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Control of Heme Homeostasis in *Corynebacterium glutamicum* by the Two-Component System HrrSA▽†

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The response regulator HrrA of the HrrSA two-component system (previously named CgtSR11) was recently found to be repressed by the global iron-dependent regulator DtxR in *Corynebacterium glutamicum*. Here, we provide evidence that HrrA mediates heme-dependent gene regulation in this nonpathogenic soil bacterium. Growth experiments and DNA microarray analysis revealed that *C. glutamicum* is able to use hemin as an alternative iron source and emphasize the involvement of the putative hemin ABC transporter HmuTUV and heme oxygenase (HmuO) in heme utilization. As a central part of this study, we investigated the regulon of the response regulator HrrA via comparative transcriptome analysis of an *hrrA* deletion mutant and *C. glutamicum* wild-type strain in combination with DNA-protein interaction studies with purified HrrA protein. Our data provide evidence for a heme-dependent transcriptional activation of heme oxygenase. Based on our results, it can be furthermore deduced that HrrA activates the expression of heme-containing components of the respiratory chain, namely, *ctaD* and the *ctaE-qcrCAB* operon encoding subunits I and III of cytochrome aa_3 oxidase and three subunits of the cytochrome bc_1 complex. In addition, HrrA was found to repress almost all genes involved in heme biosynthesis, including those for glutamyl-tRNA reductase (*hemA*), uroporphyrinogen decarboxylase (*hemE*), and ferrochelatase (*hemH*). Growth experiments with an *hrrA* deletion mutant showed that this strain is significantly impaired in heme utilization. In summary, our results provide evidence for a central role of the HrrSA system in the control of heme homeostasis in *C. glutamicum*.

Iron is a critical element for bacteria, being essential as a cofactor in a multitude of enzymes, poorly soluble in its oxidized form, and dangerous as ferrous iron by catalyzing the formation of reactive oxygen species. To avoid deprivation as well as high, toxic intracellular iron concentrations, organisms have evolved sophisticated regulatory systems. Previous studies demonstrated the transcriptional regulator DtxR as being the master regulator of iron-dependent gene expression in *Corynebacterium glutamicum*, a Gram-positive soil bacterium which is used industrially for large-scale amino acid production (13, 16). When the iron supply is sufficient, DtxR in complex with Fe^{2+} represses more than 50 genes, the majority of which are involved in iron acquisition, such as siderophore ABC transporters and siderophore binding proteins (8, 12, 49). Simultaneously, a number of genes are activated by DtxR (e.g., genes encoding the iron storage proteins ferritin and Dps). When iron becomes limiting, Fe^{2+} dissociates from DtxR and the protein loses its DNA-binding ability (14). Among the genes repressed by DtxR are also the putative hemin transport locus *hmuTUV* and the *hmuO* gene encoding heme oxygenase. Heme oxygenases are involved in the utilization of heme as an iron source by catalyzing the degradation of the tetrapyrrole ring to α-biliverdin, carbon monoxide, and free iron (40, 52). In *Corynebacterium diphtheriae*, the ABC transporter HmuTUV was shown to be required for the uptake of hemin, the oxidized form of heme as present in extracellular environments (1, 15, 26).

In *C. glutamicum*, four of the target genes repressed by DtxR under iron excess encode for themselves transcriptional regulators, which are the AraC-type regulator RipA, the ArsR-type regulators GlyR and Cg3082, and the response regulator HrrA, belonging to the HrrSA two-component system (previously named CgtSR11). Under iron limitation, when DtxR-mediated repression is relieved, RipA acts as a repressor of several genes encoding prominent iron-containing proteins (e.g., aconitase and succinate dehydrogenase) (50). GlyR was shown to activate expression of serine hydroxymethyltransferase; however, the link to iron metabolism is still not evident (41). The function of the two-component system HrrSA in *C. glutamicum* has not been studied yet. The genome of *C. glutamicum* encodes 13 two-component systems, some of which (MtrAB, PhoRS, and CitAB) have already been studied (10, 11, 25, 29, 37). Prototypical two-component systems consist of a response regulator and a cognate sensor histidine kinase; both proteins communicate via phosphorylation. Environmental signals influence the ability of the sensor protein to bring about the phosphorylation and dephosphorylation of the response regulator, which modulates gene expression (27, 45, 51).

The two-component system HrrSA of *C. diphtheriae*, which shows the highest sequence identity to *C. glutamicum* HrrSA (sensor kinases, 56%; response regulators, 86%), was shown to be involved in the heme-dependent activation of *hmuO* and also acts as repressor of *hemA* encoding glutamyl-tRNA re-

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	Source or reference
Strains		
C. glutamicum		
ATCC 13032	Biotin-auxotrophic wild type	23
13032Δ*hrrSA* mutant	In-frame deletion of the genes cg3247 and cg3248	25
13032Δ*hrrA* mutant	In-frame deletion of cg3247	This study
13032Δ*hmu* mutant	In-frame deletion of the *hmu* operon (cg0466-cg0469)	This study
E. coli		
DH5α	*supE44* Δ*lacU169* (f80*lacZ*DM15) *hsdR17 recA1 endA1 gyrA96 thi-1 relA1*	Invitrogen
BL21(DE3)	F^- *ompT* $hsdS_B(r_B^- m_B^-)$ *gal dcm* (DE3)	47
Plasmids		
pK19*mobsacB*	Kan^r; vector for allelic exchange in *C. glutamicum* (pK18 $oriV_{E.coli}$ *sacB lacZ*α)	38
pK19*mobsacB*-Δ*hrrA*	Kan^r; pK19*mobsacB* derivative containing an overlap extension PCR product covering the up- and downstream regions of *hrrA* (cg3247)	This study
pK19*mobsacB*-Δ*hmu*	Kan^r; pK19*mobsacB* derivative containing a overlap extension PCR product covering the up- and downstream regions of the *hmu* operon (*htaA hmuT hmuU hmuV* cg0466-cg0469)	This study
pK19*mobsacB*-Δ*hmuO*	Kan^r; pK19*mobsacB* derivative containing a overlap extension PCR product covering the up- and downstream regions of *hmuO* (cg2445)	This study
pMal-c	Amp^r P_{tac} *lacI*^{q} ColE1 *oriV*; *E. coli* expression vector for overproduction of *E. coli* MBP (MalE) fusion proteins without signal peptide	New England Biolabs
pMBP-HrrSΔ1-248	Amp^r; pMal-c derivative for overproduction of the HrrS kinase domain (residues 249–487) fused to the C terminus of *E. coli* MBP	This study
pET28b	Kan^r; vector for overexpression of genes in *E. coli*, adding an N-terminal or a C-terminal hexahistidine tag to the synthesized protein (pBR322 $oriV_{E. coli}$ P_{T7} *lacI*)	Novagen
pET28b-hrrA	Kan^r pET28b-Streptag derivative for overproduction of HrrA (Cg3247) with an N-terminal hexahistidine tag	This study

ductase, a heme biosynthesis enzyme (5). A second two-component system involved in heme-dependent expression of *hmuO* in *C. diphtheriae* is the ChrSA system, consisting of the response regulator ChrA and the sensor kinase ChrS (4, 5, 39). Recent studies of ChrS signal sensing postulated a mechanism by which autophosphorylation of the conserved histidine residue of ChrS is triggered by the direct interaction of heme with the N-terminal sensor domain of ChrS (6, 20). The mechanism of HrrS activation has not been studied yet.

