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Crl binds to domain 2 of σ^S and confers a competitive advantage to a natural *rpoS* mutant of *Salmonella enterica* serovar Typhi

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Running title: Crl binds to σ^S_2 and rescues a σ^S mutant protein

Key words: *Salmonella*, *crl*, *rpoS*, sigma factor, RNA polymerase

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1 **ABSTRACT**

2
3 The RpoS sigma factor (σ^S) is the master regulator of the bacterial response to a
4 variety of stresses. Mutants in *rpoS* arise in bacterial populations in the absence of stress,
5 probably as a consequence of a subtle balance between self-preservation and nutritional
6 competence. In this study, we characterized one natural *rpoS* mutant of *Salmonella enterica*
7 serovar Typhi (Ty19). We showed that the *rpoS* allele of Ty19 (*rpoS*_{Ty19}) led to the synthesis
8 of a σ^S _{Ty19} protein carrying a single glycine to valine substitution at position 282 in σ^S domain
9 4, which was much more dependent than the wild-type σ^S protein on activation by Crl, a
10 chaperone-like protein that increases the affinity of σ^S for the RNA polymerase core enzyme
11 (E). We used the bacterial adenylate cyclase two-hybrid system to demonstrate that Crl bound
12 to residues 72 to 167 of σ^S domain 2 and that G282V substitution did not directly affect Crl
13 binding. However, this substitution drastically reduced the ability of σ^S _{Ty19} to bind E in a
14 surface plasmon resonance assay, a defect partially rescued by Crl. The modelled structure of
15 the E σ^S holoenzyme suggested that substitution G282V could directly disrupt a favourable
16 interaction between σ^S and E. The *rpoS*_{Ty19} allele conferred a competitive fitness when the
17 bacterial population was wild-type for *crl*, but was outcompeted in Δ *crl* populations. Thus,
18 these results indicate that the competitive advantage of the *rpoS*_{Ty19} mutant is dependent on
19 Crl and suggest that *crl* plays a role in the appearance of *rpoS* mutants in bacterial
20 populations.

INTRODUCTION

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In bacteria, transcription depends on a RNA polymerase (RNAP) consisting of a catalytically active core enzyme (E) with a subunit structure $\alpha_2\beta\beta'\omega$, that associates with any one of several σ factors to form different $E\sigma$ holoenzymes. The σ subunit is required for specific promoter binding, and different σ factors direct RNAP to different classes of promoters, thereby modulating the gene expression patterns (17). The holoenzyme containing the σ^{70} subunit is responsible for the transcription of most genes during exponential growth (17). When cells enter stationary phase or undergo specific stress conditions (high osmolarity, low pH or high and low temperatures) during exponential growth, σ^S , which is encoded by the *rpoS* gene, becomes more abundant, associates with E, and directs the transcription of genes essential for the general stress response (17, 18, 21). In *Salmonella* and *Escherichia coli*, the σ^S regulon comprises more than 300 genes contributing to survival during stationary phase, adaptive stress responses, biofilm formation and virulence of *S. enterica* serovar Typhimurium, a wide-host range pathogen and a major cause of human gastroenteritis and food-borne disease (2, 48). However, the precise function of nearly half of the genes in the regulon is unknown.

An intriguing aspect of σ^S is the allelic variation of *rpoS* in *E. coli* and *Salmonella* (9, 13, 36, 38, 50). Because of its role in general stress resistance and its high position in the hierarchy of transcriptional regulators, one might expect σ^S to be conserved in bacteria from different environments. However, *rpoS* mutations accumulate in bacterial populations in the absence of environmental stress (31, 50). Indeed, *rpoS* is not essential in unstressed cells. But, when the culture medium is suboptimal or when bacteria are exposed to additional environmental stresses, the absence of stress resistance resulting from *rpoS* mutations becomes detrimental, and *rpoS* null mutants are outcompeted (31, 40, 50). The selection

1 pressures on *rpoS* are likely the consequence of a subtle balance between self-preservation
2 and nutritional competence (so-called SPANC balance, 13). σ^{70} is needed for vegetative
3 growth, whereas σ^S switches the cell to stress resistance and has a negative effect on the
4 expression of σ^{70} -dependent genes involved in nutrient scavenging and membrane
5 permeability (2, 34, 48). An imbalance between the two sigma factors could reduce the fitness
6 of bacteria in particular situations.

7 The concentration of σ^S is obviously the major but not the only determinant of σ^S -
8 dependent gene regulation. Sigma factors compete for a limited amount of E in the cells (16-
9 18, 32). σ^S is generally much less abundant than σ^{70} and exhibits the weakest affinity of all σ
10 factors for E *in vitro* (18). The cell uses at least two strategies to ensure the switch between
11 σ^{70} and σ^S -associated RNAP and to allow gene expression to be reprogrammed upon entry
12 into stationary phase. Several factors (Rsd, 6S RNA, ppGpp and DksA) indirectly increase σ^S
13 competitiveness by decreasing the ability of σ^{70} to bind to E or by inhibiting $E\sigma^{70}$ activity
14 (21). Furthermore, the non-conventional regulatory protein Crl increases the performance of
15 σ^S (14, 28, 35, 37, 39, 40, 46). Unlike classical regulators of transcription, Crl binds σ^S
16 instead of DNA (3, 12) and the transient interaction of Crl with σ^S increases the association
17 rate of σ^S to E (12), thereby facilitating RNAP holoenzyme $E\sigma^S$ formation (12, 14, 46).

18 We previously isolated *rpoS* mutants among clinical isolates of *Salmonella enterica*
19 serovar Typhi (36, data not shown). In the present study, we report the characterization of
20 one of these mutants, *S. Typhi* strain Ty19. This mutant initially retained our attention
21 because it produced lower levels of σ^S than wild-type strains but was highly resistant to
22 hydrogen peroxide (H_2O_2), a phenotype dependent on σ^S activity and used as a tool to screen
23 *rpoS* mutants. We here demonstrate that the low cellular activity of the σ^S_{Ty19} mutant protein
24 is partially rescued by Crl and, consistently, that the competitive fitness of the *rpoS*_{Ty19} mutant

- 1 is conditioned by the *crl* status of the bacterial population. We also report the identification of
- 2 a region of σ^S that binds Crl.

MATERIALS AND METHODS

1
2
3 **Bacterial strains, plasmids and growth conditions.** Strains and plasmids are listed in Table
4 1. *S. Typhi* clinical strains, including Ty19, were isolated from patients with septicemia and
5 provided by F. Grimont and P. Bouvet from the National Reference Center for *Salmonella*
6 (Institut Pasteur). Bacteriophage P22HT105/1*int* was used to transfer mutations between
7 *Salmonella* strains by transduction (42). Green plates, for screening for P22-infected cells or
8 lysogens, were prepared as described previously (45). Strains were routinely cultured in Luria
9 Bertani medium (LB; 41). Antibiotics were used at the following concentrations: carbenicillin
10 (Cb), $100 \mu\text{g ml}^{-1}$; chloramphenicol (Cm), $15 \mu\text{g ml}^{-1}$ for the chromosomal resistance gene
11 and $30 \mu\text{g ml}^{-1}$ for the plasmid resistance gene; kanamycin, (Km) $50 \mu\text{g ml}^{-1}$; and tetracycline
12 (Tet) $20 \mu\text{g ml}^{-1}$.

13
14 **Oxidative shock survival assay.** Cells were grown to stationary phase in LB (optical density
15 at 600 nm (OD_{600}) of 3.5 to 4), washed, resuspended in PBS and mixed with H_2O_2 at a final
16 concentration of 15 mM. Aliquots of bacteria were removed at time intervals, and numbers of
17 colony forming cells were determined on LB plates.

18
19 **DNA manipulations.** Standard molecular biology techniques were used (41).
20 Oligonucleotides were obtained from Sigma-Aldrich (France). DNA sequencing was
21 performed by Beckman Coulter Genomics (France).

