Pleiotropic effects of mutations that alter the *Sinorhizobium meliloti* cytochrome c respiratory system

Svetlana N. Yurgel,1 Jhoanna Berrocal,2 Cynthia Wilson2 and Michael L. Kahn1,2

1Institute of Biological Chemistry, Washington State University, Pullman, WA 99164-6340, USA
2School of Molecular Biosciences, Washington State University, Pullman, WA 99164-6340, USA

Using transposon mutagenesis, mutations have been isolated in several genes (*ccda*, *cycM*, *ccmC*, *ccMB* and *senC*) that play a role in *Sinorhizobium meliloti* cytochrome metabolism. As in other bacteria, mutations in the *S. meliloti* *ccda*, *ccmB* and *ccmC* genes resulted in the absence of all c-type cytochromes. However, the *S. meliloti* *ccda* mutant also lacked cytochrome oxidase *aa*3, a defect that does not appear to have been reported for other bacteria. The *aa*3-type cytochromes were also missing from a mutant strain with an insertion into the gene encoding the haem-containing subunit (SU1) of *aa*3 cytochrome c oxidase, but not in mutants unable to make SUII or SUIII, indicating that CcdA probably plays a role in assembling SU1. The cytochrome-deficient mutants also had other free-living phenotypes, including a significant decrease in growth rate on rich media and increased motility on minimal media. A *senC* mutant also had significantly decreased motility, but the motility and growth properties of the *cycM* mutant were unchanged. Unlike similar mutants in *Bradyrhizobium japonicum* and *Rhizobium leguminosarum*, an *S. meliloti* Rm1021 *cycM* mutant contained cytochrome oxidase *aa*3. Cytochrome maturation in strain Rm1021 appeared to be similar to maturation in other rhizobia, but there were some differences in the cytochrome composition of the strain, and respiration chain function and assembly.

**INTRODUCTION**

In the symbiotic interaction between legumes and rhizobia, nitrogen fixation occurs in a microaerobic environment established in the root nodule, an organ that forms after bacterial infection of the root. Like many bacteria, rhizobia have a branched electron transport chain (Delgado et al., 1998), in which the terminal oxidases have different affinities for oxygen. Reducing equivalents derived from catabolism of different energy sources are transferred first to the quinone pool, which is oxidized by the cytochrome *bc*1 complex. Electrons derived from quinol oxidation may be delivered either to cytochrome *aa*3 using transmembrane cytochrome c (CycM) as an intermediate carrier, or to cytochrome *bb*3 oxidases. Alternatively, quinol may bypass the cytochrome *bc*1 complex and directly reduce a quinol oxidase. These alternative respiratory chains allow rhizobia to adapt to various environmental conditions. In the microaerobic environment of the root nodule, it appears to be essential for nitrogen-fixing rhizobia to use a cytochrome *cb*b5 terminal oxidase with a high affinity for oxygen in order to employ electron transport and oxidative phosphorylation to meet the high ATP requirements of nitrogenase (Delgado et al., 1998).

Various *aa*3, *b*, *c* and *o* cytochromes have been observed in spectra from *Rhizobium leguminosarum* bv. *viciae*, *R. leguminosarum* bv. *trifolii*, *Rhizobium etli*, *Bradyrhizobium japonicum* (reviewed by Delgado et al., 1998) and *Sinorhizobium meliloti* strain CXM1-188 (Yurgel et al., 1998). The major rhizobial respiratory chain in aerobic conditions consists of a cytochrome *bc*1 complex, cytochrome *c*, and a cytochrome *aa*3 terminal oxidase. A respiratory chain essential in symbiosis couples the cytochrome *bc*1 complex to a cytochrome *cb*b5 terminal oxidase (Renalier et al., 1987; Delgado et al., 1998). Both of these pathways use c-type cytochromes as electron carriers; therefore, disrupting the cytochrome *c* maturation process impairs symbiotic performance (Delgado et al., 1998). One major event in the biogenesis of c-type cytochromes is the covalent attachment of haem to cysteine residues in the haem-binding motif Cys-Xaa-Xaa-Cys-His, which is found

**Abbreviations:** CycM, transmembrane cytochrome c; SU, *aa*3 cytochrome oxidase subunit; TMPD, *N*,*N*,*N*'-tetramethyl-p-phenylenediamine.
in all c-type apocytochromes. Three distinct systems for cytochrome c maturation have been identified. In bacteria, c-type cytochromes are assembled in the periplasm via system I or system II (Kranz et al., 2002; O’Brien & Thony-Meyer, 2002). Rhizobia have been reported to use system I for cytochrome c maturation. The system uses numerous genes, including ccmEFH/cycHJKL, which are thought to code for bacterial haem lyases (Cinege et al., 2004; Delgado et al., 1995; Kereszt et al., 1995; Reyes et al., 2000; Ritz et al., 1993, 1995; Schulz & Thony-Meyer, 2000; Tabche et al., 1998), ccmAB/cycVW, which encodes components of an ATP-dependent haem transport system (Aguilar & Soberon, 1996; Ramseier et al., 1991), and the cytochrome c maturation factor cycZ/ccmC (Ramseier et al., 1989; Schulz et al., 1999; Schulz & Thony-Meyer, 2000).

Recently, it has been recognized that mutations affecting bacterial cytochrome c maturation can result in phenotypes that are not easily explained by the loss of c-type cytochromes (Cianciootto et al., 2005). These phenotypes include loss of siderophore production and utilization, reduced iron uptake and intracellular growth and virulence, impaired growth on rich media, and alteration in copper sensitivity (Cianciootto et al., 2005). In addition to affecting symbiotic performance, mutations in rhizobial cytochrome c maturation have been shown to influence siderophore production, protoporphyrin IX accumulation, iron acquisition, nitrate respiration and H2 oxidation (Yeoman et al., 1997; Cinege et al., 2004; Ramseier et al., 1991).

