Repression of YdaS Toxin Is Mediated by Transcriptional Repressor RacR in the Cryptic rac Prophage of Escherichia coli K-12

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ABSTRACT Horizontal gene transfer is a major driving force behind the genomic diversity seen in prokaryotes. The cryptic rac prophage in Escherichia coli K-12 carries the gene for a putative transcription factor RacR, whose deletion is lethal. We have shown that the essentiality of racR in E. coli K-12 is attributed to its role in transcriptionally repressing toxin gene(s) called ydaS and ydaT, which are adjacent to and coded divergently to racR.

IMPORTANCE Transcription factors in the bacterium E. coli are rarely essential, and when they are essential, they are largely toxin-antitoxin systems. While studying transcription factors encoded in horizontally acquired regions in E. coli, we realized that the protein RacR, a putative transcription factor encoded by a gene on the rac prophage, is an essential protein. Here, using genetics, biochemistry, and bioinformatics, we show that its essentiality derives from its role as a transcriptional repressor of the ydaS and ydaT genes, whose products are toxic to the cell. Unlike type II toxin-antitoxin systems in which transcriptional regulation involves complexes of the toxin and antitoxin, repression by RacR is sufficient to keep ydaS transcriptionally silent.

KEYWORDS prophages, toxin-antitoxin, transcriptional repressor

Horizontal gene transfer (HGT) contributes to the vast genome diversity seen in prokaryotes. The size of the genome of Escherichia coli ranges from under 4 Mb to under 6 Mb. The core genome constitutes only ~10% of the gene families represented across these E. coli genomes. The rest of the genetic content is variable across strains and often found in genomic islands (1). Many virulence factors and determinants of antibiotic resistance are known to be horizontally acquired and encoded, for example, by genes carried on autonomously replicating plasmids and chromosomally replicating prophages (2, 3).

The genome of the laboratory strain E. coli K-12 comprises nine cryptic prophages which constitute 3.6% of its total genome. Among these prophages is a cryptic prophage called rac. The rac prophage is 23 kb long and carries 29 genes. Among these genes is a putative transcription factor called RacR, whose deletion is lethal to the cell.

In attempting to explain the essentiality of the horizontally acquired racR gene, we used a combination of genetics, biochemistry, and bioinformatics to present evidence that RacR is indeed a transcriptional repressor. We show that RacR binds to its own upstream sequence and represses the adjacent and divergently coded ydaS-ydaT gene pair. ydaS and ydaT encode toxic products. Thus, the ydaST-racR module forms a “toxin-repressor” combination, making RacR essential to the cell.
RESULTS

RacR is an essential transcriptional regulator. The rac prophage is a cryptic and mosaic prophage in E. coli. Its size and gene content vary across E. coli strains, with only a few highly conserved genes, including recE, involved in an alternative homologous recombination pathway, and trkG, a potassium ion permease (Fig. 1A). Among the less-conserved portion of the rac prophage is a gene encoding a predicted transcription factor called RacR. It contains a weak helix-turn-helix motif and at best is very distantly related to the lambda cI repressor (15% identity by Needleman-Wunsch global alignment). Its deletion is presumed to be lethal. The Keio collection of E. coli single gene deletion mutants does not contain ΔracR (4), and we were unable to delete racR by homologous recombination. Nevertheless, the entire rac prophage could be deleted (we refer to this as Δrac here), and the prophage excises at high rates in certain genetic backgrounds (5). Hence, we hypothesized that RacR could be a repressor of a toxin in the same prophage. Because the rac prophage carries a previously reported toxin called KilR, an inhibitor of cell division (6), we initiated our screen for the toxin by attempting to delete racR in the ΔkilR strain. However, we found that ΔracR could not be obtained even in a ΔkilR background.
We then deleted successively shorter segments, each containing racR, of the prophage. If a deletion attempt removed racR but not the toxin that RacR might repress, we would not recover the mutant. The smallest deletion we obtained by this approach included racR, its neighboring, divergent gene ydaS, and the common intergenic region (henceforth referred to as IGR) between them (Fig. 1B). Thus, the absence of ydaS and the common IGR between racR and ydaS is a suppressor of the lethality of ΔracR. Despite several attempts, we were unable to delete racR in the ΔydaS mutant without disturbing the IGR between them. However, we obtained ΔracR with its IGR intact in a ΔydaS-T (deletion of ydaS and ydaT and the short ~20-bp spacer separating the two ORFS) background. ydaT is encoded in tandem and downstream of ydaS and might be part of the same operon.

