The two-component system ChrSA is crucial for haem tolerance and interferes with HrrSA in haem-dependent gene regulation in Corynebacterium glutamicum

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We recently showed that the two-component system (TCS) HrrSA plays a central role in the control of haem homeostasis in the Gram-positive soil bacterium Corynebacterium glutamicum. Here, we characterized the function of another TCS of this organism, ChrSA, which exhibits significant sequence similarity to HrrSA, and provide evidence for cross-regulation of the two systems. In this study, ChrSA was shown to be crucial for haem resistance of C. glutamicum by activation of the putative haem-detoxifying ABC-transporter HrtBA in the presence of haem. Deletion of either hrtBA or chrSA resulted in a strongly increased sensitivity towards haem. DNA microarray analysis and gel retardation assays with the purified response regulator ChrA revealed that phosphorylated ChrA acts as an activator of hrtBA in the presence of haem. The haem oxygenase gene, hmuO, showed a decreased mRNA level in a chrSA deletion mutant but no significant binding of ChrA to the hmuO promoter was observed in vitro. In contrast, activation from P_{hmuO} fused to eyfp was almost abolished in an hrrSA mutant, indicating that HrrSA is the dominant system for haem-dependent activation of hmuO in C. glutamicum. Remarkably, ChrA was also shown to bind to the hrrA promoter and to repress transcription of the paralogous response regulator, whereas chrSA itself seemed to be repressed by HrrA. These data suggest a close interplay of HrrSA and ChrSA at the level of transcription and emphasize ChrSA as a second TCS involved in haem-dependent gene regulation in C. glutamicum, besides HrrSA.

INTRODUCTION

Haem plays an important role as a cofactor for proteins of various functions and is used as an alternative source of iron by many bacterial species (Andrews et al., 2003; Nobles & Maresso, 2011; Skaar, 2010). To ensure sufficient Fe^{2+} supply but also avoid toxic intracellular levels, iron uptake and utilization is usually tightly regulated at the transcriptional level (Andrews et al., 2003; Hantke, 2001; Skaar, 2010). Classical two-component systems (TCSs), composed of a sensor histidine kinase and a cognate response regulator, represent a typical regulatory module to sense extracellular environmental stimuli and transduce the information via protein phosphorylation to the level of gene expression (Krell et al., 2010; Mascher et al., 2006; Stock et al., 2000). Upon stimulus perception, the sensor kinase undergoes autophosphorylation of a conserved histidine residue; this phosphoryl group is subsequently transferred to an aspartate residue of the response regulator, which modulates gene expression by binding to the promoter region of target genes (Laub & Goulian, 2007; Stock et al., 2000; West & Stock, 2001).

The Gram-positive soil bacterium Corynebacterium glutamicum represents an important platform organism in industrial biotechnology (Burkovski, 2008; Eggeling & Bott, 2005). In total, 13 TCSs are encoded in the C. glutamicum genome (Kocan et al., 2006), several of which have been studied in more detail (Brocker et al., 2011; Bott & Brocker, 2012; Schaaf & Bott, 2007; Schelder et al., 2011). In a recent study, we demonstrated that the TCS HrRSa exhibits a central function in the control of haem homeostasis and haem utilization in C. glutamicum. In
the presence of haem, the response regulator HrrA directly represses haem biosynthesis genes and activates haem oxygenase (hmuO) as well as genes encoding haem-containing components of the respiratory chain (Frunzke et al., 2011). Expression of hrrA itself underlies control by the global iron regulator DtxR, which represses transcription from the promoter \( P_{hrrA} \), downstream of hrrS, under conditions of sufficient iron supply (Wennerhold & Bott, 2006). Under iron-limiting conditions, DtxR dissociates from the hrrA promoter, thereby enabling the utilization of alternative iron sources such as haem. Besides hrrA, DtxR directly regulates the transcription of about 60 genes involved in iron uptake and storage in response to iron availability (Boyd et al., 1990; Frunzke & Bott, 2008; Wennerhold et al., 2005; Wennerhold & Bott, 2006).

For haem utilization, *C. glutamicum*, as well as its pathogenic relative *Corynebacterium diphtheriae*, depends on a haem uptake apparatus composed of the ABC transporter HmuTUV, several cell surface haem-binding proteins (Allen & Schmitt, 2009, 2011; Drazek et al., 2000; Frunzke et al., 2011) and a haem oxygenase (HmuO), which catalyses the intracellular degradation of the tetrapyrryl ring to \( 2\)-biliverdin, free iron (Fe\(^{3+}\)) and carbon monoxide (Kunkle & Schmitt, 2007; Schmitt, 1997; Wilks & Schmitt, 1998).

Acquisition of haem, however, exposes the respective organism to the toxicity associated with high levels of haem. It was shown in a recent study that the haem-regulated ABC transport system, HrtAB, is crucial for *C. diphtheriae* to cope with elevated haem concentrations (Bibb & Schmitt, 2010). The HrtAB system consists of the permease HrtB and the ATPase HrtA and is widespread among Gram-positive bacteria (Stauff et al., 2008; Stauff & Skaar, 2009a, b). In *C. diphtheriae*, hrtBA expression was shown to be activated in the presence of haem by the TCS ChrSA (Bibb et al., 2005; Bibb & Schmitt, 2010). In previous studies, the ChrSA system was described to activate expression of hmuO and repress expression of the hemAC operon encoding haem biosynthesis enzymes (Bibb et al., 2007). Both targets, hmuO and hemAC, are also controlled by the second haem-dependent TCS, HrrSA, in *C. diphtheriae* (Bibb et al., 2005, 2007).

