JOURNAL OF BACTERIOLOGY, Nov. 1989, p. 5915–5921
0021-9193/89/115915-07$02.00/0
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Vol. 171, No. 11

Genetic Organization and Sequence of the *Pseudomonas cepacia* Genes for the Alpha and Beta Subunits of Protocatechuate 3,4-Dioxygenase

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Received 16 March 1989/Accepted 10 August 1989

The locations of the genes for the α and β subunits of protocatechuate 3,4-dioxygenase (EC 1.13.11.3) on a 9.5-kilobase-pair *PstI* fragment cloned from the *Pseudomonas cepacia* DBO1 chromosome were determined. This was accomplished through the construction of several subclones into the broad-host-range cloning vectors pRO2317, pRO2320, and pRO2321. The ability of each subclone to complement mutations in protocatechuate 3,4-dioxygenase (*pcaA*) was tested in mutant strains derived from *P. cepacia*, *Pseudomonas aeruginosa*, and *Pseudomonas putida*. These complementation studies also showed that the two subunits were expressed from the same promoter. The nucleotide sequence of the region encoding for protocatechuate 3,4-dioxygenase was determined. The deduced amino acid sequence matched that determined by N-terminal analysis of regions of the isolated enzyme. Although over 400 nucleotides were sequenced before the start of the genes, no homology to known promoters was found. However, a terminator stem-loop structure was found immediately after the genes. The deduced amino acid sequence showed extensive homology with the previously determined amino acid sequence of protocatechuate 3,4-dioxygenase from another *Pseudomonas* species.

Protocatechuate 3,4-dioxygenase (PCD; EC 1.13.11.3) catalyzes the conversion of protocatechuate to β-carboxymuconate through cleavage of the aromatic ring with the simultaneous introduction of molecular oxygen. PCD from *Pseudomonas cepacia* DBO1 has been studied extensively by Ballou and co-workers (7–10, 27, 48). The *P. cepacia* enzyme is composed of equimolar amounts of two nonidentical subunits of 23,000 daltons (α subunit) and 26,500 daltons (β subunit). Since the molecular size of the holoenzyme is 200,000 daltons, there must be four α and four β subunits that make up the intact enzyme. Analysis of the iron contained by this enzyme showed that the holoenzyme contains four ferric iron atoms. The PCD from *P. cepacia* DBO1 therefore consists of four α subunits, four β subunits, and four ferric iron atoms (8). Studies on the properties and mechanism of action of this enzyme have been performed (7, 9, 10, 48), and further studies are in progress. Crystals of PCD have been obtained by Ludwig and co-workers (27), and further studies on the fine structure of these crystals are partially dependent on knowledge of the amino acid sequence of PCD. In order to facilitate the mechanistic and structural studies and to permit future site-directed mutagenesis on *P. cepacia* PCD, we cloned the genes for both subunits of PCD (51) by using vectors that we developed specifically for use in *Pseudomonas* species (G. J. Zylstra, S. M. Cuskey, and R. H. Olsen, *in* M. Levin, R. Seidler, and P. Pritchard, ed., *Classical and Molecular Methods to Assess Environmental Applications of Microorganisms*, in press). The nucleotide sequence of the region of DNA encoding the two subunits of PCD was determined in this study.

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MATERIALS AND METHODS

Bacterial strains, plasmids, and media. The bacterial strains and plasmids used in this study are listed in Tables 1 and 2, respectively. Mutants were constructed with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine as described previously (51). Complex medium was prepared as described previously (38). The minimal medium of Vogel and Bonner (47) was used to screen for trimethoprim resistance. Minimal medium for carbon source studies (MMO) was that used previously by Stanier and co-workers (44) in their taxonomic survey of the *Pseudomonas* species. Carbon sources were added to MMO at a final concentration of 0.1% (0.05% for protocatechuate). Antibiotics were added to the media at the following concentrations: tetracycline, 25 μg/ml for *P. cepacia* and *Pseudomonas putida* and 50 μg/ml for *Pseudomonas aeruginosa*; trimethoprim, 50 μg/ml for *P. cepacia* and 600 μg/ml for *P. aeruginosa*; carbenicillin, 500 μg/ml for *P. aeruginosa* and *Escherichia coli*. *P. putida* was grown at 30°C, while all other strains were cultured at 37°C unless otherwise specified.

