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In depth analysis of the mechanism of action of metal-dependent sigma factors: characterization of CorE2 from *Myxococcus xanthus*

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ABSTRACT

Extracytoplasmic function sigma factors represent the third pillar of signal-transduction mechanisms in bacteria. The variety of stimuli they recognize and mechanisms of action they use have allowed their classification into more than 50 groups. We have characterized CorE2 from *Myxococcus xanthus*, which belongs to group ECF44 and upregulates the expression of two genes when it is activated by cadmium and zinc. Sigma factors of this group contain a Cys-rich domain (CRD) at the C terminus which is essential for detecting metals. Point mutations at the six Cys residues of the CRD have revealed the contribution of each residue to CorE2 activity. Some of them are essential, while others are either dispensable or their mutations only slightly affect the activity of the protein. However, importantly, mutation of Cys174 completely shifts the specificity of CorE2 from cadmium to copper, indicating that the Cys arrangement of the CRD determines the metal specificity. Moreover, the conserved CxC motif located between the σ2 domain and the σ4.2 region has also been found to be essential for activity. The results presented here contribute to our understanding of the mechanism of action of metal-dependent sigma factors and help to define new common features of the members of this group of regulators.

INTRODUCTION

Myxococcus xanthus is a soil δ-proteobacterium of the group of myxobacteria used as a model to study multicellular behavior and differentiation due to its unique and complex life cycle. *M. xanthus* cells feed as coordinated groups until nutrients are depleted. Upon starvation they initiate a developmental program, during which cells must produce and respond to several signals in order to aggregate and differentiate into myxospores, which are resistant to a variety of adverse conditions (1,2).

Bacteria adapt to environmental changes by using a large number of signal-transduction systems which connect extracellular inputs with the appropriate cellular responses. There are three main common and universally present signal-transduction mechanisms in bacteria: one- and two-component systems, and the extracytoplasmic function (ECF) sigma factors (3–6). Moreover, there is a fourth signal-transduction system less widespread among prokaryotes which involves Ser/Thr protein kinases and phosphatases (7,8).

ECF sigma factors belong to group 4 of the σ^{70} family of sigma factors (9). Members of this group are small proteins that contain only two of the four conserved domains found in sigma factors of groups 1 and 2, the σ2 and the σ4 domains. The σ2 domain is essential for recognition of the −10 promoter sequences and coupling with the RNA polymerase core enzyme, while the σ4.2 region (included in the σ4 domain) is required for recognition of the −35 promoter regions (10). ECF sigma factors are abundant and diverse in bacterial genomes, especially in those with a complex life cycle (11). Many ECF sigma factors function with a cognate anti-sigma factor. Anti-sigma factors are usually membrane-anchored proteins, co-expressed with their cognate sigma factor, which contain the sensor domains of these signal-transduction systems. In absence of the right environmental stimulus, anti-sigma factors sequester their sigma factors in the membrane and block the expression of specific genes. When anti-sigma factors do detect these external signals, sigma factors are released, recruiting the RNA polymerase core enzyme and binding to DNA to initiate transcription of the genes required to respond to stimuli (6,12–14).

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The mechanism of activation of ECF sigma factors, together with their sequence similarities, has allowed the classification of these transcriptional regulators into more than 50 groups (13). Even though the mechanism described above is the main mode of activation of ECF sigma factors, three other mechanisms have been reported for these regulators, in which anti-sigma factors do not participate. One of these other mechanisms is used by groups ECF32 and ECF39, which consists of direct transcription of the sigma factor (15,16). A hypothetical phosphorelay involving a Ser/Thr protein kinase co-transcribed with the sigma factor has been postulated for groups ECF43 and ECFSTK1–4 (5,17). Finally, some ECF sigma factors contain a C-terminal extension responsible for the modulation of their own activity. To date only four groups have been described with C-terminal extensions: ECF41, ECF42, ECF01-Gob and ECF44 (5,6,17,18).

Myxococcus xanthus CorE is the founding member and the only characterized sigma factor of the group ECF44. This sigma factor confers copper resistance to *M. xanthus* by regulating the expression of the P_{1B}-type ATPases CopA and CopB, and the multicopper oxidase CuoB (14,19–21). In contrast to most ECF sigma factors, CorE only partially regulates its own expression, and its activation state does not depend on an anti-sigma factor. CorE-regulated genes show a peak of expression at 2 h after copper addition that rapidly decreases due to CorE inactivation. It has been proposed that Cu(II) activates CorE, allowing DNA-binding, whereas Cu(I) inactivates the sigma factor preventing DNA binding. A conserved C-terminal Cys-rich domain (CRD) with 38 residues in CorE controls the activation and inactivation mediated by copper of this ECF sigma factor. Point mutations at each Cys residue of the CRD have revealed that certain key residues play a role in CorE activation and/or inactivation (14).

We have identified a second member of the ECF44 group in the *M. xanthus* genome, which has been named *corE2* (*MXAN_5263*). In the present work we dissect the mechanism of action of CorE2, which is activated by cadmium and zinc, and regulates the expression of at least a cation efflux pump and a glyoxal oxidase and Kelch domain containing protein. We have compared the metal responses and specificities of CorE and CorE2, and have performed an in-depth investigation of their CRD Cys arrangement to understand their differences. We have found that a change in just one Cys residue of the CRD of CorE2 shifts the response of the sigma factor from cadmium to copper. Furthermore, we have demonstrated that a CxC motif located between the σ2 domain and the σ4.2 region, conserved in all the CorE-like ECF sigma factors, is essential for the activity of CorE2.

MATERIALS AND METHODS

Bacterial strains, plasmids and growth conditions

Genotypes of *M. xanthus* and *Escherichia coli* strains, plasmids and oligonucleotides used in this study are listed in Supplementary Tables S1, 2 and 3, respectively. *E. coli* strains were grown in lysogenic broth (LB) (22) at 37°C. Agar plates contained 1.5% Bacto-agar (Difco), which were supplemented with 40 μg/ml X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside), kanamycin (25 μg/ml) and/or tetracycline (25 μg/ml) when necessary. *M. xanthus* strains were grown in CTT medium (23) at 30°C with vigorous shaking (300 rpm). CTT agar plates (1.5% agar) were supplemented with X-gal (100 μg/ml), galactose (10 mg/ml), kanamycin (80 μg/ml) and/or tetracycline (15 μg/ml). When needed, different metals were also added to the medium at the concentrations indicated in each figure.

To induce development, starvation medium CF (23) was used. Cells exponentially growing to approximately 3.0×10^8 cells/ml (optical density at 600 nm [OD_{600}] of 1) were concentrated and resuspended to an OD_{600} of 15 in TM buffer (10 mM Tris–HCl [pH 7.6], 1 mM $MgSO_4$). Ten microliter drops were spotted onto CF agar plates supplemented with the metals indicated in the figures and/or X-gal (100 μg/ml) and incubated at 30°C. Fruiting bodies were observed with an Olympus dissecting microscope.

Nucleic acid manipulations

Routine molecular biology techniques were used for nucleic acid manipulations (22). The various plasmids were introduced into *E. coli* by heat-shock transformation and into *M. xanthus* by electroporation (24). Total RNA was extracted from *M. xanthus* with the High Pure RNA Isolation kit provided by Roche. Samples were then treated with DNAse I (Sigma) to ensure removal of chromosomal DNA. Complementary DNA (cDNA) was obtained by reverse transcription (SuperScript III reverse transcriptase, Life Technologies) from the RNA template using the primer 65RT, which anneals to the gene *MXAN_5265* (Supplementary Table S3). A polymerase chain reaction (PCR) was then performed with the primers listed in Supplementary Table S3, using total RNA or cDNA as a template.

Construction of in-frame deletion mutants

The in-frame deletion mutants used in this study were obtained as previously reported (21). To generate the corresponding plasmids (listed in Supplementary Table S2), sequences upstream and downstream of the *M. xanthus* regions to be deleted were amplified by PCR with wild-type (WT) chromosomal DNA as a template, the primers listed in Supplementary Table S3, and the high-fidelity DNA-polymerase PrimeSTAR HS (Takara). The PCR products were digested and ligated to vector pBJ113 (25), which had previously been digested with the same restriction enzymes to obtain the desired plasmids (Supplementary Table S2). The resulting plasmids were introduced into *M. xanthus* strains by electroporation. Kanamycin resistant (Km^R) merodiploids were selected from CTT agar plates supplemented with this antibiotic and analyzed by Southern blot hybridization to corroborate the proper recombination events. Positive strains were grown on CTT agar plates without kanamycin and containing 1% galactose, favoring the loss of the plasmid by a second homologous recombination. Southern blot analysis was used to screen kanamycin-sensitive (Km^S) and galactose-resistant (Gal^R) colonies for the loss of the WT allele.

Construction of strains harboring *lacZ* fusions and β-galactosidase assays

Plasmids harboring *lacZ* fusions (Supplementary Table S2) were constructed as previously reported (20). In summary, to generate the corresponding plasmids, PCR was performed using chromosomal DNA of *M. xanthus* as a template and the primers listed in Supplementary Table S3. PCR products were then digested with their respective restriction enzymes and ligated into vector pKY481 (26) digested with the same enzymes to generate transcriptional *lacZ* fusions. *M. xanthus* strains were electroporated with these plasmids to generate the desired strains. The resultant Km^R recombinant strains (Supplementary Table S1) were confirmed by Southern blot analysis.

