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Vfr Directly Activates *exsA* Transcription To Regulate Expression of the *Pseudomonas aeruginosa* Type III Secretion System

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ABSTRACT

The *Pseudomonas aeruginosa* cyclic AMP (cAMP)-Vfr system (CVS) is a global regulator of virulence gene expression. Regulatory targets include type IV pili, secreted proteases, and the type III secretion system (T3SS). The mechanism by which CVS regulates T3SS gene expression remains undefined. Single-cell expression studies previously found that only a portion of the cells within a population express the T3SS under inducing conditions, a property known as bistability. We now report that bistability is altered in a *vfr* mutant, wherein a substantially smaller fraction of the cells express the T3SS relative to the parental strain. Since bistability usually involves positive-feedback loops, we tested the hypothesis that virulence factor regulator (Vfr) regulates the expression of *exsA*. ExsA is the central regulator of T3SS gene expression and autoregulates its own expression. Although *exsA* is the last gene of the *exsCEBA* polycistronic mRNA, we demonstrate that Vfr directly activates *exsA* transcription from a second promoter (P_{exsA}) located immediately upstream of *exsA*. P_{exsA} promoter activity is entirely Vfr dependent. Direct binding of Vfr to a P_{exsA} promoter probe was demonstrated by electrophoretic mobility shift assays, and DNase I footprinting revealed an area of protection that coincides with a putative Vfr consensus-binding site. Mutagenesis of that site disrupted Vfr binding and P_{exsA} promoter activity. We conclude that Vfr contributes to T3SS gene expression through activation of the P_{exsA} promoter, which is internal to the previously characterized *exsCEBA* operon.

IMPORTANCE

Vfr is a cAMP-dependent DNA-binding protein that functions as a global regulator of virulence gene expression in *Pseudomonas aeruginosa*. Regulation by Vfr allows for the coordinate production of related virulence functions, such as type IV pili and type III secretion, required for adherence to and intoxication of host cells, respectively. Although the molecular mechanism of Vfr regulation has been defined for many target genes, a direct link between Vfr and T3SS gene expression had not been established. In the present study, we report that Vfr directly controls *exsA* transcription, the master regulator of T3SS gene expression, from a newly identified promoter located immediately upstream of *exsA*.

Pseudomonas aeruginosa is an environmental bacterium typically found in soil and water. The organism is also an important opportunistic pathogen of humans, especially in those with neutropenia, severe burns, and cystic fibrosis (1, 2). Both the physical and host environments expose *P. aeruginosa* to unique stresses that challenge survival. Reprogramming gene expression is critical for adaptation. The host signals to which the bacteria respond are not entirely clear but likely include contact with host cell surfaces or host-derived macromolecules, temperature, osmolarity, pH, iron limitation, and oxidative stress (3–5). Bacterial genes induced within mammalian hosts include those important for iron acquisition, carbon utilization, and virulence factors, such as type IV pili, Xcp, and Hxc type II secretion systems, secreted factors (e.g., exotoxin A, protease IV, and elastase), and a type III secretion system (T3SS) (3, 5–8). Many of these virulence factors are directly controlled by the cyclic AMP (cAMP)-Vfr signaling (CVS) system (9, 10). The CVS pathway is a global regulatory system consisting of the CyaA and CyaB adenylate cyclases, the CpdA phosphodiesterase, and the virulence factor regulator (Vfr) transcription factor (10). Intracellular cAMP is generated by CyaA and CyaB in response to poorly defined environmental signals (9), and cAMP homeostasis is maintained by the CpdA phosphodiesterase (11). Vfr is a DNA-binding protein of the *Escherichia coli* Crp family (12) and responds directly to increased intracellular pools of cAMP to activate expression of the CVS regulon (11, 13, 14). Vfr binds to a well-characterized consensus site (5′-ANWW TGNGAWNYAGWTCACAT) within target promoters (13). Direct binding of Vfr to the promoter regions of many virulence factors has been demonstrated or can be inferred by the presence of a consensus-binding site (10). One noted exception, however, is the T3SS. Although Vfr was previously shown to regulate the T3SS (9), a mechanism has not been described, and none of the known promoter regions for T3SS genes contains a Vfr consensus-binding site.

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The *P. aeruginosa* T3SS is an important virulence determinant known to contribute to acute virulence phenotypes, such as phagocytic avoidance, cytotoxicity, and systemic spread of *P. aeruginosa* from initial sites of colonization (15). The T3SS is a multiprotein complex that forms a needle-like injectisome structure. The injectisome functions by translocating effector proteins produced within the bacterial cytoplasm into host cells, where they have antihost effects that include modulation of signal transduction, actin dynamics, inflammation, and cell death pathways (15). The primary regulator of T3SS gene expression is the DNA-binding protein ExsA (16, 17). T3SS genes are organized into 10 transcriptional units, each of which is controlled by an ExsA-dependent promoter (18). ExsA is encoded as the last gene of the *exsCEBA* operon and autoregulates its own transcription through direct activation of the P_{exsC} promoter (19). T3SS gene expression is intimately coupled to secretory activity by a partner-switching mechanism (18, 20). The partner-switching mechanism controls the DNA-binding activity of ExsA and requires three additional proteins (ExsD, ExsC, and ExsE). ExsD is an antiactivator that directly interacts with ExsA to inhibit DNA-binding activity (21). ExsC functions as both an anti-antiactivator by binding to ExsD and as a chaperone for ExsE, a secreted substrate of the T3SS (22). Type III secretory activity serves as a sensor of environmental conditions and regulates the partner-switching mechanism. In nonpermissive environments (high calcium or the absence of host cells), the secretion system is assembled but inactive (21, 23). These conditions favor formation of the ExsA-ExsD and ExsC-ExsE complexes, and T3SS gene expression is low (21, 22, 24–27). Inducing signals, which include low environmental Ca^{2+} and contact with host cells, convert the secretion system into an active conformation, leading to secretion of ExsE (25, 26, 28). The corresponding decrease in the intracellular concentration of ExsE triggers partner switching, wherein ExsC preferentially binds to ExsD and allows free ExsA to activate T3SS gene expression.

