The Regulation of the β -Ketoadipate Pathway in *Pseudomonas acidovorans* and *Pseudomonas testosteroni*

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SUMMARY

Bacteria of the acidovorans *Pseudomonas* group (*Pseudomonas acidovorans* and *P. testosteroni*) utilize β -carboxy-*cis, cis*-muconate for growth via the β -ketoadipate pathway. Since protocatechuate, the only known metabolic precursor of β -carboxy-*cis, cis*-muconate, is utilized by these bacteria via another metabolic pathway, it appears that β -carboxy-*cis, cis*-muconate can serve as a natural growth substrate.

The members of the acidovorans group are freely permeable to β -carboxy-*cis, cis*-muconate and in this respect they differ from other *Pseudomonas* species.

Unlike other bacteria (*Acinetobacter*, *Alcaligenes*, *Nocardia* and other species of *Pseudomonas*) the acidovorans group of *Pseudomonas* do not use protocatechuate or β -ketoadipate as an inducer of β -carboxy-*cis, cis*-muconate lactonizing enzyme and γ -carboxy muconolactone decarboxylase. In *Pseudomonas acidovorans* and *P. testosteroni* the inducer of these enzymes appears to be β -carboxy-*cis, cis*-muconate (or γ -carboxymuconolactone). In *P. testosteroni*, but not in *P. acidovorans*, β -ketoadipate serves as an inducer of muconolactone isomerase. Therefore strains of *P. acidovorans* and *P. testosteroni* may be identified by the unique mechanism that they employ to govern synthesis of the enzymes of the β -ketoadipate pathway.

INTRODUCTION

A number of aromatic compounds are dissimilated by micro-organisms via the diphenolic compounds protocatechuate and catechol. Bacterial oxidative metabolism of these diphenols proceeds through one of two strikingly divergent routes (Fig. 1). One of these is initiated by *meta* oxygenative cleavage to one side of the hydroxyl groups of the aromatic ring and yields α -hydroxymuconic semi-aldehydes (Dagley, Evans & Ribbons, 1960; Dagley, Geary & Wood, 1964); subsequent metabolism of these intermediates gives rise to α -keto acids (Dagley, Chapman, Gibson & Wood, 1964). The other route commences with *ortho* ring fission between the hydroxyl groups to form *cis, cis*-muconic acids which are further metabolized via β -ketoadipate to succinate and acetyl CoA (Fig. 1) (Ornston & Stanier, 1966). The enzymes of the *meta* pathway are of broad specificity and act upon a number of diphenols bearing alkyl substituents on the aromatic ring (Bayly & Dagley, 1969; Ribbons, 1970). In contrast, the enzymes of the *ortho* pathway are highly specific (Ornston, 1966*a, b*).

The *ortho* pathways are more widely distributed in bacteria than the *meta* pathways. Both protocatechuate and catechol are dissimilated via the β -ketoadipate pathway in *Acinetobacter* (Cánovas & Stanier, 1967), *Alcaligenes* (Johnson & Stanier, 1971*a*), *Nocardia* (Rann & Cain, 1969) and most species of *Pseudomonas* (Ornston 1966*c*). A few fluorescent pseudomonads also possess the genetic information required to synthesize the component enzymes of the *meta* catechol pathway (Davies & Evans, 1964; Azoulay, 1966) and in these strains the pathway that is used for the metabolism of catechol is determined by the primary growth

Fig. 1. Major pathways for the bacterial dissimilation of protocatechuate and catechol.

substrate (Cain & Farr, 1968; Feist & Hegeman, 1969). Members of the acidovorans *Pseudomonas* group (*Pseudomonas acidovorans* and *P. testosteroni*) are distinguished by their consistent use of the *meta* pathway for the utilization of protocatechuate (Stanier, Palleroni & Doudoroff, 1966; Wheelis, Palleroni & Stanier, 1967). In *Azotobacter* species protocatechuate is cleaved by *ortho* oxygenases, but the *meta* pathway is used for the dissimilation of catechol (Hardisson, Sala-Trepat & Stanier, 1969). Thus the metabolic pathway employed for the dissimilation of catechol and protocatechuate is uniform within clearly defined bacterial groups. Evolutionary relationships among bacteria that use *ortho* cleavage for both catechol and protocatechuate may be deduced from the inductive mechanisms used to govern the synthesis of the enzymes of the β -ketoadipate pathway (Cánovas, Ornston & Stanier, 1967). Members of the genera *Pseudomonas* (Ornston, 1966c), *Acinetobacter* (Cánovas & Stanier, 1967), *Alcaligenes* (Johnson & Stanier, 1971b), and *Nocardia* (Rann & Cain, 1969) may be identified by regulatory patterns that are distinctive in the extent of co-ordinate control as well as in the specific metabolites that are employed as inducers.

