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NOTES

Pseudomonas aeruginosa tssC1 Links Type VI Secretion and Biofilm-Specific Antibiotic Resistance[▽]

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Biofilm-specific antibiotic resistance is influenced by multiple factors. We demonstrated that *Pseudomonas aeruginosa tssC1*, a gene implicated in type VI secretion (T6S), is important for resistance of biofilms to a subset of antibiotics. We showed that *tssC1* expression is induced in biofilms and confirmed that *tssC1* is required for T6S.

Bacteria growing in biofilms are more resistant to antibiotics than their planktonic counterparts. Some mechanisms that contribute to the overall antibiotic resistance in a biofilm are mediated by the extracellular matrix, quorum sensing signaling, and stationary-phase stress resistance (10, 15). We have taken a genetic approach to identify genes that are important for biofilm-specific antibiotic resistance by screening for mutants of *Pseudomonas aeruginosa* with decreased resistance to antibiotics during biofilm, but not planktonic, growth (16). Initial characterization of two genetic loci, *ndvB* and PA1875 to PA1877, has led to the identification of novel antibiotic resistance mechanisms (16, 26). Here, we investigate another gene identified in the screen, PA14_01020, which is associated with a newly described type VI protein secretion (T6S) system in *P. aeruginosa* (18).

T6S systems have been studied in several pathogenic organisms, including *Vibrio cholerae*, *Francisella tularensis*, *Escherichia coli*, and *P. aeruginosa* (3, 20). They have been implicated in several diverse processes, including biofilm formation, toxin delivery, virulence, and fitness in chronic infection (3, 9, 14, 22). The *P. aeruginosa* genome contains three T6S loci, designated HSI-I, HSI-II and HSI-III (18). In the standard laboratory strain, PAO1, the HSI-I locus encompasses PA0071 to

TABLE 1. Primers used in this study

Primer name	Sequence	Use
tssC1 F1	TGTAGAATTCCGCTGCAACTGGTCTG	Deletion of *tssC1*
tssC1 R1	TTAATCTAGAGGCTGGCGAACTCACTGGT	Deletion of *tssC1*
tssC1 F2	TTACTCTAGACAACATCAACCGCTCCTTCA	Deletion of *tssC1*
tssC1 R2	GTGTAAGCTTGCACGTTCTGGCGGATGTTC	Deletion of *tssC1*
tssC1 F3	AAGGTCGATTCGCTGAACAA	Confirmation of Δ*tssC1*
tssC1 R3	ACGATGCACTTCAGGTAATG	Confirmation of Δ*tssC1*
tssC1 F4	CTCCAACGACGCGATCAAGT	qPCR
tssC1 R4	TCGGTGTTGTTGACCAGGTA	qPCR
retS F1	GTCAGAATTCGAAGGATGGCCAGGTGGTCA	Deletion of *retS*
retS R1	CAACTCTAGAGGATCACCAGCAGGTAGA	Deletion of *retS*
retS F2	CACCTCTAGAAACCTCAACCACGACATCCT	Deletion of *retS*
retS R2	GGCCAAGCTTTAGAGCACCAGCATCTTCAG	Deletion of *retS*
retS F3	ATGCTCCTGCTGCTGATGTA	Confirmation of Δ*retS*
retS R3	TTGGCCAGGATGCGCTTGAT	Confirmation of Δ*retS*
hcp1 F1	TTAAGAGCTCCGAGACCGACGAGCAACTGA	Deletion of *hcp1*
hcp1 R1	TTGGTCTAGAGGCGTGAGTCTTGTCCTTGG	Deletion of *hcp1*
hcp1 F2	TTGGTCTAGAGGCTGGAACATCCGCCAGAAC	Deletion of *hcp1*
hcp1 R2	TTGGAAGCTTGAACAGCGAAGTGGTGTTGA	Deletion of *hcp1*
hcp1 F3	TGCAGGACTGGATCCTCAAC	Confirmation of Δ*hcp1*
hcp1 R3	CAGCAGCTGGAACAGGAAGA	Confirmation of Δ*hcp1*
tssABC F1	CAAAGCTTGTGCCCGAGGGATTTCGGTTC	Complementation of *tssC1*
tssABC R1	CAGAGCTCCAGGCGCTGTCGTTGAATGCC	Complementation of *tssC1*

