Three inositol dehydrogenases involved in utilization and interconversion of inositol stereoisomers in a thermophile, Geobacillus kaustophilus HTA426

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Geobacillus kaustophilus HTA426, a thermophilic Bacillus-related species, utilizes some inositol stereoisomers, including myo-, d-chiro- and scyllo-inositols (MI, DCI and SI), as sole carbon sources. Within its genome are three paralogous genes that possibly encode inositol dehydrogenase. These genes are located in tandem within a large gene cluster containing an almost complete set of iol genes homologous to genes involved in inositol catabolism in Bacillus subtilis. Each of the three plausible inositol dehydrogenases was purified as a His8-tag fusion. The enzymes exhibited thermophilic activity, each with its own characteristic specificity for the inositol stereoisomers and cofactors. Northern blot and primer extension analyses revealed that the three enzymes were encoded by the same 5 kb polycistronic transcript and were induced simultaneously in the presence of MI. HTA426 was subjected to ethyl methanesulfonate (EMS) mutagenesis to isolate a mutant strain, PS8, which was not able to utilize MI. In PS8, inositol dehydrogenase activity was abolished along with the 5 kb transcript, suggesting that any of the three enzymes supports MI-dependent growth. Analysis of metabolites in HTA426 cells grown in the presence of MI revealed that substantial amounts of DCI and SI appeared intracellularly during the stationary phase, while only MI was present in PS8 cells, suggesting that interconversion of inositol stereoisomers may involve these three enzymes.

INTRODUCTION

A number of micro-organisms, including Bacillus subtilis (Yoshida et al., 1997), Cryptococcus melibiosus (Vidal-Leiria & van Uden, 1973), Aerobacter aerogenes (reclassified as Enterobacter aerogenes/Klebsiella mobiles) (Berman & Magasanik, 1966a, b; Anderson & Magasanik, 1971a, b), Rhizobium leguminosarum (Poole et al., 1994), Sinorhizobium meliloti (Galbraith et al., 1998), Sinorhizobium fredii (Jiang et al., 2001), Corynebacterium glutamicum (Fujita et al., 1991; Yoshida et al., 1997, 1999a, 2002). Inositol dehydrogenase, encoded by iolT, is responsible for the first step in the degradation cascade, converting MI to 2-keto-MI (2KMI), i.e. conversion of compound [1] to [4] in Fig. 1 (Fujita et al., 1991). 2KMI dehydratase, encoded by iolD, is responsible for the second step, i.e. production of 3D-(3,5,4)-trihydroxyoctahexan-1,2-dione (THcHDO; compound [6]; formerly called D-2,3-diketo-4-deoxy-epi-inositol) (Yoshida et al., 2004). The third reaction involves myo-inositol (MI) as a sole carbon source. Bacterial MI catabolism has been elucidated intensively in B. subtilis (Fujita et al., 1991; Yoshida et al., 1997, 1999a, 2002). Therefore, a large number of bacterial genes have been annotated as plausible iol genes on the basis of sequence similarity to the B. subtilis iol genes.

In B. subtilis, the iolT gene (Yoshida et al., 2002) and the iol divergon, comprising the operons iolABCDEFGHIIJ and iolRS (Yoshida et al., 1997), have been shown to be involved in MI catabolism (Yoshida et al., 2008). The iolT and iolF genes encode the primary and secondary inositol transporters, respectively, responsible for MI uptake (Yoshida et al., 2002). Inositol dehydrogenase, encoded by iolG, is responsible for the step in the degradation, converting MI to 2-keto-MI (2KMI), i.e. conversion of compound [1] to [4] in Fig. 1 (Fujita et al., 1991). 2KMI dehydratase, encoded by iolD, is responsible for the second step, i.e. production of 3D-(3,5,4)-trihydroxyoctahexan-1,2-dione (THcHDO; compound [6]; formerly called D-2,3-diketo-4-deoxy-epi-inositol) (Yoshida et al., 2004). The third reaction involves...
hydrolysis of THcHDO to yield 5-deoxy-D-glucuronic acid (5DG; compound [7]) (Yoshida et al., 2008). The fourth reaction is the isomerization of 5DG by IolB to produce 2-deoxy-5-keto-D-gluconic acid (DKG; compound [8]) (Yoshida et al., 2008). Next, in the fifth reaction, DKG is phosphorylated by IolC kinase to yield 2-deoxy-5-keto-D-gluconic acid 6-phosphate (DKGP; compound [9]) (Yoshida et al., 2008). IolR is known to be a
repressor that controls transcription of the iol operon (Yoshida et al., 1999a). In this reaction, DKGP appears to be the intermediate that acts as an inducer by antagonizing DNA binding of IolR (Yoshida et al., 2008). Finally, IolJ is the specific aldolase involved in the sixth reaction, i.e. the cleavage of DKGP into dihydroxyacetone phosphate (DHAP; compound [10]) and malonic semialdehyde (MSA; compound [11]) (Yoshida et al., 2008). The former is a known glycolytic intermediate, and the latter has previously been shown to be converted to acetyl-CoA (compound [12]) and CO₂ in a reaction catalysed by IolA. The net result of the inositol catabolic pathway in B. subtilis is thus the conversion of MI to an equimolar mixture of DHAP, acetyl-CoA and CO₂ (Fig. 1) (Yoshida et al., 2008).

