AMERICAN SOCIETY FOR MICROBIOLOGY | Applied and Environmental Microbiology

Self-Regulation and Interplay of Rsm Family Proteins Modulate the Lifestyle of *Pseudomonas putida*

Óscar Huertas-Rosales, María Isabel Ramos-González, Manuel Espinosa-Urgel

Department of Environmental Protection, Estación Experimental del Zaidín, CSIC, Granada, Spain

ABSTRACT

In the plant-beneficial bacterium *Pseudomonas putida* KT2440, three genes have been identified that encode posttranscriptional regulators of the CsrA/RsmA family. Their regulatory roles in the motile and sessile lifestyles of *P. putida* have been investigated by generating single-, double-, and triple-null mutants and by overexpressing each protein (RsmA, RsmE, and RsmI) in different genetic backgrounds. The *rsm* triple mutant shows reduced swimming and swarming motilities and increased biofilm formation, whereas overexpression of RsmE or RsmI results in reduced bacterial attachment. However, biofilms formed on glass surfaces by the triple mutant are more labile than those of the wild-type strain and are easily detached from the surface, a phenomenon that is not observed on plastic surfaces. Analysis of the expression of adhesins and exopolysaccharides in the different genetic backgrounds suggests that the biofilm phenotypes are due to alterations in the composition of the extracellular matrix and in the timing of synthesis of its elements. We have also studied the expression patterns of Rsm proteins and obtained data that indicate the existence of autoregulation mechanisms.

IMPORTANCE

Proteins of the CsrA/RsmA family function as global regulators in different bacteria. More than one of these proteins is present in certain species. In this study, all of the RsmA homologs in *P. putida* are characterized and globally taken into account to investigate their roles in controlling bacterial lifestyles and the regulatory interactions among them. The results offer new perspectives on how biofilm formation is modulated in this environmentally relevant bacterium.

Proteins belonging to the CsrA/RsmA family are small (less than 7 kDa) RNA-binding proteins that play key roles in the regulation of gene expression in diverse Gram-negative and Gram-positive bacteria. CsrA (carbon storage regulator) was first described in *Escherichia coli* (1, 2), where it plays a major role in controlling the intracellular carbon flux, acting as a negative regulator of glycogen metabolism and several enzymes involved in central carbohydrate metabolism (3, 4). Members of the CsrA/RsmA family have subsequently been found to be important elements in global posttranscriptional regulation in many other bacterial genera. In the opportunistic human pathogen *Pseudomonas aeruginosa*, the CsrA homolog RsmA (repressor of secondary metabolism) negatively regulates the production of virulence determinants, such as hydrogen cyanide, pyocyanin, or LecA (PA-1L) lectin, as well as *N*-acylhomoserine lactone (AHL) quorum-sensing signal molecules (5, 6). In this bacterium, RsmA also represses the translation of the *psl* operon, responsible for the synthesis of one of the two main exopolysaccharides (EPSs) that contribute to the extracellular matrix of biofilms in nonmucoid strains of *P. aeruginosa* (7). RsmA promotes the planktonic lifestyle of *P. aeruginosa*, functioning in opposition to the increase in the second messenger c-di-GMP, which leads to a sessile lifestyle, and some of the molecular elements connecting the two regulatory networks are being characterized (reference 8 and references therein).

RsmA homologs act posttranscriptionally, often by binding to mRNA at or near the ribosome-binding site, thus modulating translation (9, 10). For example, in *Pseudomonas fluorescens*, RsmA represses the production of hydrogen cyanide during exponential growth by reducing the translation rate of *hcnA*, the gene coding for hydrogen cyanide synthase. A specific sequence near the ribosome-binding site was shown to be required for RsmA activity to be evident on *hcnA*::*lacZ* translation (11). In *E. coli*, CsrA mediates posttranscriptional repression of glycogen biosynthesis by binding to the 5′ leader transcript of *glgC* and inhibiting its translation (12). CsrA can also act directly or indirectly as a positive regulator of gene expression: in *E. coli*, it activates genes involved in glycolysis and the glyoxylate shunt (13) and in flagellar motility. In the last case, binding of CsrA to a 5′segment of *flhDC* mRNA stimulates its translation and extends its half-life (14).

The effects of RsmA/CsrA are relieved by small regulatory RNA molecules that sequester multiple units of the proteins, thereby modulating their activity. Such antagonistic small RNAs include CsrB and CsrC in *E. coli* (1, 15); RsmB in *Erwinia carotovora* (16); and RsmX, RsmY, and RsmZ in *P. fluorescens* (17, 18). This kind of posttranscriptional control may facilitate rapid, potentially reversible regulation of diverse cellular functions.

The RsmA family proteins and their cognate small RNAs described so far in *Pseudomonas* are part of the GacS/GacA signal transduction pathway, which operates an important metabolic

Received 7 June 2016 Accepted 6 July 2016

Accepted manuscript posted online 15 July 2016

Citation Huertas-Rosales Ó, Ramos-González MI, Espinosa-Urgel M. 2016. Self-regulation and interplay of Rsm family proteins modulate the lifestyle of *Pseudomonas putida*. Appl Environ Microbiol 82:5673–5686. doi:10.1128/AEM.01724-16.

Editor: M. Kivisaar, University of Tartu

Address correspondence to Manuel Espinosa-Urgel, manuel.espinosa@eez.csic.es.

Supplemental material for this article may be found at http://dx.doi.org/10.1128/AEM.01724-16.

switch from primary to secondary metabolism in many Gram-negative bacteria and also affects enzyme synthesis and secretion (11, 19, 20). GacS is a sensor histidine kinase that responds to an as yet unidentified signal and phosphorylates the response regulator GacA, causing its activation. In *P. aeruginosa*, GacA positively regulates the quorum-sensing machinery and the expression of several virulence factors via a mechanism involving the participation of RsmA as a negative-control element (5, 21). The main regulatory targets of GacA correspond to the small RNAs of the *rsmX-rsmY-rsmZ* family that interact with RsmA (22).

In this study, we analyzed the roles and expression of the three RsmA family proteins present in the plant-beneficial bacterium *Pseudomonas putida* KT2440. Our results indicate that these proteins have different effects on motility, biofilm formation, and dispersal of *P. putida* KT2440, altering the expression of adhesins and exopolysaccharides. These effects may depend on regulatory interactions between the three Rsm proteins.

MATERIALS AND METHODS

Bacterial strains and culture conditions. The strains and plasmids used in this work are listed in Table 1. *P. putida* KT2440 is a plasmid-free derivative of *P. putida* mt-2, which was isolated from a field planted with vegetables and whose genome is completely sequenced (23, 24). Fluorescently labeled strains with a single chromosomal copy of mCherry were obtained by conjugation using the plasmid miniTn*7*Ptac-mChe (25) as detailed below. *E. coli* and *P. putida* strains were routinely grown at 37°C and 30°C, respectively, in LB medium (26) under orbital shaking (200 rpm). M9 minimal medium (27) was supplemented with trace elements (28) and glucose or citrate at the concentrations indicated in each case. For pyoverdine quantification, King's B medium was used (29). When appropriate, antibiotics were added to the media at the following concentrations: ampicillin, 100 μg/ml; kanamycin, 25 μg/ml; streptomycin, 50 μg/ml (*E. coli*) or 100 μg/ml (*P. putida*); and tetracycline, 10 μg/ml or 20 μg/ml (as indicated). Cell growth was followed by measuring turbidity at 600 nm (optical density at 600 nm [OD_{600}]) or 660 nm (OD_{660}), except for experiments done in a BioScreen apparatus with a wide-band filter (450 to 580 nm).

DNA techniques. Preparation of chromosomal DNA, digestion with restriction enzymes, dephosphorylation, ligation, and electrophoresis were carried out using standard methods (27, 30) and following the manufacturers' instructions (Roche and New England BioLabs). Plasmid DNA isolation and recovery of DNA fragments from agarose gels were done with Qiagen miniprep and gel extraction kits, respectively. The DIG-DNA labeling and detection kit (Roche) was used for Southern blots, according to the manufacturer's instructions. Electrotransformation of freshly plated *Pseudomonas* cells was performed as previously described (31). For PCR amplifications, Expand High Fidelity polymerase (Roche) was used if amplicons were used in further cloning.

Conjugation. Overnight cultures (0.5 ml) of donor, helper, and recipient bacteria were mixed, centrifuged (12,000 rpm; 2 min), washed with 1 ml of fresh LB medium to remove antibiotic traces, and resuspended in 50 μl of LB medium. The mixture was spotted onto a 0.22-μm filter placed on an LB plate and incubated overnight at 30°C. The mating mixture was then suspended in 2 ml of M9 salts and plated in M9 medium with 15 mM sodium citrate to counterselect *E. coli* strains and with the corresponding antibiotics for transconjugant selection. For strains harboring miniTn*7*Ptac-mChe, the presence of a chromosomal copy of the transposon at an extragenic location near *glmS* was checked by PCR, as described previously (32).

Generation of null mutants. The general strategy for the construction of null mutants consisted of replacement by homologous recombination of the wild-type allele with a null allele. Fragments (0.7 to 1 kb) of the regions flanking each *rsm* gene were amplified by PCR with oligonucleotides containing unique restriction sites (Table 2) and then cloned into pGEM-T Easy or pCR2.1-TOPO. The absence of missense mutations in the PCR amplicons was confirmed by sequencing. The null allele was first cloned in pUC18Not (33) and subsequently subcloned in the NotI site of plasmid pKNG101 (34), which is unable to replicate in *Pseudomonas*. The derivative plasmids of pKNG101 containing the null mutations were mobilized from *E. coli* CC118 λ*pir* into *P. putida* KT2440 by conjugation as described above, using HB101(pRK600) as a helper. Merodiploid exconjugants were first selected in minimal medium with citrate and streptomycin and then incubated in LB medium supplied with 12% sucrose to obtain clones in which a second recombination event had removed the plasmid backbone. Sm-sensitive clones were repurified, and the presence of the null mutations was checked by PCR, followed by sequencing of the corresponding chromosomal region and Southern blotting. The nomenclature of the mutants in more than one locus indicates the order in which the null alleles were introduced.

Overexpression of Rsm proteins. Plasmid pME6032 (17) was used to overexpress RsmI, RsmE, and RsmA. The three genes were amplified from *P. putida* KT2440 chromosomal DNA by PCR using oligonucleotides PP1746HistagF and PP1746HistagR, PP_3832HistagF and PP_3832HistagR, and PP_4472HistagF and PP_4472HistagR, respectively (Table 2); digested by EcoRI and XhoI; and inserted into EcoRI/XhoI-cut pME6032 to obtain pOHR40, pOHR38, and pOHR37, respectively. The integrity of all the constructs was verified by sequencing to discard any mutation in the PCR amplicons.

Construction of Rsm-LacZ translational fusions. Translational fusions were generated by PCR amplification of a fragment covering the promoter regions plus initiation codons of *rsmI*, *rsmE*, and *rsmA* designed to ensure in-frame cloning in pMP220-BamHI (35). The primers used are listed in Table 2. PCR amplicons of 222 bp (for RsmI-LacZ), 204 bp (for RsmE-LacZ with the proximal promoter), 340 bp (for RsmE-LacZ with the distal and proximal promoters), and 1,000 bp (RsmA-LacZ) containing the ribosome-binding sites, and the first 7 to 9 codons of each gene were cloned in pCR2.1-TOPO and sequenced to ensure the absence of mutations, followed by digestion with BamHI and subsequent cloning into the same site of pMP220-BamHI to yield pOHR46, pOHR47, pOHR48, and pOHR52, respectively.

