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The UDP-*N*-acetylglucosamine 1-carboxyvinyl-transferase of *Enterobacter cloacae*

Molecular cloning, sequencing of the gene and overexpression of the enzyme

Christoph Wanke[a], Rocco Falchetto[b] and Nikolaus Amrhein[a]

[a]*Institut für Pflanzenwissenschaften and* [b]*Laboratorium für Biochemie, Eidgenössische Technische Hochschule, CH-8092, Zürich, Switzerland*

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The UDP-*N*-acetylglucosamine 1-carboxyvinyltransferase (*enol*-pyruvyltransferase, EC 2.5.1.7) which catalyses the first committed step in the biosynthesis of the bacterial cell-wall peptidoglycan was purified to near homogeneity from *Enterobacter cloacae* and the NH_2-terminal amino-acid sequence determined. Using the polymerase chain reaction a 53-bp DNA fragment was synthesized; this fragment encodes the NH_2-terminal sequence of the enzyme. A clone was then isolated which contained an open reading frame of 1257 bp coding for a protein of 419 amino acids. This protein was overexpressed 100-fold in transformed *Escherichia coli* cells and shown to possess the *enol*pyruvyltransferase activity. The overall amino-acid sequence of the *enol*pyruvyltransferase is significantly similar to that of the 5-*enol*pyruvylshikimate 3-phosphate synthase, the only other enzyme known to catalyse the transfer of the *enol*pyruvate moiety of phospho*enol*pyruvate to a substrate.

*enol*Pyruvyltransferase; *Enterobacter cloacae*

1. INTRODUCTION

The first committed step in peptidoglycan biosynthesis is catalysed by the enzyme UDP-*N*-acetylglucosamine 1-carboxyvinyltransferase (*enol*pyruvyltransferase, EC 2.5.1.7) [1,2], which transfers the *enol*pyruvyl moiety of phospho*enol*pyruvate to the 3′-hydroxyl group of the glucosamine moiety of UDP-*N*-acetylglucosamine with the concomitant liberation of inorganic phosphate.

The enzyme has been studied from various sources [3–5] and purified to homogeneity from *Enterobacter cloacae* [6]. It is the target of the antibiotic phosphomycin which has been shown to bind irreversibly to a cysteinyl residue of the polypeptide [7]. The only other enzyme known to transfer the intact *enol*pyruvate moiety of phospho*enol*pyruvate to a substrate is the 5-*enol*-pyruvylshikimate 3-phosphate (EPSP) synthase (EC 2.5.1.19), the sixth enzyme of the shikimate pathway. As the target of the herbicide glyphosate [8] this enzyme has been extensively studied. Since the mechanistic relationship of the two enzymes is not straightforward [9], we decided to reinvestigate properties and mechanism of the *enol*pyruvyltransferase. Here we report the cloning, sequencing of the gene and overexpression of the *enol*pyruvyltransferase from *Enterobacter cloacae*, which will provide a basis for further investigations of this enzyme.

Abbreviations: CTAB, hexadecyltrimethylammonium bromide; DTT, 1,4-dithio-DL-threitol; EDTA, ethylene-diamine-tetraacetic acid; IPTG, isopropyl-β-D-1-thiogalactopyranoside; ORF, open reading frame; PCR, polymerase chain reaction; PEP, phospho*enol*pyruvate; UDP-GlcNAc, uridine-5′-diphospho-2′-*N*-acetyl-glucosamine.

Correspondence address: N. Amrhein, Institut für Pflanzenwissenschaften, Biochemie und Physiologie der Pflanzen, ETH-Zürich, Sonneggstr. 5, CH-8092 Zürich, Switzerland. Fax: (41) (1) 252 32 07.

2. EXPERIMENTAL

2.1. *Materials*

Enterobacter cloacae Nr. 30054 was purchased from the German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany). IPTG and PEP (Na salt) from Fluka, UDP-GlcNAc and the Na salts of phosphomycin and ampicillin from Sigma, [α-^{32}P]dATP (110 TBq/mmol), [^{35}S]dATPαS (22 TBq/mmol) and [1-^{14}C]PEP (1.04 GBq/mmol) from Amersham. All other chemicals were of the highest grade available from Fluka. DNA-modifying enzymes and their respective buffers were from different suppliers (Stratagene, Pharmacia LKB Biotechnology and New England BioLabs). Plasmid pBluescript SK(+) and its host strain *E. coli* DH5α were from Stratagene, pKK233-2 and its host strain *E. coli* JM105 from Pharmacia LKB Biotechnology.

