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GUEST COMMENTARY

Two Chlorocatechol Catabolic Gene Modules on Plasmid pJP4

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Microbial metabolism of chloroaromatic compounds has been the subject of numerous studies for more than 35 years. The paper by Plumeier et al. in this issue of the *Journal of Bacteriology* (33) finally clarifies for one of the most intensely studied environmentally relevant strains, *Ralstonia eutropha* JMP134, how different sets of enzymes are involved in the degradation of 3-chlorobenzoate and 2,4-dichlorophenoxyacetate (2,4-D). (JMP134, which was originally assigned to the species *Alcaligenes eutrophus* [now *Ralstonia eutropha*], is usually still referred to accordingly, although it is now known that it should be assigned to a different *Ralstonia* species [18].) A historical perspective will allow us to understand and assess the achievements of this contribution.

A serious investigation of the degradative pathways for chloroaromatic compounds started as early as the 1960s in the laboratories of Alexander and Evans (1, 2, 12, 13, 15, 16, 48). These groups observed that chlorophenoxyacetates may be degraded via chlorophenols to chlorocatechols, which are then cleaved between the hydroxy groups to give chloro-*cis*,*cis*-muconates. Both laboratories also observed *cis*-4-carboxymethylenebut-2-en-4-olide (the so-called *cis*-dienelactone) and its methylated or chlorinated derivatives as intermediates, which indicated that dehalogenation occurs during the cycloisomerization reaction (Fig. 1). Alexander's group had already performed some enzymatic investigations (45). However, the next outstanding contribution was made by Knackmuss's group with the isolation and detailed characterization of *Pseudomonas* sp. strain B13, a strain capable of using, besides other aromatic and haloaromatic compounds, 3-chlorobenzoate as the sole carbon source. Knackmuss's group separated the enzymes responsible for 3-chlorobenzoate catabolism via chlorocatechol from those for benzoate catabolism via catechol (9, 43). The characterization of the enzymes with respect to their substrate specificities revealed that the enzymes induced during growth with 3-chlorobenzoate convert chlorosubstituted substrates, or the dienelactones derived from them, better than the enzymes induced during growth with benzoate (10, 43). It thus became clear that a special set of enzymes is responsible for the degradation of chlorocatechol intermediates, and these enzymes were considered to have a "relaxed" or "broad" substrate specificity.

In parallel to the enzymatic approach, the groups of Pemberton and Chakrabarty started to investigate the genetic background of degradative pathways for chloroaromatic compounds. Thus, Pemberton's group (7, 30) reported on the isolation of various *Ralstonia* strains (previously known as *Alcaligenes* spp.) in which the capability to degrade phenoxyacetate herbicides is harbored on catabolic plasmids. Among them was strain JMP134. Its catabolic plasmid pJP4, later became the subject of numerous studies, including those in the article by Plumeier et al. (33). Chakrabarty's group (3, 4, 5) similarly reported on catabolic plasmids (pAC25, pAC27, and pB13) in 3-chlorobenzoate-utilizing *Pseudomonas* strains. The existence of special enzymes and thus genes for chlorocatechol degradation and the localization of these and numerous other catabolic genes on plasmids gave rise to the concept of hybrid pathways. It became possible to construct strains with new catabolic phenotypes by combining chlorocatechol genes with the genetic information for various peripheral pathways. This was initially done by Reineke and Knackmuss by transferring toluate catabolic genes into *Pseudomonas* sp. strain B13 (37, 38) and later by transferring the chlorocatechol genes into other strains by conjugation (39). After sufficient knowledge had become available, recombinant DNA techniques were used, mainly by Timmis's group, for the construction of strains with new catabolic properties, such as degradation of chlorosalicylates, dichlorobenzoates, or problematic mixtures of methylated and chlorinated aromatic compounds (22, 35, 40).

