

Nutritional Cues Control *Pseudomonas aeruginosa* Multicellular Behavior in Cystic Fibrosis Sputum^{∇†}

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Received 18 July 2007/Accepted 6 September 2007

The sputum (mucus) layer of the cystic fibrosis (CF) lung is a complex substrate that provides *Pseudomonas aeruginosa* with carbon and energy to support high-density growth during chronic colonization. Unfortunately, the CF lung sputum layer has been difficult to mimic in animal models of CF disease, and mechanistic studies of *P. aeruginosa* physiology during growth in CF sputum are hampered by its complexity. In this study, we performed chromatographic and enzymatic analyses of CF sputum to develop a defined, synthetic CF sputum medium (SCFM) that mimics the nutritional composition of CF sputum. Importantly, *P. aeruginosa* displays similar phenotypes during growth in CF sputum and in SCFM, including similar growth rates, gene expression profiles, carbon substrate preferences, and cell-cell signaling profiles. Using SCFM, we provide evidence that aromatic amino acids serve as nutritional cues that influence cell-cell signaling and antimicrobial activity of *P. aeruginosa* during growth in CF sputum.

A key concept in bacterial pathogenesis is the ability of invading pathogens to obtain sufficient carbon and energy from the host for in vivo growth. Although Garber originally proposed the host as a growth medium over 40 years ago (12), the nutritional environment of most infection sites is poorly defined and often inadequately modeled by laboratory growth media. This lack of knowledge, combined with the limited utility of many animal models, provides significant challenges for mechanistic studies aimed at examining host nutrients as mediators of colonization and disease. To overcome these challenges, it is critical both to define the nutritional composition of key infection sites and to study bacterial physiology in the context of in vivo-relevant growth substrates.

The heritable disease cystic fibrosis (CF) is an archetype for the development of nutritional models with which to study bacterial pathogenesis. A hallmark of CF disease is the accumulation of large volumes of sputum (mucus) within the lungs, which diminishes the host's ability to clear bacterial infections (17, 31). The viscous CF lung sputum provides bacteria with a nutritionally rich growth environment composed of host- and bacterial-derived factors (17, 38). The opportunistic pathogen *Pseudomonas aeruginosa* chronically colonizes the CF lung, where it often grows to high cell densities in CF sputum ($>10^9$ cells/ml sputum). Although many other bacterial species persist and grow in the CF lung, chronic *P. aeruginosa* infection is likely the most clinically relevant, as it is correlated with declining lung function (17). Mechanistically, *P. aeruginosa* colonization and progression to chronic infection is poorly understood, although potential contributing factors are high-density growth and enhanced fitness of *P. aeruginosa* in CF sputum. *P.*

aeruginosa fitness has been linked to nutritional components in CF sputum (39), thus necessitating the development of a versatile model that allows examination of CF sputum nutritional cues.

The growth environment impacts several clinically relevant phenotypes in *P. aeruginosa*. For example, individual carbon and nitrogen sources have been shown to modulate *P. aeruginosa* in vitro biofilm development (6, 23, 53, 56) and surface motility (25). *P. aeruginosa* cell-cell signaling (quorum sensing) is also influenced by nutritional cues (9, 39, 63). Growth of *P. aeruginosa* in CF sputum promotes increased synthesis of the cell-cell signaling molecule 2-heptyl-3-hydroxy-4-quinolone (the *Pseudomonas* quinolone signal [PQS]) (39), and recent studies implicate aromatic amino acids as potential mediators of this phenotype (39). However, definitive studies aimed at examining how individual CF sputum components, such as aromatic amino acids, impact *P. aeruginosa* physiology are difficult due to the complexity of sputum. As a means of circumventing this problem, we describe the development of a synthetic CF sputum medium (SCFM) that nutritionally mimics CF sputum. Studies using SCFM reveal that the expression of *P. aeruginosa* nutritionally controlled genes is similar in CF sputum and in SCFM and that CF sputum-specific phenotypes, including increased PQS production, can be recapitulated in SCFM. In addition, carbon consumption analyses provide evidence that specific amino acids support rapid/high-density growth of *P. aeruginosa* in CF sputum.

MATERIALS AND METHODS

Bacterial strains and media. *P. aeruginosa* strain UCBBP-PA14 (44) and the isogenic *pqsA* transposon insertion mutant were obtained from the MGH-Parabiosys:NHLBI Program for Genomic Applications (<http://pga.mgh.harvard.edu/cgi-bin/pa14/mutants/retrieve.cgi>). *Staphylococcus aureus* strain Xen 36 (Xenogen Biosciences), constitutively expressing the *luxABCDE* genes, was used for antimicrobial studies. *P. aeruginosa* was routinely cultured on tryptic soy agar plates, and *S. aureus* was grown in brain heart infusion (BHI) broth/agar. Morpholinepropanesulfonic acid (MOPS) glucose medium was previously described (39). Bacterial growth was assessed by monitoring optical density at 600 nm

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† Supplemental material for this article may be found at <http://jb.asm.org/>.

∇ Published ahead of print on 14 September 2007.

(OD₆₀₀). All cultures were incubated at 37°C with shaking at 250 rpm unless otherwise noted.

CF sputum sampling and preparation. Sputum samples from 12 adult patients with nonexacerbating CF with *P. aeruginosa* concentrations of $\leq 10^8$ bacteria/ml sputum were collected by expectoration into sterile containers and stored at -80°C prior to lyophilization, as previously described (39). For chromatographic analyses, powdered CF sputum was weighed and resuspended in sterile deionized water to a final concentration of 10, 20, or 25% (vol/vol). Fifty milliliters of CF sputum corresponded to approximately 2 g dry weight (39). Resuspended sputum was homogenized using a tip sonicator (Branson Ultrasonics) as previously described (39), and insoluble material was subsequently removed by centrifugation at $16,000 \times g$ for 5 min. The resulting supernatant was filtered through a 0.45- μ m-pore-size syringe filter and stored at -20°C prior to analysis.

