Flexible Survival Strategies of *Pseudomonas aeruginosa* in Biofilms Result in Increased Fitness Compared with *Candida albicans*\(^*\)\(^[S]\)

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The majority of microorganisms persist in nature as surface-attached communities often surrounded by an extracellular matrix, called biofilms. Most natural biofilms are not formed by a single species but by multiple species. Microorganisms not only cooperate as in some multispecies biofilms but also compete for available nutrients. The Gram-negative bacterium *Pseudomonas aeruginosa* and the polymorphic fungus *Candida albicans* are two opportunistic pathogens that are often found coexisting in a human host. Several models of mixed biofilms have been reported for these organisms showing antagonistic behavior. To investigate the interaction of *P. aeruginosa* and *C. albicans* in more detail, we analyzed the secretome of single and mixed biofilms of both organisms using MALDI-TOF MS/MS at several time points. Overall 247 individual proteins were identified, 170 originated from *P. aeruginosa* and 77 from *C. albicans*. Only 39 of the 131 in mixed biofilms identified proteins were assigned to the fungus whereby the remaining 92 proteins belonged to *P. aeruginosa*. In single-species biofilms, both organisms showed a higher diversity of proteins with 73 being assigned to *C. albicans* and 154 to *P. aeruginosa*. Most interestingly, *P. aeruginosa* in the presence of *C. albicans* secreted 16 proteins in significantly higher amounts or exclusively among other virulence factors such as exotoxin A and iron acquisition systems. In addition, the high affinity iron-binding siderophore pyoverdine was identified in mixed biofilms but not in bacterial biofilms, indicating that *P. aeruginosa* increases its capability to sequester iron in competition with *C. albicans*. In contrast, *C. albicans* metabolism was significantly reduced, including a reduction in detectable iron acquisition proteins. The results obtained in this study show that microorganisms not only compete with the host for essential nutrients but also strongly with the present microflora in order to gain a competitive advantage.

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Most microorganisms persist in nature by growing in surface-attached communities surrounded by an extracellular matrix, called biofilms (1). Biofilm formation is a developmental process starting from planktonic cells adhering to a surface and proceeds with a change in gene expression patterns inducing the formation of multicellular structures embedded in an extracellular matrix with distinct architecture, sometimes including cellular differentiation. Formation of biofilms is a dynamic process, which for many microorganisms is based on small, self-generated signal molecules called autoinducers, enabling chemical communication between the organisms. This process was termed *quorum sensing* (2). This form of communication includes both same-species communication and interspecies crosstalk (3). Biofilms also play an important role in human diseases. The presence of biofilms in infections and on surfaces of medical devices often results in severe complications in patient care, pronounced by an increased resistance of the biofilm-resident microorganisms to antibiotics and protection against the human immune system (4).

Two facultative pathogens, *Candida albicans* and *Pseudomonas aeruginosa*, are often found coexisting in the human host (5). Notably both organisms were found in mixed populations on intravenous catheters (6). *C. albicans* usually colonizes mucosal surfaces but can also cause recurrent mucosal and life-threatening disseminated infections associated with high mortality rates in immunosuppressed patients (7). *P. aeruginosa* is often found as part of the normal skin flora. Also, the Gram-negative bacterium is an important nosocomial germ infecting immunocompromised individuals and especially patients with cystic fibrosis.

As described above, *C. albicans* has been found to form biofilms on medical devices, such as catheters, causing severe complications in patient care (8). Both yeast and hyphal growth forms are present in these biofilms. The transition from yeast to hyphae is a crucial step in biofilm formation and pathogenicity in general (9). It has been shown that the quorum-sensing molecules farnesol and tyrosol have an important regulatory role in this morphological transition (10, 11) and also modulate biofilm formation (3, 12). Biofilm formation of *C. albicans* has been studied in great detail including the use of proteomics (13, 14) and transcriptional profiling in the presence and absence of farnesol, which confirmed the ef-
ffects of farnesol and the clear differences between planktonic and biofilm lifestyle (15–19).

*P. aeruginosa* biofilms are regulated by quorum-sensing molecules as well. It contains two interconnected acylhomoserine lactone-based quorum-sensing systems, Las and Rhl with the cognate autoinducers N-(3-oxo-dodecanoyl)-L-homoserine lactone and N-butyl-L-homoserine lactone, which also regulate the production of virulence factors (2, 20, 21). A third signaling molecule, 2-heptyl-3-hydroxy-4-quinolone, denoted PQS1 for *Pseudomonas* quinolone signal, is connected to the Las and Rhl systems as well (22). Additionally, 2-heptyl-4-quinolone, the precursor of PQS, was discovered to function as a signal molecule (23). Transcriptional profiling of *P. aeruginosa* in response to PQS revealed a marked up-regulation of genes belonging to the tightly interdependent functional groups of the iron acquisition and the oxidative stress response (24). Interestingly, one function of PQS is to work as a ferric iron chelator, which traps iron at the cell surface in order to facilitate siderophore-mediated iron uptake (23, 25). Thus, via PQS, the availability of iron is directly connected to the acylhomoserine lactone-based biofilm regulatory systems (26) (for reviews on iron uptake see (27, 28)).

The interaction of *P. aeruginosa* and *C. albicans* has been studied by several laboratories showing that they are antagonizing each other rather than acting synergistically as shown for several other mixed biofilms (29, 30). During colonization of surfaces, *P. aeruginosa* and *C. albicans* appear to compete for available binding sites, because the amount of colony forming units indicated that adherence of both microorganisms is reduced in dual-species compared with mono-species biofilms (31). *P. aeruginosa* has been described to form dense biofilms on fungal filaments resulting in killing of hyphal cells mediated by several virulence factors including pil and secreted molecules (30). However, binding of *P. aeruginosa* to the yeast form of *C. albicans* was not observed. Because of farnesol-like activities of *N-(3-oxo-dodecanoyl)-L-homoserine lactone* from *P. aeruginosa*, which represses filamentation and induces the shift to yeast growth, fungal morphology and virulence are significantly affected (30, 32). Supernatants of planktonic grown *P. aeruginosa* cells led to changes in gene expression of early *C. albicans* biofilms, mainly independent of *N-(3-oxo-dodecanoyl)-L-homoserine lactone* (33). However, farnesol modulates *Pseudomonas* behavior and virulence by altering the production of PQS (34) and thereby inducing toxic phenazines, e.g. pyocyanin in *P. aeruginosa* (35). Expression of these toxins was reported to result in a reduction of growth for both species in mixed biofilms (31). Furthermore, the phenazine derivatives pyocyanin and 1-hydroxyphenazine were shown to inhibit yeast mycelia transformation and fungal growth, suggesting a role in prevention of pulmonary candidiasis in patients colonized by *P. aeruginosa* (36). Increased levels of pyocyanin produced by *P. aeruginosa* co-cultured with *C. albicans* were found to correlate with decreased fungal viability (37). Additionally, 5-methyl-phenanzinium-1-carboxylate contributes to fungal killing by retaining toxic activity within fungal cells (38). These findings indicate that *C. albicans* and *P. aeruginosa* recognize and react to each other.

To gain more insight into how biofilm formation and interaction of *C. albicans* and *P. aeruginosa* are affected by each other, the secretomes of mixed biofilms were isolated, analyzed by LC-MALDI MS and compared with the respective single-species biofilm secretomes at different times for up to 48 h. Growing in spatial contiguity in biofilms, microorganisms may be able to influence and compete against each other by altering the composition of secreted proteins. Our findings indicate that *P. aeruginosa* rapidly increases its capability to sequester iron, which is not observed in *C. albicans*. This most likely contributes in gaining a competitive growth advantage over *C. albicans*. This adds another important point to explain the consequences of the antagonistic behavior of *C. albicans* and *P. aeruginosa* and confirms the idea that the host microflora is critical for host protection against pathogens by preventing their colonization also through nutrient limitation.

