Original Research Paper

Generation and comparison of broad-host range inducible expression vectors for use in Gram-negative bacteria including ESKAPE pathogens

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Abstract: The objective of this study is a controlled comparison of several carbohydrate inducible promoter in important bacterial species. Inducible promoter systems are invaluable for biotechnology and basic science applications. However, few inducible promoters are available on plasmids that replicate in *Saccharomyces cerevisiae,* which enables gap-repair recombination, or on broad host-range vectors, which allows replication in many Gram-negative genera. In this study we generated shuttle vectors with *S. cerevisiae* and the broad host range pBBR1 bacterial replicon. These contained a variety of inducible promoters and used a highly sensitive *luxCDABE* reporter in *Acinetobacter baumannii*, *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Pseudomonas fluorescens*, and *Serratia marcescens*. Tested carbohydrate-inducible promoters were P_{BAD}, P_{rhaB}, P_{T5}, and P_{xut}, isolated from *A. baumannii*. In the Enterobacterales the P_{BAD} and P_{rhaB} promoters demonstrated the highest levels of inducibility at 100- and 5,600-fold, respectively. For *P. aeruginosa* P_{rhaB} and P_{xut} were the most inducible at 40- and 10-fold, respectively. For *P. fluorescens* all of the carbohydrate-inducible promoters had similar inducibility, under 10-fold. None of the carbohydrate-inducible promoters were effective for *A. baumannii*; however, they could be used as constitutive promoters. Data indicated that the rhamnose-inducible promoter excelled among the carbohydrate-inducible promoters for most tested organisms, and this study highlights the need for better inducible promoters for *A. baumannii*.

Keywords: inducible promoter; expression vector; *Acinetobacter baumannii*, *Pseudomonas*; *Klebsiella pneumoniae*, *Serratia marcescens*

Introduction

Inducible promoters are particularly valuable for applications to generate proteins and other biological molecules, molecular biology, and synthetic biology. In basic science, inducible promoters are used for complementation purposes, phenotypic analysis following overexpression of a gene, expression of antisense or regulatory RNAs, and for protein purification schemes. Understanding inducible promoter strength and leakiness, that is, the transcription of a gene without the presence of a corresponding inducer, are important factors in choosing the appropriate promoter.

Yeast *in vivo* cloning, based on gap repair (Szostak et al., 1983), has been a powerful tool to manipulate DNA and clone complex constructs (Goto & Nagano, 2013; Hokanson et al., 2003; Iizasa & Nagano, 2006; Ip et al., 2020; Nickerson et al., 2021; Raymond et al., 1999; Shashikant et al., 1998; Wang et al., 2012). This allows for simultaneous cloning of multiple pieces of DNA (Iizasa & Nagano, 2006; Ip et al., 2020; Shanks et al., 2006) and single oligomer mediated deletions and amino acid changes (Shanks et al., 2009). Our group has previously adapted this technology for use in gene expression and genome manipulation in bacteria (Callaghan et al., 2020; Kalivoda et al., 2011; Lehner et al., 2020; Shanks et al., 2006; Shanks et al., 2009). In this study, we generated *S. cerevisiae* shuttle vectors that expand on the number of inducible promoters and tested them in multiple genera of bacteria. These plasmids can also be used with in vivo cloning methods such as Gibson assembly (Gibson et al., 2009).

Many commercial expression vectors for Gram-negative bacteria have either ColE1 or p15a-based replicons that have limited host ranges. In this study, we used the broad host range pBBR1 replicon so that the resulting vectors could be used in a wide range of bacterial genera (Lynch & Gill, 2006).

We have previously evaluated the P_{BAD} and P_{xut} promoters in *Escherichia coli*, *Pseudomonas aeruginosa*, and *P. fluorescens*, and used GFP as a reporter for promoter strength (Callaghan et al., 2020). GFP is a convenient reporter measurable by fluorescence, but has limited dynamic range. By contrast, luminescent reporters offer much higher signal to noise ratio and are better for detecting low levels of expression making them especially valuable for determining leakiness (Greer & Szalay, 2002). In this study, the *luxCDABE* reporter (Meighen, 1994) was used to evaluate promoter leakiness and strength of several plasmid-based inducible promoter systems in additional bacterial genera using vectors capable of being used for yeast *in vivo* cloning.

