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The Two-Component GacS-GacA System Activates *lipA* Translation by RsmE but Not RsmA in *Pseudomonas protegens* Pf-5

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In *Pseudomonas* spp., the Gac-Rsm signal transduction system is required for the production of lipases. The current model assumes that the system induces lipase gene transcription mediated through the quorum-sensing (QS) system. However, there are no reports of a QS system based upon *N*-acyl homoserine lactones or the regulation of lipase gene expression in *Pseudomonas protegens*. In this study, we investigated the regulatory mechanism acting on *lipA* expression activated by the Gac-Rsm system in *P. protegens* Pf-5 through deletion and overexpression of *gacA*, overexpression of *rsmA* or *rsmE*, expression of various *lacZ* fusions, reverse transcription-PCR analysis, and determination of whole-cell lipase activity. The results demonstrated that the GacS-GacA (GacS/A) system activates *lipA* expression at both the transcriptional and the translational levels but that the translational level is the key regulatory pathway. Further results showed that the activation of *lipA* translation by the GacS/A system is mediated through RsmE, which inhibits *lipA* translation by binding to the ACAAGGAUGU sequence overlapping the Shine-Dalgarno (SD) sequence of *lipA* mRNA to hinder the access of the 30S ribosomal subunit to the SD sequence. Moreover, the GacS/A system promotes *lipA* transcription through the mediation of RsmA inhibiting *lipA* transcription via an unknown pathway. Besides the transcriptional repression, RsmA mainly activates *lipA* translation by negatively regulating *rsmE* translation. In summary, in *P. protegens* Pf-5, the Gac-RsmE system mainly and directly activates *lipA* translation and the Gac-RsmA system indirectly enhances *lipA* transcription.

Lipases (triacylglycerol acylhydrolases; EC 3.1.1.3) are ubiquitous enzymes which are produced by various plants, animals, and microorganisms. They usually catalyze the hydrolysis of esters formed from glycerol and long-chain fatty acids in aqueous solutions. They also catalyze other reactions in micro- or nonaqueous environments, such as esterification, alcoholysis, aminolysis, or transesterification (1, 2). Owing to their versatility, lipases are widely applied in various processes, such as the manufacture of food (3, 4), detergents (5, 6), biodiesel (7, 8), and fine chemicals (9, 10) as well as waste treatment (11). Of these, the most widely used lipases are those originating from the genus *Pseudomonas*, due to their excellent properties (1, 12–15).

Although numerous bacterial lipases have been identified and characterized in past decades, rather limited reports on the molecular mechanisms regulating lipase gene expression are available. The transcription of the lipase gene is directly regulated by LipQ-LipR (LipQ/R) in *Pseudomonas alcaligenes* or CbrA-CbrB (CbrA/B) in *P. aeruginosa*. LipQ/R and CbrA/B are two-component systems of the NtrB-NtrC family that bind to a specific upstream activator sequence (UAS) with the help of RNA polymerase σ^{54} (or RpoN) (13, 16–18). In addition, two quorum-sensing (QS) systems, the *las* system and the *rhl* system, which are organized in a hierarchical manner, in which the *las* system transcriptionally activates the *rhl* system, enhance *cbrAB* transcription in *P. aeruginosa* (16, 19–22).

As we know, the global Gac-Rsm signal transduction system also plays an important role in the regulation of lipase gene expression. The two-component system GacS-GacA (GacS/A) has been reported to have a positive effect on lipase gene expression in *Pseudomonas* spp. (16, 19, 23). This highly conserved global regulatory system comprises the inner membrane-bound sensor kinase, GacS, which recognizes an as-yet-unidentified environmental signal and is activated through autophosphorylation, and the cognate response regulator, GacA, which is activated by the phosphorylated GacS via a phosphorelay mechanism. The current model assumes that the activated GacA specifically initializes the transcription of several small regulatory RNAs (sRNAs) of a common family, termed RsmY and RsmZ in *P. aeruginos*a or RsmX, RsmY, and RsmZ in *P. protegens*. Multiple single-stranded GGA motifs are a feature common to all of these sRNAs, and these motifs have a high affinity for RNA-binding protein(s) RsmA (and RsmE) of the RsmA-CsrA family. They can relieve translational repression by titrating out these RNA-binding proteins, which bind to specific motifs (usually ANGGA or GGA) overlapping or near the Shine-Dalgarno (SD) sequence of target mRNA to obstruct translation initiation (24–27). RsmA normally represses the two QS systems of *P. aeruginos*a and, consequently, inhibits expression of a variety of extracellular products, including lipase. However, RsmA also induces the production of lipase via an unknown mechanism in *P. aeruginos*a (20, 28).

Given the less well-documented scenario of the regulation of lipase gene expression, the aim of this study was to investigate in more detail the role of the Gac-Rsm signal transduction system in lipase gene expression, using the well-characterized soil bacterium *P. protegens* Pf-5 (previously called *P. fluorescens* Pf-5) (29, 30) as the model organism and lipase production as the readout. The *lipA* product, LipA, an intracellular lipase, exhibits excellent stability and high activity at moderate temperatures, under alkaline conditions, and in the presence of heavy metal ions, surfactants, and organic solvents

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TABLE 1 Strains and plasmids used in this study

