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## REVISÉD MANUSCRIPT

1

2

3 **Identification of conserved amino acid residues of the *Salmonella*  $\sigma^S$**   
4 **chaperone Crl involved in Crl- $\sigma^S$  interactions**

5

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12 Running title: Mutagenesis of the  $\sigma^S$  chaperone Crl

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

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## INTRODUCTION

1  
2  
3 In bacteria, transcription depends on a multisubunit RNA polymerase (RNAP)  
4 consisting of a catalytically active core enzyme (E) with a subunit structure  $\alpha_2\beta\beta'\omega$ , that  
5 associates with any one of several  $\sigma$  factors to form different holoenzyme ( $E\sigma$ ) species. The  $\sigma$   
6 subunit is required for specific promoter binding, and different  $\sigma$  factors direct RNAP to  
7 different classes of promoters, thereby modulating the gene expression patterns (17). The  
8 RNA polymerase holoenzyme containing the  $\sigma^{70}$  subunit is responsible for the transcription of  
9 most genes during exponential growth (17). When cells enter stationary phase or are under  
10 specific stress conditions (high osmolarity, low pH or high and low temperatures) during  
11 exponential growth,  $\sigma^S$ , which is encoded by the *rpoS* gene, becomes more abundant,  
12 associates with the core enzyme, and directs the transcription of genes essential for the  
13 general stress response and for stationary phase survival (17, 20, 25).

14 Sigma factors compete for binding to a limited amount of the core polymerase (16, 17,  
15 20, 34).  $\sigma^{70}$  is abundant throughout the growth cycle and has the highest affinity of all sigma  
16 factors for E *in vitro* (20). In contrast, levels of  $\sigma^S$  only reach about one-third of the  $\sigma^{70}$  levels  
17 upon entry into stationary phase, and  $\sigma^S$  exhibits the lowest affinity for E of all sigma factors  
18 *in vitro* (20). The cell uses at least two strategies to ensure the switch between  $\sigma^{70}$  and  $\sigma^S$ -  
19 associated RNA polymerases and to allow gene expression to be reprogrammed upon entry  
20 into stationary phase. Several factors (Rsd, 6S RNA, ppGpp and DksA) indirectly increase  $\sigma^S$   
21 competitiveness by decreasing the ability of  $\sigma^{70}$  to bind to E (25). In addition, the  
22 unconventional regulatory protein Crl increases the performance of  $\sigma^S$ .

23 The *crl* gene product is a regulator of  $\sigma^S$  activity in *Escherichia coli* (2, 14, 36, 50) and  
24 *Salmonella* (38, 40, 41). In both species, the Crl protein binds  $\sigma^S$  (2, 12) and facilitates RNA

1 polymerase holoenzyme E $\sigma^S$  formation (12, 50), thereby enhancing  $\sigma^S$  effectiveness (10, 14,  
2 27, 38, 40, 41, 50). The Crl protein of *Salmonella* binds  $\sigma^S$  with a stoichiometry 1:1 and  
3 increases the affinity of  $\sigma^S$  for the core enzyme 7-fold (12). In contrast, Crl does not bind  $\sigma^{70}$   
4 and does not modify the affinity of  $\sigma^{70}$  for the core enzyme (12). Lelong *et al.* (28) reported  
5 that, in *E. coli* W3110, Crl can interact with the ferric uptake regulator Fur, a key protein for  
6 the control of intracellular iron concentration (8 and references therein) and that Fur represses  
7 *crl* transcription (28).

8         In an attempt to gain further insight into Crl function, we searched for homologues of  
9 Crl in protein databases. Residues conserved in all members of the Crl family were  
10 substituted by site-directed mutagenesis experiments to identify residues important for Crl  
11 activity in *Salmonella*. We used the bacterial adenylate cyclase two-hybrid system (BACTH,  
12 22) to determine whether these residues and specific regions of Crl are required for efficient  
13 interaction with  $\sigma^S$ . We also used the BACTH system to assess the effect of the Crl mutations  
14 on the interaction of Crl with Fur.

## MATERIALS AND METHODS

1

2

3 **Bacterial strains, plasmids and growth conditions.** Strains and plasmids are listed in Table  
4 1. Bacteriophage P22HT105/1*int* was used to transfer mutations between *Salmonella* strains  
5 by transduction (44). Green plates, for screening for P22-infected cells or lysogens, were  
6 prepared as described previously (48). Strains were routinely cultured in Luria Bertani  
7 medium (LB; 43). Minimal medium was M9 (43) containing 20 mM glucose. For routine  
8 monitoring of multicellular behaviour (*rdar* morphotype), *Salmonella* strains were grown at  
9 28°C on LB agar plates without NaCl (LB0) supplemented with Congo Red ( $40 \mu\text{g ml}^{-1}$ ) as  
10 described (CR plates, 42). Antibiotics were used at the following concentrations: ampicillin  
11 (Ap),  $100 \mu\text{g ml}^{-1}$ ; carbenicillin (Cb),  $100 \mu\text{g ml}^{-1}$ ; chloramphenicol (Cm),  $15 \mu\text{g ml}^{-1}$  for the  
12 chromosomal resistance gene and  $30 \mu\text{g ml}^{-1}$  for the plasmid resistance gene; kanamycin,  
13 (Km)  $50 \mu\text{g ml}^{-1}$ ; and tetracycline (Tet)  $20 \mu\text{g ml}^{-1}$ .

14

15 **Oxidative shock survival assay.** Cells were grown to stationary phase in LB (optical density  
16 at 600nm ( $\text{OD}_{600}$ ) of 3.5 to 4), washed, resuspended in PBS and mixed with  $\text{H}_2\text{O}_2$  at a final  
17 concentration of 15 mM. Aliquots of bacteria were removed at time intervals, and numbers of  
18 colony forming cells were determined on LB plates.

19

20 **DNA manipulations and sequence analysis.** Standard molecular biology techniques were  
21 used (43). Oligonucleotides were obtained from Sigma-Aldrich (France). DNA sequencing  
22 was performed by Cogenics (France). DNA and amino acid sequence analyses were

1 conducted using the BLAST programs at the NCBI (National Center for Biotechnology  
2 Information).

3

4 **Cloning of the *rpoS*<sub>T2</sub> allele.** We demonstrated that the nucleotide sequence of a PCR-  
5 amplified *rpoS* gene from *Salmonella enterica* serovar Typhi strain T2 (*rpoS*<sub>T2</sub>) is identical to  
6 that in *Salmonella* ATCC14028 (41) except for a G/A mutation at position 318, which does  
7 not change the amino acid sequence, and a deletion of 8 nucleotides starting at position 109,  
8 which results in a frameshift and appearance of a premature stop codon in *rpoS*<sub>T2</sub>. Total DNA  
9 from *S. Typhi* T2 was cleaved with *Bgl*III and the resulting fragments were cloned into the  
10 *Bam*HI site of pACYC184. Recombinant plasmids were then transformed into the *E. coli*  
11 *rpoS* mutant strain MC1061K carrying an *rpoS*-dependent *spvRAB-lacZ* fusion on pSTF4  
12 (26). Selection of Cb<sup>R</sup> Cm<sup>R</sup> Lac<sup>+</sup> clones and subsequent examination of their plasmid DNA  
13 by restriction analysis and DNA sequencing of *rpoS* produced pAC*rpoS*<sub>T2</sub> containing a 6 kb  
14 *Bgl*III fragment carrying *rpoS*<sub>T2</sub>. A 3.3 kb *Sph*I-*Sca*I fragment carrying *rpoS*<sub>T2</sub> was  
15 subsequently cloned into the *Sph*I and *Sma*I sites of pUC19 to yield pUC*rpoS*<sub>T2</sub>.

16

17 **Construction of *S. Typhimurium* 2922K*rpoS*<sub>T2</sub>.** The *rpoS* allele in *S. Typhimurium*  
18 ATCC14028 was replaced by the *rpoS*<sub>T2</sub> allele using the following strategy (41). pUC*rpoS*<sub>T2</sub>  
19 contains the *rpoS*<sub>T2</sub> allele of *S. Typhi* strain T2 and the downstream genes, including a gene  
20 encoding a putative decarboxylase (named STY3047 in *S. Typhi* and STM2922 in *S.*  
21 *Typhimurium*) that is not regulated by *rpoS* (1). In pUC*rpoS*<sub>T2</sub>K, a kanamycin resistance  
22 (Km) cartridge is located in the *Sma*I site of STY3047. pUC*rpoS*<sub>T2</sub>K was introduced by  
23 electroporation into ATCC*rpoS*, where it was unstable. Recombination of the Km cartridge

1 into the host genome with simultaneous loss of pUC*rpoS*<sub>T2</sub>K resulted in recombinants that  
2 were resistant to kanamycin and sensitive to carbenicillin. A Km<sup>R</sup> Cb<sup>S</sup> Cm<sup>S</sup> recombinant was  
3 selected, checked by PCR for the presence of the STM2922::*Km* mutation and simultaneous  
4 replacement of the  $\Delta$ *rpoS*::Cm mutation by the *rpoS*<sub>T2</sub> allele. The *rpoS*<sub>T2</sub> and STM2922::*Km*  
5 alleles were then co-transduced to ATCC*rpoS*. Transductants that were Km<sup>R</sup> but Cm<sup>S</sup> were  
6 selected, and transduction of the STM2922::*Km* mutation and simultaneous replacement of  
7 the  $\Delta$ *rpoS*::Cm mutation by the *rpoS*<sub>T2</sub> allele were confirmed by PCR. One Km<sup>R</sup> Cm<sup>S</sup> strain,  
8 designated 2922K*rpoS*<sub>T2</sub>, was also checked by DNA sequencing for the presence of the  
9 *rpoS*<sub>T2</sub> allele.

10

11 **Determination of the N-terminal sequence of  $\sigma^S$ <sub>T2</sub>.** pQE*rpoS*<sub>T2</sub>, which produces the *rpoS*<sub>T2</sub>  
12 gene with a C-terminal His<sub>6</sub> extension under the control of the pQE60 IPTG-inducible  
13 promoter, was constructed as follows. Primers specific to the ends of *rpoS* (5'-  
14 CCGTCAAGAATTCACGGGTAGGAGCCACCTTATGA-3' and 5'-  
15 TGATCGAGATCTCTCGCGGAACAGCGCTTCGATATTCA-3') were used to amplify a 1  
16 kb fragment of *S. Typhi* T2 total DNA by PCR. *Eco*RI and *Bgl*III restriction sites were  
17 incorporated at its 5' and 3' ends, respectively. After digestion with *Eco*RI and *Bgl*III, the  
18 PCR-amplified fragment was ligated into the *Eco*RI and *Bgl*III sites of pQE60. The nucleotide  
19 sequence of the *rpoS* insert in pQE*rpoS*<sub>T2</sub> was verified. In this construct, *rpoS*<sub>T2</sub> is expressed  
20 from its own translation sequences. *E. coli* JM109 carrying pQE*rpoS*<sub>T2</sub> was grown in LB  
21 containing ampicillin at 37°C to an optical density of 0.9, when 0.7 mM isopropyl- $\beta$ -D-  
22 thiogalactoside (IPTG) was added. The cells were harvested after 5 h, resuspended in buffer A  
23 (50 mM Na<sub>2</sub>HPO<sub>4</sub> pH 8, 300 mM NaCl) and then disrupted in by French Press. The crude cell  
24 extract was supplemented with Benzonase (1250 U) and imidazole (10 mM) and centrifuged  
25 at 27,000 g for 30 min. The supernatant was added to 1.5 ml Ni-NTA column and washed

1 with buffer A containing 20 mM imidazole.  $\sigma_{T2}^S$ -His<sub>6</sub> was eluted with buffer A containing  
2 300 mM imidazole. After electrophoresis in a 10% polyacrylamide-SDS gel, the  $\sigma_{T2}^S$ -His<sub>6</sub>  
3 protein was transferred on a PVDF membrane, stained with Amido Black and excised from  
4 the membrane for N-terminal sequence analysis. Amino-terminal Edman degradation was  
5 carried out on an Applied Biosystems sequencer.

6

7 **Construction of a chromosomal *crl-lac* transcriptional fusion.** We previously created a  
8 chromosomal mutation in the *crl* gene of *Salmonella* ATCC14028 using PCR-generated  
9 linear DNA fragments and the  $\lambda$ Red recombination method (ATCC*crl*, 40). A single-copy  
10 *crl-lacZY* transcriptional gene fusion was constructed in ATCC*crl* using conditional plasmids  
11 containing promoterless *lacZY* genes and the FLP recognition target (FRT) site as described  
12 (11). PCR assays were used to ensure integration of the plasmids in the correct location and to  
13 exclude the presence of multiple plasmid integrants (using standard test primers, such as those  
14 described in 11). We also used flanking locus-specific primers to amplify junction fragments  
15 that were subsequently analysed by DNA sequencing. Isogenic strains were constructed by  
16 P22 HT *int* mediated transduction of the mutations into the appropriate strains.

