JOURNAL OF BACTERIOLOGY, June 2006, p. 4264–4270 Vol. 188, No. 12
0021-9193/06/$08.00+0 doi:10.1128/JB.00004-06

RcsF Is an Outer Membrane Lipoprotein Involved in the RcsCDB Phosphorelay Signaling Pathway in *Escherichia coli*

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Received 3 January 2006/Accepted 6 April 2006

The RcsCDB signal transduction system is an atypical His-Asp phosphorelay conserved in γ-proteobacteria. Besides the three proteins directly involved in the phosphorelay, two proteins modulate the activity of the system. One is RcsA, which can stimulate the activity of the response regulator RcsB independently of the phosphorelay to regulate a subset of RcsB targets. The other is RcsF, a putative outer membrane lipoprotein mediating the signaling to the sensor RcsC. How RcsF transduces the signal to RcsC is unknown. Although the molecular and physiological signals remain to be identified, the common feature among the reported Rcs-activating conditions is perturbation of the envelope. As an initial step to explore the RcsF-RcsC functional relationship, we demonstrate that RcsF is an outer membrane lipoprotein oriented towards the periplasm. We also report that a null mutation in *surA*, a gene required for correct folding of periplasmic proteins, activates the Rcs pathway through RcsF. In contrast, activation of this pathway by overproduction of the membrane chaperone-like protein DjlA does not require RcsF. Conversely, activation of the pathway by RcsF overproduction does not require DjlA either, indicating the existence of two independent signaling pathways toward RcsC.

Survival of microorganisms depends upon their capacity to efficiently adapt to a broad range of different environments. His-Asp phosphorelays, typically composed of a membrane sensor kinase and a response regulator, play essential roles in such adaptive responses, usually sensing outside parameters, transmitting the signals inside the cells, and eliciting appropriate gene expression. This is best illustrated in comparisons of different bacterial genomes for the presence of such systems. Whereas His-Asp phosphorelays are conspicuously absent in *Mycoplasma genitalium*, an obligate intracellular pathogen, *Escherichia coli*, which can live in a variety of environments outside and inside its host, has about 30 such systems (30), while about 40 are found in the cyanobacterium *Synechocystis* sp. strain PCC 6803 (31), and more than 60 are present in the genome of *Pseudomonas aeruginosa*, a ubiquitous organism found in a wide variety of environmental niches, from water and soil to plants and animals (35).

In canonical His-Asp phosphorelays, the sensor kinase autophosphorylates an invariant histidine residue present in the conserved, cytoplasmic, C-terminal domain. The phosphoryl group can then be transferred to a conserved aspartate in the N-terminal receiver domain of the response regulator.

Contrary to classical two-component systems, the Rcs phosphorelay, controlling colanic acid production in *E. coli*, comprises three proteins: RcsC, RcsD, and RcsB. RcsC is an inner membrane hybrid sensor kinase that possesses a receiver domain in addition to its His-kinase domain. The RcsD protein is also membrane associated and possesses a histidine-containing phosphotransmitter domain (Hpt). RcsB is a classical cytoplasmic response regulator composed of two domains, a receiver domain and a DNA binding domain. It has been shown (5, 40) that the phosphoryl group is transferred from the RcsC transmitter domain to the aspartate of the extra receiver domain and then to the conserved histidine of the Hpt domain of YojN/RcsD, where it is finally transferred to the conserved Asp of the RcsB receiver domain. The physiological relevance of this multistep phosphorelay is not yet understood. However, in the case of the Arc system, the Hpt domain is carried by the histidine sensor kinase ArcB itself, and it was shown that this complex phosphorelay system could be used to adjust the response as a function of different environmental conditions (27).

Together with the accessory protein RcsA, RcsB activates the transcription of the *cps* operon, which comprises the genes involved in biosynthesis and export of colanic acid. In addition to *cps* expression, the RcsC/RcsD/RcsB system was found to positively regulate a number of genes, including RprA, a small stable RNA which is a positive regulator of *rpoS* expression (25). In contrast to *cps*, *rprA* transcription depends only on RcsB and is RcsA independent (25).

Although several mutations and conditions have been described which turn on this pathway, for instance, mutations in the lipopolysaccharide (LPS) biosynthesis *rfa* operon (33) or mutations resulting in the absence of membrane-derived oligosaccharide (MDO) (10), the physiological signals for activation of the RcsC/RcsD/RcsB pathway remain elusive. The overproduction of two proteins is able to activate RcsC: one is DjlA, an inner membrane protein which belongs to the DnaJ family (3, 4); the other one is RcsF. RcsF was first identified as a regulator gene for exopolysaccharide synthesis, and it was

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TABLE 1. List of strains and plasmids used in this study

