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Identification of conserved amino acid residues of the *Salmonella* σ^S^ chaperone Crl involved in Crl-σ^S^ interactions

Véronique Monteil^1^, Annie Kolb^1^, Jacques D’Alayer^2^, Pierre Beguin^3^, and Françoise Norel^1^*

Institut Pasteur, Paris, France (1) Unité de Génétique moléculaire and CNRS URA2172; (2) Plate-Forme d’Analyse et de Microséquençage des Protéines; (3) PF5 Production de Protéines Recombinantes et d’Anticorps, 25-28, rue du Dr Roux, 75724 Paris Cedex 15, France

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*corresponding author

Institut Pasteur, Unité de Génétique Moléculaire; URA-CNRS 2172; 25 rue du Docteur Roux, 75724 Paris Cedex 15, France. E.mail : francoise.norel@pasteur.fr; Phone : 33 140613122; Fax 33 145688960.
ABSTRACT

Proteins that bind $\sigma$ factors typically attenuate $\sigma$ factor function by restricting its access to the RNA polymerase (RNAP) core enzyme. An exception to this general rule is the Crl protein that binds the stationary phase sigma factor $\sigma^S$ (RpoS) and enhances its affinity for the RNAP core enzyme, thereby increasing expression of $\sigma^S$-dependent genes. Analyses of sequenced bacterial genomes revealed that $crl$ is less widespread and less conserved at the sequence level than is $rpoS$. Seventeen residues are conserved in all members of the Crl family. Site-directed mutagenesis of the $crl$ gene from *Salmonella enterica* serovar Typhimurium and complementation of a $\Delta crl$ mutant of *Salmonella* indicated that substitution of the conserved residues Y22, F53, W56 and W82 decreased Crl activity. This conclusion was further confirmed by promoter binding and abortive transcription assays. We also used a bacterial two-hybrid system (BACTH) to show that the four substitutions in Crl abolish Crl-$\sigma^S$ interaction and that residues 1 to 71 in $\sigma^S$ are dispensable for Crl binding. In *Escherichia coli*, it has been reported that Crl also interacts with the ferric uptake regulator Fur, and that Fur represses $crl$ transcription. However, the *Salmonella* Crl and Fur proteins did not interact in the BACTH system. In addition, afur mutation did not have any significant effect on the expression level of Crl in *Salmonella*. These results suggest that the relationship between Crl and Fur is different in *Salmonella* and *E. coli*.
INTRODUCTION

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

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

The crl gene product is a regulator of $\sigma^S$ activity in Escherichia coli (2, 14, 36, 50) and Salmonella (38, 40, 41). In both species, the Crl protein binds $\sigma^S$ (2, 12) and facilitates RNA
polymerase holoenzyme EσS formation (12, 50), thereby enhancing σS effectiveness (10, 14, 27, 38, 40, 41, 50). The Crl protein of Salmonella binds σS with a stoechiometry 1:1 and increases the affinity of σS for the core enzyme 7-fold (12). In contrast, Crl does not bind σ70 and does not modify the affinity of σ70 for the core enzyme (12). Lelong et al. (28) reported that, in E. coli W3110, Crl can interact with the ferric uptake regulator Fur, a key protein for the control of intracellular iron concentration (8 and references therein) and that Fur represses crl transcription (28).

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

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

Oxidative shock survival assay. Cells were grown to stationary phase in LB (optical density at 600nm (OD_{600}) of 3.5 to 4), washed, resuspended in PBS and mixed with H_{2}O_{2} at a final concentration of 15 mM. Aliquots of bacteria were removed at time intervals, and numbers of colony forming cells were determined on LB plates.

DNA manipulations and sequence analysis. Standard molecular biology techniques were used (43). Oligonucleotides were obtained from Sigma-Aldrich (France). DNA sequencing was performed by Cogenics (France). DNA and amino acid sequence analyses were
conducted using the BLAST programs at the NCBI (National Center for Biotechnology Information).

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

Construction of \textit{S. Typhimurium} 2922K\textit{rpoS}$_{T2}$. The \textit{rpoS} allele in \textit{S. Typhimurium} ATCC14028 was replaced by the \textit{rpoS}$_{T2}$ allele using the following strategy (41). pUC\textit{rpoS}$_{T2}$ contains the \textit{rpoS}$_{T2}$ allele of \textit{S. Typhi} strain T2 and the downstream genes, including a gene encoding a putative decarboxylase (named STY3047 in \textit{S. Typhi} and STM2922 in \textit{S. Typhimurium}) that is not regulated by \textit{rpoS} (1). In pUC\textit{rpoS}$_{T2}$K, a kanamycin resistance (\textit{Km}) cartridge is located in the \textit{Sma}I site of STY3047. pUC\textit{rpoS}$_{T2}$K was introduced by electroporation into ATCC\textit{rpoS}, where it was unstable. Recombination of the \textit{Km} cartridge
into the host genome with simultaneous loss of pUCrpoST2K resulted in recombinants that were resistant to kanamycin and sensitive to carbenicillin. A Km\(^R\) Cb\(^S\) Cm\(^S\) recombinant was selected, checked by PCR for the presence of the STM2922::Km mutation and simultaneous replacement of the \(\Delta rpoS::Cm\) mutation by the rpoST2 allele. The rpoST2 and STM2922::Km alleles were then co-transduced to ATCCrpoS. Transductants that were Km\(^R\) but Cm\(^S\) were selected, and transduction of the STM2922::Km mutation and simultaneous replacement of the \(\Delta rpoS::Cm\) mutation by the rpoST2 allele were confirmed by PCR. One Km\(^R\) Cm\(^S\) strain, designated 2922KrpoST2, was also checked by DNA sequencing for the presence of the rpoST2 allele.

**Determination of the N-terminal sequence of \(\sigma^8_{T2}\).** pQE\(rpoST2\), which produces the rpoST2 gene with a C-terminal His\(_6\) extension under the control of the pQE60 IPTG-inducible promoter, was constructed as follows. Primers specific to the ends of rpoS (5’-CCGTCAAGAATTTCACGGGTAGGAGCCACCTTATGA-3’ and 5’-TGATCGAGATCTTCGCAGAACAGCGCTTATGCAC-3’) were used to amplify a 1 kb fragment of S. Typhi T2 total DNA by PCR. EcoRI and BglII restriction sites were incorporated at its 5’ and 3’ ends, respectively. After digestion with EcoRI and BglII, the PCR-amplified fragment was ligated into the EcoRI and BglII sites of pQE60. The nucleotide sequence of the rpoS insert in pQE\(rpoST2\) was verified. In this construct, rpoST2 is expressed from its own translation sequences. *E. coli* JM109 carrying pQE\(rpoST2\) was grown in LB containing ampicillin at 37°C to an optical density of 0.9, when 0.7 mM isopropyl-\(\beta\)-D-thiogalactoside (IPTG) was added. The cells were harvested after 5 h, resuspended in buffer A (50 mM Na\(_2\)HPO\(_4\) pH 8, 300 mM NaCl) and then disrupted in by French Press. The crude cell extract was supplemented with Benzonase (1250 U) and imidazole (10 mM) and centrifuged at 27,000 g for 30 min. The supernatant was added to 1.5 ml Ni-NTA column and washed
with buffer A containing 20 mM imidazole. $\sigma_{T2}^5$-His$_6$ was eluted with buffer A containing
300 mM imidazole. After electrophoresis in a 10% polyacrylamide-SDS gel, the $\sigma_{T2}^5$-His$_6$
protein was transferred on a PVDF membrane, stained with Amido Black and excised from
the membrane for N-terminal sequence analysis. Amino-terminal Edman degradation was
carried out on an Applied Biosystems sequencer.

