



Effectors of the *Stenotrophomonas maltophilia* Type IV Secretion System Mediate Killing of Clinical Isolates of *Pseudomonas aeruginosa*

Megan Y. Nas,^a Jeffrey Gabell,^a Nicholas P. Cianciotto^a

^aDepartment of Microbiology and Immunology, Northwestern University Medical School, Chicago, Illinois, USA

ABSTRACT Previously, we documented that *Stenotrophomonas maltophilia* encodes a type IV secretion system (T4SS) that allows the organism to kill, in contact-dependent fashion, heterologous bacteria, including wild-type *Pseudomonas aeruginosa*. Bioinformatic screens based largely on the presence of both a C-terminal consensus sequence and an adjacent gene encoding a cognate immunity protein identified 13 potential antibacterial effectors, most of which were highly conserved among sequenced strains of *S. maltophilia*. The immunity proteins of two of these proved especially capable of protecting *P. aeruginosa* and *Escherichia coli* against attack from the *Stenotrophomonas* T4SS. In turn, *S. maltophilia* mutants lacking the putative effectors RS14245 and RS14255 were impaired for killing not only laboratory *E. coli* but clinical isolates of *P. aeruginosa*, including ones isolated from the lungs of cystic fibrosis patients. That complemented mutants behaved as wild type did confirmed that RS14245 and RS14255 are required for the bactericidal activity of the *S. maltophilia* T4SS. Moreover, a mutant lacking both of these proteins was as impaired as a mutant lacking the T4SS apparatus, indicating that RS14245 and RS14255 account for (nearly) all of the bactericidal effects seen. Utilizing an interbacterial protein translocation assay, we determined that RS14245 and RS14255 are bona fide substrates of the T4SS, a result confirmed by examination of mutants lacking both the T4SS and the individual effectors. Delivery of the cloned 14245 protein (alone) into the periplasm resulted in the killing of target bacteria, indicating that this effector, a putative lipase, is both necessary and sufficient for bactericidal activity.

IMPORTANCE *S. maltophilia* is an increasingly important opportunistic pathogen. Inherently resistant to many antibiotics, *S. maltophilia* is often associated with lung infection, being, among other things, a complicating factor in cystic fibrosis patients. Moreover, it is a common form of coinfection in COVID-19 patients. In these various clinical settings and in natural habitats, *S. maltophilia* coexists with other pathogens, including *P. aeruginosa*. Previously, we documented that *S. maltophilia* possesses a T4SS that kills other bacteria, a notable observation given that most prior work on interbacterial competition has highlighted bactericidal effects of type VI secretion systems. By utilizing approaches ranging from bioinformatics to mutant analysis to protein translocation assays, we have now identified two substrates of the *Stenotrophomonas* T4SS that largely mediate the killing of pathogenic *P. aeruginosa*. These results represent a major advance in understanding *S. maltophilia*, the roles of T4SSs, concepts regarding clinically relevant, interbacterial competition, and activities of bactericidal effectors.

KEYWORDS *Pseudomonas aeruginosa*, *Stenotrophomonas*, *Stenotrophomonas maltophilia*, bacterial competition, bactericidal activity, cystic fibrosis, lipase, peptidoglycan hydrolases, type IV secretion

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Address correspondence to Nicholas P. Cianciotto, n-cianciotto@northwestern.edu.

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Gram-negative *Stenotrophomonas maltophilia* is a frequent isolate from water, soil, and plant material and the most characterized member of the *Stenotrophomonas* genus, which currently consists of 19 species (1, 2). *S. maltophilia* is also an increasingly important pathogen particularly in the hospital setting but also in the broader community (3–6). *S. maltophilia* infection is most often manifest as pneumonia and bacteremia (4, 7, 8). The bacterium is also notable in cystic fibrosis (CF) patients, where *S. maltophilia* infection heightens the risk of lung exacerbations (9–13). Very recent studies indicate that *S. maltophilia* often infects COVID-19 patients, suggesting that its importance is growing more pronounced (14–16). Many strains of *S. maltophilia* have resistance to a range of antibiotics, explaining, to a large extent, the clinical problem that is *S. maltophilia* (17–19). We and others have shown that *S. maltophilia* has many traits linked to virulence, including growth in the lungs of infected mice, attachment to host cells, secretion of degradative enzymes via a type II secretion system, siderophore production, and biofilm formation (20–30). Moreover, we recently documented that *S. maltophilia* encodes a type IV secretion system (T4SS) that has a profound role in the interactions of *S. maltophilia* with mammalian host cells and other types of bacteria (31).

Spanning the Gram-negative cell wall, the T4SS apparatus consists of 12 core proteins (32). Cytoplasm-facing VirD4 is a coupling protein for the recruitment of substrates to an inner membrane complex made of VirB3, VirB6, VirB8, VirB4, and VirB11. Substrates are then secreted out through a periplasm-outer membrane-spanning complex composed of VirB7, VirB9, and VirB10. VirB2 and VirB5 form a pilus-like structure designed to contact target membranes, and VirB1 helps degrade peptidoglycan during assembly of the T4SS apparatus. T4SSs are important in environmental species of *Agrobacterium*, *Lysinibacillus*, *Piscirickettsia*, *Sinorhizobium*, *Wolbachia*, and *Xanthomonas*, promoting various interactions with fish, plants, insects, and other microbes (33–41). T4SSs also play a major role for human and other mammal pathogens, including species of *Actinobacillus*, *Anaplasma*, *Bartonella*, *Bordetella*, *Brucella*, *Burkholderia*, *Coxiella*, *Ehrlichia*, *Helicobacter*, *Legionella*, *Neisseria*, and *Rickettsia* (42–57). We recently showed that environmental and clinical isolates of *S. maltophilia* encode a T4SS (31). This VirB/D4 T4SS is most similar to the T4SS of *Xanthomonas*, the genus most related to *Stenotrophomonas*. Based on analysis of a *virB10* mutant of clinical isolate K279a, along with a complemented derivative of that mutant, we showed that the *S. maltophilia* T4SS promotes the killing of human macrophages while blunting death in human epithelial cells (31). Moreover, when we cocultured, for short periods of time, strain K279a with wild-type (WT) *Pseudomonas aeruginosa*, the T4SS reduced the numbers of the heterologous bacteria, signaling that some *Stenotrophomonas* effectors have bactericidal activity (31). The effects of the T4SS required contact with the target cell. We further observed that the *S. maltophilia* T4SS killed all three *P. aeruginosa* strains tested including environmental strain 7700 and clinical isolates PAO1 and PAK and that other strains of *S. maltophilia* also could kill *P. aeruginosa* (31). The *S. maltophilia* T4SS also promoted the death of *Pseudomonas mendocina* but not *Pseudomonas fluorescens*, *Pseudomonas putida*, or *Pseudomonas stutzeri*, implying that its bactericidal effect extends to multiple, but not all, *Pseudomonas* species (31). Our findings on the bactericidal effect of the *S. maltophilia* T4SS have been confirmed by others showing that strain K279a lyses target bacteria during short-term coincubation (58). In contrast to the vast amount of data on the antibacterial effects of type VI secretion systems (T6SSs) (59–64), knowledge of the antibacterial role of T4SSs is minimal, being limited to the two studies of *S. maltophilia* and other work on the plant pathogen *Xanthomonas citri* and animal pathogen *Bartonella schoenbuchensis* (31, 58, 65, 66). Moreover, the T4SS effectors necessary for the ability of *S. maltophilia* to kill wild-type strains of other species, including *P. aeruginosa*, have remained unknown. Here, we identified two bona fide substrates of the *S. maltophilia* T4SS (to be designated TfcA and TfcB) that promote the killing of clinical isolates of *P. aeruginosa*, including those from CF lungs. These data provide novel insight into role of T4SSs in competitions between human pathogens.

RESULTS

Identification and gene distribution and expression of putative antibacterial effectors of *S. maltophilia* T4SS. Using the T4SEpre and S4TE programs that are designed to recognize proteins that have C-terminal features (e.g., glutamic acid and serine-enriched, coiled versus helical) that are similar to those of known T4SS substrates from a variety of bacteria (67, 68), we performed an initial bioinformatics screen to identify potential antibacterial effectors of the T4SS of *S. maltophilia* strain K279a. After identifying proteins containing these general C-terminal features, we narrowed in on those that were annotated as having an activity that is compatible with antibacterial function. We then focused on a subset that were encoded adjacent to an open reading frame (ORF) for a putative immunity protein, since this type of gene arrangement commonly occurs for antibacterial effectors secreted by the T4SS of *X. citri* and T6SSs (65, 69, 70). This resulted in the identification of two putative effectors, namely, those encoded by ORFs 17170 and 19100 (Fig. 1A). As a second path to finding targets for investigation, we searched the K279a genome for proteins with C-terminal sequences that had more specific similarity to the C-terminal domains in *Xanthomonas* T4SS effectors that are implicated in binding to the VirD4 coupling protein (65, 71). This yielded nine proteins that are also encoded next to putative immunity proteins, with one of those being the 19100 protein identified in the first screen and the rest being encoded by ORFs 00905, 01575, 02375, 02385, 02400, 14245, 14255, and 14405 (Fig. 1A). As a third screen, we searched the K279a genome for homologs to proteins of other *Stenotrophomonas* strains that were previously found to be akin to *Xanthomonas* T4SS substrates (65). This resulted in both the reidentification of 00905, 02400, and 14255 and the discovery of the candidate encoded by ORF 20845 (Fig. 1A). Finally, we added K279a proteins from ORFs 00510 and 01275, which had recently been proposed as T4SS substrates, based on homologies to *Xanthomonas* proteins (58). Thus, we arrived at a list of 13 possible *S. maltophilia* T4SS substrates with activity against other bacteria (Fig. 1A). Two proteins were predicted to encode lipases, two were annotated as nucleases, and three appeared to interact with peptidoglycan (Fig. 1A); i.e., these seven were compatible with antibacterial function, as best exemplified by T6SS effectors (72). The remaining six did not show sequence similarity to known proteins, suggesting they might encode novel activities (Fig. 1A). Using BLAST, we determined that 10 of the putative effectors were encoded by 90 to 93% of the 42 sequenced strains of *S. maltophilia* (including K279a) (Fig. 1A and see Table S1A in the supplemental material). The strains that lacked the proteins also lacked genes for the T4SS apparatus. The remaining three putative substrates had a prevalence within the species between 50 and 64% (Fig. 1A and Table S1A). Thirteen of the strains had all 13 putative effectors, 10 others had 12, and eight others had 11 (Table S1A), suggesting that the repertoire of K279a is representative. The prevalence of the immunity proteins mirrored that of the putative effectors (Table S1B). All 13 of the putative effectors of *S. maltophilia* had homologs in other *Stenotrophomonas* species, with five of them occurring in all 13/13 species that carry a T4SS, and seven others being present in 11/13 or 10/13 of the T4SS⁺ species (Fig. 1B). From qRT-PCR analysis, we determined that all of the putative effector genes are expressed by *S. maltophilia* strain K279a grown on LB agar. Also, all were similarly expressed when the strain was grown in the presence of competing *P. aeruginosa* for 2 to 24 h (Fig. S1). Given that all of the putative effectors were both present in the majority of *S. maltophilia* strains and similarly expressed in the presence of a competitor, we considered all of them for mutational analysis.

