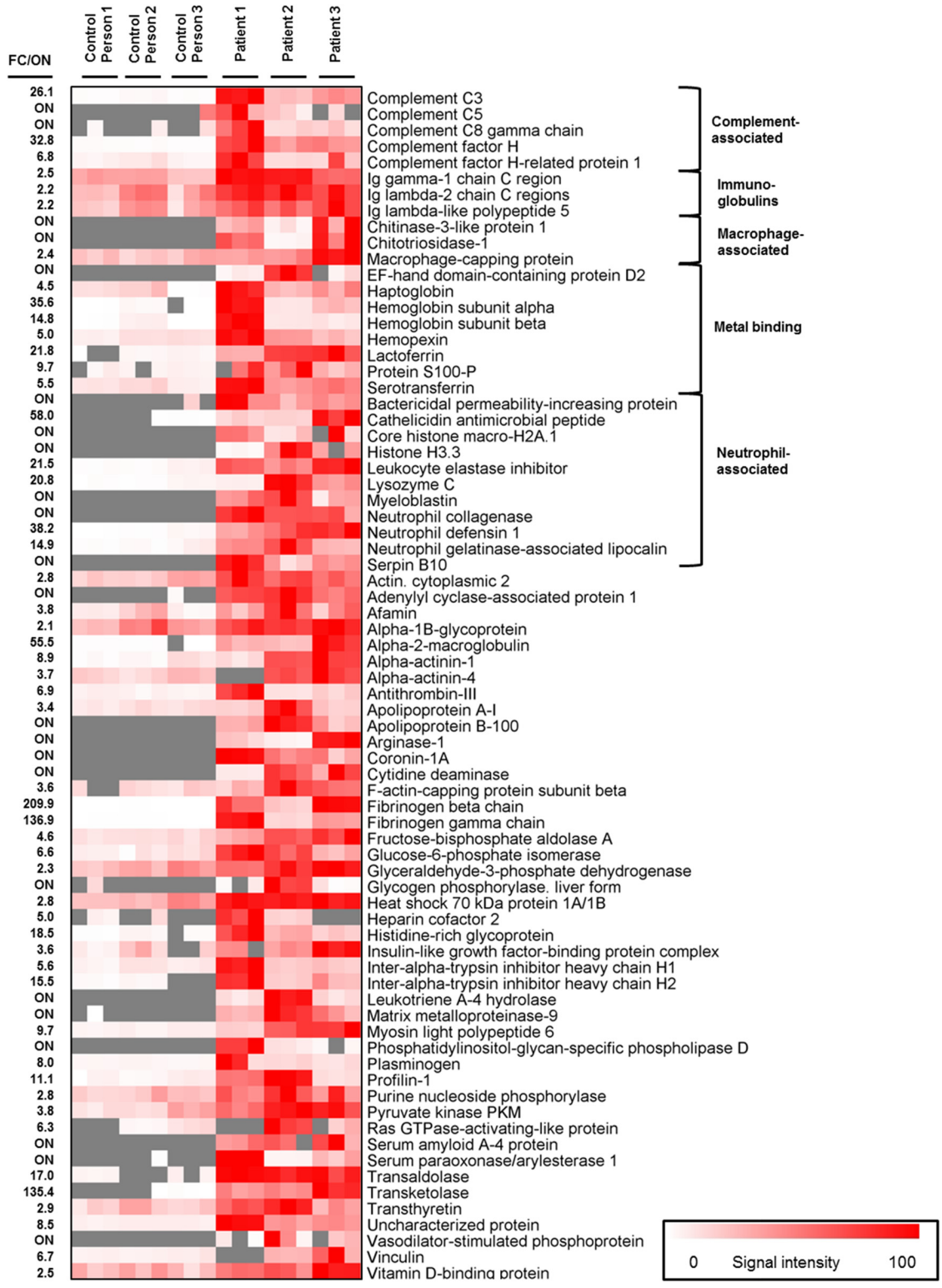

FIG. 5. **Heat map of host-derived proteins involved in innate and adaptive immunity identified in cell-free urine of three catheterized patients and three healthy control persons.** The heat map indicates the relative abundance of the given proteins which were at least twofold higher abundant ($p < 0.05$) or uniquely identified in the patients urine (corresponding proteins not detected in the control persons urine are gray).