22

1 **Cloning of the *rpoS*_{Ty19} allele.** Total DNA from *S. Typhi* Ty19 was cleaved with BglII and
2 the resulting fragments were cloned into the BamHI site of pACYC184. Recombinant
3 plasmids were then transformed into the *E. coli rpoS* mutant strain MC1061K. Transformants
4 harboring plasmids with the 6kb BglII carrying *rpoS*_{Ty19} fragment (pAC*rpoS*_{Ty19}) were
5 selected after colony hybridization with a *rpoS* probe as described previously (22). The
6 presence of *rpoS*_{Ty19} on pAC*rpoS*_{Ty19} was checked by DNA sequencing. A 3.5 kb HindIII-
7 ScaI fragment carrying *rpoS*_{Ty19} was subsequently cloned into the HindIII and SmaI sites of
8 pUC19 to yield pUC*rpoS*_{Ty19}.

9
10 **Construction of *S. Typhi* Ty19K and *S. Typhimurium* 2922K*rpoS*_{Ty19}.** pUCK3Km was
11 used to construct a $\Delta rpoS$ mutant of *S. Typhi* Ty19. After electroporation in *S. Typhi* Ty19,
12 pUCK3Km appeared to be unstable. Recombination of the *kan* cartridge into the host
13 genome, with simultaneous loss of pUCK3Km, resulted in the isolation of clones that were
14 resistant to kanamycin and sensitive to carbenicillin. The presence of the *rpoS* mutation at the
15 appropriate site in the genome of one clone, designated Ty19K, was confirmed by PCR
16 analyses. The *rpoS* allele in *S. Typhimurium* ATCC14028 was replaced by the *rpoS*_{Ty19} allele
17 using the following strategy (40). pUC*rpoS*_{Ty19} contains the *rpoS*_{Ty19} allele of *S. Typhi* strain
18 Ty19 and the downstream genes, including a gene encoding a putative decarboxylase (named
19 STY3047 in *S. Typhi* and STM2922 in *S. Typhimurium*) that is not regulated by *rpoS* (2). In
20 pUC*rpoS*_{Ty19}K, a kanamycin resistance (Km) cartridge is located in the SmaI site of
21 STY3047. pUC*rpoS*_{Ty19}K was introduced by electroporation into ATCC*rpoS*, where it was
22 unstable. Recombination of the Km cartridge into the host genome with simultaneous loss of
23 pUC*rpoS*_{Ty19}K resulted in recombinants that were resistant to kanamycin and sensitive to
24 carbenicillin. A Km^R Cb^S Cm^S recombinant was selected, checked by PCR for the presence of

1 the STM2922::Km mutation and simultaneous replacement of the $\Delta rpoS$::Cm mutation by the
2 $rpoS_{Ty19}$ allele. The $rpoS_{Ty19}$ and STM2922::Km alleles were then co-transduced to
3 ATCC $rpoS$. Transductants that were Km^R but Cm^S were selected, and transduction of the
4 STM2922::Km mutation and simultaneous replacement of the $\Delta rpoS$::Cm mutation by the
5 $rpoS_{Ty19}$ allele were confirmed by PCR. One Km^R Cm^S strain, designated 2922K $rpoS_{Ty19}$, was
6 also checked by DNA sequencing for the presence of the $rpoS_{Ty19}$ allele.

7
8 **Survival and competition in stationary phase.** For survival assays, overnight LB cultures
9 were washed, resuspended in PBS to an OD₆₀₀ of 1.0, diluted in fresh LB medium to about
10 3000 cells ml⁻¹ and incubated at 37°C with shaking. Aliquots of bacteria were removed at
11 timed intervals and numbers of viable cells were determined on LB plates. For competition
12 assays, overnight LB cultures were washed and resuspended in PBS to an OD₆₀₀ of 1.0. Two
13 strains were mixed in equal numbers in fresh LB medium to give a total of about 3000 cells
14 ml⁻¹ and the mixture was incubated at 37°C with shaking. Aliquots of bacteria were removed
15 at timed intervals and numbers of viable cells of each strain were determined on LB plates
16 containing the appropriate antibiotics.

17
18 **Enzymatic assays.** β -galactosidase activity was measured as described by Miller (27) and is
19 expressed in Miller units.

20
21 **BACTH assays.** The bacterial adenylate cyclase-based two hybrid (BACTH) assay is
22 dependent upon the functional reconstitution of the *Bordetella pertussis* adenylyl cyclase T18
23 and T25 subdomains by two interacting partners (19). The resulting cAMP binds to and
24 activates the transcription activator CRP, a positive regulator of λ *lac* and *mal* operons
25 involved in lactose and maltose catabolism. Derivatives of pUT18 and pKT25, used in the

1 BACTH assays, were constructed by cloning PCR amplified DNA fragments encoding the
2 protein of interest from *S. Typhimurium* between the PstI and EcoRI sites of pUT18 and the
3 XbaI and KpnI sites of pKT25 as previously described (Table 1, 28). All plasmids were
4 confirmed to be correct by DNA sequencing. The *E. coli cya* strain BTH101 was transformed
5 with derivatives of plasmids pKT25 and pUT18 encoding proteins fused to the T25 and T18
6 fragments of *Bordetella pertussis* adenyl cyclase. Co-transformants were plated onto
7 MacConkey maltose plates supplemented with carbenicillin, kanamycin and 0.5 mM IPTG to
8 assess the Mal⁺ phenotype and on LB plates supplemented with 5-bromo-4-chloro-indolyl- β -
9 D-galactoside (X-Gal, 40 $\mu\text{g ml}^{-1}$), carbenicillin, kanamycin and 0.5 mM IPTG to assess the
10 Lac⁺ phenotype and β -galactosidase activity. Plates were incubated at 30°C for 3 days and
11 colonies were then collected and their β -galactosidase activities were measured as described
12 by Miller (27).

13

14 **Electrophoresis and immunoblot analysis of proteins.** Whole-cell extracts were prepared
15 and SDS-polyacrylamide gel electrophoresis was carried out as described (44). The amount of
16 protein in whole-cell lysates was determined using the DC Protein Assay kit (Bio-Rad). Equal
17 amounts of protein were loaded in each slot. The molecular sizes of the proteins were
18 estimated using molecular size standards (Fermentas, France). Rabbit antibodies against the
19 σ^S protein of *S. enterica* serovar Typhimurium were from Coynault *et al.* (9). Proteins were
20 transferred to Amersham Hybond P membranes (GE Healthcare) and incubated with the
21 polyclonal rabbit antibody serum as previously described (9). Bound antibodies were detected
22 using a secondary anti-rabbit antibody linked to peroxidase and the Amersham ECL plus
23 western blotting detection system kit (GE Healthcare).