Strain or plasmid purpose and plasmid	Description	Reference or source
Strains		
E. coli		
Top10	*mcrA* Δ(*mrr-hsdRMS-mcrBC*) ϕ80*lacZ*ΔM15 Δ*lacX74 recA1 araD139*Δ(*ara-leu*)*7697 galU galK rpsL* (Str^r) *endA1 nupG*	Invitrogen
BL21(DE3)	F$^-$ *ompT hsdS*$_B$(r_B^- m_B^-) *dcm gal*(DE3)	Novagen
BL/pET-28a	BL21(DE3) with pET-28a; Km^r	This study
BL/pET-rsmE	BL21(DE3) with pET-rsmE; Km^r	This study
P. protegens		
Pf-5	Rhizosphere isolate; Ap^r	29
Pf3563	Δ*gacA* derivative of Pf-5; Ap^r	This study
Pf-5F3	pJQ003 conjugated into Pf-5; Gm^r	This study
Pf-5F4	pJQ004 conjugated into Pf-5; Gm^r	This study
Pf-5F5	pJQ005 conjugated into Pf-5; Gm^r	This study
Pf-5F6	pJQ006 conjugated into Pf-5; Gm^r	This study
Pf3563F3	pJQ003 conjugated into Pf3563; Gm^r	This study
Pf3563F4	pJQ004 conjugated into Pf3563; Gm^r	This study
Plasmids		
Triparental mating, pRK2073	Helper plasmid for triparental mating; Sp^r	37
Markerless deletion mutation		
pJQ200SK	Suicide vector with *sacB* counterselectable marker used for homologous recombination; Gm^r	33
pJQΔgacA	pJQ200SK carrying a 1.772-kb XbaI insert with a deletion in the coding region of *gacA*; Gm^r	This study
Overexpression		
pBBR1MCS-5	Broad-host-range vector; Gm^r	34
pBBR1Km	NcoI-BglII-digested kanamycin resistance cassette subcloned in pBBR1MCS-5 digested with the same endonucleases	This study
pBBRKm	pBBR1Km with a 1,280-bp BamHI-XbaI fragment harboring *lacI*^{q}-P_{lac}; Km^r	This study
pBBRKgacA	pBBRKm with a 655-bp BamHI-HindIII fragment harboring the coding region of *gacA*; Km^r	This study
pBBRKrsmA	pBBRKm carrying a 260-bp BamHI-HindIII fragment harboring the coding region of *rsmA*; Km^r	This study
pBBRKrsmE	pBBRKm carrying a 263-bp BamHI-HindIII fragment harboring the coding region of *rsmE*; Km^r	This study
pET-28a	Expression vector carrying an N-terminal His tag-thrombin-T7 tag configuration plus an optional C-terminal His tag sequence; Km^r	Novagen
pET-rsmE	pET-28a carrying a 262-bp NdeI-HindIII fragment harboring the coding region of *rsmE*; Km^r	This study
Plasmid-borne *lacZ* fusion		
pBBR001	pBBR1MCS-5 derivative with a translational *′lacZ* fusion; Gm^r	This study
pBBR002	pBBR1MCS-5 derivative with a transcriptional *lacZ* fusion; Gm^r	This study
pBBR003	pBBR001 derivative with a translational *lipA′-′lacZ* fusion; Gm^r	This study
pBBR004	pBBR002 derivative with a transcriptional *lipA-lacZ* fusion; Gm^r	This study
pBBR005	pBBR001 derivative with a translational *rsmA′-′lacZ* fusion; Gm^r	This study
pBBR006	pBBR001 derivative with a translational *rsmE′-′lacZ* fusion; Gm^r	This study
pBBR00A10U	pBBR001 derivative with a translational *lipA′*-A10U-*′lacZ* fusion; Gm^r	This study
pBBR00G11C	pBBR001 derivative with a translational *lipA′*-G11C-*′lacZ* fusion; Gm^r	This study
pBBR00G12A	pBBR001 derivative with a translational *lipA′*-G12A-*′lacZ* fusion; Gm^r	This study
pBBR00A14U	pBBR001 derivative with a translational *lipA′*-A14U-*′lacZ* fusion; Gm^r	This study
Chromosome-borne *lacZ* fusion		
pJQ003	pJQ200SK derivative with a translational *lipA′-′lacZ* fusion; Gm^r	This study
pJQ004	pJQ200SK derivative with a transcriptional *lipA-lacZ* fusion; Gm^r	This study
pJQ005	pJQ200SK derivative with a translational *rsmA′-′lacZ* fusion; Gm^r	This study
pJQ006	pJQ200SK derivative with a translational *rsmE′-′lacZ* fusion; Gm^r	This study

(31). In terms of its excellent properties, the regulatory mechanism of *lipA* expression is worthy of study and is of theoretical and practical significance. By deleting and/or overexpressing specific components of the Gac-Rsm regulatory cascade, our results demonstrated that the GacS/A system mainly activates *lipA* translation through RsmE binding to the ACAAGGAUGU sequence, which overlaps the SD sequence of *lipA* mRNA, to obstruct the interaction of the SD sequence with the 30S ribosomal subunit and, hence, translation initiation. Furthermore, the GacS/A system also promotes *lipA* transcription via a mechanism in which RsmA indirectly inhibits *lipA* transcription by an unknown pathway. In addition to the transcriptional repression, RsmA stimulates *lipA* translation by repressing the translation of *rsmE*, and we found that this translational control is the key regulatory pathway.

MATERIALS AND METHODS

Strains, plasmids, culture conditions, and general methods. The bacterial strains and plasmids used in this study are listed in Table 1. *P. protegens*

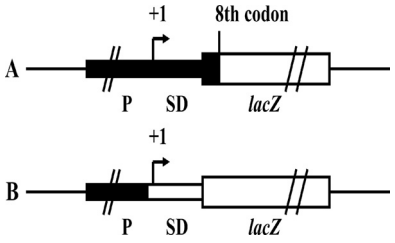


FIG 1 Schematic representation of *lacZ* translational fusion (A) and *lacZ* transcriptional fusion (B). (A) In the construct shown, the transcription of *′lacZ* is driven by the *x* promoter (P) and the mRNA produced is translated from the SD sequence of *x* mRNA to produce a hybrid protein with several N-terminal amino acids of X and a large functional C-terminal fragment of β-galactosidase. The translational fusion data can reflect the strength of the promoter and the efficiency of initiation of translation of the target gene. (B) In the construct shown, the transcription of *lacZ* is driven by the *x* promoter and the mRNA produced is translated by the SD sequence of *lacZ* mRNA to produce a wild-type β-galactosidase. The transcriptional fusion data can reflect the strength of the promoter of the target gene.

(28°C) and *Escherichia coli* (37°C) strains were propagated in liquid or solid (1.5%, wt/vol, agar) LB medium. The following antibiotics were used: for *P. protegens*, ampicillin (Ap; 100 μg/ml), gentamicin (Gm; 50 μg/ml), and kanamycin (Km; 40 μg/ml); for *E. coli*, spectinomycin (Sp; 100 μg/ml), gentamicin (50 μg/ml), and kanamycin (40 μg/ml). PrimeSTAR HS DNA polymerase, restriction enzymes, and a DNA ligation kit were purchased from TaKaRa Biotechnology (Dalian) Co., Ltd. (Dalian, China). Genomic DNA extraction, DNA gel extraction, and plasmid preparation were carried out with commercial kits (Omega Bio-Tek, Doraville, GA) according to the manufacturer's protocols. Oligonucleotide primers were synthesized by Wuhan Anygene Biological Technology Co., Ltd. (Wuhan, China). DNA sequencing was performed by Shanghai Sunny Biotechnology Co., Ltd. (Shanghai, China). All other routine manipulations were performed by standard methods (32).

Construction of suicide plasmid pJQΔgacA. PCR amplicons, including the homology arms 906 bp upstream (bp −921 to −16 relative to the translational start site) and 866 bp downstream (bp +407 to +1272 relative to the translational start site) of *gacA*, were used to produce the markerless deletion mutation cassette of the coding region by overlap PCR. An XbaI-digested mutant cassette was ligated into the suicide plasmid pJQ200SK (33) for markerless deletion of the *gacA* coding region; this was followed by *E. coli* Top10 transformation. Transformants were screened on LB plates containing 50 μg/ml gentamicin and further verified by colony PCR. Then, plasmid DNA was isolated and sequenced. The recombinant plasmid was named pJQΔgacA (Table 1).

Construction of expression plasmids. An NcoI- and BglII-digested kanamycin resistance cassette, amplified from plasmid pET-28a, was ligated into plasmid pBBR1MCS-5 (34) to create plasmid pBBR1Km (Table 1). Then, a PCR product carrying $lacI^q$-P_{lac}, obtained by using *E. coli* BL21(DE3) genomic DNA as the template, was digested with BamHI and XbaI and cloned into plasmid pBBR1Km to create plasmid pBBRKm (Table 1). Subsequently, PCR amplicons encoding the coding regions of the *gacA* (655 bp), *rsmA* (260 bp), and *rsmE* (263 bp) genes were digested with BamHI and HindIII and cloned into expression plasmid pBBRKm under the control of the *lacZ* promoter, creating recombinant plasmids pBBRKgacA, pBBRKrsmA, and pBBRKrsmE, respectively (Table 1). Similarly, the PCR amplicon encoding the coding region of the *rsmE* gene (262 bp) was digested with NdeI and HindIII and cloned into the expression plasmid pET-28a (Table 1), creating recombinant plasmid pET-rsmE (Table 1).

