Protection of the general stress response σ^S factor by the CrsR regulator allows a rapid and efficient adaptation of *Shewanella oneidensis*

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Sophie Bouillet[1], **Olivier Genest, Vincent Méjean, and Chantal Iobbi-Nivol**[2]

From the Aix-Marseille Université, CNRS, BIP UMR7281, 13402 Marseille, France

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To cope with environmental stresses, bacteria have evolved various strategies, including the general stress response (GSR). GSR is governed by an alternative transcriptional σ 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-σ domain of CrsR. Structure-based alignments of CsrR orthologs and other anti-σ factors identified a CsrR-specific region characteristic of a new family of anti-σ 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 CsrR-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)[3] is essential for survival. GSR is governed by an alternative transcriptional σ 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 and pH modifications and conversely to a decrease of this σ 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 σ^B controlling the GSR has been extensively studied in the Gram-positive bacterium *Bacillus subtilis*. σ^B is posttranslationally regulated by the RsbWV partner-switching mechanism. RsbW is an anti-σ factor that sequesters σ^B and phosphorylates RsbV when bacteria are under favorable conditions, and RsbV is an anti-σ factor antagonist that binds RsbW and frees σ^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 (see Fig. 1) (7). CrsR is a three-domain response regulator comprising a receiver domain (D1), a phosphatase domain (D2), and a kinase/anti-σ factor domain (D3), and CrsA is an anti-σ factor antagonist. In the absence of signal, σ^S is sequestered because it is bound to the anti-σ factor domain D3 of CrsR that phosphorylates the anti-σ factor antagonist CrsA (CrsA-P). When a stress arises, the phosphatase activity of $CsrR_{D2}$ dephosphorylates CrsA-P; CrsA can thus bind to the third domain of CrsR ($CrsR_{D3}$) 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, and G2 boxes) and similar structural fold (8). Among its members, bacterial anti-σ factor proteins or domains such as SpoIIAB, $CrsR_{D3}$, and RsbW constitute a subfamily of kinases that presents the conserved Bergerat ATP-binding site and a defined region of dimerization (9, 10). Moreover, the anti-σ factor can be a protein *per se* (RsbW and SpoIIAB 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

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[1] Supported by a Ministère de l'Enseignement Supérieur et de la Recherche fellowship and AMU.

[2] To whom correspondence should be addressed: Bioénergétique et Ingénierie des Protéines-Institut de Microbiologie de la Méditerranée (BIP-IMM), CNRS, 31 Chemin Joseph Aiguier, 13402 Marseille Cedex 20, France. E-mail: iobbi@imm.cnrs.fr.

[3] The abbreviations used are: GSR, general stress response; CrsA-P, phosphorylated CrsA; TMAO, trimethylamine oxide; Bis-Tris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)propane-1,3-diol; GHKL, gyrase, Hsp90, histidine kinase, and MutL.

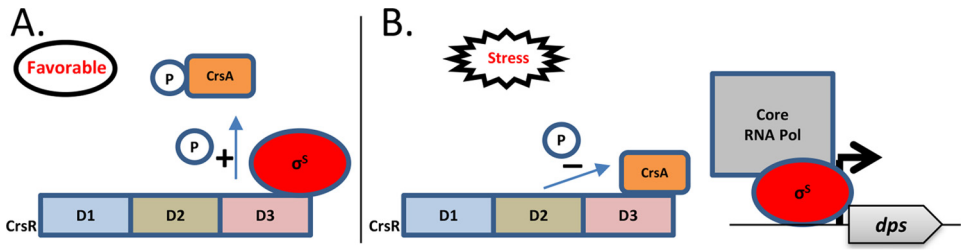

Figure 1. Model of the partner-switching mechanism involved in the regulation of σ^S in *S. oneidensis*. *A*, under favorable conditions, the third domain of CrsR (D3) phosphorylates CrsA (CrsA-P), hampering the interaction between the two proteins and leading to the sequestration of σ^S by the anti-σ 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-σ factor antagonist CrsA then interacts with the anti-σ 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 is the receiver domain of CrsR, and *P* represents the phosphoryl group.

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 $CrsR_{D3}$ belongs to a new family of anti-σ factor domains widespread in aquatic proteobacteria.