Protective effects of *S. maltophilia* T4SS immunity proteins. To focus our mutagenesis efforts, we sought to identify which putative effectors had an immunity protein that conferred protection against the T4SS's bactericidal activity. Immunity proteins normally provide self-protection to the organism encoding the T4SS (73, 74), but, for our purposes, we examined the effect of their expression in heterologous bacteria. Each of the 13 putative immunity proteins, determined based upon their arrangement in effector gene operons as described above, was cloned on a plasmid and introduced into *Escherichia coli* DH5 α and *P. aeruginosa* 7700, the strains that we had previously

A

Putative T4SS effectors of <i>S. maltophilia</i> (<i>Sm</i>)				
Strain K279a ORF* (shorthand name)	Former Locus Tag*	Predicted Activity	% Prevalence/ % Sequence Similarity in <i>Sm</i> Strains	Cognate Immunity Protein
SMLT_RS00510 (00510)	smlt0113	Novel	62/63	RS00515
SMLT_RS00905 (00905)	smlt0193	Peptidoglycan binding	93/92	RS00910
SMLT_RS01275 (01275)	smlt0273	Peptidoglycan binding	50/54	RS01280
SMLT_RS01575 (01575)	smlt0332	Novel	93/53	RS01580
SMLT_RS02375 (02375)	smlt0500	Novel	93/62	RS02380
SMLT_RS02385 (02385)	smlt0502	Novel	93/96	RS02390
SMLT_RS02400 (02400)	smlt0505	Novel	93/86	RS02405
SMLT_RS14245 (14245)	smlt2990	Lipase	93/93	RS14240
SMLT_RS14255 (14255)	smlt2992	Lysozyme-like	93/93	RS14260
SMLT_RS14405 (14405)	smlt3024	Novel	93/80	RS14410
SMLT_RS17170 (17170)	smlt3607	RNase	90/80	RS17165
SMLT_RS19100 (19100)	smlt4012	Lipase	93/52	RS19095
SMLT_RS20845 (20845)	smlt4383	Nuclease	64/49	RS20840

*As per current NCBI listings

B

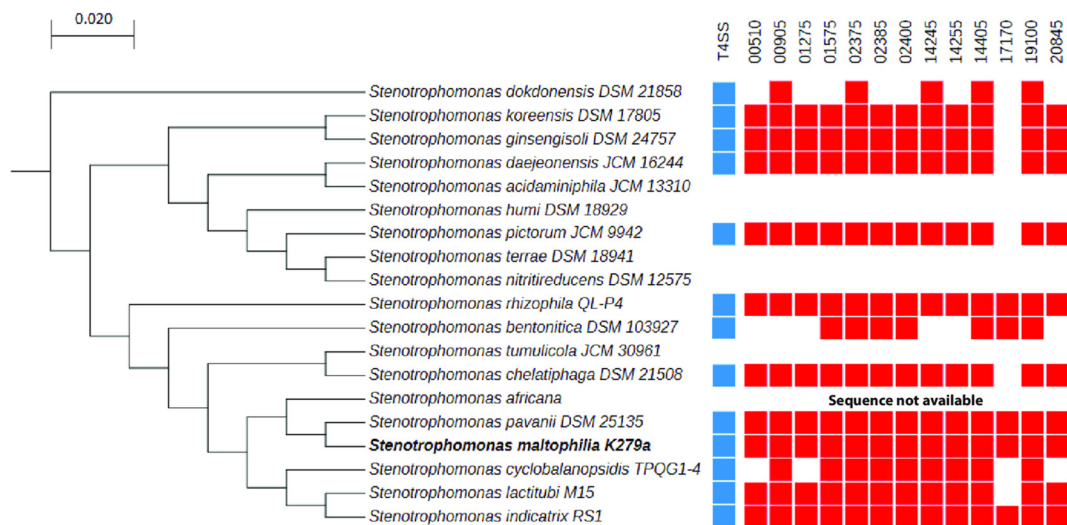


FIG 1 Putative T4SS effectors of *S. maltophilia*. (A) Thirteen putative effectors of strain K279a are presented, along with their current ORF designations and, in parentheses, the shorthand to be used throughout this paper (first column), former locus tags (second column), predicted activities based on annotations at NCBI (third column), percent prevalence among all of the sequenced strains of *S. maltophilia*/percent amino acid similarity of the proteins across the species (fourth column), and the current ORF designations for the putative cognate immunity proteins (fifth column). (B) Distribution of the putative effectors within the *Stenotrophomonas* genus. On the left side, the 19 named species of *Stenotrophomonas* are presented in a phylogenetic tree that is based on 16S rRNA sequences and scaled at 0.02 substitutions per site (2). On the right side, each column represents the distribution of one of the 13 effectors, with a red square denoting the presence of the corresponding gene and a blank denoting the absence of the gene. Also indicated in a similar manner is the presence (blue squares) or absence of the T4SS apparatus genes in the different species. No data are presented for *Stenotrophomonas africana*, since that species has not been entirely sequenced yet.

shown are sensitive to antagonism by *S. maltophilia* K279a (31). Whereas DH5 α is a laboratory strain of *E. coli*, 7700 is wild-type *P. aeruginosa* isolated from water. To begin, target *E. coli* bacteria containing or not containing the different immunity proteins were coincubated with T4SS-expressing K279a for 2 h at 37°C, and then their

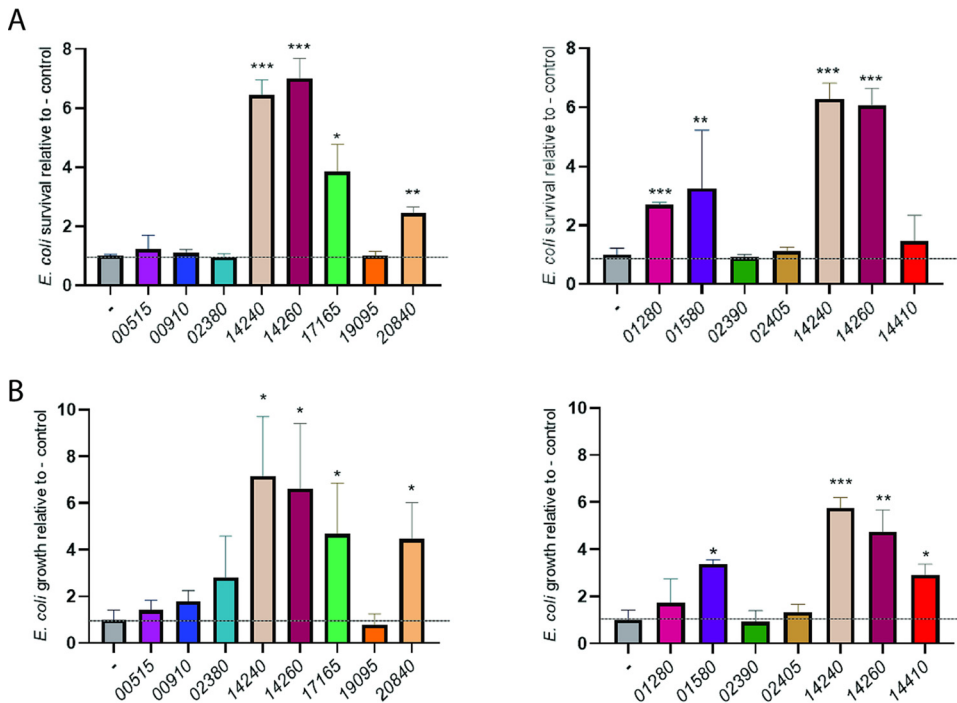


FIG 2 Ability of cloned immunity proteins to protect *E. coli* from killing by wild-type *S. maltophilia*. *E. coli* DH5 α containing either the vector pBBR1MCS alone (-) or the cloned gene encoding one of the predicted immunity proteins from *S. maltophilia* K279a, i.e., 00515, 00910, 02380, 14240, 14260, 17165, 19095, or 20840 (left panels) or 01280, 01580, 02390, 02405, 14240, 14260, or 14410 (right panels), was mixed with *S. maltophilia* K279a in a (*S. maltophilia*/*E. coli*) ratio of \sim 50:1, spotted onto LB agar, and incubated at 37°C. As added points of reference, the *E. coli* strains containing the immunity proteins 14240 and 14260 were included in both of the experiments depicted in the left and right panels. After 2 h (A) or 24 h (B) of incubation, the numbers of *E. coli* CFU were determined by plating dilutions of the entire bacterial growth area on selective medium. In the 2-h incubation, the numbers of CFU of the vector control decreased, indicative of killing, whereas in the 24-h incubation, the numbers of CFU for the control increased somewhat, indicative of the combined effect of growth and killing (Fig. S2A). Thus, the percent changes in CFU for the vector control were set to 1, and others were normalized to that control (dashed horizontal line), in order to make clearer which cloned immunity proteins afforded protection against killing by *S. maltophilia* T4SS. Asterisks indicate those cases where there were significant differences in CFU obtained for the recombinant clone versus the control (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$). Data are presented as the means and standard deviations of results from three independent experiments ($n = 3$ each).

remaining numbers were determined. As previously found (31), there is normally a decrease in *E. coli* CFU during this short incubation period. Thus, any increase in the recovery of the heterologous bacteria (above the control) is evidence of protection against the T4SS. Six of the cloned immunity proteins, i.e., 14240, 14260, 17165, 20840, 01280, and 01580, allowed DH5 α to survive better in the presence of K279a (Fig. 2A and Fig. S2A). When we extended the incubation to 24 h, 14240, 14260, 17165, 20840, and 01580 continued to give a protective effect, as one might expect (Fig. 2B and Fig. S2A). Another protein, 14410, showed a protective effect in the 24-h, but not 2-h, incubation (Fig. 2B). More significantly, when we incubated wild-type *P. aeruginosa* containing or not containing the different immunity proteins with strain K279a for 2 h at 37°C, four of the cloned proteins, i.e., 14240, 14260, 01580, and 02405, allowed the pseudomonad to survive better amid *S. maltophilia* (Fig. 3A and Fig. S2B). Among these four, 14240 and 14260 appeared to provide the greatest level of protection. Also, 14260 and 14240, but none of the others, showed clear evidence of protection when the coinoculation was extended to 24 h (Fig. 3B and Fig. S2B). The immunity protein 17165 showed a protective effect in this 24-h incubation, although it had not done so in the 2-h incubation (Fig. 3B). That the putative immunity proteins 00515, 00910, 02380, 19095, and 02390 did not appear to provide any protection means either that they are not true immunity proteins or that they were not adequately expressed and/

