myo-Inositol Catabolism in *Bacillus subtilis*

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The *iolABCDEFGHJ* operon of *Bacillus subtilis* is responsible for *myo*-inositol catabolism involving multiple and stepwise reactions. Previous studies demonstrated that IolG and IolE are the enzymes for the first and second reactions, namely dehydrogenation of *myo*-inositol to give 2-keto-*myo*-inositol and the subsequent dehydration to 3D-(3,5,4)-trihydroxycyclohexan-1,2-dione. In the present studies the third reaction was shown to be the hydrolysis of 3D-(3,5,4)-trihydroxycyclohexan-1,2-dione catalyzed by IolD to yield 5-deoxy-D-glucuronic acid. The fourth reaction was the isomerization of 5-deoxy-D-glucuronic acid by IolB to produce 2-deoxy-5-keto-D-glucuronic acid. Next, in the fifth reaction 2-deoxy-5-keto-D-glucuronic acid was phosphorylated by IolC kinase to yield 2-deoxy-5-keto-D-glucuronic acid 6-phosphate. IolR is known as the repressor controlling transcription of the *iol* operon. In this reaction 2-deoxy-5-keto-D-glucuronic acid 6-phosphate appeared to be the intermediate acting as inducer by antagonizing DNA binding of IolR. Finally, IolJ turned out to be the specific aldolase for the sixth reaction, the cleavage of 2-deoxy-5-keto-D-glucuronic acid 6-phosphate into dihydroxyacetone phosphate and malonic semialdehyde. The former is a known glycolytic intermediate, and the latter was previously shown to be converted to acetyl-CoA and CO₂ by a reaction catalyzed by IolA. The net result of the inositol catabolic pathway in *B. subtilis* is, thus, the conversion of *myo*-inositol to an equimolar mixture of dihydroxyacetone phosphate, acetyl-CoA, and CO₂.

*myo*-Inositol (MI) is abundant in soil and also common and essential in plants and animals. A number of microorganisms, including *Bacillus subtilis* (1), *Cryptococcus melibiose* (2), *Aerobacter aerogenes* (reclassified as *Enterobacter aerogenes*) (3), *Rhizobium leguminosarum* bv. *viciae* (4), *Sinorhizobium meliloti* (5), *Sinorhizobium fredii* (6), *Corynebacterium glutamicum* (7), and *Lactobacillus casei* (8) can grow on MI as the sole carbon source. MI catabolism in *A. aerogenes* was studied biochemically, and a pathway of the catabolism finally yielding acetyl-CoA and dihydroxyacetone phosphate (DHAP) was proposed (9). However, our knowledge of the molecular genetics of bacterial MI catabolism has been restricted to *B. subtilis* (1, 10–12). In *B. subtilis*, the *iol* diverge, comprising the operons *iolABCDEFGHJ* and *iolRS* (1), and the *iolT* gene (12) were shown to be required for inositol catabolism (Fig. 1). Nowadays, a large number of bacteria have genes annotated *iol* in their genome sequence, but the annotation is only based on sequence similarity to *B. subtilis* *iol* genes, as relatively few studies have been done to demonstrate the participation of the deduced *iol* genes in MI catabolism.

In *B. subtilis*, a repressor encoded by *iolR* is responsible for the regulation of all the *iol* genes (11, 12). In the absence of MI in the growth medium, the IolR repressor binds to the operator site within the promoter regions to repress the transcription. In its presence, however, MI is taken up by the cell and converted to a catabolic intermediate that acts as an inducer by antagonizing IolR, thereby inducing the *iol* divergon and *iolT* (11, 12). Consequently, inactivation of *iolR* makes the transcription of the *iol* divergon and the *iolT* gene constitutive (1).

