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# Organization and nucleotide sequence determination of a gene cluster involved in 3-chlorocatechol degradation

(gene expression/maxicell analysis/nuclease S1 mapping/*Pseudomonas* promoters)

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**ABSTRACT** **Three critical enzymes, catechol oxygenase II (chlorocatechol dioxygenase), muconate cycloisomerase II, and dienelactone hydrolase, are involved in the degradation of chlorocatechols, which are obligatory intermediates in the catabolism of chlorinated aromatic compounds. The organization and complete nucleotide sequence of the genes for these enzymes have been determined on a 4.2-kilobase-pair (kbp) *Bgl* II fragment cloned from the plasmid pAC27, based on the agreement of open reading frame lengths with apparent mobilities of polypeptides expressed in *Escherichia coli* maxicells, predicted N-terminal amino acid sequences with those of the purified proteins, and predicted total amino acid compositions with those of the purified proteins. The 4.2-kbp fragment contains the three genes and ribosome binding sites for those genes but no promoter. When placed downstream of the *tac* promoter in the broad-host-range plasmid pMMB22, this fragment directs the synthesis of all three enzymes in both *E. coli* and *Pseudomonas putida* only on induction with isopropyl β-D-thiogalactopyranoside, suggesting that the gene cluster is regulated as a single unit under the control of a single promoter. Endogenous transcription initiation of the gene cluster on pAC27, however, occurs from a site present within a 386-bp *Bgl* II fragment upstream of the 4.2-kbp fragment, and sequences 5′ to that site are similar to the sequences of other positively controlled *Pseudomonas* promoters occurring on the TOL and NAH plasmids.**

A large number of chlorinated compounds have been manufactured by the chemical industry during the last several decades and released into the environment in the form of herbicides, pesticides, solvents, refrigerants, etc., resulting in massive pollution problems (1). Highly chlorinated compounds such as polychlorinated biphenyls (PCBs), 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), and dichlorodiphenyltrichloroethane (DDT) persist in the environment because of their recalcitrance to microbial attack. In contrast, simple chlorinated compounds such as 3-chlorobenzoic acid and 2,4-dichlorophenoxyacetic acid (2,4-D) are biodegradable, and the degradative genes are borne on conjugative plasmids pAC27 and pJP4, respectively (2, 3). In both instances, the degradative genes have been cloned from the respective plasmids as parts of a 4.2-kilobase-pair (kbp) *Bgl* II fragment of pAC27 and 15-kbp *Eco*RI fragment of plasmid pJP4 (4, 5). While the plasmid pJP4 encodes the complete 2,4-dichlorophenoxyacetic acid degradative pathway, the plasmid pAC27 encodes only a chlorocatechol degradative pathway. The two enzymes that are involved in the conversion of 3-chlorobenzoate to 3-chlorocatechol are chromosomally encoded in *Pseudomonas putida* and are assumed to be the same needed for growth on benzoate. Thus, transfer of pAC27 to *P. putida* allows total degradation of 3-chlorobenzoate through both chromosomal and plasmid genes. Knackmuss and Reineke (6, 7) showed that there are three enzymes critical for chlorocatechol degradation: catechol oxygenase II (chlorocatechol dioxygenase, or pyrocatechase II), muconate cycloisomerase II, and dienelactone hydrolase (Fig. 1). Whereas the analogous chromosomally encoded enzymes involved in the oxidation of catechol (catechol oxygenase I, cycloisomerase I, and enol-lactone hydrolase) have high specificity for the nonchlorinated substrates with little activity toward chlorinated analogues, the plasmid-encoded catechol oxygenase II and cycloisomerase II are highly active toward chlorinated substrates but retain diminished activity toward the nonchlorinated substrates. Dienelactone hydrolase is, however, specific for dienelactone and has no activity toward enol-lactones. The plasmid pAC27 was demonstrated to complement *P. putida* chromosomal Ben$^-$ mutant PRS2015 (8), which is deficient in cycloisomerase I activity, to Ben$^+$ (allowing growth on benzoate as well as 3-chlorobenzoate) (9), suggesting that the plasmid-encoded enzyme can substitute for the defective chromosomal enzyme while retaining a different substrate specificity. These similarities and differences in substrate specificity raise the interesting question as to how genes encoding degradation of synthetic chlorinated compounds such as 3-chlorocatechol evolve in nature, how they are organized and regulated on a plasmid, and whether any clue to such a process can be obtained by comparing the nucleotide sequences of the newly evolved genes to the sequences for the corresponding chromosomal genes specifying degradation of nonchlorinated analogues. In this paper we report the organization and complete nucleotide sequence of the pAC27 gene cluster specifying 3-chlorocatechol degradation. We compare the promoter region of that cluster with other positively regulated *Pseudomonas* biodegradative gene functions from the TOL and NAH plasmids.