Strain or plasmid	Description and/or genotype	Source or reference
E. coli strains		
MP110	MG1655 Δ*lacIZ*(MluI) *zac3051*::Tn*10* Δ(*ara-leu*) *cps-lacZ*	This study and 8
61F	MC1061/F′ *lacI*^{q} Kan^r	21
PSG1038	MC4100 *cps-lacZ* Mud1 *rcsC52*::Tn*10*	5
GEB495	MC4100 *ara*$^+$ *cpsB-lacZ* Mud1	This study
GEB496	GEB495 *djlA*::*spc*	This study
GEB498	GEB495 *surA*::*kan*	This study
GEB499	GEB495 *surA*::*kan djlA*::*spc*	This study
GEB628	GEB495 *rcsF*::*cat sacB*	This study
GEB629	GEB495 *rcsF*::*cat sacB surA*::*kan*	This study
GEB777	GEB495 *rcsC52*::Tn*10 surA*::*kan*	This study
GEB658	MC4100 *ara*$^+$ [*rprA142p*::*lacZ*]	This study
GEB672	GEB658 *rcsF*::*cat-sacB*	This study
GEB673	GEB658 *surA*::*kan*	This study
GEB677	GEB658 *rcsF*::*cat sacB surA*::*kan*	This study
GEB775	GEB658 *rcsC52*::Tn*10*	This study
GEB776	GEB658 *rcsC52*::Tn*10 surA*::*kan*	This study
Plasmids		
pAM238	pACYC184 derivative, $lac_{UV5}p$ Spc^r	16
pHK646	pAM238 $lac_{UV5}p$::*rcsF-c-myc* Spc^r	16
pIM10	p15A derivative, Kan^r Spc^r	6
pRcsF	p15A $lac_{UV5}p$::*rcsF* Kan^r Spc^r	This study
prcsF-PhoA	$ara_{BAD}p$::*rcsF-phoA* on pSEB104 (pSC101 *ori*, Spc^r)	This study and 34
prcsF-PhoA$_{\Delta 20}$	$ara_{BAD}p$::*rcsF-phoA*$_{\Delta 20}$ on pSEB104 (pSC101 *ori*, Spc^r)	This study and 34
pPSG958	pTRC99A *tacp*::*6his-djlA*	3

proposed to be a cytoplasmic protein or inner membrane protein (11). Stimulation by RcsF of colanic acid production, which results in a mucoid phenotype on plates, was reported to be dependent upon RcsB but independent of RcsC. Hence, the authors proposed that RcsF was able to phosphorylate RcsB in the absence of RcsC. However, more recently, RcsF was inferred to be an outer membrane lipoprotein, and Hagiwara et al. (15) showed that activation by overproduction of RcsF was indeed dependent upon the presence of RcsC. These authors also showed that colonic acid production was stimulated by a combination of low temperature (20°C), zinc, and glucose, and that this stimulation was abolished in an *rcsF* mutant, suggesting an important role for RcsF in signal transduction. This was recently confirmed by Majdalani et al. (24) in the case of an *rfa* mutation. In this article, we show that mutation in the *surA* gene (encoding a periplasmic protein important for outer membrane biogenesis) leads to activation of the Rcs pathway in an RcsF-dependent manner. In addition, central to the role of RcsF in signaling outer membrane defects, we demonstrate for the first time that RcsF is an outer membrane lipoprotein oriented towards the periplasm.

MATERIALS AND METHODS

Strains, plasmids, and media. Strains and plasmids used in this study are listed in Table 1. All MC4100 derivative strains containing the *cpsB-lacZ* fusion are derivatives of SG20781, a gift from Susan Gottesman.

MP110 was constructed by introducing the allele *zac3051*::Tn*10* Δ(*ara-leu*) into strain SK1291 by P1 transduction (8).

DH303 (a gift from Nadim Majdalani) is lysogenic for λ*rprA142p*::*lacZ* and was used as a source of transducing phage to lysogenize MC4100*ara*$^+$, generating GEB658.

Relevant mutations were introduced in these two backgrounds by P1 transduction according to the method of Miller (28). The *rcsC52*::Tn*10* allele was from Susan Gottesman (SG20811). The *rcsF*::*cat sacB* allele is a gift from Nadim Majdalani. The *surA3*::*kan* allele was from R. Kolter (19).

pHK646, encoding a C-terminal c-Myc epitope-tagged version of RcsF (RcsF-c-Myc) under the control of a $lac_{UV5}p$ promoter, is a derivative of pAM238 and was a gift from Hiroshi Kadokura (16).

Plasmid p*rcsF* is a p15A vector derivative which expresses *rcsF* from the $lac_{UV5}p$ promoter and the T7 phage ϕ10 gene Shine-Dalgarno sequence. A PCR-generated *rcsF* fragment was cloned between the NdeI-BamHI cloning sites of vector pIM10 (6), the *rcsF* ATG start codon being included in the NdeI site.

Plasmid p*rcsF-phoA* encodes wild-type RcsF protein fused at the last codon to PhoA, which was deleted of its first 26 amino acids. The *rcsF*::*phoA* gene is expressed from the $ara_{BAD}p$ promoter, and the putative *rcsF* natural Shine-Dalgarno sequence (20 bp upstream of *rcsF*) and ATG were included in the construction. PCR-generated fragments were cloned in the pSC101 derivative plasmid pSEB104. The *ftsZ* moiety of the *ftsZ*::*gfp* gene fusion in pSEB104 was replaced by the *rcsF* sequence using the SacI-XbaI cloning sites. The *gfp* moiety of the *rcsF*::*gfp* fusion was then replaced by the *phoA* sequence using the XbaI-HindIII cloning sites. Plasmid p*rcsF-phoA*$_{\Delta 20}$ was similarly constructed, except that the sequence including the first 20 codons of *rcsF*, which potentially encodes RcsF signal sequence, was removed. Plasmid pPH07 was the source for PCR-generated *phoA* (13).