2.2. *Isolation and purification of the* enol*pyruvyltransferase*

Enterobacter cloacae cells were grown at 37°C under aeration in a 10-liter fermenter (0.3% Difco nutrient broth, 0.5% glucose, 0.3% meat extract and 0.5% Difco tryptone) to mid-log phase. Cells were harvested by centrifugation (5,000 × *g*, 20 min) and the cell paste (approx. 50 g) was frozen in liquid N_2 and stored at −80°C. The first steps of the protein purification (acetone extraction, ultrafiltration in an Amicon ultrafiltration cell (YM 10 membrane), anion-exchange chromatography on DE-52 (Whatman) and gel filtration on Sephadex G-75

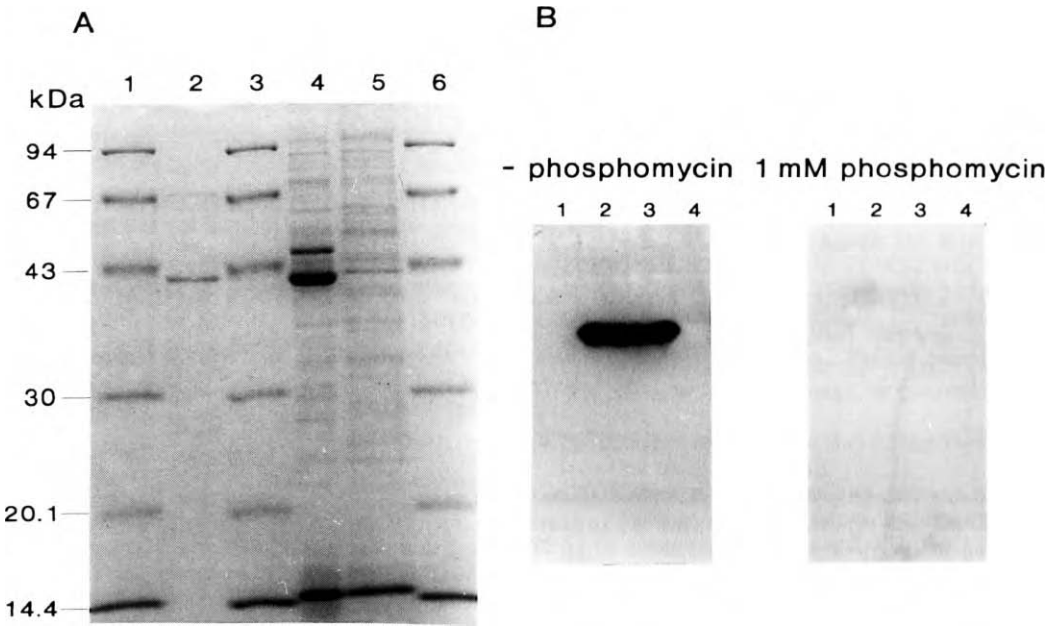

Fig. 1. A. Coomassie-stained 12.5% SDS-polyacrylamide gel. Lanes 1, 3 and 6: molecular weight standards (Pharmacia LKB Biotechnology) as indicated on the left. Lane 2, 3 μg purified *enol*pyruvyltransferase; lane 4, extract of *E. coli* (20 μg protein) overexpressing *enol*pyruvyltransferase; lane 5, extract of *Enterobacter cloacae* (20 μg protein). The band of ca. 15 kDa in lanes 4 and 5 represents lysozyme used in the protein extraction. B. Detection of *enol*pyruvyltransferase activity, after electrophoresis in 12.5% non-denaturing polyacrylamide gels, in the presence and absence of phosphomycin. In each lane extract containing 100 μg of protein was loaded. Lane 1, *E. cole* JM105 wild type; lanes 2 and 3, two independent clones of IPTG-induced *E. coli* carrying the 1.9-kb *Nco*I insert in pKK233-2 in the correct orientation; lane 4, IPTG-induced *E. coli* carrying the insert in the opposite orientation.

