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Molekulare Charakterisierung des *myo*-Inositol Metabolismus von *Salmonella enterica* serovar Typhimurium: Genetik, Transport und Regulation

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Et is wie et is,
et kütt wie et kütt,
un et hätt' noch immer jot jejange.

Rheinisches Grundgesetz

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Kröger, C. and T. M. Fuchs. Characterization of the *myo*-inositol utilization island of *Salmonella enterica* serovar Typhimurium.
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Die experimentelle Arbeit wurde ausschließlich von Carsten Kröger durchgeführt. Das Manuskript haben Thilo M. Fuchs und Carsten Kröger geschrieben.

Kröger, C., J. Stolz and T. M. Fuchs. *myo*-Inositol transport by *Salmonella enterica* serovar Typhimurium.
Microbiology. 2010 Jan;156(Pt 1):128-38. Epub 2009 Oct 15.
Die experimentelle Arbeit wurde ausschließlich von Carsten Kröger durchgeführt. Das Manuskript haben Thilo M. Fuchs und Carsten Kröger geschrieben. Jürgen Stolz hat beim Schreiben des Manuskripts mitgewirkt.

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INHALTSVERZEICHNIS

ZUSAMMENFASSUNG

In der vorliegenden Arbeit wurden die Genetik, der Transport und die Regulation des *myo*-Inositol-Metabolismus von *Salmonella enterica* serovar Typhimurium (*S.* Typhimurium) molekular charakterisiert.

Die *iol*-Gene von *S.* Typhimurium, die am *myo*-Inositol-Metabolismus beteiligt sind, liegen auf der genomischen Insel GEI4417/4436. Durch Deletionsanalysen wurden die Gene *iolA*, *iolB*, *iolC1*, *iolC2*, *iolD2*, *iolE*, *iolG1* und STM4423 als essentiell für das Wachstum mit *myo*-Inositol als einziger Kohlenstoffquelle identifiziert. Bis auf STM4423 kodieren die genannten Gene für Enzyme, die *myo*-Inositol in Dihydroxyaceton-Phosphat, Acetyl-CoA und CO_2 umwandeln. Laut RT-PCR Analyse bilden die Gene *iolA* und *iolB*, *iolC1* und *iolC2*, *iolD1* und *iolD2*, sowie *iolE* und *iolG1* jeweils transkriptionelle Einheiten. Durch Fusion der *iol*-Promotoren an die Luciferase konnte gezeigt werden, dass die Promotoren von *iolA/B*, *iolC1/C2*, *iolD1/D2* und *iolE/G1* beim Wachstum mit *myo*-Inositol als Kohlenstoffquelle stark induziert werden. In LB-Medium oder Minimalmedium mit Glukose werden die *iol*-Promotoren mit Ausnahme des *iolE/G1* Promotors durch den Regulator IolR reprimiert. In Protein-DNA Bindestudien konnte zudem gezeigt werden, dass IolR sowohl die übrigen *iol*- als auch seinen eigenen Promotor bindet und sich damit autoreguliert. Die Induktion der Gene *iolE/G1* wird höchstwahrscheinlich durch den Regulator STM4423 vermittelt, da deren Expression den Wachstumsdefekt der STM4423-Deletion supprimieren kann.

Als *myo*-Inositol Haupttransporter in *S.* Typhimurium konnte IolT1 (STM4418) identifiziert werden. In Aufnahmeexperimenten mit radioaktiv markiertem *myo*-Inositol wurde ein Aktivitätsoptimum von IolT1 bei pH 5,5 und ein K_m Wert zwischen 0,49 und 0,79 mM gemessen. Die stark reduzierte Aufnahme in Anwesenheit von Protonophoren deutet zudem darauf hin, dass IolT1 als *myo*-Inositol/H^+-Symporter operiert. Als schwachen *myo*-Inositol-Transporter konnte IolT2 (STM4419) ausgemacht werden. Die Expression beider Transporter wird während des Wachstums mit *myo*-Inositol stark induziert und in LB-Medium und in Minimalmedium mit Glukose durch IolR-Bindung an die Promotoren von *iolT1* und *iolT2* reprimiert, was durch DNA-Protein Bindestudien gezeigt wurde.

Der Begriff der biologischen Bistabilität beschreibt das unterschiedliche Verhalten isogener Organismen unter identischen Bedingungen. Ein solcher bistabiler Phänotyp zeigt sich für das Wachstum von *S.* Typhimurium in Minimalmedium mit *myo*-Inositol als einziger Kohlenstoffquelle in der Variabilität einzelner Zellen bezüglich des Übergangs von der Anlauf- in die Wachstumsphase. Auf zellulärer Ebene konnte der bistabile Phänotyp beispielhaft an der *iolE*-Promotorinduktion mit Fluoreszenzmikroskopie und Durchflusszytometrie qualitativ und quantitativ gemessen werden. Sowohl die Deletion des Repressors IolR als auch CO_2/Hydrogencarbonat im Medium heben die Bistabilität auf. Dabei wirkt CO_2/Hydrogencarbonat nicht direkt auf IolR, sondern wahrscheinlich durch Bindung an STM4423, der dann das IolR-unabhängige Operon *iolE/G1* induziert. Dieses Beispiel zeigt zum ersten Mal einen Einfluss von CO_2/Hydrogencarbonat auf die Genregulation in *S.* Typhimurium. Die doppelte Regulation durch IolR und STM4423 ist für den *myo*-Inositol-Metabolismus der bisher untersuchten Organismen einzigartig. Aus den Daten dieser Arbeit konnte ein Modell für diese Regulation des *myo*-Inositol-Metabolismus in *S.* Typhimurium erstellt werden.

SUMMARY

In this study, the genetics, the transport and the regulation of the *myo*-inositol metabolism of *Salmonella enterica* serovar Typhimurium (*S.* Typhimurium) are characterized on a molecular level.

The *iol* genes, which are involved in the *myo*-inositol metabolism, are encoded by the genomic island GEI4417/4436. By deletion, the genes *iolA*, *iolB*, *iolC1*, *iolC2*, *iolD2*, *iolE*, *iolG1* and STM4423 were identified to be essential for growth with *myo*-inositol as the sole carbon source. Except for STM4423, the mentioned genes encode the enzymes, which degrade *myo*-inositol to dihydroxyacetone phosphate, acetyl-CoA and CO_2. As investigated by RT-PRCR, the genes *iolA* and *iolB*, *iolC1* and *iolC2*, *iolD1* and *iolD2*, as well as *iolE* and *iolG1* form transcriptional units. By fusing the *iol*-promoters with the luciferase, it was shown that the promoters of *iolA/B*, *iolC1/C2*, *iolD1/D1* and *iolE/G1* are highly expressed during growth using *myo*-inositol as a C-source. In LB medium or in minimal medium containing glucose, all *iol* promoters except for the promoter of *iolE/G1* are repressed by the regulator IolR. Using DNA-protein studies, IolR was shown to bind the remaining and its own promoter indicating its auto-regulation. The induction of the genes *iolE/G1* is probably mediated by the regulator STM4423, since its expression suppresses the growth defect caused by the STM4423 deletion.

IolT1 (STM4418) could be identified to act as the main *myo*-inositol transporter in *S.* Typhimurium. An optimum of activity of IolT1 at pH 5,5 and a K_m value between 0,49 and 0,79 mM was measured in uptake experiments using radio-labelled *myo*-inositol. The strong reduction of the uptake in the presence of protonophores suggests that IolT1 operates as a *myo*-inositol/H^+ symporter. IolT2 (STM4419) was identified to be a weak *myo*-inositol transporter. The expression of these two transporters was strongly induced in cells growing with *myo*-inositol as a carbon source and repressed by IolR binding to their promoters in LB medium or minimal medium containing glucose, as shown by DNA-protein binding assays.

The term biological bistability describes the differential behaviour of isogenic organisms under identical conditions. Such a bistable phenotype could be observed for *S.* Typhimurium in minimal medium containing *myo*-inositol as the sole carbon source resulting in a variable transition of each single cell from the lag to the growth phase. On the cellular level, the bistable phenotype was measured as exemplified by the induction of the *iolE* promoter by fluorescence microscopy and flow cytometry in a qualitative and quantitative manner. The deletion of *iolR* as well as addition of CO_2/bicarbonate to the medium abolishes the bistability. CO_2/bicarbonate does not act on IolR, but probably mediates induction of the iolR-independent operon *iolE/G1* by binding STM4423. This example shows for the first time an influence of CO_2/bicarbonate on the gene expression of *S.* Typhimurium. The dual regulation of the *myo*-inositol metabolism by IolR and STM4423 is unique for all organisms investigated so far. A model of the regulation of the *myo*-inositol metabolism derived from the data presented in this study is postulated.

DIE GATTUNG *SALMONELLA*

Salmonellen sind Gram-negative, fakultativ anaerobe, peritrich begeißelte, stäbchenförmige Bakterien. Aus der Familie der Enterobacteriaceae sind aus der Gattung *Salmonella* zwei Arten bekannt: *Salmonella enterica* und *Salmonella bongori* (13). Die Salmonellen sind nach dem amerikanischen Veterinär Daniel Elmer Salmon (1850-1914) benannt, der zusammen mit seinem Kollegen Theobald Smith (1859-1934) Salmonellen (das Schweinepathogen *Salmonella choleraesuis*) zum ersten Mal beschrieb (75). Die Art *Salmonella enterica* teilt sich zudem in sechs Subspezies auf, die jeweils mit einer römischen Ziffer gekennzeichnet werden (Tabelle 1). Die jeweiligen Subspezies werden dann anhand ihrer O- und H-Antigene in Serotypen (Serovare) weiter unterteilt.

Tabelle 1: Einteilung der Salmonellen in Spezies und Subspezies nach dem Kauffmann-White Schema (68); Stand aus dem Jahr 2000 (Abbildung verändert, Quelle (13))

Salmonella Spezies und Subspezies	Anzahl der Serotypen innerhalb einer Subspezies	Habitat
S. enterica subsp. *enterica* (I)	1.454	Warmblüter
S. enterica subsp. *salamae* (II)	489	Kaltblüter und in der Umwelt
S. enterica subsp. *arizonae* (IIIa)	94	Kaltblüter und in der Umwelt
S. enterica subsp. *diarizonae* (IIIb)	324	Kaltblüter und in der Umwelt
S. enterica subsp. *houtenae* (IV)	70	Kaltblüter und in der Umwelt
S. enterica subsp. *indica* (V)	12	Kaltblüter und in der Umwelt
S. bongori	20	Kaltblüter und in der Umwelt
Insgesamt	2.463	

Die Bezeichnung des Serotyps wird dem Gattungs- und Artnamen wie folgt angeschlossen: *S. enterica* subsp. *enterica* serotype (oder serovar) Typhimurium (*S.* Typhimurium). Etwa 60% der knapp 2500 Serotypen gehören der Subspezies *S. enterica* subsp. I (*S. enterica* subsp. *enterica*) an, die für fast alle Infektionen von Salmonellen beim Menschen und bei warmblütigen Tieren verantwortlich sind. In dieser Gruppe finden sich auch die bekannten humanpathogenen Serovare *S.* Typhi, *S.* Enteritidis und *S.* Typhimurium. Während *S.* Typhi eine für Menschen und höheren Primaten spezifische Infektionskrankheit auslöst, sind *S.* Enteritidis und *S.* Typhimurium für Erkrankungen bei verschiedenen Warmblütern verantwortlich. Alle weiteren Subspezies wurden fast ausnahmslos von Kaltblütern und aus der Umwelt und nur in wenigen Ausnahmen von warmblütigen Organismen isoliert (27).

DIE INFEKTION MIT SALMONELLEN – DIE SALMONELLOSE

Die Infektion mit Salmonellen, die so genannte Salmonellose, ist eine der häufigsten Infektionskrankheiten weltweit. Salmonellosen beim Menschen werden meist durch den Verzehr von kontaminierter Nahrung, hauptsächlich Fleisch, Geflügel, Eier und Milch, aber auch in den letzten Jahren vermehrt durch Frischwaren wie Obst und Gemüse ausgelöst (12, 85). Der Verlauf der Krankheit richtet sich nach verschiedenen Faktoren, wie etwa der Prädisposition des Infizierten oder dem aufgenommen Salmonellen-Serovar.

Als häufigste klinische Isolate lösen die Serovare Typhimurium und Enteritidis beim Menschen eine meist unkomplizierte Gastroenteritis, eine lokale Infektion des terminalen Ileum und des Colons, aus, die auch ohne Behandlung mit Antibiotika nach wenigen Tagen abklingt (70, 76). Die Symptome sind in der Regel akutes Fieber, Bauchschmerzen, Diarrhöe, Übelkeit und gelegentlich Emesis. Schwerere Verläufe kann die Salmonellose jedoch bei jungen, alten und immunsupprimierten Menschen annehmen, die bei Kindern und alten Menschen mit der starken Dehydrierung durch die Diarrhöe zusammenhängen. In immunsupprimierten Menschen kann dagegen der Durchtritt der Salmonellen aus dem Darm in die Blutbahn in einer lebensbedrohlichen Bakteriämie und Sepsis resultieren (76).

Das „fact sheet N°139" der World Health Organization (WHO) von 2005 berichtet, dass es in den USA jährlich etwa 1,4 Millionen dieser nicht-typhoidalen Salmonellosen (NTS) auftreten (89). Die 1,4 Millionen Erkrankten resultieren in 168.000 Arztbesuchen, 15.000 Hospitalisierungen und 580 Opfern als Folge der Infektion, wobei Kosten von etwa 3 Milliarden US-Dollar entstehen.

Die exklusiv in Menschen oder höheren Primaten pathogenen Serovare Typhi und Paratyphi können dagegen eine systemische Erkrankung, den so genannten Typhus (auch typhiodes Fieber, enterisches Fieber oder Typhus abdominalis), bzw. Paratyphus, auslösen (66, 77). Nach der Aufnahme mit *S.* Typhi verläuft die Infektion für 7-14 Tage asymptomatisch. Danach folgen jedoch erste Anzeichen des Unwohlseins und Fieberausbrüche. Die weiteren Symptome sind durch verschiedenste im Zusammenhang mit der Infektion stehende Komplikationen sehr divers (66). Eine Studie aus dem Jahr 2004 schätzt für das Jahr 2000 ca. 20 Millionen Typhus-Fälle weltweit, wovon ca. 200.000 tödlich geendet haben, und ca. 5,5 Millionen Paratyphus-Fälle (21). Dabei sind vor allem Entwicklungsregionen betroffen. Südostasien und das zentrale Südasien verzeichnen dabei die höchste Inzidenz von mehr als 100 Fällen pro 100.000 Einwohner pro Jahr. Eine mittlere Inzidenz (10-100 Fälle pro 100.000

Einwohner pro Jahr) wurde für das restliche Asien, Afrika, die Karibik und Ozeanien (ohne Australien und Neuseeland) gemessen. Weniger als 10 Fälle pro 100.000 pro Jahr wurden im Jahr 2000 in Europa, Nordamerika, Australien und Neuseeland verzeichnet. Wie auch für andere Infektionskrankheiten hat sich die Situation für eine erfolgreiche Behandlung von Typhus mit Antibiotika durch eine Zunahme von Resistenzen und Multi-Resistenzen gegen verschiedene Wirkstoffe (z.B. Ampicillin, Chloramphenicol, Co-Trimoxazol und Quinolonen) in den letzen Jahren verschlechtert (32). Es existieren einige Impfstoffe von denen bisher jedoch keiner einen vollständigen, lang anhaltenden Schutz bietet (32, 100).

DER INFEKTIONSZYKLUS VON *SALMONELLA ENTERICA*

Nach der Aufnahme der Salmonellen durch den Wirt sind diese zunächst dem niedrigen pH im Magen ausgesetzt, den sie überstehen und so in den Darm gelangen. Im Darm können die Salmonellen auf unterschiedliche Weisen das Darmepithel überwinden, und persistieren oder vermehren und verbreiten sich in unterschiedlichen Wirtszellen. Sie induzieren ihre Aufnahmen in die M-Zellen der Peyer-Plaques im Dünndarm oder in andere nicht-phagozytierenden Darmepithelzellen (20, 49). Für die Adhäsion und Invasion benötigen sie ein Zusammenspiel aus den Genprodukten der *Salmonella* Pathogenitätsinseln 1 und 4 (SPI-1, SPI-4).

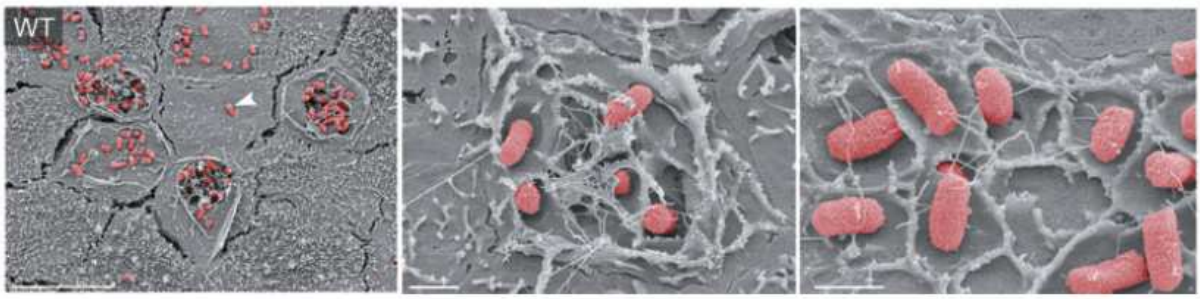

Abb.1: **Cryoelektronenmikroskop-Aufnahmen der Adhäsion und Invasion des *S*. Typhimurium Wildtyp-Stamms in polarisierte MDCK-Zellen.** Gezeigt ist die apikale Seite eines MDCK-Monolayers. Gut zu erkennen sind die Ausstülpungen der Wirtszellmembran. Die Salmonellen sind nachträglich rot eingefärbt worden. Quelle: (37)

Die SPI-1 kodiert ein Typ 3 Sekretionssystem (T3SS), welches Effektorproteine in die Wirtszelle transloziert, die dort unter anderem einer Umstrukturierung des Aktincytoskeletts der Wirtszelle führen (101). Die Wirtszellmembran bildet dadurch Ausstülpungen an seiner Oberfläche („membrane ruffling“), die die Salmonellenzelle umschließen und schließlich in

eine membrangebundene Vakuole aufnehmen („Trigger-Mechanismus“ (30, 44)). Erst kürzlich wurde jedoch gezeigt, dass die Induktion der Ausstülpungen nicht essentiell für die Aufnahme zu sein scheint (42). Die SPI-4 spielt dagegen bei der Adhäsion an die Wirtszelle eine entscheidende Rolle. Sie kodiert ein Typ 1 Sekretionssystem (T1SS) und sein Substrat SiiE, ein riesiges Adhäsin, welches den engen Kontakt zur Wirtszelle herstellt, den es für die Injektion der Effektorproteine durch das SPI-1-kodierte T3SS benötigt (Abb. 1 und (37, 39)). Beide Pathogenitätsinseln werden zudem durch dieselben globalen Transkriptionsfaktoren HilA und SirA koreguliert (38).

Von der membrangebundenen Vakuole (SCV, *Salmonella* containing vacuole) aus kann *Salmonella enterica* die Apoptose der infizierten Epithelzelle induzieren (52), wodurch zunächst keine inflammatorische Reaktion eingeleitet (29). Den Salmonellen wird so der Eintritt und die Verbreitung in tieferes Gewebe ermöglicht, wo sie von Makrophagen oder dendritischen Zellen (DC) phagozytiert werden können. In beiden Zelltypen werden die Salmonellen jedoch nicht vernichtet, sondern sie können sich in der SCV vermehren (Makrophagen) oder persistieren (DC) (14, 48). Gewöhnlich leiten infizierte Makrophagen die Pyroptose ein, eine bestimmte Art des programmierten Zelltods der Makrophagen, die eine Immunantwort einleitet. Um Zeit für die eigene Replikation zu gewinnen, verlangsamen die Salmonellen diesen Prozess (29). Für die intrazelluläre Vermehrung von *Salmonella enterica* in Epithelzellen und in Makrophagen und für die systemische Infektion, sind die Gene der SPI-2 essentiell, die unter anderem für ein weiteres T3SS und einige Effektorproteine kodieren (46, 57, 65). Das T3SS der SPI-2 transloziert Effektorproteine, welche die Physiologie der Wirtszelle verändern und die SCV aufrechterhalten, weshalb SPI-2 Mutantenstämme bezüglich der systemischen Infektion im Mausmodell hochgradig attenuiert sind (63, 80). Einige Salmonellen können außerdem direkt aus dem Darmlumen z.B. durch eingewanderte CD18-exprimirende Phagozyten Zellen aufgenommen werden, und gelangen so schon nach Minuten in die Blutbahn und somit zu den bevorzugten Replikationsorten wie die Leber und Milz (87, 93). Auch die Persistenz in dendritischen Zellen wird von *Salmonella enterica* als „Trojanisches Pferd“ benutzt, da sie die Antigenpräsentation der DC verhindern, um dem Immunsystem zu entgehen. (19).

DAS GENOM VON *S.* TYPHIMURIUM LT-2

Das Genom des Stammes *S.* Typhimurium LT-2 wurde 2001 von McClelland *et al.* sequenziert (60). Neben dem etwa 4,8 Megabasen-großen Chromosom enthält der Stamm LT-2, wie einige weitere *S.* Typhimurium Stämme, noch ein 93 Kb großes Plasmid. Dieses Plasmid trägt im Stamm LT-2 den Namen pSLT und auf ihm sind unter anderem auch einige wenige Virulenzgene kodiert sind. Je nach Wachstumsbedingungen liegt pSLT in 1-3 Kopien (40, 59). Auf dem Virulenzplasmid befinden sich 108 für Proteine kodierende Sequenzen und es besitzt einen GC-Gehalt von 53%. In anderen humanpathogenen Serovaren wie Typhi, Paratyphi A und B ist dagegen kein ähnliches Virulenzplasmid vorhanden (69).
Das Chromosom von *S.* Typhimurium LT-2 besitzt einen GC-Gehalt von 53% und beinhaltet 4489 für Proteine kodierende Sequenzen. 62 genomische Inseln bzw. Prophagen wurden identifiziert, welche sich aus einer Spanne von 4-52 Genen zusammensetzen (60). Darunter befinden sich die zum Teil detailliert charakterisierten *Salmonella* Pathogenitätsinseln (10, 31, 34, 39, 45, 46, 67, 91), die Prophagen Gifsy-1, Gifsy-2 und Fels-2, sowie weitere noch unbeschriebene genomische Inseln (15, 28, 54). Eine dieser unbeschriebenen Inseln ist die GEI4417/4436, die laut Vorhersage Gene beinhaltet, die in den *myo*-Inositol-Metabolismus involviert sein könnten. Die Vorhersage basiert dabei auf Sequenzvergleichen mit *B. subtilis*, dessen Enzyme des *myo*-Inositol-Metabolismus bereits identifiziert sind.

DER BAKTERIELLE *MYO*-INOSITOL-METABOLISMUS

Erste Reaktionen des *myo*-Inositol-Metabolismus wurden 1966 von Bermann *et al*. und 1971 von Anderson *et al*. für das Gram-negative Bakterium *Aerobacter* (heute: *Enterobacter*) *aerogenes* experimentell gezeigt (3, 4, 8, 9). Obwohl das Ausgangs- und die Endprodukte durch diese Arbeiten bekannt waren, konnten erst Yoshida *et al*. kürzlich den vollständigen Abbauweg experimentell in *B. subtilis* belegen, der in Abbildung 2 dargestellt ist und nachfolgend beschrieben wird (96): Nach der Aufnahme von *myo*-Inositol über die *myo*-Inositol-Transporter IolT und IolF in das Cytoplasma (97) wird zunächst die Hydroxylgruppe am C2-Atom durch die Inositol-Dehydrogenase (*iolG*) dehydriert, wobei 2-keto-*myo*-Inositol (2-KMI) entsteht und ein Molekül NAD^+ zu NADH reduziert wird. Im nächsten Schritt wird in einer Dehydratation ein Molekül H_2O von 2-KMI durch die 2-keto-*myo*-Inositol Dehydratase (*iolE*) abgespalten. Der Ring des daraus resultierenden 3D-(3,5/4)-Trihydroxy-

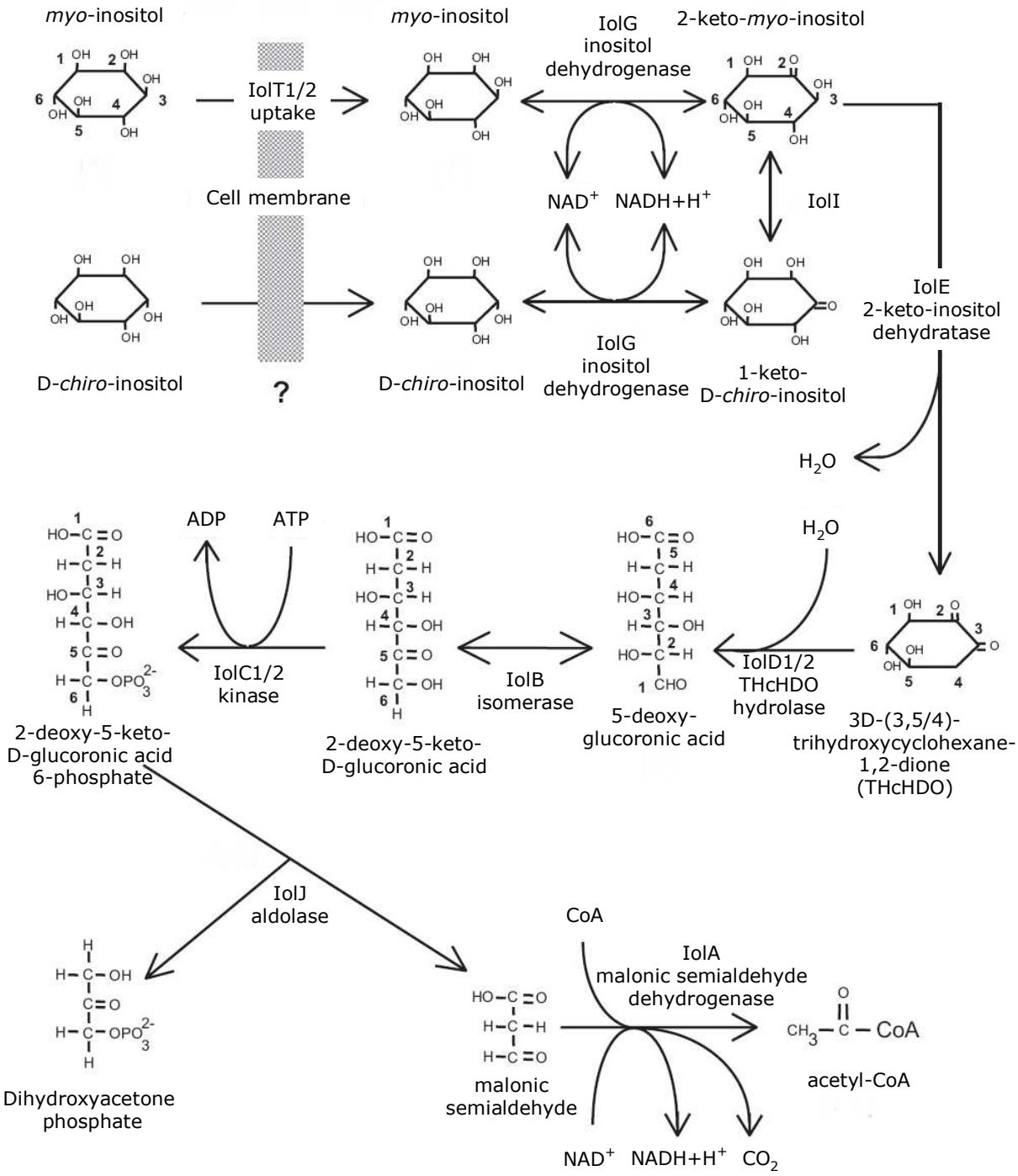

Abb.2: **Die Degradation von *myo*-Inositol.** Nach der Aufnahme von *myo*-Inositol in die Zelle wird es in sieben enyzmatischen Reaktionen schrittweise in je ein Molekül Dihydroxyaceton-Phosphat, Acetyl-CoA und CO_2 umgewandelt. Dabei werden zwei Moleküle NAD^+ reduziert und ein Molekül ATP verbraucht. Abbildung geändert; Quelle: (96).

cyclohexan-1,2-dion (THcHDO) wird dann durch die THcHDO Hydrolase (*iolD*) unter Einbau eines Moleküls H_2O gespalten, wobei 5-Deoxyglucoronsäure (5-DG) entsteht, welches dann durch die 5-DG Isomerase (*iolB*) zu 2-Deoxy-5-keto-D-Glucuronsäure (DKG) isomerisiert wird. Die membranständige DKP Kinase (*iolC*) phosphoryliert im nächsten Schritt DKG unter Verbrauch eines Moleküls ATP zu 2-Deoxy-5-keto-D-Glucoronsäure-Phosphat (DKP), welches dann durch die DKP Aldolase (*iolJ*) in Dihydroxyaceton-Phosphat (DHAP) und Malonat-Semialdehyd (MSA) gespalten wird. Während DHAP anschließend direkt in die Glykolyse einfließen kann, wird MSA noch durch die MSA Dehydrogenase (*iolA*) dehydriert, wobei ein weiteres Molekül NAD^+ zu NADH reduziert, ein Molekül CO_2 abgespalten wird und Coenzym A auf den Acetyl-Rest übertragen wird, wodurch Acetyl-CoA entsteht. Die Nettobilanz der *myo*-Inositol Degradation resultiert demnach in jeweils einem Molekül DHAP, CO_2 und Acetyl-CoA pro Molekül *myo*-Inositol, wobei ein Molekül ATP verbraucht wird und zwei Moleküle NADH entstehen (95, 96).