Cells were grown in Luria-Bertani (LB) medium supplemented with appropriate antibiotics. Antibiotics were used at the following concentrations: kanamycin and chloramphenicol, 25 μg/ml; tetracycline, 12.5 μg/ml; ampicillin, 100 μg/ml; and spectinomycin, 50 μg/ml.

Determination of alkaline phosphatase and β-galactosidase activities. Alkaline phosphatase assays were performed as described by Gutierrez et al. (12). The activity units were then calculated by the formula ($OD_{420} \times 1{,}000$)/[vol of cells (in milliliters) × time (in minutes) × OD_{600}], where OD_{420} is the optical density at 420 nm. β-Galactosidase activities were determined after overnight growth on LB plates, as described Clarke et al. (5), unless otherwise mentioned. It is to be noted that no significant differences were observed between values obtained after growth on plates and values obtained for a mid-logarithmic culture in liquid medium. Strains containing the *rprA-lacZ* fusion were grown at 37°C, and cells containing the *cps-lacZ* fusion were grown at 30°C. Activities are expressed in Miller units (28).

Cell fractionation and Western blotting analyses of total cell proteins and extracts. To determine the localization of RcsF-c-Myc, GEB658 cells containing either pAM238 (vector) or pHK646 (expressing RcsF-c-Myc) were grown at 37°C in 25 ml LB medium supplemented with spectinomycin. At an OD_{600} of approximately 0.3, isopropyl-β-D-thiogalactopyranoside (IPTG) was added at a final concentration of 500 μM and the cultures were further grown for 3 h to an

OD_{600} of approximately 3. After centrifugation and resuspension in Tris-HCl–EDTA (50 mM [pH 8.0]–5 mM) (TE), 1/10 of the culture cells were frozen for further analysis by Western blotting. The remaining cells were lysed by an osmotic shock/lysozyme treatment (1 mg/ml final) followed by DNase treatment to reduce viscosity. Unlysed cells and cell debris were removed by a 10-min centrifugation at 4,500 rpm at 4°C. The supernatant was centrifuged at 4°C for 90 min at 70,000 rpm in a Beckman TL100 bench top ultracentrifuge, using a TLA100.3 rotor. The membrane proteins (pellet) were resuspended in 500 μl of TE, an aliquot (1/10) was saved for analysis, and the suspension was loaded on a 2.5-ml sucrose step gradient prepared with 0.7 ml 70% (wt/vol) sucrose in TE layered with 1.8 ml 53% (wt/vol) sucrose in TE. The gradient was then centrifuged in a TLA100.3 rotor for 2 h at 90,000 rpm. The upper band (inner membrane proteins) and bottom band (outer membrane proteins) were collected from the top of the gradient. The material from each band was pooled, diluted to 1.5 ml in TE, and recentrifuged, and the pellet was solubilized in sodium dodecyl sulfate (SDS) sample buffer. In parallel, the total membrane fraction was resuspended in 2 ml of 50 mM Tris-HCl, pH 8, 25 mM $MgCl_2$, 1% Triton X-100 and incubated at room temperature for 1 h. Solubilized proteins and Triton X-100-insoluble materials were then separated by ultracentrifugation as described above, and the pellet and supernatant were analyzed by Western blotting, using 0.3 OD_{600} equivalents (starting material) for each sample.

After SDS-polyacrylamide gel electrophoresis (PAGE) (18) and transfer of the proteins on a polyvinylidene difluoride membrane, fractions were analyzed by Western blotting using anti-c-Myc monoclonal antibodies (at a dilution of 1:5,000; Roche) as primary antibodies and anti-mouse immunoglobulin G (IgG) antibodies coupled to alkaline phosphatase (Sigma) as secondary antibodies. To control fractionation, the various cellular fractions were also analyzed using rabbit anti-OmpA antibodies as primary antibodies and anti-rabbit IgG (Sigma) coupled to alkaline phosphatase as secondary antibodies. Detection was determined according to the methods of Clarke et al. (4).

Globomycin treatment. Four cultures of 5 ml of LB containing spectinomycin were inoculated with strain 61F, which contained pHK646 from an overnight culture, at a starting OD_{600} of approximately 0.025 and grown at 37°C to an OD_{600} of approximately 0.4. Two cultures were further grown without IPTG, and IPTG was added to two cultures at 500 μM final concentration. At the same time, globomycin was added to one induced culture and one noninduced culture at a final concentration of 50 μg/ml. All four cultures were further incubated for 90 min. Cells were then centrifuged, and the pellets were washed once in 1 ml TE buffer and finally resuspended in SDS sample buffer at a concentration of 40 OD_{600} equivalents/ml. Samples were treated for 15 min at 37°C with benzonase (Merck) in the presence of 1 mM $MgCl_2$ to reduce their viscosity before boiling for 5 min. One OD_{600} equivalent was loaded on gels for Western blot analysis as described above. Rabbit anti-SurA antibodies were used to control the amounts of cell protein loading.

