Essential histidine pairs indicate conserved haem binding in epsilonproteobacterial cytochrome c haem lyases

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Bacterial cytochrome c maturation occurs at the outside of the cytoplasmic membrane, requires transport of haem b across the membrane, and depends on membrane-bound cytochrome c haem lyase (CCHL), an enzyme that catalyses covalent attachment of haem b to apocytochrome c. Epsilonproteobacteria such as Wolinella succinogenes use the cytochrome c biogenesis system II and contain unusually large CCHL proteins of about 900 amino acid residues that appear to be fusions of the CcsB and CcsA proteins found in other bacteria. CcsBA-type CCHLs have been proposed to act as haem transporters that contain two haem b coordination sites located at different sides of the membrane and formed by histidine pairs. W. succinogenes cells contain three CcsBA-type CCHL isoenzymes (NrfI, CcsA1 and CcsA2) that are known to differ in their specificity for apocytochromes and apparently recognize different haem c binding motifs such as CX2CH (by CcsA2), CX2CK (by NrfI) and CX15CH (by CcsA1). In this study, conserved histidine residues were individually replaced by alanine in each of the W. succinogenes CCHLs. Characterization of NrfI and CcsA1 variants in W. succinogenes demonstrated that a set of four histidines is essential for maturing the dedicated multiheme cytochromes C c NrfA and McC, respectively. The function of W. succinogenes CcsA2 variants produced in Escherichia coli was also found to depend on each of these four conserved histidine residues. The presence of imidazole in the growth medium of both W. succinogenes and E. coli rescued the cytochrome c biogenesis activity of most histidine variants, albeit to different extents, thereby implying the presence of two functionally distinct histidine pairs in each CCHL. The data support a model in which two conserved haem b binding sites are involved in haem transport catalysed by CcsBA-type CCHLs.

INTRODUCTION

Bacteria employ at least two different maturation systems to synthesize c-type cytochromes. These systems commonly rely on haem b transport from the cytoplasm to the outside of the cytoplasmic membrane where covalent attachment of haem b to a haem c binding motif (HBM) of an apocytochrome c takes place (see Ferguson et al., 2008; Kranz et al., 2009 for recent reviews). This last step in cytochrome c biogenesis is catalysed by a membrane-bound cytochrome c haem lyase (CCHL) that recognizes the HBM (usually CX2CH, but other motifs such as CX2CK and CX15CH have also been described). The best-known cytochrome c maturation system is that of Escherichia coli, which is referred to as system I or the Ccm system (Richard-Fogal et al., 2009; Sanders et al., 2010). The Ccm system is present in many Gram-negative bacteria and comprises at least eight different proteins. In contrast, a maximum of four protein components arranged in system II (also known as the Ccs system) seems to be necessary to achieve cytochrome c biogenesis in Epsilonproteobacteria (for example, species of the genera Helicobacter, Campylobacter and Wolinella) and in Gram-positive bacteria such as Bacillus subtilis. The CCHL in system II is most likely to be a complex of two membrane-bound proteins: CcsB (also named ResB) and CcsA (ResC) (Ahuja et al., 2009). Notably, epsilonproteobacterial genomes encode fusion proteins called CcsBA that form 10 transmembrane domains (Frawley & Kranz, 2009). Such proteins contain an extracellular tryptophan-rich motif (designated the WWD domain, consensus sequence WGX2WXWD; possibly providing a haem b binding...
platform), which is also present in CcsA/ResC and in two system I proteins (CcmC and CcmF) (Richard-Fogal & Kranz, 2010). The CcsBA proteins from *Helicobacter pylori* and *Helicobacter hepaticus* are both able to restore cytochrome c maturation in system I-deficient *E. coli* strains, indicating that CcsBA-type enzymes function as CCHLs (Feissner et al., 2006; Richard-Fogal et al., 2007; Goddard et al., 2010).

*H. hepaticus* CcsBA as purified from *E. coli* has been shown to contain reduced haem b, and it has been suggested that the CcsBA-type CCHL mediates haem export to the periplasmic space (Frawley & Kranz, 2009; Merchant, 2009). According to this model, two pairs of conserved histidine residues (Table 1) are part of one cytoplasmic and one periplasmic haem binding pocket, and are essential in facilitating haem export and may also help to keep haem b in the reduced state. Variants of *H. hepaticus* CcsBA in which any of the four histidines is replaced by alanine are unable to support cytochrome c biogenesis, although two of them (modified at positions 2 and 4; Table 1) are still found to contain haem b, albeit in the oxidized state (Frawley & Kranz, 2009). Only the two variants lacking the histidines at positions 1 and 3 (i.e. those of the putative cytoplasmic haem b binding pocket) are complemented for cytochrome c biogenesis by adding imidazole to the culture medium, whereas the other two histidine variants are not.

