Identification of a Functional 2-keto-myo-Inositol Dehydratase Gene of *Sinorhizobium fredii* USDA191 Required for myo-Inositol Utilization

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<td>B. subtilis 60015</td>
<td>metC7 trpC2</td>
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<td>B. subtilis YF111</td>
<td>metC7 trpC2 iloE41</td>
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<td>B. subtilis KY023</td>
<td>metC7 trpC2 amyE:(cat Papac-luc2)</td>
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<td>B. subtilis KY024</td>
<td>metC7 trpC2 iloE41 amyE:(cat Papac-luc2)</td>
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<td>B. subtilis KY025</td>
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<td>E. coli JM109</td>
<td>endA1, gyrA96, thi, hsdR17, supE44, relA, Δ(lac-proAB), recA1, F[traD36, proAB+, lacIq, lacZAM15]</td>
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<td>A host strain that carries the T7 RNA polymerase gene</td>
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<td>Plasmid</td>
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<td>pCRE-test</td>
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<td>pBlueScript II SK(+)</td>
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\[ S. \text{fredii USDA191 is analogous to that in } B. \text{subtilis, we searched for the presence of 2-keto-\text{myo-inositol dehydratase in USDA191. Here we report the cloning of a functional iolE from } S. \text{fredii USDA191, and argue that it codes for active 2-keto-\text{myo-inositol dehydratase.} } \]

Materials and Methods

Bacterial strains, plasmids, and growth conditions. The bacterial strains and plasmids used in this work are listed in Table 1. Rhizobia were grown on a reciprocal shaker at 30°C in one of the following media: YEM,\(^{16}\) YEI (YEM in which 1% \text{myo-inositol} was substituted for mannotol), YE (YEM without mannotol or \text{myo-inositol}), and minimal medium, either with or without 0.02% \text{myo-inositol},\(^{17}\) containing 0.2% succinate as the carbon source. \text{B. subtilis} cells were maintained at 30°C on tryptose blood agar base plates (Becton Dickinson Microbiology Systems, Sparks, MD) supplemented with 0.18% glucose (TBABG). Cells were grown at 37°C with shaking in S6 minimal medium\(^{18}\) containing 0.02% yeast extract with or without 25 mm \text{myo-inositol}. \text{E. coli} cells were grown in Luria-Bertani (LB) medium\(^{19}\) at 37°C with shaking. When required, ampicillin (50 \mu g/ml), chloramphenicol (10 \mu g/ml), or kanamycin (50 \mu g/ml) was added to the culture media.

Cloning and nucleotide sequence analysis of iolE from \text{S. fredii USDA191.} Based on the published sequence of \text{S. meliloti 1021 iolE,}\(^{20}\) we designed two oligonucleotide primers, SMIoIE2E and SMIoIE1P (Table 2), for PCR amplification of a 0.9-kb genomic DNA fragment of USDA191. After the PCR product was trimmed with \text{EcoRI} and \text{PstI}, and ligated with an arm of pUC18 cleaved with the same enzymes, a recombinant plasmid carrying ampicillin resistance was used to transform \text{E. coli JM109.} The PCR fragment amplified from DNA of USDA191 cloned in the plasmid was sequenced and found to have high similarity to that of \text{S. meliloti} 1021 iolE. Subsequently, this fragment was used as a probe to screen a USDA191 cosmid library, constructed in the vector pLAFRI.\(^{21}\) Fifteen cosmids clones were identified that yielded positive hybridization signals. Cosmid DNA was isolated from these clones, restricted individually with \text{EcoRI}, and fractionated by agarose gel electrophoresis. The DNA was transferred to a nitrocellulose membrane and hybridized with the same probe used for the library screening. Strong hybridization to an 8.0-kb \text{EcoRI} fragment was detected. This hybridizing fragment of a cosmide clone (plol) was purified from agarose gel and cloned into pBlueScript II SK(+) to produce pHK0000. Sequencing was initiated with oligonucleotide primers corresponding to the N- and C-terminal regions of iolE and extended further with the aid of a series of primers.