## MATERIALS AND METHODS

**Bacterial Strains, Plasmids, and Phage.** All strains used in this report have been described previously: *Escherichia coli* JM103 (10) for transformation of M13 recombinants, C600F$^+$ (11) for analysis of recombinants and purification of template DNA for sequencing, and CSR603 (12) for maxicell analysis and *P. putida* PRS2015 (8) containing pAC27 (2) for isolation of RNA. The overproducing plasmid pDC100, containing the 4.2-kbp *Bgl* II fragment of pAC27, has been described (11), and subfragments of the 4.2-kbp *Bgl* II fragment were used to construct pKK223-3 (Pharmacia) derivatives pBF101 and pBF102 (Fig. 1) for maxicell analysis. M13 phage mp18 and

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Abbreviations: IPTG, isopropyl β-D-thiogalactopyranoside; ORF, open reading frame.

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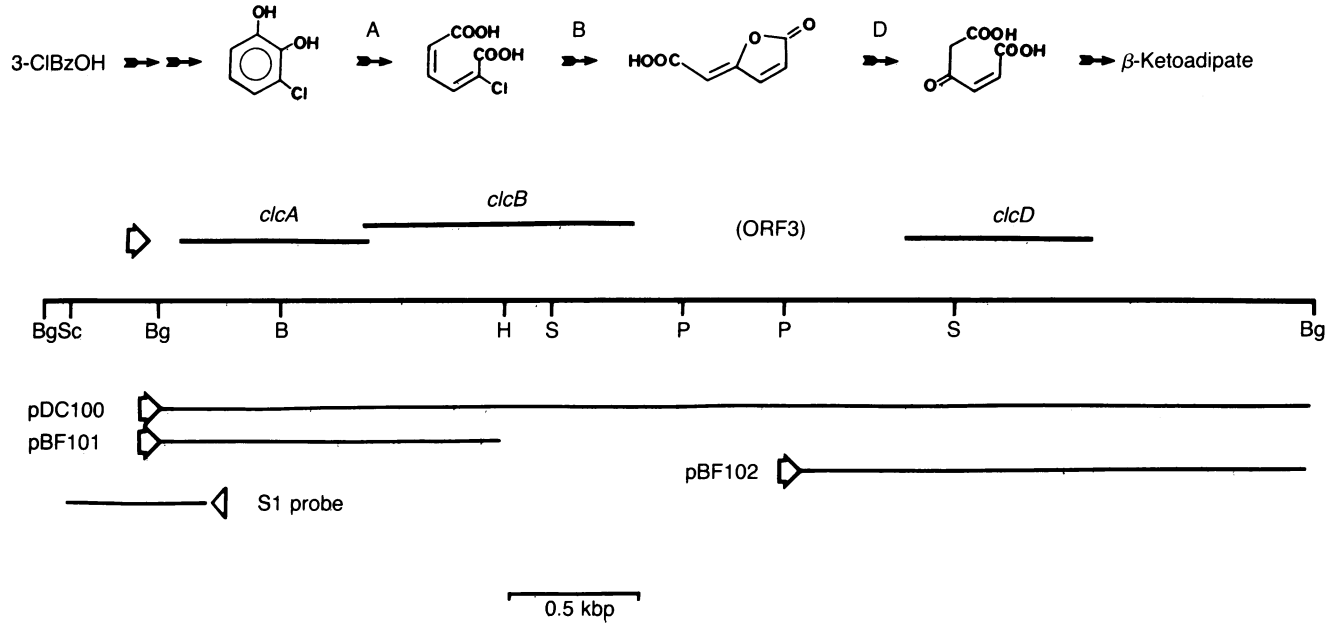


