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TOL Plasmid pWW0 in Constructed Halobenzoate-Degrading *Pseudomonas* Strains: Enzyme Regulation and DNA Structure

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WR211 and WR216 are derivatives of halobenzoate-degrading *Pseudomonas* sp. strain B13 into which the 117-kilobase TOL degradative plasmid pWW0 has been transferred from *Pseudomonas putida* mt-2. WR211 has lost the ability to grow on the TOL-specific substrate *m*-xylene but retains the ability to grow on its metabolite, *m*-toluate. An analysis of the induction of enzymes was consistent with WR211 carrying a nonfunctional regulatory gene, *xylR*. WR216 is a spontaneous derivative of WR211 which grows on none of the TOL substrates and yet expresses the nonspecific toluate oxidase, which enables it to grow on the novel substrate 4-chlorobenzoate. In addition to the *xylR* lesion inherited from WR211, WR216 appears to carry a mutation in the structural gene for catechol 2,3-oxygenase, *xylE*. The plasmids in both strains were analyzed by restriction endonuclease digestion. pWW0-1211 in WR211 has a large deletion (39 kilobases) compared with pWW0 and appears to be identical to a previously described plasmid (pWW0-8) which encodes none of the TOL degradative functions. pWW0-1216 in WR216 has undergone a major structural reorganization relative to its parent, pWW0-1211. This plasmid has a smaller deletion (19 kilobases), which is staggered relative to the deletion in pWW0-1211, and in addition it has two 3-kilobase insertions of unknown origin, one of which appears to cause the *xylE* mutation.

Pseudomonas sp. strain B13 (WR1) can grow on 3CB as the sole carbon source (13, 14); however, this strain cannot grow on other chloro-substituted benzoates (in particular 4CB), and it has been impossible to extend its range to these growth substrates by mutation or selection. The biochemical step responsible for the block appears to be the high specificity of the benzoate dioxygenase for 3CB, since WR1 can utilize both 3-chlorocatechol (the metabolite of 3CB) and 4-chlorocatechol, which would be the corresponding metabolite of 4CB (7). To circumvent this step, Reineke and Knackmuss (13, 14) transferred the TOL plasmid pWW0 from *Pseudomonas putida* mt-2 into WR1; this plasmid encodes a catabolic pathway for toluene, *m*-xylene, and *p*-xylene (Fig. 1) (19). One of the enzymes of this pathway is a nonspecific benzoate dioxygenase (17), which is also called toluate dioxygenase and is capable of oxidizing chloro-substituted benzoates (12).

The first transconjugant from the mating (WR211) which was selected for its ability to grow on the TOL-specific metabolite *m*-toluate (13), was only able to utilize the same single halobenzoate (3CB) as its parent, WR1; surprisingly, this transconjugant had lost the ability to grow on *m*-xylene. However, spontaneous derivatives of WR211, such as WR216, could be selected readily by their ability to grow on the novel substrate 4CB. All such $4CB^+$ segregants had lost the ability to grow on *m*-toluate.

In this paper we describe the biochemical regulation of the TOL-encoded enzymes in WR211 and WR216 and also document the alterations in the plasmid DNA structure which accompany the change in the TOL phenotype of these strains. In an accompanying paper Reineke et al. describe why the loss of expression of the *meta* pathway enzymes encoded by the TOL plasmid is an obligatory requirement before growth on 4CB is possible (11). (A preliminary report of some of the results was presented at the Annual Meeting of the American Society for Microbiology, Miami, Fla., 1980.)