Table 2. Oligonucleotides Used in This Study

<table>
<thead>
<tr>
<th>Oligonucleotide</th>
<th>Sequence (5'-3')*</th>
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<tr>
<td>SMIoIE2E</td>
<td>CCGGAATTCATCCGTTACGGAAACCAACC</td>
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<tr>
<td>SMIoIE1P</td>
<td>AAAATTGACGACGCGACGTATCGGAGG</td>
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<tr>
<td>SFiolE-UpB</td>
<td>CGCGATCCCTAAACACCCTTCCGCTCCA</td>
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<tr>
<td>SFiolE-B</td>
<td>CGCGATCTTCGTGGTCAATTCGCGCCTCC</td>
</tr>
<tr>
<td>NdiolE</td>
<td>CCAACATATQATCCGCCTAGAGGAAACCAACCCGG</td>
</tr>
<tr>
<td>NotiolE</td>
<td>TGGCGGCCGCGCTCGTACGAGCCGCTTCCTTCGCC</td>
</tr>
</tbody>
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*Restriction enzyme sites are underlined.
Construction of strains and plasmids. An S. fredii USDA191 iolE mutant was constructed as follows: A 2-kb omega cassette \(^1\) was inserted into a HindIII site located near the 5'-end of iolE cloned in pKH0000. The disrupted iolE region was excised from the recombinant plasmid as a 5.5-kb BamHI fragment and cloned into the corresponding site of a suicide plasmid, pJQ200 SK. \(^2\) This suicide plasmid was mobilized into USDA191 by triparental mating with helper plasmid pRK2013. \(^3\) Double recombinations were selected by plating rhizobia on minimal medium plates supplemented with 5% (wt/vol) sucrose. The mutation was confirmed by PCR analysis using a primer pair of SFiolEupB and SFiolEB \((\text{Table 2})\).

USDA191 iolE was introduced into the B. subtilis chromosome according to the following procedure: A PCR fragment containing iolE was amplified from USDA191 DNA using the SFiolEupB and SFiolEB primer pair, which generated BamHI sites at each end of the fragment. The PCR fragment was trimmed with BamHI and ligated with an arm of the pCRE-test that had been cleaved using the same restriction enzyme. The correct orientation of the ligated fragment, which placed iolE under the control of the \(\text{spa}c\) promoter, \(^4\) was confirmed by DNA sequencing. The recombinant plasmid carrying chloramphenicol resistance was linearized by PstI digestion and used to transform \(S.\) subtilis YF111. One of the transformants was obtained as KY025. Similarly, linearized pCRE-test DNA was used to transform \(S.\) subtilis 168 and YF111, providing KY023 and KY024 respectively.

Expression of \(S.\) fredii USDA191 iolE gene in \(E.\) coli. To express iolE in \(E.\) coli, the entire coding region of iolE was amplified by PCR using primers NdeiolE and NotiolE \((\text{Table 2})\). The PCR amplified fragment was cloned into pET28a \(\text{(+)}\) (Novagen, Madison, WI) vector as a NdeI and NotI fragment, resulting in pET28a(+)SFiolE carrying iolE fused with a C-terminal His\(_{6}\)-tag under the control of the \(\text{T7}\) promoter. Cells of \(E.\) coli ER2555 harboring plasmid pET28a(+)SFiolE were inoculated in 100 ml of LB containing kanamycin, and allowed to grow to an optical density at 600 nm (OD\(_{600}\)) of 0.6, and then 0.1 mM IPTG was added to the culture to induce the IolE His\(_{6}\)-tag fusion protein. After overnight incubation, cells were harvested by centrifugation, suspended in 15 ml buffer (10 mM Tris–Cl, pH 7.9, 10% glycerol, 0.5 mM NaCl, 0.1% NP40, and 5 mM DTT) and then sonicated to disrupt cell walls and membranes. The crude extract was kept on ice for 30 min, and centrifuged at 4 °C, and the supernatant was recovered. Imidazole at a final concentration of 1 mM was added to the supernatant, which was loaded onto a Ni-NTA agarose column. The column was washed with two volumes of BC100 (20% glycerol, 20 mM Tris–Cl, pH 7.9, 100 mM KCl, 5 mM DTT, and 0.5 mM PMSF) containing 20 mM imidazole followed by elution with 5 ml of BC100 containing 80 mM imidazole. The eluted fractions containing purified protein were subjected to SDS–polyacrylamide gel electrophoresis. \(^5\)