Construction of *lacZ* fusion plasmids. To construct *lacZ* translational and transcriptional fusions (Fig. 1), PCR amplicons of *′lacZ* (bp +22 to +3110 relative to the start site of translation), which lacked its own SD sequence and the first seven codons, and wild-type *lacZ* (bp −18 to +3110 relative to the start site of translation), which contained its own SD sequence, were amplified from *E. coli* BL21(DE3) genomic DNA. Then, BamHI- and HindIII-digested *′lacZ* and *lacZ* were cloned into plasmid pBBR1MCS-5 to create translational fusion plasmid pBBR001 and transcriptional fusion plasmid pBBR002, respectively (Table 1). Next, PCR amplicons of *lipA* (bp −613 to +18 relative to the start site of translation), *rsmA* (bp −517 to +7 relative to the start site of translation), and *rsmE* (bp −499 to +7 relative to the start site of translation) were digested with KpnI and HindIII and cloned into plasmid pBBR001, creating plasmids pBBR003, pBBR005, and pBBR006, respectively (Table 1). Similarly, PCR amplicons containing *lipA* (bp −613 to −12 relative to the start site of translation) were digested with KpnI and HindIII and cloned into plasmid pBBR002, and the resulting plasmid was named pBBR004 (Table 1). To clone *lipA′-′lacZ*, *lipA-lacZ*, *rsmA′-′lacZ*, and *rsmE′-′lacZ* fusions into the suicide plasmid for introduction into the chromosome, pBBR003, pBBR004, pBBR005, and pBBR006 were digested with SphI and BamHI and cloned into plasmid pJQ200SK, creating plasmids pJQ003, pJQ004, pJQ005, and pJQ006, respectively (Table 1). For the single-base substitution mutations of the ANGGAN motif in the SD sequence of *lipA* mRNA, a common forward primer and specific reverse primers carrying the desired substitutions (Table 2) were used to introduce the mutations A10U, G11C, G12A, and A14U (relative to the start site of translation) (35). Then, these amplicons were digested with KpnI and HindIII and cloned into plasmid pBBR001, creating recombinant plasmids pBBR00A10U, pBBR00G11C, pBBR00G12A, and pBBR00A14U, respectively (Table 1).

Construction of strains. A mutant with a markerless deletion of the *gacA* coding region was constructed according to a previously described method (31) through a double-crossover recombination event with a gene replacement system with plasmid pJQΔgacA (33, 36), and double-crossover colonies were confirmed by colony PCR and sequenced. All plasmids used in this study were introduced into *P. protegens* by triparental mating with pRK2073 as a helper plasmid (37).

β-Galactosidase activity assay. The β-galactosidase activity of various *P. protegens* and *E. coli* strains carrying a *lacZ* fusion on *o*-nitrophenyl-β-D-galactopyranoside (ONPG) was determined by the method of Miller (38), normalized to the optical density at 600 nm (OD_{600}) of the bacterial culture, and expressed in Miller units. To induce expression, 0.1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) was added to cultures of strains containing pBBRKm or pET-28a derivatives.

RT-PCR analysis. Total RNA was isolated from various *P. protegens* strains without a *lacZ* fusion on the chromosome ($OD_{600} \approx 6.0$) grown in LB medium at 28°C overnight using an RNApure Bacteria kit (DNase I) (CWBIO, Beijing, China) according to the manufacturer's instructions. By following the manufacturer's protocols, 2 μg of total RNA was reverse transcribed with random hexamer primers using a Thermo Scientific RevertAid first-strand cDNA synthesis kit. PCR amplifications were performed with *Taq* DNA polymerase (CWBIO) and the primers presented in Table 2. Gene expression was normalized by using *rpoD* as an internal control. Reverse transcription-PCR (RT-PCR) products were loaded on a 1.0% (wt/vol) agarose gel and visualized by ethidium bromide fluorescence. Experiments were carried out at least twice.

Lipase activity assay. As LipA has been identified to be an intracellular lipase (31), the lipase activity of LipA was expressed as whole-cell lipase activity. Cultures ($OD_{600} \approx 6.0$) of various *P. protegens* strains without a *lacZ* fusion on the chromosome were grown in LB medium at 28°C overnight. After that, whole-cell samples were prepared according to a previously described method (39). Lipase activity on *p*-nitrophenyl caprylate (*p*NPC) was determined by a previously described spectrophotometric method (40), with some modifications. The reaction mixture for the system consisted of 2.9 ml Tris-HCl buffer (50 mM, pH 9.0) and 30 μl *p*NPC (10 mM *p*NPC in acetonitrile). The mixtures were preincubated at 55°C for 5 min, and 70 μl whole-cell samples was subsequently added. After 5 min of incubation at 55°C, the reaction mixtures were centrifuged (12,000 rpm, 2 min) at 4°C and the OD_{410} was measured. One unit of lipase activity was defined as the amount of enzyme needed to release 1 μmol of

TABLE 2 Primers used in this study

Purpose and gene	Primer name	Sequence[a] (5′–3′)
Markerless deletion mutation, *gacA*	gacAU-U-XbaI	GGTCTAGAGGTGGGAGAGGATGATT
	gacAU-L	GGAAGGCTGGAATGACTTATGGTCTTTACAGGTTGC
	gacAD-U	AGTCATTCCAGCCTTCC
	gacAD-L-XbaI	GGTCTAGAGAGTTCGTCGGTCAG
	gacAF	CAAGGAGGATCAGGATG
	gacAR	AGCGGTAGGTATTCACG
Overexpression		
Km resistance	KmF-NcoI	CACCATGGATACCTGTCCGCCTTT
	KanR-BglII	CCAAGATCTACAACACTCAACCCTATCTCG
lacI^q-P_{lac}	lac_PF-BamHI	TTGGATCCAGCTGTTTCCTGTGTGAAATTGT
	lac_PR-XbaI	CCTCTAGACCATCGAATGGCGCAAAACC
gacA	gacAF-BamHI	TTGGATCCTTGATAAGGGTGCTAGTAG
	gacAR- HindIII	GGTAAGCTTGTTCGGTCATTTCAGAG
rsmA	rsmAF-BamHI	AGGGATCCATGCTGATTCTGACTCG
	rsmAR-HindIII	CCTAAGCTTCAGCTCTCCGCAACAC
rsmE	rsmEF-BamHI	TCGGATCCATGCTGATACTCACCC
	rsmEF-NdeI	CCCTCATATGCTGATACTCACCCG
	rsmER-HindIII	CCAAAGCTTCAAGGCAAACAAGACAG
lacZ fusion		
lacZ	lacZF-BamHI	GAAGGATCCGAAATACGGGCAGACAT
	′lacZR-HindIII	GGAAAGCTTCTGGCCGTCGTTTTACAA
	lacZR-HindIII	CGAAAGCTTCACACAGGAAACAGC
lipA	lipA-PF-KpnI	AAGGTACCGAGCATGAAGCGATGAAC
	lipA′-PR-HindIII	GGAAAGCTTGGCAAGCTCTTGGGACAT
	lipA-PR- HindIII	GTAAAGCTTGTGCGAAGGCAGCG
	lipA′-A10U-PR-HindIII	GGAAAGCTTCATGCGGCGACAaCCTTGTG
	lipA′-G11C-PR-HindIII	GGAAAGCTTCATGCGGCGACATgCTTGTG
	lipA′-G12A-PR-HindIII	GGAAAGCTTCATGCGGCGACATCtTTGTG
	lipA′-A14U-PR-HindIII	GGAAAGCTTCATGCGGCGACATCCTaGTG
rsmA	rsmA′-PF-KpnI	AGGGTACCACCGACTTCACC
	rsmA′-PR-HindIII	CCAAAGCTTCAGCATACCTTTCTCC
rsmE	rsmE′-PF-KpnI	TGGGTACCGACCACGACCAG
	rsmE′-PR-HindIII	CCTAAGCTTCAGCATGATCTTCTCCTT
	rt-rpoDR	TCACTTCACGGATACCCTC
RT-PCR		
lipA	rt-lipAF	CAATTCCAGCGAGGTG
	rt-lipAR	GCCGATCAGGTTGACTC
rsmX	rt-rsmXF	AGGATCAGGGAAGGTCG
	rt-rsmXR	TCCAAGACCATTATGACTTC
rsmY	rt-rsmYF	TGGACGTCGCGCAGGAAG
	rt-rsmYR	GCGGGGCTTTGCAGACTG
rsmZ	rt-rsmZF	TGTCGACGGATAGACAC
	rt-rsmZR	CCCGCCCACATTTTTCC
rsmE	rt-rsmEF	ATGCTGATACTCACCCG
	rt-rsmER	CTGCCTGAATGCGTTG
rpoD	rt-rpoDF	ATACCGACGAAGCAGCAG
	rt-rpoDR	TCACTTCACGGATACCCTC

