Isolation of *Sinorhizobium meliloti* Tn5 mutants with altered cytochrome terminal oxidase expression and improved symbiotic performance

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Abstract

The relationship between whole-cell redox potential, cytochrome composition in free-living culture and symbiotic activity of *Sinorhizobium meliloti* was studied. Three Tn5-induced mutants with increased cellular redox potential were generated. Stationary cultures of mutants Tb9 and Tb16 in contrast to the parental strain produced the b-type terminal oxidase that may be similar to the symbiotically essential cytochrome oxidase cbb3 of *Bradyrhizobium japonicum*. Increase in the symbiotic effectiveness of all three mutants and in O2 consumption rate in free-living cultures was observed. Mutants Tb1 and Tb16 were also characterized by an increase in fixNOQP gene expression. Consequently, the mutations probably affect at least two different steps of rhizobial respiratory metabolism operating both in free-living cells and endosymbiotic forms. © 1998 Federation of European Microbiological Societies. Published by Elsevier Science B.V. All rights reserved.

Keywords: *Sinorhizobium meliloti*; Cytochrome expression; fixNOQP gene

1. Introduction

The ability of rhizobia to fix dinitrogen from the atmosphere in symbiosis with legume host plants has been investigated over several decades. Symbiotic nitrogen fixation has a high energetic cost to both partners and it is important to understand how bacterial respiration relates to nitrogen fixation. Physiological and biochemical studies have aided in the identification of the electron transport components of different members of the family *Rhizobiaceae* as well as changes that occur in these during different models of growth. Free-living rhizobia express b-type and c-type cytochromes and two terminal oxidases o and aa3. In addition, *Rhizobium leguminosarum* and *Rhizobium etli* express cytochrome d oxidase [1–3]. A close relationship between respiration and symbiotic nitrogen fixation has been demonstrated by the isolation of *Bradyrhizobium japonicum* mutants affected in respiration which fail to fix N2 in symbiosis with soybean [4,5]. The fixNOQP operon is involved in the symbiotic respiration process in several *Rhizobium* species [6–10]. The endosymbiotic terminal
oxidase in *B. japonicum* belongs to the *cbb*3-type and is encoded by *fixNOQP* [11].

Although a primary function of respiration is the generation of the proton motive force, which is utilized for ATP synthesis and a number of other essential functions, the respiratory system is also necessary for the protection of oxygen-sensitive enzymes from damage and for elimination of excessive reducing equivalents by regeneration of NAD⁺ from NADH. It is possible that the excessive reducing equivalents which impose a serious challenge for free-living cells may be advantageous for bacteroids due to the extra redox requirements of the nitrogenase reaction. Theoretically, increased generation of reducing equivalents should improve energy supply to nitrogenase which might result in a more efficient legume-*Rhizobium* interaction. For example it has been observed that *R. etli* mutants which expressed the *cbb*3 terminal oxidase under free-living conditions showed an increased respiration and N₂ fixation in symbiosis with the host plant [3,12].

In this work we analyzed the cytochrome composition of *Sinorhizobium meliloti* and alteration of respiratory activity in mutants with an increased redox potential in free-living cells. Although *S. meliloti* is one of the best genetically characterized members of the family *Rhizobiacea*, up to now little has been reported concerning the composition of its respiratory chain and function in symbiosis. We thus attempted to isolate *S. meliloti* mutants with increased reducing activity using an indicator of cell redox potential, 2,3,5-triphenyltetrazolium bromide (TTB), which is used extensively to measure concentration and rate of generation of reduced metabolites in a number of in vivo systems. Here we characterize these mutants with respect to their respiratory activity, expression of cytochrome terminal oxidases and symbiotic effectiveness.

