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Quorum Sensing antagonistic activities of Azithromycin in *Pseudomonas aeruginosa* PAO1: a global approach

Yusuf Nalca[1], Lothar Jänsch[1], Florian Bredenbruch[1], Robert Geffers[2], Jan Buer[2] and Susanne Häussler[1*]

[1]Department of Cell Biology and [2]Mucosal Immunity, German Research Center for Biotechnology , Mascheroder Weg 1, D-38124 Braunschweig, Germany

Running title: Influence of azithromycin on *P. aeruginosa*

Correspondence:

PD Dr. Susanne Häussler
Head of Young Investigator Research Group
Chronic Pseudomonas Infections
German Research Center for Biotechnology
Mascheroder Weg 1
D-38124 Braunschweig
Germany
Tel.: +49-531-6181/307
Fax: +49-531-6181/444,
E-mail: susanne.haeussler@gbf.de

ABSTRACT

The administration of macrolides such as azithromycin in chronic pulmonary infection of cystic fibrosis (CF) patients has been reported to be of beneficial value. Although the mechanisms of action remain obscure, anti-inflammatory effects as well as interference of the macrolide with *Pseudomonas aeruginosa* virulence factor production have been suggested to contribute to an improved clinical outcome. In this study we used a systematic approach and analyzed the impact of azithromycin on the global transcriptional pattern and the protein expression profile of *P. aeruginosa* PAO1 cultures versus untreated controls. The most remarkable result of this study is the finding that azithromycin exhibited extensive quorum sensing antagonistic activities. In accordance with the inhibition of the quorum sensing systems, virulence factor production was diminished, the oxidative stress response was impaired, whereas the type III secretion system was strongly induced. Moreover, *P. aeruginosa* motility was reduced which probably accounts for the previously observed impaired biofilm forming capabilities of azithromycin treated cultures. The interference of azithromycin with quorum sensing dependent virulence factor production, biofilm formation and oxidative stress resistance of *P. aeruginosa* holds great promise for macrolide therapy in CF. Clearly quorum sensing antagonist macrolides should be paid more attention in the management of chronic *P. aeruginosa* infections and as a quorum sensing antagonist macrolides might gain vital importance for a more general application in chronic infections.

INTRODUCTION

There is a unique predilection of the cystic fibrosis (CF) lung for chronic *Pseudomonas aeruginosa* infection which is still the major cause of morbidity and mortality in these patients (4, 24, 36). Although in the past decades antibiotic therapy has greatly increased the life expectancy, there are only limited therapeutic options available and chronic *P. aeruginosa* infection is rarely eradicated (5, 20, 37). Hence, there is a is an urgent need to develop alternative treatment regimes to improve lung function and thus the prognosis of the disease. Although the basis of principles concerning the therapeutic strategies in the treatment of chronic lung infection has not changed significantly in the last 10 years, the use of azithromycin (AZM) for infection control and inflammation modulation is one new aspect (2, 11, 38, 44, 52). By conventional standards *P. aeruginosa* is insensitive to therapeutic concentrations of macrolides, however, recently macrolides have been reported to positively influence the clinical outcome in patients suffering from chronic *P. aeruginosa* infection in diffuse panbronchiolitis (DPB) (13, 15, 21, 40, 41). DPB was first reported in Japan and is characterised by an inflammatory cell infiltration in the respiratory bronchioles leading to their obstruction and dilatation (35). As disease progresses patients typically become colonized with mucoid strains of *P. aeruginosa* accompanied by cystic changes of the lung and by poor clinical prognosis due to a progressive deterioration of respiratory function. The remarkable parallels between DPB and CF led to the question whether macrolide antibiotics would also be of beneficial value in patients with CF and to large scale randomised controlled trials to elucidate the properties of macrolides for chronic *P. aeruginosa* infection of the CF lung (31, 34, 39, 51). The majority of clinical studies report of positive trends concerning the therapeutic potential of macrolide therapy (44). However, the mechanisms of action in chronic *P. aeruginosa* infection remain obscure (53). Immunomodulatory effects are postulated to account for some of the beneficial effects (9, 16), in addition to an altered airway epithelial chloride transport and the inhibition of *P. aeruginosa* virulence factor

production due to the interference of macrolides with interbacterial communication (33, 45). Interbacterial communication is also referred to as quorum sensing (QS) and is a very sophisticated mechanism in which signal molecules act as autoinducers and trigger a variety of biological functions when microbial populations attain certain cell densities. QS controls not only virulence factor production but also biofilm formation in *P. aeruginosa* (3) and thus significantly contributes to pathogenesis and persistence of the infection. The QS system in *P. aeruginosa* comprises two hierarchically organized systems consisting each of an autoinducer synthetase (LasI/RhlI) and a corresponding regulator protein (LasR/RhlR). Both the *las* and the *rhl* QS systems have been shown to be transcriptionally repressed by subinhibitory AZM concentrations (45). In this study we applied a systematic approach and analyzed the transcriptome and proteome profile of *P. aeruginosa* in response to subinhibitory concentrations of AZM. Using this global approach, we aimed to identify the influence of AZM on QS regulated genes/proteins and thus to gain background data on the therapy with macrolides for purposes other than their bactericide properties.

MATERIAL AND METHODS

Bacterial strains and culture conditions. For the isolation of RNA and for the preparation of the cellular and extracellular protein extracts *P. aeruginosa* PAO1 (DSM 1707) was grown in Brain-Heart-Infusion (BHI) medium at 37°C with shaking with or without the addition of 2 µg/mL AZM (Pfizer, Germany) until early stationary phase.

