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Pseudomonas aeruginosa Syntrophy in Chronically Colonized Airways of Cystic Fibrosis Patients

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Pseudomonas aeruginosa **isolates from cystic fibrosis (CF) patients undergo remarkable phenotypic divergence over time, including loss of pigmentation, hemolysis, motility, and quorum sensing and emergence of antibiotic hypersusceptibility and/or auxotrophism. With prolonged antibiotic treatment and steady decline in lung function in chronically infected patients, the divergent characteristics associated with CF isolates have traditionally been regarded as "adapted/unusual virulence," despite the degenerative nature of these adaptations. We examined the phenotypic and genotypic diversity in clonally related isogenic strains of *P. aeruginosa* from individual CF patients. Our observations support a novel model of intra-airway pseudomonal syntrophy and accompanying loss of virulence. A 2007 calendar year collection of CF *P. aeruginosa* isolates ($n = 525$) from 103 CF patients yielded *in vitro* MICs of sulfamethoxazole-trimethoprim (SMX-TMP, which typically has no activity against *P. aeruginosa*) ranging from 0.02 to >32 μg/ml (median, 1.5). Coisolation of clonally related SMX-TMP-susceptible and -resistant *P. aeruginosa* strains from the same host was common (57%), as were isogenic coisolates with mutations in efflux gene determinants (*mexR*, *mexAB-oprM*, and *mexZ*) and genes governing DNA mismatch repair (*mutL* and *mutS*). In this cohort, complete *in vitro* growth complementation between auxotrophic and prototrophic *P. aeruginosa* isogenic strains was evident and concurrent with the coding sequence mosaicism in resistance determinants. These observations suggest that syntrophic clonal strains evolve *in situ* in an organized colonial structure. We propose that *P. aeruginosa* adopts a multicellular lifestyle in CF patients due to host selection of an energetically favorable, less-virulent microbe restricted within and symbiotic with the airway over the host's lifetime.**

Pseudomonas aeruginosa is found in many natural and domestic environments, including plants, soil, and surface water, and it is particularly prevalent in environments that are moist and contain organic material such as human or animal waste (17, 37). This opportunistic pathogen infects individuals deficient in host immunity or anatomic barriers, such as patients undergoing chemotherapy or those with skin damage due to burns (27). In patients with chronic lung diseases like cystic fibrosis (CF), *P. aeruginosa* causes airway infections that persist for months or years (7, 44). Strains from newly infected CF patients are polyclonal, while those from chronically infected individual CF patients are predominately monoclonal and unique to each patient, despite considerable intraspecific diversity (6, 10, 44, 49).

P. aeruginosa strains of environmental origin and those from acute human clinical infections are resistant to many classes of antimicrobial agents, including almost uniform resistance to sulfamethoxazole-trimethoprim (SMX-TMP) (14). In fact, SMX-TMP has been excluded from the Clinical and Laboratory Standards Institute (CLSI) *P. aeruginosa* antibiotic testing tables (see the M100 series) since 2007. However, hypersusceptibility to SMX-TMP is a notable feature of many *P. aeruginosa* isolates from CF patients, and SMX-TMP was once suggested as an oral treatment option (39, 42).

SMX-TMP is a combination of two antimicrobial agents that act synergistically against a wide variety of bacteria. SMX and TMP inhibit enzymes sequentially involved in the bacterial synthesis of tetrahydrofolic acid (THF). Reduced availability of THF inhibits *de novo* bacterial synthesis of methionine from L-homocysteine and thymidine from deoxyuridine and consequently impairs DNA synthesis and other cellular functions (13). Pseudomonal resistance to SMX-TMP in CF patients is due largely to drug efflux and to a lesser extent to the acquisition of integron cassettes carrying *dfr* and *sul* determinants (50). Köhler et al. have shown that an efflux complex encoded by *mexAB-oprM* is primarily responsible for SMX-TMP resistance in *P. aeruginosa* (22). The expression of *mexAB-oprM* is regulated by its own upstream *mexR* and other cross regulators, including *mexZ*, which is intimately involved in the regulation of another polyspecific efflux pump, MexXY-OprM (25, 33).

Microbial adaptation within a highly organized multicellular biofilm that lines the airways of patients with CF has been demonstrated by comparative genomic analysis, and this adaptation is reflected by marked differences in gene expression patterns within an organized colonial structure (8, 10, 21, 44, 45). Chronic infection of the CF airway by *P. aeruginosa* leads to evolution of adapted strains with extensive phenotypic changes that include the emergence of divergent mucoid colony morphology, loss of motility and green pigmentation, quorum-sensing mutations, and amino acid biosynthetic deficiencies (auxotrophism) (3, 10,

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19, 20, 46). Auxotrophism, in particular, is often observed in strains isolated from chronically infected patients, and these auxotrophic strains are often found to coexist with their isogenic prototrophs (organisms that are able to grow in medium with glucose as the sole nutrient) (3).

Recent genomic studies have confirmed that *P. aeruginosa* isolates from CF individuals with advanced pulmonary infections show high degrees of genomic clonality that are unique to each patient but also show marked microdiversity at the level of genomic coding integrity (7, 10, 44). In the airways of CF patients, hypermutator *P. aeruginosa* strains with "relaxed" DNA mismatch repair systems associated with mutations in *mutL* and *mutS* have been found at very high frequencies (7, 10, 44). CF *P. aeruginosa* isolates in a hypermutable state appear to depart from their single-cell replicative mode of living and show a retarded growth rate and reduced virulence, as measured both by *in vitro* and *in vivo* characteristics (7, 21). Nonsynonymous and nonsense mutations arise in genes involved in DNA repair, metabolic activities, virulence, and efflux pumps, including *mexAB* (10, 44). One pattern in particular, intraspecific mutations (mutations in *P. aeruginosa* strains derived from a single founder clone) in both regulator genes of *mexR* and/or *mexZ* and the efflux structural genes *mexAB*, can be detected in simultaneous coisolates from the same CF patient that show antibiotic hypersusceptibility (10, 49, 51).

The emergence of degenerative characteristics, such as loss of pigmentation, hemolysis, motility, prototrophism, and quorum sensing, which are associated with CF *P. aeruginosa* has long been theorized to represent host airway adaptation and intraspecific gain of unusual adapted virulence (30, 45, 46). In contrast, the frequent development of antibiotic-susceptible and/or auxotrophic isogenic strains from a unique resistant prototrophic founder strain in chronically infected CF patients who undergo prolonged antibiotic treatment and exhibit steady decline in lung function seems paradoxical. In this study, coisolation of susceptible and resistant isogenic strains which show nutritional complementation suggests intra-airway evolution of a syntrophic lifestyle exhibiting reduced virulence. The resulting pseudomonal syntrophy is selected for by the enormous pressure imposed by host defense and antimicrobial treatment.

