A modular chromosomally integrated toolkit for ectopic gene expression in *Vibrio cholerae*

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**Abstract**

The ability to express genes ectopically in bacteria is essential for diverse academic and industrial applications. Two major considerations when utilizing regulated promoter systems for ectopic gene expression are (1) the ability to titrate gene expression by addition of an exogenous inducer and (2) the leakiness of the promoter element in the absence of the inducer. Here, we describe a modular chromosomally integrated platform for ectopic gene expression in *Vibrio cholerae*. We compare the broadly used promoter elements *P*ₜₐ₅ and *P*ₐ₅ₐ to versions that have an additional theophylline-responsive riboswitch (*P*ₜₐ₅-riboswitch and *P*ₐ₅ₐ-riboswitch). These constructs all exhibited unimodal titratable induction of gene expression, however, max induction varied with *P*ₜₐ₅ > *P*ₐ₅ₐ > *P*ₐ₅ₐ-riboswitch > *P*ₜₐ₅-riboswitch. We also developed a sensitive reporter system to quantify promoter leakiness and show that leakiness for *P*ₜₐ₅ > *P*ₜₐ₅-riboswitch > *P*ₐ₅ₐ; while the newly developed *P*ₐ₅ₐ-riboswitch exhibited no detectable leakiness. We demonstrate the utility of the tightly inducible *P*ₐ₅ₐ-riboswitch construct using the dynamic activity of type IV competence pili in *V. cholerae* as a model system. The modular chromosomally integrated toolkit for ectopic gene expression described here should be valuable for the genetic study of *Vibrio cholerae* and could be adapted for use in other species.

**Introduction**

Regulated promoter systems for ectopic gene expression have been widely used in bacterial systems. Two commonly employed system for ectopic gene expression are the isopropyl β-d-1-thiogalactopyranoside (IPTG)-inducible tac promoter (*P*ₜₐ₅) and the arabinose inducible araBAD promoter (*P*ₐ₅ₐ) (1, 2). Both of these systems, however, exhibit some degree of leakiness, which allows for gene expression in the absence of the inducer when cells are grown in rich LB medium. Leakiness of *P*ₐ₅ₐ can be reduced, to some extent, by addition of glucose to the
growth medium because this promoter is catabolite repressed (3), however, addition of glucose
to the growth medium can change the physiology of cells which may introduce a confounding
variable for some experiments.

Riboswitches are control elements that can regulate gene expression via direct interactions
between a small molecule ligand and mRNA. Synthetic riboswitches that are responsive to the
small molecule theophylline have recently been developed, which allow for regulated gene
expression in diverse biological systems (4, 5). These riboswitch elements likely fold the mRNA
to occlude the ribosome binding site in the absence of theophylline. And binding of theophylline
to the riboswitch alters the conformation of the mRNA to expose the ribosome binding site and
allow for translation of the downstream gene. For this reason, it is important to note that these
riboswitches likely have limited utility for controlling the expression of genetic elements like non-
coding RNAs, which do not need to be translated to exert their effect.

Generally, plasmids are employed for ectopic gene expression. However, many commonly used
plasmids are poorly maintained by *Vibrio* species and/or their copy number can vary relative to
model systems like *Escherichia coli* (6, 7). Integration of ectopic expression constructs onto the
genome can bypass these issues. For many *Vibrio* species (e.g. *Vibrio cholerae*, *Vibrio
natriegens*, *Vibrio campbellii*, *Vibrio vulnificus*, *Vibrio parahaemolyticus*, and *Vibrio fischeri*), it is
remarkably easy to integrate novel sequences into the bacterial genome by exploiting their
inherent capacity to undergo horizontal gene transfer by natural transformation (8-13), which
can be exploited for ectopic gene expression (10, 13-17).

Here, we generate a chromosomally integrated modular platform for ectopic gene expression in
*Vibrio* species based on the widely-used \( P_{\text{lac}} \) and \( P_{\text{BAD}} \) promoters in conjunction with a
previously described theophylline responsive riboswitch (5). We demonstrate that this toolkit
allows for differing levels of ectopic gene expression (i.e. max induction), and that one of these
promoter constructs (\( P_{\text{BAD}} \)-riboswitch) allows for a broad-range of titratable gene expression
without detectable leakiness. We highlight the utility of this tight expression construct to study
the dynamic surface appendages required for natural transformation in *V. cholerae*.

**Results & Discussion**

*Design of modular ectopic expression constructs*
All of the ectopic expression constructs are distinct ‘cassettes’ that can be integrated at any location in the bacterial genome (Fig. 1). We accomplish this via simple splicing by overlap extension (SOE) PCR (18) to stitch these expression cassettes to upstream and downstream regions of homology (see Fig. S1 for details) to generate linear PCR products that can then be integrated into the V. cholerae genome by chitin-induced natural transformation (8). Once an expression construct is integrated at a genomic locus, the gene of interest to be ectopically expressed can be easily exchanged by SOE PCR and natural transformation (see Fig. S1 for details). In this study, all ectopic expression constructs are integrated at the VCA0692 locus. This is a frame-shifted gene in the N16961 reference genome (19). Furthermore, the disruption of VCA0692 does not alter the fitness of V. cholerae during growth in rich medium or in environments this facultative pathogen encounters during its pathogenic life cycle (20), thus, highlighting this locus as a useful “neutral” location for the integration of novel sequences. These constructs can also be integrated at other commonly used “neutral” loci in V. cholerae including the lacZ gene, the frame-shifted transposase VC1807, or within intergenic spaces between convergently transcribed genes (21).