Previous studies in *C. glutamicum* and *C. diphtheriae* revealed the TCSs HrrSA and ChrSA to have a global function in the control of haem homeostasis; however, no studies concerning the interplay of the two systems on the transcriptional level have been performed so far. In this report, we used genome-wide transcriptome analyses, protein–DNA interaction studies and promoter fusions to identify direct target genes of ChrSA (previously named CgtSR8) and study the interaction with the homologous system HrrSA in *C. glutamicum*. Our data reveal that HrrSA is the dominant system for the haem-dependent activation of haem oxygenase in *C. glutamicum*, whereas ChrSA plays a crucial role in haem tolerance mediated by the HrtBA haem transport system. Furthermore, we provide evidence for cross-regulation of both systems, HrrSA and ChrSA, at the level of transcription.

### METHODS

#### Bacterial strains, media and growth conditions.

The bacterial strains used in this study are shown in Table S1 (available with the online version of this paper). For growth experiments, a 20 ml preculture of CGXII minimal medium containing 4 % (w/v) glucose (Keilhauer et al., 1993) was inoculated from a 5 ml BHI (brain heart infusion, Difco) culture after washing the cells with 0.9 % (w/v) NaCl. Cells were incubated overnight at 30 °C and 120 r.p.m. in a rotary shaker. The trace element solution with or without iron as well as the FeSO\(_4\) or haemin (protoporphyrin IX with Fe\(^{2+}\)) solution were added from stock after autoclaving, as indicated. Standard CGXII minimal medium contains 36 \(\mu\)M FeSO\(_4\). For the haemin stock solution, haemin (Sigma Aldrich) was dissolved in 20 mM NaOH to 250 \(\mu\)M. The main culture was inoculated from the second preculture to OD\(_{600}\) \(\approx 1\) in CGXII minimal medium containing 4 % (w/v) glucose and either FeSO\(_4\) or haemin as iron source. For cloning purposes *Escherichia coli* DH5\(\alpha\) was used; for overproduction of ChrA. *E. coli* BL21(DE3) (Studier & Moffatt, 1986). *E. coli* was cultivated in Luria–Bertani (LB) medium at 37 °C or on LB agar plates. When necessary, kanamycin was added at an appropriate concentration (50 \(\mu\)g ml\(^{-1}\) for *E. coli* and 25 \(\mu\)g ml\(^{-1}\) for *C. glutamicum*). For growth experiments on agar plates the strains were grown in a 5 ml BHI culture overnight. The stationary culture was diluted to OD\(_{600}\) \(\approx 1\) and dilution series (3 \(\mu\)l each, \(10^0\) to \(10^{-1}\)) were spotted on CGXII agar plates containing 4 % (w/v) glucose and either 2.5 or 36 \(\mu\)M FeSO\(_4\) with or without haemin. Pictures of the plates were taken after incubation for 24 h at 30 °C.

Growth experiments in microtitre scale were performed in the BioLector system (m2p-labs). Therefore, 750 \(\mu\)l CGXII containing 2 % (w/v) glucose and different concentrations of FeSO\(_4\) (2.5 or 36 \(\mu\)M) or haemin (2.5–20 \(\mu\)M) were inoculated with cells from a 20 ml CGXII preculture with iron-starved cells (0 \(\mu\)M FeSO\(_4\)) to OD\(_{600}\) \(\approx 1\) and cultivated in 48-well flowerplates (m2p-labs) at 30 °C, 1200 r.p.m. and a shaking diameter of 3 mm. The production of biomass was determined as the backscattered light intensity of sent light with a wavelength of 620 nm (signal gain factor of 10); measurements were taken in 10 min intervals. The average backscatter of non-growing wild-type cells (first 2 h of the wild-type in CGXII minimal medium with 15 \(\mu\)M haem) was used for referencing. High fluctuations of low backscatter signals (non-growing cells, Fig. 1) are due to technical limitations. For promoter fusion studies, the eYFP chromophore was excited with 510 nm and emission was measured at 532 nm (signal gain factor of 50). The specific fluorescence (au) was calculated by the eYFP fluorescence signal per backscatter signal (Kensy et al., 2009).

#### Cloning techniques.

Routine methods were performed according to standard protocols (Sambrook et al., 2001). Chromosomal DNA of *C. glutamicum* ATCC 13032 was prepared (Eikmanns et al., 1994) and utilized as template for PCR. DNA sequencing and oligonucleotide synthesis were performed by Eurofins MWG Operon (Ebersberg, Germany). Plasmids and oligonucleotides used in this work are listed in Tables S1 and S2, respectively. A detailed description of the construction of strains and plasmids is given in the supplementary material.

