Biochemical characterization of the 2-ketoacid reductases encoded by ycdW and yiaE genes in *Escherichia coli*

M. Felisa NUÑEZ, M. Teresa PELLICER, Josefa BADIA, Juan AGUILAR1 and Laura BALDOMA

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

INTRODUCTION

Glyoxylate is an important intermediate of the central microbial metabolism formed from acetate, allantoin or glycolate. Depending on the physiological conditions, glyoxylate is incorporated into the central metabolism by the combined actions of the activity of malate synthase and the d-glycerate pathway, or alternatively it can be reduced to glycolate by constitutive glyoxylate reductase activity. At present no information is available on this latter enzyme in *Escherichia coli*, although similar enzymes, classified as 2-hydroxyacid dehydrogenases, have been characterized in other organisms. A BLAST search using as the query sequence the hydroxypyruvate/glyoxylate reductase from *Cucumis sativus* identified as an orthologue the *yiaE* gene of *E. coli* encoding a ketoaldonate reductase. Use of this sequence in a subsequent BLAST search yielded the *ycdW* gene as a good candidate to encode glyoxylate reductase in this bacterium. Cloning and overexpression of the *ycdW* gene showed that its product displayed a high NADPH-linked glyoxylate reductase activity, and also catalysed the reduction of hydroxy-pyruvate with a lower efficiency. Disruption of the *ycdW* gene by a chloramphenicol acetyltransferase (‘CAT’) cassette did not totally abolish the glyoxylate reductase activity, indicating that another enzyme accomplished this function. The similarity with YiaE led us to test whether this protein was responsible for the remaining glyoxylate reductase activity. Purification of YcdW and YiaE proteins permitted their kinetic characterization and comparison. Analysis of the catalytic power (kcat/Km) disclosed a higher ratio of YcdW for glyoxylate and of YiaE for hydroxy-pyruvate.

Key words: *E. coli*, gene function assignment, glyoxylate reductase, 2-ketoacid reductase family, *ycdW* gene, *yiaE* gene.

EXPERIMENTAL

**Bacterial strains, plasmids and bacteriophages**

All the strains used were *E. coli* K-12 derivatives. Strain MC4100 (araD Δlac rpsL flbB deoC ptsF rbsR) [17] was the wild type in this study. XL1Blue (recA1 lac endA1 gyrA96 thi hsdR17 supE44 relA1 (F proAB lacP lacZ ΔM15 Tn10) (Stratagene) was used as host strain for recombinant plasmids in pBluescriptSK or pUC19. Strain JC7623 (recBC sbcB) was used for site-directed insertion mutagenesis [18], and strain TE2680 (recD::Tn10) was used to obtain single-copy lacZ fusions in the *E. coli* chromosome.
reaction was measured by monitoring the NADPH formation at standard reaction mixtures containing 100 mM potassium phosphate buffer, pH 7.2 or at 1 % (w/v) or at 1 % (w/v) for growth of transformed cells. The following antibiotic concentrations were used, unless otherwise stated: ampicillin, 100 µg/ml; tetracycline, 12.5 µg/ml; kanamycin, 50 µg/ml; and chloramphenicol, 30 µg/ml. 5-Bromo-4-chloroindol-3-yl β-D-galactopyranoside (X-Gal) and isopropyl β-D-thiogalactoside ( IPTG) were used at 30 and 10 µg/ml respectively. Extracts were prepared as described previously [23] in 20 mM potassium phosphate buffer, pH 7.2.

**Enzyme assays**

Total glyoxylate reductase activity in crude extracts was routinely determined as described by Ornston and Ornston [2]. The reduction reaction catalysed only by the YcdW enzyme was assayed by monitoring the disappearance of NADPH at 25 °C in standard reaction mixtures containing 100 mM potassium phosphate buffer, pH 7.0, 0.25 mM NADPH and 1.5 mM glyoxylate or 1.5 mM hydroxy pyruvate. For this enzyme, the oxidation reaction was measured by monitoring the NADPH formation at 25 °C in standard reaction mixtures containing glycine/NaOH buffer, pH 8.8, 1 mM NADPH and 10 mM p-glycerate or 10 mM t-glycerate. For the YiaE enzyme, the standard reaction mixtures were: (i) for the reduction reaction, 100 mM potassium phosphate buffer, pH 7.5, 0.25 mM NADPH and 10 mM 2-ketogluconate, 10 mM glyoxylate or 1.5 mM hydroxy pyruvate; and (ii) for the oxidation reaction, glycine/NaOH buffer, pH 8.8, 1 mM NADP+ and 10 mM p-glycerate or 10 mM t-glycerate.

The kinetic determinations were performed for each enzyme with eight different concentrations of substrates, bracketing those indicated in Table 5. The initial velocities obtained during the first 30 s of reaction were determined. The Km and Vmax were obtained by linear-regression analysis of the data plotted according to the method of Lineweaver and Burk [24]. One unit is defined as the amount of enzyme that catalyses the transformation of 1 µmol of substrate per min.

