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Response of sessile cells to stress : from changes in gene expression to phenotypic adaptation

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Abstract

Gaining a better understanding of the genotypic and phenotypic adaptation of sessile (biofilm-associated) microorganisms to various forms of stress is required in order to develop more effective antibiofilm strategies. This review presents an overview of what high-throughput transcriptomic analyses have taught us concerning the response of various clinically relevant microorganisms (including *Pseudomonas aeruginosa*, *Burkholderia cenocepacia* and *Candida albicans*) to treatment with antibiotics or disinfectants. In addition, several problems associated with identifying gene expression patterns in biofilms in general and their implications for identifying the response to stress are discussed (with a focus on heterogeneity in microbial biofilms and the role of small RNAs in microbial group behaviour).

Introduction

Biofilms are microbial communities containing sessile cells embedded in a self-produced extracelllular polymeric matrix (containing polysaccharides, DNA and other components). In comparison to their planktonic (free-living) counterparts, sessile cells are often much more resistant to various stress conditions (including treatment with antimicrobial agents) and this increased resistance has a considerable impact on the treatment of biofilm-related infections (Fux, 2005). Several mechanisms are thought to be involved in biofilm antimicrobial resistance including (i) slow penetration of the antimicrobial agent into the biofilm, (ii) changes in the chemical microenvironment within the biofilm, leading to zones of slow or no growth, (iii) adaptive stress responses, and (iv) presence of a small population of extremely resistant "persister" cells (Mah & O'Toole, 2001 ; Stewart & Costerton, 2001 ; Donlan & Costerton, 2002 ; Gilbert *et al*. 2002a, 2002b).

In a first part of this review I will highlight the problems associated with the study of gene expression in biofilms, using a set of studies on the human-pathogenic fungus *Candida albicans* as an example. Subsequently I will review the recent literature concerning differential gene expression in a number of microbial biofilms in response to stress (with a focus on stress related to exposure to antibiotics and reactive oxygen species) and link that to phenotypic adaptation.

The search for biofilm-specific gene expression patterns : *C. albicans* as an example

Earlier work (reviewed by Sauer [2003], Beloin & Ghigo [2005], and Lazazzera [2005]) indicated that, although gene expression patterns in biofilms often differed remarkably from those in planktonic cells, finding common biofilm gene expression patterns between different studies (even those using the same organisms) was difficult. This was attributed to the minimal overlap between functions involved in biofilm formation and the fact that sub-sets of genes expressed in biofilms are also expressed under various planktonic conditions.

C. albicans is a commensal fungus of healthy human individuals and can cause superficial and systemic infections when the immune defences are repressed or when the normal microbial flora is disturbed. *C. albicans* infections are often associated with the formation of biofilms (Douglas 2003). A first comprehensive transcriptome analysis of biofilm formation in *C. albicans* was presented by Garcia-Sanchez *et al*. (2004). In this study, gene expression in various biofilm model systems (microfermentor, catheter disks and microtiter plate) was

compared to the expression in planktonic cultures. Three different strains were tested (SC5314, CAI4 and CDB1) and several environmental parameters (medium flow, glucose concentration, aeration, time and temperature) were varied. Despite the marked differences in growth conditions, correlation coefficients for the biofilm-biofilm comparisons were high (between 0.80 and 0.97), while comparing gene expression profiles between planktonic cultures or between biofilms and planktonic cultures resulted in lower correlation coefficients (0.54 to 0.80, and 0.54 to 0.81, respectively). 325 genes were identified as being differentially expressed between biofilm and planktonic conditions (214 genes were activated in biofilms, 111 were repressed). In this set, genes involved in protein synthesis, amino acid, lipid and nucleotide metabolism, transcription, and control of cellular organisation are overrepresented. A high fraction of the 214 overexpressed genes are related to the synthesis of amino acids and many of these are homologs of genes that are under control of Gcn4p in *Saccharomyces cerevisiae*. Reduced biofilm formation in a *C. albicans gcn4/gcn4* mutant confirmed the requirement for a functional Gcn4p for normal biofilm formation. In addition, *ALS1* (thought to be involved in adhesion) was identified as the major overexpressed genes in biofilms, while other genes of the *ALS* family were underexpressed (*ALS7*) or not differentially expressed at all (*ALS5*, *ALS10*). In a second transcriptome study, Murillo *et al*. (2005) focused on gene expression in the early phases of *C. albicans* biofilm formation (30 – 390 min). 41 genes were identified as being differentially upregulated in biofilms compared to planktonic cultures, while 25 genes were downregulated in biofilms. Nine of these 41 genes encode proteins involved in sulfur metabolism, suggesting an upregulation of the entire sulfur assimilation pathway in early biofilm cultures. A second set of genes differentially upregulated in young biofilms are associated with phosphate metabolism. Marchais *et al*. (2005) identified 117 differentially expressed genes (77 overexpressed in adherent cells and 40 underexpressed). Among the overexpressed genes, 22% played a role in cellular organisation and intracellular transport, 10% were involved in amino acid and protein metabolism, 7% in carbohydrate metabolism, 5% in lipid and fatty acid metabolism, 5% in transcription, but the majority (46%) had no known function. Yeater *et al*. (2007) determined gene expression profiles in two *C. albicans* strains grown on two different substrates (denture & catheter), at three different time points (representing early, intermediate and mature biofilms). 243 genes were differentially expressed in either biofilm or planktonic specimens, over the experimental time course, while 191 genes were differentially expressed between biofilm and planktonic cells at the three developmental time points studied. Data from this study indicated that assimilation of carbohydrates (both through glycolytic and non-glycolytic routes), amino acid metabolism

and intracellular transport mechanisms are important in the early phase (6 h) of biofilm formation. During the intermediate phase (12 h), sessile cells have a high energy demand and use specific transporters for amino acids, sugars, ions, oligopeptides and lactate/pyruvate. At the 48 h time point few genes were differentially expressed.

