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Original Article

12-Methyltetradecanoic Acid, a Branched-Chain Fatty Acid, Represses the Extracellular Production of Surfactants Required for Swarming Motility in *Pseudomonas aeruginosa* PAO1

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SUMMARY: *Pseudomonas aeruginosa* is known to produce surfactants that are involved in its swarming motility behavior, such as rhamnolipids and their precursors—3-(3-hydroxyalkanoyloxy) alkanoic acids (HAAs). In *P. aeruginosa* PAO1, swarming motility is inhibited by some fatty acids, including branched-chain fatty acids and unsaturated fatty acids. In the present study, addition of 12-methyltetradecanoic acid (12-MTA, *anteiso*-C15:0) to an agar medium markedly repressed surfactant activity in the extracellular fraction of a *P. aeruginosa* culture in a drop collapse assay. Further, an extracellular fraction of a culture of *rhlA* mutant *P. aeruginosa*, which did not produce both rhamnolipids and HAAs, showed a complete loss of surfactant activity and markedly reduced swarming activity. In contrast, an extracellular fraction of a culture of *rhlB* mutant *P. aeruginosa*, which produced HAAs but not rhamnolipids, showed moderate swarming activity and weak extracellular surfactant activity that was lost on the addition of 12-MTA to the agar medium. Expression of the *rhlAB* operon from the plasmid pMR2 restored normal swarming motility on 12-MTA-containing agar medium. Taken together, these findings indicate that 12-MTA reduced extracellular surfactant activity, thus resulting in a swarming defect in *P. aeruginosa* PAO1.

INTRODUCTION

Swarming motility is flagella-dependent and has been observed among a wide range of motile bacterial species, such as *Salmonella, Vibrio, Proteus, Yersinia, Escherichia, Serratia*, and *Pseudomonas* (1,2). This type of motility is characterized by a multicellular movement in viscous environments (e.g., semisolid agar medium) and is different from flagella-dependent swimming motility, which is characterized by individual cell movement in aqueous environments. The general characteristics of swarmer cells include elongation, hyperflagellation, and the increased production of extracellular wetting agents that reduce surface tension and promote surface movement (1).

Studies on mutants showing defective swarming motility have been conducted with various bacterial species, and these studies have shown that many genes, which belong to various functional categories, are involved in swarming migration (3–6). Furthermore, transcriptome and proteome analyses have revealed that gene expression profiles in swarming cells are substantially different from those in swimming cells (7–10). These studies support the idea that swarming cells exist in a uniquely differentiated physiological state. It is noteworthy that swarming cells of pathogenic bacteria show elevated expression levels of virulence-associated genes and elevated levels of antibiotic resistance (8,10–12).

Fatty acids are ubiquitous components of cell membranes. Interestingly, exogenously added fatty acids have been reported to affect a variety of bacterial biological processes, including growth, motility, differentiation, biofilm dispersion, and production of virulence factors (13–18).

Pseudomonas aeruginosa is an opportunistic human pathogen that causes respiratory and urinary tract infections in immunocompromised hosts. Similar to the motility of other bacteria, the motility of this bacterium is associated with its virulence (19,20). In a recent study, we showed that the swarming motility of the *P. aeruginosa* strain PAO1 is inhibited by various fatty acids, including branched-chain fatty acids and unsaturated fatty acids (21). Of these, 12-methyltetradecanoic acid (12-MTA), a branched-chain fatty acid, completely inhibited swarming and slightly repressed swimming in *P. aeruginosa* PAO1 at a concentration of 10 μg/ml. The drastic inhibition of swarming by 12-MTA led us to hypothesize that 12-MTA not only influences flagellar function but also the production of wetting agents. Studies conducted to date have reported that rhamnolipids and their precursors, 3-(3-hydroxyalkanoyloxy) alkanoic acids (HAAs), show surfactant activity in *P. aeruginosa* and serve as wetting agents in swarming motility (22,23). In the present study, we used a drop

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collapse assay to investigate the effects of 12-MTA on the extracellular production of surfactants via the rhamnolipid biosynthetic pathway in *P. aeruginosa* PAO1 and its mutants.

