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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 CX_2CH (by CcsA2), CX_2CK (by NrfI) and $CX_{15}CH$ (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 multihaem cytochromes *c* NrfA and MccA, 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.

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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 CX_2CH, but other motifs such as CX_2CK and $CX_{15}CH$ 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 WGX_2WXWD; possibly providing a haem *b* binding

Abbreviations: BV, benzyl viologen; CCHL, cytochrome *c* haem lyase; HBM, haem *c* binding motif.

A supplementary figure, showing full-length alignment of selected epsilonproteobacterial CCHL primary structures, and a supplementary table, listing nucleotide primers used to modify CCHL-encoding genes, are available with the online version of this paper.

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. Imidazole is thought to functionally mimic the histidine side chain in these CCHL variants upon occupation of the cavity formed after the replacement of histidine by alanine, thus potentially re-enabling haem *b* coordination. This form of functional complementation is similar to that reported earlier for a myoglobin variant in the presence of imidazole (Barrick, 1994).

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 is specifically required to attach the unique active site haem *c* group in pentahaem cytochrome *c* nitrite reductase (NrfA), which is bound via a CX_2CK HBM (Pisa *et al.*, 2002). CcsA1 is apparently dedicated to enabling maturation of the octahaem cytochrome *c* MccA, which contains a special $CX_{15}CH$ HBM in addition to seven conventional CX_2CH 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 CX_2CH HBM and therefore corresponds to the *Helicobacter* CcsBA proteins discussed above. This hypothesis is supported by the fact that CcsA2 enables the *ccm*-deficient *E. coli* mutant strain RK103 to synthesize holocytochrome c_4 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.*

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 ε1 and ε2 are conserved only in epsilonproteobacterial CCHL enzymes. Histidine ε1 is predicted to reside in transmembrane helix 5, whereas histidine ε2 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.

CCHL	Total number of residues	Number of histidine residues	Designation and position of conserved histidine residues*					
			1	ε1	ε2	2	3	4
H. hepaticus CcsBA	936	18	83	678	691	761	858	897
H. pylori CcsBA	936	20	86	679	692	760	857	896
W. succinogenes CcsA2	910	19	**82**	651	664	**734**	**831**	**870**
W. succinogenes NrfI	902	30	**78**	**641**	654	**724**	**821**	**860**
W. succinogenes CcsA1	897	13	**84**	639	652	**722**	**820**	**859**

*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 (Kröger *et al.*, 1994; Kern & Simon, 2009b). 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.

