THE GENETIC CONTROL OF DISSIMILATORY PATHWAYS IN *PSEUDOMONAS PUTIDA*

M. L. WHEELIS* AND R. Y. STANIER

Department of Bacteriology and Immunology, University of California, Berkeley, California 94720

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WE shall present here an analysis of the linkage relationhsips among structural genes that control steps of the β-ketoadipate pathway and two other dissimilatory pathways in a fluorescent pseudomonad, *Pseudomonas putida.*

The convergent branches of the β-ketoadipate pathway (Figure 1), which exists in many taxonomic groups of aerobic bacteria, provide a means for the conversion of certain aromatic and hydroaromatic compounds to intermediates of the tricarboxylic acid cycle. The initial points of metabolic convergence are two diphenolic intermediates, catechol and protocatechuic acid: each primary substrate is oxidized to one of these intermediates through a special reaction-sequence. We shall designate such *peripheral sequences* by their primary substrates: thus, the *mandelate sequence* comprises the set of reactions by which mandelic acid is oxidized to catechol. Catechol and protocatechuic acid are further metabolized through parallel, convergent sequences to the enol-lactone of β-keto-

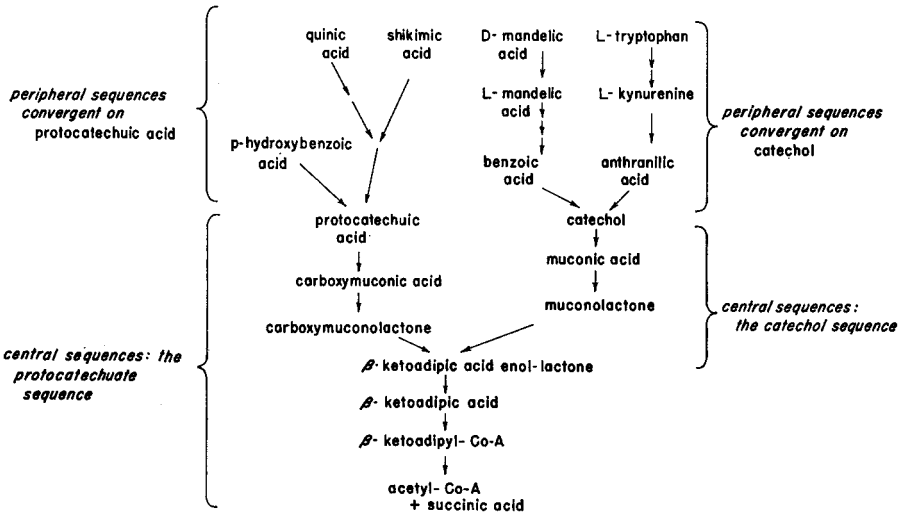

FIGURE 1.—The convergent sequences of the β-ketoadipate pathway, showing the principal aromatic and hydroaromatic compounds that are dissimilated through it by aerobic bacteria. Each arrow in this scheme represents a single enzymatic step-reaction.

* Present address: Department of Bacteriology, University of California, Davis, California 95616.

adipic acid, which is converted through a linear sequence of reactions to β-ketoadipyl-CoA. The final step-reaction (which will not be discussed in this paper) is a thiolytic cleavage of β-ketoadipyl-CoA to succinyl-CoA and acetyl-CoA. We shall term all reactions subsequent to the formation of the two diphenols the *central sequences* of the pathway. They comprise two subsets: the *catechol sequence* (conversion of catechol to the enol-lactone of β-ketoadipic acid); and the *protocatechuate sequence* (conversion of protocatechuic acid to β-ketoadipyl-CoA).

In bacteria, all sequences of the pathway are inducible. The patterns of induction are complex, however, and differ from group to group. The regulation of the enzymes operative in the central sequences was first elucidated by ORNSTON (1966c) in *Pseudomonas putida* (Figure 2), and the control pattern in *Pseudomonas aeruginosa* was later shown by KEMP and HEGEMAN (1968) to be identical. One of its characteristic features—coordinate product-induction of four enzymes

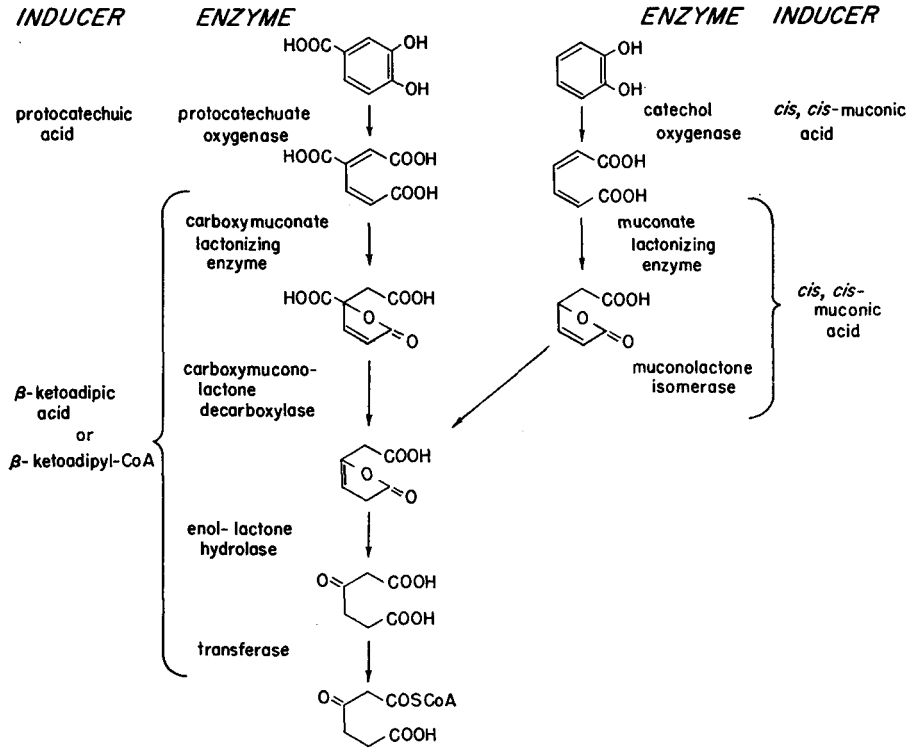

FIGURE 2.—The central sequences of the β-ketoadipate pathway and their regulation in *P. putida*. Brackets denote coordinate synthesis of enzymes.

of the protocatechuate sequence by β-ketoadipic acid or β-ketoadipyl-CoA—has been shown in several other *Pseudomonas* species (ORNSTON 1966c; KEMP and HEGEMAN 1968; unpublished data from this laboratory). In other groups of aerobic bacteria, however, the central sequences of the pathway are subject to different patterns of control (CÁNOVAS and STANIER 1967; JOHNSON, personal communication). It has been suggested that each of these control patterns constitutes a complex group-character, shared by related bacterial species, which probably reflects a common evolutionary origin of the genetic system governing the pathway (CÁNOVAS, ORNSTON and STANIER 1967).

It is convenient to describe control patterns at the phenotypic level in terms of *physiological regulatory units*. Each unit consists of an enzyme (or group of enzymes) subject to a specific mode of induction. In fluorescent pseudomonads, the eight enzymes mediating the central sequences fall into four distinct physiological regulatory units (Figure 2): the catechol unit (catechol oxygenase); the protocatechuate unit (protocatechuate oxygenase); the muconate unit (muconate lactonizing enzyme and muconolactone isomerase); and the carboxymuconate unit (carboxymuconate lactonizing enzyme, carboxymuconolactone decarboxylase, enol-lactone hydrolase and transferase).