In this study, we show via a combination of comparative transcriptomics with DNA-protein interaction studies that the response regulator HrrA of *C. glutamicum* on one hand activates expression of genes coding for heme oxygenase and heme-containing components of the respiratory chain and on the other hand represses transcription of operons encoding enzymes involved in heme biosynthesis. These results present comprehensive insights into the HrrA regulon and provide evidence for a global function of the HrrSA two-component system in the control of heme homeostasis in *C. glutamicum*.

MATERIALS AND METHODS

Bacterial strains, media, and growth conditions. The bacterial strains and plasmids used or constructed in this work are listed in Table 1. For growth experiments, 5 ml of brain heart infusion (BHI) medium (Difco Laboratories, Detroit, MI) was inoculated with *C. glutamicum* colonies from a fresh BHIS agar (BHI agar with 0.5 M sorbitol) plate and incubated overnight at 30°C and 170 rpm. This preculture was used to inoculate the main culture consisting of 50 ml CGXII minimal medium (21) with 4% (wt/vol) glucose, 250 μM ferrous iron chelator 2,2′-dipyridyl, and either 2.5 μM $FeSO_4$ or 2.5 μM hemin (Sigma-Aldrich) to an optical density at 600 nm (OD_{600}) of about 1. The trace element solution and the iron source were added after autoclaving. A 1 mM hemin stock solution was prepared in 100 mM KOH and stored at 4°C. For growth on plates, *C. glutamicum* strains grown in BHIS preculture were adjusted to an OD_{600} of about 1, and serial dilutions (10^0 to 10^{-7}) were spotted (5 μl each) on CGXII minimal medium plates, which were prepared as described for liquid cultures with an additional 1.5% (wt/vol) agar. For DNA microarray analysis, cells were harvested in the exponential growth phase at an OD_{600} of 5 to 6. *Escherichia coli* DH5α or BL21(DE3) cells were grown aerobically in LB medium on a rotary shaker (150 rpm) or on LB agar plates at 37°C (36). When appropriate, the media contained kanamycin (25 μg ml^{-1} for *C. glutamicum* or 50 μg ml^{-1} for *E. coli*) or ampicillin (100 μg ml^{-1} for *E. coli*).

Recombinant DNA work. Standard methods like PCR, restriction, or ligation were carried out according to established protocols (36). *E. coli* was transformed by the RbCl method (19). DNA sequencing was performed by Agowa (Berlin, Germany). The oligonucleotides were synthesized by Eurofins MWG Operon (Ebersfeld, Germany) and are listed in Table S1 in the supplemental material.

In-frame deletion mutants of the genes *hrrA* (cg3247) and *hmuO* (cg2445), as well as the *hmu* operon (genes *htaA* [cg0466], *hmuT* [cg0467], *hmuU* [cg0468], and *hmuV* [cg0469]), were constructed via the two-step homologous recombination procedure as described previously (31). Here, the procedure will be exemplified for *hrrA*. The deletion methods used for *hmuO* and the *hmu* operon were performed comparably; the same oligonucleotide nomenclature was used. Briefly, the corresponding up- and downstream regions were amplified with the oligonucleotide pairs DhrrA-1/DhrrA-2 and DhrrA-3/DhrrA-4. The resulting PCR products served as the template for an overlap extension PCR with the oligonucleotides DhrrA-1 and DhrrA-4. The resulting PCR products of ca. 1 kb were digested with XmaI and XbaI and cloned into the vector pK19*mobsacB*. The pK19*mobsacB* derivatives pK19*mobsacB*-Δ*hrrA*, pK19*mobsacB*-Δ*hmu*, and pK19*mobsacB*-Δ*hmuO* were then suitable for performing an allelic exchange by homologous recombination in the chromosome of *C. glutamicum* ATCC 13032 (38), resulting in the respective *C. glutamicum* Δ*hrrA*, Δ*hmu*, and Δ*hmuO* mutant strains.

For overproduction and purification of HrrA with an N-terminal hexahistidine tag, the *hrrA* coding region was cloned into the expression vector pET28b (Novagen). The gene *hrrA* (cg3247) was amplified with the oligonucleotides hrrA-for and hrrA-rev, thereby introducing restriction sites for NdeI and XhoI. The HrrA

protein encoded by the resulting plasmid, pET28b-hrrA, contains 20 additional amino acids (MGSSHHHHHHSSGLVPRGSH) at the N terminus. For overproduction, the plasmid was transferred into *E. coli* BL21(DE3).

For phosphorylation studies with the sensor kinase HrrS, the DNA region encoding the kinase domain (amino acid residues 249 to 487) was amplified with the oligonucleotides HrrS_K_for and HrrS_K_rev, thereby introducing EcoRI and HindIII restriction sites. Subsequently, the fragment was cloned into the expression vector pMal-c, resulting in the plasmid pMBP-HrrSΔ1–248, which enables the expression of the HrrS kinase domain fused to the C terminus of maltose binding protein (MBP) lacking its signal peptide.

Global gene expression analysis. DNA microarray analysis was used to compare the genome-wide mRNA levels of *C. glutamicum* grown on heme versus $FeSO_4$ as the iron source. In a second experiment, the transcriptomes of the *C. glutamicum* wild type and the Δ*hrrA* mutant were compared. For RNA preparation, the strains were first cultivated overnight in CGXII minimal medium containing 4% (wt/vol) glucose and 1 μM $FeSO_4$ as the iron source. For the main culture, cells from 1 μM $FeSO_4$ precultures were used to inoculate CGXII minimal medium with 4% (wt/vol) glucose and either 2.5 μM $FeSO_4$ or 2.5 μM hemin as the iron source. At an OD_{600} of 5 to 6, 20 ml of the cultures was poured into ice-containing tubes precooled to −20°C and cells were harvested by centrifugation (4 min, 4,200 × *g*, 4°C). The cell pellets were directly used for RNA isolation as described before (29) or stored at −20°C. All DNA microarray analyses were performed with custom-made DNA microarrays based on 70-mer oligonucleotides obtained from Operon Biotechnologies. Three independent biological replicates were performed. The experimental details for handling these microarrays were described before (18).

Overproduction and purification of HrrA. *E. coli* BL21(DE3) carrying the plasmid pET28b-*hrrA* was grown in 200 ml LB medium with 50 μg/ml kanamycin at 37°C. Expression was induced at an OD_{600} of ~0.7 with 1 mM IPTG (isopropyl-β-D-thiogalactopyranoside), and incubation was continued at 30°C. Four hours after induction, cells were harvested by centrifugation and stored at −20°C. For cell extract preparation, cells were resuspended in 3 ml of TNI-5 buffer (20 mM Tris-HCl, pH 7.9, 300 mM NaCl, 5 mM imidazole) containing Complete protease inhibitor cocktail (Roche Diagnostics). The cells were disrupted by being passed three times through a French pressure cell (SLM Aminco, Spectronic Instruments, Rochester, NY) at 207 MPa followed by centrifugation (20 min, 5,000 × *g*, 4°C). The supernatant was subjected to ultracentrifugation (1 h, 150,000 × *g*, 4°C). HrrA present in the supernatant was purified by nickel-activated nitrilotriacetic acid-agarose (Novagen). After the column had been washed with TNI-20 buffer (containing 20 mM imidazole), HrrA protein was eluted with TNI-200 buffer (containing 200 mM imidazole). Fractions containing HrrA were pooled, and the elution buffer was exchanged against bandshift buffer (20 mM Tris-HCl, pH 7.5, 50 mM KCl, 10 mM $MgCl_2$, 5% [vol/vol] glycerol, 0.5 mM EDTA).

