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Microbial synthesis of health-promoting inositols

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Highlights

- *scyllo*-Inositol and D-*chiro*-inositol are known for their health-promoting effects.
- Inexpensive mass production of these inositols is awaited.
- *Bacillus subtilis* and *Corynebacterium glutamicum* are chosen as the production hosts.
- Their inositol metabolism was manipulated to produce these inositols.

Abstract

D-*chiro*-Inositol and *scyllo*-inositol are known for their health-promoting properties and promising as ingredients for functional foods. Strains of *Bacillus subtilis* and *Corynebacterium glutamicum* were created by metabolic engineering capable of inexpensive production of these two rare inositols from *myo*-inositol, which is the most common inositol in nature. In addition, further modifications have enabled the synthesis of the two rare inositols from the much cheaper carbon sources, glucose or sucrose.

Introduction

Inositol (cyclohexane-1,2,3,4,5,6-hexanol) is a group of cyclic sugar alcohols. Epimerization of the six hydroxyl groups results in nine stereoisomers (Fig. 1). For their analysis, various methods have been reported, such as gas chromatography or HPLC [1,2]. *myo*-Inositol is the most abundant inositol in nature and available at a much lower price than the rare inositols. Its worldwide production is about 15,000 tons per year [3]. The major industrial production method nowadays is a chemical process removing the phosphate groups of phytate, but various alternative biotechnological processes for *myo*-inositol production have been described, either by fermentation or by *in vitro* enzymatic production [3].

myo-Inositol is commonly found in the brain and other mammalian tissues and is known to mediate various cell signaling pathways and plays an indispensable role as the structural basis for various inositol phosphates that function as secondary messengers in eukaryotic cells [4,5]. Furthermore, *myo*-inositol is a component of phosphatidylinositol phospholipids in cell membranes. As a result, inositol bound to lipids is found in many foods [6]. *myo*-Inositol was previously considered to belong to the vitamin B complex and was also called vitamin B8. However, *myo*-inositol was later found to be produced from glucose in the human body and is therefore no longer considered an essential nutrient [7]. In plants, *myo*-inositol also has a variety of functions in metabolism and phytic acid, the hexaphosphate of *myo*-inositol, serves as a phosphate reservoir and is accumulated in nuts, legumes, and bran-rich cereals [8]. In prokaryotes, *myo*-inositol plays functional roles in Archaea and actinobacteria [5]

Other naturally found inositol stereoisomers beside *myo*-inositol are *scyllo*-, *muco*-, D-*chiro*-, L-*chiro*, *allo*-, *epi*-, and *neo*-inositol [9]. Because they are all rare, no cheap production method has been established yet. *cis*-Inositol has not been found in nature [9]. *scyllo*-Inositol prevents the development of amyloid-beta plaques in the brains of transgenic mice, reversing memory impairment. Thus, *scyllo*-inositol has shown promise as a treatment for Alzheimer's disease [10]. D-*chiro*-Inositol and its 3-*O*-methyl derivative, D-pinitol, have an insulin-mimetic activity to lower blood glucose levels. The latter compounds are investigated for potential applications in treatments of diabetes and polycystic ovary syndrome [6] [11,12].

As *scyllo*-inositol and D-*chiro*-inositol have demonstrated health benefits, these molecules are promising candidates for functional food additives, and this review describes the development of efficient production of these isomers by two bacteria regarded generally as safe, namely *Bacillus subtilis* and *Corynebacterium glutamicum*.

Inositol metabolism in *Bacillus subtilis*

B. subtilis utilizes inositol stereoisomers, such as *myo*-, D-*chiro*-, and *scyllo*-inositol, as sole carbon sources [13]. The *iolABCDEFGHIJ* operon is responsible for the complete set of enzymes that catabolize *myo*-inositol and D-*chiro*-inositol (Fig. 2) [14]. The *iolF* and *iolT* genes encode two inositol transporters [13,15]. The *iolG* gene encodes a *myo*-inositol dehydrogenase that, as the first reaction in catabolism, converts *myo*-inositol to *scyllo*-inosose (2-keto-*myo*-inositol) with a reduction of NAD^+ to NADH [16]. IolG also reacts with D-*chiro*-inositol but not at all with *scyllo*-inositol. The promoters of the *iol* operon and *iolT* are under the control of the IolR transcriptional repressor, whose DNA binding is antagonized by 2-deoxy-5-keto-gluconate-6-phosphate produced by IolC kinase [17-19]. Two other inositol dehydrogenases, IolX and IolW, are specific for *scyllo*-inositol and require NAD^+