MATERIALS AND METHODS

Abbreviations. The following abbreviations are used in this paper: 3CB, 3-chlorobenzoate; 4CB, 4-chlorobenzoate; C23O, catechol 2,3-dioxygenase; BADH,

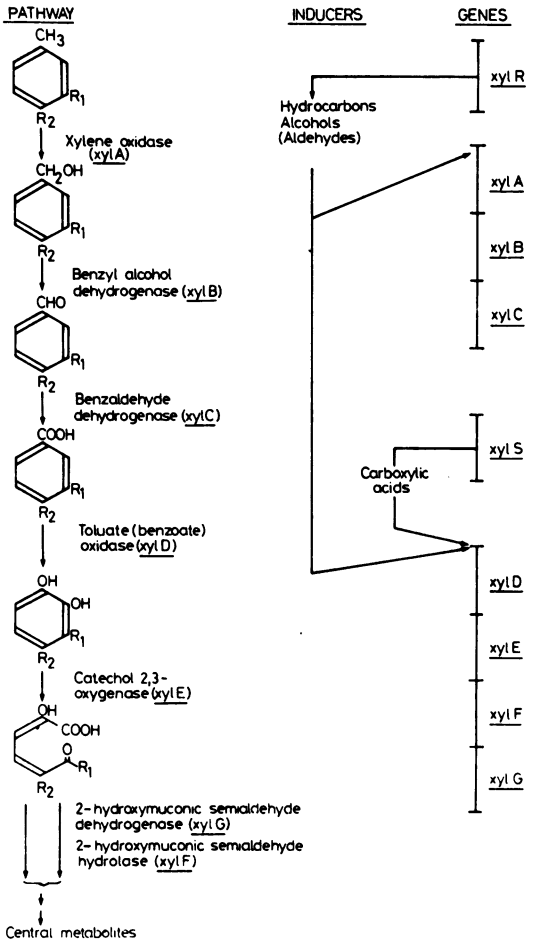

FIG. 1. TOL plasmid-encoded pathway and model proposed for its regulation in pWW0 (18). The primary metabolites of the pathway are toluene ($R_1 = R_2 = H$), *p*-xylene ($R_1 = H$; $R_2 = CH_3$), and *m*-xylene ($R_1 = CH_3$; $R_2 = H$); *m*-toluate is the carboxylic acid metabolite of *m*-xylene. In the model proposed for the regulation of expression, the hydrocarbon and alcohol substrates combine with the gene product of *xylR* to induce both the *xylABC* regulon and the *xylDEFG* regulon, whereas the carboxylic acids induce only the *xylDEFG* regulon via the *xylS* gene product (18). The model is not intended to show the spatial organization of the genes.

benzyl alcohol dehydrogenase; BZDH, benzaldehyde dehydrogenase; HMSH, 2-hydroxymuconic semialdehyde hydrolase; HMSD, 2-hydroxymuconic semialdehyde dehydrogenase; TO, toluate 1,2-dioxygenase; BO, benzoate, 1,2-dioxygenase; kb, kilobases.

Bacterial strains. The strains used in this work are listed in Table 1. All strains were maintained on minimal salts agar containing the following selective carbon sources: WR1, 5 mM 3CB; WR 211 and PaW1, 5 mM *m*-toluate; WR216, 5 mM 4CB; PaW8, 5 mM benzoate.

Media and culture conditions. The media and culture conditions used are described in the footnotes to the tables.

Preparation of cell extracts. Each cell preparation was harvested after centrifugation for 1 h at 1,000 × *g*, washed in 20 ml of 100 mM phosphate buffer (pH 7.5), centrifuged again, and stored in two portions at −20°C. The stored cells were suspended in 5-ml portions of the following buffers: phosphate-acetone (15) for C23O, HMSH, and HMSD assays and phosphate-NaCl-$MgSO_4$ (19) for BADH and BZDH assays. Each suspension was sonicated at 0°C in a 100-W ultrasonic disintegrator (Measuring & Scientific Equipment, Ltd., London) (six 30-s bursts). The cell debris was removed by centrifugation at $1.5 \times 10^5 \times g$ for 1 h at 4°C.

Enzyme assays. Enzymes were assayed by previously described procedures, using the following substrates: BADH, benzyl alcohol (19); BZDH (EC 1.2.1.28), benzaldehyde (19); C23O (EC 1.13.11.2), catechol (15); HMSH, 2-hydroxy-6-oxohepta-2,4-dienoate (17); HMSD, 2-hydroxymuconic semialdehyde (15).

Protein was determined by the biuret procedure.