A rhamnose inducible promoter, P_{rhaB} (Egan & Schleif, 1983), an isopropyl β-D-1-thiogalactopyranoside (IPTG) inducible hybrid promoter, P_{T5}-*lac* (PT5) (Bujard et al., 1987; Kitagawa et al., 2005), and a tetracycline-inducible promoter were included in this study. Data presented here demonstrate different levels of promoter inducibility, background expression, and species-specific effects that can help researchers make more informed choices for controlled gene expression. We used strains of three genera of Enterobacterales: *E. coli*, *Klebsiella pneumoniae*, and *Serratia marcescens*. In addition, *Acinetobacter baumannii* and two *Pseudomonas* species were tested. Together, these represent some of the most important bacterial species in molecular biology, basic microbiology research, and human infections.

Materials and Methods

Strain and culture conditions

Bacteria used in this study were ATCC 17978 (Piechaud & Second, 1951) and B5, a multidrug-resistant clinical isolate for *A. baumannii*, S17-1 λ *pir* for *Escherichia coli* (Miller & Mekalanos, 1988), Kp2 (ATCC43816) for *Klebsiella pneumoniae* (Lee et al., 2014), Pf0-1 for *Pseudomonas fluorescens* (Deflaun et al., 1990), UCBPP-PA14 (PA14) for *P. aeruginosa* (Rahme et al., 1995), and Db11 (Flyg et al., 1980), K904 (Kalivoda et al., 2010), and K1496 (Shanks et al., 2015) for *Serratia marcescens*. Bacteria were grown in lysogeny broth (LB) (Bertani, 1951) at 30˚C with aeration. Gentamicin was used at 10 µg/ml for *E. coli* and *P. fluorescens* and 30 µg/ml for *P. aeruginosa*.

E. coli EC100D (Lucigen Co) was transformed by electroporation using standard techniques (Dower et al., 1988), and into other organisms by conjugation. Ampicillin (150 µg/ml), chloramphenicol (30 µg/ml), nalidixic acid (20 µg/ml), or tetracycline (10 µg/ml) were used to inhibit donor *E. coli* growth following conjugation. *E. coli* strain HB101 (Kessler et al., 1992) was used as a helper strain in triparental mating.

Bacteria were grown at 30 or 37˚C under shaking conditions.

Relative light unit (RLU) determination

Bacteria were transferred from frozen stocks to LB agar plates with gentamicin. Single colonies were used to inoculate 4.5 ml of LB broth with gentamicin and inducer at the noted concentration and/or water up to 500 µl. During and after growth for either 5 h or 18-20 h with indicated concentrations of inducer added at inoculation, 0.15 ml aliquots were taken and placed in clear bottomed, black sided plates (Nunc Optical Polystyrene polymer bottom). Luminescence was read with a Spectramax L luminometer, and optical density at 600 nm (OD_{600}) was measured using a plate reader (BioTek Synergy 2 or Spectramax M3). The ratio of luminescence to optical density was used as RLU.

Molecular Biology

Several plasmids, listed in Table 1, were made for this study using yeast *in vivo* recombineering techniques as previously described (Shanks et al., 2006; Shanks et al., 2009). All primers are listed in Table 2. The linearized plasmid and amplicon were both introduced into *Saccharomyces cerevisiae* strain InvSc1 (Invitrogen), transformants were pooled, the plasmid DNA was obtained, and was used to transform electrocompetent *E. coli* S17-1. Transformants were screened by luminescence and colony PCR. The insert junctions of the resulting plasmids were verified by sequencing (University of Pittsburgh, Genomic Core) or by whole plasmid sequencing using Oxford Nanopore technology (PlasmidSaurus, Inc).

