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Characterization of the *myo*-Inositol Utilization Island of *Salmonella enterica* serovar Typhimurium▽†

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Knockout mutation of STM4432 resulted in a growth-deficient phenotype of *Salmonella enterica* serovar Typhimurium in the presence of *myo*-inositol (MI) as the sole carbon source. STM4432 is part of a 22.6-kb genomic island which spans STM4417 to STM4436 (genomic island 4417/4436) and is responsible for MI degradation. Genome comparison revealed the presence of this island in only six *Salmonella* strains and a high variability of the *iol* gene organization in gram-negative bacteria. Upon nonpolar deletion of 11 island loci, the genes involved in six enzymatic steps of the MI pathway were identified. The generation time of *S. enterica* serovar Typhimurium in minimal medium with MI decreases with higher concentrations of this polyol. Reverse transcriptase PCR showed five separate transcriptional units encompassing the genes *iolA-iolB*, *iolE-iolG1*, *iolC1-iolC2*, *iolD1-iolD2-iolG2*, and *iolI2-iolH*. Luciferase reporter assays revealed a strong induction of their promoters in the presence of MI but not glucose. The main regulator, IolR, was identified due to a reduced lag phase of a strain mutated in STM4417 (*iolR*). Deletion of *iolR* resulted in stimulation of the *iol* operons, indicating its negative effect on the *iol* genes of *S. enterica* serovar Typhimurium in rich medium at a transcriptional level. Bandshift assays demonstrated the binding of this putative repressor to promoter sequences of *iolA*, *iolC1*, and *iolD1*. Binding of IolR to its own promoter and induced *iolR* expression in an IolR-negative background demonstrate that its transcription is autoregulated. This is the first characterization of MI degradation in a gram-negative bacterium, revealing a complex transcriptional organization and regulation of the *S. enterica* serovar Typhimurium *iol* genes.

More than 60 carbon sources are known to be utilizable by *Salmonella enterica* serovar Typhimurium (10). Among them is *myo*-inositol (MI), a substrate that is ubiquitous in soil and plants, where it appears as a free form or as phospholipid derivatives. Old reported that MI utilization by *S. enterica* serovar Typhimurium strains is temperature dependent and that 95% of all strains investigated fermented MI at 25°C, although they had been designated inositol nonfermenting at 37°C (23).

In addition to *S. enterica* serovar Typhimurium, growth of gram-negative bacteria on MI has been demonstrated so far for representatives of the genera *Serratia* and *Klebsiella* (18) and for *Rhizobium leguminosarum* (24). The enzymatic steps of the MI degradation were partially analyzed in *Aerobacter* (reclassified as *Klebsiella*) *aerogenes* (3). The genetics and biochemistry of bacterial MI utilization have been described in most detail for *Bacillus subtilis* (31, 33, 35). In this organism, the *iol* divergon, comprising the operons *iolABCDEFGHIJ* and *iolRS*, and the gene *iolT*, located elsewhere on the chromosome, were shown to be responsible for MI degradation that finally results in an equimolar mixture of dihydroxyacetone phosphate, acetyl coenzyme A, and CO_2 (33). Two transporters belonging to the major facilitator superfamily have been identified in *B. subtilis* (34). Inactivation of *iolT* caused an obvious growth defect of *B. subtilis*, while a mutant with a knockout mutation of *iolF*, encoding the second MI transporter, showed a significant growth effect only when *iolT* was mutated simultaneously. IolR is a repressor that regulates the *iol* divergon of *B. subtilis*, including *iolT* (34, 36). It binds to the operator sites within the *iol* promoters in the absence of MI. If this polyol is present in the medium, it is converted to the intermediate 2-deoxy-5-keto-D-gluconic acid 6-phosphate, which acts as an inducer by antagonizing IolR DNA binding (33). Other bacteria able to grow on MI as the sole carbon source are *Corynebacterium glutamicum*, *Clostridium perfringens*, and *Lactobacillus casei* strain BL23 (14, 17, 30). In *C. perfringens*, all *iol* genes except *iolR* are unidirectionally organized, and a single transcript of 15.6 kb has been identified (14). *L. casei* BL23 was the first example of a lactic acid bacterium able to utilize MI (30). The *iol* genes in this organism are located on a 12.8-kb insertion organized in a manner similar to that in *C. perfringens.* A more complex organization of *iol* genes was reported for *Corynebacterium glutamicum*, in which a second gene cluster encodes redundant functions in MI utilization, including oxidation and transport (17). The *iol* regulon is subjected to carbon catabolite repression mediated by CcpA at least in *B. subtilis* and *L. casei* (21, 30).

The molecular genetics of MI degradation by a gram-negative bacterium have not been investigated. Here, we describe that the knockout of several genes in genomic island (GEI) 4417/4436 results in growth-negative phenotypes of *S. enterica* serovar Typhimurium on MI. The activ-

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TABLE 1. Strains and plasmids used in this study

Bacterial strain or plasmid	Description and relevant features	Source or reference
E. coli strains		
DH5α	*deoR endA1 gyrA96 hsdR17*(r_K^- m_K^+) *recA1 relA1 supE44* λ*thi-1* Δ(*lacZYA-argFV169*)	11
BL21(DE3)	F^- *ompT hsdS*$_B$ (r_B^- m_B^-) gal dcm rne131	27
S. enterica serovar Typhimurium strains		
14028	Wild-type strain ATCC 14028	ATCC
14028 STM4417::pIDM1	Insertion-duplication mutant with STM4417 knockout	This study
14028 STM4432::pIDM1	Insertion-duplication mutant with STM4432 knockout	This study
14028s	Spontaneous streptomycin-resistant mutant of 14028	This study
14028s Δ*iolR*	In-frame *iolR* (STM4417) deletion mutant	This study
14028s Δ*iolB*	In-frame *iolB* (STM4420) deletion mutant	This study
14028s Δ*iolA*	In-frame *iolA* (STM4421) deletion mutant	This study
14028s Δ*iolE*	In-frame *iolE* (STM4424) deletion mutant	This study
14028s Δ*iolG1*	In-frame *iolG1* (STM4425) deletion mutant	This study
14028s Δ*iolI1*	In-frame *iolI1* (STM4427) deletion mutant	This study
14028s Δ*iolC*	In-frame *iolC* (STM4429-30) deletion mutant	This study
14028s Δ*iolD2*	In-frame *iolD2* (STM4432) deletion mutant	This study
14028s Δ*iolG2*	In-frame *iolG2* (STM4433) deletion mutant	This study
14028s Δ*iolI2*	In-frame *iolI2* (STM4435) deletion mutant	This study
14028s Δ*iolH*	In-frame *iolH* (STM4436) deletion mutant	This study
14028s ΔSTM3253	In-frame STM3253 deletion mutant	This study
Y. enterocolitica W22703	Wild-type strain, biovar 2, serovar O:9; Nal^r Res^- Mod^+ pYV^-	6
P. luminescens subsp. *laumondii* TT01		8
Plasmids		
pKD4	Kan^r, *pir* dependent, FRT sites	CGSC, Yale (7)
pKD46	Lambda Red helper plasmid; Amp^r	CGSC, Yale (7)
pCP20	FLP recombinase plasmid; Cm^r Amp^r	CGSC, Yale (7)
pET28b	Expression vector, T7*lac* promoter; Kan^r	Novagen
pET28b-*iolR*	*iolR* cloned into pET28b for IolR overexpression and purification	This study
pIDM1	Temperature-sensitive plasmid; *repA* Tet^r	9
pBR322	Amp^r Tet^r	Fermentas
pBR322-*iolR*	*iolR* with putative promoter region cloned into pBR322 for complementation	This study
pBR322-*iolE*	*iolE* with putative promoter region cloned into pBR322 for complementation	This study
pDEW201	Promoter probe vector; Amp^r *luxCDABE*	28
pDEW201-P_{iolR}	pDEW201 with 299 bp upstream of *iolR* (STM4417)	This study
pDEW201-P_{iolA}	pDEW201 with 288 bp upstream of *iolA* (STM4421)	This study
pDEW201-P_{iolE}	pDEW201 with 321 bp upstream of *iolE* (STM4424)	This study
pDEW201-P_{iolG1}	pDEW201 with 335 bp upstream of *iolG1* (STM4425)	This study
pDEW201-P_{iolC1}	pDEW201 with 301 bp upstream of *iolC1* (STM4430)	This study
pDEW201-P_{iolD1}	pDEW201 with 325 bp upstream of *iolD1* (STM4431)	This study
pDEW201-P_{iolG2}	pDEW201 with 301 bp upstream of *iolG2* (STM4433)	This study
pDEW201-P_{iolI2}	pDEW201 with 301 bp upstream of *iolI2* (STM4435)	This study
pDEW201-P_{iolH}	pDEW201 with 299 bp upstream of *iolH* (STM4436)	This study
pDEW201-P_{argS}	pDEW201 with 244 bp upstream of *argS*	This study
pDEW201-P_{def}	pDEW201 with 350 bp upstream of *def*	This study
pDEW201-′STM0047′	pDEW201 with 350 bp of STM0047 without promoter homology	This study

