Activation of the Rcs Signal Transduction System Is Responsible for the Thermosensitive Growth Defect of the *Escherichia coli* Mutant Lacking Phosphatidylglycerol and Cardiolipin

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Abstract

Lethal effect of the *Escherichia coli pgsA* null mutation which causes a complete lack of the major acidic phospholipids, phosphatidylglycerol and cardiolipin, is alleviated by the lack of the major outer membrane lipoprotein encoded by the *lpp* gene, but the *lpp pgsA* strain shows a thermosensitive growth defect. By transposon mutagenesis we found that the thermosensitivity was suppressed by disruption of the *rcsC*, *rcsF*, and *yojN* genes that code for sensor kinase, accessory positive factor, and phosphotransmitter, respectively, of the Rcs phosphorelay signal transduction system initially identified as regulating the capsular polysaccharide synthesis genes (*cps*). Disruption of the *rcsB* gene coding for the response regulator of the system also suppressed the thermosensitivity, whereas disruption of *cpsE* did not. By monitoring the expression of a *cpsB′-lac* fusion we showed the Rcs system was activated in the *pgsA* mutant and reversed to a wild-type level by the *rcs* mutations. These results indicate that the envelope stress due to acidic phospholipid deficiency activates the Rcs phosphorelay system and thereby causes the thermosensitive growth defect independently of the activation of capsule synthesis.

Introduction

The *pgsA* gene of *Escherichia coli* codes for phosphatidylglycerophosphate synthase that catalyzes the committed step in biosynthesis of the major acidic phospholipids of the organism, phosphatidylglycerol and cardiolipin (14). A *pgsA* null mutation was lethal (25), and these phospholipids were considered essential to *E. coli*. Since a null mutation of the *cls* gene coding for cardiolipin synthase that condenses two phosphatidylglycerol molecules to form cardiolipin caused only minor growth defect (40), the lethality was attributed to the lack of phosphatidylglycerol (14). However, it was then found that in a strain defective in the *lpp* gene coding for the major outer membrane lipoprotein the *pgsA* null mutation did not result in loss of viability although it caused a complete lack of phosphatidylglycerol, indicating dispensability of this phospholipid in *E. coli* (29, 35). The precursor of Lpp is modified with diacylglycerol derived from phosphatidylglycerol (47). This modification is a prerequisite for the signal peptide processing and thus for the translocation to the outer membrane (59). When Lpp was expressed in the *pgsA* null mutant, most of the molecules remained in the inner membrane because of inefficient modification, and the covalent linkage of their C-terminal Lys to peptidoglycan led to anomalous association of the inner and outer membranes and eventually to cell lysis (54). The *lpp pgsA* strain accumulates biosynthetic intermediates of major phospholipids, phosphatidic acid and CDP-diacylglycerol, in small amounts (29). These acidic phospholipids, quantitatively very minor in wild-type cells, may substitute for major acidic phospholipids in giving negative charges to the membrane surface in *pgsA* null cells (35, 54). They were shown to serve as substrates for lipoprotein modification *in vitro*, but only inefficiently (47).

Even if defective in *lpp*, a *pgsA* null strain could not be cured of a replication-thermosensitive plasmid carrying the *pgsA* gene by elevating the growth temperature to 42°C (60). This result was misinterpreted as indicating that the *lpp pgsA* strain was inviable. However, the inability of the plasmid curing was explained by the thermosensitive growth of the *lpp pgsA* strain constructed by P1 transduction of the *pgsA*::*kan* allele into an *lpp* strain: when shifted to 42°C, the cells started to lyse in 2–3 h (29).

In this study, in order to identify the gene(s) involved in the thermosensitive defect of the *lpp pgsA* strain, we used transposon mutagenesis and screened for thermoresistant suppressor mutants, and found that disruption of the *rcsC*, *rcsF*, and *yojN* genes suppressed the defect. The *rcsC* gene codes for the sensor kinase of the Rcs phosphorelay signal transduction system, originally described as regulating the capsular polysaccharide synthesis genes (*cps*) (20, 52). RcsC activates RcsB, the response regulator of the system, by phosphoryl group transfer via phosphotransmitter YojN (55). The *rcsF* gene product was reported to activate RcsB when overproduced (19), but its function in the signal transduction was not clear until recently Hagiwara *et al.* (22) showed its involvement in Rcs signaling in response to glucose and zinc at 20°C. The Rcs system was shown to affect the expression of *tolQRA* (10), *rcsA* (15), *ftsAZ* (5), *osmC* (13), *rprA* (34) and *flhDC* (18). Recent transcriptome analyses using DNA arrays identified many other genes to be under direct or indirect control of the Rcs system (17, 22, 43).

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture media. The *E. coli* K-12 derivative strains and the plasmids used in this study are listed in Table 1. According to the DNA database (accession no. D90851) the *rcsC* gene of W3110 (Kohara) strain, from whose chromosomal DNA the ordered λ clone library was constructed (31), is disrupted by IS*2* inserted at the 2374th nucleotide (nt) of the 2799-nt coding sequence with duplication of 5 nt of 2370–2374. The C-terminal 142 residues including Asp-859 that is essential for the signal transduction (9) are missing from the gene product. However, the W3110 strain in our laboratory stock is *rcsC*$^+$: PCR analysis of its *rcsC* region gave an amplified product with no apparent difference in size from that expected for the wild type, and its derivative S330 showed a thermosensitive growth phenotype in a *rcsC*-dependent manner as described in Results.

Recently *yojN* was proposed to be renamed *rcsD* (18, EcoGene website [http://bmb.med.miami.edu/EcoGene/EcoWeb]), which may be confused with the previously reported *rcsD* mutation allelic to *dsbB* (20, 52). We use the gene symbol *yojN* in this report.

For placing the *pgsA* gene under the P_{BAD} promoter, PCR-amplified *pgsA* fragment was inserted into the *Nhe*I–*Sma*I region of pBAD24 (21), and the resultant P_{BAD}-*pgsA* on pHR702 was recombined, together with the *araC* and *bla* genes, into the chromosome at the λ attachment site by use of the λ InCh system (2). The chromosomal *pgsA* gene was deleted by Datsenko and Wanner's method utilizing λ Red and yeast FLP recombinases (12). First the entire *pgsA* gene was replaced with kanamycin resistance gene (*kan*) flanked by FLP recognition target sites (FRTs) in a strain having the *lpp* and *rcsF* mutations, which remains thermoresistant even after

the functional *pgsA* is lost. After Δ*pgsA*::FRT-*kan*-FRT was P1-transduced and confirmed for its lethality in an *lpp*$^+$ strain having P_{BAD}-*pgsA* at *att*λ, the *kan* cassette was excised to obtain the Δ*pgsA*::FRT allele.

