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Staphylococcus aureus Serves as an Iron Source for *Pseudomonas aeruginosa* during In Vivo Coculture

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***Pseudomonas aeruginosa* is a gram-negative opportunistic human pathogen often infecting the lungs of individuals with the heritable disease cystic fibrosis and the peritoneum of individuals undergoing continuous ambulatory peritoneal dialysis. Often these infections are not caused by colonization with *P. aeruginosa* alone but instead by a consortium of pathogenic bacteria. Little is known about growth and persistence of *P. aeruginosa* in vivo, and less is known about the impact of coinfecting bacteria on *P. aeruginosa* pathogenesis and physiology. In this study, a rat dialysis membrane peritoneal model was used to evaluate the in vivo transcriptome of *P. aeruginosa* in monoculture and in coculture with *Staphylococcus aureus*. Monoculture results indicate that approximately 5% of all *P. aeruginosa* genes are differentially regulated during growth in vivo compared to in vitro controls. Included in this analysis are genes important for iron acquisition and growth in low-oxygen environments. The presence of *S. aureus* caused decreased transcription of *P. aeruginosa* iron-regulated genes during in vivo coculture, indicating that the presence of *S. aureus* increases usable iron for *P. aeruginosa* in this environment. We propose a model where *P. aeruginosa* lyses *S. aureus* and uses released iron for growth in low-iron environments.**

Pseudomonas aeruginosa is a gram-negative opportunistic human pathogen commonly found in water and soil. *P. aeruginosa* causes a number of chronic and acute infections and is noted for its inherent resistance to many clinically relevant antibiotics. Two of the most common infections caused by *P. aeruginosa* are chronic colonization of the lungs of individuals with the genetic disease cystic fibrosis (CF) (19) and peritonitis in individuals undergoing continuous ambulatory peritoneal dialysis (CAPD) (25). The lungs of CF patients are commonly colonized before the age of 8, and most individuals maintain these infections throughout their lifetimes. High infection rates are also associated with CAPD, which is often used to treat end-stage renal disease.

P. aeruginosa physiology and gene expression during in vivo growth is largely unknown. Using in vivo expression technology (IVET), Wang et al. identified 19 *P. aeruginosa* genes inducible during growth in a neutropenic mouse (40). Although that study identified several new genes important for virulence in *P. aeruginosa*, it did not provide a comprehensive analysis of in vivo gene expression. Two recent studies using *Pasteurella multocida* and *Borrelia burgdorferi* have provided a more comprehensive view of in vivo bacterial gene expression by using DNA microarrays (3, 4, 33). These studies illustrate that a significant number of genes (2 to 8% of all the genes in the genomes) are differentially regulated in vivo, suggesting that the in vivo environment is distinct from normal in vitro culture conditions.

Although evaluation of the transcriptomes of in vivo-grown bacteria provides a snapshot of transcription under monoculture growth conditions, it is clear that many infections are not simply the result of colonization by one bacterium but rather the pathogenic contributions of several bacteria (10, 19, 20, 22, 46). Such is the case for *P. aeruginosa* infections, particularly in the CF lung, which often consist of a consortium of pathogenic bacteria, including *Staphylococcus aureus* and *Streptococcus pneumoniae* (19, 23). As with most bacteria, studies of *P. aeruginosa* pathogenesis have primarily focused on monoculture infections; consequently, little is known about interspecies interactions in polymicrobial infections. Although bacterium-bacterium interactions in vivo will be affected by numerous factors both spatial and temporal, it is possible that in some circumstances interspecies interactions may affect the course of disease.

To provide a more comprehensive analysis of *P. aeruginosa* gene expression in vivo, we used Affymetrix GeneChips to examine the transcriptome of *P. aeruginosa* growing as a monoculture and in coculture with *S. aureus* in the rat peritoneum. Our studies indicate that approximately 5% of all *P. aeruginosa* genes are differentially regulated during monoculture growth within the peritoneum compared to in vitro conditions. These results indicate that the peritoneum is a low-oxygen, iron-limited environment, and the presence of *S. aureus* increases usable iron for *P. aeruginosa* in vivo. We propose a model where *P. aeruginosa* lyses *S. aureus* during coculture and gains access to sequestered iron. Our data suggest that *P. aeruginosa* pathogenesis and physiology are influenced by the presence of *S. aureus*, thereby implicating the importance of studying interspecies interactions to understand *P. aeruginosa* pathogenesis.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. *P. aeruginosa* strain UCBPP-PA14 (30) and *S. aureus* strain MN8 (35) were used in these studies. *P. aeruginosa* PA14-LM1 containing a Tn*5* insertion in *pqsA* was obtained from a publicly available transposon database (http://pga.mgh.harvard.edu/cgi-bin/pa14/mutants/retrieve.cgi). Bacteria were grown in morpholinepropanesulfonic acid (MOPS) minimal medium containing 50 mM MOPS (pH 7.2), 93 mM NH_4Cl, 43 mM NaCl, 2 mM KH_2PO_4, 1 mM $MgSO_4$, 3.5 μM $FeSO_4$, and 20 mM glucose or 20

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mM sodium succinate as the sole source of carbon and energy. For in vitro growth of *S. aureus*, 0.5% yeast extract and 0.5% Casamino Acids were added to the MOPS medium. For low-iron medium, MOPS minimal medium without added $FeSO_4$ and containing 20 mM sodium succinate was chelexed two times overnight at 4°C using Chelex 100 (10 g/liter; Sigma Chemical Co., St. Louis, Mo.). For differential isolation of *P. aeruginosa* and *S. aureus* in coculture, pseudomonas isolation agar and Baird Parker agar were used, respectively (Remel, Lenexa, Kans.). Brain heart infusion (BHI; Difco, Detroit, Mich.) agar was used for coculture on petri plates.

Dialysis membrane chambers. Spectra/Por (Spectrum Medical Industries Inc., Los Angeles, Calif.) dialysis tubing with a molecular mass exclusion of molecules larger than 8,000 Da was rinsed in sterile water for 5 min and then boiled for 20 min in sterile water containing 1 mM EDTA. The bags were then rinsed with sterile water for 10 min, transferred to MOPS minimal medium to cool, and tied at one end using strict aseptic technique. Each bag was then filled with 10 ml of MOPS minimal medium containing either 2,000 *P. aeruginosa* cells/ml or a binary culture of *P. aeruginosa* and *S. aureus* each at 1,000 cells/ml. Stationary-phase cultures of MOPS medium-grown *P. aeruginosa* and *S. aureus* cells were the source of inocula for these experiments. After inoculation, the dialysis bags were tied at the other end, and excess tubing was removed from the ends of the dialysis bag. Four- to 6-week-old Sprague-Dawley rats (Sprague-Dawley Inc., Indianapolis, Ind.) were anesthetized by subcutaneous injection with a mixture of ketamine (50 mg/ml), xylazine (5 mg/ml), and acepromazine (1 mg/ml) at a dose of 1 ml/kg of body weight. The inoculated dialysis bags were then implanted into the rat's peritoneal cavity as outlined previously (1). At the desired time, the dialysis bags were explanted from the rats and rinsed with sterile MOPS minimal medium, and bacteria were removed using a sterile syringe.