Overexpression of ydaS and ydaS-T reduces growth. We tested the toxicity of ydaS, ydaT, and ydaS-T by cloning these genes under the araBAD promoter in pBAD18. Expression of these cloned genes was induced in both the wild type and Δrac mutant with 0.1% l-arabinose. We found that the expression of ydaS and ydaS-T causes rapid growth inhibition after induction in both the wild type and Δrac mutant (see Fig. S1 in the supplemental material). We collected samples at 5 h and 14 h after induction and spotted these samples onto agar plates. Cells expressing ydaS and ydaS-T from pBAD18 did not grow on these plates (Fig. 2A). The expression of ydaT alone did not have any inhibitory or lethal effect on the wild type or the Δrac prophage strain. However, we subsequently noticed that YdaT when expressed as described here accumulates in the insoluble fraction, and therefore might not be functional.

Further, we quantified the live- and dead-cell populations after the induction of ydaS, ydaT, and ydaS-T by fluorescence-activated cell sorting (FACS) using propidium iodide (PI) as a marker for dead cells. Results from six independent trials show that ydaS and ydaS-T expression, irrespective of the strain background, lead to loss of cell viability.
(Fig. 2B). We noticed that cells expressing ydaS-T were longer than the cells expressing ydaS or ydaT (Fig. S2).

Together, these results show that the expression of ydaS and ydaS-T is lethal and that ydaS and ydaT do not form a toxin-antitoxin (T-A) pair as predicted earlier (7). YdaS is critical to cell killing. While we could not successfully overexpress functional YdaT, the fact that we could isolate ΔracR only in a ΔydaS-T background indicates that YdaT is also toxic to the cell. In a separate study, we show that clustered regularly interspaced short palindromic repeat (CRISPR)-Cas-mediated knockdown of racR causes a growth defect and also results in a filamentous cell phenotype in ΔydaS cells and in ΔydaT cells, but not in ΔydaS-T cells (23). Another independent study by Campos and colleagues has shown that the ΔydaS strain from the Keio collection shows a filamentous phenotype; this the authors attribute to a polar effect on the expression of ydaT (8). It is unclear why the overexpression of ydaS, despite being toxic, does not result in a filamentous phenotype in this study.

Cooccurrence of racR and ydaS implies interaction between them. Functionally related genes tend to be conserved together across genomes (9). We examined the conservation of genes of the rac prophage across 154 E. coli genomes. Bidirectional best-hit search for orthologs confirmed the mosaic nature of the rac prophage. In fact, more than 50% of the strains have lost half of the prophage. The genes that are well conserved across the genomes are the genes, such as recE and trkG, that have documented functions in the host. Some classical phage genes like intr, pinR, stfR, tfaR, ydaF, and ydaV are conserved in more than 85% of the strains analyzed.

We observe that the known toxin genes in the prophage are lost in most of the strains, and when they are present, they are always accompanied by its cognate antitoxin genes. RalR-RalA is a known type I T-A system in the same prophage (10). We observe that the RalR toxin is conserved in only 36.3% of the strains we analyzed; the corresponding noncoding antitoxin gene was found in all these strains. KilR, previously reported as a FtsZ inhibitor, was found in 48% of the strains in this analysis; its antitoxin, if any, is unknown. YdaS is present in only 33.7% of the strains analyzed, and we observe that it always cooccurs with RacR (Fig. 1A). A few strains carried the ydaT gene in the absence of racR; however, the IGR was lost in these strains, and certain point mutations were found in the ydaT gene. Thus, genome context analysis suggests a functional interaction between RacR and YdaS(-T).