The constructs for ectopic gene expression have a modular design where all have a linked antibiotic resistance marker (AbR) (Fig. 1). This AbR facilitates selection during integration into the genome by natural transformation and can be easily altered depending on the need. Linked to this AbR, there are the gene control elements. For P\text{tac} and P\text{tac}-riboswitch constructs, this includes the Lac\text{Iq} repressor and \text{tac} promoter. By contrast the P\text{BAD} and P\text{BAD}-riboswitch constructs contain Ara\text{C} and the araBAD promoter. Both the P\text{tac} and P\text{BAD} promoter constructs can be engineered to have a user-defined ribosome binding site (Fig. S1). By contrast, the two riboswitch constructs (P\text{tac}-riboswitch and P\text{BAD}-riboswitch) contain a defined ribosome binding site within the theophylline-dependent riboswitch (riboswitch “E” in (5)) that is located immediately upstream of the gene of interest (Fig. 1 and Fig. S1).

Testing inducibility of ectopic expression constructs with GFP
To test whether these different chromosomally integrated constructs allow for inducible gene expression and to compare the maximum level of expression they support, we generated constructs for ectopic expression of gfp (22). The maximum level of gene expression varied among constructs with P\text{tac} > P\text{BAD} > P\text{BAD-riboswitch} > P\text{tac-riboswitch} (Fig. 2). Also, all of these constructs allowed for titratable gene expression (Fig. 2). This is particularly notable for P\text{BAD}, because this inducible system is known to have an “all-or-none” or autocatalytic gene
expression phenotype in wildtype strains of *E. coli* (23). This autocatalytic expression profile is
due to high affinity transport of arabinose in *E. coli*, which further stimulates increased
expression of arabinose transporters. Uncoupling this autoregulatory loop in *E. coli* can allow for
titratable gene expression (24). *V. cholerae* does not catabolize arabinose and lacks high affinity
arabinose transporters. Arabinose may be transported into *V. cholerae* nonspecifically through
one (or more) of its other carbohydrate transporters. Regardless, this low affinity transport of
arabinose in *V. cholerae* allows for titratable gene expression from *P<sub>BAD</sub>* (Fig. 2), which is
consistent with a number of previous studies (14, 25). Also, for constructs that contained the
additional riboswitch control element (*P<sub>lac</sub>*-riboswitch and *P<sub>BAD</sub>*-riboswitch), ectopic gene
expression was dependent on addition of theophylline (Fig. 2), as expected (5).

Testing ectopic expression constructs for the distribution of GFP fluorescence within single
cells.

While we observed titratable gene expression above (Fig. 2), this was assessed in bulk
cultures. Thus, titratable gene expression could be the result of bimodality in gene expression
where cells in the population exhibit either a highly fluorescent or poorly fluorescent phenotype
(similar to an ON/OFF light switch); and increased inducer simply results in a shift within the
population where a higher proportion of cells exhibit the highly fluorescent phenotype. This is in
contrast to titratable gene expression where the population responds uniformly to yield a
unimodal distribution where increased inducer simply shifts the fluorescence intensity of the
entire population (similar to a dimmer switch). Generally, for ectopic expression constructs the
latter phenotype is preferred. To distinguish between these possibilities, we assessed the
distribution of fluorescence among single cells within induced populations by epifluorescence
microscopy. In the absence of inducer, only the *P<sub>lac</sub>* construct exhibited detectable GFP
fluorescence (Fig. 2A and E), which is consistent with this construct being very leaky in *V.
cholerae*; a phenotype that is already widely appreciated. In the presence of inducer, all four
constructs exhibited unimodal distributions, which supports the latter model and suggests that
there is a uniform response to inducer among single cells within the population (Fig. 3).

Testing leakiness of ectopic expression constructs with *Flp* recombinase

A major consideration for ectopic expression constructs is leakiness, which is defined as the
basal expression of regulated genes in the absence of inducer. As mentioned above, only the
*P<sub>lac</sub>* construct exhibited detectable leakiness when using GFP fluorescence as a readout. This,
however, is a poor readout for leaky gene expression because a substantial amount of GFP
protein is required to generate an observable fluorescent readout. We sought to develop a
sensitive and easily measured phenotype for leakiness from our ectopic expression constructs.
To that end, we employed flippase (Flp), a highly-efficient recombinase that can mediate site-
specific recombination between two Flp recombinase target (FRT) sequences (26, 27). Flanking
FRT sequences can be engineered to leave behind an in-frame scar following Flp excision (28).
To generate a simple readout for Flp-mediated activity, we introduced a FRT-flanked AbR into
the lacZ gene in V. cholerae (Fig. 4A). Strains with lacZ::FRT-AbR-FRT yielded a white colony
phenotype on X-gal plates. Following Flp-mediated excision, however, the resulting lacZ gene
(containing an in-frame 81 bp insertion) is active, and strains harboring lacZ::FRT exhibit a blue
colony phenotype on X-gal plates (Fig. 4A). Thus, following Flp-mediated resolution, cells are
irreversibly converted from LacZ- (white colonies) to LacZ+ (blue colonies). To determine
whether our expression constructs exhibited leaky expression, we generated ectopic expression
constructs to drive Flp expression in strains that harbored the lacZ::FRT-AbR-FRT construct.
P_{lac}-Flp was so leaky that all cells where we introduced this construct resolved lacZ::FRT-AbR-
FRT to yield only blue colonies even in the absence of inducer. All of the other constructs (P_{lac}-
riboswitch-Flp, P_{BAD}-Flp, and P_{BAD}-riboswitch-Flp) yielded only resolved blue colonies when
grown in the presence of inducer. In the absence of inducer, both P_{lac}-riboswitch-Flp and P_{BAD}-
Flp exhibited some degree of leakiness, while P_{BAD}-riboswitch-Flp did not exhibit any detectable
leakiness in this assay (Fig. 4B). It is notable that the limit of detection of this assay is ~3 logs
below the leakiness observed from the P_{BAD} promoter, a construct that is traditionally considered
tightly repressed in the absence of inducer (2, 3). This further validates our Flp-based assay as
a highly sensitive approach to assess promoter leakiness.