In einer Studie von Gutnick *et al*. 1969 wurde ein metabolisches Profil von *S*. Typhimurium LT-2 erstellt. Etwa 600 Substanzen wurden hinsichtlich ihrer Fähigkeit als alleinige Kohlenstoff- (C-) oder Stickstoffquelle (N-) für *S*. Typhimurium LT-2 zu dienen getestet, wobei 73 verwertbare C- und 25 N-Quellen identifiziert wurden (41). Unter den C-Quellen befindet sich unter anderem auch *myo*-Inositol (in der Originalarbeit als *meso*-Inositol aufgeführt). Die Fähigkeit *myo*-Inositol bei bestimmten Temperaturen in Peptonwasser zu verwerten, wurde lange Zeit als ein Biotypisierungsmarker für Salmonellen verwendet (64).

Der *myo*-Inositol-Metabolismus wurde auch bei weiteren Bakterien untersucht. Neben *B. subtilis* und *Enterobacter aerogenes* können mit *myo*-Inositol als einziger C-Quelle auch *Corynebacterium glutamicum* (55), *Clostridium perfringens* (51), *Lactobacillus casei* BL23 (94), *Pseudomonas putida* (72), *Rhizobium leguminosarum* bv. *viciae* (33), *Sinorhizobium meliloti* (35), *Caulobacter crescentus* (11), *Klebsiella pneumoniae*, *Serratia liquefaciens* und *S. marcescens* (58) und *Yersinia enterocolitica* (56) wachsen. Da noch viele weitere Bakterien Homologe der *iol*-Gene besitzen, wird diese Auflistung sicherlich in Zukunft erweitert werden.

DIE REGULATION DES *MYO*-INOSITOL-STOFFWECHSELS IN GRAM-POSITIVEN BAKTERIEN

Bisher wurde die Regulation des *myo*-Inositol-Metabolismus ausschließlich in den Gram-positiven Organismen *B. subtilis*, *C. glutamicum* und *Clostridium perfringens* untersucht, wo

sie sich als wenig komplex erwies (51, 55, 99). Die Regulation der *iol*-Gene verläuft dabei in allen drei Arten ähnlich. Ihnen ist gemeinsam, dass die *iol*-Gene in einem langen Operon unidirektional angeordnet sind und somit von nur einem Promotor zur einer einzigen mRNA transkribiert werden (98). Die Regulation dieses Promotors steuert dadurch in einfacher und gleichzeitig effektiver Weise den Großteil Gene des *myo*-Inositol-Metabolismus der genannten Organismen. In Abwesenheit von *myo*-Inositol wird dieser Promotor durch das DNA-Bindeprotein IolR reprimiert. Nach der Aufnahme von *myo*-Inositol wird es in fünf Schritten enzymatisch zu 2-Deoxy-5-keto-D-Glucoronsäure-Phosphat konvertiert, das dann durch Bindung an IolR die Repression der *iol*-Gene freigibt (96). Die Expression einiger weniger außerhalb der großen *iol*-Operons liegenden Gene, wie die *myo*-Inositol-Transporter *iolT* in *B. subtilis* und *iolT1* und *iolT2* in *C. glutamicum*, werden ebenfalls durch IolR reprimiert (55, 97).

DIE GENOMISCHE INSEL GEI4417/4436

Wie beschrieben, wurde die Biochemie des *myo*-Inositol-Abbaus bisher nur in zwei Organismen, *Enterobacter aerogenes* und *Bacillus subtilis*, gut untersucht (3, 4, 8, 9, 96). Da die Arbeiten zu *Enterobacter aerogenes* ohne Kenntnis des genetischen Hintergrundes erfolgten, wurde die Benennung der im *myo*-Inositol-Metabolismus involvierten „*iol*"-Gene von *S.* Typhimurium dementsprechend an *B. subtilis* angelehnt (Tabelle 2 und (56)).

Im Gegensatz zu den unidirektional transkribierten, polycistronischen *iol*-Operons der Gram-positiven Bakterien, sind *iol*-Gene in Gram-negativen Bakterien divers organisiert (Abb.3). In *S.* Typhimurium kodiert die genomische Insel GEI4417/4436 für 20 Gene, die laut Vorhersage am *myo*-Inositol Stoffwechsel beteiligt sind (Tabelle 2). Etwa die Hälfte jener Gene kodiert dabei für Enzyme, die *myo*-Inositol in sieben Schritten zu je einem Molekül Dihydroxyaceton-Phosphat, Acetyl-CoA und CO_2 degradieren.

In den bisher sequenzierten Salmonellen ist diese Insel nur in *S.* Typhimurium LT-2, 14028, SL1344, *S.* Paratyphi B, *S.* Saintpaul und *S.* Weltevreden gefunden worden, während sie in den sequenzierten Stämmen der Serovare Typhi, Enteritidis, Dublin, Diarizonae, Gallinarum, Paratyphi A und C, sowie in *Salmonella bongori* nicht vorkommt.

Tabelle 2: Übersicht über die GEI4417/4436 von *S*. Typhimurium

Gen	Protein	Funktion	Essentiell*
iolR - STM4417	Transkriptionsregulator	Hauptrepressor des Inositolabbaus	nein
iolT1 - STM4418	Permease	*myo*-Inositol Transporter	nein
iolT2 - STM4419	Permease	*myo*-Inositol Transporter	nein
iolB - STM4420	Isomerase	5DG/DKG Isomerisierung	ja
iolA - STM4421	Dehydrogenase	MSA Konvertierung zu Acetyl-CoA + CO_2	ja
STM4422	unbekannt	unbekannt	n.b.[§]
STM4423	Transkriptionsregulator	Induktion des Promotors von *iolEG1*?	ja
iolE - STM4424	Dehydratase	2KMI/THcHDO Dehydratation	ja
iolG1- STM4425	Dehydrogenase	MI/2KMI Dehydrierung	ja
srfJ - STM4426	putative lysosomale Glykosylceramidase (53)	unbekannt, Induktion der Expression durch SsrB (92)	nein
iolI1 - STM4427	Isomerase	2KMI/1-keto-DCI Isomerase	nein
STM4428	Permease	unbekannt	nein
iolC2 - STM4429	Kinase	Phosphorylierung von DKG zu DKP	ja
iolC1 - STM4430	Kinase	Phosphorylierung von DKG zu DKP	ja
iolD1 - STM4431	Hydrolase	Ringbruch THcHDO/5DG	n.b.[§]
iolD2 - STM4432	Hydrolase	Ringbruch THcHDO/5DG	ja
iolG2 - STM4433	Dehydrogenase	unbekannt	nein
STM4434	Permease	unbekannt	nein
iolI2 - STM4435	Isomerase	2KMI/1-keto-DCI isomerase	nein
iolH - STM4436	Isomerase/Dehydratase	unbekannt	nein

*essentiell für das Wachstum in Minimalmedium mit *myo*-Inositol als einziger C-Quelle bei 37°C
[§] n.b. = nicht bekannt

Interessanterweise konnte ausschließlich im *E. coli*-Stamm ED1a eine zur GEI4417/4436 fast identische genomische Insel nachgewiesen werden, der nur die Homologe zu den letzten beiden Genen *iolI2* und *iolH* fehlen, was auf einen horizontalen Gentransfer des Großteils der GEI4417/4436 von *Salmonella* in *E. coli* ED1a oder umgekehrt hindeutet. Allen weiteren bisher sequenzierten *E. coli*-Stämmen, wie zum Beispiel K-12 oder auch dem enterohämorrhagischen *E. coli* O157:H7 (EHEC) fehlt diese Insel. Bemerkenswert sind auch die *iol*-Gencluster der verschiedenen *Yersinia*-Arten. Die Anordnung in *Y. enterocolitica, Y. intermedia* und *Y. frederiksenii* ist komplett anders, als in *Y. pestis* und *Y. pseudotuberculosis*. Hier wurden die beiden unterschiedlichen *iol*-Gencluster wahrscheinlich separat voneinander akquiriert und weitergegeben.

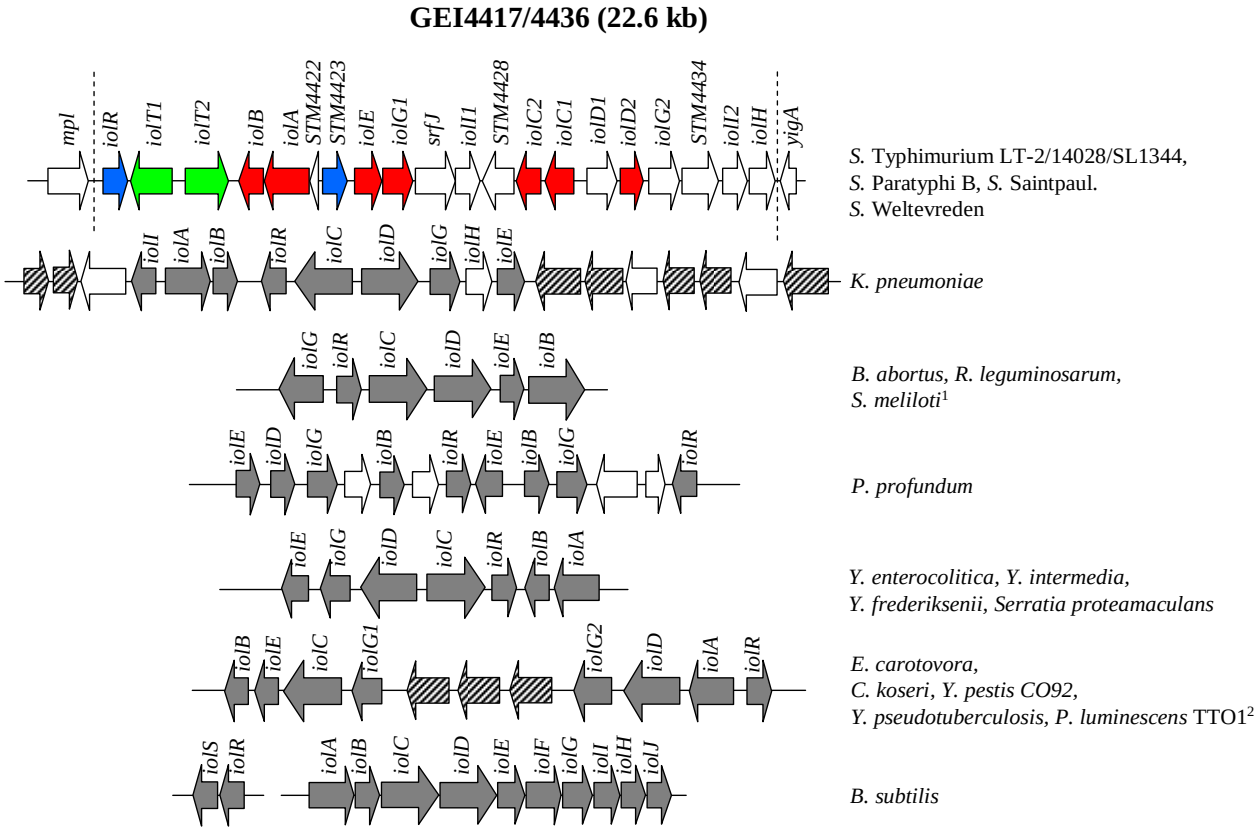

Abb. 3: **Schematische Darstellung der GEI4417/4436 von *S*. Typhimurium im Vergleich mit anderen *iol*-Divergons aus Gram-negativen Bakterien und *B. subtilis*.** Im Gegensatz zum *iol*-Operon aus *B. subtilis*, sind die *iol*-Gene in Gram-negativen Bakterien divers angeordnet. Für die GEI4417/4436 (oberste Reihe) sind die für den *myo*-Inositol-Abbau essentielle Gene aus dieser Arbeit in rot, Transkriptionsfaktoren in blau, die Gene der *myo*-Inositol-Transporter in grün und nicht essentielle Gene in weiß dargestellt. In den übrigen Reihen sind *iol*-Gene in grau eingefärbt, potentielle Gene für *myo*-Inositol-Transporter schraffiert und weitere Gene weiß. 1, In *S. meliloti* ist *iolG* umgedreht orientiert. 2, In *P. luminescens* fehlen die drei eingezeichneten Permeasen. Abbildung geändert, Quelle: (56)

Nur in den GEI4417/4436 tragenden Serovaren von *Salmonella* haben sich *iolC* und *iolD* in jeweils zwei offene Leserahmen geteilt. Sie dementsprechend als *iolC1* und *iolC2*, sowie *iolD1* und *iolD2* annotiert. Die Ursache könnte eine Mutation gewesen sein, die zu einer Leserasterverschiebung („frameshift“) geführt hat. Zudem existiert in *Salmonella* kein Gen innerhalb der GEI4417/4436, das Sequenzähnlichkeit zur DKP Aldolase IolJ aus *B. subtilis* zeigt, welche 2-Deoxy-5-keto-D-Glucoronsäure-Phosphat (DKP) in Dihydroxyaceton-Phosphat (DHAP) und Malonat-Semialdehyd (MSA) spaltet. Da dieses Protein jedoch essentiell für den *myo*-Inositol-Abbau ist, muss es eine weitere Aldolase auf dem Chromosom außerhalb der GEI4417/4436 geben, die diese Reaktion katalysiert. Die größte Sequenzähnlichkeit auf Aminosäureebene (38% Identität) mit einem Protein aus

S. Typhimurium weist IolJ aus *B. subtilis* zu einer putativen Fruktose/Tagatose Bisphosphat Aldolase (STM3253), auf. Die Deletion dieses Gens zeigte jedoch keinen Einfluss auf das Wachstum von *S.* Typhimurium in Minimalmedium mit *myo*-Inositol, womit zumindest ausgeschlossen werden kann, dass STM3253 essentiell für den *myo*-Inositol Abbau ist.

Neben den Genen, deren Produkte für die enzymatische Degradation *myo*-Inositols verantwortlich sind, liegen noch weitere Gene auf der GEI4417/4436, die etwa an der Regulation des *myo*-Inositol-Stoffwechsels (STM4417 und STM4423) oder des Transports (STM4418 und STM4419) von *myo*-Inositol beteiligt sind, sowie Gene von noch unbekannter oder unzureichend beschriebener Funktion, wie *srfJ*, das eine putative lysosomale Glykosylceramidase kodiert. Worley *et al.* konnten zeigen, dass *srfJ* durch das Zweikomponentensystem SsrAB induziert wird, welches viele Gene reguliert, die für das intrazelluläre Überleben in Makrophagen essentiell sind (36, 92). Die Funktion von *srfJ* ist bisher noch nicht experimentell gezeigt worden, jedoch führt eine Deletion zu einer leichten Attenuation bezüglich der systemischen Virulenz im Mausmodell (74).

DAS WACHSTUM VON *S.* TYPHIMURIUM MIT *MYO*-INOSITOL ALS EINZIGER KOHLENSTOFFQUELLE

Das Wachstum von *S.* Typhimurium 14028 in Minimalmedium mit *myo*-Inositol als einziger Kohlenstoffquelle (C-Quelle) ist durch mehrere Merkmale gekennzeichnet.

Besonders interessant und bisher so noch nicht in der Literatur beschrieben, ist die ungewöhnlich lange Anlaufphase (*engl.* lag-phase) von etwa 40-60 Stunden im Wachstumsverhalten von *S.* Typhimurium mit *myo*-Inositol als einziger C-Quelle. Weder ist diese lange Anlaufphase bei Untersuchungen des *myo*-Inositol-Metabolismus bei anderen Prokaryonten (51, 55, 96), noch bei Salmonellen in Verbindung mit dem Abbau anderer C-Quellen aufgetreten. Jedoch hat D.C. Old schon 1972 bei der Metabolisierung von *myo*-Inositol beobachten können, dass verschiedene Biotypen, eine damalige Einteilung von Salmonellen in Gruppen nach ihrer Fähigkeit verschiedene C-Quellen zu verwerten, unterschiedlich mit *myo*-Inositol wachsen und sie daraufhin in 21 Fermentations- oder Biotypen gruppiert. Bei einigen dieser Biotypen scheint es die lange Anlaufphase nicht zu geben, da sie nach 24h schon *myo*-Inositol in Pepton-haltigem Wasser verwerten konnten (64). Auch Sundaram berichtet nicht von einer Anlaufphase beim Wachstum mit *myo*-Inositol, allerdings bezieht sich dessen Arbeit auf *S.* Typhimurium LT-2 (84). Old berichtet

aber gleichzeitig von einem unstabilen Wachstum mit *myo*-Inositol als C-Quelle des Biotyps 9, der aus diesem Grund als „Inositol-negativ“ eingeordnet wurde. Dies muss aus heutiger Sicht als falsch bewertet werden, da er ebenfalls berichtet, dass bei diesem Stamm manchmal ein braunes Pigment im Wachstumsmedium entstand. Bisher wurde dieses Pigment noch nicht identifiziert und scheinbar konnte es nicht bei den anderen *Salmonella*-Biotypen beobachtet werden (64). Die Braunfärbung des Medium ist ein Charakteristikum des Wachstums mit *myo*-Inositol, denn es wurde in der hier vorgelegten Arbeit bei jedem der Wachstumsversuche mit *S.* Typhimurium 14028 in Minimalmedium mit *myo*-Inositol beobachtet. Das Pigment wird erst in der stationären Phase sichtbar (Abb.4) und liegt dann im Überstand vor, wobei es nicht eindeutig ist, ob es durch lysierte Zellen in den Überstand gelangt oder aktiv sekretiert wird. Es ist außerdem nicht ersichtlich, ob der Biotyp 9 aus der Arbeit von Old dem hier verwendeten *S.* Typhimurium Stamm 14028 entspricht und warum nur beim Biotyp 9 die Pigmentbildung beobachtet wurde.

Abb.4: **Bildung eines unbekannten braunen Pigments von *S.* Typhimurium**. Minimalmedium mit *myo*-Inositol als einziger C-Quelle wurde mit S. Typhimurium inokuliert und bei 37°C inkubiert. Erst in der stationären Wachstumsphase bildet sich ein braunes Pigment (rechts), das in der exponentiellen Wachstumsphase (links) noch nicht vorhanden ist.

Mittels der Deletionsmethode von Datsenko und Wanner (2000) wurden im Laufe dieser Arbeit alle Gene bis auf *iolD1* und *STM4422* systematisch deletiert (22). Alle Deletionsmutanten wurden auf Minimalmedium-Agarplatten, die nur über *myo*-Inositol als einziger C-Quelle verfügten, ausgestrichen und hinsichtlich ihres Wachstumsphänotyps bei 37°C bewertet. Die Gene *iolA*, *iolB*, *iolC1/2*, *iolD2*, *iolE*, *iolG1* und *STM4423* erwiesen sich in diesem Experiment als essentiell für das Wachstum von *S.* Typhimurium unter den

applizierten Bedingungen. Keine Beeinträchtigung des Wachstums konnte für die Deletionsmutanten Δ*srfJ*, Δ*iolG2*, Δ*iolH*, Δ*iolI1*, Δ*iolI2*, Δ*STM4428* und Δ*STM4436* festgestellt werden, die damit für die *myo*-Inositol Degradation und den Transport unter den getesteten Bedingungen entbehrlich sind. Die *S.* Typhimurium Stämme mit den Deletionen von *STM4418* (*iolT1*) und *STM4419* (*iolT2*) zeigten eine schwache (Δ*iolT2*) bzw. sehr starke Wachstumsdefizienz (Δ*iolT1*).

DIE GENEXPRESSION UND DIE TRANSKRIPTIONELLE ORGANISATION DER *IOL*-GENE

Wie beschrieben, sind in Gram-negativen Organismen die *iol*-Gene zwar in der Regel ebenfalls lokal angeordnet, jedoch liegen die Gene in Sinn- und Gegensinnrichtung, was eine komplexere transkriptionelle Organisation als in Gram-positiven Organismen impliziert.

Die Genexpression der *iol*-Gene wurde mit Hilfe des Plasmids pDEW201 ermittelt, das es ermöglicht Promotorstärken in *S.* Typhimurium quantitativ zu messen (86). Dabei werden putative Promotorsequenzen in die „multiple cloning site“ (MCS) des Vektors pDEW201 kloniert, die in 3’-Richtung der MCS das *luxCDABE*-Operon aus *Photorhabdus luminescens* trägt. Aktive Promotoren resultieren in der Transkription des *lux*-Operons, dessen Genprodukte proportional zur Stärke der Expression Photonen emittieren (Biolumineszenz). Mittels Vorhersage (BPROM, http://www.softberry.com) wurden putative Promotoren stromaufwärts einiger *iol*-Gene identifiziert und in die MCS des Vektors pDEW201 kloniert und in *S.* Typhimurium transformiert. Die Stärke der Induktion der einzelnen Promotoren wurde dann in Minimalmedium mit Glukose bzw. *myo*-Inositol als einziger C-Quelle und LB-Medium verglichen.

Dabei zeigte sich, dass die Promotoren der Gene *iolA*, *iolE*, *iolC1*, *iolD1* und *iolI2* in den Stämmen induziert wurden, die in Minimalmedium mit *myo*-Inositol wuchsen, was sinnvoll erscheint, da diese Gene - ausgenommen *iolI2* - essentiell für den Abbau von *myo*-Inositol sind. Da die übrigen für den *myo*-Inositol-Abbau essentiellen Gene (*iolB*, *iolC2*, *iolD2*, *iolG1*) ausnahmslos in 3’-Richtung der oben genannten Genen liegen, lag es nahe anzunehmen, dass *iolB* gemeinsam mit *iolA*, *iolG1* mit *iolE*, *iolC2* mit *iolC1* und auch *iolD2* zusammen mit *iolD1* transkribiert wird. Ebenfalls wurde überprüft, ob *iolG2* zusammen mit *iolD2* und *iolH* zusammen mit *iolI2* transkribiert werden. Diese Annahmen wurden experimentell mittels RT-

PCR bestätigt, indem gezeigt werden konnte, dass für die genannten Gene jeweils eine gemeinsame mRNA existiert, die in cDNA umgeschrieben werden konnte.
Die Gene dieser Operons werden während der Verstoffwechselung von *myo*-Inositol durch *S.* Typhimurium induziert, dagegen beim Wachstum in LB-Medium oder in Minimalmedium mit Glukose als einziger C-Quelle reprimiert. Die Expression erfolgt jedoch nur über die Zeitspanne des Wachstums beginnend kurz vor der logarithmischen Wachstumsphase und fällt mit dem Erreichen der stationären Phase auf das Ausgangsniveau zurück. Ausgenommen davon zeigte der Promotor des Operons *iolAB* trotz einer spezifischen Induktion während des Wachstums mit *myo*-Inositol eine starke Expression unter allen getesteten Bedingungen. Dies lässt sich möglicherweise auf eine zusätzliche, potentielle Beteiligung am Alanin-, Aspartat- und Propanoat-Stoffwechsel zurückführen (50).
Für die GEI4417/4436 wurden somit fünf transkriptionelle Einheiten nachgewiesen, was deutlich die komplexere Organisation der *iol*-Gene in Gram-negativen Bakterien zeigt. Diese komplexe Organisation der *iol*-Gene in *S.* Typhimurium unterscheidet sich damit deutlich vor allem gegenüber den untersuchten *iol*-Operons aus Gram-positiven Organismen, die meist als ein unidirektional transkribiertes Operon vorliegen (51, 55, 98).

DER *MYO*-INOSITOL TRANSPORT IN *S.* TYPHIMURIUM

Die GEI4417/4436 kodiert vier Gene, die als putative Transporter der MFS-Superfamilie annotiert sind (STM4418 (*iolT1*), STM4419 (*iolT2*), STM4428 und STM4436). Da diese Art der Transporter oftmals am Zucker- oder Zuckeralkoholtransport beteiligt sind, lag es nahe, die genannten Transporter auf ihre Fähigkeit *myo*-Inositol zu transportieren zu untersuchen. Deletionsmutanten dieser Gene resultierten in verschiedenen Phänotypen bezüglich ihrer Fähigkeit mit *myo*-Inositol als einziger C-Quelle zu wachsen. Während die Deletionen von STM4428 und STM4436 keine Wachstumsdefizienz bewirkten, zeigten die Deletionen von *iolT1* und *iolT2* einen unterschiedlichen Phänotyp. Die Deletion von *iolT2* bewirkte ein marginal schlechteres Wachstum, während die Deletion von *iolT1* in einer gravierenden Wachstumsdefizienz gegenüber dem Wildtyp mit *myo*-Inositol als einziger C-Quelle resultierte. Bei kombinierter Deletion von *iolT1* und *iolT2* zeigte diese Doppelmutante kein Wachstum mehr. Keine der Deletionsmutanten ΔSTM4418, ΔSTM4419, ΔSTM4428, ΔSTM4436 zeigte außerdem eine Wachstumsdefizienz mit anderen C-Quellen wie Glukose,

Fruktose, Xylose, Arabinose, Sorbitol oder Glycerol, so dass eine Rolle als alleiniger Transporter dieser Substrate ausgeschlossen werden konnte.

Die Expression von jeweils plasmidkodiertem *iolT1* oder *iolT2* in der Δ*iolT1-2* Doppelmutante konnte das Wachstum auf Minimalmedium-Agarplatten mit *myo*-Inositol in beiden Fällen wiederherstellen, woraus sich schließen lässt, dass *iolT1* und *iolT2* wichtig für das Wachstum von *S.* Typhimurium auf *myo*-Inositol sind. Diese Komplementation des Wachstums auf Minimalmedium-Agarplatten mit *myo*-Inositol konnte jedoch in flüssigem Minimalmedium nur mit IolT1 nach erreicht werden. Aus diesen Wachstumsexperimenten resultiert, dass IolT1 und IolT2 *myo*-Inositol mit unterschiedlicher Effizienz transportieren, wovon IolT1, zumindest unter den applizierten Bedingungen, deutlich wichtiger für den *myo*-Inositol Transport ist.

Mittels Luciferase-basierten Untersuchungen der Promotorbereiche von *iolT1* und *iolT2* konnte zudem gezeigt werden, dass beide Promotoren während der Verstoffwechselung von *myo*-Inositol stark induziert werden. Der Promotor von *iolT1* weist zudem eine relativ starke basale Aktivität auf, so dass sich auch in Anwesenheit von Glukose oder in LB-Medium eine gewisse Menge des *myo*-Inositol-Transporters IolT1 in der Cytoplasmamembran befinden sollte, um *myo*-Inositol unmittelbar aufnehmen zu können, wenn es im Medium anwesend ist. Ein ähnliches Expressionsmuster zeigt auch der außerhalb des *iol*-Operons liegende *myo*-Inositol-Transporter IolT aus *B. subtilis*, der mit *myo*-Inositol im Wachstumsmedium induziert wird (97).

MOLEKULARE CHARAKTERISIERUNG DER *MYO*-INOSITOL TRANSPORTER VON *S.* TYPHIMURIUM

Zur weiteren Charakterisierung wurden Aufnahmeexperimente mit [1,2-[^{3}H](N)]-Inositol in *S.* Typhimurium, der Δ*iolT2* Deletionsmutante und heterolog in *E. coli* DH5α durchgeführt, wobei IolT1 und IolT2 heterolog von einem Plasmid exprimiert wurden. Beide Proteine konnten in der Cytoplasmamembran von *E. coli* mittels Western Blot nachgewiesen werden. Die Aufnahme von radioaktiv markiertem *myo*-Inositol konnte dabei jedoch nur IolT1 vermitteln, während für IolT2 keine Aufnahme über zwei Stunden, auch nicht bei erhöhter spezifischer Aktivität gemessen werden konnte.

Das Aktivitätsoptimum von IolT1 befindet sich im leicht sauren pH-Bereich. Die höchste Aufnahmegeschwindigkeit von *myo*-Inositol wurde in McIlvaine's Zitronensäure-

Phosphatpuffer bei pH 5,5 gemessen. Zudem konnte die Aufnahme von *myo*-Inositol durch das Protonophor Carbonylcyanid-m-Chlorophenylhydrazon (CCCP) stark reduziert werden, was darauf hindeutet, dass IolT1 einen *myo*-Inositol/H^+-Symporter darstellt. Bei saurerem (pH 4) oder neutralem pH (pH 7) verläuft die Aufnahme deutlich schlechter und liegt nur noch bei etwa 20% der Aufnahmegeschwindigkeit verglichen mit der Rate bei pH 5,5. Der K_m-Wert für IolT1 wurde dementsprechend bei pH 5,5 gemessen und liegt im Bereich zwischen 0,49-0,79 mM. Er ähnelt damit Werten für *myo*-Inositol-Transporter aus *B. subtilis* (IolT1: K_m = 0,15 mM) oder *C. glutamicum* (IolT1: K_m = 0,33 mM; IolT2: K_m = 0,45 mM) (55, 97).

Ein IolT2 vermittelter *myo*-Inositol Transport konnte in den Aufnahmeexperimenten aus verschiedenen Gründen nicht gezeigt werden. Die Deletionsmutante *S.* Typhimurium Δ*iolT1* wächst auch nach einer Woche Inkubation in flüssigem Minimalmedium mit *myo*-Inositol (Konzentrationen von 55,5 mM bis 227,5 mM) nicht an, so dass mit ihr keine Aufnahmeexperimente durchgeführt werden konnten. Auch bei heterologer Expression von IolT2 in *E. coli* konnte keine signifikante Aufnahme von *myo*-Inositol gemessen werden. Da beide Proteine etwa über das gleiche Molekulargewicht verfügen (IolT1 = 53,4 kDa, IolT2 = 52,1 kDa), jedoch im Western Blot nicht auf gleicher Höhe eine Bande zeigen, besteht die Möglichkeit, dass IolT2 in *E. coli* teilweise degradiert wurde und so nicht mehr funktional war. Auf der anderen Seite ist ungewöhnliches Laufverhalten von Membranproteinen im SDS-Gel bekannt, das durch die Bindung von unterschiedlichen Detergenzmengen ausgelöst wird (71). Es könnte auch im zwar nah verwandten, aber doch heterologen Wirt falsch gefaltet in der Cytoplasmamembran vorliegen, was ebenfalls in einem Verlust oder einer Beeinträchtigung des *myo*-Inositol Transports resultieren könnte. Da der *myo*-Inositol-Transport von IolT2 mit Beachtung der Resultate aus den Wachstums- und Komplementationsexperimenten relativ niedrig zu sein scheint, könnte hier schon eine kleine Verschlechterung des Transports dazu führen, dass die Aufnahme nicht mehr messbar ist oder nicht mehr stattfindet. Es könnte allerdings auch möglich sein, dass IolT2 andere Versuchsbedingungen benötigt oder *myo*-Inositol nicht das Substrat darstellt, das von IolT2 mit der besten Effizienz transportiert wird. Als Hauptsubstrate könnte etwa eines oder mehrere der ebenfalls in der Natur vorkommenden Inositolisomere fungieren (72, 88). Überlappende Substratspezifität konnte für die Inositoltransporter aus *C. glutamicum* gezeigt werden, die beide neben *myo*-Inositol auch D-Fruktose transportieren können (7).