#### DNA microarrays.

The transcriptome of the deletion mutant ΔchrSA grown on haem or FeSO\(_4\) was compared with the wild-type using whole-genome-based DNA microarrays. For this purpose, cells of a BHI preculture were used for inoculation of a second preculture in CGXII medium containing 1 \(\mu\)M FeSO\(_4\). For main culture, cells were cultivated in CGXII minimal medium with 4 % glucose (w/v) containing either 2.5 \(\mu\)M FeSO\(_4\) or haemin as iron source and harvested at OD\(_{600}\) \(\approx 5\) in pre-cooled (\(\approx 20^\circ\)C) ice-filled tubes via centrifugation (9000 \(\times\) 10 min, 4 °C). The cell pellet was subsequently frozen in liquid nitrogen and stored at \(\approx 70^\circ\)C until RNA
Omnibus (GEO) database under accession no. GSE37327. & Wendisch, 2004) for further analysis and in the Gene Expression processed data were saved in the in-house microarray database (Polen were repeated in three biological replicates. The normalized and previously (Frunzke microarray purchased from Eurofins MWG Operon, as described hybridization was performed with a 70-mer custom-made DNA 25 ng total RNA from each sample was used. Labelling and 4 ng total RNA from each sample was used. Labelling and 25 ng total RNA from each sample was used. Labelling and preparation. The preparation of total RNA was performed as described previously (Mo¨ker et al., 2004). For cDNA synthesis, 25 ng total RNA from each sample was used. Labelling and hybridization was performed with a 70-mer custom-made DNA microarray purchased from Eurofins MWG Operon, as described previously (Frunzke et al., 2008). All DNA microarray experiments were repeated in three biological replicates. The normalized and processed data were saved in the in-house microarray database (Polen & Wendisch, 2004) for further analysis and in the Gene Expression Omnibus (GEO) database under accession no. GSE37327.

**Overproduction and purification of ChrA.** For the overproduction of ChrA, *E. coli* BL21(DE3) was transformed with the vector pET28b- chrA and cultivated in 200 ml LB medium. At OD$_{600}$ 0.6–0.8, the expression of chrA was induced by addition of 1 mM IPTG. After 4 h of expression at 30 °C, the cells were harvested by centrifugation (4000 g at 4 °C, 10 min). The cell pellet was stored at −20 °C until further use. For protein purification, the cell pellet was resuspended in 3 ml TNI5 buffer (20 mM Tris/HCl pH 7.9, 300 mM NaCl and 5 mM imidazole) containing Complete protease inhibitor cocktail (Roche). Cells were disrupted by passing through a French pressure cell (SLM Aino, Spectronic Instruments) twice at 207 MPa. The cell debris was removed by centrifugation (6900 g, 4 °C, 20 min), followed by ultracentrifugation of the cell-free extract for 1 h (150 000 g, 4 °C). ChrA was purified from the supernatant via Ni$^{2+}$-NTA (nickel-nitriloacetic acid) affinity chromatography as described for *C. glutamicum* HrrA (Frunzke et al., 2011). ChrA was eluted from the column with TN1100 buffer (containing 100 mM imidazole) and analysed on a 12 % SDS-polyacrylamide gel. Protein concentration was determined with Bradford reagent (Bradford, 1976). Elution fractions of ChrA were pooled and the buffer was exchanged to bandshift buffer [20 mM Tris/ HCl, pH 7.5, 50 mM KCl, 5 mM ATP, 10 mM MgCl$_2$, 5 % (w/v) glycerol, 0.5 mM EDTA, 0.005 % (w/v) Triton X-100] using a PD10 desalting column (GE Healthcare). The protein was stored in aliquots at −20 °C.

**Electrophoretic mobility shift assay (EMSA).** EMSAs were performed with purified ChrA protein and DNA fragments of the putative target genes. Promoter regions (500 bp) of the putative target genes were amplified via PCR and purified by using the Qiagen PCR purification kit. As a negative control, the promoter region of the gntK gene was used. DNA (100 ng per lane) was incubated with
different molar excesses of the purified ChrA protein at room temperature for 30 min in bandshift buffer. For phosphorylation of ChrA, 50 mM of the small phosphate donor phosphoramidate was incubated with the protein before the DNA was added. After incubation, sample buffer [0.1 % (w/v) xylene cyanol dye, 0.1 % (w/v) bromophenol blue dye, 20 % (w/v) glycerol in 1 x TBE (89 mM Tris base, 89 mM boric acid, 2 mM EDTA)] was added and samples were separated on a non-denaturing 10% polyacrylamide gel with 170 V in 1 x TBE buffer. DNA was stained using SYBR Green I (Sigma-Aldrich). For verification of the ChrA binding motif, 30 bp double-stranded oligonucleotides were assembled by hybridization of two complementary oligonucleotides. The amount of shifted DNA was quantified by using the ImageQuant TL software (GE Healthcare).

Identification of transcription start sites (TSSs) and promoter regions by RNA-Seq. A 5'-end enriched RNA-Seq library was constructed according to the following procedures. 1) Depletion of stable rRNA and enrichment of mRNA molecules were performed using the Ribo-Zero rRNA removal kit for Gram-positive bacteria (Epicentre Biotechnologies). 2) The enriched mRNA was fragmented by magnesium oxaloacetate (MgKOAc) hydrolysis. Four vols RNA solution were mixed with one vol. MgKOAc solution (100 mM KOAc and 30 mM MgOAc in 200 mM Tris/HCl, pH 8.1) and the mixture was incubated for 2.5 min at 94 °C. The reaction was stopped by adding an equal volume of 1 x TE (10 mM Tris, 1 mM EDTA, pH 8) and chilling on ice for 5 min. 3) The fragmented RNA was precipitated by addition of three vols 0.3 M NaAc in ethanol with 2 µl glycerol and incubation overnight at -20 °C. 4) The precipitated RNA fragments were dissolved in water and the 5'-end RNA fragments were enriched by using Terminator 5'-phosphate-dependent exonuclease (Epicentre Biotechnologies). 5) After RNA precipitation (as above), the triphosphates were removed using RNA 5'-polyphosphatase (Epicentre Biotechnologies). 6) After RNA precipitation (as above), the 5'-enriched, monophosphorylated RNA fragments were used to construct a cDNA library by using the Small RNA Sample Prep kit (Illumina).