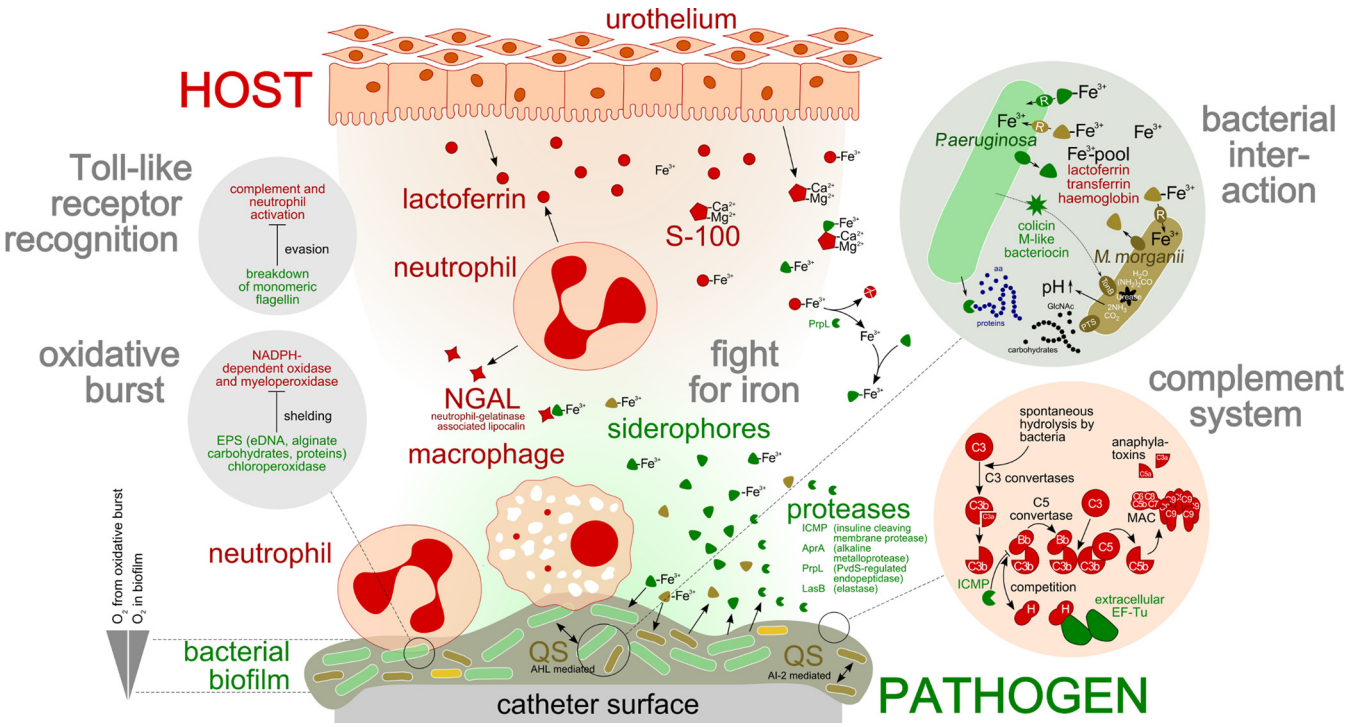

FIG. 6. **Proposed host-pathogen interactions during the catheter-associated urinary tract infection.** *P. aeruginosa* (green), *M. morganii* (brown), and *Bacteroides sp.* (yellow) form a multispecies biofilm on the catheter surface. Cell-to-cell communication, that is, *N*-acylhomoserine lactone- and autoinducer 2-mediated quorum sensing, might contribute to the expression of virulence factors and the overall biofilm physiology. Various secreted or surface-exposed virulence factors of *P. aeruginosa* could be involved in immune evasion, that is, elastase (LasB), alkaline metalloprotease (AprA), insulin cleaving membrane proteinase (ICMP), elongation factor Tu (EF-Tu), as well as in iron acquisition, that is, PvdS-regulated endoprotease (PrpL) and siderophores. Host-factors possibly involved in innate immune response have been identified, that is, various compounds of the complement system (orange circle), and proteins related to neutrophils, macrophages and the urothelium. Lactoferrin, neutrophil-gelatinase associated lipocalin (NGAL) and S100 are secreted host factors, which are known to bind siderophores and calcium. The left gray circles summarize the proposed bacterial strategies to evade immune recognition and the oxidative burst because of neutrophil activation. Suggested interspecies interactions and differential adaptation strategies of *P. aeruginosa* and *M. morganii* in the catheter biofilm are depicted in the right greenish circle. Both bacteria produce siderophores (green and brown triangles) and express specific siderophore-receptors (R) to compensate iron limitation in the bladder environment. *P. aeruginosa* expresses also receptors for heterologous siderophores (PirA, PfeA) probably enabling the uptake of siderophores produced by *M. morganii.* Moreover, *P. aeruginosa* secretes a colicin M-like bacteriocin capable of inhibiting peptidoglycan biosynthesis in *M. morganii*. The bacteriocin might be taken up by *M. morganii* via TonB-dependent transport systems (blue oval), which are known to be involved in iron acquisition. The predominant carbohydrates taken up by *P. aeruginosa* are most probably peptides and amino acids (AS) whereas *M. morganii* might utilize carbohydrates (N-acetylglucosamine) and urea.

contribute to oxygen limitation by the consumption of oxygen during ROS generation (100) (Fig. 6).

Functional Assignment of Proteins Suggests Different Adaptation Strategies of the Major Biofilm Formers—Although both dominating biofilm inhabitants employ similar strategies to overcome the major limitation in the bladder environment, that is, restriction of iron, the differential expression of carbohydrate-transporters, urease and protease(s), suggests different nutrient acquisition strategies of the two opportunistic pathogens (Fig. 6).

The finding that protein components of a PTS uptake system (N-acetylglucosamine-specific) together with proteins involved in carbohydrate metabolism were found to be strongly expressed in *M. morganii* catheter biofilms suggests that this organism gains energy from cell wall compounds of lysed bacteria taken up from the infected bladder environment. In contrast, *P. aeruginosa* is able to generate energy from the fermentation of amino acids (91), which might be released by its various secreted proteases (Fig. 6) and taken up by transporters such as an amino acid ABC transporter, which appeared to be highly abundant in the catheter biofilm (Table I). This hypothesis is supported by the finding that *P. aeruginosa* PAO1 is able to grow in AUM with peptone as the sole carbon source (21).

Moreover, our data indicate that *M. morganii* expresses urease *in vivo* and is able to metabolize urea *in vitro*, whereas no urease was assigned to *P. aeruginosa* and no urease activity was detected *in vitro*. Our results are in good agreement with the findings of Tielen *et al.* who showed that *P. aeruginosa* PAO1 is not able to grow on urea as a sole carbon source in AUM (21).

P. aeruginosa Evades Recognition by the Immune System in the Urinary Tract—In the urine various *P. aeruginosa* virulence factors involved in immune system evasion were identified,

many of which are known to exhibit proteolytic activity (Table III, Fig. 6). (1) Alkaline protease A (AprA) degrades the complement compounds C2 (101) and C3 (102) as well as IFN-gamma (103). Moreover, AprA is able to degrade monomeric but not polymeric flagella-forming flagellin thereby preventing the recognition of monomeric flagellin by TLR-5 (104). (2) Elastase (LasB) degrades pulmonary surfactant protein D (105), different surface receptors (106), and cytokines (103). (3) PvdS-regulated endoprotease (PrpL) cleaves lactoferrin and transferrin (90) and (IV) insulin-cleaving membrane proteinase (ICMP) degrades plasminogen activator, complement convertase and kallikrein (72). However, neither a protease secretion nor a proteolytic activity of the *M. morganii* culture-supernatant was observed. Therefore, we suggest that only *P. aeruginosa* is involved in the protease-dependent immune evasion and in the degradation of host proteins/tissue.

Notably, complement negative regulator factor H was found to be highly abundant on the catheter biofilm and in the corresponding urine (Fig. 5). Factor H can be bound by different human pathogens to mask themselves (107). *P. aeruginosa* is able to bind inhibitory factor H by surface-located elongation factor EF-Tu (108), too, which was found to be highly expressed in the biofilm (Fig. 6).