RESULTS

Outer membrane defects induce the Rcs phosphorelay in an RcsF-dependent manner. Several mutations affecting envelope integrity were found to activate the RcsC/RcsD/RcsB phosphorelay, resulting in capsular synthesis: deep rough (*rfa*) (33), *pgsA* (39), or *mdo* (10) mutations. Mutations in *surA* also lead to defects of the outer membrane (20, 36), as *surA* codes for a periplasmic peptidyl prolyl isomerase with chaperone activity required for correct folding and insertion of outer membrane proteins (20, 36). Missiakas et al. (29) reported that *surA* colonies were mucoid in certain conditions. Using two reporter fusions, a *cps-lacZ* fusion and an *rprA-lacZ* fusion, we examined whether a *surA* mutation could also activate the RcsC/RcsD/RcsB pathway. *rprA* encodes a small RNA that positively regulates the expression of the alternative sigma factor RpoS, and its own expression is under the control of the Rcs phosphorelay. As shown in Fig. 1, a *surA* null mutation stimulated the expression of both *rprA*- (Fig. 1A) and *cps-lacZ* (Fig. 1B) fusions seven- and threefold, respectively. The introduction of an *rcsC52*::Tn*10* null mutation abolished the induction by *surA*, indicating that the effect of *surA* mutation was mediated by the activation of the Rcs phosphorelay. It is to be noted that in the case of the *rprA-lacZ* fusion, the presence of the *rcsC* mutation led to a 2.5-fold increase in the basal level of *rprA-lacZ* expression, in agreement with previously reported results by Majdalani et al. (25) (Fig. 1A).

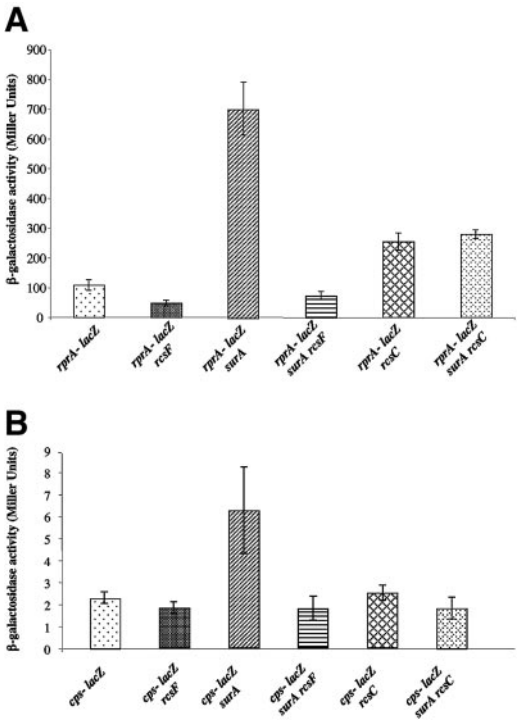

FIG. 1. Absence of SurA activates the Rcs pathway in an RcsC- and an RcsF-dependent manner. Strains carrying an *rprA-lacZ* fusion (A) and strains carrying a *cps-lacZ* fusion (B) were assayed for β-galactosidase activity as described in Materials and Methods. Bars represent the standard deviations of at least three independent measurements. (See Table 1 for complete genotypes of strains.) Relevant genotypes are indicated under each measurement.

Overproduction of RcsF leads to activation of the Rcs phosphorelay (11) in an RcsC-dependent manner (22). Majdalani et al. (24) have shown that RcsF plays a role in signal transduction from the cell envelope to RcsC, especially in the case of an *rfa* mutation. Similar results have been reported in the case of a mutation in *pgsA* necessary for synthesis of acidic phospholipids (39). We wondered whether this was also true in the case of the cell envelope defect generated by a *surA* mutation. Accordingly, we introduced an *rcsF* null allele in a *surA cps-lacZ* strain as well as in a *surA rprA-lacZ* strain. As also shown in Fig. 1A and B, induction of expression of both fusions by the *surA* mutation was completely abolished in the *rcsF* mutant, indicating that RcsC activation by *surA* was dependent upon RcsF. Similar results were obtained in the case of an *mdo* mutation, either *mdoH* or *mdoG*, indicating that absence of membrane-derived oligosaccharides activates the Rcs pathway through RcsF (data not shown).

DjlA and RcsF act on two independent pathways. DjlA is another protein whose overproduction activates the Rcs phosphorelay, leading to capsule production (3). Several RcsF-dependent inducers of *rprA-lacZ* fusion isolated by Majdalani et al. on multicopy plasmids encoded membrane proteins (22). We wondered whether RcsC/RcsD/RcsB activation by DjlA