MATERIALS AND METHODS

Bacterial strains and culture standards. Over 100 CF patients from birth to 21 years of age are cared for by the Seattle Children's Hospital CF pulmonary service annually. This study was approved by Seattle Children's Hospital Institutional Review Board as "nonhuman subject research." A total of 525 *P. aeruginosa* strains isolated from CF clinical airway secretion cultures associated with 103 patients in 2007 were evaluated anonymously. Based on the Cystic Fibrosis Foundation Clinical Practice Guidelines, routine quarterly monitoring (4 times a year) includes laboratory evaluation for assessing pulmonary status, disease progression, and response to therapy. Microbiologic studies include quantitative culture of respiratory secretions and antimicrobial susceptibility testing of pathogenic isolates, such as *Staphylococcus aureus*, *Streptococcus pneumoniae*, *Haemophilus influenzae*, *P. aeruginosa*, *Stenotrophomonas maltophilia*, *Burkholderia cepacia*, and *Achromobacter* spp. (5). Isolates of *P. aeruginosa* are maximally separated with continued subculturing for visually homogeneous growth of a single colony type. Plated on various selective and nonselective agar media, mixtures of organisms are maximally separated and isolated based on colony morphology (metallic sheen, roughness, colony size, and texture consistency), hemolysis, soluble or nonsoluble pigment production, and key biochemical reactivity patterns (5, 35). Molecular methods using real-time PCR for species identification, as well as 16S rRNA partial sequencing, are used for effective and accurate resolution of bacterial isolates to the species level (5, 35; X. Qin, unpublished methods). All 525 *P. aeruginosa* isolates included in this study were able to grow on standard laboratory agar media, such as sheep blood agar, chocolate agar, and MacConkey agar (Remel, United States). *P. aeruginosa* ATCC 27853, PA-mucQC (an established laboratory mucoid CF *P. aeruginosa* isolate), and *Escherichia coli* ATCC 25922 were used as controls for MIC or disc diffusion reference ranges on Mueller-Hinton (MH) agar. UCBPP-PA14 (PA14) and its transposon insertion mutants PA14 (*mexR*), PA14 (*mexA*), PA14 (*oprM*), and PA14 (*mexZ*) were also included as *P. aeruginosa* control strains. PA14 is a reference clinical isolate that originated from a burn patient; its fully sequenced genome was completed in 2006, and its transposon library has also been made available (http://ausubellab.mgh.harvard.edu/cgi-bin/pa14/home.cgi) (24).

***In vitro* susceptibility testing.** All CF *P. aeruginosa* isolates were tested initially with a battery of antipseudomonal agents that included aztreonam, ceftazidime, meropenem, imipenem, ticarcillin-clavulanate, amikacin, tobramycin, ciprofloxacin, and SMX-TMP. All agents were tested by Etest MIC methodology (bioMérieux, France), except for tobramycin, which was tested by disc diffusion due to the lack of approved commercial MIC methodology for measuring this agent in high concentration ranges prior to April 2011. The MICs of sulfisoxazole (SX) (investigational use only; bioMérieux) and tobramycin (TM) were tested by the Etest strips against a subset of isolates for this study (see Table 3). MH agar plates (Remel, United States) were prepared by inoculating 0.5 McFarland saline suspensions of each isolate before the application of Etest strips or antibiotic-containing filter discs (Remel). The test plates were incubated at 35°C in ambient air for 18 to 24 h for the determination of MIC or zone-of-inhibition results. According to the Clinical and Laboratory Standards Institute (CLSI) document M100-S16 (7a), isolates with SMX-TMP MICs of ≤2 μg/ml were considered susceptible and those with MICs of ≥4 μg/ml were considered resistant.

Studies of bacterial growth and auxotrophism. Nutrient minimal agar medium M9 (Teknova, Hollister, CA) containing glucose as the only carbon source was used for all nutritional studies of auxotrophism in the *P. aeruginosa* isolates. Each *P. aeruginosa* isolate, at 0.5 McFarland concentration ($\sim 1 \times 10^7$), was confluently swabbed onto an M9 agar plate. Sterile blank filter paper discs (6-mm diameter; Becton, Dickinson and Company, United States) containing 5 μl of a specific nutritional compound were used for evaluation of growth requirements. Nutritionally dependent growth of *P. aeruginosa* isolates was checked daily for a maximum of 14 days, with the first 48 h of incubation at 35°C and the remaining 12 days at room temperature (~25°C). The agar plates were wrapped in plastic bags to minimize the desiccation of agar. Common biochemically active cofactors, such as heme (Remel), menadione, and thymidine, were used for growth studies as previously described (2). Preparation of amino acid solutions was as follows. Amino acid powders (Fluka BioChemika, Steinheim, Switzerland), including L-alanine, L-arginine hydrochloride, L-asparagine, L-aspartic acid, L-cysteine, L-cystine, L-glutamic acid, L-glutamine, glycine, L-histidine hydrochloride, L-4-hydroxyproline, L-isoleucine, L-leucine, L-lysine hydrochloride, L-methionine, L-phenylalanine, L-proline, L-serine, L-threonine, L-tryptophan, L-tyrosine, and L-valine, were diluted to a final concentration of 0.1 M. Amino acid solutions were prepared using molecular-grade water (Fisher Scientific, Fairlawn NJ), with the following exceptions for the purpose of increased solubility: L-aspartic acid was prepared with 0.1 N HCl, L-tryptophan with 0.5 N HCl, L-cystine and L-tyrosine with 1 N HCl, and L-glutamic acid with 2 N HCl. All amino acid solutions were stored at −20°C. Methionine and arginine were used for the first round of auxotrophic screening. For other specific deficiencies or possible multiple amino acid deficiencies, a pooled solution of all 22 amino acids and a series of "pool minus one" solutions (where each solution contained all the amino acids except one) were tested.

Auxotrophic complementation of the nutritionally deficient strains by prototrophic "feeder" strains included either isogenic strains from the

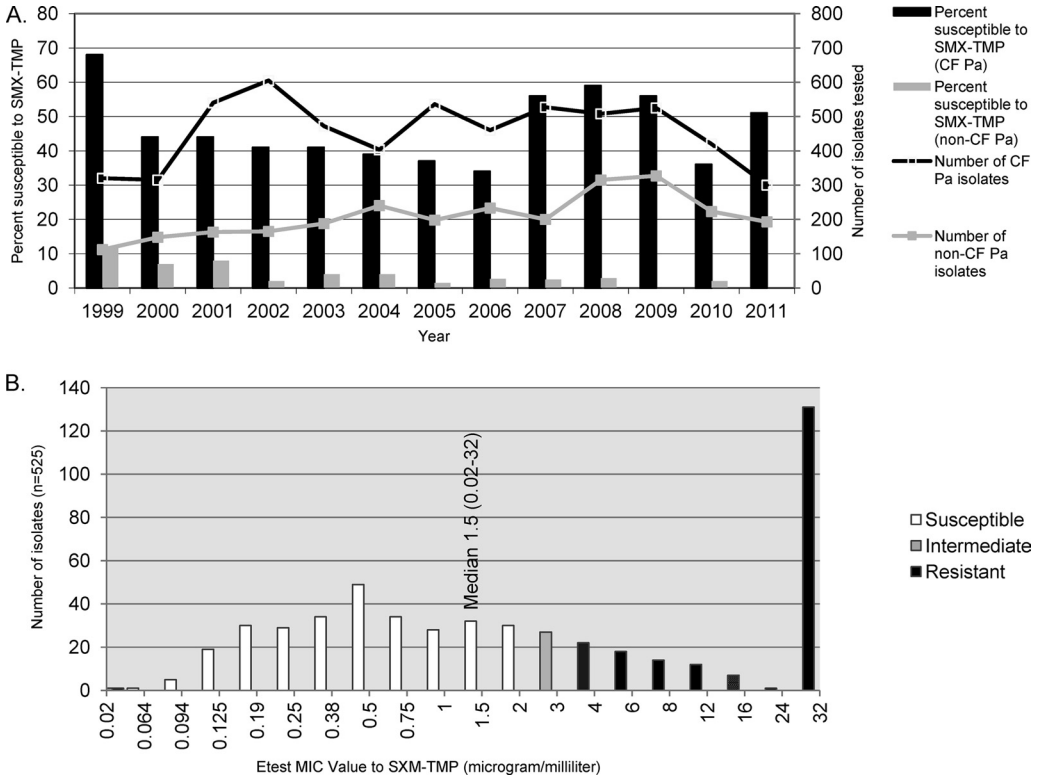

FIG 1 (A) *P. aeruginosa* susceptibility to sulfamethoxazole-trimethoprim (Seattle Children's Hospital data). (B) Sulfamethoxazole-trimethoprim MIC distribution in CF *P. aeruginosa* isolates from 2007.

same patient or nonisogenic strains from unrelated patients and quality control (QC) wild-type *P. aeruginosa* strains (ATCC 27853, PA-mucQC, and PA14) based on their growth in M9 medium. Similar to amino acid and cofactor growth complementation tests, the feeder strains were prepared at a McFarland concentration of 0.5 and 5 μl of the preparation was used on each blank filter paper disc for growth rescue of the auxotrophic isolates.