Overproduction and purification of MBP-HrrSΔ1–248. For phosphorylation studies, MBP-HrrSΔ1–248 was overproduced in *E. coli* BL21(DE3). Cultivation, cell disruption, and the preparation of the soluble supernatant were performed as described above for HrrA. MBP-HrrSΔ1–248 present in the supernatant after ultracentrifugation was purified by affinity chromatography on amylose resin (New England BioLabs) equilibrated with TNM buffer (50 mM Tris-HCl, pH 8.0, 200 mM NaCl, 5 mM $MgCl_2$). After being washed with 15 column volumes of TNM buffer, MBP-HrrSΔ1–248 was eluted with 3 column volumes of TNM buffer containing 10 mM maltose. Fractions containing MBP-HrrSΔ1–248 were pooled, the buffer was exchanged against bandshift buffer, and the purified protein was kept at 4°C and immediately used for phosphorylation studies. After storage for about 2 days, activity was significantly reduced.

***In vitro* phosphorylation assays.** To determine its autophosphorylation activity, 12 μM MBP-HrrSΔ1–248 was incubated with 0.25 μM [γ-^{33}P]ATP (10 mCi/ml; GE Healthcare) and 80 μM nonradioactive ATP. The assay mixture was incubated at room temperature, and at different time points aliquots were removed, mixed with an equal volume of 2× SDS loading buffer (124 mM Tris-HCl, pH 6.8, 20% [vol/vol] glycerol, 4.6% [wt/vol] SDS, 1.4 M β-mercaptoethanol, 0.01% [wt/vol] bromophenol blue), and kept on ice. For analysis of phosphorylation of HrrA by MBP-HrrSΔ1–248, a 2-fold molar excess of purified HrrA was added to MBP-HrrSΔ1–248 that had been preincubated for 20 min with [γ-^{33}P]ATP, and the mixture was incubated for another 60 min. At different time points, samples were taken (taking into consideration the reduced MBP-HrrSΔ1–248 concentration) and handled as described above. Subsequently, without prior heating, the samples were subjected to 12% SDS-PAGE. The dried gel was analyzed with a BAS-1800 phosphorimager (Fujifilm).

Gel shift assays. For testing the binding of HrrA to putative target promoters, purified protein (0 to 380 nM) was mixed with DNA fragments (500 bp; final concentration, 15 nM) in a total volume of 20 μl. The binding buffer contained 20 mM Tris-HCl (pH 7.5), 50 mM KCl, 10 mM $MgCl_2$, 5% (vol/vol) glycerol, 0.5 mM EDTA, and 0.005% (wt/vol) Triton X-100. Approximately 15 nM (100 ng/lane, 500-bp fragments) promoter regions of putative nontarget genes (*cytP*, cg0645, and *pck*) was used as negative controls. For phosphorylation of HrrA, the protein had been incubated for 60 min with MBP-HrrSΔ1–248 (half the concentration of HrrA) and 5 mM ATP before the DNA fragments were added. The reaction mixture was incubated at room temperature for 20 min and then loaded onto a 10% nondenaturing polyacrylamide gel. Electrophoresis was performed at room temperature and 170 V with 1× TBE (89 mM Tris base, 89 mM boric acid, 2 mM EDTA) as the electrophoresis buffer. The gels were subsequently stained with Sybr green I (Sigma-Aldrich). All PCR products used in the gel shift assays were purified with the Qiagen PCR purification kit.

Microarray data accession number. Processed and normalized data as well as experimental details conformed to the MIAME guidelines (9) and were stored in the in-house microarray database (35) for further analysis and in the Gene Expression Omnibus (GEO) repository under accession no. GSE26122.

RESULTS

The *C. glutamicum* HrrSA (CgtSR11) two-component system—genomic organization and sequence similarities. The derepression of *cgtR11* (cg3247) encoding the response regulator CgtR11 under iron limitation suggested that the CgtSR11 system (here renamed HrrSA) might be involved in some aspect related to iron acquisition (12, 49). A search for orthologous proteins of CgtR11 and CgtS11 revealed a high similarity to the two-component systems HrrSA and ChrSA of *C. diphtheriae*. CgtS11 shows 56% and 30% sequence identity to HrrS and ChrS, respectively, and CgtR11 shows 86% and 50% sequence identity to HrrA and ChrA, respectively. Both two-component systems of *C. diphtheriae* were recently shown to be involved in the heme-dependent activation of *hmuO* expression and repression of *hemA* transcription (5). Due to the high sequence similarity to HrrSA and ChrSA, an involvement of the *C. glutamicum* CgtSR11 system in the control of genes required for heme utilization appeared likely. To stay consistent with the described orthologous system of *C. diphtheriae*, we decided to rename CgtSR11 as "HrrSA" (*h*eme-*r*esponsive *r*egulator *s*ensor and *a*ctivator).

The gene *hrrS* (cg3248) encoding the sensor kinase HrrS is located upstream of and codirectional with *hrrA* (cg3247) coding for the response regulator HrrA. The two genes are separated by an intergenic region of 98 bp. We previously showed that *hrrA* is repressed by the iron-dependent regulator DtxR under conditions of sufficient iron supply and that purified DtxR binds to a DNA fragment covering the intergenic region (49). The most likely DtxR binding site (ATGAGTAAGGCT AGACTAA) is centered 97 bp upstream of the start codon of *hrrA* and partially overlaps with the coding region of *hrrS*. Thus, only expression of *hrrA*, but not that of *hrrS*, is regulated by DtxR in response to iron availability.

Heme utilization of *C. glutamicum*. In the first series of experiments, we tested whether the nonpathogenic organism *C. glutamicum* is able to use heme as an iron source. The type strain ATCC 13032 was cultivated on CGXII agar plates (Fig. 1A and B) or in liquid culture with CGXII minimal medium (Fig. 1C and D) containing either 2.5 μM $FeSO_4$ or 2.5 μM hemin as the iron source. In all experiments, 250 μM 2,2′-dipyridyl was added as a chelator of ferrous iron to ensure iron limitation of the cells. As shown in Fig. 1C and D, the liquid cultures grew with almost identical growth rates up to an OD_{600} of about 20. As high concentrations of hemin (>5 μM) are toxic for *C. glutamicum*, cells were generally grown under

