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Differential Biofilm Formation and Motility Associated with Lipopolysaccharide/Exopolysaccharide-Coupled Biosynthetic Genes in *Stenotrophomonas maltophilia*

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Stenotrophomonas maltophilia **WR-C is capable of forming biofilm on polystyrene and glass. The lipopolysaccharide/exopolysaccharide-coupled biosynthetic genes *rmlA*, *rmlC*, and *xanB* are necessary for biofilm formation and twitching motility. Mutants with mutations in *rmlAC* and *xanB* display contrasting biofilm phenotypes on polystyrene and glass and differ in swimming motility.**

Microorganisms can develop biofilms or clogging mats, causing the failure of septic tanks, systems for on-site wastewater disposal. If water in these clogged systems were contaminated by pathogens, it would pose a threat to human health. We isolated *Stenotrophomonas maltophilia* strain WR-C from a clogged septic tank system that consistently formed biofilms on sand grains, produced exopolysaccharides (EPS), and caused clogging in sand columns (33). *S. maltophilia* is a gram-negative, rod-shaped, and obligate aerobic bacterium with polar flagella in the γ-β subdivision of *Proteobacteria* (3, 14). It is found in various environments and recently has emerged as an important human pathogen. Very little is known about the mechanisms of biofilm formation by *S. maltophilia*. It has been shown to adhere to HEp-2 cells as well as abiotic surfaces, such as plastic, glass, and Teflon (7–10, 16).

To identify genes that are involved in biofilm formation by *S. maltophilia* WR-C, about 4,500 transposon mutants generated with the EZ::TN < R6Kγori/KAN-2 > Tnp transposome (Epicenter, Madison, Wis.) were screened for defects in biofilm formation in 96-well polystyrene plates (Becton-Dickinson Labware, Franklin Lakes, N.J.) by using a modified microtiter plate assay (11, 29). Briefly, wells containing 200 μl Trypticase soy (TS) broth were inoculated with overnight cultures and incubated at 30°C, 50 rpm for 2 days. Biofilm cells were stained with 0.1% crystal violet and washed, the stain remaining in the cells was solubilized with 70% ethanol, and the optical density at 590 nm was determined. Three mutants, TPH7, TPH11, and TPH13, whose growth was similar to that of the wild type but which were deficient in biofilm formation, were selected for further characterization.

The transposon flanking regions were rescued by "rescue cloning" as described by the manufacturer and sequenced by using the primers KAN-2 FP-1 and R6KAN-2 RP-1 (Table 1). The transposon-inserted genes are homologous to three genes involved in the biosynthesis of nucleotide sugar precursors of lipopolysaccharide (LPS) and EPS in *Xanthomonas campestris* pv. campestris ATCC 33913 (GenBank accession no. AE008922) (6). The genes are *rmlC* (for TPH7, 74% identity), *rmlA* (for TPH11, 78% identity), and *xanB* (for TPH13, 80% identity), which are located in the *rml* and *xan* operons of *X. campestris* pv. campestris (19, 20). As no genome sequence is available and the gene cluster involved in LPS/EPS biosynthesis has not been reported for *S. maltophilia*, the DNA segments flanking the transposon in TPH7, TPH11, and TPH13 were sequenced. The complete sequences of the *rmlBACD* and *xanAB* operons and their flanking genes were determined. The genetic organization of these genes is different from that of *X. campestris* pv. campestris (Fig. 1). The *xanA* sequence from *S. maltophilia* WR-C shares 75% identity with *xanA* in *X. campestris* pv. campestris and 72% identity with *spgM* (GenBank accession no. AY179964) in *S. maltophilia* K1014 (24).

