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Regulation of the Swarming Inhibitor *disA* in *Proteus mirabilis*

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The *disA* gene encodes a putative amino acid decarboxylase that inhibits swarming in *Proteus mirabilis*. 5′ rapid amplification of cDNA ends (RACE) and deletion analysis were used to identify the *disA* promoter. The use of a *disA-lacZ* fusion indicated that $FlhD_4C_2$, the class I flagellar master regulator, did not have a role in *disA* regulation. The putative product of DisA, phenethylamine, was able to inhibit *disA* expression, indicating that a negative regulatory feedback loop was present. Transposon mutagenesis was used to identify regulators of *disA* and revealed that *umoB* (*igaA*) was a negative regulator of *disA*. Our data demonstrate that the regulation of *disA* by UmoB is mediated through the Rcs phosphorelay.

Proteus mirabilis is a Gram-negative bacillus and a causative agent of urinary tract infections in patients with abnormal urethras or requiring long-term catheterization (1, 2, 3). *P. mirabilis* is also known for its ability to swarm, a form of flagellum-mediated surface motility (4, 5). In liquid medium, *P. mirabilis* exists as a vegetative, peritrichously flagellated swimming cell. However, 3 to 4 h after being placed on a solid surface, the vegetative swimming cells differentiate into elongated, multinucleate, aseptate, hyperflagellated swarmer cells, as reviewed in reference 6. The swarmer cells aggregate to form multicellular rafts and move concentrically away from the central inoculum for approximately 1 to 2 h before dedifferentiating back to vegetative swimming cells (7). This cycle of differentiation and consolidation gives *P. mirabilis* its characteristic bull's-eye appearance on agar plates (4).

Flagellar biogenesis is tightly controlled in *P. mirabilis* through a hierarchically tiered regulatory cascade consisting of class I, II, and III gene clusters (reviewed in reference 8). Class I consists solely of the flagellar master regulator *flhDC*. The $FlhD_4C_2$ heterohexamer is the master swarming regulator and activates transcription of class II genes (9, 10, 11). Class II is comprised of genes needed to form the hook-basal body structure of the flagella as well as *fliA*, encoding the swarming sigma factor σ^{28}, and *flgM*, the corresponding anti-sigma factor. σ^{28} is responsible for transcribing class III genes, including genes involved in chemotaxis, and the structural genes of the flagellar filament and motor. The energy expenditure to fully flagellate a swarmer cell and the cyclic aspect of swarming require that swarming be a tightly regulated process. Several signals inducing differentiation, such as the inhibition of flagellar rotation, accumulation of putrescine, and O-antigen contact with a solid surface, have been identified; however, the signals responsible for consolidation are poorly understood (12, 13, 14, 15).

A novel regulator of swarming, *disA*, that bears homology to aromatic amino acid decarboxylases was discovered by Stevenson et al. (16). Disruption of the *disA* gene resulted in a hyperswarming phenotype, whereas overexpression completely abolished swarming (16). Currently, the mechanism by which DisA inhibits swarming is unknown, but it inhibits $FlhD_4C_2$ at the posttranslational level, possibly by interfering with multimer formation (16). In addition, the mechanism of DisA-mediated inhibition is conserved in other Gram-negative enterics, in which DisA overexpression inhibited motility (17). The actual biochemical function of DisA is currently unknown, and metabolomic analysis of both the *disA* mutant and overexpressing strains did not reveal significant changes in the cellular levels of decarboxylated amino acids. In addition, the use of purified DisA and all possible amino acids did not reveal any products. However, the strong homology of DisA to tyrosine/phenylalanine decarboxylases, together with the fact that phenethylamine, but not tyramine, mimics the effect of DisA overexpression, suggests that DisA is a phenylalanine decarboxylase.

This study further defines the *disA* locus by identifying the transcriptional start site and begins the process of elucidating the regulation of *disA*. 5′ rapid amplification of cDNA ends (RACE) analysis and transcriptional *lacZ* fusions demonstrate that *disA* transcription begins at a thymine residue 70 bp upstream of the DisA start codon. Use of a *disA-lacZ* fusion demonstrated that $FlhD_4C_2$ does not have a significant role in *disA* expression. Transposon mutagenesis was used to identify UmoB as a negative regulator of *disA*. The *umoB* gene product is a negative regulator of the Rcs phosphorelay and has been previously implicated by our lab and others in swarming regulation (15, 18, 19, 20, 21, 22, 23, 24, 25). Our data indicate that the effect of the *umoB* mutation on *disA* expression is dependent upon the Rcs phosphorelay system. Taken together, our data indicate that a complex network is responsible for regulation of *disA*, allowing the cell more-precise control over the energy-intensive process of swarming.

