RpoS and RpoN are involved in the growth-dependent regulation of *rfaH* transcription and O antigen expression in *Salmonella enterica* serovar typhi

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Abstract

We reported earlier that the production of O antigen lipopolysaccharide (LPS) by *Salmonella enterica* serovar Typhi (*Salmonella typhi*) increases at the onset of stationary phase and correlates with a growth-regulated expression of the *rfaH* gene under the control of the alternative sigma factor RpoN (Microbiology 148 (2002) 3789). In this study, we demonstrate that RpoS also modulates *rfaH* promoter activity as revealed by the absence of growth-dependent regulation of an *rfaH–lacZ* transcriptional fusion and O antigen production in a *S. typhi rpoS* mutant. Introduction of a constitutively expressed *rpoN* gene into the *rpoS* mutant restored increased production of O antigen during stationary phase, suggesting that constitutive production of RpoN could overcome the RpoS defect. Similar results were observed when an *rpoS rpoN* double mutant was transformed with the intact *rpoN* gene. Thus, we conclude that both RpoS and RpoN control the *rfaH* promoter activity and concomitantly, the production of O-specific LPS in *S. typhi*.

Keywords: Lipopolysaccharide; Regulation; Sigma factors

1. Introduction

Salmonella enterica serovar Typhi (*Salmonella typhi*) is a human pathogen that causes typhoid fever, a worldwide spread systemic disease, with an estimated incidence of 16 million cases annually [1]. Lipopolysaccharide (LPS), the major component of the bacterial cell envelope, has been recognized as an important virulence factor of *Salmonella* [2–4]. LPS contributes to the pathogenesis of *S. typhi* by preventing complement-mediated bacterial killing [2] and promoting invasion of intestinal epithelial cells [5,6].

The LPS molecule is composed of three covalently-linked domains: the lipid A region which is embedded in the outer membrane, the core oligosaccharide and the O-specific polysaccharide chain (or O antigen), that is exposed to the bacterial surface [7,8]. Most of the biological effects of LPS are stimulated by lipid A, and for this reason this portion of LPS is also referred to as endotoxin [9]. Also, there is a solid evidence that the O antigen plays an important role in colonization of host tissues [10–12] as well as in resistance to components of the innate immune system [13,14].

The biosynthesis of the O-specific polysaccharide requires numerous enzymatic activities that are encoded in the *wb** gene cluster [15–17]. The transcription of the *wb** genes is subject to positive regulation by the elongation factor RfaH, a homologue of the NusG factor [18,19]. RfaH also regulates gene expression of the haemolysin operon [20–22], polysaccharide capsule genes [23], F plasmid *tra* operon [24], and a gene involved in iron acquisition [25]. RfaH-mediated regulation requires a 5′-proximally transcribed nucleic acid sequence, known as *ops* (for operon polarity suppressor [26,27]), that causes transcriptional pausing in vitro [28] and in vivo [22]. Sequences containing *ops* elements are present near the promoter region in the O-polysaccharide gene clusters of *Salmonella* and other enteric bacteria [29].

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Previous work in our laboratory demonstrated that the function of the *rfaH* gene is essential for production of the *S. typhi* Ty2 O-specific polysaccharide [30]. Also, we discovered that *rfaH* gene expression is growth phase-dependent, displaying the highest expression during stationary phase [30]. The pattern of O antigen production during the bacterial growth cycle reflects the differential *rfaH* expression [31]. Moreover, the RNA polymerase sigma factor RpoN (σ^{54} or σ^{N}) is involved in the growth-dependent regulation of *rfaH* [31]. In this study, we demonstrate that the alternative sigma factor RpoS (σ^{38} or σ^{S}), a global regulator of gene expression during the transition to stationary phase [32], also controls the expression of the *rfaH* gene, and that both RpoN and RpoS are involved in controlling the production of O antigen LPS in *S. typhi*.

2. Results and discussion

2.1. Role of RpoS in the growth phase-dependent regulation of rfaH and O antigen production

To investigate whether RpoS played any role in the growth phase-dependent regulation of *rfaH*, we constructed strain M103 (Table 1), an isogenic derivative of *S. typhi* Ty2 that carries a deletion of the *rpoS* gene. The strain M103 was transformed with plasmid pCE334 containing the *rfaH* promoter region cloned upstream of the promoterless *lacZ* operon [31]. Fig. 1 shows that the production of β-galactosidase in strain M103/pCE334 did not significantly change over time, except for only a slight increase at late logarithmic and stationary growth phases. In contrast, the control experiment with the wild type Ty2/pCE334 strain revealed that the β-galactosidase expression driven by the *rfaH* promoter increased almost four-fold when bacteria reached stationary phase. The lack of growth-dependent variations in the *rfaH* promoter activity of the *rpoS* mutant suggested that the promoter activity may be modulated by RpoS.

