Exploring the relationship between lipoprotein mislocalization and activation of the Rcs signal transduction system in *Escherichia coli*

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The Rcs phosphorelay signal transduction system controls genes for capsule production and many other envelope-related functions and is implicated in biofilm formation. We investigated the activation of the Rcs system in a *pgsA* null mutant of *Escherichia coli*, which completely lacks the major acidic phospholipids phosphatidylglycerol and cardiolipin. We found that the Rcs activation, and consequent thermosensitivity, were suppressed by overexpression of the *lgt* gene, encoding diacylglyceryltransferase, which catalyses the modification of prolipoproteins that is the first step in the maturation and localization process of lipoproteins, and is a prerequisite for the later steps. The outer-membrane lipoprotein RcsF is an essential component of Rcs signalling. This lipoprotein was poorly localized to the outer membrane in the *pgsA* null mutant, probably because of the absence of phosphatidylglycerol, the major donor of diacylglycerol in the Lgt reaction. Even in a *pgsA*⁺ background, the Rcs system was activated when RcsF was mislocalized to the inner membrane by alteration of the residues at positions 2 and 3 of its mature form to inner-membrane retention signals, or when it was mislocalized to the periplasm by fusing the mature form to maltose-binding protein. These results suggest that RcsF functions as a ligand for RcsC in activating Rcs signalling. Mislocalized versions of RcsF still responded to mutations *pgsA*, *mdoH* and *toIB*, further activating the Rcs system, although the *rfaP* mutation barely caused activation. It seems that RcsF must be localized in the outer membrane to respond effectively to stimuli from outside the cell.

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INTRODUCTION

Phosphorelay signal transduction is a sophisticated mechanism by which bacterial, archaeal and some eukaryotic cells sense and respond to environmental stresses (Stock *et al.*, 2000). The prototypical system consists of two components, a sensor histidine kinase, generally a transmembrane protein, and a response regulator protein, generally a transcription factor. Phosphotransfer takes place from the conserved His residue of the sensor kinase to the conserved Asp residue of the response regulator to transduce the signal of the external stress. Environmental stresses are sensed by the histidine kinase N-terminal sensing domain, which is exposed outside the cytoplasmic membrane. Only a very few specific ligands that directly bind to sensing domains have been identified, and the sensing mechanism remains unknown in most phosphorelay systems.

The enterobacterial Rcs signal transduction system responds to stresses that perturb the cell envelope and plays a key role in biofilm development and pathogenicity (Majdalani & Gottesman, 2005; Huang *et al.*, 2006). It is a multistep phosphorelay system (Fig. 1). These systems are found predominantly in eukaryotes, but it appears now that they are more common in prokaryotes than previously thought (Zhang & Shi, 2005). The membrane-bound histidine kinase RcsC of the system is of a hybrid type and also contains a receiver domain. When activated, RcsC autophosphorylates its His residue in the kinase domain and transfers the phosphoryl group to the Asp residue in the receiver domain. The subsequent phosphotransfer occurs to the His residue of the phosphotransmitter domain in the C-terminal region of RcsD (also called YojN), a transmembrane protein that shows high similarity to RcsC in the sensing domain and in the histidine kinase domain, although the conserved His residue in the latter is missing (Takeda *et al.*, 2001). The phosphoryl group is finally transferred to the Asp residue of the receiver domain of the RcsB response regulator, and the activated RcsB

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Abbreviation: MBP, maltose-binding protein.

Fig. 1. Rcs phosphorelay signal transduction system. Thick arrows indicate autophosphorylation of the histidine residue (H) in the sensor kinase domain of RcsC, and phosphotransfer through the aspartic acid (D) residue in the receiver domain of RcsC and histidine in the phosphotransmitter domain of RcsD to aspartic acid in the receiver domain of RcsB. Thin arrows indicate the transcription regulator function of the RcsB homodimer and RcsB–RcsA heterodimer. The dotted arrow indicates a possible direct signal transduction from the RcsF lipoprotein to the RcsC periplasmic domain. OM, outer membrane; IM, inner membrane.

functions as a transcription factor. In *Escherichia coli*, the Rcs system positively or negatively regulates the transcription of more than 150 genes, the majority of which are involved in cell envelope-related functions (Ferrières & Clarke, 2003). Some of the Rcs regulon genes, including the *cps* operon genes involved in capsular polysaccharide synthesis, are regulated by RcsB associated with the auxiliary transcription factor RcsA, and others are regulated by RcsB homodimer, independently of RcsA (Majdalani & Gottesman, 2005).

The RcsF protein was initially identified as a multicopy activator of Rcs signalling, and it was proposed that it was an inner-membrane protein that promoted the phosphorylation of RcsB (Gervais & Drapeau, 1992). However, it was later demonstrated that the Rcs activation by RcsF overproduction is dependent on RcsC (Majdalani *et al.*, 2002; Hagiwara *et al.*, 2003) and that RcsF is an outer-membrane lipoprotein (Castanié-Cornet *et al.*, 2006). A variety of environmental and mutational stresses require RcsF to stimulate Rcs signalling (Majdalani *et al.*, 2005), and RcsF is now regarded as an inherent component of the Rcs system with a crucial role in transmitting the signal to RcsC.

