Reverse Genetics of *Bacillus subtilis*:

Identification of inositol catabolic genes and their promising applications

Ken-ichi Yoshida

Department of Agrobioscience, Graduate School of Agricultural Science, Kobe University

kenyoshi@kobe-u.ac.jp

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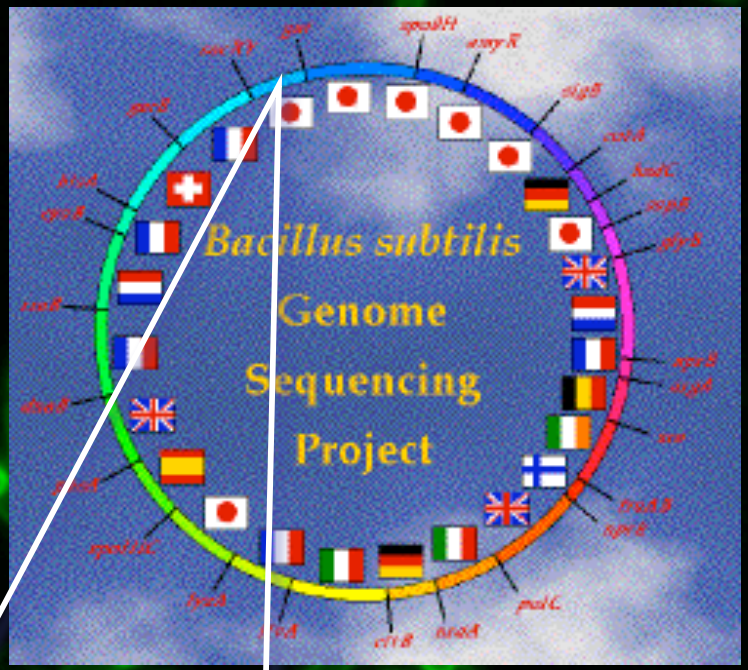
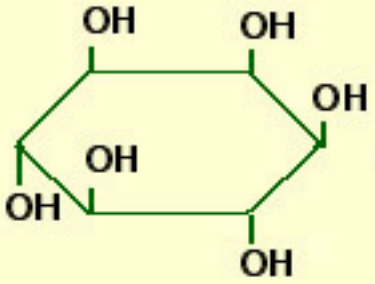
Completion of the whole genome sequence of *Bacillus subtilis* 168

Nature (1997)



There found a gene cluster for *myo*-inositol catabolism.

Inositol metabolism



myo-inositol

in bacteria

inositol dehydrogenase

inosose → DHAP + Acetyl CoA

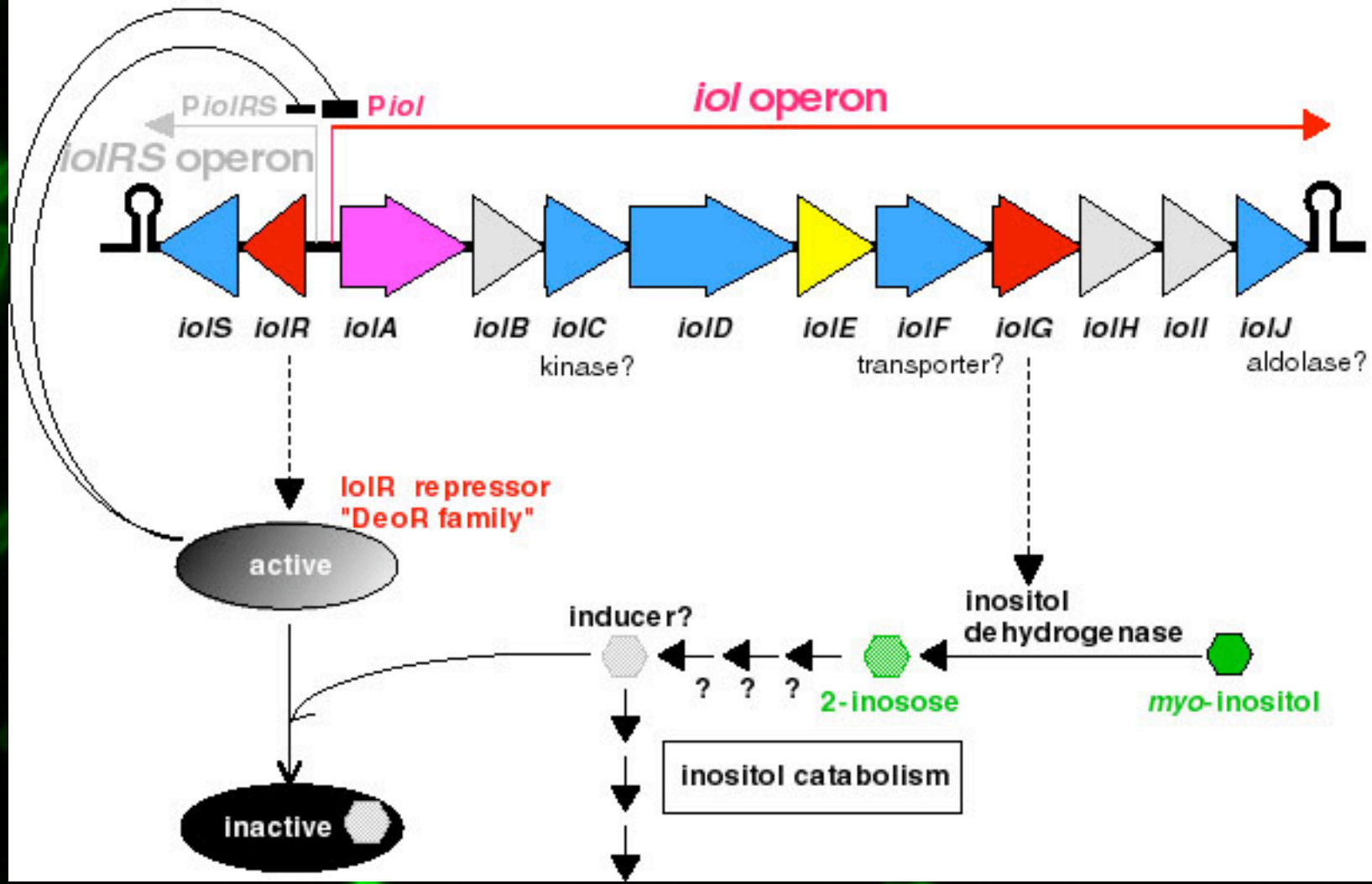
inositol oxygenase

in animals and plants

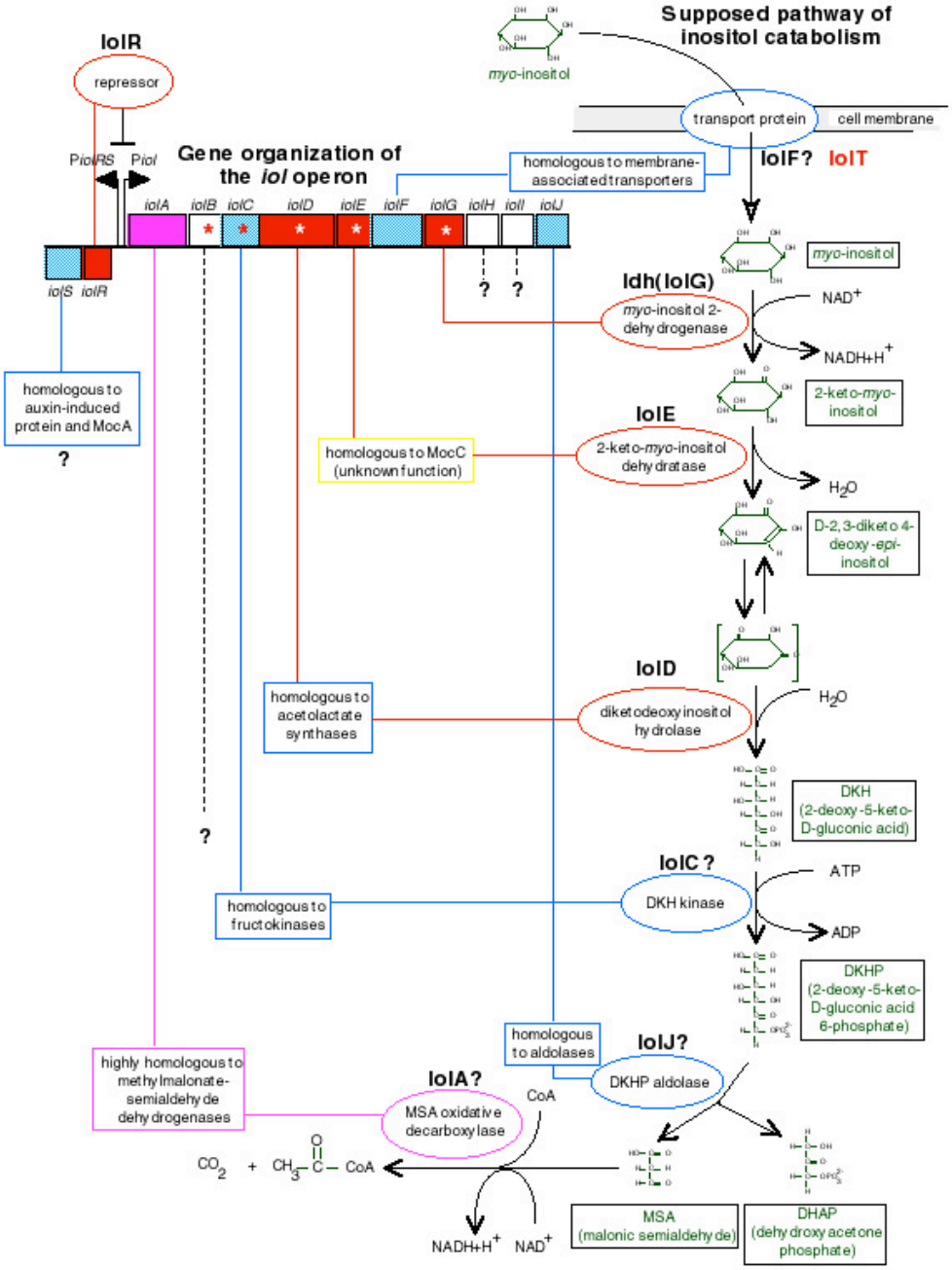
D-glucuronic acid → hexose phosphate (related metabolites)

D-glucuronic acid → pectin

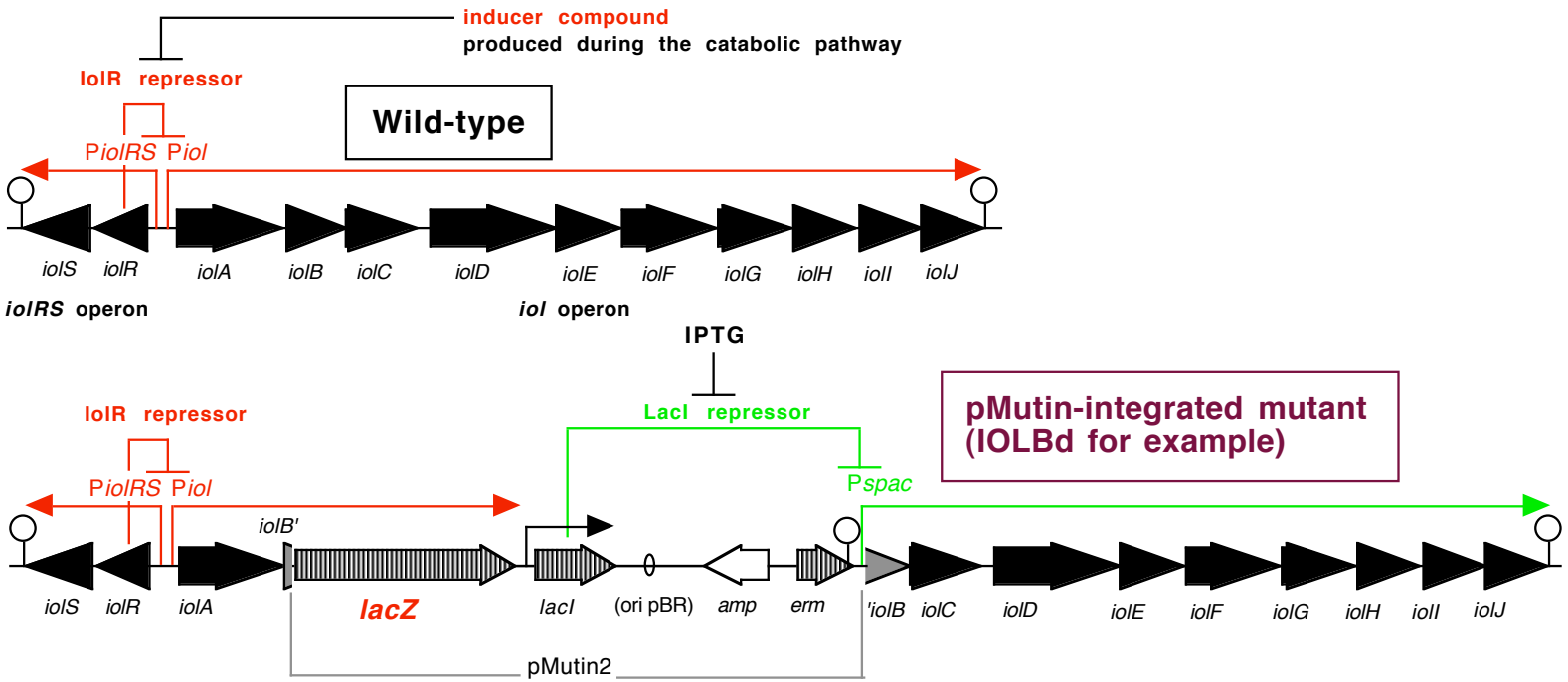
Structure and regulation of the *iol* divergon (*iol* & *iolRS* operons)



Function of the *iol* genes



pMutin-scanning:
to find the genes involved in inducer production



One of the *iol* genes was disrupted by a pMutin-integration. Expressior of the genes in the downstream of the integration point can be driven l the *spac* promoter (P*spac*).

i) If the disrupted gene is involved in the inducer production, LacZ is never produced.

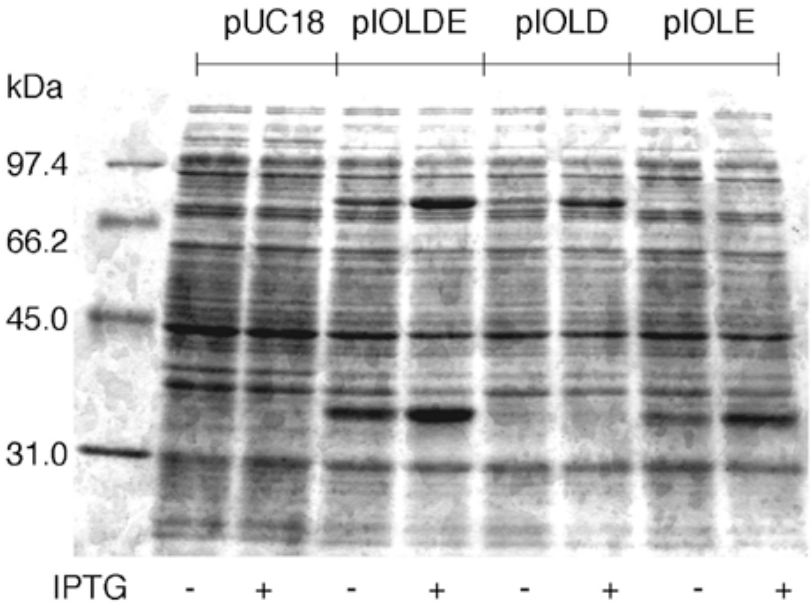
ii) If the disrupted gene is NOT involved in the inducer production, LacZ is produced upon addition of inositol or IPTG+inositol.