Bacterial mutants with altered respiration via c-type cytochromes can be identified using the Nadi cytochrome oxidase test (Marrs & Gest, 1973). Rhizobium mutants with defects in making cytochromes bc1, CycM or adaB, or in the biogenesis of c-type cytochromes are less pigmented in this test (Nadi−) (Delgado et al., 1998). The respiratory chains of B. japonicum, R. leguminosarum, R. etli and Azorhizobium caulinodans have been studied (Delgado et al., 1998; Marroqui et al., 2001). However, the only characterized S. meliloti mutants involved in c-type cytochrome biogenesis are defective in the cycHJKL genes (Cinege et al., 2004; Kereszt et al., 1995). In this study, we showed that the effects of cycM, ccmC and ccmB mutations on the cytochrome c respiratory chains were not identical to those of the corresponding mutants in other rhizobia, and identified new genes, ccda and senC, involved in rhizobial respiration. Additionally, we report that the mutations in some of these genes led to changes in motility, which is believed to be the first such report for the rhizobia.

**METHODS**

**Bacterial strains, plasmids and media.** The bacterial strains and plasmids used are listed in Table 1. S. meliloti strains were grown at 30°C on Luria–Bertani (LB) medium (Sambrook et al., 1989), minimal medium containing 1% mannitol (MM-NH4+) or 0.2% succinate (Min-succinate-NH4+), or on yeast mannitol broth (YMB) medium (Somerville & Kuhn, 1983). When indicated, 200 μg streptomycin or neomycin ml−1 was added to S. meliloti media. *Escherichia coli* strains were grown at 37°C on LB medium. As needed, kanamycin was added at a concentration of 40 μg ml−1.

**Genetic techniques.** Transduction and plasmid conjugation methods were as described by Finan et al. (1984) and Simon et al. (1983). S. meliloti strain Rm1021 was mutagenized with Tn5 using the suicide plasmid pSUP5011 (Simon et al., 1983). After overnight mating of Rm1021 with *E. coli* S17-1(pSUP501), Tn5 insertion mutants were selected on Min-succinate-NH4+ medium supplemented with neomycin. Neomycin-resistant colonies were screened under the Nadi cytochrome oxidase test (Marrs & Gest, 1973). In this test, the production of indophenol blue by a reaction between N,N,N′,N′-tetramethyl-p-phenylenediamine (TMPD) and z-naphthol depends on the amount of bacterial cytochrome c or cytochrome c oxidase. Mutants with decreased respiration via cytochrome c were isolated as colonies that remained white after staining.

**Localization of Tn5 insertions.** DNA manipulations were carried out according to standard procedures (Sambrook et al., 1989). The DNA sequences flanking Tn5 in the mutated genes were identified using the arbitrary PCR method described by Knobloch et al. (2003). The following primers were used for two steps of PCR amplification: first round, 5′-AGCCGTGCAGTACGACNNN-NNNNACGCC-3′ (AR1B), 5′-GAACCTCCTGAGCAACACTTG-3′ (transposon-specific primer); second round, 5′-GCCACCGCTGCTACTAGTAC-3′ (AR2B), 5′-ATGCGCTCCTCACTCCTGTA-3′ (transposon-specific primer). DNA sequencing was then carried out by the DyeDeoxy Terminator Cycle protocol (Applied Biosystems), using synthetic primer 5′-GAGAACACAGATTTAGCCACAG-3′ synthesized by Invitrogen. Sequencing reactions were analysed on an Applied Biosystems 373 DNA sequencer at the Washington State University Laboratory for Bioanalysis and Biotechnology. DNA sequences were then compared with the S. meliloti genome sequence to determine the site of Tn5 insertion.

**Subcloning of the S. meliloti ccmC, ccmC/D and ccmC/D/G.** Strain Rm1021 regions were amplified from chromosomal DNA using PCR with ccmC_F (5′-CATGAAGGTTATGAGTGAAAG-CAGCCTGGC-3′) as the forward primer, and ccmC/R (5′-CATGCTGCACTATGGCGTAAGCTGCCTCCGGC-3′), ccmCD_R (5′-CATGCTCAGCACTATGGCGTAAGCTGCCTCCGGC-3′) or ccmCDG_R (5′-CATGCTGCACTATGGCGTAAGCTGCCTCCGGC-3′) as the reverse primer, resulting in DNA fragments carrying the ccmC, ccmCD and ccmCD/G genes, respectively. Each primer had a 20–23 bp overlap with the S. meliloti genomic sequence, and a 10 bp 5′ extension that contained a HindIII restriction site for the forward primer and a PstI site for the reverse primer (underlined above). Following amplification, the PCR products were digested with PstI and HindIII, and ligated to PstI- and HindIII-digested pCPP30. The resulting plasmids were named pCPPccmC, pCPPccmCD and pCPPccmCDG.

**Construction of the S. meliloti Smc00010 deletion strain.** To construct an S. meliloti mutant with deletion of the entire cytochrome c oxidase polypeptide I gene (Smc00010), PCR was used to amplify 0.2 and 0.3 kb chromosomal regions flanking Smc00010, using primers Smc00010-F1 (5′-CATGAACTCGAGCTTGGCAGGAAATGACG-3′) and Smc00010-R1 (5′-CATGGATCTCGGAGGTAGAAGCGCCAT-3′) for the upstream flanking region, and primers Smc00010-F2 (5′-CATGGATCTCGGAGGTAGAAGCGCCAT-3′) or Smc00010-R2 (5′-CATGAACTCGAGCTTGGCAGGAAATGACG-3′) for the downstream flanking region. Each primer had a 20 bp overlap with the S. meliloti genomic sequence, and a 9 bp 5′ extension that contained an EcoRI restriction site for external ends and a BamHI site for internal ends (underlined above). Following amplification, the PCR products were digested with BamHI and ligated together. The resulting DNA
Table 1. Bacterial strains and plasmids