MATERIALS AND METHODS

Bacterial growth conditions. The bacterial strains and plasmids utilized are listed in Table 1. *P. mirabilis* and *Escherichia coli* were grown in Luria-Bertani (LB) broth (10 g tryptone, 5 g yeast extract, 5 g sodium chloride per liter) at 37°C with shaking at 250 rpm. For plate growth, *E. coli* and nonswarming *P. mirabilis* strains were grown on 1.5% agar; swarming strains of *P. mirabilis* were plated on 3% agar to inhibit motility. Concentrations of antibiotics for selection for *E. coli* were as follows: 25 μg/ml for streptomycin and chloramphenicol, 20 μg/ml for kanamycin, and 100 μg/ml for ampicillin. Concentrations of antibiotics for selection for *P. mirabilis* were as follows: 35 μg/ml streptomycin, 100 μg/ml chloramphenicol, 300 μg/ml ampicillin, 20 μg/ml kanamycin, and 15 μg/ml tet-

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TABLE 1 Strains and plasmids

Strain or plasmid	Genotype or characteristics[a]	Source or reference
Strains		
E. coli		
XL1	*endA1 gyrA96*(Nal^r) *thi-1 recA1 relA1 lac glnV44* F′ *hsdR17*(r_K^- m_K^+)	Laboratory stock
CC118	*araD139* Δ(*ara-leu*)*7697* Δ*lacZ74 phoA*Δ*20 galE galK thi rpsE rpoB argE*(Amp) *recA1* λ*pir*	31
SM10 λ*pir*	*thi thr leu tonA supE recA* RP4-2Tc::Mu Kan^r λ*pir*	32
EC100D	TransforMax EC100D *pir*$^+$ electrocompetent *E. coli*: *F*$^-$ *mcrA* Δ(*mrr-hsdRMS-mcrBC*) ϕ80d*lacZ*ΔM15 Δ*lacX74 recA1 endA1 araD139* Δ(*ara-leu*)*7697 galU galK* λ$^-$ *rpsL* (Str^r) *nupG pir*$^+$ (*DHFR*)	Epicentre
DH5α	F$^-$ ϕ80dlacZΔM15 Δ(lacZYA-argF)U169 endA1 recA1 hsdR17(r_K^- m_K^-) deoR thi-1 supE44 λ$^-$ gyrA96 relA1	Laboratory stock
P. mirabilis		
PM7002	Wild type; Tet^r	ATCC
PM2199	PM7002 *disA*::mini-Tn*5lacZ*; Kan^r	Laboratory stock
BB1	PM7002 *umoB*::mini-Tn*5*-Kan^r/pQF50 + P_{disA} *−1206 to +39-lacZ*	This study
BB2	PM7002 *umoB*::mini-Tn*5*-Kan^r *rcsB*::Str^r/pQF50 + P_{disA} *−1206 to +39-lacZ*	This study
BB3	PM7002 *umoB*::mini-Tn*5*-Kan^r *rcsC*::Str^r/pQF50 + P_{disA} *−1206 to +39-lacZ*	This study
BB4	PM7002 *flhC* mutant/pQF50 + P_{disA} *−1206 to +39-lacZ*	This study
BB5	PM7002 *rcsB*::Str^r/pQF50 + P_{disA} *−1206 to +39-lacZ*	This study
BB6	PM7002 *rcsC*::Str^r/pQF50 + P_{disA} *−1206 to +39-lacZ*	This study
Plasmids		
pQF50	Low copy number; Amp^r	28
pACYC184	Low copy number; Chl^r	33
pFDCH1	pACYC184 + *flhDC*	34
pKNG101	R6K-derived suicide vector; Str^r	26
pBB1	pQF50 + P_{disA} *−1206 to +39-lacZ*	This study
pBB2	pQF50 + P_{disA} *−69 to −1-lacZ*	This study
pBB3	pQF50 + P_{disA} *−1206 to +39*(Δ*-10*)*-lacZ*	This study
pUmoB	pACYC184 + *umoB*	Laboratory stock
pBB4	pKNG101 + *flhC* mutant (frameshift mutation)	This study
pUBK	pKNG101 + *umoB*::Kan^r	15
pRcsB	pKNG101 + *rcsB*::Str^r	15
pRcsC	pKNG101 + *rcsC* (internal fragment)	15
pDisA	pKNG101 + *disA* (internal fragment)	Laboratory stock
pMDA	pACYC184 + *disA*	Laboratory stock

[a] Nal^r, nalidixic acid resistant; Kan, kanamycin; Str, streptomycin; Tet, tetracycline; Amp, ampicillin; Chl, chloramphenicol.

racycline. A total of 12 μg/ml 5-bromo-4-chloro-indolyl-β-D-galactopyranoside (X-Gal) was used to observe blue and white colonies unless otherwise stated.