The *P. aeruginosa* T3SS is expressed in only a subset of cells, resulting in a property known as bistability (23, 28, 29). Observed at the single-cell level, bistability occurs when there is nonhomogeneous gene expression within a cell population. The mechanism of bistability usually involves regulatory feedback loops (30). Feedback loops that may contribute to bistable expression of the T3SS include ExsA autoregulation of its own expression (19) and the partner-switching mechanism that controls ExsA activity (18). In the present study, we report that the bistable phenotype is altered in a *vfr* mutant wherein a smaller percentage of cells are induced for T3SS gene expression than in the parental strain. We identified a Vfr-dependent promoter (P_{exsA}), located immediately upstream of *exsA*, that appears to be critical for T3SS gene expression. The P_{exsA} promoter region contains a Vfr consensus-binding site, and footprinting and binding studies confirmed that Vfr interacts with the P_{exsA} promoter region. Mutagenesis of the Vfr binding site on the chromosome significantly reduced T3SS gene expression. Unexpectedly, disruption of the Vfr binding site on the chromosome resulted in a larger defect in T3SS gene expression than that in the *vfr* mutant. We conclude that Vfr regulates T3SS gene expression through its effects on P_{exsA} promoter activity but cannot exclude the possibility that additional Vfr-controlled functions also contribute to T3SS gene expression.

MATERIALS AND METHODS

Bacterial strains, culture conditions, and sample preparation. The bacterial strains used in this study are provided in Table S1 in the supplemental material. *Escherichia coli* DH5α was used for general cloning and maintained on Luria-Bertani (LB) agar plates with gentamicin (15 μg/ml), tetracycline (12 μg/ml), or ampicillin (100 μg/ml), as appropriate. *E. coli* strain Tuner (DE3) was maintained on LB agar with ampicillin (100 μg/ml). *P. aeruginosa* PA103 strains were maintained on Vogel-Bonner minimal (VBM) medium with gentamicin (100 μg/ml) as necessary.

Plasmid and strain construction. The allelic exchange vector, reporter fusions, plasmids, and primers used in their construction are listed in Tables S1 and S2, respectively, in the supplemental material. To generate the Vfr binding site mutant (BSM) in the P_{exsA} promoter region, two overlapping PCR fragments containing upstream and downstream sequences flanking P_{exsA} and the desired Vfr binding site mutations (primer pairs 129092160-129781385 and 129781384-129092161) were amplified and joined with pEXG2Tc (digested with HindIII and SacI) in a Gibson assembly reaction (New England BioLabs). The resulting construct (pEXG2Tc P_{exsA} VBM) was conjugated from *E. coli* SM10 into the wild-type (wt) PA103 strain, and plasmid integrants were selected on VBM agar with tetracycline (50 μg/ml). Merodiploids were resolved by plating on YT agar (0.5% yeast extract and 1% tryptone) with 5% sucrose. Successful integration of P_{exsA} Vfr binding site mutations was confirmed by PCR amplification and sequencing. The P_{exsA} and P_{exsAm} reporter fusions were constructed as follows: the region containing −193 to +18 (relative to the *exsA* transcriptional start site previously mapped by transcriptome sequencing [RNA-seq] [4]) was PCR amplified from the wt PA103 strain or the PA103 P_{exsA} VBM mutant (primer pair 126323955-126639110) and joined with the mini-CTX-*lacZ* vector (precut with EcoRI and BamHI) by incubation in a Gibson assembly reaction (New England BioLabs). Reporter constructs were integrated at the *attB* site of PA103, as previously described (31). The $P_{exsD\text{-}gfp}$ reporter fusion was constructed by transferring the P_{exsD} promoter region as an EcoRI-HindIII restriction fragment from mini-CTX-$P_{exsD\text{-}lacZ}$ to the corresponding sites in pJNE05. The $P_{lacP1\text{-}gfp}$ reporter fusion was cloned as a lacP1 gBlock into EcoRI-HindIII-digested pJNE05 by Gibson assembly.

Transcriptional reporter assays. *P. aeruginosa* strains were grown overnight at 37°C on VBM plates with appropriate antibiotics. The following day, cultures were diluted to an A_{600} of 0.1 in Trypticase soy broth (TSB) with EGTA (2 mM), as described in the figure legends. Cultures were incubated at 37°C with shaking, and samples were harvested when the culture A_{600} reached 1.0. The β-galactosidase activity was assayed with the substrate *ortho*-nitrophenyl-galactopyranoside (ONPG), as previously described (22), or chlorophenol red-β-D-galactopyranoside (CPRG). CPRG activity was determined by measuring product formation at 578 nM and using an adaptation of the Miller equation, as follows: CPRG units = $[A_{578}/\text{culture } A_{600}/\text{time (min)}/\text{culture volume (ml)}] \times 1{,}000$. CPRG and Miller units are reported as the averages of the results of at least three independent experiments, with error bars representing the standard error of the mean (SEM). Statistical significance was determined by one-way analysis of variance (ANOVA) using Graphpad Prism version 5.0c for Mac OS X (GraphPad Software, La Jolla, CA).

Flow cytometry. *P. aeruginosa* strains carrying green fluorescent protein (GFP) transcriptional reporters ($P_{exoS\text{-}gfp}$ or $P_{exsD\text{-}gfp}$) were cultured overnight in TSB with gentamicin (100 μg/ml) at 37°C. The next day, bacteria were diluted to an A_{600} of 0.05 in TSB with EGTA (2 mM) and cultured at 30 or 37°C with shaking. At the indicated times, cells were collected and diluted to 1×10^7 CFU/ml in phosphate-buffered saline (PBS), and GFP fluorescence was measured by counting 10,000 bacteria per sample on a Becton Dickinson LSR II at the University of Iowa Flow Cytometry Facility.

Electrophoretic mobility shift and DNase I protection assays. Probes for the *exsA* (255 bp), *pscF* (160 bp), *exsC* (200 bp), *regA* (275 bp), and *algD* (160 bp) promoter or coding regions were amplified by PCR using primer pairs 126323955-126639110, 32179291-3349133, 22963127-

49188917, 33075941-33075940, and 85333731-85333730, respectively. The PCR products were end labeled with 10 μCi of [γ-^{32}P]ATP, as previously described (16). Vfr, apo-Vfr, and $ExsA_{His}$ were purified as previously described (11, 13, 16). Electrophoretic mobility shift assay (EMSA) reaction mixtures containing specific and nonspecific probes (0.05 nM), ExsA DNA binding buffer (10 mM Tris [pH 7.5], 50 mM KCl, 1 mM EDTA, 1 mM dithiothreitol, 5% glycerol), 25 ng/μl poly(2′-deoxyinosinic-2′-deoxycytidylic acid), and 100 μg/ml bovine serum albumin were incubated in a total volume of 18 μl for 5 min at 25°C. Where specified, cyclic AMP (cAMP; Sigma-Aldrich) was added to a final concentration of 200 nM. Vfr-cAMP, apo-Vfr, or ExsA (concentrations indicated in the figure legends) was added to a total volume of 20 μl and incubated for an additional 15 min at 25°C. Samples were analyzed by electrophoresis on 5% polyacrylamide glycine gels (10 mM Tris [pH 7.5], 380 mM glycine, 1 mM EDTA) at 4°C. Imaging was performed using an FLA-7000 phosphorimager (Fujifilm) and MultiGauge v3.0 software (Fujifilm).

