Bacillus subtilis IolQ (DegA) is a transcriptional repressor of iolX encoding NAD⁺-dependent scyllo-inositol dehydrogenase

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Abstract

Background: Bacillus subtilis is able to utilize at least three inositol stereoisomers as carbon sources, myo-, scyllo-, and D-chiro-inositol (MI, SI, and DCI, respectively). NAD⁺-dependent SI dehydrogenase responsible for SI catabolism is encoded by iolX. Even in the absence of functional iolX, the presence of SI or MI in the growth medium was found to induce the transcription of iolX through an unknown mechanism.

Results: Immediately upstream of iolX, there is an operon that encodes two genes, yisR and iolQ (formerly known as degA), each of which could encode a transcriptional regulator. Here we performed an inactivation analysis of yisR and iolQ and found that iolQ encodes a repressor of the iolX transcription. The coding sequence of iolQ was expressed in Escherichia coli and the gene product was purified as a His-tagged fusion protein, which bound to two sites within the iolX promoter region in vitro.

Conclusions: IolQ is a transcriptional repressor of iolX. Genetic evidences allowed us to speculate that SI and MI might possibly be the intracellular inducers, however they failed to antagonize DNA binding of IolQ in vitro experiments.

Keywords: Bacillus subtilis, scyllo-inositol, Inositol dehydrogenase, Transcription, Repressor

Background

Epimerization of the hydroxyl groups of cyclohexane 1,2,3,4,5,6-hexol (inositol) generates nine different stereoisomers. The most abundant form in nature is cis-1,2,3,5-trans-4,6-cyclohexanehexol (myo-inositol, MI) (Fig. 1), which is an essential component of phosphatidylinositol in the cell membranes of eukaryotes and exists as myo-inositol hexakisphosphate (phytic acid) in plant seeds [1]. Other inositol stereoisomers occur rarely in nature, although some exert specific and physiologically important effects. For example, D-chiro-inositol (DCI) (Fig. 1) and its 3-O-methyl derivative, D-pinitol, are beneficial for patients with hyperglycemia or polycystic ovary syndrome [2, 3], and scyllo-inositol (SI) (Fig. 1) directly interacts with beta-amyloid peptides to inhibit their aggregation in the brain and block the development of Alzheimer disease [4]. Bacillus subtilis efficiently utilizes inositol stereoisomers such as MI, DCI, and SI as carbon sources [5]. The iolABCDEFGHIJ operon encodes the enzymes that catabolize MI and DCI (Fig. 1). Two inositol transporters are encoded by iolF and iolT for MI and SI uptake [6, 7]. MI dehydrogenase, encoded by iolG, converts MI to scyllo-inosose (SIS) and reduces NAD⁺ in the first reaction of the catabolic pathway [8]. IolG reacts on both MI and DCI but not on SI [9]. The iol operon and iolT are regulated by the IolR transcriptional repressor, which is antagonized by the product of IolC kinase, 2-deoxy-5-keto-gluconic acid-6-phosphate [6, 10, 11]. On the other...
hand, the inositol dehydrogenases IolX and IolW are specific for SI and require NAD⁺ and NADP⁺, respectively [12]. Each enzyme converts SI to SIS, which is the same product generated from MI by IolG. Recently, IolU was found as the third SI dehydrogenase, which only can reduce SIS into SI in an NADPH-dependent manner [13].

Transcription of \textit{iolX} is induced by the addition of SI to the growth medium as the sole carbon source [12]. Transcription of \textit{iolW} is constitutive but it does not contribute to growth on SI, suggesting that IolX is essential for the catabolism of SI and that IolW is required for other reactions such as the generation of SI from SIS [5, 7].