In this paper we show that representatives of the acidovorans *Pseudomonas* group grow well at the expense of β -carboxy-*cis,cis*-muconate, despite their utilization of protocate-

chuate via the *meta* pathway. In addition, some general properties are described of the novel inductive mechanisms employed by *Pseudomonas acidovorans* and *P. testosteroni* to control the synthesis of enzymes of the β -ketoadipate pathway.

METHODS

Bacterial strains. All were obtained from R. Y. Stanier and bear the strain numbers assigned by Stanier *et al.* (1966).

Media and conditions of cultivation. Cultures were grown in 100 ml of defined mineral medium (Ornston & Stanier, 1966) contained in 500 ml growth flasks with side arms. Constant aeration at 30 °C was provided by a Gyrotary New Brunswick Environmental Incubator Shaker. Carbon sources were prepared as concentrated (0.2 to 0.5 M) stock solutions, filter-sterilized and stored at -20 °C. Stock solutions were added aseptically to give 10 mM final concentration of carbon source. Inocula were grown overnight in tubes containing 5 ml of the same medium.

Growth was followed turbidimetrically using a Klett-Summerson colorimeter with a no. 66 filter. As cultures approached the end of exponential growth they were harvested by centrifugation for 10 min at 3000 *g* in a Sorvall RC2-B refrigerated centrifuge maintained at 5 °C. The organisms were washed twice with 25 ml of 20 mM tris-HCl buffer, pH 8.0, containing 2 mM-MgCl₂ and 1 mM-disodium EDTA, resuspended in 2 ml of the same buffer and stored at -20 °C.

Preparation of cell-free extracts. Organisms were disrupted by 15 s ultrasonication using a Branson Electronics Sonifier equipped with a 1.2 cm Heat Systems Electronics probe. During this and all subsequent operations the extracts were maintained between 0° and 5 °C. Whole cells and membranes were removed from the preparations by centrifugation for 20 min at 15000*g*. The supernatant liquid was used as the crude extract for the analysis of enzyme activities. Sonic extractions and enzyme assays were performed on the same day.

Enzyme assays. Published methods were used for the assay of catechol 1,2-oxygenase (EC. 1.99.2.2) (Hegeman, 1966), *cis,cis*-muconate-lactonizing enzyme (EC. 5.5.1.1) (Ornston, 1966*b*), (+) muconolactone isomerase (Ornston, 1966*b*), protocatechuate 3,4-oxygenase (Stanier & Ingraham, 1954), β -carboxy-*cis,cis*-muconate-lactonizing enzyme (Ornston, 1966*a*), γ -carboxymuconolactone decarboxylase (Ornston, 1966*a*) and β -ketoadipate enol-lactone hydrolase (Ornston, 1966*a*). Protocatechuate 4,5-oxygenase, detected by the method of Ono, Nozaki & Hayaishi (1970), was present in very low levels in extracts of protocatechuate-grown organisms and was evidently destroyed by the extraction procedure. The technique of Hosokawa (described in Stanier *et al.* 1966) provided a reliable qualitative method for determination of protocatechuate 4,5-oxygenase in whole bacteria. All enzyme assays were performed at 22 °C in 3.0 ml silica cuvettes with a 1.0 cm light path. Changes in absorbance were measured with a Gilford 2400 recording spectrophotometer. A unit of enzyme is defined as the amount necessary to remove 1.0 μ mol of substrate/1 min under the conditions of assay. Protein concentrations were determined with biuret reagent (Weichselbaum, 1946).

