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METHODS

Xylose-Inducible Promoter Tools for *Pseudomonas* Species and Their Use in Implicating a Role for the Type II Secretion System Protein XcpQ in the Inhibition of Corneal Epithelial Wound Closure

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ABSTRACT Tunable control of gene expression is an invaluable tool for biological experiments. In this study, we describe a new xylose-inducible promoter system and evaluate it in both *Pseudomonas aeruginosa* and *Pseudomonas fluorescens*. The P_{xut} promoter, derived from the *P. fluorescens xut* operon, was incorporated into a broad-host-range pBBR1-based plasmid and was compared to the *Escherichia coli*-derived P_{BAD} promoter using *gfp* as a reporter. Green fluorescent protein (GFP) fluorescence from the P_{xut} promoter was inducible in both *Pseudomonas* species, but not in *E. coli*, which may facilitate the cloning of genes toxic to *E. coli* to generate plasmids. The P_{xut} promoter was activated at a lower inducer concentration than P_{BAD} in *P. fluorescens*, and higher *gfp* levels were achieved using P_{xut}. Flow cytometry analysis indicated that P_{xut} was leakier than P_{BAD} in the *Pseudomonas* species tested but was expressed in a higher proportion of cells when induced. D-Xylose as a sole carbon source did not support the growth of *P. aeruginosa* or *P. fluorescens* and is less expensive than many other commonly used inducers, which could facilitate large-scale applications. The efficacy of this system was demonstrated by its use to reveal a role for the *P. aeruginosa* type II secretion system gene *xcpQ* in bacterial inhibition of corneal epithelial cell wound closure. This study introduces a new inducible promoter system for gene expression for use in *Pseudomonas* species.

IMPORTANCE *Pseudomonas* species are enormously important in human infections, in biotechnology, and as model systems for investigating basic science questions. In this study, we have developed a xylose-inducible promoter system, evaluated it in *P. aeruginosa* and *P. fluorescens*, and found it to be suitable for the strong induction of gene expression. Furthermore, we have demonstrated its efficacy in controlled gene expression to show that a type II secretion system protein from *P. aeruginosa*, XcpQ, is important for host-pathogen interactions in a corneal wound closure model.

KEYWORDS *Pseudomonas*, inducible promoter, plasmids, xylose

Species of the bacterial genus *Pseudomonas* are of exceptional importance, not only as infectious agents for a broad range of organisms, including humans and plants (1–4), but also for the valuable role that species of this genus play in biotechnology and basic science (5, 6). Inducible gene expression systems are important tools in both biotechnology and basic science that enable tunable expression in the presence of an effector molecule.

Inducible plasmid systems for pseudomonads include the L-arabinose-inducible, AraC-regulated P_{BAD} promoter from *Escherichia coli*, which is inducible in *Pseudomonas aeruginosa* (7–9). Whereas this promoter has proven useful for gene expression in

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numerous studies, it has been demonstrated to be leaky without induction in *P. aeruginosa* (10). Other inducible promoter systems in *P. aeruginosa* include the *lacI*^{q}-*Ptac* and the *rhaSR*-P_{rhaB} promoter (10, 11). While *lacI*^{q}-*Ptac* and *rhaSR*-P_{rhaB} confer tightly controlled gene expression when inserted into the chromosome (10), the *rhaSR*-P_{rhaB} system is large (>2,000 bp), and the *lacI*^{q}-*Ptac* system uses a high-cost inducer. Therefore, there would be value in the development of a small-size, low-cost system for gene expression from episomal plasmids.

The goal of this study was to develop an episomal-plasmid-based inducible promoter system for *Pseudomonas* species using pseudomonad-derived DNA. This study describes the evaluation of the *Pseudomonas fluorescens xutR*-P_{xutA} promoter system (here designated P_{xut}) in both *P. aeruginosa* and *P. fluorescens*. In addition, we use this system to interrogate *P. aeruginosa* host-pathogen interactions, particularly the mechanisms underlying bacterial inhibition of corneal epithelial cell wound closure (12).

RESULTS

Identification and cloning of a putative xylose-inducible promoter from *P. fluorescens*. A putative xylose metabolism transcription factor gene, Pf101_2304, was identified in the *P. fluorescens* strain Pf0-1 genome by BLAST analysis (13). Recently, the homolog of this gene in *P. fluorescens* strain SBW25 was demonstrated to encode an activator of a xylose metabolism operon and was named XutR by Liu et al. (14). In strain SBW25, the XutR protein was demonstrated to directly and positively regulate transcriptional expression of the adjacent gene, *xutA*, in a xylose-dependent manner, and bound to a conserved operator site (AAAATC-N_{15}-GATTTTT) upstream of *xutA* (14). In the SBW25 genome, the intergenic region between *xutR* and *xutA* is 139 bp long, whereas it is 182 to 188 bp long in *P. fluorescens* strain Pf0-1, depending on whether a TTG or an ATG for *xutA* is the start codon. Other differences in the intergenic region between strains SBW25 and Pf0-1 include a direct repeat of CCAAGAACAACAA just upstream of the ribosome binding site in Pf0-1 that is present in a single copy in SBW25. A schematic diagram and sequence for the intergenic region of strain Pf0-1 are presented in Fig. 1A and B. The *xutR* and intergenic regions with the promoter for *xutA* (here designated P_{xut}) from strain Pf0-1 were cloned into a pBBR1-based broad-host-range plasmid, pMQ132 (11), with *gfp* placed under the control of P_{xut} (Table 1). A diagram of the resulting plasmid, pMQ578, is shown in Fig. 1C and Fig. S1A in the supplemental material.

The pMQ578 plasmid has an RP4 *oriT* sequence for conjugal transfer, the *aacC-1* gentamicin resistance marker, and a selectable marker and replicon for *Saccharomyces cerevisiae* to allow for *in vivo* yeast cloning (8). We used a previously described plasmid with the *E. coli* P_{BAD} promoter driving *gfp*, pMQ80 (8), as a comparison promoter, since the P_{BAD} promoter is frequently used for gene expression in *E. coli* and *Pseudomonas* species. The resulting plasmids were used to characterize P_{xut} expression in *E. coli* and two species of *Pseudomonas*.

