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Protection of the General Stress Response σ^S by CrsR Allows a Rapid and Efficient Adaptation of *Shewanella oneidensis*.

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Running title: σ^S protection in *S. oneidensis*

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Abstract

To cope with environmental stresses, bacteria have evolved various strategies, including the general stress response (GSR). GSR is governed by an alternative transcriptional sigma factor named σ^S (RpoS) that associates with RNA polymerase and controls the expression of numerous genes. Previously, we have reported that posttranslational regulation of σ^S in the aquatic bacterium *Shewanella oneidensis* involves the CrsR-CrsA partner-switching regulatory system, but the exact mechanism by which CrsR and CrsA control σ^S activity is not completely unveiled. Here, using a translational gene fusion, we show that CrsR sequesters and protects σ^S during the exponential growth phase and thus enables rapid gene activation by σ^S as soon as the cells enter early stationary phase. We further demonstrate by an *in vitro* approach that this protection is mediated by the anti-sigma domain of CrsR. Structure-based alignments of CrsR orthologs and other anti-sigma factors identified a CrsR-specific region characteristic of a new family of anti-sigma factors. We found that CrsR is conserved in many aquatic proteobacteria and most of the time it is associated with CrsA. In conclusion, our results suggest that CrsR-mediated protection of σ^S

during exponential growth enables rapid adaptation of *S. oneidensis* to changing and stressful growth conditions and this ability is probably widespread among aquatic proteobacteria.

To cope with environmental changes or stresses, bacteria develop various strategies and among them, the general stress response (GSR) is essential for survival. GSR is governed by an alternative transcriptional sigma factor named σ^S (RpoS) that associates with the RNA polymerase and thus controls the expression of numerous genes, for example its regulon contains more than 500 genes in *Escherichia coli*. As a consequence, σ^S availability is tightly regulated at transcriptional, translational and posttranslational levels, leading to an increase of σ^S in response to stresses or signals like, for instance, starvation or pH modifications, and conversely to a decrease of this sigma factor under favorable conditions (1, 2). In *E. coli*, the posttranslational regulation of σ^S is driven by the ClpXP machinery in concert with the adaptor protein RssB. During exponential phase, RssB binds to σ^S and addresses it to the protease complex (3). To counteract the role of RssB when σ^S is required, the anti-adaptor proteins IraD, IraM and IraP interact with RssB

and prevent the degradation of σ^S (4). The posttranslational regulation of sigma B controlling the GSR has been extensively studied in the gram positive bacterium, *Bacillus subtilis*. Sigma B is posttranslationally regulated by the RsbWV partner-switching mechanism. RsbW is an anti-sigma factor that sequesters sigma B and phosphorylates RsbV when bacteria are under favorable conditions, and RsbV is an anti-sigma factor antagonist that binds RsbW and frees sigma B under stressful conditions (5, 6). In the latter case, dephosphorylation of RsbV is triggered by specific phosphatases (RsbU and RsbP). In a recent study, we have shown that in *Shewanella oneidensis*, a gram negative bacterium, σ^S posttranslational regulation is also controlled by a partner-switching mechanism involving CrsR and CrsA (Fig. 1) (7). CrsR is a three-domain response regulator comprising a receiver domain (D1), a phosphatase domain (D2) and a kinase anti-sigma factor domain (D3), and CrsA is an anti-sigma factor antagonist. In the absence of signal, σ^S is sequestered since it is bound to the anti-sigma factor domain D3 of CrsR that phosphorylates the anti-sigma factor antagonist CrsA (CrsA-P). When a stress arises, the phosphatase activity of CsrR_{D2} dephosphorylates CrsA-P, CrsA can thus bind to CsrR_{D3} and liberates σ^S which can in turn interact with the RNA polymerase to allow the adaptation of bacteria to their environment. CrsR belongs to the GHKL ATPase/kinase superfamily that comprises proteins with little primary sequence homology aside from the conserved Bergerat motif (N-, G1-, G2-Boxes) and similar structural fold (8). Among its members, bacterial anti-sigma factor proteins or domains such as (9, 10)SpollAB, CsrR_{D3} or RsbW constitute a subfamily of kinases that presents the conserved Bergerat ATP-binding site and also a defined region of dimerization (9, 10). Moreover, the anti-sigma factor can be a protein *per se* (RsbW, SpollAB in *B. subtilis*) or a domain of a more complex protein (the first domain of SypE in *Vibrio fisheri* or the third domain of CrsR in *S. oneidensis* and HsbR in *Pseudomonas aeruginosa*) (7, 11–13).

In our previous study, we have unveiled the posttranslational regulation of *S. oneidensis* σ^S

by detailing the successive steps of the CrsR-CrsA partner-switching mechanism. Here, we show that this mechanism allows a rapid bacterial adaptation in versatile environments by protecting σ^S from proteolysis and thus σ^S remains available when necessary. In addition, we reveal that CsrR_{D3} belongs to a new family of anti-sigma factor domains widespread in aquatic proteobacteria.

Results

Role of CrsR in the posttranslational regulation of σ^S .

