Molecular Microbiology (2006) **60**(1), 39–50

doi:10.1111/j.1365-2958.2006.05069.x
First published online 21 February 2006

The PmrA/PmrB and RcsC/YojN/RcsB systems control expression of the *Salmonella* O-antigen chain length determinant

Mónica A. Delgado,[1†] Chakib Mouslim[1] and Eduardo A. Groisman[1,2*]

[1]*Department of Molecular Microbiology, and* [2]*Howard Hughes Medical Institute, Washington University School of Medicine, 660 S. Euclid, St. Louis, MO 63110, USA.*

Summary

The lipopolysaccharide (LPS) is the outermost component of the cell envelope in Gram-negative bacteria. It consists of the hydrophobic lipid A, a short non-repeating core oligosaccharide and a distal polysaccharide termed O-antigen. We report here that the PmrA/PmrB and RcsC/YojN/RcsB two-component systems of *Salmonella enterica* serovar Typhimurium independently promote transcription of the wzz_{st} gene, which encodes a protein that determines the chain length of the O-antigen. We show that the regulatory proteins PmrA and RcsB footprint partially overlapping regions of the wzz_{st} promoter stimulating transcription from the same start site. Induction of the PmrA/PmrB or RcsC/YojN/RcsB systems increased the fraction of LPS molecules containing 16–35 O-antigen subunits, leading to heightened resistance to serum. The LPS of a *rcsB* null mutant exhibited an altered mobility in the O-antigen subunits attached to the lipid A-core region when separated on a SDS/PAGE gel, suggesting that RcsB may regulate additional LPS genes. Inactivation of the wzz_{st} gene eliminated the enhanced swarming behaviour exhibited by the *rcsB* mutant. That multiple regulatory systems control wzz_{st} expression suggests that the Wzz_{st} protein is required under different environmental conditions.

Introduction

The lipopolysaccharide (LPS) is a complex glycolipid of Gram-negative bacteria that contributes to the stability and permeability barrier properties of the outer membrane (Raetz and Whitfield, 2002). The LPS of *Salmonella enterica* serovar Typhimurium (*S. typhimurium*) is composed of three structurally distinct regions: (i) the lipid A, a hydrophobic component composed of sugars and fatty acids that anchors the LPS to the outer membrane, (ii) a core oligosaccharide unit and (iii) the O-antigen, a polysaccharide consisting of repeating units of three to six sugars that extend from the cell surface and are attached to the outer portion of the core oligosaccharide.

The O-antigen is involved in resistance to phagocytosis, cationic peptides and serum complement (Joiner, 1985; Burns and Hull, 1998; Skurnik and Bengoechea, 2003). These activities have been correlated with the length of the O-antigen and its distribution in the outer membrane. For example, organisms with longer O-antigen are more resistant to phagocytosis and to serum than those with shorter O-antigen (Joiner *et al*., 1982a; Dasgupta *et al*., 1994; Whitfield *et al*., 1997; Murray *et al*., 2005). In *S. typhimurium*, the bimodal distribution in the number of O-antigen subunits attached to the lipid A-core is determined by two proteins: Wzz_{st} (formerly known as Cld or Rol), which mediates the production of an O-antigen modal banding pattern of 16–35 subunits (long or L-type) (Morona *et al*., 1995; Raetz and Whitfield, 2002); and Wzz_{fepE}, which is responsible for the very long modal length, containing >100 O-subunits (Murray *et al*., 2003). In *S. typhimurium,* the wzz_{st} gene is located between the UDP-glucose dehydrogenase *ugd* gene and the histidine biosynthetic *his* operon, downstream of the O-antigen biosynthesis gene cluster *rfb* (Bastin *et al*., 1993; Reeves, 1994; Franco *et al*., 1998). Although the biochemical function of the Wzz_{st} protein has been the subject of several studies (Bastin *et al*., 1993; Morona *et al*., 1995; Whitfield *et al*., 1997; Daniels and Morona, 1999; Marolda *et al*., 1999), little is known about the regulation of its expression.

The PmrA/PmrB two-component system of *S. typhimurium* is necessary for resistance to polymyxin B (Roland *et al*., 1993) and to Fe^{3+} (Wosten *et al*., 2000; Chamnongpol *et al*., 2002), and for virulence in mice (Gunn *et al*., 2000). PmrA is the response regulator that is activated by Fe^{3+}, which is the signal sensed by its cognate sensor PmrB (Wosten *et al*., 2000), and by low

Accepted 12 January, 2006. *For correspondence. E-mail groisman@borcim.wustl.edu; Tel. (+1) 314 362 3692; Fax (+1) 314 747 8228.
†Present address: INSIBIO-CONICET, Chacabuco 461, S.M. de Tucumán (4000), Provincia de Tucumán, Argentina.

Mg^{2+}, in a pathway that requires the Mg^{2+}-responsive PhoP/PhoQ two-component system and the PhoP-activated PmrD protein (Fig. 1) (Kox *et al*., 2000; Kato and Groisman, 2004). To date, all the characterized PmrA-regulated genes participate in the modification of the LPS. For example, the *pbgP* operon [also referred to as *arn* (Trent *et al*., 2001; Breazeale *et al*., 2003) and *pmrF* (Gunn *et al*., 1998)] and the *ugd* gene are responsible for the synthesis and incorporation of 4-aminoarabinose into lipid A, and the *pmrC* gene is required for the addition of phosphoethanolamine to lipid A (Groisman *et al*., 1997; Gunn *et al*., 1998; Lee *et al*., 2004). The *cptA* gene, which appears to be under PmrA control, is necessary for the addition of phosphoethanolamine to the core region of the LPS (Tamayo *et al*., 2005) (Fig. 1).

The *ugd* gene is also regulated by the Rcs phosphorelay system (Mouslim and Groisman, 2003; Mouslim *et al*., 2003; Tierrez and Garcia-del Portillo, 2004), possibly because the Ugd protein is required for the biosynthesis of colanic acid capsule, and the Rcs system controls the production of this capsular polysaccharide (see Majdalani and Gottesman, 2005 for a review). This system is composed of the sensor RcsC, the response regulator RcsB and the histidine-containing phosphotransfer protein YojN that is apparently used as an intermediary in the phosphoryl transfer from RcsC to RcsB (Majdalani and Gottesman, 2005). In *Salmonella*, the Rcs system is activated by treatments or mutations that alter the cell envelope such as growth on a solid surface (our unpubl. results), mutation of the *igaA* (Tierrez and Garcia-del Portillo, 2004) or *tolB* (Mouslim *et al*., 2003) genes, and growth of a *pmrA* mutant in the presence of low Mg^{2+} plus Fe^{3+} (Mouslim and Groisman, 2003).

In this paper, we establish that the PmrA/PmrB and RcsC/YojN/RcsB systems promote wzz_{st} transcription under different environmental conditions and independently of each other. We identify the *cis*-acting sequences recognized by the PmrA and RcsB proteins in the wzz_{st} promoter, and show that the transcriptional induction of the wzz_{st} gene increases the amount of O-antigen in the

