myo-Inositol transport by *Salmonella enterica* serovar Typhimurium

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In *Salmonella enterica* serovar Typhimurium, the genomic island GEI4417/4436 has recently been identified to be responsible for myo-inositol (MI) utilization. Here, two of the four island-encoded permeases are identified as the MI transporters of this pathogen. In-frame deletion of *iolT1* (STM4418) led to a severe growth defect, and deletion of *iolT2* (STM4419) to a slight growth defect in the presence of MI. These phenotypes could be complemented by providing the putative transporter genes *in trans*. Bioluminescence-based reporter assays demonstrated a strong induction of their promoters P_{iolT1} and P_{iolT2} in the presence of MI but not of glucose. Deletion of *iolR*, which encodes the negative regulator of most genes involved in MI degradation, resulted in upregulation of P_{iolT1} and P_{iolT2} , indicating that the expression of *iolT1* and *iolT2* is repressed by *iolR*. This finding was supported by bandshift assays using purified *iolR*. Both transporters are located in the membrane when expressed in *Escherichia coli*. Heterologously expressed *iolT1* had its optimal activity at pH 5.5. Together with the strongly reduced MI uptake in the presence of protonophores, this indicates that *iolT1* operates as a proton symporter. Using myo-[1,2-³H](N)inositol, a saturable uptake activity of *iolT1* with a K_m value between 0.49 and 0.79 mM was determined in DH5 α expressing *iolT1*, in *S. enterica* serovar Typhimurium strain 14028, and in mutant 14028 Δ *iolT2*. Phylogenetic analysis of *iolT1* identified putative MI transporters in Gram-negative bacteria also able to utilize MI.

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INTRODUCTION

myo-Inositol (MI) is a polyol abundant in soil. Its phosphorylated form, inositol hexakisphosphate or phytate, serves as a phosphorus store in plants, but can be utilized by livestock only in the presence of phytases. The capability of an increasing number of micro-organisms to grow on MI as the sole carbon source depends on the presence of a catabolic pathway that results in MI degradation to dihydroxyacetone phosphate, acetyl coenzyme A and CO₂.

Although inositol utilization was investigated extensively at the enzymic level for *Enterobacter (Aerobacter) aerogenes* decades ago (Anderson & Magasanik, 1971), the underlying genetics and regulatory mechanisms have been elucidated in most detail for *Bacillus subtilis* (Miwa & Fujita, 2001; Yoshida *et al.*, 2004, 1997). In this organism, the *iol* divergon responsible for MI utilization comprises

iolT and the operons *iolABCDEFGHJI* and *iolRS*. *IolR* acts as a repressor of the *iol* divergon by binding to the operator sites in the absence of MI. An intermediate of MI degradation, 2-deoxy-5-keto-D-gluconic acid 6-phosphate, has been shown to antagonize *IolR* binding, thus inducing the expression of *iol* genes (Yoshida *et al.*, 2008, 1999). Two proteins belonging to the major facilitator superfamily (MFS), *IolT* and *IolF*, have been identified as the major and minor inositol transporters of *B. subtilis*, and *IolR* has been revealed to inhibit the transcription of *iolT* (Yoshida *et al.*, 2002). MI degradation has also been studied in *Corynebacterium glutamicum* (Krings *et al.*, 2006), *Clostridium perfringens* (Kawsar *et al.*, 2004) and *Lactobacillus casei* (Yebra *et al.*, 2007).

So far, several Gram-negative genera have been demonstrated to utilize the polyol MI. These include species from the genera *Klebsiella*, *Caulobacter*, *Rhizobium*, *Sinorhizobium*, *Pseudomonas*, *Yersinia*, *Salmonella* and *Serratia* (Berman & Magasanik, 1966; Boutte *et al.*, 2008; Fry *et al.*, 2001; Gauchat-Feiss *et al.*, 1985; Kröger & Fuchs, 2009; Legakis *et al.*, 1976; Primrose & Ronson, 1980; Reber *et al.*, 1977). A comparison of the respective gene clusters reveals a high variability of their chromosomal organiza-

Abbreviations: CCCP, carbonyl cyanide *m*-chlorophenylhydrazon; FRT, FLP recognition target; MFS, major facilitator superfamily; MI, myo-inositol; RLU, relative light units.

A supplementary table listing the primers used is available with the online version of this paper.

tion. In *Salmonella enterica* serovar Typhimurium, the genes required for MI degradation are located on a 22.6 kb genomic island (GEI4417/4436). Identical islands are present only in the genomes of the *S. enterica* serovars Paratyphi B, Saintpaul, Weltevreden, Agona and Virchow, and are absent in serovars Typhi, Paratyphi A, Choleraesuis and many others, indicating that the utilization of MI is not a common capability of *Salmonella* strains. Interestingly, this MI utilization island is not restricted to salmonellae; we identified a nearly identical gene cluster lacking homologues of *iolI2* and *iolH* in the genome of *Escherichia coli* ED1a (NC_011745.1). In this strain, the island is flanked by two transposase-encoding genes, strongly suggesting that its distribution among Gram-negative bacteria is due to horizontal gene transfer. The genomic island of *S. enterica* includes five transcriptional units encoding enzymes involved in MI utilization, and these are induced in the presence of MI. In rich medium, the negative regulator IolR represses all but one promoter of the *iol* divergon, including its own.

