Mutations in sit B and sit D genes affect manganese-growth requirements in Sinorhizobium meliloti

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Abstract

Two transposon-induced mutants of Sinorhizobium meliloti 242 were isolated based on their inability to grow on rich medium supplemented with the metal chelator ethylenediamine di-o-hydroxyphenylacetic acid (EDDHA) and either heme-compounds or siderophores as iron sources. Tagged loci of these mutants were identified as sitB and sitD genes. These genes encode components of an ABC (ATP-binding cassette) metal-type permease in several Gram-negative bacteria. In this work, the phenotypes of these two mutants were compared with those of two siderophore-mediated iron transport mutants. The results strongly implicate a role of the sit genes in manganese acquisition when this metal is limiting in S. meliloti.

1. Introduction

Transition elements like Fe, Mn, Co, Ni, Cu and Zn are both essential and potentially toxic, therefore regulation of different routes for acquisition of these metals is critical [1]. Accordingly, bacteria have evolved low- and high-affinity transport systems expressed in response to metal availability. Although in bacteria only few metal influx systems have been studied in detail, a common feature seems to be the involvement of ABC (ATP-binding cassette) transporters [2,3]. The ABC transport system comprises four typical domains arranged in one or two integral membrane permeases and one or two ATP hydro-lases. In addition, periplasmic-binding proteins assist the membrane-associated transport machinery and confer high-affinity, specificity and directionality to the transport process [4].

Homologues to metal-type transporters are present from human to bacteria [5]. In bacteria, Köster [2] has classified ABC transporters related to iron uptake on three families: siderophore/heme/B12, ferrie iron and the recently defined ABC metal-type permeases. Not all iron transport systems are solely involved in iron acquisition, some of them have higher specificities for other metals such as zinc and manganese. Based on experimental evidence and sequence alignment studies, Claverys [3] has proposed the existence of two new clusters that belong to the family of metal-type ABC transporters, one primarily involved in the transport of Mn and the other in the transport of Zn.

Rhizobia are soil bacteria able to fix nitrogen in symbiosis with legumes. Rhizobia have evolved different strategies to adapt to these diverse environmental conditions. The ability to respond to variations in nutrient availability allows the persistence of bacteria in soil, and improves their ability to colonize and to survive in the host plant. A striking feature of Sinorhizobium meliloti 1021 genome is the high number of genes coding for ABC transport systems [6]. Over 430 ABC transporter genes are predicted based on the whole genome sequence. Half of them are located on the pSymB symbiotic megaplasmid and most of these are predicted to be import systems [7]. Although the nutritional importance of transition metals is well recognized, limited information is available concerning metal transport in rhizobia.

In this work four genes required for metal acquisition in S. meliloti 242 were identified. Data presented here demonstrate that manganese supplementation relieves deficient...
growth phenotype of sitB and sitD mutants. Metal dependent
growth and symbiotic phenotype was compared with those of two siderophore-mediated iron transport mutants. These are the first S. meliloti sitB and sitD mutants to be described and we provide further evidence that strongly supports that the SitABCD system is involved in manganese acquisition in rhizobia.

2. Materials and methods

2.1. Bacteria, plasmids, media and growth conditions

S. meliloti 242 is a spontaneous streptomycin-resistant
derivative of the Uruguayan native isolate 259 [8]. For
strains were grown at 37 °C in Luria broth.

S. meliloti strains were grown at 30 °C in tryptone-yeast
extract medium (TY) [12] or TY medium supplemented
with EDDHA – ethylenediamine-di-o-hydroxyphenylacetic
acid – (TYE). Concentrations in μM of added EDDHA is
indicated as a subscript number. EDDHA is a nonassimil-
able metal chelator extensively used to achieve iron-limiting
conditions. Heme compounds (hemoglobin, leghemo-
globin, and hemin) and different siderophores (242-
siderophore, desferal and ferrichrome) were added to ED-
DHA-supplemented media in order to provide iron sources
for rhizobia.

