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

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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 hydroxypyruvate 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 (k_{cat}/K_m) disclosed a higher ratio of YcdW for glyoxylate and of YiaE for hydroxypyruvate.

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

INTRODUCTION

Glyoxylate is an important intermediate of the central microbial metabolism in the 'glyoxylate bypass', required when the main source of carbon and energy is acetate or fatty acids [1]. Glyoxylate is also generated from glycolate or purine degradation in *Escherichia coli* [2,3], and is subsequently converted into malate. The oxidation of glycolate to glyoxylate is catalysed by glycolate oxidase, and this process is counterbalanced by constitutive glyoxylate reductase activity, reported to convert glyoxylate back into glycolate [2]. The net flux of carbon in either direction depends on the level of induction of the *glc* operon, which expresses glycolate oxidase [4], and the relative rate of the oxidative and the reductive processes. Up until now, no information on the gene encoding the glyoxylate reductase has been available, but the data derived from the *E. coli* Genome Sequencing Project has opened the possibility of searching for it by computational analysis.

In addition to the description of the glyoxylate reductase activity in *E. coli* and other bacteria [5], there are many reports of this activity in plants [6,7], algae [8] and yeast [9,10] that include information on the enzyme proteins. When studying the serine-cycle hydroxypyruvate reductase genes in *Methylobacterium extorquens* AM1, Chistoserdova and Lidstrom [11,12] found a second hydroxypyruvate-reducing enzyme with a low level of activity, but which was able to reduce glyoxylate to glycolate with high efficiency. The presence of more than one enzyme with glyoxylate reductase activity in a given organism occurs frequently. These enzymes are all related to hydroxypyruvate reductases, and they usually act on both glyoxylate and hydroxypyruvate. In plants their function has been related with photorespiration [13], and some authors proposed that the reaction catalysed by the NADPH-linked glyoxylate reductases could be a 'sink' for the excess of NADPH formed in the chloroplast during photosynthesis [6].

Several genes encoding hydroxypyruvate reductases from plants have been cloned and sequenced in the last decade [14,15]. These enzymes are related to those of the 2-hydroxyacid dehydrogenase family, such as that encoded by the *yiaE* gene of *E. coli* identified as a 2-ketoaldonate reductase. This enzyme catalyses the reduction of 2,5-dioxo-D-gluconate to 5-oxo-D-gluconate, 2-oxo-D-gluconate to D-gluconate, and 2-oxo-L-gulonate to L-idonate [16].

In the present report, we have identified and characterized two enzymes, the aforementioned 2-ketogluconate reductase (gluconate 2-dehydrogenase) and a new enzyme encoded by gene *ycdW*, which contribute to the constitutive glyoxylate reductase activity in *E. coli*.

EXPERIMENTAL

Bacterial strains, plasmids and bacteriophages

All the strains used were *E. coli* K-12 derivatives. Strain MC4100 (*araD* Δ*lac rpsL flbB deoC ptsF rbsR*) [17] was the wild type in this study. XL1Blue (*recA1 lac endA1 gyrA96 thi hsdR17 supE44 relA1* (F' *proAB lacI*^{Q} *lacZ* ΔM15 Tn*10*) (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*::Tn*10*) was used to obtain single-copy *lacZ* fusions in the *E. coli* chromosome

Abbreviations used: CAA, casein acid hydrolysate; CAT, chloramphenicol acetyltransferase; GST, glutathione S-transferase; IPTG, isopropyl β-D-thiogalactoside; LB, Luria broth; NTA, nitrilotriacetic acid; X-Gal, 5-bromo-4-chloroindol-3-yl β-D-galactopyranoside.

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Table 1 Oligonucleotides used in this study

The sequences underlined indicate the incorporated *Bam*HI or *Eco*RI restriction sites.

