Triclosan Tolerance Is Driven by a Conserved Mechanism in Diverse Pseudomonas Species

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ABSTRACT Perturbation of natural microbial communities by antimicrobials, such as triclosan, can result in selection for antibiotic tolerance, which is of particular concern when pathogens are present. Members of the genus Pseudomonas are found in many natural microbial communities and frequently demonstrate increased abundance following triclosan exposure. The pathogen and well-studied model organism Pseudomonas aeruginosa exhibits high triclosan tolerance; however, it is unknown if all Pseudomonas species share this trait or if there are susceptible strains. We characterized the triclosan tolerance phenotypes of diverse Pseudomonas isolates obtained from triclosan-exposed built environments and identified both tolerant and sensitive strains. High tolerance is associated with carriage of the enoyl-acyl carrier reductase (ENR) isozyme gene fabV, compared to the lesser protective effects of efflux or presence of ENRs. Given its unique importance, we examined fabV distribution throughout Pseudomonas species using large-scale phylogenomic analyses. We find fabV presence or absence is largely invariant at the species level but demonstrates multiple gain and loss events in its evolutionary history. We further provide evidence of its presence on mobile genetic elements. Our results demonstrate the surprising variability in triclosan tolerance in Pseudomonas and confirm fabV to be a useful indicator for high triclosan tolerance in Pseudomonas. These findings provide a framework for better monitoring of Pseudomonas in triclosan-exposed environments and interpreting effects on species and gene composition.

IMPORTANCE Closely related species are typically assumed to demonstrate similar phenotypes driven by underlying conserved genotypes. When monitoring for the effect of antimicrobials on the types of species that may be selected for, this assumption may prove to be incorrect, and identification of additional genetic markers may be necessary. We isolated several phylogenetically diverse members of Pseudomonas from indoor environments and tested their phenotypic tolerance toward the commonly used antimicrobial triclosan. Although Pseudomonas isolates are broadly regarded to be highly triclosan tolerant, we demonstrate the presence of both triclosan-tolerant and -susceptible strains, separated by a difference in tolerance of nearly 3 orders of magnitude. Bioinformatic and experimental investigation demonstrated that the presence of the gene fabV was associated with high tolerance. We demonstrate that fabV is not evenly distributed in all Pseudomonas species and that its presence could be a useful predictor of high triclosan tolerance suitable for antimicrobial monitoring efforts involving triclosan.

KEYWORDS antimicrobials, genomics, horizontal gene transfer, microbiology, phylogenetics

There is concern that anthropogenic antimicrobials are profoundly impacting microbial communities in the form of selecting for undesirable traits like antimicrobial tolerance.
or favoring the survival of antimicrobial-tolerant pathogens (1–3). Developing the capacity to predict which taxa in a naturally occurring complex community will likely tolerate antimicrobial exposure requires a detailed understanding of antimicrobial phenotypes and genotypes beyond those of select model organisms.

The intensive use of the antimicrobial triclosan [5-chloro-2-(2,4-dichlorophenoxy)phenol] has led to its widespread dissemination in both built and natural environments (4–8). Members of the cosmopolitan genus *Pseudomonas* are among those that interact most directly with environmental triclosan, affect human health (i.e., *Pseudomonas aeruginosa*), and are considered to be highly triclosan tolerant (5, 6, 8–13). Multiple studies have demonstrated genus-level increases in the total or relative abundance of *Pseudomonas* isolates present in environmental community extracts following exposure to triclosan (5, 7, 14, 15). However, it is unknown if triclosan exposure to a community containing diverse *Pseudomonas* species would select for all *Pseudomonas* species, a subset of species, or even variants of a single species, as *Pseudomonas* species have large and flexible pangenomes (16, 17). This is particularly important as selection could occur for pathogenic variants of *P. aeruginosa* or strains with unwanted metabolic capabilities (3, 18). There is some evidence that not all *Pseudomonas* species are highly tolerant, although susceptibility testing methods and taxonomic assignment are not consistent between studies (19, 20). Characterizing triclosan tolerance phenotypes in diverse *Pseudomonas* species could therefore help determine whether exposure to triclosan will result in selection for specific species, and if so, which.

Triclosan tolerance phenotypes and mechanisms in *Pseudomonas* have been principally examined in the well-studied model organism *P. aeruginosa* PAO1 (21–24). Triclosan’s sole known molecular targets are enoyl-acyl carrier reductase (ENR) isozymes, which catabolize the elongation step during fatty acid synthesis (23, 25, 26). *P. aeruginosa* PAO1 carries two of four known ENR isozymes, FabI and FabV, where FabI is highly sensitive to triclosan, while FabV is refractory and protective (23). *P. aeruginosa* PAO1 has additional protection via efflux of triclosan through resistance-nodulation-division (RND)-type multidrug efflux pumps (MDEP), including MexAB-OprM and TriABC-OpmH, that contribute various degrees of expression-dependent tolerance (21, 22, 24). Notably, the high MIC (≥128 mg/liter) of triclosan in *P. aeruginosa* PAO1 is attributed to the effect of FabV, with efflux playing a secondary role (23). Determining whether triclosan tolerance is linked to the presence or absence of these or other known factors in diverse *Pseudomonas* species could aid in determining whether triclosan-specific or multidrug tolerance genes would be selected for in triclosan-exposed communities.