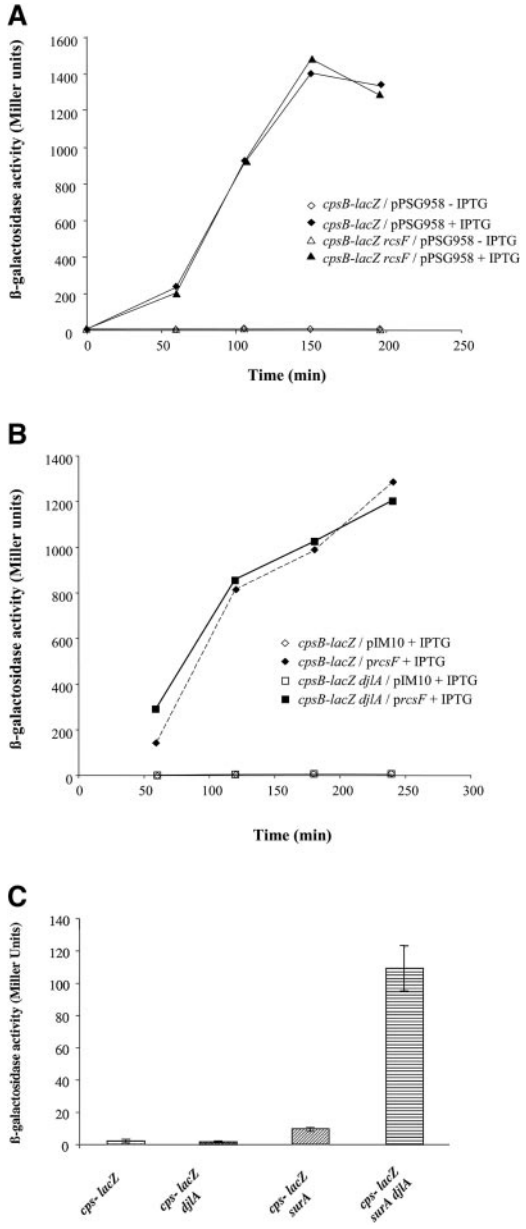

FIG. 2. DjlA and RcsF act independently. Induction of *cps-lacZ* fusion expression by DjlA (A) or by RcsF (B) overproduction. Strains GEB495 (*cps-lacZ*), GEB496 (*djlA*::*spc cps-lacZ*), and GEB628 (*rcsF*::*cat sacB cps-lac*Z) were transformed with either pPSG958, p*rcsF*, or pIM10 plasmid DNA. Cells were cultured overnight in LB in the presence of the appropriate antibiotic and then diluted to 1/1,000 into fresh LB and grown at 30°C. At an OD_{600} of approximately 0.1, 500 μM IPTG was added to some cultures, while others were left untreated. The cultures were sampled at regular intervals for assay of β-galactosidase activities. (C) The *djlA*::*spc* allele was introduced into GEB495 (*cps-lacZ*) and GEB498 (*cps-lacZ surA*::*kan*), and the resulting strains were assayed for β-galactosidase activity as described in Materials and Methods.

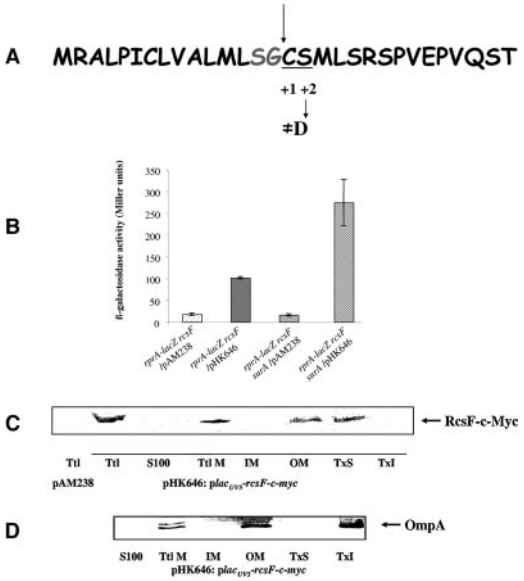

FIG. 3. Signal sequence and localization of RcsF-c-Myc. (A) Signal sequence of RcsF. The predicted cleavage site, the acylated cysteine residue at position +1, and the serine residue at position +2 are indicated. (B) Complementation of *rcsF* mutant by RcsF-c-Myc. *rprA-lacZ rcsF* mutant and *rprA-lacZ rcsF surA* double mutant strains, transformed with either pAM238 (vector) or pHK646 (encoding RcsF-c-Myc), were assayed for β-galactosidase activity. (C and D) Cells from strain GEB658 (*rprA-lacZ rcsF*$^+$) transformed with either pAM238 or pHK646 were fractionated as described in Materials and Methods, and the fractions from GEB658/pHK646 were analyzed for the presence of RcsF-c-Myc by Western blotting (C). Ttl, total fraction (whole cells); S100, soluble cytoplasmic and periplasmic content; Ttl M, total membrane fraction; IM, inner membrane fraction; OM, outer membrane fraction; TxS, Triton X-100-solubilized total membrane fraction; TxI, Triton X-100-insoluble total membrane fraction. (D) The same fractions were analyzed by Western blotting for the presence of OmpA.

was also RcsF dependent. We compared the effect on a *cps-lacZ* fusion of the induction of expression of DjlA in a wild-type strain and an *rcsF* mutant strain. As shown in Fig. 2A, induction by DjlA overproduction of the capsular operon is identical in both strains, indicating that the activation of the Rcs pathway by DjlA is RcsF independent. Reciprocally, we examined, after induction by IPTG of RcsF from a $lac_{UV5}p$ promoter, the induction of the *cps-lacZ* fusion in a *djlA*$^+$ and *djlA*-null background. The effect of RcsF was found to be independent of the presence of DjlA (Fig. 2B). These results indicate that DjlA and RcsF, two proteins whose overproduction leads to activation of the Rcs phosphorelay, are on two independent pathways. Consistent with this conclusion is the observation that the introduction of the *djlA* mutation in the *cps-lacZ surA* strain did not abolish the activation of the *cps-lacZ* expression by the *surA* mutation (Fig. 2C). In contrast, unexpectedly, the *djlA* mutation strongly potentiates the activation. Similar results were obtained with the *rprA-lacZ* fusion or when the *djlA* mutation was combined with an *rfa* mutation (data not shown). So far, we have no good explanation for the stimulation activity due to the *djlA* mutation in the *surA* or *rfa* background.

