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Abstract: Rhizobia have a versatile catabolism that allows them to compete successfully with other microorganisms for nutrients in the soil and in the rhizosphere of their respective host plants. In this study, Bradyrhizobium japonicum USDA 110 was found to be able to utilize oxalate as the sole carbon source. A proteome analysis of cells grown in minimal medium containing arabinose suggested that oxalate oxidation extends the arabinose degradation branch via glycolaldehyde. A mutant of the key pathway genes oxc (for oxalyl-coenzyme A decarboxylase) and frc (for formyl-coenzyme A transferase) was constructed and shown to be (i) impaired in growth on arabinose and (ii) unable to grow on oxalate. Oxalate was detected in roots and, at elevated levels, in root nodules of four different B. japonicum host plants. Mixed-inoculation experiments with wild-type and oxc-frc mutant cells revealed that oxalotrophy might be a beneficial trait of B. japonicum at some stage during legume root nodule colonization.

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A Link between Arabinose Utilization and Oxalotrophy in Bradyrhizobium japonicum

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Rhizobia have a versatile catabolism that allows them to compete successfully with other microorganisms for nutrients in the soil and in the rhizosphere of their respective host plants. In this study, Bradyrhizobium japonicum USDA 110 was found to be able to utilize oxalate as the sole carbon source. A proteome analysis of cells grown in minimal medium containing arabinose suggested that oxalate oxidation extends the arabinose degradation branch via glycolaldehyde. A mutant of the key pathway genes oxc (for oxalyl-coenzyme A decarboxylase) and frc (for formyl-coenzyme A transferase) was constructed and shown to be (i) impaired in growth on arabinose and (ii) unable to grow on oxalate. Oxalate was detected in roots and, at elevated levels, in root nodules of four different B. japonicum host plants. Mixed-inoculation experiments with wild-type and oxc-frc mutant cells revealed that oxalotrophy might be a beneficial trait of B. japonicum at some stage during legume root nodule colonization.

Rhizobia have a broad metabolic capacity and can use a multitude of carbon and nitrogen sources which allow them to be successful and competitive in soil and in the rhizosphere of host plants (1). Within root nodules, differentiated rhizobia (bacteroids) reduce N₂ to ammonium, which is secreted to the plant in return for C₄-dicarboxylic acids as carbon and energy sources (2). The C₄-dicarboxylic acids malate, succinate, and fumarate have been shown to be the primary carbon sources of bacteroids and can actively cross the peribacteroid membrane (3–5). In fact, transport of C₄-dicarboxylic acids is required for nitrogen fixation (6–8). C₄-dicarboxylic acids are directly fed into the tricarboxylic acid (TCA) cycle to supply the bacteroid with enough energy to perform nitrogen fixation (3).

However, despite the fact that dicarboxylic acids were shown to be the major carbon sources for N₂-fixing bacteroids, large quantities of hexose and pentose sugars are also found in nodules (9), suggesting an important role in nodule metabolism and N₂ fixation. Enzymes for hexose and pentose transport and metabolism have been reported to be present in bacteroids (10–12). The pentose l-arabinose is a well-known substrate of B. japonicum (13, 14), and this sugar is routinely used for in vitro growth studies. Previous work has shown that l-arabinose is degraded by a pathway that conceptually resembles the Entner-Doudoroff pathway (14). By analogy, l-arabinose is first oxidized to l-2-keto-3-deoxyarabonate (l-KDA) (15–17), which is then converted into α-ketoglutarate in the case of fast-growing rhizobia (18–20) or to glycolaldehyde and pyruvate in the case of slow-growing species like B. japonicum (14). While pyruvate most likely is oxidized in the TCA cycle, the fate of glycolaldehyde has not been resolved yet. In this study, through proteomic analysis of B. japonicum cells grown either in minimal medium with l-arabinose as the sole carbon and energy source or in complete medium containing l-arabinose, we identified highly expressed products of candidate genes involved in the degradation of arabinose (17). Based on protein expression data, we suggest that glycolaldehyde is oxidized to glyoxylic acid and then reduced to glyceraldehyde through a glyoxylate-carboxylase and a tartronate-semialdehyde reductase for assimilation and/or is converted to oxalate and then oxidized to formate and CO₂ through the formyl-coenzyme A (CoA) transferase (Frc), oxalyl-CoA decarboxylase (Oxc), and formate dehydrogenase reactions for energy generation (Fig. 1). Results from previous transcriptome and proteome studies in B. japonicum nodules (12, 21) suggested that glyoxylate is preferentially degraded via oxalate oxidation during symbiosis. In fact, in contrast to the glyoxylate-carboxylase and tartronate-semialdehyde reductase, the Frc and Oxc enzymes were detected in nodules where oxalate presence also could be measured. In agreement with these results, the Δfrc-oxc mutant strain constructed in the present work is not able to grow on oxalate and is partially defective in free-living growth in minimal medium containing arabinose. We also show that the mutant has a disadvantage when competing for nodule occupancy against the wild-type strain.

