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Quorum-Sensing Antagonistic Activities of Azithromycin in *Pseudomonas aeruginosa* PAO1: a Global Approach

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The administration of macrolides such as azithromycin for chronic pulmonary infection of cystic fibrosis patients has been reported to be of benefit. 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 those in 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 and 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 formation capabilities of azithromycin-treated cultures. The interference of azithromycin with quorum-sensing-dependent virulence factor production, biofilm formation, and oxidative stress resistance in *P. aeruginosa* holds great promise for macrolide therapy in cystic fibrosis. Clearly quorum-sensing antagonist macrolides should be paid more attention in the management of chronic *P. aeruginosa* infections, and as quorum-sensing antagonists, macrolides might gain vital importance for more general application against chronic infections.

The lung of cystic fibrosis (CF) patients has a unique susceptibility to chronic *Pseudomonas aeruginosa* infection, which is still the major cause of morbidity and mortality in these patients (4, 24, 36). Although in past decades antibiotic therapy has greatly increased life expectancy, only limited therapeutic options are available, and chronic *P. aeruginosa* infection is rarely eradicated (5, 20, 37). Hence, there is a is an urgent need to develop alternative treatment regimens to improve lung function and thus the prognosis of the disease. Although the principles concerning therapeutic strategies in the treatment of chronic lung infection have 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, 53).

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 (13, 15, 21, 40, 41). Diffuse panbronchiolitis was first reported in Japan and is characterized 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 progressive deterioration of respiratory function.

The remarkable parallels between diffuse panbronchiolitis and CF led to the question of whether macrolide antibiotics would also be of benefit in patients with CF and to large-scale randomized controlled trials to elucidate the properties of macrolides for chronic *P. aeruginosa* infection of the lung in CF patients (30, 34, 39, 52). The majority of clinical studies report positive trends concerning the therapeutic potential of macrolide therapy (44). However, the mechanisms of action in chronic *P. aeruginosa* infection remain obscure (54). Immunomodulatory effects are postulated to account for some of the beneficial effects (9, 16), in addition to altered airway epithelial chloride transport and inhibition of *P. aeruginosa* virulence factor production by interference with interbacterial communication (33, 45).

Interbacterial communication is also referred to as quorum sensing (QS) and is a very sophisticated mechanism by 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 contributes significantly to pathogenesis and persistence of infection. The QS system in *P. aeruginosa* comprises two hierarchically organized systems, each consisting 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 sublethal azithromycin concentrations (45). Moreover, a QS mutant was shown to be less responsive to AZM inhibition (48).

In this study we applied a systematic approach and analyzed the transcriptome and proteome profiles of *P. aeruginosa* in response to sublethal concentrations of AZM. Using this global approach, we aimed to identify the influence of AZM on QS-regulated genes and proteins and thus to gain background data on therapy with macrolides for purposes other than their bactericidal properties.

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MATERIALS AND METHODS

Bacterial strains and culture conditions. For the isolation of RNA and for the preparation of 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 four AZM-treated and untreated PAO1 cultures each, cDNA was synthesized from the RNA pooled from two 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 Affymetrix guidelines and 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 accession number GSE2430). Three independent protein extracts from pooled supernatants and cell pellets of four 150-ml AZM-treated and untreated PAO1 cultures each were prepared and used immediately for two-dimensional gel electrophoresis. The preparation of protein extracts, two-dimensional gel electrophoresis, and matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectrometry (MALDI-TOF MS) analysis were performed as described previously (51). The gels were stained with ruthenium II Tris(bathophenanthroline 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 twofold.

Lactate dehydrogenase release assay. The bacteria were grown in BHI medium with and without the addition of 2 μg/ml AZM and harvested in log phase (optical density at 600 nm [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's medium with 10% fetal calf serum and infected with 20 μl of the bacterial suspension in 200 μl fresh Dulbecco's modified Eagle's medium with 10% fetal calf serum. J774.A1 cell viability was assessed by the determination of lactate dehydrogenase in the supernatant fraction of six parallel wells by using a lactate dehydrogenase cytotoxicity detection kit according to the manufacturer's instructions (Roche, Mannheim, Germany). All experiments were performed in triplicate.

H_2O_2 sensitivity assay. The H_2O_2 sensitivity disk assay was adapted from that of 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 in 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. The swimming plates were inoculated with 15 μl of the bacterial suspension and incubated at 37°C for 24 h, whereas the twitching 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 AND DISCUSSION

Influence of sublethal azithromycin concentrations on the PAO1 transcriptome and proteome. Since 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 (1/64th of the MIC) led to a prolonged lag phase and an only minimally affected exponential and stationary phase of growth compared to that with the PAO1 control culture (Fig. 1).