Construction of plasmids and strains. All plasmids were introduced by electroporation into PM7002 as follows. PM7002 was grown in 30 ml of LB to an optical density at 600 nm (OD_{600}) of 0.4 to 0.6, and cells were harvested by centrifugation at 3,000 × *g* for 5 min at 4°C. Pellets were washed and resuspended in cold 10% glycerol with a final resuspension volume of 60 μl per electroporation. Cells were electroporated in cold cuvettes (Bio-Rad Gene Pulser cuvette; 0.2-cm diameter) using a Bio-Rad MicroPulser electroporator set to the E2 value for bacterial electroporation. Cells were recovered from the cuvette using 200 μl of prewarmed LB broth and incubated with shaking at 37°C for a minimum of 3 h for all plasmids harboring ampicillin resistance and a minimum of 1 h for all other antibiotics. Cells were plated on LB with appropriate antibiotic and agar concentrations and incubated at 37°C to allow growth of transformants.

For PCR amplification of selected genes, Phusion Hot Start II high-fidelity DNA polymerase (Thermo Scientific) was used. Validation of vector constructs and identification of transposon insertion sites were done through Beckman Coulter QuickLane sequencing according to the recommendations of the manufacturer. All primers are listed in Table 2.

To construct pBB1, primers PdisA Fwd and PdisA Rev were used to amplify the 1,245-bp region upstream of *disA*. The product was ligated into the SmaI site of pQF50, verified, and used to transform PM7002. Plasmid pBB2 was constructed by annealing 1 μg disPro Full Fwd and disPro Full Rev at 72°C for 10 min. The product was digested with SalI and BamHI and ligated into pQF50 digested with the same enzymes. The plasmid was validated by sequencing before transformation into PM7002. Plasmid pBB3 was constructed by performing site-directed mutagenesis on pBB1 using the primers dis-10Fwd and dis-10Rev. The QuikChangeII site-directed mutagenesis kit (Agilent Technologies) was used to mutate the eight base pairs at the putative −10 region upstream (TATATCAT to CGCCGGCC) as described in the user manual with the following modifications: 54 ng of pBB1 isolated from XL1 was used as a double-stranded DNA (dsDNA) template. Eighteen rounds of amplification were performed at 68°C with 1 min of extension per 500 bp (16.5 min). A total of 1 μl of 10 μM dis-10Fwd and dis-10Rev primers was used in the reactions. EC100D was transformed with 1 μl of the mutagenesis product and plated on 1.5% LB agar plates containing 100 μg/ml ampicillin. Plasmid pBB4 was constructed by digesting pBC plus *flhDC* with EcoNI, which digests once in the middle of the *flhC* gene. The product was blunt ended with T4 polymerase (Roche) as described by Promega. The blunt-ended vector was ligated to itself and digested a second time with EcoNI to enrich the sample for plasmids that had been successfully religated in the first reaction. This product was transformed into XL1 and selected by plating on chloramphenicol. Sequencing validated the presence of the single base pair insertion in *flhC* that resulted from religation. pBC with the *flhC* mutant was digested with PvuII and SalI, and the excised product was ligated to the suicide vector pKNG101 digested by SmaI with SalI. The proper *flhC* mutation in pBB4 was validated by sequencing before transformation into PM7002.

Strain BB1 was obtained by transposon mutagenesis of PM7002/pBB1 as described in "Transposon mutagenesis." BB4 was constructed by conjugating PM7002 with SM10 harboring pBB4. Exconjugants were plated on tetracycline and streptomycin to select for *P. mirabilis* containing a Campbell-type insertion of pBB4. A colony was grown in LB broth in the absence of antibiotics and plated on 10% sucrose to identify colonies that had successfully excised pKNG101. Recombinants containing the *flhC* mutant allele were validated by Southern blotting and transformed with pBB1. Strain BB1 was cured of pBB1 and then mated with SM10 containing pRcsB or pRcsC to obtain the *umoB rcsB* and *umoB rcsC* double mutants. Mutants were transformed with pBB1 to yield BB2 and BB3.

5′ RACE. 5′ RACE was performed on 5 μl PM7002 RNA according to the 5′ RACE system for rapid amplification of cDNA ends, version 2.0 (Invitrogen), methods section with a few modifications. cDNA was synthesized according to the alternative protocol for first-strand cDNA synthesis. Nested PCR was performed using the AUAP primer provided and GSP3 in 40 cycles with 30 s of annealing at 59°C and 45 s of extension using Phusion Hot Start II high-fidelity DNA polymerase. DNA was ligated to the EcoRV site of pBC and transformed into DH5α for blue/white screening. White colonies were cultured, and insertion of 5′ RACE products was verified by restriction digestion. Clones harboring inserts were sequenced using the universal T7 primer to identify potential transcriptional start sites. pBB2 and pBB3 were constructed based on the sequences of the cloned 5′ RACE products to determine the transcriptional start site.