DNA techniques. Plasmid DNA was isolated by the alkaline-sodium dodecyl sulfate technique (4, 23). DNA was quantified spectrophotometrically by assuming that an A_{260} reading of 1.0 was equivalent to a 50-μg/ml solution (29). Restriction digests were performed as recommended by the supplier (International Biotechnologies, Inc., New Haven, Conn.) and visualized by agarose gel electrophoresis in Tris acetate buffer (40 mM Tris, 1 mM EDTA [pH 7.4]). DNA was transformed into *P. aeruginosa* by the procedure of Mercer and Loutit (31), into *P. putida* by the procedure of Bagdasarian and Timmis (2), into *E. coli* by the calcium-thymidine-glycerol procedure (29, 37), and into *P. cepacia* by the procedure of Zylstra et al. (51).

DNA sequencing. DNA fragments to be sequenced were subcloned into the sequencing vector pGEM3 and transformed into *E. coli* HB101 with selection for pGEM3-encoded carbenicillin resistance. Sequencing reactions were

TABLE 1. Bacterial strains used in this study

Bacterium	Genotype or phenotype[a]	Reference or source
P. aeruginosa		
PAO1c	Prototroph	19
PAO1c.103	*pcaA103*	51
P. putida		
PPO200	Prototroph	—[b]
PPO20142	*pcaA142*	Lute[c]
P. cepacia		
DBO1	Prototroph	Ribbons[d] (48)
DBO167	*pcaA167*	51
DBO207	*pcaA207*	This work
E. coli HB101	*leuB6 thi-1 hsdR hsdM recA*	5

[a] Marker abbreviations: *pcaA*, PCD structural gene; *leu*, leucine; *thi*, thiamine; *hsdR*, restriction deficient; *hsdM*, modification deficient; *rec*, recombination deficient.
[b] —, *P. putida* PaW1 (*mt-2*) cured of the TOL plasmid in our laboratory.
[c] J. L. Lute, Ph.D. thesis, University of Michigan, Ann Arbor, 1986.
[d] D. W. Ribbons, Imperial College of Science and Technology, London, United Kingdom.

carried out by using the Klenow fragment of DNA polymerase and the appropriate primer by the dideoxynucleotide procedure of Sanger et al. (41). Reagents were furnished by Promega Biotech (Madison, Wis.). DNA samples were analyzed by electrophoresis through 5 or 8% polyacrylamide gels (acrylamide-bisacrylamide, 20:1) containing 7 M urea. Running buffer contained 0.1 M Tris, 0.1 M boric acid, and 0.2 mM sodium EDTA. In order to eliminate secondary structure, gels were run at 70°C by using a thermostatic plate and circulating water (16).

Protein sequencing. Purified PCD was prepared as described previously (8). The α and β subunits were separated from each other by chromatofocusing in the presence of 8 M urea. Samples were loaded on a polybuffer exchange column (Pharmacia Fine Chemicals, Piscataway, N.J.) previously equilibrated with 25 mM imidazole hydrochloride. The individual subunits were eluted with a 1:10 dilution of polybuffer 74–HCl (pH 5.0). N-terminal sequencing, cyanogen bromide digestion, and amino acid composition analysis were performed by the University of Michigan Protein Sequencing Facility following previously published procedures (45).

TABLE 2. Plasmids used in this study

Plasmid	Genotype or phenotype[a]	Reference or source
pRO1727	Tc^r Cb^r	13
pRO2317	Tc^r Cb^r	Zylstra et al.[b]
pRO2320	Tc^r Cb^r Tp^r	Zylstra et al.[b]
pRO2321	Tc^r Tp^r	Zylstra et al.[b]
pRO2322	Tc^r *pcaA*	51
pRO2324	Tc^r *pcaA*	51
pRO2325	Tc^r *pcaA*	This work
pRO2326	Tp^r	This work
pRO2327	Tp^r	This work
pRO2328	Tc^r	This work
pRO2329	Tc^r	This work
pRO2330	Tc^r Tp^r	This work
pRO2331	Tc^r	This work
pRO2332	Tc^r Tp^r	This work
pRO2333	Cb^r	This work
pRO2334	Cb^r Tp^r	This work
pGEM3	Cb^r	Promega[c]

[a] Marker abbreviations: *pcaA*, PCD structural gene; Tc^r, tetracycline resistance; Tp^r, trimethoprim resistance; Cb^r, carbenicillin resistance.
[b] Zylstra et al., in press.
[c] Obtained from Promega Biotech, Madison, Wis.