17

18 **Enzymatic assays.**  $\beta$ -galactosidase activity was measured as described by Miller (31) 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* adenyl cyclase T18  
23 and T25 subdomains by two interacting partners (22). The resulting cAMP binds to and  
24 activates the transcription activator CRP, a positive regulator of t□□ *lac* and *mal* operons  
25 involved in lactose and maltose catabolism. Derivatives of pUT18, pKT25 and pKNT25, used

1 in the BACTH assays, were constructed by cloning PCR amplified DNA fragments encoding  
 2 the protein of interest from *S. Typhimurium* between the *Pst*I and *Eco*RI sites of pUT18 and  
 3 pKNT25 and the *Xba*I and *Kpn*I sites of pKT25. The DNA encoding Crl was amplified using  
 4 primers Crl-Pst 5'-CA TGC CTG CAG AAG GAG ATC GCA ATG ACG TTA CCG AGT-  
 5 3' and Crl-Eco 5'-T CGA TGA ATT CGA TGC CGA CAG TTT TAC CGG CTC GTC GT-  
 6 3', restricted by *Pst*I and *Eco*RI and cloned into the *Pst*I and *Eco*RI sites of both pUT18 and  
 7 pKNT25 to yield pUTCrl and pKNTCrl, respectively. The DNA encoding RpoS was  
 8 amplified using primers RpoS-Xba 5'-TCG ACT CTA GAT ATG AGT CAG AAT ACG  
 9 CTG AAA GTT CAT-3' and RpoS-Kpn 5'-AC TTA GGT ACC TTA CTC GCG GAA CAG  
 10 CGC TTC GAT-3' restricted by *Xba*I and *Kpn*I and cloned into the *Xba*I and *Kpn*I sites of  
 11 pKT25 to yield pKTRpoS. The DNA encoding Fur was amplified using primers Fur-Pst 5'-  
 12 CAT GCC TGC AGC ATG ACT GAC AAC AAT ACC GCA-3' and Fur-Eco 5'-T CGA  
 13 TGA ATT CGA TTT AGT CGC GTC ATC GTG CGC GT-3' for cloning into the *Pst*I and  
 14 *Eco*RI sites of pUT18 (yielding pUTFur) and using Fur-Xba 5'- TCG ACT CTA GAC ATG  
 15 ACT GAC AAC AAT ACC GCA TTA AAG A-3' and Fur-Kpn 5'- AC TTA GGT ACC  
 16 TTA TTT AGT CGC GTC ATC GTG CGC GT-3' for cloning into the *Xba*I and *Kpn*I sites of  
 17 pKT25 (yielding pKTFur). pKTRpoS<sub>Δ12-71</sub> is a derivative of pKTRpoS with an in frame  
 18 deletion of an internal *Hpa*I-*Dra*I fragment. pKTRpoS<sub>Δ1-38</sub> contains a DNA fragment  
 19 amplified with primers RpoST2-Xba 5'- TCG ACT CTA GAC CTG GCT GAA GAA GAG  
 20 CTG TTA TCG CAA-3' and RpoS-Kpn (see above) and cloned into the *Xba*I and *Kpn*I sites  
 21 of pKT25. pKTRpoS<sub>Δ1-71</sub> contains a DNA fragment amplified with primers RpoS-Xba5 5'-  
 22 TCG ACT CTA GAA ACA GCC GAA GAA GAA GTC TAT TTT GCG CGT-3' and RpoS-  
 23 Kpn (see above) and cloned into the *Xba*I and *Kpn*I sites of pKT25. All plasmids were  
 24 confirmed to be correct by DNA sequencing. The *E. coli cya* strain BTH101 was transformed  
 25 with derivatives of plasmids pKT25, pKNT25, and pUT18 encoding proteins fused to the T25

1 and T18 fragments of *Bordetella pertussis* adenylyl cyclase. Co-transformants were plated onto  
 2 MacConkey maltose plates supplemented with carbenicillin, kanamycin and 0.5 mM IPTG to  
 3 assess the Mal<sup>+</sup> phenotype and on LB plates supplemented with 5-bromo-4-chloro-indolyl- $\beta$ -  
 4 D-galactoside (X-Gal, 40  $\mu\text{g ml}^{-1}$ ), carbenicillin, kanamycin and 0.5 mM IPTG to assess the  
 5 Lac<sup>+</sup> phenotype and  $\beta$ -galactosidase activity. Plates were incubated at 30°C for 3 days and  
 6 colonies were then collected and their  $\beta$ -galactosidase activities were measured as described  
 7 by Miller (31). For immunodetection of the Crl-T18 and T25- $\sigma^S$  chimeras, BTH101  
 8 derivatives were grown to stationary phase (OD<sub>600</sub>= 3.5) in LB in the presence of 2 mM  
 9 cAMP and 0.5 mM IPTG to induce fully the *lac* promoter on pKT25, pKNT25 and pUT18.

10

### 11 **Construction of plasmids with mutated *crl* genes**

12 Site-directed mutagenesis was performed using the QuikChange II Site-directed mutagenesis  
 13 kit (Stratagene) as recommended by the manufacturer. Site-directed mutagenesis of plasmid  
 14 pACcrl-1 yielded plasmids pACcrl<sub>Y22A</sub>, pACcrl<sub>F35A</sub>, pACcrl<sub>C28A</sub>, pACcrl<sub>C37A</sub>, pACcrl<sub>C41A</sub>,  
 15 pACcrl<sub>F53A</sub>, pACcrl<sub>W56A</sub>, pACcrl<sub>G74A</sub>, pACcrl<sub>G80A</sub>, pACcrl<sub>W82A</sub>, pACcrl<sub>F103A</sub>, pACcrl<sub>C28-37</sub>,  
 16 pACcrl<sub>C28-41</sub>, pACcrl<sub>C37-C41</sub> and pACcrl<sub>C28-37-41</sub> (Table 1). Plasmids pUTCrl<sub>Y22A</sub>, pUTCrl<sub>F53A</sub>,  
 17 pUTCrl<sub>W56A</sub>, pUTCrl<sub>W82A</sub> and pUTCrl<sub>G80A</sub> (Table 1) were obtained by cloning PCR amplified  
 18 DNA fragments from plasmids pACcrl<sub>Y22A</sub>, pACcrl<sub>F53A</sub>, pACcrl<sub>W56A</sub>, pACcrl<sub>W82A</sub>, and  
 19 pACcrl<sub>G80A</sub>, respectively, between the *Pst*I and *Eco*RI sites of pUT18 using primers Crl-Pst  
 20 and Crl-Eco (as described above). Plasmids pUTCrl<sub>R51A</sub>, pUTCrl<sub>E52A</sub>, pUTCrl<sub>G55A</sub>, pUTCrl<sub>W57A</sub>  
 21 were obtained by site-directed mutagenesis of plasmid pUTCrl (Table 1). All plasmids were  
 22 confirmed to be correct by DNA sequencing.

23

24 **Electrophoresis and immunoblot analysis of proteins.** Whole-cell extracts were prepared  
 25 and SDS-polyacrylamide gel electrophoresis was carried out as described by Silhavy *et al.*

1 (46). The amount of protein in whole-cell lysates was determined using the DC Protein Assay  
2 kit (Bio-Rad). Equal amounts of protein were loaded in each slot. The molecular sizes of the  
3 proteins were estimated using molecular size standards (Fermentas, France). Rabbit  
4 antibodies against the Crl protein of *Salmonella* were from Robbe-Saule *et al.* (40). Rabbit  
5 antibodies against the  $\sigma^S$  protein of *S. enterica* serovar Typhimurium were from Coynault *et*  
6 *al.* (7). Proteins were transferred to Amersham Hybond P membranes (GE Healthcare) and  
7 incubated with the polyclonal rabbit antibody serum as previously described (7). Bound  
8 antibodies were detected using a secondary anti-rabbit antibody linked to peroxidase and the  
9 Amersham ECL plus western blotting detection system kit (GE Healthcare).

10

11 **Overproduction and purification of His<sub>6</sub>-Crl variants.** A 250 ml of JM109 carrying  
12 pQE<sub>crl</sub> wild-type or mutant was grown in LB medium containing carbenicillin at 30°C to an  
13 optical density of 0.6, and then 1 mM IPTG was added. After 30 min, the temperature was  
14 lowered to 23° C. The cells were harvested after 4 h, washed, resuspended in 10 ml of buffer  
15 A (25 mM Tris pH 8.0, 1 M NaCl, 0.01% NP-40) supplemented with “Complete EDTA free  
16 anti-protease” as described by the manufacturer (Roche) and then disrupted in a French press  
17 (Aminco). The crude cell extract was centrifuged at 15,000 g for 30 min. The supernatant was  
18 adjusted to 3 mM imidazole, added to 1.5 ml of Ni-nitrilotriacetic acid agarose (QIAGEN)  
19 and gently mixed for 1 h. The slurry was packed onto an Econo-Pac column (Bio-Rad) and  
20 washed with 15 ml of buffer A containing 20 mM imidazole. His<sub>6</sub>-Crl was eluted with buffer  
21 A containing 250 mM imidazole, dialyzed against buffer B (20 mM Tris-HCl pH 8, 50 mM  
22 NaCl, 0.01% NP-40), and added to DNA-cellulose equilibrated in the same buffer (0.3 ml,  
23 Sigma D-8515). The DNA-cellulose was removed by centrifugation and the concentrations of  
24 the proteins determined by a Bradford assay.

25

1 **Gel retardation assays.** The DNA encoding *rpoS* was amplified using primers HK1 5'-  
2 AGGCTCGGATCCATGAGTCAGAATACGCTGAAAGTTCAT-3' and HK2 5'-  
3 TTCCGAAAGCTTTTACTCGCGGAACAGCGCTTCGATATT-3' and cloned into the  
4 *Bam*HI and *Hind*III sites of pQE30 to yield pQE30*rpoS*. The nucleotide sequence of the *rpoS*  
5 insert in pQE30*rpoS* was checked by DNA sequencing. *S. Typhimurium* His<sub>6</sub>-σ<sup>S</sup> produced  
6 from pQE30*rpoS* was purified using Ni-affinity chromatography as described for His<sub>6</sub>-σ<sup>S</sup><sub>T2</sub>.  
7 *E. coli* core (Epicentre) was used to reconstitute the σ<sup>S</sup>-holoenzyme, which was incubated  
8 with variant Crl proteins (wild-type or mutants) or buffer. 9 μl of the protein complexes were  
9 then added to 3 μl of [<sup>32</sup>P]-labelled *katN* fragment in buffer A (40 mM Hepes pH 8.0, 10 mM  
10 MgCl<sub>2</sub>, 100 mM K-glutamate, 2 mM DTT) containing 500 μg ml<sup>-1</sup> BSA, and incubated at  
11 28°C for 20 min. The final concentrations of core, His<sub>6</sub>-σ<sup>S</sup>, Crl and *katN* fragments were  
12 respectively 8 nM, 32 nM, 3 μM and 1 nM. After addition of 3 μl loading buffer (buffer A  
13 containing 50% sucrose, 0.025 % xylene cyanol blue and 150 μg ml<sup>-1</sup> of heparin) the mixture  
14 was loaded onto a 6 % native polyacrylamide gel run in TG buffer (25 mM Tris, 192 mM  
15 Glycine pH 8.5) at 8V/cm. The gel was dried before being autoradiographed and quantified  
16 using a PhosphorImager (Molecular Dynamics).

17

18 **Abortive initiation assays.** A mixture of the 207-bp *lacUV5* fragment (10 nM), ApA (2  
19 mM), and 80 μM [ $\alpha$ -<sup>32</sup>P] UTP was added to an equal volume of the holoenzyme (60 nM,  
20 His<sub>6</sub>-σ<sup>S</sup>: core ratio = 2.5 : 1) preincubated with buffer or Crl (wild-type or variants) at 6 μM  
21 concentration at 28° C. Aliquots were removed at various times. The aliquots were spotted  
22 onto Whatman 3MM paper prespotted with 100 mM EDTA, and chromatograms were  
23 developed as previously described (40).

24

## RESULTS

### Distribution and sequence conservation of Crl in microbial sequenced genomes

A Blast search in 1264 sequenced bacterial genomes ([www.ncbi.nlm.nih.gov](http://www.ncbi.nlm.nih.gov)) revealed 143 genomes containing a *crl* gene. They are all  $\gamma$ -Proteobacteria and contain an *rpoS* gene (Table 2). Crl was absent from many *rpoS*-containing bacteria, for example *Pseudomonas*, in which RpoS plays an important role (45). Thus, *crl* is not as widely distributed as *rpoS* and it is also less conserved at the sequence level (Table 2). Sequence comparison of the 143 Crl proteins identified 60 Crl sequences that differed by at least one amino acid. Alignment of these 60 Crl sequences by clustalW revealed 17 residues that are conserved in all members of the Crl family (Figure 1).