A total of 20 genes are located on GEI4417/4436, but a functional role has been described only for eight of them (Kröger & Fuchs, 2009). Four genes, STM4418, STM4419,

STM4428 and STM4434, encode putative, as yet uncharacterized, permeases. Their role in MI transport was investigated here by the construction of in-frame deletion mutants and complementing plasmids. The transcriptional activity of two putative transporter genes (STM4418 and STM4419), as well as the regulatory role of IolR, were monitored using the luciferase reporter system and band-shift assays. Uptake of *myo*-[1,2- ^3H](N)inositol was investigated in *S. enterica* serovar Typhimurium strain 14028, in mutant strains lacking the putative transporters, and in *E. coli* cells expressing STM4418 and STM4419, revealing a transport activity of STM4418, now termed IolT1. This study describes for the first time, to our knowledge, an MI transporter of a Gram-negative bacterium.

METHODS

Bacterial strains, plasmids and growth conditions. Bacterial strains and plasmids used in this study are listed in Table 1. *S. enterica* serovar Typhimurium and *E. coli* cultures were grown in Luria–Bertani (LB) broth (10 g tryptone l^{-1} , 5 g yeast extract l^{-1} , 5 g NaCl l^{-1}) or in minimal medium [MM; M9 medium supplemented with 2 mM MgSO_4 , 0.1 mM CaCl_2 and 55.5 mM

Table 1. Strains and plasmids used in this study

Strain or plasmid	Description and relevant features	Source or reference
Strains		
DH5 α	<i>E. coli</i> : <i>deoR</i> , <i>endA1</i> , <i>gyrA96</i> , <i>hsdR17</i> ($r_k^- m_k^+$), <i>recA1</i> , <i>relA1</i> , <i>supE44</i> , λ <i>thi-1</i> , Δ (<i>lacZYA-argFV169</i>)	Hanahan (1983)
BL21 (DE3)	<i>E. coli</i> : F^- , <i>ompT</i> , <i>hsdS_B</i> ($r_B^- m_B^-$), <i>gal</i> , <i>dcm</i> , <i>rne131</i>	Studier <i>et al.</i> (1990)
14028	Spontaneous streptomycin-resistant mutant of <i>S. enterica</i> serovar Typhimurium wild-type strain ATCC 14028	Kröger & Fuchs (2009)
14028 Δ <i>iolT1</i>	In-frame <i>iolT1</i> (STM4418) deletion mutant	This study
14028 Δ <i>iolT2</i>	In-frame <i>iolT2</i> (STM4419) deletion mutant	This study
14028 Δ <i>iolT1</i> Δ <i>iolT2</i>	In-frame deletion mutant of <i>iolT1</i> and <i>iolT2</i> (STM4418, STM4419)	This study
14028 Δ STM4428	In-frame STM4428 deletion mutant	This study
14028 Δ STM4434	In-frame STM4434 deletion mutant	This study
Plasmids		
pKD4	<i>pir</i> -Dependent, FRT sites; Kan ^r	CGSC*; Datsenko & Wanner (2000)
pKD46	Lambda-Red helper plasmid; Amp ^r	CGSC; Datsenko & Wanner (2000)
pCP20	FLP recombinase plasmid; Cm ^r Amp ^r	CGSC; Datsenko & Wanner (2000)
pET28b	Expression vector, T7 <i>lac</i> promoter; Kan ^r	Novagen
pET28b- <i>iolR</i>	<i>iolR</i> cloned into pET28b for IolR overexpression and purification	Kröger & Fuchs (2009)
pBR322	Amp ^r Tet ^r	Fermentas
pBR322- <i>iolT1</i> , pBR322- <i>iolT2</i>	<i>iolT1</i> or <i>iolT2</i> cloned into pBR322 with putative promoter region	This study
pBR322- <i>iolT1</i> -His ₆ , pBR322- <i>iolT1</i> -His ₆	As above, but encoding transporters with C-terminal His ₆ -tag	This study
pDEW201	Promoter probe vector; Amp ^r <i>luxCDABE</i>	Van Dyk & Rosson (1998)
pDEW201-P _{<i>iolT1</i>}	pDEW201 with 300 bp upstream of <i>iolT1</i> (STM4418)	This study
pDEW201-P _{<i>iolT2</i>}	pDEW201 with 300 bp upstream of <i>iolT2</i> (STM4419)	This study
pDEW201-P _{<i>argS</i>}	pDEW201 with 244 bp upstream of <i>argS</i>	Kröger & Fuchs (2009)
pDEW201-‘STM0047’	pDEW201 with intragenic 350 bp of STM0047 without promoter homology	Kröger & Fuchs (2009)

*CGSC, Coli Genetic Stock Center, Yale University.

(1%) MI, glucose, fructose, arabinose, xylose, glycerol or sorbitol]. For plasmid maintenance, the media were supplemented with the following antibiotics: ampicillin ($150 \mu\text{g ml}^{-1}$), kanamycin ($50 \mu\text{g ml}^{-1}$), chloramphenicol ($25 \mu\text{g ml}^{-1}$) or streptomycin ($50 \mu\text{g ml}^{-1}$). For solid media, 1.5% agar (w/v) was added. For all growth and promoter probe experiments, bacterial strains were grown in LB medium overnight at 37°C , washed twice in PBS and then adjusted to OD_{600} 0.005 in the desired liquid growth medium, or streaked on agar plates. Growth curves were obtained from bacterial cultures incubated at 37°C under vigorous shaking in 250 ml flasks with 50 ml MM. OD_{600} was measured at the time interval indicated.