The P_{exsA} footprinting probe (237 bp) was generated by PCR amplification with primer pair 129588340-126639110. The 129588340 primer was 5′-end modified with Amino Modifier C6 (Integrated DNA Technologies) to prevent radiolabeling at that end of the promoter probe. Vfr-mediated protection of P_{exsA} from DNase I cleavage and DNA sequencing reactions were performed as previously described (16).

mRNA collection and 5′ RACE. *P. aeruginosa* strains were grown overnight on VBM at 37°C. The following day, the bacteria were diluted to an A_{600} of 0.1 in TSB with EGTA (2 mM) and cultured at 37°C with shaking. RNA was isolated from 500-μl aliquots of cell culture using RNAprotect cell reagent (Qiagen) and the RNeasy minikit (Qiagen). cDNA was generated from the isolated RNA using SuperScript II reverse transcriptase (Invitrogen) and primer 4373912. PCRs were performed with primer pair 126323955-126639110. Genomic DNA and RNA samples not treated with reverse transcriptase served as positive and negative controls, respectively, for PCR amplification of the $P_{exsCEBA}$-specific fragment. Rapid amplification of 5′ cDNA ends (5′ RACE) was performed using the 5′ RACE system (Invitrogen). First-strand cDNA synthesis was performed using *exsA* primer 132712288 (as shown in Fig. 4C). The deoxycytidine-tailed cDNA was PCR amplified with the kit-provided abridged anchor primer and primer 132712289. Finally, nested-PCR amplification with the kit-provided abridged universal amplification primer and primer 132712290 was performed, and the resulting product was sequenced (at the Iowa Institute of Human Genetics).

RESULTS

The bistable phenotype for T3SS gene expression is altered in a *vfr* deletion mutant. The promoter regions for the ExoS effector and the ExsD antiactivator genes (P_{exoS} and P_{exsD}, respectively) are representative ExsA-dependent promoters. Previous studies found expression of $P_{exoS\text{-}lacZ}$ and$P_{exsD\text{-}lacZ}$ transcriptional reporters to be entirely dependent upon *exsA* and reduced >8-fold in the absence of *vfr* compared to the parental strain PA103 (Fig. 1A) (24). A limitation of *lacZ* reporters is that the measured activity represents the average of the entire cell population. This can obscure differences in reporter activity occurring at the single-cell level. Previous studies using the green fluorescent protein as a transcriptional readout of single-cell activity found that T3SS gene expression is bistable (23, 28, 29). Bistability results from nonhomogeneous gene expression within a cell population (30). To better understand the kinetics of T3SS gene expression at the single-cell level, we grew broth cultures of wt, *exsA*, and *vfr* strains carrying either a $P_{exoS\text{-}gfp}$ or $P_{exsD\text{-}gfp}$ transcriptional reporter overnight under nonpermissive conditions (high Ca^{2+}, without EGTA) for T3SS gene expression. Flow cytometry verified that the uninduced reporter strains had low levels of fluorescence (data not shown, although evident at the 1-h time point in Fig. 3A). The cells were then diluted to an A_{600} of 0.05 and cultured under inducing conditions for T3SS gene expression (low Ca^{2+}, with EGTA) at 37°C, and samples were harvested every hour and examined by flow cytometry. The *exsA* mutant cells had low levels of $P_{exoS\text{-}gfp}$ and $P_{exsD\text{-}gfp}$ reporter activity following 5 h of growth (Fig. 2A and B), chosen as a representative time point, and activity remained low throughout the time course (Fig. 3A). In contrast, the wt and *vfr* mutant cells had two distinct peaks of $P_{exoS\text{-}gfp}$ and $P_{exsD\text{-}gfp}$ reporter activity (Fig. 2C to F) at the 5-h time point. The first peak (coincident with the peak seen with the *exsA* mutant) represented cells with low reporter activity, and the higher-intensity peak represented cells with high reporter activity. Time course experiments demonstrated that the kinetic induction of both reporters was delayed in the *vfr* mutant compared to the wt strain (Fig. 3A). Further examination revealed that the *vfr* mutant had two observable defects in $P_{exoS\text{-}gfp}$ and$P_{exsD\text{-}gfp}$ reporter activities. First, the fluorescence intensity of the induced *vfr* mutant cells (Fig. 2E and F) was 2- to 4-fold lower than the intensity seen with the wt cells (Fig. 2C and D). Second, a smaller percentage of the *vfr* mutant cells demonstrated fluorescence over the time course (Fig. 3A and B). These findings demonstrate that the bistable phenotype is altered in the absence of *vfr*.

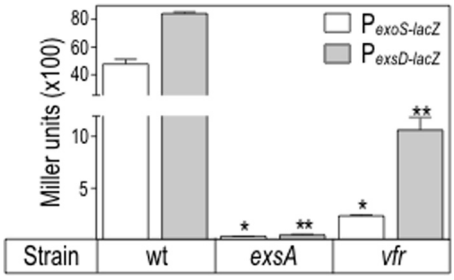

FIG 1 Vfr is required for maximal T3SS gene expression. (A) *P. aeruginosa* strains PA103 (wt), PA103 *exsA*::Ω (*exsA*), and PA103 Δ*vfr* (*vfr*) carrying chromosomally integrated $P_{exoS\text{-}lacZ}$ or $P_{exsD\text{-}lacZ}$ transcriptional reporters were grown under inducing conditions (low Ca^{2+}, with EGTA) for T3SS gene expression at 37°C to an A_{600} of 1.0 and assayed for β-galactosidase activity. The reported values (Miller units) are the means of the results of at least three experiments. *, $P < 0.0001$, compared to $P_{exoS\text{-}lacZ}$ activity in the wt strain; **, $P < 0.0001$, compared to $P_{exsD\text{-}lacZ}$ activity in the wt strain.