The fragmentation of RNA molecules (fragment sizes were 200–500 bp) and RNA concentration were monitored using the RNA 6000 Pico Assay on an Agilent 2100 Bioanalyser (Agilent). Sequencing of RNA fragments was used to construct a cDNA library by using the Small RNA Sample Prep kit (Illumina). The resulting reads were aligned to the C. glutamicum genome using the mapping software STAR (Dobin et al., 2013). Table S3. TSS and promoter regions were deduced by combining published information about promoter regions in C. glutamicum (Pátek & Nešvera, 2011) with 5'-end enriched RNA-Seq data.

RESULTS

The TCS ChrSA (previously CgtSR8): sequence similarities and genomic organization

In a previous study the TCS HrrSA was reported to play a central role in the control of haem homeoestasis in C. glutamicum (Frunzke et al., 2011). In vitro DNA binding studies with purified HrrA protein provided evidence that the response regulator HrrA binds to the upstream promoter region of an operon encoding another TCS, chrSA (cg2201–cg2200) (Kocan et al., 2006). This system consists of the genes cg2200 (chrA, previously cgtR8), encoding the response regulator ChrA, and cg2201 (chrS, previously cgtS8), encoding the sensor kinase ChrS. Sequence analysis revealed considerable similarity of ChrSA to the recently described system HrrSA of C. glutamicum. The sensor kinases, ChrS and HrrS, share a sequence identity of about 35 %, whereas the response regulators, ChrA and HrrA, exhibit a sequence identity of about 58 % at the protein level (Table 1). Both systems also share significant similarities with HrrSA and ChrSA of C. diphtheriae. A pairwise comparison is given in Table 1. In terms of consistency with the previously described orthologous system of C. diphtheriae, we renamed CgtSR8 to ‘ChrSA’ for C. glutamicum.

RNA sequencing experiments indicated that, in contrast with the hrrSA operon, where a second promoter is located upstream of hrrA, the genes chrSA form a classical operon with one promoter upstream of chrS (Table S3). The start codon of chrA overlaps with the stop codon of chrS. Divergently from chrSA (intergenic region of 110 bp) the operon hrtBA is located, encoding the permease (HrtB) and ATPase (HrtA) components of an ABC-type transport system. Microsynteny is observed at this genomic locus consisting of a classical TCS and an operon encoding a ‘haem-regulated transport system’, which is highly conserved in Gram-positive bacteria. The transporter HrtAB has been described to be involved in export of haem or degradation products thereof (Stauff & Skaar, 2009a). These findings suggest that the TCS ChrSA might interfere in the control of haem homeostasis with the recently reported system HrrSA in C. glutamicum.

Deletion of chrSA leads to increased haem sensitivity

To characterize the role of the TCS ChrSA in haem utilization, we constructed an in-frame deletion mutant lacking the genes chrA and chrSA. In first experiments, we analysed the haem tolerance of the deletion mutant ΔchrSA and the C. glutamicum wild-type. Growth of the strains was compared on agar plates or in liquid culture containing either haemin or FeSO4 as iron source. Growth in liquid culture (2.5 µM FeSO4 or 2.5–20 µM haemin) was performed in microtitre plates (48-well flowerplates, see
Growth experiments revealed a significant haemin sensitivity observed under the control of its native promoter (Fig. 1). Grown on 2.5 μM haemin, ΔchrSA revealed a strong growth defect on plates (Fig. 1a). Under iron-replete conditions, the same phenotype was observed in the presence of haem (36 μM FeSO₄ and 2.5 μM haemin), indicating that the observed phenotype is a result of the elevated haem concentration and is not influenced by the iron concentration (Fig. 1a). In liquid culture, the presence of 2.5 μM haemin resulted in a decelerated growth rate and a lower final backscatter signal for both strains. The addition of 5 μM haemin extended the lag phase and resulted in a higher final backscatter compared with cells grown on 2.5 μM haemin, indicating that iron is a limiting factor under the chosen conditions. Higher haemin concentrations (10–20 μM) led to a proportional extension of the lag phase after which cells started to grow again with a growth rate comparable to cells grown on iron (Fig. 1b). Again, the mutant strain ΔchrSA exhibited a higher sensitivity towards elevated haem concentrations (10–20 μM haemin). This delayed growth of the tested strains and the fact that the cells resume growth after the lag phase with an unaltered growth rate or final density led to the assumption that the added haemin is degraded in the culture medium over time until the concentration drops under a certain threshold. This tolerable limit would then be higher for the wild-type than for ΔchrSA. The observed phenotype of the ΔchrSA mutant was complemented by transformation with the plasmid pJC1-chrSA, expressing chrSA under the control of its native promoter (Fig. 1). Overall, these findings emphasize a central role of ChrSA in haem detoxification.