ities of *iol* gene promoters under various growth conditions were quantified using the luciferase reporter, and the complex transcriptional organization of the *iol* genes essential for MI degradation was determined by reverse transcriptase PCR (RT-PCR). IolR, encoded by STM4417, was characterized as a negative regulator of *Salmonella* MI utilization that also regulates its own expression. The binding of this repressor to all but one of the promoters controlling the MI utilization genes was demonstrated by gel mobility shift (GMS) assays.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The bacterial strains and plasmids used in this study are listed in Table 1. *S. enterica* serovar Typhimurium and *Escherichia coli* cultures were grown in Luria-Bertani (LB) broth (10 g/liter tryptone, 5 g/liter yeast extract, 5 g/liter NaCl) or in minimal medium (MM) (M9 medium supplemented with 2 mM $MgSO_4$, 0.1 mM $CaCl_2$, and 55.5 mM [1%, wt/vol] MI or 27.8 mM [0.5%, wt/vol] glucose). If necessary, the media were supplemented with the following antibiotics: ampicillin (150 μg/ml), kanamycin (50 μg/ml), chloramphenicol (25 μg/ml), or streptomycin (50 μg/ml). For solid media, 1.5% agar (wt/vol) was added. For all growth and promoter probe experiments, bacterial strains were grown in LB medium overnight at 37°C, washed

twice in phosphate-buffered saline, and then adjusted to an optical density at 600 nm (OD_{600}) of 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 rigorous shaking in 250 ml flasks with 50 ml of MM. The OD_{600} was measured at appropriate time intervals as indicated.

Standard procedures. DNA manipulations and isolation of chromosomal or plasmid DNA were performed according to standard protocols (25) and following the manufacturers' instructions. GeneRuler DNA Ladder Mix (Fermentas, St. Leon-Rot, Germany) was used as a marker for DNA analysis. Plasmid DNA was transformed via electroporation by using a Bio-Rad Gene Pulser II as recommended by the manufacturer and as described previously (15). PCRs were carried out with *Taq* polymerase (Fermentas). As a template for PCR, chromosomal DNA, plasmid DNA, or an aliquot of a single colony resuspended in 100 μl H_2O was used. Oligonucleotides used for PCRs are listed in Table S1 in the supplemental material. Strains of an *S. enterica* serovar Typhimurium mutant library were characterized as described previously (16). *S. enterica* serovar Typhimurium gene numbers refer to the LT2 annotation (NC 003197). The websites http://globin.cse.psu.edu/enterix and http://www.microbesonline.org/ were used to determinate the distribution of *S. enterica* serovar Typhimurium open reading frames in the genomes of gram-negative species. Promoter sequences located upstream of the identified genes were predicted with BPROM (Softberry, Inc.).

Phenotypic testing of carbon source utilization. A set of *S. enterica* serovar Typhimurium mutants was screened for their ability to utilize a number of 63 different substrates that are possible carbon sources for *S. enterica* serovar Typhimurium (10). For this purpose, we used a colorimetric assay based on the reduction of tetrazolium violet as final electron acceptor during respiration due to carbon catabolism (4, 22). *Salmonella* cells were grown overnight in LB medium at 37°C, washed twice with phosphate-buffered saline, resuspended in inoculation solution (M9 medium supplemented with 2 mM $MgSO_4$, 0.1 mM $CaCl_2$, 0.03% pluronic F68, 0.02% gellan gum, and 0.01% tetrazolium violet) and adjusted to an OD_{600} of 0.3; 90 μl of this cell suspension was then mixed with 10 μl (0.5 M) of each carbon source solution, pipetted in a 96-well microtiter plate, and measured after 24 and 48 h at 37°C in a microtiter plate reader (Tecan, Männedorf, Switzerland) at OD_{620}. Sucrose and lactose, which are not metabolized by *S. enterica* serovar Typhimurium, served as control substrates.

Construction of deletion mutants and complementing plasmids. In-frame STM3253, STM4417 (*iolR*), STM4420 (*iolB*), STM4421 (*iolA*), STM4424 (*iolE*), STM4425 (*iolG1*), STM4427 (*iolI1*), STM4430/4429 (*iolC1*/*iolC2*), STM4432 (*iolD2*), STM4433 (*iolG2*), STM4435 (*iolI2*), and STM4436 (*iolH*) deletion mutants were constructed by the one-step method based on the phage λ Red recombinase (7). Briefly, PCR products comprising the kanamycin resistance cassette of plasmid pKD4, including the flanking FRT sites, were generated using pairs of 70-nucleotide-long primers that included 20-nucleotide priming sequences for pKD4 as template DNA. Homology extensions of 50 bp overlapped 18 nucleotides of the 5′ end and 36 nucleotides of the 3′ end of the target gene (19). Five hundred to 1,000 ng of fragment DNA was transferred into *S. enterica* serovar Typhimurium strain 14028s cells harboring plasmid pKD46. Allelic replacement of the target gene by the kanamycin resistance cassette was controlled by PCR, and nonpolar 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 *iolR* and *iolE* plus approximately 300 bp of their upstream region were amplified from chromosomal DNA of strain 14028 with primers listed in Table S1 in the supplemental material. PCR products were digested with EcoRI and SalI (Fermentas) and ligated (T4 DNA ligase; Gibco) into the promoterless vector pBR322 to generate pBR322-*iolR* and pBR322-*iolE*, respectively. Their construction was verified by PCR and restriction analysis.