Characterization of P_{xut} expression in *E. coli*, *P. aeruginosa*, and *P. fluorescens*. To evaluate P_{xut} and P_{BAD} expression, pMQ578 and pMQ80 were introduced into *E. coli* strain S17-1, *P. aeruginosa* strain PA14, and *P. fluorescens* strain Pf0-1. Increasing doses of L-arabinose and D-xylose were added to cultures (optical density at 600 nm [OD_{600}], 0.01), and the cultures were analyzed for green fluorescent protein (GFP) fluorescence using a fluorometer after 25 h of incubation at 30°C or 37°C. After the addition of an inducer, with *E. coli*, no fluorescence was measured with P_{xut}-*gfp* compared to the absence of an inducer, whereas P_{BAD} was inducible at low concentrations (0.1 mM) of L-arabinose (Fig. 2A). In *P. aeruginosa*, both P_{xut}-based expression and P_{BAD}-based expression were dose dependent, and the P_{xut} promoter was slightly stronger than P_{BAD} at intermediate inducer doses (Fig. 2B). With *P. fluorescens*, both promoters were inducible, but the P_{xut} promoter was inducible at lower concentrations than the P_{BAD} promoter and was stronger at all doses (Fig. 2C).

The expression of both promoters was tested over time using 10 mM inducer (Fig. 3A to C). Cultures with an inducer were started at an OD_{600} of 0.01 and were allowed

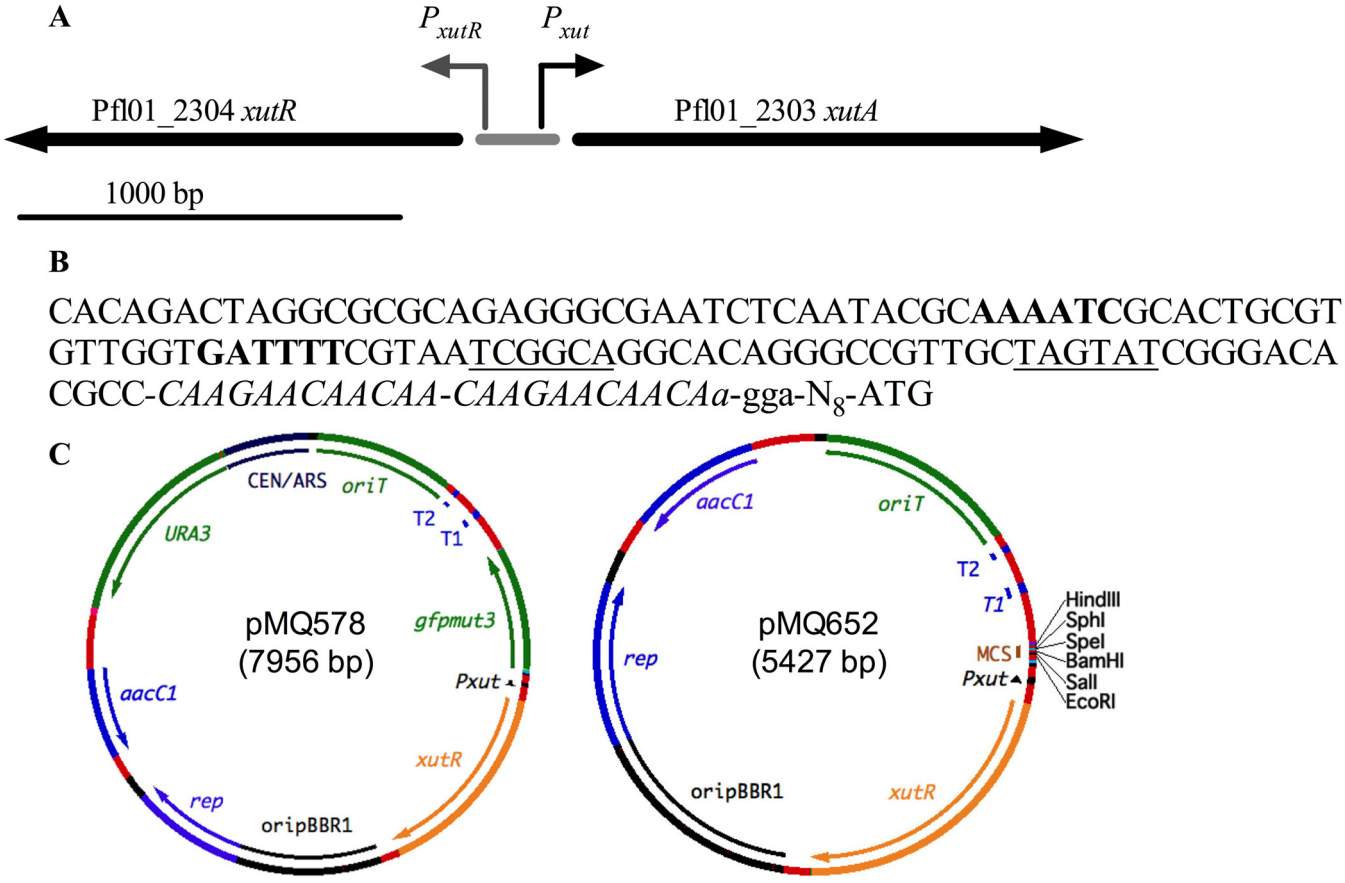

FIG 1 Xylose repressor genomic region from *P. fluorescens* strain Pf0-1. (A) Genetic map of the *xutR-xutA* region. The shaded bar represents the 188-bp intergenic region. (B) DNA sequence of the P_{xut} promoter upstream of Pf101_2303. The conserved operator inverted repeats are shown in boldface, and the –35 and –10 regions for the *xutA* promoter (P_{xut}) are underlined. A direct repeat of 13 bp is shown in italics. A putative ribosome binding site 9 to 12 bp upstream of the start codon is shown in lowercase. (C) Schematic diagrams of select plasmids used in this study.

to grow at 30°C. Samples were removed and analyzed for GFP fluorescence over time. GFP fluorescence was largely undetected until 6 h postinduction and was maximal at the final time point (25 h). The highest fluorescence levels were observed with Pf0-1 and P_{xut} (pMQ578) at 25 h (Fig. 3C).