[a] Underlining indicates restriction enzyme cutting sites, and lowercase nucleotides represent substitution mutations.

p-nitrophenol per minute by 1.0 ml sample with an OD_{600} of 1.0. The activity of the whole-cell lipase was expressed as U/ml · OD_{600}.

Statistical analysis. Statistical significance was determined by calculating the *P* values using a two-tailed, unpaired Student's *t* test, and differences with a *P* value of <0.05 were considered statistically significant.

RESULTS

GacA activates the transcription and translation of *lipA*. In *P. aeruginos*a, the response regulator GacA generally activates the transcription of lipase genes through QS systems. However, no QS system based upon *N*-acyl homoserine lactones has been found in *P. protegens* Pf-5 (41), and so far, no report on the regulation of lipase gene expression in *P. protegens* is available. To study the role of GacA in the regulation of *lipA* expression in Pf-5, a *gacA* deletion mutant, Pf3563, was constructed using a gene replacement system with plasmid pJQΔgacA through a double-crossover recombination event. The effect of GacA on the expression of *lipA* was studied by determining the β-galactosidase activities of chromosome-borne *lipA′-′lacZ* and *lipA-lacZ* fusions and the relative

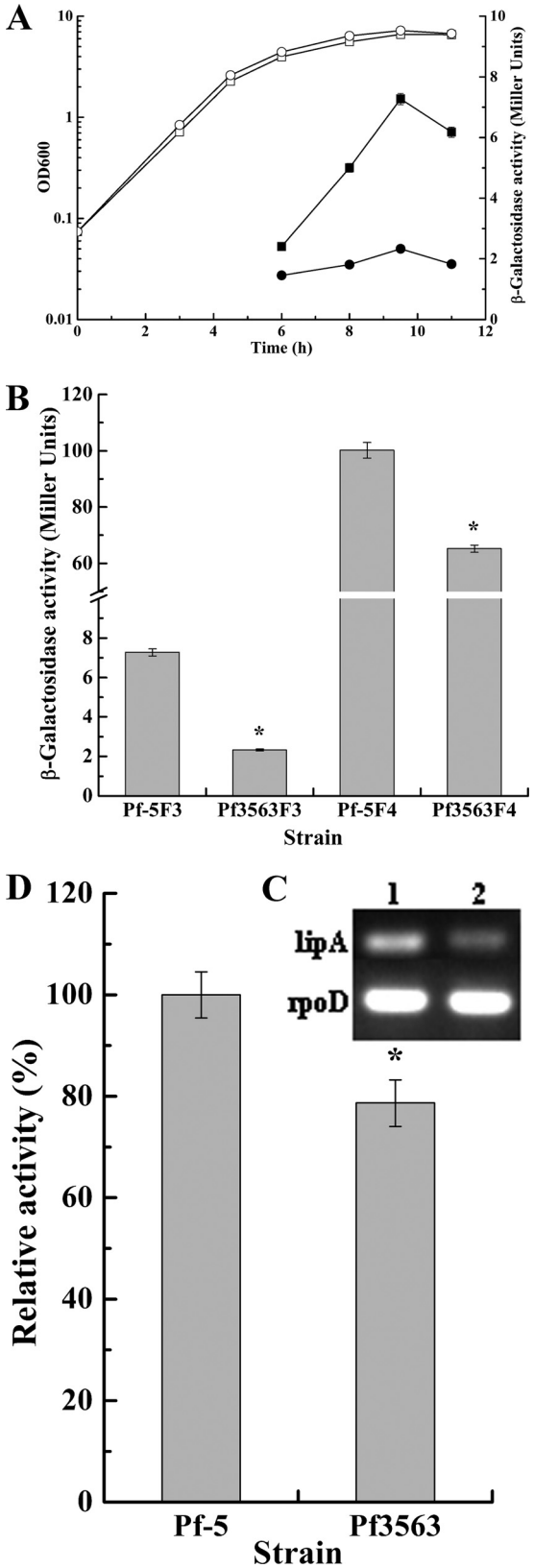


FIG 2 Effect of *gacA* mutation on *lipA* expression. (A) Influence of a *gacA* mutation on expression of a chromosome-borne *lipA′-′lacZ* translational fusion and growth of the bacteria. β-Galactosidase activity (solid symbols) and growth (open symbols) were measured in the wild-type strain (Pf-5F3; squares) and the *gacA* mutant (Pf3563F3; circles) at various time points after inoculation into 50 ml LB medium. (B) Influence of the *gacA* mutation on expression of a chromosome-borne *lipA′-′lacZ* translational fusion (wild-type strain, Pf-5F3; *gacA* mutant, Pf3563F3) and a chromosome-borne *lipA-lacZ* transcriptional fusion (wild-type strain, Pf-5F4; *gacA* mutant, Pf3563F4). The β-galactosidase activity of each strain was measured in the stationary phase after inoculation into 50 ml LB medium. (C) RT-PCR analysis of *lipA* expression in the wild-type strain (Pf-5; lane 1) and the *gacA* mutant (Pf3563; lane 2). (D) Relative whole-cell lipase activity in the wild-type strain (Pf-5) and the *gacA* mutant (Pf3563). All experiments were performed in triplicate, and the mean values ± standard deviations are indicated. *, $P < 0.05$ compared with the control.

whole-cell lipase activities of cells lacking these fusions and by performing RT-PCR analysis of *lipA* transcription in wild-type strain Pf-5 and the *gacA* mutant Pf3563. As shown in Fig. 2A, *gacA* deletion significantly repressed *lipA* expression at all stages of growth, while it slightly increased the biomass of the bacteria. However, *lipA* expression displayed similar kinetics in Pf-5 and Pf3563, with expression sharply increasing in the late exponential phase and peaking in the stationary phase. Subsequently, to investigate at which regulatory level GacA activates *lipA* expression, the expression of chromosome-borne *lipA′-′lacZ* and *lipA-lacZ* fusions was examined in the Pf-5 and Pf3563 backgrounds. As shown in Fig. 2B, GacA positively controlled *lipA* expression at both the transcriptional and the translational levels, with the translational level being the key regulatory pathway. RT-PCR analysis of *lipA* transcription as well as relative whole-cell lipase activities in Pf-5 and Pf3563 further confirmed the results described above (Fig. 2C and D).