To determine whether phenotypic differences that were observed in TPH7, TPH11, and TPH13 are due to mutations generated in the *rml* and *xan* operons, *rmlBACD*, *xanAB*, and their respective predicted promoters were cloned into pJN105 to generate p*rmlBACD* and p*xanAB* for complementation. The plasmids were electroporated into the wild type and TPH7, TPH11, and TPH13 to generate the respective complemented strains as listed in Table 2. Providing p*rmlBACD* in *trans* in TPH7 and TPH11 and p*xanAB* in TPH13 restored all of the phenotypes tested to those of the wild type (Table 3 and described below), while the introduction of pJN105 into the mutants had no effect. The presence of pJN105, p*rmlBACD*, or p*xanAB* in the wild type did not affect the phenotypes tested (not shown).

To determine whether *rmlA*, *rmlC*, and *xanB* in *S. maltophilia* WR-C play a role in LPS biosynthesis, Tris-Tricine sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis of the LPS was performed with proteinase

TABLE 1. Oligonucleotides used in this study

Oligonucleotide	Sequence (5′→3′)	Restriction site
KAN-2 FP-1	ACCTACAACAAAGCTCTCATCAACC	
R6KAN-2 RP-1	CTACCCTGTGGAACACCTACATCT	
XbaI-rml	TCTAGAACGTAATTGTGTTCAATG	XbaI
SacI-rml	GAGCTCTCAGCGCGCGGGCAGTTGTG	SacI
XbaI-xan	TCTAGAAGCGACCTGCAGGTG3	XbaI
SacI-xanB	GAGCTCTCAGGCCGGGGCGCGCCCGT	SacI

[a] Underlining indicates restriction sites.

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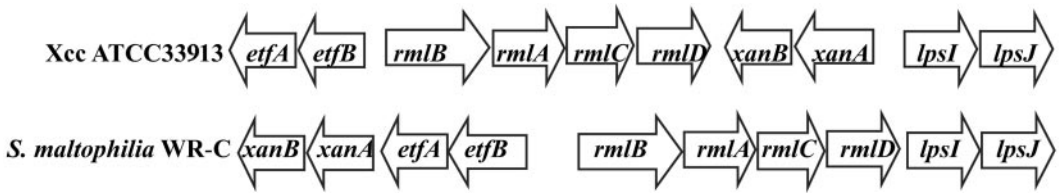

FIG. 1. Genetic organization of LPS biosynthetic genes and their flanking genes in *X. campestris* pv. campestris (Xcc) ATCC 33913 (GenBank accession no. AE008922) and *S. maltophilia* WR-C (GenBank accession no. AY956411). The genes depicted are not proportional to their respective sequence lengths.

K-digested whole-cell lysates (13). The wild type produced both the diffuse O-antigen-containing LPS and the LPS core and lipid A (Fig. 2); these results were similar to what has been observed with *X. campestris* pv. campestris (20, 39). No O-antigen-containing LPS was observed in the *rmlC*, *rmlA*, and *xanB* mutants. The *xanB* mutant produced a truncated core and lipid A. These results suggest that *rmlC* and *rmlA* are necessary for the biosynthesis of the LPS O-antigen and *xanB* for the O-antigen and LPS core. Nonmucoid morphology on TS agar and autoagglutination in TS broth were observed in the *xanB* mutant but not the *rmlC* and *rmlA* mutants and wild type (not shown). These phenotypical alterations are correlated with defective LPS in other gram-negative bacteria (4, 19, 21, 27). In addition to LPS biosynthesis, *rml* and *xanB* orthologues in *Pseudomonas aeruginosa*, *Escherichia coli*, and *X. campestris* pv. campestris are required for the biosynthesis of exopolysaccharides (19, 32). The *xanB*, *rmlA*, and *rmlC* mutants all produced lower amounts of EPS when assayed by the phenol-sulfuric acid method for total carbohydrates (13) (not shown), suggesting that the three genes are also involved in EPS biosynthesis in *S. maltophilia* WR-C. Whether *S. maltophilia* produces an EPS similar to xanthan or alginate is unknown.

