RcsA-dependent and -independent growth defects caused by the activated Rcs phosphorelay system in the *Escherichia coli pgsA* null mutant

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In the *Escherichia coli pgsA* null mutant, which lacks the major acidic phospholipids, the Rcs phosphorelay signal transduction system is activated, causing thermosensitive growth. The mutant grows poorly at 37°C and lyses at 42°C. We showed that the poor growth at 37°C was corrected by disruption of the *rcsA* gene, which codes for a coregulator protein that interacts with the RcsB response regulator of the phosphorelay system. However, mutant cells still lysed when incubated at 42°C even in the absence of RcsA. We conclude that the activated Rcs phosphorelay in the *pgsA* null mutant has both RcsA-dependent and -independent growth inhibitory effects. Since the Rcs system has been shown to positively regulate the essential cell division genes *ftsA* and *ftsZ* independently of RcsA, we measured cellular levels of the FtsZ protein, but found that the growth defect of the mutant at 42°C did not involve a change in the level of this protein.

Key words—*Escherichia coli*; FtsZ; *pgsA*; phospholipid; phosphorelay; RcsA; signal transduction

Introduction

The *Escherichia coli pgsA* null mutant defective in phosphatidylglycerophosphate synthase and thus 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 (Kikuchi et al., 2000; Matsumoto, 2001; Suzuki et al., 2002). It is likely that the requirement for acidic phospholipids in the membrane is fulfilled by the acidic intermediates, phosphatidic acid and CDP-diacylglycerol, which are accumulated in this mutant (Matsumoto, 2001). However, the Rcs phosphorelay system is constitutively activated and leads the mutant cells to lyse at 42°C (Kikuchi et al., 2000; Shiba et al., 2004).

Phosphorelay signal transduction is a major mechanism by which bacteria sense and respond to environmental stresses. The Rcs phosphorelay system of *E. coli*, initially described as positively regulating the capsular polysaccharide synthetic (*cps*) genes (Stout, 1994), is implicated in survival outside of a mammalian host (Stout, 1994) and in biofilm development (Ferrières and Clarke, 2003). Environmental stimuli are probably sensed by RcsF, a minor outer membrane lipoprotein (Hagiwara et al., 2003; Shiba et al., 2004), and mediated to sensor kinase RcsC in the inner membrane (Stout, 1994), which activates the response regulator RcsB by phosphorelay via phosphotransmitter YojN (Takeda et al., 2001). The RcsA protein is not a component of the phosphorelay but functions as a coregulator of the *cps* transcription by forming a heterodimer with phosphorylated RcsB (Wehland and Bernhard, 2000). Experiments using *lacZ* operon fusion have identified *tolQRA*, *rcsA*, *ftsAZ*, *osmC*, *rprA* and *flhDC* as the targets regulated by the Rcs system. RcsA is involved in the regulation of its own gene and *flhDC* but not of the others, for which the RcsB homodimer is a likely transcriptional regulator. Transcriptome analysis has shown that many other genes are under the direct or indirect control of the Rcs system (Ferrières and Clarke, 2003; Hagiwara et al., 2003; Oshima et al., 2002). Whether RcsA is also involved in their control is not known.

Inactivation of any one of the Rcs phosphorelay components blocks cell lysis at 42°C of *pgsA* null strains (Shiba et al., 2004), but which of the genes under control of the Rcs system are responsible for the thermosensitivity is not known. In this study we examined whether RcsA is involved in the control of the responsible gene(s), and unexpectedly found that S330, the first

pgsA null strain constructed in our laboratory (Kikuchi et al., 2000), had a spontaneous insertion mutation in the *rcsA* gene. An *rcsA*$^{+}$ *pgsA* null strain grew well at 30°C but poorly at 37°C. Disruption of the *rcsA* gene corrected the thermosensitivity at 37°C but did not prevent lysis at 42°C.