In addition, the overexpression of *gacA* in both Pf-5 and Pf3563 via the *gacA* expression plasmid pBBBRKgacA was investigated along with that in Pf-5/pBBRKm and Pf3563/pBBRKm as controls to examine the effect of *gacA* overexpression on *lipA* expression in the Pf-5 and Pf3563 backgrounds. As shown in Fig. 3, *lipA* expression was higher in Pf3563/pBBRKgacA than in Pf3563/pBBRKm or Pf-5/pBBRKm. Moreover, Pf-5/pBBRKgacA and Pf3563/pBBRKgacA exhibited similar levels of *lipA* expression, but the intensity of *lipA* expression in Pf-5/pBBRKgacA was higher than that in Pf3563/pBBRKgacA. Taken together, the intensity of *gacA* expression determines the intensity of *lipA* expression.

***gacA* deletion partly inhibits the transcription of *rsmX*, *rsmY*, and *rsmZ*.** Previous studies have shown that the transcription of *rsmX*, *rsmY*, and *rsmZ* is completely abolished by the deletion of *gacA* in the closely related strain *P. protegens* CHA0 (42, 43). However, RT-PCR analysis of *rsmX*, *rsmY*, and *rsmZ* transcription in Pf-5 and Pf3563, which was performed to investigate the effect of *gacA* deletion on their transcription, indicated that Pf3563 did initiate the transcription of *rsmX*, *rsmY*, and *rsmZ*, although at a level lower than that in Pf-5 (Fig. 4), being consistent with the partial expression of *lipA* in Pf3563 (Fig. 2).

RsmA and RsmE differentially regulate *lipA* expression. In many gammaproteobacteria, the RNA-binding proteins of the RsmA-CsrA family account for the translational control of the Gac-Rsm signal transduction system (24). Moreover, the Gac-Rsm system contains two members of the RsmA-CsrA family, RsmA and RsmE, in *P. protegens* Pf-5 (42). In practice, it is often more convenient and reliable to assess the function of the RsmA-CsrA family proteins by their overexpression than to use a com-

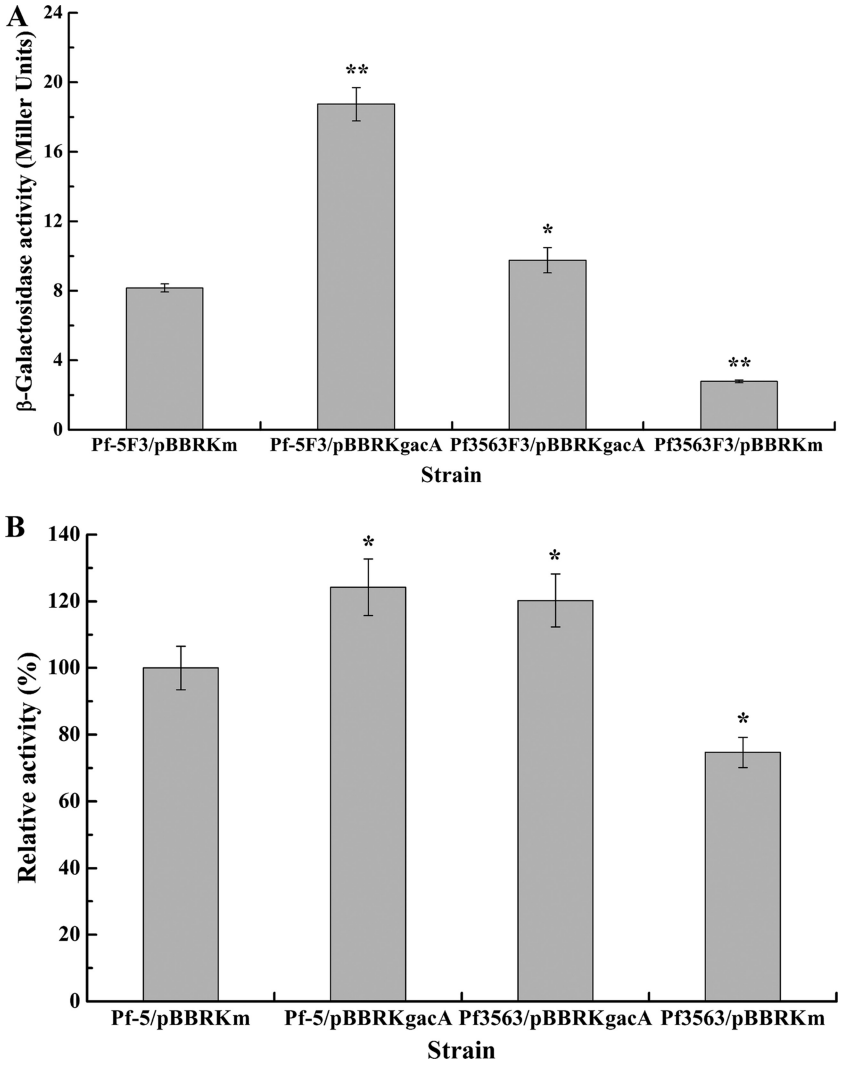


FIG 3 Effect of *gacA* overexpression on *lipA* expression. (A) Influence of *gacA* overexpression on expression of a chromosome-borne *lipA′-′lacZ* translational fusion in different strains. The β-galactosidase activity of each strain was measured in the stationary phase after inoculation into 50 ml LB medium. (B) Influence of *gacA* overexpression on relative whole-cell lipase activity in different strains. All experiments were performed in triplicate, and the mean values ± standard deviations are indicated. *, $P < 0.05$ compared with the control; **$P < 0.01$ compared with the control.