The mechanism underlying the regulation of \textit{iolX} to degrade SI is unknown. Within the \textit{B. subtilis} genome, \textit{yisR} and \textit{iolQ} (formerly known by \textit{degA}) reside upstream of \textit{iolX} and are predicted to encode transcriptional regulators that belong to the AraC/XylS and LacI families, respectively (Fig. 1). Members of the AraC/XylS family include a positive regulator such as AdaA that induce the \textit{alkA} and \textit{ada} operons in \textit{B. subtilis} [14]. In contrast, most members of the LacI family are negative regulators, such as CcpB [15], KgR [16], ExuR [17], and LacR [18] in \textit{B. subtilis}. A transcriptome analysis revealed that \textit{yisR} and \textit{iolQ} were transcribed from a single operon [19]. The function of YisR is unknown and its regulatory function has never been studied. On the other hand, IolQ (DegA) was named after the discovery that the recombinant form produced in \textit{Escherichia coli} accelerated the degradation of glutamine phosphoribosyl pyrophosphate amidotransferase, implying that it might be a protease [20]. However, its sequence similarities to regulatory proteins CytR, Lql, GalR, and PurR of \textit{E. coli} and CcpA of \textit{B. subtilis} suggest that it could have stimulated the production of a protease [20]. In the present study, we therefore investigated the possible involvement of YisR and IolQ in the regulation of \textit{iolX}. We show that \textit{iolQ} encodes a transcriptional repressor that binds to the promoter region of \textit{iolX}.

**Methods**

**Bacterial strains, plasmid and growth conditions**

The bacterial strains and plasmids used in this study are listed in Table 1. \textit{B. subtilis} strain 168 is our standard strain for the study of inositol catabolism. The mutant strain BFS3018 was constructed from strain 168 and acquired from the National Bio Resource Project, National Institute of Genetics, Japan. BFS3018 has a pMUTIN4 (\textit{lacZ lacI amp erm}) [21] integration to disrupt \textit{iolX} which allows us to monitor \textit{iolX} expression in an \textit{iolX} mutated context by \(\beta\)-galactosidase activity [12]. The other \textit{B. subtilis} mutant strains were constructed as described below. \textit{E. coli} strains DH5α (Sambrook &
Russell, 2001) and BL21 (DE3) (Merck Millipore) served as hosts for plasmid construction and expression of C-terminal His6-tagged proteins, respectively. *E. coli* strains were maintained in lysogeny broth (LB) medium and *B. subtilis* strains were maintained using a tryptose blood agar base (Becton Dickinson) or S6 liquid medium [22] containing 0.5% casamino acid (Becton Dickinson) and 0.005% L-tryptophan. Plasmids pMD20 (Takara Bio) and pET30a (Merck Millipore) served as vectors for TA-cloning and His6-tag construction, respectively. Antibiotics used as required were as follows: erythromycin (0.5 μg ml⁻¹), ampicillin (50 μg ml⁻¹), and kanamycin (50 μg ml⁻¹). Media were supplemented with 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) or 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal) as required. All bacteria were cultured at 37 °C with rotary shaking at 150 rpm.

**Construction of *B. subtilis* mutants**

CM101 (ΔyisR) and CM102 (ΔiolQ) were constructed using the marker-free approach of Morimoto et al. [23].

### Table 1 Bacterial strains and plasmids

| Strain or plasmid | Description | Source or reference |
|-------------------|-------------|--------------------|
| **E. coli**       |             |                    |
| DH5α              | supE44 ΔlacU169 (880 lacZM15) hsdR17 recA1 gyrA96 thi-1 relA | [24] |
| BL21              | F− ampR hsdS8 (rM15) dcm gal (DE3) tonA | Merck Millipore |
| **B. subtilis**   |             |                    |
| 168               | trpC2       | Laboratory stock   |
| BFS3018           | trpC2 iolXpMUTIN4 | [12] |
| CM101             | trpC2 ΔyisR | This study         |
| CM102             | trpC2 ΔiolQ | This study         |
| **Plasmid**       |             |                    |
| pMD20             | TA-cloning vector, amp | Takara Bio |
| pET-30a           | pET system expression vector, kan | Merck Millipore |
| pET-iolQ          | pET-30 derivative to express iolQ-His6 | This study |
| pET-yisR          | pET-30 derivative to express YisR-His6 | This study |