The Epsilonproteobacterium *Wolinella succinogenes* represents an extensively investigated model organism, primarily in the field of microbial energy metabolism and bioenergetics (Simon et al., 2008; Kern & Simon, 2009a). It is a microaerobic organism that grows by various modes of anaerobic respiration, including nitrate ammonification, thereby employing periplasmic enzyme systems for nitrate reduction to nitrite (Nap system) and for ammonification of nitrite (Nrf system) (Simon et al., 2000; Simon, 2002; Kern et al., 2007; Kern & Simon, 2008, 2009a; Simon & Kern, 2008). The genome of *W. succinogenes* encodes 23 different mono- and multi-haem c-type cytochromes and matures them by using cytochrome c biogenesis system II (Kern et al., 2010). Exceptionally, *W. succinogenes* forms three distinct CCHLs (NrfI, CcsA1 and CcsA2; Table 1) of the CcsBA-type that have recently been shown to differ in their HBM specificity (Hartshorne et al., 2006; Kern et al., 2010). NrfI specifically requires attachment of the active site haem c group in pentahaem cytochrome c nitrite reductase (NrfA), which is bound via a CX13CH HBM (Pisa et al., 2002). CcsA1 is apparently dedicated to enabling maturation of the octahaem cytochrome c MccA, which contains a special CX15CH HBM in addition to seven conventional CX2CH sequences (Hartshorne et al., 2007; Kern et al., 2010). Presumably, CcsA1 is needed only for haem attachment to the special HBM of MccA. In contrast to nrfI and ccsA1, the ccsA2 gene cannot be deleted from the *W. succinogenes* genome, suggesting that cytochrome c biogenesis is essential for cell survival (Kern et al., 2010). Most likely, CcsA2 recognizes the standard CX2CH HBM and therefore corresponds to the *Helicobacter* CcsBA proteins discussed above. This hypothesis is supported by the fact that CcsA2 enables the conp-deficient *E. coli* mutant strain RK103 to synthesize holocytochrome c4 from *Bordetella pertussis*, while NrfI and CcsA1 do not (Kern et al., 2010).

Here, we used established or newly created genetic systems to produce variants of NrfI, CcsA1 and CcsA2 either in *W.*

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**Table 1. Position of conserved histidine residues in CcsBA-type CCHLs from selected Epsilonproteobacteria**

A primary structure alignment is provided in Supplementary Fig. S1. Histidine residues 1 and 3 are predicted to be located at the cytoplasmic boundary of transmembrane helices 3 and 8, respectively (cytoplasmic haem b binding pocket). Histidine residues 2 and 4 are thought to be in periplasmic regions near the N-terminal end of transmembrane helix 6 and the C-terminal end of transmembrane helix 9, respectively (periplasmic haem b binding pocket). Histidine residues e1 and e2 are conserved only in epsilonproteobacterial CCHL enzymes. Histidine e1 is predicted to reside in transmembrane helix 5, whereas histidine e2 is presumably located in the periplasmic loop that connects helices 5 and 6. See Fig. 3 in Frawley & Kranz (2009) for a corresponding CcsBA topology model.

<table>
<thead>
<tr>
<th>CCHL</th>
<th>Total number of residues</th>
<th>Number of histidine residues</th>
<th>Designation and position of conserved histidine residues*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>1</td>
</tr>
<tr>
<td><em>H. hepaticus</em> CcsBA</td>
<td>936</td>
<td>18</td>
<td>83</td>
</tr>
<tr>
<td><em>H. pylori</em> CcsBA</td>
<td>936</td>
<td>20</td>
<td>86</td>
</tr>
<tr>
<td><em>W. succinogenes</em> CcsA2</td>
<td>910</td>
<td>19</td>
<td>82</td>
</tr>
<tr>
<td><em>W. succinogenes</em> NrfI</td>
<td>902</td>
<td>30</td>
<td>78</td>
</tr>
<tr>
<td><em>W. succinogenes</em> CcsA1</td>
<td>897</td>
<td>13</td>
<td>84</td>
</tr>
</tbody>
</table>

*Residues shown in bold type were substituted in this study.*
succinogenes (NrfI, CcsA1) or in E. coli (CcsA2). Thirteen histidine variants as well as two derivatives containing modified WWD domains were characterized with respect to their cytochrome c maturation capability in the presence or absence of exogenous imidazole. The present study provides the first detailed investigation, to our knowledge, of functionally distinct CcsBA-type CCHLs produced in both homologous and heterologous cellular environments.

