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Comparative Immunological Studies of Two *Pseudomonas* Enzymes

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Crystalline preparations of muconate lactonizing enzyme and muconolactone isomerase, two inducible enzymes that catalyze successive steps in the catechol branch of the β-ketoadipate pathway, were used to prepare antisera. Both enzymes were isolated from a strain of *Pseudomonas putida* biotype A. The antisera did not cross-react with enzymes of the same bacterial strain that catalyze the chemically analogous steps in the protocatechuate branch of the β-ketoadipate pathway, carboxymuconate lactonizing enzyme and carboxymuconolactone decarboxylase. The antisera gave heterologous cross-reactions of varying intensities with the muconate lactonizing enzymes and muconolactone isomerases of *P. putida* biotype B, *P. aeruginosa*, *P. stutzeri*, and all biotypes of *P. fluorescens*, but did not cross-react with the isofunctional enzymes of *P. acidovorans*, of *P. multivorans*, and of two bacterial species that belong to other genera. The evolutionary and taxonomic implications of the findings are discussed.

The β-ketoadipate pathway (Fig. 1) serves for the dissimilation of aromatic and hydroaromatic compounds by members of many groups of aerobic bacteria. The two diphenolic intermediates of the pathway, catechol and protocatechuic acid, are converted through parallel, chemically analogous sequences to a common intermediate, the enol-lactone of β-ketoadipic acid. The enzymology (10, 18, 19) and regulation (10, 20) of this segment of the pathway have been studied in detail in *Pseudomonas* species (Fig. 2). The three step-reactions of each sequence—ring opening, lactonization, and isomerization—are mediated by enzymes specific for that sequence, and the analogous enzymes of the two sequences are subject to separate regulatory control.

The two lactonizing enzymes and two isomerases of *P. putida* were extensively purified by Ornston (18, 19), who showed that the members of each analogous pair had similar molecular weights, thermostabilities, and *p*H optima. These findings suggested that the lactonizing enzymes and the isomerases might each be homologous pairs of proteins, which had arisen through gene duplication and subsequent evolutionary divergence. One test of this hypothesis is to search for possible immunological resemblances between the members of each analogous enzyme pair. To this end, we prepared antisera, using crystalline muconate lactonizing enzyme (MLE) and crystalline muconolactone isomerase (MI) from *P. putida* as immunizing antigens. These antisera have also enabled us to make a comparative study of the immunological properties of MLE and MI in other species of bacteria that synthesize the enzymatic machinery of the β-ketoadipate pathway. A preliminary account of this work has been published (23).

MATERIALS AND METHODS

Preparation of antisera. The immunizing antigens consisted of crystalline MLE and MI, isolated from *P. putida* strain 90 (19). They were stored frozen in 5 mM phosphate buffer (*p*H 6.8) containing 5 μM ethylenediaminetetraacetate (EDTA) prior to use. Each enzyme was mixed with an equal weight of methylated bovine serum albumin (15).

Antisera were prepared in male New Zealand white rabbits 3 months old. Each animal received a series of three or four intradermal injections over a period of 4 weeks, followed, after 2 weeks, by three intravenous injections at 2-day intervals. Material for the intradermal injections was emulsified in an equal volume of Freund's adjuvant (Difco), in some cases supplemented with lyophilized, phenol-killed BCG cells (4 mg/ml). The total amount of MI administered to each rabbit was 700 μg (220 μg intradermally, 480 μg intravenously). The total amount of MLE administered to each rabbit was 1,050 μg (610 μg intradermally, 440 μg intravenously). Each animal was bled on several occasions 1 to 4 weeks after the last intravenous injection, and the sera from these successive bleedings were pooled. Each of the two pooled anti-