MATERIALS AND METHODS

Bacterial strains, media, and growth conditions. The bacterial strains and plasmids used in this work are listed in Table S1 in the supplemental material. Escherichia coli cells were cultivated in Luria-Bertani medium (22) at 37°C using the following concentrations of antibiotics (in μg/ml): ampicillin (200), kanamycin (30), chloramphenicol (20), and tetracycline (10). Bradyrhizobium japonicum cells were routinely cultivated at 30°C on peptone-salts-yeast extract (PSY) medium (23) supplemented with 0.1% arabinose or in defined buffered Vincent’s minimal medium (BVM) (24, 25), referred to as minimal medium. Carbon sources used in defined
media were filter sterilized and used at a final concentration of 20 mM arabinose or 20 mM succinate. The appropriate antibiotic concentrations (in μg/ml) were added: spectinomycin (100) and kanamycin (100). Aerobic cultures for phenotypic growth analysis in PSY medium or BVM were grown in 500-ml Erlenmeyer flasks containing 25 ml medium supplemented with spectinomycin (100 μg ml⁻¹) and the respective C source on a shaker (160 rpm) at 30°C. For each strain or condition, the growth of three independent cultures was analyzed.

**Sample preparation and liquid chromatography-tandem MS (LC-MS/MS) analysis for proteomics.** Sample preparation and mass spectrometric (MS) analysis were performed as described in detail elsewhere (12). In brief, proteins extracted from three replicates of *B. japonicum* cells...
grown in complex and minimal medium with t-arabinose until mid-exponential phase were separated on a Tris-HCl polyacrylamide gel. After reduction and carboximidomethylation the proteins were digested with trypsin (Promega, Madison, WI, USA), and the resulting peptides were separated by reverse-phase high-performance liquid chromatography (RP-HPLC) and analyzed by a hybrid LTQ-Orbitrap XL mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA) interfaced with a nanoelectrospray source (12). Mass spectra were further processed with an in-house processing pipeline (26). In brief, fragment ion mass spectra were extracted from Thermo RAW files using mscanvert (ProteoWizard, version 3.0.3831), merged in one MGF file per gel and searched against a composite database consisting of 110 protein database entries grown in minimal medium, with 20 mM arabinose or 20 mM succinate as the C source, and supplemented with either Ca-oxalate or Na-oxalate ([H9004 at 30°C for 7 days. The assays were repeated three times. A cotton swab was used to streak the cells on the plate. Plates were incubated at 30°C for 7 days. The data were recorded continuously by the microcalorimeter and resampled to obtain an effective sampling rate of 1 data point every 5 min. Samples were removed from the microcalorimeter and visually inspected in order to check that bacteria did form a lawn.

**Construction of mutant strains.** DNA was isolated from the B. japonicum wild-type strain 110sp4c as previously described (36). Plasmid DNA from E. coli strains was obtained by using the NucleoSpin plasmid kit (Macherey-Nagel, Düren, Germany). Mutagenesis of genes was done by marker replacement. To construct a deletion mutant in the bll3156-3157 genes, PCR fragments of the 5' and 3' flanking regions of bll3156-3157 were amplified using the following primer pairs: bll3156-3157_1_rev (TA CGGCTGCGTCGCGCAAC) and bll3156-3157_2_for (GTACCCTCGTACCGCACG) for the 5' region of bll3156 and bll3156-3157_3_for (TGTCTCCCTGCTGTCACAATACGT) and bll3156-3157_4_rev (GTCGACGGC CACCCGCTCG) for the 3' region of bll3157. PCR products were cloned in the pGEM-T Easy vector (Promega, Madison, WI, USA), and the correct sequence was verified by sequence analysis. Up- and downstream regions were subcloned into the pSUP202pol4 vector (37), and a kanamycin resistance cassette (aphII) derived from pBSL15 (38) was inserted between both regions. The resulting plasmid, pRJ6243, was used for conjugation with B. japonicum strain 110sp4c. The correct genomic integration was verified by PCR. The resulting Δfrc-oxc deletion mutant was named 6243 (Fig. 2).