Materials and Methods

Bacterial strains, plasmids and culture media. The *E. coli* K-12 derivative strains and the plasmids used are listed in Table 1. The *rcsA*::*cam* allele, in which the chloramphenicol resistance (*cam*) gene of pACYC184 was inserted into the *Eco*RV site within the *rcsA* gene, was first constructed on the plasmid pHR704, which carries the 4.4-kb *Bam*HI fragment of λ clone 344 of the Kohara miniset library (Kohara et al., 1987) containing *rcsA*, and then crossed into the chromosome as described (Hara et al., 1997).

In pHR721 the PCR-amplified *rcsB* fragment starting from the second codon was connected to the filled-in *Nco*I site of the vector. The plasmid pHR758 was constructed by replacing the ampicillin resistance gene of pFZY1 (Koop et al., 1987) with the kanamycin resistance gene of pKC7 (Rao and Rogers, 1979). The 0.5 kb *Hin*cII fragment containing the promoter for the *cps* operon (P_{cps}) (Stout, 1996; Wehland and Bernhard, 2000) and the 1 kb *Bam*HI-*Bgl*II fragment containing the promoter for *ftsA* and *ftsZ* (P_{ftsAZ}) (Carballès et al., 1999) were cloned into pHR758 to construct pHR770 and pHR771, respectively.

Bacteria were grown in LB medium. Antibiotics were used at the following concentrations (μg ml^{-1}) 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 pGB2 and its derivatives). Cell growth was monitored with a Klett-Summerson colorimeter (no. 54 filter) or with a microbial growth recorder BioScanner 16MS (Ohtake Works) at 650 nm.

Genetic and biochemical procedures. PCR and sequencing primers were: 5′-AGTAAGTCCGCCTGCAGAAT-3′ and 5′-GTGGAGAATTCAGAGTAATC-3′ for *rcsA*; 5′-AACAATATGAACGTAATTATTGCCGATGACC-3′ and 5′-GCGGATCCTACAGGTGATTAGTCTTTATCTGCCGG-3′ for *rcsB*.

For estimation of the cellular level of FtsZ protein, whole cell extracts were separated by sodium dodecylsulfate (SDS)-gel electrophoresis with 10% polyacrylamide, and FtsZ was detected by Western immunoblotting using rabbit anti-FtsZ antiserum, which was a gift from Kengo Kanamaru of Kobe University.

The β-galactosidase assay was done in triplicate essentially as described (Koop et al., 1987). For samples with low activities, the content of permeabilized cells in the reaction mixture and reaction time were increased to up to 0.12 mg protein ml^{-1} and 120 min, respectively, to give an OD_{420} increase higher than 0.12 during the reaction. The activities were corrected for the background activities that were produced by the promoterless *lac* of the vector pHR758 in the same strains and under the same growth conditions. The results are expressed as μmol *o*-nitrophenol min^{-1} (g whole cell protein)$^{-1}$, and means and standard deviations are presented.

Results and Discussion

Disruption of rcsA does not suppress cell lysis of the pgsA null mutant at 42°C.

In our previous search for the suppressor mutations that correct the thermosensitivity of S330, which grows well at 30°C and 37°C but lyses when incubated at 42°C (Kikuchi et al., 2000), *rcsA* was not found, but the screening may not have reached a saturating level (Shiba et al., 2004). To test whether RcsA is involved in the thermosensitivity, we constructed an *rcsA*::*cam* allele and introduced it into S330 by cotransduction with *fliC*::Tn*10*. Since *pgsA* is also cotransducible with *fliC*, tetracycline-resistant transductants that remained kanamycin resistant were selected. All the transductants were thermosensitive at 42°C, irrespective of whether they received the chloramphenicol resistance or not.