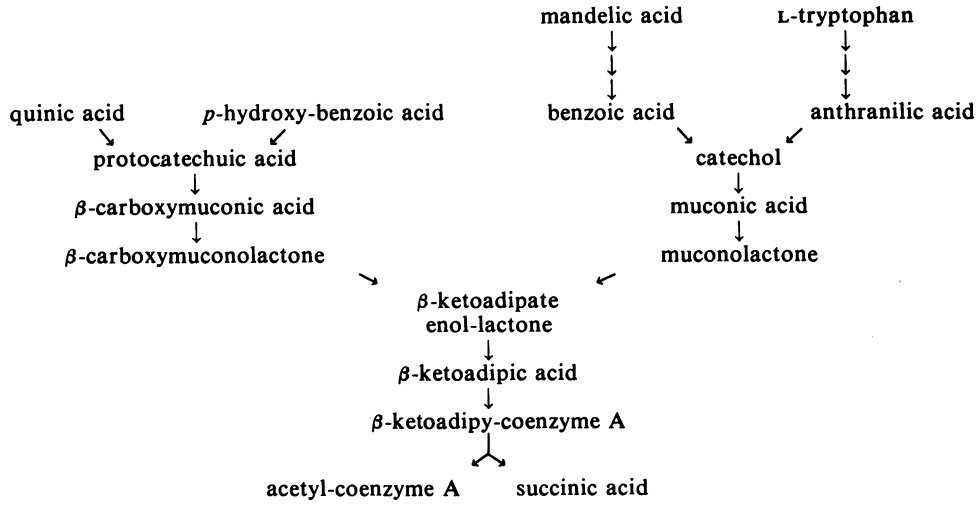

FIG. 1. *β-Ketoadipate pathway.*

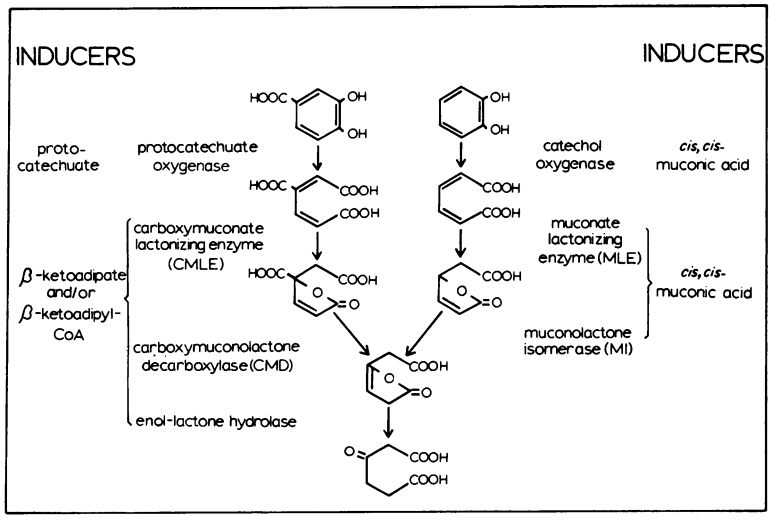

FIG. 2. *Regulation in Pseudomonas spp. of enzymes operative in the parallel, convergent sequences of the pathway leading from catechol and protocatechuic acid. Bracketed enzymes are coordinately induced.*

sera used for experiments was derived from successive bleedings of a single animal.

Preliminary immunodiffusion experiments showed that both antisera were immunologically impure. In each case, two precipitin lines formed against the homologous crystalline enzyme, as well as against a crude cell-free extract of induced (benzoate-grown) *P. putida* 90. The weaker of the two lines also formed against a crude cell-free extract of uninduced (succinate-grown) *P. putida* 90, which did not contain detectable activities of either MLE or MI. These observations suggested that the stronger precipitin line resulted from the specific enzyme-antienzyme reaction, and the weaker one from a reaction with an unknown antigen present in the purified enzyme as well as in crude extracts of both induced and uninduced cells. This second antigen is presumably a bacterial cell constituent which contaminated both immunizing antigens, despite the fact that they were crystalline and had shown only one band of protein upon starch gel electrophoresis (19).

Since an extract of succinate-grown *P. putida* 90 does not contain detectable quantities of either MLE or MI, both antisera could be purified by absorption with such an extract, without reduction of their specific antienzyme titers. The equivalence proportions between each antiserum and uninduced extract were determined by the precipitin technique (9). After appropriate absorption, each antiserum formed a single precipitin line against the homologous crystalline enzyme and against an induced extract of *P. putida* 90, but no longer formed a line against uninduced extract. These absorbed antisera were used in all experiments.

Immunodiffusion. Experiments were conducted by the Ouchterlony double-diffusion technique (25). An aqueous solution of Oxoid Ionagar No. 2 (1.7%, w/v) was diluted with an equal volume of barbital buffer, and the mixture was pipetted onto microscope slides (1 × 3 inches, 2.5 × 7.6 cm) held in an LKB immunoelectrophoresis rack. The gel was allowed to harden for at least 1 hr. An LKB gel punch (no. 6866A) was used to cut a matrix of six wells spaced at a distance of 5 mm around a center well, all wells were 3 mm in diameter. In some cases, antigen wells were enlarged, to accommodate large quantities of liquid. The barbital buffer (*p*H 8.2) contained, in 1,000 ml: 1.84 g of barbituric acid, 6.8 g of sodium acetate, 10.31 g of barbital, and 200 mg of Merthiolate.

In most experiments, the center well contained 10 μliters of undiluted, absorbed antiserum. Antigen wells furnished with cell-free extracts of induced bacteria contained approximately 5 units of MI and 1 unit of MLE, and the pure enzymes were also used at these levels. Cell-free extracts of uninduced bacteria were frequently used as controls; these antigen wells contained the same total amount of protein as the wells furnished with induced extracts of the same bacterial strain. The loaded gels were developed in a humidifier at 5 C, and were photographed after approximately 40 hr.

