Glucose and glucose 6-phosphate as carbon sources in extra- and intracellular growth of enteroinvasive *Escherichia coli* and *Salmonella enterica*

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To study the role of carbohydrates, in particular glucose, glucose 6-phosphate and mannose, as carbon substrates for extra- and intracellular replication of facultative intracellular enteric bacteria, mutants of two enteroinvasive *Escherichia coli* (EIEC) strains and a *Salmonella enterica* serovar Typhimurium isolate were constructed that were defective in the uptake of glucose and mannose ($\Delta ptsG$, $manXYZ$), glucose 6-phosphate ($\Delta uhpT$) or all three carbohydrates ($\Delta ptsG$, $manXYZ$, $uhpT$). The ability of these mutants to grow in RPMI medium containing the respective carbohydrates and in Caco-2 cells was compared with that of the corresponding wild-type strains. In the three strains, deletions of $ptsG$, $manXYZ$ or $uhpT$ resulted in considerably different levels of inhibition of growth *in vitro* in the presence of glucose, mannose and glucose 6-phosphate, respectively, but hardly reduced their capability for intracellular replication in Caco-2 cells. Even the triple mutants $\Delta ptsG$, $manXYZ$, $uhpT$ of the three enterobacterial strains were still able to replicate in Caco-2 cells, albeit at strain-specific lower rates than the corresponding wild-type strains.

INTRODUCTION

Among the *Enterobacteriaceae*, *Salmonella* and *Shigella* species, as well as the highly related enteroinvasive *Escherichia coli* (EIEC) strains, are typical intracellular pathogens, capable of survival and active replication in phagocytic (especially macrophages and dendritic) and non-phagocytic (especially epithelial) cells. Infections caused by these intracellular bacterial pathogens are multistep processes with complex metabolic adaptations. *Salmonella enterica* serovar Typhimurium (*S.* Typhimurium) and *Shigella flexneri* (as well as EIEC) are taken up preferentially by consumption of contaminated food and, in the early stages of infection, they encounter varying physico-chemical and nutritional conditions in the gut before they reach their intracellular compartments. In this early stage of infection, the bacteria grow mainly extracellularly under anoxic or microaerobic conditions

and growth may be supported by qualitatively and quantitatively changing nutrients.

Salmonella and *Shigella* cross the intestinal barrier by entering and transcytosing M-cells in the Peyer's patches (Vazquez-Torres & Fang, 2000). After reaching the underlying extracellular matrix these bacteria are taken up by phagocytic cells, i.e. dendritic cells, monocytes/macrophages or granulocytes.

Shigella triggers fast apoptosis of the infected phagocytic host cells. The released bacteria subsequently invade epithelial cells in the gut from the basolateral site. Within the gut epithelium, *Shigella* multiplies intracellularly and spreads to neighbouring cells throughout infection, thereby destroying large parts of the infected tissue (Phalipon & Sansonetti, 2007).

S. Typhimurium, sometimes in infected humans and more generally in the mouse model, becomes systemic by spreading into different peripheral organs mainly via infected phagocytes (dendritic cells, monocytes/macrophages).

In infected phagocytic and non-phagocytic host cells, *Shigella* (and EIEC) escapes from the primary phagosome into the cytosol where these pathogens actively replicate. By contrast, *S.* Typhimurium remains mainly in a specifically modified phagosomal compartment, the *Salmonella*-

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Abbreviations: CCR, carbon catabolite repression; EIEC, enteroinvasive *Escherichia coli*; FCS, fetal calf serum; Glc, glucose; Glc6P, glucose 6-phosphate; LB, Luria–Bertani; Man, mannose; PEP, phosphoenolpyruvate; p.i., post-infection; PTS, phosphoenolpyruvate:carbohydrate phosphotransferase system; SCV, *Salmonella*-containing vacuole; TSB, tryptic soy broth.

containing vacuole (SCV) (Brumell & Grinstein, 2004). Recent reports (Beuzón *et al.*, 2000, 2002) suggest, however, that even *S. Typhimurium* may survive and multiply in the cytosol of some host cells under special conditions.

The genomes of *Shigella*/EIEC (Jin *et al.*, 2002) and *S. Typhimurium* (Bell *et al.*, 2004; McClelland *et al.*, 2001) contain complete gene sets for all major catabolic and anabolic pathways – typical for prototrophic/heterotrophic micro-organisms. In accord with their genetic potential, these pathogens are able to grow in defined minimal media supplemented with a single suitable carbon source.

Glucose (Glc) is a preferred carbon source for *in vitro* growth. In *E. coli* K-12, up to 14 phosphoenolpyruvate: carbohydrate phosphotransferase systems (PTS) were identified as transporters for different carbohydrates. Six of these PTS, encoded by the genes *ptsG/crr*, *manXYZ*, *fruBA*, *malX/crr*, *scrA/crr* and *bglF* (reviewed by Postma *et al.*, 1993), were reported to accept Glc as substrate. These genes are also present in the genomes of *Shigella*/EIEC (Jin *et al.*, 2002) and some of them in part are also modified in *S. Typhimurium* (Bell *et al.*, 2004; McClelland *et al.*, 2001). PtsG represents the major Glc-specific PTS permease in *E. coli* K-12 (and in many other heterotrophic bacteria) with the highest affinity for Glc among these systems.

Glucose 6-phosphate (Glc6P) is taken up by both pathogens via the UhpT transporter. The *uhpT* gene of *Shigella*/EIEC and *S. Typhimurium* is under the control of a complex two-component system (*uhpABC*) (Verhamme *et al.*, 2002) and of carbon catabolite repression (CCR). Hence, expression of UhpT is low in the presence of Glc.

Shigella/EIEC and *S. Typhimurium* also have the metabolic gene setting for catabolizing a large variety of other carbon substrates, including C₂-, C₃-, C₄- and C₅-substrates, which may be eventually provided by the host cells as carbon substrates for intracellular bacterial growth. *S. Typhimurium* contains an additional 55 kb large gene cluster carrying all *pdu* genes for propanediol degradation and those for vitamin B₁₂ biosynthesis and ethanolamine utilization (Klumpp & Fuchs, 2007).

To obtain insights into the intracellular metabolism of the two pathogens, we constructed mutants of two EIEC strains and an *S. Typhimurium* isolate with deletions in *ptsG*, *manXYZ* and/or *uhpT* and studied their growth relative to the corresponding wild-type strains in defined culture media and in Caco-2 cells. These mutants were also recently used for determining the intracellular carbon metabolism by ¹³C-isotopologue profiling (A. Götz and other authors, unpublished data).

METHODS

Bacterial strains, cell line and plasmids. EIEC 4608-58 and EIEC HN280 were gifts from P. Sansonetti (Institut Pasteur, Paris, France) and M. Nicoletti (Dipartimento di Scienze Biomediche, Università G. D'Annunzio, Chieti, Italy). *S. Typhimurium* (*Stm*) 14028s was obtained

from M. Hensel (Mikrobiologisches Institut, Universitätsklinikum Erlangen, Germany). *E. coli* K-12 (DSM 498) and the Caco-2 cell line (ACC 169) were obtained from the DSMZ. pBR322 was obtained from New England Biolabs. All strains and mutants described in this study are listed in Table 1.

