

# Genome-Wide Identification of Genes Necessary for Biofilm Formation by Nosocomial Pathogen *Stenotrophomonas maltophilia* Reveals that Orphan Response Regulator FsnR Is a Critical Modulator

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*Stenotrophomonas maltophilia* is a Gram-negative bacterial pathogen of increasing concern to human health. Most clinical isolates of *S. maltophilia* efficiently form biofilms on biotic and abiotic surfaces, making this bacterium resistant to a number of antibiotic treatments and therefore difficult to eliminate. To date, very few studies have investigated the molecular and regulatory mechanisms responsible for *S. maltophilia* biofilm formation. Here we constructed a random transposon insertion mutant library of *S. maltophilia* ATCC 13637 and screened 14,028 clones. A total of 46 nonredundant genes were identified. Mutants of these genes exhibited marked changes in biofilm formation, suggesting that multiple physiological pathways, including extracellular polysaccharide production, purine synthesis, transportation, and peptide and lipid synthesis, are involved in bacterial cell aggregation. Of these genes, 20 putatively contributed to flagellar biosynthesis, indicating a critical role for cell motility in *S. maltophilia* biofilm formation. Genetic and biochemical evidence demonstrated that an orphan response regulator, FsnR, activated transcription of at least two flagellum-associated operons by directly binding to their promoters. This regulatory protein plays a fundamental role in controlling flagellar assembly, cell motility, and biofilm formation. These results provide a genetic basis to systematically study biofilm formation of *S. maltophilia*.

*Stenotrophomonas maltophilia* (formerly *Pseudomonas maltophilia* or *Xanthomonas maltophilia*) is an aerobic, Gram-negative bacterium belonging to the family *Xanthomonadaceae* of the *Gammaproteobacteria*. *S. maltophilia* is ubiquitous across a diverse range of ecological niches, including foods, soil, plant roots and stems, and aqueous environments. In clinical settings, *S. maltophilia* is an opportunistic nosocomial pathogen, predominantly colonizing the skin, the respiratory tract, urinary catheters, and breathing tubes, such as endotracheal tubes (1, 2). Infection generally results in pneumonia, bacteremia, urinary tract infection, or meningitis (3, 4). Over the past 3 decades, *S. maltophilia* has become an increasing threat to public health, in particular to immunosuppressed or immunocompetent patients in intensive care units (ICU) who are undergoing prolonged mechanical ventilation, tracheostomy, or broad-spectrum antibiotic therapy (5). The reported mortality rates for *S. maltophilia*-infected patients range from 23% to 77% (4, 6). Treatment of *S. maltophilia* infection has proven increasingly difficult because of the bacterium's intrinsic multidrug resistance. *S. maltophilia* is resistant to treatment with most aminoglycosides, quinolones,  $\beta$ -lactams, and  $\beta$ -lactamase inhibitors (7). Worryingly, research has shown that the susceptibility of bacterial isolates to the recommended drug for *S. maltophilia* infection, trimethoprim-sulfamethoxazole, decreased from >98% to 30 to 40% within a decade (8). Therefore, the development of more effective antibiotic agents targeting critical processes of *S. maltophilia* pathogenesis that are nonessential to survival and with minimal selective pressure will be a challenge faced by researchers in the future.

Biofilms are slime-enclosed bacterial aggregates affording protection against antibiotics, host immune responses, and multiple stresses (9). Most clinical strains of *S. maltophilia* form biofilms efficiently on various abiotic and biotic surfaces, including Teflon, plastic, glass, host tissue, and indwelling intravascular devices (10,

11). Environmental factors, including phosphate, pH, temperature, metal ions, and antibiotics, were shown to have a profound effect on *S. maltophilia* biofilm formation (3). To date, however, only a small number of genes or biochemical cascades associated with *S. maltophilia* biofilm formation have been reported, and these include the polysaccharide synthesis genes *rmlA*, *rmlC*, and *xanB* (12), diffusible signal factor (DSF)-mediated cell-cell communication (13), the small RNA modulator Hfq (14), and the ABC-type efflux pump MacABC (15). Hence, it is critical to undertake a systematic approach to elucidate the molecular and regulatory properties of *S. maltophilia* biofilm development.

In this work, we constructed a large-scale *S. maltophilia* transposon insertion mutant library which was used to screen for *S. maltophilia* mutants with altered biofilm-forming ability (16). From 14,208 clones, we identified 46 genes belonging to mutants exhibiting remarkable changes in biofilm development. Almost

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TABLE 1 Bacterial strains and plasmids used in this study

Strain or plasmid	Genotype or description <sup>a</sup>	Source or reference
<b>Strains</b>		
<i>S. maltophilia</i> ATCC 13637	WT strain	CGMCC
<i>E. coli</i> strain DH5 $\alpha$	Host strain for molecular cloning	Lab collection
<i>E. coli</i> strain BL21(DE3)	Host strain for protein expression; Kan <sup>r</sup>	Lab collection
<i>S. maltophilia</i> SM001	$\Delta$ <i>fsnR</i> ; in-frame deletion mutant of <i>fsnR</i>	This study
<i>S. maltophilia</i> SM002	$\Delta$ <i>fsnR</i> - <i>fsnR</i> ; genetic complementary strain of SM001 mutant; Str <sup>r</sup>	This study
<i>S. maltophilia</i> SM003	CK; SM001 containing a blank pHM1 vector; Str <sup>r</sup>	This study
Clones from <i>S. maltophilia</i> mutant library	EZ::TN transposon mutants of WT strain with biofilm deficiency; Kan <sup>r</sup>	See Table S1 in the supplemental material
<b>Plasmids</b>		
pK18mobsacB	Suicide vector to create mutant by double-crossover recombination; Kan <sup>r</sup>	20
pET30a	Protein expression vector; Kan <sup>r</sup>	Novagen
pHM1	Broad-host-range vector for genetic complementation; Str <sup>r</sup>	Lab collection
pHM2299	pHM1:: <i>fsnR</i> ; genetically complementary vector; Str <sup>r</sup>	This study
pET2299	pET30a:: <i>fsnR</i> ; vector for expression of <i>FsnR</i> ; Kan <sup>r</sup>	This study

<sup>a</sup> Kan<sup>r</sup>, kanamycin resistant; Str<sup>r</sup>, streptomycin resistant.

half of these genes (20 genes [43.5%]) were involved in bacterial flagellar or pilus biosynthesis, which suggested that cell motility plays a critical role in *S. maltophilia* biofilm development. We confirmed that FsnR, an orphan response regulator of the two-component signal transduction system, binds directly to the promoter regions of two gene clusters and activates their transcription. These results provide genetic information to further investigate the molecular process of *S. maltophilia* biofilm formation.