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Genotype or characteristics</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Strains</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>S. meliloti</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rm1021</td>
<td>Wild-type</td>
<td></td>
</tr>
<tr>
<td>W2</td>
<td>SMc03849/ccmC384*::Tn5-mob mutant of Rm1021</td>
<td>This work</td>
</tr>
<tr>
<td>W3</td>
<td>SMc00248/ccdA118::Tn5-mob mutant of Rm1021</td>
<td>This work</td>
</tr>
<tr>
<td>W5</td>
<td>SMc03848/ccdB251::Tn5-mob mutant of Rm1021</td>
<td>This work</td>
</tr>
<tr>
<td>W6</td>
<td>SMc01471/ccnC181::Tn5-mob mutant of Rm1021</td>
<td>This work</td>
</tr>
<tr>
<td>W8</td>
<td>SMc02897/cyeM228::Tn5-mob mutant of Rm1021</td>
<td>This work</td>
</tr>
<tr>
<td>W17</td>
<td>SMc00013/czdE549::Tn5-mob mutant of Rm1021</td>
<td>This work</td>
</tr>
<tr>
<td>W19</td>
<td>SMc00009/czdC707::Tn5-mob mutant of Rm1021</td>
<td>This work</td>
</tr>
<tr>
<td>Rm1021ASMc00010</td>
<td>SMc00101/czdA deletion mutant of Rm1021</td>
<td>This work</td>
</tr>
<tr>
<td><strong>E. coli</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S17-1</td>
<td>pro hsdR recA [RP4-2(Tc::Mu)(Km::Tn7)]</td>
<td>Simon et al. (1983)</td>
</tr>
<tr>
<td>S17-1 Δpir</td>
<td>Δpir lysogen of S17-1</td>
<td>Simon et al. (1983)</td>
</tr>
<tr>
<td><strong>Plasmids</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pSUP5011</td>
<td>pBR325::Tn5-mob, Ap′, Cm′, Km′</td>
<td>Simon et al. (1983)</td>
</tr>
<tr>
<td>pRG1SMa03849</td>
<td>ccmC pMK2030</td>
<td>Laboratory collection</td>
</tr>
<tr>
<td>pRG1SMa03848</td>
<td>ccmB pMK2030</td>
<td>Laboratory collection</td>
</tr>
<tr>
<td>pRG1SMa0248</td>
<td>ccdA pMK2030</td>
<td>Laboratory collection</td>
</tr>
<tr>
<td>pCPP33</td>
<td>IncP LacZ; Tc′</td>
<td>Huang et al. (1992)</td>
</tr>
<tr>
<td>pCPPccmC</td>
<td>pCPP30[ccmC]</td>
<td>This work</td>
</tr>
<tr>
<td>pCPPccmCD</td>
<td>pCPP30[ccmC–ccmD]</td>
<td>This work</td>
</tr>
<tr>
<td>pCPPccmCDG</td>
<td>pCPP30[ccmC–ccmD–ccmG]</td>
<td>This work</td>
</tr>
<tr>
<td>pK19mobSacB</td>
<td>pK19mob derivative sacB</td>
<td>Schafer et al. (1994)</td>
</tr>
<tr>
<td>pK19SacB-ASMc00010</td>
<td>pK19mobSacB[200–300 bp flanking SMc00010]</td>
<td>This work</td>
</tr>
</tbody>
</table>

*The numbers indicate the position of the transition from the Tn5 shoulder to the *S. meliloti* Rm1021 sequence.*

Spectral analysis of cytochrome composition. Cells were prepared, and spectra were recorded as described by Marroqui et al. (2001), with some modifications. The DNA samples were then digested with EcoRI and ligated into pK19mobsacB (Schafer et al., 1994), which had been digested with EcoRI, resulting in plasmid pK19SacB-ASMc00010. The correctness of the cloned fragment was confirmed by sequencing. The constructed deletion was transferred to the chromosome by recombination, as described by Yurgel & Kahn (2005). The *S. meliloti* strain missing SMc00010 was named Rm1021ASMc00010.

We performed spectral analysis of cytochromes in both exponential (16 h growth) and stationary (48 h growth) cultures of the parental strain and the mutants. Reduced minus oxidized spectra of exponential and stationary cultures of each strain were very similar. Therefore, Fig. 1(a) includes only the reduced minus oxidized spectra of the stationary cultures, while CO-reduced minus reduced spectra are presented for both exponential and stationary phases of the parental and the mutant strains (Fig. 1b, c).

Analysis of c-type cytochromes. Membranes were prepared from cultures grown for 24 h on Min-succinate-NH₄⁺ medium. Cells were harvested by centrifugation at 10 000 g for 15 min, washed once with 10 mM Tris-HCl, pH 8.0, and resuspended in 1/100 of the original culture volume in 10 mM Tris-HCl containing 1 mM MgSO₄ and trace amounts of DNase. The cell suspension was then sonicated three times for 3 min each at 4°C (Fisher Sonic Dismembrator 300 with the intermediate size attachment and a power setting of 60%). Identical results were obtained in separate experiments in which the cells were broken by passage through a French press. Unbroken cells were removed by centrifugation at 10 000 g for 15 min at 4°C, and discarded. Soluble and membrane fractions were separated by ultracentrifugation at 100 000 g for 2 h. The pellet membranes were resuspended in 10 mM Tris-HCl, pH 8.0. Protein concentrations were measured with a protein assay kit (Bio-Rad Laboratories), using BSA as the standard. Protein concentrations were measured with a protein assay kit (Bio-Rad Laboratories), using BSA as the standard. Protein concentrations were measured with a protein assay kit (Bio-Rad Laboratories), using BSA as the standard.
measurements. Cells (2) swarm plates containing MM-NH$_4$ medium and (b, c) CO-reduced minus reduced spectra from S. meliloti mutants. (a, b) Stationary-phase cultures grown for 48 h; (c) exponential-phase cultures grown for 12 h. Traces: 1, Rm1021; 2, W3 (ccdA); 3, W5 (ccmB); 4, W6 (senC); 5, W8 (cycM).

Fig. 1. Cytochrome difference spectra in Nadi mutants. (a) Dithionite-reduced minus ammonium-persulfate-oxidized and (b, c) CO-reduced minus reduced spectra from S. meliloti mutants. (a, b) Stationary-phase cultures grown for 48 h; (c) exponential-phase cultures grown for 12 h. Traces: 1, Rm1021; 2, W3 (ccdA); 3, W5 (ccmB); 4, W6 (senC); 5, W8 (cycM).

electroblotting to a nitrocellulose filter. Proteins containing covalently bound haem iron were visualized using chemiluminescence, as described by Vargas et al. (1993).

Respiratory activities. Oxygen uptake was determined as described by Marroqui et al. (2001). Cells were harvested after 24 h of growth at 30 °C in 10 ml Min-succinate-NH$_4$ medium, and resuspended in 10 ml 25 mM potassium phosphate buffer (pH 7.0). The oxygen uptake at 21 °C was measured using a Hansatech electrode after addition of 2 mM TMPD and 10 mM sodium ascorbate (final concentration).

