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 as 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 *iolT1* (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 *Km* value between 0.49 and 0.79 mM was determined in DH5α expressing IolT1, in *S. enterica* serovar Typhimurium strain 14028, and in mutant 14028 ΔiolT2. Phylogenetic analysis of IolT1 identified putative MI transporters in Gram-negative bacteria also able to utilize MI.

**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 *iolABCDGFHIIJ* 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), IotT 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* (Kawar 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-
lacking homologues of salmonellae; we identified a nearly identical gene cluster. Interestingly, this MI utilization island is not restricted to Salmonella. MI is not a common capability of and many others, indicating that the utilization of and are absent in serovars Typhi, Paratyphi A, Choleraesuis Paratyphi B, Saintpaul, Weltevreden, Agona and Virchow, present only in the genomes of the genomic island (GEI4417/4436). Identical islands are genes required for MI degradation are located on a 22.6 kb (Kro¨ger & Fuchs, 2009). Four genes, STM4418, STM4419, A total of 20 genes are located on GEI4417/4436, but a negative bacteria is due to horizontal gene transfer. The strong suggesting that its distribution among Gram-negative among Saintpaul, Weltevreden, Agona and Virchow, is flanked by two transposase-encoding genes, 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, 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 bandshift 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 MgSO4, 0.1 mM CaCl2 and 55.5 mM NaCl].

<table>
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<th>Table 1. Strains and plasmids used in this study</th>
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<td><strong>Strains</strong></td>
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<tr>
<td>DH5α</td>
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<tr>
<td>BL21 (DE3)</td>
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<tr>
<td>14028</td>
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<tr>
<td>14028 ΔiolT1</td>
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<td>14028 ΔiolT2</td>
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<tr>
<td>14028 ΔiolT1ΔiolT2</td>
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<td>14028 ΔSTM4428</td>
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<td>14028 ΔSTM4434</td>
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| **Plasmids** | **Description and relevant features** | **Source or reference** |
| pKD4 | pir-Dependent, FRT sites; Kan’ | CGSC; Datsenko & Wanner (2000) |
| pKD46 | Lambda-Red helper plasmid; Amp’ | CGSC; Datsenko & Wanner (2000) |
| pCP20 | FLP recombinase plasmid; Cm’ Amp’ | CGSC; Datsenko & Wanner (2000) |
| pET28b | Expression vector, T7lac promoter; Kan’ | Novagen |
| pBR322 | Amp’ Tet’ | Fermentas |
| pBR322–iolT1, pBR322–iolT2 | iolT1 or iolT2 cloned into pBR322 with putative promoter region | This study |
| pBR322–iolT1–His6, pBR322–iolT2–His6 | As above, but encoding transporters with C-terminal His6-tag | This study |
| pDEW201 | Promoter probe vector; Amp’ luxCDABE | Van Dyk & Rossom (1998) |
| pDEW201–iolT1, pDEW201–iolT2 | pDEW201 with 300 bp upstream of iolT1 (STM4418) | This study |
| pDEW201–iolT2, pDEW201–argS | pDEW201 with 300 bp upstream of iolT2 (STM4419) | This study |
| pDEW201–argS | pDEW201 with 244 bp upstream of argS | 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 μg ml⁻¹), kanamycin (50 μg ml⁻¹), chloramphenicol (25 μg ml⁻¹) or streptomycin (50 μg 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 OD₆₀₀ 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. OD₆₀₀ 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://enterix.ccbcb.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 following the primer sets listed in Supplementary Table S1. 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).