TO and BO were assayed indirectly in whole cells. The stimulation of uptake of O_2 by the addition of benzoate or 4CB was measured by Warburg respirometry. Since these enzymes catalyze the first O_2-consuming steps in the metabolism of the added substrates, the rate of uptake is a qualitative measure of whether activity is induced. 4CB is oxidized rapidly by TO but not by BO (12, 14).

Plamid isolation and digestion. Plasmid DNA was extracted by the method of Wheatcroft and Williams (16) and was digested by restriction endonucleases according to the instructions of the suppliers.

Agarose gel electrophoresis. Gels contained 700 mg of agarose and 100 μg of ethidium bromide in 100 ml of electrophoresis buffer (9). Samples were run into the gels at 100 V and 20 mA for 20 min before the gels were submerged completely in buffer and electrophoresed for 16 to 24 h at 200 V and 40 mA. DNA was viewed by placing the gels on a transilluminator (model C-62; UV Products) and was photographed with a Polaroid MP4 camera through a Wratten no. 25 filter on type 665 positive-negative film.

Chemicals and enzymes. The 2-hydroxymuconic semialdehyde and 2-hydroxy-6-oxohepta-2,4-dienoate for HMSD and HMSH assays were prepared from catechol and 3-methylcatechol, respectively, by using either a purified preparation of C23O or a heat-treated cell extract of *m*-toluate-grown *P. putida* mt-2 (PaW1) (15). Catechol and 3-methylcatechol were purified by sublimation. Benzaldehyde was redistilled and kept at −4°C under N_2. All other chemicals were from commercial sources. Restriction endonucleases were purchased from Uniscience, Cambridge, United Kingdom.

RESULTS

Induction of BO and TO. There are two distinguishable activities that are capable of oxidizing benzoate and its derivatives in the strains which we studied. BO is specific for benzoate and 3CB (12, 14), and the TOL plasmid-encoded TO shows a much broader substrate specificity for benzoate and substituted benzoates (7, 17), es-

TABLE 1. Bacterial strains

Strain	Phenotype[a]	Plasmid	Reference(s)
PaW1 (*P. putida* mt-2)	Mxy$^+$ Mtol$^+$ 3CB$^-$	pWW0	17
PaW8	Mxy$^-$ Mtol$^-$ 3CB$^-$	pWW0-8	1, 17
WR1 (*Pseudomonas* sp. B13)	Mxy$^-$ Mtol$^-$ 3CB$^+$ 4CB$^-$		3
WR211	Mxy$^-$ Mtol$^+$ 3CB$^+$ 4CB$^-$	pWW0-1211	13
WR216	Mxy$^-$ Mtol$^-$ 3CB$^+$ 4CB$^+$	pWW0-1216	13
WRB80	Mxy$^-$ Mtol$^+$ 3CB$^+$ 4CB$^-$	pWW0-1080	This paper
PRSB1 (*P. putida* A.3.12)	Mxy$^-$ Mtol$^+$ 3CB$^-$	pWW0-2001	8

[a] Mxy$^+$, Ability to grow on *m*-xylene; Mtol$^+$, ability to grow on *m*-toluate; 3CB$^+$, ability to grow on 3CB; 4CB$^+$, ability to grow on 4CB.

pecially 4CB (12). WR1 expresses only BO activity (12).

When WR211 or WR216 cells were grown on acetate, neither BO activity nor TO activity was detected (Table 2). However, after growth in the presence of 3CB, 4CB, or *m*-toluate, TO activity clearly represented a considerable proportion of the induced activity, as indicated by the ability of cells to take up O_2 at the expense of 4CB. The results with PaW1 (*P. putida* mt-2 wild type) are included for comparative purposes since TO has been indicated as the principal isoenzyme induced by *m*-toluate (17).

Induction of other TOL-specified enzymes. pWW0 encodes an inducible sequence of enzymes, the regulation of which has been analyzed by using structural and regulatory mutants. The resulting model for the induction of these enzymes is shown in Fig. 1 (18).