Construction of mutants. Bacteria were routinely cultured at 30 or 37 °C in Luria Broth [LB; 10 g tryptone l⁻¹ (Oxoid), 5 g Bacto yeast extract l⁻¹ (BD Biosciences), 5 g NaCl l⁻¹] with shaking or on LB agar (13 g l⁻¹). If needed, antibiotics and other supplements were added to the following final concentrations: 100 µg ampicillin ml⁻¹, 30 µg chloramphenicol ml⁻¹, 50 µg kanamycin ml⁻¹, 0.2% (w/v) L-arabinose. In addition, all constructed EIEC mutants were plated on tryptic soy broth (TSB) agar [30 g l⁻¹ (Sigma, contains 2 g glucose l⁻¹)] supplemented with 0.01% (w/v) Congo red and incubated at 37 °C to test their ability to bind the pigment Congo red. Only red colonies were used for further studies.

Mutant strains were generated as described by Datsenko & Wanner (2000). Oligonucleotides used to amplify the chloramphenicol- or kanamycin-resistance cassette from pKD3 or pKD4, respectively, using iProof-polymerase from Bio-Rad are listed in Table 2 (65-mers). Annealing temperature was varied between 55 and 65 °C. Colonies were screened for correct insertion of the resistance cassette by PCR using MolTaq-polymerase from Molzym according to the manufacturer's instruction. Oligonucleotides are listed in Table 2 (20-mers).

For EIEC/K-12 the following genes were deleted: *uhpT* from aa 6 to 461, *ptsG* from aa 6 to 474 and *manXYZ* from aa 2 of *manX* to 282 of *manZ*. For *Stm* 14028s, *uhpT* was deleted from aa 3 to 459, *ptsG* from aa 4 to 474, *manXYZ* from aa 4 of *manX* to 274 of *manZ* and *glk* from aa 4 to 319.

Complementation of EIEC 4608-58. For complementation experiments, the coding sequence of *uhpT* of EIEC 4608-58 was reverse-cloned into the *Bam*HI/*Sgr*AI restriction site of pBR322 using primer *uhpT*-comp-up-1 and *uhpT*-comp-down-1 (Tables 1 and 2, 30-mers). *ptsG* was reverse-cloned into the *Sal*I restriction site of pBR322 using primer *ptsG*-comp-up and *ptsG*-comp-down and *manXYZ* was cloned into the *Eco*RI site of pBR322 using primer *manXYZ*-comp-up and *manXYZ*-comp-down. Transcription of all genes was initiated at their own promoters.

Growth in minimal medium on different sugars. Cell culture medium RPMI 1640 with 2 mM L-glutamine (Gibco) was used as minimal medium. Carbon sources were added to a final concentration of 10 mM. For growth curves, bacteria from an overnight culture in TSB (EIEC) or LB (*Salmonella*) were washed twice with PBS, diluted 1:100 in minimal medium and further incubated at 37 °C with shaking. Cell density was determined every hour by OD₆₀₀ measurement.

β-Galactosidase test. The status of CCR was determined by the activity of the β-galactosidase enzyme. For this goal, an overnight culture was diluted 1:100 in fresh TSB supplemented with 5 mM lactose and further shaken for 2 h at 37 °C until OD₆₀₀ ≈ 1. An aliquot of 10⁹ bacteria was pelleted by centrifugation and resuspended in 1 ml Na⁺/K⁺-phosphate buffer (0.05 M, pH 7.4). Bacteria were lysed by incubation with 50 µl toluene and 50 µl sodium deoxycholate (1%) at 37 °C for 30 min. β-Galactosidase activity was determined at 37 °C by mixing 50 µl cell lysate with 950 µl Na⁺/K⁺-phosphate buffer and 100 µl ONPG (5 mg ml⁻¹). The reaction was stopped after 15 min by adding 40 µl Na₂CO₃ (10%) and OD₄₂₀ was measured. Protein concentration of the cell lysate was determined by Bradford assay and the specific activity of β-galactosidase was calculated as µmol min⁻¹ (mg protein)⁻¹. Statistical significance was determined with Student's unpaired *t*-test; *P* < 0.05 was considered to be significant.

Bacterial growth conditions prior to infection. EIEC strains were cultured overnight in 20 ml TSB at 30 °C and then diluted 1:100 in

Table 1. Bacterial strains and plasmids used in this study

Strain/plasmid	Genotype and property	Reference/source
<i>E. coli</i> K-12 strain		
K-12	Wild-type	DSMZ (DSM 498)
AG044	K-12 $\Delta uhpT$; sugar phosphate antiporter	This study
AG098	K-12 $\Delta ptsG$; glucose-specific PTS enzyme IICB	This study
EIEC strain		
4608-58	Wild-type, lactose ⁺	Sansonetti <i>et al.</i> (1982)
AG069	4608-58 $\Delta uhpT$	This study
AG096	4608-58 $\Delta ptsG$	This study
AG161	4608-58 $\Delta manXYZ$; mannose-specific PTS enzyme IIAB, IIC and IID	This study
AG097	4608-58 $\Delta ptsG$, $uhpT$	This study
AG157	4608-58 $\Delta ptsG$, $manXYZ$	This study
AG135	4608-58 $\Delta ptsG$, $manXYZ$, $uhpT$	This study
HN280	Wild-type, lactose ⁻	Nicoletti <i>et al.</i> (1988)
AG136	HN280 $\Delta uhpT$	This study
AG166	HN280 $\Delta ptsG$	This study
AG158	HN280 $\Delta manXYZ$	This study
AG167	HN280 $\Delta ptsG$, $uhpT$	This study
AG168	HN280 $\Delta ptsG$, $manXYZ$	This study
AG169	HN280 $\Delta ptsG$, $manXYZ$, $uhpT$	This study
<i>S. Typhimurium</i> strain		
14028s	Wild-type	ATCC14028s
AG046	14028s $\Delta uhpT$	This study
AG088	14028s $\Delta ptsG$	This study
AG094	14028s $\Delta ptsG$, $uhpT$	This study
AG223	14028s $\Delta ptsG$, $manXYZ$	This study
AG143	14028s $\Delta ptsG$, $manXYZ$, $uhpT$	This study
AG229	14028s Δglk	This study
AG230	14028s $\Delta ptsG$, $manXYZ$, glk	This study
Plasmid		
pKD3	Template plasmid contains chloramphenicol-resistance cassette, Amp ^R , Cm ^R	Datsenko & Wanner (2000)
pKD4	Template plasmid contains kanamycin-resistance cassette, Amp ^R , Kan ^R	Datsenko & Wanner (2000)
pKD46	Temperature-sensitive plasmid contains phage λ red recombinase system, Amp ^R	Datsenko & Wanner (2000)
pCP20	Temperature-sensitive plasmid contains FLP recombinase gene, Amp ^R , Cm ^R	Datsenko & Wanner (2000)
pBR322	Cloning vector, Amp ^R , Tc ^R	Bolivar <i>et al.</i> (1992), Covarrubias <i>et al.</i> (1981), New England Biolabs
pAG078	Derivate of pBR322 containing P _{<i>uhpT</i>} - <i>uhpT</i> of EIEC 4608-58 expressed from its native promoter reverse-cloned into <i>Bam</i> HI/ <i>Sgr</i> AI restriction site, Amp ^R	This study
pAG119	Derivate of pBR322 containing P _{<i>ptsG</i>} - <i>ptsG</i> of EIEC 4608-58 expressed from its native promoter reverse-cloned into <i>Sal</i> I restriction site, Amp ^R	This study
pAG124	Derivate of pBR322 containing P _{<i>manXYZ</i>} - <i>manXYZ</i> of EIEC 4608-58 expressed from its native promoter cloned into <i>Eco</i> RI restriction site, Amp ^R , Tc ^R	This study

20 ml fresh TSB and further incubated for 2 h at 37 °C with aeration. An overnight culture of *Stm* 14028s grown in 20 ml LB with 0.5 % NaCl at 37 °C was diluted 1:100 (v/v) into 20 ml fresh LB with 1 % NaCl and further shaken for 2.5 h at 37 °C. When cultures reached late exponential growth phase ($OD_{600} \approx 1$), bacteria were diluted in RPMI 1640 containing 10 % fetal calf serum (FCS) and added to the Caco-2 cells at different m.o.i. (see below).