RNA purification. Bacterial cells grown in liquid LB medium were harvested at the indicated times by centrifugation, immediately frozen with liquid nitrogen, and stored at −80°C. Alternatively, cells were collected from patches grown on LB plates for 24 or 48 h and resuspended in M9 salts prior to centrifugation and freezing. Total RNA from the mutants and the wild type was extracted by using TRI reagent (Ambion, Austin, TX, USA), as recommended by the manufacturer, except that Tripure isolation reagent was preheated at 70°C, followed by purification with an RNeasy purification kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. DNA traces were then removed from RNA samples with RNase-free DNase I (Turbo RNA-free; Ambion), as specified by the supplier. The RNA concentration was determined with a NanoDrop ND1000 spectrophotometer (NanoDrop Technologies, Inc., Wilmington, DE, USA), its integrity was assessed by agarose gel electrophoresis, and the absence of any residual DNA was checked by PCR.

qRT-PCR. Expression analyses by quantitative real-time PCR (qRT-PCR) were performed using iCycler Iq (Bio-Rad, Hercules, CA, USA) with total RNA preparations obtained from three independent cultures (three biological replicates). Total RNA (1 μg) treated with Turbo DNA free (Ambion) was retrotranscribed to cDNA with Superscript II reverse transcriptase (Invitrogen) using random hexamers as primers. Template cDNAs from the experimental and reference samples were amplified in triplicate using the primers listed in Table 2. Each reaction mixture contained 2 μl of a dilution of the target cDNA (1:10 to 1:10,000) and 23 μl of Sybr Green mix (Molecular Probes). Samples were initially denatured by heating at 95°C for 10 min, followed by a 40-cycle amplification and quantification program (95°C for 15 s, 62°C for 30 s, and 72°C for 20 s) with a single fluorescence measurement per cycle. The PCR products were between 150 and 200 bp in length. To confirm the amplification of a single

TABLE 1 Bacterial strains and plasmids

Strain or plasmid	Genotype/relevant characteristics[a]	Reference/source
E. coli		
CC118λpir	Rif^r λ*pir*	33
DH5α	*supE44 lacU169*(ϕ80*lacZ*ΔM15) *hsdR17* (r_k^- m_k^-) *recA1 endA1 gyrA96 thi-1 relA1*	50
HB101	Host of helper plasmid pRK600	V. de Lorenzo
P. putida		
KT2440	Wild type; derivative of *P. putida* mt-2 cured of pWW0	23
pvdS	Mutant in *pvdS*; defective in pyoverdine synthesis	39
ΔI	Null mutant derivative of KT2440 in PP_1746 (*rsmI*)	This study
ΔE	Null mutant derivative of KT2440 in PP_3832 (*rsmE*)	This study
ΔA	Null mutant derivative of KT2440 in PP_4472 (*rsmA*)	This study
ΔIE	Null mutant derivative of KT2440 in PP_1746 and PP_3832	This study
ΔEA	Null mutant derivative of KT2440 in PP_3832 and PP_4472	This study
ΔIA	Null mutant derivative of KT2440 in PP_1746 and PP_4472	This study
ΔIEA	Null mutant derivative of KT2440 in PP_1746, PP_3832 and PP_4472	This study
Plasmids		
pGEM-T Easy	Ap^r; PCR cloning vector with β-galactosidase α-complementation	Promega
PCR2.1 TOPO	Km^r; PCR cloning vector with β-galactosidase α-complementation	Invitrogen
pKNG101	Sm^r; *oriR6K mobRK2 sacBR*	34
pMP220-BamHI	Tc^r; pMP220 with deletion of a 238-bp BamHI fragment, removing the ribosome-binding site and 52 codons of *cat* that precede *′lacZ*	35
RK600	Cm^r; *mob tra*	V. De Lorenzo
pUC18Not	Ap^r; cloning vector; MCS of pUC18 flanked by NotI sites	33
pME6032	Tc^r; pVS1-p15A derivative; broad-host-range *lacI*^{q}-P_{tac} expression vector	17
pMMG1	Tc^r; transcriptional fusion *lapF*::*′lacZ* containing RBS and first codons	43
pMMGA	Tc^r; transcriptional fusion *lapA*::*′lacZ* containing RBS and first codons	42
pMIR125	Tc^r; transcriptional fusion *algD*::*′lacZ* containing RBS and first codons	51
pMP-bcs	Tc^r; transcriptional fusion PP_2629::*′lacZ* containing RBS and first codons	
pMP-pea	Tc^r; transcriptional fusion PP_3132::*′lacZ* containing RBS and first codons	51
pMP-peb	Tc^r; transcriptional fusion PP_1795::*′lacZ* containing RBS and first codons	51
pOHR14	Ap^r; pUC18NotI derivative with 1.9-kb NotI fragment containing the *rsmA* null allele	This study
pOHR20	Sm^r; pKNG101 derivative for the *rsmA* null allele replacement with the 1.9-kb NotI fragment of pOHR14 cloned at the same site of pKNG101	This study
pOHR30	Ap^r; pUC18NotI derivative with 1.7-kb NotI fragment containing the *rsmI* null allele	This study
pOHR33	Sm^r; pKNG101 derivative for the *rsmI* null allele replacement with the 1.7-kb NotI fragment of pOHR30 cloned at the same site of pKNG101	
pOHR32	Ap^r; pUC18NotI derivative with 2-kb NotI fragment containing the *rsmE* null allele	This study
pOHR34	Sm^r; pKNG101 derivative for the *rsmE* null allele replacement with the 2-kb NotI fragment of pOHR32 cloned at the same site of pKNG101	This study
pME6032-*rsmA*	Tc^r; pME6032 derivative for the ectopic expression of *rsmA* under the control of *lacI*^{q}-P_{tac}	This study
pME6032-*rsmE*	Tc^r; pME6032 derivative for the ectopic expression of *rsmE* under the control of *lacI*^{q}-P_{tac}	This study
pME6032-*rsmI*	Tc^r; pME6032 derivative for the ectopic expression of *rsmI* under the control of *lacI*^{q}-P_{tac}	This study
pOHR46	Tc^r; pMP220-BamHI derivative containing translational fusion RsmI-LacZ	This study
pOHR47	Tc^r; pMP220-BamHI derivative containing translational fusion RsmE-LacZ (with proximal promoter)	This study
pOHR48	Tc^r; pMP220-BamHI derivative containing translational fusion RsmE-LacZ (with proximal and distal promoters)	This study
pOHR52	Tc^r; pMP220-BamHI derivative containing translational fusion RsmA-LacZ	This study

[a] Ap, ampicillin; Cm, chloramphenicol; Km, kanamycin; Rif, rifampin; Sm, streptomycin; Tc, tetracycline; MCS, multicloning site; RBS, ribosome-binding site.

PCR product, a melting curve was obtained by slow heating from 60°C to 99.5°C at a rate of 0.5°C every 10 s for 80 cycles, with continuous fluorescence scanning. The results were normalized relative to those obtained for 16S rRNA. Quantification was based on the $2^{-\Delta\Delta CT}$ method (36).

Transcription initiation site determination. The rapid amplification of cDNA ends (RACE) system version 2.0 (Invitrogen) was used to determine the 5′ ends of *rsm* transcripts. Total RNA was extracted from *P. putida* KT2440 cultures at OD_{600}s of 0.8 and 2.8, and the RACE technique was carried out with the oligonucleotides listed in Table 2, following the manufacturer's instructions. Ten clones were sequenced for each transcript, and in all cases, 8 or more gave the same result in terms of identification of the +1 site.

Assays for β-galactosidase activity. Overnight cultures were diluted 1/100 in fresh LB medium supplemented with 10 μg/ml tetracycline and grown at 30°C for 1 h. Then, the cultures were diluted 1/10 in fresh LB medium and cultivated at 30°C for 1 more hour to allow the reduction of most of the remaining β-galactosidase accumulated in the overnight cultures. Finally, the cultures were diluted to an OD_{600} of 0.05 in fresh LB medium supplemented with 10 μg/ml tetracycline. The cells were allowed to grow at 30°C, and at the indicated time points, aliquots were taken and

TABLE 2 Primers used in this work

Primer name	Sequence (5′–3′)[a]	Comment
PP_4472UpF	TTGAGCTCCAGCATCACTACCCTGGGTC	*rsmA* null mutant construction
PP_4472UpR	TGGGATCCCATTCAGGGGTAACAGTCTTGG	
PP_4472DwF	TCGGATCCGAAGGATGAAGAGCCAAGCC	
PP_4472DwR	TCGGATCCGATTGTTGTGGATGGGAAAGC	
PP_3832UpF	GAATTCCGACCAGCACAAATACGGG	*rsmE* null mutant construction
PP_3832UpR	TCTAGACTCCTTGGTGATGTATAAGTCCG	
PP_3832DwF	TCTAGAGAAGACACACACTGAGCGTCAC	
PP_3832DwR	AAGCTTGACATCATTGGGCCTGGC	
PP_1746UpF	GAATTCCCGATGTCAACGAAGCC	*rsmI* null mutant construction
PP_1746UpR	TCTAGAGTTCCGATCCTCCTGCG	
PP_1746DwF	TCTAGAGCAGAGCAAGGCCTGAAG	
PP_1746DwR	AAGCTTCTGGCGTAGCGGCATTG	
PP_4472HistagF	GAATTCATGcatcatcatcatcatATGTTGATTCTGACTCGTCG	189-bp EcoRI/XhoI fragment for *rsmA* ectopic expression
PP_4472HistagR	CTCGAGCTATTATAAAGGCTTGGCTCTTCATCC	
PP_3832HistagF	GAATTCATGcatcatcatcatcatATGCTGATACTCACCCGTAAG	198-bp EcoRI/XhoI fragment for *rsmE* ectopic expression
PP_3832HistagR	CTCGAGCTATTATCAGTGTGTGTCTTCGTGTTTC	
PP_1746HistagF	GAATTCATGcatcatcatcatcatATGCTGGTAATAGGGCGC	180-bp EcoRI/XhoI fragment for *rsmI* ectopic expression
PP_1746HistagR	CTCGAGCTATTATCAGGCCTTGCTCTGC	
PP_4472GSPR1	GAATCAACATAGCTTTCTCCTTACGCA	+1 determination of *rsmA* by RACE-PCR
PP_4472GSPR2	TTGCCTTTGACGCCAA	
PP_3832GSPR1	AGTGTGTGTCTTCGTGTTTCTCC	+1 determination of *rsmE* by RACE-PCR
PP_3832GSPR2	TCAGTTAATAGGCACCTG	
PP_1746GSPR1	AGGCCTTGCTCTGCTTGGAC	+1 determination of *rsmI* by RACE-PCR
PP_1746GSPR2	GACCTGGAAAGCCAGCAAT	
PP_4472PromF	GGATCCGCTTCCAGGGCGTC	RsmA-LacZ fusion
PP_4472PromR	GGATCCCGACGAGTCAGAATCAA	
PP_3832ShortPromF	GGATCCCCGTTGACGGTTTGC	$RsmE_{P2}$-LacZ fusion (proximal promoter)
PP_3832ShortPromR	GGATCCCCAACCTTACGGGTG	
PP_3832LargePromF	GGATCCGGCCTTGCTGTTGTTTC	$RsmE_{P1P2}$-LacZ fusion (proximal + distal promoters)
PP_3832ShortPromR	GGATCCCCAACCTTACGGGTG	
PP_1746PromF	GGATCCGCGGCTGTATGACG	RsmI-LacZ fusion
PP_1746PromR	GGATCCCCTACTTCGCGCCCTA	
qRTAlgF	GCTTCCTCGAAGAGCTGAA	qRT-PCR; *alg* cluster (PP_1277; *algA*)[b]
qRTAlgR	CTCCATCACCGCATAGTCA	
qRTPebF	GCAATGTCTCCACAGGCAC	qRT-PCR; *peb* cluster (PP_1795)[b]
qRTPebR	TCATCTGATTGGCGACCAG	
qRTCelF	GTCGAGAGCAGCCAGCTTC	qRT-PCR; *bcs* cluster (PP_2629)[b]
qRTCelR	GCCTCATACAGTGCCAGCTC	
qRTPeaF	TGCTCAGCACGCCGACACG	qRT-PCR; *pea* cluster (PP_3132)[b]
qRTPeaR	GGTCTCGCTGTTCAGCA	
qRT16SF	AAAGCCTGATCCAGCCAT	qRT-PCR control 16S rRNA
qRT16SR	GAAATTCCACCACCCTCTACC	