Microcomplement fixation. Experiments were performed by the technique of Wasserman and Levine (12, 26). Hemolysin and guinea pig complement were obtained from BBL. Sheep red blood cells in modified Alsever's solution were obtained from the Bennet Ranch, Woodland, Calif. Bovine serum albumin powder (fraction V) was obtained from Armour Pharmaceutical Co., Kankakee, Ill. All reagents were diluted in 0.01 M tris(hydroxymethyl)aminomethane (Tris)-hydrochloride buffer containing 0.1% bovine serum albumin, 0.14 M NaCl, 0.0005 M $MgSO_4$, and 0.00015 M $CaCl_2$ (final *p*H, 7.45). The reaction volume was 7 ml, and the reaction time was 16 hr at 5 C.

The standard microcomplement-fixation reaction, to which all cross-reactions are referred, was performed with one concentration of absorbed antiserum and serial dilutions of a crude extract of induced cells of *P. putida* 90, grown with benzoate. The antibody concentration of the standard curve was adjusted so that it produced a peak at approximately 80% complement fixation. Less than 0.1 μg of each enzyme was required to produce maximal fixation of complement. In performing cross-reactions with crude extracts of induced cells prepared from other bacterial strains, the antibody concentration was usually increased until a peak at the same level of complement fixation was obtained. The measure of cross-reactivity in such cases is the factor by which the antibody concentration must be raised to produce a peak of complement fixation equal in amplitude to the standard peak: this factor is termed the *index of dissimilarity* (28).

It has been shown for other proteins that the height of the peak is linearly related to the logarithm of the antibody dilution (2, 16, 22). This is also true for the two enzymes investigated here, as shown in Fig. 3 and 4. In certain cases, where the peaks in the reference and cross-reaction curves differed in height, indices of dissimilarity were calculated from this relationship; such a derivation of the index of dissimilarity is necessary when the cross-reaction is extremely weak.

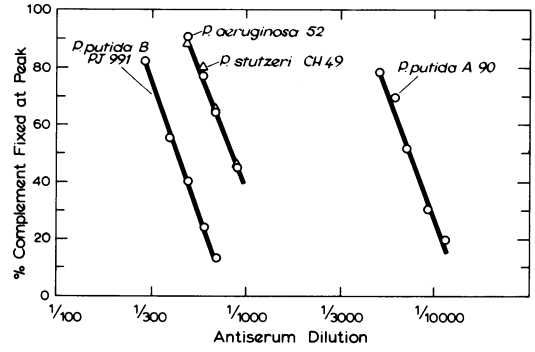

FIG. 3. *Dependence of complement fixation on the dilution of anti-MLE: data for the MLE of four different Pseudomonas spp. Serial dilutions of an extract from benzoate-grown cells were tested against different antiserum dilutions. Each point represents the peak height of a complement-fixation curve for a particular antiserum dilution.*

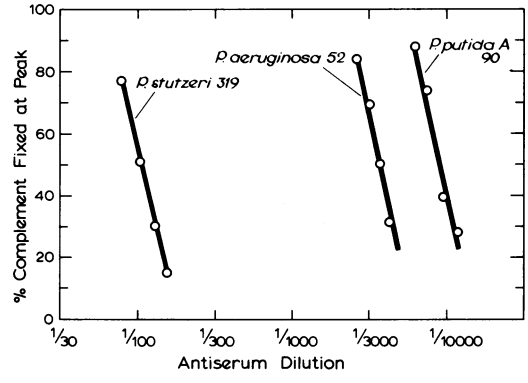

FIG. 4. *Dependence of complement fixation on the dilution of anti-MI: data for the MI of three different Pseudomonas spp. See legend to Fig. 3.*

Preliminary control experiments with anti-MLE and anti-MI showed that complement-fixation curves identical in shape and peak height could be obtained either with the pure enzymes or with crude extracts of induced cells. The degree of complement fixation is therefore not affected by the other materials present in crude cell-free extracts. In a second set of preliminary control experiments, the two absorbed antisera were tested at various dilutions with an uninduced extract of *P. putida* 90, to ascertain the range of antiserum concentrations that could be used without interference from nonspecific microcomplement fixation. It was found that anti-MI could be used at a concentration as high as 1:30, and MLE, at a concentration as high as 1:90. Since both antisera had titers of approximately 1:6,000, the largest measurable indices of dissimilarity were approximately 200 for anti-MI and 70 for anti-MLE.