When we examined the *rcsA* alleles of the transductants and their parents by PCR, the transductional donor UE33 and the chloramphenicol-resistant transductants gave a PCR product longer than the wild-type allele by 1.3-kb, the size of the inserted *cam* fragment (Fig. 1, lanes 2, 4, 8, and 10), and unexpectedly the recipient S330 and chloramphenicol-sensitive transductants were found to give a 1.2-kb longer product (Fig. 1, lanes 6, 7, and 9). Sequencing of the PCR-amplified fragment from S330 revealed the insertion of IS*5* at the 191st nucleotide (nt) of the 621-nt *rcsA* coding sequence with duplication of the 4 nt from nt 188 to 191. The orientation of the transposase gene was opposite to that of *rcsA*. Both *rcsA*::*cam* and *rcsA*::IS*5* are null alleles, whose products lack the C-terminal DNA-binding domain (Stout et al., 1991). Actually, neither S330 nor its *rcsA*::*cam* transductants were mucoid, despite the activation of the Rcs system in the *pgsA* null mutant (Shiba et al., 2004). These results indicate that RcsA is not involved in the cell lysis at 42°C of the *pgsA* null mutant.

Disruption of rcsA in the pgsA null mutant leads to better growth at 37°.

S330 was constructed by P1 transduction of the *pgsA*::*kan* allele from MDL12 (Xia and Dowhan, 1995) to S301 (Kikuchi et al., 2000), an *lpp*-defective derivative of W3110, which is wild type for *rcsA*. We suspected that the *rcsA*::IS*5* mutation arose spontaneously during the strain construction. We did the same transduction experiment again, but at 30°C, not at 37°C at

which S330 was constructed. All the kanamycin-resistant transductants were mucoid, indicating that they were *rcsA*$^+$. All showed a growth defect even at 37°C, growing poorly at this temperature and forming small colonies (Fig. 2, YS330). In LB medium the growth rate was 1.3 doublings h^{-1}, considerably lower than that of S301 (2.1 doublings h^{-1}). Incubation at 42°C resulted in cell lysis as it did in S330 (Kikuchi et al., 2000). We transduced the *rcsA*::*cam* mutation into one of these *rcsA*$^+$ *lpp pgsA*::*kan* strains, YS330. The resultant transductants were not mucoid and showed the same growth phenotype as S330: they grew well at 30°C and 37°C but lysed at 42°C (Fig. 2). When an *rcsA*-carrying plasmid, p*rcsA*, was introduced into the *rcsA*::*cam* transductant and into S330, their phenotype returned to that of YS330: mucoid and defective in growth even at 37°C. Transduction of the *rcsF*::mini-Tn*10 cam* (Fig. 2), *rcsC*::mini-Tn*10 cam*, and *rcsB*::Tn*10*Δ*16*Δ*17* mutations (not shown) into YS330, resulting in non-mucoid phenotype, corrected not only the cell lysis at 42°C, as had been observed for S330 (Shiba et al., 2004), but also the poor growth at 37°C. Transduction of the Δ*yojN*::*kan* (Takeda et al., 2001) mutation into a Δ*pgsA rcsA*$^+$ strain CL332 (Shiba et al., 2004) also corrected its poor growth at 37°C (Shiba, Y., Matsumoto, K., and Hara, H., unpubl.). These results indicate that the poor growth was due to activation of the Rcs signaling.

Thus the activated Rcs signaling in the *pgsA* null mutant (Shiba et al., 2004) causes dual growth defects, RcsA-dependent and -independent. A gene(s) under the control of the RcsA-RcsB heterodimer is involved in the growth defect at temperatures over 37°C, and a gene(s) under the control of the RcsB homodimer is responsible for the lysis phenotype at 42°C.

At present it is not known which of the Rcs-regulated genes are directly responsible for the RcsA-dependent and -independent growth defects of the *pgsA* null mutant. In addition to several genes intensively examined for their regulation by the Rcs system, recent transcriptome analyses (Ferrières and Clarke, 2003; Hagiwara et al., 2003; Oshima et al., 2002) have revealed that many genes are affected by the system. The effect of RcsA on gene expression, however, has not been examined systematically. Increased capsule synthesis due to the induction of the *cps* operon by the RcsAB heterodimer (Stout, 1994) is not involved in the RcsA-dependent defect: the *cpsE*::Tn*10* transductants of YS330 were not mucoid and showed even poorer growth than YS330 at 37°C (Fig. 2).

The pgsA null mutant incubated at 37°C acquires rcsA mutations.