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 [22]. At least three independent experiments were performed under each set of growth conditions.

Protein concentration was determined by the method of Lowry et al. [25] using BSA as standard.

**DNA manipulation**

Bacterial genomic DNA was obtained following the procedure of Silhavy et al. [26]. Plasmid DNA was routinely prepared by the boiling method [27]. For large-scale preparation, a crude DNA sample was subjected to purification on a column (Qiagen GmbH, Düsseldorf, Germany). DNA manipulations were performed essentially as described by Sambrook et al. [28]. DNA sequencing was accomplished with the dye terminator kit using an automated ABI 373 DNA sequencer. DNA fragments were amplified by PCR using as template, E. coli chromosomal DNA. If 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 under standard conditions.

**Cloning and disruption of the ycdW and yiaE genes**

The design of the primers for PCR gene amplification was on the basis of the published ycdW and yiaE nucleotide sequences (GenBank accession nos. AE000205 and AE000432 respectively).

Plasmid pFN30, bearing the ycdW gene, was constructed by cloning into pUC19 the 1752 bp PCR fragment obtained using primers GRW.1 and GRW.2 (Table 1), bearing, respectively, the sequences underlined indicate the incorporated BamHI or EcoRI restriction sites.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Orientation</th>
<th>Sequence</th>
<th>Experiment</th>
</tr>
</thead>
<tbody>
<tr>
<td>GRW.1</td>
<td>ycdW sense</td>
<td>5’-GAATTTAGTTCCGCGGGATGCAGATGTT-3’</td>
<td>ycdW cloning in pUC19</td>
</tr>
<tr>
<td>GRW.2</td>
<td>ycdW antisense</td>
<td>5’-GCGATCTGGGCGACGATATCCGAT-3’</td>
<td>ycdW cloning in pUC19</td>
</tr>
<tr>
<td>GRW.3</td>
<td>ycdW antisense</td>
<td>5’-GCGATCTGGGCGACGATATCCGAT-3’</td>
<td>ycdW promoter cloning</td>
</tr>
<tr>
<td>GRW.His1</td>
<td>ycdW sense</td>
<td>5’-TTAGAATTCGAGCTCGTATACG-3’</td>
<td>ycdW cloning in pQE-60</td>
</tr>
<tr>
<td>GRW.His2</td>
<td>ycdW antisense</td>
<td>5’-TTAGAATTCGAGCTCGTATACG-3’</td>
<td>ycdW cloning in pQE-60</td>
</tr>
<tr>
<td>GRE.1</td>
<td>yiaE sense</td>
<td>5’-GAATTTAGTTCCGCGGGATGCAGATGTT-3’</td>
<td>yiaE cloning in pBluescript</td>
</tr>
<tr>
<td>GRE.2</td>
<td>yiaE antisense</td>
<td>5’-GCGATCTGGGCGACGATATCCGAT-3’</td>
<td>yiaE cloning in pBluescript</td>
</tr>
<tr>
<td>GRE.3</td>
<td>yiaE antisense</td>
<td>5’-GCGATCTGGGCGACGATATCCGAT-3’</td>
<td>yiaE promoter cloning</td>
</tr>
<tr>
<td>GRE.4</td>
<td>yiaE antisense</td>
<td>5’-GCGATCTGGGCGACGATATCCGAT-3’</td>
<td>yiaE promoter cloning</td>
</tr>
<tr>
<td>GRE.Gst1</td>
<td>yiaE sense</td>
<td>5’-GCGATCTGGGCGACGATATCCGAT-3’</td>
<td>yiaE cloning in pGEX.3X</td>
</tr>
<tr>
<td>GRE.Gst2</td>
<td>yiaE antisense</td>
<td>5’-GCGATCTGGGCGACGATATCCGAT-3’</td>
<td>yiaE cloning in pGEX.3X</td>
</tr>
</tbody>
</table>

© 2001 Biochemical Society
**Expression and purification of the ycdW- and yiaE-encoded enzymes**

For purification of YcdW, plasmid PQE-60, which allows the incorporation of a His$_6$-tag into the C-terminus, was used to express this protein. The ycdW gene was cloned into this plasmid, previously digested with EcoRI and BamHI. Digestion with EcoRI leads to the retention of the IPTG-inducible promoter of this vector, but eliminates its ribosome-binding site. In this way, translation is directed from the signals present in the cloned gene, and the expressed protein starts with its natural methionine. For this construction, the fragment containing the ycdW gene was obtained by PCR using primers GRW.His1 and GRW.His2 (Table 1), bearing the EcoRI and BamHI restriction sites respectively.