To make pMQ670, the *luxCDABE* operon was amplified from pMQ713 (Romanowski et al., 2019) using primers 4280 and 4281 (Table 2) and the resulting amplicon was used to replace the *gfp* gene of pMQ650. The pMQ650 plasmid was cut with EcoR1 and BamH1 and used to co-transform *S. cerevisiae* along with the *luxCDABE* operon. To make pMQ688, the *rhaRS-PrhaB* region of *E. coli* strain W3110 (Bachmann, 1972) was cloned using primers 4362-3 and the amplicon was added to *S. cerevisiae* along with pMQ670 that had been digested with SfoI and SmaI. The pMQ700 plasmid was derived from pMQ457 by replacing *lacZα* with the *luxCDABE* operon from

pMQ713. The operon was amplified using primers 4283 and 4284. The pMQ457 plasmid was cut with SmaI and combined with the *luxCDABE* amplicon to transform *S. cerevisiae*. To make pMQ725, the PT5-*lac* and *lacI* region from pIM1440 (Murin et al., 2012) was amplified with primers 4647-8 and was co-transformed with pMQ688 that had been digested with PacI and PvuI.

To make pMQ782, the *luxCDABE* operon was replaced in pMQ688 with an artificial multicloning site (MCS). The pMQ688 plasmid was digested with MfeI and SpeI and the digested plasmid was mixed with an artificial double stranded DNA fragment (Integrated DNA Technology). The sequence was (5' to 3') gaccaccgcgctactgccgccaggcaaattctgttttatcagaccgcttctgc gttctgatttaatctgtatcaggctgaaaatcttctctcatccgccaaTTATT CTATTTACCAAGCTTGCATGCGACTAGTTGGA TCCTTGGGTCGACCtcctgctgaatttcattacgaccagtctaaa aagcgcctgaattcgcgaccttctcgttactgacaggaaaatgggccattgg caaccagggaaagat; the lowercase DNA to direct recombination with the plasmid and uppercase DNA that includes three translational stop sites in different frames, restriction sites, and a ribosome binding site. The resulting plasmid was screened by PCR and the entire plasmid was sequenced by PlasmidSaurus. The pMQ782 sequence was submitted to GenBank (ID: PP526039.1). A smaller variant was made by removing much of the yeast replicon by digesting pMQ782 with StuI which cuts in two places, and performing a unimolecular ligation with T4 ligase (New England Biolabs). The resulting plasmid, pMQ807, was verified by sequencing by PlasmidSaurus.

Table 1. Plasmids made in this study.

Plasmid	Description[a]	Inducer
pMQ670	P_{xut} - *luxCDABE*	D-xylose
pMQ688	P_{rhaB} – *luxCDABE*	L-rhamnose
pMQ700	P_{BAD} – *luxCDABE*	L- arabinose
pMQ725	$P_{T5\text{-}lac}$ – *luxCDABE*	IPTG
pMQ782	P_{rhaB} – MCS[b]	L-rhamnose
pMQ807	pMQ782 with yeast replicon removed	L-rhamnose

[a] all have oripBBR1 as a bacterial replicon; all have a gentamicin resistance marker, *aacC1*; plasmids have a RP4 origin of transfer.

[b] MCS, multicloning site

Table 2. Oligonucleotide primers used in this study.

Primer no.	Sequence (5' to 3')[a]
923	*CAGACCGCTTCTGCGTTCTG*
4280	ccgccaattattctatttaccaagcttgcatgcgactagtTCAACTATCAAAC GCTTCGG
4281	caagaacaacaaccaagaacaacaaggaggatttttgaATGACTAAAAAA ATTTCATTCA
4362	gccctatcggctggatcgcttttgagtggccggctaTTAATCTTTCTGCGA ATTGAGATG
4363	acctggccgttaataatgaatgaaatttttttagtcattcaaaaatcctcctttaattaaTCC TGCTGAATTTCATTACG
4283	ccgccaattattctatttaccaagcttgcatgcgactagtTCAACTATCAAAC GCTTCGG
4284	caagaacaacaaccaagaacaacaaggaggatttttgaATGACTAAAAAA ATTTCATTCA
4647	cattcaaaaatcctcctttaattaatcctgctgaaAATGAATTAATTCTGTG TGAAATTG
4648	agcccggagagcaagcccgtagggcgcattaatgcagGACACCATCGAA TGGTGCAAAAC
5635	taccactccctatcagtgatagagaaaagtgaatcggtaaggaGGTCGACCCA AGGATCC

[a] Upper case letters are for PCR amplification, lower case letters dictate recombination, and Italic letters indicate both.