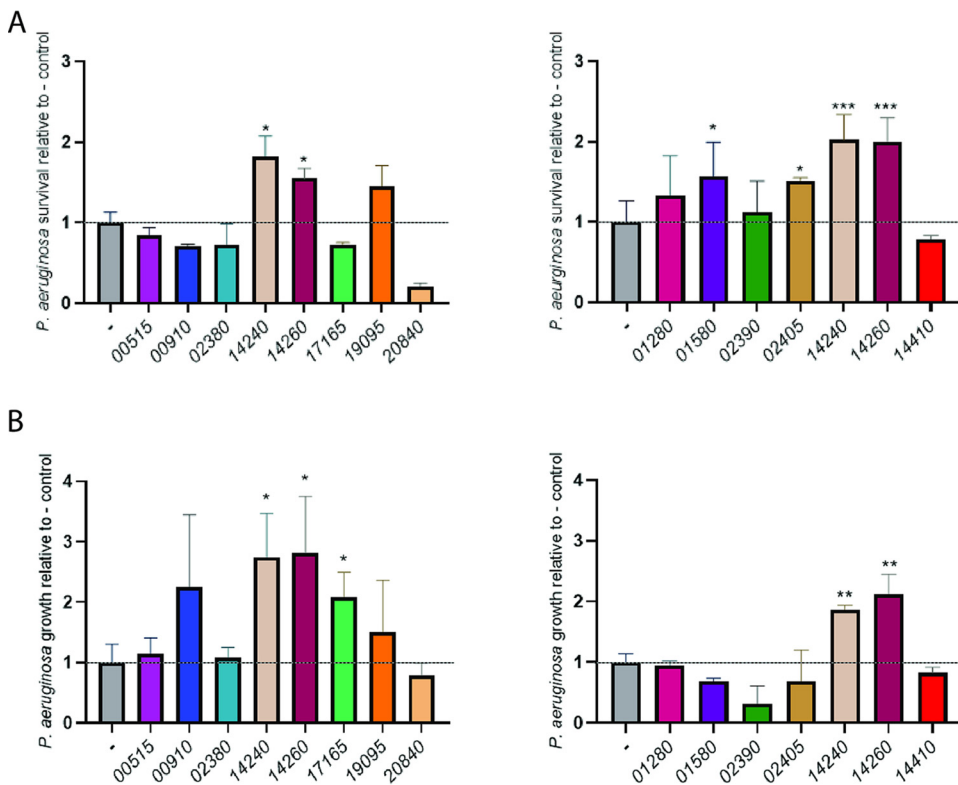


FIG 3 Ability of cloned immunity proteins to protect *P. aeruginosa* from killing by wild-type *S. maltophilia*. *P. aeruginosa* strain 7700 containing either the vector pBBR1MCS alone (-) or the cloned gene encoding one of the predicted immunity proteins from *S. maltophilia* K279a, i.e., 00515, 00910, 02380, 14240, 14260, 17165, 19095, or 20840 (left panels) or 01280, 01580, 02390, 02405, 14240, 14260, or 14410 (right panels), was mixed with *S. maltophilia* K279a in a (*S. maltophilia*/*P. aeruginosa*) ratio of ~50:1, spotted onto LB agar, and incubated at 37°C. As added points of reference, *E. coli* strains containing the immunity proteins 14240 and 14260 were included in both of the experiments depicted in the left and right panels. After 2 h (A) or 24 h (B) of incubation, the numbers of *P. aeruginosa* CFU were determined by plating dilutions of the entire bacterial growth area on selective medium. In the 2-h incubation period, the numbers of CFU of the vector control decreased, indicative of killing, whereas during the 24-h incubation, the numbers of CFU for the control increased, indicative of the combined effect of growth and killing (Fig. S2B). Thus, the percent changes in CFU for the vector control were set to 1, and the others were normalized to that control (dashed horizontal line), in order to make clearer which cloned immunity proteins afforded protection against killing by *S. maltophilia* T4SS. Asterisks indicate those cases where there were significant differences in CFU obtained for the recombinant clone versus the control (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$). Data are presented as the means and standard deviations of results from three independent experiments ($n = 3$ each).

or localized in the *E. coli* or *P. aeruginosa* cells. Nonetheless, 14240 and 14260 stood out as clearly being important, since they (i) provided the greatest protection against the *P. aeruginosa* strain, in terms of both the magnitude of the protection and that it occurred at multiple time points (Fig. 3), and (ii) also blocked killing of a second bacterium (Fig. 2).

Putative T4SS effectors 14245 and 14255 are required for the ability of *S. maltophilia* to compete against *P. aeruginosa* and *E. coli*. The analysis of the immunity proteins implicated 14245 and 14255, i.e., the corresponding effectors of 14240 and 14260 (Fig. 1A), as the most likely mediators of the (most significant) bactericidal effect of the *S. maltophilia* T4SS. These putative effectors were encoded by all 42 sequenced strains of *S. maltophilia* that encode a T4SS and were highly conserved in amino acid sequences across the strains (Fig. 1A and Table S1A). Also, they were encoded by all sequenced *Stenotrophomonas* species that carry the T4SS (Fig. 1B). Interestingly, the operon encoding 14245 maps adjacent to but in the opposite direction from the operon encoding 14255 (Fig. 4A). Both of these operons had a third ORF, which encoded a hypothetical protein (Fig. 4A). Significantly, this locus maps very close and indeed is closer than any of the other putative effector genes to the locus

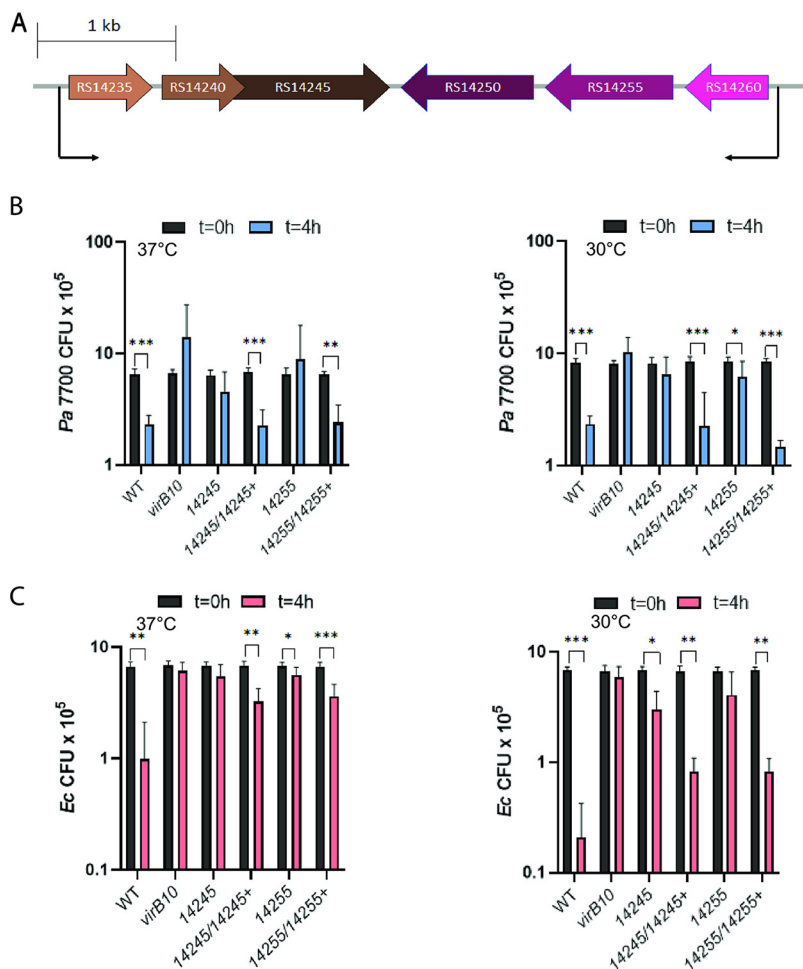


FIG 4 Bactericidal effect of *S. maltophilia* wild-type, *virB10* mutant, 14245 mutant, and 14255 mutant strains on *P. aeruginosa* and *E. coli*. (A) Representation of the region of the *S. maltophilia* K279a chromosome containing the operon with ORFs encoding effector 14245 and its cognate immunity protein (brownish horizontal arrows) and the neighboring operon encoding effector 14255 and its corresponding immunity protein 14260 (pink-purple horizontal arrows). Horizontal arrowheads indicate the direction of transcription, with the thin, bent arrows showing the approximate position of the predicted promoters. A scale, in kilobases, is presented in the upper left. (B and C) *S. maltophilia* (Sm) K279a (WT), *virB10* mutant NUS15 (*virB10*), 14245 mutant NUS17 (14245), complemented mutant NUS17 (pB14245) (14245/14245+), 14255 mutant NUS19 (14255), and complemented mutant NUS19 (pB14255) (14255/14255+) were mixed with *P. aeruginosa* strain 7700 (B) and *E. coli* strain DH5α (C) in a (*S. maltophilia*/other bacterium) ratio of ~50:1 and spotted onto LB agar, and after 4 h of incubation at either 37°C (left panels) or 30°C (right panels), the numbers of each strain were determined by plating dilutions of the entire bacterial growth area on selective medium. The results presented are the numbers of *P. aeruginosa* CFU or *E. coli* CFU at $t = 0$ h and $t = 4$ h, with asterisks indicating those cases where there were significant differences in CFU obtained at $t = 0$ versus $t = 4$ h (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$). Data are presented as the means and standard deviations of results from three independent experiments ($n = 3$ each).

encoding the T4SS apparatus, i.e., SMLT_RS14275 to SMLT_RS14330 (31). Proximity to the locus encoding the secretion apparatus is often a hallmark of secreted substrates (63, 75, 76).

To determine whether 14245 and 1455 have required roles in the antibacterial activity of the *S. maltophilia* T4SS, we constructed mutants of strain K279a that specifically lack either 14245 or 14255 and then tested them for their relative ability to kill *P. aeruginosa* 7700. As before (31), coincubation for a few hours at 37°C with K279a, but not its *virB10* T4SS mutant, led to an ~3-fold reduction in the numbers of *P. aeruginosa* (Fig. 4B, left). When we did the coincubation at 30°C, as an initial look into how interactions might occur under environmental conditions, the effect of the T4SS was still