FIG. 1. Enzymatic pathway for the degradation of 3-chlorocatechol [derived from 3-chlorobenzoic acid (3-ClBzOH)] to maleylacetate, organization of chlorocatechol-degradative genes, restriction map, and plasmids used for expression analysis and nuclease S1 protection. The pathway shows the three key steps leading to the production of maleylacetate from 3-chlorocatechol. A chromosomally encoded maleylacetate reductase is believed to convert this product to β-ketoadipate for its ultimate utilization. A, catechol oxygenase II; B, muconate cycloisomerase II; D, dienelactone hydrolase. Step C, normally used for isomerization to the enol-lactone in the nonsubstituted catechol pathway, is nonenzymatic in the chlorocatechol pathway, with the spontaneous release of chloride. The locations of the corresponding genes (A, *clcA*; B, *clcB*; D, *clcD*) and of the unassigned open reading frame ORF3 are shown above the 4.2-kbp *Bgl* II fragment. A 385-bp *Bgl* II fragment immediately precedes the 4.2-kbp *Bgl* II fragment and contains operator/promoter sequences (open arrow) for expression of the *clc* gene cluster. B, *BamHI*; Bg, *Bgl* II; H, *Hin*dIII; P, *Pst* I; S, *Sal* I; Sc, *Sac* II. pDC100, pBF101, and pBF102 represent the whole or part of the 4.2-kbp *Bgl* II fragment cloned under control of the *tac* promoter.

mp19 (13) were used for dideoxy sequencing and deletion cloning.

**DNA Sequencing and Nuclease S1 Protection Analysis.** All reagents and procedures used for sequencing and maxicell analysis have been described (11). Deletion cloning by the method of Dale *et al.* (14) was used to construct clones for sequencing and probes for S1 mapping. RNA was isolated from PRS2015 harboring pAC27, with or without induction by 5 mM 3-chlorobenzoate, by the method of Deretic *et al.* (15). The DNA probe for hybridization was prepared by primer extension of a deletion clone in the presence of [$\alpha$-$^{32}$P]dCTP, by the method of Burke (16). Nuclease S1 was purchased from Boehringer Mannheim.

## RESULTS AND DISCUSSION

**Cloning and Expression of the Chlorocatechol (*clc*) Degradative Gene Cluster.** The cloning of a 4.2-kbp *Bgl* II fragment (E fragment) from the degradative plasmid pAC27 that conferred slow growth on 3-chlorobenzoate was reported previously (4). This suggested that all the degradative enzymes were encoded by this fragment. In order to determine if such enzymes could be detected in a heterologous host such as *E. coli*, the 4.2-kbp fragment was placed (after addition of *Eco*RI linkers) downstream of the *tac* promoter on the broad-host-range plasmid pMMB22 to produce the recombinant plasmid pDC100 (Fig. 1). Introduction of pDC100 into *E. coli* maxicell strain CSR603 allowed the synthesis of three major polypeptides, of 40, 33, and 28 kDa, only upon induction with isopropyl β-D-thiogalactopyranoside (IPTG) (Fig. 2, lane 4). Similar introduction of pDC100 into other strains of *E. coli* and induction with IPTG allowed detection of all three chlorocatechol degradative enzyme activities (catechol oxygenase II, muconate cycloisomerase II, and dienelactone hydrolase) and their purification (ref. 11; K.-L. Ngai, B.F., D. K. Chatterjee, L. N. Ornston, and A.M.C., unpublished results). Subfragments of the 4.2-kbp fragment, when placed downstream of the *tac* promoter of pKK223-3 to give plasmids pBF101 and pBF102 (Fig. 1), similarly allowed the synthesis of only the 33-kDa polypeptide (Fig. 2, lane 6) and the 28-kDa polypeptide (Fig. 2, lane 8) in *E. coli* maxicells. These and the following sequencing results suggest that the catechol oxygenase II gene (*clcA*) is at the 5′ end