**Fig. 2** Schematic strategy of the marker-free deletion. **a** Positional relationship among the target deletion and regions A, B, and C contained in the PCR fragments used for construction of the pop-in construct. **b** Recombinant PCR pop-in construct ligation the fragments A, B, and C, and the mazF cassette. **c** Integrant of the mazF cassette at the target region via a double crossover at regions A and C. An intrachromosomal crossover event between the directly repeated sequences corresponding to the region B resulted in elimination of the mazF cassette together with the target deletion. **d** Final structure of the marker-free deletion.
The pop-in construction was made by ligation of three different polymerase chain reaction (PCR) fragments amplified from the 168 genome (Fig. 2a) and another one comprising the \textit{mazF} cassette [23]. The fragments were i) the first PCR fragment for region A located upstream of the deletion target, ii) the second for region B located downstream of the target, iii) the third for region C located inside the target, iv) and the \textit{mazF} cassette constituted of \textit{mazF} for suicidal toxin under the control of IPTG-inducible promoter (\textit{Pspac}), \textit{lacI} for Lac repressor controlling \textit{Pspac}, and the spectinomycin resistance gene (\textit{spc}).

For the construction of CM101, the PCR fragments of regions A, B, C, and the \textit{mazF} cassette were amplified using the primer pairs DyisRAF/DyisRAR, DyisRBF/DyisRBR, DyisRCF/DyisRCR, and MazFfw/MazFbw, respectively (Table 2). For CM102, the PCR fragments of regions A, B, C, and the \textit{mazF} cassette were amplified using the primer pairs DdegAAF/DiolQAR, DiolQBF/DiolQBR, DiolQCF/DiolQCR, and MazFfw/MazFbw, respectively (Table 2). The pop-in construction containing the regions A, B, the \textit{mazF} cassette, and region C in that order (Fig. 2b) was used to transform the parental strain 168 of \textit{B. subtilis} for spectinomycin resistance via a double crossover in the homologous regions A and C, introducing the \textit{mazF} cassette into the targeted region (Fig. 2c). The spectinomycin-resistant transformants were then screened on IPTG-containing plates for the detection of spectinomycin sensitive mutants. In such mutants, an intrachromosomal crossover event between the two direct repeat stretches corresponding to region B occurred to eliminate the \textit{mazF} cassette and resulted in the marker-free deletion of the stretch between regions A and B (Fig. 2d). Correct construction of strains CM101 and CM102 was confirmed by sequencing (data not shown).

### Enzyme assay

NAD$^+$-dependent SI dehydrogenase activities in cell extracts were measured spectrophotometrically with an increase in absorbance at 340 nm with the generation of NADH as previously described [12]. β-Galactosidase activities in cell extracts were determined as previously described [25].

### RNA techniques

\textit{B. subtilis} strains were grown at 37 °C with shaking in S6 medium containing 0.5% casamino acid, 0.005% L-tryptophan (Becton Dickinson) with or without MI or SI (10 mM each), and 10 mM glucose was added as required. Total RNAs were extracted from the cells and purified as previously described [25].

The RNA samples were subjected to a Northern blot analysis using a DIG-labeled RNA probe specific for iolX. The RNA probe was prepared as follows: A DNA fragment corresponding to part of the iolX-coding