**METHODS**

**Growth conditions for W. succinogenes and E. coli cells.** Bacterial strains used in this study are listed in Table 2. W. succinogenes cells were grown at 37 °C either by fumarate or by nitrate respiration, as described previously (Krüger et al., 1994; Kern & Simon, 2009). Brain heart infusion broth (0.5 or 1.3 %, w/v) was added where appropriate. To generate anaerobic conditions, the medium was degassed and flushed several times with dinitrogen gas. When indicated, imidazole (Serva) was added from an anaerobic stock solution [1 M, pH 7.5 (growth by nitrate respiration) or pH 8.0 (growth by fumarate respiration)]. Antibiotics were used at the following concentrations: kanamycin, 25 mg l⁻¹; chloramphenicol, 12.5 mg l⁻¹.

E. coli cells were grown in LB medium at 37 °C either aerobically with shaking at 250 r.p.m. or anaerobically in rubber-sealed tubes. Antibiotics were used at the following concentrations: ampicillin, 100 mg l⁻¹; kanamycin, 50 mg l⁻¹; chloramphenicol, 25 mg l⁻¹. Induction of CcsA2 and CycC production was achieved by the consecutive addition of IPTG and arabinose, as described previously (Kern et al., 2010). Imidazole (pH 7.0) was added to aerobic cultures during inoculation. Anaerobically grown cultures (10 ml) were grown with a few modifications. After inoculation (1 %, v/v, from a fresh overnight culture), the cells were incubated aerobically in the presence of imidazole for 3 h. Subsequently, the cells were shifted to a rubber-sealed tube and the oxygen content was reduced by alternate degassing and sparging with dinitrogen gas using a sterile filter. Then, IPTG (1 mM) was added to induce ccsA2 transcription and after 2 h, arabinose (0.2 %, w/v) was added to induce the synthesis of...

Table 2. Strains of W. succinogenes and E. coli used in this study

See Methods for details of mutant construction.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Description and/or relevant properties*</th>
<th>Reference or source</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>W. succinogenes strains</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1. Wild-type</td>
<td>Type strain DSMZ 1740</td>
<td>DSMZ†</td>
</tr>
<tr>
<td>2. ΔnrfA/ΔJ</td>
<td>Deletion mutant lacking nrfA, nrfI and part of nrfJ; KmR</td>
<td>Simon et al. (2000)</td>
</tr>
<tr>
<td>3. ΔnrfII</td>
<td>Deletion mutant lacking nrfAII and part of nrfII; KmR</td>
<td>Simon et al. (2000)</td>
</tr>
<tr>
<td>4. N3</td>
<td>Derivative of strain 2 containing a restored wild-type nrfHAIJ operon; CmR, KmR</td>
<td>Pisa et al. (2002)</td>
</tr>
<tr>
<td>5. NrfI H78A</td>
<td>Similar to strain 4 but encoding modified NrfI (H78A); CmR, KmR</td>
<td>This work</td>
</tr>
<tr>
<td>6. NrfI H641A</td>
<td>Similar to strain 4 but encoding modified NrfI (H641A); CmR, KmR</td>
<td>This work</td>
</tr>
<tr>
<td>7. NrfI H724A</td>
<td>Similar to strain 4 but encoding modified NrfI (H724A); CmR, KmR</td>
<td>This work</td>
</tr>
<tr>
<td>8. NrfI H821A</td>
<td>Similar to strain 4 but encoding modified NrfI (H821A); CmR, KmR</td>
<td>This work</td>
</tr>
<tr>
<td>9. NrfI H860A</td>
<td>Similar to strain 4 but encoding modified NrfI (H860A); CmR, KmR</td>
<td>This work</td>
</tr>
<tr>
<td>10. Δmcc kan</td>
<td>Deletion mutant lacking mccA, fbpA, mccC, mccD and ccsA1; CmR</td>
<td>This work</td>
</tr>
<tr>
<td>11. P(Δmcc)</td>
<td>Derivative of strain 10 containing a restored mcc locus under the control of the fumarate reductase promoter; CmR</td>
<td>This work</td>
</tr>
<tr>
<td>12. P(Δmcc) ΔAcc1</td>
<td>Derivative of strain 11 lacking ccsA1; CmR, KmR</td>
<td>This work</td>
</tr>
<tr>
<td>13. CcsA1 H84A</td>
<td>Similar to strain 11 but encoding modified CcsA1 (H84A); CmR, KmR</td>
<td>This work</td>
</tr>
<tr>
<td>14. CcsA1 H722A</td>
<td>Similar to strain 11 but encoding modified CcsA1 (H722A); CmR, KmR</td>
<td>This work</td>
</tr>
<tr>
<td>15. CcsA1 H820A</td>
<td>Similar to strain 11 but encoding modified CcsA1 (H820A); CmR, KmR</td>
<td>This work</td>
</tr>
<tr>
<td>16. CcsA1 H859A</td>
<td>Similar to strain 11 but encoding modified CcsA1 (H859A); CmR, KmR</td>
<td>This work</td>
</tr>
<tr>
<td>17. CcsA1 WWD</td>
<td>Similar to strain 11 but encoding modified CcsA1 (W799A, W801A, D802A); CmR, KmR</td>
<td>This work</td>
</tr>
<tr>
<td><strong>E. coli strains</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>18. RK103 pRGK332</td>
<td>Derivative of the ccm mutant strain RK103 containing two plasmids encoding B. pertussis CycC (pRGK332) and W. succinogenes CcsA2 (pWsCcsA2); CmR, KmR; AmpR</td>
<td>Kern et al. (2010)</td>
</tr>
<tr>
<td>19. RK103 CcsA2 H82A</td>
<td>Similar to strain 18 but encoding modified CcsA2 (H82A); CmR, KmR; AmpR</td>
<td>This work</td>
</tr>
<tr>
<td>20. RK103 CcsA2 H734A</td>
<td>Similar to strain 18 but encoding modified CcsA2 (H734A); CmR, KmR; AmpR</td>
<td>This work</td>
</tr>
<tr>
<td>21. RK103 CcsA2 H831A</td>
<td>Similar to strain 18 but encoding modified CcsA2 (H831A); CmR, KmR; AmpR</td>
<td>This work</td>
</tr>
<tr>
<td>22. RK103 CcsA2 H870A</td>
<td>Similar to strain 18 but encoding modified CcsA2 (H870A); CmR, KmR; AmpR</td>
<td>This work</td>
</tr>
<tr>
<td>23. RK103 CcsA2 WWD</td>
<td>Similar to strain 18 but encoding modified CcsA2 (W810A, W812A, D813A); CmR, KmR; AmpR</td>
<td>This work</td>
</tr>
</tbody>
</table>