[a] Restriction sites inserted in the primer for the cloning strategy are underlined; 6× histidine tail is in lowercase.
[b] The specific loci are given.

β-galactosidase activity was measured as described previously (37). Experiments were carried out in triplicate with two experimental replicates, and all the data represent averages and standard deviations.

Biofilm assays. Biofilm formation was analyzed in LB medium under static conditions using a microtiter plate assay described previously (38). Alternatively, biofilm development was followed during growth in LB medium in borosilicate glass tubes incubated with orbital rotation at 40 rpm at 30°C. In both cases, the OD_{600} of the cultures was adjusted to 0.02 or 0.05, respectively, at the start of the experiment. At the indicated times, the liquid was removed and nonadherent cells were washed away by rinsing with distilled water. The biofilms were stained with 1% crystal violet (Sigma) for 15 min, followed by rinsing twice with distilled water. Photographs were taken, and the cell-associated dye was solubilized with 30% acetic acid and quantified by measuring the absorbance at 580 nm (A_{580}). Assays were performed in triplicate.

For confocal laser scanning microscopy (CLSM) analysis of biofilms,

cultures were grown in LB medium diluted 1/10 in 24-well glass bottom plates (Greiner Bio-One, Germany). At the indicated times, the biofilms were visualized using a Zeiss LSM 510 Meta/AxioVert 200 confocal microscope, and three-dimensional reconstruction was performed with Imaris software (Bitplane).

Motility assays. Swimming motility was tested on LB plates containing 0.3% (wt/vol) agar. Cells from exponentially growing cultures (2 μl) were inoculated into the plates. Swimming halos were measured after 24 h of inoculation, and the area was calculated. Assays were performed three times with three replicates each. Surface motility assays were done as previously described (39) on plates containing 0.5% (wt/vol) agar.

Statistical methods. One-way analysis of variance (ANOVA), followed by Bonferroni's multiple-comparison test (set at a P value ≤ 0.05) or Student's t test for independent samples ($P \leq 0.05$), was applied using the R program for all statistical analyses.

RESULTS

CsrA/RsmA family proteins and their cognate small RNAs (sRNAs) in *P. putida* KT2440. An *in silico* analysis of the *P. putida* KT2440 genome indicated that there are three genes in the bacterium encoding CsrA/RsmA family proteins (PP_1746, PP_3832, and PP_4472), which show 46%, 70%, and 75% amino acid identity with CsrA of *E. coli*, respectively. Alignment of these protein sequences with those of RsmA of *P. aeruginosa* PAO1 and the three homologs annotated in the genome of *P. fluorescens* F113 (see Fig. S1 in the supplemental material) led to the following gene nomenclature in KT2440: PP_1746 corresponds to *rsmI*, PP_3832 to *rsmE*, and PP_4472 to *rsmA*. RsmA (62 amino acids) and RsmE (65 amino acids) of *P. putida* share 54% identical residues, while RsmI (59 amino acids) shows 43% identity with the other two proteins (see Fig. S1 in the supplemental material). RsmI is also the most divergent in terms of conserved residues that interact with RNA (see Fig. S1 in the supplemental material). None of the proteins is equivalent to RsmF/RsmN of *P. aeruginosa*, which are different in structure and sequence from other Rsm proteins, and no homolog of RsmN could be found in *P. putida* KT2440 based on sequence analysis.

In *P. fluorescens*, three noncoding sRNAs interact with Rsm proteins: *rsmX*, *rsmY*, and *rsmZ* (17, 18). Previous work had allowed the identification of the sRNAs *rsmY* and *rsmZ* in *P. putida* DOT-T1E (40). Based on sequence homology analysis, we have identified the equivalent sRNAs in KT2440 in the intergenic regions between the loci PP_0370 and PP_0371 (corresponding to *rsmY*) and between the loci PP_1624 and PP_1625 (corresponding to *rsmZ*). These sRNAs are 72% and 78% identical to their counterparts in *Pseudomonas protegens* Pf-5 (see Fig. S1 in the supplemental material). The presence or identity of *rsmX*, on the other hand, is less clear. Based on comparison with the *rsmX* sequences of *P. protegens* Pf-5 and *P. fluorescens* F113, a potential candidate could be located in the intergenic region between PP_0214 and PP_0215 (see Fig. S1 in the supplemental material), but the percent identity was much lower than that of the other two sRNAs (55%).

Generation of *rsm* single, double, and triple mutants. We generated *rsmI*, *rsmE*, and *rsmA* null mutants by complete deletion of the open reading frames without introducing antibiotic resistance cassettes, as well as the three double-mutant combinations and the triple mutant (see Materials and Methods for details). For simplicity, here, these mutants are designated ΔI, ΔE, ΔA, ΔIE, ΔEA, ΔIA, and ΔIEA.

The growth of all the mutants was analyzed in liquid cultures in rich and defined minimal media. No differences were observed in LB medium (not shown), whereas in minimal medium, the triple mutant showed an extended lag phase with respect to the wild type, regardless of the carbon source. The same delay was also observed in the ΔEA mutant growing in minimal medium with citrate as a carbon source and, although less pronounced, in the ΔA and ΔEA mutants in minimal medium with glucose as a carbon source (see Fig. S2 in the supplemental material).

Specific *rsm* mutations alter the motility of *P. putida*. In *P. fluorescens* F113, it has been reported that mutants affected in the *gacS-gacA* regulatory system show hypermotility, and a regulatory cascade involving RsmE and RsmI has been proposed (41). This, and the fact that in different bacteria the main target of the response regulator GacA is the RsmA/*rsmX-rsmY-rsmZ* regulon and that CsrA modulates flagellar genes in *E. coli* (14), led us to test the influence of *rsm* mutations on the motility of *P. putida* KT2440. As shown in Fig. 1A, the triple mutant and the ΔEA strain presented reduced swimming motility with respect to the wild type and the rest of the mutants in LB plates with 0.3% agar.

The effect of *rsm* mutations on surface motility was then analyzed on 0.5% agar plates, as previously described (39). Whereas the wild type had nearly covered the surface of the plate after 24 h, none of the mutants showed movement at that time, with the exception of ΔI (Fig. 1B), although that mutant was unable to completely cover the plate surface. This type of motility requires pyoverdine-mediated iron acquisition in KT2440 (39). This prompted us to check if any of the mutants showed altered pyoverdine production that could correlate with the defect in swarming. Pyoverdine was measured in the supernatants of cultures grown overnight in King's B medium (see Fig. S3 in the supplemental material). All the mutant strains showed a reduction in pyoverdine production with respect to the wild type that could be associated with the observed alteration in swarming motility. However, the difference in motility between ΔI and the remaining mutants cannot be explained simply in terms of pyoverdine production, which was not significantly different between them.

Rsm proteins modulate biofilm development. Previous results have shown that *gacS* is involved in the regulation of biofilm formation in *P. putida* KT2440 (42). We therefore examined the effects of *rsm* mutations on surface attachment and biofilm development. Assays were first done with cultures grown statically in polystyrene multiwell plates, following the attachment/detachment dynamics over time by staining the surface-associated biomass with crystal violet. As shown in Fig. 2A, each of the *rsm* single mutants behaved differently in these assays. The ΔI mutant initiated attachment like the wild type but showed early detachment from the surface. The ΔE and ΔA strains, on the other hand, presented reduced attachment during the first hours, reaching attached biomass values similar to those of the wild type at later times. The double and triple mutants were then analyzed (Fig. 2B). In ΔEA and ΔIEA, the attached biomass was significantly above that of the wild type after 6 h, whereas ΔIA and ΔIE kept a kinetic similar to that of the wild type, although the values for the latter were slightly below those of the wild type throughout.

Experiments were also done in borosilicate glass tubes with cultures grown under orbital rotation. We first compared the wild type and the triple mutant over time (Fig. 3A). Observation of the tubes before staining with crystal violet indicated similar attachment dynamics at early time points, with the ΔIEA mutant form-

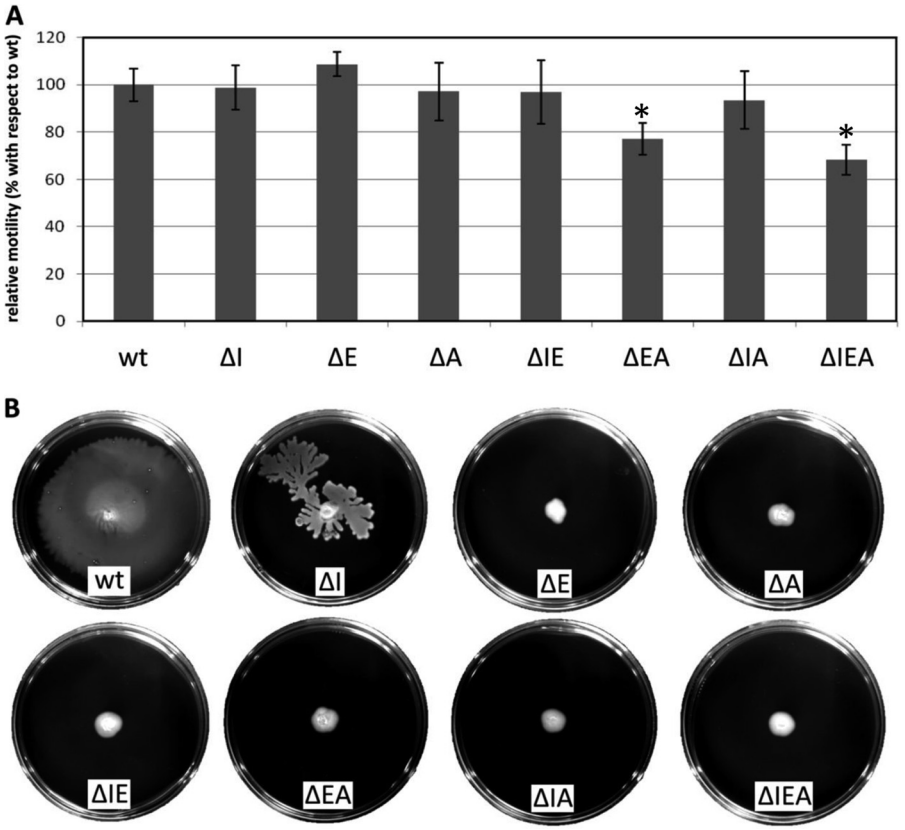

FIG 1 Influence of *rsm* mutations on motility of *P. putida*. (A) Swimming motility on LB plates with 0.3% agar. The graph indicates the areas covered by swimming halos after overnight growth. The data are the averages and standard deviations of 9 replicates. The asterisks indicate statistically significant differences (ANOVA; $P \leq 0.05$). (B) Surface motility of KT2440 and the *rsm* single, double, and triple mutants. The images were taken after 48 h of growth and show a representative experiment out of three independent replicates.