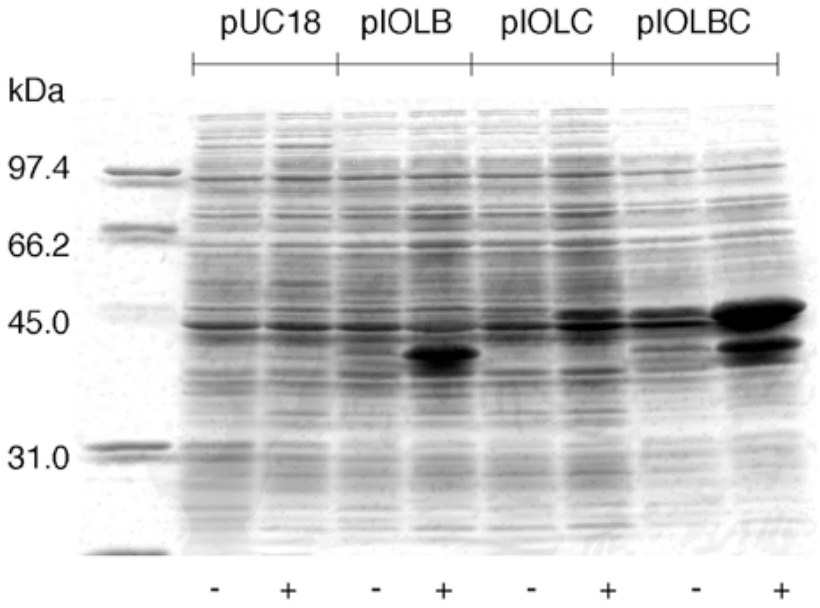
Results of pMutin-scanning on the *iol* operon

Strain	Relevant genotype	LacZ activity (nmoles/min/mg protein) in cells grown with:			
		None	iol	IPTG	iol+IPTG
60015	wild-type	0.9	0.1	1.1	0.3
IOLAd	*iolA*::pMutin2	5.6	5.1	5.5	549.2
IOLBd	*iolB*::pMutin2	4.9	5.1	4.8	6.6
IOLCd	*iolC*::pMutin2	3.8	2.4	3.0	9.9
IOLDd	*iolD*::pMutin2	2.0	2.4	2.3	2.4
IOLEd	*iolE*::pMutin2	4.5	4.0	4.5	4.0
IOLFd	*iolF*::pMutin1	8.3	74.5	8.9	192.6
IOLGd	*iolG*::pMutin1	5.7	12.2	8.3	6.1
IOLHd	*iolH*::pMutin1	11.0	580.7	6.1	587.9
IOLId	*iolI*::pMutin1	4.5	418.2	4.1	344.8
IOLJd	*iolJ*::pMutin1	7.0	498.3	6.7	349.6

When one of the *iolBCDEG* genes is disrupted, the *iol* promoter is never turned on. Thus, these five genes could be involved in the reactions until the production of a putative inducer compound.

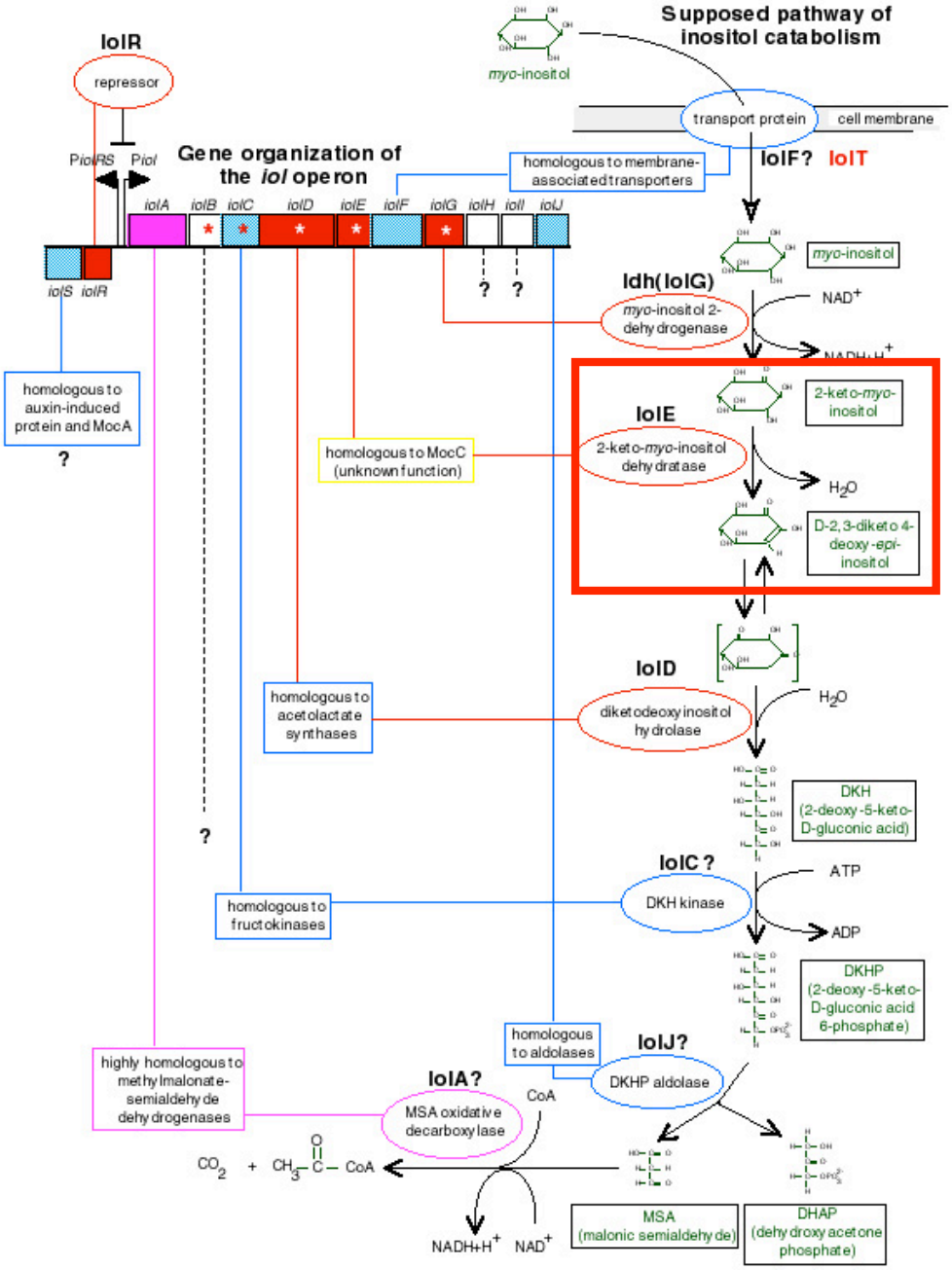
Expression of the *iol* genes in *Escherichia coli*





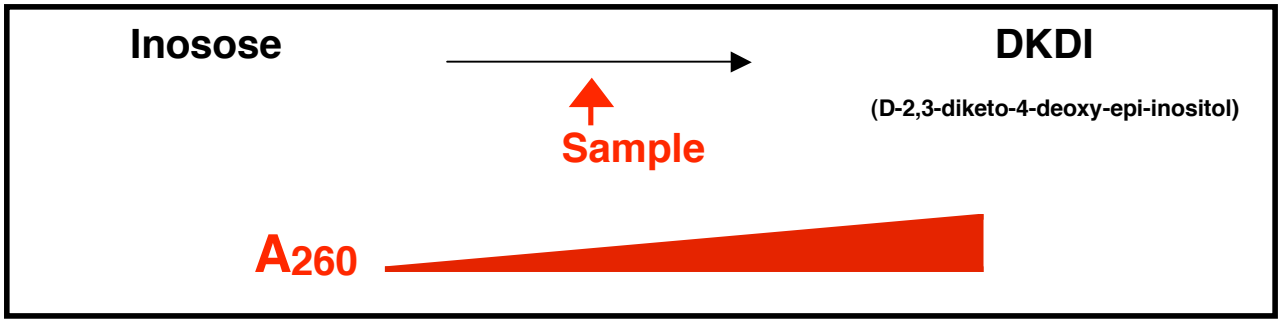
Some of the *iol* genes were cloned into the cloning site of pUC18 vector to be expressed in *E. coli* under the control of the *lac* promoter. Thus each of the cloned genes was induced in the presence of IPTG.

Function of the *iol* genes



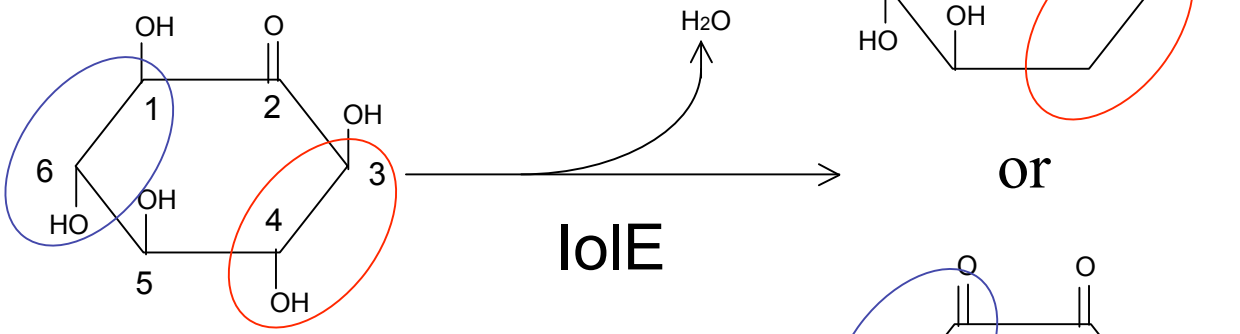
Inosose dehydratase activity in *E. coli* expressing the *iol* genes

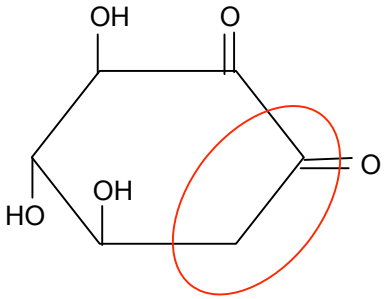
Strain	Inosose dehydratase activity (nmoles/min per mg protein) in cells grown with 1 mM IPTG
JM109/pUC18	2.9
JM109/pIOLB	1.2
JM109/pIOLC	1.0
JM109/pIOLD	4.2
JM109/pIOLE	1242.6



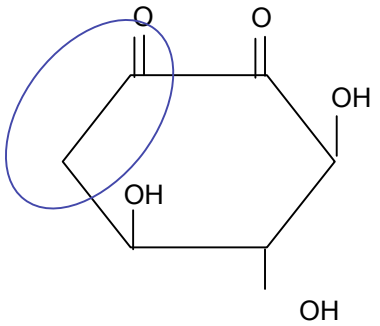
The *iolE* gene encodes inosose dehydratase*!!*

Two possible IolE reaction products



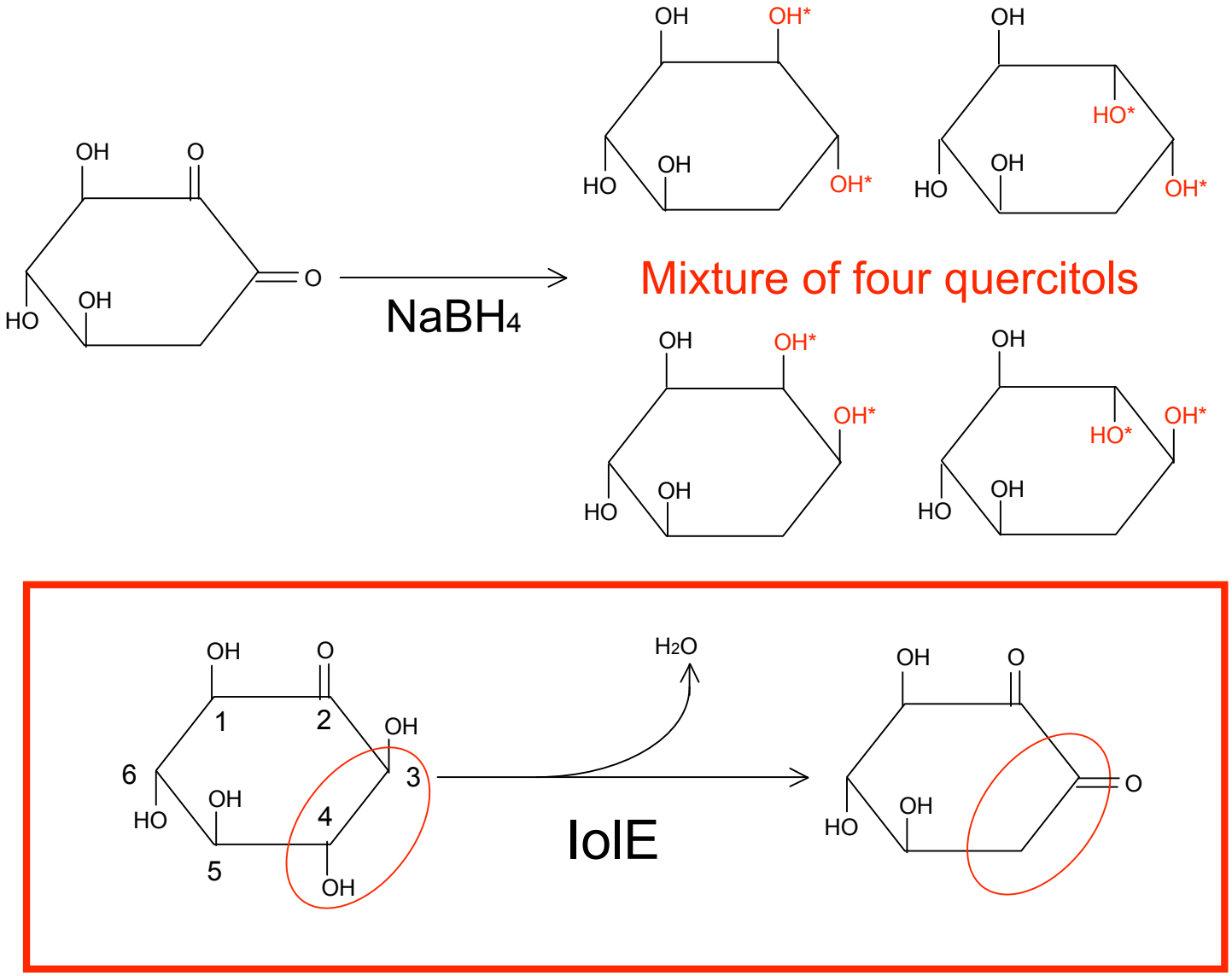


or



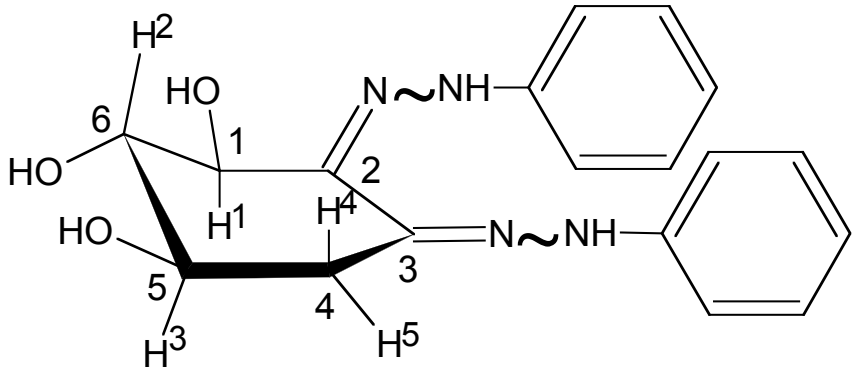
ESI-TOF/MS: $[C_6H_8O_5\text{-}H]^-$,
m/z 159.0297 (ca. m/z 159.0288)