The degradative plasmids and especially the chlorocatechol genes on them were extensively analyzed. For the archetypal plasmids pJP4, pAC27, and pP51, it was shown that the genes for all enzymes necessary to convert chlorocatechols into 3-oxoadipate form clusters which in some cases neighbor sequences for peripheral enzymes like 2,4-dichlorophenoxyacetate dioxygenase, chlorophenol hydroxylase, or toluene/chlorobenzene dioxygenase and the corresponding dihydrodiol dehydrogenase (8, 14, 17, 32, 47, 49, 50). Isofunctional enzymes of the chlorocatechol pathways of proteobacteria were shown to be homologous and more similar to each other than to the corresponding enzymes of catechol pathways, indicating a common origin of the proteobacterial chlorocatechol pathways (41). The enzymes, and specifically the chloromuconate cycloisomerase, of the gram-positive organism *Rhodococcus opacus* 1CP, in contrast, turned out to have unusual biochemical properties and sequences with relatively little similarity to proteobacterial chlorocatechol sequences, thus indicating an independent origin (11, 26, 27, 46).

With respect to pJP4 of *R. eutropha* JMP134, initial transposon mutagenesis had indicated the existence of only one chlorocatechol gene cluster (8), comprising the genes *tfdC* for a chlorocatechol dioxygenase, *tfdD* for a chloromuconate cycloisomerase, *tfdE* for a dienelactone hydrolase, and *tfdF* for an

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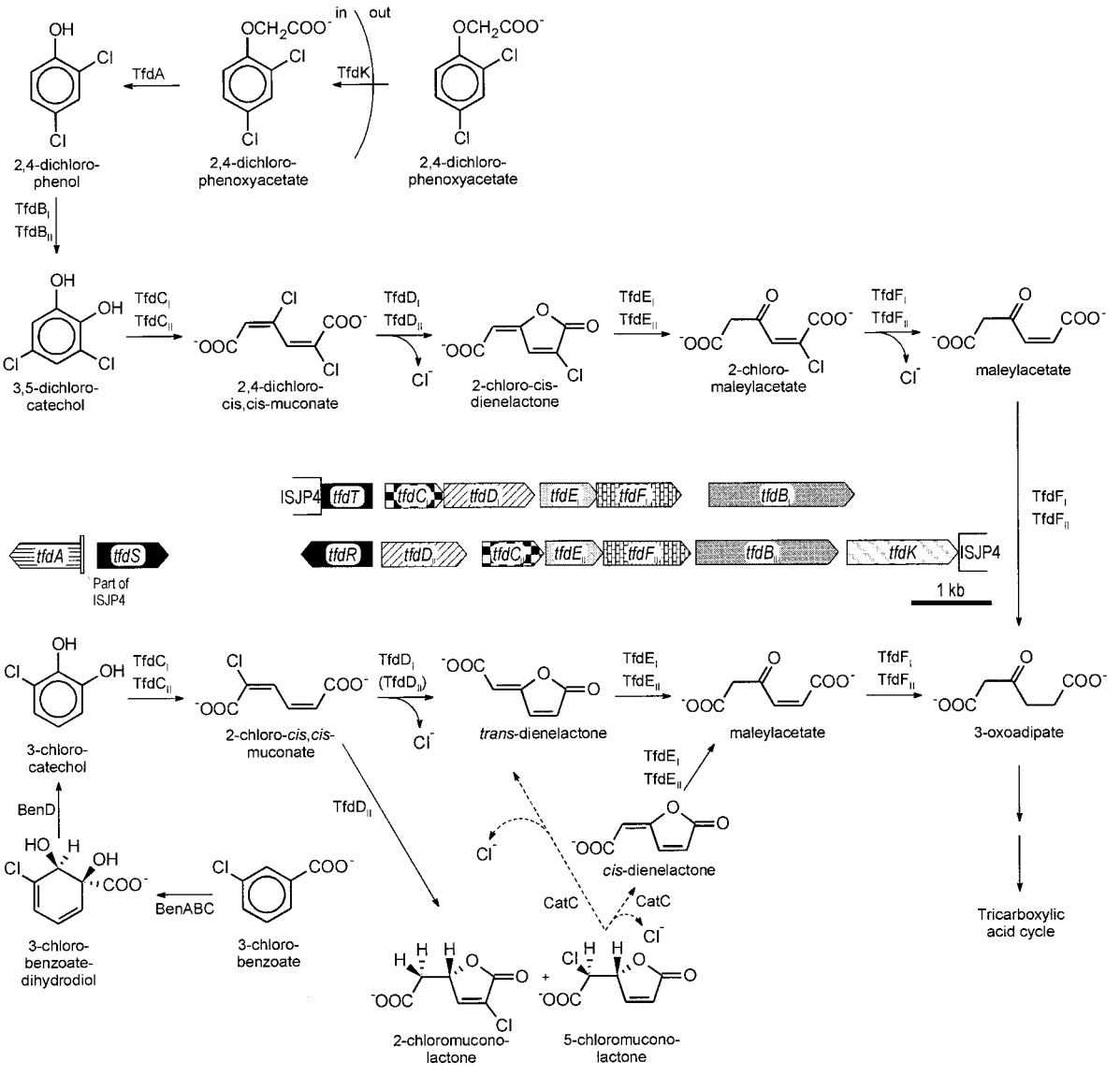