Standard procedures. DNA manipulations and isolation of chromosomal or plasmid DNA were performed according to standard protocols (Sambrook & Russell, 2001), and following the manufacturers' instructions. Plasmid DNA was transformed via electroporation by using a Bio-Rad Gene Pulser II as recommended by the manufacturer and as described previously (Klumpp & Fuchs, 2007). PCRs were carried out with *Taq* polymerase (Fermentas). As template for PCR, chromosomal DNA, plasmid DNA or cells from a single colony were used. Oligonucleotides synthesized for PCRs are listed in Supplementary Table S1. *S. enterica* serovar Typhimurium gene numbers refer to the LT2 annotation (NC 003197). The homepages <http://enteric.cbcb.umd.edu/> and <http://www.microbesonline.org/> were used to determine the distribution of *S. enterica* serovar Typhimurium ORFs in the genomes of Gram-negative species. Promoter sequences located upstream of the identified genes were predicted with BPROM (<http://www.softberry.com/>), and transmembrane domains with TOPCONS (<http://topcons.cbr.su.se/>). The cladogram was constructed with TREECON (Van de Peer & De Wachter, 1994).

Construction of deletion mutants and recombinant plasmids. In-frame deletion mutants of *iolT1* (STM4418), *iolT2* (STM4419), STM4428 and STM4434 were constructed by the one-step method based on the phage λ Red recombinase (Datsenko & Wanner, 2000). Briefly, PCR products comprising the kanamycin resistance cassette of plasmid pKD4, including the flanking FLP recognition target (FRT) sites, were generated using pairs of 70 nt primers that included 20 nt priming sequences for pKD4 as template DNA. Homology extensions of 50 bp overlapped 18 nt of the 5' end and 36 nt of the 3' end of the target gene (Link *et al.*, 1997). Fragment DNA (500–1000 ng) was transferred into *S. enterica* serovar Typhimurium strain 14028 cells harbouring plasmid pKD46. Allelic replacement of the target gene by the kanamycin resistance cassette was controlled by PCR, and non-polar deletion mutants were obtained upon transformation of pCP20. Gene deletions were verified by PCR analysis and DNA sequencing.

To complement deleted genes, the coding sequences of *iolT1* (STM4418) and *iolT2* (STM4419) plus approximately 300 bp of their upstream region were amplified from chromosomal DNA of strain 14028 with primers listed in Supplementary Table S1. PCR products were digested with *EcoRI* and *SalI* (Fermentas) and ligated (T4 DNA ligase, Gibco) into vector pBR322 to generate pBR322-*iolT1* and pBR322-*iolT2*, respectively. The plasmids were verified by PCR, restriction analysis and sequencing. To detect proteins by Western blot analysis, both genes were amplified with primers introducing a C-terminal His₆-tag and cloned as described above, resulting in pBR322-*iolT1*-His₆ and pBR322-*iolT2*-His₆.

Cloning of promoter fusions. Putative promoter regions spanning approximately 300 bp upstream of the start codons of the genes *iolT1* (STM4418) and *iolT2* (STM4419) were amplified from chromosomal DNA of *S. enterica* serovar Typhimurium 14028 by PCR using the primers listed in Supplementary Table S1. The fragments were then cloned via *EcoRI* and *BamHI* (Fermentas) upstream of the

promoterless *luxCDABE* genes into the multiple cloning site of pDEW201. After transformation into *E. coli* DH5 α cells, plasmids containing the correct transcriptional *lux* fusions were isolated and verified by PCR, restriction analysis and sequencing. pDEW201-P_{*iolT1*} and pDEW201-P_{*iolT2*} were transformed into *S. enterica* serovar Typhimurium 14028 and the Δ *iolR* mutant strain.

Quantification of promoter activity. Bioluminescence measurements were performed as previously described (Kröger & Fuchs, 2009). For growth in MM containing either 27.8 mM (0.5%) glucose or 55.5 mM MI, bacterial cells were grown at 37°C until they reached the late-exponential growth phase, e.g. for 11 h (glucose) and 70 h (MI), in 15 ml centrifuge tubes without agitation. At appropriate time points, 200 μl of each sample was transferred to a 96-well plate, and the OD_{600} and the bioluminescence, measured as relative light units (RLU), were recorded in a Wallac VICTOR³ 1420 multilabel counter (Perkin Elmer Life Sciences).

Gel mobility shift (GMS) assays. IolR-His₆ was overexpressed and purified as previously described (Kröger & Fuchs, 2009). Briefly, expression of IolR was induced by adding 0.1 mM IPTG when *E. coli* BL21 cells transformed with pET28b-*iolR* had reached OD_{600} 0.4. After incubation for 4 h, the cells were harvested and the pellet was resuspended in 1 ml buffer A (300 mM NaCl, 50 mM Na₃PO₄). The cells were lysed using a French press (SLM Aminco Instruments), and cell debris was removed by centrifugation at 4°C (20 min, $1.6 \times 10^4 \text{ g}$). After adding 10 μl of the protease inhibitor PMSF (100 mM), IolR-His₆ was purified using TALON metal affinity resin (Clontech Laboratories). A 1 ml volume of the protein extract was mixed with 1 ml resin and incubated for 1 h at room temperature. The resin was washed and eluted according to the manufacturer's protocol. Fractions containing high amounts of IolR-His₆ were pooled and the buffer was exchanged with GMS buffer [50 mM Tris/HCl, pH 7.5, 50 mM KCl, 10 mM MgCl₂, 0.5 mM EDTA, 10% (v/v) glycerol] by gel filtration using PD-10 columns (GE Healthcare) (Schaaf & Bott, 2007). The protein concentration was determined using RotiQuant solution (Carl Roth) based on the method of Bradford (1976), and the purity of eluted fractions was analysed by separation on a 15% SDS-polyacrylamide gel.