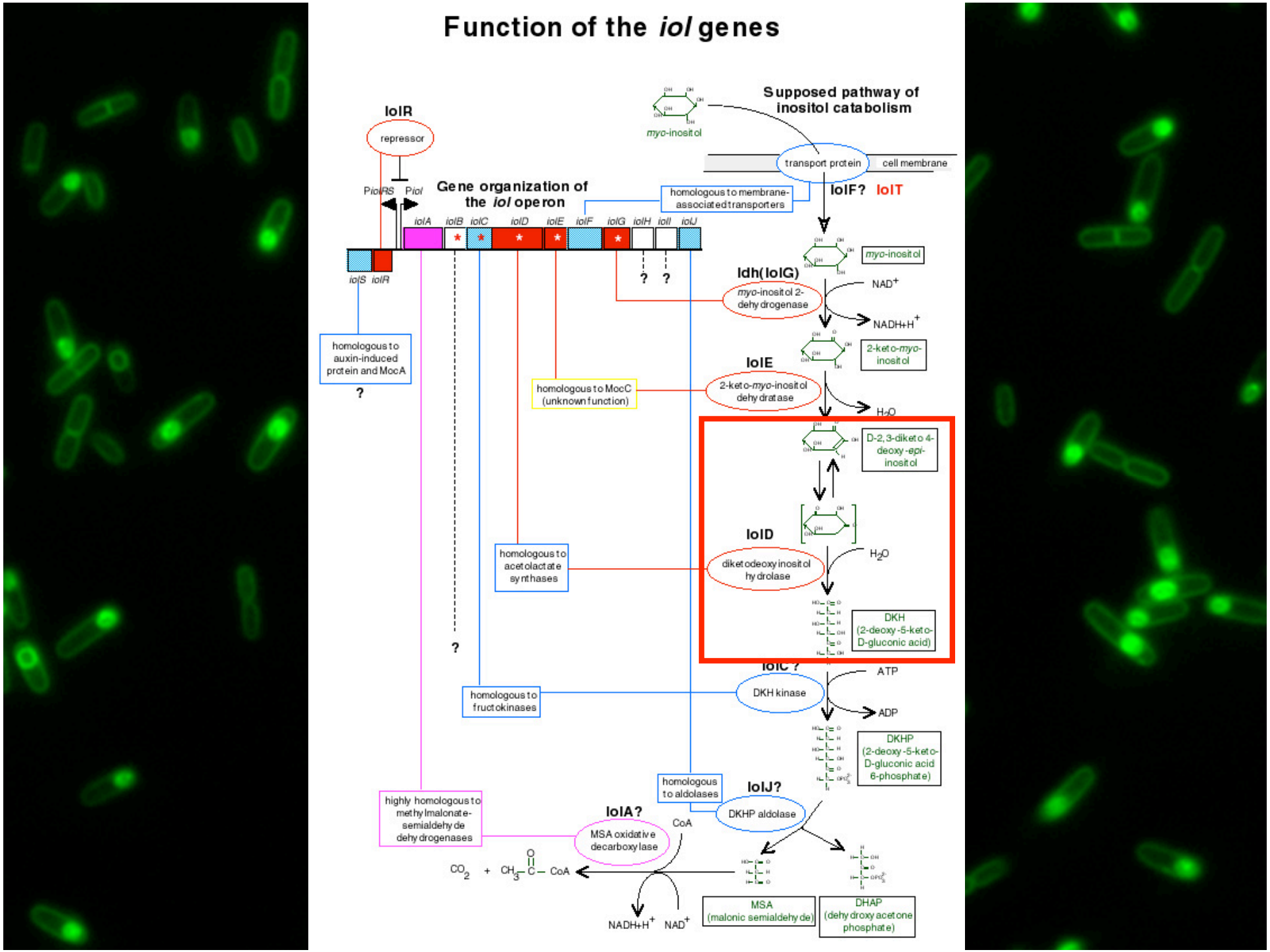
Reduction of the IolE reaction product



Identification of the IolE reaction product (bisphenylhydrazone)

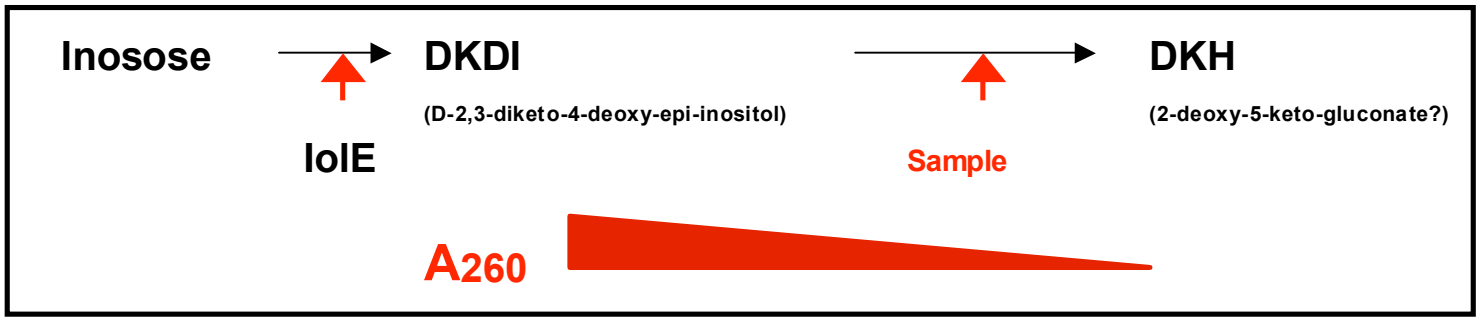
Summary of ^{1}H-NMR (300 MHz, CD_3OD)
δ4.25 (1H, d, H-1)
δ3.67 (1H, t, H-2)
δ3.88 (1H, dt, H-3)
δ2.53 (1H, dd, H-4)
δ3.07 (1H, dd, H-5)
$J_{1,2}$=7.1Hz, $J_{2,3}$=$J_{3,4}$=8.0Hz, $J_{3,5}$=6.0Hz, $J_{4,5}$=17.2Hz




Function of the iol genes
Supposed pathway of inositol catabolism
myo-inositol
transport protein
cell membrane
IolF? IolT
homologous to membrane-associated transporters
IolR
repressor
PiolRS
Piol
Gene organization of the iol operon
iolA iolB iolC iolD iolE iolF iolG iolH iolI iolJ
iolS iolR
? ?
homologous to auxin-induced protein and MocA
?
Idh(IolG)
myo-inositol 2-dehydrogenase
NAD+
NADH+H+
2-keto-myo-inositol
IolE
2-keto-myo-inositol dehydratase
homologous to MocC (unknown function)
H2O
D-2,3-diketo 4-deoxy-epi-inositol
IolD
diketodeoxyinositol hydrolase
homologous to acetolactate synthases
DKH (2-deoxy-5-keto-D-gluconic acid)
?
IolC ?
DKH kinase
ATP
ADP
homologous to fructokinases
DKHP (2-deoxy-5-keto-D-gluconic acid 6-phosphate)
homologous to aldolases
IolJ?
DKHP aldolase
highly homologous to methylmalonate-semialdehyde dehydrogenases
IolA?
MSA oxidative decarboxylase
CoA
CO2 + CH3-C(=O)-CoA
NADH+H+ NAD+
MSA (malonic semialdehyde)
DHAP (dehydroxyacetone phosphate)

Diketodeoxyinositol hydrolase activity in *E. coli* expressing the *iol* genes

Strain	Diketodeoxyinositol hydrolase activity (nmoles/min/mg protein) in cells grown with 1 mM IPTG
JM109/pUC18	14.3
JM109/pIOLB	20.2
JM109/pIOLC	23.6
JM109/pIOLD	253.0

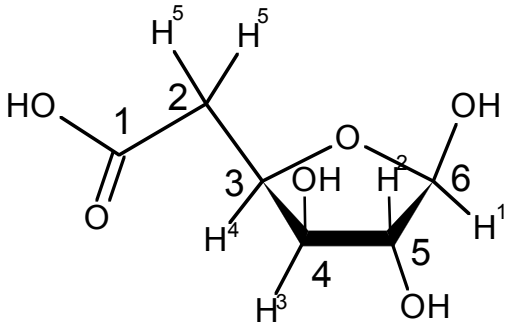


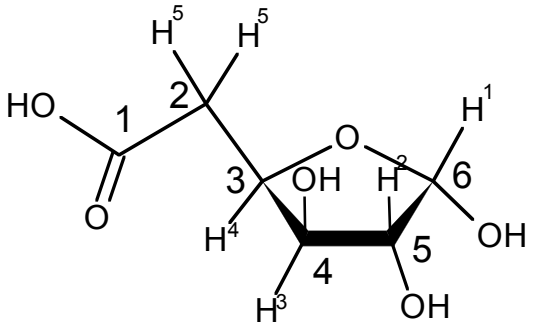
The *iolD* gene encodes DKDI hydrolase*!!*

Identification of the IolD reaction product

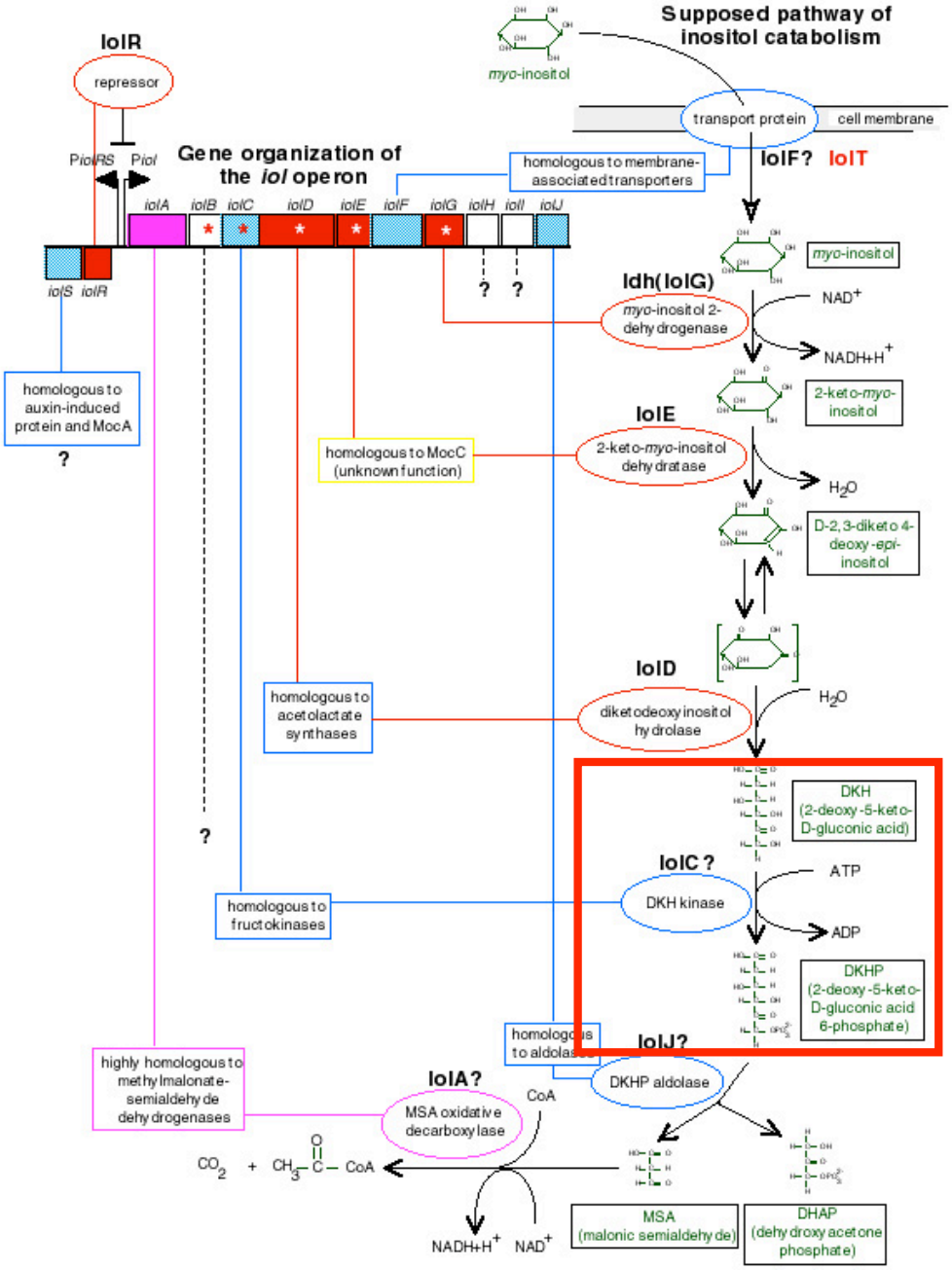
5-deoxy-glucuronate β-anomer Summary of ^{1}H-NMR (300MHz, D_2O)
δ5.28 (1H, d, H-1)
δ3.98 (1H, t, H-2)
δ4.13 (1H, dd, H-3)
δ4.47 (1H, dt, H-4)
δ2.54 (2H, dd, H-5)

5-deoxy-glucuronate α-amoner Summary of ^{1}H-NMR (300MHz, D_2O)
δ5.05 (1H, d, H-1)
δ3.96 (1H, t, H-2)
δ4.04 (1H, dd, H-3)
δ4.43 (1H, dt, H-4)
δ2.41 (2H, dd, H-5)



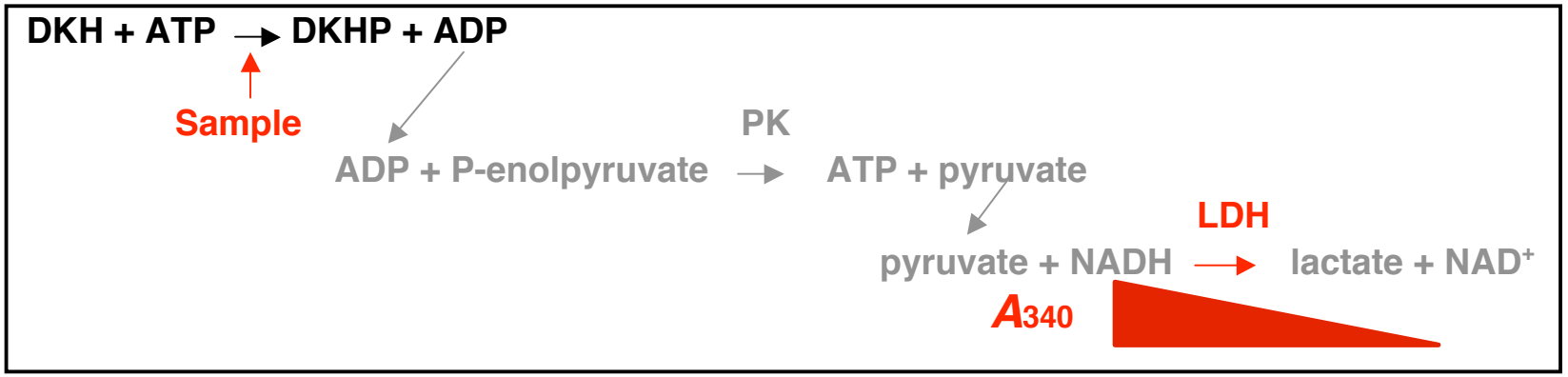


Function of the *iol* genes



DKH kinase activity in *E. coli* expressing the *iol* genes

Strain	rate: ΔA_{340}/min/mg protein without DKH	with DKH
JM109/pUC18	1.64	1.75
JM109/pIOLB	0.57	0.97
JM109/pIOLC	0.71	1.08
JM109/pIOLBC	0.40	4.55
JM109/pIOLB plus JM109/pIOLC	0.89	1.52