RESULTS

Determination of the location of the PCD genes encoded by pRO2322. It has been shown previously that PCD from *P. cepacia* consists of approximately 450 amino acids (8). This was based on the molecular weight of the subunits and on amino acid composition data. This means that only 1,350 base pairs of DNA are needed to encode for this enzyme. Since pRO2322 contains both 9.5- and 0.3-kilobase *Pst*I chromosomal DNA fragments, several subclones were constructed to determine the precise locations of the genes for the α and β subunits of PCD. The ability of the plasmid deletions to complement PCD mutations is depicted in Fig. 1, which shows only the cloned chromosomal region and not the associated vector regions. Figure 1 also shows that both pRO2324 and pRO2325 complement the PCD mutation in DBO167 and DBO207 as well as the mutation in *P. aeruginosa* PAO1c.103 and *P. putida* PPO20142. Thus, both of the subunits of PCD must be encoded by the DNA between the *Pst*I and *Sst*I sites of pRO2322. Plasmids pRO2329 and pRO2330 both complemented DBO167, but not any of the other strains. These two plasmids have in common the left *Pst*I to *Bam*HI fragment of pRO2322, so this region must encode for one of the subunits of PCD but not the other. Plasmids pRO2326, pRO2331, and pRO2332 did not complement DBO167. This suggests that the *Bgl*II and the *Eco*RI sites at the termini of these deletions must cleave the gene responsible for complementing DBO167. Neither pRO2326 nor pRO2330 complemented DBO207. Since these were overlapping subclones, one of the two possible explanations given below must be true. DBO207 could be a mutant that was missing both subunits of PCD or it could be a mutant that was missing only one subunit if the genes for both subunits were transcribed from the same promoter. If the latter is the case, then the promoter must be to the left of the *Bgl*II site, with transcription proceeding from left to right.

Determination of the number of promoters for the PCD genes. The data given in the preceding section suggested that the genes for the two subunits of PCD are transcribed from the same promoter. In order to test this hypothesis, PAO1c.103 strains were constructed that contained two plasmids, each of which possibly contained the gene for a different subunit. The vectors in each case were derived from compatible plasmids to prevent curing of one or the other because of incompatibility. The *Xho*I to *Bam*HI fragment of pRO2322 was subcloned into vector pRO1727 (13) cut with *Sal*I and *Bam*HI. The resultant plasmid (pRO2333), based on the experiments described above, should therefore encode for one of the subunits of PCD. Plasmid pRO2326 was transformed into PAO1c.103 containing pRO2333. The cloned chromosomal region of plasmid pRO2326 overlapped that of pRO2333, so that genes for each subunit were intact on one or the other plasmid. If the two genes were transcribed from separate promoters, *trans*-complementation would be seen. PAO1c.103 containing both plasmids was assayed for PCD activity under conditions described previously (51). No activity was detected. Strains containing both plasmids showed no growth on MMO agar plates containing *p*-hydroxybenzoate after 1 day, but showed growth after 2 days. Five of these colonies that regained the ability to grow