Biofilm formation by the *rmlC*, *rmlA*, and *xanB* mutants was significantly decreased on polystyrene plates (a relatively hydrophobic surface) compared to that by the wild type ($P < 0.05$; Student's two-sample *t* test and one-way analysis of variance [ANOVA]) (Fig. 3A). Interestingly, the *rmlA* and *rmlC* mutants developed more biofilm in glass tubes (a relatively hydrophilic surface) than did the wild type and *xanB* mutant ($P < 0.05$) (Fig. 3B). Differential attachment was also reported for O-antigen-deficient mutants of *Pseudomonas fluorescens* (41) and *P. aeruginosa* (22) relative to the corresponding wild-type strains and was attributed in part to a differences in cell surface hydrophobicity. By the bacterial adhesion to hexadecane assay (34), we did not observe any difference in the relative cell surface hydrophobicities displayed by the *S. maltophilia* wild type and its mutants (not shown), possibly because the strains were too hydrophilic (16) to be evaluated by this method. Alternatively, other unknown factors or mechanisms that are associated with alterations in the LPS or EPS may be involved.

TABLE 2. Bacterial strains and plasmids

Strain or plasmid	Relevant characteristics[a]	Source/reference
Strains		
E. coli		
DH5α	F$^-$ ϕ80d*lacZ*Δ*M15* Δ(*lacZYA-argF*)*U169 recA1 endA1 hsdR17*(r_K^- m_K^+) *phoA supE44* λ$^-$ *thi-1 gyrA96 relA1*	Invitrogen
DH5α λpir	DH5α λpir	A. Charkowski
S. maltophilia		
WR-C	Wild type	33
TPH7	Km^r, *rmlC*::EZTN transposon mutant, WR-C derivative	This work
TPH11	Km^r, *rmlA*::EZTN transposon mutant, WR-C derivative	This work
TPH13	Km^r, *xanB*::EZTN transposon mutant, WR-C derivative	This work
TPH41	Gm^r, WR-C harboring pJN105	This work
TPH42	Gm^r, TPH7 harboring pJN105	This work
TPH43	Gm^r, TPH11 harboring pJN105	This work
TPH44	Gm^r, TPH13 harboring pJN105	This work
TPH46	Gm^r, TPH7 harboring p*rmlBACD*	This work
TPH47	Gm^r, TPH11 harboring p*rmlBACD*	This work
TPH48	Gm^r, TPH13 harboring p*xanAB*	This work
Plasmids		
pJN105	Gm^r, arabinose-inducible expression vector	26
pGEM-T-Easy	Ap^r, *lacZ′* cloning vector	Promega
p*rmlBACD*	Gm^r, 3.9-kb *rmlBACD* with native promoter was PCR amplified with XbaI-rml and SacI-rml and cloned into the XbaI and SacI sites of pJN105	This work
p*xanAB*	Gm^r, 3.2-kb *xanAB* with predicted promoter was PCR amplified with XbaI-xan and SacI-xanB and cloned into the XbaI and SacI sites of pJN105	This work

TABLE 3. Phenotypes of *S. maltophilia* WR-C

Genotype	Biofilm[a]		LPS[b]		Relative motility		Flagella[e]	Growth characteristics		Growth (OD_{620})[f]	
	Polystyrene	Glass	O antigen	Core lipid A	Swimming[c]	Twitching[d]		Colony	Autoagglutination[d]	Without SDS	With 0.01% SDS
WT pJN105	++++	+	+	+	++	+	+	Mucoid	−	0.366 ± 0.140	0.095 ± 0.034
rmlC pJN105	++	+++	−	+	++	−	+	Mucoid	−	0.281 ± 0.065	0.106 ± 0.017
rmlA pJN105	+++	++	−	+	++	−	+	Mucoid	−	0.302 ± 0.050	0.107 ± 0.020
xanB pJN105	+	+	−	−	+	−	±	Nonmucoid	+	0.222 ± 0.043	0.002 ± 0.002[g]