Overproduction of His$_6$-tagged YcdW was achieved in transformed cells of strain M15 [pREP4] bearing the recombinant plasmid pQE-ycdW after IPTG (0.5 mM) induction in LB/ampicillin/kanamycin medium for 12 h at 37°C. The protein was purified under native conditions with Ni$_2^+$-nitrilotriacetic acid (NTA) resin (Qiagen), following the batch procedure. Centrifugations were performed at 4°C, and column chromatography was carried out at room temperature. For His$_6$-tagged YcdW purification, the cell pellet from a 50 ml culture of strain M15 [pREP4] bearing plasmid pQE-ycdW was suspended in 0.5 ml of PBS buffer, pH 8.0, containing 0.3 M NaCl and 0.5 M 2-mercaptoethanol (buffer A), and sonicated on ice. The cell lysate was centrifuged at 15000 g, and the supernatant was incubated with 5 ml of Ni$_2^+$-NTA resin for 1 h with gentle shaking. After loading the mixture into a column, the resin was first washed with 50 mM sodium phosphate buffer, pH 6.0, containing 0.3 M NaCl, 5 mM 2-mercaptoethanol and 10% (v/v) glycerol (buffer B). Stepwise elutions were performed with buffer B containing 80 mM and 150 mM imidazole to elute the contaminating proteins. Elution of His$_6$-tagged YcdW was performed with 300 mM imidazole in buffer B.

For kinetic studies, the YcdW protein was purified from cells of strain JA209 bearing plasmid pFN30 grown aerobically on CAA at 30°C. The cells were harvested by centrifugation, washed twice with 20 mM sodium phosphate buffer, pH 7.6, resuspended in the same buffer, and disrupted with an ultrasonic oscillator. Cell debris was removed by centrifugation at 15000 g for 30 min, and the supernatant was used for the purification of the YcdW enzyme. All the purification steps were performed at 4°C. Solid ammonium sulphate was added to the crude extract at 4°C to a concentration of 30% (w/v), and the suspension was equilibrated at this temperature for 20 min. Precipitated proteins were removed by centrifugation, and the supernatant was adjusted further to 60% (w/v) saturation with solid ammonium sulphate. After centrifugation, the pellet was dissolved in 20 mM sodium phosphate buffer, pH 7.6, and dialysed overnight against the same buffer. The dialysed sample was applied to a DEAE-Sepharose column (1.5 cm x 4 cm) equilibrated with 20 mM sodium phosphate buffer, pH 7.6. The column was washed with the same buffer, and proteins were eluted with a linear gradient of 0–0.3 M NaCl at a flow rate of 1 ml/min. Fractions with glyoxylate reductase activity (eluted at 0.1 M NaCl) were pooled and placed on an Affi-Gel Blue column (1 cm x 4 cm) equilibrated with 20 mM sodium phosphate buffer, pH 7.6, containing 0.6 M NaCl. The column was washed with this buffer and elution was performed with a linear gradient of 0.6–2 M NaCl in 20 mM sodium phosphate buffer, pH 7.6. The active fractions were combined, concentrated and stored at −20°C in the presence of 20% (w/v) glycerol.

Purification of YiaE was achieved using the GST gene-fusion system with recognition sites for Factor Xa cleavage. To this end, the yiaE gene was amplified by PCR with primers GRE.Gst1 and GRE.Gst2 (see Table 1), and cloned into the BamHI and EcoRI restriction sites of plasmid pGEX-3X. Primer GRE.Gst1 was designed to fuse the reported ATG start codon of yiaE [16] in-frame with GST.

Overproduction of YiaE was achieved in strain BL21 carrying the recombinant plasmid pGEX-yiaE after IPTG (0.1 mM) induction in LB/ampicillin medium for 3–4 h at 37°C, and the GST-fusion protein was purified by affinity chromatography with glutathione–Sepharose 4B resin (Pharmacia). Centrifugations were performed at 4°C, and column chromatography was performed at room temperature. For YiaE purification, the cell pellet from a 10 ml culture of strain BL21 bearing plasmid pGEX-yiaE was suspended in 0.5 ml of PBS buffer [140 mM NaCl/2.7 mM KCl/10 mM Na$_2$HPO$_4$/1.8 mM KH$_2$PO$_4$ (pH 7.3)] and sonicated on ice. The cell lysate was centrifuged at 15000 g, and the supernatant, previously diluted 10-fold, was applied to a column containing 1 ml of glutathione–Sepharose 4B resin. After the column was washed with 30 ml of PBS buffer, 10 ml of Factor Xa cleavage buffer was passed through to equilibrate the matrix before cleavage of the bound GST-fusion protein. The digestion was performed by applying to the column 1 ml of Factor Xa solution (50 units), and subsequent overnight incubation at room temperature. The cleaved YiaE protein was eluted with PBS buffer.

SDS/PAGE was performed according to the method of Laemmli [31].