**Plant material, inoculation, and cultivation.** Sterilization of soybean (Glycine max [L.] Merr. cv. Williams), mungbean (Vigna radiata), cowpea (Vigna unguiculata), and siratro (Macroptilium atropurpureum) seeds was done as described earlier (21). Plants were inoculated with cultures of B. japonicum that had been grown for 5 days in full medium and diluted to approximately 100 bacteria per plant. Nitrogenase activity was determined by using the acetylene reduction assay 21 days postinfection (dpi) for soybean, mungbean, and cowpea and 31 dpi for siratro. Bacteria were isolated from randomly selected nodules to confirm the presence of appropriate genetic markers.

**Competitiveness in symbiosis.** Soybean, mungbean, cowpea, and siratro plants were infected with a mixture of the wild type and the Δfrc-oxc mutant strain containing a total of 100 bacteria per plant. Cultures of the wild type and mutant were grown and diluted to the same CFU per milliliter. A 90:10 ratio (Δfrc-oxc to wild type) was chosen to assess symbiotic competition. Serial dilutions of the mixed inoculum were plated on selective agar to control the number of inoculated cells. All nodules from one plant were harvested at the peak of nitrogenase activity 21 (soybean, mungbean, and cowpea) and 31 (siratro) dpi. Nodules were surface sterilized (100% ethanol for 5 min) and rinsed in sterile distilled water. Nodules were then crushed in 1 ml PSY medium using a mortar, and this suspension was serially diluted and spotted on plates containing the appropriate selection for strain differentiation. The plates were incubated for 4 days at 30°C, and the ratio of the mutant to wild-type strain in nodule extracts was determined and compared to the initial inoculum ratio. As a
control, plants infected with either the wild type or mutant strain only were processed. At least three independent plants were processed per host. The nodule extracts were spotted in duplicates. Data were evaluated for statistical significance using Student’s t test and SPSS 17.0 software. 

### Results and Discussion

**Proteome analysis of B. japonicum** grown in minimal medium containing arabinose. The proteome of cells grown in minimal medium containing L-arabinose as the carbon and energy source revealed the presence of several enzymes involved in L-arabinose degradation (Fig. 1 and Table 1). Apart from detecting L-arabinose 1-dehydrogenase (Blr3205), L-arabinolactonase (Blr3207), and glycolate oxidase (Blr7540-41, Blr7543), the enzymes glyoxylate carboligase (Blr3166) as well as the tartronate semialdehyde reductase (Blr3168) (Fig. 1) were expressed abundantly in minimal medium containing L-arabinose. Remarkably, large amounts of two enzymes involved in oxalate degradation were also detected: a formyl-CoA transferase (Frc) (bll3156) and an oxalyl-CoA decarboxylase (Oxc) (bll3157) that share 71% and 78% amino acid sequence identity with the previously studied Frc and Oxc of *Oxalobacter formigenes* (39–41). This led us to speculate that glycolaldehyde is oxidized to glyoxylate, which can be fed into at least two pathways: (i) reduction to glyceraldehyde through the activity of glyoxylate carboligase (bll3166) and oxalate semialdehyde reductase, or (ii) oxidation to oxalate followed by the stepwise, complete oxidation to formate and CO2 through the activities of Oxc and Frc (43, 44) and formate dehydrogenase. We previously showed that only the enzymes Oxc and Frc involved in the second pathway were detected in bacteroids during symbiosis with all different host plants (12, 21) (Table 1). Interestingly, using qPCR analysis, an elevated expression of bll3157 and bll3156 was not observed in *B. japonicum* cultures grown in minimal medium with succinate as the carbon source. The induction factors in arabinoose-grown cells were measured as 45 for bll3157 and 116 for bll3156 compared to levels for succinate-grown cells. Thus, it appeared as if the oxalate oxidation pathway was switched on when L-arabinose was offered as the carbon source.