Adhesion assays. Caco-2 cells were cultured in RPMI 1640 with 2 mM L-glutamine and 10 mM glucose supplemented with 10 % heat-inactivated FCS (Biochrom). Before infection, cells were seeded into 24-well plates at a density of 10^5 cells well⁻¹ and grown for 2 days to 90 % confluence.

Adhesion of the bacteria to Caco-2 cells was investigated by using cytochalasin D to destroy the actin cytoskeleton and inhibit bacterial

cell invasion in an actin-dependent manner. Therefore, the cells were incubated with fresh RPMI containing 10 % FCS and 1 μ g cytochalasin D ml⁻¹ 30 min before starting the infection. Then bacteria were added at a constant m.o.i. of 17 and allowed to adhere for 1 h in the presence of 1 μ g cytochalasin D ml⁻¹ without centrifugation. After this, cells were washed six times with RPMI containing 10 % FCS and 1 μ g cytochalasin D ml⁻¹ to remove non-adherent bacteria. To count the number of adherent bacteria, cells were washed once with ice-cold PBS and lysed with ice-cold distilled water to detach the bacteria. A serial dilution was plated on LB agar.

Statistical significance was determined with Student's unpaired *t*-test; $P < 0.05$ was considered to be significant.

Invasion and replication assay. Cells were prepared as described above 2 days before infection. Bacteria were diluted in RPMI with

Table 2. Oligonucleotides used in this study

Organism	Name	Sequence 5'–3'
<i>E. coli</i>	c1	TTATACGCAAGGCGACAAGG
	c2	GATCTTCCGTCACAGGTAGG
	k1	CAGTCATAGCCGAATAGCCT
	k2	CGGTGCCCTGAATGAACTGC
	uhpT-del-up (K-12)	AGGTATTCATTTTCAGGAGTAACCCATGCTGGCTTTCTTAAGTGTAGGCTGGAGCTGCTTC
	uhpT-del-up (EIEC)	AGGTATTCATTTTCAGGAGTAACCCATGCTGGCTTTCTTAAGTGTAGGCTGGAGCTGCTTC
	uhpT-del-down	GCCGGGCAAAAACGTTACCAAAATGCACACATTTAAGCGATATTGACCATATGAATATCCTCCTTA
	ptsG-del-up	AAGCACCCATACTCAGGAGCACTCTCAATTATGTTTAAAGAATGCAGTGTAGGCTGGAGCTGCTTC
	ptsG-del-down	CTGGCTGCCTTAGTCTCCCCAAGCTCTTACGGATTAGTGGTTACGCATATGAATATCCTCCTTA
	manXYZ-del-up	TCTGGCACGTTGAGGTGTTAACGATAATAAAGGAGGTAGCAAGTGGTGTAGGCTGGAGCTGCTTC
	manXYZ-del-down	GCCAAAAGGCCCCGGTAGTGTACAACAGTCTTACAGTCCCAGCAGCATATGAATATCCTCCTTA
	uhpT-cont-up	TTGCCTGGACGCTATC
	uhpT-cont-down	TACGCTGGAAGTCACGGA
	ptsG-cont-up	TGGGCGGTGAATAACCACG
	ptsG-cont-down	TCGCGTGTATACCGACACC
	manXYZ-cont-up	CGGTGACGATCCATACTGCG
	manXYZ-cont-down	AACCCATCAGAGCCAGTGCG
	uhpT-comp-up-1	TGGAGCGCCGGTGTGTGGTTATCTCTATCGCC
	uhpT-comp-down-1	GGCGCGGATCCTGGCGGAACAAATTGAC
	ptsG-comp-up	CCAATGTCGACTGTCAAAATGTGCAACTTCTCC
	ptsG-comp-down	AAAAAGTCGACGACAAAACCTACGATACCAACG
	manXYZ-comp-up	AAAAAGAATTCCGGTGACGATCCATACTGCG
	manXYZ-comp-down	AAAAAGAATTCAACCCATCAGAGCCAGTGCG
<i>Salmonella</i>	uhpT-del-up	GCCATTTCGAGGTATAAAAAATTAGCTCAGGAGTAATCCATGCTGGGTGTAGGCTGGAGCTGCTTC
	uhpT-del-down	GCCGGGCAAAAACGTTACCAAAATGCACACATTTAAGCGATATTGACCATATGAATATCCTCCTTA
	ptsG-del-up	GTAGAAAAGCACAAATACTCAGGAGCACTCTCAATTATGTTTAAAGGTGTAGGCTGGAGCTGCTTC
	ptsG-del-down	GGCTGCCTTAATTCTCCCCAACATCATTACTGCTTAGCTGTTACGCATATGAATATCCTCCTTA
	manXYZ-del-up	AGTTGATGTGTTGACAAATAATAAAGGAGGTAGCAAGTGACCATTGGTGTAGGCTGGAGCTGCTTC
	manXYZ-del-down	ACAGCCTTACTGGCCCAGCAGTCTACGGAGTAGCCGGCGATACCCATATGAATATCCTCCTTA
	glk-del-up	AAAGACTTATTTTGACTTTAGCGGAGCAGTAGAAGAATGACAAAGGTGTAGGCTGGAGCTGCTTC
	glk-del down	TTTTGTAGGCGGATAAGGCGTTTATGCCACCATCTGGCTTATAGCATATGAATATCCTCCTTA
	uhpT-cont-up	CGCTGTCGAGGCCAC
	uhpT-cont-down	GTCGACGTTCTGGCCTG
	ptsG-cont-up	GCGTAAAGTTCACCGCCAC
	ptsG-cont-down	CTATGCGCTGGTCGATCTG
	manXYZ-cont-up	TCCATACAACGGGCTGCTGC
	manXYZ-cont-down	CTCCGTGGCTGGTTACGTTG
	glk-kont-up	GCGATTAACAGGCTTGACG
	glk-kont-down	ATGAAACCGTAAATAAGGCAGCG

*Restriction sites and primer sites are underlined.

10% FCS (or without where indicated) and added to the cells at a constant m.o.i. of 17 for invasion assays and at a different m.o.i. (as indicated) to achieve similar numbers of c.f.u. at 1 h post-infection (p.i.) for replication assays. Infected cells were centrifuged at 173 g and 37 °C for 5 min to synchronize invasion and bacteria were allowed to invade the cells for 45 min (EIEC) or 60 min (*Salmonella*). Then, the bacteria-containing medium was replaced by RPMI containing 10% FCS and 100 µg gentamicin ml⁻¹ to kill extracellular bacteria. This was defined as time point 0 h p.i. After 1 h, gentamicin concentration was decreased to 20 µg ml⁻¹. At defined time points p.i., the gentamicin-containing medium was removed, cells were washed with ice-cold PBS and lysed with ice-cold distilled water. The c.f.u. was determined by plating a serial dilution on LB agar.

RESULTS

Glucose uptake of EIEC strains depends mainly on two major PTS permeases

For this study we used two different clinical EIEC isolates, EIEC 4608-58 (Sansonetti *et al.*, 1982) and EIEC HN280 (Nicoletti *et al.*, 1988), which belong to different phylogenetic groups of *E. coli* based on the criteria described by Clermont *et al.* (2000). EIEC 4608-58 is *chuA*⁺, *yja*⁻, TspE4.C2⁻, which is characteristic for members of the phylogenetic group D strains, whereas

EIEC HN280 is *chuA*[−], *yja*[−], TspE4.C2[−], which is characteristic for group A strains.

