Appendix

CLONING AND SEQUENCE OF THE GENE ENCODING ENZYME E-1 FROM THE METHIONINE SALVAGE PATHWAY OF KLEBSIELLA OXYTOCA*

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The methionine salvage pathway converts the methylthioRibose moiety of 5′-(methylthio)-adenosine to methionine via a series of biochemical steps. One enzyme active in this pathway, a bifunctional enolase-phosphatase called E-1 that promotes oxidative cleavage of the synthetic substrate 2,3-diketo-1-phosphohexane to 2-keto-pentanoate, has been purified from Klebsiella pneumoniae and is characterized in the preceding paper (Myers, R., Wray, J., Fish, S., and Abeles, R. H. (1993) J. Biol. Chem. 268, 24785–24791). We synthesized degenerate oligonucleotides corresponding to portions of the amino terminus of E-1. These oligonucleotides were used as polymerase chain reaction primers on whole genomic DNA from Klebsiella oxytoca. This resulted in an 82-base pair DNA fragment that was used as a hybridization probe to obtain a clone of the E-1 gene from a K. oxytoca gene library. The DNA sequence of the E-1 coding region was determined, and the amino acid sequence of E-1 was deduced. E-1 appears to represent a novel class of enzymes since no homology to known enzymes was found. Cloning the gene from K. oxytoca on a multicopy plasmid leads to overproduction of E-1 enzyme that has properties indistinguishable from those of the enzyme from K. pneumoniae.

The so-called "methionine salvage pathway" converts the methylthiobisphosphate moiety of 5′-(methylthio)-adenosine to methionine in various microorganisms and higher organisms (2, 3). The enzymes and intermediates of this pathway have been most thoroughly studied in Klebsiella pneumoniae (4). 5′-(Methylthio)-adenosine is ordinarily derived from S-adenosylmethionine during polyamine biosynthesis. The pathway is of interest both for its novel enzymology (4) and for its potential use in the commercial biosynthesis of amino acids (5). In either context it is useful to obtain DNA clones of each gene involved in the pathway. The K. pneumoniae 1-phospho-2,3-diketo-1-hexene-2-hydrate enolase-1-phospho-2-hydroxy-3-keto-1-hexene phosphatase (called "E-1"), which catalyzes the fifth step in the pathway, has been purified and characterized (1).

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On the basis of a partial amino acid sequence of E-1 (1), we have synthesized DNA probes that allowed cloning of the gene that encodes this enzyme. We have sequenced the gene and deduced the entire amino acid sequence of the enolase-phosphatase enzyme.

MATERIALS AND METHODS

Bacteria were grown in LB media (6). Solid medium contained 1.5% agar. Strains containing plasmids were selected by addition of 20 µg/ml chloramphenicol to the growth medium. Although the amino-terminal sequence of E-1 was determined using enzyme isolated from K. pneumoniae strain CG253 (1), the DNA for clone bank construction was from the closely related strain, Klebsiella oxytoca UNP932, an hsdS, recA derivative of the widely used strain M5a1, formerly classified as K. pneumoniae (7). The sole taxonomic distinction between the two species is that K. oxytoca produces indole from tryptophan, whereas K. pneumoniae does not. We have determined that strain CG253 produces indole from tryptophan, so we suggest that K. pneumoniae CG253 may be properly classified as K. oxytoca.

All DNA manipulation methods were standard procedures (6). Oligonucleotides were synthesized on an Applied Biosystems model 380A or 382B, according to the manufacturer's protocols.

Double-stranded plasmid used for sequencing was purified with Qia-gen Maxi kits. DNA sequencing reactions were performed using a Sequenase version 2.0 kit (U. S. Biochemical Corp.). The double-stranded DNA templates were denatured using 1 × NaOH, and primers were annealed according to the method described (8). The labeling reactions and subsequent steps were carried out as described in the Sequenase version 2.0 protocol (6th edition) obtained from the manufacturer.