Growth measurements. To evaluate growth on various carbon substrates, S. meliloti strains were first grown in MM-NH$_4$ medium at 30 °C for 48 h to late exponential phase. Cells were then washed twice with Min-salt solution (Minimal medium without a carbon or nitrogen source), and resuspended in the same volume of Min-salt solution. Two microlitres of the cell suspension was added to 200 μl of various nutrient media to test growth rates. Growth at 30 °C was monitored for 48 h by measuring OD$_{600}$ every 30 min using a SPECTRAMax 250 Microplate Spectrophotometer system (Molecular Devices).

Motility assay. The cells were prepared as described for the growth measurements. Cells (2) were placed onto 0.3% Bacto Agar swarm plates containing MM-NH$_4^+$, Min-succinate-NH$_4^+$, YMB or 0.1× YMB medium, and incubated at 30 °C for 3 days. For each experiment, the diameter of the area occupied by the mutants was measured and normalized as a percentage of the diameter of the swarm formed by the Rm1021 parental strain. These values were used to calculate relative motility of the mutants. The experiment was repeated at least three times.

Plant tests. Alfalfa (Medicago sativa cv. Champ) was used for all nodulation studies. The plant tests were performed as described previously (McDermott & Kahn, 1992; Yurgel & Kahn, 2005).

RESULTS

Isolation of S. meliloti Nadi$^-$ mutants and localization of Tn5 insertions

A set of Tn5-mutagenized S. meliloti Rm1021 colonies was screened for decreased ability to oxidize TMPD. Two types of mutants could be distinguished. The first group of mutants had a Nadi$^-$ phenotype and remained white after staining. The second group, Nadi$^+$, was able to oxidize TMPD and develop a blue colour on indicator media, but the colour development was much slower than that seen with the wild-type strain Rm1021. The mutated genes were identified using an arbitrary PCR method to determine DNA sequences adjacent to the inserted Tn5.

Three Nadi$^-$ mutants (W2, W3 and W5) and two Nadi$^+$ mutants (W6 and W8) were chosen for detailed study. Mutants W2, W3 and W5 contained mutations in genes for proteins homologous to a family of proteins found in z- and γ-proteobacteria and in mitochondria of plants and protozoa (Thony-Meyer, 2002) and that encode proteins involved in system I-type cytochrome c maturation. Mutant W2 was mutated in ccmC (Table 1). B. japonicum cccZ/ccmC is essential for the formation of all cellular c-type cytochromes (Rameiseier et al., 1989). E. coli cytochrome c maturation factor CcmC is involved in transferring haem to the periplasmic haem chaperone CcmE (Schulz et al., 1999; Schulz & Thony-Meyer, 2000). Using phage μ12-mediated transduction, we verified that the Nadi$^-$ phenotype of the W2 mutation was caused by the Tn5 insertion, since all transductants that carried neomycin resistance (Nm$^+$) from W2 into Rm1021 also conferred a Nadi$^-$ phenotype.

The Tn5 insertion in mutant W5 was located in ccmB (Table 1). CcmB, together with CcmA, seems to be a subunit of an ATP-binding cassette (ABC) transporter involved in cytochrome c biogenesis (Thony-Meyer, 2003). The B. japonicum and R. etli CycW/CcmB proteins are thought to be involved in haem translocation required for the synthesis of mature c-type cytochromes, and for symbiotic nitrogen fixation (Aguilar & Soberon, 1996; Rameiseier et al., 1989, 1991).

The transposon in mutant W3 was located in ORF Smc00248, which has been annotated as ccsA (Galibert et al., 2001), a gene involved in type II cytochrome c maturation (Beckett et al., 2000; Hamel et al., 2003). However, the protein predicted from Smc00248 showed 49% identity and 65% similarity over its entire length to Rhodobacter capsulatus CcdA (accession no. AF156103; protein ID number AAF26218.1), which is required for type I cytochrome c biogenesis (Deshmukh et al., 2000). We therefore propose that Smc00248 be called ccdA instead of ccsA (Table 1). Phage μ12-mediated transduction of Nm$^+$ from mutant W3 into Rm1021 transferred the Nadi$^-$ phenotype to all Nm$^+$ transductants.

The Tn5 insertion in mutant W6 was located in senC (Table 1), which is similar to eukaryotic SCO1 genes...
involved in the assembly of cytochrome c terminal oxidases (Leary et al., 2004; Schulze & Rodel, 1989). A senC gene has recently been shown to be involved in the assembly of a fully functional cbb3-type cytochrome oxidase in Rh. capsulatus (Swem et al., 2005). Mutant W8 had a Tn5 insertion in Smc02897, which is predicted to encode a 25.5 kDa protein similar to B. japonicum CycM (Bott et al., 1991).

While this set of mutants may not represent all mutants with low Nadi activity, more than one mutant was isolated for many of these genes. Out of 15 Nadi− mutants, two had insertions in ccmB, two in ccmC, three in ccaA and two in senC. Mutants W17 and W19 had Tn5 insertions in Smc00013 (ctAE) and Smc00009 (ctAC), genes that have been annotated as encoding putative cytochrome c oxidase subunits (SU)III and SUII, respectively (Galibert et al., 2001). One mutation was in cycM. The other three mutations were located in genes that appeared to be regulatory; an analysis of these mutants will be reported later.

**Cytochrome composition of S. meliloti strains Rm1021.** Reduced minus oxidized difference spectra revealed that S. meliloti strain Rm1021 produced c-type cytochromes (peaks at 550 and 520 nm) and a b-type cytochrome (peak at 560 nm) in exponential (data not shown) and stationary cultures (Fig. 1, trace 1). CO-reduced minus reduced spectra showed that the strain contained only cytochrome aa3 in exponential culture (peak at 425 nm and trough at 445 nm) (Fig. 1c, trace 1), while stationary cultures of Rm1021 contained both cytochrome aa3 (peak at 429 nm and trough at 445 nm) and an o-type cytochrome (peak at 414 nm; the trough at 432 nm was masked by a peak at 429 nm corresponding to aa3 cytochrome) (Fig. 1b, trace 1).