### Table 2 Oligonucleotide primers

| Primer | Sequence (5’→ 3’)* |
|--------|---------------------|
| [FAM]iolX(+50)-R† | TAACCGAGGCTTCCTCATAATCC |
| [FAM]iolX(−250)-F† | GAGCGTCTGAGCAGCATCATCT |
| DioLQAF | TGCTCAACAGGGAAAGGTATT |
| DioLQAR | CGTCTATTAGGCGGCCATTCGTCGTTATT |
| DioLQBF | GCCGCCGTAATGAGGG |
| DioLQBR | CTAATGGAAGAGATGCGAAGACAG |
| DioLQCF | GCTGAGTCAATTCCGGTGATGAGCTCGGTGTTTTCAATAG |
| DioLQCR | CCCATCTCTTTATCGGCTG |
| ioiQ1 BamHI-R | CGCGGATCCCGGAATCCTGCAATACC |
| ioiQ1 EcoRI-R | GGAATTCTAACCAGACGAGGGATGAAG |
| ioiQ1 NdeI-R | GGGGAATTCATGACGATGTTTTTCAGAAG |
| ioiQ1 XhoI-R | CCGCCTCGATCATGTGTTGAGCGGTGATG |
| DyisRAF | TTGACAATCACAATCATCGC |
| DyisRBR | CAGCCGGAAAGTTCAATAAC |
| DioLQCF | GCTGAGTCAATTCCGGTGATGAGCTCGGTGTTTTCAATAG |
| DioLQCR | CCCATCTCTTTATCGGCTG |
| ioiQ1 BamHI-R | CGCGGATCCCGGAATCCTGCAATACC |
| ioiQ1 EcoRI-F | GGAATTCTAACCAGACGAGGGATGAAG |
| ioiQ1 NdeI-R | GGGGAATTCATGACGATGTTTTTCAGAAG |
| ioiQ1 XhoI-R | CCGCCTCGATCATGTGTTGAGCGGTGATG |
| infoX (+50)-R | TAACCGAGGCTTCCTCATAATCC |
| infoX (−1)-R | TTGAATCATCCTCCTTTTTAAGT |
| infoX (−200)-F | CTAATGGAAGAGATGCGAAGACAG |
| MazFbw | GGATGCTTGAACGACATCT |
| MazFbw | GGATGCTTGAACGACATCT |
| GM5A-Nega-F | TTTTCCGGCAGCCGTCT |
| GM5A-Nega-R | TCTAGCAGTCTGGAATAATCC |
| infoX (+50)-R | TAACCGAGGCTTCCTCATAATCC |
| infoX (−1)-R | TTGAATCATCCTCCTTTTTAAGT |
| infoX (−200)-F | CTAATGGAAGAGATGCGAAGACAG |
| MazFbw | GGATGCTTGAACGACATCT |
| MazFbw | GGATGCTTGAACGACATCT |

*Restriction enzyme recognition sites and T7 RNA polymerase promoter-tag sequence are underlined and italicized, respectively
†These primers were 5′-6-([FAM])-labeled
region was PCR-amplified using strain 168 DNA as a template and the primers Io1QXhol-F and Io1QXhol-R (Table 2) to introduce a T7 RNA polymerase promoter sequence at their 3'-termini. The PCR product was used as the template for in vitro transcription using a DIG RNA labeling kit (SP6/T7) (Roche Diagnostics, Basel, Switzerland) to produce the DIG-labeled RNA probe. Cellular RNAs were separated using gel electrophoresis, transferred to a positively charged nylon membrane (Roche Diagnostics), and hybridized using the DIG-labeled probe according to the manufacturer’s instructions. Hybrids were detected using a DIG luminescence detection kit (Roche Diagnostics).

Primer extension was performed to identify the transcriptional start site of the io1X transcript [8]. Reverse transcription initiated from the PiolX400-R primer (Table 2) was labeled at the 5'-terminus using a Megalabel kit (Takara Bio) and [γ-32P]ATP (PerkinElmer). DNA from strain 168 was used as the template for the dideoxy sequencing reactions, which initiated from the same end-labeled primer used for ladder preparation, was prepared by PCR using the primers PiolX400-F/PiolX400-R (Table 2).

Plasmid construction
DNA fragments corresponding to the coding regions of io1Q and yisR were amplified from B. subtilis 168 genomic DNA by PCR using the respective primers io1QNdel-F/io1QXhol-F and yisRNdel-F/yisRXhol-R with generation of Ndel and Xhol sites at the 5'- and 3'-termini of each amplicon, respectively (Table 2). Each PCR product was ligated to the arms of pMD20 (Takara Bio) using a Mighty TA-cloning kit (Takara Bio) and was used to transform E. coli DH5α, which was then cultured on LB plates containing ampicillin, IPTG, and X-gal. White colonies were selected and plasmid DNAs were subjected to a sequence analysis using an ABI PRISM 3100 Genetic Analyzer (Thermo Fisher Scientific). The recombinant plasmids with the correct sequences were digested using Ndel and Xhol, and the restriction fragments were ligated to the arms of Ndel/Xhol-cleaved pET-30a to generate pET-io1Q or pET-yisR, which were used to transform E. coli BL21 (DE3) to produce C-terminal His6-tagged proteins Io1Q-His6 and YisR-His6, respectively.