### 2. Materials and methods

#### 2.1. Bacterial strains and culture conditions

The strains used in the work are listed in Table 1. *S. meliloti* strains were grown in the following media: YS (per litre: K₂HPO₄ 0.5 g; KH₂PO₄ 0.5 g; MgSO₄·7H₂O 0.2 g; NaCl 0.1 g; CaCl₂ 0.04 g; yeast extract 1 g; succinate 1.35 g) and MM (per litre: MgSO₄·7H₂O, 0.2 g; FeSO₄·7H₂O, 0.12 g; EDTA, 0.02 g; KH₂PO₄, 0.6 g; CaCl₂·2H₂O, 0.05 g; biotin, 2 mg; thiamin, 2 mg; pH 6.8). Antibiotics used for selection were added at the following concentration: for *S. meliloti*: neomycin (Nm), 200 mg l⁻¹; tetracycline (Tc), 15 mg l⁻¹; gentamicin (Gm), 40 mg l⁻¹; streptomycin (Sm), 500 mg l⁻¹; rifampicin (Rf), 50 mg l⁻¹; for *Escherichia coli*: Nm, 50 mg l⁻¹; Tc, 5 mg l⁻¹; Rf, 50 mg l⁻¹.

#### 2.2. Screening for mutants with increased redox potential

*S. meliloti* strain CXM1-188 was mutagenized with the mobilizable suicide plasmid pSUP5011 carrying Tn5-mob. Neomycin resistant transconjugants were selected on plates with MM medium containing 0.003% 2,3,5-triphenyltetrazolium bromide (TTB), 1 mM glutamate as nitrogen source and 20 mM lactate as carbon source. After 5 days of growth, colonies of mutants with enhanced ability to reduce TTB developed.

Table 1

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Relevant characteristics</th>
<th>Source or reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Strains</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Sinorhizobium meliloti</em> CXM1-188</td>
<td>Spontaneous Sm' mutant of strain 425a</td>
<td>[13]</td>
</tr>
<tr>
<td><em>Escherichia coli</em> S17-1</td>
<td>pro res mod pRP4-(Tc::Mu) (Km::Tn7) integrated into the chromosome, Tc', Sm'</td>
<td>[14]</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>[14]</td>
</tr>
<tr>
<td>pRK2013</td>
<td>helper plasmid</td>
<td>[16]</td>
</tr>
</tbody>
</table>
oped a much deeper red color than colonies of the parental strain. The mutants were denoted as Red$^{++}$ (enhanced reduction).

2.3. Genetic and DNA manipulations

Tn5 mutagenesis was carried out as described by Simon et al. [14]. Transduction using bacteriophage $\phi$U12 was done according to Sharypova et al. [17]. Total and plasmid DNA were isolated using standard protocols [18]. Southern transfer of DNA to nylon membrane and hybridization were performed using the Boehringer Mannheim DIG DNA Labeling and Detection Kit according to the manufacturer’s instructions.

2.4. Respiration rate of free-living cells

Determination of TMPD ($N,N,N',N'$-tetramethyl-$p$-phenylenediamine) oxidase activity was carried out as described by Wu et al. [19].

2.5. Spectral analysis of cytochromes

Cells were cultured aerobically using YS medium. Cells were harvested by centrifugation, washed and suspended to 30% (w/v) in 50 mM Tris-hydrochloride (pH 7.4), 5 mM CaCl$_2$, 5 mM MgCl$_2$. Cytochrome spectra of whole cells were recorded on a SLM Aminco Midan II spectrophotometer. Samples were reduced with dithionite (few grains), or oxidized with ammonium-persulfate (few grains). Carbon monoxide (CO) difference spectra were obtained by bubbling CO (2 min) to a reduced cell sample and the spectrum recorded against a reduced cell sample. Spectra were obtained at room temperature with 1.0 cm light path cuvettes.

2.6. Determination of fixNOQP expression in S. meliloti mutants

Plasmid pGMI931 containing a R. meliloti fixN-$\text{lacZ}$ translational gene fusion [15] was introduced into the Red mutants and the wild-type strain in a riparental cross, using helper plasmid pRK2013 [16]. The transconjugants obtained were cultured in YS medium. $\beta$-galactosidase activity was determined as Miller units [18].

2.7. Symbiotic properties of S. meliloti strains

These were tested under gnotobiotic conditions on alfalfa plants (Medicago sativa cv Wega) as described by Sharypova et al. [17]. Each mutant was tested on 12 alfalfa plants grown for 28 days after inoculation of seedling in 60 ml tubes with vermiculite.