RNA preparation and RT-PCR. RNA was isolated according to the modified single-step method of Chomczynski and Sacchi (5). Briefly, 15 ml of an *S. enterica* serovar Typhimurium culture grown in MM supplemented with MI to an OD_{600} of ~0.4 was centrifuged, and the cell pellet was resuspended in 1 ml of Trizol (Invitrogen, Karlsruhe, Germany). The cells were disrupted in a Ribolyzer (Hybaid, Heidelberg, Germany) as described recently (12). Following chloroform extraction, nucleic acids were precipitated, washed, and resuspended in 30 μl diethyl pyrocarbonate-treated H_2O. DNase treatment was performed with RQ1 DNase I (Promega, Mannheim, Germany) according to the manufacturer's instruction. Annealing of reverse primers (see Table S1 in the supplemental material) was performed in a total volume of 10 μl containing 75 ng of total RNA, 10 pmol reverse primer, and 20 mM deoxynucleoside triphosphate mix using the following protocol: 75°C for 2 min, 70°C for 1 min, 65°C for 1 min, 55°C for 1 min, 50°C for 1 min, 45°C for 1 min, and 42°C for 60 min. Immediately after the mixture reached 42°C, 10 μl RT mix (Promega) with 0.1 M dithiothreitol and 200 U RT (Promega) was added to generate cDNA. Heat inactivation of RT was performed by incubation at 70°C for 15 min, and 2 μl of this sample was then used as the PCR template.

Cloning of promoter fusions. Putative promoter regions spanning approximately 300 bp upstream of the start codons of the genes *iolR* (STM4417), *iolA* (STM4421), *iolE* (STM4424), *iolG1* (STM4425), *iolC1* (STM4430), *iolD1* (STM4431), *iolG2* (STM4433), *iolI2* (STM4435), *iolH* (STM4436), *argS* (STM1909), and *def* (STM3406) and an intragenic fragment of STM0047 without promoter homology were amplified from chromosomal DNA of *S. enterica* serovar Typhimurium 14028 by PCR using the primers listed in Table S1 in the supplemental material. The fragments were then cloned via EcoRI and BamHI or EcoRI and KpnI (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.

Quantification of promoter activity. Bioluminescence measurements were performed in 96-well plates. For growth in MM containing either 27.8 mM glucose or 55.5 mM MI, bacterial cells were grown at 37°C 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 the 96-well plate, and the OD_{600} and the bioluminescence, measured as relative light units (RLU), were recorded in a Wallac Victor^3 1420 multilabel counter (Perkin-Elmer Life Sciences, Turku, Finland).

Overexpression of *iolR*. The *iolR* gene without its stop codon was cloned into plasmid pET28b using the restriction sites XhoI and NhoI, thus introducing a C-terminal fusion of a His_6 tag for protein purification purposes. pET28b-*iolR* was transformed into *E. coli* BL21, and the expected clone was verified by restriction analysis. An overnight culture of this strain was diluted 1:100 in 100 ml LB medium supplemented with 150 μg/ml ampicillin and incubated for 3 h at 37°C at 180 rpm. Heterologous expression of *iolR* was then induced by adding 0.1 mM isopropyl-β-D-1-thiogalactopyranoside (IPTG). After incubation for 4 h at 37°C and 180 rpm, the cells were harvested by centrifugation at 4°C (30 min, 10^4 rpm), and the pellet was resuspended in 1 ml buffer A (300 mM NaCl, 50 mM Na_3PO_4). The cells were subsequently lysed by two passages through a French press (SLM Aminco Instruments, Rochester, NY), and residual cell debris was removed by centrifugation at 4°C (20 min, 1.4×10^4 rpm). After addition of 10 μl of the protease inhibitor phenylmethylsulfonyl fluoride (100 mM), 10 μl of the supernatant containing soluble proteins mixed with 10 μl 2× Laemmli buffer was applied to sodium dodecyl sulfate-polyacrylamide gels to verify IolR-His_6 overexpression, and separated proteins were stained with Coomassie blue.

Purification of IolR-His_6 and GMS assays. Protein IolR-His_6 was purified using Talon metal affinity resin (Clontech Laboratories, Mountain View, CA). One milliliter of the protein extract was mixed with 1 ml of the resin and incubated for 1 h at room temperature. The probe was then washed 10 times with 0.5 ml buffer A and 5 times with 0.5 ml buffer B (buffer A containing 7.5 mM imidazole). IolR-His_6 was eluted 10 times using 0.5 ml buffer C (buffer A with 150 mM imidazole). Fractions containing large 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 $MgCl_2$, 0.5 mM EDTA, and 10% [vol/vol] glycerol) by gel filtration using PD-10 columns (GE Healthcare, Munich, Germany) (26). The protein concentration was determined in a Nanodrop spectrophotometer (Thermo Fischer Scientific, Langenselbold, Germany), and the purity of eluted fractions was analyzed by separation on a 15% sodium dodecyl sulfate-polyacrylamide gel.

For the GMS assays, putative promoter regions of *iolR*, *iolA*, *iolE*, *iolG1*, *iolC1*, *iolD1*, *iolG2*, *iolI2*, and *iolH* were amplified as described above, and 100 ng of DNA was then mixed with increasing amounts of purified IolR-His_6 in GMS buffer. As a control, 100 ng of competitor DNA was added, resulting in a total volume of 20 μl. After incubation for 45 min at room temperature, the samples were loaded with 4 μl of 6× loading dye (Fermentas) on a 9.5% native polyacrylamide gel and separated at 120 V for 3 h in 1× Tris-borate-EDTA buffer precooled at 4°C. DNA was stained in ethidium bromide solution and visualized by UV irradiation.