Chromatographic and enzymatic analyses of CF sputum. Anion concentrations in CF sputum supernatants were determined by anion-exchange chromatography using a Dionex DX5000 system and an AS-4A column. Cation concentrations were determined by cation-exchange chromatography using a Dionex DX500 system and a CA12A analytical ion-exchange column. Data analysis was performed using Peaknet software (Dionex Corporation).

Free amino acid levels in CF sputum supernatants were determined by cation-exchange chromatography. CF sputum supernatants were prepared for free amino acid analysis as follows. CF sputum supernatant (100 μ l) was mixed with 100 nmol of the internal standard β -(2-thienyl)-DL-alanine and an equal volume of 6% trichloroacetic acid. Samples were vortexed for 30 s and incubated at 89°C for 1 min prior to centrifugation for 10 min at $1,500 \times g$. Supernatants were removed and extracted three times with 600 μ l ethyl acetate, and organic fractions were discarded. Aqueous fractions were subsequently filtered through 0.45- μ m-pore-size spin filter columns by centrifugation at $16,000 \times g$ for 5 min. Spin filter columns were prewashed with 0.01 N HCl. Filtrates were evaporated to dryness by vacuum centrifugation (Eppendorf) and resuspended in 200 μ l 0.01 N HCl. Cation-exchange chromatography and data analysis were performed at the University of Oklahoma Health Sciences Center Molecular Biology-Proteomics Facility, using a Beckman system Gold model 126 high-performance liquid chromatography amino acid analyzer. Lactate levels in CF sputum supernatants were measured with the lactate assay kit A-108 (Biomedical Research Service Center, SUNY at Buffalo, NY). This assay measures both D- and L-isomers of lactate. Glucose levels were measured with a glucose (hexokinase) assay kit (Sigma).

SCFM. SCFM was developed from the average concentrations of ions, free amino acids, glucose, and lactate in CF sputum samples. For amino acids, the percentage of total free amino acids in each CF sputum sample was determined for individual amino acids. The average percentage of each amino acid was extrapolated to a final total amino acid concentration of 19 mM for SCFM (for raw data, see Table S2 in the supplemental material). Amino acids were maintained as 100-mM stocks in deionized water and stored in the dark at 4°C. Tyrosine, aspartate, and tryptophan were resuspended in 1.0 M, 0.5 M, and 0.2 M NaOH, respectively. Lactate stocks were adjusted to a pH of 7.0 with NaOH.

For SCFM, amino acids were added from 100-mM stocks to a buffered base (6.5 ml 0.2 M NaH₂PO₄, 6.25 ml 0.2 M Na₂HPO₄, 0.348 ml 1 M KNO₃, 0.122 g NH₄Cl, 1.114 g KCl, 3.03 g NaCl, 10 mM MOPS, 779.6 ml deionized water) in the following volumes: L-aspartate, 8.27 ml; L-threonine, 10.72 ml; L-serine, 14.46 ml; L-glutamate · HCl, 15.49 ml; L-proline, 16.61 ml; L-glycine, 12.03 ml; L-alanine, 17.8 ml; L-cysteine · HCl, 1.6 ml; L-valine, 11.17 ml; L-methionine, 6.33 ml; L-isoleucine, 11.2 ml; L-leucine, 16.09 ml; L-tyrosine, 8.02 ml; L-phenylalanine, 5.3 ml; L-ornithine · HCl, 6.76 ml; L-lysine · HCl, 21.28 ml; L-histidine · HCl, 5.19 ml; L-tryptophan, 0.13 ml; and L-arginine · HCl, 3.06 ml. SCFM was adjusted to pH 6.8 and filter sterilized through a 0.2- μ m-pore-size filter. After sterilization, the following sterile components were added per liter: 1.754 ml 1 M CaCl₂, 0.606 ml 1 M MgCl₂, and 1 ml 3.6 mM FeSO₄ · 7H₂O. Three milliliters 1 M D-glucose and 9.3 ml 1 M L-lactate were added for gene expression and carbon consumption experiments. For some experiments, aromatic amino acids were removed from SCFM and replaced with an equimolar amount of serine (referred to as SCFM-aromatics).

Carbon consumption analyses. Carbon consumption was examined during *P. aeruginosa* growth in CF sputum and in SCFM. For CF sputum, *P. aeruginosa* was grown overnight in SCFM and diluted to an OD₆₀₀ of 0.1 in fresh SCFM. At an OD₆₀₀ of 0.4, cells were pelleted by centrifugation and washed twice in a carbon-free MOPS-buffered medium (39). Washed cells were used to inoculate 20% CF sputum medium (39) to an OD₆₀₀ of 0.05. Sputum samples from two individuals with CF were independently examined. At the time of inoculation and at multiple points during growth, samples were removed for carbon analysis. Samples were centrifuged at $16,000 \times g$ for 5 min and filtered through 0.45- μ m-pore-size syringe filters immediately after harvesting. Amino acid, glucose, and lactate

analyses were performed as described above for CF sputum. All incubations in CF sputum were performed at 37°C with shaking at 250 rpm. For SCFM, *P. aeruginosa* was grown overnight in SCFM and diluted to an OD₆₀₀ of 0.05 in fresh SCFM. At an OD₆₀₀ of 0.5, cells were pelleted by centrifugation and washed twice in carbon-free SCFM. Cells were incubated in carbon-free SCFM for 30 min and then used to inoculate fresh SCFM to an OD₆₀₀ of 0.04. Samples were removed at the time of inoculation and at multiple points throughout growth for carbon analysis, as performed for CF sputum. Incubations in SCFM for carbon consumption experiments were carried out at 37°C with shaking at 160 rpm.