Lipoproteins are synthesized as precursors containing a consensus sequence called a lipobox around the signal peptide cleavage site. After being translocated across the

inner membrane by the Sec machinery, they are processed to mature forms in a sequence of steps on the periplasmic side of the inner membrane (Tokuda & Matsuyama, 2004). First, prolipoprotein diacylglycerol transferase (Lgt) modifies the Cys residue in the lipobox with diacylglycerol, using phosphatidylglycerol as the major donor of diacylglycerol (Sankaran & Wu, 1994). This modification is a prerequisite for the next signal peptide cleavage step by prolipoprotein signal peptidase (LspA). Then the N-terminal diacylglyceryl-Cys residue is *N*-acylated by apolipoprotein *N*-acyltransferase (Lnt). The matured lipoproteins are transported to the outer membrane by the Lol system or retained in the inner membrane depending on the lipoprotein sorting signal located at position 2 of the mature forms. Asp at position 2 functions as an inner-membrane retention signal (Tokuda & Matsuyama, 2004). RcsF has Ser at position 2 and is transported to the outer membrane (Castanié-Cornet *et al.*, 2006).

An *E. coli* *pgsA* null mutant defective in phosphatidylglycerophosphate synthase and completely lacking the major acidic phospholipids, phosphatidylglycerol and cardiolipin, is viable, if it also lacks the major outer-membrane lipoprotein encoded by the *lpp* gene, but shows a thermosensitive growth defect at 42 °C (Kikuchi *et al.*, 2000; Matsumoto, 2001; Suzuki *et al.*, 2002). A transposon mutagenesis experiment searching for the suppressor mutation of the thermosensitivity revealed that disruption of any one of *rscF*, *rscC*, *rscD* and *rscB* genes suppresses the thermosensitivity. It was shown that the Rcs system is constitutively activated in the *pgsA lpp* mutant, which leads the cells to lyse at 42 °C (Shiba *et al.*, 2004). Disruption of *rscA* does not suppress the thermosensitivity at 42 °C, but does correct the poor growth phenotype of the mutant at 37 °C (Nagahama *et al.*, 2006).

In this study we further searched for transposon-induced mutations that suppress the thermosensitivity at 42 °C, and identified a mini-Tn10 *cam* (Kleckner *et al.*, 1991) insertion upstream of the *lgt* gene with the *cam* gene in the same orientation as *lgt*. In a separate trial to clone multicopy suppressors of the thermosensitivity on a plasmid, chromosomal fragments containing the *lgt* gene were repeatedly cloned. Treatment of *pgsA*⁺ *lpp* cells with globomycin, a specific inhibitor of LspA, caused Rcs activation, and disruption of the *rscF* gene suppressed the activation. These results suggest that the Rcs activation in the *pgsA lpp* mutant involves a defective maturation process of lipoproteins, and RcsF in particular. We carefully examined the role of RcsF in the activation of Rcs signalling.

METHODS

Bacterial strains, plasmids and culture media. The *E. coli* strains and the plasmids used for this study are listed in Table 1. DNA primers used for this study are listed in Table 2. For construction of *pptsP* and *plgt*, PCR-amplified fragments were cloned into the multiple cloning site of pGB2 (Churchward *et al.*, 1984) in the same orientation as that of the spectinomycin-resistance gene (*aadA*) of the vector. Plasmid pHR725 was constructed by ligating *Nco*I-digested

Table 1. Bacterial strains and plasmids used in this study

Abbreviations: MCS, multiple cloning site; ΔSS, deletion of signal sequence.