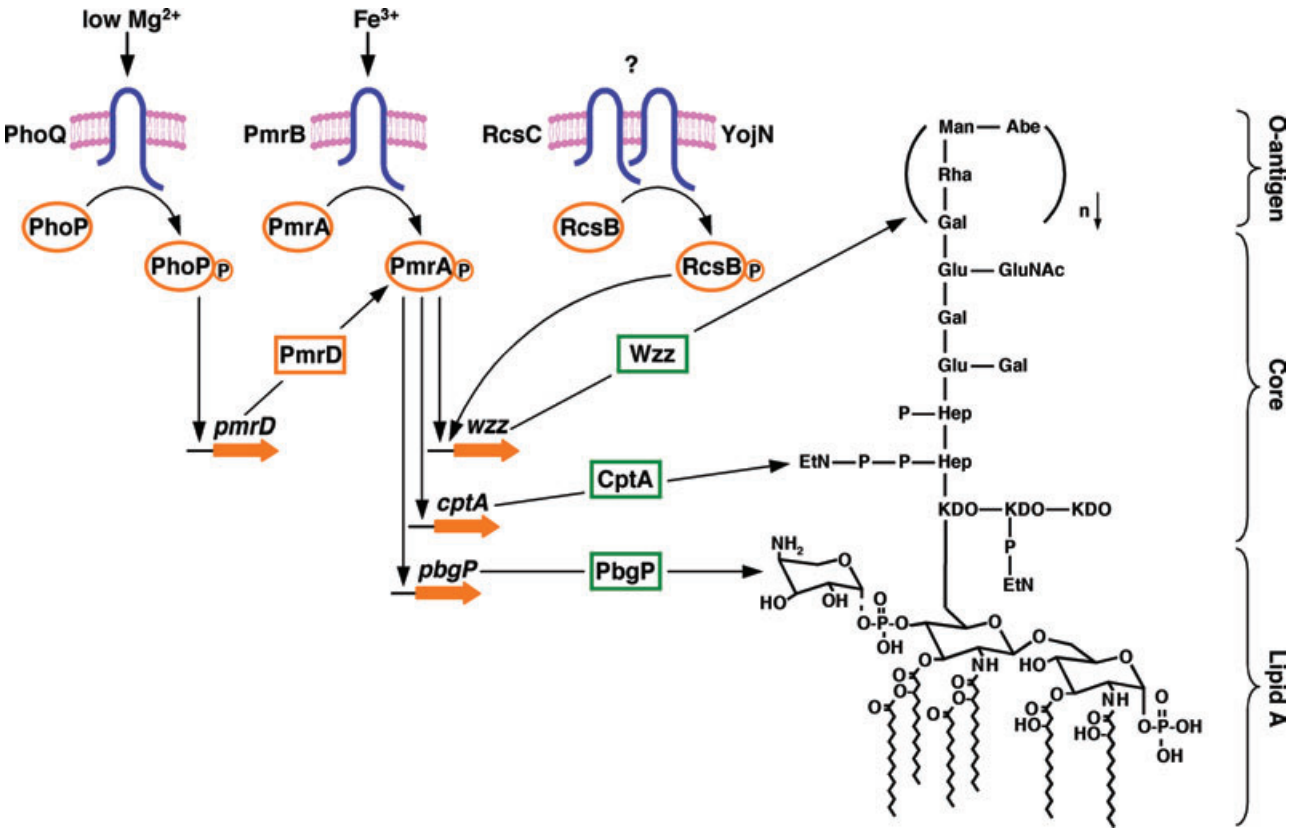


Fig. 1. Signals and pathways promoting transcription of the *Salmonella* wzz_{st} gene. Low Mg^{2+} is sensed by the PhoQ protein, which activates its cognate regulator PhoP. Activation of PhoP results in expression of the PmrD protein, which activates the PmrA protein post-translationally. Fe^{3+} is recognized by the sensor protein PmrB, which activates the PmrA protein. The activated PmrA protein binds to the wzz_{st} and *pbgP* promoters and activates their transcription. The *pbgP* locus encodes proteins responsible for the synthesis and incorporation of 4-aminoarabinose in lipid A. The wzz_{st} gene encodes the O-antigen chain length determinant. The *cptA* gene mediates the modification of the core region with phosphoethanolamine. Activation of the RcsC/YojN/RcsB system promotes transcription of the wzz_{st} gene independently of the PhoP/PhoQ and PmrA/PmrB systems.

LPS leading to heightened resistance to serum complement. In addition, we report new phenotypes for mutants defective in the *rcsB* and *wzz*$_{st}$ genes.

Results

The PmrA/PmrB two-component system promotes transcription of the wzz$_{st}$ *gene*

Because the PmrA/PmrB system controls the expression of genes mediating the modification of lipid A and because ligation of the O-antigen depends on the structure of the lipid A-core (Whitfield *et al*., 1997), we investigated whether the *wzz*$_{st}$ gene was PmrA/PmrB-regulated. Thus, we determined the β-galactosidase activity produced by a strain harbouring a *wzz*$_{st}$ *–lacZY* transcriptional fusion in the chromosome following growth in media of low or high Mg^{2+}, which are conditions that activate and repress expression of PmrA-regulated genes respectively (Kox *et al*., 2000). Transcription of the *wzz*$_{st}$ gene was 14-fold higher in the wild-type strain grown in low Mg^{2+} compared with organisms experiencing high Mg^{2+} (Fig. 2A). *wzz*$_{st}$ transcription was reduced, but not eliminated, in *phoP* and *pmrA* mutants (Fig. 2A). These results indicate that *wzz*$_{st}$ expression is induced in low Mg^{2+}, possibly via the PhoP/PhoQ–PmrD–PmrA/PmrB pathway (Fig. 1), and suggest that an additional regulator(s) participate(s) in *wzz*$_{st}$ expression.

The RcsC/YojN/RcsB system mediates the PmrA-independent wzz$_{st}$ *transcription experienced in low* Mg^{2+} *plus* Fe^{3+}

Growth in the presence of low Mg^{2+} plus Fe^{3+} also induced *wzz*$_{st}$ transcription (Fig. 2A). Although Fe^{3+} is the specific signal sensed by the PmrB protein, this induction was not abolished in the *pmrA* mutant (Fig. 2A). This is reminiscent of *ugd* transcription, which is decreased but not elim-

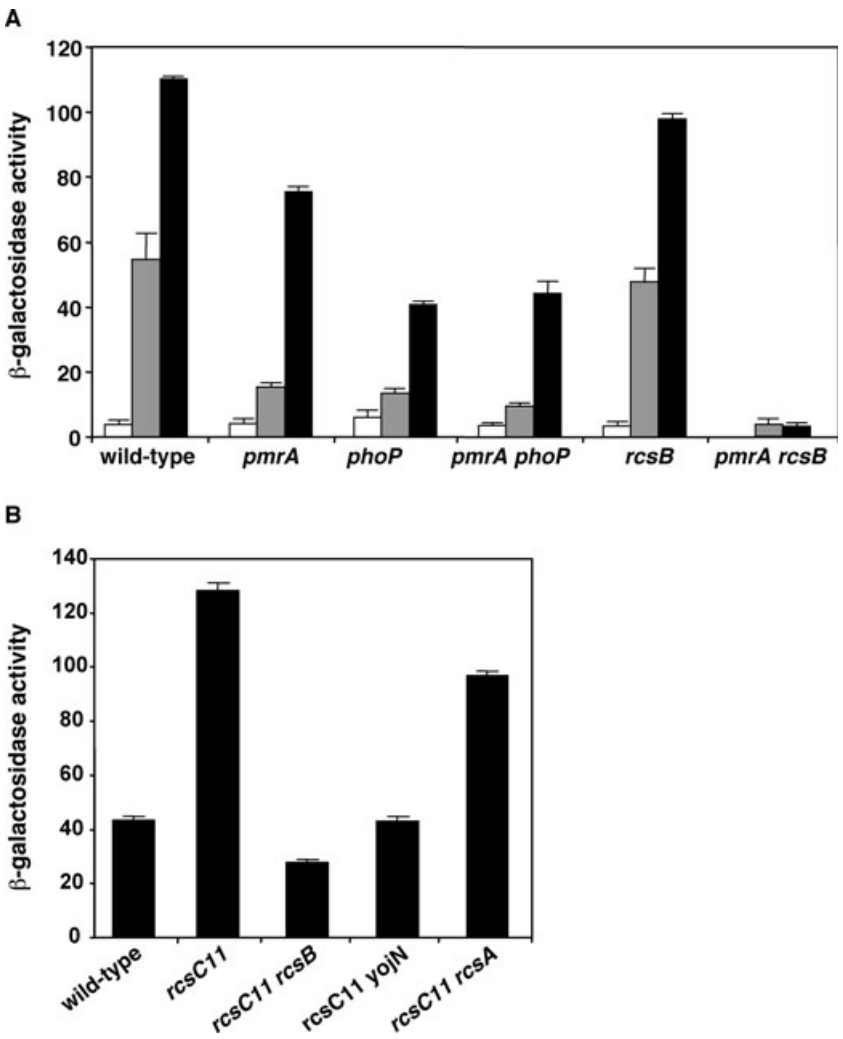


Fig. 2. The PmrA/PmrB and RcsC/YojN/RcsB systems regulate *wzz*$_{st}$ transcription.
A. β-Galactosidase activity (Miller units) expressed by strains harbouring chromosomal *lacZY*-transcriptional fusions to the *wzz*$_{st}$ gene following growth in minimal medium pH 7.4 containing 10 mM (white bars) $MgCl_2$, 10 μM $MgCl_2$ (grey bars) or 10 μM $MgCl_2$ 100 μM $FeSO_4$ (black bars). The transcriptional activity was investigated in six genetic backgrounds: wild-type (EG14930), *pmrA* (EG16362), *phoP* (EG16365), *pmrA phoP* (EG16364), *rcsB* (EG15410) and *pmrA rcsB* (EG16363).
B. β-Galactosidase activity (Miller units) expressed by strains harbouring chromosomal *lacZY*-transcriptional fusions to the *wzz*$_{st}$ gene grown in LB broth. The transcriptional activity was investigated in five genetic backgrounds: wild-type (EG14930), *rcsC11* (EG15407), *rcsC11 rcsB* (EG16368), *rcsC11 yojN* (EG15408) and *rcsC11 rcsA* (EG16371). Data correspond to mean values of three independent experiments performed in duplicate. Error bars correspond to the standard deviation.