For GMS assays, putative promoter regions of *iolT1*, *iolT2*, *iolR*, *iolH* and *argS* as competitor DNA were amplified with oligonucleotides listed in Supplementary Table S1, and 100 ng DNA was mixed with increasing amounts of purified IolR-His₆ in GMS buffer. After incubation for 45 min at room temperature, the samples were loaded on a 9.5% native polyacrylamide gel prepared in 1 $\times$ Tris/borate/EDTA buffer and separated at 120 V for 3 h. DNA was then stained with ethidium bromide solution and visualized by UV irradiation.

Membrane isolation and Western blot analysis. DH5 α cells transformed with pBR322-*iolT1*-His₆ and pBR322-*iolT2*-His₆ were grown to stationary phase. Isolation of *E. coli* membranes was then performed as described previously (Kreutzenbeck *et al.*, 2007) but using a French press (SLM Aminco Instruments) to lyse the cells. Protein concentrations were measured using RotiQuant solution. Samples (15 μg) of membrane protein were separated on a 12.5% SDS-polyacrylamide gel, and Western blot analysis of the His₆-tagged proteins IolT1 and IolT2 was performed according to standard procedures with 1:1000 diluted monoclonal anti-His₆ antibodies (dianova) and 1:15 000 diluted alkaline phosphatase-conjugated anti-mouse antibodies (dianova). Phosphatase activity was detected with nitro blue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCiP) as substrates.

Uptake of myo-[1,2-³H](N)inositol. *E. coli* DH5 α cells transformed with pBR322-*iolT1*, pBR322-*iolT2* and pBR322 were grown in

30 ml LB medium containing ampicillin ($100 \mu\text{g ml}^{-1}$) to OD_{600} 1.0. Then, 20 ml was harvested by centrifugation (4°C and $1 \times 10^4 \text{ g}$) and resuspended in McIlvaine's buffer [$0.1 \text{ M Na}_2\text{HPO}_4$, 0.05 M citric acid mixed to obtain the desired pH (McIlvaine, 1921)] to OD_{600} 4.0 and stored on ice. Cell suspension ($250 \mu\text{l}$) was mixed with $230 \mu\text{l}$ McIlvaine's buffer and $12.5 \mu\text{l}$ 2.2 M (40%) glucose, and stirred in a water bath at 37°C for 2 min. The protonophore carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) was added 2 min before the start of the experiment at a final concentration of $50 \mu\text{M}$. The uptake experiment was started by adding $7 \mu\text{l}$ of a mixture of unlabelled MI and *myo*-[1,2- ^3H](N)inositol [specific activity 30 Ci (1110 GBq) mmol^{-1} ; Biotrend]. In experiments to determine the pH optimum of MI uptake, this mix contained 1 mM unlabelled and 26.67 nM tritiated substrate. The K_m value was determined at pH 5.5 with a mix containing the same amount of labelled substrate but decreasing amounts of unlabelled substrate ($1, 0.5, 0.2$ and 0.1 mM). Aliquots of $60 \mu\text{l}$ were removed, rapidly filtered through a Pall GN6 $0.45 \mu\text{m}$ pore-size nitrocellulose filter, washed once with 5 ml 150 mM NaCl and transferred to a scintillation vial. After addition of 3 ml scintillation cocktail (Carl Roth), the radioactivity associated with the filters was counted in a Perkin Elmer Tri-Carb scintillation counter. The uptake activity was constant for the first 50 s, and this interval was used to determine uptake velocities. Uptake experiments with *S. enterica* serovar Typhimurium were performed in a similar manner, but cells were cultivated in MM with 55.5 mM MI.

RESULTS

Identification of two putative transporters involved in MI uptake

The genomic island GEI4417/4436 of *S. enterica* serovar Typhimurium responsible for degradation of MI carries four genes (STM4418, STM4419, STM4428 and STM4434), whose products, annotated as permeases or sugar transporters of the MFS, represent candidate MI transporters. Strain 14028 and in-frame deletion mutants of all four putative transporter genes were tested for their ability to grow on MM agar plates, or in liquid MM, containing 55.5 mM MI. The doubling time of the wild-type strain was lower in glucose (growth rate $\mu=0.56 \text{ h}^{-1}$) than in MI ($\mu=0.37 \text{ h}^{-1}$). In contrast to the mutants 14028 $\Delta\text{STM4428}$ and 14028 $\Delta\text{STM4434}$, the deletion of STM4418 or STM4419 resulted in altered growth phenotypes; these genes were therefore named *iolT1* (STM4418) and *iolT2* (STM4419). The deletion of *iolT1* abolished growth in liquid minimal medium containing MI for up to 74 h (Fig. 1a), and the mutant strain 14028 ΔiolT1 showed