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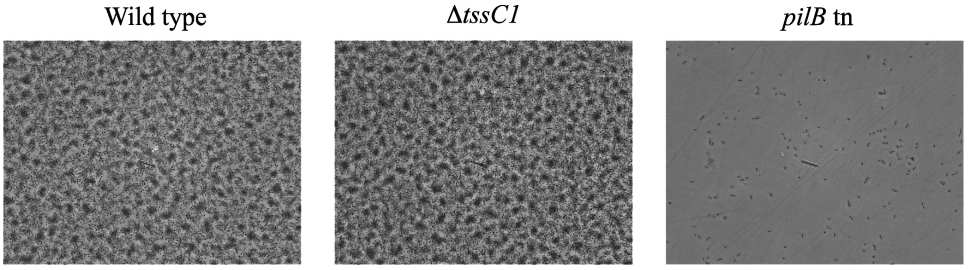

FIG. 1. Deletion of *tssC1* does not affect biofilm formation. The results of an air-liquid interface assay of biofilm formation of the PA14 wild type and Δ*tssC1* and *pilB* transposon (negative control for biofilm formation) mutants are shown (19). Images (magnification, ×400) of the biofilms were taken after 7 h of growth in M63 medium supplemented with glucose (0.2%), Casamino Acids (0.5%), and $MgSO_4$ (1 mM).

PA0091 and includes PA0084, the ortholog of PA14_01020 (13, 18). This gene is highly conserved among T6S gene clusters and has been designated *tssC1* (13, 25). Genes in the HSI-I cluster are negatively regulated by RetS, which also controls expression of several chronic virulence factors (12). Recent work has shown that HSI-I is involved in the secretion of a toxin to bacteria (13). Although *tssC1* has not been studied in *P. aeruginosa*, homologs of *tssC1* are necessary for T6S (2, 7, 27). In *V. cholerae*, the TssB1 and TssC1 homologs (VipA and VipB) form a complex similar to a bacteriophage tail sheath (5). Since we identified *tssC1* in a screen designed to identify genes important for biofilm-specific antibiotic resistance, we wanted to confirm that *tssC1* was involved in both biofilm-specific antibiotic resistance and T6S.

***tssC1* is expressed in biofilms and important for biofilm-specific antibiotic resistance.** The original *tssC1* mutant isolated from the screen was a PA14 Tn*5* mutant (16). In order to avoid any possible polar effects, we constructed a *P. aeruginosa* PA14 mutant with an unmarked deletion of *tssC1* by allelic exchange (8) (using pEX18Gm), as described previously (16), with the primers listed in Table 1. Loss of *tssC1* had no effect on the growth rate of this mutant (data not shown). Since T6S has been implicated in biofilm formation (1), we assessed the ability of the Δ*tssC1* mutant to form biofilms at the air-liquid interface of a six-well microtiter plate (Fig. 1) (17). Mutation of *tssC1* had no effect on biofilm formation compared to that of the wild-type strain.

To further explore the phenotype of the Δ*tssC1* mutant, we compared the antibiotic resistance phenotype of the Δ*tssC1* mutant strain with that of the PA14 wild-type strain. We determined the minimal bactericidal concentration for planktonic cells (MBC-P) and the minimal bactericidal concentration for biofilm cells (MBC-B) for tobramycin, gentamicin, and ciprofloxacin (antibiotics used to treat *P. aeruginosa* infections in cystic fibrosis patients [11, 21]), using the 96-well microtiter dish system (Table 2) (16). We found that deletion of *tssC1* resulted in a 2- to 4-fold reduction in resistance in the MBC-B assay to all three antibiotics. In the MBC-P assay, deletion of *tssC1* had a minor effect on planktonic resistance; however, results from an MIC assay (a more sensitive assay that measures planktonic antibiotic resistance) (Table 3) revealed that there was no defect in planktonic antibiotic resistance in the Δ*tssC1* strain. Together, these results confirmed the importance of *tssC1* in biofilm-specific antibiotic resistance. Hcp is an important component of T6S (3, 20). Deletion of *hcp1* (HSI-I version of *hcp*) resulted in a strain that also had a slight defect in biofilm-specific antibiotic resistance (Table 2), suggesting that the HSI-1 T6S system is involved in biofilm-specific antibiotic resistance.

The intact PA14 *tssC1* gene was cloned into a broad-host-range vector, pJB866, to create pJB866-*tssC1*. This vector carries the *Pm* promoter, and expression from this promoter is induced by *m*-toluic acid (4, 26). Compared with PA14 wild-type planktonic cells carrying the vector alone, the cells that carried pJB866-*tssC1* (preinduced with *m*-toluic acid) showed 2- to 4-fold-higher MIC values for tobramycin and gentamicin, but not ciprofloxacin, suggesting that overexpression of *tssC1* in planktonic cells increases antibiotic resistance.