RESULTS

The MI utilization genes of *S. enterica* serovar Typhimurium are located on a GEI. A total of 177 clones of a mutant library of strain 14028 had been characterized with respect to the site of an insertional knockout (15). Fifty mutated genes were

predicted to be involved in carbohydrate metabolism and transport, and each mutant was therefore tested for its ability to respire in the presence of one of 66 carbon sources, including MI. One mutant was not able to use this substrate as the sole carbon source, and a second mutant appeared to metabolize MI within 24 h in comparison to the wild-type strain, which showed a significant respiration signal only after 48 h. The strains are mutated in the genes STM4432 and STM4417, which belong to a GEI of 22.6 kb that starts with STM4417 and ends with STM4436 (20). Homology searches using BLAST programs (1) revealed 12 genes to be possibly involved in MI metabolism by *S. enterica* serovar Typhimurium (Fig. 1A). The island also carries four genes coding for putative sugar transporters, two hypothetical regulatory genes, one gene of unknown function, and *srfJ*. The last one is regulated by the two-component system SsrAB, that also controls the expression of *Salmonella* pathogenicity island 2 genes (29). The annotation of the MI utilization genes of *S. enterica* serovar Typhimurium shown in Fig. 1A is essentially based on homologies to *B. subtilis* and is also in line with a comparative annotation performed recently (30). Frameshifts have split the *S. enterica* serovar Typhimurium *iolC* and *iolD* homologues into two open reading frames annotated *iolC1/2* and *iolD1/2*. The frameshift in *iolC* might result in two functional proteins, because IolC2 represents an intact enzyme domain with a putative kinase function. The enzymes IolG2, IolD1, and IolD2 are predicted to require thiamine pyrophosphate as a cofactor. The GC content of GEI 4417/4436 (50.7%) does not significantly differ from that of the whole genome (52.2%), a finding that argues against a recent acquisition of the *Salmonella* MI utilization island by horizontal gene transfer.

***iol* genes of gram-negative bacteria.** GEI 4417/4436 is present in the genomes of the *S. enterica* serovar Typhimurium strains LT2, 14028, SL1344; *S. enterica* serovar Saintpaul strain SARA23; *S. enterica* serovar Weltevreden strain HI_N05-537; and *S. enterica* serovar Paratyphi B strain SPB7. Most genes of this island are absent in the genomes of the *E. coli* strains K-12, O157:H7, CFT073, 042, and E2348/69; of *Shigella flexneri*, *Shigella dysenteriae* M131649, and *Shigella sonnei* 53G; of eight *S. enterica* serovars (serovars Typhi CT18/Ty2, Paratyphi A/C, Enteritidis, Dublin, Gallinarum, and Diarizonae); of *Salmonella bongori*, and of *Vibrio cholerae*. A chromosomal fragment of *Klebsiella pneumoniae* carries nine genes involved in MI degradation and seven genes encoding putative transporters. Permeases that might play a role in MI uptake were also found in an *iol* gene cluster present in *Erwinia carotovora*, *Citrobacter koseri*, *Yersinia pestis*, *Yersinia pseudotuberculosis*, and *Photorhabdus luminescens*. *Yersinia enterocolitica* and two nonpathogenic *Yersini*a species share the same organization of *iol* genes, as do *Brucella abortus*, *Rhizobium leguminosarum*, and *Sinorhizobium meliloti*.

We therefore tested *Y. enterocolitica* and *P. luminescens* for their ability to utilize MI as the only carbon source. Indeed, we observed growth of psychrotrophic *Y. enterocolitica* at 15°C and 22°C, but not at 37°C, and of *P. luminescens* at 30°C (data not shown). This result shows that six genes, as exemplified by *Yersinia* spp., are sufficient for MI degradation by gram-negative species.

The pathway of MI degradation in *S. enterica* serovar Typhimurium is depicted in Fig. 1B. The annotation and the functional assessment of the genes essentially follow enzymological studies of *B. subtilis* and *K. aerogenes* (2, 3).

Growth properties of *S. enterica* serovar Typhimurium in the presence of MI. We investigated the growth behavior of strain 14028 in MM supplemented with various concentrations of MI. Three features appeared to be characteristic of *S. enterica* serovar Typhimurium growth under these conditions: (i) the maximum OD_{600} in the presence of MI parallels that of the strain grown in glucose (data not shown), (ii) the growth is dose dependent, and (iii) the lag phase is prolonged to approximately 60 h (Fig. 2). As already reported by Old, the wild-type strain produced abundant amounts of a brown pigment (23). This pigmentation appeared only in stationary phase. It was absent when the strain was cultivated anaerobically and might therefore result from metabolite oxidation.

Phenotypes of deletion mutants. To experimentally demonstrate that genes of GEI 4417/4436 are responsible for MI degradation by *S. enterica* serovar Typhimurium, nonpolar deletions of *iolB*, *iolA*, *iolE*, *iolG1*, *iolI1*, *iolC1/2*, *iolD2*, *iolG2*, *iolI2*, *iolH*, and STM3253 were constructed as described above. Growth of these 11 mutants was monitored at least for 5 days, or until the cultures reached stationary phase. No growth deficiencies of the mutants were observed in LB medium or in MM supplemented with glucose (data not shown). The mutants 14028s Δ*iolIB*, 14028s Δ*iolA*, 14028s Δ*iolE*, 14028s Δ*iolG1*, 14028s Δ*iolC*, and 14028s Δ*iolD2* did not grow in liquid MM containing MI as the sole carbon source, clearly demonstrating the role of the deleted genes in MI utilization. In contrast, growth deficiencies of the mutants 14028s Δ*STM3253*, 14028s Δ*iolI1*, 14028s Δ*iolI2*, 14028s Δ*iolH*, and 14028s Δ*iolG2* in comparison to the wild type were not observed under these conditions. IolI1 and IolI2 might be functionally redundant, and the role of IolH remains to be disclosed. Two genes coding for proteins with homology to IolG from *B. subtilis* (IolG1) and to a putative MI dehydrogenase of *Lactobacillus plantarum* WCFS1 (IolG2) are present in GEI 4417/4436, a redundancy that might explain the wild-type-like growth of the *iolG2* mutant. STM3253 encodes a protein with a significant homology of 38% to *B. subtilis* IolJ. IolJ is responsible for the formation of dihydroxyacetone phosphate and malonate semialdehyde from 2-deoxy-5-keto-D-gluconic acid 6-phosphate (Fig. 1B), but GEI 4417/4436 does not encode such a biphosphate aldolase. The wild-type-like phenotype of the deletion mutant (data not shown), however, excludes a role of STM3253 in MI degradation. All mutants were also streaked on MM agar plates containing 55.5 mM MI and incubated for 64 h. The phenotype of the *iolE* deletion mutant could be complemented with pBR322-*iolE* as shown by growth on MM agar plates and in liquid medium (data not shown). These results indicate that *iolB*, *iolA*, *iolD2*, *iolE*, *iolG1*, and *iolC1/2* are required for MI degradation as indicated in Fig. 1A, thus confirming the pathway reconstruction in Fig. 1B.