In WR211 the levels of all the enzymes in both uninduced (acetate-grown) and *m*-toluate-induced cells were virtually identical to the levels in PaW1 (Table 3), indicating that the role and efficacy of *m*-toluate as an inducer were not altered by the transfer of the plasmid into the strain B13 background. The same qualitative pattern of induction has been found with PRSB1, a transconjugant of WR211 into a different recipient strain, *P. putida* A.3.12 (8).

Growth of WR211 (and PRSB1) in the presence of *m*-xylene produced an induction pattern fundamentally different from the pattern in PaW1 since there was no induction of BADH and BZDH (Table 3). There is one problem in interpreting the significance of the low levels of these activities since many *Pseudomonas* species which degrade aromatic compounds carry chromosomally encoded BADH and BZDH activities; WR1 is no exception to this, and both activities can be detected under the growth conditions used in these experiments. It is possible to distinguish these chromosomal enzymes from the pWW0 isoenzymes by their respective substrate specificities, using *m*- and *p*-methyl-substituted substrates (19). By this criterion both the BADH and the BZDH present in uninduced cells of WR211 and WR216 appear to be the plasmid-specified activities, which indicates that these strains are not structural gene mutants in either *xylB* or *xylC* (data not shown).

The later enzymes of the *meta* pathway (C23O, HMSH, and HMSD) are also not induced in WR211 cells grown in the presence of *m*-xylene (Table 3). In this respect WR211 differs from Mxy$^-$ mutants of PaW1 which are blocked in structural genes *xylA* and *xylB*, where *m*-xylene does induce these enzymes (18). It appears that the characteristics of WR211 can best be explained by the hypothesis that WR211 carries a mutation in the *xylR* regulatory gene

TABLE 2. Rates of oxygen uptake by washed cell suspensions of PaW1, WR211, and WR216[a]

Growth substrate(s)	Rate of oxygen uptake[b]					
	PaW1		WR211		WR216	
	Benzoate[c]	4CB	Benzoate	4CB	Benzoate	4CB
Acetate (5 mM)	<0.2	<0.2	<0.2	<0.2	<0.2	<0.2
Acetate (5 mM) + 3CB (5 mM)	5.1	2.7	14.7	9.0	1.5	1.1
Acetate (5 mM) + 4CB (5 mM)	6.7	2.3	2.9	1.0	3.5	2.7
Acetate (5 mM) + *m*-toluate (5 mM)	3.7	3.3	6.6	4.0	2.3	1.1

[a] Strains were grown overnight on 20 mM acetate, and the suspensions were incubated for 4 h with the growth substrate(s) before being assayed for BO and TO.

[b] In microliters of O_2 per minute per 2 ml of a cell suspension of absorbance 10 at 546 nm, corrected for endogeneous uptake.

[c] Assay substrate.

TABLE 3. Specific activities of plasmid-encoded enzymes in cell extracts of *Pseudomonas* strains

Strain	Growth substrate(s)[a]	Sp act (mU/mg of protein)				
		BADH	BZDH	C23O	HMSD	HMSH
PaW1	Acetate	16	8	130	3	22
	m-Toluate	46	140	9,800	250	860
	Acetate + *m*-xylene	885	440	10,500	230	950
WR211	Acetate	18	9	130	2	8
	m-Toluate	38	100	4,200	167	540
	Acetate + *m*-xylene	20	15	190	7	35
PRSB1	Acetate	9	12	140	5	36
	m-Toluate	9	61	5,450	165	500
	Acetate + *m*-xylene	13	19	130	5	14
WR216	Acetate	17	7	<0.5	17	66
	Acetate + *m*-toluate	11	10	<0.5	31	140
	Acetate + *m*-xylene	16	7	<0.5	26	130
WRB80	Acetate	26	8	250	17	55
	m-Toluate	26	48	3,100	125	545
	Acetate + *m*-xylene	20	7	160	12	54

[a] Cells were prepared by growing 2-liter batch cultures at 30°C for 18 h under forced aeration; 20 ml of nutrient broth-grown cells were inoculated into growth medium containing minimal salts and 10 mM acetate or 5 mM *m*-toluate. *m*-Xylene (as an inducer) was introduced into acetate cultures for the entire growth period as a vapor by passing the air supply through a flask containing liquid *m*-xylene. Because WR216 accumulated toxic intermediates in the presence of aromatic substrates, cells were grown on 10 mM acetate for 14 h, and the inducer was added for only the last 4 h before harvest.

which confers an induction pattern similar to that of *xylR* mutant PaW210 (18).