Some enzymes involved in MI catabolism have been characterized. Inositol dehydrogenase encoded by *iolG* is responsible for the first step of the degradation cascade by converting MI to 2-keto-MI (2KMI), conversion of compound [1] to [3] see (Fig. 1) (10). Recently IolG was also shown to be able to act on 1,2-dihydroxyinositol (DCI, compound [2]) to yield 1-keto-DCI (compound [4]), and Ioll interconverts 1-keto-DCI and 2KMI, indicating that not only MI but also DCI is metabolized through the MI catabolic pathway (Fig. 1) (14). In addition, pinitol (3-O-methyl-DCI) contained in soybean appeared to be an alternative substrate of IolG, and this substrate was degraded depending on the presence of functional *iol* genes (15). 2KMI dehydratase, encoded by *iolE*, is responsible for the second step to produce 3D-(3,5,4)-trihydroxycyclohexan-1,2-dione (THcHDO, compound [5]), formerly called 2,3-diketo-4-deoxy-epi-inositol) (13). The *iolT* and *iolF* genes encode the primary and secondary inositol transporters, respectively (Fig. 1) (12). The *iolA* gene was shown to encode malonic semialdehyde (MSA) dehydrogenase (16), supposed to be involved in MI catabolism based on the assumption that the catabolic pathway...
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**TABLE 1**

Bacterial strains and plasmids used in this study

| Strains | Genotype | Reference or source |
|---------|----------|---------------------|
| **B. subtilis** | trpC2 metC7 | Laboratory collection |
| 60015 | | 1 |
| YF244 | trpC2 metC7 iolR:cat | This work |
| YF258 | trpC2 metC7 iolR:cat iolB52 | This work |
| YF259 | trpC2 metC7 iolR:cat iolB88 | This work |
| YF260 | trpC2 metC7 iolR:cat iolC62 | This work |
| **E. coli** | recA1 endA1 gyrA96 thi-1 hsdR17(rK m-m, +) e14: (mcrA-) supE44 relA1 | 20 |
| JM109 | Δ(lac-proAB)/F^T7traD36 proAB ^lac^IolZ ΔM15 | |
| BL21(DE3) | F^*:amp F^lacS41^r^m-m^ dcm gal (DE3)^tonA | Novagen |
| **Plasmids** | | |
| pDGHisC | amp PT7^His^-tag | K. Asai |
| pET30a | kan | Novagen |
| pPiolF | amp Plac-iolB | 11 |
| pPiolB | amp Plac-iolBC | 13 |
| pPiolBC | | |
| pPiolB-His | amp PT7-iodB-his | This work |
| pPiolC | amp Plac-iolC | 13 |
| pPiolD | amp Plac-iodD | 13 |
| pPiolDE | amp Plac-iodE | 13 |
| pPiolJ-His | amp PT7-iodJ-his | This work |
| pPiolL3 | amp Plac-iodR | 11 |
| pg-KJ84 | cat dnak dual gpeE groES groEL | 24 |
| pUC18 | amp lacZM15 | 20 |
| pUC19 | amp lacZM15 | 20 |

is similar to that proposed by Anderson and Magasanik for *A. aerogenes* (9). But the reaction step producing MSA has not been identified. Homology searches indicated possible functions for the products of the *iolC* and *iolI* genes (13), but none has been identified experimentally. In the present studies we tried to identify the functions of *iolD*, *iolB*, *iolC*, and *iolI*, aiming to define the whole pathway of MI catabolism and to understand how it is linked to the transcriptional regulation of the *iol* genes.

**EXPERIMENTAL PROCEDURES**

Bacterial Strains, Plasmids, and Growth Conditions—Bacterial strains and plasmids used in this study are listed in Table 1. *B. subtilis* 60015 (*trpC2 metC7*) is our standard laboratory strain. *B. subtilis* strains YF244, YF258, YF259, and YF260 are 60015 derivatives. YF244 possesses the *iolR:*cat mutation inactivating the IolR repressor to allow constitutive expression of 60015 derivatives. YF244 possesses the *iolR:*cat mutation inactivating the IolR repressor to allow constitutive expression of 60015 derivatives. YF244 possesses the *iolR:*cat mutation inactivating the IolR repressor to allow constitutive expression of 60015 derivatives. YF244 possesses the *iolR:*cat mutation inactivating the IolR repressor to allow constitutive expression of 60015 derivatives. YF244 possesses the *iolR:*cat mutation inactivating the IolR repressor to allow constitutive expression of 60015 derivatives. *E. coli* JM109 was used as the hosts for plasmid constructions and expression of C-terminal His<sub>6</sub>-tag fusion proteins, respectively. *E. coli* cells were grown in LB (21) and TGA (22) media at 37 °C. Plasmids pUC18, pUC19 (20), pET30a (Novagen), and pDGHisC (a gift from Kei Asai, Saitama University) were used as cloning vectors. pDGHisC is a pDG148 (23) derivative designed to express a desired gene as a C-terminal His<sub>6</sub>-tag fusion under the control of T7 promoter in *E. coli*. pG-KJ84 (24) was used for supplying chaperons to stabilize overproduced fusion proteins. When required, media were supplemented with ampicillin (50 μg/ml), arabinose (1 mg/ml), chloramphenicol (15 and 35 μg/ml for *B. subtilis* and *E. coli*, respectively), IPTG (0.5 mm), kanamycin (50 μg/ml), and tetracycline (5 ng/ml).