3. Results

3.1. Isolation of Tn5 mutants in S. meliloti

By screening a population of S. meliloti CXM1-188 Tn5-mutagenized colonies for increased ability to reduce TTB to a red compound formazan (Red$^{++}$-phenotype), three mutants were identified. These mutants, named Tb1, Tb9, and Tb16 were assumed to have an altered cell redox potential. Genomic DNA from these mutants was digested with either HindIII or EcoRI and probed with DIG-labelled plasmid pSUP5011 containing Tn5 and pBR325. The patterns of hybridization showed that all mutants had single Tn5 insertions in different genomic sites. $\phi$U12-mediated transduction verified that the Red$^{++}$-phenotype of all three mutants was linked to the Tn5 in all mutants, since more than 90% of the Km$^r$ transconjugants in each mating were shown to have a Red$^{++}$-phenotype.

### Table 2

<table>
<thead>
<tr>
<th>Strain</th>
<th>O$_2$ consumption$^a$ (nmol min$^{-1}$ (mg protein)$^{-1}$)</th>
<th>fixN-$\text{lacZ}$ $\beta$-galactosidase activity$^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td>CXM1-188</td>
<td>43</td>
<td>382</td>
</tr>
<tr>
<td>Tb1</td>
<td>50</td>
<td>1089</td>
</tr>
<tr>
<td>Tb9</td>
<td>100</td>
<td>812</td>
</tr>
<tr>
<td>Tb16</td>
<td>223</td>
<td>1300</td>
</tr>
</tbody>
</table>

$^a$ Representative results of three independent experiments with a variation of 20% or less.

$^b$ Activities are expressed as Miller units per milligram of protein. Representative results of two independent experiments with a variation of 18% or less.
3.2. Two Red$^{++}$ mutants showed increased respiratory activity in free-living cultures

To study the respiration of the mutants, TMPD oxidase activity was measured. Mutant Tb9 had at least 2-fold higher ascorbate-TMPD oxidase activity than the wild-type strain. Moreover whole cells of the strain CXM1-188 had TMPD oxidase activity of less than 20% that observed with cells of mutant Tb16 (Table 2). Mutant Tb1 did not differ significantly from the strain CXM1-188.

3.3. Free-living cells of S. meliloti express c- and b-type cytochromes and the terminal oxidases $aa_3$ and $o$

Dithionite-reduced minus ammonium-persulfate-oxidized spectrum revealed that S. meliloti strain CXM1-188 produces $c$-type cytochromes (shoulder 420 nm, peaks 522 nm and 551.8 nm) $b$-type cytochromes (peak 429 nm and 530.2 nm, shoulder at 563 nm) and the cytochrome $aa_3$ terminal oxidase (shoulder at 445 nm and peak 603 nm) in exponential culture (12 h) (see Fig. 1A, trace 1). In stationary (36 h) culture this strain demonstrated a similar cytochrome content, but the shoulder at 445 nm was not present (this shoulder corresponded to cytochrome $aa_3$) (see Fig. 1A, trace 2).

CO-reduced minus reduced spectrum revealed that the exponential culture of CXM1-188 produced cytochrome $aa_3$ (peak 432 nm, trough at 445 nm and 610 nm) (see Fig. 1B, trace 1). Stationary culture showed that this strain repressed cytochrome $aa_3$ (no trough at 445 nm or at 610 nm), under these conditions only cytochrome $o$ was expressed (peak at 416 nm and troughs at 430 nm and 559 nm, see Fig. 1B, trace 2). Thus the CO-reduced minus reduced spectrum clearly showed that cytochrome

![Fig. 1. Dithionite-reduced minus ammonium-persulfate-oxidized (A) and CO-reduced minus reduced (B) spectra from S. meliloti Red$^{++}$ mutants. CMX1-188 cultured for 12 h (trace 1) and 36 h (trace 2), Tb1 cultured 36 h (trace 3), Tb9 cultured 36 h (trace 4), Tb16 cultured 36 h (trace 5).](https://academic.oup.com/femsle/article-abstract/165/1/167/623731)
aa3 was repressed, although in the dithionite-reduced minus ammonium-persulfate-oxidized spectrum the 603 nm peak was derepressed. The absorbance found at 603 nm in reduced minus oxidized spectrum could be due to catalase which absorbs at similar wavelength [20]. Therefore the repression of cytochrome aa3 in stationary cultures of S. meliloti is similar to what occurs in a number of other Rhizobium species [2-4].

