Significance of MccR, MccC, MccD, MccL and 8-methylmenaquinone in sulfite respiration of *Wolinella succinogenes*

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## ABSTRACT

Reduction of sulfite to sulfide is an essential step in the biogeochemical sulfur cycle. The Epsilonproteobacterium *Wolinella succinogenes* uses the copper-containing octahaem cytochrome *c* sulfite reductase MccA to respire sulfite. MccA is encoded by the first gene of the *mcc* gene cluster, whose transcription is apparently induced by the two-component regulatory system MccRS. It has been proposed that the iron-sulfur protein MccC, the putative quinol dehydrogenase MccD, the copper chaperone MccL as well as menaquinone-6 (MK_6) and/or 8-methylmenaquinone-6 ($8-MMK_6$) are involved in the electron transport chain of *W. succinogenes* sulfite respiration. Here, non-polar *W. succinogenes* mutants were constructed that lacked MccC, MccD, MccL or the 8-MMK_6-producing MK_6 methyltransferase MqnK. Each mutant possessed a frameshift-corrected *mccR* gene, thus inducing *mcc* expression in the presence of a mixture of fumarate and sulfite as terminal electron acceptors. Under these conditions, growth by sulfite respiration of cells lacking MccA, MccC or MccD was found to be abolished. However, cells lacking MccL or 8-MMK_6 still coupled formate oxidation to sulfite reduction and grew by sulfite respiration to some extent. The results indicate that MccR, MccC, MccD, MccL and 8-MMK_6 are essential or significant components of *W. succinogenes* sulfite respiration.

1. Introduction

The sulfite (SO_3^{2-}) and hydrogen sulfite (HSO_3^-) anions are reactive and hence toxic substances that can cause damage to proteins, nucleic acids and lipids of living organisms. Hence, sulfite is widely used as disinfectant, antioxidant and food preservative. Nonetheless, many microorganisms reduce sulfite in assimilatory and/or dissimilatory metabolism, thereby contributing to the biogeochemical sulfur cycle on Earth [1–5]. Canonical cytoplasmic assimilatory and dissimilatory sulfite reductases (aSir/dSir) contain a coupled sirohaem-[4Fe-4S] cluster cofactor and catalyse the six-electron reduction of sulfite to yield sulfide according to Eq. (1).

$$HSO_3^- + 6\,e^- + 6\,H^+ \rightleftharpoons HS^- + 3\,H_2O; \quad E_0{'}\,(HSO_3^-/HS^-) = -116\;mV \tag{1}$$
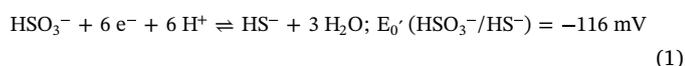
Dissimilatory sulfite reduction is a key step in sulfate reducing bacteria and archaea, for example in *Desulfovibrio* and *Archaeoglobus* species. The dSir enzyme of these organisms is a sirohaem-dependent DsrAB complex and several high-resolution structures of dSir enzymes helped to elucidate the reaction mechanism of sulfite reduction [6–10]. In sulfate reducing bacteria, electron transport from membranous quinols to DsrAB most likely involves a membrane-bound DsrMKJOP complex as well as DsrC, a protein that has been shown to bridge a sulfite-derived sulfur atom via two conserved cysteine residues [2,11,12].

Although unable to respire sulfate various prokaryotes are capable to grow at the expense of sulfite reduction by employing a respiratory and presumably also detoxifying sulfidogenic process [4]. Examples comprise Epsilonproteobacteria such as *Wolinella succinogenes*, *Sulfurospirillum deleyianum* and several *Campylobacter* species [13,14] as well as gammaproteobacterial *Shewanella* spp. [15] and Firmicutes, for example species of the genus *Desulfitobacterium*. Organisms that lack DsrAB, such as *W. succinogenes* and *Shewanella oneidensis*, have been reported to contain an alternative respiratory sulfite reductase, namely a periplasmic octahaem cytochrome *c* designated MccA (or SirA in case of *S. oneidensis*) [13,15,16]. The high-resolution crystal structure of *W. succinogenes* MccA revealed that this enzyme is an unprecedented haem *c*-copper(I) sulfite reductase [17]. The heterobimetallic active site of MccA was found to contain a Cu(I) ion juxtaposed to haem 2 at a Fe–Cu distance of 4.4 Å and the oxidation-labile Cu(I) centre did not seem to undergo a redox transition during catalysis. At haem 2, MccA tightly bound SO_2, a dehydration product of the substrate sulfite. Another distinctive feature of MccA is the presence of two different haem *c* binding motifs, namely seven canonical CX_2CH motifs and one unique $CX_{15}CH$ motif binding haem 8 [16–19]. MccA proteins are predicted in

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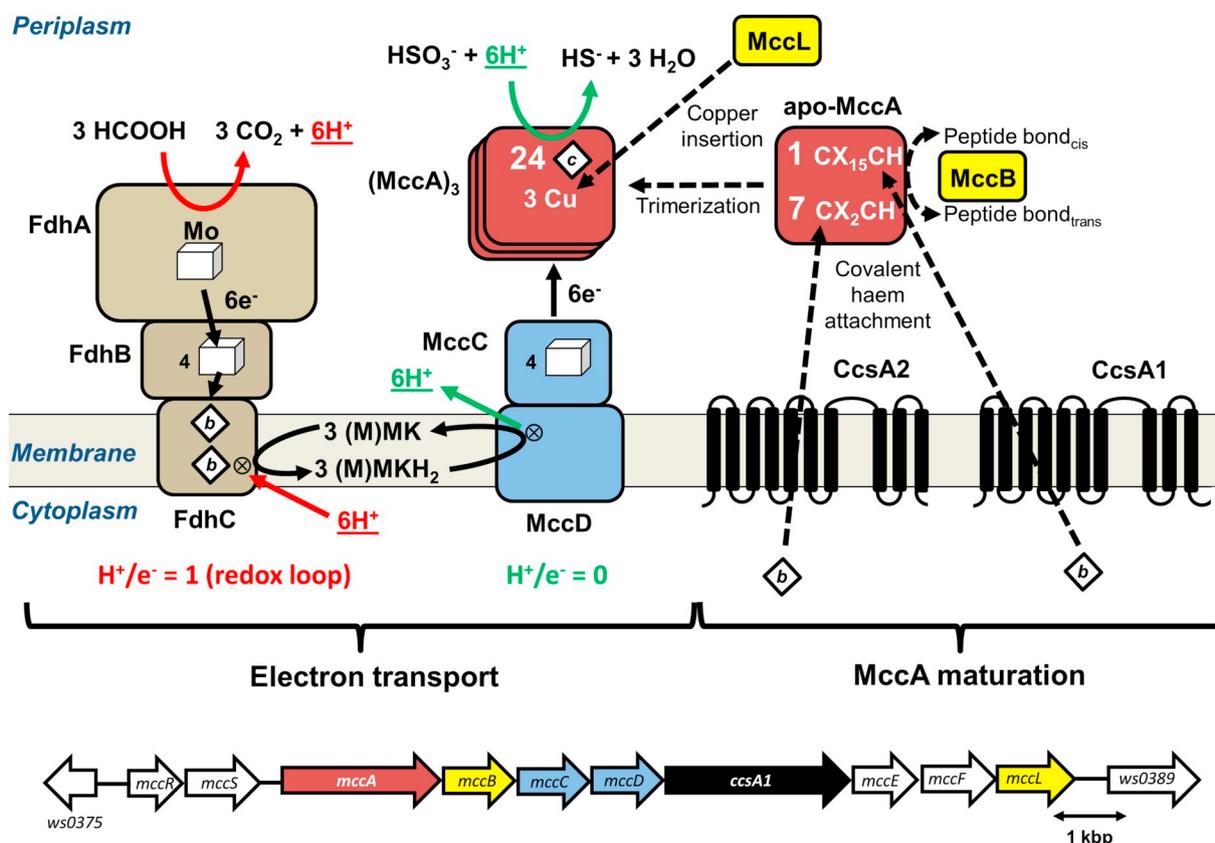 *E-mail address:* simon@bio.tu-darmstadt.de (J. Simon).