RNA extraction and preparation of protein samples. Total RNA was extracted from 10 mL of 4 AZM-treated and untreated PAO1 cultures each, cDNA was synthesized from the RNA pooled from 2 independent cultures and subsequently two GeneChips were hybridized for each culture condition. RNA isolation, cDNA generation, fragmentation, biotinylation and GeneChip hybridization and analysis were performed according to the Affimetrix guidelines and are conform to the MIAME requirements (Minimum Information About a Microarray Experiment; experimental details are available at http://www.ncbi.nlm.nih.gov/projects/geo/submission/login/ under the accession number: GSE2430). Three independent protein extracts from pooled supernatants and cell pellets of 4 150-mL AZM-treated and untreated PAO1 cultures each were prepared and used immediately for 2-dimensional gel electrophoresis. The preparation of protein extracts, 2-D gel electrophoresis and matrix-assisted laser desorption / -ionisation time of flight mass spectrometry (MALDI-TOF MS) analysis were performed as described previously (50). The gels were stained with Ruthenium II tris (bathophenantroline disulfonate) (RuBPS) (22) and differentially expressed proteins as detected in duplicate gels were quantified by ProteomWeaver 2.1 (DEFINIENS). Proteins were considered to be significantly affected when their spot intensities changed at least 2-fold.

Lactate dehydrogenase (LDH) release assay. The bacteria were grown in BHI medium with and without the addition of 2 µg/mL AZM and harvested in log- (OD_{600} ~ 0.6) and stationary-

phase (OD_{600} ~ 2.7) respectively. J774.A1 cells were grown to confluence in flat-bottom 96-well plates in Dulbecco's Modified Eagle Medium (DMEM) + 10 % Fetal Calf Serum (FCS) medium and infected with 20 μL of the bacterial suspension in 200 μL fresh DMEM + 1% FCS. Cell viability was assessed by the determination of LDH in the supernatant fractions by using an LDH cytotoxicity detection kit according to the manufacturer's instructions (Roche, Mannheim, Germany).

H_2O_2 sensitivity assay. The H_2O_2 sensitivity disk assay was adapted from Hassett et al. (7). Briefly, *Pseudomonas* PAO1 was grown at 37°C in BHI medium for various incubation periods with and without the addition of 2 μg/mL AZM. 100 μL of the bacterial culture was suspended in 3 mL of LB soft agar at 40°C (0.6% (wt/vol) agar), mixed and poured on LB agar plates with 1.5% wt/vol agar. Sterile filter paper disks were placed on the soft solid agar and the disks were spotted with 8 μL of 30% H_2O_2. Plates were incubated at 37°C for 24 h and the diameter of the zone of growth inhibition was measured. All experiments were performed in triplicate.

Motility Assays. Media for swimming and twitching assays were LB containing 0.3% (wt/vol) and 1.5% (wt/vol) Bacto-agar (Difco) with and without the addition of 2 μg/mL AZM. Plates were inoculated with bacteria from an overnight LB culture grown in the presence and absence of 2 μg/mL AZM respectively. The swimming agar plates were inoculated with 15 μL of the bacterial suspension and incubated at 37°C for 24 h whereas the twitching agar plates were inoculated with a sterile toothpick at the bottom of the petri dish and incubated at 37°C for at least 24 h.

RESULTS

Influence of subinhibitory azithromycin concentrations on the PAO1 transcriptome and proteome. Since an interference with virulence factor production in *P. aeruginosa* has been postulated to be responsible for the observed beneficial effect of macrolide therapy, we aimed to analyze the effect of AZM on bacteria at the early stationary growth phase, when virulence factor production is high. As observed previously (45) the addition of 2 µg/mL azithromycin led to a prolonged lag phase, whereas exponential- and stationary-phase of growth were only minimally affected as compared to the PAO1 control culture (Fig. 1).

A comparison of the gene expression profile when the PAO1 strain cultured with and without exogenous AZM reached an OD_{600} of 2.8 revealed 107 genes that were differentially expressed (78 of those genes were shown to be up-regulated and 29 genes were repressed), representing 1.9% of the entire genome (Table 1). Many of the AZM induced genes were genes encoding for the ribosomal subunits. In addition, an initiation factor (*infA* PA2619) and an elongation factor (*efp* PA2851) were over-expressed in AZM treated cultures. Since the macrolides exhibit their antibacterial activity by binding to the 50S ribosomal subunit resulting in the blockage of transpeptidation and/or translation, this implies these genes may be over-expressed to compensate for an impaired translation process due to subinhibitory AZM concentrations.

In a complementary approach to the identification of the global transcriptional pattern we analysed the protein expression profile of AZM treated in comparison to non-treated PAO1 cultures. Comparative analyses of the secretome and of cellular extracts of AZM treated versus non-treated PAO1 cultures disclosed a total of 43 differentially expressed proteins (Table 2). 11 proteins were up-regulated and 15 proteins were down-regulated in the secretome of the PAO1 cultures after AZM addition (Fig. 2). In contrast to the marked differences in the expression profiles of the secretome, the 2-D gels of the cellular extracts exhibited relatively constant protein profiles (data not shown). 6 proteins were up-regulated

and 11 proteins were down-regulated in the cellular fraction of the PAO1 cultures after AZM addition.

The finding that only 8 genes / proteins (Table 3) were shown to be affected at both the transcriptional and the protein level, emphasizes that transcriptomics and proteomics are complementary approaches, and by combining their particular strengths a maximum of relevant results is to be expected. Major differences between proteome and transcriptome data have been documented in previous studies to map the *P. aeruginosa* QS regulon: the 2 proteome studies of Arevalo-Ferro et al. (1) and Nouwens et al.(29) identified 47 and 27 QS regulated proteins and only 11 and 8 respectively of the corresponding genes were found to be regulated at the transcriptional level in 3 independent transcriptome studies analyzing QS dependent *P. aeruginosa* gene expression (8, 42, 48).