Co-expression of *iolB* and *iolC* is needed for the full activity of DKH kinase?*!!*

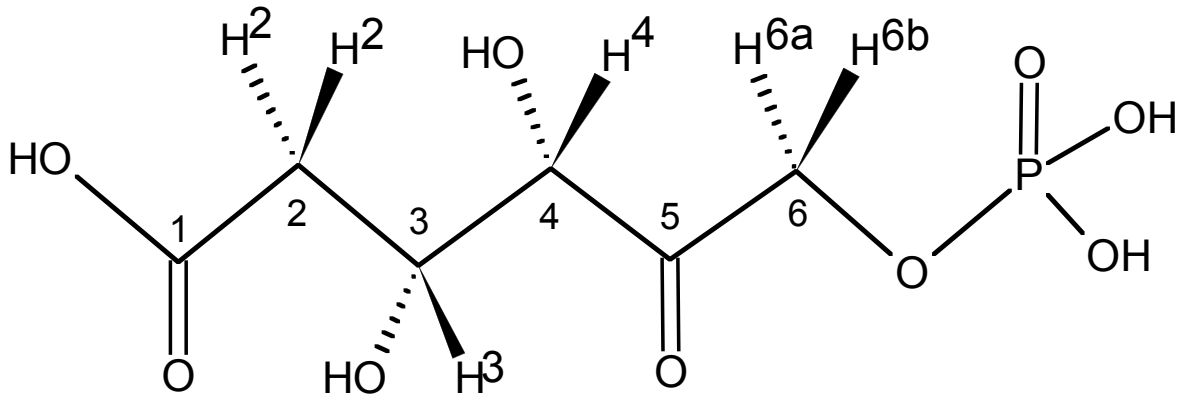
DKH kinase activity in strains of *B. subtilis*

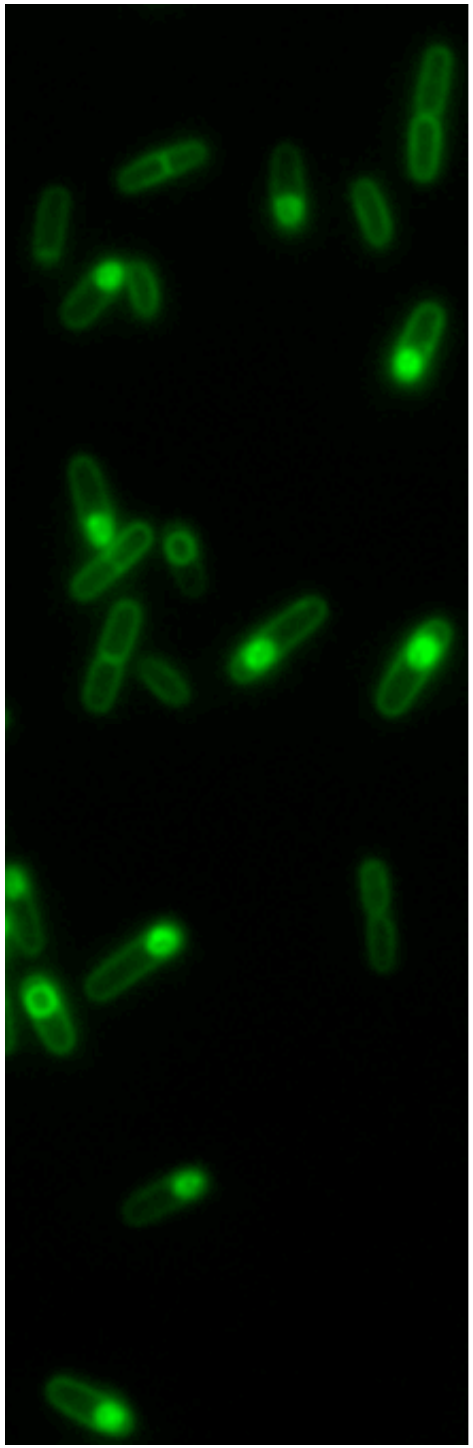
Strain	Relevant genotype	Grown with 10 mM inositol	Rate: ΔA_{340}/min/mg protein without DKH	with DKH
60015	wild-type	-	0.12	0.11
		+	0.18	0.37
YF244	*iolR::cat*	-	0.23	0.88
		+	0.27	0.82
YF258	*iolR::cat iolB52*(Q137OCH)	-	0.15	0.31
YF259	*iolR::cat iolB58*(E162K)	-	0.12	0.31
YF260	*iolR::cat iolC62*(A269T)	-	0.17	0.11

Both of the functional IolB and IolC are indispensable for DKH kinase activity *!!*

Identification of the IolB/C reaction product

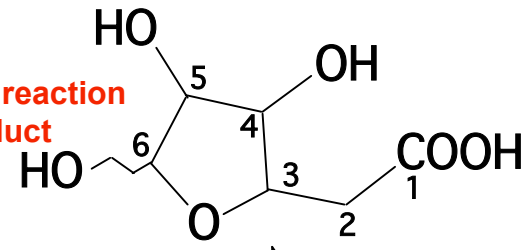
2-deoxy-5-keto-D-gluconate 6-phosphate
Summary of ^{1}H-NMR (300MHz, D_2O)
δ2.42 (2H, d, H-2)
δ4.35 (1H, dt, H-3)
δ4.33 (1H, d, H-4)
δ4.65 (1H, dd, H-6a)
δ4.75 (1H, dd, H-6b)

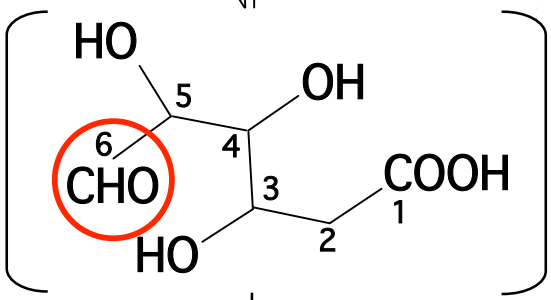




IolB reaction: working hypothesis

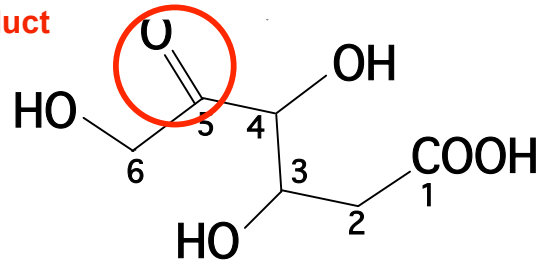
IolD reaction product

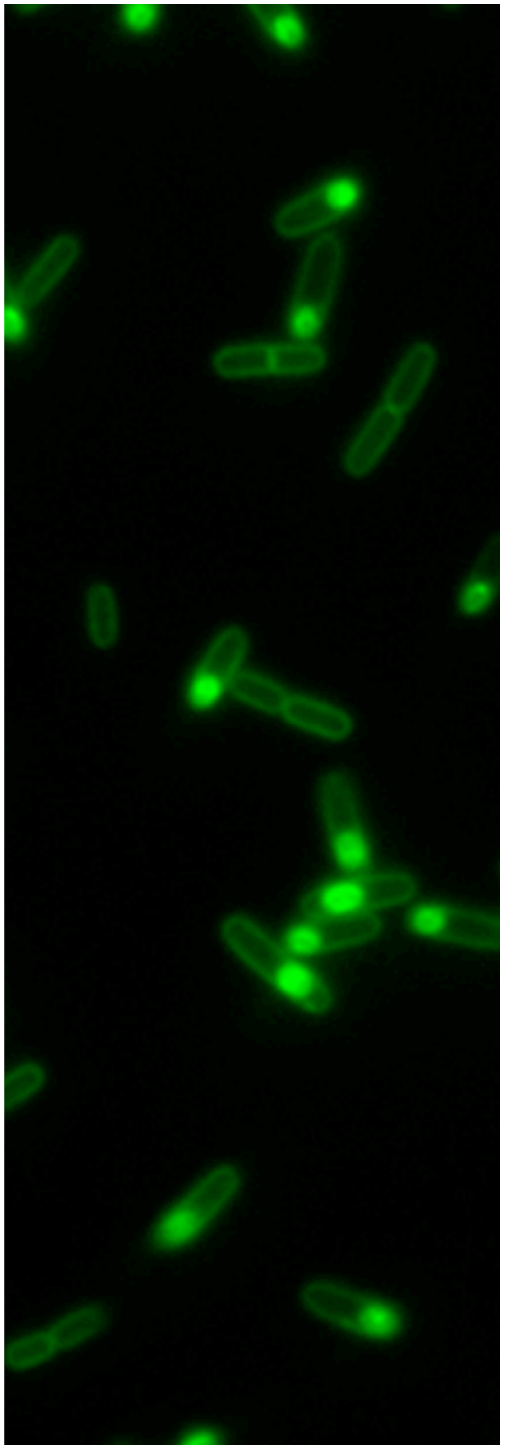




IolB isomerase?

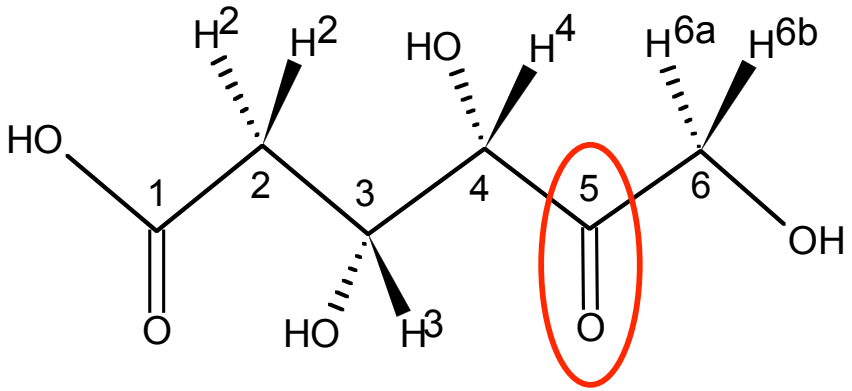
IolB reaction product

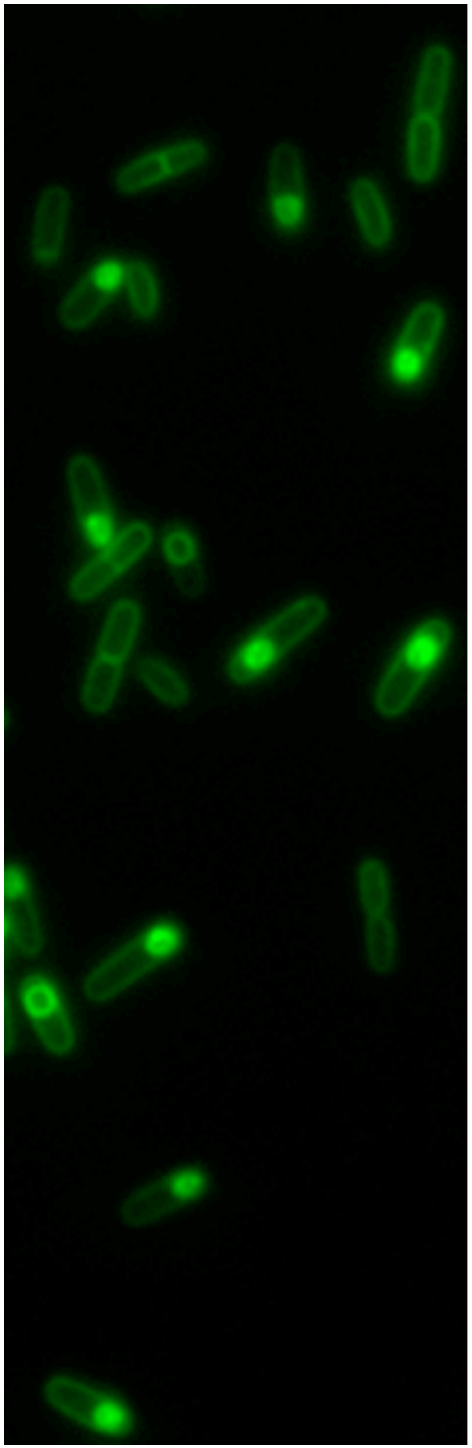




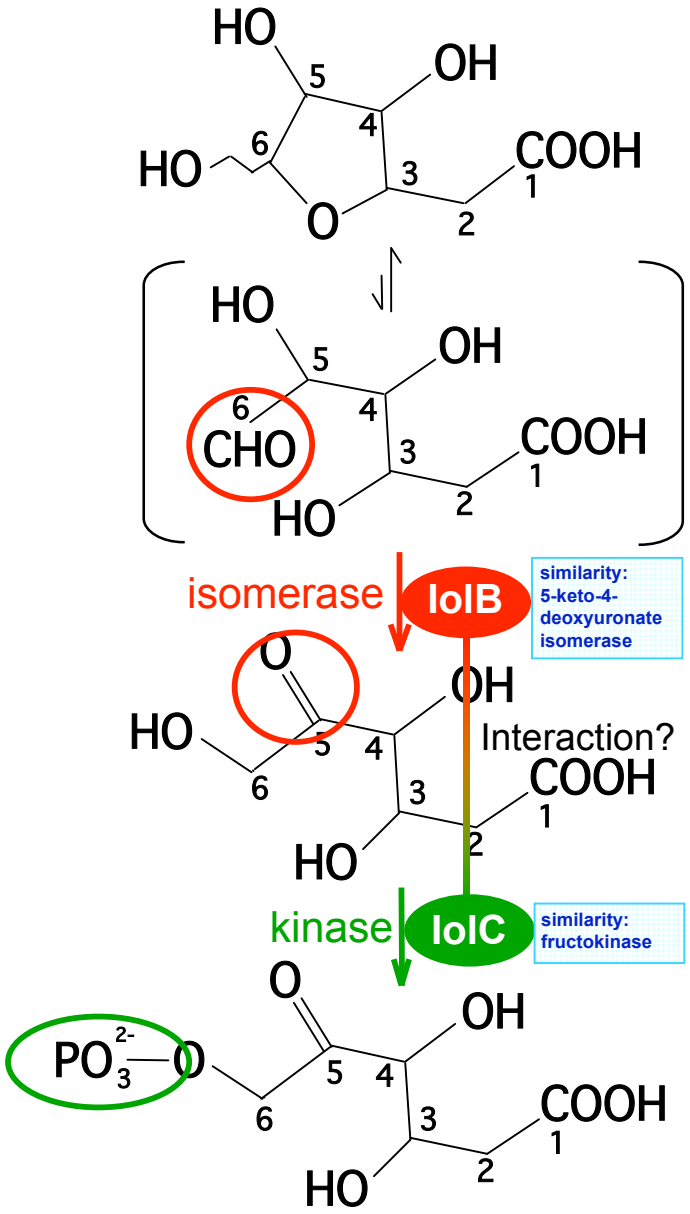
Identification of the IolB reaction product