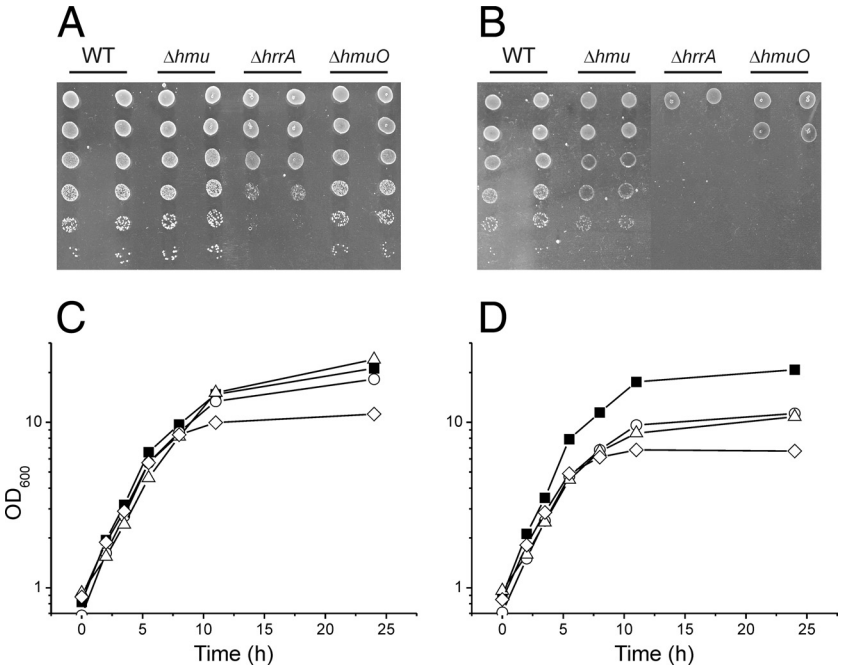

FIG. 1. Growth of the *C. glutamicum* ATCC 13032 wild-type (WT) strain and Δ*hmu*, Δ*hrrA*, and Δ*hmuO* deletion mutants on agar plates (A and B) or in liquid culture (C and D). For panels A and B, serial dilutions of the indicated strains were spotted on CGXII minimal medium plates containing either 2.5 μM $FeSO_4$ (A) or 2.5 μM hemin (B) as the iron source. The plates were incubated at 30°C for 48 h. For panels C and D, the *C. glutamicum* wild-type strain (filled squares) and Δ*hrrA* (triangles), Δ*hmuO* (diamonds), and Δ*hmu* (circles) mutants were cultivated in CGXII minimal medium with 4% (wt/vol) glucose and either 2.5 μM $FeSO_4$ (C) or 2.5 μM hemin (D) as the iron source. When CGXII minimal medium without an iron source was inoculated with wild-type cells, growth stopped at an OD_{600} of about 3 (data not shown).

iron limitation to compare different iron sources. Under nonlimiting conditions (36 μM $FeSO_4$), a typical *C. glutamicum* wild-type culture reaches a final OD_{600} of about 60. Further experiments could show that *C. glutamicum* is also able to extract heme iron from heme proteins such as hemoglobin (data not shown). In control experiments with CGXII medium lacking an iron source, cells stopped growing after approximately 1 to 2 duplications at an OD_{600} of about 3. This clearly demonstrated that growth on hemin (Fig. 1D) is not just a result of consumption of stored iron from the $FeSO_4$ preculture but that *C. glutamicum* is able to extract heme iron when hemin is added as the sole iron source.

Influence of heme on global gene expression. To identify genes of *C. glutamicum* necessary for heme utilization, we compared the transcriptomes of wild-type cells grown with either heme or $FeSO_4$ as the iron source. Table 2 shows a selection of genes upregulated in heme-grown cells (also see Table S2 in the supplemental material). Among the genes showing the highest upregulation on heme was the putative hemin transport operon cg0466-cg0469, including the putative hemin ABC transporter *hmuTUV* and the gene encoding the secreted hemin-binding protein HtaA. The genes *htaB* (cg0470) and *htaC* (cg0471), located downstream of the *hmu* operon, and *htaD* (cg3156) encode putative secreted hemin-binding proteins and also showed a significant upregulation in heme-grown cells. The mRNA level of *hmuO* encoding heme oxygenase, an enzyme necessary for the release of iron from the tetrapyrrol ring, was increased more than 10-fold on heme. In recent studies, the ABC transporter HrtAB was shown to be required for dealing with toxic heme concentrations and a role in heme efflux was postulated in several Gram-positive species (2). The orthologous genes *hrtA* and *hrtB* of *C. glutamicum* showed a high upregulation in heme-grown cells, consistent

TABLE 2. Genes differentially expressed in the *C. glutamicum* wild type grown with heme instead of iron sulfate as the sole iron source[a]

Gene ID	Gene name	Annotation	Heme/Fe ratio[b]
cg0465		Hypothetical protein	8.01
cg0466	*htaA*	Secreted heme transport-associated protein	8.66
cg0467	*hmuT*	Hemin-binding periplasmic protein precursor	7.67
cg0468	*hmuU*	Hemin transport system, permease protein	6.43
cg0469	*hmuV*	Hemin transport system, ATP-binding protein	5.55
cg0470	*htaB*	Secreted heme transport-associated protein	12.37
cg0471	*htaC*	Secreted heme transport-associated protein	5.71
cg1734	*hemH*	Ferrochelatase precursor	0.52
cg2202	*hrtB*	ABC-type transport system, permease component	3.45
cg2204	*hrtA*	ABC-type transport system, ATPase component	4.97
cg2444		Hypothetical protein	3.15
cg2445	*hmuO*	Heme oxygenase	10.88
cg3156	*htaD*	Secreted heme transport-associated protein	18.24

[a] This table shows genes with a putative heme-related function, such as heme uptake, utilization, or biosynthesis. A list of all genes with a ≥2-fold-altered mRNA level is provided in Table S2 in the supplemental material.

[b] Average mRNA ratio of genes showing a ≥2-fold-altered mRNA level (heme/Fe) of *C. glutamicum* cells grown in CGXII minimal medium with 2.5 μM hemin or 2.5 μM $FeSO_4$ as the iron source. The mRNA ratios represent average values from three independent DNA microarray experiments ($P \leq 0.05$).

with a similar function of this system in *C. glutamicum*. The only downregulated gene having a function related to heme metabolism is *hemH* encoding ferrochelatase, a protein involved in protoheme IX biosynthesis. Other genes of heme biosynthesis enzymes show no significantly changed mRNA ratio.

Role of *hmu* genes for heme utilization. Deletion of the *hmu* operon (cg0466-cg0469; *htaA*, *hmuT*, *hmuV*, and *hmuU*) encoding a putative hemin ABC transporter resulted in a slight but significant growth defect when hemin was supplied as the iron source (Fig. 1B and D). Both the growth rate and the final OD_{600} were reduced compared to those of the wild type in heme-containing media. A mutant with deletion of *hmuO* encoding heme oxygenase was significantly impaired when grown on hemin (Fig. 1B and D). These findings support a role for the Hmu ABC transporter in heme uptake and highlight the importance of heme oxygenase in recycling of heme iron, which apparently is also of relevance in $FeSO_4$-grown cells facing iron starvation. A *C. glutamicum* mutant lacking *htaB* and *htaC* in addition to the *hmu* operon showed the same growth phenotype as the Δ*hmu* mutant (data not shown).