We also examined the effect of temperature on expression of the $P_{exoS\text{-}gfp}$ or$P_{exsD\text{-}gfp}$ reporters by comparing cells grown at 30°C or 37°C for 5 h under permissive conditions for T3SS gene expression. Although most of the wt cells were positive for GFP expression when cultured at 37°C, only ~30% were positive when grown at 30°C (Fig. 3). A similar trend was seen with the *vfr* mutant, in that more GFP-positive cells were observed at 37°C than at 30°C. We concluded that the requirement for *vfr* is more stringent for cells grown at 30°C.

Bistability generally involves positive feedback regulation (30). One candidate regulator for bistable control of T3SS gene expression is Vfr itself, which directly autoregulates its own expression (13). To determine whether the Vfr regulon is subject to bistable expression, we constructed a $P_{lacP1\text{-}gfp}$ reporter. The P_{lacP1} reporter was previously shown to be Vfr dependent and a reliable surrogate for expression of the Vfr regulon (32). When assayed in the wt and *vfr* strains, however, the fluorescence intensity was uniform, suggesting that the Vfr regulon is not expressed in a bistable manner (see Fig. S1 in the supplemental material).

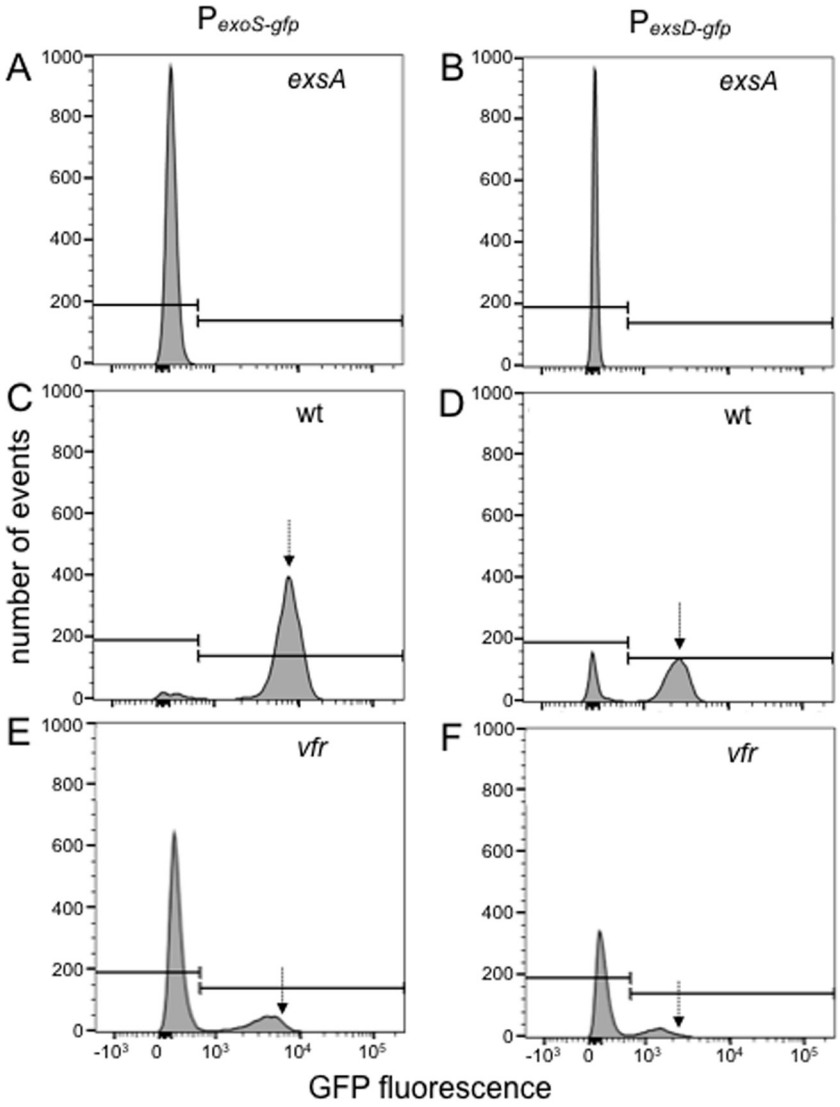

FIG 2 T3SS gene expression is bistable. (A through F) wt strain PA103 (C and D), the *exsA* mutant (A and B), and the *vfr* mutant (E and F) carrying either the $P_{exoS\text{-}gfp}$ (A, C, and E) or $P_{exsD\text{-}gfp}$ (B, D, and F) transcriptional reporter were cultured under inducing conditions at 37°C for 5 h and examined for GFP expression at the single-cell level by flow cytometry. The reported values are the relative numbers of cells (events on the *y* axis) at each level of GFP fluorescence as shown on the *x* axis. Horizontal bars indicate the fluorescence intensities gated as negative and positive for GFP fluorescence. The peak GFP fluorescence for the wt strain (indicated with an arrow in panels C and D) is also shown for reference for the *vfr* mutant (in panels E and F). The reported data are from a representative experiment.

Another potential role for Vfr in bistability could be a direct effect on *exsA* expression, which is controlled by the ExsA-dependent P_{exsC} promoter. To test this possibility, we generated a radiolabeled P_{exsC} promoter probe and tested for Vfr binding in an EMSA experiment. Although purified Vfr bound to the previously characterized promoter region for *regA* (33) (see lanes 11 to 13 in Fig. S2A in the supplemental material), binding to the P_{exsC} probe was not detected (see lanes 1 to 4 in Fig. S2A). We also considered the possibility that Vfr requires ExsA for binding. Whereas $ExsA_{His}$ bound to the P_{exsC} promoter probe, further addition of Vfr had no effect on $ExsA_{His}$ binding activity (see lanes 5 to 8 in Fig. S2). We concluded that Vfr does not regulate T3SS gene expression through direct effects on P_{exsC} promoter activity.

Identification of the P_{exsA} promoter. A recent RNA-seq study of *P. aeruginosa* strain PA14 grown at 37°C identified a transcript with a 5′ triphosphate originating in the *exsB-exsA* intergenic region (IR) (4). Prior to that finding, no promoters were known to exist in the IR. To independently verify the RNA-seq finding, we performed 5′ RACE with RNA isolated from strain PA103 cultured under inducing conditions for T3SS gene expression and identified the same transcriptional start site to within 2 nucleotides of the previous report (Fig. 4A and B). No additional start sites were mapped within the IR, suggesting that the region contains a single promoter (designated herein as P_{exsA}). The region upstream of the transcription start site has several putative −10 TATAAT boxes but lacks an obvious −35 box (Fig. 4B), suggesting a potential requirement for a transcriptional activator. In support of that conclusion, we found a strong match to the Vfr consensus-binding site that was centered at the −41 position (Fig. 4B).