ing a significantly denser biofilm than the wild type at later time points. However, at these times, the biofilm of the mutant proved to be more labile than that of the wild type, so that it was washed from the surface during staining with crystal violet. A follow-up of this phenomenon in the remaining mutants indicated that ΔEA behaved like the triple mutant and was washed from the surface during staining after 7 h of growth (Fig. 3B). The ΔA mutant was also partly removed at this time, whereas the remaining mutants showed this phenotype at later times, while the biofilm of the wild type remained stainable.

The development of biofilms of KT2440 and the triple mutant tagged with mCherry was also followed in microscopy-ready multiwell plates by CLSM during growth under static conditions. The results presented in Fig. 4 show that under these conditions, ΔIEA started colonizing the surface faster than the wild type and also detached earlier.

Overexpressing RsmE or RsmI, but not RsmA, causes reduced biofilm formation in KT2440. To further explore the role of Rsm proteins in biofilm formation, each protein was cloned independently in an expression vector that is stable in *Pseudomonas* (17) under the control of IPTG (isopropyl-β-D-thiogalactopyranoside). These constructs were introduced into the wild-type KT2440, and biofilm formation was analyzed during growth in LB medium in the presence or absence of IPTG. As shown in Fig. 5, overexpressing RsmE caused a reduction in biofilm formation. The increase in RsmI had a similar, although less pronounced, effect, whereas the increase in RsmA had no obvious influence on biofilm formation. Quantification of the attached biomass after solubilization of the dye revealed 70% and 50% reductions due to overexpression of RsmE and RsmI, respectively (see Fig. S4 in the supplemental material).

To investigate the potential interplay between the different Rsm proteins, we also introduced each of the expression constructs in all the mutants and analyzed biofilm formation on borosilicate glass tubes (Fig. 5; see Fig. S4 in the supplemental material). As with the wild type, overexpression of RsmA had no significant effect regardless of the genetic background, whereas overexpression of RsmE resulted in decreased biofilm formation in all the strains, with small differences between them. Interestingly, the effect observed in the wild type when RsmI was overexpressed was nearly lost in the triple mutant. Analysis of the remaining mutants showed that the presence of intact *rsmA* was required for the reduced biofilm phenotype associated with RsmI overexpression. It should be noted that at the time at which this analysis was done (5 h after inoculation), the reduction in crystal violet staining was correlated with the visual observation of attached biomass, and it was not a consequence of the biofilm lability that can be observed at later times (shown in Fig. 3).

Influence of Rsm proteins on expression of structural elements of *P. putida* biofilms. The results described so far could suggest the possibility that Rsm proteins participate in biofilm formation by altering the surface characteristics of *P. putida* cells,

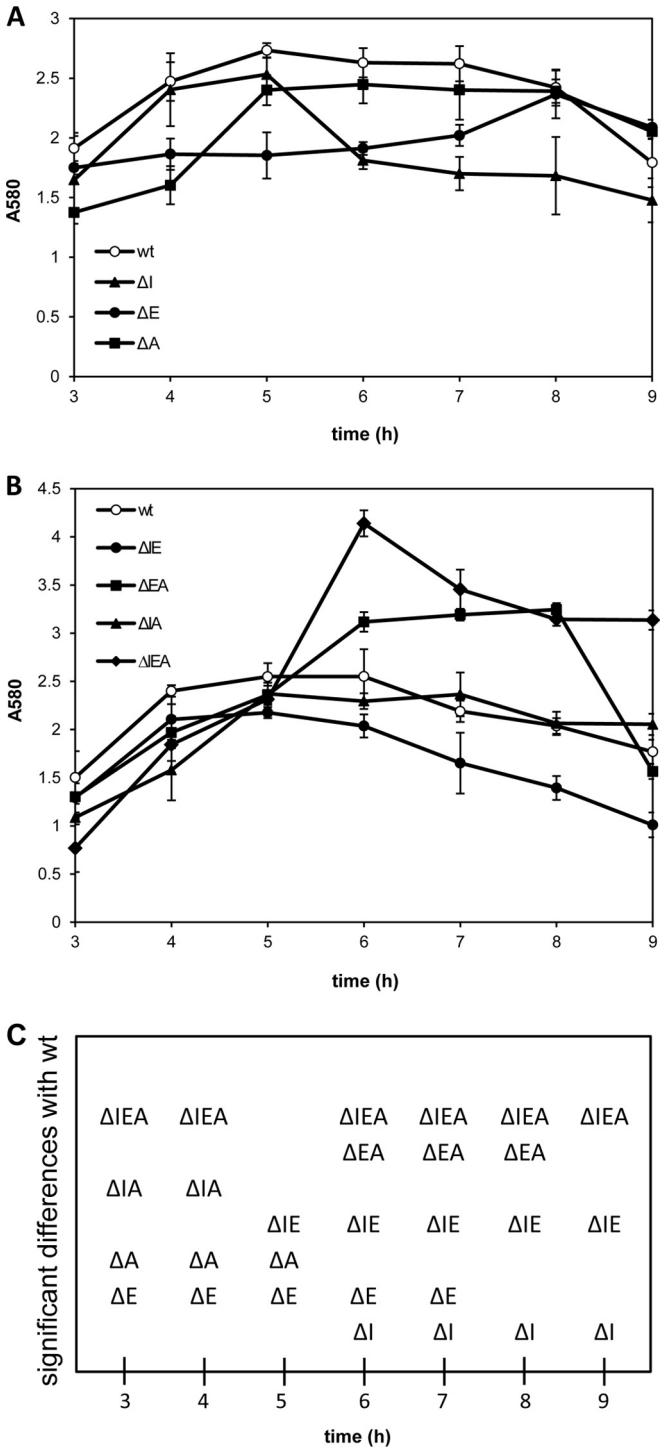

FIG 2 Biofilm formation by KT2440 and *rsm* mutant derivatives in polystyrene multiwell plates. Overnight cultures grown in LB medium were diluted to an OD_{660} of 1, and 5 μl was added to each well containing 200 μl of LB medium. Attachment was followed by removing the liquid from the wells at the indicated times and staining with crystal violet. The data correspond to the measurement of absorbance at 580 nm (A_{580}) after solubilization of the dye and are the averages and standard deviations of three independent experiments, each with three replicates per strain. (A) Wild type and single mutants. (B) Wild type, double mutants, and triple mutant. (C) Mutants showing significant differences from the wild type at a given time point (Student's *t* test; $P \leq 0.05$).

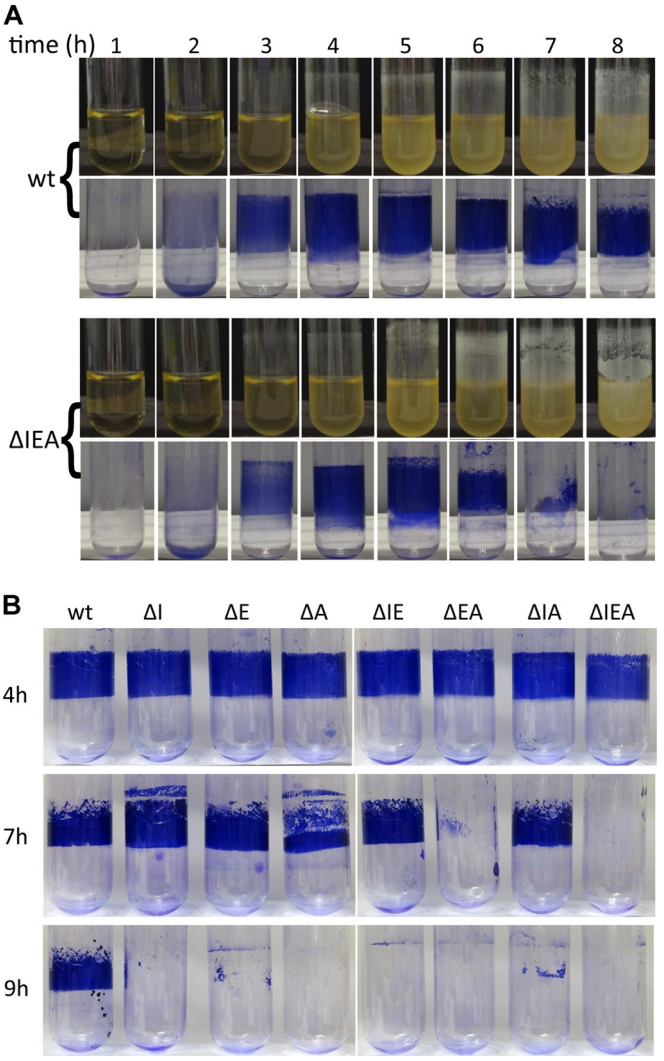

FIG 3 Kinetics of biofilm formation. (A) Biofilm formation by KT2440 and the *rsm* triple mutant (ΔIEA) growing in LB medium in borosilicate glass tubes under orbital rotation. At the indicated times, tubes were removed and images (top) were taken before discarding planktonic cells and staining with crystal violet (bottom). (B) Evaluation of the surface-attached biomass in KT2440 and the seven *rsm* mutants at different times of biofilm development. Growth conditions were as for panel A. Images from a representative experiment out of three replicates are shown (different experiments are represented in panels A and B).

which would explain why the triple mutant showed thicker but more labile biofilms on glass surfaces while remaining attached on plastic surfaces. This, and the fact that in *P. aeruginosa* RsmA modulates expression of the Psl exopolysaccharide (7), prompted us to investigate if any of the mutations caused changes in the expression of structural elements known to participate in the buildup of *P. putida* biofilms under different conditions, namely, the two large adhesins LapA and LapF and the exopolysaccharides Pea, Peb, cellulose (Bcs), and alginate (Alg).