Prolonged incubation of YS330 at 37°C gave rise to faster growing, non-mucoid derivatives, most of which were still thermosensitive at 42°C. Many of these suppressor mutants had insertion mutations in the *rcsA* gene according to PCR analysis. We sequenced four of these newly isolated *rcsA* alleles, and identified IS*5* insertions at nt 49 and 191 (the transposase gene was in the same orientation as *rcsA* in these two alleles) and IS*1* insertions at nt 274 and 620 (the orientation of the transposase gene was the same in the former and the opposite in the latter). The IS*1* insertion at nt 620 was interesting in that it was at the last nucleotide but one of the *rcsA* coding sequence, causing an extension by only 6 residues after the normal C terminus. All of the other 21 insertion alleles of *rcsA* that we examined seemed to contain IS*5*, judging from the fragment sizes of the PCR products digested with *Eco*T14I, which cuts IS*5* at 4 sites. It is not known why suppressor mutations in other *rcs* genes, which should have conferred thermoresistance at 42°C, were less frequent than the mutations in *rcsA*.

It seems that the *rcsA*::IS*5* mutation in S330 has been similarly acquired and selected for during or just after the transduction procedure of Kikuchi et al. who constructed the strain at 37°C (Kikuchi et al., 2000). The *rcsA*$^+$ *pgsA* null cells do not lyse but remain viable at 37°C, and acquisition of an *rcsA* mutation leads to formation of normal-sized colonies (see Fig. 2). This is a possible explanation for the low frequency of P1 transduction of *pgsA*::*kan* into *lpp* strains at 37°C, which was about an eighth of the frequency of the internal control marker *lacY*::Tn*9* (Kikuchi et al., 2000). In contrast, lysis at 42°C would have precluded the *pgsA*::*kan lpp* mutant from easily acquiring a suppressor mutation in *rcsF*, *rcsC*, *yojN*, or *rcsB* during a trial to cure a replication-thermosensitive, *pgsA*-carrying plasmid by elevating the growth temperature to 42°C (Xia and Dowhan, 1995).

The thermosensitive defect of the pgsA null mutant at 42°C does not involve a change in the level of FtsZ protein.

Among the Rcs-regulated genes, only *ftsA* and *ftsZ* have been proven to serve an indispensable function. Their products are pivotal components of the cell division apparatus

(Buddelmeijer and Beckwith, 2002). They are the last two genes but one in the 16-gene cluster of cell division/cell envelope biosynthesis genes, which has a complex transcriptional organization with multiple promoters (Joseleau-Petit et al., 1999). Among them, a promoter located just upstream of *ftsA*, P_{ftsAZ}, has been shown to be positively regulated by RcsB, independently of RcsA (Carballès et al., 1999; Gervais and Drapeau, 1992). We initially suspected that *ftsAZ* hyperexpression due to the Rcs activation might be responsible for the growth defect of the *pgsA* null strain at 42°C. It should not be directly responsible for the growth defect at 37°C, which was dependent on RcsA.

We examined if the Rcs activation in the *pgsA* null mutant resulted in an increased level of the FtsZ protein. Immunoblot analysis using anti-FtsZ serum showed that the level of FtsZ in the *pgsA* null strain S330 grown at 30°C (Fig. 3) and 37°C (not shown) was the same as in the $pgsA^+$ parent strain S301. To test a possibility of higher *ftsZ* expression at higher temperature, we shifted the mid-exponential phase culture of S330 from 30°C to 42°C and continued incubation until the turbidity tripled. After the incubation no difference in the amount of FtsZ was observed (Fig. 3). The level of FtsZ was also unchanged also in the *rcsC*::mini-Tn*10 cam* and *rcsF*::mini-Tn*10 cam* derivatives of S330, which were thermoresistant.

