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Identification of a repressor for the two *iol* operons required for inositol catabolism in *Geobacillus kaustophilus*

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Abstract

Geobacillus kaustophilus HTA426, a thermophilic Gram-positive bacterium, feeds on inositol as its sole carbon source, and an *iol* gene cluster required for inositol catabolism has been postulated with reference to the *iol* genes in *Bacillus subtilis*. The *iol* gene cluster of *G. kaustophilus* comprises two tandem operons induced in the presence of inositol; however, the mechanism underlying this induction remains unclear. *B. subtilis iolQ* is known to be involved in the regulation of *iolX* encoding *scyllo*-inositol dehydrogenase, and its homologue in HTA426 was found two genes upstream of the first gene (*gk1899*) of the *iol* gene cluster and was termed *iolQ* in *G. kaustophilus*. When *iolQ* was inactivated in *G. kaustophilus*, not only cellular *myo*-inositol dehydrogenase activity due to *gk1899* expression but also the transcription of the two *iol* operons became constitutive. IolQ was produced and purified as a C-terminal histidine (His)-tagged fusion protein in *Escherichia coli* and subjected to an *in vitro* gel electrophoresis mobility shift assay to examine its DNA-binding property. It was observed that IolQ bound to the DNA fragments containing each of the two *iol* promoter regions and that DNA binding was antagonized by *myo*-inositol. Moreover, DNase I footprinting analyses identified two tandem binding sites of IolQ within each of the *iol* promoter regions. By comparing the sequences of the binding sites, a consensus sequence for IolQ binding was deduced to form a palindrome of 5′-RGWAAGCGCTTSCY-3′ (where R=A or G, W=A or T, S=G or C, and Y=C or T). IolQ functions as a transcriptional repressor regulating the induction of the two *iol* operons responding to *myo*-inositol.

INTRODUCTION

There has been extensive research on the biochemical pathway and the regulation of inositol catabolism in *Bacillus subtilis*, representing Gram-positive bacteria. *B. subtilis* possesses a complete set of *iol* genes required for its inositol catabolism, including the *iolABCDEFGHIJ* operon, *iolRS* operon, *iolQ*, *iolT*, *iolU*, *iolW* and *iolX* [1–6].

The *iolABCDEFGHIJ* operon encodes the enzymes responsible for the primary pathway of inositol catabolism [7, 8], whereas *iolT* encodes the major inositol transporter [4]. A repressor encoded by *iolR* is the major transcription factor belonging to the DeoR family, and it regulates transcription of the *iolABCDEFGHIJ* operon and *iolT* [2, 4]. In the absence of inositol in the culture medium, the IolR repressor binds to the respective promoter regions to arrest the initiation of transcription. However, in the presence of inositol, one of the metabolic intermediates appearing in the catabolic pathway, 2-deoxy-D-glucuronic acid 6-phosphate produced in the IolC reaction acts as an inducer *in vivo* to antagonize the repressor function of IolR, leading to the transcriptional induction of the *iolABCDEFGHIJ* operon and *iolT* [1, 2, 4]. The process of regulation of *iol* genes by IolR orthologues is conserved in a number of bacterial species including not only Gram-positive but also Gram-negative bacteria. For instance, the alphaproteobacterium *Sinorhizobium meliloti* was shown to possess the *iol* genes regulated by its IolR orthologue [9], and in the gammaproteobacterium *Salmonella enterica*, its IolR orthologue was found to regulate not only the transcription of *iol* genes [10] but also an orphan response regulator encoded by *reiD* involved in *myo*-inositol utilization [11].

On the other hand, a recent study demonstrated that in *B. subtilis*, an additional minor repressor encoded by *iolQ* that belongs to the LacI family regulates *iolX*, which encodes an NAD^+-dependent *scyllo*-inositol dehydrogenase [3]; however, the mechanisms underlying the regulation of *iolU* and *iolW*,

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Abbreviations: DIG, digoxigenin; 6-FAM, 6-carboxyfluorescein; 5-FOA, 5-fluoroorotic acid; 5′-RACE, 5′-rapid amplification of cDNA ends.

Two supplementary figures are available with the online version of this article.

both of which encode an $NADP^+$-dependent *scyllo*-inositol dehydrogenase [5, 6], have not been elucidated because they appeared to be almost constitutive. Genetic evidence suggests that *scyllo*-inositol and *myo*-inositol are intracellular inducers interacting with IolQ; however, both failed to antagonize its DNA-binding activity *in vitro* [3].

Geobacillus kaustophilus HTA426 is a Gram-positive, thermophilic, facultatively anaerobic bacterium isolated from deep-sea sediment collected from the Mariana Trench in the western Pacific Ocean [12, 13]. It can grow at temperatures from 48 to 74 °C (optimal temperature: 60 °C) [13]. Its entire genome has been sequenced [13], and some methods for its genetic manipulation have also been established [14–16]. Interestingly, this bacterium was found to possess a gene cluster with a composition similar to the complete set of *iol* genes in *B. subtilis* [17]. In fact, it has been demonstrated that *G. kaustophilus* metabolizes at least three inositol stereoisomers: *myo*-inositol, *scyllo*-inositol and D-*chiro*-inositol [17]. In the *G. kaustophilus* genome, the *iol* gene cluster is separated into two operons, both of which are induced in parallel in the presence of inositol: (1) a 5 kb long operon containing four genes (*gk1896–1899*) and (2) a 12 kb long operon containing 10 genes (*gk1885–1894*) [17] (Fig. 1c). However, to the best of our knowledge, to date, no experimental study has elucidated how their induction is regulated.

The best candidate for an *iolR* orthologue in *G. kaustophilus* was found to be *gk1840* belonging to the DeoR family and annotated as a fructose operon transcriptional repressor [13]. However, it shares only low homology with *iolR* [28% identity; Bit-score=102 bits (253); *E*-value=1e-26]. Therefore, in the present study, we focused on *gk1901* of *G. kaustophilus* that is homologous to the *iolQ* gene of *B. subtilis*, which is presumed to be a repressor belonging to the LacI family and located close upstream of the *iol* cluster. We explored the function of *gk1901* showing that it encodes a repressor, which binds to the two promoter regions within the *iol* cluster and is responsible for their transcriptional induction in the presence of *myo*-inositol.

