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Induction of RpoS Degradation by the Two-Component System Regulator RstA in *Salmonella enterica*▽

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Bacterial survival in diverse and changing environments relies on the accurate interplay between different regulatory pathways, which determine the design of an adequate adaptive response. The proper outcome depends on a precise gene expression profile generated from the finely tuned and concerted action of transcriptional factors of distinct regulatory hierarchies. *Salmonella enterica* serovar Typhimurium harbors multiple regulatory systems that are crucial for the bacterium to cope with harsh extra- and intracellular environments. In this work, we found that the expression of *Salmonella* RstA, a response regulator from the two-component system family, was able to downregulate the expression of three RpoS-controlled genes (*narZ*, *spvA*, and *bapA*). Furthermore, this downregulation was achieved by a reduction in RpoS cellular levels. The alternative sigma factor RpoS is critical for bacterial endurance under the most-stressful conditions, including stationary-phase entrance and host adaptation. Accordingly, RpoS cellular levels are tightly controlled by complex transcriptional, translational, and posttranslational mechanisms. The analysis of each regulatory step revealed that in *Salmonella*, RstA expression was able to promote RpoS degradation independently of the MviA-ClpXP proteolytic pathway. Additionally, we show that RstA is involved in modulating *Salmonella* biofilm formation. The fact that the RpoS-modulated genes affected by RstA expression have previously been demonstrated to contribute to *Salmonella* pathogenic traits, which include biofilm-forming capacity, suggests that under yet unknown conditions, RstA may function as a control point of RpoS-dependent pathways that govern *Salmonella* virulence.

RstA is a member of the OmpR subfamily of two-component system (TCS) response regulators (RRs), characterized by the display of a winged-helix-turn-helix motif in the DNA-binding domain. Several lines of evidence demonstrated that in *Escherichia coli*, the encoding gene, *rstA*, belongs to the PhoP/PhoQ regulon (39) showing downregulation of *rstAB* (which codes for the putative RstA/RstB TCS putative pair) in a *phoPQ* mutant strain and direct PhoP control over *rstA* expression by a footprinting assay (34). Yamamoto et al. showed that RstB, the putative RstA-associated histidine kinase, was able to transfer in vitro its phosphoryl group to RstA (53), suggesting that in *E. coli*, RstB and RstA constitute an orthodox TCS regulatory pair. Phenotypic screenings, either systematically deleting the genes that code for TCS or overexpressing all RRs, indicated that *E. coli rstAB* was involved in bacterial resistance to ketoprofen, pridinol, and troleandomycin (54) and that RstA overexpression conferred low-level β-lactam resistance (20). More recently, it was shown that in *E. coli*, the transient expression of RpoE (sigma E) in stationary phase downregulated *rstA* and *rstB* transcription (22) and that multiple copies of *rstA* suppressed the lethal phenotype of a deletion in *yjeE*, encoding an ATPase with undetermined function (6). While this report was in preparation, Ogasawara et al. (39) reported that *E. coli* RstA upregulated the expression of *asr* (acid shock RNA) but that RstA overexpression repressed *csgD* (a regulator of curli fimbriae). On the other hand, in *Salmonella enterica* serovar Typhimurium, the regulation of *rstA* by PhoP was shown by bioinformatics analysis (55, 56). Intriguingly, in this bacterium, *rstA* and *rstB* are separated by 3,585 bp in the chromosome, in contrast to the adjacent localization displayed in *E. coli*. Apart from this knowledge, both the identity of the members of the *Salmonella* RstA regulon and the physiological relevance of its function remain unclear.

In this work, we searched for RstA gene targets with the aim of understanding the role of the RstA regulon in *S. enterica* serovar Typhimurium physiology. This screening led us to uncover that RstA was able to downregulate RpoS-dependent genes and that this modulation was achieved by decreasing the intracellular levels of the alternative sigma factor. RpoS (also known as sigma S or sigma 38) plays a central role in bacterial adaptation to starvation and countering a variety of stressful conditions, including low pH, heat or cold shock, oxidative damage, hyper- or hypo-osmolarity, and DNA damage (19). It has also been demonstrated that RpoS plays an essential role in *S. enterica* serovar Typhimurium pathogenesis: the *rpoS* mutant displays a decreased ability to colonize murine Peyer's patches and a severe defect in the systemic phase of the infection (11, 17, 37). The fine-tuning of the cellular concentration of this sigma factor underlies an intricate regulatory network fed by multiple inputs, involving transcriptional, translational, and posttranslational controls (comprehensively reviewed in reference 19). Among the posttranslational processes, RpoS degradation, which maintains the sigma factor at low levels in

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TABLE 1. Bacterial strains, vectors, and plasmids used in this study