In order to explain the specificity of *tssC1* in biofilm but not planktonic resistance, we measured the gene expression of *tssC1* in cells grown as planktonic cultures or biofilms by quantitative real-time PCR (qPCR) (Table 1 and Fig. 2A). Planktonic cultures were grown in M63 medium (supplemented with 0.4% arginine and 1 mM $MgSO_4$ to an optical density at 600 nm [OD_{600}] of 0.6), while biofilms were grown as colonies on M63 agar plates (6). We observed that the *tssC1* gene was 18-fold more highly expressed in biofilm cells than in plank-

TABLE 2. MBCs for the *P. aeruginosa* PA14 wild-type strain and Δ*tssC1* and Δ*hcp1* mutants[a]

Strain	Tobramycin		Gentamicin		Ciprofloxacin	
	MBC-P	MBC-B	MBC-P	MBC-B	MBC-P	MBC-B
PA14	16	100	32	800	2	20–40
PA14 Δ*tssC1*	8–16	25	32	400	1	10
PA14 Δ*hcp1*	8–16	50	32	200	2	10–20

[a] MBCs (in micrograms per milliliter) represent the modes of at least six replicates.

TABLE 3. MICs for the *P. aeruginosa* PA14 wild-type strain and Δ*tssC1* and Δ*hcp1* mutants

Strain	MIC[a] (μg/ml)					
	Tobramycin		Gentamicin		Ciprofloxacin	
	M63	LB	M63	LB	M63	LB
PA14	2	2	2	2	0.125	0.5
PA14 Δ*tssC1*	2	2	2	2	0.125	0.5
PA14 Δ*hcp1*	2	2	2	2	0.125	0.5

[a] MICs were determined for strains grown in M63 or LB medium.

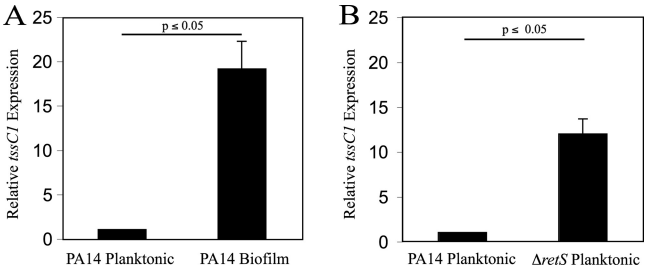

FIG. 2. *tssC1* is differentially expressed in PA14 wild-type and Δ*retS* planktonic and biofilm cultures. Expression of *tssC1* in wild-type planktonic and biofilm cells (A) or wild-type and Δ*retS* planktonic cells (B) was measured by real-time PCR (qPCR) using primers specific for *tssC1* (Table 1). Expression of *tssC1* in planktonic cells was set at 1. Expression of the housekeeping gene, *rpoD*, was used as an internal control. Experiments were performed in triplicate. Error bars represent the standard deviations.

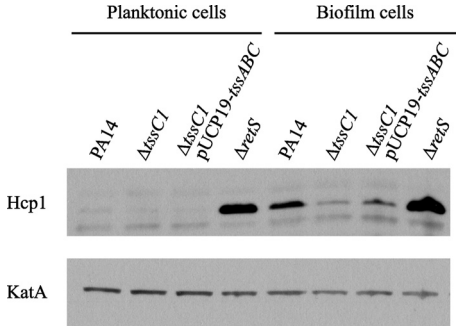

FIG. 4. Hcp1 levels are greater in PA14 biofilm cells than in planktonic cells. Whole-cell lysates of strains grown as biofilms or planktonic cultures were resolved on a 10% SDS-PAGE gel and assayed for Hcp1 and KatA expression by Western blotting. KatA expression served as a loading control. Cell suspensions were centrifuged, and cell pellets were suspended in Tris-buffered saline and sonicated prior to SDS-PAGE analysis.

tonic cells. RetS is a negative regulator of T6S gene expression (12). To confirm that RetS controls expression of *tssC1* in PA14 planktonic cells, we constructed a *retS* deletion strain using PA14 and measured *tssC1* expression in the mutant strain by qPCR. As expected, *tssC1* was highly expressed in the strain that lacked RetS (Fig. 2B).