Molecular amplification and sequencing of *mexR*, *mexAB-oprM*, *mexZ*, *mutL*, and *mutS*. Primers for amplification and sequencing of the pertinent genetic determinants were optimally selected by using Primer3 (http://frodo.wi.mit.edu/primer3/). The gene coding regions of *mexR*, *mexAB-oprM*, *mutL*, and *mutS* were amplified, and some were fully sequenced. DNA sequencing was carried out using a BigDye terminator cycle sequencing ready reaction DNA sequencing kit and an ABI Prism 310 genetic analyzer (Applied Biosystems). The sequencing thermocycling steps used were 96°C for 30 s, 25 cycles of 96°C for 10 s, and 60°C for 10 s. Sequencing chromatographs generated from both forward and reverse strands of the PCR template were edited using Seqman, DNASTAR (DNASTAR, Inc.).

PCR product cloning and transformation. Cloning of heterogeneous *mexAB* PCR product was carried out by gel purification of an ~1,500-bp amplification product containing 131 bp of the 3′ end of *mexA* and 1,341 bp of the 5′ half of the *mexB* from all isolates, followed by plasmid ligation and transformation into *E. coli* Qiagen EZ competent cells (Qiagen, Germany). Heterogeneous *mexAB* products were successfully cloned from two isolates, PA-A5 (four clones) and PA-B8 (two clones) (see Fig. S1 in the supplemental material). The *E. coli* transformants were selected on Luria-Bertani (LB) agar containing ampicillin (100 μg/ml) (Remel, United States). Plasmid DNA was extracted from the *E. coli* transformants by using a QIAprep spin miniprep kit (Qiagen, Germany) and sequenced individually with dense primer coverage for each cloned product.

Bacterial genome typing for clonality. Genetic DNA fingerprinting of *P. aeruginosa* strains was generated by pulsed-field gel electrophoresis (PFGE) using SpeI restriction fragmentation (service provided by IEH Laboratories & Consulting Group, Lake Forest Park, WA, United States).

RESULTS

***P. aeruginosa* clinical isolates and their patterns of susceptibility to SMX-TMP.** We examined the antimicrobial susceptibilities in 525 archived CF *P. aeruginosa* isolates collected from 103 patients in 2007. A subset of 498 (95%) isolates was obtained from 76 patients who had an average of 3 cultures per patient over the year with a median of 2 (range, 2 to 24) isolates per patient. The remaining 27 *P. aeruginosa* isolates were obtained from single-isolate cultures from 27 patients.

Antimicrobial susceptibility results generated from isolates of CF patients have been analyzed separately from those of non-CF patients in our hospital over the last 13 years using CLSI M100-S16 interpretive criteria (7a). The *P. aeruginosa* pseudomonal susceptibility results demonstrated overall higher rates of resistance to beta-lactams, aminoglycosides, and quinolones than did the results for *P. aeruginosa* isolates of non-CF origin (data not shown). However, an unusual proportion of CF *P. aeruginosa* isolates (34 to 68% from 1999 to 2011) was susceptible to SMX-TMP (Fig. 1A).

We observed a wide range of *in vitro* MICs of many antimicrobial agents among the 525 CF *P. aeruginosa* isolates, including SMX-TMP (Fig. 1B and unpublished data). SMX-TMP MICs ranged from 0.02 to ≥32 μg/ml (median, 1.5 μg/ml) using Etest methodology (Fig. 1B). Of these 525 isolates, 293 (56%) showed SMX-TMP MICs in the susceptibility range of ≤2 μg/ml, with a median of 0.38 μg/ml. The distribution of SMX-TMP MICs generated by the 525 *P. aeruginosa* isolates is nearly continuous in the

TABLE 1 Groups of patient based on the number(s) of *P. aeruginosa* isolates with divergent SMX-TMP MICs

Patient group[a]	No. (%) of patients with[b]:		
	Coexisting isolates exhibiting S/I/R SMX-TMP MICs (0.002–32 μg/ml)	Isolate(s) exhibiting S/I SMX-TMP MICs (≤3 μg/ml)	Isolate(s) exhibiting R SMX-TMP MICs only (≥4 μg/ml)
Two or more isolates ($n = 76$)	43 (57)	21 (28)	12 (16)
Single isolate ($n = 27$)	NA	15 (56)	12 (44)
All patients ($n = 103$)	43 (42)	36 (35)	24 (23)

[a] Patients are grouped based on cultures of their respiratory secretions and the number of *P. aeruginosa* isolates obtained in 2007 with divergent SMX-TMP MICs using CLSI M100-S6 breakpoints.
[b] S, susceptible, I, intermediate; R, resistant; NA, not applicable .

concentration range measurable by Etest (Fig. 1B). Coexisting *P. aeruginosa* isolates exhibiting a wide range of SMX-TMP MICs (0.02 to ≥32 μg/ml) were present in CF airway secretions from 43 patients (57%) (Table 1). Vector-borne and integron-associated SMX-TMP resistance was excluded by negative *int1* and *sul1* amplification results obtained from these 525 *P. aeruginosa* isolates after they were divided into smaller sample pools (5 isolates in each PCR mix).

Coisolation of patient-specific clonal *P. aeruginosa* strains with divergent susceptibilities to SMX-TMP. In order to understand the resistance and functional divergence associated with potentially chronically colonized patients, three sets of *P. aeruginosa* isolates ($n = 27$) with widely divergent SMX-TMP MICs from three patients (patients A, B, and C) were chosen for further molecular analysis and *in vitro* nutrient requirement studies (Table 2). In each set, organisms had been isolated from three to five sputum cultures collected over 2007. Two *P. aeruginosa* strains, *P. aeruginosa* ATCC 27853 and PA-mucQC (a laboratory-validated mucoid CF *P. aeruginosa* type strain), were used as susceptibility testing controls, and both consistently showed SMX-TMP MICs of ≥32 μg/ml and correlated sulfisoxazole (SX) MICs of ≥1,024 μg/ml. Strain PA14 and its corresponding *mex* gene-inactivated derivatives were used as additional controls for SMX-TMP MIC determinations (24). The MICs for PA14 mutational derivatives correlated with the functional predictions of their mutated genes; *mexR* mutation resulted in an elevated SMX-TMP MIC of ≥32 μg/ml (the PA14 wild-type MIC is 16 μg/ml), while *mexA* and *oprM* mutations resulted in significantly reduced of SMX-TMP MICs (0.19 to 0.25 μg/ml) (Table 2). For both control and patient isolates, TMP alone showed no activity against any of the *P. aeruginosa* isolates regardless of whether the isolate was susceptible to SX, although TMP appeared to have a synergistic effect *in vitro*, as was evident from MIC and spatial antibiotic disc approximation in the 10- to 15-mm range, suggesting that the folic acid biosynthetic pathway was intact (Table 2 and Fig. 2).

Divergent antimicrobial susceptibilities to our standard panel of antimicrobial agents were observed in these 27 CF *P. aeruginosa* isolates, as shown by wide MIC distributions (Table 3). The SMX-TMP susceptibilities exhibited by CF *P. aeruginosa* isolates correlated closely with their susceptibilities to SX alone (see the results for PA-A1 to PA-C7 in Table 2). In addition, antimicrobial pan-susceptible strains were found in all three patients (see the results for PA-A2, -A3, -B6, and -C1 in Table 3). Despite divergence in resistance patterns, isolates of *P. aeruginosa* from a chronically infected individual are often closely related based on DNA fingerprinting patterns, which was confirmed by representative isolates from these three patients (Fig. 3) (6–8).