**N-terminal amino acid sequencing of the ycdW gene product**

In order to identify the translational start codon of the ycdW gene, purified C-terminal His$_6$-tagged protein (2 µg) was electrophoresed on an SDS/polyacrylamide gel [31], and electroblotted...
Figure 1  Nucleotide sequence of the ycdW gene encoding glyoxylate/hydroxypyruvate reductase

The gene ycdW has been translated into the one-letter amino acid code; amino acid symbols are written below the first nucleotide of the corresponding codon. The stop codon is indicated by an asterisk. The ten residues of the YcdW enzyme determined by N-terminal amino acid sequencing are underlined. The GTG start codon proposed in the E. coli Genome Sequencing Project is boxed, and the putative ribosome-binding site is double underlined. The primers GRW.1 and GRW.2 used for PCR cloning are shown by arrows. The insertion of the CAT cassette in the HindIII restriction site is indicated by a downwards-pointing arrowhead.

© 2001 Biochemical Society
on to a PVDF transfer membrane for 2 h at 60 V (or 400 mA) in a Bio-Rad Trans-Blot apparatus. The PVDF membrane was stained with 0.1% (w/v) Coomassie Blue in 50% (v/v) methanol for 2 min, destained and washed with milliQ water to eliminate the glycine present in the transfer buffer. The N-terminal amino acid sequence of this immobilized protein was determined by Edman degradation using a Beckman LF3000 sequencer equipped with a phenylthiohydantoin derivative analyser (System Gold; Beckman Instruments, Fullerton, CA, U.S.A.).

Product analyses of the enzyme reactions

YcdW reaction mixtures (1 ml each) containing 50 mM potassium phosphate buffer, pH 7.0, 5 mM NADPH, 5 mM glyoxylate or hydroxyxpyruvate and 5 units of pure YcdW enzyme were incubated at 37°C for 1 h. YiaE reactions (1 ml each) were performed in parallel containing, in this case, 50 mM potassium phosphate buffer, pH 7.5, 10 mM glyoxylate or 5 mM hydroxyxpyruvate, 10 mM or 5 mM NADPH, depending on the substrate, and 5 units of pure YiaE enzyme. Control reactions were carried out with 0.5 mg of BSA instead of YcdW or YiaE enzymes. The disappearance of NADPH was monitored by the decrease in absorption at 340 nm. No decrease was detected in the BSA control reaction mixtures. Reactions were heated to 80°C for 15 min and then mixed with activated charcoal (10%, w/v). Extraction of NADPH and NADP⁺ was performed with gentle shaking at room temperature for 1 h. Charcoal was removed by centrifugation (10 min, 2500 g) and the reaction products (glycolate and glycerate) were analysed by paper chromatography (Whatman No. 3). Chromatograms were developed in freshly mixed propan-2-ol/ammonia (65:35, v/v) at room temperature [32], air-dried and stained with a 1% (w/v) acidine solution in ethanol [33]. Glycolate (Rₖ 0.48) and glycerate (Rₖ 0.43) yielded yellow spots that were fluorescent in response to UV light. The reaction substrates glyoxylate and hydroxyxpyruvate were not stained by this method.

Identification of the products was also accomplished by using them as substrates for E. coli glycolate oxidase or glycerate kinase enzymes, overexpressed from the corresponding genes cloned in our laboratory (plasmids pTP24 and pglxK). These activities were assayed as described previously [4,21] using, as substrate in this case, 0.25 ml of the extracted products obtained from YcdW or YiaE reductase reactions.

Construction of lacZ fusions to analyse gene expression

Transcriptional fusions were constructed by inserting the DNA fragments into plasmid pRS550 [20]. This plasmid carried a cryptic lac operon and genes that confer resistance to both kanamycin and ampicillin. To construct the ycdW–lacZ fusion, primers GRW.1 (described above) and GRW.3 (Table 1) were used to amplify a 697 bp DNA fragment comprising 381 bp upstream of the ycdW translational start site. To construct the yiaE–lacZ operon fusion, primers GRE.3 and GRE.4 (Table 1) were used to amplify a 822 bp DNA fragment comprising 436 bp upstream of the yiaE translational start site. Both fragments were digested with BamHI and EcoRI, and cloned into pRS550.

After introduction of the recombinant plasmids into the tetracycline-resistant strain XL1Blue, blue colonies on LB plates containing X-Gal, ampicillin and kanamycin were isolated. Plasmid DNA was sequenced by using the M13 primer to ensure that no mutations had been introduced into the desired fragment. Single-copy fusions on the E. coli chromosome were obtained by using the method of Elliot [19]. Plasmids containing the different lacZ fusions were linearized with XhoI and used to transform strain TE2680. Owing to the presence in strain TE2680 of the recD::Tn10 mutation and sequences inserted into the trp operon that are homologous with sequences in pRS plasmids, this strain recombines linear pRS550-based plasmids into its chromosome. The transformants were selected for kanamycin resistance and screened for sensitivity to ampicillin and chloramphenicol. P1vir lysates were prepared to transduce the fusions into the desired genomic backgrounds.