Zakikhany *et al*. (2007) and Nett *et al*. (2009) took the study of gene expression in *C. albicans* biofilms to the next level by performing transcriptome analyses on biofilms grown in more elaborate model systems, that more-closely mimic human infections. Zakikhany *et al*. compared the expression in sessile *C. albicans* cells grown on reconstituted human oral epithelium (RHE) for various time points (1 to 24 h post inoculation) to that in planktonic cells (grown to mid-exponential phase). It turned out that 15% of the appr. 4300 reliably expressed genes were ≥ 2-fold upregulated at one or more time points. One hour post-inoculation, 164 genes were upregulated, of which 29 were only upregulated at this time point. The majority of these "early-only" genes (21/29) had no known function while others were involved in cellular functions such as transcription. 38 genes were significantly overexpressed throughout the entire experiment (1 to 24 h). Several of these were hyphae-specific or at least hyphae-associated (including *HWP1* and *ALS3*), indicating that contact with the epithelial cells induces hyphae formation. Identification of genes that were only upregulated in later stages (12 or 24 h post inoculation) showed that these were mainly involved in metabolic functions and suggested a shift towards the use of other molecules than glucose as carbon source (e.g. lipid-derived two-carbon compounds). Interestingly, when the results were compared with results obtained with mRNA recovered from 11 HIV-positive patients with pseudomembranous candidiasis, 38 genes that were increased at all time points in the RHE model also showed an increased expression in the patient samples. These 38 genes included hyphae-associated genes (including *HWP1* and *ALS3*) as well as genes involved in the utilisation of two-carbon compounds via the glyoxylate cycle (Zakikhany *et al*., 2007). In the study of Nett *et al*. biofilms were grown on catheters inserted in the jugular vein of rats (Andes *et al*., 2004). Samples taken from these central venous catheters at two time points (12 h, intermediate growth and 24 h, mature) were compared to in vitro grown planktonic cells. 124 genes were upregulated in biofilms at both time points, compared to the expression in planktonic cells. The majority of these genes were involved in transcription and protein synthesis (13%), energy and metabolism (12%), carbohydrate synthesis and processing (10%), and transport (6%), while 35% of the 124 genes had no function assigned to it. 27 genes were downregulated at both time points ; 30% of these genes were involved in DNA processing.

Besides the above-descibed transcriptomics studies, several research groups have used proteomics to identify differentially expressed proteins. Thomas *et al*. (2006) identified nine differentially expressed cell-surface associated proteins, seven (Hsp70, pyruvate decarboxylase, inositol-1-phosphate synthase, enolase [ENO1], O-acetylhomoserine O-acetylserine sulfhydrylase [MET15], alcohol dehydrogenase 1 and inosine-5'-monophosphate dehydrogenase) were overexpressed, while two (alchol dehydrogenase 2 and malate dehydrogenase) were downregulated in biofilms. Interestingly, not a single surface-associated protein was identified as being solely expressed in sessile or planktonic cells. Nineteen proteins were significantly overexpressed in *C. albicans* biofilms grown in 24-well microtiterplates, compared to planktonic cultures, and in contrast to the results obtained by Thomas *et al*. (2006), ENO1 was two-fold underexpressed . Highly significant overexpression was observed for citrate synthase (14.45 – fold) and several proteins involved in oxidative stress, including alkyl hydroperoxide reductase AHP1 and several other reductases (GRP2, MCR1, TSA1, PST1 and TRX1), were also overexpressed. Proteomics has also been used for a three-way comparison of planktonic yeast cells, planktonic hyphae and sessile cells (Martinez-Gomariz *et al*., 2009). 175 cytoplasmic and 70 cell surface – associated proteins were differentially expressed between sessile and planktonic yeast cells, while these numbers were 218 and 51, respectively, when sessile cells were compared to planktonic hyphae. The fold over- or underexpression varied considerably depending on the comparison made. For example, MET15 was downregulated in biofilms when compared to planktonic yeast cells, but upregulated when biofilms were compared to planktonic hyphae, confirming that morphology is an important factor. Futher complicating the comparison of protein expression is the presence of various isoforms of the same protein. E.g. six isoforms of pyruvate decarboxylase were identified by Martinez-Gomariz *et al*. : isoforms 1, 2, 5 and 6 are underexpressed in biofilms compared to planktonic yeast cells, while isoforms 3 and 4 are overexpressed.

A detailed analysis of the results obtained in the studies summarised above reveals that, although generally representatives of particular classes of genes are differentially expressed between planktonic and sessile cells (Fig. 1) there is very little overlap between *C. albicans* genes identified as differentially expressed in different studies and the same is true for other microorganisms. The observation that the experimental conditions for culturing the cells prior to RNA extraction are often variable (Table 1) offers a first explanation. There is a wide range of biofilm model systems available, and few studies have used the same model system. Similarly, planktonic cells are cultured in a variety of ways (Table 1). In addition, there is

growing evidence that there are marked differences in gene expression between different stages of biofilm formation and as such the comparison of gene expression profiles obtained in a particular model system at a particular time point with an expression profile obtained at another time point in another model system is probably of little relevance. In addition, while most studies with *C. albicans* were carried out with the reference isolate SC5314, a wider variety of isolates has been included in this kind of studies for other organisms. For example, for *Escherichia coli* strains MG1655 (Schembri *et al*., 2003 ; Ito *et al*., 2009a, 2009b), TG and TG1 (Beloin *et al*., 2004), JM109 and ATCC 25404 (Ren *et al*., 2004), BW25113 (Domka *et al*., 2007) and PHL628 (Junker *et al*., 2007) have been used, as well as clinical isolates recovered from asymptomatic bacteriuria (Hancock & Klemm, 2007). Although several of these strains are listed as "K12", subtle differences between them may confound the comparison of gene expression data. It is important to keep this in mind when looking for genotypic and/or phenotypic adaptation to stress in sessile cells, as the differential expression of particular genes due to differences in the environmental conditions in the test and control situation may introduce bias and lead to erroneous conclusions.

Stress-induced gene expression in biofilms and phenotypic adaptation

Adaptation of sessile *Pseudomonas aeruginosa* cells to exposure to antibiotics

P. aeruginosa was one of the first organisms in which gene expression in biofilms was studied, but surprisingly, when Whiteley *et al*. (2001) compared gene expression levels between cells grown on granite pebbels in a chemostat and cells grown in liquid culture medium in the same chemostat, very few genes showed differential expression. When gene expression in untreated sessile *P. aeruginosa* PAO1 cells was compared with the expression in sessile cells treated with high doses of tobramycin (seven times the minimal inhibitory concentration [MIC] for planktonic cells), only 20 genes were differentially expressed (14 were activated and 6 repressed). Ten of these genes code for hypothetical proteins with no known function, two additional genes code for hypothetical proteins of a Pf1-like bacteriophage. Upregulated genes include those involved in stress response (*dnaK*, *groES*) and efflux systems, while downregulated genes include both hypothetical phage proteins as well as the β-subunit of urease (Table 2). The *tolA* gene, whose product affects LPS structure in such a way that the outer membrane has a decreased affinity for aminoglycoside antibiotics, was overexpressed in untreated sessile cells compared to planktonic cells, possibly leading to

decreased aminglycoside susceptibility in biofilms. Genes encoding cytochrome c oxidases (subunits I, II and III, encoded by PA0106, PA0105 and PA0108, respectively) on the other hand were downregulated (2.7 to 2.9 fold) in untreated sessile cells when compared to planktonic cells. As cytochrome c oxidase is the terminal electron acceptor during aerobic growth and as aminoglycoside transport is coupled with terminal electron transport (Bryan *et al*., 1980), this downregulation is likely to confer reduced susceptibility as well.