(Pharmacia LKB Biotechnology), G-75 instead of G-100, were adopted from [3]. Active fractions from the gel filtration were concentrated by ultrafiltration (see above) and loaded on a Mono Q HR 5/5 column equilibrated with 25 mM Tris–HCl pH 7.4, 1 mM DTT (buffer A), connected to a FPLC-System (Pharmacia LKB Biotechnology). Protein was eluted (flow rate 1 ml/min) with a 30 ml step gradient in buffer A from 0 to 0.25 M NaCl (buffer B). Within the first 3 ml the concentration of buffer B was raised to 40% and held constant for another 9 ml. Within the next 8 ml, buffer B was raised to 50% and reached 100% by the end of the run. Active fractions were pooled and dialysed twice against 2 l of 50 mM Tris–HCl pH 7.4, 1 mM DTT (buffer C). The desalted material was loaded on a Reactive Yellow 86-Agarose column (Sigma, 1.5 × 18 cm, flow rate 30 ml/h) equilibrated in buffer C. After washing the column, protein was eluted with a linear gradient (150 ml) in buffer C from 0 to 0.5 M NaCl. Active fractions were collected and dialysed twice against 2 l buffer C. The material was then chromatofocused on a Mono P HR 5/5 column connected to an FPLC-System. Protein was eluted with a linear pH gradient from 6 to 4 (flow rate 1 ml/min) according to the manufacturer's instructions and neutralised with 50 mM Tris–HCl pH 8.0.

2.3. *Protein analysis*

Protein concentrations were determined according to [10]. *enol*Pyruvyltransferase activity was assayed as described [3], with the exception that inorganic phosphate was measured by the method described in [11]. The molecular weight of the native enzyme was determined using a Superose 12 HR 10/30 column which was connected to an FPLC-System. The column was equilibrated with buffer C (flow rate 0.2 ml/min) and calibrated with commercially available standard proteins (Pharmacia LKB Biotechnology). For NH_2-terminal amino-acid sequencing the chromatofocused material was separated by SDS-PAGE [12] and blotted onto a PVDF membrane (Immobilon, Millipore). Coomassie-stained bands were cut out and sequenced with an Applied Biosystems 470A Sequencer and a 120A phenylthiohydantoin derivative analyser.

Soluble protein of recombinant *E. coli* JM 105 was analysed by

Table I

Purification of the *enol*pyruvyltransferase of *Enterobacter cloacae* from ca. 50 g (wet weight) cell material

Fraction	Protein (mg)	Total activity (nkat)	Spec. activity (nkat/mg)
Crude extract	410	–[a]	–
DE-52	117	43.1	0.37
G-75	40.1	36.7	0.92
Mono Q	12.6	34.7	2.75
Reactive Yellow 86-Agarose	1.2	15.6	13.0
Mono P pH 6–4	0.3	8.9	29.7

[a]Difficulties were encountered in determining the activity in crude extracts using ^{14}C-labelled PEP as described in [3].

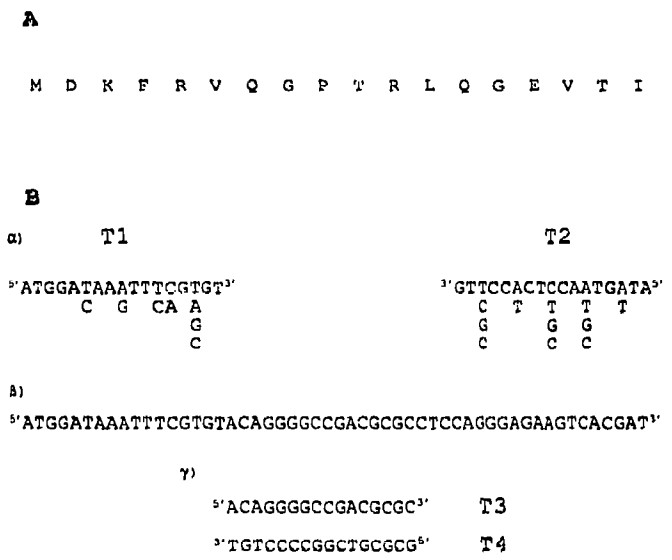

Fig. 2. A: NH_2-terminal amino acid sequence of the *enol*pyruvyltransferase obtained by sequencing of the protein. B: degenerate primers used in PCR amplification (α), DNA sequence coding for the NH_2-terminus obtained after PCR amplification (β), and primers used for DNA sequencing (γ).