Strain	Description and/or relevant properties*	Reference or source
***W. succinogenes* strains**		
1. Wild-type	Type strain DSMZ 1740	DSMZ†
2. Δ*nrfAIJ*	Deletion mutant lacking *nrfA*, *nrfI* and part of *nrfJ*; Km^R	Simon *et al.* (2000)
3. Δ*nrfIJ*	Deletion mutant lacking *nrfI* and part of *nrfJ*; Km^R	Simon *et al.* (2000)
4. N3	Derivative of strain 2 containing a restored wild-type *nrfHAIJ* operon; Cm^R, Km^R	Pisa *et al.* (2002)
5. NrfI H78A	Similar to strain 4 but encoding modified NrfI (H78A)‡; Cm^R, Km^R	This work
6. NrfI H641A	Similar to strain 4 but encoding modified NrfI (H641A)‡; Cm^R, Km^R	This work
7. NrfI H724A	Similar to strain 4 but encoding modified NrfI (H724A)‡; Cm^R, Km^R	This work
8. NrfI H821A	Similar to strain 4 but encoding modified NrfI (H821A)‡; Cm^R, Km^R	This work
9. NrfI H860A	Similar to strain 4 but encoding modified NrfI (H860A)‡; Cm^R, Km^R	This work
10. Δ*mcc kan*	Deletion mutant lacking *mccA*, *fkpA*, *mccC*, *mccD* and *ccsA1*§; Km^R	This work
11. P_{frd}-*mcc*	Derivative of strain 10 containing a restored *mcc* locus under the control of the fumarate reductase promoter§; Cm^R	This work
12. P_{frd}-*mcc* Δ*ccsA1*	Derivative of strain 11 lacking *ccsA1*; Cm^R, Km^R	This work
13. CcsA1 H84A	Similar to strain 11 but encoding modified CcsA1 (H84A)‡; Cm^R	This work
14. CcsA1 H722A	Similar to strain 11 but encoding modified CcsA1 (H722A)‡; Cm^R	This work
15. CcsA1 H820A	Similar to strain 11 but encoding modified CcsA1 (H820A)‡; Cm^R	This work
16. CcsA1 H859A	Similar to strain 11 but encoding modified CcsA1 (H859A)‡; Cm^R	This work
17. CcsA1 WWD	Similar to strain 11 but encoding modified CcsA1 (W799A, W801A, D802A)‡; Cm^R	This work
***E. coli* strains**		
18. RK103 pRGK332 pWsCcsA2	Derivative of the *ccm* mutant strain RK103 containing two plasmids encoding *B. pertussis* CycC (pRGK332) and *W. succinogenes* CcsA2 (pWsCcsA2); Cm^R, Km^R; Amp^R	Kern *et al.* (2010)
19. RK103 CcsA2 H82A	Similar to strain 18 but encoding modified CcsA2 (H82A)‡; Cm^R, Km^R; Amp^R	This work
20. RK103 CcsA2 H734A	Similar to strain 18 but encoding modified CcsA2 (H734A)‡; Cm^R, Km^R; Amp^R	This work
21. RK103 CcsA2 H831A	Similar to strain 18 but encoding modified CcsA2 (H831A)‡; Cm^R, Km^R; Amp^R	This work
22. RK103 CcsA2 H870A	Similar to strain 18 but encoding modified CcsA2 (H870A)‡; Cm^R, Km^R; Amp^R	This work
23. RK103 CcsA2 WWD	Similar to strain 18 but encoding modified CcsA2 (W810A, W812A, D813A)‡; Cm^R, Km^R; Amp^R	This work

*Cm^R, Km^R and Amp^R 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). Nitrite reductase activity was determined by spectrophotometrically recording the rate of benzyl viologen (BV) radical oxidation by nitrite, 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 (50 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 QuikChange 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* Δ*nrfAIJ* upon integration of pBR-N3 derivatives, resulting in a restored *nrf* 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* Δ*nrfAIJ* 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 *nrfH* gene was confirmed by PCR, and each mutation was verified by sequencing an appropriate PCR product.