Enzyme assays. A culture of \(S.\) fredii USDA191 was grown to OD\(_{600}\) of 0.6 in 250 ml of YEM. The cells were harvested by centrifugation, resuspended in two 250 ml flasks of minimal medium with or without 0.02% \(\text{myo}\)-inositol, and then incubated for 24 h. \(\text{myo}\)-Inositol dehydrogenase and 2-keto-\(\text{myo}\)-inositol dehydratase activities in protein extracts and the 2-keto-\(\text{myo}\)-inositol dehydratase activity of the purified protein were measured as described previously. \(^6\)

**Results**

\(\text{myo}\)-Inositol dehydrogenase and 2-keto-\(\text{myo}\)-inositol dehydratase activities in free-living \(S.\) fredii USDA191 cells

\(\text{myo}\)-Inositol dehydrogenase encoded by \(\text{idhA}\) is required for \(S.\) fredii USDA191 \(\text{myo}\)-inositol utilization. \(^7\) If USDA191 possesses a \(S.\) subtilis-like \(\text{myo}\)-inositol catabolic pathway, then the organism is likely to express 2-keto-\(\text{myo}\)-inositol dehydratase to catalyze the second step in this pathway. \(^8\) In order to measure the activities of the two \(\text{myo}\)-inositol catabolic enzymes, we cultured USDA191 in YEM medium, harvested the cells, and then re-suspended them in a minimal medium containing 0.2% succinate as the carbon source and 0.02% \(\text{myo}\)-inositol as an inducer. The initial growth and subsequent transfer were necessary, as USDA191 did not grow to a sufficient culture density on \(\text{myo}\)-inositol as the sole carbon source to yield enough cells for the enzyme assays (data not shown). As previously reported, \(^7\) induction of \(\text{myo}\)-inositol dehydrogenase was observed. Cells grown in the presence or absence of \(\text{myo}\)-inositol gave activities of 23.8 ± 1.5 and 1.3 ± 0.5 nmoles/min per mg protein respectively (values are means ± SD of three independent measurements). Similarly, the activity of 2-keto-\(\text{myo}\)-inositol dehydratase was 16-fold higher in cells grown on \(\text{myo}\)-inositol than in those cultured in its absence. In the presence and absence of \(\text{myo}\)-inositol it gave activities of 81.6 ± 2.1 and 4.8 ± 1.1 nmoles/min per mg protein respectively. These results clearly indicate the expression and induction of \(\text{myo}\)-inositol dehydrogenase and 2-keto-\(\text{myo}\)-inositol dehydratase in \(S.\) fredii USDA191.

Cloning and nucleotide sequencing of \(S.\) fredii USDA191 iolE region

To clone the gene encoding 2-keto-\(\text{myo}\)-inositol dehydratase of \(S.\) fredii USDA191, we synthesized PCR primers, SMiolE2E and SMiolE1P \((\text{Table 2})\), based upon 5' and 3' regions of the published \(S.\) meliloti 1021 iolE sequence respectively. \(^9\) The primer pair was
utilized to amplify a 0.9-kb fragment from USDA191 genomic DNA. The PCR product was cloned into pUC18 vector, sequenced, and found to be very similar to the corresponding segment of the *S. meliloti* 1021 *iolE* coding region (data not shown). A genomic cosmid library of USDA191 was screened using this PCR fragment as a hybridization probe, and 15 positive cosmids were identified. Southern blot analysis revealed that these cosmids shared an 8.0-kb EcoRI fragment that hybridized to the probe (data not shown). The 8.0-kb EcoRI fragment was subcloned for determination of the nucleotide sequences flanking the previously cloned 0.9-kb region. A contiguous 3.5-kb sequence that displayed two complete ORFs and one ORF lacking the amino terminus was recognized (Fig. 1A).
Purification and characterization of S. fredii USDA191 iolE product