The question we posed was what happens to σ^S when *S. oneidensis* is under favorable conditions? In a previous work, we have clearly identified the protein CrsR as a σ^S anti-sigma factor. Indeed, it was shown that CrsR is bound to σ^S when the bacterium is under favorable environment while under stressful conditions CrsR frees RpoS and binds the anti-sigma factor antagonist CrsA (Fig. 1). σ^S can thus act as a transcriptional regulator for its regulon. σ^S activity can be followed in vivo by using the *dps-lacZ* fusion as previously shown (7). During exponential growth, the transcription level of the *dps-lacZ* fusion is at a basal level while at stationary phase it increases drastically (Fig. 2). Moreover, we had observed that during exponential growth, the level of transcription of the fusion was lower in the absence of CrsR (strain $\Delta crsR$ harboring *dps-lacZ* fusion) than in its presence (strain WT harboring *dps-lacZ* fusion). Thus, we wonder whether σ^S could be protected by CrsR during the exponential growth of the bacterium in order to be quickly available in case a stress signal arises. To answer this question, an in vivo experiment measuring σ^S activity was done. To this end, a *dps-lacZ* chromosomal fusion, previously shown to be σ^S dependent (7 and Fig. 2A), was introduced in a *crsR*-deleted strain (Fig. 2). As a control, the mutated strain was complemented by a chromosomal insertion of the wild type copy of *crsR*. As expected, during the exponential growth, a basal level of β -galactosidase activity was measured in the three strains with that of *crsR*-deleted strain as low as that of the *rpoS* mutant. At early

stationary phase (10 hours), the activity increased strongly under the control of σ^S in the wild type and complemented strains, whereas in the $\Delta crsR$ strain a significant increase of β -galactosidase activity was observed only at late stationary phase (Fig. 2A) with it reaching a plateau of lower value (Fig. 2A). This result indicates that in the absence of CrsR, σ^S activity is delayed. In contrast, in the presence of CrsR, the adaptation of the bacteria is probably faster since the σ^S -dependent regulation is more rapidly effective. Moreover, when the *crsR* deletion was complemented, no time shift was detected and induction levels were similar to that of the wild type strain. It is noteworthy that the growth of the three strains was similar and that the delay in the activity was thus not correlated to the growth stage of the bacteria (Fig. 2B). This result is in favor of a protective role of CrsR towards σ^S (Fig. 2A), and we therefore wanted to look at the level of σ^S in the presence or the absence of CrsR.

Unfortunately, we were unable to detect σ^S by western blot in the wild type strain during exponential phase. We thus decided to overproduce σ^S . For this purpose, σ^S was produced from a plasmid introduced in wild type *S. oneidensis* (MR1), $\Delta crsR$ and $\Delta crsA$ strains. The crude extracts of the three exponentially grown strains were then submitted to SDS-PAGE and the presence of σ^S was revealed by western blot. A band corresponding to σ^S was observed for each strain but the amount of σ^S are much higher for the wild type and $\Delta crsA$ strains, than that obtained in the absence of CrsR (Fig. 3A). To confirm these results, we then tested the stability of σ^S by an in vitro approach. Purified σ^S was incubated with the crude extract of strain MR1 carrying either the control vector (ptac) or the pCrsR plasmid allowing the overproduction of CrsR, and σ^S stability was followed as a function of time by western blot. We found that after 2 hours of incubation, the band corresponding to σ^S almost disappeared when σ^S was incubated with the control crude extract, whereas the intensity of the band was less reduced when σ^S was incubated with the extract overproducing CrsR (Fig. 3B). Since in *E. coli* σ^S degradation depends on the Clp

machinerie, we tested whether in *S. oneidensis*, the Clp proteases are also involved in σ^S stability. Purified σ^S was incubated with the crude extract of MR1 or $\Delta clpP$ strains, and stability of σ^S was followed as above (Fig. 3C). It appears that under these conditions, there is no differences in the pattern of degradation in the presence or in the absence of the ClpP protease. This suggests that another protease is involved in *S. oneidensis* σ^S proteolysis. Altogether, these experiments confirm that CrsR protects σ^S against degradation (Fig. 3).

CrsR and the CrsR-CrsA partner-switch are widespread in aquatic proteobacteria

To determine if the novel regulation of σ^S we found in *S. oneidensis* could be conserved in other bacteria, we searched for CrsR-like proteins in bacterial genomes. This bioinformatics analysis revealed more than six hundreds CrsR homologs, all sharing the same domain organization (i. e. a receiver, a phosphatase domain and a kinase/anti-sigma factor domain). Strikingly, all of the CrsR homologs were found in Proteobacteria, with the exception of 5 homologs belonging to the Nitrospirae (one) and to the Deferribacteres (four). These CrsR homologs were mainly present in the γ -Proteobacteria class, although several representatives also appeared in the α -, β -, δ - and ϵ -Proteobacteria classes. Interestingly, no CrsR homolog was identified in the *Enterobacteriales*. Indeed, they were rather found in several other orders of the γ -Proteobacteria, three of them containing about 80% of the CrsR homologs (namely the *Alteromonadales* to which the *Shewanellaceae* belongs, the *Pseudomonadales* and the *Vibrionales*). A phylogenetic tree was then constructed using a subset of representative CrsR homologs (see Materials and Methods) (Fig. 4). The genetic environment (DNA length ≤ 20 kb) of the corresponding *crsR* genes was then analyzed seeking for *crsA*. We identified genes encoding CrsA homologs nearby the *crsR* genes in the majority of the bacterial genome analyzed (45 out of 59 CrsR homologs, Fig. 4). These results lead us to propose that the CrsR-CrsA partner-switching system is widespread among the Proteobacteria, and thus suggest that this mechanism of posttranslational

regulation of σ is almost general in aquatic proteobacteria.