Carbohydrate inducible promoters evaluated in this study

Four carbohydrate inducible promoters (Figure 1) were cloned upstream of the *luxCDABE* reporter operon in a broad host-range vector with a pBBR1 replicon. Of the inducible promoters including regulatory transcription factor genes, P_{BAD} was the shortest (under 1,200 bp) and P_{rhaB} was the longest at over 2,000 bp. All of the regulatory genes originated from *E. coli* except *xutR,* which was isolated from *P. fluorescens*. The $P_{T5\text{-}lac}$ promoter, noted as P_{T5} throughout, is a hybrid bacteriophage and *E. coli* promoter. The plasmids were introduced into six different species and tested for induction over a 1,000-fold range of inducer and luminescence was measured at 18-20 hours and normalized by culture turbidity to

generate relative luminescence units (RLU). In all cases RLU values for bacteria without the *luxCDABE* reporter were less than 10^3. This time point was chosen, because it is typical for many of the stationary phase-harvest studies done in our laboratory.

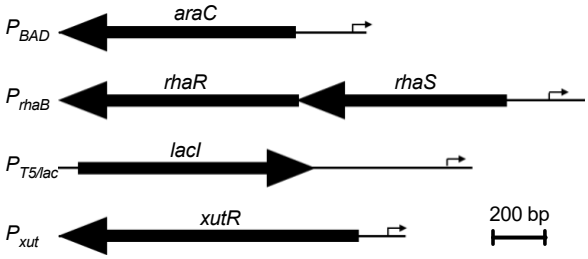

Figure 1. Schematic maps for the carbohydrate-inducible promoters used in this stud. Regulatory regions for the inducible promoter system cloned upstream of the *luxCDABE* reporter shown to scale.

Results

Evaluation of carbohydrate inducible promoters in E. coli

In this study, *E. coli* was used as a control to validate the plasmids and our system, as these promoters have been characterized previously in this species using plasmids with different replicons and copy numbers. Our series of plasmids with the same pBBR1-replicon and diverse promoters were introduced into *E. coli* strain S17-1 and tested for induction over a 1,000-fold range of inducer and luminescence was measured at 18-20 hours and normalized by culture turbidity to generate relative luminescence units (RLU). P_{xut} was not induced in *E. coli*, whereas P_{T5}, P_{BAD}, and P_{rhaB} had dose dependent inducible profiles (Figure 2A). Plasmid leakiness (expression without inducer) was lowest in P_{BAD} ($3.7x10^4$ RLU) and P_{rhaB} ($3.4x10^4$ RLU) and higher in P_{T5} ($7.3x10^4$ RLU) and P_{xut} ($7.5x10^4$ RLU). P_{rhaB} had the highest promoter strength at all tested inducer concentrations and highest overall inducibility when 10 mM of inducer was compared to the absence of inducer (672-fold) (Figure 2A). By contrast the fold-induction for the other promoters were 99.4 for P_{BAD}, 7.7 for P_{T5} and 0.8 for P_{xut}.