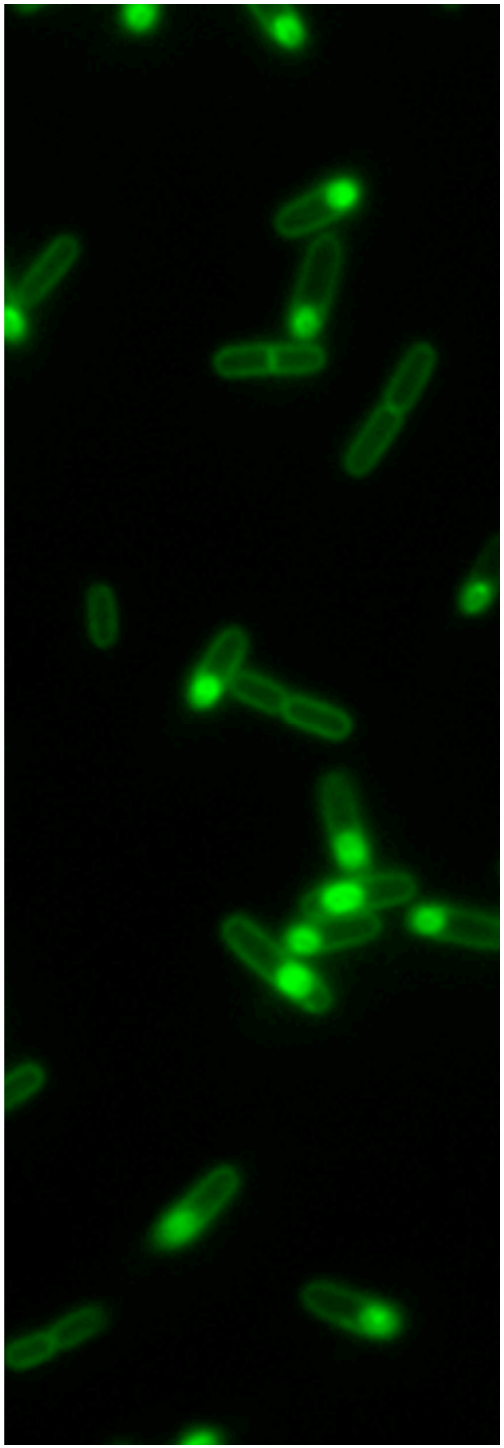
2-deoxy-5-keto-D-gluconate Summary of ^{1}H-NMR (300MHz, D_2O)
δ2.44 (2H, d, H-2)
δ4.27 (1H, dt, H-3)
δ4.23 (1H, d, H-4)
δ4.41 (1H, d, H-6a)
δ4.53 (1H, d, H-6b)

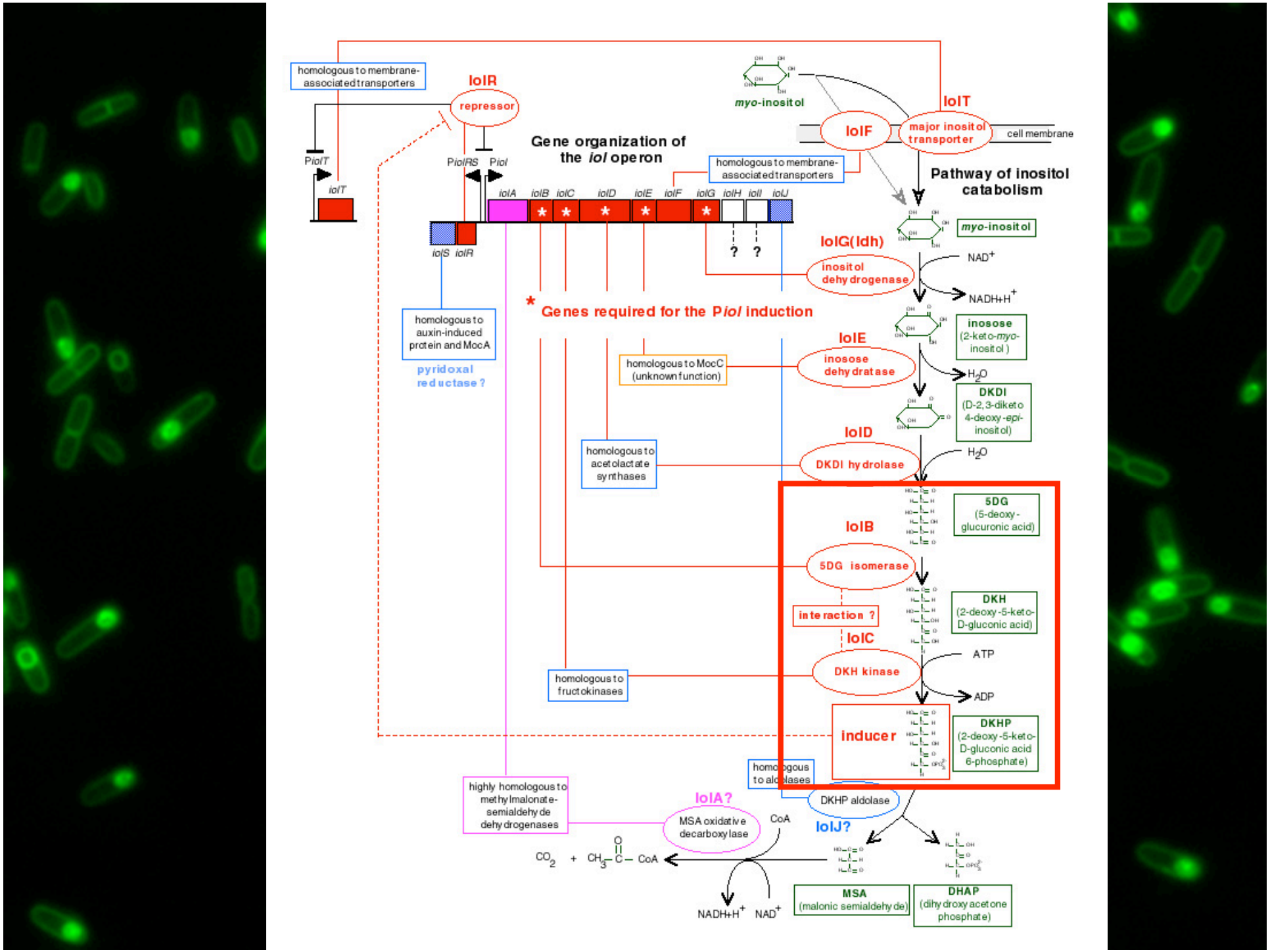




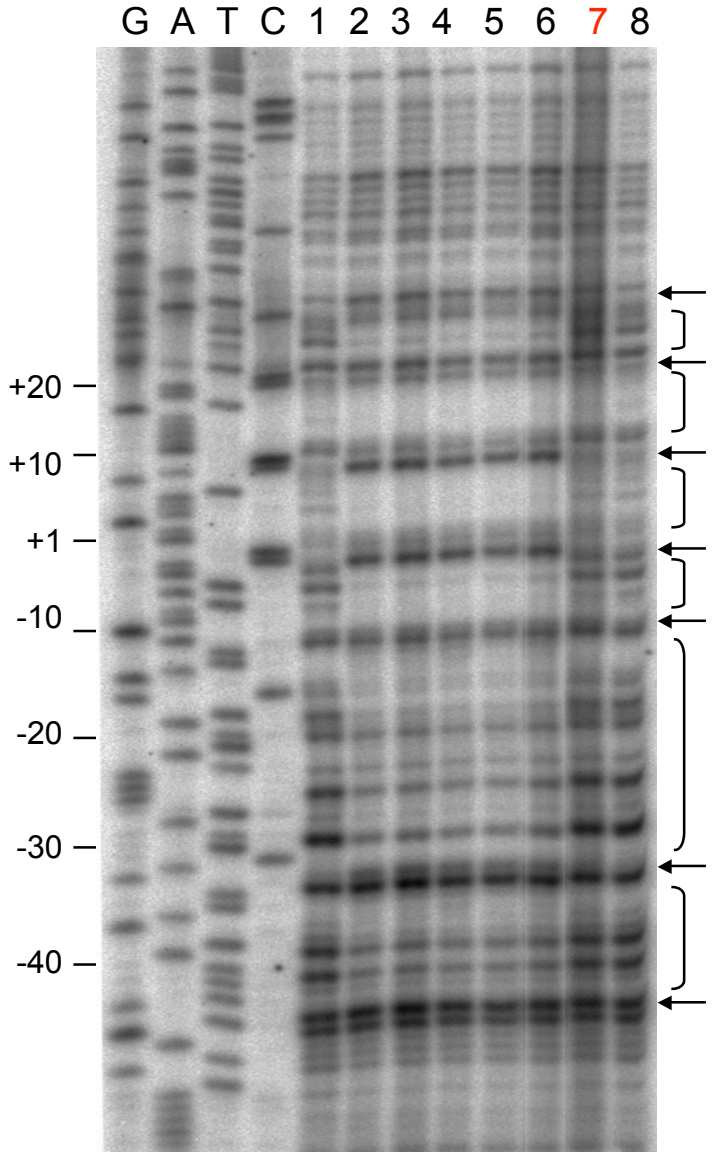
IolBC reaction






homologous to membrane-associated transporters
IolR
repressor
PiolT
iolT
PiolRS
Piol
iolS
iolR
Gene organization of the iol operon
iolA iolB iolC iolD iolE iolF iolG iolH iolI iolJ
homologous to membrane-associated transporters
myo-inositol
IolT
major inositol transporter
cell membrane
IolF
Pathway of inositol catabolism
myo-inositol
IolG(Idh)
inositol dehydrogenase
NAD+
NADH+H+
inosose (2-keto-myo-inositol)
IolE
inosose dehydratase
H2O
DKDI (D-2,3-diketo 4-deoxy-epi-inositol)
IolD
DKDI hydrolase
H2O
5DG (5-deoxy-glucuronic acid)
IolB
5DG isomerase
DKH (2-deoxy-5-keto-D-gluconic acid)
interaction ?
IolC
DKH kinase
ATP
ADP
DKHP (2-deoxy-5-keto-D-gluconic acid 6-phosphate)
inducer
* Genes required for the Piol induction
homologous to auxin-induced protein and MocA
pyridoxal reductase ?
homologous to MocC (unknown function)
homologous to acetolactate synthases
homologous to fructokinases
homologous to aldolases
DKHP aldolase
IolA?
MSA oxidative decarboxylase
highly homologous to methylmalonate-semialdehyde dehydrogenases
CoA
IolJ?
CO2 + CH3-C(=O)-CoA
NADH+H+
NAD+
MSA (malonic semialdehyde)
DHAP (dihydroxyacetone phosphate)

Identification of catabolic intermediate acting as the inducer by IolR DNase I footprinting on the *iol* operator region



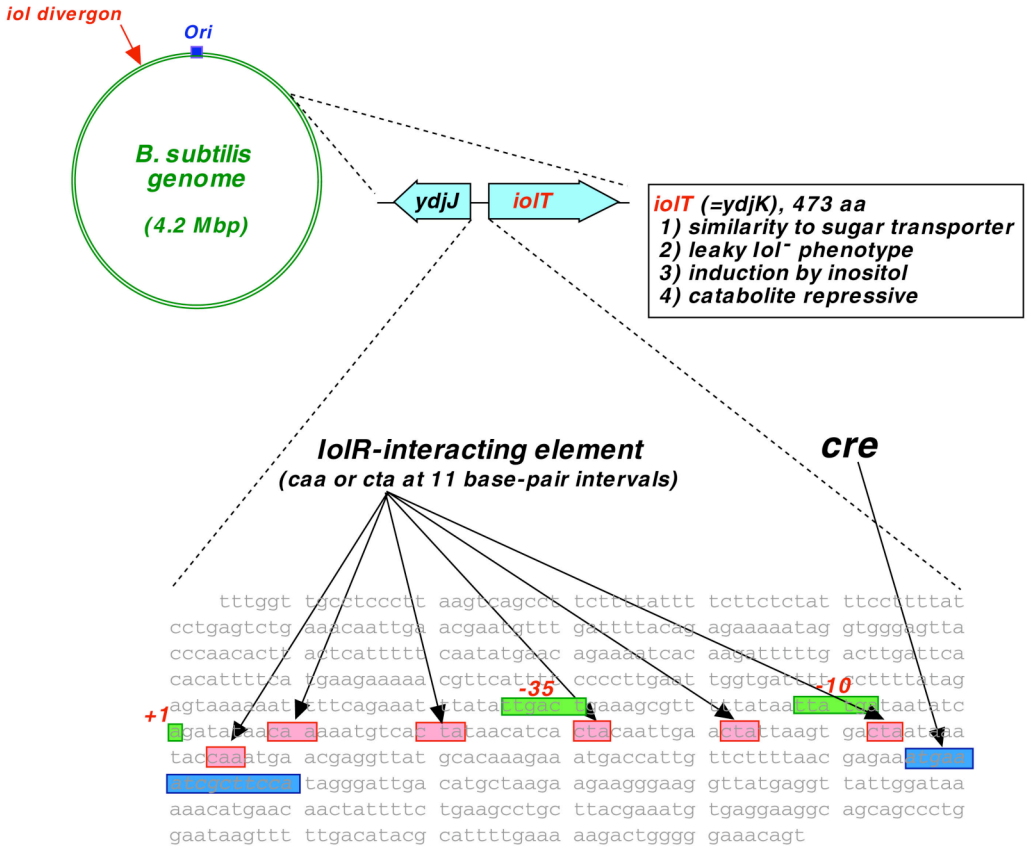
Lane assignment

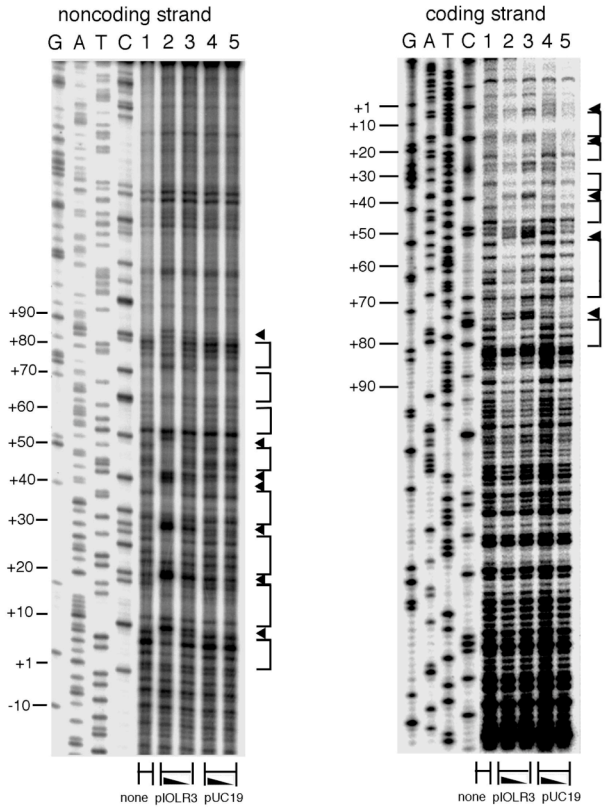
1. without any protein extract
2. pIOLR extract
3. pIOLR extract + 10 mM *myo*-inositol
4. pIOLR extract + 10 mM 2-inosose
5. pIOLR extract + 10 mM 2,3-diketo-4-deoxy-*epi*-inositol
6. pIOLR extract + 10 mM 5-deoxy-glucuronate/ 2-keto-5-deoxy-gluconate (1/2)
7. pIOLR extract + 10 mM 2-keto-5-deoxy-gluconate 6-phosphate
8. pUC18 extract

2-keto-5-deoxy-gluconate 6-phosphate is identified as a catabolic intermediate acting as the inducer that antagonizes IolR/operator interaction!

