The gene *yghK* linked to the *glc* operon of *Escherichia coli* encodes a permease for glycolate that is structurally and functionally similar to L-lactate permease

M. Felisa Núñez, M. Teresa Pellicer, Josefa Badía, Juan Aguilar and Laura Baldomà

Author for correspondence: Juan Aguilar. Tel: +34 93 403 4496. Fax: +34 94 402 4520.
e-mail: jaguilar@farmacia.far.ub.es

Department of
Biochemistry, Faculty of
Pharmacy, University of
Barcelona, Diagonal 643,
08028 Barcelona, Spain

In *Escherichia coli* the *glc* operon involved in glycolate utilization is located at 67.3 min and formed by genes encoding the enzymes glycolate oxidase (*glcDEF*) and malate synthase G (*glcB*). Their expression from a single promoter upstream of *glcD* is induced by growth on glycolate and regulated by the activator encoded by the divergently transcribed gene *glcC*. Gene *yghK*, located 350 bp downstream of *glcB*, encodes a hydrophobic protein highly similar to the L-lactate permease encoded by *lldP*. Expression studies have shown that the *yghK* gene (proposed name *glcA*) is transcribed from the same promoter as the other *glc* structural genes and thus belongs to the *glc* operon. Characterization of a *glcA::cat* mutant showed that GlcA acts as glycolate permease and that glycolate can also enter the cell through another transport system. Evidence is presented of the involvement of L-lactate permease in glycolate uptake. Growth on this compound was abolished in a double mutant of the paralogous genes *glcA* and *lldP*, and restored with plasmids expressing either GlcA or LldP. Characterization of the putative substrates for these two related permeases showed, in both cases, specificity for the 2-hydroxy-monocarboxylates glycolate, L-lactate and D-lactate. Although both GlcA and LldP recognize D-lactate, mutant analysis proved that L-lactate permease is mainly responsible for its uptake.

Keywords: gene function assignment, bacterial transport, LctP transporter family, paralogue genes

INTRODUCTION

Escherichia coli uses glycolate as a carbon and energy source in aerobic conditions. This compound is abundant in nature and especially in algae or plants, where it is an important metabolite of photorespiration (Friedrich *et al.*, 1991; Stewart & Codd, 1981). Glycolate is metabolized in *E. coli* through oxidation to glyoxylate (Hansen & Hayashi, 1962; Kornberg & Sadler, 1961) in a reaction catalysed by glycolate oxidase (Lord, 1972; Ornston & Ornston, 1969). Glyoxylate is a branching point in the metabolic pathway since it is metabolized by two divergent reactions. One, catalysed by malate synthase G, condenses glyoxylate with acetyl-CoA

(Vanderwinkel & DeVlieghere, 1968), while the other condenses two molecules of glyoxylate in a process catalysed by glyoxylate carboligase, which simultaneously decarboxylates the intermediate to tartronic semialdehyde (Chang *et al.*, 1993).

The locus *glc* (67.3 min), associated with glycolate utilization in *Escherichia coli*, is known to contain *glcB*, encoding malate synthase G, the genes *glcDEF* needed for glycolate oxidase activity, and *glcG*, of unknown function (Molina *et al.*, 1994; Pellicer *et al.*, 1996). However, the *gcl* gene encoding glyoxylate carboligase is not linked to the *glc* locus and is located at 12 min (Chang *et al.*, 1993). The structural genes of the *glc* locus are organized as an operon (*glcDEFGB*) and are transcribed as a single message from a promoter located at the 5' end of *glcD*. Expression of this operon is induced by growth on glycolate and is under the control of an

Abbreviations: CAA, casein acid hydrolysate; CAT, chloramphenicol acetyltransferase; LB, Luria–Bertani broth; IHF, integration host factor.

activator protein encoded by the divergently transcribed gene *glcC* (see Fig. 1) Expression is strongly dependent on the integration host factor (IHF) and is repressed by the global respiratory regulator ArcA-P. *In vitro* gel-shift experiments demonstrated direct binding of the promoter DNA to IHF and ArcA-P (Pellicer *et al.*, 1999).

As glycolate (pK_a 3.8) exists primarily as an anion at physiological pH it has been postulated that a protein is required for its transport across the cell membrane. Transporters for glycolate have been described in chloroplasts (Howitz & McCarty, 1991) and in several algae (Wilson & Tolbert, 1991), but not in bacteria. In eukaryotic cells a monocarboxylate transporter family has been proposed (Halestrap & Price, 1999) and in mammals, the members of this family are involved in the proton-linked transport of lactate, pyruvate, ketone bodies and acetate in different tissues. Kinetic studies have shown that these transporters can use glycolate as substrate with different affinities (Jackson & Halestrap, 1996). In bacteria, several lactate permeases have been reported (Matin & Konings, 1973; Sensen *et al.*, 1996), including a proton-linked activity from *E. coli* (Collins *et al.*, 1976). However, none of these bacterial permeases displays sequence similarities to the members of the eukaryotic monocarboxylate transporter family.

L-Lactate permease of *E. coli* is encoded by the *lld* operon (formerly labelled *lct*) (Berlyn, 1998). This operon, located at 81.4 min, contains three overlapping genes encoding the permease (*lldP*), the regulator protein (*lldR*) and the L-lactate dehydrogenase (*lldD*). Its transcription is activated by growth on L-lactate from the single promoter upstream of *lldP* (Dong *et al.*, 1993). No information is available on the substrate specificity of this permease.