USDA191 iolE was cloned into pET28a(+) for expression in E. coli, and the encoded protein was subsequently purified as the C-terminal His$_8$-tag fusion, SF191IolE-His. The purified SF191IolE-His protein formed a 35-kDa band when resolved on SDS–polyacrylamide gel, coinciding with the mass predicted from the amino acid sequence data. Assays of 2-keto-myo-inositol dehydratase activity were conducted using the purified protein under conditions described previously. A Lineweaver-Burk plot gave an apparent $K_m$ of 0.410 ± 0.026 mM for 2-keto-myo-inositol, approximately 4-fold lower than that of the B. subtilis enzyme. The optimal pH for SF191IolE-His was 6.0–7.0, while that for B. subtilis enzyme was 7.5–8.0. The differences in the pH optima and $K_m$ values between these 2-keto-myo-inositol dehydratase isomers from the two species possibly reflect the dissimilar environmental conditions in which these microorganisms exist.

USDA191 iolE complemented an iolE deficient mutant of B. subtilis

B. subtilis YF111 does not utilize myo-inositol as a carbon source, because the iolE41 mutation minimizes 2-keto-myo-inositol dehydratase activity in the cell. USDA191 iolE was placed under the control of the spac promoter and introduced into the B. subtilis YF111 amyE locus. The introduced USDA191 iolE was able to complement the mutated B. subtilis iolE, facilitating growth on myo-inositol (Fig. 2), and confirming that USDA191 iolE encodes biologically active 2-keto-myo-inositol dehydratase, but the complementation was partial, ostensibly due to insufficient expression of the introduced USDA191 iolE and/or to differences among the biochemical properties of the IolE enzymes of the two bacterial species.

**Fig. 2.** Complementation of an iolE-Deficient B. subtilis Mutant by Expressing USDA191 iolE.

Strains of B. subtilis, 60015 (wild type, open circles), YF111 (iolE41, closed circles), KY023 (amyE::cat, open squares), KY024 (iolE41 amyE::cat, closed squares), and KY025 (iolE41 amyE::cat P$_{spac}$-USDA191iolE), open triangles], were grown in a minimal medium containing 25 mM myo-inositol as the sole carbon source (see text). Cell growth was followed by OD$_{600}$. The experiment was repeated at least three times, with similar results.

AmyE could be required for myo-inositol utilization of free-living S. fredii USDA191

Cells of wild-type S. fredii USDA191 and the iolE mutant were inoculated into YEM, YEI, or YE media and incubated for 72 h (Fig. 3). USDA191 grew well in YEM medium, which provides mannitol and yeast extract as carbon and nitrogen sources respectively. USDA191 grew on YE medium without mannitol, but growth stalled after 24 h, suggesting that the organism was able to use yeast extract as a carbon source to some extent (Fig. 3A). USDA191 growth in YEI medium containing myo-inositol was significantly greater than in YE medium, indicating that the organism could utilize myo-inositol as carbon source. Growth of the iolE mutant and USDA191 in YEM and YE was comparable. Additionally, the iolE mutant did not show increased growth on YEI over that on YE media. These results are indicative of the mutant being compromised in the utilization of myo-inositol, but the inactivation of iolE might have affected downstream genes such as iolB in
case these genes were contained in an operon (Fig. 1A), which might be involved in the myo-inositol catabolic pathway. Nevertheless, as we have shown, iolE encodes 2-keto-myoinositol dehydratase complementing the mutated B. subtilis iolE for the second step in the myo-inositol catabolic pathway (Fig. 2), while as found in B. subtilis, iolB might function for the latter steps in the pathway.13) These results provide evidence that iolE might be indispensable for myo-inositol utilization.