**The HrtBA transporter confers resistance towards haem toxicity**

Growth experiments revealed a significant haemin sensitivity of the ΔchrSA mutant. As outlined in the Introduction, the genes hrtBA, located divergently to chrSA, encode a putative ‘haem regulated transporter’ (Bibb & Schmitt, 2010; Stauff & Skaar, 2009b). Thus, a lowered expression of hrtBA in the ΔchrSA mutant could be a reason for the observed haem sensitivity of the ΔchrSA mutant. In order to investigate the function of the putative transport system HrtBA in C. glutamicum, an in-frame deletion mutant of the genes hrtB and hrtA was constructed. As observed for ΔchrSA, the growth of ΔhrtBA was not affected when FeSO₄ was added as sole iron source. In the presence of haemin, ΔhrtBA exhibited a significant growth defect, both on agar plates and during liquid cultivation (Fig. 1). This phenotype was complemented by transformation of ΔhrtBA with the plasmid pEKE2-hrtBA carrying the hrtBA operon under the control of the IPTG-inducible P_tac promoter, which allows a basal gene expression even in the absence of IPTG. The strain ΔhrtBA/pEKE2-hrtBA showed wild-type-like tolerance towards high haemin concentrations (Fig. 1). Induction of hrtBA expression by addition of IPTG led to a strong growth defect (data not shown). In the next step, we tested our hypothesis that reduced expression of hrtBA might be the reason for the observed growth phenotype of the ΔchrSA mutant and examined whether plasmid-driven expression of hrtBA in ΔchrSA could restore wild-type-like growth. In fact, the cross-complemented strain ΔchrSA/pEKE2-hrtBA exhibited wild-type-like growth on agar plates containing 2.5 μM haemin (Fig. 1a). These data indicate that HrtBA plays a key function in haem detoxification in *C. glutamicum* and suggest a role of ChrSA in the control of hrtBA expression.

**Transcriptome analysis of a ΔchrSA mutant strain**

To identify additional potential target genes of ChrSA we assessed the influence of ChrSA on global gene expression via comparative transcriptome analysis of the ΔchrSA mutant and *C. glutamicum* wild-type grown in CGXII minimal medium with 4% glucose and either 2.5 μM FeSO₄ or 2.5 μM haemin as iron source. Genes whose mRNA level showed a more than twofold alteration in either experiment (FeSO₄ or haemin) are listed in Table 2. In cells grown on FeSO₄, the deletion of chrSA had no significant influence on global gene expression. When cultivated with haemin as an iron source, the relative expression level of hrtBA (coding for the putative haem transport system HrtBA) was two- to threefold decreased in the ΔchrSA mutant.

Likewise, the expression of hmuO, encoding the haem oxygenase, was nearly sevenfold decreased in the presence of haemin, but showed no difference on iron as well. Expression of hmuO is also described as being under control of the global iron regulator DtxR in *C. glutamicum* (Wennerhold & Bott, 2006). In our studies, the ΔchrSA mutant showed a slightly reduced expression (1.3- to 2-fold) of several DtxR target genes (Table 2) composing the typical iron starvation response. Among those, we found the operon hmuTUV encoding a haem uptake system as well as htaA, htaC and htaD encoding putative haem-binding proteins. However, hmuO expression was significantly decreased even more than the other DtxR targets.

Remarkably, the mRNA level of hrrA encoding the response regulator of the TCS HrrSA was slightly increased (approx. 1.5-fold) in the ΔchrSA mutant. Together with the observed derepression of chrSA in a ΔhrrA mutant (Frunzke et al., 2011) these data hint at a cross-regulation of both systems at the level of transcription. Further genes exhibiting an altered mRNA level include a regulator of unknown function (cg3303) and the redox-sensing regulator qorR, whose DNA-binding activity was reported to be affected by oxidants (Ehira et al., 2009).

**Identification of direct target genes of the response regulator ChrA**

To test for direct binding of the response regulator ChrA to putative target promoters, we performed *in vitro* EMSA experiments using purified ChrA protein and probes representing potential target promoters.
Table 2. Comparative transcriptome analysis of ΔchrSA and C. glutamicum wild-type

This table shows all genes that revealed a $\geq$ twofold altered relative mRNA (P-value $\leq$ 0.06) level in at least two of three independent DNA microarrays of C. glutamicum ΔchrSA versus wild-type grown on CGXII minimal medium with 4 % (w/v) glucose and 2.5 $\mu$M FeSO$_4$ or haem as iron source.

<table>
<thead>
<tr>
<th>Gene ID</th>
<th>Gene</th>
<th>Annotation</th>
<th>Ratio 2.5 $\mu$M FeSO$_4^*$</th>
<th>Ratio 2.5 $\mu$M haem$^*$</th>
</tr>
</thead>
<tbody>
<tr>
<td>cg3247</td>
<td>hrrA</td>
<td>TCS, response regulator</td>
<td>1.03</td>
<td>1.45</td>
</tr>
<tr>
<td>cg3248</td>
<td>hrrS</td>
<td>TCS, signal transduction histidine kinase</td>
<td>1.01</td>
<td>0.86</td>
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<tr>
<td>Haem homeostasis-related genes</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>cg2202</td>
<td>hrbT</td>
<td>ABC-type transport system, permease component</td>
<td>1.05</td>
<td>0.64</td>
</tr>
<tr>
<td>cg2204</td>
<td>hrtA</td>
<td>ABC-type transport system, ATPase component</td>
<td>1.17</td>
<td>0.33</td>
</tr>
<tr>
<td>cg2445</td>
<td>hmuO</td>
<td>Haem oxygenase</td>
<td>0.96</td>
<td>0.16</td>
</tr>
<tr>
<td>cg0466</td>
<td>htaA</td>
<td>Secreted haem transport-associated protein</td>
<td>0.97</td>
<td>0.48</td>
</tr>
<tr>
<td>cg0467</td>
<td>hmuT</td>
<td>Haemin-binding periplasmic protein precursor</td>
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<td>0.68</td>
</tr>
<tr>
<td>cg0468</td>
<td>hmuU</td>
<td>Haemin transport system, permease protein</td>
<td>1.03</td>
<td>0.66</td>
</tr>
<tr>
<td>cg0469</td>
<td>hmuV</td>
<td>Haemin transport system, ATP-binding protein</td>
<td>0.98</td>
<td>n.d.</td>
</tr>
<tr>
<td>Others</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>cg0018</td>
<td>qorR</td>
<td>Redox-sensing transcriptional regulator</td>
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<td>2.02</td>
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<tr>
<td>cg1552</td>
<td></td>
<td></td>
<td>1.00</td>
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<td>Putative secreted protein</td>
<td>1.01</td>
<td>2.03</td>
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<td>cg2845</td>
<td>pstC</td>
<td>ABC-type phosphate transport system, permease component</td>
<td>0.93</td>
<td>2.17</td>
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<tr>
<td>cg3303</td>
<td></td>
<td>Transcriptional regulator, PadR-like family</td>
<td>0.95</td>
<td>2.20</td>
</tr>
</tbody>
</table>