A comparison of the gene expression profiles 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 ribosomal subunits. In addition, an initiation factor (*infA* PA2619) and an elongation factor (*efp* PA2851) were overexpressed 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 overexpressed to compensate for impaired translation due to sublethal AZM concentrations. Moreover, the ribosome modulation factor *rmf* was strongly repressed upon AZM addition. The PAO1 *rmf* gene exhibits high homology to the *rmf* gene in *Escherichia coli*, in which *rmf* has been shown to be important for survival under stationary-phase conditions (56).

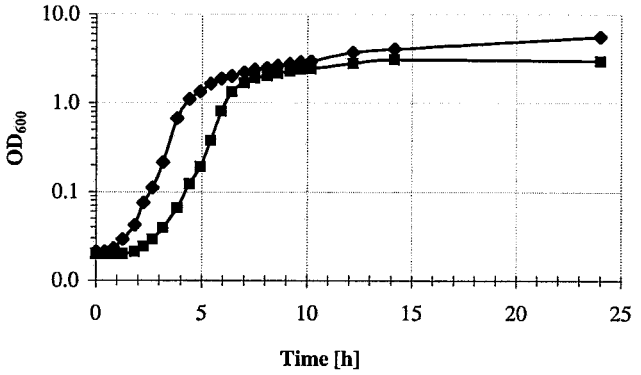

FIG. 1. Growth of PAO1 in LB broth with (■) and without (◆) the addition of 2 μg/ml azithromycin.

An exposure-dependent bactericidal activity of macrolides has been described previously (46) and was observed in this study (after 24 h and 48 h of incubation, significantly fewer bacteria were isolated from the AZM-treated culture than from controls; paired *t* test, $P < 0.001$). In this context the AZM-dependent down-regulation of *rmf* might contribute to the observed impaired survival of AZM-treated PAO1 under stationary-phase conditions.

In a complementary approach to the identification of the global transcriptional pattern we analyzed the protein expression profile of AZM-treated in comparison to nontreated PAO1 cultures. Comparative analyses of the secretome and of cellular extracts of AZM-treated versus nontreated 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). Whereas the two-dimensional gels of the secretome comprised approximately 645 protein spots, 714 protein spots were detected within the cellular fraction. Six 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 eight genes and proteins (*ahpC*, *cbpD*, *cysP*, *pcrV*, *minD*, *tsaA*, *sodB*, and *groEL*) were shown to be affected at both the transcriptional and the protein levels emphasizes that transcriptomics and proteomics are complementary approaches, and combining their particular strengths should give maximal relevant results. Major differences be-

TABLE 1. Azithromycin-regulated genes in PAO1

Gene[a]	Gene name	Up-regulated (+) or down-regulated (−) in response to AZM (fold)	Protein
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 phosphoribosyltransferase
PA0651	*trpC*	+5.90	Indole-3-glycerolphosphate 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[d]	*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,d]	*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-coenzyme A dehydrogenase
PA2016		+8.72	Probable transcriptional regulator
PA2023	*galU*	+4.90	UTP-glucose-l-phosphate uridylyltransferase
PA2191[b]	*exoY*	+20.12	Adenylate cyclase ExoY
PA2193[e]	*hcnA*	+2.62	Hydrogen cyanide synthase HcnA
PA2423[e]		+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[d]	*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[f]		+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

Continued on following page

TABLE 1—*Continued*

Gene[a]	Gene name	Up-regulated (+) or down-regulated (−) in response to AZM (fold)	Protein
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[d]	*ahpC*	−2.97	Alkyl hydroperoxide reductase subunit C
PA0586		−4.61	Conserved hypothetical protein
PA0852[d,e]	*cpbD*	−3.31	Chitin-binding protein CbpD precursor
PA1048		−2.81	Probable outer membrane protein precursor
PA1244		−6.62	Hypothetical protein
PA1871[e]	*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[e]	*chiC*	−4.38	Chitinase
PA2564[e]		−3.77	Hypothetical protein
PA2565		−3.23	Hypothetical protein
PA3049	*rmf*	−12.49	Ribosome modulation factor
PA3478[e]	*rhlB*	−4.33	Rhamnosyltransferase chain B
PA3529[d]		−4.86	Probable peroxidase
PA3533		−3.98	Conserved hypothetical protein
PA4078[f]		−18.35	Probable nonribosomal peptide synthetase
PA4205	*mexG*	−4.78	Hypothetical protein
PA4206	*mexH*	−3.53	Probable RND efflux membrane fusion protein precursor
PA4236[g]	*katA*	−10.78	Catalase KatA
PA4306[e]		−3.63	Hypothetical protein
PA4366[d,g]	*sodB*	−4.42	Superoxide dismutase
PA4377		−6.80	Hypothetical protein
PA4385[d]	*groEL*	−4.07	GroEL protein
PA4386	*groES*	−4.09	GroES protein
PA4611		−4.46	Hypothetical protein