TABLE 1 Strains and plasmids used in this study

Strain or plasmid	Description	Reference or source
Strains		
S17-1 λ-*pir*	*E. coli* laboratory strain	46
PIC3611	*S. marcescens*, wild type	Presque Isle Cultures
PAO1	*P. aeruginosa*, wild type	47
PA14	*P. aeruginosa* UCBPP-PA14, wild type	48
PAC	*P. aeruginosa*, keratitis isolate	49
Pf0-1	*P. fluorescens* wild-type strain	50
Plasmids		
pMQ80	Shuttle vector, pRO1600 with P_{BAD}-*gfpmut3*	8
pMQ132	Shuttle vector, pBBR1; P_{lac}-*lacZ aacC-1*	11
pMQ414	Shuttle vector, RSF1010; P_{nptII}-*tdtomato aacC-1*	43
pMQ578	pMQ132 with *xutR*-P_{xut}-*gfp* replacing P_{lac}-*lacZ*	This study
pMQ643	pMQ578 with *tdtomato* replacing *gfp*	This study
pMQ644	pMQ578 with *xcpQ* from PA14 replacing *gfp*	This study
pMQ650	pMQ578 with multicloning site replacing *gfp*	This study
pMQ652	pMQ650 with yeast replicon removed	This study

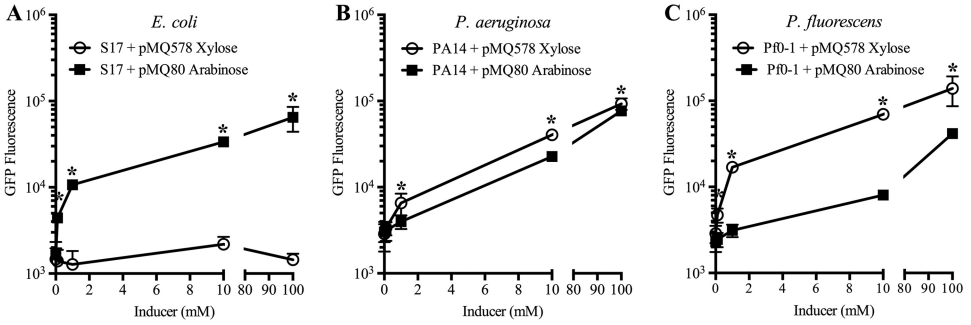

FIG 2 Comparison of the P_{BAD} and P_{xut} promoters in *E. coli* and *Pseudomonas* species. Shown is the expression of *gfpmut3* from the two different promoters in a pBBR1-based plasmid. Cultures were prepared in LB medium with a range of inducer concentrations and were grown at 30°C for 25 h. Means and standard deviations are shown (n, $\geq$5 independent cultures). P_{xut} was not inducible in *E. coli* and was inducible at lower inducer concentrations than P_{BAD} in *P. fluorescens*. The pMQ578 plasmid has P_{xut}-*gfp*, and pMQ80 has P_{BAD}-*gfp*. Asterisks indicate statistically significant differences ($P < 0.05$) between groups by Student's t test.

The fold induction of GFP fluorescence was calculated for cultures with 10 mM inducer relative to no inducer. For P_{BAD}, the highest induction was for *E. coli*, with a 46 $\pm$ 36-fold increase, compared to 19 $\pm$ 3-fold and 27 $\pm$ 9-fold for Pf0-1 and PA14, respectively. With P_{xut}, GFP levels were unchanged for *E. coli*, with a ratio of 1.03 $\pm$ 0.2 for 10 mM xylose relative to no xylose, but were 51 $\pm$ 22-fold and 38 $\pm$ 20-fold increased for Pf0-1 and PA14, respectively.

The P_{xut} promoter was evaluated in additional *P. aeruginosa* isolates: the widely used strain PAO1 and the clinical keratitis isolate PAC. With 10 mM xylose, GFP fluorescence in both strains was induced and notably higher than that observed for strain PA14 at the same concentration (compare Fig. S2A with Fig. 5B). The fold induction was calculated and was found to be highest for PAO1 among the three strains, at 55-fold with xylose relative to no inducer (Fig. S2B).

Because *P. aeruginosa* is often studied at 37°C due to its role as a human pathogen, we tested the inducibility of P_{xut} at 37°C with xylose (10 mM) by using strains PAO1 and PA14. In results similar to those obtained at 30°C, xylose induced GFP production at 37°C. No significant differences were found between the two temperatures in terms of fold induction for either strain, with >50-fold induction for PAO1 and 23- to 35-fold induction for PA14 (Fig. S3).

The utilization of an inducer as a carbon source by bacteria can reduce the efficacy of the inducer in a culture over time. We tested the abilities of PA14 and Pf0-1 to grow

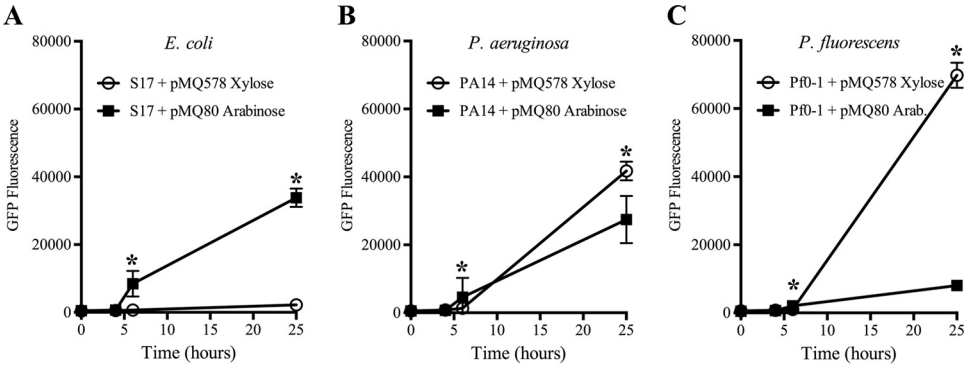

FIG 3 Expression of the P_{BAD} and P_{xut} promoters in *Pseudomonas* species and *E. coli* over time with 10 mM inducer. Shown is the expression of *gfpmut3* over time from the two different promoters in a pBBR1-based plasmid. Cultures were prepared in LB medium with an inducer (10 mM) and were grown at 30°C. Means and standard deviations are shown (n, $\geq$4 independent cultures for *E. coli*; n, 6 for *Pseudomonas* species). The pMQ578 plasmid has P_{xut}-*gfp*, and pMQ80 has P_{BAD}-*gfp*. Xylose was used as an inducer for pMQ578, and arabinose was used for pMQ80. Asterisks indicate statistically significant differences ($P < 0.05$) between groups by Student's t test.

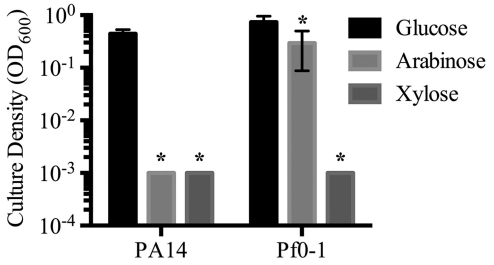

FIG 4 The *Pseudomonas* species tested do not use D-xylose as a sole carbon source. Cultures were incubated in M9 minimal medium with the indicated sugars at 10 mM for 25 h at 30°C. Means and standard deviations are shown (*n*, 6 independent cultures). Asterisks indicate significant differences from the glucose group ($P < 0.05$) by ANOVA with Tukey's posttest.

in minimal medium with L-arabinose or D-xylose as a sole carbon source at 10 mM. Glucose at 10 mM was used as a positive control (Fig. 4). After 24 h of growth at 30°C, culture density was measured with a spectrophotometer. Pf0-1 grew with arabinose or glucose, but not with xylose, as a sole carbon source. PA14 grew only with glucose as a carbon source.