*The mRNA ratio represents the mean value of three independent DNA microarray experiments.

studies with purified ChrA. To this end, ChrA was overproduced in E. coli containing an N-terminal hexahistidine tag and purified by affinity chromatography. Purified ChrA was phosphorylated by the addition of the small-molecule phosphate donor phosphoramidate, which led to an approximately two- to threefold increased affinity of ChrA-P to the tested DNA fragments.

In our assays, a clear binding of ChrA to the intergenic region of chrSA and hrtBA was detected (Fig. 2a). A complete shift was observed upon addition of a 30- to 50-fold molar excess of phosphorylated ChrA. Under these conditions neither the negative control (gntK, cg2732) nor the promoter region of htaA was bound by ChrA (data not shown). Binding of ChrA to a DNA fragment covering the promoter of hrrA was also observed, however, with a lower affinity than binding to hrtBA–chrSA. Notably, the promoter region of hmuO whose expression level was significantly decreased (sevenfold) in the ΔchrSA mutant was not bound by ChrA in this assay.

In further EMSA assays, the binding region of ChrA to the promoters of hrtBA–chrSA and hrrA was narrowed down to DNA fragments of about 30 bp. Positive subfragments covering the binding motif of ChrA showed a comparable shift from the originally tested fragments (Fig. 2b). For the hmuO promoter region EMSA assays with a subfragment covering the region upstream of the DtxR-binding region (~45 bp upstream of the TSS) showed a slightly different picture to the negative control, suggesting very low affinity binding of ChrA in vitro. Whether this binding is of physiological relevance has to be verified in further studies.

**Mutational analysis of the ChrA-binding motif**

Sequence analysis of the 30 bp DNA fragment in the intergenic region of hrtBA and chrSA revealed a small inverted repeat (CGACcaaaGTCG). To assess the relevance of this repeat for ChrA binding we performed mutational analysis of the whole 30 bp fragment. For this purpose, three to four nucleotides were exchanged for the complementary ones and the mutated fragments were tested in gel retardation analysis. The exchange of small inverted repeats abolished ChrA binding, whereas the exchange of adjacent nucleotides or the four nucleotides in between the repeat led to reduced ChrA affinity towards the particular DNA fragment (Fig. 3). Mutations outside of the motif did not affect ChrA binding. Overall, the mutational analysis supported the relevance of the inverted repeat for binding of ChrA and revealed the sequence AgTaCGACcaaaGTCG as binding motif in the intergenic region of hrtBA–chrSA. A motif with considerable sequence identity was also found in the promoter region of hrrA (Fig. 4). A 30 bp fragment covering this predicted motif exhibited a clear binding by ChrA in EMSA assays (Fig. 2b).

Fig. 4 illustrates the position of the ChrA binding sites in relation to the TSS of the respective target gene. The TSS
has been determined by RNA sequencing of the C. glutamicum transcriptome (see Table S3). In the hrtBA–chrSA intergenic region the ChrA motif is located in between the −35 regions of hrtBA and chrSA, a position that would be in agreement with ChrA having an activating function on the expression of both operons. In the case of hrrA, which showed a slightly increased mRNA level in the ΔchrSA mutant, the ChrA binding site is located close to the TSS and would support a proposed repressor function of ChrA interfering at this locus with the binding of the RNA polymerase (Madan Babu & Teichmann, 2003).

**HrrSA and ChrSA interfere in haem-dependent gene regulation**

Previous studies revealed binding of the response regulator HrrA to the hrtBA–chrSA intergenic region. In view of the data reported in this study, HrrA and ChrA likely interfere in the transcription control of hrtBA and/or chrSA. To study the influence of both TCSs in vivo we constructed promoter fusions of the intergenic region of hrtBA–chrSA fused to eyfp in both possible directions (PchrSA and PhrtBA). While the wild-type containing the reporter plasmids (WT/pJC1-P_hrtBA-eyfp, WT/pJC1-P_chrSA-eyfp) exhibited no fluorescence when grown on iron, cells grown on haem showed a significantly increased fluorescence signal in the lag and early exponential phase (Fig. 5a). The ΔchrSA strain transformed with the promoter fusion plasmids (ΔchrSA/pJC1-P_hrtBA-eyfp, ΔchrSA/pJC1-P_chrSA-eyfp) showed no

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**Fig. 2.** DNA–protein interaction studies of ChrA and putative target promoters. (a) For gel retardation assays, 500 bp DNA fragments covering the promoter regions of hrtBA–chrSA, hrrA and hmuO were incubated without or with different molar excesses of phosphorylated ChrA (0– to 50-fold). The promoter region of gntK served as control fragment. For phosphorylation, purified ChrA protein was preincubated with 50 mM phosphoramidate (see Methods). Samples were separated on a 10 % non-denaturing polyacrylamide gel and stained with SYBR green I. (b) As described in (a), 30 bp DNA fragments covering the putative binding site of ChrA. Samples were separated on a 15 % non-denaturing polyacrylamide gel.