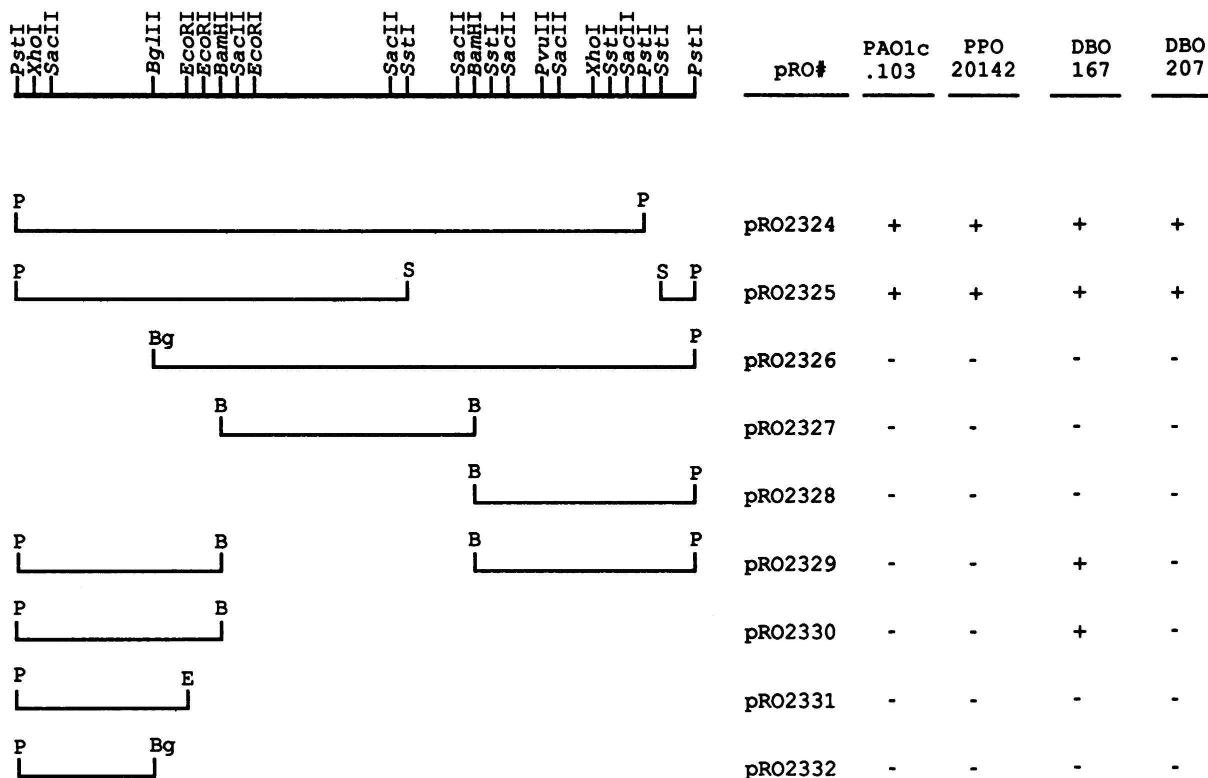

FIG. 1. Ability of pRO2322 subclones to complement PCD mutants. Only the cloned chromosomal region is shown. Abbreviations: B, *Bam*HI; Bg, *Bgl*II; E, *Eco*RI; P, *Pst*I; S, *Sst*I.

on *p*-hydroxybenzoate were examined. Each strain contained a new plasmid that resulted from a recombinational crossover event in the region of homology between the two plasmids, essentially reconstructing the cloned region of the parental plasmid pRO2322. This and the foregoing observations suggest that both subunits are transcribed from a single promoter. If this is the case, then it is likely that the genes for the two subunits are contiguous.

DNA sequencing of the *P. cepacia* PCD genes. The region of DNA postulated above to encode for the two subunits was sequenced as described in Materials and Methods. A series of overlapping subclones of this region was constructed in the sequencing vector pGEM3. Both strands of the DNA were sequenced from independently derived subclones. The nucleotide sequence is depicted in Fig. 2.

The nucleotide sequence data show that there are two open reading frames, 196 and 235 amino acids long, within the region studied (Fig. 2). Each reading frame was of the expected size needed to encode for one of the two subunits of *P. cepacia* PCD. Their locations were also consistent with the subclone complementation data presented above (Fig. 1). The first open reading frame was cleaved by both *Bgl*II and *Eco*RI and was to the left of the *Bam*HI site. The second reading frame was separated from the first one by only three nucleotides, suggesting that both reading frames are transcribed from the same promoter.

Protein sequencing of PCD. The amino acid sequence of regions of the α and β subunits of PCD were also determined in order to verify the DNA sequence. The α subunit and four peptides generated by cyanogen bromide cleavage of the α subunit were subjected to N-terminal sequence analysis and amino acid composition analysis. The β subunit and three peptides generated by cyanogen bromide cleavage of the β subunit were also subjected to the same analysis. The data derived from the N-terminal peptide sequencing agree with the amino acid sequence predicted by the DNA sequence (Fig. 2). The results of amino acid composition analyses also agree with those predicted by the DNA sequence (data not shown). N-terminal analysis of the PCD holoenzyme yielded two amino acids per cycle in equimolar quantities. These holoenzyme protein sequence data confirm the previous observation by Bull and Ballou (8) that the α and β subunits are in a one-to-one relationship in the holoenzyme. However, the N-terminal methionine of the α subunit was removed, while the N-terminal methionine of the β subunit was not.

