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Molecular Cloning and Expression of the 3-Chlorobenzoate-Degrading Genes from *Pseudomonas* sp. Strain B13

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The genes specifying the utilization of 3-chlorobenzoate by *Pseudomonas* sp. strain B13 WR1 have been cloned by using a broad-host-range cosmid cloning system. Analysis of the catabolic products of the enzymatic reactions encoded by two hybrid cosmids, pMW65 and pMW90, by thin-layer and high-performance liquid chromatography demonstrated that both encoded the genes for the complete catabolism of 3-chlorobenzoate. Physical analysis of one of the cosmid derivatives, pMW65, by restriction endonuclease mapping and subcloning demonstrated that the pathway genes are encoded on a fragment no larger than 11 kilobases.

One of the consequences of increased industrial and agricultural activity during recent decades has been the release of large amounts of aromatic compounds into the environment. Many of these compounds are metabolized by soil microorganisms as part of the carbon cycle. However, certain man-made compounds such as halosubstituted aromatics tend to resist microbial attack (18, 28). A number of bacteria have now been isolated which, remarkably, have evolved the ability to completely metabolize xenobiotic compounds such as 2,4-dichlorophenoxy-acetic acid and 3-chlorobenzoate (3CB) (14, 24).

One such organism is *Pseudomonas* sp. strain B13 WR1, a bacterium which has the ability to utilize 3CB, 4-fluorobenzoate, and 4-chlorophenol (11, 24, 33). Biochemical analysis of these pathways has demonstrated that this organism has evolved a number of enzymes which specifically catalyze catabolism of the halogenated substrates (12, 13, 32). Comparison of the catabolic pathway for 3CB and that of benzoate in strain WR1 (Fig. 1) revealed that the initial and final steps are catalyzed by the same enzymes. However, the intermediate stages of ring fission, lactonization, isomerization, and delactonization of the nonsubstituted substrate are carried out by the enzymes pyrocatechase I, muconate cycloisomerase, isomerase, and 4-carboxymethylbut-3-en-4-olide hydrolase, whereas the halogenated substrate is metabolized by an analogous but clearly different set of enzymes: pyrocatechase II, chloromuconate cycloisomerase, 4-carboxymethylenebut-2-en-4-olide hydrolase, and maleylacetate reductase (13, 28, 32). Pyrocatechase I or muconate cycloisomerase is highly specific for nonsubstituted catechol and *cis,cis*-muconate and shows no significant activity with halogenated intermediates (12, 13, 32).

Despite the detailed biochemical analysis of haloaromatic utilization by WR1, the genetic basis of this activity has not been delineated beyond the demonstration that the pathway genes are transmissible (29, 31) and the isolation of small quantities of plasmid DNA, sufficient to perform just a few restriction digests (5, 6). In a recent paper, Chatterjee and Chakrabarty (6) have described cloning a 16.8-kilobase (kb) fragment of DNA, which encodes gene specifying 3CB utilization, from a strain of *Pseudomonas putida*. However, the organization of the genes on this fragment and which enzymes they specify have not been elucidated.

We are at present analyzing the genetic basis of haloaromatic utilization by WR1. In this report we describe the molecular cloning of the pathway genes and their expression in *Escherichia coli* and *Alcaligenes eutrophus*.

MATERIALS AND METHODS

Strains. The bacterial strains and plasmids used in this work are listed in Table 1.

All *E. coli* strains were cultivated at 37°C; the *Pseudomonas* and *Alcaligenes* strains were cultivated at 30°C. Liquid cultures were incubated with aeration on an orbital shaker platform. L-nutrient broth (NB) was routinely used as a rich medium, and minimal salts (9) containing 30 mM acetate, 5 mM 3CB (E. Merck AG, Darmstadt, Federal Republic of Germany), or 5 mM benzoate (Merck) was used as the minimal medium. Antibiotics were incorporated into media at the following final concentrations: kanamycin (Km), 50 μg/ml; tetracycline (Tc), 25 μg/ml; streptomycin (Sm), 1,000 μg/ml. When necessary, the minimal medium was supplemented with 2 μg each of proline, leucine, tryptophan, and thiamine per ml.

Strain WR1313 was isolated from a mating between B13 and the benzol-degrading *P. putida* strain DTG. Strains B13 and DTG were plated together on an L-broth plate and incubated overnight, and the mixed strains were transferred to a plate with mineral medium described by Dorn et al. (11). The plates were incubated at 30°C in a desiccator. Chlorobenzene was supplied as the carbon source through the vapor phase (3 μl of liquid chlorobenzene per liter of gas volume in the desiccator) without direct contact of the hydrocarbon with the medium (30). The isolated strain WR1313 can degrade (as can the parent strain DTG) benzene and nicotinate, which cannot be degraded by B13, but also chlorobenzene and 3CB as a result of transfer of the 3CB-