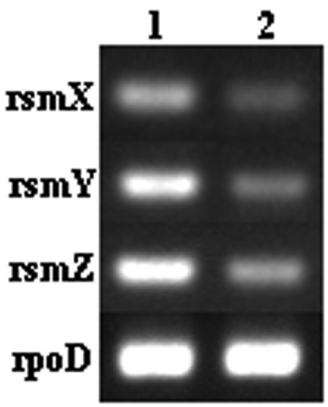
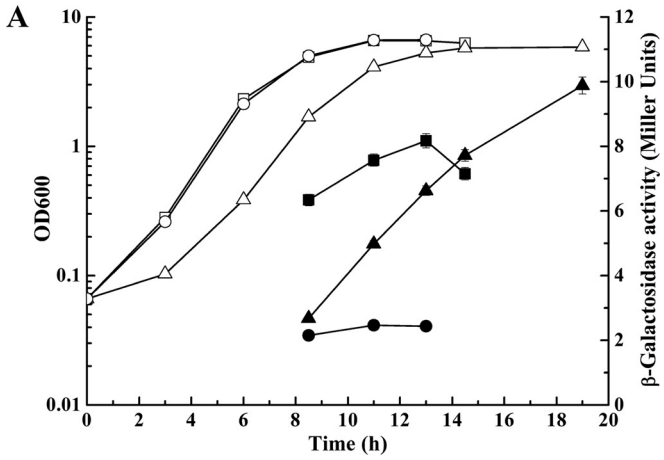
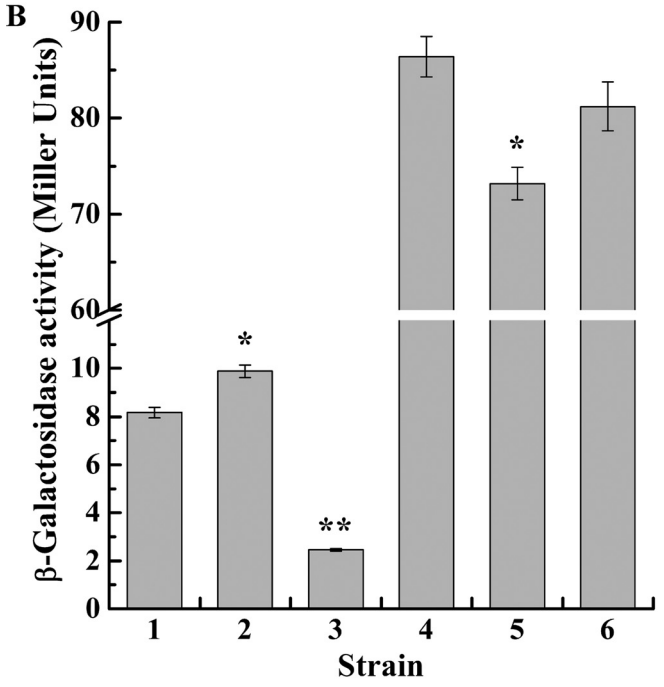
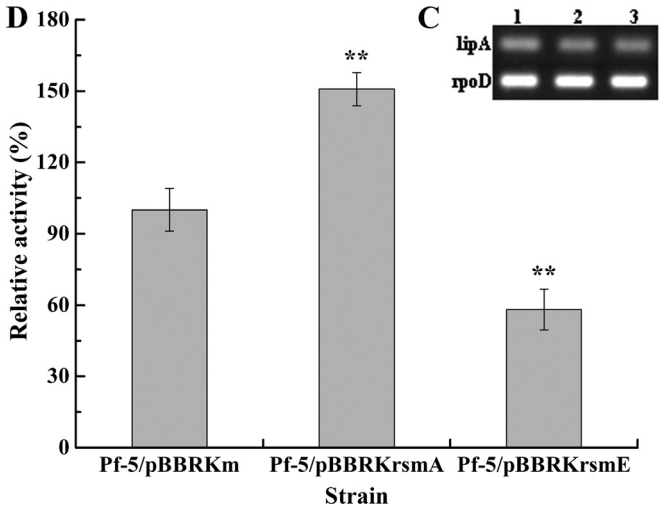


FIG 4 RT-PCR analysis of *rsmX*, *rsmY*, and *rsmZ* expression in the wild-type strain (Pf-5; lane 1) and the *gacA* mutant (Pf3563; lane 2).

parison of the wild-type strain and the mutants (24, 35). Therefore, to examine how Rsm proteins act on the regulation of *lipA* expression, strains overexpressing *rsmA* and *rsmE* were constructed by triparental mating, and the roles of the Rsm proteins in regulating *lipA* expression were explored by measuring the β-galactosidase activities of chromosome-borne *lipA′-′lacZ* and *lipA-lacZ* fusions and the relative whole-cell lipase activities of cells lacking these fusions and by carrying out RT-PCR analysis of *lipA* transcription in Pf-5/pBBRKm, Pf-5/pBBRKrsmA, and Pf-5/pBBRKrsmE. As shown in Fig. 5A, overexpressed RsmA significantly enhanced *lipA* expression when bacteria entered the late stationary phase, whereas overexpressed RsmE notably inhibited *lipA* expression throughout the growth cycle. In addition, *rsmA* overexpression resulted in bacteria exhibiting a delayed growth cycle and slightly decreased the biomass. To further determine the regulatory mechanisms of RsmA and RsmE in *lipA* expression, the expression of chromosome-borne *lipA′-′lacZ* and *lipA-lacZ* fusions was investigated in the Pf-5/pBBRKm, Pf-5/pBBRKrsmA







and Pf-5/pBBRKrsmE backgrounds. RsmA transcriptionally repressed but also translationally stimulated *lipA* expression, and the translational control was found to be the key regulatory pathway. In contrast, RsmE almost repressed *lipA* translation and also weakly inhibited *lipA* transcription (Fig. 5B and C). The relative whole-cell lipase activities in Pf-5/pBBRKm, Pf-5/pBBRKrsmA, and Pf-5/pBBRKrsmE further demonstrated that RsmA activated *lipA* expression, while RsmE inhibited *lipA* expression (Fig. 5D). As stated above, in *P. protegens* Pf-5, RsmA and RsmE differentially regulated *lipA* expression and exhibited different regulatory modes in some aspects.

RsmA activates *lipA* translation by inhibiting *rsmE* translation. RsmA has been proven to repress the expression of *rsmE* in *P. protegens* CHA0 (42), suggesting that the activation of *lipA* translation by RsmA may be mediated by RsmE. Consequently, we next investigated the effect of RsmA on *rsmE* expression by analyzing the expression of the chromosome-borne *rsmE′-′lacZ* fusion and by implementing RT-PCR analysis of *rsmE* transcription in Pf-5/pBBRKm and Pf-5/pBBRKrsmA. As shown in Fig. 6A, overexpressed RsmA notably repressed *rsmE* expression. The RT-PCR analysis further showed that RsmA did not regulate *rsmE* transcription (Fig. 6B). Taken together, these results indicate that RsmA represses *rsmE* translation, confirming the suggestion made above.

RsmE represses *lipA* translation by binding to the SD sequence of *lipA* mRNA. It has been reported that RsmE-mediated translational repression involves its binding to an (A/U)CANGG ANG(U/A) consensus sequence, which overlaps the SD sequence of target mRNA, blocking the recruitment of the 30S ribosomal subunit and, hence, translation initiation (24, 35, 44). It is worth noting that the ACAAGGAUGU sequence overlapping the SD sequence of *lipA* mRNA fully matches the consensus sequence. In addition, RsmE from *P. protegens* Pf-5 is 100% identical to that from *P. protegens* CHA0, and the protein-RNA interaction between RsmE and the consensus sequence has been studied in *P. protegens* CHA0 (35, 42). To mimic the interaction between RsmE and the ACAAGGAUGU sequence, four single-base mutations at nucleotides A10, G11, G12, and A14 of *lipA* mRNA were con-