Transposon mutagenesis. Strain BB1 was transformed by electroporation with EZ-Tn5 <Kan-2> Tnp Transposome (Epicentre). Transformants were selected on 3% LB containing ampicillin, kanamycin, and X-Gal (60 μg/ml). Colonies with increased or decreased blue color were cultured as described above and assayed for β-galactosidase activity (see below). Southern blotting was performed to identify the segment of the

TABLE 2 Primers

Name	Sequence (5′–3′)	Use
GSP1	GGATGACGTGCAATCGCCATCGGCAG	5′ RACE
GSP2	GCTGATAATGTTTTTAAC	5′ RACE
GSP3	CCAGCAGCTAATGAATAA	5′ RACE
disPro Full Fwd	CCCAAGCGTCGACCGTAAAATAAACTCAATTCTGATTAAAATTGATAAC AAAAATTTATATATGGATCCTTGGC	$P_{disA^{-69-1}}$::*lacZ*
disPro Full Rev	GCCAAGGATCCATATATAAATTTTTGTTATCAATTTTAATCAGAATTGAG TTTATTTTACGGTCGACGCTTGGG	$P_{disA^{-69-1}}$::*lacZ*
PdisA Fwd	ATCAAGGATCCATGAAGATATCGCTTTACCG	$P_{disA^{-1206-+39}}$::*lacZ*
PdisA Rev	ATCAAGCGTCGACCTGCAGCACTCAGACAGG	$P_{disA^{-1206-+39}}$::*lacZ*
dis-10Fwd	GATTCTTACTCATCATGTAGCGCCGGCCTTAAAAAGAGACTAATTATT	$P_{disA^{-1206-+39\Delta-10}}$::*lacZ*
dis-10Rev	AATAATTAGTCTCTTTTTAAGGCCGGCGCTACATGATGAGTAAGAATC	$P_{disA^{-1206-+39\Delta-10}}$::*lacZ*

chromosome where the transposon was inserted. This region was subcloned into pBC and sequenced using primers provided with the transposome to identify the specific site of insertion.

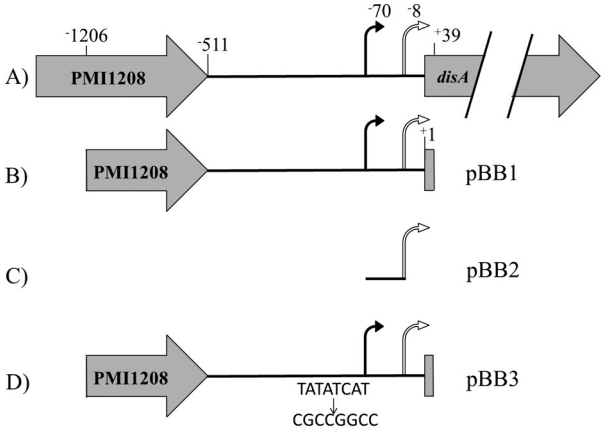
Construction of an *umoB* disruption by a Campbell-type insertion. An *umoB* disruption was constructed in PM2199 *disA*::mini-Tn*5lacZ* by cloning a PCR-derived fragment internal to the *umoB* coding region obtained using the primers 5′-CGTCATCTAGAGCGGTAGAGATCCATA TTCC and 5′-CGTCAGGATCCGGCCCTTGCTTGATAACATG into the suicide plasmid pKNG101 (26). The construct was then mobilized into PM2199 by a filter mating with *E. coli* SM10 containing the plasmid. Exconjugants were selected on LB plates containing 35 μg/ml streptomycin and 15 μg/ml tetracycline. The correct disruption of *umoB* was verified by Southern blotting.

β-Galactosidase assays. Overnight cultures were grown, and optical densities (ODs) were normalized to the lowest density culture. A total of 200 μl of normalized culture was spread on a 1.5% LB plate and incubated at 37°C for 2 or 4 h. These time points were chosen to assess expression before (T2) and at the peak of (T4) swarming. Cells were collected from plates either by resuspension in 1 ml of fresh LB (two-hour harvest) or by washing one-quarter of the plate with 500 μl of fresh LB (four-hour harvest). The OD_{600} was recorded, and a portion of the culture was pelleted and frozen at −20°C overnight. Pellets were lysed by chloroform/SDS treatment and assayed as previously described (27). Plates with 25 mM phenethylamine (PEA) were made using an appropriate volume of phenethylamine (Sigma-Aldrich catalog no. 241008 [50 ml] or Acros Organics catalog no. 156491000), and pH was adjusted to 7.