RESULTS

Inactivation of *iolQ* (*gk1901*) rendered *myo*-inositol dehydrogenase constitutive

A recent study showed that *B. subtilis iolX* is regulated by a repressor encoded by *iolQ* [3]. A conventional homology search using BLASTP [18] indicated that the best candidate for an *iolQ* counterpart in *G. kaustophilus* was *gk1901*, which encodes a transcriptional regulator of the LacI family [13] and shares high homology with *B. subtilis iolQ* [51% identity; Bit-score=353 bits (905); *E*-value=2e-122]. Furthermore, *gk1901* is located close (only two genes upstream) to the first gene of the *iol* gene cluster (*gk1899*) (Fig. 1c). The gene *gk1900* lies between *gk1899* and *gk1901*, which is transcribed oppositely to the *iol* genes and *gk1901*, predicted to encode a transposase, and which is unlikely to be involved in transcriptional regulation [13]. Therefore, we focused on *gk1901* as the possible candidate for the regulator of *iol* genes in *G. kaustophilus*. We created a mutant strain YS202 from the parental strain MK72 (a derivative of HTA426 lacking both functional *pyrF* and *pyrR*, which had been constructed for the counter selection system [15]), in which almost the entire coding region of *gk1901* was eliminated by an in-frame internal deletion to leave the first two codons connected to the last two (Table 1).

It is known that NAD^+-dependent *myo*-inositol dehydrogenase is encoded by *gk1899*, which is the first gene of the *iol* cluster and transcribed from a promoter located upstream to be induced in the presence of inositol in the growth medium [17]. As shown in Table 2, the activity of NAD^+-dependent *myo*-inositol dehydrogenase in MK72 was repressed in the absence of *myo*-inositol but induced in its presence. In contrast, its activity became completely constitutive in YS202 lacking *gk1901*. These results suggested that *gk1901* is involved in the induction mechanism of *iol* genes including *gk1899*. Consequently, we renamed *gk1901* as *iolQ* of *G. kaustophilus* hereafter.

On the other hand, the enzyme activities that were induced in MK72 and were constitutive in YS202 were not repressed in the presence of additional glucose in the medium (Table 2); this finding suggested that the *iol* genes in *G. kaustophilus* might not be under catabolite repression.

iolQ encodes a regulator involved in the transcriptional induction of the two *iol* operons of *G. kaustophilus*

The constitutive activity of NAD^+-dependent *myo*-inositol dehydrogenase in YS202 lacking *iolQ* suggested that transcription of the *iol* genes in *G. kaustophilus* is regulated by *iolQ*. To validate this hypothesis, YS202 and its parental strain were grown in the presence or absence of *myo*-inositol, and their total RNAs were extracted and subjected to northern blot analyses (Fig. 1a, b). Short sequences (about 400 bp long) internal to the coding regions of *gk1899* and *gk1894* were used as probes (Fig. 1c) to detect the transcripts of 5 and 12 kb operons, respectively [17]. In MK72, the specific transcripts of the two operons were detected only in the presence of *myo*-inositol, as previously found in HTA426 [17], whereas in YS202, both were completely constitutive irrespective of the presence or absence of *myo*-inositol (Fig. 1). Our results clearly indicated that *iolQ* was involved in the transcriptional regulation, particularly in induction responding to *myo*-inositol, of the two *iol* operons.

IolQ bound to DNA fragments representing two promoter regions within the *iol* gene cluster

Northern blot analyses showed that *iolQ* was involved in the repression of the two operons of *iol* genes in the absence of *myo*-inositol. Therefore, we examined whether IolQ can bind to the promoter regions of the two *iol* operons. For this, *iolQ* was cloned into pET30a(+) to be expressed as a C-terminal His-tagged fusion protein (IolQ-his) and purified in *Escherichia coli* BL21(DE3). The production and purification of IolQ-his (approximately 35 kDa) were confirmed (Fig. S1, available in the online version of this article), after which IolQ-his was