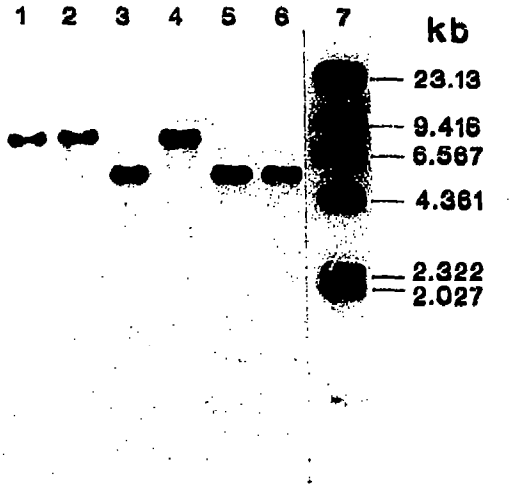

Fig. 3. Southern blot of chromosomal DNA of *Enterobacter cloacae* (5 μg DNA/lane) separated on a 0.8% agarose gel and hybridised with the 53 bp PCR fragment. Digests: *Eco*RI (lane 1), *Hin*dIII (lane 2), *Bam*HI (lane 3), *Eco*RI/*Hin*dIII (lane 4), *Eco*RI/*Bam*HI (lane 5), *Bam*HI/*Hin*dIII (lane 6). Lane 7 contains ^{32}P-labelled lambda DNA digested with *Hin*dIII as a molecular size marker (as indicated on the right).

resuspending pelleted cells in 50 mM Tris–HCl pH 8.0, 1 mM EDTA, 0.1% Triton X-100 and 0.1 M NaCl (3 ml/g cells). For each gram of cells, 100 μl lysozyme (10 mg/ml) (Boehringer) was added and incubated for 20 min on ice. Cells were broken by sonication and insoluble material was removed by centrufigation (15,000 × *g*, 20 min). The supernatant was desalted on PD-10 fast desalting columns (Pharmacia LKB Biotechnology) run with buffer C.

For visualising *enol*pyruvyltransferase activity directly in non-denaturing gels (prepared and run as described for SDS-PAGE with the exception that SDS was replaced by 1 mM DTT) the modified method of Cutting and Roth [13] as described in [6] was used.

2.4. *Molecular cloning and DNA sequencing*

Recombinant DNA techniques were essentially adopted from [14]. Recombinant bacteria were grown in 2 YT medium containing 100 μg ampicillin/ml at 37°C. Chromosomal DNA was purified by the CTAB-method [15]. PCR was carried out in a DNA Thermal Cycler (Perkin Elmer Cetus) (92°C for 45 s, 50°C for 2 min, 72°C for 30 s, 4 mM $MgCl_2$, 30 cycles). Products were analysed and separated by electrophoresis in low gelling agarose (FMC) before being cloned into pBluescript SK(+). DNA was sequenced by the dideoxy-chain termination method [16] with the ^{T7}Sequencing Kit (Pharmacia LKB Biotechnology). The PCR fragment was radiolabelled according to [17] except that the degenerate primers T1 and T2 (Fig. 2) were used instead of random hexanucleotide primers. Southern blot analysis and screening of recombinant colonies were performed on Hybond N+ and Hybond N membranes (Amersham), respectively, according to the manufacturer's instructions. Filters were hybridised for 12 h at 65°C in 5 × SET (0.75 M NaCl, 0.15 M Tris-HCl pH 8.0, 5 mM EDTA), 0.5% SDS, 2.5 × Denhardt's reagent 0.05% (w/v) bovine serum albumin (Serva), 0.05% (w/v) Ficoll (Pharmacia LKB Biotechnology), 0.05% (w/v) polyvinylpyrolidone and 100 μg/ml boiled salmon sperm DNA (Sigma). Washing was performed twice at room temperature for 5 min in 1 × SSC (0.15 M NaCl, 0.015 M Na_3-citrate), 0.1% SDS and finally for 30 min at 65°C in the same solution.

