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Tracking Polymicrobial Metabolism in Cystic Fibrosis Airways: *Pseudomonas aeruginosa* Metabolism and Physiology Are Influenced by *Rothia mucilaginosa*-Derived Metabolites

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ABSTRACT Due to a lack of effective immune clearance, the airways of cystic fibrosis patients are colonized by polymicrobial communities. One of the most widespread and destructive opportunistic pathogens is *Pseudomonas aeruginosa*; however, *P. aeruginosa* does not colonize the airways alone. Microbes that are common in the oral cavity, such as *Rothia mucilaginosa*, are also present in cystic fibrosis patient sputum and have metabolic capacities different from those of *P. aeruginosa*. Here we examine the metabolic interactions of *P. aeruginosa* and *R. mucilaginosa* using stable-isotope-assisted metabolomics. Glucose-derived ^{13}C was incorporated into glycolysis metabolites, namely, lactate and acetate, and some amino acids in *R. mucilaginosa* grown aerobically and anaerobically. The amino acid glutamate was unlabeled in the *R. mucilaginosa* supernatant but incorporated the ^{13}C label after *P. aeruginosa* was cross-fed the *R. mucilaginosa* supernatant in minimal medium and artificial-sputum medium. We provide evidence that *P. aeruginosa* utilizes *R. mucilaginosa*-produced metabolites as precursors for generation of primary metabolites, including glutamate.

IMPORTANCE *Pseudomonas aeruginosa* is a dominant and persistent cystic fibrosis pathogen. Although *P. aeruginosa* is accompanied by other microbes in the airways of cystic fibrosis patients, few cystic fibrosis studies show how *P. aeruginosa* is affected by the metabolism of other bacteria. Here, we demonstrate that *P. aeruginosa* generates primary metabolites using substrates produced by another microbe that is prevalent in the airways of cystic fibrosis patients, *Rothia mucilaginosa*. These results indicate that *P. aeruginosa* may get a metabolic boost from its microbial neighbor, which might contribute to its pathogenesis in the airways of cystic fibrosis patients.

KEYWORDS *Pseudomonas aeruginosa*, *Rothia mucilaginosa*, metabolite cross-feeding, microbial interactions, polymicrobial infections, stable-isotope-assisted metabolomics

Cystic fibrosis (CF) patients experience persistent polymicrobial colonization of their airways. *Rothia mucilaginosa* and *Pseudomonas aeruginosa* are microbes frequently detected in CF patient airways, and their cooccurrence has been observed in CF patient sputum (1–4). Microbes within polymicrobial infections display complex interactions, such as metabolite cross-feeding (5). For example, *P. aeruginosa* inefficiently metabolizes host-derived mucins. Rather, *P. aeruginosa* utilizes mucin degradation products from oral anaerobes to support its growth (6, 7). Still, many studies of CF-associated microbes are conducted under artificial conditions that fail to take into account the nutrient and oxygen gradients found in CF patient airways (8, 9, 25). The lack of overlap between laboratory conditions and CF patient airways is reflected by the differences in growth rates, with estimates of bacterial doubling times being 100-fold times lower in