MATERIALS AND METHODS

Bacterial strains and growth conditions: Bacterial strains used were *P. aeruginosa* PAO1, *Escherichia coli* DH5α as a host for construction of plasmids, and *E. coli* SM10*lacI*^{q} (24,25) as a mobilizer strain. These strains were routinely cultured at 37°C in Luria-Bertani (LB) broth or on LB agar, unless otherwise mentioned. When required, antibiotics were used at the following concentrations: for *E. coli*, ampicillin (Ap) (50–100 μg/ml), gentamicin (Gm) (16–32 μg/ml), and tetracycline (Tc) (25 μg/ml); and for *P. aeruginosa*, carbenicillin (Cb) (100–200 μg/ml), Gm (32–64 μg/ml), and Tc (62.5–125 μg/ml). To examine the effects of 12-MTA, an ethanol solution of 12-MTA (Sigma, St Louis, Mo., USA) was prepared and added to an agar medium; agar medium with an equal amount of ethanol (without 12-MTA) was prepared to serve as a control.

Swarming motility assay: The bacterial strains were incubated overnight at 37°C either on LB agar, LB-Gm agar, or LB-Tc agar. The colonies growing on the agar plates were picked up with a toothpick and inoculated on the surface of swarm plates containing 0.8% nutrient broth (NB) (Difco Laboratories, Detroit, Miss., USA), 0.5% glucose, and 0.5% agar (Wako Pure Chemical Industries, Osaka, Japan), with or without 12-MTA. The swarm plates were incubated overnight at 33°C and observed for swarming.

Construction of *rhlA* and *rhlB* mutants: Site-specific insertional mutagenesis of *P. aeruginosa* PAO1 was performed according to the method described by Hoang et al. (26).

(i) *rhlA* mutant: A 1.9-kbp DNA fragment containing the *rhlA* region of PAO1 was amplified by polymerase chain reaction (PCR) using the primers rhlA-del-1 (5′-G**GAATTC**CTGACCCTCGAGTTCTCCAATAC - 3′) and rhlA-del-2 (5′-GC**TCTAGA**GCTAGGGAATCCC GTACTTCTCG-3′) and digested with *Eco*RI and *Xba*I. The digested *Eco*RI-*Xba*I fragment was ligated to *Eco*RI- and *Xba*I-digested pUC19 to form pUC19-*rhlA*. The Gm^R-*GFP* cassette was cleaved from pPS858 using *Bam*HI and further ligated to the *Bam*HI-digested and dephosphorylated pUC19-*rhlA*. The *rhlA*::Gm^R-*GFP* region in the pUC19-*rhlA*::Gm^R-*GFP* was amplified using the primers rhlA-del-1 and rhlA-del-2, 5′-phosphorylated, and ligated to *Sma*I-digested and dephosphorylated pEX100T containing the *sacB* gene to produce the plasmid pERAG6. *E. coli* SM10*lacI*^{q} was transformed using pERAG6, and the plasmid was transferred from SM10*lacI*^{q} to PAO1 by conjugation. Colonies grown on VBMM agar (27) containing Gm and Cb were used to inoculate VBMM-Gm agar supplemented with 10% sucrose. Next, Cb-sensitive and Gm-resistant clones were selected, and a representative of these clones was designated MRA1. Insertional mutagenesis of the *rhlA* gene in MRA1 was verified by PCR.

(ii) *rhlB* mutant: The *rhlB* mutant was generated as described above. A 1.8-kbp DNA fragment containing the *rhlB* region of PAO1 was amplified by PCR using the primers rhlB-FW (5′-GA**AGGCCT**TGGCCATCTG CTCAACGAGAC-3′) and rhlB-RV (5′-GA**AGGCCT** TATACGGCAAAATCATGGCAAC-3′). The PCR product was 5′-phosphorylated and then ligated to *Sma*I-digested and dephosphorylated pUC19 to yield pUC19-*rhlB*. The Gm^R cassette prepared from *Sma*I digests of pMS255 (28) was ligated to *Nru*I- and *Sma*I-digested and dephosphorylated pUC19-*rhlB*. The resulting plasmid was digested with *Stu*I, and the fragment containing the *rhlB*::Gm^R region was ligated to *Sma*I-digested and dephosphorylated pEX100T to yield the plasmid pERBG22. Transformation of SM10*lacI*^{q} with pERBG22 and conjugative transfer of pERBG22 into PAO1 were performed. The *rhlB*-deleted clones were selected as described above. One of the clones was designated RBG21. We used PCR to confirm the insertion of the Gm^R cassette into the *rhlB* gene of the RBG21 genome.

PAO1 harboring a plasmid encoding the *rhlAB* genes: A plasmid vector, pME6032 (29), and its derivative plasmid, pMR2, carrying the *rhlAB* operon (30) (kindly provided by Taichiro Tanikawa) were introduced into PAO1 by electroporation (31). The transformants were selected on LB-Tc agar.