Protein production and purification
E. coli BL21 (DE3) transformed with pET-io1Q or pET-yisR was inoculated into LB medium containing kanamycin and cultured at 37 °C with shaking. The recombinant proteins were induced using 1 mM IPTG when the optical density of the culture reached OD600 = 0.35, and the culture was further incubated for 2 h at 37 °C with shaking; the cells were harvested and disrupted by sonication. Io1Q-His6 and YisR-His6 were purified from cell lysates using a TALON metal-affinity resin (Takara Bio) according to the manufacturer’s instructions.

Gel mobility shift assay
Gel mobility shift assays were performed according to a previous study [26]. DNA fragments of the 200-bp sequences of the io1X and yisR-io1Q promoter regions were PCR-amplified using the specific primers io1X (−200)-F/io1X (−1)-R and yisR (−200)-F/yisR (−1)-R, respectively (Table 2). A negative control of a 100 bp fragment representing a segment of the io1W coding region was amplified using the primers GMSA-Nega-F/GMSA-Nega-R (Table 2). Each DNA fragment (0.155 pmol) was incubated in 0.02 ml of binding buffer [10 mM Tris-HCl (pH 8.0), 1 mM DTT, 10 mM KCl, 5 mM MgCl2, 10% glycerol, 5 μg ml−1 poly d(I-C), and 50 μg ml−1 bovine serum albumin] at 37 °C for 30 min with varying amounts of Io1Q-His6 or YisR-His6. DNA protein complexes were separated using nondenaturing polyacrylamide gels in TAE buffer. The DNA fragments in the gel were stained using SYBR Green for 30 min and the bands were visualized using Chemi Doc XRS+ with Image Lab software (Bio-Rad).

DNase I footprint assay
PCR reactions were used to amplify 5’-6-[FAM]-labeled DNA fragments containing the io1X promoter region (300 bp) from the DNA of strain 168 using the specific primers [FAM]io1X(−250)-F/io1X (+50)-R and io1X (−250)-F/[FAM]io1X(+50)-R for labeling the sense and antisense strands, respectively (Table 2). Each differentially 5’-6-[FAM]-labeled DNA fragment (0.45 pmol) was incubated in 0.2 ml of binding buffer with varying amounts of Io1Q-His6 at 37 °C for 30 min. 0.75 units of DNase I (Takara Bio) was added to digest the DNA for 5 min, and the reaction was stopped by adding 0.2 ml of 0.5 M EDTA. DNAs were extracted using a PCR purification kit (Promega). DNA sequencing of the sense and antisense strands employed the primers io1X (−250)-F and io1X (+50)-R, respectively, using the Thermo Sequenase Dye Primer Manual Cycle Sequence Kit (USB). The DNA samples were analyzed by Sigma-Aldrich using an ABI 3130xl Genetic Analyzer and ABI Gene Mapper Software Ver. 4.0 (Thermo Fisher Scientific).

Results
SI and MI induce the transcription of io1X
As shown in Fig. 3a, in the standard strain 168, NAD+-dependent SI dehydrogenase activity was induced in the presence of SI up to 40-fold more than its absence, while it completely disappeared in strain BSF3018 with the inactivation of io1X through pMU-TIN4 integration (Fig. 3b). It was previously reported that BSF3018 did not grow when depending on SI as the sole carbon source [12]. In B. subtilis, there are at least two NADP+-dependent SI dehydrogenases, Io1W and Io1U, however neither of them functions to
dehydrogenate SI to degrade it as the carbon source [12, 13]. Therefore, SI induced iolX to produce NAD^+-dependent SI dehydrogenase that was responsible for the physiological utilization of SI in *B. subtilis*. Although iolX does not play a role in the MI catabolism [12], MI was also able to induce NAD^+-dependent SI dehydrogenase activity up to 20-fold more than in its absence, indicating that MI also could induce iolX (Fig. 3a).