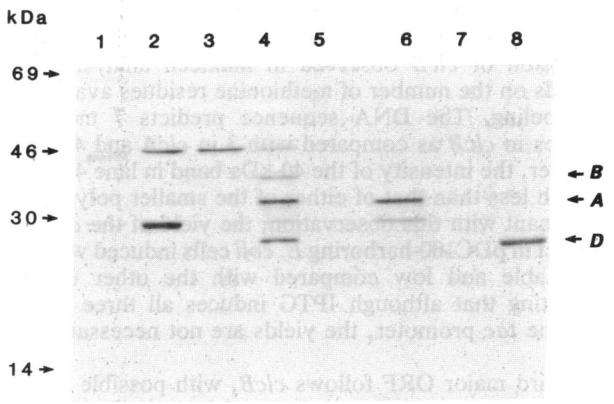


FIG. 2. Autoradiogram of L-[$^{35}$S]methionine-labeled proteins after UV irradiation of *E. coli* maxicells carrying various plasmids. Lanes: 1, $^{14}$C-labeled protein standards; 2, pMMB22 induced with IPTG; 3, pDC100 uninduced; 4, pDC100 induced with IPTG; 5 and 7, pKK223-3; 6, pBF101; 8, pBF102. Expression of polypeptides in pKK223-3 derivatives is constitutive in the maxicell strain CSR603 and thus independent of IPTG induction. The 30-kDa polypeptide present in pKK223-3 for ampicillin resistance is not detectable in pBF102 because that construct contains putative transcriptional termination sequences following the coding region for the 28-kDa polypeptide, which may prevent read-through from the *tac* promoter. Arrows with letter designations indicate associated *clc* genes responsible for expression of the polypeptides shown.

and the dienelactone hydrolase gene (*clcD*) is at the 3′ end of the 4.2-kbp fragment.

**Nucleotide Sequence Analysis of *clc* Genes.** Plasmid pAC27 contains a 385-bp *Bgl* II fragment, immediately upstream of the 4.2-kbp *Bgl* II E fragment (Fig. 1), that was previously unrecorded. The recombinant plasmid (pDC25) carrying these fragments was reported to elicit slow growth on 3-chlorobenzoate (4) but occasionally gave rise to larger, faster-growing colonies that were shown to have undergone as much as an 8-fold amplification of the 4.2-kbp segment. Amplification was seen as a direct consequence of the severe selective pressure on cells carrying a recombinant plasmid devoid of necessary regulatory signals to grow on 3-chlorobenzoate as a sole source of carbon and energy and was suggested to allow growth on that substrate by a gene-dosage effect (4). To determine the presence (or absence) of regulatory and structural gene elements within the two fragments, we determined the nucleotide sequences of the two fragments and performed nuclease S1 protection analysis to determine the site of mRNA initiation.