B. subtilis can utilize not only MI but also two other inositol stereoisomers, i.e. D- and L-chiro-inositol (DCI; compound [2]) and scylo-inositol (SI; compound [3]), as carbon sources. IolG also acts on DCI to yield 1-keto-DCI (1KDCI; compound [4]), indicating that not only MI but also DCI is metabolized through the MI catabolic pathway (Fig. 1a) (Yoshida et al., 2006). B. subtilis has been shown to possess two additional and distinct inositol dehydrogenases, i.e. IolX and IolW, which specifically act on SI with NAD⁺ and NADP⁺ reduction, respectively (Morinaga et al., 2010a). Each of these enzymes converts SI to 2KMI, which is the same compound as that produced from MI by IolG and is readily degraded further via the metabolic pathway described above. These two additional pathways have been applied to enable bioconversion for the production of DCI and SI from MI; the former and the latter are drug candidates for the control of diabetes and Alzheimer’s disease, respectively (Yoshida et al., 2006; Yamaoka et al., 2011).

Geobacillus kaustophilus HTA426 is a thermophilic and aerobic endospore-forming Bacillus-related species, whose upper temperature limit for growth is 74 °C (optimally at 60 °C), isolated from the deepest sediment of the Mariana Trench (Takami et al., 2004a). Its genome is composed of a 3.54 Mb chromosome and a 47.9 kb plasmid, and it is thus used in comparative genomic studies with other mesophilic bacilli to facilitate the study of the thermophily and thermostability of proteins, because this was the first genomic sequence from a thermophilic Bacillus-related species (Takami et al., 2004b). Within its genome there is a large cluster of genes (range gk1885-gk1899) (Fig. 1) which contains a set of iol homologues. Interestingly, this cluster also contains three paralogous genes that possibly encode inositol dehydrogenase. These genes are located in tandem so that they are expressed simultaneously.

In this study, we distinguished the enzymic properties of these three paralogues, confirmed their simultaneous expression, and found evidence that they may be involved in interconversion of inositol stereoisomers.

**METHODS**

**Bacterial strains, plasmids and growth conditions.** Bacterial strains and plasmids used in this study are listed in Table 1. Strains of G. kaustophilus (Takami et al., 2004a) were typically maintained on Luria–Bertani broth (LB) (Sambrook & Russell, 2001) medium and were grown in a minimal medium containing 18.7 mM NH₄Cl, 2.79 mM Na₂HPO₄, 1.72 mM K₂SO₄, 1.62 mM MgSO₄.7H₂O, 1.39 μM ZnSO₄.7H₂O, 162 nM H₂BO₃, 238 nM CoCl₂.6H₂O, 800 nM CuSO₄.5H₂O, 42 nM NiCl₂.6H₂O, 744 nM disodium EDTA, 11.1 μM MnCl₂.4H₂O, 34.1 μM CaCl₂.2H₂O, 25.9 μM FeCl₃.6H₂O, 10.0 mM K-MOPS (pH 8.0) and a carbon source, including 0.5% Casamino acids (Becton Dickinson), 25 mM glucose, 12.5 mM sucrose, 25 mM MI (Sigma-Aldrich), 25 mM SI (Tokyo Chemical Industry) or 25 mM DCI (Tokyo Chemical Industry), at 60 °C with shaking. *Escherichia coli* strains DH5α (Sambrook & Russell, 2001) and BL21(DE3) (Merck) were used as hosts for plasmid construction and expression of C-terminal His₆-tag fusion proteins, respectively. *E. coli* cells were grown in LB medium at 37 °C with shaking. Plasmid pMD20-t (Takara Bio) and pET28b+ (+) (Merck) were used for TA cloning of PCR fragments and His₆-tag fusion protein expression in *E. coli*, respectively. When required, medium was supplemented with ampicillin (50 μg ml⁻¹), kanamycin (50 μg ml⁻¹) and IPTG (0.5 mM).