and $NADP^+$, respectively [19]; IolU was identified as the third *scyllo*-inositol dehydrogenase, but it only reduces *scyllo*-inosose into *scyllo*-inositol in an NADPH-dependent manner, whose physiological significance is still unclear (Fig. 2) [20]. The *iolX* gene is induced only when *scyllo*-inositol is used as the carbon source [21]. On the other hand, expression of *iolW* is almost constitutively expressed but does not contribute to the growth depending on *scyllo*-inositol [22]. Therefore, IolX is responsible for the catabolism of *scyllo*-inositol, while IolW and IolU may function for other purposes, such as *scyllo*-inositol production. It is noteworthy that *iolI* encodes an inosose isomerase. Inosose isomerase converts *scyllo*-inosose to 1-keto-D-*chiro*-inositol, which can serve as an additional substrate for IolG, being reduced to D-*chiro*-inositol (Fig. 2) [17].

B. subtilis **cell factory for conversion of** ***myo*****-inositol into D-*chiro*-inositol**

D-*chiro*-Inositol has value as a therapeutic agent for diabetes and polycystic ovary syndrome, although its actions are not yet fully understood. It has been shown to improve insulin efficiency and also promote ovulation. *B. subtilis* was engineered to produce D-*chiro*-inositol from *myo*-inositol as follows [23]. In the first and second steps of the *myo*-inositol catabolic pathway, *myo*-inositol is converted to *scyllo*-inosose by IolG and then to 3D-(3,5/4)-trihydroxycyclohexane-1,2-dione by IolE (Fig. 2). As mentioned above, *iolI* encodes an inosose isomerase, converting *scyllo*-inosose to 1-keto-D-*chiro*-inositol, and IolG reduces 1-keto-D-*chiro*-inositol to D-*chiro*-inositol. Upon inactivation of *iolE*, *scyllo*-inosose accumulated, which was further converted to D-*chiro*-inositol through the coupling reactions of IolI and IolG, allowing at least 6% of the input *myo*-inositol to be converted to D-*chiro*-inositol.

B. subtilis **cell factories for the conversion of** ***myo*****-inositol into** ***scyllo*****-inositol**

As mentioned above, *scyllo*-inositol has shown promise as a therapeutic agent for Alzheimer's disease. We have modified *B. subtilis* to develop a series of cell factories that efficiently convert *myo*-inositol to *scyllo*-inositol as follows. We constructed the first generation of cell factories by deleting three genes, including *iolR*, *iolX*, and *iolI,* and introducing the missense mutation *iolE41*. The purpose of these mutations was to make the expression of the *iolABCDEFGHIJ* operon constitutive, to stop the dehydrogenation of *scyllo*-inositol, and to minimize the isomerization and dehydrogenation of *scyllo*-inosose. In this modified cell, after 72 h of cultivation, about 5 g L^{-1} of *myo*-inositol was converted into *scyllo*-inositol [13]. The second generation was to delete all *iolR*, *iolX*, and *iolABCDEFHIJ* and to overexpress *iolG* and *iolW* simultaneously [21]. Thus, all the input 10 g L^{-1} of *myo*-inositol was converted into *scyllo*-inositol within 48 h. However, when the initial concentration of *myo*-inositol increased to 50 g L^{-1}, it produced only 15.1 g L^{-1} of *scyllo*-inositol. Therefore, we constructed the third generation further to overexpress *iolT*, the principal transporter of *myo*-inositol, and *pntAB*, the membrane-intrinsic nicotinamide nucleotide transhydrogenase of *E. coli*. However, conversion

efficiency did not improve dramatically. Nevertheless, conversion was enhanced when the concentration of nitrogen source in the medium (Soytone) was increased to 4% to improve the *scyllo*-inositol production up to 27.6 g L^{-1} in 48 h [22].