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

Enzymatic assays. $\beta$-galactosidase activity was measured as described by Miller (31) and is
expressed in Miller units.

BACTH assays. The bacterial adenylate cyclase-based two hybrid (BACTH) assay is
dependent upon the functional reconstitution of the Bordetella pertussis adenyl cyclase T18
and T25 subdomains by two interacting partners (22). The resulting cAMP binds to and
activates the transcription activator CRP, a positive regulator of $\lambda$$\lambda$ lac and mal operons
involved in lactose and maltose catabolism. Derivatives of pUT18, pKT25 and pKNT25, used
in the BACTH assays, were constructed by cloning PCR amplified DNA fragments encoding
the protein of interest from S. Typhimurium between the PstI and EcoRI sites of pUT18 and
pKNT25 and the XbaI and KpnI sites of pKT25. The DNA encoding Crl was amplified using
primers Crl-Pst 5'-CA TGC CTG CAG AAG GAG ATC GCA ATG ACG TTA CCG AGT-
3' and Crl-Eco 5'-T CGA TGA ATT GCA TGG CAG TTT TAC CGG CTC GTC GT-
3', restricted by PstI and EcoRI and cloned into the PstI and EcoRI sites of both pUT18 and
pKNT25 to yield pUTCrl and pKNTCrl, respectively. The DNA encoding RpoS was
amplified using primers RpoS-Xba 5'-TCG ACT CTA GAT ATG AGT CAG AAT ACG 8
CTG AAA GTT CAT-3' and RpoS-Kpn 5'-AC TTA GGT ACC TTA CTC GCG GAA CAG
CGC TTC GT-3' restricted by XbaI and KpnI and cloned into the XbaI and KpnI sites of
pKT25 to yield pKTRpoS. The DNA encoding Fur was amplified using primers Fur-Pst 5'-
CAT GCC TGC AG C ATG ACT GAC AAC AAT ACC GCA-3' and Fur-Eco 5'-T CGA
TGA ATT GCA TTT AGT CGC GTC ATC GTG CGC GT-3' for cloning into the PstI and
EcoRI sites of pUT18 (yielding pUTFur) and using Fur-Xba 5'- TCX ACT CTA GAC ATG
ACT GAC AAC AAT ACC GCA TTA AAG A-3' and Fur-Kpn 5'- AC TTA GGT ACC
TTA TTT AGT CGC GTC GT-3' for cloning into the XbaI and KpnI sites of
pKT25 (yielding pKTFur). pKTRpoS\(_{\Delta 12-71}\) is a derivative of pKTRpoS with an in frame
deletion of an internal HpaI-DraI fragment. pKTRpoS\(_{\Delta 1-38}\) contains a DNA fragment
amplified with primers RpoST2-Xba 5'- TCG ACT CTA GAC CTG GCT GAA GAA GAG
CTG TTA TCG GAA-3' and RpoS-Kpn (see above) and cloned into the XbaI and KpnI sites
of pKT25. pKTRpoS\(_{\Delta 1-71}\) contains a DNA fragment amplified with primers RpoS-Xba5 5'-
TCG ACT CTA GAA ACA GCC GAA GAA GAA TAT TTT CGG GT-3' and RpoS-
Kpn (see above) and cloned into the XbaI and KpnI sites of pKT25. All plasmids were
confirmed to be correct by DNA sequencing. The E. coli cya strain BTH101 was transformed
with derivatives of plasmids pKT25, pKNT25, and pUT18 encoding proteins fused to the T25
and T18 fragments of *Bordetella pertussis* adenyl cyclase. Co-transformants were plated onto MacConkey maltose plates supplemented with carbenicillin, kanamycin and 0.5 mM IPTG to assess the Mal\(^+\) phenotype and on LB plates supplemented with 5-bromo-4-chloro-indolyl-β-D-galactoside (X-Gal, 40 µg ml\(^{-1}\)), carbenicillin, kanamycin and 0.5 mM IPTG to assess the Lac\(^+\) phenotype and β-galactosidase activity. Plates were incubated at 30°C for 3 days and colonies were then collected and their β-galactosidase activities were measured as described by Miller (31). For immunodetection of the Crl-T18 and T25-σ\(^5\) chimeras, BTH101 derivatives were grown to stationary phase (OD600= 3.5) in LB in the presence of 2 mM cAMP and 0.5 mM IPTG to induce fully the lac promoter on pKT25, pKNT25 and pUT18.

**Construction of plasmids with mutated crl genes**

Site-directed mutagenesis was performed using the QuikChange II Site-directed mutagenesis kit (Stratagene) as recommended by the manufacturer. Site-directed mutagenesis of plasmid pAC\(_{crl}\)-1 yielded plasmids pAC\(_{crl}\)Y22A, pAC\(_{crl}\)F35A, pAC\(_{crl}\)C28A, pAC\(_{crl}\)C37A, pAC\(_{crl}\)C41A, pAC\(_{crl}\)F53A, pAC\(_{crl}\)W56A, pAC\(_{crl}\)G74A, pAC\(_{crl}\)G80A, pAC\(_{crl}\)W82A, pAC\(_{crl}\)F103A, pAC\(_{crl}\)C28-37, pAC\(_{crl}\)C28-41, pAC\(_{crl}\)C37-C41 and pAC\(_{crl}\)C28-37-41 (Table 1). Plasmids pUTCrl\(_{Y22A}\), pUTCrl\(_{F53A}\), pUTCrl\(_{W56A}\), pUTCrl\(_{W82A}\) and pUTCrl\(_{G80A}\) (Table 1) were obtained by cloning PCR amplified DNA fragments from plasmids pAC\(_{crl}\)Y22A, pAC\(_{crl}\)F53A, pAC\(_{crl}\)W56A, pAC\(_{crl}\)W82A, and pAC\(_{crl}\)G80A, respectively, between the *Pst*I and *Eco*RI sites of pUT18 using primers Crl-*Pst* and Crl-*Eco* (as described above). Plasmids pUTCrl\(_{R51A}\), pUTCrl\(_{E52A}\), pUTCrl\(_{G55A}\), pUTCrl\(_{W57A}\) were obtained by site-directed mutagenesis of plasmid pUTCrl (Table 1). All plasmids were confirmed to be correct by DNA sequencing.

**Electrophoresis and immunoblot analysis of proteins.** Whole-cell extracts were prepared and SDS-polyacrylamide gel electrophoresis was carried out as described by Silhavy *et al.*
The amount of protein in whole-cell lysates was determined using the DC Protein Assay kit (Bio-Rad). Equal amounts of protein were loaded in each slot. The molecular sizes of the proteins were estimated using molecular size standards (Fermentas, France). Rabbit antibodies against the Crl protein of *Salmonella* were from Robbe-Saule *et al.* (40). Rabbit antibodies against the $\sigma^S$ protein of *S. enterica* serovar Typhimurium were from Coynault *et al.* (7). Proteins were transferred to Amersham Hybond P membranes (GE Healthcare) and incubated with the polyclonal rabbit antibody serum as previously described (7). Bound antibodies were detected using a secondary anti-rabbit antibody linked to peroxidase and the Amersham ECL plus western blotting detection system kit (GE Healthcare).