The nucleotide sequence of the 385-bp and 4.2-kbp *Bgl* II fragments revealed four major open reading frames (ORFs), the first initiating 23 bp downstream from the 5′ *Bgl* II end of the 4.2-kbp segment (Fig. 3, base 402). N-terminal amino acid sequence analysis of purified catechol oxygenase II from IPTG-induced pDC100 in *E. coli* agreed with the predicted amino acid sequence from the DNA, and the total amino acid composition of the purified protein agreed, within experimental error, with that predicted by the DNA (K.-L. Ngai, B.F., D. K. Chatterjee, L. N. Ornston, and A.M.C., unpublished data). Thus, we designated ORF1 as *clcA*. The molecular mass of the protein predicted by the DNA sequence is, however, 4 kDa lower than that consistently observed in $NaDodSO_4$/PAGE. Similar to other dioxygenases, catechol oxygenase II complexes non-heme $Fe^{3+}$ ions as cofactors (17), and thus the anomalous migration may be due to incomplete denaturation of the protein (K.-L. Ngai, personal communication).

The termination codon for *clcA* at base 1182 overlaps the initiation codon of the second major ORF (Fig. 3), which by agreement with N-terminal amino acid sequence analysis and total amino acid composition of the purified protein, we have designated *clcB*, the gene for muconate cycloisomerase II. This overlap may play a role in negative interference of the expression of *clcB* observed in maxicell analysis, which depends on the number of methionine residues available for $^{35}S$ labeling. The DNA sequence predicts 7 methionine residues in *clcB* as compared with 3 in *clcA* and 4 in *clcD*; however, the intensity of the 40-kDa band in lane 4 of Fig. 2 is much less than that of either of the smaller polypeptides. Consonant with this observation, the yield of the *clcB* gene product in pDC100-harboring *E. coli* cells induced with IPTG is variable and low compared with the other enzymes, suggesting that although IPTG induces all three enzymes from the *tac* promoter, the yields are not necessarily coordinate.

A third major ORF follows *clcB*, with possible initiation codons at bases 2320 and 2464, although relatively poor accompanying ribosome-binding sequences appear to be present. No enzyme function has been identified with this coding region and the sequence is not translated in Fig. 3. There is no major corresponding polypeptide directed in *E. coli* maxicells, although a very light band is observed below the 30-kDa product of the ampicillin-resistance gene.

The fourth major ORF, initiating at base 3325, predicts a polypeptide of molecular mass 26.5 kDa, again slightly lower than that observed in $NaDodSO_4$/PAGE. The N-terminal coding sequence of ORF4 matched the N-terminal sequence of purified dienelactone hydrolase from *Pseudomonas* sp. B13 harboring the plasmid pWR1, a plasmid shown to be extensively homologous to pAC27 (18), and the total amino acid composition of dienelactone hydrolase purified from pDC100-harboring, IPTG-induced cells matched the composition predicted by the DNA sequence. Thus, we designated ORF4 as *clcD* (11).

All three genes (*clcA*, *-B*, and *-D*) are preceded by potential ribosome-binding sequences with the conserved sequence GGAGA appearing in each case. The G+C content of each coding region is maintained at ≈60%, resembling that of the *P. putida* chromosome (19), and a pronounced preference for codons ending in guanosine or cytidine is universal. Base preference for G or C in the wobble position has been observed in a variety of organisms with high G+C content (11) and is useful in identifying reading frames within a given sequence.