When required, 50 μg kanamycin ml⁻¹, 50 μg neomycin
ml⁻¹ or 100 μg streptomycin ml⁻¹ were added to the me-
dia.

Siderophore production was detected by using the
chrome azurol sulfonate (CAS) agar plate method [13].

2.2. Random transposon mutagenesis and screening for
iron-acquisition mutants

Tn5-1063a was introduced into the S. mellioti
genome by triparental conjugation [14] with E. coli
DH5α (pRL1063a) and E. coli DH5α (pRK2013) as a
helper strain. Transconjugants screening was performed
on solid TY medium supplemented with neomycin and
streptomycin, on TYE500 and on TYE500 supplemented
with 4 μM hemoglobin (TYEHb).

2.3. Bioassays and growth in the presence of different metal
sources

Bioassay experiments were carried out as previously de-
scribed [15]. Stock solutions of inorganic metal sources
used were: 10 mM FeCl₃, 10 mM MnCl₂ and 10 mM
ZnCl₂. Iron-porphyrins used as iron sources for rhizobia
– hemoglobin (Hb), hemin (Hm), leghemoglobin (Lb) –
were used at the following concentrations: 0.23 mM,
1 mM, and 0.25 mM, respectively. Fifty mM protoporphyr-
n IX (PPIX) was used as a control for tetrapyrrrole re-
quirements. The siderophore produced by S. meliloti 242
strain – 242-siderophore (S) – and two exogenous sidero-
phores used for iron acquisition by this strain – ferri-
chrome (Fc) and desferal (D) – were used at the following
concentrations: 2.5 mM and 7.6 mM, respectively. Briefly,
20 μl (for results shown in Table 1) or 7 μl (for results
shown in Table 3) of the stock solutions to be tested were
added in holes performed on solid TYE medium (15 g
agar l⁻¹) containing about 10⁶ CFU rhizobia ml⁻¹. Plates
were incubated for at least 48 h at 30°C and the growth
halo around the holes was measured.

For growth experiments in liquid medium, tubes con-
taining 6 ml of TY, TYE200 and TYE200 containing differ-
ent metal sources were inoculated with 100 μl of early
stationary-phase cultures. When indicated, 5 μM hemoglo-
in or 50 μM of the chloride salts of metal ions tested
were added. Tubes were incubated with shaking at 30°C.
Growth was determined by measuring optical density at
620 nm.

2.4. DNA isolation and manipulation

Total DNA was isolated from S. meliloti strains accord-
ing to de Bruijn et al. [16]. Digestion with restriction en-
donucleases, DNA ligation, Southern-blotting experiments
and mini-preparation of plasmid DNA was performed es-
tentially as described by Sambrook et al. [17]. Labeling of
DNA probes and DNA hybridizations were performed
using a biotinylated DNA labeling and detection kit ac-
cording to the manufacturer’s instructions (New England
Biolabs).

2.5. DNA sequence analysis

To characterize Tn5-1063a interrupted genes, each
tagged locus was cloned in E. coli DH5α cells. For this
purpose, total DNA was digested with EcoRI, a restriction
enzyme that does not excise the transposon. Digestion
mixture was ligated using T4 DNA ligase, and this mixture
was used to transform E. coli competent cells. This was
possible as transposon construction has an E. coli oriV
[10]. Southern blot analysis of EcoRI-digested plasmids
recovered from kanamycin-resistant E. coli colonies
was used to confirm the presence of the transposon in cloned
DNA. Region flanking the Tn5-1063a insertion was se-
quenced using unique primers corresponding to both
ends of the transposon according to Milcamps et al.
[14]. DNA sequences obtained with each primer were
fused into single nucleotide sequences (500–800 nucleo-
tides) using the Gene Runner program (Hasting Software).
Amino acid and nucleotide homologies were identified by
BLAST searches in non-redundant databases [18,19]. Se-
quence similarities with the S. meliloti 1021 genome were
studied using the S. meliloti BLAST server at http://
sequence.toulouse.inra.fr/meliloti.html.
2.6. Plant assays