Analysis of global gene expression using Affymetrix GeneChips. Dialysis bags containing *P. aeruginosa* or *P. aeruginosa-S. aureus* cocultures were explanted from the rats at 18 h (optical density at 600 nm [OD_{600}] = 0.3 to 0.4). In vivo-grown bacteria were removed from the dialysis bags by using a sterile syringe and mixed 1:1 with the RNA-stabilizing agent RNALater (Ambion, Austin, Tex.). To serve as an in vitro comparison, *P. aeruginosa* was grown aerobically in MOPS minimal medium containing either 20 mM glucose or 20 mM succinate to an OD_{600} of 0.4 and mixed 1:1 with RNALater. DNA-free RNA was isolated from in vitro- and in vivo-grown *P. aeruginosa* as outlined elsewhere, except for cocultures of *P. aeruginosa* and *S. aureus*, where the lysozyme-lysostaphin treatment was omitted (36). RNA integrity was monitored by gel electrophoresis of glyoxylated samples. Preparation of labeled cDNA and processing of the *P. aeruginosa* GeneChip arrays was performed as previously described (36). Washing, staining, and scanning of the GeneChips were performed by the University of Iowa DNA Core Facility using an Affymetrix fluidics station. GeneChips were performed in duplicate or triplicate for each culture condition. Data were analyzed using Microarray Suite software, and only genes exhibiting regulation levels of fivefold or greater are reported. Verification of GeneChip data was performed with semiquantitative reverse transcription-PCR (RT-PCR) using Superscript II (Invitrogen, Carlsbad, Calif.) as described previously (31). PA2426 (*pvdS*), PA2247 (*bkdA1*), and PA1717 (*pscD*) were confirmed using PA1802 (*clpX*) as the constitutively expressed control.

Lysis of *S. aureus*. Lysis of *S. aureus* on petri plates was performed by thoroughly swabbing a BHI plate with an overnight culture of *S. aureus* diluted to an OD_{600} of 0.1. After drying, 5 μl of an overnight culture of *P. aeruginosa* was spotted onto the petri plate, dried, and incubated at 37°C for 24 h. Plates were imaged using an Alpha-Innotech documentation system.

***P. aeruginosa* growth yields.** For growth yield experiments using *S. aureus* as a source of iron, overnight bacterial cultures were centrifuged at 5,000 × *g* for 5 min and washed (three times) in chelexed MOPS minimal succinate medium. *P. aeruginosa* and *S. aureus* were then resuspended in chelexed MOPS minimal succinate medium (no added $FeSO_4$) to an OD_{600} of 2.0 and 100, respectively. *P. aeruginosa* was then mixed 1:1 with *S. aureus* or chelexed MOPS minimal succinate medium (as a no-iron control). A sterile 25-mm polycarbonate membrane was placed onto a chelexed MOPS minimal succinate plate solidified with 1% agarose and allowed to dry. Five-microliter aliquots of these mixtures were placed onto the polycarbonate membrane, allowed to dry, and incubated at 37°C for 24 h (12). Membranes were resuspended in 1 ml MOPS minimal medium, and serial dilutions were performed. Viable bacteria were quantitated using pseudomonas isolation agar and Baird Parker agar plates. *S. aureus* does not grow on MOPS minimal succinate medium due to amino acid auxotrophy. To examine if *P. aeruginosa* could use lysed *S. aureus* as a source of iron, *S. aureus* (1 ml of OD_{600} = 100) was mechanically lysed by bead beating for 30 min in a Biospec Products minibead beater as outlined by the manufacturer (Biospec Products, Bartlesville, Okla.) with 0.1-mm beads. After filter sterilization, chelexed minimal succinate agarose plates containing mechanically lysed *S. aureus* (1/14 dilution) were used to grow *P. aeruginosa* as outlined above. To ensure the chelexed succinate plates were iron limited, *P. aeruginosa* was also grown with 10 μM $FeSO_4$ added to the solidified medium.

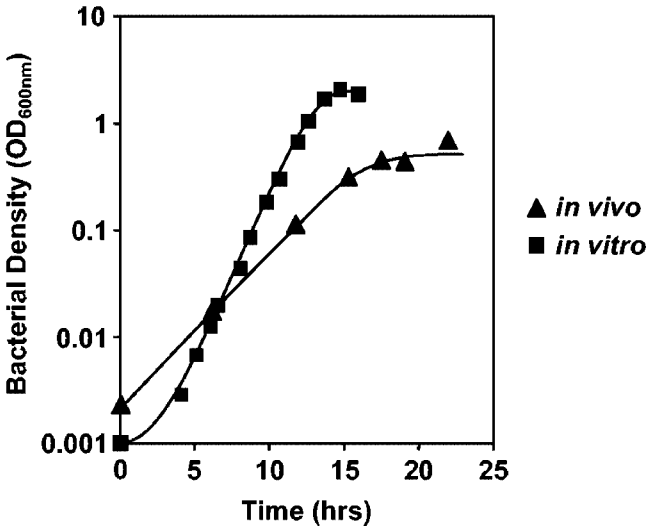

FIG. 1. Growth of *P. aeruginosa* in vitro in MOPS minimal medium with 20 mM glucose (■) and in vivo in the DMC in the rat (▲). Representative growth curves are shown. Maximum doubling times were approximately 40 min in vitro and 50 min in vivo.

RESULTS

Monitoring growth of *P. aeruginosa* in vivo. The opportunistic human pathogen *P. aeruginosa* primarily causes infections in individuals with compromised immune systems. These infections often occur in the lungs of individuals with the heritable disease CF (19) and the peritoneum of individuals undergoing CAPD (25). Although a number of in vivo models exist to study *P. aeruginosa* lung and peritoneal pathogenesis (14, 29, 43–45), most of these models do not possess the versatility to perform genome-scale gene expression studies and study multispecies consortia. To begin to understand in vivo gene expression, we grew *P. aeruginosa* in a dialysis membrane chamber (DMC) implanted into the peritoneal cavity of a rat (1). In this in vivo model, *P. aeruginosa* undergoes a typical planktonic growth curve with a doubling time similar to that of in vitro glucose-grown bacteria (approximately 50 min in the DMC and 40 min in glucose minimal medium) (Fig. 1). Final bacterial densities achieved by *P. aeruginosa* in the DMC were greater than 10^{10} bacteria. Although the DMC model does not allow direct interactions with host cells, *P. aeruginosa* is growing on peritoneal contents; thus, we will refer to this model as an in vivo batch culture model.

Identification and classification of *P. aeruginosa* genes differentially expressed in the peritoneum. Although most laboratory experiments evaluating bacterial gene expression are conducted in vitro, in vivo growth conditions are difficult to mimic in the laboratory. To begin to understand the physiology of in vivo-grown *P. aeruginosa*, we performed transcriptome analyses of DMC-grown and in vitro-grown *P. aeruginosa* using Affymetrix GeneChips. As in vitro comparisons, *P. aeruginosa* was grown in MOPS minimal medium containing 20 mM glucose or 20 mM sodium succinate as the sole source of carbon