The two EIEC strains multiplied in RPMI 1640 containing 10 mM Glc with similar generation times (48 ± 0.2 and 49 ± 1 min, respectively) to *E. coli* K-12 (46 ± 0.5 min), taken as a control (Fig. 1). To obtain Glc-uptake mutants of both EIEC strains, we first deleted *ptsG* encoding EIICB of the major Glc-specific PTS permease of *E. coli* K-12 (Stock *et al.*, 1982). The resulting $\Delta ptsG$ mutants grew – as expected – more slowly with Glc. The increase in doubling times was, however, less significant for the two EIEC $\Delta ptsG$ mutants (70 ± 1 min for EIEC 4608-58 and 89 ± 1 min for EIEC

HN280) than for the *E. coli* K-12 mutant (133 ± 1 min), suggesting that an additional Glc uptake system(s) is more efficient in the EIEC strains than in *E. coli* K-12. Growth of the mutants in the presence of mannose (Man) or Glc6P was not altered compared to the wild-type strains (data not shown).

Next, *manXYZ*, which encodes EIIB, IIC and IID of the major PTS^{Man}, but is also able to transport Glc (Erni & Zanolari, 1985; Erni *et al.*, 1987; Postma *et al.*, 1993), was deleted in both EIEC wild-type strains and their $\Delta ptsG$ mutants. The $\Delta manXYZ$ mutants grew with identical doubling times to the wild-type strains on Glc and Glc6P but much more slowly on Man (not shown). However,

Fig. 1. Growth of EIEC 4608-58 (a, e, i), EIEC HN280 (b, f, j), *E. coli* K-12 (c, g) and *Stm* 14028s (d, h, k) wild-type strains (WT) and their Glc- and/or Glc6P-uptake mutants in RPMI 1640 with 10 mM Glc (a, b, c, d, i, j, k) or Glc6P (e, f, g, h, i, j, k). As a control, growth of the WT in RPMI 1640 medium without carbohydrate (w/o) is shown. Generation times were calculated during exponential growth in the first 3 h. Generation times given in the text and on the figure are mean values $\pm$ SD of three independent experiments.

growth of the $\Delta ptsG$, $manXYZ$ mutants of both EIEC strains (and *E. coli* K-12, data not shown) in the presence of 10 mM Glc was very low (Fig. 1a, b). The $\Delta ptsG$, $manXYZ$ mutant of strain 4608-58 showed some residual growth with 10 mM Glc (reaching an OD₆₀₀ of 0.4 after 13 h) but not with 2 mM Glc, suggesting an additional low-affinity Glc transport system(s) in this strain.

Growth of the $\Delta ptsG$, $manXYZ$ mutants in the presence of 10 mM Glc6P was as efficient as that of the wild-type strains, while growth in the presence of 10 mM Man was as low as observed with the $\Delta manXYZ$ mutants (not shown).

The phenotype of the *uhpT* deletion mutants of the two EIEC strains is different

The two EIEC wild-type strains grew equally well in RPMI medium containing 10 mM Glc6P as did *E. coli* K-12 (Fig. 1e, f, g). The $\Delta uhpT$ mutants of EIEC HN280 and *E. coli* K-12 were unable to replicate in the presence of 10 mM Glc6P, indicating that UhpT is the only transporter for Glc6P in these strains (Fig. 1f, g). However, the corresponding $\Delta uhpT$ mutant of EIEC 4608-58 was still able to grow under these conditions, albeit at an elevated generation time (81 ± 0.4 min) compared to that of the wild-type strain (48 ± 1 min) (Fig. 1e).

A *uhpT* gene duplication in this EIEC strain could be ruled out by Southern blot analyses. Using two different probes comprising nt 50–622 and 216–1179 of the *uhpT* coding sequence, a single hybridizing band was detected with each probe in *EcoRI*-restricted genomic DNA of the wild-type strain, which was missing in the $\Delta uhpT$ mutant (data not shown).

Since recent reports (Schwoppe *et al.*, 2002, 2003) showed that the Glc6P sensor UhpC is also able to transport Glc6P in the absence of UhpT, we deleted *uhpC* in the EIEC 4608-58 wild-type strain and its $\Delta uhpT$ mutant. However, this deletion did not alter growth of these two strains in the presence of 10 mM Glc6P.

GlpT, the glycerol-3-phosphate antiporter, and PgtP, a putative phosphoenolpyruvate (PEP) transporting protein (Johnson *et al.*, 2007; Welch *et al.*, 2002) that shows sequence similarity with UhpT, were also tested for their ability to function as an additional Glc6P transporter in EIEC 4608-58 by deleting these genes. However, deletion of none of these genes in the wild-type strain or the $\Delta uhpT$ mutant affected Glc6P transport. As expected, *uhpT* mutants of both EIECs grew similarly to the wild-type strains in the presence of 2 and 10 mM Glc or Man (data not shown).

In vitro growth of EIEC mutants deficient in the major Glc and Glc6P uptake systems in the presence of Glc and/or Glc6P

Deletion of *ptsG*, $manXYZ$ and *uhpT* abolished growth of EIEC HN280 completely in the presence of 10 mM Glc and Glc6P (Fig. 1j) as well as Man. The $\Delta ptsG$, $manXYZ$, *uhpT* mutant of EIEC 4608-58 grew more slowly and reached a lower cell density in Glc- (and Man-) containing RPMI 1640 medium than the $\Delta ptsG$, $manXYZ$ mutant (Fig. 1i) due to partial autolysis, which occurred even in TSB (not shown).

However, in the presence of 2 and 10 mM Glc6P, the $\Delta ptsG$, $manXYZ$, *uhpT* mutant still replicated with the same generation time as the $\Delta uhpT$ mutant (Fig. 1i). This result rules out the possibility that an extracellular phosphatase(s) hydrolysing Glc6P to Glc may be responsible for the residual growth of the $\Delta uhpT$ mutant with Glc6P.

The growth defects of the $\Delta ptsG$, $manXYZ$, *uhpT* mutant of EIEC 4608-58 in RPMI medium with Glc, Man and Glc6P could be restored by *trans*-complementation with the plasmids pAG119 (carrying P_{ptsG} -*ptsG*), pAG124 (carrying P_{manXYZ} -*manXYZ*) and pAG078 (carrying P_{uhpT} -*uhpT*), respectively. Replication of the $\Delta ptsG$, $manXYZ$, *uhpT* mutant reached almost wild-type levels in the presence of Glc and Glc6P when complemented with pAG119 and pAG078, respectively (Fig. 2a, c), while pAG124 restored growth of the $\Delta ptsG$, $manXYZ$, *uhpT* mutant to a level

Fig. 2. Complementation of EIEC 4608-58 $\Delta ptsG$, $manXYZ$, *uhpT*. The Glc/Glc6P-uptake mutant of EIEC 4608-58 was *trans*-complemented with (a) pAG119 (carrying P_{ptsG} -*ptsG*), (b) pAG124 (carrying P_{manXYZ} -*manXYZ*) and (c) pAG078 (carrying P_{uhpT} -*uhpT*), as described in Methods. The *trans*-complemented strains were grown in RPMI 1640 with 10 mM Glc (a, b) or Glc6P (c). The WT and mutant strains transformed with the pBR322 vector alone were used as controls.

similar to that of the $\Delta ptsG$ mutant in the presence of Glc (Fig. 2b).