RESULTS

Identification of the putative glyoxylate reductase gene in the E. coli genome

Given that the E. coli Genome Sequencing Project has been completed [34], we attempted to identify the gene encoding glyoxylate reductase by searching for putative 2-hydroxyacid dehydrogenases among unidentified proteins encoded by E. coli genes. In plants, several glyoxylate and hydroxyxpyruvate reductases have been reported and their characterization showed that both kinds of enzymes catalyse the reduction of either glyoxylate or hydroxyxpyruvate [6,7,14]. Since no entries for glyoxylate reductase were found in the GenBank database, we chose the amino acid sequence of a hydroxyxpyruvate reductase from Cucumis sativus (P13443) [14] as the query sequence for a BLAST search in the E. coli genome database. As a result, four genes encoding previously characterized 2-hydroxyacid dehydrogenases were identified. These four genes encoded a 2-ketoalcohol reductase (yiaE), the fermentative L-lactate dehydrogenase (ldhA), a 3-d-phosphoglycerate dehydrogenase (serA) and a 4-phosphoerythronate dehydrogenase (pdxB). A subsequent search using the amino acid sequence of each of the previously identified proteins yielded another candidate only when the yiaE-encoded protein, the most similar to P13443, was the query sequence for another BLAST search. In this way, a new putative 2-hydroxyacid dehydrogenase gene (ycdW) was found at 23 min on the E. coli chromosome (AE000205, nt 2393 to 3370) (Figure 1). Alignment of YcdW (P75913) and YiaE (P37666) amino acid sequences showed that both proteins displayed similarity only at the C-terminal-end moieties that include the coenzyme-binding site.

Characterization of the ycdW-encoded protein

To characterize the protein encoded by ycdW, the gene was amplified by PCR with DNA from strain MC4100 as the template, and cloned into pUC19, as described in the Experimental section, yielding pFN30. Cell extracts of recombinant cells of strain MC4100 harbouring plasmid pFN30 grown on CAA displayed high NADPH-linked glyoxylate reductase activity (Table 2). This enzyme also catalysed the reduction of hydroxyxpyruvate with NADPH as the preferred electron donor, although the activity levels displayed with this substrate were 4-fold lower than those with glyoxylate (Table 2). These results showed that the enzyme encoded by ycdW was a 2-ketoacid reductase that recognized glyoxylate and hydroxyxpyruvate as substrates.

The open reading frame corresponding to ycdW has been proposed by the E. coli Genome Project to start with the GTG at nt 394–396 (see Figure 1), yielding a protein of 324 amino acid residues. However, the determination of the N-terminal 10-amino-acid sequence (Met-Asp-Ile-Ile-Phe-Tyr-His-Pro-Trp-Phe) of the His₃-tagged purified protein (Figure 2A) has shown that the functional start codon was the ATG located 36 nt downstream, which is preceded by a ‘good’ ribosome-binding
Table 2  Effect of insertional disruption of ycdW or yiaE genes on glyoxylate reductase activity

<table>
<thead>
<tr>
<th>Strain*</th>
<th>Relevant genotype</th>
<th>Enzyme activity (m-units/mg of protein)†</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Glyoxylate reductase</td>
</tr>
<tr>
<td>MC4100</td>
<td>Wild-type</td>
<td>35</td>
</tr>
<tr>
<td>JA206</td>
<td>ycdW::cat</td>
<td>12</td>
</tr>
<tr>
<td>JA207</td>
<td>yiaE::cat</td>
<td>18</td>
</tr>
<tr>
<td>JA209</td>
<td>ycdW::cat yiaE::cat</td>
<td>0</td>
</tr>
<tr>
<td>JA209 (pFN30)</td>
<td>ycdW::cat yiaE::cat</td>
<td>9500</td>
</tr>
<tr>
<td>JA209 (pFN32)</td>
<td>ycdW::cat yiaE::cat</td>
<td>2250</td>
</tr>
</tbody>
</table>

* Strains were grown aerobically on casein acid hydrolysate.
† Activities were measured with NADPH as electron donor, as described by Ornston and Ornston [2].