Fig. 1. Growth of *S. enterica* serovar Typhimurium strains in the presence of 55.5 mM MI. (a) Growth curves of strains in liquid MM supplemented with MI. $\blacklozenge$, 14028 (growth rate $\mu=0.37 \text{ h}^{-1}$); $\square$, 14028 ΔiolT2 ($\mu=0.28 \text{ h}^{-1}$); $\triangle$, 14028 $\Delta\text{STM4428}$ ($\mu=0.38 \text{ h}^{-1}$); $\circ$, 14028 $\Delta\text{STM4434}$ ($\mu=0.42 \text{ h}^{-1}$). Zero growth phenotype: $\blacktriangle$, 14028 ΔiolT1 ; $\times$, 14028 $\Delta\text{iolT1}\Delta\text{iolT2}$. The average data from two independent experiments are shown. (b) Strains were streaked on MM agar plates containing 55.5 mM MI and grown at 37°C for 5 days. Above, strain 14028, the double mutant 14028 $\Delta\text{iolT1}\Delta\text{iolT2}$ with plasmid pBR322, and the complementing constructs pBR322-*iolT1* and pBR322-*iolT2*; below, deletion mutants lacking either *iolT1* or *iolT2*. *iolT1* was shown to have a bigger effect on growth with MI, but a significant growth defect was also observed for 14028 ΔiolT2 . Each mutant was complemented by providing the respective gene *in trans*. The scale of all photographs is identical.

only very weak growth on solid medium after 128 h (Fig. 1b). The combined deletion of *iolT1* and *iolT2* completely abolished growth in the presence of MI (Fig. 1a, b). To unequivocally demonstrate the contribution of *iolT1* to MI utilization, plasmid pBR322-*iolT1* was transformed into strains 14028 $\Delta iolT1$ and 14028 $\Delta iolT1\Delta iolT2$, resulting in wild-type-like growth of both mutants (Fig. 1b).

Only slightly reduced growth of 14028 $\Delta iolT2$ (STM4419) was observed in liquid medium, but a significant growth deficiency on solid medium was observed. Plasmid pBR322-*iolT2* complemented this phenotype (Fig. 1a, b). The presence of plasmid pBR322-*iolT2* only partially restored the growth behaviour of 14028 $\Delta iolT1\Delta iolT2$ to that of strain 14028, and retarded growth in comparison with 14028 $\Delta iolT1\Delta iolT2$ /pBR322-*iolT1* was observed (Fig. 1b). Strain 14028 $\Delta iolT1$ did not grow in liquid medium even with 277.5 mM MI, nor with plasmid pBR322-*iolT2*, indicating that *iolT2* does not provide sufficient substrate for growth under these conditions for as yet unknown reasons.

We also tested the growth of mutants 14028 $\Delta iolT1$ and 14028 $\Delta iolT2$ in the presence of other carbon sources, namely fructose, xylose, glucose, arabinose, sorbitol and glycerol, but did not observe significant growth differences between the mutants and strain 14028 (data not shown). These results show that *iolT1* and *iolT2* are both involved in MI utilization and that *iolT1* appears to make the bigger contribution. In contrast, STM4428 and STM4434 do not contribute to growth on MI, at least under the conditions examined here.

Expression and regulation of genes involved in MI transport

In order to investigate the regulation of *iolT1* and *iolT2*, the 300 bp upstream sequence of each gene was cloned into vector pDEW201, which contains the *lux* operon of *Photobacterium luminescens*. The promoter sequence of *argS* encoding arginyl-tRNA synthase and a 350 bp intragenic

fragment of STM0047 lacking any promoter homology served as controls. All recombinant constructs, as well as pDEW201, were transformed into strain 14028. The optical density and the bioluminescence of the strains were measured during growth in MM supplemented with glucose or MI (Table 2). The background level determined from the control construct with the intragenic fragment 'STM0047' ranged from 2.19×10^4 to 2.41×10^4 RLU per OD₆₀₀ unit under all conditions tested. In MM with 27.8 mM glucose, the upstream region of *iolT2* resulted in light emission only slightly above the background (3.38×10^4 RLU per OD₆₀₀ unit), while the predicted *iolT1* promoter showed a maximal bioluminescence of 8.52×10^5 RLU per OD₆₀₀ unit. When glucose was replaced by MI, a 13.5-fold (P_{iolT1}) and a 22.2-fold (P_{iolT2}) induction was observed. These data indicate that *S. enterica* serovar Typhimurium adapts to the presence of MI by increasing the amount of specific transporters whose expression is repressed in the absence of MI.

Because several promoters of genes involved in MI utilization by *S. enterica* serovar Typhimurium are negatively regulated by the repressor IolR, the pDEW201 constructs were also tested in strain 14028 $\Delta iolR$ during growth in MM containing glucose (Table 1). The lack of IolR increased the transcriptional activity of P_{iolT1} and of P_{iolT2} by factors of 11.2 and 42.6, respectively, thus resembling the expression pattern for both transporters in the presence of MI. Therefore, the expression of *iolT1* and *iolT2* is probably repressed by IolR when *S. enterica* serovar Typhimurium is grown in a rich medium.

The repressor IolR binds to the promoters of *iolT1* and *iolT2*

To strengthen the hypothesis that IolR acts as a repressor of P_{iolT1} and P_{iolT2} , as has already been shown for the promoters of *iola*, *iolC1*, *iolD1* and *iolR* (Kröger & Fuchs, 2009), its binding to the *iolT1* and *iolT2* promoter regions

Table 2. Quantification of promoter activities of the MI transporter genes *iolT1* and *iolT2*

Samples were taken from the late exponential phase corresponding to maximal promoter activity.