For qualitative β-galactosidase activity analyses, cells were concentrated to an OD_{600} of 15 and spotted onto CTT or CF agar plates containing 100 μg/ml X-gal and the additives indicated in each figure. For quantitative analysis, cells grown on CTT liquid medium were concentrated at an OD_{600} of 15 and spotted onto CTT or CF agar plates. β-galactosidase-specific activity was determined in cell extracts obtained at various time points by sonication as previously reported (21), and is expressed as nmol of *o*-nitrophenol produced per min and mg of protein. All extracts were assayed in triplicate, and values were averaged from three independent measurements.

Site-directed mutagenesis

Single amino acid substitutions in CorE and CorE2 were performed using the QuikChange II site-directed mutagenesis kit (Agilent) as recommended by the manufacturer. Plasmids pNG00 and pMT00 (Supplementary Table S2), containing the WT *corE* and *corE2* sequences, respectively, were used as templates. The oligonucleotides used as primers (listed in Supplementary Table S3) were designed using the QuikChange Primer Design Program (http://www.genomics.agilent.com/). The amplified plasmids were digested with DpnI and transformed into *E. coli* to obtain the mutant plasmids listed in Supplementary Table S2. All plasmids were sequenced to confirm the presence of the desired mutations and the absence of unwanted mutations, and were introduced by electroporation into *M. xanthus* JM51EBZY (*cuoB-lacZ-*Δ*corE*) for mutations in CorE, and into *M. xanthus* JM52IF3ZY5 (*5265-lacZ-*Δ*corE2*) for point mutations in CorE2. The mutant strains obtained are listed in Supplementary Table S1. Plasmid pMT00 was also electroporated in JM52IF3ZY5 to construct the strain JM52SDM00, which was used as a control for CorE2 mutation analyses. All Tet^R and Km^R recombinants were analyzed by Southern blot. As in previous studies for the CorE mutation, strain JM00BZY was used as a control (14).

Studies on the stability of the proteins harboring point mutations

In order to determine whether the proteins harboring point mutations of CorE and CorE2 were stable in *M. xanthus*, genes encoding them and the WT genes were cloned with an N-terminal S tag and under control of the constitutive *oar* promoter (27). A 781-bp fragment upstream of the *oar* gene (*MXAN_1450*) was amplified by PCR, using the appropriate oligonucleotide pair listed in Supplementary Table S3 as primers. The PCR product was introduced in-frame with the coding sequence of the S tag present in the pRSFDuet-1 vector (Novagen). The resulting plasmid was named pPOar-S and used to amplify the *oar* promoter fused to the S tag. Next, the WT *corE* and *corE2* genes were amplified from the *M. xanthus* chromosomal DNA, while the corresponding plasmids (Supplementary Table S2) were used to amplify *corE* and *corE2* genes harboring point mutations and were cloned in-frame with the S-tagged *oar* promoter in the pBJ113 vector (25). The resulting plasmids were introduced by electroporation into a Δ*corE* (those containing the WT *corE* or point mutations in *corE*) or Δ*corE2* *M. xanthus* strain (those containing the WT *corE2* or point mutations in *corE2*). Several kanamycin-resistant (Km^R) colonies were analyzed by PCR to confirm the proper recombination event. To detect the S-tagged proteins, the different strains were grown for 24 h in CTT supplemented with 200 μM of $ZnNO_3$, after which the cells were harvested in TM buffer containing a protease inhibitor cocktail (Promega) and disrupted by sonication. The sonicated samples were centrifuged and the protein concentration of the supernatants was determined by the Bio-Rad protein assay kit using bovine serum albumin as standard. Proteins were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis and transferred onto a membrane of PVDF. S-tagged proteins were detected using an S-protein HRP Conjugate (Novagen) antibody, which is conjugated with horseradish peroxidase, using 1-Step Ultra TMB Blotting Solution (Pierce) as the substrate, following the instructions specified by the manufacturer.

Bioinformatic analysis

The list of CorE-like ECF sigma factors was updated, as previously reported (14), by BLASTP analysis of all genome and protein sequences deposited in the database of the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/genome/browse/). Protein sequence alignments were performed using the ClustalW program (28), and a graphic representation of the results was generated with ESPript.cgi Version 3.0 (29) (http://espript.ibcp.fr/ESPript/cgi-bin/ESPript.cgi). The domain architecture of proteins was analyzed against the Pfam database (30).

RESULTS

CorE-like ECF sigma factors in myxobacteria

A BLASTP analysis in search of CorE-like ECF sigma factors resulted in the identification of 67 of these regulators in bacteria, 17 of which are found in species of the order *Myxococcales* (Supplementary Figure S1). Interestingly, all the myxobacterial genomes so far sequenced encode at least one CorE-like sigma factor, with the exception of several species of the genus *Anaeromyxobacter* (where one is only found in the strain Fw109) and *Haliangium ochraceum*. The *Myxococcus stipitatus* genome harbors three sigma factors of this type (Supplementary Figure S1). In the case of *M. xanthus*, in addition to two complete CorE-like sigma factors,

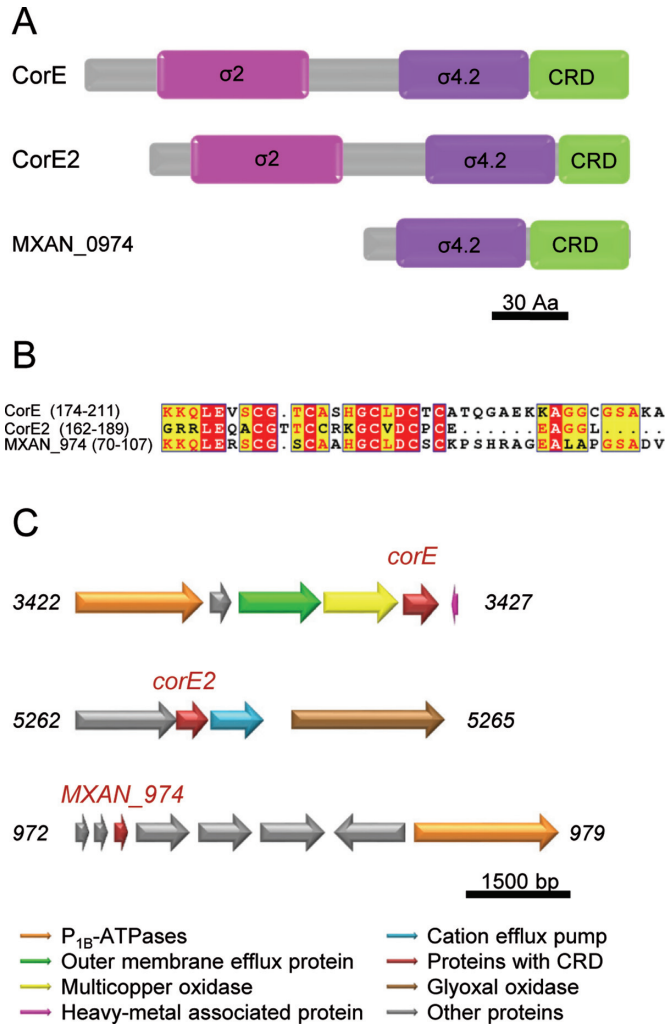

Figure 1. The *Myxococcus xanthus* genome contains three genes with conserved C-terminal CRD domains. (**A**) Domain architecture of the three proteins with a CRD domain. CorE and CorE2 have the σ2 domain (Sigma70_r2, PF04542) and the σ4.2 region (sigma70_r4_2, PF08281) typical of ECF sigma factors. MXAN_0974 shows sequence similarities of 40.4% with CorE and 36.5% with CorE2 within the σ4.2 region. (**B**) Sequence alignment of the three CRDs showing the conserved Cys residues. Numbers indicate the position of the first and last residue of the sequences shown for each protein. Identical residues in two proteins are written in red and highlighted in yellow, and those that are identical in the three proteins are written in white and highlighted in red. (**C**) Genetic environment of the three genes with a CRD (represented in red). Genes of these regions that encode proteins associated with metals are drawn following the color code indicated at the bottom of the panel.

a third gene (*MXAN_0974*) is present which encodes a protein with a region resembling that of σ4.2 of sigma factors and with a CRD (Figure 1A and B). However, this protein is not expected to function as an ECF sigma factor because it lacks the σ2 domain. The paralog of CorE found in *M. xanthus* has been designated as CorE2 (MXAN_5263), and in this report we have focused on its characterization. Analysis of the genetic context of *corE2* has revealed a gene upstream encoding a hypothetical lipoprotein (*MXAN_5262*) which has been predicted to be the cognate anti-sigma factor of CorE2 (12). Moreover, downstream of *corE2* there are two genes which encode proteins associated with metals. MXAN_5264 (with Pfam PF01545) is similar to cation efflux systems, such as CzcD of *Cupriavidus metalidurans* (31), while MXAN_5265 corresponds to a metalloenzyme with a Kelch domain and a glyoxal oxidase domain (PF07250 and PF01344, respectively), which resembles the developmental protein FbfB of *Stigmatella aurantiaca* (32) (Figure 1C). Genes encoding proteins involved in metal homeostasis and detoxification are also found in the proximity of *corE* and *MXAN_0974* (Figure 1C). This observation, along with the presence of a CRD, suggests that CorE2, like CorE, might be metal-responsive.