[a] Only open reading frames which were found in all four GeneChip pairings defined by the Affymetrix microarray suite software as having significant changes in their signal intensities and were at least twofold up- or down-regulated in each of the four pairings (the arithmetic middle of all four pairings is given) are listed. Genes identified previously as being QS regulated are in boldface.
[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] Genes/proteins that were shown to be regulated by both proteomics and transcriptomics.
[e] General QS regulon, based on the results of three microarray studies: Hentzer et al. (8), Schuster et al. (42), and Wagner et al. (49).
[f] Conditional QS-induced genes; identified as QS regulated by Hentzer et al. (8).
[g] Identified as QS induced by Hassett et al. (7).

tween proteome and transcriptome data have been documented in previous studies to map the *P. aeruginosa* QS regulon: the two proteome studies of Arevalo-Ferro et al. (1) and Nouwens et al. (29) identified 47 and 27 QS-regulated proteins, respectively, and only 11 and 8, respectively, of the corresponding genes were found to be regulated at the transcriptional level in three independent transcriptome studies analyzing QS-dependent *P. aeruginosa* gene expression (8, 42, 49).

Effect of azithromycin on quorum sensing in PAO1. Eight out of 77 genes (10.4%) of the general QS regulon (identified as QS-dependent genes in all three recent microarray studies (8, 42, 49) 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 microarray 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, but five genes were up-regulated. An up-regulation is opposite to what is expected when assuming that AZM inhibits QS. However, QS-regulated proteins have previously been found to be oppositely regulated in a comparison between two previous proteomic studies (1, 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 QS-controlled *katA* and *sodB* (7) to be AZM dependent.

In addition to the identification of a common subset between

TABLE 2. Azithromycin-regulated proteins in PAO1

Group and spot no.[a]	Gene[b]	Gene name	Protein	Up- or down-regulation[c]		
				This study	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[d]	PA1493	*cysP*	Sulfate-binding protein of ABC transporter	↑		
45	PA1673		Hypothetical protein	↑		
64[d]	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	*tufA*	Elongation factor Tu, TufA	↑		
	PA4277	*tufB*	Elongation factor Tu, TufB			
41[d]	PA0139	*ahpC*	Alkyl hydroperoxide reductase subunit C	↓		
1, 2, 3	PA0572		Hypothetical protein	↓	↓	
29, 30, 32[d]	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[d]	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[d]	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[d]	PA3529	*tsaA*	Probable peroxidase	↓		
78[d]	PA4366	*sodB*	Superoxide dismutase SodB	↓		
72	PA5173	*arcC*	Carbamate kinase ArcC	↓		

[a] See 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 differential protein expression, the spot intensities of the entirety of the fragments of one protein were compared between AZM-treated and nontreated PAO1 cultures. Except for protein spots that were detected only in gels of cultures with or without AZM, the differences in protein spot intensity were between 2- and 10-fold.
[d] Genes shown to be regulated by both proteomics and transcriptomics.

QS- and AZM-regulated genes, we compared differential protein expression due to AZM treatment with QS-dependent protein expression in the two 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 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. (50), who reported the constant expression of QS-regulated virulence factors under various culture conditions.

Since chronic *P. aeruginosa* infection in CF is rarely eradicated despite intensive antimicrobial therapy, interference with or blocking of QS systems has been considered an attractive