The Multispecies Biofilm Seems to Induce an Innate Immune Response—Proteins associated with the complement system, one of the central effectors of the innate immune system, are highly abundant in our metaproteomics data set and suggest an important role of the complement system in the defense of catheter-associated urinary tract infections. The innate immune system works clinically silently (30), which might explain why CAUTIs are mostly asymptomatic. The comparison of urine protein profiles of three patients and three control persons revealed that the expression of most of these proteins is at least associated with the presence of a catheter biofilm (Fig. 5). Neutrophils, known to be the first defense line of the immune system have been proven to be active during kidney infections and urinary tract infections (29, 30, 38). The high abundance of neutrophil-associated proteins suggests that neutrophils are also active during catheter-associated asymptomatic urinary infections.

Our finding that "acute-phase" and "negative acute-phase" proteins were significantly more abundant in patient urine compared with urine from healthy control persons might suggest a so far uncharacterized pronounced immune response to the catheter biofilm. However, because of the limited number of analyzed samples from CAUTI patients and healthy control persons, these results can only be considered as first evidence for a specific function of the innate immune system during CAUTIs. Moreover, additional controls, that is, urine from noncatheterized patients suffering from UTI or patients prior to catheterization, should be analyzed to verify that the observed immune response is specific for catheter-associated infections.

CONCLUSIONS

Combining metaproteomics analyses of a selected multispecies urinary tract catheter biofilm and the associated urine together with *in vitro* proteome analyses of *P. aeruginosa* and *M. morganii* isolated from the biofilm enabled us to unravel different molecular adaptation strategies of these important uropathogens to the urinary tract and co-infecting biofilm inhabitants as well as the response of the human innate immune system to the asymptomatic infection process. Our proteomics analysis strongly supports earlier studies that have demonstrated that iron restriction is one of the major limiting factors in the bladder environment. *P. aeruginosa* overcomes iron restriction by the host and cocolonizing competitors by the expression of proteins involved in siderophore-production, lactoferrin-degradation, and heme- and siderophore uptake. Moreover, the identification of complement-degrading proteases *in vivo* indicates that *P. aeruginosa* is able to evade the human immune response. Interestingly, *M. morganii* is not involved in protease secretion and might therefore often be considered as a harmless opportunistic pathogen. Our study indicates differential adaptation strategies of *P. aeruginosa* and *M. morganii* in nutrient acquisition in the urinary tract. Although *P. aeruginosa* seems to express secreted and surface-exposed proteases to metabolize amino acids, *M. morganii* is able to take up sugars and to degrade urea. Highly abundant proteins associated with neutrophils, eosinophils, and the complement system found in the biofilm and urine might suggest an activation of the innate immune system in response to the catheter biofilm. Even though extended clinical proteomics studies are urgently needed to consolidate our hypothesis, our data provides first evidence for a sophisticated interplay between the different members of the mixed biofilm and the host and will serve as a good basis for further studies aiming at a deeper insight into detailed molecular mechanisms of synergistic or competitive interactions observed in the urinary tract.

Acknowledgments—We thank the BMBF for financial support (UroGenOmics, grant 0315833B) and Holger Kock, Alexander Graf and Stefanie Dietz for critical reading of the manuscript.

S This article contains supplemental Files S1 to S6.

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REFERENCES

1. Stamm, W. E. (1991) Catheter-associated urinary tract infections: Epidemiology, pathogenesis, and prevention. *Am. J. Med.* **91,** 65S–71S
2. Warren, J. W. (1991) The catheter and urinary tract infection. *Med. Clin. North. Am.* **75,** 481–493
3. Tambyah, P. A., and Maki, D. G. (2000) Catheter-associated urinary tract infection is rarely symptomatic: a prospective study of 1497 catheterized patients. *Arch. Intern. Med.* **160,** 678–682
4. Warren, J. W., Tenney, J. H., Hoopes, J. M., Muncie, H. L., and Anthony, W. C. (1982) A prospective microbiologic study of bacteriuria in patients with chronic indwelling urethral catheters. *J. Infect. Dis.* **146,** 719–723