Employing ectopic expression constructs to study the dynamic activity of type IV competence
pili in V. cholerae
Horizontal gene transfer by natural transformation in V. cholerae is dependent on type IV
competence pili (29, 30). These pili extend from the bacterial surface, bind to DNA in the
environment, and then retract to pull DNA across the outer membrane (29). This ingested DNA
can then be translocated into the cytoplasm and integrated into the bacterial genome by
homologous recombination. PilB is the motor ATPase that is required for extension of type IV
competence pili (31). To study the role of PilB in dynamic pilus activity, we sought to establish a
strain that allowed for titratable and tightly regulated control of pilus extension. To that end, we
generated strains where the native copy of pilB was deleted, and pilB expression was
ectopically driven by our expression constructs. We then tested whether these strains were
naturally transformable when grown without any inducer added. Only leaky expression of pilB would allow for natural transformation in this assay because ΔpilB mutants are not transformable (Fig. 5A). As observed using our Flp reporter readout, in the absence of inducer, P_{tac}, P_{tac-riboswitch}, and P_{BAD} all exhibited leaky expression of pilB as evidenced by detectable natural transformation, while there was no detectable leakiness observed for P_{BAD-riboswitch} because no transformants were obtained in this background without inducer added (Fig. 5A). Importantly, this experiment was performed in plain LB medium, thus, induction of catabolite repression (by the addition of glucose to the medium) was not necessary to prevent leaky gene expression. As expected, all strains transformed at high rates in the presence of inducer (Fig. 5A). These results suggest that leaky expression of pilB from P_{tac}, P_{BAD}, and P_{tac-riboswitch} allow for some degree of pilus assembly even without inducer added, while pilus assembly is completely inhibited in the P_{BAD-riboswitch} construct when no inducer is present. To test this idea further, we deleted the retraction ATPase pilT in these strains. This prevents extended pili from being easily retracted and sensitizes the direct observation of pilus assembly via a recently developed pilus labeling approach (32). To determine whether leaky pilB expression allowed for pilus assembly, we assessed piliation in strains that were grown in the absence of inducer. Indeed, for P_{tac}, P_{BAD}, and P_{tac-riboswitch}, we observed cells that contained extended pilus fibers (Fig. 5B), which is consistent with the leaky expression detected using our Flp recombinase reporter (Fig. 4B). We did not, however, observe extended pili in strains with P_{BAD-riboswitch} when grown without inducer (Fig. 5B), which is consistent with a lack of leaky expression for this construct (Fig. 4B). As expected, all strains generated extended pili when grown with the appropriate inducer (Fig. 5B).

Together, these data indicate that only our newly generated P_{BAD-riboswitch-pilB} construct allows for tightly regulated and titratable control of pilus biogenesis / extension in V. cholerae. This provides a valuable resource that will be critical for addressing diverse questions related to type IV pilus biology, which will be the focus of future work.

Methods

Bacterial strains and growth conditions

All strains used in this study are derivatives of E7946, an El Tor isolate of V. cholerae (33). See Table S1 for a complete list of strains used in this study. Strains were routinely grown at 30°C or 37°C in LB Miller broth and agar (BD Difco). When appropriate, media was supplemented with...
carbenicillin (20 µg/mL), spectinomycin (200 µg/mL), kanamycin (50 µg/mL), trimethoprim (10 µg/mL), chloramphenicol (1 µg/mL), or erythromycin (10 µg/mL).

Construction of mutant strains

All strains were generated by SOE PCR and chitin-induced natural transformation exactly as previously described (8, 21). See Fig. S1 for details on how the ectopic expression constructs were assembled and Table S2 for a detailed list of all primers used to generate all of the mutant constructs in this study. The araC – P_{BAD} region for our P_{BAD} and P_{BAD-riboswitch} constructs was amplified from pBAD18-Kan (3). The P_{tac}-riboswitch construct was amplified from DNA generously provided by Kim Seed (15).

GFP fluorescence assays

Cells harboring ectopic expression constructs driving gfp were grown rolling at 30°C to mid-log in LB medium. Then, inducer was added as indicated in each experiment and cells were grown for two additional hours rolling at 30°C. Next, cells were washed and resuspended to the same optical density in instant ocean medium (7 g/L; Aquarium Systems) and fluorescence was measured on a Biotek H1M plate reader: excitation 500 nm / emission 540 nm exactly as previously described (34). The parent strain lacking any ectopic expression construct was assayed alongside and used to subtract the background fluorescence of cells.

To image cells for GFP fluorescence, cultures were grown rolling at 30°C in LB medium in the presence of the indicated inducers for 5 hours to late-log. Then, cells were washed in instant ocean medium and mounted on 0.2% gelzan pads made with instant ocean medium exactly as previously described (35).

Flp recombinase assays

Cells harboring ectopic expression constructs driving Flp and lacZ::FRT-Spec^{R,-FRT} were struck out onto LB + X-gal (40 µg/mL) + spectinomycin (200 µg/mL) plates. Single white colonies were picked, inoculated into plain LB medium, and grown overnight rolling at 30°C. Then, each culture was plated quantitatively on LB+Xgal plates to determine the frequency of blue colonies within the population. Empirically, we determined that we could only reliably detect blue colonies at a rate of ~1 in 1,000,000 cells or 0.0001%. This equated to scoring for blue colonies on 100 µL spread plates of a dilution of 10^{-3} or greater. If no blue colonies were observed at 10^{-3}, we
assumed a single blue colony was present to define the limit of detection for that sample. Data are reported as the % Resolution = (CFU/mL blue colonies / CFU/mL total colonies) x 100.

Natural transformation assays

Cells harboring ectopic expression constructs driving pilB and the native pilB gene deleted were tested for rates of natural transformation. All of these strains also harbored P<sub>constitutive</sub>-tfoX and ∆luxO mutations, which rendered these strains constitutively competent. Strains were tested for natural transformation using chitin-independent transformation assays exactly as previously described (36). The transforming DNA using in these experiments was 100 ng of a 6 kb ∆VC1807::Erm<sup>R</sup> PCR product.