We also compared the production of LPS by M103 (Δ*rpoS*) and Ty2 at various times during growth. In all of these experiments, the LPS preparations were normalized by cell mass (Section 3) to avoid artifacts due to the different growth rates of the *rpoS* deletion mutant and the parental strain, especially when reaching stationary phase (Fig. 1). Therefore, despite that the optical densities reached at the mid-logarithmic and stationary phases in M103 and wild type Ty2 strains differed, they corresponded to identical growth stages. As with β-galactosidase expression, no significant variation in the levels of O antigen production at mid-logarithmic and stationary phases of growth was detected in strain M103 (Fig. 2A: lanes 3 and 4). Densitometric analysis of lanes 3 and 4 (Fig. 2B) showed that the ratios of O antigen to the lipid A–core band were not significantly different. In contrast, the production of O antigen in the wild type *S. typhi* Ty2 during stationary phase increased approximately 2.5-fold relative to the amount of

Table 1
S. typhi strains and plasmids used in this study

Strain or plasmid	Relevant properties	Source or reference
Salmonella typhi		
Ty2	*Salmonella enterica* serovar Typhi, wild-type	ISP[a]
M103	Ty2 Δ*rpoS*, Cam^R	This study
M161	Ty2 Δ*rpoN*, Cam^R	[31]
M265	Ty2 Δ*rpoN*Δ*rpoS*, Cam^R	This study
Plasmids		
PGEM-T Easy	Cloning vector, Amp^R	Promega
pKD46	*bla* P_{BAD} *gam bet exo* pSC101 oriTS	[36]
pKD3	*bla* FRT *cat* FRT PS1 PS2 ori R6K	[36]
pCP20	*bla cat cI*857 λP_R *flp* pSC101 oriTS	[37]
pSM103	PGEM-T Easy containing the wild type *rpoS* gene from *S. typhi* Ty2. Amp^R	This study
pSM161	PGEM-T Easy containing the wild type *rpoN* gene from *S. typhi* Ty2. Amp^R	This study
pCE334	PFZY1 containing a 334 bp fragment of the *yigC–rfaH* intergenic region from *S. typhi* Ty2. Amp^R	[31]
PKHT19	pBluescript KSII + containing the wild type *rfaH* gene from *S. typhi* Ty2. Amp^R	[30]

Cam, chloramphenicol; Amp, ampicillin.
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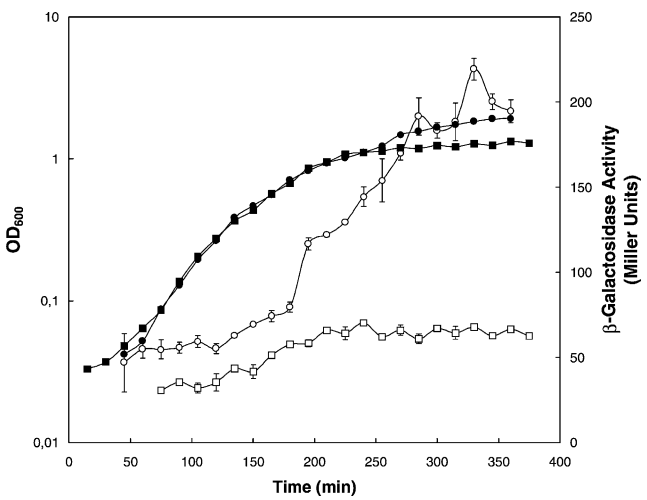

Fig. 1. Growth and expression of *rfaH–lacZ* transcriptional fusion in *S. typhi* strains Ty2/pCE334 and M103(Δ*rpoS*)/pCE334. Growth (OD_{600}) of Ty2/pCE334 (●) and M103/pCE334 (■) and expression of *rfaH–lacZ* in Ty2/pCE334 (○) and M103/pCE334 (□), measured as β-galactosidase activity in Miller units. Data are the mean ± SD of three independent assays.