Strain or plasmid	Relevant genotype or description	Construction, source or reference
Strains		
DH5	<i>recA1 endA1 hsdR17</i>	Laboratory collection
S330	W3110 <i>ksgB1 lpp-2 pgsA30::kan rcsA330::IS5</i>	Kikuchi <i>et al.</i> (2000); Nagahama <i>et al.</i> (2006)
S330S21	S330 <i>zgd::mini-Tn10 cam</i>	Mini-Tn10 mutagenesis; Shiba <i>et al.</i> (2004); this study
YAS006	S330 <i>zgd::mini-Tn10 cam</i>	T4GT7(S330S21) × S330
UE29	MC4100 <i>cpsB10::lac-Mu-immλ lpp-2</i>	Shiba <i>et al.</i> (2004)
CL330	UE29 <i>pgsA30::kan</i>	Shiba <i>et al.</i> (2004)
CL022	CL330 <i>zgd::mini-Tn10 cam</i>	T4GT7(S330S21) × CL330
SG20803	MC4100 <i>cpsB10::lac Mu-immλ rcsC137 ompC::Tn5</i>	Brill <i>et al.</i> (1988)
S330A21	S330 <i>rcsC::mini-Tn10 cam</i>	Mini-Tn10 mutagenesis; Shiba <i>et al.</i> (2004)
CL062	UE29 <i>rcsC::mini-Tn10 cam</i>	P1(S330A21) × UE29
S330K41	S330 <i>rcsF::mini-Tn10 cam</i>	Mini-Tn10 mutagenesis; Shiba <i>et al.</i> (2004)
CL050	UE29 <i>rcsF::mini-Tn10 cam</i>	P1(S330K41) × UE29
BL21(DE3)	<i>ompT hsdSB (λ cIts857 Sam7 nin5 lacUV5-T7 gene 1)</i>	Studier & Moffatt (1986)
ST001Δ <i>rscF</i>	Δ <i>rscF::kan</i>	Hagiwara <i>et al.</i> (2003)
CL053	UE29 Δ <i>rscF::kan</i>	P1(ST001Δ <i>rscF</i>) × UE29
CL063	UE29 Δ <i>rscF::kan rcsC::mini-Tn10 cam</i>	P1(S330A21) × CL053
CL047	CL330 <i>rcsF::mini-Tn10 cam</i>	P1(S330K41) × CL330
CL034	UE29 <i>mdoH200::Tn10</i>	Shiba <i>et al.</i> (2006)
CL044	CL034 <i>rcsF::mini-Tn10 cam</i>	P1(S330K41) × CL034
JW5100	BW25113 Δ <i>tolB::kan</i>	Keio collection strain*
CL122	UE29 Δ <i>tolB::kan</i>	P1(JW5100) × UE29
CL138	CL122 <i>rcsF::mini-Tn10 cam</i>	P1(S330K41) × CL122
JW3605	BW25113 Δ <i>rfaP::kan</i>	Keio collection strain*
CL095	UE29 Δ <i>rfaP::kan</i>	P1(JW3605) × UE29
CL107	CL095 <i>rcsF::mini-Tn10 cam</i>	P1(S330K41) × CL095
Plasmids		
pGB2	pSC101 derivative; Sp ^r	Churchward <i>et al.</i> (1984)
pptsP	pGB2 <i>ptsP</i>	This study
p <i>lgt</i>	pGB2 <i>lgt</i>	This study
pACYC184	p15A derivative; Cm ^r , Tc ^r	Chang & Cohen (1978)
pHR718	pGB2 <i>lacI^q P_{trc}-MCS</i>	Shiba <i>et al.</i> (2004)
pHR725	pHR718 <i>P_{trc}-lgt</i>	This study
pET22b(+)	pBR322 derivative; <i>lacI P_{T7lac}-MCS-His₆</i> ; Ap ^r	Novagen
pYAS001	pET22b(+) <i>P_{T7lac}-rcsFΔSS-His₆</i>	This study
pYAS003	pHR718 <i>P_{trc}-rcsF</i>	This study
pYAS010	pHR718 <i>P_{trc}-rcsFS2D</i>	This study
pYAS013	pHR718 <i>P_{trc}-rcsFS2DM3Q</i>	This study
pMal-p2	pBR322 derivative; <i>lacI^q P_{tac}-male'-MCS-lacZα</i> ; Ap ^r	New England Biolabs
pMal-rcsF	pMal-p2 <i>P_{tac}-male'-rcsFΔSS</i>	This study
pHM001	pHR718 <i>P_{tac}-male</i>	This study
pHM002	pHR718 <i>P_{tac}-male'-rcsFΔSS</i>	This study

*From the National BioResource Project (NIG, Japan): *E. coli*.

pHR718 and the PCR-amplified fragment, which were first blunt-ended and then digested with *Bam*HI.

As the starting material of site-directed mutagenesis, we used pYAS003, in which wild-type *rcsF* was cloned under the *P_{trc}* promoter on a low-copy-number plasmid containing the *lacI^q* gene. In the presence of 20 μg IPTG ml⁻¹ this plasmid produced about the same amount of RcsF as the chromosomal *rcsF* gene.

Plasmid pMal-rcsF was constructed by inserting a PCR-amplified fragment into the *Eco*RI–*Hind*III region of pMal-p2. For construction

of pHM001 and pHM002, *Mlu*I (within the *lacI^q* gene)–*Hind*III fragments of pMal-p2 and pMal-rcsF, respectively, were substituted for the *Mlu*I (within the *lacI^q* gene)–*Hind*III region of pHR718.

Luria–Bertani (LB) medium and buffered LB medium (Shiba *et al.*, 2004) were used. For plates, media were solidified with 1.5% agar. When appropriate, antibiotics were included at the following concentrations (in μg ml⁻¹) for multicopy and single-copy resistance genes, respectively: ampicillin, 50 (for multicopy); chloramphenicol, 50 and 10; kanamycin, 50 and 20; tetracycline, 3 (for single copy); spectinomycin, 50 (for multicopy). X-Gal was used at 40 μg ml⁻¹ to

Table 2. PCR and sequencing primers used for this study

Primer	Sequence (5'→3')*	Purpose
cat-up	CTACTGACGGGGTGGTGCCTAACGGC	Determination of position and orientation of the mini-Tn10 <i>cam</i> insertion
cat-dn	CGATATGATCATTATTCTGCCTCCC	
ptsP- <i>Sa</i> II	GTGATGTCgACCGTAGGGTAATG	
ptsP- <i>Eco</i> RI	GTGCGAAATtTAGCACAAGGGGACC	<i>ptsP</i> construction
lgt- <i>Sa</i> II	gtCGaCAACCAGCCAGGTCCCT	<i>p</i> lgt construction
lgt- <i>Eco</i> RI	GaaTTCCGGTTCGGGTACGGT	
lgt-f	ATGACCACTAGCTATCTG	
T7-r	taatacgactcactatagggcgaTCAGGAAACGTGTTGC	Preparation of probe for <i>lgt</i> dot blot hybridization
hisano-p	GACTAAGAGATCATAGCGACCAACCCG	Identification of cloned fragment in the <i>Bam</i> HI site of pACYC184
naomi-p	CGGTGATGTCGGCGATATAGG	
lgt5	ACCAGTAGCTATCTGCATTTTCCGG	<i>p</i> HR725 construction
lgt3B	ATGGaTCCTCAGGAAACGTGTTGCTGTGGGC	
rcsFNdeI	TGCTAAGCcatatgTCCATGTTAAGC	<i>p</i> YAS001 construction
rcsFHindIII	AAACaagctfTTTCGCCGTAATG	<i>p</i> YAS003 construction
rcsFSNcoI	GACGCCaTgGTCATACATTGAGGA	
rcsFAsHindIII	ATAAaGCtTATTTGCTCG	<i>p</i> YAS010 construction
rcsFS2D	GCACTCATGCTAAGCGGCTGTgatATGTTAAGCAGATCCCC	
rcsFAsS2D	GGGGATCTGCTTAACATatcACAGCCGCTTAGCATGAGTGC	<i>p</i> YAS013 construction
rcsFS2DSM3Q	CATGCTAAGCGGCTGTGATcaGTTAAGCAGATCCCCTGTGCG	
rcsFS2DAsM3Q	CGACAGGGGATCTGCTTAACtgATCACAGCCGCTTAGCATG	<i>p</i> Mal-rcsF construction, together with rcsFAsHindIII
rcsFSCys	cgaattCTGTTCCATGTTAAGCAGATCCCC	