Results

Role of CrsR in the posttranslational regulation of σ^S

The question we posed is: 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-σ factor. Indeed, it was shown that CrsR is bound to σ^S when the bacterium is in a favorable environment, whereas under stressful conditions CrsR frees RpoS and binds the anti-σ 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 shown previously (7). During exponential growth, the transcription level of the *dps-lacZ* fusion is at a basal level, whereas 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 Δ*crsR* harboring *dps-lacZ* fusion) than in its presence (strain WT harboring *dps-lacZ* fusion). Thus, we wondered whether σ^S could be protected by CrsR during the exponential growth of the bacterium to be quickly available in case a stress signal arises. To answer this question, an *in vivo* experiment measuring σ^S activity was performed. To this end, a *dps-lacZ* chromosomal fusion, previously shown to be σ^S-dependent (Ref. 7 and Fig. 2*A*), 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 exponential growth, a basal level of β-galactosidase activity was measured in the three strains with that of the *crsR*-deleted strain as low as that of the *rpoS* mutant. At early stationary phase (10 h), the activity increased strongly under the control of σ^S in the wild-type and complemented strains, whereas in the Δ*crsR* strain a significant increase of β-galactosidase activity was observed only at late stationary phase (Fig. 2*A*) with it reaching a plateau of lower value (Fig. 2*A*). 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 because the σ^S-dependent regulation is more rapidly effective. Moreover, when the *crsR*

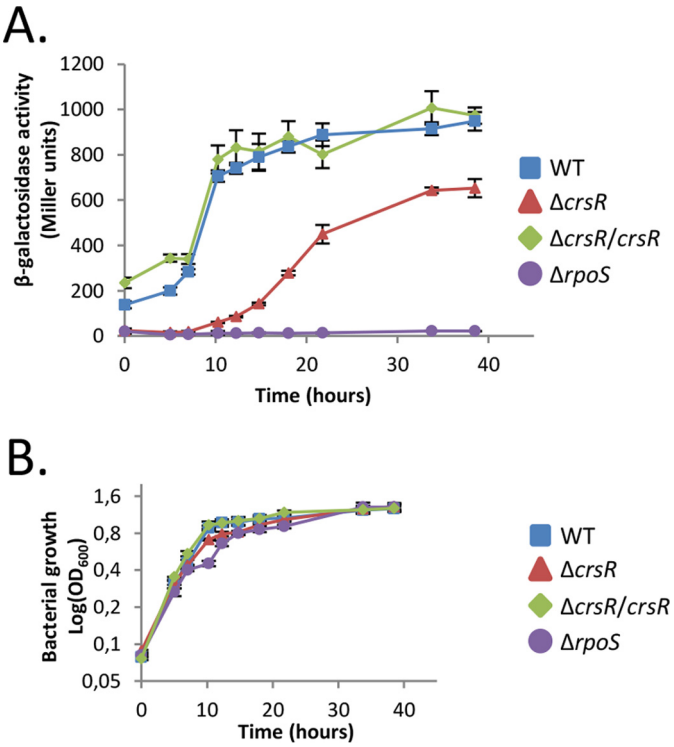

Figure 2. Effects of CrsR on σ^S *in vivo*. *A*, the absence of CrsR delays the σ^S-dependent *dps* induction in stationary phase. Strains WT, Δ*rpoS*, Δ*crsR*, and Δ*crsR*/*crsR* harboring the *dps-lacZ* fusion were grown until stationary phase anaerobically with TMAO. β-Galactosidase activities were measured at different times. *B*, growth of WT, Δ*rpoS*, Δ*crsR*, and Δ*crsR*/*crsR* strains is similar. Curves represent the A_{600} of strains used in *A* as a function of time. For *A* and *B*, averages and S.D. (*error bars*) from three independent experiments are shown.

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. 2*B*). This result is in support of a protective role of CrsR toward σ^S (Fig. 2*A*), and we therefore wanted to look at the level of σ^S in the presence or absence of CrsR.

Unfortunately, we were unable to detect σ^S by Western blotting 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), Δ*crsR*, and Δ*crsA* strains. The crude extracts of the three exponentially grown strains were then subjected to SDS-PAGE, and