There is some controversy about the presence of haem o in rhizobia (reviewed by Delgado et al., 1998). Since the spectroscopic properties of cytochrome o cannot be distinguished readily from those of a high-spin b-type cytochrome (Poole, 1994), it is possible that the cytochrome o absorption peak corresponds to a part of a cytochrome bb3 complex (reviewed by Delgado et al., 1998). When we refer to cytochrome o, there is the possibility that this is a high-spin cytochrome b, as suggested by Delgado et al. (1998).

W2 and W5 (ccmB and ccmC). The cytochrome spectra of mutants W2 and W5 were very similar; so, in Fig. 1 we present only spectra of the W5 mutant (Fig. 1a–c, traces 3). In contrast to the parental strain, the mutants produced no c-type cytochromes (reduced minus oxidized spectrum, no peaks at 550 and 520 nm) (Fig. 1a, trace 3). This is in agreement with the speculation that the mutations in W2 (ccmB) and W5 (ccmC) block cytochrome c maturation. CO-reduced minus reduced spectra showed that exponential cultures of the mutants still contained an aa3 cytochrome (peak at 425 nm and trough at 445 nm) (Fig. 1c, trace 3), and stationary cultures contained cytochromes o (peak at 414 nm) and aa3 (peak at 429 nm and trough at 445 nm) (Fig. 1b, trace 3).

**W6 (senC).** We could not detect any differences between the spectra of W6 and wild-type Rm1021. Like Rm1021, the W6 mutant produced c-type cytochromes (reduced minus oxidized spectra, peaks at 550 and 520 nm) and a b-type cytochrome (peak at 560 nm in reduced minus oxidized spectra) (Fig. 1a, trace 4). CO-reduced minus reduced spectra of W6 showed that, like the parent strain, the W6 mutant produced cytochromes aa3 (peak at 425 nm and trough at 445 nm) and o (peak at 414 nm) (Fig. 1b, trace 4) in stationary culture, and cytochromes aa3 (peak at 429 nm and trough at 445 nm) (Fig. 1c, trace 4) in exponential culture.

**W8 (cycM).** The reduced minus oxidized spectrum of mutant W8 revealed that it produced less cytochrome c (low ratio between peaks at 550 and 560 nm, corresponding to cytochrome b) (Fig. 1a, trace 5). However, the production of mature aa3 cytochrome by the mutant remained unchanged [peak at 425 nm and trough at 445 nm (Fig. 1b, trace 5), and peak at 429 nm and trough at 445 nm (Fig. 1c, trace 5)].

**W3 (ccdA).** Like mutants W2 and W5, mutant W3 did not produce c-type cytochromes (no peaks at 550 and 520 nm in reduced minus oxidized spectrum) (Fig. 1a, trace 2). However, the CO-reduced minus reduced spectrum showed that the mutant probably lacked aa3 cytochrome in both exponential (no peak at 425 nm and no trough at 445 nm) (Fig. 1c, trace 2) and stationary culture (no peak at 429 nm and no trough at 445 nm) (Fig. 1b, trace 2). In contrast to strain Rm1021, the mutant expressed cytochrome o in exponential culture (peak at 416 nm and trough at 432 nm) (Fig. 1c, trace 2) and produced only an o-type cytochrome in stationary culture (peak at 414 nm and trough at 432 nm) (Fig. 1b, trace 2).

**aa3 cytochrome c oxidase mutants**

aa3 cytochrome oxidase is composed of several subunits but only one of these, SUI, actually contains haem groups (Iwata et al., 1995). To show that the absence of a trough at 445 nm and a peak at 425 nm in the W3 cytochrome spectra was caused by a lack of aa3-type cytochromes, we analysed the cytochrome composition of mutants W17 and W19, which carry transposon mutations in the catE and catC genes annotated as encoding putative aa3-type cytochrome c oxidase SUII and SUIII. Surprisingly, CO-reduced minus reduced spectra of both mutants were still similar to those of strain Rm1021, in having a peak at 429 nm and a trough at 445 nm (Fig. 2, traces 1–3), indicating that both mutants still produced significant amounts of aa3-type cytochrome. This suggests that S. meliloti does not need either SUII or SUIII for proper assembly of SUI. We then generated a deletion of the S. meliloti Smc00010 gene, which encodes SUI, and analysed the cytochrome spectra of the mutant.
This mutant, Rm1021ΔSMc00010, exhibited a Nadi\textsuperscript{2} phenotype and did not produce spectroscopically detectable aa\textsubscript{3} cytochrome oxidase. CO-reduced minus reduced spectra of the mutant were similar to those of mutant W3, showing no peak at 429 nm and no trough at 446 nm (Fig. 2, traces 4 and 5), which confirmed that the mutant W3 lacked aa\textsubscript{3}-type cytochromes.

**Cytochrome c production and respiration of S. meliloti strains**

Iron present in haem groups that are covalently bound to proteins, such as c-type cytochromes, can be visualized by using a sensitive chemiluminescence assay (Feissner et al., 2003). Haem staining of electrophoretically fractionated soluble and membrane preparations of strain Rm1021 revealed three bands of about 12, 42 and 46 kDa in the soluble fraction (Figs. 3 and 4), and three bands of approximately 42, 45 and 49 kDa in the membrane fraction (Fig. 4). The profile of bands in the senC mutant W6 was similar to that of the parental strain Rm1021 (Fig. 4). Profiles from the soluble fraction of the W2 (ccmC) and W3 (ccdA) mutants showed that they lacked soluble c-type cytochromes (Fig. 3). Similarly, we could not detect any c-type cytochromes in membrane fractions of the ccmC and ccdA mutants (data not shown). These results suggest strongly that CcmC and CcdA are needed in cytochrome c synthesis and, based on observations in other systems, most likely in cytochrome c maturation. The W8 (cycM) mutant lacked two bands in the soluble fraction, at 42 and 46 kDa, and two bands in the membrane fraction, at 45 and 49 kDa (Fig. 4). These bands were much larger than the predicted size of CycM (25 kDa), and were unlikely to correspond to CycM itself. The membrane band at 42 kDa was still present in the extracts from the W8 mutant.