**EXPERIMENTAL PROCEDURES**

**Strains and Culture Conditions—** *C. albicans* strain SC5314 was used in this study, propagated in YPD medium (10 g yeast extract, 20 g peptone, 20 g glucose per liter) at 30 °C (39). The *P. aeruginosa* strain PAO1, kindly provided by Barbara H. Iglewski (University of Rochester, School of Medicine and Dentistry), was propagated in LB medium (10 g bacto tryptone, 5 g yeast extract, 5 g NaCl per liter, pH 7.0) at 37 °C (40). Three overnight cultures of each strain were harvested at 4200 g for 10 min, washed with YNBNP (1.7 g Difco Yeast Nitrogen Base without amino acids and ammonium sulfate, 25 mM phosphate buffer pH 7.0, 2.5 mM *N*-acetylglucosamine per liter) (32) and adjusted to an optical density of 1.0 (Jasco V-630 Spectrophotometer; 3 × 10⁶ cells/ml for *C. albicans*; 1 × 10⁸ cells/ml for *P. aeruginosa*) in YNBNP. For biofilm formation, 30 ml of three independent cell suspensions (SC5314, PAO1, SC5314 + PAO1) were incubated in Petri dishes (order number 82.1473.001; Sarstedt, Germany) at 37 °C without shaking. After adherence for 90 min, the supernatant was replaced by 30 ml of fresh medium and incubated for an additional 1.5, 3, 4.5, 6, 24, or 48 h.

**Isolation of Secreted Proteins—** For the identification of secreted proteins, supernatants of the biofilms were collected and bacterial and yeast cells were removed by filtration (PES membrane, 0.2-μm pore size; Sarstedt). Proteins in the supernatant were concentrated by ultrafiltration (Amicon® Ultra-15 Centrifugal filter units, MWCO: 10 kDa; Millipore, Germany) at room temperature and stored at −20 °C. Protein concentration was measured using DC protein microplate assay (Bio-Rad, Germany) (41).

**Identification of Secreted Proteins by Mass Spectrometry—** One microgram of total protein of three biological replicates were pooled for all conditions (SC5314, PAO1, SC5314 + PAO1) and time points (1.5, 3, 4.5, 6, 24, 48 h) and reduced by 5 mM tris(2-carboxyethyl)-phosphine in 0.5 M triethylammoniumbicarbonate at 60 °C for 60 min followed by alkylation using 3.5 mM iodoacetamide for 30 min at room temperature in the dark. The proteins were digested by adding trypsin.
Survival of *P. aeruginosa* and *C. albicans* in Mixed Biofilms (sequencing grade modified trypsin; Promega) in a ratio (w/w) of 1:50 and incubation for 16 h at 37 °C. After stopping the reaction by vaporizing, the peptide mixtures were resolubilized in 0.1% trifluoroacetic acid and 2 μg were used for reversed-phase Nano Liquid Chromatography (Ultimate 3000 nanoflow LC system; Dionex). Peptides were desalted at a flow rate of 20 μl/min (Acclaim PepMap 100, C18, 5-μm inner diameter × 5 mm; Dionex) and subsequently fractionated on an analytical column (Acclaim PepMap100, C18, 5 μm, 100 Å, 75-μm inner diameter × 25 cm) with a linear gradient of 5%–50% diluent (80% acetonitrile in 0.1% trifluoroacetic acid) with a flow rate of 300 nl/min for 180 min at 40 °C. The column end was directly connected to the spotting tip of a Proteineer fc fraction collector (Bruker Daltonics, Germany). α-Cyano-4-hydroxycinnamic acid matrix solution was prepared by adding 1/40 of saturated α-cyano-4-hydroxycinnamic acid solution (90% acetonitrile, 0.1% trifluoroacetic acid) to 1 mm NH₄HPO₄ in 90% acetonitrile and 0.1% trifluoroacetic acid, which was pumped to the spotting tip and therein mixed with the column elution. The mixture was deposited onto a 384-well anchorChip target (Bruker Daltonics) every 20 s between minutes 20 to 148. To minimize carryover, one idle circuit was integrated after each sample. The calibration mixture (Peptide Calibration Standard II; Bruker Daltonics) with matrix was afterward manually plotted on the calibration spots.

The monoisotopic mass of the peptides was determined by mass spectrometry (MS) using matrix-assisted laser desorption ionization-time-of-flight (MALDI-TOF) (Ultraflex II TOF/TOF 200; Bruker Daltonics) with the mass spectrometer scanning a mass range from 700 to 5000 Da. Mass spectra were acquired in reflector mode and processed using WARP-LC, FlexControl, and FlexAnalysis 3.3 software (Bruker Daltonics). Masses for MS/MS measurement (signal/noise >20, maximal 30 MS/MS measurements per fraction) were selected from the mass lists generated before using WARP-LC software. Fragment spectra of the peptides for MS/MS analysis were generated using the LIFT mode of the instrument.

For the identification of proteins, MS/MS spectra were submitted to the database search program MASCOT 2.2.07 (Matrix Science, UK) by BioTools (Bruker Daltonics). A protein database based on Assembly 21 of translated open reading frames of the *C. albicans* SC5314 genome from the Candida genome database (http://www.candidagenome.org/) and on NC002516 of strain PA01 from the Pseudomonas genome database (http://www.pseudomonas.com) (42) containing 11,774 entries was used with the following search parameters: allowing up to one missed cleavage, tolerance of 100 ppm for peptides and 0.5 Da for MS/MS (MALDI-TOF/TOF, enzyme: semi-trypsin, variable modifications: oxidation (M), fixed modifications: carbamidomethyl (C)). Probability-based MASCOT scores were used to evaluate protein identifications. Only peptides with p values < 0.05 for random occurrence were considered to be significant. To estimate potential false-positive correlations, measurements were assigned to an equal-sized synthetic database containing randomized protein sequences. The average false-positive rate of assignments was approximately 2.8%. This suggests that in samples of *P. aeruginosa* biofilms, 17 proteins related to *C. albicans* genome database (http://www.pseudomonas.com) and on NC002516 of strain PAO1 from the *Candida* genome database (candidagenome.org/) and on NC002516 of strain PAO1 from the *Candida* genome database (candidagenome.org/) and on NC002516 of strain PAO1 from the *Candida* genome database (candidagenome.org/), and 10 fungal proteins were potential false positives in biofilms, 17 proteins related to *C. albicans* genome database (http://www.pseudomonas.com) and on NC002516 of strain PAO1 from the *Candida* genome database (candidagenome.org/) and on NC002516 of strain PAO1 from the *Candida* genome database (candidagenome.org/) and on NC002516 of strain PAO1 from the *Candida* genome database (candidagenome.org/), and 10 fungal proteins were potential false positives in biofilms.
their respective quorum-sensing molecules (34). To better understand this interaction, we attempted to analyze the secretome of both organisms growing either as single-species biofilms or in combination. To do that, we first defined the biofilm growth condition to obtain comparable results.