Fig. 1. Working model of the electrogenic *W. succinogenes* electron transport chain that catalyses formate oxidation by sulfite and genomic organisation of the corresponding *mcc* gene cluster. The electron transport chain catalysing the six-electron reaction of sulfite reduction by formate (formic acid) comprises the heterotrimeric formate dehydrogenase complex (FdhABC), the (methyl)menaquinone pool, the putative MccCD quinol dehydrogenase complex and the periplasmic copper-containing cytochrome *c* sulfite reductase MccA. Formate and sulfite are drawn in their protonated forms to illustrate the bioenergetics of sulfite respiration based on the coupled turnover of protons (underlined) and electrons. While formate-dependent quinone reduction has been shown to be electrogenic, quinol oxidation by sulfite is assumed to be electroneutral. The distinct function of two cytochrome *c* synthase isoenzymes (CcsA1 and CcsA2) in periplasmic MccA maturation is depicted. Both Ccs enzymes contain ten transmembrane segments and are assumed to transport haem *b* across the membrane [19,26,28–30]. It is assumed that CcsA1 and CcsA2 handle the CX_2CH and $CX_{15}CH$ haem *c* binding motifs respectively. Furthermore, the putative peptidyl *cis-trans* isomerase MccB and the assumed copper chaperone MccL are thought to be involved in MccA maturation. For simplicity, only monomeric enzyme forms are shown with the exception of homotrimeric MccA [16,17]. Solid black arrows indicate electron transport reactions, dashed black arrows mark protein maturation steps, red and green arrows designate reactions involving electrogenic and electroneutral proton turnover respectively. Diamonds denote haem *b* or *c* groups; cubes designate iron-sulfur clusters containing four or three iron atoms and ⊗ marks quinone/quinol binding sites. Mo, molybdenum cofactor; (M)MK, menaquinone-6 or 8-methylmenaquinone-6; (M)MKH_2, menaquinol-6 or 8-methylmenaquinol-6. See Fig. 3 for a visualization of the *mccE* and *mccF* gene products. Adapted from Simon and Kroneck [4].

the genome sequences of several non-sulfate reducing Beta-, Gamma-Delta- and Epsilonproteobacteria (genera *Burkholderia*, *Parasutterella*, *Sutterella*, *Shewanella*, *Edwardsiella*, *Ferrimonas*, *Anaeromyxobacter*, *Campylobacter*, *Sulfurospirillum* and *Wolinella*) and a diverse range of corresponding *mcc* gene clusters has been described [4,13].

Using formate as electron donor *W. succinogenes* cells grow by sulfite (hydrogen sulfite) respiration according to reaction Eq. (2) [13].

$$3\,HCO_2^- + HSO_3^- + 3\,H^+ \rightleftharpoons 3\,CO_2 + HS^- + 3\,H_2O;\ \Delta G_0'$$
$$= -57\,kJ\,mol^{-1}\,formate \tag{2}$$
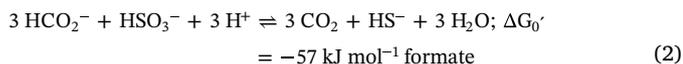
A model of the electron transport chain of *W. succinogenes* sulfite respiration has been established based on the composition of the *mcc* gene cluster (Fig. 1). In this model, energy conservation is accomplished through the redox loop mechanism of the membrane-bound formate dehydrogenase complex. In contrast, sulfite-dependent quinol oxidation by the Mcc system is envisaged to be electroneutral, i.e. non-proton motive. The corresponding electron transport pathway has been postulated to comprise the iron-sulfur protein MccC as immediate redox partner protein of MccA and the quinol dehydrogenase MccD, a member of the NrfD/PsrC family of polytopic membrane proteins [20–22]. However, it remained unresolved whether menaquinone-6 (MK_6), 8-methylmenaquinone-6 (8-MMK_6) or both could serve in mediating

electrons between the formate dehydrogenase complex and the sulfite reductase system. *W. succinogenes* cells have been shown to produce the two menaquinone species during fumarate or polysulfide respiration [23,24] and recently the methyltransferase MqnK, which catalyses 8-MMK_6 formation via MK_6 methylation, has been identified and characterized [24]. Compared to MK/MKH_2 ($E_0' \approx -80\,mV$), the midpoint redox potential of 8-MMK/8-$MMKH_2$ is about 70 mV more negative, thus placing the standard redox potential at pH 7 of the hydrogen sulfite/sulfide pair (Eq. (1)) right in the middle between those of 8-MMK/8-$MMKH_2$ and MK/MKH_2 [25]. Apart from MK_6 and 8-MMK_6, no other quinone has been described to be present in *W. succinogenes* cells.

Downstream of *mccD*, the *W. succinogenes mcc* gene cluster contains four more genes: *ccsA1*, *mccE* (formerly annotated as *ws0384*), *mccF* (*ws0385*) and *mccL* (*ws0387*) (Fig. 1). CcsA1 is a typical epsilonproteobacterial cytochrome *c* synthase of the so-called cytochrome *c* biogenesis system II that has been shown to be required for MccA synthesis [16,19,26,27]. While the function of MccE and MccF is not clear (see Discussion for more details), the *mccL* gene product is a putative copper chaperone that resembles NosL encoded in nitrous oxide reductase (*nos*) gene clusters. The start codon of the *mccL* gene had been misannotated in the *W. succinogenes* genome sequence and therefore, initially, *mccL* was not considered as a genuine component of the *mcc* gene cluster

[13,31]. Later, however, the MccL protein has been extended at the N-terminus by 46 amino acid residues to the length of 349 residues (NCBI reference WP_041571694 as opposed to GenBank entry CAE09533).

A previous characterization of the respiratory Mcc system from *W. succinogenes* made use of genetically engineered cells in which the *mcc* gene cluster was expressed under the control of a fumarate reductase promoter element (P_{frd}) [13]. This was due to the finding that *W. succinogenes* wild-type cells commenced to reduce sulfite only about 8 h after the exhaustion of other electron acceptors of anaerobic respiration such as fumarate or nitrate. Any sulfite reduction activity was in accord with the amount of cellular MccA detected by haem staining of SDS polyacrylamide gels in which appropriate *W. succinogenes* cell homogenates had been separated [13]. However, the underlying regulatory mechanism was not resolved. Here, it is demonstrated that the expression of the *mcc* gene cluster depends on the response regulator MccR whose gene was found to be truncated in wild-type cells. Restoration of an intact *mccR* gene led to a sulfite-inducible Mcc system in the presence of fumarate. This phenotype was exploited to investigate the role of MccC, MccD, MccE, MccF, MccL and 8-MMK$_6$ in sulfite respiration by characterizing appropriate mutants.