5. Warren, J. W. (2001) Catheter-associated urinary tract infections. *Int. J. Antimicrob. Agents* **17,** 299–303
6. Warren, J. W., Damron, D., Tenney, J. H., Hoopes, J. M., Deforge, B., and Muncie, H. L., Jr. (1987) Fever, bacteremia, and death as complications of bacteriuria in women with long-term urethral catheters. *J. Infect. Dis.* **155,** 1151–1158
7. Stickler, D. J. (2008) Bacterial biofilms in patients with indwelling urinary catheters. *Nat. Clin. Pract. Urol.* **5,** 598–608
8. Donlan, R. M. (2001) Biofilms and device-associated infections. *Emerg. Infect. Dis.* **7,** 277–281
9. Hall-Stoodley, L., Costerton, J. W., and Stoodley, P. (2004) Bacterial biofilms: from the natural environment to infectious diseases. *Nat. Rev. Microbiol.* **2,** 95–108
10. Donlan, R. M. (2002) Biofilms: microbial life on surfaces. *Emerg. Infect. Dis.* **8,** 881–890
11. Stewart, P. S., and Costerton, J. W. (2001) Antibiotic resistance of bacteria in biofilms. *Lancet* **358,** 135–138
12. Lewis, K. (2001) Riddle of biofilm resistance. *Antimicrob. Agents Chemother.* **45,** 999–1007
13. Bonkat, G., Widmer, A. F., Rieken, M., van der Merwe, A., Braissant, O., Müller, G., Wyler, S., Frei, R., Gasser, T. C., and Bachmann, A. (2012) Microbial biofilm formation and catheter-associated bacteriuria in patients with suprapubic catheterisation. *World J. Urol.* **31,** 565–557
14. Macleod, S. M., and Stickler, D. J. (2007) Species interactions in mixed-community crystalline biofilms on urinary catheters. *J. Med. Microbiol.* **56,** 1549–1557
15. Vebø, H. C., Solheim, M., Snipen, L., Nes, I. F., and Brede, D. A. (2010) Comparative genomic analysis of pathogenic and probiotic *Enterococcus faecalis* isolates, and their transcriptional responses to growth in human urine. *PloS One* **5,** e12489
16. Alteri, C. J., and Mobley, H. L. (2007) Quantitative profile of the uropathogenic *Escherichia coli* outer membrane proteome during growth in human urine. *Infect. Immun.* **75,** 2679–2688
17. Roos, V., and Klemm, P. (2006) Global gene expression profiling of the asymptomatic bacteriuria *Escherichia coli* strain 83972 in the human urinary tract. *Infect. Immun.* **74,** 3565–3575
18. Pearson, M. M., Yep, A., Smith, S. N., and Mobley, H. L. (2011) Transcriptome of *Proteus mirabilis* in the murine urinary tract: virulence and nitrogen assimilation gene expression. *Infect. Immun.* **79,** 2619–2631
19. Snyder, J. A., Haugen, B. J., Buckles, E. L., Lockatell, C. V., Johnson, D. E., Donnenberg, M. S., Welch, R. A., and Mobley, H. L. (2004) Transcriptome of uropathogenic *Escherichia coli* during urinary tract infection. *Infect. Immun.* **72,** 6373–6381
20. Hagan, E. C., Lloyd, A. L., Rasko, D. A., Faerber, G. J., and Mobley, H. L. (2010) *Escherichia coli* global gene expression in urine from women with urinary tract infection. *PLoS Pathog.* **6,** e1001187
21. Tielen, P., Rosin, N., Meyer, A. K., Dohnt, K., Haddad, I., Jänsch, L., Klein, J., Narten, M., Pommerenke, C., Scheer, M., Schobert, M., Schomburg, D., Thielen, B., and Jahn, D. (2013) Regulatory and metabolic networks for the adaptation of *Pseudomonas aeruginosa* biofilms to urinary tract-like conditions. *PloS One* **8,** e71845
22. O'Hara, C. M., Brenner, F. W., and Miller, J. M. (2000) Classification, identification, and clinical significance of *Proteus*, *Providencia*, and *Morganella*. *Clin. Microbiol. Rev.* **13,** 534–546
23. Williams, E. W., Hawkey, P. M., Penner, J. L., Senior, B. W., and Barton, L. J. (1983) Serious nosocomial infection caused by *Morganella morganii* and *Proteus mirabilis* in a cardiac surgery unit. *J. Clin. Microbiol.* **18,** 5–9
24. Falagas, M. E., Kavvadia, P. K., Mantadakis, E., Kofteridis, D. P., Bliziotis, I. A., Saloustros, E., Maraki, S., and Samonis, G. (2006) *Morganella morganii* infections in a general tertiary hospital. *Infection* **34,** 315–321
25. Khatri, I., Dureja, C., Raychaudhuri, S., and Subramanian, S. (2013) Draft genome sequence of the opportunistic human pathogen *Morganella morganii* SC01. *Genome Announc.* **1,** e00051–12
26. Tucci, V., and Isenberg, H. D. (1981) Hospital cluster epidemic with *Morganella morganii. J. Clin. Microbiol.* **14,** 563–566
27. Young, G. M., Amid, D., and Miller, V. L. (1996) A bifunctional urease enhances survival of pathogenic *Yersinia enterocolitica* and *Morganella morganii* at low pH. *J. Bacteriol.* **178,** 6487–6495
28. Poirel, L., Guibert, M., Girlich, D., Naas, T., and Nordmann, P. (1999) Cloning, sequence analyses, expression, and distribution of ampC-ampR from *Morganella morganii* clinical isolates. *Antimicrob. Agents Chemother.* **43,** 769–776
29. Weichhart, T., Haidinger, M., Hörl, W. H., and Säemann, M. D. (2008) Current concepts of molecular defence mechanisms operative during urinary tract infection. *Eur. J. Clin. Invest.* **38,** 29–38
30. Zasloff, M. (2007) Antimicrobial peptides, innate immunity, and the normally sterile urinary tract. *J. Am. Soc. Nephrol.* **18,** 2810–2816
31. Agace, W. W., Hedges, S. R., Ceska, M., and Svanborg, C. (1993) Interleukin-8 and the neutrophil response to mucosal gram-negative infection. *J. Clin. Invest.* **92,** 780–785
32. Haraoka, M., Hang, L., Frendéus, B., Godaly, G., Burdick, M., Strieter, R., and Svanborg, C. (1999) Neutrophil recruitment and resistance to urinary tract infection. *J. Infect. Dis.* **180,** 1220–1229
33. Hedges, S., Anderson, P., Lidin-Janson, G., de Man, P., and Svanborg, C. (1991) Interleukin-6 response to deliberate colonization of the human urinary tract with gram-negative bacteria. *Infect. Immun.* **59,** 421–427
34. Selsted, M. E., and Ouellette, A. J. (2005) Mammalian defensins in the antimicrobial immune response. *Nat. Immunol.* **6,** 551–557
35. Chromek, M., Slamova, Z., Bergman, P., Kovacs, L., Podracka, L., Ehren, I., Hökfelt, T., Gudmundsson, G. H., Gallo, R. L., Agerberth, B., and Brauner, A. (2006) The antimicrobial peptide cathelicidin protects the urinary tract against invasive bacterial infection. *Nat. Med.* **12,** 636–641
36. Håversen, L. A., Engberg, I., Baltzer, L., Dolphin, G., Hanson, L. A., and Mattsby-Baltzer, I. (2000) Human lactoferrin and peptides derived from a surface-exposed helical region reduce experimental *Escherichia coli* urinary tract infection in mice. *Infect. Immun.* **68,** 5816–5823
37. Bates, J. M., Raffi, H. M., Prasadan, K., Mascarenhas, R., Laszik, Z., Maeda, N., Hultgren, S. J., and Kumar, S. (2004) Tamm-Horsfall protein knockout mice are more prone to urinary tract infection: rapid communication. *Kidney Int.* **65,** 791–797
38. Chowdhury, P., Sacks, S. H., and Sheerin, N. S. (2004) Minireview: functions of the renal tract epithelium in coordinating the innate immune response to infection. *Kidney Int.* **66,** 1334–1344
39. Walport, M. J. (2001) Complement. First of two parts. *N. Engl. J. Med.* **344,** 1058–1066
40. Hopkins, W. J., Uehling, D. T., and Balish, E. (1987) Local and systemic antibody responses accompany spontaneous resolution of experimental cystitis in cynomolgus monkeys. *Infect. Immun.* **55,** 1951–1956
41. Jones-Carson, J., Balish, E., and Uehling, D. T. (1999) Susceptibility of immunodeficient gene-knockout mice to urinary tract infection. *J. Urol.* **161,** 338–341
42. Bradford, M. M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72,** 248–254
43. Brooks, T., and Keevil, C. W. (1997) A simple artificial urine for the growth of urinary pathogens. *Lett. Appl. Microbiol.* **24,** 203–206
44. Laemmli, U. K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227,** 680–685
45. Neuhoff, V., Arold, N., Taube, D., and Ehrhardt, W. (1988) Improved staining of proteins in polyacrylamide gels including isoelectric focusing gels with clear background at nanogram sensitivity using Coomassie Brilliant Blue G-250 and R-250. *Electrophoresis* **9,** 255–262
46. Teeling, H., Fuchs, B. M., Becher, D., Klockow, C., Gardebrecht, A., Bennke, C. M., Kassabgy, M., Huang, S., Mann, A. J., Waldmann, J., Weber, M., Klindworth, A., Otto, A., Lange, J., Bernhardt, J., Reinsch, C., Hecker, M., Peplies, J., Bockelmann, F. D., Callies, U., Gerdts, G., Wichels, A., Wiltshire, K. H., Glöckner, F. O., Schweder, T., and Amann, R. (2012) Substrate-controlled succession of marine bacterioplankton populations induced by a phytoplankton bloom. *Science* **336,** 608–611
47. Okuda, S., Tsuchiya, Y., Kiriyama, C., Itoh, M., and Morisaki, H. (2012) Virtual metagenome reconstruction from 16S rRNA gene sequences. *Nat. Commun.* **3,** 1203
48. Silva, J. C., Gorenstein, M. V., Li, G. Z., Vissers, J. P., and Geromanos, S. J. (2006) Absolute quantification of proteins by LCMSE: a virtue of parallel MS acquisition. *Mol. Cell. Proteomics* **5,** 144–156
49. Vizcaino, J. A., Deutsch, E. W., Wang, R., Csordas, A., Reisinger, F., Rios, D., Dianes, J. A., Sun, Z., Farrah, T., Bandeira, N., Binz, P. A., Xenarios, I., Eisenacher, M., Mayer, G., Gatto, L., Campos, A., Chalkley, R. J., Kraus, H. J., Albar, J. P., Martinez-Bartolomé, S., Apweiler, R., Omenn, G. S., Martens, L., Jones, A. R., and Hermjakob, H. (2014) ProteomeXchange provides globally coordinated proteomics data submission and dissemination. *Nat. Biotechnol.* **32,** 223–226