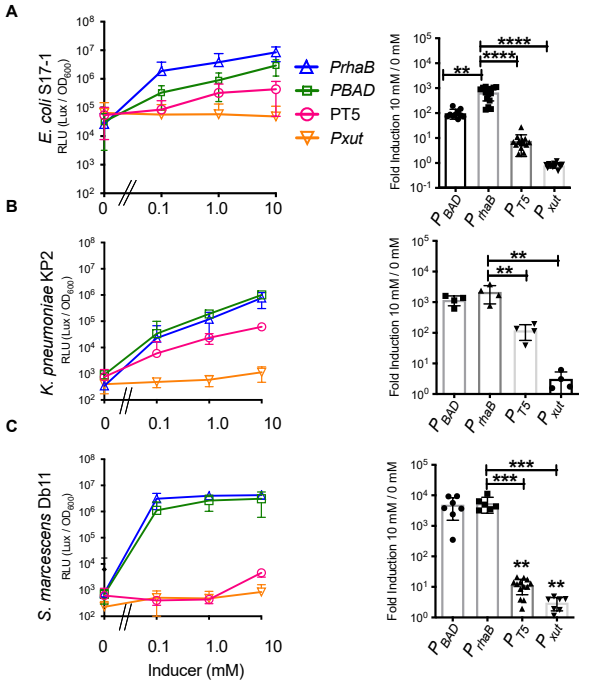

Figure 2. Analysis of inducible promoter activity with variable inducer concentrations for three Enterobacterales bacteria. The average and standard deviation are shown, n≥4 independent replicates for each data point. P_{T5} refers to the $P_{T5\text{-}lac}$ promoter. **A.** *E. coli* strain S17-1. **B.** *K. pneumoniae* strain KP2. **C.** *S. marcescens* strain Db11. Left column shows relative luminescence units (RLU) and right column depicts fold change between RLU from higher inducer to no inducer. Means and SD are shown. Bacteria were grown at 30˚C. Asterisks indicate significant differences by ANOVA with Tukey's post-test. **, p<0.01; ***, p<0.001; ****, p<0.0001. For *S. marcescens* the asterisks over P_{T5} and P_{xut} show differences from P_{BAD}.

Evaluation of sugar inducible promoters in K. pneumoniae

P_{xut} was poorly induced in *K. pneumoniae* strain KP2, up to 3-fold with maximal inducer, whereas P_{T5}, P_{BAD}, and P_{rhaB} had pronounced dose dependent inducible profiles (Figure 2B). The P_{BAD} and P_{rhaB} promoters were largely indistinguishable. Plasmid leakiness (expression without inducer) was highest in P_{BAD} (911 RLU) and P_{T5} (758 RLU) and lowest with P_{rhaB} (345 RLU) and P_{xut} (401 RLU). P_{BAD} and P_{rhaB} had the highest promoter strength at all tested inducer

concentrations, whereas P_{rhaB} had the highest overall inducibility when 10 mM of inducer was compared to the absence of inducer (2,174-fold) (Figure 2A). This was followed by P_{BAD} with 1,184-fold and P_{T5} with 120-fold induction (Figure 2B).

Evaluation of carbohydrate inducible promoters in S. marcescens strain Db11

E. coli and *K. pneumoniae* are *Enterobacteriaceae*; to test another family member of the order Enterobacterales, we used *S. marcescens*, which is in the Yersiniaceae family.

In *S. marcescens* strain Db11, expression of P_{BAD} and P_{rhaB} was similar (Figure 2C). P_{T5} and P_{xut} were poorly induced with only 8 and 3-fold higher expression when comparing the maximum inducer concentration compared to no inducer. Plasmid leakiness was low in all plasmids with a maximum of 685 RLU for P_{BAD}. P_{rhaB} had highest expression at all inducer concentrations and the highest overall inducibility when 10 mM of inducer was compared to the absence of inducer (5,612-fold); inducibility of P_{BAD} was also high at 4,927-fold. (Figure 2C).

Unlike the other Enterobacterales bacteria tested, the P_{T5} was not strongly inducible in *S. marcescens* Db11. *S. marcescens* strains are generally unable to ferment lactose, although some biotypes such as A8c are lactose fermenters (Grimont & Grimont, 1978). The P_{T5} promoter was also not inducible in strain K904 that cannot use lactose as a sole carbon source (Figure 3). We tested whether the P_{T5} construct would be useful in a lactose fermenting *S. marcescens* isolate, strain K1496. Notably, the P_{T5} reporter plasmid showed a 358-fold induction with IPTG at 10 mM compared to 0 mM, whereas there was a less than 10-fold induction in strains unable to utilize lactose as a sole carbon source at the highest concentration (Figure 3).

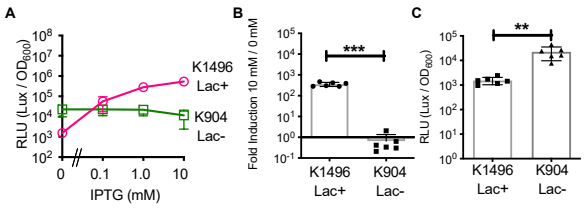

Figure 3. Analysis of $P_{T5\text{-}lac}$ in additional *S. marcescens* strains. Strain K1496 can utilize lactose, and strain K904 does not utilize lactose. The average and standard deviation are shown, n≥6 independent replicates for each data point. **A.** Fluorescence across a range of inducer concentration. **B.** Fold induction of RFU for 10 mM / 0 mM. **C.** Background fluorescence with 0 mM inducer. Asterisks indicate significant differences by Student's T-test. **, p<0.01; ***, p<0.001. Bacteria were grown at 30˚C.