Expression of ydaS is kept silent under normal physiological conditions. In order to examine the expression of RacR and YdaS in vivo, we tagged these two genes with C-terminal 3×FLAG (DYKDDDDK). Western blotting using an anti-FLAG antibody showed that RacR was expressed throughout batch growth. However, YdaS expression could not be detected in our experimental conditions (Fig. 3A). An absolute protein quantification study performed by Li et al. (11) also shows a low copy number for YdaS. Analysis of various publicly available and in-house transcriptome sequencing (RNA-seq) data showed that the expression of ydaS is comparable to that of bglG, a well-characterized transcriptionally silent cryptic gene (12). racR was among the most highly expressed genes in the rac prophage, but only to a level comparable to that of the lac repressor gene (Fig. S3). These results show that YdaS is not expressed in E. coli and, in light of the genetic experiments reported above, lead to the hypothesis that RacR is a repressor of this toxin.

Binding of RacR in the IGR. RacR comprises a helix-turn-helix (HTH) motif, and hence, we investigated whether it binds to DNA. The 123-bp IGR between racR and ydaS contains three slightly variant repeats of GCCTAA and its inverse TTAGGC (Fig. 3B). This is similar to the regulatory region of lambda phage, which is bound by CI and Cro, even though the exact sequences bound by the proteins are different. To test for the binding of RacR to the IGR, we first performed a thermal shift assay with purified RacR and various nucleic acid sequences. The thermal shift assay measures the thermal denaturation temperature (Tm) of a test protein. A change in this temperature in the presence of a ligand might argue in favor of an interaction between the protein and
ligand. We found that the $T_D$ of RacR increased by $\sim 5^\circ C$ in the presence of racR-IGR-ydaS or a 189-bp sequence upstream of ydaS and including the IGR (Fig. 3C). The extended 189-bp region, including a portion of the racR gene, was chosen for this experiment because this included an additional half-site of the above-mentioned palindrome.

We then performed a chromatin immunoprecipitation (ChIP) of RacR::3×FLAG to test for the binding of RacR to the IGR in vivo. By performing quantitative PCR (qPCR) against the DNA thus recovered, we found that the IGR was 2.5-fold enriched in comparison to a random region (Fig. S4A). Finally, we performed electrophoretic mobility shift assay (EMSA) to investigate the binding of purified RacR to the IGR. RacR formed three distinct complexes in the presence of the IGR (Fig. 3D). EMSA with a 49-bp DNA upstream of ydaS, containing a single copy of the repeat, also showed binding to RacR (Fig. S4B). Consistent with the view that the three palindromic repeats might be the sites to which RacR binds, we found only a single protein DNA complex with the 49-bp segment of the IGR. Thus, we show binding of RacR to the intergenic region between racR and ydaS both in vitro and in vivo.
Transcriptional repression of \( ydaS \) is mediated by RacR binding to the IGR.

Finally, to test whether the binding of RacR represses \( ydaS \), we cloned the IGR upstream of \( gfp-mut2 \) in pUA66. We monitored the promoter activity of pUA66::IGR-\( gfp-mut2 \) in \( \Delta ydaS-T \) cells and in \( \Delta racR \Delta ydaS-T \) cells for 25 h. We observed that the \( ydaS \) promoter is active only in \( \Delta racR \Delta ydaS-T \) cells; no fluorescence from \( gfp-mut2 \) could be detected in \( \Delta ydaS-T \) cells (Fig. 4A). The maximal \( ydaS \) promoter activity was observed in the log phase (optical density at 600 nm \( [OD_{600}] \) of ~0.2 to 0.3). We tested the expression of \( gfp-mut2 \) from these strains grown to mid-exponential phase using FACS. The distribution of fluorescence from pUA66::IGR-\( gfp-mut2 \) in \( \Delta ydaS-T \) cells was similar to that of the promoterless control where most cells were green fluorescent protein (GFP) negative. In contrast, in \( \Delta racR \Delta ydaS-T \) cells, nearly 80% of the cells were GFP positive (Fig. 4B). Thus, single-copy availability of RacR from the chromosome appears to be sufficient to suppress the activity of the \( ydaS \) promoter from a multicopy (three or four) plasmid. Thus, RacR represses transcription of \( ydaS \).