RESULTS

Identification of the *disA* promoter. The 5′ end of the *disA* transcript was identified by 5′ RACE performed on total RNA harvested from wild-type PM7002. Sequencing of the 5′ RACE PCR products returned two potential transcriptional start sites, one located 8 bp upstream of the *disA* open reading frame (ORF) (Fig. 1A, open arrow, designated −8) and a second site located 70 bp upstream of the *disA* ORF (Fig. 1A, closed arrow, designated −70). To determine if active promoters were present upstream of these potential start sites, various fragments were cloned into pQF50 (28) to create transcriptional *lacZ* fusions. These constructs were transformed into PM7002 and assayed for β-galactosidase activity (Fig. 2).

Plasmid pBB1 contains a fragment extending from −1,206 to +39 relative to the ATG start of the *disA* gene (Table 1; Fig. 1B). This fragment contains both potential transcriptional start sites as well as part of the *disA* and PMI1208 open reading frames (Fig. 1B). The expression of β-galactosidase from pBB1 was 816-fold and 50-fold higher than in cells containing the pQF50 vector with no promoter inserted at 2 and 4 h after plating, respectively



E)

GACTATGGTAAAAATATTATAATAAGTTTTGAAAAA

TAGCATAAATCAAAATTGATAATGAAATTTATAAAC

TAATTAATAAAACTAAATCATTTATCATGAAAAAGT

AAAAACTTTAGAACGAACTTGGAAAATCTTATCTG

ATATCAATAAAATAAAGAAGATTCTTACTCATCATG

TAGTATATCATTTAAAAAGA**G**ACTAATTATTTACGT

AAAATAAACTCAATTCTGATTAAAATTGATAACAAA

AATTTATATA**T**AGGTGCGATG

FIG 1 Construction of transcriptional *lacZ* fusions. (A) The region upstream of *disA* is shown with the noncoding region comprised of 511 bp, designated −511 to +1 with the *disA* ORF beginning at +1. The bent arrows indicate potential transcriptional start sites identified by 5′ RACE. The black arrow (−70) is located 70 bp upstream of the *disA* ORF. The open arrow (−8) is located eight base pairs upstream of the *disA* ORF. (B) Plasmid pBB1 contains a region extending from 39 bp into the *disA* gene to 1,206 bp upstream of the *disA* gene (including 695 bp from the PMI1208 gene) that is fused to a promoterless *lacZ* gene in pQF50. (C) Plasmid pBB2 contains the region from −69 to −8 that is fused to *lacZ* in pQF50. (D) Plasmid pBB3 is identical to pBB1 but contains an 8-bp substitution at the putative −10 region, changing the sequence from TATATCAT to CGCCGGCC (see Materials and Methods) (Table 1). (E) Sequence upstream of the *disA* open reading frame. The start codon is underlined, and the putative transcriptional start sites located 8 bp and 70 bp upstream of the ATG start codon are shown in bold font. The proposed −10 promoter element is shaded. Three Rcs binding sites with homology to the *E. coli* consensus sequence (TAAGAATAATCCTA) are underlined, and mismatched bases are noted with lighter font.

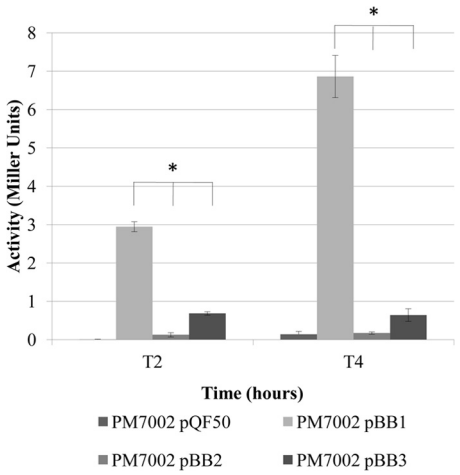


FIG 2 Transcriptional activity of promoter fragments. 5′ RACE returned two potential transcriptional start sites, −70 and −8 (Fig. 1). Various promoter fragments were cloned upstream of a promoterless *lacZ* gene in pQF50 as shown in Fig. 1. LB agar plates were inoculated with overnight cultures of each strain that were adjusted to identical optical densities, and cells were harvested off plates at 2 and 4 h after plating. The data shown are representative of two independent experiments, with samples assayed in triplicate. An asterisk indicates a *P* value of <0.05.