2. Material and methods

2.1. Growth of bacterial cells

Strains and mutants of *W. succinogenes* used in this study are listed in Table 1. *W. succinogenes* cells were grown in liquid media containing formate (100 mM) and fumarate as energy substrates as described previously by Kröger et al. [32] except that 45 mM instead of 90 mM fumarate was used. 10 mM sodium sulfite (final concentration) was added when indicated. For growth by sulfite respiration in the absence of fumarate the medium described by Kern et al. [13] was used. Growth by nitrate respiration was achieved in medium containing 80 mM sodium formate and 50 mM potassium nitrate [33,34]. Growth with N_2O as terminal acceptor was performed as described previously [35,36]. Media for N_2O respiration contained 100 mM formate as electron donor and were gassed with pure N_2O. Routinely, media for growth by nitrate and N_2O respiration contained 5 mM fumarate serving as carbon source. Where required, fumarate was replaced by 5 mM sodium succinate. The media were degassed and flushed with nitrogen gas several times to reduce the oxygen content. Media were supplemented with 0.5% (w/v) brain-heart infusion broth (Sigma). Kanamycin (25 mg l^{-1}), chloramphenicol (12.5 mg l^{-1}) and/or apramycin (30 mg l^{-1}) were added where appropriate. Molar cell yields for *W. succinogenes* sulfite respiration were determined using the method described by Hein et al. [35]. An OD$_{578}$ value of one corresponded to 0.73 g dry cell mass. Cells of *Escherichia coli* XL-1 Blue (Agilent Technologies) were used for plasmid construction and amplification. The cells were grown aerobically at 37 °C in LB medium (Lennox) in the presence of kanamycin (50 mg l^{-1}), chloramphenicol (25 mg l^{-1}) or apramycin (60 mg l^{-1}) for plasmid maintenance.

2.2. Construction of W. succinogenes mutants

Standard genetic procedures were used [37]. Genomic DNA from *W. succinogenes* was isolated using the DNeasy Tissue Kit (Qiagen). PCR was carried out using Q5 High Fidelity DNA polymerase (NEB) for cloning and sequencing procedures or One*Taq* DNA polymerase (NEB) for mutant and plasmid screening with standard amplification protocols. Plasmid DNA and PCR fragments were purified using the GenElute HP Plasmid Miniprep Kit or the GenElute PCR Clean-Up Kit (Sigma Aldrich).

Plasmids used for constructing *W. succinogenes* mutants were assembled from up to five PCR fragments using different cloning strategies (see Tables S1 and S2 for compilations of primers and plasmids). Each plasmid contained two DNA stretches that were identical with regions in the *W. succinogenes* genome and served in double homologous recombination (see Fig. S2 for length and localization of these regions). Successful construction of each plasmid was confirmed by DNA sequencing. Mutants 4 and 10–12 (Table 1) were constructed with plasmids obtained by conventional cloning using DNA restriction endonucleases [24,38]. In contrast, the plasmids serving to construct mutants 5–9 were assembled from PCR fragments containing suitable 20 bp overlaps by one-step isothermal assembly according to Gibson et al. [39] using the NEBuilder HiFi DNA Assembly Master Mix (NEB). These plasmids were derivatives of pASK-IBA3 (IBA GmbH). Between the two DNA fragments designed for homologous recombination one or more PCR products were introduced. One of those fragments was an antibiotic resistance gene cassette [either *kan* (originally from pUC4K) or *apr* from pAC1A as described by Cameron and Gaynor [40]]. If appropriate, an *mccA* promoter element was inserted downstream of the antibiotic resistance gene cartridge to avoid polar effects in *W. succinogenes* mutants. The plasmids used for constructing *W. succinogenes* *mccR*$^+$ Δ*mccA*::*kan*, *W. succinogenes* *mccR*$^+$ Δ*mccD*::*kan* and *W. succinogenes* *mccR*$^+$ Δ*mccEF*::*apr* were derivatives of pkomp_mccBCD and pkomp_mccEF, respectively (Table S2). A specific primer pair was designed to amplify

Table 1
Strains and mutants of *W. succinogenes* used in this study.

Strain/mutant[a]	Relevant properties[b]	Reference
1. Wild-type	Type strain DSM 1740^T containing a truncated version of *mccR*	DSMZ; [31]
2. *nosZ*$^+$	Derivative of the wild-type strain containing an intact *nosZ* gene (note that *nosZ* in wild-type cells is disrupted by a copy of the insertion element IS*1302*; [31,41])	[36]
3. Δ*mqnK*::*kan*	Derivative of the wild-type strain; the *mqnK* gene encoding the menaquinone methyltransferase MqnK is replaced by a kanamycin resistance gene cassette (*kan*); Km^R	[24]
4. *mccR*$^+$ *cat*	Derivative of the wild-type strain carrying a corrected frameshift in *mccR*, thus encoding the full-length response regulator of the MccRS two-component regulatory system; Cm^R	This work
5. *mccR*$^+$ Δ*mccA*::*kan*	Derivative of mutant 4; part of the *mccA* gene (nucleotides 502–2073) is replaced by *kan* and an *mcc* promoter element (P$_{mcc}$); Km^R; Cm^R	This work
6. *mccR*$^+$ komp *mccBCD*	Derivative of mutant 4; the *mccB*, -*C* and -*D* genes are replaced by *kan*, P$_{mcc}$ and *mccB*, -*C*, -*D*; Km^R; Cm^R	This work
7. *mccR*$^+$ Δ*mccC*::*kan*	Derivative of mutant 4; the *mccB*, -*C* and -*D* genes are replaced by *kan*, P$_{mcc}$, *mccB* and *mccD* (the entire *mccC* gene is lacking except for 24 bp at the 3′-end); Km^R; Cm^R	This work
8. *mccR*$^+$ Δ*mccD*::*kan*	Derivative of mutant 4; the *mccB*, -*C* and -*D* genes are replaced by *kan*, P$_{mcc}$, *mccB*, *mccC* and a partial *mccD* gene (lacking nucleotides 1–429 at the 5′-end); Km^R; Cm^R	This work
9. *mccR*$^+$ Δ*mccEF*::*apr*	Derivative of mutant 4; the genes *mccE* (nucleotides 4–531) and *mccF* (nucleotides 1–829) are replaced by *apr* and an *mcc* promoter element (P$_{mcc}$); Cm^R; Apr^R	This work
10. *mccR*$^+$ Δ*mccL*::*kan*	Derivative of mutant 4; the entire *mccL* is replaced by *kan*; Km^R; Cm^R	This work
11. *mccR*$^+$ Δ*mqnK*::*kan*	Derivative of mutant 4; the entire *mqnK* gene is replaced by *kan* as decribed by Hein et al. [24]; Km^R; Cm^R	This work
12. Δ*mccRS*::*kan*	Derivative of the wild-type strain; the entire *mccR* gene and part of *mccS* (nucleotides 1–1046) are replaced by *kan*; Km^R	This work

[a] See Fig. S1 for partial genome maps of the wild-type strain and mutants 4–12.
[b] Km^R, Cm^R and Apr^R denote resistance against kanamycin, chloramphenicol and apramycin respectively.

pkomp_mccBCD omitting *mccC* or part of *mccD* or to amplify pkomp_mccEF omitting most of *mccE* and *mccF*. Obtained PCR products were phosphorylated with T4 polynucleotide kinase (NEB) and ligated using T4 DNA ligase (Invitrogen).

Transformation of nitrate-grown *W. succinogenes* cells with the constructed plasmid was performed by electroporation as described previously [38]. Transformants were selected on agar plates in the presence of kanamycin (25 mg l^{-1}) or apramycin (30 mg l^{-1}) and intended double homologous recombination events were verified by sequencing suitable PCR products.

2.3. Determination of sulfite, sulfide and formate

Formate was measured as described [42]. Sulfite was determined according to Pachmayer [43] using a slightly modified protocol: 100 ml of fuchsine reagent (40 mg fuchsine dissolved in 87.5 ml deionized water and 12.5 ml concentrated H_2SO_4) was mixed with 800 ml sample (containing up to 0.1 mM sulfite). After 10 min of incubation at room temperature 10 μl formaldehyde [37% (w/v)] was added. The optical density at 570 nm was determined after 90 min against a fuchsine reagent blank. Sulfide was quantified following methylene blue formation according to King and Morris [44]. Formate was measured following the reduction of NAD$^+$ by formate dehydrogenase as described by Bergmeyer [45].

