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

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and Laura Baldoma

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 abund-
ant 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 simul-
taneously 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 glc 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 trans-
scribed 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 Escherichia coli (Collins et al., 1976). However, none of these bacterial permeases displays sequence similarities to the members of the eukaryotic monocarboxylate transporter family.

$\ell$-Lactate permease of E. coli is encoded by the $\ellld$ operon (formerly labelled $\ellct$) (Berlyn, 1998). This operon, located at 81-4 min, contains three overlapping genes encoding the permease ($\ellldp$), the regulator protein ($\ellldR$) and the $\ell$-lactate dehydrogenase ($\ellldD$). Its transcription is activated by growth on $\ell$-lactate from the single promoter upstream of $\ellldP$ (Dong et al., 1993). No information is available on the substrate specificity of this permease.

In this report we identify the $ygbK$ gene product as a permease for glycolate uptake and characterize it as a transporter structurally and functionally similar to $\ell$-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 hydrolysat (CAA), 0.5%. The following antibiotic concentrations were used unless otherwise noted: ampicillin, 100 $\mu$g ml$^{-1}$; tetracycline, 12.5 $\mu$g ml$^{-1}$; kanamycin, 50 $\mu$g ml$^{-1}$; and chloramphenicol, 30 $\mu$g ml$^{-1}$. X-Gal and IPTG were used at 30 and 10 $\mu$g ml$^{-1}$, respectively.

**Transport and enzyme assays.** For uptake assays, chloramphenicol was added at a final concentration of 40 $\mu$g 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 $^\circ$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$^{-1}$), ICN] to a suspension of 10$^8$ 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. $\ell$-Lactate permease assays were performed in the same conditions with $\ell$-[14C]lactate [80 mCi mmol$^{-1}$ (2960 MBq mmol$^{-1}$), ICN]. Specific activities were expressed in mmol min$^{-1}$ mg total protein$^{-1}$. To identify competitors for the glycolate or $\ell$-lactate uptake, the assays were performed in the presence of a 20-fold excess of unlabelled carboxylate compounds, unless otherwise specified.

Specific $\beta$-galactosidase activity was assayed at 28 $^\circ$C in cells permeablized with chloroform and SDS, with o-nitrophenyl $\beta$-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 MgCl$_2$. 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 Ornst & Ornst (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-dichlorophenolindophenol.

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 A67 (b221 elts857 rex::Tn5 Oam29 Pam80), as described by Bruijn & Lupski (1984). Tn5 insertion mutants in the $\ellld$ operon were obtained from strain MC4100 and selected by their inability to grow on $\ell$-lactate.

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

In the gene-inactivation experiments the chloromphenicol-resistance gene cassette CAT19 (Fuqua, 1992; Winans et al., 1985) was inserted into the internal HindIII site of the $ygbK$ gene in plasmid pFN11. The plasmid carrying the inactivated gene was linearized by digestion with NruI and used to transform strain JC7623 to chloromphenicol resistance (Cm$^r$). This strain efficiently recombines linear DNA into its chromosome (Winans et al., 1985). P$\lambda$vir 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

<table>
<thead>
<tr>
<th>Strain/Plasmid</th>
<th>Description</th>
<th>Source or reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>XL-1 Blue</td>
<td>recA1 lac endA1 gyrA96 thi hsdR17 supE44 relA1 (F lac proAB lacZ AM15 Tn10)</td>
<td>Stratagene</td>
</tr>
<tr>
<td>MC4100</td>
<td>araD Δlac rpsL I bbB deoC ptsF rhsR</td>
<td>Casadaban (1976)</td>
</tr>
<tr>
<td>TE2680</td>
<td>F- Δ (rnlD–rnlE) ΔlacX74 rpsL galK2 recD::Tn10d-tet trpDC700::putA13033::(Kan' Cm' lac)</td>
<td>Elliot (1992)</td>
</tr>
<tr>
<td>JC7623</td>
<td>arg thi thr leu pro bis strA recB21 recC22 sbcB15</td>
<td>Wackernagel (1973)</td>
</tr>
<tr>
<td>CH1827</td>
<td>araD139 Δ araBC-leu7687 Δ lacX74 galU galK hsdR (Rc tns) rpsL160 thi zce-726::Tn10</td>
<td>This work</td>
</tr>
<tr>
<td>CH1828</td>
<td>CH1827 rne-50'</td>
<td>Mudd et al. (1990)</td>
</tr>
<tr>
<td>JA200</td>
<td>MC4100 glcA::cat</td>
<td>This work</td>
</tr>
<tr>
<td>JA201</td>
<td>MC4100 llDP::Km</td>
<td>This work</td>
</tr>
<tr>
<td>JA202</td>
<td>MC4100 glcA::cat llDP::Km</td>
<td>(JA201) P1 × JA200</td>
</tr>
<tr>
<td>pBluescript SK</td>
<td>Ap′ cloning vector</td>
<td>Stratagene</td>
</tr>
<tr>
<td>pBR322</td>
<td>Ap′ Te′ cloning vector</td>
<td>Boehringer Mannheim</td>
</tr>
<tr>
<td>pCAT19</td>
<td>Ap′ Cm′; source of cat gene</td>
<td>Fuqua (1992)</td>
</tr>
<tr>
<td>pRS550</td>
<td>Ap′ Kmr′; lacZ fusion vector</td>
<td>Simons et al. (1987)</td>
</tr>
<tr>
<td>pFN11</td>
<td>glcABG′ (7 kb EcoRI-NruI fragment) in pBR322</td>
<td>This work</td>
</tr>
<tr>
<td>pFN12</td>
<td>glcABG+41 kb SflI–NsiI fragment in pBR322</td>
<td>This work</td>
</tr>
<tr>
<td>pFN20</td>
<td>llDP in pBR322</td>
<td>This work</td>
</tr>
</tbody>
</table>

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 pfu DNA polymerase in standard conditions. DNA fragments 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′-GGAGATCCAGAAGCTTGAAGATGC-3′) and primer LctP2 (5′-GGACACGACCGAGAAGATGC-3′) bearing respectively the BamHI and EcoRI restriction sites (underlined).