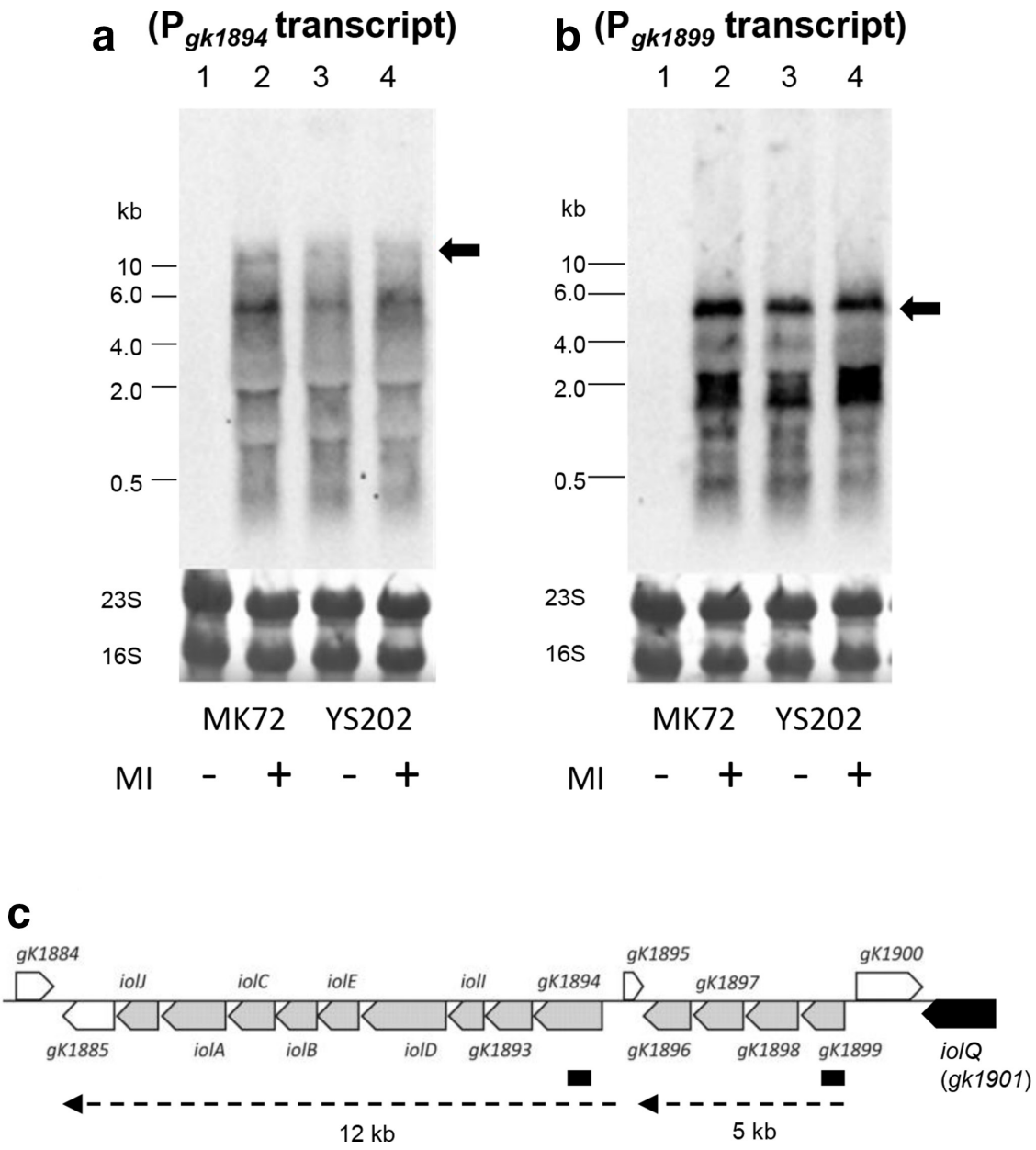

Fig. 1. Northern blot analyses of the two *iol* operons. Northern blot analyses for transcripts containing *gk1894* (a) and *gk1899* (b) were conducted using RNA samples prepared from cells of strains MK72 (lanes 1 and 2) and YS202 (lanes 3 and 4) grown with (lanes 2 and 4) and without (lanes 1 and 3) 10 mM *myo*-inositol (MI). Each of the lanes contained 20 µg of RNA samples. The 5 and 12 kb transcripts are indicated by arrows. Loading controls of rRNA (16S and 23S) are shown at the bottom. (c) Schematic organization of the *iol* gene cluster in *G. kaustophilus*. The probes are indicated with solid boxes beneath the arrowheads representing *gk1894* and *gk1899*.

subjected to gel electrophoresis mobility shift analyses to check whether it binds to the upstream sequences of the two *iol* operons *in vitro*.

In brief, the transcriptional start site of the former 5-kb-long operon containing *gk1896–1899* was previously defined 136 bp upstream from the start codon of *gk1899* [17], and the corresponding −35 and −10 regions were predicted as the P_{gk1899} region (Fig. 2a) [17]. To determine the transcriptional start site of the latter 12-kb-long operon for *gk1885–1894*, we conducted a 5′-rapid amplification of cDNA ends (5′-RACE) analysis using a sample of total RNA prepared from strain MK72 grown in the presence of *myo*-inositol. Based on the results, we identified a transcriptional start site 210 bp upstream from the start codon of *gk1894* (Fig. 2b) and the corresponding −35 and −10 regions were predicted to be the P_{gk1894} region.

We performed gel electrophoresis mobility shift analyses using three DNA fragments: P_{gk1894} fragment containing DNA

Table 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Description and relevant properties	Source or reference
G. kaustophilus		
HTA426	Wild-type	[13]
MK72	Δ*pyrF* Δ*pyrR*	[15]
YS202	Δ*iolQ*Δ*pyrF* Δ*pyrR*	This work
E. coli		
BL21(DE3)	F⁻ *ompT hsdSB*(rB⁻ mB⁻) *gal*(λ*cI857 ind1 Sam7 nin5 lacUV5-T7gene1*) *dcm*(DE3)	Takara Bio
BR408	Donor strain for conjugative plasmid transfer	[15]
DH5α	F⁻ Φ80d *lacZ*Δ*M15* Δ(*lacZYA-argF*)U169, *deoR recA1 endA1 hsdR17*(rK⁻ mK⁺) *phoA supE44* λ⁻ *thi-1 gyrA96 relA1*	Takara Bio
Plasmid		
pET30a(+)	*kan*	Takara Bio
pETiolQhisGK	*kan* P*t7-IolQ-his*	This work
pGKE25	*oriT pyrF amp*	[39]
pGKEdiolQ	A derivative of pGKE25	This work
pMD20	*amp*	Takara Bio

corresponding to −400 to −100 of the *gk1894* start codon, P_{gk1899} fragment DNA corresponding to −250 to +50 of the *gk1899* start codon, and negative control fragment corresponding to an internal coding sequence of *gk1894* containing stretches of +959 to +1109 from the start codon. A fixed amount of DNA fragments was reacted with increasing concentrations of IolQ-his *in vitro* and loaded onto the polyacrylamide gel. Both P_{gk1894} and P_{gk1899} fragments exhibited an obvious gel electrophoresis mobility shift, forming DNA–protein complexes in a concentration-dependent manner with an increase in IolQ-his concentration, whereas the negative control fragment did not exhibit such a result (Fig. 3a, b). On the other hand, when either *myo*-inositol, *scyllo*-inositol, *scyllo*-inosose or ribose was added to the DNA–protein reaction mixture, the gel electrophoresis mobility shifts of P_{gk1894} and P_{gk1899} reduced partially but significantly only in the presence of *myo*-inositol (Fig. 3c, d). These results indicated that IolQ-his bound to each of the P_{gk1894} and P_{gk1899} fragments specifically and that *myo*-inositol could antagonize the interaction between each of the DNA fragments and IolQ-his.