Production of *scyllo*-inositol from glucose by *B. subtilis*

The biosynthesis of *myo*-inositol is conserved evolutionary, involving the stepwise reactions as follows. Glucose is phosphorylated to glucose-6-phosphate by glucose phosphorylation, the isomerization of glucose-6-phosphate to *myo*-inositol-1-phosphate by *myo*-inositol-1-phosphate synthase (MI1PS) encoded by *ino1*, and the formation of *myo*-inositol by inositol monophosphatase trimming a phosphate from *myo*-inositol-1-phosphate. The key enzyme in the biosynthesis is MI1PS. MI1PS is present in numerous bacteria and archaea, including *Mycobacterium tuberculosis*, which is known to synthesize *myo*-inositol [24]. However, *B. subtilis* and its close relatives do not have a gene supposed to encode MI1PS but do have a gene that encodes a functional inositol monophosphatase [25]. And the substrate of MI1PS, glucose-6-phosphate, is always available in *B. subtilis.* Thus, if MI1PS functions and *myo*-inositol is converted to *scyllo*-inositol as described above, *scyllo*-inositol biosynthesis from glucose may occur in *B. subtilis*. Therefore, *M. tuberculosis ino1* was introduced into *B. subtilis*. The introduced enzyme was produced as designed but showed no detectable activity. Importantly, MI1PS requires NAD^+-NADH as an essential cofactor. Although the mechanism is not yet understood, we found that the inactivation of *pbuE*, which encodes a purine base/nucleoside efflux pump, increased intracellular NAD^+-NADH levels. Consequently, the mycobacterial enzyme functioned enough when *pbuE* was inactivated and converted glucose-6-phosphate into *myo*-inositol-1-phosphate, which was then dephosphorylated to *myo*-inositol by YktC, the endogenous inositol monophosphatase in *B. subtilis*. *myo*-Inositol was readily isomerized into *scyllo*-inositol via the previously established pathway involving the two inositol dehydrogenases, IolG and IolW [22]. In addition, *glcP* and *glcK*, encoding glucose transporter and glucose kinase, respectively, were simultaneously overexpressed to provide more glucose-6-phosphate and promote *scyllo*-inositol production. Ultimately, an effective *B. subtilis* cell factory was constructed that produced 2 g L^{-1} *scyllo*-inositol from 20 g L^{-1} glucose [26].

Inositol metabolism in *Corynebacterium glutamicum*

Like *B. subtilis*, *C. glutamicum* can utilize *myo*-, *scyllo*-, and D-*chiro*-inositol as sole carbon sources [27,28]. Their import is catalyzed by the secondary transporters IolT1 and IolT2, which have similar kinetic properties [27,28]. The degradation pathways follow the same routes as shown in Fig. 2, with an initial oxidation of *myo*- and *scyllo*-inositol to *scyllo*-inosose (2-keto-*myo*-inositol) catalyzed by one or several of the four NAD^+-dependent inositol dehydrogenases IolG, OxiD, OxiB, and OxiE, which have recently been characterized [28]. *scyllo*-Inosose is further catabolized via six enzymes

(IolE, IolD, IolB, IolC, IolJ, IolA) to dihydroxyacetone phosphate, acetyl-CoA, and CO_2. The majority of genes involved in inositol metabolism are present in two clusters on the genome (Fig. 3A), one of which contains the operon *iolCJABDEGH*-cg0206-*iolW* including all genes involved in *myo*-inositol degradation and is essential for growth on inositols [27]. This operon and the *iolT1* gene are induced the GntR-type transcriptional regulator IolR when inositols are present [29]. The second cluster includes an operon of four genes, *oxiC*-cg3390-*oxiD*-*oxiE*. Whereas OxiC is presumably an inactive inositol dehydrogenase with an unknown function, OxiD oxidizes *myo*- and D-*chiro*-inositol and OxiE oxidizes *scyllo*-inositol [28]. Cg3390 is a putative sugar phosphate isomerase and was shown to be highly active on *scyllo*-inososе leading to the formation of dark brown-colored products [30]. *C. glutamicum* does not only consume inositols, but also has the intrinsic capability to synthesize *myo*-inositol from glucose-6-phosphate via *myo*-inositol phosphate synthase (Ino1, Cg3323) and a phosphatase (ImpA, Cg2298) [31]. Expression of the *ino1* gene is activated by the LacI-type transcriptional regulator IpsA [31] when the cytoplasmic concentration of *myo*-inositol is insufficient for the synthesis of important cellular constitutents such as mycothiol, phosphatidylinositol, and more complex lipids of the cell envelope [31].