Differential expression of genes involved in MI degradation. In order to investigate the regulation of the genes in GEI4417/4436, fragments of approximately 300 bp located upstream of the start codons of STM4417, *iolA*, *iolE*, *iolG1*, *iolC1*, *iolD1*, *iolG2*, *iolI2*, and *iolH* were cloned into the promoter probe vector pDEW201 carrying the *luxCDABE* cassette. Promoter fragments of *def* and *argS*, encoding peptide deformylase and arginyl-tRNA synthetase, respectively, were cloned as positive

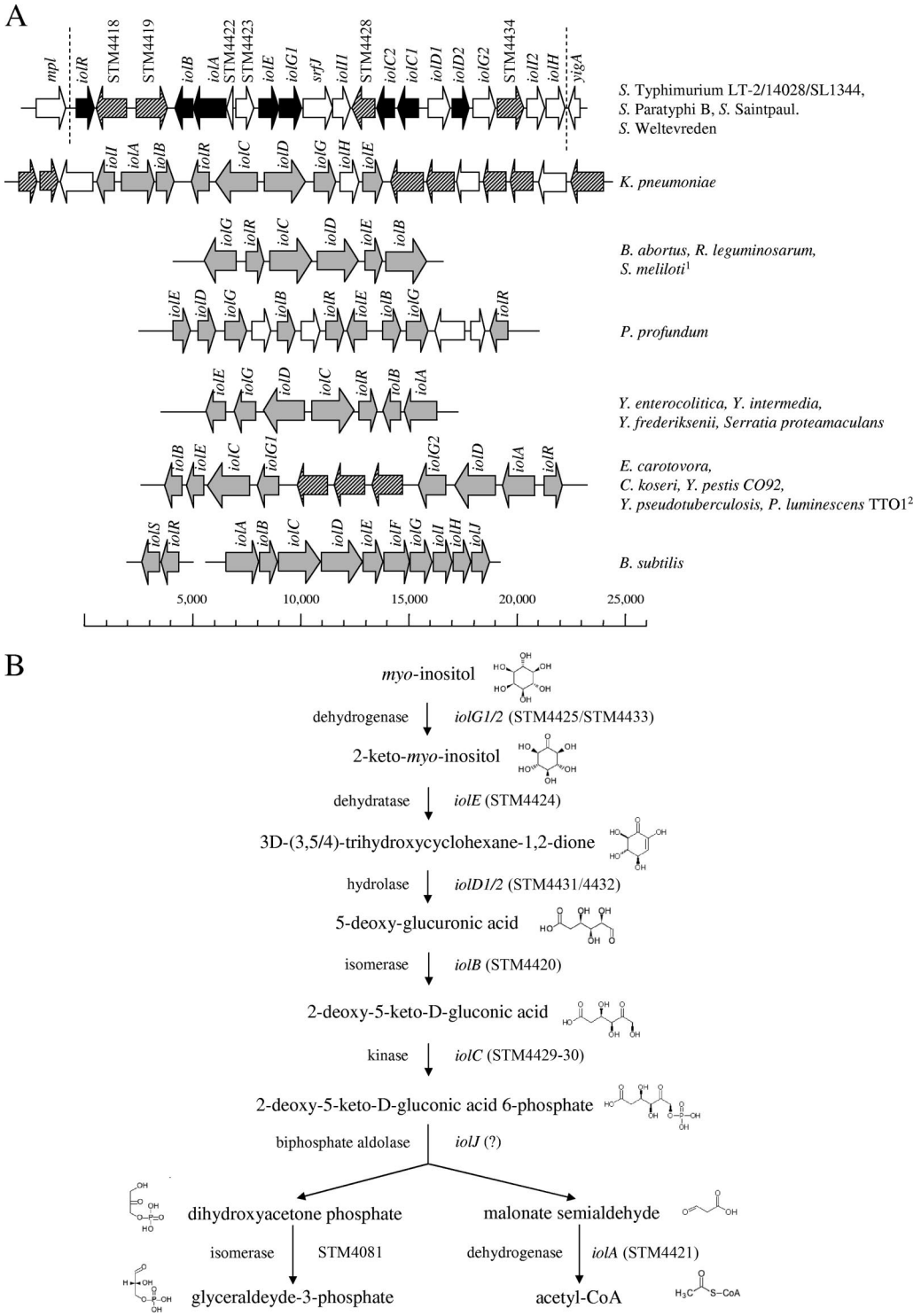

FIG. 1. (A) Examples of *iol* divergons. GEI 4417/4436 (22.6 kb) of *S. enterica* serovar Typhimurium is presented in comparison to the structural organization of *iol* genes from *B. subtilis* and several gram-negative bacteria. *Salmonella* genes experimentally demonstrated in this study to belong to the inositol divergon are depicted in black; their homologues in other pathogens are shown in gray. Genes encoding putative permeases are hatched. 1, in this organism, *iolG* is transcribed in the same orientation as the other genes; 2, the *iol* cluster of *P. luminescens* is similar to that of *E. carotovora* but lacks two of three putative permease genes. (B) Reconstruction of the pathway for MI degradation in *S. enterica* serovar Typhimurium. Seven stepwise reactions are involved in MI degradation to glyceraldehyde-3-phosphate and acetyl coenzyme A (acteyl-CoA). None of the genes from GEI 4417/4436 encodes a homologue of a biphosphate aldolase. Chemical structures were taken from the Kyoto encyclopedia of genes and genomes (13).

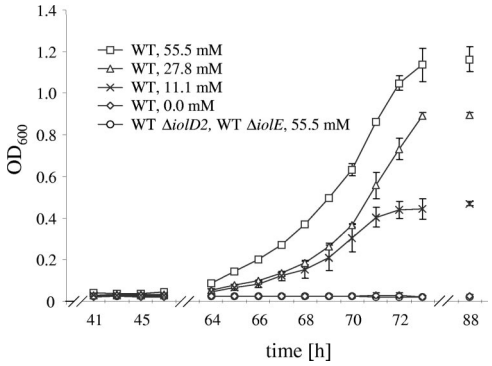

FIG. 2. Growth curves of the *S. enterica* serovar Typhimurium wild-type strain 14028 in MM without or with increasing concentrations of MI. The *iolD2* and *iolE* deletion mutants were cultivated in the presence of 55.5 mM MI. Zero growth of the wild-type strain and the two mutants in the absence of this carbon source was monitored for at least 100 h after inoculation. Standard deviations from at least three independent experiments are shown. The molarity of MM with respect to MI is indicated. WT, wild type.

controls, and a 350-bp intragenic fragment of STM0047 without promoter homology served as a negative control. No promoter sequences could be found upstream of *iolB*, *iolC2*, and *iolD2*. Recombinant plasmids were transformed into strain 14028s and a mutant with a deletion of STM4417, and bioluminescence was measured during growth experiments in LB broth or in MM containing either MI or glucose until the cells reached stationary phase. Regardless of carbon source and growth phase, the reporter did not respond to the fragments upstream of *iolG1*, *iolG2*, and *iolH* in a wild-type-like background in comparison to negative controls with a nonsense sequence cloned into pDEW201 or the empty vector pDEW201 (Table 2). In the presence of glucose and in rich medium, the promoters of STM4417 and *iolC1* were transcriptionally active, emitting approximately 2×10^5 to 3×10^5 RLU/OD_{600} unit. The putative *iolD1* promoter region resulted in light emission slightly above the threshold level set by the control construct with the nonsense fragment STM0047, whose activity ranges from 1.25×10^4 to 1.87×10^4 RLU/OD_{600} unit. The strong induction of the *iolA* promoter under the same growth conditions is probably due the role of IolA in alanine, aspartate, and propanoate metabolism (13). In MM with MI, the promoters of STM4417, *iolE*, *iolC1*, *iolD1*, and *iolI2* were at least 10-fold induced and the *iolA* promoter 3-fold induced during the exponential growth phase. A similar promoter induction pattern was observed when a mutant lacking STM4417 was grown in MM with glucose, indicating a STM4417-mediated negative regulation of the genes required for MI degradation. Due to its obvious negative regulatory function in MI degradation, we annotated STM4417 as *iolR* in accordance with the MI repressor protein IolR of gram-positive bacteria. The *iolE* and *iolI2* promoters are also strongly induced in the *iolR*-negative background in comparison to their transcriptional activity in the presence of glucose. However, their absolute RLU/OD_{600} unit values are at least 2 orders of magnitude lower than those of the IolR-regulated promoters of *iolA*, *iolC1*, and *iolD1*. The high induction rates of P_{iolE} and P_{iolI2} in the presence of MI compared to glucose, however, point to a role of both promoters in MI utilization, and they might indirectly have been repressed by IolR. The *iolR* promoter itself is induced in the absence of IolR in the presence of glucose as well as MI, indicating an autoregulatory activity of this repressor. Taken together, these findings indicate that the promoters of *iolR*, *iolE*, *iolC1*, *iolD1*, and *iolI2* are strongly induced in MM with MI during the exponential growth phase, while being repressed in the presence of glucose or in rich medium, and the *iolA* promoter is active under each condition tested here.