*CmR, KmR and AmpR denote resistance to chloramphenicol, kanamycin and ampicillin, respectively.
†DSMZ, Deutsche Sammlung von Mikroorganismen und Zellkulturen.
§See Table 1 for the position of the histidine residue within the primary structure.
§§See Fig. 1.
apo-CycC. Cells were harvested after incubation for an additional 4 h at 37 °C.

**Cell fractionation and determination of protein concentrations.** *W. succinogenes* cells harvested in the exponential or early stationary growth phase were suspended (10 g cell protein l⁻¹) in an anoxic buffer (pH 8.0) containing 50 mM Tris/HCl. The suspension was passed through a high-pressure cell disruption system (Constant Systems) at 135 MPa. The resulting cell homogenate was centrifuged for 15 min at 5000 g to remove cell debris. The periplasmic protein fraction of *E. coli* cells was obtained as described previously (Feissner et al., 2006). Protein was measured using the Biuret method with KCN (Bode et al., 1968) or the Bradford assay.

**Cytochrome c detection and determination of specific activities.** Samples of cell proteins were subjected to SDS-PAGE using either a reducing (for *W. succinogenes* samples) or a non-reducing (for *E. coli* samples) loading buffer (Roth). Proteins were transferred to a PVDF membrane by Western blotting, and cytochromes c were detected using the SuperSignal West Pico chemiluminescence substrate (Thermo Scientific) and exposure to X-ray film (CL-XPosure film, Thermo Scientific). Nitrile reductase activity was determined by spectrophotometrically recording the rate of benzyl viologen (BV) radical oxidation by nitrile, as described previously (Kern et al., 2010). One unit of enzyme activity is defined as the oxidation of 2 μmol BV min⁻¹. The electron transport activity from formate to nitrite was measured with intact cells of *W. succinogenes* according to the method described by Simon et al. (2001) with a few modifications. In brief, washed cells were suspended (2–5 g protein l⁻¹) in an anoxic buffer (pH 8.5) containing 150 mM Bicine and 0.5 M mannitol. A cell aliquot was incubated for 5 min at 37 °C prior to the addition of sodium formate (30 mM final concentration). The reaction was started by adding 10 mM potassium nitrite and samples were taken after various time intervals (total duration up to 30 min). Electron transport activity was calculated from the nitrite production rate. One unit of electron transport activity (U) is equivalent to the consumption of 1 μmol formate min⁻¹.