Fig. 3. Molecular analysis of the wzz_{st} promoter. S1 mapping of wzz_{st} transcripts produced by logarithmically growing wild-type (14028s) and *rcsC11* (EG14873) *Salmonella* strains grown in LB broth (A), and by wild-type (14028s), *rcsB* (EG13308), *pmrA* (EG13307) and *pmrA rcsB* (EG13310) *Salmonella* strains grown in minimal medium pH 7.4 containing 10 μM $MgCl_2$ or 10 μM $MgCl_2$ 100 μM $FeSO_4$ (B). The S1 protection assay was performed as described in *Experimental procedures*. Lane AG corresponds to the Maxam–Gilbert DNA ladder of the target sequence. The single transcription start site is marked with *arrows*. + and – refer to the presence of Fe^{3+} in the media used to grow the organisms before harvesting the RNA. (C) DNA sequence corresponding to the 140 bp region upstream of the start codon of the wzz_{st} gene, which includes the *cis*-acting elements required for transcription promoted by the PmrA/PmrB and RcsC/YojN/RcsB systems. +1 corresponds to the transcription start site of the wzz_{st} promoter. The sequences underlined by solid blue and dotted red lines represent the DNA regions footprinted by the PmrA and RcsB proteins respectively. (D) Alignment of the regulatory sequences of the PmrA-activated wzz_{st} gene and a conserved PmrA motif. The conserved sequences corresponding to the putative PmrA binding motif are boxed. (E) Alignment of the RcsB-dependent regulatory sequences of *S. enterica* (*Se*, complementary sequence) wzz_{st}, *S. enterica* (*Se*) *ugd*, *Erwinia amylovora* (*Ea*) *ams*, *E. coli* (*Ec*) *fts* and *E. coli* (*Ec*) *cps* genes. The conserved sequence corresponding to the putative RcsB binding motif is boxed. DNase footprinting analysis of the wzz_{st} promoter region with the PmrA-His6 (F) or RcsB-His6 (G) proteins was performed with the end-labelled fragments corresponding to the coding and non-coding strands using 0, 5, 10, 40 and 80 pmol (from left to right) of protein. Solid blue bars represent the PmrA-protected regions (F) and red dote line the RcsB-protected regions (G). The position of the binding was determined by comparison with an A + G sequence ladder, generated with the same labelled primers as those utilized for the probe.

inated in a *pmrA* mutant experiencing low Mg^{2+} plus Fe^{3+} (Mouslim *et al*., 2003). Because *ugd* transcription is dependent on the RcsC/YojN/RcsB and PhoP/PhoQ two-component systems in the *pmrA* mutant experiencing low Mg^{2+} plus Fe^{3+} (Mouslim *et al*., 2003), we examined the possibility that these systems might be responsible for transcription of the wzz_{st} gene. There was no wzz_{st} transcription in *pmrA rcsB* (Fig. 2A), *pmrA rcsC* and *pmrA yojN* double mutants (data not shown), indicating that the Rcs system is required for this activation. On the other hand, the *rcsB* single mutant displayed wild-type levels of wzz_{st} expression (Fig. 2A), possibly due to the presence of a functional PmrA/PmrB system. Likewise, wzz_{st} transcription was similar in *pmrA* and *pmrA phoP* mutants (Fig. 2A), consistent with the notion that the PhoP/PhoQ system is not necessary for this activation.

RcsB-regulated genes have been divided into two groups depending on whether they require the RcsA protein for transcription (Majdalani and Gottesman, 2005). Those that are RcsA-dependent are involved in the production of colanic acid capsule, whereas those that are RcsA-independent participate in other cellular activities (Mouslim *et al*., 2003). To assess the role of the RcsA protein in wzz_{st} transcription, we used the *rcsC11* mutant strain, which expresses RcsB-regulated genes under non-inducing conditions (Costa and Anton, 2001). wzz_{st} transcription was strongly diminished in both *rcsC11 rcsB* and *rcsC11 yojN* double mutants to less than one-third of the levels exhibited by the *rcsC11* strain (Fig. 2B), but not in the *rcsC11 rcsA* strain, which retained >75% of the expression of the *rcsC11* strain (Fig. 2B). These results demonstrate that the RcsA protein is not absolutely required for the RcsC/YojN/RcsB phosphorelay-mediated expression of the wzz_{st} gene.

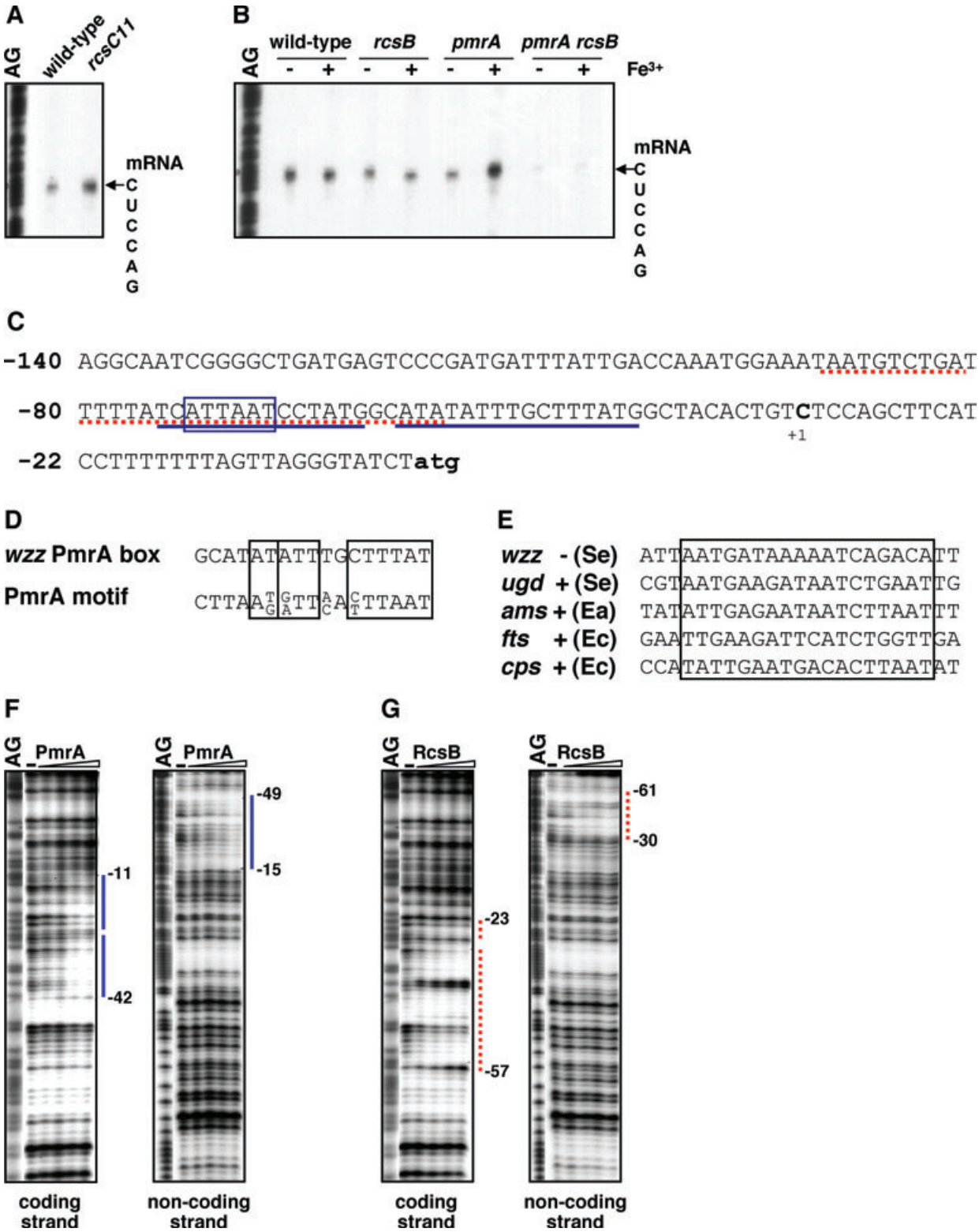
The PmrA/PmrB and Rcs systems promote wzz_{st} *transcription from the same start site*

The wzz_{st} gene is located immediately downstream and in the same orientation as the *ugd* gene, which is also regulated by the PmrB/PmrA and RcsC/YojN/RcsB systems (Mouslim and Groisman, 2003; Mouslim *et al*., 2003). Yet, the *ugd* and wzz_{st} genes do not form an operon because deletion of the 238 bp region immediately upstream of the *ugd* start codon and harbouring the *ugd* promoter did not affect wzz_{st} transcription (data not shown).

We mapped the transcription start site for the wzz_{st} promoter using mRNA harvested from wild-type, *pmrA*, *rcsB*, *pmrA rcsB* and *rcsC11* strains. A single S1 product was detected in the wild-type strain grown in low Mg^{2+} (Fig. 3A), which is an inducing condition for the PmrA/PmrB system (Kox *et al*., 2000), and in the *rcsC11* mutant (Fig. 3A) or in a *pmrA* mutant experiencing low Mg^{2+} plus Fe^{3+} (Fig. 3B), which are inducing conditions for the RcsC/YojN/RcsB system (Mouslim *et al*., 2003). The S1 product was slightly reduced in *pmrA* and *rcsB* mutants, and strongly reduced in the *pmrA rcsB* double mutant (Fig. 3B). On the other hand, the levels of the S1 product were higher in the *rcsC11* mutant (Fig. 3A) or in the *pmrA* mutant grown in the presence of low Mg^{2+} plus Fe^{3+} (Fig. 3B) due to activation of the RcsC/YojN/RcsB system. These results were further verified in real-time polymerase chain reaction (PCR) experiments demonstrating that functional *pmrA* and *rcsB* genes were required for wzz_{st} transcription, which is in contrast to the specific requirement for *pmrA* in the case of the *pmrC* gene and of *rcsB* for the *wzc* gene (data not shown). These results demonstrate that the PmrA and RcsB proteins can independently promote wzz_{st} transcription from the same start site (Fig. 3C).