DISCUSSION

We have shown that the expression of \( ydaS \) and \( ydaS-T \) is lethal, and we attribute the essentiality of \( racR \) to its role in repressing the expression of this toxin. Earlier studies have shown the presence of two toxins, KilR and RalR, in the \( rac \) prophage (6, 10). In the present work, we suggest that that YdaS-YdaT (YdaS-T) is yet another toxin(s) encoded by the \( rac \) prophage. We do not know how this toxin affects cell killing and whether other genes in the operon to which \( ydaS \) and \( ydaT \) belong contribute to cell killing. The YdaS-T module could be compared to the orphan toxin OrtT for which the antitoxin counterpart is not known (13). In general, essential transcription factors are rare in \( E. coli \). The essentiality of RacR is purely by virtue of its role in keeping a toxin transcriptionally silent. RacR is unlikely to have too many additional targets, because its expression level, based on RNA-seq, is very similar to that of the highly specific Lac repressor. Among the few essential transcription factors in \( E. coli \) is the antitoxin MazE. MazE and MazF are carried on the same operon, unlike \( racR-ydaS \), which make a divergent gene pair. The antitoxin activity of MazE is primarily by protein-protein interactions with the toxin MazF. In fact, the binding of MazE to DNA is enhanced when in complex with MazF (14). Yet another essential transcription factor is the antitoxin MqsA, which again sequesters its cognate toxin MqsR. Unlike the conditional cooperativity displayed by MazE and MazF in binding to the DNA, the high stability of the MqsR-MqsA (MqsR-A) complex makes the protein-protein interaction mutually exclusive of MqsA-DNA interactions (15). In both these cases, it is apparent that the activity of the transcriptional repressor does not entirely prevent the expression of the toxin. In our case, however, we could not detect the presence of the YdaS protein, the expression level of the \( ydaS \) transcript is comparable to that of a bona fide cryptic gene across
tens of RNA-seq data sets, and we cannot detect any activity from the ydaS promoter fused to gfp-mut2 in the presence of RacR. The expression of YdaS-T is toxic, independent of the presence of RacR (wild type versus Δrac mutant), which argues against the possibility of RacR interacting physically with YdaS-T/ΔdaS in suppressing its activity.

RacR is a repressor of ydaS-T, and this module is an example of a “toxin-repressor” system, where the toxicity of YdaS-T is repressed totally at the transcriptional level. The fact that the activity of the toxin is totally suppressed at the level of transcription initiation itself might render posttranslational killing downstream of the loss of the module impossible.

We propose that RacR could be functionally similar to the CI repressor of lambda phage. The rac prophage has lost many of its structural genes compared to the lambda phage (see Fig. S5 in the supplemental material). However, the organization of regulatory elements in the rac prophage (Fig. 3B) is similar to the cl-Cro switch of lambda phage (16). There are three repeat elements in the IGR, which might be the operator of this prophage. Our observation of the formation of three distinct DNA-protein complexes of the 123-bp IGR with increasing concentrations of RacR suggests that the IGR might act as a complex regulatory switch that resembles the regulatory region of cl-cro of lambdoid phages (17).