**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 strains 14028 with primers listed in Supplementary Table S1. The plasmids 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 DH5α cells, plasmids containing the correct transcriptional lux fusions were isolated and verified by PCR, restriction analysis and sequencing. pDEW201-PiolT1 and pDEW201-PiolT2 were transformed into S. enterica serovar Typhimurium 14028 and the ΔiolR 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 OD₆₀₀ and the bioluminescence, measured as relative light units (RLU), were recorded in a Wallac VICTOR² 1420 multilabel counter (PerkinElmer 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 OD₆₀₀ 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 Amino Instruments), and cell debris was removed by centrifugation at 4 °C (20 min, 1.6 x 10⁴ 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 x 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.** DH5α 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 (Kreutzbeek et al., 2007) but using a French press (SLM Amino 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]inositol.** E. coli DH5α cells transformed with pBR322–iolT1, pBR322–iolT2 and pBR322 were grown in
30 ml LB medium containing ampicillin (100 μg ml⁻¹) to OD₆₀₀ 1.0. Then, 20 ml was harvested by centrifugation (4 °C and 1 x 10⁴ g) and resuspended in McIlvaine’s buffer [0.1 M Na₂HPO₄, 0.05 M citric acid mixed to obtain the desired pH (McIlvaine, 1921)] to OD₆₀₀ 4.0 and stored on ice. Cell suspension (250 μl) was mixed with 230 μl McIlvaine’s buffer and 12.5 μl 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 μM. The uptake experiment was started by adding 7 μl 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 Kₘ 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 μl were removed, rapidly filtered through a Pall GN6 0.45 μm 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 μ=0.56 h⁻¹) than in MI (μ=0.37 h⁻¹). In contrast to the mutants 14028 ΔSTM4428 and 14028 ΔSTM4434, 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
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 ΔiolT1 and 14028 ΔiolT1ΔiolT2, resulting in wild-type-like growth of both mutants (Fig. 1b).

Only slightly reduced growth of 14028 ΔiolT2 (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 ΔiolT1ΔiolT2 to that of strain 14028, and retarded growth in comparison with 14028 ΔiolT1ΔiolT2/pBR322-iolT1 was observed (Fig. 1b). Strain 14028 ΔiolT1 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 ΔiolT1 and 14028 ΔiolT2 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 Photorhabdus 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 × 10^6 RLU per OD_600 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 OD_600 unit), while the predicted iolT1 promoter showed a maximal bioluminescence of 8.52 × 10^5 RLU per OD_600 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 ΔiolR 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 |
|---------------------------------|-------------------|-------------------|-------------------|
| Fragment cloned into pDEW201   | 14028 grown in MM + glucose | 14028 grown in MM + MI | 14028 ΔiolR grown in MM + glucose |
|                                | RLU per OD_600 unit* | SD (%)  | RLU per OD_600 unit* | SD (%)  | Fold induction^1 | RLU per OD_600 unit* | SD (%)  | Fold induction^1 |
| 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             |

^1Data are the mean RLU derived from three independent experiments.

^1Fold induction was calculated with respect to the RLU per OD_600 unit values of strain 14028 grown in glucose.
was tested by GMS 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.

**Fig. 2.** GMS 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.

**IoIT1 mediates MI uptake when expressed in E. coli**

IoIT1 and IoIT2, 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* DH5α 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 IoIT1 and IoIT2 to study their MI transport activity. *E. coli* DH5α cells were transformed with pBR322- *iolT1* and pBR322- *iolT2*, and uptake assays were performed as detailed in Methods. In a first experiment, DH5α cells expressing IoIT1 or IoIT2 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). IoIT1-expressing cells showed high levels of MI uptake, which were linear for 50 s after substrate addition (Fig. 3a).
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.

Fig. 3. MI uptake by heterologously expressed IolT1 and IolT2. (a) E. coli DH5α cells containing plasmids pBR322-iolT1 (●), pBR322-iolT2 (□), or pBR322 (○, negative control) were grown in LB medium, harvested by centrifugation, resuspended in McIlvaine’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 DH5α/pBR322-iolT1 (●), 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 μg membrane protein was subjected to SDS-PAGE and Western blot analysis using monoclonal anti-His6 antibodies. The samples were obtained from E. coli DH5α cells containing pBR322-iolT1–His6 (left), pBR322-iolT2–His6 (centre) or pBR322 (right, negative control).

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 ΔiolT2 (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
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 IolT1 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. IolT1 and IolT2 are labelled in bold type. *The IolT1 homologue is clustered with the genes of the *iol* divergon; other 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 ΔiolT2 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 ΔiolT1ΔiolT2 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 that of IolT, and IolF can support growth on MI only partially. 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 *Kₘ* 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*.

**ACKNOWLEDGEMENTS**

We thank Siegfried Scherer for financial support of this study. Patrick Schiewek is acknowledged for technical assistance, and Gabor Kottra for help with data analysis.

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Edited by: G. H. Thomas