Primer	Orientation	Sequence	Experiment
GRW.1	*ycdW* sense	5′-GATGGATCCCCGGTGTGTGCAATATACG-3′	*ycdW* cloning in pUC19
GRW.2	*ycdW* antisense	5′-GCGAATTCCGGCCAGATACGCATGTT-3′	*ycdW* cloning in pUC19
GRW.3	*ycdW* antisense	5′-GCGAATTCCAGCAGAGTCATTATCTCCG-3′	*ycdW* promoter cloning
GRW.His1	*ycdW* sense	5′-GCGAATTCGCCTTTGTAGATCATAACG-3′	*ycdW* cloning in PQE-60
GRW.His2	*ycdW* antisense	5′-TTAGGATCCGTAGCCGCGTGCGCG-3′	*ycdW* cloning in PQE-60
GRE.1	*yiaE* sense	5′-GATGGATCCGTCACGACCTGAACATGC-3′	*yiaE* cloning in pBluescript
GRE.2	*yiaE* antisense	5′-GCGAATTCCAGGCGTTTAATCAGGC-3′	*yiaE* cloning in pBluescript
GRE.3	*yiaE* sense	5′-GGAGGATCCATGCCGAACAATGTGACC-3′	*yiaE* promoter cloning
GRE.4	*yiaE* antisense	5′-GCGAATTCTTCTGCTACCTCCACAACC-3′	*yiaE* promoter cloning
GRE.Gst1	*yiaE* sense	5′-GATGGATCCTTATGAAGCCGTCCGTTATC-3′	*yiaE* cloning in pGEX.3X
GRE.Gst2	*yiaE* antisense	5′-GCGAATTCCGCAGTCGCGGCTTAGTC-3′	*yiaE* cloning in pGEX.3X

[19]. Strains M15 [pREP4] (Qiagen) and BL21 (Pharmacia Biotech) were used to express the His_6-tag YcdW and the glutathione S-transferase (GST)–YiaE proteins respectively. Strains JA206 (MC4100 *ycdW::cat*), JA207 (MC4100 *yiaE::cat*) and JA209 (MC4100 *ycdW::cat yiaE::cat*) were constructed in this study (see below).

Plasmids used were: pBluescriptSK, cloning vector carrying ampicillin resistance (Stratagene); pUC19, cloning vector carrying ampicillin resistance (Biolabs); pRS550, for transcriptional fusions to *lacZ* carrying ampicillin and kanamycin resistance [20]; pQE60, vector for expression of C-terminal His_6-tag proteins carrying ampicillin resistance (Qiagen); and pGEX-3X, vector for GST-fusion proteins carrying ampicillin resistance (Pharmacia Biotech). The recombinant plasmids pTP24 [4] and pglxK [21] were used for the overproduction of the *E. coli* glycolate oxidase and glycerate kinase enzymes respectively.

Bacteriophage P1 transduction experiments were performed as described by Miller [22].

Cell growth and preparation of cell extracts

Cells were grown aerobically on Luria broth (LB) or minimal medium and harvested at the end of the exponential growth phase, as described previously [23]. For growth on minimal medium, carbon sources were added at 60 mM carbon concentration for aerobic growth and 120 mM for anaerobic growth, unless otherwise specified. Casein acid hydrolysate (CAA) was used at 0.5 % (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 hydroxypyruvate. 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 $NADP^+$ and 10 mM D-glycerate or 10 mM L-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 hydroxypyruvate; and (ii) for the oxidation reaction, glycine/NaOH buffer, pH 8.8, 1 mM $NADP^+$ and 10 mM D-glycerate or 10 mM L-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 K_m and V_{max} 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 377 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

*Bam*HI and the *Eco*RI restriction sites (see Figure 1). Plasmid pFN32, containing the *yiaE* gene, was constructed by inserting into plasmid Bluescript the 1383 bp PCR fragment made with primers GRE.1 and GRE.2 (Table 1). All constructions obtained by PCR were sequenced to confirm that no mutations had been introduced.

Disruption of *ycdW* in plasmid pFN30 was performed by inserting the chloramphenicol acetyltransferase (CAT) resistance gene cassette CAT19 [29,30] into the internal *Hin*dIII restriction site (see Figure 1). For disruption of the *yiaE* gene cloned in pFN32, the CAT cassette was inserted into the internal *Bss*HII restriction site (525 nt downstream of the ATG start codon of *yiaE*). The plasmids carrying the inactivated genes were linearized by digestion with *Sca*I, and used to transform strain JC7623 [18] to chloramphenicol resistance. This strain efficiently recombines linear DNA into its chromosome [30]. P1 *vir* lysates obtained from the selected chloramphenicol-resistant recombinants were used to transduce the CAT insertions into strain MC4100, yielding strain JA206 (*ycdW::cat*) and strain JA207 (*yiaE::cat*). Chromosomal insertions were confirmed by PCR.

To construct the double *ycdW::cat yiaE::cat* mutant (strain JA209) the following strategy was used: first a Tn10 marker close to the *yiaE* gene (*yiaJ::Tn10*), present in our laboratory collection strain JA205, was transduced into strain JA207, which contained a CAT insertion into the *yiaE* gene. Tetracycline-resistant transductants that retained the CAT insertion in *yiaE* were selected and one of these transductants (strain JA208) was subsequently used to transduce the two linked markers into *ycdW* mutant strain JA206, yielding strain JA209.