Genes of the *corE2* region, but not *corE2*, are upregulated by metals

To analyze the metal response of genes in the *corE2* region, four genes (*MXAN_5262*, *corE2*, *MXAN_5264* and *MXAN_5265*) were tested for metal regulation. Plasmids containing transcriptional fusions between these four genes and *E. coli lacZ* (Figure 2A) were electroporated into the WT strain of *M. xanthus*. The resulting Km^R strains JM52ZY2 (*5262-lacZ*), JM52ZY3 (*corE2-lacZ*), JM52ZY4 (*5264-lacZ*) and JM52ZY5 (*5265-lacZ*) (Supplementary Table S1) were confirmed by Southern blot. The strains containing the fusions, and the WT strain as a negative control, were spotted onto CTT (growth) and CF (development) agar plates containing 100 μg/ml of X-gal and several metals to qualitatively screen for gene expression. Surprisingly, none of the four genes was upregulated by copper (Figure 2B), as happened with CorE-regulated genes (14). However, two genes downstream of *corE2* were upregulated in the presence of cadmium and zinc (Figure 2B). Both genes exhibit an expression profile with cadmium in which a plateau is reached several hours after addition of the metal (Figure 2C). In contrast, the expression of these two genes rapidly increases after the addition of zinc, exhibiting a maximum at 2 h. Thereafter the expression levels slightly decrease, remaining quite high for an extended period (Figure 2D). None of the metals tested was able to affect *corE2* and *MXAN_5262* expression (Figure 2B). However, an upregulation of the four genes was observed during development in the absence of metals (Figure 2E), although at different levels (Figure 2F), indicating regulation of all these genes during fruiting-body formation.

Due to their proximity and orientation, and the dual expression profile of the genes in the cluster, co-expression of these four genes was examined under two different conditions: growth on CTT agar plates with 0.1 mM cadmium and development on CF agar plates with no metals added. Total RNA was extracted after 48 h of incubation under these two conditions, and it was used as a template for synthesis of cDNA using primer 65RT (Supplementary Figure S2). Using the two cDNAs as templates and the strategy depicted in Supplementary Figure S2A, it was found that two differentially regulated operons are found in the *corE2* region, one containing the four genes (*MXAN_5262* through *MXAN_5265*), which is expressed during development (Supplementary Figure S2B), and a second one, containing only two genes (*MXAN_5264* and *MXAN_5265*), which is upregulated by cadmium (Supplementary Figure

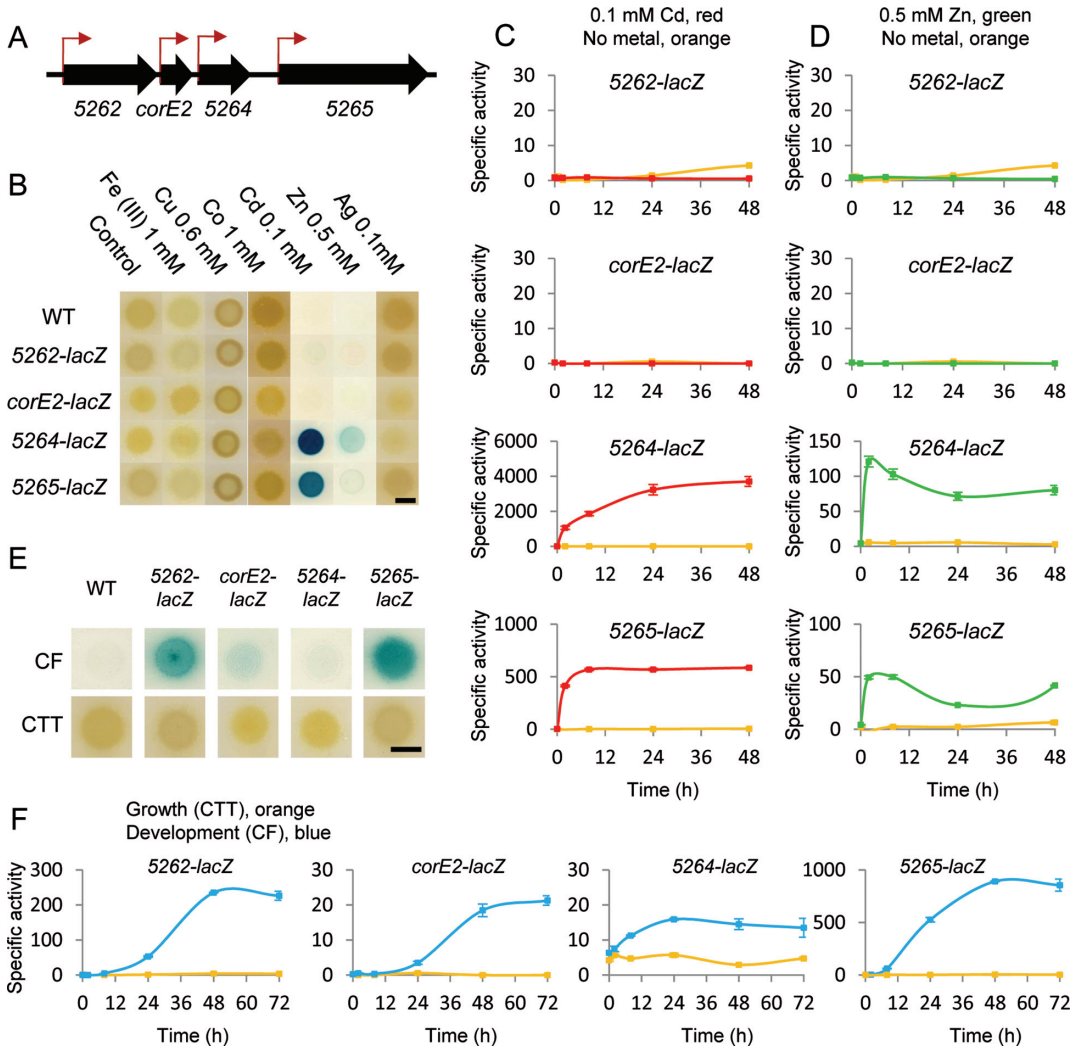

Figure 2. Expression of genes located in the proximity of *corE2*. (**A**) Schematic representation of the *corE2* context. Red arrows indicate *Escherichia coli lacZ* transcriptional fusions. (**B**) Qualitative analysis of *lacZ* fusions for genes of the *corE2* region in the presence of various metals. Cells harboring fusions between genes of the *corE2* cluster and *lacZ* were inoculated on CTT medium (growth) containing X-gal and different metals. Pictures were taken after 96 h of incubation. (**C** and **D**) Quantification of β-galactosidase-specific activity of the four fusions with *lacZ*. Cells were grown on CTT without metals (orange lines) or containing either 0.1 mM cadmium (C, red lines) or 0.5 mM zinc (D, green lines), and samples were harvested at the time points indicated in each figure. Specific activity was determined as indicated in 'Materials and Methods' section. (**E**) Qualitative analysis of gene expression of the *corE2* cluster during development. Cells were spotted onto both CTT agar plates (growth) and CF agar plates (development) containing X-gal and no metals. Pictures were taken after 96 h of incubation. Bar in panels B and E represents 0.5 mm. (**F**) Quantification of β-galactosidase-specific activity during growth (orange lines) and development (blue lines) in the absence of metals. Cells of each strain were spotted onto CF agar plates, and at the time points indicated in the figures they were harvested and analyzed for β-galactosidase activity. Error bars in panels C, D and F indicate standard deviations. Extracts were assayed in triplicate, and values were averaged from three independent measurements. Please note that the graphs have different scales.

S2C). These data are in good agreement with the data presented in Figure 2.

The mutant Δ*corE2* is more sensitive to cadmium and zinc than the WT strain, and exhibits delay in development

To determine whether CorE2 is involved in metal detoxification, an in-frame deletion mutant (Δ*corE2*) was constructed, lacking the essential σ2 domain of the sigma factor (Figure 3A), and the phenotype of the mutant was analyzed for metal sensitivity. When the mutant Δ*corE2* and the WT strain were cultured in liquid CTT medium, no significant difference in growth was observed between the two strains when several concentrations of zinc or cadmium were tested (Supplementary Figure S3). Since metal homeostasis mechanisms require preadaptation to be fully active (33), WT and Δ*corE2* strains were first grown for 24 h in CTT medium containing either 10 μM cadmium or 0.2 mM zinc. Cells were then transferred to CTT liquid cultures containing varying concentrations of metals. As shown in Figure 3B, zinc tolerance was reduced in the Δ*corE2* preadapted cells. In contrast, cadmium tolerance was only slightly reduced in the mutant (Figure 3C). These data indicate that CorE2 is involved in conferring resistance to some metals, and suggest that this sigma factor could be regulating the expression of the two genes located downstream of itself, which are upregulated by these two metals.