## MATERIALS AND METHODS

**Bacterial strains, plasmids, and culture conditions.** The bacterial strains and plasmids used in the present study are described in Table 1. *Escherichia coli* DH5 $\alpha$  was used as the host in molecular cloning procedures and was routinely cultured at 37°C in Luria-Bertani (LB) medium. *S. maltophilia* ATCC 13637, obtained from stocks of the China General Microbiological Culture Collection Center (CGMCC), was cultured at 28°C in NYG medium (5 g/liter tryptone, 3 g/liter yeast extract, 20 g/liter glycerol; pH 7.0). Competent cells of *S. maltophilia* were prepared by culture in 210 medium (4 g/liter yeast extract, 8 g/liter casein enzymatic hydrolysate, 5 g/liter sucrose, 3 g/liter K<sub>2</sub>HPO<sub>4</sub>, 0.3 g/liter MgSO<sub>4</sub> · 7H<sub>2</sub>O; pH 7.0) and washed in ice-cold glycerol (10%). Antibiotics were used at the following concentrations: ampicillin, 100  $\mu$ g/ml; kanamycin, 50  $\mu$ g/ml; spectinomycin, 100  $\mu$ g/ml; and streptomycin, 200  $\mu$ g/ml. Electroporation conditions for the transformation of both *S. maltophilia* and *E. coli* were set at 18 kV cm<sup>-1</sup>, 25  $\mu$ F, and 200  $\Omega$ , and experiments were conducted in a Bio-Rad Pulser XCell electroporation system (Bio-Rad, Hercules, CA).

**Construction of insertion mutant library and genetic manipulation of bacterial strains.** The *S. maltophilia* transposon insertion mutant library was constructed using an EZ::TN <KAN-2> Tnp transposome kit (Epicentre) as described previously and in accordance with the manufacturer's protocol (17, 18). A total of 14,208 kanamycin-resistant transformants were selected and stored in 37 384-well plates at -80°C until use. Southern blot hybridization was used to determine the transgene copy number. In brief, total DNAs were extracted from bacterial strains, and the electrophoresed fragments were transferred to a Hybond N<sup>+</sup> membrane and hybridized with an [ $\alpha$ -<sup>32</sup>P]dCTP-labeled PCR probe (Prime-A-Gene; Promega). PCR primers used to amplify the kanamycin resistance sequence and used as DNA probes are listed in Table 2.

General molecular cloning procedures, including PCR, DNA extraction and ligation, restriction enzyme digestion, and Southern blotting, were conducted per the methods of Sambrook and Russell (19). All primers used for mutagenesis, protein expression, and reverse transcription-

PCR (RT-PCR) are listed in Table 2. The in-frame deletion mutant of *fsnR* was constructed by homologous double-crossover recombination using the suicide vector pK18mobsacB (20). Correction of the in-frame deletion mutant was verified by PCR and sequencing. A genetically complementary strain was constructed as outlined below. EcoRI and HindIII restriction sites were introduced into full-length *fsnR*, the gene sequence was amplified by PCR, and the amplicon was ligated with the pHM1 broad-host-range vector and electroporated into *S. maltophilia* strains. In this construct, *fsnR* is under the control of the *lacZp* promoter.

**Biofilm assay and quantification.** The crystal violet staining method for the quantification of biofilm formation was adapted from a previously described method (21, 22). In brief, *S. maltophilia* cultures were inoculated quantitatively into NYG medium within 96-well polystyrene plates and incubated at 28°C for 8 h without shaking. The optical density at 600 nm (OD<sub>600</sub>) for each well was measured on a Tecan Infinite 200 Pro microplate reader. The wells were washed rigorously with water prior to staining with 0.1% crystal violet solution for 20 min. The wells were washed, the crystal violet stain was solubilized in absolute ethanol, and biofilm formation was determined by measuring the OD<sub>590</sub> on a microplate reader. Each data point was the average for at least four replicates.

**Swimming motility and transmission electron microscopy.** For the swimming motility assay, bacterial strains were inoculated into 0.1% NYG agar with a toothpick and cultured for 36 h at 28°C, and the diameters of the swimming zones were measured.

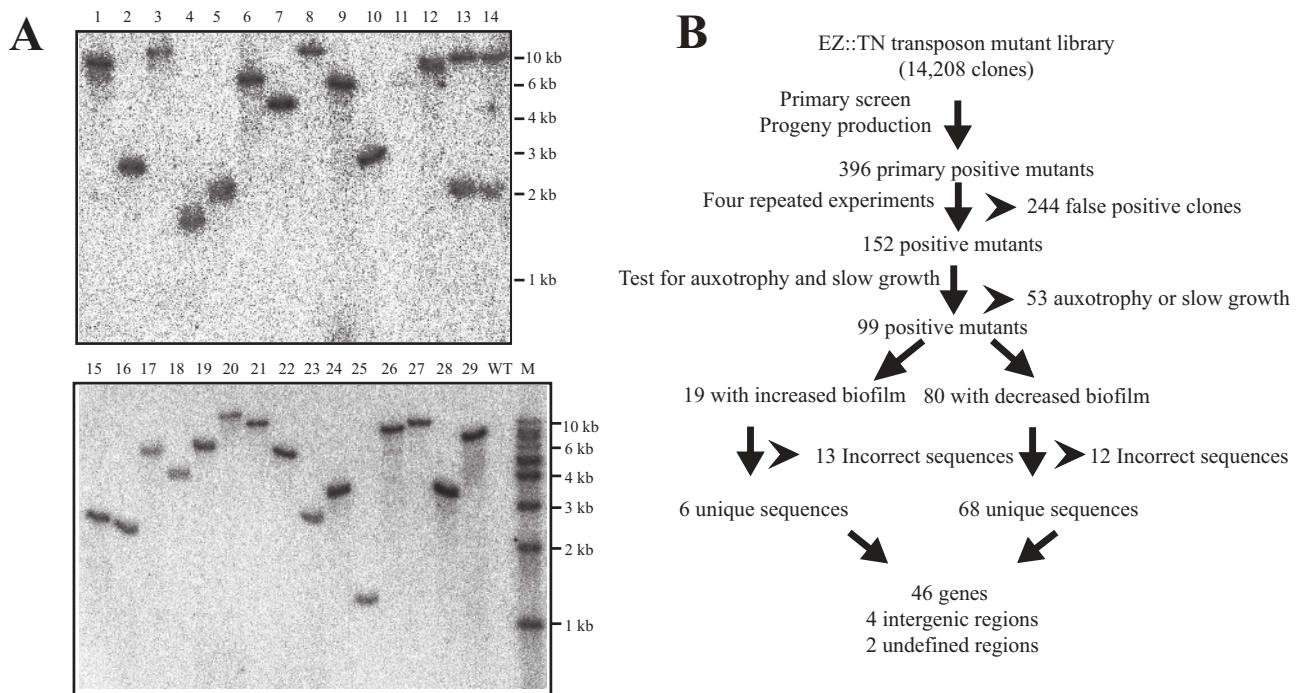
Transmission electron microscopy was used to observe flagellar morphology. Briefly, bacterial suspensions prepared from cultures of *S. maltophilia* were placed directly on glow-discharged carbon-coated grids, stained with 2.0% uranyl acetate for 2 min, washed twice with water, and air dried. Bacterial flagella were then visualized using a JEDL JEM-1400 transmission electron microscope (JEOL).