MATERIALS AND METHODS

**Media, strains, and plasmid construction.** E. coli K-12 MG1655 from CGSC was used and grown at 37°C in Luria broth (LB) or LB agar (HiMedia). The antibiotic-resistant strains were grown in antibiotics wherever required; ampicillin (100 μg/ml), kanamycin (50 μg/ml), or chloramphenicol (30 μg/ml) was used. All the knockout strains were constructed by the one-step inactivation method described by Datsenko and Wanner using pKD13 as the template plasmid for the kanamycin resistance cassette amplification (18). Tagging of racR with 3×FLAG at the C-terminal end was done using the pSU11 plasmid (19). Ectopic expression of racR, ydaS, ydaT, and ydaST were achieved by cloning them between the EcoRI and Sall sites of pBAD18; this brings the genes under the arabinose-inducible araBAD promoter.

The plasmid for the promoter activity was constructed by cloning the intergenic region (IGR) in the lambda phage (see Fig. S5 in the supplemental material). However, the organization of regulatory elements in the rac prophage (Fig. 3B) is similar to the cl-Cro switch of lambda phage (16). There are three repeat elements in the IGR, which might be the operator of this prophage. Our observation of the formation of three distinct DNA-protein complexes of the 123-bp IGR with increasing concentrations of RacR suggests that the IGR might act as a complex regulatory switch that resembles the regulatory region of cl-cro of lambdoid phages (17).

**Growth curve and spotting assay.** Growth curve was monitored in a 96-well plate with the final volume of 200 μl using a Tecan F200 reader. Overnight culture was inoculated in the ratio of 1:100 and allowed to grow until an optical density at 600 nm (OD600) of 0.4 was reached. This was further diluted in fresh medium to an OD600 of 0.01 with or without 0.1% l-arabinose, and A600 was recorded for 14 h. For the spotting assay, appropriate overnight cultures were inoculated in LB broth containing 100 μg/ml ampicillin (diluted 1:100) with or without 0.2% l-arabinose. The cells were collected after 5 h and 14 h of inoculation, serially diluted, and spotted on LB agar plates containing ampicillin without arabinose.

**FACS.** Overnight cultures of the respective strains were inoculated in LB broth at 1:100 dilution with or without 0.2% l-arabinose. Samples were collected after 5 h of induction, pelleted, washed, and resuspended in 500 μl of saline (0.9% sodium chloride [wt/vol]). Exponentially growing cells were used as a live-cell control, and cells subjected to 80°C for 10 min were used as a dead-cell control. Propidium iodide (PI) solution (5 μl of a 1-mg/ml solution) was added to all the vials 10 min before acquisition of data in a BD FACSCalibur. Around 20,000 cells were acquired for each sample using a 488-nm excitation laser, and the emission was recorded from FL2 channel that uses a 585/42 band-pass (BP) filter to collect the PI intensity. The intermediate population in this study is described as the cells that fall between the region of live unstained control and dead control.

Exponential culture of ΔydaS-T and ΔaraR ΔydaT ΔydaS-T bacteria containing pUA66:IGR-gfp-mut2 were pelleted, washed, and resuspended in saline. Green fluorescent protein (GFP) intensity was monitored using FL1 channel that uses a 530/30 BP filter. A strain containing empty pUA66:gfp-mut2 was used to set the background fluorescence, and GFP intensity above this background was marked as positive. Data were analyzed using Flowing software (Cell Imaging Core of the Turku Centre for Biotechnology [http://www.flowingsoftware.com/]).

**Bidirectional search for orthologous genes.** The genomes of 154 completely sequenced E. coli strains were downloaded from NCBI refseq ftp site. A bidirectional search for orthologous genes of the rac prophage, excluding pseudogenes, was performed using phmmer (version 3.1). The E-value threshold used was 10−20. An ortholog presence-absence matrix was hierarchically clustered based on Euclidean distance with centroid linkage. Clustering was performed using Cluster 3 (http://bonsai.hgc.jp/~mdehoon/software/cluster/), and the heat map was generated using matrix2png (http://www.chibi.ubc.ca/matrix2png/).