If each physiological regulatory unit is controlled by a single operon (JACOB and MONOD 1961), genetic analysis should reveal close linkage between any two structural genes within a complex physiological regulatory unit (for example, the carboxymuconate unit), but not necessarily between any two structural genes operative in different physiological regulatory units. The first genetic analysis of the pathway was conducted by KEMP and HEGEMAN (1968) on *P. aeruginosa*, using the generalized transducing phage F116. Close linkage was observed between the two structural genes of the muconate unit, and among the three genes of the carboxymuconate unit for which markers were available. Unexpectedly, however, a considerable degree of clustering of genes specifying enzymes of other physiological regulatory units of the pathway with both of these complex units was discovered. Thus, the structural gene for *p*-hydroxybenzoate hydroxylase (operative in a peripheral sequence convergent on protocatechuate) was cotransducible with the genes of the carboxymuconate unit; and structural genes belonging to no less than three physiological regulatory units associated with the catechol branch (the genes for catechol oxygenase, anthranilate oxidase, and benzoate oxidase) were cotransducible with the genes of the muconate unit. Subsequently, ROSENBERG and HEGEMAN (1969) have observed that two additional structural genes, belonging to different physiological regulatory units of the mandelate sequence, are also cotransducible with members of the latter gene cluster. Accordingly, the twelve structural genes governing steps of the β-ketoadipate pathway in *P. aeruginosa* that have so far been examined fall into only three linkage groups (defined in terms of cotransducibility), but are associated with no less than nine physiological regulatory units.

The primary goal of the present study was to determine whether analogous or homologous gene clusters controlling enzymes of the β-ketoadipate pathway occur

in *P. putida*, for which a transducing phage is now available (CHAKRABARTY, GUNSALUS and GUNSALUS 1968). A secondary goal was the extension of the genetic analysis to other dissimilatory pathways in this species.

MATERIALS AND METHODS

Biological materials: The wild-type strain of *Pseudomonas putida* used in this work was strain PRS1 (ATCC 12633; strain 90 of STANIER, PALLERONI and DOUDOROFF 1966). Table 1 shows the genotypes and origins of the mutants derived from PRS1. The nutritional and enzymatic properties of mutants with lesions in the β-ketoadipate pathway are shown in Table 2.

TABLE 1

Description of strains

Strain	Previous designation	Genotype*	Parent	Derived by†	Reference
PRS1	90	wild	....	....	STANIER *et al.* 1966
PRS2	rac⁻	*mdlA1001*	PRS1	UV	HEGEMAN 1966b
PRS3	md⁻	*mdlB1002*	PRS1	EMS	HEGEMAN 1966b
PRS4	NG-22	*catB1001*	PRS1	NMG	ORNSTON 1966c
PRS5	A14	*pcaD1001*	PRS1	NMG	ORNSTON 1966c
PRS6	A202	*pcaB1002*	PRS1	NMG	ORNSTON 1966c
PRS9		*mdlA1001*, *catC1004*	PRS2	EMS	
PRS10		*pcaE1003*	PRS1	EMS	
PRS12		*mdlA1001*, *pcaE1004*	PRS2	EMS	
PRS14		*ben-1001*	PRS1	NMG	
PRS18		*pcaA1005*	PRS1	NMG	
PRS19		*pcaA1006*	PRS1	NMG	
PRS20		*pobA1001*	PRS1	NMG	
PRS21		*pobA1002*	PRS1	NMG	
PRS40		*pcaB1002*, *catC1006*	PRS6	NMG	
PRS41		*pcaA1006*, *pcaE1007*	PRS19	NMG	
PRS50	cs-III-2	*catB1008*	PRS1	EMS	CONDON and INGRAHAM 1967
PRS60		*ben-1002*	PRS1	NMG	
PRS81		*qui-1001*	PRS1	NMG	
PRS86		*pal-1002*	PRS1	NMG	
PRS87		*pal-1003*	PRS1	NMG	
PRS90		*pac-1001*	PRS1	NMG	
PRS93		*pal-1005*	PRS1	NMG	
PRS503		*hut-1003*	PRS1	NMG	
PRS504		*hutH1004*	PRS1	NMG	
PRS505		*hut-1005*	PRS1	NMG	

* The genetic nomenclature conforms to the suggestions of DEMEREC *et al.* (1966), although cistron assignments must be considered tentative, since there is as yet no complementation system available for *P. putida*.

† Abbreviations used: UV = ultraviolet irradiation; EMS = ethylmethane sulfonate treatment; NMG = N-methyl-N′-nitro-N-nitrosoguanidine treatment.

TABLE 2

Nutritional and enzymatic phenotypes of mutants with lesions in the β-ketoadipate pathway

Strain	Growth with					Enzymatic deficiency
	Mandelic acid	Benzoic acid	*p*-Hydroxy-benzoic acid	Quinic acid	Shikimic acid	
PRS1	+	+	+	+	+	none
PRS2	—	+	+	+	+	mandelate racemase
PRS3	—	+	+	+	+	L-mandelate dehydrogenase
PRS4	—	—	+	+	+	muconate lactonizing enzyme
PRS5	—	—	—	—	—	enol-lactone hydrolase
PRS6	+	+	—	—	—	carboxymuconate lactonizing enzyme
PRS9	—	—	+	+	+	mandelate racemase and muconolactone isomerase
PRS10	—	—	—	—	—	transferase
PRS12	—	—	—	—	—	mandelate racemase and transferase
PRS14	—	—	+	+	+	benzoate oxidase
PRS18	+	+	—	—	—	protocatechuate oxygenase
PRS19	+	+	—	—	—	protocatechuate oxygenase
PRS20	+	+	—	+	+	*p*-hydroxybenzoate hydroxylase
PRS21	+	+	—	+	+	*p*-hydroxybenzoate hydroxylase
PRS40	—	—	—	—	—	carboxymuconate lactonizing enzyme and muconolactone isomerase
PRS41	—	—	—	—	—	protocatechuate oxygenase and transferase
PRS50 30°C	+	+	+	+	+	none
PRS50 10°C	—	—	+	+	+	muconate lactonizing enzyme
PRS60	—	—	+	+	+	benzoate oxidase
PRS81	+	+	+	—	+	not determined

Media and conditions of cultivation: The mineral base described by STANIER *et al.* (1966) was routinely used for the preparation of synthetic media. In the preparation of liquid media, carbon sources were added to a final concentration of 20mM for succinate and 10mM for other compounds. These concentrations were reduced by half in the preparation of solid media. Media were solidified by the addition of Bacto-Agar to a final concentration of 1% (w/v).

Cultures were routinely incubated at 30°C. Liquid cultures were grown on a New Brunswick gyrotory shaker. Stock cultures were maintained on yeast extract agar slants at 4°C, and transferred at monthly intervals.

Isolation of mutants: Certain of the mutants examined had been isolated previously by other workers after UV irradiation (PRS2, HEGEMAN 1966b), treatment with ethylmethane sulfonate (PRS3, HEGEMAN 1966b; PRS9 and PRS12, HEGEMAN, unpublished; and PRS50, CONDON and INGRAHAM, 1967), or N-methyl-N′-nitro-N-nitrosoguanidine (PRS4 through 6, ORNSTON 1966c; PRS18 through 20, HOSOKAWA, unpublished). Additional mutants were obtained after treatment with N-methyl-N′-nitro-N-nitrosoguanidine as described by ORNSTON (1966c). Following such treatment, the cells were diluted 1:1000 into minimal medium containing 10mM succinate, divided into ten aliquots, and grown overnight to allow for nuclear segregation. Appropriate dilutions were then plated on a medium which permitted the recognition of mutants blocked in a specific catabolic pathway. The plating medium contained a low concentration of succinate (1mM), sufficient to allow the formation of very small colonies, together with a higher concentration (5mM)

of the primary substrate of the pathway in question. After incubation for five days at 30°C, small colonies were picked and transferred to 10mM succinate plates. The existence of a specific catabolic lesion was confirmed by replication on plates containing the primary substrate of the pathway in question. Between 20 and 40% of the small colonies proved to be the desired type of mutant. After verification of the phenotype, each mutant was purified by restreaking. Only one representative of any given phenotype was kept from each of the ten cultures in which segregation occurred.