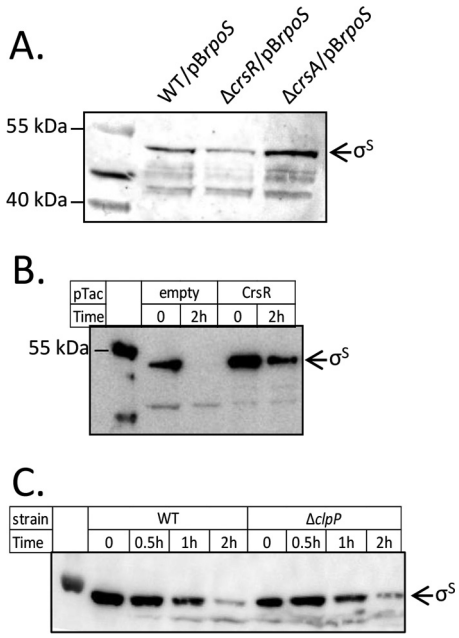

Figure 3. CrsR protects σ^S from degradation *in vivo* and *in vitro*. *A*, σ^S is protected from degradation by CrsR *in vivo* during exponential phase. Strains WT, Δ*crsA*, and Δ*crsR* carrying pBRpoS were grown until exponential phase aerobically with 0.02% arabinose to induce σ^S production. Crude extracts were subjected to SDS-PAGE, and σ^S was revealed by Western blotting 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 $t_{2\,h}$ and subjected to SDS-PAGE, and σ^S was detected by a StrepTactin antibody. *C*, stability of σ^S in the absence of ClpP protease. The same experiment as above was performed except that crude extracts were prepared from MR1 and Δ*clpP* cells. Collection times of the samples are indicated in the figure.

the presence of σ^S was revealed by Western blotting. A band corresponding to σ^S was observed for each strain, but the amounts of σ^S are much higher for the wild-type and Δ*crsA* strains than that obtained in the absence of CrsR (Fig. 3*A*). 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 blotting. We found that after 2 h 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. 3*B*). Because in *E. coli* σ^S degradation depends on the Clp machinery, 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 Δ*clpP* strains, and stability of σ^S was followed as above (Fig. 3*C*). It appears that under these conditions there are no differences in the pattern of degradation in the presence or 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 whether 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 600 CrsR homologs, all sharing the same domain organization (*i.e.* a receiver, a phosphatase domain, and a kinase/anti-σ factor domain). Strikingly, all of the CrsR homologs were found in Proteobacteria with the exception of five homologs belonging to the Nitrospirae (one) and 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 with 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 "Experimental procedures") (Fig. 4). The genetic environment (DNA length $\leq$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 genomes analyzed (45 of 59 CrsR homologs; Fig. 4). These results led us to propose that the CrsR-CrsA partner-switching system is widespread among the Proteobacteria and thus to suggest that this mechanism of posttranslational regulation of σ is almost general in aquatic proteobacteria.

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

When searching for CrsR homologs in the sequence data bank as we did for the phylogenic study, the well studied anti-σ factors such as SpoIIAB, RsbW, and SypE were not hits. A possible explanation for this is that SpoIIAB 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-σ factors and $CrsR_{D3}$ homologs obtained from the phylogenic tree highlights an additional region present in $CrsR_{D3}$. Interestingly, this region is conserved in anti-σ factor domains of CrsR homologs presented in Fig. 4 (Fig. 5*A*). The extra region stretches from Leu-469 to Ser-496 (*S. oneidensis* CrsR numbering) between the N and G1 boxes, which are conserved motifs of the GHKL ATPase/kinase superfamily (Fig. 5*A*). On the basis of solved structures of anti-σ factor proteins, the predictive model of the 3D structure of $CrsR_{D3}$ was designed using the I-TASSER program (14). The structural organization of the stretch of 28 amino acids described above was simulated as an unfolded loop (from Leu-469 to Asp-488) followed by a short α-helix (from Ser-489 to Arg-493) at the surface of the protein (Fig. 5*B*). This additional region defines a new class of anti-σ factors.

Discussion

We have recently shown that σ^S is regulated by a partner switch in *S. oneidensis* (7). $CrsR_{D3}$ 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