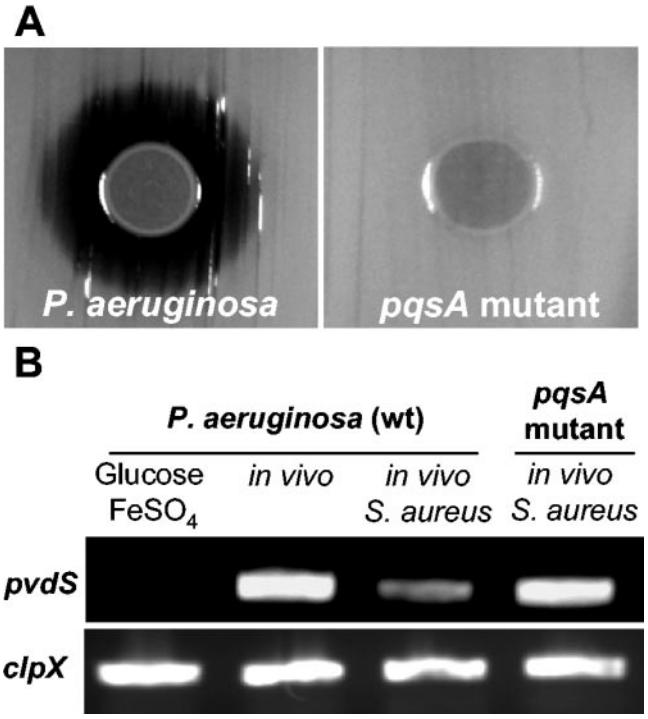

FIG. 2. Lysis of *S. aureus* is necessary for iron acquisition by *P. aeruginosa* during coculture. (A) A BHI petri plate was swabbed with a confluent lawn of *S. aureus*, and 5 μl of an overnight culture of wild-type *P. aeruginosa* PA14 and *P. aeruginosa* PA14-LM1 containing a Tn*5* insertion in *pqsA* were spotted on the plate and incubated at 37°C for 24 h. A zone of clearing (indicating lysis of *S. aureus)* is visible around the wild-type *P. aeruginosa* colony. Similar lysis results were also observed in test tube coculture experiments (data not shown). Although not shown here, approximately 20% of the time *P. aeruginosa* PA14-LM1 produces a small zone of lysis in this assay. (B) Semiquantitative RT-PCR analysis of the low-iron-inducible gene *pvdS* and the constitutively expressed *clpX* gene (42). *P. aeruginosa* was grown in vitro in glucose minimal medium containing excess $FeSO_4$ (3.5 μM) or in vivo as a monoculture or in coculture with *S. aureus*. *P. aeruginosa* PA14-LM1, which is unable to lyse *S. aureus* but possesses no observed growth defects under iron-limited conditions, was also grown in vivo in coculture with *S. aureus*.

and energy. To eliminate carbon source-specific genes from our analysis, only genes differentially expressed in vivo compared to glucose- and succinate-grown bacteria are reported.

Bacteria were harvested for GeneChip analysis at mid-logarithmic phase (OD_{600} = 0.3 to 0.5) under both in vitro and in vivo conditions, and gene expression profiles were compared using Affymetrix Microarray Suite software. On average, 71% of the gene-specific tiles present on the Affymetrix GeneChip hybridized at levels sufficient for statistical analysis; thus, we were able to compare expression profiles of approximately 4,000 genes. A total of 316 genes (approximately 5% of all *P. aeruginosa* genes) were differentially regulated at least fivefold during growth of *P. aeruginosa* in vivo compared to the in vitro controls. Of these genes, the majority were up-regulated (238 genes), while a smaller number were repressed (78 genes). As validation of our GeneChip results, we observed similar regulation patterns for PA2426 (*pvdS*), PA2247 (*bkdA1*), and PA1717 (*pscD*) using semiquantitaive RT-PCR (Fig. 2 and data not shown).

Genes in the sequenced *P. aeruginosa* genome are grouped into a number of classes based on functionality (www.pseudomonas.com). Classification of our in vivo-regulated genes revealed that most of these genes (33%) are of unknown function (Table 1). This is not surprising, given the observation that 45% of the 5,570 predicted *P. aeruginosa* genes have little homology to known proteins (37). The remaining genes are included in several classes, some of which possess distinct patterns of regulation. Most of the classes primarily include genes which are induced during in vivo growth, while all the genes in one group (bacteriophage/transposon) were repressed (Table 1). One of the most striking features of these data is that 29 transcriptional activators were induced during growth in vivo, indicating specific responses to the peritoneal environment. Only one gene involved in motility and attachment was differentially regulated during in vivo growth, indicating that these processes are quite similar with regard to transcription during in vitro and in vivo growth. Although no genes involved in lipopolysaccharide (LPS) biosynthesis were differentially expressed, it should be noted that most of the genes involved in O-antigen biosynthesis did not hybridize at levels sufficient for analysis. This is not surprising given the fact that we are not using the sequenced *P. aeruginosa* PAO1 strain, and O-antigen genes are highly divergent among *P. aeruginosa* strains (32).

Correlation of GeneChip and IVET data. Using IVET and a neutropenic mouse model, Wang et al. identified 19 genes induced during growth of *P. aeruginosa* in vivo (40). An examination of our in vivo-regulated genes indicated that of these 19 IVET genes, 6 did not hybridize at levels sufficient for GeneChip analysis, 10 were not differentially regulated, 2 were induced over fivefold in our DMC model (Table 2), and 1 was induced threefold in the DMC (PA4115). The two IVET genes induced over fivefold in our DMC model, *np20* (PA5499) and

TABLE 1. Classes of in vivo-induced genes

Functional class[a]	No. activated[b]	No. repressed[b]
Adaptation, protection	10	2
Amino acid biosynthesis and metabolism	17	6
Biosynthesis of cofactors	7	3
Carbon compound catabolism	3	2
Cell wall-LPS	1	0
Central intermediary metabolism	4	1
Chaperones and heat shock protein	2	0
Energy metabolism	25	6
Membrane proteins	2	0
Motility and attachment	1	0
Nucleotide biosynthesis and metabolism	1	0
Protein-secretion-export apparatus	4	0
Putative enzymes	15	2
Related to phage, transposon, plasmid	0	11
Secreted factors	2	0
Transcriptional regulators	29	2
Translation, posttranslational modification, degradation	1	0
Transport of small molecules	32	18
Two-component regulatory systems	3	0
Hypothetical proteins	65	19
Unknown (conserved hypothetical)	14	6

[a] Functional classes are from the *P. aeruginosa* genome website (www.pseudomonas.com).
[b] Genes that were activated or repressed in the DMC at least fivefold compared to cells grown in vitro in MOPS medium.

TABLE 2. *P. aeruginosa* genes differentially regulated during in vivo growth

ORF[a]	Gene	Homology or function[a]	Fold regulation[b]
0149		Probable sigma-70 factor	7
0171		Hypothetical protein	−8
0200		Hypothetical protein	7
0280	*cysA*	Sulfate transport protein	−11
0281	*cysW*	Sulfate transport protein	−17
0282	*cysT*	Sulfate transport protein	−11
0283	*sbp*	Sulfate-binding protein precursor	−15
0284		Hypothetical protein	−17
0440*		**Probable oxidoreductase**	**7**
0466		Hypothetical protein	−7
0509*	***nirN***	**Probable *c*-type cytochrome**	**7**
0510		Probable uroporphyrin III *c*-methyltransferase	13
0511*	***nirJ***	**Heme d1 biosynthesis protein**	**7**
0512		Conserved hypothetical protein	8
0513		Probable transcriptional regulator	8
0514*	***nirL***	**Heme d1 biosynthesis protein**	**11**
0515		Probable transcriptional regulator	9
0516*	***nirF***	**Heme d1 biosynthesis protein**	**8**
0517*	***nirC***	**Probable *c*-type cytochrome precursor**	**11**
0518*	***nirM***	**Cytochrome c_{551} precursor**	**12**
0519*	***nirS***	**Nitrate reductase precursor**	**15**
0521		Probable cytochrome *c* oxidase subunit	6
0523*	***norC***	**Nitric oxide reductase subunit C**	**32**
0524*	***norB***	**Nitric oxide reductase subunit B**	**68**
0525*		**Probable denitrification protein**	**15**
0587		Conserved hypothetical protein	5
0613		Hypothetical protein	−5
0614		Hypothetical protein	−5
0617		Probable bacteriophage protein	−10
0621		Conserved hypothetical protein	−5
0622		Probable bacteriophage protein	−8
0627		Conserved hypothetical protein	−5
0629		Conserved hypothetical protein	−7
0631		Hypothetical protein	−6
0632		Hypothetical protein	−7
0633		Hypothetical protein	−8
0635		Hypothetical protein	−11
0636		Hypothetical protein	−8
0639		Conserved hypothetical protein	−9
0641		Probable bacteriophage protein	−19
0674		Hypothetical protein	25
0675		Probable sigma-70 factor	5
0713		Hypothetical protein	26
0714		Hypothetical protein	24
0781		Hypothetical protein	18
0782†	***putA***	**Proline dehydrogenase**	**8**
0865†	***hpd***	**4-Hydroxyphenylpyruvate dioxygenase**	**5**
0872†	***phhA***	**Phenylalanine-4-hydroxylase**	**31**
0910		Hypothetical protein	−18
0911		Hypothetical protein	−6
0921		Hypothetical protein	−6
0984		Colicin immunity protein	7
1123		Hypothetical protein	5
1178	*oprH*	Outer membrane protein H1 precursor	−17
1179	*phoP*	Two-component response regulator	−5
1195		Hypothetical protein	6
1196		Probable transcriptional regulator	5
1382		Probable type II secretion system protein	12
1414		Hypothetical protein	5
1516		Hypothetical protein	5
1537		Probable short-chain dehydrogenase	6
1540		Conserved hypothetical protein	7
1541		Probable drug efflux transporter	8
1552		Probable cytochrome *c*	−12
1553		Probable cytochrome *c* oxidase subunit	−13
1554		Probable cytochrome oxidase subunit	−5
1673		Hypothetical protein	5
1742		Probable amidotransferase	7
1746		Hypothetical protein	9
1837		Hypothetical protein	−13
1838	*cysI*	Sulfite reductase	−10