*S. meliloti* 242 mutants were screened for their symbiotic phenotype on alfalfa plants. Bacteria were grown to early stationary phase on TY liquid medium. Washed cells were used to inoculate *Medicago sativa* by Creola plants at a final concentration of $5 \times 10^7$ CFU per plant. Alfalfa plants were grown aseptically in 15 ml of N-free Jensen medium [20] solidified with 15 g agar l$^{-1}$. Plant growth medium was supplemented with 60 $\mu$M FeCl$_3$ or 25 $\mu$M EDDHA. Plants were maintained at $21 \pm 2^\circ C$ in a controlled light room with a photoperiod of 12 h. At least 12 tubes (two plants per tube) were used for each strain inoculation. Complete experiment was independently repeated twice. Plant dry weight 90 days after inoculation was determined. Mutant phenotype of bacteria recovered from nodules was tested on TY and TYE$_{500}$ solid medium.

3. Results

3.1. Transposon mutagenesis and sequence analysis of *S. meliloti* Tn5-1063a tagged loci

Previously we showed that heme compounds could be used as iron sources for *S. meliloti* and other rhizobia [15]. In order to isolate mutants defective in siderophore and/or heme-mediated iron acquisition systems, a population of Tn5 mutants was screened for the ability to use Hb as an iron source. To do this, transconjugants were grown on TY, TYE$_{500}$ and TYE$_{500}$ supplemented with Hb as an iron source. Siderophore production was tested in CAS-plates. Out of 5000 mutants analyzed, three groups of mutants were obtained (Table 1). The first group comprised mutants defective in siderophore production. They did not produce a visible halo on CAS plates and were able to grow on TYE$_{500}$Hb but not on TYE$_{500}$. Mutants in the second group produced a halo in CAS-plate larger than that of the wild-type, but they were apparently unable to use the iron siderophore as an iron source, indicating a defect in siderophore internalization. The third group was comprised by two mutants that were unable to grow on TYE$_{500}$Hb but produced a normal CAS halo. One mutant from group I (strain H38), one mutant from group II (strain H21) and the two mutants from group III (strains H26 and H36) were selected for further studies (Table 1).

Southern blot analysis of EcoRI-digested genomic DNA using biotinylated pRL1063a as a probe, revealed a single Tn5-1063a insertion in each of the four mutants selected (data not shown).

DNA sequences of transposon flanking regions were determined and results obtained from BLAST searches are summarized in Table 2. Tagged loci in mutant strain H21 showed 98% identity (462 nucleotides analyzed) with the rhtA gene of *S. meliloti* 1021. RhtA is the receptor for rhizobactin, the di-hydroxamate siderophore produced by *S. meliloti* 1021 [21].

Tagged sequence in strain H38 (691 nucleotides analyzed) was found to be highly homologous to rhrA, an araC-like gene. RhrA is located upstream rhizobactin biosynthetic genes in the *S. meliloti* 1021 rhizobactin regulon. According to Lynch et al. [21], RhrA is a transcriptional activator for siderophore biosynthetic genes as well as for the transcription of the rhizobactin receptor in *S. meliloti* 2011. It is worthwhile to note that *S. meliloti* 1021 and *S. meliloti* 1102 are streptomycin-resistant derivatives of *S. meliloti* SU47 [22], indicating that the siderophore and siderophore receptor expressed should be the same in both derivatives.

Transposon-flanking regions in strains H26 (532 nucleotides analyzed) and H36 (875 nucleotides analyzed) were almost identical to nucleotide sequences of *S. meliloti* 1021 sitD and sitB genes, respectively (Table 2). SitB and SitD are components of ABC metal-type permeases found in many bacteria [3]. SitD is an integral membrane protein while SitB has been implicated in coupling ATP hydrolysis to the transport of the substrates across the inner membrane.