*Mismatched nucleotides are in lower-case type and restriction sites are in italic type.

examine β -galactosidase levels of colonies on LB agar medium. Cell growth was monitored with a Klett–Summerson colorimeter equipped with a no. 54 filter.

Genetic and recombinant DNA procedures. These were based on standard methods (Miller, 1992; Sambrook & Russell, 2001). The insertion positions and orientations of mini-Tn10 *cam* were determined as described by Shiba *et al.* (2004). Generalized T4GT7 transduction was done according to Young & Edlin (1983). Total cellular RNA preparation and dot blot hybridization were done as described by Nagahama *et al.* (2007). For isolation of multicopy suppressors of the thermosensitivity of S330, chromosomal DNA of S330 was digested by *Sau*3AI, and 2–5 kb fragments were ligated with *Bam*HI-digested pACYC184. Ligation products were introduced into S330 by transformation, and chloramphenicol-resistant transformants grown at 42 °C were picked up. Plasmids were isolated and suppression was confirmed by reintroducing them into S330. Cloned fragments were identified by sequencing. Site-directed mutagenesis was done using the QuikChange Site-Directed Mutagenesis kit (Stratagene) according to the manufacturer's instructions.

Biochemical procedures. Cellular phospholipids were extracted and analysed as described previously (Okada *et al.*, 1994). The β -galactosidase assay method using ONPG as substrate and the unit definition were as described by Wang & Doi (1984). Outer-membrane/inner-membrane localization of RcsF and its derivative was examined by sucrose density gradient centrifugation essentially as described previously (Suzuki *et al.*, 2002). Periplasmic and spheroplast fractions were prepared as described by Boeke & Model (1982). ECL Plus (Amersham) was used for immunodetection in Western blotting experiments according to the manufacturer's instructions.

Preparation of anti-RcsF antiserum. We constructed a pET22b(+)-based plasmid, *p*YAS001, which encodes C-terminally His₆-tagged RcsF lacking its signal sequence and the N-terminal Cys of the mature form. The product was purified using Ni-nitrilotriacetic acid agarose (Qiagen) according to the manufacturer's instructions. A rabbit polyclonal antiserum against the product was raised by Tanpaku Seisei Kogyo (Isezaki, Gumma, Japan). Anti-maltose-binding protein (MBP) antiserum was purchased from New England Biolabs. Anti-OmpA and anti-EnvZ antisera were kind gifts from Yasuhiro Anraku and Masayori Inouye, respectively.

RESULTS

Transposon insertions upstream of the *lgt* gene suppress the thermosensitivity of the *pgsA* null mutant

In a previous study we isolated 44 thermoresistant suppressor mutants of the *pgsA lpp* strain S330 by transposon mutagenesis using mini-Tn10 *cam* (Kleckner *et al.*, 1991). Forty-one of them had the transposon insertions in the *rcsC*, *rcsF* or *rcsD* gene as judged by PCR analysis (Shiba *et al.*, 2004). By cloning and sequencing of the chromosomal fragments containing the inserted minitransposons (chloramphenicol-resistant), two of the other three suppressor mutants were shown to have mini-Tn10 *cam* inserted at 2 nt downstream of the termination codon of the *ptsP* gene. We found that 9 nt (the last 4 nt of *ptsP* plus the termination codon plus the downstream 2 nt) were duplicated (Fig. 2a). Phage T4GT7 lysate (Young &

Fig. 2. (a) Mini-transposon insertions upstream of the *lgt* gene that suppress the thermosensitivity of the *pgsA* null mutant. (b) Dot blot hybridization experiment for *lgt* expression. Ten microlitres of $0.2 \mu\text{g } \mu\text{l}^{-1}$ total cellular RNA preparations isolated from S330 and its *zgd::mini-Tn10 cam* transductant grown to mid-exponential phase (~ 100 Klett units) were spotted onto a positively charged nylon membrane (Hybond-N+, Amersham) and hybridized with a DIG-labelled *lgt* probe.