DISCUSSION

Codon usage and G+C% of the PCD genes. The entire region sequenced was 67.2% G+C. This is similar to that reported previously for *P. cepacia* (66.9%) and *Pseudomonas multivorans* (67.6%) by Ballard and co-workers (3). *P. multivorans* is considered to be a pseudonym for *P. cepacia* (3). The coding regions of the sequence were 68% G+C, while the region preceding the two open reading frames was only 64% G+C. The latter was due to the abundance of A-T base pairs in this region which may promote melting of the DNA, allowing RNA polymerase to enter and initiate transcription. The high G+C content of the DNA was due primarily to a preference of guanine- and cytosine-terminated codons (Table 3). A total of 89% of the variable third-base positions were guanine or cytosine rather than adenine or thymine. Notable exceptions to this preference were the histidine and glutamate codons. A total of 6 histidine codons ended in thymine, while 8 ended in cytosine

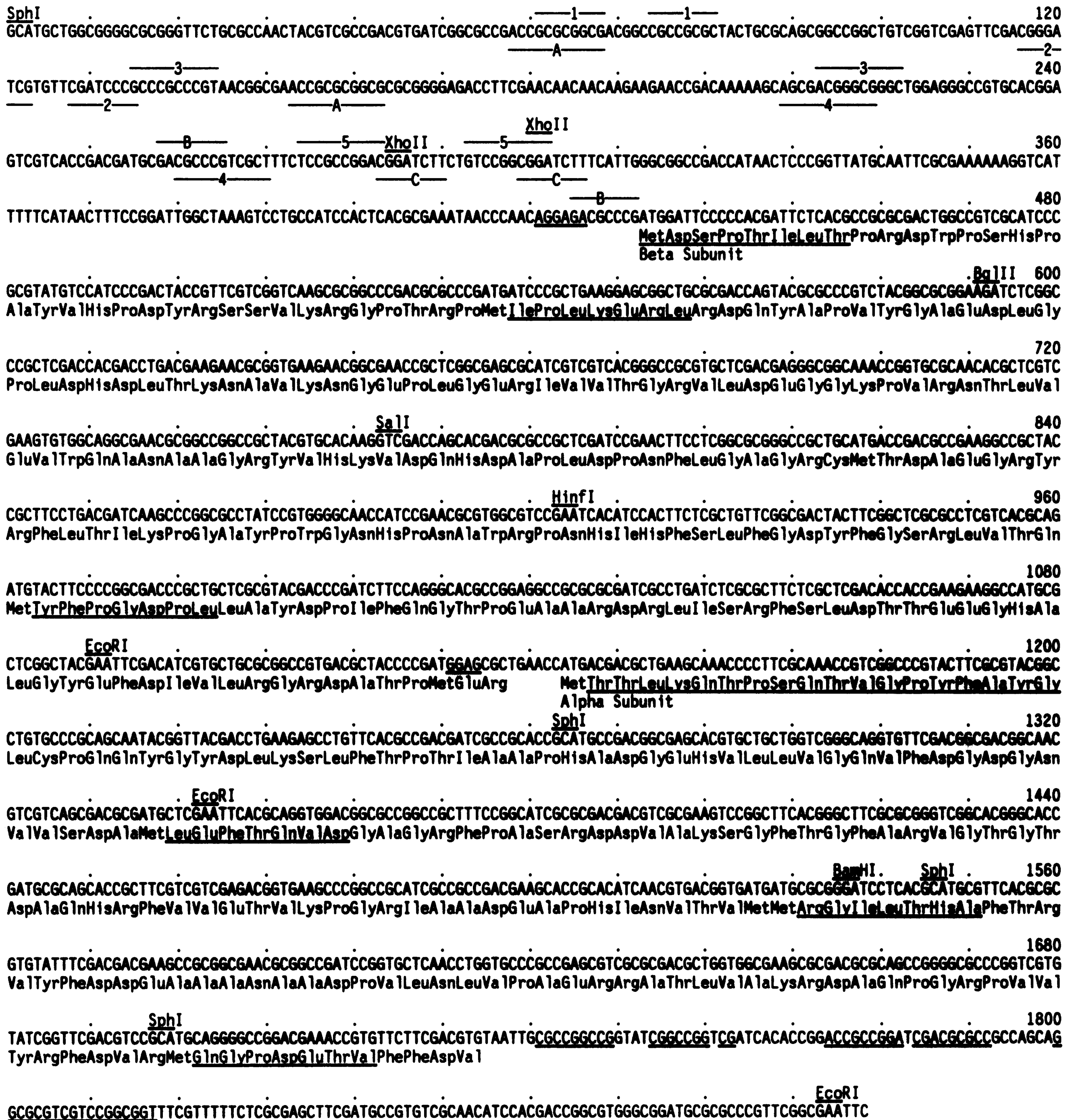

FIG. 2. Nucleotide sequence of the region encoding for the α and β subunits of PCD. Direct repeats in the promoter region are marked with lines and letters. Indirect repeats in the promoter region are marked with lines and numbers. The restriction sites used to generate subclones for sequencing are indicated. The amino acid sequences confirmed by N-terminal sequencing of cyanogen bromide-generated peptides are underlined. Sequences corresponding to possible ribosome-binding sites as determined by Shine and Dalgarno (43) are underlined. The stem-loop structure at the end of the operon is underlined.

and 11 glutamate codons ended in adenine, while only 8 ended in guanine. The preference for guanine and cytosine also extended to the first codon position where there was a choice. All 31 leucine codons started with cytosine rather than with thymine, and all 34 arginine codons started with cytosine rather than adenine. These codon