The PmrA and RcsB proteins bind to the wzz_{st} *promoter*

Analysis of the wzz_{st} promoter region revealed the presence of potential binding sites for the PmrA (Fig. 3D) and RcsB proteins (Fig. 3E), suggesting that these proteins promote wzz_{st} transcription directly by binding to the wzz_{st} promoter. DNase I footprinting analysis of the 219 bp region upstream of the wzz_{st} open reading frame demon-



strated that the PmrA protein binds to the wzz_{st} promoter protecting positions –42 to –29 and –26 to –11 on the coding strand and –49 to –15 on the non-coding strand relative to the transcription start site (Fig. 3F). The protected sequences include the predicted PmrA binding site (Fig. 3D), and also an additional region that resembles one half of a PmrA box (Fig. 3C, blue box). Chromatin immunoprecipitation (ChIP) experiments using a strain

expressing an epitope-tagged PmrA protein demonstrated enhanced binding to the wzz_{st} promoter in bacteria experiencing low (10 μM) Mg^{2+} compared with in those grown in high (10 mM) Mg^{2+} (data not shown), indicative that the PmrA protein binds to the wzz_{st} promoter *in vivo*.

The RcsB protein footprinted the wzz_{st} promoter protecting the –61 to –30 region in the non-coding strand and the –57 to –23 region in the coding strand relative to the transcription start site (Fig. 3G). The protected region includes the predicted RcsB box (Fig. 3E). Cumulatively, these results show that the PmrA and RcsB proteins exert their regulatory effect directly by binding to the wzz_{st} promoter.

Regulation of O-antigen modal length by the PmrA/PmrB and RcsC/YojN/RcsB systems

To examine the phenotypic consequences of PmrA and RcsB regulation of the wzz_{st} gene, we investigated the O-antigen chain length distribution in the wild-type strain grown under repressing conditions (high Mg^{2+}), and in wild-type, *pmrA*, *rcsB*, *pmrA rcsB*, *rcsC11* and wzz_{st} strains grown under inducing conditions (low Mg^{2+} plus Fe^{3+}). We established that activation of the PmrA/PmrB or RcsC/YojN/RcsB systems results in an increase in the amount of O-antigen of 16–35 subunits in length (i.e. L-type) in wild-type, *rcsB* and *pmrA* mutants (Fig. 4A and B). In contrast, the L-type O-antigen was absent from the *pmrA rcsB* double mutant, which exhibited the same profile as the wzz_{st} mutant (Fig. 4B). We also noticed that the LPS of the *rcsB* mutant had a slightly slower mobility in those bands containing 6–10 and 16–22 O-subunits attached to the lipid A-core (Fig. 4B). These results indicate that activation of the PmrA/PmrB and RcsC/YojN/RcsB systems increases the amount of L-type O-antigen in the LPS.

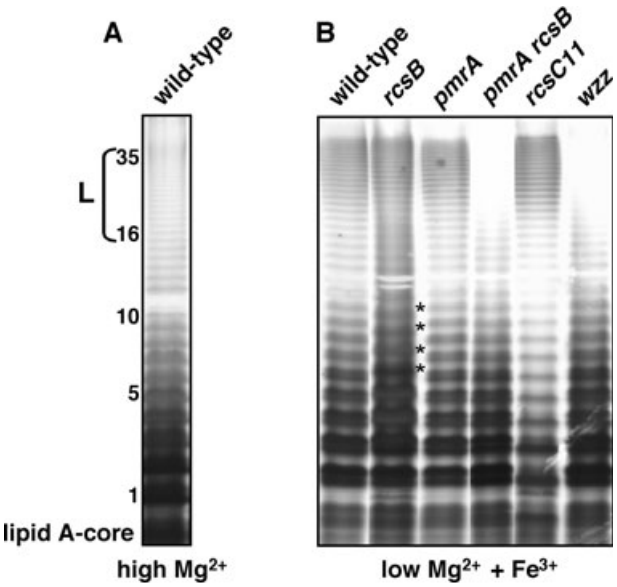


Fig. 4. Production of L-type O-antigen in response to inducing conditions for the PmrA/PmrB and Rcs systems. SDS-PAGE analysis of LPS isolated from ≈1×10^8 cells corresponding to a wild-type *Salmonella* strain (14028s) grown in minimal medium pH 7.4 containing 10 mM $MgCl_2$ (A), and from wild-type (14028s), *rcsB* (EG13308), *rcsB pmrA* (EG13310), *pmrA* (EG13307), *rcsC11* (EG14873) and wzz_{st} (EG14929) strains, grown in minimal medium pH 7.4 with 10 μM $MgCl_2$ 100 μM $FeSO_4$ (B). The L-type O-antigen (L) and the number of O-subunits attached to the lipid A-core are indicated on the left. Under the utilized experimental conditions, the very long antigen that is produced by wzz_{fepE} was not detected, possibly because there is no wzz_{fepE} transcription (data not shown). Asterisk (*) indicate O-antigen bands of a different mobility in the *rcsB* mutant. Close examination revealed that the mobility O-antigen bands of 16–22 subunits is also altered in the *rcsB* mutant.

The pmrA *and* rcsB *genes are required for resistance to complement-mediated killing*

Resistance to complement is associated with the presence of a complete O-antigen, which sterically hinders the formation of an effective membrane attack complex (Joiner *et al*., 1982a,b). We examined the serum susceptibility of a set of isogenic strains grown under PmrA/PmrB- and RcsC/YojN/RcsB-inducing conditions. Growth in low Mg^{2+} plus Fe^{3+} resulted in increased survival of the wild-type strain relative to organisms grown in low Mg^{2+} (Fig. 5). Likewise, the enhanced survival of the *rcsC11* mutant relative to the wild-type strain (Fig. 5) demonstrated that activation of the RcsB-dependent pathway increases resistance to complement. Although complement resistance has been ascribed to the presence of capsule in certain strains of *Escherichia coli* (Cross *et al*., 1986; Burns and Hull, 1998) and the RcsB/YojN/RcsC system controls the production of colanic acid capsule in *Salmonella* (Majdalani and Gottesman, 2005), RcsB-mediated complement resistance appears to be due solely to expression of the wzz_{st} gene because resistance was abolished in the *rcsC11* wzz_{st} double mutant but not in the *rcsC11 cps* double mutant (Fig. 5), which is defective in capsule formation. Resistance to complement was lower in *pmrA* and *rcsB* mutants than in the wild-type strain, and the *pmrA rcsB* double mutant was as sensitive as the wzz_{st} mutant (Fig. 5). These results indicate that the *pmrA* and *rcsB* genes are required for resistance to complement.

Inactivation of the wzz_{st} *gene prevents the enhanced swarming behaviour of the* rcsB *mutant*

We investigated whether the wzz_{st} gene was involved in bacterial swarming because: first, the RcsC/YojN/RcsB phosphorelay system has been implicated in the modulation of swarming behaviour of *E. coli* cells growing on an agar surface (Takeda *et al*., 2001); and second, the *Salmonella* O-antigen is required for swarm colony expansion

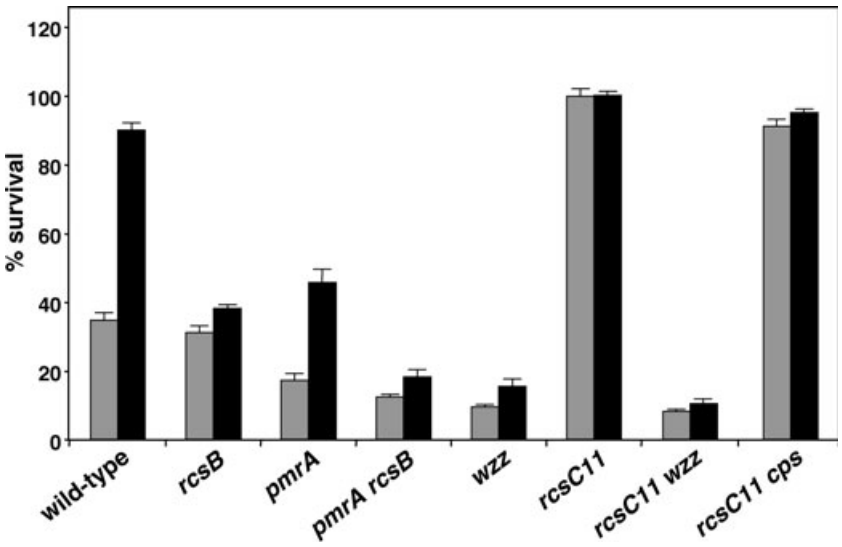


Fig. 5. Activation of the PmrA/PmrB and Rcs systems confers resistance to complement-mediated killing. The wild-type (14028s), *rcsB* (EG13308), *rcsB pmrA* (EG13310), *pmrA* (EG13307), wzz_{st} (EG14929), *rcsC11* (EG14873), *rcsC11* wzz_{st} (EG15407) and *rcsC11 cps* (EG14876) strains grown in minimal medium pH 7.4 containing 10 μM $MgCl_2$ (grey bars) or 10 μM $MgCl_2$ 100 μM $FeSO_4$ (black bars) were exposed to 10% of human serum for 1 h and the cfu ml^{-1} was determined by plating dilutions onto LB agar plates, incubation at 37°C overnight and counting the colonies. % survival was determined as described in *Experimental procedures*. Data correspond to mean values of three independent experiments performed in duplicate. Error bars correspond to the standard deviation.