50. Schneider, T., Schmid, E., de Castro, J. V., Jr., Cardinale, M., Eberl, L., Grube, M., Berg, G., and Riedel, K. (2011) Structure and function of the symbiosis partners of the lung lichen (*Lobaria pulmonaria L. Hoffm.)* analyzed by metaproteomics. *Proteomics* **11,** 2752–2756
51. Schäffer, A. A., Wolf, Y. I., Ponting, C. P., Koonin, E. V., Aravind, L., and Altschul, S. F. (1999) IMPALA: matching a protein sequence against a collection of PSI-BLAST-constructed position-specific score matrices. *Bioinformatics* **15,** 1000–1011
52. Eddy, S. R. (2011) Accelerated Profile HMM Searches. *PLoS Comput. Biol.* **7,** e1002195
53. Tatusov, R. L., Fedorova, N. D., Jackson, J. D., Jacobs, A. R., Kiryutin, B., Koonin, E. V., Krylov, D. M., Mazumder, R., Mekhedov, S. L., Nikolskaya, A. N., Rao, B. S., Smirnov, S., Sverdlov, A. V., Vasudevan, S., Wolf, Y. I., Yin, J. J., and Natale, D. A. (2003) The COG database: an updated version includes eukaryotes. *BMC Bioinformatics* **4,** 41
54. Haft, D. H., Selengut, J. D., Richter, R. A., Harkins, D., Basu, M. K., and Beck, E. (2013) TIGRFAMs and Genome Properties in 2013. *Nucleic Acids Res.* **41,** D387–D395
55. Finn, R. D., Bateman, A., Clements, J., Coggill, P., Eberhardt, R. Y., Eddy, S. R., Heger, A., Hetherington, K., Holm, L., Mistry, J., Sonnhammer, E. L., Tate, J., and Punta, M. (2014) Pfam: the protein families database. *Nucleic Acids Res.* **42,** D222-D230
56. Bernhardt, J., Michalik, S., Wollscheid, B., Völker, U., and Schmidt, F. (2013) Proteomics approaches for the analysis of enriched microbial subpopulations and visualization of complex functional information. *Curr. Opin. Biotechnol.* **24,** 112–119
57. Camarinha-Silva, A., Wos-Oxley, M. L., Jáuregui, R., Becker, K., and Pieper, D. H. (2012) Validating T-RFLP as a sensitive and high-throughput approach to assess bacterial diversity patterns in human anterior nares. *FEMS Microbiol. Ecol.* **79,** 98–108
58. Camarinha-Silva, A., Jáuregui, R., Chaves-Moreno, D., Oxley, A. P., Schaumburg, F., Becker, K., Wos-Oxley, M. L., and Pieper, D. H. (2014) Comparing the anterior nare bacterial community of two discrete human populations using Illumina amplicon sequencing. *Environ. Microbiol.* **16,** 2939–2952
59. Lane, D. J. (1991) 16S/23S rRNA sequencing. In: Stackebrandt E., Goodfellow M., eds. *Nucleic acid techniques in bacterial systematics*. Chichester, United Kingdom: John Wiley and Sons; 1991. pp. 115–175
60. Altschul, S. F., Gish, W., Miller, W., Myers, E. W., and Lipman, D. J. (1990) Basic local alignment search tool. *J. Mol. Biol.* **215,** 403–410
61. O'Toole, G. A., and Kolter, R. (1998) Initiation of biofilm formation in *Pseudomonas fluorescens* WCS365 proceeds via multiple, convergent signaling pathways: a genetic analysis. *Mol. Microbiol.* **28,** 449–461
62. Savoia, D., and Zucca, M. (2007) Clinical and environmental *Burkholderia* strains: biofilm production and intracellular survival. *Curr. Microbiol.* **54,** 440–444
63. Bauer, A. W., Kirby, W. M., Sherris, J. C., and Turck, M. (1966) Antibiotic susceptibility testing by a standardized single disk method. *Am. J Clin. Pathol.* **45,** 493–496
64. Mobley, H. L., and Warren, J. W. (1987) Urease-positive bacteriuria and obstruction of long-term urinary catheters. *J. Clin. Microbiol.* **25,** 2216–2217
65. Hanlon, G. W., and Hodges, N. A. (1981) Bacitracin and protease production in relation to sporulation during exponential growth of *Bacillus licheniformis* on poorly utilized carbon and nitrogen sources. *J. Bacteriol.* **147,** 427–431
66. Holloway, B. W. (1955) Genetic recombination in *Pseudomonas aeruginosa. J. Gen. Microbiol.* **13,** 572–581
67. Schneider, T., Keiblinger, K. M., Schmid, E., Sterflinger-Gleixner, K., Ellersdorfer, G., Roschitzki, B., Richter, A., Eberl, L., Zechmeister-Boltenstern, S., and Riedel, K. (2012) Who is who in litter decomposition? Metaproteomics reveals major microbial players and their biogeochemical functions. *ISME J.* **6,** 1749–1762
68. Schneider, T., and Riedel, K. (2010) Environmental proteomics: analysis of structure and function of microbial communities. *Proteomics* **10,** 785–798
69. Lichtman, J. S., Marcobal, A., Sonnenburg, J. L., and Elias, J. E. (2013) Host-centric proteomics of stool: a novel strategy focused on intestinal responses to the gut microbiota. *Mol. Cell. Proteomics* **12,** 3310–3318
70. Verberkmoes, N. C., Russell, A. L., Shah, M., Godzik, A., Rosenquist, M., Halfvarson, J., Lefsrud, M. G., Apajalahti, J., Tysk, C., Hettich, R. L., and Jansson, J. K. (2009) Shotgun metaproteomics of the human distal gut microbiota. *ISME J.* **3,** 179–189