FIG 5 Effects of *rsmA* and *rsmE* overexpression on *lipA* expression. (A) Influences of *rsmA* and *rsmE* overexpression on expression of a chromosome-borne *lipA′-′lacZ* translational fusion and growth of bacteria. β-Galactosidase activity (solid symbols) and growth (open symbols) were measured in the wild-type strain (Pf-5F3/pBBRKm; squares), an *rsmA*-overexpressing strain (Pf-5F3/pBBRKrsmA; triangles), and an *rsmE*-overexpressing strain (Pf-5F3/pBBRKrsmE; circles) at various time points after inoculation into 50 ml LB medium. (B) Influences of *rsmA* and *rsmE* overexpression on expression of a chromosome-borne *lipA′-′lacZ* translational fusion (bar 1, wild-type strain Pf-5F3/pBBRKm; bar 2, *rsmA*-overexpressing strain Pf-5F3/pBBRKrsmA; bar 3, *rsmE*-overexpressing strain Pf-5F3/pBBRKrsmE) and a chromosome-borne *lipA-lacZ* transcriptional fusion (bar 4, wild-type strain Pf-5F4/pBBRKm; bar 5, *rsmA*-overexpressing strain Pf-5F4/pBBRKrsmA; bar 6, *rsmE*-overexpressing strain Pf-5F4/pBBRKrsmE). The β-galactosidase activity of each strain was measured in the stationary phase after inoculation into 50 ml LB medium. (C) RT-PCR analysis of *lipA* expression in wild-type strain Pf-5/pBBRKm (lane 1), *rsmA*-overexpressing strain Pf-5/pBBRKrsmA (lane 2), and *rsmE*-overexpressing strain Pf-5/pBBRKrsmE (lane 3). (D) Relative whole-cell lipase activity in wild-type strain Pf-5/pBBRKm, *rsmA*-overexpressing strain Pf-5/pBBRKrsmA, and *rsmE*-overexpressing strain Pf-5/pBBRKrsmE. All experiments were performed in triplicate, and the mean values ± standard deviations are indicated. *, $P < 0.05$ compared with the control; **$P < 0.01$ compared with the control.

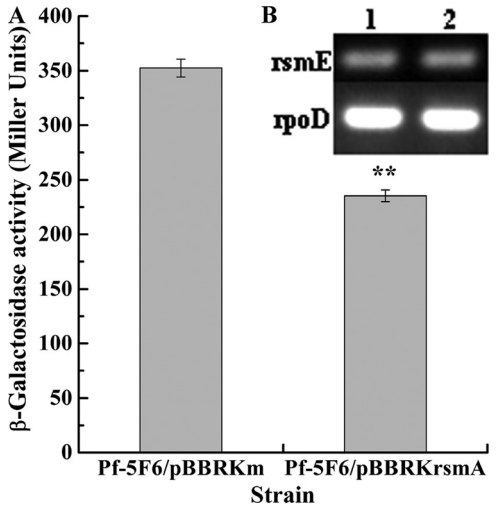


FIG 6 Effect of *rsmA* overexpression on *rsmE* expression. (A) Influence of *rsmA* overexpression on expression of a chromosome-borne *rsmE′-′lacZ* translational fusion (wild-type strain, Pf-5F6/pBBRKm; *rsmA*-overexpressing strain, Pf-5F6/pBBRKrsmA). The β-galactosidase activity of each strain was measured in the stationary phase after inoculation into 50 ml LB medium. (B) RT-PCR analysis of *rsmE* expression in wild-type strain Pf-5/pBBRKm (lane 1) and *rsmA*-overexpressing strain Pf-5/pBBRKrsmA (lane 2). All experiments were performed in triplicate, and the mean values ± standard deviations are indicated. $^{**}P < 0.01$ compared with the control.

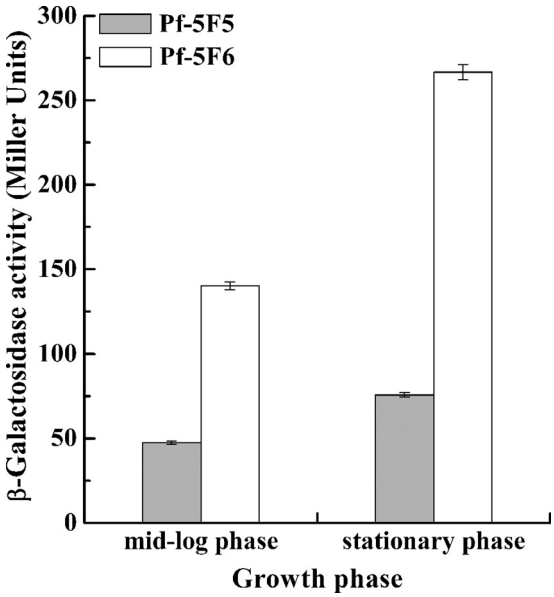


FIG 7 Expression of a chromosome-borne *rsmA′-′lacZ* translational fusion (Pf-5F5) and a chromosome-borne *rsmE′-′lacZ* translational fusion (Pf-5F6) at different growth phases. The β-galactosidase activity of each strain was measured in the mid-log and stationary phases after inoculation into 50 ml LB medium.

structed in the plasmid-borne *lipA′-′lacZ* fusions, and the fusions were expressed in *E. coli* BL21(DE3) with plasmid pET-rsmE or pET-28a. The effects of the A10U, G11C, G12A, and A14U mutations on translation regulation by RsmE are listed in Table 3. All these mutations influenced the regulatory control of RsmE, but the mutation of G11, G12, and A14 significantly eliminated the inhibitory effect of RsmE on the expression of the *lipA′-′lacZ* fusion. In short, these results demonstrate that RsmE inhibits *lipA* translation via binding to the ACAAGGAUGU sequence.

Expression of *rsmA* and *rsmE*. As mentioned above, overexpressed RsmA inhibited *rsmE* translation, thereby enhancing *lipA* translation. However, GacA activated *lipA* translation via the GacA-sRNAs-RsmE-LipA pathway rather than repress *lipA* translation through the GacA-sRNAs-RsmA-RsmE-LipA pathway, implying that the intensity of expression of *rsmE* is higher than that of *rsmA*. To assess the intensity of *rsmA* and *rsmE* expression, the β-galactosidase activities of chromosome-borne *rsmA′-′lacZ* and *rsmE′-′lacZ* fusions in the Pf-5 background were measured. As shown in Fig. 7, the intensity and variation of *rsmA* expression were less than those of *rsmE* expression. In addition, the expression of both *rsmA* and *rsmE* was enhanced with increasing cell density.

TABLE 3 Effects of mutations in the SD sequence of *lipA* mRNA on translational repression by RsmE

Plasmid	β-Galactosidase activity (Miller units) for strain: BL/pET-28a	BL/pET-rsmE	Repression factor
pBBR003	1,312.5 ± 106.4	818 ± 75.3	1.60[a]
pBBR00A10U	1,323.9 ± 110.8	1,018.6 ± 95.4	1.30[a]
pBBR00G11C	611.5 ± 56.9	541.2 ± 42.8	1.13
pBBR00G12A	712 ± 69.5	608.5 ± 60.7	1.17
pBBR00A14U	1,025.6 ± 101.4	967.5 ± 98.3	1.06

[a] $P < 0.05$ compared with the control.

DISCUSSION

In many gammaproteobacteria, the Gac-Rsm system regulates several unrelated pathways, such as primary and secondary metabolic pathways, biofilm formation, quorum sensing, oxidative stress, exoproduct formation, virulence, and pathogenesis (24, 45). Published studies on the regulation of lipase gene expression by the Gac-Rsm signal transduction system demonstrate that this system activates lipase gene transcription by mediation of the QS system and the CbrA/B two-component system in *P. aeruginosa* (16, 19). In the present study, a novel regulatory mechanism acting on lipase gene expression activated by the Gac-Rsm system was characterized in *P. protegens* Pf-5, which does not have a QS system based upon *N*-acyl homoserine lactones.

