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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 *Pst*I 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 pP51 (59.4% identical positions), *tfdF* of pJP4 (55.1%), and *tftE* 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-oxoadipates 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 biochemistry 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, pP51 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-dioxygenase, chloromuconate cycloisomerase, and (chloro)dienelactone hydrolase, 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), corresponding to bases 676 to 696 of the *clcD* gene of pAC27 (6), and primer TK-11 [5′-GG(A/C/G/T)GG(A/C/G/T)C(G/T)(A/C/G/T)CC(A/G)TGC CA(A/C/G/T)GC(G/A)TC], derived from the tryptic peptide MAR6 (9). The other PCR made use of primers M1 [5′-CGA (T/C)GG(C/T)GCGACGATGCA] and M2 [5′-ATGGCACA GCTTGTG(G/A)TG(T/C)A], 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 *tftE* genes. Its cloning into the *Srf*I site of vector pCR-Script(Amp)KS+ yielded plasmid pPCR31-13. The 548-bp PCR product was cloned into the *Eco*RV site of pBluescriptIIKS+, 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 [^{32}P] dCTP-labeled PCR products as probes. *Pst*I fragments of B13 DNA were ligated into pBluescriptIIKS+ (3,167-bp insert), yielding plasmid pB13P containing the relevant gene.

The complete nucleotide sequence of the 3,167-bp *Pst*I 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 512 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-

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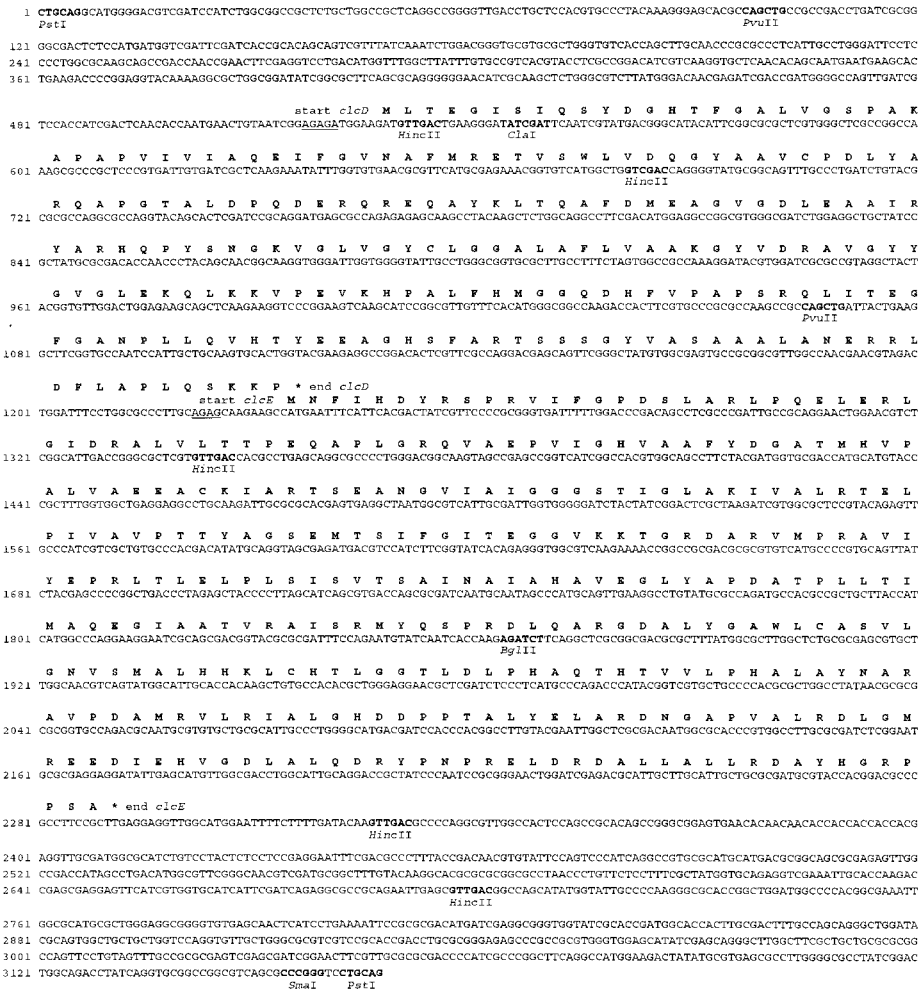

FIG. 1. DNA sequence and deduced amino acid sequences of the 3,167-bp *Pst*I fragment from *Pseudomonas* sp. strain B13 as present on the recombinant plasmid pB13P. Possible ribosome binding sites are underlined. Stars represent the stop codons. Bold letters indicate the main restriction sites.

ditional nucleotides, which, if translated, would result in frameshifts 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 pBluescriptIIKS+ 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); TftE (AC1100), sequence predicted for the *tftE* 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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