DIE REGULATION DES *MYO*-INOSITOL-METABOLISMUS IN *S.* TYPHIMURIUM

Die GEI4417/4436 kodiert für zwei Proteine, die als Transkriptionsfaktoren (STM4417 und STM4423) annotiert sind. Beide verfügen jeweils über ein helix-turn-helix (HTH)-Motiv (DNA-Bindemotiv), unterscheiden sich jedoch in ihrer zweiten Domäne.

Das Protein STM4417 (31,6 kDa) besitzt ihr HTH-Motiv im N-Terminus und zusätzlich eine SIS-(sugar isomerase) Domäne, die in der Regel Zuckerphosphate bindet (6). Diese Art der Transkriptionsfaktoren ist häufig in der Regulation von Stoffwechselvorgängen beteiligt, indem durch die Bindung eines Zuckerphosphats, meist ein Intermediat eines bestimmten Stoffwechselweges, die Konformation des Proteins verändert wird und so zum Beispiel das Protein von der DNA und dadurch eine Repression von Zielgenen gelöst wird (6). Eine katalytische Aktivität konnte für diese Art der Transkriptionsfaktoren bisher noch nicht gezeigt werden. Sie kommen sowohl in Bakterien als auch Archaebakterien und Eukaryoten vor, was ihre universelle Bedeutung im Phosphozuckermetabolismus unterstreicht.

Die Deletion des Gens STM4417 resultierte in einer deutlichen Verkürzung der Anlaufphase beim Wachstum von *S.* Typhimurium in Minimalmedium mit *myo*-Inositol als einziger C-Quelle. Dies war ein Hinweis, dass STM4417 den Abbau von *myo*-Inositol reprimiert und zum größten Teil an der ungewöhnlich langen Anlaufphase verantwortlich ist. In Anlehnung an die Repressorproteine IolR des *myo*-Inositol-Stoffwechsels aus *B. subtilis* und *C. glutamicum,* wurde das Gen STM4417 ebenfalls *iolR* getauft. Um die Bedeutung von IolR für die Genexpression der *iol*-Gene eingehend zu untersuchen, wurden die oben beschriebenen Promotor-Reporterplasmide im Hintergrund der *iolR*-Deletion im Vergleich mit dem Wildtyp in Minimalmedium mit Glukose getestet. Hierbei zeigte sich, dass sich das Genexpressionsmuster der *iol*-Gene in der *iolR*-Deletionsmutante deutlich von dem des Wildtyps unterscheidet. Die Promotoren der Gene *iolR, iolA, iolD1, iolC1, iolT1* und *iolT2* wurden im *iolR*-Deletionsstamm im Gegensatz zum Wildtyp-Stamm in Minimalmedium mit Glukose sehr stark induziert, was zum einen im Wildtyp-Stamm auf eine Autoregulation von *iolR* hindeutet, als auch auf eine Repression der vier für den *myo*-Inositol Abbau essentiellen Gene bzw. Operons *iolA/B, iolD1/D2* und *iolC1/2* und der *myo*-Inositol-Transportergene hinweist. Diese Ergebnisse wurden durch entsprechende DNA-Bindestudien untermauert, in denen gezeigt werden konnte, dass gereinigtes IolR-Protein *in vitro* an die Promotoren von *iolR, iolA, iolC1, iolD1, iolT1* und *iolT2* bindet.

Interessanterweise wird der Promotor der für den *myo*-Inositol-Abbau essentiellen Gene *iolE/G1* nicht von IolR reguliert. Die Genexpressionsmuster des *iolE*-Promotors im Wildtyp-Stamm und *iolR*-Deletionsstamm in allen getesteten Medien unterscheiden sich nicht signifikant voneinander, und auch in DNA-Bindestudien konnte keine Interaktion von IolR mit dem *iolE*-Promotor gezeigt werden. Aufgrund der räumlichen Nähe des zweiten, ebenfalls auf der GEI4417/4436 lokalisierten Transkriptionsfaktors (STM4423) zum Gen *iolE* (STM4424), wurde die Vermutung aufgestellt, dass der Promotor von *iolE/G1* durch STM4423 induziert werden könnte.

DIE BETEILIGUNG VON STM4423 AM *MYO*-INOSITOL-METABOLISMUS

Der Transkriptionsfaktor STM4423 (31,5 kDa) wurde bisher ausschließlich in Salmonellen, die die GEI4417/36 besitzen und in *E. coli* ED1a gefunden und stellt damit eine Ausnahme im bakteriellen *myo*-Inositol-Metabolismus dar. Das vom Gen STM4423 kodierte Protein ist der Vorhersage nach ein aus zwei Domänen bestehender Transkriptionsregulator vom AraC-Typ. Im C-Terminus befindet sich ein DNA-Bindemotiv (helix-turn-helix) und im N-Terminus eine so genannten Cupin-Domäne, die aus einem kleinen β-Fass besteht und funktionell sehr vielseitig ist. Bisher konnten 18 verschiedene funktionelle Klassen für Proteine der Cupin-Superfamilie gezeigt werden, so dass eine Funktionsvorhersage generell schwierig ist (25). Transkriptionsregulatoren vom AraC-Typ sind in der Regel Proteine, die deren Zielgene induzieren (26, 78).

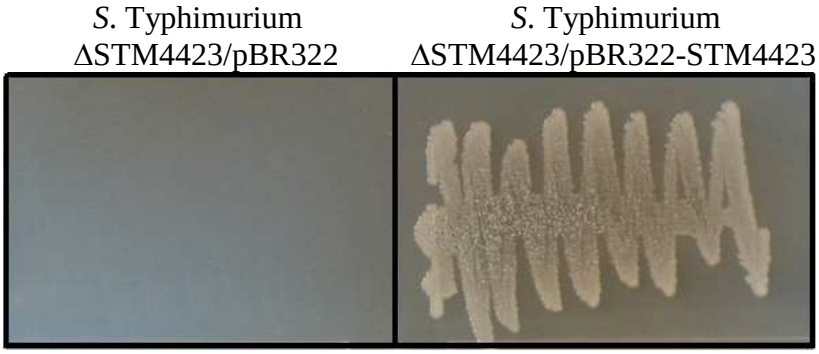

Abb. 5: **STM4423 ist essentiell für den Abbau von *myo*-Inositol in *S*. Typhimurium.** Die Deletion von STM4423 führt bei *S*. Typhimurium zu einem Wachstumsdefekt auf Minimalmedium-Agarplatten mit *myo*-Inositol als einziger C-Quelle (links). Dieser Phänotyp kann durch Expression von STM4423 vom Plasmid pBR322-STM4423 komplementiert werden (rechts).

Die Deletion von STM4423 resultierte darin, dass die Mutante in Minimalmedium mit *myo*-Inositol als einziger C-Quelle nicht mehr wachsen konnte, wobei keine generelle Wachstumsdefizienz in LB-Medium oder Minimalmedium mit Glukose auftrat. Durch Komplementation mit STM4423 auf dem Plasmid pBR322 wurde dieser Wachstumsdefekt aufgehoben, womit auch die essentielle Funktion von STM4423 für den *myo*-Inositol Abbau bewiesen wurde (Abb.5). Wie oben beschrieben, wurde vermutet, dass STM4423 das für den *myo*-Inositol Abbau essentielle Operon *iolE*/*iolG1* induziert, da es nicht unter der Repression von IolR unterliegt und die räumliche Nähe eine Interaktion von STM4423 mit dem Promotor von *iolE*/*iolG1* (STM4424/STM4425) nahe legt. Durch die Deletion von STM4423 scheint die Induktion des *iolE*/*iolG1*-Operons zu fehlen, dessen Genprodukte für die beiden initialen Degradationsschritte im *myo*-Inositol-Metabolismus verantwortlich sind. Dadurch könnte *myo*-Inositol nicht mehr abgebaut werden.

Diese Vermutung konnte indirekt durch eine Suppression des Wachstumsdefekts, ausgelöst durch die STM4423-Deletion, untermauert werden. In einer Dreifachmutante, der drei für den *myo*-Inositol Abbau essentiellen Gene fehlen (ΔSTM4423, Δ*iolE*, Δ*iolG1*), konnte das Wachstum durch *in trans* Expression der beiden an der Degradation beteiligten Protein IolE und IolG1 wiederhergestellt werden und somit der Wachstumsdefekt der STM4423-Deletion supprimiert werden. Da eine direkte Interaktion von STM4423 mit dem *iolE*-Promotor bisher noch nicht gezeigt wurde, kann zumindest geschlussfolgert werden, dass STM4423 an der Expression von *iolE*/*iolG1* beteiligt zu sein scheint. Dabei ist außerdem noch unklar, auf welches Signal STM4423 reagiert oder mit welchem Induktor er interagiert, um die Genexpression von *iolE*/*iolG1* zu induzieren.

Interessanterweise konnten Rollenhagen und Bumann zeigen, dass STM4423 im Gegensatz zum Typhus-Mausmodell spezifisch im Enteritis-Mausmodell stark induziert wird (73). Normalerweise entwickeln Mäuse, die mit *S*. Typhimurium infiziert werden, eine systemische Infektion, die dem Krankheitsbild des Typhus beim Menschen ähnelt (Infektion mit *S*. Typhi). Eine der Infektion vorangehende Verfütterung von Streptomycin an die Mäuse löst in der Maus jedoch nur eine Enteritis aus, die der Enteritis beim Menschen nach Infektion mit *S*. Typhimurium nahe kommt (5, 43). Demnach könnte es sein, dass der Abbauweg von *myo*-Inositol im Darm induziert wird.

DIE BISTABILITÄT IM *MYO*-INOSITOL-METABOLISMUS

Vergleicht man das Wachstumsverhalten von *S.* Typhimurium in Minimalmedium mit *myo*-Inositol als einziger C-Quelle mit dem anderer Bakterien, so zeigt sich neben vielen Gemeinsamkeiten auch ein wesentlicher Unterschied. Wie schon beschrieben, konnte bisher ausschließlich bei *S.* Typhimurium eine ungewöhnlich lange Anlaufphase (engl. *lag-phase*) beobachtet werden. Die Dauer der Anlaufphase ist trotz gleicher Bedingungen in parallelen Experimenten variabel. Der Start der exponentiellen Wachstumsphase in flüssigem Minimalmedium mit *myo*-Inositol beginnt im Durchschnitt erst nach einer Inkubation von etwa 50-60 h. Auf Minimalmedium-Agarplatten mit *myo*-Inositol kann in der Regel nach 40-50 h Wachstum beobachtet werden. Hier scheinen die Bedingungen dem Wachstum von Salmonellen mit *myo*-Inositol eher gegeben zu sein. Auf Minimalmedium-Agarplatten zeigt sich zudem ein ungewöhnlicher Phänotyp. Einzelne Kolonien sind erkennbar, bevor der Rest der Zellen zu einem Rasen gleichmäßig anwächst. Ein kleiner Teil der isogenen Gesamtzellpopulation verhält sich somit unter gleichen Bedingungen anders als der restliche Teil. Ein Verhalten, dass phänotypische Variation oder biologische Bistabilität genannt wird und erstmals bei der Verwertung von Laktose von *E. coli* beobachtet wurde (24, 62, 83). Um auszuschließen, dass es sich bei den früher in die exponentielle Wachstumsphase eintretenden Zellen um Mutanten handelt, wurden 18 Kolonien gepickt, drei Mal in LB-Medium passagiert und dann erneut auf Minimalmedium-Agarplatten mit *myo*-Inositol ausgestrichen. In jedem Fall waren der bistabile Phänotyp und die lange Anlaufphase reproduzierbar, so dass eine Mutation oder eine irreversible Phasenvariation ausgeschlossen werden konnte. Eine einzelne Passage in LB-Medium reichte hingegen noch nicht aus, um den bistabilen Phänotyp sowie die lange Anlaufphase erneut zu generieren. Hier scheinen der Stoffwechsel und die regulatorische Anpassung an das Wachstum mit *myo*-Inositol als C-Quelle noch nicht vollständig revidiert worden zu sein.

DER NACHWEIS DER BISTABILITÄT AUF ZELLULÄRER EBENE

Um eine differentielle Expression einer isogenen Zellpopulation unter identischen Bedingungen zu messen, wird in der Literatur die Genexpression einzelner Zellen meist mit

Hilfe der Fluoreszenzmikroskopie oder Durchflusszytometrie qualitativ und quantitativ bestimmt (83). Dazu wurde der Promotor des Operons *iolE/G1* vor das grün fluoreszierende Proteins (GFP) in den Vektor pPRONE-NT kloniert (61). Mit Hilfe dieses Plasmids kann der Übergang aus der Anlauf- in die Wachstumsphase untersucht werden, da die Induktion von *iolE* den Beginn des Wachstums der Salmonellen mit *myo*-Inositol charakterisiert. Die Expression des Operons *iolE/G1* beginnt erst wenige Stunden vor dem Ende der Anlaufphase, erreicht einen Maximalwert in der Mitte der exponentiellen Wachstumsphase und fällt in der stationären Phase wieder ab (Abb.6). Mit beiden Methoden konnte gezeigt werden, dass zum Ende der Anlaufphase nur ein Teil der Zellen den *iolEG1*-Promotor induziert, der den Anteil der Zellen darstellt, der früher aus der Anlaufphase in die exponentielle Wachstumsphase übertritt. Aus diesen Zellen werden vermutlich die distinkten Kolonien, die auf Minimalmedium-Agarplatten mit *myo*-Inositol früher anwachsen als der Rest der Population. Die Bistabilität im *myo*-Inositol-Metabolismus konnte also auf zellulärer Ebene bestätigt werden.

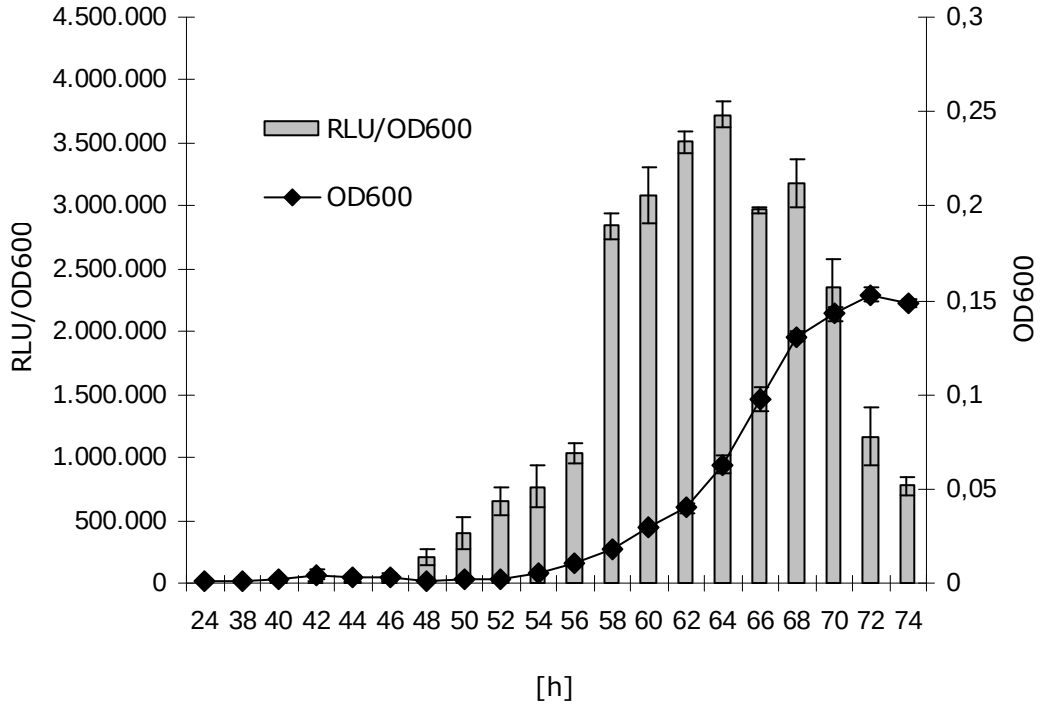

Abb.6: **Das Expressionsprofil des P_{iolEG1}-Promotors in *S*. Typhimurium mit Luciferase-Reporterplasmid pDEW201-P_{iolEG1}.** Das Diagramm zeigt den zeitlichen Verlauf der Expression des Operons *iolE/iolG1* in Minimalmedium mit *myo*-Inositol. Die Expression beginnt wenige Stunden bevor die Salmonellen anfangen sich zu vermehren, steigt auf ein Maximum während der mittleren exponentiellen Phase und fällt dann in der stationären Phase wieder ab. RLU = relative light units, OD600 = Optische Dichte bei 600nm

AUFHEBUNG DER BISTABILITÄT DURCH *IOLR*-DELETION UND CO_2/HYDROGENCARBONAT

Wie beschrieben, ist die starke Repression des *myo*-Inositol-Abbaus, die das Repressorprotein IolR vermittelt, eine wesentliche Ursache für das verzögerte Anwachsen der Salmonellen. Die Anlaufphase wird jedoch nur teilweise verkürzt, so dass es nahe liegt, dass neben IolR noch ein oder mehrere weitere Faktoren den Start in die exponentielle Wachstumsphase aufschieben könnten. Zur Identifizierung dieser Faktoren wurden die Wachstumsbedingungen und die Zusammensetzung des Minimalmediums verändert. Dabei stellte sich heraus, dass der pH-Wert des Mediums (pH 2-11) und niedrige Phosphatkonzentrationen (Induktions-bedingungen der Gene der SPI-2 (23)) im Minimalmedium keinen Einfluss auf die Länge der Anlaufphase haben. Interessanterweise konnte jedoch bei der Inkubation von *S.* Typhimurium auf Minimalmedium-Agarplatten mit *myo*-Inositol mit erhöhten CO_2-Werten (5%) der bistabile Phänotyp aufgehoben und zusätzlich die Anlaufphase etwa zu gleichen Maße verkürzt werden, wie im *iolR*-Deletionsstamm (Abb.7). In weiteren Versuchen konnte gezeigt werden, dass die minimal benötigte CO_2-Konzentration für die Reversion der Bistabilität in der Atmosphäre bei etwa 0,55% CO_2 liegt, wobei 0,2% CO_2 nicht ausreichen.
Durch Diffusion gelangt CO_2 in die Zellen, wo Carboanhydrasen die reversible Hydratation von CO_2 katalysieren [$CO_2+H_2O \leftrightarrow HCO_3^- + H^+$] (82). Der Auslöser für die Auflösung der

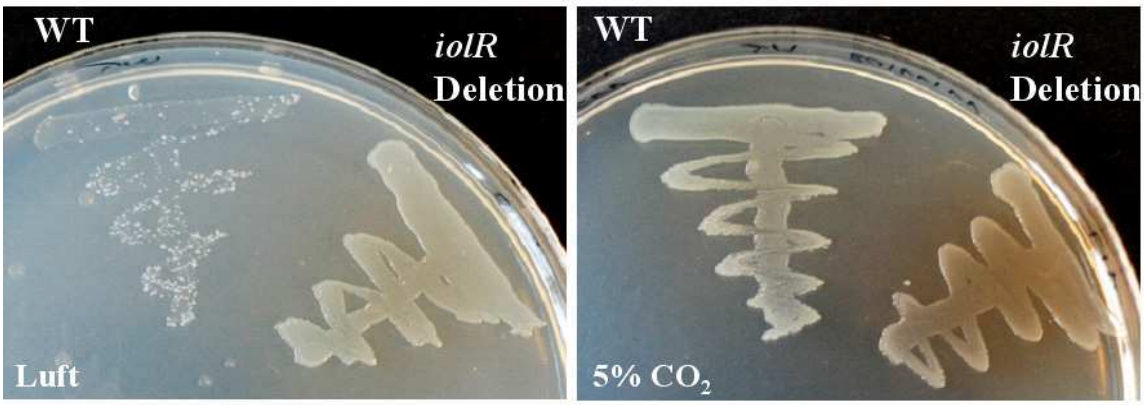

Abb. 7: **Vergleich des Wachstums des *S*. Typhimurium Wildtyp-Stammes (WT) mit dem *iolR*-Deletionsstamm (*iolR* Deletion) auf Minimalmedium-Agarplatten mit *myo*-Inositol nach 51 h.** Auf dem linken Bild wurde die Platte bei 37°C bei atmosphärischem CO_2 Niveau (Luft), auf der rechten Seite wurde die Platte bei 37°C mit 5% CO_2 inkubiert. Auf dem linken Bild kann man den bistabilen Phänotyp des *S.* Typhimurium Wildtyp-Stammes und die Aufhebung der Bistabilität im *S.* Typhimurium Stamm mit der *iolR* Deletion gut erkennen. Auf dem rechten Bild kann die Aufhebung der Bistabilität des *S.* Typhimurium Wildtyp-Stammes durch CO_2 beobachtet werden.

Bistabilität könnte also molekulares CO_2 oder Hydrogencarbonat sein. In flüssigem Minimalmedium mit *myo*-Inositol wurde die Verkürzung der Anlaufphase bestätigt. Eine Konzentration von 11.9 mM (0.1%) $NaHCO_3$ im Medium stellte sich dabei als optimal heraus, da hier die Anlaufphase am stärksten verringert wurde. Die Kombination der beiden Faktoren, die die Anlaufphase etwa in gleichem Maße verkürzen (jeweils 10-20 Stunden), resultierte in einer weiteren deutlichen Verringerung der Anlaufphase, so dass eine minimale Anlaufphase von etwa 15 Stunden gemessen werden konnte. Dies lässt zwei Schlüsse ziehen: (i) Die Effekte der *iolR*-Deletion der Anwesenheit von CO_2/HCO_3^- addieren sich und (ii) IolR und CO_2/HCO_3^- scheinen nicht miteinander zu interagieren.

CO_2/HYDROGENCARBONAT ALS INDUKTOR DER GENEXPRESSION IN PROKARYOTEN

Die Genregulation durch CO_2 oder HCO_3^- wurde in den letzten Jahren vor allem bei pathogenen Bakterien untersucht, da diese beiden Moleküle als Signal angesehen werden, dass sich die pathogenen Bakterien im Wirt, vor allem im Darm befinden. Zum einen besteht aus CO_2/HCO_3^- das Hauptpuffersystem des Körpers und die relativen Level dieser Moleküle beeinflussen viele zelluläre, biochemische und physiologische Prozesse, wobei Hydrogencarbonatkonzentrationen von 15-40 mM in extrazellulären Flüssigkeiten gemessen wurden (18, 81). Zum anderen gibt die Bauchspeicheldrüse Hydrogencarbonat beim Übergang vom Magen in den Darm ins Duodenum ab, um die Magensäure zu neutralisieren, wobei Konzentrationen von 70-150 mM erreicht werden (79).

Für einige Organismen konnte die Induktion von Virulenzgenen durch CO_2/HCO_3^- bereits gezeigt werden. In *B. anthracis* werden die Toxingene, die für die Toxinfaktoren, den Lethalitätsfaktor, den Ödemfaktor und das protektive Antigen kodieren neben der Temperatur auch durch Hydrogencarbonat reguliert (81). Eine Deletion des Hydrogencarbonat-Transporters führt zudem zur Avirulenz von *B. anthracis* in Mäusen (90). In *Vibrio cholerae* induziert CO_2/HCO_3^- die Expression des Regulatorproteins ToxT, das dann unter anderem Transkription der Cholera-Toxingene aktiviert (2), und in enterohämorrhagischen *E. coli* O157:H7 wird der Hauptinduktor Ler des „locus of enterocyte effacement“ (LEE) in Anwesenheit von Hydrogencarbonat exprimiert, in dem viele Virulenzgene lokalisiert sind (1). Der Hauptvirulenzfaktor in Gruppe A Streptokokken (*Streptococcus pyrogenes*), das M

Protein, sowie das Antigenprofil von *Borrelia burgdorferi* werden ebenfalls durch CO_2/HCO_3^- reguliert (16, 47).

EIN MODELL FÜR DIE REGULATION DES *MYO*-INOSITOL-METABOLISMUS IN *S.* TYPHIMURIUM

Aus den in dieser Arbeit generierten Daten lässt sich ein Modell für die Regulation des *myo*-Inositol-Metabolismus in *S.* Typhimurium 14028 postulieren, welches in Abbildung 8 dargestellt ist. In Anwesenheit von *myo*-Inositol im umgebenden Medium, nimmt *S.* Typhimurium aufgrund der basalen Expression des *myo*-Inositol-Transporters IolT1 kleine Mengen an *myo*-Inositol auf, dass dann zu 2-Deoxy-5-keto-D-Glucoronsäure-Phosphat (DKP) umgewandelt wird. DKP bindet dann an den Repressor IolR und löst ihn von den Promotoren der *iol*-Gene, was deren Expression induziert und einen positiven Feedback-Loop startet. Zunächst sind aber nur suboptimale Mengen an DKP vorhanden, so dass nicht jede einzelne Zelle zur gleichen Zeit die *iol*-Gene induziert, wodurch der beschriebene bistabile Phänotyp entsteht. Diese IolR-gesteuerte Regulation gilt jedoch nur für die Gene *iolA/B*, *iolC1/2* und *iolD1/2*. Die Gene *iolE/G1*, deren Proteine die ersten beiden Reaktionsschritte im *myo*-Inositol-Metabolismus katalysieren, unterliegen nicht der IolR-vermittelten Repression. Der Promotor dieser Gene wird wahrscheinlich durch einen zweiten Regulator (STM4423) in Abhängigkeit von CO_2/HCO_3^- induziert. Diese doppelte Regulation ist einzigartig für den *myo*-Inositol-Metabolismus aller untersuchten Organismen und kommt so nur Salmonellen und eventuell auch im *E. coli* Stamm ED1a vor.

Die in dieser Arbeit präsentierten Daten deuten darauf hin, dass *myo*-Inositol im Wirt als C-Quelle genutzt werden könnte. Stärkstes Indiz dafür ist, dass der für den *myo*-Inositol-Abbau essentielle Regulator STM4423 im Enteritis-Infektionsmodell der Maus induziert wird (73). Zudem konnte ein positiver Effekt auf die Induktion des *myo*-Inositol-Abbaus durch CO_2/HCO_3^- festgestellt werden, was in der Regel als ein Signal für den Aufenthalt im Wirt angesehen wird. In einem anderen Enteritis-Infektionsmodell, bei dem Schweine mit Salmonellen infiziert wurden, identifizierten Carnell *et al*. mehrere bei der Kolonisierung des Schweinedarms attenuierte Mutanten (17). Eine dieser Mutanten trug eine Mutation im Gen STM4433 (*iolG2*), das zwar in dieser Arbeit als nicht essentiell für den *myo*-Inositol-Metabolismus beschrieben worden ist, jedoch ist eine wichtige Funktion unter anderen Bedingungen wie z.B. im Darm des Wirts denkbar.

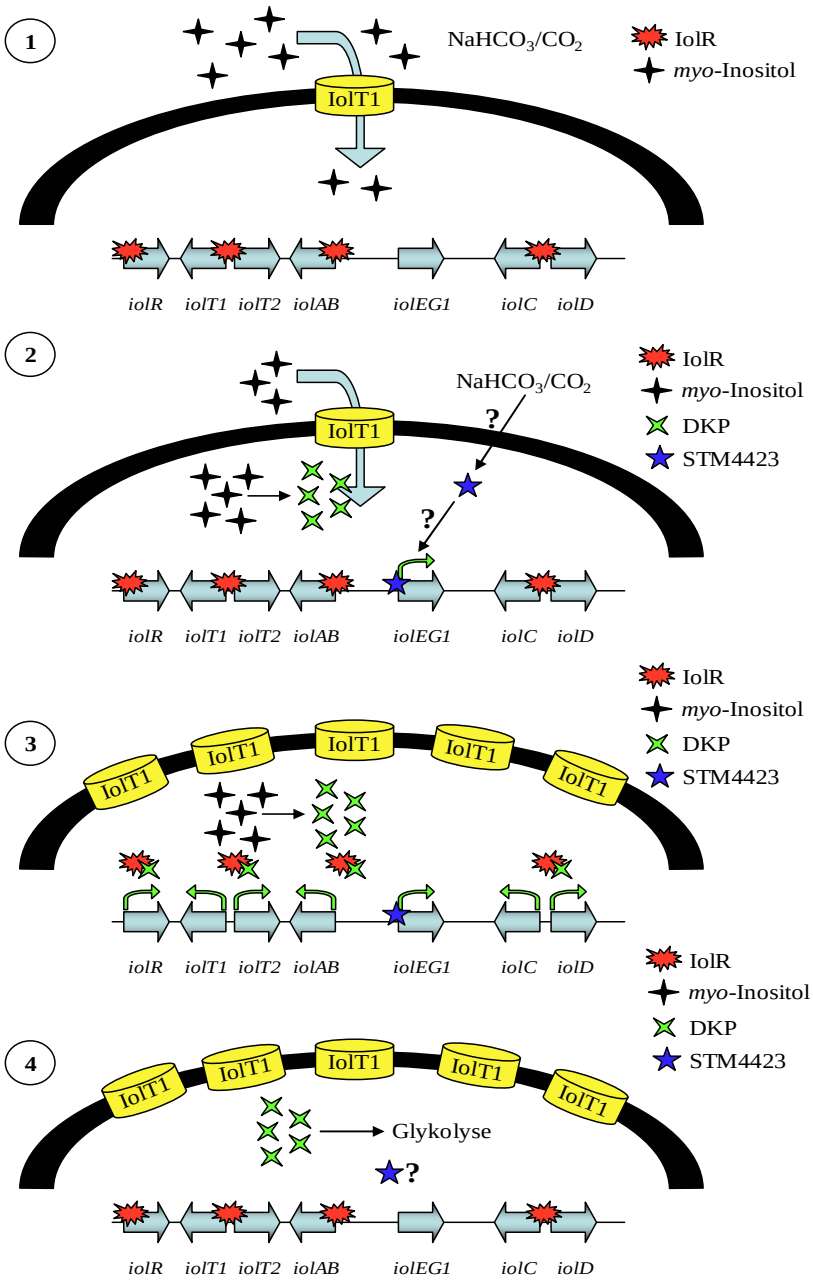

Abbildung 8: **Schematische Darstellung der Regulation der *iol*-Gene in *S*. Typhimurium.** (1) Aus dem extrazellulären Medium werden geringe Mengen an *myo*-Inositol über IolT1 aufgenommen. IolR ist an die Promotoren der Gene *iolR*, *iolT1/T2*, *iolA/B*, *iolC1/C2* und *iolD1/D2* gebunden und reprimiert deren Expression.
(2) Ist CO_2/HCO_3^- im Medium, kann es entweder in die Zelle diffundieren (CO_2) oder durch einen putativen Hydrogencarbonat-Transporter aufgenommen werden, und könnte dort die Genexpression von *iolE/G1* induzieren. Durch die Expression von *iolE/G1* und die niedrige Expression der übrigen *iol*-Gene werden kleine Mengen *myo*-Inositol in 2-Deoxy-5-keto-D-Glucoronsäure-Phosphat (DKP) umgewandelt, das nun an IolR binden kann und die Repression der *iol*-Gene löst.
(3) Alle *iol*-Gene werden stark exprimiert. Die Menge an *myo*-Inositol Transportern in der Cytoplasmamembran ist dadurch erhöht, so dass mehr *myo*-Inositol in die Zelle transportiert wird, das sofort degradiert werden kann, um Energie für die Zelle zu generieren.
(4) Nachdem *myo*-Inositol aus dem Medium aufgebraucht ist und intrazellulär das restliche DKP in die Glykolyse eingeflossen ist, kann IolR wieder an die oben genannten Promotoren binden und so die Expression wieder reprimieren. Wie sich STM4423 in dieser Phase verhält ist unklar.