Cloning and Expression of the iol Genes in *E. coli* and Purification of the Gene Product—The native *iolB*, *iolC*, *iolD*, and *iolE* genes were cloned into the pUC18 vector and expressed in *E. coli* cells grown in TGA medium as described previously (13). To express *iolB* and *iolI* and produce the corresponding proteins as C-terminal His<sub>6</sub>-tag fusions, pPiolB-His and pPiolJ-His, respectively, were constructed as follows. DNA fragments covering the *iolB* or *iolI* coding regions, associated with their Shine-Dalgarno sequence and a short linker enabling the C-terminal His<sub>6</sub> tag fusion at the head and tail, respectively, were amplified from DNA of strain 60015 by PCR using the specific primers. The primers for the *iolB* PCR fragment were iolBE (CCTCGAATTCATAGAAAGAGGGGGTTTCTC, an EcoRI site is indicated in bold) and iolBH: (CCTCGAATTCATTCTTAATAATTTTCTAG, a Clal site is in bold), and those for the *iolI* fragment were iolIE (CCTCGAATTCATTCTTAGCCTGTTAATGGATAAAAGG, an EcoRI site is in bold) and iolH: (CCTCGAATTCATTCTTAGCCTGTTAATGGATAAAAGG, an EcoRI site is in bold). The *iolB* fragment was trimmed with EcoRI and
Phosphorylation triggered by the addition of 5-deoxy-glucuronic acid (5DG) was monitored as follows. The reaction substrate, 5DG, was prepared by the enzymatic conversion of 2KMI and purified as described below. JM109 cells carrying pUC18, pIOLB, pIOLC, or pIOLBC were grown with IPTG, and the protein extracts were prepared as described (13). B. subtilis cells were grown with and without inositol, and the extracts were prepared in the same way as described for the inositol dehydrogenase assay (1). Phosphorylation was monitored by the procedures modified from those reported previously (9). 0.9 ml of assay mixture was prepared to contain 50 mM Tris-Cl (pH 8.0), 2.5 mM MgCl₂, 1.25 mM phosphoenolpyruvate, 0.5 mM ATP, 3.0 mM KCl, 10 mM 2-mercaptoethanol, 10 μg/ml of pyruvate kinase, 5 μg/ml of lactate dehydrogenase, 0.25 mM NADH, and 100 μl of protein extract (~200–300 μg of protein). 0.1 ml of 20 mM 5DG or H₂O was added to the mixture, and the rate of decrease in the absorbance of NADH at 340 nm was measured.

IolJ aldolase activity was measured as follows. The substrate was the reaction product of the IolBC reaction as described below. IolJ-His was purified as indicated above and used for the enzyme assay. The IolJ reaction was supposed to produce DHAP, and the production of DHAP was monitored by the procedures modified from those reported previously (9). The purified IolJ-His protein was serially diluted in test tubes, each containing the reaction mixture (100 mM Tris-Cl (pH 7.6), 3.3 mM DKG, 1.43 mM NADH, and glycerol-1-phosphate dehydrogenase (0.1 unit/μl)), and incubated at 24 °C for 30 min. Each of the reaction mixtures was diluted 25 times immediately after the reaction to measure the absorbance of NADH at 340 nm.

The IolJ reaction was supposed to produce MSA besides DHAP, and MSA would be the substrate of IolA MSA dehydrogenase, which was shown to convert MSA to acetyl-CoA and CO₂ with the specific reduction of NAD⁺ to NADH (16). Thus, MSA was detected as follows by the procedures modified from those reported previously (9). The purified IolJ-His protein was serially diluted in test tubes, each containing the reaction mixture (100 mM Tris-Cl (pH 8.0), 3.3 mM DKG, IolA-His (5.7 μg), 3.0 mM CoA, and 3.0 mM NAD⁺), and incubated at 24 °C for 60 min. Each of the reaction mixtures was diluted 25 times immediately after the reaction to measure the absorbance of NADH at 340 nm.