W. succinogenes Δ*mcc kan* was constructed through double homologous recombination of the wild-type genome with a deletion plasmid (pΔmcc) designed to replace the consecutive genes *mccA*, *fkpA*, *mccC*, *mccD* and *ccsA1* with the kanamycin-resistance gene cartridge (*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′-GCGAATTCTTG-GCATTGAGGTTGAGG-3′ and 5′-CCGGATCCGTTTCCTCCTTT-CAAAACAC-3′ for amplifying the upstream fragment, and 5′-GCGGATCCAAACTCTATCTAAGGAGGTGTG-3′ and 5′-CGCCA-TGGGATAGATCACTCCACCAAAAAAGAG-3′ for the downstream fragment (black bars in Fig. 1). Primers carried *Eco*RI, *Bam*HI or *Nco*I restriction sites (underlined) for cloning. Both fragments as well as *kan* (obtained by *Bam*HI excision from pUC4K) were consecutively inserted into the high-copy-number plasmid pPR-IBA1 (IBA BioTAGnology) 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* P_{frd}-*mcc*, the complete *mcc* gene cluster was restored on the genome of *W. succinogenes* Δ*mcc kan* upon integration of plasmid pP_{frd}-mcc cat (Fig. 1). This plasmid contained the deleted *mcc* region downstream of the fumarate reductase promoter (P_{frd}) 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 pP_{frd}-mcc cat, the *kan* gene was replaced by *cat* in pΔmcc, resulting in pΔmcc cat. A P_{frd} fragment amplified using the primer pair 5′-AAAGGGGTTTGGACGGGG-TTTTG-3′ and 5′-CTGTTTCCCCTGTGCAGTATTGGAGTTTC-3′ was blunt end-ligated with a linear plasmid fragment obtained by PCR from pΔmcc cat with the primer pair 5′-AAACTCTATCTA-AGGAGGTGTGATGC-3′ and 5′-CCGGATCCCGGTTTTTGTTAA-TCCGC-3′, resulting in pP_{frd} Δmcc cat. In a second step, the f1 origin of pP_{frd} 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 *cat*/P_{frd} region flanked with the upstream and downstream fragments was amplified by PCR and blunt end-ligated with the SC101 origin (obtained by *Bam*HI/*Afe*I excision from pSC101 and subsequent filling in of recessed 3′ ends by the Klenow fragment of *E. coli* DNA polymerase I), resulting in $p(lc)P_{frd}$ Δmcc cat. In the last step of pP_{frd}-mcc cat construction, the *mcc* region, comprising the consecutive genes *mccA*, *fkpA*, *mccC*, *mccD* and *ccsA1*, was inserted downstream of P_{frd}. The *mcc* fragment was amplified using the primer pair 5′-ATGAAATATTGGGACAA-AGCGTTGCTGAGTC-3′ and 5′-TCAAATCGTCCCCACTTTTCGG-CG-3′, and blunt end-ligated into a linear plasmid fragment obtained by PCR from $p(lc)P_{frd}$ Δmcc cat. The corresponding mutant strain *W. succinogenes* P_{frd}-*mcc* was constructed by transforming *W. succinogenes* Δ*mcc kan* with pP_{frd}-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* Δ*mcc kan* upon integration of pP_{frd}-mcc cat derivatives containing a mutated *ccsA1* gene. Site-directed mutagenesis of pP_{frd}-mcc cat was performed using appropriate primer pairs (Supplementary Table S1). Mutant *W. succinogenes* P_{frd}-*mcc* Δ*ccsA1* was obtained after transformation of *W. succinogenes* P_{frd}-*mcc* with pΔccsA1::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 pRGK332 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

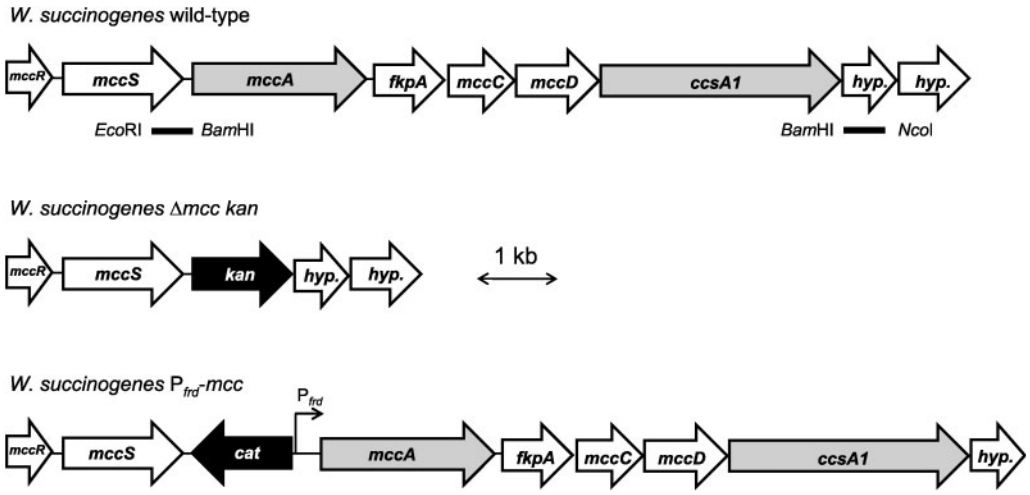

Fig. 1. Physical maps of the *mcc* loci in *W. succinogenes* wild-type cells and in mutant strains Δ*mcc kan* and P_{frd}-*mcc*. Black bars indicate regions used for homologous recombination during mutant construction (see Methods for details). P_{frd} and the accompanying arrow indicate the position of the fumarate reductase promoter and the corresponding transcriptional direction. The *ccsA1* gene encodes a CcsBA-type CCHL that is specifically required for the maturation of the octahaem cytochrome *c* MccA.

(Supplementary Table S1). Transformation of *E. coli* RK103 pRGK332 with these plasmids resulted in strains 19–23 (Table 2).