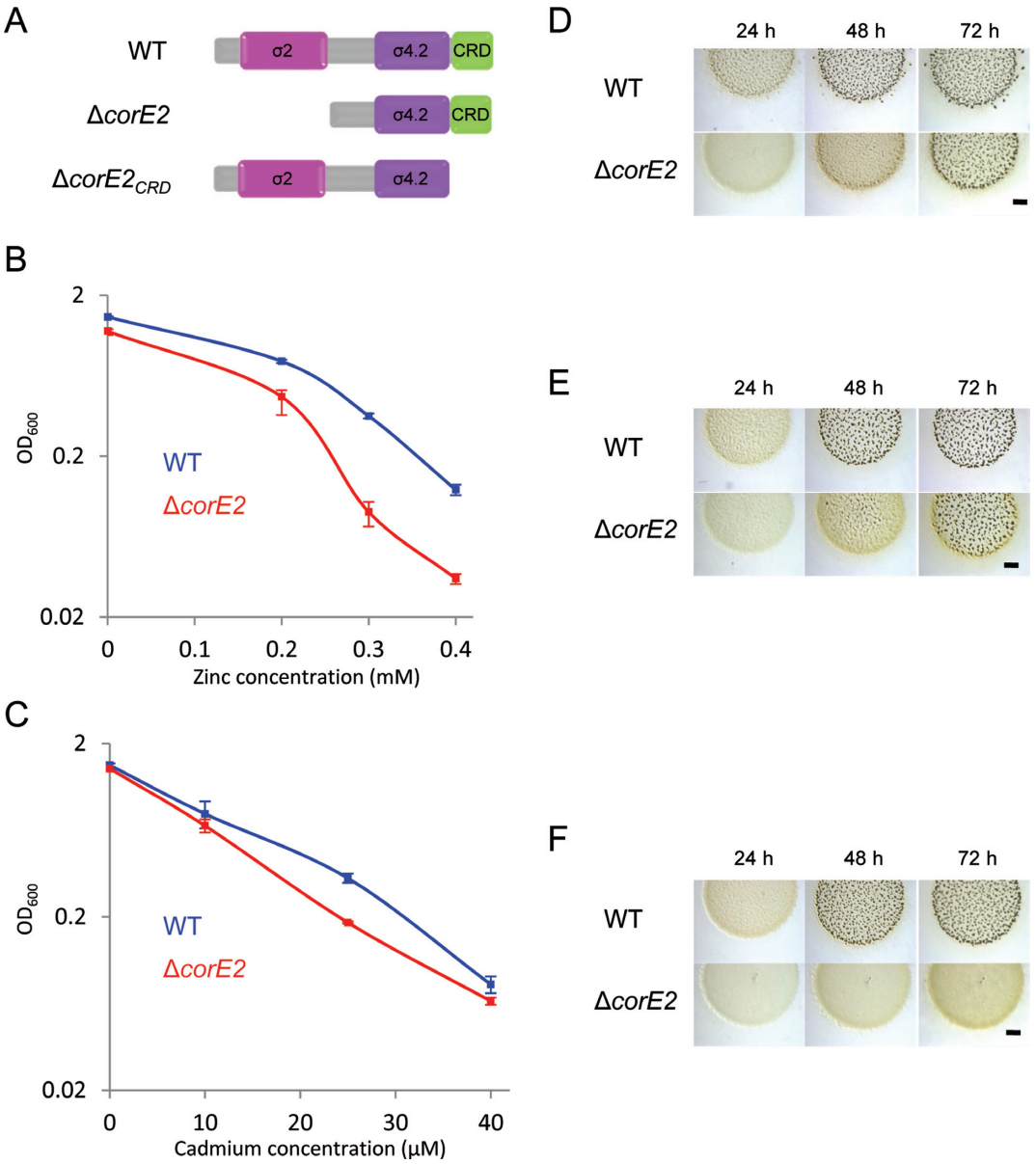

Figure 3. Phenotype of the Δ*corE2* mutant. (**A**) Domains present in CorE2 in the WT strain and in the mutants Δ*corE2* and $\Delta corE2_{CRD}$. (**B**) Cells of the WT strain (blue line) and the Δ*corE2* mutant (red line) were grown for 24 h in CTT containing 0.2 mM zinc and then diluted to an OD_{600} of 0.05 in CTT medium containing 0, 0.2, 0.3 and 0.4 mM zinc. OD_{600} was determined after 32 h of incubation. (**C**) As in panel B, cells were grown for 24 h in 10 μM cadmium and then diluted to an OD_{600} of 0.05 in CTT medium containing 0, 10, 25 and 40 μM cadmium. OD_{600} was determined after 32 h of incubation. Error bars in panels B and C indicate standard deviations. Values are averages of three experiments. (**D**) Fruiting body formation of the WT strain and the Δ*corE2* mutant in the absence of metals. (**E**) Fruiting body formation of the WT strain and the Δ*corE2* mutant on CF medium with 200 μM zinc. (**F**) Fruiting body formation of the WT strain and the Δ*corE2* mutant on CF medium with 20 μM cadmium. Pictures in panels D, E, and F were taken at the time indicated. Bar in panels D, E, and F represents 1 mm.

As the four genes of the operon are induced during development, it was also tested whether the Δ*corE2* mutant exhibits developmental defects. When dropped onto CF starvation medium the Δ*corE2* mutant exhibited a clear delay in development, although normal fruiting bodies were observed at 72 h of incubation (Figure 3D). This delay in development indicates that this sigma factor must be regulating the expression of some genes required for the proper timing of fruiting body formation. When development was analyzed in the presence of zinc, a slightly longer delay in fruiting body formation was observed in both the WT strain and the mutant (Figure 3E). In contrast, fruiting bodies were not observed in the mutant strain spotted on CF medium containing 20 μM cadmium even after 72 h of incubation, while the WT strain developed almost normally at 48 h (Figure 3F). These results corroborate that CorE2 plays a role in metal detoxification as well as in development.

CorE2 is controlling the cadmium- and zinc-dependent expression of genes *MXAN_5264* and *MXAN_5265*

As we have demonstrated, genes *MXAN_5264* and *MXAN_5265* form an operon which is upregulated by cadmium and zinc (Figure 2 and Supplementary Figure S2). To elucidate whether CorE2 is responsible for the metal-dependent expression of this operon, the Δ*corE2* mutant was used as genetic background to introduce the transcriptional fusion *5265-lacZ*, to obtain the strain JM52IF3ZY5 (*5265-lacZ*-Δ*corE2*). This strain was used to compare the upregulation of the gene *MXAN_5265* by cadmium and zinc to the WT strain. As shown in Figure 4A and B, lack of a functional CorE2 results in the absence of both cadmium- and zinc-dependent expression of the gene *MXAN_5265*, demonstrating that the metal induction of this operon is controlled by this sigma factor. It remains to be elucidated why the phenotype of the Δ*corE2* mutant during growth is more dramatic with zinc than with cadmium (Figure 3), whereas genes regulated by this sigma factor exhibit higher expression levels with cadmium (Figure 2). However, one explanation could be that the proteins responsible for metal detoxification regulated by CorE2 exhibit a higher affinity for zinc than for cadmium.

As the four genes are induced during development in the absence of metal, it was also tested whether CorE2 was also responsible for this expression. The strains *5265-lacZ*-Δ*corE2* and the WT harboring the same *lacZ* fusion were plated onto CF agar without metals. As shown in Figure 4C, CorE2 is not responsible for the expression of gene *MXAN_5265* during development. This result supports the notion of a complex regulation of this operon, with several transcriptional regulators involved.

Investigating the mode of action of CorE-like ECF sigma factors

In this section, the mechanism of action of CorE2 will be compared with that reported for CorE, in order to identify common features for the whole ECF44 group of sigma factors and also the peculiarities of each particular regulator.

CorE2 does not regulate its own expression. As shown above (Figure 2 and Supplementary Figure S2), *corE2* is part of an operon that is only induced during development, but not by metals. Moreover, the expression of the gene *MXAN_5265* during development, representing the operon, does not depend on CorE2, indicating that CorE2 does not regulate its own expression, in contrast to what has been reported for other ECF sigma factors, including CorE (9,14). However, due to the low expression levels of *corE2*, β-galactosidase activity was directly quantified in the strain JM52ZY3 (*corE2-lacZ*) during development, both in the presence and in the absence of 7.5 μM cadmium. As shown in Figure 5A, *corE2* expression is not upregulated by cadmium, as no significant difference between the two conditions was observed. Furthermore, to support these data, the strain JM52IF3ZY3 (*corE2-lacZ*-Δ*corE2*) was constructed introducing the *corE2-lacZ* transcriptional fusion into the Δ*corE2* mutant. Quantitative and qualitative analyses of this strain during development with and without cadmium rendered similar expression patterns to those of the WT