Effect of azithromycin on quorum sensing in PAO1. 8 out of 77 genes (10.4%) of the general QS regulon (identified as QS dependent genes in all 3 recent micro-array studies (8, 42, 48)) were identified as being influenced by AZM addition (Table 1). Overall, 15 AZM and QS dependent genes (identified as QS dependent in at least one of the three micro-array studies) were found. Ten of the 15 QS and AZM dependent genes were shown to be down-regulated in this study in response to AZM, however 5 genes were up-regulated. An up-regulation is opposite to what is expected assuming that AZM inhibits QS. However, QS regulated proteins have previously been found to be oppositely regulated in a comparison between 2 previous proteomic studies (1, 29, 29) and it was speculated that some of the observed discrepancies are caused by differences in experimental conditions. Further work will be required to address this issue. Moreover, we found genes of the QS dependent type III secretion system (TTSS) (10) and the QS controlled *katA* and *sodB* (7) to be AZM dependent. In addition to the identification of a common subset between QS and AZM regulated genes, we compared the differential protein expression due to AZM treatment with the QS

dependent protein expression of the 2 previous proteome studies. There was a large common subset of QS and AZM regulated proteins: 15 proteins that were shown to be affected by AZM addition were among the total of 47 proteins previously identified as being QS dependent (31.9%) (Table 2). The best congruence was found in the secretome supporting the recent findings of Wagner et al. (49) who reported the constant expression of QS regulated virulence factors even under various culture conditions.

Azithromycin enhances the expression of the type III secretion system. One of the major findings of this study is that AZM treatment of PAO1 led to an increased expression of the TTSS. We found an increased expression of 10 out of 36 genes of the TTSS gene cluster (PA1690-PA1725) in the AZM treated cultures. Moreover, the expression of genes encoding for the secreted effector proteins ExoT, ExoY and ExoS, that are located outside the TTSS gene cluster, was enhanced. The *pcrV* gene encoding for a TTSS secreted protein exhibited the highest differential gene expression (91.9 fold) and accordingly PcrV was overproduced in the AZM treated cultures.

In order to test whether TTSS overproduction in *P. aeruginosa* has a biological effect, we determined the *in vitro* cytotoxicity of AZM pre-treated bacteria on the murine macrophage cell line J774.A1. As shown in Fig. 3 *P. aeruginosa* PAO1 that was cultured in medium containing 2 µg/mL AZM until log- and early-stationary phase of growth exhibited an increased cytotoxicity in comparison to the bacteria cultured without AZM.

Azithromycin affects P. aeruginosa motility. AZM pre-treated cultures exhibited a reduced expression of various proteins required for flagella biosynthesis (Table 2) and demonstrated a reduced flagella driven motility on swimming agar plates. The mean value of the radius of the untreated PAO1 and AZM pre-treated PAO1 cultures was 3.58 (± 0.17) cm and 1.18 (± 0.09) cm respectively, $p<0.01$. Although AZM has been previously demonstrated to inhibit not

only swimming motility (14) but also twitching motility (14, 53), we observed an increased expression of *pilA* and *pilH* involved in type IV pili biogenesis (Table 1). No effects of sub-inhibitory AZM concentrations on type IV pili could be observed by proteomics. Analysis of twitching motility of AZM pre-treated PAO1 revealed that their twitching motility was significantly reduced. The mean value of the radius of the untreated PAO1 and AZM pre-treated PAO1 cultures was 0.67 (± 0.06) cm and 0.51 (± 0.06) cm, respectively, $p<0.01$. While flagella mediated motility has been implicated to be required to bring *P. aeruginosa* within proximity of a surface, type IV pili by virtue of twitching motility enable *P. aeruginosa* to migrate across a surface, recruit cells from adjacent monolayers and form cell aggregates (30) thus contributing to biofilm formation. The observed impaired swimming and twitching motility of AZM treated PAO1 could explain the previous observations that AZM delays biofilm formation, as evidenced by a decreased biomass (6) and impaired alginate production (12, 27).

Azithromycin affects the oxidative stress response in PAO1. Another major finding of this study is that AZM treatment obviously led to an impaired oxidative stress response in *P. aeruginosa*. The superoxide dismutase SodB, the catalase KatA and the alkylhydroperoxide reductase AhpC, all of which contribute significantly to the stress response in *P. aeruginosa* (32), were shown to be repressed at the transcriptional (*sodB, ahpC, katA*) and the protein level (SodB and AhpC) upon AZM addition. We also observed an up-regulation of the *fur* gene in response to subinhibitory AZM concentrations. *Fur* is involved in the regulation of iron uptake under iron limiting conditions. However, *fur* has also been shown to be up-regulated under oxidative stress (32) and the simultaneous over-expression of the *bfrB* gene indicates a sufficient intercellular iron storage (47). Another important element affected by oxidative stress is sulfur, since iron-sulfur proteins have been shown to play a protective role in oxidative stress (18). The sulfate binding protein of an ABC transporter (CysP) was one of

the eight proteins that were demonstrated to be up-regulated upon AZM addition both at the transcriptional and at the protein level. PA3262, a putative peptidyl-proplyl isomerase that probably corrects misfolding caused by the damage of reactive oxygen intermediates, was up-regulated at the transcriptional level. Similar, the thiol-disulfide oxireductase (DsbA) which has been shown to be responsible for protein thiol modifications in *Escherichia coli* (23), was up-regulated in the secretome of AZM treated PAO1. Thiol-disulfide interconversion plays a crucial role in the control of the cellular redox potential and the prevention of oxidative damage. PA3529, a probable peroxidase, as well as the genes encoding the heat shock proteins GroEL (also shown to be differentially expressed at the protein level) and GroES were down-regulated by AZM treatment. Moreover, the ribosome modulation factor *rmf* was the most strongly repressed gene upon AZM addition. This gene exhibits a high homology to the *rmf* gene in *Escherichia coli*, where *rmf* has been shown to be important for survival under stationary phase conditions (55). The observed effects of AZM on several genes / proteins that are involved in the oxidative stress response implicated that AZM might affect long term survival of *P. aeruginosa* during chronic infection. Thus, we tested whether AZM exposed cultures were more sensitive to H_2O_2 treatment as compared to the untreated controls. As shown in Fig. 4 bacteria that were pre-treated with AZM were significantly more susceptible when exposed to H_2O_2 on solid agar. Prolonged growth of PAO1 in AZM supplemented cultures increased the bacterial susceptibility towards H_2O_2.