Growth properties of *S. Typhimurium* mutants with deletions in $\Delta ptsG$, $manXYZ$ and/or glk or $uhpT$

We also introduced similar deletions as described for the two EIEC strains into *S. Typhimurium* 14028s. This wild-type strain (abbreviated as *Stm* 14028s) grew on Glc or Glc6P with generation times of about 40 ± 2 min (Fig. 1d and h). The $ptsG$ deletion led to a doubling time of 53 ± 1 min in the presence of 10 mM Glc (Fig. 1d) but did not affect growth on Glc6P or Man.

Deleting $manXYZ$ inhibited growth on 10 mM Man but not on Glc or Glc6P (not shown). The $\Delta ptsG$, $manXYZ$ mutant did not grow on Man and grew more slowly on Glc (generation time 63 ± 1 min) than the wild-type strain and the $\Delta ptsG$ mutant, whereas growth on Glc6P was unaffected. However, in contrast with the corresponding EIEC mutants, the $\Delta ptsG$ and $\Delta ptsG$, $manXYZ$ mutants of *Stm* 14028s reached a similar cell density in Glc-containing RPMI during the stationary phase to the wild-type (Fig. 1d) even if the concentration was lowered to 2 mM. This suggests that other glucose-transporting PTS or other non-PTS glucose transporters, e.g. GalP and MglABC (Death & Ferenci, 1993; Postma, 1977), are active in these mutants. This was confirmed by deleting glk encoding a glucokinase, which phosphorylates glucose that is taken up by these non-PTS systems (Meyer *et al.*, 1997). The resulting $\Delta ptsG$, $manXYZ$, glk mutant showed a strong growth reduction in RPMI with 10 mM Glc (Fig. 1d), whereas the deletion of glk alone had no effect (not shown).

The $uhpT$ deletion strongly reduced growth in the presence of Glc6P (Fig. 1h) without affecting growth on Glc or Man. The $\Delta ptsG$, $manXYZ$, $uhpT$ mutant was unable to grow on Glc6P or Man as expected but could still grow on Glc with a doubling time similar to the $\Delta ptsG$, $manXYZ$ mutant (Fig. 1k).

Growth of the two EIEC strains and *Stm* 14028s on C_3 -substrates

Recently, it was shown that glycerol and possibly other C_3 -substrates may serve as preferred carbon sources for intracellular growth of *Listeria monocytogenes* (Eylert *et al.*, 2008). We therefore also tested the growth capacity of the EIEC strains, the *Stm* 14028s isolate and the generated carbohydrate uptake mutants on various C_3 -substrates.

All wild-type strains and their $\Delta ptsG$, $manXYZ$, $uhpT$ mutants were able to grow on 10 mM glycerol, lactate or pyruvate. As shown in Fig. 3 (for EIEC 4608-58 as an example), all wild-type strains and mutants grew on these substrates with generation times ranging from 60 to 70 min. The highest bacterial density was always reached in RPMI medium with a carbon source of glycerol, followed by lactate and pyruvate.

Fig. 3. Growth of EIEC 4608-58 WT on different C_3 -substrates at 10 mM; similar results to those shown here for EIEC 4608-58 WT were also found for the other two WT strains and the mutants. Glycerol, $\square$; lactate, $\circ$; pyruvate, $\triangle$; without substrate, ∇ .

Interestingly, the $\Delta ptsG$, $manXYZ$, $uhpT$ mutant of EIEC 4608-58 no longer exhibited autolysis when growing in the presence of either of these C_3 -substrates or on sugars other than Glc or Man.

Carbohydrate-uptake mutants of the two EIEC strains and of *Stm* 14028s show altered adhesion, invasion and intracellular replication rates in *Caco-2* cells

To test the influence of the defects in Glc and Glc6P uptake on the intracellular growth cycle of the analysed enterobacterial pathogens, we compared adhesion, internalization and intracellular generation times of the above-described mutants (determined as described in Methods) to those of the corresponding wild-type strains, using epithelial colorectal adenocarcinoma (*Caco-2*) cells as host cells.

As shown in Fig. 4 (EIEC 4608-58) and Table 3 (EIEC HN280 and *Stm* 14028s), the rates of internalization of the bacteria by *Caco-2* cells correlated in general with their adhesion efficiencies to these human cells. EIEC 4608-58 was about fivefold less adherent and invasive than EIEC HN280. However, a significant increase in adherence and invasiveness was observed with the $\Delta ptsG$ mutant as well as with the $\Delta ptsG$, $manXYZ$ and $\Delta ptsG$, $manXYZ$, $uhpT$ mutants of EIEC 4608-58. CCR is partially relieved due to the $\Delta ptsG$ mutation as indicated by a more than 10-fold increase of the β -galactosidase activity of the $\Delta ptsG$ mutants (Fig. 4). Thus, there might be a connection between the relaxed CCR and the increased adherence and subsequent internalization of the $\Delta ptsG$ mutants of EIEC 4608-58.

Only a slight increase in adherence and invasiveness was observed in the $\Delta ptsG$ mutant of EIEC HN280 but the $\Delta ptsG$, $manXYZ$ and $\Delta ptsG$, $manXYZ$, $uhpT$ mutants showed a sharp drop in both properties. No significant difference in adherence and invasiveness was observed between the *Stm* 14028s wild-type strain and the Glc and Glc6P uptake mutants (Table 3).

Fig. 4. Adhesion (light grey bars) and invasiveness (dark grey bars) of EIEC 4608-58 WT strain and mutants determined with Caco-2 cells (for details see Methods). The numbers on the right-hand y-axis represent the percentage of adherent or internalized bacteria relative to the total number of bacteria used for infection (3×10^6 c.f.u.). The β -galactosidase activity (left-hand y-axis; hatched bars) was also measured (after induction with lactose for 2 h) to monitor the state of CCR of the WT and the carbohydrate uptake mutants. * $P < 0.05$.

To determine the intracellular replication efficiency of the mutants in comparison to the wild-type strains, the numbers of bacteria used for infecting Caco-2 cells (90 % confluent cell layer) were adjusted according to their adhesion/invasion values (Fig. 4; Table 3) to reduce the intracellular bacterial count of the more invasive strains at the beginning of the intracellular growth period. Internalization by the Caco-2 cells was performed with bacteria resuspended in RPMI medium with and without 10 % FCS, as described in Methods. Intracellular bacterial growth was monitored over a time period of 8 h p.i.. After

8 h replication, the EIEC strains reached a stationary phase but no apoptosis of the infected cells occurred. Growth of *Stm* 14028s had not yet reached stationary phase at this time point but replicated considerably more slowly after 8 h p.i.

When the infection was carried out in RPMI medium in the presence of 10 % FCS, the EIEC 4608-58 wild-type as well as the $\Delta uhpT$ and $\Delta manXYZ$ mutants replicated with mean generation times of 47 ± 8 min (shown in Fig. 5a for the wild-type strain). The $\Delta ptsG$, $\Delta ptsG$, $manXYZ$ and $\Delta ptsG$, $uhpT$ mutants replicated at wild-type level until 3 h p.i., but their generation times dropped significantly in the next 3 h (shown in Fig. 5a for $\Delta ptsG$) compared with those of the wild-type strain (77 ± 12 min versus 53 ± 6 min). The $\Delta ptsG$, $manXYZ$, $uhpT$ mutant showed a very low generation time (215 ± 36 min) under these conditions (Fig. 5a). However, when the internalization of the EIEC 4608-58 wild-type and the mutants was carried out in FCS-free RPMI medium, the generation time of most mutants resembled that of the wild-type strain more, and even the $\Delta ptsG$, $manXYZ$, $uhpT$ mutant replicated only slightly more slowly than the wild-type strain (Fig. 5b).