2.5. *Overexpression of the enzyme*

A 1.9-kb *Nco*I fragment containing the entire putative coding sequence (Fig. 4A) was cloned into the expression vector pKK233-2 in both orientations and used to transform *E. coli* JM105. Cells from overnight cultures were diluted 1:25 with fresh medium. After growing for 2 h, protein expression was induced by exposing bacteria to 0.1 mM IPTG for 2–3 h.

2.6. *Computer analysis*

DNA and protein sequences were analysed using the Genetic Computer Group Inc. (GCG) Package version 7.0 on a VAX 8700.

3. RESULTS

The *enol*pyruvyltransferase of *Enterobacter cloacae* was purified to near homogeneity (Fig. 1A with a yield of ca. 20% according to the protocol shown in Table 1. SDS-PAGE showed a major band corresponding to an apparent molecular weight of 42.1 kDa. Under non-denaturing conditions a molecular weight of about 37

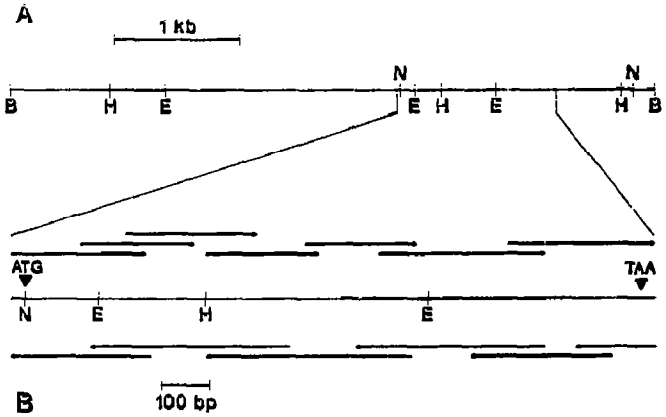

Fig. 4. A: restriction map of the cloned 5.2 kb *Bam*HI fragment. B: strategy for sequencing the *enol*pyruvyltransferase gene. Restriction sites: B, *Bam*HI; E, *Eco*RV; H, *Hin*cII; N, *Nco*I.