DISCUSSION

Both antimicrobial and anti-inflammatory effects of AZM have been implicated to be responsible for the improvement in CF patient outcome. The results of this study clearly indicate that there is an antipseudomonal effect of AZM that is linked to a reduced virulence factor production, biofilm formation and survival under stressful conditions due to an interference with QS in *P. aeruginosa*. We identified a large common subset between QS and

AZM regulated genes / proteins in particular within the secretome comprising many virulence factors. Moreover, the TTSS - that was previously shown to be negatively regulated by QS (10) - was induced upon AZM addition, whereas in accordance with the results of a study reporting the QS control of genes essential for relieving oxidative stress (7), we found a markedly increased sensitivity of AZM pre-treated PAO1 cultures to H_2O_2. Moreover, our data on *P. aeruginosa* motility are in accordance with the observation that AZM retards biofilm formation (6) which has been reported to be dependent on the QS systems. Our *in vitro* data implicate that the QS antagonistic activity of AZM contributes to the improvement of CF patient health. Apart from the reduced expression of virulence factor production, the interference with the bacterial oxidative stress response might be of major relevance. One vitally adaptive response of *P. aeruginosa* is the ability to resist oxidative stress that is induced during phagocytosis, when the bacteria are confronted with reactive oxygen intermediates (ROI) such as H_2O_2, O_2^-, and OH^- from the respiratory burst of human phagocytes. Polymorphonuclear cells are the major effector cells responsible for the clearance of *P. aeruginosa* from the site of infection, and the inflammatory response in the chronically infected CF lung in particular is accompanied by very high levels of ROIs that the bacteria must survive to be able to persist. Thus the impaired oxidative stress response might account for the observed beneficial effects of AZM treatment and for the significant reduction of PAO1 viability after prolonged incubation with subinhibitory AZM concentrations that has been reported previously (46).

Several studies have demonstrated that macrolide antibiotics suppress the expression of substances that contribute to *P. aeruginosa* virulence such as exoenzymes, exopolysaccharides and pigments (14, 17, 25, 26) and it has been hypothesized that CF patients benefit from an AZM treatment due to the negative effect on virulence factor production. However, it has also been demonstrated that pre-treatment of *P. aeruginosa* with macrolides, including AZM, enhances virulence in mice significantly (19). An involvement

of acute toxic effects rather than multiplication of the bacteria was suggested. The results of our study implicate that the enhanced expression of the TTSS might account for these previously observed effects as the TTSS has been shown to enhance *P. aeruginosa* virulence significantly (43). However, only the inoculation of macrolide pre-treated bacteria had been shown to be associated with an increased mortality in mice, whereas the administration of macrolides after the inoculation of *P. aeruginosa* did not increase mortality (19, 28). The impact of the TTSS induced cytotoxicity for therapeutic macrolide administration remains to be clarified in future investigations.

Since chronic *P. aeruginosa* infection in CF is rarely eradicated despite intensive antimicrobial therapy, interference or blocking of QS systems have been considered as attractive alternative therapeutic strategies. Recently, halogenated furanones have been shown to control *P. aeruginosa* infections in animal models (8, 54). The finding that QS antagonists are effective is of considerable importance, since it demonstrates that QS is a useful and promising drug target *in vivo*. For the treatment in humans, macrolides seem to be a promising alternative drug to the toxic halogenated furanones, that might block the QS systems within therapeutic concentration ranges. How the macrolides precisely interfere with the transcription of QS regulated genes remains poorly defined and will be an important task to be addressed in the future. Macrolides inhibit protein synthesis at the ribosomal level and it is conceivable that yet unidentified stress responses, bacterial regulons or signal transduction processes are responsible for the observed effects of subinhibitory concentrations on gene expression. Furthermore, future studies will have to elucidate whether the observed effects of subinhibitory AZM concentrations are relevant also within the *in vivo* situation. However, the results of this *in vitro* study and the fact that AZM exhibits beneficial effects in the treatment of CF patients give reasons to assume that the administration of AZM in CF has a great impact on the management of chronic infection due to its interference with *P. aeruginosa* QS

and thus virulence factor production, biofilm formation, and persistence during chronic infection.

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TABLE 1. Azithromycin regulated genes in PAO1

PA number[a]	Gene name	Up-regulated (+) and down-regulated (-) in response to AZM (fold)	Proteins as described by http://www.pseudomonas.com
PA0044[b]	***exoT***	+13,54	exoenzyme T
PA0263[c]	***hcpC***	+3,83	secreted protein Hcp
PA0409	*pilH*	+3,24	twitching motility protein PilH
PA0422		+6,27	conserved hypothetical protein
PA0456		+3,38	probable cold-shock protein
PA0555	*fda*	+5,16	fructose-1,6-bisphosphate aldolase
PA0649	*trpG*	+12,08	anthranilate synthase component II
PA0650	*trpD*	+7,38	anthranilate phosphoribosyl transferase
PA0651	*trpC*	+5,90	indole-3-glycerol-phosphate synthase
PA0805		+8,74	hypothetical protein
PA0943		+3,51	hypothetical protein
PA0996	*pqsA*	+3,78	probable coenzyme A ligase
PA1440		+5,38	hypothetical protein
PA1493	*cysP*	+3,50	sulfate-binding protein of ABC transporter
PA1494		+2,61	conserved hypothetical protein
PA1564		+4,64	conserved hypothetical protein
PA1696[b]	***pscO***	+8,38	translocation protein in type III secretion
PA1706[b]	***pcrV***	+91,88	type III secretion protein PcrV
PA1707[b]	***pcrH***	+14,16	regulatory protein PcrH
PA1708[b]	***popB***	+6,77	translocator protein PopB
PA1709[b]	***popD***	+6,25	Translocator outer membrane protein PopD precursor
PA1710[b]	***exsC***	+8,99	exoenzyme S synthesis protein C precursor
PA1711[b]		+11,87	hypothetical protein
PA1712[b]	***exsB***	+8,59	exoenzyme S synthesis protein B
PA1714[b]		+4,45	hypothetical protein
PA1715[b]	***pscB***	+16,46	type III export apparatus protein
PA1754	*cysB*	+3,24	transcriptional regulator CysB