Edlin, 1983) was prepared from one of these two suppressor mutants (these strains were resistant to phage P1 for unknown reasons) and used to infect S330 cells. All the chloramphenicol-resistant transductants were thermoresistant, indicating that the minitransposon insertion was responsible for the suppression. The insertion was named *zgd::mini-Tn10 cam* according to its chromosomal position, min 63.9 (Chumley *et al.*, 1979). The third suppressor mutant had the insertion at nucleotide 1321 of the 2238 nt coding sequence of *ptsP*, and the 9 nt from position 1313 to position 1321 were duplicated (Fig. 2a). In all three suppressor mutants, the *cam* gene of the minitransposon was in the same orientation as the *ptsP* and *lgt* genes, the latter's initiation codon starting at 151 nt downstream of the *ptsP* termination codon. Introduction of a *ptsP*-carrying plasmid, *pptsP*, to these suppressor mutants did not reverse the thermoresistant phenotype, indicating that it was not a defect in *ptsP* that was responsible for the suppression. An *lgt*-carrying plasmid, *plgt*, did not reverse the thermoresistance either, whereas introduction of the plasmid to the parent S330 strain led to thermoresistant growth at 42°C . We suspected that increased expression of *lgt* through transcription that started from the promoter of the *cam* gene might be responsible for the suppression by *zgd::mini-Tn10 cam*. Indeed, in a dot blot hybridization experiment, the level

of *lgt* transcript in a *zgd::mini-Tn10 cam* transductant was higher than in the parent S330 (Fig. 2b).

In a separate trial conducted to isolate multicopy suppressors of the thermosensitivity of S330, 23 clones of pACYC184-derived suppressing plasmids were isolated. Six of them were shown to carry chromosomal fragments containing the *lgt* gene. We concluded that overexpression of *lgt* corrects the thermosensitive defect of the *pgsA* null strain. One of the suppressing plasmids carried the *yrfF* gene, the homologue of the *Salmonella igaA* gene that encodes a negative regulator of the Rcs system (Domínguez-Bernal *et al.*, 2004). Others carried various chromosomal fragments, and we did not study them further.

lgt overexpression suppresses Rcs activation of the *pgsA* null mutant

Since the thermosensitivity of the *pgsA* null strain can be ascribed to activation of the Rcs system (Shiba *et al.*, 2004; Nagahama *et al.*, 2007), we examined the effect of *lgt* overexpression on the system. The activation level was monitored by the expression level of the *cps* genes for capsular polysaccharide synthesis, using a *cpsB'-lac* transcriptional fusion.

The *pgsA lpp cpsB'-lac* fusion strain CL330 showed high β -galactosidase activity, which was suppressed by disruption of *rcsF*, *rcsC*, *rcsD* or *rcsB*, indicating that the high β -galactosidase activity was due to an activated Rcs system (Shiba *et al.*, 2004). A *zgd::mini-Tn10 cam* transductant of CL330 grew and developed a pale-blue colour at 42°C on LB agar containing X-Gal. The cells grown in buffered LB medium at 30°C showed β -galactosidase activity lower than one half of that of CL330, although still higher than in the *pgsA⁺ lpp cpsB'-lac* fusion strain UE29 (Fig. 3a). Phospholipid analysis of the transductant cells showed that they lacked detectable phosphatidylglycerol and cardiolipin, as did the parent CL330 cells (Fig. 4).

When the low-copy-number plasmid pHR725, in which *lgt* is under the control of an IPTG-inducible *trc* promoter, was introduced into CL330, cells grew well and formed white colonies at 42°C on LB agar containing X-Gal and 0.5 mM IPTG. In the absence of the inducer, cells grew poorly and developed a pale-blue colour at 42°C . The cells grown in the presence of 0.1 mM IPTG at 30°C showed β -galactosidase activity that was about half that of CL330 harbouring the vector plasmid pHR718 (Fig. 3b). Thus, *lgt* overexpression suppresses the activation of Rcs signalling in the *pgsA* null mutant.

When pHR725 was introduced into the *rcsC137* mutant SG20803, in which the Rcs system is constitutively activated (Brill *et al.*, 1988), suppression of the Rcs activation was not observed on LB agar containing X-Gal and 0.5 mM IPTG: the blue colony colour did not differ from that of the mutant carrying the vector plasmid pHR718 (data not shown). This suggests that *lgt* overexpression does not affect the Rcs signal transduction pathway downstream of RcsC.

Fig. 3. Activation levels of Rcs signalling monitored via a *cpsB'*-*lacZ* transcriptional fusion. Cells were grown in buffered LB medium at 30 °C to mid-exponential phase (~100 Klett units), and β -galactosidase activity was measured. The means and SEMs of at least three measurements are shown. (a) Effect of *zgd::mini-Tn10 cam*. (b) Effect of *lgt* overexpression. The medium contained 0.1 mM IPTG. (c) Effect of globomycin. +G, globomycin dissolved in DMSO was added at a final concentration of 10 μ g ml⁻¹; +D, DMSO was added instead of globomycin solution. (d) Effect of mutations in the lipoprotein sorting signal of RcsF. IPTG was added at 20 μ M. (e) Activation by MBP-RcsF fusion protein. The IPTG concentration (in μ M) is shown in brackets. (f–i) Response of sorting signal mutants to the *pgsA*, *mdoH*, *tolB* and *rfaP* mutations. The medium contained 20 μ M IPTG. (j) Response of MBP-RcsF to the *pgsA*, *mdoH*, *tolB* and *rfaP* mutations. No IPTG was added.