2.4. Determination of turnover rates

W. succinogenes cells were harvested in the late exponential growth phase, washed and resuspended (15–20 g protein l^{-1}) in anoxic buffer (pH 7.3) containing 100 mM Tris/HCl and 0.3 M sucrose. Aliquots of the cell suspension were incubated for 5 min at 37 °C before the addition of sodium formate (40 mM final concentration) and sodium sulfite (10 mM). Samples were taken every 5 min and concentrations of sulfite, sulfide, and formate were determined.

2.5. Quinone extraction and analysis

Solvent extraction of quinones from *W. succinogenes* membranes was performed as described previously [24]. After solvent evaporation dry quinones were dissolved in methanol. The quinones were separated by reversed phase HPLC using an OmniSpher 5 C18 150 × 4.6 mm column (Agilent) on a Hitachi LaChrom Elite system. Pure methanol was used as an eluent at a flow rate of 1 ml min^{-1}. UV spectra of eluted quinones were recorded using the L-2450 diode array detector of the HPLC system [35].

3. Results

3.1. Substrate-induced sulfite respiration and characterization of W. succinogenes mccR$^+$ cat

The genome of *W. succinogenes* wild-type cells carried a frameshift mutation in the *mccR* gene (Fig. 1). Consequently, *mccR* encoded a truncated and most likely inactive response regulator due to an incomplete C-terminal DNA-binding domain (see Fig. S1 for details). Based on primary structure alignments using MccR proteins encoded in *mccR*, -*S* gene arrangements from various Epsilonproteobacteria the underlying frameshift mutation in *mccR* was corrected by deleting one deoxyadenosine monophosphate nucleotide, yielding mutant *W. succinogenes mccR$^+$ cat* (see Table 1 for a compilation of all strains and mutants used in this study and Fig. S2 for detailed depictions of corresponding *mcc* loci). The restored *mccR* gene encoded a typical response regulator of 229 amino acid residues containing a signal receiver domain including a conserved phosphorylation site, a dimerization interface and a canonical DNA-binding domain (Fig. S1).

Compared with wild-type cells or cells lacking *mccA* (mutant *W. succinogenes mccR$^+$ ΔmccA::kan*), *W. succinogenes mccR$^+$ cat* cells reached a higher optical density at 578 nm (OD$_{578}$) in the stationary phase after growth in anoxic medium containing 45 mM fumarate and

10 mM sodium sulfite as terminal electron acceptors (Table 2; see Fig. S3A for corresponding growth curves), indicating simultaneous fumarate and sulfite respiration. The observed OD$_{578}$ increase of around 0.12 is attributed to growth by sulfite respiration and this value is in accord with the final OD$_{578}$ of a *W. succinogenes mccR$^+$ cat* culture grown with 10 mM sulfite as sole electron acceptor (OD$_{578}$ difference of about 0.09 as compared with mutant *W. succinogenes mccR$^+$ ΔmccA::kan*; Table 2). Apparently, the presence of 10 mM sulfite did not affect growth by fumarate respiration since non-sulfite converting wild-type cells reached about the same optical density after reduction of 45 mM fumarate irrespective of the presence or absence of sulfite (Table 2). Based on the OD$_{578}$ increase ascribed to sulfite respiration, a molar cell yield of 6.6–8.8 g dry cells per mole sulfite (about 2.2–2.9 g dry cells per mole formate) was estimated, which corresponds to 31–41% of the values determined for fumarate or nitrate respiration [35]. As expected, the presence of MccA in *W. succinogenes mccR$^+$ cat* cells grown in the presence of sulfite was confirmed by haem staining (Fig. S4). Notably, when *W. succinogenes mccR$^+$ cat* cells were grown by nitrate respiration in a medium containing 80 mM formate, 50 mM nitrate and 10 mM sulfite, formation of MccA was not observed (not shown).

In medium containing fumarate and sulfite, growing cells of *W. succinogenes mccR$^+$ cat* consumed 10 mM sulfite within 8 h (Fig. 2A). At the time point of sulfite exhaustion approximately 45 mM formate had been converted, supporting the view that fumarate and sulfite were reduced at the same time. Specific consumption rates of formate or sulfite as well as the production rate of sulfide were determined using washed cell suspensions of *W. succinogenes mccR$^+$ cat* (Table 2). The observed rates indicated a functional respiratory Mcc system and suggested a formate per sulfite stoichiometry of three, indicating that sulfite respiration operated according to reaction Eq. (2). The turnover values were similar to those reported previously for wild-type cells grown by sulfite respiration in the absence of fumarate [13]. Growth by sulfite respiration as well as formate-dependent sulfite reduction was abolished in the absence of MccA or MccRS (mutants *W. succinogenes mccR$^+$ ΔmccA::kan* and *W. succinogenes ΔmccRS::kan*; Tables 1 and 2; Fig. 2B and C, S2, S3A and S4).

3.2. The role of MccC, MccD, MccE, MccF, MccL and 8-MMK$_6$ in sulfite respiration

Individual deletions of *mccC*, *mccD* (partial) or *mccL* were introduced in the *mcc* gene cluster of *W. succinogenes mccR$^+$* cells by double homologous recombination (mutants 7, 8 and 10 in Table 1; Fig. S2). In case of the *mccC* and *mccD* deletions an additional *mcc* promoter was inserted downstream of the inserted kanamycin resistance gene cassette (*kan*) to avoid polar effects. As a control for this strategy, mutant *W. succinogenes mccR$^+$ komp mccBCD* was constructed (Table 1; Fig. S2). In the genome of this mutant, all *mcc* genes downstream of *mccA* were put under the control of the introduced *mcc* promoter situated between *kan* and *mccB*. Cells of this mutant showed about wild-type properties with respect to sulfite respiration and sulfite turnover to sulfide (Table 2; Figs. 2B and S3A). The deletions of *mccC* or *mccD* resulted in cells with similar phenotypes; cell suspensions of *W. succinogenes mccR$^+$ ΔmccC::kan* and *W. succinogenes mccR$^+$ ΔmccD::kan* did not show any notable formate and sulfite conversion despite the presence of MccA in amounts similar to that of the parental *W. succinogenes mccR$^+$* mutant (Table 2; Figs. 2E and F and S4). Accordingly, growth experiments in medium with 45 mM fumarate and 10 mM sulfite did not suggest growth by sulfite respiration (Table 2; Fig. S3B). Likewise, the two mutants did not grow by sulfite respiration in the absence of fumarate (Table 2; Fig. S3C).

Lack of the putative copper chaperone MccL or the 8-MMK$_6$-producing menaquinone methyltransferase MqnK allowed growth by sulfite respiration to a minor degree in the presence or absence of fumarate and the corresponding mutants *W. succinogenes mccR$^+$ ΔmccL::kan* and

Table 2

Characterization of sulfite respiration as well as substrate turnover in suspensions of *W. succinogenes* wild-type cells and various mutants.