FIG. 1. Degradative pathways of 2,4-D (top) and 3-chlorobenzoate (bottom) by *R. eutropha* JMP134 (pJP4) and the two chlorocatechol gene clusters of pJP4. The two clusters, which are contiguous on pJP4, are shown separately to make comparisons easier.

enzyme later suggested to be a (chloro)maleylacetate reductase (44) (Fig. 1). However, after the work of Don et al. (8), evidence of two chlorocatechol dioxygenase genes and two chloromuconate cycloisomerase genes, respectively, on pJP4 was obtained (17, 28). van der Meer's group (25) then reported on the existence of a complete second gene cluster, the tfd_{II} cluster, which in addition to genes homologous to those of the tfd_I cluster comprises a gene, *tfdK*, for active 2,4-D uptake (Fig. 1). The tfd_{II} cluster together with the identical regulatory genes *tfdS* and *tfdR* (28, 54) is located on a composite transposon flanked by ISJP4 and a remnant of a second copy of it between *tfdA* and *tfdS* (24). Expression studies with mRNA from induced chemostat cultures showed that both clusters of enzyme-encoding genes are expressed while expression of the regulatory genes *tfdR* and *tfdS* remains low (23). Laemmli et al. (21) recently suggested that the $tfdD_{II}C_{II}E_{II}F_{II}B_{II}K$ cluster forms an operon which is expressed in response to the presence of 2,4-D. By expression in *Escherichia coli* they also showed that functional enzymes may be formed from the tfd_{II} cluster. Due to its highly unusual sequence, this was especially interesting for the cycloisomerase $TfdD_{II}$, which turned out to convert 2-chloro-*cis*,*cis*-muconate less efficiently than 3-chloro- or 2,4-dichloro-*cis*,*cis*-muconate. However, the question of whether the second chlorocatechol gene cluster is, in fact, important for *R. eutropha* JMP134 could not be satisfactorily answered by the experiments designed by Laemmli et al. (21), and this question is especially interesting, since the original transposon mutagenesis (8) had not indicated its existence. Nevertheless, the authors suggested three possible reasons for the maintenance of almost isofunctional genes on pJP4. (i) Since the predicted sequence of the maleylacetate reductase $TfdF_{II}$ contains the N terminus of the previously purified enzyme (44), $TfdF_{II}$ could be necessary especially for 2-chloromaleylacetate conversion and dechlorination, while $TfdF_I$ may not be sufficient. (ii) The

transporter TfdK, which occurs only in the operon of the tfd_{II} cluster (Fig. 1), may be advantageous, though not indispensable. (iii) The regulatory genes *tfdR* and *tfdS* would be lost by a loss of the composite transposon, though a functional regulator could be restored, if the tfd_{II} cluster were lost by recombination between *tfdR* and *tfdT*.