AUSBLICK

Die in dieser Arbeit generierten Ergebnisse haben eine breite Grundlage für das Verständnis des *myo*-Inositol-Metabolismus in *S.* Typhimurium gelegt. Jedoch sind einige Aspekte des *myo*-Inositol-Metabolismus noch unklar und verlangen so nach weiteren Experimenten.

Die Induktion von STM4423 durch CO_2/Hydrogencarbonat wurde bisher nur indirekt gezeigt. Eine direkte Interaktion von STM4423 mit dem Promotor des Operons *iolE/G1* müsste mit Hilfe von Protein-DNA Bindestudien gezeigt werden. Falls kein weiterer, unbekannter Faktor involviert ist, müsste STM4423 in Anwesenheit von Hydrogencarbonat an den Promotor von *iolE/G1* binden.

Die vorstellten Daten deuten darauf hin, dass *myo*-Inositol in Wirt, wahrscheinlich im Darm, als C-Quelle verwertet werden kann. Um die Relevanz des *myo*-Inositol-Abbaus im Wirt zu untersuchen, müssten Infektionsversuche mit Mutanten des *myo*-Inositol-Stoffwechsels im Enteritis-Mausmodell durchgeführt werden.

Interessant ist auch die Tatsache, dass das Gen *srfJ* auf der GEI4417/4436 liegt. Da dieses Gen vom Zweikomponentensystem SsrAB induziert wird, dass vor allem Gene reguliert, die für das intrazelluläre Überleben der Salmonellen essentiell sind, stellen sich die Fragen: Was die genaue Funktion von *srfJ*? Ist *srfJ* auf irgendeine Weise im *myo*-Inositol-Metabolismus involviert? Werden die *iol*-Gene ebenfalls durch SsrAB reguliert?

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Characterization of the *myo*-inositol utilization island of *Salmonella enterica* serovar Typhimurium

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ABSTRACT

Knockout mutation of *STM4432* resulted in a growth-deficient phenotype of *Salmonella enterica* serovar Typhimurium (*S.* 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 from *STM4417* to *STM4436* (GEI 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 *iol* gene organization in Gram-negative bacteria. Upon non-polar deletion of eleven island loci, the genes involved in six enzymatic steps of the MI pathway were identified. The generation time of *S.* Typhimurium in minimal medium with MI decreases with higher concentrations of this polyol. RT-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.* 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 demonstrates that its transcription is autoregulated. Taken together, this is the first characterization of MI degradation in a Gram-negative bacterium, revealing a complex transcriptional organization and regulation of the *S.* Typhimurium *iol* genes.

INTRODUCTION

More than 60 carbon sources are known to be utilizable by *S.* 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. D. C. Old reported that MI utilization by *S.* Typhimurium strains is temperature-dependent, and that 95% of all strains investigated were fermenting MI at 25°C, although they had been designated inositol-nonfermenting at 37°C (23).

Besides *S.* 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 analysed 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-CoA, 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 knockout mutant 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 that acts as inducer by antagonizing IolR DNA binding (33). Other bacteria able to grow on MI as sole carbon source are *Corynebacterium glutamicum*, *Clostridium perfringens*, and *Lactobacillus casei* strain BL23 (14, 17, 30). In *C. perfringens*, all *iol* genes with the exception of *iolR* are unidirectionally organized, and one 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 similar manner as in *C. perfringens.* A more complex organisation 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 has not been investigated. Here, we describe that the knockout of several genes in GEI 4417/4436 results in growth-negative phenotypes of *S.* Typhimurium on MI. The activities of *iol* gene promoters under varying growth conditions are quantified using the luciferase reporter, and the complex transcriptional organisation of the *iol* genes essential for MI degradation is determined by RT-PCR. IolR encoded by *STM4417* is characterized as a negative regulator of *Salmonella* MI utilization that also regulates its own expression. The binding of this repressor to all but one promoters controlling the MI utilization genes is demonstrated by gel mobility shift assays.

MATERIALS AND METHODS

Bacterial strains, plasmids and growth conditions. Bacterial strains and plasmids used in this study are listed in Table 1. *S.* Typhimurium and *Escherichia coli* cultures were grown in Luria-Bertani (LB) broth (10 g/l tryptone, 5 g/l yeast extract, 5 g/l NaCl) or in minimal medium (MM; M9 medium supplemented with 2 mM $MgSO_4$, 0.1 mM $CaCl_2$ and 55.5 mM (1% w/v) MI or 27.8 mM (0.5% w/v) glucose, respectively. 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 (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 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 in 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^{TM} 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). Polymerase chain reactions (PCRs) were carried out with Taq polymerase (Fermentas). As 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. Strains of a *S.* Typhimurium mutant library were characterized as described previously (16). *S.* Typhimurium gene numbers refer to the LT2 annotation (NC 003197). The homepages http://globin.cse.psu.edu/enterix and http://www.microbesonline.org/ were used to determinate the distribution of *S.* 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/).

Phenotypic testing of carbon source utilization. A set of *S.* Typhimurium mutants was screened for their capability to utilize a number of 63 different substrates that are possible carbon sources for *S.* Typhimurium (10). For this purpose, we applied a colourimetric 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 PBS, 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 microtitre plate and measured after 24 and 48 h at 37°C in a microtitre plate reader (Tecan, Männedorf, Switzerland) at OD_{620}. Sucrose and lactose which are not metabolized by *S.* Typhimurium served as control substrates.

Construction of deletion mutants and complementing plasmids. In-frame deletion mutants of *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*) 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 nucleotides-long primers that included 20 nucleotides 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). 500-1000 ng of fragment DNA was transferred into *S.* Typhimurium strain 14028s 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 *iolR* and *iolE* plus approx. 300 bp of their upstream region were amplified from chromosomal DNA of strain 14028 with primers listed in Table S1. PCR products were digested with *Eco*RI and *Sal*I (Fermentas) and ligated (T4 DNA ligase; Gibco, Hudson, USA) 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 Reverse Transcriptase (RT)-PCR. RNA was isolated according to the modified single-step method of Chomczynski and Sacchi (5). Briefly, 15 ml of a *S.* Typhimurium culture grown in MM supplemented with MI to an OD_{600} ~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 DEPC treated H_2O. DNase treatment was performed with RQ1 DNaseI (Promega, Mannheim, Germany) according to the manufacturer's instruction. Annealing of reverse primers (Table S1) was performed in a total volume of 10 µl containing 75 ng of total RNA, 10 pmol reverse primer and 20 mM dNTP 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 reaching 42°C, 10 µl RT mix (Promega) with 0.1 M dithiothreitol (DTT), and 200 U RT (Promega) was added to generate cDNA. Heat inactivation of RT was performed by incubation at 70°C for 15 min. 2 µl of this sample was then used as PCR template.

Cloning of promoter fusions. Putative promoter regions spanning approximately 300 base pairs 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*), *def* (*STM3406*) and an intragenic fragment of *STM0047* without promoter homology were amplified from chromosomal DNA of *S.* Typhimurium 14028 by PCR using the primers listed in Table S1. The fragments were then cloned *via Eco*RI and *Bam*HI or *Eco*RI and *Kpn*I (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 timepoints, 200 µl of each sample was transferred to the 96-well plate, and the optical density at 600 nm 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 *Xho*I and *Nho*I, thus introducing a C-terminal fusion of a 6x His-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 3h 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, USA), and residual cell debris was removed by centrifugation at 4°C (20 min, $1.4x10^4$ rpm). After adding 10 µl of protease inhibitor phenylmethanesulphonylfluoride (PMSF; 100 mM), 10 µl of the supernantant containing soluble proteins mixed with 10 µl 2x Laemmli buffer were applied to SDS-PAGE to verify IolR-His_6 overexpression, and seperated proteins were stained with Coomassie blue.

Purification of IolR-His_6 and gel mobility shift assays. Protein IolR-His_6 was purified using TALON metal affinity resin (Clontech Laboratories, Mountain View, USA). 1 ml 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 ten times with 0.5 ml buffer A and five times with 0.5 ml buffer B (buffer A containing 7.5 mM imidazole). IolR-His_6 was eluted ten times using 0.5 ml buffer C (buffer A with 150 mM imidazole). 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 $MgCl_2$, 0.5 mM EDTA and 10% [vol/vol] glycerol) by gel filtration using PD-10 columns (GE Healthcare, München, Germany) (26). The protein concentration was determined in a Nanodrop spectrophotometer (Thermo Fischer Scientific, Langenselbold, Germany), and the purity of eluted fractions was analysed by separation on a 15% SDS polyacrylamide gel.

For the gel mobility shift (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 were 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 6x loading dye (Fermentas) on a 9.5% native polyacrylamide gel and separated at 120 V for 3 h in 1x TBE buffer precooled at 4°C. DNA was stained in ethidium bromide solution and visualized by UV irradiation.

RESULTS

The MI utilization genes of *S*. Typhimurium are located on a genomic island. 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 capability to respire in the presence of one of 66 carbon source 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 that showed a significant respiration signal only after 48 h. The strains are mutated in the genes *STM4432* and *STM4417*, which belong to a genomic island (GEI) of 22.6 kb that starts with *STM4417* and ends with *STM4436* (20). Homology searches using BLAST programs (1) revealed twelve genes to be possibly involved in MI metabolism of *S.* 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 latter one is regulated by the two-component system SsrAB that also controls the expression of *Salmonella* pathogenicity island 2 (SPI-2) genes (29). The annotation of the MI utilization genes of *S.* 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 splitted the *S.* Typhimurium *iolC* and *iolD* homologues into two ORFs 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 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.* Typhimurium strains LT2, 14028, SL1344, *S.* Saintpaul strain SARA23, *S.* Weltevreden strain HI_N05-537, and also *S.* 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* spp. *S. flexneri*, *S. dysenteriae* M131649 and *S. sonnei* 53G, of eight *S. enterica* serovars (Typhi CT18/Ty2, Paratyphi A/C, Enteritidis, Dublin, Gallinarum, Diarizonae), of *S. bongori*, and of *Vibrio cholerae*. A chromosomal fragment of *K. 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*

TABLE 1. Strains and plasmids used in this study.

Bacterial strains	**Description and relevant features**	**Source or literature**
DH5α	*E. coli: deoR, endA1, gyrA96, hsdR17*(r_k-m_{k+}), *recA1, relA1*, s*upE44, λthi-1,* Δ(*lacZYA-argFV169*)	(11)
BL21 (DE3)	*E. coli: F*$^-$*, omp*T, *hsd*S_B ($r_B^-m_B^-$), *gal, dcm, rne*131	(27)
14028	*S.* Typhimurium wild-type strain ATCC14028	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*	Non-polar *iolR* (*STM4417*) deletion mutant	This study
14028s-Δ*iolB*	Non-polar *iolB* (*STM4420*) deletion mutant	This study
14028s-Δ*iolA*	Non-polar *iolA* (*STM4421*) deletion mutant	This study
14028s-Δ*iolE*	Non-polar *iolE* (*STM4424*) deletion mutant	This study
14028s-Δ*iolG1*	Non-polar *iolG1* (*STM4425*) deletion mutant	This study
14028s-Δ*iolI1*	Non-polar *iolI1* (*STM4427*) deletion mutant	This study
14028s-Δ*iolC*	Non-polar *iolC* (*STM4429-30*) deletion mutant	This study
14028s-Δ*iolD2*	Non-polar *iolD2* (*STM4432*) deletion mutant	This study
14028s-Δ*iolG2*	Non-polar *iolG2* (*STM4433*) deletion mutant	This study
14028s-Δ*iolI2*	Non-polar *iolI2* (*STM4435)* deletion mutant	This study
14028s-Δ*iolH*	Non-polar *iolH* (*STM4436*) deletion mutant	This study
14028s-Δ*STM3253*	Non-polar *STM3253* deletion mutant	This study
Y. enterocolitica W22703	Wild-type strain. Biovar 2, serovar O:9, Nal^r, Res^- Mod^+, pYV^-	(6)
P. luminescens	*P. luminescens* ssp. *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

gene cluster present in *Erwinia carotovora*, *Citrobacter koseri*, *Y. pestis*, *Y. pseudotuberculosis*, and *Photorhabdus luminescens*. *Y. enterocolitica* and two non-pathogenic *Yersini*a species share the same organization of *iol* genes, as well as *Brucella abortus*, *R. leguminosarum*, and *Sinorhizobium meliloti*.

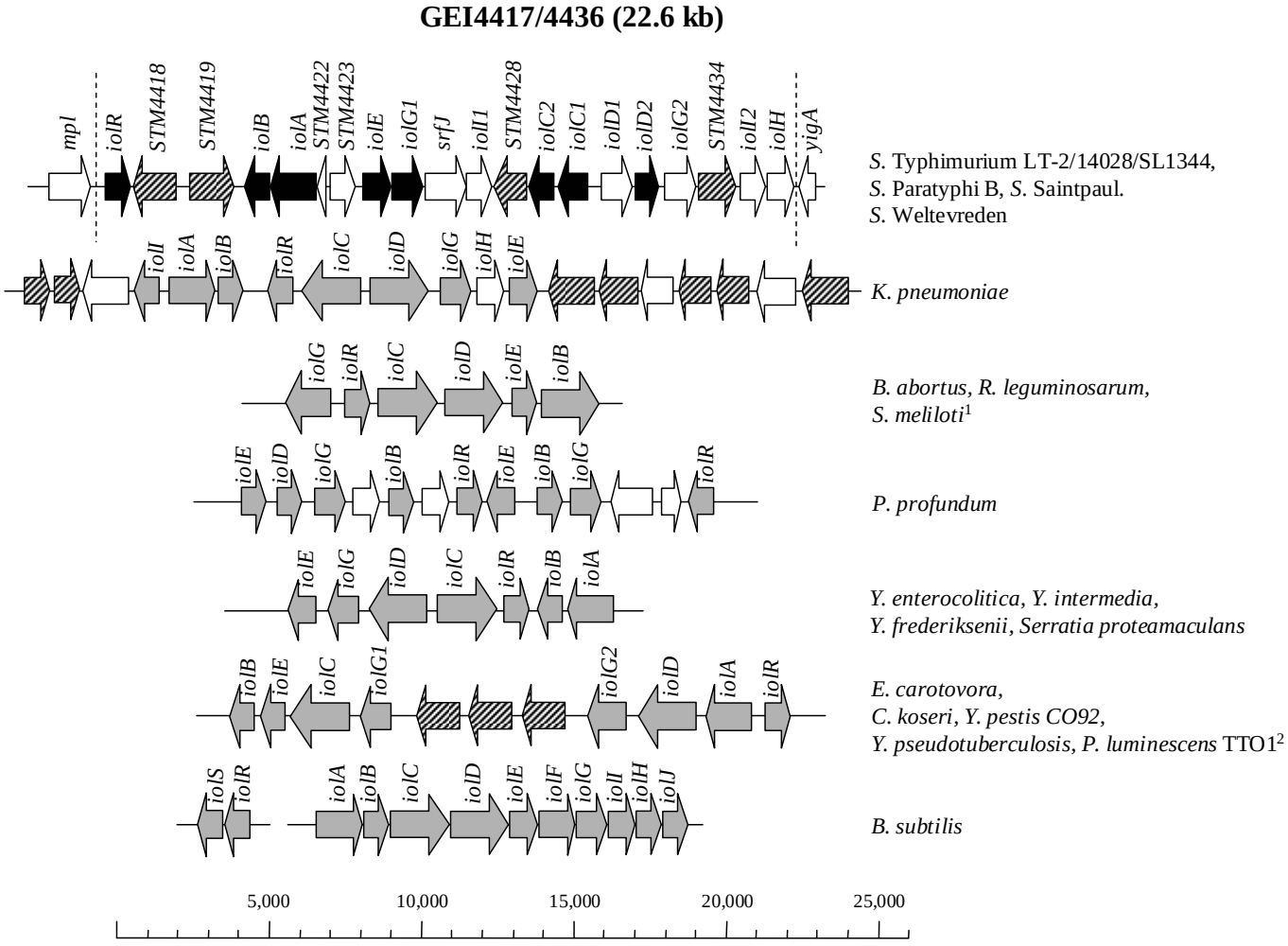

FIG. 1. (A) Examples of *iol* divergons. Genomic island GEI 4417/4436 of *S.* Typhimurium is presented in comparison to the structural organization of *iol* genes from B. subtilis, and several Gram-negative bacteria. *Salmonella* genes experimentally demonstrated in this study to belong to the inositol divergon are depicted in black, their homologues in other pathogens in grey. 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.

We therefore tested *Y. enterocolitica* and *P. luminescens* for their capability 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.* 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).

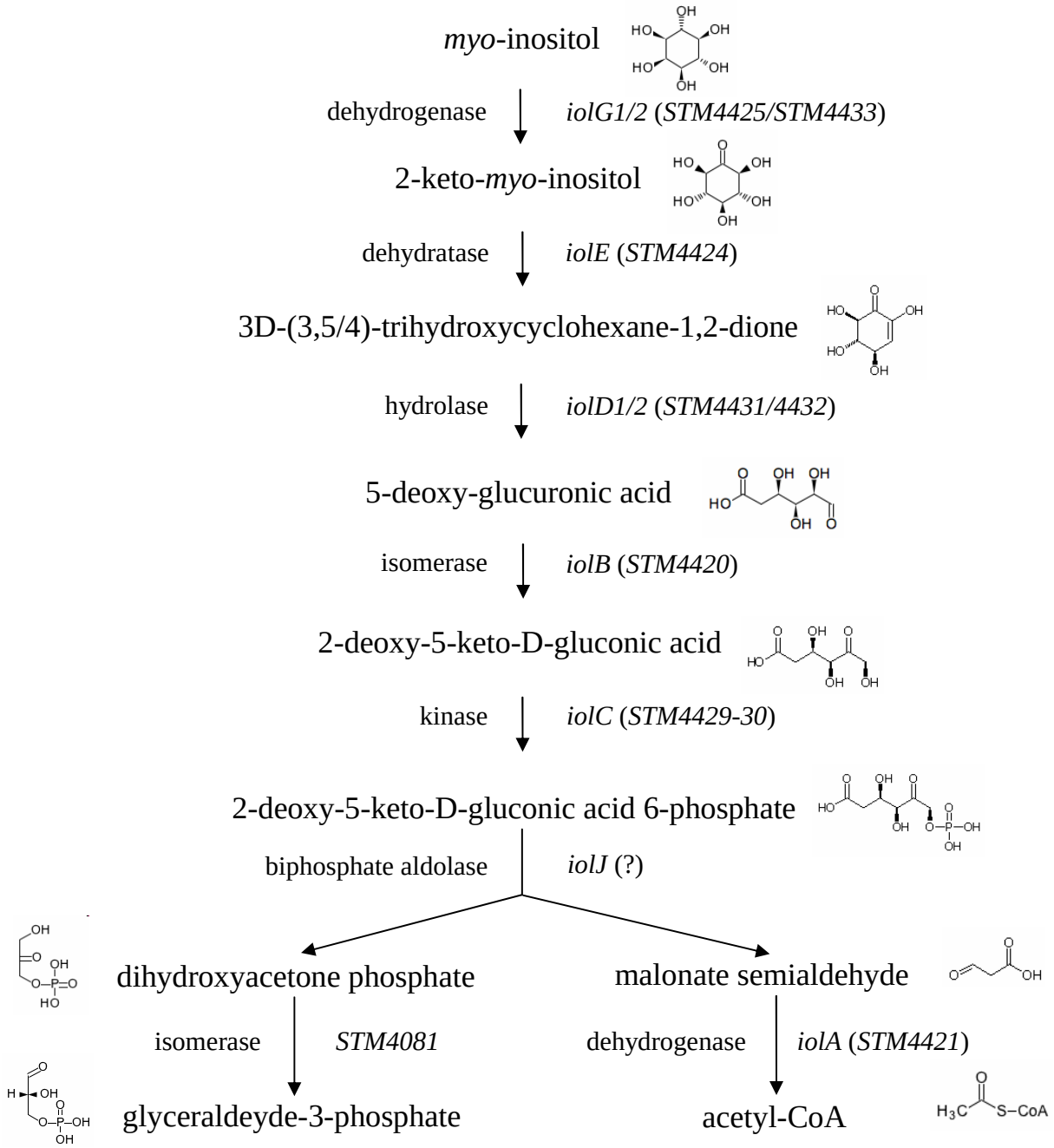

FIG. 1. (B) Pathway reconstruction of MI degradation in *S.* Typhimurium. Seven stepwise reactions are involved in MI degradation to glyceraldehyde-3-phosphate and acteyl-CoA. None of the genes from GEI 4417/4436 encodes a homolog of a biphosphate aldolase.

Growth properties of *S.* Typhimurium in the presence of MI. We investigated the growth behaviour of strain 14028 in MM supplemented with varying concentrations of MI. Three features appeared to be characteristic for *S.* 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 D. C. Old, the wild-type strain produced abundant amounts of a brown pigment (23). This pigmentation appeared only in stationary phase. It was missed when the strain was cultivated anaerobically and might therefore result from metabolite oxidation.

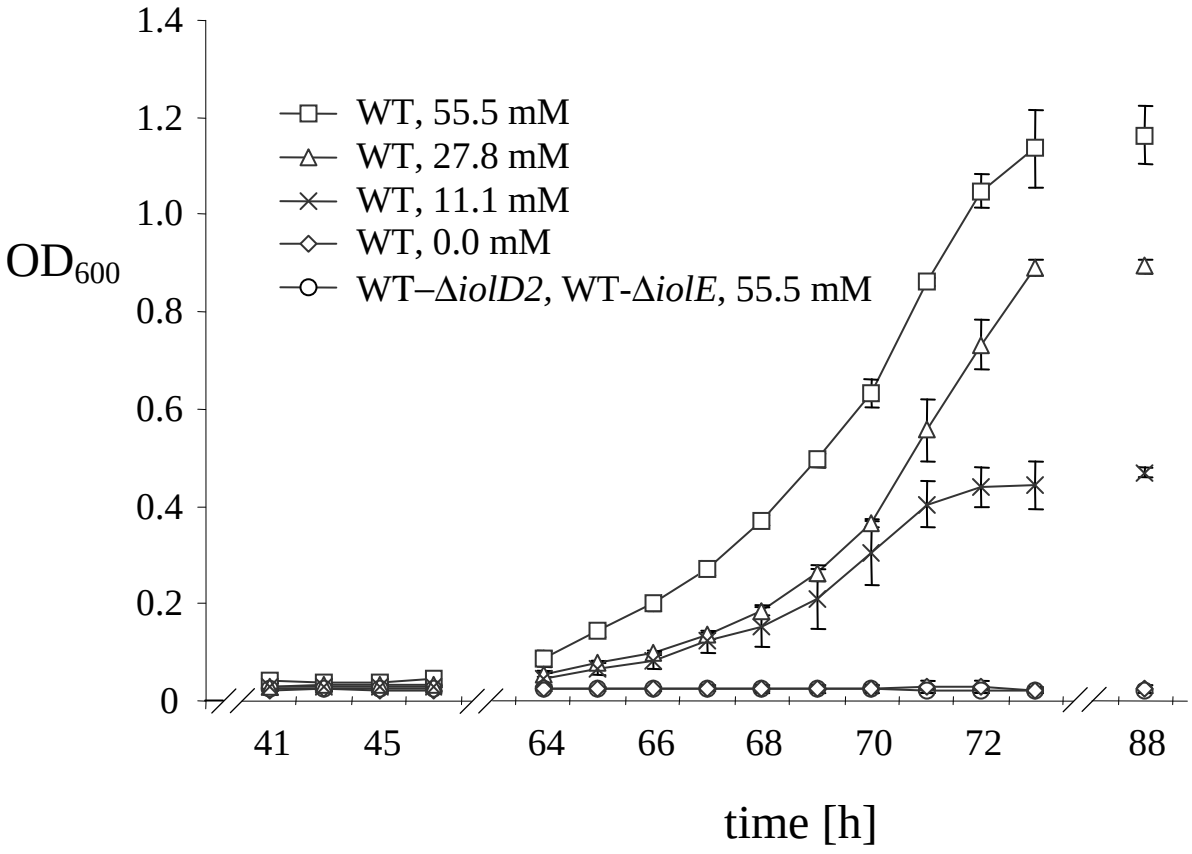

FIG. 2. Growth curves of the *S.* Typhimurium wild-type strain 14028 in MM without or with increasing concentrations of MI. The *iolD2* and *iolE* deletion mutants were cultivated in the presence of 55.5 mM MI. Zero growth of the wild-type strain and the two mutants in the absence of this carbon source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

Phenotypes of deletion mutants. To experimentally demonstrate that genes of GEI 4417/4436 are responsible for MI degradation by *S.* Typhimurium, non-polar deletions of *iolB, iolA, iolE, iolG1, iolI1, iolC1/2, iolD2, iolG2, iolI2, iolH* and *STM3253* were constructed as described. Growth of these eleven mutants was monitored at least for five 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*, -Δ*iolA*, -Δ*iolE*, -Δ*iolG1*, -Δ*iolC* and-Δ*iolD2* did not grow in liquid MM containing MI as sole carbon source, clearly demonstrating the role of the deleted genes in MI utilization. In contrast, growth deficiencies of the mutants 14028s-Δ*STM3253*, -Δ*iolI1*, -Δ*iolI2*, -Δ*iolH* and -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 *L. 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 were cloned as positive controls, 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 strains 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. Irrespective 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 2-3x10^5 RLU/OD_{600}. 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 1.25x10^4 to 1.87x10^4 RLU/OD_{600}. 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 ten-fold and the *iolA* promoter three-fold induced during 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 iolR* in accordance to 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 MI. However, their absolute RLU/ OD_{600} values are at least two 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 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, 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 organisation 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 promotor (Table 2), suggesting a co-transcription of *iolB* with *iolA*, of *iolG1* with *iolE*, of *iolC2* with *iolC1*, and of *iolD2*/*iolG2* with *iolD1*. To reveal the transcriptional organisation of the *iol* genes within GEI4417/4436, we performed RT-PCR

TABLE 2. Quantification of *iol* promoter activities shown as relative light units (RLU) per OD_{600}. Fold induction was calculated with respect to the RLU/OD_{600} values of the wild-type strain grown in glucose. Samples were taken from the late exponential phase. Data are the average RLU derived from three independent experiments. SD, standard deviation.

	14028s grown in		14028s grown in			14028s-Δ*iolR* grown		
fragment cloned into pDEW201	MM+glucose [RLU/OD_{600}]	SD [%]	MM+MI [RLU/OD_{600}]	SD [%]	fold induction	in MM+glucose [RLU/OD_{600}]	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	3202.8	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	16864.2	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

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 300-500 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 template revealed a DNA fragment whose length corresponds to the PCR fragments amplified from genomic DNA (Fig. 3). In line with the data of the luciferase reporter fusions, *iolG2* and *iolH* are under 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.* Typhimurium comprise all genes required for MI utilization as well as three functionally dispensable or redundant genes, *iolG2*, *iolI2* and *iolH*.