present (Fig. 4B, right). As predicted by our analysis of the immunity proteins, the 14245 mutant had a diminished ability to impede the survival of *P. aeruginosa*, and this was evident at 37°C and 30°C (Fig. 4B). Reintroduction of the 14245 gene into the mutant restored its capacity to kill strain 7700, confirming that the 14245 protein is required for the ability of *S. maltophilia* to kill a heterologous bacterium. The 14255 mutant was also impaired for the ability to blunt the survival of *P. aeruginosa*, although the mutant phenotype was slightly more evident at 37°C than at 30°C (Fig. 4B). At both temperatures, a complemented 14255 mutant had restored activity, confirming that the 14255 protein, like the 14245 protein, is needed for the ability of *S. maltophilia* to maximally counteract its competitor. As we had observed previously for the wild type (WT) and the *virB10* mutant (31), the numbers of 14245 mutant and 14255 mutant did not change during the incubation period (Fig. S3A), indicating that the effects on *P. aeruginosa* depicted in Fig. 4B were not simply or indirectly due to alterations in growth of the *S. maltophilia* strains. The complemented mutants did show a drop in CFU during the coinubation at 37°C (but not at 30°C), which we suspect is due to elevated levels of the plasmid-encoded effectors not being sufficiently blocked by chromosomally encoded immunity proteins. Nonetheless, as noted above, the complemented mutants behaved as the wild type did in terms of impeding the survival of strain 7700, affirming that 14245 and 14255 are necessary for *S. maltophilia* to fully kill *P. aeruginosa*. The killing effects of 14245 and 14255 were evident whether the coinubations had a starting ratio of *S. maltophilia* to *P. aeruginosa* equal to 50 (as in Fig. 4B) or equal to 10 or 1 (Fig. S4). When we did the competition assay using strain DH5 α , we confirmed the inhibitory effect of the T4SS on *E. coli* at 37°C (31) and further observed that the inhibition of the *E. coli* strain, like the inhibition of *P. aeruginosa* 7700, was present and somewhat greater at 30°C (Fig. 4C). Most importantly, the 14245 mutant and 14255 mutant were again impaired for the ability to kill the competitor, whereas their complements were better at countering *E. coli*, especially at 30°C (Fig. 4C). When we monitored the numbers of *S. maltophilia* strains during these coinubations, the wild type and the mutants displayed no changes in CFU but the complemented mutants showed some reduction (Fig. S3A), as we had observed for the incubations with *P. aeruginosa*. Thus, these data confirmed that 14245 and 14255 are necessary for the ability of *S. maltophilia* to optimally kill two types of heterologous bacteria. In some cases, e.g., during the 30°C incubation with *P. aeruginosa* 7700 or the 37°C incubation with *E. coli* DH5 α , the 14245 mutant appeared to be as impaired as the *virB10* mutant was (Fig. 4B and C). In other cases, e.g., during 37°C incubation with strain 7700, the behavior of the 14255 mutant most closely matched the behavior of the *virB10* mutant (Fig. 4C). Thus, it appears that 14245 and 14255 are (the) major mediators of the bactericidal effects of the *S. maltophilia* T4SS, at least against the species tested here.

To begin to discern if the other putative T4SS effectors have antibacterial activity, we constructed and tested three more mutants. We examined 20845, since it was annotated as a nuclease and is prevalent among *S. maltophilia* strains and *Stenotrophomonas* species (Fig. 1) and its immunity protein (20840) had partly protected *E. coli* from killing (Fig. 2A). We also tested 19100, since it was annotated as a lipase and is also well conserved (Fig. 1). Finally, we included 02375, as an example of a novel protein that is also well conserved (Fig. 1). Although each of the three newly made mutants survived just as well as the wild type did when coinubated with either *P. aeruginosa* 7700 or *E. coli* DH5 α at 30°C and 37°C (Fig. S3B), none of them had an impaired ability to kill the heterologous bacteria upon short-term incubation (Fig. 5A and B). Since the immunity protein of 20845 had also afforded protection to *E. coli* during a 24-h coinubation with *S. maltophilia* (Fig. 2B), we further analyzed its corresponding mutant by extending the *E. coli* coinubation to 24 h. In this case, the 20845 mutant did show an impaired ability to compete against *E. coli*, and this was evident at both 37°C and 30°C (Fig. 5C). These data suggest that 20845 might also confer an antibacterial effect, although the effect of 20845 would appear to be bacteriostatic and more

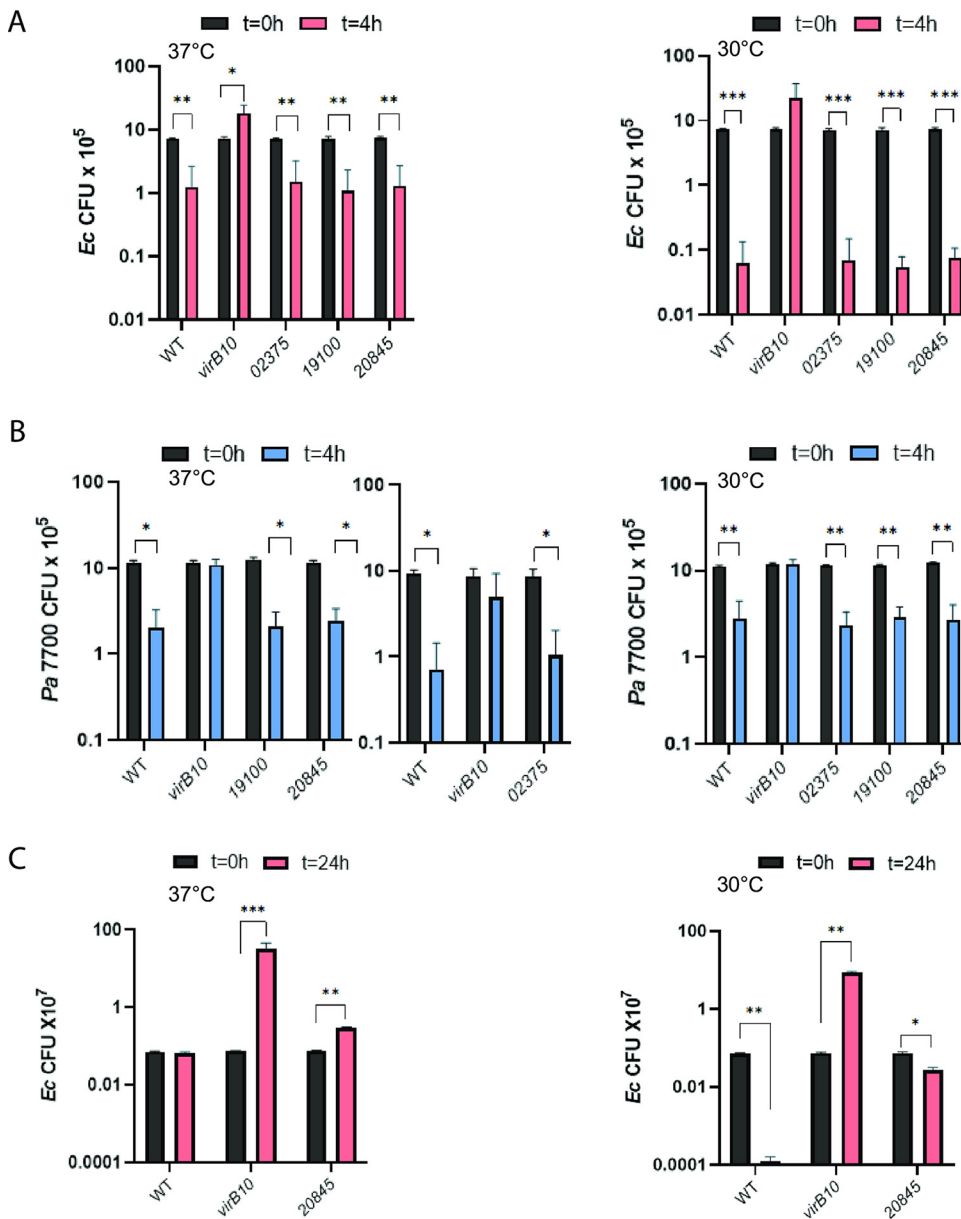


FIG 5 Effects of *S. maltophilia* wild-type, *virB10* mutant, 02375 mutant, 19100 mutant, and 20845 mutant strains on *E. coli* and *P. aeruginosa*. (A and B) *S. maltophilia* (Sm) K279a (WT), *virB10* mutant NUS15 (*virB10*), 02375 mutant NUS21 (02375), 19100 mutant NUS23 (19100), and 20845 mutant NUS24 (20845) were mixed with *E. coli* strain DH5 α (A) and *P. aeruginosa* strain 7700 (B) in a (*S. maltophilia*/other bacterium) ratio of ~50:1 and spotted onto LB agar, and after 4 h of incubation at either 37°C (left panels) or 30°C (right panels), the numbers of each strain were determined by plating dilutions of the entire bacterial growth area on selective medium. (C) *S. maltophilia* K279a WT, *virB10* mutant NUS15, and 20845 mutant NUS23 were mixed with *E. coli* DH5 α at a ratio of ~50:1 and spotted onto LB agar, and after 24 h of incubation at either 37°C (left panel) or 30°C (right panel), the numbers of CFU for each strain were determined. Results presented are the numbers of *E. coli* CFU or *P. aeruginosa* CFU at $t = 0$ h, $t = 4$ h, or $t = 24$ h, with asterisks indicating instances where there were significant differences in CFU obtained at $t = 0$ versus $t = 4$ or 24 h (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$). Data are presented as the means and standard deviations of results from three independent experiments ($n = 3$ each).

limited in terms of targets compared to 14245 and 14255. Thus, we returned our focus to the examination of 14245 and 14255, which had potent killing activities and were active against both *E. coli* and *P. aeruginosa*.

14245 and 14255 are bona fide substrates of the *S. maltophilia* T4SS. To determine if 14245 and 14255 translocate into competing bacteria in a T4SS-dependent

manner, we performed a Cre recombinase-mediated interbacterial protein translocation assay that has been used to study other T4SSs (66, 77). We generated translational fusions between the *S. maltophilia* effectors and the Cre recombinase in transfer-deficient plasmids and then introduced the constructs, along with the separate introduction of the vector control, into wild-type K279a and its *virB10* mutant. The different *S. maltophilia* strains were incubated with an *E. coli* strain that harbors a plasmid containing a *loxP*-flanked DNA segment which prevents the expression of a kanamycin resistance (*Kan*^r) gene; i.e., *Kan*^r would be expressed only if, upon coinubation with *S. maltophilia*, the effector translocates Cre into the recipient leading to Cre-mediated excision of the floxed sequence. When we incubated the *S. maltophilia* donors with the *E. coli* recipient for 4 h at 37°C, the frequency of *Kan*^r resulting from incubation with wild-type K279a carrying either the 14245 or 14255 fusion was greater than that obtained upon incubation with wild-type *S. maltophilia* carrying the vector control that lacks an effector-Cre fusion (Fig. 6A), indicating that 14245 and 14255 are capable of translocating into a heterologous bacterium. Importantly, this translocation event was abolished when the donor was the *virB10* T4SS mutant (Fig. 6A). The same observations were made when we allowed the donor and recipient to incubate for 16 h (Fig. 6B). This indicated that 14245 and 14255 are translocated in a T4SS-dependent manner from *S. maltophilia* to a target bacterium. In order to gain further support for this conclusion, we constructed a double mutant inactivated for both *virB10* and 14245 and a double mutant lacking both *virB10* and 14255 and then tested the double mutants for their relative ability to kill *E. coli* and *P. aeruginosa*. We first observed that the *virB10* 14245 mutant was not any more defective than the *virB10* mutant was (Fig. 7A), indicating that VirB10 (i.e., the T4SS apparatus) and 14245 operate in the same functional pathway. That the double mutant was more impaired than the 14245 mutant was compatible with there being more antibacterial effectors, such as 14255. Indeed, in the next experiment, we saw that the *virB10* 14255 double mutant was not any more defective than the *virB10* mutant was (Fig. 7B), indicating that VirB10 and 14255 are functioning in the same pathway. These data, together with the results of the Cre recombinase assay, document that 14245 and 14255 are bona fide effectors of the *S. maltophilia* T4SS. With this confirmation, we sought to further investigate the degree to which 14245 and 14255 explain the bactericidal effect of the T4SS, as measured against *P. aeruginosa* 7700. Thus, we made and tested a double mutant lacking both 14245 and 14255. This mutant was more defective for killing *E. coli* and *P. aeruginosa* 7700 than the 14245 mutant and 14255 mutant were (Fig. 7C), indicating that the two effectors promote independent killing mechanisms. However, the 14245 14255 mutant displayed a defect for killing similar to the *virB10* mutant (Fig. 7C), suggesting that 14245 and 14255 are required for all or nearly all T4SS-dependent killing that is occurring in this assay.