Transcriptional organization of *iol* genes. In contrast to the unidirectional organization of *iol* genes of *B. subtilis* and *C. perfringens*, the genes involved in MI degradation by gram-negative bacteria are not transcribed polycistronically (Fig. 1A). As demonstrated above, *iolB*, *iolG1 iolC2*, and *iolD2* are obligate for MI degradation but do not possess a separate

TABLE 2. Quantification of *iol* promoter activities[a]

Fragment cloned into pDEW201	14028s grown in MM + glucose		14028s grown in MM + MI			14028s Δ*iolR* grown in MM + glucose		
	RLU/OD_{600} unit[b]	SD (%)	RLU/OD_{600} unit	SD (%)	Fold induction[c]	RLU/OD_{600} unit	SD (%)	Fold induction
P_{iolR}	1.92×10^5	3.1	2.01×10^6	2.8	10.5	2.46×10^6	5.3	12.8
P_{iolA}	4.16×10^6	2.4	1.27×10^7	7.8	3.1	1.78×10^7	3.7	4.3
P_{iolE}	9.27×10^2	67.9	2.97×10^6	0.8	3,202.8	2.16×10^4	17.8	23.3
P_{iolG1}	4.93×10^3	7.4	1.55×10^4	7.4	3.2	2.65×10^4	10.4	5.4
P_{iolC1}	2.91×10^5	2.8	9.88×10^6	6.7	33.9	1.90×10^7	2.2	65.3
P_{iolD1}	2.95×10^4	7.3	5.72×10^6	2.6	194.0	7.42×10^6	5.4	251.7
P_{iolG2}	5.61×10^2	14.0	6.29×10^3	27.0	11.2	1.69×10^4	26.3	30.1
P_{iolI2}	8.32×10^2	24.2	1.40×10^7	0.7	16,864.2	3.25×10^4	11.9	39.0
P_{iolH}	1.66×10^3	58.9	1.17×10^3	60.2	0.7	1.83×10^3	32.5	1.1
Controls								
P_{argS}	2.96×10^6	4.0	1.85×10^6	18.3	0.6	3.41×10^6	10.1	1.2
P_{def}	1.13×10^6	2.9	1.11×10^6	5.5	1.0	1.35×10^6	2.8	1.2
′STM0047′	1.62×10^4	6.6	1.25×10^4	3.9	0.8	1.87×10^4	4.0	1.2
None	1.58×10^3	9.0	1.36×10^3	11.3	0.9	1.00×10^3	50.4	0.6

[a] Samples were taken from the late exponential phase.
[b] Data are averages from three independent experiments.
[c] Fold induction was calculated with respect to the RLU/OD_{600} unit values for the wild-type strain grown in glucose.

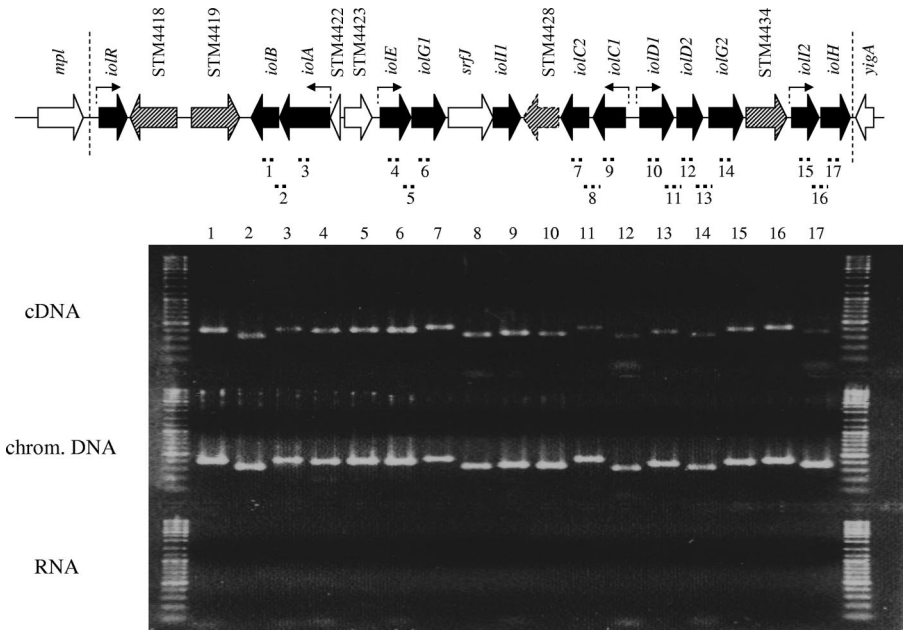

FIG. 3. Transcriptional organization of *S. enterica* serovar Typhimurium MI utilization genes. Strain 14028s was grown in MM with 55.5 mM MI at 37°C, and mRNA was extracted at an OD_{600} of 0.4. cDNA was amplified with reverse primers listed in Table S1 in the supplemental material. RT-PCR was performed with primer pairs specific for the indicated regions 1 to 17. All PCR products were separated by 2% agarose gel electrophoresis. As controls, PCR amplification products with genomic DNA and DNase-treated RNA samples as template are shown. Line numbers correspond to PCR product numbers depicted above the gel lanes. Arrows indicate promoters identified in this study.

promoter (Table 2), suggesting a cotranscription of *iolB* with *iolA*, of *iolG1* with *iolE*, of *iolC2* with *iolC1*, and of *iolD2*/*iolG2* with *iolD1*. To reveal the transcriptional organization of the *iol* genes within GEI4417/4436, we performed RT-PCR with RNA isolated from strain 14028 grown in MM with MI. The RNA was demonstrated to be DNA free, and cDNA of 17 regions spanning approximately 300 to 500 bp was amplified. Two oligonucleotides hybridizing to STM4434 and *iolI2* did not result in a PCR product from cDNA, thus validating the approach. All PCRs with cDNA as the template revealed a DNA fragment whose length corresponds to the PCR fragments amplified from genomic DNA (Fig. 3). In line with the data for the luciferase reporter fusions, *iolG2* and *iolH* are under the control of the promoters located upstream of *iolE* and *iolI2*. In summary, *iolA-iolB*, *iolE-iolG1*, *iolC1-iolC2*, *iolD1-iolD2-iolG2*, and *iolI2-iolH* are transcriptionally coupled. Thus, these five operons of *S. enterica* serovar Typhimurium comprise all genes required for MI utilization as well as three functionally dispensable or redundant genes, *iolG2*, *iolI2*, and *iolH*.