24

1 ***In vitro* transcription.** Single round transcription assays were carried out using pJCD01
2 plasmid derivatives, which harbour the promoter of interest cloned upstream of the *rrnB* T1
3 terminator (40). pJCD*poxB* was constructed as follows: the *S. Typhimurium poxB* fragment
4 (extending from -97 to +81 relative to the transcription start) was generated by PCR from
5 chromosomal DNA of strain ATCC14028 using primers 5'-
6 GTCAGCGAATTCGGGCGATTTACCACTCGC-3' and 5'-
7 CCAGACCTGCAGACGCCAGCCTGTTCCAGC -3'. The fragment was cleaved by EcoRI
8 and PstI and inserted into the pJCD01 vector cleaved by EcoRI and PstI. *E. coli* core enzyme
9 and *S. Typhimurium* Crl were prepared according respectively to Lederer *et al.*, (25) and
10 England *et al.* (12). The DNA encoding *rpoS*_{Ty19} was amplified using primers HK1 5'-
11 AGGCTCGGATCCATGAGTCAGAATACGCTGAAAGTTCAT-3' and HK2 5'-
12 TTCCGAAAGCTTTTACTCGCGGAACAGCGCTTCGATATT-3' and cloned into the
13 BamHI and HindIII sites of pQE30 to yield pQE30*rpoS*_{Ty19}. The nucleotide sequence of the
14 *rpoS* insert in pQE30*rpoS*_{Ty19} was checked by DNA sequencing. His₆-σ^S_{WT} and His₆-σ^S_{Ty19}
15 were purified from JM109 carrying plasmids pQE*rpoS* and pQE*rpoS*_{Ty19} as described in
16 Monteil *et al.* (28), except that the temperature of the cultures were maintained respectively at
17 28°C and 19°C after 1 mM IPTG addition. For run-off assays, 5 µl of RNA polymerase (σ^S:
18 300 nM; core: 120 nM) were incubated in buffer A (40 mM Hepes pH 8.0, 10 mM MgCl₂,
19 100 mM K-glutamate, 2 mM DTT supplemented with 500 µg/ml BSA) with 5 µl of buffer
20 with or without Crl (6 µM) for 20 min at 30°C. 5 µl of DNA template were then added at a
21 final concentration of 10 nM and the mixture incubated for 12 min at 30°C. Elongation was
22 started by the addition of a 5 µl mixture containing 1 mM CTP, 1 mM GTP and 1 mM UTP,
23 100 µM ATP, 1 µCi of [α -³²P]-ATP and 600 µg ml⁻¹ heparin. After 10 min the reactions were
24 stopped by the addition of formamide containing 10 mM EDTA, xylene cyanol, bromophenol
25 blue and 1 % sodium dodecyl sulfate (SDS). After heating to 90°C, samples were subjected to

1 electrophoresis on a 7% polyacrylamide sequencing gel and the transcripts were quantified
2 using a PhosphorImager (Molecular Dynamics).

3

4 **Molecular modelling.** The structure of σ^S was modelled using the σ^{70} of the *T. thermophilus*
5 holoenzyme structure (chain F, PDB code 1IW7, 47) as a template, with the CPHmodels 3.0
6 Server (30) and was manually corrected based on sequence alignment. The structure was
7 energy minimized in 100 steps with a dielectric constant of 1, using the program CNS (4).
8 The model spans residues 53 to 314. The modelled σ^S was docked onto E by superimposing
9 the σ^S coordinates with those of σ^{70} from the *T. thermophilus* holoenzyme structure (1IW7).
10 The figures were generated using PyMOL (10).

11

12 **Surface plasmon resonance.** Experiments were performed as previously described (12),
13 using a Biacore 2000 instrument (GE Healthcare) equilibrated at 25°C in buffer A
14 supplemented with 0.034% Tween 20. The monoclonal antibody 4RA2 (Neoclone), directed
15 against RNAP, was covalently immobilized on two flowcells of a CM5 sensorchip (GE
16 Healthcare). 2000-2500 resonance units (RU, \approx pg/mm²) were captured on one of the
17 surfaces, while the other was left unliganded and used as a reference. σ_{WT}^S or σ_{Ty19}^S (20 nM to
18 2.5 μ M), alone or pre-equilibrated with Crl (5 μ M), were then injected over the two surfaces
19 for 3 min at 20 μ l/min, and the dissociation was then followed for 5 min. All the association
20 and dissociation profiles were double-referenced using the Scrubber 2.0 software (BioLogic
21 software) (i.e. both the signals from the reference surface, and from blank injections of Crl or
22 running buffer were subtracted).

23

24

RESULTS

Characterization of $rpoS_{Ty19}$, a natural $rpoS$ mutant allele of *S. Typhi*

During a search for $rpoS$ mutants in clinical isolates of *Salmonella* (36 and data not shown), our attention was drawn by *S. Typhi* strain Ty19, a strain isolated from human blood. *S. Typhi* Ty19 produced a low amount of σ^S (Figure 1, lane 2), compared to wild-type isolates of *S. Typhi* (Figure 1 lane 1, and 36). Surprisingly, Ty19 was resistant to hydrogen peroxide (H_2O_2) in stationary phase (Figure 2A, lanes 1 and 3), a phenotype dependent on σ^S (Figure 2A lanes 1 and 5). The sequence of the promoter and leader regions of the $rpoS$ gene in Ty19 ($rpoS_{Ty19}$) was wild-type but the open-reading frame contained a G/T mutation at position 845, which resulted in a glycine to valine amino-acid substitution at residue 282 (G282V) in the σ^S_{Ty19} protein.

The chaperone-like protein Crl increases the performance of σ^S but its impact on the H_2O_2 resistance level of *S. Typhimurium* in stationary phase is hardly detectable in standard growth conditions (37, 40). Most interestingly, a crl mutation affected the ability of *S. Typhi* Ty19 to resist to H_2O_2 (Figure 2A, lanes 3-4), whereas, as expected, no significant effect of the crl mutation was detected on the H_2O_2 resistance level of strain 5959 (Figure 2A, lanes 1-2), a *S. Typhi* strain wild-type for $rpoS$ (36). At low cell density, the effect of the crl mutation on the H_2O_2 resistance level of Ty19 was drastic and not highly different from that of the $rpoS$ deletion (Figure 2B). These results suggested that the σ^S activity in Ty19 was more dependent on Crl activation than in strain 5959.

The activity of σ^S_{Ty19} is highly dependent on Crl

To characterize the $rpoS_{Ty19}$ allele in an otherwise isogenic background, the $rpoS$ gene from *S. Typhimurium* ATCC14028 was replaced by the $rpoS_{Ty19}$ allele yielding strain

1 2922K*rpoS*_{Ty19}. As previously observed in *S. Typhi*, σ^S_{Ty19} was detected in lower amounts
2 than the wild-type σ^S protein (Figure 1 lanes 4 and 7). Interestingly, levels of H₂O₂ resistance
3 were more dependent on Crl in the *rpoS*_{Ty19} mutant than in the wild-type strain (Figure 2C).
4 Consistent with this finding, the expression level of a *lacZ* gene fusion in *katE*, a σ^S -
5 dependent gene encoding a catalase required for the H₂O₂ resistance of *Salmonella* in
6 stationary phase (37), was affected by the *crl* mutation in the *rpoS*_{Ty19} mutant but not in the
7 wild-type strain (Figure 2D lanes 4-5 and 1-3). σ^S_{Ty19} production levels were not lowered in
8 the absence of Crl (Figure 1, lanes 7-8). These results suggested that the activity of σ^S_{Ty19} was
9 highly dependent on Crl activation. At low cell density, the *rpoS*_{Ty19} mutant was slightly less
10 resistant to H₂O₂ than the wild-type strain (Figure 2C) and expressed the *katE-lacZ* fusion to
11 slightly lower levels (Figure 2D).

12 In agreement with the *in vivo* data, *in vitro* transcription experiments using three
13 different σ^S -dependent promoters, *katE*, *katN* and *poxB*, demonstrated that the activity of the
14 σ^S_{Ty19} protein was lower, and much more dependent on Crl activation, than that of the wild-
15 type σ^S protein (Figure 3A). In these assays, addition of Crl rescued σ^S_{Ty19} activity to a level
16 similar to that obtained using the wild-type σ^S protein in the absence of Crl (Figure 3A). In
17 conclusion, the G282V substitution in σ^S_{Ty19} both decreased the activity of σ^S and increased
18 its dependency to Crl activation.

19

20 σ^S_{Ty19} depends on Crl for binding to RNAP core

21 *In vitro* transcription assays showed that the activity of σ^S_{Ty19} is impaired, and that this
22 defect is rescued by Crl (Figure 3A). Because Crl favors σ^S binding to the RNAP core
23 enzyme E, one possibility to explain this finding is that Crl compensates a low E-binding
24 propensity of σ^S_{Ty19} .