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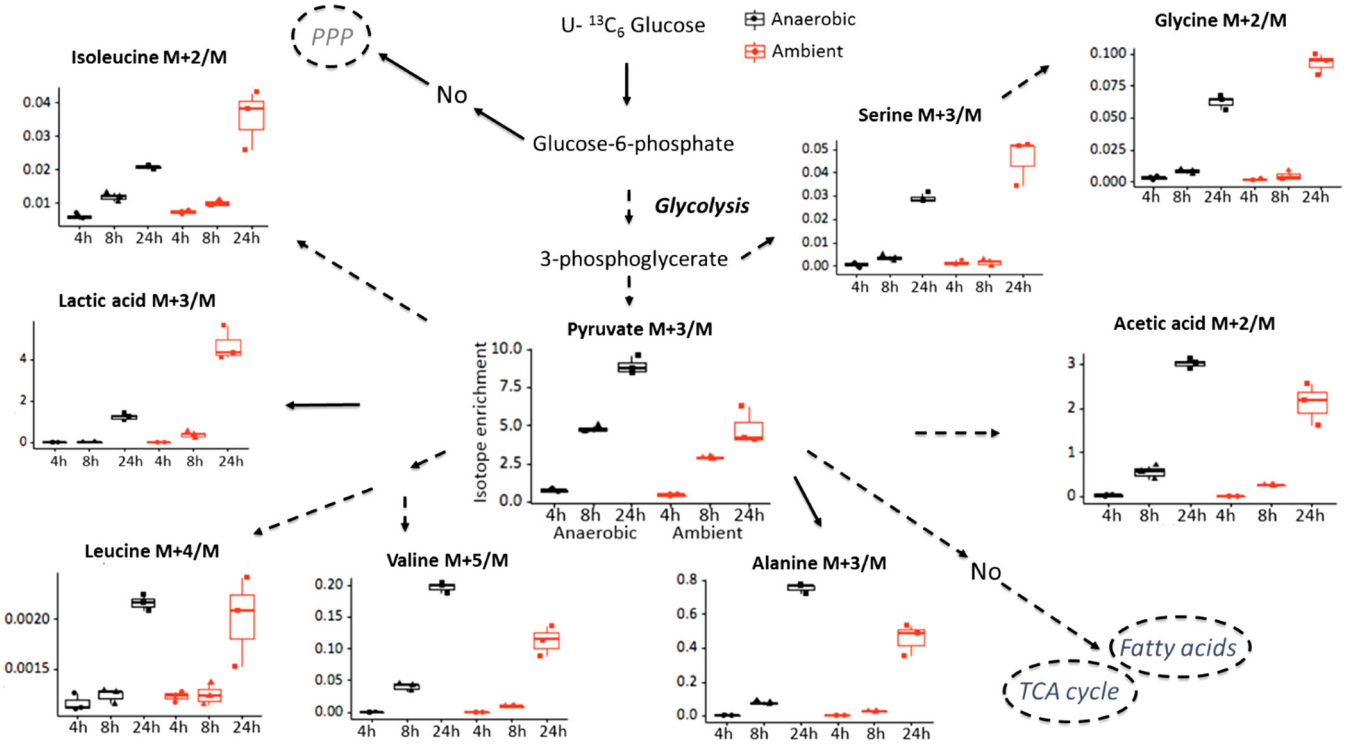

FIG 1 Glucose-derived ^{13}C was incorporated into pyruvate, lactate, acetate, alanine, valine, serine, glycine, leucine, and isoleucine in *R. mucilaginosa* under both anaerobic and ambient-oxygen conditions. M+2, M+3, M+4, and M+5 indicate compounds that contained 2, 3, 4, and 5 ^{13}C atoms, respectively. Isotope enrichment means an abundance of labeled ion/unlabeled ion (corrected for natural abundance). Isotope enrichment was greater at 24 h than at 8 h or 4 h. For pyruvate, alanine, valine, and acetate, greater isotope enrichment was observed under anaerobic conditions at 24 h. For lactate, glycine, serine, and isoleucine, greater isotope enrichment was observed under ambient-oxygen conditions at 24 h. The incorporation of glucose-derived ^{13}C into leucine biosynthesis was not affected by oxygen conditions. Dashed lines and solid lines indicate multiple steps and one metabolic step(s) needed to obtain the metabolite, respectively. Error bars, means ± standard deviations (SD) (n = 3 bacterial cultures per group); TCA, citric acid cycle; PPP, pentose phosphate pathway.

sputum than in standard medium (10). Furthermore, most CF studies focus on single microbes. One primary reason for this is the lack of a robust model to examine the microbial interactions. Stable-isotope-assisted metabolomics analyzes the fate of heavy atoms from stable-isotope-labeled precursors to products, which makes it a suitable approach for monitoring metabolites produced by one microbe when cross-fed to a second microbe. In order to further explore cross-feeding interactions between two CF microbes in a relevant environment, we cross-fed labeled glycolysis products from *R. mucilaginosa* to *P. aeruginosa* (8). Both strains were isolated from the sputa of CF patients. We believe that our *P. aeruginosa* strain is representative of CF strains, as its core genome is similar to that of *P. aeruginosa* strain PA17 and other CF isolates (11). In an effort to mimic the CF airway environment, *R. mucilaginosa* was fed labeled glucose in anaerobic and aerobic artificial-sputum media, and the *R. mucilaginosa* supernatant was fed to *P. aeruginosa* in nutrient-rich (artificial-sputum medium) under low-nutrient (M9 minimal medium) conditions. As *P. aeruginosa* lacks some glucose utilization capacities, including a key enzyme involved in glycolysis, phosphofructokinase, we postulated that cross-feeding metabolites from *R. mucilaginosa* impacts the metabolism of *P. aeruginosa* (12).