In this report we identify the *yghK* gene product as a permease for glycolate uptake and characterize it as a transporter structurally and functionally similar to L-lactate permease. It is also shown that these two paralogous proteins exhibit a common substrate specificity for 2-hydroxymonocarboxylates.

METHODS

Bacterial strains, plasmids and phages. All the strains used were *E. coli* K-12 derivatives. The genotypes and sources of the relevant bacterial strains and plasmids are given in Table 1.

Cell growth. Cells were grown aerobically on Luria-Bertani broth (LB) or minimal medium (Boronat & Aguilar, 1979). For growth on minimal medium, carbon sources were added at the following concentrations unless otherwise specified: D-xylose, 12 mM; D-glucose, 10 mM; glycolate and acetate, 30 mM; D-lactate, L-lactate and glycerol, 20 mM; and casein acid hydrolysate (CAA), 0.5%. The following antibiotic concentrations were used unless otherwise noted: ampicillin, 100 µg ml⁻¹; tetracycline, 12.5 µg ml⁻¹; kanamycin, 50 µg ml⁻¹; and chloramphenicol, 30 µg ml⁻¹. X-Gal and IPTG were used at 30 and 10 µg ml⁻¹, respectively.

Transport and enzyme assays. For uptake assays, chloramphenicol was added at a final concentration of 40 µg ml⁻¹ to cultures at the end of exponential phase. After 15 min, the cells

were collected, washed twice and suspended in minimal medium (Boronat & Aguilar, 1979). Glycolate permease activity was determined at 24 °C from the rate of [¹⁴C]-glycolate [35 mCi mmol⁻¹ (1295 MBq mmol⁻¹), ICN] uptake by whole cells. The assay was started by adding glycolate at 0.03 mM concentration [25 mCi mmol⁻¹ (925 MBq mmol⁻¹)] to a suspension of 10⁹ cells ml⁻¹. After 5 s (5 s uptake times reflect initial velocities), samples of 100 µl were taken and filtered through a 0.45 µm-pore-size filter. The filters were washed with 5 ml minimal medium, and processed for counting in a scintillator. L-Lactate permease assays were performed in the same conditions with L-[¹⁴C]lactate [80 mCi mmol⁻¹ (2960 MBq mmol⁻¹), ICN]. Specific activities were expressed in nmol min⁻¹ (mg total protein)⁻¹. To identify competitors for the glycolate or L-lactate uptake, the assays were performed in the presence of a 20-fold excess of unlabelled carboxylate compounds, unless otherwise specified.

Specific β -galactosidase activity was assayed at 28 °C in cells permeabilized with chloroform and SDS, with *o*-nitrophenyl β -D-galactopyranoside being used as substrate, and expressed as Miller units (Miller, 1992).

Malate synthase (EC 4.1.3.2) and glycolate oxidase (EC 1.1.3.15) activities were assayed in crude extracts prepared as described by Boronat & Aguilar (1979), in 10 mM Tris/HCl, pH 8.0 containing 1 mM MgCl₂. Total activity of malate synthase, and the relative concentrations of the two isozymes malate synthase A and malate synthase G, were determined as described by Ornston & Ornston (1969). Immunological determination of malate synthase G was carried out by immunoelectrophoresis as described previously (Pellicer *et al.*, 1999). Activity of glycolate oxidase was determined spectrophotometrically according to the method of Lord (1972). As indicated by that author, the 30% ammonium sulfate fraction resuspended in 10 mM phosphate buffer was routinely used as a source of enzyme to avoid non-specific reduction of 2,6-dichloroindophenol.

Uptake and activity values reported are the means of at least three separate experiments performed in duplicate. Protein concentration was determined by the Lowry method using bovine serum albumin as standard.

Mutagenesis and genetic techniques. Phage P1 transduction experiments were performed as described by Miller (1992). Tn5 insertion mutagenesis was carried out by infection with phage λ 467 (*b221 cts857 rex::Tn5 Oam29 Pam80*), as described by Bruijn & Lupski (1984). Tn5 insertion mutants in the *lld* operon were obtained from strain MC4100 and selected by their inability to grow on L-lactate.

Primer TN5A (5'-TGGAAAACGGGAAAGGTTCCG-3'), corresponding to an internal sequence of the transposon, was used to amplify and sequence the insertion.

In the gene-inactivation experiments the chloramphenicol-resistance gene cassette CAT19 (Fuqua, 1992; Winans *et al.*, 1985) was inserted into the internal *Hind*III site of the *yghK* gene in plasmid pFN11. The plasmid carrying the inactivated gene was linearized by digestion with *Nru*I and used to transform strain JC7623 to chloramphenicol resistance (Cm^r). This strain efficiently recombines linear DNA into its chromosome (Winans *et al.*, 1985). P1vir lysates obtained from the selected Cm^r recombinants were used to transduce the *cat* insertions into strain MC4100. Chromosomal insertions were confirmed by PCR.