IolR acts as transcriptional repressor. Homology searches with the protein sequence of IolR (STM4417) revealed a putative transcriptional regulator with an HTH-6 motif belonging to the RpiR family. This domain is N terminal to a sugar isomerase domain that is predicted to bind phosphosugars. No homologies to the characterized IolR proteins of *B. subtilis* or *C. glutamicum* were observed, but there were homologies to putative regulators which might play a similar role in regulation of MI degradation by *K. pneumoniae*, *Y. enterocolitica*, *Y. pestis*, and *P. luminescens* (Fig. 1). In MM supplemented with MI, a nonpolar deletion of *iolR* resulted in a lag phase of 10 h shorter than the one measured for the wild-type strain (Fig. 4). The 14028s Δ*iolR* phenotype could be complemented by gene expression of *iolR* from pBR322 (Fig. 4). Induction of the promoters of *iolR*, *iolA*, *iolC1*, and *iolD1* in the *iolR* deletion mutant in the presence of glucose, and the identification of an HTH motif in the IolR sequence, prompted us to perform promoter binding studies. For that purpose, IolR was overexpressed in *E. coli* BL21(DE3) and purified as described above. The putative promoter fragments of *iolR*, *iolA*, *iolE*, *iolG1*, *iolC1*, *iolD1*, *iolG2*, *iolI2*, and *iolH* fragments were incubated without or with increasing amounts of the purified IolR pro-

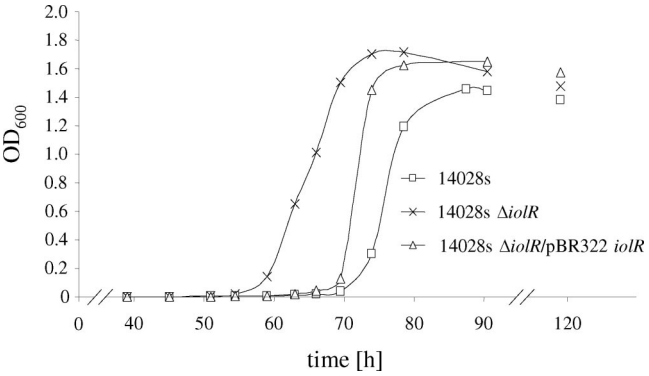

FIG. 4. Growth curve of strain 14028s Δ*iolR*. Exponential growth of the strain lacking the repressor IolR starts approximately 10 h earlier than that of the wild-type strain. The phenotype of 14028s Δ*iolR* could partially be complemented upon in *trans* expression of *iolR* via pBR322. Average values from three independent experiments are shown. Standard deviations are not given due to a variable lag phase, and all growth curves were normalized to a lag phase ending 60 h after inoculation.

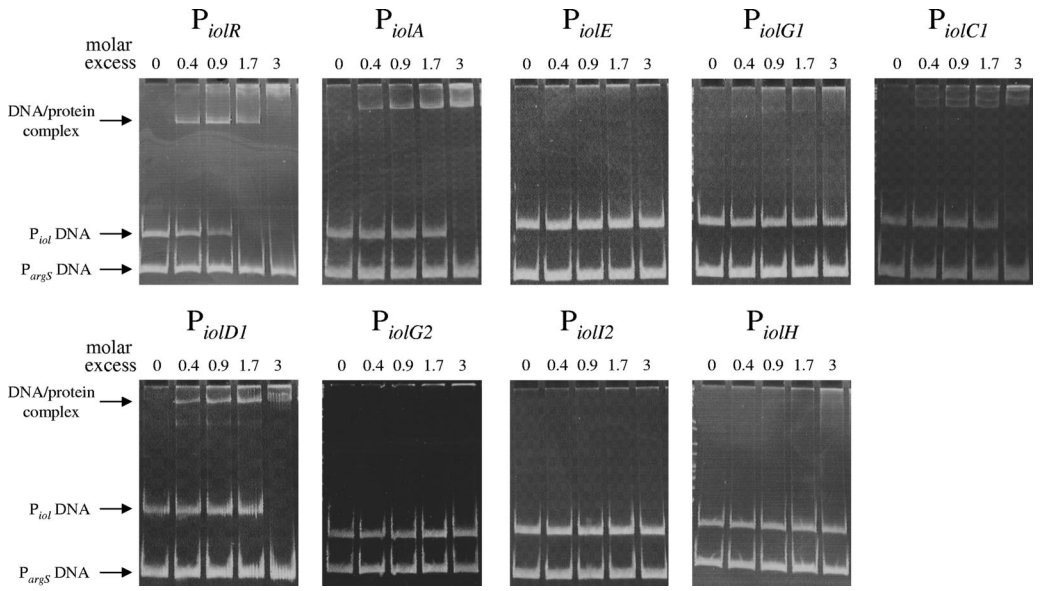

FIG. 5. Promoter binding activity of IolR. The interaction of IolR with the regulatory region of nine genes of GEI 4417/4436 is shown. One hundred nanograms of DNA was used in each experiment. The promoter fragments were incubated without or with increasing amounts (7 ng, 14 ng, 28 ng, and 49 ng [221 fM, 442 fM, 884 fM, and 1547 fM, respectively]) of the purified IolR protein. No bandshift was observed when a maximum of 210 to 280 ng IolR, corresponding to a maximal 17-fold molar excess, was incubated with promoter DNAs of *iolE*, *iolG2*, *iolI*, and *iolH* (data not shown). Protein-DNA complexes were separated on a 9.5% native polyacrylamide gel. A 200-bp sequence of the *argS* promoter served as a negative control.

teins, and the protein-DNA complexes were separated on a 9.5% native polyacrylamide gel (Fig. 5). A retarded DNA band with decreased motility representing the IolR-DNA complex was observed with the *iolR*, *iolA*, *iolC*, and *iolD1* fragments. Thus, the binding of IolR to the respective promoters results in repression of these genes during growth of *S. enterica* serovar Typhimurium in glucose-rich medium. Binding of IolR to its own promoter demonstrates its autoregulatory function. In contrast, complex formation was observed neither with a control fragment of the *argS* promoter indicating the IolR binding specificity, nor with fragments upstream of *iolE*, *iolI2*, *iolH*, *iolG1*, and *iolG2*. IolI2 is not required for MI degradation (see above), and its expression might therefore not be regulated by IolR. The bandshift experiments with the *iolE* and *iolI2* promoters are in line with the data in Table 2.