Strain genotype, vector, or plasmid	Original name	Genotype and/or comments	Source or reference
Strains			
Salmonella enterica serovar Typhimurium			
ATCC 14028s		Wild type	ATCC
Δ*rstA*	PB3145	ATCC 14028s Δ*rstA*::Cm^r	This work
rstA-lacZ	PB3582	ATCC 14028s Δ*rstA-lacZY*$^+$ Kn^r	This work
rstA-lacZ rpoS	PB4033	ATCC 14028s Δ*rstA-lacZY*$^+$ Kn^r Δ*rpoS*::Cm^r	This work
narZ-lacZ	PB3899	ATCC 14028s *narZ::MudJ*	This work
Δ*rstA narZ-lacZ*	PB3900	ATCC 14028s *narZ*::MudJ Δ*rstA*::Cm^r	This work
Δ*rpoS narZ*::*lacZ*	PB4128	ATCC 14028s *narZ*::MudJ Δ*rpoS*::Cm^r	This work
spvA-lacZ	PB3901	ATCC 14028s *spvA*::MudJ	This work
Δ*rstA spvA-lacZ*	PB3902	ATCC 14028s *spvA*::MudJ Δ*rstA*::Cm^r	This work
Δ*rpoS spvA-lacZ*	PB4127	ATCC 14028s *spvA*::MudJ Δ*rpoS*::Cm^r	This work
bapA-lacZ	PB4361	ATCC 14028s STM2689::MudJ	This work
Δ*rstA bapA-lacZ*	PB4362	ATCC 14028s STM2689::MudJ Δ*rstA*::Cm^r	This work
Δ*rpoS bapA-lacZ*	PB4363	ATCC 14028s STM2689::MudJ Δ*rpoS*::Cm^r	This work
rpoS-lacZ	PB4744	ATCC 14028s Δ*rpoS-lacZY*$^+$ Kn^r	This work
rstA rpoS-lacZ	PB4807	ATCC 14028s Δ*rpoS-lacZY*$^+$ Kn^r Δ*rstA*::Cm^r	This work
clp	PB5681	ATCC 14028s Δ*rstA narZ*::MudJ *clp*::Tn*10*Tc^r	This work
mviA	PB5682	ATCC 14028s Δ*rstA narZ*::MudJ *mviA*::Kn^r	This work
hslV	PB6216	Δ*hslV*::Cm^r	This work
lon	PB6214	Δ*lon*::Cm^r	This work
hflB	PB6213	LB5010 Δ*hflB*::Cm^r	This work
UK1	SF530		12
rpoS177-lacZ	JF3205	UK1 *rpoS-lacZ* (protein fusion)	1
rpoS177-lacZ hfq	PB5857	UK1 *rpoS-lacZ* (protein fusion) *hfq*::Cm^r	This work
clp rpoS177-lacZ	JF4097	UK1 *rpoS-lacZ* (protein fusion) *clpP1*::Tn*10*Tc^r	1
mviA rpoS177-lacZ	PB5922	UK1 *rpoS-lacZ* (protein fusion) *mviA*::Kn^r	This work
Vectors and plasmids			
pKD3		*bla* FRT *cat* FRT PS1 PS2 *oriR6K*	14
pCP20		*bla cat* cI857 lPR *flp* pSC101 *oriTS*	10
pCE36		*ahp* FRT *lacZY1* t_{his} *oriR6K*	16
pUHE21-2 *lacI*^{q}		rep$_{pMB1}$ Ap^r *lacI*^{q}	43
pRstA		rep$_{pMB1}$ Ap^r *lacI*^{q} *rstA*$^+$	This work
pGolS		rep$_{pMB1}$ Ap^r *lacI*^{q} STM0354$^+$	8
pPhoP		rep$_{pMB1}$ Ap^r *lacI*^{q} *phoP*$^+$	28

exponentially growing cells, has been described to be dependent exclusively on the action of the MviA-mediated ClpXP protease complex (35, 36, 41, 42).

The results presented here demonstrate that RstA is able to promote RpoS degradation in *Salmonella*, downregulating the expression of RpoS-modulated genes and altering biofilm formation. The data shown in this work also underscore the existence of an RpoS degradation process that is independent of the MviA-ClpXP proteolytic machinery.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The bacterial strains and plasmids used in this study are listed in Table 1. Bacteria were grown at 37°C in Miller's Luria broth (LB) (10 g/liter NaCl, 5 g/liter yeast extract, and 10 g/liter tryptone). Ampicillin was used at 100 μg ml^{-1}, kanamycin at 50 μg ml^{-1}, chloramphenicol at 10 μg ml^{-1}, and spectinomycin at 100 μg ml^{-1}. IPTG (isopropyl-β-D-thiogalactopyranoside) was used at 0.5 mM, when indicated.