DNA manipulation. Bacterial genomic DNA was obtained as described by Silhavy *et al.* (1984). Plasmid DNA was routinely

Table 1. Strains and plasmids used in this work

Description		Source or reference
<i>E. coli</i> strains		
XL-1 Blue	<i>recA1 lac endA1 gyrA96 thi hsdR17 supE44 relA1</i> (F' <i>proAB lacI^a lacZ</i> Δ M15 Tn10)	Stratagene
MC4100	<i>araD Δlac rpsL flbB deoC ptsF rbsR</i>	Casadaban (1976)
TE2680	F ⁻ λ^- IN(<i>rrnD-rrnE</i>) Δ <i>lacX74 rplS galK2</i> <i>recD::Tn10d-tet trpDC700::putA13033::</i> (Kan ^r Cm ^r <i>lac</i>)	Elliot (1992)
JC7623	<i>arg thi thr leu pro his strA recB21 recC22 sbcB15</i>	Wackernagel (1973)
CH1827	<i>araD139 Δ(<i>araBC-leu</i>)7687 Δ<i>lacX74 galU galK hsdR</i> (<i>r_k⁻ m_k⁺</i>) <i>rpsL160 thi zce-726::Tn10</i></i>	
CH1828	CH1827 <i>rne-50^{ts}</i>	Mudd <i>et al.</i> (1990)
JA200	MC4100 <i>glcA::cat</i>	This work
JA201	MC4100 <i>lldP::Km</i>	This work
JA202	MC4100 <i>glcA::cat lldP::Km</i>	(JA201) P1 $\times$ JA200
Plasmids		
pBluescript SK	Ap ^r cloning vector	Stratagene
pBR322	Ap ^r Tc ^r cloning vector	Boehringer Mannheim
pCAT19	Ap ^r Cm ^r ; source of <i>cat</i> gene	Fuqua (1992)
pRS550	Ap ^r Km ^r ; <i>lacZ</i> fusion vector	Simons <i>et al.</i> (1987)
pFN11	<i>glcABG'</i> (7 kb <i>EcoRI-NruI</i> fragment) in pBR322	This work
pFN12	<i>glcAB''</i> 4.1 kb <i>SalI-NsiI</i> fragment) in pBR322	This work
pFN20	<i>lldP</i> in pBR322	This work

prepared by the boiling method (Holmes & Quigley, 1981). For large-scale preparation, a crude DNA sample was subjected to purification on a column (Qiagen). DNA manipulations were performed essentially as described by Sambrook *et al.* (1989). DNA sequencing was done by using an automated ABI 377 DNA sequencer and fluorescent dye termination methods. DNA fragments were amplified by PCR using *E. coli* chromosomal DNA as template. When necessary, specific restriction sites were incorporated at the 5' end of the primers to facilitate the cloning of the fragments in the appropriate vector. PCR reactions were performed with *pfu* DNA polymerase in standard conditions.

Plasmid pFN20, bearing the *lldP* gene, was constructed by cloning into pBR322 a 2.7 kb PCR fragment containing the entire coding region. The insert of this plasmid was made by using primer LctP1 (5'-GGAGGATCCAGAAGCTGATGAAGATGC-3') and primer LctP2 (5'-GCGAATTCACCAGATACATCCGCTGAC-3') bearing respectively the *Bam*HI and the *Eco*RI restriction sites (underlined).

Isolation of RNA and Northern blot hybridization. For preparation of total RNA, cells of a 25 ml culture grown to an OD₆₅₀ of 0.5 were collected by centrifugation at 5000 g and processed according to Belasco *et al.* (1985). Northern blot hybridization was performed with each RNA sample (10 μ g) by the procedure described by Moralejo *et al.* (1993).

Construction of *lacZ* fusions to analyse promoter function. Transcriptional fusions were constructed by inserting the DNA fragments into plasmid pRS550 (Simons *et al.*, 1987). This plasmid carries a cryptic *lacZ* operon and genes that confer resistance to both kanamycin and ampicillin. To construct the *glcA-lacZ* fusion, the 1382 bp *NsiI-Bst*XI fragment was excised from plasmid pFN11 and ligated into the *Bam*HI site of plasmid pRS550. This fusion comprises

1145 bp upstream of the translational start site of *glcA*. To construct a *lld-lacZ* operon fusion, primers LctP1 (described above) and LctP3 (5'-GCGAATTCAGCGATGCTATCAGACTGG-3') were used to amplify a 0.6 kb DNA fragment comprising 541 bp upstream of the *lldP* translational start site. The fragment was digested with *Bam*HI and *Eco*RI and cloned into pRS550.

After introduction of the recombinant plasmids into the tetracycline-resistant strain XL-1 Blue, blue colonies were isolated on LB plates containing X-Gal, ampicillin and kanamycin. Plasmid DNA was sequenced by using the M13 primer to ensure that the desired fragment was inserted in the correct orientation. Single-copy fusions on the *E. coli* chromosome were obtained by the method of Elliot (1992). Plasmids containing the different *lacZ* fusions were linearized with *Xho*I and used to transform strain TE2680. Due to the presence in strain TE2680 of the *recD::Tn10* mutation and sequences inserted into the *trp* operon that are homologous to sequences in pRS plasmids, this strain recombines linear pRS550- or pRS551-based plasmids into its chromosome. The transformants were selected for kanamycin resistance and screened for sensitivity to ampicillin and chloramphenicol. P1vir lysates were made to transduce the fusions into the desired genomic backgrounds.

RESULTS

Characterization of the *yghK* gene

Sequences flanking the *glc* locus region made available from the *E. coli* genome project (Blattner *et al.*, 1997) displayed the entire open reading frame *f*560 (*yghK* gene), which was truncated in clone pLB10 described in our previous *glc* operon study (Pellicer *et al.*, 1996) (Fig.