**Fig. 3.** Mutational analysis of the ChrA binding site in the intergenic region of hrtBA–chrSA. To analyse the relevance of different nucleotides for ChrA binding, a 30 bp DNA fragment covering the putative ChrA binding site in the hrtBA–chrSA intergenic region was mutated by an exchange of 3 to 4 bp to the complementary base pairs, as indicated, and analysed via EMSA studies. After incubation, the samples were separated on a 15 % non-denaturing polyacrylamide gel and stained with Sybr Green I. +, Fragments that were shifted with unaltered affinity; (+), a shift with lower affinity; −, fragments that were not shifted. The amount of shifted DNA is given as a percentage and was quantified by using ImageQuant TL (GE Healthcare) from three experimental replicates (mean ± SD).
Fig. 4. Localization of ChrA binding sites in the hrtBA–chrSA intergenic region and the hrrA promoter. Promoters were derived from RNA sequencing experiments; the corresponding −10 and −35 regions are given in Table S3. The TSS is indicated as +1 and the ChrA binding sites are shown as a black box, the DtxR binding site is shown as shaded box. The number below the ChrA box indicates the distance to the TSS. The mRNA ratios were obtained from DNA microarray analysis [ΔchrSA mutant versus wild-type (WT) grown on 2.5 μM haemin, see also Table 2]. Nucleotides conserved in both motifs are shaded in black.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Genetic organization</th>
<th>Ratio ΔchrSA/WT haemin</th>
<th>Translational start site</th>
<th>ChrA motif</th>
</tr>
</thead>
<tbody>
<tr>
<td>cg2202/cg2201</td>
<td>chrSA</td>
<td>0.64:0.33</td>
<td>Leaderless</td>
<td>GCA...CAATTGAGTCAC</td>
</tr>
<tr>
<td>cg3247</td>
<td>chrSA</td>
<td>1.45</td>
<td>+83</td>
<td>AAGCTA...CAATTGAGTCAC</td>
</tr>
</tbody>
</table>

Fig. 5. Promoter studies of P_{chrSA}, P_{hrtBA} and P_{hmuro} in wild-type and TCS mutants. For promoter studies, the promoters of chrSA, hrtBA and hmuro were fused to eyfp. C. glutamicum wild-type (black), ΔchrSA (blue), and ΔhrrA (red) were cultivated in CGXII minimal medium with 2 % glucose in microtitre plates (a) with 2.5 μM FeSO₄ (dotted lines) or 2.5 μM haemin (solid lines) as iron source or (b) with 36 μM FeSO₄ with (solid lines) or without (dotted lines) 2.5 μM haemin. In the BioLector system, the growth (backscatter signal of 620 nm light) and eYFP fluorescence (excitation 510 nm/emission 532 nm) were monitored over 10 min intervals. The specific fluorescence was calculated as fluorescence signal per backscatter signal (given in arbitrary units, au). Shown are representative experiments of three to four independent replicates.
significant fluorescent signal (Fig. 5a), indicating that ChrSA is crucial for the haem-dependent activation of both promoters. A similar response was observed under iron-excess conditions (36 μM FeSO₄) in the presence of haem (Fig. 5b). These data are in line with a positive autoregulation of chrSA and a ChrA-dependent activation of hrtBA in haem-grown cells. In a ΔchrSA strain, lacking the genes hrrA and hrrS of the TCS HrrSA, a higher signal was detected for both promoters (P_hrtBA and P_chrSA) under iron limitation in comparison with the wild-type, supporting the postulated repressor function of HrrA on chrA (Fig. 5). Remarkably, under iron-replete conditions, the activity of P_hrtBA and P_chrSA in the ΔhrrSA strain remained high throughout the exponential and stationary growth phase and did not decline to the background level.

**DISCUSSION**

Many bacterial species rely on haem or haem proteins as alternative sources of iron. Here, we showed that the TCS ChrSA is the crucial regulatory system for resistance towards haem toxicity in the non-pathogenic soil bacterium *C. glutamicum*. We identified the putative haem exporter hrtBA and hrrA, which encode the response regulator HrrA of the homologue TCS HrrSA, as direct target genes of the response regulator ChrA. The highest binding affinity of purified ChrA was observed in the presence of the phosphate donor phosphoramidate, indicating that ChrA follows the classical model and is active in its phosphorylated state (Gao et al., 2007; Stock et al., 2000). This is consistent with recent studies where the phosphotransfer from the soluble kinase domain of ChrS to the response regulator ChrA was described for the *C. diphtheriae* ChrSA system (Burgos & Schmitt, 2012). The autophosphorylation of ChrS was shown to occur in the presence of haemin in purified *E. coli* proteoliposomes, indicating a direct interaction of ChrS with haem (Ito et al., 2009).