Enzyme Assays—THcHDO hydrolase activity was measured as follows. The reaction substrate, THcHDO, was prepared by the enzymatic conversion of 2KMI and purified as described (13). JM109 cells carrying pUC18, pIOLB, pIOLC, or pIOLD were grown with IPTG, and the protein extracts were prepared as described (13). The hydrolase assay was performed by the procedures modified from those previously reported (3). 0.98 ml of assay mixture was prepared to contain 50 mM Tris-Cl (pH 8.0), 0.1 mM glutathione, 0.05 mM CoCl₂, and sufficient THcHDO (giving an absorbance of 0.6 at 260 nm; ~0.1 mm). After adding 20 μl of the protein extract containing ~60 μg of protein to the mixture, the rate of decrease in the absorbance at 260 nm was measured, and consumption of the substrate THcHDO was calculated from its estimated molar absorption coefficient of 6000 (3).
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and then centrifuged for 10 min at 5000 rpm. The supernatant solution was saved, concentrated, and dried. The dried solution was dissolved in 20 ml of methanol/ethyl acetate (4:1, v/v), and the solution was passed through a column filled with 12 ml silica gel (Merck) to remove the remaining substrate and intermediates. The eluent was concentrated once again and dried to obtain 119 mg of pale yellow compound. The $^1$H NMR spectrum of this compound was obtained in D$_2$O.

The IolBC reaction product was identified as follows. JM109 cells carrying pIOLBC (13) were grown in 100 ml of LB containing ampicillin and IPTG for 16 h, harvested, suspended in 2.2 ml of water, treated with lysozyme, disrupted by sonication, and centrifuged to obtain the supernatant solution containing both the IolB and IolC enzymes. The enzyme solution (~3 ml) and 222 mg of 5DG were mixed to make 13 ml of reaction mixture (pH 8.0, adjusted with NaOH) containing 90 mM ATP, 3 mM MgCl$_2$, and 3 mM KCl. The mixture was incubated at 26 °C for 16 h. The reaction was terminated by the addition of H$_2$SO$_4$ to lower the pH to 2–3. After being concentrated to 10 ml and mixed with 40 ml of methanol, the mixture was centrifuged to recover the supernatant solution. The solution was concentrated to 10 ml, neutralized with NaOH to adjust the pH to 7.0, and combined and mixed well with 2.5 ml of BaCl$_2$. The mixture was centrifuged to obtain the white sediment, which was suspended in 10 ml of water, treated with lysozyme, disrupted by sonication, and centrifuged to save the supernatant solution, which was concentrated and dried to obtain the product of the IolE reaction. In A. aerogenes, the third reaction

The substrate of the third reaction is THcHDO, which is the product of the IolE reaction. In A. aerogenes, the third reaction was demonstrated to be ring scission (hydrolysis) of the substrate to yield 2-deoxy-5-keto-D-gluconic acid (DKG, compound [7]) in Fig. 1) as the product (25). Assuming that the third reaction in B. subtilis could be similar to that in A. aerogenes, THcHDO hydrolyzing activity in the protein extracts of the E. coli cells expressing iolB, iolC, or iolD was measured (Table 2). The results clearly indicated that only the extract prepared from cells expressing iolD contained a large amount of the THcHDO hydrolyzing activity. From these results, we concluded that iolD encoded the B. subtilis THcHDO hydrolase. The ESI-TOF mass spectrum of the isolated product gave a major peak at m/z 177.0484 (data not shown). It was likely that this anion could correspond to the molecular ion of [C$_6$H$_{10}$O$_6$-32P]-, suggesting that it might be DKG. However, its $^1$H NMR spectra revealed that the product of the B. subtilis enzyme reaction was not DKG but 5DG (compound [6]) in Fig. 1). Actually, the product was an ~1:1 mixture of compounds [12] and [13], α- and β-anomers of the five-membered ring compounds (Fig. 2), indicating that these were produced after spontaneous formation of a hemiacetal linkage between the C1 aldehyde and the C4 hydroxyl groups of 5DG (compound [6]). Therefore, the IolD THcHDO hydrolase of B. subtilis could cleave the C2–C3 bond of THcHDO to yield 5DG (Fig. 1).