**Overproduction and purification of His$_6$-Crl variants.** A 250 ml of JM109 carrying pQEcrl wild-type or mutant was grown in LB medium containing carbenicillin at 30°C to an optical density of 0.6, and then 1 mM IPTG was added. After 30 min, the temperature was lowered to 23°C. The cells were harvested after 4 h, washed, resuspended in 10 ml of buffer A (25 mM Tris pH 8.0, 1 M NaCl, 0.01% NP-40) supplemented with “Complete EDTA free anti-protease” as described by the manufacturer (Roche) and then disrupted in a French press (Aminco). The crude cell extract was centrifuged at 15,000 g for 30 min. The supernatant was adjusted to 3 mM imidazole, added to 1.5 ml of Ni-nitrilotriacetic acid agarose (QIAGEN) and gently mixed for 1 h. The slurry was packed onto an Econo-Pac column (Bio-Rad) and washed with 15 ml of buffer A containing 20 mM imidazole. His$_6$-Crl was eluted with buffer A containing 250 mM imidazole, dialyzed against buffer B (20 mM Tris-HCl pH 8, 50 mM NaCl, 0.01% NP-40), and added to DNA-cellulose equilibrated in the same buffer (0.3 ml, Sigma D-8515). The DNA-cellulose was removed by centrifugation and the concentrations of the proteins determined by a Bradford assay.
**Gel retardation assays.** The DNA encoding rpoS was amplified using primers HK1 5’-AGGCTCGGATCCATGAGTCAGAATACGCTGAAAGTTCAT-3’ and HK2 5’-TTCCGAAAGCCTTTTACTCGGGAACACCGCTTGCATATT-3’ and cloned into the BamHI and HindIII sites of pQE30 to yield pQE30rpoS. The nucleotide sequence of the rpoS insert in pQE30rpoS was checked by DNA sequencing. S. Typhimurium His6-σS produced from pQE30rpoS was purified using Ni-affinity chromatography as described for His6-σS T2.

*E. coli* core (Epicentre) was used to reconstitute the σS-holoenzyme, which was incubated with variant Crl proteins (wild-type or mutants) or buffer. 9 μl of the protein complexes were then added to 3 μl of [32P]-labelled katN fragment in buffer A (40 mM Hepes pH 8.0, 10 mM MgCl2, 100 mM K-glutamate, 2 mM DTT) containing 500 μg ml−1 BSA, and incubated at 28°C for 20 min. The final concentrations of core, His6-σS, Crl and katN fragments were respectively 8 nM, 32 nM, 3 μM and 1 nM. After addition of 3 μl loading buffer (buffer A containing 50% sucrose, 0.025 % xylene cyanol blue and 150 μg ml−1 of heparin) the mixture was loaded onto a 6 % native polyacrylamide gel run in TG buffer (25 mM Tris, 192 mM Glycine pH 8.5) at 8V/cm. The gel was dried before being autoradiographed and quantified using a PhosphorImager (Molecular Dynamics).

**Abortive initiation assays.** A mixture of the 207-bp lacUV5 fragment (10 nM), ApA (2 mM), and 80 μM [α-32P] UTP was added to an equal volume of the holoenzyme (60 nM, His6-σS: core ratio = 2.5 : 1) preincubated with buffer or Crl (wild-type or variants) at 6 μM concentration at 28° C. Aliquots were removed at various times. The aliquots were spotted onto Whatman 3MM paper prespotted with 100 mM EDTA, and chromatograms were developed as previously described (40).
RESULTS

Distribution and sequence conservation of Crl in microbial sequenced genomes

A Blast search in 1264 sequenced bacterial genomes (www.ncbi.nlm.nih.gov) revealed 143 genomes containing a crl gene. They are all γ-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) 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 σS 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 RpoSTM and the RpoS proteins from the Enterobacteria Proteus mirabilis and Providencia stuartii is 43% and 40% for amino acids 1-55 compared to 87% and 86% for amino acids 56-330, respectively. These first 55 residues define a specific domain of σS that is not well conserved in other sigma factors besides σ70 (corresponding roughly to region σ1.1, see also Discussion section and 32, 51), and might thus play a role in the specificity of recognition of
σS by Crl. In addition, σ1.1 and Crl might play complementary roles in the formation of EσS. Crl increases the affinity of σS for the core enzyme (12). σ1.1 stabilizes the interaction between σ70 and core RNAP (18) and might play a similar role in σS-core interaction. These possibilities prompted us to determine whether this domain of σS is required for σS activity and for Crl activation.

We first examined the sensitivity to Crl activation of a σS mutant lacking the first 39 residues of σS (σ5T2, Figures 2 and 3) and thus lacking most of region σ1.1 (32, 51). rpoST2 is a natural rpoS allele from S. Typhi strain T2 that contains a deletion of eight nucleotides in the 5′ end of the coding sequence resulting in the appearance of a premature stop codon (Figure 2A). Surprisingly, rpoST2 was able to direct the synthesis of a 35 kDa σS protein (σ5T2, Figure 3A). N-terminal sequence analysis indicated that σ5T2 has a N-terminal Met residue followed by a sequence that is identical to that downstream of residue 39 in native σS (Figure 2B). We inferred that σ5T2 is encoded from the mutant gene in which translation initiates at a GTG start codon preceding codon 40 of σS in rpoST2, and upstream of which is a putative ribosome binding site (SD, Figure 2B). Translational reinitiation within the E. coli rpoS gene has also been reported previously (15). To facilitate characterization of the rpoST2 allele in isogenic backgrounds, the rpoS gene from S. Typhimurium ATCC14028 was replaced by rpoST2 yielding strain 2922K rpoST2. During exponential growth, σ5T2 was produced in higher amounts than the wild-type σS protein (OD600 0.1 to 1.4, Figure 3B), likely because the N-terminal 11 residues of σS involved in ClpX recognition and σS proteolysis by ClpP (13, 49) were not present in σ5T2. σS is required for bacterial resistance to various stresses during stationary phase (the so-called general stress resistance) (20,25). The σS-dependent gene katE encodes a catalase, an enzyme that detoxifies hydrogen peroxide (H2O2) and contributes to the resistance of Salmonella to oxidative stress in stationary phase (38). Despite the high amount
of $\sigma^S_{T2}$ produced, strain 2922KrpoS$_{T2}$ was slightly less resistant to H$_2$O$_2$ than the wild-type strain 2922K and exhibited lower expression of the katE-lacZ fusion (Figure 2CD). This suggested that $\sigma^S_{T2}$ is less active than wild type $\sigma^S$. In vitro transcription experiments with $\sigma^S_{T2}$ confirmed this hypothesis (data not shown). Interestingly, the levels of H$_2$O$_2$ resistance and the expression of the catalase gene katE were more dependent on Crl activation in 2922KrpoS$_{T2}$ than in the wild-type strain (Figure 2CD). This indicated that the $\sigma^S_{T2}$ protein is able to interact with Crl and that its activity is highly dependent on Crl.