**RNA extraction and semiquantitative RT-PCR.** Total bacterial RNA was extracted using TRIzol (Invitrogen) according to the manufacturer's instructions. The extracted RNA was quantified with a NanoDrop spectrophotometer (Thermo Fisher). Total RNA was treated with DNA-free DNase (Life Technologies) to remove contaminating DNA. The first strand of cDNA was synthesized using random primers (Promega) and Superscript III reverse transcriptase (Invitrogen). Controls used in the semiquantitative RT-PCR assay were as follows: DNA templates of the wild-type (WT) strain were included as the positive control for PCR, transfer-messenger RNA (tmRNA) amplification was the loading control for RT-PCR, and samples that lacked reverse transcriptase during cDNA synthesis were included as negative controls to evaluate potential DNA contamination. Primers used for the amplification of sample genes and tmRNA genes are listed in Table 2.

TABLE 2 Primers used in this study

Primer	Direction of primer	Sequence (5'–3') <sup>a</sup>	Purpose
SP1		GATAGATTGTGCGCACCTGATTG	Specific primer 1 for TAIL-PCR
SP2		AAGACGTTTCCCCTTGAATATG	Specific primer 2 for TAIL-PCR
SP3		GCAATGTAACATCAGAGATTTTGAG	Specific primer 3 for TAIL-PCR
AD1		NTCGASTWTSGWGTT	Arbitrary primer for TAIL-PCR
AD4		TGWGNANCASAGA	Arbitrary primer for TAIL-PCR
AD5		AGWGNAGWANCAWAGG	Arbitrary primer for TAIL-PCR
AD6		CAWCGICNGAIASGAA	Arbitrary primer for TAIL-PCR
AD8		STTGNTASTNCTNTGC	Arbitrary primer for TAIL-PCR
Kan	Sense Antisense	GCAATCAGGTGCGACAATC AAGTCAGCGTAATGCTCTGC	DNA probe used in Southern blotting
IFD2299	Sense-W Antisense-X Sense-Y Antisense-Z	GAATTCTTACTCCAGCTCGTGCTG ACTAGTAGCAACAAGGAAATCGCC ACTAGTGCCATCAGGACCACGTC AAGCTTGTGCGAGTTCTCATCGTC	$\Delta$ <i>fsnR</i> mutant construction
C2299	Sense Antisense	AAGCTTGTGCGAGTTCTCATCGTC GAATTCCTACTCCAGCTCGTGCTG	$\Delta$ <i>fsnR-fsnR</i> strain construction
P2299	Sense Antisense	CGCCATATGCGAGTTCTCATCGTCGAC AAGCTTCTCCAGCTCGTGCTGATG	FsnR protein expression
Pb2303	Sense Antisense	GTGATTCTCCTGGATCC CTCTTTCAGCGCTGTACCT	EMSA probe for <i>Smlt2303</i>
Pb2318	Sense Antisense	GGCTGCTGTATCGGCGGGGT GGCGCCTCCTGTGATGG	EMSA probe for <i>Smlt2318</i>
0706	Sense Antisense	GTGCCGTTGCCGGTTCGTAC GGACCTGGTTGTTCCGAGC	Semiquantitative RT-PCR analysis of <i>Smlt0706</i>
0710	Sense Antisense	CATCAACCGCCGTGGCC CTACGGTCTGCTGCAGGG	Semiquantitative RT-PCR analysis of <i>Smlt0710</i>
2274	Sense Antisense	GTGGGAGCATTTCATGGTC GAAATGAAGGAGAGCGAG	Semiquantitative RT-PCR analysis of <i>Smlt2274</i>
2283	Sense Antisense	GAGAACAGCATCAGCAGG CTTCACCCAGTCCAAGCAC	Semiquantitative RT-PCR analysis of <i>Smlt2283</i>
2290	Sense Antisense	GACCATCACCTTGGCGAG CTGCCGGGCACGGTGCTG	Semiquantitative RT-PCR analysis of <i>Smlt2290</i>
2297	Sense Antisense	GACGGTGGACTCGTGCATG AGTACGAACAGGCGCTGG	Semiquantitative RT-PCR analysis of <i>Smlt2297</i>
2303	Sense Antisense	GCTGACGTAGCCGTCGAC TGGGACTGCAGACGCGTG	Semiquantitative RT-PCR analysis of <i>Smlt2303</i>
2306	Sense Antisense	GAGATCTGCAGGCCGGAG TCCTTCACCAGCCAGCTG	Semiquantitative RT-PCR analysis of <i>Smlt2306</i>
2318	Sense Antisense	GTTCTCGCCTGAGGCGTG GTGCTCGATCCGGCGATG	Semiquantitative RT-PCR analysis of <i>Smlt2318</i>
2319	Sense Antisense	CAACACCAGGTGGAGGT CCGGGCAGCGTCACGCTG	Semiquantitative RT-PCR analysis of <i>Smlt2319</i>
2324	Sense Antisense	TCGTCTTCTGCAGGGAG CAGCTTCCATGCCGGCCTG	Semiquantitative RT-PCR analysis of <i>Smlt2324</i>
tmRNA	Sense Antisense	GGGGGTGCACTGGTTTCG TGGTGGAGGTGGCGGAAT	Semiquantitative RT-PCR analysis of tmRNA

<sup>a</sup> The letters used are standard IUB/IUPAC nucleic acid codes.