(Fig. 2). PM7002 containing plasmid pBB2 with the *disA* region from −69 to −1 exhibited no β-galactosidase activity, indicating the absence of a promoter upstream of the −8 transcriptional start site (Fig. 1C and 2). This indicated that the functional *disA* promoter region was upstream of the transcriptional start site originating 70 bp upstream of the *disA* ATG start codon. To verify this, site-directed mutagenesis was used to change base pairs at the −10 sequence from TATATCAT to CGCCGGCC. The resulting plasmid pBB3 contains these altered base pairs in the context of the full-length *disA* region present in pBB1. Plasmid pBB3 exhibited a 4.3-fold reduction and a 10.7-fold reduction in β-galactosidase activity 2 and 4 h after being plated on agar surfaces, respectively, compared to pBB1, indicating that altering nucleotides in the −10 region severely decreased the overall promoter activity.

$FlhD_4C_2$ does not regulate *disA*. The class I activator $FlhD_4C_2$ has a central role in activating gene expression during swarming, and our lab previously demonstrated that *disA* expression increases during swarming (16). To address the role of $FlhD_4C_2$ in regulating *disA* expression, a null allele in *flhC* was constructed as described above (Materials and Methods), resulting in strain BB4. As expected, BB4 was unable to swarm (data not shown). The loss of *flhC* did not have a statistically significant effect on the expression of a *disA-lacZ* fusion (pBB1) when cells were assayed either 2 or 4 h after plating on agar surfaces (data not shown). Furthermore, when *flhDC* was overexpressed from a medium-copy-number plasmid, *disA* expression was not altered in a statistically significant manner (data not shown). These data indicate that $FlhD_4C_2$, the master regulator of swarming, does not have a role in regulating *disA* expression.

Role of phenethylamine and autoregulation in *disA* expression. The predicted product of the DisA decarboxylase is phenethylamine (PEA), and previous work demonstrated that exogenous PEA inhibited swarming and flagellar gene expression in a manner similar to *disA* overexpression (16). We assessed the effect of PEA on *disA* expression in PM2199, containing a single-copy transcriptional *disA-lacZ* fusion generated by the insertion of mini-Tn*5lacZ1* into the chromosomal copy of *disA*. This strain was used because the *disA* gene is inactivated, thereby reducing the intracellular levels of the putative product phenethylamine and allowing for a more sensitive assessment of the effects of exogenous phenethylamine. The presence of various concentrations of phenethylamine decreased *disA* expression in a dose-dependent manner at T4, with 3.1-fold repression seen at 25 mM, 2.7-fold repression at 16 mM, 1.7-fold repression at 8 mM, and 1.3-fold repression at 4 mM (Fig. 3A). The presence of phenethylamine had little effect in cells at T2 (Fig. 3). To determine if *disA* expression was subject to autoregulation, the *disA* gene was overexpressed in *trans* (Fig. 3B). The overexpression of *disA* in PM2199 decreased *disA-lacZ* expression 1.3-fold at 4 h after plating on agar but had no effect at 2 h.

UmoB is a negative regulator of *disA*. To identify potential regulators of *disA*, transposon mutagenesis was used to create random mutations in PM7002/pBB1 and colonies were screened on X-Gal plates for those with increased expression from the *disA*-

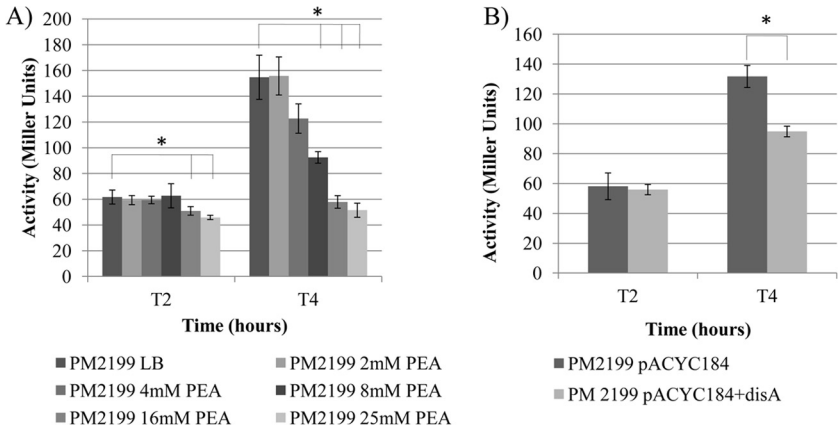


FIG 3 Role of autoregulation in *disA* expression. (A) The effect of phenethylamine on *disA* expression was assayed in PM2199 containing a single-copy *disA-lacZ* fusion generated by an insertion of mini-Tn*5lacZ1* into the *disA* coding region. In this strain, the *disA* gene is inactivated by the transposon insertion. (B) *disA-lacZ* expression is measured in PM2199 cells containing either the vector pACYC184 or pACYC184 plus *disA*. Cells were harvested off plates as described for Fig. 2. Data shown are representative of two independent experiments, with samples assayed in triplicate. An asterisk indicates a *P* value of <0.05.