Both BADH and BZDH also appear to be uninduced in WR216 as a result of growth in the presence of *m*-xylene (Table 3). The three later enzymes (C23O, HMSH, and HMSD) behave differently in WR216. C23O was completely absent under all conditions of growth examined; since the assay for this enzyme is particularly sensitive, we must conclude that WR216 has a mutation in *xylE*, the structural gene for C23O. The defect also extends to the subsequent enzymes HMSH and HMSD, which are detectable but are not induced significantly by either *m*-xylene or *m*-toluate. The induction in WR216 could be explained in terms of the following three mutations: *xylR*, inherited from WR211 and leading to the loss by *m*-xylene of its functional role as inducer; *xylE*, the structural gene for C23O; and a third mutation leading to the uninducibility of HMSH and HMSD by *m*-toluate. However, a simpler explanation of this latter effect is that the *xylE* mutation is polar and affects the expression of HMSH and HMSD. This explanation is given further weight by the observation that the operon is transcribed in the order *xylDEGF* (T. Nakazawa, personal communication).

Structure of plasmid DNA in WR211 and WR216. Figure 2 shows agarose electrophoresis gels containing *Xho*I and *Hin*dIII digests of the plasmid DNAs from WR211 (pWW0-1211) and WR216 (pWW0-1216), together with digests of the parent plasmid (pWW0) extracted from PaW1. *Xho*I cleaves pWWO into 10 fragments (*Xho*I fragments A to J) (4). Of these, only fragments corresponding to *Xho*I-A,-B, and -H were retained in pWW0-1211. When these digests, the *Hin*dIII digests, and the *Xho*I-*Hin*dIII double digests were analyzed (Table 4), the results were consistent with a single deletion of about 39 kb extending from 56 to 95 kb on the pWW0 map at one extreme or from 58 to 97 kb at the other extreme (Fig. 3). In fact, pWW0-1211 is indistinguishable after single or double digestion or after digestion with *Eco*RI (data not shown) with plasmid pWW0-8 (5), the plasmid found in "cured" strain PaW8 (1, 17).

A similar analysis (Table 4) of digests of pWW0-1216 led to the map shown in Fig. 3. Considering that WR216 arose from WR211 by nutritional selection for a $4CB^+$ phenotype, a complex series of recombinational events appear to have taken place. pWW0-1216 is about 26 kb larger than pWW0-1211. It has a smaller deletion (only 19 kb), which consists of a well-defined 16.5-kb region (*Hin*dIII-*Xho*I double digestion fragments U through O) that extends an additional 2.5 kb into either *Hin*dIII-*Xho*I-F or *Hin*dIII-*Xho*I-C, generating a novel 15-kb fragment from them. Therefore, the deletion is staggered relative to the deletion in pWWO1211; the region from *Hin*dIII-*Xho*I-P through *Hin*dIII-*Xho*I-C (about 27 kb) is regained, and *Hin*dIII-*Xho*I-I through *Hin*dIII-*Xho*I-O (about 7 kb) is lost in the change. In addition two novel fragments are generated. These can best be observed as *Xho*I-I′ (5.5 kb) in the *Xho*I digest and