PA2015		+2,78	probable acyl-CoA dehydrogenase
PA2016		+8,72	probable transcriptional regulator
PA2023	*galU*	+4,90	UTP--glucose-1-phosphate uridylyltransferase
PA2191[b]	***exoY***	+20,12	adenylate cyclase ExoY
PA2193[d]	***hcnA***	+2,62	hydrogen cyanide synthase HcnA
PA2423[d]		+5,55	hypothetical protein
PA2464		+3,42	hypothetical protein
PA2619	*infA*	+18,96	initiation factor
PA2747		+4,85	hypothetical protein
PA2755	*eco*	+4,69	ecotin precursor
PA2830	*htpX*	+6,97	heat shock protein HtpX
PA2851	*efp*	+4,60	translation elongation factor P
PA2895		+2,70	hypothetical protein
PA2900		+5,85	probable outer membrane protein precursor
PA2901		+14,03	hypothetical protein
PA3244	*minD*	+5,71	cell division inhibitor MinD
PA3262		+4,72	probable peptidyl-prolyl cis-trans isomerase, FkbP-type
PA3369		+6,90	hypothetical protein
PA3370		+6,28	hypothetical protein
PA3371[e]		+11,50	hypothetical protein
PA3531	*bfrB*	+4,88	bacterioferritin B
PA3656	*rpsB*	+4,74	30S ribosomal protein S2
PA3686	*adk*	+5,59	adenylate kinase
PA3742	*rplS*	+2,88	50S ribosomal protein L19
PA3841[b]	***exoS***	+9,56	exoenzyme S
PA3842		+14,72	probable chaperone
PA3976	*thiE*	+7,89	thiamine-phosphate pyrophosphorylase
PA3988		+2,67	hypothetical protein
PA4004		+4,36	conserved hypothetical protein
PA4006	*nadD*	+4,19	nicotinic acid mononucleotide adenylyltransferase
PA4059		+6,47	hypothetical protein

PA4114		+8,51	spermidine acetyltransferase
PA4235	*bfrA*	+4,35	Bacterioferritin A
PA4263	*rplC*	+3,56	50S ribosomal protein L3
PA4441		+3,18	hypothetical protein
PA4460		+3,56	conserved hypothetical protein
PA4495		+3,45	hypothetical protein
PA4525	*pilA*	+4,89	type 4 fimbrial precursor PilA
PA4567	*rpmA*	+5,05	50S ribosomal protein L27
PA4568	*rplU*	+9,85	50S ribosomal protein L21
PA4605		+8,72	conserved hypothetical protein
PA4670	*prs*	+7,09	ribose-phosphate pyrophosphokinase
PA4671		+3,70	probable ribosomal protein L25
PA4751	*ftsH*	+3,21	cell division protein FtsH
PA4764	*fur*	+3,06	ferric uptake regulation protein
PA4765	*omlA*	+8,35	Outer membrane lipoprotein OmlA precursor
PA5130		+11,23	conserved hypothetical protein
PA5191		+2,79	hypothetical protein
PA5316	*rpmB*	+5,23	50S ribosomal protein L28
PA5481[c]		+5,46	hypothetical protein
PA5482		+6,22	hypothetical protein
PA0139	*ahpC*	-2,97	alkyl hydroperoxide reductase subunit C
PA0586		-4,61	conserved hypothetical protein
PA0852[d]	***cpbD***	-3,31	chitin-binding protein CbpD precursor
PA1048		-2,81	probable outer membrane protein precursor
PA1244		-6,62	hypothetical protein
PA1871[d]	***lasA***	-8,33	LasA protease precursor
PA2031		-3,88	hypothetical protein
PA2146[c]		-6,30	conserved hypothetical protein
PA2171[c]		-7,61	hypothetical protein
PA2190[c]		-3,13	conserved hypothetical protein
PA2259	*ptxS*	-2,93	transcriptional regulator PtxS
PA2274		-2,73	hypothetical protein

PA2300[d]	***chiC***	-4,38	chitinase
PA2564[d]		-3,77	hypothetical protein
PA2565		-3,23	hypothetical protein
PA3049	*rmf*	-12,49	ribosome modulation factor
PA3478[d]	***rhlB***	-4,33	rhamnosyltransferase chain B
PA3529		-4,86	probable peroxidase
PA3533		-3,98	conserved hypothetical protein
PA4078[e]		-18,35	probable nonribosomal peptide synthetase
PA4205	*mexG*	-4,78	hypothetical protein
PA4206	*mexH*	-3,53	probable RND efflux membrane fusion protein precursor
PA4236[f]	***katA***	-10,78	catalase KatA
PA4306[d]		-3,63	hypothetical protein
PA4366[f]	***sodB***	-4,42	superoxide dismutase
PA4377		-6,80	hypothetical protein
PA4385	*groEL*	-4,07	GroEL protein
PA4386	*groES*	-4,09	GroES protein
PA4611		-4,46	hypothetical protein

[a] Only those ORFs are listed, which were found in all four Gene Chip pairings defined by the Affymetrix Microarray Suite Software as having significant changes in their signal intensities and are at least 2-fold regulated in each of the four pairings (the arithmetic middle of all four pairings is given in column 3); **Bold:** genes identified previously as being quorum sensing regulated

[b] The TTSS was identified as QS-repressed by Hogardt et al. (10)

[c] Conditional QS-induced genes; identified as QS-regulated by Schuster et al. (42)

[d] General QS regulon, based on the results of three micro-array studies: Hentzer et al. (8), Schuster et al. (42), Wagner et al. (49)