Continued on following page

TABLE 2—*Continued*

ORF[a]	Gene	Homology or function[a]	Fold regulation[b]
1871	*lasA*	LasA protease precursor	6
1887		Hypothetical protein	11
1888		Hypothetical protein	10
1892		Hypothetical protein	−9
1894		Hypothetical protein	−9
1895		Hypothetical protein	−10
1896		Hypothetical protein	−9
1897		Hypothetical protein	−35
1911		Probable transmembrane sensor	6
1912		Probable sigma-70 factor	7
1922		Probable TonB-dependent receptor	11
1924		Hypothetical protein	9
1925		Hypothetical protein	8
1999†		**Probable CoA transferase, subunit A**	**35**
2000†		**Probable CoA transferase, subunit B**	**44**
2001	*atoB*	Acetyl-CoA acetyltransferase	13
2003	*bdhA*	3-Hydroxybutyrate dehydrogenase	6
2004		Conserved hypothetical protein	8
2016		Probable transcriptional regulator	5
2114		Probable MFS transporter	−7
2202		Probable amino acid permease	−7
2204		Probable binding protein component of ABC transporter	−10
2223		Hypothetical protein	6
2247†	***bkdA1***	**2-Oxoisovalerate dehydrogenase (alpha subunit)**	**38**
2248†	***BkdA2***	**2-Oxoisovalerate dehydrogenase (beta subunit)**	**14**
2249†	***bkdB***	**Branched-chain alpha-keto acid dehydrogenase (lipoamide component)**	**15**
2250†	***lpdV***	**Lipoamide dehydrogenase (−Val)**	**9**
2466		Probable TonB-dependent receptor	5
2534		Probable transcriptional regulator	5
2567		Hypothetical protein	5
2648	*nuoM*	NADH dehydrogenase I chain	−5
2686	*pfeR*	Two-component response regulator	6
2687	*pfeS*	Two-component sensor	5
2753		Hypothetical protein	7
2807		Hypothetical protein	−19
2825		Probable transcriptional regulator	5
2826		Probable glutathione peroxidase	5
2929		Hypothetical protein	5
2931		Probable transcriptional regulator	19
2932	*morB*	Morphinone reductase	71
2933		Probable MFS transporter	31
2934		Probable hydrolase	16
2945		Conserved hypothetical protein	−6
3038		Probable porin	−6
3118†	***leuB***	**3-Isopropylmalate dehydrogenase**	**−5**
3120†	***leuD***	**3-Isopropylmalate dehydratase small subunit**	**−6**
3121†	***leuC***	**3-Isopropylmalate dehydratase large subunit**	**−6**
3126	*ibpA*	Heat shock protein	7
3188		Probable permease of ABC sugar transporter	−382
3189		Probable permease of ABC sugar transporter	−79
3195	*gapA*	Glyceraldehyde-3-phosphate dehydrogenase	−6
3249		Probable transcriptional regulator	5
3309		Conserved hypothetical protein	6
3313		Hypothetical protein	−5
3391*	***nosR***	**Regulatory protein**	**54**
3392*	***nosZ***	**Nitrous oxide reductase precursor**	**25**
3393*	***nosD***	**NosD protein**	**11**
3394*	***nosF***	**NosF protein**	**16**
3395*	***nosY***	**NosY protein**	**8**
3396*	***nosL***	**NosL protein**	**8**
3404		Probable secretion protein	7
3405	*hasE*	Metalloprotease secretion protein	32
3406	*hasD*	Transport protein	16
3415		Probable dihydrolipoamide acetyltransferase	9
3416		Probable pyruvate dehydrogenase E1 component, beta chain	36
3417		Probable pyruvate dehydrogenase E1 component, alpha subunit	7
3418†	***ldh***	**Leucine dehydrogenase**	**8**
3441		Probable molybdopterin-binding protein	−12
3442		Probable ATP-binding component of ABC transporter	−21
3443		Probable permease of ABC transporter	−43
3444		Conserved hypothetical protein	−161

Continued on following page

TABLE 2—*Continued*

ORF[a]	Gene	Homology or function[a]	Fold regulation[b]
3445		Conserved hypothetical protein	−62
3446		Conserved hypothetical protein	−19
3450		Probable antioxidant protein	−8
3516		Probable lyase	−5
3581	*glpF*	Glycerol uptake facilitator protein	9
3582	*glpK*	Glycerol kinase	6
3584	*glpD*	Glycerol-3-phosphate dehydrogenase	44
3598		Conserved hypothetical protein	7
3600		Conserved hypothetical protein	139
3601		Conserved hypothetical protein	83
3719		Hypothetical protein	21
3720		Hypothetical protein	30
3721		Probable transcriptional regulator	5
3837		Probable permease of ABC transporter	−5
3862		Hypothetical protein	6
3870	*moaA1*	Molybdopterin biosynthetic protein	7
3871		Probable peptidyl-prolyl *cis-trans* isomerase	7
3872*	***narI***	**Respiratory nitrate reductase gamma chain**	**9**
3873*	***narJ***	**Respiratory nitrate reductase delta chain**	**11**
3874*	***narH***	**Respiratory nitrate reductase beta chain**	**24**
3875*	***narG***	**Respiratory nitrate reductase alpha chain**	**19**
3876*	***narK2***	**Nitrite extrusion protein 2**	**10**
3877*	***narK1***	**Nitrite extrusion protein 1**	**6**
3914	*moeA1*	Molybdenum cofactor biosynthetic protein	7
3915	*moaB1*	Molybdopterin biosynthetic protein	5
3922		Conserved hypothetical protein	−6
3931		Conserved hypothetical protein	−16
3933		Probable choline transporter	10
3935	*tauD*	Taurine dioxygenase	−16
3936		Probable permease of ABC taurine transporter	−35
3937		Probable ATP-binding component of ABC taurine transporter	−25
3938		Probable periplasmic taurine-binding protein precursor	−21
4063		Hypothetical protein	15
4064		Probable ATP-binding component of ABC transporter	7
4065		Hypothetical protein	5
4156		Probable TonB-dependent receptor	10
4160	*fepD*	Ferric enterobactin transport protein	5
4167		Probable oxidoreductase	13
4168		Probable TonB-dependent receptor	17
4181		Hypothetical protein	16
4182		Hypothetical protein	10
4197		Probable two-component sensor	10
4288		Probable transcriptional regulator	8
4333		Probable fumarase	−5
4364		Hypothetical protein	113
4365		Probable transporter	54
4442†	***cysN***	**ATP sulfurylase GTP-binding subunit, APS kinase**	**−22**
4443†	***cysD***	**ATP sulfurylase small subunit**	**−10**
4577		Hypothetical protein	5
4588†	***gdhA***	**Glutamate dehydrogenase**	**−6**
4610		Hypothetical protein	9
4621		Probable oxidoreductase	5
4657		Hypothetical protein	5
4738		Conserved hypothetical protein	−6
4739		Conserved hypothetical protein	−9
4834		Hypothetical protein	18
4835		Hypothetical protein	16
4836		Hypothetical protein	32
4837		Probable outer membrane protein	38
4838		Hypothetical protein	7
5024		Conserved hypothetical protein	−13
5027		Hypothetical protein	5
5088		Hypothetical protein	7
5100†	***hutU***	**Urocanase**	**7**
5102		Hypothetical protein	−8
5106		Conserved hypothetical protein	6
5170†	***arcD***	**Arginine-ornithine antiporter**	**11**
5171†	***arcA***	**Arginine deiminase**	**9**
5172†	***arcB***	**Ornithine carbamoyltransferase, catabolic**	**10**
5173†	***arcC***	**Carbamate kinase**	**6**
5302†	***dadX***	**Catabolic alanine racemase**	**6**