Chemicals. Protocatechuate, *p*-hydroxybenzoate and catechol were purchased from Sigma Chemical Co., St Louis, Missouri. Chemical syntheses were used to prepare *cis,cis*-muconic acid (Elvidge *et al.* 1950) and β -ketoadipate (Riegel & Lilienfeld, 1954). β -Carboxy-*cis,cis*-muconate was synthesized enzymatically by the procedure of Meagher, McCorkle, Ornston & Ornston (1972) and (+) muconolactone was synthesized enzymatically by the method of Ornston & Stanier (1966). β -Carboxy-*cis,cis*-muconate was measured as the amount of

material absorbing at 270 nm that was removed by purified β -carboxy-*cis,cis*-muconate lactonizing enzyme (MacDonald, Stanier & Ingraham, 1954). Purified enzymes used in the assay procedures were prepared as described by Ornston (1966*a, b*).

RESULTS

*The chemical stability of β -carboxy-*cis,cis*-muconate*

In neutral solution the half-life of β -carboxy-*cis,cis*-muconate is 3 min at 100 °C, and 11 h at 45 °C; the compound is transformed into the chemically stable *cis,trans* isomer which is not a substrate for β -carboxy-*cis,cis*-muconate lactonizing enzyme (MacDonald *et al.* 1954). The data of MacDonald *et al.* (1954) indicate that the activation energy for the isomerization is high (22.6 Kcal/mol) and the Arrhenius equation predicts that the half-life of the *cis,cis* isomer is 3 days at 30 °C, 3 weeks at 15 °C and 3 months at 5 °C. By measuring the rate of disappearance of β -carboxy-*cis,cis*-muconate from neutral solutions, we have confirmed that this is indeed the case. Therefore it is possible for substantial quantities of β -carboxy-*cis,cis*-muconate to accumulate in the natural environment at low temperatures.

*Growth of Pseudomonas acidovorans and P. testosteroni strains at the expense of β -carboxy-*cis,cis*-muconate*

All six strains studied (three of *Pseudomonas acidovorans* and three of *P. testosteroni*) grew immediately and rapidly at the expense of β -carboxy-*cis,cis*-muconate: in exponential growth the doubling time for different strains ranged from 60 to 80 min. As reported by Robert-Gero, Poiret & Stanier (1969) all six strains grew at the expense of *cis,cis*-muconate.

Patterns of induction in Pseudomonas acidovorans

The inductive response of *Pseudomonas acidovorans* strain 14 to growth at the expense of intermediates in the β -ketoadipate pathway is described in Table 1. The inducible nature of the enzymes is demonstrated by their low activities in succinate-grown bacteria: β -ketoadipate enol-lactone hydrolase was the only enzyme studied that was present at detectable levels (Table 1). As shown in Table 1, a qualitative test indicated that protocatechuate elicited the synthesis of the enzymes of the *meta* cleavage pathway; none of the enzymes of the *ortho* pathway was induced by protocatechuate, confirming that the metabolism of this diphenol in *P. acidovorans* proceeds exclusively by the *meta* mechanism. As is the case in a permeability mutant strain of *P. putida* (Meagher *et al.* 1972), growth of *P. acidovorans* strain 14 with β -carboxy-*cis,cis*-muconate specifically induced the three enzymes that convert this compound to β -ketoadipate, but did not induce *cis,cis*-muconate lactonizing enzyme or muconolactone isomerase (Table 1). After growth on *cis,cis*-muconate, *P. acidovorans* strain 14 contained induced levels of the three enzymes that give rise to β -ketoadipate from *cis,cis*-muconate, but undetectable levels of β carboxy-*cis, cis*-muconate lactonizing enzyme and γ -carboxymuconolactone decarboxylase (Table 1). Since the last two enzymes are induced by growth with β -carboxy-*cis,cis*-muconate but not by growth with *cis,cis*-muconate, it is evident that either β -carboxy-*cis,cis*-muconate or γ -carboxymuconolactone is the inducer. In this respect *P. acidovorans* differs markedly from *P. putida* (Ornston, 1966*c*), *P. aeruginosa* (Ornston, 1966*c*; Kemp & Hegeman, 1968), and *P. cepacia* (= *P. multivorans*) (Ornston, 1966*c*) in which during growth with *cis,cis*-muconate, β -ketoadipate gratuitously induces β -carboxy-*cis,cis*-muconate lactonizing enzyme and γ -carboxymuconolactone decarboxylase. That β -ketoadipate does not serve as a product inducer of these two enzymes in *P. acidovorans* was evident from the fact that their activities were not detectable in β -ketoadipate-grown bacteria (Table 1).