To elucidate the function of the two chlorocatechol clusters directly in *Ralstonia*, Pérez-Pantoja et al. (31) constructed clones of *R. eutropha* JMP222, a pJP4-free derivative of JMP134, which contained the single module I ($tfdC_ID_IE_IF_I$) or the single module II ($tfdD_{II}C_{II}E_{II}F_{II}$), both under the control of *tfdR*. With these constructs they measured relatively high activities for the Tfd_I enzymes (except $TfdF_I$), for $TfdC_{II}$, for $TfdF_{II}$, and with 2,4-dichloro-*cis,cis*-muconate also for $TfdD_{II}$ directly in *R. eutropha*. However, the dienelactone hydrolase $TfdE_{II}$ gave low activities, as did the cycloisomerase $TfdD_{II}$ with 2-chloro-*cis*,*cis*-muconate, the major chloromuconate isomer during 3-chlorobenzoate degradation. Corresponding to the higher enzyme activities, *R. eutropha* JMP222 harboring module I grew faster with 3-chlorobenzoate than the strain containing module II. In a *Pseudomonas putida* background, module I was not sufficient for growth with 3-chlorobenzoate, an observation which was interpreted as showing that the maleylacetate reductase of module II, $TfdF_{II}$, is required for efficient growth. Overall, Pérez-Pantoja et al. (31) identified possible bottlenecks in both modules ($TfdF_I$ in module I and $TfdD_{II}$ and $TfdE_{II}$ in module II). However, their study still discussed constructs with single modules carried on a medium-copy-number vector and did not directly address the complexity of wild-type *R. eutropha* JMP134. In addition, Pérez-Pantoja et al. (31) concentrated on growth with 3-chlorobenzoate and not with 2,4-D. Finally the possible contribution of the chromosomal maleylacetate reductase of strain JMP222 or JMP134 (42) and the effects on the results of possible instabilities of the enzymes in cell extracts were not experimentally addressed.

The study reported by Plumeier et al. (33) in this issue of the *Journal of Bacteriology* represents a new approach to elucidating the complex metabolic network for chloroaromatic compounds in *R. eutropha* JMP134. The authors partially purified all the relevant enzymes, i.e., the chlorocatechol dioxygenases, the chloromuconate cycloisomerases, the dienelactone hydrolases, and the maleylacetate reductases from 3-chlorobenzoate-grown *R. eutropha* strains carrying the single modules. Using two different chromatographic principles and several buffers, they determined the stability of the enzymes under the respective conditions as well as their retention behavior. The latter information could then be used to assign activity peaks in chromatograms of 2,4-D-grown wild-type cells of *R. eutropha* JMP134 to specific enzymes. The information on the stability could be used to estimate the relative importance of module I versus module II activity in the 2,4-D-grown wild type. While for dioxygenase and cycloisomerase the module II enzymes contributed ca. 20% of the overall activities, the dienelactone hydrolase $TfdE_{II}$ appeared to contribute only 5%.

Plumeier et al. (33) also finally elucidated the roles of the different maleylacetate reductases of *R. eutropha* JMP134. This question arose especially because JMP134, in addition to $TfdF_I$ and $TfdF_{II}$, also harbors a chromosomal maleylacetate reductase (42) and because the enzyme of module I, $TfdF_I$, had previously shown very low activities in cell extracts (31). In the study by Plumeier et al. (33), $TfdF_I$ initially turned out to be relatively active in vivo, as suggested by the lack of maleylacetate excretion during incubation with 3-chlorobenzoate of cells harboring only module I. The authors then showed that $TfdF_I$ is in fact a functional enzyme like $TfdF_{II}$, stable in extracts with phosphate buffer but unstable in extracts with Tris buffer. The protein chromatographic experiments proved that the chromosomal maleylacetate reductase is not recruited for growth with 3-chlorobenzoate or 2,4-D. By combination of anion-exchange chromatography, two-dimensional electrophoresis, and N-terminal sequencing, positive proof that $TfdF_I$ is, in fact, the maleylacetate reductase which allows growth with 3-chlorobenzoate of *R. eutropha* JMP222 containing module I was obtained. Anion-exchange chromatography with phosphate buffer surprisingly showed that $TfdF_I$, during growth of wild-type JMP134 with 2,4-D, contributes as much as roughly half of overall maleylacetate reductase activity.

Protein chromatography was also essential for differentiating the activities encoded by the two modules of pJP4 from chromosomally encoded activities of catechol catabolism. During growth with 3-chlorobenzoate, the background from chromosomal catechol enzymes turned out to be much higher in *R. eutropha* JMP222 carrying module II than in a strain carrying module I. High-pressure liquid chromatography assays by Plumeier et al. showed that during growth with 3-chlorobenzoate, *R. eutropha* JMP222 harboring module II accumulates considerably larger quantities of 2-chloromuconate than the strain with module I, due to the previously observed low activity of chloromuconate cycloisomerase $TfdD_{II}$ with this substrate (21, 31). The authors argue convincingly that the increased expression of catechol enzymes in strain JMP222 with module II is due to the higher accumulation of 2-chloro-*cis*,*cis*-muconate, which may act on the presumed catechol regulator.