[a] Results indicate relative biofilm formation (+ to ++++) in polystyrene microtiter plates and glass tubes.
[b] Results are from an SDS-PAGE analysis of LPS from proteinase K-digested whole-cell lysates of *S. maltophilia*. + and −, presence and absence of O antigen, core, or lipid A.
[c] + and ++, relative degree of swimming motility.
[d] + and −, presence and absence of twitching motility or autoagglutination. Autoagglutination was determined in 3-ml TS broth cultures in 13- by 100-mm borosilicate glass tubes (Fisher Scientific, Hampton, N.H.) incubated at 30°C, 200 rpm for 24 h.
[e] +, flagella present; −, flagella usually absent in the *xanB* mutant but occasionally present when cells were not linked.
[f] Susceptibility of *S. maltophilia* WR-C to SDS. Results represent the means and the standard deviations of three independent experiments. There was a significant difference ($P < 0.05$; one-way ANOVA) between all cultures without and with 0.01% SDS. OD_{620}, optical density at 620 nm.
[g] There was a significant difference ($P < 0.05$; one-way ANOVA) relative to other cultures with 0.01% SDS.

Flagellum-mediated swimming is involved in biofilm development (28, 36). Alterations in LPS were reported to interfere with the production, export, or assembly of flagella in *E. coli*, *Helicobacter pylori*, *P. aeruginosa*, and *Salmonella enterica* serovar Typhimurium (2, 12, 18, 30). The swimming motility of our strains was determined on TrA (1% tryptone, 0.5%NaCl, and 0.25% Bacto agar) (35). Overnight cultures (5 μl) were spotted on TrA plates (60 by 15 mm, containing 8 ml TrA) and incubated at 30°C. Only the *xanB* mutant was less motile than the wild type (Fig. 4A). One possible cause for the impairment in swimming motility in the *xanB* mutant could be a lack of flagellar formation as a result of the truncated LPS core. Cells of all strains were harvested from the edge of a 2-day swimming culture on TrA and stained with crystal violet by using a method described by Mayfield and Inniss (23) and modified by Kearns and coworkers (17). Flagella were observed consistently in the wild type and *rmlC* and *rmlA* mutants (Fig. 5), all of which existed predominantly as single cells. In contrast, most cells of the *xanB* mutant were linked into chains of three or more bacteria that were devoid of flagella. This likely contributed to the autoagglutination by the *xanB* mutant that was observed in liquid medium (not shown). Occasionally single flagellated cells were observed.

Type IV pilus-mediated twitching motility is involved in bio-

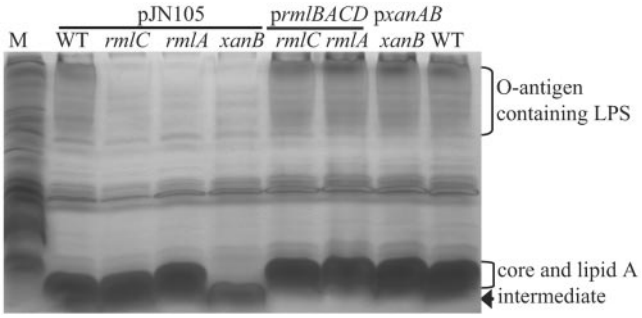

FIG. 2. SDS-PAGE analysis of LPS from proteinase K-digested whole-cell lysates of *S. maltophilia*. The *rmlC*, *rmlA*, and *xanB* mutants were defective in biosynthesis of LPS O-antigen. The *xanB* mutant was also defective in LPS core. The wild-type (WT) LPS profile was restored in the complemented strains. M, LPS standard from *Salmonella enteritidis* serovar Typhimurium (12.5 μg; Sigma).