Kinase Activity Triggered by 5DG Addition Requires Both IolB and IolC—In A. aerogenes, the next step is phosphorylation of the product of the third reaction, DKG, and the reaction is mediated by a kinase requiring ATP (9). In B. subtilis, the product of the third step appeared to be 5DG, and we were not sure if such phosphorylation would occur on 5DG itself. Nevertheless, we tried to detect the possible kinase activity, which might be triggered by 5DG addition, in the protein extracts of the E. coli cells expressing iolB and/or iolC. In this detection system, the activity was judged spectrophotometrically as described (9) by the difference between the rates of decrease in

RESULTS

The Third Step Reaction of the MI Catabolic Pathway Is Catalyzed by IolD Hydrolyzing THcHDO to 5DG—Previous studies revealed that IolB, IolC, IolD, IolE, and IolG were all required for the initial reaction steps of MI catabolism to produce an intermediate acting as the inducer in the cell to antagonize DNA binding of IolR to induce transcription of the iol operon (13). Because IolG and IolE catalyze the first and second reactions, respectively (10, 13), either IolB, IolC, IolD, or combinations of these must be required for the third reaction.

The substrate of the third reaction is THcHDO, which is the product of the IolE reaction. In A. aerogenes, the third reaction was demonstrated to be ring scission (hydrolysis) of the substrate to yield 2-deoxy-5-keto-β-gluconic acid (DKG, compound [7]) in Fig. 1) as the product (25). Assuming that the third reaction in B. subtilis could be similar to that in A. aerogenes, THcHDO hydrolyzing activity in the protein extracts of the E. coli cells expressing iolB, iolC, or iolD was measured (Table 2). The results clearly indicated that only the extract prepared from cells expressing iolD contained a large amount of the THcHDO hydrolyzing activity. From these results, we concluded that iolD encoded the B. subtilis THcHDO hydrolase. The ESI-TOF mass spectrum of the isolated product gave a major peak at m/z 177.0484 (data not shown). It was likely that this anion could correspond to the molecular ion of [C$_6$H$_{10}$O$_6$-32P]-, suggesting that it might be DKG. However, its $^1$H NMR spectra revealed that the product of the B. subtilis enzyme reaction was not DKG but 5DG (compound [6]) in Fig. 1). Actually, the product was an ~1:1 mixture of compounds [12] and [13], α- and β-anomers of the five-membered ring compounds (Fig. 2), indicating that these were produced after spontaneous formation of a hemiacetal linkage between the C1 aldehyde and the C4 hydroxyl groups of 5DG (compound [6]). Therefore, the IolD THcHDO hydrolase of B. subtilis could cleave the C2–C3 bond of THcHDO to yield 5DG (Fig. 1).
FIGURE 1. MI catabolic pathway and functional activities of the *B. subtilis* *iol* genes. *B. subtilis* *iol* genes proven to encode the enzymes involved in the various reaction steps of the MI catabolic pathway are shown. Compounds: [1], MI; [2], DCI; [3], 2KMI; [4], 1-keto-DCI; [5], THcHDO; [6], 5DG; [7], DKG; [8], DKGP; [9], DHAP; [10], MSA; [11], acetyl-CoA. Carbon numbering is defined for MI, 2KMI, THcHDO, SDG, DKG, and DKGP; for the former three the definition from previous studies is applied (3, 25).
absorbance of NADH at 340 nm in the presence and absence of 5DG in the reaction mixture. The kinase reaction converts ATP to ADP, which will result in the oxidation of NADH through the coupling reactions involving pyruvate kinase and lactate dehydrogenase (9). Therefore, a higher kinase activity triggered by 5DG addition will result in a more pronounced decrease in absorbance at 340 nm. This was indeed observed. As shown (Table 3), every protein extract prepared from E. coli cells gave a decrease in the absorbance of NADH, and this did not depend on 5DG. However, only the extract of the cells with pIOLBC expressing both IolB and IolC exhibited an activity almost 2.5 times higher in the presence of 5DG than in its absence (Table 3). Extracts from B. subtilis cells were also prepared and subjected to the measurement of the kinase activity similarly as above. The activity found in the extracts of B. subtilis was lower than that in E. coli. When the cells were grown in the presence of MI, the wild-type strain 60015 was judged to contain the higher activity triggered by 5DG, whereas YF244, an iolR::cat mutant allowing constitutive expression of the iol operon, always exhibited the higher activities triggered by 5DG regardless of growth conditions. These results suggested that the kinase could be encoded within the iol operon and induced in the presence of MI (Table 3). On the other hand, none of the other three iolR::cat mutants, each of which had an additional defect either in iolB or iolC, exhibited an activity as high as YF244 did (Table 3), suggesting that the mutations in iolB and iolC might diminish and abolish the kinase activity, respectively. Taken together, we concluded that both IolB and IolC were required for the kinase activity triggered by 5DG addition.