On the other hand, in strain BFS3018, iolX was inactivated but its transcription was monitored by the expression of lacZ for β-galactosidase activity instead (Fig. 3b). As shown in Fig. 3c, in the presence of SI and MI, β-galactosidase activity was induced up to 50- and 10-fold more than in their absence, respectively, indicating that both SI and MI are able to induce iolX at the transcription level without functional iolX. As shown in Fig. 1, SI and MI are degraded to produce the same set of intermediates [11, 12], and we can consider that none of them could be made from SI when iolX was inactivated, as BSF3018 did not grow when depending on SI as the sole carbon source [12]. Consequently, it is unlikely that any of the intermediates were involved in the transcriptional induction of iolX.

We previously reported that not only MI but also SI was mainly imported by the IolIT transporter [7]. As the expression of iolT is controlled by IolR [6], it
is thus induced when MI or SI is degraded down to the product of the IolC reaction (Fig. 1), 2-deoxy-5-keto-gluconic acid-6-phosphate, which antagonizes DNA binding of IolR [11]. Since SI can never be converted into the IolC-reaction product in BFS3018 due to the inactivation of iolX, the results suggest that SI uptake supported by the basal expression of iolT could be enough to allow induction of iolX. On the other hand, in BFS3018, MI is degraded involving IolG, thus allowing the induction of iolT. Therefore, the induction of β-galactosidase activity of BFS3018 in response to MI could be achieved due to the elevated levels of MI uptake. Nevertheless, the activity was still less than that produced in response to SI.

As shown in Fig. 4, the Northern blot analysis confirmed that the transcription of iolX in strain 168 was induced in the presence of SI or MI. The induction of NAD⁺-dependent SI dehydrogenase activity in strain 168 in the presence of SI or MI was abolished by additional glucose, suggesting that iolX could be under catabolite repression (Fig. 3a). In addition, the induction of β-galactosidase activity of BFS3018 in response to SI and MI was also abolished by additional glucose. These results indicated that the induction and catabolite repression of iolX occurred at the transcription level (Fig. 3c).

Expression of iolQ is required to regulate iolX transcription in response to SI

Immediately upstream of iolX, there is an operon that encodes two genes, yisR and iolQ [19], each of which could encode a transcriptional regulator; yisR and iolQ were predicted to encode transcriptional regulators that belong to the AraC/XylS and LacI families, respectively (Fig. 1). To determine whether YisR and IolQ regulate iolX, we generated the mutant strains CM101 and CM102 (Fig. 3a). In CM101 (ΔyisR), yisR was deleted to avoid the polar effect on iolQ downstream of it, while in CM102 (ΔiolQ), iolQ was alternatively deleted. Therefore, only iolQ was expressed under the control of the original yisR-iolQ promoter in CM101 whereas only yisR was expressed in CM102.

In CM101 (ΔyisR), the NAD⁺-dependent SI dehydrogenase activity of IolX was repressed in the absence of SI or MI and induced in their presence, while in CM102 (ΔiolQ) it became constitutive to be almost 50-fold higher than that in strain 168 in the absence of SI or MI (Fig. 3a). The activities in CM101 and CM102 in the presence of SI and MI seemed higher than those in strain 168 by unknown reasons. On the other hand, the activities in both CM101 and CM102 were repressed in the presence of glucose. These results suggest that induction of iolX could be regulated by IolQ but not by YisR. In addition, neither IolQ nor YisR could be involved in the catabolite repression of iolX.

The Northern blot analyses revealed that, in CM102 without functional iolQ, iolX was transcribed in the absence of SI and MI (Fig. 4). However, the transcription was shut off in CM101 (ΔyisR) when SI and MI were absent, and it was obviously induced in response to SI and MI. These results indicate that the transcriptional regulation of iolX in response to SI and MI depended on iolQ but not on yisR.