Strain/mutant	OD_{578} value in the stationary growth phase[a]	Doubling time [min][a]	Formate consumption rate[b]	Sulfite consumption rate[b]	Sulfide production rate[b]
			$[nmol\ min^{-1}\ mg\ protein^{-1}]$		
Fumarate/sulfite respiration (45 mM fumarate, 10 mM sulfite)					
Wild-type	0.463 ± 0.020	65 ± 17	< 0.1	< 0.1	< 0.1
mccR[+] *cat*	0.597 ± 0.012	63 ± 9	239 ± 20	68 ± 7	63 ± 4
mccR[+] Δ*mccA*::*kan*	0.476 ± 0.016	73 ± 18	< 0.1	< 0.1	< 0.1
mccR[+] komp *mccBCD*	0.566 ± 0.011	72 ± 11	225 ± 19	61 ± 6	57 ± 4
mccR[+] Δ*mccC*::*kan*	0.484 ± 0.007	69 ± 4	< 0.1	< 0.1	< 0.1
mccR[+] Δ*mccD*::*kan*	0.487 ± 0.004	67 ± 2	< 0.1	< 0.1	< 0.1
mccR[+] Δ*mccEF*::*apr*	0.548 ± 0.011	72 ± 2	210 ± 9	63 ± 3	51 ± 3
mccR[+] Δ*mccL*::*kan*	0.511 ± 0.021	77 ± 9	155 ± 17	45 ± 6	35 ± 4
mccR[+] Δ*mqnK*::*kan*	0.514 ± 0.010	72 ± 2	118 ± 8	33 ± 1	26 ± 2
Δ*mccRS*::*kan*	0.481 ± 0.002	77 ± 13	< 0.1	< 0.1	< 0.1
Fumarate respiration (45 mM fumarate)					
Wild-type	0.468 ± 0.018	69 ± 6	< 0.1	< 0.1	< 0.1
mccR[+] *cat*	0.486 ± 0.016	61 ± 6	< 0.1	< 0.1	< 0.1
Sulfite respiration (10 mM sulfite)					
mccR[+] *cat*	0.149 ± 0.013	216 ± 59	187 ± 14	63 ± 5	51 ± 4
mccR[+] Δ*mccA*::*kan*	0.056 ± 0.004	∞ (no growth)	< 0.1	< 0.1	< 0.1
mccR[+] komp *mccBCD*	0.152 ± 0.009	297 ± 33	213 ± 11	67 ± 5	57 ± 4
mccR[+] Δ*mccC*::*kan*	0.053 ± 0.004	∞ (no growth)	< 0.1	< 0.1	< 0.1
mccR[+] Δ*mccD*::*kan*	0.054 ± 0.001	∞ (no growth)	< 0.1	< 0.1	< 0.1
mccR[+] Δ*mccEF*::*apr*	0.153 ± 0.006	274 ± 26	214 ± 9	68 ± 3	56 ± 1
mccR[+] Δ*mccL*::*kan*	0.109 ± 0.011	343 ± 24	127 ± 9	43 ± 4	36 ± 4
mccR[+] Δ*mqnK*::*kan*	0.082 ± 0.008	447 ± 112	111 ± 7	32 ± 2	28 ± 3

Mean values and standard deviations from at least three biological replicates are shown.

[a] The OD_{578} value was determined after 24 h of incubation in the indicated medium. Refer to Fig. S3 for corresponding growth curves.

[b] Determined using concentrated cell suspensions as described in Experimental procedures.

W. succinogenes mccR[+] Δ*mqnK*::*kan* contained MccA in wild-type amounts (Table 2; Figs. S3B, C and S4). Formate consumption as well as sulfite conversion to sulfide was observed in growing cultures and non-growing cell suspensions amounting to values from 55 to 70% (*W. succinogenes mccR*[+] Δ*mccL*::*kan*) and from 41 to 59% (*W. succinogenes mccR*[+] Δ*mqnK*::*kan*) of those of mutant *W. succinogenes mccR*[+] (Table 2; Fig. 2H and I). These results indicated that cells lacking MccL or MqnK were still capable of catalysing electron transport from formate to sulfite.

In contrast to the mutants described above, the characterization of the mutant lacking the open reading frames *mccE* and *mccF* (*W. succinogenes mccR*[+] Δ*mccEF*::*apr*, Table 1; Fig. S2) did not reveal any notable difference to the parental mutant (Table 2; Figs. 2G, S3B and C and S4).

3.3. The proportion of MK₆ and 8-MMK₆ in W. succinogenes wild-type and mutant cells grown by different modes of anaerobic respiration

Wild-type or *W. succinogenes mccR*[+] *cat* cells grown with fumarate, sulfite or a mixture of both contained about equal amounts of MK_6 and 8-MMK_6 (between 43 and 64% 8-MMK_6; Table 3). Deletion of *mqnK* in *W. succinogenes mccR*[+] *cat* cells abolished 8-MMK_6 formation; similar to what has been reported previously for wild-type cells [24]. In contrast, the portion of MK_6 relative to 8-MMK_6 in *W. succinogenes* cells grown by nitrate or N_2O respiration was found to be significantly elevated (85% MK_6 or above; Table 3). Note that cells of *W. succinogenes nosZ*[+] have been used for the latter measurements since wild-type cells did not grow by N_2O respiration due to a disrupted *nosZ* gene [35,36,46].

4. Discussion

This work refines the knowledge of the dedicated electron transport chain and the biogenesis apparatus underlying MccA-dependent sulfite respiration of *W. succinogenes* as illustrated in Fig. 1. To date, as far as we know, no other Mcc system or *mcc* gene cluster has been characterized in such detail.

4.1. Sulfite induction of the respiratory Mcc system depends on the regulatory two-component system MccRS

The construction of mutant *W. succinogenes mccR*[+] *cat* allowed exploring sulfite respiration under sulfite-responsive conditions for the first time. Our current model of *mcc* gene cluster upregulation involves the MccRS regulatory two-component system, of which the response regulator MccR has now been shown to be essential to establish an active Mcc system in fumarate-respiring cultures. It is remarkable that even *W. succinogenes mccR*[+] *cat* cells did not produce the Mcc system in the presence of nitrate, implying the hierarchical use of electron acceptors that has also been described for *Escherichia coli* [47]. In fact, RNA-seq data from fumarate- and nitrate-grown *W. succinogenes nosZ*^{+} cells (*mccR*[−] genotype) revealed similar low transcript abundances of genes organized in the *mccA* cluster (S. Hein and J. Simon, unpublished data). Similar experiments will be performed in the future using sulfite-grown *W. succinogenes mccR*[+] *cat* cells to characterize the sulfite regulon. Notably, the membrane-bound histidine kinase MccS is predicted to contain a conserved periplasmic monohaem cytochrome *c* domain that could play a role in sulfite (and/or SO_2) sensing and the initiation of a corresponding signal transduction process [19].

Cells used in the previous study by Kern et al. [13] apparently lacked a functional regulatory two-component system MccRS (*mccR*[−] genotype; Fig. S1) and hence it is conceivable that the supposed sulfite regulon had been severely affected. In the absence of functional MccR, another transcriptional activator might have caused *mccA* gene cluster upregulation following the complete consumption of fumarate (or nitrate) and this assumption could explain the previously described derepression phenotype of *mccR*^{−} cells [see Introduction and Kern et al. [13]].

4.2. Bioenergetics of W. succinogenes sulfite respiration and the composition of the electron transport chain connecting the quinone pool and MccA