Selection of Growth Conditions for Secretome Analysis—To compare single- and mixed-species biofilms, we tested several growth conditions including substrate, initial amount of cells, and media for biofilm formation of C. albicans and P. aeruginosa. The medium YNBNP was chosen, which was described earlier for interaction studies of C. albicans and P. aeruginosa (30) because it supports biofilm formation for both microorganisms in mono and mixed cultures and because it does not contain complex protein mixtures that would make analysis of the secretome much more difficult. After an initial incubation period of 90 min, non-adherent cells were removed and replaced by fresh medium to exclude planktonically growing cells. Because the formation of biofilms is based on the amount of cells remaining attached to the surface, the number of cells was compared for the different conditions used. To estimate the number of cells, we used CNF-labeled P. aeruginosa cells and the XTT reduction assay for C. albicans as described in the Experimental Procedures section. At the concentration of cells used for seeding the biofilms, our analyses revealed for C. albicans >80% of the XTT activity in mixed biofilms (10% background activity of P. aeruginosa was subtracted) whereas for P. aeruginosa, no statistically significant difference was observed. These results confirm a similar starting point of the experiments with regard to cell population making the results obtained in the following experiments comparable (Fig. 1). Using different seeding and growth conditions as well as other strains, a significant reduction of adhering cells of both organisms in dual-species biofilms was noted compared with mono-species biofilms (31).

Because early phases of biofilm formation including the initial adherence steps are crucial steps in colonization of medical devices or patients, early time points (1.5, 3, 4.5, 6 h) were analyzed. During these time points of biofilm formation, C. albicans also undergoes the morphological switch from yeast to hyphae. It was shown previously that interactions of P. aeruginosa and C. albicans alter the fungal morphology early on (30). Therefore, we hypothesized that responses of P. aeruginosa and C. albicans sensing the counterpart become most apparent at early phases. To analyze how the presence of C. albicans and P. aeruginosa affect more mature biofilms, 24- and 48-h time points were included.

Morphology of Single-species and Mixed Biofilms—To visualize differences in biofilm formation of C. albicans and P. aeruginosa in single-species and mixed biofilms, scanning electron microscopy was performed for all time points analyzed (Fig. 2). Both microorganisms built distinct biofilms. Biofilms of P. aeruginosa at time point 6 h formed a monolayer of connected cells. In 48-h-grown biofilms, the amount of cells enclosed in this layer and the development of microfibers increased resulting in the development of a three-dimensional structure (Figs. 2C and 2D). This is in agreement with previous observations of P. aeruginosa biofilms grown under static conditions (31, 50). For C. albicans, after the initial adherence phase resulting in a monolayer of yeast-form cells, the fungus started to build three-dimensional structures already after 6 h through the development of thriving hyphae. During these first 6 h, the hyphae and pseudohyphae elongated to build structured yeast-hyphae networks merged with microfibers. Yeast cells originally plated remained attached to the surface while the three-dimensional architecture consisted mainly of hyphae also at later time points (Figs. 2A and 2B). This observation of biofilm development under static conditions agrees with previous reports (51, 52).

Visualizing mixed biofilms consisting of C. albicans and P. aeruginosa, it was striking to see that fungal and bacterial
cells built preferably layered biofilms. Yeast cells remained at the plate surface covered by an accumulating network of hyphae that is roofed by a bacterial layer merged with microfibers. In the intersection of both biofilms, *P. aeruginosa* cells are observed covering fungal cells, mostly hyphae but remarkably also yeast cells (Figs. 2E and 2F). In these mixed biofilms, a much larger fraction of *C. albicans* yeast cells was observed, which is in agreement with the finding that *P. aeruginosa* blocks hyphal development in *C. albicans* (30, 31). However, at early time points, long filaments of *C. albicans* were observed similar to those in single fungal biofilms and some hyphae persisted after 48 h of co-incubation. This confirms that regulation of hyphal elongation and biofilm formation of *C. albicans* is altered in the presence of *P. aeruginosa*. These conditions provided the basis for further analyses.

**Comparison of Metabolic Activity of C. albicans between Single-species and Mixed Biofilms**—Because the appearance of *C. albicans* was different in the presence of *P. aeruginosa* compared with single-species biofilms and the negative impact on *C. albicans* growth was observed previously (30), we analyzed how strongly the fungal activity was hampered during initial and late phases in mixed biofilms using the XTT assay. This assay measures the overall XTT throughput, generally related to the overall metabolic activity of a cell population. Using a protocol optimized for *C. albicans* as described in the Experimental Procedures section, we observed only limited formazan production by *P. aeruginosa* at about 10% of the fungal activities in early time points (Fig. 3, dark gray bars) giving a rough estimate of the background activity of the assay in the presence of this organism (see also Fig. 1).
We could confirm that in mixed biofilms of *C. albicans* and *P. aeruginosa* the overall XTT throughput of *C. albicans* is reduced compared with purely fungal biofilms (Fig. 3). Thus, the overall metabolic activity of *C. albicans*, either by reduction of cell numbers or the reduction of individual cellular metabolism is diminished in mixed biofilms. For the early time points, reduced activities between 60% and 70% were detected for *C. albicans*, whereas in later time points, a reduction of up to 80% was observed (24 h), but at a significantly lower level of XTT activities. After 72 h, the XTT levels dropped both in single and mixed biofilms to approximately 10% of the initial activity (Fig. 3), because of metabolization of the nutrients initially provided.

**Total Amount of Protein Secreted by Single-species and Mixed Biofilms**—To unravel some of the differences in the development of single-species and mixed biofilms observed in morphology (Fig. 2) and metabolism (Fig. 3), we set out to identify their secretomes. At the time points mentioned above, up to 48-h supernatants of the biofilms were removed, filtered to remove contaminating cells, and concentrated (see Experimental Procedures). In a first step, the total protein concentration released from the biofilm was determined. Fig. 4A shows the total amount of protein determined for the different samples averaged of three independent replicates. The protein concentration in the supernatant increased for *C. albicans*, *P. aeruginosa*, and mixed biofilms over time. From *P. aeruginosa* biofilm supernatants, total protein up to 23 μg in 30 ml biofilm medium could be recovered after 48 h (Fig. 4A, dark gray bars) whereas from *C. albicans* biofilms, 58 μg could be recovered (Fig. 4A, light gray bars). Remarkably, from mixed biofilms we could recover more protein than the sum of single-species biofilms. After 48 h growth, 124 μg total protein was isolated (Fig. 4A, black bars). This observation could be attributable to secretion of a more complex mixture of proteins in mixed biofilms than in single-species biofilms, or to quantitative increase in protein secretion. Also, increased lysis of cells may be possible resulting in a significant increase in protein concentration in the supernatant. To address these questions, we analyzed the proteins in the supernatant using MALDI-TOF MS/MS.

**Analysis of Proteins Secreted by Mixed Biofilms and Comparison to Single *C. albicans* and *P. aeruginosa* Biofilms**—The general setup of the experiment is determined by the total amount of proteins in the supernatant in early biofilms. Be-
cause the concentration of proteins at early time points is rather low (see Fig. 4A), a strategy of pooling biological replicates was performed. Previous studies have shown that the statistical difference of the results from technical, biological, or pooled replicates in general are small both in microarray and proteomic experiments (53, 54). In an initial experiment, we evaluated whether this could be confirmed for our approach. Therefore, we compared the results obtained from pooled biological replicates and individual analysis of biological replicates. For this purpose, an equal amount of proteins from three biological replicates of 48 h grown biofilms were tryptic digested, separated by reverse-phase liquid chromatography, and identified by MS/MS. In parallel, identical protein amounts of pooled biological replicates were used for tryptic digestions, separation, and identification, performing technical duplicates. Consistency of biological and technical replicates was verified by t tests. No significant differences (p < 0.01) in peptide counts of the mean of biological replicates and the mean of technical replicates were found for >95% of the identified proteins. This was verified for two different samples. This reflects the general reproducibility of proteome analysis by MS, as shown by others (43, 55). These results are summarized in supplemental material 2. Because of the small amount of total protein in samples of early biofilms (Fig. 4A), technical duplicates of pooled biological replicates were used for further analysis of all time points.