Inhibition of LspA activates Rcs signalling

The fact that *lgt* overexpression in the *pgsA* null mutants suppressed the activation of Rcs signalling implied that a defective lipoprotein maturation process might be responsible for Rcs activation. We thus treated cells of the *pgsA*⁺ *lpp cpsB'*-*lac* fusion strain (UE29) with globomycin, a specific inhibitor of LspA (Tokuda & Matsuyama, 2004), at a concentration of 10 μ g ml⁻¹. At this concentration cell growth was barely inhibited (Hussain *et al.*, 1980). We found that β -galactosidase activity increased in globomycin-treated cells, although only to

a small extent (Fig. 3c). This can be attributed to activation of the Rcs system, since disruption of *rcsC* suppressed the increase. Disruption of *rcsF* also suppressed the increase, providing further evidence that impaired processing of RcsF lipoprotein causes the Rcs activation.

RcsF is accumulated in the inner membrane of the *pgsA* null mutant

The above results suggest that the Rcs activation in the *pgsA* null mutant involves a defective lipoprotein maturation

Fig. 4. Insertion of *zgd::mini-Tn10 cam* does not suppress the loss of phosphatidylglycerol and cardiolipin of the *pgsA* null mutant. UE29 (lane 1), CL330 (lane 2) and its *zgd::Tn10 cam* transductant (lane 3) were grown at 30 °C to mid-exponential phase (~100 Klett units) in LB medium. The lipids were extracted and separated by TLC, and spots of phospholipids were visualized with Dittmer–Lester reagent. F, solvent front; CL, cardiolipin; PG, phosphatidylglycerol; PE, phosphatidylethanolamine; O, chromatographic origin. The faint spots at the positions between CL and PG in lanes 2 and 3 are phosphatidic acid, which accumulates in small amounts in *pgsA* null cells (Kikuchi *et al.*, 2000).

tion process, of RcsF in particular. We have previously shown that *lpp* expression in the *pgsA lpp* mutant leads to poor modification of the product because of the lack of phosphatidylglycerol, the major diacylglycerol donor in the Lgt reaction, and consequently to accumulation of the precursor form in the inner membrane, which causes cell lysis (Suzuki *et al.*, 2002). However, there were two reasons that we could not be sure that the maturation of RcsF was also retarded. (i) Phosphatidic acid and CDP-diacylglycerol, intermediates in phospholipid biosynthesis, are detectably accumulated in the *pgsA lpp* mutant (Kikuchi *et al.*, 2000), and these two lipids can serve as diacylglycerol donors in the Lgt reaction, although much less efficiently than phosphatidylglycerol (Sankaran & Wu, 1994). In fact, when *lpp* was expressed from a plasmid in the *pgsA lpp* mutant, a small fraction of Lpp was detected as mature lipoprotein (Suzuki *et al.*, 2002). (ii) In contrast to Lpp, which is the most abundant protein in *E. coli* (7×10^5 molecules per cell) (Nikaido, 1996), the other lipoproteins are present in minor quantities only. The absence of Lpp in the *pgsA lpp* mutant might make it possible to save Lgt and other maturation enzymes for minor lipoproteins. For these reasons we examined the outer-membrane/inner-membrane localization of RcsF by sucrose density gradient centrifugation followed by Western analysis.

In *pgsA*⁺ UE29 cells, RcsF was found in the outer-membrane fractions together with OmpA protein (Fig. 5a), as reported by Castanié-Cornet *et al.* (2006). In contrast, in

pgsA null CL330 cells a substantial amount of RcsF was recovered in the inner-membrane fractions together with succinate dehydrogenase activity (Fig. 5b). We believe that this result indicates that lack of phosphatidylglycerol, the major donor of diacylglycerol in the Lgt reaction, causes retardation of the maturation process of the quantitatively minor lipoprotein RcsF, although we did not directly analyse diacylglycerol modification.

RcsF with modified lipoprotein sorting signal is mislocalized to the inner membrane and activates the Rcs system in *pgsA*⁺ cells

Localization of matured lipoproteins depends on the sorting signal at position 2 of the mature form. Asp at position 2 leads to inner-membrane retention of the lipoprotein, and the residue at position 3 affects the retention efficiency (Tokuda & Matsuyama, 2004). We first changed the position 2 residue of RcsF from Ser to Asp by site-directed mutagenesis. When this RcsFS2D was expressed from *P_{trc}* promoter on a low-copy-number plasmid at about the same level as chromosomally encoded RcsF (with 20 µM IPTG) (Fig. 6a), *pgsA*⁺ cells showed activation of the Rcs system (Fig. 3d). RcsF has Met at position 3. Asp and Met at positions 2 and 3, respectively, do not function as a strong inner-membrane retention signal: only about 60 % was retained in an *in vitro* assay system using Lpp as a model lipoprotein (Tokuda & Matsuyama, 2004). Asp, Glu and Gln at position 3 together with Asp at position 2 produce the strongest retention signals, with a retention efficiency of 100 % in the *in vitro* system. We next changed the position 3 residue of RcsFS2D from Met to Gln. The resulting RcsRS2DM3Q caused higher activation of the Rcs system than RcsFS2D (Fig. 3d). Activation by these RcsF mutants was dependent on RcsC. In sucrose density gradient centrifugation experiments most of the RcsFS2DM3Q molecules were recovered in the inner-membrane fractions together with the inner-membrane protein EnvZ (Fig. 5c). We note that inner-membrane retention of RcsFS2D causing Rcs activation has recently been reported in *Salmonella enterica* (Farris *et al.*, 2010).