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 *Eco*RI and *Bam*HI. Digestion with *Eco*RI 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 *Eco*RI and *Bam*HI 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 25 °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 2.5 ml of 50 mM sodium phosphate buffer, pH 8.0, containing 0.3 M NaCl and 5 mM 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 eliminate contaminant 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 × 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 × 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 *Bam*HI and *Eco*RI 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_2HPO_4/1.8 mM KH_2PO_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*, purified C-terminal His_6-tagged protein (2 μg) was electrophoresed on an SDS/polyacrylamide gel [31], and electroblotted

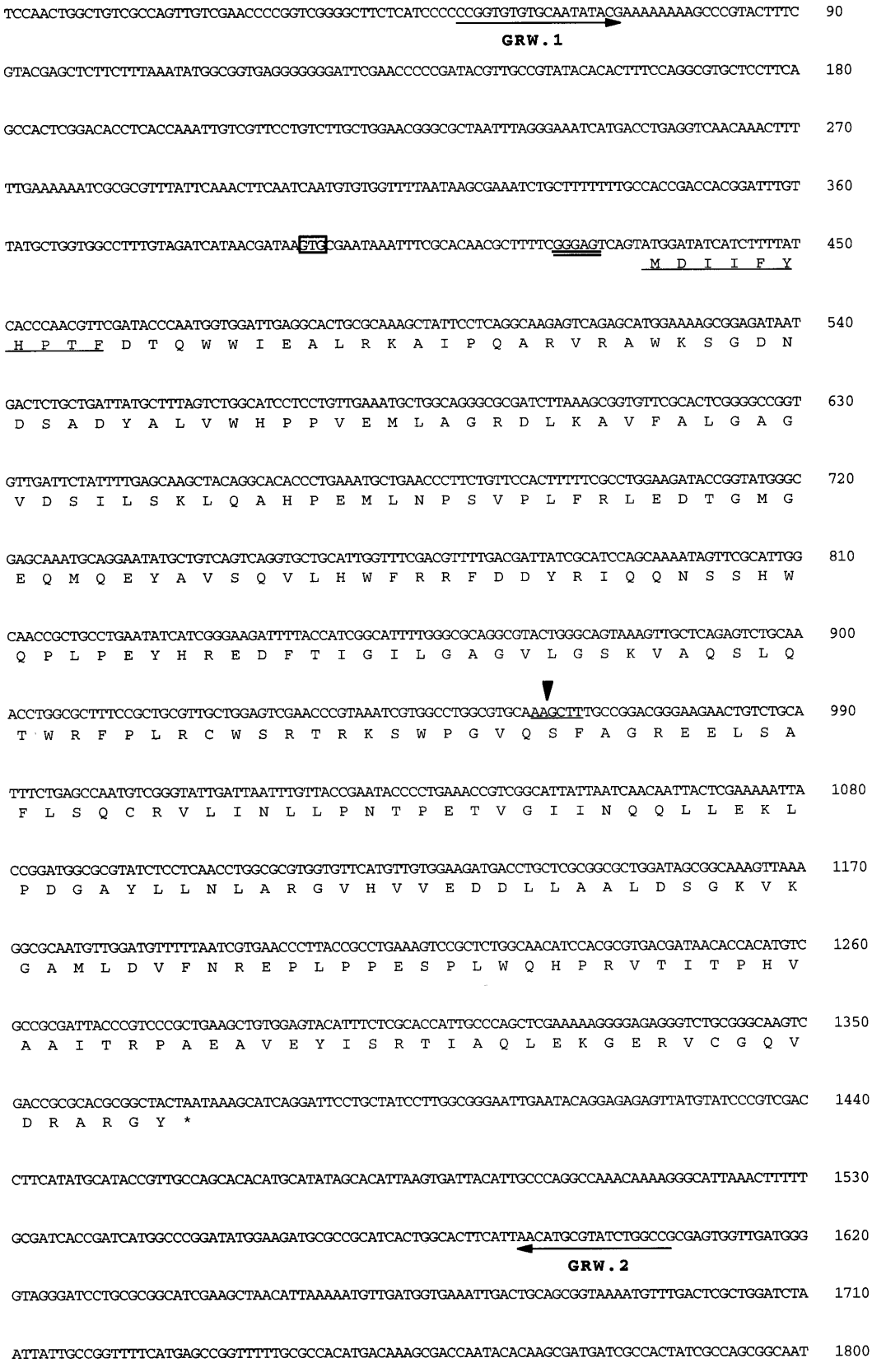

Figure 1 Nucleotide sequence of the *ycdW* gene encoding glyoxylate/hydroxypyruvate reductase

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 *Hin*dIII restriction site is indicated by a downwards-pointing arrowhead.