The presented data confirmed the results of an earlier study inasmuch as MccC and MccD proved to be obligatory constituents of the electron transport chain delivering electrons to MccA [13]. This
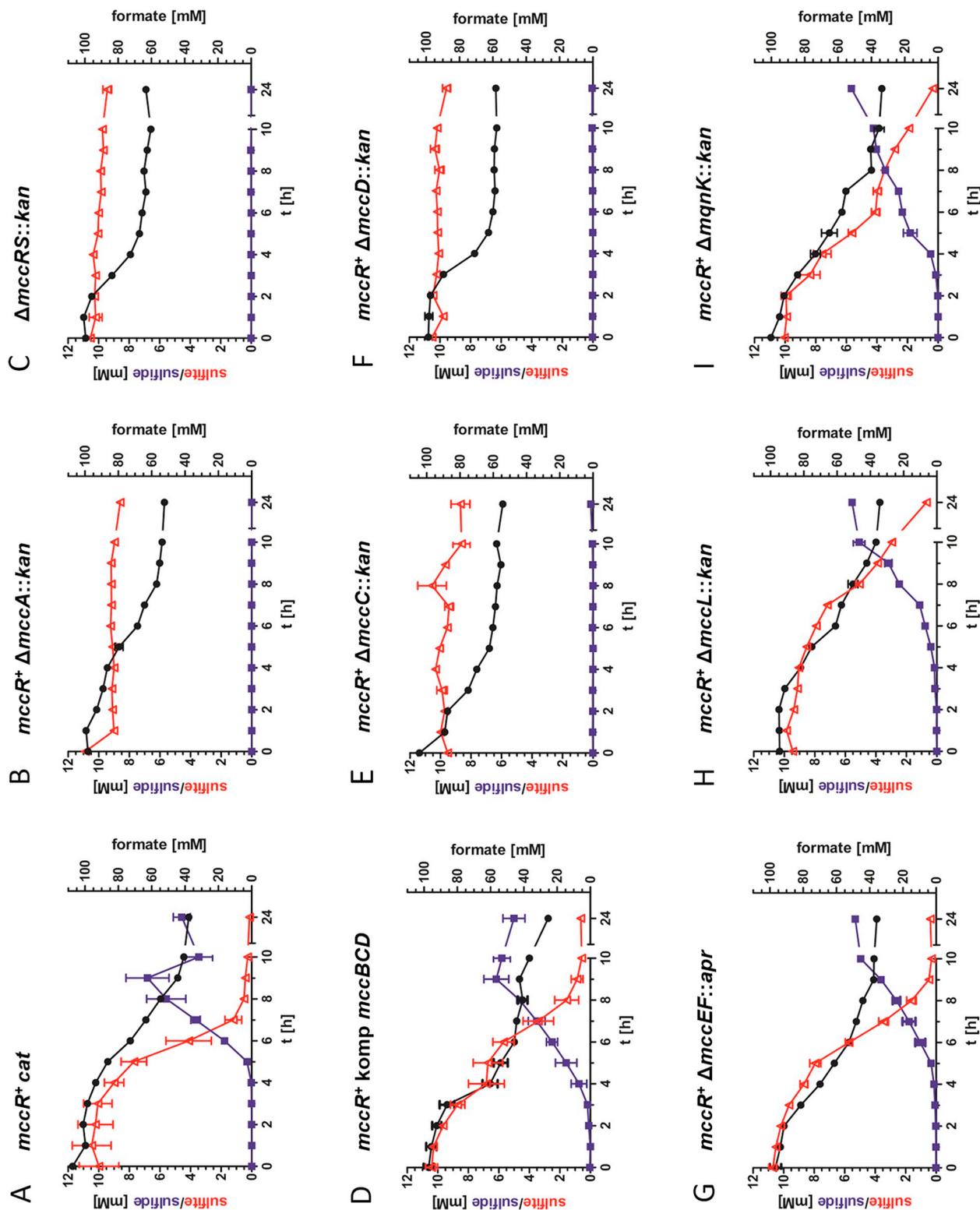
Fig. 2. Substrate turnover in growing *W. succinogenes* cultures. The medium contained 100 mM formate, 45 mM fumarate and 10 mM sulfite as energy substrates (see Experimental procedures for details). Traces of formate (circles), sulfite (triangles) and sulfide (squares) concentrations are shown.

Table 3
Proportion of MK_6 and $8\text{-}MMK_6$ in membranes of *W. succinogenes* wild-type and mutant cells.

Strain/mutant	Terminal electron acceptor(s)/ [carbon source[a]]	MK_6 [%]	$8\text{-}MMK_6$ [%]
Wild-type	Fumarate (45 mM)	36	64
$mccR^+$ *cat*	Fumarate (45 mM)	47	53
$nosZ^+$	Fumarate (45 mM)	57	43
Wild-type	Fumarate (45 mM); sulfite (10 mM)	46	54
$mccR^+$ *cat*	Fumarate (45 mM); sulfite (10 mM)	44	56
$mccR^+$ *cat* $\Delta mqnK::kan$	Fumarate (45 mM); sulfite (10 mM)	100	0
$nosZ^+$	Fumarate (45 mM); sulfite (10 mM)	56	44
$mccR^+$ *cat*	Sulfite (10 mM)/[Fumarate]	53	47
$mccR^+$ *cat*	Sulfite (10 mM)/[Succinate]	54	46
$nosZ^+$	N_2O/[Fumarate]	85[b]	15[b]
$nosZ^+$	N_2O/[Succinate]	98[b]	2[b]
$nosZ^+$	Nitrate (50 mM)/[Fumarate]	88	12
$nosZ^+$	Nitrate (50 mM)/[Succinate]	88	12

The amounts of MK_6 and $8\text{-}MMK_6$ were estimated from HPLC data (see Experimental procedures). Mean values of least three biological replicates are shown. The standard deviation was < 2% in each case.

[a] Sodium fumarate or sodium succinate was added to a final concentration of 5 mM.

[b] Calculated from data reported by Hein et al. [35].

suggests that, even under sulfite-responsive conditions, electrons are linearly transported via MccD and MccC to MccA and that neither of these proteins can be replaced in *W. succinogenes* sulfite respiration (Fig. 1). However, since the *mccC* and *-D* genes are not conserved throughout the many *mcc* gene cluster variations, other modes of electron transport to MccA might exist [4,13]. The assumed ratio of protons translocated across the membrane per electron transported from formate to sulfite (H^+/e^-) is expected to be one, due to the well-established redox loop mechanism operated by the quinone-reactive membrane-bound formate dehydrogenase complex FdhABC (Fig. 1) [22,48,49]. In contrast, electron transport from quinol to sulfite is assumed to be electroneutral as illustrated in Fig. 1. An H^+/e^- value of one is rather close to the estimated theoretical H^+/e^- maximum for formate-dependent sulfite reduction of around two, calculated according to Eq. (3).

$$(H^+/e^-)_{max} = \Delta E/\Delta p = 0.316\ V/0.15\ V = 2.11 \qquad (3)$$

Here, standard redox potentials at pH 7 (E_0') of $-432\ mV$ (CO_2/HCO_2^-) and $-116\ mV$ (HSO_3^-/HS^-) [50] were taken into account and an electrochemical proton potential across the cytoplasmic membrane (the *proton motive force, pmf*) of $-150\ mV$ (outside positive) was assumed. Thus, under standard conditions at pH 7, sulfite respiration appears thermodynamically demanding but achievable. Thus, if the FdhABC-catalysed redox loop were functional as described, the electron transport chain from quinol to sulfite almost certainly will not contribute to the *pmf*.

On the other hand, the experimentally determined cell yield of sulfite respiration turned out to be only about half of that of fumarate-respiring *W. succinogenes* cells for which a H^+/e^- of one has been firmly established in the past [35,49,51,52]. Thus, it cannot be excluded that the actual H^+/e^- of sulfite respiration is less than one and this situation is reminiscent of *W. succinogenes* polysulfide respiration, in which the bioenergetic constraints are even more unfavourable than in sulfite respiration given an E_0' for the redox couple polysulfide/hydrogen sulfide of about $-275\ mV$ [53]. According to Eq. (3) this would result in a $(H^+/e^-)_{max}$ value of 1.05 when formate is used as electron donor, implying that the actual H^+/e^- is well below one. In fact, a value of 0.5 has been proposed for polysulfide respiration based on the cell yield of 3.2 g dry cells per mole of formate [53,54].