In this study, we aimed to determine whether diverse *Pseudomonas* species demonstrated variability in triclosan tolerance, and if they did, what genetic factors drove the observed variation and whether they could be predictive of high tolerance. To this end, we characterized the triclosan tolerance phenotypes of phylogenetically diverse *Pseudomonas* isolates obtained from two environments commonly exposed to triclosan: indoor dust and hospital sink drains. Using a combined experimental and bioinformatic approach, we examined the phylogenetic context of triclosan tolerance variability and its association with various genetic factors. We establish *fabV* as a useful marker for inferring high triclosan tolerance in *Pseudomonas* and demonstrate evidence of its association with multiple mobile genetic elements. Our study broadly highlights the unexpected variation in antimicrobial tolerance in closely related species that differ from a few known type strains and the utility of gene-level identification rather than taxonomic assignment to predict tolerance phenotypes.

**RESULTS**

The built environment contains phylogenetically diverse *Pseudomonas* isolates from multiple established clades. Thirty spatially distinct *Pseudomonas* isolates were isolated from two separate built environment source types, indoor dust (n = 25) and hospital sink drains (n = 5), assumed to come in contact with triclosan (Table 1; see supplemental text in the supplemental material) (5, 6, 27). Whole-genome sequencing (WGS) was performed on all study isolates to characterize study isolate diversity by
phylogenetic analysis and whole-genome average nucleotide identity (ANI) to 160 known type strains. The WGS characteristics of the study isolates are listed in Table 2. A multilocus sequence analysis (MLSA) maximum likelihood phylogeny was constructed from 34 single-copy genes found in all 30 study isolates, 160 Pseudomonas type strains, and a Cellvibrio japonicus outgroup (see Table S1 and supplemental data in the supplemental material). The resulting tree topology agreed well with those generated in prior phylogenetic and phylogenomic studies of Pseudomonas, based on the presence and preservation of the 13 previously described monophyletic clades with well-supported bootstrap values (Fig. 1) (28–30). All study isolates fell into six clades: P. putida, P. stutzeri, P. fluorescens, P. oryzihabitans, P. aeruginosa, and P. syringae. Eleven and eight study isolates were placed in clades P. putida and P. stutzeri, respectively, and were distributed throughout their member clade’s branches. The four study isolates found in the P. fluorescens clade, which we further divided into five subclades, were all placed within subclade 1. The remaining seven study isolates were placed in clades P. oryzihabitans, P. aeruginosa, and P. syringae.

### Table 1: Study isolate and reference strain triclosan-related phenotypes and genotypes and phylogenomic characteristics

<table>
<thead>
<tr>
<th>Isolate ID</th>
<th>Count of homolog detected</th>
<th>MIC (mg/liter)</th>
<th>Phylogenetic clade</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>FabV</td>
<td>FabI</td>
<td>MexB</td>
</tr>
<tr>
<td>Dust</td>
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</tr>
<tr>
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</tr>
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<sup>a</sup> Shown are MICs for triclosan (TCS) alone or with 40 μg/ml phenylalanine-arginine-β-naphthylamide (PAβN).

<sup>b</sup> ANI, average nucleotide identity.

<sup>c</sup> Clades designated in Fig. 1.

<sup>d</sup> ND, not determined. Because the inhibitor affected the growth control, the MIC was not calculated.

<sup>e</sup> NA, not applicable.

**Conserved Mechanism Drives Pseudomonas TCS Tolerance Applied and Environmental Microbiology**

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\begin{table}
\centering
\begin{tabular}{|c|c|c|c|c|c|c|c|c|c|c|}
\hline
Isolate ID & Total length (bp) & Total no. of contigs & \( N_o \) & GC content & No. of ORFs & Coding density per Mb & Completeness (%)\(^a\) & Contamination (%)\(^a\) & Fold coverage & NCBI accession no. \\
\hline
4A7 & 4,384,099 & 63 & 200,315 & 0.65 & 3,833 & 874.30 & 99.57 & 0.43 & 176.78 & GCA_013523165.1 \\
6C6 & 4,766,710 & 114 & 97,492 & 0.64 & 4,252 & 892.02 & 99.25 & 0.48 & 81.15 & GCA_013523125.1 \\
8A1 & 4,387,320 & 22 & 658,584 & 0.63 & 4,011 & 914.23 & 98.85 & 0.23 & 137.94 & GCA_013523115.1 \\
10A6 & 5,011,469 & 74 & 143,141 & 0.66 & 4,582 & 914.30 & 98.28 & 0.51 & 106.98 & GCA_013523075.1 \\
20A1 & 5,957,387 & 67 & 216,552 & 0.61 & 5,427 & 910.97 & 100 & 0.96 & 112.32 & GCA_013523055.1 \\
31A8 & 4,933,281 & 64 & 140,977 & 0.62 & 4,472 & 906.50 & 100 & 0.14 & 150.84 & GCA_013523065.1 \\
34A1 & 5,218,387 & 41 & 227,787 & 0.62 & 4,714 & 914.23 & 98.85 & 0.23 & 137.94 & GCA_013523035.1 \\
39A1 & 5,149,393 & 73 & 212,731 & 0.65 & 3,833 & 874.30 & 99.57 & 0.43 & 176.78 & GCA_013523165.1 \\
45C2 & 5,744,003 & 48 & 430,146 & 0.59 & 4,990 & 868.73 & 100 & 0.41 & 102.52 & GCA_013522975.1 \\
56A10 & 5,310,052 & 73 & 162,085 & 0.61 & 4,386 & 920.86 & 100 & 0.53 & 145.27 & GCA_013522965.1 \\
62A4 & 4,274,196 & 38 & 216,335 & 0.63 & 3,939 & 921.58 & 99.17 & 0.35 & 156.18 & GCA_013522915.1 \\
66C3 & 5,433,829 & 45 & 322,747 & 0.62 & 4,913 & 904.15 & 98.72 & 1.19 & 123.42 & GCA_013522925.1 \\
69C1 & 5,024,421 & 27 & 352,420 & 0.55 & 5,318 & 948.21 & 99.04 & 1.04 & 168.02 & GCA_013522775.1 \\
89C1 & 5,608,481 & 108 & 114,000 & 0.62 & 4,672 & 935.59 & 98.53 & 3.42 & 150.17 & GCA_013522825.1 \\
96A1 & 6,346,948 & 71 & 157,681 & 0.6 & 5,694 & 897.12 & 100 & 0.24 & 80.21 & GCA_013522805.1 \\
97C1 & 5,024,241 & 37 & 364,598 & 0.66 & 4,597 & 914.93 & 98.6 & 0.61 & 143.27 & GCA_013522775.1 \\
99A1 & 4,929,078 & 33 & 548,892 & 0.61 & 4,613 & 935.87 & 99.28 & 1.04 & 108.41 & GCA_013522755.1 \\
109A1 & 5,134,915 & 194 & 194,981 & 0.63 & 4,812 & 937.11 & 99.89 & 2.52 & 113.41 & GCA_013522695.1 \\
114A4 & 5,725,822 & 84 & 125,350 & 0.61 & 5,210 & 909.91 & 100 & 0.08 & 149.02 & GCA_013522705.1 \\
115A1 & 4,824,973 & 29 & 381,757 & 0.63 & 4,430 & 918.14 & 98.96 & 0.65 & 124.02 & GCA_013522725.1 \\
119A3 & 5,260,717 & 42 & 292,402 & 0.62 & 4,749 & 902.73 & 98.72 & 1.19 & 97.81 & GCA_013522655.1 \\
HS1 & 6,514,732 & 85 & 185,275 & 0.6 & 5,907 & 906.71 & 98.7 & 0.22 & 141.47 & GCA_013522665.1 \\
HS2 & 6,508,831 & 99 & 184,619 & 0.6 & 5,903 & 906.92 & 100 & 0.88 & 83.64 & GCA_013522615.1 \\
HS3 & 6,888,403 & 121 & 160,961 & 0.66 & 6,365 & 924.02 & 99.68 & 0.13 & 128.79 & GCA_013522595.1 \\
HS4 & 6,878,130 & 131 & 129,918 & 0.66 & 6,426 & 934.27 & 99.68 & 0.4 & 55.57 & GCA_013522575.1 \\
HS5 & 6,415,930 & 123 & 170,289 & 0.61 & 5,971 & 930.65 & 100 & 0.25 & 122.29 & GCA_013522605.1 \\
\hline
\end{tabular}
\end{table}