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Genotype</th>
<th>Reference or source</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>G. kaustophilus</strong> strains</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HTA426</td>
<td>trpC2 metC7</td>
<td>Takami et al. (2004b)</td>
</tr>
<tr>
<td>PS8</td>
<td>iol</td>
<td>This study</td>
</tr>
<tr>
<td><strong>E. coli</strong> strains</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BL21(DE3)</td>
<td>F⁻ ampT hsdS₂(r₂ m₂) dcm gal BL21(DE3) tonA</td>
<td>Sambrook &amp; Russell (2001)</td>
</tr>
<tr>
<td>DH5α</td>
<td>F⁺ 800 dlacZAM15 Δ(lacZYA-argF) U169 deoR recA1 endA1 hsdR17(rK₃ mK₃) phoA supE44 ñc thi-1 gyrA96 relA1</td>
<td>Merck</td>
</tr>
<tr>
<td><strong>Plasmids</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pET28h(+)</td>
<td>kan</td>
<td>Merck</td>
</tr>
<tr>
<td>pHis-gk1897</td>
<td>kan</td>
<td>This study</td>
</tr>
<tr>
<td>pHis-gk1898</td>
<td>kan</td>
<td>This study</td>
</tr>
<tr>
<td>pHis-gk1899</td>
<td>kan</td>
<td>This study</td>
</tr>
<tr>
<td>pMD20-t</td>
<td>amp lacZ</td>
<td>Takara Bio</td>
</tr>
</tbody>
</table>
Cloning and expression of *gk1897*, *gk1898* and *gk1899* in *E. coli* and purification of the gene products. PCR fragments corresponding to the reading frames (without the termination codon) of *gk1897*, *gk1898* and *gk1899* were amplified from the chromosomal DNA of strain HTA246 using specific primers (Table 2), which were designed to generate additional unique restriction enzyme cutting sites at either end of the fragments, as indicated (Table 2). Each of the fragments was separately ligated into pMD20-t by TA cloning, and were transformed into *E. coli* DH5α to yield the respective recombinant plasmids conferring ampicillin resistance. Subsequently, the recombinant plasmids were digested with specific pairs of restriction enzymes, and the insert fragments were recovered and inserted into pET28b (+) so that each of *gk1897*, *gk1898* and *gk1899* could be expressed as an N-terminal His<sub>6</sub>-tag fusion protein. The three plasmids constructed in this manner were designated pHis-gk1897, pHis-gk1898 and pHis-gk1899, and were introduced into *E. coli* BL21(DE3) to produce the proteins His-GK1897, His-GK1898 and His-GK1899, respectively.

BL21(DE3) cells carrying pHis-gk1897, pHis-gk1898 or pHis-gk1899 were inoculated into LB medium containing kanamycin and grown at 37 °C with shaking. When cultures reached OD<sub>600</sub> 0.35, production of the N-terminal His<sub>6</sub>-tag fusion proteins was induced for 2 h by the addition of IPTG. The cells were harvested, treated with lysozyme and disrupted by brief sonication, and then the soluble fraction was obtained by centrifugation. The His<sub>6</sub>-tag fusion proteins were purified from the soluble fraction using the His-Bond system (Merck), employing the procedure recommended by the supplier. The purified proteins, His-GK1897, His-GK1898 and His-GK1899, were subjected to Sephadex G-25 (GE Healthcare) gel filtration to remove imidazole and salts. The purity of the His<sub>6</sub>-tag fusion proteins was verified by SDS-PAGE.

Enzyme assays. The purified His-GK1897, His-GK1898 and His-GK1899 were subjected to a spectrophotometric enzyme assay for inositol dehydrogenase as follows. Under standard conditions, 5 μg of the purified enzyme was combined with a 1 ml reaction mixture composed of 100 mM phosphate buffer (pH 8.0) containing the substrate (MI, SI or DCI) at 50 mM and the cofactor NAD(P)⁺ at 0.5 mM. The mixture was incubated at 40 °C and any increase in absorbance at 340 nm associated with NAD(P)⁺ reduction was measured to calculate the reaction rates, as described by Fujita et al. (1991). Alternatively, the mixture was incubated at various temperatures as indicated to test the effect of temperature on the stability and activity of the enzymes. In order to analyse the reaction products, 25 μg purified enzyme was combined with a 0.1 ml reaction mixture composed of 100 mM phosphate buffer (pH 8.0) containing 80 mM 2KMI and 40 mM NAD(P)H, and subsequently incubated at 40 °C for 1 h. After centrifugation at 4 °C, the supernatant was subjected to gas chromatography/time-of-flight MS (GC-TOF-MS) analysis as described below.

**GC-TOF-MS analysis.** Intracellular metabolites were extracted and analysed as follows. Bacterial cells were inoculated in minimal medium containing 0.5 % Casamino acids and 25 mM MI, and allowed to grow with shaking. At OD<sub>600</sub> 1.0, the cells in 40 ml of culture were harvested by centrifugation and washed twice with ice-cold water. The cell pellet was frozen in liquid nitrogen and dried under vacuum. Ten milligrams of the dried cells was dissolved in 0.9 ml extraction mixture consisting of water, chloroform and methanol (2 : 2 : 5) and vigorously mixed together with 0.2 ml of zirconia/silica beads (diameter, 0.6 mm) and one larger zirconia bead (diameter, 5.0 mm) using a high-speed homogenizer (Shake Master NEO, Bio Medical Science) for 5 min at 1500 r.p.m. and 4 °C. After centrifugation for 3 min at 11 800 g and 4 °C, 0.63 ml of the clear supernatant was withdrawn and mixed with 0.28 ml pure water. After further centrifugation, 0.3 ml of the supernatant was transferred to another tube and allowed to dry completely.