***S. maltophilia* T4SS effector 14245 is sufficient to kill heterologous bacteria.**

Given the validation of the importance of 14245 and 14255, we sought to determine if expression of these effectors alone within another bacterium would be sufficient to affect viability or growth of that competitor. Given data from T6SS studies (74, 78) as well as the putative enzyme activities of 14245 and 14255 (Fig. 1A), we posited that the effectors act in the target cell's periplasm. Thus, following a method that is long established in the T6SS field (79–81), we joined full-length 14245 and 14255 to an N-terminal periplasmic localization signal derived from the periplasmic protein PelB and then placed the genes' expression under the control of an arabinose-inducible (pBRA) promoter, all in the vector pBAD18. *E. coli* DH5 α containing these constructs as well as versions of 14245 and 14255 lacking the PelB signal were incubated on LB agar with and without arabinose at 37°C and 30°C. Cloned 14255, with or without periplasmic localization, did not alter the survival of *E. coli*, whether grown with or without inducing arabinose and whether grown at 37°C or 30°C (Fig. 8A), suggesting that 14255, although necessary for optimal killing by the *S. maltophilia* T4SS (as determined by mutant analysis), may not be sufficient to kill on its own and that it might have an

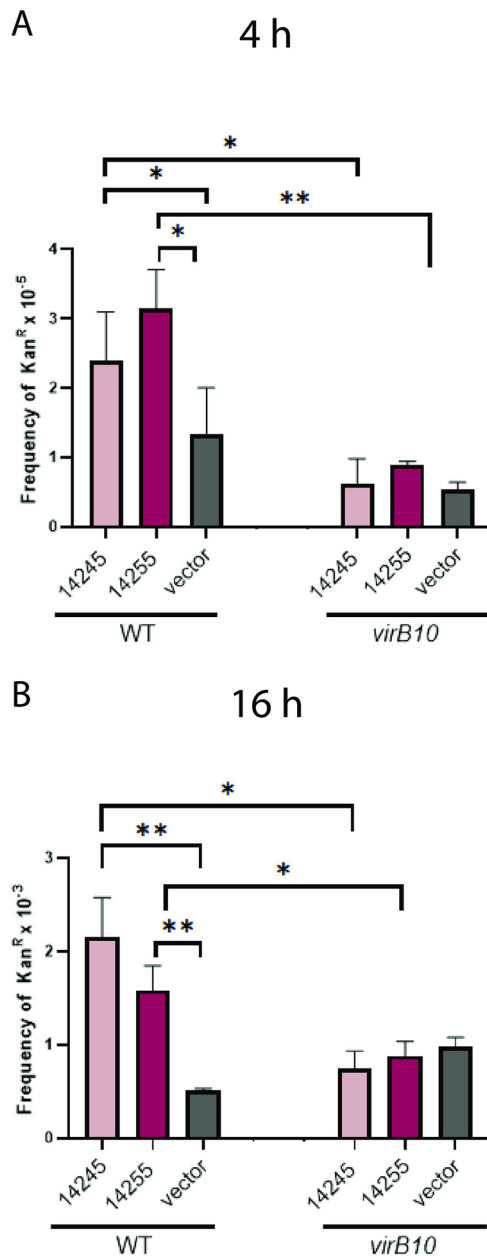


FIG 6 Translocation of effectors 14245 and 14255 from *S. maltophilia* wild-type and *virB10* mutant strains to heterologous bacteria. *S. maltophilia* K279a (WT) and *virB10* mutant NUS15 (*virB10*) either expressing a fusion between protein 14245 and the Cre recombinase (14245) or protein 14255 and the Cre recombinase (14255) or carrying only the pZL180Cre control (vector) were mixed with recipient *E. coli* XL1-Blue carrying pZL184 at a ratio of 1:50 (*S. maltophilia*/*E. coli*) and plated onto LB agar. After incubating at 37°C for 4 h (A) and 16 h (B), the entire spot of bacterial growth was resuspended and plated onto selective medium in order to identify excisants in which there was Cre-mediated excision of a floxed DNA sequence giving kanamycin resistance (Kan^R). The transfer frequency was represented by the frequency of Kan^R CFU per donor bacterium. Indicative of effector protein translocation, asterisks indicate transfer frequencies from WT strains that were greater than that of either the vector control or those from the *virB10* mutant (*, $P < 0.05$; **, $P < 0.01$). Data are presented as the means and standard deviations of results from three independent experiments ($n = 3$ each).

interacting partner. It is also possible that the presence of the PelB sequence may have altered the activity of the 14255 protein. In contrast, clones containing induced, periplasmic 14245 showed greatly impaired survival at 37°C and 30°C, with the efficiency of plating being ~4 orders of magnitude less than that of control *E. coli* (Fig. 8A, right).

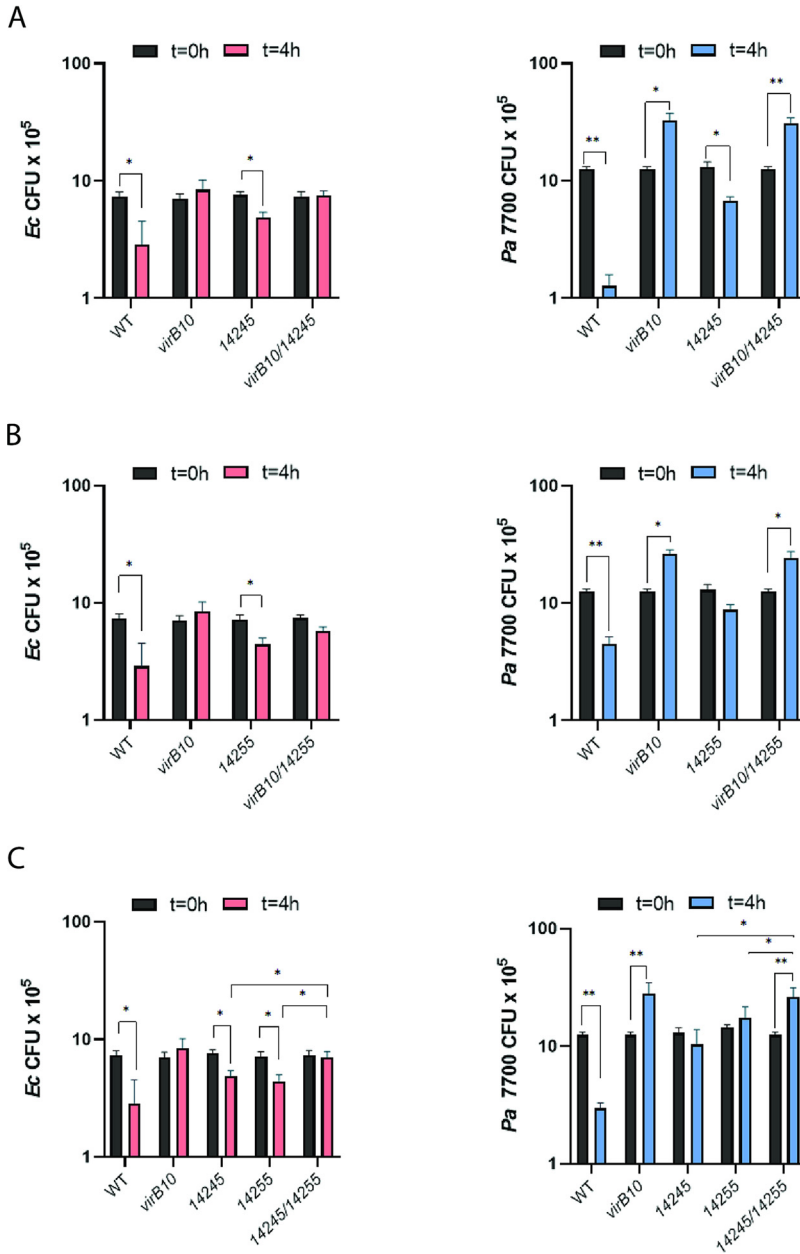


FIG 7 Bactericidal effect of *S. maltophilia* *virB10* 14245 mutant, *virB10* 14255 mutant, and 14245 14255 mutant strains on *E. coli* and *P. aeruginosa*. *S. maltophilia* K279a (WT) and *virB10* mutant NUS15 (*virB10*) (A to C) as well as either the 14245 mutant NUS17 (14245) and *virB10* 14245 mutant NUS25 (*virB10* 14245) (A), 14255 mutant NUS19 (14255) and *virB10* 14255 mutant NUS26 (*virB10* 14255) (B), or 14245 mutant, 14255 mutant, and 14245 14255 mutant NUS27 (14245 14255) (C) were mixed with *E. coli* DH5 α (left panels) and *P. aeruginosa* strain 7700 (right panels) in a (*S. maltophilia*/other bacterium) ratio of ~50:1 and spotted onto LB agar, and after 4 h of incubation at 37°C, the numbers of each strain were determined by plating dilutions of the entire bacterial growth area on selective medium. Results presented are the numbers of *E. coli* or *P. aeruginosa* CFU at *t* = 0 h and *t* = 4 h, with asterisks indicating cases where there were significant differences in CFU obtained at the two time points (*, *P* < 0.05; **, *P* < 0.01). Data are presented as the means and standard deviations of results from three independent experiments (*n* = 3 each).