While screening a library of appr. 4000 random *P. aeruginosa* PA14 transposon insertion mutants, Mah *et al*. (2003) identified a mutant that had decreased tobramycin susceptibility when grown in biofilms, but was otherwise indistinguishable from the wild-type strain (i.e. no differences in tobramycin susceptibility when grown planktonically). The mutation was mapped to PA1163 (*ndvB*), coding for a periplasmic glucosyltransferase required for the synthesis of cyclic-β-(1,3)-glucans. Through a series of elegant experiments, the authors were able to demonstrate that the cyclic glucans synthesised by *ndvB* can sequester various antibiotics (including tobramycin, gentamycin and ciprofloxacin) and as such interfere with the movement of the antibiotics through the periplasmic space. Semi-quantitative PCR confirmed that *ndvB* is preferentially expressed in sessile cells. In addition, further screening of this Tn5 insertion mutant bank resulted in the identification of a novel efflux pump (PA1874-1877) that was more highly expressed in biofilm cells than in planktonic cells and was contributed to the increased resistance of sessile populations to tobramycin, gentamycin and ciprofloxacin (Zhang & Mah, 2008) (Table 2).

In *P. aeruginosa* biofilms treated with 1 µg/ml of the β-lactam antibiotic imipenem (a concentration below the MIC), 336 genes were induced or repressed at least twofold (Bagge *et al*., 2004). Not surprisingly, *ampC* (encoding a chromosomal β-lactamase) showed the strongest differential expression (150-fold on day 3). Several genes involved in alginate biosynthesis (including the *algD* to *algA* cluster and the *algU-mucABC* gene cluster) were also upregulated, while in younger biofilms treated with a subinhibitory concentration of imipenem, downregulation of motility-associated genes (*flgC* to *flgI* cluster, *pilA*, *pilB, pilM* to *pilQ*) was observed. The upregulation of alginate-related genes was associated with a drastic (up to 20-fold) increase in alginate production. Imipenem treatment also resulted in significant differences in biofilm structure, with treated biofilms containing more biomass per area and being thicker, but having a smoother surface, leading to a lower surface-to-volume ratio. The overexpression of *ampC* and genes involved in alginate biosynthesis probably allows the more efficient neutralisation of imipenem : the AmpC β-lactamase is secreted in

membrane vesicles and the accumulation of this enzyme in the matrix permits the rapid hydrolysis of β-lactams as they penetrate the matrix.

Exposure of *P. aeruginosa* PAO1 biofilms to sub-MIC levels of azithromycin (2 µg/ml) for 4 days, resulted in differential expression (≥5-fold difference) of 274 genes compared to untreated control biofilms (Gillis *et al*., 2005). Several of the upregulated genes encode RND efflux pumps, including *mexC* (x 94.8), *oprJ* (x 19.3), *nfxB* (x 14.5), *mexD* (x 12.7) and *oprN* (x 6.7). The expression of *mexAB-oprM* genes was not altered, but these genes are constitutively expressed at high levels already. By creating RND efflux pump mutants and transcriptional fusions, Gillis *et al*. (2005) showed that the *mexAB-oprM* and *mexCD-oprJ* RND efflux pumps are required for the formation of azithromycin-resistant *P. aeruginosa* biofilms. Also, the various efflux pumps showed different expression patterns : while *mexA* was expressed continuously throughout the biofilm regardless of the presence of azithromycin, *mexC* was expressed only in biofilms (but not in planktonic cells) in the presence of azithromycin and expression levels appeared to be highest in central parts of the biofilm (it should be noted that in an earlier study expression of *mexAB-oprM* and *mexCD-oprJ* was found to be highest at the biofilm substratum, not the centre [de Kievit *et al*., 2001]). Interestingly, genes PA0105, PA0106 and PA0108 (encoding cytochrome c oxidase subunits) were significantly down-regulated in response to azithromycin treatment, suggesting that also for macrolides there may be a coupling between electron transport and susceptibility, as already observed for tobramycin (Whiteley *et al*., 2001) (Table 2).

When *P. aeruginosa* PA14 biofilms formed on cystic fibrosis-derived airway epithelial cells are treated with 500 µg/ml tobramycin (approximately half of the minimum bactericidal concentration under these conditions) for 30 min, 338 transcripts were upregulated and 500 were downregulated (Anderson *et al*., 2008). Tobramycin treatment reduced the virulence of the bacteria towards the epithelial cells and several virulence-related genes were downregulated. Conversely, several genes involved in alginate biosynthesis were upregulated (*algU, mucA, algZ*), but as core *alg* biosynthetic genes were not upregulated, it is uncertain whether this leads to increased alginate production. The transcript levels for most resistance-related genes were only slightly altered (PA1541, *mexB, mexR*) or remained unchanged, suggesting that the expression of other, yet unknown, factors is important for resistance under these conditions.

Comparing the data reported in the various studies revealed that very few differentially expressed genes are common between the different studies (Table 2). Analysing the expression data reported by Whiteley *et al*. (2001) and Bagge *et al*. (2004) revealed that only

PA2703 (encoding a hypothetical protein) and PA3819 (encoding a hypothetical membrane protein) are overexpressed as a result of both tobramycine and imipenem treatment (Table 2). The only two genes that were upregulated by imipenem (Bagge *et al*., 2004) and tobramycine (cystic fibrosis-derived airway epithelial cell model, Anderson *et al*., 2008) (PA5261 and PA5162) are both involved in alginate biosynthesis. Also when a treatment with imipenem (Bagge *et al*., 2004) is compared to treatment with azithromycin (Gillis *et al*., 2005), two genes are found to be regulated in the same way : while PA4306 (encoding a type IVb pilin) is downregulated as a consequence of both treatments, PA5170 (encoding an arginine-ornithine antiporter) is upregulated (Table 2). When comparing these studies, it also becomes obvious that the expression of particular genes can be induced or repressed, depending on the antibiotic used (Table 2). PA2367 is downregulated by azithromycin, and it is upregulated by imipenem. Similarly, PA3049 is downregulated by azithromycin and upregulated by tobramycin, while PA5216 is downregulated by tobramycin and upregulated by azithromycin (Table 2).