RcsF fused to periplasmic MBP strongly activates the Rcs system in *pgsA*⁺ cells

We fused the mature form of RcsF (starting from the N-terminal Cys) to periplasmic MBP (the *malE* gene product). When cells expressing this MBP–RcsF fusion protein from the *P_{tac}* promoter on a low-copy-number plasmid were spheroplasted, more than half were recovered in the supernatant periplasmic fraction together with chromosomally encoded MBP (Fig. 6b). MBP–RcsF strongly activated the Rcs system (Fig. 3e), even at very low expression levels (Fig. 6c). Farris *et al.* (2010) have also reported periplasmic mislocalization of mature RcsF fused to the PagC signal sequence and consequent Rcs activation in *S. enterica*.

Fig. 5. Outer-/inner-membrane localization of RcsF. Cells were grown in LB medium at 30 °C to mid-exponential phase (~100 Klett units). Total membranes were prepared and separated by sucrose density gradient centrifugation. The amounts of RcsF, OmpA (an outer-membrane protein) and EnvZ (an inner-membrane protein) were examined by SDS-PAGE followed by immunoblotting using specific antisera. The amount of succinate dehydrogenase (SDH; an inner-membrane protein) was estimated by a specific enzyme assay. (a) UE29 (*pgsA*⁺); (b) CL330 (*pgsA*); (c) CL050 (*rcsF*) harbouring pYAS013 (*rcsFS2DM3Q*). The medium contained 20 μ M IPTG in (c). In (c), OmpA appears in two populations (in the outer membrane and in an intermediate fraction); the reason for this is not known.

Part of the MBP-RcsF fusion protein was recovered in the spheroplast fraction, in contrast to MBP, which was recovered totally in the periplasmic fraction (Fig. 6b). We expected that the presence of the fusion protein in the spheroplast fraction might be caused by interaction between RcsF and RcsC/RcsD, but this was not the case. Disruption of the *rcsC* or *rcsD* gene did not affect the fractionation pattern (our unpublished data). RcsF has two moderately hydrophobic segments in the C-terminal region (Gervais & Drapeau, 1992), whose interaction with membrane lipids may result in the recovery of a part of the fusion protein in the spheroplast fraction. These molecules are

Fig. 6. Expression of modified versions of RcsF. (a) Expression levels of RcsF mutants with modified lipoprotein sorting signals. Cells were grown in buffered LB medium containing 20 μ M IPTG at 30 °C to mid-exponential phase (~100 Klett units), and equivalent amounts were analysed by SDS-PAGE, followed by immunoblotting using anti-RcsF antiserum. Triplicate samples used for the β -galactosidase assay in the upper half of Fig. 3(d) were analysed. (b) CL050 cells (*rcsF*) harbouring pHM002 (*malE*'-*rcsF*) were grown in buffered LB containing 10 μ M IPTG and 0.2% maltose (the latter for inducing the chromosomal *malE*), and separated into periplasmic and spheroplast fractions. Equivalent amounts were analysed by SDS-PAGE, followed by immunoblotting using anti-RcsF and anti-MBP antisera. (c) Expression levels of MBP-RcsF fusion protein. Cells were grown in buffered LB medium containing IPTG at the indicated concentrations at 30 °C to mid-exponential phase (~100 Klett units), and equivalent amounts were analysed by SDS-PAGE, followed by immunoblotting using anti-RcsF antiserum. Triplicate samples used for the β -galactosidase assay in Fig. 3(e) were analysed.

probably associated with the inner membrane and may also contribute to Rcs activation, together with periplasmic fusion proteins.

Mislocalized versions of RcsF respond differentially to various envelope mutations

A variety of mutational stresses stimulate Rcs signalling only in the presence of RcsF. These include stresses caused by mutations in: (i) the *pgsA* gene, encoding phosphatidylglycerophosphate synthase (Shiba *et al.*, 2004); (ii) the *mdoH* gene for glucosyltransferase, responsible for the synthesis of periplasmic membrane-derived oligosaccharides (Ebel *et al.*, 1997; Shiba *et al.*, 2004); (iii) the *tolB* gene for a periplasmic component of the Tol system, involved in maintenance of cell envelope integrity (Mouslim & Groisman, 2003); and (iv) the *rfaP* and *rfaG* genes, encoding phosphoryl- and glucosyltransferase, respectively, responsible for the assembly of the lipopolysaccharide core (Parker *et al.*, 1992; Majdalani *et al.*, 2005).

We examined whether mislocalized versions of RcsF would respond to these mutations to see if outer-membrane localization was important for the response. When RcsFS2D and RcsFS2DM3Q were expressed in the *pgsA* null mutant at about the same level as chromosomally encoded RcsF (with 20 μ g IPTG ml⁻¹), they activated the Rcs system more than the wild-type RcsF (Fig. 3f). RcsFS2DM3Q, which was mislocalized to the inner membrane, responded to the stimulus by the *pgsA* null mutation [compare 59.2 units of β -galactosidase activity in CL047 (*pgsA rcsF*)/pYAS013 (*rcsFS2DM3Q*) in Fig. 3(f) with 20.1 units in CL053 (*rcsF*)/pYAS013 in Fig. 3(d)]. In the *mdoH* null mutant, RcsFS2D and RcsFS2DM3Q led to higher activation of the Rcs system than the wild-type RcsF (Fig. 3g). In the *tolB* null mutant, RcsFS2D produced a slightly lower activation than the wild-type RcsF, and the activation by RcsFS2DM3Q was lower again, but both still elicited a substantial activation level (Fig. 3h). In the *rfaP* null mutant, RcsFS2D caused about half the activity of wild-type RcsF. In contrast, RcsFS2DM3Q showed very low β -galactosidase activity (Fig. 3i). It seems that RcsFS2DM3Q did not respond to *rfaP* [compare 16.3 units of β -galactosidase activity in CL107 (*rfaP rcsF*)/pYAS013 (*rcsFS2DM3Q*) in Fig. 3(i) with 20.1 units in CL053 (*rcsF*)/pYAS013 in Fig. 3(d)].