Bacterial genetic and molecular biology techniques. Phage P22-mediated transductions were performed as described previously (15). Plasmid DNA was introduced into bacterial strains by electroporation using a Bio-Rad apparatus according to the manufacturer's recommendations. The deletion of various genes and the concomitant insertion of an antibiotic resistance cassette were carried out using Lambda Red-mediated recombination (14, 16) in strain LB5010 (4). The mutations resulting from this procedure were transferred to the wild-type ATCC 14028s background by P22 transduction. To introduce the *lacZ* reporter gene, antibiotic resistance cassettes were removed using the temperature-sensitive plasmid pCP20 carrying the FLP recombinase (10). pCE36 was used to introduce the transcriptional *lacZ* fusion as previously described (16). The oligonucleotides used to perform these procedures were purchased from Bio-Synthesis, Inc. (Lewisville, TX). Their sequences are listed in Table 2. β-Galactosidase assays were carried out as described previously (33).

Stability assays. Overnight, aerated cultures grown in LB were cultured (1:100) in fresh LB medium. When cultures reached late exponential phase (4.5 h), IPTG was added to a final concentration of 0.5 mM in order to induce RstA expression. Cultures were incubated at 37°C for thirty additional minutes. Then, spectinomycin was added to a final concentration of 1.0 mg/ml. The 0-min sample was taken immediately before the addition of spectinomycin. Subsequent samples were taken at the indicated time points (see Fig. 4). One-milliliter aliquots were resuspended in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) loading buffer in a volume in milliliters equal to 0.1 optical density unit at 600 nm (OD_{600}) and subjected to SDS-PAGE and Western blot analysis.

Western blot analysis. Except for the stability assay samples, 1.0-ml samples were prepared by pelleting whole cells and immediately resuspending them in SDS loading buffer in a volume with an OD_{600} of 10. After resuspension in loading buffer, all samples from the stability assays and other experiments were treated in the same fashion. Equal volumes (5.0 μl for *clp* and *mviA* samples withdrawn from stationary phase and 20 μl for the rest of the assays) were loaded onto 12% polyacrylamide denaturing gels. After electrophoresis, proteins were transferred onto nitrocellulose membranes and probed with a 1:1,000 dilution of anti-RpoS monoclonal antibody (Neoclone). Goat anti-mouse immunoglobulin G–horseradish peroxidase conjugate (Amersham Pharmacia Biotech) was used at a 1:2,500 dilution as a secondary antibody. The bands were detected with the

TABLE 2. Oligonucleotide primers used in this study

Primer[a]	Sequence (5′→3′)
rstAP1 (F)	GGGAAAACAGAGGCGGTGTATGTTGGCGTTTTCTATTCTCCATTTATAATGTGTAGGCTGGAGCTGCTTCG
rstAP2 (R)	GAGCATCGCCACTATGACTTGATAAAGGCAGGTAAAATCTGTCGGCTAACCATATGAATATCCTCCTTA
rstA (F)	AGTGGATCCATATGAACCGCATTG
rstA (R)	GATAAGCTTATCCCGTCGTTTCGAC
rstBP1 (F)	GTGTAGGCTGGAGCTGCTTCGCGCGCGATAGAGCTAATGTATTGAGATCCGGTGGGCGTTG
rstBP2 (R)	CATATGAATATCCTCCTTAATAGCACAGTTCCCGCCGCTCAACCAGCGTGCAAAATGCG
rpoSP1 (F)	TTGCTAGTTCCGTCAAGGGATCACGGGTAGGAGCCACCTTGTGTAGGCTGGAGCTGCTTCG
rpoSP2 (R)	AAGGCCAGTCGACAGACTGGCCTTTTTTTGACAAGGGTACCATATGAATATCCTCCTTA
lonP1 (F)	ATCTGATTACCTGGCGGACACTAAACTAAGAGAGAGCTCTGTGTAGGCTGGAGCTGCTTCG
lonP2 (R)	TGCCAGCCCTGTTTTTATTAGCGCTATTTGCGCGAGGTCACATATGAATATCCTCCTTAG
hslVP1 (F)	CGGGGCCGCAATTCAGCATTAGTAACCAAGGGGTCTGCTCGTGTAGGCTGGAGCTGCTTCG
hslVP2 (R)	TGACAATTTCGCGTGGGGTCATTTCAGACATGAGAGGTCCCATATGAATATCCTCCTTAG
hflBP1 (F)	TTTAACACAGTTGTAATAAGAGGTTAATCCCTTGAGTGACGTGTAGGCTGGAGCTGCTTCG
hflBP2 (R)	GTTTCGGTGAGCGCTAAACATAATGTTGTAAAACAAATGCCATATGAATATCCTCCTTAG
hfqP1 (F)	GCTAAGGGGCAATCTTTACAAGATCCGTTCCTGAACGCAGTGTAGGCTGGAGCTGCTTCG
hfqP2 (R)	ATTCAGTCTCTTCGCTGTCCTGTTGCGCAGTAGACCCCTGCCATATGAATATCCTCCTTA

[a] F, forward primer; R, reverse primer.

ECL antibody detection kit (GE Healthcare). In the experiments performed with the *clp* mutant strains, the exposure time was adjusted to avoid signal saturation. Immunoblotting results were scanned densitometrically to produce quantitative determinations. The immunoblots shown in the figures are representative results from at least three independent assays.