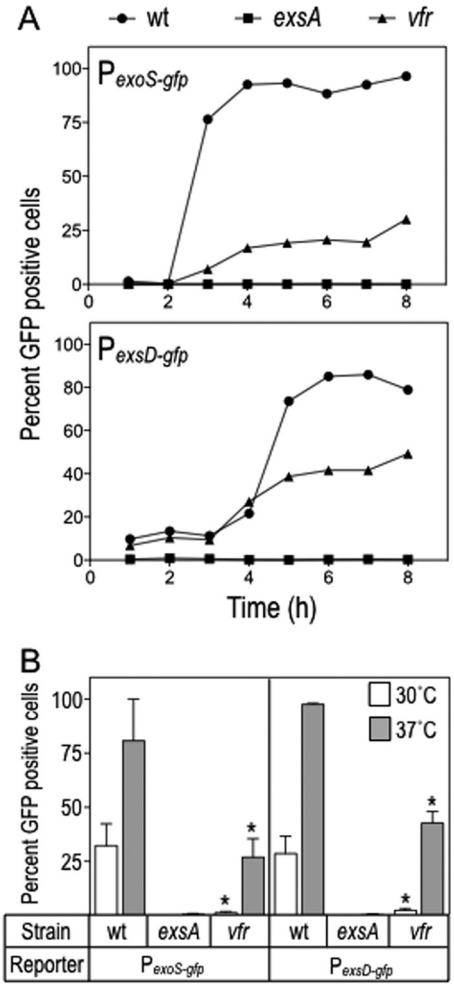

FIG 3 The bistable phenotype for T3SS gene expression is altered in the *vfr* mutant. (A) The indicated strains carrying plasmid-based $P_{exoS\text{-}gfp}$ or $P_{exsD\text{-}gfp}$ transcriptional reporters were cultured at 37°C from an initial A_{600} of 0.05. Samples were collected at the indicated times (1 to 8 h), diluted 100-fold into PBS, subjected to flow cytometry, and gated, as shown in Fig. 2. The percentage of cells demonstrating a positive GFP phenotype is reported for each time point. Data reported are from a representative experiment. (B) The indicated strains carrying plasmid-based $P_{exoS\text{-}gfp}$ or $P_{exsD\text{-}gfp}$ transcriptional reporters were cultured at 30°C or 37°C from an initial A_{600} of 0.05. After 5 h, culture aliquots were diluted 100-fold into PBS, subjected to flow cytometry, and scored for GFP-positive cells, as indicated in Fig. 2. The reported values are the means of the results of at least three experiments. *, $P < 0.0001$, when comparing the *vfr* mutant to the wt strain for each reporter and the corresponding growth temperature.

To determine whether the P_{exsA} promoter is controlled by Vfr, a transcriptional reporter ($P_{exsA\text{-}lacZ}$, −193 to +18 region relative to the P_{exsA} transcription start site [Fig. 4A]) was introduced into wt cells and the *vfr* mutant. Weak $P_{exsA\text{-}lacZ}$ reporter activity was observed in the wt background (Fig. 5A), and that activity was significantly reduced in the *vfr* deletion mutant. $P_{exsA\text{-}lacZ}$ reporter activities were similar under both noninducing and inducing conditions for T3SS gene expression. Because $P_{exsA\text{-}lacZ}$ reporter activity was low, we included an additional reporter lacking a cloned promoter and found that both the promoterless and $P_{exsA\text{-}lacZ}$ reporters had similar activities in the *vfr* mutant (Fig. 5A). Exogenous expression of *vfr* from a plasmid stimulated $P_{exsA\text{-}lacZ}$ reporter activity in both the wt and *vfr* backgrounds to levels higher than those seen in the wt strain carrying a vector control (Fig. 5B). This finding suggests that Vfr activity is limiting in wt cells. These combined data suggest that P_{exsA} reporter activity is entirely Vfr dependent and is not influenced by the addition of EGTA to the growth medium (T3SS-inducing conditions).

P_{exsA} promoter activity is not subject to autoregulatory control by ExsA. Previous studies concluded that ExsA autoregulates its own expression through activation of the P_{exsC} promoter and generation of a polycistronic mRNA for *exsCEBA* (Fig. 4A) (19). The discovery of the P_{exsA} promoter raised the possibility that the transcript originating from the P_{exsC} promoter terminates prior to reaching *exsA* and that *exsA* transcription is driven entirely from the P_{exsA} promoter. To test this possibility, we isolated RNA from wt and *exsA* mutant cells, generated cDNA, and performed a PCR using the primers outlined in Fig. 4C. The PCR primers were positioned upstream and downstream of the P_{exsA} transcription start site and should only have yielded a 255-bp product from transcripts originating upstream of P_{exsA}. The expected product was detected by PCR when using RNA isolated from wt cells but not from the *exsA* mutant cells (Fig. 6). These findings are consistent with previous data concluding that *exsCEBA* are transcribed as an operon from the ExsA-dependent P_{exsC} promoter. Nevertheless, it seemed possible that ExsA also influences P_{exsA} promoter activity as a further mechanism of autoregulation. This does not appear to be the case, however, as $P_{exsA\text{-}lacZ}$ reporter activity is not significantly altered in an *exsA* mutant (see Fig. S2B in the supplemental material) or under T3SS-inducing conditions (Fig. 5A), and ExsA binding to the P_{exsA} promoter probe alone or in combination with Vfr was not detected (see Fig. S2C in the supplemental material).

Vfr directly controls P_{exsA} promoter activity. To determine whether Vfr controls P_{exsA} promoter activity directly, we performed EMSA experiments with a radiolabeled P_{exsA} promoter probe and purified Vfr. Incubation of the P_{exsA} probe with increasing amounts of Vfr resulted in dose-dependent formation of a Vfr-P_{exsA} promoter probe complex (Fig. 7A, lanes 3 to 8). The apparent affinity for complex formation (apparent equilibrium binding constant [*K*eq], ~100 nM) is similar to those for other Vfr interactions that have been measured previously (11). The mobility of a nonspecific probe (derived from *pscF*) was not altered in the presence of Vfr, demonstrating that binding to the P_{exsA} probe is specific (Fig. 7A). Although the DNA binding activity of Vfr is usually cAMP dependent, Vfr binding to the *lasR* promoter region was previously shown to be cAMP independent (13). The cAMP requirement for P_{exsA} binding was examined using apo-Vfr, as previously described (13). Binding of apo-Vfr to the P_{exsA} probe was strictly dependent upon cAMP addition (Fig. 7A, compare lanes 9 and 10). The interaction of Vfr with the P_{exsA} probe, therefore, is typical of most Vfr promoter interactions in being cAMP dependent.