Analysis of the 16 completed genomic sequences of *Salmonella* ([www.ncbi.nlm.nih.gov](http://www.ncbi.nlm.nih.gov)) indicated that the Crl sequence is highly conserved (Figure 1 and Table 2). Interestingly, the *crl* gene in *S. enterica* serovar Paratyphi B SPB7 lacks a G at position 73 in the open-reading frame, causing the appearance of a premature stop codon and the likely production of a truncated, non-functional Crl protein.

### The first 71 amino acids of $\sigma^S$ are non-essential for Crl binding

Crl is less conserved than the full length RpoS protein (Table 2). However, the first 55 residues of RpoS are poorly conserved, whereas the remaining 275 residues of RpoS are highly conserved (data not shown). For instance, the level of sequence identity between RpoS<sub>STM</sub> and the RpoS proteins from the *Enterobacteria* *Proteus mirabilis* and *Providencia stuartii* is 43% and 40% for aminoacids 1-55 compared to 87% and 86% for amino acids 56-330, respectively. These first 55 residues define a specific domain of  $\sigma^S$  that is not well conserved in other sigma factors besides  $\sigma^{70}$  (corresponding roughly to region  $\sigma_{1.1}$ , see also Discussion section and 32, 51), and might thus play a role in the specificity of recognition of

1  $\sigma^S$  by Crl. In addition,  $\sigma_{1.1}$  and Crl might play complementary roles in the formation of  $E\sigma^S$ .  
 2 Crl increases the affinity of  $\sigma^S$  for the core enzyme (12).  $\sigma_{1.1}$  stabilizes the interaction  
 3 between  $\sigma^{70}$  and core RNAP (18) and might play a similar role in  $\sigma^S$ -core interaction. These  
 4 possibilities prompted us to determine whether this domain of  $\sigma^S$  is required for  $\sigma^S$  activity  
 5 and for Crl activation.

6 We first examined the sensitivity to Crl activation of a  $\sigma^S$  mutant lacking the first 39  
 7 residues of  $\sigma^S$  ( $\sigma_{T2}^S$ , Figures 2 and 3) and thus lacking most of region  $\sigma_{1.1}$  (32, 51). *rpoS<sub>T2</sub>* is a  
 8 natural *rpoS* allele from *S. Typhi* strain T2 that contains a deletion of eight nucleotides in the  
 9 5' end of the coding sequence resulting in the appearance of a premature stop codon (Figure  
 10 2A). Surprisingly, *rpoS<sub>T2</sub>* was able to direct the synthesis of a 35 kDa  $\sigma^S$  protein ( $\sigma_{T2}^S$ , Figure  
 11 3A). N-terminal sequence analysis indicated that  $\sigma_{T2}^S$  has a N-terminal Met residue followed  
 12 by a sequence that is identical to that downstream of residue 39 in native  $\sigma^S$  (Figure 2B). We  
 13 inferred that  $\sigma_{T2}^S$  is encoded from the mutant gene in which translation initiates at a GTG start  
 14 codon preceding codon 40 of  $\sigma^S$  in *rpoS<sub>T2</sub>*, and upstream of which is a putative ribosome  
 15 binding site (SD, Figure 2B). Translational reinitiation within the *E. coli rpoS* gene has also  
 16 been reported previously (15). To facilitate characterization of the *rpoS<sub>T2</sub>* allele in isogenic  
 17 backgrounds, the *rpoS* gene from *S. Typhimurium* ATCC14028 was replaced by *rpoS<sub>T2</sub>*  
 18 yielding strain 2922K*rpoS<sub>T2</sub>*. During exponential growth,  $\sigma_{T2}^S$  was produced in higher  
 19 amounts than the wild-type  $\sigma^S$  protein (OD600 0.1 to 1.4, Figure 3B), likely because the N-  
 20 terminal 11 residues of  $\sigma^S$  involved in ClpX recognition and  $\sigma^S$  proteolysis by ClpP (13, 49)  
 21 were not present in  $\sigma_{T2}^S$ .  $\sigma^S$  is required for bacterial resistance to various stresses during  
 22 stationary phase (the so-called general stress resistance) (20,25). The  $\sigma^S$ -dependent gene *katE*  
 23 encodes a catalase, an enzyme that detoxifies hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) and contributes to the  
 24 resistance of *Salmonella* to oxidative stress in stationary phase (38). Despite the high amount

1 of  $\sigma^S_{T2}$  produced, strain 2922*KrpoS*<sub>T2</sub> was slightly less resistant to H<sub>2</sub>O<sub>2</sub> than the wild-type  
 2 strain 2922K and exhibited lower expression of the *katE-lacZ* fusion (Figure 2CD). This  
 3 suggested that  $\sigma^S_{T2}$  is less active than wild type  $\sigma^S$ . *In vitro* transcription experiments with  
 4  $\sigma^S_{T2}$  confirmed this hypothesis (data not shown). Interestingly, the levels of H<sub>2</sub>O<sub>2</sub> resistance  
 5 and the expression of the catalase gene *katE* were more dependent on Crl activation in  
 6 2922*KrpoS*<sub>T2</sub> than in the wild-type strain (Figure 2CD). This indicated that the  $\sigma^S_{T2}$  protein is  
 7 able to interact with Crl and that its activity is highly dependent on Crl.

8 We previously used the bacterial two-hybrid system (BACTH system, 22) to detect  
 9 interaction between Crl and  $\sigma^S$  (12). C-terminal fusions of *S. Typhimurium*  $\sigma^S$  to T25 (T25-  
 10  $\sigma^S$ ) and N-terminal fusions of *S. Typhimurium* Crl to T18 (Crl-T18) were produced in the *E.*  
 11 *coli cya lac*<sup>+</sup> strain BTH101 from pKTRpoS and pUTCrl, respectively, and the resulting  $\beta$ -  
 12 galactosidase activities were measured (Table 3). As expected (12), T25- $\sigma^S$  interacted with  
 13 Crl-T18 (Table 3). In this system, the first 38 residues of  $\sigma^S$  were dispensable for efficient  
 14 binding of Crl (compare T25- $\sigma^S$  and T25- $\sigma^S_{\Delta 1-38}$  in the presence of Crl-T18, Table 3).  
 15 Moreover, deletion of residues 1 to 38 from  $\sigma^S$  resulted in increased levels of the chimera and  
 16  $\beta$ -galactosidase activity in the BACTH assay (Figure 3C lanes 2 and 5, Table 3). This is  
 17 probably again due to the absence of the ClpX recognition site in T25- $\sigma^S_{\Delta 1-38}$ . Interestingly,  
 18 deletion of residues 1 to 71 in T25- $\sigma^S_{\Delta 1-71}$ , and deletion of residues 12 to 71, which does not  
 19 remove the ClpX recognition site (13, 49) in T25- $\sigma^S_{\Delta 12-71}$ , had a more pronounced effect on  
 20  $\sigma^S$  levels and  $\beta$ -galactosidase activity than deletion of residues 1 to 38 (in T25- $\sigma^S_{\Delta 1-38}$ ) (Figure  
 21 3C lanes 4-6, Table 3). This indicated that the first 71 residues in  $\sigma^S$  are non-essential for Crl  
 22 binding, and further suggested that the deletion of this region induces a conformational  
 23 change in  $\sigma^S$  that favors its stability. Altogether, these results suggested that Crl interacts with  
 24 the conserved amino acid region (72-330) of  $\sigma^S$ .

1

## 2 **Identification of conserved residues important for Crl activity**

3 Alanine substitution mutagenesis was performed to investigate the functional  
4 relevance of the conserved residues in Crl. The conserved residues are located in four regions  
5 of Crl (one third of them are aromatic amino-acids, Figure 1). As a first step, one or two  
6 residues in each conserved region were mutated. We also mutated the three cysteine residues  
7 in Crl. pACcrl-1 expresses the *crl* gene from *S. Typhimurium* under the control of the vector  
8 *cat* promoter, and is able to complement a  $\Delta crl$  mutant of *Salmonella* (40). Site-directed  
9 mutagenesis of pACcrl-1 yielded eleven derivatives expressing altered Crl proteins (Table 1  
10 and Figure 4A). The ability of the mutated *crl* genes to complement the *Salmonella*  $\Delta crl$   
11 mutant was assessed using three different tests (Figures 4 and 5). The development of the rdar  
12 morphotype depends on Crl (40) and was used as a qualitative test (Figure 5). This colony  
13 morphology is caused by production of curli and cellulose and is correlated with biofilm  
14 formation and expression of the regulatory gene *csgD* (42). In ATCC14028, the expression  
15 level of a *csgD-lacZ* fusion and the H<sub>2</sub>O<sub>2</sub> resistance level at 28°C both depend on Crl (38, 40),  
16 and were used as quantitative assays (Figure 4BC).

17 Derivatives of pACcrl-1 expressing the Crl proteins with F35A, C28A, C37A, G74A,  
18 G80A and F103A substitutions complemented  $\Delta crl$  as well as did pACcrl-1 (Figures 4-5 and  
19 data not shown). Two substitutions, W56A and F53A, located in a conserved motif of Crl  
20 (residues 48 to 57, Figure 1), substantially diminished complementation in all three tests  
21 (Figure 4 and Figure 5 spots 7-8). Two additional substitutions, Y22A and W82A, also  
22 decreased the complementation levels but to a lesser extent (Figure 4 and Figure 5 spots 3 and  
23 11). These four substitutions did not significantly decrease the cellular level of Crl (Figure  
24 6A) and, thus, likely affected its activity.

1           The *Salmonella* Crl protein contains three cysteines, (C28, C37, C41), one of which  
2 (C41) is conserved in all Crl members. Alanine substitution of cysteine residues (C28A,  
3 C37A and C41A) did not significantly affect the levels of Crl protein or its activity (Figures 4,  
4 5 and 6A). Only a slight effect of C41A on H<sub>2</sub>O<sub>2</sub> resistance was observed (Figure 4B).  
5 Interestingly, substitution of all three cysteine residues substantially decreased Crl levels  
6 (Figure 6A lanes 17 and 20). Examination of the Crl family members indicated that their all  
7 contain at least 2 cysteine residues. Altogether, these results suggested that the presence of at  
8 least one, and of probably two, cysteine residue(s) is required for Crl stability. Double  
9 mutations in the cysteine residues in Crl (Crl<sub>C28-37</sub>, Crl<sub>C28-41</sub>, Crl<sub>C37-41</sub>) were constructed to  
10 determine what combination of cysteine substitution affected Crl stability. The combinations  
11 of C28A and C37A substitutions and C28A and C41A substitutions did not significantly  
12 affected the levels of Crl protein (Figure 6A lanes 14 and 15). In contrast, alanine substitution  
13 of both C37 and C41 substantially decreased Crl levels (Figure 6A lane 16) suggesting that  
14 these two cysteine residues are important for Crl stability. Levels of the triple Crl<sub>C28-37-41</sub>  
15 mutant protein were lower than that of Crl<sub>C37-41</sub> (Figure 6A, lanes 19-20). This suggests that  
16 the C28A substitution further decreased the stability of Crl<sub>C37-41</sub>.

17

#### 18 **Screening of the *crl* mutations in the BACTH system and *in vitro* assays**

19           We next determined whether the mutations selected above affected Crl- $\sigma^S$  interaction.  
20 DNA encoding proteins with Y22A, F53A, W56A or W82A substitutions or the neutral  
21 G80A substitution were introduced into pUTCrl and the ability of the Crl-T18 derivatives to  
22 interact with  $\sigma^S$  was assessed in the presence of T25- $\sigma^S$  (Table 3). Interestingly, the Y22A,  
23 F53A, W56A, W82A substitutions, but not the G80A substitution, all abolished the  
24 interaction between Crl-T18 and T25- $\sigma^S$  (Table 3). These substitutions did not affect levels of  
25 Crl-T18 (Figure 6B). The altered Crl-T18 hybrid proteins were also unable to interact with

1 the T25- $\sigma^S$  derivative lacking amino acids 12 to 71 (data not shown). In conclusion, the  
2 Y22A, F53A, W56A and W82A substitutions all abolished the interaction between Crl and  $\sigma^S$   
3 either directly or indirectly through conformational changes in Crl.