We previously used the bacterial two-hybrid system (BACTH system, 22) to detect interaction between Crl and $\sigma^S$ (12). C-terminal fusions of S. Typhimurium $\sigma^S$ to T25 (T25-$\sigma^S$) and N-terminal fusions of S. Typhimurium Crl to T18 (Crl-T18) were produced in the E. coli cya lac$^+$ strain BTH101 from pKTRpoS and pUTCrl, respectively, and the resulting $\beta$-galactosidase activities were measured (Table 3). As expected (12), T25-$\sigma^S$ interacted with Crl-T18 (Table 3). In this system, the first 38 residues of $\sigma^S$ were dispensable for efficient binding of Crl (compare T25-$\sigma^S$ and T25-$\sigma^S_{\Delta1-38}$ in the presence of Crl-T18, Table 3). Moreover, deletion of residues 1 to 38 from $\sigma^S$ resulted in increased levels of the chimera and $\beta$-galactosidase activity in the BACTH assay (Figure 3C lanes 2 and 5, Table 3). This is probably again due to the absence of the ClpX recognition site in T25-$\sigma^S_{\Delta1-38}$. Interestingly, deletion of residues 1 to 71 in T25-$\sigma^S_{\Delta1-71}$, and deletion of residues 12 to 71, which does not remove the ClpX recognition site (13, 49) in T25-$\sigma^S_{\Delta12-71}$, had a more pronounced effect on $\sigma^S$ levels and $\beta$-galactosidase activity than deletion of residues 1 to 38 (in T25-$\sigma^S_{\Delta1-38}$) (Figure 3C lanes 4-6, Table 3). This indicated that the first 71 residues in $\sigma^S$ are non-essential for Crl binding, and further suggested that the deletion of this region induces a conformational change in $\sigma^S$ that favors its stability. Altogether, these results suggested that Crl interacts with the conserved amino acid region (72-330) of $\sigma^S$. 
Identification of conserved residues important for Crl activity

Alanine substitution mutagenesis was performed to investigate the functional relevance of the conserved residues in Crl. The conserved residues are located in four regions of Crl (one third of them are aromatic amino-acids, Figure 1). As a first step, one or two residues in each conserved region were mutated. We also mutated the three cysteine residues in Crl. pACcrl-1 expresses the crl gene from S. Typhimurium under the control of the vector cat promoter, and is able to complement a ∆crl mutant of Salmonella (40). Site-directed mutagenesis of pACcrl-1 yielded eleven derivatives expressing altered Crl proteins (Table 1 and Figure 4A). The ability of the mutated crl genes to complement the Salmonella ∆crl mutant was assessed using three different tests (Figures 4 and 5). The development of the rdar morphotype depends on Crl (40) and was used as a qualitative test (Figure 5). This colony morphology is caused by production of curli and cellulose and is correlated with biofilm formation and expression of the regulatory gene csgD (42). In ATCC14028, the expression level of a csgD-lacZ fusion and the H$_2$O$_2$ resistance level at 28°C both depend on Crl (38, 40), and were used as quantitative assays (Figure 4BC).

Derivatives of pACcrl-1 expressing the Crl proteins with F35A, C28A, C37A, G74A, G80A and F103A substitutions complemented ∆crl as well as did pACcrl-1 (Figures 4-5 and data not shown). Two substitutions, W56A and F53A, located in a conserved motif of Crl (residues 48 to 57, Figure 1), substantially diminished complementation in all three tests (Figure 4 and Figure 5 spots 7-8). Two additional substitutions, Y22A and W82A, also decreased the complementation levels but to a lesser extent (Figure 4 and Figure 5 spots 3 and 11). These four substitutions did not significantly decrease the cellular level of Crl (Figure 6A) and, thus, likely affected its activity.
The *Salmonella* Crl protein contains three cysteines, (C28, C37, C41), one of which (C41) is conserved in all Crl members. Alanine substitution of cysteine residues (C28A, C37A and C41A) did not significantly affect the levels of Crl protein or its activity (Figures 4, 5 and 6A). Only a slight effect of C41A on \( \text{H}_2\text{O}_2 \) resistance was observed (Figure 4B). Interestingly, substitution of all three cysteine residues substantially decreased Crl levels (Figure 6A lanes 17 and 20). Examination of the Crl family members indicated that their all contain at least 2 cysteine residues. Altogether, these results suggested that the presence of at least one, and of probably two, cysteine residue(s) is required for Crl stability. Double mutations in the cysteine residues in Crl (Crl\(_{C28-37}\), Crl\(_{C28-41}\), Crl\(_{C37-41}\)) were constructed to determine what combination of cysteine substitution affected Crl stability. The combinations of C28A and C37A substitutions and C28A and C41A substitutions did not significantly affected the levels of Crl protein (Figure 6A lanes 14 and 15). In contrast, alanine substitution of both C37 and C41 substantially decreased Crl levels (Figure 6A lane 16) suggesting that these two cysteine residues are important for Crl stability. Levels of the triple Crl\(_{C28-37-41}\) mutant protein were lower than that of Crl\(_{C37-41}\) (Figure 6A, lanes 19-20). This suggests that the C28A substitution further decreased the stability of Crl\(_{C37-41}\).

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

We next determined whether the mutations selected above affected Crl-\( \sigma^S \) interaction. DNA encoding proteins with Y22A, F53A, W56A or W82A substitutions or the neutral G80A substitution were introduced into pUTCrl and the ability of the Crl-T18 derivatives to interact with \( \sigma^S \) was assessed in the presence of T25-\( \sigma^S \) (Table 3). Interestingly, the Y22A, F53A, W56A, W82A substitutions, but not the G80A substitution, all abolished the interaction between Crl-T18 and T25-\( \sigma^S \) (Table 3). These substitutions did not affect levels of Crl-T18 (Figure 6B). The altered Crl-T18 hybrid proteins were also unable to interact with
the T25-σ^S^ derivative lacking amino acids 12 to 71 (data not shown). In conclusion, the
Y22A, F53A, W56A and W82A substitutions all abolished the interaction between Crl and σ^S^ either directly or indirectly through conformational changes in Crl.

The activities of the variant proteins were also probed in vitro by gel retardation and abortive initiation assays, based on the ability of Crl to increase the amount of promoter-complexes formed by the σ^S^-RNA polymerase (Eσ^S^) and activate transcription initiation (12, 40). As shown on the autoradiogram of the native polyacrylamide gel (Figure 7A lane 3), Eσ^S^ binding to the [^32^P]-labelled katN promoter was stimulated 2-fold by wild-type Crl. The Y22A and W82A variants were still able to increase the formation of Eσ^S^-katN complexes by about 1.8 and 1.6 fold (Figure 7A lanes 4 and 6). In contrast, the F53A and W56A variants of Crl (Figure 7A lanes 5 and 7) had hardly any effect, in agreement with their highly reduced ability to stimulate csgD-lacZ expression or hydrogen peroxide resistance. The gel retardation methodology is only semi-quantitative, however, and the addition of heparin to disrupt non-specific core-DNA binding before electrophoresis might have disturbed weak interactions between Crl variants and σ^S^. Therefore, abortive initiation assays, which do not require heparin addition and can be exploited for more quantitative studies, were conducted using the well-characterised lacUV5 promoter, which can be transcribed in vitro by Eσ^70^ and Eσ^S^ and, unlike katN, has the advantage of producing high amounts of abortive products (29). As expected, Eσ^S^ produced less ApApUpU tetranucleotide from lacUV5 in the presence of the Crl variants than with the wild-type protein (Figure 7B). The results were more obvious than in gel retardation assays since wild-type Crl stimulated Eσ^S^-lacUV5 abortive transcription by 4-fold under our conditions. After 30 min incubation at 28°C, the observed decrease in ApApUpU synthesis was 35% for Y22A, 40% for W82A and 90% for F53A. The addition of W56A Crl resulted in an even lower activity than in the absence of Crl, suggesting a slight inhibitory effect of this variant on σ^S^-dependent transcription. Altogether, these results
emphasise the role of the REF-GWW motif (Figure 1) in Crl activation of $\sigma^S$-dependent promoters. Consistent with this hypothesis, the R51A, W57A substitutions and to a lesser extent the E52A and G55A substitutions affected the interaction between Crl and $\sigma^S$ in the BACTH assay (Table 3) but did not affect levels of Crl-T18 (Figure 6B).