Table 1. *The influence of growth substrate on the specific activity of enzymes in extracts of Pseudomonas acidovorans (strain 14)*

Protocatechuate 4,5-oxygenase was measured qualitatively in whole organisms. Induction of an enzyme is indicated by the symbol (+) when the specific activity of the enzyme exceeded by at least threefold the value found in extracts of uninduced bacteria, symbolized by (—).

Growth substrate	Specific activity (units/mg protein)				
	Proto-catechuate 4-5-oxygenase (<i>meta</i> cleavage)	Protocatechuate 3,4-oxygenase (<i>ortho</i> cleavage)	β -Carboxy- <i>cis,cis</i> -muconate lactonizing enzyme	γ -Carboxymuconolactone decarboxylase	β -Ketoadipate enol-lactone hydrolase
Succinate	(—)	< 0.02 (—)	< 0.004 (—)	< 0.04 (—)	0.04 (—)
Protocatechuate	(+)	< 0.02 (—)	< 0.004 (—)	< 0.04 (—)	0.04 (—)
β -Carboxy- <i>cis,cis</i> -muconate	(—)	< 0.02 (—)	0.83 (+)	2.94 (+)	0.13 (+)
<i>cis,cis</i> -Muconate	(—)	< 0.02 (—)	< 0.004 (—)	< 0.04 (—)	0.18 (+)
β -Ketoadipate	(—)	< 0.02 (—)	< 0.004 (—)	< 0.04 (—)	0.04 (—)
				<i>cis,cis</i> -Muconate lactonizing enzyme	Muconolactone isomerase (+)
				< 0.002 (—)	< 0.02 (—)
				< 0.002 (—)	< 0.02 (—)
				< 0.002 (—)	< 0.02 (—)
				0.25 (+)	0.80 (+)
				< 0.002 (—)	< 0.02 (—)

After growth of *Pseudomonas acidovorans* strain 14 with β -ketoadipate, the level of enol-lactone hydrolase was identical with that in succinate-grown bacteria (0.04 units/mg protein, Table 1). This indicates that the hydrolase was not induced by β -ketoadipate and thus appears to contradict the conclusion of Robert-Gero *et al.* (1969) that β -ketoadipate induced enol-lactone hydrolase in *P. acidovorans*. The basis of the discrepancy lies not so much in the level of enol-lactone hydrolase in extracts of β -ketoadipate-grown bacteria (Robert-Gero *et al.* (1969) reported a specific activity of 0.06 unit/mg protein) but in the specific activity of the enzyme in succinate grown cultures: whereas we observed a rather high basal level of the enzyme (0.04 unit/mg protein), Robert-Gero *et al.* (1969) found it to be undetectable (< 0.01 unit/mg protein). This difference might be attributed to the fact that we examined strain 14 of *P. acidovorans* rather than the strain 13 studied by Robert-Gero *et al.* (1969), but we do not believe this to be the sole explanation. We found the levels of enol-lactone hydrolase in succinate-grown cultures of *P. acidovorans* strains 24 and 102 to be comparable to those in strain 14; in our studies and in those of Robert-Gero *et al.* (1969) specific activity of the hydrolase in fully induced cultures of *P. acidovorans* never exceeded 0.20 unit/mg protein, i.e. only 20 % of the level in fully induced cultures of *P. testosteroni* (Table 2). Thus it appears that induction of the hydrolase may have been obscured by inadequate procedures of extraction or assay. Until these experimental obstacles are removed, no conclusion can be drawn about the inducer of β -ketoadipate enol-lactone hydrolase in *P. acidovorans*.

Patterns of induction in Pseudomonas testosteroni

In many respects the inductive patterns in *Pseudomonas testosteroni* resemble those in *P. acidovorans*. As shown in Table 2, the enzymes of the β -ketoadipate pathway are not induced by protocatechuate, which is cleaved by the inducible protocatechuate 4,5-oxygenase of the *meta* pathway. As with *P. acidovorans*, growth of *P. testosteroni* with β -carboxy-*cis,cis*-muconate induces the three enzymes that convert it to β -ketoadipate (Table 2). The level of β -carboxy-*cis,cis*-muconate-lactonizing enzyme in these extracts is low, reflecting its probable destruction during the preparation of extracts, but nevertheless the induction of the enzyme is clearly indicated by the increase of its specific activity to a level over 10 times that found in uninduced cells. *Pseudomonas testosteroni* resembles *P. acidovorans* in that growth with *cis,cis*-muconate did not induce β -carboxy-*cis,cis*-muconate-lactonizing enzyme or γ -carboxymuconolactone decarboxylase.