1 To study the interaction between $\sigma_{\text{Ty19}}^{\text{S}}$ and E, we used a surface plasmon resonance
2 (SPR) assay that we had previously set-up (12). A monoclonal antibody specific for the C
3 terminus of the RNAP α subunit (α -CTD; which plays no role in the association of σ with E)
4 was covalently immobilized to the dextran surface of a sensor chip and used to capture E
5 noncovalently.

6 We observed that, in the absence of Crl, no binding of $\sigma_{\text{Ty19}}^{\text{S}}$ (up to 2.5 μM) to E could
7 be detected (Figure 3B). On the contrary, in the presence of Crl, the ability of $\sigma_{\text{Ty19}}^{\text{S}}$ to bind to
8 E was restored to a level similar to that observed with the wild-type σ^{S} protein in the absence
9 of Crl (Figure 3B), in agreement with the *in vitro* transcription data (Figure 3A). Altogether,
10 these results showed that $\sigma_{\text{Ty19}}^{\text{S}}$ is impaired for E binding and that this defect is alleviated by
11 Crl.

12

13 **G282 is located in a flexible loop in region 4 of σ^{S}**

14 Sequence alignments of σ^{70} family members, to which σ^{S} belongs, have revealed that
15 they are constituted of four conserved regions (regions 1-4, Figure 4B) (26, 29, 33). Among
16 these, region 2 (subregions 2.1 to 2.4) and region 4 (subregions 4.1 and 4.2) contain DNA-
17 binding domains that mediate recognition of the conserved -10 and -35 elements of σ^{70} -
18 dependent promoters, respectively. The linear division of σ^{70} factors into functionally distinct
19 regions has been largely confirmed by structural data, which revealed that primary sigma
20 factors have four flexibly linked domains, $\sigma_{1.1}$, σ_2 , σ_3 and σ_4 , containing regions 1.1, 1.2-2.4,
21 3.0-3.1, and 4.1- 4.2, respectively (6, 7, 26, 29, 33, Figure 4AB). The $\sigma_{1.1}$ region is
22 unstructured in all available crystal structures. Additionally, the linker between σ_3 and σ_4
23 corresponds to region 3.2 (Figure 4AB). Regions 2 and 4 are not only involved in DNA
24 binding but also contain critical determinants for binding the β' and β subunits of the RNAP,

1 respectively (1, 29) (Figure 5A). The G282V substitution is located in region 4 of σ^S_{Ty19}
2 (Figure 4B).

3 In the $E\sigma^{70}$ holoenzyme, the σ subunit stretches across the upstream face of the
4 enzyme, making extensive contacts with subunits β and β' of the RNAP and with its DNA
5 recognition elements positioned to contact the promoter (29). A flexible flap domain of
6 subunit β of the RNAP (β -flap), interacts with region 4 of σ^{70} , involving mainly residues
7 belonging to the flexible loop between helix H2 and helix H3 of σ_4 , positioning region 4.2 to
8 interact with the -35 promoter element, α C-terminal domain and activators (11) (Figure 5).
9 This interaction depends on a hydrophobic patch on one face of the short helix stretch located
10 at the tip of the flap domain, called the β -flap-tip helix (15). The contact between the β -flap-
11 tip helix hydrophobic patch and the σ hydrophobic region is essential for the stable interaction
12 of the β -flap-tip helix with the H2-H3 loop. The σ_4 domain (region 4.1-4.2) is C-shaped, with
13 a concave pocket coated with hydrophobic residues of region 4.1. In the holoenzyme, the β -
14 flap-tip helix fits into this concave pocket (Figure 5).

15 In the structure of the modelled σ^S , based on the structure of *T. thermophilus* σ^{70} (47),
16 the G282 residue is located at the top of the H2-H3 flexible loop (residues L280-E289, Figure
17 4A). When docked onto E, this loop lies in a cleft formed by the β -flap on one side and the β'
18 zinc finger on the other side (Figure 5). Interestingly, both the β -flap and the β' zinc finger
19 region sequences are highly conserved between *Thermus thermophilus* and *Salmonella*
20 *enterica*. In contrast, the residues corresponding to G282 in σ^{70} are either an aspartate or a
21 methionine, whose side chains easily accommodate into the E cleft as observed in the *T.*
22 *thermophilus* $E\sigma^{70}$ holoenzyme (47). Substitution G282V could directly disrupt the interaction
23 between σ^S and E through two different mechanisms. First, G282V could modify the
24 conformation of the H2-H3 loop by increasing its rigidity and thus reducing its capacity to

1 interact with the β -flap. Second, the interaction of the loop with the β -flap could be sterically
2 destabilized because of the presence of the valine side-chain.

3

4 **Crl binds to domain 2 of σ^S**

5 So far, the σ^S domain involved in Crl binding remained unknown. To understand
6 better the effect of the G282V substitution on the interaction between Crl and σ^S , the bacterial
7 two-hybrid system (BACTH system, 19) was used. In this system, the T25- σ^S and Crl-T18
8 hybrid proteins were shown to interact, yielding levels of β -galactosidase activity higher than
9 those detected in negative controls (Figure 4B and 28). We previously showed that the first 71
10 residues of σ^S were not required for Crl binding since the T25- σ^S_{72-330} and Crl-T18 chimeras
11 interacted efficiently (28 and Figure 4B). The level of β -galactosidase activity detected with
12 T25- σ^S_{72-330} was actually higher than with T25- σ^S , which might be due to the higher
13 expression of T25- σ^S_{72-330} compared with T25- σ^S , as detected by immunoblotting with a
14 polyclonal σ^S antibody (28, Figure 4B and data not shown). For the T25- σ^S_{Ty19} chimera,
15 levels of β -galactosidase activity were also higher than for the T25- σ^S chimera (Figure 4B),
16 but in this case the amount of T25- σ^S_{Ty19} detected by the σ^S antibody was lower than that of
17 T25- σ^S (data not shown).

18 To determine whether the C-terminal domain of σ^S interacts with Crl, truncated
19 variants of the T25- σ^S chimera were assessed in the BACTH. The T25- σ^S_{90-330} , T25- $\sigma^S_{169-330}$,
20 and T25- $\sigma^S_{238-330}$ chimeras, did not yield significant β -galactosidase activity (Figure 4B)
21 although the protein amounts were similar to that of the T25- σ^S_{72-330} chimera, as assessed by
22 immunodetection with the σ^S antibody (data not shown). In contrast, the five chimeras T25-
23 σ^S_{1-254} , T25- σ^S_{72-254} , T25- σ^S_{1-167} , T25- σ^S_{56-167} , and T25- σ^S_{72-167} yielded levels of β -
24 galactosidase activity in the BACTH that were higher than that detected with the T25- σ^S

1 chimera, showing that they were able to interact with the Crl-T18 protein (Figure 4B). These
2 chimeras were barely or not detectable by the σ^S polyclonal antibody (data not shown),
3 suggesting either that their amounts were low or that these chimeras did not react efficiently
4 with the σ^S antibody. Altogether, these results demonstrated that amino acids 72 to 167 in σ^S
5 are sufficient for interaction with Crl. Interestingly, the G282V substitution in T25- σ^S_{90-330}
6 and T25- $\sigma^S_{238-330}$ did not allow them to interact with Crl-T18 (Figure 4B). Thus this
7 substitution likely favors the interaction of T25- σ^S_{Ty19} with Crl-T18 indirectly, through
8 conformational changes of T25- σ^S_{Ty19} .

9
10 ***rpoS_{Ty19}* confers a competitive fitness to *Salmonella*, conditional to the *crl* status of the**
11 **bacterial population.**

12 One likely hypothesis to explain the appearance of the *rpoS_{Ty19}* allele in natural
13 isolates of *S. Typhi* is that this mutant allele confers a competitive fitness. We previously set
14 up a survival assay with mixed populations of *Salmonella* in which the Δcrl mutation
15 increased the competitive fitness of *Salmonella* in stationary phase (40). We used this assay to
16 assess the competitive fitness of strains carrying the *rpoS_{Ty19}* allele.