The lack of change in the FtsZ level was rather unexpected because more than 50-fold P_{ftsAZ} stimulation by RcsB has been reported (Carballès et al., 1999). We re-examined the stimulation of P_{ftsAZ} by overproduction of RcsB and compared it with the effect of the *pgsA* null mutation. A single-copy P_{ftsAZ}-*lac* operon fusion plasmid pHR771 was introduced into a strain UE86 harboring pHR721 in which the only functional *pgsA* gene was under control of the L-arabinose-regulatable P_{BAD} promoter and the plasmid-borne *rcsB* was under the IPTG-inducible P_{trc} promoter. A *lac* operon fusion of the *cps* promoter, P_{cps}, (Stout, 1996; Wehland and Bernhard, 2000) was similarly constructed (pHR770) and examined. As shown in Table 2, overproduction of RcsB by addition of IPTG led to elevated transcription from both P_{ftsAZ} and P_{cps}, as expected. P_{ftsAZ} was much weaker, exhibiting β-galactosidase activity of about a twentieth of that exhibited by P_{cps}. When *pgsA* expression was repressed by removal of arabinose, transcription from P_{cps} was activated, albeit much more weakly than by RcsB

overproduction, whereas no activation was detectable for P_{ftsAZ}. The repression of P_{BAD}-controlled *pgsA* may not produce a complete null phenotype (Shiba et al., 2004). Actually, when we introduced P_{cps}-*lac* into a Δ*pgsA* strain UE87, 8-fold higher β-galactosidase activity was detected than in UE86(pHR721) with its *pgsA* gene repressed. However, P_{ftsAZ}-*lac* in UE87 did not produce significantly increased β-galactosidase activity.

P_{ftsAZ} was a weak promoter, and the *pgsA* null mutation had only a moderate effect even on the strong P_{cps} promoter compared with the activation by RcsB overproduction (Table 2) or by the *rcsC137* mutation (Shiba et al., 2004) which impairs the negative regulatory function of RcsC (Brill et al., 1988; Stout, 1994). The contribution of P_{ftsAZ} to the *ftsZ* expression was estimated to be about 4% (Flärdh et al., 1998). Thus it may not be surprising that the level of FtsZ was not changed in the *pgsA* null mutant. It can be considered certain that the growth defect due to the activated Rcs phosphorelay in the mutant does not involve an increased level of FtsZ. We note that this is not the first example of a mutant with activated Rcs signaling and unchanged FtsZ level. A *Salmonella enterica igaA* mutant described by Cano et al. (2002) exhibits the same characteristic.

Although moderate, the Rcs activation in the *pgsA* null mutant causes dual growth defects, RcsA-dependent and -independent, whereas no apparent growth defect is caused by the higher Rcs activation in the $pgsA^+$ background due to RcsB overproduction or due to the *rcsC137* mutation. The thermosensitive growth defects of the *pgsA* null mutant seem to be multifactorial. In order to identify the responsible genes, transcriptome analysis of the *pgsA* null strains is currently under way with special attention to those genes affected by growth temperature in the mutants.

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Table 1. Bacterial strains and plasmids used.