[e] Conditional QS-induced genes; identified as QS-regulated by Hentzer et al. (8)

[f] Identified as QS-induced by Hassett et al. (7)

TABLE 2. Azithromycin regulated proteins in PAO1

Spot name [a]	PA number [b]	Gene name	Proteins as described by http://www.pseudomonas.com	Regulation [c]		
				This study	Previous studies	
					Nouwens et al. (29)	Arevalo-Ferro et al. (1)
Secretome						
51, 63	PA0026		hypothetical protein	↑	↑	
65	PA0888	*aotJ*	arginine/ornithine binding protein AotJ	↑	↑	↑
54	PA1065		conserved hypothetical protein	↑		
66	PA1342		probable binding protein component of ABC transporter	↑		↑
60	PA1493	*cysP*	sulfate-binding protein of ABC transporter	↑		
45	PA1673		hypothetical protein	↑		
64	PA1706	*pcrV*	type III secretion protein PcrV	↑		
55	PA4175	*prpL*	Pvds-regulated endoprotease, lysyl class	↑	↓	↑
69	PA5489	*dsbA*	thiol:disulfide interchange protein DsbA	↑	↑	
59	PA4265 PA4277	*tufA /* *tufB*	elongation factor Tu, tufA; elongation factor Tu, tufB	↑		
41	PA0139	*ahpC*	alkyl hydroperoxide reductase subunit C	↓		
1, 2, 3	PA0572		hypothetical protein	↓	↓	
29, 30, 32	PA0852	*cbpD*	chitin-binding protein CbpD precursor	↓	↓	↑
4, 5, 6, 8, 19, 20, 44	PA1086	*flgK*	flagellar hook-associated protein 1 FlgK	↓	↑	
18	PA1087	*flgL*	flagellar hook-associated protein type 3 FlgL	↓		↑

13, 14, 15, 16, 17, 21, 22, 23, 24, 25, 46, 47, 48, 49, 50, 57, 58, 67, 68	PA1092	*fliC*	flagellin type B	↓	↑	↑
26, 27, 28, 37, 38, 39, 52, 53, 56	PA1094	*fliD*	flagellar capping protein FliD	↓		↑
33	PA1158		probable two-component sensor	↓		
7	PA1294	*aprA*	alkaline metalloproteinase precursor AprA	↓	↓	
42	PA1784		hypothetical protein	↓		
9	PA2939	*pepB*	probable aminopeptidase	↓	↓	↑
31, 34, 35, 36	PA3724	*lasB*	elastase LasB	↓	↓	↑
40	PA3746	*ffh*	signal recognition particle protein Ffh	↓		
10, 12	PA4385	*groEL*	GroEL protein	↓		
11	PA5192	*pckA*	phosphoenolpyruvate carboxykinase PckA	↓		
Cytosolic proteins						
82	PA0609	*trpE*	anthranilate synthase (EC 4.1.3.27) alpha chain	↑		
81	PA1596	*htpG*	heat shock protein HtpG	↑		
86	PA3244	*minD*	cell division inhibitor MinD	↑		
87	PA3397	*fpr*	ferredoxin-NADP+ reductase	↑		
84	PA3635	*eno*	enolase	↑		
85	PA4602	*glyA3*	serine hydroxymethyltransferase GlyA3	↑		
83	PA0139	*ahpC*	alkyl hydroperoxide reductase subunit C	↓		
73	PA0230	*pcaB*	3-carboxy-cis,cis-muconate cycloisomerase PcaB	↓		
71	PA0766	*mucD*	serine protease MucD precursor	↓		

76	PA0837	*slyD*	peptidyl-prolyl cis-trans isomerase SlyD	↓	
74	PA1337	*ansB*	glutaminase-asparaginase AnsB	↓	
75	PA1344	*yvaG*	probable short-chain dehydrogenase	↓	
79	PA1584	*sdhB*	succinate dehydrogenase (B subunit) SdhB	↓	
80	PA1900	*phzB2*	probable phenazine biosynthesis protein PhzB2	↓	↑
77	PA3529	*tsaA*	probable peroxidase	↓	
78	PA4366	*sodB*	superoxide dismutase SodB	↓	
72	PA5173	*arcC*	carbamate kinase ArcC	↓	

[a] Spot name refers to the numbers shown in Fig. 2.

[b] Data generated from peptide mass maps were compared to the complete translated open reading frames of the PAO1 genome (www.pseudomonas.com)

[c] In order to detect a differential in protein expression the spot intensity of the entirety of the fragments of one protein were compared between the AZM treated versus the non-treated PAO1 cultures.

TABLE 3. ORFs that were shown to be regulated by both proteomics and transcriptomics

ORF	Gene name	Proteins as described by http://www.pseudomonas.com	Transcriptome	Secretome	Cellular extracts
PA0139	*ahpC*	alkyl hydroperoxide reductase subunit C	↓	↓	↓
PA0852	*cbpD*	chitin-binding protein CbpD precursor	↓	↓	-
PA1493	*cysP*	sulfate-binding protein of ABC transporter	↑	↑	-
PA1706	*pcrV*	type III secretion protein PcrV	↑	↑	-
PA3244	*minD*	cell division inhibitor MinD	↑	-	↑
PA3529	*tsaA*	probable peroxidase	↓	-	↓
PA4366	*sodB*	superoxide dismutase	↓	-	↓
PA4385	*groEL*	GroEL protein	↓	↓	-

Figure legends

FIG. 1. Growth of PAO1 in LB broth with (■) and without (♦) the addition of 2 µg/mL azithromycin.

FIG. 2. Secretome of the PAO1 cultures with (A) and without (B) the addition of 2 µg/mL AZM. Forty-three differentially expressed protein spots (1-43) were identified by mass spectrometry from the gels of the secretome of the AZM treated PAO1 cultures (A) and twenty-six protein spots (44-69) were identified from the gels of the PAO1 control secretome (B).