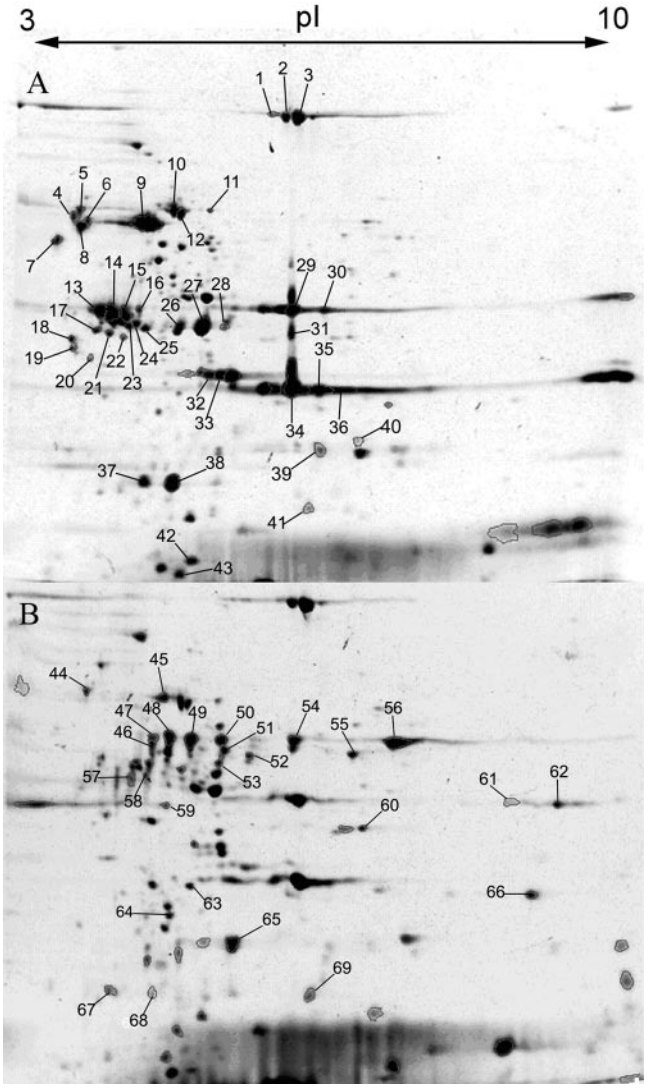

FIG. 2. Secretome of PAO1 cultures without (A) and with (B) the addition of 2 μg/ml AZM. Forty-three differentially expressed protein spots (1 to 43) were identified by mass spectrometry from the gels of the secretome of the PAO1 control cultures (A) and 26 protein spots (44 to 69) were identified from the gels of the AZM-treated PAO1 secretome (B).

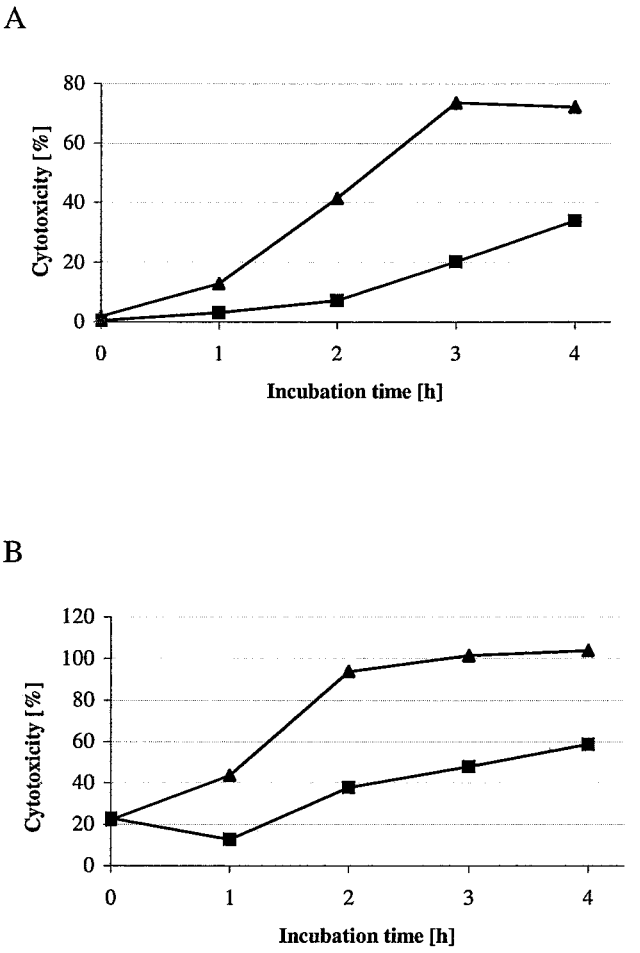

FIG. 3. Cytotoxicity as determined by lactate dehydrogenase release of J774.A1 cells of AZM-treated bacteria (▲) versus the untreated PAO1 control (■). The bacteria were harvested from the log phase of growth (A) and the stationary phase of growth (B). One representative experiment out of three is shown.

alternative therapeutic strategy. Recently, halogenated furanones have been shown to control *P. aeruginosa* infections in animal models (8, 55). 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 treatment of humans, macrolides seem to be a promising alternative to the toxic halogenated furanones that might block the QS systems within therapeutic concentration ranges. How precisely the macrolides interfere with the transcription of QS-regulated genes remains poorly defined and will be an important question to be addressed in the future.

Azithromycin enhances the expression of the type III secretion system. Several studies have demonstrated that macrolide antibiotics suppress the expression of substances that contribute to *P. aeruginosa* virulence, such as exoenzymes, exopolysaccharides, and pigments (17, 25, 26, 48), and it has been hypothesized that CF patients benefit from AZM treatment due to the negative effect on virulence factor production. However, one of the major findings of this study is that AZM treatment of PAO1 led to increased expression of the TTSS. We found increased expression of 10 of 36 genes of the TTSS gene cluster (PA1690 to PA1725) in the AZM-treated cultures. Moreover, the expression of genes encoding the secreted effector proteins ExoT, ExoY, and ExoS, which are located outside the TTSS gene cluster, was enhanced. The *pcrV* gene, encoding 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-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 the log and early stationary phases of growth exhibited increased cytotoxicity in comparison to bacteria cultured without AZM. These effects were not observed when PAO1 was grown with

sublethal gentamicin or ceftazidime concentrations (data not shown). Remarkably, it has previously been demonstrated that treatment of *P. aeruginosa* with macrolides, including AZM, significantly enhances virulence in mice (19). An involvement of acute toxic effects rather than multiplication of the bacteria was suggested.

The results of our study imply that 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 inoculation of macrolide-treated bacteria had been shown to be associated with increased mortality in mice, whereas the administration of macrolides after inoculation of *P. aeruginosa* did not increase mortality (19, 28). The impact of the TTSS-induced cytotoxicity on therapeutic macrolide administration remains to be clarified in future investigations.