Transductions: All transductions were performed with phage pf16h2 (CHAKRABARTY *et al.* 1968). The technique used by these workers was employed, except that recipient cells were grown on the mineral base described by STANIER *et al.* (1966), with succinate as sole source of carbon and energy.

Extraction of cells and enzyme assays: In order to determine the specific enzymatic lesions in mutants blocked in the β-ketoadipate pathway, cells were grown with succinate (10mM) and an appropriate inducer (5mM). They were then harvested by centrifugation, resuspended in 0.2M Tris-HCl pH 8.0 containing 10 μM MgEDTA, and treated for three minutes in a Raytheon 10kc sonicator. The extract was centrifuged, and the soluble fraction was used for the determination of enzymatic constitution. The following enzymes were assayed by published procedures: *p*-hydroxybenzoate hydroxylase (HOSOKAWA and STANIER 1966); protocatechuate 3,4-oxygenase (STANIER and INGRAHAM 1954); β-carboxy-*cis,cis*-muconate lactonizing enzyme, γ-carboxymuconolactone decarboxylase, and β-ketoadipate enol-lactone hydrolase (ORNSTON 1966a); β-ketoadipate succinyl-CoA transferase (CÁNOVAS and STANIER 1967); catechol 1,2-oxygenase (HEGEMAN 1966a); *cis,cis*-muconate lactonizing enzyme and muconolactone isomerase (ORNSTON 1966b).

Since there is no enzymatic assay for the system ("benzoate oxidase") which converts benzoate to catechol, mutants blocked in this step were identified by two more indirect criteria. One was their failure to synthesize enzymes of the catechol sequence after growth with succinate (10mM) and benzoate (5mM); the other was their failure to accumulate catechol (readily detectable visually as its blue iron chelate) when grown under these conditions.

Histidase was assayed by the technique of LESSIE and NEIDHARDT (1967) on extracts in 0.1M Tris H_2SO_4 (pH 7.4), prepared as described above.

Other physical and chemical measurements: Manometric experiments were conducted as described by WHEELIS, PALLERONI and STANIER (1967). Protein concentration was measured by the method of LOWRY *et al.* (1951).

All chemicals were commercial products of the highest purity obtainable, with the following exceptions. Succinyl-CoA was synthesized by the method of STADTMAN (1957), and *cis,cis*-muconate by the method of ELVIDGE *et al.* (1950). Both (+)-muconolactone and β-carboxy-*cis,cis*-muconate were prepared enzymatically (ORNSTON and STANIER 1966). Purified preparations of β-carboxy-*cis,cis*-muconate lactonizing enzyme, *cis,cis*-muconate lactonizing enzyme, (+)-muconolactone isomerase and β-ketoadipate enol-lactone hydrolase were prepared as described by ORNSTON (1966a, 1966b).

RESULTS

Since certain of the markers examined controlled steps in metabolic sequences that were poorly known in *P. putida*, some preliminary experiments were conducted in order to obtain information about the metabolic function and regulatory control of the genes in question. These results will be summarized prior to the presentation of the genetic data.

Biochemistry and regulation of the quinate sequence: P. putida dissimilates the hydroaromatic compounds, quinic and shikimic acids, through a peripheral sequence of the β-ketoadipate pathway, shown in Figure 3 (YOSHIDA 1964). In Acinetobacter, a single enzyme appears to dehydrogenate both hydroaromatic

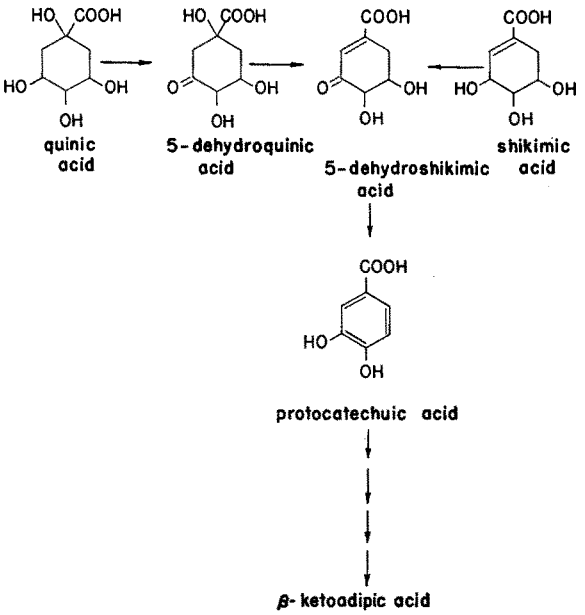

FIGURE 3.—The quinate and shikimate sequence of the β-ketoadipate pathway.

acids (CÁNOVAS, TRESQUERRES and DE TORRONTEQUI 1969); this peripheral sequence may, accordingly, be mediated by only three enzymes. Manometric experiments with intact cells of the wild type of *P. putida* show that the enzymes of the sequence are all induced by growth with either quinate or shikimate, but not with *p*-hydroxybenzoate or protocatechuate (Table 3). Mutant PRS81 has lost the ability to grow with quinate, but grows with either shikimate or *p*-hydroxybenzoate. The ability to oxidize shikimate is not induced when succinate-grown cells of this mutant are exposed to a mixture of quinate and protocatechuate (Figure 4). From these facts, it can be concluded that quinic acid is not an inducer for the enzymes of this peripheral sequence; the most probable

TABLE 3

Rates of oxygen consumption by PRS1 after growth with aromatic or hydroaromatic compounds

Cells grown with	Qo_2 (μl O_2/hr mg protein) with Shikimic acid	Quinic acid	*p*-Hydroxybenzoic acid	Protocatechuic acid
Shikimic acid	465	491	105	NT
Quinic acid	495	497	112	NT
p-Hydroxybenzoic acid	68	53	304	NT
10mM Succinic acid + 5mM Protocatechuic acid	63	27	NT	280
Succinic acid	11	7	NT	NT

NT: not tested

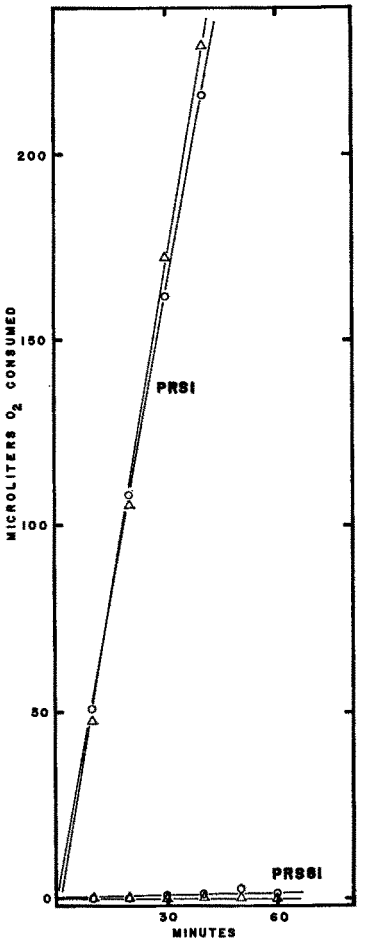

FIGURE 4.—Oxygen consumption with quinic (△) and shikimic (○) acids by PRS1 and PRS81 after exposure to quinic (5mM) and protocatechuic (5mM) acids for two hours (approximately two generations) during growth with 10mM succinic acid.

inducer is 5-dehydroshikimic acid, though an inductive role for either 5-dehydroquinic or shikimic acid cannot be rigorously excluded.