Pilus labeling

All strains harbored the indicated ectopic expression construct, a cysteine amino acid substitution in the major pilin subunit PilA (PilA<sup>S56C</sup>), a deletion of the native copy of pilB (ΔpilB), P<sub>constitutive</sub>-tfoX, a ∆luxO mutation, and a deletion of the retraction ATPase pilT (ΔpilT). Strains were grown and labeled with Alexa fluor 488-maleimide exactly as previously described (36). And mounted on 0.2% gelzan pads made with instant ocean medium.

Microscopy

Phase contrast and fluorescence images were collected on a Nikon Ti-2 microscope using a Plan Apo ×60 objective, a GFP filter cube, a Hamamatsu ORCAFlash 4.0 camera and Nikon NIS Elements imaging software. For Fig. 2E, the lookup tables for each phase and fluorescent image were adjusted to the same range so that they can be compared between samples. Fluorescence intensity of cells was determined using the MicrobeJ plugin (37) in Fiji (38).

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References
1. de Boer HA, Comstock LJ, Vasser M. 1983. The tac promoter: a functional hybrid derived from the trp and lac promoters. Proc Natl Acad Sci U S A 80:21-5.

2. Lee N, Francklyn C, Hamilton EP. 1987. Arabinose-induced binding of AraC protein to aral2 activates the araBAD operon promoter. Proc Natl Acad Sci U S A 84:8814-8.

3. Guzman LM, Belin D, Carson MJ, Beckwith J. 1995. Tight regulation, modulation, and high-level expression by vectors containing the arabinose PBAD promoter. J Bacteriol 177:4121-30.

4. Lynch SA, Gallivan JP. 2009. A flow cytometry-based screen for synthetic riboswitches. Nucleic Acids Res 37:184-92.

5. Topp S, Reynoso CM, Seeliger JC, Goldlust JS, Desai SK, Murat D, Shen A, Puri AW, Komeili A, Bertozzi CR, Scott JR, Gallivan JP. 2010. Synthetic riboswitches that induce gene expression in diverse bacterial species. Appl Environ Microbiol 76:7881-4.

6. Tschirhart T, Shukla V, Kelly EE, Schultzhaus Z, NewRingeisen E, Erickson JS, Wang Z, Garcia W, Curl E, Egbert RG, Yeung E, Vora GJ. 2019. Synthetic Biology Tools for the Fast-Growing Marine Bacterium Vibrio natriegens. ACS Synth Biol 8:2069-2079.

7. Delavat F, Bidault A, Pichereau V, Paillard C. 2018. Rapid and efficient protocol to introduce exogenous DNA in Vibrio harveyi and Pseudoalteromonas sp. J Microbiol Methods 154:1-5.

8. Dalia AB. 2018. Natural Cotransformation and Multiplex Genome Editing by Natural Transformation (MuGENT) of Vibrio cholerae. Methods Mol Biol 1839:53-64.

9. Simpson CA, Podicheti R, Rusch DB, Dalia AB, van Kessel JC. 2019. Diversity in Natural Transformation Frequencies and Regulation across Vibrio Species. mBio 10.

10. Dalia TN, Hayes CA, Stolyar S, Marx CJ, McKinlay JB, Dalia AB. 2017. Multiplex Genome Editing by Natural Transformation (MuGENT) for Synthetic Biology in Vibrio natriegens. ACS Synth Biol 6:1650-1655.

11. Chimalapati S, de Souza Santos M, Servage K, De Nisco NJ, Dalia AB, Orth K. 2018. Natural Transformation in Vibrio parahaemolyticus: a Rapid Method To Create Genetic Deletions. J Bacteriol 200.

12. Brooks JF, 2nd, Gyllborg MC, Kocher AA, Markey LE, Mandel MJ. 2015. TfoX-based genetic mapping identifies Vibrio fischeri strain-level differences and reveals a common lineage of laboratory strains. J Bacteriol 197:1065-74.

13. Visick KL, Hodge-Hanson KM, Tischler AH, Bennett AK, Mastrodomenico V. 2018. Tools for Rapid Genetic Engineering of Vibrio fischeri. Appl Environ Microbiol 84.

14. Lo Scrudato M, Blokesch M. 2012. The regulatory network of natural competence and transformation of Vibrio cholerae. PLoS Genet 8:e1002778.

15. McKitterick AC, Seed KD. 2018. Anti-phage islands force their target phage to directly mediate island excision and spread. Nat Commun 9:2348.

16. Borgeaud S, Blokesch M. 2013. Overexpression of the tcp gene cluster using the T7 RNA polymerase/promoter system and natural transformation-mediated genetic engineering of Vibrio cholerae. PLoS One 8:e53952.

17. Dalia AB, Lazinski DW, Camilli A. 2014. Identification of a membrane-bound transcriptional regulator that links chitin and natural competence in Vibrio cholerae. MBio 5:e01028-13.
Horton RM, Hunt HD, Ho SN, Pullen JK, Pease LR. 1989. Engineering hybrid genes without the use of restriction enzymes: gene splicing by overlap extension. Gene 77:61-8.

Heidelberg JF, Eisen JA, Nelson WC, Clayton RA, Gwinn ML, Dodson RJ, Haft DH, Hickey EK, Peterson JD, Umayam L, Gill SR, Nelson KE, Read TD, Tettelin H, Richardson D, Ermolaeva MD, Vamathevan J, Bass S, Qin H, Dragoi I, Sellers P, McDonald L, Utterback T, Fleishmann RD, Nierman WC, White O, Salzberg SL, Smith HO, Colwell RR, Mekalanos JJ, Venter JC, Fraser CM. 2000. DNA sequence of both chromosomes of the cholera pathogen Vibrio cholerae. Nature 406:477-83.

Kamp HD, Patimalla-Dipali B, Lazinski DW, Wallace-Gadsden F, Camilli A. 2013. Gene fitness landscapes of Vibrio cholerae at important stages of its life cycle. PLoS Pathog 9:e1003800.

Dalia AB, McDonough E, Camilli A. 2014. Multiplex genome editing by natural transformation. Proc Natl Acad Sci U S A 111:8937-42.