Growth phenotype of an *hrrA* deletion mutant. In order to assess the function of the response regulator HrrA in iron-dependent gene regulation, an *hrrA* deletion mutant was constructed. In liquid culture, this Δ*hrrA* mutant showed a similar growth phenotype to the Δ*hmu* mutant (Fig. 1C): i.e., a reduced growth rate and final OD_{600} in heme-containing medium. However, when spotted on hemin plates, the Δ*hrrA* mutant showed an even stronger growth delay than the mutant lacking heme oxygenase and was also impaired when $FeSO_4$ was added as the iron source (Fig. 1A and B). These results suggest a key function of HrrA in the control of heme utilization and metabolism.

Transcriptome analysis of a Δ*hrrA* mutant. To identify the regulon of the response regulator HrrA, the transcriptomes of the Δ*hrrA* mutant and the wild type grown in CGXII minimal medium with either $FeSO_4$ or hemin were compared. Genes showing a more than 2-fold-altered mRNA level in the Δ*hrrA* mutant in $FeSO_4$- or heme-grown cells are summarized in Table S3 in the supplemental material. In Table 3, all genes with a putative function in heme metabolism are shown. Generally, the microarray data indicated that the Δ*hrrA* mutant shows a stronger iron starvation response than the wild type when cultivated with heme as the iron source. Several genes belonging to the DtxR regulon showed higher mRNA levels in the mutant strain, whereas genes of the RipA regulon coding for iron-containing proteins (e.g., succinate dehydrogenase or nitrate reductase) showed lower mRNA levels in the Δ*hrrA* mutant (see Table S3 in the supplemental material). This is most likely a result of the downregulation of genes involved in heme utilization, such as heme oxygenase. The corresponding *hmuO* gene, despite being a target of DtxR repression, showed a 20-fold-decreased mRNA level in the Δ*hrrA* mutant compared to the wild type when the cells were grown with heme as the iron source, whereas the *hmuO* mRNA level was 10-fold decreased on $FeSO_4$. Similar to *hmuO*, the mRNA levels of the *hmu* operon, *htaA*, and *htaD*, all of which are targets of DtxR, also are lowered in the Δ*hrrA* mutant. In contrast, the expression of *htaB* (cg0470) and *htaC* (cg0471), coding for putative heme binding proteins which are both DtxR targets, also showed an upregulation which could be explained by the increased iron starvation response in the mutant. Keeping in mind that the mRNA level of other DtxR targets was increased in the Δ*hrrA* mutant strain (see Table S3 in the supplemental material), this result supported our assumption that the HrrSA system is involved in heme-dependent activation of *hmuO* expression and might also be involved in the heme-dependent regulation of heme uptake systems.

Two genes belonging neither to the DtxR regulon nor to the typical iron starvation response are *hrtA* and *hrtB*, whose mRNA level was significantly increased in the Δ*hrrA* mutant. In several Gram-positive species, these genes are activated in a heme-dependent manner by a two-component system located in close vicinity to *hrtAB*. In fact, in *C. glutamicum* the genes encoding the two-component system CgtSR8 are found nearby and, interestingly, both genes show a 2-fold upregulation in the Δ*hrrA* mutant. Thus, the altered mRNA level of *hrtAB* might not be a direct effect of the *hrrA* deletion but rather an indirect effect caused by the altered mRNA level of *cgtSR8*.

Besides *hmuO*, several other genes involved in heme metabolism were differentially expressed in the Δ*hrrA* mutant. Almost all genes known to be involved in heme biosynthesis showed a ca. 2-fold-increased mRNA level in the mutant, during both growth with heme and that with $FeSO_4$. Among those are the genes encoding ferrochelatase (*hemH*), glutamyl-tRNA reductase (*hemA*), and uroporphyrinogen decarboxylase (*hemE*), which are the first genes of the heme biosynthesis operons *hemA-hemC* and *hemEYL*-cg0519-cg0520-*ccsA*-cg0523-*ccsB*, respectively (see also Fig. 4) (for operon prediction, see http://coryneregnet.cebitec.uni-bielefeld.de) (7). Even so, these genes are more or less equally upregulated in heme- and $FeSO_4$-grown cells; these data reveal that HrrA is involved in the repression of the heme biosynthesis machinery in *C. glutamicum*.

Another group of genes related to heme metabolism which showed an altered expression in the Δ*hrrA* mutant were those coding for subunits I and III of cytochrome aa_3 oxidase (*ctaD* and *ctaE*, respectively) and the three subunits of the cytochrome bc_1 complex (*qcrCAB*), which presumably form an operon with *ctaE* (31). In the Δ*hrrA* mutant, the *ctaE-qcrCAB* and *ctaD* mRNA levels showed a stronger decrease in heme-grown cells (2- to 3-fold) than in cells cultivated with $FeSO_4$ as the iron source (1.5- to 2-fold). These data suggest that expression of these genes might be positively regulated by the HrrSA system in the presence of heme in a direct or indirect manner. The cytochrome P450 and the Rieske-type iron-sulfur protein Cg3369 showed a similar expression pattern to that described for *ctaE-qcrCAB* and *ctaD*. Only the gene *hmp* encoding a flavohemoglobin showed an increased mRNA level in the Δ*hrrA* mutant. According to the results described above, HrrA might carry out a dual function in *C. glutamicum* by acting as an activator of genes for heme oxygenase and heme-containing proteins and as a repressor of genes involved in heme biosynthesis.

Phosphorylation of HrrA. In nearly all described cases, the response regulator of a two-component system is activated by phosphorylation of a conserved aspartate residue in the receiver domain. To test the phosphorylation activities of the HrrSA system, the kinase domain of HrrS (MBP-HrrSΔ1–248) and the full-length HrrA were overexpressed in *E. coli* and purified via affinity chromatography. To monitor auto-

TABLE 3. Comparative transcriptome analysis of *C. glutamicum* WT and the Δ*hrrA* mutant[a]