It has been reported that *sitABCD* from *S. typhimurium*...
and its homologous in Yersinia pestis – yfeABCD – are involved in the utilization of chelated iron. This observation was supported by the fact that expression of these operons in an enterobactin-deficient E. coli strain allows its growth in iron-chelated medium [23,24].

No significant homologies could be detected with H26 and H36-tagged loci and fhuDCB genes, an ABC iron transporter found in Rhizobium leguminosarum [25].

3.2. RhtA and RhrA are required for 242-siderophore-mediated iron acquisition

To investigate whether a mutation in rhtA or rhrA genes could have effects other than a deficiency in siderophore-mediated iron uptake, we tested the ability of H21 and H38 mutants to use different iron sources in bioassays. As shown in Table 1, a bacterial growth halo produced by 242, H38 and H21 strains was observed around wells containing hemoglobin, leghemoglobin, hemin and ferriochrome, indicating that these compounds could be used as iron sources under iron-depleted conditions. The ability of mutant strain H21 to accumulate siderophores in the growth medium (large CAS-halo) together with the poor cell growth in iron-limited medium (Tables 1 and 3) support the genetic evidence that the tagged locus in this mutant encodes the putative receptor for the 242-siderophore. Moreover this result suggests that Fc, Hm and Hb use other outer-membrane receptors.

Siderophore production by H38 mutant could not be detected in CAS plates. The phenotype of this mutant, together with the genetic data obtained, indicates that, like in S. meliloti 2011 [21], an AraC-like protein (RhrA) acts as a transcriptional activator for siderophore biosynthesis in S. meliloti 242.

3.3. SitB and SitD proteins are involved in manganese-growth requirements

As it is shown in Tables 1 and 3, H26 and H36 mutants presented normal growth on TY medium but were unable to grow on media containing EDDHA regardless of the iron sources added. Neither FeCl3, heme compounds (Lb, Hm, Hb) or hydroxamate compounds (S, Fc) could restore growth of strains H26 and H36 on TYE medium. Although EDDHA has been widely used as a specific ferric iron chelator we considered the possibility that some other essential metal was chelated by this compound. Therefore, we tested the ability of H26 and H36 mutants to grow on TYE supplemented with different chloride metal salts. In bioassays performed on TYE250, growth of these mutants was detected only when Mn2+ was added (Table 3). This result indicates that a supply of manganese to the medium is required to recover growth deficiencies associated with mutations in sitB and sitD genes.

The metal-dependent growth phenotype of the mutant strains H21, H26 and H36 was compared with wild-type growth in liquid medium (Fig. 1). Under these conditions, a low cell yield was obtained in TYE200 for H21, H26 and H36 mutants. However, cell yield of the H21 mutant was clearly improved when the culture was supplemented with an iron source – FeCl3 or hemoglobin. On the other hand, cell yield of H26 and H36 cultures were almost equivalent to that of the wild-type strain when cultures were supplemented with manganese. These results indicate that ED-

### Table 2

<table>
<thead>
<tr>
<th>Strain</th>
<th>Tagged loci</th>
<th>Function of similar gene</th>
<th>Identity percentage</th>
<th>Location of similar gene in S. meliloti 1021 genome</th>
</tr>
</thead>
<tbody>
<tr>
<td>H21</td>
<td>rhtA</td>
<td>Rhizobactin receptor</td>
<td>98</td>
<td>Rhizobactin regulon in pSymA</td>
</tr>
<tr>
<td>H38</td>
<td>rhrA</td>
<td>AraC-type regulatory protein</td>
<td>95</td>
<td>Rhizobactin regulon in pSymA</td>
</tr>
<tr>
<td>H26</td>
<td>sitD</td>
<td>ABC-transporter/membrane protein</td>
<td>96</td>
<td>SitABCD operon in chromosome</td>
</tr>
<tr>
<td>H36</td>
<td>sitB</td>
<td>ABC-transporter/ATP-binding protein</td>
<td>98</td>
<td>SitABCD operon in chromosome</td>
</tr>
</tbody>
</table>

*Identity percentage obtained using the blast program at the S. meliloti nucleotide database.