Table 2. NAD⁺-dependent *myo*-inositol dehydrogenase activity in strains of *G. kaustophilus*

Strain	NAD⁺-dependent *myo*-inositol dehydrogenase activity (nmol min⁻¹ mg protein⁻¹)		
	None*	*myo*-Inositol*	*myo*-Inositol + glucose*
MK72	10.6±4.3	287.2±13.0	256.4±31.7
YS202	357.9±39.5	400.5±26.8	377.8±11.7

*Bacterial strains were grown in liquid minimal medium containing both 0.1% casamino acids and 1 µg uracil ml⁻¹ and additionally supplemented with the carbon sources as indicated (each at 10 mM). Values are shown as mean±SD of three independent measurements.

Two IolQ-binding sites within each of the *iol* promoter regions

All the above-described results suggested that IolQ can be a transcriptional repressor of the two *iol* operons that are induced in the presence of *myo*-inositol. We conducted DNase I footprinting analyses to define the IolQ-binding sites within the two promoter regions. DNA fragments corresponding to each of the promoter regions were prepared using the respective 5′-6-carboxyfluorescein (6-FAM)-labelled primers and reacted with IolQ-his under conditions similar to those used in the gel electrophoresis mobility shift analyses. The DNA–protein complexes were treated with DNase I, and the digested DNA was subjected to DNA fragment size analysis to determine the regions protected from DNase I by the bound IolQ-his (Fig. 4). The results suggested that there are two protected regions within each of the two *iol* promoter regions (Figs 2 and 4, the nucleotide sequences of the upper and lower strands of the protected regions are shown in red), although the protection in the lower strand of the P_{gk1894} fragment was less evident than that in the others for an unknown reason (Fig. 4d). The protected regions could be considered the IolQ-binding sites, and a comparison of the nucleotide sequences among the four binding sites allowed deduction of a conserved palindromic sequence of 5′-RGWAAGCGCTTSCY-3′ (where R=A or G, W=A or T, S=G or C, and Y=C or T) recognized by IolQ.

Fig. 2. Nucleotide sequences of the two *iol* promoter regions (a, P_{gk1899}; b, P_{gk1894}). The –35 and –10 regions, corresponding to transcriptional start sites (+1) and translational start codons (fMet), are indicated in the upper strand. The nucleotides protected from DNase I by the bound IolQ-his are indicated in red letters in the sequence, and the region of consensus sequences is surrounded by dotted boxes. The primers used for reverse transcription of P_{gk1894} mRNA in 5′-RACE and P_{gk1899} mRNA in primer extension [17] are indicated with arrows beneath the lower strand sequences.