Fur-Crl interactions in *Salmonella*

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

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

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

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

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

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

Of the 16 Salmonella strains whose genome sequence is completed, one, S. Paratyphi SB7, contains a frameshift mutation in crl that results in the appearance of a premature stop codon. This observation is reminiscent of previous findings that rpoS null mutants can be found in natural isolates of S.Typhi (7, 37, 39), and is consistent with our previous observation that a $\Delta$crl mutation increases the competitive fitness of Salmonella by attenuating $\sigma^S$ activity (41). The selection of rpoS mutants in bacterial populations likely results from a growth advantage of rpoS mutants in the absence of environmental stress (24, 33, 34). However, in environments where bacteria encounter mild stress, $\Delta$rpoS mutants are outcompeted by the wild-type strain, whereas rpoS attenuated mutants exhibit increased
fitness (33, 41, 53). We can predict that the beneficial effect of a crl mutation is significant when the survival strategy requires “enough but not too much” σS activity.

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

The F53A and W56A substitutions are located in a conserved motif in the middle part of the protein (REF-GWW Figure 1). Analysis of four additional substitutions (R51A, E52A, G55A W57A) in the BACTH assay confirmed the role of the REF-GWW motif in Crl-σS interactions (Table 3, Figure 6B). The two other substitutions, Y22A and W82A, are outside of this motif. At least some of these mutations likely affect Crl binding to σS indirectly through conformational changes. The three-dimensional structure of Crl would provide new insight into this issue. Unfortunately, our attempts to crystallize Crl have been unsuccessful.

In addition, deletion experiments in the BACTH system did not allow us to delineate a sub-region of Crl involved in σS binding. Crl might spread along σS or, more likely, a specific conformation of Crl might be required for efficient interaction with σS.

The primary determinants of σS involved in the binding of Crl are unknown, but the lack of interaction between Crl and σ70 (12) suggests that the interaction involves sequence determinants or structural features that are specific to σS. The vast majority of σ factors, including σS, belong to the so-called σ70 family, reflecting their relationship to the principal σ
factor of *E. coli*, $\sigma^{70}$. Sequence alignments of $\sigma^{70}$ family members of groups 1 and 2, to which $\sigma^8$ belongs, reveal that they have four conserved regions (regions 1-4) (32, 35). Among these, region 2 (subregions 2.1 to 2.4) and region 4 (subregions 4.1 and 4.2) contain DNA-binding domains that mediate recognition of the conserved –10 and –35 elements of $\sigma^{70}$-dependent promoters respectively. The linear division of $\sigma^{70}$ factors into functionally distinct regions is largely confirmed by structural data, which revealed that primary sigma factors have four 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 4.1-4.2, respectively (5, 32). Free $\sigma$ factors generally do not specifically bind promoter DNA, and the N-terminal $\sigma_{1.1}$ region is autoinhibitory (4, 9). $\sigma_{1.1}$ might act indirectly to inhibit promoter binding by stabilizing a compact conformation of $\sigma$ that is incompatible with promoter recognition (47). Binding to core RNAP induces large movements of the $\sigma$ domains (3), converting $\sigma$ into an active conformation in which the DNA binding determinants in $\sigma_2$ and $\sigma_4$ are exposed (32).

$\sigma_{1.1}$ shows little conservation between $\sigma^{70}$ and $\sigma^8$ and is not found in the alternative sigma factors of the $\sigma^{70}$ family (32, 35). Therefore, the specificity of recognition between Crl and $\sigma^8$ might involve $\sigma_{1.1}$. However, our findings that (1) $\sigma^8_{T2}$, a protein lacking residues 1 to 39, including most of region $\sigma_{1.1}$ in native $\sigma^8$ (32, 51) is dependent on Crl activation (Figure 2), and (2) deletion of residues 1 to 71 of $\sigma^8$, which removes region $\sigma_{1.1}$ and part of subregion 1.2 (32, 51) does not abolish Crl-$\sigma^8$ interaction (Table 3), ruled out this possibility. Experiments are in progress to determine which region of $\sigma^8$ is involved in Crl binding. In addition to its autoinhibitory role, $\sigma_{1.1}$ stabilizes the interaction between $\sigma^{70}$ and core RNAP (18). Our finding that $\sigma^8_{T2}$ is less active but more dependent on Crl activation than wild-type $\sigma^8$ (Figure 2CD and data not shown) is consistent with a similar role for $\sigma_{1.1}$ in $\sigma^8$-core
interaction. Crl increases the affinity of $\sigma^3$ for core RNAP (12) and might partially compensate for the absence of a full region $\sigma_{1,1}$ in $\sigma_{T2}^s$.

Acknowledgments:

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50. Typas, A. C., Barembruch, A., Possling, and R. Hengge. 2007. Stationary phase reorganisation of the *Escherichia coli* transcription machinery by Crl protein, a fine-tuner of $\sigma^S$ activity and levels. The EMBO J. **26**: 1569-1578.


Table 1: Bacterial strains and plasmids used in this study

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**Plasmids**

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13. pSTF4
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20. pQE30
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25. pQEcrl\_F53A
    - pQEcrl with mutation F53A in Crl
1. pQEcr1W56A: pQEcr1 with mutation W56A in Crl
2. pQEcr1W82A: pQEcr1 with mutation W82A in Crl
3. pKT25: BACTH vector designed to express a given polypeptide fused in frame at its N-terminal end with T25 fragment, p15 ori, Km^R
4. pKNT25: BACTH vector designed to express a given polypeptide fused in frame at its C-terminal end with T25 fragment, p15 ori, Km^R
5. pUT18: BACTH vector designed to express a given polypeptide fused in frame at its C-terminal end with T18 fragment, ColE1 ori, Ap^R
6. pUTCrl: pUT18 expressing Crl-T18
7. pUTCrlY22A: pUT18 expressing Crl-T18 with mutation Y22A in Crl
8. pUTCrlR51A: pUT18 expressing Crl-T18 with mutation R51A in Crl
9. pUTCrlE52A: pUT18 expressing Crl-T18 with mutation E52A in Crl
10. pUTCrlF53A: pUT18 expressing Crl-T18 with mutation F53A in Crl
11. pUTCrlG55A: pUT18 expressing Crl-T18 with mutation G55A in Crl
12. pUTCrlW56A: pUT18 expressing Crl-T18 with mutation W56A in Crl
13. pUTCrlW82A: pUT18 expressing Crl-T18 with mutation W82A in Crl
14. pKNTCrl: pKNT25 expressing Crl-T25
15. pKTRpoS: pKT25 expressing T25-RpoS
16. pKTRpoS_{\Delta}^{12-71}: pKTRpoS with in frame deletion of residues 12-71 in RpoS
17. pKTRpoS_{\Delta}^{1-38}: pKTRpoS with deletion of residues 1-38 in RpoS
<p>| | | |</p>
<table>
<thead>
<tr>
<th></th>
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</thead>
<tbody>
<tr>
<td>1</td>
<td>pKTRpoS&lt;sub&gt;Δ1-71&lt;/sub&gt;</td>
<td>pKTRpoS with deletion of residues 1-71 in RpoS</td>
</tr>
<tr>
<td>2</td>
<td>pKT25 Fur</td>
<td>pKT25 expressing T25-Fur</td>
</tr>
<tr>
<td>3</td>
<td>pUT18 Fur</td>
<td>pUT18 expressing Fur-T18</td>
</tr>
</tbody>
</table>

*This study, unless otherwise noted.*

\(^{b}\) WHO Reference Center for *Salmonella* (Institut Pasteur, Paris)

\(^{c}\) American Type Culture Collection
Table 2: Distribution and sequence conservation of Crl in bacterial sequenced genomes.