Ribose was also shown to be an inducer of P_{xut} in strain SBW25 (14); therefore, we evaluated ribose as an inducer of bacteria with pMQ578 (Fig. 5). After 25 h of growth at 30°C, PA14 showed a 19% increase in GFP fluorescence with 10 mM ribose relative to no ribose, and a 93% increase was observed with 100 mM ribose (1,723 ± 219 relative fluorescence units [RFU] without ribose, 2,050 ± 265 RFU with 10 mM, and 3,335 ± 464 RFU with 100 mM). For Pf0-1, we observed a 25% increase in fluorescence at 10 mM and a 41% increase at 100 mM ribose (2,074 ± 172 RFU without ribose, 2,597 ± 200 RFU with 10 mM, and 2,919 ± 219 RFU with 100 mM). These findings suggest that ribose would have limited utility as an inducer of P_{xut} relative to xylose in the two strains tested unless controlled, low levels of gene expression are required for an experiment (Fig. 5A).

We further tested whether ribose would have a negative impact on induction by xylose (Fig. 5B). *P. aeruginosa* with pMQ578 was exposed to xylose and ribose independently or as a mixture, and the GFP fluorescence levels of cultures incubated with xylose alone or both xylose and ribose at 10 mM were indistinguishable (*P*, >0.05 by

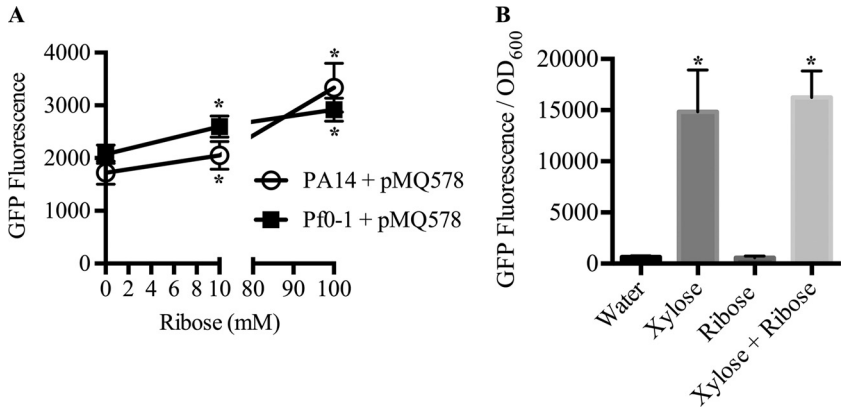

FIG 5 Evaluation of ribose for induction of the P_{xut} promoter in two *Pseudomonas* species. Shown is the expression of *gfpmut3* in a pBBR1-based plasmid. Cultures were prepared in LB medium with a range of inducer concentrations and were grown at 30°C for 25 h. Means and standard deviations are given (*n*, 6 independent cultures). (A) P_{xut}-based GFP expression was minimally induced by different ribose concentrations. Asterisks indicate significant differences compared to the no-ribose group as determined by Student's *t* test ($P < 0.05$). (B) Ribose (10 mM) did not alter the ability of xylose (10 mM) to activate P_{xut}-based GFP expression in *P. aeruginosa* strain PA14. Asterisks indicate significant differences compared to the water group as determined by ANOVA with Tukey's posttest ($P < 0.05$).

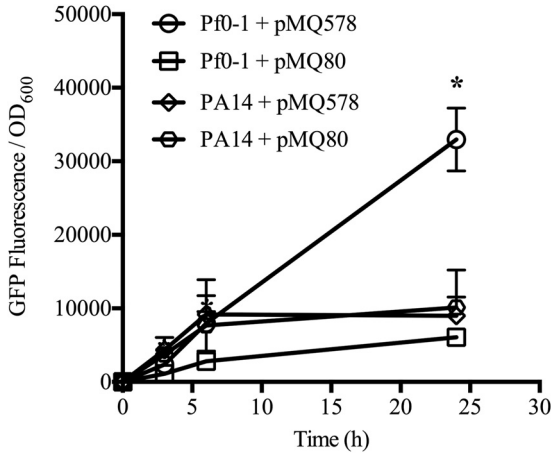

FIG 6 Stationary-phase induction of the P_{BAD} and P_{xut} promoters in *Pseudomonas* species. Bacteria were grown overnight in LB broth, washed, and adjusted to an OD_{600} of 2.0 in PBS with an inducer at 10 mM. GFP fluorescence was measured over time. Means and standard deviations are given (*n*, 3 to 4 independent cultures). P_{xut} was more highly inducible in *P. fluorescens* than in *P. aeruginosa* during stationary phase. P_{xut} and P_{BAD} were indistinguishable in *P. aeruginosa*. The pMQ578 plasmid has P_{xut}-*gfp*, and pMQ80 has P_{BAD}-*gfp*. The asterisk indicates a statistically significant difference ($P < 0.05$) by Student's *t* test between normalized fluorescence levels for Pf0-1 with different plasmids; there were no differences between plasmids in fluorescence levels for PA14. At 25 h, Pf0-1 with pMQ578 produced significantly more fluorescence than PA14.

analysis of variance [ANOVA]), demonstrating that ribose does not prevent the induction of the *xut* promoter by xylose under the conditions tested.

A similar lack of induction of the P_{xut} promoter was observed with the *Serratia marcescens* laboratory strain PIC3611. GFP fluorescence was measured at 727 ± 144 RFU with xylose (10 mM) and at 488 ± 320 RFU without xylose ($P = 0.22$; $n = 4$). Similar results were observed in preliminary experiments with *Klebsiella pneumoniae* and another *E. coli* strain, MIC4100. These data suggest that, for reasons unknown at this time, P_{xut} is not expressed in the *Enterobacteriaceae*.