**Single-species Biofilms**—The proteins identified from *P. aeruginosa* biofilms are summarized in supplemental material 1. Of all *P. aeruginosa* proteins detected, 66 contain a characteristic export signal sequence (37%), which represents the percentage of predicted secreted proteins (38%) of the whole *P. aeruginosa* proteome (56). This percentage did not vary significantly between single-species and mixed biofilms. In previous studies of secreted proteins using 2DE, approximately 50% of the identified proteins were marked for secretion (57). Also in other studies, several proteins without export sequence have been found located extracellular previously, like HasAp, or were identified in other microorganisms as so-called moonlighting proteins (58). Proteins corresponding to surface components such as flagellin-associated proteins, have been identified in the secretome before (57). This could be due to ejection of these appendages in biofilms or to natural degradation by protein turnover during growth. In purely bacterial biofilms proteins related to central metabolic activity, among others, ribosomal proteins were found represented by limited peptides that were not identified in mixed biofilms (supplemental material 1). Some other cytosolic proteins such as ArcB (PA5172) were observed with more peptides in pure bacterial biofilms. Further on, PA0620, PA0622, and PA0623 were found represented by significantly more peptides (p values for random occurrence of peptide accumulation 0.009, 0.000, 0.009) in single bacterial biofilms (Table I). These proteins are related to R-type pyocin, a cell death causing bacteriocin (59). This might suggest increased cell lysis in bacterial biofilms compared with mixed biofilms. However, most of the cytosolic proteins observed are represented by a limited amount of peptides, indicating low abundances in the supernatant permitting only limited lysis during the analyzed growth conditions.

For *C. albicans*, 55 proteins are predicted secretory proteins (68%) with an N-terminal secretory signal sequence (60) including 22 glycosylphosphatidylinositol (GPI)-anchored proteins (61). Six additional proteins have cell wall or membrane-related functions or locations, or have been found in supernatants before such as Tsa1A/Tsa1B (62) or Grp2 (14). In earlier studies, membrane-associated and predicted GPI-anchored proteins have been identified in culture supernatants as well (46, 63). Wall-associated proteins might have been released during cell wall remodeling or before their attachment to membrane polysaccharides.

For both single-species secretomes, more proteins were identified for the consecutive time points, except for the first time point in *P. aeruginosa* biofilms (Fig. 4B), although the same amount of total protein was used. This effect may be attributable to a larger number of proteins being represented in amounts that are below the detection/identification level at earlier time points. Because the increase in identified peptides cannot be attributed to differences in the amount of protein used for the individual measurements, which was additionally confirmed by UV spectra recorded during fractionation by nano-HPLC (supplemental material 3), we assume that the actual complexity of proteins is reduced at later time points. The result would be a higher concentration of individual proteins, which can be more easily detected, as observed by us.

With maturation of biofilms, different cell layers within the three-dimensional structure will take over different functions, resulting in changing expression patterns. The number of proteins identified at 1.5 h grown *P. aeruginosa* biofilms is conspicuously high compared with 3 and 6 h samples. A noticeable large number of *P. aeruginosa* proteins identified at this early time point are represented by just one peptide, while proteins showing high abundances are exceptional cases. In the course of biofilm development, secreted proteins agglomerated or were restricted to a defined phase. In *C. albicans* biofilms, the pattern of protein secretion clearly allows discrimination between early (1.5 to 6 h) and mature biofilms (24 and 48 h). Secretion of, for example, extracellular operating proteins such as Sun41 (orf19.3642, p value 0.004) and members of the Sap family (orf19.5542 and orf19.756) significantly increased in mature biofilms (Table II).

**Dual-species Biofilm**—Although the entire amount of secreted proteins detected increased in mixed-species biofilms, the diversity of proteins observed that were secreted by the individual species in mixed biofilms was reduced, if compared with the single-species biofilms (Table III, Fig. 4B). The number of *C. albicans* proteins detected in single-species biofilms amounts to 73 whereas only 39 different *C. albicans* proteins
### TABLE I

**Proteins assigned to *P. aeruginosa* that have been identified secreted in specific patterns either during the time courses of mono- or mixed-species biofilms or compared between mono- and mixed-species biofilms**

Numbers represent the average amount of peptides of two technical replicates that have been found in *P. aeruginosa* biofilms (upper rows) or mixed biofilms (lower rows). Proteins with predicted secretion domains are marked with an asterisk.