**FIG 1** Construction and screening of a random transposon insertion mutant library of *S. maltophilia*. (A) Determination of randomness and insertion copy numbers of mutants. Twenty-nine randomly chosen colonies of EZ::TN transposon insertion transformants and the WT strain were analyzed by Southern blotting. Total DNAs were extracted from the bacterial strains, digested with *Sma*I, and fractionated in a 1% agarose gel. A PCR product of the EZ::TN transposon was labeled with [ $\alpha$ - $^{32}$ P]dCTP and used as the DNA hybridization probe. M, 1-kb DNA ladder. (B) Pipeline of screening for mutants with biofilm deficiency. The number of mutants obtained after each round of screening is shown. Details of the screens are described in Materials and Methods.

#### Protein expression and electrophoretic mobility shift assay (EMSA)

Recombinant FsnR-His<sub>6</sub> protein was expressed in *E. coli* BL21(DE3) cells transformed with a recombinant pET30a (Novagen) expression vector. PCR primers used for the construction of the recombinant vector are listed in Table 2. The FsnR-His<sub>6</sub> protein was purified by Ni-nitrilotriacetic acid (Ni-NTA) affinity chromatography according to the manufacturer's instructions (Novagen). The purified protein was concentrated using Centricon YM-10 columns (Millipore, Australia), and the buffer was exchanged with storage buffer (50 mM Tris-HCl, 0.5 mM EDTA, 50 mM NaCl, 5% glycerol; pH 8.0) for further use.

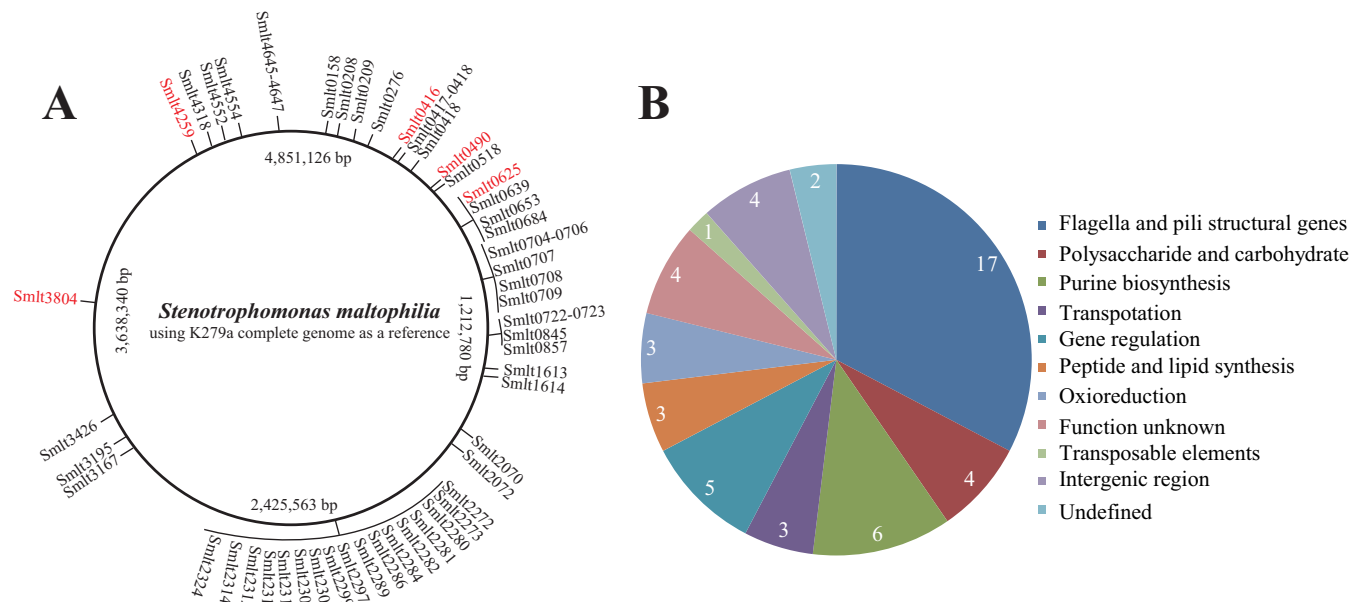
For EMSA, promoter regions of the *Smlt2303* and *Smlt2318* operons were amplified by PCR (see Table 2 for primer sequences). The PCR products (299 bp and 267 bp, respectively) were labeled with [ $\alpha$ - $^{32}$ P]dCTP by use of a Prime-A-Gene kit (Promega), and free [ $\alpha$ - $^{32}$ P]dCTP was removed by use of a ProbeQuant G-50 column (GE). DNA probes (4 fmol) and proteins (1  $\mu$ g) were coinubated in reaction buffer containing 10 mM Tris (pH 7.0), 50 mM KCl, 1 mM dithiothreitol (DTT) (pH 7.5), 2.5% glycerol, 5  $\mu$ l MgCl<sub>2</sub>, 50 ng/ $\mu$ l poly(dI-dC), 0.05% NP-40, and 10 mM EDTA for 20 min at room temperature. A 4- $\mu$ l aliquot of DNA loading buffer (0.25% bromophenol blue, 40% sucrose) was added to stop the reaction, and the samples were loaded into a 5% native PAGE gel. Electrophoresis was performed at 120 V for about 40 min, using 0.5 $\times$  Tris-borate-EDTA (TBE) buffer. The gel was placed in a ziplock bag, and phosphorimaging screens were used for signal detection. Unlabeled probes were used in the competitive experiments.

## RESULTS

**Construction and screening of random insertion mutant library of *S. maltophilia*.** *S. maltophilia* ATCC 13637 is a clinical strain that was isolated from the oropharyngeal region of a patient with mouth cancer. It was chosen for use in the current study because it rapidly forms biofilms, within several hours. A random Tn5 trans-

poson insertion mutant library was constructed with the EZ::TN transposome system (16). The library comprises 37 384-well plates and 14,208 kanamycin-resistant transformants. To evaluate the randomness and proportion of single-copy insertions, total DNAs from 29 randomly chosen clones were extracted, digested with *Sma*I, and analyzed by Southern blotting. As shown in Fig. 1A, 27 clones (93%) contained single-copy insertions, while 2 clones (7%) (lanes 13 and 14) simultaneously received two EZ::TN transposon insertions. Additionally, six clones cultivated in NYGB medium in the absence of kanamycin for 20 generations maintained their resistance to kanamycin, confirming the stability of the transposon inserts (see Fig. S1 in the supplemental material).