Drop collapse assay: The drop collapse assay was performed as described by Caiazza et al. (22). Unless otherwise mentioned, an overnight culture grown in LB or LB-Gm broth, was diluted 10^6-fold with NB, and 50 μl of the diluted culture was spread on plates containing NB, 0.5% glucose, and 1% agar, which were then incubated at 33°C for 24 h. The colonies grown on agar plates were scraped, and the cells were suspended in phosphate-buffered saline (PBS). The optical density (OD) of the cell suspensions was measured at 600 nm (OD_{600}) using a spectrophotometer (Novaspec II; Pharmacia Biotech, Uppsala, Sweden) and adjusted to OD_{600} values of 5, 10, or 15 using PBS. The suspensions were centrifuged, and the supernatants were filtered through a 0.22-μm PVDF membrane (Millipore, Billerica, Mass., USA). The filtrate was designated as a "surface prep." These surface preps were serially diluted with PBS, and 25-μl drops were placed on the underside of the lid of a 96-well polystyrene microtiter plate. When the samples contained surfactants, the diameter of the bead was larger than that of PBS or no bead formation was observed.

RESULTS AND DISCUSSION

Effects of agar concentration on surfactant production: Swarming motility is highly dependent on the agar concentration in the medium (32). To observe swarming motility in *P. aeruginosa*, a semisolid agar medium with an agar concentration in the range 0.5–0.7% is routinely used (1). To roughly assess the effect of agar concentration on surfactant production in *P. aeruginosa* PAO1, NB medium containing 0.5% glucose and either 0.5%, 1.0%, or 1.5% agar was used to prepare the surface preps. As shown in Fig. 1, drop collapse was clearly observed in all the 2-fold-diluted surface preps, indicating that even under non-swarming conditions (1.0% and 1.5% agar media), substantial amounts of surfactants were produced. It is particularly interesting to note that the extracellular surfactant production in a 24-h culture

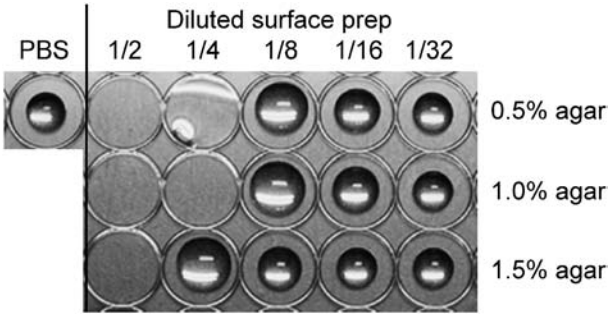

Fig. 1. Drop collapse assay. Preparation of surface preps from swarming cells: PAO1 cells were incubated on swarm plate at 33°C for 18 h. Preparation of surface preps from non-swarming cells: PAO1 overnight culture was diluted 10^6-fold with NB; 50 μl of the diluted culture was spread on NB plus 0.5% glucose containing 1% or 1.5% agar and incubated at 33°C for 24 h. PAO1 cultures grown on these agar plates were scraped and suspended in PBS to obtain an OD_{600} of 10. Surface preps and the diluted solutions were prepared as described in Materials and Methods.

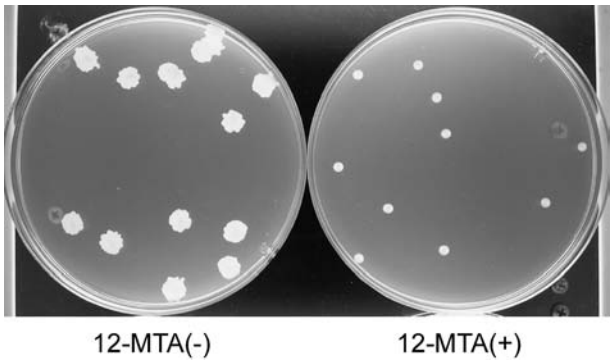

Fig. 2. Colonies of *P. aeruginosa* PAO1 grown on NB plus 0.5% glucose medium solidified with 1% agar, with or without 12-MTA (5 μg/ml).