General stress response in *E. coli* biofilms

The studies by Schembri *et al*. (2003), Beloin *et al*. (2004), Ren *et al*. (2004), Domka *et al*. (2007) and Hancock & Klemm (2007) revealed that stress-related genes are often overexpressed in sessile *E. coli* populations compared to planktonic cultures, even in the absence of antibiotics (Wood, 2009). When comparing 40 h-old *E. coli* biofilms grown in a flow cell with exponentially growing planktonic cultures, Schembri *et al*. (2003) noted that 46% (30/65) of *rpoS*-controlled genes were differentially expressed during biofilm growth (most were upregulated) and a *rpoS* mutant turned out to be incapable of forming a biofilm in the flow system. In addition, *yeaGH* were also overexpressed ; these genes are *rpoS*-regulated in *Salmonella enterica* and may also be associated with a stress response. Ito *et al*. (2008, 2009a) confirmed that *rpoS*-mediated stress responses contribute to biofilm-specific phenotypes (including ampicillin resistance). Also in 8 d-old *E. coli* TG1 biofilms grown in a microfermentor, stress-related genes were upregulated, including SOS response genes, chaperones, general stress response genes, heat shock proteins and genes involved in DNA repair and envelope stress response (Beloin *et al*., 2004). This last group of genes includes *cpxAR* (sensor-regulator components of the *cpx* two-component system) and the phage shock protein operon (*pspABCDE*), although no biofilm-related phenotype was obvious in a *psp* operon mutant. In addition, a TG1 *recA* mutant was no longer capable of forming mature biofilms, confirming the importance of stress responses in biofilm formation. In *E. coli*

biofilms grown on glass wool, stress genes are also induced, including *hslS, hslT, hha, soxS* and b1112 (Ren *et al*., 2004). *hslST* are involved in response to heat shock and superoxide stress, while *soxS* is involved in the response to superoxide. Gene b1112 (also known as *ycfR* or *bhsA*), encoding a putative outer membrane protein, plays an important role in stress response and biofilm formation as it mediates stress response by a mechanism that involves increased synthesis of the signal molecule indole (Zhang *et al*., 2007 ; Wood, 2009). Cells in urine-grown biofilms formed by isolates recovered from asymptomatic bacteriuria cases also exhibit an overexpression of stress genes (Hancock & Klemm, 2007). Among the most upregulated genes are cold and heat shock proteins including *cpsAGH* and *hslS*, and *soxS, yfiD* and *pphA*. The temporal data from Domka *et al*. (2007) revealed that various cold and heat-shock proteins (*cspABFGI*) were upregulated in young but not in older biofilms. If, to what extent and how these general stress genes protect *E. coli* biofilms remains to be determined.

Adaptation of sessile *P. aeruginosa* and *Burkholderia cenocepacia* cells to oxidative stress

In several Gram-negative bacteria coordinated regulation of many genes associated with oxidative stress is mediated by the transcriptional regulator OxyR (Zheng *et al*., 2001 ; Ochsner *et al*., 2001). In *P. aeruginosa*, oxidized OxyR increases the expression of *ahpCF* and *katB* (both encoding cytoplasmic enzymes) and of *ahpB* (encoding a periplasmic enzyme) (Ochnser *et al*., 2001). Panmanee & Hassett (2009) recently showed that these OxyR-controlled antioxidant enzymes have differential roles in planktonic and sessile *P. aeruginosa* cells. While exposure to H_2O_2 results in the upregulation of the *katB* gene in planktonic cells, no such upregulation is observed in sessile cells. In contrast, treatment of planktonic cultures with H_2O_2 does not result in a differential expression of *ahpC*, while this gene is significantly upregulated in sessile cells treated with high (25 mM) H_2O_2 concentrations. A possible explanation for this is that, due to iron starvation, catalase activity in biofilm cells is extremely low, making the increased expression of *ahpCF* a necessity for survival under these growth conditions (Panmanee & Hassett, 2009).

B. cenocepacia is a Gram-negative bacterium that is notorious for causing respiratory infections in people with cystic fibrosis (Coenye & Vandamme, 2003 ; Mahenthiralingam *et al*., 2008). Most *B. cenocepacia* strains readily form biofilms on various surfaces, and sessile *B. cenocepacia* cells are highly resistant against antibiotics and disinfectants (Peeters *et al*., 2008, 2009). While studying the resistance of sessile *B. cenocepacia* cells against disinfection procedures implemented in various infection control guidelines, it was noticed that these

sessile cells are highly resistant against H_2O_2 and NaOCl (Peeters *et al.*, 2008). This observation not only has implications for infection control practices but, as these oxidative agents are being produced by neutrophils as part of the endogenous defence against microorganisms (MacDonald & Speert, 2007), may also have implications for pathogenesis. When the transcriptional response of treated vs. untreated *B. cenocepacia* biofilms was compared, it was observed that the exposure to H_2O_2 and NaOCl resulted in an upregulation of 315 (4.4%) and 386 (5.4%) genes, respectively (Peeters *et al*., 2010). Transcription of 185 (2.6%) and 331 (4.6%) genes was decreased in response to H_2O_2 or NaOCl treatments, respectively. Not surprisingly, many of the upregulated genes in the treated biofilms are involved in (oxidative) stress responses, emphasizing the importance of the efficient neutralization and scavenging of reactive oxygen species. In addition, multiple upregulated genes encode proteins that are necessary to repair reactive oxygen species-induced cellular damage. Similar to what was observed for *P. aeruginosa*, *ahpC* and *ahpF* were highly upregulated, while *katB* was only modestly upregulated (upregulations of 41.3, 15.5 and 1.8-fold, respectively, after 30 min of treatment with H_2O_2) (Peeters *et al*., 2010). However, biofilms formed by a *B. cenocepacia katB* mutant (which still contains a functional *ahpCF*) were nevertheless highly susceptible to H_2O_2, and there already is substantial expression of *katB* in untreated biofilms. This clearly indicates that, unlike in *P. aeruginosa*, this catalase is crucial for the protection of sessile cells against exogenous H_2O_2 although its expression is not increased following exposure to reactive oxygen species. Treatments with H_2O_2 or NaOCl also resulted in the increased transcription of several organic hydroperoxide resistance (*ohr*) genes, including BCAS0085. Interestingly, in addition to the upregulation of BCAS0085 (49.3-fold), also a marked increase in the expression of BCAS0086 (encoding an exported lipase) was observed (96.6-fold), probably due to cotranscription of both genes. As a result of the marked overexpression of BCAS0086 an increased extracellular lipase activity was observed in treated biofilms. BCAS0085 and BCAS0086 orthologs in other *Burkholderia* genomes are organised in a similar operon-like fashion, and increased lipase activity was also observed in the supernatant of H_2O_2-treated biofilms of *B. cenocepacia* C5424, HI2424 and AU1054, *Burkholderia multivorans* LMG 17588, *Burkholderia ambifaria* LMG 19182 and *Burkholderia dolosa* AU0158 (Peeters *et al*., 2010). It remains to be determined whether this increased lipase activity has a protective effect or is merely the consequence of the cotranscription of a lipase-encoding gene.