Fig. 1. Restriction map and gene organization of the *glc* operon. The open bar represents the genomic fragment inserted in clone λ 1G7 of the Kohara library, and the black bar represents the phage DNA. Relevant restriction sites are shown along the *E. coli* DNA as follows: B, *Bam*HI; Bx, *Bst*XI; H, *Hind*III; N, *Nru*I; Ni, *Nsi*I; S, *Sal*I. Open arrows indicate the direction and position of the *glc* genes. The inserts of the plasmids used in this study are indicated by thin lines below the map. The grey line over *glcA* corresponds to the gene fragment used as probe in the Northern experiments.

1). Here, this gene, located 350 bp downstream of *glcB*, is referred to as *glcA* for its involvement in glycolate utilization. The corresponding amino acid sequence (Q46839) was used as the query sequence for a BLASTP search of the GeneBank database using the NCBI BLAST server. High similarity was found between this sequence and the L-lactate permease encoded by *lldP* in *E. coli* (P33231). Alignment of the GlcA and LldP primary sequences showed an identity of 65 % and a similarity of 80 %. According to the protein structure prediction of the NCBI search program, the *glcA* gene was shown to encode a highly hydrophobic protein with 13 potential transmembrane segments, suggesting a membrane location for GlcA. It seems likely that this protein corresponds to a membrane permease which could be involved in glycolate transport.

We next cloned the *glcA* gene from clone λ 1G7 of the Kohara library (Kohara *et al.*, 1987). By restriction site analysis and subsequent digestion and ligation a 7 kb *Eco*RI–*Nru*I fragment containing *glcA* was inserted into pBR322, yielding plasmid pFN11 (Fig. 1). To obtain a recombinant plasmid encoding only *glcA* but not the neighbouring genes, further subcloning was performed giving rise to plasmid pFN12 (Fig. 1).

Expression of *glcA*

Expression of *glcA* was studied by Northern blot analysis and by transcriptional fusion experiments of the putative promoter region. For Northern analysis, total RNA was prepared from cultures of wild-type strain MC4100 grown on CAA in the absence or presence of the inducer glycolate. Using a *Bst*XI internal probe of *glcA*, hybridization bands were only detected with RNA preparations obtained from cultures grown in inducing conditions. In these experiments several bands of 1.6–4.0 kb were observed, possibly due to polycistronic message decay (not shown). To circumvent such a possibility, we grew the RNase E temperature-sensitive mutant strain CH1828 and its isogenic parent CH1827 on CAA in the presence of glycolate to compare the results of Northern blots using the same *glcA* internal

Fig. 2. Northern blot analysis of *glcA* expression. Total RNA was obtained from wild-type strain CH1827 and its isogenic derivative mutant strain CH1828 with temperature-sensitive RNase E as follows. Cells were grown at 30 °C to an OD₆₅₀ of 0.5, and a sample of each culture was withdrawn (strain CH1827, lane 2; strain CH1828, lane 4) before shifting the growth temperature to 45 °C. A second sample of each culture (strain CH1827, lane 1; strain CH1828, lane 3) was taken after 45 min. Hybridization was performed with a 1073 bp *glcA*-specific probe (*Bst*XI internal fragment).

probe. Only RNA preparations of mutant strain CH1828 grown at the restrictive temperature showed an mRNA of 8.5 kb corresponding to the full-length transcript of the *glc* system (Fig. 2). The same polycistronic mRNA was detected using either *glcD* or *glcB* as a probe (not shown). Since no internal promoters had been found in the *glcDEFG* gene cluster (Pellicer *et al.*, 1999), the presence of multiple bands in these RNA preparations could only be assigned to message decay.

Transcription of the *glcA* gene together with genes *glcDEFG* as a single unit was further supported by the absence of promoter activity in the 5'-flanking region of

Table 2. Growth and glycolate uptake in wild-type and mutant strains

Strain	Relevant genotype	Doubling time (h)*	[¹⁴ C]Glycolate uptake (nmol min ⁻¹ mg ⁻¹)†
MC4100	Wild-type	5	23.1 ± 1.4
JA200	<i>glcA::cat</i>	8.5	7.1 ± 0.8
JA201	<i>lldP::Tn5</i>	6.5	10.3 ± 0.7
JA202	<i>glcA::cat lldP::Tn5</i>	–	< 0.3

* Growth rates were measured in minimal medium plus glycolate.

† Due to the inability of strain JA202 to grow on minimal medium plus glycolate, uptake measurements were performed in cells grown on CAA in the presence of 30 mM glycolate.

glcA shown by *lacZ* fusion experiments. To test any possible promoter function activating this gene expression, a 1382 bp *NsiI*–*BstXI* fragment, containing the 350 bp intergenic region upstream of the *glcA* start codon, was fused to *lacZ*. The construction was introduced as a single copy in strain MC4100 and the resulting merodiploid was grown on CAA with or without glycolate as inducer. No β -galactosidase activity was detected in any of the conditions, indicating the absence of promoter activity in this intergenic fragment.

Role of the *glcA* gene product in glycolate transport

Disruption of *glcA* by a CAT cassette inserted in the *HindIII* restriction site and transfer of the mutant gene to the MC4100 genome yielded strain JA200. Growth of this mutant on glycolate was not totally abolished but its rate was much lower than that of the parental strain, with a doubling time of 8.5 h for strain JA200 and 5 h for wild-type strain MC4100 (Table 2). Control cultures in CAA, acetate, L-lactate, glucose or glycerol did not display significant differences between JA200 and MC4100.