<table>
<thead>
<tr>
<th>Genus</th>
<th>identity with Crl&lt;sub&gt;STM&lt;/sub&gt; (%)&lt;sup&gt;a&lt;/sup&gt;</th>
<th>identity with RpoS&lt;sub&gt;STM&lt;/sub&gt; (%)&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Salmonella</td>
<td>96-100</td>
<td>99-100</td>
</tr>
<tr>
<td>Citrobacter</td>
<td>90</td>
<td>100</td>
</tr>
<tr>
<td>Escherichia</td>
<td>83-84</td>
<td>98-99</td>
</tr>
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<td>Shigella</td>
<td>83-84</td>
<td>99</td>
</tr>
<tr>
<td>Enterobacter</td>
<td>74-77</td>
<td>97</td>
</tr>
<tr>
<td>Klebsiella</td>
<td>75-76</td>
<td>98</td>
</tr>
<tr>
<td>Serratia</td>
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<td>94</td>
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<tr>
<td>Erwinia</td>
<td>66</td>
<td>95</td>
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<td>Yersinia</td>
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<td>84-97</td>
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<td>Pectobacterium</td>
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<td>Proteus</td>
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<td>Providencia</td>
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<tr>
<td>Aeromonas</td>
<td>43-44</td>
<td>85-84</td>
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<td>Vibrio</td>
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<td>72-82</td>
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<tr>
<td>Unclassified Vibrionales</td>
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<td>Photobacterium</td>
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<td>79-80</td>
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<td>Moritella</td>
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<td>76</td>
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<tr>
<td>Psychromonas</td>
<td>35-44</td>
<td>77</td>
</tr>
</tbody>
</table>

<sup>a</sup> bacterial sequenced genomes containing a crl gene
Range of percentage of identity between the aminoacid sequence of the Crl proteins in strains belonging to the indicated genus and that of the S. Typhimurium Crl protein (CrlSTM, 133 residues, NP-459316 Figure 1 line 16).

Range of percentage of identity between the aminoacid sequence of the RpoS proteins in strains belonging to the indicated genus and that of the S. Typhimurium RpoS protein (RpoSTM, 330 residues, X77752, 26).
Table 3: BACTH analysis of Crl interactions with $\sigma^S$ and Fur.

<table>
<thead>
<tr>
<th>Co-expressed proteins</th>
<th>$\beta$-galactosidase activity $^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>5 T18 + T25</td>
<td>47 ± 6</td>
</tr>
<tr>
<td>6 Crl-T18 + T25</td>
<td>47 ± 6</td>
</tr>
<tr>
<td>7 T18 + T25-$\sigma^S$</td>
<td>43 ± 3</td>
</tr>
<tr>
<td>8 Crl-T18 + T25-$\sigma^S$</td>
<td>263 ± 38</td>
</tr>
<tr>
<td>9 Crl-T18 + T25-$\sigma^S_{A1-38}$</td>
<td>455 ± 33</td>
</tr>
<tr>
<td>10 Crl-T18 + T25-$\sigma^S_{A1-71}$</td>
<td>1830 ± 186</td>
</tr>
<tr>
<td>11 Crl-T18 + T25-$\sigma^S_{A12-71}$</td>
<td>2352 ± 146</td>
</tr>
<tr>
<td>12 Crl$^{22A}$-T18 + T25-$\sigma^S$</td>
<td>44 ± 5</td>
</tr>
<tr>
<td>13 Crl$^{51A}$-T18 + T25-$\sigma^S$</td>
<td>59 ± 3</td>
</tr>
<tr>
<td>14 Crl$^{52A}$-T18 + T25-$\sigma^S$</td>
<td>102 ± 12</td>
</tr>
<tr>
<td>15 Crl$^{53A}$-T18 + T25-$\sigma^S$</td>
<td>48 ± 5</td>
</tr>
<tr>
<td>16 Crl$^{55A}$-T18 + T25-$\sigma^S$</td>
<td>138 ± 19</td>
</tr>
<tr>
<td>17 Crl$^{56A}$-T18 + T25-$\sigma^S$</td>
<td>44 ± 4</td>
</tr>
<tr>
<td>18 Crl$^{57A}$-T18 + T25-$\sigma^S$</td>
<td>74 ± 4</td>
</tr>
<tr>
<td>19 Crl$^{580A}$-T18 + T25-$\sigma^S$</td>
<td>205 ± 27</td>
</tr>
<tr>
<td>20 Crl$^{82A}$-T18 + T25-$\sigma^S$</td>
<td>55 ± 6</td>
</tr>
<tr>
<td>21 Crl-T18 + T25-Fur</td>
<td>43 ± 1</td>
</tr>
<tr>
<td>22 Fur-T18 + Crl-T25</td>
<td>58 ± 6</td>
</tr>
<tr>
<td>23 Fur-T18 + T25-Fur</td>
<td>243 ± 32</td>
</tr>
<tr>
<td>24 Fur-T18 + T25</td>
<td>55 ± 3</td>
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<tr>
<td></td>
<td>Protein Combination</td>
</tr>
<tr>
<td>---</td>
<td>---------------------</td>
</tr>
<tr>
<td>1</td>
<td>T18 + T25-Fur</td>
</tr>
<tr>
<td>2</td>
<td>T18 + Crl-T25</td>
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</tbody>
</table>

a The efficiencies of functional complementation between the indicated proteins were quantified by measuring β-galactosidase activities in *E. coli* BTH101 cells harboring the corresponding plasmids as described in Material and Methods. β-galactosidase activity was measured according to the method of Miller (31).
FIGURE 1. Alignment of the proteins from the Crl family.

Alignment of the Crl protein sequences was performed with ClustalW. Residues that are identical in all of the Crl proteins are shown in black boxes and position of these conserved residues in the Crl sequence from *S. enterica* serovar Typhimurium (CrlSTM, 133 residues, NP-459316, line 16) is shown below the alignment. The accession numbers for the Crl sequences are as follows: 

- **Salmonella**: ZP-02346845 (14), ZP-02663633 (15)NP-459316 (16), YP-215307 (17), YP-001571691 (18);
- **Citrobacter**: YP-001454497 (19); **Escherichia**: NP-285957 (22), NP-414775 (23), YP-001742400 (25), YP-002383844 (26), YP-002327819 (27), NP-752324 (28), YP-539315 (29), ZP-02902139 (30);
- **Shigella**: YP-309300 (20), YP-402173 (21), NP-706240 (24);
- **Enterobacter**: YP-001175503 (31), ZP-03281271 (32), YP-001439177 (35);
- **Klebsiella**: YP-001333935 (33), YP-002240279 (34);
- **Serratia**: YP-001477200 (13);
- **Erwinia**: YP-001908518 (36);
- **Yersinia**: YP-001007379 (6), ZP-00829910 (7), ZP-00821198 (8), ZP-00826726 (9), ZP-00833806 (10), YP-001402111 (11), NP-668295 (12);
- **Pectobacterium**: YP-051554 (37);
- **Proteus**: YP-002150140 (38);
- **Providencia**: ZP-03313695 (39), ZP-03317513 (40), ZP-02958311 (41);
- **Aeromonas**: YP-857911 (42), YP-001140774 (43);
- **Vibrio**: ZP-01235672 (1), NP-231906 (44), ZP-01957597 (45), ZP-01980856 (46), NP-759329 (47), NP-933650 (48), ZP-01065753 (49), YP-002418001 (50), ZP-00993088 (51), ZP-01870715 (53), NP-797054 (54), ZP-01260295 (55), YP-001444378 (56), ZP-01987497 (57), ZP-02197346 (58);
- **Unclassified Vibrionales**: ZP-01813308 (52);
- **Photobacterium**: ZP-01159718 (2), YP-129053 (3), ZP-01218949 (4);
- **Moritella**: ZP-01898329 (5);
- **Psychromonas**: YP-944255 (59), ZP-01216084 (60).
FIGURE 2. Characterization of the \textit{rpoS} mutant allele \textit{rpoS}_{T2}.