**RNA-seq data analysis.** Raw reads from 15 different transcriptome sequencing (RNA-seq) studies (with a total of 61 fastq files) were obtained either in-house or from the NCBI GEO or EBI array express databases (Table S3). The SRA files from GEO were converted to fastq using fastq dump. Reads from the fastq file were aligned to NC_000913.3 genome using bwa. The aligned files were sorted using sam
(sequence alignment/map) tools. Further, these sam files were used to get read counts per nucleotide, from which read counts per gene was generated. RPMK (reads per kilobase of transcript per million mapped reads) was calculated by normalizing the raw read counts to the length of the gene and further by the total number of mapped reads for each fastq file. The distribution of RPMK values of the rac prophage genes was plotted as a boxplot, along with those of the bgl operon genes and lacI as reference genes. Because differential expression was not a goal of this study, more state-of-the-art normalization methods such as those used by EdgeR or DEseq were not required.

**Western blotting.** Total protein from *E. coli* K-12 cells was prepared and quantified using a bicinchoninic acid (BCA) assay, and 20 μg of total protein was loaded in a 15% SDS-polyacrylamide gel. The gel was subjected to electrophoresis at 120 V for 1 h, and proteins were transferred to a nitrocellulose membrane. Monoclonal anti-FLAG antibody (Sigma) was used to bind the specific protein to which the FLAG is tagged, and the signal was detected using horseradish peroxidase (HRP)-conjugated anti-mouse IgG. HRP luminescence was further detected by West Dura reagent (Thermo Scientific). Digital images of the blots were obtained using an LAS-3000 Fuji imager.

**Chromatin immunoprecipitation.** Immunoprecipitation was performed by the method of Kahramanoglu et al. (20) except that cell lysis and DNA shearing were coupled together using a Bioruptor (Diagenode) with 35 cycles (1 cycle consisting of 30 s on and 30 s off) at a high setting. Immunoprecipitated samples were quantified with specific primers for the 123-bp intergenic region and a random primer (wso), which is not part of the rac prophage, using quantitative PCR. The fold enrichment was calculated using 2−ΔΔCt by the method of Mukhopadhyay et al. (21).

**RacR purification.** RacR was cloned between the Ndel and XhoI restriction sites in a pET28a expression vector with the C-terminal His tag. After confirmation of its sequence and orientation, this plasmid was transformed in the expression strain C41(DE3). A single colony of the C41 strain containing the pET28a:racR plasmid was inoculated in 5 ml LB containing 100 μg/ml ampicillin. This overnight culture was diluted to a 1:100 ratio in 10 ml of fresh LB for raising the secondary inoculum. When the secondary culture reached an OD600 of 0.4, it was seeded into 1 liter of fresh LB in a 3-liter baffled flask at 37°C. The culture reached an OD600 of 0.6, RacR expression was induced by adding isopropyl-β-D-thiogalactopyranoside (IPTG) at a final concentration of 100 μM, and the flask was incubated at 25°C for 5 h. The culture was harvested, and the cells were resuspended in 100 ml of lysis buffer (50 mM Tris [pH 8.5], 500 mM NaCl, 5% glycerol, 1% NP-40, 1× Sigma protease inhibitor cocktail). The resuspended cells were sonicated for 30 cycles (1 cycle consists of 30 s on and 30 s off). Further, the lysate was passed through equilibrated 1 ml prepacked Histrap column (Invitrogen) at a flow rate of 0.5 ml/min. The column was then washed with 50 ml of elution buffer (50 mM Tris [pH 8.5], 500 mM NaCl, 5% glycerol) containing 10 mM imidazole and then with 20 ml of elution buffer containing 50 mM imidazole and 100 mM imidazole. Finally, RacR was eluted with 10 ml of elution buffer containing 250 mM imidazole. Purified RacR was further passed through a Superdex 200 10/300 size exclusion column, which was preequilibrated with the same elution buffer without imidazole.