17 Two strains of *Salmonella* were mixed in equal cell numbers in LB liquid medium,
18 and the numbers of each were monitored for several days (Figure 6B). The *rpoS_{Ty19}* mutant
19 showed a competitive advantage during stationary phase over wild-type strain ATCC14028
20 (Figure 6B panel c). Two days after inoculation of the medium, *rpoS_{Ty19}* mutant cells
21 represented more than 80% of the total population. However, the gain of fitness afforded by
22 the *rpoS_{Ty19}* allele was lost in a population carrying a deletion of the *crl* gene. Indeed, in Δcrl
23 context, strains carrying the *rpoS_{Ty19}* allele were outcompeted by strains carrying a wild-type
24 *rpoS* allele (Figure 6B, panel d). In a similar way, the $\Delta rpoS$ mutant was outcompeted by the
25 wild-type strain (40, Figure 6B) suggesting that the gain of fitness afforded by the *rpoS_{Ty19}*

1 mutation is conditional upon the presence of *crl*. Vice-versa, the Δcrl mutant also showed a
2 competitive advantage over the wild-type strain (Figure 6B panel e) but this gain of fitness
3 was lost in populations carrying the *rpoS*_{Ty19} allele (Figure 6B, panels f and h). In control
4 experiments, wild-type strain ATCC14028 showed similar fitness as wild-type strain 2922K
5 (Figure 6B panel a) and the Km or Cm resistance cartridges, harbored by some of the strains,
6 had no effect (Figure 6B panels a and g and 40). Finally, we observed no significant
7 differences when comparing the ability of wild-type and mutant strains to survive in
8 monocultures under the same conditions (Figure 6A).

9

DISCUSSION

1
2
3 In this study, we show that the natural *rpoS*_{Ty19} mutant allele of *S. Typhi* Ty19 leads to
4 the synthesis of a σ^S _{Ty19} protein with a very low E-binding propensity due to a G282V
5 substitution in the σ_4 domain. All σ_4 domains of the σ^{70} family have a common structural fold
6 but present different electrostatic surface potentials (24); σ^S domain 4 is more acidic than its
7 σ^{70} counterpart and has been shown to bind with a higher apparent affinity to the β -flap of
8 RNAP in a bacterial two-hybrid assay (23). Since the overall affinity of σ^S for E is lower than
9 that of σ^{70} (18), it is possible that the interaction between σ^S_4 and the β -flap plays a crucial
10 role in the stability of $E\sigma^S$ and that any substitution (such as G282V) weakening this
11 interaction would thus impede the formation of the holoenzyme. The findings that Crl can
12 bind and rescue σ^S _{Ty19} efficiently (Figures 3 and 4B), indicate that residue G282 is not in itself
13 crucial for the interaction with E, but rather that σ^S _{Ty19} is blocked in a conformation
14 incompatible for E binding.

15 Production of σ^S is tightly regulated through a combination of regulatory mechanisms
16 operating at different levels (reviewed in 21). Rapid degradation of σ^S in growing cells
17 depends on the ClpXP protease and the RssB protein which is required to deliver σ^S to
18 ClpXP. In stationary phase, there is a reduction of σ^S proteolysis and accumulation of σ^S (21).
19 The steady state levels of σ^S _{Ty19} in stationary phase were lower than those of σ^S (Figure 1) but
20 the half-lives of the proteins were not significantly different (data not shown). However, in the
21 exponential phase, the amounts of σ^S _{Ty19} were below the level of detection (data not shown)
22 and we cannot exclude a negative effect of the G282V substitution on the stability of the
23 protein. Alternatively, the mutation in *rpoS*_{Ty19} may affect the efficiency of translation and/or
24 the stability of the mRNA. Clearly, additional experiments are required to address this issue.

1 Crl has a dual role in σ^S proteolysis (35, 46). On one side, Crl stimulates the σ^S -
2 dependent transcription of *rssB*, and thereby increases σ^S degradation (46). On the other side,
3 σ^S is protected from degradation within the holoenzyme (51) and the function of Crl in favour
4 of $E\sigma^S$ formation results in increased σ^S stability (46). This positive effect of Crl on σ^S
5 stability, which would result in higher levels of σ^S in the presence of Crl, is masked by the
6 effect of Crl on *rssB* expression and cellular amounts of σ^S are higher in the absence of Crl (35,
7 46, 39, Figure 1 lanes 4-5). Surprisingly, no effect of the *crl* mutation was detected on the
8 cellular amounts of σ_{Ty19}^S (Figure 1, lanes 7-8). It is likely that, in the *rpoS_{Ty19}* mutant, the
9 inverse effects of Crl on σ_{Ty19}^S stability, through $E\text{-}\sigma_{Ty19}^S$ formation and induction of the
10 σ_{Ty19}^S -dependent *rssB* transcription, fully compensate each other.

11 Negative control of gene expression by σ^S appears to be as significant as positive
12 control (2, 34) and to result in a growth advantage of *rpoS* mutants and their selection in
13 bacterial populations (13, 31, 40, 50). Indirect mechanisms, such as activation of repressors or
14 sigma factor competition for E-binding, are presumably involved in the negative regulation by
15 σ^S . σ^S is needed for competitive fitness in stationary phase (40, 50, Figure 6B) but the lower
16 than wild-type functionality of σ^S in the *rpoS_{Ty19}* mutant (Figure 2CD, Figure 3 and data not
17 shown) resulted in gain of competitive fitness (Figure 6B). This is probably because, in the
18 conditions used, there is a good compromise in this strain between reduction of expression of
19 σ^S -dependent genes required for survival and stress resistance on the one hand, and increased
20 expression of σ^{70} -dependent genes required for persistence and/or growth on the other hand.
21 Interestingly, the gain of fitness afforded by the *rpoS_{Ty19}* mutation was conditional upon the
22 presence of *crl* (Figure 6B), suggesting that, under these conditions, the level of σ_{Ty19}^S activity
23 in the absence of Crl is below the threshold required for *Salmonella* fitness. This flexibility of
24 σ_{Ty19}^S activity might allow bacteria to adjust the level of σ^S activity in the *rpoS_{Ty19}* mutant to

1 varying environments through regulation of *crl* expression or secondary mutations in *crl*. In
2 this regard, the *rpoS*_{Ty19} mutant provides a genetic tool that may facilitate the identification of
3 *crl* mutants and/or mutants affected in the regulation of *crl* expression. These results suggest a
4 role for *crl* in the appearance of *rpoS* mutants and raise the possibility that a number of *rpoS*
5 mutations in bacterial populations have escaped our attention due to the capacity of Crl to
6 rescue the activity of the corresponding σ^S mutant proteins.

7 There is no 3D-structure available for full-length free σ but several lines of evidence
8 suggest that free σ adopts a compact (closed) conformation, incompatible with promoter
9 recognition and different from its open structure in $E\sigma$ complexes (7, 43). Indeed, binding to
10 E induces large movements of the σ domains (5, 7, 29), switching σ into an active
11 conformation in which the DNA binding determinants in σ_2 and σ_4 are exposed (29). The
12 regulation of many key biological processes in bacteria is fine-tuned by the interplay between
13 σ factors and their cognate anti- σ factors (7). Proteins that bind σ factors typically reduce
14 their efficiency by restricting their access to E. In contrast, the transient interaction of Crl
15 with σ^S increases the association rate of σ^S to E (12), thereby facilitating RNA polymerase
16 holoenzyme $E\sigma^S$ formation (12, 14, 46). One attractive model is that Crl acts as a unique,
17 chaperone-like protein, favouring an open conformation of σ^S with a high E-binding
18 propensity.