(Toguchi *et al*., 2000). The wild-type strain and wzz_{st} mutants displayed very similar swarming behaviour, migrating at a speed of $\approx 1.5 \times 10^{-2}$ mm min^{-1} and $\approx 1.4 \times 10^{-2}$ mm min^{-1} respectively. In contrast, the *rcsB* mutant exhibited a noticeable increase in swarming migration at $\approx 2.2 \times 10^{-2}$ mm min^{-1} (Fig. 6), which was abrogated by mutating wzz_{st} as the *rcsB* wzz_{st} double mutant migrated at $\approx 1.4 \times 10^{-2}$ mm min^{-1} (Fig. 6).

Discussion

We have established that transcription of the *Salmonella* wzz_{st} gene is induced by distinct signals via three different two-component regulatory systems (Fig. 1). Low Mg^{2+} promotes wzz_{st} transcription in a process that depends on the Mg^{2+}-responsive PhoP/PhoQ system, likely via the PmrD shunt protein and the PmrA/PmrB system. Fe^{3+} induces wzz_{st} expression via the iron-responsive PmrA/PmrB system and independently of the PhoP/PhoQ system. Activation of the RcsC/YojN/RcsB phosphorelay system by the constitutive *rcsC11* allele or in a *pmrA* mutant exposed to low Mg^{2+} plus Fe^{3+} also induced transcription of the wzz_{st} gene.

The PmrA and RcsB proteins promote transcription of the wzz_{st} gene independently of each other because: first, under inducing conditions for the PmrA/PmrB system, the increase in wzz_{st} transcription was dependent on PmrA but not on RcsB (Fig. 2). Second, under RcsC/YojN/RcsB-inducing conditions, wzz_{st} transcription was promoted in a RcsB-dependent but PmrA-independent manner (Fig. 2). Third, when both the *pmrA* and *rcsB* genes were inactivated, there were background levels of wzz_{st} transcription (Fig. 2) that correlated with the absence of L-type O-antigen (Fig. 4). And fourth, the amount of L-type O-antigen produced by *pmrA* and *rcsB* single mutants was similar to that of the wild-type strain (Fig. 4), consistent with the notion that, under the investigated conditions, wzz_{st} expression can be promoted by one regulator when the other one is absent.

The PmrA/PmrB and RcsC/YojN/RcsB systems exert their control directly, via the PmrA and RcsB proteins binding to partially overlapping sequences in the wzz_{st} promoter and initiating transcription from the same start site (Fig. 3). This regulation is analogous to that controlling the *osmCp1* promoter of *E. coli*, which is induced independently by the NhaR and RcsB regulatory proteins, shown to bind to overlapping sequences and to initiate *osmC* transcription from the same start site (Sturny *et al*., 2003). The regulation of wzz_{st} transcription is also reminiscent, in part, of that controlling *ugd* expression in that both the wzz_{st} and *ugd* genes are controlled by the PmrA/PmrB and RcsB/YojN/RcsC systems. However, expression of these genes differs in that the transcription taking place in a *pmrA* mutant experiencing low Mg^{2+} plus Fe^{3+}

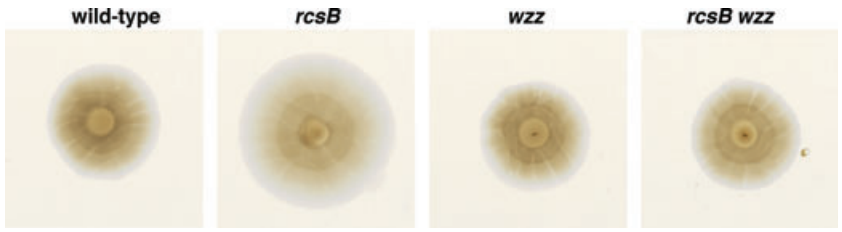


Fig. 6. The wzz_{st} gene is required for swarming of a *rcsB* null mutant. Swarming behaviour of wild-type (14028s), *rcsB* (EG13308), wzz_{st} (EG14929) and *rcsB* wzz_{st} (EG15410) strains in LB plate containing 0.5% agar and 0.5% glucose. All images were captured after 24 h of incubation at 37°C.

requires the PhoP protein for *ugd* (Mouslim *et al*., 2003), but not for wzz_{st} (Fig. 2A); and that the PmrA and RcsB proteins promote wzz_{st} transcription from the same start site (Fig. 3A and B), but use two different start sites to transcribe the *ugd* gene (Mouslim and Groisman, 2003; Mouslim *et al*., 2003).

A *Salmonella rcsC11* mutant forms mucoid colonies due to constitutive activation of the RcsC/YojN/RcsB system (Costa and Anton, 2001) and the ensuing expression of the *cps* capsule synthesis operon. An *rcsC11* wzz_{st} double mutant retained the ability to form mucoid colonies (our unpubl. results), indicating that the wzz_{st} gene product is not required for the synthesis of colanic acid. Consistent with the notion that only those RcsB-regulated genes that participate in the synthesis of the capsular polysaccharide are RcsA-dependent (Mouslim *et al*., 2003), transcription of the wzz_{st} gene was RcsA-independent (Fig. 2B).

The regulation of wzz_{st} expression by the PmrA/PmrB and RcsC/YojN/RcsB systems is physiologically significant because growth conditions or mutations that altered the ability of these systems to transcribe the wzz_{st} gene modified the amount of L-type O-antigen (Fig. 4) and affected the susceptibility to complement (Fig. 5). For example, the hyperactive *rcsC11* mutant exhibited higher levels of complement resistance than the wild-type strain (Fig. 5). This phenotype is due entirely to the control of the wzz_{st} gene because the *rcsC11* wzz_{st} mutant was as susceptible to serum as a wzz_{st} mutant (Fig. 5). Our results also indicate that the Rcs-regulated colanic acid capsule does not participate in serum resistance because the non-mucoid *rcsC11 cps* mutant retained the *rcsC11* levels of serum resistance (Fig. 5), and because the serum-sensitive *rcsC11* wzz_{st} mutant was as mucoid as the *rcsC11* mutant (our unpubl. results).

The present demonstration that the Mg^{2+}-responsive PhoP/PhoQ system is a positive regulator of the O-antigen modal distribution (by virtue of activating the PmrA/PmrB system) would appear to be in conflict with the observation that a *Salmonella* $PhoP^c$ strain, which expresses PhoP-activated genes even under non-inducing conditions, has a shorter O-antigen than wild-type *Salmonella* (Baker *et al*., 1999). These discrepancies could be due to the fact that we studied the widely used wild-type strain 14028s while the previous experiments used derivatives of strain C5 (Baker *et al*., 1999). Moreover, a $PhoP^c$ strain often displays completely different phenotypes from those exhibited by wild-type *Salmonella* experiencing inducing conditions, which is most vividly observed in its inability to survive within macrophages and to cause a lethal infection in mice (Miller and Mekalanos, 1990).

The RcsC/YojN/RcsB system was originally identified because of its role in the regulation of colanic acid capsule (Majdalani and Gottesman, 2005). However, it is now becoming increasingly clear that this system controls the expression of other surface molecules such as the flagella (Ferrieres and Clarke, 2003; Francez-Charlot *et al*., 2003). We have now determined that the Rcs system regulates the production of a normal LPS as the LPS profile of the *rcsB* mutant exhibited an abnormal migration in the bands containing 6–10 and 16–22 O-subunits attached to the lipid A-core (Fig. 4B). This indicates that RcsB regulates at least two aspects of the LPS: the chain length determinant wzz_{st} gene as well as the one(s) responsible for the wild-type mobility of the O-antigen bands.