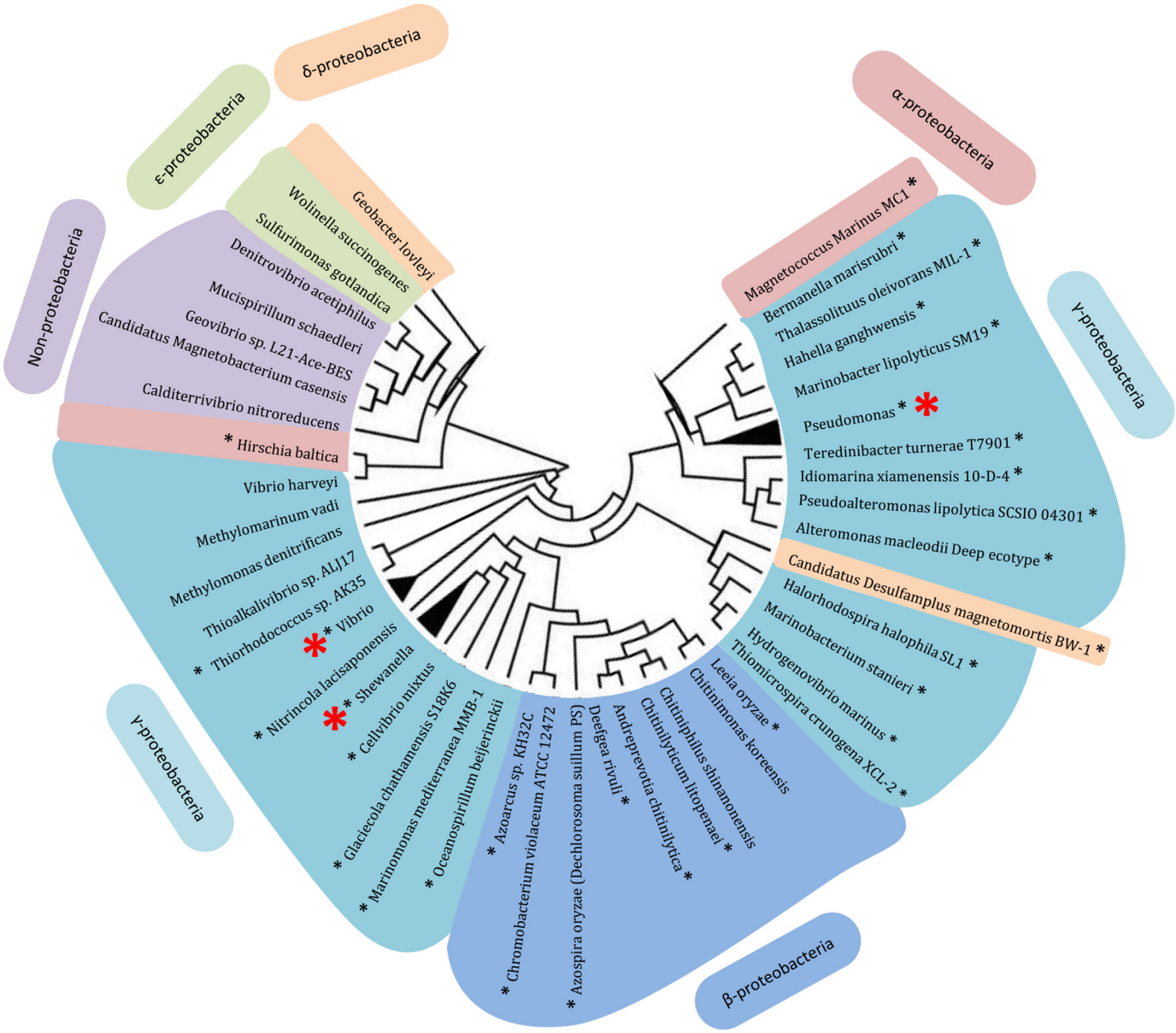

Figure 4. Occurrence of CrsR homologs in bacteria. Searches for proteins homologous to *S. oneidensis* CrsR were done using the bioinformatics BLAST tool, and the sequences were assembled using the program Phylogeny. The homologs were found in α-Proteobacteria, β-Proteobacteria, δ-Proteobacteria, ϵ-Proteobacteria, Deferribacteres (eubacteria except Proteobacteria), and γ-Proteobacteria (Alteromonadales, Chromatiales, Methylococcales, Oceanospirillales, Pseudomonadales, Thiotrichales, and 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 *red asterisk* indicates a genus. Among the genus *Pseudomonas*, the species *P. aeruginosa*, *Pseudomonas putida*, *Pseudomonas chlororaphis*, *Pseudomonas fluorescens*, *Pseudomonas syringae*, and *Pseudomonas stutzeri* were selected. The genus *Vibrio* includes *Vibrio mimicus*, *Vibrio cholerae*, and *Vibrio vulnificus*, and the genus *Shewanella* includes *Shewanella xiamenensis*, *Shewanella decolorationis*, *Shewanella* sp. HN-41, *Shewanella baltica* OS185, *Shewanella* sp. ANA-3, *Shewanella* sp. MR-7,*Shewanella putrefaciens*, and *S. oneidensis* MR-1.

inactive, 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 stationary phase. It will be interesting to confirm this possible on/off mechanism using other σ^S-dependent genes and various stresses, 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. These data confirm that the posttranslational 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,