Global expression profiling. *P. aeruginosa* cultures grown in SCFM were harvested at an OD₆₀₀ of 0.1 to 0.2 and mixed 1:1 with the RNA stabilizing agent RNeasy lysis buffer (Ambion). RNA was isolated using RNeasy minicolumns (QIAGEN), and cDNA was prepared for hybridization to Affymetrix GeneChip microarrays as previously described (34, 39, 45, 52). DNA contamination of RNA samples was assessed by PCR amplification of the *P. aeruginosa* *rplU* gene with the primers *rplU*-for (5'-CGCAGTGATTGTTACCGGTG-3') and *rplU*-rev (5'-AGGCTGAATGCCGGTGATC-3'). The *rplU* gene is commonly used to monitor DNA contamination in *P. aeruginosa* RNA samples (27, 29, 68). Agarose gel electrophoresis was used to assess RNA integrity. Washing, staining, and scanning of GeneChips was performed at the University of Iowa DNA core facility, using an Affymetrix fluidics station. Transcriptome data for SCFM-grown *P. aeruginosa* were compared to that of CF sputum medium-grown bacteria and MOPS-glucose-grown bacteria (39). GeneChip analyses were performed in duplicate (SCFM and MOPS-glucose medium) or triplicate (CF sputum medium) for each condition tested, and data were analyzed using GeneChip operating software version 1.4. Results were reported as differentially regulated based on pairwise comparisons of all GeneChips ($P \leq 0.05$).

Quinolone and pyocyanin analyses. To quantify PQS, *P. aeruginosa* was grown to an OD₆₀₀ of 0.1 in SCFM or SCFM-aromatics (SCFM in which aromatic amino acids had been replaced with an equimolar amount of serine). Cultures were extracted three times with an equal volume of acidified ethyl acetate (600 μ l glacial acetic acid/4 liters ethyl acetate), and the organic layer was removed and evaporated under a continuous stream of N₂. Extracts were analyzed by thin-layer chromatography as previously described (11, 35, 40). Synthetic PQS was used as a standard and *n*-fold differences in PQS levels were determined by spot densitometry using a FluorChem 8900 gel imager (Alpha Innotech). To quantify other quinolones, *P. aeruginosa* was grown as outlined above to an OD₆₀₀ of 0.8, and 40-ml volumes were extracted with an equal volume of acidified ethyl acetate. Organic extracts were dried by rotoevaporation, and quinolones were quantified by liquid chromatography-mass spectrometry as described previously (30, 35) at the University of Oklahoma Health Sciences Center Molecular Biology-Proteomics Facility. Briefly, dried extracts were resuspended in 500 μ l of a 30% dimethylformamide-30% acetonitrile-1% acetic acid solution, and 125 μ l was applied to a Michrom Paradigm model high-performance liquid chromatography system. Elution was performed with a 30 to 100% gradient of a 97% acetonitrile-2% water-1% acetic acid solution over 40 min with a flow rate of 40 μ l/min. Quinolone compounds were quantified with a precursor ion scan of 172 (30, 35). To quantify pyocyanin, *P. aeruginosa* was grown for 24 h in SCFM or SCFM-aromatics. Samples (10 ml) were removed and extracted with 5 ml chloroform, and chloroform fractions were subsequently extracted with 1 ml 0.01 N HCl. Pyocyanin levels in the aqueous phase were measured spectrophotometrically (*A*₅₂₀) as previously described (8, 35, 65).

***S. aureus* antimicrobial assays.** For antimicrobial assays, *P. aeruginosa* culture supernatants were prepared by growing bacteria at 37°C for 18 h in SCFM or SCFM-aromatics. Bacteria were then removed by centrifugation at $6,000 \times g$ for 15 min followed by filtration through 0.22- μ m-pore-size syringe filters. Exponential *S. aureus* Xen-36 was diluted to an OD₆₀₀ of 0.1 in BHI, and 50- μ l samples were added to wells of a 96-well plate. Plates were incubated for 15 min at 37°C before addition of 150 μ l of *P. aeruginosa* culture supernatants, 3% H₂O₂, or sterile BHI. *S. aureus* luminescence was monitored at 2-min intervals for 10 min, using a FLUOstar luminometer (BMG). The internal temperature within the luminometer was maintained at 37°C, and plates were shaken at 150 rpm for 15 s prior to luminescence measurement.

RESULTS

Construction of a defined medium that nutritionally mimics CF sputum. To create a defined CF sputum medium, we collected and analyzed CF sputum samples from multiple individuals for levels of free amino acids, cations, anions, glucose, and lactate. CF sputum samples utilized for these analyses

TABLE 1. Composition of SCFM

Components	Concn (mM) ^a
Ions	
Na ⁺	66.6
K ⁺	15.8
NH ₄ ⁺	2.3
Ca ²⁺	1.7
Mg ²⁺	0.6
Cl ⁻	79.1
NO ₃ ⁻	0.35
PO ₄ ²⁻	2.5
SO ₄ ²⁻	0.27
Amino acids	
Serine.....	1.4
Threonine.....	1.0
Alanine.....	1.8
Glycine.....	1.2
Proline.....	1.7
Isoleucine.....	1.1
Leucine.....	1.6
Valine.....	1.1
Aspartate.....	0.8
Glutamate.....	1.5
Phenylalanine.....	0.5
Tyrosine.....	0.8
Tryptophan.....	0.01
Lysine.....	2.1
Histidine.....	0.5
Arginine.....	0.3
Ornithine.....	0.7
Cysteine.....	0.2
Methionine.....	0.6
Other	
Glucose.....	3.2
Lactate.....	9
FeSO ₄ (μM) ^b	3.6

^a SCFM component concentrations are based on the average concentrations measured in CF sputum samples (see Tables S1 to S3 in the supplemental material for raw data). Amino acid values do not include those present within peptides but instead represent “free” levels in CF sputum. Values in this table have been rounded for clarity.