Remarkably, growth of *Pseudomonas testosteroni* with β -carboxy-*cis,cis*-muconate induced the gratuitous synthesis of muconolactone isomerase. In this respect *P. testosteroni* is quite unlike *P. acidovorans* or, for that matter, any other bacterium examined, except *Nocardia opaca* (Rann & Cain, 1969). Growth with β -ketoadipate induced both the isomerase and enol-lactone hydrolase in *P. testosteroni*. Since the hydrolytic reaction that gives rise to β -ketoadipate is highly exergonic, it is probable that the inducer of both muconolactone isomerase and β -ketoadipate enol-lactone hydrolase in *P. testosteroni* is β -ketoadipate (or β -ketoadipyl CoA). Of the growth substrates examined, only *cis,cis*-muconate elicited synthesis of *cis,cis*-muconate lactonizing enzyme (Table 2). Thus the inducer of this enzyme appears to be either *cis,cis*-muconate or (+) muconolactone.

The inductive controls in *Pseudomonas testosteroni* strains 79 and 138 do not differ from those in strain 16. Therefore the regulatory pattern summarized in Table 2, including the extraordinary induction of muconolactone isomerase by β -ketoadipate (or β -ketoadipyl CoA), appears to be characteristic of the species.

Table 2. *The influence of growth substrate on the specific activity of enzymes in extracts of Pseudomonas testosteroni (strain 16)*

Protocatechuate 4,5-oxygenase was measured qualitatively in whole bacteria. Induction of an enzyme is indicated in the symbol (+) when the specific activity of the enzyme exceeded by at least threefold the value found in extracts of uninduced bacteria, symbolized by (—).

Growth substrate	Specific activity (units/mg protein)					
	Proto-catechuate 4,5-oxygenase (<i>meta</i> cleavage)	Protocatechuate 3,4-oxygenase (<i>ortho</i> cleavage)	β -Carboxy- <i>cis,cis</i> muconate lactonizing enzyme	γ -Carboxymuconolactone decarboxylase	β -Ketoadipate enol-lactone hydrolase	<i>cis,cis</i> -muconate lactonizing enzyme
Succinate	(—)	< 0.02 (—)	< 0.004 (—)	< 0.04 (—)	< 0.01 (—)	< 0.002 (—)
Protocatechuate	(+)	< 0.02 (—)	< 0.004 (—)	< 0.04 (—)	< 0.01 (—)	< 0.002 (—)
β -Carboxy- <i>cis,cis</i> -muconate	(—)	< 0.02 (—)	0.04 (+)	0.90 (+)	0.80 (+)	< 0.002 (—)
<i>cis,cis</i> -Muconate	(—)	< 0.02 (—)	< 0.004 (—)	< 0.04 (—)	1.00 (+)	0.20 (+)
β -Ketoadipate	(—)	< 0.02 (—)	< 0.004 (—)	< 0.04 (—)	0.28 (+)	< 0.002 (—)
						Muconolactone isomerase
						< 0.02 (—)
						< 0.02 (—)
						1.50 (+)
						1.00 (+)
						1.03 (+)

DISCUSSION

Most of the intermediates in the β -ketoadipate pathway do not readily permeate the membrane of wild-type strains of *Pseudomonas putida* (Ornston, 1966c), *P. aeruginosa* (Kemp & Hegeman, 1968), *Acinetobacter calcoaceticus* (Cánovas & Stanier, 1967), *Alcaligenes eutrophus* (Johnson & Stanier, 1971b) or *Nocardia opaca* (Rann & Cain, 1969). For this reason it has been generally believed that the biological role of the β -ketoadipate pathway was restricted to the dissimilation of aromatic compounds (Ornston & Stanier, 1966). That this is not the case was first demonstrated by Robert-Gero *et al.* (1969) who isolated cultures of *P. acidovorans* by enrichment with *cis,cis*-muconate or *cis,trans*-muconate as sole carbon and energy source. A survey of previously isolated representatives of the acidovorans *Pseudomonas* group revealed that they grew well with the *cis* isomers of muconate and that they metabolized these compounds via β -ketoadipate (Robert-Gero *et al.* 1969). Robert-Gero *et al.* (1969) concluded that the primary physiological role of the muconate branch of the β -ketoadipate pathway in the acidovorans *Pseudomonas* group is to permit the utilization of muconic acids as growth substrates. Robert-Gero *et al.* (1969) also noted that representatives of the acidovorans group are freely permeable to *cis, cis*-muconate and that in this respect they differ markedly from any other *Pseudomonas* species.