Biofilm assay. In order to test different strains for their ability to produce a biofilm, 2.0 μl of an overnight culture was used to inoculate 2.0 ml of LB, in borosilicate tubes. The cultures were incubated at room temperature in static conditions for 6 days, as described previously (27). The cultures were removed, and the tubes were rinsed with phosphate-buffered saline three times. After drying the tubes at room temperature, 3.0 ml of crystal violet (1%) was added for 20 min. The stained biofilms were rinsed several times with distilled water and visualized as a colored ring in the air-broth interface. In order to quantify the biofilm formation, adhered crystal violet was solubilized by the addition of 3.0 ml of 95% ethanol and then 3.0 ml of 30% acetic acid. The OD_{575} was measured.

RESULTS

Search for RstA-regulated genes. With the aim of analyzing the *Salmonella* RstA regulon, we constructed *lacZ* reporter fusions to *yfiA*, *cspC*, and *entE*. These genes were among the RstA-activated genes found previously by microarray analysis in *E. coli* (39). Surprisingly, we found that the expression of the aforementioned genes was not altered either by expressing RstA from a medium-copy-number, IPTG-inducible plasmid (pRstA) or in an *rstA* deletion mutant (Δ*rstA*), compared with the wild-type strain (data not shown). This result suggested that the *Salmonella* RstA regulon would not entirely overlap with its counterpart in *E. coli.* Because the RstA-inducing signal was undetermined, we decided to search for *Salmonella* RstA-regulated genes by screening a random insertional MudJ library in a Δ*rstA* strain harboring pRstA. The screening was carried out in LB–X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) replica plates, with or without the addition of IPTG, as described previously (44). Among the 10,000 mutants screened, we found three MudJ insertions in which *lacZ* expression was downregulated by RstA. A DNA sequence analysis of the regions adjacent to the insertions and a subsequent search of the *Salmonella* genome database (32) showed that they were located in *narZ*, *spvA*, and STM2689. *narZ* is the first gene of the *Salmonella narZYWV* operon. Spector et al. showed that in *S. enterica*, *narZ* was required for carbon-starvation-inducible thermotolerance and that it was induced inside epithelial cells, while a *narZ* deletion raised approximately 10-fold the 50% lethal dose of the wild-type strain in a mouse virulence assay (45). *spvA* is the first gene of the *spvABCD* operon located in the *Salmonella* virulence plasmid. This operon is essential for *Salmonella* to establish a successful systemic infection, and this phenotype has been ascribed mainly to *spvB* expression (7, 9, 18, 25). STM2689 has recently been renamed *bapA* and codes for a large proline-threonine-rich protein involved in biofilm formation and invasion (27). It should be mentioned that we found *narZ*, *spvA*, and *bapA* transcriptional levels to be repressed in a strain expressing RstA from pRtsA, while they were unaltered in a Δ*rstA* mutant, compared to a wild-type background under the growth conditions tested (Fig. 1). These results remained unchanged when tested in an *rstB* deletion mutant background (data not shown). These last findings suggest that RstA overexpression could functionally mimic the effect generated by the switch of the regulator to an activated state, as it occurs with other two-component RRs, such as PhoP, UphB, and OmpR (13, 26, 29).

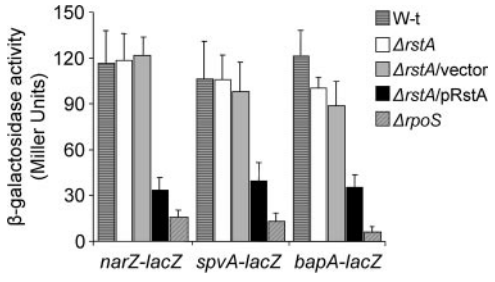

FIG. 1. The RpoS-dependent genes *narZ*, *spvA*, and *bapA* are downregulated by the expression of RstA. β-Galactosidase activities from *lacZ* transcriptional fusions to *narZ*, *spvA*, and *bapA* were measured in the wild type (W-t), the Δ*rstA* strain, the Δ*rstA* strains harboring pUHE21-2 *lacI*^{q} (vector) and pUHE21-2 *lacI*^{q}::*rstA* (pRstA), and the otherwise isogenic Δ*rpoS* background. Cells were grown overnight in LB, with the addition of 0.5 mM IPTG when plasmids were present, as indicated. The data shown are averages from at least three independent assays performed in duplicate. Error bars, standard deviations.