Comparison of P_{BAD} and P_{xut} for induction of genes during stationary phase. Next, we investigated whether the P_{BAD} and P_{xut} promoters were responsive to inducers for cultures in the stationary phase (Fig. 6). We tested the expression of P_{BAD}-*gfp* and P_{xut}-*gfp* in stationary-phase cultures to which an inducer was added (10 mM) as a function of time. Pf0-1 and PA14 cultures were grown overnight, washed with phosphate-buffered saline (PBS) to remove residual LB medium, and diluted to an OD_{600} of 2 in PBS with an inducer. While we do not expect researchers to culture bacteria in PBS for gene expression experiments, PBS was used to prevent significant additional growth and to keep the bacteria in a stationary-phase-like state. Modest induction was shown by 6 h in stationary-phase cultures incubated in PBS relative to actively growing cultures (Fig. 6). At the final time point, P_{xut} in Pf0-1 demonstrated the greatest induction of GFP fluorescence, with 5.5 ± 1.4-fold higher fluorescence than Pf0-1 with P_{BAD} (Fig. 6). This suggests that the P_{xut} promoter could be used in stationary-phase cultures, and perhaps biofilms, for the expression of desired genes.

Flow cytometry analysis of promoter activity in *Pseudomonas* species. Previous studies have indicated that under many conditions, the P_{BAD} promoter is expressed only in a subset of cells of an induced population of *E. coli* (15–17). Here, we evaluated the P_{BAD} and P_{xut} promoters in PA14 and Pf0-1 by using flow cytometry analysis to assess promoter leakiness and to determine the frequency of cells in which gene expression is activated upon the addition of a moderate level of an inducer (10 mM). The data in Fig. 7 and Fig. 8A demonstrate a higher level of leakiness for P_{xut} than for P_{BAD} in both PA14 and Pf0-1. For both species, the P_{xut} promoter produced higher fluorescence intensity in induced GFP-positive cells than the P_{BAD} promoter (2.5 ± 8-fold and 2.3 ± 0.5-fold for *P. fluorescens* and *P. aeruginosa*, respectively)

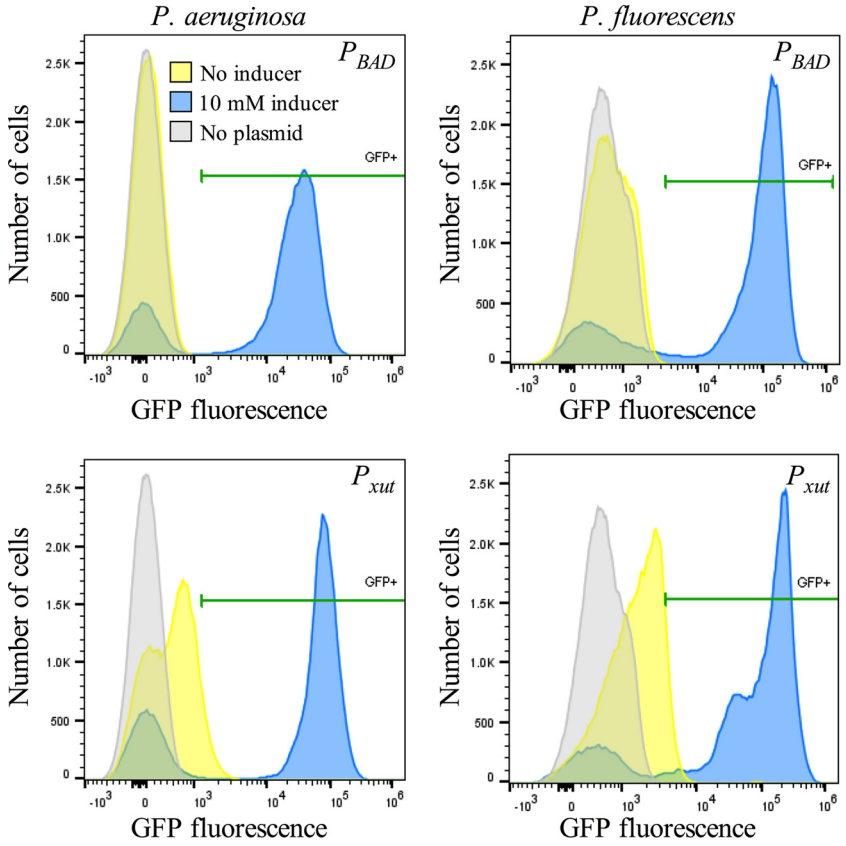

FIG 7 Flow cytometry analysis of P_{BAD} and P_{xut} promoter-driven GFP expression in *Pseudomonas* species indicates increased leakiness and increased proportions of bacteria expressing GFP with an inducer. The expression of *gfpmut3* from the two different promoters was measured by flow cytometry. Cultures were prepared in LB medium with an inducer (10 mM) and were grown at 30°C. Gray areas represent PA14 or Pf0-1 with no plasmid, used to determine background levels. Yellow peaks represent bacteria with pMQ578 or pMQ80 without an inducer, used to indicate leakiness. Blue (and green) peaks represent bacteria with pMQ578 or pMQ80 and with an inducer. The pMQ578 plasmid has P_{xut}-*gfp*, and pMQ80 has P_{BAD}-*gfp*. Xylose was used as an inducer for pMQ578, and arabinose was used for pMQ80. Levels of GFP fluorescence above those for bacteria without a plasmid were used to determine the positive cutoff (noted as GFP+); horizontal green bars indicate positive values. Results of a representative experiment are shown (*n*, ~100,000 cells per group).

(Fig. 8B). Higher fluorescence intensity was observed for Pf0-1 with both promoters, but this may be due to the larger cell size of Pf0-1 than of PA14 (P, <0.001 by ANOVA with Tukey's posttest). The cell length for PA14 was measured at 1.8 ± 0.5 or 1.6 ± 0.4 μm when the strain was grown in LB with xylose or arabinose at 10 mM, respectively (n, $\geq$50 cells per group). The cell length for Pf0-1 was measured at 2.5 ± 0.7 or 2.4 ± 0.6 μm when the strain was grown in LB with xylose or arabinose at 10 mM, respectively (n, $\geq$50 cells per group).

Additional shuttle vectors with P_{xut}. The pMQ578 vector with P_{xut}-*gfp* did not have a convenient restriction site at the 5′ end of the *gfp* gene, which would impede the replacement of *gfp* with another gene using traditional cloning methods. To make more user-friendly constructs, the *gfp* gene was replaced with *tdtomato* flanked by restriction sites to produce pMQ643 and with a multicloning site containing six unique restriction sites to produce pMQ650 (Table 1 and Fig. S1). The pMQ643 plasmid was introduced into Pf0-1, which was grown with or without xylose (10 mM). The culture with xylose was significantly brighter than the culture without xylose (P, 0.016 by an unpaired t test); tdTomato fluorescence/OD_{600} values for cultures with xylose were $1.3 \times 10^5 \pm 7.2 \times 10^4$ versus $2.0 \times 10^3 \pm 1.7 \times 10^3$ for the same culture conditions without xylose.