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 (Fig. 2a). Such a phenotype was also found in the Δ*nrfIJ* 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 CX_2CK-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, Δ*nrfAIJ*, Δ*nrfIJ*, 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

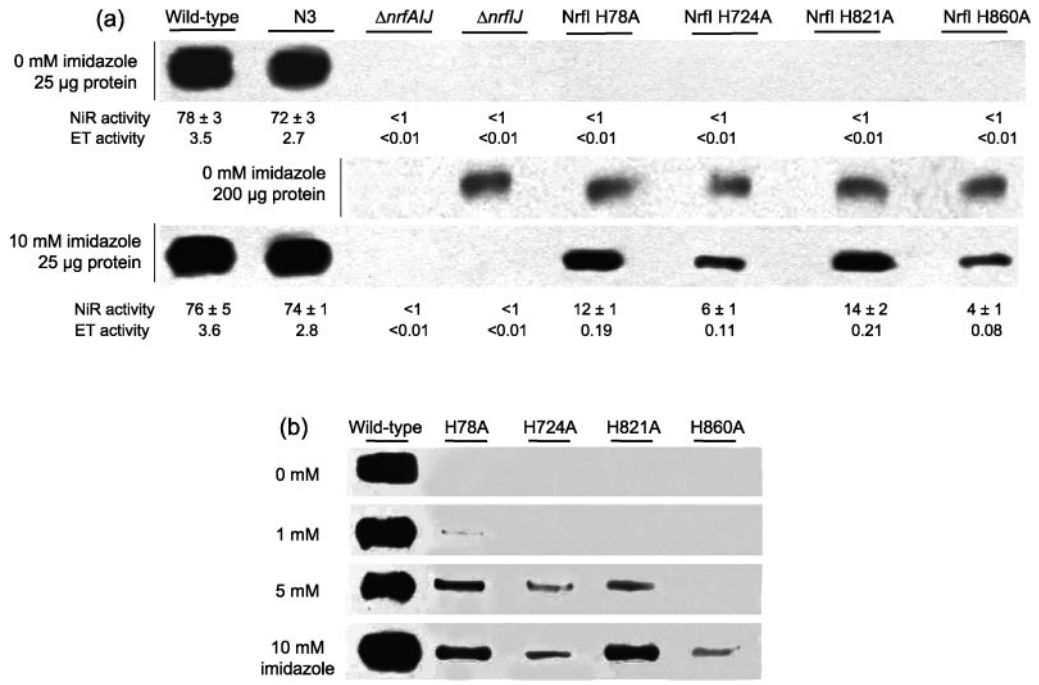

Fig. 2. Detection of cytochrome *c* nitrite reductase (NrfA) by haem staining in cell homogenates of different *W. succinogenes* strains. Cell homogenates (protein amounts as indicated) of nitrate-grown cells were separated by SDS-PAGE and blotted onto a PVDF membrane. Only the gel region containing 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.

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 (P_{frd}) (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* Δ*mcc 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 P_{frd} upstream of *mccA* (Fig. 1). The resulting strain (*W. succinogenes* P_{frd}-*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 P_{frd}-*mcc* after deletion of the *ccsA1* gene (Fig. 3, lane 2).

Derivatives of *W. succinogenes* P_{frd}-*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).

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.

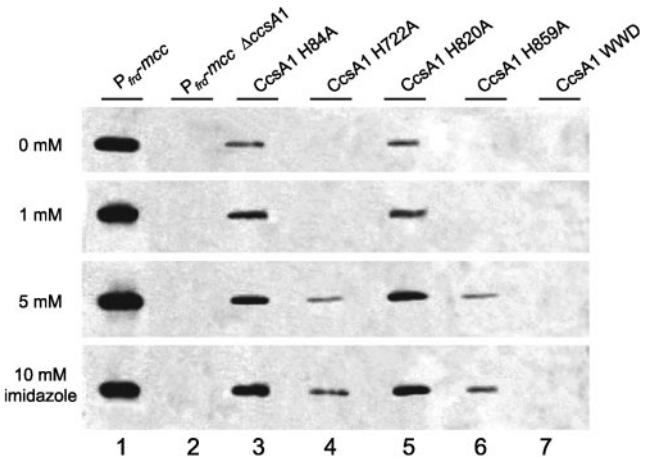

Fig. 3. Detection of MccA by haem staining in cell homogenates of different *W. succinogenes* strains. Fumarate-grown cells were separated by SDS-PAGE (100 μg protein applied per lane), blotted onto a PVDF membrane and subjected to haem staining. Only the gel region containing MccA (at about 75 kDa) is shown. Initial imidazole concentrations added to the cultures are shown at the left of each gel image.