DNA fragments were purified by preparative agarose gel electrophoresis using either Qiaex (Qiagen) or Gene Clean (Bio 101, La Jolla, CA), as instructed by the manufacturers. Polymerase chain reactions (PCR) were performed with the Perkin-Elmer Cetus PCR kit using a Perkin-Elmer Cetus DNA thermal cycler. For the first 4 cycles melting was at 94 °C for 30 s and annealing at 37 °C for 30 s, followed by linear temperature rise to 72 °C over 2 min, with polymerization continued at 72 °C for 1 min. For the subsequent 25 cycles the annealing temperature was changed to 50 °C for 30 s, rising to 72 °C over 30 s, with continued polymerization at 72 °C for 30 s, and a final extension for 10 min at 72 °C. Colony screening by PCR was done with the toothpick method (9).

Hybridization probe of high specific radioactivity was produced in a 16-L PCR reaction containing the following reagents: 5 µl of [α-32P]ATP stock solution (3000 Ci/mmol); DuPont NEN NEG-021H; final dATP concentration, 1.7 µM; 1.5 µl of dTTP, dCTP, and dGTP mix, containing each at 125 µM; 1.0 µl of Perkin-Elmer Cetus PCR buffer 1 (equivalent to buffer II plus 15 mM MgCl2); 1.0 µl of mixed primers, each at 10 µM; 0.5 µl of Taq polymerase mix, diluted to 1/10 concentration of stock (0.25 units); and 1.0 µl of template solution, containing about 0.1 ng of template. The reaction was covered with 10 µl of mineral oil and run with the program described above (29 cycles) (10). About three-quarters of the radioactivity was incorporated into PCR product. When products from similar reactions (performed without radiolabel) are visualized in ethidium bromide-stained agarose gels, a distinct band of full-length product is visible with a pronounced shadow of...
smaller products. Both full-length and partial products should hybridize to the target sequence and are useful as reporters. Initially, all cloning work involving Klebsiella DNA was performed under BL2 conditions. During this work, the NIH Recombinant DNA Guidelines were amended, and work with clones from strain M5A1 and its derivatives was classified as requiring only BL1 conditions.

RESULTS

Primers—Based on the amino acid sequence of the first 26 residues of the purified E-1 from strain CG253 (1), two mixed oligonucleotide PCR primers were designed to amplify an 82-base pair DNA region that corresponded to most of the known protein sequence (see Fig. 1). Each primer consisted of an 11-base pair sequence corresponding to the protein sequence. At mixed base sites all relevant bases were incorporated in equimolar amounts. In addition, each primer featured an extension of six or seven bases, not based on protein sequence, to facilitate cloning. Similar primers that incorporated inosine at the ambiguous base positions did not amplify the target.

Clone Bank—Genomic DNA from strain UNF932 was cleaved partially with Sau3A1, and fragments of 5–10 kilobases were isolated by preparative agarose gel electrophoresis. The purified fragments were ligated into a phosphatase-treated, BamHI-digested pACYC184 plasmid vector (11). Escherichia coli strain 294 was transformed with the resulting DNA, and clones resistant to chloramphenicol (20 μg/ml) were selected. About 2,000 transformant colonies were pooled and stored in 50% glycerol at -80 °C. Aliquots of the pool were diluted and plated at a density of about 1,000 colonies/plate. Replicas were made onto nitrocellulose filters, which were then used for 32P-labeled DNA probe hybridization (6).

Hybridization Probe—Using the oligonucleotide primers described above (Fig. 1), PCR amplification was performed with 1 μg of UNF932 genomic DNA as target. The PCR products were separated electrophoretically on a 2% agarose gel stained with ethidium bromide. No amplification products were observed after 29 cycles of amplification, so the 29-cycle program was repeated. An 82-base pair fragment was then produced using UNF932 DNA as the target but not from E. coli DNA treated under the same conditions. The 82-base pair fragment was excised and purified with Gene Clean. A sample of the 82-base pair fragment was itself used as a target for PCR amplification. The reamplified fragment was purified by preparative acrylamide gel electrophoresis, cloned into the unique Smal site of the 27,000 dalton size of the purified enzyme, as measured by SDS-polyacrylamide gel electrophoresis (1).