3.4. The Red++ mutants showed altered production of cytochrome terminal oxidases

We performed spectral analyses of cytochromes both in exponential and stationary cultures, but found that the most significant differences between the mutants and the parental strain were detected in stationary cultures. Dithionite-reduced minus ammonium-persulfate-oxidized and CO-reduced minus reduced spectra of their stationary cultures are presented in Fig. 1.

Dithionite-reduced minus ammonium-persulfate-oxidized spectrum of mutant Tb1 in Fig. 1 (trace 3 in Fig. 1A and B) showed that strain Tb1 retained the shoulder at 445 nm in contrast with the parental strain CXM1-188 in 36 h cultures (see Fig. 1A, trace 2). CO-reduced minus reduced spectrum showed that the mutant produced cytochrome aa3 (trough 445 and 610 nm), in contrast with CXM1-188 (Fig. 1B). The mutant produced cytochrome o as did the parental strain (peak at 416 nm and troughs at 430 nm and 559 nm, see Fig. 1B, trace 3). From these data we concluded that Tb1 expressed cytochrome aa3 in stationary cultures in contrast with the strain CXM1-188, and no other apparent effect was observed.

Dithionite-reduced minus ammonium-persulfate-oxidized spectra revealed that the mutant Tb9 had a very different ratio of expression of c-type versus b-type cytochromes in contrast with the parental strain (see the ratio of peaks at 551 nm and 562 nm respectively, trace 4 in Fig. 1A). Mutant Tb1 showed no difference in the ratio of expression of c-type versus b-type cytochromes with the strain CXM1-188 (see Fig. 1A). As in the parental strain, there was no evidence for cytochrome aa3 production in 36 h cultures of these mutants since the shoulder at 445 nm was not present. CO-reduced minus reduced spectra showed that these strains did not produce cytochrome aa3 (no trough at 610 nm, see trace 4 in Fig. 1B). Interestingly, we found a trough at 440 nm which is absent in strain CXM1-188. This CO-reactive pigment could be related to cbb3 (symbiotic) terminal oxidase since similar spectral signal was found for the purified B. japonicum oxidase [10], but additional evidence is required to confirm this. To conclude, the mutant Tb9 was derepressed for expression of b-type terminal oxidase that is not expressed in free-living stationary phase cultures of the parental strain. The expression of cytochrome aa3 was unaffected.

Similarly to mutant Tb9, strains Tb16 (trace 5 in Fig. 1A and B) had an altered ratio of expression of c-type versus b-type (the peak at 557 nm was present, see Fig. 1A). However, in contrast with it, Tb16 produced cytochrome aa3 (shoulder at 445 nm in dithionite-reduced minus ammonium-persulfate-oxidized spectrum and 610 nm in the CO-reduced minus reduced spectrum, see Fig. 1B). Thus, we concluded that this mutant is derepressed for the expression of both the b-type oxidase that is expressed in Tb9 as well as the aa3 terminal oxidase.

3.5. The Red++ mutants are characterized by increased level of the fixNOQP expression

Spectral analysis showed that strains Tb9 and Tb16 produced a CO-reactive cytochrome and a b-type cytochrome that could be related to the symbiotic cbb3 terminal oxidase. Therefore, we decided to evaluate the expression of the structural genes for this terminal oxidase, the fixNOQP, in these strains using a fixN-lacZ reporter carried on a wide host range plasmid. We performed analyses of fixNOQP gene expression in different points of exponential and stationary cultures, but found that the most significant differences between the mutants and the parental strain were detected in stationary cultures. Table 2 shows that strain CXM1-188 was characterized by a low level of the fixNOQP expression in 36 h cultures. In contrast, mutants Tb1 and TB16 showed 2.8- and 3.4-fold higher levels of this operon expression. Mutant Tb9 had a 2-fold increased expression of fixNOQP as compared with the parental strain.
3.6. The Red++ mutants show enhanced symbiotic activity

The symbiotic properties of the mutants were studied in plant tests. After alfalfa seedlings were inoculated with both parent and mutant bacteria, all of them formed pink nitrogen-fixing nodules and plants grew well in tubes with N-free medium and vermiculite. All three mutants had higher symbiotic activity than their parental strain. The plants inoculated with mutants Tb1, Tb9, and Tb16 exceeded the plants inoculated with the parental strain CXM1-188 in shoot dry mass by more than 56% (Table 3).