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

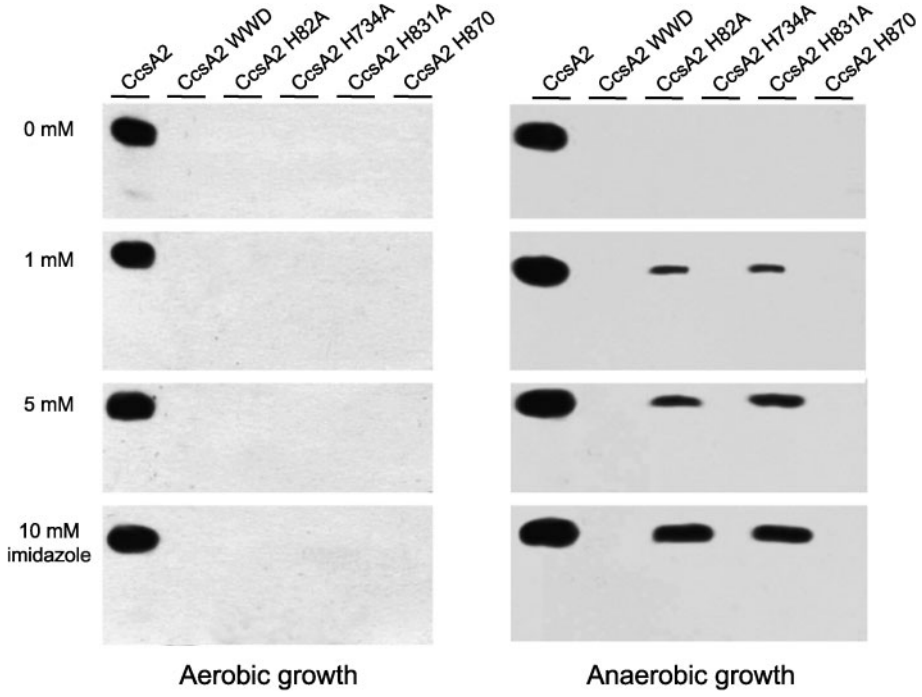

Fig. 4. Detection of *B. pertussis* cytochrome c_4 (CycC) in periplasmic extracts of different *E. coli* strains. Samples from cells grown under aerobic or anaerobic conditions were separated by SDS-PAGE, blotted onto a PVDF membrane and subjected to haem staining (50 μg protein applied per lane). Only the gel region containing cytochrome c_4 (at about 24 kDa) is shown. Concentrations of imidazole added to the cultures are shown at the left of the figure.

acquired evidence suggests that CcsBA-type CCHLs mediate haem export with the help of two haem *b* 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 *b* 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 *H. hepaticus* CcsBA histidine variants when produced in *E. coli* RK103 (Frawley & Kranz, 2009). However, this effect was only achieved 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 *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 *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 *H. hepaticus* or CcsA2 from *W. succinogenes*) were produced in *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 *Chlamydomonas reinhardtii* and the WWD domain-containing CcmF from *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 *H. hepaticus* CcsBA and for CcsA from *C. reinhardtii* (Hamel *et al.*, 2003; Frawley & Kranz, 2009). The WWD domains of the *E. coli* CcmC and CcmF proteins have also been subjected to modification (Schulz *et al.*, 2000; 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 *W. succinogenes* CCHL isoenzymes in order to attempt future purification by affinity chromatography. As CcsBA from *H. hepaticus* as well as *B. subtilis* ResB seem to be prone to proteolytic degradation in *E. coli*, it might be beneficial to use *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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