To determine if *rsm* mutations had an influence on the mRNA levels of genes involved in exopolysaccharide biosynthesis, qRT-PCR was done with RNA extracted from the wild-type and ΔIEA strains grown on solid medium for 24 or 48 h, using primers that correspond to the loci PP_1277 (Alg), PP_1795 (Peb), PP_2629

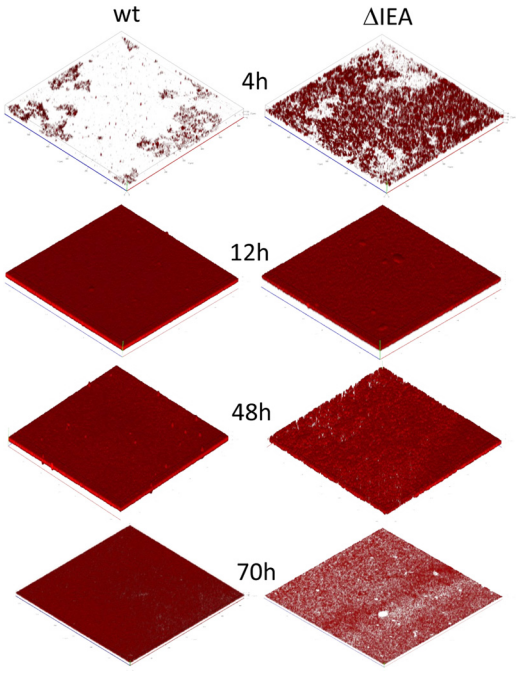

FIG 4 CLSM follow-up of biofilm formation by KT2440 and the *rsm* triple mutant, ΔIEA. Strains were tagged with miniTn*7*Ptac-mChe (miniTn*7*:: mCherry) in single copy at an intergenic location in the chromosome. Bacterial cells were grown in LB medium diluted 1:10 in microscopy-ready multiwell plates. Three-dimensional reconstructions, generated with Imaris, of representative fields are shown.

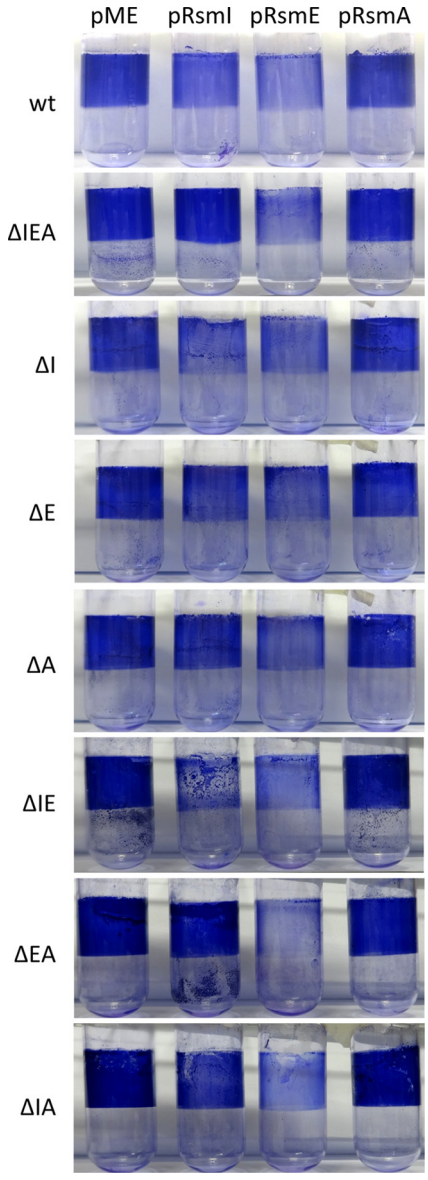

FIG 5 Effects of overexpressing each Rsm protein on biofilm formation by KT2440 and the seven *rsm* mutant derivatives. The genes *rsmI*, *rsmE*, and *rsmA* were cloned in the broad-host-range expression vector pME6032. The resulting constructs (pRsmI, pRsmE, and pRsmA), as well as the empty vector (pME) as a control, were introduced into all the strains. Experiments were done as for Fig. 3. The images show attached biomass stained with crystal violet after 5 h of growth in the presence of 0.1 mM IPTG and 20 μg/ml tetracycline. wt, wild type.

(Bcs), and PP_3132 (Pea). As shown in Fig. 6A, there was a significant increase in the mRNAs corresponding to Peb (2.5-fold) and Bcs (5-fold) in the triple mutant with respect to the wild type, whereas no significant differences were observed for Pea (PP_3132) and Alg (PP_1277). Analysis of these differences in the single mutants after 48 h indicated that in the case of Peb, the lack of either RsmE or RsmA had an effect similar to that observed in the triple mutant, while RsmI did not appear to influence its expression (Fig. 6B). For Bcs, increased expression was observed in ΔE and ΔA, but in neither case did it reach the levels observed in ΔIEA, suggesting a cumulative effect of both proteins.

Since Rsm proteins may regulate translation with or without effects on mRNA transcript levels, we decided to expand this analysis by checking the expression patterns of fusions of the first gene in each EPS cluster (including the promoter and first codons) with the reporter gene *lacZ* devoid of its own promoter, as described elsewhere (51). The results obtained comparing the wild type with the ΔIEA mutant are shown in Fig. 7. In all cases, the pattern of expression showed alterations at different times during growth in liquid medium, with an overall increase in activity in the mutant. In the case of the fusion corresponding to Pea, the differences were significant only between 4 and 8 h of growth, which could explain why qRT-PCR at 24 at 48 h did not reveal alterations in the ΔIEA mutant. Analysis of the fusions in all the mutant backgrounds indicated that in all cases except Peb, the combination of *rsmA* and *rsmE* mutations was responsible for most of the observed changes in the triple mutant (Fig. 7A, C, and D), whereas no single or double mutant had the same pattern as ΔIEA in the case of Peb (data not shown).

Next, expression of the two adhesins was followed during growth by measuring the β-galactosidase activity of *lapA*::′*lacZ* and *lapF*::′*lacZ* fusions carried on plasmids pMMGA and pMMG1, respectively (42, 43), in the wild type and all the *rsm* mutants. No great differences were observed for *lapA* in the different genetic backgrounds, except for a slight overall increase in ΔIEA (Fig. 7E) that was not significant. In contrast, expression of *lapF*::*lacZ* was clearly influenced by the three Rsm proteins, and the triple mutant showed earlier induction and increased β-galactosidase activity with respect to the wild type (Fig. 7F). Detailed analysis in each of the single mutants indicated that the absence of

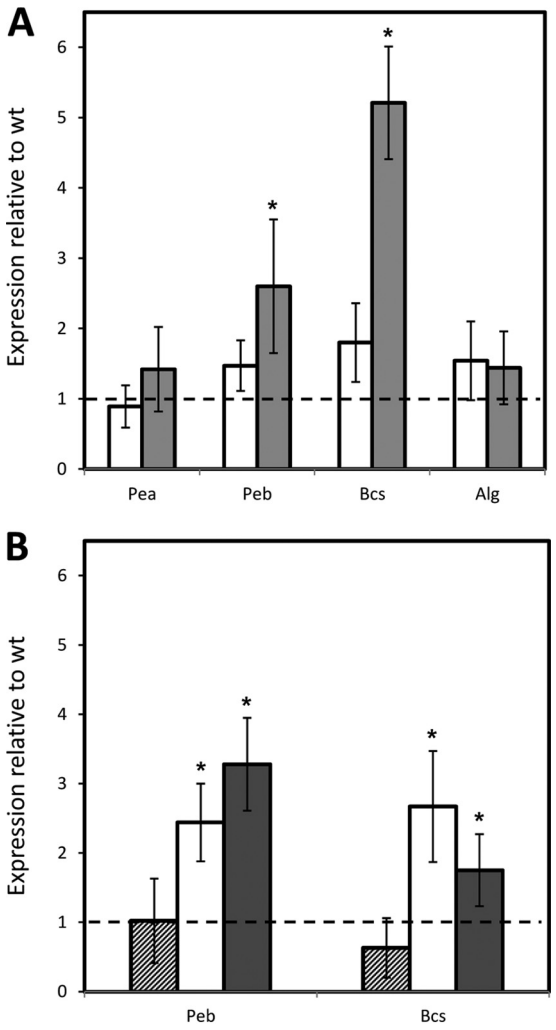

FIG 6 Influence of *rsm* mutations on mRNA levels of EPS-encoding genes in cultures grown in solid medium. (A) mRNA levels of EPS genes in the *rsm* triple mutant relative to the wild type analyzed by qRT-PCR. RNA was isolated from samples grown on LB agar plates after 24 h (white bars) or 48 h (gray bars). A value of 1 (dashed line) indicates expression levels identical to those of the wild type. (B) Relative expression of genes corresponding to cellulose (Bcs) and the specific EPS Peb in mutants ΔI (hatched bars), ΔE (gray bars), and ΔA (white bars) with respect to the wild type after 48 h of growth on LB agar plates. The data are the averages and standard deviations from three biological replicates with three technical replicates. The values significantly different from those of the wild type are indicated by asterisks (Student's *t* test; $P \leq 0.05$).

RsmA and RsmE had a cumulative influence on the overall increase in expression, while the lack of RsmI mostly contributed to the earlier peak of activation (see Fig. S5 in the supplemental material).

Expression patterns of *rsm* genes. RACE was used to determine the transcription initiation sites of the three *rsm* genes. In *rsmI* and *rsmA*, the +1 site was located 63 and 245 bp upstream of the ATG, respectively, while in *rsmE*, two transcription initiation sites were identified at bases −53 (proximal +1 site) and −183 (distal +1 site) (see Fig. S6 in the supplemental material). The same initiation sites were identified in transcripts from cultures in the exponential or stationary phase of growth.

Based on this information, two translational fusions were constructed with the reporter gene *lacZ* in plasmid pMP220-BamHI containing the *rsmI* and *rsmA* regions upstream of the +1 site (pOHR46 and pOHR52), respectively, and two with *rsmE*, a fragment containing the proximal +1 site closer to the ATG (pOHR47) and a larger one including both the proximal and distal transcription start sites (pOHR48). All the constructs included the ribosome-binding site and translation initiation codon. The constructs were introduced in KT2440, and β-galactosidase activity was followed during growth in LB medium. The activity of the RsmI-LacZ fusion was very low and was detected only when cultures had already reached the stationary phase (Fig. 8A). The results for RsmA-LacZ (Fig. 8B) showed a gradual increase in activity during exponential growth and the early stationary phase, followed by a decrease later. In the case of RsmE, both constructs showed an increase in expression in midexponential phase (Fig. 8C and D). The difference between the construct harboring the proximal promoter region (P2) and the construct with both promoter regions can be explained if the activity of the distal promoter (P1) is maintained longer than that of P2.

The observations made in different genetic backgrounds with respect to biofilm formation and expression of the extracellular matrix components suggested the existence of cross-regulation between *rsm* genes. This possibility was first explored by introducing the above-mentioned constructs in the triple mutant. Analysis of β-galactosidase activity showed, in all cases, altered patterns and/or increased levels of expression with respect to the wild type (Fig. 8). The different fusions were then introduced in the single and double mutants. The results obtained with ΔE and ΔA indicated that both RsmE (Fig. 9A) and RsmA (Fig. 9B) have a clear negative effect on their own expression, with the self-repression effect of RsmA during the late stationary phase especially evident (Fig. 9B), while in the remaining combinations of mutations and reporter fusions, the observed changes were minor or nonexistent (data not shown).