Strain or plasmid	Relevant genotype or description	Construction, source or reference
Strains		
S301	W3110 *ksgB1 lpp-2*	Kikuchi et al., 2000
S330	S301 *pgsA30*::*kan rcsA330*::IS*5*[a]	Kikuchi et al., 2000; this study
S330A21	S330 *rcsC*::mini-Tn*10 cam*	Shiba et al., 2004
S330K41	S330 *rcsF*::mini-Tn*10 cam*	Shiba et al., 2004
SG20043	MC4100 Δ*lon-100 cpsE*::Tn*10*	Stout, 1996; NIG[b] collection
SG20797	MC4100 *cpsB10*::*lac*-Mu-*imm*λ Δ*lon-510 rcsB11*::Tn*10*Δ*16*Δ*17*	Brill et al., 1988
UE33	MG1655 *fliC*::Tn*10*[c] *rcsA*::*cam*	This study
MDL12	*pgsA30*::*kan* Φ(*lacOP-pgsA*$^{+}$)*1 lacZ lacY*::Tn*9*	Xia and Dowhan, 1995
YS330	S301 *pgsA30*::*kan rcsA*$^{+}$	P1(MDL12) × S301
YS331	YS330 *rcsA*::*cam*	P1(UE33) × YS330
YS332	YS330 *rcsB11*::Tn*10*Δ*16*Δ*17*	P1(SG20797) × YS330
YS333	YS330 *rcsC*::mini-Tn*10 cam*	P1(S330A21) × YS330
YS334	YS330 *rcsF*::mini-Tn*10 cam*	P1(S330K41) × YS330
YS340	YS330 *cpsE*::Tn*10*	P1(SG20043) × YS330
UE83	MC4100 *lpp-2* Δ*ara714*	This study
UE42[d]	MG1655 Δ*ara714* Δ(λ*attL-lom*)::(*bla araC* P_{BAD}-*pgsA*) *pgsA30*::*kan*	Shiba et al., 2004
UE84	MC4100 *lpp-2* Δ*ara714* Δ(λ*attL-lom*)::(*bla araC* P_{BAD}-*pgsA*)	P1(UE42) × UE83
UE60[d]	MG1655 Δ*ara714* Δ(λ*attL-lom*)::(*bla araC* P_{BAD}-*pgsA*) Δ*pgsA*::FRT[e]-*kan*-FRT	Shiba et al., 2004
UE85	MC4100 *lpp-2* Δ*ara714* Δ(λ*attL-lom*)::(*bla araC* P_{BAD}-*pgsA*) Δ*pgsA*::FRT-*kan*-FRT	P1(UE60) × UE84
UE86	MC4100 *lpp-2* Δ*ara714* Δ(λ*attL-lom*)::(*bla araC* P_{BAD}-*pgsA*) Δ*pgsA*::FRT	Yeast FLP recombinase procedure (Datsenko and Wanner, 2000) in UE85

UE62[d]	MG1655 Δ*ara714* Δ(λ*attL-lom*)::(*bla araC* P_{BAD}-*pgsA*) Δ*pgsA*::FRT *fliC*::Tn*10*	Shiba et al., 2004
UE87	MC4100 *lpp-2* Δ*ara714* Δ*pgsA*::FRT *fliC*::Tn*10*	P1(UE62) × UE83
UE88	MC4100 *lpp-2* Δ*ara714 fliC*::Tn*10*	P1(UE62) × UE83
Plasmids		
pHR704	pACYC177 *'fliO···rcsA···yedP'*	This study
pHR707	pHR704 *rcsA*::*cam*	This study
pGB2	pSC101 derivative; Spc^r	Churchward et al., 1984
p*rcsA*	pGB2 *rcsA*	This study
pHR758	mini-F-based promoter-probe vector; promoterless *lac*, Km^r	This study
pHR770	pHR758 P_{cps}-*lac*	This study
pHR771	pHR758 P_{ftsAZ}-*lac*	This study
pHR718	pGB2 $lacI^q$ P_{trc}	Shiba et al., 2004
pHR721	pGB2 $lacI^q$ P_{trc}-*rcsB*	This study

[a] The *rcsA330*::IS*5* mutation in S330 was identified in this study (see text).

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

[c] The *fliC*::Tn*10* allele was derived from YK4516 (NIG collection). It was used as a selective marker in constructing isogenic $rcsA^{\pm}$ transductants. *fliC* is hardly expressed in the *pgsA* mutant (Nishino et al., 1993) in which the flagellar master operon *flhDC* is repressed (Kitamura et al., 1994), probably due to the activated Rcs phosphorelay system (Francez-Charlot et al., 2003), and the *fliC* mutation is unlikely to affect the growth of the *pgsA* mutant.

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

[e] FRT, yeast FLP recombinase recognition target site.

Table 2. Effect of the Δ*pgsA* mutation and RcsB overproduction on the activities of the *cps* and *ftsAZ* promoters.[a]

Host strain	Genotype	Addition	β-Galactosidase activity μmol min^{-1} (g protein)$^{-1}$	
			P_{cps}-*lac*	P_{ftsAZ}-*lac*
UE86 (pHR721)	Δ*pgsA* P_{BAD}-*pgsA* P_{trc}-*rcsB*	0.2% arabinose	0.71 ± 0.02	0.17 ± 0.03
		None	2.15 ± 0.05	0.12 ± 0.01
		1 mM IPTG	350 ± 9	16.5 ± 0.2
UE88	*pgsA*$^+$	None	0.18 ± 0.03	0.21 ± 0.08
UE87	Δ*pgsA*	None	17.0 ± 0.2	0.17 ± 0.01

[a] UE86 harboring pHR721, UE88, and UE87 were transformed with pHR758, pHR770, and pHR771. They were grown to about 160 Klett units at 30°C, and β-galactosidase activities were assayed. Precultures of transformants of UE86(pHR721) were prepared in the presence of L-arabinose and washed with LB medium before inoculation.