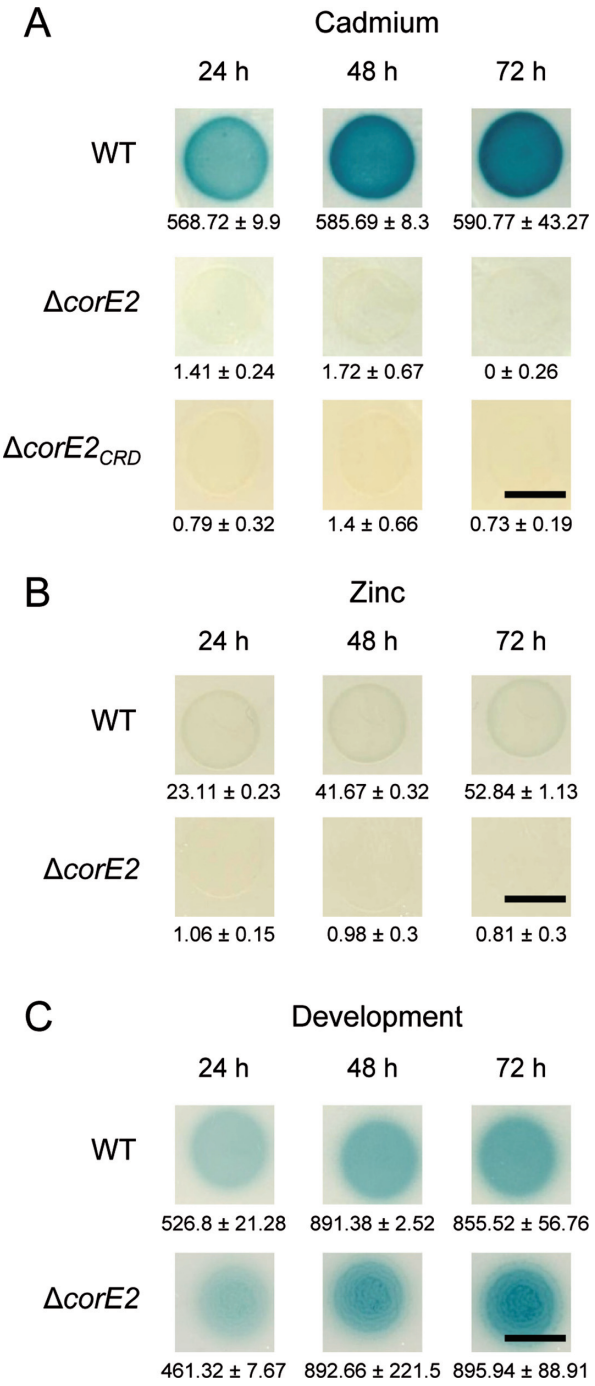

Figure 4. CorE2 regulation of gene expression. (**A** and **B**) Expression of the gene *MXAN_5265* in the WT strain and the mutants Δ*corE2* and Δ$corE2_{CRD}$. The strains were incubated on CTT agar plates containing either 0.1 mM cadmium (A) or 0.5 mM zinc (B), and X-gal to monitor β-galactosidase activity. Pictures were taken at the time points indicated at the top of each picture. Numbers below each picture indicate β-galactosidase-specific activity obtained for each strain. (**C**) Expression of *MXAN_5265* during development in the WT strain and the Δ*corE2* mutant. Cells were spotted onto CF agar plates without metals. As in panels A and B, the time at which each picture was taken is shown above it, and numbers below the pictures indicate β-galactosidase-specific activity at that time point. Values are average of three experiments. Bar represents 0.5 cm.

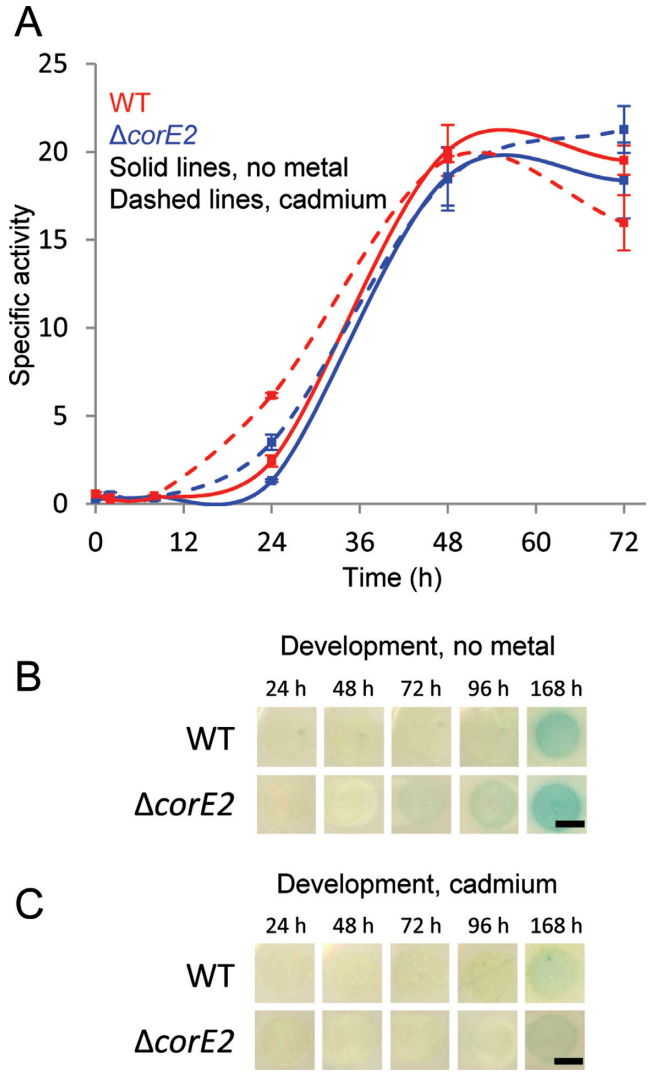

Figure 5. *corE2* auto-regulation. (**A**) Quantitative analysis of β-galactosidase-specific activity of the *corE2-lacZ* fusion in the WT (red line) and the Δ*corE2* (blue line) strains during development without metals (solid lines) and with 7.5 μM cadmium (dashed lines). Experiments were carried out in triplicate, and error bars indicate standard deviations of three measurements. (**B** and **C**) Qualitative analysis of β-galactosidase activity of both strains on CF agar plates with X-gal and no metals (B), and plates supplemented with X-gal and 7.5 μM cadmium (C). Bar represents 0.5 cm.

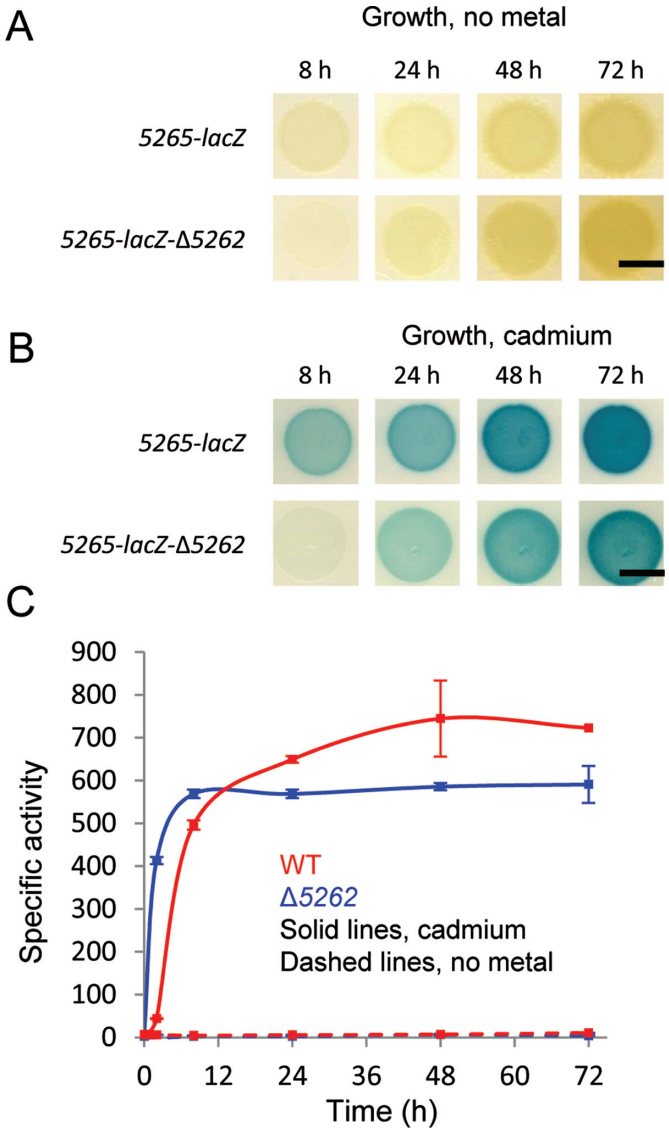

Figure 6. The lipoprotein encoded by the gene *MXAN_5262* does not function as the anti-sigma factor of CorE2. The WT and Δ*5262* strains harboring the *5265-lacZ* fusion were spotted onto CTT agar plates without metals (**A**) and containing 0.1 mM cadmium (**B**). For qualitative assays, plates also contain X-gal to monitor β-galactosidase activity. (**C**) Quantitative analysis of the cadmium-dependent expression of the CorE2 regulated gene *MXAN_5265* in the WT and Δ*5262* genetic backgrounds. The WT strain (blue lines) and the Δ*5262* mutant (red lines) were incubated on CTT agar plates without metals (dashed lines) and containing 0.1 mM cadmium (continuous lines). β-galactosidase-specific activity was determined in cell extracts harvested at the time points indicated in the figure. Values are averages of three experiments. Error bars indicate standard deviations. Bar represents 0.5 cm.

strain (Figure 5). These data all support the conclusion that the expression of *corE2* is not auto-regulated.

MXAN_5262 does not function as an anti-sigma factor for CorE2. Even though it has been postulated that the activity of CorE-like sigma factors is only regulated by their own CRD (13,14), it has been proposed that a membrane lipoprotein encoded by a gene co-expressed with *corE2* might function as an anti-sigma factor for CorE2 (12). If the cognate anti-sigma factor of an ECF sigma factor is knocked out, genes regulated by such an ECF sigma factor would be expressed even in the absence of the stimulus (14). In order to test whether MXAN_5262 acts as the cognate anti-sigma factor for CorE2, a new in-frame deletion strain, JM52IF2 (Δ*5262*), was obtained. Next, the fusion *5265-lacZ* was introduced into this mutant. When β-galactosidase activity was determined in the strain JM52IF2ZY5 (*5265-lacZ*-Δ*5262*), both qualitatively and quantitatively, and compared with that of the WT cells carrying the *5265-lacZ* fusion, it was observed that deletion of *MXAN_5262* does not increase CorE2 activity, even in the absence of cadmium (Figure 6A and C), demonstrating that this membrane protein does not function as a

cognate anti-sigma factor for CorE2. However, deletion of the gene *MXAN_5262* delays the upregulation of the gene *MXAN_5265* in the presence of cadmium (Figure 6B and C), indicating that this lipoprotein must somehow participate in the same signal-transduction pathway as CorE2.