The dried pellet was dissolved and the substances present in it were derivatized with methoxyamine hydrochloride in pyridine at 30 °C for 90 min, with vigorous shaking. After the addition of N-methyl-N-(trimethylsilyl)trifluoroacetamide (GL Science), the derivatized substances were further incubated at 37 °C for 30 min and then injected via an Agilent 7683B autosampler into an Agilent 7890A gas chromatograph (Agilent Technologies) coupled to a Pegasus HT time-of-flight mass spectrometer (Leco) with the settings as described previously (Yamaoka et al., 2011). Peak deconvolution, identification and quantification were performed using the Pegasus ChromaTOF software package v. 4.21 (Leco). Commercially available pure inositols were also derivatized and analysed in parallel with the experimental samples. The mass spectra and retention times obtained were used to identify the stereoisomers, and ion intensities were used to calculate the concentration of the stereoisomers. Substances contained in the *in vitro* enzymic reactions were also analysed by GC-TOF-MS, as described previously (Yamaoka et al., 2011).

**RNA techniques.** Cells of strains of *G. kaustophilus* were grown in minimal medium containing 0.5 % (w/v) Casamino acids and with

<table>
<thead>
<tr>
<th>Oligonucleotide</th>
<th>Sequence (5’–3’) *</th>
</tr>
</thead>
<tbody>
<tr>
<td>gk1897NdeI</td>
<td>GGAATTCATATGATACGGTTTGCTCAGGT</td>
</tr>
<tr>
<td>gk1897Xhol</td>
<td>CCGCTCGAGCTACGGTTTGCTCATG</td>
</tr>
<tr>
<td>gk1898Nhel</td>
<td>CTAGCTAGCATAAAGAGAGAAAAAACAATCC</td>
</tr>
<tr>
<td>gk1898Xhol</td>
<td>CGGCCCTCGAGCTACGGTTTGCTCATG</td>
</tr>
<tr>
<td>gk1899NdeI</td>
<td>GAATTCATATGATACGGTTTGCTCAGGT</td>
</tr>
<tr>
<td>gk1899NotI</td>
<td>ATAAAGATGCGCGGCGTTATTTTGACCGGAGCTTGT</td>
</tr>
<tr>
<td>gk pro-F</td>
<td>GCTGATGCTAGGGTTT</td>
</tr>
<tr>
<td>gk pro-R</td>
<td>GCGTCCCCAAAATCCCTAC</td>
</tr>
<tr>
<td>nGK1894</td>
<td>CAATCAGGCAAAGGGCACTCA</td>
</tr>
<tr>
<td>nGK1894DIG</td>
<td>TAATACGACTCACTATAGAGGTGTTGCTTCCATCCTCATT</td>
</tr>
<tr>
<td>nGK1899</td>
<td>CCCCATATGGTGCTCATGTA</td>
</tr>
<tr>
<td>nGK1899DIG</td>
<td>TAATACGACTCACTATAGAGGTGTTGCTTCCATCCTCATT</td>
</tr>
</tbody>
</table>

*Restriction sites are underlined, and T7 promoter tag sequences are in italic type.*
and without supplementation with 10 mM MI up to OD\text{\textsubscript{600}} 1.0. Cells in 45 ml of culture were harvested by centrifugation and washed twice with 10 ml ice-cold Tris/HCl (pH 7.4). Total RNA was extracted from the cells as described previously (Yoshida et al., 1999b).

The RNA samples were subjected to Northern blot analysis, performed in a manner similar to that described previously (Yoshida et al., 1997); however, DIG-labelled RNA probes were used. These were prepared as follows: DNA fragments corresponding to the target genes were amplified from HTA426 DNA by PCR, and a T7 RNA promoter tag was generated at the downstream end. The specific primer pairs used were nGK1894 and nGK1894D1GR for the gk1894 probe, and nGK1899 and nGK1899D1GR for the gk1899 probe (Table 2). The PCR product was used as the template for \textit{in vitro} transcriptions using DIG RNA Labeling kit (SP6/17) (Roche Diagnostics). The RNA samples (15 μg) prepared from the cells were electrophoresed in an agarose gel (Yoshida et al., 1997), transferred to a positively charged nylon membrane (Roche Diagnostics), and hybridized with the DIG-labelled RNA probe under conditions recommended by the supplier. The hybridized probes were detected on the membrane using a DIG Luminescent Detection kit (Roche Diagnostics).