C. glutamicum cell factories for the synthesis of *scyllo*-inositol

C. glutamicum was engineered to serve as cell factory for *scyllo*-inositol. Interestingly, when cultivated with *myo*-inositol, already the parental strain produces some *scyllo*-inositol, which is then degraded again [30]. As a first engineering step, the two gene clusters involved in inositol metabolism were deleted in strain MB001(DE3) to avoid degradation of inositols (Fig. 3A). MB001(DE3) is a derivative of ATCC 13032 lacking the prophage genes and containing genomically encoded the T7 RNA polymerase gene under control of the *lacUV*5 promoter as well as the *lacI*^{q} gene [32]. Strain MB001(DE3)Δ*iol1*Δ*iol2*, which lacks the gene clusters cg0196-cg0212 and cg3389-cg3392 involved in inositol metabolism (Fig. 3A), was transformed with the T7-based expression plasmid pIolGW encoding the NAD^+-dependent *myo*-inositol dehydrogenase IolG (Cg0204) and the *scyllo*-inositol dehydrogenase IolW (Cg0207) that reduces *scyllo*-inososе to *scyllo*-inositol with NADPH (Fig. 3B). In minimal medium, 4.4 g/L *scyllo*-inositol and 2.7 g L^{-1} *scyllo*-inososе were formed from 20 g L^{-1} *myo*-inositol within 72 h [30]. In rich BHI medium, the strain formed 18 g L^{-1} *scyllo*-inositol within 72 h from 18 g L^{-1} consumed *myo*-inositol, suggesting that the conversion in minimal medium possibly suffered from NADPH limitation [30].

To enable *scyllo*-inositol production from glucose and sucrose, plasmid pSI containing the synthetic operon *ino1-impA-iolG-iolW* under control of the T7 promoter was used (Fig. 3C). In BHI medium strain MB001(DE3)Δ*iol1*Δ*iol2* transformed with pSI formed 1.8 g L^{-1} *scyllo*-inositol from 20 g L^{-1} glucose within 72 h and even 4.4 g L^{-1} *scyllo*-inositol from 20 g L^{-1} sucrose, suggesting that sucrose

is a superior carbon source for *scyllo*-inositol production [30].

C. glutamicum cell factories for the synthesis of D-*chiro*-inositol

The synthesis of D-*chiro*-inositol from *myo*-inositol involves three reactions: the oxidation of *myo*-inositol to *scyllo*-inosose, the isomerization to 1-keto-D-*chiro*-inositol, and the reduction of the latter to D-*chiro*-inositol (Fig. 2). As a first step in the development of *C. glutamicum* strains for D-*chiro*-inositol production, a search for enzymes with inosose isomerase activity was performed that identified Cg0212 and Cg2312 [33]. Also Cg3390 had the corresponding activity, but in addition led to the formation of other unknown products [33]. For the conversion of *myo*-inositol to D-*chiro*-inositol, a derivative of strain MB001(DE3)Δ*iol1*Δ*iol2* was used in which also the *idhA3* gene (cg2313, Fig. 3A) encoding another putative inositol dehydrogenase was deleted. This strain, MB001(DE3)ΔIOL, when transformed with the T7-based expression plasmid pIolG-Cg0212 (Fig. 4A), formed 1.1 g/L D-*chiro*-inositol from 6 g/L consumed *myo*-inositol, corresponding to a yield of 18% [33]. In this reaction, both the oxidation and the reduction are catalyzed by IolG and the yield is limited by the unfavorable reduction of 1-keto-D-*chiro*-inositol to D-*chiro*-inositol with NADH.

To shift the equilibrium in favor of D-*chiro*-inositol production, a novel production route was established that was based on the substrate promiscuity of two plant enzymes identified in *Medicago truncatula* [33]. MtOEPa naturally oxidizes a methylated derivative of *myo*-inositol, D-ononitol (4-*O*-methyl-*myo*-inositol) with NAD^+ to 4-*O*-methyl-D-*myo*-1-inosose, whereas MtOEPb reduces this keto intermediate with NADPH to D-pinitol (3-*O*-methyl-D-*chiro*-inositol) (Fig. 4B) [34]. Codon-optimized *MtOEPa* and *MtOEPb* genes were expressed in *C. glutamicum* MB001(DE3)ΔIOLΔISO, a strain that differs from MB001(DE3)ΔIOL by the additional deletion of five genes encoding isomerases (cg0502, cg2312, cg2716, cg2822, cg2917), including the ones with proven inosose isomerase activity cg0212 and cg2312 [33]. With plasmid pOEPa-b (Fig. 4C), 1.6 g L^{-1} D-*chiro*-inositol were formed from 2 g L^{-1} consumed *myo*-inositol, showing that the plant enzymes also use the non-methylated *myo*-inositol and *myo*-1-inosose as substrates [33]. The advantage of this pathway is the use NAD^+ for oxidation and of NADPH for reduction.