Bacterial strains. Most of the *Pseudomonas* strains examined have been described by Stanier et al. (24), and will be designated here by the same strain num-

bers. Strains bearing numbers with the prefix PJ were studied by Jessen (8), and strains bearing numbers with the prefix Ch were isolated by N. J. Palleroni. The PJ and Ch strains were identified taxonomically by Palleroni (*personal communication*), using the methodology of Stanier et al. (25).

Conditions of cultivation. For the preparation of cell-free extracts, the strains were grown at 30 C in mechanically agitated liquid cultures, and were harvested during exponential growth. All media were prepared from a standard mineral base which contained, in 1,000 ml: 40 ml of 1 M Na_2HPO_4-KH_2PO_4 buffer (*p*H 6.8), 1.0 g of $(NH_4)_2SO_4$, and 20 ml of Hutner's vitamin-free mineral base (5). To this was added an appropriate carbon source (final concentration: 10 mM). Uninduced cells were grown with succinate as carbon source; induced cells, with a substrate decomposed through the reactions of the β-ketoadipate pathway. For specific induction of the enzymes of the protocatechuate branch, *p*-hydroxybenzoate was used as the growth substrate. For specific induction of enzymes of the catechol branch, benzoate was most commonly used. However, some of the strains examined could not utilize benzoate, and in such cases the enzymes of the catechol branch were induced by growth with either L-tryptophan (some strains of *P. fluorescens*) or *cis,cis*-muconate (*P. acidovorans*).

Enzymological methods. Pellets of bacterial cells harvested by centrifugation were resuspended in Tris-hydrochloride buffer (20 mM, *p*H 8.0) containing 130 mM NaCl and 0.01 mM Mg-EDTA, and were treated for 3 min at 0.5 C in a Raytheon sonic disintegrator. The extracts were then centrifuged in the cold for 20 min at 35,000 × *g* to remove coarse debris and unbroken cells, and the supernatant fluids were immediately assayed for MLE and MI activity. Thereafter, the extracts were stored at −10 C until required for immunological experiments. MLE and MI were assayed spectrophotometrically by the methods of Ornston (19). The protein content of cell-free extracts was determined by the method of Lowry et al. (13).

RESULTS

Specificities of anti-MLE and anti-MI. An immunodiffusion experiment was performed in which each antiserum was tested with five potentially cross-reacting preparations, all derived from *P. putida* strain 90: the two purified enzymes and crude, cell-free extracts from cells grown with benzoate, with *p*-hydroxybenzoate, and with succinate (Fig. 5). Precipitin lines formed between each antiserum and only two of the five potential antigens: the homologous crystalline enzyme and the crude extract of benzoate-grown cells. With each antiserum, a single sharp precipitin line was produced against each of these antigens, and the lines coalesced without spur formation. For the interpretation of these results, it is necessary to know the enzymatic constitution of *P. putida* 90 after growth with the three substrates employed (Table 1).

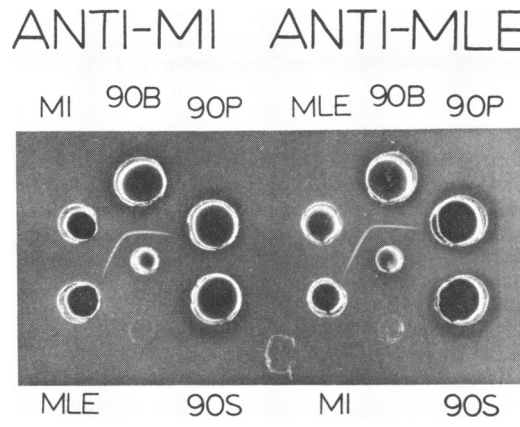

FIG. 5. *Immunodiffusion experiment demonstrating the specificity of anti-MLE and anti-MI. Anti-MI, left center well; anti-MLE, right center well. Contents of surrounding antigen wells: MI, crystalline muconolactone isomerase (5 units); MLE, crystalline muconate lactonizing enzyme (1 unit); 90B, crude extract, benzoate-grown P. putida 90; 90P, crude extract, p-hydroxybenzoate-grown P. putida 90; 90S, crude extract, succinate-grown P. putida 90.*

TABLE 1. *Specific activities of muconate lactonizing enzyme (MLE), muconolactone isomerase (MI), carboxymuconate lactonizing enzyme (CMLE), and carboxymuconolactone decarboxylase (CMD) in crude extracts of P. putida strain 90 grown with different carbon sources*[a]

Enzyme	Cells grown with		
	Succinate	Benzoate	*p*-Hydroxybenzoate
MLE	<0.002[b]	1.09	<0.002[b]
MI	<0.02[b]	0.39	<0.02[b]
CMLE	0.02	0.68	0.95
CMD	0.05	2.02	2.82

[a] Specific activities are expressed as units per milligram of protein. Data are from Ornston (20).