^b The iron level is adapted from previous studies (6, 46, 57, 58).

were obtained from adults by expectoration and contained *P. aeruginosa* concentrations of $\leq 10^8$ bacteria/ml sputum. From these analyses, a defined medium referred to as SCFM was devised (Table 1). A detailed description of SCFM is provided in Materials and Methods and in Tables S1 and S2 in the supplemental material. As observed in previous studies (49), CF sputum chloride levels measured in this study varied over an approximate fivefold range; consequently, this anion was used to balance the salts base of SCFM. Therefore, the final chloride concentration in SCFM (79.1 mM) is higher than the average determined in this study (54.6 mM), although still within the range of CF sputum chloride levels (Table 1 and see Table S1 in the supplemental material). An initial pH of 6.8 was chosen for SCFM, since the average pH of submucosal gland fluid in CF patients is ~ 6.6 to 7.0 (19, 55), measurements of airway mucus in explanted CF lungs have detected mucus pH values ranging from ~ 6.0 to 6.9 (69), and the pH of lyophilized CF sputum samples resuspended in deionized water varies from 6.8 to 7.4 (data not shown). Since SCFM does not possess the native buffering system of the lung, it was supple-

mented with 10 mM MOPS to supply additional buffering capacity.

***P. aeruginosa* growth and gene expression in SCFM.** *P. aeruginosa* displays distinct phenotypes during growth in CF sputum (39, 66), and nutritional cues within sputum have been proposed to mediate several of these phenotypes. The goal of this study was to develop a versatile nutritional model for *P. aeruginosa* growth in CF sputum. The utility of SCFM as a model for CF sputum necessitates that the growth and expression of nutritionally controlled genes in SCFM are similar to those in CF sputum but distinct from common laboratory media. Examination of growth in SCFM and in CF sputum reveals that *P. aeruginosa* grows well in SCFM with a doubling time (~ 32 min) similar to that observed for CF sputum (Fig. 1). For gene expression analyses, the transcriptome of SCFM-grown *P. aeruginosa* was compared to that of glucose-grown and CF sputum-grown bacteria. The comparison to glucose-grown bacteria provides an evaluation of nutritionally regulated genes similar to that performed previously for CF sputum (39), and the comparison to CF sputum-grown bacteria allows identification of nutritional genes differentially regulated in CF sputum and SCFM. Previous studies of CF sputum and glucose-grown *P. aeruginosa* revealed 147 genes differentially regulated during growth in CF sputum (39). Of these 147 genes, 80 (54%) were also differentially regulated during growth in SCFM compared to that in glucose (Table 2i also see Table S3 in the supplemental material). Importantly, genes involved in amino acid catabolism and production of the quorum sensing signaling molecule PQS were significantly up-regulated during growth in SCFM (Table 2), to levels that were similar to those observed during growth in CF sputum (39). Of the 67 remaining genes, most were iron regulated or involved in chemotaxis/flagellar motility (see Table S3 in the supplemental material). Since each of these processes has been shown to be impacted by specific host factors in the CF lung, including host chelators and neutrophil elastase (7, 20, 46, 54, 57, 58, 62), it is not surprising that their expression patterns in CF sputum were distinct from those in SCFM. Comparisons of CF sputum and SCFM transcriptomes revealed that 137 genes were differentially regulated at least fivefold (see Table S6 in the supplemental material). Of these genes, few have been implicated in *P. aeruginosa* nutrient acquisition and catabolism. The acetyl-coenzyme A synthetase (*acsA*) gene was down-regulated fivefold during growth in SCFM compared to that in CF sputum

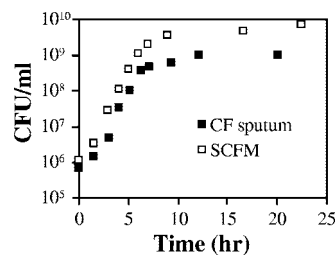


FIG. 1. Growth of *P. aeruginosa* in SCFM and in CF sputum medium. Growth was monitored by dilution plating for viable cell counts, and data are shown as CFU/ml. Bacteria were harvested at $\sim 10^8$ CFU/ml for Affymetrix GeneChip analyses. Representative growth curves are shown. The CF sputum growth curve data are from Palmer et al. (39).