The library was then screened for mutants with altered biofilm-forming capabilities by using the crystal violet staining assay. Bacterial growth was also quantified to eliminate potential growth-associated contributions to changes in biofilm formation. The primary screening round identified a total of 396 clones exhibiting changes in the quantity of biofilm on the polypropylene surface (Fig. 1B). These clones were selected, propagated in 96-well plates, and subjected to four additional rounds of screening by biofilm assay. Of the 396 clones selected, 244 were identified as being false positive, because their phenotypic change failed to be replicated stably throughout the screening process. Additionally, 53 clones with poor or no growth were excluded from subsequent analyses because the decrease in biofilm formation may have been caused by auxotrophy or slow growth. Thermal asymmetric inter-laced PCR (TAIL-PCR) was used to identify genomic sequences flanking the EZ::TN transposon insertion sites for 99 clones ex-



**FIG 2** Mapping of insertion sites on the genome of *S. maltophilia*. (A) Schematic view of insertion sites on the genome of *S. maltophilia*, using the complete genome of K279a as a reference. Coding sequences (CDSs) were labeled per the genomic annotation for K279a. Genes carrying mutations which resulted in decreases and increases in biofilm formation are depicted in black and red, respectively. (B) Functional classification of mutated genes. Detailed information on the mutated genes in both panels is provided in Table S1 in the supplemental material.

hibiting decreased (80 clones) and increased (19 clones) biofilm formation.

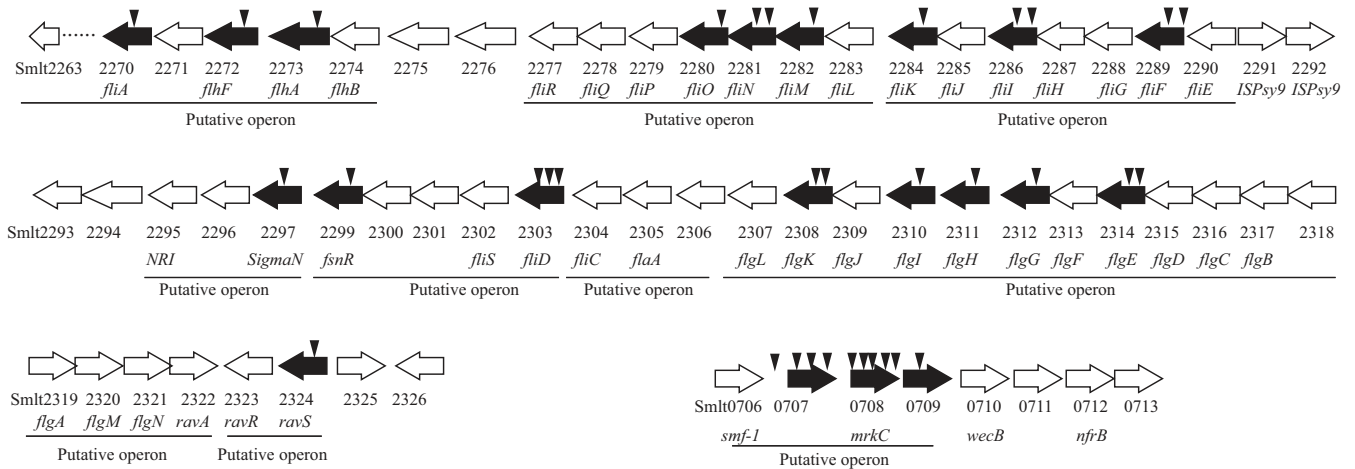
**Identification of genes responsible for biofilm formation emphasizes the role of flagellar biosynthesis.** TAIL-PCR analysis successfully identified the transposon insertion sites in each of the clones. A total of 46 nonredundant genes, 4 intergenic regions, and 2 undefined insertion sites within a multicopy locus were identified (Fig. 2A; see Table S1 in the supplemental material). Because the genome of *S. maltophilia* ATCC 13637 has not been sequenced, the inserted genes were mapped onto the complete genome of *S. maltophilia* reference strain K279a (1). The mutant genes can be functionally classified into the following nine categories: (i) flagellum and pilus assembly; (ii) polysaccharide biosynthesis, e.g., *xanA*; (iii) peptide and lipid synthesis; (iv) purine biosynthesis, e.g., *purE*, *purK*, *guaA*, and *purC*; (v) transmembrane transport; (vi) gene expression regulation; (vii) oxidoreduction; (viii) transposable elements; and (ix) unknown function (Fig. 2B; see Table S1). Overall, mutations identified in 5 genes were associated with an increase in biofilm formation, while the remaining 41 genes showed deficits. Southern blotting revealed that all the above-mentioned mutants contained single-copy transposons (see Fig. S2).

Almost half of the genes identified (20 genes [43.5%]) were associated with flagellar structure and regulation, which is highly suggestive of the flagellum playing a prominent role in *S. maltophilia* biofilm formation. As shown in Fig. 3, these genes were organized into 10 putative operons in the genome of *S. maltophilia* reference strain K279a. These operons encode structural and assembly proteins associated with different stages of flagellar assembly. FlhA, FlhO, FlhN, FlhM, FlhI, FlhF, FlgI, FlgH, and FlgG are structural components of the basal body. FlhD is the flagellar filament capping protein, while FlhK controls flagellar hook length during assembly. FlhF is a putative GTPase that is often necessary

for assembly and placement of polar flagella. FlhJ acts as a chaperone, exporting flagellar proteins from the cytoplasm into the periplasm. FlgL, FlgK, FlgJ, and FlgE are components of the flagellar hook and assist in connecting the basal body to the filament.

It is noteworthy that five regulatory genes were identified within or in the vicinity of flagellar gene clusters. *Smlt2270* (*fliA*) and *Smlt2297* encode  $\sigma^{28}$  and  $\sigma^{54}$ , respectively. These alternative sigma factors are involved in regulating flagellar gene transcription. *Smlt0158* encodes a histidine kinase (NRII or GlnL) which may phosphorylate the putative cognate response regulator *Smlt0159* (NRI or GlnG). *Smlt2324* encodes a PAS/PAC domain-containing histidine kinase that is orthologous to RavS of *Xanthomonas campestris* (23). In the vicinity of *ravS*, there is another histidine kinase gene (*ravA* or *Smlt2322*) and a GGDEF/EAL domain-containing response regulator gene, *Smlt2323* (an ortholog of *ravR*). Additionally, a response regulator gene, *Smlt2299*, was also identified. *Smlt2299* encodes a putative protein product with 210 amino acid residues. The protein contains an N-terminal receiver domain for accepting phosphoryl groups and a C-terminal LuxR-type DNA-binding domain. In the absence of a histidine kinase gene near *Smlt2299*, its product, *Smlt2299*, was considered an “orphan” response regulator. To our knowledge, *Smlt2299* and its orthologs have not been reported previously. As this gene is not an ortholog of genes for known flagellum-associated response regulators, such as the FlgR proteins of *Campylobacter jejuni* and *Helicobacter pylori*, we proposed “FsnR,” for flagellum biosynthesis regulator, as a more suitable name. The regulatory role of FsnR was investigated further in the current study.