To construct plasmids carrying the *pgsA*, *rcsC*, and *rcsF* genes, PCR-amplified fragments were cloned into the multiple cloning sites of pGB2 (6) in the same orientation as that of the spectinomycin resistance gene (*aadA*) of the vector. Low-copy number vectors carrying P_{trc} and its weakened derivative P_{204} (pHR718 and pHR719) were constructed by inserting 1.8-kb *Nde*I (filled in)–*Hin*dIII fragment containing l*acI*^{q} and the promoters of pTrc99A (1) and pDSW204 (58), respectively, into the *Eco*RI (filled in)–*Hin*dIII region of pGB2. PCR-amplified *yojN* and *rcsB* fragments were inserted into the *Nco*I (filled in)–*Bam*HI region of pHR718 to construct pHR722 and pHR737, respectively. pHR741 was constructed by inserting PCR-amplified *djlA* fragment into pHR719 in a similar way but without filling the *Nco*I site, and the second residue of the product was changed from Gln to Glu.

LB medium (38), buffered LB medium in which the NaCl content was reduced to 3.5 g/liter and 10 ml of buffered salt solution (BSS) (24) per liter was included, and M9 medium (38) were used as rich and minimal media. For plates, media were solidified with 1.5% agar. When appropriate, antibiotics were included at the following concentrations (in μg/ml) for multicopy and single-copy resistance genes, respectively: ampicillin, 50 and 20; chloramphenicol, 50 and 10; kanamycin, 50 and 20; tetracycline, 3 (for single copy); spectinomycin, 50 (for multicopy). 5-Bromo-4-chloro-3-indolyl-β-D-galactoside (X-Gal) was used at 40 μg/ml to 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

(38, 46). Mutagenesis with mini-Tn*10 cam* (30) was performed essentially as described by Cao *et al.* (4). Briefly, S330 harboring pNK2884 in early exponential phase was incubated for 30 min with 0.1 mM isopropyl-β-D-thiogalactoside (IPTG) to induce the expression of transposase. Cells were collected and resuspended in IPTG-free medium for preparation of phage P1 lysate. We estimated the number of transposed mini-Tn*10 cam* per chromosome to be about 5 by comparing the transduction frequencies of kanamycin and chloramphenicol resistance markers. Chromosomally inserted minitransposons with their flanking regions were cloned by digesting the chromosomal DNA of the insertion mutants with *Hin*dIII or *Pst*I, or partially with *Sau*3AI, inserting the fragments into the *Hin*dIII, *Pst*I, or *Bam*HI site of pBR322, and screening for those conferring chloramphenicol resistance. The insertion positions and orientations were determined by sequencing the cloned fragments in either direction from inside the transposon, using the primers 5′-CTACTGACGGGGTGGTGCGTAACGGC-3′ and 5′-CGATATGATCATTTAT-TCTGCCTCCC-3′ corresponding to 315–341 nucleotides [nt] upstream of the initiation codon and 253–278 nt downstream of the termination codon, respectively, of the chloramphenicol resistance gene (*cam*). DNA sequencing was performed with a dye terminator cycle sequencing kit and model 310 capillary DNA sequencer (Applied Biosystems).

PCR primers were: 5′-GGGGT*AAGCTT*ACTGACAACAG-3′ (48–27 nt upstream of the initiation codon with a substitution [underlined] to introduce a restriction site [italic]) and 5′-ggg*gatCC*TGGCGCGATGAGTCAACG-3′ (68–48 nt downstream of the termination codon plus additional nucleotides [lower case]) for cloning *pgsA* on pGB2; 5′-ACTGACAACA*GCTA-GC*TACCCGTC-3′ (27–4 nt upstream of the initiation codon) and 5′-CCC*GAATTC*ATCAAG-

CAATCAG-3′ (156–135 nt downstream of the termination codon) for placing *pgsA* under P_{BAD} promoter; 5′-TAATCTTACTGACAACAGATAGTTACCCGTCATTATG-3′ (33 upstream nt plus the initiation codon) joined to P4, a priming sequence for pKD13 (12), and 5′-TGATCG-TTTGCTGAAAATTACGCCGAAACGATCACT-3′ (31 nt downstream–2 nt upstream of the termination codon) joined to priming sequence P1 for deleting the chromosomal *pgsA*; 5′-ggg*gaag-cTT*CGGTAATGGGGGCAAGTTCTGC-3′ (484–461 nt upstream of the initiation codon) and 5′-gc*ggaTCC*GGCAGATAAAGACTAATCACCTGTAGG-3′ (215–186 nt downstream of the termination codon) for *rcsC*; 5′-gc*ctgcaG*ACTCCGGCGAAAGACGTATCTTT-3′ (297–274 nt upstream of the initiation codon) and 5′-g*gaattC*GAGCGAATAACGCCTATTTGCTC-3′ (38–15 nt downstream of the termination codon) for *rcsF*; 5′-CA*CTGCAG*GATGATAAATATCA-CGGG-3′ (126–101 nt upstream of the initiation codon) and 5′-TTTGC*GAATTC*CGAACAAG-AC-3′ (79–59 nt downstream of the termination codon) for PCR analysis of *yojN*; 5′-CGTCAG-AAAGAGACAACGGCC-3′ (starting with the second codon) and 5′-gc*ggatCC*TTGCTACAG-CAAGCTCTTGAC-3′ (5 nt downstream–15 nt upstream of the termination codon) for cloning *yojN*; 5′-ATTG*AAGCTT*AACTGGCGCAGGAAGAG-3′ (352–326 nt upstream of the initiation codon) and 5′-ggg*gatCC*TGACGTTATATGCCGAGAG-3′ (249–229 nt downstream of the termination codon) for PCR analysis of *rcsB*; 5′-AACAATATGAACGTAATTATTGC-CGATGACC-3′ (starting with the second codon) and 5′-gc*ggatCC*TACAGGTGATTAGTCT-TTATCTGCCGG-3′ (11 nt downstream–15 nt upstream of the termination codon) for cloning

rcsB; and 5′-catg*ccATGG*AGTATTGGGGAAAAATCATTGGCG-3′ (28 nt starting with the initiation codon) and 5′-ggc*aagCTT*TAATCGTGGGCAGTTACTCAGC-3′ (38–14 nt downstream of the termination codon) for *djlA*.

Biochemical procedures. Cellular phospholipids were extracted and analyzed as described previously (41). β-Galactosidase assay method using *o*-nitrophenyl-β-D-galactoside as substrate and the unit definition were as described by Wang and Doi (57).

RESULTS

Suppression of the thermosensitive growth defect of the *pgsA* null mutant by disruption of the *rcsC*, *rcsF*, *yojN*, and *rcsB* genes. A *pgsA* null strain S330 lacking detectable phosphatidylglycerol and cardiolipin grows well at 30°C and 37°C but lyses when incubated at 42°C (29). Introduction of plasmid pHR693 carrying the *pgsA* gene corrected this thermosensitive defect. We sought suppressor mutations of this defect by transposon mutagenesis. Mini-Tn*10 cam* (30) was transposed randomly into the chromosome of S330 from plasmid pNK2884 by inducing the *tac* promoter-controlled transposase gene on the plasmid. Phage P1 lysates prepared from 16 independently transposition-induced cultures were used to infect S330, and chloramphenicol-resistant transductants were selected at 42°C on LB agar medium. Several transductants were picked from each independent experiment and examined for growth at 42°C in LB liquid medium. Forty-four transductants confirmed for the suppression of the thermosensitivity were analyzed for phospholipid composition. They all lacked detectable phosphatidylglycerol and cardiolipin as did S330.