TABLE 2. Gene expression profiles in CF sputum and SCFM^a

ORF function	ORF	Gene	Function or class	Fold change in gene expression	
				CF sputum versus glucose ^b	SCFM versus glucose ^c
Amino acid transport and degradation	PA0782	<i>putA</i>	Proline dehydrogenase PutA	4.3	18
	PA0865	<i>hpd</i>	4-hydroxyphenylpyruvate dioxygenase	66	45
	PA0866	<i>aroP2</i>	Aromatic amino acid transport protein	13	9
	PA0870	<i>phhC</i>	Aromatic amino acid aminotransferase	9	6
	PA0871	<i>phhB</i>	Pterin-4- α -carbinolamine dehydratase	5	4.3
	PA0872	<i>phhA</i>	Phenylalanine-4-hydroxylase ^d	32	23
	PA0897	<i>anuG</i>	Arginine/ornithine succinyltransferase AII subunit	3	3.7
	PA0898	<i>anuD</i>	Succinylglutamate-5-semialdehyde dehydrogenase	2.7	3.4
	PA2001	<i>atoB</i>	Acetyl-coenzyme A acetyltransferase	16	9
	PA2007	<i>maiA</i>	Maleylacetoacetate isomerase	8	8
	PA2008	<i>fahA</i>	Fumarylacetoacetase	9	9
	PA2009	<i>hmgA</i>	Homogentisate 1,2-dioxygenase	11	10
	PA2247	<i>bkdA1</i>	2-oxoisovalerate dehydrogenase, α -subunit	20	7
	PA2248	<i>bkdA2</i>	2-oxoisovalerate dehydrogenase, β -subunit	19	6
	PA2249	<i>bkdB</i>	Branched-chain α -keto acid dehydrogenase	13	5
	PA2250	<i>lpdV</i>	Lipoamide dehydrogenase-Val	19	6
	PA3766		Probable aromatic amino acid transporter	2.8	3.6
	PA4470	<i>fumC1</i>	Fumarate hydratase	6	3.2
	PA5302	<i>dadX</i>	Catabolic alanine racemase	9	10
	PA5304	<i>dadA</i>	D-amino acid dehydrogenase, small subunit	20	15
	Glucose transport and metabolism	PA2322		Gluconate permease	-5.5
PA2323			Probable glyceraldehyde-3-phosphate dehydrogenase	-3.8	-35
PA3181			2-keto-3-deoxy-6-phosphogluconate aldolase	-3	-6
PA3186		<i>oprB</i>	Carbohydrate outer membrane porin	-2.7	-6
PA3195		<i>gapA</i>	Glyceraldehyde-3-phosphate dehydrogenase	-3.2	-6
Flagellar synthesis and chemotaxis	PA1092	<i>fliC</i>	Flagellin type B	-21	NC
	PA2867		Probable chemotaxis transducer	-8	NC
	PA4307	<i>pctC</i>	Chemotactic transducer PctC	-8	NC
	PA4310	<i>pctB</i>	Chemotactic transducer PctB	-23	NC
<i>Pseudomonas</i> quinolone signaling	PA0996	<i>pqsA</i>	Probable coenzyme A ligase	18	6
	PA0997	<i>pqsB</i>	β -keto-acyl-acyl-carrier protein synthase	17	8
	PA0998	<i>pqsC</i>	β -keto-acyl-acyl-carrier protein synthase	19	7
	PA0999	<i>pqsD</i>	3-oxoacyl-[acyl-carrier-protein] synthase III	17	6
	PA1000	<i>pqsE</i>	Quinolone signal response protein	19	5
	PA1001	<i>phnA</i>	Anthranilate synthase component I	22	7
	PA1002	<i>phnB</i>	Anthranilate synthase component II	14	3.9

^a Open reading frame (ORF), gene name, and function data were obtained from the *P. aeruginosa* genome website (www.pseudomonas.com).

^b Regulation (*n*-fold change) of genes differentially expressed during *P. aeruginosa* growth in CF sputum medium compared to that in glucose; a positive number indicates an up-regulation of the gene during growth in sputum. Data are from Palmer et al. (39).

^c Regulation (*n*-fold change) of genes differentially expressed during *P. aeruginosa* growth in SCFM as compared to growth in glucose; a positive number indicates an up-regulation of the gene during growth in SCFM. NC indicates no change in mRNA levels as determined by GeneChip operating software version 1.4.

^d Phenylalanine-4-hydroxylase is involved in the synthesis of tyrosine and the degradation of phenylalanine.

and is important for *P. aeruginosa* acetate catabolism (26). The PA3758, PA3759, and PA3761 genes were also down-regulated in SCFM and may be involved in catabolism of *N*-acetylglucosamine and glucosamine (41). Finally, the dehydroorotase *pyrQ* (*pyrC2*) gene was down-regulated in SCFM; however, this gene plays a redundant role in de novo pyrimidine biosynthesis and has no known role in nucleotide catabolism (3).

***P. aeruginosa* exhibits similar carbon preferences during growth in SCFM and in CF sputum.** Since carbon catabolism impacts *P. aeruginosa* virulence and biofilm formation (39, 43, 47, 50, 53), carbon utilization profiles were examined in SCFM

and in CF sputum medium to determine the primary carbon sources consumed by *P. aeruginosa* during growth in these media. For these experiments, concentrations of individual carbon sources were monitored throughout *P. aeruginosa* growth in CF sputum medium and in SCFM. Results from these experiments revealed that *P. aeruginosa* carbon consumption profiles are remarkably similar in CF sputum medium and in SCFM (see Tables S4 and S5 in the supplemental material), with six carbon sources utilized first in both medium types, i.e., proline, alanine, arginine, glutamate, aspartate, and lactate (Table 3). It was not possible to distinguish a preference among these six carbon sources, and the results suggest they

TABLE 3. Preferred carbon sources in CF sputum and SCFM^a

Carbon source	% ± SD of remaining carbon after growth in:	
	CF sputum	SCFM
Proline	18 ± 0	20 ± 9
Alanine	26 ± 13	31 ± 10
Arginine	37 ± 3	0
Lactate	45 ± 3	54 ± 8
Glutamate	46 ± 20	41 ± 9
Aspartate	51 ± 10	24 ± 9

^a The six carbon sources quantitatively consumed first by *P. aeruginosa* during growth in 20% CF sputum medium and in SCFM. Shown are the percentages ± standard deviations (SD) of the initial carbon source remaining after *P. aeruginosa* growth in CF sputum (2 h) and in SCFM (3 h). A later time point was used for SCFM since it contains 5 times more carbon than 20% CF sputum. See Materials and Methods for experimental details and Tables S4 and S5 in the supplemental material for data on all available carbon sources.

are consumed concomitantly. These studies indicate that *P. aeruginosa* carbon preferences are similar in CF sputum and in SCFM and that SCFM serves as an *in vivo*-relevant medium with which to mechanistically evaluate carbon substrate utilization by *P. aeruginosa*.