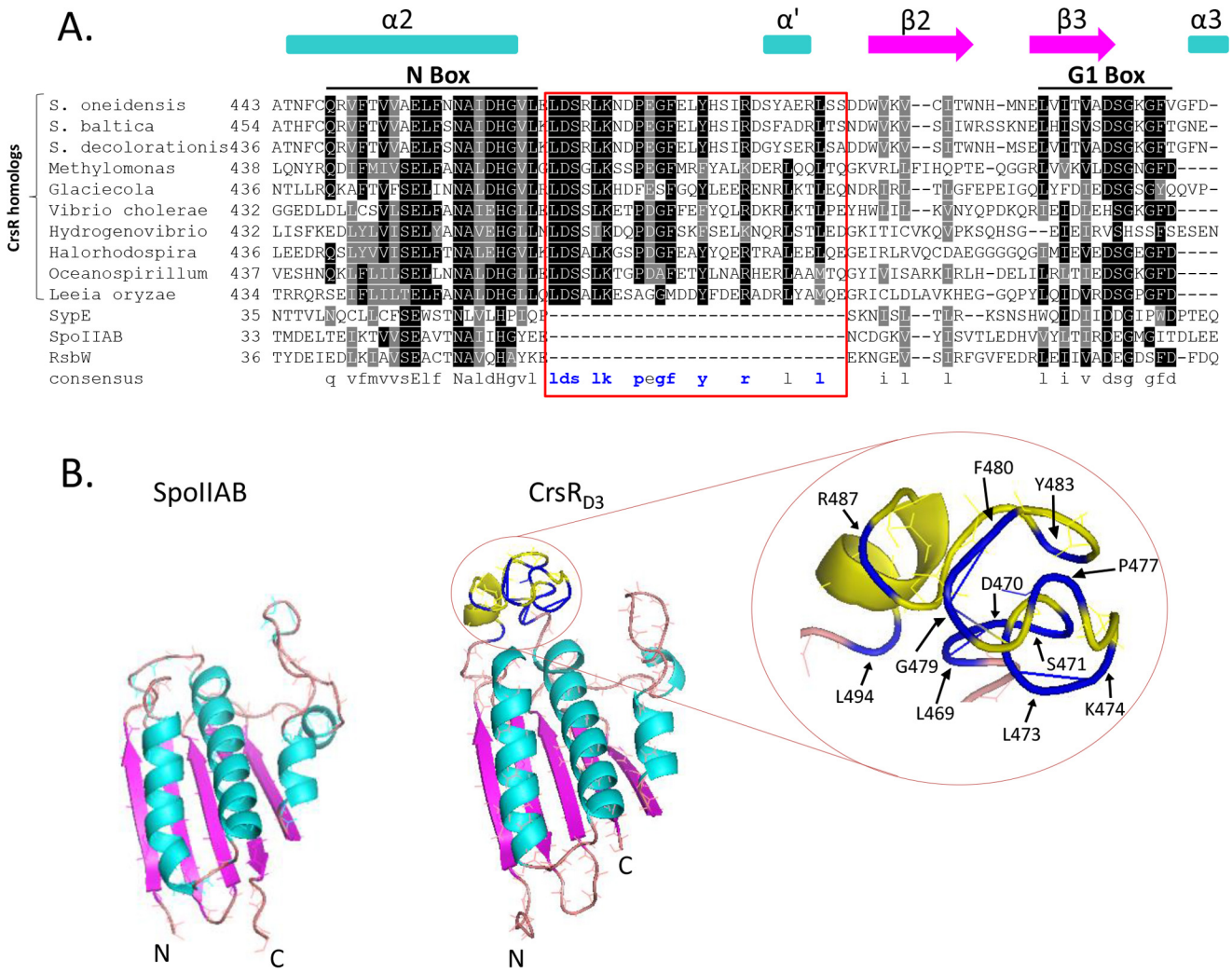

Figure 5. The GHKL ATPase/kinase domain of CrsR possesses an additional region. *A*, sequence alignment of CrsR$_{D3}$ from amino acids 447 to 527 of *S. oneidensis* and CrsR$_{D3}$ homologs from γ-proteobacteria *Methylomonas denitrificans*, *Hydrogenovibrio marinus*, *Oceanospirillum beijerinckii*, *Halorhodospira halophila* SL1, *Glaciecola chathamensis*, *V. cholerae* N16961, *S. baltica* OS185, *S. 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 *B. subtilis* (9, 10) and the predicted structure of CrsR$_{D3}$. Helices are *green*, sheets are *pink*, loops are *orange*, and the extra region is *yellow* and *blue* and is enlarged in the *circle*. The highly conserved residues in the extra region appear in *blue* and are annotated in the enlarged *box*. The CrsR$_{D3}$ structure was predicted using the I-TASSER program.

reaching its maximum during the late stationary phase (1, 2, 15). In *S. oneidensis*, σ^S is always available and can quickly activate 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-σ factor was previously described for σ^T in the Gram-positive *Streptomyces coelicolor* (16). The preservation of the σ 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 σ factor FliA, which is protected by the anti-σ 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 stresses 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 α-Proteobacteria do not possess a σ^S homolog, but instead have a 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 σ factors.