The EIEC HN280 strain and most of its mutants multiplied in Caco-2 cells until 6 h p.i. with similar generation times (55 ± 3 min) regardless of whether the internalization occurred in FCS-free or FCS-containing RPMI medium (Fig. 5c, d). Only the $\Delta ptsG$, $manXYZ$ and $\Delta ptsG$, $manXYZ$, $uhpT$ mutants showed reduced intracellular generation times (70 ± 9 min) that hardly changed in the absence of FCS (Fig. 5c, d).

The $\Delta uhpT$, $\Delta manXYZ$ and Δglk mutants of *Stm* 14028s replicated in Caco-2 cells with the same intracellular doubling time as the wild-type strain (as shown in Fig. 5f for the Δglk mutant) while the $\Delta ptsG$ and even more the $\Delta ptsG$, $manXYZ$ and $\Delta ptsG$, $manXYZ$, $uhpT$ mutants replicated more slowly (Fig. 5e). As expected, the generation times of *Stm* 14028s and the mutants (between

Table 3. Adhesion and invasion rates of EIEC HN280 and *Stm* 14028s wild-type strains and mutants obtained with Caco-2 cells

Values are given as percentage of adherent or internalized bacteria relative to the total number of bacteria used for infection (3×10^6 c.f.u.). ND, Not determined.

	EIEC HN280		<i>Stm</i> 14028s invasion (%)
	Adhesion (%)	Invasion (%)	
WT	2.51 ± 0.65	1.37 ± 0.58	1.93 ± 0.74
$\Delta uhpT$	2.64 ± 0.62	1.19 ± 0.64	2.09 ± 1.21
$\Delta ptsG$	2.23 ± 1.12	1.19 ± 0.59	1.73 ± 0.74
$\Delta manXYZ$	2.63 ± 0.48	1.36 ± 0.72	ND
$\Delta ptsG$, $uhpT$	2.65 ± 1.13	1.22 ± 0.34	1.82 ± 0.91
$\Delta ptsG$, $manXYZ$	$0.13 \pm 0.03^*$	$0.22 \pm 0.07^*$	ND
$\Delta ptsG$, $manXYZ$, $uhpT$	$0.63 \pm 0.10^*$	$0.07 \pm 0.02^*$	1.80 ± 0.86

* $P < 0.05$.

Fig. 5. Intracellular growth of EIEC 4608-58 (a, b), EIEC HN280 (c, d) and *Stm* 14028s (e, f) WT and their Glc⁻ and/or Glc6P-uptake mutants in Caco-2 cells. Internalization was carried out in the presence (a, c, e, f) or absence (b, d) of 10% FCS with different amounts of bacteria as described in Methods to reduce the intracellular bacterial counts (first time point p.i.) of the highly invasive $\Delta ptsG$ mutants. Time point 0 h p.i. (indicated by arrows) marks the addition of gentamicin. Each experiment was performed three times in triplicate (mean $\pm$ SD shown); a representative experiment is shown.

150 and 170 min) were considerably longer than those observed with the EIEC wild-type strains and most of their mutants. Even the $\Delta ptsG$, *manXYZ*, *glk* mutant was still able to replicate in Caco-2 cells with a similar generation time to the $\Delta ptsG$, *manXYZ*, *uhpT* (Fig. 5f). These latter data were unexpected since a similar $\Delta ptsG$, *manXYZ*, *glk* mutant of strain *S. Typhimurium* 4/74 showed a much stronger growth inhibition when replicating in RAW 264.7 macrophages (Bowden *et al.*, 2009).

DISCUSSION

Glucose (Glc), mannose (Man) and glucose 6-phosphate (Glc6P) are the preferred carbon sources for growth of *E. coli* and the entire family of *Enterobacteriaceae*. These carbon sources may be also encountered within mammalian host cells by those family members that are able to invade and actively replicate in these host cells, mainly *Salmonella* spp. and *Shigella* spp. (as well as the closely related EIEC).

The transport of these carbohydrates has been extensively studied in the model enteric bacteria *E. coli* K-12 and *S. Typhimurium* LT2 where several PTS and non-PTS transporters for glucose were identified (Death & Ferenci, 1993; Henderson *et al.*, 1977; Postma *et al.*, 1993). PtsG/Crr is the major transporter for Glc, but ManXYZ, primarily a Man PTS (Erni & Zanolari, 1985; Erni *et al.*, 1987), can also

efficiently transport Glc in both species. The major transporter for Glc6P is the P_i/Glc6P antiporter UhpT (Island *et al.*, 1992).

As expected, deletions of *ptsG* and *manXYZ* in *E. coli* K-12 abolish growth entirely when the $\Delta ptsG$, *manXYZ* mutant is cultured in RPMI medium with low (2 mM) or high (10 mM) concentrations of Glc or Man as carbon source, and the *E. coli* K-12 $\Delta uhpT$ mutant is unable to multiply in RPMI with Glc6P. The well-defined RPMI medium is utilized in this study for *in vitro* growth, since uptake and replication of facultative intracellular bacteria in mammalian cells (including those reported here) is most frequently carried out in this medium.

The results obtained with the facultative intracellular enteric bacteria, two enteroinvasive *E. coli* strains (EIEC 4608-58 and EIEC HN280), one *S. Typhimurium* strain (*Stm* 14028s) and the corresponding mutants of these wild-type strains show, however, a more differentiated picture than *E. coli* K-12 with respect to Glc and Glc6P transport.

Deletion of *ptsG* alone leads to strong growth inhibition in the presence of Glc as sole carbon source in EIEC HN280 only, which is comparable to, but not as evident as, the inhibition of *E. coli* K-12; however, it has relatively little effect on growth of EIEC 4608-58 and *Stm* 14028s, even at low Glc concentration (2 mM). This Glc concentration can be expected in the cytosol of Caco-2 cells where the two

EIEC strains replicate, but the Glc concentration in the SCV where *Stm* 14028s multiplies is basically unknown under the applied culture conditions.

While deletion of *ptsG* and *manXYZ* inhibits *in vitro* growth at low Glc concentration in both EIEC strains, the Δ *ptsG*, *manXYZ* mutant of *Stm* 14028s shows still substantial growth under these conditions and reaches the same cell density as the wild-type strain in the stationary growth phase, suggesting that other Glc transporters, possibly the non-PTS permeases GalP and MglABC (Death & Ferenci, 1993; Postma, 1977), may function as efficient Glc transporters in *Stm* 14028s.

Growth differences between the two EIEC isolates and *E. coli* K-12 are especially remarkable with Glc6P as carbon source. While the Δ *uhpT* mutant of EIEC HN280, similar to the corresponding *E. coli* K-12 mutant, is unable to grow in the presence of Glc6P, the Δ *uhpT* mutant of EIEC 4608-58 can still grow with about a doubled generation time and reaches the same cell density as the wild-type strain in the stationary phase. Our results show that this growth capacity of the Δ *uhpT* mutant is not caused by a second *uhpT* gene. Transport of Glc6P by UhpC, which may occur in *E. coli* K-12 in the absence of UhpT (Schwoppe *et al.*, 2002, 2003), or by other UhpT-homologous transporters, like the glycerol-3-phosphate antiporter GlpT, or the putative PEP transporter PgtP (Johnson *et al.*, 2007; Welch *et al.*, 2002), can be ruled out too, since deletion of these genes does not alter the growth properties of the EIEC 4608-58 wild-type strain or the *uhpT* mutant in the presence of Glc6P. These data thus suggest that EIEC 4608-58 may possess another type of Glc6P transporter in addition to UhpT.