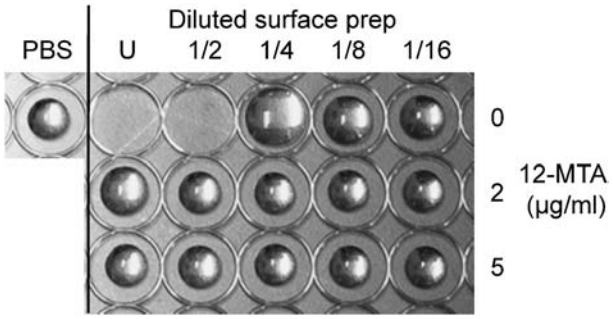

Fig. 3. Drop collapse assay. *P. aeruginosa* PAO1 cultures, grown on agar plates with or without 2 or 5 μg/ml of 12-MTA at 33°C for 24 h, were scraped and suspended in PBS to obtain an OD_{600} of 5. Surface preps and the diluted solutions were prepared as described in Materials and Methods. U, undiluted.

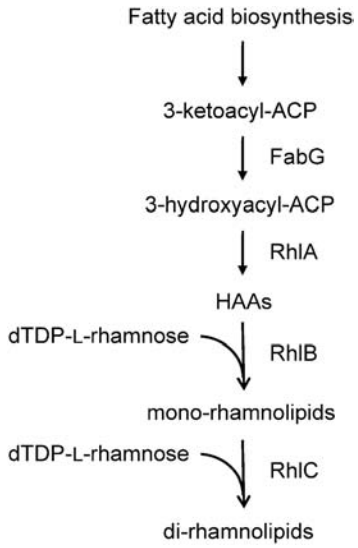

Fig. 4. Rhamnolipid biosynthetic pathway (33,34).

on 1.0% agar medium is similar to that in an 18-h swarming culture on 0.5% agar medium (swarm plate). In the subsequent experiments, 1.0% agar medium was used to prepare surface preps.

Effects of 12-MTA on surfactant production: In an earlier study, our group had reported that a branched-chain fatty acid, 12-MTA, strongly inhibits the swarming motility and slightly represses the swimming motility of *P. aeruginosa* PAO1 (21). Rhamnolipids and HAAs, which show surfactant activity, have been reported to be involved in the swarming motility of *P. aeruginosa* (22,23). Hence, we investigated the effect of 12-MTA on the production of extracellular surfactants. Figure 2 shows colonies of PAO1 on agar medium with or without 12-MTA. Addition of 12-MTA to the agar medium considerably decreased the colony size of the PAO1 strain. Surface preps were prepared from these culture plates. As shown in Fig. 3, beads of undiluted surface preps did not collapse when the surface preps were prepared from agar cultures grown on media containing 2 or 5 μg/ml of 12-MTA; this indicated that surfactant production by PAO1 was greatly inhibited by the addition of a small amount of 12-MTA to the agar medium.

Effects of *rhlA* and *rhlB* mutations on swarming motility and surfactant production: Figure 4 shows the biochemical pathway by which rhamnolipids and their precursor forms, the HAAs, are synthesized in *P. aeruginosa* (33,34). Previous studies showed that mutations in the *rhlA* locus result in decreased swarming and extracellular surfactant activity (22,23). To confirm this, we first constructed the *rhlA* deletion mutant of PAO1 by allelic exchange mutagenesis. This mutant is expected to lack the ability to synthesize both rhamnolipids and HAAs. Compared with PAO1, the mutant MRA1 showed a remarkable decrease in the swarming activity (Fig. 5A). On the same note, the cells showed a complete loss of extracellular surfactant activity (Fig. 5B). Considering these findings together with the results in Fig. 3, it appers that 12-MTA is likely to repress extracellular production of rhamnolipids and HAAs. In addition, no other extracellular surfactants seemed to be produced under the present culture conditions. Compared to the complete swarming inhibition induced by 12-MTA (21), the MRA1 strain in our study still possessed a weak spreading ability on a swarm plate. This suggests that in addition to interfering with the rhamnolipid biosynthetic pathway, 12-MTA impacts other molecular targets and biological processes related to