**Construction of *W. succinogenes* mutants.** Standard genetic procedures were used (Sambrook et al., 1989). Genomic DNA was isolated from *W. succinogenes* using the DNeasy Tissue kit (Qiagen). PCR was carried out using Phusion High Fidelity DNA polymerase (Finnzymes) (for cloning procedures) or Biotaq Red DNA polymerase (Bioline) (for mutant and plasmid screening) with standard amplification protocols. Site-directed mutagenesis was performed using the QuickChange Site-Directed Mutagenesis kit (Stratagene) or the Phusion Site-Directed Mutagenesis kit (Finnzymes) with specifically synthesized primer pairs (Supplementary Table S1). *W. succinogenes* mutants producing NrfI variants (strains 5–9 in Table 2) were obtained from *W. succinogenes* ΔnrfAIJ upon integration of pBR-N3 derivatives, resulting in a restored nrfI operon (Pisa et al., 2002). Site-directed mutagenesis of nrfI was performed with pBR-N3 as template and a pair of complementary primers (Supplementary Table S1). Transformation of *W. succinogenes* ΔnrfAIJ with the resulting plasmids was performed by electroporation, as described previously (Simon et al., 1998). Transformants were selected in the presence of kanamycin (25 mg l⁻¹) and chloramphenicol (12.5 mg l⁻¹). The desired integration of the plasmid into the genome via the nrfIH gene was confirmed by PCR, and each mutation was verified by sequencing an appropriate PCR product.

*W. succinogenes* Δmcc kan was constructed through double homologous recombination of the wild-type genome with a deletion plasmid (pΔmcc) designed to replace the consecutive genes mccA, fpA, mccC, mccD and ccsA1 with the kanamycin-resistance gene cassette (*kan*). For homologous recombination, the respective deletion plasmid contained *kan* flanked by two DNA segments obtained by PCR that were identical to appropriate regions in the *W. succinogenes* genome (Fig. 1). The two PCR fragments were synthesized using the following primer pairs: 5′-GCGAAATTCCTG-GCATCTAGGTGAGG-3′ and 5′-CCGATCCGGTTCCTCCTTTT-CAAAACAC-3′ for amplifying the upstream fragment, and 5′-GGGATCCCAAATCTATCATAAGGGTTTG-3′ and 5′-CCGCA-TGGGATAGTCATCCTCCACCAAAAAGG-3′ for the downstream fragment (black bars in Fig. 1). Primers carried EcoRI, BamHI or NcoI restriction sites (underlined) for cloning. Both fragments as well as *kan* (obtained by BamHI excision from pUC4K) were consecutively inserted into the high-copy-number plasmid pPR-IBA1 (IBA BioTARGnomics) using appropriate restriction enzymes. PCR analysis was used to confirm that the plasmid contained *kan* in the same orientation as the mcc sequence. Transformants of *W. succinogenes* were selected in the presence of kanamycin (25 mg l⁻¹). The desired deletion in the transformant genome was confirmed by PCR, and the integrity of DNA stretches involved in recombination events was confirmed by sequencing suitable PCR products.

To construct *W. succinogenes* Pnfrd-mcc, the complete mcc gene cluster was restored on the genome of *W. succinogenes* Δmcc kan upon integration of plasmid pPfrd-mcc cat (Fig. 1). This plasmid contained the deleted mcc region downstream of the furamate reductase promoter (Pfrd) and flanked by the upstream and downstream fragments of pΔmcc. In addition, the chloramphenicol-resistance gene cassette (*cat*) was inserted between the upstream fragment and the mcc region. In a first step to synthesize pPfrd-mcc cat, the *kan* gene was replaced by cat in pΔmcc, resulting in pPfrd cat. A Pfrd fragment amplified using the primer pair 5′-AAAGGGAATTCCGAGGGGGTTTTG-3′ and 5′-CTGTTTCCCCTGTGAGATTG-3′ was blunt end-ligated with a linear plasmid fragment obtained by PCR from pΔmcc cat with the primer pair 5′-AAACTCTATCCTGAGGGTTTTGGAAC-3′ and 5′-CCGATCCGGTTCCTCCTTTT-3′, resulting in pPfrd Δmcc cat. In a second step, the f1 origin of pPfrd Δmcc cat was replaced by a SC101 origin, which lowered the plasmid number per cell (this was necessary to allow cloning of the large mcc region later on). The catPfrd region flanked with the upstream and downstream fragments was amplified by PCR and blunt end-ligated with the SC101 origin (obtained by BamHI/AflII excision from pSC101) and subsequent filling in of recessed 3′ ends by the Klenow fragment of *E. coli* DNA polymerase I), resulting in p(lk)Pfrd Δmcc cat. In the last step of pPfrd-mcc cat construction, the mcc region, comprising the consecutive genes mccA, fpA, mccC, mccD and ccsA1, was inserted downstream of Pfrd. The mcc fragment was amplified using the primer pair 5′-ATGAAATATTTGGACAAAGCTTGCAGTG-3′ and 5′-TCAATCTCACCCTCCACCTTTTGCG-3′, and blunt end-ligated into a linear plasmid fragment obtained by PCR from p(lk)Pfrd Δmcc cat. The corresponding mutant strain *W. succinogenes* Pnfrd-mcc was constructed by transforming *W. succinogenes* Δmcc kan with pPfrd-mcc cat. Transformants were selected in the presence of chloramphenicol (12.5 mg l⁻¹), and the desired double homologous recombination was verified by PCR. The *W. succinogenes* strains producing CcsA1 variants (strains 13–17 in Table 2) were obtained from *W. succinogenes* Δmcc kan upon integration of pPfrd-mcc cat derivatives containing a mutated ccsA1 gene. Site-directed mutagenesis of pPfrd-mcc cat was performed using appropriate primer pairs (Supplementary Table S1). Mutant *W. succinogenes* Pnfrd-mcc ΔccsA1 was obtained after transformation of *W. succinogenes* Pnfrd-mcc with ΔccsA1;kan (Hartshorne et al., 2007) and selection in the presence of kanamycin.