Gene ID	Gene name	Annotation	Δ*hrrA*/wild-type mRNA ratio[b]	
			Heme	$FeSO_4$
Two-component systems				
cg3247	*hrrA*	Two-component system, response regulator	0.02	0.02
cg2200	*cgtR8*	Two-component system, response regulator	1.79	2.13
cg2201	*cgtS8*	Two-component system, signal transduction histidine kinase	1.99	2.42
Heme transport/utilization				
cg0466	*htaA*	Secreted heme transport-associated protein	0.46	1.02*
cg0467	*hmuT*	Hemin-binding periplasmic protein precursor	0.57	1.03*
cg0468	*hmuU*	Hemin transport system, permease protein	0.60	1.04*
cg0469	*hmuV*	Hemin transport system, ATP-binding protein	0.86*	1.24*
cg0470	*htaB*	Secreted heme transport-associated protein	9.00	8.71*
cg0471	*htaC*	Secreted heme transport-associated protein	8.06	5.09
cg2202	*hrtB*	ABC-type transport system, permease component	10.90	40.79
cg2204	*hrtA*	ABC-type transport system, ATPase component	10.47	43.93
cg2445	*hmuO*	Heme oxygenase	0.06	0.12
cg3156	*htaD*	Secreted heme transport-associated protein	0.26	0.63*
Heme-containing proteins				
cg0645	*cytP*	Cytochrome P450	0.20	0.32
cg2403	*qcrB*	Cytochrome *b*, membrane protein	0.35	0.47
cg2404	*qcrA*	Rieske iron-sulfur protein	0.36	0.47
cg2405	*qcrC*	Cytochrome c_1	0.36	0.48
cg2406	*ctaE*	Cytochrome aa_3 oxidase, subunit 3	0.34	0.46
cg2780	*ctaD*	Cytochrome aa_3 oxidase, subunit 1	0.51	0.62
cg3141	*hmp*	Flavohemoprotein	2.74	1.95
cg3369		Rieske-type iron-sulfur protein	0.57	0.75*
Heme biosynthesis				
cg0497	*hemA*	Glutamyl-tRNA reductase	1.77*	1.44*
cg0498	*hemC*	Porphobilinogen deaminase	1.62*	1.19*
cg0516	*hemE*	Uroporphyrinogen decarboxylase	1.87	1.99
cg0517	*hemY*	Protoporphyrinogen oxidase	1.90*	2.04
cg0518	*hemL*	Glutamate-1-semialdehyde 2,1-aminomutase	1.77	2.10
cg0519		Putative phosphoglycerate mutase	1.59*	1.74
cg0520		Secreted thiol-disulfide isomerase or thioredoxin	1.51*	1.70
cg0522	*ccsA*	Cytochrome *c* biogenesis protein membrane protein	1.34*	1.53
cg0523		Membrane protein required for cytochrome *c* biosynthesis	1.37*	1.75
cg0524	*ccsB*	Cytochrome *c* assembly membrane protein	1.39*	1.90
cg1734	*hemH*	Ferrochelatase precursor	2.41*	2.20

[a] This table includes all genes with a putative heme/iron-related function that are differentially expressed in the Δ*hrrA* mutant. A list of all genes with a ≥2-fold-altered mRNA level is provided in Table S2 in the supplemental material. Genes and operons shown to be directly regulated by HrrA have all been included in this table, irrespective of their relative expression.

[b] Average ratio of genes showing a ≥2-fold-altered mRNA level (Δ*hrrA*/wild-type mRNA ratio) of *C. glutamicum* cells grown on CGXII minimal medium with 2.5 μM heme or 2.5 μM $FeSO_4$ as the iron source. The mRNA ratios represent average values from three independent DNA microarray experiments ($P \leq 0.05$). *, $P > 0.05$.

phosphorylation of MBP-HrrSΔ1–248, the protein was incubated with [γ-^{33}P]ATP and samples were taken at different time points and analyzed via SDS-PAGE and autoradiography. As shown in Fig. 2, MBP-HrrSΔ1–248 showed significant autophosphorylation after approximately 20 min. When HrrA was added, an efficient transfer of the phosphoryl group occurred and was detectable already after 1 min (Fig. 2). The two bands below MBP-HrrSΔ1–248 are most likely cleavage products which are often observed in the case of MBP fusion proteins.

Identification of direct target genes of HrrA. In order to test whether some of the genes showing an altered mRNA level in the Δ*hrrA* mutant are direct target genes of this response regulator, binding of purified HrrA protein to the promoter regions of selected genes with a putative heme-related function was tested in gel shift assays. As shown in Fig. 3A, HrrA was able to bind to the promoter regions of *hmuO*, *hemA*, *hemE*, *hemH*, *ctaD*, and *ctaE*. Depending on the DNA fragment tested, a complete shift was observed at a 20- to 40-fold molar excess (300 to 600 nM) of HrrA. No binding was observed when HrrA was incubated with the promoter regions of other genes which showed an altered mRNA level in the Δ*hrrA* mutant, including *htaA*, *htaB*, and *htaD* (putative heme binding proteins), cg0640 (ferredoxin), and *cytP* (cytochrome P450), or with the promoter regions of genes (*sdhC*, *narK*, and *pck*) that served as a negative control. A weak but significant binding was also observed with the promoter region of *cgtS8*. However, further experiments have to verify whether HrrA directly regulates expression of the *cgtSR8* two-component system (Fig. 3). All gel shifts shown in Fig. 3A were carried out with HrrA protein that had been phosphorylated by preincubation with ATP and the purified HrrS kinase domain (MBP-HrrSΔ1–

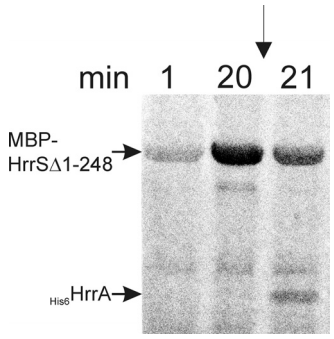

FIG. 2. Autophosphorylation of the kinase domain MBP-HrrSΔ1–248 and phosphoryl group transfer to the response regulator HrrA. Purified MBP-HrrSΔ1–248 (12 μM final concentration) was incubated with [γ-^{33}P]ATP for 20 min (A). Then the purified response regulators HrrA was added (12 μM final concentration), resulting in a 2-fold dilution of MBP-HrrSΔ1-248 and the ATP. The samples were incubated at room temperature for a further 60 min. At the indicated time points, samples were taken, mixed with SDS loading buffer, and stored on ice (see Materials and Methods). Finally, these samples were separated by SDS-PAGE and the dried gels were analyzed with a phosphorimager.

248), as described in Materials and Methods. We decided to do so since we observed an approximately 2-fold-increased binding affinity of the phosphorylated HrrA protein in comparison to that of unphosphorylated HrrA for the DNA fragments tested (Fig. 3B). Figure 3B shows two examples: *ctaE*, which is presumably activated by HrrA; and *hemH*, which is presumably repressed by HrrA. In both cases, the difference is not striking but significant and was also observed with the other HrrA target promoters. The comparatively weak effect might be due to an incomplete phosphorylation of HrrA upon incubation with MBP-HrrSΔ1–248. Nevertheless, *in vivo* small differences in the binding affinity of a transcriptional regulator may already significantly alter gene expression.