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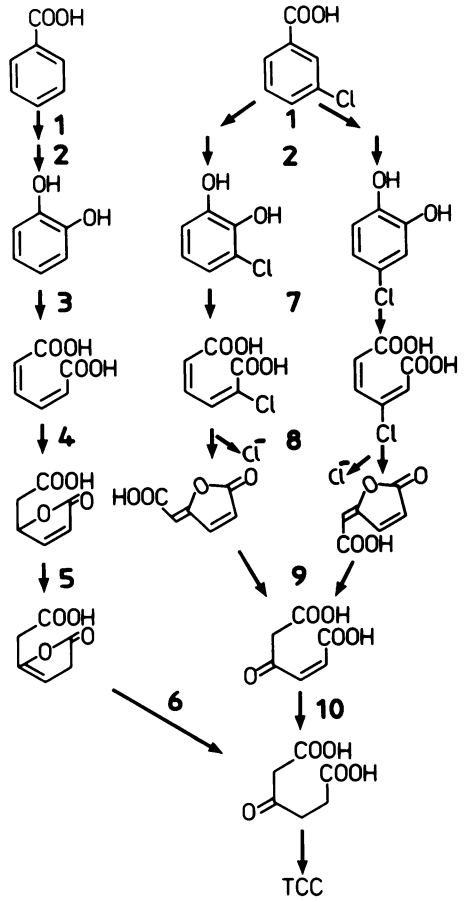

FIG. 1. Pathways for degradation of benzoate and 3CB by *Pseudomonas* sp. strain B13 WR1. Enzymes: 1, BO; 2, DHBDH; 3, pyrocatechase I; 4, muconate cycloisomerase; 5, isomerase; 6, 4-carboxymethylbut-3-en-4-olide hydrolase; 7, pyrocatechase II; 8, chloromuconate cycloisomerase; 9, 4-carboxymethylenebut-2-en-4-olide hydrolase; 10, maleylacetase reductase. Pyrocatechase I and muconate cycloisomerase are highly specific for nonsubstituted substrates.

degrading abilities from B13 (W. Reineke, unpublished data).

Construction and screening of the WR1 gene library. Two aliquots of vector pMMB33 were digested with *Eco*RI or *Sst*I as described previously (16), dephosphorylated, and pooled. A 20-μg portion of the total DNA from WR1 was partially digested with 1 U of *Sau*3A for 5 min at 37°C, ligated to 2 μg of the pMMB33 cosmid "arms," packaged in phage λ as described previously (26), and infected into CF503. Strain CF503 was grown in 20 ml of prewarmed NB with 0.2% maltose and 10 mM $MgCl_2$ to an optical density at 590 nm of 2.5. A 200-μl amount of this culture and 110 μl of in vitro packaged cosmids were mixed and incubated for 15 min at room temperature, 3 ml of NB was added, and the mixture was incubated at 37°C on an orbital shaker for 50 min. The mixture was then plated on AM3-agar plates with kanamycin.

The single colonies were isolated and incubated for 6 h in 96-well microtiter plates (Costar) containing 200 μl of NB with kanamycin and 50% glycerin and then stored. Two batches were prepared; one was stored at −70°C and the second was stored at −20°C. Packaging efficiency was tested with 0.8 μg of Charon 4A DNA and was 7.6×10^7 PFU/μg of DNA.

Screening of the bank was made by mobilization of the individual clones into *Pseudomonas* sp. mutants MWS28 and MWS489 and *A. eutrophus* strains. Mutants MWS28 and MWS489 defective in the 3CB degradation pathway (dihydrodihydroxybenzoate dehydrogenase [DHBDH] and 4-carboxymethylenebut-2-en-4-olide hydrolase defect) were isolated by mutagenesis and by screening for colonies which could no longer grow on 3CB (M. P. Weisshaar, unpublished data). These strains and the benzoate dioxygenase (BO)-defective mutant PaW95 (25) were used as recipients as well as strain JMP222 (11), cured from plasmid pJP4 and therefore unable to grow on 3CB. These strains were grown to mid-log phase on NB, and 200-μl aliquots were plated onto NB-agar plates. Small aliquots (1 μl) of the individual clones of the gene bank in strain CF503 were transferred from the microtiter plates onto the NB-agar plates containing the recipients with a replicating device. The plates were then incubated at 30°C for 6 h and replicated on the corresponding selection medium: benzoate for strain PaW95, kanamycin plus streptomycin for strain JMP222, and 3CB for mutants MWS28 and MWS489. Screening was then done for growth on 3CB and kanamycin-tetracycline.

The control of mobilization efficiency was done with one clone and the streptomycin-resistant recipients from *P. putida* and *Alcaligenes* sp. on a filter, selecting for kanamycin and streptomycin (50 μg/ml and 1,000 μg/ml). The mobilization frequency was very high, 8.4×10^{-1} per donor into *P. putida* and 6×10^{-1} per donor into *Alcaligenes* sp.

HPLC. Analysis of 3CB utilization and accumulation of pathway intermediates were monitored by using high-performance liquid chromatography (HPLC) and a reverse-phase Hibar column (Merck) for a period of 72 h after addition of 3CB (5 mM final concentration) to cultures of bacteria which had been pregrown overnight in succinate minimal medium or NB. Phosphoric acid (10 mM) containing 50% (vol/vol) acetonitrile was used as the solvent system. Peaks were detected in a variable-wavelength model 635 spectrometric detector (Varian-Techtron, Melbourne, Victoria, Australia) and were identified with authentic samples by their retention times.

TLC. For a quick identification of the 3CB pathway intermediates accumulated in the medium by mutants or recombinant clones, the thin-layer chromatography (TLC) method was used as it allows the screening of 10 to 12 clones at the same time. Silica gel TLC plates (F254; Merck) were used with the following solvent system: diisopropyl-etherformic acid-water, 250:7:3 (vol/vol/vol). The clones were incubated on acetate or NB overnight and supplemented with 3CB. Every hour a drop of the culture was applied to a TLC plate and chromatographed for 10 to 15 min. Bands were located under UV light and were identified with authentic samples of their R_f values. The following metabolites and their corresponding R_f values were identified: 3CB, R_f 0.84; 3-chlorocatechol, R_f 0.73; 4-chlorocatechol, R_f 0.694; 3-chloro-*cis*-muconate, R_f 0.694; *cis*-dien-lactone, R_f 0.58; *trans*-dien-lactone, R_f 0.209. Maleylacetic acid is not detectable under these conditions.