The phosphorylated product of the kinase reaction involving both IolB and IolC was extracted as a barium salt form and found to give a very complicated ESI-TOF mass spectrum, probably due to its lower purity as well as differential ionization (data not shown). However, its 1H NMR spectra clearly revealed that the product was DKGP (compound [8]) in Figs. 1 and 3; 1H

**TABLE 2**

| Plasmid in JM109 | THcHDO hydrolyzing activity* | umol/min/mg of protein |
|-----------------|-----------------------------|------------------------|
| pUC18           |                             | 14.3 ± 14.2            |
| pIOLB           |                             | 20.2 ± 12.1            |
| pIOLC           |                             | 26.6 ± 11.4            |
| pIOLD           |                             | 253.0 ± 17.7           |

* THcHDO hydrolyzing activity in E. coli JM109 carrying the plasmids grown in TGA medium containing ampicillin; IPTG was determined. Mean values ± S.D. of three independent measurements are shown.

**FIGURE 2. Structure determination of the product of the IolD reaction.**

IolD reaction products turned out to be an equimolar mixture of compound [12] and [13] (with carbon and proton numbering). These compounds are α- and β-anomers of 5DG forming a five-membered ring made by a hemiacetal linkage between the C1 aldehyde and the C4 hydroxyl groups (Fig. 1). Summary tables of their 1H NMR spectrum analysis (right) and defined structural formulas (left) are given.

**FIGURE 3. Structure determination of the product of the IolBC reaction.**

The IolBC reaction product was identified as compound [8], DKGP (with carbon and proton numbering). A higher kinase activity triggered by 5DG addition resulted in a more pronounced decrease in absorbance at 340 nm. Only a set of representative data is shown, but at least three independent measurements were repeated with similar results.

The kinase activity triggered by 5DG addition was investigated in the protein extracts of the cells. Values shown are the rates of decrease in absorbance of NADH at 340 nm (ΔA340/min/mg of protein) in the presence of 5DG (+5DG) and in its absence (−5DG). A higher kinase activity triggered by 5DG addition resulted in a more pronounced decrease in absorbance at 340 nm. Only a set of representative data is shown, but at least three independent measurements were repeated with similar results.

**TABLE 3**

| Plasmid in JM109* | ΔA340/min/mg of protein |
|-------------------|-------------------------|
|                   | −5DG | +5DG |
| pUC18             | 1.40 | 0.99 |
| pIOLB             | 0.71 | 0.58 |
| pIOLC             | 0.91 | 1.41 |
| pIOLD             | 0.79 | 2.10 |

| Strain (relevant genotype*) | ΔA340/min/mg of protein |
|-----------------------------|-------------------------|
| 60015 (wild type)           | 0.12 | 0.11 |
| +MI                         | 0.18 | 0.37 |
| YF244 (iolR::cat)           | 0.23 | 0.88 |
| −MI                         | 0.27 | 0.82 |
| YF258 (iolR::cat iolB58)    | 0.15 | 0.31 |
| YF259 (iolR::cat iolB58), −MI | 0.12 | 0.31 |
| YF260 (iolR::cat iolC62), −MI | 0.14 | 0.13 |

* Kinase activity triggered by 5DG addition was investigated in the protein extracts of the cells. Values shown are the rates of decrease in absorbance of NADH at 340 nm (ΔA340/min/mg of protein) in the presence of 5DG (+5DG) and in its absence (−5DG). A higher kinase activity triggered by 5DG addition resulted in a more pronounced decrease in absorbance at 340 nm. Only a set of representative data is shown, but at least three independent measurements were repeated with similar results.

* E. coli JM109 cells carrying the indicated plasmid were grown in TGA medium containing ampicillin and 1 mM IPTG.

* B. subtilis cells were grown in S6 medium with (+) or without (−) 10 mM MI as indicated.
NMR summary shown in Fig. 3, which turned out to be the same compound as in *A. aerogenes* (9).