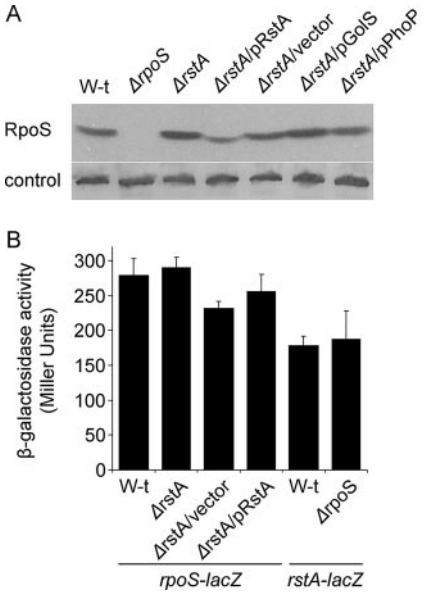

FIG. 2. (A) Expression of RstA specifically downregulates RpoS levels. Cells were grown to late stationary phase (18 h) in LB alone or in LB plus 0.5 mM IPTG when plasmids were present. Equivalent aliquots of each culture (standardized by OD_{600}) were analyzed by using immunoblots developed with monoclonal anti-RpoS antibodies (upper panel) or polyclonal anti-PhoQ antibodies (lower panel). (B) RstA and RpoS expression levels are transcriptionally independent. β-Galactosidase activities from *rstA-lacZ* or *rpoS-lacZ* transcriptional fusions, in the indicated genetic backgrounds, were measured as described in Materials and Methods. Cells were grown overnight in LB, with the addition of 0.5 mM IPTG when plasmids were present, as indicated. The data shown are averages from three independent assays performed in duplicate. Error bars, standard deviations. W-t, wild type.

RstA modulates RpoS-dependent genes. Previous work demonstrated that the expression of both *narZ* and the *spv* operon was activated by RpoS (9, 24, 45). Using a Δ*rpoS* isogenic strain, we corroborated that in stationary phase both *narZ* expression and *spvA* expression were dependent on the intactness of *rpoS* (Fig. 1). Moreover, we verified that *bapA* expression was also under RpoS control (Fig. 1). In light of these results, we investigated whether RpoS and RstA regulatory pathways were interconnected.

To assess whether RstA expression was altering the cellular RpoS content, RpoS steady-state levels were measured by immunoblot analysis using stationary phase bacterial cultures (the growth condition in which RstA-dependent downregulation of the reporter genes was detected). As shown in Fig. 2A, upon RstA expression, cellular RpoS levels were greatly diminished with respect to the levels found in the wild type or the Δ*rstA* strains (i.e., a 40% reduction as determined by densitometric analysis). A quantitatively equivalent expression of a related RR of the two-component family (PhoP) or of the unrelated transcriptional MerR-type metalloregulator GolS (8) did not significantly modify RpoS levels (Fig. 2A, upper panel). In parallel, an internal control experiment was carried out by developing the Western blot with polyclonal anti-PhoQ antibodies (Fig. 2A, lower panel). These results suggest that the observed downregulation of RpoS-dependent genes was due specifically to an RstA-induced decrease of the cellular levels of the sigma factor. Therefore, we determined at which stage of RpoS expression (transcriptional, translational, or protein stability) the RstA-mediated effect was taking place. First, we generated a chromosomal *rpoS-lacZ* transcriptional fusion. Figure 2B shows that *rpoS* transcriptional levels remained essentially unaltered either in a Δ*rstA* strain or in an RstA-expressing strain. We also performed the counterpart experiment by assaying an *rstA-lacZ* transcriptional reporter in either a wild-type or Δ*rpoS* strain to rule out that *rstA* transcriptional levels could be influenced by RpoS (Fig. 2B).

Next, to investigate whether RstA was acting on *rpoS* translation, we used an *rpoS-lacZ* translational reporter (9) in which the *lacZ* fusion occurs at codon 177 of *rpoS*, truncating the essential target motif for RpoS degradation. This reporter was previously demonstrated to be a useful tool reflecting the sum of transcriptional and translational controls (46, 50). Because of the lack of a selectable marker to perform the transduction of this construction to the ATCC 14028s strain, we carried out this set of determinations in the *Salmonella enterica* strain UK1 original background (1, 12). As shown in Fig. 3A, RstA-induced expression was not able to modify *rpoS* translational levels, simultaneously corroborating that *rpoS* transcription was unaffected as well. Control experiments were performed to test the *rpoS177-lacZ* fusion in *clpP* or *mviA* isogenic backgrounds to verify the inability of the MviA-ClpXP machinery to accomplish the degradation of the corresponding reporter fusion protein, or in a strain harboring a null mutation in *hfq*, which codes for an RNA chaperone that positively regulates RpoS translation (30) (see Fig. 3A).