Adaptation of sessile *C. albicans* cells to exposure to antifungal agents

The molecular mechanisms of antifungal resistance in *C. albicans* have been extensively studied and changes in the expression of genes have frequently been reported in resistant clinical isolates (White, 1997 ; White *et al*., 1998 ; Sanglard, 2002). Azole antifungal drugs (including fluconazole, miconazole and itraconazole) target the P450 mono-oxygenase encoded by the *ERG11* gene. This enzyme is involved in the conversion of lanosterol into ergosterol by mediating 14-α-demethylation, a key step in ergosterol biosynthesis (White *et al*., 1998). Resistance to fluconazole, the most commonly used antifungal agent, is associated with overexpression of *ERG11* but changes in the expression of other *ERG* genes (including *ERG3* and *ERG25*) have also been associated with azole resistance (Franz *et al*., 1998 ; Lopez-Ribot *et al*., 1998; Henry *et al*., 2000). In addition, in fluconazole-resistant isolates, genes encoding efflux pumps (including *MDR1*, *CDR1* and *CDR2*) are often upregulated, resulting in increased efflux (Lopez-Ribot *et al*., 1998 ; White *et al*., 2002 ; Rogers & Barker, 2003). Polyene antifungal agents, including amphotericin B and nystatin, are fungicidal and bind to ergosterol in the fungal cell membrane, leading to membrane damage and oxidative stress (Brajtbrug *et al*., 1990 ; Beggs, 1994). In vitro exposure of planktonic cells to amphotericin B often leads to a repression of *ERG3* and *ERG11* expression and a concomitant decrease in ergosterol levels in the membrane, indicating that changes in the sterol composition are important for amphotericin B resistance in *C. albicans* (Liu *et al*., 2005). Furthermore, changes in the expression of genes involved in β-1,6-glucan biosynthesis (including *SKN1* and *KRE1*) have also been proposed as a resistance mechanism against polyene antifungals (Gale, 1986 ; Mio *et al*., 1997 ; Liu *et al*., 2005).

Antifungal resistance in *C. albicans* biofilms is a complex phenomenon, and like in planktonic cells, multiple mechanisms appear to be involved (Kuhn & Ghannoum, 2004). It was reported that efflux pumps are highly expressed in young biofilms (Ramage *et al*., 2002 ; Mukherjee *et al*., 2003 ; Mateus *et al*., 2004), even in the absence of an antifungal agent. However, the expression of genes encoding efflux pumps (*CDR* and *MDR* family) seems to be model-system and/or strain-dependent as *CDR* and *MDR* genes were not found to be overexpressed in the transcriptome studies of Garcia-Sanchez *et al*. (2004) and Murillo *et al*. (2005). Nevertheless, some genes (including *QDR1* and *CDR4*) appeared overexpressed in the study by Yeater *et al*. (2007) and other genes (including *CDR2* at 12 h and *MDR1* at 12 and at 24 h) were overexpressed in the in vivo model described by Nett *et al*. (2009). Reduced ergosterol levels (combined with increased levels of other sterols) also provide a possible resistance mechanism in biofilms (Mukherjee *et al*., 2003) and changes in the expression

levels of *ERG* genes were observed in several studies (Yeater *et al*., 2007 ; Nett *et al*., 2009). These changes probably lead to changes in the sterol composition of the cell membrane and may have a profound impact on antifungal resistance. Khot *et al*. (2006) and LaFleur *et al.* (2006) showed that resistant subpopulations ("persisters") are present in *C. albicans* biofilms. Using untreated biofilms, Khot *et al.* (2006) compared the less-resistant, shear-removed, fraction of the biofilm with the basal blastospore subpopulation. In the latter, a marked downregulation of the *ERG1* gene was observed, probably resulting in an overall downregulation of the ergosterol biosynthesis (remarkably, the expression of *ERG11* was not altered). *SKN1* and *KRE1* were markedly upregulated in this resistant subpopulation. These changes in gene expression likely contributed to the observed amphotericin B resistance. When *C. albicans* biofilms in various stages of growth were treated with very high doses of fluconazole, an overexpression of genes involved in the ergosterol biosynthesis (*ERG1, 3, 11* and *25*) was observed, whereas after exposure to amphotericin B an upregulation of *SKN1* and *KRE1* was observed. The transcriptional changes in sessile *C. albicans* cells in the presence of an antifungal agent likely result in an upregulation of the associated biosynthetic pathways, thereby contributing to a resistant biofilm phenotype. These data suggested that young and mature biofilms show a rapid and antifungal-specific transcriptional response to exposure to antifungal agents. This drug-specific molecular adaptation could help to explain the high resistance of *C. albicans* biofilms towards antifungal agents (Nailis *et al*., 2010)

Role of differential expression of phage-related genes

Overexpression of phage-related genes in sessile cells compared to planktonic cells and/or increased expression in response to stress have been observed in several species. The most-highly overexpressed *P. aeruginosa* PAO1 genes in the study of Whiteley *et al*. (2001) were proteins from a Pf1-like bacteriophage (now designated Pf4 ; Webb *et al*., 2004) and this was confirmed by a 100-1000-fold greater abundance of phage particles in the biofilm reactor compared to planktonic cultures. In *Bacillus subtilis,* 17 genes involved in production of the defective prophage PBSX are overexpressed in biofilms (Stanley *et al*., 2003). In *B. cenocepacia* biofilms, a prolonged treatment (30 or 60 min) with H_2O_2 resulted in an increased transcription of genes belonging to a BcepMu prophage (BCAS0540-BCAS0554), located on one of the *B. cenocepacia* genomic islands (genomic island 14) (Peeters *et al*., 2010). One of these genes (BCAS0547, encoding a putative DNA-binding phage protein) was also found to be upregulated during growth in cystic fibrosis sputum (Drevinek *et al*., 2008).