Characterization of critical mutations in efflux determinants *mexR*, *mexAB-oprM*, and *mexZ*, as well as the bacterial hypermutable state associated with *mutL* and *mutS* inactivation. The 27 *P. aeruginosa* isolates with various MICs to SMX-TMP from the three selected patients were subjected to amplification and sequence examination, focusing primarily on *mexR*, *mexAB-oprM*, and *mexZ*, as well as on *mutL* and *mutS* coding regions. Among the isolates, we identified critical mutations in efflux determinants, such as *mexR*, *mexAB*, and *mexZ*, as well as bacterial hypermutable states associated with *mutL* and *mutS* inactivation. These results are summarized in Table 2. Despite the patient-specific clonal nature of these isolates, it is evident that their genetic integrity has undergone a process of erosion, as shown by deletions and insertions, as well as single-nucleotide polymorphisms, in *mexR*, *mexAB*, *mexZ*, *mutL*, and *mutS*, but not in *oprM* (affected only by a few silent mutations; data not shown). The patterns of micromutation consisted largely of small deletions/insertions resulting in frameshift and premature stop codons, as well as both synonymous (SS, or silent mutations) and nonsynonymous substitutions (NSS, or missense mutations) (Table 2; also see Fig. S1 in the supplemental material).

Molecular amplification produced seemingly homogeneous sequences for *mexR*, *oprM*, and for the most part, *mexA* from these 27 patient isolates (Table 2). In contrast, despite many *in vitro* passages for colony purification, the molecular amplification products associated with the coding sequences of *mexB*, *mexZ*, *mutL*, and *mutS* in all 27 isolates appeared to be composed of mixed or heterogeneous sequences which required cloning for resolution (Table 2; also see Fig. S1 in the supplemental material). Single or compound mutations, either in efflux regulatory genes such as *mexR* (or *mexZ*) or structural gene(s) such as *mexAB* or in both, were common in these isolates, which serves to explain their intraspecific divergent and continuous SMX-TMP MIC distribution (Table 2). For example, the three SMX-TMP-resistant strains (isolates PA-A2, PA-A4 to -A7, and PA-A9 to -A11) from patient A all contained a critical single base G deletion (Table 2, ΔG near 5′) that caused a frameshift resulting in an immediate premature termination codon in *mexR*, a well-studied negative regulator that controls *mexAB-oprM* expression (33, 49). In patient B, while *mexR* remained intact in all nine isolates (PA-B1 to -B9), a 7-bp deletion near the 5′ end of *mexA* (Table 2, Δ7 bp near the 5′) which was identical in each instance was found in three isolates (PA-B4, -B5, and -B8) that showed significantly reduced MICs to SMX-TMP. Compound mutations involving *mexR*, *mexZ*, and/or *mexAB* were common to all isolates (Table 2; also see Fig. S1). Moreover, mutations associated with the *mex* gene determinants clearly showed specific patterns indicative of isogenic origin associated with each patient (Table 2, ΔG near the 5′ in *mexR* and Δ7 bp near the 5′ in *mexA*, associated with isolates from patient A and B, respectively). Such massive *mex* gene sequence degeneration has led us to hypothesize that pseudomonal isogenic divergence is not limited to antimicrobial susceptibility alone. Indeed, heterogeneous mutations found simultaneously in genes that govern

TABLE 2 Characteristics of the 27 clinical *P. aeruginosa* strains isolated from cultures in 2007[a]

Isolate	Muc/pig[b]	MIC (μg/ml)		Amino acid auxotrophy	Growth complementation by other *P. aeruginosa* strains	Sequencing result[c]					
		SMX-TMP	SX			*mexR*	*mexA*	*mexB*	*mexZ*	*mutL*	*mutS*
PA-ATCC	N/g	>32	>1,024	Prototroph	NA						
PAmucQC	M/n	>32	>1,024	Prototroph	NA						
PA14	N/g	16	128	Prototroph	NA						
PA14 (*mexR*)	N/g	>32	>1,024	Prototroph	NA	*mexR*					
PA14 (*mexA*)	N/g	0.190	6	Prototroph	NA		*mexA*				
PA14 (*oprM*)	N/g	0.250	6	Prototroph	NA						
PA14 (*mexZ*)	N/g	>32	>1,024	Prototroph	NA				*mexZ*		
PA-A1	N/n	0.75	16	Lys, met	A3, B1, B3, B4, C6	WT	Δ18 bp near 3′ end	m	NSS	m	Δ12 bp near 3′ end
PA-A2	N/g	0.25	3	Arg	A3, B4, C6	ΔG near 5′ end	WT	m	m	m	m
PA-A3	N/n	1	48	Prototroph	NA	WT	WT	m	m	m	m
PA-A4[d]	N/g	0.5	12	Arg	A3, B4, C6	ΔG near 5′ end	WT	m	m	m	m
PA-A5[d]	M/n	0.38	12	Lys, met	C6, PAmucQC	ΔG near 5′ end	Hetero: SS & NSS	Hetero: SS & NSS	m	m	m
PA-A6[d]	N/g	>32	>1,024	Arg	All prototrophs	ΔG & Δ10 bp	WT	m	m	m	m
PA-A7[d]	N/n	>32	>1,024	Arg	All prototrophs	ΔG near 5′ end	WT	m	m	m	m
PA-A8	N/n	0.5	3	Unknown	A3, B1, B3, B4, C6	WT	WT	m	m	m	m
PA-A9[d]	N/g	>32	>1,024	Arg	All prototrophs	ΔG near 5′ end	WT	m	m	m	m
PA-A10	N/n	0.5	12	Arg	All prototrophs	ΔG near 5′ end	WT	m	m	m	m
PA-A11	M/n	0.5	16	Arg	All prototrophs	ΔG near 5′ end	WT	m	m	m	m
PA-B1	N/n	>32	>1,024	Prototroph	NA	WT	WT	m	Missing	m	m
PA-B2	M/n	8	64	Prototroph	NA	WT	WT	m	m	m	m
PA-B3	N/g	3	24	Prototroph	NA	WT	WT	m	m	m	m
PA-B4	N/n	4	512	Prototroph	NA	WT	Δ7 bp near 5′ end	m	m	m	m
PA-B5	N/n	1	12	Ile, val	A3, B4	WT	Δ7 bp near 5′ end	m	m	m	Δ12 bp near 5′ end
PA-B6	N/n	3	64	Ala, trp	A3, B4	WT	WT	m	m	m	m
PA-B7	M/g	6	48	Prototroph	NA	WT	WT	m	m	m	m
PA-B8	N/n	0.75	32	Ile, leu, val	A3, B4	WT	Δ7 bp near 5′ end	Hetero: SS & NSS, bp+	m	m	Δ12 bp near 5′ end
PA-B9	M/n	>32	>1,024	Prototroph	NA	WT	WT	m	m	m	m
PA-C1	M/n	0.750	16	Met	All prototrophs	WT	WT	m	6 bp+ & NSS	WT	m
PA-C2	N/n	>32	>1,024	Arg	C6, A3, B1, B4	WT	WT	m	m	ΔT near 5′ end	m
PA-C3	M/n	2.000	32	Met	All prototrophs	WT	WT	m	m	WT	m
PA-C4	N/n	>32	>1,024	Arg	C6, A3, B1, B4	WT	WT	m	m	ΔT near 5′ end	m
PA-C5	M/n	1.500	48	Met	All prototrophs	WT	WT	m	m	WT	m
PA-C6[d]	N/n	>32	>1,024	Prototroph[d]	NA	WT	WT	m	m	ΔT near 5′ end	m
PA-C7	N/n	0.380	32	Arg, leu	C6, B4	WT	WT	m	m	ΔT near 5′ end	m

[a] Summary of the SMX-TMP and SX MICs, growth properties, nutritional requirements, and sequencing results of efflux and DNA mismatch repair genes of the strains.
[b] Muc/pig, bacterial growth on agar media exhibiting mucoid (Muc) or pigmented (pig) colonies; M, mucoid; N, nonmucoid; g, green pigment; n, no pigment.
[c] WT, wild type, discounting any synonymous point mutation that resulted in no amino acid change; NA, not applicable; SS, a synonymous mutation due to a nucleotide mutation that resulted in no amino acid substitution; NSS, a nonsynonymous mutation due to a nucleotide mutation that resulted in amino acid substitution; Δ bp, deletion of indicated number of base pair(s); ΔT or ΔG, deletion of indicated nucleotide; bp+, base pair insertion; m, PCR-amplified materials that were mixed or heterogeneous; Hetero, cloned sequences show heterogeneity.
[d] Isolate shows show nonhomogeneous colonies under ×60 stereoscope.

bacterial hypermutable states, namely, *mutL* and *mutS*, further support the concept that organized gene decay is associated with the process of pseudomonal symbiotic adaptation selected for by the persistent CF airway environment (Table 2).