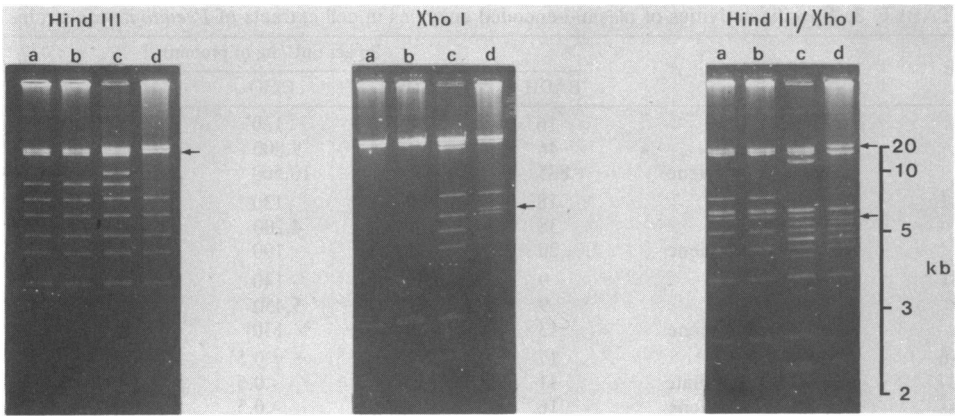

FIG. 2. Agarose gel electrophoresis of *Hin*dIII digests, *Xho*I digests, and *Hin*dIII-*Xho*I double digests of TOL plasmid pWW0 (lanes c) and its derivative (pWW0-8 [lanes a], pWW0-1211 [lanes b], and pWW0-1216 [lanes d]). The nomenclature and size estimates for the *Hin*dIII-*Xho*I double digestion fragments are shown in Table 4, and the positions of the fragments on the plasmid are shown in Fig. 3. The arrows indicate the novel fragments on pWW0-1216 generated by the addition of insertions (see text).

as *Hin*dIII-C′ (about 22 to 23 kb) in the *Hin*dIII digest, running as an unresolved double band coincident with *Hin*dIII-B (Fig. 2). The simplest explanation is that these fragments result from 3- to 3.5-kb insertions into *Hin*dIII-*Xho*I-Q (identical to *Xho*I-I [2.3 kb]) and into *Hin*dIII-*Xho*I-A (identical to *Hin*dIII-C [19 kb]). This analysis has been confirmed by *Eco*RI digestion since the corresponding *Eco*RI fragments from the unpublished map (P. Broda, personal communication) are correspondingly larger. Additional confirmation in the case of the *Hin*dIII-*Xho*I-Q (*Xho*I-I) insertion comes from reversion of WR216 to Mtol$^+$. Revertants, which have a phenotype identical to that of WR211, contain plasmids that are indistinguishable from pWW0-1216, except that *Hin*dIII-*Xho*I fragment Q′(*Xho*I-I′) has disappeared and is replaced by a fragment the same size as *Hin*dIII-*Xho*I-Q (*Xho*I-I), presumably as a result of spontaneous loss of the 3-kb insertion (data not shown). Since these revertants, (exemplified by WRB80), express C23O normally and show full inducibility of the other *meta* pathway enzymes (Table 3), the mutagenic role of that insertion on *xylE* and its polar effect upon the later *meta* pathway enzymes are confirmed.

DISCUSSION

WR211 and WR216 were constructed so that the expression of a nonspecific TO in a halobenzoate-degrading strain could extend their substrate specificity. Not only was this goal achieved with the 4CB$^+$ phenotype of WR216 and the ability of a subsequent derivative (WR941) to degrade 3,5-dichlorobenzoate (13), but the rationale for carrying out the conjugation was proved correct since the TO activity of pWW0 appears to be expressed in both WR211 and WR216 (Table 2). In addition, WR211 and WR216 have supplied further information about the regulation, structure, and behavior of pWW0.

The mutation in WR211 which accounts for its Mxy$^-$ phenotype agrees adequately with the proposed model for regulation (18), since it is typical of a *xylR* mutation. The pleiotropic negative character reinforces the evidence that the *xylR* gene product is a positive regulator (6, 10, 18). The most surprising feature of pWW0-1211 is its identity with pWW0-8. Whereas strains carrying pWW0-8 (such as PaW8 [1, 17]) are irreversibly Mxy$^-$ Mtol$^-$ and express none of the TOL enzymes, WR211 is Mtol$^+$. An accompanying paper (8) demonstrates that a considerable region of pWW0 DNA is located elsewhere in the genome of WR211, thus accounting for this discrepancy. However, the recovery of about 27 kb of TOL DNA missing from pWW0-1211 in plasmid pWW0-1216 was the first experimental clue as to the existence of this alternatively located DNA.