RcsF is localized in the outer membrane. Although RcsF is predicted to be an outer membrane lipoprotein (15) based on its N-terminal sequence (Fig. 3A), its localization has not yet been determined experimentally. To determine RcsF localization, we used a c-Myc epitope C-terminal-tagged version of RcsF. The plasmid pHK646 is a derivative of pAM238 and encodes RcsF-c-Myc under the control of a $lac_{UV5}p$ promoter

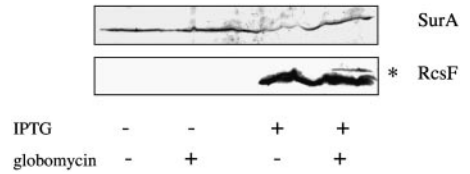

FIG. 4. Inhibition of processing of RcsF-c-Myc by globomycin. Strain 61F/pHK646 was grown in LB supplemented with spectinomycin. RcsF-c-Myc production was induced with IPTG in some cultures and left uninduced in others, and some cultures were treated with globomycin while others were not. Proteins from each culture were separated by SDS-PAGE. After transfer, the upper part of the membrane was immunoblotted with SurA antibodies as a loading control (upper panel), and the bottom part of the membrane was immunoblotted with c-Myc-specific monoclonal antibodies (lower panel; see Materials and Methods). The upper band detected in globomycin-treated cultures, indicated by an asterisk, corresponds to the unprocessed RcsF-c-Myc precursor.

(16). This plasmid, or the vector plasmid, was introduced into strain GEB658 carrying an *rprA-lacZ* fusion (Table 1). RcsF-c-Myc could easily be detected in whole-cell extracts using anti-c-Myc antibodies (see Materials and Methods) (Fig. 3C). It is to be noted that, due to the absence of a *lacI*^{q} allele in this strain background, the protein could be detected even in the absence of IPTG induction. As shown in Fig. 3B, the introduction of pHK646 in the *rprA-lacZ rcsF* strain stimulates the *rprA-lacZ* expression, suggesting that RcsF-c-Myc is able to activate the Rcs system. In addition, the observation that expression of the *rprA-lacZ* fusion was higher when pHK646 was introduced in the *rprA-lacZ surA rcsF* double mutant indicates that RcsF-c-Myc complements the *rcsF* mutation and that the activation of the Rcs phosphorelay is not due to an indirect effect of overproducing the engineered protein.

GEB658 (*rprA-lacZ*)/pAM38 and GEB658 (*rprA-lacZ*)/pHK646 whole cells were fractionated as described in Materials and Methods, and each fraction was analyzed by Western blotting for the presence of RcsF-c-Myc. As shown in Fig. 3C, no RcsF-c-Myc was detected in cells carrying the empty vector pAM238, whereas a band migrating as a 17-kDa protein (the expected size for RcsF) was detected in the strain carrying pHK646. In addition to the total cell fraction, RcsF-c-Myc was found to be present in the total membrane fraction and, after fractionation on a sucrose gradient of this membrane fraction, absent from the inner membrane fraction and present only in the outer membrane fraction. The observation that RcsF was detected in the same fractions as those containing the major outer membrane protein OmpA is indicative of the quality of the fractionation (Fig. 3D) and is in agreement with the conclusion that RcsF is an outer membrane protein.

Integral β-barreled outer membrane proteins are insoluble in Triton X-100, contrary to lipoproteins, which are inserted into the membrane via an acyl residue. When the total membrane fraction from GEB658/pHK646 was treated with 1% Triton X-100, RcsF-c-Myc was recovered only in the Triton X-100-soluble fraction, also consistent with the prediction that RcsF is a lipoprotein (Fig. 3C). In contrast, OmpA was insoluble in Triton X-100 (Fig. 3D), as expected for an integral outer membrane protein.

RcsF is a lipoprotein. The fact that RcsF is both in the outer membrane and solubilized by Triton X-100 suggested that it is a lipoprotein, in conformity with computer predictions. Using [^{3}H]palmitate labeling, we failed to detect a specific RcsF-labeled band in the outer membrane fraction, presumably because ^{3}H-RcsF was masked by one or several lipoproteins of the same size present in higher amounts. Maturation of lipoproteins, i.e., cleavage of their signal sequences, is specifically inhibited by the antibiotic globomycin, an inhibitor of leader peptidase II. pHK646 was introduced in strain 61F (Table 1), which carries a *lacI*^{q} allele on an F′ plasmid. In this strain background, the RcsF-c-Myc protein was not detected in the absence of IPTG (Fig. 4). Cells were grown to an OD_{600} of approximately 0.4, and then IPTG (500 μM) was added. Growth was pursued for 90 min in the absence or in the presence of globomycin (50 μg/ml), and total cell extracts were analyzed for the presence of RcsF-c-Myc by Western blotting. As shown in Fig. 4, treatment by globomycin resulted, in addition to the appearance of wild-type RcsF (mature protein), in the appearance of a slower migrating protein corresponding to the unprocessed RcsF-c-Myc protein, as would be expected in the case of a lipoprotein.