Discussion

myo-Inositol dehydrogenase and 2-keto-myoinositol dehydratase are responsible for the first and second steps of the myo-inositol catabolic pathway respectively, as demonstrated in B. subtilis.13) Earlier, we found that idhA of S. fredii USDA191 encoded myo-inositol dehydrogenase.7) In the current study, we found that iolE encoded a protein exhibiting 2-keto-myoinositol dehydratase activity. DNA sequence analysis of the iolE region of USDA191 revealed putative iolD and iolB flanking iolE (Fig. 1A). B. subtilis requires both iolD and iolB for myo-inositol catabolism.13) In the S. meliloti 1012 genome, a gene cluster, iolDEB, similar to that in USDA191 was observed. An additional gene homologous to B. subtilis iolC is located just upstream of iolD in S. meliloti.20) Other iol genes that share sequence similarities to those found in B. subtilis, including idhA (iolG) and iolA, are also present in the genome of S. meliloti 1021.20) These results indicate that the pathway of myo-inositol catabolism in S. meliloti 1021 and S. fredii USDA191 might be similar to that of B. subtilis.

myo-Inositol catabolism may play a role in the competition among bacteria for nodulation. Mutants of R. leguminosarum bv. viciae compromised in myo-inositol catabolism showed a competitive disadvantage in nodulation, while transport mutants were less impaired and still maintained slightly reduced competitive ability with wild-type strains.28) An idhA mutant of S. fredii USDA191 was found to be impaired in its nodulation and nitrogen fixing ability.7) Evidence suggests that the competitive disadvantage occurs early in the process, ostensibly during infection-thread formation.28) MocA and MocC of S. meliloti L5-30, which exhibit significant similarities to IolG and IolE of B. subtilis, are involved in the degradation of rhizopine (L-3-O-methyl-scyllio-inosamine), a symbiosis-specific compound found in alfalfa nodules.127) It has been proposed that myo-inositol catabolism is linked to rhizopine utilization.6) Mutants of R. leguminosarum bv. trifolii compromised in rhamnose catabolism exhibit a competitive disadvantage in clover nodulation,29) but mutants affecting the catabolism of sorbitol and adonitol vie successfully with the wild-type strain. Perhaps rhamnose plays a role in infection-thread formation and the inability of the mutant to utilize rhamnose can lead to diminished competitiveness.

We are currently trying to establish a system to investigate the composition of metabolites contained in

Fig. 3. Requirement of the iolE Function for Better Growth on myo-Inositol.
USDA191 (A) and USDA191 iolE mutant (B) were grown in YEM (containing mannitol as carbon source, open circles), YEI (myo-inositol as carbon source, solid circles), and YE media (without supplemental carbon source; open squares). Cell growth was followed by optical density at 600 nm. The experiment was repeated at least three times, with similar results.
nODULES. Although expression of iolE in the nodules has not been clarified, at least idhA is significantly induced in the nodules,\(^7\) and their defect might be reflected by a change in the metabolites contained in the nodules. Our preliminary results revealed that soybean nodules appeared to contain at least pinitol (3-O-methyl-D-chiro-inositol), D-chiro-inositol, scylo-inositol, glucose, sucrose, and further unidentified substances (K. Yoshida, unpublished). In B. subtilis, the iol genes are involved in metabolism not only of myo-inositol but also of pinitol and D-chiro-inositol.\(^{30,31}\) In addition, the iol genes are induced in the presence of each of these compounds. We characterized S. fredii USDA191 iolE encoding 2-keto-myoinositol dehydratase specific for the second step in the reaction in the myo-inositol catabolic pathway, suggesting that USDA191 might possess a pathway analogous to that of B. subtilis. Therefore, the pathway might involve not only myo-inositol but also other compounds, such as pinitol and D-chiro-inositol contained in the nodules. Further studies are underway to elucidate the role that myo-inositol catabolism plays in the competition among bacteria for nodulation.

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**References**