| Accession | Name                                | Function                              | 1.5 h | 3 h  | 4.5 h | 6 h  | 24 h | 48 h |
|-----------|-------------------------------------|---------------------------------------|-------|------|-------|------|------|------|
| PA0049*   | Hypothetical protein                |                                       | 1.5   | 3.0  |       |      |      |      |
| PA0146*   | Conserved hypothetical protein      |                                       | 2.0   | 6.0  |       |      |      |      |
| PA0300*   | spuD                               | Polyamine transport protein           | 0.5   | 1.0  | 3.5   |      |      | 1.5  |
| PA0423*   | pasP                               | PasP protease                         | 9.0   | 6.0  | 12.5  | 4.5  | 11.5 | 12.0 |
| PA0468*   | Hypothetical protein                |                                       | 4.5   | 9.0  | 5.0   | 4.5  | 12.0 | 19.0 |
| PA0572*   | Hypothetical protein                |                                       | 3.0   | 1.0  | 5.0   | 3.5  |      |      |
| PA0620*   | Probable bacteriophage protein      |                                       | 1.0   | 4.5  | 2.5   | 4.5  | 29.5 | 16.5 |
| PA0622    | Probable bacteriophage protein      |                                       |       |      | 0.5   | 2.0  |      |      |
| PA0623    | Probable bacteriophage protein      |                                       | 1.0   | 1.0  | 6.5   | 6.0  |      |      |
| PA0807    | ampDh3                              | AmpDh3 N-acetyl-anhydro-muramyl-L-alanine amidase | 3.5   | 2.5  | 6.5   | 3.5  | 9.0  | 13.5 |
| PA0852*   | cbpD                                | Chitin-binding protein CbpD precursor | 10.0  | 1.0  | 1.5   | 0.5  | 4.5  | 6.5  |
| PA0888*   | aotJ                                | Arginine/ornithine-binding protein AotJ | 3.5   | 0.5  | 1.0   | 1.0  | 4.0  |      |
| PA0962    | oprL                                | Probable DNA-binding stress protein   | 0.5   | 1.5  | 1.5   | 2.0  | 5.5  | 7.5  |
| PA0973*   | oprL                                | Peptidoglycan-associated lipoprotein OprL precursor | 0.5   | 1.5  | 1.5   | 2.0  | 5.5  | 7.5  |
| PA1074*   | braC                                | Branched-chain amino acid transport protein BraC | 1.0   | 0.5  | 1.0   | 2.0  | 1.5  | 1.0  |
| PA1092    | fiIC                                | Flagellin type B                      | 5.5   | 2.5  | 1.5   | 2.0  | 1.5  | 1.5  |
| PA1094    | fiID                                | Flagellar capping protein FiID        | 1.0   | 1.0  | 1.5   | 3.0  | 13.0 | 20.0 |
| PA1148*   | toxA                                | Exotoxin A precursor                  | 4.0   | 3.5  | 1.5   | 5.0  | 5.5  | 21.5 |
| PA1249    | aprA                                | Alkaline metalloprotease precursor    | 6.5   | 11.0 | 47.5  | 48.0 | 64.0 | 67.0 |
| PA1342*   | Probable binding protein component of ABC transporter | 4.0   | 3.5  | 1.5   | 5.0  | 12.0 | 15.0 |
| PA1777*   | oprF                                | Major porin and structural outer membrane porin OprF precursor | 1.0   | 1.5  | 1.5   | 4.5  | 6.5  | 6.5  |
| PA1871*   | lasA                                | LasA protease precursor               | 1.5   | 0.5  | 2.0   | 3.0  | 2.0  | 1.0  |
| PA1982*   | exaA                                | Quinoprotein ethanol dehydrogenase    | 1.0   | 8.5  | 14.5  |      |      |      |
| PA2451    | Hypothetical protein                |                                       | 8.5   | 4.5  | 5.5   | 24.5 | 20.0 | 8.5  |
| PA2452*   | Hypothetical protein                |                                       | 0.5   | 1.0  | 2.0   | 33.0 | 25.5 | 18.0 |
| PA2453*   | Hypothetical protein                |                                       | 0.5   | 0.5  | 3.0   | 3.0  |      |      |
| PA2462*   | Hypothetical protein                |                                       | 1.0   | 0.5  | 1.0   | 0.5  | 0.5  | 1.5  |
| PA2623    | icd                                 | Isocitrate dehydrogenase              | 5.0   | 2.0  | 2.0   | 1.0  |      |      |
| PA2787*   | cpg2                                | Carboxypeptidase G2 precursor         | 0.5   | 1.0  | 0.5   | 0.5  |      |      |
| PA2862*   | lipA                                | Lactonizing lipase precursor          | 1.0   | 3.0  | 12.0  |      |      |      |
| PA2939*   | Probable aminopeptidase             |                                       | 6.0   | 1.5  | 11.5  | 28.5 | 33.5 | 25.5 |
were detected in mixed-species biofilms. From the total of 77 C. albicans proteins detected over all time points and conditions, four fungal proteins were detected only in mixed biofilms (Table IV), but only with low amounts of peptides identified for the individual proteins, whereas 38 proteins were exclusively detected in single-species biofilms. In addition, the number of excreted fungal proteins stagnated between 23 and 27 at all time points, implying a bacterial prevalence (Table III, Fig. 4B). Therefore, in mixed biofilms, the increase in the number of proteins identified in the secretome over time is largely dependent on proteins secreted by P. aeruginosa. For P. aeruginosa, the number of proteins detected in the supernatant of single-species biofilms was 154, also much higher than the 92 proteins detected in mixed-species biofilms. However, in total, 16 proteins were detected only in mixed biofilms and 10 were represented by significantly more pep-
| Accession     | Name               | Function                                   | 1.5 h | 3 h  | 4.5 h | 6 h  | 24 h | 48 h |
|--------------|--------------------|--------------------------------------------|-------|------|-------|------|------|------|
| orf19.1097*  | Als2               | ALS family protein                         | 2.5   | 2.0  | 1.5   | 1.0  |      |      |
| orf19.1442*  | Plb4.5             | Putative phospholipase                     | 1.5   | 3.0  | 4.0   | 3.5  | 4.0  | 5.5  |
| orf19.1490   | Msb2               | Mucin family, adhesin-like protein         | 1.5   | 3.0  | 4.0   | 2.5  | 5.5  | 6.0  |
| orf19.1671*  | Utr2               | Putative glycosidase                       | 1.0   | 3.0  | 2.0   | 3.5  | 5.0  | 7.0  |
| orf19.1690   | Tos1               | Protein similar to alpha-agglutinin anchor subunit | 11.0  | 23.5 | 27.0  | 19.0 | 27.0 | 24.5 |
| orf19.1779*  | Mp65               | Cell surface mannoprotein                  | 6.0   | 15.5 | 18.5  | 15.0 | 19.5 | 22.5 |
| orf19.1816*  | Als3               | Adhesin                                    | 2.5   | 5.0  | 5.5   | 5.5  | 7.0  | 10.5 |
| orf19.2060*  | Sod5               | Copper- and zinc-containing superoxide dismutase | 0.5   | 1.0  | 1.0   | 2.0  | 3.5  | 3.5  |
| orf19.220*   | PIR1               | Structural protein of cell wall             | 0.5   | 0.5  | 0.5   |      |      |      |
| orf19.2451*  | Pga45              | Cell wall protein                          | 0.5   | 0.5  | 1.5   | 1.5  | 3.0  | 2.5  |
| orf19.2706*  | Chrl1              | GPI-anchored cell wall transglycosylase     | 0.5   | 2.0  | 1.5   | 2.5  | 4.5  |      |
| orf19.2770*  | Sod1               | Copper- and zinc-containing superoxide dismutase | 2.0   | 1.0  | 1.0   | 1.0  | 1.0  | 1.0  |
| orf19.2990*  | Xog1               | Exo-1,3-beta-glucanase                     | 0.5   | 2.0  | 1.5   | 2.0  | 4.0  |      |
| orf19.3010*  | Ecm33              | GPI-anchored cell wall protein             | 1.0   | 1.0  | 1.0   | 1.0  | 1.0  | 1.0  |
| orf19.3097*  | Ub3                | Fusion of ubiquitin with the S34 protein of the small ribosomal subunit | 3.0   | 4.5  |      |      |      |      |
| orf19.3117*  | Csa2               | Extracellular-associated protein            | 1.5   | 2.5  | 2.5   |      |      |      |
| orf19.3374*  | Ece1               | Hyphal-specific cell wall protein           | 1.5   | 2.5  | 4.5   | 3.5  | 2.5  | 1.0  |
| orf19.3499*  | orf19.3499         | Secreted protein                           |       |      | 3.5   | 3.5  |      |      |
| orf19.3642*  | Sun41              | Putative cell wall glycosidase             | 2.5   | 6.0  | 9.0   | 5.0  | 13.0 | 12.0 |
| orf19.3829*  | Phr1               | Glycosidase of cell surface                | 6.5   | 12.0 | 14.5  | 12.0 | 10.0 | 8.5  |
| orf19.3895*  | Cht2               | GPI-linked chitinase                       | 0.5   | 1.5  | 0.5   | 1.0  | 2.5  | 2.0  |
| orf19.395    | Enol1              | Enolase                                    | 0.5   | 3.0  | 3.5   | 3.5  | 6.0  | 4.5  |
| orf19.4035*  | Pga4               | GPI-anchored cell surface protein           | 1.0   | 3.5  | 4.0   | 2.5  | 4.0  | 6.5  |
| orf19.4393   | CIt1               | Citrate synthase                           | 3.0   | 2.0  | 1.0   | 1.0  | 1.0  | 1.0  |
| orf19.4565*  | Bgl2               | 1,3-beta-glucosyltransferase               | 1.0   | 3.0  | 3.0   | 2.0  | 3.0  | 4.0  |
| orf19.4899   | Gca1/Gca2          | Predicted extracellular glucoamylase       | 1.0   | 0.5  | 0.5   | 1.5  | 1.0  | 9.0  |
| orf19.4975*  | Hyr1               | Hyphal-induced GPI-anchored cell wall protein | 1.0   | 1.5  | 1.5   | 1.5  | 1.0  | 2.0  |
| orf19.5063*  | Coi1               | Secreted protein                           | 3.5   | 5.0  |      |      |      |      |
| orf19.5542*  | Sap6               | Secreted aspartyl proteinase               | 0.5   | 1.0  | 6.0   | 7.5  |      |      |
| orf19.5636*  | Rbt5               | GPI-anchored cell wall protein involved in hemoglobin utilization | 0.5   | 0.5  | 0.5   | 0.5  | 0.5  | 0.5  |
| orf19.5741*  | Als1               | Adhesin                                    | 6.0   | 9.5  | 6.5   | 6.5  | 5.5  | 9.0  |
| orf19.5806   | Ald5               | NAD-aldehyde dehydrogenase                 | 5.5   | 8.5  | 8.5   | 7.0  | 3.0  | 4.0  |