Azithromycin affects *P. aeruginosa* motility. AZM-treated PAO1 cultures exhibited reduced expression of various proteins required for flagellum biosynthesis (Table 2) and demonstrated reduced flagellum-driven motility on swimming agar plates. The mean value of the radius of the untreated PAO1 and AZM-treated PAO1 cultures was 3.58 (±0.17) cm and 1.18 (±0.09) cm, respectively ($P < 0.01$). Although macrolides have been previously demonstrated to inhibit not only swimming motility (14) but also twitching motility (14, 54), we observed increased expression of *pilA* and *pilH*, involved in type IV pilus biogenesis (Table 1). No effects of sublethal AZM concentrations on type IV pili could be observed by proteomics.

Analysis of the twitching motility of AZM-treated PAO1 revealed that twitching motility was significantly reduced. The mean value of the radius of the untreated PAO1 and AZM-treated PAO1 cultures was 0.67 (±0.06) cm and 0.51 (±0.06) cm, respectively ($P < 0.01$). While flagellum-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 (31), 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 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 level (*sodB*, *ahpC*, and *katA*) and the protein level (SodB and AhpC) upon AZM addition. We also observed an up-regulation of the *fur* gene in response to sublethal 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 simultaneous overexpression of the *bfrB* gene indicates a sufficient intracellular iron storage (47).

Another important element affected by oxidative stress is sulfur, since iron-sulfur proteins have been shown to play a protective role against 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 level and at the protein level. PA3262, a putative peptidyl-prolyl 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 cellular redox potential and the prevention of oxidative damage.

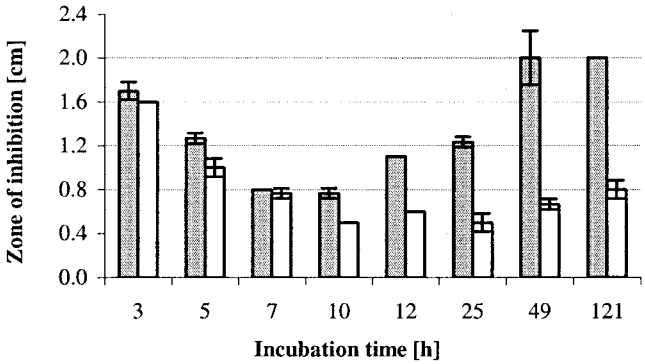

FIG. 4. Growth inhibition by H_2O_2 as determined by agar diffusion assays. Prolonged cultivation (≥10 h) of PAO1 in AZM-supplemented medium significantly increased sensitivity to H_2O_2 (*t* test; $P < 0.0017$). AZM-treated PAO1 (gray bars) was compared with untreated PAO1 (white bars). Results are given as the mean ± standard deviation of three determinations.

PA3529, encoding 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. The observed effects of AZM on several genes and proteins that are involved in the oxidative stress response implied 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 than the untreated controls. As shown in Fig. 4 bacteria that were 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 bacterial susceptibility to H_2O_2, whereas gentamicin or ceftazidime pretreatment had no effect (data not shown).

Concluding remarks. Both the antimicrobial and anti-inflammatory effects of AZM have been implicated as being 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 reduced virulence factor production, biofilm formation, and survival under stressful conditions due to interference with QS in *P. aeruginosa*. We identified a large common subset of QS- and AZM-regulated genes/proteins, in particular within the secretome, comprising many virulence factors. Moreover, the TTSS, which 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 QS control of genes essential for relieving oxidative stress (7), we found markedly increased

sensitivity of AZM-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 imply that the QS-antagonistic activity of AZM contributes to the improvement of CF patient health. Apart from the reduced expression of virulence factors, interference with the bacterial oxidative stress response might be of major relevance. One vitally adaptive response of *P. aeruginosa* is the ability to resist the oxidative stress that is induced during phagocytosis, when the bacteria are confronted with reactive oxygen intermediates 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 reactive oxygen intermediates 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 sub-MIC concentrations of AZM that has been reported previously (46).

Macrolides inhibit protein synthesis at the ribosomal level, and it is conceivable that unidentified stress responses, bacterial regulons, or signal transduction processes are responsible for the observed effects of sublethal concentrations on gene expression. Furthermore, future studies will have to elucidate whether the observed effects of sublethal AZM concentrations are also relevant in vivo. However, the results of this in vitro study and the fact that AZM exhibits beneficial effects in the treatment of CF patients give us reason to assume that the administration of AZM for CF will have a great impact on the management of chronic infection due to its interference with *P. aeruginosa* QS and thus with virulence factor production, biofilm formation, and persistence during chronic infection.