DNA isolation. Total DNA from WR1 was isolated by standard procedures. An 80-ml amount of NB overnight culture of WR1 was centrifuged and suspended in 6 ml of lysis solution (25% sucrose in 50 mM Tris hydrochloride, pH 8, 1 ml of lysozyme [5 mg/ml], 2.5 ml of 0.25 mM EDTA, 2.5 ml of 20% sodium dodecyl sulfate). The suspension was kept at room temperature until lysis was complete. The DNA was

TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Relevant characteristics[a]	Reference or source
Strains		
Pseudomonas sp.		
B13 WR1	3Cb$^+$ 4HB$^+$ Bzt$^+$ 4FB$^+$ P$^+$ 4CP$^+$	10
B13 MWS28	3Cb$^-$ 4HB$^+$ Bzt$^-$ 4FB$^-$ P$^+$ 4CP$^-$	EMS-induced mutant from WR1 DHB-dase defect (Weisshaar, unpublished data)[b]
B13 MWS489	3Cb$^-$ 4HB$^+$ Bzt$^+$ 4FB$^-$ P$^+$ 4CP$^-$	EMS-induced mutant from WR1 hydrolase II defect mutant (Weisshaar, Ph.D. thesis)
P. putida DTG	Bz$^+$	D. T. Gibson (University of Texas, Austin)
P. putida WR1313	CBz$^+$	Transconjugant from WR1 and DTG (W. Reineke, University of Wuppertal)
P. putida mt-2	TOL$^+$ 3Cb$^-$ *rmo*$^+$ *mod*$^+$	33
P. putida PaW95	Bzt$^-$ *rmo*$^+$ *mod*$^+$	24
A. eutrophus JMP222	2,4D$^-$ 3Cb$^-$ Sm$^+$	9
E. coli		
SK1592	*hsdR*$^+$ *hsdM*$^+$ *gal thi* T1^h *endA*	3
HB101	*hsdS20 recA13 ara-14 proA2 lacY1 galK2 rpsL20 pro leu thi*	
CF503	HB101 with pLG223	15
Plasmids		
pMMB33	Km^r *cos*	15
pLG223	ColIb *drd-1*:Tn*10* Tc^r	4
pKT210	Cm^r Sm^r Mob$^+$	1
pWWO	TOL	33
pJP4	2,4D$^+$ 3Cb$^+$ Hg^r	9

[a] Designations used for relevant genotypes and phenotypes are as follows: 3Cb, 3-Chlorobenzoate; 4HB, 4-hydroxybenzoate; Bzt, benzoate; 4FB, 4-fluorobenzoate; P, phenol, 4CP, 4-chlorophenol; Bz, benzene; CBz, chlorobenzene; TOL, toluene; 2,4D, 2,4-dichlorophenoxyacetate; *rmo*$^+$, host-specific restriction; *mod*$^+$, host-specific modification; *hsdR*$^+$, host-specific restriction; *hsdM*$^+$, host-specific modification; *hsdS*, deficient in both restriction and modification; Km^r, enhanced resistance to kanamycin; Cm^r, enhanced resistance to chloramphenicol; Sm^r, enhanced resistance to streptomycin; Hg^r, mercuric ion resistance.

[b] EMS, Ethyl methanesulfonate.

then gently extracted with an equal volume of phenol-chloroform (1:1, vol/vol) and centrifuged for 20 min. The phenol extraction was repeated five times. The DNA was precipitated with 1 volume of isopropanol, and the precipitated DNA was recovered on a glass rod, dissolved in TE buffer, and further purified on a CsCl gradient (1.25 g/g of solution).

Plasmid DNA from *Pseudomonas* sp. was isolated by the method of Hansen and Olsen (20). Recombinant plasmids were isolated by either the cleared lysate method of Clewell and Helinski (7) or the "mini-prep" method described by Holmes and Quigley (21).

Analysis of plasmid DNA by restriction endonuclease digestion and agarose gel electrophoresis has been described previously (16). Electron microscopic and heteroduplex analyses were done as described previously (9). Southern blotting and hybridization experiments were done as described by Maniatis et al. (26). Hybridization was done overnight at 65°C.

RESULTS

Construction and screening of the WR1 cosmid library. Approximately 5,000 clones were isolated in CF503 by using the broad-host-range cosmid pMMB33 (16). A random sample of 12 was then checked to determine the average size of the cloned fragment. In all cases the recombinant plasmids were 40 to 50 kb in size and contained different DNA inserts, as judged by restriction endonuclease analysis. This established that the DNA sequences representing the genes encoding 3CB catabolism would in all probability be present in the library. However, recent studies have shown that genes from *Pseudomonas* spp. are generally poorly expressed in *E. coli* (15). To overcome this problem, 1,000 clones were transferred by mobilization, as described in Materials and Methods, from CF503 to JMP222, a strain of *A. eutrophus* unable to utilize 3CB because it is cured from the pJP4 plasmid encoding 2,4-dichlorophenoxyacetic acid and 3CB

