Cloning, Characterization, and Sequence Analysis of the clcE Gene Encoding the Maleylacetate Reductase of Pseudomonas sp. Strain B13

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A 3,167-bp PstI fragment of genomic DNA from Pseudomonas sp. strain B13 was cloned and sequenced. The gene clcE consists of 1,059 nucleotides encoding a protein of 352 amino acids with a calculated mass of 37,769 Da which showed maleylacetate reductase activity. The protein had significant sequence similarities with the polypeptides encoded by tcbF of pPS1 (59.4% identical positions), tfdF of pJP4 (55.1%), and clcE of Burkholderia cepacia AC1100 (53.1%). The function of TcbF as maleylacetate reductase was established by an enzyme assay. The aerobic mineralization of various chloroaromatic compounds by bacteria often produces chlorocatechols as central intermediates. After several steps in the modified ortho pathway (chloro)maleylacetates are formed, and these are further converted to 3-oxoacetates by a maleylacetate reductase. The same type of enzyme is also present in the 2,4,5-trichlorophenoxyacetate degradation pathway via (chlorinated) 1,2,4-trihydroxybenzene of Burkholderia cepacia AC1100 (2). Maleylacetate reductases have recently been purified from various microbial strains (3, 8, 11, 12, 14, 18). They reduce carbon-carbon double bonds while using NADH as the cosubstrate. In addition, dechlorinating activity has been documented. The biochemical of the maleylacetate reductases has been intensively investigated with purified bacterial enzymes (13, 14).

The enzymes of the modified ortho pathway are usually encoded by degradative plasmids, such as pJP4 from the 2,4-dichlorophenoxyacetate-utilizing strain Ralstonia eutropha JMP134, pPS1 from the 1,2,4-trichlorobenzene-degrading Pseudomonas sp. strain P51, and pAC27 from the 3-chlorobenzoate-catabolizing strain Pseudomonas putida AC858 (1, 4, 20). The genes encoding the enzymes of the modified ortho pathway are located in operons having similar structures (6, 15, 20). While the genes tcbCDE, tfdCDE, and clcABD have long been known to encode chlorocatechol 1,2-dioxynasase, chlorocateconuclomase cycloisomerase, and (chloro)dienealactone hydrolyase, respectively, information on the functions of the TcbF, TfdF, and ClcE gene products were obtained more recently. After it had been reported that TcbF and TfdF are homologous to iron-containing alcohol dehydrogenases (19), our groups provided evidence that these proteins and ClcE encoded by pAC27 are in fact maleylacetate reductases (9, 16, 18). This paper deals with the isolation of the maleylacetate reductase gene from Pseudomonas sp. strain B13, its characterization and functional analysis, the comparison of this gene with other genes, and the function of the tcbF gene product.

From 3-chlorobenzoate-grown Pseudomonas sp. strain B13 (5) we have successfully amplified parts of the maleylacetate reductase gene by using two different PCR strategies. One PCR yielded a 1,050-bp product by employing primer TK-9 (5′-GATTTCCTGGCGCCCTTGCAG). The other PCR made use of primers M1 [5′-GG(A/C/G/T)GG(A/C/G/T)C(G/T)CC(A/G)TGC CA(A/C/G/T)GG(A/G)TC], derived from the tryptic peptide MAR6 (9). The other PCR made use of primers M1 [5′-CGA(T/C)GG(A/G/C/T)GC(T/A)TC] and M2 [5′-ATGGCACA GCTTGTG(G/A)TGTG(T/A)TC], which corresponded to regions conserved in both tfdF and tcbF (15, 20) (bases 186 to 203 and 710 to 729 of tfdF, respectively). This PCR resulted in a 548-bp fragment containing the middle part of the clcE gene. The PCR-amplified 1,050-bp fragment was nearly the same size as the tfdF, tcbF, and clcE genes. Its cloning into the SrfI site of vector pCR-Script(Amp)KS+ yielded plasmid pCR31-13. The 548-bp PCR product was cloned into the EcoRV site of pBluescriptIKS+, yielding plasmid pVS1.

To clone the whole gene for the maleylacetate reductase of Pseudomonas sp. strain B13, the genomic DNA was cleaved with various restriction endonucleases, separated by electrophoresis, and analyzed by Southern hybridization with [32P]dCTP-labeled PCR products as probes. PstI fragments of B13 DNA were ligated into pBluescriptIKS+ (3,167-bp insert), yielding plasmid pB13I containing the relevant gene.