This massive killing was not seen when there was no induction of gene expression; i.e., in the absence of arabinose (Fig. 8A, left). Moreover, it did not occur when the induced, cloned protein lacked a periplasmic localization signal (Fig. 8A, right). These data indicated that 14245, when delivered into the periplasm, is sufficient to kill *E. coli*. To bolster this conclusion, we tested the effect of the pBRA-PelB-14245 and pBRA-14245

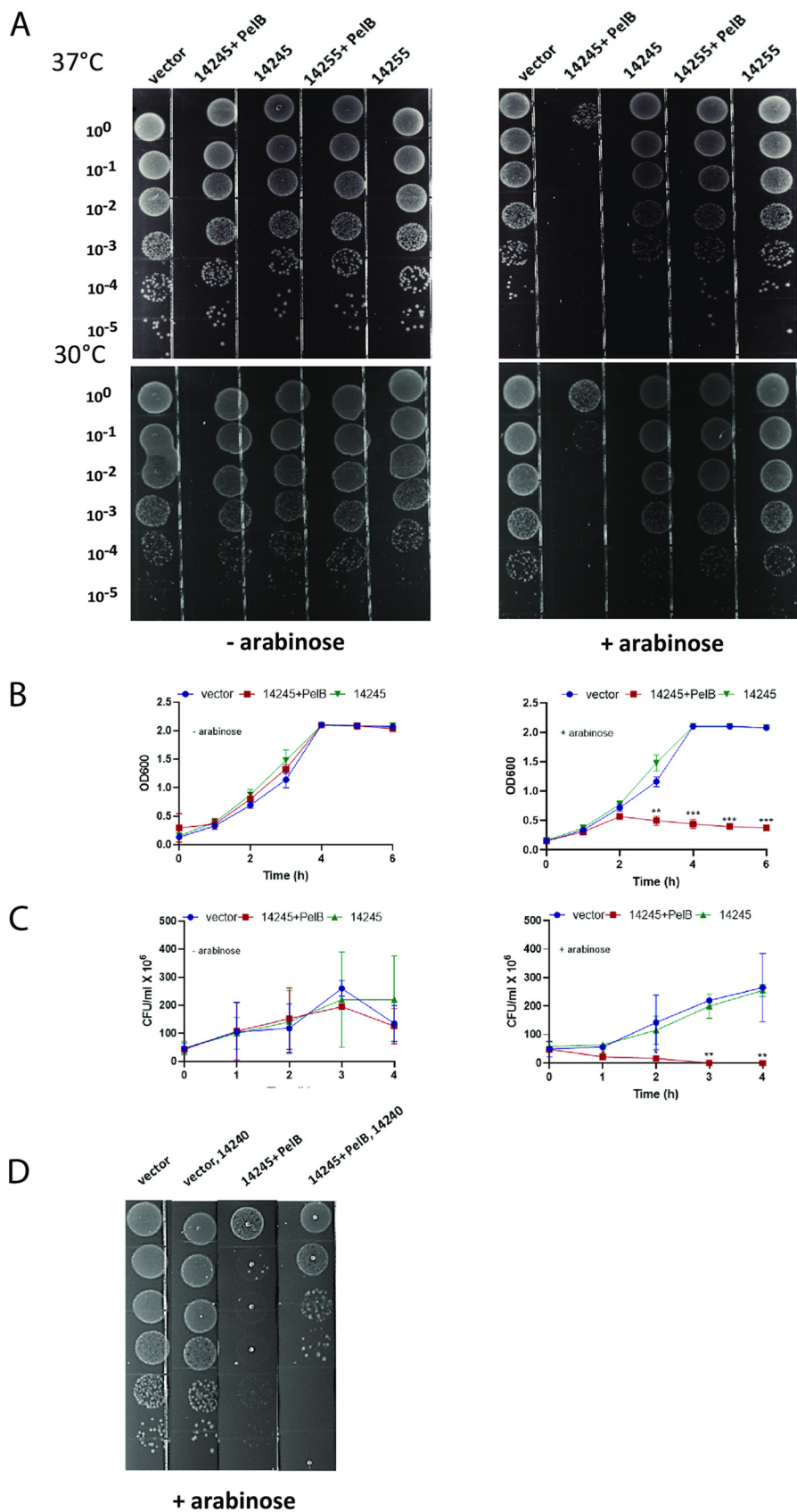


FIG 8 Effects of cloned T4SS effectors 14245 and 14255 on the viability of heterologous bacteria. (A) *E. coli* DH5 α containing either the pBAD18 vector (vector), pBAD18-pelB14245 expressing 14245 (Continued on next page)

constructs, along with the pBRA control, on *E. coli* survival in broth. When there was no induction of gene expression, the optical densities (OD) of the different *E. coli* cultures were similar, but when arabinose was present, the periplasmically localized 14245 resulted in a sharp drop in the OD (Fig. 8B). The impact of 14245 was also evident when we assayed for CFU in the presence of arabinose; whereas there was an ~5-fold increase in CFU in 4 h for the strain containing the vector or the 14245 gene lacking the PelB signal, the PelB-14245 fusion resulted in an immediate and steady decline in CFU (Fig. 8C, right). Finally, we observed that the coexpression of the immunity protein 14240 reversed to a large extent the killing activity of the periplasmic PelB-14245 (Fig. 8D). In sum, these data indicate that the T4SS effector 14245 is both necessary and sufficient to kill heterologous bacteria.

T4SS effectors 14245 and 14255 promote the ability of *S. maltophilia* to kill clinical isolates of *P. aeruginosa*. With the confirmation that 14245 and 14255 are indeed T4SS substrates that help mediate the killing of and competition with an environmental isolate of *P. aeruginosa*, we sought to determine if the two have activity against clinical isolates of *P. aeruginosa*. Based upon testing the *virB10* mutant and its complement, we had previously reported that *S. maltophilia* T4SS promotes the killing of *P. aeruginosa* strain PAO1, a human wound isolate (82), and *P. aeruginosa* PAK, another well-studied clinical isolate (83, 84). Shortly thereafter (58), it was reported that the *Stenotrophomonas* T4SS also impedes *P. aeruginosa* PA14, a human burn wound isolate (85, 86). When our *S. maltophilia* strains were competed against these three *P. aeruginosa* strains in parallel, the *virB10* mutant again showed an impaired ability to kill each of the strains and to comparable degrees (Fig. 9A). More importantly to the present study, the 14245 mutant and 14255 mutant were each as impaired as the *virB10* mutant was against the three targets (Fig. 9A), further implicating 14245 and 14255 as having roles in the bactericidal effects of the *S. maltophilia* T4SS. As before, the numbers of mutant bacteria were constant during the incubation (Fig. S5A), indicating that the effects on *P. aeruginosa* shown in Fig. 9A were not simply due to alterations in growth of the *S. maltophilia* strains. Since PAO1, PAK, and PA14 were isolated more than 40 years ago and have been passaged a great deal in laboratories, we studied, at both 37°C and 30°C, the bactericidal effect of the *S. maltophilia* T4SS and effectors 14245 and 14255 against four strains of *P. aeruginosa* that were recently isolated from the lungs of four CF patients. Based upon mutant analysis, the T4SS promoted killing of two of these isolates, i.e., C073 and X209, and in both cases, the killing was greater at 37°C than at 30°C, and effectors 14245 and 14255 continued to play a major role, particularly at 37°C (Fig. 9B, two leftmost panels). When we monitored the numbers of the *S. maltophilia* strains during the coinoculations, the wild type and the mutants behaved similarly, aside from a slight reduction for the 14245 mutant at 30°C (Fig. S5B), indicating that the more notable effects on *P. aeruginosa* depicted in Fig. 9B

FIG 8 Legend (Continued)

with a PelB periplasmic-localization signal (14245 + PelB), pBAD18-14245 expressing 14245 without a periplasmic-localization signal (14245), pBAD18-pelB14255 expressing 14255 with a PelB periplasmic-localization signal (14255 + PelB), or pBAD18-14255 expressing 14255 without a periplasmic-localization signal (14255) was serially diluted (as indicated) and spotted onto LB agar without (left panel) and with (right panel) 0.4% arabinose, which induces expression of the cloned proteins from the vector's pBRA promoter. Images present bacterial survival and growth after 18 h of incubation at 37°C (top row) or 30°C (bottom row) and are representative of the results seen in three independent experiments. (B and C) The *E. coli* strains indicated above in panel A were incubated at 37°C in LB broth without (left panels) and with (right panels) arabinose, and then at the indicated time points, bacterial survival and growth were assessed by measuring the OD₆₀₀ of the cultures (B) and by determining the numbers of CFU by plating (C). Asterisks indicate time points at which the clone expressing periplasmically localized 14245 behaved differently from clones expressing the vector control or the cytoplasmically localized 14245 (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$). (D) *E. coli* DH5 α containing either pBAD18 (vector), pBAD18-pelB14245 (14245 + PelB), pBAD18 and a second vector expressing the 14240 immunity protein (vector, 14240), or pBAD18-pelB14245 and the immunity protein-encoding plasmid (14245 + PelB, 14240) was serially diluted and spotted as indicated in panel A. Clones with the 14240 construct were plated on LB plates that also contained kanamycin. Data are presented as the means and standard deviations of results from three independent experiments ($n = 3$ each).

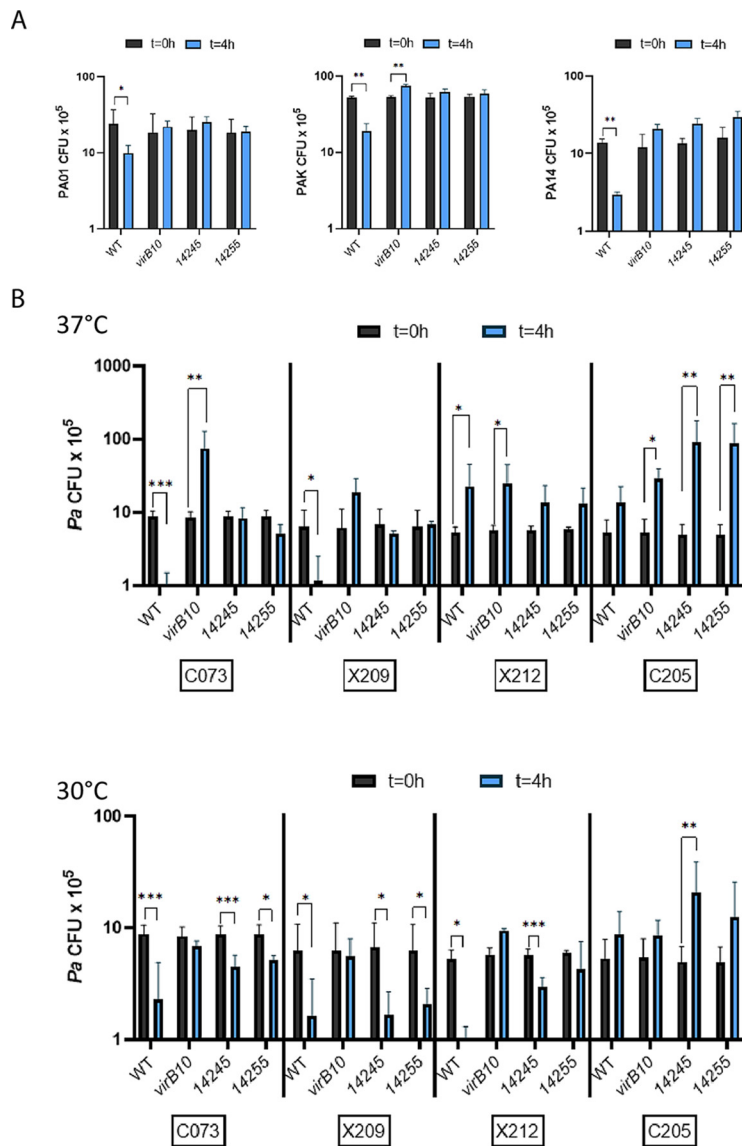


FIG 9 Bactericidal effect of *S. maltophilia* wild-type, *virB10* mutant, 14245 mutant, and 14255 mutant strains on clinical isolates of *P. aeruginosa*. (A) *S. maltophilia* K279a (WT), *virB10* mutant NUS15 (*virB10*), 14245 mutant NUS17 (14245), and 14255 mutant NUS19 (14255) were mixed with *P. aeruginosa* strains PAO1, PAK, and PA14 in a (*S. maltophilia*/other bacterium) ratio of ~50:1 and spotted onto LB agar, and after 4 h of incubation at 37°C, the numbers of each strain were determined by plating dilutions of the entire bacterial growth area on selective medium. (B) The four *S. maltophilia* strains in panel A were mixed with *P. aeruginosa* CF lung isolates C073, X209, X212, and C205 in a ratio of ~50:1 and spotted onto LB agar, and after 4 h of incubation at 37°C (top) or 30°C (bottom), the numbers of each strain were determined by plating. The results presented are the numbers of *P. aeruginosa* CFU at *t* = 0 h and *t* = 4 h, with asterisks indicating those cases where there were significant differences in CFU obtained at *t* = 0 versus *t* = 4 h (*, *P* < 0.05; **, *P* < 0.01; ***, *P* < 0.001). Values that are not sufficiently clear from the figure are as follows: at 37°C, C073 CFU when competed against WT = 7.1×10^4 ; at 30°C, X212 CFU when competed against WT = 5.8×10^4 . Data are presented as the means and standard deviations of results from three independent trials (*n* = 3 each).