\(^a\)CheckM estimate.
FIG 1 Maximum likelihood phylogeny of *Pseudomonas* type strains and new built environment isolates using 34 single-copy genes. The tree includes 160 *Pseudomonas* type strains and 30 study isolates. (Continued on next page)
Thirteen study isolates demonstrated ≥95% ANI, a commonly accepted threshold for species delineation with a type strain (Table 1) (31). These were phylogenetically placed within clades P. putida, P. oryzihabitans, P. stutzeri, and P. aeruginosa. Two study isolates, HS3 and HS4, had ≥95% ANI with P. aeruginosa PAO1. The remaining 17 study isolates shared lower than 91% ANI with any type strain, but were phylogenetically placed within established clades. Only two study isolates, 119A3 and 66C3, were not embedded deeply within a clade. These were placed on the outermost branch of clade P. putida with type strains of P. coleopterorum and P. rhizosphaerae. Taken together, phylogenetic and ANI analyses demonstrate that despite occurrences of novel whole-genome diversity relative to established type strains, placement of all study isolates is consistent with established Pseudomonas phylogenies (28–30).

High- and low-triclosan-tolerance isolates were found among phylogenetically diverse Pseudomonas isolates. The triclosan tolerance phenotypes of all 30 study isolates, along with reference controls P. aeruginosa PAO1 and P. putida KT2440, were characterized using broth microdilution. A strongly bimodal MIC distribution emerged, with 15 (50%) study isolates demonstrating a median MIC of 0.5 mg/liter and the other 15 a median MIC of 128 mg/liter (Fig. 2A and Table 1). These two triclosan tolerance phenotypes were subsequently termed the low-MIC group and high-MIC group isolates.

Low-MIC group study isolates had an invariably higher percentage of growth inhibition at all tested concentrations of triclosan compared to high-MIC group study isolates, highlighting their increased susceptibility. At 0.25 mg/liter, the lowest common tested triclosan concentration, the average percentage of growth inhibition of low-MIC isolates was 80.25% (minimum, 51.6%; maximum, 99.4%) compared to 2.33% inhibition (minimum, 0%; maximum, 7.65%) of high-MIC isolates (Fig. 2C). At 4 mg/liter, all low-MIC group study isolates had ≥95% growth inhibition, compared to high-MIC study isolates, with an average of 8.51% (minimum, 0%; maximum, 29.6%). While high-MIC group isolates generally began to demonstrate increases in percentage of growth inhibition starting at 4 to 8 mg/liter triclosan; only one study isolate, 6C6, ever reached ≥95% growth inhibition in the tested range. While there were minor within-group differences in MICs and percentages of growth inhibition, the dramatically larger differences between groups suggested a significant mechanistic underpinning warranting further examination.