*Numbers represent the average amount of peptides of two technical replicates identified in C. albicans biofilms (upper rows) or mixed biofilms (lower rows). Predicted secretory domains are indicated by an asterisk, predicted GPI anchors by a plus.*
tides than in single-species biofilms (Table IV), whereas 78 proteins were detected exclusively in single-species biofilms. Proteins that are not detected do not have to be absent but may be below the detection/identification level. Nevertheless, the total amount of protein isolated from mixed biofilms as described above was higher (124 µg) than the sum of protein isolated from individual biofilms (81 µg), indicating the massive production of individual proteins in response to the other

| Accession | Name | Function | 1.5 h | 3 h | 4.5 h | 6 h | 24 h | 48 h |
|-----------|------|----------|-------|-----|-------|-----|------|------|
| orf19.6081*+ | Phr2 | Glycosidase | 1.0 | 1.0 | 1.0 | 1.0 | 2.0 | 2.0 |
| orf19.6202* | Rbf4 | Protein with similarity to plant pathogenesis-related proteins | 2.0 | 4.0 | 1.0 | 2.0 | 4.0 | 0.5 |
| orf19.6274* | Pbr1 | Protein required for cohesion, adhesion, and biofilm formation | 1.0 | 1.0 | 3.5 | 4.0 | 3.5 | 4.0 |
| orf19.6673* | Hex1 | beta-N-acetyl-hexosaminidase/chitobiase | 5.5 | 10.0 | 8.0 | 6.5 | 5.0 | 5.0 |
| orf19.6844 | Icl1 | Isocitrate lyase | 3.5 | 3.5 | 2.5 | 1.5 | 3.0 | 3.5 |
| orf19.7114*+ | Csa1 | Surface antigen on elongating hyphae and buds | 2.0 | 4.0 | 2.0 | 1.0 |
| orf19.7218* | Pbe1 | Cell wall protein | 1.0 | 3.5 | 2.5 | 1.5 | 3.0 | 3.5 |
| orf19.756* | Sap7 | Member of the secreted aspartyl proteinase family | 1.0 | 2.0 | 4.0 | 1.0 |
| orf19.7574 | Uncharacterized orf | 0.5 | 0.5 |
| orf19.903 | Gpm1 | Phosphoglycerate mutase | 1.5 | 3.0 |

Table III

Number of proteins detected in the supernatant assigned to C. albicans and P. aeruginosa in comparison between single-species and mixed biofilms

| 1.5 h | 3 h | 4.5 h | 6 h | 24 h | 48 h |
|-------|-----|-------|-----|------|------|
| C. albicans proteins | Single-species biofilms | 26 | 37 | 42 | 40 | 48 | 61 |
| Mixed biofilms | 23 | 27 | 26 | 23 | 23 | 25 |
| P. aeruginosa proteins | Single-species biofilms | 63 | 27 | 46 | 25 | 70 | 105 |
| Mixed biofilms | 22 | 37 | 31 | 26 | 34 | 71 |

Table IV

Proteins exclusively identified in mixed biofilms

Numbers indicate the average amount of peptides of two technical replicates. Predicted secreted proteins are marked with an asterisk, predicted GPI-anchored proteins with pluses.

| Accession | Name | Function | 1.5 h | 3 h | 4.5 h | 6 h | 24 h | 48 h |
|-----------|------|----------|-------|-----|-------|-----|------|------|
| orf19.1589 | orf19.1589 | Putative DNA-binding transcription factor | 0.5 | 0.5 |
| orf19.3445* | Hoc1 | Protein with similarity to mannosyltransferase | 1.0 | 1.0 |
| orf19.5674*+ | Pga10 | Plasma membrane protein involved in heme-iron utilization | 0.5 | 0.5 |
| orf19.5716* | Sap4 | Secreted aspartyl proteinase | 1.0 | 1.0 |
| PA0002 | dnaN | DNA polymerase III, beta chain | 2.5 | 2.5 |
| PA0283* | sbp | Sulfate-binding protein precursor | 1.5 | 1.5 |
| PA1733 | Conserved hypothetical protein | 0.5 | 0.5 |
| PA2377* | Hypothetical protein | 3.5 | 2.5 |
| PA2398* | FpvA | Ferricytochrome d receptor | 0.5 | 0.5 |
| PA2758 | Probable transcriptional regulator | 0.5 | 0.5 |
| PA3117 | Asd | Aspartate semialdehyde dehydrogenase | 1.0 | 1.0 |
| PA3301 | Hypothetical protein | 0.5 | 0.5 |
| PA3407 | HasAp | Heme acquisition protein HasAp | 10.0 | 14.5 | 28.0 | 28.0 |
| PA3441 | Probable molybdenopterin-binding protein | 0.5 | 0.5 |
| PA4228 | PchD | Pyochelin biosynthesis protein PchD | 0.5 | 0.5 |
| PA4266 | fusA1 | Elongation factor G | 2.0 | 2.0 |
| PA4694 | ilvC | Ketol-acid reductoisomerase | 0.5 | 0.5 |
| PA4761 | dnaK | DnaK-protein, chaperone | 2.5 | 2.5 |
| PA5288 | glnK | Nitrogen regulatory protein P-II | 1.0 | 1.0 |
| PA5288 | glnK | Nitrogen regulatory protein P-II | 1.0 | 1.0 |
organism or the occurrence of cell lysis. Interestingly, the amount of potential cytosolic proteins containing no secretory domain that are involved in central metabolism is not increased for both species in mixed biofilms. Thus, increased lysis of both microorganisms within the timeframe observed is not likely to occur. However, we identified a set of *P. aeruginosa* proteins showing a massive increase in peptide numbers (Table I). The most prominent effect was observed for five proteins (*p* values 0.000 to 0.003): exotoxin A ToxA (PA1148), quinoprotein ethanol dehydrogenase ExaA (PA1982), heme acquisition protein HasAp (PA3407), and two unknown proteins related to ferric enterobactin esterases, PA2451 and PA2452. Three of the five proteins are involved in iron utilization. The heme acquisition protein HasAp (PA3407) and proteins related to ferric enterobactin esterases (PA2451 and PA2452) constitute known iron acquisition systems, indicating a lack of iron in co-cultures with *C. albicans*. The exotoxin ToxA (PA1148), a known virulence factor in *P. aeruginosa*, was specifically secreted in the presence of *C. albicans*. Its expression was previously observed under iron starvation conditions as well (64). Secretion of the periplasma localized quinoprotein ethanol dehydrogenase ExaA (PA1982) is induced at later time points indicating the appearance of alcohols in the medium (65).

For *C. albicans*, we could not observe a strong induction of individual proteins in the supernatant. On the contrary, we observed fewer peptides matching proteins of the hemoglobin-receptor gene family, namely Rbt5 (orf19.5636), Csa1 (orf19.7114), and Csa2 (orf19.3117), in co-cultures with *P. aeruginosa*. Its expression was previously observed under iron starvation conditions as well (64). Secretion of the periplasma localized quinoprotein ethanol dehydrogenase ExaA (PA1982) is induced at later time points indicating the appearance of alcohols in the medium (65).