IolQ binds to the iolX promoter region

IolQ-His₆ and YisR-His₆ (Fig. 5) were tested for their binding to DNA fragments containing either promoter region of the iolX or yisR-iolQ operon. Gel mobility shift assays revealed that IolQ-His₆ formed complexes with the DNA fragment of the iolX promoter region (Fig. 5). The IolQ-DNA complexes formed distinct two bands, the lower and the higher molecular weight bands. As the
concentrations of IolQ-His6 were elevated, the former appeared first at the lower concentrations, which shifted to form the latter exclusively as the concentrations increased further (Fig. 5). The results indicate that the iolX promoter fragment may contain at least two IolQ-binding sites with different affinities (Fig. 5); the lower molecular weight band could correspond to the IolQ-DNA complex formed by IolQ binding only to a higher affinity site while the higher molecular weight one was formed by its binding to both higher and lower affinity sites. Neither SI nor MI (at higher concentrations up to 20 mM) affected the specific DNA binding of IolQ-His6 in vitro (data not shown). In addition, another set of gel mobility shift experiments involving not only IolQ-His6 but also YisR-His6 was conducted. Nevertheless, neither SI, MI, nor SIS caused any effect on DNA binding of IolQ-His6 in the additional presence of YisR-His6 (data not shown).

On the other hand, IolQ did not interact with the yisR-iolQ promoter region, and we failed to detect YisR-His6 binding to either fragment of the iolX or yisR-iolQ promoter region in the presence and absence of YisR-His6 (data not shown).

Identification of the two IolQ-binding sites within the iolX promoter region

The primer extension experiment (Fig. 6) determined two transcriptional start sites downstream of the promoters P1 and P2 for the iolX transcript. Only a small amount of the reverse transcript corresponding to promoter P1 was detected in the absence of SI, but it was significantly induced in response to SI together with the additional transcript corresponding to P2. Their respective −35 and −10 regions were deduced to serve as the iolX promoters P1 and P2 (Fig. 7). Another reverse transcript was found to be as strong as the one corresponding to promoter P1 but was shorter by 6 bp. This was considered to be due to a truncated product derived from the P1 transcript, since there are no consensus −35 and −10 sequences corresponding to this 5′ end.

IolQ-binding sites within the iolX promoter region identified using a DNase I footprint analysis revealed that IolQ bound with different affinities to the two regions (Fig. 7). The stretches with sequences TCTT TTGAGAAACGCTTTGCGCAAAAT (spanning +4 to +30 bp, position numbers assigned relative to the transcription start site of the promoter P2) and AGA-GAAACGCTTTCTCAAG (spanning +68 to +88 bp) were protected from DNase I at lower and higher concentrations of IolQ, respectively (Fig. 7). Therefore, the former and the latter stretches were judged as the higher and lower affinity regions, respectively (Fig. 7). In addition, we could also predict another plausible cre site within the higher affinity region.

Discussion

B. subtilis strains possess at least three types of SI dehydrogenases encoded by iolX, iolW [12], and iolU [13].
IolX requires NAD\(^+\) and both IolW and IolU need NADP\(^+\) as a cofactor. It is known that iolx plays an indispensible role in the utilization of SI as a carbon source for growth [12], and we showed here that iolx was induced more than 40-fold in the presence of SI (Figs. 3 and 4). The transcription of iolw is constitutive, and IolW can convert SI into SIS in vitro but does not contribute to growth depending on the availability of SI as the carbon source [12]. IolU is also produced constitutively and generally at low levels [19] and was not able to dehydrogenate SI but only reduce SIS into SI with oxidation of NADPH [5]. How- ever, we failed to identify any good candidates. Although IolW is constitutively produced, it only inefficiently converts SI into SIS with the predominating reverse reaction [12]. We previously demonstrated that MI was converted into SI through the coupling reactions involving IolG and IolW; the former dehydrogenates MI into SIS with a reduction of NAD\(^+\), and the latter reduces SIS into SI with oxidation of NADPH [5]. However, the conversion was detected only when the intermediate SIS was accumulated by the additional inactivation of iolE, which encodes SIS dehydratase acting on SIS for further degradation of this intermediate (Fig. 1) [5]. Another NADP\(^+\)-dependent SI dehydrogenase encoded by ioll was recently identified [13]. Although this enzyme is not as active as IolW, it is able to convert SIS into SI but only when overexpressed. Therefore, IolU is unlikely to be involved in the possible conversion of MI into SI. All of these observations led us to speculate that mainly SI and secondarily MI could be the intracellular inducers interacting with IolQ to antagonize its DNA binding, allowing the induction of ioIx, however they failed to antagonize DNA binding of IolQ-His\(_6\) in vitro. The C-terminal His-tag fusion might affect effector binding.