Evaluation of carbohydrate inducible promoters in P. fluorescens

RLU measurements from *P. fluorescens* strain Pf0-1 with the inducible plasmid is shown in Figure 4A. All of the plasmids were inducible, although none were above 10-fold (Figure 4A). The least leaky carbohydrate-inducible promoter was P_{BAD} ($1.7x10^5$ RLU). The other promoters had higher levels of expression in the absence of inducer: $9.9x10^5$ RLU for P_{rhaB}, $4.9x10^5$ RLU for P_{T5}, and $1.8x10^6$ RLU for P_{xut}. Fold induction of 10 mM compared to 0 mM was highest for P_{BAD} and P_{rhaB} at 6.1 and 6.4-fold induction, and lower for P_{xut} and P_{T5} at 4.3 and 2.6-fold, respectively (Figure 4A).

Evaluation of carbohydrate inducible promoters in P. aeruginosa

All tested promoters were inducible in *P. aeruginosa* strain PA14, with P_{BAD}, P_{xut} and P_{rhaB} all demonstrating similar high promoter strength at the highest level of inducer (Figure 4B). All of the promoters were leaky in the absence of the inducer. P_{BAD} and P_{xut} promoters were the highly leaky ($3.2x10^6$ RLU and $8.2x10^5$ RLU respectively), whereas the P_{T5} ($2.9x10^5$ RLU) and P_{rhaB} ($1.8x10^5$ RLU) had relatively lower levels of expression in the absence of inducer. The P_{rhaB} promoter had the highest inducibility at 10

mM of inducer compared to 0 mM of inducer, which was 44-fold (Figure 4B). The maximum induction level dropped to 11-fold for P_{xut} and 4-fold for both P_{BAD} and P_{T5}.

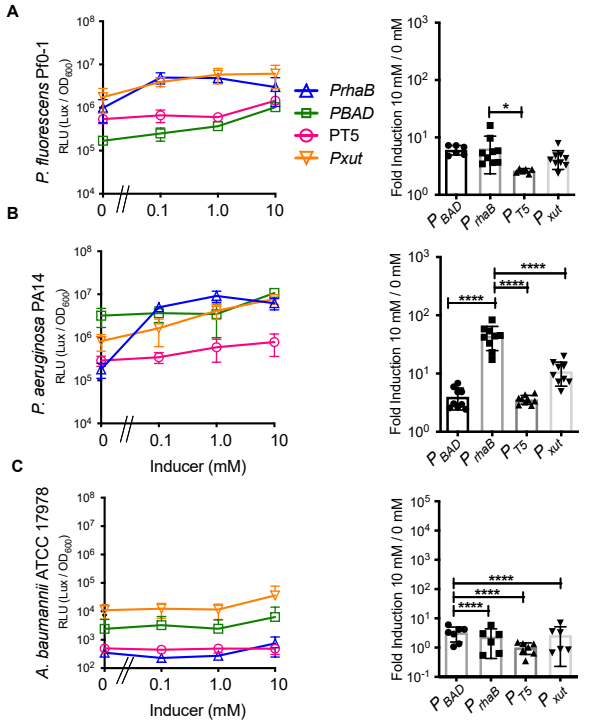

Figure 4. Analysis of inducible promoter activity with variable inducer concentration for two *Pseudomonas* species and *A. baumannii*. The average and standard deviation are shown, n≥6 independent replicates for each data point. **A.** *P. fluorescens* strain Pf0-1. **B.** *P. aeruginosa* strain PA14. **C.** *A. baumannii* ATCC 17978. Left column shows relative luminescence units (RLU) and right column depicts fold change between RLU from higher inducer to no inducer. Means and SD are shown. Asterisks indicate significant differences by ANOVA with Tukey's post-test. *, p<0.05; ****, p<0.0001. Bacteria were grown at 30˚C.