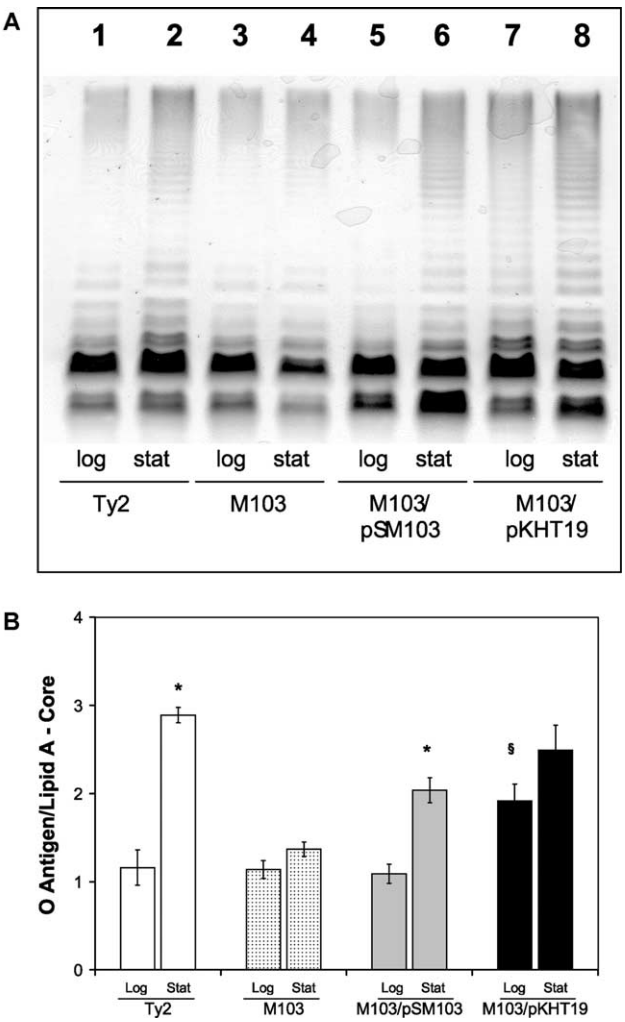

Fig. 2. Effect of an *rpoS* mutation on LPS production in *S. typhi*. (A) LPS profiles of cells grown in LB to the following OD_{600}: lane 1, *S. typhi* Ty2: 0.223; lane 2, *S. typhi* Ty2: 1.663; lane 3, M103 (Δ*rpoS*): 0.139; lane 4, M103 (Δ*rpoS*): 1.123; lane 5, M103/pSM103: 0.195; lane 6, M103/pSM103: 1.195; lane 7, M103/pKHT19: 0.208; lane 8, M103/pKHT19: 1.375. (B) Densitometric analysis of the gel in Fig. 2A. Bars represent the O antigen levels relative to levels of the lipid A-core region in each lane. (*indicates significant differences in the amount of O antigen produced by each strain grown to mid-logarithmic and stationary phase, $p < 0.01$ $n = 4$, one way ANOVA and Tukey post test. §indicates significant differences in the amount of O antigen produced by Ty2 strain and M103/pKHT19 grown to mid-logarithmic phase, $p < 0.01$ $n = 4$, one way ANOVA and Tukey post test).

O antigen in mid-logarithmic phase (Fig. 2A, lanes 1 and 2 and Fig. 2B). When strain M103 was complemented with pSM103 carrying the intact *rpoS* gene (Table 1), the proportion of O antigen to lipid A–core in cells grown to stationary phase was 1.8-fold higher than in cells reaching mid-logarithmic phase (Fig. 2A, lanes 5 and 6 and Fig. 2B). Therefore, the reduction in O antigen production during stationary phase in the *rpoS*-defective mutant reflects the lack of growth-dependent transcriptional activation of *rfaH*. This conclusion is supported by the observation of high O antigen expression in both logarithmic and stationary phases when the *rpoS* mutant was transformed with the high-copy number plasmid pKHT19 (Table 1), harboring the *rfaH* gene under the control of the *Plac* promoter (Fig. 2A, lanes 7 and 8 and Fig. 2B).