Amino acid auxotrophism in CF *P. aeruginosa*. Compensatory effects of a multicellular mode of living are likely to allow nucleotide mismatch during DNA polymerization, which in turn may promote rapid intraspecific diversification within the colony (7, 10). Isolates of CF *P. aeruginosa* are well known to exhibit extraordinary *in vitro* growth and morphological divergence over the course of chronic infection (Tables 2 and 3 and Fig. 4) (21, 44). Typical *P. aeruginosa* isolates from chronically infected CF patient are mostly less or nonpigmented colonies with variable mucoid presentation and generally show a small colony phenotype resulting from a retarded growth rate on any nutrient medium, including Mueller-Hinton agar (see all seven isolates from patient C in Fig. 4). Nutritional deficiency, specifically amino acid auxotrophism, is common in CF *P. aeruginosa* isolates (3). Among the 27 isolates from the three patients, 19 were auxotrophic, as shown by their inability to grow on M9 medium containing only a simple

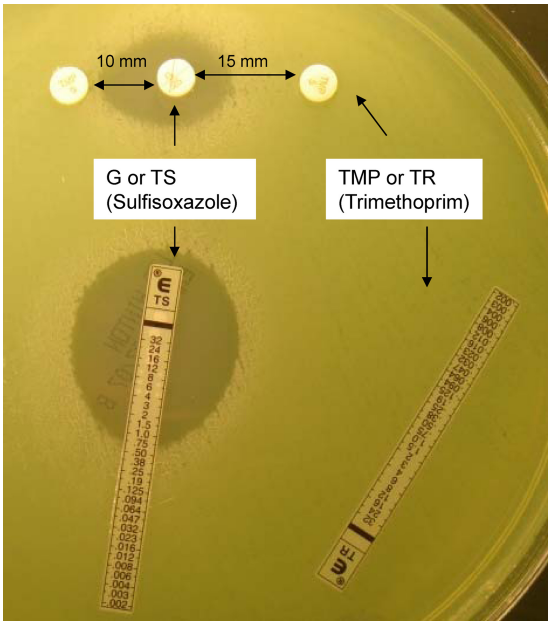

FIG 2 An example of a CF *P. aeruginosa* isolate tested for its susceptibility to SMX-TMP and to TMP or sulfisoxazole alone. A green-pigmented CF *P. aeruginosa* isolate is showing susceptibility to SMX-TMP (TS) at a MIC of 0.75 μg/ml by Etest and to TMP (TR) alone at a MIC of >32 μg/ml. Disc diffusion with sulfisoxazole (G, 500 μg) in the top center approximated by TMP (5 μg) discs measured at 10 mm to the left and 15 mm to the right.

sugar, glucose (Table 2). The remaining eight isolates (at least one from each patient) and all control organisms, including PA14 and its altered *mex* gene derivatives, were able to grow on M9 minimal medium (prototrophic). In isolates from patient A, a growth requirement for arginine alone was found in seven isolates (PA-A2, -A4, -A6, -A7, and -A9 to -A11) and for both lysine and methionine in two isolates (PA-A1 and -A5). In isolates from patient B, a growth requirement for both alanine and tryptophan was found in one (PA-B6), for both isoleucine and valine in one (PA-B5), and for isoleucine, leucine, and valine in one (PA-B8). For isolates from patient C, a growth requirement for methionine alone was found in three isolates (PA-C1, -C3, and -C5), for arginine alone in two (PA-C2 and -C4), and for both arginine and leucine in one (PA-C7). To rule out oxidative respiration deficiencies and/or folic acid biosynthetic deficiencies which may result in the bacterial small colony variant (SCV) phenotype and antimicrobial resistance to SMX-TMP, all 19 auxotrophic *P. aeruginosa* isolates were screened and determined not to require thymidine, heme, or menadione for growth. Moreover, no association was found between SMX-TMP susceptibility and amino acid auxotrophism, nor was there correlation with any other *in vitro* growth morphological phenotype (Table 2).

Complementation of auxotrophism by isogenic and nonisogenic *P. aeruginosa* prototrophic isolates. Intraspecific morphological, metabolic, and genetic heterogeneity in chronic CF isolates all suggest that growth complementation by isogenic and nonisogenic *P. aeruginosa* prototrophic isolates may reflect pseudomonal division of labor and a primitive multicellular lifestyle (36, 43). Prototrophic feeder strain(s) from each patient (Table 2) were tested on blank filter discs for growth complementation of the auxotrophic strains inoculated onto M9 minimal medium. Growth complementation was determined both by visual (Fig. 5A to D) and stereoscopic (Fig. 5E and F; ×30 magnification) examination. Auxotrophic isolates deficient in various specific amino acids from each patient were complemented by at least one of the prototrophic isogenic isolates as a feeder from the same patient, except for PA-A5. Some auxotrophs were complemented by the prototrophic nonisogenic feeder isolates from unrelated CF patients or even from control organisms with prolonged incubation (Table 2). The wild-type ATCC 27853 and PA14 appeared to be ineffective as feeder strains for growth complementation for many of the auxotrophs even with prolonged incubation (Fig. 5A and B). Most of the isogenic feeder effects were readily visible and diffusible into a radius ranging up to ~15 mm within 7 days (Fig. 5C and D). The growth patterns with feeder complementation were highly diverse, as satellite colonies of the auxotrophs grown around the feeders showed integration with the feeder strain in pairs between isogenic strains (Fig. 5C and E) or showed reverse density radiating from the feeder strain in pairs between nonisogenic strains (Fig. 5D and F). The latter pattern was prominent during early examination (e.g., <7 days after plating). Moreover, the deterrence of growth of wild-type PA14 by contact inhibition in the presence of CF *P. aeruginosa*, such as by the auxotrophic PA-C4 on M9 medium, was apparent after 14 days of incubation (Fig. 5G). Nonhomogeneous growth on M9 medium was rather common among several patient strains (PA-C6, -A4, -A5, -A6, -A7, and -A9) and could be resolved only by stereoscopy at ×60 magnification (Table 2 and Fig. 5H). In addition, for isolates associated with patient C, none of the auxotrophic isolates deficient for arginine (PA-C2 and -C4) or methionine (PA-C1 and -C3) alone were able to grow on M9 medium, whereas either a mixture of PA-C1 and PA-C2 or a mixture of PA-C3 and PA-C4 was able to grow on M9 with prolonged incubation at room temperature for up to 14 days. *In vitro* nutrient and growth complementation between isogenic strains suggests pseudomonal development of a multicellular codependent lifestyle during the course of chronic infection in the CF airway.