71. Ochsner, U. A., Vasil, A. I., and Vasil, M. L. (1995) Role of the ferric uptake regulator of *Pseudomonas aeruginosa* in the regulation of siderophores and exotoxin A expression: purification and activity on iron-regulated promoters. *J. Bacteriol.* **177,** 7194–7201
72. Fricke, B., Parchmann, O., Kruse, K., Rücknagel, P., Schierhorn, A., and Menge, S. (1999) Characterization and purification of an outer membrane metalloproteinase from *Pseudomonas aeruginosa* with fibrinogenolytic activity. *Biochim. Biophys. Acta* **1454,** 236–250
73. Jacobsen, S. M., Stickler, D. J., Mobley, H. L., and Shirtliff, M. E. (2008) Complicated catheter-associated urinary tract infections due to *Escherichia coli* and *Proteus mirabilis. Clin. Microbiol. Rev.* **21,** 26–59
74. Griffith, D. P., Musher, D. M., and Itin, C. (1976) Urease. The primary cause of infection-induced urinary stones. *Invest. Urol.* **13,** 346–350
75. Gruys, E., Toussaint, M. J., Niewold, T. A., and Koopmans, S. J. (2005) Acute phase reaction and acute phase proteins. *J. Zhejiang Univ. Sci. B.* **6,** 1045–1056
76. Becker, D., Selbach, M., Rollenhagen, C., Ballmaier, M., Meyer, T. F., Mann, M., and Bumann, D. (2006) Robust *Salmonella* metabolism limits possibilities for new antimicrobials. *Nature* **440,** 303–307
77. Twine, S. M., Mykytczuk, N. C., Petit, M. D., Shen, H., Sjöstedt, A., Wayne Conlan, J., and Kelly, J. F. (2006) *In vivo* proteomic analysis of the intracellular bacterial pathogen, *Francisella tularensis*, isolated from mouse spleen. *Biochem. Biophys. Res. Commun.* **345,** 1621–1633
78. Kruh, N. A., Troudt, J., Izzo, A., Prenni, J., and Dobos, K. M. (2010) Portrait of a pathogen: the *Mycobacterium tuberculosis* proteome *in vivo*. *PloS One* **5,** e13938
79. Mestas, J., and Hughes, C. C. (2004) Of mice and not men: differences between mouse and human immunology. *J. Immunol.* **172,** 2731–2738
80. Fux, C. A., Shirtliff, M., Stoodley, P., and Costerton, J. W. (2005) Can laboratory reference strains mirror "real-world" pathogenesis? *Trends Microbiol.* **13,** 58–63
81. Bainton, D. F., Ullyot, J. L., and Farquhar, M. G. (1971) The development of neutrophilic polymorphonuclear leukocytes in human bone marrow. *J. Exp. Med.* **134,** 907–934
82. Bjerregaard, M. D., Jurlander, J., Klausen, P., Borregaard, N., and Cowland, J. B. (2003) The *in vivo* profile of transcription factors during neutrophil differentiation in human bone marrow. *Blood* **101,** 4322–4332
83. Tanca, A., Palomba, A., Deligios, M., Cubeddu, T., Fraumene, C., Biosa, G., Pagnozzi, D., Addis, M. F., and Uzzau, S. (2013) Evaluating the impact of different sequence databases on metaproteome analysis: insights from a lab-assembled microbial mixture. *PloS One* **8,** e82981
84. Purschke, F. G., Hiller, E., Trick, I., and Rupp, S. (2012) Flexible survival strategies of *Pseudomonas aeruginosa* in biofilms result in increased fitness compared with *Candida albicans. Mol. Cell. Proteomics* **11,** 1652–1669
85. Schaible, U. E., and Kaufmann, S. H. (2004) Iron and microbial infection. *Nat. Rev. Microbiol.* **2,** 946–953
86. Ghysels, B., Ochsner, U., Möllman, U., Heinisch, L., Vasil, M., Cornelis, P., and Matthijs, S. (2005) The *Pseudomonas aeruginosa* pirA gene encodes a second receptor for ferrienterobactin and synthetic catecholate analogs. *FEMS Microbiol. Lett.* **246,** 167–174
87. Dean, C. R., and Poole, K. (1993) Cloning and characterization of the ferric enterobactin receptor gene (pfeA) of *Pseudomonas aeruginosa. J. Bacteriol.* **175,** 317–324
88. Cherayil, B. J. (2011) The role of iron in the immune response to bacterial infection. *Immunol. Res.* **50,** 1–9
89. Singh, P. K., Parsek, M. R., Greenberg, E. P., and Welsh, M. J. (2002) A component of innate immunity prevents bacterial biofilm development. *Nature* **417,** 552–555
90. Wilderman, P. J., Vasil, A. I., Johnson, Z., Wilson, M. J., Cunliffe, H. E., Lamont, I. L., and Vasil, M. L. (2001) Characterization of an endoprotease (PrpL) encoded by a PvdS-regulated gene in *Pseudomonas aeruginosa. Infect Immun.* **69,** 5385–5394
91. Borregaard, N., and Cowland, J. B. (2006) Neutrophil gelatinase-associated lipocalin, a siderophore-binding eukaryotic protein. *Biometals* **19,** 211–215
92. Peek, M. E., Bhatnagar, A., McCarty, N. A., and Zughaier, S. M. (2012) Pyoverdine, the Major Siderophore in *Pseudomonas aeruginosa*, Evades NGAL Recognition. *Interdiscip. Perspect. Infect. Dis.* doi: 10.