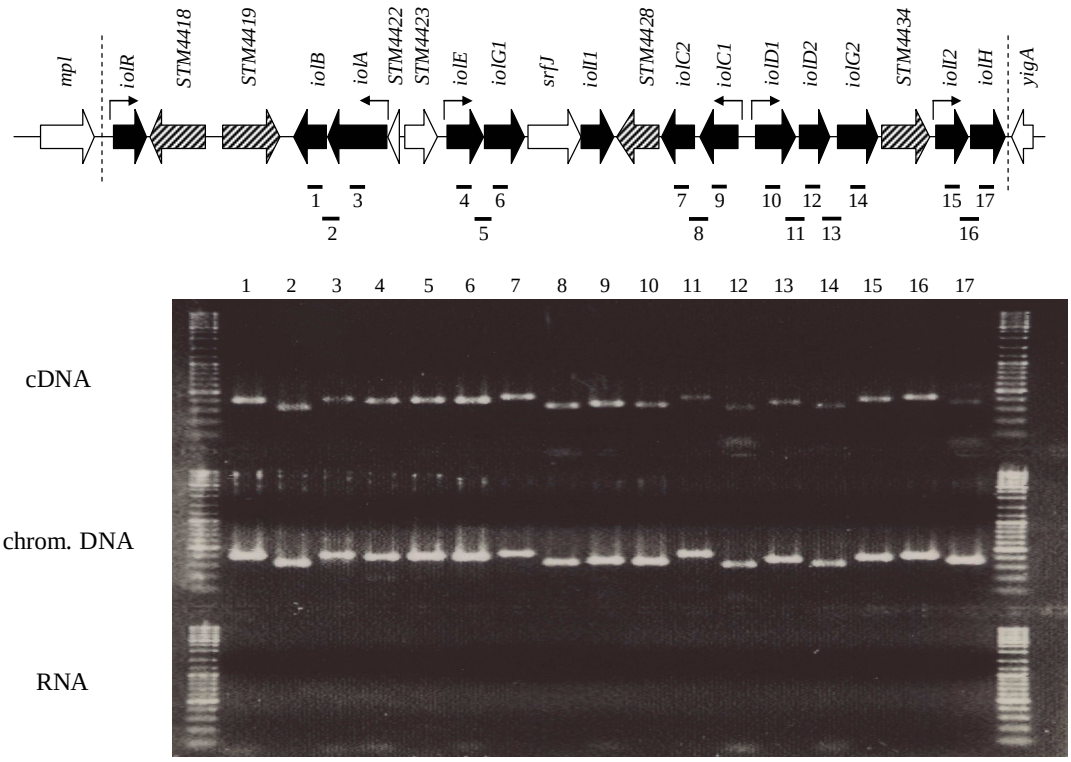

FIG. 3. Transcriptional organization of *S.* Typhimurium MI utilization genes. Strain 14028s was grown in MM with 55.5 mM MI at 37°C, and mRNA was extracted at OD_{600}= 0.4. cDNA was amplified with reverse primers listed in Table S1. RT-PCR was performed with primer pairs specific for the indicated regions 1-17. All PCR products were separated by 2% agarose gel electrophoresis. As control, 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.

IolR acts as transcriptional repressor. Homology searches with the protein sequence of IolR (STM4417) revealed a putative trancriptional regulator with a HTH-6 motif belonging to the RpiR family. This domain is N-terminated to a sugar isomerase (SIS) domain that is predicted to bind phosphosugars. No homologies to the characterized IolR proteins of *B. subtilis* or *C. glutamicum* were observed, but 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 non-polar deletion of *iolR* resulted in a lag-phase 10 h shorter in comparison to the wild-type strain (Fig. 4). The 14028s-Δ*iolR* phenotype could be complemented by gene expression of *iolR* from pBR322 (Fig. 4).

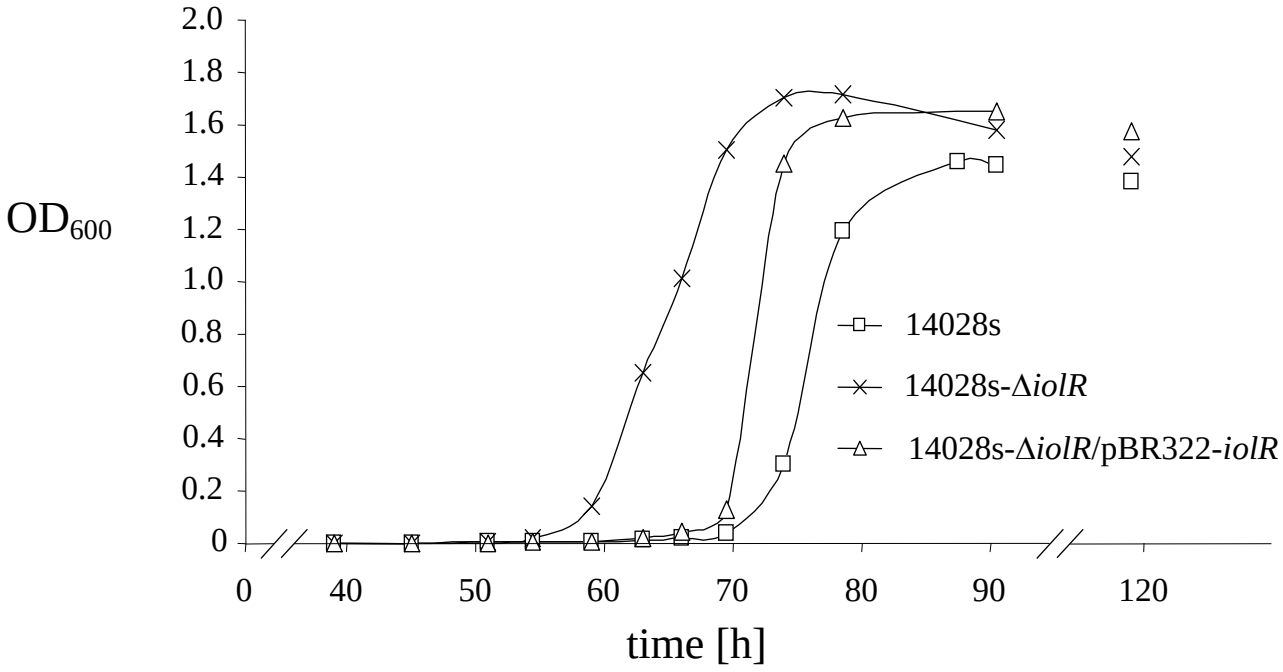

FIG. 4. Growth curve of strain 14028s-Δ*iolR*. Exponential growth of the strain lacking the repressor IolR starts approximately 10 h earlier than that of the wild-type strain. The phenotype of 14028s-Δ*iolR* could partially be complemented upon *in trans* expression of *iolR via* pBR322. Average values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

Induction of the promoters of *iolR, iolA, iolC1* and *iolD1* in the *iolR* deletion mutant in the presence of glucose, and the identification of a 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*, *lA*, *iolE*, *iolG1*, *iolC1*, *iolD1*, *iolG2*, *iolI2* and *iolH* fragments were incubated without or with increasing amounts of the purified IolR proteins, and the protein-DNA complexes were separated on a 9.5% native polyacrylamide gel (Fig. 5).

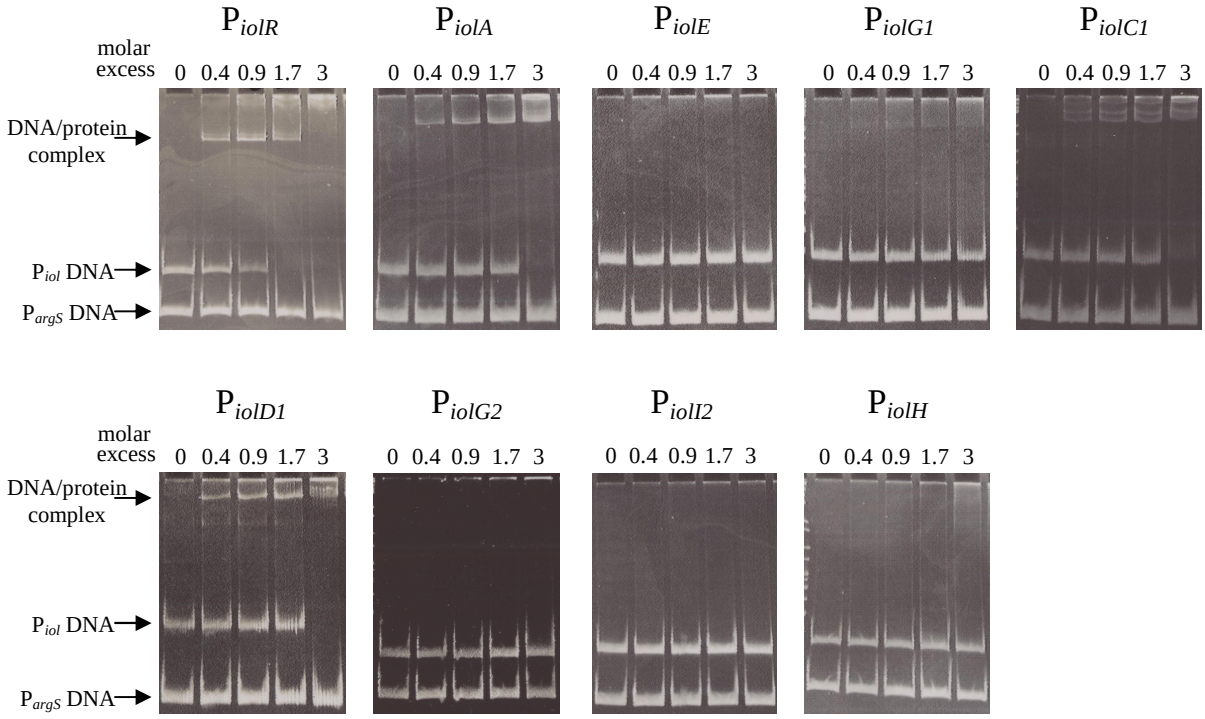

FIG. 5. Promoter binding activity of IolR. The interaction of IolR with the regulatory region of nine genes of GEI 4417/4436 is shown. 100 ng of DNA were used in each experiment. The promoter fragments were incubated without or with increasing amounts (7 ng, 14 ng, 28 ng, and 49 ng or 221 fM, 442 fM, 884 fM, and 1547 fM, respectively) of the purified IolR protein. No bandshift was observed when a maximum of 210-280 ng IolR corresponding to a maximal 17-fold molar excess was incubated with promoter DNA 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.

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.* 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 of 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 organisation in comparison to their Gram-positive counterparts. The systematic knockout of GEI 4417/4436 genes revealed that this *S.* 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 a 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.

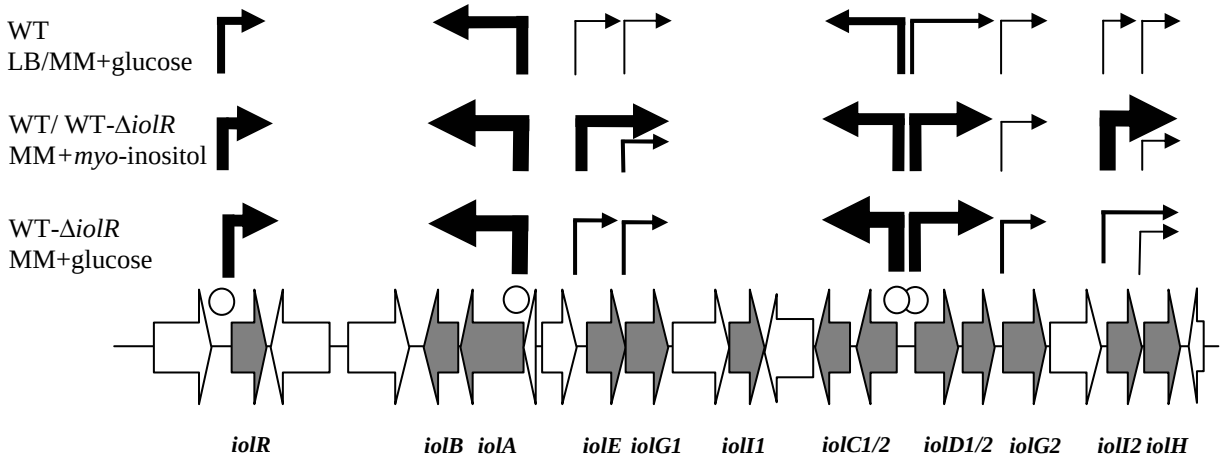

FIG. 6. Regulation of MI utilization in *S.* Typhimurium. The wild-type strain and an *iolR* deletion mutant carrying recombinant pDEW201-constructs were grown in LB, or in MM with MI or glucose. Promoter induction [RLU/OD_{600}] is depicted by arrows of different size: ¾ pt., $<10^4$ RLU/OD_{600}; 1½ pt., $<10^5$ RLU/OD_{600}; 3 pt., $<10^6$ RLU/OD_{600}; 4½ pt., $<10^7$ RLU/OD_{600}; 6 pt., $<10^8$ RLU/OD_{600}. Induction of *iol* genes was similar in both strains in the presence of MI. Binding sites of IolR are indicated by open circles.

S. 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 the stationary phase. However, a remarkable difference is the extended lag phase of approximately 60 h in the presence of MI as 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 few hours after inoculation (17). 2-deoxy-5-keto-D-gluconic acid has since long been considered a key step in MI degradation (2). Only recently, 2-deoxy-5-keto-D-gluconic acid 6-phosphate (DKGP), 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.* 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 that they are under catabolite repression. *iolE* encoding the dehydratase that catalyzes the second step in MI degradation might be positively induced by MI or a related substance rather than by an antagonistically acting intermediate such as DKGP.

RT-PCR, reporter fusions and GMS revealed a consistent picture of the transcriptional organisation, 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 cluster required for MI utilization by *S.* Typhimurium, namely *iolA*/*iolB*, *iolC1*/*iolC2*, and *iolD1*/*iolD2*/*iolG2*. A lack of IolR binding to the putative promoter of the *iolE*/*iolG1* operon that encodes the first two steps in MI degradation hints to an additional, IolR-independent regulatory mechanism. The autoregulatory activity of the MI repressor and substrate antagonism might explain that *iolR* of *S.* Typhimurium is induced under conditions at 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* cluster 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.* 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 *P. profundum* genome (*iolE, iolG*) that 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 cluster, 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 addressed in *S.* 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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SUPPLEMENTARY MATERIAL

TABLE S1. Primers used in this study.

primer name	target gene	modification	5’ – 3’ sequence
*construction of non-polar deletion mutants**			
del_3253_for	*STM3253*		TTTAATTTCGAAAGTTATAAACGGAGACCACTATGTTCATTATTTCCAGTGTGTAGGCTGGAGCTGCTTC
del_3253_rev			TCACAGGCGATTCCTCACAGTTGCCCTTCACAGCCGCAAACATGAATTACCATATGAATATCCTCCTTA
del_4417_for	*STM4417* (*iolR*)		TACGAAATTTTCGTTCTATTAGAGTATCATGCATGTCTAAACATCAAACTGTGTAGGCTGGAGCTGCTTC
del_4417_rev			ATCGGCTTGTTTTTTTACTCCGTCGCCAGCGCCAGTGAAACCGCTAACGTCATATGAATATCCTCCTTA
del_4420_for	*STM4420* (*iolB*)		TCGGTTAATACGCTTTCAGGAAGGAGAAGATTATGGCCAACTTGCTGAGTGTGTAGGCTGGAGCTGCTTC
del_4420_rev			CACTCAACGAAGTGTTATCGCGGGTAGTCTGGTGAGTTAATCCACGCGTGCATATGAATATCCTCCTTA
del_4421_for	*STM4421* (*iolA*)		CGTCAACATCGGTTTAAGAGGATAAAAAAGAGATGGAAACAGTCGGCAATGTGTAGGCTGGAGCTGCTTC
del_4421_rev			CACTCAACGAAGTGTTATCGCGGGTAGTCTGGTGAGTTAATCCACGCGTGCATATGAATATCCTCCTTA
del_4424_for	*STM4424* (*iolE*)		CATCGCGGTATGCAGTAAAAGCGTTTCATTAAGTGGGAGCCAGCAATATGGTGTAGGCTGGAGCTGCTTC
del_4424_rev			TGTTTCCTTTTCAATTAAAGATAATTTTTCAGTAACTCATCAATATGCTTCATATGAATATCCTCCTTA
del_4425_for	*STM4425* (*iolG1*)		AAAATTATCTTTAATTGAAAAGGAAACAGAAAATGACTTTAAAAGCAGGTGTGTAGGCTGGAGCTGCTTC
del_4425_rev			AATACGCGGAAGGTTTATTTGTAGAAATCAGGTTTTGAGGGTAATTCAACCATATGAATATCCTCCTTA
del_4427_for	*STM4427* (*iolI1*)		GTACGTTGCTATGGCGGCAGGAGTCGATCTGAATGATGAAGCTGGGATTTGTGTAGGCTGGAGCTGCTTC
del_4427_rev			TTGCTGCGGCAACATTAAGCTGTTAAGAAGGGGCGAGTCTTTTCCGCCCCCATATGAATATCCTCCTTA
del_4430-29_for	*STM4429-30* (*iolC2-1*)		AGCAAAAATAACACCGTCTTCTGTTAGGGGATATGTCAATGAATAAAGCAGTGTAGGCTGGAGCTGCTTC
del_4430-29_rev			GTGGCGCGATACCACTAACGCGCCGCAGGCGTTGCATAACGGCAGGCTTGCATATGAATATCCTCCTTA
del_4432_for	*STM4432* (*iolD2*)		GTGAGACTATTCACCGCGTCCGCTCGCAATATATGGCGGAAGTTGAGCGCGTGTAGGCTGGAGCTGCTTC
del_4432_rev			CGACGGCAAATTAATCAGTACTGCCGGGCCTGTCCGATATGTTCTTCAAGCATATGAATATCCTCCTTA
del_4433_for	*STM4433* (*iolG2*)		AAATTTACTATTCATGAGATTAAGAGGTACGAATGAAAAAGCTTCGATGTGTGTAGGCTGGAGCTGCTTC
del_4433_rev			ATTAATAGATGCAGTTAAAGATAAATATCGCTTGTTATTTTCACCGGCAGCATATGAATATCCTCCTTA
del_4435_for	*STM4435* (*iolI2*)		TTACCCACTCATATACGTTCAGGGAGAAAATAATGAATATCGAAAAAACAGTGTAGGCTGGAGCTGCTTC
del_4435_rev			GGAAATTAGCTGTATCATTGCAGCAGCAATGACACGCTGCGATTAATTTGCATATGAATATCCTCCTTA
del_4436_for	*STM4436* (*iolH*)		CCGTTTTCAGTTTCCGCAGCGTGGTATTTACAATGAAAATTGCTTTTGATGTGTAGGCTGGAGCTGCTTC
del_4436_rev			AAATCCACCCGTTTTTAACGTCCGAGTAGCTCCCGCTCGATCCGCTCGCGCATATGAATATCCTCCTTA
*test of insertion of kanR and gene deletion***			
test_3253	*STM3253*		TCACCGAATGCTCAATG
test_4417	*STM4417*		TATGTTCAGTTCATTTGTGC
test_4420	*STM4420*		CCAGCGAGTGCGCACAGG
test_4421	*STM4421*		TACCCTCCAATATCCG
test_4424	*STM4424*		CGTTACTAACCACGGATG
test_4425	*STM4425*		AAGCATGTCGCCTCGC
test_4427	*STM4427*		TACGATAATCTTCTGGAAG
test_4429-30	*STM4429-30*		GAAATTTATCTTCCATGCG

test_4432	*STM4432*		CGTACAGATGCAGGG
test_4433	*STM4433*		GTTCAGGTTGCAGAAGTG
test_4435	*STM4435*		GCGCTGGTCTCCGTTC
test_4436	*STM4436*		ATATCGCGGTATTTACGC
kanR3	*kanR*		GCGCTGCGAATCGGG
*construction of pBR322-complementation vectors***			
com_4417_EcoRI	*STM4417*	*Eco*RI	CCGGAATTCGTGATTTATAAACGTCATC
com_4417_SalI	*STM4417*	*Sal*I	TTTTGTCGACCTGATTAAGTTTCACCAC
com_4424_EcoRI	*STM4424*	*Eco*RI	CCGGAATTCAATATCGCAAGGACTATC
com_4424_SalI	*STM4424*	*Sal*I	TTTTGTCGACATCGGAGCCGATCATG
*construction of pET28b-iolR overexpression vector***			
pu_4417_for_NcoI	*STM4417*	*NcoI*	AAACCATGGATGTCTAAACATCAAACTCAAC
pu_4417_rev_XhoI	*STM4417*	*XhoI*	AAACTCGAGCTCCGTCGCCAGCGCC
*primers used in RT-PCR***			
RT_4420-21_for	*STM4420-21*		CCAGTCGGTTGAAGCCG
RT_4420-21_rev	*STM4420-21*		ACATACTCCCAGCCTGC
RT_4420_for	*STM4420*		AAGTGACAGCCGAAACCG
RT_4420_rev	*STM4420*		GTGGTAGCCCTTAGGTAC
RT_4421_for	*STM4421*		TTTAACGTCGGCTCAGGC
RT_4421_rev	*STM4421*		AGATCTGCATCCGGCATG
RT_4424_for	*STM4424*		TAATGCTTCGCGGACTGG
RT_4424_rev	*STM4424*		CCCGGTATCAAACAGCAG
RT_4424-25_for	*STM4424-25*		GGCTGGATTGTTGTTGAG
RT_4424-25_rev	*STM4424-25*		CATTTAGCGCGGCAACG
RT_4425_for	*STM4425*		GTGATTGAAGCAGAGCAG
RT_4425_rev	*STM4425*		GTGAATGTCGTAGCCATAC
RT_4429_for	*STM4429*		ATCATCCACTCGATGCAG
RT_4429_rev	*STM4429*		CATCGTTAAGCTGGCCG
RT_4429-30_for	*STM4429-30*		ACGTTCTGAACGTTCTGG
RT_4429-30_rev	*STM4429-30*		CGCCGACTTCATTTGCG
RT_4430_for	*STM4430*		CTGTCGGATCGCGATCG
RT_4430_rev	*STM4430*		CTGGAGCTGTTCGGTAAC
RT_4431_for	*STM4431*		CCATCAGATTTATGCCTGC
RT_4431_rev	*STM4431*		TTCACCCTGCACATCCTG
RT_4431-32_for	*STM4431-32*		TCAAGCTGGATGGCGTAC
RT_4431-32_rev	*STM4431-32*		CTTCGTAGCCCATACAGG
RT_4432_for	*STM4432*		ATGATGCTGCACTCTGAG
RT_4432_rev	*STM4432*		ATGTCTATCAGCGTGCTC
RT_4432-33_for	*STM4432-33*		TGCTTGAAGAACATATCGG
RT_4432-33_rev	*STM4432-33*		CGAATAAAGCCAATCGCTC

RT_4433_for	*STM4433*		GAATGAGGATGAGCAGGC
RT_4433_rev	*STM4433*		GCTGGGATCAATCAGCG
RT_4435_for	*STM4435*		AGCGGCAGCGTTACTGATG
RT_4435_rev	*STM4435*		ATGATGGAAGGTATCCAGC
RT_4435-36_for	*STM4435-36*		CCCAGTTGGCTTCCTGG
RT_4435-36_rev	*STM4435-36*		ATCTCAATCATCCGCTTCC
RT_4436_for	*STM4436*		CCAAATGGTACGGCAGG
RT_4436_rev	*STM4436*		CTCGTTCCACGATCGGC
*cloning, testing and sequencing of promoter fusions pDEW201-luxCDABE***			
5'P4417_for	*STM4417*	*Eco*RI	GGAATTCACGAAAAGAGCCAGTTCG
3'P4417_rev	*STM4417*	*Bam*HI	CGGATCCGTTTAGACATGCATGATAC
5'P4421_for	*STM4421*	*Eco*RI	GGAATTCTTCCTCGTCACAACAG
3'P4421_rev	*STM4421*	*Bam*HI	CGGATCCCGACTGTTTCCATCTC
5'P4424_for	*STM4424*	*Eco*RI	GGAATTCTCAATATCGCAAGGACTATC
3'P4424_rev	*STM4424*	*Bam*HI	CGGATCCTGGCTCCCACTTAATGAAAC
5'P4425_for	*STM4425*	*Eco*RI	GGAATTCCATGCCGCTACTGAGTAAAC
3'P4425_rev	*STM4425*	*Kpn*I	CGGTACCTTAAAGTCATTTTCTGTTTCC
5'P4430_for	*STM4430*	*Eco*RI	GGAATTCTTCATTTATGGGAAGG
3'P4430_rev	*STM4430*	*Bam*HI	CGGATCCATTCATTGACATATCC
5'P4431_for	*STM4431*	*Eco*RI	GGAATTCCAAGATCACAGAAATGGC
3'P4431_rev	*STM4431*	*Bam*HI	CGGATCCTTTTCATGTACCCCACC
5'P4433_for	*STM4433*	*Eco*RI	GGAATTCAAGTACCTGAGCTGGTGG
3'P4433_rev	*STM4433*	*Bam*HI	CGGATCCGCTTTTTCATTCGTACCTCT
5'P4435_for	*STM4435*	*Eco*RI	GGAATTCATTTCGTTGGGCCAGCG
3'P4435_rev	*STM4435*	*Bam*HI	CGGATCCCGATATTCATTATTTTCTCC
5'P4436_for	*STM4436*	*Eco*RI	GGAATTCCTGGTGCATTTGTCCGGT
3'P4436_rev	*STM4436*	*Bam*HI	CGGATCCCAATTTTCATTGTAAATACCAC
5'PargS_for	*argS*	*Eco*RI	CCGGAATTCCCATATCAGGACGCTC
3'PargS_rev	*argS*	*Kpn*I	CGGGGTACCTCACCGGAATACCTTAC
5'Pdef_for	*def*	*Eco*RI	CCGGAATTCTCGGTACGGGCCATC
3'Pdef_rev	*def*	*Kpn*I	CGGGGTACCGAAGGCGCTCGTCCG
5'Pnonsense_for	*STM0047*	*Eco*RI	CCGGAATTCGGATACGGTAGGGCTG
3'Pnonsense_rev	*STM0047*	*Kpn*I	CGGGGTACCCCAGTACTATCAGCGC
luxC_rev****	*luxC*		AATCACGAATGTATGTCC
*construction of control fragment in GMS assay***			
GMS_argS_for	*argS*		CAACCTTTGATTTGATTGG
GMS_argS_rev	*argS*		AAGAGCCTGAATATTCAC

* template DNA: pKD4
** template DNA: chromosomal DNA of *S*. Typhimurium
*** template DNA: pDEW201

Myo-inositol transport by *Salmonella enterica* serovar Typhimurium

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Abbreviations: MI, *myo*-inositol; MFS, major facilitator superfamily; RLU, relative light units
A supplementary table listing the primers used is provided.

ABSTRACT

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 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 of P_{iolT2} in the presence of MI but not of glucose. Deletion of *iolR* encoding the negative regulator of most genes involved in MI degradation resulted in upregulation of P_{iolT1} and of 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-^{3}H(N)]inositol, a saturable uptake activity of IolT1 with a K_m value between 0.49 mM and 0.79 mM was determined in DH5α 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.

INTRODUCTION

myo-inositol (MI) is a polyol abundant in soil. Its phosphorylated form, inositol hexakisphosphate or phytate, serves as a phosphorus storage in plants, but can be utilized by livestock only in the presence of phytases. The capability of an increasing number of microorganisms 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 CO_2.

Although inositol utilization has been investigated extensively on the enzymatic level for *Klebsiella* (*Aerobacter*) *aerogenes* decades ago (2), the underlying genetics and regulatory mechanisms have been elucidated in most detail for *Bacillus subtilis* (20, 29, 32). In this organism, the *iol* divergon responsible for MI utilization comprises *iolT* and the operons *iolABCDEFGHIJ* 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 (30, 33). 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 was revealed to inhibit the transcription of *iolT* (31). MI degradation has also been studied in *Corynebacterium glutamicum* (14), *Clostridium perfringens* (11), and *Lactobacillus casei* (28).

So far, several genera comprising Gram-negative bacteria have been demonstrated to utilize the polyol MI. These include species from the genera *Klebsiella*, *Caulobacter*, *Rhizobium*, *Sinorhizobium*, *Pseudomonas*, *Yersinia*, *Salmonella*, and *Serratia* (4, 5, 8, 9, 15, 17, 21, 22). A comparison of the respective gene clusters revealed a high variability of their chromosomal organization. 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, but absent in serovars Typhi, Parathypi 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, but we identified a nearly identical gene cluster lacking homologues of *iolI2* and *iolH* in the genome of *E. coli* ED1a (NC_011745.1) by a homology search. In this strain, the island is flanked by two transposase-encoding genes, strongly suggesting its distribution among Gram-negative bacteria is due to horizontal gene transfer. The genomic island includes five transcriptional units encoding enzymes involved in MI utilization, which 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 (15). Four genes, STM4418, STM4419, STM4428, and STM4434, encode putative, 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, was monitored using the luciferase reporter system and bandshift assays. Uptake of *myo*-[1,2-^{3}H(N)]inositol was investigated in *Salmonella 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, in both organisms. This study describes for the first time a 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/l tryptone, 5 g/l yeast extract, 5 g/l NaCl) or in minimal medium (MM; M9 medium supplemented with 2 mM $MgSO_4$, 0.1 mM $CaCl_2$ and 55.5 mM (1%) MI, glucose, fructose, arabinose, xylose, glycerol or sorbitol, respectively). For plasmid maintenance, 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 (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 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 vigorous shaking in 250 ml flasks with 50 ml of MM. The OD_{600} was measured in timed intervals as indicated.

Standard procedures. DNA manipulations and isolation of chromosomal or plasmid DNA were performed according to standard protocols (23), 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 (12). Polymerase chain reactions (PCRs) were carried out with Taq polymerase (Fermentas, St. Leon-Rot, Germany). As template for PCR, chromosomal DNA, plasmid DNA, or cells from a single colony was used. Oligonucleotides synthesized for PCRs are listed in Table S1. *S. enterica* serovar Typhimurium gene numbers refer to the LT2 annotation (NC 003197). The homepages http://globin.cse.psu.edu/enterix 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 (26).

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 (7). Briefly, PCR products comprising the kanamycin resistance cassette of plasmid pKD4 including the flanking FRT sites were

generated using pairs of 70 nucleotides-long primers that included 20 nucleotides 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 (18). 500-1000 ng of fragment DNA 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 Table S1. PCR products were digested with *Eco*RI and *Sal*I (Fermentas) and ligated (T4 DNA ligase; Gibco, Hudson, USA) into vector pBR322 to generate pBR322-*iolT1* and pBR322-*iolT2*, respectively. Their construction was verified by PCR, restriction analysis and sequencing. To detect proteins by Western Blot analysis, both genes were amplified with primers introducing a C-terminal His_6-tag and cloned as described, resulting in pBR322-*iolT1*-His_6 and pBR322-*iolT2*-His_6.

Cloning of promoter fusions. Putative promoter regions spanning approximately 300 base pairs 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 Table S1. The fragments were then cloned *via Eco*RI and *Bam*HI (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-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 described recently (15). 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 optical density at 600 nm 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).