RstA promotes RpoS degradation in an MviA-ClpXP-independent manner. While active proteolysis maintains RpoS at low levels in exponentially growing cells, RpoS stability increases at the entry into stationary phase and when starvation challenges the bacterial cell (19, 31). Two factors are described to be essential for RpoS turnover: the ClpXP protease complex and MviA (known as RssB in *E. coli*). MviA associates with RpoS and delivers the sigma factor to the ClpXP degradation complex (35, 36, 41, 42) (compare also cellular RpoS levels in wild-type and *clpP* or *mviA* backgrounds [Fig. 3B]). In order to assess whether RstA was triggering this pathway, we compared the effects of RstA expression on RpoS levels in strains carrying a deletion mutation in either *clpP* or *mviA*. As shown in Fig. 3B, RstA expression was able to lower cellular RpoS levels in both mutant backgrounds (an average 50% reduction of RpoS levels, as determined by the densitometric analysis of three independent experiments). An equivalent effect was observed when samples taken from either exponential or late stationary phase were analyzed (Fig. 3B, compare the lower and upper panels). This result clearly indicates that the action of RstA over RpoS was independent of the ClpXP-MviA degradation pathway. In a parallel experiment, we determined *narZ* transcriptional activity in the otherwise wild-type Δ*rstA* strain compared to that of the *clpP* or *mviA* isogenic strains when RstA was expressed. We observed that, consistent with the previous result, RstA expression downregulated this RpoS-dependent gene independent of the integrity of the ClpXP/MviA machinery (Fig. 3C).

Finally, we tested whether the observed downregulation of RpoS was due to RstA-promoted induction of the degradation of the sigma factor. With this aim, de novo protein synthesis

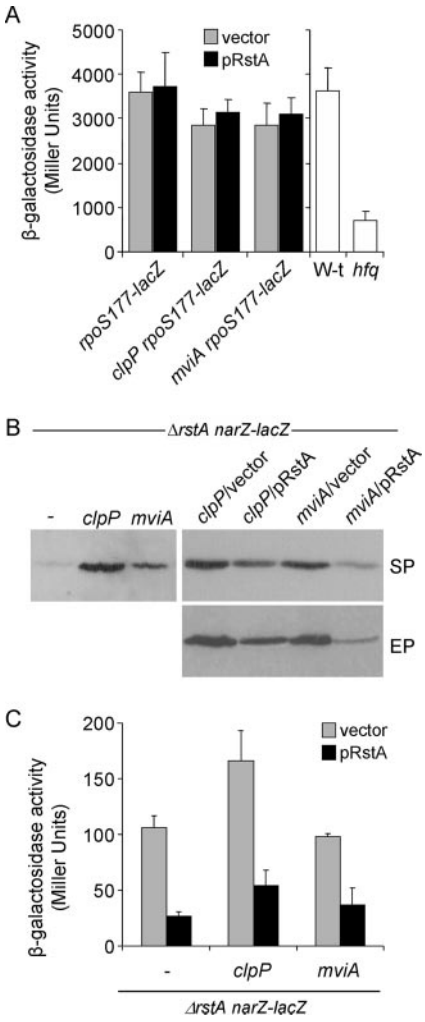

FIG. 3. RstA expression downregulates RpoS at a posttranslational level, independently of the MviA-ClpXP degradation pathway. (A) β-Galactosidase activities were determined from an *rpoS177-lacZ* translational fusion in the *Salmonella* UK1 wild type (W-t) and the *clpP*, *mviA* and *hfq* strains harboring the vector or the derived pRstA plasmids, as indicated. (B) The Δ*rstA narZ-lacZ* strain and the otherwise isogenic *clpP* and *mviA* strains were grown to exponential phase (EP) or to late stationary phase (SP; 18 h) in LB or LB plus 0.5 mM IPTG when the cells harbored pUHE21-2 *lacI*^{q} (vector) or pUHE21-2 *lacI*^{q}::*rstA* (pRstA), as indicated. -, no additional mutations. Equivalent aliquots (20 μl for EP and 5.0 μl for SP) of each culture (standardized by OD_{600}) were analyzed by using immunoblots developed with monoclonal anti-RpoS antibodies. (C) β-Galactosidase activities from the Δ*rstA narZ-lacZ* strain and from the otherwise isogenic *clpP* and *mviA* strains were measured as described in Materials and Methods. -, no additional mutation. Cells were grown overnight in LB, with the addition of 0.5 mM IPTG when plasmids were present, as indicated. The data shown are averages from three independent assays performed in duplicate. Error bars, standard deviations.