Levels of glycolate oxidase activity and immunologically detected malate synthase G protein were similar in strains JA200 and MC4100 when grown in the presence of glycolate (not shown). These results indicated that expression of the structural genes of the *glc* system was not affected by the *cat* insertion. Consistently, expression of a transcriptional fusion of the *glc* operon, Φ (*glcD*–*lacZ*) (Pellicer *et al.*, 1999), in the genetic background of strain JA200 yielded the same β -galactosidase activity as strain MC4100 (not shown). Thus, the slow growth of strain JA200 on glycolate was not due to a reduced metabolic rate and may be explained by lower transport activity. The rate of entry of radioactive glycolate was measured in mutant strain JA200 and its parental strain grown in the presence of glycolate. The rate of glycolate uptake in the *glcA* mutant was not null but diminished to one-third of the value determined in wild-type cells (Table 2).

Specific dependence of radioactive glycolate uptake on the transporter function was ascertained by performing the experiments in cells where the metabolic carbon incorporation was made negligible. Glycolate oxidase mutants, deficient in metabolism of the substrate, could

Fig. 3. Dependence of glycolate transport on the presence of the functional GlcA or LldP proteins. Time course of [¹⁴C]glycolate uptake by whole cells grown in the presence of glycolate was performed at 10 °C for strains MC4100 (○), JA200 (●), JA201 (□) and JA202 (■).

not be used for this purpose because they yielded artifactual results due to the accumulation of endogenous glycolate and overexpression of the *glc* system. Instead we minimized the metabolic contribution to radioactive incorporation by carrying out the transport experiments at 10 °C. In these conditions the time course of uptake is asymptotic and proportional to the transporter function, with an initial rate for mutant JA200 about one-third of that found for wild-type cells (Fig. 3).

These results support the hypothesis that *glcA* encodes a glycolate permease and suggest that glycolate also enters the cell through another transport protein. Consistent with the role of *glcA* in the transport of glycolate is the fact that the growth rate on this carbon source was restored when the *glcA::cat* mutant strain JA200 was transformed with plasmid pFN12.

Glycolate transport through L-lactate permease

The high similarity between the L-lactate and glycolate permeases indicated above, which led some authors to classify them in the same family (Saier, 2000), opens the possibility that the L-lactate permease could be the alternative transport system for glycolate. This hypothesis was explored in experiments in which glycolate was used as competitor for L-lactate uptake in cells of strain JA200 induced by growth on L-lactate. Activities

Table 3. Competition of different carboxylates with the uptake of glycolate through GlcA or with the uptake of L-lactate through LldP

Competing carboxylate	[¹⁴ C]Glycolate uptake in strain JA201*		L-[¹⁴ C]Lactate uptake in strain JA200*	
	Rate†	% of JA201 control	Rate†	% of JA200 control
None	10.1 ± 0.8	100	3.6 ± 0.4	100
Glycolate	—	—	2.2 ± 0.3	60
L-Lactate	1.8 ± 0.3	18	—	—
D-Lactate	2.4 ± 0.4	24	1.5 ± 0.3	43
L-Glycerate	7.1 ± 0.8	70	3.4 ± 0.3	95
D-Glycerate	7.3 ± 0.7	72	3.6 ± 0.2	99
Acetate	10.6 ± 0.9	105	3.8 ± 0.4	105
Malate	11.1 ± 1.0	110	3.8 ± 0.4	105
Succinate	9.9 ± 0.8	99	3.5 ± 0.3	97
Citrate	10.3 ± 0.7	102	3.6 ± 0.4	99

* Uptake was measured using 0.03 mM [¹⁴C]glycolate or L-[¹⁴C]lactate in the presence of 0.6 mM competing carboxylate.

† Rate is expressed as nmol min⁻¹ (mg total protein)⁻¹.

of L-lactate permease were indeed inhibited by increasing concentrations of glycolate in the assay mixture. When added at a concentration 10-fold higher than that of the labelled substrate, glycolate caused a 25% inhibition with respect to the rate of L-lactate uptake in the absence of any competitor.

Further evidence of the involvement of L-lactate permease in glycolate uptake was obtained with a Tn5 insertion mutant in *lldP* obtained as described in Methods. To rule out the possibility of multiple transposon insertions, this mutation was P1-transduced into the wild-type strain MC4100 and again selected for the same phenotype (strain JA201). Location of the Tn5 insertion in *lldP* was first mapped by PCR amplification of JA201 chromosomal DNA using the specific transposon primer TN5A and oligonucleotides LctP1 and LctP2 flanking the *lldP* gene. The precise location was determined by sequencing the region adjacent to the Tn5 joining site using primer TN5A. Analysis of this sequence showed that the Tn5 transposon was inserted 341 nucleotides downstream of the start codon of gene *lldP*. A double mutant defective in both L-lactate permease and glycolate permease was constructed by introducing the *lldP*::Tn5 mutation into the genomic background of strain JA200, yielding strain JA202.

Strain JA201 displayed a glycolate uptake around 50% lower than the wild-type cells. Consistently, the growth rate of strain JA201 on glycolate was slightly lower than wild-type, with a doubling time of 6.5 h. The double mutant strain JA202 was unable to grow on glycolate and displayed undetectable glycolate uptake when grown on CAA in the presence of glycolate (Table 2). This double mutant recovered the ability to grow on glycolate when transformed with plasmids pFN12 or pFN20, expressing GlcA or LldP respectively.