### Table 3

Symbiotic properties of *S. meliloti* Red++ mutants

<table>
<thead>
<tr>
<th>Strain</th>
<th>Shoot dry mass</th>
<th>% to CXM1-188</th>
</tr>
</thead>
<tbody>
<tr>
<td>CXM1-188</td>
<td>34.3</td>
<td>100</td>
</tr>
<tr>
<td>Tb1</td>
<td>58.5*</td>
<td>170</td>
</tr>
<tr>
<td>Tb9</td>
<td>54.6*</td>
<td>159</td>
</tr>
<tr>
<td>Tb16</td>
<td>53.4*</td>
<td>156</td>
</tr>
<tr>
<td>LSD_{(0.05)}*</td>
<td>12.2</td>
<td></td>
</tr>
</tbody>
</table>

*Significantly greater than the value for CXM1-188.
*LSD, least significant difference.

4. Discussion

Spectral analysis of cytochromes indicates that *S. meliloti* strain CXM1-188 expresses b-type and c-type cytochromes and two terminal oxidases o and aa3 in free-living cultures. These data are consistent with spectral analyses of other *Rhizobium* species. The repression of cytochrome aa3 expression in post-exponential phase of growth is not unique to *S. meliloti* and has been previously characterized for other rhizobia [3,12]. The main focus of the work here was to examine how the alteration of redox potential of free-living cells may be related to the cytochrome composition of the *S. meliloti* electron transport chain and to its symbiotic performance.

The cell redox potential indicator TTB was used for detection of *S. meliloti* mutants. Since it has been shown previously that reducing agents for tetrazolium are NADH and NADPH [21], generated mainly by the TCA cycle, the capability of our mutants to enhance TTB reduction may be attributed to the generation of excessive reducing equivalents. Although a primary function of respiration is the generation of proton motive force, the respiratory system is also necessary to eliminate excess reducing equivalents. In agreement with this, mutants Tb9 and Tb16 manifested significant increases in the rate of O2 consumption. A possible cell response to this may be an alteration of the electron transfer chain content. This was confirmed by spectral analysis of bacterial cytochromes. Stationary cultures of Tb9, and Tb16, in contrast to CXM1-188, were characterized by the presence of b-type terminal oxidase. This oxidase is possibly a homolog to the *B. japonicum* symbiotically essential cbb3 oxidase [13]. In aerobic culture, mutant Tb16 had 3.4 higher level of the expression of fixNOQP genes. Evidently mutant Tb16 and probably Tb9 in contrast with the wild-type strain derepressed the cbb3 terminal oxidase under free-living conditions. These findings and an increase of symbiotic activity provide evidence that mutations in Tb9 and Tb16 affect both free-living and endosymbiotic metabolism. Further molecular and genetic analysis of these mutants will allow us to understand the general metabolic ways of *Rhizobium* in greater detail.

All Red++ mutants had altered expression of cytochrome terminal oxidases with respect to the parental strain CXM1-188. Mutations in all mutants were due to different Tn5 insertions and there are differences in their phenotypes. Mutants Tb9 and Tb16 but not Tb1 consumed O2 at a higher rate than the parental strain. In stationary culture, mutant Tb1 derepressed expression of cytochrome aa3 although no alteration in expression of b-type terminal oxidase occurred. Nevertheless the level of *fixNOQP* expression under aerobic culture conditions was increased 2.8-fold. Mutant Tb1 also showed improved symbiotic performance. The mutation in Tb1 may affect regulation of *fixNOQP* gene expression. Molecular cloning and sequencing of the gene mutated in strain Tb1 could be helpful in understanding regulation of symbiotically essential genes expression.

It is noteworthy that our approach to isolating Red++ mutants could be used for generation of *S. meliloti* strains with improved symbiotic activity.
Acknowledgments

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References