Evaluation of carbohydrate inducible promoters in A. baumannii

No significant differences were measured between the promoters in *A. baumannii* strain ATCC 17978 with respect to the fold difference between 0 and 10 mM inducer (Figure 4C). The magnitude of inducibility between 0 and 10 mM inducer was minimal: highest with P_{BAD} (3.3-fold) and lowest for P_{T5} (1.0-fold). Overall promoter strength at maximum inducer ($3.7x10^4$ RLU) and background ($1.1x10^4$ RLU) were highest for P_{xut}, intermediate for P_{BAD} ($6.3x10^3$ RLU at 10 mM and $2.4x10^3$ RLU at 0 mM L-arabinose), and negligible for P_{rhaB} and P_{T5} (<10^3 RLU).

Evaluation of background expression of each carbohydrate inducible promoter

Background expression of promoters without inducer is often undesirable. The RLU for each promoter under each condition was evaluated in the absence of inducer (Figure 5). In general, these were highest in *Pseudomonas* species and lowest in *A. baumannii*, *K. pneumoniae*, and *S. marcescens*.

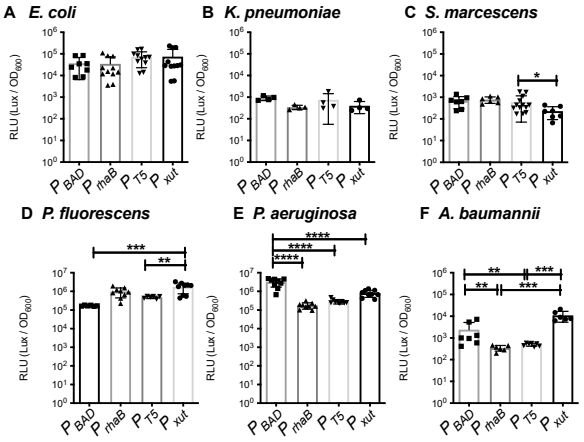

Figure 5. Background luminescence with no inducer. Mean and SD are shown, n≥4. Asterisks indicate significant differences by ANOVA with Tukey's post-test. *, p<0.05; **, p<0.01; ***, p<0.001; ****, p<0.0001.

Evaluation of P_{rhaB} over time at 37˚C

Prior experiments evaluated various promoters at a single time point after overnight growth at 30˚C. To expand on this, experiments were performed to evaluate earlier time with a subset of organisms when grown at 37˚C. Bacteria were grown overnight without induction and subcultured with

inducer at 0.1 mM for rhamnose added at time 0 h. Luminescence and optical density were read over time 5h and the next day (Figure 6). For *S. marcescens* strain Db11 with P_{rhaB}, luminescence increased starting at 3h (190-fold) and continued to increase through 5h where it was over 25,000-fold higher than at 0h (Figure 6). In the absence of inducer, luminescence increased 9.2-fold by 5h, which was remarkably lower than with 0.1 mM rhamnose. Rhamnose did not change growth of *S. marcescens* in these experiments (Figure 6).

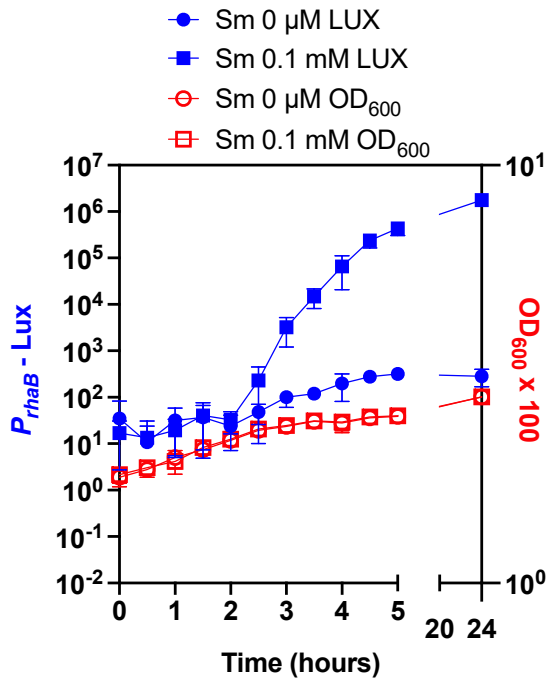

Figure 6. Induction of P_{rhaB} promoter over time at 37°C in *S. marcescens* Db11. P_{rhaB}-dependent luminescence and growth of *S. marcescens* strain Db11 (Sm) over time. Inducer and vehicle negative control were added at time = 0. Mean and SD are shown, n=4-6.