When MBP–RcsF fusion protein was expressed in the absence of IPTG, the *pgsA* and *tolB* null mutations led to high activation of the Rcs system, and the *mdoH* null mutation led to limited activation (Fig. 3j), indicating that the nature of the lipoprotein and its outer-membrane localization are not necessary for responding to these mutational stresses. In contrast to the other mutations, however, the *rfaP* null mutation barely caused activation in the cells expressing MBP–RcsF (Fig. 3j), as was also the case for RcsFS2DM3Q, indicating a localization requirement.

DISCUSSION

In this study, we have shown that in a *pgsA* null mutant lacking phosphatidylglycerol, which serves as the major

Fig. 7. Mislocalization of RcsF activates the Rcs system. The precursor form of RcsF in a *pgsA* mutant, the diacylglycerol-modified but unprocessed form in globomycin-treated cells, and mature forms of RcsFS2D and RcsFS2DM3Q are retained in the inner membrane. The MBP–RcsF fusion protein is freely diffusible in the periplasm. It is likely that these mislocalized versions of RcsF are easily accessible to the periplasmic sensor domain of RcsC and activate the phosphorelay system (thick arrows). We suggest that the wild-type RcsF functions similarly as a ligand for RcsC in activating the Rcs system (dotted arrow).

donor of diacylglycerol in the modification of precursors of lipoproteins by the Lgt enzyme, a considerable amount of RcsF is localized to the inner membrane, although RcsF is a quantitatively minor outer-membrane lipoprotein. We infer that this mislocalization causes the activation of Rcs signalling in the *pgsA* null mutant from the following observations. (i) Overexpression of the *lgt* gene from the promoter of the *cam* gene in mini-Tn10 *cam* insertions upstream of *lgt* or from a plasmid carrying the *lgt* gene partially suppressed the Rcs activation. (ii) Inhibition of LspA, lipoprotein signal peptidase, by the specific inhibitor globomycin in the *pgsA*⁺ strain caused Rcs activation, and disruption of the *rcsF* gene suppressed the activation. (iii) Inducing retention of RcsF in the inner membrane via expression of RcsFS2D and RcsFS2DM3Q, which carry modified lipoprotein sorting signals, activated the Rcs system in the *pgsA*⁺ strain.

The retention of RcsF in the inner membrane should make it more accessible to the periplasmic sensor domain of the sensor kinase RcsC, which is an inner-membrane protein. This presumably leads to the activation of the Rcs system. MBP–RcsF fusion protein, which is probably freely diffusible in the periplasmic space, should be even more accessible to the RcsC periplasmic domain, and this would explain the high activation of Rcs signalling (Fig. 7). RcsF thus seems to function as a ligand for RcsC in activating the Rcs system. In the wild-type cells the outer-membrane lipoprotein RcsF is anchored to the outer membrane with N-terminal acyl chains, and the 114-residue mature protein region is exposed to the periplasm (Castanié-Cornet *et al.*,

2006). RcsC has a large periplasmic domain of 272 residues in its N-terminal region (Stout & Gottesman, 1990). In response to environmental or mutational stimuli RcsF may change its conformation, leading to direct interaction with the RcsC periplasmic domain and to activation of Rcs signalling. A similar mechanism for Rcs activation has been proposed by Farris *et al.* (2010). RcsD shows high similarity to RcsC, except for the C-terminal phosphotransmitter domain (Takeda *et al.*, 2001). It has a 266-residue periplasmic domain in its N-terminal region (Majdalani & Gottesman, 2005). RcsF may also interact with RcsD in response to Rcs-activating stimuli.

RcsFS2DM3Q, which was mislocalized to the inner membrane, still responded to the stimulus by the *pgsA* null mutation (Fig. 3f). MBP-RcsF, which is not affected by failure of the Lgt reaction, responded strongly to the *pgsA* null mutation (Fig. 3j). Thus, the inner-membrane mislocalization of RcsF is not the sole cause of the Rcs activation in the *pgsA* null mutant. It seems that the lack of major acidic phospholipids provokes a response from RcsF in addition to that stemming from its inner-membrane mislocalization.

RcsF and its inner membrane- and periplasm-mislocalized versions all responded to mutations of *pgsA*, *mdoH* and *tolB*, although to varying degrees. This makes it conceivable that the exact characteristics of RcsF as an outer-membrane lipoprotein are not required for responding to stimuli. In contrast, the *rfaP* mutation did not activate the Rcs system through RcsFS2DM3Q or MBP-RcsF. Whereas *pgsA*, *mdoH* and *tolB* mutations affect membranes and the periplasmic space, the *rfaP* mutation affects the structure of the lipopolysaccharide outside the outer membrane. We conclude that in order to respond effectively to environmental stimuli that originate outside the cell, it does seem important that RcsF is anchored to the outer membrane as a lipoprotein.

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