FIG. 3. Cytotoxicity as determined by LDH release of J774.A1 cells of AZM pre-treated bacteria (▲) versus the PAO1 control (■). The bacteria were harvested from log phase of growth (A) and stationary phase of growth (B). Results are given as the mean of a 6-fold determination.

FIG 4. Growth inhibition by H_2O_2 as determined by agar diffusion assays. Prolonged cultivation ($\geq$ 10 h) of PAO1 in AZM supplemented medium significantly increased the sensitivity to H_2O_2 (t-test; p value < 0.0017). AZM-pretreated PAO1 (gray bars) in comparison with untreated PAO1 (white bars). Results are given as the mean $\pm$ SD of a triplicate.

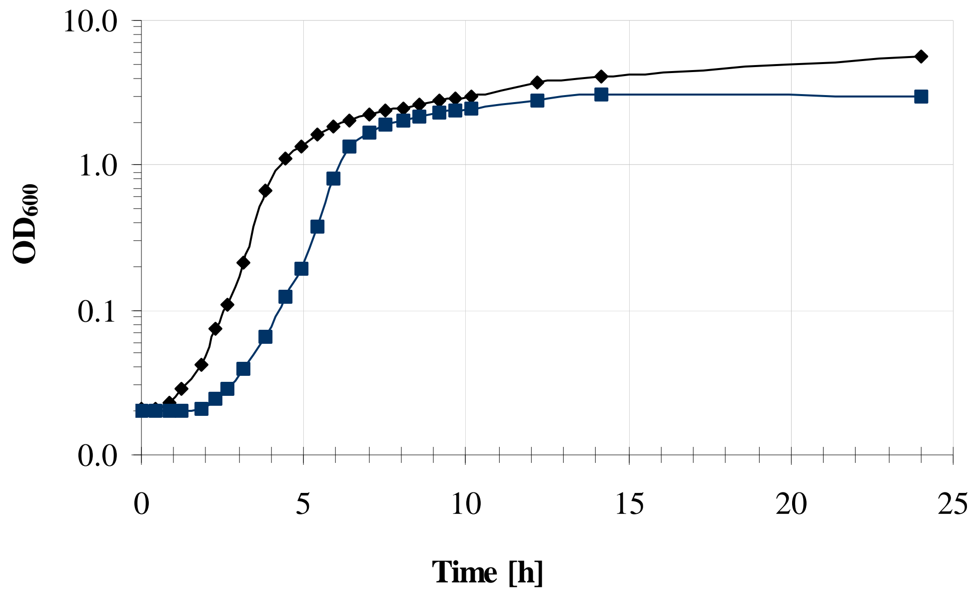


Fig. 1.

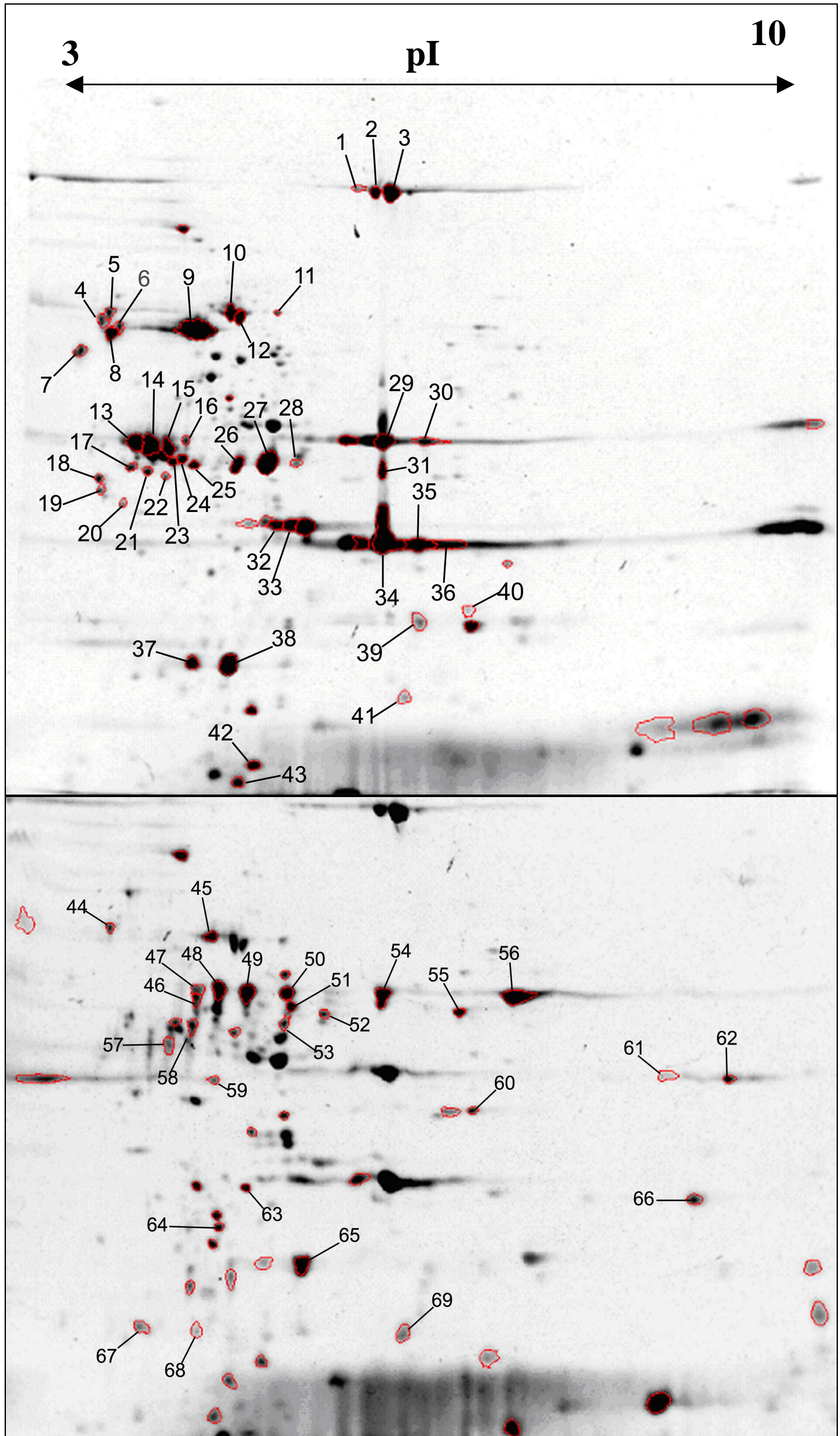
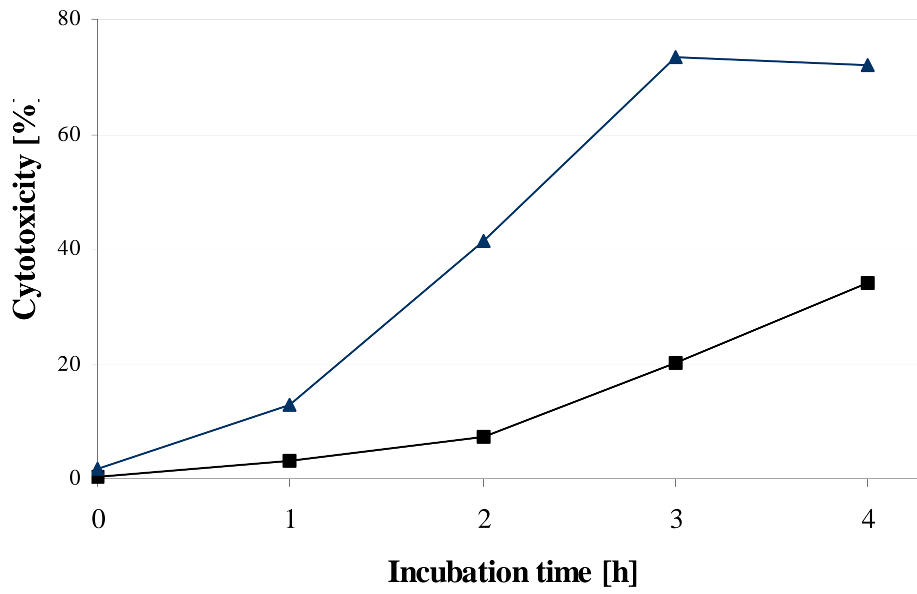