Some of the purified 82-base pair fragment was also reamplified using [α-32P]dATP to produce a radioactive probe specific for the 82-base pair sequence. This probe was hybridized to filter replicas of E. coli MM294 colonies containing the UNF932 clone bank (see above). Eight positively hybridizing colonies were picked from the master plate and named DIOX 1 through DIOX 8 (at that time, the encoded enzyme was thought to be a dioxygenase). The first four of these and one non-hybridizing colony (named X) were assayed for enolase-phosphatase activity as described in the accompanying paper (1). One isolate, DIOX 2, gave a level of enzyme activity more than 100-fold higher than that of the others and the negative control, and 20-fold higher than that of a Klebsiella strain (Table 1). All eight isolates were streaked for single colonies, and three to eight colonies from each were screened by PCR using the toothpick method (8) for templates able to direct synthesis of the 82-base pair fragment. Only one colony from isolate DIOX 2 was positive. The plasmid isolated from this colony was named pDiox-2. The other isolates were further screened by hybridization of radioactive probe to filter replicas of single colonies. The colonies that gave the strongest positive signals were screened by PCR, but none was positive by that test.

Strain CG253 was transformed with pDiox-2 by electroporation using a Bio-Rad Electroporator according to the manufacturer’s instructions. Colonies resistant to chloramphenicol (20 μg/ml) were selected. An extract of the transformed CG253 contained about 6 times more enolase-phosphatase activity per mg of protein than the untransformed strain (1). On the basis of these results, we concluded that pDiox-2 contains a gene that encodes the enolase-phosphatase.

Mapping by restriction endonuclease digestion showed that the Klebsiella DNA insert of pDiox-2 was about 6 kilobases (Fig. 2). pDiox-2 DNA was cleaved with various restriction enzyme combinations and analyzed by Southern blotting (6) using the 32P-labeled 82-base pair fragment described above, whose sequence represents a portion of the amino-terminal region of the protein. The probe hybridized to all DNA fragments that contained the end of the cloned insert nearest to the 5' end of the tetracycline resistance gene of pACYC184 (Fig. 2). The fragment generated by BglI cleavage was particularly informative, placing the 5' end of the enolase-phosphatase gene within about 800 base pairs of the end of the cloned insert.

Starting from the middle of the 82-base pair sequence, the entire enolase-phosphatase gene was sequenced by the “primer walking” strategy. The DNA sequence of the entire coding region was determined for both strands. The resulting sequence (Fig. 3) encodes a polypeptide containing 229 amino acids. The deduced molecular weight of 25,616 is in good agreement with the 27,000 dalton size of the purified enzyme, as measured by SDS-polyacrylamide gel electrophoresis (1).

![Fig. 1. K. oxytoca enolase-phosphatase enzyme sequence, primers, and polymerase chain reaction product.](image-url)
Enolase-phosphatase activity of transformants containing clones that hybridized to the E-1 specific probe. Transformant "X" is a non-hybridizing isolate taken as a negative control. UnF932 is the K. oxytoca strain from which the clone bank was constructed, taken as a positive control. The enzyme assay monitored release of formate from the artificial substrate, 2,3-diketo-l-phosphohexane, as described in the accompanying paper (1).

| Heat strain | Transformant designation | Enolase-phosphatase activity nmol/mg protein/min |
|-------------|--------------------------|-----------------------------------------------|
| UnF932      | X                        | 8.16                                          |
| MM294       | DIOX1                    | >1                                            |
| MM294       | DIOX2                    | 179                                           |
| MM294       | DIOX3                    | <1                                            |
| MM294       | DIOX4                    | <1                                            |
| MM294       | X                        | <1                                            |

FIG. 2. Diagram of pDiox-2. pDiox-2 was isolated from a K. oxytoca clone bank made by cloning DNA partially digested with Sau3AI into the unique BamHI site of the Tet gene of pACYC184. The insert is shown as a linear segment. The locations of cleavage sites for various restriction endonucleases are indicated. In the case of the endonucleases EcoRI and EcoRV, additional unmapped cleavage sites occur within the insert to the left of the sites indicated in the diagram. The dashed lines indicate restriction fragments that hybridized to the 82-base pair E-1 probe in Southern blots (see text for details).

Just upstream from the coding region of enolase-phosphatase gene sequence elements characteristic of typical Gram-negative bacterial promoters, namely a ribosome binding site (12), a "-10 region," and a "-35 region" (13) (see Fig. 3). Just downstream from the enolase-phosphatase coding region and overlapping the enolase-phosphatase coding region by 1 base pair is a second open reading frame that extends past the end of the DNA sequence that has been determined so far. This arrangement of genes is typical of translationally coupled genes in prokaryotic operons.

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