Bacterial stress responses can increase the mobility of bacteriophages (reviewed by Miller, 2001) and it has been proposed that prophage production may have a role in generating genetic diversity in the biofilm (e.g. production of Pf4 in *P. aeruginosa* biofilms is correlated with the emergence of small-colony variants) (Webb *et al*., 2004). When faced with unstable environmental conditions, communities are protected by diversity, a principle known as the "insurance hypothesis" (Boles *et al*., 2004) and the diversity generated by the induction of prophages may contribute to biofilm resilience.

An emerging picture

From the examples above, it is clear that sessile cells have various ways of coping with the stress imposed on them by treatment with antibiotics or disinfectants.

A first defense mechanism is the upregulation of genes encoding efflux pumps, resulting in an increased efflux of the antimicrobial agent. In some organisms particular efflux pumps appear to be biofilm specific. The increased production of enzymes that can degrade antibiotics or reactive oxygen species is an important defense mechanism in various bacteria. While some of these enzymes appear equally important for protecting planktonic and sessile cells (e.g. *katB* in *B. cenocepacia*), some appear biofilm specific (e.g. *ahpCF* in *P. aeruginosa*). Phenotypic adaptations resulting in reduced transport of antimicrobial agents in biofilms and/or reduced permeability of the cell have also been reported. These include the increased production of extracellular matrix (e.g. alginate) and/or other components that can sequester antibiotics (e.g. cyclic glucans), and the differential expression of genes affecting cellular uptake (e.g. *tolA*). Finally, altering the expression of genes coding for the target of the antimicrobial agent (e.g. *ERG* genes in *C. albicans*) and/or activating alternative pathways can also result in decreased susceptibility.

Interestingly, in various organisms the expression of genes thought to be involved in stress resistance is altered in sessile cells compared to planktonic cells, even in the absence of the stress, leading to "innate resistance" of sessile cells. Examples include the upregulation of several genes coding for efflux pumps in *C. albicans*, the upregulation of *tolA* in *P. aeruginosa*, the downregulation of cytochrome c oxidase genes in *P. aeruginosa* and the upregulation of heat-shock proteins in *E. coli*. Generating diversity by the induction of prophages may also contribute to the intrinsic resistance of biofilm populations.

Problems associated with reliably identifying gene expression patterns in biofilms and implications for identifying the response to stress

Heterogeneity in microbial biofilms

It is a common misconception that all cells in a biofilm are exposed to the same conditions. Contrary, differences in metabolic activities combined with differencs in transport of molecules in a biofilm result in gradients of nutrients, oxygen, signalling molecules and metabolic end products. As a result of these gradients, considerable structural, chemical and biological heterogeneity can be found within a biofilm (Stewart & Franlin, 2008). For example, tomographic fluorescence imaging using silica nanoparticle sensors showed that within an *E. coli* biofilm pH values can vary from 5 to > 7, due to low rates of diffusion of acidic metabolites or accumulation of fermentation products in oxygen-limited parts of the biofilm (Hidalgo *et al*., 2009). As a consequence of this diversity, harvesting entire biofilm populations will only allow the identification of genes as being differentially expressed if these genes are uniquely expressed in biofilms and will result in an "average" picture of gene expression (Stewart & Franklin, 2008). Unfortunately, few alternatives are at our disposal. Reporter genes fused to promotor regions of a gene of interest can be used to microscopically monitor the expression of that gene in a biofilm (Stewart & Franklin, 2008). A recent example of such a study is that of Ito *et al*. (2009b) who used a *rpoS-gfp* transcriptional fusion mutant to monitor *rpoS* expression in *E. coli* biofilms. Their results confirmed the existence of localised expression profiles, with *rpoS* being expressed in the majority of cells in early phases of biofilm formation, while in later stages of biofilm formation, *rpoS* expression appeared limited to cells at the outside of the biofilm. Although useful, this approach requires the use of genetically-manipulated microorganisms and is at present not suitable for the simultaneous analysis of a large number of genes. Lenz *et al*. (2008) described the use of laser capture microdissection microscopy (LCMM) to recover cells from spatially resolved sites within biofilms. RNA can then be isolated from these cells, allowing the study of gene expression by real-time quantitative PCR. Their proof-of-concept study confirmed that this approach is feasible and demonstrated that mRNA levels for particular genes are not uniform throughout the biofilm. The issue of heterogeneity is particularly relevant for *C. albicans*, which has multiple morphological forms (yeast, hyphae, pseudo-hyphae) (Calderone & Fonzi, 2001). The fraction of filaments in a biofilms is highly dependent on the biofilm model system and the stage of biofilm formation (Nailis *et al*., 2009) and as a number of genes are considered to be hyphae-specific (or at least hyphae-associated), including *ALS3* and *HWP1*

(Hoyer *et al*., 1998 ; Sundstrom, 2002), interpretation of differential expression of genes under conditions that affect filamentation should take this into account.

It should be pointed out that also in planktonic cultures there can be considerable heterogeneity. Laser-diffraction particle-size scanning and microscopy of "planktonic" cultures of *P. aeruginosa* indicated that up to 90% of the entire culture was present in aggregates of 10 to 400 µm, rather than as individual cells, and these planktonic cultures actually are more similar to "suspended biofilms" (Schleheck *et al*., 2009). How this growth phenotype influences gene expression is at present unclear, but this observation illustrates that a careful validation of both model systems (biofilm and planktonic) prior to comparing gene expression is warranted.

Role of small RNAs (sRNA) in microbial group behaviour

sRNA-mediated post-transcriptional control at the mRNA or protein level plays a pivotal role in mediating bacterial adaptation to changing conditions (Papenfort & Vogel, 2009 ; Waters & Storz, 2009). The regulation exerted by sRNAs is often negative, as protein levels are repressed through translational inhibition, mRNA degradation or both. Most require the RNA chaperone Hfq to facilitate RNA-RNA interactions and to stabilise unpaired sRNAs. A given sRNA can regulate multiple targets and this means that a single sRNA can globally modulate a particular physiological response in much the same manner as a conventional transcription factor, but at the posttranscriptional level (Papenfort & Vogel, 2009 ; Vogel, 2009 ; Waters & Storz, 2009). Modeling studies have clearly indicated that, when fast response to external signals are required (like in the case of a stress response), sRNA-based regulation is advantageous over protein-based regulation. sRNAs are also better than transcription factors in filtering out the noise in input signals. Taken together the data from modeling studies suggest that there is a particular "niche" for sRNAs in allowing the quick and reliable transition between distinct states (Levine *et al*., 2007 ; Shimoni *et al*., 2007 ; Mehta *et al*., 2008). Conventional transcriptomic analyses rely on microarray-based identification of gene expression, and are inherently biased as only expression levels of genes for which probes are included on the array can be measured. As our knowledge about the occurrence of sRNAs in various organisms is still limited, the number of probes directed against intergenic regions (containing sRNAs) is often small, precluding the identification of transcripts coming from intergenic regions. In addition, reverse transcription of sRNAs is often suboptimal (due to their small size and pronounced secondary structure) and probe labeling can also be hampered by the intrinsic structure of the sRNA (Huttenhoffer & Vogel, 2006 ; Sharma & Vogel, 2009).