The Mtol$^-$ phenotype of WR216 is an obligatory result of selection for 4CB$^+$ (11, 14), but the complexity of the DNA structural modification accompanying this phenotypic change was surprising. Not only do recombinational events to reintroduce pWW0 DNA back into the plasmid occur, but two insertions of unknown origin, each about 3 kb long, appear. The insertion into *Hin*dIII-*Xho*I-Q appears to be in *xylE*, which in addition to inactivating C23O, the gene product, can also be interpreted as having a polar effect on *xylF* and *xylG*. However, the expression of TO (*xylD*) does not appear to be affected in WR216. If the regulatory model is correct (Fig.

TABLE 4. Sizes of the *Hin*dIII-*Xho*I double digestion fragments from pWW0 and its derivatives

*Hin*dIII-*Xho*I fragment	Size (kb) in:			
	pWW0[a]	pWW0-8[a]	pWW0-1211	pWW0-1216
A′				22.4[b]
A	19.0	19.0	19.0	
B	16.0	16.0	16.0	16.0
C′				15.0[c]
C	11.8			
d		7.2[d]	7.2[d]	
D[e]	6.2	6.2	6.2	6.2
E[e]	6.1	6.1	6.1	6.1
F	5.8	5.8	5.8	
Q′				5.5[f]
G	5.3			5.3
H	5.0	5.0	5.0	5.0
I[g]	4.7			
J[g]	4.7			4.7
K	4.4	4.4	4.4	4.4
L	4.2			
M[h]	3.6	3.6	3.6	3.6
N[h]	3.6			3.6
O	2.6	2.6	2.6	
P	2.3			2.3
Q	2.25			
R	2.1	2.1	2.1	2.1
S	1.56			1.56
T	1.23			
U	1.03			
V	0.79			
W	0.60			
X	0.55			
Y[i]	0.50			
Z[i]	0.50			0.50[j]
A1	0.28	0.28	0.28[j]	0.28[j]
B1	0.20	0.20	0.20[j]	0.20[j]
C1	0.13	0.13	0.13[j]	

[a] Sizes taken from references 4 and 5.
[b] Novel fragment derived from a 3.4-kb insertion into *Hin*dIII-*Xho*I-A.
[c] Novel fragment derived from residues of *Hin*dIII-*Xho*I-C and -F: runs as double with *Hin*dIII-*Xho*I-B.
[d] Novel fragment derived from residues of *Hin*dIII-*Xho*I-I and -J.
[e] In all plasmids, *Hin*dIII-*Xho*I-D and -E were a double band.
[f] Novel fragment derived from a 3.2-kb insertion into *Hin*dIII-*Xho*I-Q.
[g] In pWW0, *Hin*dIII-*Xho*I-I and -J were a double band.
[h] In pWW0 and pWW0-1216, *Hin*dIII-*Xho*I-M and -N were a double band.
[i] In pWW0, *Hin*dIII-*Xho*I-Y and -Z were a double band.
[j] These fragments were not detected on gels because of their small sizes but were assumed to be present because of the presence of surrounding fragments.

1) and *xylDEFG* is a coordinate operon, then our results indicate that the order of genes from the promoter is *xylDE(F,G)*; the 3-kb insertion in *xylE* would not affect TO expression but would have a polar effect on HMSH and HMSD. This hypothesis based on physiological evidence agrees well with the gene order *xylDEGF* derived from cloning experiments (Nakazawa, personal communication).

The 3-kb insertion in *Hin*dIII-*Xho*I-A has no obvious function. It is outside the 39-kb region delineated by the region missing from pWW0-8 and pWW0-1211, which we consider to carry all of the TOL catabolic genes. We have obtained no strains with plasmids which have lost this insertion which we can use for phenotypic comparison.