4 The activities of the variant proteins were also probed *in vitro* by gel retardation and  
5 abortive initiation assays, based on the ability of Crl to increase the amount of promoter-  
6 complexes formed by the  $\sigma^S$ -RNA polymerase ( $E\sigma^S$ ) and activate transcription initiation (12,  
7 40). As shown on the autoradiogram of the native polyacrylamide gel (Figure 7A lane 3),  $E\sigma^S$   
8 binding to the [<sup>32</sup>P]-labelled *katN* promoter was stimulated 2-fold by wild-type Crl. The  
9 Y22A and W82A variants were still able to increase the formation of  $E\sigma^S$ -*katN* complexes by  
10 about 1.8 and 1.6 fold (Figure 7A lanes 4 and 6). In contrast, the F53A and W56A variants of  
11 Crl (Figure 7A lanes 5 and 7) had hardly any effect, in agreement with their highly reduced  
12 ability to stimulate *csgD-lacZ* expression or hydrogen peroxide resistance. The gel retardation  
13 methodology is only semi-quantitative, however, and the addition of heparin to disrupt non-  
14 specific core-DNA binding before electrophoresis might have disturbed weak interactions  
15 between Crl variants and  $\sigma^S$ . Therefore, abortive initiation assays, which do not require  
16 heparin addition and can be exploited for more quantitative studies, were conducted using the  
17 well-characterised *lacUV5* promoter, which can be transcribed *in vitro* by  $E\sigma^{70}$  and  $E\sigma^S$  and,  
18 unlike *katN*, has the advantage of producing high amounts of abortive products (29). As  
19 expected,  $E\sigma^S$  produced less ApApUpU tetranucleotide from *lacUV5* in the presence of the  
20 Crl variants than with the wild-type protein (Figure 7B). The results were more obvious than  
21 in gel retardation assays since wild-type Crl stimulated  $E\sigma^S$ -*lacUV5* abortive transcription by  
22 4-fold under our conditions. After 30 min incubation at 28°C, the observed decrease in  
23 ApApUpU synthesis was 35 % for Y22A, 40% for W82A and 90% for F53A. The addition of  
24 W56A Crl resulted in an even lower activity than in the absence of Crl, suggesting a slight  
25 inhibitory effect of this variant on  $\sigma^S$ -dependent transcription. Altogether, these results

1 emphasise the role of the REF-GWW motif (Figure 1) in Crl activation of  $\sigma^S$ -dependent  
2 promoters. Consistent with this hypothesis, the R51A, W57A substitutions and to a lesser  
3 extent the E52A and G55A substitutions affected the interaction between Crl and  $\sigma^S$  in the  
4 BACTH assay (Table 3) but did not affect levels of Crl-T18 (Figure 6B).

5

#### 6 **Fur-Crl interactions in *Salmonella***

7 To determine whether the four substitutions studied above affected Crl- $\sigma^S$  interaction  
8 specifically, we assessed their effect on the interaction of Crl with another partner. In a  
9 previous study using *E. coli* W3110, an interaction between Crl and the ferric uptake regulator  
10 Fur was detected by co-purification (28). To investigate the interaction between the  
11 *Salmonella* Crl and Fur proteins in the BACTH assay, we constructed plasmids pUTFur  
12 encoding a Fur-T18 protein and pKTFur encoding a T25-Fur protein. Significant interactions  
13 between the Crl- and the Fur- hybrid proteins were not detected (Table 3). The Fur hybrid  
14 proteins were stable, as indicated by the fact that the expected dimerization of Fur-T18 and  
15 T25-Fur (8 and references therein) gave a positive signal (Table 3).

16 *crl* transcription in *E. coli* W3110 is repressed by Fur (by 100-fold) and by Crl (by  
17 more than 10-fold) (28). To assess the possible relationship between Crl and Fur in  
18 *Salmonella* ATCC14028, we examined the effects of Fur and Crl on *crl* expression. We first  
19 compared Crl levels in the wild-type and *fur* strains by immunodetection of the Crl protein  
20 (Figure 6C). Cells were grown to stationary phase in LB at 30°C and 37°C, and also on LB  
21 agar at 30°C, a growth condition used to study the regulation of *crl* expression by Fur in *E.*  
22 *coli* W3110 (28). The amount of Crl detected in the *fur* strain was the same as in the wild-type  
23 under all three conditions (Figure 6C, compare lanes 1 and 2, 4 and 5, 7 and 8). In addition,  
24 the levels of *crl-lacZ*-encoded  $\beta$ -galactosidase were not significantly affected either by the *fur*

1 mutation or by the production of Crl *in trans* from plasmid pACcrl-1 (Figure 8AB). These  
2 results suggested that *crl* transcription is not repressed by Fur and Crl in ATCC14028.

3 In the presence of iron, Fur negatively controls transcription of a small RNA, RyhB,  
4 which facilitates degradation of the *sdhCDBA* mRNA encoding succinate dehydrogenase  
5 (30). Consistent with this finding, *sdhA-lacZ* -encoded  $\beta$ -galactosidase in ATCC14028 was  
6 dependent on both iron and Fur (Figure 8C). This demonstrated that regulation by Fur is  
7 indeed functional in ATCC14028. Fur did not exert any effect on the *crl-lacZ* fusion (Figure  
8 8C). Also, no effect of Fur on *crl-lacZ* expression was detected in LB supplemented with iron  
9 (data not shown).

10 *E. coli* W3110 has many genes that are differentially expressed in *E. coli* MG1655  
11 (52), and many laboratory stocks of W3110 contain mutations in *rpoS* (21, 52 and  
12 [www.ncbi.nlm.nih.gov](http://www.ncbi.nlm.nih.gov)). These findings prompted us to evaluate the effect of the *fur* mutation  
13 on *crl* expression in an *rpoS* background. Crl production and *crl-lacZ*-encoded  $\beta$  galactosidase  
14 levels were not affected by the *fur* mutation in the *Salmonella rpoS* mutant (Figure 6C lanes  
15 12 and 14, and Figure 8D). As previously shown (38), Crl production was increased by the  
16 *rpoS* mutation (Figure 6C lanes 10, 12, 15) by a mechanism that likely operates at the post-  
17 transcriptional level (Figure 8D).

18 Altogether, these results suggest that, contrary to *E. coli* W3110, Crl and Fur do not  
19 interact in *Salmonella*.

## DISCUSSION

1  
2 Analysis of the protein sequence databases revealed the narrow distribution of Crl  
3 homologues in bacteria. In contrast, RpoS homologues are found in many gram-negative  
4 bacteria of the  $\gamma$ -,  $\delta$ - and  $\beta$ - subdivisions ([www.ncbi.nlm.nih.gov](http://www.ncbi.nlm.nih.gov)). Several hypotheses might  
5 explain the absence of Crl in bacteria containing a RpoS homologue. Crl compensates for a  
6 low affinity of  $\sigma^S$  for the E, and might be dispensable in bacteria expressing a  $\sigma^S$  protein with  
7 a high affinity for E. Moreover, the physiological impact of Crl is greatest at low  
8 concentrations of  $\sigma^S$  (40, 41), and the environment can modulate the impact of Crl by  
9 affecting  $\sigma^S$  level (38). Thus, depending on the bacterial lifestyles and environment  
10 encountered, cellular physiology and the mechanisms of regulation of *rpoS* expression, Crl  
11 might be dispensable. Alternatively, functional homologues of Crl might exist in some *rpoS*-  
12 containing bacteria or some species might use alternative strategies to favor  $\sigma^S$  interaction  
13 with E. Interestingly, we did not find Crl homologues in bacteria that do not contain *rpoS*.  
14 This is consistent with previous work suggesting that the main function of Crl is to favor  $\sigma^S$   
15 interaction with RNA polymerase(41, 50).

16 Of the 16 *Salmonella* strains whose genome sequence is completed, one, *S. Paratyphi*  
17 SB7, contains a frameshift mutation in *crl* that results in the appearance of a premature stop  
18 codon. This observation is reminiscent of previous findings that *rpoS* null mutants can be  
19 found in natural isolates of *S. Typhi* (7, 37, 39), and is consistent with our previous  
20 observation that a  $\Delta$ *crl* mutation increases the competitive fitness of *Salmonella* by  
21 attenuating  $\sigma^S$  activity (41). The selection of *rpoS* mutants in bacterial populations likely  
22 results from a growth advantage of *rpoS* mutants in the absence of environmental stress (24,  
23 33, 34). However, in environments where bacteria encounter mild stress,  $\Delta$ *rpoS* mutants are  
24 outcompeted by the wild-type strain, whereas *rpoS* attenuated mutants exhibit increased

1 fitness (33, 41, 53). We can predict that the beneficial effect of a *crl* mutation is significant  
2 when the survival strategy requires “enough but not too much”  $\sigma^S$  activity.

3 We showed that four conserved residues (Y22, F53, W56, W82) are important for Crl  
4 activity and for Crl- $\sigma^S$  interaction but not for Crl stability. In contrast, a Crl protein in each of  
5 its three cysteine residues was substituted was unstable. The Y22A and W82A substitutions  
6 had a more dramatic effect in the BACTH system than in the complementation tests and the *in*  
7 *vitro* assays (Table 3 and Figures 4, 5 and 7). The IPTG-inducible *lac* promoter on pKT25 and  
8 pUT18 is induced by cAMP and, thus, a mutation that decreases the efficiency of the  
9 interaction between T25- $\sigma^S$  and Crl-T18 also lowers the expression level of the hybrid  
10 proteins, thereby amplifying the effect of the mutation (24 and Daniel Ladant personal  
11 communication).

12 The F53A and W56A substitutions are located in a conserved motif in the middle part  
13 of the protein (REF-GWW Figure 1). Analysis of four additional substitutions (R51A, E52A,  
14 G55A W57A) in the BACTH assay confirmed the role of the REF-GWW motif in Crl- $\sigma^S$   
15 interactions (Table 3, Figure 6B). The two other substitutions, Y22A and W82A, are outside  
16 of this motif. At least some of these mutations likely affect Crl binding to  $\sigma^S$  indirectly  
17 through conformational changes. The three-dimensional structure of Crl would provide new  
18 insight into this issue. Unfortunately, our attempts to crystallize Crl have been unsuccessful.  
19 In addition, deletion experiments in the BACTH system did not allow us to delineate a sub-  
20 region of Crl involved in  $\sigma^S$  binding. Crl might spread along  $\sigma^S$  or, more likely, a specific  
21 conformation of Crl might be required for efficient interaction with  $\sigma^S$ .

22 The primary determinants of  $\sigma^S$  involved in the binding of Crl are unknown, but the  
23 lack of interaction between Crl and  $\sigma^{70}$  (12) suggests that the interaction involves sequence  
24 determinants or structural features that are specific to  $\sigma^S$ . The vast majority of  $\sigma$  factors,  
25 including  $\sigma^S$ , belong to the so-called  $\sigma^{70}$  family, reflecting their relationship to the principal  $\sigma$

1 factor of *E. coli*,  $\sigma^{70}$ . Sequence alignments of  $\sigma^{70}$  family members of groups 1 and 2, to which  
 2  $\sigma^S$  belongs, reveal that they have four conserved regions (regions 1-4) (32, 35). Among these,  
 3 region 2 (subregions 2.1 to 2.4) and region 4 (subregions 4.1 and 4.2) contain DNA-binding  
 4 domains that mediate recognition of the conserved -10 and -35 elements of  $\sigma^{70}$ -dependent  
 5 promoters respectively. The linear division of  $\sigma^{70}$  factors into functionally distinct regions is  
 6 largely confirmed by structural data, which revealed that primary sigma factors have four  
 7 flexibly linked domains,  $\sigma_{1.1}$ ,  $\sigma_2$ ,  $\sigma_3$  and  $\sigma_4$ , containing regions 1.1, 1.2-2.4, 3.0-3.1, and 4.1-  
 8 4.2, respectively (5, 32). Free  $\sigma$  factors generally do not specifically bind promoter DNA, and  
 9 the N-terminal  $\sigma_{1.1}$  region is autoinhibitory (4, 9).  $\sigma_{1.1}$  might act indirectly to inhibit promoter  
 10 binding by stabilizing a compact conformation of  $\sigma$  that is incompatible with promoter  
 11 recognition (47). Binding to core RNAP induces large movements of the  $\sigma$  domains (3),  
 12 converting  $\sigma$  into an active conformation in which the DNA binding determinants in  $\sigma_2$  and  
 13  $\sigma_4$  are exposed (32).

14  $\sigma_{1.1}$  shows little conservation between  $\sigma^{70}$  and  $\sigma^S$  and is not found in the alternative  
 15 sigma factors of the  $\sigma^{70}$  family (32, 35). Therefore, the specificity of recognition between Crl  
 16 and  $\sigma^S$  might involve  $\sigma_{1.1}$ . However, our findings that (1)  $\sigma_{T2}^S$ , a protein lacking residues 1 to  
 17 39, including most of region  $\sigma_{1.1}$  in native  $\sigma^S$  (32, 51) is dependent on Crl activation (Figure  
 18 2), and (2) deletion of residues 1 to 71 of  $\sigma^S$ , which removes region  $\sigma_{1.1}$  and part of subregion  
 19 1.2 (32, 51) does not abolish Crl- $\sigma^S$  interaction (Table 3), ruled out this possibility.  
 20 Experiments are in progress to determine which region of  $\sigma^S$  is involved in Crl binding. In  
 21 addition to its autoinhibitory role,  $\sigma_{1.1}$  stabilizes the interaction between  $\sigma^{70}$  and core RNAP  
 22 (18). Our finding that  $\sigma_{T2}^S$  is -less active but -more dependent on Crl activation than wild-type  
 23  $\sigma^S$  (Figure 2CD and data not shown) is consistent with a similar role for  $\sigma_{1.1}$  in  $\sigma^S$ -core

1 interaction. Crl increases the affinity of  $\sigma^S$  for core RNAP (12) and might partially  
2 compensate for the absence of a full region  $\sigma_{1.1}$  in  $\sigma_{T2}^S$ .