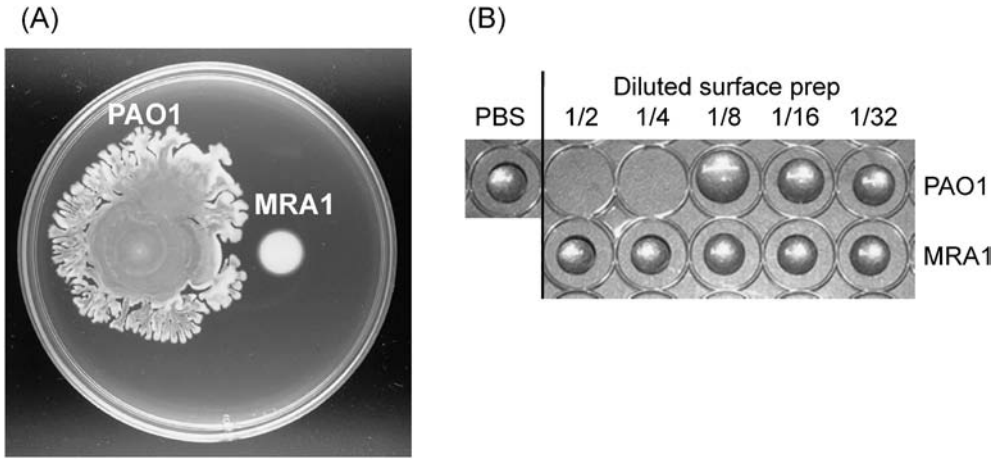

Fig. 5. (A) Swarming motility of PAO1 and its *rhlA* mutant, MRA1. (B) Drop collapse assay. PAO1 and MRA1 cultures grown on agar plates were scraped and suspended in PBS to obtain an OD_{600} of 10. Surface preps and the diluted solutions were prepared as described in Materials and Methods.

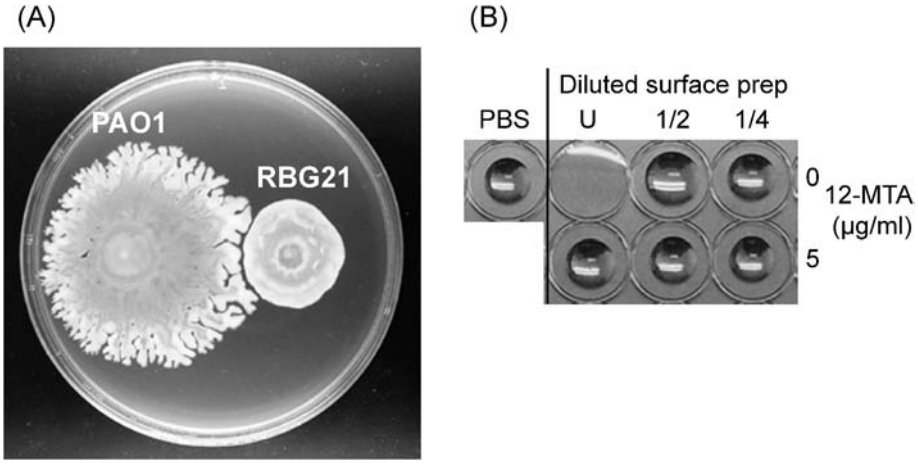

Fig. 6. (A) Swarming motility of PAO1 and its *rhlB* mutant, RBG21. (B) Drop collapse assay. RBG21 cultures, grown on agar plates with or without 12-MTA (5 μg/ml), were scraped and suspended in PBS to obtain an OD_{600} of 15. Surface preps and the diluted solutions were prepared as described in Materials and Methods. U, undiluted.

swarming motility, such as flagellar biosynthesis and function, and/or production of other swarm-promoting materials such as the acidic polysaccharide observed in *Proteus* spp. (1). *P. aeruginosa* is known to produce three types of exopolysaccharides (35).

On the basis of the pathway shown in Fig. 4, the *rhlB* deletion mutant would be expected to produce HAAs but not rhamnolipids. Figure 6 shows the swarming motility and surfactant production in the *rhlB* mutant RBG21. RBG21 is capable of swarming, but its swarming activity is lower than that of PAO1 (Fig. 6A). This reduced level of swarming motility might be due to the expected defect in rhamnolipid production. In the drop collapse assay, an undiluted bead obtained using the RBG21 mutant collapsed (Fig. 6B), suggesting that this mutant produced a small amount of surfactants, probably HAAs, which is consistent with the observations of Caiazza et al. (22). Addition of 12-MTA to the agar medium completely impaired the surfactant production, confirming that 12-MTA represses extracellular production of HAAs as well as of rhamnolipids.