2.2. Interplay of RpoS and RpoN in the growth phase-dependent regulation of rfaH and O antigen production

In a previous study, we demonstrated that expression of *rfaH* in *S. typhi* Ty2 is regulated, either directly or indirectly, by the alternative sigma factor RpoN and that inactivation of the *rpoN* gene abolishes the growth-phase-dependent induction of *rfaH* expression [31]. The results described above indicated that RpoS is an additional sigma factor involved in *rfaH* expression. To investigate a possible relationship between these two sigma factors in the transcription of the *rfaH* gene, we constructed an *rpoS rpoN* double mutant of *S. typhi* Ty2. The resulting strain, designated M265 (Ty2 Δ*rpoN* Δ*rpoS*, Table 1), was transformed with plasmid pCE334 and the production of β-galactosidase was determined at mid-logarithmic, late logarithmic and stationary phases of growth. As shown in Fig. 3, the *rfaH–lacZ* expression did not vary in response to the bacterial growth phase; the units of enzyme activity were similar to the levels determined in the *rpoS* and the *rpoN* single mutants.

These results suggested that RpoS and RpoN could both be involved in controlling the expression of the *rfaH* promoter, and indirectly, the synthesis of O-specific LPS. To investigate the interplay of RpoS and RpoN, we introduced pSM103 into the *rpoN* mutant. This plasmid contains a wild-type *rpoS* gene cloned in pGEM-T (pSM103) that makes *rpoS* expression independent of *rpoN*. No significant increase in O-specific LPS expression during stationary phase was detected in strain M161/pSM103 (Fig. 4A, lanes 1 and 2 and Fig. 4B).

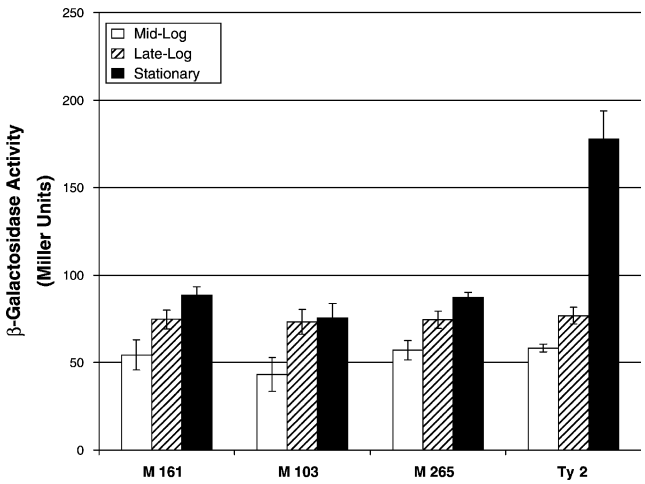

Fig. 3. Effect of *rpoN*, *rpoS* and *rpoN rpoS* mutations on transcription from the *rfaH* promoter. *rfaH–lacZ* expression, measured as β-galactosidase activity, was monitored in strains *S. typhi* Ty2, M161 (Δ*rpoN*), M103 (Δ*rpoS*) and M265 (Δ*rpoN*Δ*rpoS*) grown to mid-logarithmic (open bars), late-logarithmic (dashed bars) and stationary phase (filled bars) of growth.