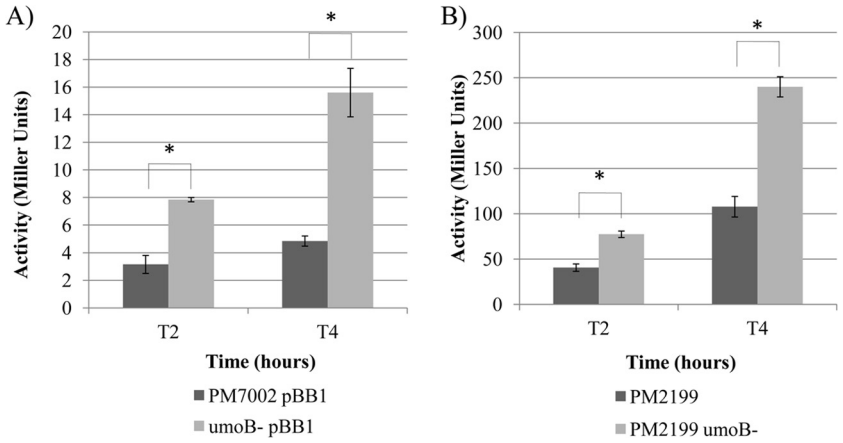


FIG 4 *disA* expression in *umoB* mutants. (A) The expression of *disA-lacZ* was monitored from plasmid pBB1 in wild-type PM7002 and an *umoB*::Kan mutant. (B) The effect of the *umoB* mutation on a single-copy *disA-lacZ* fusion was examined in PM2199 or the corresponding *umoB*::Str mutant. The expression of *disA* was measured by β-galactosidase expression in cells harvested 2 and 4 h after plating on agar plates as described for Fig. 2. Data shown are representative of two independent experiments, with samples assayed in triplicate. An asterisk indicates a *P* value of <0.05.

lacZ fusion. This yielded an insertion in the *umoB* (*igaA*) gene encoding an integral membrane protein that has been shown in members of the *Enterobacteriaceae* to act as an inhibitor of the Rcs phosphorelay (reviewed in reference 29). The transposon insertion in *umoB* resulted in a 2.5- to 3-fold increase in *disA* expression (Fig. 4). The levels of *disA* expression were reduced to those of the wild type in the presence of a plasmid containing the cloned *umoB* gene (data not shown). An *umoB* mutation (*umoB*::Str) was also independently constructed in PM2199, in which the *disA-lacZ* fusion is in a single copy, and a similar 2.5-fold increase in expression was observed (Fig. 4).

The *umoB* mutation alters *disA* expression via the Rcs phosphorelay. The effect of UmoB on *disA* expression, as well as its established role as an inhibitor of the Rcs phosphorelay, led us to investigate if the Rcs phosphorelay was involved in regulating *disA*. Single mutations in *rcsB* and *rcsC*, the Rcs response regulator and sensor kinase, and the *rcsB umoB* and *rcsC umoB* double mutants were constructed and designated BB5, BB6, BB2, and BB3, respectively (Table 1). Activity of *disA* was measured in samples harvested 2 h and 4 h after plating by β-galactosidase assays, as described earlier. The data demonstrate that an *rcsB* or *rcsC* single mutant had no effect on *disA* expression; however, the *umoB rcsB* and *umoB rcsC* double mutants mitigated the effect of an *umoB* single mutant, returning *disA* expression to wild-type levels (Fig. 5).

DISCUSSION

In this study, regulation of the *disA* locus was characterized by using both single-copy and plasmid-based transcriptional *lacZ* fusions, which allowed us to (i) identify the promoter region, (ii) determine that $FlhD_4C_2$ does not have a role in *disA* regulation, (iii) address the role of autoregulation via phenethylamine and *disA* overexpression, and (iv) identify a regulatory mutation in *umoB* that alters *disA* expression via the Rcs phosphorelay. Interestingly, the levels of β-galactosidase from the single-copy *disA-lacZ* fusion in PM2199 were higher than those from the *disA-lacZ* fusion in multicopy (pBB1) (compare Fig. 2 and 3). There are several possible explanations for this. First, the *lacZ* gene is translated from different ribosome binding sites (RBS) in each construct; the RBS in mini-Tn*5lacZ* is from the *trp* operon of *E. coli*, and in pBB1 (pQF50 vector), it is derived from the *lpp* gene. Second, there may be *cis*-acting regulatory sequences that are missing from the promoter region cloned into pBB1 that are required for full expression.