preferences may reflect the relative amounts of the different tRNAs in this strain. Many of the *Pseudomonas* genes that have been sequenced show this codon preference. Plasmid-encoded genes such as the mercury resistance genes of Tn*501* (6, 34), the toluene degradation genes of the TOL plasmid (32, 35), and the camphor degradation genes of the CAM plasmid (46) all have a relatively high G+C% and a preference for codons ending in guanine or cytosine. Chromosomally encoded genes such as anthranilate synthase (12), exotoxin A (17), tryptophan synthase (18), and carboxypeptidase G_2 (33) also

TABLE 3. Codon usage of the PCD α- and β-subunit genes

Codon	Amino acid	No. of times used	Codon	Amino acid	No. of times used	Codon	Amino acid	No. of times used	Codon	Amino acid	No. of times used
TTT	Phe	1	TCT	Ser	0	TAT	Tyr	4	TGT	Cys	0
TTC	Phe	21	TCC	Ser	2	TAC	Tyr	13	TGC	Cys	2
TTA	Leu	0	TCA	Ser	0	TAA	Ter	1	TGA	Ter	1
TTG	Leu	0	TCG	Ser	9	TAG	Ter	0	TGG	Trp	4
CTT	Leu	0	CCT	Pro	1	CAT	His	6	CGT	Arg	4
CTC	Leu	15	CCC	Pro	8	CAC	His	8	CGC	Arg	27
CTA	Leu	0	CCA	Pro	0	CAA	Gln	3	CGA	Arg	0
CTG	Leu	16	CCG	Pro	27	CAG	Gln	11	CGG	Arg	3
ATT	Ile	1	ACT	Thr	0	AAT	Asn	1	AGT	Ser	0
ATC	Ile	11	ACC	Thr	8	AAC	Asn	11	AGC	Ser	2
ATA	Ile	0	ACA	Thr	0	AAA	Lys	1	AGA	Arg	0
ATG	Met	10	ACG	Thr	21	AAG	Lys	11	AGG	Arg	0
GTT	Val	0	GCT	Ala	1	GAT	Asp	6	GGT	Gly	1
GTC	Val	18	GCC	Ala	12	GAC	Asp	30	GGC	Gly	33
GTA	Val	0	GCA	Ala	3	GAA	Glu	11	GGA	Gly	0
GTG	Val	19	GCG	Ala	24	GAG	Glu	8	GGG	Gly	4

have a high G+C% and a preference for codons ending in guanine and cytosine. This bias toward high G+C% also extends to *Pseudomonas* phages such as Pf1 (28). One exception to this generality of a high G+C% through codon preference in *Pseudomonas* species is the pilin genes of *P. aeruginosa* (42). The pilin genes from both *P. aeruginosa* PAO and PAK are homologous and have only about 50% G+C. In these two examples there is a preference for thymine rather than cytosine in the third codon position.