Cormack BP, Valdivia RH, Falkow S. 1996. FACS-optimized mutants of the green fluorescent protein (GFP). Gene 173:33-8.

Siegele DA, Hu JC. 1997. Gene expression from plasmids containing the araBAD promoter at subsaturating inducer concentrations represents mixed populations. Proc Natl Acad Sci U S A 94:8168-72.

Khlebnikov A, Risa O, Skaug T, Carrier TA, Keasling JD. 2000. Regulatable arabino-inducible gene expression system with consistent control in all cells of a culture. J Bacteriol 182:7029-34.

Judson N, Mekalanos JJ. 2000. TnAraOut, a transposon-based approach to identify and characterize essential bacterial genes. Nat Biotechnol 18:740-5.

Broach JR, Guarascio VR, Jayaram M. 1982. Recombination within the yeast plasmid 2mu circle is site-specific. Cell 29:227-34.

Cox MM. 1983. The FLP protein of the yeast 2-microns plasmid: expression of a eukaryotic genetic recombination system in Escherichia coli. Proc Natl Acad Sci U S A 80:4223-7.

Baba T, Ara T, Hasegawa M, Takai Y, Okumura Y, Baba M, Datsenko KA, Tomita M, Wanner BL, Mori H. 2006. Construction of Escherichia coli K-12 in-frame, single-gene knockout mutants: the Keio collection. Mol Syst Biol 2:2006 0008.

Ellison CK, Dalia TN, Vidal Ceballos A, Wang JC, Biais N, Brun YV, Dalia AB. 2018. Retraction of DNA-bound type IV competence pili initiates DNA uptake during natural transformation in Vibrio cholerae. Nat Microbiol 3:773-780.

Seitz P, Blokesch M. 2013. DNA-uptake machinery of naturally competent Vibrio cholerae. Proc Natl Acad Sci U S A 110:17987-92.

Ellison CK, Kan J, Chlebek JL, Hummels KR, Panis G, Violler PH, Biais N, Dalia AB, Brun YV. 2019. A bifunctional ATPase drives tad pilus extension and retraction. Sci Adv 5:eaa2591.

Ellison CK, Dalia TN, Dalia AB, Brun YV. 2019. Real-time microscopy and physical perturbation of bacterial pili using maleimide-conjugated molecules. Nat Protoc doi:10.1038/s41596-019-0162-6:In Press.
33. Miller VL, DiRita VJ, Mekalanos JJ. 1989. Identification of toxS, a regulatory gene whose product enhances toxR-mediated activation of the cholera toxin promoter. J Bacteriol 171:1288-93.

34. Dalia AB. 2016. RpoS is required for natural transformation of Vibrio cholerae through regulation of chitinases. Environ Microbiol 18:3758-3767.

35. Dalia AB, Dalia TN. 2019. Spatiotemporal Analysis of DNA Integration during Natural Transformation Reveals a Mode of Nongenetic Inheritance in Bacteria. Cell 179:1499-1511 e10.

36. Chlebek JL, Hughes HQ, Ratkiewicz AS, Rayyan R, Wang JC, Herrin BE, Dalia TN, Biais N, Dalia AB. 2019. PilT and PilU are homohexameric ATPases that coordinate to retract type IVa pili. PLoS Genet 15:e1008448.

37. Ducret A, Quardokus EM, Brun YV. 2016. MicrobeJ, a tool for high throughput bacterial cell detection and quantitative analysis. Nat Microbiol 1:16077.