DISCUSSION

The regulatory cascade involving posttranscriptional regulators of the Rsm family and their cognate small RNAs has gained increasing relevance because, beyond its role in secondary metabolism, it is arising as a central element in bacterial gene expression regulation. RsmA homologs are present in diverse bacteria, from generalist species able to adapt to a variety of environments, like *P. putida*, to highly specialized bacteria with relatively few regulatory proteins, such as the human gastric pathogen *Helicobacter pylori*, where RsmA controls virulence and the stress response (44). It is noticeable that in the former, which has been the focus of this work, and in related species, such as *P. fluorescens* or *P. protegens*, there are three very similar Rsm proteins. Although this could imply the existence of regulatory redundancy or something like a "backup system," our data suggest that there is a combination of differentiated and cumulative effects attributable to these proteins. This is exemplified by the fact that RsmA and RsmE have roles in swimming motility that can be detected only when both are deleted, whereas the lack of RsmI has no detectable influence on swimming. On the other hand, swarming is completely abolished in all the mutants except ΔI. Other members of the RsmA/CsrA family have been previously described as relevant elements for swarming motility in different bacteria, such as *P. aeruginosa* (45), *Serratia marcescens* (46), and *Proteus mirabilis* (47).

We have shown that Rsm proteins function as negative regula-

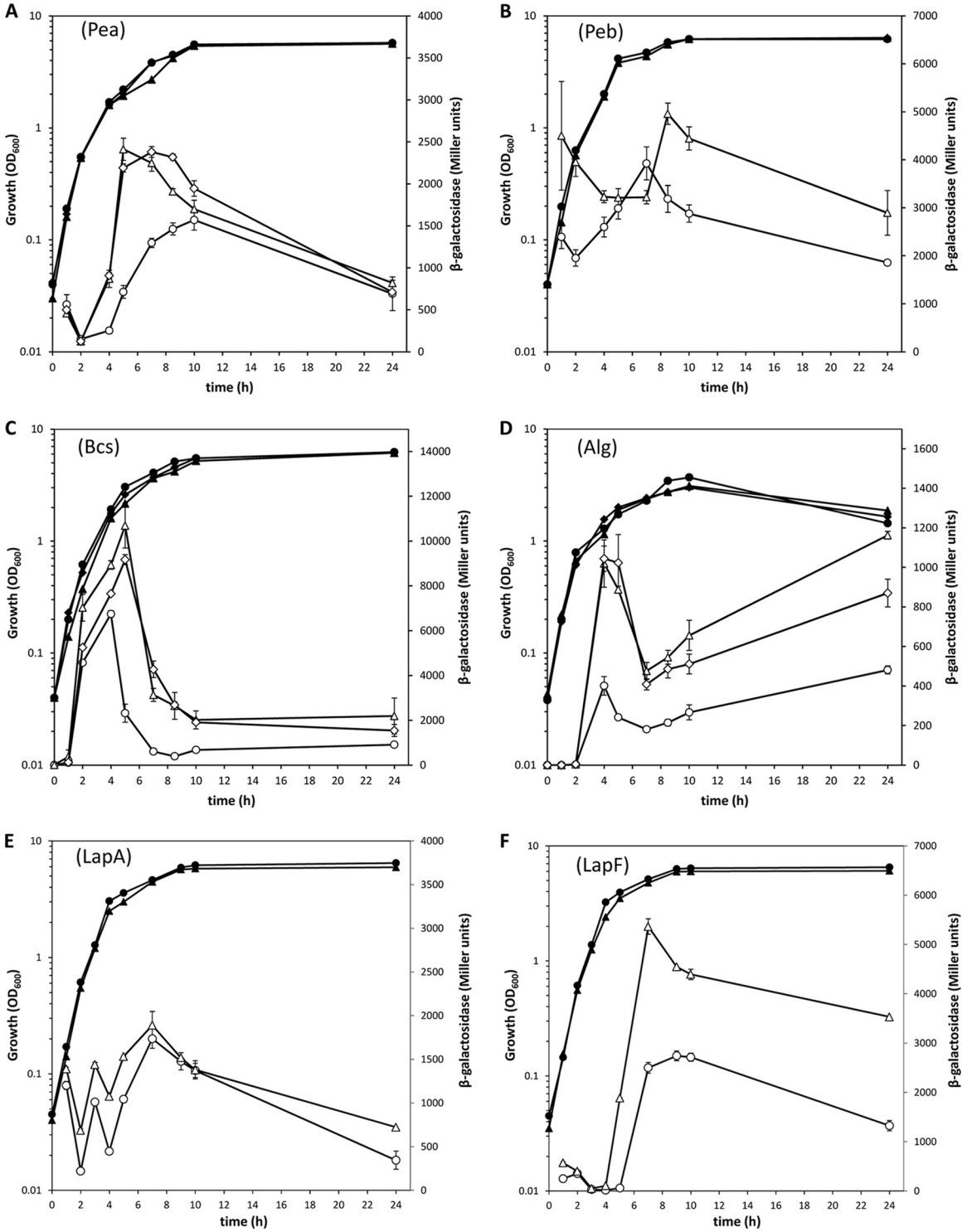

FIG 7 Influence of *rsm* mutations on expression of EPS- and adhesin-encoding genes during growth in liquid medium. Growth (solid symbols) and β-galactosidase activity (open symbols) of KT2440 (circles), ΔIEA (triangles), and ΔEA (diamonds) carrying reporter fusions corresponding to Pea (PP_3132::′*lacZ*) (A), Peb (PP_1795::′*lacZ*) (B), Bcs (PP_2629::′*lacZ*) (C), Alg (*algD*::′*lacZ*) (D), *lapA*::*lacZ* (E), and *lapF*::*lacZ* (F) were followed over time. The data are averages and standard deviations from three biological replicates with two technical repetitions. Statistically significant differences between the wild type and ΔIEA were detected from 4 to 8 h (A); at 2, 4, 8, 10, and 24 h (B); from 2 to 10 h (C); from 4 h onward (D); and from 5 h onward (F) (Student's *t* test; $P \leq 0.05$). (D) D-Cycloserine (75 μg/ml) was added after 2 h of growth, since in *P. putida*, the *algD* promoter is silent in liquid medium in the absence of cell wall stress (M. I. Ramos-González, unpublished data).

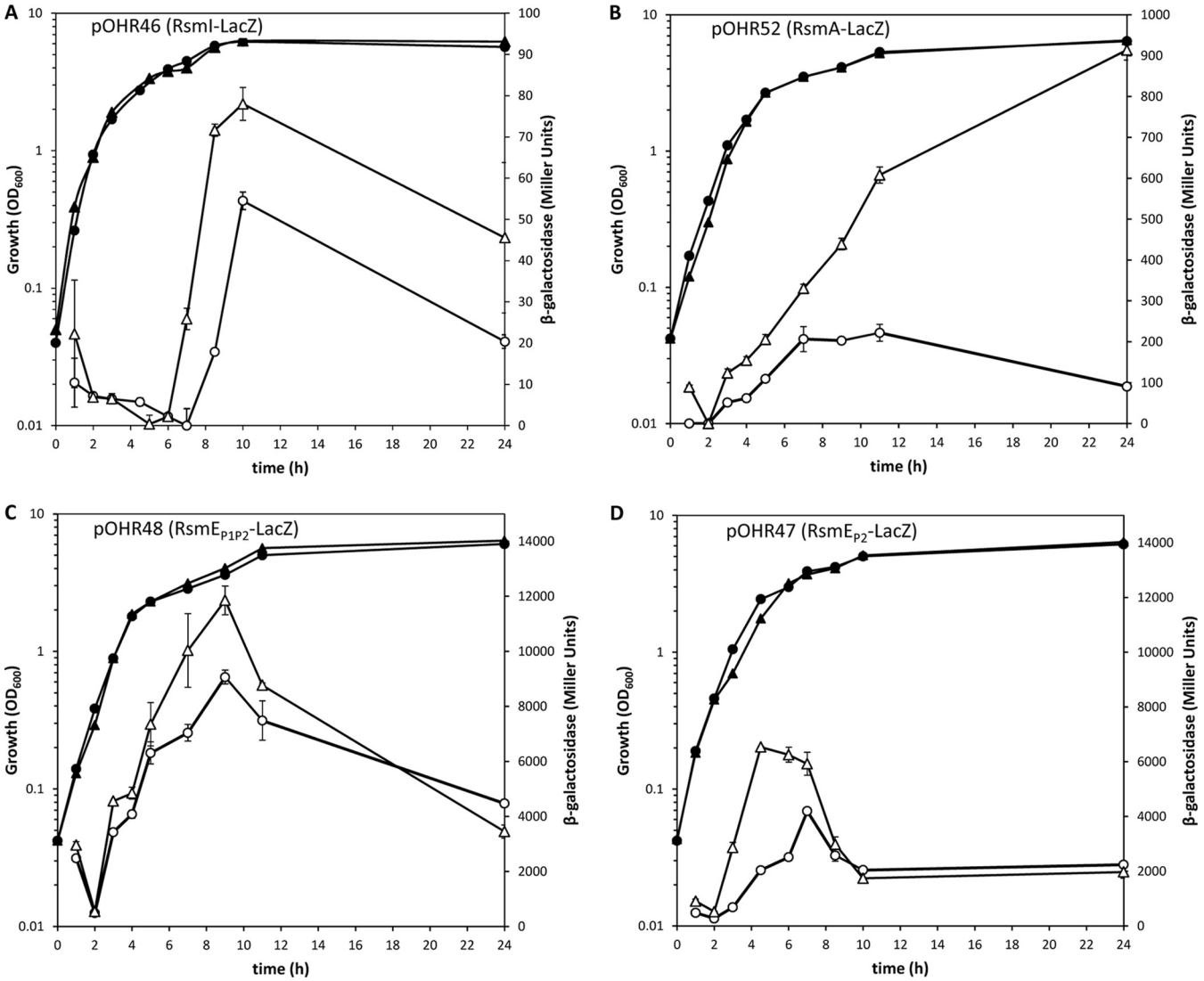

FIG 8 Expression patterns of Rsm proteins in the wild-type KT2440 and the *rsm* triple mutant ΔIEA. Growth (solid symbols) and β-galactosidase activity (open symbols) of KT2440 (circles) and ΔIEA (triangles) carrying the different Rsm-LacZ fusions are indicated in each panel. The RsmE$_{P1P2}$-LacZ fusion contains both distal and proximal promoters, and the RmsE$_{P2}$-LacZ fusion contains only the proximal promoter. The data are averages and standard deviations from three biological replicates with two technical repetitions. Statistically significant differences between the wild type and ΔIEA were detected from 7 h onward for pOHR46, from 7 h onward for pOHR52, at 7 and 8 h for pOHR48, and from 3 to 7 h for pOHR47 (Student's *t* test; $P < 0.05$).

tors in the process of biofilm development: the lack of all three proteins causes an increase in biofilm formation, but the robustness of the biofilm in terms of bacterial association with the surface is reduced, resulting in relatively easy and early detachment (Fig. 3 and 4). This effect is observed on glass surfaces, especially with ΔEA and ΔIEA, but it is not evident on plastic, where these strains remain attached to the surface. Under these conditions the single ΔE and ΔA mutants show delayed attachment relative to the wild type, whereas the ΔI mutant presents early detachment.