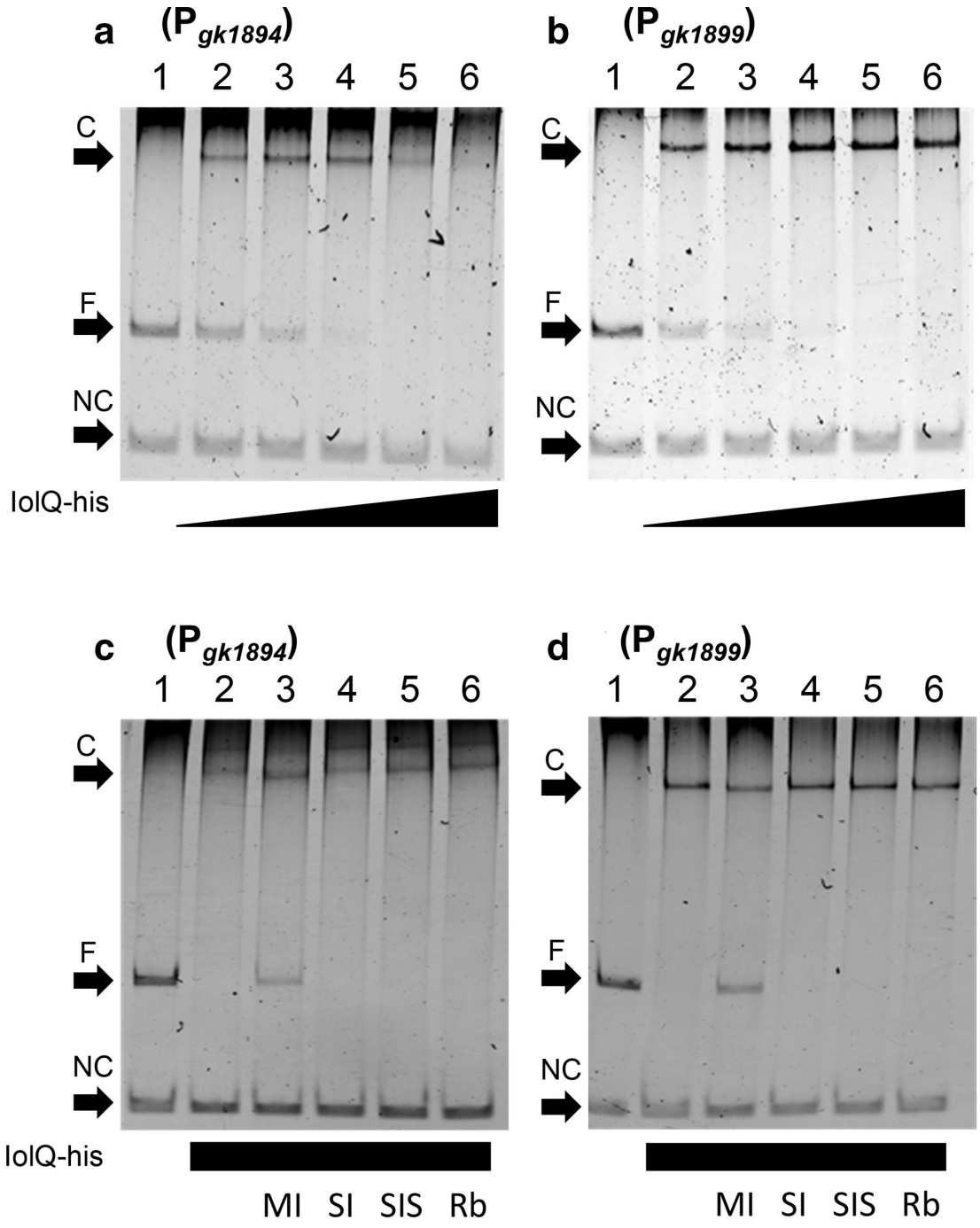

Fig. 3. Gel electrophoresis mobility shift of DNA fragments containing the *iol* promoter by IolQ binding. The DNA fragment (2.3 nM) containing P_{gk1894} (a) or P_{gk1899} (b) was combined with the negative control fragment (2.3 nM) and incubated with various concentrations of IolQ-his (for lanes 1–6, 0, 5.7, 11.4, 22.8, 45.7, and 91.4 nM as monomer, respectively) and subjected to 6% polyacrylamide gel electrophoresis. The DNA fragment (2.3 nM) containing P_{gk1894} (c) or P_{gk1899} (d) was combined with the negative control fragment (2.3 nM) and incubated without (lane 1) and with 91.4 nM IolQ-his (lanes 2–6) in the absence (lane 2) and presence of one of the inducer candidate chemicals [lanes 3–6, *myo*-inositol (MI), *scyllo*-inositol (SI), *scyllo*-inosose (SIS) and ribose (Rb), respectively, each 1 mM]. The positions of IolQ–DNA complex (C), free DNA (F) and negative control fragment (NC) are indicated by arrows.

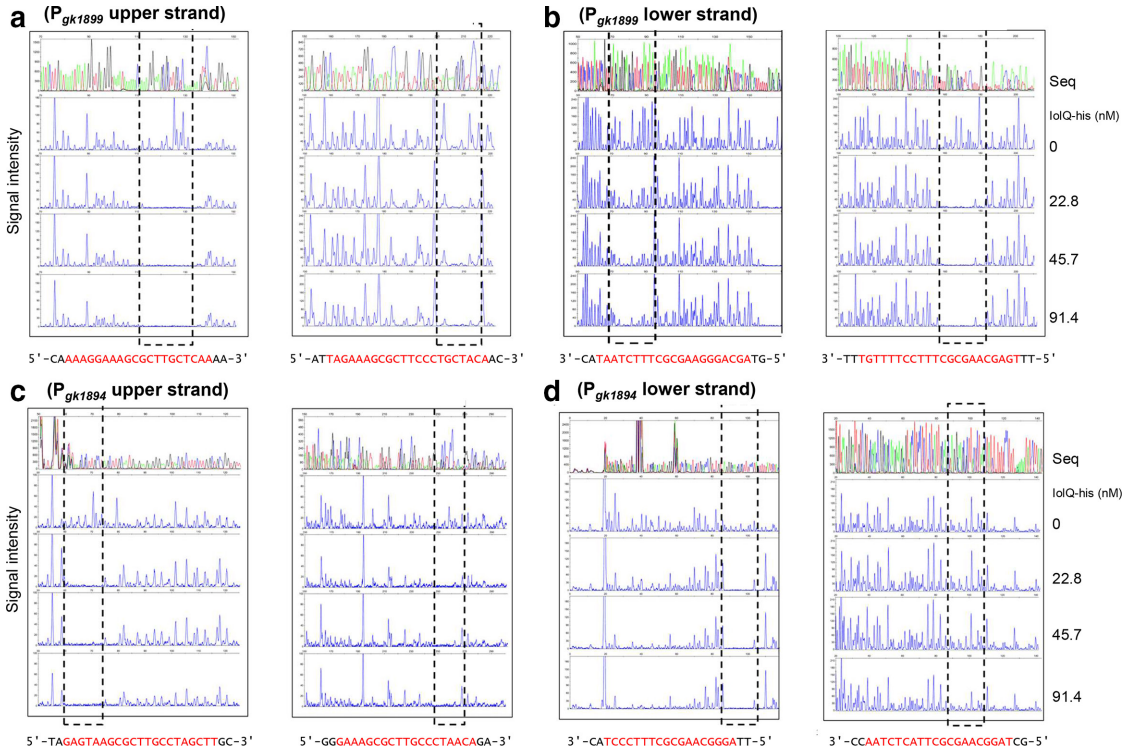

Fig. 4. DNase I footprints of IolQ on P_{gk1899} and P_{gk1894}. The results of DNase I footprinting analysis of IolQ on P_{gk1899} (a, b) and P_{gk1894} (c, d) are shown. For clarity, each of the panels has two sets of chromatograms, which are the two sections of a continuous chromatogram; the left is the former part and the right is the latter. Next, 2.3 nM of DNA fragments alternatively labelled with 6-FAM at the 5′-end of the upper (a and c) or lower strand (b and d) were reacted with IolQ-his and digested by DNase I before the fragment analysis was performed. Each of the panels presents five chromatograms as follows: on the top, the sequencing chromatogram (Seq) and the second from the top to the fifth, the fragmentation patterns with various concentrations of IolQ-his (0, 22.8, 45.7 and 91.4 nM, respectively). The regions protected from DNase I by the bound IolQ-his are surrounded by dotted boxes and indicated as red letters in the nucleotide sequences beneath the respective chromatograms; for panels a and c, sequences of upper strands (5′ to 3′) are shown, whereas for b and d, those of lower strands (3′ to 5′) are shown.

DISCUSSION

In the present study, we have demonstrated that the two *iol* operons of *G. kaustophilus* are regulated by IolQ and that the DNA-binding activity of IolQ is antagonized exclusively by *myo*-inositol *in vitro*. Inactivation of *iolQ* made the transcription of *iol* operons completely constitutive (Fig. 1), indicating that the *iolR* homologue (*gk1840*) does not play the major role in the regulation of *iol* genes as shown in other microorganisms [1, 2, 4, 9, 10]. *G. kaustophilus* can grow not only on *myo*-inositol but also on other inositol stereoisomers, including *scyllo*-inositol and D-*chiro*-inositol [17], suggesting that these inositol isomers also induce the *iol* operons. In *B. subtilis*, *iolW* and *iolI* (the respective homologues of *gk1898* and *iolI* of *G. kaustophilus*) are required to metabolize *scyllo*-inositol and D-*chiro*-inositol, respectively [19, 20]. Moreover, studies have shown that these genes, together with *iolG* (the counterpart of *gk1899* of *G. kaustophilus*), are involved in interconversion among *myo*-inositol, *scyllo*-inositol and D-*chiro*-inositol [19, 20]. In *G. kaustophilus*, *gk1898* and *gk1899* are contained in the former 5 kb operon, whereas *iolI* is the third gene of the latter 12 kb operon. Transcriptional repression by IolQ might allow basal expression of *iol* genes to a certain extent (Table 2). This may allow *gk1898*, *iolI* and *gk1899* to convert *scyllo*-inositol and D-*chiro*-inositol into *myo*-inositol in the cell to induce the *iol* operons.