To enable D-*chiro*-inositol production from glucose, expression plasmids containing in addition to *MtOEPa* and *MtOEPb* also the *ino1* gene were constructed, including the use of a novel bicistronic T7 expression vector. Strain MB001(DE3)ΔIOLΔISO with plasmid pBiT7-InoDCI (Fig. 4D) formed 1.2 g L^{-1} D-*chiro*-inositol and 0.23 g L^{-1} *myo*-inositol from 20 g L^{-1} glucose after 72 h, confirming that D-*chiro*-inositol can be produced from glucose [33].

Conclusions and outlook

D-*chiro*-Inositol and *scyllo*-inositol may become ingredients for functional foods due to their pharmacological properties [11]. In particular, they are expected to impact on preventing and overcoming diseases that are emerging in rapidly ageing societies. Therefore, strains of *B. subtilis* and *C. glutamicum* were created by metabolic engineering that are capable of inexpensive production of these two rare inositols from *myo*-inositol. Furthermore, overexpression of MI1PS enabled the synthesis of D-*chiro*-inositol and *scyllo*-inositol from the much cheaper carbon sources glucose or sucrose. Future studies should be directed to increase the yields of the rare inositols by further metabolic and enzyme engineering approaches [35,36]. When using glucose or sucrose as carbon sources, an increased availability of glucose-6-phosphate, a higher activity of *myo*-inositol phosphate synthase, and an increased NADPH availability would be desirable. Further targets are the optimization of upscaled fermentation conditions and the development of cheap downstream processing processes.

Declaration of interest: MB has a patent pending for DCI production.

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Figure legends

Fig. 1. Nine inositol stereoisomers
Inositol is a group of hexahydric alcohols, and epimerization of the six hydroxyl groups results in nine stereoisomers, with *myo*-inositol being the most abundant in nature. The other stereoisomers found naturally are *scyllo*-, *muco*-, D-*chiro*-, and *neo*-inositol, but they are rare.

Fig. 2. Inositol metabolism in *B. subtilis*
The inositol degradation pathway in microorganisms such as *B. subtilis* is shown schematically by connecting the chemical reactions indicated by arrows. The name of the enzyme catalyzing each reaction is given; those used selectively for interconversion of inositol isomers are shown in red. The chemical structures are depicted for some of the intermediates involved in the initial steps. The reaction pathways involved in the interconversion of inositol isomers are circled in red.

Fig. 3. Pathways and enzymes for the production of *scyllo*-inositol from *myo*-inositol (A) or glucose 6-phosphate (B) using endogenous genes of *C. glutamicum* and the chassis strain *C. glutamicum* MB001(DE3)Δ*iol1*Δ*iol2*. In panel C, the gene clusters *iol1* and *iol2* involved in inositol metabolism are shown. The genes *iolG* (purple) and *iolW* (blue) are required for conversion of *myo*-inositol to *scyllo*-inositol, the gene cg0212 (orange) is an isomerase catalyzing the conversion of *scyllo*-inosose to 1-keto-D-*chiro*-inositol. Genes shown in green have a known function, genes in white have a yet unknown function.

Fig. 4. Pathways and enzymes for the production of D-*chiro*-inositol from *myo*-inositol (A, C) or glucose (D) using either endogenous enzymes (A) or the plant-derived inositol dehydrogenases MtOEPa and MtOEPb (C, D) in the chassis strain *C. glutamicum* MB001(DE3)ΔIOLΔISO. The plant enzymes MtOEPa and MtOEPb naturally catalyze the conversion of D-ononitol to D-pinitol (B).