Adhesion of all three intracellular pathogens to Caco-2 cells correlates with their internalization by Caco-2 cells. Similar adhesion and invasion efficiencies are observed for *Stm* 14028s and EIEC HN280, while those of EIEC 4608-58 are more than fivefold lower. Loss of Glc and Glc6P transport does not significantly affect adhesion and invasiveness of *Stm* 14028s, but leads to significant variations in both properties of the EIEC strains.

Deletion of *ptsG* (but not of *manXYZ* or *uhpT*) results in a more than fivefold increased adherence and invasiveness of EIEC 4608-58, but not of EIEC HN280. Although the deletion of *ptsG* has only a moderate effect on Glc-mediated growth *in vitro* (Fig. 1a, b), it seems to lead to partial relaxation of CCR as indicated by the increased β -galactosidase activity of the Δ *ptsG* mutant. Expression of the IpaBC complex, mainly responsible for invasion of *Shigella* and EIEC (Cossart & Sansonetti, 2004) is not under CCR control (Dagberg & Uhlin, 1992), suggesting that an additional, CCR-regulated factor(s) may stimulate adhesion and subsequently invasion of EIEC 4608-58 to Caco-2 cells.

While the deletion of *ptsG* or *manXYZ* in EIEC HN280 has no effect on adhesion and invasion, the deletion of both

ptsG and *manXYZ* leads to a strong decrease of both properties of this strain. The reason for this decrease is presently unknown. A possible explanation might be an impaired synthesis and/or translocation of effector molecules (via the type III secretion system), necessary for adhesion/invasion (Cossart & Sansonetti, 2004; Lafont & van der Goot, 2005; Scherer *et al.*, 1997). The better adherence of the corresponding EIEC 4608-58 mutant might compensate for this impairment.

Although the Δ *ptsG*, *manXYZ* mutant of EIEC HN280 is unable to grow with Glc as sole carbon source *in vitro*, this mutant (similar to the Δ *ptsG*, *manXYZ*, *uhpT* mutant) replicates only slightly more slowly in Caco-2 cells than the wild-type strain (within the 8 h period of infection where intracellular growth was monitored and where no apoptosis of the infected cells occurred, the wild-type strain underwent eight, and the mutants six, cell divisions).

A similar picture is observed with *Stm* 14028s: here, the wild-type strain (replicating more slowly in the SCV) underwent four and the mutants, including the Δ *ptsG*, *manXYZ*, *glk* mutant, underwent three cell divisions, although these mutants (in particular the Δ *ptsG*, *manXYZ*, *glk* mutant) showed drastically reduced growth rates *in vitro* in the presence of low (2 mM) Glc and Glc6P, respectively.

The Δ *ptsG* mutant of EIEC 4608-58 exhibited only a moderate reduction in viable bacterial counts in Caco-2 cells compared with the wild-type strain when the bacteria were treated with 10% FCS prior to infection, but the Δ *ptsG*, *manXYZ*, *uhpT* mutant showed a strong reduction. This reduction seems to have been caused, however, mainly by increased killing of the FCS-treated mutant bacteria, since the untreated bacteria showed much better growth in Caco-2 cells, closely resembling that of the corresponding EIEC HN280 mutant. A higher fragility of the Δ *ptsG*, *manXYZ*, *uhpT* mutant of EIEC 4608-58 is also indicated by the increased autolysis in the stationary phase during extracellular growth.

These data show that intracellular growth of the two EIEC strains and the *S. Typhimurium* strain analysed in this study does not depend exclusively on Glc (or Glc6P) when replicating in Caco-2 cells. Indeed, an in-depth analysis of the intracellular carbon metabolism of the two EIEC strains and *Stm* 14028s by ^{13}C -isotopologue analysis with uniformly labelled [^{13}C]glucose revealed that Glc (but not Glc6P) is the preferred carbon source for EIEC HN280 and *Stm* 14028s when replicating in Caco-2 cells. But, both strains can readily switch to C_3 -carbon sources when Glc becomes unavailable (A. Götz and other authors, unpublished data). Thus, the relatively minor reduction of intracellular growth in Caco-2 cells of mutants lacking the ability to utilize Glc, compared with the strong growth inhibition of these mutants in RPMI medium with Glc probably reflects the capacity of the mutants to grow intracellularly on alternative carbon substrates in the absence of Glc. There may be, however, bacterial strain-

and/or host cell-specific differences with respect to the dependency on Glc for intracellular growth of *S. Typhimurium*. Recently, Bowden *et al.* (2009) reported that a $\Delta ptsG$, $manXYZ$, glk mutant of the *S. Typhimurium* 4/74 strain when replicating in RAW 264.7 macrophages exhibits a much stronger growth inhibition in these macrophages (Bowden *et al.*, 2009) than the corresponding mutant of strain *Stm* 14028s in Caco-2 cells analysed in this study.

Interestingly, neither the results reported here nor the ^{13}C -isotopologue analysis (A. Götz and other authors, unpublished data) show a significant contribution of Glc6P to intracellular replication of the EIEC strains (replicating in the cytosol) or *S. Typhimurium* (replicating in the SCV), although transcription of the *uhpT* gene is induced under these conditions (Hautefort *et al.*, 2008; Lucchini *et al.*, 2005). Possibly, the observed transcriptional *uhpT* induction is simply caused by a decreased CCR of the intracellular growing bacteria due to the lower Glc concentration in the host cells compared with the extracellular medium, but this has no major impact on the intracellular carbon metabolism of these enteric bacteria.