Primer extension analysis was performed as described previously (Yoshida et al., 1997). Reverse transcription was initiated from the gk pro-R primer (Table 2), which had been labelled at its 5’ terminus using a Megalab kit (Takara Bio) and [\textit{\textit{\textalpha}}\textsuperscript{\textit{\textit{\texti}}\textsuperscript{\textit{\textp}}}]\textit{\textit{\textATP}} (MP Biomedicals). A template for the dideoxy sequencing reactions for ladder preparation starting from the same end-labelled primer was prepared by PCR using the gk pro-F and gk pro-R primer pair (Table 2) and DNA from HTA426 as template.

\textbf{Ethyl methanesulfonate (EMS) mutagenesis and penicillin screening.} \textit{G. kaustophilus} HTA426 was grown in LB medium to OD\text{\textsubscript{600}} 0.5. The cells in 20 ml of culture were harvested by centrifugation, suspended in 10 ml LB medium containing 80 mM EMS, and allowed to grow for 30 min at 42 °C with shaking. All cells were then harvested, washed twice with minimal medium supplemented with 25 mM MI, and suspended in 50 ml of the same medium in a 500 ml Sakaguchi flask. The cells were allowed to grow at 60 °C for 16 h with vigorous shaking (180 r.p.m.). During growth, 7000 U penicillin G ml\textsuperscript{-1} (Sigma-Aldrich) was added to the culture at OD\text{\textsubscript{600}} 0.5. All cells were harvested by centrifugation, washed three times with LB medium, and suspended in 5 ml LB medium. Aliquots of the suspension were spread on minimal medium agar plates containing both 0.1 % Casamino acids and 10 mM MI, and incubated at 60 °C for 48 h. Smaller colonies appearing on the plates were replicated on two types of agar plates: one consisted of minimal medium containing various carbon sources: none (○), 25 mM glucose (□), 25 mM MI (■), 25 mM SI (▲) and 25 mM DCI (△). Growth was monitored by the increase in OD\text{\textsubscript{600}}.

Representative data from three independent experiments with similar results are shown.

\textbf{RESULTS AND DISCUSSION}

\textit{G. kaustophilus} HTA426 possesses an \textit{iol} gene cluster

In \textit{B. subtilis}, the \textit{iol} operon (including \textit{iolABCDEFGHIJ}) and some other independently expressed \textit{iol} genes, such as \textit{iolX} and \textit{iolW}, are known to encode inositol catabolic enzymes; this set of \textit{iol} genes confer the ability to utilize the three inositol stereoisomers, namely MI, DCI and SI, as sole carbon sources (Yoshida et al., 1997, 2006, 2008; Morinaga 1996).

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{Fig2.png}
\caption{Growth of \textit{G. kaustophilus} using various carbon sources. Cells of \textit{G. kaustophilus} HTA426 were grown with shaking at 60 °C in minimal medium containing various carbon sources: none (○), 25 mM glucose (□), 25 mM MI (■), 25 mM SI (▲) and 25 mM DCI (△). Growth was monitored by the increase in OD\text{\textsubscript{600}}. Representative data from three independent experiments with similar results are shown.}
\end{figure}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{Fig3.png}
\caption{Phylogenetic tree of inositol dehydrogenases of \textit{G. kaustophilus} and \textit{B. subtilis}. Amino acid sequences of GK1897, GK1898 and GK1899 were aligned using CLUSTAL W with those of the products of \textit{iolG} (BSiolG), \textit{iolW} (BSiolW) and \textit{iolX} (BSiolX) from \textit{B. subtilis} 168 (Thompson et al., 1994) to draw the unrooted tree illustrating the relatedness of the six inositol dehydrogenases. Sequence identity (percentage of overlapping amino acid residues) is shown for each of the neighbouring pairs of GK1897/BSiolX, GK1898/BSiolW and GK1899/BSiolG.}
\end{figure}
et al., 2010a). *G. kaustophilus* HTA426 was also able to grow in the presence of each of these three inositol stereoisomers (Fig. 2). Moreover, within its genome is a cluster of 15 genes (range gk1885–gk1899) containing an almost complete set of iol genes homologous to genes found in *B. subtilis* (Takami et al., 2004b) (Fig. 1). Therefore, the inositol catabolic pathways in *B. subtilis* and *G. kaustophilus* are possibly similar, involving the enzymes encoded by the genes indicated in Fig. 1.

Compared with the *B. subtilis* iol operon, it was notable that the *G. kaustophilus* gene cluster contained three paralogous genes in tandem, gk1897–gk1899; each of these genes was predicted to encode MI 2-dehydrogenase or oxidoreductase, which could be responsible for the initial oxidation of inositol (Fig. 1). Amino acid sequences of the three gene products were aligned with those of the three genes of *B. subtilis* characterized to encode inositol dehydrogenase, namely iolG, iolX and iolW, using the CLUSTAL W (1.81) program with default settings (Thompson et al., 1994). As shown in the resulting phylogenetic tree (Fig. 3), gk1897 and iolX were the most similar to each other (sharing 70 % identity in an overlap of 331 amino acid residues), while gk1899 and gk1898 were more similar to iolG (24 % identity in 223 amino acid residues) and iolW (22 % identity in 250 amino acid residues), respectively.