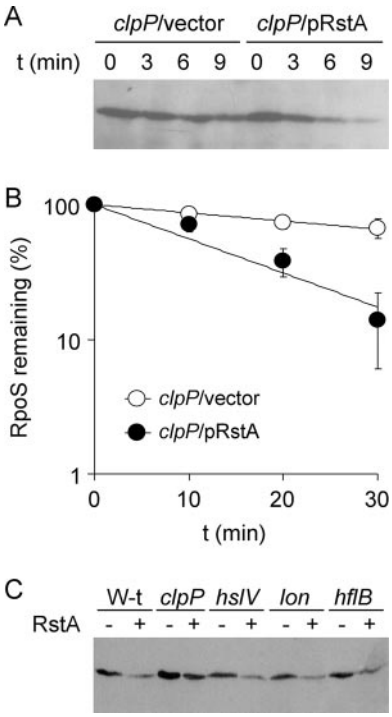

FIG. 4. RstA stimulates RpoS degradation through a ClpXP-independent pathway. (A) Δ*rstA clpP* strains harboring pUHE21-2 *lacI*^{q} (vector) or pUHE21-2 *lacI*^{q}::*rstA* (pRstA) were grown in LB to late exponential phase (OD_{600} of 1.3). IPTG (0.5 mM) was added to the culture medium, and cells were grown for an additional 30 min to allow for RstA expression. Translation was stopped by the addition of spectinomycin (1.0 mg/ml). Equivalent aliquots were withdrawn at 0, 3, 6, and 9 min after the spectinomycin addition and analyzed by using immunoblots developed with polyclonal anti-RpoS antibodies, as described in Materials and Methods. (B) Densitometric quantifications at various time points of each band shown in panel A are shown. The density for samples at time zero was set to 100% for each experiment. (C) Equivalent aliquots of overnight cultures (standardized by OD_{600}) of the wild type (W-t) and the *clpP*, *hslV*, *lon*, and *hflB* strains harboring the vector (-) or pRstA (+), grown to late stationary phase (18 h) in LB plus 0.5 mM IPTG, were analyzed by SDS-PAGE, followed by using immunoblots developed with monoclonal anti-RpoS antibodies. Δ*clpP* strain samples were diluted (1/4) with respect to the rest in order to avoid signal saturation. The results shown are representative of four identical independent assays.

was halted by the addition of spectinomycin to the bacterial cultures, and RpoS decay in the *clp* mutant strain harboring either the empty vector or pRstA was followed over time by immunodetection (see Materials and Methods for details). As shown in Fig. 4A and B, once translation was stopped, the degradation of preexisting RpoS was estimated to be three times faster in the RstA-expressing strain. This last result demonstrates that in *Salmonella*, RstA is able to upregulate RpoS degradation through a pathway distinct from the MviA-ClpXP-dependent one.

We then examined whether other major cellular proteases present in *Salmonella* could be involved. With this purpose, we generated *lon*, *hslV*, and *hflB* deletion mutant strains (5). As shown in Fig. 4C, none of these deletions affected RstA-induced degradation of RpoS. In a parallel experiment, we corroborated that the transcriptional activity of *narZ* was not affected in any of these protease deletion backgrounds (data not shown).

RstA expression inhibits biofilm formation. The production of thin aggregative fimbriae, together with cellulose, capsule, extracellular polysaccharides, and BapA, has been demonstrated to form a matrix that contributes to *Salmonella* biofilm development (27, 52). We showed that RstA downregulates

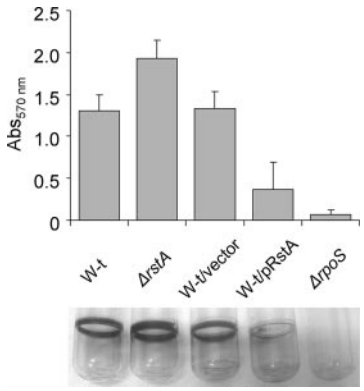

FIG. 5. Static biofilm formation is modulated by *rstA*. Cultures were grown in LB medium, in borosilicate tubes, for 6 days at room temperature with no agitation. The biofilm that adhered to the glass surface was stained with crystal violet, and the tubes were photographed (lower panel) or the bound stain was quantified by solubilizing crystal violet and measuring the OD_{570} (upper panel), as described in Materials and Methods. The data shown are the averages of four identical independent assays. Error bars, standard deviations. W-t, wild type.

bapA expression (Fig. 1). Moreover, it was previously demonstrated that RpoS induces the expression of AgfD (known also as CsgD), a LuxR-type regulator that controls the expression of all the aforementioned factors (27, 52). Therefore, we examined whether RstA expression affected biofilm formation. As shown in Fig. 5, expression of RstA diminishes the biofilm formation visualized by crystal violet staining of the ring that remained firmly adhered to the culture tubes (upper panel) or by quantification of the solubilized stain (lower panel). Accordingly, a moderate but consistent increase in the adhered biofilm was detected in the Δ*rstA* strain compared to the wild-type strain. As expected, no detectable biofilm formation was observed in the Δ*rpoS* strain. These results suggest the possibility that *rstA* is involved in a regulatory pathway that controls the *Salmonella* biofilm differentiation process.

DISCUSSION

With the aim of deciphering the role of the RstA regulon in *Salmonella*, we undertook a genetic screening in search of RstA-regulated genes. In this approach, we assumed that the induced expression of the RR from a medium-copy-number plasmid circumvented the need for activation by the associated sensor protein—as we demonstrated to be the case for the homologous PhoP transcriptional regulator (29)—or by other possible phosphodonors, such as acetyl phosphate (23, 49).