Isolation of RNA and Northern blot hybridization. For preparation of total RNA, cells of a 25 ml culture grown to an OD<sub>600</sub> 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 µg) 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–BsrXI fragment was excised from plasmid pFN11 and ligated into the BamHI 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′-GGAGATCCAGAAGCTTGAAGATGC-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 BamHI and EcoRI 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 XhoI 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 f560 (yghK gene), which was truncated in clone pLB10 described in our previous glc operon study (Pellicer et al., 1996) (Fig.
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 \( \lambda \)-lactate permease encoded by \( ldp \) in \( E. coli \) (P33231). Alignment of the \( GlcA \) and \( Ldp \) 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 \( \lambda 1G7 \) of the Kohara library (Kohara et al., 1987). By restriction site analysis and subsequent digestion and ligation a 7 kb \( EcoRI-NruI \) 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 \( BstXI \) 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 1-6-40 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 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 \( glcDEFGB \) 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 \( glcDEFGB \) as a single unit was further supported by the absence of promoter activity in the 5′-flanking region of

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**Fig. 1.** Restriction map and gene organization of the \( glc \) operon. The open bar represents the genomic fragment inserted in clone \( \lambda 1G7 \) of the Kohara library, and the black bar represents the phage DNA. Relevant restriction sites are shown along the \( E. coli \) DNA as follows: \( B, \) \( BamHI; \) \( Bx, \) \( BstXI; \) \( H, \) \( HindIII; \) \( N, \) \( NruI; \) \( Ni, \) \( NsiI; \) \( S, \) \( SalI. \) 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.

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**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 \( OD_{650} \) 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 (\( BstXI \) internal fragment).
Expression of the structural genes of the glycolate system was similar in strains JA200 and MC4100 when grown in the presence of glycolate (not shown). These results indicated that levels of glycolate oxidase activity and immunologically detected malate synthase G protein were similar in strains JA200 and MC4100.

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

<table>
<thead>
<tr>
<th>Strain</th>
<th>Relevant genotype</th>
<th>Doubling time (h)</th>
<th>[14C]Glycolate uptake (nmol min⁻¹ mg⁻¹) †</th>
</tr>
</thead>
<tbody>
<tr>
<td>MC4100</td>
<td>Wild-type</td>
<td>5</td>
<td>23.1 ± 1.4</td>
</tr>
<tr>
<td>JA200</td>
<td>glcA::cat</td>
<td>8.5</td>
<td>7.1 ± 0.8</td>
</tr>
<tr>
<td>JA201</td>
<td>lldP::Tn5</td>
<td>6.5</td>
<td>10.3 ± 0.7</td>
</tr>
<tr>
<td>JA202</td>
<td>glcA::cat lldP::Tn5</td>
<td>—</td>
<td>&lt; 0.3</td>
</tr>
</tbody>
</table>

* 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 maltose 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 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 to compete for L-lactate uptake in cells of strain JA200 induced by growth on L-lactate.
Table 3. Competition of different carboxylates with the uptake of glycolate through GlcA or with the uptake of l-lactate through LldP

<table>
<thead>
<tr>
<th>Competing carboxylate</th>
<th>% of JA201 control</th>
<th>% of JA200 control</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Glycolate</td>
<td>49</td>
<td>95</td>
</tr>
<tr>
<td>l-Lactate</td>
<td>72</td>
<td>99</td>
</tr>
<tr>
<td>d-Lactate</td>
<td>99</td>
<td>99</td>
</tr>
<tr>
<td>d-Glycerate</td>
<td>72</td>
<td>99</td>
</tr>
<tr>
<td>Succinate</td>
<td>72</td>
<td>99</td>
</tr>
<tr>
<td>Citrate</td>
<td>102</td>
<td>99</td>
</tr>
</tbody>
</table>

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 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, and 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, maleate) 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

<table>
<thead>
<tr>
<th>Carbon source</th>
<th>β-Galactosidase activity (Miller units)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MC4100 Φ(glcd-lacZ)</td>
</tr>
<tr>
<td>CAA</td>
<td>125 ± 10</td>
</tr>
<tr>
<td>D-Xylose</td>
<td>97 ± 7</td>
</tr>
<tr>
<td>Glycolate</td>
<td>3158 ± 83</td>
</tr>
<tr>
<td>L-Lactate</td>
<td>94 ± 8</td>
</tr>
<tr>
<td>D-Lactate</td>
<td>639 ± 34</td>
</tr>
</tbody>
</table>

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-hydroxy monocarboxylate compound as substrate when appropriately overexpressed.

Cross-induction of glc and lld operons by related 2-hydroxy monocarboxylates

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 Φ(glcd-lacZ) or Φ(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 ygbK 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 ygbK gene product a function of L-lactate permease. In this work we present evidence that the ygbK 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 hydroxy monocarboxylates. 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 \textit{glcA-} and \textit{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).

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