CRD is essential for the activity of all CorE-like ECF sigma factors. As a member of group ECF44, CorE2 contains a CRD consisting of 28 residues (as opposed to the 38 amino acids of the CRD of CorE). It also contains 6 Cys, but in a different arrangement from that of CorE (Figure 1B). To test whether the CorE2 CRD is essential for CorE2 activity, an in-frame deletion mutant, JM52IF3DCRD ($\Delta corE2_{CRD}$), was generated in which most of the CRD was deleted (Figure 3A). This mutant was used to electroporate the fusion *5265-lacZ* to obtain the strain JM52IF3DCRDZY5 (*5265-lacZ*-$\Delta corE2_{CRD}$). Although $CorE2_{\Delta CRD}$ harbors the essential σ2 domain and σ4.2 region of ECF sigma factors, it remains inactive even in the presence of 0.1 mM cadmium, as shown by the lack of β-galactosidase activity in the strain JM52IF3DCRDZY5 (Figure 4A). Since the CRD of CorE is also required for activity (14), it is plausible to postulate that all the sigma factors of the group ECF44 exhibit a similar mechanism of action.

In view of the differences between the Cys arrangement in the CRD domain of CorE2 and CorE, the metal activation by each sigma factor, and the expression profile of genes regulated by each regulator, it is plausible to expect each Cys of the CRD of CorE2 to play a different role in CorE2 activity from the one reported for CorE (14). To analyze which Cys are responsible for CorE2 activity, all six residues were mutated individually to Ala by site-directed mutagenesis (Figure 7A). Each mutated *corE2* was then introduced into the JM52IF3ZY5 strain (*5265-lacZ*-Δ*corE2*) to evaluate the expression profile of the gene *MXAN_5265* in the presence and absence of cadmium. Moreover, the WT *corE2* gene was also electroporated into the strain JM52IF3ZY5 (strain JM52SDM00) to allow a reliable comparison between expression of *MXAN_5265* by the WT protein and the six mutant proteins. Although the strain JM52SDM00 will be considered as a WT in these experiments, it should be remembered that it is not the same WT strain used in the previous experiments, so the results that we may obtain with JM52SDM00 will not necessarily be identical to those shown in Figures 2–6. The analysis revealed that the expression profile in the mutant C183A was nearly identical to that of the WT *corE2'* strain (Figure 7B), indicating that this residue is not crucial for CorE2 activity. Some upregulation by cadmium still remained in the C173A and C181A mutants. However, the maximum expression levels achieved in these two mutants were only about 15% of that of the WT strain (Figure 7C). These results indicate that Cys173 and 181 are important, although not essential, for CorE2 activation by cadmium. Interestingly, when Cys174 was mutated to Ala, there was a constitutive expression of *MXAN_5265* in the absence of cadmium, which did not significantly change in the presence of this metal (Figure 7D). These data indicate that Cys174 is involved in both activation of CorE2 in the presence of cadmium and inactivation when the metal is absent. Interestingly, Cys169 and Cys178 were revealed to be essential for CorE2 activity, because no expression was observed in both mutants even in the presence of cadmium (Figure 7E).

To rule out the possibility that the lack of activity of some point-mutated proteins might be due to instability, all the mutated genes were cloned under control of the constitutive *oar* promoter and fused at the N-terminal region to an S tag, as described in 'Materials and Methods' section. Plasmids containing the hybrid genes were electroporated into *M. xanthus* Δ*corE2*, and the different strains thus obtained (Supplementary Table S1) were analyzed to investigate whether the mutated proteins are produced and soluble. The results obtained have revealed that all the CorE2 point-mutated proteins are stable (Supplementary Figure S4).

Cys arrangement in the CRD of metal-dependent sigma factors determines the metal specificity. As CRDs have been postulated to be the metal-recognition site of CorE-like ECF sigma factors (14 and data shown above), differences between the CRDs of CorE and CorE2 were analyzed for residues that might be responsible for recognition of copper by CorE and of cadmium by CorE2. Even though the Cys motif is well conserved within the ECF44 group of sigma factors, there is one Cys (Cys174 in CorE2) that represents a key difference between the two characterized sigma factors within this group. The CRD of CorE lacks this Cys and an Ala is found in this position (Figures 1B and 8A). As shown above, cadmium recognition was impaired in the CorE2 C174A mutant (Figure 7D), so it was plausible to think that this mutant sigma factor might respond to copper, in the same way as CorE. To test this possibility, the CorE2 C174A mutant was assayed for copper upregulation of the gene *MXAN_5265*. Interestingly, the mutant C174A, which does not respond to cadmium, exhibits a change in metal specificity, and the expression of *MXAN_5265* reaches quite high levels in the presence of copper (Figure 8B). When other metals were tested, it was observed that this mutated CorE2 is only activated by copper (Supplementary Figure S5A). It remains to be elucidated whether CorE2 C174A responds to Cu(I) or Cu(II). According to these data, a change of just one residue in the CRD of CorE2 is sufficient to completely shift the metal specificity of the sigma factor from cadmium to copper.

To learn more about the significance of the residue located in position 174 in CorE2 in metal recognition, CorE CRD was also mutated, in this case to replace Ala185 with a Cys, in order to make the CorE CRD more similar to that of CorE2 (Figures 1B and 8A). As shown in Figure 8C, the CorE A185C mutant was not significantly impaired in terms of copper induction of *cuoB* (one of the genes regulated by this sigma factor). However, upregulation of *cuoB* expression in the presence of cadmium was three times higher in the mutant A185C than in the WT strain (compare continuous lines in Figure 8C), indicating that this mutant likely binds both metals. The effect observed with cadmium was not obtained with any of the other metals tested (Supplementary Figure S5B). As the mutated CorE A185C is also stable (Supplementary Figure S4B), these results confirm that differences in only one residue in the Cys arrangement of the CRD of sigma factors of the group ECF44 af-

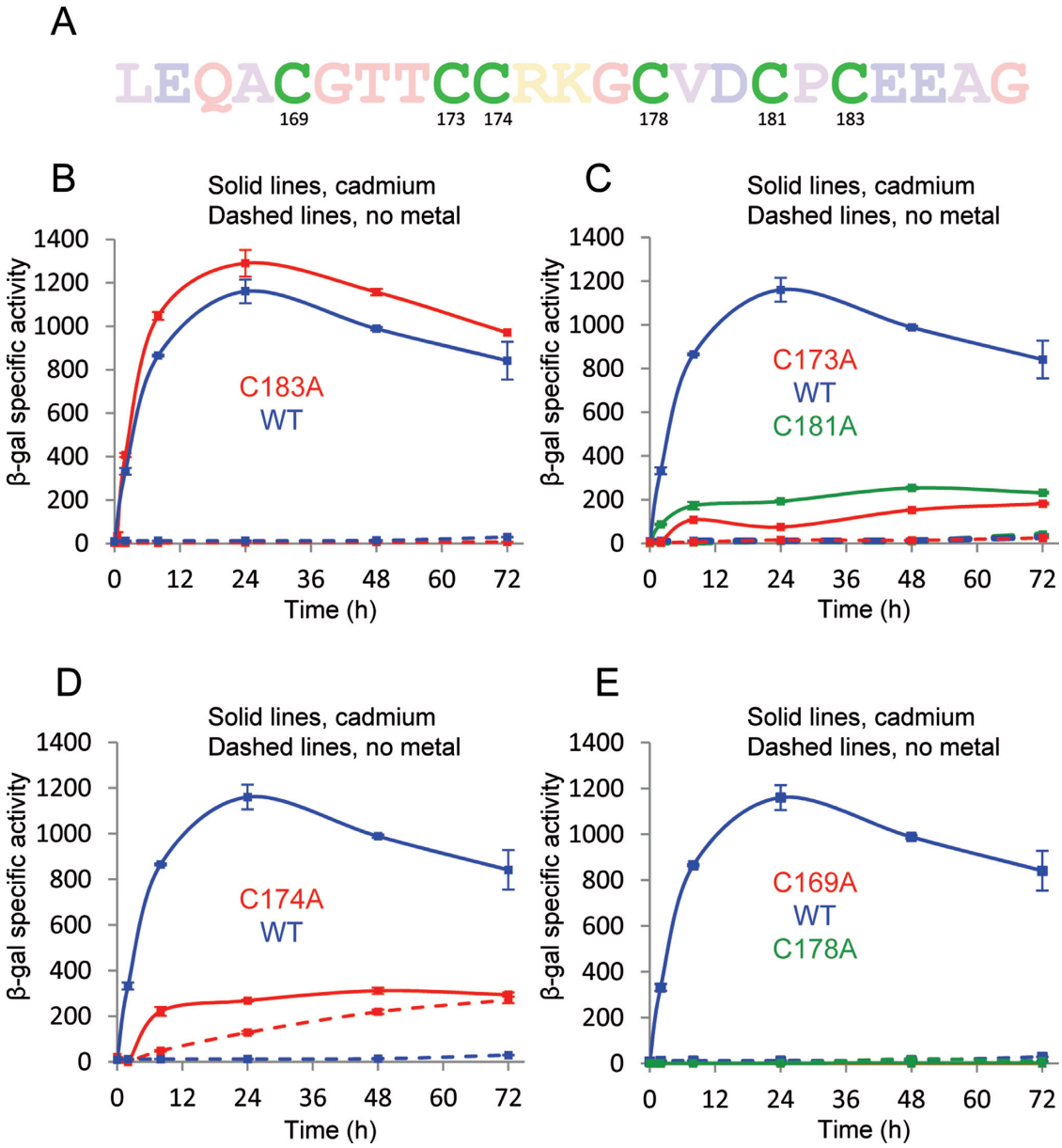

Figure 7. Analysis of the CRD of CorE2. (**A**) Sequence of CorE2 CRD, highlighting the Cys subjected to site-directed mutagenesis. (**B**–**E**). Expression analysis of the gene *MXAN_5265* in the Cys-to-Ala mutants (red or green lines, indicated in each panel) and the WT strain (blue lines). Cell extracts were harvested at the time points indicated in the figures from CTT agar plates without metals (dashed lines) and with 0.1 mM cadmium (continuous lines). β-galactosidase-specific activity was determined as indicated in 'Materials and Methods' section. The residue mutated is indicated in each panel with the same color code as the line for β-galactosidase-specific activity. Values are the averages of three independent experiments and error bars indicate standard deviations.

fect metal recognition and/or responsiveness of this RNA polymerase subunit.