Biochemistry and regulation of the histidine pathway: The pathway of histidine dissimilation by *P. putida* (Figure 5) was established by TABOR (1955); and the regulation of this metabolic sequence has been studied in *P. aeruginosa* by LESSIE and NEIDHARDT (1967). In *P. aeruginosa*, all enzymes of the pathway are induced by urocanic acid, the first metabolic intermediate. A nonmetabolizable analog, dihydrourocanic acid, was shown to induce histidase, urocanase and enzyme IV, the only enzymes of the pathway for which there are specific assay methods. The synthesis of enzyme IV is not, however, coordinate with the synthesis of histidase and urocanase, which suggests that there are two physiological regulatory units, both subject to induction by the same effector. We have con-

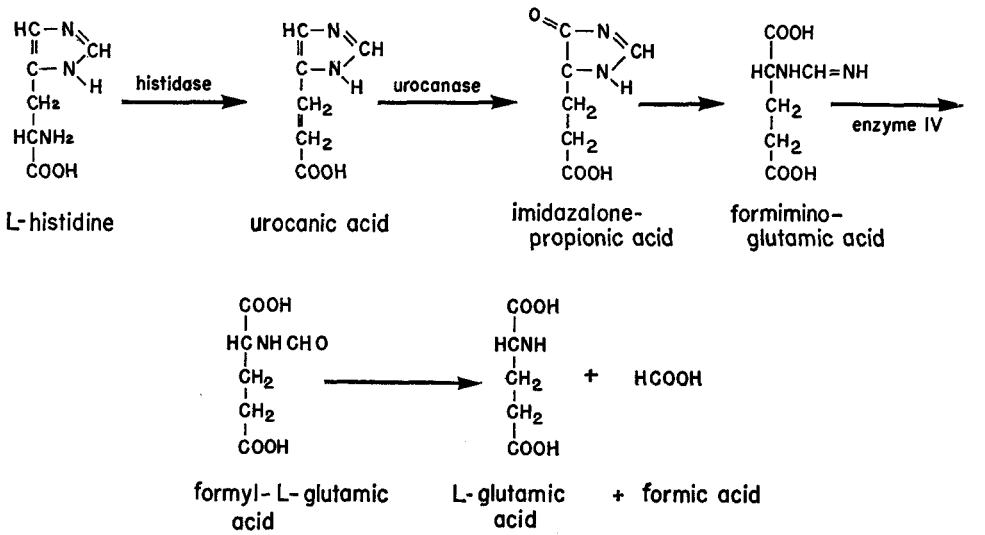

FIGURE 5.—The pathway of dissimilation of histidine by *P. putida*.

firmed for *P. putida* certain features of the regulatory system described by LESSIE and NEIDHART: specifically, the induction of histidase by either urocanic acid or dihydrourocanic acid (Table 4).

Three histidine-negative mutants were isolated. PRS504 lacks histidase; PRS-503 and PRS505 are blocked at undetermined steps below urocanic acid.

Pathways of phenylacetate and phenylalanine dissimilation: The available biochemical evidence shows that phenylacetic acid, phenylalanine and tyrosine are not metabolized through the β-ketoadipate pathway by bacteria. The pathways for their dissimilation are still unclear; two different diphenolic acids, homogentisic acid and homoprotocatechuic acid, have been suggested as intermediates (DAGLEY, FEWSTER and HAPPOLD 1952; KUNITA 1955a, 1955b, 1956; BLAKLEY *et al.* 1967). Our observations on the properties of PRS1 and of mutants derived from it suggest that phenylalanine is dissimilated via tyrosine, and that this sequence subsequently converges with the pathway of phenylacetic acid dissimilation (Figure 6).

As shown in Table 5, the attack on all three compounds by PRS1 is inducible,

TABLE 4

Specific activities of histidase after growth of PRS1 with a variety of carbon sources

Growth substrate	Specific activity of histidase μmoles/minute mg protein
20mM Succinic acid	0.003
10mM Histidine	0.340
10mM Urocanic acid	0.540
5mM Succinic acid + 10mM Dihydrourocanic acid	0.110

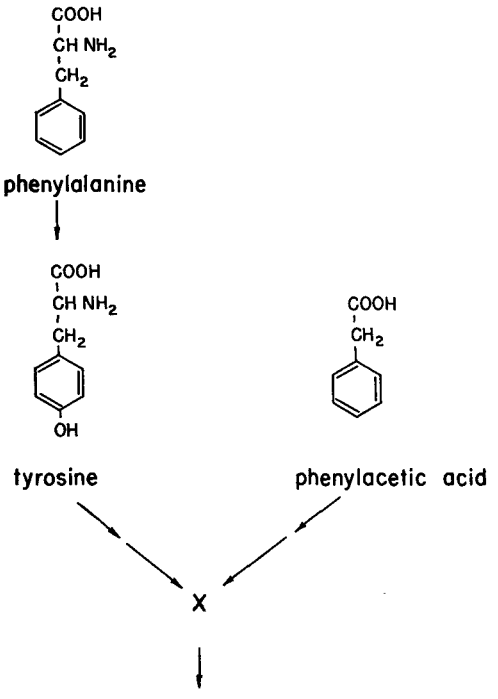

FIGURE 6.—The probable pathways of dissimilation of phenylacetic acid and phenylalanine by *P. putida*.

and the initial inductive events involved in phenylacetic acid and phenylalanine metabolism are distinct. Four nutritional phenotypes occurred among mutants blocked in the metabolism of these compounds (Table 6). From their nutritional properties, mutants of class one can be interpreted as being unable to convert phenylacetic acid to intermediate *X*; mutants of class two as being unable to convert phenylalanine to tyrosine; mutants of class three as being unable to convert tyrosine to intermediate X; and mutants of class four as being blocked below the point of metabolic convergence.

Genetic experiments—preliminary comments: Cotransducibility of markers can be demonstrated either by donor phenotype selection (CLOWES 1958), or by depression of the frequency of recovery of wild-type transductants (FARGIE and

TABLE 5

Rates of oxygen consumption by PRS1 after growth with phenylacetate and related compounds

Cells grown with:	Q_{O_2} (μl O_2/hr mg protein) with Phenylacetic acid	Phenylalanine	Tyrosine
Succinic acid	11	8	14
Phenylacetic acid	285	0	11
Phenylalanine	41	75	248
Tyrosine	6	16	322

TABLE 6

Nutritional phenotypes of phenylacetate and phenylalanine mutants

Class	Growth with			Number of mutants
	Phenylacetic acid	Phenylalanine	Tyrosine	
1	—	+	+	9
2	+	—	+	1
3	+	—	—	1
4	—	—	—	1

HOLLOWAY 1965). The latter method is in principle more useful, since there are no restrictions on the crosses that can be performed. However, KEMP and HEGEMAN (1968) found that in *P. aeruginosa* the variations in the transducing potency of phage preparations derived from different mutants, as well as in the effectiveness with which these mutants can be transduced by phage derived from the wild type, were so great that reliable quantitative linkage data could not be obtained by this method. We therefore used exclusively the technique of donor phenotype selection, which has the following restrictions. Firstly, in crosses involving genes that operate in a linear metabolic sequence, the recipient must be blocked at a later step in the sequence than the donor; secondly, intermediary metabolites that are chemically unstable or to which the cells are impermeable (e.g., most of the nonaromatic intermediates of the β-ketoadipate pathway) cannot be used for selection of transductants.

Mapping of genes that control steps in the central sequences of the β-ketoadipate pathway: Markers for six structural genes were examined (Table 7). The

TABLE 7

Transductional analysis of genes controlling reactions of the central sequences of the β-ketoadipate pathway

Genotypes*		Markers		Number of transductants examined	Percent cotransduction
Donor	Recipient	Selected	Unselected		
catB1008	*catC1006*	Benzoate+ @ 30°C	Benzoate− @ 11°C	108	57
pcaA1005	*pcaD1001*	Benzoate+	*p*-Hydroxybenzoate+	154	16
pcaA1006	*pcaD1001*	Benzoate+	*p*-Hydroxybenzoate+	216	11
pcaB1002	*pcaD1001*	Benzoate+	*p* Hydroxybenzoate+	61	95
pcaA1005	*pcaE1003*	Benzoate+	*p*-Hydroxybenzoate+	52	15
pcaA1006	*pcaE1003*	Benzoate+	*p*-Hydroxybenzoate+	32	3
pcaA1005	*pcaE1004*	Benzoate+	*p*-Hydroxybenzoate+	48	15
pcaA1006	*pcaE1004*	Benzoate+	*p*-Hydroxybenzoate+	48	19
pcaB1002	*pcaE1003*	Benzoate+	*p*-Hydroxybenzoate+	31	13
pcaB1002	*pcaE1004*	Benzoate+	*p*-Hydroxybenzoate+	67	69
wild	*pcaA1006*, *pcaE1007*	Benzoate+	*p*-Hydroxybenzoate+	33	50
catB1002	*pcaD1001*	*p*-Hydroxybenzoate+	Benzoate−	117	0
catC1004	*pcaD1001*	*p*-Hydroxybenzoate+	Benzoate−	106	0

* Only that portion of the genotype relevant to selection or scoring is given.