Figure 2  SDS/PAGE of the different steps in the purification of the enzymes encoded by the ycdW and yiaE genes

(A) Purification of the His6-tagged YcdW from strain M15 [pREP4] bearing plasmid pQE-ycdW. Lane 1, molecular-mass markers (Gibco BRL); lane 2, crude extract of cells induced with IPTG; lane 3, fraction not bound to the column; lane 4, eluate at pH 6.0; lane 5, eluate at 150 mM imidazole; lane 6, eluate at 300 mM imidazole. (B) Purification of YcdW from strain JA209 bearing plasmid pFN30. Lane 1, molecular-mass markers (Gibco BRL); lane 2, crude extract of cells grown on CAA; lane 3, ammonium sulphate fraction (30–60%, w/v); lane 4, active fractions after DEAE-Sepharose chromatography; lane 5, active fractions after Affi-Gel Blue chromatography. (C) Purification of YiaE from strain BL21 bearing plasmid pGEX-yiaE. Lane 1, molecular-mass markers (Gibco BRL); lane 2, crude extract of cells induced with IPTG; lane 3, eluate from the glutathione–Sepharose 4B column after digestion with Factor Xa.

Disruption of the ycdW gene provides evidence for a second gene for glyoxylate reductase activity in the E. coli genome

Chromosomal ycdW gene was disrupted by insertion of a CAT cassette, as described in the Experimental section, yielding strain JA206. This insertional mutation did not completely abolish glyoxylate reductase activity. Crude extracts of strain JA206 displayed activity levels that were approximately one-third of those expressed by the parental strain MC4100 (Table 2), suggesting the presence of another glyoxylate reductase enzyme.

Since yiaE-encoded protein displayed high similarity either to several hydroxypyruvate reductases/glycerate dehydrogenases or to YcdW, we analysed whether this protein was responsible for the remaining glyoxylate reductase activity. The yiaE gene was cloned in Bluescript (plasmid pFN32), and was shown to express high levels of glyoxylate reductase activity. In contrast with YcdW, the YiaE protein displayed higher activity on hydroxypyruvate than on glyoxylate (Table 2). That yiaE accounted for the remaining activity was confirmed by introducing the yiaE::cat mutation in strain JA206. The double mutant (strain JA209) displayed null activity when grown in different carbon sources, such as CAA, glycerol or succinate, indicating that no other enzymes performing this activity were present in the corresponding cell extracts (Table 2). Consistently, strain JA207 (yiaE::cat) displayed reduced glyoxylate reductase activity that corresponded to the YcdW function.

The constitutive expression of the 2-ketoacid reductase activity described above was confirmed by expression studies of ycdW or yiaE transcriptional fusions. As shown in Table 3, β-galactosidase activity of the transcriptional fusions displayed no significant differences in any of the indicated carbon sources, thus showing that both enzymes are permanently present in the cell cytoplasm.
and may complement each other’s functional role. A slight increase was observed in the presence of hydroxypyruvate.

**Kinetic characterization and comparison of ycdW- and yiaE-encoded enzymes**

Since both enzymes showed reactivity towards glyoxylate and hydroxypyruvate, a kinetic analysis was performed in an attempt to identify the optimal substrate for each protein. To this end, YcdW and YiaE proteins were purified. YiaE was purified using the GST gene-fusion system that incorporated recognition sites YcdW and YiaE proteins were purified. YiaE was purified using Affi-Gel Blue from crude extracts of strain MC4100 grown under different conditions.

Table 3 Expression analysis of the ycdW and yiaE genes

<table>
<thead>
<tr>
<th>Carbon source</th>
<th>$\Phi$ (ycdW–lacZ)</th>
<th>$\Phi$ (yiaE–lacZ)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CAA</td>
<td>170</td>
<td>250</td>
</tr>
<tr>
<td>Glucose</td>
<td>80</td>
<td>280</td>
</tr>
<tr>
<td>Glycolate</td>
<td>120</td>
<td>200</td>
</tr>
<tr>
<td>Xylose</td>
<td>160</td>
<td>200</td>
</tr>
<tr>
<td>Xylose + glyoxylate</td>
<td>150</td>
<td>220</td>
</tr>
<tr>
<td>Xylose + hydroxypyruvate</td>
<td>370</td>
<td>470</td>
</tr>
<tr>
<td>Anaerobic conditions</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Xylose</td>
<td>150</td>
<td>145</td>
</tr>
<tr>
<td>Xylose + nitrate</td>
<td>100</td>
<td>120</td>
</tr>
<tr>
<td>Glycolate + nitrate</td>
<td>165</td>
<td>155</td>
</tr>
<tr>
<td>LB + glucose</td>
<td>70</td>
<td>60</td>
</tr>
</tbody>
</table>

Table 4 Purification of YcdW from cell extracts of E. coli strain JA209 harbouring pFN30

<table>
<thead>
<tr>
<th>Step</th>
<th>Protein (mg)</th>
<th>Total activity (units)</th>
<th>Specific activity (units/mg)</th>
<th>Yield (%)</th>
<th>Fold purification</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell extract</td>
<td>130</td>
<td>1148</td>
<td>8.83</td>
<td>100</td>
<td>1</td>
</tr>
<tr>
<td>Ammonium sulphate (30–60%-satn)</td>
<td>55.5</td>
<td>885</td>
<td>15.9</td>
<td>77</td>
<td>1.8</td>
</tr>
<tr>
<td>DEAE-Sepharose</td>
<td>16</td>
<td>723</td>
<td>45.2</td>
<td>63</td>
<td>5.1</td>
</tr>
<tr>
<td>Affi-Gel Blue</td>
<td>4.7</td>
<td>361</td>
<td>76.8</td>
<td>31.5</td>
<td>8.7</td>
</tr>
</tbody>
</table>