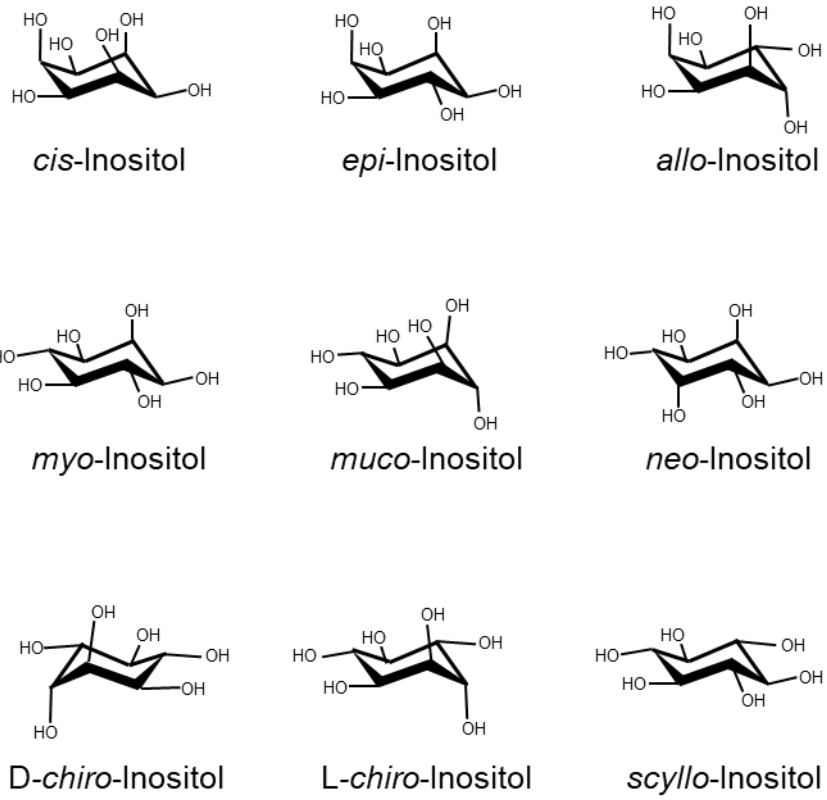
cis-Inositol
epi-Inositol
allo-Inositol
myo-Inositol
muco-Inositol
neo-Inositol
D-*chiro*-Inositol
L-*chiro*-Inositol
scyllo-Inositol

Fig. 1

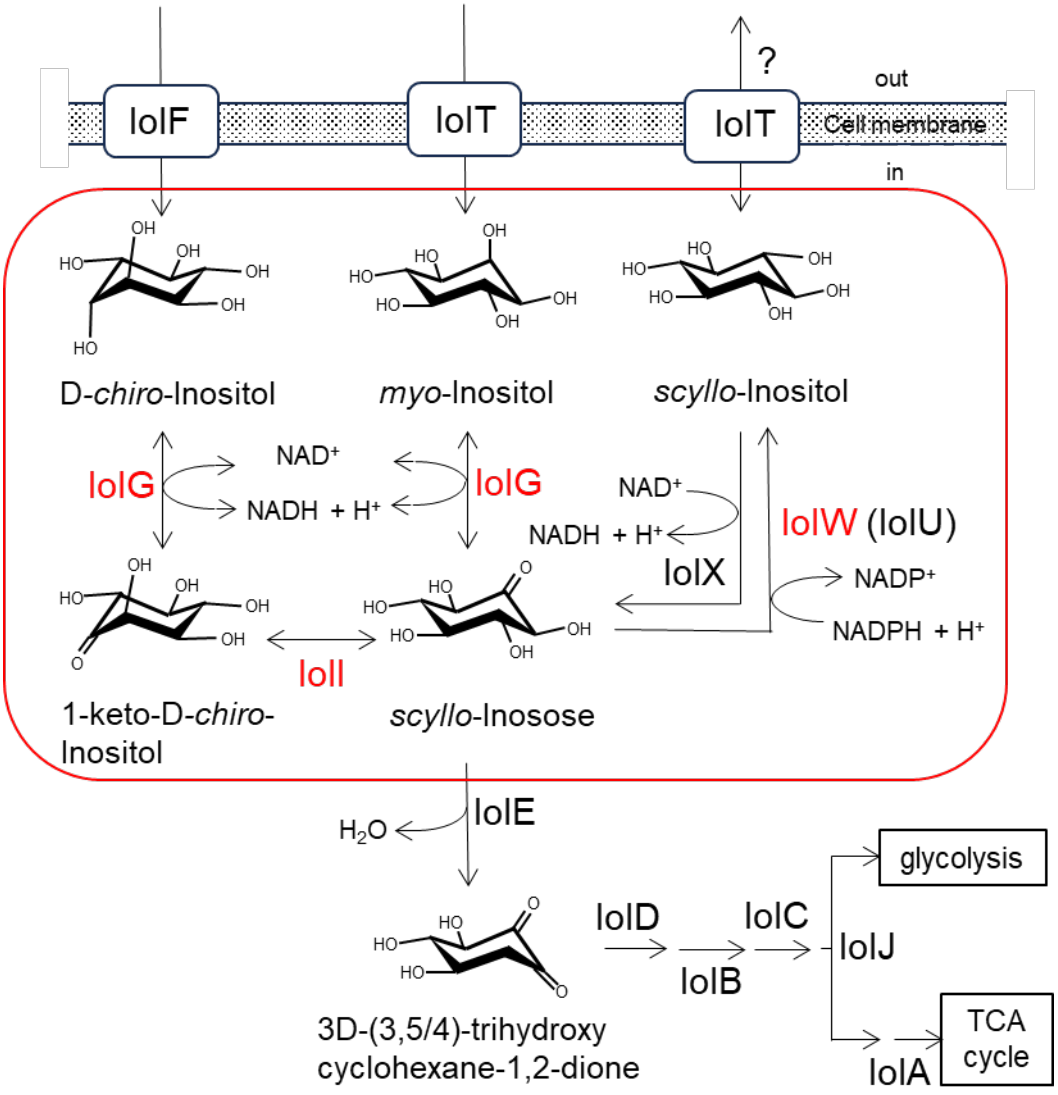
IolF
IolT
IolT
?
out
Cell membrane
in
D-chiro-Inositol
myo-Inositol
scyllo-Inositol
IolG
NAD+
NADH + H+
IolG
NAD+
NADH + H+
IolW (IolU)
IolX
NADP+
NADPH + H+
1-keto-D-chiro-Inositol
IolI
scyllo-Inosose
H2O
IolE
3D-(3,5/4)-trihydroxy cyclohexane-1,2-dione
IolD
IolB
IolC
IolJ
IolA
glycolysis
TCA cycle