Fragment cloned into pDEW201	14028 grown in MM + glucose		14028 grown in MM + MI			14028 $\Delta iolR$ grown in MM + glucose		
	RLU per OD ₆₀₀ unit*	SD (%)	RLU per OD ₆₀₀ unit*	SD (%)	Fold induction†	RLU per OD ₆₀₀ unit*	SD (%)	Fold induction†
P_{iolT1}	8.52×10^5	1.4	1.15×10^7	3.8	13.5	9.57×10^6	18.6	11.2
P_{iolT2}	3.38×10^4	18.1	7.50×10^5	7.6	22.2	1.44×10^6	4.9	42.6
Controls								
P_{argS}	3.62×10^6	4.1	3.42×10^6	3.9	0.94	3.16×10^6	18.5	0.87
'STM0047'	2.41×10^4	10.4	2.19×10^4	14.8	0.91	2.21×10^4	4.7	0.92
None	4.36×10^3	43.6	2.33×10^3	13.7	0.53	1.60×10^3	31.5	0.37

*Data are the mean RLU derived from three independent experiments.

†Fold induction was calculated with respect to the RLU per OD₆₀₀ unit values of strain 14028 grown in glucose.

was tested by EMS assays. For that purpose, IolR was overexpressed in *E. coli* BL21 (DE3) and purified. PCR products containing the putative promoters of *iolR*, *iolT1*, *iolT2* and *iolH* were incubated with or without increasing amounts of the purified IolR protein, and the protein–DNA complexes were separated on 9.5% native polyacrylamide gels. A DNA band with decreased motility representing the IolR–DNA complex was observed with the *iolT1* and *iolT2* fragments, indicating that IolR binds to the promoter elements of these two MI transporter genes (Fig. 2). Binding of IolR to its own promoter served as a positive control, and complex formation was not observed with a fragment located upstream of *iolH* encoding a protein that is not required for MI degradation (Kröger & Fuchs, 2009). Together with the transcriptional analysis described above, these data demonstrate that IolR is a DNA-binding protein that negatively regulates the expression of *iolT1* and *iolT2* during growth of *S. enterica* serovar Typhimurium in a medium lacking MI. A conserved motif in the upstream regions of the two genes could not be identified.

IolT1 mediates MI uptake when expressed in *E. coli*

IolT1 and IolT2, with molecular masses of 53.4 and 52.1 kDa, respectively, appear to be typical members of the MFS (Law *et al.*, 2008), and are predicted to possess 12 transmembrane (TM) domains by multiple prediction algorithms. Since *E. coli* DH5 α is not able to grow on MI, a finding that is in line with the absence of *iol* genes in the genome of *E. coli* K-12, it was chosen for heterologous expression of IolT1 and IolT2 to study their MI transport activity. *E. coli* DH5 α cells were transformed with pBR322-*iolT1* and pBR322-*iolT2*, and uptake assays were performed as detailed in Methods. In a first experiment, DH5 α cells expressing IolT1 or IolT2 were compared with cells containing an empty plasmid. The assays were performed with an initial extracellular MI concentration of 1 mM, which is higher than the K_m value of the related MI transporters from *C. glutamicum* (Krings *et al.*, 2006). IolT1-expressing cells showed high levels of MI uptake, which were linear for 50 s after substrate addition (Fig. 3a)

Fig. 2. EMS assays to study DNA binding activity of IolR. The interaction of IolR with the promoter region of four genes of GEI 4417/4436 is shown. Promoter DNA (100 ng) was incubated with or without increasing amounts (81, 162, 324, 678 and 810 ng) of purified IolR. Protein–DNA complexes were separated on 9.5% native polyacrylamide gels. As a control, 100 ng competitor DNA comprising the *argS* promoter was added.

Fig. 3. MI uptake by heterologously expressed IolT1 and IolT2. (a) *E. coli* DH5 α cells containing plasmids pBR322-*iolT1* ($\blacklozenge$), pBR322-*iolT2* ($\square$) or pBR322 ($\times$; negative control) were grown in LB medium, harvested by centrifugation, resuspended in Mcllvaine's buffer (pH 5.5) at OD₆₀₀ 4.0 and energized by the addition of 55.5 mM glucose (final concentration). The uptake experiment was started by the addition of a mixture of 1 mM MI and 26.67 nM *myo*-[1,2-³H](N)inositol. Aliquots of the cell suspension were taken 10, 30 and 50 s after the addition of MI. In experiments with DH5 α /pBR322-*iolT1* ($\diamond$), the protonophore CCCP was added 2 min before starting the uptake experiment. (b) Membrane localization of IolT1-His₆ and IolT2-His₆. For each sample, 15 μ g membrane protein was subjected to SDS-PAGE and Western blot analysis using monoclonal anti-His₆ antibodies. The samples were obtained from *E. coli* DH5 α cells containing pBR322-*iolT1*-His₆ (left), pBR322-*iolT2*-His₆ (centre) or pBR322 (right, negative control).

and levelled off at later time points (data not shown). This uptake activity was drastically reduced in the presence of the protonophore CCCP (Fig. 3a). In contrast to IolT1-producing cells, cells expressing IolT2 did not significantly differ from vector controls and showed no MI uptake (Fig. 3a). This situation did not change even when this experiment was continued for up to 2.5 h, nor in the presence of 2 mM MI at higher specific activity (data not shown).