TABLE 2. Optical density achieved by a range of clones carrying the genes encoding 3CB degradation when grown on 3CB minimal medium

Strain	Optical density at 546 nm	% Growth yield[a]
Pseudomonas sp. strain B13 WR1	0.67	100
A. eutrophus[b]		
JMP222	NG[c]	
JMP222(pMW65)	0.23	34
JMP222(pMW377)	0.09	14
JMP222(pMW433)	0.10	16
JMP222(pMW279)	0.14	22
JMP222(pMW432)	0.13	19
JMP222(pMW104)	0.12	17
JMP222(pMW272)	0.13	19
E. coli K-12[d]		
HB101	NG	
HB101(pMW433)	0.05	9
HB101(pMW916)	0.07	11
HB101(pMW90)	0.28	42
HB101(pMW385)	0.15	23

[a] Expression means growth yield compared with that of the parental *Pseudomonas* sp. strain B13 WR1 over the same period of time.

[b] Clones grown at 30°C on 3CB minimal medium containing kanamycin. The optical density was followed for a period of 1 to 2 weeks on a Klett-Summerson colorimeter.

[c] NG, No growth detected.

[d] Clones grown on 3CB minimal media supplemented with tryptophan, proline, and leucine (20 μM).

TABLE 3. 3CB utilization and accumulation of pathway intermediates by *E. coli* HB101 and *A. eutrophus* JMP222 carrying plasmid pMW65 or pMW90[a]

Strain	Growth substrate	3CB uptake (%)	Accumulated product(s)[b] on day:	
			1	2 to 3
HB101	NB		—	
HB101(pMW90)	NB	46	ND	CMS + Ccat
HB101(pMW90)	NB + 3CB	5	Ccat	CMS + Ccat
JMP222	S	38	Ccat	Ccat
JMP222(pMW90)	NB	12	MA + CMS + Ccat	CMS + Ccat
JMP222(pMW90)	NB + 3CB	10	MA + CMS + Ccat	CMS + Ccat
JMP222(pMW90)	S	27	ND	MA
JMP222(pMW90)	B + 3CB	22	—	—
JMP222(pMW90)	AC	2	ND	ND
JMP222(pMW65)	S	99	MA	MA

[a] Clones were grown overnight on nutrient broth (NB), succinate (S), benzoate (B), or acetate (AC) before addition of 3CB. Uptake of the 3CB added to the pregrown cultures was monitored by HPLC as described in Materials and Methods.
[b] CMS, Chloromuconate; Ccat, chlorocatechol; MA, maleylacetate; ND, not measured; —, no accumulation of products.

degradation (10). The clones were transferred at high efficiency, around 6×10^{-1} per donor cell. Clones exhibiting the ability to catabolize 3CB were detected by selecting transconjugants on 3CB minimal agar containing kanamycin. Of the 1,000 clones mobilized from CF503, 7 supported growth on 3CB ($3Cb^+$). In parallel to the mobilization, the *E. coli* clones were also screened on 3CB minimal agar supplemented with proline, leucine, and tryptophan. We had anticipated that this would not enable us to detect $3Cb^+$ clones. Surprisingly, however, several clones were detected which showed slight growth.

Growth characteristics of the clones specifying 3CB utilization. All seven 3CB-utilizing JMP222 transconjugants required a period of 7 days of incubation at 30°C to produce small colonies on 3CB minimal agar. The growth of the transconjugants compared with that of WR1 was then determined in 3CB minimal media. Inspection of the results (Table 2) reveals that none of the transconjugants attained the optical density achieved by a WR1 culture incubated for the same period of time (10 to 14 days). All but one grew to only 15 to 20% of the optical density reached by the wild-type organism. The transconjugant containing plasmid pMW65 was the exception; it reached 34% of wild-type optical density. From Table 2 it can be seen that a similar result was obtained with the four $3Cb^+$ *E. coli* derivatives, with most achieving around 10 to 20% of the WR1 level. One clone, that containing pMW90, grew to 42% of the WR1 level. However, prolonged incubation of the $3Cb^+$ *E. coli* cultures on 3CB minimal agar or medium resulted in the colony/culture turning brown/black in color and was accompanied by cell death. It was assumed that this was due to accumulation of chlorocatechol in the cells. This was later confirmed by HPLC (see below). The induction of the enzymes appeared to be too low to allow a quick turnover of 3-chlorocatechol, which is a poison for the cell; when the culture was supplemented again (after 1/10 dilution) with 3CB, the bacteria stopped growing after 6 h (data not shown). The concentration of 3CB was too high for the rate of degradation by the cells; as a consequence, dichlorocatechol was accumulated in the cells, and the culture stopped growing.

Following transfer by mobilization of pMW90 and pMW65 into JMP222, the ability of bacterial clones containing pMW65 and pMW90 to catabolize 3CB was further investigated by HPLC. Cultures were grown overnight in either succinate minimal medium or NB before 3CB was added to a final concentration of 5 mM. Its utilization and the accumulation of pathway intermediates were then monitored for the next 72 h (Table 3). From the results it may be seen that the host strain is capable of carrying out the first two catabolic steps converting some of the added 3CB into chlorocatechol. This is due to the activity of a chromosomally encoded BO and DHBDH (3,5-cyclohexadiene-1,2-diol-1-carboxylate dehydrogenase), normally functioning in the degradation of benzoate. However, further breakdown is not possible, as the specificity of pyrocatechase in JMP222 is similar to that of the pyrocatechase I of *Pseudomonas* sp. strain B13 in that it does not exhibit activity with 3-substituted chlorocatechol (12, 13). The JMP222 strain car-