**Construction and growth analysis of a Delta frc-oxc deletion mutant.** To further analyze the hypothesis that oxalate degradation is linked to arabinoose utilization, a Delta frc-oxc mutant strain was constructed and grown in vitro in minimal (BVM) and complex (PSY) media containing L-arabinose (the two rightmost columns) or were extracted from bacteroids of different host plants (12, 21). The proteomics data summarize expression from three independent biological replicates.
First, we compared the ability of both the wild type and the \( \Delta frc-oxc \) mutant to catabolize oxalate using plate assays and microcalorimetry (see Materials and Methods). We initially aimed at monitoring oxalotrophic growth in liquid cultures using Ca- or Na-oxalate as the sole source of carbon. Even though various concentrations (5, 10, and 20 mM) of oxalate were used, these cultivation attempts failed. Therefore, we examined growth on Schlegel's minimal medium agar plates in the presence of 20 mM Na-oxalate or 35 mM Ca-oxalate as the sole carbon source (data not shown). For this test, strains were first grown in minimal medium and then streaked out on agar plates. This test revealed that the \( B. japonicum \) wild type was able to grow on plates supplemented with 20 mM Na-oxalate, whereas the \( \Delta frc-oxc \) mutant did not grow. Likewise, microcalorimetric analyses on Angle's medium supplemented with 35 mM Ca-oxalate revealed that the \( B. japonicum \) wild type was able to grow on Ca-oxalate, whereas no growth was observed for the \( \Delta frc-oxc \) mutant (Fig. 3C). The metabolic heat production resulting from the oxidation of oxalate according to the reaction \( 2\text{C}_2\text{H}_2\text{O}_4/\text{H}_1{\text{O}} \rightarrow 4\text{CO}_2 + 2\text{H}_2\text{O} \) with a reaction enthalpy \( (\Delta H_{\text{rxn}}) \) of \(-499 \text{ KJ/mol} \) is directly proportional to the oxalate consumption rate. At this point, it must be noted that biomass production can easily be neglected (34), because biomass yield is extremely low with oxalate. Thus, each curve represents the overall metabolic activity related to the growth of \( B. japonicum \). Such measurements can be compared using common metabolic assays, such as the 2,3,5-triphenyltetrazolium chloride assay, for example (45). The metabolic activity pattern for the \( B. japonicum \) wild-type strain was similar to what could be observed in other studies (34).

When grown on minimal medium with \( l\)-arabinose as the carbon source, the \( \Delta frc-oxc \) mutant displayed a diminished growth rate compared to that of the wild type (Fig. 3A). The mean generation time of the parental strain was 23.4 h, compared to 37.3 h for the \( \Delta frc-oxc \) mutant. Moreover, wild-type cells reached a final optical density of 5.6, while the mutant strain did not exceed an OD of 2. In contrast, the \( \Delta frc-oxc \) strain exhibited growth behavior similar to that of the wild-type strain when cultivated either in minimal medium with the dicarboxylic acid succinate (Fig. 3B) or in complex medium (data not shown). Thus, the results have shown that a deletion of two specific oxalate degradation genes caused a partial defect in growth with arabinose. This can be explained in at least two different ways: (i) in the mutant, \( l\)-arabino- 

\[ \text{Oxalate content of root material and root nodules of cowpea, mungbean, siratro, and soybean plants infected with the } B. japonicum \text{ wild type} \]

<table>
<thead>
<tr>
<th>Host plant</th>
<th>Oxalate level (mg g(^{-1}) wet wt) in:</th>
<th>Root</th>
<th>Root nodules</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cowpea</td>
<td>0.10 ± 0.01</td>
<td>0.37 ± 0.08</td>
<td></td>
</tr>
<tr>
<td>Mungbean</td>
<td>0.09 ± 0.01</td>
<td>0.31 ± 0.05</td>
<td></td>
</tr>
<tr>
<td>Siratro</td>
<td>0.09 ± 0.01</td>
<td>0.22 ± 0.01</td>
<td></td>
</tr>
<tr>
<td>Soybean</td>
<td>0.03 ± 0.01</td>
<td>0.18 ± 0.02</td>
<td></td>
</tr>
</tbody>
</table>

*Results are presented as means ± standard deviations where \( n \geq 2 \).
oxalate, glyoxylate, and glycolaldehyde and found these compounds to be elevated by a factor of up to 2 in the mutant compared to the wild type (data not shown). Whether or not this slight increase is inhibitory to growth has not been further investigated. Regardless of which of the two explanations is true, the degradation of oxalate appears to be a necessary requirement for the complete utilization of arabinose for growth and biomass formation.