We have also analyzed the effect of overexpressing each Rsm protein in the wild type and in each mutant background. Our results pinpoint RsmE as the main element modulating bacterial attachment, with RsmI having a less pronounced effect that is dependent on the presence of an intact RsmA, which indicated that there is a regulatory interplay between Rsm proteins, so that they may act in a concerted way on their targets. Expression of the translational fusions constructed in the different genetic backgrounds indicated the existence of self-regulation in *rsmA* and *rsmE* and also revealed sequential activation of the different *rsm* genes. Thus, *rsmE* and *rsmA* would be the first to be expressed during growth in rich medium at 30°C, followed by *rsmI* in stationary phase. Expression of each gene also seems to be turned off sequentially: first *rsmA*, then *rsmE*, and finally *rsmI*. It is also worth mentioning the presence in *rsmE* of two distinct promoters with different expression dynamics. Although the expression sequence of *rsm* genes in the same bacterium had not been previously investigated, small RNAs are known to be sequentially expressed. In *P. fluorescens* (now *P. protegens*) CHA0, the expression patterns of the three sRNAs that interact with RsmA homologs have been described, with *rsmX* and *rsmY* showing a linear increase during growth while *rsmZ* is expressed at a later time (18). However, a possible correlation with the expression patterns of Rsm proteins

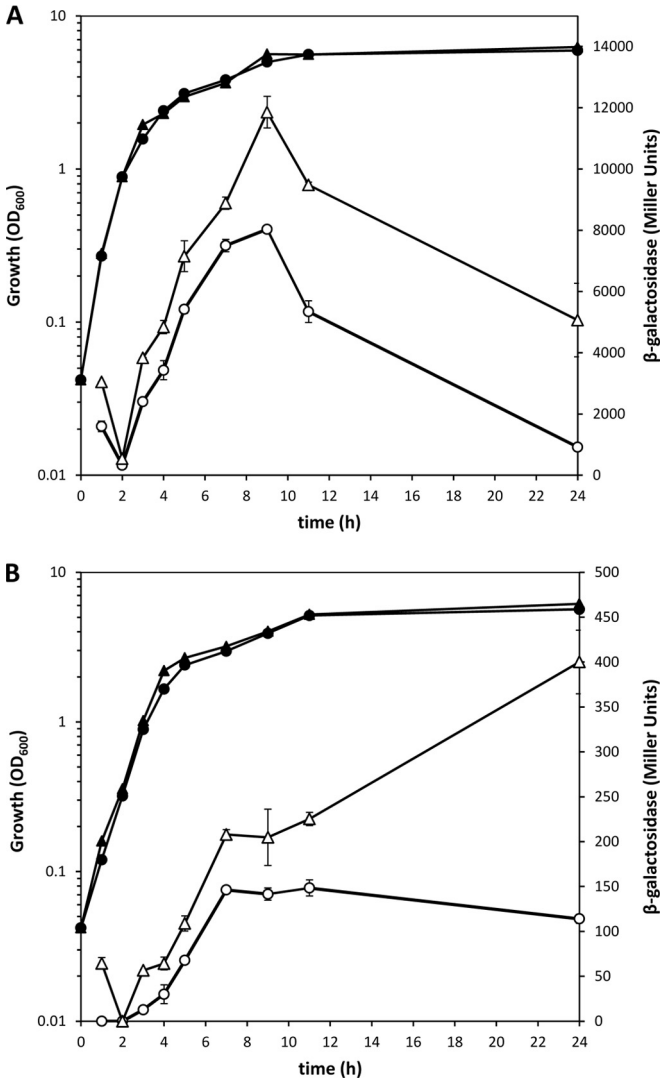

FIG 9 Self-repression exerted by Rsm proteins. Growth (solid symbols) and β-galactosidase activity (open symbols) of KT2440 (circles) and single mutants (triangles) carrying $RsmE_{P1P2}$-LacZ (ΔE mutant) (A) and RsmA-LacZ (ΔA mutant) (B) fusions. The data are averages and standard deviations from three biological replicates with two technical repetitions. Statistically significant differences between the wild type and the mutant were detected from 3 h onward for $RsmE_{P1P2}$-LacZ and from 7 h onward for RsmA-LacZ (Student's *t* test; $P \leq 0.05$).

has not been studied. It may also be that different environmental conditions cause alterations in the progression of expression of the various elements. Detailed analysis of these aspects will be of great interest in terms of the responses of *P. putida* to different environmental conditions.

To have a clearer picture of the molecular basis for the role of Rsm proteins in biofilm formation, we have examined their influence on the expression of elements that are required for surface attachment (LapA), cell-cell interactions (LapF), and extracellular matrix composition (both adhesins and EPS). We have shown that the lack of Rsm proteins causes earlier and increased expression of LapF and also increases expression of cellulose and the strain-specific EPS Peb. Previous work has supported the notion that Pea (the other strain-specific EPS) and, to a minor extent, Peb are the exopolysaccharides with the main structural roles in *P. putida* biofilms grown under conditions similar to those used here, while alginate and cellulose would have a role in different environmental situations (48, 49). Therefore, removing Rsm proteins (particularly RsmE and RsmA) likely promotes cell-cell interactions mediated by LapF, giving rise to thicker biofilms, and causes alterations in the balance and composition of the biofilm extracellular matrix. This probably also has consequences for characteristics such as hydrophobicity, which would explain the lability of the biofilms formed by the triple mutant, despite their increased biomass on glass surfaces, and the differences observed with plastic surfaces. In *P. aeruginosa*, RsmA negatively influences the expression of *psl*, one of its two species-specific EPS operons, involved in the architecture of biofilms (7). Similarly, RsmA and RsmE influence the expression of one of the two *P. putida*-specific EPSs, Peb. However, the modulation of biofilm formation by the Rsm system appears to be far more complex in this bacterium, not only because of the existence of three proteins, but also because of their influence on additional elements involved in surface colonization that are absent in *P. aeruginosa* (LapF and cellulose).

The results obtained here provide evidence of the regulatory complexities associated with the adaptation of a versatile bacterium like *P. putida* KT2440 to different environmental conditions. The tools generated in this work (mutants, overexpression constructs, and reporter fusions) and the knowledge gained will be of great importance for further dissection of the elements involved in posttranscriptional control of expression in *P. putida*.

ACKNOWLEDGMENTS

We thank M. Cámara and S. Heeb for plasmid pME6032, general advice, and discussion of results. We also acknowledge the comments made by the reviewers, which have helped improve the original manuscript.

This work was supported by Ministerio de Economía y Competitividad and EFDR through grants BFU2010-17946 and BFU2013-43469-P and an FPI predoctoral scholarship (EEBB-BES-2011-047539) to Ó.H.-R.

FUNDING INFORMATION

This work, including the efforts of Óscar Huertas-Rosales, María Isabel Ramos-González, and Manuel Espinosa-Urgel, was funded by Ministerio de Economía y Competitividad and EFDR through grants BFU2010-17946 and BFU2013-43469-P and through a fellowship of the FPI program (EEBB-BES-2011-047539) to Óscar Huertas-Rosales.

REFERENCES

1. **Romeo T, Gong M, Liu MY, Brun-Zinkernagel AM.** 1993. Identification and molecular characterization of *csrA*, a pleiotropic gene from *Escherichia coli* that affects glycogen biosynthesis, gluconeogenesis, cell size, and surface properties. J Bacteriol **175:**4744–4755.
2. **Romeo T.** 1998. Global regulation by the small RNA-binding protein CsrA and the non-coding RNA molecule CsrB. Mol Microbiol **29:**1321–1330. http://dx.doi.org/10.1046/j.1365-2958.1998.01021.x.
3. **Yang H, Liu MY, Romeo T.** 1996. Coordinate genetic regulation of glycogen catabolism and biosynthesis in *Escherichia coli* via the *csrA* gene product. J Bacteriol **178:**1012–1017.
4. **Sabnis NA, Yang H, Romeo T.** 1995. Pleiotropic regulation of central carbohydrate metabolism in *Escherichia coli* via the gene *csrA*. J Biol Chem **270:**29096–29104. http://dx.doi.org/10.1074/jbc.270.49.29096.
5. **Pessi G, Haas D.** 2001. Dual control of hydrogen cyanide biosynthesis by the global activator GacA in *Pseudomonas aeruginosa* PAO1. FEMS Microbiol Lett **200:**73–78. http://dx.doi.org/10.1111/j.1574-6968.2001.tb10695.x.
6. **Pessi G, Williams F, Hindle Z, Heurlier K, Holden MTG, Cámara M, Haas D, Williams P.** 2001. The global posttranscriptional regulator RsmA modulates production of virulence determinants and *N*-acylhomoserine