DISCUSSION

Although a large number of putative *iol* genes are present in gram-negative genomes, little is known about their functionality and their organization in comparison to their gram-positive counterparts. The systematic knockout of GEI 4417/4436 genes revealed that this *S. enterica* serovar Typhimurium island encodes all the enzymatic activities required for MI catabolism, leading to the production of acetyl coenzyme A and dihydroxyacetone phosphate. The genes *iolR*, *iolB*, *iolA*, *iolE*, *iolG1*, *iolC1/2*, and *iolD2* provide an MI-negative phenotype upon deletion and encode the key functions to utilize MI. These genes are also present in the genomes of *K. pneumoniae*, *Yersinia* spp., and *P. luminescens*, while homologues of other GEI 4417/4436 *iol* genes could not be identified in these strains. As experimentally demonstrated in this study, these genes enable *Y. enterocolitica* and *P. luminescens* to catabolize MI. *iolI* and *iolH* were found only in the genome of *K. pneumoniae*. The putative inosose isomerase IolI has been demonstrated to convert 2-keto-MI to 1-keto-D-*chiro*-inositol (32). Together with its very strong induction in the presence of MI, these results suggest a role of IolI in providing IolE substrates. The function of IolH remains to be elucidated.

S. enterica serovar Typhimurium exhibits similar growth phenotypes in MM containing glucose or MI with respect to the generation time during exponential phase and the OD_{600} in stationary phase. However, a remarkable difference is the extended lag phase of approximately 60 h in the presence of MI as the sole carbon source. Such a retarded metabolic switch has not been reported for gram-positive bacteria able to grow on MI. For example, growth of *C. glutamicum* in MI-containing medium starts within a few hours after inoculation (17). 2-Deoxy-5-keto-D-gluconic acid has long been considered a key step in MI degradation (2). Only recently, 2-deoxy-5-keto-D-gluconic acid 6-phosphate, another intermediate of MI degradation (Fig. 1B) was identified to antagonize IolR binding to the promoter of the *B. subtilis iol* operon (33). Thus, the long lag phase of *S. enterica* serovar Typhimurium in the presence of MI might be the result of a tighter repression of its *iol* genes, but other mechanisms of IolR antagonizing or an additional regulatory factor cannot be excluded. This assumption is supported by the obviously IolR-independent *iolE* and *iolI2* regulation as shown by the bandshift experiments (Fig. 5). Addition of glucose during exponential phase had no effect on the promoter activity of *iolE* and *iolI2* in the Δ*iolR* background (data not shown), excluding the possibility that they are under catabolite repression. *iolE*, encoding the dehydratase that catalyzes the

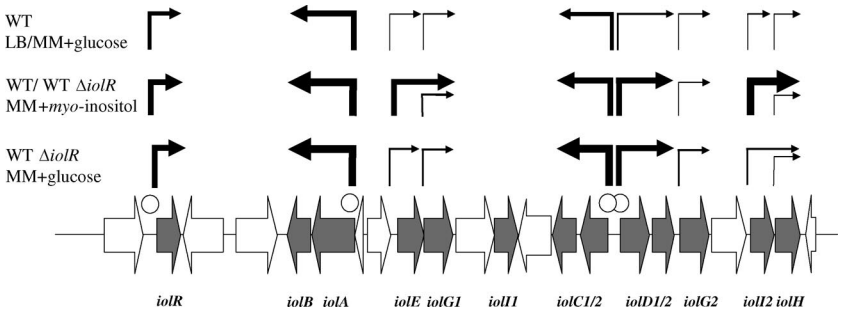

FIG. 6. Regulation of MI utilization in *S. enterica* serovar Typhimurium. The wild-type strain and an *iolR* deletion mutant carrying recombinant pDEW201-constructs were grown in LB or in MM with MI or glucose. Promoter induction is depicted by arrows of different sizes (from smallest to largest): $<10^4$ RLU/OD_{600} unit, $<10^5$ RLU/OD_{600} unit, $<10^6$ RLU/OD_{600} unit, $<10^7$ RLU/OD_{600} unit, and $<10^8$ RLU/OD_{600} unit. Induction of *iol* genes was similar in both strains in the presence of MI. Binding sites of IolR are indicated by open circles. WT, wild type.

second step in MI degradation, might be positively induced by MI or a related substance rather than by an antagonistically acting intermediate such as 2-deoxy-5-keto-D-gluconic acid 6-phosphate.

RT-PCR, reporter fusions, and GMS revealed a consistent picture of the transcriptional organization, the regulation, and the promoter activities of the *Salmonella iol* genes. The data obtained are summarized in Fig. 6. IolR binds to the *iolR* promoter and regulates its own expression (Fig. 4 and 5). This is in line with the finding that in *B. subtilis*, inactivation of *iolR* results in a constitutive transcription of the *iol* divergon including *iolJ* (33). IolR was demonstrated in this study to negatively regulate the transcription of three gene clusters required for MI utilization by *S. enterica* serovar Typhimurium, namely, *iolA-iolB*, *iolC1-iolC2*, and *iolD1-iolD2-iolG2*. A lack of IolR binding to the putative promoter of the *iolE-iolG1* operon, which encodes the first two steps in MI degradation, hints at an additional, IolR-independent regulatory mechanism. The autoregulatory activity of the MI repressor and substrate antagonism might explain that *iolR* of *S. enterica* serovar Typhimurium is induced under conditions in which the genes involved in MI degradation are transcribed, an observation already described for *C. perfringens* (14). However, *iolR* of *C. glutamicum* is not upregulated in the presence of MI (17).

In gram-positive bacteria, the genes encoding enzymes for MI degradation are mostly unidirectionally organized, resulting in polycistronically transcribed operons (14, 30). Two *iol* clusters putatively encoding redundant functions were identified in *C. glutamicum* and *L. plantarum* (17, 30). Genome comparison revealed a more complex transcriptional organization of *iol* genes in *S. enterica* serovar Typhimurium and other gram-negative bacteria belonging to the genera *Yersinia*, *Photorhabdus*, *Citrobacter*, *Erwinia*, *Brucella*, *Photobacterium*, and *Rhizobium* (Fig. 1A). Besides *Salmonella*, genes encoding putative redundant enzymatic functions have been found only in the *Photobacterium profundum* genome (*iolE* and *iolG*), which also carries an *iolR* duplication, and in the island exemplified by *E. carotovora*. Remarkably, *Y. enterocolitica*, *Y. intermedia*, and *Y. frederiksenii* on the one hand and *Y. pestis* and *Y. pseudotuberculosis* on the other hand carry distinct *iol* gene clusters, suggesting their independent acquisition by *Yersinia* ancestor strains. Taken together, these data support a repeated acquisition and chromosomal rearrangement of *iol* genes in gram-negative bacteria.

Open questions that are currently being addressed for *S. enterica* serovar Typhimurium are the transport mechanisms for MI or derivatives, further regulatory mechanisms contributing to MI utilization, and the identification of MI-related substrates metabolized by the Iol enzymes.

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