**Nuclease S1 Protection Analysis.** Because the coding sequence of the *clcA* gene initiates close to the 5′ end of the 4.2-kbp *Bgl* II fragment, sufficient space for promoter/operator sequence appeared to be absent within this fragment. Also, we observed that growth on 3-chlorobenzoate by *P. putida* PRS2015 harboring pDC100 was dependent on IPTG induction. This observation indicated that the 4.2-kbp *Bgl* II fragment lacks a functional promoter. That a functional promoter is present on the upstream, 385-bp *Bgl* II fragment was inferred from the fact that cloning of this fragment upstream of a promoterless streptomycin phosphotransferase gene in plasmid pKT240 led to enhanced streptomycin resistance (A. Berry and B.F., unpublished observations). In order to localize the site of mRNA transcription initiation, a 1.8-kbp *Bam*HI fragment carrying DNA overlapping the 385-bp *Bgl* II fragment and spanning the 4.2-kbp *Bgl* II fragment to base 845 (Fig. 3) was subcloned from pDC10 (4) into M13 vectors. Deletion clones from the *Bam*HI site (base 845) toward the 385-bp *Bgl* II fragment were generated by the method of Dale *et al.* (14). One of these deletion clones, with base 559 at the 3′ end, was used to produce a single-stranded labeled DNA probe by primer extension (16) followed by digestion with *Sac* II (site at base 72). The labeled fragment was excised from a 5% polyacrylamide (60:1 acrylamide/*N,N′*-methylenebisacrylamide weight ratio) gel after electrophoresis under denaturing conditions and then was hybridized at 65°C with 60 μg of total RNA from *P. putida* cells harboring pAC27 grown with 3-chlorobenzoate. The results of digestion with 900 units of nuclease S1 are shown on the autoradiogram in Fig. 4. No protection was observed when the probe was hybridized with RNA isolated from cells grown with glucose (data not shown). Because the sequencing ladder bands used as size markers in the analysis contain the 17-base M13 primer as well as 18 nucleotides generated in cloning, a correction of 35 bases in the 3′ direction of the RNA-complementary strand (SL) is necessary. Assignment of RNA initiation was correlated with the most intense (**) band after nuclease treatment, although a short series of minor bands around the central band is seen. This localized the major transcription initiation site at a deoxycytosine residue (base 356) on the 385-bp *Bgl* II fragment upstream of the 4.2-kbp *Bgl* II E fragment. There is some evidence that the *clc* gene cluster is under positive control, presumably by a trans-acting element (4). When the DNA sequence 5′ to the transcription initiation site is compared to promoters of the *nah* (20), *sal* (20), and *xyl* (21) operons, sequences in the −10 region show considerable similarity (Fig. 5). Raibaud and Schwartz (22) demonstrated that the most highly conserved residues in positively regulated *E. coli* promoters are the adenine and thymine nucleotides in the −10 region and that these residues probably provide a minimal requirement for participation of RNA polymerase. This requirement appears to have been met also in the *Pseudomonas* promoters shown in Fig. 5, while very little similarity is seen in the −35 region.