CrsR belongs to a new family of anti-sigma factor proteins

When searching for CrsR homologs in sequence data bank as we did for the phylogenetic study, we did not hit the well-studied anti-sigma factors such as SpollAB, RsbW and SypE. An explanation can be that SpollAB and RsbW are organized as a single domain and the organization of the three domains of SypE is different from that of CrsR. In fact, analysis of the sequence alignment of CrsR_{D3} with these three anti-sigma factors and CrsR_{D3} homologs obtained from the phylogenetic tree highlights an additional region present in CrsR_{D3}. Interestingly, this region is conserved in anti-sigma factor domains of CrsR homologs present in figure 4 (Fig. 5A). The extra region stretches from Leu469 to Ser496 (*S. oneidensis* CrsR numbering) between the N- and G1-Boxes which are conserved motifs of the GHKL ATPase/kinase superfamily (Fig. 5A). On the basis of solved structures of anti-sigma factor proteins, the predictive model of the 3D-structure of CrsR_{D3} was designed using I-Tasser program (14). The structural organization of the stretch of 28 amino acids described above was simulated as an unfolded loop (from Leu469 to Asp488) followed by a short alpha helix (from Ser489 to Arg493) at the surface of the protein (Fig. 5B). This additional region defines a new class of anti-sigma factors.

Discussion

We have recently shown that σ^S is regulated by a partner switch in *S. oneidensis* (7). The third domain of CrsR (CrsR_{D3}) is an anti- σ factor domain that sequesters σ^S in the absence of stress. In starvation condition (stationary phase), σ^S is released from CrsR due to the binding of the anti- σ factor antagonist CrsA to CrsR_{D3}. Phosphorylation or dephosphorylation of CrsA results from the action of either the kinase of CrsR_{D3} or the phosphatase of CrsR_{D2},

respectively (Fig. 1). In the absence of stress, CrsR_{D2} is inactive while CrsA is phosphorylated by CrsR_{D3} and CrsA-P cannot bind CrsR.

In this study, we show that CrsR protects σ^S against proteolysis under no stress condition. We also observed that σ^S induced *dps* during the early stationary phase (Fig. 2) and the induction level remains constant from early to late stationary phases. This result strongly suggests that the entire pool of σ^S is released from CrsR when cells enter into the stationary phase. It will be interesting to confirm this possible on/off mechanism using other σ^S -dependent genes and various stress, although transcriptional regulation could partially contribute to σ^S regulon induction. Another striking point is that *dps* is also induced in a Δ *crsR* mutant but the induction level increased slightly during the stationary phase and it did not reach that of the wild type strain. We thus propose that an additional regulatory mechanism operates during the stationary phase, possibly by inactivating the protease targeting σ^S or by protecting σ^S with a specific escort protein produced during the stationary phase. In contrast to σ^S of *E. coli*, σ^S of *S. oneidensis* is not degraded by the Clp protease in our experimental condition. This data confirms that the post-translational regulations of σ^S of *E. coli* and *S. oneidensis* have no similarity. In *E. coli*, σ^S is degraded in the absence of stress. Therefore, σ^S must be synthesized *de novo* during stressful conditions and the response is thus delayed reaching its maximum during the late stationary phase (1, 2, 15). In *S. oneidensis*, σ^S is always available and can activate quickly the target genes in the presence of a stress, allowing an efficient cell adaptation. We suppose that, when the stress disappears, CrsR could again sequester σ^S . If true, this partner switch allows a rapid and reversible answer with a low energy cost.

Interestingly, the protective role of an anti-sigma factor was previously described for σ^T in the gram positive *Streptomyces coelicolor* (16). The preservation of the sigma factor even when no signal is present could be an efficient way to adapt for bacteria living in versatile biotopes. A similar effect was also observed for

the flagellar sigma factor FliA, which is protected by the anti-sigma factor FlgM (17).

Using a bioinformatics approach, we identified a large family of proteobacterial proteins homologous to CrsR of *S. oneidensis*. It is striking that among the analyzed bacteria including, *Shewanella* sp., *Pseudomonas* sp. and *Vibrio* sp., many live in aquatic environments and have to deal with a wider range of stress than *E. coli* and other enterobacteria that live in more restricted habitats. In addition, the CrsR-CrsA partner-switch homologs could be involved in the regulation of other alternative σ factors. Indeed, although Alphaproteobacteria do not possess a σ^S homolog, but instead a sigma σ^{EcfG} factor, CrsR-CrsA partner-switch is conserved in some of them (18–20). For example, *Magnetococcus marinus* MC1 does not encode the NepR-PhyR proteins that usually regulate σ^{EcfG} , but possesses *crsA* and *crsR* homologs (Fig. 4). Taken together, these data suggest that the CrsR-CrsA partner-switch is a widespread regulatory system involved in the posttranslational regulation of GSR sigma factors.