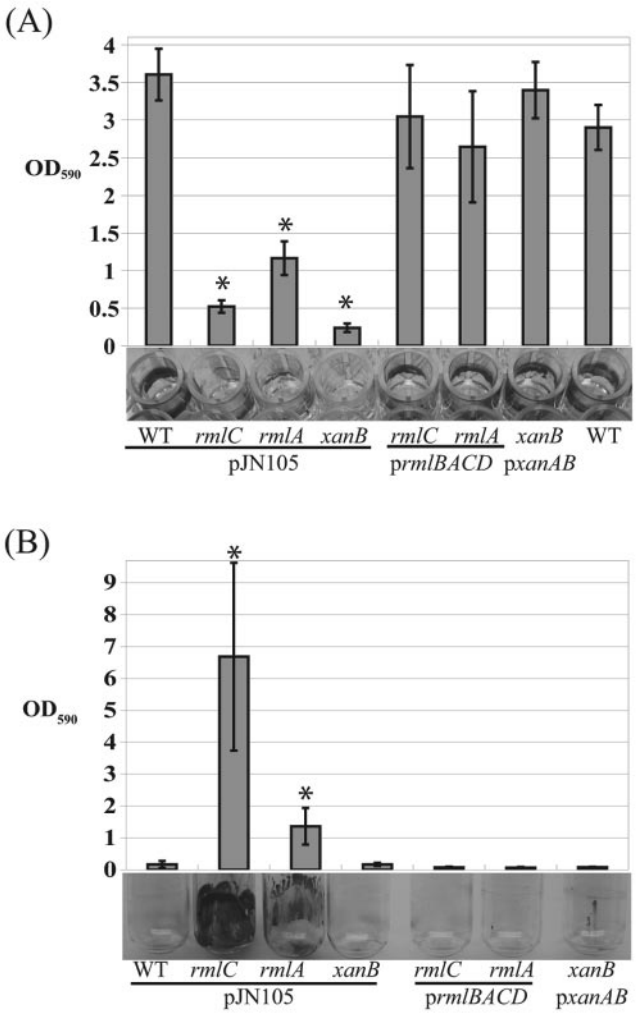

FIG. 3. Biofilm formation by *S. maltophilia.* (A) Formation in a 96-well polystyrene microtiter plate. (B) Formation in a borosilicate glass tube. All experiments were performed at least three times. Results represent the mean and standard deviation (error bars) of a representative experiment. ∗, significant difference ($P < 0.05$, analyzed by Student's two-sample *t* test and one-way ANOVA) relative to biofilm formation by other strains. WT, wild type; OD_{590}, optical density at 590 nm.

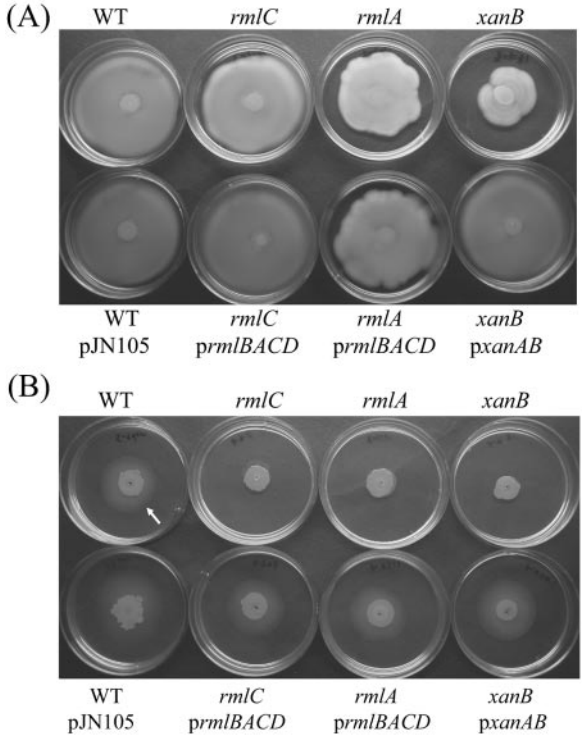

FIG. 4. Motility of *S. maltophilia.* (A) Swimming motility. Only *xanB* mutant had reduced swimming motility. (B) Twitching motility. The *rmlC*, *rmlA*, *xanB* mutants were all defective in twitching motility. The arrow indicates the edge of twitching movement away from the colony center. WT, wild type.