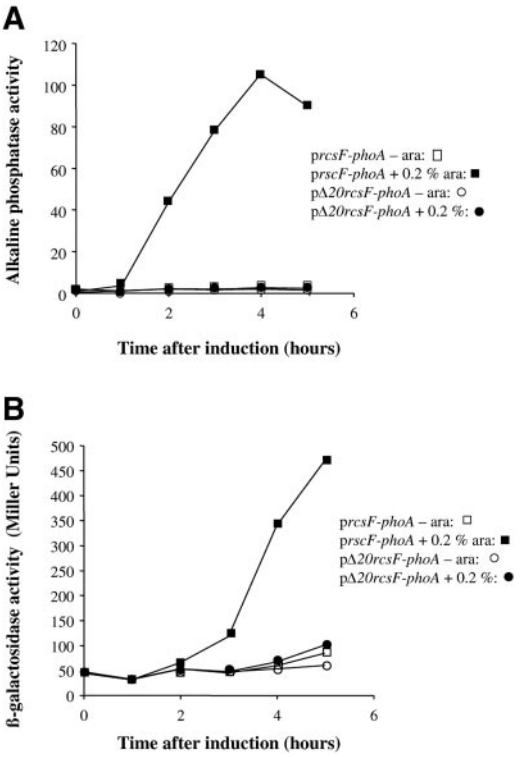

FIG. 5. RcsF-PhoA is periplasmic. MP110 (*cps-lacZ*) strains transformed either with p*rcsF-phoA* or p*rcsF-phoA*$_{\Delta 20}$ were grown overnight in LB in the presence of spectinomycin and then diluted 1,000-fold in fresh LB and grown to an OD_{600} of ~0.1 at 37°C. At time zero, 0.2% arabinose was added to some cultures but not to others, and the cultures were sampled at regular intervals to assay either for alkaline phosphatase activity (A) or for β-galactosidase activity (B).

RcsF faces the periplasm. Alkaline phosphatase is an enzyme active only when present in the oxidizing periplasmic compartment, and as such it is widely used as a reporter of membrane protein topology (26). To further determine the topology of RcsF, we generated alkaline phosphatase fusions to the C-terminal end of two versions of RcsF: wild-type RcsF

and RcsF deleted of its first 20 amino acids (i.e., its putative signal sequence and the first 5 amino acids of the mature sequence [$RcsF_{\Delta 20}$]). *rcsF-phoA* and *rcsF-phoA*$_{\Delta 20}$ were placed under the control of the *ara*$_{BAD}$*p* promoter (14) and introduced into the *E. coli* Δ(*ara-leu*) strain MG1655 *cps-lacZ* (MP110; Table 1). Expression of fusion proteins was checked by SDS-PAGE. Coomassie staining indicates that both proteins are equally expressed (data not shown). Cells were grown in the presence or in the absence of arabinose (0.2%) and assayed at regular time intervals both for alkaline phosphatase activity (Fig. 5A) and for β-galactosidase activity (Fig. 5B), as described in Materials and Methods.

After induction of production of the RcsF-PhoA fusion protein, phosphatase activity accumulated, indicating that the PhoA moiety of the fusion was localized in the periplasm (Fig. 5A). This is in contrast to what was obtained in the case of the RcsF-PhoA$_{\Delta 20}$ fusion, which was found to be totally inactive, as would be expected if the mutant protein is retained in the cytoplasm.

Importantly, the RcsF-PhoA fusion was found to be able to induce the expression of the *cps-lacZ* fusion present in the reporter strain (Fig. 5B), indicating that this protein still possesses signaling activity, whereas RcsF-PhoA$_{\Delta 20}$ does not, suggesting that appropriate localization of RcsF in the periplasmic compartment is necessary to activate the Rcs pathway.

DISCUSSION

The actual, physiological signal of the Rcs phosphorelay pathway has remained elusive. However, studies of mutations leading to activation of this signaling pathway have suggested that perturbations in the cell envelope induce the Rcs regulon (23). For instance, *rfa* mutations, which cause a defect in LPS (33), *mdo* mutations, leading to the absence of periplasmic membrane-derived oligosaccharides (10), or a *pgsA* mutation (39), which causes a complete lack of the major acidic phospholipids phosphatidylglycerol and cardiolipin, all lead to activation of the Rcs pathway. *surA* encodes a periplasmic peptidyl prolyl isomerase with chaperone activity and is important for the proper folding and insertion of outer membrane proteins (1, 20, 36). Here, we show that *surA* inactivation causes induction of the Rcs pathway in an RcsC-dependent manner. Importantly, disruption of *surA* also increases the extracytoplasmic, RpoE-mediated stress response (36), which itself was reported to regulate *surA* expression (7, 29). An *rfaD* mutant was likewise reported to exhibit higher RpoE activity (29). This is to be expected, since abnormal LPS would lead, like *surA* disruption, to accumulation of misfolded outer membrane proteins in the periplasm, a known inducer of the RpoE-mediated response (41). Thus, *rfa* and *surA* mutations, resulting in a defective outer membrane, lead to the activation of both the extracytoplasmic RpoE-mediated pathway and the Rcs pathway. It will be interesting to know whether these two pathways share additional signals. However, it is likely that the Rcs pathway senses a consequence of an outer membrane defect other than the accumulation of misfolded intermediates of outer membrane proteins, the specific signal inducing the RpoE pathway.