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 miliQ 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 hydroxypyruvate 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 hydroxypyruvate, 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 A_{340}. 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 #3). Chromatograms were developed in freshly mixed propan-1-ol/ammonia (65:35, v/v) at room temperature [32], air-dried and stained with a 1 % (w/v) acridine solution in ethanol [33]. Glycolate (R_F 0.48) and glycerate (R_F 0.43) yielded yellow spots that were fluorescent in response to UV light. The reaction substrates glyoxylate and hydroxypyruvate 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 *Bam*HI and *Eco*RI, 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 *Xho*I and used to transform strain TE2680. Owing to the presence in strain TE2680 of the *recD*::Tn*10* 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. P1*vir* 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 hydroxypyruvate reductases have been reported and their characterization showed that both kinds of enzymes catalyse the reduction of either glyoxylate or hydroxypyruvate [6,7,14]. Since no entries for glyoxylate reductase were found in the GenBank database, we chose the amino acid sequence of a hydroxypyruvate 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-ketoaldonate 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 hydroxypyruvate 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 hydroxypyruvate 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 His_6-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

Strain*	Relevant genotype	Enzyme activity (m-units/mg of protein)†	
		Glyoxylate reductase	Hydroxypyruvate reductase
MC4100	Wild-type	35	50
JA206	*ycdW::cat*	12	35
JA207	*yiaE::cat*	18	15
JA209	*ycdW::cat yiaE::cat*	0	0
JA209 (pFN30)	*ycdW::cat yiaE::cat*	9500	2600
JA209 (pFN32)	*ycdW::cat yiaE::cat*	2250	4950

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

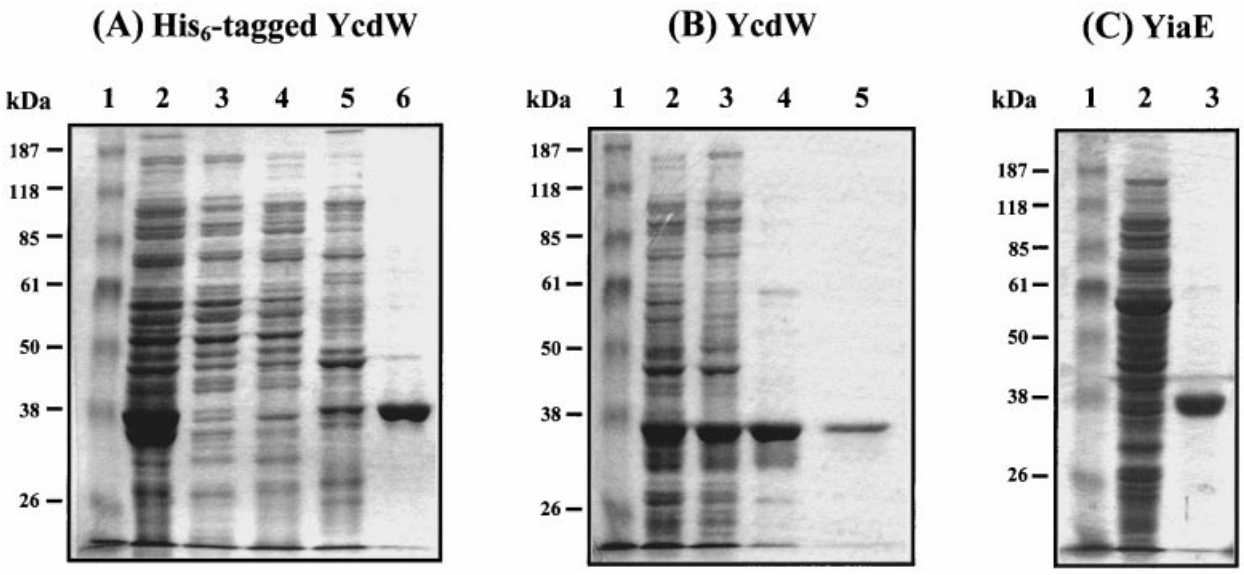

Figure 2 SDS/PAGE of the different steps in the purification of the enzymes encoded by the *ycdW* and *yiaE* genes

(**A**) Purification of the His_6-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.

site (Figure 1). According to these results the *ycdW* gene encodes a protein of 312 amino acids.

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

Table 3 Expression analysis of the *ycdW* and *yiaE* genes

Expression of the *ycdW–lacZ* and *yiaE–lacZ* transcriptional fusions were studied in wild-type strain MC4100 grown under different conditions.