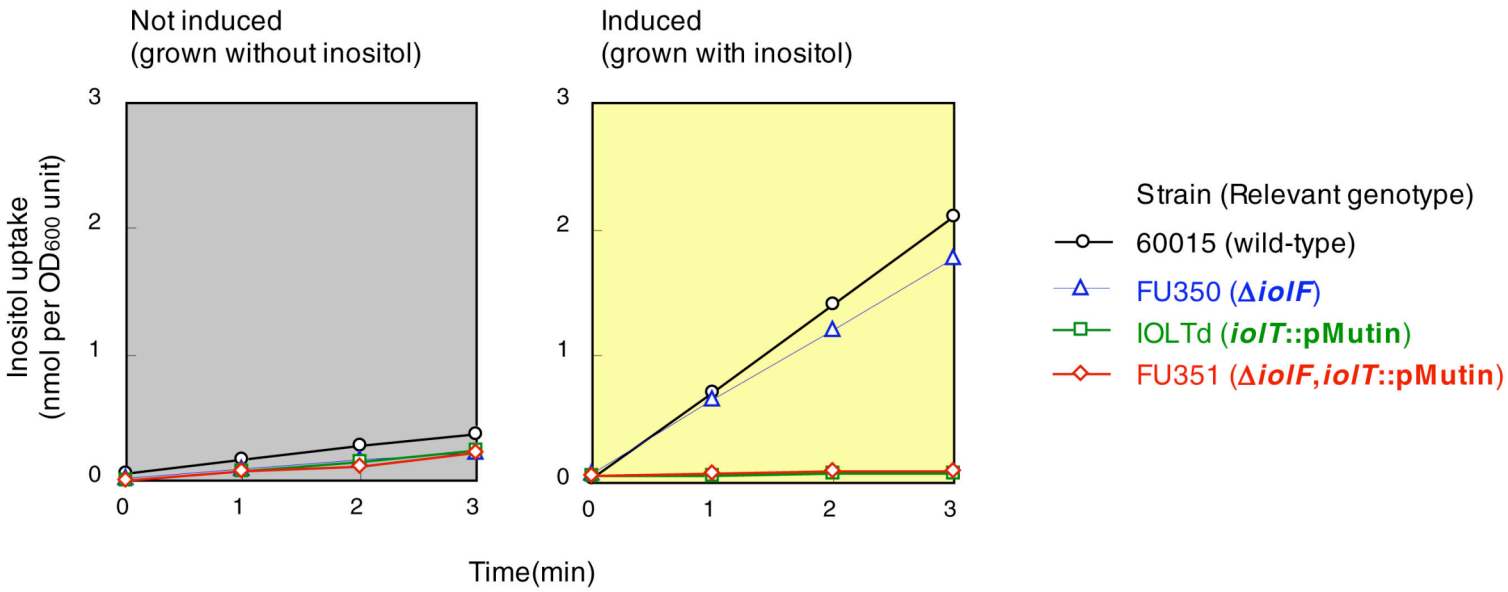

DNA microarray analysis: *iolR* mutant vs. wild type
IolR targets:
iolABCDEFGHIJ, *iolRS* and *iolT*
The 3rd target!!

iolT: a new member of "the iol regulon"





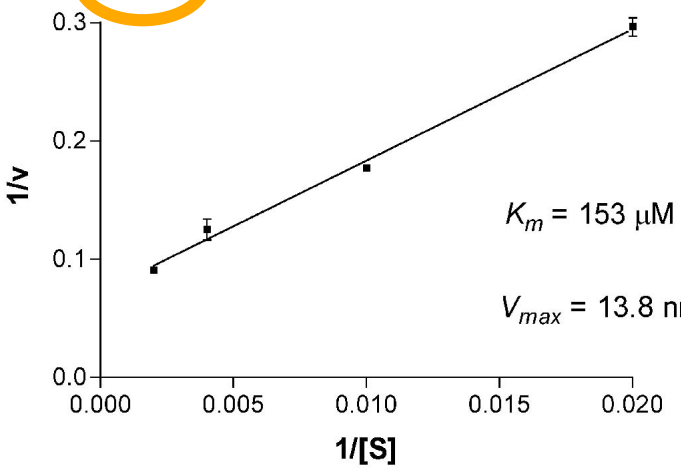
Inositol-uptake by strains of *B. subtilis*



!! The *iolT* and *iolF* genes encode major and minor inositol transporters, respectively !!

Determination of the K_m and V_{max} values of inositol transporters

IolT



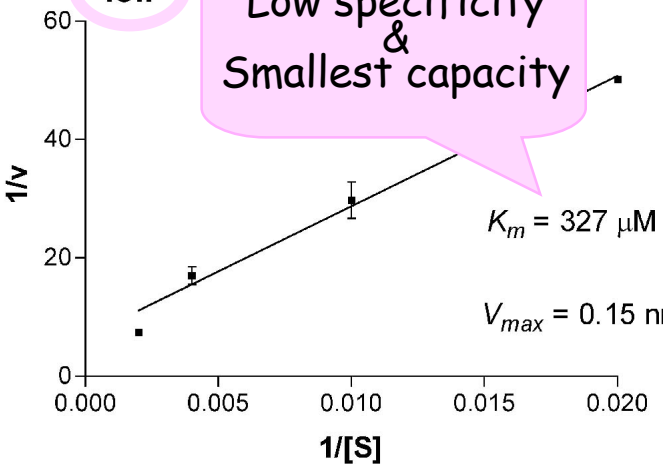
K_m = 153 μM

V_{max} = 13.8 nmol/min/OD_{600}

High specificity
&
Large capacity

IolF

Low specificity
&
Smallest capacity

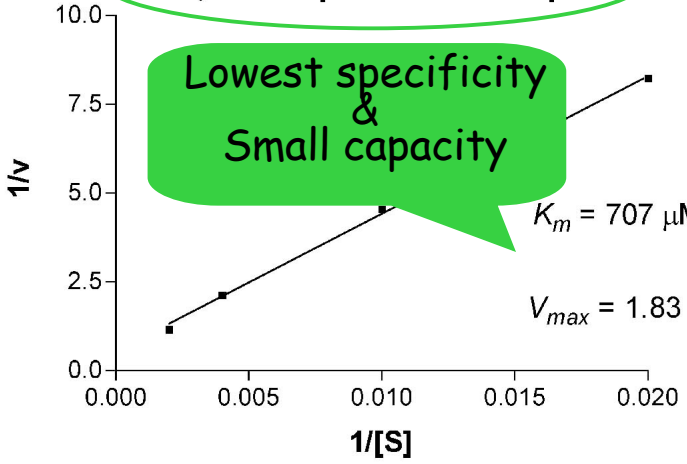


K_m = 327 μM

V_{max} = 0.15 nmol/min/OD_{600}

IolT, F-independent transport

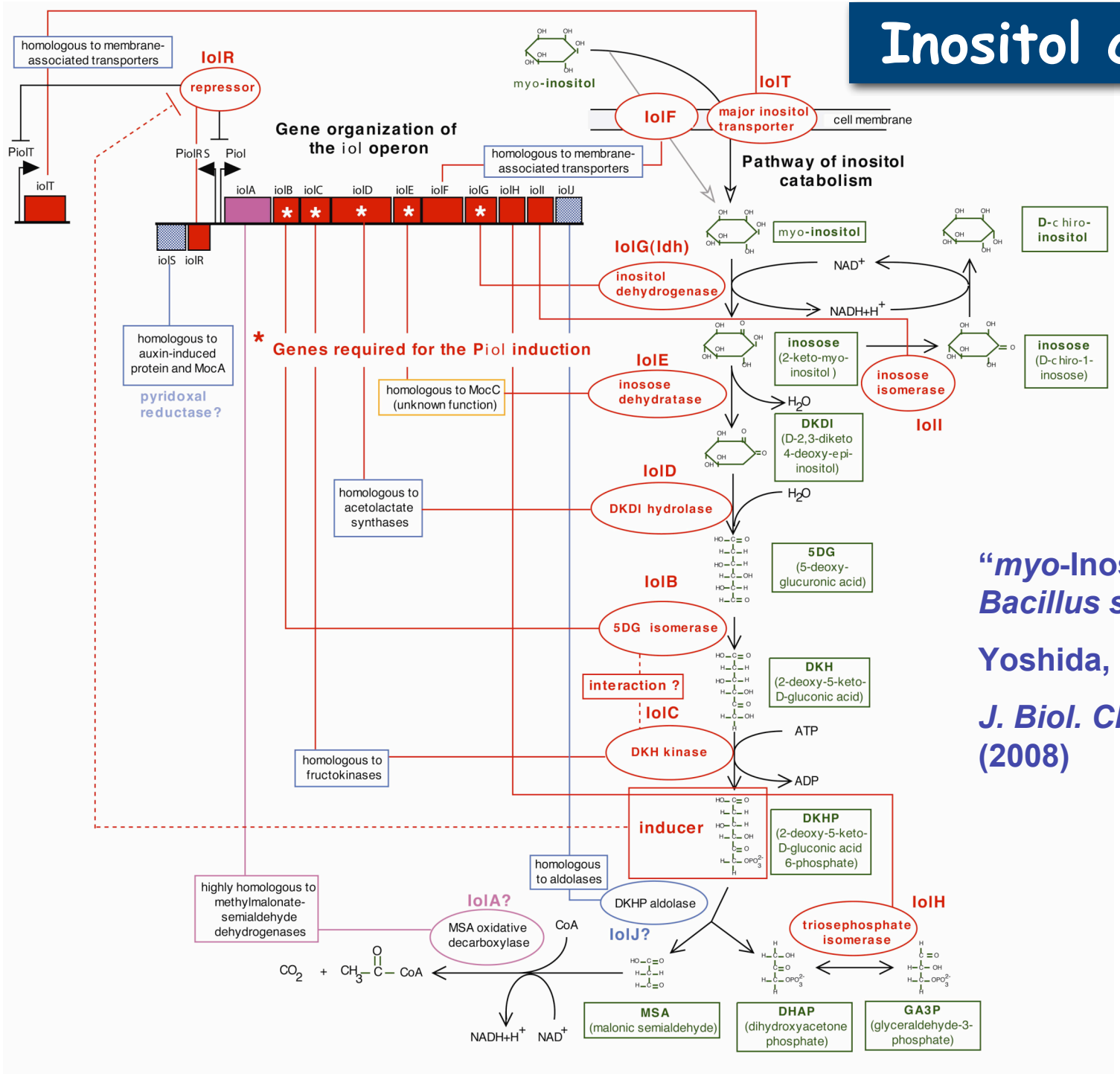
Lowest specificity
&
Small capacity



K_m = 707 μM

V_{max} = 1.83 nmol/min/OD_{600}

Inositol catabolism

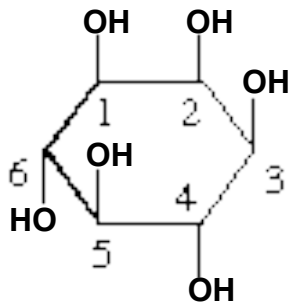


"*myo*-Inositol catabolism in *Bacillus subtilis*."

Yoshida, K. *et al.*

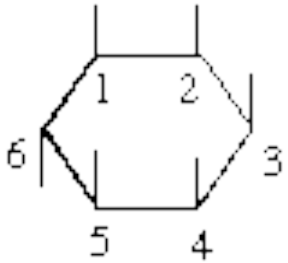
J. Biol. Chem. 283, 10415-24 (2008)

Inositol stereoisomers

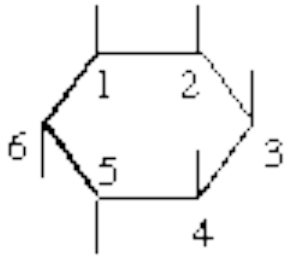


***myo*-inositol**

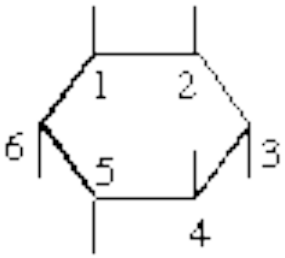
- 1,2,3,4,5,6-Cyclohexanehexol
- Nine stereoisomers
- Natural ingredients found in plants and animals
- Various biological functions



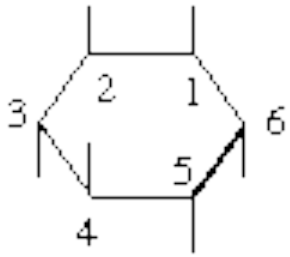
***epi*-inositol**



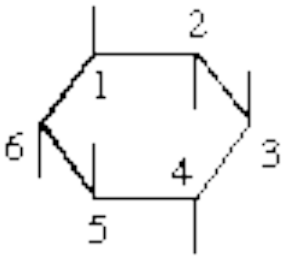
***allo*-inositol**



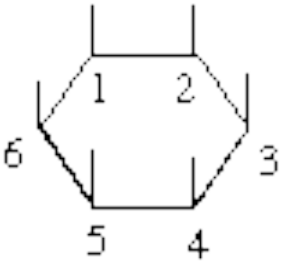
L-***chiro*-inositol**



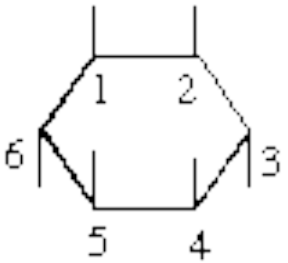
D-***chiro*-inositol**



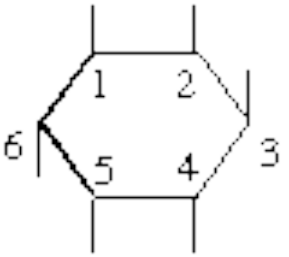
***scyllo*-inositol**



***cis*-inositol**

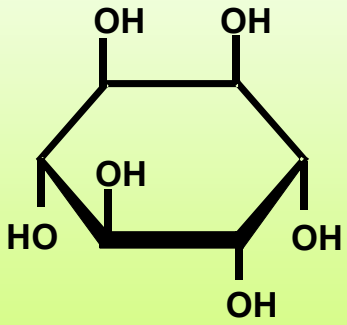


***muco*-inositol**



***neo*-inositol**

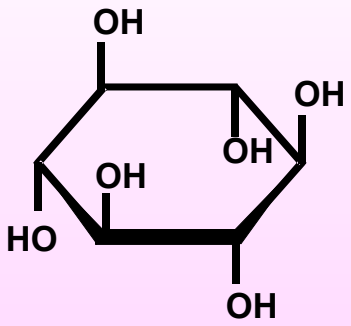
D-*chiro*-inositol (DCI)



We need an efficient way to produce DCI!

- **DCI lowers serum glucose in STZ rats, diabetes-model animals (Kawa *et al.*, 2003).**
- **A drug candidate for the treatment of type 2 diabetes and polycystic ovary syndrome, but too expensive at present.**

scyllo-inositol (SI)



They say “it can be a sweet solution...”