***P. aeruginosa* signaling is similar in CF sputum and in SCFM.** We previously observed increased PQS production during growth in CF sputum (39). Since genes involved in PQS biosynthesis were significantly up-regulated during growth in SCFM (Table 2), we anticipated that PQS levels of SCFM-grown *P. aeruginosa* would resemble those observed for CF sputum-grown bacteria. Analysis of PQS levels revealed that, as in CF sputum, *P. aeruginosa* produces high levels of PQS during growth in SCFM (Fig. 2A).

The SCFM-mediated increase in PQS biosynthesis suggested that nutritional components within CF sputum enhance PQS biosynthesis. Since SCFM is a defined medium, the im-

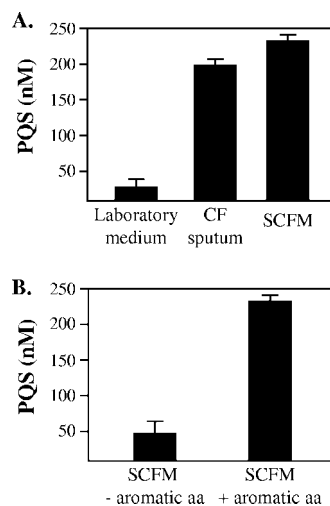


FIG. 2. PQS production is enhanced during growth in SCFM and in CF sputum. (A) PQS levels were assessed for *P. aeruginosa* grown in MOPS-glucose laboratory medium (39), SCFM, and CF sputum medium. Bacteria were sampled in exponential phase at an OD₆₀₀ of 0.1. (B) *P. aeruginosa* was grown in SCFM and in SCFM without aromatic amino acids to an OD₆₀₀ of 0.1, and PQS levels were determined. Error bars represent standard deviations.

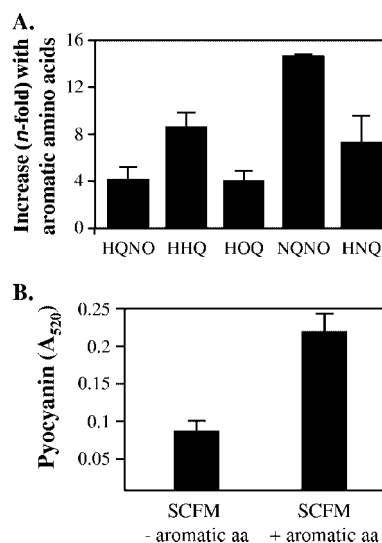


FIG. 3. Aromatic amino acids impact quinolone and pyocyanin production in *P. aeruginosa*. (A) *P. aeruginosa* cultures grown to an OD₆₀₀ of 0.8 in SCFM or SCFM lacking aromatic amino acids were extracted with acidified ethyl acetate, and quinolone levels were analyzed by liquid chromatography-mass spectrometry. Shown are 4-hydroxy-2-heptylquinoline *N*-oxide (HQNO), 4-hydroxy-2-heptylquinoline (HHQ), 4-hydroxy-2-octylquinoline (HOQ), 4-hydroxy-2-nonylquinoline *N*-oxide (NQNO), and 4-hydroxy-2-nonylquinoline (HNQ). (B) Pyocyanin levels in *P. aeruginosa* cultures grown for 24 h in SCFM or in SCFM lacking aromatic amino acids. Pyocyanin levels were determined after extraction by monitoring A₅₂₀ as outlined in Materials and Methods. Error bars represent standard deviations.

act of individual nutritional components on PQS biosynthesis can be assessed by simply removing them from the medium. Since previous studies implicated aromatic amino acids as modulators of PQS biosynthesis (9, 39), we began by examining their role in PQS biosynthesis. Removal of aromatic amino acids from SCFM and replacement with equimolar levels of serine, which does not impact PQS production (39), resulted in a significant decline in PQS production (Fig. 2B), to levels similar to those observed for glucose-grown bacteria. In addition, levels of several other quinolone molecules that share biosynthetic components with PQS were also significantly reduced upon removal of aromatic amino acids from SCFM (Fig. 3A). It is important to note that no differences in growth rate were observed upon removal of aromatic amino acids (data not shown), and samples were removed for analyses at equivalent bacterial densities.

Due to enhanced PQS biosynthesis, CF sputum-grown *P. aeruginosa* increases the production of several PQS-controlled factors, including the secondary metabolite pyocyanin (39). Based on these results, we reasoned that the presence of aromatic amino acids would significantly enhance pyocyanin production during growth in SCFM. As observed with CF sputum (39), *P. aeruginosa* produced high levels of pyocyanin during growth in SCFM, and removal of aromatic amino acids decreased pyocyanin levels by approximately 2.5-fold (Fig. 3B).