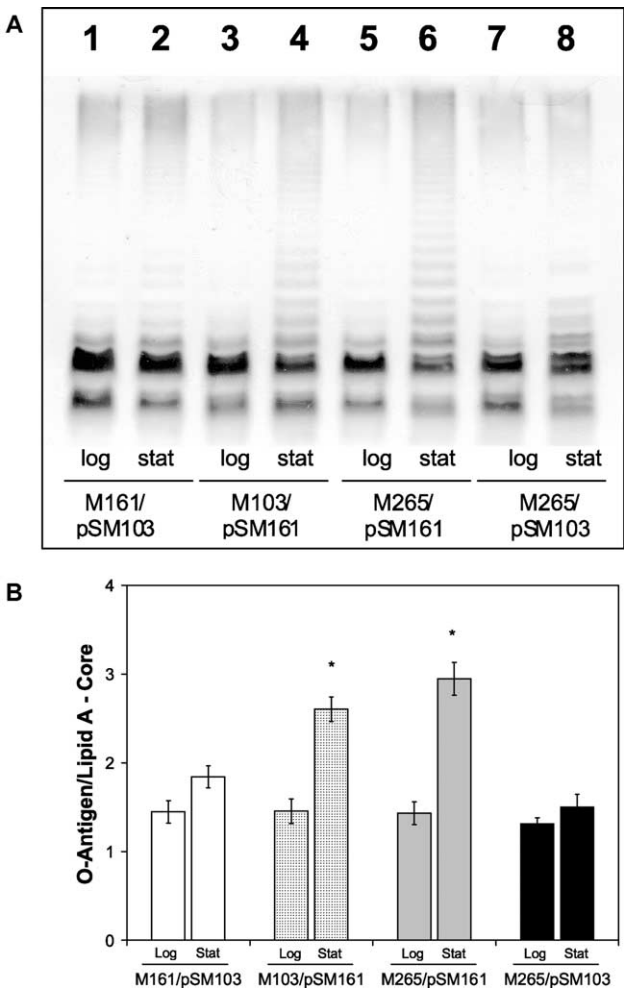

Fig. 4. Role of RpoN and RpoS on O antigen expression in *S. typhi*. (A) LPS profiles of cells grown in LB to the following OD_{600}: lane 1, M161/pSM103: 0.125; lane 2, M161/pSM103: 1.436; lane 3, M103/pSM161: 0.204; lane 4, M103/pSM161: 1.579; lane 5, M265/pSM161: 0.149; lane 6, M265/pSM161: 1.687; lane 7, M265/pSM103: 0.129; lane 8, M265/pSM103: 1.659. (B) Densitometric analysis of the gel in Fig. 4A. Bars represent the O antigen levels relative to levels of the lipid A–core region in each lane. (*indicates significant differences in the amount of O antigen produced by each strain grown to mid-logarithmic and stationary phase, $p < 0.01$ $n = 4$, one way ANOVA and Tukey post test).

In contrast, when the *rpoS* mutant was complemented with a wild-type copy of the *rpoN* gene, also cloned in pGEM-T (pSM161), a significant increase in the amount of O antigen expressed during stationary phase was observed (Fig. 4A, lanes 3 and 4 and Fig. 4B), suggesting that constitutive production of RpoN could overcome the RpoS deficiency. Similar LPS profiles were obtained when the *rpoS rpoN* double mutant (M265) was transformed with the intact *rpoN* gene (Fig. 4A, lanes 5 and 6 and Fig. 4B) but not when M265 was transformed with the *rpoS* gene (Fig. 4A, lanes 7 and 8 and Fig. 4B).

These results suggest that the RpoS effect on *rfaH* expression, as well as on the production of O antigen, is mediated through RpoN. This notion is consistent with the presence of sequences that resemble the −12 and −24 recognition regions for the alternative sigma factor RpoN in the *rfaH* gene promoter region [31]. Furthermore, preliminary band shift experiments using a DNA fragment encompassing the *rfaH* promoter region incubated with crude extracts from the *rpoN* strain M161, have showed a qualitative modification of the migration pattern obtained with extracts from the wild-type strain *S. typhi* Ty2 (unpublished results).

2.3. Concluding remarks

Recently, Hübner et al. [33] have reported that the expression of *Borrelia burgdorferi* lipoproteins, OspC and DbpA, is controlled by an RpoN–RpoS regulatory pathway. In this network, RpoN controls the expression of RpoS, which in turn, governs the expression of these lipoproteins. Unlike OspC or DbpA lipoproteins, production of O antigen depends on a large number of enzymatic activities and assembly functions catalyzed by proteins whose genes, for the most part, are located within *wb** gene cluster, under the control of *rfaH*. Therefore, changes in *wb** gene expression are not necessarily causing quantitative changes in the O antigen production. However, our results indicate that the effect of RpoN/RpoS in *rfaH* transcription is of sufficient magnitude to impact on O antigen expression in a growth-dependent manner that is quantifiable by examining the differential ratio of O antigen polysaccharide relative to lipid A core oligosaccharide.

Our data support a model of interplay between these two general stress sigma factors, whereby the involvement of RpoN in a regulatory cascade appears to be downstream from RpoS, most likely by modulating the activity of the *rfaH* promoter. A unique feature of RpoN-dependent transcription is that the core RNA polymerase complexed with RpoN catalyzes strand separation only with the help of a transcriptional activator [34]. Because the expression of *rpoN* is constitutive [35], it is tempting to speculate that RpoS could control the expression of some transcriptional activator needed for RpoN-mediated transcription, during entry into stationary phase. We are in the process of identifying the protein(s) that bind to the *rfaH* promoter region to characterize in detail the molecular mechanisms involved in its regulation.

3. Materials and methods

Bacterial strains, plasmids, media and growth conditions. Table 1 summarizes the properties of the bacterial strains and plasmids used in this study. Bacteria were grown in Luria-Bertani medium (LB: bacto tryptone 10 g/l; bacto yeast extract 5 g/l; NaCl 5 g/l). Ampicillin (Amp, 100 μg/ml) and chloramphenicol (Cam, 20 μg/ml) were added when appropriate.