On the other hand, the inositol transporters in *G. kaustophilus* have not yet been identified; however, some genes included in the two *iol* operons, such as *gk1893*, *1894* and *1895* possibly composing an ABC transporter and *gk1885* encoding a member of the major facilitator superfamily [13], might serve as *myo*-inositol transporter(s) with a broader specificity to uptake any of these inositol isomers. The broader specificity of the inositol transporters was reported for *Caulobacter crescentus*, which transports not only *myo*-inositol but also ribose [21]. Although ribose might be an additional substrate of the putative inositol transporters, it is unlikely that ribose acts as an inducer in *G. kaustophilus* because a gel electrophoresis mobility shift assay showed that IolQ binding to the DNA fragments of the *iol* promoter regions was not antagonized by ribose (Fig. 3), and to the best of our knowledge, there is no report describing any pathway involved in converting ribose to *myo*-inositol.

The transcription of *iol* genes in a number of bacterial species, including *B. subtilis*, *Clostridium perfringens*, *Lactobacillus casei*, *Salmonella enterica* and *Sinorhizobium meliloti*, is repressed in the presence of glucose and thus is under carbon catabolite repression [10, 22–26]. Catabolite repression in Gram-positive bacteria such as *B. subtilis* involves transcriptional repression through CcpA/P-Ser-HPr binding to the specific chromosomal sites with *cre* sequence [26]. Within the *G. kaustophilus* genome, because *gk2810* and *gk3082* were found to be highly homologous to *ccpA* and *hpr* of *B. subtilis*, respectively [13], it was considered that carbon catabolite repression might function in *G. kaustophilus* in a similar manner as that in *B. subtilis*. However, the *iol* genes in *G. kaustophilus* were not subjected to catabolite repression (Table 2). This finding implied that inositol catabolism in *G. kaustophilus* might have a physiological significance other than being a simple strategy to utilize this carbon source, and a similar finding has been discussed with respect to another Gram-positive bacterium, *Corynebacterium glutamicum* [27, 28]. As already mentioned, *G. kaustophilus* HTA426 was isolated from the deep-sea sediment collected from the Mariana Trench in the western Pacific Ocean [12, 13]. Reportedly, inositols are often found on the deep seafloor [29] and could be considered osmotic regulators (osmolytes) [30, 31]. Deep-sea organisms are known to accumulate osmolytes to increase their intracellular osmotic pressure to adapt to the specific environment in the deep sea [32]. Moreover, *scyllo*-inositol is known in particular as a chemical chaperone that interferes with the aggregation of denatured proteins such as amyloid beta accumulating in the brain, resulting in the development of Alzheimer's disease [33, 34]. Through the possible interconversion among inositol stereoisomers [17, 20], some *scyllo*-inositol might be produced in *G. kaustophilus* to prevent the aggregation of proteins denatured under conditions of lower temperatures and high pressure in the deep sea. The *iol* genes in *G. kaustophilus* might have been excluded from the general control of carbon catabolite repression to be responsible for such a specific cellular function.

Each of the two *iol* promoter regions (P_{gk1899} and P_{gk1894}) has a pair of IolQ-binding sites that were found to share a palindromic consensus sequence of 5′-RGWAAGCGCTTSCY-3′ (Figs 3 and 4). Within the genome sequence of *G. kaustophilus*, the consensus sequence can be found in the intergenic regions preceding *gk0016* (a hypothetical protein), *gk0786* (a transposase), *gk1017* (a hypothetical protein), *gk2450* (a 50S ribosomal protein L33), *gk2733* (a hypothetical protein) and *gk3430* (a glycyl-tRNA synthetase) [13]. However, the presence of pairwise IolQ-binding sites is specific to the two *iol* promoter regions, suggesting that the closely located two binding sites are required to establish transcriptional repression via IolQ binding. In addition, the DNA fragments containing the two binding sites exhibited a single-step shift, even at the lowest concentration of IolQ (Fig. 3), implying that the two binding sites might function cooperatively for IolQ binding even if each site might have different affinities as suggested by the DNase I footprinting analyses (Fig. 4). In fact, IolQ belongs to the transcription factors of the LacI family, which is represented by LacI of *E. coli* forming a dimeric structure to bind to the two operator sites of the lactose operon [35], and the two DNA-bound LacI dimers associate into a tetramer to form a DNA loop structure [36, 37].

In *B. subtilis*, IolQ was found to act as a transcriptional repressor of *iolX*, encoding NAD^+-dependent *scyllo*-inositol dehydrogenase [3]. *B. subtilis* IolQ binds to the two sites with a consensus sequence of 5′-AGAAARCGCTTKCKCAAA-3′ (where K=G or T) in the *iolX* promoter region [3]. *scyllo*-Inositol and *myo*-inositol are possible intracellular inducers; however, neither of them antagonized its DNA-binding activity *in vitro* [3]. In this study, we clearly demonstrated that *G. kaustophilus* IolQ bound to the four binding sites with the palindromic consensus sequence 5′-RGWAA-GCGCTTSCY-3′, and its DNA-binding activity was antagonized by *myo*-inositol. These two repressor proteins share a certain similarity in terms of their amino acid sequences (Fig. S2), and the N-terminal regions corresponding to the helix–turn–helix LacI-type domain (amino acids 1–57) are more conserved than the latter regions, in agreement with the finding that the consensus nucleotide sequences required for their binding are essentially very similar palindromes. Although both are functionally related based on their involvement in the regulation of the genes required for inositol catabolism, *B. subtilis* IolQ regulates only *iolX*, encoding an enzyme only for *scyllo*-inositol catabolism, whereas *G. kaustophilus* IolQ controls the entire *iol* genes. The reduced conservation in their amino acid sequences in the latter regions might be related to the difference in their regulatory functions distinguishing the ligands. Further investigation of the similarities and differences between the two IolQs may yield insight into the evolution of regulatory genes with related functions.