### Table 3

<table>
<thead>
<tr>
<th>Strain</th>
<th>Growth halo (cm) on TYE250a</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Fe</td>
</tr>
<tr>
<td>H21</td>
<td>1.1</td>
</tr>
<tr>
<td>H26</td>
<td>0</td>
</tr>
<tr>
<td>H36</td>
<td>0</td>
</tr>
</tbody>
</table>

*aAverage radius of growth halo around wells containing different compounds. Results are an average of three independent experiments. Standard deviation is less than 0.3.

bD, desferal.

*S, 242-siderophore.
DHA is able to limit not only iron but also manganese availability and corroborate results obtained on solid media. Our findings demonstrate that sitB and sitD genes are involved in manganese acquisition in S. meliloti.

3.4. sitB, sitD, rhtA and rhrA loci are not essential for nodulation and plant growth promotion

Alfalfa plants inoculated with wild-type and mutant strains formed effective nodules. Phenotypic evaluation (growth on TY and TYE500 solid medium supplemented with the appropriate antibiotic) of bacteria recovered from nodules, indicated that the phenotype of the inoculant (mutants and wild-type) was retained. Number, size and time of appearance of the nodules produced by the different inocula tested were similar. Mutants did not induce significant differences in the values of plant dry weights compared to those of the wild-type strain. The aerial plant dry weight was similar from plants grown with or without an iron chelator (data not shown). These data indicate that the metal transport systems impaired in these four mutants are not essential for symbiosis under the conditions examined.

4. Discussion

In the current work we studied the behavior of four Tn5-1063a mutants of S. meliloti 242 whose tagged loci correspond to genes involved in metal acquisition. The nucleotide sequences of the tagged loci were almost identical to the corresponding sequence of the genome of strain 1021, even though the two strains have distant geographical origins.

Sequence analysis indicates that Tn5-1063a tagged loci of H26 and H36 mutants map in sitD and sitB genes, respectively. These genes belong to a family of ABC transporters that are reported to mediate iron transport from the periplasm across the cytoplasmic membrane in a siderophore-independent fashion [23]. However, our work showed that strains H26 and H36 were complemented by addition of Mn to metal-deficient media, suggesting that the sitB and sitD genes are involved in manganese acquisition. Furthermore, while this work was under review, Kehres et al. [26] reported that SitABCD system of S. typhimurium is also involved in Mn2+ transport. Collectively, the data strongly suggest that the S. meliloti sitABCD operon is involved in manganese acquisition under conditions where the metal is limiting.

The genome of S. meliloti strain 1021 reveals a putative fur gene next to the sitABCD genes and in the opposite orientation. Fur is a global regulator of iron metabolism in many bacteria. Interestingly, upstream regions share a perfect palindromic sequence TGCAAATGXXXXX-CATTGGCA. These observations suggest a coordinately regulated mechanism for fur and sitABCD transcription. This would not be unprecedented, as it was reported that Fe and Mn through Fur or SirR, respectively, regulate the expression of sitC in Staphylococcus epidermidis and yfeA in Y. pestis [3].
In summary, we have detected in _S. meliloti_ 242 four different loci involved in metal acquisition. Results obtained strongly support the idea that two of them are required for high-affinity iron transport and the other two for high-affinity manganese transport. None of these loci were essential for symbiosis with alfalfa under the conditions evaluated in this work. We cannot rule out the possibility that an alternative metal transporter could compensate this defect during the symbiosis with the host.

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References