Gel mobility shift assays. IolR-His$_6$ was overexpressed and purified as described recently (15). Briefly, expression of IolR was induced by adding 0.1 mM isopropyl-β-D-1-thiogalactopyranoside (IPTG) when *E. coli* BL21 cells transformed with pET28b-*iolR* had reached an OD_{600} of 0.4. After incubation for 4 h, the cells were harvested and the pellet was resuspended in 1 ml of buffer A (300 mM NaCl, 50 mM Na_3PO_4). The cells were lysed using a French Press (SLM Aminco Instruments, Rochester, USA), and cell debris was removed by centrifugation at 4°C (20 min, 1.6×10^4 g). After adding 10 µl of protease inhibitor phenylmethanesulphonylfluoride (PMSF; 100 mM), IolR-His$_6$ was purified using TALON metal affinity resin (Clontech Laboratories, Mountain View, USA). 1 ml of the protein extract was mixed with 1 ml of the 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-His$_6$ 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) (24). The protein concentration was determined using RotiQuant solution (Carl Roth GmbH, Karlsruhe, Germany) based on the method of Bradford (6), and the purity of eluted fractions was analysed by separation on a 15% SDS polyacrylamide gel.

For gel mobility shift (GMS) assays, putative promoter regions of *iolT1*, *iolT2*, *iolR*, *iolH*, and *argS* as competitor DNA, were amplified with oligonucleotides from Table S1, and 100 ng of DNA was mixed with increasing amounts of purified IolR-His$_6$ 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 in ethidium bromide solution and visualized by UV irradiation.

Membrane isolation and Western Blot analysis. DH5α cells transformed with pBR322-*iolT1*-His$_6$ and pBR322-*iolT2*-His$_6$ were grown to stationary phase. Isolation of *E. coli* membranes was then performed as described previously (13) but using a French Press (SLM Aminco Instruments, Rochester, NY) to lyse the cells. Protein concentrations were measured using RotiQuant solution. Fifteen µg of membrane protein were separated on a 12.5 % SDS polyacrylamide gel, and Western Blot analysis of the His$_6$-tagged proteins IolT1 and IolT2 was performed according to standard procedures with 1:1,000 diluted monoclonal anti-His$_6$

antibodies (dianova, Hamburg, Germany) and 1:15,000 diluted alkaline phosphatase-conjugated anti-mouse antibodies (dianova). Phosphatase activity was detected with nitroblue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCiP) as substrates.

Uptake of *myo*-[1,2-^{3}H(N)]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) to an OD_{600} of 1.0. Then, 20 ml were harvested by centrifugation (4°C and 1 x 10^4 g) and resuspended in McIlvaine's buffer (0.1 M Na_2HPO_4, 0.05 M citric acid mixed to obtain the desired pH (19)) to an OD_{600} of 4.0 and stored on ice. 250 µl of the cell suspension were mixed with 230 µl of McIlvaine's buffer and 12.5 µl of 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 minutes 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-^{3}H(N)]inositol (specific activity: 30 Ci/mmol; Biotrend, Cologne, Germany). In experiments to determine the pH optimum of MI uptake, this mix contained 1 mM of unlabelled and 26.67 nM of tritiated substrate. The K_m value was determined at pH 5.5 with a mix containing the same amount of labeled substrate but decreasing amounts of unlabelled substrate (1 mM, 0.5 mM, 0.2 mM, and 0.1 mM). Aliquots of 60 µl were removed, rapidly filtered through a Pall GN6 0.45-µm nitrocellulose filter, washed once with 5 ml of 150 mM NaCl and transferred to a scintillation vial. After addition of 3 ml scintillation cocktail (Carl Roth GmbH), 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 at several time points. Uptake experiments with *S. enterica* serovar Typhimurium were performed in a similar manner, but cells were cultivated in MM with 55.5 mM MI.

TABLE 1. Strains and plasmids used in this study.

Bacterial strains	**Description and relevant features**	**Source or literature**
DH5α	*E. coli: deoR, endA1, gyrA96, hsdR17*(r_k-m_{k+}), *recA1, relA1*, s*upE44*, *λthi-1*, Δ(*lacZYA-argFV169*)	(10)
BL21 (DE3)	*E. coli: F*$^-$*, omp*T, *hsd*S_B ($r_B^-m_B^-$), *gal, dcm, rne*131	(25)
14028	Spontaneous streptomycin-resistant mutant of *S. enterica* serovar Typhimurium wild-type strain ATCC14028	(15)
14028 Δ*iolT1*	In-frame *iolT1* (STM4418) deletion mutant	This study
14028 Δ*iolT2*	In-frame *iolT2* (STM4419) deletion mutant	This study
14028 Δ*iolT1*Δ*iolT2*	In-frame deletion mutant of *iolT1* and *iolT2* (STM4418, STM4419)	This study
14028 ΔSTM4428	In-frame STM4428 deletion mutant	This study
14028 ΔSTM4434	In-frame STM4434 deletion mutant	This study
Plasmids		
pKD4	*pir*-dependent, FRT sites; Kan^r	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	(15)
pBR322	Amp^r Tet^r	Fermentas
pBR322-*iolT1*, pBR322-*iolT2*	*iolT1* or *iolT2* cloned into pBR322 with putative promoter region	This study
pBR322-*iolT1*-His_6, pBR322-*iolT1*-His_6	As above, but encoding transporters with C-terminal His_6-tag	This study
pDEW201	Promoter probe vector; Amp^r *luxCDABE*	(27)
pDEW201-P_{iolT1}	pDEW201 with 300 bp upstream of *iolT1* (STM4418)	This study
pDEW201-P_{iolT2}	pDEW201 with 300 bp upstream of *iolT2* (STM4419)	This study
pDEW201-P_{argS}	pDEW201 with 244 bp upstream of *argS*	(15)
pDEW201-`STM0047´	pDEW201 with intragenic 350 bp of STM0047 without promotor homology	(15)

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 $\mu = 0.56/h$) than in MI ($\mu = 0.37/h$). In contrast to the mutants 14028 ΔSTM4428 and 14028 ΔSTM4434, the deletion of STM4418 or STM4419 resulted in growth phenotypes; these genes were therefore named *iolT1* (STM4418) and *iolT2* (STM4419). The deletion of *iolT1* abolished the growth in liquid minimal medium containing MI for up to 74 h (Fig. 1a), and the mutant strain 14028 Δ*iolT1* showed only very weak growth on solid medium after 128 h (Fig. 1b). The combined deletion of *iolT1* and *iolT2* completely abolished the 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 Δ*iolT1* and 14028 Δ*iolT1*Δ*iolT2*, resulting in wildtype-like growth of both mutants (Fig. 1b).

An only slightly reduced growth of 14028 Δ*iolT2* (STM4419) 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 Δ*iolT1*Δ*iolT2* to that of strain 14028, and a retarded growth in comparison to 14028 Δ*iolT1*Δ*iolT2*/pBR322-*iolT1* was observed (Fig. 1b). 14028 Δ*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 yet unknown reasons.

We also tested the growth of mutants 14028 Δ*iolT1* and 14028 Δ*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 have the bigger contribution. In contrast, STM4428 and STM4434 do not contribute to growth on MI at least under the conditions applied here.

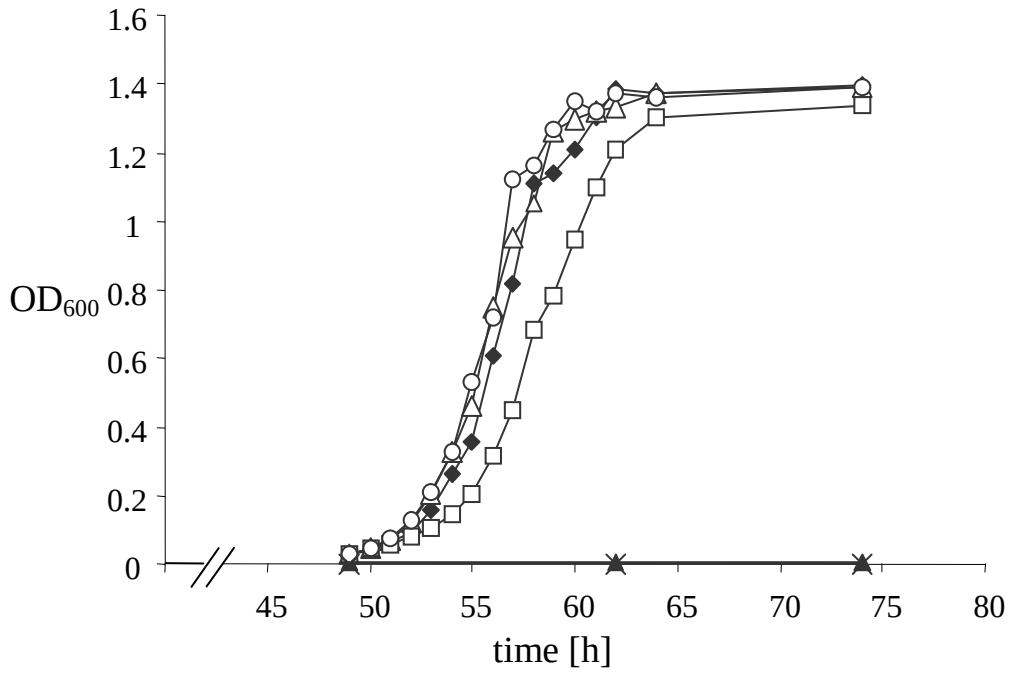


FIG. 1. (A) Growth of *Salmonella enterica* serovar Typhimurium strains in the presence of 55.5 mM MI. Growth curves of strains in liquid MM supplemented with MI. —◆— 14028 (growth rate μ = 0.37/h), —□—14028 Δ*iolT2* (μ = 0.28/h), —△— 14028 ΔSTM4428 (μ = 0.38/h), —○— 14028 ΔSTM4434 (μ = 0.42/h). Zero growth phenotype: —▲— 14028 Δ*iolT1*, —✕— 14028 ΔiolT1Δ*iolT2*. The average data of two independent experiments are shown.

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 that 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 by the control construct with the intragenic fragment `STM0047´ ranged from 2.19×10^4 to 2.41×10^4 RLU/OD_{600} 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/OD_{600}), while the predicted *iolT1* promoter showed a maximal bioluminescence of 8.52×10^5 RLU/OD_{600}. When glucose was exchanged 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.

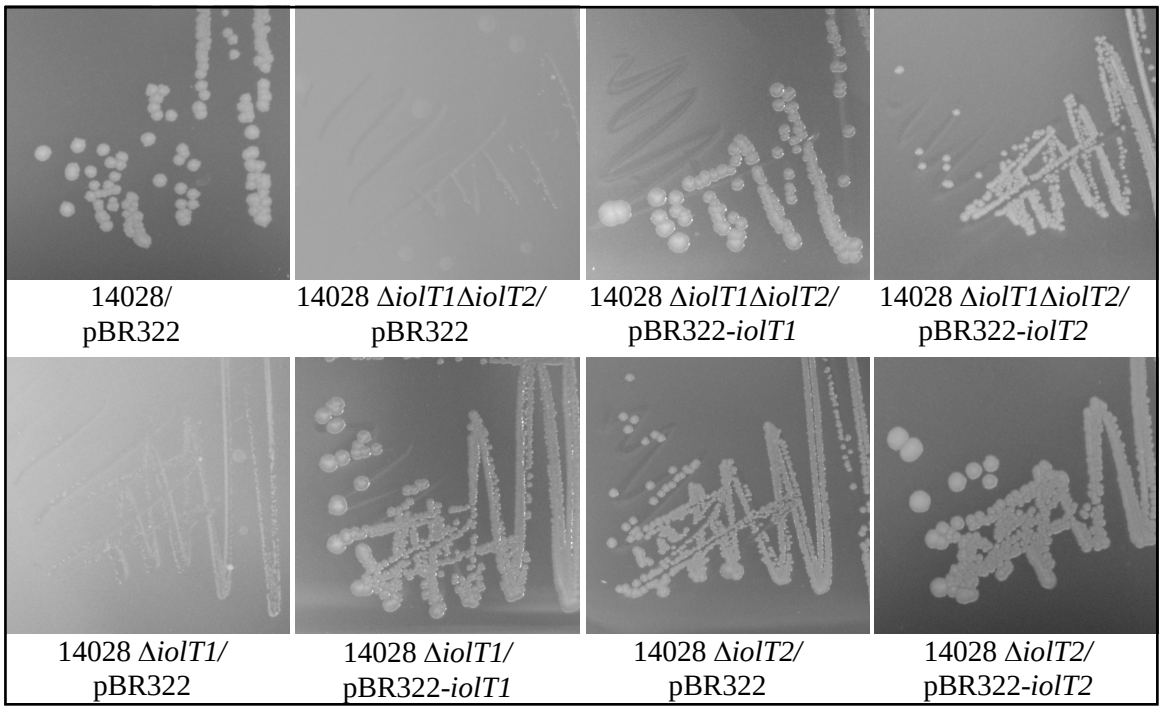


FIG. 1. (B) Growth of *Salmonella enterica* serovar Typhimurium strains in the presence of 55.5 mM MI. Strains were streaked on MM agar plates containing 55.5 mM MI and grown at 37°C for 5 days. Above: Strain 14028, and the double mutant 14028Δ*iolT1*Δ*iolT2* with plasmid pBR322 and the complementing constructs pBR322-*iolT1* and pBR322-*iolT2*. Below: Deletion mutants lacking either *iolT1* or *iolT2*. IolT1 revealed to have a bigger impact on growth with MI, but a significant growth defect was also observed for 14028 Δ*iolT2*. Each mutant was complemented by providing the respective gene *in trans*. The scale of all photographs is identical.

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 Δ*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 a factor 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.

TABLE 2. Quantification of promoter activities of the MI transport genes *iolT1* and *iolT2*[a]

Fragment cloned into pDEW201	14028 grown in MM + glucose		14028 grown in MM + MI			14028 Δ*iolR* grown in MM + glucose		
	[RLU/OD_{600}]	SD [%]	[RLU/OD_{600}]	SD [%]	Fold induction	[RLU/OD_{600}]	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

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

[b]Data are the average RLU derived from three independent experiments.

[c]Fold induction was calculated with respect to the RLU/OD_{600} values of strain 14028 grown in glucose.

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 of P_{iolT2} as already shown for the promotors of *iolA*, *iolC1*, *iolD1* and *iolR* (15), its binding on the *iolT1* and *iolT2* promoter regions was tested by gel mobility shift 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 without or with 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 (15). 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.* 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 a molecular weight of 53.4 kDa and 52.1 kDa, respectively, appear to be typical members of the MFS (16) and are predicted to possess twelve 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* K12, it was chosen for heterologous expression of IolT1 and IolT2 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 the methods section. In a first experiment, DH5α cells expressing IolT1 or IolT2 were compared to cells containing an empty plasmid. The assays were performed with an initial extracellular MI concentration of 1 mM, which is higher than the *Km* value of the related MI transporters from *C. glutamicum* (14). IolT1 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 carbonyl cyanide *m*-chlorophenylhydrazone (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 has 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 tested against monoclonal anti-His_6 antibodies.

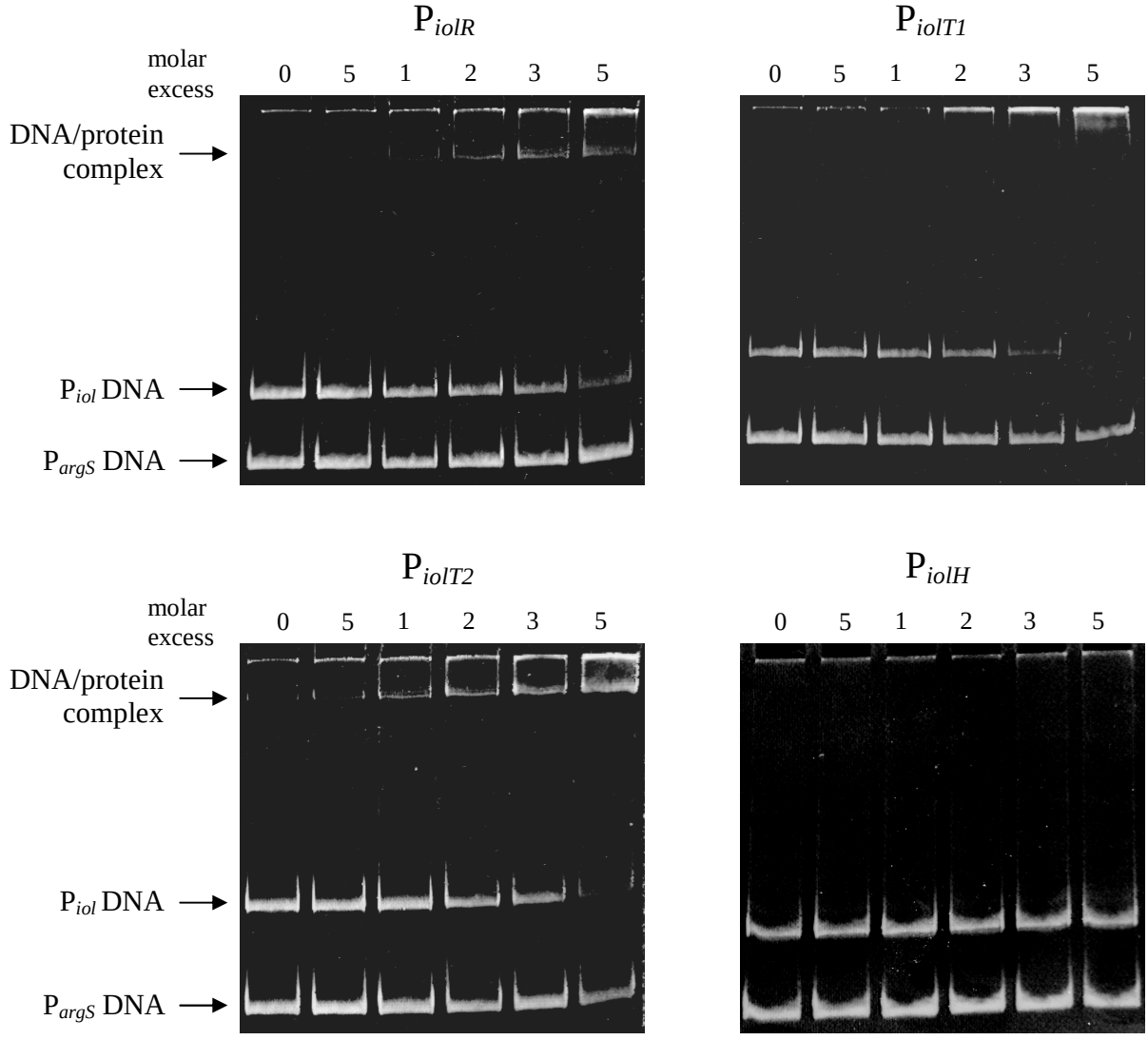


FIG. 2. GMS assays to study DNA binding activity of IolR. The interaction of IolR with the promoter region of four genes of GEI 4417/4436 is shown. 100 ng of promoter DNA were incubated without or with increasing amounts (81 ng, 162 ng, 324 ng, 678 ng and 810 ng) of purified IolR. Protein-DNA complexes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

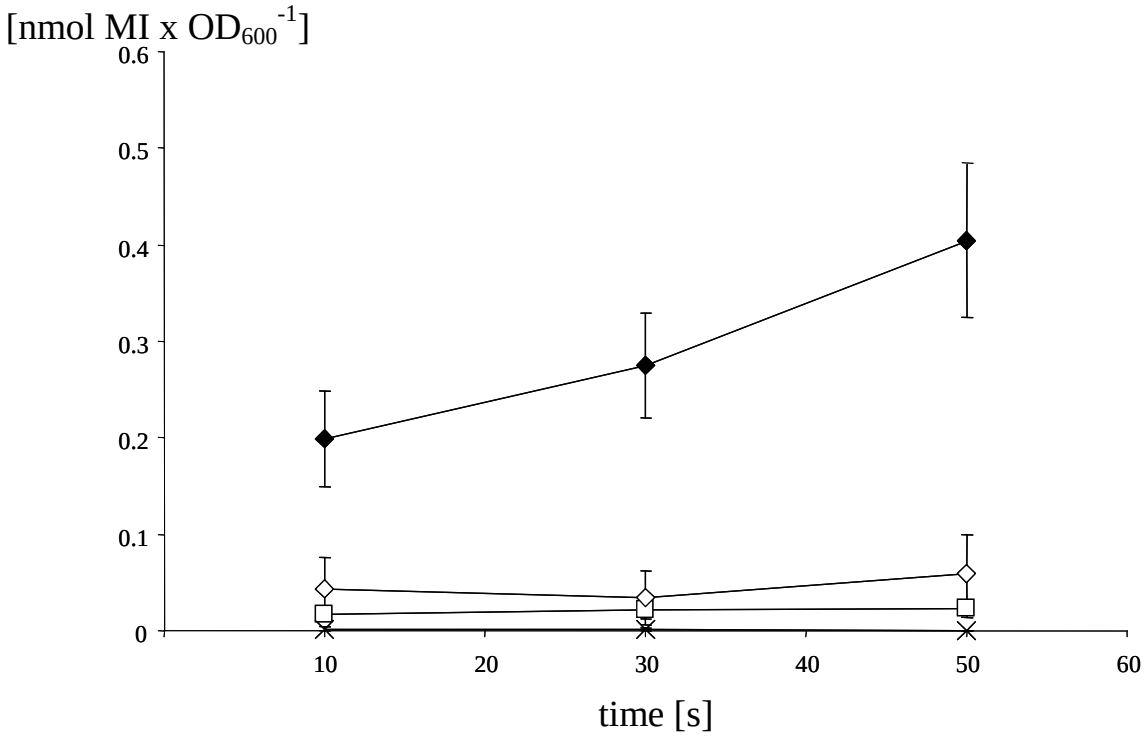
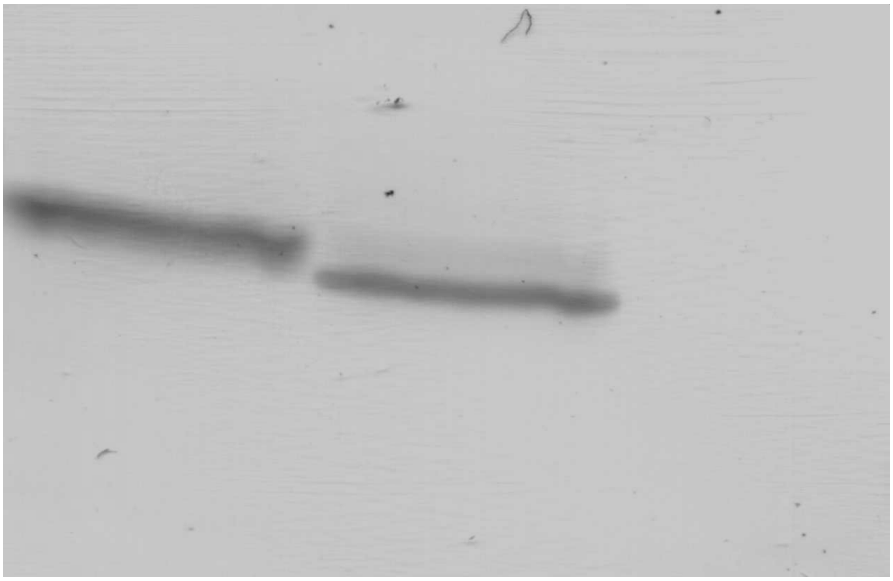


FIG. 3. (A) MI uptake by heterologously expressed IolT1 and IolT2. *E. coli* DH5α cells containing plasmids pBR322-*iolT1*(—◆—), pBR322-*iolT2* (—□—)or pBR322 (—×—; negative control) were grown in LB medium, harvested by centrifugation, resuspended in McIlvaine's buffer (pH 5.5) at an OD_{600} of 4.0 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-^{3}H(N)]inositol. Aliquots of the cell suspension were taken 10, 30 and 50 seconds after the addition of MI. In experiments with DH5α/pBR322-*iolT1* (—◇—), the protonophore CCCP was added 2 min minutes before starting the uptake experiment.

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 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 a MI/proton symporter.



DH5α/ pBR322-*iolT1*-His_6 | DH5α/ pBR322-*iolT2*-His_6 | DH5α/ pBR322

FIG. 3. (B) Membrane localisation of IolT1-His_6 and IolT2-His_6. For each sample, fifteen µg of membrane protein was subjected to SDS-PAGE and Western Blot analysis using monoclonal His_6 antibodies. The samples were obtained from *E. coli* DH5α cells containing pBR322-*iolT1*-His_6 (left), pBR322-*iolT2*-His_6 (middle) or pBR322 (right, negative control).

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 genetically 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 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, a K_m value of 0.71 mM, 0.51 and 0.38 mM was calculated, resulting in an average K_m value of 0.56 ± 0.2 mM for IolT1 expressed in *E. coli*. Taken together, IolT1 appears as the predominant MI transporter of *S. enterica* serovar Typhimurium with functional similarities to the two MI facilitators characterized in *C. glutamicum* (14).

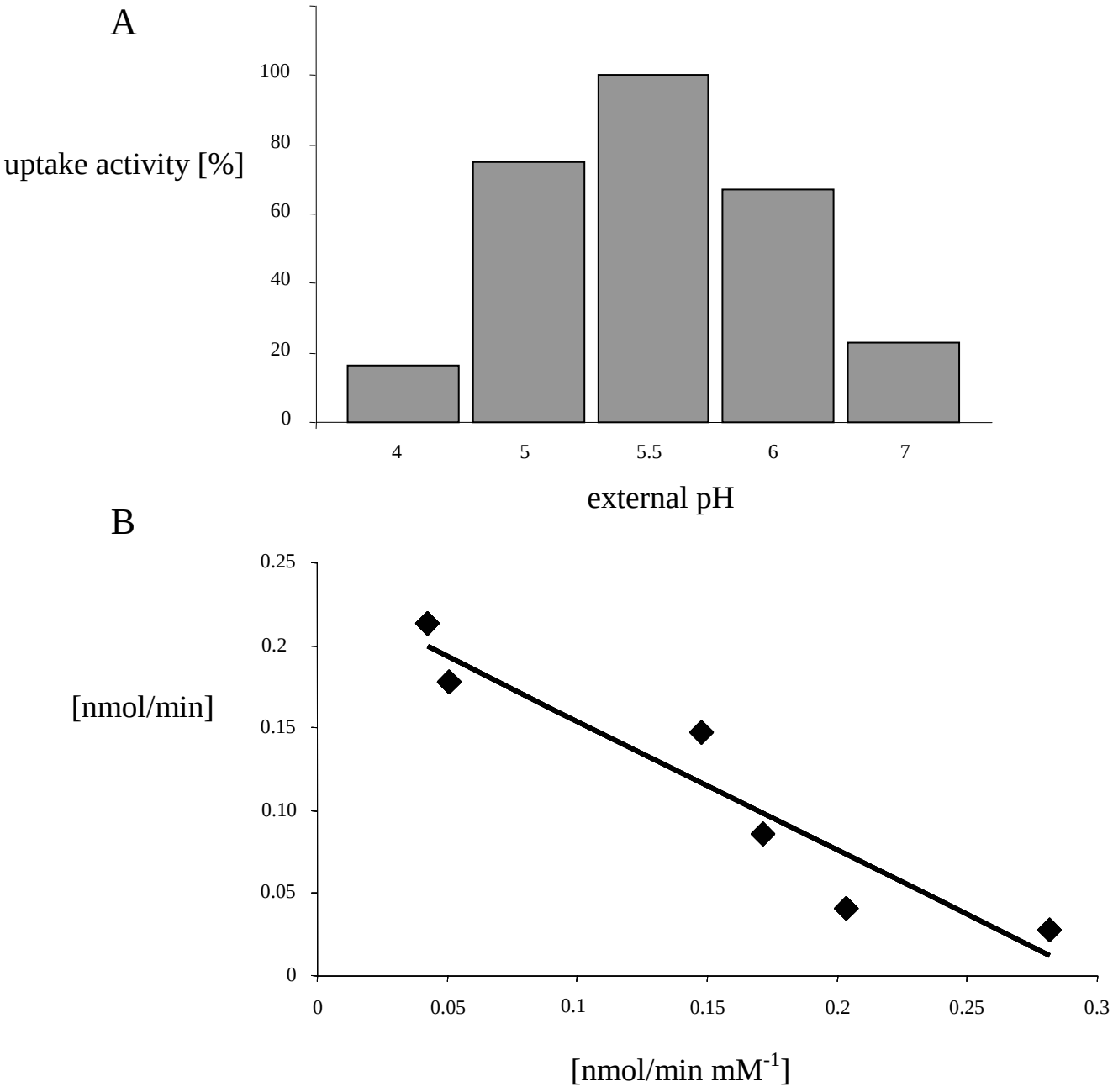


FIG. 4. pH optimum and saturation kinetics of IolT1-mediated uptake of MI. The uptake assays with were performed as described in the legend to Fig. 3.

(A) Determination of the pH optimum of MI uptake with DH5α/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 at pH 5.5.

(B) The graph shows an Eadie-Hofstee plot of an MI uptake experiment with the *S. enterica* serovar Typhimurium strain 14028. Substrate concentrations were 5 mM, 3.5 mM, 1 mM, 0.5 mM, 0.2 mM, and 0.1 mM. A K_m value of 0.79 mM was calculated from three measurements.

IolT1-like proteins involved in MI utilization. According to genome sequence data, a large number of bacteria carry genes with homology to *iol* genes, such as *Brucella abortus*, *Photobacter profundum*, *Yersinia* spp., *Citrobacter koseri* and *Erwinia carotovora*. Genetic comparison and computational analysis of *iol* divergons has been performed recently (5, 15). A phylogenetic analysis of *iol* genes from *L. casei* is also available (28). In the following, we investigated whether and to which degree of similarity IolT homologs 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 of either protein to IolT1 or IolT2. Homology searches using BLAST (1) 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 homologs of *E. coli* ED1a, *Y. frederiksenii*, *S. 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 homologs of several species unable to utilize MI, such as *E. coli*, *V. angustum*, *Photobacterium* sp. or *Pedibacillus* sp., are also grouped, indicating that these proteins transport other sugars or sugar alcohols. Interestingly, the *B. cereus* IolT1 homolog of this group is not identical to the major MI transporter of *B. subtilis* (31), but is more closely related to homologs from Gram-negative species. On the other hand, IolT1 homologs of *Y. intermedia*, *Y. enterocolitica* and *K. 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 homologs of species such as *Y. pseudotuberculosis*, *Y. pestis*, *E. carotovora* or *P. luminescens.* These homologs are located outside the *iol* divergons which carry genes encoding permeases, suggesting yet unknown MI transporters in these bacteria. Consistent with this interpretation, Boutte *et al.* identified an ABC transporter for MI in *S. meliloti* and five other α-proteobacteria, thus defining a novel class of MI facilitators (5).