19 Among the structurally characterized anti- σ factors, all but the AsiA interact with two
20 or more structural domains of σ simultaneously (7, 49). In each case, the complex that is
21 formed precludes a functional interaction between σ and E. In contrast, AsiA, which functions
22 in the context of $E\sigma^{70}$, interacts only with domain 4 of σ^{70} , selectively inhibiting the
23 utilization of $-10/-35$ promoters. Our results reveal that Crl interacts with domain 2 of σ^S
24 (Figure 4B). We cannot rule out the possibility that Crl contacts transiently and/or weakly an

1 additional domain of σ^S , besides the σ^{S_2} domain, thus making this association difficult to
2 detect in the BACTH assay. However, the affinity of Crl for σ^S is low (around 2 μM) and the
3 Crl- σ^S complex has a short half-life (less than 3sec). These findings suggest that Crl does not
4 form extended interfaces with σ^S and are consistent with the binding of Crl to a single domain
5 of σ^S . Experiments are in progress to determine which amino acids of the σ^{S_2} residues 72 to
6 167 are directly involved in Crl binding and to investigate the mechanism of action of Crl in
7 greater detail by structural and biophysical approaches.

8

9

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1 **Table 1: Bacterial strains and plasmids used in this study**

2

3	Strain or plasmid	Characteristics	Source or reference ^a
4	<i>Escherichia coli</i>		
5	MC1061K	<i>araD139 Δ(ara-leu)-767 Δ(lacIPOZY)X74 rpsL</i>	22
6		<i>galU galK rpoS::kan</i>	
7	JM109	<i>recA1 supE44 endA1 hsdR17 gyrA96 relA1 thi</i>	41
8		<i>Δ(lac-proAB) F'(traD36 proAB⁺ lacF^q lacZΔM15)</i>	
9	BTH101	F- <i>cya-99 araD139 galE15 galK16 rpsL1 (Str^R)</i>	D. Ladant
10		<i>hsdR2 mcrA1 mcrB1</i>	
11	<i>Salmonella</i> serovar Typhi		
12	5959	Wild-type, isolated from human blood	CNR ^b
13	5959 <i>crl</i>	5959 <i>Δcrl::Cm</i>	
14	Ty19	Wild-type	CNR ^b
15	Ty19K	Ty19 <i>ΔrpoS::Km</i>	
16	Ty19 <i>crl</i>	Ty19 <i>Δcrl::Cm</i>	
17	<i>Salmonella</i> serovar Typhimurium		
18	ATCC14028	Wild-type	ATCC ^c
19	ATCC <i>rpoS</i>	ATCC14028 <i>ΔrpoS::Cm</i>	39
20	ATCC <i>crl</i>	ATCC14028 <i>Δcrl::Cm</i>	39
21	ATCC <i>Δcrl</i>	ATCC14028 <i>Δcrl</i>	39
22	2922K	ATCC14028 <i>ΔSTM2922::Km</i>	40

1	2922K <i>crl</i>	ATCC14028 Δ STM2922:: <i>Km</i> Δ <i>crl</i> :: <i>Cm</i>	40
2	2922K Δ <i>crl</i>	ATCC14028 Δ STM2922:: <i>Km</i> Δ <i>crl</i>	
3	2922K <i>rpoS</i>	ATCC14028 Δ STM2922:: <i>Km</i> Δ <i>rpoS</i> :: <i>Cm</i>	40
4	2922K <i>rpoS</i> _{Ty19}	ATCC14028 Δ STM2922:: <i>Km</i> <i>rpoS</i> _{Ty19}	
5	2922K <i>rpoS</i> _{Ty19} <i>crl</i>	2922K <i>rpoS</i> _{Ty19} Δ <i>crl</i> :: <i>Cm</i>	
6	2922K <i>rpoS</i> _{Ty19} Δ <i>crl</i>	2922K <i>rpoS</i> _{Ty19} Δ <i>crl</i>	
7	2922K <i>katE-lacZ</i>	2922K Tn5B21-2.4	40
8	2922K <i>rpoS katE-lacZ</i>	2922K <i>rpoS</i> Tn5B21 -2.4	40
9	2922K <i>crl katE-lacZ</i>	2922K <i>crl</i> Tn5B21 -2.4	40
10	2922K <i>rpoS</i> _{Ty19} <i>katE-lacZ</i>	2922K <i>rpoS</i> _{Ty19} Tn5B21-2.4	
11	2922K <i>rpoS</i> _{Ty19} <i>crl katE-lacZ</i>	2922K <i>rpoS</i> _{Ty19} Δ <i>crl</i> :: <i>Cm</i> Tn5B21-2.4	
12			
13	Plasmids		
14	pACYC184	cloning vector, <i>Cm</i> ^R , <i>Tet</i> ^R	8
15	pUCK3 <i>Km</i>	pUC19 with <i>S. Typhimurium</i> Δ <i>rpoS</i> :: <i>Km</i> , <i>Cb</i> ^R <i>Km</i> ^R	22
16	pUC4K	source of <i>Km</i> resistance cartridge	Pharmacia
17	pAC <i>rpoS</i> _{Ty19}	pACYC184 with a 6 kb <i>Bgl</i> III fragment	
18		carrying <i>rpoS</i> _{Ty19} , <i>Cm</i> ^R	
19	pUC <i>rpoS</i> _{Ty19}	pUC19 with a 3.5 kb <i>Hind</i> III- <i>Sca</i> I fragment	
20		carrying <i>rpoS</i> _{Ty19} , <i>Cb</i> ^R	
21	pUC <i>rpoS</i> _{Ty19} K	pUC <i>rpoS</i> _{Ty19} with STY3047:: <i>Km</i> , <i>Cb</i> ^R <i>Km</i> ^R	
22	pQE30	vector for expression of His-tagged proteins, <i>Cb</i> ^R	Qiagen
23	pQE30 <i>rpoS</i>	pQE30:: <i>rpoS</i> expresses a His ₆ - σ^S protein, <i>Cb</i> ^R	28
24	pQE30 <i>rpoS</i> _{Ty19}	pQE30:: <i>rpoS</i> expresses a His ₆ - σ^S _{Ty19} protein, <i>Cb</i> ^R	
25	pKT25	BACTH vector designed to express a given	20

1		polypeptide fused in frame at its N-terminal end with	
2		T25 fragment, p15 ori, Km ^R	
3	pUT18	BACTH vector designed to express a given	20
4		polypeptide fused in frame at its C-terminal end with	
5		T18 fragment, ColE1 ori, Ap ^R	
6	pUTCrl	pUT18 expressing Crl-T18	28
7	pKTRpoS	pKT25 expressing T25- σ^S	28
8	pKTRpoS _{Ty19}	pKT25 expressing T25- σ^S_{Ty19}	
9	pKTRpoS $_{\Delta 1-71}$	pKT25 expressing T25- σ^S_{72-330}	28
10	pKTRpoS ₉₀₋₃₃₀	pKT25 expressing T25- σ^S_{90-330}	
11	pKTRpoS _{90-330Ty19}	pKT25 expressing T25- $\sigma^S_{90-330Ty19}$	
12	pKTRpoS ₁₆₉₋₃₃₀	pKT25 expressing T25- $\sigma^S_{169-330}$	
13	pKTRpoS ₂₃₈₋₃₃₀	pKT25 expressing T25- $\sigma^S_{238-330}$	
14	pKTRpoS _{238-330Ty19}	pKT25 expressing T25- $\sigma^S_{238-330Ty19}$	
15	pKTRpoS ₁₋₂₅₄	pKT25 expressing T25- σ^S_{1-254}	
16	pKTRpoS ₇₂₋₂₅₄	pKT25 expressing T25- σ^S_{72-254}	
17	pKTRpoS ₁₋₁₆₇	pKT25 expressing T25- σ^S_{1-167}	
18	pKTRpoS ₅₆₋₁₆₇	pKT25 expressing T25- σ^S_{56-167}	
19	pKTRpoS ₇₂₋₁₆₇	pKT25 expressing T25- σ^S_{72-167}	

20 ^a This study, unless otherwise noted.