**FsnR controls biofilm development and flagellar biosynthesis.** Since *fsnR* is putatively located in an operon and transposon insertion may lead to polar effects to complicate genetic analysis, we constructed a nonpolar, in-frame deletion mutant of *fsnR* via homologous double-crossover recombination. This mutant strain

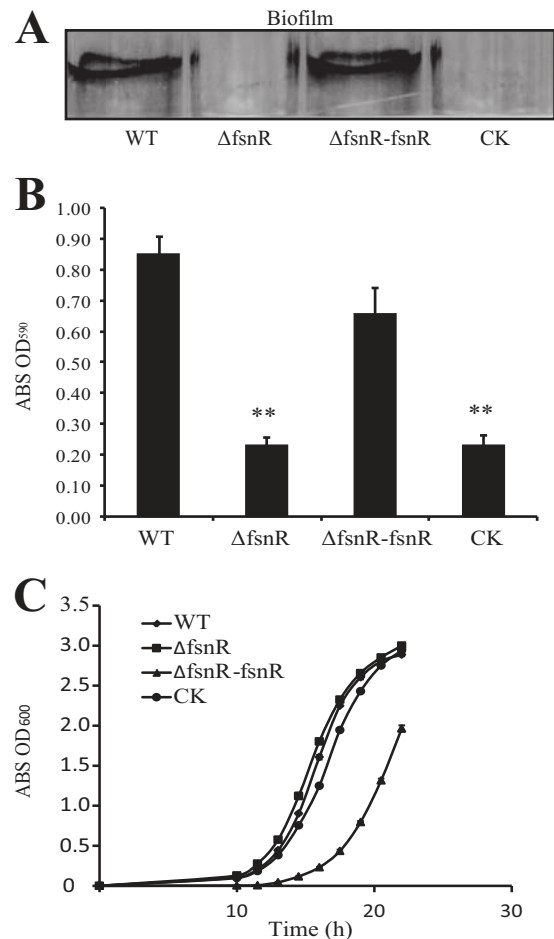


**FIG 3** Transposon insertions in gene clusters associated with flagellar or pilus assembly. White arrows indicate CDSs and the direction in which they are transcribed. Black arrows indicate genes disrupted by transposon insertions. Black triangles indicate independent EZ::TN transposon insertions. Virtual Institute of Microbial Stress and Survival (VIMSS) operon predictions are illustrated below.

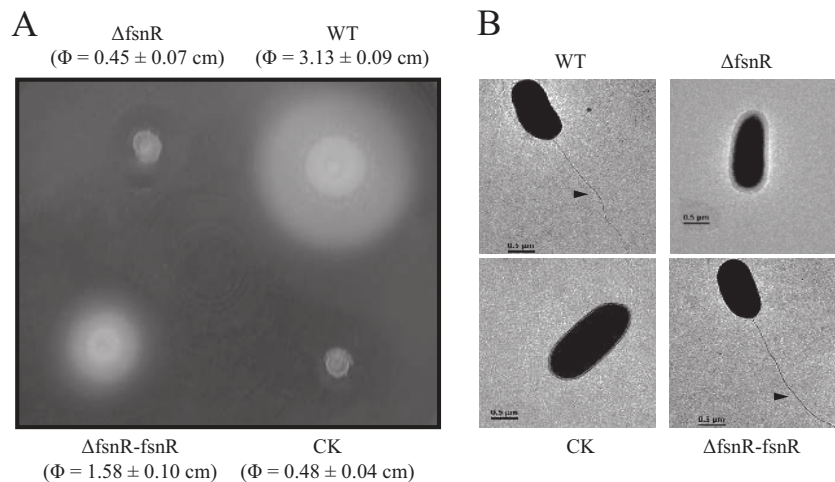
was labeled SM001 ( $\Delta fsnR$ ). To generate the complementary gene mutation, the full-length sequence of *fsnR* was amplified by PCR, cloned into the broad-host-range vector pHM1, and transformed into SM001, giving rise to strain SM002 ( $\Delta fsnR$ -*fsnR*). In this construct, *fsnR* was under the control of the *lacZp* promoter. As shown in Fig. 4A and B, SM001 biofilm production decreased significantly, to 27.5% of the WT level, while genetic complementation substantially restored the bacterium's biofilm-forming ability (to 77.6% of the WT level). Similar growth curves were observed for the SM001 and WT strains, while SM002 grew more slowly (Fig. 4C). The disparity in growth curves may have been caused by overexpression of the *fsnR* gene for SM002. These results demonstrated that mutations in the *fsnR* gene were responsible for deficiencies in bacterial biofilm formation and that the phenotypic change was not due to changes in growth rate.

Because *fsnR* resides in an operon with *fliS*, *fliD*, and two as yet functionally unknown genes, *Smlt2300* and *Smlt2301*, we surmised that *Smlt2299* may be required for the regulation of flagellar biosynthesis and bacterial motility. As such, swimming motility and flagellar morphology were investigated for each of the bacterial strains. The SM001 mutant was found to be nonmotile on semisolid NYG agar plates containing 0.1% agar, while genetic complementation partially restored the swimming ability of SM002 to 50.5% of the WT strain level (Fig. 5A). Transmission electron microscopy confirmed the presence of clearly visible polar, single flagella for most WT and SM002 cells, while SM001 cells lacked flagella (Fig. 5B). These results demonstrate that the response regulator FsnR controls flagellar biosynthesis and cell motility.

**FsnR directly binds to promoters of flagellar genes and activates their transcription.** Semiquantitative RT-PCR was used to establish whether FsnR is a critical transcription factor in controlling flagellar gene expression. The transcription levels of 11 genes located in the 5' regions of operons associated with flagellar assembly (Fig. 3) were measured. As shown in Fig. 6A, with the exception of *Smlt0710*, whose expression was undetectable, the quantities of mRNA for these representative genes were markedly reduced in the *fsnR* mutant. In contrast, the transcriptional levels of all genes were either restored or increased to levels greater than



**FIG 4** Mutations in *fsnR* substantially reduced biofilm formation. (A) Crystal violet-stained biofilms formed by bacterial strains. (B) Quantification of bacterial biofilm formation by OD<sub>590</sub> measurements. Error bars represent standard deviations ( $n = 4$ ). \*\*,  $P < 0.01$ , calculated by Student's  $t$  test. WT, wild-type strain;  $\Delta fsnR$ , in-frame *fsnR* deletion mutant SM001;  $\Delta fsnR$ -*fsnR*, complementary strain SM002; CK,  $\Delta fsnR$  strain containing blank pHM1 vector (SM003). ABS, absorbance. (C) Growth curves for bacterial strains in rich NYG medium. Error bars represent standard deviations ( $n = 4$ ).