Legends to Figures

Fig. 1. PCR analysis of the *rcsA* alleles of S330 and its *rcsA*::*cam* transductants.

PCR products were subjected to 1% agarose gel electrophoresis. Lane 1, pHR704 carrying the wild-type *rcsA*; lane 2, pHR707 carrying *rcsA*::*cam*; lane 3, MG1655; lane 4, UE33, a transductional donor; lane 5, W3110; lane 6, S330; lanes 7 and 9, *fliC*::Tn*10* transductants of S330; lanes 8 and 10, *fliC*::Tn*10 rcsA*::*cam* transductants of S330. Twenty nanogram of plasmid DNA preparations or 1 μl of overnight cultures were included in the 20 μl PCR mixture. Molecular sizes of standards are indicated in kb on the left.

Fig. 2. Growth characteristics of the *pgsA* null strains.

(Upper panels) Overnight cultures in LB medium of S301 (*pgsA*$^+$), YS330 (*pgsA*::*kan*), YS331 (*pgsA*::*kan rcsA*), YS334 (*pgsA*::*kan rcsF*), and YS340 (*pgsA*::*kan cpsE*) were diluted to 10 Klett units and then tenfold serially down to 10^{-5} of that, and 5 μl each was spotted on LB agar plates. The plates were incubated at 30°C, 37°C, and 42°C for ca. 16 h. (Lower panels) Early exponential phase cultures in LB medium of S301 (open circles), YS330 (closed circles), YS331 (open squares), YS334 (closed squares), and YS340 (open triangles) were diluted to 0.8 Klett units and grown at 30°C and 37°C, and at 42°C after incubation at 30°C for $2^3/_4$ h. Their growth was monitored with a microbial growth recorder BioScanner 16MS (Ohtake Works). Transmittance readings at 650 nm <97.5% (OD_{650}>0.01) are plotted. In the shift-up experiment it took about 30 min before the temperature in the growth chamber reached 42°C; readings at 3 h are not plotted. YS332 (*pgsA*::*kan rcsB*) and YS333 (*pgsA*::*kan rcsC*) showed essentially the same growth characteristics as YS334.

Fig. 3. Levels of FtsZ protein in the *pgsA* null mutant cells.

S301 (lanes 1 and 2), S330 (3 and 4), S330A21 (5 and 6), and S330K41 (7 and 8) grown in LB to 100 Klett units at 30°C were diluted two-fold and incubated at 30°C (odd-numbered lanes) and 42°C (even-numbered) until the turbidity reached 150 Klett units. During the incubation at 42°C the S330 cells did not start to lyse. Cell extracts (36 μg protein) were

analyzed by SDS-gel electrophoresis followed by Western immunoblotting using anti-FtsZ antiserum. The arrowhead indicates the position of FtsZ protein, which was confirmed by pZAQ (Ward and Lutkenhaus 1985)-directed overproduction (not shown). Molecular sizes of standards (in kDa) and the dye front (F) are indicated on the left.

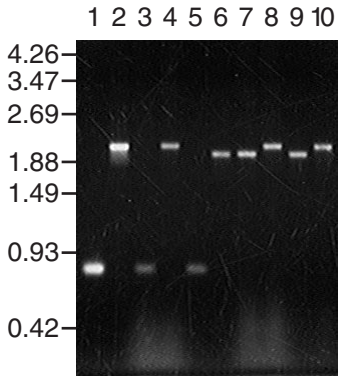


Fig. 1. PCR analysis of the *rcsA* alleles of S330 and its *rcsA*::*cam* transductants.