Time-course experiments performed at 10 °C showed for mutant JA201 an initial rate one-half of that found for the wild-type cells and a null incorporation for the double mutant JA202, thus proving again the specific dependence of the measured transport on the activity of the proposed transporters (Fig. 3). To show that the mutant strain JA202 had no other metabolic functions altered, we tested the growth rates on other carbon sources such as L-rhamnose, glycerol, D-xylose or acetate, and also determined the proton-linked L-rhamnose uptake (not shown). No differences were observed with respect to the wild-type strain.

Substrate specificity of GlcA and LldP transport proteins

The common recognition of glycolate by both permeases led us to study their specificity for other substrates. This was approached by using a set of different mono-, di- and tricarboxylate compounds as competitors of the physiological substrate uptake.

Putative substrates for GlcA were tested in cells of strain JA201, defective in L-lactate permease. These mutant cells were grown on glycolate and uptake of labelled glycolate was measured in the presence of a 20-fold excess of each of the competitors. None of the dicarboxylates (succinate, malate) or the tricarboxylate (citrate) tested inhibited glycolate uptake. Among the monocarboxylates, only 2-hydroxymonocarboxylates were good competitors and among them D- and L-lactate were the most effective (75 and 80% inhibition respectively) (Table 3). No significant changes in the uptake rate were observed in the presence of acetate.

The putative substrates for the L-lactate permease were defined in a parallel competition experiment with cells

Table 4. Expression of *glc* and *lld* operon fusions

Carbon source	β -Galactosidase activity (Miller units)	
	MC4100 $\Phi(\text{glcD-lacZ})$	MC4100 $\Phi(\text{lldP-lacZ})$
CAA	125 $\pm$ 10	220 $\pm$ 9
D-Xylose	97 $\pm$ 7	205 $\pm$ 5
Glycolate	3158 $\pm$ 83	270 $\pm$ 9
L-Lactate	94 $\pm$ 8	2020 $\pm$ 91
D-Lactate	639 $\pm$ 34	1053 $\pm$ 42

of GlcA-deficient mutant strain JA200 grown on L-lactate. The pattern of competition with L-lactate uptake was similar to that described above for glycolate uptake except for L- and D-glycerate, which caused uptake inhibition only for glycolate permease (Table 3). Nevertheless, when tested at higher concentrations (100-fold) these compounds also inhibited L-lactate uptake. Thus, both GlcA and LldP transporters share the specificity for 2-hydroxymonocarboxylates.

Identification of the permease for D-lactate

As D-lactate competed with the uptake mediated by GlcA and LldP, identification of the transport system accounting for the D-lactate uptake was undertaken by testing the growth of mutant strains JA200, JA201 and JA202 on this carbon source. The *glcA* mutation (strain JA200) did not reduce the growth rate on D-lactate, while the *lldP::Tn5* mutation (strain JA201) impaired growth on this carbon source, indicating that the transport of this compound was mainly carried out by the *lldP* product.

Growth of double mutant strain JA202 on D-lactate was restored by plasmids expressing GlcA (pFN12) or LldP (pFN20), indicating that both permeases transport this 2-hydroxycarboxylate compound as substrate when appropriately overexpressed.

Cross-induction of *glc* and *lld* operons by related 2-hydroxymonocarboxylates

Since genetic experiments have shown that L-lactate permease is involved in the transport of D-lactate and glycolate across the cell membrane, we analysed the ability of these compounds to induce the expression of the *lld* operon. Induction of the *glc* operon by growth in the presence of both lactate isomers was also analysed. To this end, cells of wild-type-strain MC4100 bearing $\Phi(\text{glcD-lacZ})$ or $\Phi(\text{lldP-lacZ})$ were grown on glycolate, D-lactate, L-lactate, CAA or D-xylose. Levels of β -galactosidase activity indicated that glycolate and L-lactate were highly specific, inducing only their own operons (Table 4). D-Lactate displayed a weak induction of the *glc* operon and a significant induction of the *lld* operon to a level approximately one-half of that observed with L-lactate (Table 4).

DISCUSSION

The *E. coli* genes *yghK* of unknown function and *lldP* encoding L-lactate permease have been classified as paralogues by computational analysis. On the basis of this similarity the SWISS-PROT database tentatively assigned to *yghK* gene product a function of L-lactate permease. In this work we present evidence that the *yghK* gene (*glcA*) is closely linked to the *glc* genetic system and its gene product has a role in the transport of glycolate. The 350 bp intergenic region between *glcA* and the end of the previously described *glc* operon suggests the presence of a specific promoter for the expression of this gene. However, transcriptional fusion experiments failed to identify any promoter activity in the intergenic region, while Northern blot analysis revealed a band corresponding to the full-length polycistronic message, thus including *glcA* in the *glc* operon. This is in accordance with the participation of GlcA in the metabolism of glycolate, a finding further supported by the fact that the disruption of this gene significantly affected glycolate dissimilation.

Sequence analysis suggested that GlcA is a membrane protein. The high similarity with L-lactate permease further indicated its role in the transport of substrates structurally related to hydroxycarboxylates. Results presented in this study indeed confirmed that GlcA was involved in glycolate transport when the cells were induced by growth on this carbon source. Nevertheless, the ability of strain JA200 (*glcA::cat*) to utilize glycolate indicated that glycolate enters the cell through another transport system, most likely the system encoded by the paralogous gene *lldP*. Our experiments of *lldP* disruption and characterization of the double mutant *glcA::cat lldP::Tn5* showed that glycolate enters the cell simultaneously via the two permeases. Although glycolate did not induce *lld*, the basal expression of this operon appears to be sufficient for uptake of glycolate at rates which can support growth.