***R. mucilaginosa* metabolism under aerobic and anaerobic conditions.** *R. mucilaginosa* was grown aerobically and anaerobically in artificial-sputum medium (see Text S1 in the supplemental material). Under both anaerobic and aerobic conditions, glucose-derived ^{13}C was incorporated into glycolysis metabolites, namely, lactate and acetate, and some amino acid biosynthesis pathways in *R. mucilaginosa* (Fig. 1; Table S1). The labeled glucose was not incorporated into the tricarboxylic acid (TCA) cycle, pentose phosphate pathway, or long-chain fatty acid biosynthesis pathways. For most

metabolites, ^{13}C incorporation rates were different under different oxygen conditions. For pyruvate, alanine, valine, and acetate, greater label ratios were observed under anaerobic conditions at 24 h. In contrast, lactate, glycine, serine, and isoleucine had greater label ratios under aerobic conditions at 24 h. The incorporation of [U-$^{13}C_6$]glucose into leucine biosynthesis was not impacted by oxygen conditions. Carbon fate in *R. mucilaginosa* diverged after 3-phosphoglycerate. The ^{13}C label was incorporated into serine and glycine, or into pyruvate, the precursor for lactate, acetate, and some amino acids.

Cross-feeding interactions between *R. mucilaginosa* and *P. aeruginosa*. In order to study the impact of *R. mucilaginosa* metabolites on *P. aeruginosa*, we cross-fed supernatant from an aerobic 48-h *R. mucilaginosa* culture to *P. aeruginosa* grown under low-nutrient conditions (M9 minimal medium) and nutrient-rich conditions (artificial-sputum medium). *P. aeruginosa* was grown for 120 h before the cells were harvested in order to recapitulate the low growth rates of bacteria in CF patient sputa (10). The *R. mucilaginosa* supernatant included labeled lactate, pyruvate, and alanine (Fig. 2A; Fig. S1; Table S2). *P. aeruginosa* utilized *R. mucilaginosa*-derived metabolites to produce metabolites in M9 minimal medium and artificial-sputum medium. For example, although labeled lactate was found in the *R. mucilaginosa* supernatant, it was not detected in *P. aeruginosa* cultures, suggesting that *P. aeruginosa* consumed *R. mucilaginosa*-derived lactate (Fig. 2A; Fig. S1; Table S2). *P. aeruginosa* utilization of lactate and other fermentation products has been observed in other studies (6, 13). Since lactate levels have been reported as an indicator of CF patient response to antibiotic therapy, the finding that *P. aeruginosa* consumes lactate derived from another CF microbe may have clinical implications (14).

Labeled metabolites detected in *P. aeruginosa* cells grown in minimal medium included pyruvate, alanine, valine, serine, glycine, leucine, and isoleucine (Fig. 2A; Fig. S1; Table S2). In addition, isotope enrichment for serine, glycine, leucine, and isoleucine was greater in *P. aeruginosa* cells than in the supernatant of *R. mucilaginosa*, indicating that *P. aeruginosa* biosynthesized those metabolites. In contrast, when *P. aeruginosa* was grown in artificial-sputum medium, *P. aeruginosa* had higher levels of a single isotope-enriched amino acid (isoleucine) than occurred in the *R. mucilaginosa* supernatant (Fig. 2A; Fig. S1; Table S2). Interestingly, although the *R. mucilaginosa* supernatant contained only unlabeled glutamate (Fig. 2B and C; Fig. S2; Table S3), labeled glutamate was detected in both *P. aeruginosa* cultures (Fig. 2B, D, and E; Fig. S2; Table S3). This suggests that *P. aeruginosa* biosynthesized glutamate from ^{13}C sources in the *R. mucilaginosa* supernatant even in a nutrient-rich background with initially freely available glutamate (Text S1).