1155/2012/843509
93. Barreteau, H., Tiouajni, M., Graille, M., Josseaume, N., Bouhss, A., Patin, D., Blanot, D., Fourgeaud, M., Mainardi, J. L., Arthur, M., van Tilbeurgh, H., Mengin-Lecreulx, D., and Touzé, T. (2012) Functional and structural characterization of PaeM, a colicin M-like bacteriocin produced by *Pseudomonas aeruginosa. J. Biol. Chem.* **287,** 37395–37405
94. Cascales, E., Buchanan, S. K., Duché, D., Kleanthous, C., Lloubes, R., Postle, K., Riley, M., Slatin, S., and Cavard, D. (2007) Colicin biology. *Microbiol. Mol. Biol. Rev.* **71,** 158–229
95. Grinter, R., Milner, J., and Walker, D. (2012) Ferredoxin containing bacteriocins suggest a novel mechanism of iron uptake in *Pectobacterium spp. PloS One* **7,** e33033
96. Baughn, A. D., and Malamy, M. H. (2004) The strict anaerobe *Bacteroides fragilis* grows in and benefits from nanomolar concentrations of oxygen. *Nature* **427,** 441–444
97. Schobert, M., and Jahn, D. (2010) Anaerobic physiology of *Pseudomonas aeruginosa* in the cystic fibrosis lung. *Int. J. Med. Microbiol.* **300,** 549–556
98. Trunk, K., Benkert, B., Quäck, N., Münch, R., Scheer, M., Garbe, J., Jänsch, L., Trost, M., Wehland, J., Buer, J., Jahn, M., Schobert, M., and Jahn, D. (2010) Anaerobic adaptation in *Pseudomonas aeruginosa*: definition of the Anr and Dnr regulons. *Environ. Microbiol.* **12,** 1719–1733
99. Dahlgren, C., and Karlsson, A. (1999) Respiratory burst in human neutrophils. *J. Immunol. Methods.* **232,** 3–14
100. Jesaitis, A. J., Franklin, M. J., Berglund, D., Sasaki, M., Lord, C. I., Bleazard, J. B., Duffy, J. E., Beyenal, H., and Lewandowski, Z. (2003) Compromised host defense on *Pseudomonas aeruginosa* biofilms: characterization of neutrophil and biofilm interactions. *J. Immunol.* **171,** 4329–4339
101. Hong, Y. Q., and Ghebrehiwet, B. (1992) Effect of *Pseudomonas aeruginosa* elastase and alkaline protease on serum complement and isolated components C1q and C3. *Clin. Immunol. Immunopathol.* **62,** 133–138
102. Laarman, A. J., Bardoel, B. W., Ruyken, M., Fernie, J., Milder, F. J., van Strijp, J. A., and Rooijakkers, S. H. (2012) *Pseudomonas aeruginosa* alkaline protease blocks complement activation via the classical and lectin pathways. *J. Immunol.* **188,** 386–393
103. Parmely, M., Gale, A., Clabaugh, M., Horvat, R., and Zhou, W. W. (1990) Proteolytic inactivation of cytokines by *Pseudomonas aeruginosa. Infect. Immun.* **58,** 3009–3014
104. Bardoel, B. W., van Kessel, K. P., van Strijp, J. A., and Milder, F. J. (2012) Inhibition of *Pseudomonas aeruginosa* virulence: characterization of the AprA-AprI interface and species selectivity. *J. Mol. Biol.* **415,** 573–583
105. Alcorn, J. F., and Wright, J. R. (2004) Degradation of pulmonary surfactant protein D by *Pseudomonas aeruginosa* elastase abrogates innate immune function. *J. Biol. Chem.* **279,** 30871–30879
106. Leduc, D., Beaufort, N., de Bentzmann, S., Rousselle, J. C., Namane, A., Chignard, M., and Pidard, D. (2007) The *Pseudomonas aeruginosa* LasB metalloproteinase regulates the human urokinase-type plasminogen activator receptor through domain- specific endoproteolysis. *Infect. Immun.* **75,** 3848–3858
107. Zipfel, P. F., and Skerka, C. (2009) Complement regulators and inhibitory proteins. *Nat. Rev. Immunol.* **9,** 729–740
108. Kunert, A., Losse, J., Gruszin, C., Hühn, M., Kaendler, K., Mikkat, S., Volke, D., Hoffmann, R., Jokiranta, T. S., Seeberger, H., Moellmann, U., Hellwage, J., and Zipfel, P. F. (2007) Immune evasion of the human pathogen *Pseudomonas aeruginosa*: elongation factor Tuf is a factor H and plasminogen binding protein. *J. Immunol.* **179,** 2979–2988
109. Bernhardt, J. F., S.; Hecker, M.; Siebourg, J. (2009) Sixth international symposium on Voronoi diagrams in science and engineering. *Computer Society Digital Library CSDL*, 233–241
110. Folders, J., Tommassen, J., van Loon, L. C., and Bitter, W. (2000) Identification of a chitin-binding protein secreted by *Pseudomonas aeruginosa. J. Bacteriol.* **182,** 1257–1263
111. Mougous, J. D., Cuff, M. E., Raunser, S., Shen, A., Zhou, M., Gifford, C. A., Goodman, A. L., Joachimiak, G., Ordonez, C. L., Lory, S., Walz, T., Joachimiak, A., and Mekalanos, J. J. (2006) A virulence locus of *Pseudomonas aeruginosa* encodes a protein secretion apparatus. *Science* **312,** 1526–1530
112. Porra, R. J., Langman, L., Young, I. G., and Gibson, F. (1972) The role of ferric enterochelin esterase in enterochelin-mediated iron transport and ferrochelatase activity in *Escherichia coli. Arch. Biochem. Biophys.* **153,** 74–78
113. Wohlfarth, S., Hoesche, C., Strunk, C., and Winkler, U. K. (1992) Molecular genetics of the extracellular lipase of *Pseudomonas aeruginosa* PAO1. *J. Gen. Microbiol.* **138,** 1325–1335
114. Obernesser, H. J., Doring, G., and Botzenhart, K. (1981) Extracellular toxins of *Pseudomonas aeruginosa.* I. Purification and characterization of two exoproteases. *Zentralbl. Bakteriol. A* **249,** 76–88
115. Heck, L. W., Alarcon, P. G., Kulhavy, R. M., Morihara, K., Russell, M. W., and Mestecky, J. F. (1990) Degradation of IgA proteins by *Pseudomonas aeruginosa* elastase. *J. Immunol.* **144,** 2253–2257
116. Pham, C. T. (2006) Neutrophil serine proteases: specific regulators of inflammation. *Nat. Rev. Immunol.* **6,** 541–550
117. Baggiolini, M., Hirsch, J. G., and De Duve, C. (1969) Resolution of granules from rabbit heterophil leukocytes into distinct populations by zonal sedimentation. *J. Cell Biol.* **40,** 529–541
118. Odeberg, H., and Olsson, I. (1976) Microbicidal mechanisms of human granulocytes: synergistic effects of granulocyte elastase and myeloperoxidase or chymotrypsin-like cationic protein. *Infect. Immun.* **14,** 1276–1283
119. Ohlsson, K., and Olsson, I. (1977) Neutral proteases of human granulocytes. IV. Interaction between human granulocyte collagenase and plasma protease inhibitors. *J. Lab. Clin. Med.* **89,** 269–277
120. Olsson, I., and Venge, P. (1974) Cationic proteins of human granulocytes. II. Separation of the cationic proteins of the granules of leukemic myeloid cells. *Blood* **44,** 235–246
121. Baggiolini, M., Bretz, U., Dewald, B., and Feigenson, M. E. (1978) The polymorphonuclear leukocyte. *Agents Actions* **8,** 3–10
122. Campanelli, D., Melchior, M., Fu, Y., Nakata, M., Shuman, H., Nathan, C., and Gabay, J. E. (1990) Cloning of cDNA for proteinase 3: a serine protease, antibiotic, and autoantigen from human neutrophils. *J. Exp. Med.* **172,** 1709–1715
123. Schwarting, A., Hagen, D., Odenthal, M., Brockmann, H., Dienes, H. P., Wandel, E., Rumpelt, H. J., Zum Buschenfelde, K. H., Galle, P. R., and Mayet, W. (2000) Proteinase-3 mRNA expressed by glomerular epithelial cells correlates with crescent formation in Wegener's granulomatosis. *Kidney Int.* **57,** 2412–2422
124. Campanelli, D., Detmers, P. A., Nathan, C. F., and Gabay, J. E. (1990) Azurocidin and a homologous serine protease from neutrophils. Differential antimicrobial and proteolytic properties. *J. Clin. Invest.* **85,** 904–915
125. Weiss, J., and Elsbach, P. (1977) The use of a phospholipase A-less Escherichia coli mutant to establish the action of granulocyte phospholipase A on bacterial phospholipids during killing by a highly purified granulocyte fraction. *Biochim. Biophys. Acta* **466,** 23–33
126. Zeya, H. I., and Spitznagel, J. K. (1963) Antibacterial and enzymic basic proteins from leukocyte lysosomes: separation and identification. *Science* **142,** 1085–1087
127. Zeya, H. I., and Spitznagel, J. K. (1966) Antimicrobial specificity of leukocyte lysosomal cationic proteins. *Science* **154,** 1049–1051
128. Zeya, H. I., Spitznagel, J. K., and Schwab, J. H. (1966) Antibacterial action of PMN lysosomal cationic proteins resolved by density gradient electrophoresis. *Proc. Soc. Exp. Biol. Med.* **121,** 250–253
129. Segal, A. W., and Jones, O. T. (1979) Identification of a previously undescribed cytochrome b in human neutrophils and its relationship to phagocytosis-induced oxidase activity. *Biochem. Soc. Trans.* **7,** 187–188
130. Masson, P. L., Heremans, J. F., and Schonne, E. (1969) Lactoferrin, an iron-binding protein in neutrophilic leukocytes. *J. Exp. Med.* **130,** 643–658
131. Abrink, M., Larsson, E., Gobl, A., and Hellman, L. (2000) Expression of lactoferrin in the kidney: implications for innate immunity and iron metabolism. *Kidney Int.* **57,** 2004–2010
132. Agerberth, B., Charo, J., Werr, J., Olsson, B., Idali, F., Lindbom, L., Kiessling, R., Jörnvall, H., Wigzell, H., and Gudmundsson, G. H. (2000) The human antimicrobial and chemotactic peptides LL-37 and alpha-defensins are expressed by specific lymphocyte and monocyte populations. *Blood* **96,** 3086–3093
133. Bals, R., Goldman, M. J., and Wilson, J. M. (1998) Mouse beta-defensin 1 is a salt-sensitive antimicrobial peptide present in epithelia of the lung and urogenital tract. *Infect. Immun.* **66,** 1225–1232
134. Hasty, K. A., Pourmotabbed, T. F., Goldberg, G. I., Thompson, J. P., Spinella, D. G., Stevens, R. M., and Mainardi, C. L. (1990) Human