METHODS

Bacterial strains, plasmids, primers and growth conditions

The strains and plasmids used in this study are detailed in Table 1, and the primers are depicted in Table 3. *E. coli* strains were grown aerobically at 37 °C in LB medium containing 25 µg ampicillin ml^{-1} or 25 µg kanamycin ml^{-1} as needed. *G. kaustophilus* strains were grown aerobically at 60 °C in LB medium or minimal medium containing 1.72 mM K_2SO_4, 1.39 µM $ZnSO_4$, 162 nM H_3BO_3, 238 nM $CoCl_2$, 800 nM $CuSO_4$, 42 nM $NiCl_2$, 744 nM EDTA-2Na, 2.79 mM Na_2HPO_4, 11.1 µM $MnCl_2$, 1.62 mM $MgSO_4$, 34.1 µM $CaCl_2$, 25.9 µM $FeCl_3$, 18.7 mM NH_4Cl, 10.0 mM K-MOPS (pH 8.0) and carbon sources (10 mM *myo*-inositol, 10 mM glucose and 0.1% casamino acids). When required, 1 or 100 µg uracil ml^{-1} and 50 µg 5-fluoroorotic acid (5-FOA) ml^{-1} were added.

Plasmid construction

pGKE25diolQ is a plasmid carrying an in-frame deletion of *iolQ*. The in-frame internal deletion was from the third to the 336th codons of the *iolQ* coding region to connect the first two codons to the last two. In brief, a 1.0 kb region corresponding

Table 3. Primers used in this study

Primer	Sequence (5′–3′)
gk1901-UF	GACGGCCAGTGAATTAGGAAATAACATCGCTTTATG
gk1901-UR	ACATCCTTACGATTTTTTCATCGCTCATCATTCTC
gk1901-DF	AAATCGTAAGGATGTGAGC
gk1901-DR	TGATTACGCCAAGCTATGAACAGACCGAGGCCC
pGKE25-ER	AATTCACTGGCCGTCGTTTTAC
pGKE25-HF	AGCTTGGCGTAATCATGGTC
gk1901-F	AAGGAGATATACATAATGAAAGTAACCATTTACGATG
gk1901-R	GACGGAGCTCGAATTCGATTTCACTTTAGCGATATG
n-gk1894-F	CTATGTGCTGGAAATGATAGAC
n-gk1894-R	TAATACGACTCACTATAGGGGTTGCCTCGTCTTTTTGAC
n-gk1899-F	AGTAGGGATTTTGGGAGC
n-gk1899-R	TAATACGACTCACTATAGGGGTTTATACACCGACGCATACC
EMSA-UF	CCTTTTTCGACATAAGCC
EMSA-UR	ACCTTGGCAATCCCTCC
EMSA-DF	GGTTAGAGTAAGCGCTTGC
EMSA-DR	TAACAAAATTACCTTATTTAGCGC
EMSA-IF	GACGATCCGCTCGACGC
EMSA-IR	TGCAAAAAGCCTGCCTTC
EMSA-12UF	TGGCAGACGTTCATAAAATAG
RT	CATGTTCGGAACAGGGC
S1	GGGAAATCGACATTGATG
S2	AAGAGTTGAAAGTCTCCACG
A1	GAACCGTGCCTCTTTTAAC
A2	ACCGTCTAACGCTTTGAC

to the upstream region of *iolQ* in the *G. kaustophilus* HTA426 chromosome was amplified by PCR using the primer pair gk1901-UF/gk1901-UR (Table 3). Similarly, another 1.0 kb stretch corresponding to the downstream region of *iolQ* was amplified using the primer pair gk1901-DF/gk1901-DR (Table 3). pGKE25 [38] was linearized by inverse PCR using pGKE25-ER and pGKE25-HF as primers (Table 3). The PCR fragments of the upstream and downstream regions of *iolQ* and the linearized pGKE25 were incubated with Gibson Assembly Master Mix (New England Bio) at 50 °C for 15 min and transformed into *E. coli* DH5α (Table 1) to yield the recombinant plasmid pGKE25diolQ, with correct construction confirmed by DNA sequencing.

pETiolQhisGK was designed to express *IolQ-his* and to purify the gene product as a C-terminal His-tagged fusion protein in *E. coli*. In brief, the coding region of *iolQ* was amplified by PCR from the HTA426 chromosome using the primer pair gk1901-F/gk1901-R (Table 3). The PCR fragment and DNA of pET30a(+) previously cleaved using *Nde*I and *Eco*RI were mixed and incubated for 15 min at 50 °C with Gibson Assembly Master Mix to produce pETiolQhisGK, with accurate construction confirmed by DNA sequencing.

Strain construction

G. kaustophilus YS202 is a derivative of MK72 [15], which was constructed to introduce an in-frame deletion of *iolQ*. Briefly, the recombinant plasmid pGKE25diolQ was introduced into *E. coli* BR408 (Table 1) and transferred into MK72 by conjugation as described previously [15] to form colonies on minimal plate medium containing 0.1% casamino acids and 1% glucose without uracil at 60 °C. One of the resulting uracil-non-demanding transconjugants was proliferated once in LB medium at 60 °C, and an aliquot of the culture was inoculated and allowed to grow in minimal medium containing 0.1% casamino acids, 1% glucose, 1 µg uracil ml^{-1} and 50 µg 5-FOA ml^{-1} for 24 h. From this culture, colonies were formed on minimal medium plates containing 0.1% casamino acids, 1% glucose, 1 µg uracil ml^{-1} and 50 µg 5-FOA ml^{-1}, and one of them was selected as strain YS202, with correct construction confirmed by DNA sequencing.

myo-Inositol dehydrogenase assay

G. kaustophilus strains were grown aerobically in liquid minimal medium containing 0.1% casamino acids and 1 µg uracil ml^{-1} and additionally supplemented (or not) with 0 mM *myo*-inositol. Bacterial cells were harvested by centrifugation and washed once in a buffer prepared using 1 M NaCl and 50 mM Tris-HCl (pH 8.0). Cells were suspended in 100 µl of 100 mM Tris-HCl (pH 8.0) and transferred into a microtube containing 5 µl of lysozyme at 10 mg ml^{-1} in 100 mM Tris-HCl (pH 8.0). Cells were incubated at 37 °C, after which they were disrupted completely by a Bioruptor UCD-250 (Cosmo Bio). After centrifugation, the supernatant was stored as the enzyme solution. Next, the activity of NAD^+-dependent *myo*-inositol dehydrogenase in the enzyme solution was measured spectrophotometrically as described previously [17].