The location of the Vfr binding site in the P_{exsA} promoter region was determined by DNase I footprinting. In the presence of Vfr, a 24-bp area of protection was observed, and that region was centered on the predicted Vfr consensus-binding site (Fig. 4B and 7C). Furthermore, the nucleotides located in the core of the conserved regions demonstrated hypersensitivity to DNase I cleavage (Fig. 7C, indicated with an asterisk). This hypersensitivity pattern is a signature typical of Vfr binding interactions and likely results from Vfr-induced DNA bending (11, 13).

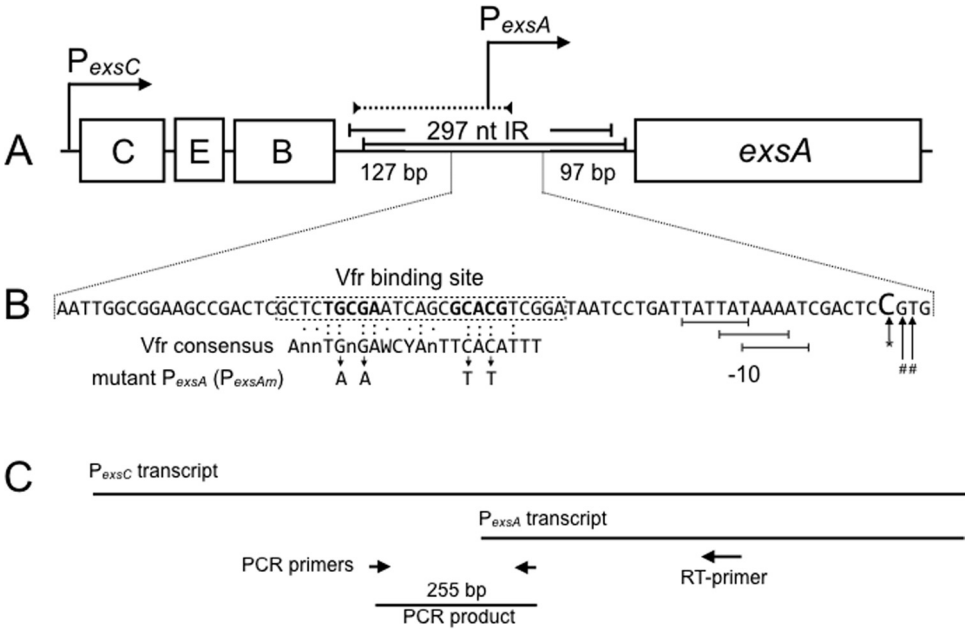

FIG 4 Diagram of the 297-bp *exsB-exsA* intergenic (IR) region and the P_{exsA} promoter region. (A) The promoter regions that control transcription of the *exsCEBA* operon (P_{exsC}) and *exsA* (P_{exsA}) are indicated by arrows. The dashed line with arrowheads at the ends corresponds to the region used to construct the $P_{exsA\text{-}lacZ}$ transcriptional reporter used in Fig. 5 and the EMSA/footprinting probes used in Fig. 6. (B) Sequence of the P_{exsA} promoter region showing the Vfr binding site mapped by DNase I footprinting (dashed rectangle), the match to the Vfr consensus-binding site, the nucleotide substitutions used to construct the mutant P_{exsAm} promoter, and the three potential −10 TATAAT boxes. The asterisk and number sign indicate the transcriptional start sites previously mapped by RNA-seq (4) and 5′ RACE in this study, respectively. (C) Strategy used to demonstrate that the transcript originating from the P_{exsC} promoter includes *exsA*. cDNA was generated from total cellular RNA using the reverse transcriptase (RT) primer (longer arrow) followed by PCR amplification using primers that spanned the P_{exsA} transcription start site (shorter arrows).

To further verify that Vfr interacts directly with the P_{exsA} promoter region, we generated a transcriptional reporter and a promoter probe, referred to as $P_{exsAm\text{-}lacZ}$ and P_{exsAm}, respectively, in which the Vfr consensus-binding site was mutagenized, as outlined in Fig. 4B. $P_{exsAm\text{-}lacZ}$ reporter activity was the same in both the wt and *vfr* mutant backgrounds and was similar to the activity seen for the wt $P_{exsA\text{-}lacZ}$ reporter when measured in the *vfr* mutant (Fig. 5A). In contrast to our findings with the $P_{exsA\text{-}lacZ}$ reporter, $P_{exsAm\text{-}lacZ}$ reporter activity was not stimulated by expressing *vfr* from a plasmid (Fig. 5B). Finally, binding of Vfr to the P_{exsAm} promoter probe could not be detected in EMSA experiments (Fig. 7B). These combined findings confirm that Vfr contributes to T3SS gene expression through a direct binding interaction with the P_{exsA} promoter region. To determine whether regulation of P_{exsA} promoter activity by Vfr fully accounts for reduced T3SS gene expression in the *vfr* deletion mutant, we introduced the same Vfr binding site mutations depicted in Fig. 4B onto the chromosome of the wt PA103 strain, resulting in strain BSM (for binding site mutant). ExsA-dependent $P_{exoS\text{-}lacZ}$ and $P_{exsD\text{-}lacZ}$ reporter activities were examined under T3SS-inducing growth conditions. Compared to the *vfr* mutant, the BSM strain had a reproducible reduction in $P_{exoS\text{-}lacZ}$ activity (though it did not reach significance) and a significant defect in $P_{exsD\text{-}lacZ}$ reporter activity (Fig. 8). These findings were contrary to our expectation that the BSM strain would phenocopy the *vfr* mutant. One possible explanation is that the mutations used to disrupt the Vfr binding site also interfere with the inherent basal activity of the promoter (i.e., Vfr-independent activity). The typical way to test this is to measure wt and mutant promoter activity in the absence of the activator (i.e., *vfr*). Unfortunately, the promoter is entirely devoid of activity in the absence of *vfr* (Fig. 5A), making it impossible to ascertain the effect of the mutations on inherent promoter activity. Thus, our data demonstrate that Vfr regulates T3SS gene expression through its effects on P_{exsA} promoter activity and suggest that an additional Vfr-controlled activity and/or an unknown function of the *exsB-exsA* intergenic region also contributes to T3SS gene expression.