DISCUSSION

The rates of antimicrobial resistance of CF *P. aeruginosa* strains reported in the past have been consistently higher than those of their non-CF counterparts (32, 47). Most reports used MIC aggregates generated from a cohort of CF clinical isolates grouped solely by interpretive MIC (or diameter of inhibition zone) criteria, which were established primarily for non-CF bacterial infections, and were expressed as "percent susceptible" rather than by MIC distribution. This clinical practice hinders appreciation of the concept that pseudomonal chronic infection in the CF airway results in a resistant syntrophy. This bacterial syntrophy is composed of a less free-living, less virulent, and intraclonally highly divergent bacterial population developed in each patient over his or her life time. Since environmental and non-CF *P. aeruginosa* strains are almost invariably SMX-TMP resistant, SMX-TMP-susceptible strains of *P. aeruginosa* must arise from a resistant parental clone via mutation. The wide range and continuous distribution of *in vitro* SMX-TMP MICs (similar MIC distribution patterns apply to all other agents tested; data not shown) in this *P. aeruginosa* collection confirmed that this resistance is not due to a single acquired determinant, such as integron-associated *sul*, which results in an "all-or-none" resistance pattern. Instead, our findings are consistent with independent, patient-specific clonal

TABLE 3 Antimicrobial susceptibilities of the *P. aeruginosa* control isolates and 27 patient isolates measured by Etest

Isolate	MIC (μg/ml)[a]									
	SMX-TMP	SX	TLc	AT	TZ	MP	IP	AK	TM	CI
PA-ATCC 27853	>32	>1,024	16	16	1.5	0.25	2	3	0.5	0.19
PAmucQC	>32	>1,024								
PA14	16	128	16	3	1	0.125	1	2	0.38	0.094
PA14 (*mexR*)	>32	>1,024	48	16	3	1.5	1.5	3	0.5	0.38
PA14 (*mexA*)	0.190	6	0.38	0.19	0.5	0.047	0.75	1.5	0.38	0.032
PA14 (*oprM*)	0.250	6	0.5	0.25	0.75	0.047	1	1.5	0.38	0.016
PA14 (*mexZ*)	>32	>1,024								
PA-A1	0.75	16	>256	16	>256	0.5	1.5	>256	64	0.5
PA-A2	0.25	3	1.5	0.25	0.75	0.094	1.5	16	2	0.38
PA-A3	1	48	0.38	0.19	0.75	0.032	0.5	12	1.5	1
PA-A4	0.5	12	1.5	0.5	1	0.064	1.5	48	4	0.094
PA-A5	0.38	12	0.75	0.75	0.5	0.064	0.38	48	3	0.38
PA-A6	>32	>1,024	>256	12	3	>32	12	32	3	0.75
PA-A7	>32	>1,024	>256	12	3	0.5	1.5	32	4	0.38
PA-A8	0.5	3	96	12	2	3	>32	>256	24	4
PA-A9	>32	>1,024	>256	12	2	>32	>32	32	3	0.19
PA-A10	0.5	12	1.5	0.5	1.5	8	1.5	>256	8	0.38
PA-A11	0.5	16	0.5	0.19	0.5	0.064	0.38	>256	4	0.25
PA-B1	>32	>1,024	16	4	2	0.75	3	2	0.38	0.125
PA-B2	8	64	8	3	4	0.19	0.75	32	3	0.5
PA-B3	3	24	3	0.25	1.5	0.094	2	32	4	0.75
PA-B4	4	512	16	4	2	0.25	2	32	8	0.38
PA-B5	1	12	0.5	0.25	2	0.064	0.38	>256	32	0.5
PA-B6	3	64	12	2	2	0.38	2	8	1.5	0.5
PA-B7	6	48	3	1.5	0.75	0.064	0.5	12	2	0.19
PA-B8	0.75	32	0.5	0.25	1.5	0.094	0.38	>256	32	0.5
PA-B9	>32	>1,024	8	2	3	0.19	0.75	48	4	1
PA-C1	0.750	16	1	0.19	0.75	0.19	1.5	12	0.75	0.75
PA-C2	>32	>1,024	>256	24	>256	6	>32	>256	192	16
PA-C3	2.000	32	0.5	0.5	1	1.5	>32	24	1.5	0.5
PA-C4	>32	>1,024	>256	6	>256	4	>32	>256	64	6
PA-C5	1.500	48	1.5	1	1	1.5	>32	24	1.5	0.75
PA-C6	>32	>1,024	48 F	6	6	16 f	>32	>256	>1,024	4
PA-C7	0.380	32	>256	32	>256	>32	>32	>256	192	>32

[a] SMX-TMP, sulfamethoxazole-trimethoprim; SX, sulfisoxazole; TLc, ticarcillin-clavulanate; AT, aztreonam; TZ, ceftazidime; MP, meropenem; IP, imipenem; AK, amikacin; TM, tobramycin; CI, ciprofloxacin.

divergence involving both efflux regulation and structural determinants, primarily in *mexR/mexAB-oprM* and *mexZ* (Table 2 and Fig. 1B) (33, 49).

CF *P. aeruginosa* isogenic strains studied to date have long been regarded as "pure" cultures. However, poor reproducibility of *in vitro* susceptibility results in a single set of isolates suggests phenotypic variability (12). In addition to phenotypic variability, we have for the first time demonstrated that CF *P. aeruginosa* isogenic isolates are in fact not homogeneous despite the conventional practice of repeat single-colony passages *in vitro*. This conclusion is based on both the heterogeneous growth patterns (Fig. 5H) and heterogeneous mutations associated with *mex* gene products demonstrating sequence mosaicism generated from a "single colony" (see Fig. S1 in the supplemental material). Perhaps for these reasons, correlations between *in vitro* antimicrobial testing results and *in vivo* efficacy are inadequate to guide clinical practice in management of CF infection (11). Divergent intraclonal antimicrobial resistance patterns may in part reflect the complexity of treatment strategies that are uniquely employed in CF, as both parenteral and inhaled forms of antibiotics are used to control the bacterial burden in CF airways (40). These treatments, coupled with host defense mechanisms, lead to the formation of a pseudomonal biofilm or multicellular configuration in which susceptible and resistant isogenic organisms appear to coexist. Within this colonial lifestyle, intraspecific genetic variation may confer role differentiation between the bacteria closest to the host mucosal cells (in immediate contact with host defenses and oral/parenterally administered drugs) and those closest to the airway lumen (in contact with inhaled drugs) (Fig. 6). We hypothesize that divergently resistant *P. aeruginosa* isogenic strains evolve *in situ* to create a microenvironment that protects and fosters the existence of highly susceptible isogenic strains (specialized in other colonial functions) despite prolonged antimicrobial exposure.

Isogenic *P. aeruginosa* strains with divergent SMX-TMP susceptibilities could not be distinguished from each other based on their colonial morphology, auxotrophism, or other *in vitro* growth properties. Similar to bacterial SCVs of *Staphylococcus aureus* and *Escherichia coli*, CF *P. aeruginosa* isolates often show slow growth that results in small colonies on standard agar media (Fig. 4) (18). The bacterial SCV phenotype is frequently associated with chronic infections, even in non-CF immunocompetent hosts. Bacterial SCVs are characterized by their retarded growth rate *in vitro*, due

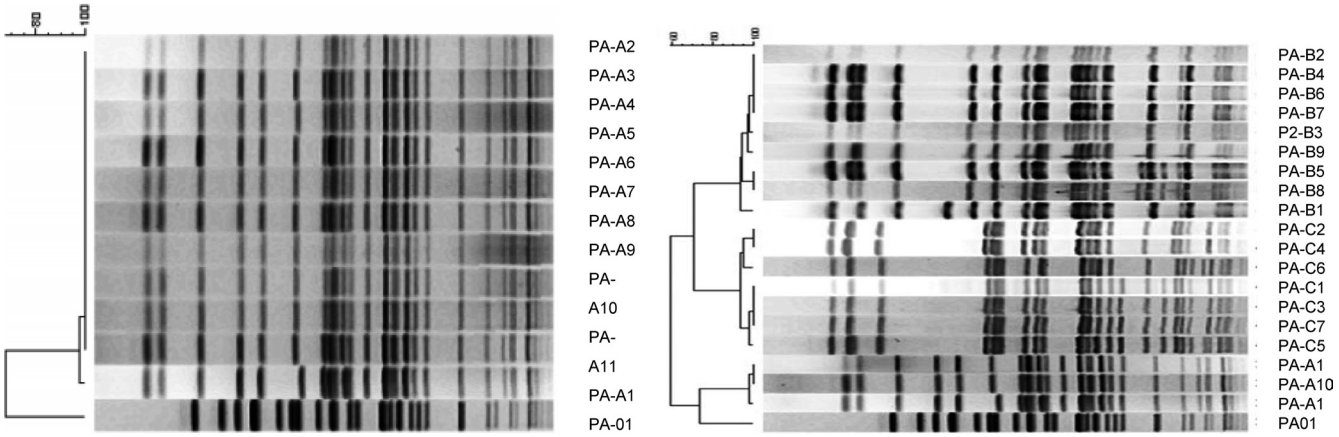

FIG 3 Genetic DNA fingerprinting patterns of *P. aeruginosa* strains from patients A, B, and C were generated by pulsed-field gel electrophoresis (PFGE) using SpeI restriction fragmentation.

in part to deficiencies in electron transport chain components and/or blockage in the folic acid biosynthetic pathway, which require not only heme or menadione but both methionine and thymidine to restore rapid growth (2, 34). However, auxotrophism in our CF *P. aeruginosa* isolates differs from other bacterial SCV phenotypes in that none of the 19 auxotrophic isolates, including those that were methionine dependent (PA-A1, -A5, -C1, -C3, and -C5), showed a thymidine, heme, or menadione requirement for growth (18). Therefore, the folic acid biosynthetic pathway appears to be intact in CF *P. aeruginosa*. Instead, multicellular metabolic and resistance interdependency maturating from a single *P. aeruginosa* clone appears to be the result of evolution under host and antimicrobial pressures and may be vital to promote long-term chronic airway colonization via symbiosis.