The results obtained in the competition experiments with other candidate substrates show that both glycolate and L-lactate permeases display specificity for L-lactate, D-lactate and glycolate. Consistently, clones expressing GlcA or LldP restored growth of strain JA202 on glycolate or on D-lactate. The ability of these plasmids to restore growth on L-lactate could not be analysed due to polar effects of the Tn5 insertion in *lldP* on expression of the downstream gene encoding L-lactate dehydrogenase. Nevertheless, physiological uptake of both lactate isomers is carried out mainly by LldP, since disruption of *glcA* did not reduce the growth rate on these carbon sources. Former studies (Matin & Konings, 1973) suggested that D- and L-lactate share a permease other than the dicarboxylate transport system, although no precise assignment to a specific protein was proposed at that time for D-lactate. For these reasons, our findings of D-lactate recognition by L-lactate permease and the induction of the *lld* operon by this isomer could be of physiological importance in providing an efficient transport system for this compound.

Finally, on the basis of sequence similarity, it is worth mentioning that *glcA*- and *lldP*-encoded proteins have been included in the LctP family of secondary transporters (<http://www.biology.ucsd.edu/~ipaulsen/transport>). Here we have shown that these two transporters specifically recognize 2-hydroxymonocarboxylates. However, unlike the 2-hydroxycarboxylate transporters of lactic acid bacteria (Bandell *et al.*, 1997), they do not transport malate and, in contrast to the monocarboxylate transporter family, they do not recognize acetate (Halestrap & Price, 1999).

ACKNOWLEDGEMENTS

This work was supported by grant PB97-0920 from the Dirección General de Enseñanza Superior e Investigación Científica, Madrid, Spain, and partially by the help of the 'Comissionat per Universitats i Recerca de la Generalitat de Catalunya'. F.N. is the recipient of a predoctoral fellowship from the Generalitat de Catalunya.