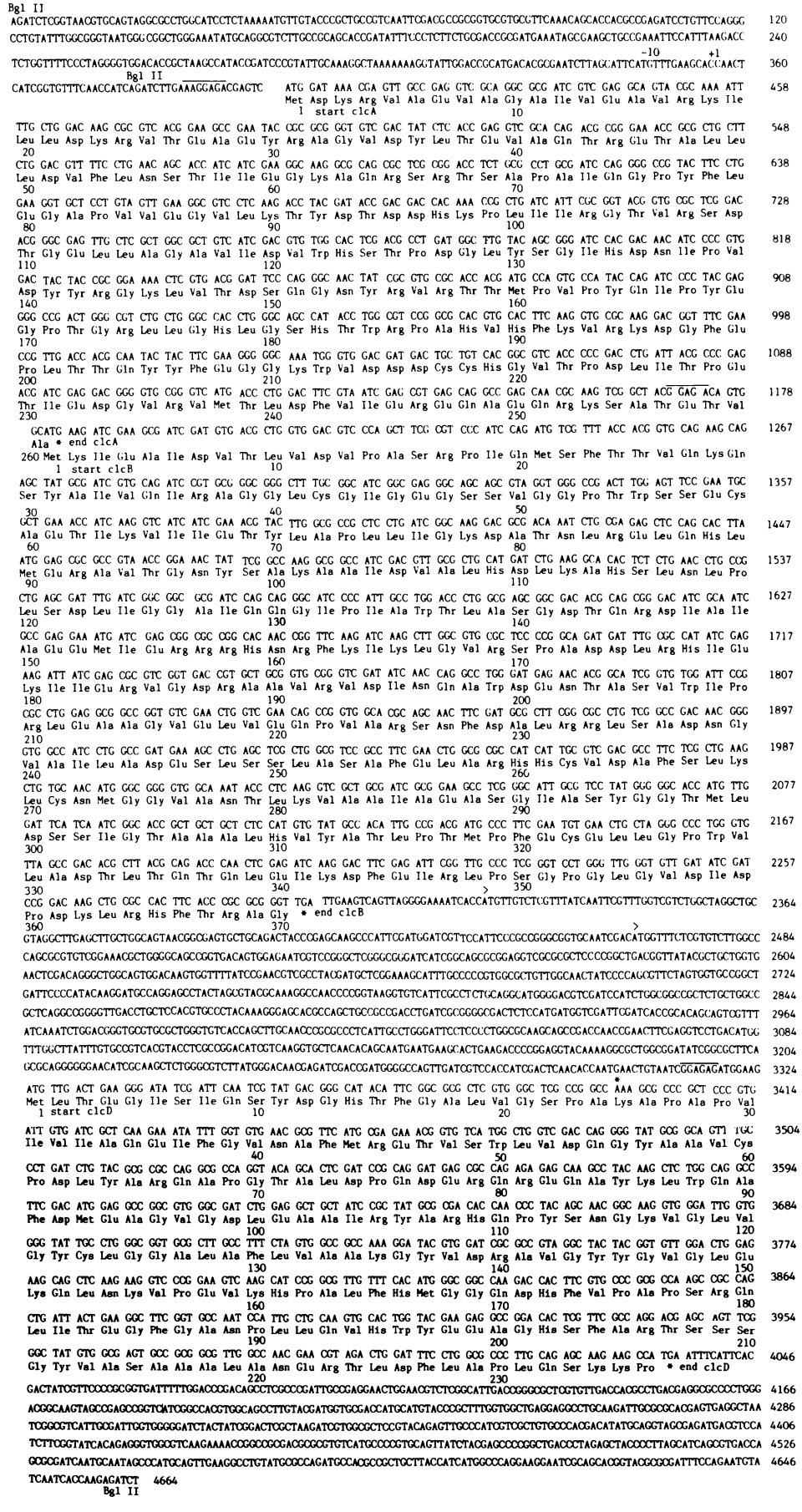

FIG. 3. Nucleotide sequence of chlorocatechol-degradative genes and DNA 5′ to the initiation codon of the first gene. mRNA initiation site mapped by nuclease S1 protection is indicated at +1. *Bgl* II sites shown in Fig. 1 are indicated above the sequence. The first amino acid residue for each coding sequence identified by direct comparison with N-terminal amino acid sequence analysis of the purified protein is designated no. 1, and stop codons are indicated with asterisks. Ribosome-binding sequences for each gene are overlined. Possible start codons for ORF3 are indicated (>).

The complete nucleotide sequence of the *clc* genes revealed the homology of a pentapeptide sequence near a cysteine residue (Cys-60) of the dienelactone hydrolase (*clcD* gene product) with the chromosomally encoded enol-lactone hydrolase, which otherwise shows no appreciable homology (11). Nucleotide sequence analysis of the chromosomal gene