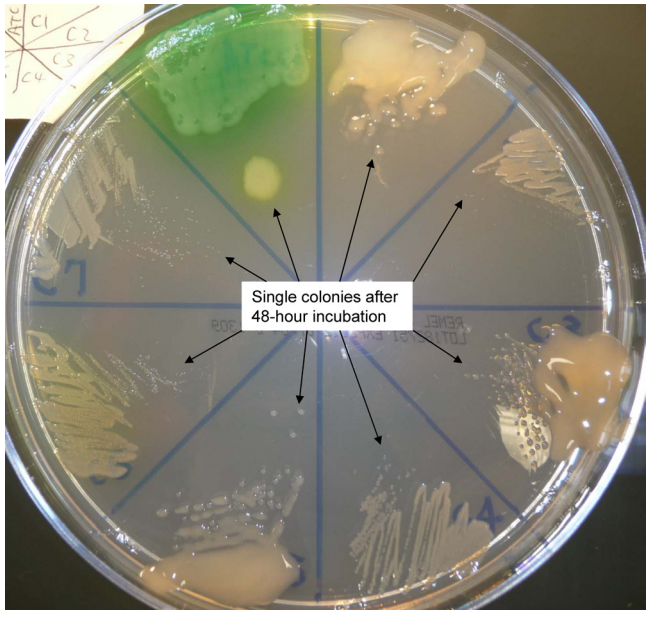

FIG 4 Comparison of growth properties of *P. aeruginosa* isolates on Mueller-Hinton agar after a full 48-h incubation in 35°C ambient air. In clockwise sequence from the green wild-type ATCC 27853 at top left, the isolates are PA-C1 (mucoid), PA-C2, PA-C3 (mucoid), PA-C4, PA-C5 (mucoid), PA-C6, and PA-C7.

Despite divergence in resistance patterns, isolates of *P. aeruginosa* from a single individual are often closely related based on DNA fingerprinting patterns (6–8). Nutritional complementation of *in vitro* growth in M9 minimal medium suggests that the survival of these auxotrophic strains is also possible *in vivo* because CF-adapted *P. aeruginosa* engages in a cooperative multicellular lifestyle. Bacterial intracolonial organization and cooperative metabolic interdependency is termed "syntrophy," and this condition can only be supported by a multicellular mode of living (23, 43). Notably, the *in situ* development of a pseudomonal conglomerate resembles a multicellular organism that is highly tolerant of host defenses and antibiotic inhibitory actions through division of labor (36, 43). Auxotrophic strains may in fact be more common than has been observed because the severe auxotrophism associated with highly adapted organisms may prohibit their viability *in vitro* and culture-based microbiology may thus underrepresent these highly adapted auxotrophs. If bacterial syntrophy is responsible for the evolution of antibiotic-susceptible and/or auxotrophic bacteria in multicellular conglomerates found in airways of CF patients, these adaptive changes must offer some advantage to *P. aeruginosa*. For example, the net result of syntrophy may be bacterial energy conservation, better resource utilization, and/or, most importantly, antimicrobial and host tolerance.

The selective loss of certain amino acid biosynthesis capabilities in CF *P. aeruginosa* cannot be explained by the nutrient abundance in the diseased airway environment. Host immunodefense must play a role in selection of pseudomonal auxotrophism and in counterselection of bacterial virulence. Amino acid auxotrophism can be found in up to 86% of adult CF patients who were colonized by *P. aeruginosa*, with arginine and methionine being the most common auxotrophisms detected (1, 3, 30). Arginine auxotrophism is directly beneficial to the adapting organism for evasion of a harsh ancient mechanism of host defense, as L-arginine is the common substrate for nitric oxide (NO) synthases and arginase (15, 26, 31, 38). Pseudomonal development of arginine auxotrophism may thus explain a highly studied but underappreciated airway immunodeficiency in CF patients, who typically show decreased concentrations of exhaled nitric oxide (NO) (16). The elimination of methionine biosynthesis in CF *P. aeruginosa* is also unlikely to be the result of random events but may instead reflect host counterselection against bacterial virulence factors and their