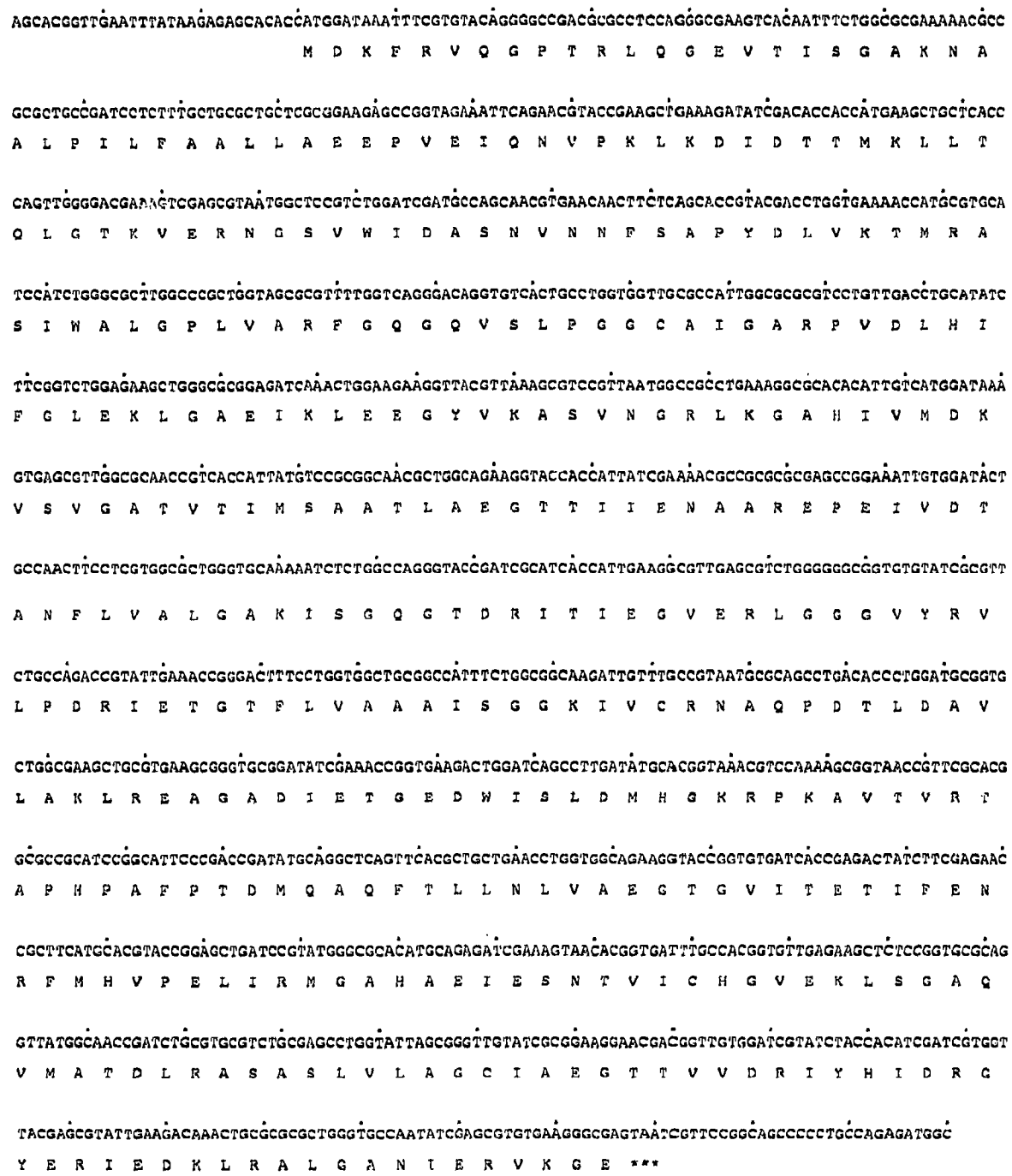

Fig. 5. DNA sequence of the *UDP-N*-acetylglucosamine 1-carboxyvinyl-transferase gene of *Enterobacter cloacae* and the deduced amino acid sequence of the enzyme.

kDa was determined. When analysed by automated gas-phase amino-acid sequencing, this 42.1 kDa band yielded the partial NH_2-terminal sequence shown in Fig. 2. Based on this sequence two degenerate 17-mer primers were designed for PCR-amplification of the corresponding DNA sequence. A 53-bp fragment was isolated which corresponded to the NH_2-terminus of the protein (Fig. 2). Southern blot analysis of chromosomal DNA digested with a number of restriction enzymes showed a single *Bam*HI fragment of 5.2 kb, hybridising with the labelled PCR fragment (Fig. 3). *Bam*HI digested chromosomal DNA in the range of 4 to 6 kb in size was cloned into pBluescript SK(+) and used to transform *E. coli* DH5α. Four positive clones were isolated by screening ca. 5000 colonies. The 5.2-kb *Bam*HI insert was then mapped with *Eco*RV and *Hin*cII. The

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  1 MDKFRVQGPTRLQGEVTISGAKNAALPILFAALLAEEPVEIQNVPKLKDI  50
  1 MESLTLQPIARVDGTINLPGSKTVSNRALLLAALAHGKTVLTNLLDSDDV  50