Continued on folliwing page

TABLE 2—*Continued*

ORF[a]	Gene	Homology or function[a]	Fold regulation[b]
5303		Conserved hypothetical protein	9
5304†	***dadA***	**D-Amino acid dehydrogenase, small subunit**	**9**
5351		Rubredoxin	−5
5372†	***betA***	**Choline dehydrogenase**	**12**
5373†	***betB***	**Betaine aldehyde dehydrogenase**	**12**
5374	*betI*	Transcriptional regulator	11
5427	*adhA*	Alcohol dehydrogenase	6
5446		Hypothetical protein	13
5475		Hypothetical protein	5
5481		Hypothetical protein	−7
5482		Hypothetical protein	−8
5499‡	***np20***	**Transcriptional regulator**	**6**
5500	*znuC*	Zinc transport protein	6
5532		Hypothetical protein	8
5534		Hypothetical protein	13
5535		Conserved hypothetical protein	15
5536		Conserved hypothetical protein	52
5538	*amiA*	*N*-Acetylmuramoyl-L-alanine amidase	28
5539		Hypothetical protein	8
5540		Hypothetical protein	17
5541		Probable dihydroorotase	11

[a] From the *P. aeruginosa* genome website, www.pseudomonas.com. Genes involved in anaerobic metabolism (*), transport and metabolism of amino acids (†), or identified previously using IVET (‡) are listed in bold.

[b] Fold change in *P. aeruginosa* mRNA level when grown in vivo in the DMC compared to that in bacteria grown in glucose minimal medium in vitro. Positive numbers represent induction, and negative numbers indicate repression in the DMC. All genes listed were also >5- fold regulated when DMC-grown and succinate-grown *P. aeruginosa* cultures were compared.

fptA (PA4221), have been partially characterized in *P. aeruginosa* (2, 39, 40). The *np20* gene was up-regulated approximately sixfold in the DMC and encodes a polypeptide with homology to a number of transcriptional regulators. The *np20* locus is important for pathogenesis of *P. aeruginosa* (40). The *fptA* gene encodes a receptor for the siderophore pyochelin (discussed below) and is up-regulated approximately 81-fold in the DMC model (2).

Iron acquisition in vivo. Most bacteria require iron for growth, and acquisition of iron is a significant challenge in most in vivo environments where little free iron exists. *P. aeruginosa* and many other bacteria produce high-affinity iron chelators called siderophores to acquire iron in vivo (7). These extracellular chelators are able to scavenge iron from in vivo sources and deliver it to the bacterium. *P. aeruginosa* produces two well-studied siderophores, pyoverdine and pyochelin, and the genes important for their syntheses are induced in low-iron environments (7). Ochsner et al. recently reported a transcriptome analysis of *P. aeruginosa* grown in vitro under low- and high-iron conditions (28). Of the 113 genes which were induced at least fivefold in this study, under low-iron conditions, 82 were also induced at least fivefold in our DMC model (Table 3). Included within these in vivo-induced genes were genes involved in synthesis and binding of pyoverdine and pyochelin, as well as multiple genes important for heme uptake (Table 3). Heme may serve as a source of iron in vivo, and the *hasAp* gene involved in heme utilization was the most highly induced in vivo gene (over 2,000-fold induction).

Oxygen levels in the peritoneum. *P. aeruginosa* grows anaerobically by using nitrate and its reduced derivatives as terminal electron acceptors. If nitrate is not present, arginine may be metabolized fermentatively by *P. aeruginosa* (38). The genes encoding proteins critical for anaerobic nitrate and arginine metabolism are positively regulated under low-oxygen conditions (34). An examination of our in vivo GeneChip data indicates that several *P. aeruginosa* operons involved in utilization of nitrate, nitrite, nitric oxide, nitrous oxide, and arginine were highly up-regulated during in vivo growth compared to aerobic glucose-grown cells (Table 2). Thus, we hypothesize that *P. aeruginosa* would possess the enzymatic capabilities of anaerobically grown bacteria, including the ability to reduce nitrate via the nitrate reductase enzyme. An evaluation of the nitrate reductase activity of in vivo-grown *P. aeruginosa* revealed that in vivo-grown *P. aeruginosa* was capable of reducing exogenously added nitrate to levels below detection (data not shown).

***P. aeruginosa* physiology and metabolism in vivo.** To cause more than a transient infection, *P. aeruginosa* must survive and grow within the host environment. However, the growth environment within the host is not well understood, other than the observation that *P. aeruginosa* mutants unable to synthesize purine precursors grow poorly in mouse models (40). Thus, it is critical to understand the growth environment of *P. aeruginosa* in vivo. Our in vitro conditions required *P. aeruginosa* to metabolize glucose or succinate as the sole carbon source and synthesize all essential metabolic precursors de novo. Under these in vitro conditions as our GeneChip control, our data indicate that genes encoding proteins involved in transport and metabolism of amino acids, particularly aromatic (PA0865 and PA0872) and branched-chain (PA2247 to PA2250) amino acids, are highly induced during growth in the DMC (Table 2). These data suggest that *P. aeruginosa* is using amino acids as a carbon source in the peritoneum.

Growth of *P. aeruginosa* and *S. aureus* cocultures in vitro and in vivo. It is clear that a number of bacterial infections are not simply the result of colonization by one microorganism, but of the pathogenic contributions of several organisms (13, 46). Such is the case with chronic lung infections and peritoneal infections in patients undergoing dialysis, where multispecies consortia, often including *P. aeruginosa* and *S. aureus* (10, 19,

TABLE 3. Expression of *P. aeruginosa* iron-regulated genes during monoculture and coculture growth in vivo