**Construction of *E. coli* mutants.** Strains used in this study were derived from *E. coli* RK103 pRKG332 pWSCcsA2, which contains two plasmids encoding *B. pertussis* CycC and *W. succinogenes* CcsA2, respectively (Kern et al., 2010). To obtain plasmids encoding CcsA2 variants, pWSCcsA2 was modified using suitable primer pairs.
RESULTS

Production and characterization of NrfI variants in W. succinogenes

A previously described genetic system allowed site-directed mutagenesis of the nrfI gene on a plasmid in E. coli as well as expression of the mutated gene in the context of the entire nrfHAIJ operon in W. succinogenes (Pisa et al., 2002). Here, five NrfI histidine residues (H78, H641, H724, H821, and H860; Table 1) were individually replaced by alanine and the corresponding proteins were produced in W. succinogenes (mutants 5–9 in Table 2). Strain W. succinogenes N3 (a control containing wild-type nrfI) and strain NrfI H641A had wild-type properties with respect to growth by both nitrate and nitrite respiration, while the other four mutants reduced nitrate to nitrite but did not grow with nitrite as electron acceptor (results not shown). These four mutants contained only very small amounts of NrfA (as judged by haem staining) and did not show any detectable nitrite reductase activity measured with reduced BV as artificial electron donor (results not shown). These four mutants contained only very small amounts of NrfA (as judged by haem staining) and did not show any detectable nitrite reductase activity measured with reduced BV as artificial electron donor (results not shown). Such a phenotype was also found in the ΔnrfIJ mutant (Fig. 2a) as well as in W. succinogenes stopI, which contained an nrfI gene inactivated by several stop codons (Pisa et al., 2002). For W. succinogenes stopI, it was shown that NrfA possessed only four covalently bound haem groups and lacked the active site C3,CK-bound haem which is essential for nitrite reduction (Pisa et al., 2002). Therefore, it appears that the histidine residues 78, 724, 821 and 860 are obligatory for NrfI function and/or stability.

In another experiment, wild-type cells and mutants W. succinogenes N3, ΔnrfAIJ, ΔnrfIJ, H78A, H724A, H821A and H860A were grown by nitrate respiration in the same medium as before but in the presence of exogenous imidazole (10 mM final concentration in the medium). Under these conditions, considerably larger amounts of NrfA were detected in the four histidine mutants along with the recovery of substantial nitrite reductase activity (between 5 and 19 % relative to strain N3) (Fig. 2a). This activity was also reflected in restored electron transport activities from formate to nitrite (between 3 and 8 % relative to strain N3) (Fig. 2a). Notably, the mutants could be grouped into two pairs (H78/H821 and H724/H860, corresponding to positions 1/3 and 2/4 in Table 1) based on similar enzyme activities and protein contents. Lowering the initial imidazole concentrations in the medium resulted in decreasing amounts of NrfA (Fig. 2b) and, apparently, the H78/H821 variants tolerated the presence of low imidazole concentrations to a greater extent than the H724/H860 variants. The addition of 10 mM imidazole slightly impaired the growth rate of all four histidine mutants in a medium containing 50 mM formate and 10 mM nitrate as energy substrates but the cells reached final optical densities similar to those of control cultures grown in the absence of imidazole (results not shown). Addition of 15 or 25 mM imidazole almost completely abolished growth of W. succinogenes cells during nitrate respiration.