On the other hand, the iol gene cluster of *G. kaustophilus* contained no homologue of iolF or iolT, each of which encodes an inositol transporter belonging to the major

Table 3. Enzymic parameters of His-GK1897, His-GK1898 and His-GK1899

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Substrate</th>
<th>Cofactor</th>
<th>$K_m^*$ (mM)</th>
<th>$V_{max}^\dagger$ (µmol min⁻¹ mg⁻¹)</th>
<th>$V_{max}/K_m$</th>
</tr>
</thead>
<tbody>
<tr>
<td>His-GK1897</td>
<td>MI</td>
<td>0.5 mM NAD⁺</td>
<td>29.4 ± 5.62</td>
<td>0.857 ± 0.176</td>
<td>0.029</td>
</tr>
<tr>
<td></td>
<td>SI</td>
<td></td>
<td>21.4 ± 5.12</td>
<td>0.758 ± 0.184</td>
<td>0.035</td>
</tr>
<tr>
<td></td>
<td>DCI</td>
<td></td>
<td>48.4 ± 7.98</td>
<td>0.093 ± 0.022</td>
<td>0.002</td>
</tr>
<tr>
<td>His-GK1898</td>
<td>MI</td>
<td>0.5 mM NADP⁺</td>
<td>24.8 ± 3.54</td>
<td>0.969 ± 0.121</td>
<td>0.039</td>
</tr>
<tr>
<td></td>
<td>SI</td>
<td></td>
<td>18.1 ± 2.37</td>
<td>1.42 ± 0.284</td>
<td>0.078</td>
</tr>
<tr>
<td></td>
<td>DCI</td>
<td></td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>His-GK1899</td>
<td>MI</td>
<td>0.5 mM NAD⁺</td>
<td>13.1 ± 1.69</td>
<td>2.56 ± 0.366</td>
<td>0.195</td>
</tr>
<tr>
<td></td>
<td>SI</td>
<td></td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>DCI</td>
<td></td>
<td>28.8 ± 1.56</td>
<td>1.21 ± 0.243</td>
<td>0.042</td>
</tr>
</tbody>
</table>

*Values are mean ± SD (mM) of three independent measurements.
†Values are mean ± SD (µmol min⁻¹ (mg protein)⁻¹) of three independent measurements.
facilitator superfamily (Yoshida et al., 2002; Morinaga et al., 2010b). Instead, the three genes gk1893, gk1894 and gk1896 were deduced to encode a permease, an ATP-binding protein and a sugar-binding protein subunit of a putative sugar ABC transporter, respectively, suggesting that the three gene products may compose an ABC transporter for inositol uptake.

Characterization of the three inositol dehydrogenases of *G. kaustophilus*

The products of gk1897, gk1898 and gk1899 were expressed in *E. coli* as N-terminal His-tag fusion proteins and purified to homogeneity, as assessed by SDS-PAGE (data not shown).

The three purified proteins, His-GK1897, His-GK1898 and His-GK1899, were subjected to an inositol dehydrogenase assay using MI as a substrate and NAD$^+$ as a cofactor; NAD$^+$ was used as a cofactor to monitor the increase in A$_{340}$ that was associated with reduction of NAD$^+$ to NADH. Under standard conditions, His-GK1897 and His-GK1899 demonstrated specific activities of 857 and 2560 nmol min$^{-1}$ (mg protein)$^{-1}$, respectively, while His-GK1898 exhibited no detectable activity (data not shown). When NADP$^+$ was employed as the cofactor instead of NAD$^+$, however, His-GK1898 alone demonstrated a significant specific activity of 969 nmol min$^{-1}$ (mg protein)$^{-1}$. These results indicated that all three proteins indeed functioned as inositol dehydrogenases and were able to dehydrogenate MI with different cofactor requirements: His-GK1897 and His-GK1899 depended on NAD$^+$, while only His-GK1898 depended on NADP$^+$. For comparison, the principal MI dehydrogenase IolG of *B. subtilis* has been reported to exhibit a high activity of up to 34.4 μmol min$^{-1}$ (mg protein)$^{-1}$ under certain conditions (Ramaley et al., 1979). On the other hand, we previously demonstrated that the two SI dehydrogenases of *B. subtilis*, namely IolW and IolX, were also able to act on MI, with lower activities of 390 and 340 nmol min$^{-1}$ (mg protein)$^{-1}$, respectively (Morinaga et al., 2010a).

Because *G. kaustophilus* is a thermophile, we also investigated the effect of temperature on the three enzymes (Fig. 4). His-GK1897, His-GK1898 and His-GK1899 demonstrated the highest activity at 55, 65 and 75 °C, respectively. Moreover, His-GK1897 and His-GK1898 retained >60% activity up to approximately 60 °C, while His-GK1899 was the most thermostable up to 70 °C. These results clearly indicated that all these enzymes were functional at the physiological temperatures necessary for the growth of *G. kaustophilus*, although they were more stable at temperatures lower than 55 °C.