We found the disruption of the *rcsC*, *rcsF*, and *yojN* genes in one, four, and one independent suppressor mutants, respectively, by cloning and sequencing of the chromosomal fragments containing the inserted minitransposons. In the *rcsC* mutant the insertion was at the 2318th nt of the 2799-nt coding sequence, and the 9 nt of 2310–2318 were duplicated. The *cam* gene was in the same orientation as that of *rcsC*. In the four *rcsF* mutants the insertion positions and orientations were the same: at the 43rd of the 399-nt coding sequence with duplication of nt 35–43, and in the orientation opposite to that of *rcsF*. In the *yojN* mutant the transposon was inserted at 1358th of 2670-nt coding sequence in the same orientation. Although the

transposition was catalyzed by altered target specificity (ATS) transposase (30), the 9-nt target sequences for these insertions matched with the consensus sequence 5′-NGCTNAGCN-3′ for the wild-type Tn*10* or mismatched only at a single AT base pair. Insertions of 1.5 kb in the *rcsC*, *rcsF*, and *yojN* genes in these suppressor mutants were also shown by PCR amplification of the respective genes.

Screening by PCR of the other 35 suppressor mutants identified 1.5-kb insertions in either of the *rcsC*, *rcsF*, or *yojN* regions in all but three mutants. In the following transduction experiments we used the mini-Tn*10 cam* insertion alleles with defined insertion positions that we first identified. Three mutants, two of which were originated from one P1 lysate, did not have insertions in the genes known to be involved in the Rcs signal transduction system and are now under investigation.

Mini-Tn*10 cam* insertions in *rcsC*, *rcsF*, and *yojN* were transduced to S330 by phage P1 again, and all the chloramphenicol-resistant transductants were thermoresistant (Fig. 1A–D). Their phospholipid composition was not altered from that of the parent strain S330 (see Fig. 3). When the *rcsF*::mini-Tn*10 cam* and *yojN*::mini-Tn*10 cam* transductants were transformed with plasmids carrying *rcsF* and *yojN*, respectively, they became thermosensitive again, indicating that the disruption of *rcsF* and *yojN* were responsible for the suppression. Introduction of an *rcsC*-carrying plasmid to the *rcsC*::mini-Tn*10 cam* transductant resulted in much poorer growth at 42°C, but not in complete thermosensitivity. In this plasmid the cloned fragment contained the putative promoter for the *rcsC* gene. Moreover, the *rcsC* gene was placed downstream of the *aadA* gene of the vector so that the transcription from the *aadA* promoter flows into *rcsC*. Although the plasmid has a low copy number, it is likely to moderately overexpress *rcsC*. When S330 was transformed with the same plasmid, it grew, albeit very poorly, at 42°C on LB agar

medium. Sensor kinase RcsC was suggested to control the activity of response regulator RcsB not only positively but also negatively presumably via dephosphorylation (3, 20, 34, 52). We supposed that the *rcsC* disruption was responsible for suppression of the thermosensitive growth while the *rcsC* overexpression only partially suppressed the thermosensitivity.

Among the suppressor mutants we isolated, none was found to have a minitransposon insertion in the *rcsB* region by PCR screening. However, when we transduced the *rcsB*::Tn*10*Δ*16*Δ*17* mutation (3) into S330, the transductants were thermoresistant on LB agar medium while remaining devoid of the major acidic phospholipids (see Fig. 3). This suppression was reversed by introduction of an *rcsB*-carrying plasmid. Thus the disruption of *rcsB* also suppresses the thermosensitivity of S330.

In these complementation experiments the *yojN* and *rcsB* genes were cloned under the *trc* promoter and expressed at the basal level in the absence of IPTG. Induction with 0.5 mM IPTG of either gene was inhibitory to the growth of S330 even at low temperatures.

Activation of the Rcs phosphorelay signal transduction system in the *pgsA* null mutant. Since disruption of the genes for the components of the Rcs phosphorelay system suppressed the thermosensitive growth defect of S330, we supposed that this signal transduction system was activated in the *pgsA* null mutant and responsible for the growth defect. The Rcs system positively regulates the *cps* genes for capsular polysaccharide synthesis (20, 52), and the *cpsB′-lac* transcriptional fusion (3, 56) provides a sensitive assay of the level of activation of the system. First we constructed a *cpsB′-lac* fusion strain in which the only functional *pgsA* gene was under control of the arabinose-regulatable P_{BAD} promoter. This strain UE46 was grown in the presence of the inducer and plated on LB agar medium containing X-Gal. In the absence of arabinose the colonies developed blue color, whereas in its presence the colony color was white,

indicating that the Rcs system was activated when expression of the *pgsA* gene was repressed.

Although the P_{BAD} promoter is tightly regulated (21), its repression may not produce the complete null phenotype, especially in the case of genes normally expressed at low levels (23). In the absence of arabinose UE46 formed minute colonies at 42°C. Hence, we constructed a *pgsA* null, *cpsB′-lac* fusion strain CL330, and used it for quantitative measurement of β-galactosidase activity. This strain was thermosensitive as was S330 and formed no colony at 42°C. Cells were grown at 30°C and assayed for the enzyme activity at 28°C as described by Wang and Doi (57). As shown in Fig. 2A the *cps* transcription in CL330 was more than ten times as high as in the *pgsA*$^+$ parent strain UE29. This transcription level was about a third of that found in SG20803 having the *rcsC137* mutation, which causes constitutive activation of the Rcs system (3, 20, 52). On LB agar medium containing X-Gal the colonies of CL330 developed blue color at 30°C and at 37°C.

Transformation with a *pgsA*-carrying plasmid and transduction of the disrupted alleles of *rcsC*, *rcsF*, and *rcsB* corrected the thermosensitive growth defect of CL330 and lowered the *cps* transcription to the wild-type level (Fig. 2B), demonstrating a correlation of activation of the Rcs phosphorelay system with the thermosensitivity. CL330 contained no detectable phosphatidylglycerol or cardiolipin as did S330, and the suppressor mutations of the thermosensitivity did not alter the phospholipid composition (Fig. 3).