Production of CcsA1 variants in W. succinogenes cells overproducing MccA

CcsA1 has been shown previously to be dedicated to the maturation of the octahaem cytochrome c MccA, whose
function in \( W. \) succinogenes is not known (Hartshorne et al., 2007; Kern et al., 2010). To assess the role of single amino acid residues of CcsA1 in \( W. \) succinogenes, a genetic test system was established that allowed site-directed mutagenesis of \( ccsA1 \) in the background of a strain whose \( mcc \) gene cluster was expressed from the fumarate reductase promoter (Pfrd) (Fig. 1). The same frd promoter element has been shown previously to be able to initiate expression of the \( mcc \) locus (Hartshorne et al., 2007). In a first step, a deletion mutant (\( W. \) succinogenes \( Dmcc \) kan) was constructed that lacked the consecutive genes \( mccA, fkpA, mccC, mccD \) and \( ccsA1 \) (Table 2). The entire \( mccA \) locus was then restored on the genome of the deletion mutant by double homologous recombination between its genome and a suitable plasmid that carried Pfrd upstream of \( mccA \) (Fig. 1). The resulting strain (\( W. \) succinogenes \( Pfrd-mcc \)) was found to produce MccA under fumarate-respiring growth conditions (Fig. 3, lane 1). The formation of MccA under these conditions was already known to depend on the presence of \( ccsA1 \) (Hartshorne et al., 2007), and therefore it was not surprising that MccA could not be detected by haem staining in cells of strain \( Pfrd-mcc \) after deletion of the \( ccsA1 \) gene (Fig. 3, lane 2).

Derivatives of \( W. \) succinogenes \( Pfrd-mcc \) were constructed that produced variants of CcsA1 (strains 13–17 in Table 2). Each of the histidine residues H84, H722, H820 and H859 of CcsA1 was replaced by alanine, and a further CcsA1 variant was constructed that contained an AGAA sequence instead of WGWD within the WWD domain (mutant \( W. \) succinogenes CcsA1 WWD). In the absence of exogenous imidazole, low amounts of MccA were detected in strains CcsA1 H84A and CcsA1 H820A, whereas MccA was not found in the other three mutants (Fig. 3, lanes 3–7). Increasing amounts of added imidazole ultimately resulted in the detection of MccA in all four histidine mutants, and two mutant pairs were identified whose phenotypes were apparently identical (Fig. 3). These pairs (H84/H820 and H722/H859) corresponded to CcsA1 variants with modified histidines at positions 1/3 and 2/4, similar to the results observed for NrfI (see above). In contrast to the histidine variants, CcsA1 containing the modified WWD domain was not able to produce detectable MccA, irrespective of the presence of imidazole (Fig. 3).

**Fig. 2.** Detection of cytochrome c nitrite reductase (NrfA) by haem staining in cell homogenates of different \( W. \) succinogenes strains. Cell homogenates (protein amounts as indicated) of nitrate-grown cells were separated by SDS-PAGE and blotted onto a PVDF membrane. Only the gel region containing NrfA (at about 55 kDa) is shown. Numbers below the gel refer to specific activities [U (mg protein)\(^{-1}\)] measured in the corresponding cell homogenate. At least three independent cultures were used for activity determination and the SD is given in the case of nitrite reductase activity, whereas representative values are shown for electron transport activities. Initial imidazole concentrations added to the cultures are shown at the left of each gel section. NIR activity, specific nitrite reductase activity; ET activity, specific electron transport activity from formate to nitrite.

**Characterization of \( W. \) succinogenes CcsA2 variants using ccm-deficient \( E. \) coli RK103 cells**

As CcsA2 proved to be essential for growth of \( W. \) succinogenes cells (Kern et al., 2010), it was not possible to produce CcsA2 variants in a homologous system.
Instead, *W. succinogenes* CcsA2 was synthesized using the heterologous cytochrome *c* maturation test system, employing *E. coli* RK103 as host cells that also produced *B. pertussis* dihaem cytochrome *c*4 (CycC) as reporter protein (Feissner *et al.*, 2006). Using this approach, wild-type CcsA2 has been shown previously to mature CycC (Kern *et al.*, 2010). Here, each of the CcsA2 histidines located at the conserved positions 1–4 (Table 1) was replaced by alanine, and a WWD domain variant similar to that of CcsA1 was also constructed. Each of these variants was functionally tested using the respective *E. coli* RK103 cells (strains 19–23 in Table 2). A strain that produced wild-type CcsA2 served as control. Grown under either aerobic or anaerobic growth conditions, none of the five CcsA2 variants was found to be capable of CycC maturation, in contrast to wild-type CcsA2 (Fig. 4). When grown in the presence of imidazole, however, mutants CcsA2 H82A and CcsA2 H831A (histidine positions 1 and 3) produced stable holo-CycC, the amount of which increased with higher imidazole concentrations (Fig. 4). Notably, this effect was observed only in anaerobically grown cells. In the other three mutants, CycC maturation was not restored by imidazole, irrespective of the oxygen content of the cultures (Fig. 4).