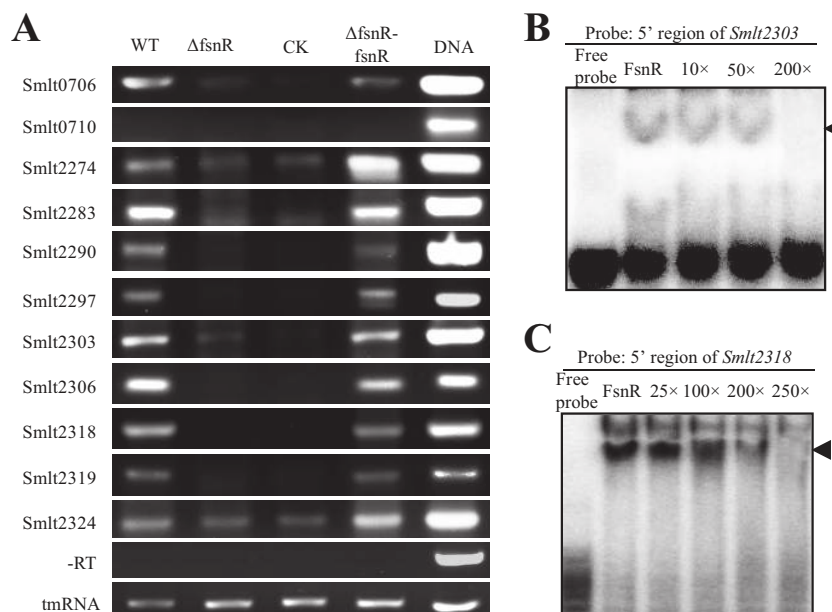


**FIG 5** *fsnR* mutation resulted in deficiencies in swimming motility and flagellar development. (A) Swimming motilities of bacterial strains. All strains were inoculated into 0.1% NYG agar and grown for 36 h. WT, wild-type strain;  $\Delta fsnR$ , in-frame *fsnR* deletion mutant SM001;  $\Delta fsnR$ -*fsnR*, complementary strain SM002; CK,  $\Delta fsnR$  strain containing blank pHM1 vector (SM003). (B) *fsnR* mutation caused abnormalities in flagellar assembly. Bacterial morphology was observed with a scanning transmission electron microscope. Magnification,  $\times 4,000$ . Black arrows indicate the presence of flagella.

those for the WT for the complementary strain SM002. This result suggests that FsnR is a canonical positive regulator directly or indirectly controlling the transcription of most flagellar genes.

FsnR has a C-terminal LuxR-type DNA-binding domain and may act as a transcription factor. A recombinant FsnR-His<sub>6</sub> protein expressed in *E. coli* and purified by Ni-NTA affinity chromatography was used in EMSAs to establish whether FsnR binds to the 5' promoters of the *Smlt2303* and *Smlt2318* operons. The pro-

motor region of the *Smlt2303* operon was selected because *fsnR* is putatively located within this operon. If FsnR binds to the *cis*-regulatory elements of the *Smlt2303* operon, it will form an auto-regulation loop. Conversely, the 5' promoter sequence of the *Smlt2318* operon was selected because the operon contains 12 genes (*Smlt2307* to *Smlt2318*) and is the largest gene cluster of all flagellum-associated operons. FsnR-His<sub>6</sub> formed stable protein-DNA complexes with the promoter sequences of both *Smlt2303*



**FIG 6** FsnR controls expression of flagellar genes and directly binds to promoter regions. (A) Semi-quantitative RT-PCR assays of flagellum-associated gene transcription. The bands represent RT-PCR amplification of cDNAs synthesized from total RNA of bacterial strains. -RT, negative control in which reverse transcriptase was absent during cDNA synthesis. Amplification using bacterial DNA and cDNA for tmRNA as templates were used as the positive control and loading control, respectively. WT, wild-type strain;  $\Delta fsnR$ , in-frame *fsnR* deletion mutant;  $\Delta fsnR$ -*fsnR*, complementary strain. (B and C) EMSAs detected FsnR binding directly to promoter regions of the *Smlt2303* (B) and *Smlt2318* (C) operons. Four femtomoles of [ $\alpha$ -<sup>32</sup>P]dCTP-labeled double-stranded DNA corresponding to the *Smlt2303* or *Smlt2318* promoter region was used in each reaction mixture. Increasing amounts of unlabeled probes were used as competitors (10 $\times$  to 250 $\times$ ) for binding to 1  $\mu$ g of FsnR-His<sub>6</sub> recombinant protein. Black triangles indicate the protein-DNA binding complexes.

(Fig. 6B) and *Smlt2318* (Fig. 6C). The addition of unlabeled probes effectively competed with the binding of FsnR-His<sub>6</sub> to isotope-labeled probes. These results demonstrated that FsnR specifically binds to the promoter regions of the *Smlt2303* and *Smlt2318* operons and activates their transcription, which may be a critical regulatory process during *S. maltophilia* biofilm development.

## DISCUSSION

The molecular process of *S. maltophilia* biofilm formation is important in defending this emergent pathogen (3). Here we identified 46 genes containing mutations associated with changed biofilm formation (Fig. 2; see Table S1 in the supplemental material). As these genes are involved in multiple biochemical and regulatory cascades, we surmised that *S. maltophilia* biofilm formation is a tightly controlled and highly complex process. With the exception of a few genes, such as *xanA*, which is involved in polysaccharide biosynthesis (12), all remaining genes had not previously been reported, and hence their roles in biofilm development require further investigation. We provided evidence that an orphan response regulator, FsnR, directly binds to promoters of at least two flagellum-associated operons, activating their transcription (Fig. 6). Inactivation of *fsnR* resulted in deficiencies in flagellar assembly, swimming motility, and biofilm formation (Fig. 4 and 5). These results identify FsnR as a pivotal modulator of *S. maltophilia* biofilm formation and, as such, a desirable target in the development of novel antibacterial agents.