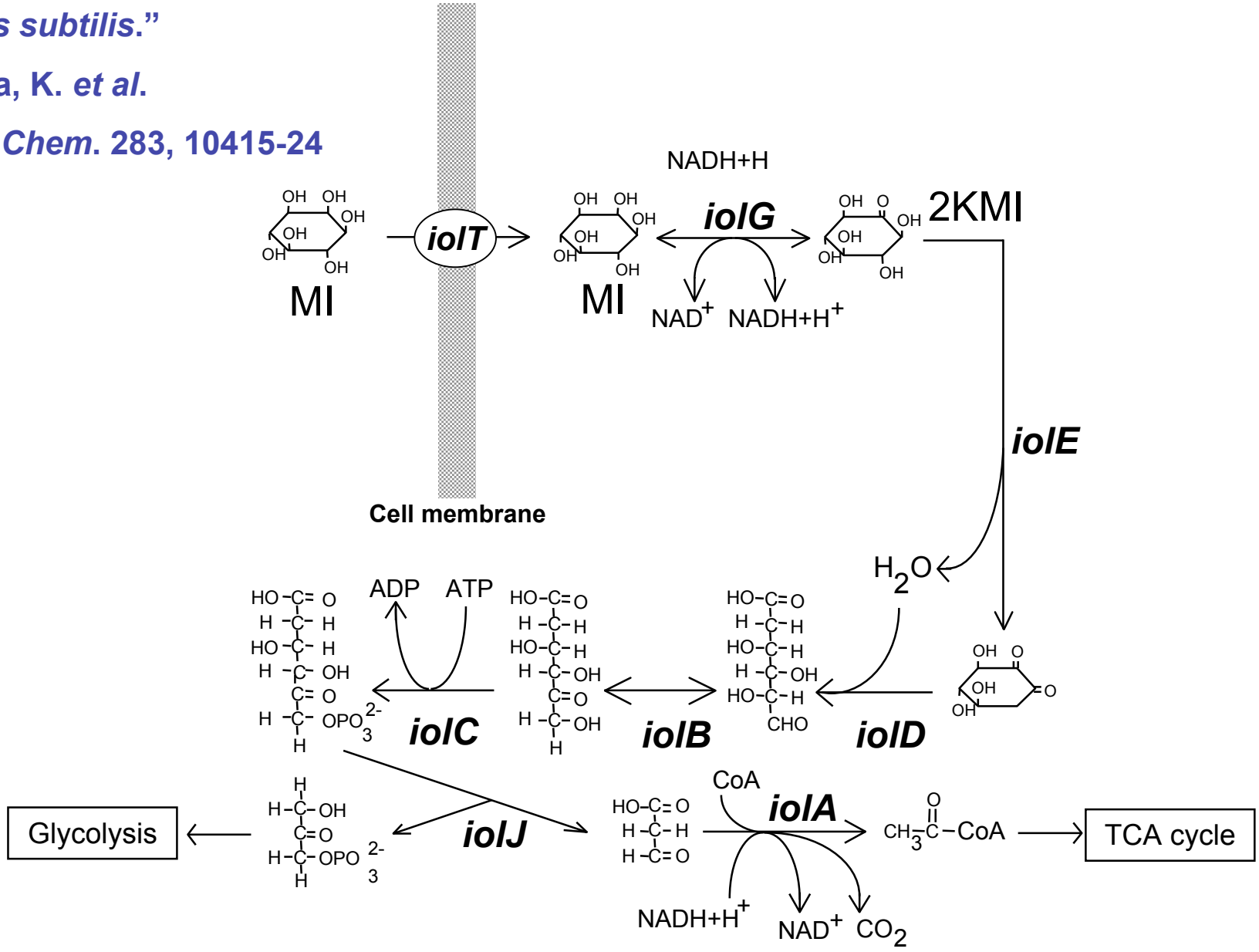
- **Some types of inositol prevented the accumulation of amyloid â deposits, a hallmark of Alzheimer's disease. *scyllo*-Inositol treatment also improved cognitive abilities in the mice and allowed them to live a normal lifetime. (Nature Medicine on June 11, 2006.)**

Inositol metabolism in *B. subtilis*

"*myo*-Inositol catabolism in *Bacillus subtilis*."

Yoshida, K. *et al*.

J. Biol. Chem. 283, 10415-24 (2008)



Inositol metabolism in *B. subtilis*

"Identification of two *scyllo*-inositol dehydrogenases in *Bacillus subtilis*."

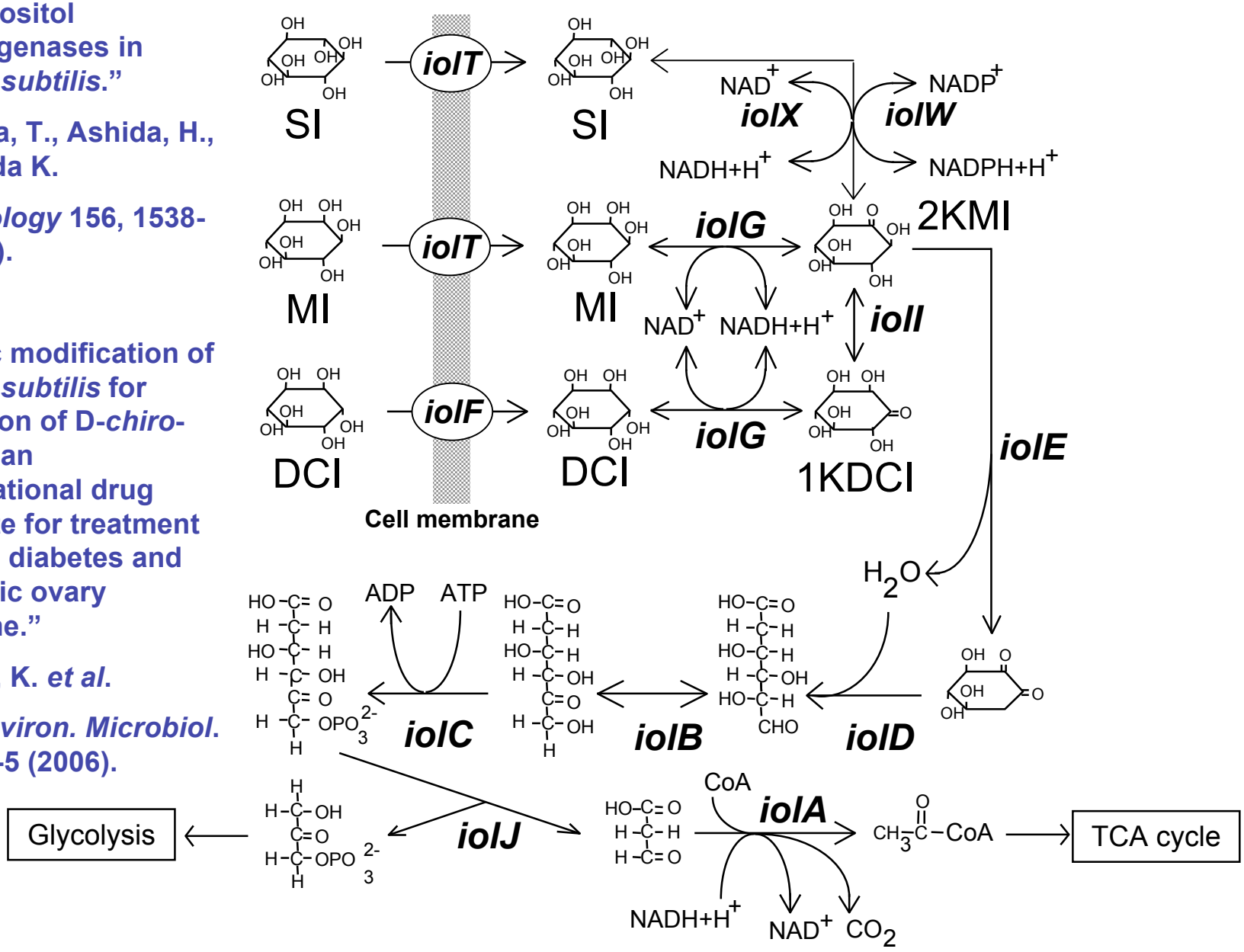
Morinaga, T., Ashida, H., & Yoshida K.

***Microbiology* 156, 1538-46 (2010).**

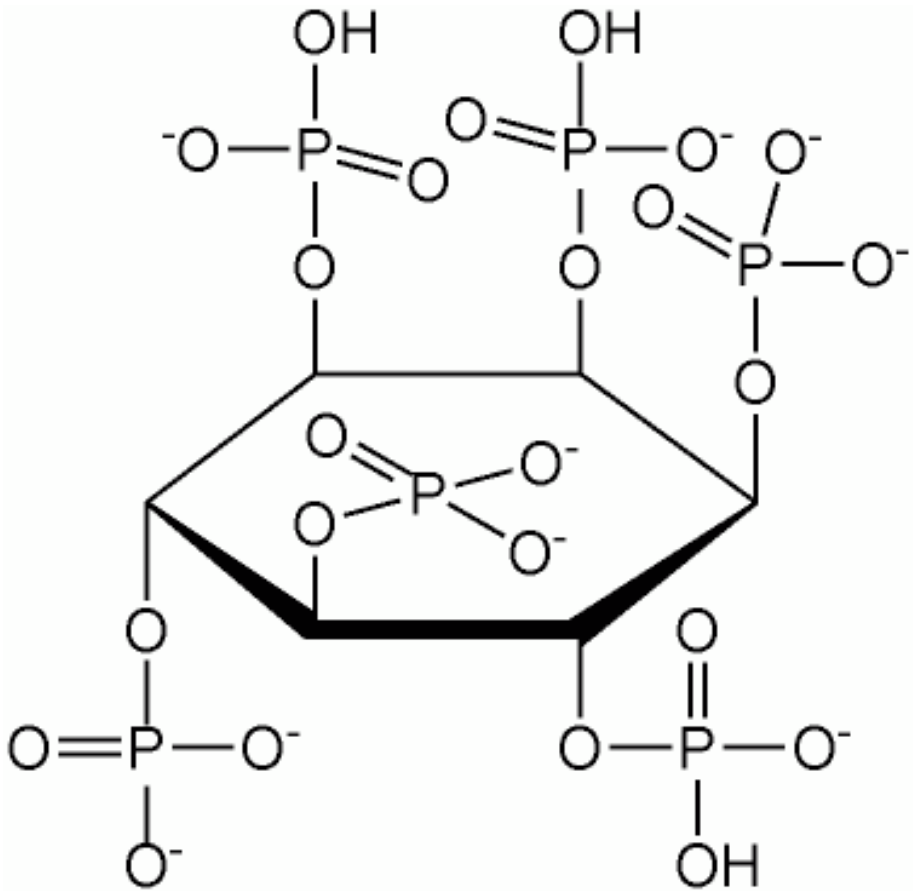
"Genetic modification of *Bacillus subtilis* for production of D-*chiro*-inositol, an investigational drug candidate for treatment of type 2 diabetes and polycystic ovary syndrome."

Yoshida, K. *et al*.

***Appl. Environ. Microbiol*. 72, 1310-5 (2006).**



Rice bran is rich in phytic acid (up to 5% of dry-weight).



Phytic acid (known as *myo*-inositol hexakisphosphate (IP_6), or phytate when in salt form) is the principle storage form of phosphorus in many plant tissues, especially bran and seeds.

Inositol metabolism in *B. subtilis*

Rice bran (phytate)

SI

iolT

SI

NAD$^+$ *iolX* NADH+H$^+$

NADP$^+$ *iolW* NADPH+H$^+$

2KMI

MI

iolT

MI

iolG

NAD$^+$ NADH+H$^+$

iolI

DCI

iolF

DCI

iolG

1KDCI

iolE

Cell membrane

H_2O

ADP ATP

iolC

iolB

iolD

Glycolysis

iolJ

CoA

iolA

TCA cycle

NADH+H$^+$ NAD$^+$ CO_2

Inositol metabolism in *B. subtilis*

Rice bran (phytate)

SI

MI

DCI

2KMI

1KDCI

iolT

iolF

iolX

iolW

iolG

iolI

iolE

iolC

iolB

iolD

iolJ

iolA

NAD+

NADH+H+

NADP+

NADPH+H+

ADP

ATP

H_2O

CoA

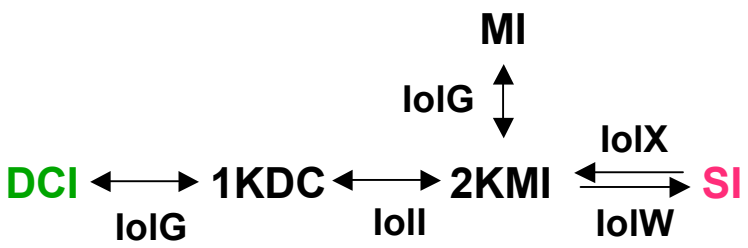
CO_2

Glycolysis

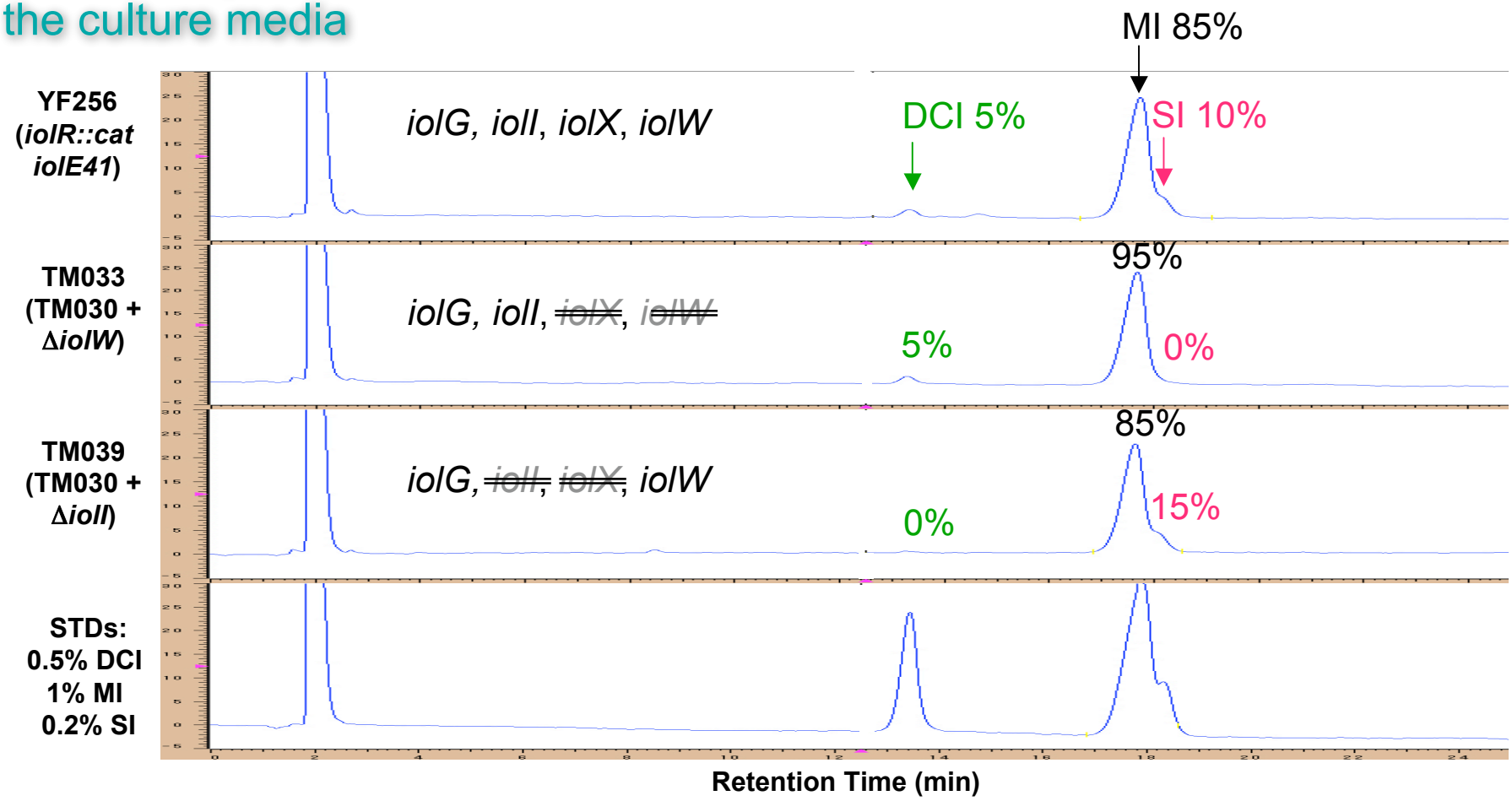
TCA cycle

Cell membrane

Inositol bioconversion: from MI to DCI and SI



HPLC analysis of the culture media

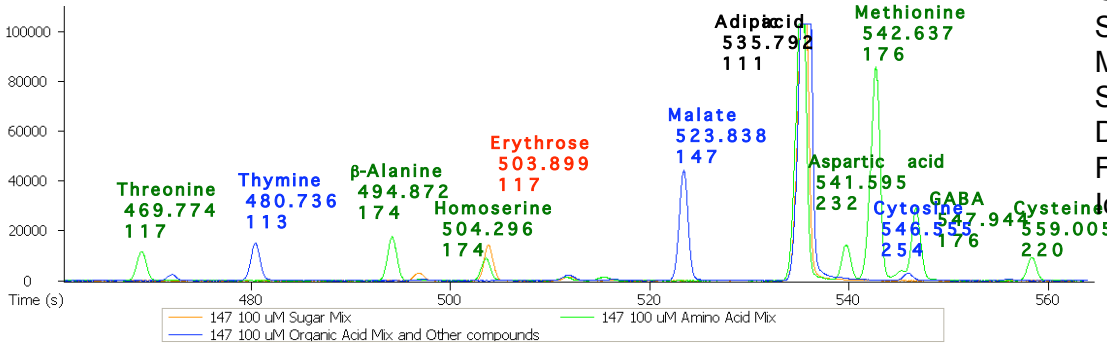


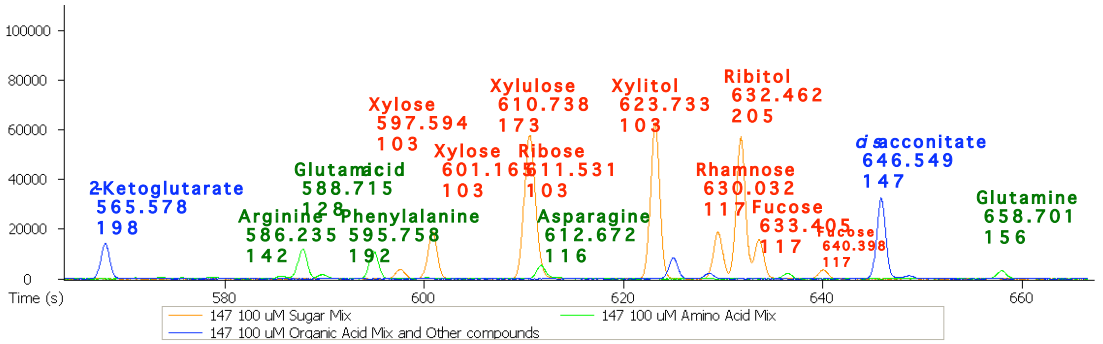
メタボローム解析(多成分一斉分析系の構築)