**DISCUSSION**

Haem is synthesized in the bacterial cytoplasm and needs to be exported in order to serve as a CCHL substrate. For the bacterial cytochrome *c* biogenesis system II, recently
acquired evidence suggests that CcsBA-type CCHLs mediate haem export with the help of two haem binding sites located on different sides of the membrane (Frawley & Kranz, 2009; Kranz et al., 2009; Merchant, 2009; Goddard et al., 2010). According to this model, two pairs of histidines arranged in cytoplasmic and periplasmic haem binding pockets, respectively, serve in axial haem b ligation during the export process. This hypothesis is supported by the fact that imidazole addition to the growth medium leads to restored cytochrome c maturation activity of \textit{H. hepaticus} CcsBA histidine variants when produced in \textit{E. coli} RK103 (Frawley & Kranz, 2009). However, this effect was only obtained with variants H83A and H858A, which were modified at histidine positions 1 and 3 (cytoplasmic haem b binding site; Table 1). Here, we have shown that imidazole complementation can be achieved in variants of all four conserved histidine residues in two CcsBA-type CCHLs (NrfI and CcsA1) from \textit{W. succinogenes}, a fact that supports the idea of separate haem b binding sites. It needs to be emphasized, however, that neither histidine variant used in this study showed a cytochrome-maturing activity equivalent to that of the wild-type protein, even at the highest imidazole concentration used. The results depicted in Figs 2 and 3 suggest that the function of the cytoplasmic haem b binding site can be restored more effectively by imidazole than that of the periplasmic site in both NrfI and CcsA1 from \textit{W. succinogenes}. Likewise, the cytoplasmic haem b binding site also seemed to be more accessible to imidazole complementation when CcsBA-type CCHLs (either CcsBA from \textit{H. hepaticus} or CcsA2 from \textit{W. succinogenes}) were produced in \textit{E. coli}. Interestingly, anaerobic growth conditions apparently enhanced the imidazole complementation effect, which might be due to the fact that haem is more reduced under these conditions. Histidine residues essential for cytochrome c biogenesis have also been reported for CcsB and CcsA from \textit{Chlamydomonas reinhardtii} and the WWD domain-containing CcmF from \textit{E. coli} (Ren et al., 2002; Dreyfuss et al., 2003; Hamel et al., 2003).

Variants of CcsBA-type CCHLs from Epsilonproteobacteria carrying modifications of the WWD domain were generally inactive in cytochrome c maturation, thus demonstrating the importance of this motif. The results obtained for both CcsA1 and CcsA2 in this study were similar to those reported previously for \textit{H. hepaticus} CcsBA and for CcsA from \textit{C. reinhardtii} (Hamel et al., 2003; Frawley & Kranz, 2009). The WWD domains of the \textit{E. coli} CcmC and CcmF proteins have also been subjected to modification (Schulz et al., 2009; Ren et al., 2002; Richard-Fogal & Kranz, 2010). It these cases, the exchange of one conserved tryptophan residue typically resulted in loss of cytochrome c maturation activity. The molecular function of the WWD domain is not understood, but it is thought to be located near the periplasmic haem b binding pocket, where it possibly plays a role in CCHL function subsequent to haem b export, for example in proper presentation of haem in order to facilitate the haem lyase reaction.

Taken together, the results presented in this study suggest that CcsBA-type CCHLs from different Epsilonproteobacteria share a common architecture that comprises 10 conserved transmembrane segments, four essential histidine residues involved in binding haem b, and a WWD domain that might play a crucial role during the formation of the covalent thioether bridges from haem b and a suitable apocytochrome c.

The genetic strategies presented in this study offer the opportunity to attach a short affinity tag (His- or Strep-tag) to each of the \textit{W. succinogenes} CCHL isoenzymes in order to attempt future purification by affinity chromatography. As CcsBA from \textit{H. hepaticus} as well as \textit{B. subtilis} ResB seem to be prone to proteolytic degradation in \textit{E. coli}, it might be beneficial to use \textit{W. succinogenes} as production host (Ahuja et al., 2009; Frawley & Kranz, 2009). Although polytopic membrane-bound proteins or protein complexes cannot be easily purified, let alone crystallized, successful large-scale preparation of an intact CCHL will hopefully lead to a structural model in the future that might elucidate the many poorly understood molecular details of CCHL function.

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unconventional covalent haem binding.


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