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## REFERENCES

- 1  
2  
3
- 4 1. **Bang, I.-S., J. G. Frye, M. McClelland, J. Velayudhan, and F.C. Fang.** 2005.  
5 Alternative sigma factor interactions in *Salmonella*:  $\sigma^E$  and  $\sigma^H$  promote antioxidant defences  
6 by enhancing  $\sigma^S$  levels. *Mol. Microbiol.* **56**: 811-823.  
7
- 8 2. **Bougdoor, A., C. Lelong, and J. Geiselmann.** 2004. Crl, a low temperature-induced  
9 protein in *Escherichia coli* that binds directly to the stationary phase sigma subunit of RNA  
10 polymerase. *J. Biol. Chem.* **279**:19540-50.  
11
- 12 3. **Callaci S., E. Heyduk, and T. Heyduk.** 1999. Core RNA polymerase from *E. coli* induces  
13 a major change in the domain arrangement of the sigma 70 subunit. *Mol. Cell.* **3**: 229-138.  
14
- 15 4. **Camarero, J. A., A. Shekhtman, E.A. Campbell, M. Chlenov, T. M. Gruber, D. A.**  
16 **Bryant, S. A. Darst, D. Cowburn, and T. W. Muir.** 2002. Autoregulation of a bacterial  $\sigma$   
17 factor explored by using segmental isotopic labeling and NMR. *Proc. Natl. Acad. Sci USA*  
18 **99**: 8536-8541.  
19
- 20 5. **Campbell, E. A., L. F. Westblade, and S. A. Darst.** 2008. Regulation of bacterial RNA  
21 polymerase  $\sigma$  factor activity: a structural perspective. *Current Opin. Microbiol.* **11**: 1-7.  
22
- 23 6. **Chang, A. C., and S. N. Cohen.** 1978. Construction and characterization of amplifiable  
24 multicopy DNA cloning vehicles derived from the P15A cryptic miniplasmid. *J. Bacteriol.*  
25 **134**: 1141-56.

1

2 7. **Coynault, C., V. Robbe-Saule, and F. Norel.** 1996. Virulence and vaccine potential of  
3 *Salmonella typhimurium* mutants deficient in the expression of the RpoS (sigma S) regulon.  
4 Mol. Microbiol. **22**:149-60.

5

6 8. **D'Autréaux, B., L. Pecqueur, A. Gonzalez de Peredo, R. E. M. Diederix, C. Caux-**  
7 **Thang, L. Tabet, B. Bersch, E. Forest and I. Michaud-Soret.** 2007. Reversible Redox- and  
8 Zinc-dependent dimerization of the *Escherichia coli* Fur protein. Biochemistry **46**: 1329-  
9 1342.

10

11 9. **Dombroski A. J., W. A. Walter, M. T. Jr Record, D. A. Siegele, and C. A. Gross.** 1992.  
12 Polypeptides containing highly conserved regions of transcription initiation factor sigma 70  
13 exhibit specificity of binding to promoter DNA. Cell. **70**: 501-512.

14

15 10. **Dong, T., M. G. Kirchhof, and H. E. Schellhorn.** 2008. RpoS regulation of gene  
16 expression during exponential growth of *Escherichia coli* K12. Mol. Genet. Genomics **279**:  
17 267-77.

18

19 11. **Ellermeier, C. D., A. Janakiraman, and J. M. Slauch.** 2002. Construction of targeted  
20 single copy *lac* fusions using lambda Red and FLP-mediated site-specific recombination in  
21 bacteria. Gene **290**:153-61.

22

23 12. **England, P., L. F. Westblade, G. Karimova, V. Robbe-Saule, F. Norel, and A. Kolb.**  
24 2008. Binding of the unorthodox transcription activator, Crl, to the components of the  
25 transcription machinery. J Biol Chem. 283:33455-64.

1

2 13. Flynn J. M., S. B. Neher, Y-I. Kim, R. T. Sauer, and T. A. Baker. 2003. Proteomic  
3 discovery of cellular substrates of the ClpXP protease reveals five classes of ClpX-  
4 recognition signals. *Mol. Cell* **11**: 671-683.

5

6 14. Gaal, T., M. J. Mandel, T. J. Silhavy, and R. L. Gourse. 2006. Crl facilitates RNA  
7 polymerase holoenzyme formation. *J. Bacteriol.* **188** : 7966-70.

8

9 15. Gowrishankar, J., K. Yamamoto, P. R. Subbarayan, and A. Ishihama. 2003. In vitro  
10 properties of RpoS ( $\sigma^S$ ) mutants of *Escherichia coli* with postulated N-terminal subregion 1.1  
11 or C-terminal region 4 deleted. *J. Bacteriol.* **185**: 2673-2679.

12

13 16. Grigorova, I. L., N. J. Phleger, V. K. Mutalik, and C. A. Gross. 2006. Insights into  
14 transcriptional regulation and  $\sigma$  competition from an equilibrium model of RNA polymerase  
15 binding to DNA. *Proc. Natl. Acad. Sci. USA* **103**: 5332-5337.

16

17 17. Gruber, T. M., and C. A. Gross. 2003. Multiple sigma subunits and the partitioning of  
18 bacterial transcription space. *Annu. Rev. Microbiol.* **57**: 441-466.

19

20 18. Hinton, D. M., S. Vuthoori, and R. Mulamba. 2006. The bacteriophage T4 inhibitor and  
21 coactivator AsiA inhibits *Escherichia coli* RNA polymerase more rapidly in the absence of  
22  $\sigma^{70}$  region 1.1: Evidence that region 1.1 stabilizes the interaction between  $\sigma^{70}$  and core. *J.*  
23 *Bacteriol.* **188**: 1279-1285.

24

- 1 19. **Ikeda, J. S., A. Janakiraman, D. G. Kehres, M. E. Maguire, and J. M. Slauch.** 2005.  
2 Transcriptional regulation of *sitABCD* of *Salmonella enterica* serovar Typhimurium by MntR  
3 and Fur. *J. Bacteriol.* **187**: 912-922.  
4
- 5 20. **Ishihama, A.** 2000. Functional modulation of *Escherichia coli* RNA polymerase. *Annu.*  
6 *Rev. Microbiol.* **54**:499-518.  
7
- 8 21. **Jishage, M., and A. Ishihama.** 1997. Variation in RNA polymerase sigma subunit  
9 composition within different stocks of *Escherichia coli* W3110. *J. Bacteriol.* **179**: 959-963.  
10
- 11 22. **Karimova G., J. Pidoux, A. Ullmann, and D. Ladant.** 1998. A bacterial two-hybrid  
12 system based on a reconstituted signal transduction pathway. *Proc. Natl. Acad. Sci. USA.* **95**:  
13 5752-5756.  
14
- 15 23. **Karimova, G., C. Robichon, and D. Ladant.** 2009. Characterization of YmgF, a 72-  
16 residue inner membrane protein that associates with the *Escherichia coli* cell division  
17 machinery. *J. Bacteriol.* **191**: 333-346.  
18
- 19 24. **King, T., A. Ishihama, A. Kori, and T. Ferenci.** 2004. A regulatory trade-off as a source  
20 of strain variation in the species *Escherichia coli*. *J. Bacteriol.* **186**: 5614-5620.  
21
- 22 25. **Klauck, E., A. Typas, and R. Hengge.** 2007. The  $\sigma^S$  subunit of RNA polymerase as a  
23 signal integrator and network master regulator in the general stress response in *Escherichia*  
24 *coli*. *Sci. Prog.* **90**: 103-127.  
25

- 1 26. **Kowarz, L., C. Coynault, V. Robbe-Saule, and F. Norel.** 1994. The *Salmonella*  
2 *typhimurium katF (rpoS)* gene: cloning, nucleotide sequence, and regulation of *spvR* and  
3 *spvABCD* virulence plasmid genes. *J. Bacteriol.* **176**: 6852-60.
- 4
- 5 27. **Lelong, C., K. Aguiluz, S. Luche, L. Kuhn, J. Garin, T. Rabilloud, and J.**  
6 **Geiselmann.** 2007. The Crl-RpoS regulon of *Escherichia coli*. *Mol. Cell. Proteomics* **6**: 648-  
7 659.
- 8
- 9 28. **Lelong, C., M. Rolland, M. Louwagie, J. Garin, and J. Geiselmann.** 2007. Mutual  
10 regulation of Crl and Fur in *Escherichia coli* W3110. *Mol. Cell. Proteomics* **6**: 660-668.
- 11
- 12 29. **Malan, T. P., A. Kolb, H. Buc, and W. R. McClure.** 1984. Mechanism of CRP-cAMP  
13 activation of *lac* operon transcription initiation activation of the P1 promoter. *J. Mol. Biol.*  
14 **180**: 881-909.
- 15
- 16 30. **Massé, E., and S. Gottesman.** 2002. A small RNA regulates the expression of genes  
17 involved in iron metabolism in *Escherichia coli*. *Proc. Natl. Acad. Sci. USA* **99**: 4620-4625.
- 18
- 19 31. **Miller, J. H.** 1972. *Experiments in Molecular Genetics*, Cold Spring Harbour, N. Y.
- 20
- 21 32. **Murakami K.S., and S. A. Darst.** 2003. Bacterial RNA polymerase: the whole story.  
22 *Current Opin. Struct. Biol.* **13**: 31-39.
- 23

- 1 33. **Notley-McRobb, L., T. King, and T. Ferenci.** 2002. *rpoS* mutations and loss of general  
2 stress resistance in *Escherichia coli* populations as a consequence of conflict between  
3 competing stress responses. *J. Bacteriol.* **184**: 806-811.  
4
- 5 34. **Nyström T.** 2004. Growth versus maintenance: a trade-off dictated by RNA polymerase  
6 availability and sigma factor competition? *Mol Microbiol.* **54**: 855-62.  
7
- 8 35. **Paget , M. S. B., and J. Helmann.** 2003. The  $\sigma^{70}$  family of sigma factors. *Genome Biol.*  
9 **4**: 203  
10
- 11 36. **Pratt, L. A., and T. J. Silhavy.** 1998. Crl stimulates RpoS activity during stationary  
12 phase. *Mol. Microbiol.* **29**: 1225-36.  
13
- 14 37. **Robbe-Saule, V., G. Algorta, I. Rouilhac, and F. Norel.** 2003. Characterization of the  
15 RpoS status of clinical isolates of *Salmonella enterica*. *Appl. Environ. Microbiol.* **69**: 4352-  
16 4358.  
17
- 18 38. **Robbe-Saule, V., I. Carreira, A. Kolb, and F. Norel.** 2008. Effect of growth  
19 temperature on Crl-dependent regulation of  $\sigma^S$  activity in *Salmonella enterica* serovar  
20 Typhimurium. *J. Bacteriol.* **190**: 4453-4459.  
21
- 22 39. **Robbe-Saule V., C. Coynault, and F. Norel** 1995. The live oral typhoid vaccine Ty21a  
23 is a *rpoS* mutant and is susceptible to various environmental stresses. *FEMS Microbiol. Lett.*  
24 **126**: 171-176.  
25

- 1 40. **Robbe-Saule, V., V., Jaumouille, M. C., Prevost, S., Guadagnini, C. Talhouarne, H.**  
2 **Mathout, A., Kolb A, and F. Norel.** 2006. Crl activates transcription initiation of RpoS-  
3 regulated genes involved in the multicellular behavior of *Salmonella enterica* serovar  
4 Typhimurium. J Bacteriol. **188** : 3983-94.
- 5
- 6 41. **Robbe-Saule, V., M. D., Lopes, A., Kolb, and F. Norel.** 2007. Physiological effects of  
7 Crl in *Salmonella* are modulated by  $\sigma^S$  level and promoter specificity. J. Bacteriol. **189**: 2976-  
8 2987.
- 9
- 10 42. **Römling, U.** 2005. Characterization of the rdar morphotype, a multicellular behaviour in  
11 Enterobacteriaceae. Cell. Mol. Life Sci. **62**:1234-46.
- 12
- 13 43. **Sambrook, J., E. F. Fritsch and T. Maniatis.** 1989. Molecular cloning: a laboratory  
14 manual (2nd edn.), Cold Spring Harbor, N. Y.
- 15
- 16 44. **Schmieger, H.** 1972. Phage P22-mutants with increased or decreased transduction  
17 abilities. Mol. Gen. Genet. **119**: 75-88.
- 18
- 19 45. **Schuster, M., A. C. Hawkins, C. S. Harwood, and E. P. Greenberg.** 2004. The  
20 *Pseudomonas aeruginosa* RpoS regulon and its relationship to quorum sensing. Mol.  
21 Microbiol. **51**: 973-985.
- 22
- 23 46. **Silhavy, T. J., M. L. Bernman and L. W. Ernqvist.** 1984. Experiments with Gene  
24 Fusions, Cold Spring Harbour, N. Y.
- 25