Fig. 2

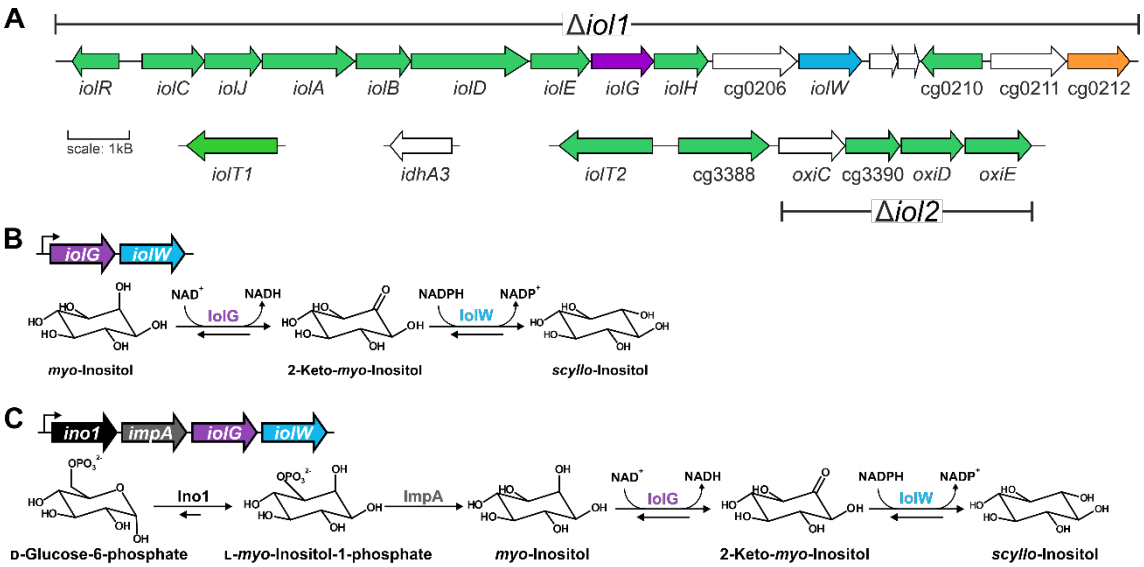
A
Δiol1
iolR iolC iolJ iolA iolB iolD iolE iolG iolH cg0206 iolW cg0210 cg0211 cg0212
scale: 1kB
iolT1
idhA3
iolT2 cg3388 oxiC cg3390 oxiD oxiE
Δiol2
B
iolG iolW
NAD+ NADH
IolG
NADPH NADP+
IolW
myo-Inositol
2-Keto-myo-Inositol
scyllo-Inositol
C
ino1 impA iolG iolW
Ino1
ImpA
D-Glucose-6-phosphate
L-myo-Inositol-1-phosphate
myo-Inositol
2-Keto-myo-Inositol
scyllo-Inositol