Finally, in this study, we identified a region of the D3 domain specific to anti-σ factors of the CrsR family comprising a loop between the N and G1 conserved boxes found in kinase

sequences (Fig. 5*A*). This 28-amino acid loop is characteristic of CrsR homologs presenting the same three-domain organization and thus could be the trademark of a new family of anti-σ factors found in various classes of the phylum Proteobacteria (Fig. 4). The structure prediction of this region suggests no particular fold, whereas the rest of the domain can be modeled following SpoIIAB structure (9). This potentially disordered extension located on the surface of the domain is reminiscent of that observed in NepR of α-Proteobacteria; however, although NepR and CrsR are both anti-σ 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 whether 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 toward σ^S during the exponential phase of bacterial growth. Indeed, it is now clear that, in the absence of stress, the interaction between the two proteins leads to protection of σ^S. The latter can be released from CrsR as soon as CrsA is dephosphorylated when an environmental stress signal is detected by a 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 and 37 °C for *S. oneidensis* and *E. coli*, respectively (22). When appropriate, antibiotics were used at the following concentrations: kanamycin, 25 μg/ml; streptomycin, 100 μg/ml; and chloramphenicol, 25 μg/ml. All *S. oneidensis* strains used in this study (WT, Δ*clpP*, WT *dps-lacZ* fusion, Δ*rpoS dps-lacZ*, Δ*crsR dps-lacZ* fusion, and Δ*crsR*/*crsR dps-lacZ* fusion) are derivatives of the MR1-R strain referred as WT (7, 23). Complementation in *trans* of SO2119 (*crsR*) named Δ*crsR*/*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 SalI 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 ($TTGACAN_{17}TATAAT$) and a consensus ribosome-binding site sequence (AGGAGA) into modified pKNG101, introduced into *E. coli* CC118λpir, and then transferred to Δ*crsR* as described before for deletion mutants (7). The pKNG101 vector containing the *dps-lacZ* fusion was then transferred to Δ*crsR*/*crsR* strain by conjugation as described previously (7).

The following plasmids were used in this study. pBrpoS corresponds to the pBAD33 vector carrying the *rpoS* (SO3432) coding sequence in-frame with an N-terminal StrepTagII sequence. pETσ^S-52 vector corresponds to the pET-52b vector carrying the *rpoS* (SO3432) sequence (7). pTCrsR vector corresponds to the p33Tac vector (pBAD33 derivative vector with *ara* promoter replaced by *lac* promoter) carrying the *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 pETσ^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 the final electron acceptor (26). Samples of cultures were collected at different times, and β-galactosidase activities were measured in Miller units as described previously (22).

In vitro degradation systems

CrsR protein was produced from MR1-R strains containing the plasmids p33Tac and pTCrsR. At an A_{600} of 0.4, isopropyl 1-thio-β-D-galactopyranoside (1 mM) was added to overproduce the protein. Cells were then grown for an additional 2 h, collected by centrifugation, washed with Tris-HCl pH 7.6 buffer, and lysed by adding 1:10 PopCulture® reagent (Novagen®) and lysozyme (1 mg/ml final concentration). The crude extracts were collected by centrifugation at 13,000 rpm for 15 min (27). MR1-R and Δ*clpP* strains were harvested during exponential growth, and crude extracts were prepared as above.

Crude extracts were then diluted to 5 mg/ml total proteins in Tris-HCl pH 7.6 buffer, and reactions were started by adding 0.5 μM σ^S protein. Samples were incubated at 25 °C, and aliquots were collected at times 0 and 2 h or 0, 0.5, 1, and 2 h. Loading buffer was added, and samples were heated for 5 min at 95 °C before migration by electrophoresis using a Bolt™ 4–12% Bis-Tris gel (Invitrogen). Proteins were then visualized after Western blotting using StrepTactin 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 pBRpoS. Cells were grown for 1 h before 0.02% arabinose was added, and cells were incubated for 2 h under shaking. The crude extracts were prepared and treated as described above. σ^S was visualized after Western blotting using anti-σ^S rabbit antibody (a 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 sequence database using the protein BLAST search tool. For phylogenetic tree construction, the searches were made independently on the different classes of Proteobacteria (α-, β-, γ-, δ-, and 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 comprising 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 Phylogeny.fr software in the "one-click" mode, *i.e.* with the default parameters optimized by the authors (28) (http://www.phylogeny.fr/).[4] 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 the PhyML version 3.1 method using 100 bootstrap replicates. For the tree rendering step, we used the software FigTree version 1.4.2 (29) (http://tree.bio.ed.ac.uk/software/figtree/)[4] in which we entered the result in Netwick 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, Microbial Genome Annotation and Analysis Platform (MaGe) (30) (https://www.genoscope.cns.fr/agc/microscope/home/),[4] and Kyoto Encyclopedia of Genes and Genome (KEGG) (31) (http://www.genome.jp/).[4]