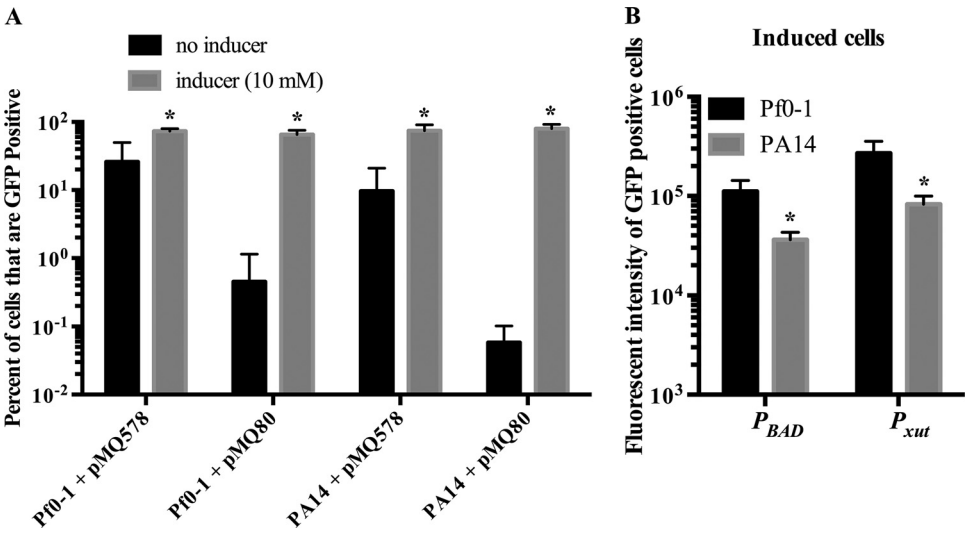

FIG 8 Analysis of promoter leakiness and strength using flow cytometry. GFP expression in *Pseudomonas* species was analyzed by flow cytometry ($n = 7$). (A) The percentage of GFP-positive cells was determined relative to the level for bacteria without a GFP plasmid. Means and standard deviations are shown. pMQ578 has P_{xut}, and pMQ80 has P_{BAD}, driving *gfp* expression. Asterisks indicate significant differences ($P < 0.05$) from the no-inducer group by Student's *t* test. (B) Fluorescence intensity of GFP-positive cells that have been induced (inducer concentration, 10 mM). Means and standard deviations are shown. Asterisks indicate significant differences ($P < 0.05$) between species by Student's *t* test.

The pMQ650 plasmid was additionally modified by removing the yeast replicon for the benefit of researchers who do not use yeast recombineering. The resulting plasmid, pMQ652, is 5.4 kbp rather than 7.3 kbp (Table 1, Fig. 1C, and Fig. S1B).

Use of the P_{xut} promoter system to evaluate the impact of *P. aeruginosa* corneal epithelial cell wound closure *in vitro*. A previous study demonstrated that secreted factors from *P. aeruginosa* strain PA14 could inhibit corneal cell migration and wound healing (12). To gain insight into the mechanism by which a secreted factor(s) from *P. aeruginosa* inhibits corneal wound healing, we first heat-treated normalized secretomes from strain PA14. Whereas unheated secretomes inhibited wound healing, those heated for 10 min at 95°C were unable to inhibit corneal cell migration (data not shown). This result suggested that the inhibitory secreted factor was a heat-labile molecule, such as a protein.

P. aeruginosa has numerous secretion systems. We reasoned that the secreted inhibitor factor was unlikely to be secreted by type III or type VI secretion systems, because contact between *P. aeruginosa* and the corneal cells was not necessary for the cell migration phenotype (only culture filtrates were used). Because many enzymes are secreted through the type II secretion system of *P. aeruginosa* (18), we used a strain deficient in XcpQ to test whether the type II secretion system was required for inhibiting cell migration. The XcpQ protein is an essential component of the type II secretion system and forms part of the outer membrane pore (18).

Unlike wild-type PA14 culture filtrates, those from a previously described Δ*xcpQ* derivative of PA14 (19) were unable to block cell migration (Fig. 9). Expression of *xcpQ*, but not *gfp* (used as a negative control) from P_{xut}, was able to restore the cell migration inhibition phenotype to the *xcpQ* mutant in a D-xylose-dependent manner; that is, 50 mM (but not 5 mM) D-xylose was sufficient to complement the cell migration inhibition phenotype (Fig. 9). Importantly, as a control, D-xylose (50 mM) did not restore the cell migration inhibition phenotype to the Δ*xcpQ* mutant that did not have a plasmid, indicating that D-xylose alone was not responsible for the phenotype in the absence of the *xcpQ* plasmid (data not shown). These results indicate that the *P. aeruginosa* type II secretion system is necessary for the inhibition of corneal wound healing and demonstrate the utility of the P_{xut} system for studying *Pseudomonas* biology.