38. Schindelin J, Arganda-Carreras I, Frise E, Kaynig V, Longair M, Pietzsch T, Preibisch S, Rueden C, Saalfeld S, Schmid B, Tinevez JY, White DJ, Hartenstein V, Eliceiri K, Tomancak P, Cardona A. 2012. Fiji: an open-source platform for biological-image analysis. Nat Methods 9:676-82.
Fig. 1 – Diagram of ectopic expression constructs. The four ectopic expression constructs characterized in this study are indicated. All have a linked antibiotic resistance cassette (Ab\textsuperscript{R}) to facilitate selection during integration into the genome by natural transformation. The gene encoding a transcription factor (lac\textit{Iq} or ara\textit{C}) and the promoter (\textit{P}\text{\textsubscript{tac}} or \textit{P}\text{\textsubscript{BAD}}) required for inducible gene regulation are indicated. The presence of a theophylline-dependent riboswitch is demarcated by a loop before the gene of interest (\textit{gene x}). For details on how these constructs were assembled, see Fig. S1.
Fig. 2 – Testing inducibility of ectopic expression constructs with GFP. (A-D) Cells harboring ectopic expression constructs driving gfp integrated at the VCA0692 locus were grown with inducer as indicated and assessed for GFP fluorescence in bulk cultures on a plate reader. (A) Cells with a P_{bad}-gfp construct were grown with the indicated amount of IPTG. (B) Cells with a P_{bad}-gfp construct were grown with the indicated amount of arabinose. (C) Cells with a P_{tac}-riboswitch construct were grown with increasing doses (denoted by a triangle below bars) of IPTG (from left to right: 0 µM, 1 µM, 5 µM, 25 µM, 125 µM, 500 µM) or theophylline (from left to right: 0 mM, 0.018 mM, 0.054 mM, 0.16 mM, 0.5 mM, 1.5 mM). 'Max' below bars denotes that cells were incubated with the highest concentration of IPTG (500 µM) or theophylline (1.5 mM) as indicated, while 'none' indicates that none of that inducer was added. (D) Cells with a P_{bad}-riboswitch construct were grown with increasing doses (denoted by a triangle below bars) of arabinose (from left to right: 0%, 0.0016%, 0.008%, 0.04%, 0.2%, 1%) or theophylline (from left to right: 0 mM, 0.018 mM, 0.054 mM, 0.16 mM, 0.5 mM, 1.5 mM). 'Max' below bars denotes that cells were incubated with the highest concentration of arabinose (1%) or theophylline (1.5 mM) as indicated, while 'none' indicates that none of that inducer was added. All data in A-D are from at least two independent biological replicates and shown as the mean ± SD. (E) Representative phase and epifluorescence images of cells with the indicated ectopic expression construct grown without any inducer added. Scale bar, 4 µM.
Fig. 3 – Testing ectopic expression constructs for the distribution of GFP fluorescence within single cells. Cells harboring the indicated ectopic expression constructs were grown with inducer as indicated and assessed for GFP fluorescence in single cells via epifluorescence microscopy (Theo = theophylline; Ara = arabinose). The distribution of fluorescence among cells in the population is indicated on the plotted histograms. Data are from >1000 cells per condition tested and representative of two independent experiments.
**Fig. 4 – Testing leakiness of ectopic expression constructs with Flp recombinase.** (A) Diagram of the approach used to test leakiness in gene expression with Flp recombinase. Wildtype *V. cholerae* cells have intact *lacZ* and form blue colonies on X-gal plates (top). Cells with *lacZ*::FRT-Ab<sup>R</sup>-FRT have inactive LacZ and are white on X-gal plates (middle). Flp recombination resolves the FRT-Ab<sup>R</sup>-FRT cassette within *lacZ* (making *lacZ*::FRT), which restores LacZ activity (bottom). (B) Single colonies of cells harboring the indicated ectopic expression constructs integrated at the VCA0692 locus and *lacZ*::FRT-Ab<sup>R</sup>-FRT were grown in LB medium without any inducer overnight. Then, % resolution of *lacZ*::FRT-Ab<sup>R</sup>-FRT was determined by plating for quantitative culture on X-gal plates. Percent resolution is defined as the number of blue colonies / total CFUs. Data in B are from 6 independent biological replicates and shown as the mean ± SD. Statistical comparisons were made by one-way ANOVA with Tukey’s post-test. LOD, limit of detection, *** = *P* < 0.001.
Fig. 5 – Employing ectopic expression constructs to study the type IV competence pilus extension ATPase PilB. (A) Natural transformation of the indicated strains was tested. All ectopic expression constructs in these strains were integrated at the VCA0692 locus. IPTG (100 µM), arabinose (0.2%), and/or theophylline (1.5 mM) were added to reactions as indicated below each bar. Data are from at least 3 independent biological replicates and shown as the mean ± SD. Statistical comparisons were made by one-way ANOVA with Tukey’s post-test. LOD, limit of detection, *** = P < 0.001. (B) Representative images of surface piliation. All strains harbor ΔpilB and ΔpilT mutations at the native locus and the indicated ectopic pilB expression construct. Where indicated, cells were grown with inducer as follows: 100 µM IPTG for P_{tac}, 0.2% arabinose for P_{BAD}, 100 µM IPTG + 1.5 mM theophylline for P_{tac}-riboswitch, and 0.2% arabinose + 1.5 mM theophylline for P_{BAD}-riboswitch. Examples of extended pili in no inducer samples are indicated by white arrows. Scale bars, 1 µm.
Supporting Information for

A modular chromosomally integrated toolkit for ectopic gene expression in *Vibrio cholerae*

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  - Fig. S1
  - Tables S1-S2
**Fig. S1 - Details on how to assemble the ectopic expression constructs and swap out genes of interest.** A generic diagram of the ectopic expression construct is shown with the location of different primers needed to initially construct each expression construct (P1, P2, P3, P6, P7, and P8). As well as the primers to insert novel genes of interest into established expression constructs (P1, P4, P5, P6, P7, and P8). Ab^R_ = antibiotic resistance cassette, reg = regulatory gene (lacIq or araC), P_x_ = the promoter (P_{tac}, P_{BAD}, P_{tac}-riboswitch, or P_{BAD}-riboswitch), and gene X = the gene of interest.

**Establishing ectopic expression constructs at new genomic locations:** The UP arm of homology is generated with P1 and P2, while the DOWN arm of homology is amplified with P7 and P8. In this example, these primers amplify homology arms to integrate the ectopic expression constructs into *V. cholerae* ChII. P2 and P7 contain tails that overlap any of the 4 ectopic expression constructs. To move intact expression constructs to new locations in the genome, they can be amplified with P3 and P6. This can serve as a MIDDLE arm to stitch to the UP and DOWN arms of homology.

**To insert new genes of interest:**
Once an ectopic expression construct has been introduced to a genetic locus, the gene of interest can be swapped out.
-To amplify a gene of interest to place within $P_{\text{tac}}$-riboswitch or $P_{\text{BAD}}$-riboswitch the forward P5-1 primer must have a 5’ overlap region as indicated. Immediately after this overlap should be the start codon for the gene of interest (highlighted in green) + additional sequence to serve as a primer for the gene of interest (denoted by ‘XXXXXX’). P5-1 overlaps with P4-1 (blue highlighted sequence), which sits within the theophylline-dependent riboswitch. Thus, the same overlap can be used to make either $P_{\text{tac}}$-riboswitch or $P_{\text{BAD}}$-riboswitch constructs. The reverse P6 primer for the gene of interest must have the tail indicated, which should be immediately followed by the stop codon of the gene (highlighted in purple – TAA stop codon in this example) + additional sequence to serve as a primer for the gene of interest (denoted by ‘YYYYYY’).

-To amplify a gene of interest to place within $P_{\text{tac}}$ or $P_{\text{BAD}}$ the forward P5-2 primer must have the 5’ overlap region indicated. Immediately after this overlap should be the desired ribosome binding site (indicated in bold and underline – the ‘optimal’ RBS AGGAGGT is used in this example), which should be followed by a 6 bp spacer (this spacing between the ribosome binding site and the start codon is essential for optimal translation and can be derived from the native gene) + the start codon (highlighted in green) + additional sequence to serve as a primer for the gene of interest (denoted by ‘XXXXXX’). P5-2 overlaps with P4-2 (blue highlighted sequence), which sits downstream of the two promoter elements. Thus, the same overlap can be used to make either $P_{\text{tac}}$ or $P_{\text{BAD}}$ constructs. The reverse P6 primer for the gene of interest can be made exactly as described above.