A) Relevant portion of the \textit{rpoS} sequence in the wild-type \textit{rpoS} allele and the \textit{rpoS}_{T2} mutant allele. The deletion of eight nucleotides in \textit{rpoS}_{T2}, compared to \textit{rpoS} wild-type, is shown. This mutation results in the appearance of a premature stop codon in \textit{rpoS}_{T2}. B) Determination of the N-terminal sequence of \(\sigma^S_{T2}\). Production of \(\sigma^S_{T2}\) likely results from translational reinitiation in \textit{rpoS}_{T2} at the GTG codon that is preceded by a putative ribosome binding site (Shine-Dalgarno sequence SD). C) Resistance to hydrogen peroxide of the \textit{S. Typhimurium} strains indicated. Cells were grown to stationary phase in LB at 37°C, washed, resuspended in PBS to an \(\text{OD}_{600}\) of 0.1 and \(\text{H}_2\text{O}_2\) 15 mM was added. A representative experiment is shown. Similar results were obtained in repeat experiments. D) Expression of a \textit{katE-lacZ} gene fusion in \textit{Salmonella} carrying the wild-type \textit{rpoS} and mutant \textit{rpoS}_{T2} alleles. 1: 2922K\textit{katE-lacZ}, 2: 2922K\textit{rpoS katE-lacZ}, 3: 2922K\textit{crl katE-lacZ}, 4: 2922K\textit{rpoS}_{T2}\textit{crl katE-lacZ}. \(\beta\)-galactosidase activity was measured in overnight LB cultures at 37°C according to the method of Miller (31).

FIGURE 3. Expression of \(\sigma^S\) wild-type and mutant proteins.

A) Detection of \(\sigma^S_{T2}\) produced from the \textit{rpoS}_{T2} allele. Overnight LB cultures at 37°C were analysed by western blotting with anti-\(\sigma^S\) antibodies. 5 \(\mu\)g of total protein was loaded in each slot. 1: \textit{S. Typhimurium} wild-type strain C52, 2: \textit{S. Typhimurium} \(\Delta\textit{rpoS}\) mutant C52K, 3: \textit{S. Typhi} strain T2, 4: \textit{E.coli} MC1061K harboring plasmid pAC\textit{rpoS}_{T2}. B) Expression of \(\sigma^S\) and \(\sigma^S_{T2}\) as a function of bacterial cell growth. \textit{Salmonella} strains 2922K and 2922K\textit{rpoS}_{T2} were grown in LB at 37°C. Exponential-phase cultures (optical density at 600 nm = 0.5) were diluted into LB pre-warmed at 37°C to prolong the exponential phase. Aliquots were removed at various time intervals and analysed by western blotting with anti-\(\sigma^S\) antibodies. 10 \(\mu\)g of
total protein was loaded in each slot. The growth phase was determined by measuring culture
turbidity at an optical density of 600 nm (OD600). C1: 2922K\textit{rpoS} (OD600=4), C2: 2922K
(OD600=1.4). C) Expression of wild-type and truncated T25-\textit{\sigma}^S hybrid proteins. The \textit{E. coli}
cy\textit{a} strain BTH101 harboring pKT25 and its derivatives were grown to stationary phase
(OD600= 3.5) in LB in the presence of 2 mM cAMP and 0.5 mM IPTG to fully induce the \textit{lac}
promoter on pKT25. 10 \mu g of total protein was loaded in each slot and analysed by western
blotting with anti-\textit{\sigma}^S antibodies. 1: no plasmid, 2: pKTR\textit{rpoS}, 3: pKT25, 4: pKTR\textit{rpoS}_{\Delta 12-71}, 5:

\textbf{FIGURE 4} Site-directed mutagenesis of the \textit{Salmonella} \textit{crl} gene and characterization of
the \textit{crl} mutant alleles.

Site-directed mutagenesis of pAC\textit{crl}-1 was performed, yielding eleven derivatives expressing
Crl proteins in which residues Y22, F35, C28, C37, C41, F53, W56, G74, G80, W82, and
F103 are substituted by alanine. The ability of the mutated \textit{crl} genes to complement the
\textit{Salmonella} \Delta\textit{crl} mutant was assessed using three different tests: the H\textsubscript{2}O\textsubscript{2} resistance level at
28°C, the expression level of a \textit{csgD-lacZ} fusion, and development of the rdar morphotype.
A) Summary of the results obtained: (+) complementation level similar to that obtained with
pAC\textit{crl}-1 expressing the wild-type Crl protein, (-) complementation level similar to that
obtained with the negative control pACYC184, and (\pm) partial complementation level.
Complementation experiments for the rdar morphotype are shown in Figure 5. B)
Complementation of the \Delta\textit{crl} mutant for resistance to H\textsubscript{2}O\textsubscript{2}. ATCC\textit{crl} derivatives containing
the indicated plasmids were grown to stationary phase in LB at 28°C, washed, resuspended in
PBS to an OD\textsubscript{600} of 1 and H\textsubscript{2}O\textsubscript{2} 15 mM was added. A representative experiment is shown.
Similar results were obtained in repeat experiments. C) Complementation of the \Delta\textit{crl} mutant
for expression of a \textit{csgD-lacZ} fusion. ATCC\textit{crl} \textit{csgD-lacZ} derivatives containing the
indicated plasmids were grown in LB0 (LB without NaCl) at 28°C. Exponential-phase cultures (optical density at 600 nm = 0.5) of *Salmonella* were diluted into LB0 pre-warmed at 28°C to prolong the exponential phase. Aliquots were removed at various time intervals and β-galactosidase activity measured (lines) according to the method of Miller (31). The growth phase was determined by measuring the culture turbidity at an optical density of 600 nm (dashed line, the growth curve was similar for all the strains). The measurements were repeated twice, and a representative experiment is shown.

**FIGURE 5.** Rdar morphotypes of *Salmonella* ATCCcr1 harbouring different plasmids.

The ∆crl mutant harbouring the vector pACYC184, the wild-type crl gene on pACcrl-1 and mutated pACcrl-1 derivatives (Table 1) were grown five days on CR plates at 28°C. Plasmids are 1: pACYC184, 2: pACcrl-1, 3: pACcrlY22A, 4: pACcrlF35A, 5: pACcrlC37A, 6: pACcrlC41A, 7: pACcrlF53A, 8: pACcrlW56A, 9: pACcrlG74A, 10: pACcrlG80A, 11: pACcrlW82A, 12: pACcrlF103A, 13: pACcrlC28A, 14: pACcrlC28-37-41. Complementation of the crl mutation was observed with pACcrl-1 and its derivatives containing mutations F35A, C28A, C37A, C41A, G74A, G80A, F103A.