The *yojN*::mini-Tn*10 cam* allele unexpectedly activated Rcs signaling at 30°C. Transduction of the *yojN*::mini-Tn*10 cam* allele into CL330 also suppressed the thermosensitive defect without suppressing the defect in the major acidic phospholipid synthesis (Fig. 3). However, the *cpsB′-lac* transcription in the transductant cells grown at 30°C was as high as that in the *rcsC137* mutant (Fig. 2C). When we examined the transductants on X-Gal-containing LB

agar medium, they formed blue colonies at 30°C, but the colony color was very pale at 37°C and white at 42°C, indicating that *yojN*::mini-Tn*10 cam* suppressed activation of the Rcs system at the high temperature restrictive for the growth of the *pgsA* null mutant. We then transduced this *yojN* allele into UE29 and found that it caused very high *cps* transcription at 30°C even in this *pgsA*$^+$ strain (Fig. 2C). When examined on X-Gal-containing agar medium, the β-galactosidase activity was very low at 37°C and almost at the wild-type level at 42°C. A similar result was found in the *yojN*::mini-Tn*10 cam* transductants of the *lpp*$^+$ parent of UE29, SG20781. Thus high *cps* expression in the cells having this *yojN* allele was irrelevant to the *pgsA* or *lpp* defect and observed only at low temperature.

Transcription of the inserted *cam* gene might flow into the downstream *rcsB* gene and increase its expression, which could result in the increased *cps* expression (3, 20). However, introduction of the *yojN*-carrying plasmid into a *yojN*::mini-Tn*10 cam* derivative of SG20781 reduced the β-galactosidase activity at 30°C to the wild-type level as examined on X-Gal-containing agar medium, indicating that the high *cps* expression was due to the *yojN* mutation, not to increase of the *rcsB* expression. Transduction of *rcsB*::Tn*10*Δ*16*Δ*17* abolished the *cps* induction, and the induction was likely due to activation of RcsB. The truncated product encoded by this transposon-inserted *yojN* allele might have an unusual function at 30°, although it was recessive to the wild type.

The unexpected activation of RcsB at 30°C was peculiar to this mini-Tn*10 cam*-inserted allele. The Δ*yojN*::*kan* allele constructed by Takeda *et al.* completely abolish Rcs signaling stimulated in response to DjlA overproduction at 30°C (55) and to glucose and zinc ion at 20°C (22). CL330 is resistant to kanamycin, and its Δ*yojN*::*kan* transductants cannot be selected. Hence we constructed a *pgsA* deletion allele having no antibiotic resistance marker and

substituted it for the *pgsA*::*kan* allele in CL330. The resultant strain CL332 showed the same phenotypes as CL330: lack of the major acidic phospholipids, the thermosensitive growth defect, and the elevated *cpsB′-lac* transcription at 30°C, all of which were complemented by the plasmid-borne *pgsA* gene. When transduced into CL332, Δ*yojN*::*kan* corrected the growth defect and lowered *cps* transcription to the wild-type level (Fig. 2D). Thus the thermosensitivity of the *pgsA* null mutant was correlated with activation of the Rcs phosphorelay system including YojN.

Capsular polysaccharide synthesis is not involved in the thermosensitivity of the *pgsA* null mutant. The Rcs phosphorelay system was activated and caused the thermosensitive growth defect in CL330 and CL332. These strains were unable to produce capsule because the *lac* fusion inactivated the *cpsB* gene and probably exerted a polar effect on the downstream *cps* genes. The *cpsB* gene is the 14th in the 19-gene *cps* operon (51). Thus the thermosensitive defect was not due to an increase in the amount of capsule. However, the first 13 genes of the operon were probably induced when the Rcs system was activated, and the imbalance in expression among the *cps* genes might lead to accumulation of a capsule biosynthetic intermediate(s) and have a deleterious effect on the bacterial growth as suggested by Brill *et al.* (3). We then examined the effect of the *cpsE*::Tn*10* mutation (53, 56). The *cpsE* gene is positioned upstream of the other genetically identified *cps* genes including *cpsB* (53), and according to the Profiling of *E. coli* Chromosome database (http://www.shigen.nig.ac.jp/ecoli/pec/) it is the first gene of the operon. When *cpsE*::Tn*10* was transduced into S330, all the transductants remained thermosensitive (Fig. 1E). We concluded that activation of the capsule synthesis pathway was not involved in the thermosensitivity of the *pgsA* null mutant.

Defective modification of membrane-derived oligosaccharides is not responsible for activation of the Rcs phosphorelay system. Phosphatidylglycerol is a substrate for phosphoglycerol modification of osmoregulated periplasmic glucans, which were hence named membrane-derived oligosaccharides (MDOs) (28). The *pgsA3* point mutation leads to low phosphatidylglycerol content and to reduced phosphoglycerol modification of MDOs (39). The *pgsA* null cells are probably devoid of phosphoglycerol-modified MDOs. Ebel *et al.* (16) reported that disruption of *mdoH* activated the Rcs system, which we confirmed in *mdoH*::Tn*10* transductants of UE29 (Fig. 2E), and we first suspected that the defective MDOs could be responsible for Rcs activation in the *pgsA* null mutant. When the *mdoH*::Tn*10* transductants were plated on LB agar medium containing X-Gal, colony color was blue at 30°C but white at 37°C and 42°C, which was in contrast to the blue color of the colonies of CL330 at 37°C. The *mdoH* mutation leads to loss of glucosyltransferase and the mutant cells lack MDOs (33); the *mdoB* mutation leads to loss of phosphoglycerol transferase and the mutant cells produce MDOs of the normal amount but lacking phosphoglycerol substitutions (26). The *pgsA* null cells are presumably defective in the latter enzyme reaction because of lack of the phosphoglycerol donor. We transduced *mdoB*::Tn*10* into UE29 and found that the loss of phosphoglycerol modification did not activate the Rcs system (Fig. 2E). We concluded that defective modification of MDOs was not responsible for activation of the Rcs system and thus for the thermosensitive growth defect of the *pgsA* null mutant.

Involvement of RcsF in activation of the Rcs phosphorelay system. RcsF protein was first reported to activate RcsB when overproduced (19) and recently to be essential for Rcs signaling in response to glucose and zinc ion at 20°C (22). In this study we showed that this protein was essential for Rcs activation in the *pgsA* null mutant. Rcs activation in the *mdoH* null

strain was also dependent on RcsF: transduction of *rcsF*::mini-Tn1*0 cam* into an *mdoH*::Tn*10* derivative of UE29 abolished high *cpsB′-lac* expression (Fig. 2E). By contrast, Rcs activation in the cells overproducing DjlA, membrane-anchored DnaJ-like protein (8, 27), was observed in the absence of RcsF. IPTG-induced expression of *djlA* cloned under a weakened derivative of the P_{trc} promoter led to elevated transcription of the *cpsB′-lac* fusion in UE29, and introduction of *rcsF*::mini-Tn1*0 cam* by P1 transduction did not lower the transcription (Fig. 2F).

We were next interested in activation of the Rcs system by a psychotropic drug chlorpromazine at sublethal concentrations (11). Chlorpromazine is a cationic amphipathic compound and potentially has an affinity to acidic phospholipids (48). When challenged by 70–90 μg/ml chlorpromazine, SG20781 showed several fold induction of the *cps* expression, whereas no induction was observed in the *rcsF*::mini-Tn1*0* transductant (Fig. 4), indicating involvement of RcsF in the stimulation of Rcs signaling by the drug. The *rcsF* transductant was slightly more sensitive to the drug than the $rcsF^+$ parent strain.