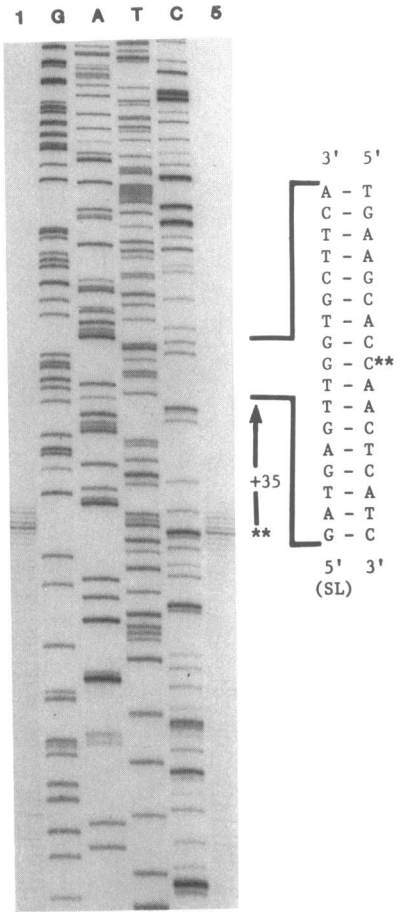


FIG. 4. Nuclease S1 mapping of the transcription initiation site of the *clc* gene cluster. Lanes 1 and 5: DNA fragments protected by RNA from nuclease S1 digestion. Lanes G, A, T, and C: products of sequencing reactions of the same recombinant used to make the probe. The sequencing ladder (SL) is read 5′ to 3′ from bottom to top (shown enlarged) and represents the mRNA-complementary strand. Because the ladder bands each contain M13 primer nucleotides as well as intervening bases generated in deletion cloning, a correction of 35 bases in the 3′ direction is necessary to align mRNA initiation with the sequence in the ladder. Double asterisk indicates the major protected band and is aligned after correction with the appropriate nucleotide on the DNA sense strand (+1 or base 356 in Fig. 3). To save space, the 487-base DNA probe, which migrates extremely high in the gel, is not shown.

*catB* (encoding cycloisomerase I) from *P. putida* demonstrated a high degree of homology (52%) with the *clcB* gene. Although the two enzymes encoded by these genes show divergent substrate specificities, long regions of amino acids are conserved throughout the two proteins, with the most homologous stretches localized to the middle portions of both (23). Similarly, catechol oxygenases from divergent backgrounds—namely, protocatechuate 3,4-dioxygenase and catechol oxygenase II—have been compared at the amino acid level, and homologous regions thought to be involved in the iron-binding domains of these proteins appear to be conserved (K.-L. Ngai, B.F., D. K. Chatterjee, L. N. Ornston, and A.M.C., unpublished results). Thus, comparison of nucleotide sequences of the plasmid-borne *clc* structural and regulatory genes with those of chromosomal genes specifying analogous reaction mechanisms or with other plasmid genes specifying catabolic activities against natural aromatic hydrocarbons is expected to throw considerable light on the mode of evolution of new degradative functions against new synthetic compounds.

| | -35 | -10 |
|---|---|---|
| nah | ATTGACAAATAAAAGCACGCTCACCATCATCGCGAATACA | |
| sal | TGTATTTATCAATATTGTTTGCTCCGTTATCGTTATTAACA | |
| xylABC | CGGTATAAGCAATGGCATGGCGGTTGCTAGCTATACGAGA | |
| xylDEFG | TGGCTATCTCTAGAAAGGCCTACCCCTTAGGCTTTATGCA | |
| clcABD | ACCGCATGACACGCGAATCTTAGCATTCATGTTTGAAGCACC | |
| E. coli | TTGACA (17±2) | TATAAT (5-8) |
| PRCS (E. coli) | | A T |
| PRCS (Pseudomonas) | | C T A* T A |

FIG. 5. Comparison of five positively regulated promoters from *Pseudomonas* plasmids involved in metabolism of aromatic compounds. In each case the transcription initiation site was identified by either nuclease S1 or reverse transcriptase mapping [*nah* and *sal* (20); *xylABC* and *xylDEGF* (21)]. The most conserved residues, which are similar to those found in the *E. coli* consensus sequence, are clustered in the −10 region and are shown in boldface. PRCS (*E. coli*), positively regulated conserved sequence from *E. coli* promoters (discussed in ref. 22). PRCS (*Pseudomonas*), positively regulated conserved sequence derived by comparison of the promoters shown in the figure. Asterisk indicates deoxyadenosine residue conserved in all cases.

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