**Thermal shift assay.** DNA (0.3 μM) ydaS with 189 bp upstream of it, including a portion of racR, racR-IGR-ydaS or random DNA was mixed with 3 μM purified RacR in the presence of 20× Sypro orange (Sigma-Aldrich), and the final volume of the reaction mixture was adjusted to 20 μl with RacR elution buffer. Three replicates of each sample were loaded in a 384-well plate and sealed with an optical adhesive cover. The fluorescence spectrum in the 635-nm–640-nm bin was recorded using ABI Via7 PCR with the standard melt curve experiment setting in which the temperature ranged from 20°C to 95°C at the rate of 1°C per min. The denaturation temperature (Td) was reported as the temperature at which the maximum dF/dT was recorded, where dF/dT is the rate of change in Sypro orange fluorescence (F) with respect to the temperature (T). The data were processed and plotted using a custom R script to calculate dF/dT.

**Electrophoretic mobility shift assay.** The entire 123-bp IGR was PCR amplified and gel purified. A 6% polyacrylamide gel was prepared from 40% acrylamide-bisacrylamide (80:1) stock and allowed to bind buffer (10 mM Tris [pH 8], 10 mM EDTA, 1 M NaCl, 1 mM dithiothreitol [DTT], 50% glycerol, 0.1 mg/ml bovine serum albumin [BSA]) in a final volume of 20 μl in 0.2-ml PCR tubes. These tubes were incubated at room temperature for 1 h. After incubation, samples were mixed with 2.2 μl of 10× loading dye (10 mM Tris [pH 8], 1 mM EDTA, 50% glycerol, 0.001% bromophenol blue, 0.001% xylene cyanol) and run at 70 V in room temperature for 90 min. The gel was stained using SYBR green (Thermo Scientific) for 15 min. The gel was subjected to electrophoresis at 120 V for 1 h, and proteins were transferred to a nitrocellulose membrane. Monoclonal anti-FLAG antibody (Sigma) was used to bind the specific protein to which the FLAG is tagged, and the signal was detected using horseradish peroxidase (HRP)-conjugated anti-mouse IgG. HRP luminescence was further detected by West Dura reagent (Thermo Scientific). Digital images of the blots were obtained using an LAS-3000 Fuji imager.

**Promoter activity.** The promoter activity of the ydaS IGR was monitored by transforming the pUA66:IGR-gfp-mut2 construct in *E. coli* K-12 cells and examining the resulting colonies on LB plates containing 100 μg/ml ampicillin. Overnight culture containing the plasmid in the respective background strain was inoculated at a ratio of 1:100 in a 96-well flat transparent black plate (Corning) in wells in a total volume of 200 μl. The optical density (OD600) and the GFP intensity (excitation at 485 nm and emission at 510 nm) were measured using the Tecan multimode reader at every 16-min interval with continuous shaking in between at 37°C. The background optical density is subtracted by using the optical density obtained from the blank well. The background fluorescence intensity was subtracted by using the intensity obtained from the strain that has the promoterless empty vector. Promoter activity (PA) was calculated as the rate of change in the GFP intensity normalized by the average OD for the given time point as follows: PA = smoothed dGFP/dt / smoothed (OD600) (22). Data processing and analysis were done using custom R script.
Accession number(s). RNA-seq data have been deposited in the GEO database under accession number GSE104504. Additional accession numbers are listed in Table S3 in the supplemental material.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/mSphere.00392-17.

FIG S1, TIF file, 1.2 MB.
FIG S2, TIF file, 2.5 MB.
FIG S3, TIF file, 1.5 MB.
FIG S4, TIF file, 0.6 MB.
FIG S5, PDF file, 0.3 MB.
TABLE S1, PDF file, 0.1 MB.
TABLE S2, PDF file, 0.1 MB.
TABLE S3, PDF file, 0.1 MB.

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