Like *cis,cis*-muconate, β -carboxy-*cis,cis*-muconate does not permeate the membrane of most bacteria (Ornston, 1966c). For this reason, and because of its chemical instability, it has been assumed that β -carboxy-*cis,cis*-muconate cannot serve as a growth substrate in the natural environment (Ornston & Stanier, 1966; Ornston, 1971). In fact three lines of evidence indicate that β -carboxy-*cis,cis*-muconate is a naturally occurring growth substrate. First, the rate of isomerization of the compound is low at the temperatures that prevail in the soil. Secondly, permeability mutant strains of *Pseudomonas putida*, selected for growth at the expense of β -carboxy-*cis,cis*-muconate, possess an inducible uptake system for the compound (Meagher *et al.* 1972). This suggests that natural selection has endowed the fluorescent pseudomonads with the genetic capability to grow at the expense of β -carboxy-*cis,cis*-muconate. Thirdly, the six representatives of the acidovorans *Pseudomonas* group that we examined have no known mechanism for the endogenous formation of β -carboxy-*cis,cis*-muconate, but nevertheless possess all of the genetic information necessary for growth at its expense. It is difficult to imagine how the metabolic potential for growth with β -carboxy-*cis, cis*-muconate could have been selected and maintained if the compound did not occur in the natural environment.

With the findings of Robert-Gero *et al.* (1969), our observations support the general conclusion that the physiological function of the β -ketoadipate pathway in the acidovorans *Pseudomonas* group is to permit growth with muconic and carboxymuconic acids, rather than with the aromatic precursors of these compounds. This property distinguished the acidovorans group from other *Pseudomonas* species which appear to use the β -ketoadipate pathway primarily for the utilization of aromatic growth substrates. The basis for the nutritional difference lies in the permeability of the members of the acidovorans group to the polycarboxylic acids of the β -ketoadipate pathway. Representatives of the acidovorans group may also be distinguished from other *Pseudomonas* species by their pathways of aromatic hydroxylation (Stanier *et al.* 1966; Wheelis *et al.* 1967) and their mechanism of malate oxidation (Francis, Hughes, Kornberg & Phizackerly, 1963; Stanier *et al.* 1966; Tiwari & Campbell, 1969). Since both transport mechanisms and electron transport systems are often intimately associated with the membrane, it seems possible that a fundamental step in the

evolution of the acidovorans group was the acquisition of genetic determinants for a distinctive membrane structure.

One novel property serves to distinguish the regulatory mechanism used to govern the β -ketoadipate enzymes in the acidovorans group from those of other bacteria: in *Pseudomonas acidovorans* and *P. testosteroni* neither protocatechuate nor β -ketoadipate serves as an inducer of β -carboxy-*cis,cis*-muconate lactonizing enzyme and γ -carboxymuconolactone decarboxylase. The inducer in the acidovorans group appears to be either β -carboxy-*cis,cis*-muconate or γ -carboxymuconolactone. In accord with comparative studies of the means of regulation of the β -ketoadipate pathway in other bacteria (Cánovas *et al.* 1967), the unique inductive control in the acidovorans group may be taken as evidence of the evolutionary isolation of these bacteria, a conclusion that is supported by a considerable amount of biochemical evidence (Stanier *et al.* 1966; Palleroni, Ballard, Ralston & Doudoroff, 1972). A more remarkable disparity in control mechanisms is evidenced by *P. testosteroni* and *P. acidovorans*; β -ketoadipate is an inducer of muconolactone isomerase in the former but not the latter species. The two species share many nutritional properties and in this respect appear to be closely related (Stanier *et al.* 1966). On the other hand, the difference in the guanine + cytosine content of their DNA is 5 %, indicating that there is a considerable evolutionary distance between them (Mandel, 1966). At least with regard to the portion of the genome associated with the utilization of *cis,cis*-muconate, the latter conclusion appears justified.

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