TABLE 8

Transductional analysis of genes controlling reactions of the mandelate sequence

Genotypes* Donor	Recipient	Markers Selected	Unselected	Number of transductants examined	Percent cotransduction
mdlA1001	*catB1001*	Benzoate+	D-Mandelate-	22	9
mdlB1002	*catB1001*	Benzoate+	D-Mandelate-	27	8
mdlA1001	*catC1006*	Benzoate+	D-Mandelate-	43	23
mdlB1002	*catC1006*	Benzoate+	D-Mandelate-	59	32
wild	*mdlA1001*, *catC1004*	Benzoate+	D-Mandelate-	42	2
mdlA1001	*ben-1001*	Benzoate+	D-Mandelate-	41	5
mdlA1001	*ben-1002*	Benzoate+	D-Mandelate-	75	3
mdlB1002	*ben-1001*	Benzoate+	D-Mandelate-	98	8
mdlB1002	*pcaE1004*	Benzoylformate+	D-Mandelate-	95	0

* Only that portion of the genotype relevant to selection or scoring is given.

data show that *catB* and *catC* (the two genes of the muconate unit) are linked, as are *pcaA* (protocatechuate unit), *pcaB*, *pcaD*, and *pcaE* (carboxymuconate unit). Neither *catB* nor *catC* (muconate unit) shows detectable linkage to *pcaD* (carboxymuconate unit).

Mapping of genes that control steps in the mandelate sequence, convergent on catechol: In *P. putida*, the conversion of D-mandelic acid to catechol is mediated by six enzymes, which fall into two physiological regulatory units: five coordinately induced enzymes which convert D-mandelic acid to benzoic acid, governed by *mdl* genes; and the benzoate oxidase system, governed by *ben* genes (HEGE-

TABLE 9

Transductional analysis of genes controlling reactions of sequences convergent on protocatechuate

Genotypes* Donor	Recipient	Markers Selected	Unselected	Number of transductants examined	Percent cotransduction
pobA1001	*pcaA1005*	Quinate+	*p*-Hydroxybenzoate-	138	0
pobA1001	*pcaD1001*	Benzoate+	*p*-Hydroxybenzoate-	279	14
pobA1002	*pcaD1001*	Benzoate+	*p*-Hydroxybenzoate-	151	25
pobA1001	*pcaE1003*	Benzoate+	*p*-Hydroxybenzoate-	33	18
pobA1002	*pcaE1003*	Benzoate+	*p*-Hydroxybenzoate-	31	3
pobA1001	*pcaE1004*	Benzoate+	*p*-Hydroxybenzoate-	26	40
pobA1002	*pcaE1004*	Benzoate+	*p*-Hydroxybenzoate-	34	3
qui-1001	*pobA1001*	*p*-Hydroxybenzoate+	Quinate-	66	3
qui-1001	*pobA1002*	*p*-Hydroxybenzoate+	Quinate-	103	4
qui-1001	*pcaA1005*	*p*-Hydroxybenzoate+	Quinate-	58	21
qui-1001	*pcaA1006*	*p*-Hydroxybenzoate+	Quinate-	38	37
qui-1001	*pcaD1001*	*p*-Hydroxybenzoate+	Quinate-	139	6
qui-1001	*pcaE1003*	*p*-Hydroxybenzoate+	Quinate-	59	12
qui-1001	*pcaE1004*	*p*-Hydroxybenzoate+	Quinate-	43	16

* Only that portion of the genotype relevant to selection or scoring is given.

MAN 1966b, 1966c; ORNSTON 1966c). Markers for three structural genes (*mdlA*, *mdlB*, and *ben*) were examined (Table 8). Both *mdlA* and *mdlB* are linked to *catB* and *catC*, as well as to *ben*. No linkage between *mdlB* and *pcaE* could be detected.

Mapping of genes that control steps in peripheral sequences convergent on protocatechuate: Markers for two structural genes operative in different physiological regulatory units were examined: *pobA* (the structural gene of *p*-hydroxybenzoate hydroxylase) and *qui* (one of the structural genes of the quinate sequence). Both show linkage to one another and to *pcaD* and *pcaE*; in addition, *qui* shows linkage to *pcaA* (Table 9).

Mapping of genes that control steps in this histidine pathway: Three markers were examined. One, *hutH*, affects the structural gene for histidase, while the other two affect biochemically unidentified steps subsequent to urocanic acid formation. As shown in Table 10, these three markers are cotransducible with one another, as well as with genes governing steps in the protocatechuate sequence of the β-ketoadipate pathway in the peripheral sequences convergent on protocatechuate. No linkage between *hut-1005* and *mdlA1001* could be detected.

Mapping of genes that control steps in the metabolism of phenylacetic acid and phenylalanine: Four markers affecting steps in the dissimilation of these compounds were examined: *pac-1001* (phenylacetic acid negative, phenylalanine and tyrosine positive); *pal-1003* (phenylalanine negative, tyrosine and phenylacetic acid positive); *pal-1002* (negative for both phenylalanine and tyrosine, positive for phenylacetic acid); and *pal-1005* (phenylalanine, tyrosine, and phenylacetic acid negative). These four mutants represent the four nutritional classes decribed in Table 6. All proved to be cotransducible with other markers studied. Although *pal-1002* and *pal-1003* were not cotransducible with *pcaA*, they were

TABLE 10

Transductional analysis of genes controlling histidine dissimilation

Genotypes*		Markers		Number of transductants examined	Percent cotransduction
Donor	Recipient	Selected	Unselected		
hutH1004	*hut-1003*	Urocanate+	Histidine−	56	14
hutH1004	*hut-1005*	Urocanate+	Histidine−	33	45
hutH1004	*pobA1001*	*p*-Hydroxybenzoate+	Histidine−	87	9
hutH1004	*pcaA1005*	*p*-Hydroxybenzoate+	Histidine−	138	20
hutH1004	*pcaD1001*	Benzoate+	Histidine−	139	3
hutH1004	*pcaE1004*	Benzoate+	Histidine−	64	2
pobA1001	*hutH1004*	Histidine+	*p*-Hydroxybenzoate−	198	7
pcaA1001	*hutH1004*	Histidine+	*p*-Hydroxybenzoate−	111	14
pcaB1002	*hutH1004*	Histidine+	*p*-Hydroxybenzoate−	55	58
pcaD1001	*hutH1004*	Histidine+	Benzoate−	156	4
pcaE1004	*hutH1004*	Histidine+	Benzoate−	174	0
mdlA1001	*hut-1005*	Histidine+	D-Mandelate−	39	0
qui-1001	*hut-1005*	Histidine+	Quinate−	109	0
pcaB1002	*hut-1005*	Histidine+	*p*-Hydroxybenzoate−	117	24

* Only that portion of the genotype relevant to selection or scoring is given.

with *pobA*, *pcaD*, *pcaE*, and *hutH*. The *pac-1001* and *pal-1005* lesions were cotransducible with these markers and, in addition, with *pcaA* (Table 11).