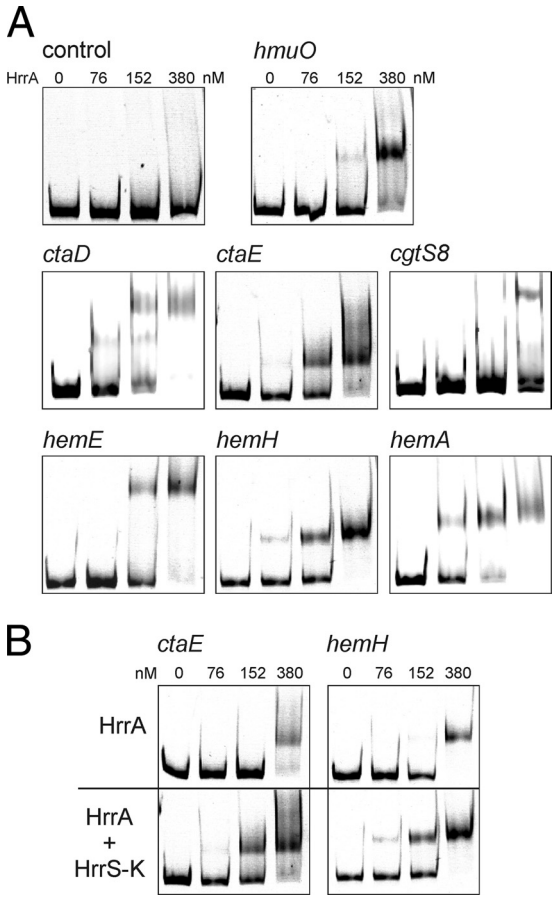

FIG. 3. Identification of direct target genes of HrrA in *C. glutamicum*. (A) DNA fragments (500 bp) covering the promoter region of *hmuO*, *ctaE*, *cgtS8*, *hemE*, *hemH*, and *hemA* were incubated without or with a 5, 10, and 25 molar excess of (partially) phosphorylated HrrA protein (0 to 380 nM), as described in Materials and Methods. After incubation, samples were separated on a 10% nondenaturating polyacrylamide gel and stained with Sybr green I. DNA fragments covering the promoter region of *cytP* or *pck* served as a negative control. (B) DNA fragments containing the promoter region of a gene postulated to be activated by HrrA (*ctaE*) and one repressed by HrrA (*hemH*) were incubated with equal amounts of unphosphorylated HrrA (upper gels) or HrrA that had been phosphorylated by preincubation with MBP-HrrSΔ1–248 (HrrS-K) and ATP. These examples clearly demonstrate increased binding affinity of the phosphorylated response regulator to its target promoters.

DISCUSSION

Most organisms have evolved complex regulatory networks ensuring an adequate supply of iron, which is essential for central metabolic processes, such as the tricarboxylic acid (TCA) cycle, respiration, and DNA biosynthesis. Especially in pathogenic bacteria, several heme-responsive regulatory systems have been described consisting of classic transcriptional regulators (30, 33, 53), two-component systems (5, 20, 34, 42, 43), and extracytoplasmic sigma factors (22, 24, 48). In this work, we investigated the role of the HrrSA two-component system in the regulation of iron homeostasis in the Gram-positive soil bacterium *C. glutamicum*. Recent studies on the homologous system HrrSA in *C. diphtheriae* provided detailed insights into the heme-dependent regulation of *hmuO* and *hemA*, which were both postulated to be direct targets of the response regulator HrrA (4, 5, 39). We used DNA microarray analysis of a Δ*hrrA* deletion mutant and DNA-protein interaction studies with purified HrrA protein to demonstrate a direct regulation of at least 20 genes encoding proteins involved in heme metabolism in *C. glutamicum*. Based on our results, it can be deduced that the response regulator HrrA directly activates expression of the *hmuO* gene coding for heme oxygenase, an enzyme catalyzing the degradation of the tetrapyrrole ring to release iron. Furthermore, HrrA acts as a heme-dependent activator of *ctaD* and the putative operon *ctaE-qcrBAC*. These genes encode subunits I (CtaD) and III (CtaE) of cytochrome aa_3 oxidase and the three subunits of the cytochrome bc_1 complex (QcrCAB). Previous studies revealed that these two complexes form a supercomplex in *C. glutamicum* (32). Each of the proteins CtaD, QcrB, and QcrC contains two heme moieties (heme *a* and a_3 in the case of CtaD, two heme *b* moieties in the case of QcrB, and two heme *c* moieties in the case of QcrC). Thus, HrrSA not only is involved in the release of heme iron by the activation of heme oxygenase but also activates expression of heme-containing protein complexes of the respiratory chain when heme is available.

A regulation of heme-containing proteins has not yet been described for the *C. diphtheriae* HrrSA system; however, repression of the gene *hemA* encoding glutamyl-tRNA reductase, the first enzyme of heme biosynthesis, was recently reported (5). Besides the *hemA-hemC* operon, our results provide evidence that HrrA, in fact, is involved in the repression of almost the whole set of genes involved in heme biosynthesis in *C. glutamicum* (except *hemB* and *hemD*). Based on these data, it can be assumed that HrrSA downregulates energy-demanding heme biosynthesis in *C. glutamicum* when heme can be acquired by the uptake of extracellular heme sources.

Interestingly, transcriptome analysis and *in vitro* gel shift experiments suggested direct repression of the two-component system CgtSR8 by HrrA. In a number of distinct Gram-positive bacteria, a microsynteny is observed at this genomic locus (43): two genes encoding a prototypical two-component system are located in close vicinity to *hrtAB* encoding an ABC transporter that was supposed to be required for coping with high, toxic hemin concentrations (42–44). The two-component systems encoded by these genes, HssRS in *Staphylococcus aureus* and *Bacillus anthracis* and ChrSA in *C. diphtheriae*, were recently shown to be involved in the activation of *hrtAB* expression in the presence of heme (6, 42, 44). Sequence analysis revealed a high sequence identity of CgtSR8 to the ChrSA and HrrSA systems of *C. diphtheriae*: thus, a similar function of CgtSR8 in control of *hrtAB* transcription (cg2204 and cg2202) and potentially other heme-related targets can be envisaged. Since the potential HrrA binding site seems to be located in the intergenic region of *cgtS8-hrtA*, a direct regulation of *hrtAB* by HrrA cannot be excluded based on our data. However, the significance of HrrA binding to this region needs to be confirmed by additional studies as our gel shift experiments revealed a comparably low binding affinity of HrrA to the corresponding fragment. Further experiments will focus on the investigation how *C. glutamicum* HrrSA and CgtSR8 are involved in transcription control of their own promoters as well as *hrtAB* in response to heme availability.

As expected for a heme-dependent two-component system, our transcriptome studies with a Δ*hrrA* deletion mutant revealed a stronger effect on the mRNA level of *hmuO*, *ctaD*, and *ctaE-qcrCAB* in heme-grown cells than in cells cultivated on $FeSO_4$, when the system is supposed to be less active (Table 3). This was not the case for the mRNA ratio observed for heme biosynthesis genes or *cgtSR8*, which are also supposed to be directly regulated by HrrSA, but show rather equally altered mRNA levels in both experiments. In *C. diphtheriae*, *hmuO* and *hemA* expression is regulated by intensive cross talk of the two-component systems HrrSA and ChrSA (5). In *C. glutamicum*, a putative cross talk with other systems, such as CgtSR8, might also play an important role in heme-dependent regulation of some target genes and might partially compensate for *hrrSA* deletion.

Although the *C. diphtheriae* and *C. glutamicum* HrrSA proteins show high similarity with regard to sequence identity and function, a striking difference between the systems is that in *C. glutamicum hrrA* expression is repressed by the global iron regulator DtxR, which also downregulates expression of the *hmu* operon, *htaB*, *htaC*, and *htaD* (49) (Fig. 4). In contrast, neither *hrrSA* nor *chrSA* in *C. diphtheriae* was reported to be controlled by DtxR and inspection of the corresponding promoter regions failed to reveal the presence of a DtxR binding motif.