DISCUSSION

In this study we showed that the Rcs phosphorelay signal transduction system was activated in the *pgsA* null mutant lacking phosphatidylglycerol and cardiolipin and that the activation was responsible for the thermosensitive growth defect of the mutant. The growth defect could be interpreted as an indication of an essential role of these major acidic phospholipids at high temperatures. However, when the Rcs signaling was blocked by disruption of the *rcsC*, *rcsF*, *yojN*, and *rcsB* genes, the *pgsA* null mutant became thermoresistant while remaining devoid of these phospholipids, indicating dispensability of the major acidic phospholipids even at high temperatures. Acidic phospholipids are considered to be required for essential cellular functions including recruitment of peripheral membrane proteins to the negatively charged membrane surface (14, 35). Minimum requirements for such functions can probably be fulfilled by acidic intermediates, phosphatidic acid and CDP-diacylglycerol, which are accumulated, albeit in small amounts, in the *pgsA* null mutant (29, 35, 54).

The disrupted alleles of *rcsF*, *rcsC*, and *yojN*. The suppressor mutations of the thermosensitivity were isolated by minitransposon mutagenesis. In the *rcsF*::mini-Tn*10 cam* allele five codons of unnatural sequence followed by two consecutive termination codons were introduced after the 13th codon, and thus this interrupted *rcsF* is a null allele. In the *rcsC*::mini-Tn*10 cam* allele two consecutive termination codons were introduced just after the 773rd codon. This mutation may leave intact the kinase domain including the active center His-463 (9, 20, 52, 55), although we do not know whether the truncated product is stable in the cells. Even if it is stable, it is devoid of the receiver domain including Asp-859 (9, 55), and loss of Rcs signaling due to this mutation can be taken as an indication of importance of the receiver domain in the

phosphorelay (9).

In the *yojN*::mini-Tn*10 cam* allele two consecutive termination codons were introduced just after the 453rd codon. The truncated product lacks the C-terminal phosphotransmitter domain including His-842 that is essential for the phosphorelay (55). Surprisingly this mutation stimulated the *cps* transcription in the wild-type background in the apparent absence of an external stimulus at 30°C. The truncated product may be stable in the cells and manifest the unusual phenotype at low temperatures. This phenotype was dependent on the intact *rcsB* gene, indicating involvement of activation of RcsB. The Rcs signal transduction system is postulated to relay a phosphoryl group from the autophosphorylated kinase domain of the sensor protein RcsC to its receiver domain, then to the YojN transmitter domain, and finally to the RcsB receiver domain to activate this response regulator (9, 20, 52, 55). In the presence of the transmitter domain-truncated YojN encoded by the minitransposon-inserted allele identified in this study, however, RcsB may be activated by direct phosphotransfer from the RcsC kinase domain. Although the YojN truncation is artificial, a natural occurrence of a similar shortcut phosphotransfer in a multistep phosphorelay system has been reported for the Arc signal transduction system (36). YojN protein has a structural similarity to RcsC except that the His residue to be autophosphorylated is missing, and has been suggested to form a heteromeric complex with RcsC in the cytoplasmic membrane (55). Truncation of YojN after residue 453 may not completely impair its ability to interact with RcsC, and RcsC complexed with the truncated YojN may acquire an unusual activity of autophosphorylation without an external stimulus and of direct phosphotransfer to RcsB from its kinase domain. Probably the truncated YojN is weaker in interaction with RcsC than the wild-type protein and displaced from the complex in the presence of the wild type. Thus, a plasmid-borne wild-type *yojN* corrected the

unusual phenotype of *yojN*::mini-Tn*10 cam* at 30°C. At higher temperatures the truncated YojN is likely to be unstable, and the phenotype disappeared at 42°C.

In our minitransposon mutagenesis experiments, suppressor mutations were isolated repeatedly in the *rcsC*, *rcsF*, and *yojN* genes, but none in the *rcsB* gene. However, transposon-inserted *rcsB* (3) suppressed the thermosensitivity of the *pgsA* null mutant. Thus our screening for the suppressors has not reached a saturating level, and further screening can be expected to lead to identification of a gene(s) for an unknown factor(s) involved in the regulation of Rcs signaling or of a gene(s) whose Rcs-induced expression is responsible the thermosensitive defect.

Rcs activation is responsible for the thermosensitive growth defect. Suppression of activation of the Rcs signal transduction system and of the thermosensitive growth defect by disruption of the *rcs* genes were well correlated in the *pgsA* null mutant, indicating the responsibility of Rcs activation for the defect. In the case of *yojN*, the deletion allele constructed by Takeda *et al.* (55) showed clear-cut correlation. Although the *yojN*::mini-Tn*10 cam* allele that we isolated showed an unusual Rcs-activating phenotype at 30°C, it blocked activation of the Rcs system at high temperatures and suppressed the thermosensitivity.

Although the Rcs system positively regulates expression of the *cps* genes, capsular polysaccharide synthesis is not involved in the growth defect as revealed by inability of the *cpsB* or *cpsE* disruption to suppress the defect. In addition to the *cps* genes and several other genes whose regulation by the Rcs system was fairly well investigated (5, 10, 13, 15, 18, 34), many genes were recently identified as putative Rcs regulon members (17, 22, 43). Some are positively and the others are negatively regulated. It is yet to be elucidated which of the members is directly responsible for the thermosensitivity of the *pgsA* null mutant. The responsible gene(s) might be found among suppressor mutations or multicopy suppressors.

Altered gene expression under the activated Rcs signaling causes the growth defect only in the absence of the major acidic phospholipids. In the *pgsA*$^+$ background Rcs activation does not cause the defect. SG20803, an *rcsC137 cpsB′-lac* fusion strain (3), grew well and formed blue colonies on X-Gal-containing agar medium at 42°C. Although overproduced DjlA is toxic in a *pgsA*$^+$ strain even at low temperatures (7), the toxic effect cannot be ascribed solely to activation of the Rcs system, because in our preliminary experiment the growth inhibition due to overexpression of *djlA* cloned under a strong inducible promoter P_{trc} was not suppressed by disruption of *rcsC* or *yojN*.

The growth defect was observed only at high temperatures. The *pgsA* null mutant may be vulnerable to an adverse effect of Rcs activation at high temperatures only. Alternatively, the stimulus to the Rcs system in the *pgsA* null mutant may be stronger at higher temperatures, causing higher Rcs activation. At 30°C the system was not fully activated as compared with the activation in the *rcsC137* mutant, and overexpression of *rcsF* (Y. Shiba, K. Matsumoto, and H. Hara, unpublished data), *yojN*, or *rcsB* (this study), which would further stimulate Rcs signaling, caused a growth defect even at 30°C.