The results described in this study support the prediction that *C. glutamicum* ChrSA has a key function in activating the expression of the divergently located operon hrtBA in the presence of haem. In fact, this function of ChrSA was expected due to the conserved microsynteny of this genomic locus where an operon of a TCS system is found in divergent orientation to hrtBA encoding a putative ‘haem-regulated’ ABC-transport system. This genomic organization is highly conserved among Gram-positive bacteria and homologous HrtAB transport systems were described as being required for coping with toxic haem concentrations for the species *C. diphtheriae*, *Staphylococcus aureus* and *Bacillus anthracis* (Bibb & Schmitt, 2010; Stauff et al., 2008). So far, this transport system has mainly been described in pathogenic species where it is of major importance during host infection, when the bacteria are exposed to high haem concentrations in the blood. The presence of hrtBA in the genome of the non-pathogenic soil bacterium *C. glutamicum* might be a relic of evolution, as *C. glutamicum* is closely related to several pathogenic Corynebacteria, such as *C. diphtheriae* or *Corynebacterium ulcerans* (Yukawa et al., 2007). However, high haem tolerance might also be of benefit in the soil, where haem is present in decaying organic material and represents an attractive alternative iron source for aerobic bacteria (Andrews et al., 2003). An alternative regulatory mechanism of transcriptional regulation of hrtBA has recently been reported for the Gram-positive communal bacterium *Lactococcus lactis*. Here, the cytoplasmic one-component regulator HrtR was described as a crucial factor for the haem-dependent activation of hrtBA (Lechardeur et al., 2012). This mechanism is conserved among different Gram-positive commensals and contrasts with the TCS-mediated control described for several pathogenic species as well as *C. glutamicum*.

By using gel retardation assays and mutational analysis, we identified an imperfect inverted repeat (AgTaCGACcaaaG-TCGgAtT) as a ChrA binding site within the hrtBA-chrSA intergenic region. Five of the eight nucleotides composing the inverted repeat are conserved in the binding site in the hrrBA promoter. A genome-wide motif search did not reveal candidates for additional, putative binding sites of haem-relevant genes, probably due to the poor conservation of the motif. The motif revealed only weak similarities to the identified ChrA binding motif upstream of *C. diphtheriae* hrtBA (CatATCAAccggGTTGATtgG) or with the motif of the ChrA orthologues HsrR from *S. aureus* and *B.
C. diphtheriae to be present in global iron regulator DtxR under iron sufficiency and is 2 promoter (hmuO is located upstream of the DtxR binding site in the sequence similarity, the genomic organization differs. In – a question which will be addressed in following studies. be speculated whether both regulators might bind to and HrrA share significant sequence identity (62 % sequence 2 (upstream of hmuO is also likely to be located in this promoter region (upstream of –35). As the two response regulators ChrA and HrrA share significant sequence identity (62 % sequence identity in the DNA-binding helix–turn–helix motif), it can be speculated whether both regulators might bind to overlapping or even identical regions with different affinities – a question which will be addressed in following studies.

Although the HrrSA and ChrSA systems share high sequence similarity, the genomic organization differs. In contrast with chrSa, hrrA expression is repressed by the global iron regulator DtxR under iron sufficiency and is derepressed when iron becomes limiting (Wennerhold & Bott, 2006) (Fig. 6). This control of hrrA by DtxR seems not to be present in C. diphtheriae (Bibb et al., 2007). Variations in the regulatory network composition in these closely related species may be surprising; however, sequence analysis revealed striking differences between Corynebacterium species regarding their TCS equipment (Bott & Brocker, 2012). With respect to HrrSA and ChrSA, several corynebacterial genomes contain only one of the two systems; both systems together were identified in the C. glutamicum species C. diphtheriae, C. pseudotuberculosis and C. lipophiloflavum (Bott & Brocker, 2012; Cerdeno-Tarraga et al., 2003; Kalinowski et al., 2003; Trost et al., 2010; Yukawa et al., 2007). These findings indicate significant variation at the species level and suggest an individual network constitution meeting the requirements of each particular species.

The fact that both TCSs HrrSA and ChrSA are involved in haem-dependent gene regulation already suggests that the two systems might interact with each other. Here, we provided evidence for a cross-regulation of HrrSA and ChrSA at the level of transcription. In our previous studies, we observed a weak binding of HrrA to the intergenic region of hrtBA–chrSA (Frunzke et al., 2011). This result is further supported by the finding that expression from PchrSa is increased in a ΔhrrSA mutant indicating repression of chrSa by the homologous system (Fig. 5). This effect is especially obvious under iron limitation where HrrSA seems to be the dominating system ensuring additional iron supply from haem by the activation of haem oxygenase (Fig. 5). Additionally, our data suggest haem-dependent repression of hrrA by ChrA. Our current model shown in Fig. 6 emphasizes that this cross-regulation acts as a balancing act to avoid toxic levels on the one hand and ensure iron acquisition on the other hand. Remarkably, this cross-regulation only affects the expression of hrrA and not hrrS, which seems to be expressed at a constitutively low level; no significant difference in the level of hrrS mRNA was observed in transcriptome comparisons of ΔchrSA and wild-type. A further level of interaction was suggested in previous studies of the C. diphtheriae systems, which provided evidence for in vivo cross-talk by phosphoryl transfer of HrrSA and ChrSA (Bibb et al., 2005, 2007). Altogether, these data provide striking evidence for a close link between the HrrSA and ChrSA systems and further studies are required to understand the interplay between these TCSs and the physiological relevance thereof.

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