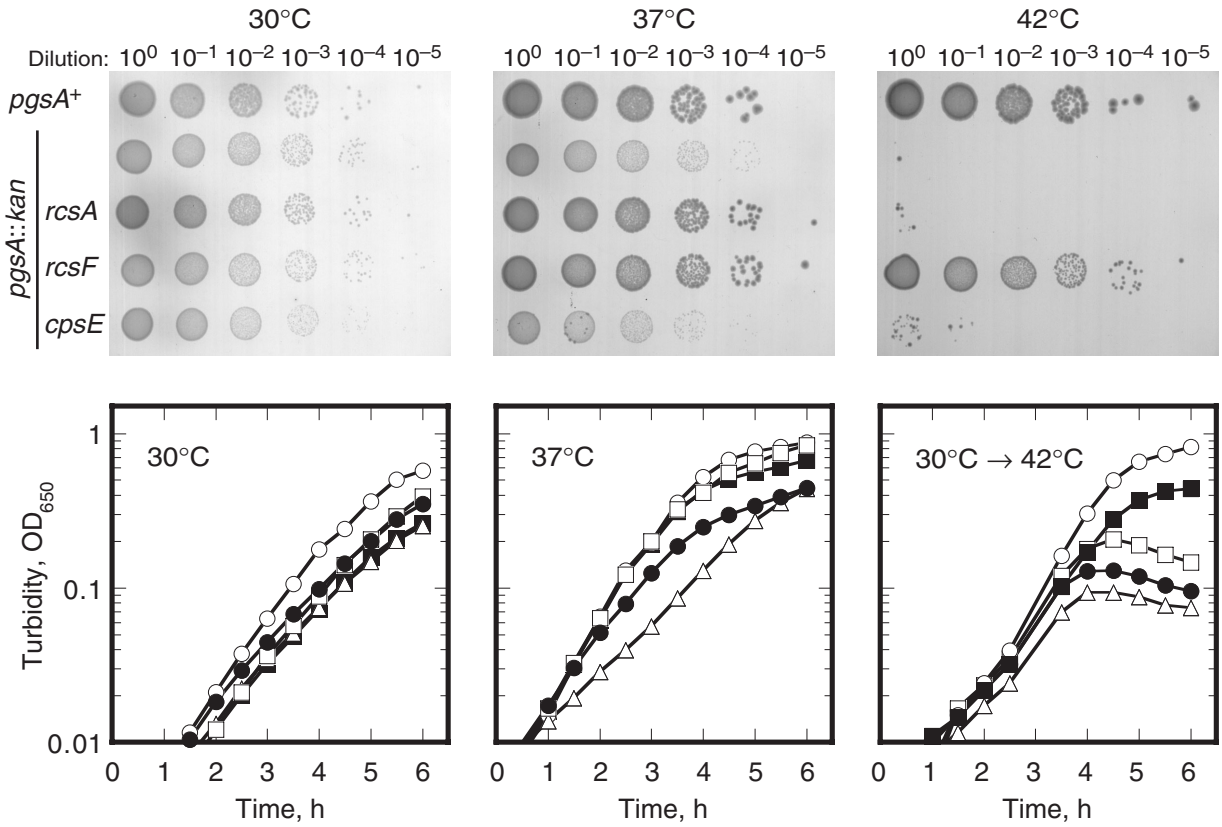
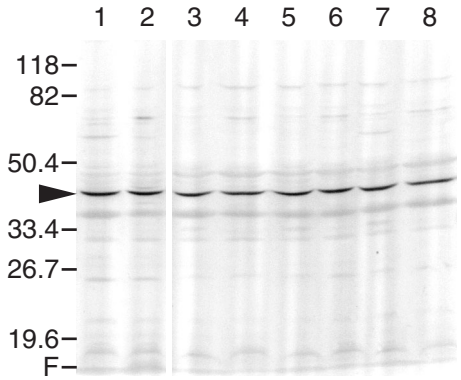


Fig. 2. Growth characteristics of the *pgsA* null strains.



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