DISCUSSION

Tentative maps: The 17 structural genes examined fall into two linkage groups, defined in terms of the cotransducibility of markers. Tentative maps of these two gene clusters are shown in Figures 7 and 8. The map distances are calculated from the data in Tables 7, 8, 9, and 10, using the formula of Wu (1966): $d = L[1-(cf)^{1/3}]$, where d represents distance, L the length of a transducing fragment, and cf the cotransduction frequency; an arbitrary value of 100 units is assigned to L. The map distances are only first approximations, since the technique of donor phenotype selection for the most part precluded reciprocal crosses, and no three-point crosses were performed.

Gunsalus *et al.* (1968) have calculated that phage pf16, of which we used a host-range mutant, carries an amount of DNA that corresponds at most to five percent of the Pseudomonas chromosome. The smaller gene cluster (Figure 7), with a total map length of about 70 units, could therefore span at most 3–4% of the bacterial chromosome; and the larger gene cluster (Figure 8), with a total map length of about 100 units, at most 4–5% of the chromosome.

TABLE 11

Transductional analysis of genes controlling steps in phenylacetate and phenylalanine dissimilation

Genotypes*		Markers		Number of transductants examined	Percent cotransduction
Donor	Recipient	Selected	Unselected†		
pac-1001	*pobA1001*	*p*-Hydroxybenzoate+	Phenylacetate-	34	21
pac-1001	*pcaA1005*	*p*-Hydroxybenzoate+	Phenylacetate-	68	6
pac-1001	*pcaD1001*	*p*-Hydroxybenzoate+	Phenylacetate-	97	1
pac-1001	*pcaE1004*	*p*-Hydroxybenzoate+	Phenylacetate-	124	1.6
pac-1001	*hutH1004*	Histidine+	Phenylacetate-	213	41
pal-1002	*pobA1001*	*p*-Hydroxybenzoate+	Phenylalanine-	200	68
pal-1002	*pcaA1005*	*p*-Hydroxybenzoate+	Phenylalanine-	181	0
pal-1002	*pcaD1001*	*p*-Hydroxybenzoate+	Phenylalanine-	77	30
pal-1002	*pcaE1004*	*p*-Hydroxybenzoate+	Phenylalanine-	94	36
pal-1002	*hutH1004*	Histidine+	Phenylalanine-	72	7
pal-1003	*pobA1001*	*p*-Hydroxybenzoate+	Phenylalanine-	188	72
pal-1003	*pcaA1005*	*p*-Hydroxybenzoate+	Phenylalanine-	193	0
pal-1003	*pcaD1001*	*p*-Hydroxybenzoate+	Phenylalanine-	114	21
pal-1003	*pcaE1004*	*p*-Hydroxybenzoate+	Phenylalanine-	23	8
pal-1005	*pobA1001*	*p*-Hydroxybenzoate+	Phenylacetate-	190	70
pal-1005	*pcaA1005*	*p*-Hydroxybenzoate+	Phenylacetate-	280	37
pal-1005	*pcaE1004*	*p*-Hydroxybenzoate+	Phenylacetate-	140	98
pal-1005	*hutH1004*	Histidine+	Phenylacetate-	183	46

* Only that portion of the genotype relevant to selection or scoring is given.

† Scoring of transductants on phenylalanine was often difficult due to the fact that this is a very poor carbon source for PRS1. In all cases, revertants were also scored, and the growth of transductants was evaluated relative to the growth of the revertants.

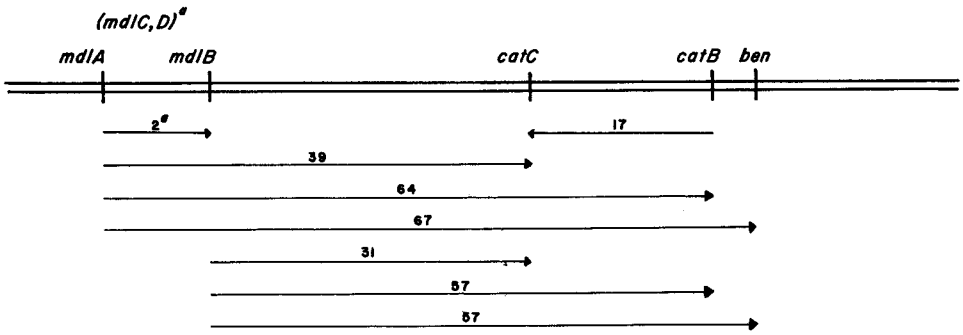

FIGURE 7.—Tentative map of genes of the mandelate and catechol sequences of the β-ketoadipate pathway in *P. putida*. a) Data from CHAKRABARTY and GUNSALUS (1969).

A consistent linear map can be constructed for the smaller gene cluster. However, attempts to order the genes of the larger cluster on a linear map lead to serious inconsistencies. The crosses that appear to give inconsistent results are those in which *pcaD1001*, *pcaE1004*, and *hutH1004* served as recipients; in all cases, the distances between donor and recipient markers are much larger than those suggested by other crosses involving these markers. Thus *pac-1001*,

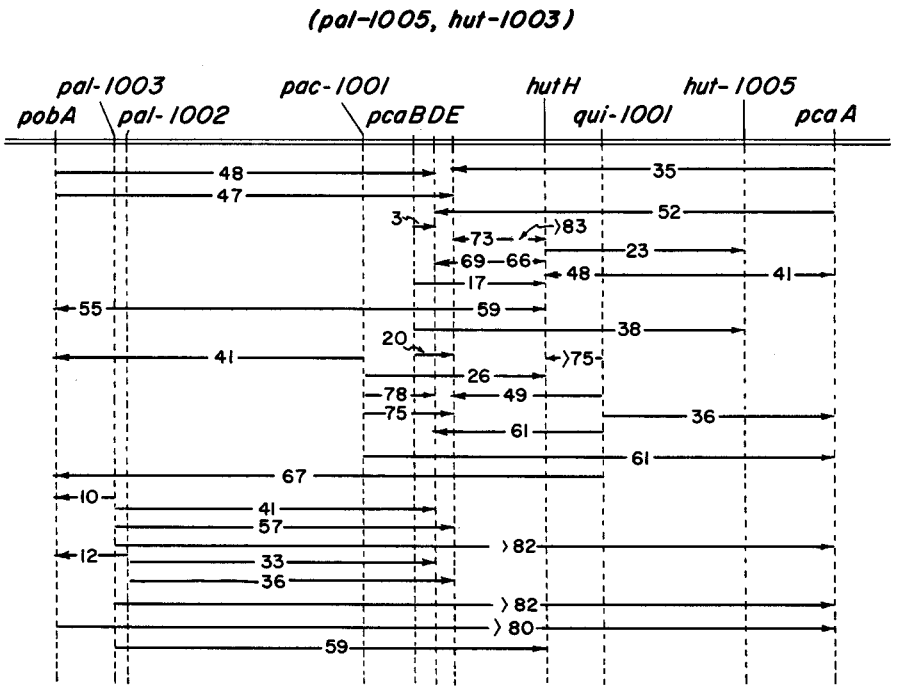

FIGURE 8.—Tentative map of genes of *p*-hydroxybenzoic acid, quinic acid, histidine, phenylacetic acid, and phenylalanine dissimilation in *P. putida*. The markers in parentheses cannot be ordered relative to other markers, but are known to be cotransducible with genes of this cluster.

hutH1004 and *qui-1001→pcaD1001; pac-1001, hutH1004, qui-1001→pcaE1004;* and *pcaD1001, pcaE1004, qui-1001→hutH1004* all yield cotransduction frequencies an order of magnitude less than those expected from the other linkage data.

These inconsistencies can be largely eliminated by the construction of a circular map, which allows the placement of two groups of markers—*pcaB,D,E;* and *qui, hutH, pac*—between *pobA* and *pcaA*, but distant from one another. However, some preliminary three-factor crosses are inconsistent with a circular map. The topology of the map of this linkage group and the precise ordering of its constituent genes therefore remain unclear. It is evident, however, that *pobA* and *pcaA* lie at the two ends of the linkage group, and that the remaining markers are distributed between them. Figure 8 shows the most satisfactory linear arrangement deducible from two-factor crosses.