To study the respiration in the mutants, TMPD oxidase activity was measured. All mutants except W6 (senC) had significantly lower O\textsubscript{2} consumption than that of Rm1021. In their original isolation using the Nadi test, W6 and W8 developed blue colour more slowly than the wild-type, which is consistent with the retention of some cytochrome c respiration in these mutants (Table 2).
Motility and growth measurement of the mutants

Analysis of motility of the mutants revealed that W3 and W2 had significantly decreased motility on YMB medium compared to that of the Rm1021 parental strain, but they had increased motility on Min-succinate-NH₄⁺ medium (Table 3). A mutation in ccmB (W5) also showed decreased motility on YMB medium (Table 3), but no changes in motility were observed on Min-succinate-NH₄⁺ medium. Mutant W6 (senC) had decreased motility on both Min-succinate-NH₄⁺ and MM-NH₄⁺ media. No significant changes in motility of the W8 mutant (cycM) (Table 3) or of mutants with defects in the SUI, SUII or SUIII of aa₃-type cytochrome c oxidase were detected (data not shown).

One possibility for decreased motility of the mutants could be their impaired growth on the tested media. To test this, we measured the growth rate of the strains in liquid cultures. Mutants W3 and W5 had significantly lower growth rates on YMB medium, and the W2 mutant did not grow on YMB medium. However, the growth rate of all mutants was similar to that of the parental strain on Min-succinate-NH₄⁺ (Fig. 5) and MM-NH₄⁺ media (data not shown). Adding yeast extract to the MM-NH₄⁺ medium decreased the growth rate to one that was similar to the rate observed on YMB medium (data not shown). This indicated that the slower growth on YMB medium was caused by an inhibitory effect of yeast extract.

Complementation of the phenotype of the mutants by addition of exogenous copper

SenC has been proposed to act as a copper chaperone that delivers copper to the CuA site of cytochrome c oxidase (Glerum et al., 1996). In support of this, Swem et al. (2005) have found that extra copper in the medium reverses the phenotype of their senC mutant in Rh. capsulatus. To test the possibility that SenC is involved in copper metabolism in S. meliloti, we tested various copper concentrations to

<table>
<thead>
<tr>
<th>Table 2. Symbiotic properties and ascorbate-TMPD oxidase activity of S. meliloti strains</th>
</tr>
</thead>
<tbody>
<tr>
<td>The shoot dry mass of the plants without inoculation was 7.25±0.36 mg. Values represent mean±SD from three replicate experiments.</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Strain</th>
<th>Plant shoot dry mass (mg)</th>
<th>O₂ consumption [nmol min⁻¹ (mg protein)⁻¹]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rm1021</td>
<td>19.7±4.16</td>
<td>80.6±12.51</td>
</tr>
<tr>
<td>W2</td>
<td>7.1±1.32*</td>
<td>46.7±1.63*</td>
</tr>
<tr>
<td>W3</td>
<td>7.1±0.20*</td>
<td>46.8±2.40*</td>
</tr>
<tr>
<td>W5</td>
<td>7.9±0.23*</td>
<td>37.6±2.68*</td>
</tr>
<tr>
<td>W6</td>
<td>21.5±5.04</td>
<td>69.8±4.31</td>
</tr>
<tr>
<td>W8</td>
<td>21.1±2.08</td>
<td>56.2±2.90*</td>
</tr>
</tbody>
</table>

*Significantly different from Rm1021.

<table>
<thead>
<tr>
<th>Table 3. Relative motility of S. meliloti strains</th>
</tr>
</thead>
<tbody>
<tr>
<td>The values shown are a percentage relative to the motility of Rm1021 (diameter, mm) on YMB (13±1.0); 1/10 YMB (13±1.2); MM-NH₄⁺ (23±1.9); and Min-succinate-NH₄⁺ (12±1.4). Values represent mean±SD from at least three replicate experiments.</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Strain</th>
<th>Medium</th>
<th>YMB</th>
<th>1/10 YMB</th>
<th>MM-NH₄⁺</th>
<th>Min-succinate-NH₄⁺</th>
</tr>
</thead>
<tbody>
<tr>
<td>W2</td>
<td></td>
<td>67±7.5*</td>
<td>93±11.7</td>
<td>124±12.1*</td>
<td>156±20.4*</td>
</tr>
<tr>
<td>W2(pRG1SMa03849)</td>
<td></td>
<td>100±5.6</td>
<td>100±5.6</td>
<td>136±19.8*</td>
<td>182±8.5*</td>
</tr>
<tr>
<td>W2(pCPPccmC)</td>
<td></td>
<td>100±5.6</td>
<td>90±11.4</td>
<td>136±4.3*</td>
<td>182±8.5*</td>
</tr>
<tr>
<td>W2(pCPPccmCD3)</td>
<td></td>
<td>100±5.6</td>
<td>82±17.7</td>
<td>136±19.8*</td>
<td>182±8.5*</td>
</tr>
<tr>
<td>W2(pCPPccmCDG)</td>
<td></td>
<td>100±5.6</td>
<td>100±12.7</td>
<td>128±4.2*</td>
<td>150±11.3*</td>
</tr>
<tr>
<td>W3</td>
<td></td>
<td>67±8.8*</td>
<td>70±11.8*</td>
<td>104±9.8</td>
<td>133±13.2*</td>
</tr>
<tr>
<td>W3(pRG1SMa00248)</td>
<td></td>
<td>100±5.6</td>
<td>100±5.6</td>
<td>143±3.5*</td>
<td>170±2.8*</td>
</tr>
<tr>
<td>W5</td>
<td></td>
<td>82±12.2*</td>
<td>90±11.4</td>
<td>89±15.6</td>
<td>88±12.7</td>
</tr>
<tr>
<td>W6</td>
<td></td>
<td>88±10.7*</td>
<td>102±25.1</td>
<td>80±15.5*</td>
<td>80±12.5*</td>
</tr>
<tr>
<td>W8</td>
<td></td>
<td>98±8.7</td>
<td>97±8.8</td>
<td>94±10.1</td>
<td>97±9.4</td>
</tr>
</tbody>
</table>

*Significantly different from Rm1021.
determine the lowest level of copper that could complement
the Nadi± phenotype of the mutant. We found that 5 μM
CuCl₂ restored the rate of pigment formation by senC
mutant W6 to within the wild-type range. In contrast,
ccmB, ccmC, ccdA and cycM mutants remained Nadi−, even when
the concentration of CuCl₂ was increased up to 40 μM.