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

of total protein was loaded in slots 18 to 20. 19: pACcr[C37-41], 20: pACcr[C28-37-41], 19: a stationary phase culture of ATCC14028 in LB at 37°C was used as control. B) Expression of wild-type and altered Crl-T18 hybrid proteins. The E. coli cya strain BTH101 harboring pUT18 and its derivatives were grown to stationary phase (OD600= 3) in LB in the presence of 2 mM cAMP and 0.5 mM IPTG to fully induce the lac promoter on pUT18. 5 µg of total protein was loaded in each slot and analysed by western blotting with anti-Crl antibodies. 1: no plasmid, 2: pUT18, 3: pUTCrl, 4: pUTCrlY22A, 5: pUTCrlF53A, 6: pUTCrlW56A, 7: pUTCrlG80A, 8: pUTCrlW82A, 9: pUTCrlE52A, 10: pUTCrlG55A, 11: pUTCrlW57A. C) Detection of Crl in Salmonella ATCC14028 (1, 4, 7, 10, 15) and its mutant derivatives ATCCfur (2, 5, 8, 13), ATCCcr (3, 6, 9, 11), ATCCrpoS (12), and ATCCrpoSfur (14). Cultures were grown in different conditions, as indicated, and analysed by western blotting with anti-Crl antibodies. 1 µg of total protein was loaded in each slot. LB 37°C: overnight LB cultures at 37°C. LB 30°C: overnight LB cultures at 30°C. LB agar 30°C: colonies grown on LB agar at 30°C as described (28). 15

FIGURE 7. Effects of the Crl variants on EσS promoter binding and abortive transcription.
A) Band shift analysis of EσS binding to the katN labelled fragment in the absence of Crl (lanes 2 and 8) and in the presence of wild-type Crl (lane 3) or its variants Y22A (lane 4), F53A (lane 5), W82A (lane 6) and W56A (lane 7), lane 1: no protein. A typical autoradiogram is shown. The bands corresponding to free and bound DNAs are indicated by arrows and the percentage of bound DNA indicated below each lane is the average of two experiments. B) Abortive initiation assays. EσS was preincubated in the absence of Crl or in the presence of wild-type Crl or its variants Y22A, F53A, W82A and W56A. After addition of a mixture containing the lacUV5 fragment, the ApA dinucleotide and [α-32P]-UTP, the
incorporation of labeled [α-32P]UMP into abortive transcripts was monitored as a function of time. The measurements were repeated twice, and a representative experiment is shown.


Expression of the gene fusions in the Salmonella strains indicated was determined in cultures grown in different conditions. LB 37°C: overnight LB cultures at 37°C. LB 30°C: overnight LB cultures at 30°C. LB agar 30°C: colonies grown on LB agar at 30°C as described (28). M9: Minimal M9 medium with 20 mM glucose. M9 + FeCl2: M9 with 100 μM ferrous chloride. M9 + DP: M9 with the iron chelator 2,2’-Dipyridyl (100 μM). β-galactosidase activity was measured according to the method of Miller (31). A) Expression of the crl-lacZ gene fusion in ATCC crl-lacZ strains harboring pACYC184 and pACcrl-1 that expresses the crl gene under the control of the cat promoter of pACYC184 (40). B) Expression of the crl-lacZ gene fusion in ATCC crl-lacZ and ATCCfur crl-lacZ. C) Expression of the sdhA-lacZ gene fusion in ATCC sdhA-lacZ-k and ATCCfur sdhA-lacZ-k and expression of the crl-lacZ gene fusion in ATCC crl-lacZ and ATCCfur crl-lacZ. D) Expression of the crl-lacZ gene fusion in ATCC crl-lacZ, ATCCrpoS crl-lacZ, ATCCfur crl-lacZ and ATCCrpoSfur crl-lacZ.
<table>
<thead>
<tr>
<th>conserved residues</th>
<th>G</th>
<th>P</th>
<th>Y-R</th>
<th>FD</th>
<th>C</th>
<th>P--REF-GWW</th>
<th>G</th>
<th>G-W</th>
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<tr>
<td>position in Cr1STM</td>
<td>20</td>
<td>24</td>
<td>35</td>
<td>36</td>
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<td>80</td>
<td>82</td>
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</table>
A

\( rpoS \) ... 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

\( rpoS_{T2} \) ... GAA CCC AGT G-- --- --- CT TGC TGA
  STOP

B

\[ \text{SD} \]

TTTGAGAAGAAAAGGCTTTGAGTGAAGAGGAACCCAGTG CTG GCT GAA GAA GAG CTG TTA
  \( \sigma^{S}_{T2} \) 1 2 3 4 5 6 7 8

C

\( \text{H}_2\text{O}_2 \) 15 mM

D

\( katE-lacZ \)

\( \beta\)-galactosidase

\( rpoS \) + - + T2 T2

Crl + + - + -
A

\[ \sigma^S \text{ WT } - \ T2 \ T2 \]

\[ \begin{array}{cccc}
M & 1 & 2 & 3 & 4 \\
(kDa) & 43 & & & \\
& 34 & & & \\
\end{array} \]

\[ \sigma^S \quad \sigma^S_{T2} \]

\text{OD600}

\[ \begin{array}{ccccccc}
0.1 & 0.3 & 0.5 & 1.4 & 2.6 & 4 & C1 \\
\end{array} \]

\text{2922K}

\[ \sigma^S \]

\text{B}

\text{OD600}

\[ \begin{array}{ccccccc}
0.1 & 0.3 & 0.5 & 1.5 & 2.7 & 4 & C1 \ C2 \\
\end{array} \]

\text{2922KrpoS}_{T2}

\[ \sigma^S \quad \sigma^S_{T2} \]

\text{C}

\[ \begin{array}{ccccccc}
\sigma^S & - & \text{WT} & - & \Delta12-71 & \Delta1-38 & \Delta1-71 \\
M (kDa) & 1 & 2 & 3 & 4 & 5 & 6 \\
& 72 & & & & & \\
& 55 & & & & & \\
\end{array} \]

\[ \text{T25-}\sigma^S \]
### A

<table>
<thead>
<tr>
<th></th>
<th>Y22</th>
<th>F35</th>
<th>C28</th>
<th>C37</th>
<th>C41</th>
<th>F53</th>
<th>W56</th>
<th>G74</th>
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<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>±</td>
<td>+</td>
</tr>
<tr>
<td>csgD-lacZ</td>
<td>±</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>±</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>±</td>
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<tr>
<td>H$_2$O$_2^R$</td>
<td>±</td>
<td>+</td>
<td>+</td>
<td>±</td>
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<td>±</td>
<td>-</td>
<td>+</td>
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</table>

### B

**H$_2$O$_2$ 15 mM**

**Bacterial Survival (%) over Time (min)**

- pACYC184
- pACcrl-1
- pACcrl-1 Y22A
- pACcrl-1 W82A
- pACcrl-1 C41A
- pACcrl-1 F53A
- pACcrl-1 W56A

### C

**csgD-lacZ**

**OD$_{600}$ over Time (min)**

- pACYC184
- pACcrl-1
- pACcrl-1 Y22A
- pACcrl-1 W82A
- pACcrl-1 C41A
- pACcrl-1 F53A
- pACcrl-1 W56A
A

Bound DNA →

Free DNA →

B

% katN bound

no Crl
WT
Y22A
F53A
W82A
W56A

no Crl
WT
Y22A
F53A
W82A
W56A

0 10 20 30

time (min)