21 ^b National Reference Center for *Salmonella* (Institut Pasteur, Paris)

22 ^c American Type Culture Collection

23

LEGENDS TO FIGURES

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FIGURE 1. Detection of σ^S and σ_{Ty19}^S in *S. Typhi* (STY) and *S. Typhimurium* (STM). Overnight LB cultures at 37°C were analysed by western blotting with anti- σ^S antibodies. 5 μ g of total protein was loaded in each slot. 1: 5959, 2: Ty19, 3: Ty19K, 4: 2922K, 5: 2922Kcrl, 6: 2922KrhoS, 7: 2922KrhoS_{Ty19}, 8: 2922KrhoS_{Ty19}crl.

FIGURE 2. *In vivo* characterization of the *rpoS*_{Ty19} mutant allele. ABC) Resistance to hydrogen peroxide of *Salmonella* strains carrying wild-type or *rpoS*_{Ty19} mutant alleles. A) *S. Typhi* strains 1: 5959, 2: 5959crl, 3: Ty19, 4: Ty19crl, 5: Ty19K. B) *S. Typhi* strains 5959 (WT), Ty19, Ty19crl, Ty19K. C) *S. Typhimurium* strains 2922K (WT), 2922K Δ crl, 2922KrhoS_{Ty19}, 2922KrhoS_{Ty19} Δ crl, 2922KrhoS. Cells were grown to stationary phase in LB at 37°C, washed, resuspended in PBS to an OD₆₀₀ of 1 (A) and 0.1 (BC) and H₂O₂ 15 mM was added. Representative experiments are shown in BC. Similar results were obtained in repeat experiments. D) Expression of a *katE-lacZ* gene fusion in *S. Typhimurium* carrying the wild-type *rpoS* and mutant *rpoS*_{Ty19} alleles. 1: 2922KkatE-lacZ, 2: 2922KrhoS katE-lacZ, 3: 2922Kcrl katE-lacZ, 4: 2922KrhoS_{Ty19} katE-lacZ, 5: 2922KrhoS_{Ty19}crl katE-lacZ. β -galactosidase activity was measured in overnight LB cultures at 37°C according to the method of Miller (27).

FIGURE 3. *In vitro* characterization of the *rpoS*_{Ty19} mutant allele. A). Single round run-off transcripts of *katE* (lanes 1-4), *katN* (lanes 5-8) and *poxB* (lanes 9-12) promoters. RNA polymerases reconstituted with His₆- σ_{Ty19}^S (lanes 1-2, 5-6 and 9-10) or His₆- σ_{WT}^S (lanes 3-4, 7-8 and 11-12) in the absence (lanes 1, 3, 5, 7, 9 and 11) or presence of Crl (lanes 2, 4, 6, 8, 10 and 12) were incubated with plasmid templates for 12 min at 30°C before addition of a

1 mixture of heparin and XTPs as described in Materials and Methods. The ^{32}P -labelled
 2 transcripts were analysed on a 7 % sequencing gel and the band intensities quantified as
 3 indicated below each lane. B). Real-time SPR experiments showing the effect of Crl (5 μM)
 4 on the binding of His₆- $\sigma^{\text{S}}_{\text{Ty19}}$ (left; 625 nM) or His₆- $\sigma^{\text{S}}_{\text{WT}}$ (right; 625 nM) to the immobilized
 5 RNAP core. The sensorgrams shown in blue and cyan correspond to the binding of σ^{S} in the
 6 absence of Crl, and those in red and magenta to that in the presence of Crl. No E-binding of
 7 His₆- $\sigma^{\text{S}}_{\text{Ty19}}$ can be detected in the absence of Crl.

8
 9 **FIGURE 4. Crl binds to σ^{S} domain 2.** A) Model of the σ^{S} structure. The structural domains
 10 σ_2 (residues 53–163), σ_3 (residues 164-216), linker (residues 217-244) and σ_4 (residues 245-
 11 314) are represented in light green, yellow, orange and blue, respectively. The G282 is
 12 represented in red. B) BACTH analysis of Crl interactions with σ^{S} , $\sigma^{\text{S}}_{\text{Ty19}}$ and truncated σ^{S}
 13 proteins. A schematic representation of the four regions of the σ^{S} protein showing highly
 14 conserved amino acid sequence with the σ^{70} family members (7, 26) is shown at the top of the
 15 figure. The efficiencies of functional complementation between the indicated hybrid proteins
 16 were quantified by measuring β -galactosidase activities in *E. coli* BTH101 cells harboring the
 17 corresponding plasmids as described in Material and Methods. β -galactosidase activity was
 18 measured according to the method of Miller (27).

19
 20 **FIGURE 5. Position of the G282V substitution that affects the interaction of $\sigma^{\text{S}}_{\text{Ty19}}$ with**
 21 **the RNAP core enzyme.** A) Structure of the modelled σ^{S} positioned in the *T. thermophilus*
 22 enzyme core. The αI , αII , β , β' and ω subunits are represented in pale yellow, wheat, dark
 23 red, pink and pale orange, respectively. The region corresponding to the β -flap is coloured in

1 orange, and the region corresponding to the β' zinc finger in cyan. B) Close-up view of the
2 interaction between the core enzyme and σ_4 . A valine is shown at position 282 of the σ^S .

3

4 **FIGURE 6. Survival and competitive fitness of *Salmonella* strains during stationary**
5 **phase.** (A) Survival in stationary-phase cultures in LB medium at 37°C. Cells from overnight
6 LB cultures of *S. Typhimurium* (a) and *S. Typhi* (b) strains indicated were washed,
7 resuspended in PBS to an OD₆₀₀ of 1.0, diluted in fresh LB medium and incubated at 37°C
8 with shaking. Aliquots of bacteria were removed at timed intervals and numbers of viable
9 cells were determined on LB plates. One hundred percent survival corresponds to the number
10 of cells in cultures grown overnight (day 1). Representative experiments are shown. Similar
11 results were obtained in repeat experiments. (B) Competition assays between *S.*
12 *Typhimurium* (abcdefg) and *S. Typhi* (h) strains. Overnight LB cultures were washed and
13 resuspended in PBS to an OD₆₀₀ of 1.0. In each of the eight experiments, the two strains
14 indicated were mixed in equal cell numbers in fresh LB medium to give a total of about 3000
15 cells ml⁻¹ (time zero) and the mixtures were incubated at 37°C with shaking. Aliquots of
16 bacteria were removed at timed intervals and numbers of viable cells of each strain were
17 determined. Numbers of cells of each strain are reported as a percentage of the total number
18 of viable cells in the culture.