The presence of fabV associates with high triclosan tolerance in phylogenetically diverse Pseudomonas isolates. To identify genetic factors driving group-level differences in triclosan tolerance, all 30 isolates were searched for the presence of eight experimentally verified triclosan tolerance determinants, including ENRs and efflux pumps, similar to previous studies (see Tables S2 and S3 in the supplemental material) (32). A phylogeny-aware pangenome-wide association test demonstrated that carriage of a fabV homolog was 100% specific and sensitive to the 15 isolates demonstrating high triclosan tolerance (Fisher’s exact test, Bonferroni corrected $P = 6.45 \times 10^{-10}$) (Fig. 2A and C; see Table S4 in the supplemental material). Carriage of a P. aeruginosa triB homolog component belonging to the two-part membrane fusion TriABC-OpmH RND efflux system, known to have high triclosan affinity, was also found in all 15 high-MIC group isolates, as well as in 8 low-MIC group isolates (24). While not 100% specific, it nevertheless demonstrated statistical significance (Fisher’s exact test, Bonferroni-corrected $P = 0.01$) (24). All identified FabV enzymes had ≥58% amino acid identity and ≥98% coverage with P. aeruginosa PAO1 FabV and contained the typical YX$_K$K motif. Importantly, no high-MIC isolates carried fabI containing any substitutions known to confer increased triclosan tolerance or additional fab variants (33). These results supported the hypothesis that the presence of fabV was the distinguishing characteristic between high- and low-MIC group study isolates, despite the potential contributions of other factors such as efflux (33).

FIG 1 Legend (Continued)
isolates. Phylogeny is rooted to C. japonicus. Bootstrap support values of <99 are annotated. The right-hand side displays clade assignments based on those from Hesse et al. (28). The P. fluorescens clade has five additional subclades designated by this study.
Isolates with \textit{fabV} demonstrate higher triclosan tolerance, even with efflux inhibition. We next tested the impact of efflux inhibition on isolates with and without \textit{fabV}. Microbroth susceptibility testing was conducted using triclosan and a constant concentration of 40 \text{mg/ml} phenylalanine-arginine-\(\beta\)-naphthylamide (\text{PA\(\beta\)N}), a widely used efflux pump inhibitor (34, 35). Four \textit{fabV}-negative study isolates were excluded from MIC calculations because the concentration of \text{PA\(\beta\)N} used impacted growth at 0 \text{mg/liter} triclosan (Table 1). All study isolates demonstrated reduced triclosan tolerance in the presence of \text{PA\(\beta\)N}, as determined by MIC and/or percentage of growth inhibition (Fig. 2C and D). The effect of \text{PA\(\beta\)N} was more notable within the low-MIC group, where \text{PA\(\beta\)N}-treated study isolates had a median triclosan MIC of 0.0625 \text{mg/liter} compared to an untreated median triclosan MIC of 0.5 \text{mg/ml}. Comparatively, most, but not all, high-MIC group study isolates also demonstrated reductions in triclosan MIC. \text{PA\(\beta\)N}-treated high-MIC group study isolates had a median triclosan MIC of 8 \text{mg/liter}, compared to an untreated median triclosan MIC of 128 \text{mg/ml}. There is a possibility that \text{PA\(\beta\)N} treatment at this concentration resulted in not just efflux inhibition but also membrane permeabilization (36). However, even in the context of this unintended synergy, the protective effect of \textit{fabV} carriage is still observed.

\textbf{Efflux pump and \textit{fabI} expression contribute to triclosan tolerance.} Functional screens using transposon mutagenesis were performed to identify additional or unknown positive or negative regulators of triclosan tolerance not identified by our bioinformatic screen. Transposon mutants from two high-MIC group study isolates,
56A10 and 96A1, belonging to clades *P. putida* and *P. fluorescens*, were screened for the absence of growth in 128 mg/liter triclosan. From a total of 10,000 transposon mutants generated, only three mutants (0.03%) were unable to grow at 128 mg/liter. Transposon mutants 56A10-A12, 56A10-H5, and 96A1-B4 were found to have triclosan MICs of 8 to 16 mg/liter, compared to wild-type MICs of $128 \text{ mg/liter}$ (Table 3). All insertions for the three mutants were within an operon that is most homologous to *P. aeruginosa* PAO1 RND MDEP MexAB-OprM (see Fig. S1A in the supplemental material) (37). Because both 56A10 and 96A1 carry *fabV* as their sole ENR, no *fabV* interruption mutants were expected to be generated, as that would be lethal to the strain (23). Transposon mutant saturation amounted to $\approx 9\%$ of potential dinucleotide insertions.

Ten thousand transposon mutants were generated from the *fabV*-negative, low-MIC group study isolate 57B2, which was embedded within clade *P. stutzeri*, resulting in 12 mutants (0.12%) with two distinct genetic interruptions. The first type, represented by 57B2-A1, had a transposon insertion upstream of an RND MDEP homologous to *P. aeruginosa* PAO1 MexJK. The second type, represented by 57B2-C3, had an insertion upstream of *fabI* (Fig. S1B). Triclosan MICs in both mutants ranged from 8 to 16 mg/liter, compared to the 4-mg/liter wild-type MIC. The increased triclosan MICs for both mutants were attributable to insertion of the transposon cassette's outward-facing *Ptac* promoter in an orientation that could drive expression of a downstream target (supplemental material text and Fig. S1C). Transposon mutant saturation amounted to $\approx 18\%$ of potential dinucleotide insertions.

Overall, both positive and negative screens returned known tolerance systems, namely, efflux ablation/overexpression and *fabI* overexpression (21). Therefore, no new genes or mechanisms that could confound the separation of MIC by *fabV* status were identified. However, it is possible that increasing transposon mutant saturation could identify additional mechanisms.

**fabV** demonstrates evidence of multiple independent acquisition events. The demonstrated importance of FabV in contributing to high triclosan tolerance across different clades led us to ask how *fabV* was distributed throughout *Pseudomonas* strains. Homology searches for FabV were performed for all 160 *Pseudomonas* type strains and in the *C. japonicus* outgroup and mapped onto the existing phylogeny. Strikingly, *fabV* carriage was not monophyletic; instead, *fabV* appeared in multiple clusters and was loosely concordant with clade designation (Fig. 3i). Ancestral state reconstruction for *fabV* using a maximum likelihood model was performed to quantify the probabilities of its presence or absence. The resulting reconstruction broadly demonstrates $<0.5$ posterior probability for the presence of *fabV* at parent nodes leading to multiple *fabV*-carrying strains, providing support for four ancestral *fabV* gain events (Fig. 3i to iv) and one loss event in clade *P. fluorescens* subclade 2 (Fig. 3iv).