Fig. 2.

A



B

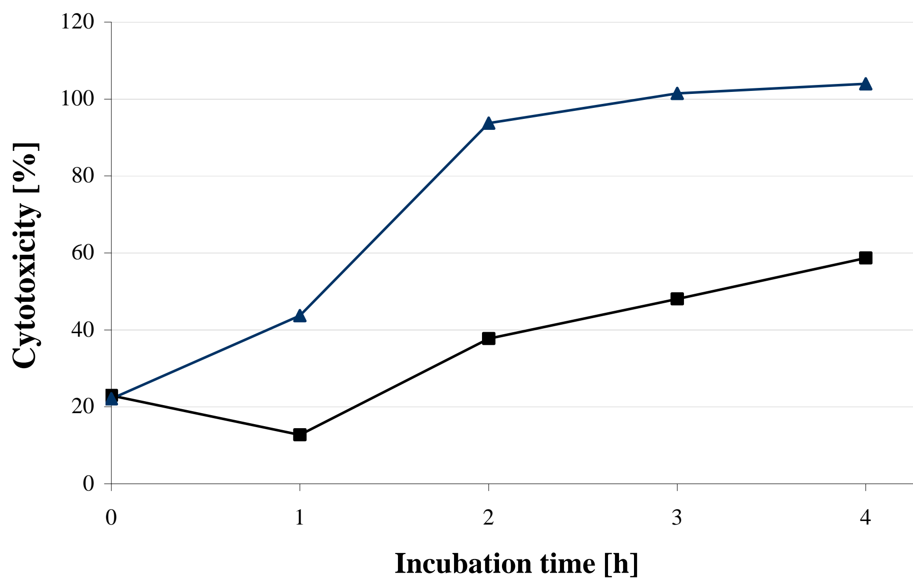


Fig. 3.

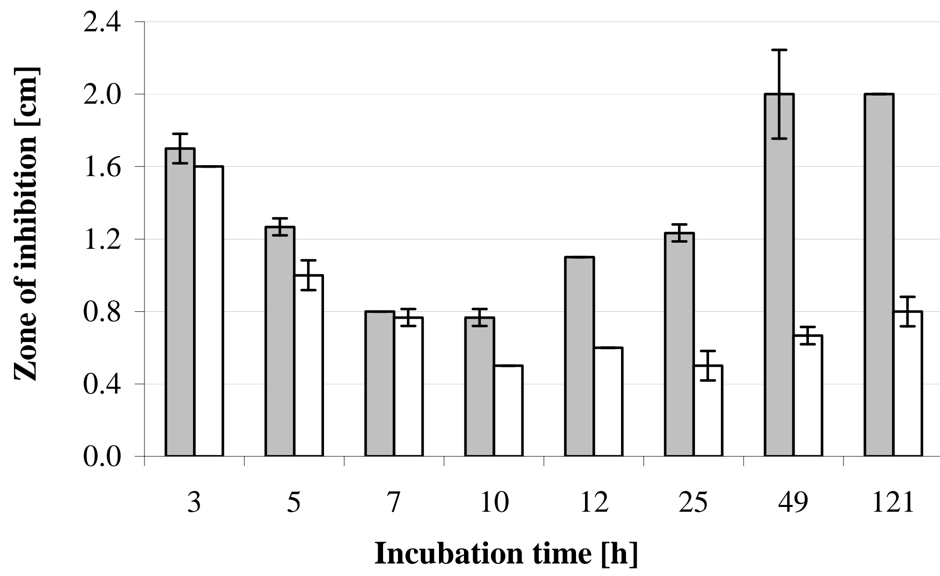


Fig. 4.