In the current study, we identified 17 structural and 3 regulatory genes associated with flagellar assembly (see Table S1 in the supplemental material). The vital role that bacterial flagella play in biofilm development is well recognized (24). Flagella not only contribute to cell motility during biofilm formation and dispersal but also take part in surface sensing and colonization (25). Consequently, subtle regulation of flagellar biosynthesis and cell motility behavior is important for generating and maintaining the bacterial biofilm matrix. In general, regulatory genes of flagella can be assigned to one of three groups: group I regulates flagellar gene expression and assembly, group II regulates flagellum-associated chemotaxis, and group III regulates modes of flagellar operation (24). To date, these regulatory genes have not been studied in *S. maltophilia*. Here we identified a number of regulatory genes. *fsnR* was characterized as a group I transcription regulator responsible for activation of a number of flagellar genes of *S. maltophilia*. BLASTP sequence alignment revealed that FsnR shared the greatest homology with *E. coli* UvrY ( $e = 1e-40$ ; identity = 38%; 100% coverage). Inactivation of *uvrY* caused deficiencies in biofilm formation and virulence for a number of enterobacterial species. A *uvrY* gene mutation downregulated type 1 and Pap fimbriae in *E. coli* (26). Teplitski et al. reported that *uvrY* (*sirA*) inactivation caused repression of the master regulator of flagellar genes, *flhDC*, in *Salmonella enterica* (27). The present study provides molecular evidence that FsnR is a transcription activator directly binding to the promoter regions of flagellar genes (Fig. 6B). Collectively, these results indicate that a UvrY/FsnR-like response regulator may modulate flagellar gene expression on multiple levels or that these response regulators have experienced functional differentiation of signal rewiring during bacterial evolution. Furthermore, FsnR is an “orphan” response regulator whose cognate histidine kinase remains unknown. Identification of its cognate histidine kinase by use of computational methods for prediction of protein-protein interactions, together with phosphorylation profiling (28,

29), will improve our understanding of the effects that environmental stimuli have on FsnR-mediated flagellar assembly.

Four other regulatory genes (*Smlt2270*, *Smlt0158*, *Smlt2297*, and *Smlt2324*) associated with *S. maltophilia* biofilm formation were identified in the current study. *Smlt2270* (*fliA*) encodes the flagellar sigma factor FliA ( $\sigma^{28}$ ). The  $\sigma^{28}$ -containing holoenzyme of *S. enterica* serovar Typhimurium was shown to selectively bind to the  $-35$  regions downstream of *fliC* and the anti-sigma-factor gene *fliM*, activating their transcription at a late stage of flagellar assembly (30). Furthermore,  $\sigma^{28}$  was shown to control type III secretion system gene expression in *S. Typhi* (31), suggesting that it is a global regulator connecting different physiological pathways. The regulatory gene *Smlt2297* resides in the *fsnR* operon, and hence its transcription is also modulated by FsnR autoregulation. *Smlt2297* encodes an alternative sigma factor ( $\sigma^{54}$ ) that is known to regulate flagellar gene expression in a diverse range of bacterial species, including *Enterococcus faecalis* (32), *Vibrio fischeri* (33), *Pseudomonas aeruginosa* (34), and *Burkholderia cenocepacia* (35). Consequently, inactivation of  $\sigma^{28}$  or  $\sigma^{54}$  has been shown to result in deficiencies in motility, virulence, and biofilm formation. To date, the consensus binding motifs of  $\sigma^{28}$  and  $\sigma^{54}$  in most of the aforementioned bacteria, including *S. maltophilia*, remain unclear.

As with *fsnR*, the regulatory genes *glnL* and *ravS* encode histidine kinase sensors belonging to two-component signal transduction systems. GlnL (*Smlt0158* or NRII) is a putative histidine kinase with a predicted transmembrane domain. The presence of a PAS domain in the N-terminal region of GlnL suggests that GlnL has the capacity to detect intracellular redox potentials, oxygen, or other stimuli. Not surprisingly, *glnL* is situated with a putative cognate response regulator gene, *glnG* (*Smlt0159*). GlnG (NRI) is an NtrC family transcription factor containing the receiver AAA and a helix-turn-helix output domain. Future studies will focus on the GlnL-GlnG regulon and its relationship to *S. maltophilia* biofilm formation. The fourth regulatory gene identified in the present study was *Smlt2324*, an ortholog of the *ravS* gene of *X. campestris*. In *X. campestris*, *ravS* encodes a PAS domain-containing histidine kinase and appears to constitute a “three-component” signal transduction system (RavSAR) with the histidine kinase RavA and the response regulator RavR (23, 36). The RavSAR system seems to regulate bacterial gene expression by affecting turnover of the second messenger c-di-GMP, which is conceivable because RavR contains both putative diguanylate cyclase (GGDEF) and c-di-GMP phosphodiesterase (EAL) domains (23). RavSAR gene inactivation markedly attenuated the virulence of *X. campestris* (23, 36). However, comparatively little is known of RavSAR regulation, and it would be worthwhile to investigate the role of RavS during RavA-RavR phosphotransfer, as well as the nature of the signals detected by RavS and RavA, and to establish how these three proteins constitute a dynamic protein complex during regulation.

Gene mutations causing increased biofilm formation have been identified in other bacterial species, including lipopolysaccharide-related genes in *E. coli* (37), an S-ribosylhomocysteinase gene (*luxS*) in *Listeria monocytogenes* (38), and two-component signaling system genes in *P. aeruginosa* (39). We identified five genes whose mutants exhibited substantial increases in biofilm formation compared to that of the WT strain (Fig. 2A; see Table S1 in the supplemental material). Four of these genes, *Smlt3804*, *Smlt4259*, *Smlt0490*, and *Smlt0625*, encode enzymes, while



*Smlt0416* encodes a protein of unknown function. Insufficient information was obtained in the current study to elucidate a relationship between biofilm formation and the functionality of these genes. For example, *Smlt3804* encodes a glyceraldehyde-3-phosphate dehydrogenase (GAPDH) which is involved in glycolysis. Studies have suggested a role for GAPDH in bacterial biofilm formation. GAPDH expression was altered in mutants with biofilm deficiencies for *Streptococcus suis* and *Streptococcus mutans* (40, 41). In *Streptococcus oralis*, cell surface GAPDH acts as the binding site for biofilm formation by the periodontopathic bacterium *Porphyromonas gingivalis* (42, 43). These studies indicated that GAPDH has a role in bacterial biofilms. However, further experimental evidence is needed to elucidate why mutations in the five genes listed above resulted in increases in *S. maltophilia* biofilm production.

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