Generation of a PrhaB vector with a multicloning site

The plasmids in this study have limited restriction sites for standard cloning approaches. While we have previously generated plasmids with P_{BAD} and P_{xut} promoters that contain multicloning sites (Callaghan et al., 2020; Shanks et al., 2009), we have not made one for P_{rhaB}. To address this, the *luxCDABE* operon was replaced with an artificial multicloning site with unique restriction sites for BamHI, HinDIII, SalI, SpeI, and SphI, a ribosome binding site, and stop codons in three frames to make pMQ782. The resulting plasmid was further altered by removal of the *URA3* and *CEN6/ARSH4* region to reduce its size by digestion with StuI followed by unimolecular ligation to generate pMQ807.

Discussion

Carbohydrate inducible promoters are widely used and helpful for numerous applications; however, they can have limitations (Brautaset et al., 2009). These include potential alteration of the catabolite repression system of the host bacterium which can result in pleiotropic effects (McMackin et al., 2021), the utilization of the inducer for energy which reduces the concentration of this molecule, and induced expression in only a subset of bacteria in a given population (Callaghan et al., 2020; Khlebnikov et al., 2000). These limitations were not evaluated in this study due to the use of a luminescent reporter, and will have to be evaluated using fluorescent reporters in the future. Luminescent reporters provide exceptional sensitivity and dynamic range, but like all reporters are indirect readouts for transcription and do not take into consideration the half-life of a given transcript. Expression of individual genes will have to be determined in a case-by-case manner.

The results reported here demonstrate that for Enterobacterales, P_{BAD} and P_{rhaB} are useful inducible promoters that can be highly tuned based on inducer concentration. The P_{T5} promoter worked well with *K. pneumoniae*, but was moderately effective in *E. coli* and strain-dependent in *S. marcescens*. The copy number of the plasmid likely differs between species, so no quantitative comparisons were made between species.

For *P. aeruginosa*, the P_{rhaB} promoter was more inducible than the widely used P_{BAD} and conferred very strong expression at 0.1 and 1.0 mM inducer concentration. P_{BAD} and P_{xut} were also strong promoters in *P. aeruginosa*, but the P_{rhaB} promoter had better sensitivity and the lowest leakiness. None of the inducible promoters were particularly strong for *P. fluorescens*. While P_{xut} was the strongest, it was poorly inducible. P_{rhaB} and P_{BAD} had the highest inducibility and P_{BAD} had the lowest leakiness.

For *A. baumannii*, none of the promoters were highly inducible. This highlights the need for better inducible systems for this important opportunistic pathogen.

Among the tested carbohydrate-inducible promoters, the P_{rhaB} promoter arguably performed the best in the assays based on maximum induction and low leakiness for most tested bacteria. Induction levels between no inducer and the lowest inducer concentration were generally greatest for P_{rhaB}. In addition, rhamnose and xylose are relatively inexpensive compared to arabinose and IPTG. One minor negative of P_{rhaB} is the larger size of the promoter and regulator genes; this yields larger vectors that may have lower transformation efficiency.

In all cases, easily measurable levels of transcript were detected in the absence of inducer. This background could be reduced by using lower copy replicons such as RK2 or integration of the promoter/regulator genes into the bacterial chromosome. Another issue not tested was whether the promoter is expressed in every cell or just a subset of cells as has been demonstrated for P_{BAD} (Siegele & Hu, 1997) and P_{xut} (Callaghan et al., 2020); as these assays are easier with fluorescent reporters. The use of carbohydrates as inducers also has limitations. Carbohydrates can be catabolized by wild-type organism changing inducer concentration over time and affecting carbon regulation systems that could have unintended effects. This study was also limited by the chosen inducer concentrations, time of inducer addition, and temperatures. Nevertheless, we have generated broad host-range vectors with convenient restriction sites that should be effective for controlled gene expression in a number of microbes, they were effective in evaluating the different promoters in a number of genera.

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