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

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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 aa3, a defect that does not appear to have been reported for other bacteria. The aa3-type cytochromes were also missing from a mutant strain with an insertion into the gene encoding the haem-containing subunit (SU) of aa3 cytochrome c oxidase, but not in mutants unable to make SUII or SUIII, indicating that CcdA probably plays a role in assembling SU. 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 aa3. 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 cytochrome bc1 complex. Electrons derived from quinol oxidation may be delivered either to cytochrome aa3 using transmembrane cytochrome c (CycM) as an intermediate carrier, or to cytochrome cbb3 oxidases. Alternatively, quinol may bypass the cytochrome bc1 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 cbb3 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 aa3, 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 bc1 complex, cytochrome c, and a cytochrome aa3 terminal oxidase. A respiratory chain essential in symbiosis couples the cytochrome bc1 complex to a cytochrome cbb3 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
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/ccmHIJKL, 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/cycVV, 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 (Cianciotto 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 (Cianciotto 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 ada, 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 cycHIKL 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(pSUP5011), Tn5 insertion mutants were selected on Min-succinate-NH₄⁺ medium supplemented with neomycin. Neomycin-resistant colonies were screened by screening the PCR products using 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’-ACGCCTGCACTAGTACNNNNN-NNNNACGCC-3’ (ARB1), 5’-GAACTCCTGAGCCAACTTG-3’ (transposon-specific primer); second round, 5’-GCCACGCGTCG-CTAGTAC-3’ (ARB2), 5’-ATGGCTTCATACCTCTGTA-3’ (transposon-specific primer). DNA sequencing was then carried out by the DyeDeoxy Terminator Cycle protocol (Applied Biosystems), using synthetic primer 5’-GAGAACACAGATTTAGGCAAGCTCTGTTGGAGG-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’-CATGAAGCTTATGAGTGAAG-CAGCCTGCG-3’) as the forward primer, and ccmC_R (5’-CATGCTGCAAGCTATGGGTAAGCTGTCCTCCCGC-3’) as the reverse primer, and subsequent PCR amplification (transposon-specific primer). Following amplification, the PCR products were digested with *S. meliloti* genomic sequence, and a 10 bp 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 pCPPcmC, pCPPcmCD and pCPPcmCDG.

**Construction of the *S. meliloti* Smc00010 deletion mutants.** 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’-CATGAATTCGCCCTCCGAG-CAAGATC-3’) and Smc00010-R1 (5’-CATGGAATTCGCGTCAA-GCTCCACTGAT-3’) for the upstream flanking region, and primers Smc00010-F2 (5’-CATGGAATTCGCCCTCCGAG-CAAGATC-3’) and Smc00010-R2 (5’-CATGAATTCGCCCTCCGAG-CAAGATC-3’) for the downstream flanking region. Each primer had a 20–23 bp overlap with the *S. meliloti* genomic sequence, and a 10 bp 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 pCPPcmC, pCPPcmCD and pCPPcmCDG.
Table 1. Bacterial strains and plasmids

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

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).

**Spectral analysis of cytochrome composition.** Cells were prepared, and spectra were recorded as described by Marroqui et al. (2001), with some modifications. S. meliloti was grown in Min-succinate-NH4+ medium at 30°C for 48 h. The cells were harvested by centrifugation, washed in 0.1 M phosphate buffer (pH 7.4), and resuspended to 10% (v/v) in 0.1 M phosphate buffer (pH 7.4) with 30% (v/v) glycerol. Cytochrome spectra were recorded at room temperature using a PerkinElmer Lambda 18 UV/VIS spectrophotometer. When indicated, the samples were reduced with a few granules of sodium dithionite or oxidized with ammonium persulfate. Reduced minus oxidized spectra were obtained by recording differences between the spectra of the dithionite-reduced sample and the ammonium persulfate-oxidized sample. Reduced minus oxidized spectra were obtained by recording differences between the spectra of the dithionite-reduced sample and the ammonium persulfate-oxidized sample. CO difference spectra (CO-reduced minus CO-dissolved) were obtained by bubbling CO through a suspension of cells reduced with dithionite for 2 min, and recording differences between the spectra of this sample and those of a dithionite-reduced sample.

**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. The membrane suspension was incubated for 15 min at 46°C with an equal volume of 124 mM Tris, pH 7.0, 20% (v/v) glycerol and 4.6% SDS. Proteins were separated by electrophoresis through 10% SDS-polyacrylamide gels, and transferred by...
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* cycZ/ccmC is essential for the formation of all cellular c-type cytochromes (Ramseier *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; Ramseier *et al.*, 1989, 1991).