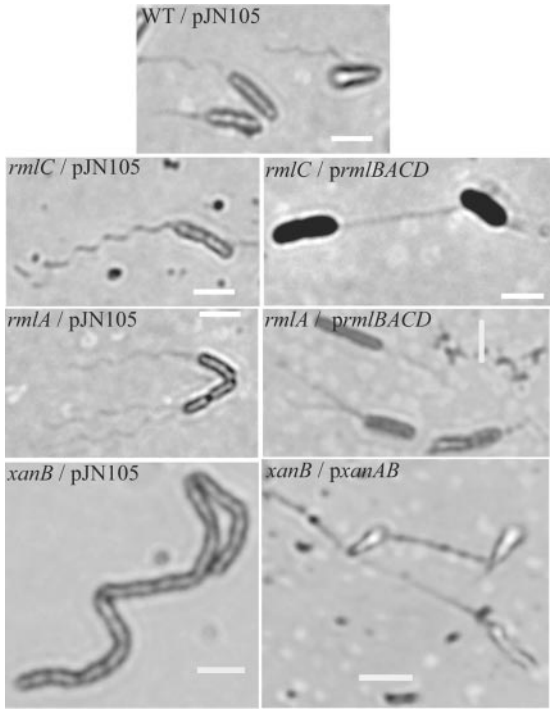

FIG. 5. Flagellar formation by *S. maltophilia*. The *xanB* mutant formed long chains devoid of flagella. The complemented strain was mostly dispersed as flagellated single cells as in other strains. WT, wild type. Bar, 2 μm.

film formation by *P. aeruginosa* (36). Defects in O-antigen polysaccharides affected twitching motility in *P. aeruginosa* (40) and prevented the export and assembly of the toxin-coregulated pilus subunit, a type IV pilin of *Vibrio cholerae* O1 (15). The involvement of *rml* and *xan* operons in twitching motility has not been examined. Twitching motility was determined in plates containing 1% TS and 1% Noble agar (36). Overnight cultures (5 μl) were stabbed to the bottom of the agar and incubated at 30°C. All three mutants were defective in twitching motility (Fig. 4B). Twitching in *S. maltophilia* is positively correlated with biofilm development in polystyrene microtiter plates, which is in agreement with that observed in *P. aeruginosa* by Chiang and Burrows (5) and O'Toole and Kolter (28) but is in contrast to the results of Singh et al. (37).

LPS is important as a barrier to antimicrobial agents and neutral detergents (31, 38) and for maintaining cell function and integrity (1, 25, 38). Growth was slightly decreased to similar extents (62 to 74%) in the wild type and the *rmlA* and *rmlC* mutants when 0.01% SDS, an anionic detergent, was added; however, the *xanB* mutant was particularly susceptible (Table 3). The alteration of LPS affected the type II secretory system in *P. aeruginosa* (26). We observed that protease activity was significantly reduced in the *xanB* mutant, while hemolytic and lecithinase activities were not affected in the *rmlA*, *rmlC*, and *xanB* mutants (not shown). LPS biosynthetic genes in *S. maltophilia* may have effects on the secretory machinery or expression of certain putative virulence factors by this opportunistic pathogen.

rmlA, *rmlC*, and *xanB* in *S. maltophilia* WR-C contribute to an LPS/EPS-coupled biosynthetic pathway. The formation of *S. maltophilia* WR-C biofilm on a polystyrene surface requires EPS and intact LPS. Alteration in LPS caused by the *rmlAC* and *xanB* mutations may contribute to changes in outer membrane components and apparatuses, such as flagella and type IV pili; this in turn interferes with motility, attachment, and biofilm formation. Whether the differences in biofilm phenotype of *rmlAC* and *xanB* mutants on glass is due to differences in polysaccharide compositions remains to be determined.

Nucleotide sequence accession numbers. The sequences obtained in this study were deposited in the GenBank database (accession no. AY956411).

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