REFERENCES

- Bandell, M., Ansanay, V., Rachidi, N., Dequin, S. & Lolkema, J. S. (1997). Membrane potential-generating malate (MleP) and citrate (CitP) transporters of lactic acid bacteria are homologous proteins. Substrate specificity of the 2-hydroxycarboxylate transporter family. *J Biol Chem* **272**, 18140–18146.
- Belasco, J. G., Beatty, T., Adams, C. W., von Gabain, A. & Cohen, S. N. (1985). Differential expression of photosynthesis genes in *R. capsulata* results from segmental differences in stability within the polycistronic *rxsA* transcript. *Cell* **40**, 171–181.
- Berlyn, M. K. B. (1998). Linkage map of *Escherichia coli* K12, edition 10: the traditional map. *Microbiol Mol Biol Rev* **62**, 814–984.
- Blattner, F. R., Plunkett, G., III, Bloch, C. A. & 14 other authors (1997). The complete genome sequence of the *Escherichia coli* K-12. *Science* **277**, 1453–1462.
- Boronat, A. & Aguilar, J. (1979). Rhamnose-induced propanediol oxidoreductase in *Escherichia coli*: purification, properties, and comparison with the fucose-induced enzyme. *J Bacteriol* **140**, 320–326.
- Bruijn, F. J. & Lupski, J. R. (1984). The use of transposon Tn5 mutagenesis in the rapid generation of correlated physical and genetic maps of DNA segments cloned into multicopy plasmids. *Gene* **27**, 131–149.
- Casadaban, M. J. (1976). Transposition and fusion of the *lac* genes to selected promoters in *Escherichia coli* using bacteriophage lambda and Mu. *J Mol Biol* **104**, 541–555.
- Chang, Y. Y., Wang, A. Y. & Cronan, J. E., Jr (1993). Molecular cloning, DNA sequencing, and biochemical analysis of *Escherichia coli* glyoxylate carboxylase. *J Biol Chem* **268**, 3911–3919.
- Collins, S. H., Jarvis, A. W., Lindsay, R. J. & Hamilton, W. A. (1976). Proton movements coupled to lactate and alanine transport in *Escherichia coli*: isolation of mutants with altered stoichiometry in alanine transport. *J Bacteriol* **126**, 1232–1244.
- Dong, J. M., Taylor, J. S., Latour, D. J., Iuchi, S. & Lin, E. C. C. (1993). Three overlapping *lct* genes involved in L-lactate utilization by *Escherichia coli*. *J Bacteriol* **175**, 6671–6678.
- Elliot, T. (1992). A method for constructing single copy *lac* fusions in *Salmonella typhimurium* and its application to the *hemA*–*prfA* operon. *J Bacteriol* **174**, 245–253.
- Friedrich, M., Laderer, U. & Schink, B. (1991). Fermentative degradation of glycolic acid by defined syntrophic cocultures. *Arch Microbiol* **156**, 398–404.
- Fuqua, W. C. (1992). An improved chloramphenicol resistance gene cassette for site-directed marker replacement mutagenesis. *Biotechniques* **12**, 223–225.
- Halestrap, A. P. & Price, N. T. (1999). The proton-linked monocarboxylate transporter (MCT) family: structure, function and regulation. *Biochem J* **343**, 281–299.
- Hansen, R. W. & Hayashi, J. A. (1962). Glycolate metabolism in *Escherichia coli*. *J Bacteriol* **83**, 679–687.
- Holmes, D. S. & Quigley, M. (1981). A rapid boiling method for the preparation of bacterial plasmids. *Anal Biochem* **114**, 193–197.
- Howitz, K. T. & McCarty, R. E. (1991). Solubilization, partial purification, and reconstitution of the glycolate/glycerate transporter from chloroplast inner envelope membranes. *Plant Physiol* **96**, 1060–1069.
- Jackson, V. N. & Halestrap, A. P. (1996). The kinetics, substrate, and inhibitor specificity of the monocarboxylate (lactate) transporter of rat liver cells determined using the fluorescent intracellular pH indicator, 2',7'-bis(carboxyethyl)-5(6)-carboxyfluorescein. *J Biol Chem* **271**, 861–868.
- Kohara, Y., Akiyama, K. & Isono, K. (1987). The physical map of the whole *Escherichia coli* chromosome. Application of a new strategy for rapid analysis and sorting of a large genomic library. *Cell* **50**, 495–501.
- Kornberg, H. L. & Sadler, J. R. (1961). The metabolism of C₂ compounds in microorganisms. 8. A dicarboxylic acid cycle as a route for the oxidation of glycolate by *Escherichia coli*. *Biochem J* **81**, 503–513.
- Lord, J. M. (1972). Glycolate oxidoreductase in *Escherichia coli*. *Biochim Biophys Acta* **267**, 227–237.
- Matin, A. & Konings, W. N. (1973). Transport of lactate and succinate by membrane vesicles of *Escherichia coli*, *Bacillus subtilis* and a *Pseudomonas* species. *Eur J Biochem* **34**, 58–67.
- Miller, J. H. (1992). *A Short Course in Bacterial Genetics*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- Molina, I., Pellicer, M.-T., Badia, J., Aguilar, J. & Baldomà, L. (1994). Molecular characterization of *Escherichia coli* malate synthase G. Differentiation with the malate synthase A isoenzyme. *Eur J Biochem* **224**, 541–548.
- Moralejo, P., Egan, S. M., Hidalgo, E. & Aguilar, J. (1993). Sequencing and characterization of a gene cluster encoding the enzymes for L-rhamnose metabolism in *Escherichia coli*. *J Bacteriol* **175**, 5585–5594.
- Mudd, E. A., Krisch, H. M. & Higgins, C. F. (1990). RNAseE, an endoribonuclease, has a general role in the chemical decay of *Escherichia coli* mRNA: evidence that *rne* and *ams* are the same genetic locus. *Mol Microbiol* **4**, 2127–2135.
- Ornston, L. N. & Ornston, M. K. (1969). Regulation of glyoxylate metabolism in *Escherichia coli* K12. *J Bacteriol* **98**, 1098–1108.
- Pellicer, M.-T., Badia, J., Aguilar, J. & Baldomà, L. (1996). *glc* locus of *Escherichia coli*: characterization of genes encoding the subunits of glycolate oxidase and the *glc* regulator protein. *J Bacteriol* **178**, 2051–2059.
- Pellicer, M.-T., Fernandez, C., Badia, J., Aguilar, J., Lin, E. C. C. & Baldomà, L. (1999). Cross-induction of *glc* and *ace* operons of

Escherichia coli attributable to pathway intersection. Characterization of the *glc* promoter. *J Biol Chem* **274**, 1745–1752.

Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989). *Molecular Cloning: a Laboratory Manual*, 2nd edn. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.

Sensen, C. W., Klenk, H. P., Singh, R. K. & 10 other authors (1996). Organizational characteristics and information content of an archaeal genome: 156 kb of sequence from *Sulfolobus solfataricus* P2. *Mol Microbiol* **22**, 175–191.

Saier, M. H., Jr (2000). Families of transmembrane sugar transport proteins. *Mol Microbiol* **35**, 699–710.

Silhavy, T. J., Berman, M. L. & Enquist, L. (1984). *Experiments with Gene Fusions*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.

Simons, R. W., Houman, F. & Kleckner, N. (1987). Improved single and multicopy *lac*-based cloning vectors for protein and operon fusions. *Gene* **53**, 85–96.

Stewart, R. & Codd, G. A. (1981). Glycollate and glyoxylate excretion by *Sphaerocystis Schroeteri* (Chlorophyllaceae). *Br Phycol J* **16**, 177–182.

Vanderwinkel, E. & De Vlieghere, M. (1968). Physiologie et génétique de l'isocitritase et des malate synthases chez *Escherichia coli*. *Eur J Biochem* **5**, 81–90.

Wackernagel, W. (1973). Genetic transformation in *E. coli*: the inhibitory role of the *recBC* DNase. *Biochem Biophys Res Commun* **51**, 306–311.

Wilson, B. J. & Tolbert, N. E. (1991). The transport of glycolic acid by *Chlamydomonas reinhardtii*. *FEBS Lett* **279**, 313–315.

Winans, S. C., Elledge, S. J., Krueger, J. H. & Walker, G. C. (1985). Site-directed insertion and deletion mutagenesis with cloned fragments in *Escherichia coli*. *J Bacteriol* **161**, 1219–1221.

Received 22 September 2000; revised 11 December 2000; accepted 18 December 2000.