[b] No detectable activity; values represent the minimal specific activities that could have been detected by the assay methods used.

Since neither antiserum cross-reacts with the heterologous purified enzyme, it is evident that MLE and MI are immunologically unrelated to each other. Furthermore, neither of these enzymes is immunologically related to carboxymuconate lactonizing enzyme (CMLE) or carboxymuconolactone decarboxylase (CMD), the enzymes that catalyze chemically analogous steps in the protocatechuate branch of the pathway. As shown in Table 1, both benzoate- and *p*-

hydroxybenzoate-grown cells contain high levels of CMLE and CMD. If these enzymes were cross-reacting proteins, single precipitin lines would be formed between each antiserum and the extract of *p*-hydroxybenzoate-grown cells, and double precipitin lines between each antiserum and the extract of benzoate-grown cells. Figure 5 shows that neither expectation is met.

Experiments with heterologous systems. The two antisera were used to explore the immunological relationships of the MLE and MI of *P. putida* 90 to the isofunctional enzymes of other strains and species. The disposition of immunodiffusion experiments, shown in Fig. 6, permitted a determination on one slide of the cross-reactivity of both MLE and MI from two heterologous strains. The two central wells contained anti-MI (left) and anti-MLE (right). The three upper antigen wells adjacent to each antibody well contained crude cell-free extracts of induced cells of the homologous strain (center) and of two heterologous strains (left and right). The corresponding lower antigen wells contained extracts of uninduced (succinate-grown) cells of the same three strains. The three antigen wells furnished with induced extracts contained approximately the same amounts of MLE (1 unit) and of MI (5 units). Each well furnished with uninduced extract contained the same amount of protein as the corresponding well furnished with induced extract.

The extract from uninduced cells served to control the specificity of a heterologous cross-reaction observed with the extract from induced cells. Since MLE and MI are strictly inducible enzymes in all bacteria so far examined, cross-reactions attributable to these enzymes should occur only with extracts prepared from cells grown under conditions known to elicit their synthesis. As can be seen in the representative experiments portrayed in Fig. 7–11, no cross-reactions occurred between the antisera and extracts from succinate-grown cells of any strains examined.

Immunodiffusion experiments with other strains of P. putida. In their taxonomic treatment of the aerobic pseudomonads, Stanier et al. (24) assigned nearly all fluorescent strains that were gelatinase-negative and did not give an egg yolk reaction to one species, *P. putida*. The species was subdivided on the basis of minor nutritional differences into two biotypes, A and B. The major biotype, A, includes *P. putida* 90. Mandel (14) found that the two biotypes differ slightly but significantly in mean deoxyribonucleic acid (DNA) base composition as measured by buoyant density. He obtained values of 62.5 ± 0.9 moles per cent guanine plus cytosine for six strains

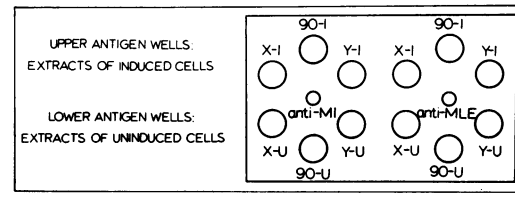

FIG. 6. *Topology of comparative immunodiffusion experiments, specific examples of which are shown in Fig. 7–11. Antigen wells designated X and Y contained extracts of two different heterologous strains; the antigen wells designated 90 contained extracts of the homologous strain (P. putida 90). The suffix I (e.g., X-I) indicates an extract prepared from induced cells, the suffix U (e.g., X-U), an extract prepared from uninduced cells.*

of biotype A, and 60.7 ± 1.1 for eight strains of biotype B.

Extracts of 14 strains of biotype A which had been studied by Stanier et al., together with a few strains received from other laboratories and taxonomically identified by us as representatives of this biotype, were tested with both antisera. All gave immunodiffusion patterns which indicated identity or near identity of their MLE and MI with the enzymes of strain 90. At most, a small spur directed towards the heterologous antigen well was detected with one or both of the antisera (Fig. 7).

Extracts of eight strains of biotype B showed an entirely different immunodiffusion pattern. A markedly heterologous response (heavy spurring) occurred with anti-MLE. There was either no cross-reaction or a very diffuse, barely perceptible one with anti-MI (Fig. 8).

Immunodiffusion experiments with strains of other fluorescent Pseudomonas species. Four strains of *P. aeruginosa* showed heterologous cross-reactions of considerable strength with both antisera. Spurring was always much heavier with anti-MLE than with anti-MI (Fig. 9).