Especially for organisms with a pathogenic lifestyle, heme and heme-containing proteins such as hemoglobin represent important iron sources during host colonization. In this work, it was demonstrated for the first time that the nonpathogenic soil bacterium *C. glutamicum* is able to use hemin and hemoglobin (data not shown) as the sole iron source, thereby reaching the same growth rate and final OD_{600} as cells cultivated on an equivalent concentration of $FeSO_4$. Among the genes upregulated in heme-grown *C. glutamicum* cells was the *hmu* operon encoding a putative ABC hemin transporter, HmuTUV (17). The system shows striking similarity to the HmuTUV transporter of *C. diphtheriae*, which was shown to be important for the utilization of heme or hemoglobin as the iron source (15, 26). Recently, further hemin binding proteins (HtaA and HtaB) were identified that are involved in heme uptake of *C. diphtheriae* (1). Our DNA microarray analysis showed an upregulation of four putative *h*eme-*t*ransport *a*ssociated protein genes (*hta*-like), namely *htaA* (cg0466), *htaB* (cg0470), *htaC* (cg0471), and *htaD* (cg3156). Common features of Hta-like proteins are an N-terminal signal peptide, one or two HtaA domains (PF04213; two in the case of Cg0466), and a putative transmembrane helix at their C terminus. These typical structural features were found in *C. glutamicum* HtaB and HtaC as well as *C. diphtheriae* HtaA and HtaB or the heme binding proteins Shr and Shp of *Streptococcus pyogenes* (3, 28). In the case of *C. glutamicum* HtaA, a C-terminal transmembrane helix is predicted; none is present in HtaD. Based on the studies with *C. diphtheriae*, we can assume that these Hta proteins act as membrane-anchored or -associated hemin receptors which directly scavenge hemin from the environment and transfer it to the HmuT lipoprotein. HmuT then passes hemin to the permease HmuU, which together with the ATPase HmuV, catalyzes hemin uptake. Surprisingly, a *C. glutamicum* mutant missing the whole *hmu* operon and the genes *htaB* and *htaC* was still able to grow on hemin as the iron source (Fig. 1). However, for many organisms, including *C. diphtheriae*, *S. pyogenes*, and *Yersinia enterolytica*, it has been reported that mutations in the hemin transport systems did not result in a complete loss of the ability to use heme as the iron source (1, 3, 46). This indicates the existence of alternative heme uptake possibilities that can compensate for the absence of the Hmu ABC transporter to some extent. Besides heme uptake, heme oxygenase is required to make use of heme iron, and our analysis of a Δ*hmuO* mutant confirmed that this enzyme is important for utilization of exogeneous heme, but probably also for recycling of heme synthesized by *C. glutamicum* itself.

So far, several systems have been described which control expression of genes involved in heme utilization dependent on heme availability. Based on our current model, the response regulator HrrA of *C. glutamicum* functions as an activator of genes involved in heme utilization (heme oxygenase) and of genes encoding heme-containing components of the respiratory chain. On the other hand, HrrA represses transcription of heme biosynthesis genes (Fig. 4). Altogether, the investigation of the HrrA regulon adds to our understanding of the hierarchical regulatory network controlling iron homeostasis in *C.*

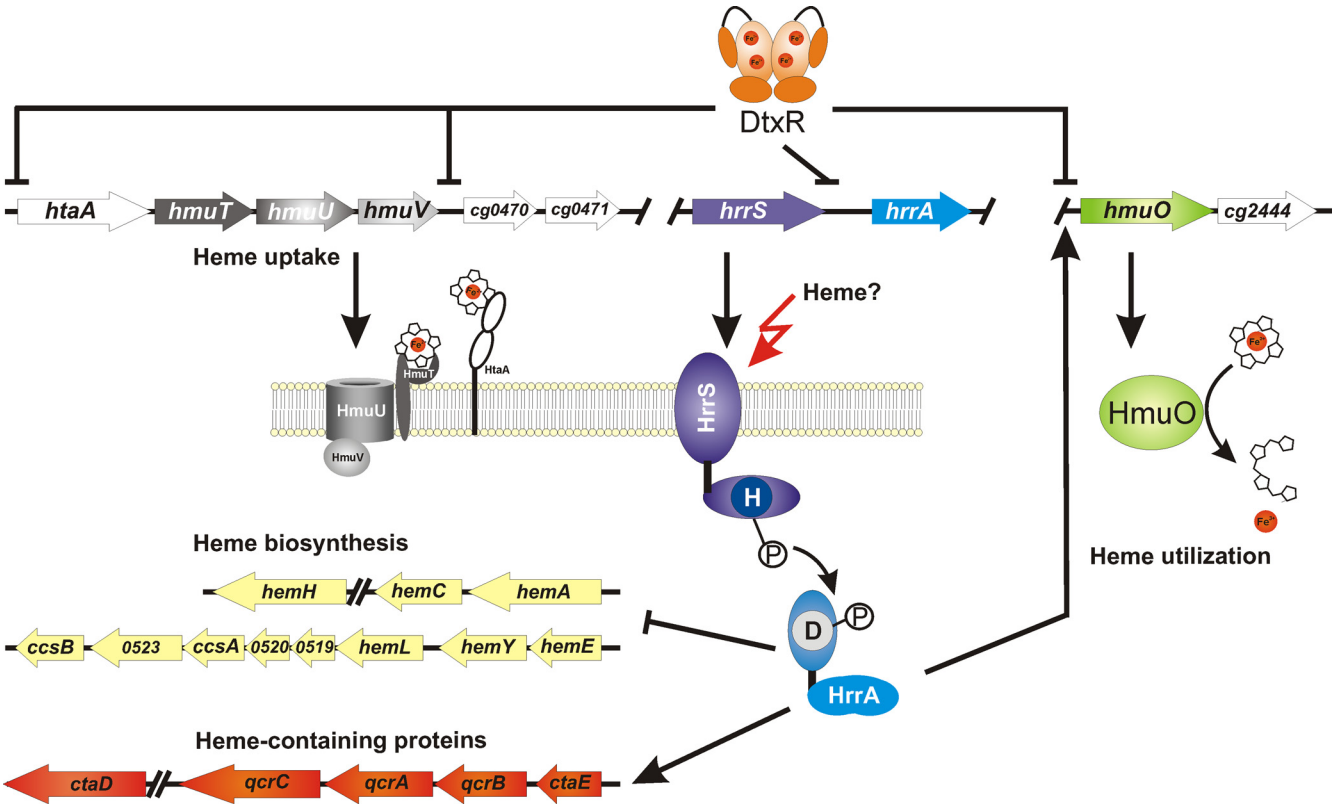

FIG. 4. Model for the regulation of heme homeostasis by the two-component system HrrSA in *C. glutamicum*. Under conditions of sufficient iron supply, the global iron regulator DtxR represses expression of the response regulator *hrrA*, heme uptake systems (*htaA* and *hmuTUV*), and heme oxygenase (*hmuO*), an enzyme involved in the release of heme iron. Under iron limitation, expression of *hrrA* increases. In this study, we could show that the two-component system HrrSA of *C. glutamicum* is involved in the heme-dependent activation of *hmuO* and genes encoding heme-containing protein complexes of the respiratory chain. Furthermore, HrrA directly represses transcription of genes coding for heme biosynthesis enzymes (for operon prediction, see http://coryneregnet.cebitec.uni-bielefeld.de). The signal sensed by the sensor kinase HrrS is not yet known, but heme itself or a heme-related metabolite is likely. Not included in this model is the postulated repression of the CgtSR8 two-component system by HrrSA, which is currently under investigation and might disclose a further level of complexity in this regulatory network.

glutamicum and highlights the importance of heme-dependent gene regulation in nonpathogenic bacteria.

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