Envelope stresses that activate the Rcs signal transduction system. The Rcs signal transduction system is activated in response to environmental stresses such as desiccation (42), osmotic shock (49), treatment with chlorpromazine (11), and during growth in the presence of glucose and zinc ion at 20°C (22) and on a solid surface (17). Thus the system is implicated in survival outside of a mammalian host (52) and in biofilm formation (17). Genetic modifications leading to a defect in lipopolysaccharides (45), overproduction of DjlA (8, 27), and lack of MDOs (16) also activate the Rcs system. Lack of phosphoglycerol substitutions in MDOs was not the cause of Rcs activation in the *pgsA* null mutant because a defect of the phosphoglycerol

transferase MdoB had no stimulatory effect on the Rcs system. The environmental and genetic conditions that activate the Rcs system can be regarded as perturbing the cell envelope. Lack of the major acidic phospholipids in the *pgsA* null mutant is another example of such an envelope stress. It greatly decreases the negative charge on the membrane surfaces, which could affect the functions of peripheral and integral membrane proteins (14, 35). Decrease in the negative charge of MDOs because of the loss of phosphoglycerol residues could also affect periplasmic and membrane proteins (28).

Mileykovskaya and Dowhan (37) reported that the Cpx phosphorelay signal transduction system was activated in the *pssA* null mutant, which completely lacks the major zwitterionic phospholipid, phosphatidylethanolamine, and contains the major acidic phospholipids in its place (14). We suspected that this dramatic alteration in phospholipid composition could also be an envelope stress to activate the Rcs phosphorelay system, but the *pssA* null mutation did not activate the Rcs system (A. Ito, K. Matsumoto, and H. Hara, unpublished data). Besides zwitterionic versus anionic head groups, phosphatidylethanolamine and phosphatidylglycerol have distinct structural features: phosphatidylethanolamine has a propensity to form a nonbilayer phase owing to its small polar head group, whereas phosphatidylglycerol prefers to form a bilayer phase. Cardiolipin also has a nonbilayer-forming propensity, but only in the presence of high concentrations of divalent cations (14). Intrinsic curvature of the membrane with higher content of nonbilayer-forming lipids would result in lower packing density of head groups at the membrane surface. The *pssA* null mutant lacks nonbilayer-forming phosphatidylethanolamine, whereas the *pgsA* null mutant has a higher phosphatidylethanolamine content than the wild type (29). The physical properties of the membranes of the *pgsA* and *pssA* null mutants might produce distinct responses of the Rcs and

Cpx phosphorelay systems. Activation of the Rcs system by treatment with chlorpromazine can also be ascribed to the altered physical properties of the membrane (11). This cationic amphipathic compound intercalates into the lipid bilayer and induces a curvature stress (48).

The Cpx signal transduction system is implicated in biofilm formation as is the Rcs system. The Cpx system is activated upon attachment to abiotic surfaces and the mutants defective in the system show a decreased level of attachment (44). The Cpx activation occurs immediately upon attachment probably in the initial stage of biofilm formation. In contrast, the Rcs system is induced during growth on a solid surface and affects the cell surface composition, and is required for maturation of the biofilm (17). Along with development of the biofilm, cell-to-surface and cell-to-cell interactions may modulate the physical property of the cell membrane, thereby eliciting responses of the phosphorelay systems appropriate for the biofilm developmental stages.

Involvement of RcsF in Rcs signaling. RcsF is an essential component of the Rcs signal transduction system when it responds to glucose and zinc ion at 20°C (22), lack of the major acidic phospholipids, lack of MDOs, and treatment with chlorpromazine. RcsF is predicted to be a lipoprotein located in the outer membrane because it has a lipobox sequence at the end of the putative signal peptide, followed by a Ser residue (59). The stresses that stimulate Rcs signaling might affect the physical property of the outer membrane and be sensed by RcsF. Among the Rcs-stimulating stresses that we examined, overproduction of DjlA was the only example that was not dependent on RcsF. This is reasonable considering that DjlA is a cytoplasmic membrane protein whose major part is in the cytoplasmic side (7). The overproduced DjlA presumably interacts with RcsC to activate the signal transduction.

RcsF was first identified as a protein whose overproduction stimulated Rcs signaling (19).

In the Cpx signal transduction system overproduction of a minor outer membrane lipoprotein NlpE has a stimulatory effect (50). While this protein is not involved in Cpx activation in the *pssA* null mutant (37), it plays an essential role in the response to attachment to abiotic surfaces (44). It was suggested that in this response NlpE senses a stress in the outer membrane and mediates the signal to the sensor kinase CpxA in the cytoplasmic membrane (44). RcsF is likely to play a similar role in Rcs activation.

For the *pgsA* null mutant there is another possible explanation as to how RcsF is involved in Rcs activation. Phosphatidylglycerol missing in the mutant normally serves as a diacylglycerol donor for modification of the lipobox Cys residue, which is prerequisite for maturation of lipoproteins (59). Phosphatidic acid and CDP-diacylglycerol that the mutant accumulates in small amounts can also serve as the donor, but only inefficiently (47). When the major outer membrane lipoprotein Lpp was expressed in the mutant, the product was poorly modified and the precursor form remained in the cytoplasmic membrane (54). RcsF may be also retarded in maturation and thus in localization to the outer membrane, which may lead to stimulation of RcsC in the cytoplasmic membrane. But we do not know how much this quantitatively minor lipoprotein is affected by the lack of phosphatidylglycerol.

A complete lack of a particular lipid species in the membrane is an artificial situation for wild-type bacteria. However, alteration of the properties of the membrane due to the altered lipid composition may mimic envelope perturbation brought about by environmental stresses, as we speculated for the *pgsA* and *pssA* null mutants of *E. coli*. We expect that the mutants defective in lipid synthesizing enzymes can be useful models for elucidation of how phosphorelay signal transduction systems and other stress-responsive systems sense and respond to the stresses exerted on the envelope.

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Legends to Figures

FIG. 1. Suppression of the thermosensitive growth defect of the *pgsA* null mutant. The minitransposon insertions in *rcsC*, *rcsF*, and *yojN* identified in the screening for suppressor mutants were transduced by phage P1 to S330 again. Overnight cultures of S330 (A) and its *rcsC*::mini-Tn*10 cam* (B), *rcsF*::mini-Tn*10 cam* (C), *yojN*::mini-Tn*10 cam* (D), and *cpsE*::Tn*10* (E) transductants were diluted to 3–5 Klett units in LB medium and grown at 37°C (open circles) and at 42°C (closed circles), and the culture turbidities were monitored.

FIG. 2. Activation of the Rcs phosphorelay signal transduction system in the *pgsA* null mutant. Cells were grown in buffered LB medium at 30°C to mid-exponential phase (*ca.* 100 Klett units) and measured for β-galactosidase activity. The means and standard errors of at least three measurements are shown. The mutations transduced into UE29, CL330 or CL332 were *rcsC*::mini-Tn*10 cam* (*rcsC*), *rcsF*::mini-Tn*10 cam* (*rcsF*), *rcsB*::Tn*10*Δ*16*Δ*17* (*rcsB*), *yojN*::mini-Tn*10 cam* (*yojN*), Δ*yojN*::*kan* (Δ*yojN*), *mdoH*::Tn*10* (*mdoH*), and *mdoB*::Tn*10* (*mdoB*). Three transductants were isolated and examined. They exhibited the essentially the same results. In (F), cells were grown in the presence of 1 mM IPTG.