The genetic homologies between P. putida *and* P. aeruginosa*:* Map distances for genes operative in the β-ketoadipate pathway which have been determined both in *P. putida* and in *P. aeruginosa* (KEMP and HEGEMAN 1968; ROSENBERG and HEGEMAN 1969) are compared in Figure 9. A high degree of similarity with respect to genes governing the central sequences is evident. The one major difference concerns the location of *pcaA* (the structural gene for protocatechuate oxygenase). In *P. putida*, it is relatively distant from, but cotransducible with, the other *pca* genes; in *P. aeruginosa*, it is not. This difference is not necessarily significant, since the linkage of *pcaA* to the other *pca* genes in *P. aeruginosa* may

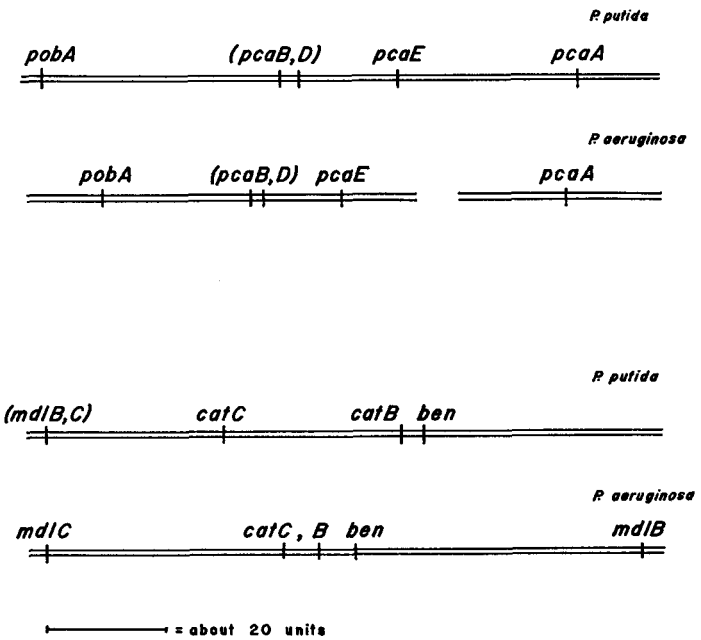

FIGURE 9.—Comparative genetic maps showing genes of the β-ketoadipate pathway in *P. putida* and *P. aeruginosa*. The distances for *P. aeruginosa* were calculated from the data given in KEMP and HEGEMAN (1968) and ROSENBERG and HEGEMAN (1969).

not be quite close enough to have been detected with the transducing phage employed. The genetic homologies between the two species support the hypothesis that the central sequences of the pathway have had a common evolutionary origin in *P. aeruginosa* and *P. putida*, a hypothesis originally proposed on the basis of similarities of control patterns (CÁNOVAS, ORNSTON and STANIER 1967) and immunological cross-reactivity of homologous enzymes (STANIER 1968).

An interesting feature of the two maps is the evident lack of homology with respect to *mdl* genes. In *P. putida*, *mdlB* and *mdlC* are close together, on one side of the *catC–catB–ben* cluster; in *P. aeruginosa*, they are widely separated, on each side of this cluster. The two wild-type strains also differ with respect to both the enzymology and the regulation of the mandelate sequence. In the wild-type strain of *P. putida*, the conversion of D-mandelic acid to benzoic acid is mediated by a total of five enzymes, which are induced coordinately (HEGEMAN 1966b, 1966c). The wild-type strain of *P. aeruginosa* cannot attack the D-isomer of mandelic acid; and the conversion of L-mandelic acid to benzoic acid is mediated by three enzymes, belonging to two regulatory units (ROSENBERG and HEGEMAN 1969). These differences with respect to the enzymology, regulation and genetic control of the mandelate sequence contrast strikingly with the essential homology of the central sequences of the pathway in the two species. The mandelate sequence might have arisen by largely independent evolution in the two species, as an adjunct to the preexisting, homologous central sequences.

Possible interpretations of the observed gene clustering: Among the genes of *P. putida* governing steps of the β-ketoadipate pathway that were mapped, three sets govern groups of enzymes each belonging to a single physiological regulatory unit, and hence subject to coordinate induction. These sets are: *mdlA* and *mdlB; catB* and *catC; pcaB*, *pcaD*, and *pcaE*. It can be seen (Figures 7 and 8) that the genes of each set map adjacent to one another, and that the map distances between them are relatively small. Each of the corresponding multi-enzyme physiological regulatory units may therefore be controlled by a single, complex operon. These three specific cases of gene clustering can be interpreted plausibly in terms of the operon hypothesis.

However, each linkage group includes structural genes that belong to several different physiological regulatory units. Thus, the smaller linkage group contains genes operative in three metabolically related physiological regulatory units, all associated with the catechol branch of the pathway: *mdlA-mdlB* and *ben*, operative in the two physiological regulatory units of the peripheral mandelate sequence; and *catB–catC*, operative in the catechol sequence. The larger linkage group contains genes operative in no less than four different physiological regulatory units associated with the protocatechuate branch of the pathway: *qui*, operative in the quinate sequence; *pobA*, operative in the *p*-hydroxybenzoate sequence; and 4 *pca* genes, operative in the two physiological regulatory units of the protocatechuate sequence. Such supra-operonic clustering cannot be a result of chance, assuming a random distribution of operons on the chromosome. If a single transducing particle can transfer 5% of the chromosome, the probability that any four operons randomly distributed on the chromosome will prove

to be cotransducible is of the order of 0.0002. Although *pobA* and *pcaA* are not cotransducible, their distances from markers between them indicate that they are probably separated by less than 5% of the chromosome. It must therefore be concluded that the order on the Pseudomonas chromosome of the genes controlling the β-ketoadipate pathway is highly nonrandom. Furthermore, we have observed that at least two genes controlling histidine dissimilation, and four controlling the dissimilation of phenylacetate and phenylalanine, are cotransducible with the genes operative in the protocatechuate branch of the β-ketoadipate pathway. This suggests the existence of a higher order of gene clustering on the chromosome of *P. putida*, which results in association of genes controlling several biochemically nonconvergent dissimilatory pathways. However, the data indicative of this particular feature of chromosomal organization are minimal, and we shall discuss primarily the possible interpretations of the well established supraoperonic clustering of genes that control the β-ketoadipate pathway, a phenomenon characteristic of both *P. putida* and *P. aeruginosa*.

Two specific features of the clustering should be emphasized, since each must be accounted for by any explanatory hypothesis. The first, already alluded to, is the *physiological* significance of the two cotransducible clusters: each contains a group of genes operative in metabolically related sequences of one of the two major branches of the β-ketoadipate pathway. The second feature is the relatively loose association between genes belonging to separate physiological regulatory units having related metabolic functions. For example, *pobA* and *pcaA*, which control two sequential steps in the protocatechuate sequence, are both separated by approximately 50 map units from the *pcaBDE* cluster, which controls subsequent steps of this sequence (Figure 8). It is therefore obvious that a large number of other genes, specifying unknown functions, must be intercalated between the chromosomal sites of *pobA*, *pcaBDE* and *pcaA*.

The two major clusters could be interpreted as reflections of the evolution of each of the two branches of the pathway by repeated gene duplications, followed by functional and regulatory differentiation of the duplicated genes. This would imply that the enzymes of the pathway controlled by each gene cluster are homologous proteins. In the specific case of the β-ketoadipate pathway, this hypothesis is not attractive, since the enzymes under the control of each gene cluster are known to be highly diverse in physical, catalytic and immunological respects. In fact, the only enzymes of the entire pathway which do resemble one another in their physical and catalytic properties are the pairs of enzymes which catalyze chemically analogous steps in the catechol and protocatechuate sequences: for example, the two lactonizing enzymes, for which the respective substrates are muconic acid and carboxymuconic acid (Ornston 1966a, 1966b). These two enzymes do not, however, show any immunological relationship (Stanier 1968). Furthermore, since the two lactonizing enzymes are controlled by genes belonging to different clusters, their similarities cannot in any case be invoked as support for the notion of an origin of the enzymes controlled by each cluster through gene duplication. The available evidence, though limited, points

rather to a complete absence of homology among the enzymes of the catechol branch and among those of the protocatechuate branch.