Stanier et al. (24) placed all gelatin-liquefying fluorescent pseudomonads except *P. aeruginosa* in one species, *P. fluorescens*. Within this species, five distinct and well-defined major biotypes (A to E) could be recognized. Of these, biotypes D and E are phenazine-producers, which had previously been accorded specific status as *P. chlororaphis* and *P. aureofaciens*, respectively. Extracts from at least three representative strains of each biotype were examined, and all showed grossly similar immunodiffusion patterns, very similar to that characteristic of *P. putida* biotype B. There is a relatively strong heterologous reaction with anti-MLE, though accompanied by a heavy spur, and there is a very weak and

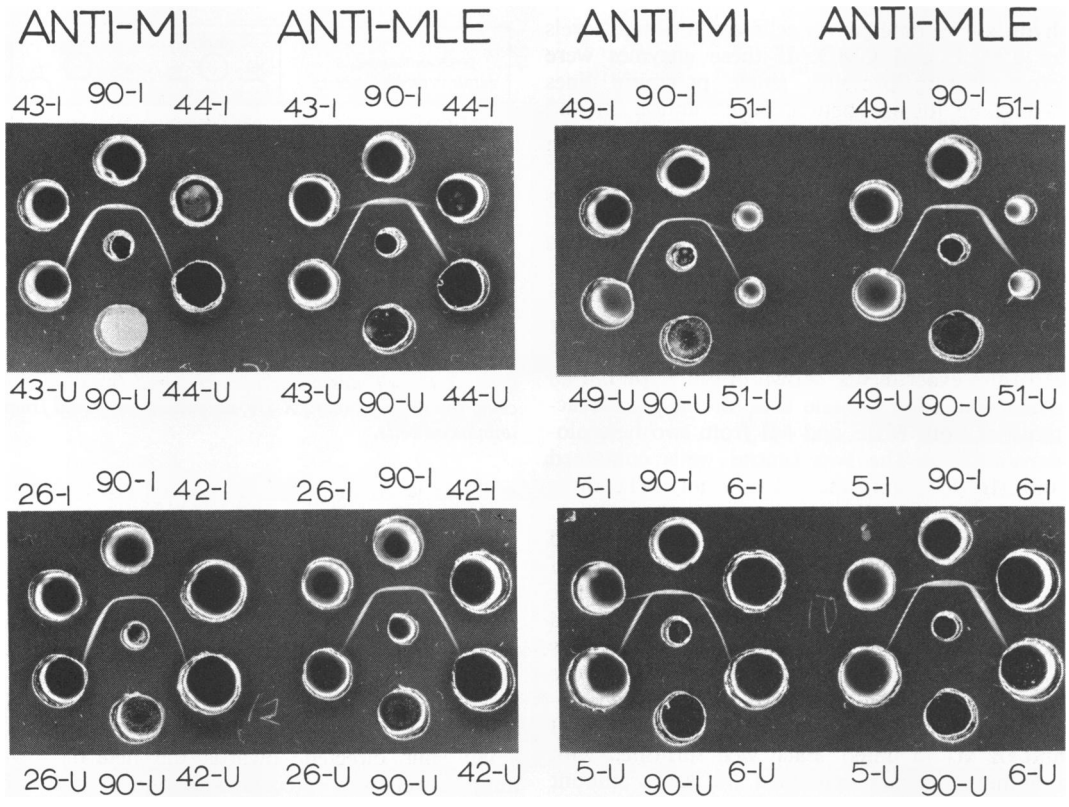

FIG. 7. *Comparative immunodiffusion experiments with extracts of eight other strains of P. putida biotype A; for key, see Fig. 6. Extracts from induced cells from some strains do not spur against either antiserum, whereas extracts of others form weak spurs against one or both of the antisera.*

diffuse reaction (often barely detectable) with anti-MI (Fig. 10).

Immunodiffusion experiments with other species. The nonfluorescent pseudomonad *P. stutzeri*, of which five strains were examined, contains MLE and MI that cross-react with the antisera (Fig. 11). Extracts of single strains of two other *Pseudomonas* species that synthesize MLE and MI—*P. multivorans* and *P. acidovorans*—did not cross-react with either antiserum. *P. acidovorans* is markedly different in both nutritional and biochemical respects from the fluorescent group: it does not use the β-ketoadipate pathway for the dissimilation of aromatic compounds, but metabolizes muconic acids via the catechol branch (21).

No cross-reactions with either antiserum were obtained with extracts from single strains of two bacteria belonging to other genera in which the β-ketoadipate pathway serves for the dissimilation of aromatic compounds: *Acinetobacter calcoaceticus* (3) and *Alcaligenes* (*Hydrogenomonas*) *eutrophus* (6).

Microcomplement-fixation experiments. Quantitative measurements of cross-reactivity were made with extracts of selected strains representative of all cross-reacting species and biotypes. The results, expressed as indices of dissimilarity, are shown in Table 2.