- 1 47. **Sorenson M. K., and S.A. Darst.** 2006. Disulfide cross-linking indicates that FlgM-  
2 bound and free  $\sigma^{28}$  adopt similar conformations. Proc. Natl. Acad. Sci USA 103: 16722-  
3 16727.
- 4
- 5 48. **Sternberg, N. L., and R. Maurer.** 1991. Bacteriophage-mediated generalized  
6 transduction in *Escherichia coli* and *Salmonella typhimurium*. Methods Enzymol. **204**: 18-43.
- 7
- 8 49. **Stüdemann, A., M. Noirclerc-Savoye, E. Klauck, G. Becker, D. Schneider, and R.**  
9 **Hengge.** 2003. Sequential recognition of two distinct sites in  $\sigma^S$  by the proteolytic targeting  
10 factor RssB and ClpX. EMBO J. **22**: 4111-4120.
- 11
- 12
- 13 50. **Typas, A. C., Barembruch, A., Possling, and R. Hengge.** 2007. Stationary phase  
14 reorganisation of the *Escherichia coli* transcription machinery by Crl protein, a fine-tuner of  
15  $\sigma^S$  activity and levels. The EMBO J. **26** : 1569-1578.
- 16
- 17 51. **Vassylyev D. G., S-I. Sekine, O. Laptenko, J. Lee, M. N. Vassylyeva, S. Borukhov,**  
18 **and S. Yokoyama.** 2002. Crystal structure of a bacterial RNA polymerase holoenzyme at  
19 2.6A resolution. Nature **417**: 712-719.
- 20
- 21 52. **Vijayendran, C., T. Polen, V. F. Wendisch, K. Friehs, K. Niehaus, and E. Flaschel.**  
22 2007. The plasticity of global proteome and genome expression analysed in closely related  
23 W3110 and MG1655 strains of a well-studied model organism, *Escherichia coli*-K12. J.  
24 Biotechnol. **128**: 747-761.
- 25

1

2 53. **Zambrano, M. M., and R. Kolter.** 1996. GASPing for life in stationary phase. *Cell*  
3 **86:**181-184.

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

2	3	4	5
Strain or plasmid	Characteristics	Source or reference <sup>a</sup>	
4	<b><i>Escherichia coli</i></b>		
5	MC1061K	<i>araD139 Δ(ara-leu)-767 Δ(lacIPOZY)X74 rpsL</i>	26
6		<i>galU galK rpoS::kan</i>	
7	JM109	<i>recA1 supE44 endA1 hsdR17 gyrA96 relA1 thi</i>	43
8		$\Delta(lac-proAB)$ F'(traD36 proAB <sup>+</sup> lacI <sup>f1</sup> lacZΔM15)	
9	BTH101	F- <i>cya-99 araD139 galE15 galK16 rpsL1 (Str<sup>R</sup>)</i>	D. Ladant
10		<i>hsdR2 mcrA1 mcrB1</i>	
11	<b><i>Salmonella</i> serovar Typhi</b>		
12	T2	Wild-type	CIS <sup>b</sup>
13	<b><i>Salmonella</i> serovar Typhimurium</b>		
14	C52	Wild-type	26
15	C52K	C52 $\Delta rpoS::kan$	26
16	ATCC14028	Wild-type	ATCC <sup>c</sup>
17	ATCCrpoS	ATCC14028 $\Delta rpoS$	41
18	ATCCcrl	ATCC14028 $\Delta crl::Cm$	40
19	JS402	$\Delta fur-41::cat$	19
20	ATCCfur	ATCC14028 $\Delta fur-41::cat$	
21	ATCCrpoSfur	ATCC14028 $\Delta rpoS \Delta fur-41::cat$	
22	ATCCcrl csgD-lacZ	ATCC14028 $\Delta crl::cm csgD-lacZY$	40
23	ATCCsdhA-lacZ-k	ATCC14028 <i>sdhA-lacZY-Km</i>	41

1	ATCC <i>fur</i> <i>sdhA-lacZ-k</i>	ATCC14028 $\Delta$ <i>fur-41::cat</i> <i>sdhA-lacZY-Km</i>	
2	ATCC <i>crl-lacZ</i>	ATCC14028 <i>crl-lacZY-Km</i>	
3	ATCC <i>rpoS</i> <i>crl-lacZ</i>	ATCC14028 $\Delta$ <i>rpoS::Cm</i> <i>crl-lacZY-Km</i>	
4	ATCC <i>fur</i> <i>crl-lacZ</i>	ATCC14028 $\Delta$ <i>fur-41::cat</i> <i>crl-lacZY-Km</i>	
5	ATCC <i>rpoSfur</i> <i>crl-lacZ</i>	ATCC14028 $\Delta$ <i>rpoS</i> $\Delta$ <i>fur-41::cat</i> <i>crl-lacZY-Km</i>	
6	2922K	ATCC14028 $\Delta$ STM2922:: <i>Km</i>	41
7	2922K <i>crl</i>	ATCC14028 $\Delta$ STM2922:: <i>Km</i> $\Delta$ <i>crl::Cm</i>	41
8	2922K <i>rpoS</i>	ATCC14028 $\Delta$ STM2922:: <i>Km</i> $\Delta$ <i>rpoS::Cm</i>	41
9	2922K <i>rpoS</i> <sub>T2</sub>	ATCC14028 $\Delta$ STM2922:: <i>Km</i> <i>rpoS</i> <sub>T2</sub>	
10	2922K <i>rpoS</i> <sub>T2</sub> <i>crl</i>	2922K <i>rpoS</i> <sub>T2</sub> $\Delta$ <i>crl::Cm</i>	
11	2922K <i>katE-lacZ</i>	2922K Tn5B21-2.4	41
12	2922K <i>rpoS</i> <i>katE-lacZ</i>	2922K <i>rpoS</i> Tn5B21 -2.4	41
13	2922K <i>crl</i> <i>katE-lacZ</i>	2922K <i>crl</i> Tn5B21 -2.4	41
14	2922K <i>rpoS</i> <sub>T2</sub> <i>katE-lacZ</i>	2922K <i>rpoS</i> <sub>T2</sub> Tn5B21-2.4	
15	2922K <i>rpoS</i> <sub>T2</sub> <i>crl</i> <i>katE-lacZ</i>	2922K <i>rpoS</i> <sub>T2</sub> $\Delta$ <i>crl::Cm</i> Tn5B21-2.4	
16			
17	<b>Plasmids</b>		
18	pACYC184	cloning vector, <i>Cm</i> <sup>R</sup> , <i>Tet</i> <sup>R</sup>	6
19	pAC <i>crl</i> -1	pACYC184 with the promoterless	40
20		<i>crl</i> gene cloned into the <i>cat</i> gene	
21		( <i>crl</i> is transcribed from the <i>cat</i> promoter), <i>Tet</i> <sup>R</sup>	
22	pAC <i>crl</i> <sub>Y22A</sub>	pAC <i>crl</i> -1 with mutation Y22A in <i>Crl</i>	
23	pAC <i>crl</i> <sub>F35A</sub>	pAC <i>crl</i> -1 with mutation F35A in <i>Crl</i>	
24	pAC <i>crl</i> <sub>C28A</sub>	pAC <i>crl</i> -1 with mutation C28A in <i>Crl</i>	
25	pAC <i>crl</i> <sub>C37A</sub>	pAC <i>crl</i> -1 with mutation C37A in <i>Crl</i>	

1	pACcrl <sub>C41A</sub>	pACcrl-1 with mutation C41A in Crl	
2	pACcrl <sub>F53A</sub>	pACcrl-1 with mutation F53A in Crl	
3	pACcrl <sub>W56A</sub>	pACcrl-1 with mutation W56A in Crl	
4	pACcrl <sub>G74A</sub>	pACcrl-1 with mutation G74A in Crl	
5	pACcrl <sub>G80A</sub>	pACcrl-1 with mutation G80A in Crl	
6	pACcrl <sub>W82A</sub>	pACcrl-1 with mutation W82A in Crl	
7	pACcrl <sub>F103A</sub>	pACcrl-1 with mutation F103A in Crl	
8	pACcrl <sub>C28-37</sub>	pACcrl-1 with mutation C28A, C37A in Crl	
9	pACcrl <sub>C28-41</sub>	pACcrl-1 with mutation C28A, C41A in Crl	
10	pACcrl <sub>C37-41</sub>	pACcrl-1 with mutation C37A, C41A in Crl	
11	pACcrl <sub>C28-37-41</sub>	pACcrl-1 with mutation C28A, C37A, C41A in Crl	
12	pUC4K	source of Km resistance cartridge	Pharmacia
13	pSTF4	<i>spvRAB-lacZ</i> fusion in pQF50, Cb <sup>R</sup>	26
14	pACrpoS <sub>T2</sub>	pACYC184 with a 6 kb <i>Bgl</i> III fragment	
15		carrying <i>rpoS</i> <sub>T2</sub> , Cm <sup>R</sup>	
16	pUCrpoS <sub>T2</sub>	pUC19 with a 3.3 kb <i>Sph</i> I- <i>Sca</i> I fragment	
17		carrying <i>rpoS</i> <sub>T2</sub> , Cb <sup>R</sup>	
18	pUCrpoS <sub>T2</sub> K	pUCrpoS <sub>T2</sub> with STY3047::Km, Cb <sup>R</sup> Km <sup>R</sup>	
19	pQE60	vector for expression of His-tagged proteins, Cb <sup>R</sup>	Qiagen
20	pQE30	vector for expression of His-tagged proteins, Cb <sup>R</sup>	Qiagen
21	pQE <sub>rpoS</sub> <sub>T2</sub>	pQE60:: <i>rpoS</i> <sub>T2</sub> expresses a $\sigma^{\text{S}}_{\text{T2}}$ -His <sub>6</sub> protein, Cb <sup>R</sup>	
22	pQE30 <sub>rpoS</sub>	pQE30:: <i>rpoS</i> expresses a His <sub>6</sub> - $\sigma^{\text{S}}$ protein, Cb <sup>R</sup>	
23	pQE <sub>crl</sub>	pQE30:: <i>crl</i> expresses a His <sub>6</sub> -Crl protein, Cb <sup>R</sup>	40
24	pQE <sub>crl</sub> <sub>Y22A</sub>	pQE <sub>crl</sub> with mutation Y22A in Crl	
25	pQE <sub>crl</sub> <sub>F53A</sub>	pQE <sub>crl</sub> with mutation F53A in Crl	

1	pQEcr1 <sub>W56A</sub>	pQEcr1 with mutation W56A in Crl	
2	pQEcr1 <sub>W82A</sub>	pQEcr1 with mutation W82A in Crl	
3	pKT25	BACTH vector designed to express a given	23
4		polypeptide fused in frame at its N-terminal end with	
5		T25 fragment, p15 ori, Km <sup>R</sup>	
6	pKNT25	BACTH vector designed to express a given	23
7		polypeptide fused in frame at its C-terminal end with	
8		T25 fragment, p15 ori, Km <sup>R</sup>	
9	pUT18	BACTH vector designed to express a given	23
10		polypeptide fused in frame at its C-terminal end with	
11		T18 fragment, ColE1 ori, Ap <sup>R</sup>	
12	pUTCrl	pUT18 expressing Crl-T18	
13	pUTCrl <sub>Y22A</sub>	pUT18 expressing Crl-T18 with mutation Y22A in Crl	
14	pUTCrl <sub>R51A</sub>	pUT18 expressing Crl-T18 with mutation R51A in Crl	
15	pUTCrl <sub>E52A</sub>	pUT18 expressing Crl-T18 with mutation E52A in Crl	
16	pUTCrl <sub>F53A</sub>	pUT18 expressing Crl-T18 with mutation F53A in Crl	
17	pUTCrl <sub>G55A</sub>	pUT18 expressing Crl-T18 with mutation G55A in Crl	
18	pUTCrl <sub>W56A</sub>	pUT18 expressing Crl-T18 with mutation W56A in Crl	
19	pUTCrl <sub>W57A</sub>	pUT18 expressing Crl-T18 with mutation W57A in Crl	
20	pUTCrl <sub>G80A</sub>	pUT18 expressing Crl-T18 with mutation G80A in Crl	
21	pUTCrl <sub>W82A</sub>	pUT18 expressing Crl-T18 with mutation W82A in Crl	
22	pKNTCrl	pKNT25 expressing Crl-T25	
23	pKTRpoS	pKT25 expressing T25-RpoS	
24	pKTRpoS <sub>Δ12-71</sub>	pKTRpoS with in frame deletion of residues 12-71 in RpoS	
25	pKTRpoS <sub>Δ1-38</sub>	pKTRpoS with deletion of residues 1-38 in RpoS	

1	pKTRpoS <sub>Δ1-71</sub>	pKTRpoS with deletion of residues 1-71 in RpoS
2	pKTFur	pKT25 expressing T25-Fur
3	pUTFur	pUT18 expressing Fur-T18

4

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5 <sup>a</sup> This study, unless otherwise noted.