Distance between the coding regions for the two subunits of PCD. Analysis of the sequence also revealed that there were only three bases between the stop codon for the β subunit and the start codon for the α subunit (Fig. 2). Other proteins containing α and β subunits have been studied in *Pseudomonas* species. The anthranilate synthase subunit genes overlap by 23 bases (12), and the tryptophan synthase subunit genes overlap by 4 bases (18). The 7β-(4-carboxyhutanamido)cephalosporanic acid acylase from a *Pseudomonas* strain is translated as a single peptide which is cleaved to form the α and β subunits (30). The genes for the α and β subunits of the iron sulfur protein component of toluene dioxygenase are separated by 114 bases (G. J. Zylstra and D. T. Gibson, J. Biol. Chem., in press). Figure 2 also shows that there is a ribosome-binding site resembling that determined by Shine and Dalgarno (43) for *P. aeruginosa* before the coding region for each subunit. Two ribosome-binding sites have also been found for the overlapping genes for tryptophan synthase (18). Similar ribosome-binding sites have been found preceding the genes from the *Pseudomonas* species discussed above.

Analysis of the promoter and terminator regions. No homology was found to known promoters (21, 22, 32, 40) in the region upstream from the PCD genes. Analysis of the promoter region revealed the presence of numerous direct and indirect repeats as well as stem-loop structures (Fig. 2). This region was also rich in adenine and thymine nucleotides. There was a string of five adenines starting at position 202. There was also a string of six adenines starting at position 349 that was followed by a string of five thymines in a row starting at position 360. It is probable that the relatively high concentration of A-T base pairs in this region is significant because of the high G+C content of *P. cepacia*. Analysis of the promoter region also revealed two peculiar base sequences. The sequence CCCGCCCGCCCG at position 132 repeats the base sequence CCCG three times. The sequence GAACAACAACAAGAAGAAC alternates two adenines with either a guanine or a cytosine. The significance of these peculiar sequences as well as the direct repeats, indirect repeats, and stem-loop structures is not known.

Analysis of the region immediately following the coding region for the two subunits revealed the presence of two consecutive stem-loop structures (Fig. 2). These are illustrated in Fig. 3. Both of these stem-loop structures are rich in G-C base pairs, and the second stem-loop structure would be followed by a string of uridine residues in the resultant mRNA. This structure resembles the Rho-independent transcription terminators of *E. coli* (1, 39). Such a terminator structure has also been found in the DNA sequence following the exotoxin A gene of *P. aeruginosa* (17).

Comparison of the PCD protein sequence from *P. aeruginosa* and *P. cepacia*. One other PCD has been sequenced

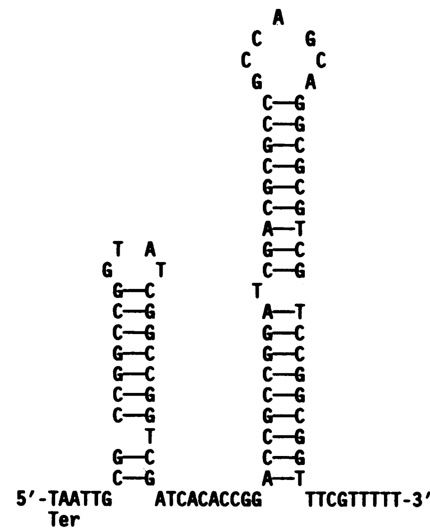

FIG. 3. Possible Rho-independent terminator in mRNA. Ter marks the end of the α subunit-coding region. The sequence shown corresponds to nucleotide positions 1735 to 1826 from Fig. 2.

A.

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PIELLPETPSQTAGPYVHIGLALEAAGNPTRDQEIWNRLAKPDAPGEHILLLGQVYDGD
 TTLKQTPSQTVGPYFAYGLCPQQYGYDLKSLFTPTI AAPHADGEHVLLVGQVFDGD

GHLVRDSFLEVWQADADGEYQDAYNLENA   FNSFGRTATTFDAGEWTLH TVKPGVV
GNVVSDAMLEFTQVDGAGRFP ASRDDVAKSGFTGFARVGTGTDAQHRFVVETVKPGRI

NNAAGVPMAPHINISLFARGINIHLHTRLYFDDEAQANAKCPVLNLIEQPQRRETLIAK
 AADE  APHINVTVMMRGILTHAFTRVYFDDEAAANAADPVLNLVPAE RRATLVAK

RCEVDGKTA YRFDIRIQGEGETVFFDF
RDAQPGRPVVYRFDVRMQGPDETVFFDV
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B.