FIG. 3. The suppressor mutations of the thermosensitivity of the *pgsA* null mutant do not suppress the loss of phosphatidylglycerol and cardiolipin. UE29 (lane 1), CL330 (lane 2), and its *rcsC*::mini-Tn*10 cam* (lane 3), *rcsF*::mini-Tn*10 cam* (lane 4), *yojN*::mini-Tn*10 cam* (lane 5), and *rcsB*::Tn*10*Δ*16*Δ*17* (lane 6) transductants were grown to mid-exponential phase (*ca.* 100 Klett units) in LB medium. Lipids were extracted and separated by thin layer chromatography,

and spots of phospholipids were visualized with Dittmer-Lester reagent. F, solvent front; CL, cardiolipin; PG, phosphatidylglycerol; PE, phosphatidylethanolamine; O, chromatographic origin. Faint spots at the position between CL and PG in lanes 2–6 are phosphatidic acid, which is accumulated in a small amount in *pgsA* null cells (29).

FIG. 4. RcsF is essential for the Rcs signal transduction in response to treatment with chlorpromazine. The experimental procedure was essentially as described by Conter *et al.* (11). A *cpsB′-lac* fusion strain SG20781 (circles) and its *rcsF*::mini-Tn*10 cam* transductant (squares) were grown in NaCl-free LB medium to *ca.* 25 Klett units at 37°C, and chlorpromazine was added to the indicated concentrations. After 2 h turbidity (open symbols) and β-galactosidase activity (closed symbols) were measured. The turbidity was normalized to the value of the culture that was not treated with the drug for each strain (*ca.* 200 Klett units) and is shown in percentage.

TABLE 1. Bacterial strains and plasmids used in this study

Strains or plasmids	Relevant genotype or description	Construction, source or reference
Strains		
S301	W3110 *ksgB1 lpp-2*[a]	29
S330	S301 *pgsA30*::*kan*	29
SG20043	MC4100 Δ*lon-100 cpsE3*::Tn*10*	53, 56, NIG[b] collection
SG20781	MC4100 *cpsB10*::*lac*-Mu-*imm*λ	3
SG20797	MC4100 *cpsB10*::*lac*-Mu-*imm*λ Δ*lon-510 rcsB11*::Tn*10*Δ*16*Δ*17*	3
SG20803	SG20781 *rcsC137 ompC*::Tn*5*	3
UE29	SG20781 *lpp-2*	This study
UE44	SG20781 *lpp-2* Δ*ara714*[c]	This study
DHB6501	$\lambda^- \lambda^s$ *supF58*	2
UE38	DHB6501 Δ(λ*attL-lom*)::(*bla araC* P_{BAD}-*pgsA*)	λ InCh procedure (2) using pHR702
UE35	MG1655 Δ*ara714*	This study
UE39	MG1655 Δ*ara714* Δ(λ*attL-lom*)::(*bla araC* P_{BAD}-*pgsA*)	P1(UE38) × UE35
UE42[d]	MG1655 Δ*ara714* Δ(λ*attL-lom*)::(*bla araC* P_{BAD}-*pgsA*) *pgsA30*::*kan*	P1(S330) × UE39
UE45	SG20781 *lpp-2* Δ*ara714* Δ(λ*attL-lom*)::(*bla araC* P_{BAD}-*pgsA*)	P1(UE42) × UE44
UE46	SG20781 *lpp-2* Δ*ara714* Δ(λ*attL-lom*)::(*bla araC* P_{BAD}-*pgsA*) *pgsA30*::*kan*	P1(UE42) × UE45
MDL12	*pgsA30*::*kan* Φ(*lacOP-pgsA*$^+$)*1 lacZ lacY*::Tn*9*	60

CL330	SG20781 *lpp-2 pgsA30*::*kan*	P1(MDL12) × UE29
UE54	MG1655 *lpp-2* Δ*ara714 rcsF*::mini-Tn*10 cam* Δ*pgsA*::FRT-*kan*-FRT	λ Red recombinase procedure (12)
UE60[d]	MG1655 Δ*ara714* Δ(λ*attL-lom*)::(*bla araC* P_{BAD}-*pgsA*) Δ*pgsA*::FRT-*kan*-FRT	P1(UE54) × UE39
UE61[d]	MG1655 Δ*ara714* Δ(λ*attL-lom*)::(*bla araC* P_{BAD}-*pgsA*) Δ*pgsA*::FRT	Yeast FLP recombinase procedure (12)
YK4516	*fliC*::Tn*10*	NIG collection
UE62[d]	MG1655 Δ*ara714* Δ(λ*attL-lom*)::(*bla araC* P_{BAD}-*pgsA*) Δ*pgsA*::FRT *fliC*::Tn*10*	P1(YK4516) × UE61
CL332	SG20781 *lpp-2* Δ*pgsA*::FRT *fliC*::Tn*10*	P1(UE62) × CL330
ST261	Δ*yojN*::*kan*	55
NFB216	*mdoH200*::Tn*10*	33
NFB732	*mdoB214*::Tn*10*	32
Plasmids[e]		
pNK2884	pBR322 derivative, mini-Tn*10 cam* P_{tac}-ATS transposase[f]	30, ATCC
pGB2	pSC101 derivative, Spc^r	6
pHR693	pGB2 *pgsA*	This study
p*rcsC*	pGB2 *rcsC*	This study
p*rcsF*	pGB2 *rcsF*	This study
pHR718	pGB2 *lacI*^{q} P_{trc}	This study
pHR719	pGB2 *lacI*^{q} P_{204} (down mutation in the –35 region of P_{trc})	This study
pHR722	pGB2 *lacI*^{q} P_{trc}-*rcsB*	This study
pHR737	pGB2 *lacI*^{q} P_{trc}-*yojN*	This study

pHR741	pGB2 *lacI*^{q} P_{204}-*djlA*	This study
pBAD24	pBR322 derivative, *araC* P_{BAD}, Ap^{r}	21
pHR702	pBAD24 *pgsA*	This study

[a] Formerly called *lpo-5508*.