All amplified genes of interest serve as MIDDLE arms in SOE reactions with an UP arm amplified with P1 and P4, and a DOWN arm amplified with P7 and P8.
| Name | Relevant figure(s) | Full genotype | Reference / Strain# |
|------|-------------------|----------------|---------------------|
| parent | Fig. 2E | E7946 lacZ::FRT-SpecR::FRT | This study / SAD035 |
| \( P_{\text{lac-gfp}} \) | Fig. 2A, E; Fig. 3 | E7946 lacZ::FRT-SpecR::FRT, \( \Delta VCA0692::P_{\text{lac-gfp}} \) CarbR | This study / TND2290 (SAD2780) |
| \( P_{\text{BAD-gfp}} \) | Fig. 2B, E; Fig. 3 | E7946 lacZ::FRT-SpecR::FRT, \( \Delta VCA0692::P_{\text{BAD-gfp}} \) CarbR | This study / TND2291 (SAD2781) |
| \( P_{\text{lac-riboswitch-gfp}} \) | Fig. 2C, E; Fig. 3 | E7946 lacZ::FRT-SpecR::FRT, \( \Delta VCA0692::P_{\text{lac-riboswitch-gfp}} \) CarbR | This study / TND2293 (SAD2782) |
| \( P_{\text{BAD-riboswitch-gfp}} \) | Fig. 2D, E; Fig. 3 | E7946 lacZ::FRT-SpecR::FRT, \( \Delta VCA0692::P_{\text{BAD-riboswitch-gfp}} \) CarbR | This study / TND2292 (SAD2783) |
| wildtype | Fig. 4A | E7946 | (25) / SAD031 |
| \( P_{\text{BAD-Flp}} \) | Fig. 4A-B | E7946 lacZ::FRT-SpecR::FRT, \( \Delta VCA0692::P_{\text{BAD-Flp}} \) CarbR | This study / TND2083 (SAD2784) |
| \( P_{\text{lac-riboswitch-Flp}} \) | Fig. 4B | E7946 lacZ::FRT-SpecR::FRT, \( \Delta VCA0692::P_{\text{lac-riboswitch-Flp}} \) CarbR | This study / TND2289 (SAD2785) |
| \( P_{\text{BAD-riboswitch-Flp}} \) | Fig. 4B | E7946 lacZ::FRT-SpecR::FRT, \( \Delta VCA0692::P_{\text{BAD-riboswitch-Flp}} \) CarbR | This study / TND2086 (SAD2786) |
| \( \Delta \text{pilB} \) | Fig. 5A | E7946 SmR, \( P_{\text{const-tfoX}}, \Delta \text{luxO}, \text{pilA} \ S67C, \text{lacZ}::\text{FRT-KanR-FRT}, \Delta VCA1807::\text{CmR}, \Delta \text{pilB} \) | This study / TND2373 (SAD2787) |
| \( P_{\text{lac-pilB}} \Delta \text{pilB} \) | Fig. 5A | E7946 SmR, \( P_{\text{const-tfoX}}, \Delta \text{luxO}, \text{pilA} \ S67C, \Delta \text{lacZ}::P_{\text{lac-pilB}} \text{SpecR}, \Delta VCA1807::\text{CmR}, \Delta \text{pilB} \) | This study / JLC769 (SAD2788) |
| \( P_{\text{BAD-pilB}} \Delta \text{pilB} \) | Fig. 5A | E7946 SmR, \( P_{\text{const-tfoX}}, \Delta \text{luxO}, \text{pilA} \ S67C, \text{lacZ}::\text{FRT-KanR-FRT}, \Delta VCA1807::\text{CmR}, \Delta \text{pilB}, \Delta VCA0692::P_{\text{BAD-pilB}} \text{CarbR} \) | This study / TND2043 (SAD2789) |
| \( P_{\text{lac-riboswitch-pilB}} \Delta \text{pilB} \) | Fig. 5A | E7946 SmR, \( P_{\text{const-tfoX}}, \Delta \text{luxO}, \text{pilA} \ S67C, \text{lacZ}::\text{FRT-KanR-FRT}, \Delta VCA1807::\text{CmR}, \Delta \text{pilB}, \Delta VCA0692::P_{\text{lac-riboswitch-pilB}} \text{CarbR} \) | This study / TND2402 (SAD2790) |
| \( P_{\text{BAD-riboswitch-pilB}} \Delta \text{pilB} \) | Fig. 5A | E7946 SmR, \( P_{\text{const-tfoX}}, \Delta \text{luxO}, \text{pilA} \ S67C, \text{lacZ}::\text{FRT-KanR-FRT}, \Delta VCA1807::\text{CmR}, \Delta \text{pilB}, \Delta VCA0692::P_{\text{BAD-riboswitch-pilB}} \text{CarbR} \) | This study / TND2378 (SAD2791) |
| \( P_{\text{lac-pilB}} \Delta \text{pilT} \) | Fig. 5B | E7946 SmR, \( P_{\text{const-tfoX}}, \Delta \text{luxO}, \text{pilA} \ S67C, \Delta \text{lacZ}::P_{\text{lac-pilB}} \text{SpecR}, \Delta VCA1807::\text{CmR}, \Delta \text{pilB}, \Delta \text{pilT}::\text{ZeoR} \) | This study / TND2498 |
| \( P_{\text{BAD-pilB}} \Delta \text{pilT} \) | Fig. 5B | E7946 SmR, \( P_{\text{const-tfoX}}, \Delta \text{luxO}, \text{pilA} \ S67C, \text{lacZ}::\text{FRT-KanR-FRT}, \Delta VCA1807::\text{CmR}, \Delta \text{pilB}, \Delta VCA0692::P_{\text{BAD-pilB}} \text{CarbR}, \Delta \text{pilT}::\text{ZeoR} \) | This study / TND2501 |
| Strain Configuration | Fig. | Description |
|----------------------|------|-------------|
| \( P_{\text{tac}} \)-riboswitch-\( \Delta \)pilB \( \Delta \)pilT | 5B   | E7946 Sm\(^R\), \( P_{\text{const}} \)-tfoX, \( \Delta \)luxO, pilA S67C, lacZ::FRT-Kan\(^R\)-FRT, \( \Delta \)VC1807::CmR, \( \Delta \)pilB, \( \Delta \)VCA0692::\( P_{\text{tac}} \)-riboswitch-\( \Delta \)pilB Carb\(^R\), \( \Delta \)pilT::Zeo\(^R\) | This study / TND2500 |
| \( P_{\text{BAD}} \)-riboswitch-\( \Delta \)pilB \( \Delta \)pilT | 5B   | E7946 Sm\(^R\), \( P_{\text{const}} \)-tfoX, \( \Delta \)luxO, pilA S67C, lacZ::FRT-Kan\(^R\)-FRT, \( \Delta \)VC1807::CmR, \( \Delta \)pilB, \( \Delta \)VCA0692::\( P_{\text{BAD}} \)-riboswitch-\( \Delta \)pilB Carb\(^R\), \( \Delta \)pilT::Zeo\(^R\) | This study / TND2499 |
### Table S2 – Primers used in this study