Plumeier et al. (33) also extensively purified the chloromuconate cycloisomerase $TfdD_{II}$, which had previously been shown to have a very unusual amino acid sequence (21, 28). The investigation of its substrate specificity by spectrophotometric assays confirmed previous findings that the enzyme is active with 3-chloro-*cis*,*cis*-muconate but that 2-chloro-*cis*,*cis*-muconoate is a poor substrate (21, 31). Poor activity was also observed with unsubstituted *cis*,*cis*-muconate. $TfdD_{II}$ with respect to substrate specificity thus resembles the independently evolved enzyme of *R. opacus* 1CP (46) and is also similar to $TfdD_I$ of pJP4 which likewise showed a preference, though less pronounced, for 3-chloro- versus 2-chloro-*cis*,*cis*-muconate as a substrate (20, 52). Most interestingly, $TfdD_{II}$ during high-pressure liquid chromatography analyses of product formation turned out to convert 2-chloro-*cis*,*cis*-muconate initially to an equilibrium mixture of the substrate and 2-chloromuconolactone as well as 5-chloromuconolactone. Only upon longer incubation were *trans*-dienelactone and minor amounts of *cis*-dienelactone detected. In this respect the catalytic properties of $TfdD_{II}$ are intermediate between those of known muconate and chloromuconate cycloisomerases of proteobacterial origin: The former convert 2-chloro-*cis*,*cis*-muconate only to the equilibrium mixture and do not dehalogenate this substrate or the chloromuconolactones (51), while the latter are able to catalyze efficient dehalogenation of 2-chloromuconate or 5-chloromuconolactone (43, 53). The formation of chloromuconolactones instead of dienelactones may be regarded as an

insufficient adaptation of $TfdD_{II}$ to 2-chloro-*cis*,*cis*-muconate conversion, and the formation of catechol enzymes due to 2-chloro-*cis*,*cis*-muconate accumulation as an unnecessary burden. However, as discussed by the authors, the combination of both effects may result in a dehalogenation of 5-chloromuconolactone to *cis*- or *trans*-dienelactone by muconolactone isomerase (34), thus providing complete degradation by an intricate network of chromosomally encoded and plasmid-encoded activities.

Overall, by using a comprehensive experimental approach, Plumeier et al. (33) obtained new insights into the catalytic diversity of the cycloisomerases and especially into their dehalogenation capabilities, and it will be interesting to learn which key residues in the cycloisomerases are responsible for substrate specificities and dehalogenation. Most importantly, however, the authors provided a plausible answer to the question of why two chlorocatechol gene clusters are maintained on pJP4. They acknowledge possible roles of the transporter encoded by *tfdK*, for which there is no homologue in module I, and of the transcriptional activator encoded by *tfdR*, of which the homologue on module I has been inactivated by ISJP4 (21). However, Plumeier et al. argue convincingly that given an increase in activity by 25%, the gene dosage effect may provide for a sufficient selective pressure to maintain also the other genes of module II in addition to the almost isofunctional counterparts of module I on pJP4. They give examples of amplifications of chlorocatechol genes from *Pseudomonas* sp. strain B13 (36) and in *R. eutropha* JMP134 (6) as well as in constructed strains (19) to emphasize the importance of gene dosage. New results from my laboratory concerning the occurrence of two different chlorocatechol gene clusters in *R. opacus* 1 CP (29; Moiseeva et al, submitted for publication) also support this idea. They show at the same time that the central question investigated by Plumeier et al., the importance of the two almost isofunctional gene clusters, is important generally and not just for plasmid pJP4.

The paper by Plumeier et al. (33) and those on amplification of chlorocatechol genes have suggested the importance of gene dosage in the metabolism of haloaromatic compounds by soil bacteria. A number of questions arise from these studies. Given the short duration of high mRNA levels during induction of the chlorocatechol genes of pJP4 (23), what is the relative importance of gene dosage for the kinetics of mRNA synthesis and for maximum mRNA levels reached? What is the ecological advantage of maintaining two sets of very similar genes under conditions in which bacteria may not grow at a high rate? How general is the effect of gene dosage in the metabolism of nonaromatic halogenated compounds or in the metabolism of other xenobiotics? These questions are open avenues in research on the metabolism of xenobiotic compounds, and novel and exciting findings are still to come.

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