In this context it is important to note that reconstituted *W. succinogenes* polysulfide respiration has been shown to strictly depend on the presence of $8\text{-}MMK_6$ in the proteoliposomal membrane [23]. This finding appears reasonable since the E_0' value of MK/MKH_2 is about 200 mV more positive than that of polysulfide/hydrogen sulfide. It cannot be excluded, however, that in living cells the existence of a mixture of MK_6 and $8\text{-}MMK_6$ was sufficient to account for any observed electron transport rates [55]. The membrane-bound protein PsrC has been proposed to be the quinol-reactive subunit of the heterotrimeric *W. succinogenes* PsrABC complex [23]. Apparently, polysulfide reductase receives electrons from reduced $8\text{-}MMK_6$ bound to PsrC. Along with MccD, PsrC belongs to the NrfD/PsrC family, of which only one member has been structurally explored. PsrC from *Thermus thermophilus* forms eight transmembrane domains and contains a putative quinone/quinol binding site near the periplasmic surface of the membrane [20]. This site was experimentally shown to be occupied by MK_7 or the inhibitor pentachlorophenol in corresponding crystal structures of the *T. thermophilus* PsrABC complex. Regarding the mechanism of *W. succinogenes* polysulfide respiration, a hypothesis has been put forward that assumes diffusion and collision of the FdhABC complex and the $8\text{-}MMK_6$-containing PsrABC assembly in the membrane [23,53]. This scenario implies that the redox loop of FdhABC is affected to lower the H^+/e^- from one to the thermodynamically feasible value of 0.5. The corresponding mechanism has not been elucidated. The formation of a stabilized anionic hydroquinone form of $8\text{-}MMK_6$ might be involved whose oxidation by polysulfide could be driven by the Δp across the membrane.

We propose that MccD is the quinol-oxidizing protein in *W. succinogenes* sulfite respiration. In line with the thermodynamic constraints it seems plausible that MccD is able to oxidize the reduced forms of both MK_6 and $8\text{-}MMK_6$, which could explain the stimulating effect of $8\text{-}MMK_6$ on the electron transport rate and growth by sulfite respiration. In this context, it is notable that most of the organisms predicted to possess MccA also encode an MK methyltransferase, with the exception of some *Edwardsiella* and *Anaeromyxobacter* species (S. Hein and J. Simon, unpublished data). In any case, the functionality of sulfite respiration with MK_6 in the absence of $8\text{-}MMK_6$ clearly distinguishes polysulfide from sulfite respiration. In line with what has been proposed for polysulfide respiration, the (transient) complex formation between FdhABC and MccACD cannot be excluded but currently there is no experimental evidence for this. Attempts to purify MccC, MccD or a MccAC complex have not been successful (J. Eller and J. Simon, unpublished data).

Under the described conditions, the $8\text{-}MMK_6$ content is not upregulated in polysulfide- or sulfite-grown *W. succinogenes* cells as compared with fumarate-grown cells (Table 3). In this respect, it is notable that the *W. succinogenes* genome encodes an $8\text{-}MMK_6$-dependent fumarate reductase complex in addition to the canonical MK_6-dependent fumarate reductase FrdABC [52,56]. On the other hand, $8\text{-}MMK_6$ synthesis might be repressed when the cells use nitrate or laughing gas as electron acceptor, i.e. substances with positive redox potentials $[E_0'(NO_3^-/NO_2^-) = +433\ mV; E_0'(N_2O/N_2) = +1355\ mV]$. In principle, the maintenance of a certain $MK_6/8\text{-}MMK_6$ ratio might involve the regulation of the amount and/or specific activity of the $8\text{-}MMK_6$-producing MqnK enzyme [24]. Furthermore, an $8\text{-}MMK_6$ demethylase might be involved although such an enzyme has not yet been described. Such a scenario is reminiscent to the varying 2-demethylmenaquinone-8 (DMK_8)/MK_8 ratios found in response to the redox potential of the terminal electron acceptor in *E. coli* [57,58].

4.3. The role of accessory proteins encoded in the W. succinogenes mcc gene cluster

Apparently, five accessory gene products are encoded in the *W. succinogenes mcc* gene cluster, namely MccB, CcsA1, MccE, MccF and MccL (Figs. 1 and 3). As stated in the Introduction, MccA proteins are most
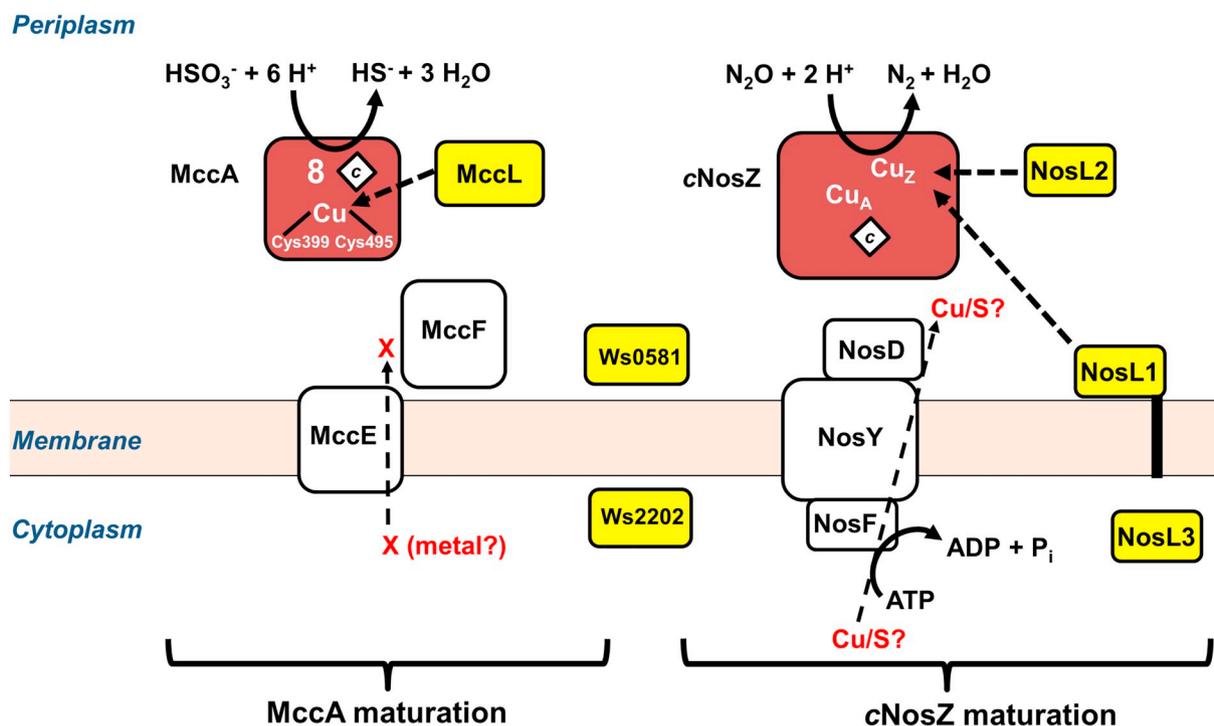
Fig. 3. Working model illustrating the putative functionality of different *W. succinogenes* copper chaperones as well as of MccE and MccF in MccA and *c*NosZ biogenesis respectively. NosL1 is predicted to be a membrane-anchored lipoprotein. For clarity only one monomer of the periplasmic (MccA)₃ and (*c*NosZ)₂ oligomers are shown. *c*NosZ, cytochrome *c* nitrous oxide reductase; NosDFY, ABC transporter of unknown function. Dashed arrows indicate speculative protein-protein interactions or transport processes. See Fig. 1 for further explanations, Table 4 for the prediction of the cellular localization and the Discussion for details on individual proteins.