Although the GacS/A two-component system has previously been reported to activate the production of lipase (16, 19, 23), the effect of the GacS/A system on lipase gene expression in *P. protegens* is still unclear. To determine the status of the GacS/A system in *P. protegens* Pf-5, a *gacA* deletion mutant, Pf3563, and strains overexpressing *gacA* in the Pf-5 and Pf3563 background were constructed. On the basis of the available literature, GacA positively controls lipase gene transcription. However, the results in this study demonstrated that GacA mainly activates *lipA* translation (Fig. 2 and 3). It is worth noting that GacA also enhanced *lipA* transcription (Fig. 2B), which is consistent with the results of RT-PCR analysis of *lipA* transcription in Pf-5 and Pf3563 (Fig. 2C). Unlike the results obtained with the closely related strain *P. protegens* CHA0 (42, 43), the deletion of *gacA* in *P. protegens* Pf-5 partly abolished the transcription of *rsmX*, *rsmY*, and *rsmZ* (Fig. 4), which is in agreement with the regulatory effect of GacA on *lipA* expression. In addition, the deletion of *gacA* slightly increased

the biomass of the bacteria (Fig. 2A), while the overexpression of *gacA* slightly decreased the biomass of the bacteria (data not shown), a result similar to that reported for other *Pseudomonas* species (24, 46). The role of GacA in regulating the biomass of bacteria may be due to the fact that the Gac-Rsm system is a part of the QS machinery (46–48).

Given that the Rsm system governs translational control in the Gac-Rsm regulatory cascade (27, 45), *rsmA* and *rsmE* overexpression strains Pf-5/pBBRKrsmA and Pf-5/pBBRKrsmE were constructed to investigate the regulatory mechanisms of Rsm proteins in *lipA* expression. The results showed that RsmA and RsmE differentially controlled the expression of *lipA*. RsmA regulated *lipA* expression in a biphasic manner, inhibiting its transcription but stimulating its translation, whereas RsmE repressed *lipA* translation (Fig. 5). To our knowledge, this is the first time that Rsm proteins have been reported to play such roles in the regulation of *lipA* expression.

In general, Rsm proteins inhibit gene expression mainly through the translational pathway (24, 45). However, RsmA represses *lipA* transcription in *P. protegens* Pf-5, but the mechanism remains to be elucidated. We suggest that the regulatory network of *lipA* expression may include transcriptional factors controlled by RsmA. It has also been suggested that translational activation by Rsm proteins may involve their binding to mRNAs, which somehow stabilize them and/or facilitate their translation initiation, ultimately resulting in a positive effect on gene expression (20, 49). Similarly, it has been reported that binding of the ortholog of Rsm proteins, CsrA, to the highly structured untranslated leader of mRNA results in translational activation either by causing structural changes in the RNA (45) or by interfering with the 5′ end-dependent RNase E cleavage pathway of the transcript (50). However, the mechanism underlying the activation of *lipA* translation by RsmA differs from that proposal. It has been reported that RsmA inhibits *rsmE* expression in *P. protegens* CHA0 (42), suggesting that the mechanism by which RsmA realizes the activation of *lipA* translation involves translational repression of *rsmE* expression by RsmA, which is consistent with the effect of overexpressed RsmA on *rsmE* expression (Fig. 6). Although RsmA has been reported to positively control the ability to produce extracellular lipase in *P. aeruginosa* (20), the molecular mechanism remains unclear.

Translational repression by Rsm proteins generally involves their binding to multiple sites in the untranslated leader and/or the initially translated region of target mRNAs, one of which overlaps the cognate SD sequence (26, 27). It has been reported that RsmE from *P. protegens* CHA0, which is identical to that from *P. protegens* Pf-5, recognizes an (A/U)CANGGANG(U/A) consensus sequence overlapping the SD sequence of target mRNAs (35, 42). The ACAAGGAUGU sequence, which is located at the SD sequence of *lipA* mRNA, was found to fully match the consensus sequence, suggesting that RsmE represses *lipA* translation by binding to the sequence. Mutational analysis of the SD sequence of *lipA* mRNA verified that RsmE interacts with the SD sequence and, hence, inhibits the translation of *lipA* mRNA (Table 3), which demonstrates that the activation of *lipA* translation by GacA is mediated through RsmE binding to the SD sequence of *lipA* mRNA, and then the Gac-RsmE system in *P. protegens* Pf-5 directly activates *lipA* translation. However, in *P. aeruginosa* the Gac-Rsm system stimulates the expression of QS systems and the CbrA/B two-component system, thereby enhancing lipase gene transcription (16, 19). This may be due to the binding of RsmE to the SD sequence of *lipA* mRNA and the absence of a QS system based upon *N*-acyl homoserine lactones in *P. protegens* Pf-5 (41).

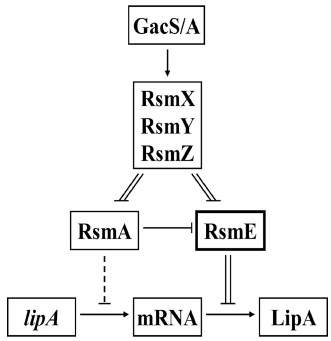


FIG 8 Schematic diagram depicting the activation of *lipA* expression by the Gac-Rsm system in *P. protegens* Pf-5. Transcription of three sRNAs (RsmX, RsmY, and RsmZ) depends on the presence of GacA. The function of sRNAs is to relieve translational repression of two RNA-binding proteins, RsmA and RsmE. RsmA mainly activates *lipA* translation by inhibiting *rsmE* translation, but it also represses *lipA* transcription via an unknown pathway. However, RsmE inhibits *lipA* translation by binding to the SD sequence of *lipA* mRNA. Among these regulatory pathways, the RsmE-mediated pathway plays a major role in the regulation of *lipA* expression. Solid line, direct regulation; dotted line, indirect regulation; double line, physical interaction; arrow, positive effect; bar, negative effect.

The question remains as to why RsmE rather than RsmA plays a major role in the regulatory network of *lipA* expression. Possible reasons are as follows. (i) The 5′ untranslated region of *lipA* mRNA contains only a GGA motif, which is in the ACAAGG AUGU sequence and which can be bound by RsmE but not RsmA. The interaction between RsmA and RNA is complex, as it depends on at least two RNA recognition sequences, as well as their spatial relationship and, potentially, their binding cooperativity (35). (ii) In our study, the intensity and variation of *rsmE* expression were higher than those of *rsmA* expression (Fig. 7), which may also be the reason why the Gac-Rsm system activates *lipA* translation via the Gac-RsmE-LipA pathway rather than represses *lipA* translation through the Gac-RsmA-RsmE-LipA pathway. In contrast, the level of *rsmE* expression is less than that of *rsmA* expression in *P. protegens* CHA0 (42). (iii) RsmE probably plays a role in the termination of GacA-controlled gene expression (42).

In conclusion, in *P. protegens* Pf-5 the Gac-Rsm signal transduction system mainly and directly activates *lipA* translation via the Gac-RsmE-LipA pathway and, in addition, indirectly enhances *lipA* transcription through the of Gac-RsmA-?-LipA pathway (Fig. 8).

ACKNOWLEDGMENTS

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