Similar to *rfa*, *mdo*, and *pgsA* mutations (24, 39), activation of the Rcs pathway by *surA* inactivation is also RcsF dependent, confirming the importance of this protein in the signaling cascade upstream of RcsC. However, not all signals activating the Rcs pathway pass through RcsF. Overproduction of the inner membrane DnaJ-like chaperone DjlA leads to activation of RcsC (3, 17). Kelley and Georgopoulos (17) reported that activation by DjlA overproduction was partially reduced in an *rcsF* mutant. In contrast, here we show that activation by DjlA is totally independent of RcsF, consistent with results obtained by Shiba et al. (39). Such results did not exclude the possibility that DjlA acts downstream of RcsF. However, we found that reciprocally, activation of the Rcs phosphorelay by overproduction of RcsF is also independent of DjlA. Hence, those two positive regulators are on two independent activating pathways. Consistent with this conclusion is the fact that activation of the Rcs phosphorelay by a *surA* mutation is independent of DjlA (Fig. 2C).

Overproduction of DjlA was found to increase sensitivity to novobiocin and EDTA (2). Such increased sensitivity is often associated with outer membrane defects. Accordingly, DjlA overproduction could indirectly activate the RcsC phosphorelay due to such a defect. The fact that DjlA acts in an RcsF-independent manner makes such a model unlikely, as the RcsF-dependent pathway is itself activated by outer membrane defects.

Analysis of the predicted amino acid sequence of RcsF shows the presence of 15 N-terminal amino acids with the feature of a lipoprotein signal sequence (Fig. 3A). Such signal sequences are specifically cleaved by the leader peptidase, signal peptidase II, leaving a cysteine residue at the N-terminal sequence of the mature protein that can be modified by acylation.

Lipoproteins can be localized in the periplasmic face of the inner membrane or in the periplasmic face of the outer membrane, depending upon the second residue of the mature sequence, according to the so-called +2 rule, which states that the presence of an aspartate residue at the +2 position (D + 2) immediately following the fatty acylated cysteine triggers the retention of the protein in the inner membrane, whereas other residues (often a serine residue) at this position results in the sorting of the protein to the outer membrane (38). Hence, RcsF was predicted to localize to the outer membrane. Fractionation and solubilization experiments in this study demonstrated that RcsF is an outer membrane protein. In addition, the appearance of the uncleaved precursor of RcsF following the inhibition of the signal peptidase II activity by globomycin demonstrated that RcsF is a lipoprotein. Prolipoproteins are first modified by transfer of a diacylglyceryl group from phosphatidylglycerol (PG) to the sulfhydril group of the invariant cysteine residue at position +1 of the mature lipoprotein. This initial step is required for further processing of the prolipoprotein by signal peptidase II (37). Mutation of *pgsA* activates the Rcs pathway in an RcsF-dependent manner. Since *pgsA* mutants are totally devoid of PG, it would be interesting to investigate processing and localization of RcsF in this mutant in relation to the role of RcsF in signaling. Finally, using an alkaline phosphatase fusion to RcsF, we showed that RcsF is oriented toward the periplasm and that its periplasmic localization is important for activity (Fig. 5).

Hence, RcsF is the second example of an outer membrane lipoprotein involved in signal transmission and activation of a

phosphorelay, the first one being NlpE. NlpE senses surface attachment and in response activates the Cpx pathway, another extracytoplasmic stress response pathway (32). Other factors inducing the Cpx pathways (i.e., alkaline pH) were found to be independent of NlpE (9). Similarly, RcsF seems to be specific to signals generated at the outer membrane level, consistent with its localization, but other types of signals, such as overproduction of DjlA, are independent of RcsF. The nature of the actual signal sensed by RcsF remains obscure.

How does RcsF activate RcsC? It is still not known whether RcsF can directly interact with RcsC. If this were the case, the interaction would certainly take place in the periplasm between the periplasmic domain of RcsF and the periplasmic domain of RcsC. We are currently testing this possibility.

ACKNOWLEDGMENTS

We thank Nadim Majdalani and Susan Gottesman for gifts of strains, helpful discussions, and communicating results prior to publication and Leonora Poljak for helpful discussions on the manuscript. We also thank Sunishi Miyakoshi from Sankyo Co. (Japan) for the gift of globomycin. M.-P.C.-C. and K.C. thank Claude Gutierrez for his constant support in this work. A.J. is grateful to Philippe Bouloc and all the members of the SRRB laboratory at the IGM for discussions and a supportive and stimulating atmosphere.

This work was supported in part by a grant from the microbiology program of the Génopôle of Toulouse.

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