lactones in *Pseudomonas aeruginosa*. J Bacteriol **183:**6676–6683. http://dx.doi.org/10.1128/JB.183.22.6676-6683.2001.
7. **Irie Y, Starkey M, Edwards AN, Wozniak DJ, Romeo T, Parsek MR.** 2010. *Pseudomonas aeruginosa* biofilm matrix polysaccharide Psl is regulated transcriptionally by RpoS and post-transcriptionally by RsmA. Mol Microbiol **78:**158–172. http://dx.doi.org/10.1111/j.1365-2958.2010.07320.x.
8. **Colley B, Dederer V, Carnell M, Kjelleberg S, Rice SA, Klebensberger J.** 2016. SiaA/D interconnects c-di-GMP and RsmA signaling to coordinate cellular aggregation of *Pseudomonas aeruginosa* in response to environmental conditions. Front Microbiol **7:**179. http://dx.doi.org/10.3389/fmicb.2016.00179.
9. **Liu MY, Yang H, Romeo T.** 1995. The product of the pleiotropic *Escherichia coli* gene *csrA* modulates glycogen biosynthesis via effects on mRNA stability. J Bacteriol **177:**2663–2672.
10. **Liu MY, Romeo T.** 1997. The global regulator CsrA of *Escherichia coli* is a specific mRNA-binding protein. J Bacteriol **179:**4639–4642.
11. **Blumer C, Heeb S, Pessi G, Haas D.** 1999. Global GacA-steered control of cyanide and exoprotease production in *Pseudomonas fluorescens* involves specific ribosome binding sites. Proc Natl Acad Sci U S A **96:**14073–14078. http://dx.doi.org/10.1073/pnas.96.24.14073.
12. **Baker CS, Morozov I, Suzuki K, Romeo T, Babitzke P.** 2002. CsrA regulates glycogen biosynthesis by preventing translation of *glgC* in *Escherichia coli*. Mol Microbiol **44:**1599–1610. http://dx.doi.org/10.1046/j.1365-2958.2002.02982.x.
13. **Pernestig A-K, Georgellis D, Romeo T, Suzuki K, Tomenius H, Normark S, Melefors Ö.** 2003. The *Escherichia coli* BarA-UvrY two-component system is needed for efficient switching between glycolytic and gluconeogenic carbon sources. J Bacteriol **185:**843–853. http://dx.doi.org/10.1128/JB.185.3.843-853.2003.
14. **Wei BL, Brun-Zinkernagel AM, Simecka JW, Prüß BM, Babitzke P, Romeo T.** 2001. Positive regulation of motility and *flhDC* expression by the RNA-binding protein CsrA of *Escherichia coli*. Mol Microbiol **40:**245–256. http://dx.doi.org/10.1046/j.1365-2958.2001.02380.x.
15. **Weilbacher T, Suzuki K, Dubey AK, Wang X, Gudapaty S, Morozov I, Baker CS, Georgellis D, Babitzke P, Romeo T.** 2003. A novel sRNA component of the carbon storage regulatory system of *Escherichia coli*. Mol Microbiol **48:**657–670. http://dx.doi.org/10.1046/j.1365-2958.2003.03459.x.
16. **Liu Y, Cui Y, Mukherjee A, Chatterjee AK.** 1998. Characterization of a novel RNA regulator of *Erwinia carotovora* ssp. *carotovora* that controls production of extracellular enzymes and secondary metabolites. Mol Microbiol **29:**219–234. http://dx.doi.org/10.1046/j.1365-2958.1998.00924.x.
17. **Heeb S, Blumer C, Haas D.** 2002. Regulatory RNA as mediator in GacA/RsmA-dependent global control of exoproduct formation in *Pseudomonas fluorescens* CHA0. J Bacteriol **184:**1046–1056. http://dx.doi.org/10.1128/jb.184.4.1046-1056.2002.
18. **Kay E, Dubuis C, Haas D.** 2005. Three small RNAs jointly ensure secondary metabolism and biocontrol in *Pseudomonas fluorescens* CHA0. Proc Natl Acad Sci U S A **102:**17136–17141. http://dx.doi.org/10.1073/pnas.0505673102.
19. **Heeb S, Haas D.** 2001. Regulatory roles of the GacS/GacA two-component system in plant-associated and other Gram-negative bacteria. Mol Plant Microbe Interact **14:**1351–1363. http://dx.doi.org/10.1094/MPMI.2001.14.12.1351.
20. **Hyytiainen H, Montesano M, Palva ET.** 2001. Global regulators ExpA (GacA) and KdgR modulate extracellular enzyme gene expression through the RsmA-*rsmB* system in *Erwinia carotovora* subsp. carotovora. Mol Plant Microbe Interact **14:**931–938. http://dx.doi.org/10.1094/MPMI.2001.14.8.931.
21. **Reimmann C, Beyeler M, Latifi A, Winteler H, Foglino M, Lazdunski A, Haas D.** 1997. The global activator GacA of *Pseudomonas aeruginosa* PAO positively controls the production of the autoinducer *N*-butyryl-homoserine lactone and the formation of the virulence factors pyocyanin, cyanide, and lipase. Mol Microbiol **24:**309–319. http://dx.doi.org/10.1046/j.1365-2958.1997.3291701.x.
22. **Lapouge K, Schubert M, Allain FH, Haas D.** 2008. Gac/Rsm signal transduction pathway of γ-proteobacteria: from RNA recognition to regulation of social behaviour. Mol Microbiol **67:**241–253.
23. **Nakazawa T.** 2002. Travels of a *Pseudomonas*, from Japan around the world. Environ Microbiol **4:**782–786. http://dx.doi.org/10.1046/j.1462-2920.2002.00310.x.
24. **Nelson K, Weinel C, Paulsen I, Dodson R, Hilbert H, Martins dos Santos V, Fouts D, Gill S, Pop M, Holmes M.** 2002. Complete genome sequence and comparative analysis of the metabolically versatile *Pseudomonas putida* KT2440. Environ Microbiol **4:**799–808. http://dx.doi.org/10.1046/j.1462-2920.2002.00366.x.
25. **Tecon R, Binggeli O, Van der Meer JR.** 2009. Double-tagged fluorescent bacterial bioreporter for the study of polycyclic aromatic hydrocarbon diffusion and bioavailability. Environ Microbiol **11:**2271–2283. http://dx.doi.org/10.1111/j.1462-2920.2009.01952.x.
26. **Lennox ES.** 1955. Transduction of linked genetic characters of the host by bacteriophage P1. Virology **1:**190–206. http://dx.doi.org/10.1016/0042-6822(55)90016-7.
27. **Sambrook J, Russell DW.** 2001. Molecular cloning: a laboratory manual, 3rd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
28. **Yousef-Coronado F, Travieso ML, Espinosa-Urgel M.** 2008. Different, overlapping mechanisms for colonization of abiotic and plant surfaces by *Pseudomonas putida*. FEMS Microbiol Lett **288:**118–124. http://dx.doi.org/10.1111/j.1574-6968.2008.01339.x.
29. **King EO, Ward MK, Raney DE.** 1954. Two simple media for the demonstration of pyocyanin and fluorescin. J Lab Clin Met **44:**301–307.
30. **Ausubel F, Brent R, Kingston R, Moore D, Seidman J, Smith J, Struhl K.** 1987. Current protocols in molecular biology. Wiley, New York, NY.
31. **Enderle PJ, Farwell MA.** 1998. Electroporation of freshly plated *Escherichia coli* and *Pseudomonas aeruginosa* cells. Biotechniques **25:**954–958.
32. **Koch B, Jensen LE, Nybroe O.** 2001. A panel of Tn*7*-based vectors for insertion of the *gfp* marker gene or for delivery of cloned DNA into Gram-negative bacteria at a neutral chromosomal site. J Microbiol Methods **45:**187–195. http://dx.doi.org/10.1016/S0167-7012(01)00246-9.
33. **Herrero M, de Lorenzo V, Timmis KN.** 1990. Transposon vectors containing non-antibiotic resistance selection markers for cloning and stable chromosomal insertion of foreign genes in gram-negative bacteria. J Bacteriol **172:**6557–6567.
34. **Kaniga K, Delor I, Cornelis GR.** 1991. A wide-host-range suicide vector for improving reverse genetics in Gram-negative bacteria: inactivation of the *blaA* gene of *Yersinia enterocolitica*. Gene **109:**137–141. http://dx.doi.org/10.1016/0378-1119(91)90599-7.
35. **Matilla MA, Travieso ML, Ramos JL, Ramos-González MI.** 2011. Cyclic diguanylate turnover mediated by the sole GGDEF/EAL response regulator in *Pseudomonas putida*: its role in the rhizosphere and an analysis of its target processes. Environ Microbiol **13:**1745–1766. http://dx.doi.org/10.1111/j.1462-2920.2011.02499.x.
36. **Livak KJ, Schmittgen TD.** 2001. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. Methods **25:**402–408. http://dx.doi.org/10.1006/meth.2001.1262.
37. **Miller JH.** 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
38. **O'Toole GA, Kolter R.** 1998. Initiation of biofilm formation in *Pseudomonas fluorescens* WCS365 proceeds via multiple, convergent signalling pathways: a genetic analysis. Mol Microbiol **28:**449–461. http://dx.doi.org/10.1046/j.1365-2958.1998.00797.x.
39. **Matilla MA, Ramos JL, Duque E, Alché JD, Espinosa-Urgel M, Ramos-González MI.** 2007. Temperature and pyoverdine-mediated iron acquisition control surface motility of *Pseudomonas putida*. Environ Microbiol **9:**1842–1850. http://dx.doi.org/10.1111/j.1462-2920.2007.01286.x.
40. **Molina-Santiago C, Daddaoua A, Gómez-Lázaro M, Udaondo Z, Molin S, Ramos JL.** 2015. Differential transcriptional response to antibiotics by *Pseudomonas putida* DOT-T1E. Environ Microbiol **17:**3251–3262. http://dx.doi.org/10.1111/1462-2920.12775.
41. **Martínez-Granero F, Navazo A, Barahona E, Redondo-Nieto M, Rivilla R, Martín M.** 2012. The Gac-Rsm and SadB signal transduction pathways converge on AlgU to downregulate motility in *Pseudomonas fluorescens*. PLoS One **7:**e31765. http://dx.doi.org/10.1371/journal.pone.0031765.
42. **Martínez-Gil M, Ramos-González MI, Espinosa-Urgel M.** 2014. Roles of cyclic di-GMP and the Gac system in transcriptional control of the genes coding for the *Pseudomonas putida* adhesins LapA and LapF. J Bacteriol **196:**1287–1313. http://dx.doi.org/10.1128/JB.01287-13.
43. **Martínez-Gil M, Yousef-Coronado F, Espinosa-Urgel M.** 2010. LapF, the second largest *Pseudomonas putida* protein, contributes to plant root colonization and determines biofilm architecture. Mol Microbiol **77:**549–561. http://dx.doi.org/10.1111/j.1365-2958.2010.07249.x.
44. **Barnard FM, Loughlin MF, Fainberg HP, Messenger MP, Ussery DW,**

Williams P, Jenks PJ. 2004. Global regulation of virulence and the stress response by CsrA in the highly adapted human gastric pathogen *Helicobacter pylori*. Mol Microbiol **51:**15–32.

45. **Heurlier K, Williams F, Heeb S, Dormond C, Pessi G, Singer D, Cámara M, Williams P, Haas D.** 2004. Positive control of swarming, rhamnolipid synthesis, and lipase production by the posttranscriptional RsmA/RsmZ system in *Pseudomonas aeruginosa* PAO1. J Bacteriol **186:**2936–2945. http://dx.doi.org/10.1128/JB.186.10.2936-2945.2004.
46. **Ang S, Horng YT, Shu JC, Soo PC, Liu JH, Yi WC, Lai HC, Luh KT, Ho SW, Swift S.** 2001. The role of RsmA in the regulation of swarming motility in *Serratia marcescens*. J Biomed Sci **8:**160–169.
47. **Liaw SJ, Lai HC, Ho SW, Luh KT, Wang WB.** 2003. Role of RsmA in the regulation of swarming motility and virulence factor expression in *Proteus mirabilis*. J Med Microbiol **52:**19–28. http://dx.doi.org/10.1099/jmm.0.05024-0.
48. **Nielsen L, Li X, Halverson LJ.** 2011. Cell-cell and cell-surface interactions mediated by cellulose and a novel exopolysaccharide contribute to *Pseudomonas putida* biofilm formation and fitness under water-limiting conditions. Environ Microbiol **13:**1342–1356. http://dx.doi.org/10.1111/j.1462-2920.2011.02432.x.
49. **Nilsson M, Chiang WC, Fazli M, Gjermansen M, Givskov M, Tolker-Nielsen T.** 2011. Influence of putative exopolysaccharide genes on *Pseudomonas putida* KT2440 biofilm stability. Environ Microbiol **13:**1357–1369. http://dx.doi.org/10.1111/j.1462-2920.2011.02447.x.
50. **Woodcock DM, Crowther PJ, Doherty J, Jefferson S, DeCruz E, Noyer-Weidner M, Smith SS, Michael MZ, Graham MW.** 1989. Quantitative evaluation of *Escherichia coli* host strains for tolerance to cytosine methylation in plasmid and phage recombinants. Nucleic Acids Res **17:**3469–3478. http://dx.doi.org/10.1093/nar/17.9.3469.
51. **Molina-Henares MA, Ramos-González MI, Daddaoua A, Fernández-Escamilla AM, Espinosa-Urgel M.** 2016. FleQ of *Pseudomonas putida* KT2440 is a multimeric cyclic diguanylate binding protein that differentially regulates expression of biofilm matrix components. Res Microbiol, in press. http://dx.doi.org/10.1016/j.resmic.2016.07.005.