neutrophil collagenase. A distinct gene product with homology to other matrix metalloproteinases. *J. Biol. Chem.* **265,** 11421–11424
135. Bundgaard, J. R., Sengelov, H., Borregaard, N., and Kjeldsen, L. (1994) Molecular cloning and expression of a cDNA encoding NGAL: a lipocalin expressed in human neutrophils. *Biochem. Biophys. Res. Commun.* **202,** 1468–1475
136. Cowland, J. B., Sorensen, O. E., Sehested, M., and Borregaard, N. (2003) Neutrophil gelatinase-associated lipocalin is up-regulated in human epithelial cells by IL-1 beta, but not by TNF-alpha. *J. Immunol.* **171,** 6630–6639
137. Egesten, A., Alumets, J., von Mecklenburg, C., Palmegren, M., and Olsson, I. (1986) Localization of eosinophil cationic protein, major basic protein, and eosinophil peroxidase in human eosinophils by immunoelectron microscopic technique. *J. Histochem. Cytochem.* **34,** 1399–1403
138. Peters, M. S., Rodriguez, M., and Gleich, G. J. (1986) Localization of human eosinophil granule major basic protein, eosinophil cationic protein, and eosinophil-derived neurotoxin by immunoelectron microscopy. *Lab. Invest.* **54,** 656–662