Fig. 3

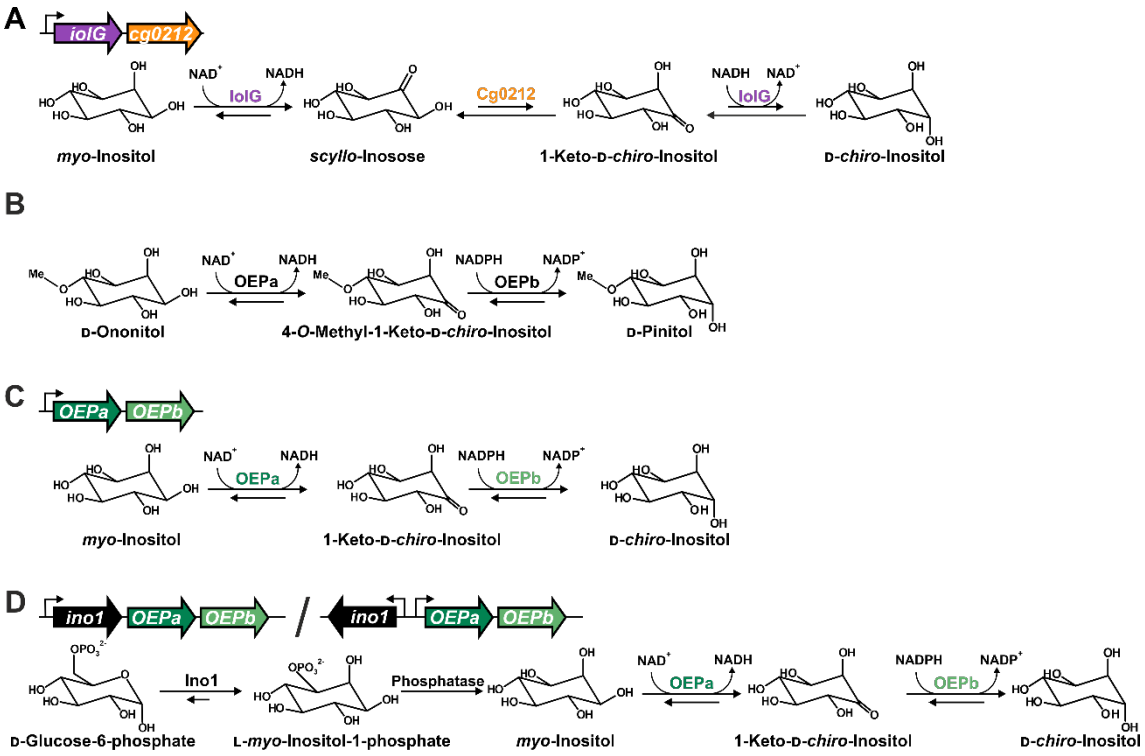
A
iolG
cg0212
NAD+
NADH
IolG
Cg0212
NADH
NAD+
IolG
myo-Inositol
scyllo-Inosose
1-Keto-D-chiro-Inositol
D-chiro-Inositol
B
NAD+
NADH
OEPa
NADPH
NADP+
OEPb
D-Ononitol
4-O-Methyl-1-Keto-D-chiro-Inositol
D-Pinitol
C
OEPa
OEPb
NAD+
NADH
OEPa
NADPH
NADP+
OEPb
myo-Inositol
1-Keto-D-chiro-Inositol
D-chiro-Inositol
D
ino1
OEPa
OEPb
/
ino1
OEPa
OEPb
Ino1
Phosphatase
NAD+
NADH
OEPa
NADPH
NADP+
OEPb
D-Glucose-6-phosphate
L-myo-Inositol-1-phosphate
myo-Inositol
1-Keto-D-chiro-Inositol
D-chiro-Inositol

Fig. 4