Table 5 Kinetic parameters of YcdW and YiaE enzymes

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Substrate</th>
<th>$K_m$ (mM)</th>
<th>$V_{max}$ (units/mg)</th>
<th>$k_{cat}$ ($s^{-1}$)</th>
<th>$k_{cat}/K_m$ ($s^{-1} \cdot M^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>YcdW</td>
<td>Glyoxylate</td>
<td>0.6</td>
<td>120</td>
<td>70.6</td>
<td>1.2 $\cdot 10^3$</td>
</tr>
<tr>
<td>YcdW</td>
<td>Hydroxypyruvate</td>
<td>1</td>
<td>20</td>
<td>11.8</td>
<td>1.2 $\cdot 10^3$</td>
</tr>
<tr>
<td>YiaE</td>
<td>Glyoxylate</td>
<td>6.6</td>
<td>345</td>
<td>203</td>
<td>3.0 $\cdot 10^4$</td>
</tr>
<tr>
<td>YiaE</td>
<td>Hydroxypyruvate</td>
<td>0.7</td>
<td>123</td>
<td>72.5</td>
<td>1.0 $\cdot 10^4$</td>
</tr>
<tr>
<td>YiaE</td>
<td>2-Oxo-o-glucarate</td>
<td>1.5</td>
<td>69</td>
<td>40.7</td>
<td>2.7 $\cdot 10^4$</td>
</tr>
</tbody>
</table>

Kinetic parameters were determined for purified YcdW and YiaE enzymes in the presence of saturating concentrations of NADPH. Each assay was performed in triplicate and the values for $K_m$, $V_{max}$ were calculated using the Lineweaver-Burk plot [24] with linear-regression analysis (correlations in the range of 0.99–0.97). The substrate concentrations were: glyoxylate, 0.2–20 mM; hydroxypyruvate, 0.2–10 mM; and 2-oxo-o-glucarate, 0.4–20 mM.

Identification of the products of YcdW- and YiaE-catalysed reactions

Two lines of evidence support the conclusion that both YcdW and YiaE reductases converted glyoxylate and hydroxypyruvate into glycolate and glycylate. Only d- and t-glycylate revealed trace levels of oxidative activity with NADP$^+$ (1 to 2 % of the reductive activity values). Kinetic constants for both glyoxylate and hydroxypyruvate substrates were investigated by extrapolation of the reciprocal plots at saturating concentrations of NADPH. Values of $K_m$, $V_{max}$ and $K_m$, as well as the ratio $k_{cat}/K_m$ are given in Table 5. As shown, the $K_m$ values for both substrates are very similar, whereas significant differences are observed in $V_{max}$ values, which displayed 6-fold higher activity when glyoxylate is the substrate. Thus the catalytic efficiency defined by the $k_{cat}/K_m$ parameter was better for glyoxylate.

In the case of YiaE enzyme, previously reported as 2-ketogluconate reductase [16], the activity on either glyoxylate or hydroxypyruvate displayed the same optimal pH of 7.5 with NADPH, as described for the substrate 2-oxo-o-glucarate. As found for YcdW, activity with NADH was one-tenth of that obtained with NADPH. 2-Oxoglutarate, oxalacetate or pyruvate were not used as substrates, and the oxidative reaction analyses revealed trace levels of activity only with d-glycylate (0.4 % of that determined for the reduction reaction). Kinetic constants for both glyoxylate and hydroxypyruvate substrates were investigated by extrapolation of the reciprocal plots at saturating concentrations of NADPH (Table 5). For this enzyme, the $K_m$ for hydroxypyruvate was significantly lower than for glyoxylate, and the $k_{cat}/K_m$ value showed greater catalytic efficiency for hydroxypyruvate. As the physiological role of YiaE has been linked to the catabolism of 2-oxo-o-glucarate, kinetic parameters for this substrate were also analysed. The catalytic efficiency of this enzyme was lower for 2-keto-d-glucarate than for hydroxypyruvate (Table 5).
First, we analysed the YcdW and YiaE products by paper chromatography (see the Experimental section). In both cases, a single spot corresponding to glycolate ($R_g \; 0.48$) was detected only when glyoxylate was used as substrate, and a single spot at the same position of glycerate ($R_g \; 0.43$) was observed only for the reduction of hydroxyoxypuruvate. No spots appeared in the control reactions performed with BSA. Secondly, we detected the presence of glycolate and glycerate in the extracted products by testing their transformation as specific substrates of the E. coli enzymes glycolate oxidase and glycerate kinase respectively. Taken together, these data strongly suggest that glycolate is the product of the glyoxylate reductase reaction catalysed by both YcdW and YiaE, and that glycerate is the product of the two hydroxyoxypuruvate reductase reactions, although it was not possible to discern whether the D- or the L-isomer was formed.