Finally, we identified in this study a region of the D3 domain specific of anti-sigma factors of the CrsR family. It is a loop comprised between the N- and G1 conserved boxes found in kinase sequences (Fig. 5A). This loop comprising 28 amino acid residues is characteristic of CrsR homologs presenting the same three-domain organization and thus could be the trademark of a new family of anti-sigma factors found in various classes of the phylum of proteobacteria (Fig. 4). The structure prediction of this region suggests no particular fold while the rest of the domain can be modelled following SpoIIAB structure (9). This potentially disordered extension located on the surface of the domain is reminiscent of that observed in NepR of *Alphaproteobacteria* although, even NepR and CrsR are both anti-sigma factor proteins, they are not related. The disordered region of NepR was shown to participate in the binding of its substrates (PhyR and σ^{EcfG}) (19, 21). It would be interesting to determine if the additional extension is also involved in the binding of CrsR partners. Unfortunately, so far any

modification of this region leads to instable variants allowing no conclusion about the role of this extra region.

In conclusion, this study demonstrates the role of CrsR towards σ^S during the exponential phase of the bacterial growth. Indeed, it is now clear that in the absence of stress, the interaction between the two proteins leads to a protection of σ^S . This latter can be released from CrsR as soon as CrsA is dephosphorylated when an environmental stress signal is detected by an yet unknown signal transduction pathway. This mechanism, which is highly conserved among proteobacteria, could allow a faster adaptation of the bacteria under versatile conditions.

Experimental procedures

Medium, growth conditions, strains, and plasmids- Strains were routinely grown in LB medium at 28°C and 37°C for *S. oneidensis* and *E. coli*, respectively (22). When appropriated, antibiotics were used at the following concentrations: kanamycin (25 $\mu\text{g}/\text{mL}$), streptomycin (100 $\mu\text{g}/\text{mL}$), and chloramphenicol (25 $\mu\text{g}/\text{mL}$). All *S. oneidensis* strains used in this study (WT, ΔclpP , WT *dps-lacZ* fusion, $\Delta\text{rpoS dps-lacZ}$, $\Delta\text{crsR dps-lacZ}$ fusion and $\Delta\text{crsR}/\text{crsR dps-lacZ}$ fusion) are derivatives of the MR1-R strain referred as WT (7, 23). Complementation *in trans* of SO2119 (*crsR*) named $\Delta\text{crsR}/\text{crsR}$ was done by cloning two 500 bp fragments containing *XmaI* and *XhoI* restriction sites and flanking the site of insertion (between the genes SO2126 and SO2127). The fragment was cloned into the pKNG101 suicide vector (24) at the *Sall* and *SpeI* restriction sites as described before (25). The coding sequence of *crsR* (SO2119) was then cloned in frame after a consensus σ^{70} promoter sequence (TTGACAn₁₇TATAAT) and a consensus RBS sequence (AGGAGA) into modified pKNG101 and was introduced into *E. coli* CC118 λpir and then transferred to ΔcrsR as described before for deletion mutants (7). The pKNG101 vector containing the *dps-lacZ* fusion was then transferred to $\Delta\text{crsR}/\text{crsR}$ strain by conjugation as previously described (7).

The plasmids used in this study are the followings. pBrp σ^S corresponds to the pBAD33 vector carrying *rpoS* (SO3432) coding sequence in frame with N-terminal StrepTagII sequence, pET σ^S -52 vector corresponds to the pET-52b vector carrying *rpoS* (SO3432) sequence (7). pTCrsR vector corresponds to the p33Tac vector (pBAD33 derivative vector with *ara* promoter replaced by *lac* promoter) carrying *crsR* coding sequence (SO2119).

Expression and purification of recombinant σ^S protein- Recombinant protein Strep- σ^S was produced and purified from *E. coli* BL21(DE3) strain containing the plasmid pET σ^S -52 as described before (7).

In vivo assays- To follow the activity of the *dps-lacZ* fusion in stationary phase, the strains were grown at 28 °C anaerobically in LB medium supplemented with trimethylamine oxide (TMAO, 10 mM) as final electron acceptor (26). Samples of cultures were collected at different times and β -galactosidase activities were measured in Miller units as previously described (22).

In vitro degradation systems- CrsR, protein was produced from MR1-R strains containing the plasmids p33Tac and pTCrsR. At OD₆₀₀=0.4, IPTG (1 mM) was added to overproduce the protein. Cells were then grown for 2 additional hours, collected by centrifugation, washed with Tris-HCl pH 7.6 buffer and lysed by adding 1:10 PopCulture[®] reagent (Novagen[®]) and lysozyme (1mg/mL final concentration). The crude extracts were collected by centrifugation at 13000 rpm for 15 minutes (27). MR1-R and Δ *clpP* strains were harvested during exponential growth and crude extract were prepared as above.