With eight other strains of *P. putida* biotype A, the indices of dissimilarity were in general close to unity, and two strains (49 and PJ 395) showed immunological identity with the reference strain. PJ 395, derived from the collection of Jessen (8), is a somewhat atypical representative of biotype A in terms of the taxonomic criteria proposed by Stanier et al. (24), and its assignment to this group was tentative (Palleroni, *personal communication*). The microcomplement-fixation data confirm its taxonomic position. Strain 5 gave unusually high indices of dissimilarity with both antisera (1.56 and 2.75). This particular strain was isolated over 40 years ago by den Dooren de Jong (7), and has since been maintained in culture on complex media. It may, therefore, have undergone many genetic changes.

Microcomplement-fixation data for five strains of *P. putida* biotype B confirm the marked im-

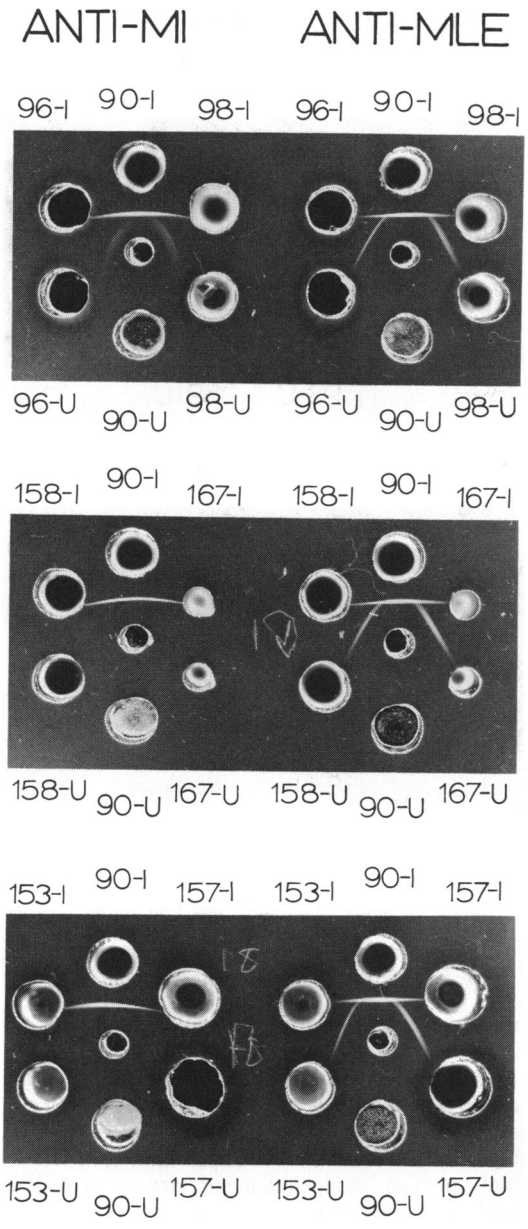

FIG. 8. *Comparative immunodiffusion experiments with extracts of six strains of P. putida biotype B; for key, see Fig. 6. Cross-reactions with anti-MI are very weak or undetectable, and cross-reactions with anti-MLE show heavy spurring.*

munological differences of its MLE and MI from those of biotype A. The indices of dissimilarity for MLE ranged from 13 to 17, and complement fixation was undetectable with anti-MI.

In three strains of *P. aeruginosa*, the MI closely resembles that of *P. putida* biotype A (index of dissimilarity, approximately 3), whereas MLE is much more distant (index of dissimilarity, 11 to 12).

Most strains of biotypes A to E of *P. fluorescens* did not show complement fixation with anti-MI, and the indices of dissimilarity for MLE were high and variable (even within one biotype). The lowest indices for MLE were found in biotypes D and E (*P. chlororaphis* and *P. aureofaciens*), and in these two biotypes very weak complement fixation with anti-MI was also consistently observed.

The MLE of *P. stutzeri* has an index of dissimilarity of approximately 10, and the MI, of more than 100. The quantitative picture is similar to that for biotypes D and E of *P. fluorescens*.