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REFERENCES

1. **Arevalo-Ferro, C., M. Hentzer, G. Reil, A. Gorg, S. Kjelleberg, M. Givskov, K. Riedel, and L. Eberl.** 2003. Identification of quorum-sensing regulated proteins in the opportunistic pathogen *Pseudomonas aeruginosa* by proteomics. Environ. Microbiol. **5:**1350–1369.
2. **Carr, R. R., and M. C. Nahata.** 2004. Azithromycin for improving pulmonary function in cystic fibrosis. Ann. Pharmacother. **38:**1520–1524.
3. **Davies, D. G., M. R. Parsek, J. P. Pearson, B. H. Iglewski, J. W. Costerton, and E. P. Greenberg.** 1998. The involvement of cell-to-cell signals in the development of a bacterial biofilm. Science **280:**295–298.
4. **Davies, J. C.** 2002. *Pseudomonas aeruginosa* in cystic fibrosis: pathogenesis and persistence. Paediatr. Respir. Rev. **3:**128–134.
5. **Gibson, R. L., J. L. Burns, and B. W. Ramsey.** 2003. Pathophysiology and management of pulmonary infections in cystic fibrosis. Am. J. Respir. Crit. Care Med. **168:**918–951.
6. **Gillis, R. J., and B. H. Iglewski.** 2004. Azithromycin retards *Pseudomonas aeruginosa* biofilm formation. J. Clin. Microbiol. **42:**5842–5845.
7. **Hassett, D. J., J. F. Ma, J. G. Elkins, T. R. McDermott, U. A. Ochsner, S. E. West, C. T. Huang, J. Fredericks, S. Burnett, P. S. Stewart, G. McFeters, L. Passador, and B. H. Iglewski.** 1999. Quorum sensing in *Pseudomonas aeruginosa* controls expression of catalase and superoxide dismutase genes and mediates biofilm susceptibility to hydrogen peroxide. Mol. Microbiol. **34:**1082–1093.
8. **Hentzer, M., H. Wu, J. B. Andersen, K. Riedel, T. B. Rasmussen, N. Bagge, N. Kumar, M. A. Schembri, Z. Song, P. Kristoffersen, M. Manefield, J. W. Costerton, S. Molin, L. Eberl, P. Steinberg, S. Kjelleberg, N. Hoiby, and M. Givskov.** 2003. Attenuation of *Pseudomonas aeruginosa* virulence by quorum sensing inhibitors. EMBO J. **22:**3803–3815.
9. **Hirakata, Y., M. Kaku, R. Mizukane, K. Ishida, N. Furuya, T. Matsumoto, K. Tateda, and K. Yamaguchi.** 1992. Potential effects of erythromycin on host defense systems and virulence of *Pseudomonas aeruginosa*. Antimicrob. Agents Chemother. **36:**1922–1927.
10. **Hogardt, M., M. Roeder, A. M. Schreff, L. Eberl, and J. Heesemann.** 2004. Expression of *Pseudomonas aeruginosa exoS* is controlled by quorum sensing and RpoS. Microbiology **150:**843–851.
11. **Hoiby, N.** 2002. New antimicrobials in the management of cystic fibrosis. J. Antimicrob. Chemother. **49:**235–238.
12. **Ichimiya, T., K. Takeoka, K. Hiramatsu, K. Hirai, T. Yamasaki, and M. Nasu.** 1996. The influence of azithromycin on the biofilm formation of *Pseudomonas aeruginosa* in vitro. Chemotherapy **42:**186–191.
13. **Kadota, J., H. Mukae, H. Ishii, T. Nagata, H. Kaida, K. Tomono, and S. Kohno.** 2003. Long-term efficacy and safety of clarithromycin treatment in patients with diffuse panbronchiolitis. Respir. Med. **97:**844–850.
14. **Kawamura-Sato, K., Y. Iinuma, T. Hasegawa, T. Horii, T. Yamashino, and M. Ohta.** 2000. Effect of subinhibitory concentrations of macrolides on expression of flagellin in *Pseudomonas aeruginosa* and *Proteus mirabilis*. Antimicrob. Agents Chemother. **44:**2869–2872.
15. **Keicho, N., and S. Kudoh.** 2002. Diffuse panbronchiolitis: role of macrolides in therapy. Am. J. Respir. Med. **1:**119–131.