**Oxalate content in roots and root nodules induced by B. japonicum.** Since both Frc and Oxc had been detected in soybean, cowpea, and siratro bacteroids using a global proteomics approach (12, 21) (Table 1), we became interested in exploring whether oxalate is present in nodules as a potential C source for the bacteroids. Notably, it has been hypothesized previously that oxalate is a potential energy-yielding substrate sufficient to sustain nitrogen fixation in *Vicia faba* (46).

The oxalate content of roots and root nodules of four *B. japonicum* host plants (soybean, siratro, cowpea, and mungbean) was analyzed using an oxalate oxidase assay (Table 2). In general, three times higher oxalate concentrations were detected in root nodules than in the roots of uninfected plants (Table 2). This showed that oxalate is indeed present in nodules and might be available as a C source together with other compounds, such as succinate and malate.

**Plant symbiosis and competition for nodule occupancy.** The fact that *B. japonicum* host plants displayed different concentrations of soluble oxalate prompted us to investigate the role of the oxalate degradation genes in symbiosis. The symbiotic efficiency of the wild type and the Δfrc-oxc mutant was tested on all plant hosts. Plants infected with Δfrc-oxc grew as healthy as the wild type, displaying green leaves, normal nodule development, and wild-type-like nitrogenase activity 21 dpi for soybean, mungbean, and cowpea and 31 dpi for siratro (see Table S2 in the supplemental material). Moreover, the same number of nodules was observed in plants inoculated with the Δfrc-oxc mutant and with the parental strain. Resolation of bacteroids from plants used in this test revealed comparable viable cell counts for wild-type and mutant strains (data not shown).

It was previously shown that mutations in genes responsible for the catabolism of carbon sources, such as rhamnose and myo-inositol, and other used nutrients, such as mimose, are correlated with a decreased ability to compete for nodule occupancy (47–50). Therefore, we investigated if the presence of the oxalate catabolic pathway could represent a competitive advantage for nodulation occupancy on all host plants. In order to test nodule colonization, 50 CFU/ml of *B. japonicum* Δfrc-oxc and wild-type cells were mixed at a 90:10 ratio and used for inoculation of soybean, siratro, cowpea, and mungbean seedlings. After 21 dpi for soybean, mungbean, and cowpea and 31 dpi for siratro, all nodules from one plant were collected. In total, three independent experiments including at least three plants were performed per strain and host. To isolate bacteroids, root nodules were homogenized and the wild-type-to-mutant ratio was investigated by comparing the recovered viable cell counts of Δfrc-oxc and wild-type strains using genetic markers. In all host plants the Δfrc-oxc mutant was affected in nodule occupancy when competing with the parental strain (Fig. 4), despite the fact that the mutant was present in 10-fold excess over the wild type in the inoculant mixture. A possible impact of the antibiotic resistance cassette (aphII in strain Δfrc-oxc) on the nodulation competition phenotype had been excluded by results presented recently (51), where an ACC deaminase mutant containing the identical aphII antibiotic resistance cassette could compete as well as the wild type for nodule occupancy.

**Concluding remarks.** In this study, we demonstrated that *B. japonicum* is capable of entertaining an oxalotrophic lifestyle. Sequence analyses indicated that the oxc and frc genes involved in oxalate degradation are present and conserved in other *Bradyrhizobium* strains (52) but not in fast-growing rhizobia such as *Sinorhizobium meliloti*, *Rhizobium leguminosarum*, and *R. etli*. In this study, based on proteomics and growth analysis on different carbon sources, we showed that the degradation of D-arabinose creates intermediates that most likely are fed into the oxalate degradation pathway (Fig. 1). Disrupting the oxalate degradation branch by mutation leads to compromised growth on arabinose, because either the additive benefit from reductant formation in the formate dehydrogenase reaction is lacking or inhibitory amounts of oxalate and its precursors build up in the mutant. While the presence of functional formyl-CoA transferase (frc) and oxalyl-CoA decarboxylase (oxc) genes in *B. japonicum* is dispensable for the establishment of an effective symbiosis, these enzymes nevertheless seem to provide an advantage in the process of root-nodule colonization by *B. japonicum*. It can be speculated that at some point during rhizobial infection and nodule occupancy the ability to degrade oxalate, which is present in roots and root nodules, represents a beneficial trait for *B. japonicum*.

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