**Identification and Observation of Production of Pyoverdine**—During mixed biofilm formation, it was noticed that the color of the supernatant changed to yellowish if compared with single-species biofilms. Absorbance of the supernatants was measured showing a maximum at 400 nm (Fig. 5A). Performing scans of excitation and emission, it was observed that a compound present in the supernatant emits at 455 nm when excited at 405 nm. These absorbance and fluorescence data are characteristic for the presence of pyoverdine, a siderophore secreted by *P. aeruginosa* (66). To confirm this, MALDI MS was used to identify the specific mass of the molecule. The characteristic mass peak of pyoverdine at 1333.62 Da [M + H] was identified and verified the presence of pyoverdine (Fig. 5B) (67).

The amount of pyoverdine increased rapidly in mixed biofilms within the first 20 h followed by slow decrease over the following 50 h (Fig. 6A). In single biofilms of *P. aeruginosa*, some fluorescent signals were measured at least partially because of auto-fluorescence of this bacterium. However, signals detected in *P. aeruginosa* biofilms were significantly lower at all time points compared with mixed biofilms. Addition of iron abolished production of pyoverdine also in the presence of *C. albicans* to background levels (Fig. 6B), showing a clear response of *P. aeruginosa* to iron-limiting conditions in mixed biofilms, which is in agreement with the increased expression of iron sequestering proteins.

**DISCUSSION**

Mixed biofilms of *C. albicans* and *P. aeruginosa* have been investigated by several groups in recent years and present an interesting example of competition between two opportunistic pathogens of the human mucosal flora (3, 30–32, 68). To address the question of how biofilm formation of *P. aeruginosa* and *C. albicans* is affected by the presence of each other, we, for the first time, analyzed the secretome of individual and mixed biofilms also with the idea of examining molecules mediating communication between these species.

Interestingly, strong differences in the amount and identity of proteins released to the medium were identified. Noticeably only 39 of the 131 in mixed-biofilms-identified proteins were assigned to the fungus whereby the remaining 92 proteins belonged to *P. aeruginosa*. Just four *C. albicans* proteins were exclusively found in the bacterial presence, but *P. aeruginosa* excreted 16 proteins in significantly higher amounts or exclusively sensing the fungus. This is in agreement with previous observations that *C. albicans* is outcompeted by *P. aeruginosa* (30). In single-species biofilms, both organisms showed a higher diversity of proteins with 73 being assigned to *C. albicans* and 154 to *P. aeruginosa*.

These numbers already indicate that both organisms react to each other over the time course analyzed by changing the nature and the amount of the proteins released. In addition, it could be observed that *C. albicans* shows a reduced activity against XTT, indicating an overall reduced metabolism in mixed biofilms compared with fungal biofilms whereas *P. aeruginosa* raises the production of specific proteins to defeat the fungus. The overall reduced activity of *C. albicans* in mixed biofilms is consistent with the reduced amount of proteins detected.

Additionally, the number of proteins identified varies significantly over the individual time points (Fig. 4B), although the same amount of total protein was used for each sample. This effect may be attributable to a larger number of proteins being represented in amounts that are below the detection/identification level at earlier time points. Because the increase in identified peptides cannot be attributed to differences in the amount of protein used for the individual measurements, we assume that the actual complexity of proteins is reduced at later time points, resulting in a higher concentration of the individual proteins in the mixture, which can be more easily detected. This would be in agreement with a reduced availability of nutrients over time and a more fierce competition focusing on the remaining resources required for essential metabolic pathways as discussed below.

For both single- and mixed-species biofilms we observed proteins without secretory signal peptides or cell wall proteins.
Survival of *P. aeruginosa* and *C. albicans* in Mixed Biofilms

**FIG. 5.** A, absorption scan of filter sterilized supernatants of 24-h-grown biofilms built by *C. albicans* (light gray line), *P. aeruginosa* (dark gray line), and mixed biofilms (black line). B, mass spectrum of supernatants of mixed biofilms reveals the specific mass of pyoverdine at 1333.62 Da [M + H].
not expected in the secretome. Recently, so-called moonlighting proteins have been identified, including proteins having multiple localizations in the cell (58). Several transport processes are discussed for unconventional secretion, such as autophagy or specific transporters. However, cell lysis might also contribute to this as discussed below.

For C. albicans, the major amount of proteins was identified from single-species biofilms. Interestingly, GPI-anchored proteins were found in the supernatant. We hypothesize that these proteins, because of incomplete anchoring or shedding, might be released from the cell wall. Particularly, the ALS (agglutinin-like sequence) family plays a major role in biofilm development of C. albicans. Furthermore, ALS genes were shown to be involved in interactions between C. albicans and bacteria (48, 69). In our experiments in C. albicans biofilms, peptides derived from Als1 (orf19.5741), Als2 (orf19.1097), and Als3 (orf19.1816) were found at different levels over the different time points. Als3 has been shown to be important for adhesion and biofilm formation. Its transcript is induced >10 times in initial stages during adhesion and down-regulated in maturating 48-h-grown biofilms (70). This parallels the increase of Als3-derived peptides over time found in our study, suggesting that the protein is stable, which is compatible with a structural function (Table II). Also, ALS1 is expressed in biofilms (70). We found Als1 present as one of the proteins with higher abundancy over all time points (Table II). Also in mixed-species biofilms, both ALS proteins were detected at high abundancy. However, in later phases of mixed biofilms, this abundancy decreased paralleling the general reduction of metabolism in C. albicans. Work by Holcombe et al. (33), dissecting the effects of P. aeruginosa supernatants on gene expression in early stages of C. albicans biofilm formation (6 h) using transcriptional analysis, revealed down-regulation of gene expression of the ALS genes as well. Other proteins with predicted adhesion-like functions, such as Als4 (orf19.4555), the pheromone-induced biofilm regulator Pbr1 (orf19.6274),
Survival of *P. aeruginosa* and *C. albicans* in Mixed Biofilms

which is also required for adhesion and cohesion, and Sim1 (orf19.5032) were detected in *C. albicans* biofilms but not in mixed biofilms. Transcript levels of ALS4 also were found to be down-regulated in *C. albicans* biofilms treated with *P. aeruginosa* (33), paralleling our results.

Proteins that are involved in morphogenesis including Pga45 (orf19.2451), Pir1 (orf19.220), Ece1 (orf19.3374), and the RBT proteins Rbt4 (orf19.6202) and Rbt5 (orf19.5636) were found represented by more peptides in single fungal biofilms compared with mixed biofilms. It was shown before that transcript levels of morphology-related genes such as RBT4 and RBT5 are affected by *P. aeruginosa* as well, confirming our results (33). Grown in mixed biofilms together with *P. aeruginosa*, *C. albicans* seems not only to alter the expression of adhesins but cell wall composition in general, confirming also on a protein level that the morphological switch is affected.

Similarly, in *P. aeruginosa* biofilms, a large number of proteins identified in this work were reported previously to be involved in biofilm formation and adhesion. For example, it was reported that flagellar and twitching motility are necessary for biofilm development (71). Proteins assigned to flagella (PA1086 FilK, PA1087 FlgL, PA1092 FilC, PA1094 FilD) and Typ IV pili (PA4525 PilA and PA4554 PilY1) were identified in the secretome accumulating with maturation. Type IV pili are involved in twitching motility and adherence to eukaryotic cells, and mutants defective in pilY1 are not able to form biofilms. Genes for the synthesis of pili and flagella were found to be repressed in maintained biofilms compared with planktonic-grown cells (72). Attended by *C. albicans*, *P. aeruginosa* released the proteins associated with flagella and pili in smaller amounts, inferred from the amount of peptides assigned to the respective protein. Thus, a flagellar structure seems not to confer an advantage in mixed biofilms.