Nevertheless, a limited number of studies have focused on the potential role of sRNAs in biofilm formation and phenotypic adaptation to stress. One of the bacterial regulatory systems involving sRNA is the carbon storage regulator (Csr) system (Romeo, 1998). CsrA is a small RNA-binding protein that represses the expression of many stationary phase genes, while inducing expression of exponential-phase pathways (including glycogen synthesis and catabolism, glycolysis and gluconeogenesis). The second component of the Csr system is the sRNA CsrB. CsrB can bind 18 CsrA molecules simultaneously and as such antagonizes the effect of CsrA (Romeo, 1998). Jackson *et al*. (2002a) showed that in *E. coli*, biofilm formation is increased in a *csrA* mutant and that there is no biofilm formation in a *csrB* mutant. *CsrB* and *CsrC* sRNAs modulate protein activity by mimicking mRNA and sequester away the CsrA protein from mRNA leaders. Moreover, induction of *csrA* expression induces biofilm dispersal. Additional studies have shown that the role of CsrA is consistent under diverse growth conditions and in a variety of enterobacterial strains and species (Jackson *et al*., 2002b ; Agladze *et al*., 2003). The link between the *csrA/B* system and biofilm formation was found to be the cell-bound polysaccharide adhesin poly-β-1,6-N-acetyl-glucosamine (PGA) (Wang *et al*., 2005), as CsrA post-transcriptionally represses the gene required for PGA production, while there is also an indirect repression through inhibition of *glgCAP* expression (necessary for the stationary phase carbon flux into glycogen and subsequent conversion to glucose-1-phosphate required to generate a PGA precursor). In addition, the expression of *luxS* in *E. coli* (encoding the key enzyme in the biosynthesis of the autoinducer-2 quorum sensing molecule) is negatively regulated by the sRNA CyaR (De Lay & Gottesman, 2009). This downregulation results in a decreased AI-2 production ; under glucose-limited conditions this system probably decreases biofilm formation while increasing planktonic behaviour and as such may trigger the organisms to move in search of nutrients. Also in *P. aeruginosa* social behaviour is co-regulated by sRNA molecules (Heurlier *et al*., 2004 ; Kay *et al*., 2006 ; Luchetti-Miganeh *et al*., 2008 ; Lapouge *et al*., 2008). RsmA (a sRNA-binding regulatory protein) negatively controls the production of *N*-acyl homoserine lactone signalling molecules but this RsmA-based repression is antagonised by the GacA-dependent sRNAs RsmY and RsmZ. In addition, an *rsmY rsmZ* double mutant shows enhanced biofilm formation compared to the wild type, suggesting that both genes jointly influence biofilm formation.

Recently, a significant upregulation of transcriptional activity stemming from intergenic regions was noted when *B. cenocepacia* J2315 biofilms were treated with oxidising agents (Peeters *et al*., 2010). Treatment with H_2O_2 or NaOCl resulted in the upregulation of 37 and

56 intergenic regions, respectively, compared to untreated biofilms. Several of these intergenic regions were located in close proximity of genes with a similar expression pattern, suggesting co-transcription. However, other intergenic regions demonstrated markedly different expression patterns compared to their flanking genes and the basal expression levels of several of these regions was high. Several of these putative sRNAs were previously predicted by an *in silico* approach (Coenye *et al*., 2007), while others were found to be differentially expressed in *B. cenocepacia* grown in sputum (Drevinek *et al*., 2008) or in soil-like conditions (Yoder-Himes *et al*., 2009). While the function of most of these putative sRNAs remained elusive, one had a marked similarity to the 6S RNA consensus structure, indicating its potential involvement in regulating gene expression.

Outlook

Traditionally, microarrays are used to identify changes in gene expression in high-throughput analyses, but there are several drawbacks associated with their use. Probably the most relevant drawback is that this approach is inherently biased (i.e. you can only measure what is known and hence represented on the array). This can be circumvented by using high-throughput parallel sequencing ("RNA sequencing"). This novel, unbiased, approach will not only reveal changes in the expression level of protein coding genes, but will also lead to the discovery of changes in sRNA expression. Several sequencing technologies are currently available, including pyrosequencing (454 sequencing) and Illumina "sequencing-by-synthesis" (Mardis, 2008 ; Shendure & Hanlee, 2008 ; Petterson *et al*., 2009). These techniques present a vast improvement over microarray-based transcriptome analysis, but still rely on the generation of cDNA prior to sequencing, which may be the source of various types of errors. Ozsolak *et al*. (2009) recently described an entirely novel approach called "direct RNA sequencing". Direct RNA sequencing is based on Helicos BioSciences' "True Single Molecule Sequencing" technology and allows the sequencing of femtomole quantities of RNA without the need for prior cDNA generation. This approach would allow the unbiased whole-transcriptome analysis of a low number of cells and would provide a snapshot of the response in various parts of the biofilms.

Despite advances in transcriptomics, the challenge for the future will remain the same, i.e. to link the changes in gene expression to phenotypic changes and (i) to determine whether differential gene expression really results in an observable altered phenotype and (ii) to

determine whether this differential gene expression and the resulting phenotype is attributable to the stress conditions applied.