19

STY**STM****1****2****3****4****5****6****7****8** σ^S

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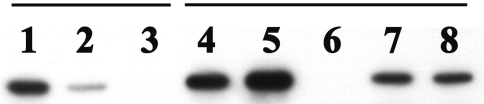
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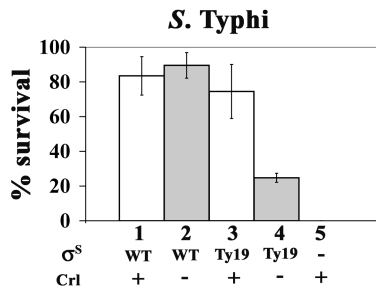
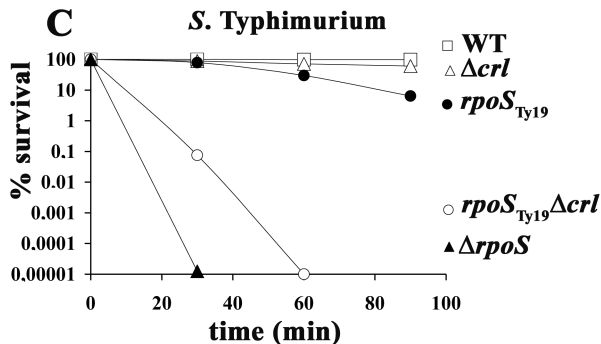
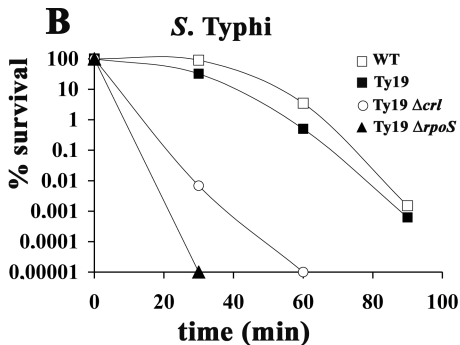
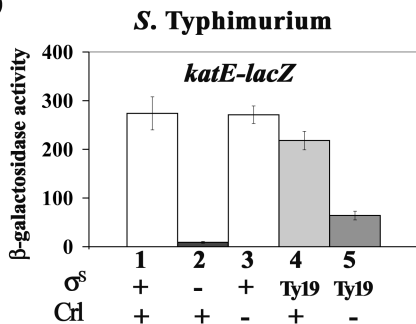
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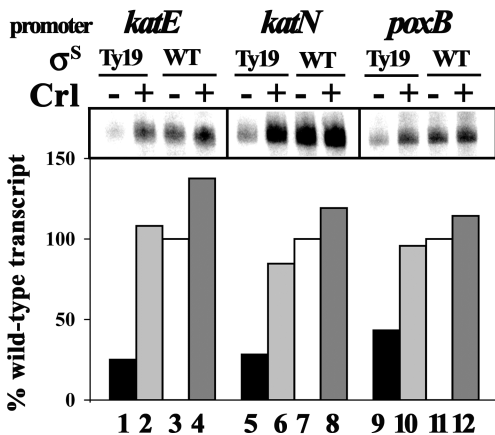
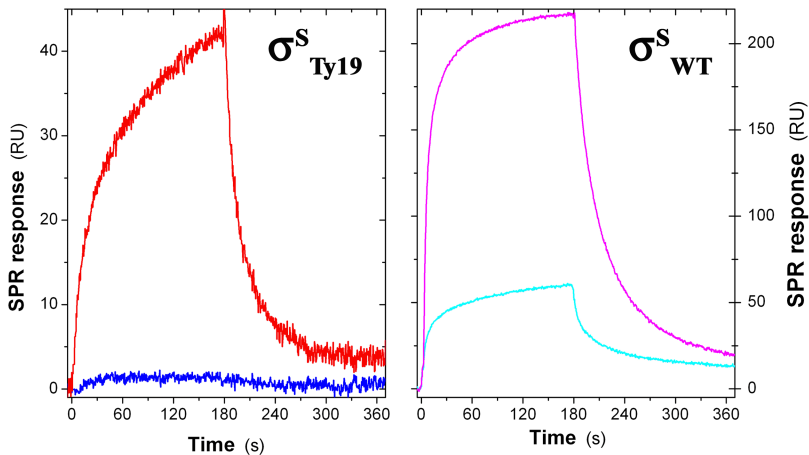
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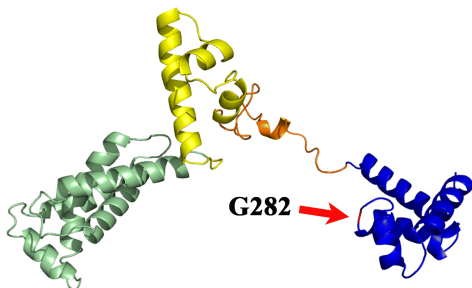
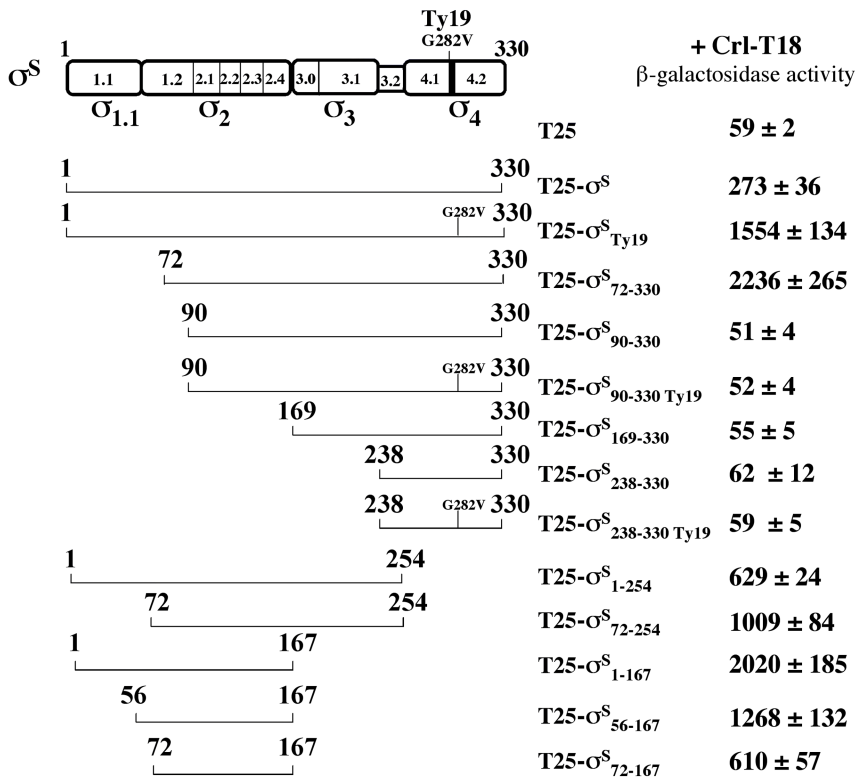
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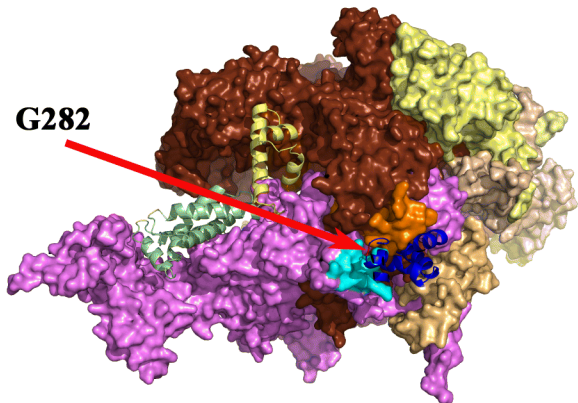
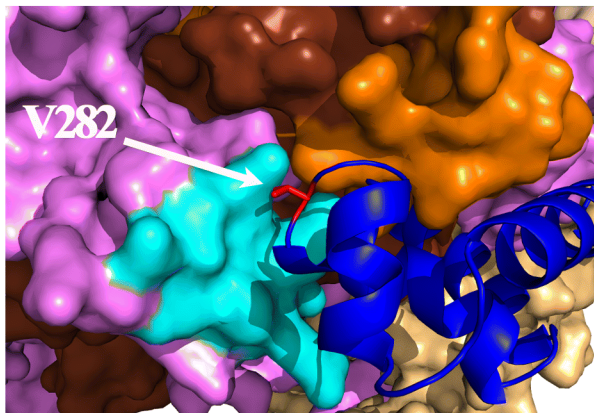
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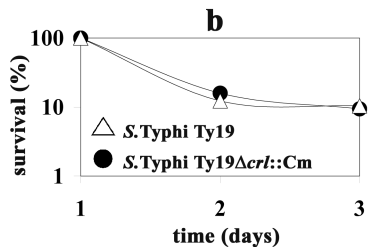
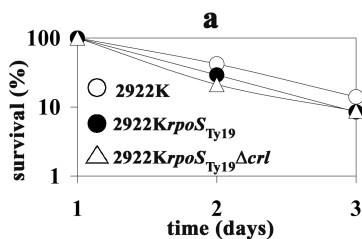


A**C****B****D**

A**B**

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