**Complementation of ccmB, ccmC and ccdA
mutations**

Since ccmC is the first gene in a probable ccmC–ccmD–ccmG
operon, polarity of a Tn5 insertion in ccmC might decrease
the expression of ccmD or ccmG, and this proposed inhibition could contribute to the phenotype of the
ccmC::Tn5 mutant. To test this possibility, we com-
plemented the ccmC mutation in W2 by introducing plasmids
carrying constitutively expressed copies of ccmC, ccmC–
ccmD, or ccmC–ccmD–ccmG genes. We also complemented
ccmB, ccmC and ccdA mutations by homologous recombi-
nation of pRG1SMa03848, pRG1SMa03849 and
pRG1SMa00248 carrying the respective genes into the
chromosome of W5, W2 or W3.

The presence of pRG1SMa03848, pRG1Sma03849 and
pRG1SMa00248 restored the ability of the corresponding
mutants (W5, W2 and W3) to oxidize TMPD, to grow in
YMB medium (data not shown), and to be motile on rich
media (Table 3). However, none of the plasmids that carried
ccmC could prevent the increased motility of mutant W2 on
minimal medium supplemented with succinate or mannitol
(Table 3), and actually appeared to slightly increase
motility. Introducing pRG1SMa00248 into W3 also
appeared to increase motility of the mutant on minimal
media (Table 3), rather than reversing the increase of
motility observed on Min-succinate-NH₄⁺ medium.
pRG1SMa00248 or pRG1Sma03849 did not increase the
motility of strain Rm1021 on either rich or minimal media
data not shown).

**Symbiotic phenotype of the mutants**

The symbiotic phenotypes of the mutants were studied
using alfalfa as the host plant. Mutants W6, W8, W17, W19
and Rm1021ΔSMc00010 formed pink, nitrogen-fixing
nodules on the inoculated plants. The masses of the
plants inoculated with the mutants W6 and W8 were
similar to those of plants inoculated with parental strain
Rm1021 (Table 2). In contrast, mutants W2, W3 and W5
formed small white nodules. Plants inoculated with these
mutants were yellow, and had shoot dry masses resembling
plants that had not been inoculated (Table 2).

**DISCUSSION**

Respiration plays a very important role in the ability of
rhizobia to establish effective nitrogen-fixing symbiotic
associations with their host plants. The ability of rhizobia to
adapt their respiration to the microaerobic conditions in
nitrogen-fixing legume root nodules, and the key role played
by bacterial respiration in fulfilling the high ATP require-
ment of nitrogenase, make a greater understanding of the
operation of respiration a research priority. Several studies
of rhizobial respiratory chain function and its genetic determinants have been carried out in rhizobia that form large nodules, making it easier to study the physiology and biochemistry of symbiotic bacteria (Delgado et al., 1998). However, similar information does not exist for S. meliloti, a rhizobial species with good genetic systems, and for which the genome of one strain has recently been determined (Galibert et al., 2001). In this paper, we report the characterization of mutants of S. meliloti with defects in cytochrome assembly, showing that several genes implicated in cytochrome assembly in other systems are also important in S. meliloti. However, there are subtle differences in the physiology of these mutants, suggesting either that the functions carried out by these genes may be blocked to different extents in the mutants, or that the genes may have additional roles in rhizobial physiology.

The cytochrome composition of S. meliloti Rm1021 differs from that of several other rhizobia. For example, several reports indicate that cytochrome aa₃ is repressed in stationary cultures of some Rhizobium species (O’Brian & Maier, 1989; Soberon et al., 1999; Yurgel et al., 1998). S. meliloti CXM1-188 does not contain much cytochrome aa₃ in stationary-phase culture (Yurgel et al., 1998). However, the CO-reduced minus reduced spectra clearly showed that cytochrome aa₃ was present in both stationary- and exponential-phase cultures of S. meliloti Rm1021 and, even after 48 h growth, it still contained a cytochrome aa₃ terminal oxidase.

The chemiluminescent staining procedure used to visualize protein bands in Figs. 3 and 4 detects bound iron, and is generally considered to specifically stain proteins that contain c-type cytochromes, since only the c-type cytochromes contain covalently bound haem groups. The number of proteins found was surprising. In addition to a 12 kDa soluble c-type cytochrome, the gel contained at least two high-molecular-mass soluble proteins of about 42 and 46 kDa, and these were not present in the cycM mutant. Relatively few S. meliloti genes are predicted to produce proteins that contain the CXXCH haem-binding motif, and are in this molecular mass range. These include the product of SMb20402, which is predicted to be a 50 kDa protein with a 25 aa N-terminal sequence that could be a cleavable leader sequence; SMb20848 and SMb21292, membrane proteins that are about 46 kDa; SMb21367 (cycA), a 40 kDa cytochrome protein; Smc00401, a predicted 48 kDa peroxidase; Smc01814, a predicted 48 kDa glutamate synthase (GOGAT) subunit; Smc01815, a 48 kDa protein with homology to dihydroyperimidine dehydrogenase; Smc02858, a 41 kDa DnaJ-type protein; Smc03937, a 47 kDa adenylate/guanolate cyclase (cyaG); and Smc03831, a 46 kDa protein without predicted function. Only the product of Smc01814 has been detected in a recent analysis of the S. meliloti proteome (Djordjevic et al., 2003).

cycM mutations lead to a loss of cytochrome aa₃ in B. japonicum (Bott et al., 1991) and R. leguminosarum bv. viciae (Wu et al., 1996), but the S. meliloti cycM mutant reported here contained spectroscopically detectable cytochrome aa₃. This indicated that proper assembly of cytochrome aa₃ oxidase in S. meliloti did not depend on CycM. CycM has been proposed to be important for cytochrome aa₃ assembly in B. japonicum and R. leguminosarum (Bott et al., 1991; Wu et al., 1996). In addition, our finding of cytochrome aa₃ in ccmC and ccmB mutants also indicated that, in contrast to B. japonicum (Bott et al., 1991; Thony-Meyer et al., 1989), assembly of this terminal oxidase does not depend on a functional cytochrome bc₁ complex, since this was affected in the corresponding mutants that we isolated.