GC-TOFMSによる水溶性代謝産物の網羅的解析

酵母細胞内主要代謝産物

糖・糖アルコール	21化合物	同時分析
アミノ酸	27化合物	
有機酸	13化合物	
核酸など	9化合物	





Agilent 7890N Gas Chromatograph

Column : CP-Sil 8 CB Low Bleed 30 m × 0.25 mm i.d.
Carrier gas: Helium
Gas Flow: 1 ml/min
Injected volume: 1 ml
Split ration: 1:10
Inlet temperature: 230oC
Oven temperature ramp: 80oC (2 min hold),
15.0oC/min, increment to 330oC (6 min hold)
Transfer line temperature: 250℃

Leco Pegasus HT Time-of-flight Mass Spectrometry

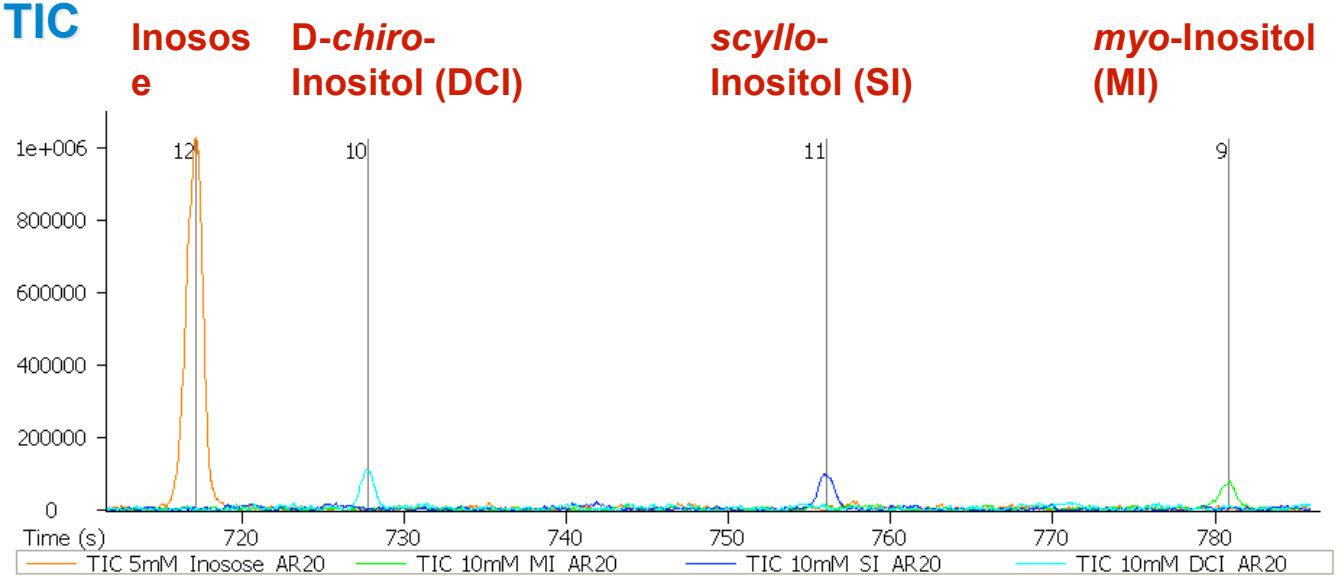
Solvent delay: 230 sec
Mass range: 85-500 u
Scan rate: 20 spectra/sec
Detector voltage: 1650V
Filament bias voltage: -70V
Ion source temperature: 200℃

Collaboration:

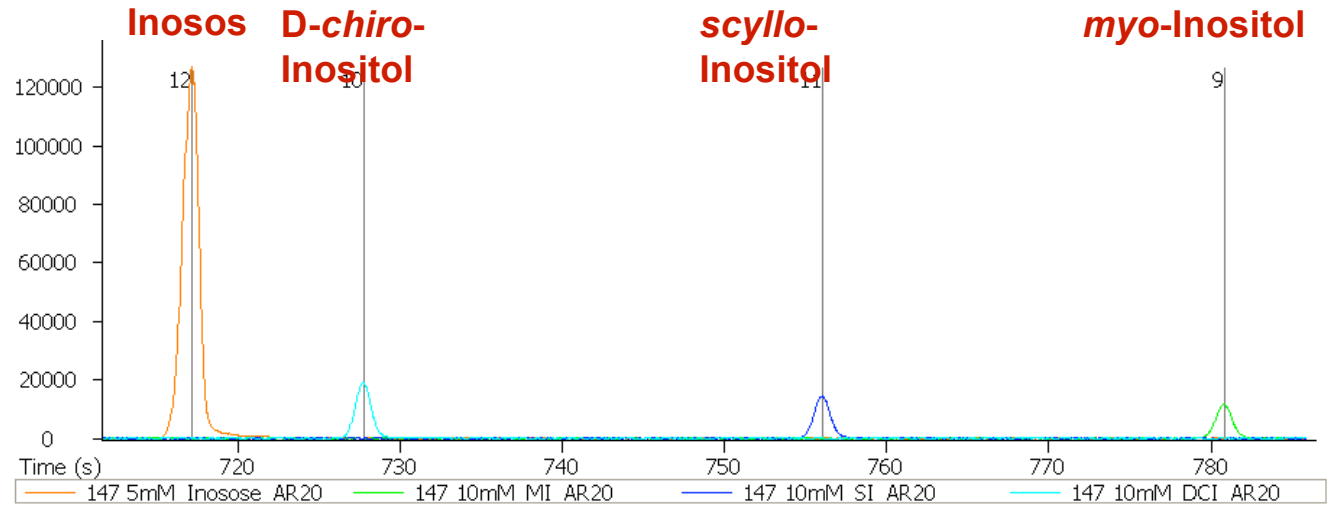
Dr. Tomohisa Hasunuma, Kobe Univ.

Detection of ions for inositol derivatives

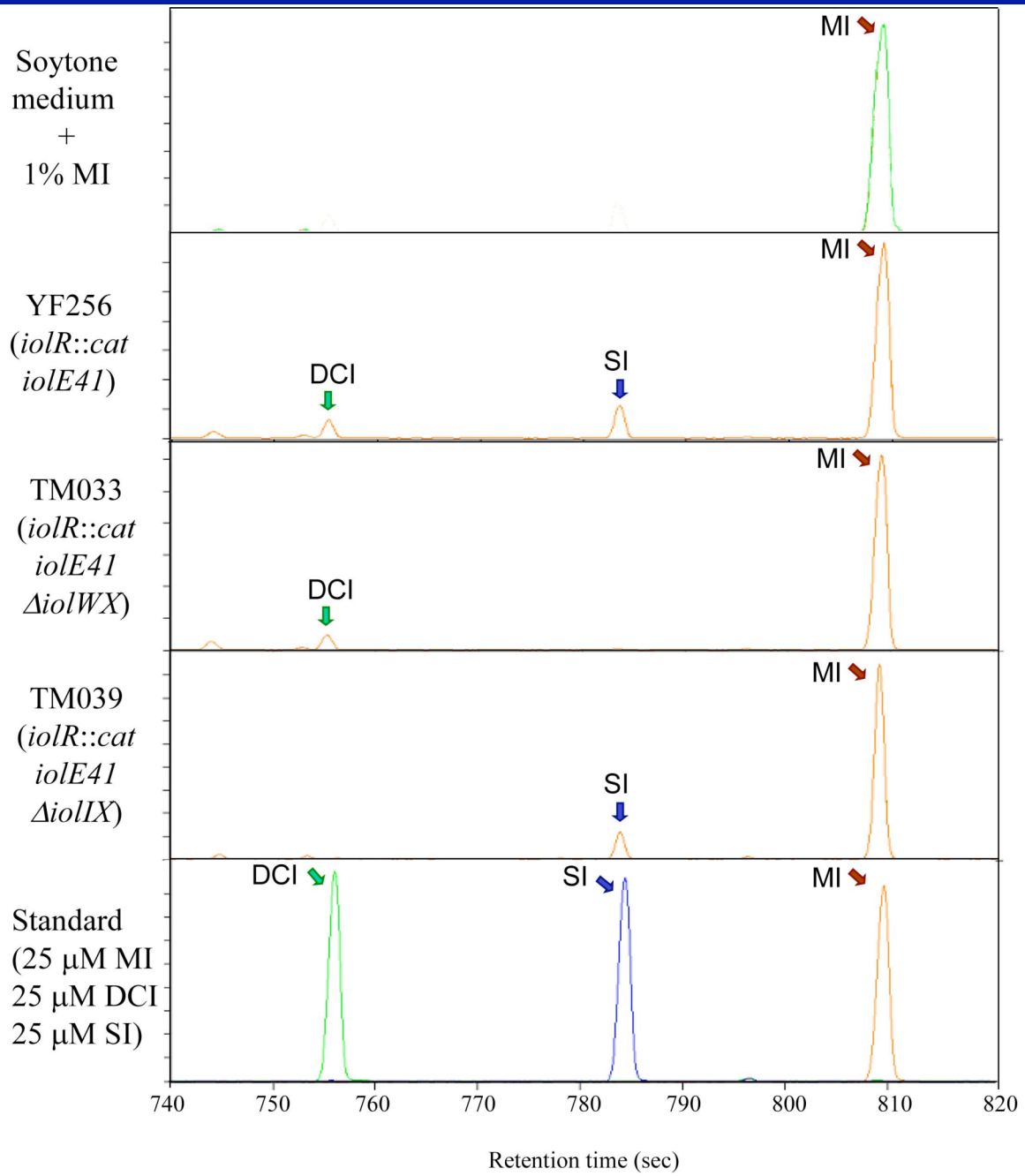
TIC



EIC (Mass = 147)



GC-TOFMS analysis of bioconversion media



MI + SI + DCI (85:10:5)

MI + DCI (95:5)

Selective bioconversion is achieved!

MI + SI (85:15)

GC-TOFMS analysis of intracellular products

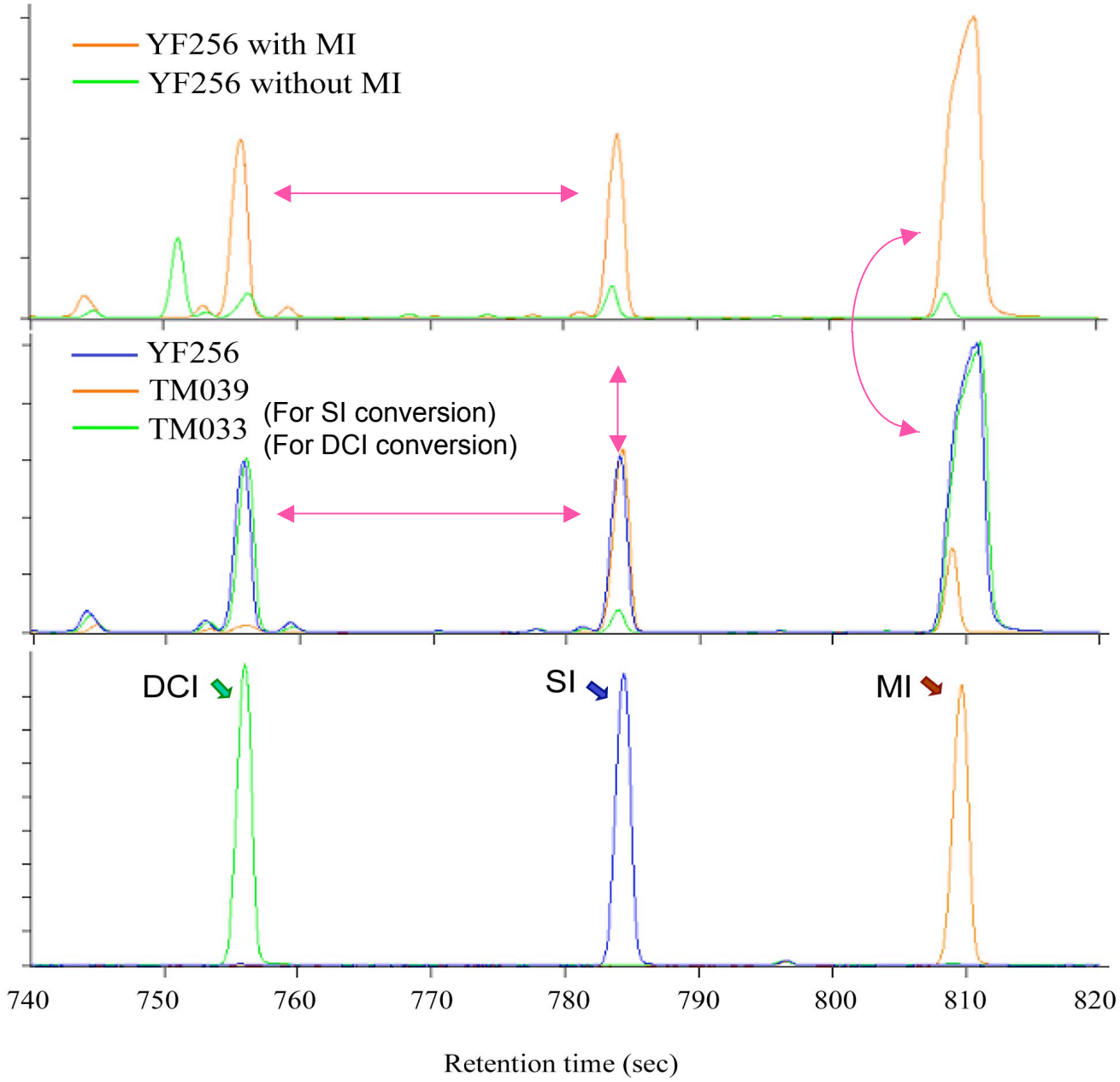
Intracellular products (1):

With/without conversion

Intracellular products (2):

Different conversion

Standards



Merci beaucoup de votre attention…