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PAQDNSRFVIRDRNWHPKALTPDYKTSIARSPRQALVSIPQSISETTGPNFSHLGFGAH
 MDSPTILTPRDWPSHPAYVHPDYRSSVKRGPTRPMIPLKERLRDQYAPVYGAEDLGPL

DHDLLLNFNNGGLPIGERIIVAGRVVDQYGKPVPNTLVEMWQANAGGRYRHKNDRYLAP
DHDLTKNAVKNGEPLGERIVVTGRVLDEGGKPVRNTLVEVWQANAAGRYVHKVDQHDAP

LDPNFGGVGRCLTDSDGYYSFRTIKPGPYPWRNGPNDWRPAHIHFGISGPSIATKLITQ
LDPNFLGAGRCMTDAEGRYRFLTIKPGAYPWGNHPNAWRPNHIHFSLFGDYFGSRLVTQ

LYFEGDPLIPMCPIVKSIANPEAVQQ LIAK  LDMNNANPMNCLAYRFDIVLRGQRKT
MYFPGDPLLAYDPIFQGT  PEAARDRLISRFSLDTTEEGHA  LGYEFDIVLRGRDAT

HFENC
PMER
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FIG. 4. Comparison of the protein sequence of PCD from *P. aeruginosa* and *P. cepacia*. The uppermost sequence in both cases is the *P. aeruginosa* amino acid sequence. A bold line indicates homology. The sequences were aligned to show maximum homology. (A) α-Subunit comparison. (B) β-Subunit comparison.

completely by protein sequencing techniques (15, 24, 25, 36, 50). This sequence was derived from the enzyme isolated from a strain of *Pseudomonas* (B-10). This strain is reported to be *P. aeruginosa* by the investigators who performed the sequencing, but the strain is listed as *P. putida* by the American Type Culture Collection (ATCC 23975). This sequence shows much homology to the sequence determined here for *P. cepacia* PCD. Figure 4 illustrates the two protein sequences aligned to show maximum homology. The N terminus of both subunits seems to have diverged much farther than the C terminus.

PCD has also been isolated from *Rhizobium trifolii* (11), *Azotobacter vinelandii* (14), *Acinetobacter calcoaceticus* (20), *Nocardia erythropolis* (26), *Brevibacterium fuscum* (49), and *Thiobacillus* sp. strain A2 (M. Wells, Ph.D. thesis, University of Texas, Austin, 1972). These different PCDs have different subunit molecular weights, holoenzyme compositions, pH optimums, and K_m values for protocatechuate. A detailed comparative study of these enzymes will reveal more information concerning their relatedness as well as their mechanisms of action. Toward this end, we are analyzing the DNA which encodes PCD from *P. aeruginosa* PAO1c and *P. putida* PPO200. The DNA sequence of PCD from *Acinetobacter calcoaceticus* has also been reported (C. Hartnett, R. C. Doten, K. L. Ngai, and L. N. Ornston, Abstr. Annu. Meet. Am. Soc. Microbiol. 1987, R22, p. 244). It will be interesting to compare these sequences at both the DNA and protein levels. A comparison of these DNA sequences will yield information about the evolution of these genes, particularly since *Pseudomonas* species have about 70% G+C in their DNA, while *A. calcoaceticus* has only about 50% G+C. Comparisons of the amino acid sequences along with studies on the mechanistic and structural aspects of PCD will allow future experiments to be designed in which site-directed mutagenesis can be used to study the mechanism of oxygen activation and reaction with the protocatechuate catalyzed by this enzyme.

ACKNOWLEDGMENTS

We thank C. Mountjoy for purifying PCD from *P. cepacia* DBO1. We also thank C. Batie for helpful discussions.

This work was supported in part by a grant from the Michigan Biotechnology Institute, cooperative agreement CR-812679 from the U.S. Environmental Protection Agency (to R.H.O.), and Public Health Service grant GM-20877 from the National Institutes of Health (to D.P.B.). One of us (G.J.Z.) was supported by a grant from the University of Michigan Program in Protein Structure and Design.

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