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Table 1. Growth conditions for sessile and planktonic *C. albicans* SC5314 cells as reported in various transcriptomic and proteomic studies

Reference	Biofilm model	Planktonic model	Medium	Flow	Temp (°C)	Sampling point
Garcia-Sanchez *et al*. (2004)	Microfermentor		YNB* +0.4% glucose	Continuous	37	48 h
	Microtiter plate		YNB+0.4% glucose	Limited	37	48 h
	Catheter disks		YNB+0.4% glucose	Limited	37	72 h
	Microfermentor		YNB+2% glucose	Continuous	30	72 h
	Microfermentor		YNB+0.4% glucose	Continuous	37	72 h
		Flask	YNB+0.4% glucose	Limited	37	20 h
		Flask	YNB+0.4% glucose	Limited	37	48 h
		Flask	YNB+2% glucose	Limited	30	48 h
		Microtiter plate	YNB+0.4% glucose	Limited	37	48 h
Murillo *et al*. (2005)	Polystyrene petri dish	Polypropylene flask	F12 medium	Limited	37	30, 90, 150, 270 & 390 min
Thomas *et al*. (2006)	Tissue culture flasks	Liquid medium	RPMI-1640	Limited	37	24 h
Yeater *et al*. (2007)	Denture acrylic coated with saliva	Microtiter plate	YNB+50 mM glucose	Limited	37	6, 12 & 48 h
Zakikhany *et al*. (2007)	RHE†			Limited	37	1, 3, 6, 12 & 24 h
		Liquid medium	YPD+	Limited	37	mid-log phase
Seneviratne *et al*. (2008)	Microtiter plate	Liquid medium	YNB+100mM glucose	Limited	37	48 h
Nett *et al*. (2009)	Rat central venous catheter			Continuous	37	12 and 24 h
		Liquid medium	YPD	Limited	37	12 and 24 h
Martinez-Gomariz *et al*. (2009)	PMMA‡ strips		YPD+50 mM glucose	Continuous	37	48 h
		Liquid medium	YPD+50 mM glucose		37	48 h
		Liquid medium	YPD+50 mM glucose		37	1.5 - 2 h

* YNB, Yeast nitrogen base growth medium
† RHE, Reconstituted human epithelium
+ YPD, Yeast peptone dextrose growth medium
‡ PMMA, poly-methyl-methacrylate

Table 2. Selection of *P. aeruginosa* genes identified as being differentially expressed in treated vs. untreated sessile cells. Genes in bold were identified as differentially expressed in more than one study.

Gene	Function	Treatment	Up(+)/ Down(-)	Reference
PA0105-0106	Cytochrome c oxidase subunits	Azithromycin	-	Gillis *et al*. (2005)
PA0108	Cytochrome c oxidase subunit	Azithromycin	-	Gillis *et al*. (2005)
PA0376	RpoH	Imipenem	+	Bagge *et al*. (2004)
PA0721	Hypothetical protein (phage Pf1)	Tobramycin	-	Whitely *et al*. (2001)
PA0725	Hypothetical protein (phage Pf1)	Tobramycin	-	Whitely *et al*. (2001)
PA0762-0764	Alginate biosynthesis	Tobramycin	+	Anderson *et al*. (2008)
PA0762-0765	Alginate biosynthesis	Imipenem	+	Bagge *et al*. (2004)
PA0913	MgtE	Tobramycin	+	Anderson *et al*. (2008)
PA0996-1000	PQS biosynthesis	Tobramycin	-	Anderson *et al*. (2008)
PA1078-1081	Flagellar synthesis	Imipenem	+	Bagge *et al*. (2004)
PA1163	NvdB	Tobramycin	+	Mah *et al*. (2003)
PA1172-1175	Periplasmic nitrate reductase	Azithromycin	-	Gillis *et al*. (2005)
PA1178-1180	OprH, PhoP, PhoQ	Imipenem	+	Bagge *et al*. (2004)
PA1431	RsaL	Azithromycin	-	Gillis *et al*. (2005)
PA1432	LasI	Azithromycin	-	Gillis *et al*. (2005)
PA1541	Probable drug-efflux protein	Tobramycin	+	Whitely *et al*. (2001)
PA1703-1722	TT3S related genes	Azithromycin	+	Gillis *et al*. (2005)
PA1874-1877	Efflux pump	Tobramycin	+	Zhang & Mah (2008)
PA2191	Adenylate cyclase ExoY	Azithromycin	+	Gillis *et al*. (2005)
PA2367	Hypothetical protein	Imipenem	+	Bagge *et al*. (2004)
		Azithromycin	-	Gillis *et al*. (2005)
PA2703	Hypothetical protein	Tobramycin	+	Whitely *et al*. (2001), Bagge *et al*. (2004)
PA3049	Ribosome modulation factor	Tobramycin	+	Whitely *et al*. (2001)
		Azithromycin	-	Gillis *et al*. (2005)
PA3540-3551	Alginate biosynthesis	Imipenem	+	Bagge *et al*. (2004)
PA3574	Probable transcriptional regulator	Tobramycin	+	Whitely *et al*. (2001)
PA3819	Conserved hypothetical protein	Tobramycin	+	Whitely *et al*. (2001), Bagge *et al*. (2004)
PA3841-3843	Exoenzyme S & chaperone	Azithromycin	+	Gillis *et al*. (2005)
PA3920	Probable metal-transporting	Tobramycin	+	Whitely *et al*. (2001)

		P-type ATPase			
	PA4110	AmpC	Imipenem	+	Bagge *et al*. (2004)
	PA4306	Type IVb pilin	Imipenem	-	Bagge *et al*. (2004)
			Azithromycin	-	Gillis *et al*. (2005)
	PA4386	GroES	Tobramycin	+	Whitely *et al*. (2001)
	PA4407-4417	Cell wall biosynthesis, cell division	Imipenem	+	Bagge *et al*. (2004)
	PA4525-4526	Pili biosynthesis	Imipenem	-	Bagge *et al*. (2004)
	PA4597-4599	MexCD-OprJ efflux pump	Azithromycin	+	Gillis *et al*. (2005)
	PA4635	Hypothetical MgtC protein	Tobramycin	+	Anderson *et al*. (2008)
	PA4761	DnaK	Tobramycin	+	Whitely *et al*. (2001)
	PA4825	MgtA	Tobramycin	+	Anderson *et al*. (2008)
	PA4867	Urease β subunit	Tobramycin	-	Whitely *et al*. (2001)
	PA5040-5044	Pili biosynthesis	Imipenem	-	Bagge *et al*. (2004)
	PA5170	Arginine-ornithine antiporter	Imipenem,	+	Bagge *et al*. (2004)
			Azithromycin	+	Gillis *et al*. (2005)
	PA5216	Permease of ABC Fe transporter	Tobramycin	-	Anderson *et al*. (2008)
			Azithromycin	+	Gillis *et al*. (2005)
	PA5253-5255	Alginate biosynthesis	Tobramycin	+	Anderson *et al*. (2008)
	PA5261-5262	Alginate biosynthesis	Imipenem	+	Bagge *et al*. (2004)
			Tobramycin	+	Anderson *et al*. (2008)
	PA5348	Probable DNA binding protein	Tobramycin	+	Whitely *et al*. (2001)

Figure 1. *C. albicans* genes differentially expressed between planktonic and sessile cells, as identified in various studies. Data are expressed as fraction of the total number of differentially expressed genes identified in each study. The total number of genes identified as differentially expressed is also indicated.