RNA sample preparation

G. kaustophilus strains were grown aerobically in liquid minimal medium containing 0.1% casamino acids and 1 µg uracil ml^{-1} and additionally supplemented (or not) with 10 mM *myo*-inositol. The bacterial cells were collected and disrupted to extract total RNA as described previously [17].

Northern blot analysis

RNA samples were subjected to northern blot analyses using DIG-labelled RNA probes specific for *gk1894* and *gk1899*, as described previously [3]. The RNA probes were prepared as follows: the DNA fragments corresponding to the part of the *gk1894*- and *gk1899*-coding regions were amplified by PCR using HTA426 DNA as a template and the primer pairs n-gk1894-F/n-1894-R and n-gk1899--F/n-1899-R, respectively (Table 3) to introduce a T7 RNA polymerase promoter sequence in the tail. The PCR product was used as the template for *in vitro* transcription using the DIG RNA labelling kit (SP6/T7; Roche Diagnostics) to produce DIG-labelled RNA probes. Cellular

RNAs were separated by gel electrophoresis, transferred to a positively charged nylon membrane (Roche Diagnostics), hybridized using the DIG-labelled probes and detected using the DIG Luminescence Detection Kit (Roche Diagnostics).

5′-RACE

5′-RACE was performed to identify a transcriptional start site using the 5′-Full RACE Core Set (Takara Bio) according to the supplier's protocol. Briefly, the first strand of cDNA was synthesized from the RNA sample prepared from the cells of MK72 grown in liquid minimal medium containing 0.1% casamino acids, 1 µg uracil ml^{-1} and 10 mM *myo*-inositol by reverse-transcription reaction using a 5′-end phosphorylated RT primer (Table 3) and treated with RNase H to liberate the single-stranded cDNA. The single-stranded cDNA was cyclized by T4 RNA ligase and subjected to two-step nested inverse PCR using two PCR pairs, A1/S1 and A2/S2 (Table 3), successively. The resulting PCR fragments were cloned into the T vector pMD20 (Takara Bio) and sequenced to identify the transcriptional start site.

IolQ-his purification

E. coli BL21(DE3) carrying pETiolQhisGK was grown aerobically in LB liquid medium containing kanamycin, and 1 mM IPTG was added to the growing culture to induce *IolQ-his* expression. Bacterial cells were harvested, suspended in a buffer prepared using 50 mM phosphate buffer (pH 8.0), 20% glycerol and 0.5 M NaCl, and disrupted completely by using a Bioruptor UCD-250 (Cosmo Bio). After centrifugation, the supernatant was subjected to purification of His-tagged fusion protein using TALON Metal affinity resins (Takara Bio) according to the supplier's instructions. The eluted fractions were subjected to SDS-PAGE, and the purity of IolQ-his was confirmed.

Gel electrophoresis mobility shift assay

A gel electrophoresis mobility shift assay was performed basically as described previously [38]. DNA fragments were prepared by PCR amplification from HTA426 DNA. Regarding preparation of the P_{gk1894} fragment, P_{gk1899} fragment and negative control fragment corresponding to an internal coding sequence of *gk1894*, the primer pairs EMSA-DF/EMSA-DR, EMSA-UF/EMSA-UR and EMSA-IF/EMSA-IR were used, respectively (Table 3). A fixed amount of DNA fragments and various amounts of the purified IolQ-his were mixed in a binding buffer containing 20 mM Tris-HCl (pH 8), 100 mM KCl, 0.5 mM $MgCl_2$, 0.5 mM DTT, 0.005 mg poly[d(I-C)] ml^{-1}, 0.05 mg BSA ml^{-1} and 0.05% polyethylene glycol in an ice bath and reacted at 37 °C for 20 min. The reaction mixture was subjected to 6% (or 12%) PAGE in 40 mM Tris-acetate (pH 8)/2 mM EDTA. After the completion of electrophoresis, the gel was soaked in 0.1 µg cybergreen ml^{-1} for 30 min and the DNA bands were detected by the Gel Doc XR+system (Bio-Rad).

DNase I footprinting analysis

DNase I footprinting analysis was conducted essentially as described previously [3]. PCR procedures were used to amplify 5′-6-FAM-labelled DNA fragments containing the P_{gk1899} region from the DNA of strain HTA426 using the specific primers [6-FAM]EMSA-UF/EMSA-UR and EMSA-UF/[6-FAM]EMSA-UR for labelling the sense and antisense strands, respectively (Table 3). Similarly, 5′-6-FAM-labelled DNA fragments containing the P_{gk1894} region were amplified using the specific primer pairs [6-FAM]EMSA-12UF/EMSA-DR and EMSA-12UF/[6-FAM]EMSA-DR for labelling the sense and antisense strands, respectively (Table 3). Each 5′-6-FAM-labelled DNA fragment was incubated with varying concentrations of IolQ-his and digested by DNase I to be analysed using the ABI 3130xl Genetic Analyzer with the ABI GeneMapper Software Ver. 4.0 (Thermo Fisher Scientific) together with the corresponding DNA sequencing reactions.

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Author contributions
Y.S., R.N. and K.F. conducted most experiments and analysed results under the supervision of K.Y., and S.I. K.Y. conceived the idea for the project and wrote the final manuscript with S.I. All authors read and approved the final manuscript.

Conflicts of interest
The authors declare that there are no conflicts of interest.

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