Glutamate provides a link between nitrogen and carbon metabolism by serving as a major amine group donor in transamination reactions for the synthesis of additional amino acids and nucleosides. In *Escherichia coli*, up to 88% of the total nitrogen that ends up in a biomass comes from glutamate, and the cellular glutamate pool needs to be kept high to drive the transamination reactions (15). In *P. aeruginosa* specifically, glutamate is a component of the cell wall and may play a role in *P. aeruginosa* virulence (16). Glutamate enhanced the yield of a virulence factor, exotoxin A (17), and induced swarming motility in *P. aeruginosa* on semisolid surfaces (18). More recently, glutamate-induced dispersion via c-di-GMP signaling pathways has been suggested (19). Glutamate might be derived from glutamine or alpha-ketoglutarate (20–22). However, the abundance of these two compounds was below the limit of quantification in this study. Future studies are needed to examine the biosynthesis pathways of glutamate and its role in the metabolism and physiology of *P. aeruginosa*. In summary, this study provides evidence that metabolite cross-feeding exists between *R. mucilaginosa* and *P. aeruginosa*, two common microorganisms found in polymicrobial communities in CF patient airways. The results from our study provide evidence that the physiology of CF pathogens can be influenced by the metabolic capabilities of other nearby microor-

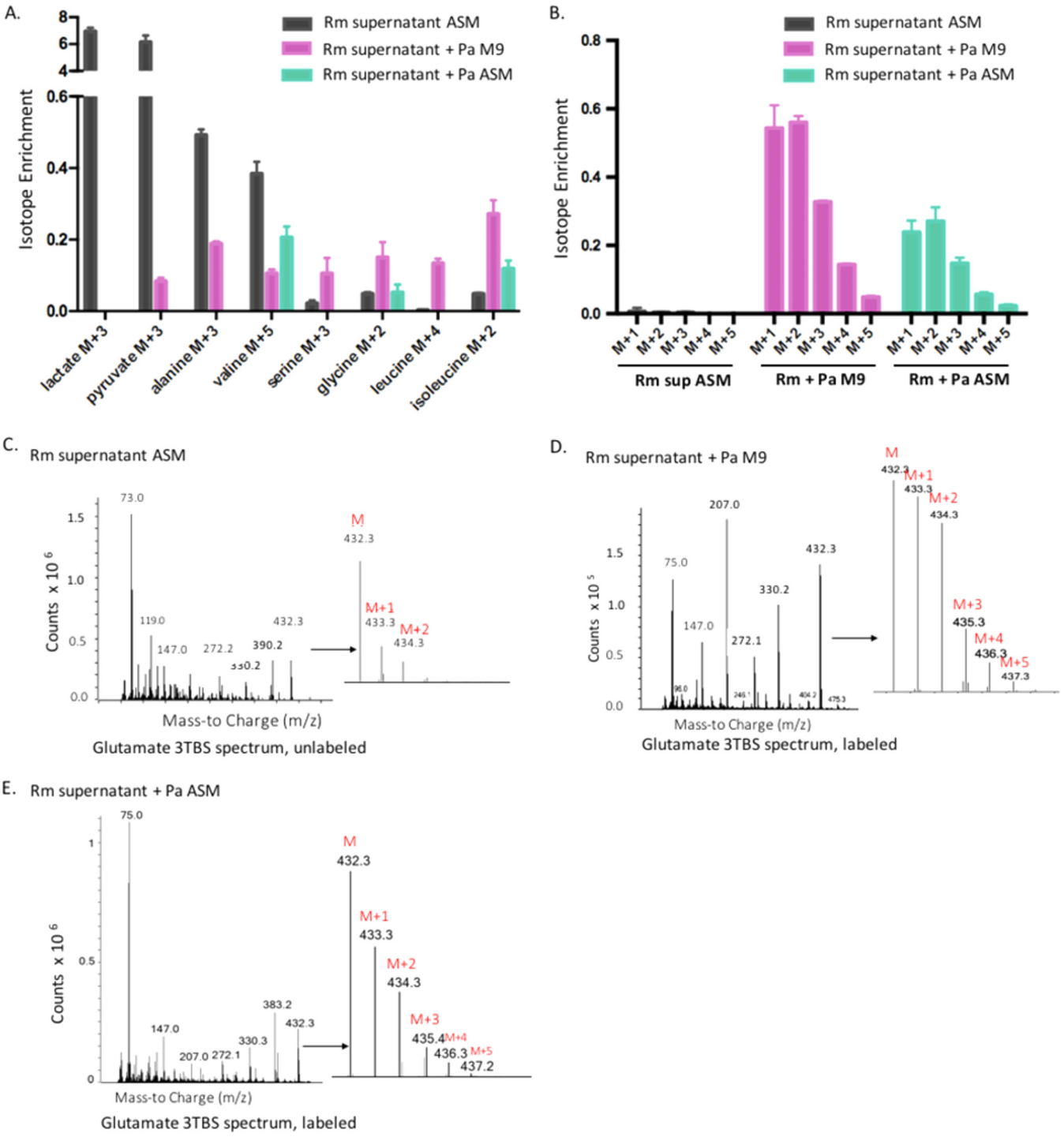

FIG 2 Cross-feeding interactions between *R. mucilaginosa* and *P. aeruginosa*. M+1, M+2, M+3, M+4, and M+5 indicate compounds that contained 1, 2, 3, 4, and 5 ^{13}C atoms, respectively. Error bars, means ± SD (n = 3 bacterial cultures per group). (A) Labeled lactate was found in the *R. mucilaginosa* (Rm) supernatant but not in *P. aeruginosa* (Pa) cells. In M9 minimal medium, *P. aeruginosa* cells contained isotopically enriched pyruvate, alanine, valine, serine, glycine, leucine, and isoleucine. In artificial-sputum medium, *P. aeruginosa* cells contained isotopically enriched valine, glycine, and isoleucine. (B) Although the *R. mucilaginosa* supernatant contained only unlabeled glutamate, labeled glutamate was detected in