From these results pWW0 appears to have a flexible structure in the strain B13 background. There appear to be four sites at which deletion and recombination events can occur, namely, the termini of the 39-kb deletion in pWW0-1211 and the termini of the 19-kb deletion in pWW0-1216. It should be stated here that the structural change from pWW0-1211 to pWW0-1216 can be reproduced, since independent $4CB^+$ segregants of WR211 which have a plasmid structure identical to that of

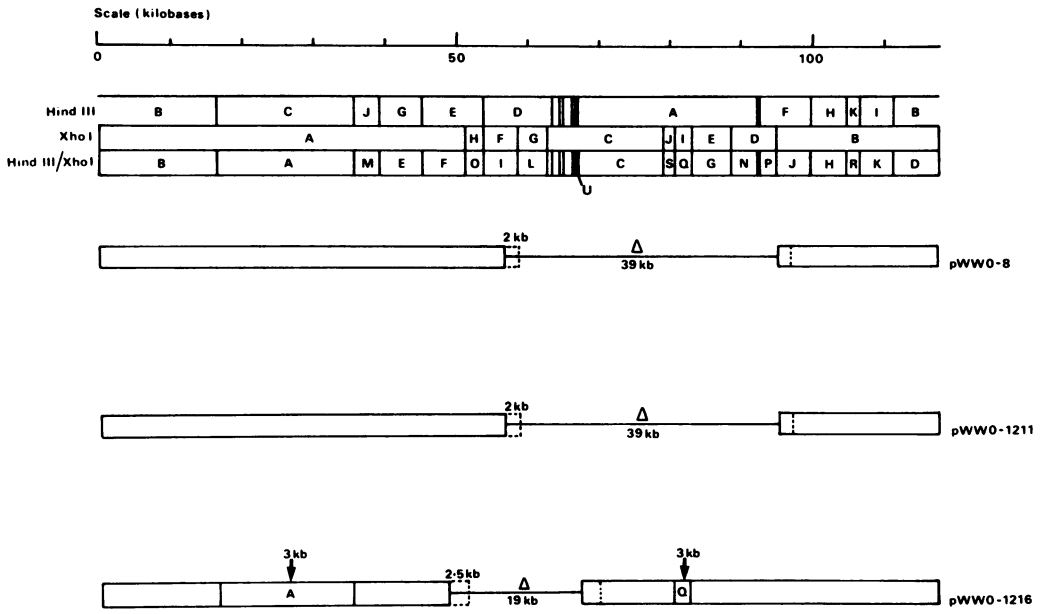

FIG. 3. Structures of pWW0 and its derivatives. These structures are presented in linear form from the *Xho*I site between *Xho*I fragments A and B. The positions of the cutting sites and the nomenclature of the fragments are taken from Downing and Broda (4), except that the order of *Xho*I fragments E, I, and J is reversed to conform with the order of these fragments in RP4::TOL plasmid pTN2 (10); we have independent evidence that the latter order is correct (unpublished data). The structure of pWW0-8 is as described previously (5). The structures of pWW0-1211 and pWW0-1216 were derived from an analysis of the hydrolysates of plasmid DNA by using single digestion with *Hin*dIII and *Xho*I separately and double digestion with the two enzymes together (Table 4) and comparison with the pWW0 map (4). The broken lines at the ends of the deletions represent the areas of positional uncertainty inherent in this analysis; however, as the sizes of the deletions could be determined quite accurately, they must run from either the right ends of both boxes (broken lines) at one extreme or from the left ends (solid lines) at the other. The vertical arrows represent the insertions of DNA of unknown origin into the specified double *Hin*dIII-*Xho*I digestion fragments, but the exact locations within the fragments are unknown. The maps were checked by comparing *Eco*RI digests of pWW0-1211 and pWW0-1216 with the unpublished *Eco*RI map (P. Broda, personal communication).

pWW0-1216 have been isolated. It is remarkable that nutritional selection pressures result in such complex changes in DNA organization when point mutations could be expected to produce the same result; the implication is that these changes occur at a higher frequency than point mutations. It could be that the facility of these changes reflects the stability of pWW0 in *Pseudomonas* B13 or that they result from the particular selective pressures which are possible in this strain. This might serve as an indication that the rapid evolution of plasmids might be determined in part by their passage through hosts in which minor selective pressures result in major modifications of the DNA structure.

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