Sequences alignment and tertiary structure prediction

Representative sequences of CrsR from different classes, orders, and genera were selected. HATPase domains from these proteins and SypE (from *V. fisheri*) and RsbW and SpoIIAB (from *B. subtilis*) were aligned using the Clustal Omega program (European Molecular Biology Laboratory), and the highlighted and conserved amino acid residues were generated using the BoxShade (ExPASy) server. The secondary structure of $CrsR_{D3}$ was predicted using the PSIRED server (32) (http://bioinf.cs.ucl.ac.uk/psipred/).[4] The structure of $CrsR_{D3}$ was predicted using the I-TASSER server, and the model having the highest C-score (−1.14) was annotated and is shown in Fig. 5*B* (14).

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References

1. Hengge, R. (2011) The general stress response in Gram-negative bacteria, in *Bacterial Stress Responses* (Storz, G., and Hengge, R., eds) 2nd Ed., pp. 251–290, American Society for Microbiology (ASM) Press, Washington, D. C.
2. Battesti, A., Majdalani, N., and Gottesman, S. (2011) The RpoS-mediated general stress response in *Escherichia coli*. *Annu. Rev. Microbiol.* **65,** 189–213
3. Hengge, R. (2009) Proteolysis of σS (RpoS) and the general stress response in *Escherichia coli*. *Res. Microbiol.* **160,** 667–676
4. Battesti, A., Hoskins, J. R., Tong, S., Milanesio, P., Mann, J. M., Kravats, A., Tsegaye, Y. M., Bougdour, A., Wickner, S., and Gottesman, S. (2013) Anti-adaptors provide multiple modes for regulation of the RssB adaptor protein. *Genes Dev.* **27,** 2722–2735
5. Dufour, A., and Haldenwang, W. G. (1994) Interactions between a *Bacillus subtilis* anti-σ factor (RsbW) and its antagonist (RsbV). *J. Bacteriol.* **176,** 1813–1820
6. Price, C. W. (2011) The general stress response in *Bacillus subtilis* and related Gram-positive bacteria, in *Bacterial Stress Responses* (Storz, G., and Hengge, R., eds) 2nd Ed., pp. 301–318, American Society for Microbiology (ASM) Press, Washington, D. C.
7. Bouillet, S., Genest, O., Jourlin-Castelli, C., Fons, M., Méjean, V., and Iobbi-Nivol, C. (2016) The general stress response σS is regulated by a partner switch in the Gram-negative bacterium *Shewanella oneidensis*. *J. Biol. Chem.* **291,** 26151–26163
8. Dutta, R., and Inouye, M. (2000) GHKL, an emergent ATPase/kinase superfamily. *Trends Biochem. Sci.* **25,** 24–28
9. Campbell, E. A., Masuda, S., Sun, J. L., Muzzin, O., Olson, C. A., Wang, S., and Darst, S. A. (2002) Crystal structure of the *Bacillus stearothermophilus* anti-σ factor SpoIIAB with the sporulation σ factor σF. *Cell.* **108,** 795–807
10. Masuda, S., Murakami, K. S., Wang, S., Anders Olson, C., Donigian, J., Leon, F., Darst, S. A., and Campbell, E. A. (2004) Crystal structures of the ADP and ATP bound forms of the *Bacillus* anti-σ factor SpoIIAB in complex with the anti-anti-σ SpoIIAA. *J. Mol. Biol.* **340,** 941–956
11. Morris, A. R., and Visick, K. L. (2013) The response regulator SypE controls biofilm formation and colonization through phosphorylation of the syp-encoded regulator SypA in *Vibrio fischeri*. *Mol. Microbiol.* **87,** 509–525
12. Houot, L., Fanni, A., de Bentzmann, S., and Bordi, C. (2012) A bacterial two-hybrid genome fragment library for deciphering regulatory networks of the opportunistic pathogen *Pseudomonas aeruginosa*. *Microbiology* **158,** 1964–1971
13. Bhuwan, M., Lee, H.-J., Peng, H.-L., and Chang, H.-Y. (2012) Histidine-containing phosphotransfer protein-B (HptB) regulates swarming motility through partner-switching system in *Pseudomonas aeruginosa* PAO1 strain. *J. Biol. Chem.* **287,** 1903–1914
14. Roy, A., Kucukural, A., and Zhang, Y. (2010) I-TASSER: a unified platform for automated protein structure and function prediction. *Nat. Protoc.* **5,** 725–738
15. Becker, G., Klauck, E., and Hengge-Aronis, R. (2000) The response regulator RssB, a recognition factor for σS proteolysis in *Escherichia coli*, can act like an anti-σS factor. *Mol. Microbiol.* **35,** 657–666
16. Mao, X.-M., Ren, N.-N., Sun, N., Wang, F., Zhou, R.-C., Tang, Y., and Li, Y.-Q. (2014) Proteasome involvement in a complex cascade mediating SigT degradation during differentiation of *Streptomyces coelicolor*. *FEBS Lett.* **588,** 608–613
17. Barembruch, C., and Hengge, R. (2007) Cellular levels and activity of the flagellar σ factor FliA of *Escherichia coli* are controlled by FlgM-modulated proteolysis. *Mol. Microbiol.* **65,** 76–89
18. Österberg, S., del Peso-Santos, T., and Shingler, V. (2011) Regulation of alternative σ factor use. *Annu. Rev. Microbiol.* **65,** 37–55
19. Fiebig, A., Herrou, J., Willett, J., and Crosson, S. (2015) General stress signaling in the Alphaproteobacteria. *Annu. Rev. Genet.* **49,** 603–625
20. Francez-Charlot, A., Kaczmarczyk, A., Fischer, H.-M., and Vorholt, J. A. (2015) The general stress response in Alphaproteobacteria. *Trends Microbiol.* **23,** 164–171
21. Herrou, J., Willett, J. W., and Crosson, S. (2015) Structured and dynamic disordered domains regulate the activity of a multifunctional anti-σ factor. *mBio* **6,** e00910–15
22. Miller, J. H. (1972) *Experiments in Molecular Genetics*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
23. Bordi, C., Iobbi-Nivol, C., Méjean, V., and Patte, J.-C. (2003) Effects of ISSo2 insertions in structural and regulatory genes of the trimethylamine oxide reductase of *Shewanella oneidensis*. *J. Bacteriol.* **185,** 2042–2045
24. Herrero, M., de Lorenzo, V., and Timmis, K. N. (1990) Transposon vectors containing non-antibiotic resistance selection markers for cloning and