O-antigen assembly requires the flippase Wzx, the polymerase Wzy and the chain length modulator Wzz_{st} (Whitfield *et al*., 1997; Raetz and Whitfield, 2002). The *wzx* and *wzy* genes do not appear to be regulated by the PmrA and RcsB proteins because the *pmrA rcsB* double mutant was not defective for O-antigen biosynthesis and assembly (Fig. 4). That the wzz_{st} gene is expressed independently of the O-antigen biosynthetic genes raises the possibility of the Wzz_{st} protein participating in additional activities unrelated to O-antigen length control.

Salmonella mutants lacking O-antigen are defective for swarming, presumably because the O-antigen increases the 'wettability' of the agar-swarming surface (Toguchi *et al*., 2000). As the wzz_{st} null mutant exhibited the same swarming behaviour as the wild-type strain (Fig. 6), it appears that it is the absence of the O-antigen (as opposed to the lack of O-antigen of a particular chain length) that is responsible for the swarming defect of LPS mutants (Toguchi *et al*., 2000). On the other hand, inactivation of the wzz_{st} gene overcame the precocious cell-swarming behaviour of the *rcsB* mutant (Fig. 6). In this context, the Wzz_{st} protein might be critical only when the bacterium is hyperflagellated because the RcsB protein is a transcriptional repressor of the master flagellar operon *flhDC* (Francez-Charlot *et al*., 2003), and flagella are required for swarming (Harshey and Matsuyama, 1994).

Finally, our current findings reinforce the notion that the PmrA/PmrB system is a major regulator of LPS structure as it controls genes that encode products that affect not only the lipid A, such as *pbgP*, *ugd* and *pmrC* (Gunn *et al*., 1998; Lee *et al*., 2004), but also the O-antigen chain length (this work) and the core region as well (i.e. *cptA*) (Tamayo *et al*., 2005) (Fig. 1).

Experimental procedures

Bacterial strains, molecular techniques and growth conditions

Bacterial strains used in this study are listed in Table 1. Mutations were introduced into different strains by phage P22-mediated transduction as described (Davis *et al*., 1980).

Table 1. Bacterial strains and plasmids used in this study.

Strain or plasmid	Description[a]	Reference or source
S. enterica serovar Typhimurium		
14028s	Wild-type	Fields *et al*. (1986)
EG13307	Δ*pmrA*	Mouslim and Groisman (2003)
EG13308	Δ*rcsB*	Mouslim and Groisman (2003)
EG13310	Δ*pmrA rcsB*::Km	Mouslim and Groisman (2003)
EG14873	*rcsC11*	Mouslim *et al*. (2004)
EG14929	Δwzz_{st}	This work
EG14930	Δwzz_{st}::*lacZY*	This work
EG15407	Δwzz_{st}::*lacZY rcsC11*	This work
EG15408	Δwzz_{st}::*lacZY rcsC11*Δ*yojN*::Cm	This work
EG15410	Δwzz_{st}::*lacZY*Δ*rcsB*	This work
EG16362	Δwzz_{st}::*lacZY*Δ*pmrA*	This work
EG16363	Δwzz_{st}::*lacZY*Δ*pmrA*Δ*rcsB*::Cm	This work
EG16364	Δwzz_{st}::*lacZY*Δ*pmrA phoP7953*::Tn*10*	This work
EG16365	Δwzz_{st}::*lacZY phoP7953*::Tn*10*	This work
EG16368	Δwzz_{st}::*lacZY rcsC11*Δ*rcsB*	This work
EG16370	Δwzz_{st}::*lacZY*Δ*ugd* promoter	This work
EG16371	Δwzz_{st}::*lacZY rcsC11 rcsA*::Tn*10*	This work
EG14876	*rcsC11 cps*::MudJ	Mouslim *et al*. (2004)
E. coli		
BL21(DE3)	*dcm ompT hsd gal* ϕ(DE3)	Studier *et al*. (1990)
EG12708	BL21(DE3)/pT7-7-*rcsB*-His6	This work
Plasmid		
pT7-7	rep_{pMBI} pT7, Ap^r	Tabor and Richardson (1985)
pT*7-7-rcsB*-His6	rep_{pMBI} pT7-*rcsB*-His6, Ap^r	This work

a. Gene designations are summarized by Sanderson *et al*. (1995).

Recombinant DNA techniques were performed according to standard protocols (Sambrook *et al*., 1989). Bacteria were grown at 37°C in Luria–Bertani (LB) (Sambrook *et al*., 1989) or in N-minimal medium [50 mM Trizma base, 50 mM bis-Tris, 50 mM KCl, 75 mM $(NH4)_2SO_4$ and 10 mM KH_2PO_4, pH 7.4] (Snavely *et al*., 1991), supplemented with 0.1% casamino acids, 38 mM glycerol and the indicated concentrations of $MgCl_2$ and $FeSO_4$. Kanamycin was used at a final concentration of 50 μg ml^{-1} and chloramphenicol at 25 μg ml^{-1}.

Introduction of gene fusions and mutations in the chromosomal wzz_{st} *locus*

The one-step gene-inactivation method (Datsenko and Wanner, 2000) was used to construct a strain deleted for the wzz_{st} gene. Construction of a chromosomal wzz_{st}::*lacZY* fusion strain was done as described (Ellermeier *et al*., 2002). Briefly, a Cm^R cassette was amplified by using pKD3 plasmid DNA as template and primers 3817 (5′-CACT GTCTCCAGCTTCATCCTTTTTTTAGTTAGGGTATCTTGTAG GCTGGAGCTGCTTCG-3′) and 3818 (5′-ATTTTACCT GTCGTAGCCGACCACCATCCGGCAAAGAAGCCATATGAA TATCCTCCTTAG-3′), and integrated into the chromosome. The junction region of the wzz_{st} gene and the Cm^R cassette was amplified from the chromosome and confirmed by direct nucleotide sequencing. After removing the Cm^R cassette, the *lacZY*-transcriptional fusion plasmid pCE36 was integrated into the FLP recombination target sequence immediately downstream of the wzz_{st} gene by FLP-mediated recombination.

β-Galactosidase assays

β-Galactosidase assays correspond to mean values of three independent experiments done in duplicate, and the activity was determined as described (Miller, 1972). Error bars correspond to the standard deviation.

S1 mapping of the wzz_{st} *promoter*

The S1 mapping assay was performed as described (Garcia Vescovi *et al*., 1996) with RNA harvested from mid-exponential phase cultures (OD_{600} of 0.4–0.6) grown in LB media or in minimal media containing 10 μM $MgCl_2$ or 10 μM $MgCl_2$ 100 μM $FeSO_4$. Total RNA was isolated with the RNA SV total isolation kit (Promega) according to the manufacturer's specifications, and the same amount of RNA was used in each S1 reaction. A PCR product generated with primers 4829 (5′-CCGCTCGAGTAAGGCAATCGGGGCTGA-3′) and 5646 (5′-TGTAGCAATAACTCAATCAAATCAATC-3′) and chromosomal DNA from *S. typhimurium* strain 14028s as template was used as probe. This probe was labelled at the 5′ end by phosphorylation with [γ^{32}P]-ATP using T4 polynucleotide kinase (Gibco BRL) as described (Mouslim and Groisman, 2003).

Real-time polymerase chain reaction

Primer pairs for quantitative analysis were designed using the Applied Biosystems (Foster City, CA) Primer Express software, which generated a list of primer pairs selected with Tm values between 58°C and 60°C, a total amplicon size between 60 and 80 bp, and no more than two Gs and/or Cs

in the last five bases. wzz_{st} was amplified using primer 5998 (5′-GGTGGTAATGCCCCCAAA-3′) and primer 6000 (5′-GATAATGCGGAAAACGCAGAGC-3′); for *pmrC* forward primer 3021 (5′-CATGATCGCCAATATGATGGAT-3′) and reverse primer 3022 (5′-GGCGTCATCAGCGCAAA-3′), and for *wzc* forward primer #5332 (5′-GGGCAATCCGAC CGATCT-3′) and reverse primer #5333 (5′-CTGCATCATG GCGAAATGC-3′). A master mix for each primer set was prepared so that each well contained the following: 2× SYBR Green Master Mix (Applied Biosystems) that contained all the nucleotides, polymerase, reaction buffer and SYBR Green dye; forward and reverse primers at concentrations of 50, 300 or 900 nM depending on previously determined optimal concentrations; and nuclease-free water to a total of 23 μl per well. To this, 2.0 μl of each sample or standard was added, and the plate was briefly centrifuged and placed in the thermocycler for analysis. Species-specific real-time quantitative PCR was performed using the Applied Biosystems Prism 7000 sequence detection system, with fluorescence detection of SYBR Green dye. Amplification consisted of an initial hold for 10 min followed by 40–50 cycles at 95°C for 15–25 s and at 58–60°C for 60–90 s. Standards were run on the same plate in triplicate, and the amount of DNA of the target species was calculated as a percentage of the total DNA added to the wells (determined spectrophotometrically). In general, approximately 2–20 ng of target cDNA from same RNA samples used in the S1 mapping assay was loaded in each well and standards serially diluted in 1:5 increments.