Despite the multiple gain and loss events, study isolates did not exhibit unusual *fabV* carriage given their phylogenetic placement: 13 of 15 study isolates absent for *fabV* were firmly embedded within clades that were exclusively absent for *fabV*, such as within clades *P. stutzeri* and *P. oryzihabitans*. The singular exception was *fabV*-negative study isolates 119A3 and 66C3, which grouped within clade *P. putida*, which

<table>
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<tr>
<th>Transposon mutant strain ID</th>
<th>Genotype</th>
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<tr>
<td>96A1-B4</td>
<td><em>oprM</em>::Himar1</td>
<td>8</td>
<td>8</td>
</tr>
<tr>
<td>57B2-A1</td>
<td>G7015_RS12405::Himar1</td>
<td>8</td>
<td>2</td>
</tr>
<tr>
<td>57B2-C3</td>
<td>G7015_RS08705::Himar1</td>
<td>16</td>
<td>4</td>
</tr>
</tbody>
</table>

*TCS, triclosan.

$^a$Fold decrease compared to the wild type.

$^b$Fold increase compared to the wild type.

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**TABLE 3** Transposon mutant genotype and corresponding triclosan phenotype

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contains mostly type strains carrying \textit{fabV}. Importantly, 119A3 and 66C3 branch with other \textit{fabV}-negative type strains of clade \textit{P. putida}, suggesting a within-clade gain event.

To further assess the potential shared (or dissimilar) origins of \textit{fabV}, a pruned MLSA phylogeny containing all \textit{fabV}-positive genomes (15 study isolates and 59 type strains) was compared to a \textit{fabV} maximum likelihood phylogeny. The resulting FabV phylogeny was qualitatively and quantitatively distinct from that of the MLSA phylogeny (normalized Robinson-Foulds distance = 0.51, Kendall-Colijn distance = 120.5), and similarly supports at least three (and possibly four) distinct origins for the ancestral \textit{Pseudomonas fabV} acquisitions (Fig. 4). Two gain events are observed at the bifurcation between \textit{P. fluorescens} clade FabV and \textit{P. aeruginosa}/\textit{P. resinovorans} FabV. A distinct third (and potentially fourth) gain event is apparent in a separate branch containing FabV from clades \textit{P. straminea} and \textit{P. putida}.

Taken together, ancestral state reconstruction and FabV phylogeny provide evidence that \textit{fabV} carriage in \textit{Pseudomonas} is characterized by multiple, potentially independent, gain events followed by vertical lineage transmission. Importantly, these results indicate that clade alone is not predictive of \textit{fabV}.

\textbf{FIG 3} Ancestral state reconstruction of \textit{fabV} within the phylogeny of 160 \textit{Pseudomonas} type strains and 30 study isolates. Only pie charts at nodes with log likelihoods for the presence/absence of \textit{fabV} between 0.1 and 0.9 are displayed. Stars indicate hypothesized \textit{fabV} gain events. Clade assignments are identical to those in Fig. 1. (i) \textit{fabV} likelihood for the MLSA phylogeny. (ii) Inset of clades \textit{P. aeruginosa}, \textit{P. oryzihabitans}, and \textit{P. resinovorans}. (iii) Inset of clade \textit{P. putida}. (iv) Inset of clade \textit{P. fluorescens}. 
Large-scale genome analysis shows fabV presence is largely invariant within *Pseudomonas* species groups. We examined whether fabV was present in strains in single copies and whether highly similar strains (ANI of ≥95%) were likely to share similar fabV status. We greatly increased the scope of our genomic screening to 7,163 *Pseudomonas* genomes from NCBI. All genomes were assigned species groups based on a ≥95% ANI threshold match with one of the 160 *Pseudomonas* type strains (see Data Set S2 in the supplemental material). FabV BLAST searches for each strain showed that its fabV status was almost always identical to that of its representative type strain, with a significant odds ratio (OR) that a strain will carry fabV given its representative type strain is also carrying fabV (OR = 4.5 × 10^15; 95% confidence interval [CI], 8.4 × 10^3 to 4.5 × 10^15; P < 2.2 × 10^-16) (see Fig. S3 in the supplemental material). In fact, there were only nine total instances (0.12%) in which a strain from a species group differed from the type strain’s fabV status. These differences were in the form of no fabV detected when expected (n = 2), fabV detected in multiple copies (n = 4), and fabV detected when no fabV was expected (n = 3). Examination of the NCBI BioSample metadata indicated that the seven strains with unusual fabV content relative to the type strain were each geographically and temporally distinct. Since their species membership was standardized to the highest type strain ANI, fabV discrepancies are not the results of taxonomic misassignments, but could be either contamination (biological or...
computational), incomplete or incorrect genome assemblies, or true \textit{de novo} duplication or loss events that were distinct from the previously identified \textit{fabV} gain/loss events.

**Evidence of \textit{fabV} on mobile genetic elements and within genomic islands.** We sought to identify whether \textit{fabV} from the seven strains with unusual \textit{fabV} copy number demonstrated evidence of chromosomal mobility. \textit{FabV} sequences from all 4,914 \textit{fabV}-positive strains were used to construct an expanded \textit{FabV} phylogeny, which maintained a structure consistent with that constructed from 59 type strains and 15 study isolates (Fig. 5A). Their overall similarity further supports that \textit{fabV} has been gained independently in multiple clade-level lineages, followed by vertical dissemination. A conspicuous exception was a singular branch in clade \textit{P. fluorescens} that contained seven \textit{fabV} sequences belonging to seven previously identified strains with an unusual \textit{fabV} copy number given their type strain (Fig. 5A).