A CxC conserved motif located between the σ2 domain and the σ4.2 region is essential for CorE2 activity. When the amino acid sequences of the 67 members of the ECF44 group of sigma factors were aligned, besides the CRD that is present in all members, an additional conserved CxC motif was found located between the σ2 domain and the σ4.2 region (Supplementary Figure S1). As Cys have been demonstrated to be important for the activity of these sigma factors (14 and Figures 7 and 8B), we determined the role of this conserved motif in CorE2 activation. For this purpose, Cys108 and 110 of CorE2, and Cys120 and 122 of CorE (Figure 9A) were individually mutated to Ala in the same way as reported above for point mutations in the CRD of CorE2. The two mutated *corE2* genes were then introduced into the JM52IF3ZY5 strain to obtain the mutants JM52SDM08 and JM52SDM10. They were then tested for cadmium upregulation of the *MXAN_5265* gene. Similarly, the two mutated *corE* genes were introduced into the strain JM51EBZY to obtain the mutants JM51SDM20 and JM51SDM22, which were also tested for upregulation of *cuoB* by copper. Surprisingly, neither of the mutated sigma

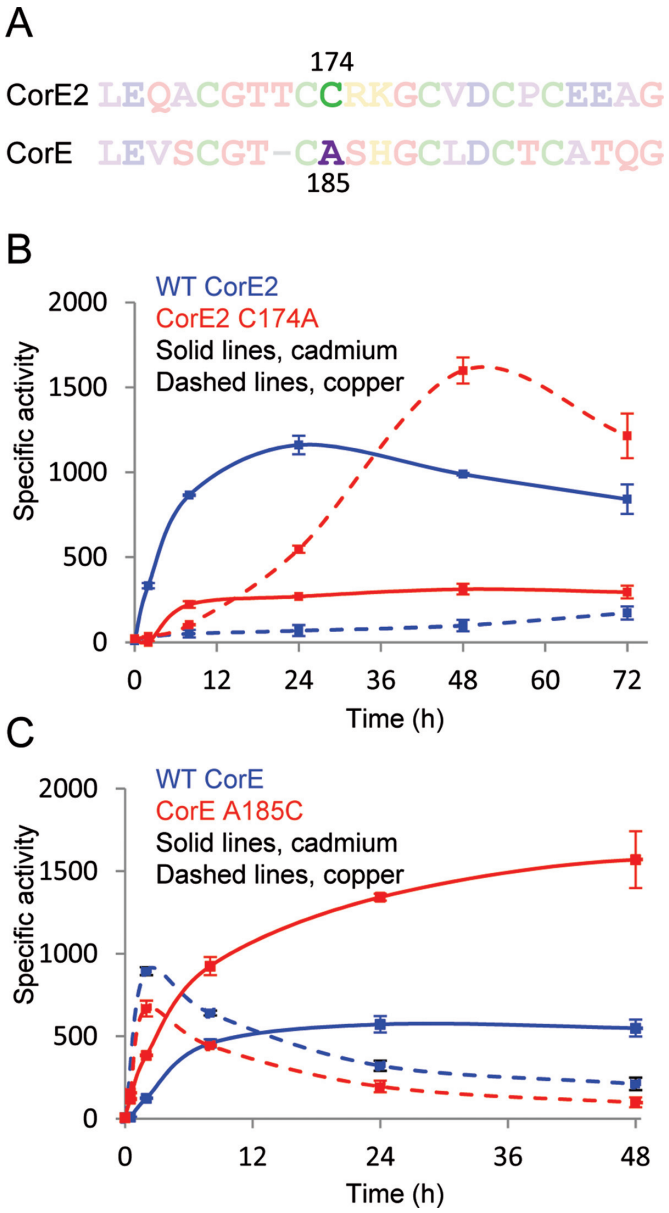

Figure 8. The Cys distribution in the CRD motif of metal-binding sigma factors determines the metal specificity. (**A**) Sequence of CorE and CorE2 CRDs, where Ala185 in CorE and Cys174 in CorE2 are highlighted. (**B**) Upregulation of the gene *MXAN_5265* in the CorE2 WT strain (blue line) and the CorE2 C174A mutant (red line) by cadmium (continuous lines) and copper (dashed lines). (**C**) Upregulation of *cuoB* in the CorE WT strain (blue line) and the CorE A185C mutant (red line) by cadmium (continuous lines) and copper (dashed lines). In panels B and C cells grown on CTT medium containing 0.1 mM cadmium or 0.3 mM copper were harvested at the time points indicated in the figures and their β-galactosidase-specific activity was determined. Experiments were performed in triplicate. Error bars indicate standard deviations.

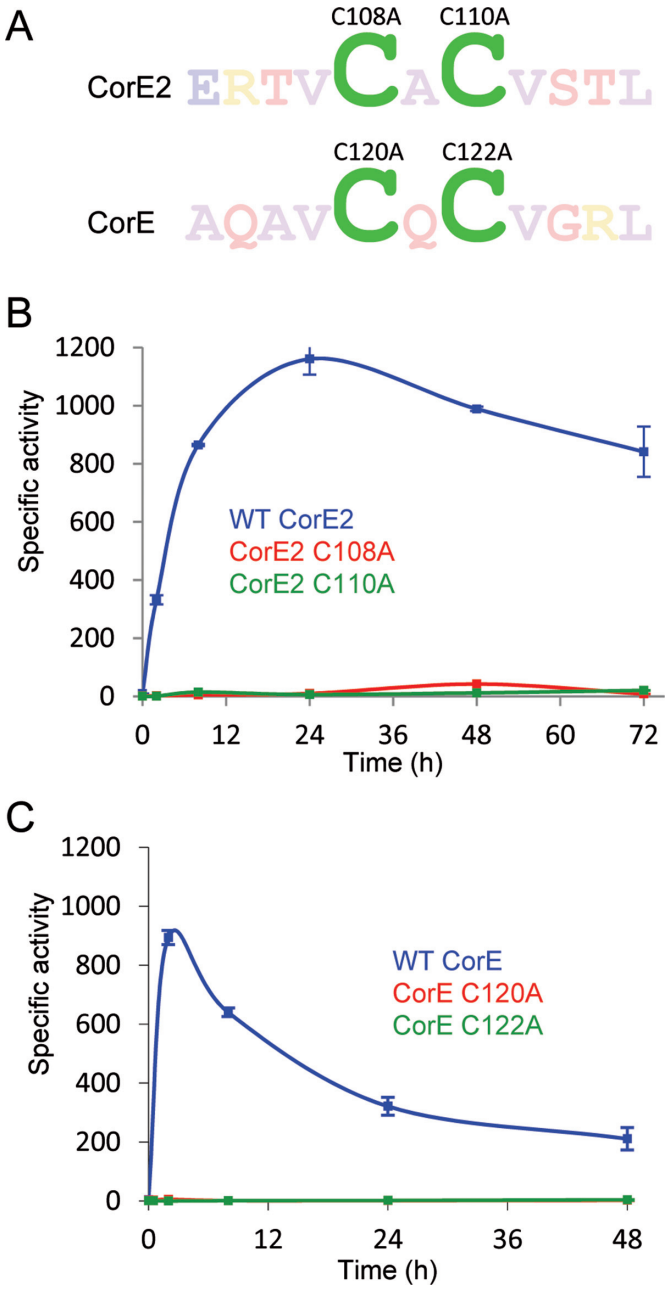

Figure 9. Analysis of the role of the CxC motif in CorE and CorE2 activity. (**A**) Sequence of the CorE2 and CorE CxC motif between the σ2 domain and the σ4.2 region where both Cys are highlighted. (**B**) β-galactosidase-specific activity of the CorE2 WT strain JM52SDM00 (blue line) and mutants JM52SDM08 (mutation C108A, red line) and JM52SDM10 (mutation C110A, green line) grown in CTT medium containing 0.1 mM cadmium. (**C**) β-galactosidase-specific activity of the CorE WT strain JM00BZY (in blue) and mutants JM51SDM20 (mutation C120A, in red) and JM51SDM22 (mutation C122A, in green) grown in CTT medium containing 0.3 mM copper. In panels B and D, cells were grown on CTT medium with metal and harvested at the time points indicated in each figure. β-galactosidase-specific activity was determined in triplicate as indicated in 'Materials and Methods' section. Error bars indicate standard deviations.

factors was able to respond to its corresponding metal, since no β-galactosidase activity was detected in the mutant strains grown in the presence of cadmium in the case of the CorE2 mutants (Figure 9B), or copper in the case of the CorE mutants (Figure 9C). The four mutant proteins at the CxC motif were also tested for stability, and the results obtained revealed that all of them are stable (Sup-

plementary Figure S4). This finding indicates not only that the Cys of the CRD are required for the activity of metal-dependent sigma factors, but also that the CxC motif, found in all members of this group of factors, is essential for this activity.