***tssC1* is involved in type VI secretion.** T6S systems are characterized by secretion of Hcp into culture supernatants, which can be used as an indicator of functional T6S (20). In *P. aeruginosa*, there is no secretion of Hcp1 in planktonically grown wild-type strains but Hcp1 is present in the supernatant of Δ*retS* mutants (18). It has been demonstrated that *tssC1* homologs are required for Hcp1 secretion, but this has not been confirmed in *P. aeruginosa*. In order to determine if *tssC1* is involved in T6S, we tested whether *tssC1* inactivation affected Hcp1 secretion. We reasoned that if *tssC1* were involved in T6S, then Hcp1 would not be secreted from a Δ*retS* Δ*tssC1* double mutant. We constructed this double mutant strain in a PA14 background and assayed the supernatants from wild-type, Δ*tssC1*, Δ*retS*, and Δ*retS* Δ*tssC1* cultures by Western blotting with an antibody to Hcp1 as previously described by Mougous et al. (18) (Fig. 3A). Supernatants were isolated by centrifugation of planktonic cultures grown to late exponential phase in LB medium. As expected, Hcp1 was not secreted by the wild-type PA14 or the Δ*tssC1* strains but was secreted by the Δ*retS* strain. However, Hcp1 was not secreted by the Δ*retS* Δ*tssC1* double mutant, suggesting that *tssC1* is required for T6S. The presence of Hcp1 in cell-associated fractions of the Δ*retS* Δ*tssC1* double mutant (Fig. 3A) indicated that the *tssC1* mutation abolished secretion but not expression of Hcp1. The Δ*retS* Δ*tssC1* mutant produced slightly less Hcp1 than the Δ*retS* mutant. Complementation of *tssC1* in a Δ*retS* Δ*tssC1* mutant restored Hcp1 secretion (Fig. 3B, pUCP19-*tssABC*). The vector was constructed by cloning a DNA fragment containing the promoter and open reading frame sequences of *tssC1* (as well as the upstream operon genes *tssA1* and *tssB1*) into the medium-copy-number plasmid pUCP19 (24).

The secretion assay described above was performed using planktonic cultures in which Hcp1 expression is strongly repressed by RetS (18). Our antibiotic sensitivity and gene expression data suggested that *tssC1* plays a role in antibiotic resistance of wild-type biofilms. To test whether growth in biofilms leads to increased Hcp1 protein expression, we compared cell-associated Hcp1 levels from planktonic and biofilm cultures of the PA14 parent and Δ*tssC1* and Δ*retS* mutant strains (Fig. 4). Planktonic cultures were grown to an OD_{600} of 0.3 in M63 medium supplemented with 0.4% arginine and 1

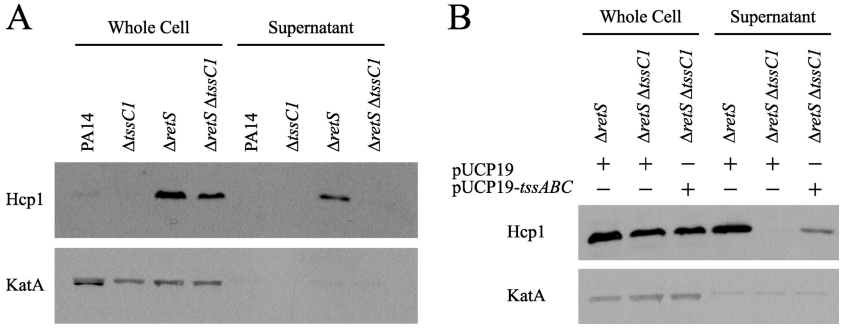

FIG. 3. TssC1 is required for secretion of Hcp1 into culture supernatants. Whole-cell lysates and supernatant fractions were resolved on a 10% SDS-PAGE gel and assayed for Hcp1 and KatA expression by Western blotting. Levels of the cell-associated protein KatA served as a loading control for the whole-cell fractions and a lysis control for the secreted fractions. (A) Planktonic cultures of the PA14 wild-type strain and Δ*tssC1*, Δ*retS*, and Δ*retS* Δ*tssC1* mutants were grown in LB medium to late exponential phase prior to analysis. (B) Hcp1 secretion was partially restored in the Δ*retS* Δ*tssC1* strain carrying the complementation vector pUCP19-*tssABC*.

mM $MgSO_4$. Static biofilms were grown in the same medium for 48 h in six-well microtiter plates, washed with 0.9% NaCl, and scraped from the sides of the wells. For each strain, Hcp1 levels were significantly higher in biofilm cells than in planktonic cells, although expression was again slightly reduced in the *tssC1* mutant. This result, combined with the *tssC1* expression data (Fig. 2), suggests that genes encoding components of the T6S system are induced during biofilm growth.

The *P. aeruginosa* HSI-I T6S system has been implicated in fitness during chronic infections and toxin delivery to bacteria (13). We have shown that an essential component of this secretion system, *tssC1*, promotes antibiotic resistance in biofilms. To our knowledge, this is the first evidence linking a component of a T6S system to antibiotic resistance. Although the resistance mechanism is unclear, it likely involves an uncharacterized effector of this secretion system. Evidence in *P. aeruginosa* and other bacteria suggest a role for T6S in mediating bacterial cell-cell interactions (13, 23). Since bacteria in biofilms are in close contact, secretion of an effector between bacteria in biofilms might lead to an antibiotic-resistant state. Investigation of this mechanism could reveal insights into both the function of T6S in *P. aeruginosa* and the mechanisms responsible for the enhanced resistance of biofilms.

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