peculiar as they contain an unconventional haem *c* binding motif (consensus sequence $CX_{15 \text{ or } 17}CH$) that is uniquely found in this type of cytochrome *c* [13,16–18]. For *W. succinogenes* MccA, it has been shown by Hartshorne et al. [16] that haem 8 is bound by both cysteine residues of the $CX_{15}CH$ motif. Even slight alterations of this motif led to protein destabilization and/or folding defects precluding MccA detection in a haem stain assay [13]. The extended region between the two cysteine residues of the $CX_{15}CH$ motif forms a loop with a short helical turn in direct vicinity to another loop harbouring the only non-proline *cis* peptide in the enzyme, between residues G508 and F509 [17]. Formation of this *cis* peptide might require the essential peptidyl isomerase MccB (Fig. 1). MccB is a conserved protein commonly encoded in *mcc* gene clusters. It was originally annotated as a peptidyl *cis-trans* isomerase and shown to be essential for MccA synthesis/stability in *W. succinogenes* [13]. It has been speculated that the *cis* peptide could be a prerequisite for correct loop folding during haem 8 attachment, which is likely to require the dedicated cytochrome *c* synthase CcsA1 (Fig. 1) [18,19,27]. Haem 8 is exposed to the protein surface and appears to be a suitable entry point for electrons presumably delivered by MccC (Fig. 1). Notably, the structure of the $CX_{15}CH$ haem *c* binding motif disrupts the general parallel/perpendicular haem *c* stacking sequence and rotates the haem group out of plane, possibly to optimize the interaction with MccC. A homologue of CcsA1, designated CcsA2, is required in *W. succinogenes* cytochrome *c* biogenesis and this cytochrome *c* synthase is likely to covalently attach haem to the canonical CX_2CH haem *c* binding motif (Fig. 1) [27]. CcsA1 and CcsA2 are typical proteins belonging to the so-called cytochrome *c* biogenesis system II. However, MccA is also encoded in genomes of organisms employing the cytochrome *c* biogenesis system I, for example in some *Shewanella* species [4,15]. Interestingly, the corresponding *mcc* gene loci encode homologues of NrfE, -F and -G from *E. coli* that have been postulated to form a dedicated cytochrome *c* synthase involved in the maturation of the cytochrome *c* nitrite reductase NrfA [59,60]. Thus, the *Shewanella* NrfE, -F and -G proteins might be a functional counterpart to CcsA1 during MccA biogenesis.

The astounding and unexpected finding that *W. succinogenes* MccA contained a Cu(I) ion in its active site raised the question as to how this copper atom is incorporated into MccA [17]. A copper chaperone is usually needed to accomplish this task and the *mccL* gene product is an obvious candidate. Compared with other members of the NosL family, *W. succinogenes* MccL is a large copper chaperone that contains two putative copper binding motifs and is presumably exported to the periplasm by the Sec apparatus (Table 4). Although supportive of the view that MccL is involved in the formation of active MccA, the phenotype of the *W. succinogenes* mutant lacking MccL suggests that at least one other protein might also play a role in MccA copper insertion.

In general, free copper ions are toxic due to their high chemical reactivity and produce reactive oxygen species by Fenton-like chemistry [61]. Therefore, all forms of life have to ensure copper homeostasis. In prokaryotes copper transport (import/export) and delivery/metallation systems such as cytoplasmic and periplasmic copper chaperones contribute to this tightly controlled and highly balanced process. Copper toxicity is usually circumvented by fixing Cu(I) in the form of stable Cu(I)-thiol complexes through copper-binding proteins and/or low molecular thiols such as glutathione. Copper chaperones often use cysteine residues arranged in a CX_2C motif as Cu(I) ligands resulting in exceptionally low K_D values in the femto- to zeptomolar (10^{-15} to 10^{-21}) range [62]. Bacterial cuproenzymes are usually located in the periplasm or in the cytoplasmic membrane with the catalytic site oriented to the periplasm, thus avoiding the necessity of copper accumulation in the cytoplasm. Besides MccA, the most prominent copper-binding respiratory enzymes in *W. succinogenes* are the membranous cytochrome cbb_3 oxidase and the periplasmic cytochrome *c* nitrous oxide reductase *c*NosZ. Such respiratory enzymes often harbour conserved specific copper arrangements such as the Cu_B-haem a_3 site in cytochrome oxidase or the binuclear Cu_A and tetranuclear Cu_Z centres in nitrous oxide reductase [17,63]. In this respect, MccA seems to be exceptional as it contains a single redox-inactive Cu(I) ion. The *W. succinogenes* genome predicts a sophisticated copper homeostasis network and encodes six

Table 4
Putative copper chaperones encoded in the *W. succinogenes* genome.

Designation (locus tag/accession number)	Genetic context	Length (amino acids)	Predicted cellular location and type of signal peptide[a]	Potential copper-binding motif (CX$_2$CGM)
MccL (Ws0387; WP_041571694)	*mcc* gene cluster	349	Periplasm. Signal peptide of 19 residues; no lipoprotein signal peptide	CPNCGM, CPICGM
NosL1 (Ws0925; CAE10029)	*nos* gene cluster	151	Membrane-anchored periplasmic protein. Signal peptide of 23 residues or potential lipoprotein (cleavage after 19 residues)	CERCKM
NosL2 (Ws0927; CAE10031)[b]	*nos* gene cluster	165	Periplasm? Weak signal peptide of 24 residues; no lipoprotein signal peptide	Only one cysteine in processed protein[b]
NosL3 (Ws0928; CAE10032)	*nos* gene cluster	147	Cytoplasm. No signal peptide; no lipoprotein signal peptide	CALCGM
Ws0581 (CAE09713)	Orphan gene	230	Periplasm. Signal peptide of 19 residues; no lipoprotein signal peptide	CPVCGM
Ws2202 (CAE11192)	Orphan gene	244	Cytoplasm. No signal peptide; no lipoprotein signal peptide	CPNCGM

[a] Based on predictions made by programs SignalP and LipoP.

[b] NosL2 is related to NosL proteins from, for example, *Achromobacter cycloclastes* and *Paracoccus denitrificans* ([70,71]). In these proteins Cu(I) is thought to be coordinated by two sulfur ligands (Cys/Met or Met/Met) and one nitrogen/oxygen. Note that the corresponding *nos* gene clusters do only encode one NosL-type protein, in contrast to the *W. succinogenes nos* gene cluster ([46,64]).

putative copper chaperones of which at least three are predicted to bind copper in the periplasm (Fig. 3; Table 4). The copper chaperones include MccL and three NosL-type proteins encoded by three of the four terminal open reading frames of the N$_2$O reductase-encoding *nos* gene cluster [46,64–66]. However, our knowledge on the functional and structural properties of copper chaperones is scarce and the specificity of individual copper chaperones for their copper-accepting interaction partners is not known.

Many different *mcc* gene clusters, though not all, contain an *mccL* orthologue [4]. Moreover, a conserved *mccL-nosD-nosF-nosY* gene assembly is present in the genomes of various *Shewanella* species suggesting a functional relationship between MccL and a putative NosDFY-type ABC transporter located in the cytoplasmic membrane (Fig. 3). Such a transporter has been shown to be involved in the biogenesis of N$_2$O reductase in Proteobacteria such as *Pseudomonas stutzeri* and it has been proposed that it might help to translocate copper ions and/or an unresolved "sulfur compound" to the periplasm [63,67–69]. Notably, *nosD, -F* and *-Y* genes are also present in the genome of *W. succinogenes* as part of the *nos* gene cluster and a functional crosstalk between the respiratory Mcc and Nos systems cannot be excluded. Another possibility is that MccE and MccF are functionally equivalent to the NosDFY complex as hypothesized in Fig. 3. MccE is a putative integral membrane protein predicted to form four transmembrane segments. It belongs to the CopD superfamily and might be involved in metal ion transport. MccF belongs to the Sel1 superfamily and contains a Sec-dependent signal peptide, seven CX$_7$C motifs and multiple Sel1-like repeats. However, homologues of the *mccE* and *-F* genes are found only in *mcc* gene clusters from some epsilonproteobacterial species of the genera *Campylobacter*, *Sulfurospirillum* and *Wolinella* [4]. The lack of MccE and MccF did not affect the *W. succinogenes* Mcc system, confirming results obtained previously with similar deletion mutants in an *mccR$^-$* background [mutants *W. succinogenes* Δ*ws0384/5::kan* and *W. succinogenes* P$_{frd}$*-mcc* Δ*ws0384/5::kan*; Kern et al. [13]]. Thus, similar to MccL, functional redundancy is expected.

Transparency document

The Transparency document associated with this article can be found, in online version.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbabio.2018.10.002.

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