Crude extracts were then diluted at 5 mg/mL of total proteins in Tris-HCl pH 7.6 buffer and reactions were started by adding 0.5 μ M of σ^S protein. Samples were incubated at 25°C and aliquots were collected at times 0 and 2 hours or 0, 0.5, 1 and 2 hours. Loading buffer was added and samples were heated for 5 minutes at 95°C before migration by electrophoresis with a Bolt™ 4-12% Bis-Tris gel (Invitrogen). Proteins were then visualized after western

blot using Strep-Tactin probe HRP conjugated antibody (IBA).

In vivo production of σ^S protein- σ^S protein was produced from MR1-R, Δ *crsR* and Δ *crsA* strains containing the plasmid pBrp σ^S . Cells were grown for 1 hour before 0.02% arabinose was added and cells were incubated for 2 hours under shaking. The crude extracts were prepared and treated as described above. σ^S was visualized after western blot using anti- σ^S rabbit antibody (gift from Susan Gottesman), followed by anti-rabbit HRP-conjugated antibody (Sigma Aldrich).

Bioinformatics analyses- The proteins sharing homologies with CrsR were found in the NCBI non-redundant protein sequences database using the protein BLAST search tool. For the phylogenetic tree construction, the searches were made independently on the different classes of Proteobacteria (α -, β -, γ -, δ -, ϵ -Proteobacteria) as well as on bacteria, with the exclusion of the Proteobacteria. For the γ -Proteobacteria, the searches were made separately on each order composing this class. One representative sequence for each genus was subsequently selected, except for Shewanella, Vibrio and Pseudomonas. We chose the proteins sharing the highest E-value with CrsR on the whole length of the proteins. For the phylogenetic analysis, we used the “Phylogeny.fr” software in the “one-click” mode, i.e. with the default parameters optimized by the authors (28) (<http://www.phylogeny.fr/>). The main steps performed by this software correspond to multiple alignments of the CrsR homologs using the MUSCLE version 3.8.31 method, alignment curation by GBlocks version 0.9b and phylogeny using PhyML version 3.1 method using 100 bootstrap replicates. For the tree rendering step, we used the software “FigTree” version 1.4.2 (<http://tree.bio.ed.ac.uk/software/figtree/>) in which we entered the result in Newick format obtained with “Phylogeny”. After the first phylogenetic analysis, we manually removed the unnecessary sequences and a second phylogenetic analysis was performed. The neighborhood of the genes coding for the CrsR homologs was extracted from the databases

using NCBI, MAGE (<https://www.genoscope.cns.fr/agc/microscope/home/>) and KEGG (<http://www.genome.jp/>).

Sequences alignment and tertiary structure prediction– Representative sequences of CrsR from different classes, orders and genus were selected. HATPase domains from these proteins and SypE (from *Vibrio fischeri*), RsbW and SpoIIAB (from *Bacillus subtilis*) were

aligned using the Clustal Omega program (EMBL) and the highlighted and conserved amino acid residues were generated using the BoxShade (ExPASy) server. The secondary structure of CrsR_{D3} was predicted by using the PSIPRED server (<http://bioinf.cs.ucl.ac.uk/psipred/>). Structure of CrsR_{D3} was predicted by using I-Tasser server and the model having the highest C-score (-1.14) was annotated and shown in Fig. 5B (14) (<http://zhanglab.Ccmb.med.umich.edu/I-TASSER/>).

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Author contributions:

All authors were involved in conduction of experiments, analysis of the results, and the writing of the paper.

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Figure legends**Figure 1. Model of the partner-switching mechanism involved in the regulation of σ^S in *S. oneidensis***

A. Under favorable conditions, the third domain of CrsR (D3) phosphorylates CrsA (CrsA-P), hampering the interaction between the two proteins and leading to the sequestration of σ^S by the anti-sigma factor domain (D3) of CrsR. Thus, σ^S is unable to promote the transcription of the genes from its regulon.

B. Under stress conditions, CrsR dephosphorylates CrsA-P *via* its phosphatase domain (D2). The anti-sigma factor antagonist CrsA then interacts with the anti-sigma factor domain of CrsR, driving the release of σ^S . σ^S can thus bind the core of the RNA polymerase (Core RNA-Pol) and promotes the transcription of the genes involved in GSR, including the *dps* gene. D1 represents the receiver domain of CrsR and P the phosphoryl group.

Figure 2. Effects of CrsR on σ^S in vivo.

A. The absence of CrsR delays the σ^S -dependent *dps* induction in stationary phase. Strains WT, $\Delta rpoS$, $\Delta crsR$ and $\Delta crsR/crsR$ harboring the *dps-lacZ* fusion were grown until stationary phase, anaerobically with TMAO. β -galactosidase activities were measured at different times.

B. Growth of WT, $\Delta rpoS$, $\Delta crsR$ and $\Delta crsR/crsR$ strains is similar. Curves represent the OD₆₀₀ of strains used in A as a function of time.