DISCUSSION

The *exsCEBA* genes were originally characterized as an operon transcribed from an ExsA-dependent promoter located upstream of *exsC* (19). Data from two recent studies suggest that *exsA* transcription is more complex than previously appreciated and controlled by at least one additional promoter. The first study identified a transcript originating in the *exsB-exsA* intergenic region using an RNA-seq approach (4), and the second identified a VqsM-dependent promoter located in the same region (34). In the present study, we identified and characterized a Vfr-dependent promoter (P_{exsA}), also located within the *exsB-exsA* intergenic region. In 5′ RACE experiments, we identified two nucleotides as candidate transcription start sites for the P_{exsA} promoter (Fig. 4B). Those nucleotides are adjacent to the start site previously mapped by RNA-seq (4). Although it is unlikely that all three nucleotide represent authentic start sites, we conclude that they collectively represent the general start site for P_{exsA} transcription initiation. In support of this, there are several strong matches to −10 TATAAT boxes appropriately positioned just upstream of the candidate start sites (Fig. 4B). A start site for the VqsM-dependent promoter

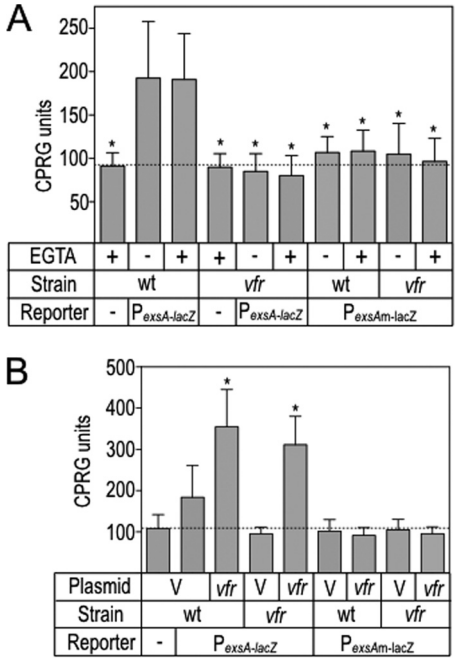

FIG 5 Vfr is required for P_{exsA} promoter activity. (A and B) The wt PA103 strain and the *vfr* mutant carrying either a promoterless *lacZ* transcriptional reporter (−), $P_{exsA\text{-}lacZ}$, or $P_{exsAm\text{-}lacZ}$ (containing a mutated Vfr binding site) were cultured at 37°C under noninducing (without EGTA) or inducing (with EGTA) conditions for T3SS gene expression and assayed for β-galactosidase activity. The experiments shown in panel B were conducted in the same manner under inducing conditions (with EGTA) but included a vector control (V) or a *vfr* expression vector (*vfr*). Arabinose (0.1%) was added to these cultures to induce *vfr* expression from the plasmids. Strains carrying the promoterless reporter were used to determine the background level of activity (indicated by the horizontal dashed lines). β-Galactosidase activity was measured using the fluorescent CPRG substrate. The indicated statistical differences (in panels A and B) are relative to the wt strain carrying the $P_{exsA\text{-}lacZ}$ reporter; *, $P < 0.01$.

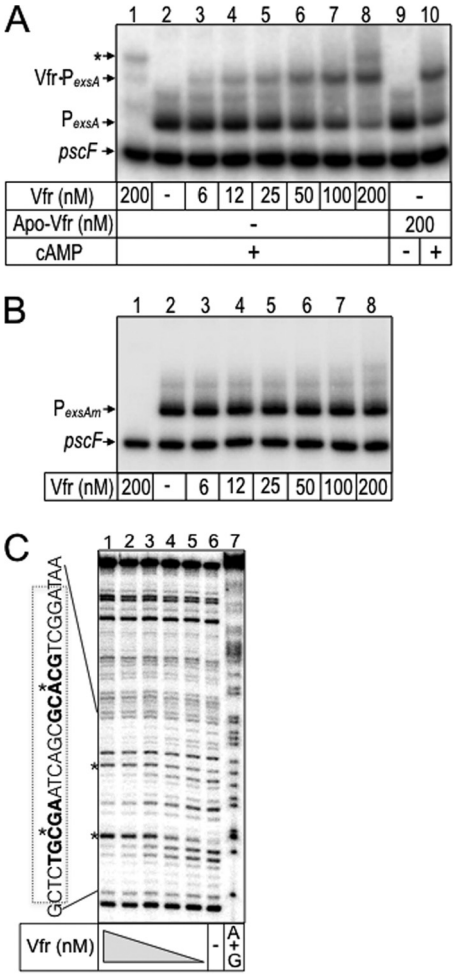

FIG 7 Vfr directly interacts with the P_{exsA} promoter. (A and B) Vfr or apo-Vfr was incubated with radiolabeled P_{exsA} (A) or P_{exsAm} (B) promoter probes and a nonspecific probe (*pscF*) for 15 min at 25°C. cAMP was added to the indicated samples (A) or to all of the samples (B). Arrows indicate the positions of unbound P_{exsA} (A) and P_{exsAm} (B) probes, the nonspecific *pscF* probe, and the Vfr-P_{exsA} promoter probe complex. The band indicated with an asterisk represents nonspecific formation of a Vfr-*pscF* complex when using >100 nM Vfr. (C) DNase I footprint of the P_{exsA} promoter region by Vfr (2-fold dilutions from 400 to 25 nM). The protected region (outlined with a dashed rectangle) includes a Vfr consensus-binding site, the core of which is indicated in bold. Sites hypersensitive to DNase I cleavage are indicated with asterisks. Lane 7 contains a Maxam-Gilbert A+G sequencing reaction.

was not determined in the previous study, but the VqsM binding site was mapped by DNase I footprinting (34). The mapped binding site, however, is centered ~12 bp downstream of the start sites mapped by 5′ RACE and RNA-seq. This position is inconsistent with VqsM having a positive effect on P_{exsA} promoter activity and suggests that the VqsM- and Vfr-dependent promoters are distinct from one another.