The transposon in mutant W3 was located in ORF Smc00248, which has been annotated as *cssA* (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 *cssA* (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 *ccdA* 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 subunit (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. 1a, 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**

It is suggested 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\(^-\) phenotype and did not produce spectroscopically detectable \(a_{a_3}\) 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 \(a_{a_3}\)-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 \(O_2\) 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).

![Fig. 2. CO-reduced minus reduced spectra from S. meliloti mutants. The spectra were taken from stationary-phase cultures grown for 48 h. Traces: 1, Rm1021; 2, W17 (ctaE); 3, W19 (ctaC); 4, W3 (ccdA); 5, Rm1021ΔSMc00010 (ctaD).](image)

![Fig. 3. \(c\)-type cytochrome in soluble fractions of Rm1021 and W3 (ccdA) and W2 (ccmC) mutants. Proteins were separated by electrophoresis, transferred to a membrane filter, and visualized using Coomassie blue and chemiluminescent methods. Left panel, proteins on membrane were stained with Coomassie blue after chemiluminescent detection; right panel, chemiluminescent localization of iron (20 min exposure).](image)

![Fig. 4. \(c\)-type cytochrome in soluble and membrane fractions of Rm1021, W6 (\(senC\)) and W8 (\(cycM\)). Left panel, proteins on membrane were stained with Coomassie blue after chemiluminescent detection; right panel, chemiluminescent localization of iron (20 min exposure). Lanes: 1, membrane fractions; 2, soluble fractions.](image)
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-NH$_4^+$ 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-NH$_4^+$ medium. Mutant W6 (senC) had decreased motility on both Min-succinate-NH$_4^+$ and MM-NH$_4^+$ 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 aa$_3$-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-NH$_4^+$ (Fig. 5) and MM-NH$_4^+$ media (data not shown). Adding yeast extract to the MM-NH$_4^+$ 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 2. Symbiotic properties and ascorbate-TMPD oxidase activity of S. meliloti strains

The shoot dry mass of the plants without inoculation was 7.25±0.36 mg. Values represent mean±SD from three replicate experiments.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Plant shoot dry mass (mg)</th>
<th>O$_2$ 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 3. Relative motility of S. meliloti strains

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-NH$_4^+$ (23±1.9); and Min-succinate-NH$_4^+$ (12±1.4). Values represent mean±SD from at least three replicate experiments.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Medium</th>
<th>YMB</th>
<th>1/10 YMB</th>
<th>MM-NH$_4^+$</th>
<th>Min-succinate-NH$_4^+$</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(pCPPccmCD)</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 \(\mu\)M CuCl\(_2\) 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 CuCl\(_2\) was increased up to 40 \(\mu\)M.

**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 complemented 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 recombination of pRG1SMa03848, pRG1SMa03849 and pRG1SMa00248 carrying the respective genes into the chromosome of W5, W2 or W3.

The presence of pRG1SMa03848, pRG1Sma03849 and pRG1SMa0024 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-NH\(_4^+\) 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 Rm1021DSMc00010 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 requirement 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 dihydropyrimidinidene dehydrogenase; SMc02858, a 41 kDa DnaJ-type protein; SMc03937, a 47 kDa adenylate/guanylate cyclase (cyG1); 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 (Bardischewsky & 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 synthesis or haem 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 senC does not appear to have been studied previously in the rhizobia. senC is homologous to the human SCO1 gene, the product of which is required for cytochrome c oxidase activity. SCO1, together with SCO2, encodes a protein with metallochaperone activity that is essential in delivering copper to cytochrome c terminal oxidase (Leary et al., 2004). Mutation in the Rh. capsulatus senC gene affects both respiration and the induction of photosynthesis (Bugg & Bauer 1995; Swem et al., 2005). We did not see any changes in the S. meliloti senC mutant, other than the original Nadi phenotype on plates and decreased motility. In particular, the senC mutant had a normal cytochrome composition, and was able to form nitrogen-fixing nodules on alfalfa. Since cbb3 oxidases are essential for S. meliloti to form a nitrogen-fixing symbiosis (Renalier et al., 1987), this suggests that, in contrast to Rh. capsulatus, senC was not involved in the assembly of the cytochrome cbb3 terminal oxidases. We suspect that the decreased motility of the senC mutant was not due to defects in cytochrome c-related respiration. In Rh. capsulatus, it has been proposed that reduction of cytochrome c oxidase activity in a 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 Rhodobacter sphaeroides, SenC has been proposed to act as a signal mediator between cytochrome cbb3 oxidase and a sensor kinase RegB that is involved in controlling expression of a number of processes, such as photosynthesis and respiration (Oh et al., 2004). Interaction of S. meliloti SenC with global regulation of this type could influence mobility. We plan to continue this analysis to understand the role of senC in this aspect of rhizobial physiology.

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

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