For A and B, averages and SD from three independent experiments are shown.

Figure 3. CrsR protects σ^S from degradation in vivo and in vitro.

A. σ^S is protected from degradation by CrsR *in vivo* during exponential phase. Strains WT, $\Delta crsA$ and $\Delta crsR$ carrying pBRpoS were grown until exponential phase aerobically with 0.02% arabinose to induce σ^S production. Crude extracts were submitted to SDS-PAGE and σ^S was revealed by western blot with σ^S antibodies.

B. σ^S is protected in vitro by CrsR. Crude extracts of MR1 cells harvested during exponential phase and overproducing CrsR were incubated with purified Strep- σ^S protein. Samples were collected at t_0 and t_{2h} , submitted to SDS-PAGE and σ^S was detected by a StrepTactin antibody.

C. Stability of σ^S in the absence of ClpP protease. Same experiment as above except that crude extracts were prepared from MR1 and $\Delta clpP$ cells. Collection time of the samples are indicated on the figure.

Figure 4. Occurrence of CrsR homologs in Bacteria.

Searches for homologous proteins to *S. oneidensis* CrsR were done using the bioinformatics BLAST tool and the sequences were assembled using the program "Phylogeny". Bacteria are: Alpha-proteobacteria, Beta-proteobacteria, Delta-proteobacteria, Epsilon-proteobacteria, Deferribacteres (eubacteria except Proteobacteria), Gamma-proteobacteria (*Alteromonadales*, *Chromatiales*, *Methylococcales*, *Oceanospirillales*, *Pseudomonadales*, *Thiotrichales*, *Vibrionales*). The symbol * indicates the presence of a homolog of the *S. oneidensis* gene *crsA* in the vicinity (≤ 20 kb) of the *crsR* homolog in the tied species. The symbol * indicates a genus. Among the genus *Pseudomonas*, the species *P. aeruginosa*, *P. putida*, *P. chlororaphis*, *P. fluorescens*, *P. syringae*, *P. stutzeri* were selected. The genus *Vibrio* includes *V. mimicus*, *V. cholerae*, *V. vulnificus*, and the genus *Shewanella* includes *S.*

xiamenensis, *S. decolorationis*, *S. sp. HN-41*, *S. baltica OS185*, *S. sp. ANA-3*, *S. sp. MR-7*, *S. putrefaciens* and *S. oneidensis MR-1*.

Figure 5. The GHKL ATPase/kinase domain of CrsR possesses an additional region.

A. Sequence alignment of the third domain of CrsR (CrsR_{D3}) from amino acids 447 to 527 of *S. oneidensis* and CrsR_{D3} homologs from γ -proteobacteria *Methylomonas denitrificans*, *Hydrogenovibrio marinus*, *Oceanospirillum beijerinckii*, *Halorhodospira halophila SL1*, *Glaciecola chathamensis*, *Vibrio cholerae N16961*, *Shewanella baltica OS185*, *Shewanella decolorationis* and β -proteobacterium *Leeia oryzae*, with the anti- σ factors RsbW and SpoIIAB of *B. subtilis* (Firmicutes), and the anti- σ domain of SypE (from *V. fisheri*, γ -proteobacterium). The conserved ATP binding Bergerat-fold N- and G1- boxes are indicated. The additional region is framed in red and the secondary structure prediction of CrsR_{D3} is drawn above the alignment. Conserved residues in the additional region are in blue.

B. Comparison of the tertiary structure of SpoIIAB from *Bacillus subtilis* (9, 10) and the predicted structure of CrsR_{D3}. Helix are in green, sheets in pink, loops in orange and the extra region in yellow and blue, and zoomed in the circle. The highly conserved residues in the extra region appear in blue and are annotated in the zoomed box. CrsR_{D3} structure was predicted using I-Tasser program.

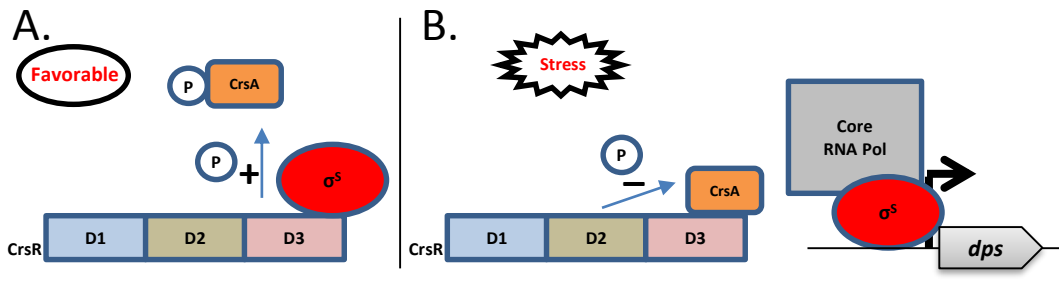
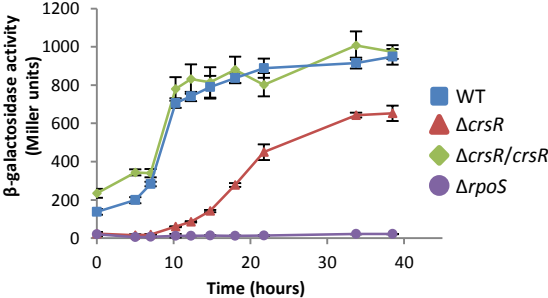


Figure 1

A.