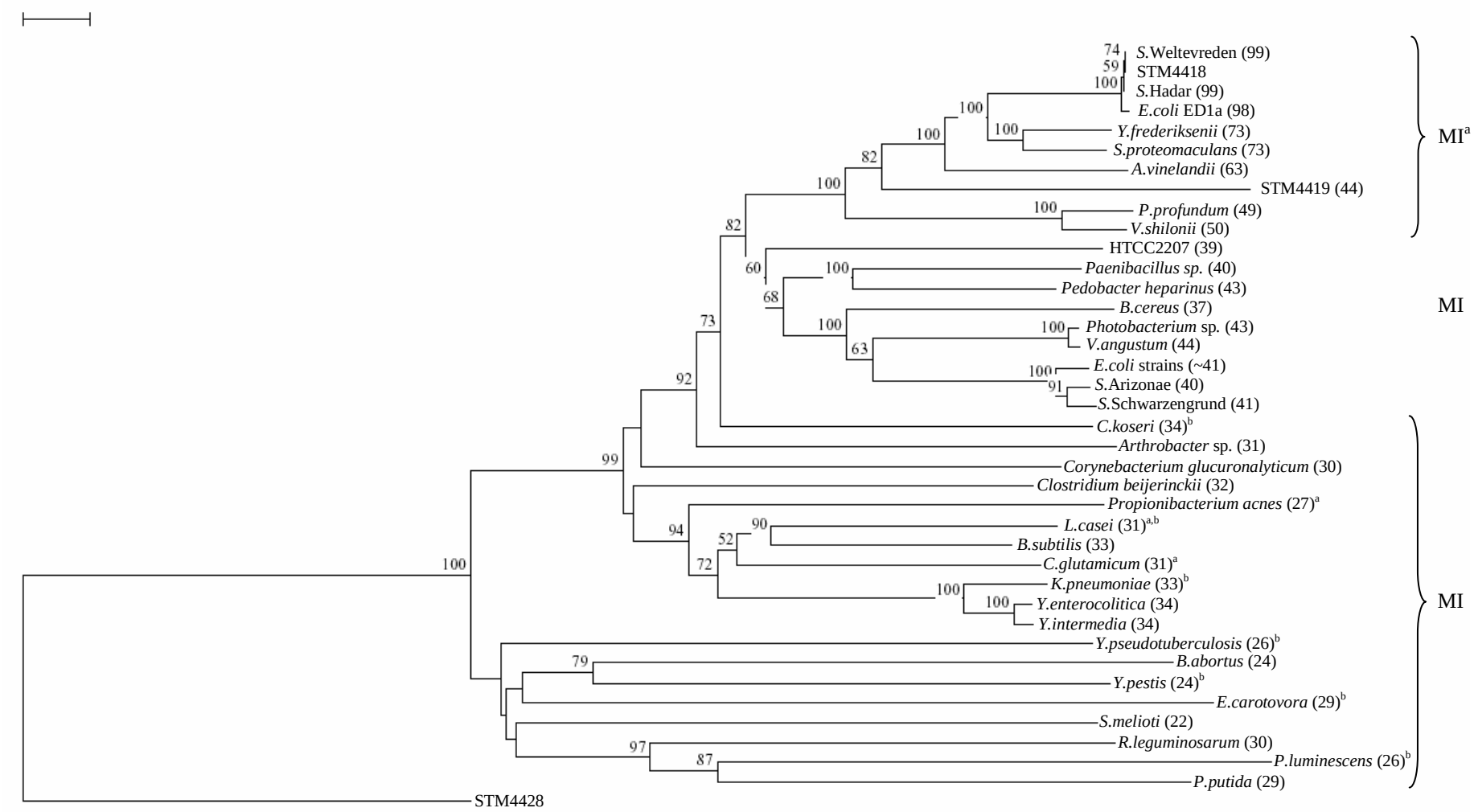


FIG. 5. Cladogram based on 37 proteins with homology to IolT1 of *S. enterica* serovar Typhimurium. Identities in percent are indicated in brackets. The putative permease STM4428 served as outgroup. The phylogenetic analysis was performed with the neighbour-joining method and calculated using the Poisson correction. Values on each branch indicate the occurrence (%) of the branching order in 500 bootstrapped trees. Bar represents 10% sequence divergence. HTCC2207 is a marine γ proteobacterium. MI, capable to utilize MI or presence of *iol* genes in the genome. IolT1 and IolT2 are depicted in bold letters. [a]the IolT1 homolog is clustered with the genes of the *iol* divergon; [b]other putative permeases are present within the *iol* gene cluster.

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, an only 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* (14, 31). In *B. subtilis*, the minor transporter IolF was shown to have a lower substrate affinity than IolT, and IolF could 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 comparable kinetic properties and a sequence identity of 55%. Overexpression of *iolT1* and *iolT2* of the same organism led to a twofold increase of the D-fructose uptake rate, but both transporters showed a lower specificity towards this sugar than to MI (3). However, an apparent growth defect of *iolT* mutants of neither *B. subtilis* nor *S. enterica* serovar Typhimurium in the presence of a variety of sugars was observed (31). A major difference between MI utilization by Gram-positive bacteria and *S. enterica* serovar Typhimurium is the extended lag phase of this Gram-negative pathogen in medium containing MI as sole carbon source and energy source (Fig. 1a). The molecular mechanism underlying this growth retardation by at least two days is not yet completely understood. The lag phase has been shown to be shortened by approximately ten hours in a *iolR* deletion mutant, indicating a key role of IolR in this phenomenon (15). 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 as a repressor of *iolT1* and *iolT2* transcription (Table 2). The absolute RLU/OD 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 MI transport activity of IolT2 was not detectable. Because IolT2 appears with a significant lower molecular weight than IolT1, it might be assumed that IolT2 is misfolded in the *E. coli* membrane or degradated 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. In contrast to *B. subtilis* and *C. glutamicum*, the putative minor transporter IolT2 supports growth of *S. enterica* serovar Typhimurium on solid medium only, and with a higher generation time in comparison to strain 14028. 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 (22). The K_m values of 0.49 mM and 0.79 mM reported here for IolT1 of *S. enterica* serovar Typhimurium is comparable to that 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 (31). 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 organisation and also functionality, while several aspects of MI utilization still remain to be discovered. Open questions regarding the MI metabolism that are currently addressed in *S. enterica* sv. 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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TABLE S1. Primers used in this study.

primer name	target gene	modification	5’ – 3’ sequence
*construction of non-polar deletion mutants**			
del_4418_for	STM4418 (*iolT1*)		TAGATGAAGTTTATTCTGGAGATAATCTACCTATGTCCACATCAGATAGTGTGTAGGCTGGAGCTGCTTC
del_4418_rev			CATTGTGGTGAAACTTAATCAGAATAACGTTCGGTTTGAATTGGCTGCAGCATATGAATATCCTCCTTA
del_4419_for	STM4419 (*iolT2*)		GTGTTGTTTCCTCAACAGGCAAGGGAGGGATTATGTCTCAGAGAAGTAAGGTGTAGGCTGGAGCTGCTTC
del_4419_rev			ATATTGGAGAGGTATCAGGCTATTACATCGCGACGTTTCCCCTGAATTTCCATATGAATATCCTCCTTA
del_4418-19_for	STM4418-19 *(iolT1-2)*		GTATTTAGCGATCATCGGCATTGTGGTGAAACTTAATCAGAATAACGTTCGTGTAGGCTGGAGCTGCTTC
del_4418-19_rev			ATATTGGAGAGGTATCAGGCTATTACATCGCGACGTTTCCCCTGAATTTCCATATGAATATCCTCCTTA
del_4428_for	STM4428		ATCGGACAAGGGGCGTGTTCAAGGACGATTCCATGCCAGCTCTCACACAAGTGTAGGCTGGAGCTGCTTC
del_4428_rev			CCCTTCTTAACAGCTTAATGTTGCCGCAGCAAGAGATGGACAACCGCCGCCATATGAATATCCTCCTTA
del_4434_for	STM4434		TGTGAGAGCTTTTCTATACCGAGGATAATAAAATGAAAGAAATGCAGGCAGTGTAGGCTGGAGCTGCTTC
del_4434_rev			AAGAATATTTGAAGCTATTTCTCGCCAGGCACTTTAAGAAACTGCACCAGCATATGAATATCCTCCTTA
*test of insertion of kanR and gene deletion***			
test_4418	*iolT1*		TCACTGGCGCTGGCGACG
test_4419	*iolT2*		GCGACAGTACCTGAACTC
test_4418-19	*iolT1-2*		TTCCGTTCTCAGGTCGCG
test_4428	STM4428		CCTGCCATGCTGGCTC
test_4434	STM4434		GATGAAGTCAGCGAAGTC
kanR3	*kanR*		GCGCTGCGAATCGGG
*construction and sequencing of pBR322-complementation vectors and his_6-tag fusions***			
4418_EcoRI	*iolT1*	*Eco*RI	GGAATTCTTGAGTTCAGGTACTGTCG
4418_SalI	*iolT1*	*Sal*I	GGTCGACCGTATTTAGCGATCATCGG
4419_EcoRI	*iolT2*	*Eco*RI	GGAATTCTCTCGTTTCACAACCTATG
4419_SalI	*iolT2*	*Sal*I	GGTCGACGTTGTGTAACCAGCGGAG
4418_SalI_his_6	*iolT1*	*Sal*I	GGTCGACTCAGTGGTGGTGGTGGTGGTGATCAGAATAACGTTCGGTTTG
4419_SalI_his_6	*iolT2*	*Sal*I	GGTCGACTCAGTGGTGGTGGTGGTGGTGGGCTATTACATCGCGACG
seq_pBR322_for****			TGCCACCTGACGTCTAAG
seq_4418_for1	*iolT1*		GTCCACATCAGATAGTTG
seq_4418_for2	*iolT1*		GCATCGATTGCAGCGG
seq_4418_for3	*iolT1*		GCACTATCGGCAGCATC
seq_4418_for4	*iolT1*		GCGCATTTCCAATGTGG
seq_4419_for1	*iolT2*		TCTTTCCTGGAACATCTC
seq_4419_for2	*iolT2*		CGCTTCTGATCATTGCG
seq_4419_for3	*iolT2*		GGGCTGGAAGACTATGC
seq_4419_for4	*iolT2*		GCTATGGCCGTATACCG
seq_4419_for5	*iolT2*		TGCTATGTCTTTATTTCTCG

*cloning, testing and sequencing of promoter fusions pDEW201-luxCDABE***			
5'P4418_for	*iolT1*	*Eco*RI	CGAATTCATCAATAAAATCAAGTAACTTC
3'P4418_rev	*iolT1*	*Bam*HI	CGGATCCATGTGGACATAGGTAGATTA
5'P4419_for	*iolT2*	*Eco*RI	CGAATTCAACTTATGTTTTGTTATGGGTATC
3'P4419_rev	*iolT2*	*Bam*HI	CGGATCCTCTGAGACATAATCCCTCCC
5'PargS_for	*argS*	*EcoRI*	CCGGAATTCCCATATCAGGACGCTC
3'PargS_rev	*argS*	*KpnI*	CGGGGTACCTCACCGGAATACCTTAC
5'Pnonsense_for	*STM0047*	*EcoRI*	CCGGAATTCGGATACGGTAGGGCTG
3'Pnonsense_rev	*STM0047*	*KpnI*	CGGGGTACCCCAGTACTATCAGCGC
luxC_rev****	*luxC*		AATCACGAATGTATGTCC
*generation of fragments used in GMS assays***			
GMS_argS_for	*argS*		CAACCTTTGATTTGATTGG
GMS_argS_rev	*argS*		AAGAGCCTGAATATTCAC
bs_4418_for	*iolT1*		AGGTAGATTATCTCCAG
bs_4418_rev	*iolT1*		AACGATAAAAAACGCCAG
bs_4419_for	*iolT2*		AAGATGCTGCGTTTTACG
bs_4419_rev	*iolT2*		AATCCCTCCCTTGCCTG
*construction of pET28b-iolR overexpression vector***			
pu_4417_for_NcoI	STM4417		AAACCATGGATGTCTAAACATCAAACTCAAC
pu_4417_rev_XhoI	STM4417		AAACTCGAGCTCCGTCGCCAGCGCC

* template DNA: pKD4
** template DNA: chromosomal DNA of *S*. Typhimurium
*** template DNA: pDEW201
**** template DNA: pBR322

Bicarbonate-dependent bistability in *myo*-inositol utilization by *Salmonella enterica* serovar Typhimurium

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Running title: Bistability in *myo*-inositol degradation by *Salmonella typhimurium*

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ABSTRACT

The capability of *Salmonella enterica* serovar Typhimurium to utilize *myo*-inositol (MI) is determined by the genomic island GEI4417/4436 carrying the *iol* genes encoding enzymes, transporters and a regulator responsible for this catabolic pathway. In contrast to all gram-negative and gram-positive bacteria investigated so far, *S. enterica* serovar Typhimurium strain 14028 growing on MI as sole carbon source is characterized by a remarkable long lag phase of 40-60 hours. Here, we report that on solid and in liquid medium, this human pathogen exhibits a bistable phenotype that is abolished when cells reach the logarithmic growth phase. This heterogeneity is reversible and therefore not caused by mutation. On the single cell level, fluorescence microscopy and flow cytometry analysis revealed a gradual switch of the *iolE* promoter from the "off" to the "on" state at the end of the lag phase. A bistable phenotype was not observed in the presence of at least 0.55% CO_2, nor in the absence of the *iol* gene repressor IolR. Early growth start of strain 14028 in medium with MI is induced by *iolR* deletion and addition of 0.1% $NaHCO_3$. Bicarbonate/CO_2, however, does not act on IolR, suggesting the involvement of a yet unknown regulatory protein. The positive feedback loop *via* repressor release and positive induction by bicarbonate/CO_2 allow strain 14028 to adapt to rapidly changing environments. This is a novel example of bistability in substrate degradation, and, to our knowledge, the first example of gene regulation by bicarbonate/CO_2 in *Salmonella*.

INTRODUCTION

Phenotypic variation is widespread among prokaryotes, and its underlying molecular mechanisms include genetic changes such as genomic inversion and strand-slippage mechanisms, epigenetic variations depending on DNA methylation, and feedback-based multistability characterized by at least two distinct phenotypes within an isogenic population (35). Per definition, bistability must arise stochastically at the cellular level (9). A common regulatory arrangement exhibiting bistability includes a positive feedback loop with a cooperative response to an activator (10). This was firstly described for the lactose utilization by *Escherichia coli* in which a population is splitted into cells highly or not all expressing the *lac* operon (11, 27). Further prominent examples are the bistable switch between lysogeny and lysis of bacteriophage lambda depending on the fragile balance between the regulators Cro and CI (4), and the *Bacillus subtilis* K-state (competence) system in which 10-20% of the cells in stationary phase highly express genes permitting transformation competence (24, 34). The survival of *Staphylococcus aureus* against antibiotic treatment requires that some cells called persisters enter a condition of reduced growth (5), and population heterogeneity is also observed during *B. subtilis* sporulation (13). Only recently, it could be demonstrated that biofilm formation by *S. enterica* serovar Typhimurium involves the bistable expression of CsgD, the major biofilm regulator (14). Even more intriguing, when this pathogen infects hosts, a stochastic switch takes place that results in a self-destructive fraction and one that benefits from the dying one (3).

myo-inositol (MI) is a polyol abundant in soil. The *iol* genes of *Salmonella enterica* serovar Typhimurium responsible for utilization of MI as carbon and energy source are located on a 22.6-kb genomic island (GEI4417/4436) which is absent in all but six *Salmonella* genomes sequenced so far (21). All genes on this island necessary for MI degradation are induced in the presence of this substrate. The catabolic pathway requires IolG and IolE converting MI to 3D-trihydroxycyclohexane-1,2-dione that is then hydrolysed by IolD. 5-deoxy-glucuronic acid is isomerized by IolB to 2-deoxy-5-keto-D-gluconic acid that is then phosphorylated by the kinase IolC and further degraded to dihydroxyacetone phosphate, acetyl coenzyme A, and CO_2. In rich medium, the negative regulator IolR represses all but one promoter of the *iol* divergon including its own (21). A protein belonging to the major facilitator superfamily (MFS), IolT1, has recently been identified as the predominant MI transporter of *S. enterica* serovar Typhimurium, and IolR was revealed to inhibit the transcription of *iolT1* (22).

The ability to degrade MI is known for the gram positive species *Bacillus subtilis* (26, 38, 40), *Corynebacterium glutamicum* (20), *Clostridium perfringens* (17), and *Lactobacillus casei BL23* (37), and for several gram-negative species such as *Serratia*, *Klebsiella*, and *Pseudomonas* (6, 12, 23, 29). Although a comparison of the respective gene clusters revealed a high variability of their chromosomal organization, the negative regulation of the *iol* genes by the IolR repressor is a common feature of all MI divergons investigated so far. An intermediate of MI degradation, 2-deoxy-5-keto-D-gluconic acid 6-phosphate (DKP), has been shown to antagonize IolR binding, thus inducing the expression of *iol* genes (39, 41).

A remarkable and unique property of *S. enterica* serovar Typhimurium is its long lag phase in the presence of MI. Here, we show for the first time that phenotypic variation is involved in this growth phenomenon, and that deletion of IolR and addition of bicarbonate induce rapid growth of the pathogen in MI. A model of the molecular mechanism underlying bistability in the presence of MI is proposed.

MATERIALS AND METHODS

Bacterial strains, plasmids and growth conditions. Bacterial strains and plasmids used in this study are listed in Table 1. *S. enterica* serovar Typhimurium cultures were grown at 37°C in Luria-Bertani (LB) broth (10 g/liter tryptone, 5 g/liter yeast extract, 5 g/liter NaCl) or in minimal medium (MM). MM is M9 medium supplemented with 2 mM $MgSO_4$, 0.1 mM $CaCl_2$, 55.5 mM (1%, wt/vol) MI, or 27.8 mM (0.5%, wt/vol) glucose. For plasmid maintenance, the media were supplemented with the antibiotics ampicillin (150 µg/ml) or kanamycin (50 µg/ml). For solid media, 1.5% agar (wt/vol) was added and the cultures were incubated at an atmospheric CO_2 concentration or with elevated CO_2 concentrations in a cell culture incubator. 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 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 without agitation in 250 ml bulb flasks with 50 ml of MM. The OD_{600} was measured in time intervals as indicated.

Standard procedures. DNA manipulations and isolation of chromosomal or plasmid DNA were performed according to standard protocols (31), 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 (18). Polymerase chain reactions (PCRs) were carried out with Taq polymerase (Fermentas, St. Leon-Rot, Germany). As template for PCR, chromosomal DNA, plasmid DNA, or cells from a single colony was used. *S. enterica* serovar Typhimurium gene numbers refer to the LT2 annotation (NC 003197).

Quantification of promoter activity. Bioluminescence measurements were performed in 96-well plates. For growth in MM containing 55.5 mM MI, bacterial cells were grown at 37°C 74 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 Victor3 1420 multilabel counter (Perkin-Elmer Life Sciences, Turku, Finland).

Cloning of the *iolE* promoter into the *gfp* reporter plasmid pPROBE-NT. The *iolE* promoter is located within the first 300 bp upstream of the start codon of the *iolE*/*iolG1*

operon (21). This region was amplified from chromosomal DNA of wild-type strain 14028 by PCR using listed in Table 1. The fragment was then cloned *via* HindIII and EcoRI (Fermentas) upstream of *gfp* into the multiple cloning site of pPROBE-NT. After transformation into 14028 cells, plasmids containing the correct transcriptional *gfp*-fusions were isolated and verified by PCR, restriction analysis and sequencing. pNT-P_{iolE} was then transformed into strain 14028 and the Δ*iolR* mutant.

Fluorescence microscopy and flow cytometry. Fluorescence microscopy was performed using an Olympus BX51 microscope equipped with a F-view camera. Flow cytometry was carried out on a Becton Dickinson LSR II cytometer at 488 nm excitation wavelength. Light emission was measured between 515 and 545 nm. The population of bacteria was gated in forward and side scatter, which were both amplified logarithmically. For all assays, an overnight culture of strain 14028 carrying pNT-P_{iolE} was washed twice in PBS and inoculated in 50 ml MM containing 55.5 mM MI and kanamycin (50 µg/ml) in 250 ml bulbed flasks at OD_{600} = 0.08. The cells were harvested at different time points and concentrated by centrifugation (1 min at 2000 x g). For fluorescence microscopy, 5 µl of a concentrated cell suspension was applied to a glass slide. In flow cytometry experiments, about 10,000 events were measured and the collected data were analysed using winMDI (v2.9).

RESULTS

***S. enterica* serovar Typhimurium shows a bistable phenotype in the presence of MI.** To investigate the genetics of MI utilization by *S. enterica* serovar Typhimurium, strain 14028 was streaked on MM agar plates with 55.5 mM MI and grown for at least 100 hours at 37°C, or diluted 1:1,000 into liquid MM containing MI and shaken with 180 rpm for at least 100 hours. Under these conditions, two interesting phenotypes repeatedly appeared: (i) on agar plates, a small portion of the whole cell population grew earlier than the residual cell population forming distinct colonies on a background of a thin bacterial lawn (Fig. 1), and (ii) in liquid cultures, the end of the lag phase was highly variable, a phenomenon that could not be reduced despite various efforts to apply reproducible conditions. In average, a lag phase of 40-60 hours was determined, but in some experiments growth was observed not at all. The same bistable phenotype on solid medium was obtained when deletion mutants of *iolI1*, *iolG2*, and *iolH* were tested, indicating no role of these functional unknown genes in the underlying molecular mechanism (Fig. 1).

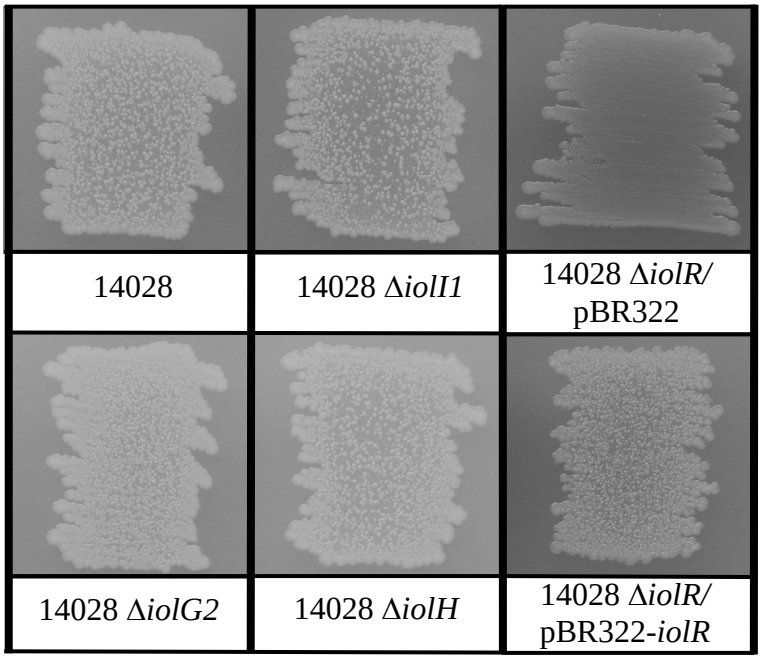


Fig. 1: Bistable phenotype on agarplates, and involvement of *iolR*. *S. enterica* serovar Typhimurium strain 14028 and its deletion mutants 14028 Δ*iolI*, 14028 Δ*iolG2* and 14028 Δ*iolH* were streaked on MM agar plates with 55.5 mM MI. Distinct colonies on a bacterial background lawn manifesting a bistable phenotype are visible. Deletion of the repressor IolR (14028 Δ*iolR*/pBR322) abolish this phenotype, complementation with *iolR* (14028 Δ*iolR*/pBR322-*iolR*) restores bistability.

Reversibility of bistability. To exclude the possibility that the observed phenotype is the result of a mutation or an irreversible phase variation, eighteen distinct colonies grown on MI agar plates with MI as described were isolated to inoculate liquid LB medium, and cultivated overnight at 37°C, followed by two additional passages in LB medium. Cells were then spread on MM agar plates with MI, and all cultures again showed the phenotype described above. Also, a growth phenotype with delayed start of the logarithmic growth phase in liquid medium was observed. One intermediate cultivation in LB medium, however, was not sufficient to reverse the regulatory arrangement required for a bistable phenotype under these conditions. These data indicate that bistability of *S. enterica* serovar Typhimurium cultures in the presence of MI is reversible, and that it is not caused by any kind of mutation.

Involvement of the repressor IolR. IolR has been characterized as a repressor of *iol* gene expression and is known to bind to all but one promoter of the genes required for MI degradation including its own promoter. Furthermore, its deletion resulted in a significantly reduced lag phase during growth in liquid medium (21). We therefore investigated the role of IolR by plating the deletion mutant 14028 Δ*iolR* on agar plates with MM and MI. Instead of distinct colonies reflecting a bistable phenotype, a homogenous bacterial lawn identical to that of a wild-type strain growing on LB agar plates was observed (Fig. 1). Complementation of the *iolR* deletion using plasmid pBR322-*iolR* restored the phenotype of strain 14028, clearly suggesting a role of IolR in bistability in the presence of MI.

Visualization and quantification of bistability. Bistability is caused by the existence of two states of gene expression in an isogenic cell population under identical conditions. For a single cell analysis, the promoter P_{iolE} was chosen that drives the expression of IolE and IolG1 required for the initial enzymatic steps of MI degradation by *S. enterica* serovar Typhimurium. In a previous study, P_{iolE} showed a very low basal expression in MM with glucose or LB, but a high induction during growth in MM with MI (21). A complete expression profile of P_{iolE} was derived during growth of strain 14028 in MM with MI (Fig. 2A). The logarithmic growth phase started only 2-4 h after *iolE* induction, demonstrating the feasibility of P_{iolE} to investigate bistability on a single cell level during growth in medium with MI.

For this purpose, MM containing MI was inoculated with 14028 cells harbouring a *gfp* fusion to P_{iolE} on the low copy plasmid pPROBE-NT. The reporter response during growth of several cultures was monitored by fluorescence microscopy. Images of cells taken from the early growth phase and the late lag phase of two representative experiments are shown in Fig. 2. In

Table 1. Strains, plasmids and oligonucleotides used in this study

Bacterial strains	**Description and relevant features**	**Source or literature**
14028	Spontaneous streptomycin-resistant mutant of *S. enterica* serovar Typhimurium wild-type strain ATCC14028	(21)
14028 Δ*iolR*	In-frame *iolR* (STM4417) deletion mutant	(21)
14028 Δ*iolI1*	In-frame *iolI1* (STM4427) deletion mutant	(21)
14028 Δ*iolG2*	In-frame *iolG2* (STM4433) deletion mutant	(21)
14028 Δ*iolH*	In-frame *iolH* (STM4436) deletion mutant	(21)
Plasmids		
pBR322	Amp^r Tet^r	Fermentas
pBR322-*iolR*	*iolR* cloned into pBR322 with putative promoter region	(21)
pPROBE-NT	Promoter probe vector, pBBR1 replicon, *gfp*-reporter, Km^r	(25)
pNT-P_{iolE}	pPROBE-NT with 300 bp of *iolE* upstream sequence cloned in front of the *gfp*- reporter	This study
pDEW201-P_{iolE}	pDEW201 with 321 bp upstream of *iolE* (STM4424)	(21)
Oligonucleotides		
P_{iolE}_*Hind*III	5'-TTTAAGCTT AGGACTATCGTAAAGC -3', cloning oligonucleotide	This study
P_{iolE}_*Eco*RI	5'-TTGAATTCTTAATGAAACGCTTTTACTG-3', cloning oligonucleotide	This study
control	5'-CCGTATGTTGCATCACC-3', sequencing primer	This study

comparison to a probe yielded 49 hours after inoculation near the end of the lag phase at OD_{600} = 0.089 that shows only four cells with fluorescent activity, a much higher number of cells taken four hours later at OD_{600} = 0.107 obviously express IolE and IolG1 and replicate. During early logarithmic growth, three distinct cell populations with no, low or high fluorescence were detectable, demonstrating the bistable phenotype on a cellular level. As a control, strain 14028/pNT-P_{iolE} was grown in MM with glucose, but no cells expressing *iolE* could be observed before or during exponential growth.

For quantitative analysis, aliquots of a 14028/pNT-P_{iolE} culture in MM with MI were taken at appropriate time points and analysed by flow cytometry. In a representative experiment, only 1% of 14028 cells expressed GFP 41.5 hours after inoculation at an OD_{600} = 0.086. The fraction with the *iolE* promoter in "on" state then increased to 19.35% four hours later at OD_{600} = 0.09 and, 60 hours after inoculation, to 91.70 % at OD_{600} = 0.316 (Fig. 3). These data correlate well with the fluorescence images of Fig. 2.