The enzymic parameters were determined using the three inositol stereoisomers MI, SI and DCI as alternative substrates (Table 3). Among the three enzymes, only His-GK1897 was capable of dehydrogenation of all three isomers, while His-GK1898 and His-GK1899 could not act on DCI and SI, respectively. SI was the preferred substrate for both His-GK1897 and His-GK1898, resulting in the smallest $K_m$ and the largest $V_{max}/K_m$ values. Given that all six hydroxyl groups are equatorial in SI (Fig. 1), His-GK1897 and His-GK1898 appeared to be able to act on equatorial hydroxyl groups. On the other hand, MI has only one axial hydroxyl group, while DCI has two in tandem (Fig. 1). Because His-GK1899 demonstrated no activity on SI, it is possible that it could not react with the equatorial hydroxyl groups. However, for all three enzymes, the more axial hydroxyl groups an inositol isomer contained, the less preferred as a substrate it became. In case of His-GK1898, DCI was probably rejected because of its two tandem axial hydroxyl groups, which may cause some steric hindrance in the interaction with the substrate-binding pocket of the enzyme.

To assess the possible stereospecificity of these enzymes further, 2KMI reduction by each of the enzymes in the presence of reduced forms of the respective cofactors was evaluated, and the reaction products were analysed by GC-TOF-MS (Fig. 5). The reactions were performed at 40 °C, taking into account the difference in thermal stability of the enzymes. 2KMI has a single carbonyl group and five equatorial hydroxyl groups (Fig. 1), and thus could potentially be reduced to MI and SI by axial and equatorial hydroxyl group-targeting enzymes, respectively. We found that His-GK1899 produced MI almost exclusively, but both

![Fig. 5. GC-TOF-MS analysis of the reaction products appearing after 2KMI reduction. 2KMI reduction by His-GK1897, His-GK1898 or His-GK1899 was performed as described in the text. GC-TOF-MS charts are shown that follow the reduction by (a) His-GK1897, (b) His-GK1898 and (c) His-GK1899; (d) standards of MI, SI, DCI, 2KMI and ε-inositol (EI).](image-url)
His-GK1897 and His-GK1898 produced an approximately 50:50 mixture of MI and SI. These results verified that His-GK1899 acted only on axial hydroxyl groups, while both His-GK1897 and His-GK1898 have the potential to react with axial or equatorial hydroxyl groups. However, it is likely that these enzymes typically encounter equatorial hydroxyl groups, because inositol stereoisomers with axial hydroxyl groups are less preferred substrates in dehydrogenation, as described above.

**Transcription of the iol gene cluster of *G. kaustophilus***

Total RNA samples were prepared from *G. kaustophilus* HTA426 cells grown in the presence and absence of MI in the growth medium, and were subjected to Northern blot and primer extension analyses. For Northern blot analysis, probes specific for *gk1894* and *gk1899* were used (Fig. 6). The *gk1894* probe detected a weak band of a 12 kb long transcript, possibly covering the 10 genes from *gk1885* to *gk1894*, with smeared bands of smaller RNA species probably due to degradation of the 12 kb one. The *gk1899* probe detected a 5 kb transcript, which corresponded to the four genes from *gk1896* to *gk1899*. The *gk1895* gene was oriented in a direction opposite to that of both of these transcripts, and its expression was not investigated further. Both the transcripts were detected only in the presence of MI and were thus most probably induced by MI. Primer extension analysis was performed to determine the transcription start site of the 5 kb transcript immediately

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**Fig. 6.** Northern blot analysis of the *iol* gene cluster in *G. kaustophilus*. Northern blot analysis was performed using total RNA samples extracted from cells of strains HTA426 (lanes 1 and 2) and PS8 (lanes 3 and 4) grown without (lanes 1 and 3) or with 10 mM MI (lanes 2 and 4), utilizing probes specific for *gk1894* (a) and *gk1899* (b). Positions of size markers are indicated on the left. Arrows indicate the specific bands of the transcripts. Plausible assignment of the transcripts on the *iol* gene cluster is shown schematically at the bottom of the figure.
upstream of \( gk1899 \); the transcription start site was associated with the plausible \(-10\) and \(-35\) sequences of a promoter presumably recognized by the housekeeping sigma factor (Fig. 7). Therefore, the 5 kb transcript must be polycistronic to cover \( gk1896-gk1899 \), including the three inositol dehydrogenase genes that are induced simul-aneously in the presence of their common substrate, MI.