Mutagenesis of S. typhi rpoS and rpoN genes. Mutagenesis was performed by the method described by Datsenko and

Wanner [36] to create chromosomal mutations using PCR products. To disrupt the rpoS gene *S. typhi* Ty2 cells carrying the thermosensitive plasmid pKD46, that expresses the λ Red recombinase system, were transformed with a PCR product that was generated using plasmid pKD3, carrying a chloramphenicol acetyl transferase (*cat*) gene flanked by FRT sites, as template. Primers used were: WrpoS1: 5′-AATACGCTGAAAGTTCATGATTTAA-ATGAAGACGCGGGTGTAGGCTGGAGCTGCTTCG-3′ and WrpoS2: 5′-GCGCAGGTATACGTTCAGCTCTT-TAACAATGTGAATCATATGAATATCCTCCTTAG-3′. Transformants were plated on LB agar plates containing chloramphenicol, replica plated in the absence of antibiotic selection at 43 °C, and finally assayed for ampicillin sensitivity to confirm the loss of pKD46.

To construct the *rpoS rpoN* double mutant, the *cat* cassette inserted in the *rpoN* gene in mutant M161 [31] was removed by transforming this strain with plasmid pCP20 that expresses the FLP recombinase [37]. Transformants were plated on LB agar plates at 37 °C. Individual colonies were tested for loss of the *cat* cassette and of plasmid pCP20 by replica plating onto LB plates containing Cam and onto LB plates containing Amp. The Cam sensitive and Amp sensitive colonies were purified. The loss of the *cat* cassette was confirmed by PCR analysis. One of the transformants, strain M162(Δ*rpoN*), was mutagenized as described above to disrupt the *rpoS* gene. The resulting strain was designated M265 (Ty2 Δ*rpoN* Δ*rpoS*).

Cloning of the rpoS and rpoN genes. PCR amplification of the *S. typhi rpoS* and *rpoN* genes was carried out in a Perkin Elmer 2400 GeneAmp PCR system (55 °C annealing temperature and an elongation period of 90 s). The primers used were: rpoN1: 5′-AGACGAACACGTTAAGCGTG-3′ and rpoN2: 5′-GTCGTCACAAATTCACGCAG-3′ to amplify a DNA fragment containing the promoterless *rpoN* gene and NrpoS: 5′-GGAATTCCGATCACGGG-TAGGAGCCACC-3′ and CrpoS: 5′-GGGGTACCCCACT-TACTCGCGGAACAGCGC-3′ to amplify a DNA fragment containing the promoterless *rpoS* gene. The amplicons were cloned into pGEM-T Easy as recommended by the supplier. The recombinant plasmids were introduced in *E. coli* DH5α and recombinant clones were selected on LB agar plates containing Amp and X-Gal.

LPS analysis. Culture samples were adjusted to an $OD_{600} = 2.0$ in a final volume of 100 μl. Then, proteinase K-digested whole cell lysates were prepared as described [38] and LPS was separated on 14% acrylamide gels using a tricine–sodium dodecylsulfate (SDS) buffer system [39]. Gel loadings were normalized so that each sample represented the same number of cells. Each well was loaded with approximately 1×10^8 CFU. Gels were silver stained by a modification of the procedure of Tsai and Frasch [40]. LPS bands in all samples were compared by densitometric analysis with the UN-SCAN-IT gel software (Silk Scientific). The ratio of the relative intensity of the lipid A–core band with the average intensity of all the additional bands containing O antigen was calculated by quantifying the pixels in a narrow window across the center of each lane. The densitometric analysis was calibrated by determining the ratio of the relative intensity of the lipid A–core band with respect to the average intensity of the O antigen bands using a range of loading volumes of Ty2 LPS. Similar ratios were obtained over the loading range used in this study. The statistical significance of differences in the data was determined using the one way ANOVA test and comparing all pairs of columns using the Tukey post test.

β-galactosidase assays. Bacteria were grown overnight in LB, subcultured and grown in 100 ml of the same medium on an orbital shaker. Every 30 min, a 2 ml sample was withdrawn to measure the bacterial growth (OD_{600} and CFU/ml) and the β-galactosidase activity according to Ref. [41]. Enzyme activities (Miller units), normalized for cell density (OD_{600}), were calculated using the equation $((OD_{420} - 1.75\ OD_{550}) \times 1000)/(\text{reaction time (min)} \times \text{culture volume (ml)} \times OD_{600})$. Each sample was analyzed in triplicate during at least three independent experiments.

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