The first round of screening pinpointed three genes as targets of RstA downregulation: *narZ*, *spvA*, and *bapA*. Remarkably, these genes were demonstrated to be transcriptionally controlled by RpoS (24, 27, 46) (see Fig. 1). Having also verified that RstA expression is not RpoS dependent, we hypothesized that RstA could be able to modulate intracellular RpoS levels. RpoS, the stationary-phase alternative sigma factor, has a large impact on the ability of enteric and nonenteric gram-negative bacteria to cope with adverse conditions, such as nutrient starvation, medium acidification, and peroxide resistance (19), by remodeling the expression pattern of more than 100 genes (21, 40, 48, 51). Accordingly, diverse regulatory factors, from transcriptional ones to small RNAs, proteases (19), antiadaptor proteins (3), and even other alternative sigma factors (2), are involved in the complex control of the cellular RpoS content. Here we demonstrated that RstA expression lowered steady-state RpoS levels and that this effect was not associated with a general stress effect promoted by protein overexpression but was due specifically to the expression of RstA (see Fig. 2A). To define where the regulatory action of RstA was occurring, we sequentially focused on each regulatory stage that determines RpoS expression. We showed that RstA expression had no impact on either RpoS transcription or translation by monitoring the expression of a transcriptional fusion that disrupts the RpoS region that is essential for MviA interaction (46). It has been largely demonstrated that the RpoS degradation pathway, active mostly to maintain low levels of the sigma factor when bacteria are growing in exponential phase, depends on the sequential recognition of RpoS by MviA (named also SprE or RssB in *E. coli*), which delivers it to the ClpXP proteolytic complex. We therefore asked whether RstA was stimulating this cascade or, alternatively, it could conceivably replace MviA in the function of delivery to the protease complex. By assessing the RpoS levels in *clpP* and *mviA* deletion mutants, we showed that the RstA ability to downregulate RpoS expression was independent of this degradation pathway, regardless of the growth phase. This result was substantiated by showing that RstA-mediated repression of the identified target genes also remained MviA-ClpXP independent. We also demonstrated that apart from ClpXP, none of the other major cellular proteases, i.e., Lon, HslUV, or HflB, are responsible for the RstA-induced degradation of RpoS. Finally, RpoS degradation was monitored after blocking de novo protein synthesis while inducing RstA expression. This experiment established that in *Salmonella*, RstA is able to promote RpoS turnover, activating its degradation in a ClpXP-MviA-independent manner. This last result uncovered clearly the existence of a yet unknown RpoS degradation mechanism triggered by the two-component RR RstA.

Further work will be required to elucidate the mechanism by which RstA promotes RpoS degradation. We foresee several potential alternatives: (i) RstA induces the expression of a gene(s) that encodes an unknown protease (or protease complex) involved in RpoS turnover, (ii) RstA itself chaperones RpoS to a degradation apparatus different from ClpXP, or (iii) RstA directly or indirectly counteracts the effect of a factor that protects RpoS from proteolysis.

It was demonstrated previously that *rstA* is transcriptionally induced in extracellular Mg^{2+} limitation (a PhoP/PhoQ system-inducing condition). However, this stimulus was insufficient to promote RstA-dependent modulation of *narZ*, *spvA*, or *bapA* (data not shown). With this finding and the fact that identical results were obtained when the assays shown in this work were done in an *rstB* background, it becomes apparent that an additional and yet unidentified signal is required to trigger the switch of RstA to an activated state, which in our experiments is bypassed by RstA-induced expression. That the RstA-activating signal is not present in the culture conditions used (LB medium, vigorous shaking, 37°C) would also explain the lack of significant differences detected between the wild type and the Δ*rstA* strains in the modulation of RpoS levels

and, in consequence, in the transcriptional activity of the RpoS-dependent genes.

Interestingly, Tu et al. recently showed that IraP, a small protein under the transcriptional control of PhoP, stabilizes RpoS by preventing MviA binding, thus blocking ClpXP-mediated degradation (47). Because PhoP upregulates both *rstA* and *iraP* transcription, we can predict that in an environment where not only PhoP- but also RstA-activating signals are simultaneously present, cellular RpoS levels will be finely adjusted by the opposing effects of the two circuits.

Finally, biofilm formation was enhanced in the Δ*rstA* strain, while it was repressed in the RstA-expressing bacteria, compared to the wild-type strain. Because we were able to detect a differential phenotype between the wild type and the Δ*rstA* strains when biofilm was developed, we can speculate that the assay conditions that promote multicellular behavior in *Salmonella* (i.e., static growth over an extended period of time and cell-cell and cell-abiotic surface contacts) would generate a signal which physiologically modulates RstA activity. RstA-dependent regulation of biofilm was not unexpected in light of the RstA-dependent modulation of cellular RpoS levels and the knowledge that this sigma factor stimulates AgfD expression. This regulator controls the production of essential constituents of the extracellular matrix that leads to biofilm development. Indeed, the recent work of Ogasawara et al. (38) revealed that in *E. coli*, overexpression of RstA downregulates *csgD* (*agfD* in *Salmonella*) expression by direct binding to its promoter region. As a result, and because *agfD* also exhibits the described RstA-binding motif (as determined by our visual inspection of the *agfD* promoter region), RstA would display a double control over *Salmonella* biofilm formation, simultaneously repressing the two master regulatory factors, RpoS and AgfD.

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