**IolB Isomerizes 5DG to DKG, and IolC Phosphorylates DKG to DKGP**—The kinase reaction involving both IolB and IolC produced DKGP from 5DG. On the other hand, a homology search revealed that IolB exhibited similarities to a number of 4-deoxy-L-threo-5-hexulose uronate isomerases, and its amino acid sequence contains the motif of 5-keto-4-deoxyuronate isomerase as suggested in the BSORF data base. These facts prompted us to test whether IolB might be an isomerase converting 5DG to DKG. IolB was produced in *E. coli* as a C-terminal His6 tag fusion protein and purified as IolB-His. The purified IolB-His was incubated with 5DG, and the reaction product was obtained. Although its ESI-TOF mass analysis was not performed, its 1H NMR spectrum was clear enough to indicate that it contained an equimolar mixture of 5DG and DKG (compound [7]) in Figs. 1 and 4; 1H NMR summary shown in Fig. 4).

These results allowed us to conclude that IolB encodes the isomerase responsible for the fourth-step reaction, thereby converting 5DG to DKG reversibly. The amino acid sequence of IolC exhibits significant similarity to fructo-kinases and in its amino acid sequence contains a carbohydrate kinase motif of the pfkB family as listed in the BSORF data base, suggesting that IolC could be a kinase phosphorylating the C6 hydroxyl group of DKG (Fig. 4). Thus, the conversion of 5DG to DKGP appeared to involve two successive reactions, namely the fourth reaction of the pathway catalyzed by IolB isomerizing 5DG to DKG and then the fifth reaction by IolC, thereby phosphorylating DKG to DKGP.

**The Product of the IolC Reaction, DKGP, Antagonizes IolR Binding to DNA**—IolG, IolE, IolD, IolB, and IolC appeared to catalyze the initial five reaction steps of the MI catabolic pathway in this order (Fig. 1), and previously all of these proteins were also shown to be required for the production of an intermediate acting as the inducer in cell (13). These findings suggested that the product of the IolC reaction might be the inducer that antagonizes DNA binding of IolR. It is known that the IolR repressor binds to DNA containing the iol promoter region, thereby altering the DNase I digestion pattern (11). Therefore, each of the intermediates appearing in the catabolic pathway was tested for its ability to interfere with DNA binding of IolR (Fig. 5). None of the compounds MI, 2KMI, THcHDO, the mixture of α- and β-anomers of 5DG, or DKG altered the
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DNase I digestion pattern upon IolR binding (11), whereas only DKGP rendered the digestion pattern almost the same as that without IolR binding. The results clearly indicated that the reaction product of the IolC reaction, DKGP, antagonized IolR for its DNA binding, revealing DKGP as the intermediate that functions as the inducer in the cell.

IolJ Is an Aldolase Acting on DKGP to Produce DHAP and MSA—In A. aerogenes it was shown that an aldolase converted DKGP into two C3 compounds, DHAP (compound [9]) in Fig. 1) and MSA (compound [10]) in Fig. 1). The amino acid sequence of IolJ shares high similarities with those of many aldolases (1). IolJ was produced in E. coli as a C-terminal His tag fusion protein and purified as IolJ-His. IolJ-His was incubated with DKGP to test its aldolase activity, employing the assay system previously described (9). The aldolase reaction would produce DHAP, which is known to serve as the specific substrate for glycerol-1-phosphate dehydrogenase involving the oxidation of NADH. Under the conditions we used, the more IolJ-His was contained in the reaction mixture, the more DHAP was produced (Fig. 6A), clearly indicating that IolJ-His had the aldolase activity producing DHAP from DKGP. When fructose bisphosphate was used as another substrate in the same system as above, no production of DHAP was observed (data not shown), suggesting that IolJ might not function as fructose-bisphosphate aldolase as annotated in databases.