**DISCUSSION**

Several observations have suggested the conversion of glyoxylate into glycolate in E. coli, although the molecules responsible for this conversion have not been identified up until now. Among these observations, it is worth noting that mutants lacking both malate synthase G and malate synthase A activity induce the glc operon when grown on substrates yielding glyoxylate, such as acetate or CAA [35]. This induction suggests the formation of glycolate from glyoxylate in these conditions.

In the present paper we demonstrate for the first time that the glyoxylate reductase constitutively expressed in E. coli is the overlapping activity of two different enzymes encoded by genes *ycdW* and *yiaE*, which also catalyse the reduction of hydroxyoxypuruvate to glycerate. Levels of glyoxylate/hydroxyoxypuruvate reductase activity were only totally abolished in the double *ycdW/yiaE* defective mutant. It is thus shown experimentally that both proteins complement each other and that no other enzyme performing these activities is present in E. coli in these conditions. Nevertheless, the kinetic characterization of the purified enzymes showed that YcdW displays greater catalytic efficiency ($k_{cat}/K_M$) for the reduction of glyoxylate, whereas YiaE might be more effective in the reduction of hydroxyoxypuruvate. The YiaE gene product has been previously characterized as 2-ketoadonate reductase involved in the metabolism of 2-oxo-$\alpha$-gluconate; nevertheless, the kinetic characterization of the purified enzyme presented in this work suggests that the physiological role of YiaE may be the reduction of hydroxyoxypuruvate. In this context, the reduction of 2-oxo-$\alpha$-gluconate could be an alternative physiological role of this enzyme required under certain conditions and allowed by its rather broad substrate specificity.

Glyoxylate and hydroxyoxypuruvate reductases are widely found in prokaryotes and eukaryotes, and are generally encoded by more than one gene. This redundancy is likely to derive from an evolutionary pressure generated by metabolic requirements that have to be guaranteed. We would like to emphasize, for instance, that the presence of several glyoxylate reductases could be a safeguard against toxic accumulation of the chemically reactive glyoxylate. Glyoxylate has been described to be an inhibitor of the malic enzyme of *Pseudomonas* [36], and it has been suggested that glyoxylate reductase in algae mitochondria protects the tricarboxylic acid cycle from the inhibitory effect of glyoxylate [8]. In plants disposal of glyoxylate is a key process, as this compound is permanently formed in photorespiration.

Hydroxyoxypuruvate reductases are involved in the biosynthesis of serine in methylotrophic bacteria [12]. In E. coli, a hydroxyoxypuruvate isomerase encoded by a gene closely linked to genes involved in glyoxylate metabolism has been described recently.

This isomerase catalyses the conversion of the intermediate tartronate semialdehyde into hydroxyoxypuruvate, which could serve as a precursor of serine [37]. The hydroxyoxypuruvate not used for serine biosynthesis might be transformed into glycerate by the hydroxyoxypuruvate reductases, and in this way incorporated into general metabolism. In humans, the role of hydroxyoxypuruvate reductases has been proposed to be the production of the gluconeogenic precursor D-glycerate from hydroxyoxypuruvate, and thus acting as a mechanism to prevent the formation of oxalate. The inherited disease primary hyperoxaluria type 2, which causes endogenous overproduction of oxalate, has been related with a reduced cytosolic hydroxyoxypuruvate reductase activity. Recently, a human liver cDNA encoding a protein with NADPH-linked glyoxylate and hydroxyoxypuruvate reductase activities has been cloned and expressed. Analysis of its biochemical properties indicated that this enzyme could be the isoform related to hyperoxaluria type 2 [38].

We conclude that the widespread presence of multiple genes encoding enzymes of this type, as well as their constitutive expression, may be a consequence of the multiple metabolic roles that they fulfil.

We thank E.C.C. Lin for helpful discussion and Robin Rycroft for editorial assistance. We thank the Servei de Sequenciació de Proteïnes, Institut de Biologia Fonamental, UAB, for assistance in N-terminal sequencing. This work was supported by grant PB97–0920 from the Direcció General de Ensenyament Superior i Investigació Científica, Madrid, Spain, and by the help of the Comissionat per Universitats i Recerca de la Generalitat de Caralunya. M.F.N. is a recipient of a Formació de Personal Investigador (‘FPI’) scholarship from the Generalitat de Catalunya.

**REFERENCES**

715 ycdW and yiaE encode 2-ketoacid reductases in Escherichia coli