 51 DTTMKLLTQLGT....KVERNGSVWIDASNVNNFSAPYDLVKTMRASIWA  96
 51 RHMLNALTALGVSYTLSADRTRCEIIGNGGPLHAEGALELF..LGNAGTA  98

 97 LGPLVA..RFGQGQVSLPGGCAIGARPVDLHIFGLEKLGAEIKL..EEGY 142
 99 MRPLAAALCLGSNDIVLTGEPRMKERPIGHLVDALRLGGAKITYLEQENY 148

143 VKASVNGRLKGAHIVMD.KVSVGATVTIMSAATLAEGTTII...ENAARE 188
149 PPLRLQGGFTGGNVDVDGSVSSQFLTALLMTAPLAPEDTVIRIKGDLVSK 198

189 PEIVDTANFLVALGAKISGQGTDRITIEGVERL.GGGVYRVLPDRIETGT 237
199 PYIDITLNLMKTFGVEIENQHYQQFVVKGGQSYQSPGTYLVEGDASSASY 248

238 FLVAAAISGGKI....VCRNAQPDT..LDAVLAKLTEAGADIETGEDWIS 281
249 FLAAAAIKGGTVKVTGIGRNSMQGDIRFADVLEKM...GATICWGDDYIS 295

282 LDMHGKRPKAVTVRTAPHPAFPTDMQAQFTLLNLVAEGTGVITETIFENR 331
296 CTRGELNAIDMDMNHIPDAAMTIATAA.....LFAKGTTRLRN.IYNWR 338

332 .......FMHVPELIRMGAHAEIESNTVICHGVEKLSGAQVMA.TDLRAS 373
339 VKETDRLFAMATELRKVGAEVEEGHDYIRITPPEKLNFAEIATYNDHRMA 388

374 ASLVLAGCIAEGTTVVDRIYHIDRGYERIEDKLRALGAN 412
389 MCFSLVALSDTPVTILDPKCTAKTFPDYFEQLARISQAA 427
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Fig. 6. Alignment of the deduced amino acid sequences of the *enol*pyruvyltransferase of *Enterobacter cloacae* (this work, upper line) and the EPSP synthase of *E. coli* ([20], lower line) using the GCG program BestFit [21]. Vertical lines between the sequences connect identical amino acids, while colons connect amino acids with similar side chains.

relative location of the *enol*pyruvyltransferase gene was determined by Southern blot analysis and by sequencing of subcloned restriction fragments using also the primers T3 and T4 (Fig. 2). Both strands of the entire coding region were sequenced by the nested deletion method as illustrated in Fig. 4. An ORF of 1257 bp coding for a protein of 419 amino acids was obtained (Fig. 5). To verify that this ORF indeed codes for a protein with *enol*pyruvyltransferase activity, a 1.9-kb *Nco*I fragment containing the entire putative coding sequence was cloned into the expression vector pKK233-2 in both orientations. Protein extracts of IPTG-induced recombinant *E. coli* JM105 were analysed by SDS-PAGE for protein (Fig. 1A) and after electrophoresis on a non-denaturing gel for *enol*pyruvyltransferase activity (Fig. 1B). Out of 18 randomly picked clones 7 showed high expression of *enol*pyruvyltransferase activity and a greatly increased amount of the 42-kDa protein. DNA sequencing of these positive clones confirmed that they contained the insert in the correct orientation. After induction with IPTG the specific activity of the *enol*pyruvyltransferase was more than 100-fold higher compared to *Enterobacter cloacae* as determined by analysis of the respective extracts after anion-exchange chromatography on DE-52. As shown in Fig. 1B, the activity of *enol*pyruvyltransferase in induced recombinant *E. coli* was also greatly increased compared to wild-type cells.

4. DISCUSSION

A purification scheme for the *enol*pyruvyltransferase from *Enterobacter cloacae* has previously been reported [6]. However, we were not able to reproduce this procedure. Critical to this procedure is an affinity chromatography step using a presumed feedback inhibitor of the enzyme, UDP-*N*-acetyl-muramyl-L-Ala-D-Glu-*meso*-diaminopimelate (UDP-muramyltripeptide), covalently attached to a sepharose matrix. The *enol*pyruvyltransferase is, however, unlikely to have a high affinity for this material, because (a) the K_i for this inhibitor is in the mM concentration range (confirmed in [18]), and (b) the UDP-muramyltripeptide accumulates in penicillin-treated *Enterobacter cloacae* NRC 392 [19] which makes it an improbable negative modulator of its synthesis. For these reasons a new and reproducible purification scheme was developed (Table I).

The purified *enol*pyruvyltransferase showed an apparent molecular weight of 41.1 kDa in SDS-PAGE compared to a value of about 37 kDa under non-denaturing conditions, indicating that the enzyme is active as a monomer. The molecular weight derived from the deduced amino-acid sequence is 44,776 Da which is slightly higher than that determined by SDS-PAGE.

When comparing the deduced amino-acid sequence of the *enol*pyruvyltransferase with known protein sequences, we found significant similarity to EPSP synthase sequences from different organisms. As an example, the alignment of the deduced *enol*pyruvyltransferase sequence with that of the EPSP synthase of *E. coli* [20] is shown in Fig. 6. Using the GCG program BestFit [21] we determined an identity of 25% and a similarity of 47% of the amino-acid sequences over their entire length (EPSP synthase sequences from other organisms showed values in the same range). It is tempting to speculate therefore that the two proteins are related and that their reaction mechanisms may, in consequence, be more similar than presently assumed.

The nucleotide sequence reported in this paper has been submitted to the GenBank/EMBL Data Bank.

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