the *P. aeruginosa* cells grown in artificial-sputum medium and M9 minimal medium. (C to E) Glutamate spectrum for the *R. mucilaginosa* supernatant (C), *P. aeruginosa* grown in M9 minimal medium spiked with the *R. mucilaginosa* supernatant (D), and *P. aeruginosa* grown in artificial-sputum medium spiked with the *R. mucilaginosa* supernatant (E).

ganisms, even in a nutrient-rich environment, which can be tracked with stable-isotope-labeled metabolomics.

Culture conditions and metabolomics. The bacterial strains chosen for this study were isolated from CF patients at the UCSD Adult CF Clinic: *Pseudomonas aeruginosa*

PaFLR01 and *Rothia mucilaginosa* RmFLR01 (11, 23). First, we took time points from *R. mucilaginosa* cultures to examine the kinetics of metabolites in glycolysis, the TCA cycle, amino acid biosynthesis, short- and long-chain fatty acid biosynthesis, and the pentose phosphate pathway in *R. mucilaginosa*, which was grown in triplicate in artificial-sputum medium (24) spiked with 100 mM [U-$^{13}C_6$]D-glucose (Sigma-Aldrich and Cambridge Isotope Laboratory) under anaerobic and aerobic oxygen conditions (5% CO_2) at 37°C. *R. mucilaginosa* cells were harvested at 4 h, 8 h, and 24 h. For the metabolite cross-feeding study, *R. mucilaginosa* was grown in the same medium aerobically for 48 h. The *R. mucilaginosa* supernatant was collected by filtering the culture, and the supernatant was diluted 10-fold in M9 minimal medium supplemented with succinate and in fresh artificial-sputum medium. *P. aeruginosa* was grown in triplicate aerobically, and the cells were harvested at 120 h. Metabolite extraction and data acquisition were carried out by following West Coast Metabolomics Center standard operating procedures (Text S1). Agilent MassHunter quantitative analysis software (v. B.07.00) was used for raw data processing. Natural abundance was corrected when isotope enrichment was calculated.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/mSphere.00151-18.

TEXT S1, PDF file, 0.2 MB.
FIG S1, PDF file, 0.2 MB.
FIG S2, PDF file, 0.2 MB.
TABLE S1, XLSX file, 0.04 MB.
TABLE S2, XLSX file, 0.04 MB.
TABLE S3, XLSX file, 0.04 MB.

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