6 <sup>b</sup> WHO Reference Center for *Salmonella* (Institut Pasteur, Paris)

7 <sup>c</sup> American Type Culture Collection

1 **Table 2: Distribution and sequence conservation of Crl in bacterial sequenced genomes.**

2

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3 Genus<sup>a</sup>                      identity with Crl<sub>STM</sub> (%)<sup>b</sup>      identity with RpoS<sub>STM</sub> (%)<sup>c</sup>

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4

5	<i>Salmonella</i>	96-100	99-100	
6	<i>Citrobacter</i>	90	100	
7	<i>Escherichia</i>	83-84	98-99	
8	<i>Shigella</i>	83-84	99	
9	<i>Enterobacter</i>	74-77	97	
10	<i>Klebsiella</i>	75-76	98	<i>Enterobacteriaceae</i>
11	<i>Serratia</i>	69	94	
12	<i>Erwinia</i>	66	95	
13	<i>Yersinia</i>	63-66	84-97	
14	<i>Pectobacterium</i>	64	91	
15	<i>Proteus</i>	49	79	
16	<i>Providencia</i>	40-45	78	
17	<i>Aeromonas</i>	43-44	85-84	
18	<i>Vibrio</i>	38-50	72-82	
19	Unclassified <i>Vibrionales</i>	46	81	
20	<i>Photobacterium</i>	42-49	79-80	
21	<i>Moritella</i>	38	76	
22	<i>Psychromonas</i>	35-44	77	

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23 <sup>a</sup> bacterial sequenced genomes containing a *crl* gene

1 <sup>b</sup> Range of percentage of identity between the aminoacid sequence of the Crl proteins in  
2 strains belonging to the indicated genus and that of the *S. Typhimurium* Crl protein (Crl<sub>STM</sub>,  
3 133 residues, NP-459316 Figure 1 line 16).

4 <sup>c</sup> Range of percentage of identity between the aminoacid sequence of the RpoS proteins in  
5 strains belonging to the indicated genus and that of the *S. Typhimurium* RpoS protein  
6 (RpoS<sub>STM</sub>, 330 residues, X77752, 26).

7

1 **Table 3: BACTH analysis of Crl interactions with  $\sigma^S$  and Fur.**

2

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3 Co-expressed proteins  $\beta$ -galactosidase activity<sup>a</sup>

4

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5	T18 + T25	47 ± 6
6	Crl-T18 + T25	47 ± 6
7	T18 + T25- $\sigma^S$	43 ± 3
8	Crl-T18 + T25- $\sigma^S$	263 ± 38
9	Crl-T18 + T25- $\sigma^S_{\Delta 1-38}$	455 ± 33
10	Crl-T18 + T25- $\sigma^S_{\Delta 1-71}$	1830 ± 186
11	Crl-T18 + T25- $\sigma^S_{\Delta 12-71}$	2352 ± 146
12	Crl <sub>Y22A</sub> -T18 + T25- $\sigma^S$	44 ± 5
13	Crl <sub>R51A</sub> -T18 + T25- $\sigma^S$	59 ± 3
14	Crl <sub>E52A</sub> -T18 + T25- $\sigma^S$	102 ± 12
15	Crl <sub>F53A</sub> -T18 + T25- $\sigma^S$	48 ± 5
16	Crl <sub>G55A</sub> -T18 + T25- $\sigma^S$	138 ± 19
17	Crl <sub>W56A</sub> -T18 + T25- $\sigma^S$	44 ± 4
18	Crl <sub>W57A</sub> -T18 + T25- $\sigma^S$	74 ± 4
19	Crl <sub>G80A</sub> -T18 + T25- $\sigma^S$	205 ± 27
20	Crl <sub>W82A</sub> -T18 + T25- $\sigma^S$	55 ± 6
21	Crl-T18 + T25-Fur	43 ± 1
22	Fur-T18 + Crl-T25	58 ± 6
23	Fur-T18 + T25-Fur	243 ± 32
24	Fur-T18 + T25	55 ± 3

1	T18 + T25-Fur	42 ± 2
2	T18 + CrI-T25	52 ± 5

3

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4 <sup>a</sup> The efficiencies of functional complementation between the indicated proteins were  
5 quantified by measuring  $\beta$ -galactosidase activities in *E. coli* BTH101 cells harboring the  
6 corresponding plasmids as described in Material and Methods.  $\beta$ -galactosidase activity was  
7 measured according to the method of Miller (31).

1 **LEGENDS TO FIGURES**

2

3 **FIGURE 1. Alignment of the proteins from the Crl family.**

4 Alignment of the Crl protein sequences was performed with ClustalW. Residues that are  
 5 identical in all of the Crl proteins are shown in black boxes and position of these conserved  
 6 residues in the Crl sequence from *S. enterica* serovar Typhimurium (Crl<sub>STM</sub>, 133 residues,  
 7 NP-459316, line 16) is shown below the alignment. The accession numbers for the Crl  
 8 sequences are as follows: *Salmonella*: ZP-02346845 (14), ZP-02663633 (15)NP-459316 (16),  
 9 YP-215307 (17), YP-001571691 (18); *Citrobacter*: YP-001454497 (19); *Escherichia*: NP-  
 10 285957 (22), NP-414775 (23), YP-001742400 (25), YP-002383844 (26), YP-002327819 (27),  
 11 NP-752324 (28), YP-539315 (29), ZP-02902139 (30); *Shigella*: YP-309300 (20), YP-402173  
 12 (21), NP-706240 (24); *Enterobacter*: YP-001175503 (31), ZP-03281271 (32), YP-001439177  
 13 (35); *Klebsiella*: YP-001333935 (33), YP-002240279 (34); *Serratia*: YP-001477200 (13);  
 14 *Erwinia*: YP-001908518 (36); *Yersinia*: YP-001007379 (6), ZP-00829910 (7), ZP-00821198  
 15 (8), ZP-00826726 (9), ZP-00833806 (10), YP-001402111 (11), NP-668295 (12);  
 16 *Pectobacterium*: YP-051554 (37); *Proteus*: YP-002150140 (38); *Providencia*: ZP-03313695  
 17 (39), ZP-03317513 (40), ZP-02958311 (41); *Aeromonas*: YP-857911 (42), YP-001140774  
 18 (43); *Vibrio*: ZP-01235672 (1), NP-231906 (44), ZP-01957597 (45), ZP-01980856 (46), NP-  
 19 759329 (47), NP-933650 (48), ZP-01065753 (49), YP-002418001 (50), ZP-00993088 (51),  
 20 ZP-01870715 (53), NP-797054 (54), ZP-01260295 (55), YP-001444378 (56), ZP-01987497  
 21 (57), ZP-02197346 (58); Unclassified *Vibrionales*: ZP-01813308 (52); *Photobacterium*: ZP-  
 22 01159718 (2), YP-129053 (3), ZP-01218949 (4); *Moritella*: ZP-01898329 (5);  
 23 *Psychromonas*: YP-944255 (59), ZP-01216084 (60).

24

25

26

1

2 **FIGURE 2. Characterization of the *rpoS* mutant allele *rpoS*<sub>T2</sub>.**

3 A) Relevant portion of the *rpoS* sequence in the wild-type *rpoS* allele and the *rpoS*<sub>T2</sub> mutant  
 4 allele. The deletion of eight nucleotides in *rpoS*<sub>T2</sub>, compared to *rpoS* wild-type, is shown. This  
 5 mutation results in the appearance of a premature stop codon in *rpoS*<sub>T2</sub>. B) Determination of  
 6 the N-terminal sequence of  $\sigma^S_{T2}$ . Production of  $\sigma^S_{T2}$  likely results from translational  
 7 reinitiation in *rpoS*<sub>T2</sub> at the GTG codon that is preceded by a putative ribosome binding site  
 8 (Shine-Dalgarno sequence SD). C) Resistance to hydrogen peroxide of the *S. Typhimurium*  
 9 strains indicated. Cells were grown to stationary phase in LB at 37°C, washed, resuspended in  
 10 PBS to an OD<sub>600</sub> of 0.1 and H<sub>2</sub>O<sub>2</sub> 15 mM was added. A representative experiment is shown.  
 11 Similar results were obtained in repeat experiments. D) Expression of a *katE-lacZ* gene fusion  
 12 in *Salmonella* carrying the wild-type *rpoS* and mutant *rpoS*<sub>T2</sub> alleles. 1: 2922K*katE-lacZ*, 2:  
 13 2922K*rpoS katE-lacZ*, 3: 2922K*crl katE-lacZ*, 4: 2922K*rpoS<sub>T2</sub> katE-lacZ*, 5: 2922K*rpoS<sub>T2</sub>crl*  
 14 *katE-lacZ*.  $\beta$ -galactosidase activity was measured in overnight LB cultures at 37°C according  
 15 to the method of Miller (31).

16

17 **FIGURE 3. Expression of  $\sigma^S$  wild-type and mutant proteins.**

18 A) Detection of  $\sigma^S_{T2}$  produced from the *rpoS*<sub>T2</sub> allele. Overnight LB cultures at 37°C were  
 19 analysed by western blotting with anti- $\sigma^S$  antibodies. 5  $\mu$ g of total protein was loaded in each  
 20 slot. 1: *S. Typhimurium* wild-type strain C52, 2: *S. Typhimurium*  $\Delta$ *rpoS* mutant C52K, 3: *S.*  
 21 *Typhi* strain T2, 4: *E. coli* MC1061K harboring plasmid pAC*rpoS*<sub>T2</sub>. B) Expression of  $\sigma^S$  and  
 22  $\sigma^S_{T2}$  as a function of bacterial cell growth. *Salmonella* strains 2922K and 2922K*rpoS*<sub>T2</sub> were  
 23 grown in LB at 37°C. Exponential-phase cultures (optical density at 600 nm = 0.5) were  
 24 diluted into LB pre-warmed at 37°C to prolong the exponential phase. Aliquots were removed  
 25 at various time intervals and analysed by western blotting with anti- $\sigma^S$  antibodies. 10  $\mu$ g of

1 total protein was loaded in each slot. The growth phase was determined by measuring culture  
2 turbidity at an optical density of 600 nm (OD<sub>600</sub>). C1: 2922K*rpoS* (OD<sub>600</sub>=4), C2: 2922K  
3 (OD<sub>600</sub>=1.4). C) Expression of wild-type and truncated T25- $\sigma^S$  hybrid proteins. The *E. coli*  
4 *cya* strain BTH101 harboring pKT25 and its derivatives were grown to stationary phase  
5 (OD<sub>600</sub>= 3.5) in LB in the presence of 2 mM cAMP and 0.5 mM IPTG to fully induce the *lac*  
6 promoter on pKT25. 10  $\mu$ g of total protein was loaded in each slot and analysed by western  
7 blotting with anti- $\sigma^S$  antibodies. 1: no plasmid, 2: pKTRpoS, 3: pKT25, 4: pKTRpoS $_{\Delta 12-71}$ , 5:  
8 pKTRpoS $_{\Delta 1-38}$ , 6: pKTRpoS $_{\Delta 1-71}$ .