Mutations in *rcsB* and *rcsC* in conjunction with an *umoB* mutation demonstrated that the increased *disA* expression observed in an *umoB* mutant background was completely abrogated when

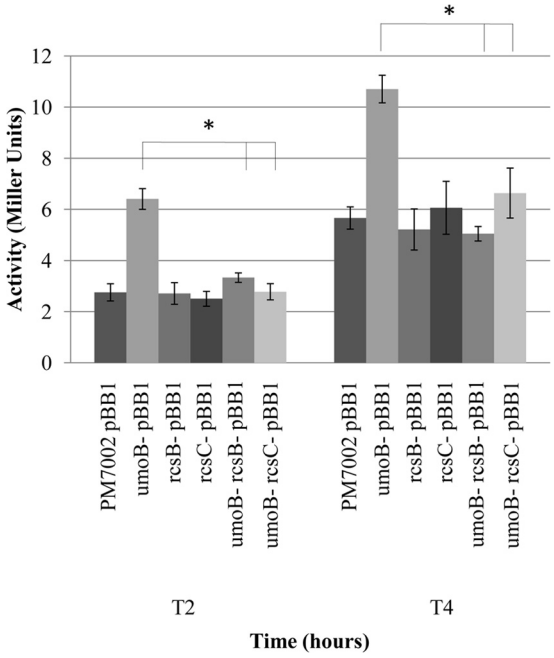


FIG 5 Regulation of *disA* via UmoB is through the Rcs phosphorelay. Expression of *disA* was measured by β-galactosidase expression from plasmid pBB1 in wild-type PM7002, an *umoB*::Kan mutant (BB1), an *rcsB*::Str mutant (BB5), an *rcsC*::Str mutant (BB6), and *rcsB umoB* and *rcsC umoB* double mutants (BB2 and BB3, respectively). Data shown are representative of at least two independent experiments, with samples assayed in triplicate. An asterisk indicates a *P* value of <0.05.

the Rcs phosphorelay was nonfunctional. The observation that *disA* expression was not decreased in either an *rcsB* or *rcsC* single mutant was unexpected but may be due to the presence of the *disA-lacZ* fusion in pQF50, where the effect of the Rcs phosphorelay, and specifically the RcsB response regulator, on *disA* expression may be masked by the multicopy nature of the *disA-lacZ* fusion. The increased RcsB activity in the *umoB* mutant may be enough to still see regulatory changes in the multicopy *disA-lacZ* fusion. In support of this, recent data from our lab have identified *disA* as an RcsB-activated gene by RNA-Seq analysis (our unpublished data). The fact that RcsB activated $FlhD_4C_2$ yet $FlhD_4C_2$ did not have a role in *disA* regulation indicates that RcsB may directly bind *disA* to mediate regulation. In support of this, there are three possible RcsB binding sites upstream of the *disA* −10 region (Fig. 1E).

Previous research and sequence homology led to the prediction that phenethylamine, the decarboxylated form of phenylalanine, or a similar molecule was the product of DisA (16). We have provided evidence that a negative feedback loop is present for *disA* regulation. First, *disA* expression was decreased in PM2199 when *disA* was overexpressed (Fig. 3). Second, the addition of exogenous phenethylamine inhibited *disA* expression (Fig. 3). However, the magnitude of the repression differed under each condition, 3-fold with phenethylamine at 25 mM versus 1.4-fold with *disA* overexpressed. This may be due to differences in the intracellular levels of phenethylamine, with higher levels present during growth with 25 mM and potentially lower levels when *disA* is overexpressed. Consistent with this, a strong dose-dependent effect on *disA* repression was observed with phenethylamine concentrations ranging from 4 to 25 mM (Fig. 3).

One purpose of this negative feedback loop may be to downregulate *disA* expression after the peak levels of expression have been reached at 3 to 4 h into the swarming cycle. The subsequent decrease in DisA activity would then prepare cells for the next cycle of swarming by relieving the inhibition of $FlhD_4C_2$. It is also possible that extracellular phenethylamine encountered in the environment may have a role in regulating *disA* expression. It has been proposed that phenethylamine concentrations in the low millimolar range may be encountered in the intestinal tract and that this may influence swarming in the intestine (30). A similar mechanism for the control of *disA* expression may exist in the urinary tract. However, this is highly speculative because, to our knowledge, the levels of phenethylamine in the urinary tract are unknown. The ability to fine-tune *disA* expression by both a negative feedback loop and the Rcs phosphorelay would provide more-precise control of *disA* expression required for the transition between the swarming and consolidation phases.

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