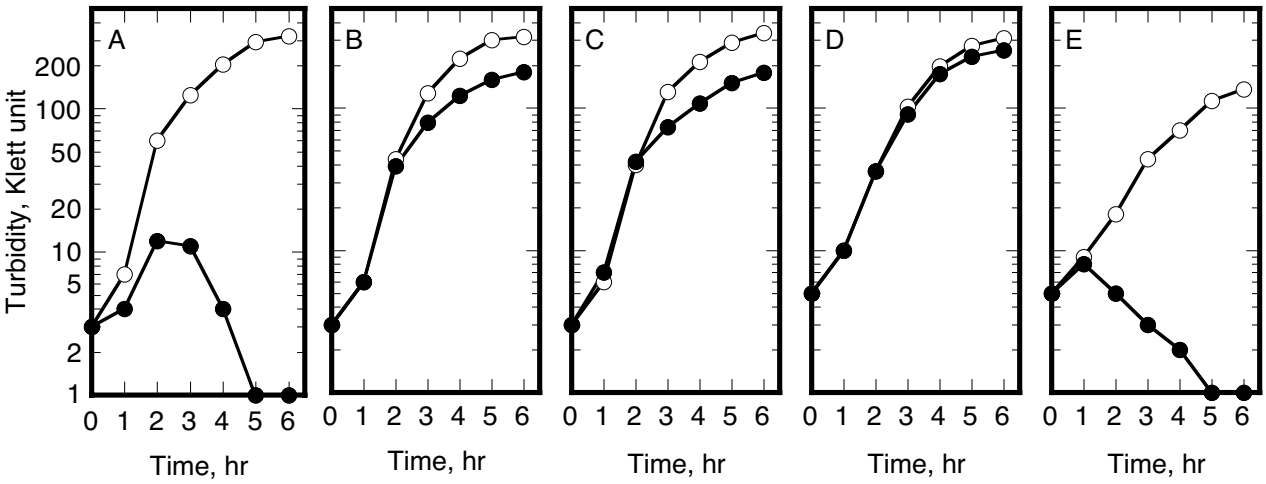
[b] NIG, National Institute of Genetics, Mishima, Japan.

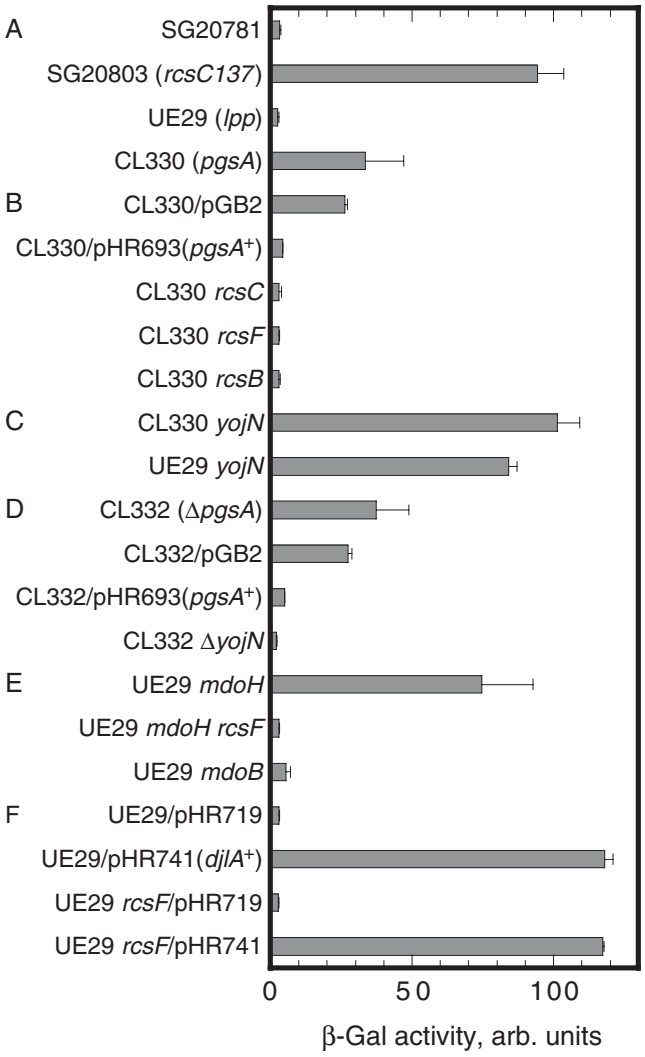
[c] SG20781, a derivative of MC4100, is sensitive to L-arabinose because of the *araD139* mutation. Its Δ*ara714* derivatives are not sensitive to L-arabinose.

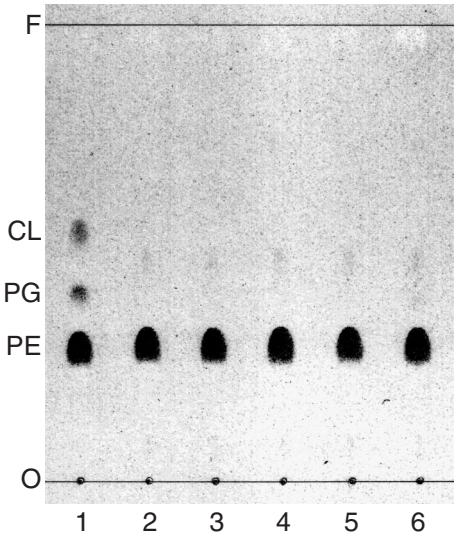
[d] These strains grow only in the presence of L-arabinose which induces transcription from the P_{BAD} promoter.

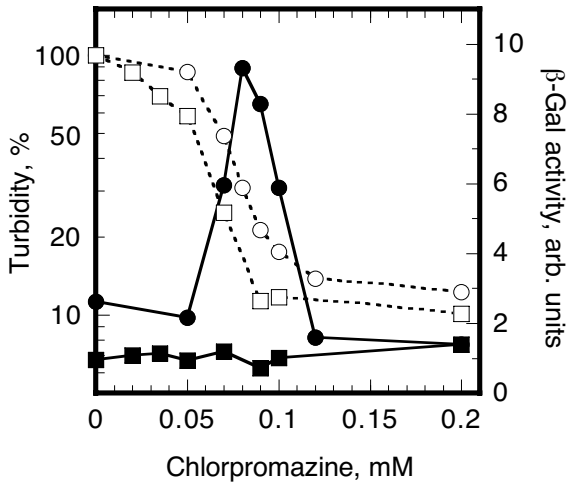
[e] For construction of the plasmids, see Materials and Methods.

[f] ATS transposase, altered target specificity transposase (30).


A
B
C
D
E
Turbidity, Klett unit
200
100
50
20
10
5
2
1
0 1 2 3 4 5 6
Time, hr


A
SG20781
SG20803 (rcsC137)
UE29 (lpp)
CL330 (pgsA)
B
CL330/pGB2
CL330/pHR693(pgsA+)
CL330 rcsC
CL330 rcsF
CL330 rcsB
C
CL330 yojN
UE29 yojN
D
CL332 (ΔpgsA)
CL332/pGB2
CL332/pHR693(pgsA+)
CL332 ΔyojN
E
UE29 mdoH
UE29 mdoH rcsF
UE29 mdoB
F
UE29/pHR719
UE29/pHR741(djlA+)
UE29 rcsF/pHR719
UE29 rcsF/pHR741
0
50
100
β-Gal activity, arb. units


F
CL
PG
PE
O
1
2
3
4
5
6


100
50
20
10
Turbidity, %
10
8
6
4
2
0
β-Gal activity, arb. units
0
0.05
0.1
0.15
0.2
Chlorpromazine, mM