Whole-genome alignments of the seven strains provide evidence of seven mobilizable \textit{fabV} genes. Four strains, AK6U, 105819, HBP1, and Aramo J, have two copies of \textit{fabV}. For each, one \textit{fabV} copy is located within a near-identical 120,000-bp region and predicted to be a type IV integrative and conjugative transfer element (ICE) containing 23 predicted mobility-associated genes, including \textit{traI}, \textit{traG}, \textit{traU}, and \textit{traC} (Fig. 5B) (38). The other copy of \textit{fabV} is located elsewhere in the genome, where all strains share similar surrounding gene content, including several metabolic genes, but no mobile elements (Fig. S3).

Three strains, P12, ZBG1, and 48C10, carry only a single copy of \textit{fabV}. The regions surrounding these \textit{fabV} genes do not demonstrate similar genetic content between each other (no shared locally colinear blocks beyond that attributed to \textit{fabV} and an

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**FIG 5** Identification of seven \textit{fabV} genes with strong evidence of recent horizontal transfer. (A) Maximum likelihood tree of all \textit{FabV} enzymes identified from 7,163 \textit{Pseudomonas} genomes. Clade assignments are identical to those used in Fig. 1. Triangles indicate collapsed branches. The inset demonstrates the positioning of outlying \textit{fabV} genes. Shown is the stylized Mauve output of seven whole-genome alignments with \textit{fabV} genes of likely HGT origin. Locally colinear blocks between genomes are linked with identically colored vertical lines. Genes related to HGT are annotated or colored in. Each genome is identified by the ANI species group they were assigned, their NCBI strain name, and the RefSeq identifier. (B) The \textit{fabV} gene region within genomes that carry two copies. The outlying \textit{fabV} from panel A is visualized. (C) The \textit{fabV} gene region within genomes whose species group did not typically carry \textit{fabV}. The gene region is visualized over a 21,000-bp region.
immediately adjacent site-specific recombinase). The genomic region surrounding these fabV genes was predicted to be a genomic island, which explains the lack of genetic synteny despite close phylogenetic proximity. Interestingly, these strains also share a large homologous region with the second fabV copy in the previous four strains, where the only difference is the absence of fabV, offering evidence of a loss of fabV (Fig. S3).

**DISCUSSION**

Here, we present evidence that phylogenetically distinct *Pseudomonas* species can exhibit a difference in triclosan tolerance of almost 3 orders of magnitude that is likely driven by carriage of fabV. While there are multiple additional mechanisms that can independently or synergistically increase triclosan tolerance, we confirm fabV to be a useful phylogeny-independent marker of high triclosan tolerance in *Pseudomonas*.

Both ENR and efflux pump homologs known to confer triclosan tolerance were detected in our 30 study isolates. While searches for all known ENRs FabI, FabV, FabK, and FabL were performed, only fabI and fabV genes were detected, and they are likely the predominant ENR genes of *Pseudomonas*. This was not unexpected as functional homologs of FabK and FabL in *Pseudomonas* have not been identified (23). Comparatively, a large diversity of RND-type efflux pump homologs of mexB and mexD were observed in all study isolates. Interestingly, the presence of the TriABC-OpmH membrane fusion gene, triB, whose expression is triclosan specific, was significantly associated with high triclosan tolerance, but the gene was also present in sensitive strains (24). Despite the multitude of potential triclosan tolerance factors, only the singular presence of fabV was associated with a difference in tolerance of 3 orders of magnitude between high- and low-MIC groups.

Differences in efflux capacity resulted in notable decreases in triclosan tolerance in both fabV-negative and -positive isolates. Percentage of growth inhibition was variable for fabV-negative study isolates, potentially due to differences in either efflux pump diversity or expression, which were further confirmed by the mexJK overexpression transposon mutant 57B2-C3. However, differences in fabI expression could also play a role, as shown by overexpression transposon mutant 57B2-A1. Treatment of fabV-positive study isolates with PAβN caused a uniform reduction in percentage of growth inhibition. Variations in reductions could be due to differences in efflux capability. The impact of efflux in fabV-positive isolates was further demonstrated in mexAB-opmR transposon mutant interruption strains 56A10-A12, 56A10-H5, and 96A1-B4, which resulted in an 8- to 16-fold decrease in triclosan tolerance. However, the strong explanatory effect of fabV suggests that it provides the majority of triclosan tolerance in *Pseudomonas*, with efflux contributing a lesser effect, similar to *P. aeruginosa* PAO1.

Our finding that tolerance in *Pseudomonas* can differ by several orders of magnitude carries specific suggestions for monitoring of *Pseudomonas* in natural and laboratory microbial communities exposed to triclosan. Principally, because increases in genus-level abundances may not correspond to all *Pseudomonas* species present in the community, using methods that offer species-level resolution, such as WGS or multilocus sequence typing, can offer better health- and study-relevant insights. For example, when monitoring a triclosan-exposed environment containing *Pseudomonas*, including *P. aeruginosa*, selection for fabV would carry implications for human health. In a different scenario, selection of high tolerance to the degree conferred by FabV and the detection of a *Pseudomonas* species that does not typically carry fabV could be particularly meaningful. We show that while an ANI of ≥95% to a type strain is strongly predictive of fabV status, there are instances where fabV was sometimes present but other times absent. fabV-negative species exhibiting high-level tolerance could indicate selection for one or a combination of factors, including increased efflux pump or fabI expression, acquisition of a tolerant ENR isozyme, a triclosan-metabolizing enzyme, or an unknown genetic factor. Of particular importance would be increased efflux capacity through increased expression or assortment of RND-type efflux pumps, as they can
accommodate clinically relevant antibiotic substrates, inadvertently selecting for increased antibiotic tolerance (21). Alternatively, the presence of fabV in Pseudomonas species not known to typically carry fabV would suggest evidence of horizontal gene transfer (HGT), the mobility of which we have provided evidence for on a type IV ICE (39).