ORF[a]	Gene	Homology or function[a]	Fold regulation[b]	
			In vivo vs in vitro	Coculture vs monoculture
0471		Probable transmembrane sensor	7	−17
0472		Probable sigma-70 factor	8	−34
0500	*bioB*	Biotin synthase	−9	NC
0672		Hypothetical protein	44	−44
0707	*toxR*	Transcriptional regulator	6	−4
1245		Hypothetical protein	5	−3
1300		Probable sigma-70 factor	35	−37
1301		Probable transmembrane sensor	13	−12
2033		Hypothetical protein	20	−23
2034		Hypothetical protein	20	−16
2384		Hypothetical protein	41	−46
2385		Probable acylase	34	−30
2386	*pvdA*	L-Ornithine N^5-oxygenase	85	−74
2389		Conserved hypothetical protein	9	−9
2390		Probable ATP-binding/permease fusion ABC transporter	7	−8
2391		Probable outer membrane protein	5	−7
2392		Hypothetical protein	18	−12
2393		Probable dipeptidase precursor	53	−127
2394		Probable aminotransferase	57	−60
2395		Hypothetical protein	13	−38
2396		Hypothetical protein	13	−7
2397	*pvdE*	Pyoverdine biosynthesis protein	44	−25
2398	*fpvA*	Ferripyoverdine receptor	30	−86
2399	*pvdD*	Pyoverdine synthetase	40	−23
2400		Probable nonribosomal peptide synthetase	94	−28
2401		Probable nonribosomal peptide synthetase	94	−40
2402		Probable nonribosomal peptide synthetase	46	−28
2403		Hypothetical protein	58	−22
2404		Hypothetical protein	43	−39
2405		Hypothetical protein	43	−37
2406		Hypothetical protein	24	−21
2407		Probable adhesion protein	14	−18
2408		Probable ATP-binding component of ABC transporter	38	−11
2411		Probable thioesterase	84	−111
2412		Conserved hypothetical protein	197	−203
2413		Probable class III aminotransferase	51	−136
2424		Probable nonribosomal peptide synthetase	112	−53
2425		Probable thioesterase	44	−51
2426	*pvdS*	Sigma factor	66	−117
2427		Hypothetical protein	14	−8
2451		Hypothetical protein	9	−9
2452		Hypothetical protein	73	−110
2467		Probable transmembrane sensor	5	−8
2468		Probable sigma-70 factor	6	−13
3407	*hasAp*	Heme acquisition protein	2,180	−724
3408	*hasR*	Heme acquisition protein	74	−74
3409		Probable transmembrane sensor	9	−7
3410		Probable sigma-70 factor	14	−18
3530		Conserved hypothetical protein	9	−39
3899		Probable sigma-70 factor	17	−26
3900		Probable transmembrane sensor	7	−11
3901	*fecA*	Fe(III) dicitrate transport protein	9	−10
4158	*fepC*	Ferric enterobactin transport protein	13	−13
4218		Probable transporter	40	−22
4219		Hypothetical protein	109	−66
4220		Hypothetical protein	288	−48
4221	*fptA*	Fe(III) pyochelin receptor precursor	81	−92
4222		Probable ATP-binding component of ABC transporter	34	−13
4223		Probable ATP-binding component of ABC transporter	26	−15
4224		Hypothetical protein	58	−34
4225	*pchF*	Pyochelin synthetase	52	−65
4226	*pchE*	Dihydroaeruginoic acid synthetase	53	−46
4227	*pchR*	Transcriptional regulator	21	−76
4228	*pchD*	Pyochelin biosynthesis protein	74	−66
4229	*pchC*	Pyochelin biosynthesis protein	99	−163
4230	*pchB*	Salicylate biosynthesis protein	126	−118
4231	*pchA*	Salicylate biosynthesis isochorismate synthetase	122	−89
4359		Conserved hypothetical protein	5	−10
4467		Hypothetical protein	21	−10
4468	*sodM*	Superoxide dismutase	40	−48

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TABLE 3—*Continued*

ORF[a]	Gene	Homology or function[a]	Fold regulation[b]	
			In vivo vs in vitro	Coculture vs monoculture
4469		Hypothetical protein	48	−53
4470	*fumC1*	Fumarate hydratase	60	−106
4471		Hypothetical protein	100	−131
4570		Hypothetical protein	37	−25
4708		Hypothetical protein	9	−6
4709		Probable hemin degrading factor	9	−8
4710		Probable outer membrane hemin receptor	19	−30
4895		Probable transmembrane sensor	12	−13
4896		Probable sigma-70 factor	14	−25
4973	*thiC*	Thiamin biosynthesis protein	−14	NC
5312		Probable aldehyde dehydrogenase	6	NC
5313		Probable pyridoxal-dependent aminotransferase	7	NC

[a] From the *P. aeruginosa* genome website, www.pseudomonas.com.
[b] Regulation of *P. aeruginosa* iron-regulated genes (28) as determined by Affymetrix GeneChip analysis. Two conditions were compared: monoculture growth in vivo versus monoculture in vitro growth in glucose minimal medium with added $FeSO_4$ (positive numbers represent induction in vivo); and in vivo coculture growth versus in vivo monoculture growth (positive numbers represent induction during coculture growth). NC, no change.

20, 22, 46), are associated with disease. Although these bacteria may reside in the same pathogenic environment, essentially nothing is known about if or how they interact in vivo. To begin to understand the interactions between *P. aeruginosa* and *S. aureus* in vivo, these bacteria were grown in monoculture and in coculture in the DMC model. When grown in monoculture, *S. aureus* possesses a faster doubling time than *P. aeruginosa* in the DMC (30 min for *S. aureus* versus 50 min for *P. aeruginosa)*. When *P. aeruginosa* and *S. aureus* are started at identical densities and grown in the DMC model, *P. aeruginosa* grows similar to that under monoculture conditions, reaching densities of 5×10^8 bacteria/ml after 15 to 18 h. *S. aureus* reaches slightly lower growth yields at this time during coculture in the DMC (4×10^9 bacteria/ml) compared to monoculture growth (10^{10} bacteria/ml).

Gene expression of *P. aeruginosa* cocultured in vivo with *S. aureus*. To determine the impact of *S. aureus* on *P. aeruginosa* physiology during growth in the peritoneum, we isolated RNA from *P. aeruginosa* grown in vivo with *S. aureus*. As mentioned above, *P. aeruginosa* reaches similar densities after 15 to 18 h in the DMC when growing as a monoculture or in coculture with *S. aureus*; thus, we sampled bacteria for RNA isolation at these time points under both conditions. To enrich for *P. aeruginosa* RNA during isolation, *S. aureus* lysis was prevented by omitting the lysozyme-lysostaphin treatment. If any contaminating *S. aureus* RNA were present, it would likely not cross-hybridize because of the significant differences in the GC content of these bacteria and the built-in mismatch controls on the Affymetrix GeneChips.

We compared the transcriptomes of *P. aeruginosa* grown in vivo in monoculture to those of *P. aeruginosa* grown in coculture with *S. aureus* in vivo. A total of 178 genes were differentially expressed at least fivefold during in vivo growth with *S. aureus*, with the majority of these genes (131) repressed in the coculture situation. One of the most striking features of these data is the fact that over 95% (78 of 82) of the genes repressed during in vivo growth with *S. aureus* are regulated by iron availability (Table 3). This includes genes involved in pyoverdine and pyochelin biosynthesis and suggests that *P. aeruginosa* growing in coculture with *S. aureus* perceives its environment as high in iron, in contrast to the monoculture in vivo situation, where the perceived environment is low in iron (Table 3). In fact, no differences in iron-regulated genes were observed when the transcriptomes of high-iron, in vitro-grown *P. aeruginosa* were compared to those of *P. aeruginosa* grown in vivo with *S. aureus* (data not shown). This suggests that the presence of *S. aureus* must locally concentrate useful iron for *P. aeruginosa* or increase the concentration of free iron in the peritoneum. The former is likely, since the molecular weight exclusion of the dialysis membranes prevents diffusion of most *S. aureus* toxins and most in vivo iron is chelated by transferrin or lactoferrin.