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REFERENCES

- Bell, K. S., Sebaihia, M., Pritchard, L., Holden, M. T., Hyman, L. J., Hovav, M. C., Thomson, N. R., Bentley, S. D., Churcher, L. J. & other authors (2004). Genome sequence of the enterobacterial phytopathogen *Erwinia carotovora* subsp. *atroseptica* and characterization of virulence factors. *Proc Natl Acad Sci U S A* **101**, 11105–11110.
- Beuzón, C. R., Meresse, S., Unsworth, K. E., Ruiz-Albert, J., Garvis, S., Waterman, S. R., Ryder, T. A., Boucrot, E. & Holden, D. W. (2000). *Salmonella* maintains the integrity of its intracellular vacuole through the action of SifA. *EMBO J* **19**, 3235–3249.
- Beuzón, C. R., Salcedo, S. P. & Holden, D. W. (2002). Growth and killing of a *Salmonella enterica* serovar Typhimurium *sifA* mutant strain in the cytosol of different host cell lines. *Microbiology* **148**, 2705–2715.
- Bolivar, F., Rodriguez, R. L., Greene, P. J., Betlach, M. C., Heynker, H. L., Boyer, H. W., Crosa, J. H. & Falkow, S. (1992). Construction and characterization of new cloning vehicles. II. A multipurpose cloning system. 1977. *Biotechnology* **24**, 153–171.
- Bowden, S. D., Rowley, G., Hinton, J. C. & Thompson, A. (2009). Glucose and glycolysis are required for the successful infection of macrophages and mice by *Salmonella enterica* serovar Typhimurium. *Infect Immun* **77**, 3117–3126.
- Brumell, J. H. & Grinstein, S. (2004). *Salmonella* redirects phagosomal maturation. *Curr Opin Microbiol* **7**, 78–84.
- Clermont, O., Bonacorsi, S. & Bingen, E. (2000). Rapid and simple determination of the *Escherichia coli* phylogenetic group. *Appl Environ Microbiol* **66**, 4555–4558.
- Cossart, P. & Sansonetti, P. J. (2004). Bacterial invasion: the paradigms of enteroinvasive pathogens. *Science* **304**, 242–248.
- Covarrubias, L., Cervantes, L., Covarrubias, A., Soberon, X., Vichido, I., Blanco, A., Kupersztosch-Portnoy, Y. M. & Bolivar, F. (1981). Construction and characterization of new cloning vehicles. V. Mobilization and coding properties of pBR322 and several deletion derivatives including pBR327 and pBR328. *Gene* **13**, 25–35.
- Dagberg, B. & Uhlin, B. E. (1992). Regulation of virulence-associated plasmid genes in enteroinvasive *Escherichia coli*. *J Bacteriol* **174**, 7606–7612.
- Datsenko, K. A. & Wanner, B. L. (2000). One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. *Proc Natl Acad Sci U S A* **97**, 6640–6645.
- Death, A. & Ferenci, T. (1993). The importance of the binding-protein-dependent Mgl system to the transport of glucose in *Escherichia coli* growing on low sugar concentrations. *Res Microbiol* **144**, 529–537.
- Erni, B. & Zanolari, B. (1985). The mannose-permease of the bacterial phosphotransferase system. Gene cloning and purification of the enzyme IIMan/IIIMan complex of *Escherichia coli*. *J Biol Chem* **260**, 15495–15503.
- Erni, B., Zanolari, B. & Kocher, H. P. (1987). The mannose permease of *Escherichia coli* consists of three different proteins. Amino acid sequence and function in sugar transport, sugar phosphorylation, and penetration of phage lambda DNA. *J Biol Chem* **262**, 5238–5247.
- Eylert, E., Schar, J., Mertins, S., Stoll, R., Bacher, A., Goebel, W. & Eisenreich, W. (2008). Carbon metabolism of *Listeria monocytogenes* growing inside macrophages. *Mol Microbiol* **69**, 1008–1017.
- Hautefort, I., Thompson, A., Eriksson-Ygberg, S., Parker, M. L., Lucchini, S., Danino, V., Bongaerts, R. J., Ahmad, N., Rhen, M. & Hinton, J. C. (2008). During infection of epithelial cells *Salmonella enterica* serovar Typhimurium undergoes a time-dependent transcriptional adaptation that results in simultaneous expression of three type 3 secretion systems. *Cell Microbiol* **10**, 958–984.
- Henderson, P. J., Giddens, R. A. & Jones-Mortimer, M. C. (1977). Transport of galactose, glucose and their molecular analogues by *Escherichia coli* K12. *Biochem J* **162**, 309–320.
- Island, M. D., Wei, B. Y. & Kadner, R. J. (1992). Structure and function of the *uhp* genes for the sugar phosphate transport system in *Escherichia coli* and *Salmonella typhimurium*. *J Bacteriol* **174**, 2754–2762.
- Jin, Q., Yuan, Z., Xu, J., Wang, Y., Shen, Y., Lu, W., Wang, J., Liu, H., Yang, J. & other authors (2002). Genome sequence of *Shigella flexneri* 2a: insights into pathogenicity through comparison with genomes of *Escherichia coli* K12 and O157. *Nucleic Acids Res* **30**, 4432–4441.
- Johnson, T. J., Kariyawasam, S., Wannemuehler, Y., Mangiamale, P., Johnson, S. J., Doetkott, C., Skyberg, J. A., Lynne, A. M., Johnson, J. R. & Nolan, L. K. (2007). The genome sequence of avian pathogenic *Escherichia coli* strain O1:K1:H7 shares strong similarities with human extraintestinal pathogenic *E. coli* genomes. *J Bacteriol* **189**, 3228–3236.
- Klumpp, J. & Fuchs, T. M. (2007). Identification of novel genes in genomic islands that contribute to *Salmonella typhimurium* replication in macrophages. *Microbiology* **153**, 1207–1220.
- Lafont, F. & van der Goot, F. G. (2005). Bacterial invasion via lipid rafts. *Cell Microbiol* **7**, 613–620.
- Lucchini, S., Liu, H., Jin, Q., Hinton, J. C. & Yu, J. (2005). Transcriptional adaptation of *Shigella flexneri* during infection of macrophages and epithelial cells: insights into the strategies of a cytosolic bacterial pathogen. *Infect Immun* **73**, 88–102.
- McClelland, M., Sanderson, K. E., Spieth, J., Clifton, S. W., Latreille, P., Courtney, L., Porwollik, S., Ali, J., Dante, M. & other authors

- (2001). Complete genome sequence of *Salmonella enterica* serovar Typhimurium LT2. *Nature* **413**, 852–856.
- Meyer, D., Schneider-Fresenius, C., Horlacher, R., Peist, R. & Boos, W. (1997). Molecular characterization of glucokinase from *Escherichia coli* K-12. *J Bacteriol* **179**, 1298–1306.
- Nicoletti, M., Superti, F., Conti, C., Calconi, A. & Zagaglia, C. (1988). Virulence factors of lactose-negative *Escherichia coli* strains isolated from children with diarrhea in Somalia. *J Clin Microbiol* **26**, 524–529.
- Phalipon, A. & Sansonetti, P. J. (2007). *Shigella*'s ways of manipulating the host intestinal innate and adaptive immune system: a tool box for survival? *Immunol Cell Biol* **85**, 119–129.
- Postma, P. W. (1977). Galactose transport in *Salmonella typhimurium*. *J Bacteriol* **129**, 630–639.
- Postma, P. W., Lengeler, J. W. & Jacobson, G. R. (1993). Phosphoenolpyruvate:carbohydrate phosphotransferase systems of bacteria. *Microbiol Rev* **57**, 543–594.
- Sansonetti, P. J., d'Hauteville, H., Formal, S. B. & Toucas, M. (1982). Plasmid-mediated invasiveness of “*Shigella*-like” *Escherichia coli*. *Ann Microbiol (Paris)* **133**, 351–355.
- Scherer, C. A., Hantman, M. J. & Miller, S. I. (1997). *Salmonella* invasion and delivery of protein effectors to mammalian cell cytoplasm. *Trends Microbiol* **5**, 127–129.
- Schwoppe, C., Winkler, H. H. & Neuhaus, H. E. (2002). Properties of the glucose-6-phosphate transporter from *Chlamydia pneumoniae* (HPTcp) and the glucose-6-phosphate sensor from *Escherichia coli* (UhpC). *J Bacteriol* **184**, 2108–2115.
- Schwoppe, C., Winkler, H. H. & Neuhaus, H. E. (2003). Connection of transport and sensing by UhpC, the sensor for external glucose-6-phosphate in *Escherichia coli*. *Eur J Biochem* **270**, 1450–1457.
- Stock, J. B., Waygood, E. B., Meadow, N. D., Postma, P. W. & Roseman, S. (1982). Sugar transport by the bacterial phosphotransferase system. The glucose receptors of the *Salmonella typhimurium* phosphotransferase system. *J Biol Chem* **257**, 14543–14552.
- Vazquez-Torres, A. & Fang, F. C. (2000). Cellular routes of invasion by enteropathogens. *Curr Opin Microbiol* **3**, 54–59.
- Verhamme, D. T., Postma, P. W., Crielgaard, W. & Hellingwerf, K. J. (2002). Cooperativity in signal transfer through the Uhp system of *Escherichia coli*. *J Bacteriol* **184**, 4205–4210.
- Welch, R. A., Burland, V., Plunkett, G., III, Redford, P., Roesch, P., Rasko, D., Buckles, E. L., Liou, S. R., Boutin, A. & other authors (2002). Extensive mosaic structure revealed by the complete genome sequence of uropathogenic *Escherichia coli*. *Proc Natl Acad Sci U S A* **99**, 17020–17024.

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