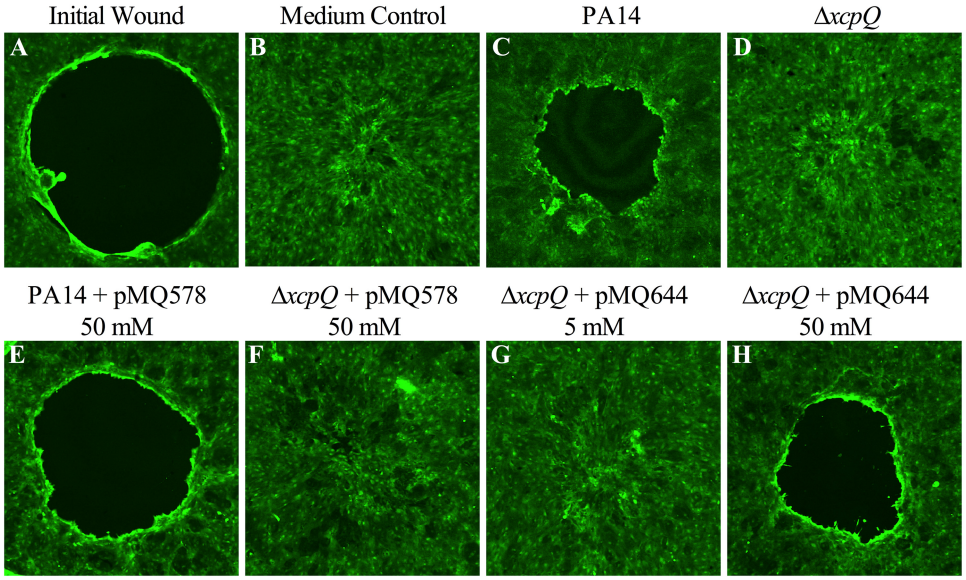

FIG 9 XcpQ is required for the inhibition of stratified corneal cell migration *in vitro*. Shown are representative images of cells stained with the vital stain Calcein AM and imaged by confocal microscopy. (A to C) A cell-free zone (black circle) in layers of a stratified human corneal cell line (A) closes rapidly when treated with LB medium and incubated for 24 h (B), but this migration is inhibited by filtrates from PA14 cultures grown in LB medium (C). (D) Filtrates from isogenic Δ*xcpQ* mutants were unable to impede cell migration. (E and F) D-Xylose (50 mM)-induced expression of *gfp* from pMQ578 did not alter the migration phenotypes of cells exposed to PA14 or Δ*xcpQ* mutant filtrates. (G and H) Culture filtrates from the Δ*xcpQ* mutant with the wild-type *xcpQ* gene on a plasmid (pMQ644) that had been grown in LB medium with 5 mM D-xylose were unable to inhibit cell migration (G), but those grown with 50 mM D-xylose were complemented for the cell migration inhibition-defective phenotype (H). All cultures were grown to stationary phase and were adjusted to an OD_{600} of 2 with fresh LB, and bacteria were removed by centrifugation and filtration.

DISCUSSION

This study introduces a new inducible promoter system for pseudomonads. The new P_{xut} promoter had no detectable function in *E. coli*. This could be considered a disadvantage of the P_{xut} promoter, because one cannot use the same plasmid construct to express genes in *E. coli* and a *Pseudomonas* species. However, since plasmids are almost exclusively passed through *E. coli* before moving into *Pseudomonas* species, genes that are toxic to *E. coli* are likely to be more easily cloned with P_{xut} than with other promoter systems that might have leaky expression in *E. coli*. For *P. aeruginosa*, the P_{xut} promoter appeared to be largely equivalent to P_{BAD}, although it was leakier, and there was a tendency toward higher expression with P_{xut} on a population basis (Fig. 2) and in GFP-positive fluorescent cells, but this did not reach significance (Fig. 7 and 8). In *P. fluorescens*, the P_{xut} promoter performed better than P_{BAD} with respect to maximal expression levels and required a lower concentration of inducer for expression (Fig. 2C, 7, and 8). One caveat in comparing the P_{xut} and P_{BAD} promoters in these *Pseudomonas* species is that pMQ80 has a pRO1600 origin of replication and pMQ578 has a pBBR1 origin of replication. While these have been reported to support similar copy numbers (20–22), this feature is an additional variable. In addition to xylose, P_{xut} can be activated by ribose (14), raising the possibility that the P_{xut} promoter can be fine-tuned with these alternative carbohydrate inducers.

Pseudomonas species have been suggested for industrial production and bioremediation of molecules, including, but not limited to, β-peptides (23), polyhydroxyalkanoates (24), phenazine-1-carboxyamide (25), proteases (26, 27), pseudofactin (28), rhamnolipids (29–31), silver nanoparticles (32), and toluene (33). The ability of P_{xut} to activate gene expression in the majority of cells and its relative strength in *P. fluorescens* compared to P_{BAD} suggest that it may be useful for large-scale gene synthetic applications. These advantages are further strengthened by the low cost of D-xylose relative to other inducers; L-arabinose and isopropyl-β-D-thiogalactopyranoside (IPTG) cost

TABLE 2 Primers and synthetic DNA sequences used in this study

Oligonucleotide primer	Sequence[a]
3821 Pxut	ccagtgaaaagttcttctccttttactcatatgtatatcTCCTTGTTGTTCTTGGTTGTTG
3822 Pxut	atgcagctggcacgacaggtttcccgactggaaagcggCAGGACACGGCGCGTATTCTCC
4127 Pxut-tdtomato	aacagccaagcttgcatgcctgcagactagtcggatccTTACTTGTACAGCTCGTCCATG
4128 Pxut-tdtomato	caagaacaacaaccaagaacaacaaggagaattcttttttATGGTGAGCAAGGGCGAGGAG
4132 xcpQ	gccaaaacagccaagcttgcatgcctgcagactagtTTATTCCGTCTTCAGTTCGCGCAG
4133 xcpQ	acaacaaccaagaacaacaaggagatataATGTCCCAACCTTTGCTCCGCGCCCTGTTTG
4168 Pxut-MCS	ctactgccgccaggcaaattctgttttatcagaccgcttctgcgttctgatttaatctgtatcaggctgaaaatcttctctcatccgccaaTTATTCTATTTACCAAGCTTGCA TGCGACTAGTTGGATCCTTGGGTCGACCGAAGAATTCAAAAATCctccttgttgttcttggttgttgttcttgggcgtgtcccgatactagcaacggccctgt gcctgccgattacgaaaatcaccaacacgcagtgcgattttgcgtattgagattcgccctctgcgcgcctagtctgtgcagaccgcctctacggcaagaggcccggaacaag cctgaaaacaatgaaaaccgtaccgcctgttcaccgcatcgccctgttgttcaacggcagcaagatctatgaccgcggcatcatcagcggcatcggcaactacctgagcag cacgcgcgcgtcttgggacctgtttctggaagaggattttctctgccgcttgaaaggta

[a]Capital letters indicate sequence that directs priming, except for oligonucleotide 4168, where capital letters indicate the sequence that replaced GFP on pMQ578. Lowercase letters indicate sequence that directs recombination.

approximately 10 times more to induce a culture (with 10 mM sugar or 0.1 mM IPTG). An additional benefit of D-xylose is that it is not usable as a carbon source by either of the *Pseudomonas* species tested, indicating that the D-xylose inducer will not be catabolized for energy and thereby eliminated from the culture over time.

Xylose-inducible plasmid systems have been developed for a number of other Gram-negative bacteria, such as *Caulobacter crescentus* (34), and for Gram-positive species, including *Bacillus subtilis*, *Clostridioides difficile*, *Lactococcus lactis*, *Staphylococcus xylosus*, and *Streptomyces lividans* (35–39). The xylose-responsive transcription factors for these systems include members of the AraC family, such as XutR, used in this study, LacI, MarR, and the ROK family. The fold induction ranges from ~10-fold, as is the case with *C. crescentus*, up to several orders of magnitude for *B. subtilis* and *C. difficile*. While the fold induction values for the different systems are not clearly comparable, because the developers used different reporters, media, induction times, and other variables, taken at face value, they put P_{xut} in the low to middle range of these expression systems.