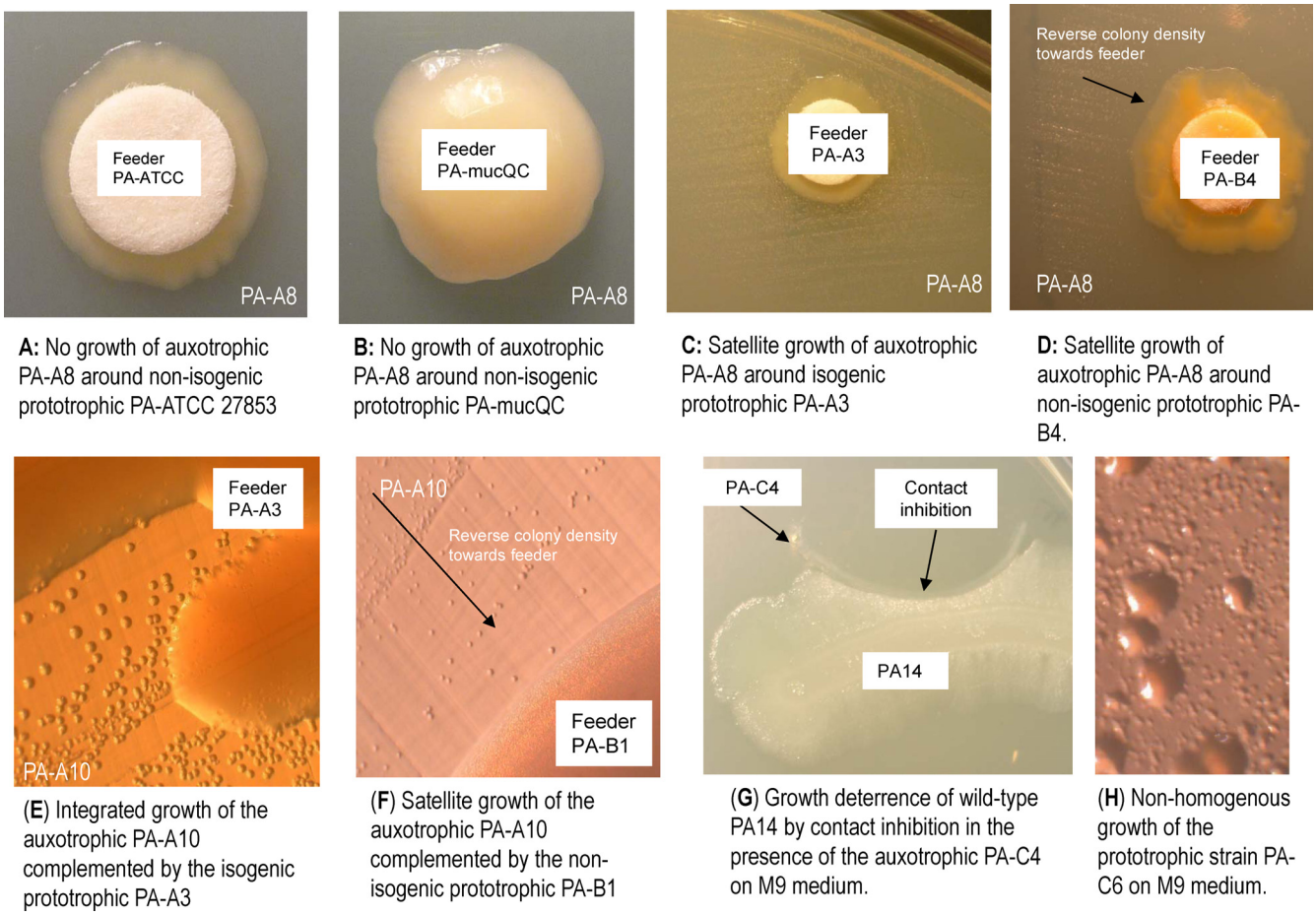

FIG 5 Growth complementation of auxotrophism by isogenic and nonisogenic *P. aeruginosa* prototrophic isolates (over a 6-mm sterile absorbent filter disc labeled "Feeder") inoculated onto M9 agar medium. (A to F) Examples of negative (A and B) and positive (C, D, E, and F) growth complementation of an auxotrophic strain by various prototrophic *P. aeruginosa* strains. (E and F) Examples of close-up views (stereoscopy at ×30 magnification) of growth complementation patterns of an auxotrophic strain by prototrophic strains that were either isogenic (E) or nonisogenic (F). (G) An example of contact inhibition of wild-type PA14 growth by the auxotrophic PA-C4. (H) An example of nonhomogeneous growth of PA-C6 on M9 agar medium (×60 magnification).

upstream regulators, such as the quorum-sensing molecule homoserine lactone and its precursor molecule methionine (19). The emergence and accumulation of quorum-sensing-negative mutants (*lasR*) in 30 to 63% of CF isolates further supports the concept of host counterselection against virulence during chronic infection (10, 20, 44). Clearly, gene retention or loss, as well as the processes by which gene decay proceeds in CF *P. aeruginosa*, cannot be random events but, rather, reflect a regulated pathway that gives rise to a spectrum of coding mosaicism and functional divergence during an unfinished pseudomonal journey to symbiosis.

Pseudomonal clonal success in the CF airway may protect the host from repeat acquisition of unrelated strains from the environment. Pseudomonal clonal discrimination and intraspecific coadaptation are supported by complete growth complementation *in vitro* between prototrophic and auxotrophic isogenic *P. aeruginosa* strains, as opposed to partial or no complementation or growth inhibition between clonally unrelated *P. aeruginosa* strains (Fig. 5D, F, and G). As they compete for the same pool of nutrient resources, free-living bacteria usually produce secondary metabolites and other weaponry, affecting both inter- and intrabacterial species (4, 48, 52). Such inhibitory properties are visible even in the presence of nutrient complementation between nonisogenic strains *in vitro*. Cellular interactions of this nature may preserve a strict *P. aeruginosa* clonal monopoly in the host airway. Thus, the absence of environmental nonisogenic strains of *P. aeruginosa* in the airway secretions of CF patients who are chronic CF *P. aeruginosa* carriers may not be explained simply by acquired host immunity to *P. aeruginosa*.

In many respects, the adaptations that occur in CF *P. aeruginosa* mimic aspects of evolving symbiosis, chiefly involving genomic degenerative patterns that are found in myriad forms of animal symbionts (29, 41). For example, the animal symbionts studied to date are in various stages of adaptation and show degrees of genome degradation typically involving *mutL* and *mutS*. Pseudomonal adaptation via multicellularity associated with CF airway chronic infection may provide a model for a common pathway by which evolution of pseudogenes and minimal genomes in ancient animal symbionts can be achieved (28). Many ancient bacterial symbionts provide their insect animal hosts with essential nutrients or ancillary benefits, such as resistance to environmental stress or natural enemies, and even affect host reproduction to favor their own matriline (9). However, during the early phases of symbiotic evolution that are possible over the

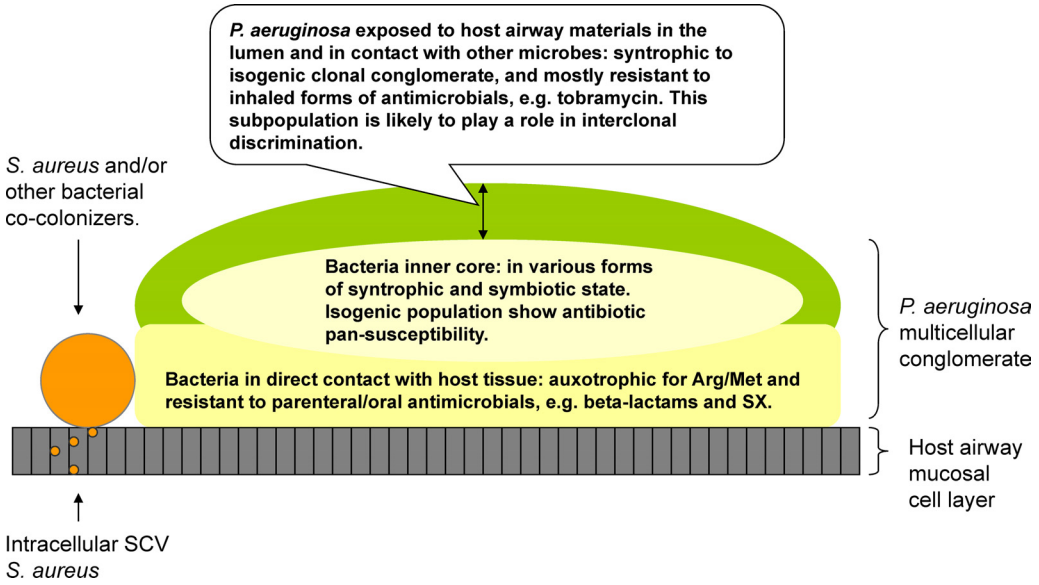

FIG 6 Diagram of CF airway-adapted *P. aeruginosa*: a multicellular syntrophy with specialized functional divergence within a colonial architecture.

life span of a CF patient, adaptation of CF *P. aeruginosa* does not prevent irreversible damage to the host airway and neither is it able to proceed further down the symbiotic pathway in order to yield benefit to the host. Moreover, the highly matured CF airway-specific *P. aeruginosa* conglomerate would have minimal chance of either horizontal or vertical transmission due to its inability to compete with wild-type *P. aeruginosa* outside the CF airway, the rarity of CF-affected hosts in the general population (1/2,500 in the Caucasian population), and a relatively short average life expectancy of these hosts, 38.3 years (http://www.cff.org/UploadedFiles/LivingWithCF/CareCenterNetwork/PatientRegistry/2010-Patient-Registry-Report.pdf).

In conclusion, pseudomonal persistence and antimicrobial resistance properties in the CF airway are consistent with a model based on the principle of bacterial syntrophy. The *in vitro* hypersusceptibility to SMX-TMP and to other antimicrobials associated with individual isogenic strains may not predict the therapeutic potential of SMX-TMP against the *P. aeruginosa* multicellular conglomerate *in vivo*. The net result is a biologically less free-living and less virulent *P. aeruginosa* strain which is selected by host defense, as this bacterial parasite possesses features symbiotic with the CF pulmonary environment. In some respects, a uniclonal and highly adapted *P. aeruginosa* syntrophy in the airway of chronically infected CF patients may benefit the host by preventing the acquisition of another environmental wild-type strain, which would result in a deleterious replay of the infectious process in the same host airway. These traits suggest that genetic manipulation of CF pulmonary *P. aeruginosa* into a probiotic symbiont, as opposed to eradication of the organism, may be an alternative therapeutic strategy in the clinical management of CF and related disorders.

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