| Primer Name | Primer Sequence (5’→3’) | Description |
|-------------|--------------------------|-------------|
| BBC832      | GCTTTTTGCTACAACGACCG     | Replace VCA0692 w/ an ectopic expression construct UP ARM F1 (aka P1 in **Fig. S1**) |
| BBC828      | caccataccacgccgaaccaACGTGATGTACCGAATCGGAC | Replace VCA0692 w/ an ectopic expression construct UP ARM R1 (aka P2 in **Fig. S1**) |
| BBC830      | gaagcagctacagcactaGTTGAGTTGGATGCACGCACC | Replace VCA0692 w/ an ectopic expression construct DOWN ARM F2 (aka P7 in **Fig. S1**) |
| BBC834      | CACAATTCTCCTGTTAAATGTCC  | Replace VCA0692 w/ an ectopic expression construct DOWN ARM R2 (aka P8 in **Fig. S1**) |
| BBC243      | TTGTTTCGCGCGGGGATGATGGTG | Amplify any ectopic expression construct to move to a new genetic locus F (aka P3 in **Fig. S1**) |
| BBC203      | CCCGGGATCCTGTGTGAAATTG   | Primer to clone new ectopic genes into P_tac or P_BAD constructs R1 (aka P4-2 in **Fig. S1**) |
| BBC2137     | CTTGTTTCTACCTCCTTAGCAGG  | Primer to clone new ectopic genes into P_tac-riboswitch or P_BAD-riboswitch constructs R1 (aka P4-1 in **Fig. S1**) |
| BBC252      | caatttcacacaggtccggAGGAGGTAACGTAATCGCGTAAGAGGAGAAGAAC | F Primer to amplify up gfp to clone into P_tac or P_BAD constructs (aka P5-2 in **Fig. S1**) |
| BBC2182     | cctgtaaggaggttaacacaagATGCCTGGTTAAGGAGAAGAAGAATTTTTCAC | F Primer to amplify up gfp to clone into P_tac-riboswitch or P_BAD-riboswitch constructs (aka P5-1 in **Fig. S1**) |
| BBC254      | tgtaggctgagctctTTAGTTGTATAGGCTCATGCG | R Primer to amplify up gfp to clone into any ectopic expression construct (aka P6 in **Fig. S1**) |
| BBC2427     | caatttcacacaggtccggAGGAGGTTTTTGTATGGCACAATTGGATATATTAG | F Primer to amplify up Flp to clone into P_tac or P_BAD constructs (aka P5-2 in **Fig. S1**) |
| BBC2455     | cctgtaaggaggttaacacaagATGCCACAATTGGATATATTGAAAC | F Primer to amplify up Flp to clone into P_tac-riboswitch or P_BAD-riboswitch constructs (aka P5-1 in **Fig. S1**) |
| BBC2428     | tgtaggctgagctctTTATATGCGTCTATTTATGTAGGATG | R Primer to amplify up Flp to clone into any ectopic expression construct (aka P6 in **Fig. S1**) |
| BBC1952     | caatttcacacaggtccggAGGAGGTTAAGGACTAATGCCTCACAACGTGGTTG | F Primer to amplify up pilB to clone into P_tac or P_BAD constructs (aka P5-2 in **Fig. S1**) |
| BBC2806     | cctgtaaggaggttaacacaagATGCCTCACCACCATGTTGC | F Primer to amplify up pilB to clone into P_tac-riboswitch or P_BAD-riboswitch constructs (aka P5-1 in **Fig. S1**) |
|   | Sequence                                              | Description                                      |
|---|-------------------------------------------------------|--------------------------------------------------|
| BBC1953 | tgtaggctggagctgccTTAAAAGTAGAGCACACGCTG              | R Primer to amplify up pilB to clone into any ectopic expression construct (aka P6 in **Fig. S1**) |
| ABD253  | GCGAACCACCACCGATGGG                                   | lacZ::FRT-AbR-FRT F1                           |
| ABD263  | gtggacggatccgggaatatAACTGATCCAATTTTTTCAGCGCATATTTTG   | lacZ::FRT-AbR-FRT R1                           |
| ABD262  | gaagcagctccagctacaTGCCGAGGAAAACC GCCCCCTCTaATC        | lacZ::FRT-AbR-FRT F2                           |
| ABD256  | CCCAAATACGGCAGAACTTGGCG                               | lacZ::FRT-AbR-FRT R2                           |
| ABD123  | ATTCGGGGGATCCGTCGAC                                   | Amplify any AbR cassette F                       |
| ABD124  | TGTAGGCTGGAGGCTTGCTTC                                  | Amplify any AbR cassette R                       |