With regard to corneal epithelial wound closure, previous work has implicated lipopolysaccharide from *E. coli* and *Serratia marcescens* in the inhibition of corneal wound healing (12, 40). Unlike that of *P. aeruginosa*, the secreted inhibitory factor from *S. marcescens* was heat stabile (12). Work with the P_{xut} promoter in this study strongly implicated the type II secretion system of *P. aeruginosa* strain PA14 in allowing the bacterium to inhibit corneal epithelial wound closure, which may increase its ability to establish ocular infections.

In summary, we have created an episomal-plasmid-based, xylose-inducible expression system and tested it in *P. aeruginosa* and *P. fluorescens*. The P_{xut} promoter region, including the ribosome binding site and regulatory gene, spans 1,356 bp, in contrast to >2,042 bp for the *rhaSR*-P_{rhaB} system, making P_{xut} well suited for use on an episome. The P_{xut} system is likely leakier without an inducer than *lacI*^{q}-P_{tac} and *rhaSR*-P_{rhaB}, but this has yet to be tested in controlled experiments. Nevertheless, the P_{xut} system presented here should be useful for the inducible expression of genes in *Pseudomonas* species by itself or in conjunction with other inducible systems.

MATERIALS AND METHODS

Strains, media, and growth conditions. Bacterial strains and plasmids are listed in Table 1. Bacteria were grown in lysogeny broth (LB) (41) and were aerated on a TC-7 tissue culture roller (New Brunswick, Inc). *P. aeruginosa* strains are listed in Table 1. Gentamicin was used at 10 μg/ml in *E. coli* and 30 μg/ml in *Pseudomonas* species to select for plasmids. Bacterial culture density was measured with a 1-cm cuvette using a SpectraMax M3 plate reader, except where noted below.

Plasmid construction. Plasmids are listed in Table 1, and oligonucleotide primers and synthetic DNA sequences are listed in Table 2. Plasmids were made using yeast homologous recombination (8), except for pMQ652, and were verified by PCR and sequencing of junctions. The *xutR* gene and the intergenic region between *xutR* and *xutA* from the *Pseudomonas fluorescens* Pf0-1 genome were cloned along with *gfpmut3* (*gfp*) (42) from pMQ80 (8) to replace P_{lac} and *lacZ*α from pMQ132 (11). The resulting plasmid, pMQ578, and all plasmids made in this study are listed in Table 1. One variant of pMQ578 was

made in which *gfp* was replaced with *tdtomato* from pMQ414 (43) using primers 4127 and 4218; this variant was named pMQ643. For another variant, pMQ650, an artificial DNA sequence containing six restriction enzyme sites (EcoRI, SalI, BamHI, SpeI, SphI, and HindIII) and a sequence with stop codons in three frames was introduced using a 499-bp double-stranded DNA sequence listed as primer 4168 (gBlock; IDT, Inc.). The pMQ650 plasmid was sequenced using the PacBio platform (coverage of 32) by SNPSaurus (Eugene, OR). Last, pMQ652 was made by the digestion of pMQ650 with SspI and StuI, which excises a 1.8-kba region containing the yeast replicon and the *URA3* gene, followed by recircularization with T4 DNA ligase.

Fluorescence assays. Bacteria were grown as described above, and at various time points, samples were obtained for analysis. Aliquots (150 μl) were read in 96-well plates with black opaque sides and clear bottoms (catalog no. 165305; Thermo Scientific Nunc). GFP fluorescence and tdTomato fluorescence were read with a BioTek Synergy 2 plate reader using 485/20 excitation and 516/20 emission filters for GFP and 545/40 excitation and 590/20 emission filters for tdTomato. Optical density was read at 600 nm.

Flow cytometry analysis of GFP fluorescence. Bacteria were grown overnight with or without 10 mM inducer, adjusted to an OD_{600} of 2, washed twice, and adjusted to an OD_{600} of 0.02 in phosphate-buffered saline (PBS) that had been filtered with a 0.22-μm filter. Flow cytometric analysis was performed on a CytoFLEX LX instrument (Beckman Coulter, USA). Filtered PBS was used as a negative control for gating bacteria by forward scatter (FSC) and side scatter (SSC). Approximately 10^5 cells were analyzed per genotype, and the experiments were repeated on five occasions. Bacteria of each species without the fluorescent plasmid were used as negative controls to determine the FSC threshold, as well as for background fluorescence cutoff. Software analysis was performed using FlowJo, version 10 (Becton, Dickinson, USA).

Corneal cell migration analysis. *In vitro* epithelial cell wound closure assays were performed as described previously (12) using human corneal limbal epithelial (HCLE) cells (44) and a commercial wound-healing assay kit (Oris; product no. CMAU101; Platypus Technologies, LLC). After stratified cell layers of HCLE cells were established (45), they were challenged with 50 μl of normalized bacterial culture filtrates or LB medium that was added to 100 μl of culture medium overlaid on the cells. The tissue culture medium consisted of stratification medium or keratinocyte serum-free medium (catalog no. 10724-011; Gibco) supplemented with bovine pituitary extract (25 μg/ml), epidermal growth factor (0.2 ng/ml), and penicillin and streptomycin at 100 μg/ml. Bacterial culture filtrates were made using overnight cultures of *P. aeruginosa* grown in LB medium with or without xylose and antibiotic and were incubated at 30°C. Cultures were adjusted to an OD_{600} of 1.0 using fresh LB medium and were filtered with a 0.22-μm filter (Mellex-GV polyvinylidene difluoride [PVDF]). After 20 to 24 h, HCLE cells were rinsed with PBS and were stained using 0.5 μM Calcein AM (catalog no. C3099; Invitrogen) for 15 min. Cells were imaged using an Olympus FluoView FV-1000 laser scanner confocal microscope with a 4× (numerical aperture [NA], 0.3) objective and were analyzed with FluoView image-viewing software.

Bacterial length analysis. Bacteria were grown in LB medium with or without inducer (arabinose or xylose at 10 mM) for 24 h. Bacteria were then imaged as described above, but using a 60× objective. Bacteria were measured using ImageJ software, and at least 50 cells were counted from two different experiments per group.

Statistical analysis. Experiments were performed at least three times, and data were analyzed with GraphPad Prism software using ANOVA, two-way ANOVA, and Student *t* tests.

Data availability. The annotated pMQ650 plasmid sequence has been deposited in GenBank under accession number MT344942. The DNA sequences of the *xutR* and P_{xut} regions from pMQ578, corresponding to genome coordinates 2656767 to 2656949 from the Pf0-1 genome, have been deposited in GenBank under accession number MN857504.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 1 MB.

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