We must therefore ask whether any selective pressure can be envisaged which would lead to the loose clustering on the chromosome of functionally related but nonhomologous genes. A possible selective pressure can perhaps be found in the mechanisms of bacterial gene transfer, which typically result in the transfer of relatively small chromosomal fragments and occasional larger ones, from a donor to a recipient cell. If any such mechanisms of gene transfer operate frequently in natural Pseudomonas populations, a loose clustering of metabolically related operons would obviously favor the transfer *en bloc* of the determinants for a complete metabolic pathway, or of determinants for a segment of a pathway sufficiently extensive to be physiologically functional. If, on the other hand, metabolically related operons were widely separated on the bacterial chromosome, the dissemination of complex metabolic functions through the population would be relatively inefficient, since a single operon governing only one segment of such a sequence would be physiologically functionless upon transfer to a recipient cell. Natural selection would therefore tend to favor translocations which brought metabolically related operons into sufficient chromosomal propinquity to make their simultaneous transfer probable.

The simultaneous transfer of gene clusters determining two or more different catabolic functions might also have had considerable selective value, and could therefore perhaps explain the association on the Pseudomonas chromosome of genes operative in different pathways (e.g., the histidine and β-ketoadipate pathways) that is suggested by our work. The operation of a similar mode of natural selection in a slightly different genetic context can be deduced in the evolution of the resistance transfer factors of the enteric group of bacteria. The available evidence (ANDERSON 1967; WATANABE 1963) suggests that natural selection has produced a very rapid clustering on these episomic elements of genes that determine resistance to antibiotics. The analogy is particularly striking, because the gene clustering on resistance transfer factors is clearly supra-operonic, and is thus intelligible only in terms of gene function, not of gene expression.

Indeed, the selective force which we propose can also be invoked to explain the genesis of complex operons. Gene clustering which is selected for primarily in terms of the achievement of simultaneous transmissibility can be expected on rare occasions to bring two functionally related simple operons into immediate juxtaposition on the chromosome, a situation which then permits imposition on the two structural genes in question of a common mechanism of regulation. Given sufficient time, an entire pathway could thus be brought under the control of a large complex operon. This situation probably exists with respect to the β-ketoadipate pathway in *Acinetobacter calcoaceticus* (CÁNOVAS and STANIER 1967; CÁNOVAS, WHEELIS and STANIER 1968), where a degree of coordinate control far greater than that characteristic of *Pseudomonas* spp. governs both branches of the pathway (Figure 10). In the protocatechuate branch, all the

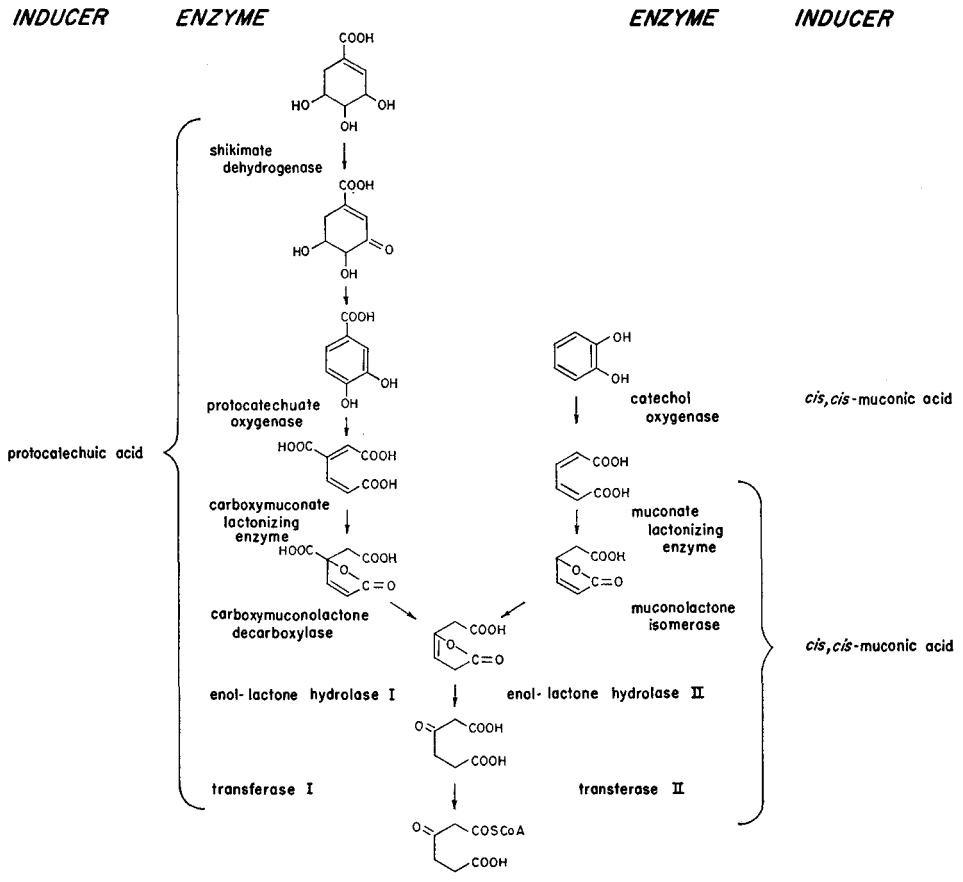

FIGURE 10.—The shikimate and central sequences of the β-ketoadipate pathway and their regulation in *Acinetobacter calcoaceticus*. Brackets denote coordinate synthesis of enzymes.

enzymes responsible for the conversion of shikimic acid to β-ketoadipyl-CoA are elicited by one inducer, protocatechuic acid, and appear to be subject to coordinate induction. In the catechol branch, all the enzymes mediating the conversion of *cis, cis*-muconic acid to β-ketoadipyl-CoA are induced by *cis-cis*-muconic acid, and their synthesis is coordinate under most physiological conditions. This extension of coordinate regulatory control has necessitated the evolution in Acinetobacter of two separate sets of enzymes governing the terminal common steps of the pathway; one set is linked by regulation with the enzymes of the protocatechuate branch, and the other with the enzymes of the catechol branch.

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SUMMARY

The genes specifying enzymes of the β-ketoadipate pathway in *Pseudomonas*

putida were mapped by transduction. Those whose products were synthesized in a coordinate fashion were found to be cotransducible at high frequencies, consistent with the notion that they are organized into complex operons. Genes operative in this pathway whose products are related functionally, but regulated independently, were found to be cotransducible, generally at lower frequencies. All genes studied which code for enzymes of the catechol branch of the β-ketoadipate pathway were located by transduction in a single linkage group. All genes coding for enzymes of the protocatechuate branch of the pathway were located in a second linkage group. Cotransduction between the two linkage groups could not be demonstrated. Six additional markers, affecting enzymes in the pathways for dissimilation of histidine, phenylalanine, and phenylacetic acid were also analyzed by tranduction. All proved to be cotransducible with the cluster of genes that controls enzymes of the protocatechuate branch of the β-ketoadipate pathway. It is proposed that the selective advantage of such clustering, not explicable by the operon hypothesis, arises from the possibility of the simultaneous transfer of related nutritional determinants by the mechanisms of genetic transfer characteristic of bacteria. These mechanisms usually mediate the transfer of relatively small fragments of the genome; thus close linkage would be required for the cotransfer of the determinants of a metabolic pathway, any of which alone would be physiologically functionless in a recipient cell.

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