Aromatic amino acids enhance *P. aeruginosa* antimicrobial activity in SCFM. Along with increased production of PQS and PQS-controlled factors, recent studies in our laboratory revealed that *P. aeruginosa* exhibits increased lysis of the CF lung

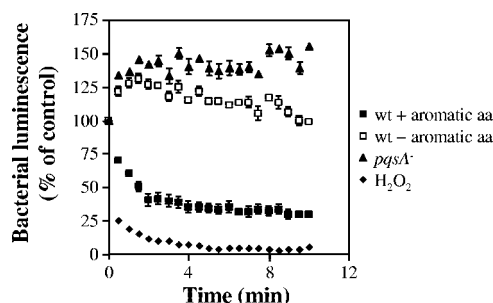


FIG. 4. Aromatic amino acids enhance the antimicrobial activity of *P. aeruginosa* cultures. *P. aeruginosa* was grown in SCFM or in SCFM lacking aromatic amino acids, and culture supernatants were applied to the luminescent *S. aureus* strain Xen 36. *S. aureus* luminescence was followed over time using a luminometer. Decreases in luminescence correlates with antimicrobial activity. Data are shown relative to the luminescence of a control to which fresh medium (BHI) had been added. Hydrogen peroxide (3%) was used as a positive control, and supernatants from the SCFM-grown *P. aeruginosa* *pqsA* mutant, which does not exhibit lysis of *S. aureus*, was used as a negative control. Error bars represent standard deviations and in some cases are too small to be seen.

coinhabitant *S. aureus* during growth in CF sputum (39). *P. aeruginosa* lysis of *S. aureus* requires a functional quinolone biosynthesis system (34), and it is likely mediated by multiple factors, including pyocyanin and antimicrobial quinolones. Given the impact of aromatic amino acids on quinolone and pyocyanin levels (Fig. 2 and 3), we hypothesized that high levels of *P. aeruginosa* antimicrobial activity would be observed for SCFM and that this activity would be dependent on the presence of aromatic amino acids. To test this hypothesis, we examined the antistaphylococcal activity of *P. aeruginosa* supernatants during growth in SCFM in the presence and absence of aromatic amino acids. For these experiments, *P. aeruginosa* supernatants were added to a luminescent *S. aureus* strain constitutively expressing *luxABCDE*, and the impact of these supernatants on light production was examined. Light production has previously been used as a marker for antimicrobial activity (1, 42, 60, 61). In this assay, decreases in light production correlate with increased antimicrobial activity. Supernatants derived from the SCFM-grown *P. aeruginosa* exhibited significant antimicrobial activity, similar to that observed for the antimicrobial H₂O₂; however, removal of aromatic amino acids from SCFM resulted in complete loss of this activity (Fig. 4).

DISCUSSION

While it is clear that the nutritional environment significantly impacts *P. aeruginosa* physiology and gene expression, little is known about the CF lung bacterial growth environment. In general, studies utilizing laboratory media supplemented with CF sputum have sought to identify *P. aeruginosa* virulence genes differentially expressed in response to sputum (39, 64, 66). Two studies have provided crude, nondialyzed sputum as the sole source of carbon and energy for *P. aeruginosa* (38, 39); however, for nutritional studies, this approach is hampered by the complexity of CF sputum. Other groups have developed surrogate in vitro growth media to mimic CF spu-

tum. A complex medium designed to foster *P. aeruginosa* mucoidy with components either directly measured or presumed to be in CF sputum was developed by Ghani and Soothill (14) and further modified by Sriramulu et al. (56). Although these media have utility for assessing *P. aeruginosa* phenotypes such as microcolony formation (56), the undefined nature of these media limits their use for assessing the roles of specific nutritional cues.

This study describes the development of a defined medium that nutritionally mimics CF sputum. Levels of specific components, including sodium, chloride, potassium, calcium, ammonium, and magnesium resemble those previously reported (13, 21, 24, 48, 49). Although concentrations of individual amino acids, glucose, and lactate have not previously been reported for CF sputum, the total free amino acid concentrations measured in this study are comparable to those previously published by Thomas et al. (59). More importantly, *P. aeruginosa* exhibits gene expression profiles, carbon consumption patterns, and cell-cell signaling profiles during growth that are similar in SCFM and in CF sputum.

P. aeruginosa carbon preference is poorly understood, despite the fact that the carbon source has profound effects on virulence-associated phenotypes, including toxin production and formation of antibiotic-resistant biofilms (4, 5, 15, 23, 37, 50, 53). Our results clearly show that *P. aeruginosa* exhibits preferences for carbon substrates that are similar during growth in SCFM and in CF sputum (Table 3i also see Tables S4 and S5 in the supplemental material). The mechanism of carbon source preference is not known, although novel factors are likely involved, since mutation of the carbon repression control (*crc*) gene, encoding a protein critical for small organic acid preference (16, 32), has no impact on *P. aeruginosa* carbon preference in SCFM (data not shown). It could be argued that since CF sputum contains a range of potential carbon sources not present in SCFM (such as mucin, lipids, nucleotides, and intact proteins), our studies may not accurately reflect carbon consumption in vivo. Although we cannot completely discount this possibility, several results support similar carbon preferences in CF sputum and in SCFM: growth rates in CF sputum and SCFM are similar (Fig. 1), the expression levels of carbon catabolism genes are similar in CF sputum and in SCFM (Table 2i also see Table S6 in the supplemental material), and the disappearance of specific carbon sources is temporally and quantitatively similar in both CF sputum and SCFM (see Tables S4 and S5 in the supplemental material). Together these points suggest that although other carbon-containing compounds are present in CF sputum, they likely contribute little to *P. aeruginosa* growth since growth rates and carbon consumption profiles are not drastically different in the presence (CF sputum) and absence (SCFM) of these alternative carbon sources. In addition, genes involved in pathways such as fatty acid β oxidation (*fadD1* and *fadD2* [2]), ribose catabolism (*rbsK* [18]), and nucleic acid catabolism (*dht* [22] and PA5019 [10]) were not differentially expressed when transcriptomes of SCFM-grown and CF sputum-grown cultures were compared (see Table S6 in the supplemental material). In fact, the only differentially expressed carbon catabolism genes between SCFM-grown and CF sputum-grown bacteria are putatively involved in the degradation of *N*-acetylglucosamine to acetyl-coenzyme A (PA3758-9, PA3761, and *acsA* [www.pseudomonas.com]). Thus,

while we do not exclude the possibility that *P. aeruginosa* metabolizes these substrates for carbon and energy in CF sputum, our data support the conclusion that SCFM contains the primary carbon sources utilized by *P. aeruginosa* in vivo.