16. **Kita, E., M. Sawaki, F. Nishikawa, K. Mikasa, Y. Yagyu, S. Takeuchi, K. Yasui, N. Narita, and S. Kashiba.** 1990. Enhanced interleukin production after long-term administration of erythromycin stearate. Pharmacology **41:**177–183.
17. **Kita, E., M. Sawaki, D. Oku, A. Hamuro, K. Mikasa, M. Konishi, M. Emoto, S. Takeuchi, N. Narita, and S. Kashiba.** 1991. Suppression of virulence factors of *Pseudomonas aeruginosa* by erythromycin. J. Antimicrob. Chemother. **27:**273–284.
18. **Kobayashi, K., and S. Tagawa.** 2004. Activation of SoxR-dependent transcription in *Pseudomonas aeruginosa*. J. Biochem. (Tokyo) **136:**607–615.
19. **Kobayashi, T., K. Tateda, T. Matsumoto, S. Miyazaki, A. Watanabe, T. Nukiwa, and K. Yamaguchi.** 2002. Macrolide-treated *Pseudomonas aeruginosa* induces paradoxical host responses in the lungs of mice and a high mortality rate. J. Antimicrob. Chemother. **50:**59–66.
20. **Koch, C., and N. Hoiby.** 2000. Diagnosis and treatment of cystic fibrosis. Respiration **67:**239–247.
21. **Kudoh, S., A. Azuma, M. Yamamoto, T. Izumi, and M. Ando.** 1998. Improvement of survival in patients with diffuse panbronchiolitis treated with low-dose erythromycin. Am. J. Respir. Crit. Care Med. **157:**1829–1832.
22. **Lamanda, A., A. Zahn, D. Roder, and H. Langen.** 2004. Improved Ruthenium II tris (bathophenantroline disulfonate) staining and destaining protocol for a better signal-to-background ratio and improved baseline resolution. Proteomics **4:**599–608.
23. **Leichert, L. I., and U. Jakob.** 2004. Protein thiol modifications visualized in vivo. PLoS Biol. **2:**e333.
24. **Lyczak, J. B., C. L. Cannon, and G. B. Pier.** 2002. Lung infections associated with cystic fibrosis. Clin. Microbiol. Rev. **15:**194–222.
25. **Molinari, G., C. A. Guzman, A. Pesce, and G. C. Schito.** 1993. Inhibition of *Pseudomonas aeruginosa* virulence factors by subinhibitory concentrations of azithromycin and other macrolide antibiotics. J. Antimicrob. Chemother. **31:**681–688.
26. **Molinari, G., P. Paglia, and G. C. Schito.** 1992. Inhibition of motility of *Pseudomonas aeruginosa* and *Proteus mirabilis* by subinhibitory concentrations of azithromycin. Eur. J. Clin. Microbiol. Infect. Dis. **11:**469–471.
27. **Nagino, K., and H. Kobayashi.** 1997. Influence of macrolides on mucoid alginate biosynthetic enzyme from *Pseudomonas aeruginosa*. Clin. Microbiol. Infect. **3:**432–439.
28. **Nicolau, D. P., M. A. Banevicius, C. H. Nightingale, and R. Quintiliani.** 1999. Beneficial effect of adjunctive azithromycin in treatment of mucoid *Pseudomonas aeruginosa* pneumonia in the murine model. Antimicrob. Agents Chemother. **43:**3033–3035.
29. **Nouwens, A. S., S. A. Beatson, C. B. Whitchurch, B. J. Walsh, H. P. Schweizer, J. S. Mattick, and S. J. Cordwell.** 2003. Proteome analysis of extracellular proteins regulated by the *las* and *rhl* quorum sensing systems in *Pseudomonas aeruginosa* PAO1. Microbiology **149:**1311–1322.
30. **Ordonez, C. L., M. Stulbarg, H. Grundland, J. T. Liu, and H. A. Boushey.** 2001. Effect of clarithromycin on airway obstruction and inflammatory markers in induced sputum in cystic fibrosis: a pilot study. Pediatr. Pulmonol. **32:**29–37.
31. **O'Toole, G. A., and R. Kolter.** 1998. Flagellar and twitching motility are necessary for *Pseudomonas aeruginosa* biofilm development. Mol. Microbiol. **30:**295–304.
32. **Palma, M., D. DeLuca, S. Worgall, and L. E. Quadri.** 2004. Transcriptome