\textit{G. kaustophilus} strain HTA426 was subjected to EMS mutagenesis, followed by penicillin screening in order to select mutants unable to utilize MI as the sole carbon source. A mutant strain, PS8, in which inositol dehydrogenase activity was almost completely abolished, was isolated (data not shown). Moreover, it appeared that the two transcripts covering the \( iol \) gene cluster were not induced in this strain even in the presence of MI (Fig. 6). The lack of inositol dehydrogenase activity in PS8 was probably due to the absence of the 5 kb polycistronic transcript covering \( gk1897-gk1899 \). PS8 appeared to be unable to utilize SI or DCI (data not shown). Taken together, these results suggested that any of the three enzymes could be responsible for the substantial inositol dehydrogenase activity that enables utilization of MI, SI and DCI as carbon sources. We did not find any mutation within the 15-gene cluster of PS8, including the promoter region determined above. At present, we are therefore unable to determine the genetic reason(s) for the loss of the \( iol \) transcripts in PS8; however, inositol uptake may be impaired in PS8, as described below.

**Intracellular interconversion of inositol stereoisomers**

Metabolites in the cells of strains HTA426 and PS8, grown to the stationary phase in the presence of MI, were analysed by GC-TOF-MS (Fig. 8). In HTA426 cells, not only MI but also SI and DCI were present along with traces of unknown minor substances, while only a limited amount of MI alone was observed in PS8 cells. These results suggested that intracellular interconversion of inositol stereoisomers could have occurred involving the three inositol dehydrogenases expressed simultaneously in HTA426. As described above, MI is the common substrate for the three enzymes and can be converted into a mixture of inosose (2,3,4,5,6-pentahydroxycyclohexanone), including 2KMI and 1KDCI (Fig. 1). It could be speculated that during the stationary phase, for currently unknown reasons, the inosose was not catabolized further but instead was readily reduced by any of the three inositol dehydrogenases into a mixture of inositol stereoisomers, including MI, SI and DCI. \( gk1898 \) may be particularly important for this reduction because it is orthologous to \( B. subtilis \) \( IolW \) (Fig. 3), which has been shown to reduce 2KMI to SI efficiently with NADPH oxidation (Yamaoka et al., 2011). In addition, \( iol \) of HTA426 may play a role in the interconversion by isomerizing inosose; its role is similar to that of \( B. subtilis \) \( iol \) in isomerization of 2KMI to 1KDCI, which was further converted to DCI (Fig. 1) (Yoshida et al., 2006). In PS8 cells, the intracellular amount of MI was markedly lower than that in HTA426 cells, which implied that inositol uptake may be impaired in PS8. This could be relevant to the absence of transcripts for the putative ABC transporter genes \( gk1893 \), \( gk1894 \) and \( gk1896 \) (Fig. 6), but also suggested the possible presence of other minor transport systems.

Of the nine inositol stereoisomers in which the six hydroxyl groups are epimerized, MI is by far the most prevalent in nature (Turner et al., 2002) and is indispensable in animals and plants, while the other inositol isomers occur relatively rarely. We previously demonstrated the artificial interconversion of MI to DCI and SI in genetically manipulated strains of \( B. subtilis \), which was only possible when \( iolE \), which encodes 2KMI dehydratase, was inactivated.

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**Fig. 7.** Determination of the transcription start site of the 5 kb \( iol \) transcript in \( G. kaustophilus \). (a) Total RNA samples extracted from \( G. kaustophilus \) HTA426 grown in minimal medium containing 0.5% Casamino acids and without (lane 1) or with supplementation with 10 mM MI (lane 2) were reverse-transcribed to generate cDNA. Lanes C, T, A and G are dideoxy sequencing ladders generated using the same primer as that used for reverse transcription. The partial nucleotide sequence around the transcription start site is shown on the left; the 5’ end of the transcript is indicated by an arrow. (b) Organization of the putative promoter region. The transcription start site (+1) is indicated by an arrow, and –35 and –10 regions of the corresponding promoter are boxed.
leading to accumulation of intracellular 2KMI (Yoshida et al., 2006; Yamaoka et al., 2011). The natural occurrence of the intracellular interconversion of inositol stereoisomers in wild-type HTA426 was unexpected, because neither SI nor DCI has ever been observed in standard B. subtilis strain 168, grown under similar conditions but at lower temperatures (data not shown). The isomer produced most efficiently was SI (Fig. 8), which is known to exert inhibitory effects on self-aggregation of proteins into amyloid fibrils, a pathological hallmark of numerous diseases, such as Alzheimer’s disease, type II diabetes and prion-related disorders (Li et al., 2012; Chiti & Dobson, 2006). It is conceivable that such protein aggregation may also be provoked under thermal and pressure stresses. Because G. kaustophilus HTA426 is a thermophile and was isolated from deep-sea sediment, we may suppose that it has developed this SI generation system as a means of preventing protein aggregation. Intriguingly, an earlier report indicated that the two rarer isomers, i.e. SI and DCI, often occur in organic-rich marine sediments (White & Miller, 1976). Some micro-organisms in marine sediments may be involved in the generation of minor inositol isomers, such as SI, whose biological significance remains to be elucidated.

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