The iolA gene product was previously characterized as MSA dehydrogenase (16), which acts on MSA to convert it into acetyl-CoA and CO₂ with reduction of NAD⁺ to NADH. Because MSA is very unstable and its authentic standard for NMR analysis is not available, production of MSA was also shown in the enzymatic way employing IolA. IolA was produced in E. coli and purified as a C-terminal His tag fusion protein, IolA-His. When the IolJ aldolase reaction was performed in the presence of IolA-His together with CoA and NAD⁺, the specific reduction of NAD⁺ to NADH was observed, suggesting that MSA produced by the aldolase reaction could serve as the substrate for the successive IolA reaction. The more IolJ-His was contained in the reaction mixture, the more MSA was produced (Fig. 6B), although the production was as low as 10% that of DHAP. Probably under the conditions we used, most of MSA was lost due to its instability and possible side reactions. Nevertheless the results clearly indicated that IolJ-His functioned to produce MSA from DKGP. We, thus, concluded that IolJ was the aldolase acting on DKGP, thereby converting it into DHAP and MSA. DHAP is a known glycolytic intermediate, and MSA is converted into acetyl-CoA and CO₂ with reduction of NAD⁺ by the MSA dehydrogenase of the iolA gene product.

DISCUSSION

The work described here together with previously published works revealed that MI catabolism of B. subtilis comprises seven stepwise reactions (Fig. 1). The first step is the dehydrogenation of MI to 2KMI, which is catalyzed by IolG, and the second step is the successive dehydration by IolE to give THcHDO. The third step is ring scission of THcHDO, catalyzed by IolD, to yield 5DG, and the fourth step is the isomerization of 5DG by IolB to produce DKGP. The fifth step is the phosphorylation of DKGP by IolC, yielding DKG, and the sixth step is the aldolase reaction by IolJ, cleaving DKG into DHAP and MSA. The last, seventh, step is catalyzed by IolA, thereby converting MSA to acetyl-CoA and CO₂. The net result of the inositol catabolic pathway in B. subtilis is the conversion of one MI molecule to yield one DHAP, one acetyl-CoA, and one CO₂ molecule, with acquisition of two NADH molecules and the consumption of an ATP molecule.

In A. aerogenes, the ring scission of THcHDO, was initially reported to give 4-deoxy-5-ketohexonic acid as the product (25). This was reinvestigated 5 years later, however, with the result that the product should be DKG (26). This suggests...
that the C2–C3 bond of THcHDO (compound [5]) in Fig. 1) was hydrolyzed upon the ring scission to yield DKG in a single step, taking into account the possible keto-enol tautomerization of THcHDO (26). In \textit{B. subtilis}, our results indicated that the product of the ring scission catalyzed by IolD was not DKG but 5DG (compound [6]) in Fig. 1). After its isolation, 5DG was found in an equimolar mixture of compounds [12] and [13] (Fig. 2), which were \( \alpha - \) and \( \beta - \) anomers produced after the spontaneous formation of a hemiacetal linkage between its C1 aldehyde and the C4 hydroxyl groups, suggesting that both in \textit{A. aerogenes} and \textit{B. subtilis} the C2–C3 bond of THcHDO was cut but the product was different. However, we showed that in \textit{B. subtilis} 5DG was successively isomerized by IolB enzyme to yield DKG, which was the same product identified in \textit{A. aerogenes} after the ring scission. Therefore, the conversion of THcHDO to DKG in \textit{B. subtilis} requires the successive hydrolysis and isomerization reactions corresponding to the third and fourth steps of the pathway catalyzed by IolD and IolB, respectively (Fig. 1). The previous studies on MI catabolism in \textit{A. aerogenes} were performed in a biochemical way mostly using crude extracts or partially purified enzymes prepared from mutant strains (3, 9, 25, 26). It is possible that both the active counterparts for IolD hydrolase and IolB isomerase were contained together in the crude extract and enzyme preparations, resulting in DKG as the major product so that the interme-

Two \textit{iol} genes, \textit{iolH} and \textit{iolS}, have remained uncharacterized. IolH is paralogous to both IolI and IolE. The \textit{iol} gene product was shown to be 2KMI/1-keto-DCI isomerase involved in DCI metabolism (14), and IolE is known as 2KMI dehydratase indispensable for MI catabolism (13). This suggests that IolH may also act on a certain ketose as its isomerase or dehydratase. It is unlikely, however, that IolH serves as another 2KMI dehydratase because the \textit{iolE}-defective mutant did not possess this enzyme activity at all and, thus, could not grow on inositol (13). Finally, IolS was reported to be homologous to pyridoxal reductase of \textit{Schizosaccharomyces pombe} (28). The corresponding gene was cloned and expressed in \textit{E. coli}, and this actually showed some pyridoxal reductase activity. This fact may imply a possible relationship between the MI and vitamin B6 metabolism, which would require further studies to be elucidated.

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