Lysis of *S. aureus* is required for iron acquisition. Given the observation that *P. aeruginosa* lyses several bacteria, including *S. aureus* (11, 17, 26), we hypothesized that the increased iron levels perceived by *P. aeruginosa* during in vivo coculture may be partially explained by *S. aureus* lysis and subsequent release of intracellular iron. To test this hypothesis, we cocultured *S. aureus* in vivo with *P. aeruginosa* PA14 and *P. aeruginosa* PA14-LM1, which contains a Tn*5* insertion in *pqsA* and exhibits reduced lysis of *S. aureus* (Fig. 2A). The *pqsA* gene is involved in biosynthesis of several quinolone molecules in *P. aeruginosa* important for cell-cell signaling and lysis of *S. aureus* but possesses no known involvement in regulation of low-iron-inducible genes (data not shown). We used the low-iron-inducible gene *pvdS* as a marker gene for iron limitation and evaluated transcript levels of *pvdS* in these cocultures using RT-PCR. As expected from our GeneChip data, the wild-type PA14 perceives the in vivo environment as high in iron when cocultured with *S. aureus* (low levels of *pvdS* transcript), while PA14-LM1 perceives its environment as low in iron (high levels of *pvdS* mRNA) in coculture (Fig. 2B).

***P. aeruginosa* can use *S. aureus* as an iron source.** These data suggest that *P. aeruginosa* can use *S. aureus* as an iron source. If this were true, *P. aeruginosa* should grow to higher densities in iron-limited media when grown in coculture with *S. aureus* or when grown in the presence of lysed *S. aureus* cells. To test this hypothesis, we grew *P. aeruginosa* PA14 in iron-replete medium in vitro as a monoculture, as a monoculture in the presence of mechanically lysed *S. aureus*, and in coculture with viable *S. aureus*. Final *P. aeruginosa* growth yields showed that the wild-type strain grew to higher densities when grown in

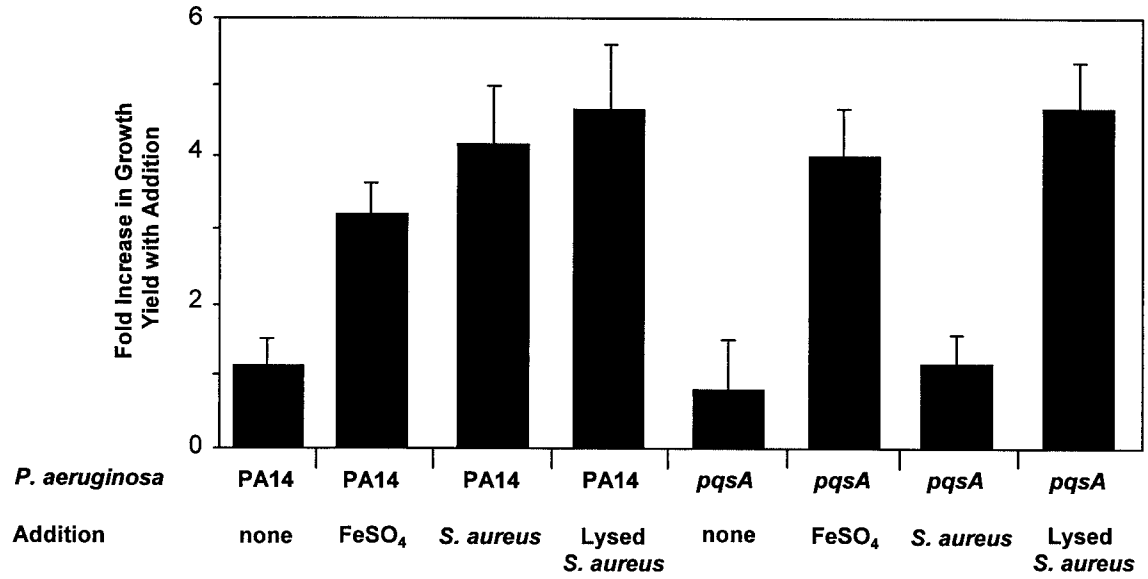

FIG. 3. Growth yields of wild-type *P. aeruginosa* and *P. aeruginosa* PA14-LM1 grown in iron-limited MOPS minimal medium (see Materials and Methods) as a monoculture, with 10 μM $FeSO_4$, in coculture with *S. aureus*, and with the addition of mechanically lysed *S. aureus*. Data are expressed as (the ratio of *P. aeruginosa* cell yield in the presence of the addition)/(monoculture cell yields with no addition) (numbers >1 indicate increased cell yield during growth with the indicated addition). For the two no-addition controls, the ratio was calculated by pair-wise comparisons of four individual replicates. *S. aureus* does not grow in this minimal medium due to amino acid auxotrophy. Less than 10^3 *S. aureus* cells were present in PA14-*S. aureus* coculture colonies at the time of sampling, while $>10^9$ remained in the PA14-LM1-*S. aureus* cocultures. Error bars represent the standard deviation for three to four experimental repeats.

coculture with *S. aureus* or in the presence of *S. aureus* lysate (Fig. 3), indicating that viable and lysed *S. aureus* can be used as a source of iron by wild-type *P. aeruginosa*. To assess if lysis is required for acquisition of iron from viable *S. aureus* cells, we grew *P. aeruginosa* PA14-LM1 in the presence and absence of viable and lysed *S. aureus* (Fig. 3). Growth of PA14-LM1 in the presence of lysed *S. aureus* greatly increased the growth yields over monoculture growth, indicating that this mutant can use lysed *S. aureus* as an iron source. However, significantly increased growth yields were not observed for PA14-LM1 when cocultured with viable *S. aureus*, suggesting that the inability of PA14-LM1 to effectively lyse *S. aureus* impairs its growth under low-iron coculture conditions.

DISCUSSION

The goal of this study was to examine gene expression of *P. aeruginosa* during in vivo growth in order to better understand the physiology of this bacterium during infection. We used a rat DMC model in conjunction with DNA microarrays to identify *P. aeruginosa* genes differentially regulated during growth in vivo as a monoculture and in coculture with *S. aureus*. This model involves growing *P. aeruginosa* in a dialysis bag implanted in the peritoneum of the rat. The DMC model does not allow direct interactions with host molecules larger than 8 kDa, including host cells; thus, genes differentially regulated by such interactions will not be detected. However, this model makes microarray and proteomic approaches viable and is easily manipulated to observe interactions between bacteria in an in vivo growth environment. *P. aeruginosa* grows well in the DMC, with a doubling time of less than 1 h and reaching final bacterial densities of over 10^{10} bacteria, illustrating that the peritoneum is a high-nutrient growth environment.

To identify in vivo-specific genes, we compared the transcriptome of in vivo-grown *P. aeruginosa* to bacteria grown in vitro in MOPS minimal medium with glucose or succinate as the sole source of carbon and energy. These carbon sources were chosen for comparison since most *P. aeruginosa* metabolic studies have focused on growth using these substrates. By comparing our in vivo transcriptome results to two in vitro growth conditions, we were able to decrease the number of in vivo-regulated genes by 30 to 40% compared to single in vitro growth conditions. We believe this analysis effectively eliminated many carbon source-specific genes from our analysis. It should be noted that both of our in vitro growth conditions require *P. aeruginosa* to synthesize all anabolic precursors de novo, thus allowing us to make predictions about the nutritional environment of the peritoneum. Our data indicate that amino acids are likely a substrate for growth in the peritoneum, since genes involved in catabolism and transport of branched-chain and aromatic amino acids are induced during growth in the DMC (Table 2). Although further studies are necessary to determine the specific growth substrates, we feel the DMC model provides an easily manipulatable preliminary model for these studies. Metabolic genes identified as important for growth in the DMC can then be tested in more relevant animal systems.