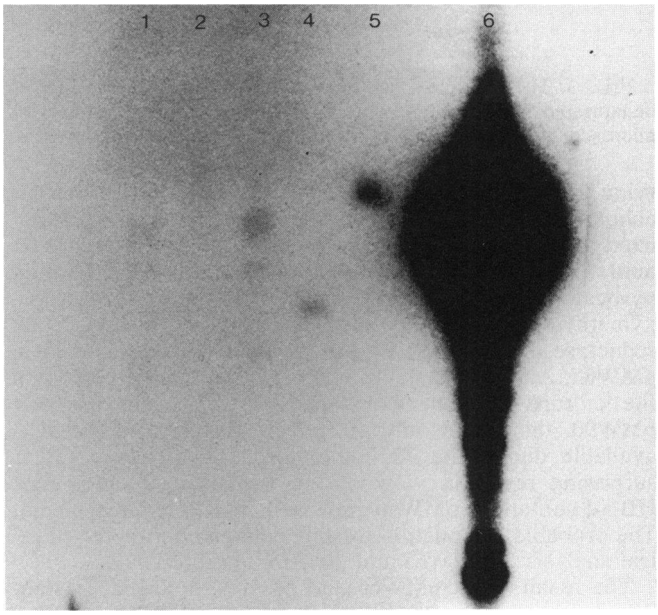

FIG. 2. DNA hybridization. Total DNAs from strains WR1, WR1313, and DTG were isolated as described in Materials and Methods, digested with *Xho*I, and blotted on a nitrocellulose filter together with plasmids pWWO and pJP4 digested with *Hin*dIII. The hybridization was done with the nick-translated pMW90 plasmid under high-restringency conditions (25). Lanes: 1, WR1313; 2, DTG; 3, WR1; 4, pJP4; 5, pWWO; 6, pMW90.

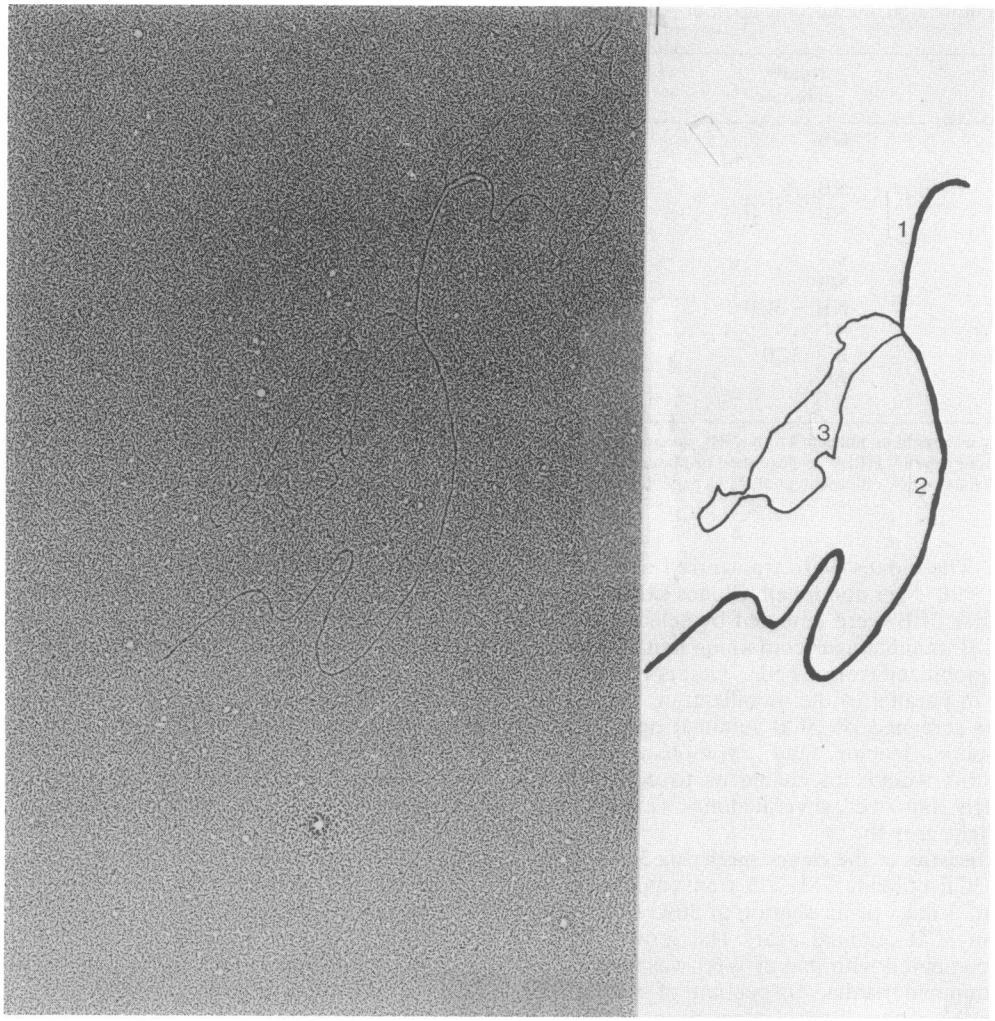

FIG. 3. Heteroduplex analysis of pMMB33 and pMW65. Both plasmids were linearized with *Hin*dIII mixed in a 1:1 ratio, relation denaturated for 2 min at 150°C, and incubated for 7 min at 37°C to allow renaturation. The droplet method (8) was used for electron microscopic analysis. The length of the fragments is as follows: 1 + 2 + 3 = 25.81 kb; 1 + 2 = 14.42 kb = pMMB33.