We showed here that IolQ bound with different affinities to the two sites within the ioIx promoter region. The high affinity site was located from positions +4 to +30 of the promoter P2 within the sequence TCTTTTGTGAGAAAGCGCTTGCGCAAAAT, and the low affinity site was located from +68 to +88 within the sequence GAAAGCGCTTGCGCAAAAT, and the low affinity site was located from +68 to +88 within the sequence
AGAGAAACGCTTTCTCAAAG (Fig. 7). Most members of the LacI family preferentially require a palindromic sequence within their DNA binding sites [28]. A comparison between the sequences of the two IolQ binding sites identified the relatively conserved sequence AGAAAR CGCTTKCK, which may suggest the potential perfect palindrome could be AGAAAGCGCTTTCT. However, this perfect palindrome is not present in either of the two binding sites that differ in two and one positions in the higher and lower affinity binding sites, respectively. Therefore, the consensus palindrome is not the only determinant of IolQ binding, although the sequences extending from the conserved stretch may contribute to high affinity binding of IolQ to its target sequence. Within the B. subtilis genome, there are 22 sites with a sequence similar to the conserved consensus sequence (maximum of two different positions, data not shown). At least seven of the 22 sites are located close to promoter regions, including the one of the iolX promoter. Thus, IolQ may regulate six additional promoters and therefore drive the transcription of at least the following genes (products): glpT (glycerol-3-phosphate permease), ycsA (putative enzyme similar to 3-isopropylmalate dehydrogenase), acor (transcriptional activator of acetoin utilization genes), yrbE (another member of the Gfo/Idh/MocA family paralogs including iolG, iolU, iolW, and iolX) [13], menA (1,4-dihydroxy-2-naphthoate octaprenyltransferase), and bglS (endo-β-1,3–1,4 glucanase). Our future course will focus on determining the
mechanisms of transcriptional regulation of these genes and their involvement in SI metabolism.

Expression of iolX for NAD⁺-dependent SI dehydrogenase activity in strain 168 as well as the β-galactosidase activity in strain BFS3018 was almost completely repressed in response to glucose even in the presence of SI and MI, indicating that iolX is under catabolite repression (Fig. 3a and c). The plausible cre site predicted as overlapping the lower affinity region for IolQ binding (Fig. 7) might be involved in catabolite repression. We noticed that part of the conserved sequence AGAAARCGCTTCKCK for IolQ binding was quite similar to the one WNGAANCGNTTNCW for CcpA/P-Ser-HPr biding [31]. In addition, the sequence AGAAAGCGCTTGCGC within the higher affinity site for IolQ binding was also similar to the cre site consensus (Fig. 7). Both or either of the two IolQ-binding sites might also function as the binding site of CcpA/P-Ser-HPr in the presence of glucose. Since iolX functions for the catabolism of SI as a minor alternative carbon source, it makes sense that this gene is regulated by global catabolite repression involving CcpA/P-Ser-HPr [31].

Conclusion

In *B. subtilis*, both SI and MI induce iolX expression for NAD⁺-dependent SI dehydrogenase activity. The iolX expression became constitutive in an iolQ background, and IolQ binds to two sites upstream of iolX where two transcription start sites were located. Genetic evidences allowed us to speculate that SI and MI might possibly be the intracellular inducers; however they failed to antagonize DNA binding of IolQ in in vitro experiments.

Abbreviations

DCI: D-chiro-inositol; IPTG: isopropyl β-D-1-thiogalactopyranoside; LB: lysogeny broth; MI: myo-inositol; oNp: o-nitrophenol; PCR: polymerase chain reaction; Pspc: spc promoter; SI: scyllo-inositol; SIS: scyllo-inosose; X-gal: 5-bromo-4-chloro-3-indolyl-β-D-galactoside.

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Availability of data and materials

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors’ contributions

DMK and TM conducted most of the experiments and analyzed the results under the supervision of KT and ST. CM conducted experiments with the mutant strains of *B. subtilis*. KY conceived the idea for the project and wrote the final manuscript with SI. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

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Competing interests

The authors declare that they have no competing interests.

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