Our transcriptome analysis of in vivo monocultures did not show activation of most of the *P. aeruginosa* genes identified by Wang et al. as in vivo-induced by IVET (Tables 2 and 3). There are a number of reasons to account for this difference, the most obvious being the differences in the mouse models used. Wang et al. used a neutropenic mouse model and harvested bacteria from the mouse liver, whereas our peritoneal model is a much simpler in vivo batch growth model. Our study also used glu-

cose- or succinate-grown *P. aeruginosa* as an in vitro control, whereas Wang et al. used a common complex medium for IVET library construction. Regardless of the differences in the studies, it is an important observation that three genes were common between the studies. Of these three genes, much is known about the FptA pyochelin receptor, which is important for iron acquisition in low-iron environments. Much less is known about *np20*, other than it is induced in vivo and is important for production of the extracellular virulence factor pyocyanin, and nothing is known about the third gene (PA4115), which was only induced threefold during growth in the peritoneum. Our data in combination with the previous IVET analysis indicate that these three genes are members of a core set of genes inducible during in vivo growth in at least two disparate animal models. The *np20* gene is the most intriguing, given its homology with a number of transcriptional regulators. Further study of these genes should provide clues to the in vivo environment and potentially identify processes important for in vivo growth and pathogenesis.

Our transcriptome analysis of in vivo *P. aeruginosa* monocultures revealed that the peritoneal environment is low in iron (Table 3). These data are not surprising and coincide with a number of studies evaluating bacterial growth in vivo (21, 24, 40). It is interesting that the in vitro response of *P. aeruginosa* to low iron reported by Ochsner et al. (28) is remarkably similar to our in vivo results, indicating that in vitro studies evaluating the response to low iron are applicable to understanding in vivo growth and pathogenesis. It should be noted that although our data indicate that the peritoneal environment is low in free iron, we do not believe that iron is severely limiting growth, since *P. aeruginosa* grows near its maximum doubling rate in the rat (50-min doubling time). Thus, it appears that although low in free iron, as expected *P. aeruginosa* is well adapted to growth in the peritoneum.

Our transcriptome data also indicate that the peritoneum is low in oxygen, since many genes known to be regulated by anaerobiosis were highly induced during growth in vivo (Table 2). Based on the fast growth rate of *P. aeruginosa* in the DMC, it is likely that sufficient levels of oxygen and/or nitrate are available in the peritoneum for growth, since the maximum doubling time anaerobically in vitro using arginine is approximately 5 h (data not shown). Nitrate is present within the peritoneum, as we detected low micromolar levels of nitrate from an uninoculated DMC chamber (data not shown). Anaerobic growth in the peritoneum is relevant to pathogenesis, given the recent evidence that the ability of *P. aeruginosa* to form antibiotic-resistant biofilms is enhanced in low-oxygen environments (18). Many *P. aeruginosa* infections, including those in the CF lung and in the peritoneum, are a consequence of biofilm formation (8, 9, 18). The observation that *P. aeruginosa* growing in the peritoneum perceives its environment as low in oxygen enhances the importance of this model to pathogenic studies, including biofilm studies. Although in this study we focused on planktonic bacteria, we have preliminary evidence that *P. aeruginosa* readily forms antibiotic-resistant biofilms on catheter tubing placed in the DMC (data not shown).

The most interesting observation from this study is that wild-type *P. aeruginosa* perceives its environment as high in iron when grown with *S. aureus* in the DMC. Our data suggest that the change in iron perception by *P. aeruginosa* during coculture with *S. aureus* is dependent on lysis of *S. aureus*, since *P. aeruginosa* PA14-LM1, which is unaffected in iron acquisition or growth in vivo but cannot effectively lyse *S. aureus* (Fig. 2A), perceives its environment as low in iron during coculture growth with *S. aureus* (Fig. 2B). This would not be surprising, since *P. aeruginosa* produces several extracellular antimicrobial molecules (many controlled by the *pqsA-E* operon) known to be important for lysis of *S. aureus* (11, 17, 26, 27). The DMC coculture experiments were also supported by in vitro data, which showed that growth yields of *P. aeruginosa* were increased in iron-limited media when grown in coculture with *S. aureus* (Fig. 3). This again was dependent on lysis of *S. aureus*, since growth yields of PA14-LM1 were not increased in coculture. This deficiency in growth by PA14-LM1 cannot be explained by competition with *S. aureus* for iron, since *S. aureus* is auxotrophic and will not grow in the minimal succinate medium used in these experiments. We believe these data together suggest that *P. aeruginosa* lyses *S. aureus* and can use it as an iron source.

S. aureus is capable of undergoing autolysis during in vitro growth (5, 6, 15, 16). Our data do not distinguish whether *P. aeruginosa* is causing direct lysis of *S. aureus* or inducing autolysis; however, our results with *P. aeruginosa* PA14-LM1 indicate that *P. aeruginosa*-independent autolysis by *S. aureus* is most likely not the primary mechanism of iron acquisition during coculture. We are currently dissecting the specific mechanism(s) of *S. aureus* lysis, and our preliminary work and the work of others indicate that it is most likely multifactorial (11, 26). Lysis of *S. aureus* by *P. aeruginosa* is not unique to the laboratory strains used in this study. Our laboratory *P. aeruginosa* strain exhibited visible lysis of seven *S. aureus* strains tested, and 24 of 29 (83%) *P. aeruginosa* CF lung isolates tested produced visible lysis of our laboratory *S. aureus* strain (data not shown).

Although lysis appears to be important for iron acquisition during coculture in vitro and in vivo, the source of the iron is unknown. Our experiments indicate that *P. aeruginosa* can use mechanically lysed *S. aureus* as a source of iron, and it is likely that iron-containing proteins released from the lysed *S. aureus* cells serve as the source of iron. The DMC dialysis membrane will prevent diffusion of proteins larger than 8,000 Da, which may act to concentrate iron-containing proteins; thus, we cannot be certain whether iron acquisition through *S. aureus* lysis is important for *P. aeruginosa* in vivo. However, it is clear that the physiology of *P. aeruginosa* with regard to iron availability is dramatically altered during coculture growth with *S. aureus* in the DMC. Iron acquisition through siderophore biosynthesis and response is an energy-intensive process; thus, increasing local iron levels through lysis of *S. aureus* during growth in low-iron environments, such as those encountered in vivo, might be beneficial. We do not believe our data are limited to *P. aeruginosa*-*S. aureus* interactions. *P. aeruginosa* also lyses several other gram-positive pathogens, including *S. pneumoniae* and *Bacillus anthracis* (data not shown), which it may also encounter during infection.

Although we have shown that *P. aeruginosa* may obtain iron through lysis of *S. aureus*, our results do not preclude other mechanisms of iron acquisition during coculture. *P. aeruginosa* may also obtain iron by binding and uptake of siderophores produced by other bacteria. A recent study of cocultures of *P.*

aeruginosa and *Burkholderia cepacia* indicated that *P. aeruginosa* specifically responds to the presence of *B. cepacia* siderophores during coculture (41). It is likely that the mechanistic details of iron acquisition will vary depending on the constituents of the coculture, but our results indicate that antagonistic interactions between species may help define community structure. Spatial and temporal factors will also affect these interactions, and it is likely that *P. aeruginosa* iron acquisition through *S. aureus* lysis will only be important when these bacteria are in very close proximity, such as in multispecies biofilms.

Our transcriptome analysis provides a more comprehensive view of in vivo gene expression of *P. aeruginosa*. Our data suggest that the peritoneum is a low-oxygen, iron-limited environment with sufficient nutrients to support large numbers of *P. aeruginosa*. Our studies verified three previously described in vivo-induced *P. aeruginosa* genes and identified a large number of other genes differentially expressed during growth in the peritoneum. Our data also provide a genomic analysis of a common pathogenic coculture and indicate that the physiology of *P. aeruginosa* in vivo is drastically altered in regards to iron availability during coculture with *S. aureus*. These results may have significant ramifications for in vivo growth of *P. aeruginosa* in polymicrobial communities and underscore the need for analysis of multispecies infections. Although we focused on iron acquisition in this study, it is plausible that other nutrients or cofactors may be obtained through competitive interactions. It is likely that the benefits of such interactions are not confined to pathogenesis but instead reflect processes important for survival and growth in many polymicrobial environments.

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