B.

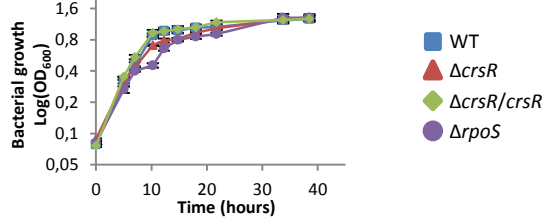


Figure 2

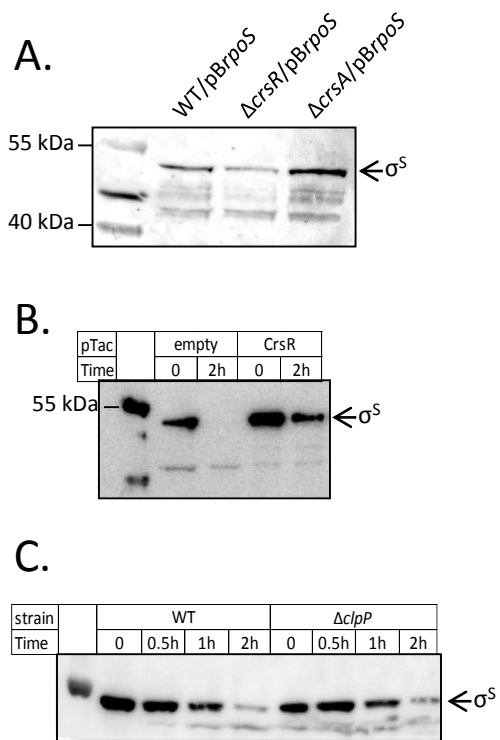


Figure 3