Analysis of mutants with defects in genes shown in other bacteria to be involved in cytochrome c maturation revealed that S. meliloti CcmB, CcmC and CcdA were essential for the formation of all cellular c-type cytochromes, and were required for symbiotic nitrogen fixation. CcdA functions in transmembrane transfer of thiol-reducing equivalents from the bacterial cytoplasm to the periplasm (Kadokura et al., 2003; Le Brun et al., 2000; Ortenberg & Beckwith, 2003), and is related to the DsbD/DipZ family of membrane proteins (Kimball et al., 2003). Mutation in ccdA in other bacteria results in a deficiency in c-type cytochromes, presumably by blocking reduction of apocytochrome c thiols in the haem binding site (Bardschewsky & Friedrich 2001; Deshmukh et al., 2000). Mutant W3 (ccdA) exhibited an unexpected phenotype in lacking cytochrome aa₃ terminal oxidases in both exponential and stationary cultures. We cannot attribute the lack of cytochrome aa₃ terminal oxidases in the ccdA mutant to the absence of c-type cytochromes since, as indicated above, two other cytochrome c maturation mutants, W2 (ccmC) and W5 (ccmB), both contained cytochrome aa₃ oxidase. This might indicate that CcdA, which was shown to be important in cytochrome c assembly, plays a broader role in rhizobial respiration, such as involvement in assembly of aa₃ cytochrome c oxidase. S. meliloti strains carrying mutations in aa₃ cytochrome oxidase SUII or SUIII contained spectroscopically detectable aa₃-type cytochromes in both mutants, indicating that neither SUII nor SUIII was essential for proper assembly of the haem-containing SUI. Mutation in ccdA resulted in the loss of a-type cytochromes, a phenotype similar to that of the SUI deletion mutant, which narrows the possible involvement of ccdA in cytochrome aa₃ assembly to the level of haem a synthesis or haem a incorporation in SUI.

Additionally, S. meliloti ccmC, ccmB and ccdA mutations reduced the ability of the mutants to grow on rich media. A similar effect of ccmC mutations has been reported for Paracoccus denitrificans (Page & Ferguson, 1999), although unlike in P. denitrificans, in S. meliloti, a ccmB mutation also resulted in failure to grow on rich media. Mutations in S. meliloti ccmB and ccdA also increased motility on minimal media, which is difficult to explain as a direct consequence of the lack of c-type cytochromes. Introducing additional intact copies of these genes into the mutant strains, in an attempt to complement the mutations, accentuated the
mobility phenotype of the mutants, but had little effect on the wild-type.

A mutation in \textit{senC} does not appear to have been studied previously in the rhizobia. \textit{senC} is homologous to the human SCO1 gene, the product of which is required for cytochrome \textit{c} oxidase activity. SCO1, together with SCO2, encodes a protein with metallochaperone activity that is essential in delivering copper to cytochrome \textit{c} terminal oxidase (Leary et al., 2004). Mutation in the \textit{Rh. capsulatus} \textit{senC} gene affects both respiration and the induction of photosynthesis (Buggy & Bauer 1995; Swem et al., 2005). We did not see any changes in the \textit{S. meliloti senC} mutant, other than the original Nadi phenotype on plates and decreased motility. In particular, the \textit{senC} mutant had a normal cytochrome composition, and was able to form nitrogen-fixing nodules on alfalfa. Since \textit{cbb3} oxidases are essential for \textit{S. meliloti} to form a nitrogen-fixing symbiosis (Renali et al., 1987), this suggests that, in contrast to \textit{Rh. capsulatus}, \textit{senC} was not involved in the assembly of the cytochrome \textit{cbb3} terminal oxidases. We suspected that the decreased motility of the \textit{senC} mutant was not due to defects in cytochrome \textit{c}-related respiration. In \textit{Rh. capsulatus}, it has been proposed that reduction of cytochrome \textit{c} oxidase activity in a \textit{senC} mutant affects the oxidation/reduction state of the ubiquinone pool, leading to alteration of photosystem and respiratory gene expression (Swem et al., 2005). In \textit{Rhodobacter sphaeroides}, \textit{SenC} has been proposed to act as a signal mediator between cytochrome \textit{cbb} oxidase and a sensor kinase \textit{RegB} that is involved in controlling expression of a number of processes, such as photosynthesis and respiration (Oh et al., 2004). Interaction of \textit{S. meliloti} \textit{SenC} with global regulation of this type could influence mobility. We plan to continue this analysis to understand the role of \textit{senC} in this aspect of rhizobial physiology.

In summary, several \textit{S. meliloti} Rm1021 mutants that had severe defects in the ability to reduce a dye using a cytochrome \textit{c}-coupled reaction were analysed. Most of these were mutated in one of several genes that have been linked to cytochrome \textit{c} or cytochrome \textit{c} oxidase assembly in other bacteria. The defects that these mutations caused are similar to those seen when the corresponding mutations are studied in other rhizobia. Various data suggest that in the \textit{\alpha}, \beta- and \gamma-proteobacteria, \textit{Ccm} proteins perform one or more functions that are critical to bacterial physiology and growth (Cianciotto et al., 2005). \textit{S. meliloti} \textit{ccmB}, \textit{ccmC}, \textit{ccDA} and \textit{senC} mutations caused pleiotropic effects which cannot simply be explained to result from the decreased efficiency of the cytochrome \textit{c} respiratory chain. For example, all these mutants, including \textit{ccyM}, were impaired in respiration. However, a \textit{ccyM} mutant did not display the increased motility associated with \textit{ccmC} or \textit{ccDA} mutations, or the decreased motility associated with a \textit{senC} mutation. It also survived well on rich media, in contrast to the cytochrome \textit{c} maturation mutants. Based on the results obtained in this study, we conclude that proteins involved in rhizobial cytochrome \textit{c} respiration, such as \textit{ccmB/C}, \textit{ccDA} and \textit{senC}, directly participate in several distinct physiological processes.

\textbf{ACKNOWLEDGEMENTS}

We thank Isaac Forquer and Dr David Kramer for assistance in determining the cytochrome spectra and respiration rates. This work was supported by grants DE-FG03-96ER20225 from the Energy Biosciences Program at the US Department of Energy, and MCB-0131376 from the US National Science Foundation. We also acknowledge support from the Agriculture Research Center at Washington State University.

\textbf{REFERENCES}


Page, M. D. & Ferguson, S. J. (1999). Mutational analysis of the Paracoccus denitrificans c-type cytochrome biosynthetic genes ccmABCDO: disruption of ccmC has distinct effects suggesting a role for CcmC independent of CcmAB. Microbiology 145, 3047–3057.


Edited by: C. W. Ronson