Effects of *rhlAB* expression on swarming inhibition by 12-MTA: As described above, rhamnolipid production is a probable target of 12-MTA. To examine whether *rhlA* and *rhlB* expression is sufficient to complement the swarming defect observed on a 12-MTA-containing medium, we used the *rhlAB*-encoding plasmid pMR2. As shown in Fig. 7, PAO1 containing the plasmid vector pME6032 showed defective swarming migration in the presence of 12-MTA, whereas PAO1 carrying pMR2 exhibited normal swarming activity under the same conditions. The colony spreading pattern of PAO1 harboring the pME6032 or pMR2 plasmids appeared to be different from that of PAO1. The reason for this is currently unknown. However, swarming involves many biological processes (3–6), and the presence of the plasmid vector might affect some of these processes and lead to alterations in the spreading pattern. These results indicate that the targets of 12-MTA are the production and/or functioning of the RhlA and RhlB proteins. Thus, 12-MTA could possibly affect the enzymatic activities of RhlA and RhlB. Since both the *rhlA* and *rhlB* genes are organized in an operon, another possibility would be that 12-MTA causes the down-regulation of *rhlAB* gene expression. It is also possible that 12-MTA inhibits the extracellular secretion of sur-

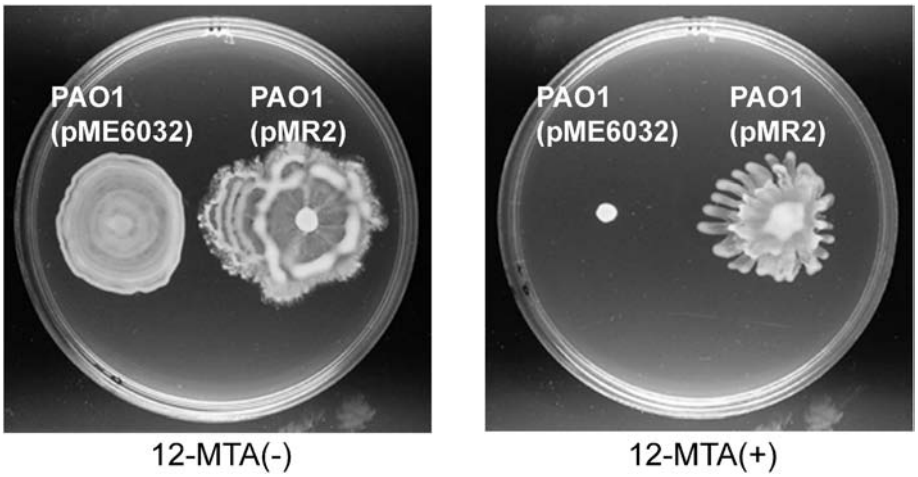

Fig. 7. Swarming assays of PAO1 (pME6032) and PAO1 (pMR2). Bacterial cells grown overnight on LB-Tc agar were picked up with a toothpick, used to inoculate swarm plates with or without 12-MTA (5 μg/ml), and incubated at 33°C for 20 h.

factants produced within a cell. However, as mentioned above, since many genes encoding various cellular processes are involved in swarming motility (3,4), we cannot exclude the possibility that certain biological processes other than the rhamnolipid biosynthetic pathway are also affected by 12-MTA.

In this study, we present results indicating that a mono-methyl fatty acid, 12-MTA, represses the extracellular production of surfactants, including rhamnolipids and HAAs, thus causing alterations in the swarming activity of *P. aeruginosa*. The present results are different from the previous data obtained using a conventional rhamnolipid plate assay, in which addition of 12-MTA caused no significant reduction in rhamnolipid production (21,36). This may be due to differences in culture conditions, such as medium components, incubation temperature, and incubation time.

In addition to serving as wetting agents in swarming, rhamnolipids are also considered to be virulence factors of *P. aeruginosa*. Soberón-Chávez et al. (33), in their review, have discussed the various biological activities of rhamnolipids against host tissues. In addition, a recent microarray analysis of *P. aeruginosa* revealed that compared to the cultures subjected to swimming conditions, those subjected to swarming conditions showed upregulation of certain virulence-associated genes, such as genes involved in the type III secretion system and related exoenzymes and those encoding extracellular proteases and siderophore biosynthesis, and antibiotic resistance was concomitantly elevated (8). In the early stages of bacterial infection, bacterial cells may be exposed to viscous environments such as the mucous layer covering epithelial cells, and exposure to these environments may increase the swarming activity and the production of virulence-associated factors. In this context, we could speculate that fatty acids have the potential to reduce swarming motility and simultaneously decrease the production of virulence factors in *P. aeruginosa* infection. Further studies are needed to understand the role of swarming motility in bacterial infections and analyze the effects of fatty acids in the infectious processes.

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Conflict of interest None to declare.

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