analysis of the response of *Pseudomonas aeruginosa* to hydrogen peroxide. J. Bacteriol. **186:**248–252.
33. **Pechere, J. C.** 2001. Azithromycin reduces the production of virulence factors in *Pseudomonas aeruginosa* by inhibiting quorum sensing. Jpn. J. Antibiot. **54**(Suppl. C)**:**87–89.
34. **Pirzada, O. M., J. McGaw, C. J. Taylor, and M. L. Everard.** 2003. Improved lung function and body mass index associated with long-term use of macrolide antibiotics. J. Cyst. Fibros. **2:**69–71.
35. **Poletti, V., M. Chilosi, G. Casoni, and T. V. Colby.** 2004. Diffuse panbronchiolitis. Sarcoidosis Vasc. Diffuse Lung Dis. **21:**94–104.
36. **Rajan, S., and L. Saiman.** 2002. Pulmonary infections in patients with cystic fibrosis. Semin. Respir. Infect. **17:**47–56.
37. **Ratjen, F.** 2001. Changes in strategies for optimal antibacterial therapy in cystic fibrosis. Int. J. Antimicrob. Agents **17:**93–96.
38. **Saiman, L.** 2004. The use of macrolide antibiotics in patients with cystic fibrosis. Curr. Opin. Pulmon. Med. **10:**515–523.
39. **Saiman, L., B. C. Marshall, N. Mayer-Hamblett, J. L. Burns, A. L. Quittner, D. A. Cibene, S. Coquillette, A. Y. Fieberg, F. J. Accurso, and P. W. Campbell III.** 2003. Azithromycin in patients with cystic fibrosis chronically infected with *Pseudomonas aeruginosa*: a randomized controlled trial. JAMA **290:** 1749–1756.
40. **Sawaki, M., R. Mikami, K. Mikasa, M. Kunimatsu, S. Ito, and N. Narita.** 1986. The long term chemotherapy with erythromycin in chronic lower respiratory tract infections—first report: comparison with amoxicillin. Kansenshogaku Zasshi **60:**37–44.
41. **Sawaki, M., R. Mikami, K. Mikasa, M. Kunimatsu, S. Ito, and N. Narita.** 1986. The long term chemotherapy with erythromycin in chronic lower respiratory tract infections—second report: including cases with Pseudomonas infections. Kansenshogaku Zasshi **60:**45–50.
42. **Schuster, M., C. P. Lostroh, T. Ogi, and E. P. Greenberg.** 2003. Identification, timing, and signal specificity of *Pseudomonas aeruginosa* quorum-controlled genes: a transcriptome analysis. J. Bacteriol. **185:**2066–2079.
43. **Shaver, C. M., and A. R. Hauser.** 2004. Relative contributions of *Pseudomonas aeruginosa* ExoU, ExoS, and ExoT to virulence in the lung. Infect. Immun. **72:**6969–6977.
44. **Southern, K. W., P. M. Barker, and A. Solis.** 2004. Macrolide antibiotics for cystic fibrosis. Cochrane Database Syst. Rev. CD002203. [Online.]
45. **Tateda, K., R. Comte, J. C. Pechere, T. Kohler, K. Yamaguchi, and C. Van Delden.** 2001. Azithromycin inhibits quorum sensing in *Pseudomonas aeruginosa*. Antimicrob. Agents Chemother. **45:**1930–1933.
46. **Tateda, K., Y. Ishii, T. Matsumoto, N. Furuya, M. Nagashima, T. Matsunaga, A. Ohno, S. Miyazaki, and K. Yamaguchi.** 1996. Direct evidence for antipseudomonal activity of macrolides: exposure-dependent bactericidal activity and inhibition of protein synthesis by erythromycin, clarithromycin, and azithromycin. Antimicrob. Agents Chemother. **40:**2271–2275.
47. **Vasil, M. L., and U. A. Ochsner.** 1999. The response of *Pseudomonas aeruginosa* to iron: genetics, biochemistry and virulence. Mol. Microbiol. **34:**399–413.
48. **Wagner, T., G. Soong, S. Sokol, L. Saiman, and A. Prince.** 2005. Effects of azithromycin on clinical isolates of *Pseudomonas aeruginosa* from cystic fibrosis patients. Chest **128:**912–919.
49. **Wagner, V. E., D. Bushnell, L. Passador, A. I. Brooks, and B. H. Iglewski.** 2003. Microarray analysis of *Pseudomonas aeruginosa* quorum-sensing regulons: effects of growth phase and environment. J. Bacteriol. **185:**2080–2095.
50. **Wagner, V. E., R. J. Gillis, and B. H. Iglewski.** 2004. Transcriptome analysis of quorum-sensing regulation and virulence factor expression in *Pseudomonas aeruginosa*. Vaccine **22**(Suppl. 1)**:**S15–S20.
51. **Wehmhoner, D., S. Haussler, B. Tummler, L. Jansch, F. Bredenbruch, J. Wehland, and I. Steinmetz.** 2003. Inter- and intraclonal diversity of the *Pseudomonas aeruginosa* proteome manifests within the secretome. J. Bacteriol. **185:**5807–5814.
52. **Wolter, J., S. Seeney, S. Bell, S. Bowler, P. Masel, and J. McCormack.** 2002. Effect of long term treatment with azithromycin on disease parameters in cystic fibrosis: a randomised trial. Thorax **57:**212–216.
53. **Wolter, J. M., S. L. Seeney, and J. G. McCormack.** 2002. Macrolides in cystic fibrosis: is there a role? Am. J. Respir. Med. **1:**235–241.
54. **Wozniak, D. J., and R. Keyser.** 2004. Effects of subinhibitory concentrations of macrolide antibiotics on *Pseudomonas aeruginosa*. Chest **125:**62S–69S.
55. **Wu, H., Z. Song, M. Hentzer, J. B. Andersen, S. Molin, M. Givskov, and N. Hoiby.** 2004. Synthetic furanones inhibit quorum-sensing and enhance bacterial clearance in *Pseudomonas aeruginosa* lung infection in mice. J. Antimicrob. Chemother. **53:**1054–1061.
56. **Yamagishi, M., H. Matsushima, A. Wada, M. Sakagami, N. Fujita, and A. Ishihama.** 1993. Regulation of the *Escherichia coli rmf* gene encoding the ribosome modulation factor: growth phase- and growth rate-dependent control. EMBO J. **12:**625–630.