To verify that the transformation of the two plasmids had led to the production and cytoplasmic membrane insertion of the IolT proteins, similar plasmids were constructed in which the reading frames of the two genes were extended by a C-terminal His-tag. Both transporter genes were transcribed from their own promoters. Membrane extracts from the transformed cells were separated by SDS-PAGE, blotted onto a nitrocellulose membrane and probed with monoclonal anti-His₆ antibodies. Two bands, corresponding to IolT1 and IolT2, were revealed that were absent in the control strain with pBR322 (Fig. 3b). This analysis showed that the promoters of *iolT1* and *iolT2* are active in *E. coli*, leading to the production of both proteins at similar levels, and that they are inserted into the *E. coli* membrane. Thus, the lack of inositol uptake in IolT2-expressing cells is unlikely to be caused by a lack of gene expression.

pH optimum and kinetic properties of IolT1-dependent MI transport

We continued with the analysis of the IolT1 activity and determined its pH optimum by performing uptake assays at various external pH values. The IolT1-driven MI uptake displayed a sharp pH optimum with a maximum at pH 5.5, and significantly reduced uptake rates at higher or lower pH (Fig. 4a). Together with the inhibition of MI uptake by CCCP (Fig. 3a), this indicates that IolT1 operates as an MI/proton symporter.

To determine the kinetic properties of IolT1-dependent MI uptake by *S. enterica* serovar Typhimurium, transport assays were performed at pH 5.5 with varying amounts of substrate. The uptake of MI was saturable, and a K_m value of 0.79 mM was determined. The Eadie-Hofstee plot of these data provides no indication that two kinetically different proteins contribute to MI uptake by *S. enterica* serovar Typhimurium (Fig. 4b). A similar K_m value (0.49 mM) was obtained with strain 14028 Δ *iolT2* (data not shown), again demonstrating the bigger contribution of IolT1 to MI uptake. To exclude the possibility that the presence of 1% glucose used for cell energizing had a detrimental effect on MI transport, the experiment was

Fig. 4. pH optimum and saturation kinetics of IolT1-mediated MI uptake. The uptake assays were performed as described in the legend to Fig. 3. (a) Determination of the pH optimum of MI uptake with DH5 α /pBR322-*iolT1*. The cells were resuspended in McIlvaine's buffer at the indicated pH values. The figure shows the result of one of three repetitions that all confirmed the pH optimum to be pH 5.5. (b) Eadie-Hofstee plot of an MI uptake experiment with *S. enterica* serovar Typhimurium strain 14028. Substrate concentrations were 5, 3.5, 1, 0.5, 0.2 and 0.1 mM. A K_m value of 0.79 mM was calculated from three measurements.

performed with strain 14028 in the absence of glucose, but no significant differences were observed (data not shown). In experiments with heterologously expressed IolT1, K_m values of 0.71, 0.51 and 0.38 mM were calculated, resulting in an average K_m value of 0.53 ± 0.2 mM for IolT1 expressed in *E. coli*. Taken together, IolT1 appears to be the predominant MI transporter of *S. enterica* serovar Typhimurium, with functional similarities to the two characterized MI facilitators of *C. glutamicum* (Krings *et al.*, 2006).

IolT1-like proteins involved in MI utilization

According to genome sequence data, a large number of bacteria carry genes with homology to *iol* genes, including *Brucella abortus*, *Photobacter profundum*, *Yersinia* spp., *Citrobacter koseri* and *Erwinia carotovora*. Genetic comparison and computational analysis of *iol* divergons have been performed recently (Boutte *et al.*, 2008; Kröger & Fuchs, 2009). A phylogenetic analysis of *iol* genes from *L. casei* is also available (Yebra *et al.*, 2007). We investigated whether, and to what degree of similarity, IolT homologues are present in other bacterial genomes. IolT1 and IolT2 of *S. enterica* serovar Typhimurium exhibit an identity of 44 % and a similarity of 66 % over the whole protein length. No significant sequence identity was found between the two

predicted permeases STM4428 or STM4434, or between either protein and IolT1 or IolT2. Homology searches using BLAST (Altschul *et al.*, 1997) revealed many uncharacterized bacterial proteins with significant similarity to IolT1, among them the major MI transporter of *B. subtilis*, IolT, with an identity of 33 %. A cladogram of a representative selection of these proteins, many of which are annotated as putative xylose transporters, is shown in Fig. 5. The IolT1 homologues of *E. coli* ED1a, *Yersinia frederiksenii*, *Serratia proteamaculans*, *Vibrio shiloi*, *P. profundum* and *Azotobacter vinelandii* are clustered with the genes involved in MI degradation, suggesting an MI transport function for these proteins. The IolT homologues of several species unable to utilize MI, such as *E. coli*, *Vibrio angustum*, *Photobacterium* sp. and *Paenibacillus* sp., are also grouped, indicating that these proteins transport other sugars or sugar alcohols. Interestingly, the *Bacillus cereus* IolT1 homologue of this group is not identical to the major MI transporter of *B. subtilis* (Yoshida *et al.*, 2002), but is more closely related to homologues from Gram-negative species. On the other hand, IolT1 homologues of *Yersinia intermedia*, *Yersinia enterocolitica* and *Klebsiella pneumoniae* are more closely related to transporter proteins of Gram-positive bacteria, making it difficult to trace the evolutionary origin of *iolT1* and the gene clusters it belongs to. The most distantly related group comprises IolT1