9

10 **FIGURE 4 Site-directed mutagenesis of the *Salmonella crl* gene and characterization of**  
11 **the *crl* mutant alleles.**

12 Site-directed mutagenesis of pAC*crl*-1 was performed, yielding eleven derivatives expressing  
13 Crl proteins in which residues Y22, F35, C28, C37, C41, F53, W56, G74, G80, W82, and  
14 F103 are substituted by alanine. The ability of the mutated *crl* genes to complement the  
15 *Salmonella*  $\Delta$ *crl* mutant was assessed using three different tests: the H<sub>2</sub>O<sub>2</sub> resistance level at  
16 28°C, the expression level of a *csgD-lacZ* fusion, and development of the rdar morphotype.  
17 A) Summary of the results obtained: (+) complementation level similar to that obtained with  
18 pAC*crl*-1 expressing the wild-type Crl protein, (-) complementation level similar to that  
19 obtained with the negative control pACYC184, and ( $\pm$ ) partial complementation level.  
20 Complementation experiments for the rdar morphotype are shown in Figure 5. B)  
21 Complementation of the  $\Delta$ *crl* mutant for resistance to H<sub>2</sub>O<sub>2</sub>. ATCC*crl* derivatives containing  
22 the indicated plasmids were grown to stationary phase in LB at 28°C, washed, resuspended in  
23 PBS to an OD<sub>600</sub> of 1 and H<sub>2</sub>O<sub>2</sub> 15 mM was added. A representative experiment is shown.  
24 Similar results were obtained in repeat experiments. C) Complementation of the  $\Delta$ *crl* mutant  
25 for expression of a *csgD-lacZ* fusion. ATCC*crl csgD-lacZ* derivatives containing the

1 indicated plasmids were grown in LB0 (LB without NaCl) at 28°C. Exponential-phase  
 2 cultures (optical density at 600 nm = 0.5) of *Salmonella* were diluted into LB0 pre-warmed at  
 3 28°C to prolong the exponential phase. Aliquots were removed at various time intervals and  
 4  $\beta$ -galactosidase activity measured (lines) according to the method of Miller (31). The growth  
 5 phase was determined by measuring the culture turbidity at an optical density of 600 nm  
 6 (dashed line, the growth curve was similar for all the strains). The measurements were  
 7 repeated twice, and a representative experiment is shown.

8

9 **FIGURE 5. Rdar morphotypes of *Salmonella* ATCCcrl harbouring different plasmids.**

10 The  $\Delta$ crl mutant harbouring the vector pACYC184, the wild-type *crl* gene on pACcrl-1 and  
 11 mutated pACcrl-1 derivatives (Table 1) were grown five days on CR plates at 28°C.  
 12 Plasmids are 1 : pACYC184, 2 : pACcrl-1, 3 : pACcrl<sub>Y22A</sub>, 4 : pACcrl<sub>F35A</sub>, 5 : pACcrl<sub>C37A</sub>, 6 :  
 13 pACcrl<sub>C41A</sub>, 7 : pACcrl<sub>F53A</sub>, 8 : pACcrl<sub>W56A</sub>, 9 : pACcrl<sub>G74A</sub>, 10 : pACcrl<sub>G80A</sub>, 11 :  
 14 pACcrl<sub>W82A</sub>, 12 : pACcrl<sub>F103A</sub>, 13 : pACcrl<sub>C28A</sub>, 14 : pACcrl<sub>C28-37-41</sub>. Complementation of the  
 15 *crl* mutation was observed with pACcrl-1 and its derivatives containing mutations F35A,  
 16 C28A, C37A, C41A, G74A, G80A, F103A.

17 **FIGURE 6. Expression of Crl wild-type and mutant proteins.**

18 A) Detection of Crl produced from pACcrl-1 and its mutant derivatives in *Salmonella*  
 19 ATCCcrl. ATCCcrl strains, harboring the vector pACYC184 and the pACcrl-1 derivatives as  
 20 indicated below, were grown to stationary phase (OD<sub>600</sub>=4) in LB at 37°C and analysed by  
 21 western blotting with anti-Crl antibodies. 1  $\mu$ g of total protein was loaded in slots 1 to 17. 1:  
 22 pACcrl-1, 2: pACYC184, 3: pACcrl<sub>Y22A</sub>, 4: pACcrl<sub>F35A</sub>, 5: pACcrl<sub>C37A</sub>, 6: pACcrl<sub>C41A</sub>, 7:  
 23 pACcrl<sub>F53A</sub>, 8: pACcrl<sub>W56A</sub>, 9: pACcrl<sub>G74A</sub>, 10: pACcrl<sub>G80A</sub>, 11: pACcrl<sub>W82A</sub>, 12: pACcrl<sub>F103A</sub>,  
 24 13: pACcrl<sub>C28A</sub>, 14: pACcrl<sub>C28-37</sub>, 15: pACcrl<sub>C28-41</sub>, 16: pACcrl<sub>C37-41</sub>, 17: pACcrl<sub>C28-37-41</sub>. 5  $\mu$ g

1 of total protein was loaded in slots 18 to 20. 19: pACcrl<sub>C37-41</sub>, 20: pACcrl<sub>C28-37-41</sub>, 19: a  
 2 stationary phase culture of ATCC14028 in LB at 37°C was used as control. B) Expression of  
 3 wild-type and altered Crl-T18 hybrid proteins. The *E. coli cya* strain BTH101 harboring  
 4 pUT18 and its derivatives were grown to stationary phase (OD<sub>600</sub>= 3) in LB in the presence  
 5 of 2 mM cAMP and 0.5 mM IPTG to fully induce the *lac* promoter on pUT18. 5 µg of total  
 6 protein was loaded in each slot and analysed by western blotting with anti-Crl antibodies. 1:  
 7 no plasmid, 2: pUT18, 3: pUTCrl, 4: pUTCrl<sub>Y22A</sub>, 5: pUTCrl<sub>F53A</sub>, 6: pUTCrl<sub>W56A</sub>, 7:  
 8 pUTCrl<sub>G80A</sub>, 8: pUTCrl<sub>W82A</sub>, 9: pUTCrl<sub>R51A</sub>, 10: pUTCrl<sub>E52A</sub>, 11: pUTCrl<sub>G55A</sub>, 12: pUTCrl<sub>W57A</sub>.  
 9 C) Detection of Crl in *Salmonella* ATCC14028 (1, 4, 7, 10, 15) and its mutant derivatives  
 10 ATCC<sub>fur</sub> (2, 5, 8, 13), ATCC<sub>crl</sub> (3, 6, 9, 11), ATCC<sub>rpoS</sub> (12), and ATCC<sub>rpoSfur</sub> (14).  
 11 Cultures were grown in different conditions, as indicated, and analysed by western blotting  
 12 with anti-Crl antibodies. 1 µg of total protein was loaded in each slot. LB 37°C: overnight LB  
 13 cultures at 37°C. LB 30°C: overnight LB cultures at 30°C. LB agar 30°C: colonies grown on  
 14 LB agar at 30°C as described (28).

15

16 **FIGURE 7. Effects of the Crl variants on Eσ<sup>S</sup> promoter binding and abortive**  
 17 **transcription.**

18 A) Band shift analysis of Eσ<sup>S</sup> binding to the *katN* labelled fragment in the absence of Crl  
 19 (lanes 2 and 8) and in the presence of wild-type Crl (lane 3) or its variants Y22A (lane 4),  
 20 F53A (lane 5), W82A (lane 6) and W56A (lane 7), lane 1: no protein. A typical  
 21 autoradiogram is shown. The bands corresponding to free and bound DNAs are indicated by  
 22 arrows and the percentage of bound DNA indicated below each lane is the average of two  
 23 experiments. B) Abortive initiation assays. Eσ<sup>S</sup> was preincubated in the absence of Crl or in  
 24 the presence of wild-type Crl or its variants Y22A, F53A, W82A and W56A. After addition  
 25 of a mixture containing the *lacUV5* fragment, the ApA dinucleotide and [α-<sup>32</sup>P]-UTP, the

1 incorporation of labeled [ $\alpha$ - $^{32}$ P]UMP into abortive transcripts was monitored as a function of  
2 time. The measurements were repeated twice, and a representative experiment is shown.

3

4 **FIGURE 8. Expression of *crl-lacZ* and *sdhA-lacZ* gene fusions in *Salmonella*.**

5 Expression of the gene fusions in the *Salmonella* strains indicated was determined in cultures  
6 grown in different conditions. LB 37°C: overnight LB cultures at 37°C. LB 30°C: overnight  
7 LB cultures at 30°C. LB agar 30°C: colonies grown on LB agar at 30°C as described (28).  
8 M9: Minimal M9 medium with 20 mM glucose. M9 + FeCl<sub>2</sub>: M9 with 100  $\mu$ M ferrous  
9 chloride. M9 + DP: M9 with the iron chelator 2,2'-Dipyridyl (100  $\mu$ M).  $\beta$ -galactosidase  
10 activity was measured according to the method of Miller (31). A) Expression of the *crl-lacZ*  
11 gene fusion in ATCC *crl-lacZ* strains harboring pACYC184 and pAC*crl*-1 that expresses the  
12 *crl* gene under the control of the *cat* promoter of pACYC184 (40). B) Expression of the *crl*-  
13 *lacZ* gene fusion in ATCC *crl-lacZ* and ATCC*fur* *crl-lacZ*. C) Expression of the *sdhA-lacZ*  
14 gene fusion in ATCC *sdhA-lacZ*-k and ATCC*fur* *sdhA-lacZ*-k and expression of the *crl-lacZ*  
15 gene fusion in ATCC *crl-lacZ* and ATCC*fur* *crl-lacZ*. D) Expression of the *crl-lacZ* gene  
16 fusion in ATCC *crl-lacZ*, ATCC*CrpoS* *crl-lacZ*, ATCC*fur* *crl-lacZ* and ATCC*CrpoSfur* *crl-lacZ*.  
17

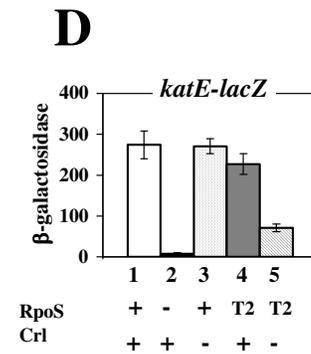
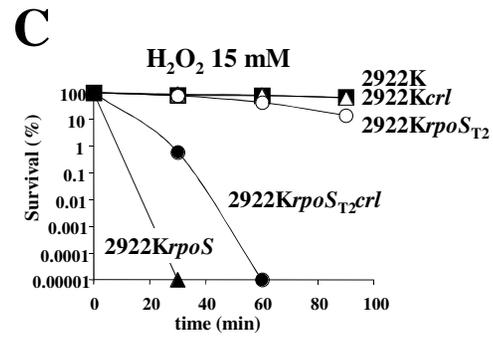


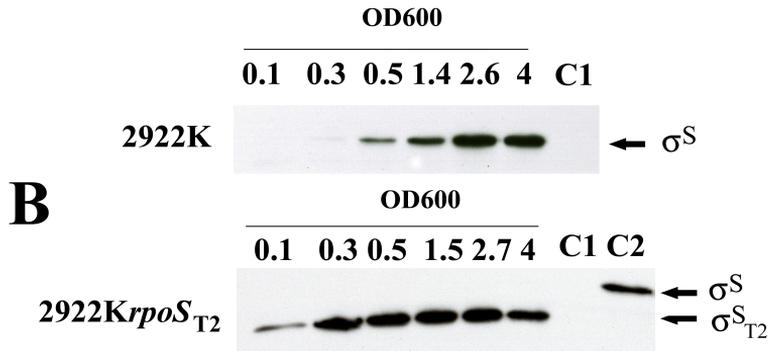
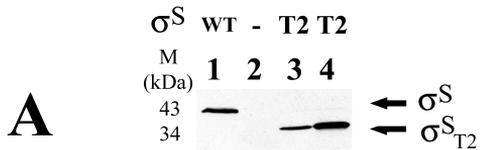
**A**

	34		40
<i>rpoS</i>	... GAA CCC AGT GAT AAC GAC CTG GCT GAA GAA GAG CTG TTA		
	E P S D N D L A E E E L L		
<i>rpoS</i> <sub>T2</sub>	... GAA CCC AGT G-- --- --- CT GGC TGA	Δ	STOP

**B**

	SD								
	TTTGACGAAAAAGCCTTGAGTGAAGAGGAACCCA	GTG	CTG	GCT	GAA	GAA	GAG	CTG	TTA
$\sigma^S_{T2}$	M	L	A	E	E	E	L	L	
	1	2	3	4	5	6	7	8	

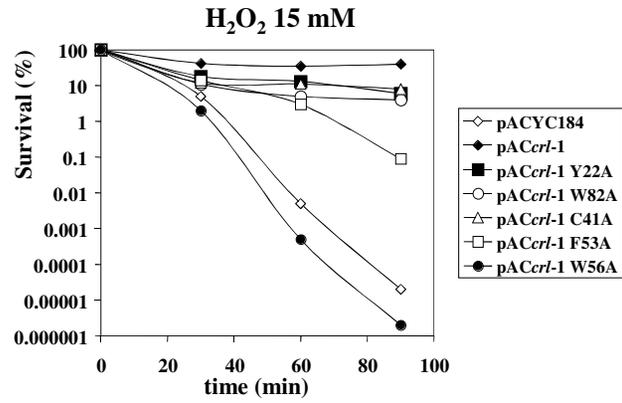




**A**

	Y22	F35	C28	C37	C41	F53	W56	G74	G80	W82	F103
<b>Rdar</b>	±	+	+	+	+	-	-	+	+	±	+
<i>csgD-lacZ</i>	±	+	+	+	+	±	-	+	+	±	+
$H_2O_2^R$	±	+	+	+	±	±	-	+	+	±	+

**B**



**C**