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stable chromosomal insertion of foreign genes in Gram-negative bacteria. *J. Bacteriol.* **172,** 6557–6567
25. Baraquet, C., Théraulaz, L., Iobbi-Nivol, C., Méjean, V., and Jourlin-Castelli, C. (2009) Unexpected chemoreceptors mediate energy taxis towards electron acceptors in *Shewanella oneidensis*. *Mol. Microbiol.* **73,** 278–290
26. Lemaire, O. N., Honoré, F. A., Jourlin-Castelli, C., Méjean, V., Fons, M., and Iobbi-Nivol, C. (2016) Efficient respiration on TMAO requires TorD and TorE auxiliary proteins in *Shewanella oneidensis*. *Res. Microbiol.* **167,** 630–637
27. Thibodeau, S. A., Fang, R., and Joung, J. K. (2004) High-throughput β-galactosidase assay for bacterial cell-based reporter systems. *BioTechniques* **36,** 410–415
28. Dereeper, A., Guignon, V., Blanc, G., Audic, S., Buffet, S., Chevenet, F., Dufayard, J.-F., Guindon, S., Lefort, V., Lescot, M., Claverie, J.-M., and Gascuel, O. (2008) Phylogeny.fr: robust phylogenetic analysis for the non-specialist. *Nucleic Acids Res.* **36,** W465–W469
29. Drummond, A. J., Suchard, M. A., Xie, D., and Rambaut, A. (2012) Bayesian phylogenetics with BEAUti and the BEAST 1.7. *Mol. Biol. Evol.* **29,** 1969–1973
30. Vallenet, D., Calteau, A., Cruveiller, S., Gachet, M., Lajus, A., Josso, A., Mercier, J., Renaux, A., Rollin, J., Rouy, Z., Roche, D., Scarpelli, C., and Médigue, C. (2017) MicroScope in 2017: an expanding and evolving integrated resource for community expertise of microbial genomes. *Nucleic Acids Res.* **45,** D517–D528
31. Kanehisa, M. (2000) *Post-genome Informatics*, Oxford University Press, Oxford, UK
32. Jones, D. T. (1999) Protein secondary structure prediction based on position-specific scoring matrices. *J. Mol. Biol.* **292,** 195–202