DNase I footprinting assay

DNase I protection assays were carried out using the appropriately labelled primers. DNA fragments used for DNase I footprinting were amplified by the PCR using chromosomal DNA from *S. typhimurium* strain 14028s as template. Prior to the PCR, primers 5646 (5′-TGTAGCAATAACTCAAT CAAATCAATC-3′) and 4829 (5′-CCGCTCGAGTAAG GCAATCGGGGCTGA-3′), which anneal to the coding and non-coding strand of wzz_{st} respectively, were labelled with T4 polynucleotide kinase and [γ^{32}P]-ATP. The wzz_{st} promoter region was amplified with the labelled primers 5646 and 4829 for the coding strand or with the labelled primers 4829 and 5646 for the non-coding strand. Approximately 25 pmol of labelled DNA and 0, 5, 10, 40 or 80 pmol of PmrA-H6 or RcsB-H6 proteins were incubated in 100 μl of 2 mM Hepes (pH 7.9)/10 mM KCl/20 μM EDTA/500 μg ml^{-1} BSA/20 μg ml^{-1} poly(dI-dC)/2% glycerol for 20 min at room temperature. DNase I (Gibco/BRL) (0.01 units), 100 μM $CaCl_2$ and 100 μM $MgCl_2$ were added and incubated for 3 min at room temperature. The reaction was stopped by the addition of 100 μl of phenol chloroform, and the aqueous phase was precipitated with ethanol. The precipitate was dissolved in sequence-loading buffer and electrophoresed on a 6% acrylamide/7 M urea gel together with a sequence ladder initiated with the appropriate primer by using the T7 Sequenase 2.0 DNA-sequencing kit (Amersham Biosciences).

Purification of the PmrA and RcsB proteins

The histidine-tagged PmrA protein was purified as described (Wosten *et al*., 2000). The histidine-tagged RcsB protein was expressed in *E. coli* BL21(DE3) containing plasmid pT7.7-*rcsB*-His6 (EG12708). Cells were grown in 500 ml of LB broth without NaCl at 30°C to an OD_{600} of 0.6. Then, NaCl was added to a final concentration of 0.3 M, and incubation was continued for 4 h. Cells were harvested and suspended in 10 ml of buffer A (50 mM NaH_2PO_4, pH 8.0, 300 mM NaCl and 10 mM imidazole) and disrupted by sonication. Cell debris was pelleted by centrifugation at 3000 rpm for 15 min, then 3 ml of 50% nickel-nitrilotriacetic acid was added to the supernatant and incubated for 1 h at 4°C on a rocking platform. The RcsB-His6 protein was washed four times with 10 ml of buffer A containing imidazole (50 mM). The protein was eluted with 4 ml of buffer A containing imidazole (250 mM).

Lipopolysaccharide analysis

Lipopolysaccharide was purified from strains grown in N-minimal media containing 10 mM $MgCl_2$ or 10 μM $MgCl_2$ 100 μM $FeSO_4$ as described (Marolda *et al*., 1990). Cultures samples were adjusted to OD_{600} 1.0 in a final volume of 100 μl, and LPS samples normalized to the number of cells were separated on 12% acrylamide gels using a Tris-Glycine/SDS buffer system and stained by a modification of the conventional silver staining method (Fomsgaard *et al*., 1990).

Serum complement sensitivity assay

Salmonella typhimurium cells were grown in N-minimal media containing 10 mM $MgCl_2$ or 10 μM $MgCl_2$ 100 μM $FeSO_4$, until OD_{600} 0.25, harvested and suspended in PBS buffer. A total of 10^4 cells ml^{-1} were incubated at 37°C for 1 h with PBS or PBS 10% of human serum (Sigma). Samples were serially diluted, plated on LB agar plate and incubated overnight at 37°C to determine the cfu ml^{-1}. The per cent survival to serum was calculated as 100× (cfu ml^{-1} after 1 h in the presence of serum)/(cfu ml^{-1} at time 0 h in PBS).

Swarming assays

Swarming assays were conducted as described (Kim and Surette, 2004). The values of the distances from the centre to the edge of migrating colonies (in mm) were plotted against the times (in min) of incubation to estimate the average speed of migration (in mm min^{-1}).

Acknowledgements

We thank T. Latifi for technical assistance, C. L. Marolda for protocols on the purification and staining of the LPS; and C. Materon for comments on an earlier version of the manuscript. M. Delgado was a Pew Latin American Fellow. This work was supported, in part, by Grant 42336 from the NIH to E.A.G., who is an Investigator of the Howard Hughes Medical Institute.

References

Baker, S.J., Gunn, J.S., and Morona, R. (1999) The *Salmonella typhi* melittin resistance gene *pqaB* affects intracellu-

lar growth in PMA-differentiated U937 cells, polymyxin B resistance and lipopolysaccharide. *Microbiology* **145:** 367–378.

Bastin, D.A., Stevenson, G., Brown, P.K., Haase, A., and Reeves, P.R. (1993) Repeat unit polysaccharides of bacteria: a model for polymerization resembling that of ribosomes and fatty acid synthetase, with a novel mechanism for determining chain length. *Mol Microbiol* **7:** 725–734.

Breazeale, S.D., Ribeiro, A.A., and Raetz, C.R. (2003) Origin of lipid A species modified with 4-amino-4-deoxy-L-arabinose in polymyxin-resistant mutants of *Escherichia coli*. An aminotransferase (ArnB) that generates UDP-4-deoxyl-L-arabinose. *J Biol Chem* **278:** 24731–24739.

Burns, S.M., and Hull, S.I. (1998) Comparison of loss of serum resistance by defined lipopolysaccharide mutants and an acapsular mutant of uropathogenic *Escherichia coli* O75:K5. *Infect Immun* **66:** 4244–4253.

Chamnongpol, S., Dodson, W., Cromie, M.J., Harris, Z.L., and Groisman, E.A. (2002) Fe(III)-mediated cellular toxicity. *Mol Microbiol* **45:** 711–719.

Costa, C.S., and Anton, D.N. (2001) Role of the *ftsA1p* promoter in the resistance of mucoid mutants of *Salmonella enterica* to mecillinam: characterization of a new type of mucoid mutant. *FEMS Microbiol Lett* **200:** 201–205.

Cross, A.S., Kim, K.S., Wright, D.C., Sadoff, J.C., and Gemski, P. (1986) Role of lipopolysaccharide and capsule in the serum resistance of bacteremic strains of *Escherichia coli*. *J Infect Dis* **154:** 497–503.

Daniels, C., and Morona, R. (1999) Analysis of *Shigella flexneri wzz* (Rol) function by mutagenesis and cross-linking: *wzz* is able to oligomerize. *Mol Microbiol* **34:** 181–194.

Dasgupta, T., de Kievit, T.R., Masoud, H., Altman, E., Richards, J.C., Sadovskaya, I., *et al.* (1994) Characterization of lipopolysaccharide-deficient mutants of *Pseudomonas aeruginosa* derived from serotypes O3, O5, and O6. *Infect Immun* **62:** 809–817.

Datsenko, K.A., and Wanner, B.L. (2000) One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. *Proc Natl Acad Sci USA* **97:** 6640–6645.

Davis, R.W., Bolstein, D., and Roth, J.R. (1980) *Advanced Bacterial Genetics*. Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press.

Ellermeier, C.D., Janakiraman, A., and Slauch, J.M. (2002) Construction of targeted single copy lac fusions using lambda Red and FLP-mediated site-specific recombination in bacteria. *Gene* **290:** 153–161.

Ferrieres, L., and Clarke, D.J. (2003) The RcsC sensor kinase is required for normal biofilm formation in *Escherichia coli* K-12 and controls the expression of a regulon in response to growth on a solid surface. *Mol Microbiol* **50:** 1665–1682.

Fields, P.I., Swanson, R.V., Haidaris, C.G., and Heffron, F. (1986) Mutants of *Salmonella typhimurium* that cannot survive within the macrophage are avirulent. *Proc Natl Acad Sci USA* **83:** 5189–5193.

Fomsgaard, A., Freudenberg, M.A., and Galanos, C. (1990) Modification of the silver staining technique to detect lipopolysaccharide in polyacrylamide gels. *J Clin Microbiol* **28:** 2627–2631.

Francez-Charlot, A., Laugel, B., Van Gemert, A., Dubarry, N., Wiorowski, F., Castanie-Cornet, M.P., *et al.* (2003) RcsCDB His-Asp phosphorelay system negatively regulates the *flhDC* operon in *Escherichia coli*. *Mol Microbiol* **49:** 823–832.

Franco, A.V., Liu, D., and Reeves, P.R. (1998) The *wzz* (cld) protein in *Escherichia coli*: amino acid sequence variation determines O-antigen chain length specificity. *J Bacteriol* **180:** 2670–2675.

Garcia Vescovi, E., Soncini, F.C., and Groisman, E.A. (1996) Mg^{2+} as an extracellular signal: environmental regulation of *Salmonella* virulence. *Cell* **84:** 165–174.