were not due to alterations in growth of the *S. maltophilia* strains. A third *P. aeruginosa* isolate from the CF lung, strain X212, was sensitive to killing by the *S. maltophilia* T4SS and the 14245 effector, but this was seen only at the lower temperature (Fig. 9B), and in the case of the effector, could not be simply ascribed to there being greater growth of the mutant (Fig. S5B). Further examination of complemented mutants confirmed the importance of the 14245 and 14255 effectors in the killing of these three clinical

isolates (Fig. S6). In marked contrast, the *S. maltophilia* T4SS (and its effectors) did not promote the killing of the fourth isolate, strain C205, at either temperature (Fig. 9B), even though the *S. maltophilia* strains were largely able to maintain their numbers (Fig. S5B). Together, these data confirm that the *S. maltophilia* T4SS and effectors 14245 and 14255 are active against multiple clinical isolates of *P. aeruginosa*, including contemporary isolates obtained from the lungs of CF patients.

DISCUSSION

The present study represents the first documentation of a bactericidal effector of the recently described *S. maltophilia* T4SS and one of only a few such effectors from T4SSs overall. We designate the gene encoding 14245 as *tfcA*, for type four secreted bactericidal effector A, and hence the protein as TfcA. Furthermore, we designate the gene encoding 14255 as *tfcB*, for type four secreted bactericidal effector B, and hence the protein as TfcB. Discovering these roles for TfcA and TfcB further validates a bioinformatic screen that was developed for the study of *Xanthomonas* T4SS (65). While analyzing TfcA and TfcB, we also documented, for the first time, that the T4SS of *S. maltophilia* is active against recent clinical isolates, namely *P. aeruginosa* from the lungs of CF patients. The analysis of the bactericidal T4SS of *S. maltophilia* remains the only such examination of a human pathogen, since other experimental work on T4SS-mediated interbacterial competition involves *Xanthomonas* and *Bartonella* species that afflict plants and animals, respectively (65, 66). Yet, our findings have implications for not only the uncharacterized T4SSs that exist in other species of *Stenotrophomonas* but also T4SSs beyond *Stenotrophomonas* (31, 87).

The ~46-kDa TfcA effector was both necessary and sufficient to kill heterologous bacteria. Based on the assays utilizing the PelB localization sequence, TfcA likely kills when it is injected by the T4SS into the periplasm of target bacteria. Compatible with this hypothesis is that the cognate immunity protein of TfcA carries a lipoprotein signal sequence, which would carry it to the periplasm of *S. maltophilia* where its protective effect is normally needed against “accidental leakage” of TfcA. Such a scenario is akin to that of many bactericidal effectors of T6SS (79–81). TfcA has very high sequence similarity to a large number of hypothetical proteins encoded by a wide range of bacteria, with the greatest levels of similarity occurring with proteins from the human pathogen *Acinetobacter baumannii* and environmental *Streptomyces* and *Xanthomonas* species (see Fig. S7A in the supplemental material). Importantly, the T4SS effector also has significant similarity to known lipases produced by five fungal species and three (Gram-negative) bacterial species (Fig. S7A), including members of the class III family of lipases and di- and triacylglycerol lipases (88–94). That TfcA might be a lipase is supported by structural prediction obtained using Phyre2 (Fig. S7B). Thus, we hypothesize that, following delivery by the T4SS, TfcA acts on the target’s lipid-containing, outer membrane and/or inner membrane, resulting in death of the competitor. Although ~37-kDa TfcB was necessary for optimal killing by the *S. maltophilia* T4SS, it alone was not sufficient to kill targets whether expressed in the periplasm (due to a fused PelB sequence) or in the cytoplasm (when lacking the PelB tag). Thus, we posit that TfcB might have a T4SS-dependent, interacting partner that is needed for it to act. Alternately, it is possible that our assay system did not properly deliver (sufficient levels of) TfcB in order to see its toxic effect. Regardless of whether TfcB acts alone or in combination, we strongly suspect that its bactericidal activity is associated with a predicted lysozyme-like activity. TfcB has very high sequence similarity to a large number of hypothetical proteins that are encoded by a wide range of bacteria and are variously annotated as putative chitinases, putative lytic enzymes, and putative peptidoglycan-binding proteins (Fig. S8A). The greatest levels of similarity occurred with proteins from *A. baumannii*, *P. aeruginosa*, and environmental *Streptomyces*, *Mesorhizobium*, and *Xanthomonas* species. Yet, this T4SS effector also has significant similarity to at least two characterized enzymes, i.e., a T4SS-dependent and bactericidal peptidoglycan hydrolase from *X. citri* and a chitinase from *Streptomyces griseus* (Fig. S8A) (65, 95, 96). Compatible with these various BLAST results, Phyre2 software

predicts a structure that fits into the family of endolysins (Fig. S8B). Thus, we posit that, following injection into the target cell, TfcB acts to degrade peptidoglycan, resulting in the death of the target cell. Compatible with this hypothesis is that the cognate immunity protein of TfcB is homologous to known peptidoglycan hydrolase inhibitors (65, 97). That *S. maltophilia* would utilize more than one effector to kill a single target, whether that is *E. coli* or *P. aeruginosa*, is compatible with the literature involving other antibacterial systems such as the T6SSs (73, 98). On the other hand, it is also conceivable that the importance of TfcA and TfcB and perhaps other putative effectors varies with the target. Future work should confirm how these newly defined, major effectors kill heterologous bacteria.

Aside from our extensive analysis of TfcA and TfcB, we gained evidence for there being antibacterial activities associated with other putative effectors of the *S. maltophilia* T4SS. The supporting data were greatest in the case of 20845, a putative nuclease that is well conserved among *S. maltophilia* strains and confers at least a bacteriostatic activity when delivered into some target bacteria. The putative effectors 01275, 01575, 02400, and 17170 might each also encode a bactericidal activity; however, mutant analysis obviously needs to be done in order to affirm the role of these proteins. That 17170 might prove to be important even though it lacks the typical C-terminal secretion signal of the other effectors indicates that there is utility to continuing to use multiple bioinformatic screens when studying *S. maltophilia* T4SS. The last other protein to mention as a bacteriostatic factor is the 14405 protein. This last case is supported by a recent study which determined that 14405 (i) is translocated from *S. maltophilia* into a laboratory strain of *E. coli* in a T4SS-dependent manner, (ii) is inhibitory to *E. coli* growth when expressed as a cloned protein within the periplasm, and (iii) is inhibitory to *E. coli* growth when delivered by the T4SS of *X. citri*, with this inhibitory effect being reversed by the presence of the cognate immunity protein (58). Thus, despite the clear role of TfcA and TfcB in the bactericidal activity of *S. maltophilia*, there are benefits to confirming if other proteins identified by bioinformatic analysis are bona fide substrates of the T4SS and, if they are, to characterizing their activities. The possibility that the *S. maltophilia* T4SS might elaborate as many as 13 (or perhaps more) effectors is compatible with the output of some of the other T4SSs (99, 100).

S. maltophilia and *P. aeruginosa* are important human pathogens that can coexist both in environmental niches, including water systems in hospital settings (101–108), and within the human host, most notoriously in the CF lung (3, 11, 109–113). Reports have varied as to whether each organism benefits from the other's presence or not (11, 113). The VirB/D4 T4SS and the effectors TfcA and TfcB that we have defined obviously act to antagonize *P. aeruginosa*. For several reasons, we infer that this antagonism is biologically relevant. First, the T4SS and its effectors were active against a range of wild-type *P. aeruginosa* strains, including recent isolates from CF lungs. Second, the bactericidal effect was evident both at multiple temperatures, indicative of environmental habitats and the human host, and at bacterial ratios as low as 1:1. Third, based on our prior study (31), other strains of *S. maltophilia* can kill a *P. aeruginosa* competitor. Interestingly, one out of the four CF isolates tested (i.e., C205) was not impeded by *S. maltophilia* K279a. This could be due to that bacterium being resistant to the action of the T4SS and/or because it has its own antibacterial tools that weaken the *Stenotrophomonas* competitor (63, 114, 115). Given our results, it will be worthwhile to test an even larger panel of *P. aeruginosa* strains for sensitivity to *S. maltophilia* effectors. Moreover, it will be instructive to examine this impact of the T4SS under different assay conditions, such as in environmental samples and biofilms, on host cell surfaces, or in lungs of experimental animals. Although we featured in our studies the impact of the *S. maltophilia* T4SS on *Pseudomonas* species, the analysis is worth expanding to other species, especially those that naturally coexist with *S. maltophilia* in humans, whether it be in the CF lung, the SARS-CoV-2-infected lung, or other sites of mixed infection (14–16). Moreover, since we previously discovered that the *S. maltophilia* T4SS modulates death pathways in human cells (31), it will be important to ascertain if

any of the effectors or putative effectors defined here (also) mediate interactions with mammalian targets. In conclusion, our results provide fresh insights into understanding the emerging pathogen *S. maltophilia*, the expanding role of T4SSs and their myriad effectors, and the principles of clinically relevant, interbacterial competition.

MATERIALS AND METHODS

Bacterial strains and media. *S. maltophilia* K279a (American Type Culture Collection [ATCC] BAA-2423) was the primary wild-type strain of *S. maltophilia* used in this study as well as the parent for the *virB10* mutant NUS15 (31) and newly made mutants (see below). In addition to strain 7700 (ATCC 7700) (31), strains of *P. aeruginosa* used in the bacterial competition assays were PAO1, PAK, PA14, and CF lung isolates C073, C205, X209, and X212 (obtained from Alan Hauser, Northwestern University). In addition to also being employed in the competition assays, as before (31), *E. coli* DH5 α served as the host for recombinant plasmids. *E. coli* S17-1 was used for conjugation during mutagenesis, as before (31), whereas *E. coli* XL1-Blue was used in the protein translocation assay, as previously described (77). All strains were maintained or tested at either 37°C or 30°C on Luria-Bertani (LB) agar (Becton, Dickinson). Wild-type and mutant *S. maltophilia* and recombinant DH5 α were also grown and monitored in liquid cultures by using LB broth.