Fig. 5. Cladogram based on 37 proteins with homology to lolT1 of *S. enterica* serovar Typhimurium. Percentage identities are indicated in parentheses. The putative permease STM4428 served as the outgroup. The phylogenetic analysis was performed with the neighbour-joining method and calculated using the Poisson correction. Values on each branch indicate the occurrence (%) of the branching order in 500 bootstrapped trees. Bar, 10 % sequence divergence. HTCC2207 is a marine γ -proteobacterium. MI, capability of utilizing MI or presence of *iol* genes in the genome. lolT1 and lolT2 are labelled in bold type. ^aThe lolT1 homologue is clustered with the genes of the *iol* divergon; ^bother putative permeases are present within the *iol* gene cluster.

homologues of species such as *Yersinia pseudotuberculosis*, *Yersinia pestis*, *Erwinia carotovora* and *P. luminescens*. These homologues are located outside the *iol* divergons, which carry genes encoding permeases, suggesting as yet unknown MI transporters in these bacteria. Consistent with this interpretation, Boutte *et al.* (2008) identified an ABC transporter for MI in *Sinorhizobium meliloti* and five other α -proteobacteria, thus defining a novel class of MI facilitators.

DISCUSSION

Of the four permeases located within GEI4417/4436, two were identified as MI transporters of *S. enterica* serovar Typhimurium and termed IolT1 and IolT2. While deletion of *iolT1* severely affected growth of strain 14028, only a slight growth deficiency was observed when 14028 Δ *iolT2* was grown in liquid MM containing MI (Fig. 1a). Two MI transporters with major and minor transport activity have also been identified in *B. subtilis* and *C. glutamicum* (Krings *et al.*, 2006; Yoshida *et al.*, 2002). In *B. subtilis*, the minor transporter IolF has been shown to have a lower substrate affinity than that of IolT, and IolF can support growth on MI only partially. The double deletion mutant 14028 Δ *iolT1* Δ *iolT2* could be complemented by providing *iolT2* *in trans* only on solid, but not in liquid medium. No significant MI transport activity of IolT2 could be detected in uptake experiments with *E. coli*. In *C. glutamicum*, the two MI transporters IolT1 and IolT2 have similar kinetic properties and a sequence identity of 55%. Overexpression of *iolT1* and *iolT2* of the same organism leads to a twofold increase of the D-fructose uptake rate, but both transporters show a lower specificity towards this sugar than towards MI (Bäumchen *et al.*, 2009). However, an apparent growth defect of *iolT* mutants of *B. subtilis* or *S. enterica* serovar Typhimurium in the presence of a variety of sugars has not been observed (Yoshida *et al.*, 2002). A major difference between MI utilization by Gram-positive bacteria and that of *S. enterica* serovar Typhimurium is the extended lag phase of this Gram-negative pathogen in medium containing MI as sole carbon and energy source (Fig. 1a). The molecular mechanism underlying this growth retardation by at least 2 days is not yet completely understood. The lag phase has been shown to be shortened by approximately 10 h in an *iolR* deletion mutant, indicating a key role of IolR in this phenomenon (Kröger & Fuchs, 2009). The binding of a cofactor or an intermediate of MI degradation to IolR, or an external signal, might release the tight repression of *iol* genes in *S. enterica* serovar Typhimurium.

Luciferase reporter assays revealed IolR to be a repressor of *iolT1* and *iolT2* transcription (Table 2). The absolute RLU per OD unit values show that in MM with MI, *iolT1* is expressed to an approximately 15-fold higher degree than *iolT2*. When both transporter genes were overexpressed in *E. coli*, similar protein levels were detected by Western blot analysis (Fig. 3b), but the MI transport activity of IolT2

was not detectable. Because IolT2 appears to have a significantly lower molecular mass than IolT1, it might be assumed that IolT2 is misfolded in the *E. coli* membrane or degraded to a stable yet inactive form. Also, it cannot be excluded that IolT2 activity requires different assay conditions. However, the tests performed in *S. enterica* serovar Typhimurium strongly indicate that IolT2 supports growth on MI, albeit with a longer generation time. This and the fact that the putative minor transporter IolT2 supports growth of *S. enterica* serovar Typhimurium on solid medium only are in contrast to findings in *B. subtilis* and *C. glutamicum*. It is possible that the preferred substrate of IolT2 is an unknown, MI-related compound or MI catabolite and that MI does not represent the physiologically relevant substrate of the protein (Reber *et al.*, 1977). The K_m values of 0.49 and 0.79 mM reported here for IolT1 of *S. enterica* serovar Typhimurium are similar to those of the major MI transporters in *B. subtilis* (0.15 mM, IolT) and *C. glutamicum* (0.33/0.45 mM, IolT1/IolT2). In *S. enterica* serovar Typhimurium, the transcriptional activities of the *iolT1* and *iolT2* promoters are approximately 10–40-fold induced in the presence of MI, or upon deletion of the repressor gene *iolR*. This pattern is similar to the results of *iolT::lacZ* fusion assays performed with *B. subtilis* under similar growth conditions (Yoshida *et al.*, 2002). Together with the finding that the expression of MI transporters is negatively controlled by IolR, these data indicate a highly conserved regulatory mechanism of MI transporter expression in Gram-positive and Gram-negative bacteria.

Taken together, the growing number of *iol* gene sequences and of bacterial species able to grow on MI revealed a high variation of gene content, genetic organization and functionality, while several aspects of MI utilization still remain to be discovered. Open questions regarding MI metabolism that are currently addressed in *S. enterica* serovar Typhimurium are further regulatory mechanisms contributing to MI utilization, the identification of MI-related substrates metabolized by the Iol enzymes, and the relevance of this pathway *in vivo*.

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