Transcriptional reporter assays showed that $P_{exsA\text{-}lacZ}$ reporter activity is significantly reduced in a *vfr* mutant compared to the parental strain (Fig. 5A). It is important to note that P_{exsA} promoter activity is quite weak (~200 CPRG units). Compared to a promoterless reporter, which served as a negative control, $P_{exsA\text{-}lacZ}$ reporter activity was only ~2-fold above the background level of activity (Fig. 5A). Disruption of the Vfr binding site reduced $P_{exsA\text{-}lacZ}$ reporter activity to background levels, leading us to conclude that P_{exsA} promoter activity is entirely Vfr dependent. The Vfr effect on P_{exsA} promoter activity results from a direct binding interaction, as determined in EMSA experiments (Fig. 6A) and DNase I cleavage assays (Fig. 6B), where Vfr protected the −52 to −27 portion of the promoter region (Fig. 4B). Binding of Vfr to this region of the promoter, coupled with the lack of a strong match to a −35 consensus site, is consistent with a mech-

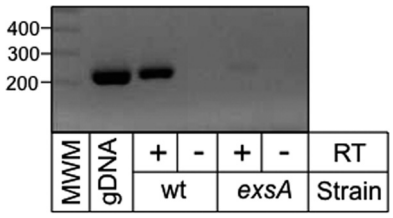

FIG 6 The transcript originating from the P_{exsC} promoter contains *exsA*. mRNA prepared from wt PA103 and the *exsA* mutant grown under inducing conditions (2 mM EGTA) for T3SS gene expression was converted to cDNA with reverse transcriptase, as shown in Fig. 4C. Primers were used to generate a 255-bp product that spanned the P_{exsA} transcription start site. Genomic DNA (gDNA) served as a positive control for the PCR amplification, and samples that were not treated with reverse transcriptase served as a negative control. MWM, DNA molecular size markers in base pairs.

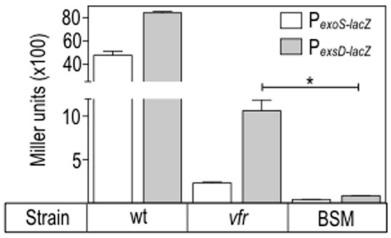

FIG 8 Disruption of the Vfr binding site on the chromosome prevents T3SS gene expression. *P. aeruginosa* strains PA103 (wt), the *vfr* mutant, and the *vfr* binding site mutant (BSM) carrying chromosomally integrated $P_{exoS\text{-}lacZ}$ or $P_{exsD\text{-}lacZ}$ transcriptional reporters were grown under inducing conditions (low Ca^{2+}, with EGTA) for T3SS gene expression at 37°C to an A_{600} of 1.0 and assayed for β-galactosidase activity. The data for the wt strain and the *vfr* mutant are the same as those shown in Fig. 1A. The reported values (Miller units) for all of the data in this figure are the means of results of at least three experiments. *, $P < 0.001$.

anism wherein Vfr stimulates P_{exsA} promoter activity by assisting in the recruitment of RNA polymerase.

Identification of the P_{exsA} promoter prompted us to reexamine the autoregulatory properties of ExsA. Although ExsA-dependent control of the P_{exsC} promoter has been well established (16, 19), evidence that the transcript originating from the P_{exsC} promoter includes *exsA* was lacking. Using a PCR approach, we identified a signal for an mRNA species that minimally spanned the P_{exsA} transcription start site and included *exsA* (Fig. 4C and 7). Consistent with previous reports that ExsA autoregulates its own expression, the mRNA was not detected in an *exsA* mutant. We took this as further evidence that *exsA* is included on a transcript originating upstream of the P_{exsA} promoter. Because no additional start sites were detected within the *exsCEB* coding regions in the previous RNA-seq experiment (4), the transcript likely is derived from the P_{exsC} promoter. Binding of ExsA to P_{exsA} promoter probe was not detected, and $P_{exsA\text{-}lacZ}$ reporter activity was not significantly altered in an *exsA* mutant. The relative contribution of P_{exsA} to the absolute level of *exsA* transcript is not entirely clear, but given the weak expression of $P_{exsA\text{-}lacZ}$ relative to the $P_{exsC\text{-}lacZ}$ reporter, a difference of at least 20-fold (data not shown), we propose that most *exsA*-containing mRNA in the cell is derived from the P_{exsC} promoter. Taken together, these data further support the model that ExsA autoregulates its own expression solely through activation of the P_{exsC} promoter.

Autoregulation of *exsA* expression by ExsA and the control of ExsA activity by the ExsCED regulatory cascade result in a finely balanced regulatory mechanism. The current working model is that ExsD prevents high levels of ExsA-dependent transcription under noninducing conditions (21). Inherent to the system seems to be a stochastic component wherein the negative regulatory activity of ExsD is relieved in only a fraction of the cells under inducing conditions. The latter feature likely accounts for the bistable phenotype and could be altered by factors that influence the expression, synthesis, and/or activity of ExsA. For example, factors such as osmolarity and the metabolic status of cells were previously shown to alter the bistable phenotype for T3SS gene expression (23). A key finding from the latter study was that the percentage of cells expressing the T3SS was positively correlated with intracellular levels of cAMP. Our findings that bistability is altered in the *vfr* mutant and that Vfr controls *exsA* expression are consistent with the idea that fluctuations in cAMP influence the bistable phenotype. Bistability does not appear to be an inherent property of Vfr-dependent control, however, because the $P_{lacP1\text{-}gfp}$ reporter demonstrated uniform expression in single-cell studies (see Fig. S1 in the supplemental material). Rather, Vfr-dependent stimulation of *exsA* expression likely alters the stochastic balance of the system, resulting in a larger proportion of wt cells than *vfr* mutant cells being positive for T3SS gene expression.

Two unresolved questions remain. First, aside from altering the bistable phenotype, *vfr* deletion also resulted in a 2- to 4-fold reduction in the fluorescence intensity of the $P_{exoS\text{-}gfp}$ and $P_{exsD\text{-}gfp}$ reporters (Fig. 2). Given the weak activity of the P_{exsA} promoter, we had expected that the Vfr requirement for *exsA* expression would be alleviated in cells induced for T3SS gene expression (i.e., GFP positive) owing to ExsA-dependent expression of the *exsCEBA* mRNA from the much stronger P_{exsC} promoter (Fig. 4A). The second question is why the BSM strain has a larger defect in $P_{exoS\text{-}lacZ}$ and $P_{exsD\text{-}lacZ}$ reporter activity than the *vfr* mutant (Fig. 5C). Several distinct roles have now been described for the *exsB-exsA* intergenic region. The region contains Vfr-and VqsM-dependent promoters (34) and contributes to posttranscriptional regulation of ExsA expression by the DeaD RNA-dependent helicase (35) and the small RNA binding protein RsmA (36). The nucleotide substitutions used to disrupt the Vfr binding site, therefore, may have also altered *exsCEBA* mRNA stability or posttranscriptional effects on ExsA synthesis or may have impaired the inherent activity of the P_{exsA} promoter (i.e., Vfr independent). Understanding the contributions of the *exsB-exsA* intergenic region to T3SS gene expression will be the subject of future studies.

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