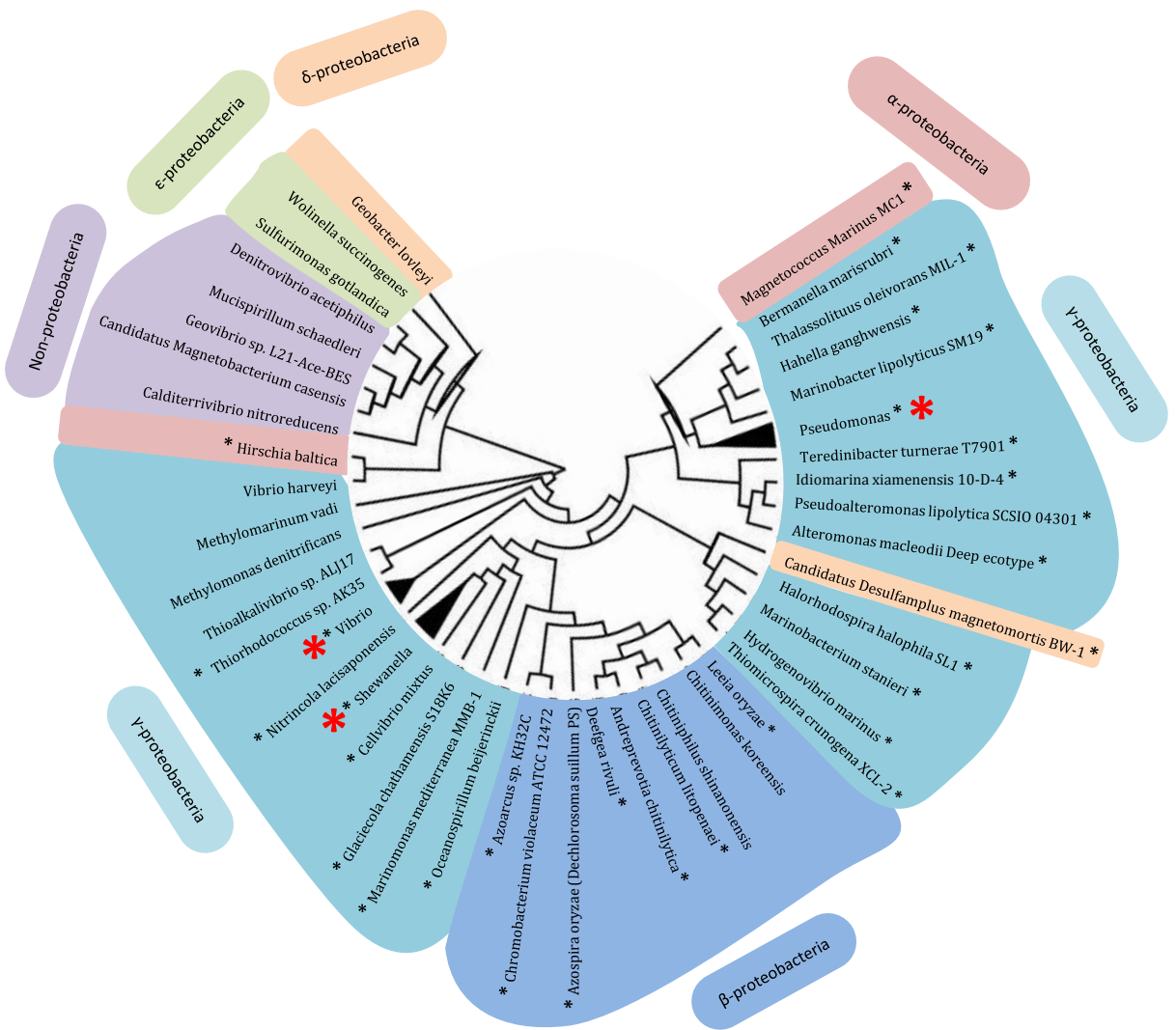


Figure 4

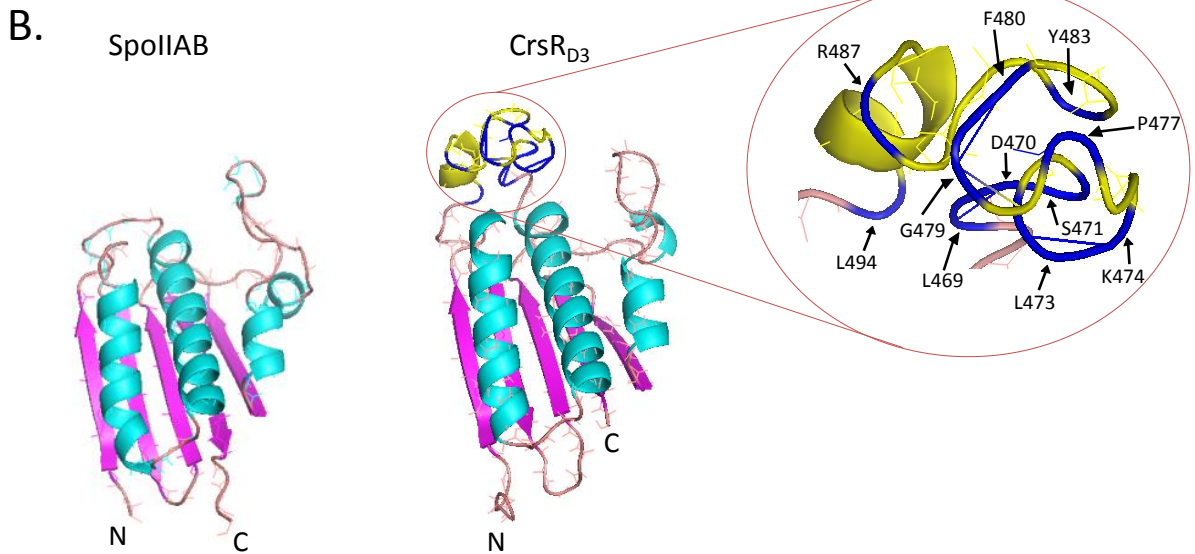
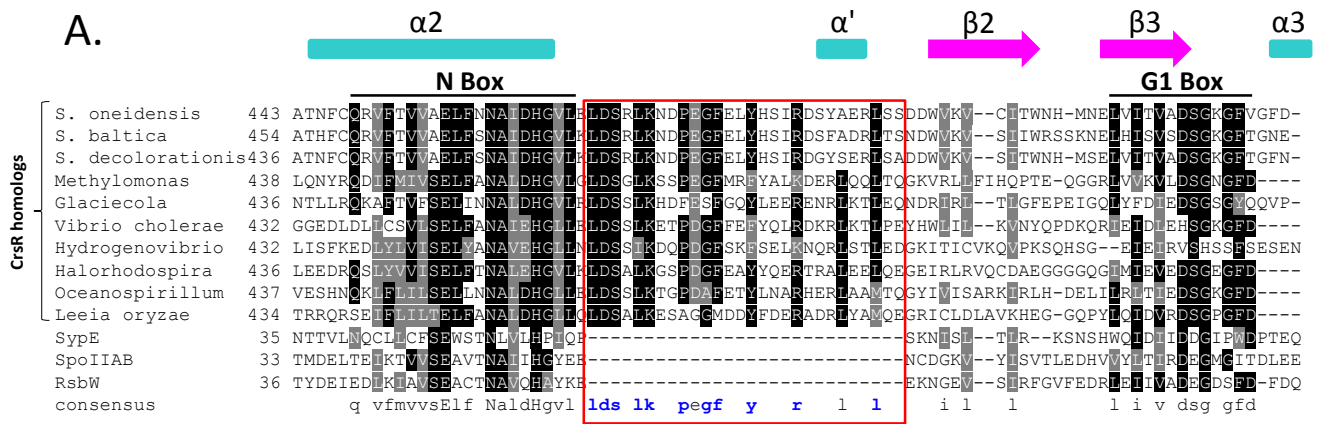


Figure 5