rying pMW65 showed no such accumulation of chlorocatechol; instead, the chlorobenzoate was completely metabolized with some intermediary accumulation of maleylacetic acid. The result indicated that the genes encoding pyrocatechase II, chloromuconate cycloisomerase, 4-carboxymethylene-but-2-en-4-olide hydrolase, and maleylacetate reductase are encoded by the segment of DNA cloned in pMW65. Although maleylacetic acid was also detectable in the culture medium from the JMP222 strain carrying pMW90, this strain only utilized around 30% of the 3CB available during the 72 h monitored. This was a rather surprising result in view of the earlier observation that HB101 containing pMW90 grew well on 3CB minimal media. The probable explanation for this emerged during the physical analysis of pMW65 and pMW90 plasmid DNA.

The results with pMW65 and pMW90 in JMP222 established that the genes for the degradation of chlorocatechol through 3-oxoadipate were encoded on these plasmids. The presence of the host-encoded BO and DHBDH made it impossible to determine if the analogous 3CB pathway genes were present on pMW65 and pMW90. Two approaches were used to resolve this problem. First, pMW65 was mobilized into *P. putida* PaW95. This strain is unable to grow on benzoate as sole carbon and energy source due to a mutation in the gene encoding BO (25). The introduction of pMW65 into PaW95 restored the ability of PaW95 to grow on benzoate, thereby demonstrating that the gene encoding BO is located on pMW65. A similar conclusion was reached for pMW90; the initial growth studies with the CF503 strain containing this plasmid had resulted in what was apparently weak growth on 3CB and accumulation of very low quantities of chlorocatechol. This was confirmed by HPLC analysis of HB101 containing pMW90 (Table 3). The results show that, while the HB101 host does not detectably catabolize 3CB, the HB101 (pMW90) strain utilizes 46% of the available substrate and accumulates chlorocatechol and chloromuconate in very low amounts, confirming that genes encoding BO and DHBDH are located on pMW90. The accumulation of low quantities of chlorocatechol and chloromuconate indicates that the induction of enzymes responsible for their catabolism is below the normal level. At present it is not possible to explain this observation, although it may possibly be a result of the instability of pMW90 (see below). Evidence that DHBDH is also encoded by pMW65 was obtained by complementation of a DHBDH-defective mutant of WR1 (MWS28). Further evidence that the 4-carboxymethyl-