We identified both triclosan-tolerant and -sensitive Pseudomonas species in the built environment. However, we cannot determine whether tolerance was indeed selected for by triclosan. Given that environmental triclosan levels are typically in the pg/liter-to-ng/liter range, it is unclear whether the highly protective ($\geq 128$ mg/liter) effect of fabV is necessary for survival or whether lesser levels of tolerance conferred by efflux and/or other factors are sufficient (4, 40). A metagenomic analysis found fabV copy number to be higher in presumed triclosan-exposed environments compared to unexposed environments, suggesting that fabV-positive species are selected for (41). However, a retrospective study found non-fabV mechanisms may also be undergoing selection, while another study found the presence of horizontally transferred fabI (42, 43). Additionally, it is still unknown how fabV-carrying species react to low levels of triclosan, as the presence of fabV does not necessarily prohibit additional adaptations like increased efflux pump expression. It is likely that the utility of FabV levels of triclosan tolerance is dependent on the environment and the level and rate of triclosan exposure. It is also important to consider that ENRs and efflux pumps have additional metabolic roles that are also under selection (44, 45). Therefore, while further investigation is needed on multiple fronts, our study suggests that determining the fabV status of a Pseudomonas species in a triclosan-exposed microbial community is a useful first step in assessing potential selective effects.

More broadly, our findings highlight that supposedly conserved traits are not as well conserved as expected in even closely related species, highlighting the limitations of extrapolating knowledge from a few type species to whole genera common to amplicon sequencing. To better estimate the effect of antimicrobials on microbial communities, increased phenotypic and genotypic investigation of non-model, non-laboratory strains is necessary. Additionally, methods like WGS and metagenomic sequencing that provide species- and gene-level resolution can help to identify new and unexpected ways in which taxa may overcome antimicrobial challenges.

**MATERIALS AND METHODS**

**Isolate collection, identification, and susceptibility testing.** Dust sample isolates originated from a culture isolate library obtained from a previous study (6). These were screened for Pseudomonas by patch plating on Pseudomonas isolation agar composed of Pseudomonas agar base (Thermo Fisher Scientific), 10 ml/liter glycerol, and CFC selective supplement (Thermo Fisher Scientific) containing 10 mg/liter cetrimide, 10 mg/liter fucidin, and 50 mg/liter cephalosporin (6). Isolates obtained from Rush University Medical Center sink drains were collected by first swabbing the inner rim of the sink drain and diluting in 1× phosphate-buffered saline (PBS). Colonies were picked and purity streaked onto tryptic soy agar (TSA) plates and further validated using Pseudomonas-specific primers (Table 4). Detailed methods for DNA extraction and PCR can be found in the supplemental text. Samples were submitted
Whole-genome sequencing, assembly, and annotation. All isolate genomic DNA extracts were prepared to a minimum concentration of 2 ng/µl and a minimum mass of 60 ng, for a target coverage of 1.5 Gbp (see supplemental text for details on genomic DNA extraction and quantification). Genomic DNA was shipped to the Broad Institute for additional DNA quantification using a fluorescent dye-based method followed by paired-end short-read (2 x 150 bp) sequencing using a Nextera XT DNA Library kit (Illumina) with an Illumina HiSeq system. The resulting sequencing read BAM files were converted to fastq format with SAtools v1.6 (48). Sequencing adapters were trimmed, and low-quality reads (quality score of <30 (<20Q)) were removed using fastq 0.20.0 (49). High-quality read pairs were merged with BBmap v38.69 and assembled using SPAdes v3.13.0 (50, 51). Assembly quality statistics were obtained using QUAST v5.0.2, and estimates of assembly completeness and contamination were acquired with CheckM v1.07 (52, 53). Open reading frames (ORFs) were predicted with Prodigal v2.6.3 (54).

Transposon mutant generation. Conjugation was performed via biparental mating of the recipient isolate and donor E. coli SM10(pir) carrying the Himar1C9 delivery vector pBT20 (55). Isolates and SM10(pir) cells were streaked onto tryptic soy agar (TSA) plates and incubated at 30°C for 20 to 24 h. Three colonies per strain were picked and grown in 5 ml tryptic soy broth (TSB) for 20 to 24 h at 30°C and 170 rpm. For biparental mating, a 1:1 volume of isolate and SM10(pir) cultures were obtained, plated onto TSA, and incubated at 37°C. Subsequent mating spots were picked with cotton tip swabs and resuspended in phosphate-buffered saline (PBS). Fifty microliters was plated onto SM10(pir)-containing auxotrophic minimal medium M63 agar selection plates containing 30 µg/ml gentamicin (Alfa Aesar) and incubated for 24 to 48 h at 37°C. M63 agar plates containing the following: 1x M63 salts (2 g/liter ammonium sulfate (Millipore Sigma), 2 g/liter, 13.6 g/liter potassium phosphate monobasic (Millipore Sigma), 0.5 mg/liter ferrous ammonium sulfate heptahydrate (Millipore Sigma), pH 7), 15 g/liter bacteriological agar (Millipore Sigma), 20 mM sodium succinate (Alfa Aesar), 20 mM L-glutamic acid (Millipore Sigma), 20 mM magnesium chloride (Millipore Sigma), and 10 mM ferrous ammonium sulfate heptahydrate.