	β-Galactosidase activity (Miller units)	
Carbon source	Φ(*ycdW–lacZ*)	Φ(*yiaE–lacZ*)
Aerobic conditions		
CAA	170	250
Glucose	80	280
Glycerol	120	200
Xylose	160	200
Xylose + glyoxylate	150	220
Xylose + hydroxypyruvate	370	470
Anaerobic conditions		
Xylose	150	145
Xylose + nitrate	100	120
Glycerol + nitrate	165	155
LB + glucose	70	60

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 for Factor Xa cleavage as described above. As the YcdW-fused protein could not be digested by Factor Xa, and C-terminal His_6-tagged YcdW displayed different kinetic parameters, this protein was purified to homogeneity by ammonium sulphate precipitation and column chromatography on DEAE-Sepharose and Affi-Gel Blue from crude extracts of *E. coli* cells bearing plasmid pFN30 (Table 4). Figure 2 shows SDS/PAGE analysis of these purification procedures, and reveals the presence of a single band for the purified proteins stained with Coomassie Blue (lane 5 in panel B, and lane 3 in panel C). The molecular mass of YcdW correlated well with that of 35343 Da deduced from the corresponding nucleotide sequence.

The reductase activity of YcdW on either glyoxylate or hydroxypyruvate displayed an optimal pH of 7.0 in 100 mM phosphate buffer at 25 °C, with NADPH as the preferred electron donor. Activity with NADH was very low (one-tenth of that obtained with NADPH). 2-Ketoacids other than glyoxylate or hydroxypyruvate were tested as putative substrates of this enzyme. YcdW was found to be inactive towards 2-oxo-D-gluconate, 2-oxoglutarate, oxalacetate or pyruvate. The reverse reaction was also tested with 1 mM NAD^+ or $NADP^+$ and 1, 5 or 10 mM of the following substrates: D- and L-lactate, D- and L-glycerate, and glycolate. Only D- and L-glycerate 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_{cat}, 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.

Table 5 Kinetic parameters of YcdW and YiaE enzymes

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 and 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-D-gluconate, 0.4–20 mM.

Enzyme	Substrate	K_m (mM)	V_{max} (units/mg)	k_{cat} (s^{-1})	k_{cat}/K_m ($s^{-1} \cdot M^{-1}$)
YcdW	Glyoxylate	0.6	120	70.6	$1.2 \cdot 10^5$
YcdW	Hydroxypyruvate	1	20	11.8	$1.2 \cdot 10^4$
YiaE	Glyoxylate	6.6	345	203	$3.0 \cdot 10^4$
YiaE	Hydroxypyruvate	0.7	123	72.5	$1.0 \cdot 10^5$
YiaE	2-Oxo-D-gluconate	1.5	69	40.7	$2.7 \cdot 10^4$

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-D-gluconate. 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-glycerate (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-D-gluconate, kinetic parameters for this substrate were also analysed. The catalytic efficiency of this enzyme was lower for 2-keto-D-gluconate than for hydroxypyruvate (Table 5).

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 glycerate respectively.

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

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

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_F 0.48) was detected only when glyoxylate was used as substrate, and a single spot at the same position of glycerate (R_F 0.43) was observed only for the reduction of hydroxypyruvate. 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 hydroxypyruvate 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 hydroxypyruvate to glycerate. Levels of glyoxylate/hydroxypyruvate 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 hydroxypyruvate. The *yiaE* gene product has been previously characterized as 2-ketoaldonate reductase involved in the metabolism of 2-oxo-D-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 hydroxypyruvate. In this context, the reduction of 2-oxo-D-gluconate could be an alternative physiological role of this enzyme required under certain conditions and allowed by its rather broad substrate specificity.

Glyoxylate and hydroxypyruvate 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 *Paracoccus denitrificans* [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.

Hydroxypyruvate reductases are involved in the biosynthesis of serine in methylotrophic bacteria [12]. In *E. coli*, a hydroxypyruvate 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 hydroxypyruvate, which could serve as a precursor of serine [37]. The hydroxypyruvate not used for serine biosynthesis might be transformed into glycerate by the hydroxypyruvate reductases, and in this way incorporated into general metabolism. In humans, the role of hydroxypyruvate reductases has been proposed to be the production of the gluconeogenic precursor D-glycerate from hydroxypyruvate, 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 hydroxypyruvate reductase activity. Recently, a human liver cDNA encoding a protein with NADPH-linked glyoxylate and hydroxypyruvate 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.

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