In biofilms of *P. aeruginosa* also cytosolic proteins related to protein and amino acid biosynthesis were identified. The presence of cytosolic proteins in *P. aeruginosa* may be due to the reported prophage-mediated cell lysis that contributes to void formation inside microcolonies, benefiting differentiation and dispersion of subpopulations (73). The filamentous Pf1-like prophage in the *P. aeruginosa* genome was shown to be involved in biofilm cell lysis (73, 74). Also other factors, *i.e.*, other bacteriophages, were discussed to be involved in cell lysis in biofilms (75). In our study, we identified proteins with high homology to proteins of phage phi CTX (PA0620) and phage PS17 (PA0622 and PA0623), which are related to the bacteriocin R2 pyocin (59). Remarkably these proteins were detected at higher levels in 24 h and 48 h grown *P. aeruginosa* biofilms compared with mixed biofilms paralleled by a higher number of peptides associated with potentially cytosolic proteins, such as ArcB (PA5172) in single-species biofilm. It was shown before that pyocins are produced by *P. aeruginosa* biofilms under anaerobic conditions (76). Despite the general believe that microorganisms are insensitive to self-produced bacteriocins, the opposite was found for the *P. aeruginosa* pyocin, which was linked to autolysis (77, 78). In mixed biofilms, significantly fewer peptides matching pyocin were found paralleled by the identification of a reduced amount of cytosolic proteins involved in central metabolic activities such as ribosomal proteins compared with single-species biofilms. This indicates that prophage-mediated cell lysis of *P. aeruginosa* in mixed biofilms with *C. albicans* is rather repressed than induced.

In mixed biofilms, one immediately apparent feature was a change of color of the supernatant into yellowish. The yellowish compound could be identified as the *Pseudomonas*-specific siderophore pyoverdine using MS and photometric analysis. In *P. aeruginosa*, pyoverdine functions as a high-affinity iron acquisition system, which is synthesized and secreted during iron starvation (79). In our experimental setup, biofilms were grown in iron-containing media (ferric chloride, 200 µg/ml). In single-species biofilms of *C. albicans* and *P. aeruginosa*, there was no evidence suggesting iron limitation. Only when grown in co-culture with *C. albicans*, *P. aeruginosa* released the virulence associated siderophore pyoverdine, indicating limiting iron concentrations most likely due to the cumulative consumption of both organisms. This could be confirmed by adding additional iron (10 mM FeCl₃) to the medium, which resulted in a complete block of pyoverdine production. It was shown previously that a functional pyoverdine system is necessary for biofilm maturation under iron limitation (23, 80, 81). Further evidence for iron-limiting conditions is the identification of the heme acquisition protein HasAp (PA3407), which was found with increasing peptide numbers during the time course in supernatants of mixed biofilms. Expression of the hemoprotein again is induced by iron limitation and is secreted by an ABC transporter. *P. aeruginosa* uses heme and hemoglobin as a source of iron via binding to HasAp (82). Two hypothetical proteins of unknown functions, PA2451 and PA2452, have been found secreted in mixed biofilms with increasing peptide numbers over time (Table I). The proteins seem to be organized in one operon as predicted by DOOR (83). Interestingly, only PA2452 contains a known secretion domain; however, secretion of the other protein seems to be occurring in a similar fashion. Homology searches identified domains related to enterobactin/ferric enterobactin esterase (84). Enterobactin functions as another siderophore in Gram-negative bacteria such as *Escherichia coli* and can be utilized by *P. aeruginosa* (85).

The production of important virulence factors, namely the exotoxin A ToxA and the proteases PrpL and AprA, was also shown to be induced under conditions of iron starvation through pyoverdine-dependent signal transduction pathways (64). Consequently, secretion of the pyoverdine-dependent exotoxin ToxA (PA1148) was observed starting after 6 h growth in mixed biofilms and was found represented by 22 peptides after 48 h. In single-species biofilms, only one peptide matching ToxA was found. Exotoxin A is an important
virulence factor produced by P. aeruginosa that is responsible for local tissue damage, bacterial invasion, and immunosuppression within the eukaryotic host. Precursors of AprA (PA1249) were identified in the secretome as well.

The massive induction of iron acquisition systems by P. aeruginosa results in a dramatic reduction in overall activity of C. albicans, as measured by the XTT assay. Although C. albicans is able to utilize siderophores produced by other organisms (86), this does not seem to be the case in this study. This is paralleled by a general decrease in peptide numbers and protein species identified. The activity of C. albicans was reduced in the presence of P. aeruginosa compared with single fungal biofilms starting at the first time point with the strongest effect at 24 h (>80% reduction). This effect could even be underestimated because of the finding that the activity of the C. albicans XTT reductase is induced in low-iron conditions compared with iron-replete conditions (87). The reduced activity of C. albicans observed in our experimental setup, however, is not due to cell lysis caused by P. aeruginosa, because we could not detect a higher amount of cytosolic proteins in mixed biofilms compared with C. albicans biofilms. This indicates that the cell integrity is not affected within the time frame observed. Also, no cellular debris was visible in electron microscopic pictures of mixed biofilms as well, but a substantial amount of apparently intact, predominantly yeast-form Candida cells (Fig. 2). Previous studies using different growth conditions and other laboratory strains did observe dead and lysed Candida cells during co-culture with P. aeruginosa.

The reduced XTT activity is paralleled by the reduced expression of almost all proteins identified in mixed biofilms, including the GPI-anchored cell-surface proteins of the hemoglobin-receptor gene family, which are involved in hemin- and hemoglobin-iron utilization if compared with C. albicans–only biofilms. Although peptides matching members of this gene family, namely Rbt5 (orf19.5636), Csa1 (orf19. 7114), and Csa2 (orf19.3117) (88), were found both in single and mixed biofilms, they were found at lower levels in mixed biofilms, indicating a strong impact of P. aeruginosa, possibly by a reduction of iron levels below the threshold required for induction of these proteins.

P. aeruginosa previously has been shown to efficiently block growth of other microorganisms. It was shown, for example, that supernatants of P. aeruginosa co-cultured with Aeromonas hydrophila contained rhamnolipids and the siderophores pyoverdine and pyocyanin, which caused inactivation of A. hydrophila (89). A combination of pyocyanin and 1-hydroxyphenazine was shown to inhibit yeast mycelia transformation and growth of C. albicans and Aspergillus fumigatus (36).

In our experimental setup, biofilms of P. aeruginosa react to the presence of C. albicans by powering up iron acquisition systems. On the one hand, it synthesizes the siderophore pyoverdine to bind ferric ions and prohibit the availability of the essential metal to the fungus. On the other hand, the bacterium raises pathways responsible for utilization of iron bound to chelators used by the host such as hemoglobin or potential competitive microorganisms such as enterobactin. Additionally, it raises production of virulence factors such as exotoxin A. Others have reported the presence of antimicrobials such as phenazine derivatives effectively blocking proliferation of microbial competitors. C. albicans in face of this armory seems unable to counteract efficiently resulting in the general reduction of metabolic activity, up to cell death, as observed by others. Thus, by a combination of different means, including antimicrobials and sequestration of iron, P. aeruginosa is able to gain prevalence against C. albicans. The results obtained in this study confirm that microorganisms not only compete with the host for essential nutrients but also strongly with the present microflora in order to gain a competitive advantage.

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This article contains supplemental material 1 to 3.

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