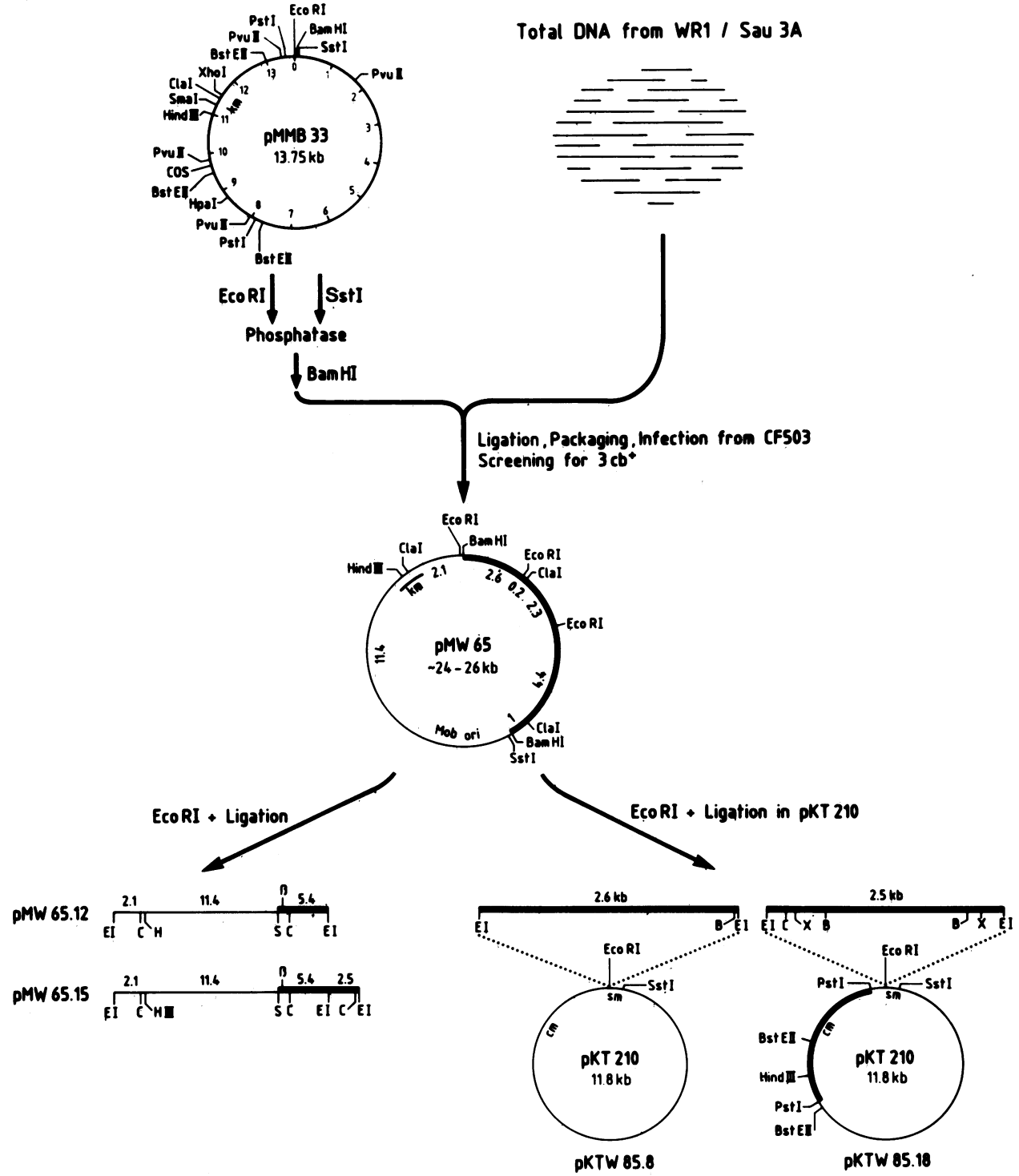

FIG. 4. Physical map of plasmid pMW65 construction of the library and construction of the deletion mutants and subclones in pKT210. The dark thick line represents the region encoding the 3CB degradation genes isolated from *Pseudomonas* sp. strain B13 WR1.

enebut-2-en-4-olide hydrolase gene is encoded by both pMW65 and pMW90 was obtained by complementation of a hydrolase-defective mutant, MWS489.

From the data obtained from growth, TLC, and HPLC studies, it was concluded that both pMW65 and pMW90 encode genes which specify all enzymes of the 3CB pathway of WR1.

From this HPLC analysis it could also be concluded that not all growth substrates are well adapted for such induction experiments. Succinate appeared to be the best when the recipient was JMP222, and NB was best for HB101. Similar results were observed when induction of the 3CB pathway in WR1 itself was analyzed (Weisshaar, unpublished data; E. Schmidt, personal communication). Succinate was also the

best growth substrate for WR1 and allowed induction by 3CB already 1 h after the inductor was added, while with the other substrates the induction came very late or not at all. Another point was that the successive addition of growth and induction substrates inhibited the uptake of the inductor. The results in Table 3 show the same effect when NB and 3CB are added at the same time. The procedure for induction by 3CB seems to be, then, the same as in wild-type WR1.

Colony hybridization with a nick-translated pB1313 plasmid (117 kb) isolated from strain WR1313, which received the 3CB genes from WR1 when selecting for a transconjugant able to degrade chlorobenzol, confirmed also that pMW90 and pMW65, as well as the other clones mentioned, contain the genes for 3CB degradation (data not shown). Plasmid pB1313 hybridized with the WR1 total DNA. A Southern blot of the total DNAs from WR1, WR1313, and the parent strain DTG was hybridized with the nick-translated pMW90 plasmid. The genes encoded by pMW90 are on WR1 as well as on WR1313 (Fig. 2), but not on DTG. Another interesting point is that plasmid pMW90 also hybridizes with *Hin*dIII fragment 4 (7 kb) from plasmid pWWO and *Hin*dIII fragment 5 from pJP4. The results are not surprising as the pJP4 plasmid also encodes the 3CB degradation genes, but what kind of functions are encoded by fragment 4 is not yet known. Similar results were found by Chakrabarty et al. after analysis of the 3CB-degrading plasmids pAC25 and pJP4 (17).

Physical analysis of recombinant plasmids. Plasmid DNA was isolated from HB101 carrying pMW65 and pMW90. The DNA was then characterized by restriction endonuclease digestion, agarose gel electrophoresis, and electron microscopy. By agarose gel electrophoresis pMW65 was found to have a molecular weight of 24,250, but electron microscopic analysis showed a molecular weight of 25,810. Unfortunately, pMW90 appears to be extremely unstable; plasmid DNA isolated on several occasions was found to consist of a mixture of molecules ranging from about 14 to 42 kb, with the highest proportion (19:2) at the lower value. This observation probably explains the results obtained above. As a result of the difficulties with pMW90, pMW65 was chosen for further analysis.

Comparison of the molecular weight of pMW65 and that of the parental cosmid pMMB33 and heteroduplex analysis with pMMB33 (Fig. 3) revealed the size of the insert DNA to be 11.39 kb. This is rather small for a cosmid clone, but it was expected that with such big inserts instability could appear during further analysis of the clones (M. Bagdasarian, personal communication). It is therefore conceivable that some DNA sequences were deleted from pMW65 and, unlike pMW90, pMW65 is now stable. A restriction map of pMW65 was constructed based on the restriction endonucleases *Hin*dIII, *Bam*HI, *Eco*RI, *Cla*I, and *Sst*I (Fig. 4). The accurate physical location of the 3CB pathway genes on pMW65 has yet to be determined; however, some preliminary data have been obtained. The construction and analysis of a deletion derivative have provided a preliminary position for three pathway genes. Plasmids pMW65.12 and pMW65.15 (Fig. 4) were constructed by in vitro deletion of the two *Eco*RI fragments from pMW65. The ability of pMW65.12 and pMW65.15 to complement the BO mutation in PaW95 and the DHBDH mutation in *Pseudomonas* sp. strain B13 MWS28 was then determined. Both mutations were complemented, indicating that the BO and DHBDH genes are located on the 5.4-kb *Bam*HI-*Eco*RI fragment of pMW65.