DNA, RNA, and protein analysis. *S. maltophilia* K279a DNA and RNA were isolated as before (21, 22). DNA sequence analysis and primer design were done using SnapGene (GSL Biotech). qRT-PCR analysis was performed as previously described (116), with the primers targeting putative T4SS effectors designed using the Primer-BLAST tool at NCBI. All primers (Integrated DNA Technology) are listed in Table S2 in the supplemental material. The T4SEpre and S4TE programs were utilized to search the K279a genome in GenBank at NCBI for putative T4SS effectors based on the presence of C-terminal sequence patterns (67, 68). Additionally, BLAST was used to search the K279a database for proteins bearing sequence similarity to GLXRIDHV and FAVQGxxDPAHxRAHV, the conserved C-terminal domains in *Xanthomonas* T4SS substrates (65, 71). The ORFs for the identified proteins were visualized on NCBI to determine whether the ORFs' operons contained an associated immunity protein. Annotations of the putative effectors and immunity proteins were obtained using NCBI BLAST. A reciprocal best BLAST strategy and HHpred (117) were utilized to find orthologs of the *S. maltophilia* K279a T4SS effectors and immunity proteins within genomes deposited in GenBank. In that analysis, an E value cutoff of 1E-5 was used to remove highly divergent sequences. Structural homology and structural predictions for 14245 and 14255 were done by inputting the FASTA sequences for the proteins into the Phyre2 software (118).

Mutant and complemented mutant constructions. We introduced deletion mutations into the *S. maltophilia* K279a genes encoding 14245, 14255, 02375, 19100, and 20845, as we did before for various other genes (31). DNA fragments consisting of the FLP recombination target (FRT)-flanked chloramphenicol cassette bounded on the one side by the ~600 bp of sequences upstream of the effector gene and flanked on the other side by the ~600 bp of sequences downstream of the effector gene were synthesized by Twist Bioscience. Each of the constructs was ligated into pEX18Tc, and then the resultant plasmids were transformed into *E. coli* strain S17-1 followed by transfer to *S. maltophilia* K279a by conjugation. The desired gene deletions were confirmed by sequencing and PCR using target-gene specific primers (Table S2). Two independent 14245 mutants were obtained and were designated strains NUS17 and NUS18. Two independent 14255 mutants were made and were named strains NUS19 and NUS20. The two mutants inactivated for 02375 was designated strains NUS21 and NUS22. Finally, the 19100 mutant was named NUS23, and the 20845 mutant was named NUS24. For *trans*-complementation of the 14245 mutant NUS17 and the 14255 mutant NUS19, PCR fragments containing the 14245 and 14255 ORFs and their promoters were PCR amplified from K279a DNA using primer pairs MN58 and MN59 and MN60 and MN61, respectively (Table S2). These fragments were digested by EcoRI and XbaI and cloned into pBBR1MCS, yielding pB14245 and pB14255, which were electroporated into their corresponding mutants. Transformants were selected on chloramphenicol-containing LB agar, and clones were confirmed as carrying pB14245 and pB14255 by PCR using M13 primers (Table S2). Double mutants lacking either *virB10* (i.e., SMLT_RS14310) and 14245 or *virB10* and 14255 were constructed by mobilizing pEX Δ *virB10::frt-cat-frt* carried by *E. coli* S17-1 (31) into either the newly made 14245 mutant NUS17 or 14255 mutant NUS19. The *virB10* 14245 double mutant was designated strain NUS25, and the *virB10* 14255 double mutant was designated NUS26. A double mutant lacking both 14245 and 14255 (designated NUS27) was constructed by mobilizing pEX Δ 14245::*frt-cat-frt* carried by the *E. coli* S17-1 strain (above) into the NUS19 mutant containing the 14255 deletion.

Assays for interbacterial killing and competition. Aside from utilizing a range of incubation temperatures, interbacterial killing and competition were assessed as before (31). Briefly, strains to be tested were grown in LB broth at either 37°C or 30°C, and then the different cell suspensions brought to equivalent CFU/ml were combined to yield ratios of *S. maltophilia* to the heterologous bacterium approximating 50:1, 10:1, and 1:1. Fifty microliters of the mixture was spotted onto LB agar plates and incubated at 37°C or 30°C, either for 2 to 4 h in order to gauge bactericidal effects or for 24 h in order to judge effects on bacterial growth. Bacterial survival was determined by measuring the numbers of different CFU in the spot by plating resuspended material on LB agar. In order to test the complemented mutants in this assay and induce the expression of the plasmid-encoded *S. maltophilia* genes, 1 μ M IPTG was present in both the LB broth used for the precultures and the LB agar. In order to measure the ability of strain K279a immunity proteins to protect heterologous bacteria against the *S. maltophilia* T4SS, the proteins'

coding regions were either synthesized by Twist Bioscience or PCR amplified by primers MN62 to MN71 (Table S2), digested by EcoRI and XbaI, and ligated into pBBR1MCS. The constructs were then transformed into *E. coli* DH5 α and *P. aeruginosa* 7700, and the resultant transformants were incorporated into the bacterial killing and competition assays as described above.

Cre recombinase-based assay for measuring bacterial translocation of T4SS effectors. To measure the T4SS-dependent transfer or translocation of proteins from *S. maltophilia* strain K279a to a competing bacterium, we adapted a protein translocation assay developed to study the T4SS of *Legionella pneumophila* (77). DNA fragments containing either the ORF encoding 14245 or the ORF for 14255 were PCR amplified using primer pairs MN72 and MN73, and MN74 and MN75, respectively (Table S2), digested with BglII and KpnI, and then ligated to the 3' end of *cre* on pZL180, which lacks an origin of transfer (*oriT*) (77). The resultant pZL180Cre14245 and pZL180Cre14255, along with vector control pZL180Cre, were separately transformed into both wild-type K279a and its *virB10* mutant derivative. The newly made *S. maltophilia* donor strains and *E. coli* XL1-Blue carrying pZL184, a derivative of *mob*-pBBRMCS that carries a *loxP*-flanked KanR (77), were each grown to mid-log phase, washed twice with phosphate-buffered saline (PBS), and then resuspended to an OD₆₀₀ of 0.3 in LB broth. The two cell suspensions were then combined, at a ratio of 1:50 (*S. maltophilia*/*E. coli*) in a volume of 1 ml. Fifty microliters of the mixtures was plated onto LB agar containing 1 μ M IPTG and incubated at 37°C. After 4 h and 16 h of incubation, the entire spot was swabbed from the plate and resuspended in 1 ml of PBS. The desired excisants were then selected on LB agar containing 5% (wt/vol) sucrose, 30 μ g/ml kanamycin, and 30 μ g/ml chloramphenicol, i.e., a condition that kills both the donor *S. maltophilia* and *E. coli* recipients still carrying a floxed kanamycin cassette. The amount of donor *S. maltophilia* was determined by plating appropriate dilutions onto LB agar containing 100 μ g/ml carbenicillin. Ultimately, the transfer frequency was represented by the frequency of kanamycin resistance CFU (i.e., the number of excisants) per donor bacterium, as previously defined (77).

Assay for the bactericidal activity of cloned T4SS effectors. In order to measure the bactericidal activity of *S. maltophilia* T4SS effectors, we utilized a method that was established in the T6SS field and also permits the identification of effectors that kill by acting within the periplasm (79–81). DNA fragments containing the full-length 14245 gene or the entire 14255 gene were PCR amplified using pairs MN58 and MN59 and MN60 and MN61, respectively (Table S2), digested with EcoRI and XbaI, and then ligated into pBAD18 (119) such that the expression of the cloned genes was under the control of the arabinose-inducible pBRA promoter. Once transformed into target *E. coli*, these constructs, designated pBAD18-14245 and pBAD18-14255, would allow us to determine if the K279a proteins promoted cell death when they are expressed in the cytoplasm of the heterologous bacterium. By using primer pairs MN76 and MN59 and MN77 and MN61 (Table S2), the two *S. maltophilia* genes were cloned into pBAD18 without their start codon and instead aligned with the periplasmic localization sequence (ATGAAATACCTGCTGCCGACCGTGTCTGCTGCTCTCGCTGCCAGCCGGCGATGGCC) of PelB, a well-studied periplasmic protein (120). Once transformed into target *E. coli*, these constructs, designated pBAD18-pelB14245 and pBAD18-pelB14255, would allow us to determine if the K279a proteins promoted killing when they are expressed in the periplasm of the bacterial competitor. pBAD18-14245, pBAD18-pelB14245, pBAD18-14255, and pBAD18-pelB14255, along with the control pBAD18 vector, were separately transformed into *E. coli* DH5 α . Following growth overnight at 37°C on LB plates containing 100 μ g/ml ampicillin, each of the selected clones was resuspended in LB broth to an OD₆₀₀ of 0.3, further diluted in LB broth in 10-fold increments, and then spotted, as 10- μ l aliquots, onto ampicillin-containing LB agar with and without 0.4% arabinose to induce the pBRA promoter. Bacterial survival and growth within the spots were visually observed after 18 h of incubation at 37°C. In order to also assess the effect of the cloned *S. maltophilia* proteins on *E. coli* in liquid cultures, the various clones were resuspended to an OD₆₀₀ of 0.1 in LB broth with and without arabinose and then allowed to grow for 6 h at 37°C with shaking at 200 rpm (C25KC incubator; New Brunswick Scientific). Once every hour, bacterial survival and growth were assessed by measuring the OD₆₀₀ of the culture and by determining CFU by plating. To determine the protective nature of the cognate immunity protein of 14245 in this assay, a plasmid encoding the immunity protein 14240 (see above) was transformed into *E. coli* DH5 α clones harboring either pBAD18-pelB14245 or the control pBAD18 vector. Following growth overnight at 37°C on LB plates containing 100 μ g/ml ampicillin and 50 μ g/ml kanamycin, both clones were resuspended in LB broth to an OD₆₀₀ of 0.3 and dilution plated in 10- μ l aliquots as described above onto ampicillin- and kanamycin-containing LB agar with 0.4% arabinose to induce expression of pelB14245. Bacterial survival and growth within the spots were visually observed after 18 h of incubation at 37°C.

Statistical procedures. In all experiments, each sample or condition was assessed using at least three technical replicates. All experiments were repeated at least three times. The resultant values obtained were presented as the means and standard deviations from the three independent experiments, and statistical analysis was applied using the Student *t* test, as appropriate. *P* values are presented in the figure legends.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

FIG S1, TIF file, 0.3 MB.

FIG S2, TIF file, 0.7 MB.

FIG S3, TIF file, 0.5 MB.

FIG S4, TIF file, 0.3 MB.

FIG S5, TIF file, 0.7 MB.

FIG S6, TIF file, 0.3 MB.

FIG S7, TIF file, 0.7 MB.

FIG S8, TIF file, 0.7 MB.

TABLE S1, PDF file, 0.2 MB.

TABLE S2, PDF file, 0.1 MB.

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