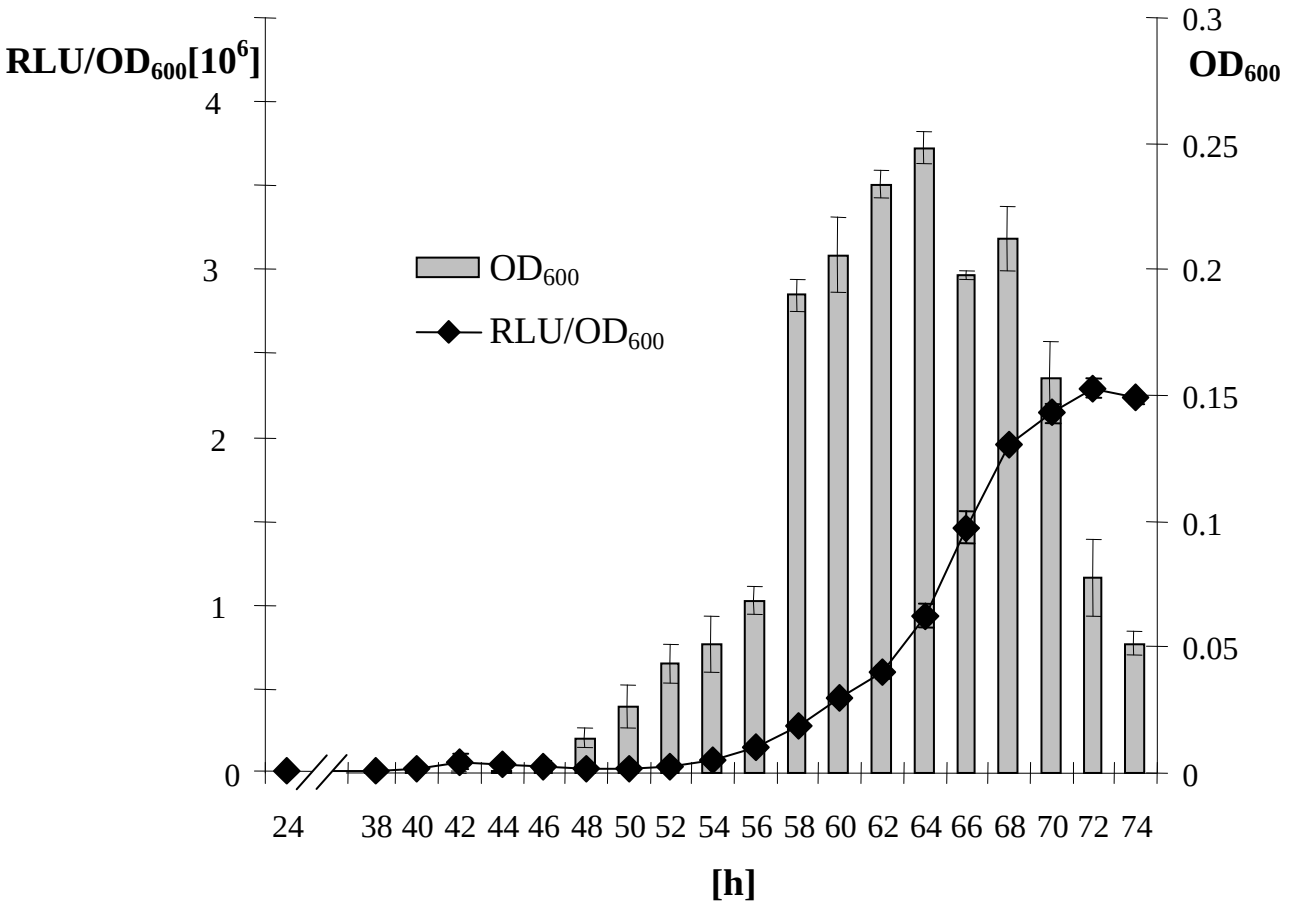
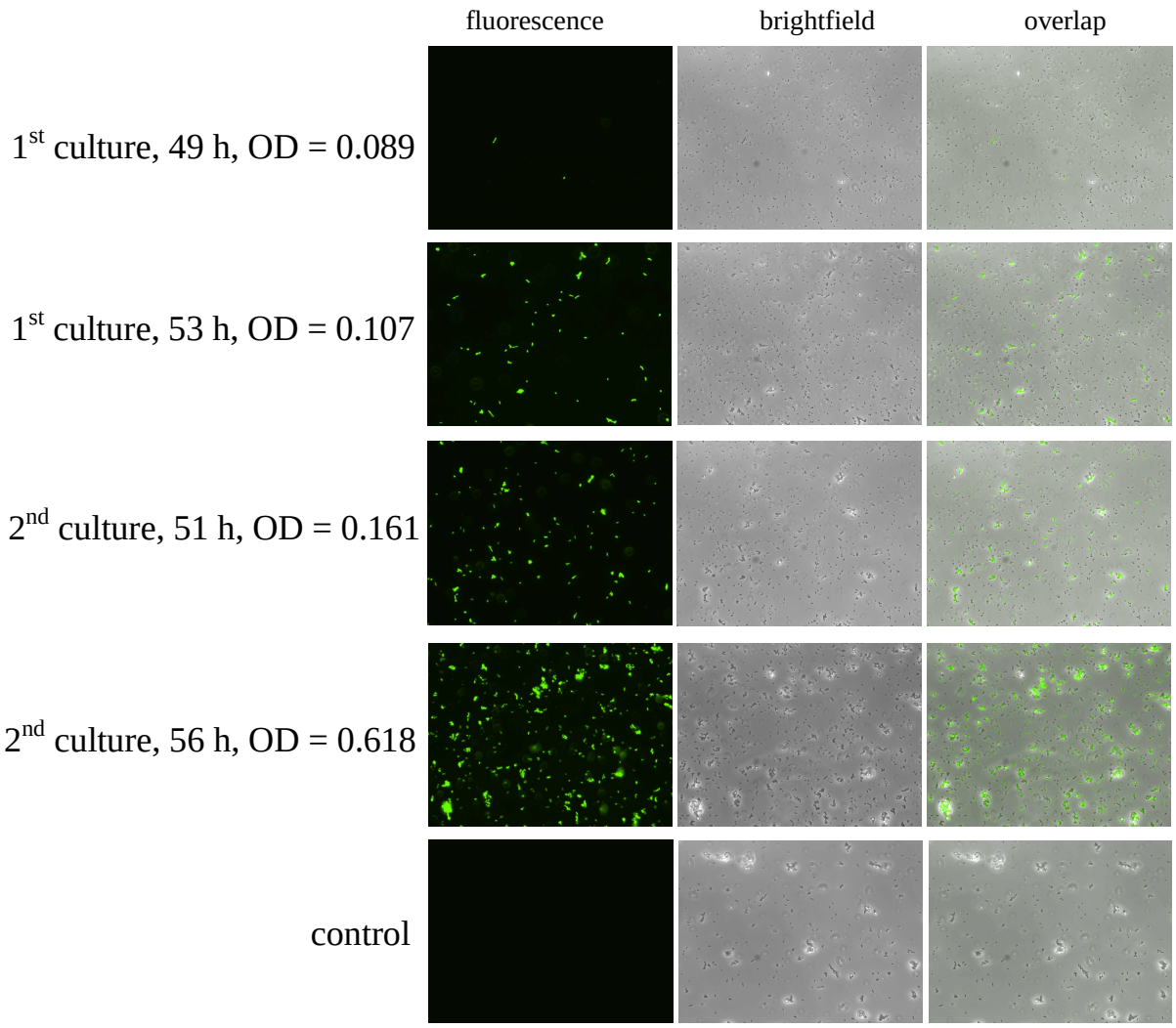


Fig. 2: Transcriptional activity of P_{iolE} during growth of strain 14028 in MM with MI. (A) Growth curve of 14028/pDEW201-P_{iolE} and expression profile of P_{iolE}. OD_{600} and bioluminescence [RLU] were measured in parallel.



(B) Aliquots of two 14028/pNT-P_{iolE} cultures independently inoculated in MM with MI were analysed by fluorescence microscopy. Cells without or with low and high fluorescence activity were observed. A lack of simultaneous growth reflects the random switch between the "off" and the "on" state of the *iolE* promoter. Magnification is 400-fold. The time point post inoculation, and the OD_{600} are indicated. No fluorescence was observed at any time point when strain 14028/pNT-P_{iolE} was cultivated in MM with glucose as control. The respective images show cells in the late exponential growth phase eight hours post inoculation.

The bistable phenotype is abolished by carbon dioxide. Several growth conditions were tested for their effect on *Salmonella* bistability in liquid MM media with MI, but without any measurable change of the growth phenotype. Examples are pH modifications from pH 2 to pH 12 in 0.5 intervals, or low phosphate concentrations known to induce virulence properties (8). However, a significantly reduced lag phase of strain 14028 was observed when cells were grown on MM agar plates with MI in the presence of 5% CO_2. To determine a carbon dioxide threshold, strain 14028 was grown in the presence of 0% to 5% CO_2 (Fig. 4A). While a concentration of 0.2% CO_2 still resulted in a bistable phenotype, a

concentration of 0.55% CO_2 was identified as the minimal CO_2 concentration required to release bistability of strain 14028 on solid medium. As expected, no bistable phenotype was observed when the *iolR* deletion mutant was grown in the presence of 5% CO_2. However, when the complementing plasmid pBR322-*iolR* was present in the mutant 14028 Δ*iolR*, a bistable phenotype appeared (Fig. 4B), indicating that the IolR overexpression in this strain neutralizes the effect of 5% CO_2 on *iol* gene regulation.

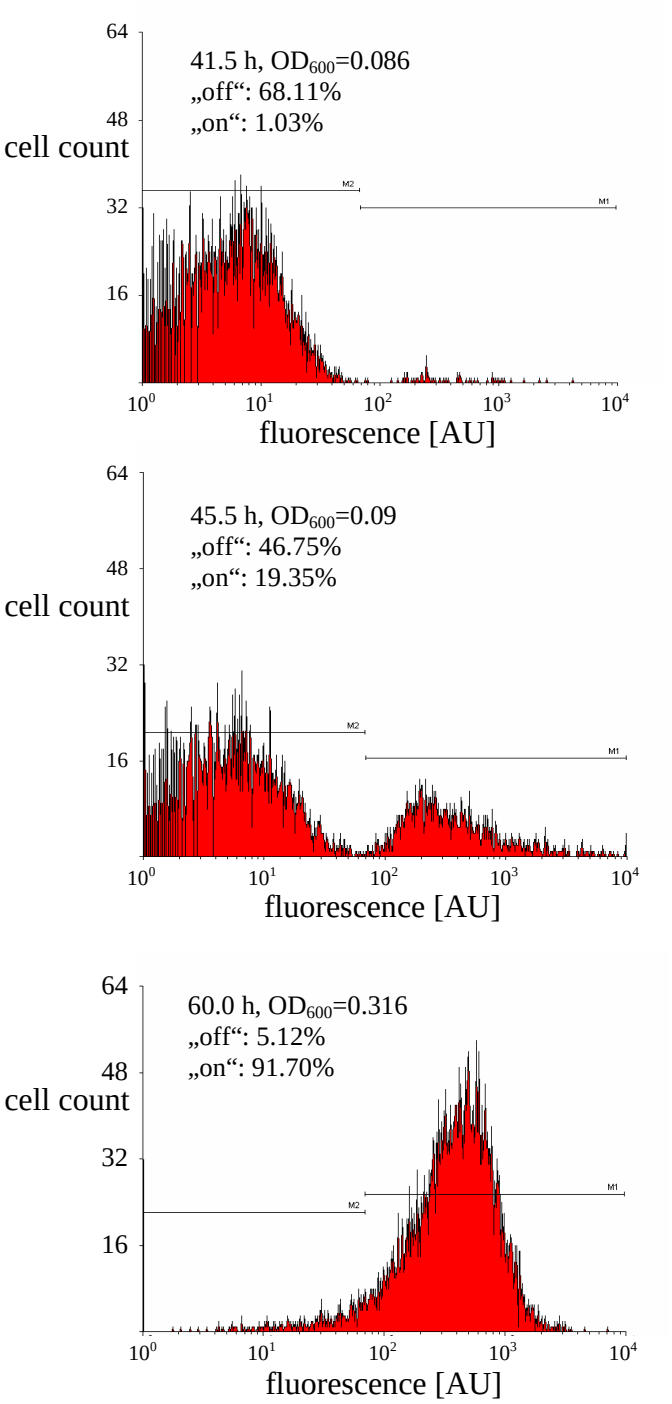


Fig. 3: Quantification of bistability by flow cytometry. 14028/pNT-P_{iolE} was grown in MM containing MI. Cells expressing GFP were quantified using a LSR II flow cytometer. The abscissas of the histograms represent the green fluorescence intensity at 515 to 545 nm as arbitrary units (AU) in a logarithmic scale, and the ordinates the cell count or events. Time points, OD_{600} and percentage of cells in "off" state (left fraction M2) and "on" state (right fraction M1) are indicated. The initial peak adding to 100% summarizes events out of range and might represent dead cells or cells in undefined state.

Influence of bicarbonate as growth signal. Carbon dioxide diffuses into the cell where it is converted to bicarbonate by the activity of bacterial carboanhydrases (CAs). CA candidates of *S. enterica* serovar Typhimurium are encoded by *yadF* (STM0171) or the virulence plasmid-encoded gene PSLT046. In order to investigate the effect of bicarbonate on the length of the lag phase, we diluted an overnight culture of strain 14028 into MI containing MM without $NaHCO_3$, or with 0.01%, 0.1%, 0.18%, 0.4%, 0.8% and 1% $NaHCO_3$. Incubation was performed without shaking because agitation, for unknown reasons, reduced the reproducibility with respect to the start of the logarithmic growth phase. The shortest lag phase of the *Salmonella* cells was obtained with 0.1% $NaHCO_3$ (Fig. 5A), while it was significantly longer when the medium contained higher or lower concentrations of $NaHCO_3$ (data not shown). To our knowledge, this is the first example for *Salmonella enterica* that bicarbonate effects gene regulation of this pathogen.

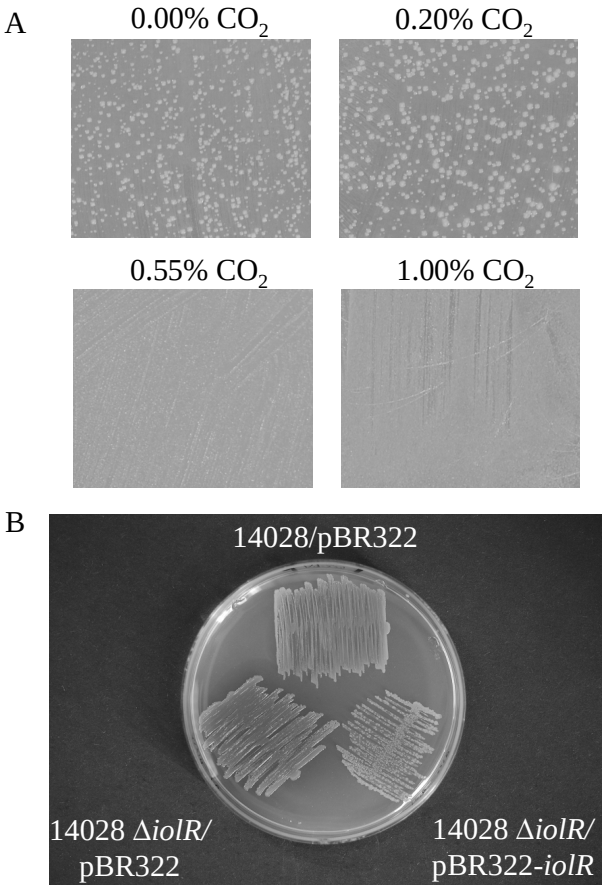


Fig. 4: Effect of CO_2. (A) MM agar plates with 55.5 mM MI were incubated for 100 hours in the presence of varying concentrations of CO_2. A loss of bistability was observed in the presence of at least 0.55% CO_2. (B) A concentration of 5% CO_2 was applied in this experiment. Upon complementation with pBR322-*iolR*, the bistable phenotype is restored, thus compensating the CO_2 effect. Both experiments were repeated several times.

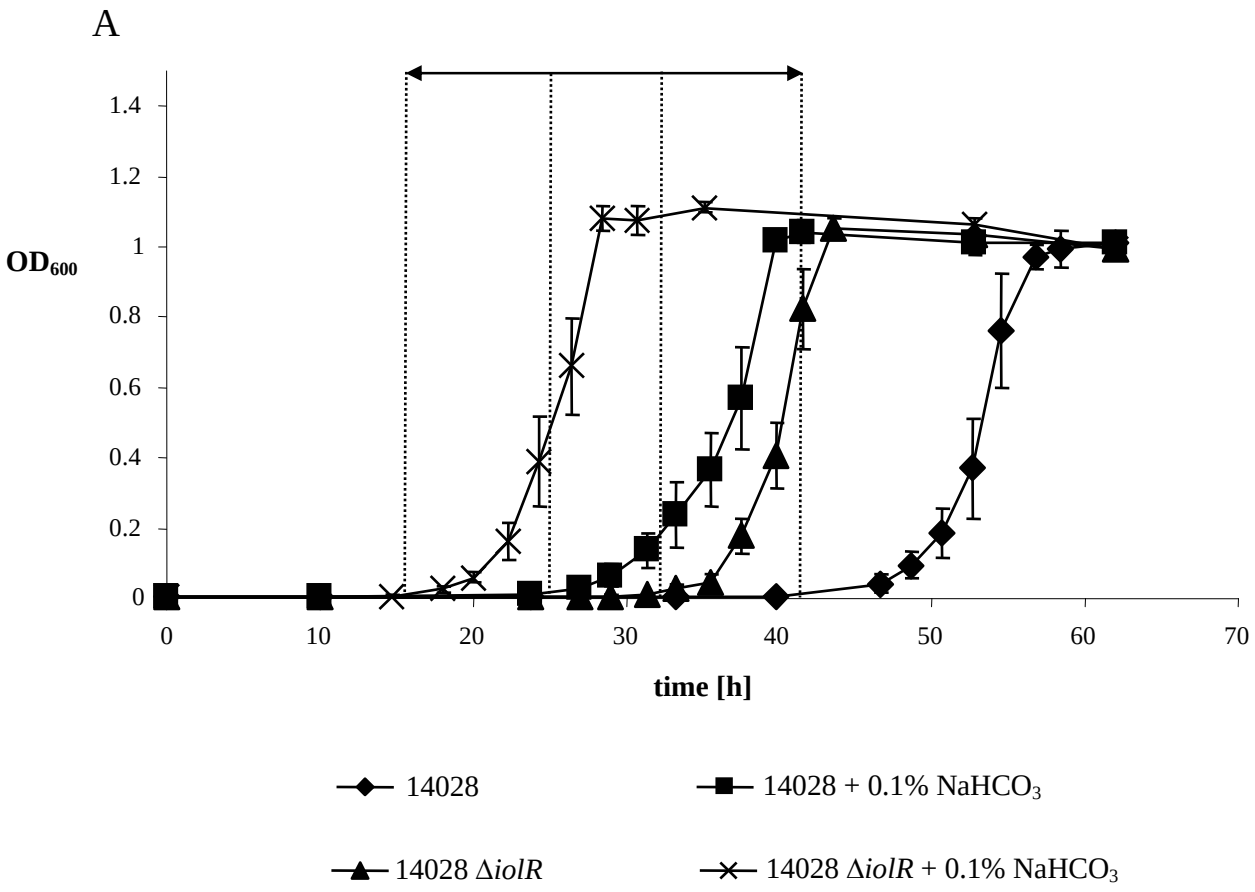
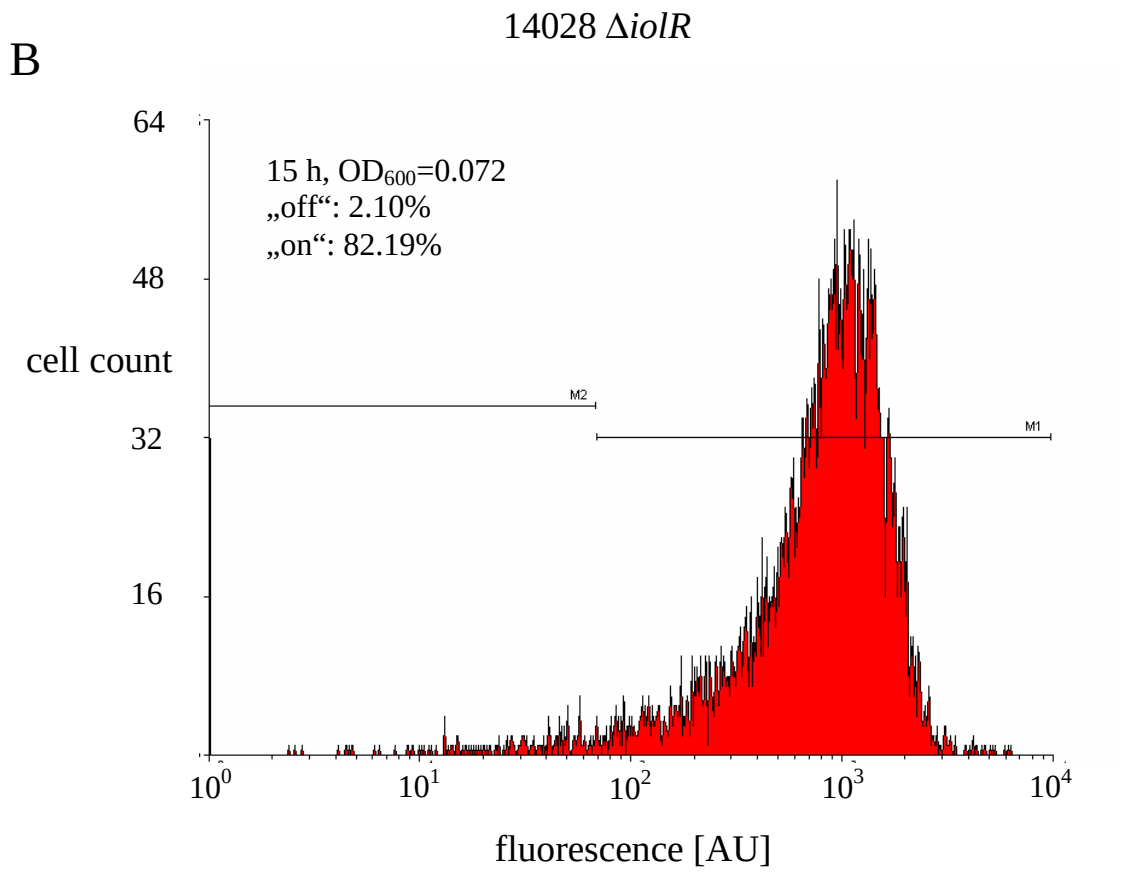


Fig. 5: Early growth start of mutant 14028 Δ*iolR* in MM with MI in the presence of 0.1% $NaHCO_3$. (A) Growth curves of strains 14028 and 14028 Δ*iolR*. Average values of three independent experiments are shown, and standard deviations are indicated. Representative graphs are shown due to the highly variable start points of the logarithmic phase, which are indicated by dashed lines. The arrow depicts the maximal reduction of the lag phase.

Deletion of *iolR* and presence of 0.1% bicarbonate result in a minimal lag phase. Bistability of *S. enterica* serovar Typhimurium growing with MI could be abolished by *iolR* deletion, or by the addition of bicarbonate. This effect was quantified by monitoring the growth of 14028 and 14028 Δ*iolR* in MM with 55.5 mM MI without agitation. Under these conditions, deletion of IolR or addition of 0.1% (11.9 mM) bicarbonate reduced the lag phase by approximately 10-20 hours in each case. An additive effect on lag phase reduction was obtained when 14028 Δ*iolR* was incubated in MM with MI and bicarbonate. Here, the logarithmic growth phase started approximately 30 hours earlier than that of the wild-type strain in the absence of bicarbonate, and only approximately 10 hours later than that of strain 14028 in MM with glucose (Fig. 5A). 14028 cells precultivated in MM with glucose showed pre-adaptation to MM and thus a further reduced lag phase (data not shown). Flow cytometry



(B) Flow cytometry analysis of mutant 14028 Δ*iolR* grown in MM with MI and 0.1% $NaHCO_3$. See figure legend 3 for details.

analysis of a 14028 Δ*iolR* culture in a MM with MI and 0.1% bicarbonate confirmed this observation. Already 15 hours post inoculation and near the end of the lag phase, a majority of 82.19% of all cells shows an active *iolE* promoter (Fig. 5B), in contrast to only 19.35% in the wild-type in MM with MI and without bicarbonate (Fig. 3). These data allow two conclusions: (i) the regulatory impact of bicarbonate is comparable to that of IolR with respect to the switch of a *Salmonella* population from an “off” to an “on” state in MI utilization, and (ii) bicarbonate does not seem to interact with IolR because addition of $NaHCO_3$ further reduced the lag phase of the *iolR* deletion mutant.

DISCUSSION

Bistability is defined as the occurence of two stable subpopulations within an overall genetically homogenous population (35). The underlying mechanism is assumed as a specific feedback that acts in combination with a nonlinear response within a network generating a bistable phenotype (35). In this study, we have introduced a metabolic property, MI degradation, as a novel example of such a phenotypic variation. MI utilization in *S. enterica* serovar Typhimurium strain14028 is characterized by a extraordinarily long lag phase on MI that is absent in all other bacteria investigated so far with respect to this metabolic pathway, indicating a tight regulation of the responsible genes. The early appearance of a few, distinct colonies, as well as the weak reproducibility of growth curves reflecting the stochastical variability of the two cellular states within a population, are characteristic features of bistability of strain 14028 in the presence of MI. This phenotypic variation reminds of an observation by D. C. Old who reported unstable and "leaky" growth of *S. enterica* serovar Typhimurium biotype 9 strains in peptone water with MI (28). Both phenotypes disappeared when the repressor IolR was released from *iol* gene promoters possibly by binding the MI degradation intermediate DKP as shown for *B. subtilis* (39).

Interestingly, a similar effect was obtained when the cells were cultivated in the presence of higher concentrations of CO_2 or HCO_3^-. Slight overexpression of IolR in the *iolR* deletion mutant restored the bistable phenotype even in the presence of 5% CO_2 (Fig. 4B), probably resulting in a tighter repression of the *iol* genes. The results summarized in Fig. 5A clearly indicate that HCO_3^- does not interact with IolR, and that two distinct molecular mechanisms regulate MI utilization. The promoter P_{iolE} used for fluorescence analysis in this study drives the expression of IolE and IolG1 required for the first two enzymatic steps of MI degradation, and IolR has been demonstrated not to bind to P_{iolE}. We therefore postulate that HCO_3^- interacts with P_{iolE} directly or, more probably, indirectly *via* binding to a second regulatory protein, thus inducing the transcription of *iolE* and *iolG1*. A promising candidate for such a positive regulator is STM4423, an AraC-like regulator, due to its expression in the gut of infected mice (30) and its localization directly upstream of *iolE*, STM4424.

An increasing number of reports demonstrate the regulatory role of bicarbonate in pathogenic bacteria. This environmental chemical is involved in virulence gene transcription of *Bacillus anthracis*, in a change of the antigenic profile of *Borrelia burgdorferi*, and in M protein expression in group A streptococci (7, 15, 19, 33). Regulation of virulence factors by bicarbonate may especially occur in enteropathogenic bacteria, because HCO_3^- is secreted from the pancreas for stomach acid neutralization. Experimental examples are the activity of

RegA on the two putative virulence determinants *adcA* and *kfc* of *Citrobacter rodentium*, the cholera toxin induction in *Vibrio cholerae* by ToxT, and the expression of locus of enterocyte effacement-encoded genes in enterohemorrhagic *Escherichia coli* O157:H7 (1, 2, 36). In the duodenum, HCO_3^- might reach concentrations between 70 and 150 mM (32) exceeding 11.9 mM (0.1%) used in our experiments. To our knowledge, an effect of CO_2/HCO_3^- on gene regulation in *Salmonella* has not been described so far.

The following model for the regulation of MI utilization by *S. enterica* serovar Typhimurium is suggested (Fig. 6): In environments lacking MI, most *iol* gene promoters are repressed by IolR (21). When MI is added, only few molecules will enter the cell due to the repression of *iolT1* encoding the major facilitator of this molecule (22). MI is degraded to intermediates such as DKP, which is now present at suboptimal levels but induces bistability by binding to IolR. Later on, the DKP level reaches a threshold concentration that releases more IolR from its promoters. Thus, a positive feedback loop is initiated by inducing *iolT1* expression and the MI degradation enzymes, and most cells reach the "on" state with respect to MI catabolism. Interestingly, HCO_3^- might act as an environmental signal stimulating MI degradation by inducing *iolE/G1 via* a second regulator predicted above. In case that this signal is not appropriate due to the absence of MI, IolR activity will ensure that most *Salmonella* cells remain in the "off" state.

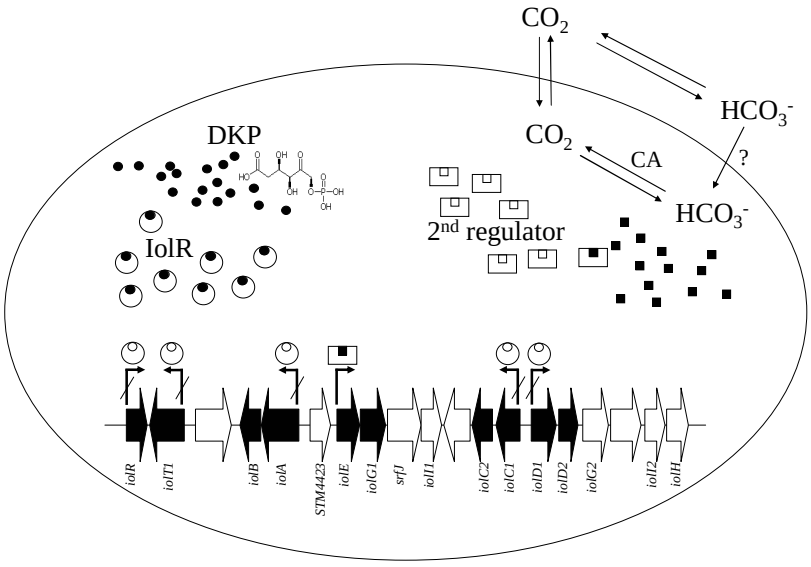


Fig. 6: Model of the regulatory mechanism of MI utilization. *Salmonella* genes experimentally demonstrated to be involved in MI transport and degradation, or their regulation, are depicted in black, functional unknown genes in white. Crossed out arrows indicated gene repression. A bicarbonate transporter has not been identified in *Salmonella*. DKP, 2-deoxy-5-keto-D-gluconic acid 6-phosphate; CA, carboanhydrase. See discussion for details.

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WEITERE PUBLIKATIONEN UND POSTERPRÄSENTATIONEN

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