DISCUSSION

CorE-like sigma factors are a recently discovered group of ECF sigma factors (13), and to date only one member has been characterized (14). In this work we have aimed to explore in more detail the mechanism of action of another CorE-like metal regulator by studying CorE2 from *M. xanthus*.

Comparison of the sequences and genomic context of these two characterized sigma factors with others of the group ECF44 allows us to infer some features that may be common to all these transcriptional regulators. The 67 sigma factors of this group so far identified exhibit a CorE-like architecture, with a highly conserved Cys arrangement located at the C-terminus of the protein which is essential for the activation by metals of both CorE and CorE2. Four Cys in this domain are conserved in nearly all the proteins (Supplementary Figure S1, highlighted in red). However, although one would expect the four Cys to play important roles in protein activity, the results obtained for CorE and CorE2 reveal that point mutations at these four residues alter the activity of the two proteins in differing manners (14, Figure 7), indicating that the presence of other residues in this domain, including other Cys, determines the specific role of each Cys. In the case of CorE and CorE2, it is tempting to speculate that the different roles of these four Cys in each regulator are related to differences in metal recognition. In contrast, Cys189 in CorE (14) and its equivalent in CorE2 Cys178 (Figure 7E) are essential. Interestingly, it is mainly delta-proteobacteria and acidobacteria that conserve a Cys in this position (highlighted in green Supplementary Figure S1), while other groups of bacteria, mostly alfa-proteobacteria, lack that Cys, suggesting that this residue is indispensable for the proper functioning of all ECF44 sigma factors found in delta-proteobacteria and acidobacteria. Another interesting residue is Cys174, which is found in CorE2 and other ECF sigma factors, but not in CorE. As shown in Figure 8, the amino acid found in this position determines the metal specificity of CorE2. To our knowledge, this is the clearest example of a change in a single residue drastically modifying the metal specificity of a protein. Analysis of the sequences of the other sigma factors of group ECF44 (Supplementary Figure S1) reveals that 14 out of the 67 proteins (including CorE2) exhibit a Cys at that position, suggesting that they might also be activated by divalent metals such as cadmium and zinc.

It is known that cells restrict the numbers of metal atoms within the cytoplasm to avoid competition between metals in metalloproteins. To do so, cells have metal-specific transporters and metal sensors that frequently regulate those transporters (34). In the cases of CorE and CorE2, the transporters seem to correspond to the P_{1B}-type ATPases CopA and CopB, and the cation efflux family protein encoded by the gene *MXAN_5264*, respectively (14 and Figures 1C and 4). To maintain the correct proportion of metal, it is crucial that metal sensors correctly distinguish between the inorganic elements in order for metalloproteins to acquire the right metals (35). In fact, one of the ongoing challenges in metal sensor research is to determine which metal (or metals) each homologue detects within its respective organism (36). Usually, the roles of regulators in sensing specific metals are inferred from protein-sequence similarity. This is partially true in our case, because we have found that both CRDs, with very similar sequences and Cys arrangements, are able to recognize different metals. However, our results for the metal specificity of CorE and CorE2 confirm that an accurate and precise analysis of key residues is required. Data obtained with other metalloregulators that use Cys to coordinate metals (such as CueR and GolS, which respond to monovalent metals, and ZntR, which responds to divalent metals) exhibit Cys arrangements that differ from those observed in CorE and CorE2 and from one another (37,38), which supports the notion that the Cys arrangement of a protein determines the metal specificity.

Genes under control of both CorE and CorE2 are upregulated by metals (copper and cadmium, respectively), and some of them are involved in, or resemble others which are involved in, metal homeostasis (14, and Figures 1C and 4). As all the genes known to be regulated by these two sigma factors are located in the same region as the genes for *corE* and *corE2*, the genomic environment surrounding the regions where sigma factors of group ECF44 are encoded in other bacteria was analyzed. As shown in Supplementary Table S4, in 52 out of the 65 sigma factors analyzed (excluding CorE and CorE2), genes involved in metal homeostasis or metal-related functions are found in the proximity of the gene for the ECF sigma factor, indicating that most members of this group might be involved in metal homeostasis and detoxification.

Comparison of the expression profiles of genes regulated by CorE2 and CorE during growth has revealed clear differences. For simplicity, we will consider only the expression profiles of these genes during growth because it has been reported that, for a reason yet to be uncovered, developing *M. xanthus* cells are more sensitive to metals than growing cells (21). Moreover, as zinc is a biological metal while cadmium is not, we will compare the expression profiles of genes regulated by CorE2 with zinc with that of genes regulated by CorE with copper. For CorE-regulated genes, expression levels peak at 2 h, dropping afterward to low basal levels (14). In contrast, although genes regulated by CorE2 also reach maximum expression levels at 2 h after the addition of the metal, their levels decrease slowly thereafter, remaining quite close to the maximum even at 48 h (Figure 2D). This difference can be explained by the fact that copper exhibits two redox states, Cu(I) and Cu(II), and that Cu(II) activates CorE while Cu(I) inactivates it (14). Conversely, zinc only has one redox state, which activates CorE2. Accordingly, it has been proposed that when copper is added to the medium, the metal rapidly enters into the cytoplasm as Cu(II), activating CorE and upregulating the expression of those genes that are under the control of this sigma factor. However, after a short time the metal will be reduced to Cu(I) due to the reducing environment in the cytoplasm, inactivating CorE and drastically diminishing the expression levels of the genes under its control

(14). In the case of zinc, we propose that it also rapidly enters the cytoplasm and activates CorE2. This activation will be maintained through time because the redox state of zinc will be unaltered. However, upregulation by CorE2 of the gene *MXAN_5264*, which encodes a protein with similarities to the cation diffusion facilitators which extrude, among other metals, zinc (31), will diminish the concentration of this metal in the cytoplasm, explaining why the expression levels of genes regulated by CorE2 slightly decrease after they peak.

Unlike most ECF sigma factors, in which the sigma factors themselves completely regulate their own expression (39), *corE2* expression is completely under the control of another regulator, which does not respond to metals (Figure 5). In the case of CorE, it was reported that its expression level in the presence of copper was approximately halved in the Δ*corE* mutant, indicating that CorE expression is only partially auto-regulated (14). In this respect, and although the data about auto-regulation of *corE* and *corE2* are not identical, both seem to differ from most characterized ECF sigma factors. Characterization of other members of the ECF44 group will clarify whether the absence of auto-regulation is a common feature of this group.

corE2, but not *corE*, is upregulated during development in the absence of metals. As a consequence of this developmental expression, fruiting body formation is delayed in the Δ*corE2* mutant, and nearly blocked in the presence of cadmium (Figure 3D and F). Interestingly, the gene *MXAN_5265* is also upregulated during development in a CorE2-independent manner (Figures 2 and 4, and Supplementary Figure S2). This gene encodes a protein that exhibits 37.25% identity with *S. aurantiaca* FbfB, which has been reported to play an important role in proper fruiting body formation in this myxobacterium (32). Identification of the genes regulated by CorE2 during development and their roles in fruiting body formation, as well as the regulator of the *corE2* operon during development and the exact function of genes *MXAN_5262*, *MXAN_5264* and *MXAN_5265* during both life stages, will help us to understand the complex lifestyle of myxobacteria.

Most ECF sigma factors are co-expressed with their cognate anti-sigma factors, and *corE2* forms an operon with the gene *MXAN_5262*, which encodes a lipoprotein that has been suggested to function as the anti-sigma factor of CorE2 (12). However, an in-frame deletion of the gene for this lipoprotein does not cause expression of genes regulated by CorE2 in the absence of metals (Figure 6). These data, along with the fact that that the activity of CorE does not depend on an anti-sigma factor either, indicate that the mechanism of action of the ECF44 group differs from that of most ECF sigma factors. In group ECF44, the C-terminal extension rich in Cys is an element responsible for sensing metal fluctuations and determining the activation and inactivation of the ECF sigma factor. Although the lipoprotein encoded by the gene *MXAN_5262* does not function as the anti-sigma factor of CorE2, it is likely that they work together in the same signal-transduction pathway, since it is also involved in cadmium-induced transcription of gene *MXAN_5265*, as it has been previously reported for other ECF sigma factors that function without an anti-sigma factor (16).

In addition to CRD, all CorE-like ECF sigma factors share a common CxC motif between the σ2 domain and the σ4.2 region (Supplementary Figure S1), which is indispensable for their functioning (Figure 9). Since both CRD and CxC are distinctive sequences of this ECF44 group of sigma factors and Cys are important to metal coordination (40,41), it is likely that they work together to coordinate the correct metal, providing the sigma factor with the conformational changes necessary for its activation. Conversely, when the protein is not bound to metals or it binds a metal which does not allow folding in the active conformation, the sigma factor will remain inactive. Taking into consideration the location of the CxC motif (between the σ2 domain and the σ4.2 region) and the fact that the mutant proteins are stable, it is plausible to think that mutations in this region prevent these sigma factors from interacting with the core RNA polymerase.

Although the work done on CorE and CorE2 has shed light on the mechanism of activation of this new group of ECF sigma factors, characterization of other members of the group along with studies about metal coordination of these proteins and determination of their crystal structure will reveal the exact mechanism of activation and inactivation of these regulators and their physiological roles.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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