For negative selection, individual transposon mutant colonies were picked onto 96-well plates containing TSB and grown for 20 to 24 h at 30°C and 170 rpm. Next, a sterile replicator pin was used to transfer contents from each well onto a TSA selection plate containing 128 mg/liter triclosan and incubated for 24 to 48 h at 37°C. Transferred contents that did not exhibit growth were traced back to the 96-well plate to confirm growth in the original well. If the original well had growth, contents from the well D4 were streaked onto a new TSA plate containing 30 µg/ml gentamicin and incubated for 24 to 24 h at 37°C. For positive selection, mating spots resuspended in PBS were plated onto M63 agar plates containing 30 µg/ml gentamicin and either 0.5, 1, 2, 4, 8, or 16 mg/liter triclosan. Insertions were identified using inverse PCR (supplemental text).

Transposon saturation was calculated as number of colonies per strain/number of TA dinucleotides in the genome.

Bioinformatic screen for triclosan tolerance factors. BLAST v2.7.1 + searches were performed against a database of functionally verified homologs (Table S2) (56). All hits were filtered for ≥40% amino acid identity and an alignment length of ≥80%. FabI and FabV hit sequences were confirmed for the distinguishing presence of their respective YXK and YX,K catalytic motifs using JalView v2.11 (26, 33, 57). OrthoFinder v2.4.1 was used to cluster hits into orthologous groups and inputted in Scoary v1.16.6 to test for orthogroups associated with ≥128 mg/liter tolerance (58, 59).

MLSA phylogenetic analysis and pairwise ANI calculations. Assemblies of 160 Pseudomonas type strains and one Cellibrio japonicus type strain were downloaded from NCBI on 4 January 2020 and had ORFs identified using Prodigal (Data Set S2) (54). All type strains and study isolates underwent homology searching with BLAST for 34 single-copy genes used by Hesse et al. (28) by using ≥40% amino acid identity and ≥80% coverage thresholds and filtering for the highest bit score (Table S3). Nucleotide sequences were aligned individually with MAFFT v7.313 using the L-INS-I option and trimmed with TrimAl v1.4. rev15 using the “automated1” parameter. Individual gene alignments were concatenated, and a maximum likelihood phylogenetic tree was built using RAxML v8.2.12 using the -m GTRGAMMA substitution model and 100 bootstraps (60). The resulting tree was rooted to the C. japonicus outgroup with the ape package and visualized using the ggtree function from the ape package. Pairwise ANI calculations for study isolates and type strains was performed using fastANI v1.3 (31). Clade assignments were based on those from Hesse et al. (28).

Ancestral state reconstruction and FabV phylogeny tree comparison. Ancestral state reconstruction for FabV was performed using the “ace” function from the ape R package v5.4 using the “ML” method and “ARD” model (63). Visualizations were made using ggtree (62). All FabV amino acid sequences found in all type strains and study isolates were aligned and trimmed with MAFFT and TrimAl as described previously. A maximum likelihood phylogeny was constructed using RAxML with the PROTGAMMAAUTO substitution model and 100 bootstraps (60). The normalized Robinson-Foulds distance was calculated using the RF.dist function, with normalize and rooted options set to TRUE, from the phangorn R package v2.5.5 (64). The Kendall-Colijn distance was calculated using the treeDist function from the treeseq R package v1.1.3.2 (65). Topological differences were visualized using the plotTreeDist function from the treeseq R package.

Large-scale Pseudomonas genome FabV analysis. A total of 9,620 assemblies matching the “Pseudomonas” genus tag were downloaded from the NCBI RefSeq database on 27 March 2020. Quality filtering was performed using QUAST and CheckM as described above. Assemblies were filtered for
≤300 contigs, $≥10\text{ kb}$ at $N_{\text{cp}}, ≥90\%$ completeness, ≤10% contamination, and ≤50% overall quality (defined as completeness $– 5\times$ contamination), retaining 8,742 assemblies (52, 53, 66). FastANI was used for pairwise ANI calculations between all 160 type strains (31). Type strains with $≥95\%$ pairwise ANI were grouped (designated with the suffix "_group"). Afterward, the ANI between each type strain and the remaining assemblies was calculated. Species group assignments were based on the highest match and $≥95\%$ ANI. This process resulted in 7,163 genomes used in the rest of the analyses (Data Set S2). FabV homology searching, alignment, trimming, and maximum likelihood tree construction were performed as previously described. The resulting tree was visualized using ggtree, with branches collapsed using the “max” parameter (62). The odds ratio for fabV presence versus absence given type strain status was obtained via a one-tailed Fisher’s exact test using the fisher.test function in R.

HGT prediction. GenBank format assemblies from genomes with outlying fabV composition were downloaded from the NCBI RefSeq database and aligned and visualized with Mauve v2.4.0 using the progressiveMauve option (67, 68). Locally colinear blocks are defined by progressiveMauve. The IslandViewer FTP site was used for genomic island prediction (69). The IceFinder web portal was used for ICE region identification (70, 71).

Data availability. All analyzed bacterial genomes are publicly available on the NCBI website and downloaded from the RefSeq database (68). Study isolate genome assemblies are available under BioProject accession no. PRJNA606080. Supplemental data and analytical scripts are available at https://github.com/hartmann-lab/fabv_paper under the MIT license (72). All strains are available upon request (erica.hartmann@northwestern.edu).

SUPPLEMENTAL MATERIAL
Supplemental material is available online only.

**SUPPLEMENTAL FILE 1**, PDF file, 3.4 MB.

**SUPPLEMENTAL FILE 2**, XLSX file, 0.03 MB.

**SUPPLEMENTAL FILE 3**, XLSX file, 0.5 MB.

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We declare that we have no conflicts of interest.

REFERENCES


Conserved Mechanism Drives Pseudomonas TCS Tolerance

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