An intriguing phenotype of CF sputum-grown *P. aeruginosa* is enhanced production of the cell-cell signal PQS (39). Previous studies suggested that this phenotype is nutritionally mediated (9, 39), and our results with SCFM provide evidence that the presence of high levels of phenylalanine and tyrosine in CF sputum likely enhances PQS production (Fig. 3B). Recent studies indicate that high (high-micromolar to millimolar) levels of tryptophan also increase PQS production (9); however, our CF sputum chromatographic analyses revealed very little “free” tryptophan (10 μ M) in CF sputum (Table 1), and this level was insufficient to induce PQS production (data not shown). In fact, tryptophan levels were often below the limit of detection (see Table S2 in the supplemental material); thus, it is unlikely that “free” tryptophan is critical for enhanced PQS production in CF sputum. Although the impact of enhanced PQS production in vivo is unknown, we speculate that this phenotype may be important during the initial stages of *P. aeruginosa* colonization. *P. aeruginosa* normally colonizes the CF lung after other bacteria such as *S. aureus* have established infections (17). Upon entering the CF lung, *P. aeruginosa* competes with and displaces the current bacterial inhabitants. Several factors likely contribute to this displacement, including production of antimicrobial factors by *P. aeruginosa* (33, 39). Our results suggest that aromatic amino acids are important inducers of antimicrobial compounds, as *P. aeruginosa* exhibits no antimicrobial activity against *S. aureus* in the absence of aromatic amino acids (Fig. 4).

Not all previously reported CF sputum-specific phenotypes were observed for SCFM-grown *P. aeruginosa*. Several studies have shown that iron-regulated genes are differentially expressed during growth in CF sputum (39, 64), indicating that CF sputum is an iron-limited environment. Although SCFM contains a physiologically relevant concentration of iron (7, 46, 57, 58), readily available iron is likely high in SCFM and low in CF sputum, since iron is bound by host/bacterial chelators in the CF lung. Also, genes involved in flagellar motility were previously reported to be differentially expressed by CF sputum-grown *P. aeruginosa* (39, 66). Recent studies indicate that the host factor neutrophil elastase mediates the loss of flagella and the reduction of *P. aeruginosa* flagellar gene expression observed during growth in CF sputum (20, 54). SCFM could easily be modified with the addition of host iron chelators or neutrophil elastase to study these phenotypes in a nutritionally relevant context.

SCFM is presented as a tool to examine the impact of the CF lung nutritional environment on *P. aeruginosa* physiology and gene expression. One important utility of this medium is the capacity for manipulation of components used in this study to demonstrate the impact of aromatic amino acids on *P. aeruginosa* cell-cell signaling. The observation that nutritionally controlled CF sputum phenotypes are observed in SCFM provides advantages over complex commercially available laboratory media such as Luria-Bertani and tryptic soy broth. This is an important point, as discrepancies in experimental results, particularly in regard to *P. aeruginosa* quorum sensing and biofilm

formation, are often attributed to differing media conditions (23, 51–53, 63, 65).

While biofilm formation was not examined in this study, SCFM may have utility as a model growth substrate for *P. aeruginosa* biofilms. The carbon growth substrate has been shown to significantly impact *P. aeruginosa* biofilm formation (53); thus, SCFM provides a tool for examining the roles of specific CF sputum nutritional parameters in biofilm formation. One potential caveat to these studies is that CF sputum has an inherent viscosity provided by both high mucin content and dehydration of CF mucus (36) that is not present in SCFM. It is within this viscous mucus that *P. aeruginosa* forms microcolony structures in vivo (28, 67); thus, SCFM biofilm studies would necessitate mucin addition to mimic the natural viscosity.

It is critical to note that SCFM was developed from sputum samples provided by patients with nonexacerbating CF; thus, this medium represents a specific stage of CF lung infection. It is possible that the CF sputum nutritional environment may vary depending on the stage of disease and the clinical treatment methodologies. However, the approach of examining CF sputum nutritional content can be extended to early childhood, when *P. aeruginosa* infections are intermittent, or for individual patients before, during, and after exacerbation. Information from such in vitro studies will provide new information regarding the roles of nutritional cues in distinct stages of infection and allow for the design of focused studies to examine mechanistic details in vivo.

ACKNOWLEDGMENTS

We thank Nathan Dalleska and Anne Spain for help with cation and anion analyses of CF sputum.

This work was supported by a grant from the Cystic Fibrosis Foundation (WHITEL06G0 to M.W.). K.P. was a University of Oklahoma graduate predoctoral fellow.

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ERRATUM

Nutritional Cues Control *Pseudomonas aeruginosa* Multicellular Behavior in Cystic Fibrosis Sputum

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Volume 189, no. 22, p. 8079–8087, 2007. Page 8080, column 1, fifth paragraph, second and third lines, the parenthetical expression should read as follows: “(6.5 ml 0.2 M NaH_2PO_4 , 6.25 ml 0.2 M Na_2HPO_4 , 0.348 ml 1 M KNO_3 , 1.084 ml 0.25 M K_2SO_4 , 0.122 g NH_4Cl , 1.114 g KCl , 3.03 g NaCl , 10 mM MOPS, 779.6 ml deionized water).”