The complete nucleotide sequence of the 3,167-bp PstI fragment and the deduced amino acid sequences are shown in Fig. 1. The DNA sequence comprises one incomplete open reading frame (ORF) from positions 1 to 52 and, colinear with it, two complete ORFs which overlap by 4 bp. The sequence of the first complete ORF (positions 528 to 1238) is identical to the previously determined sequence of clcD of pAC27 (7), and thus it is assumed that this ORF is clcD. The second complete ORF, termed clcE, extends from positions 1235 to 2293 and codes for a protein of 352 amino acid residues with a predicted mass of 37,769 Da. The predicted ClcE sequence contains all tryptic peptides of the previously purified maleylacetate reductase of Pseudomonas sp. strain B13 (9), allowing for a few discrepancies at the beginning or end of the peptides. A potential ribosome binding site upstream of clcE starting at position 1222 was observed.

While the incomplete ORF and clcD of Pseudomonas sp. strain B13 were identical to the corresponding genes of pAC27, the clcE sequence was not completely identical to the 633-bp partial sequence reported for the region downstream of the clcABD cluster of plasmid pAC27 (6). The latter sequence differs from the one presented in Fig. 1 in four missing or ad-
ditional nucleotides, which, if translated, would result in frame-shifts as well as three base exchanges. However, resequencing of the first 300 bp of the pAC27 clcE gene, as present on the recombinant plasmid pDC100 (7), showed the pAC27 sequence to be in fact identical to the strain B13 sequence. In addition, the latter, with respect to the reading frame, is supported by the previously reported sequences of tryptic peptides (9).

The deduced protein sequence of maleylacetate reductase ClcE of *Pseudomonas* sp. strain B13 has 59.4% identical positions with the sequence of TcbF (20), 55.1% identical positions with that of TfdF (15), and 53.1% identical positions with that of TftE (2). An alignment of the ClcE sequence with these protein sequences shows several regions which are completely conserved (Fig. 2), thus indicating that these proteins all function as maleylacetate reductases.

The sequences of the maleylacetate reductases have ca. 30% identical positions with the iron-containing (type III) alcohol dehydrogenases (19), a group of enzymes able to reduce carbonylic functions or to oxidize alcoholic groups by use of NADH or NAD, respectively. Interestingly, no convincing similarity of ClcE to proteins that are able to reduce carbon-carbon double bonds by consuming NADH (EC 1.3.1 group) was found.

The expression of the clcE gene was analyzed with an *Escherichia coli* strain carrying plasmid pB13P and with different subclones by using the lacZ promoter located on the pBlue-scriptIIKS vector and that on the pCR-Script(Amp)KS vector, respectively, as described by the manufacturer. The activity of the crude extract was tested according to the standard assay of Kaschabek and Reineke (12) with an “anaerobic” modification. The reaction mixture contained 6 mM dithiothreitol, all solutions were saturated with nitrogen, and the reaction was performed in a stream of nitrogen to prevent unintended oxidation of NADH. Strains carrying plasmid pB13P or subcloned plasmids with an intact clcE gene showed activity of maleylacetate reductase, while no activity was found in strains carrying plasmids with an incomplete clcE gene from B13 or pAC27, such as pDC100 (7). Further enzyme expression experiments were performed to prove that TcbF has the capability to reduce maleylacetate. In the case of a strain carrying the tcbF gene on the recombinant plasmid pTCB86 (20) we observed that the gene product TcbF was able to convert maleylacetate and its chlorinated analogs. Interestingly, the enzyme was found to be unstable, with a half-life of 10 h (10), a fact which has also been observed with TfdF (17).

The chemistry, enzymology, and genetics of the modified ortho pathway have now been studied. However, the use of chlorocatechols as carbon and energy sources by bacterial cells...
FIG. 2. Comparison of the predicted protein sequences of various maleylacetate reductases. ClcE (B13), protein sequence predicted for the clcE gene product of Pseudomonas sp. strain B13; TcbF (pP51), sequence predicted for the product of the tcbF gene of plasmid pP51 from Pseudomonas sp. strain P51 (20); TfdF (pJP4), sequence predicted for the product of the tfdF gene of plasmid pJP4 from R. eutropha JMP134 (15); Tfe (AC1100), sequence predicted for the tfe gene product of B. cepacia AC1100 (2). Stars mark positions conserved in all four sequences.

seems to require the last two enzymes of the β-ketoadipate pathway. Work to provide clear-cut proof of the involvement of these enzymes in the degradation of chloroaromatics is in progress.

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REFERENCES


