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 e2 domain and the e4.2 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 σ70 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 e2 and the e4 domains. The σ2 domain is essential for recognition of the −10 promoter sequences and coupling with the RNA polymerase core enzyme, while the e4.2 region (included in the e4 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).
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 pBJI13 (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 KmR recombinant strains (Supplementary Table S1) were confirmed by Southern blot analysis.

For qualitative β-galactosidase activity analyses, cells were concentrated to an OD<sub>600</sub> 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<sub>600</sub> 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-ΔcorE) for mutations in CorE, and into M. xanthus JM52IF3ZY5 (5265-lacZ-ΔcorE2) 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<sup>R</sup> and Km<sup>R</sup> recombinants were analyzed by Southern blot. As in previous studies for the CorE mutation, strain JM00BZY was used as a control (14).

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/brow se/). Protein sequence alignments were performed using the ClustalW program (28), and a graphic representation of the results was generated with ESPript (http://escript.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 Halalium 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,
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 metallidurans (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 FbdB 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 corE, 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 were electroporated into the WT strain of *M. xanthus*. The resulting Km<sup>r</sup> strains JMS2ZY2 (5262-lacZ), JMS2ZY3 (corE2-lacZ), JMS2ZY4 (5264-lacZ) and JMS2ZY5 (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 with no metals added. Genes encoding proteins involved in metal homeostasis and detoxification are also found in the proximity 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.

To qualitatively screen for gene expression, severalMETALs were used as templates 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 S2C).}

**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 (Sigma70σ2, PF04542) and the σ4.2 region (sigma70_σ4.2, 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...
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) Quantitative analysis of β-galactosidase 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 ΔcorE2 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 (ΔcorE2) 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 ΔcorE2 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 ΔcorE2 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 ΔcorE2 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 ΔcorE2 mutant. (A) Domains present in CorE2 in the WT strain and in the mutants ΔcorE2 and ΔcorE2,CRD. (B) Cells of the WT strain (blue line) and the ΔcorE2 mutant (red line) were grown for 24 h in CTT containing 0.2 mM zinc and then diluted to an OD₆₀₀ of 0.05 in CTT medium containing 0, 0.2, 0.3 and 0.4 mM zinc. OD₆₀₀ 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 OD₆₀₀ of 0.05 in CTT medium containing 0, 10, 25 and 40 μM cadmium. OD₆₀₀ 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 ΔcorE2 mutant in the absence of metals. (E) Fruiting body formation of the WT strain and the ΔcorE2 mutant on CF medium with 200 μM zinc. (F) Fruiting body formation of the WT strain and the ΔcorE2 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 ΔcorE2 mutant exhibits developmental defects. When dropped onto CF starvation medium the ΔcorE2 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 ΔcorE2 mutant was used as genetic background to introduce the transcriptional fusion 5265-lacZ, to obtain the strain JM52IF3ZY5 (5265-lacZ-ΔcorE2). 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 ΔcorE2 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-ΔcorE2 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-ΔcorE2) was constructed introducing the corE2-lacZ transcriptional fusion into the ΔcorE2 mutant. Quantitative and qualitative analyses of this strain during development with and without cadmium rendered similar expression patterns to those of the WT.
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 ΔcorE2 (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) 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.

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 (13). In order to test whether MXAN_5262 acts as the cognate anti-sigma factor for CorE2, a new in-frame deletion strain, JM52IF2 (Δ5262), was obtained. Next, the fusion 5265-lacZ was introduced into this mutant. When β-galactosidase activity was determined in the strain JM52IF2ZY5 (5265-lacZ-Δ5262), 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
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-
A CxC conserved motif located between the $\sigma$2 domain and the $\sigma$4.2 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 $\sigma$2 domain and the $\sigma$4.2 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 couB 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.

Factors were 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 CxG 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 P1B-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 GofS, 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.
CORRECTION

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 (5). In the case of CorE, it was reported that its expression is only partially auto-regulated (14). In this respect, and although the data about auto-regulation of \( corE2 \) 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 \( \Delta corE2 \) 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 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 \( \sigma_2 \) domain and the \( \sigma 4.2 \) 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 \( \sigma_2 \) domain and the \( \sigma 4.2 \) 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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