During the course of our cloning studies, we discovered to our surprise that the *E. coli* SK1592 host we were using, unlike HB101, accumulated small quantities of chlorocatechol when grown on L-broth in the presence of 3CB. Generally, it is assumed that *E. coli* does not metabolize benzoate-derived compounds; however, strains have been isolated which can catabolize 3- or 4-hydroxyphenylacetate (8). When the 2.5-kb *Eco*RI fragment from pMW65 was subcloned into the broad-host-range vector pKT210 and recombinant plasmid pKTW65.18 was introduced into SK1592, it was observed that growth in the presence of 3CB no longer resulted in the accumulation of chlorocatechol. Analysis of the culture media by TLC revealed the presence of chloromuconate. From this it may be concluded that pKTW65.18 encodes the gene for pyrocatechase II. Predictably, when plasmid pKTW65.8, which contains the pMW65 2.6-kb *Eco*RI fragment cloned into pKT210 (Fig. 4), was introduced into SK1592 and the strain was grown in the presence of 3CB, there was an accumulation of chlorocatechol. In view of the fact that SK1592 (pKTW65.18) accumulates chloromuconate but is unable to metabolize this further, it is likely that pKTW65.8 encodes genes specifying enzymes from the lower part of the pathway; however, further study is required to confirm this.

DISCUSSION

In this communication we report the molecular cloning of genes from *Pseudomonas* sp. strain B13 WR1 which specify the catabolism of 3CB. By screening a cosmid library of the bacterium, we identified a number of clones which confer this ability on both *E. coli* and *A. eutrophus* hosts. Growth studies revealed that none of these strains was capable of utilizing 3CB as efficiently as WR1. At present we have no complete explanation for this. However, there are two strong possibilities. First, studies with other aromatic catabolic pathways such as TOL (15) and NAH (19) have demonstrated that the pathways are positively regulated. Hence, the removal of the regulatory gene activity by mutation or by separate cloning of the structural gene results in low-level noninducible pathway gene expression (15). The nature of the regulation of the 3CB pathway is unknown, but it might be possible that it is also positively regulated. The low levels of 3CB turnover exhibited by the clones will then indicate that the structural genes have been cloned in the absence of their regulator gene. A second possibility is that genes from soil pseudomonads are generally poorly expressed in *E. coli* (15) due to differences in the DNA sequences and organization of their promoter regions from those of *E. coli* (22, 27; F. C. H. Franklin, unpublished observations). Whether *Pseudomonas* genes are expressed efficiently in other bacteria is unknown, and while *A. eutrophus* and *Pseudomonas* sp. strain B13 are thought to be closely related, there is no direct evidence that genes from *Pseudomonas* sp. strain B13 are expressed in *A. eutrophus* at the levels found in their natural genetic background. Further investigation of the clones will be required to determine if either or both of these possibilities are responsible for the observed results.

Two of the clones, pMW65 and pMW90, were analyzed further by HPLC. Both were found to encode all of the genes necessary to catabolize 3CB to tricarboxylic acid cycle intermediates. One of the clones, pMW65, was then physically mapped by restriction endonuclease analysis, and a number of derivatives were isolated by generating deletions in vitro or by subcloning into the broad-host-range plasmid

pKT210. Further evidence that pMW65 does indeed encode all of the 3CB degradation pathway was obtained by using plasmid pMW65 and its derivatives to complement various 3CB pathway mutants. These studies also permitted preliminary mapping of some of the pathway genes on the pMW65 genome.

These results indicate that, in common with other biodegradative pathways which have been analyzed at the molecular level such as TOL (15), NAH (19), and phenol utilization (Franklin et al., submitted for publication), the genes for 3CB catabolism are clustered on a segment of DNA not larger than 11 kb. This finding is similar to that of Chatterjee, Chakrabarty, and co-workers (6, 17) and further supports the proposal that genes specifying biodegradation pathways are clustered. To this end it will be interesting to compare the organization and homology of the genes from the *Pseudomonas* sp. strain B13 pathway with those of *P. putida* studied by Chatterjee and Chakrabarty. Many degradative pathways are plasmid encoded (10, 15, 19; M. P. Weisshaar, Ph.D. thesis, University of Georg-August, Göttingen, Federal Republic of Germany, 1984); tentative evidence suggests that this may also be the case for 3CB catabolism by WR1 (6), confirmed by the hybridization obtained in this work with plasmid pB1313 (Fig. 2). However, it is interesting to note that, of 1,000 cosmid clones tested, 10 showed detectable 3CB catabolism in JMP222 or *E. coli*. This is a considerably higher value than might be expected if the genes were present as a single copy on the WR1 chromosome, and one explanation is that the genes are present on a plasmid which is present in several copies within the WR1 cell. However, this cannot be the case as the plasmid is very difficult to isolate and the yields are very low; for pB1313, only 5 μg of plasmid per liter could be isolated. Hybridization with plasmid pWWO may shed some light on this problem. It is known that a deletion of 39 kb appears very often in the TOL plasmid (2, 23, 34). To this region belongs *Hin*dIII fragment 4, which hybridizes with plasmid pMW90. This deletion occurs also when plasmid pWWO is introduced into WR1, and it was found by hybridization that the pWWO plasmid hybridizes with the chromosome from the WR1 derivative WR211 (23). This may indicate that the 3CB genes are a kind of translocatable element integrated into the chromosome, perhaps in several copies, explaining the high number of clones encoding 3CB we obtained in this work.

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