Groisman, E.A., Kayser, J., and Soncini, F.C. (1997) Regulation of polymyxin resistance and adaptation to low-Mg2+ environments. *J Bacteriol* **179:** 7040–7045.

Gunn, J.S., Lim, K.B., Krueger, J., Kim, K., Guo, L., Hackett, M., and Miller, S.I. (1998) PmrA-PmrB-regulated genes necessary for 4-aminoarabinose lipid A modification and polymyxin resistance. *Mol Microbiol* **27:** 1171–1182.

Gunn, J.S., Ryan, S.S., Van Velkinburgh, J.C., Ernst, R.K., and Miller, S.I. (2000) Genetic and functional analysis of a PmrA-PmrB-regulated locus necessary for lipopolysaccharide modification, antimicrobial peptide resistance, and oral virulence of *Salmonella enterica* serovar Typhimurium. *Infect Immun* **68:** 6139–6146.

Harshey, R.M., and Matsuyama, T. (1994) Dimorphic transition in *Escherichia coli* and *Salmonella typhimurium*: surface-induced differentiation into hyperflagellate swarmer cells. *Proc Natl Acad Sci USA* **91:** 8631–8635.

Joiner, K.A. (1985) Studies on the mechanism of bacterial resistance to complement-mediated killing and on the mechanism of action of bactericidal antibody. *Curr Top Microbiol Immunol* **121:** 99–133.

Joiner, K.A., Hammer, C.H., Brown, E.J., Cole, R.J., and Frank, M.M. (1982a) Studies on the mechanism of bacterial resistance to complement-mediated killing. I. Terminal complement components are deposited and released from *Salmonella minnesota* S218 without causing bacterial death. *J Exp Med* **155:** 797–808.

Joiner, K.A., Hammer, C.H., Brown, E.J., and Frank, M.M. (1982b) Studies on the mechanism of bacterial resistance to complement-mediated killing. II. C8 and C9 release C5b67 from the surface of *Salmonella minnesota* S218 because the terminal complex does not insert into the bacterial outer membrane. *J Exp Med* **155:** 809–819.

Kato, A., and Groisman, E.A. (2004) Connecting two-component regulatory systems by a protein that protects a response regulator from dephosphorylation by its cognate sensor. *Genes Dev* **18:** 2302–2313.

Kim, W., and Surette, M.G. (2004) Metabolic differentiation in actively swarming *Salmonella*. *Mol Microbiol* **54:** 702–714.

Kox, L.F., Wosten, M.M., and Groisman, E.A. (2000) A small protein that mediates the activation of a two-component system by another two-component system. *EMBO J* **19:** 1861–1872.

Lee, H., Hsu, F.F., Turk, J., and Groisman, E.A. (2004) The PmrA-regulated *pmrC* gene mediates phosphoethanolamine modification of lipid A and polymyxin resistance in *Salmonella enterica*. *J Bacteriol* **186:** 4124–4133.

Majdalani, N., and Gottesman, S. (2005) The Rcs Phosphorelay: a complex signal transduction system. *Annu Rev Microbiol* **59:** 379–405.

Marolda, C.L., Welsh, J., Dafoe, L., and Valvano, M.A. (1990) Genetic analysis of the O7-polysaccharide biosynthesis region from the *Escherichia coli* O7:K1 strain VW187. *J Bacteriol* **172:** 3590–3599.

Marolda, C.L., Feldman, M.F., and Valvano, M.A. (1999) Genetic organization of the O7-specific lipopolysaccharide biosynthesis cluster of *Escherichia coli* VW187 (O7:K1). *Microbiology* **145:** 2485–2495.

Miller, J.H. (1972) *Experiments in Molecular Genetics.* Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

Miller, S.I., and Mekalanos, J.J. (1990) Constitutive expression of the *phoP* regulon attenuates *Salmonella* virulence and survival within macrophages. *J Bacteriol* **172:** 2485–2490.

Morona, R., van den Bosch, L., and Manning, P.A. (1995) Molecular, genetic, and topological characterization of O-antigen chain length regulation in *Shigella flexneri*. *J Bacteriol* **177:** 1059–1068.

Mouslim, C., and Groisman, E.A. (2003) Control of the *Salmonella ugd* gene by three two-component regulatory systems. *Mol Microbiol* **47:** 335–344.

Mouslim, C., Latifi, T., and Groisman, E.A. (2003) Signal-dependent requirement for the co-activator protein RcsA in transcription of the RcsB-regulated *ugd* gene. *J Biol Chem* **278:** 50588–50595.

Murray, G.L., Attridge, S.R., and Morona, R. (2003) Regulation of *Salmonella typhimurium* lipopolysaccharide O antigen chain length is required for virulence; identification of FepE as a second Wzz. *Mol Microbiol* **47:** 1395–1406.

Murray, G.L., Attridge, S.R., and Morona, R. (2005) Inducible serum resistance in *Salmonella typhimurium* is dependent on *wzz*(*fepE*)-regulated very long O antigen chains. *Microbes Infect* **7:** 1296–1304.

Raetz, C.R., and Whitfield, C. (2002) Lipopolysaccharide endotoxins. *Annu Rev Biochem* **71:** 635–700.

Reeves, P.R. (1994) In biosynthesis and assembly of lipopolysaccharide. *New Compr Biochem* **27:** 281–314.

Roland, K.L., Martin, L.E., Esther, C.R., and Spitznagel, J.K. (1993) Spontaneous *pmrA* mutants of *Salmonella typhimurium* LT2 define a new two-component regulatory system with a possible role in virulence. *J Bacteriol* **175:** 4154–4164.

Sambrook, J., Fritsch, E.F., and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual.* Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

Sanderson, K.E., Hessel, A., and Rudd, K.E. (1995) Genetic map of *Salmonella typhimurium*, edition VIII. *Microbiol Rev* **59:** 241–303.

Skurnik, M., and Bengoechea, J.A. (2003) The biosynthesis and biological role of lipopolysaccharide O-antigens of pathogenic *Yersiniae*. *Carbohydr Res* **338:** 2521–2529.

Snavely, M.D., Gravina, S.A., Cheung, T.T., Miller, C.G., and Maguire, M.E. (1991) Magnesium transport in *Salmonella typhimurium*. Regulation of *mgtA and mgtB* expression. *J Biol Chem* **266:** 824–829.

Studier, F.W., Rosenberg, A.H., Dunn, J.J., and Dubendorff, J.W. (1990) Use of T7 RNA polymerase to direct expression of cloned genes. *Methods Enzymol* **185:** 60–89.

Sturny, R., Cam, K., Gutierrez, C., and Conter, A. (2003) NhaR and RcsB independently regulate the *osmCp1* promoter of *Escherichia coli* at overlapping regulatory sites. *J Bacteriol* **185:** 4298–4304.

Tabor, S., and Richardson, C.C. (1985) A bacteriophage T7 RNA polymerase/promoter system for controlled exclusive expression of specific genes. *Proc Natl Acad Sci USA* **82:** 1074–1078.

Takeda, S., Fujisawa, Y., Matsubara, M., Aiba, H., and Mizuno, T. (2001) A novel feature of the multistep phosphorelay in *Escherichia coli*: a revised model of the RcsC $\rightarrow$ YojN $\rightarrow$ RcsB signalling pathway implicated in capsular synthesis and swarming behaviour. *Mol Microbiol* **40:** 440–450.

Tamayo, R., Choudhury, B., Septer, A., Merighi, M., Carlson, R., and Gunn, J.S. (2005) Identification of *cptA*, a PmrA-regulated locus required for phosphoethanolamine modification of the *Salmonella enterica* serovar Typhimurium lipopolysaccharide core. *J Bacteriol* **187:** 3391–3399.

Tierrez, A., and Garcia-del Portillo, F. (2004) The *Salmonella* membrane protein IgaA modulates the activity of the RcsC-YojN-RcsB and PhoP-PhoQ regulons. *J Bacteriol* **186:** 7481–7489.

Toguchi, A., Siano, M., Burkart, M., and Harshey, R.M. (2000) Genetics of swarming motility in *Salmonella enterica* serovar Typhimurium: critical role for lipopolysaccharide. *J Bacteriol* **182:** 6308–6321.

Trent, M.S., Ribeiro, A.A., Doerrler, W.T., Lin, S., Cotter, R.J., and Raetz, C.R. (2001) Accumulation of a polyisoprene-linked amino sugar in polymyxin-resistant *Salmonella typhimurium* and *Escherichia coli*: structural characterization and transfer to lipid A in the periplasm. *J Biol Chem* **276:** 43132–43144.

Whitfield, C., Amor, P.A., and Koplin, R. (1997) Modulation of the surface architecture of gram-negative bacteria by the action of surface polymer: lipid A-core ligase and by determinants of polymer chain length. *Mol Microbiol* **23:** 629–638.

Wosten, M.M., Kox, L.F., Chamnongpol, S., Soncini, F.C., and Groisman, E.A. (2000) A signal transduction system that responds to extracellular iron. *Cell* **103:** 113–125.