The independent taxonomic studies of Jessen (8) and Stanier et al. (24) have shown that, although *P. aeruginosa* is a remarkably uniform and easily definable species, the other fluorescent pseudomonads are not. Stanier et al. (24) were unable to assign all of the fluorescent strains that they examined to well-defined species or biotypes. A miscellany of gelatin-liquefying strains was assembled in a provisional biotype (G) of *P. fluorescens*. Several nonliquefying strains could not be placed in either biotype of *P. putida*, and were not assigned to a species. Table 2 presents microcomplement-fixation data for a few of these taxonomically indeterminate strains. The five strains of *P. fluorescens* clearly fall into two different categories: three show indices of dissimilarity comparable to those characteristic of the well-defined biotypes of this species, and two show relatively low indices (less than 7) with both antisera. The data for indeter-

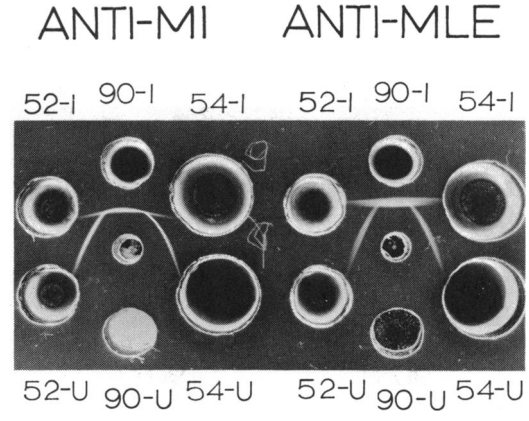

FIG. 9. *Comparative immunodiffusion experiments with extracts of two strains of P. aeruginosa; for key, see Fig. 6. The cross-reactions wtih both antisera are strong, and spurring is more pronounced with anti-MLE than with anti-MLI.*

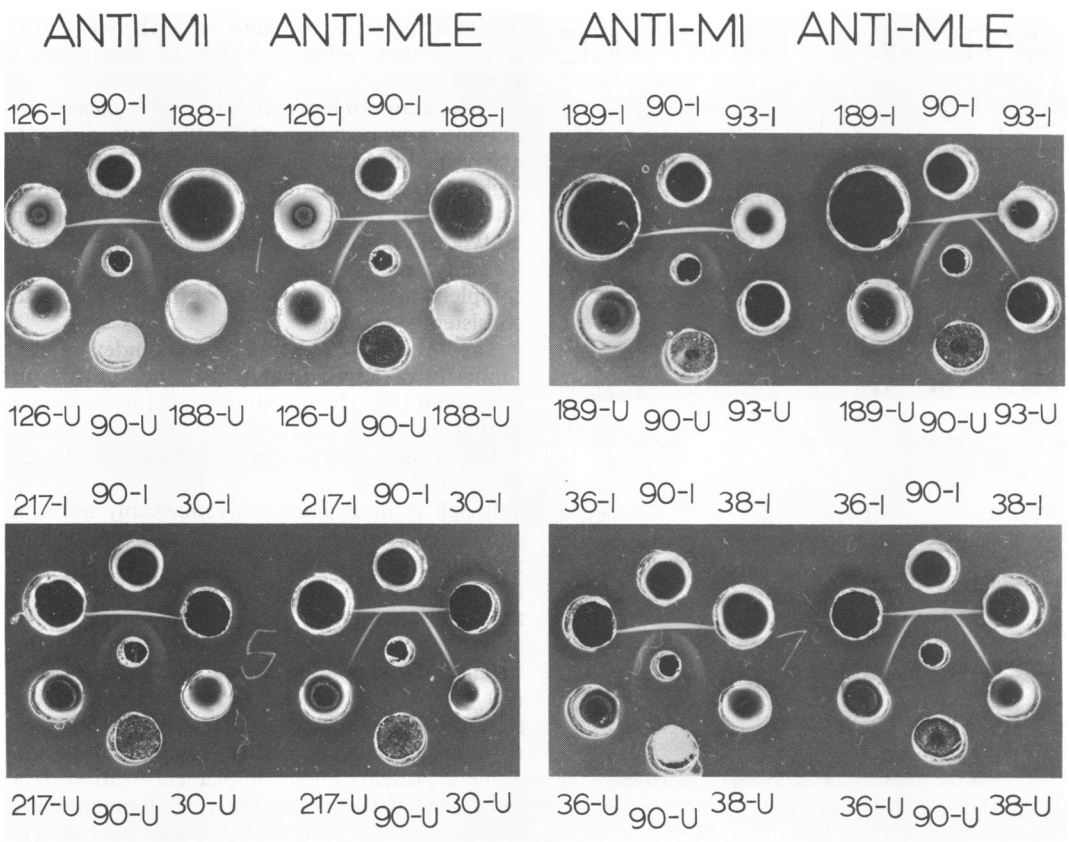

FIG. 10. *Comparative immunodiffusion experiments with extracts of strains representative of the major biotypes of P. fluorescens; for key, see Fig. 6. The biotype assignments of the strains used are: biotype A, 126, 188 and 189; biotype B, 93; biotype C, 217; biotype D (P. chlororaphis), 30, 31, and 32; biotype E (P. aureofaciens), 36 and 38. The immunodiffusion patterns of all biotypes are qualitatively indistinguishable, and very similar to that characteristic of P. putida biotype B.*

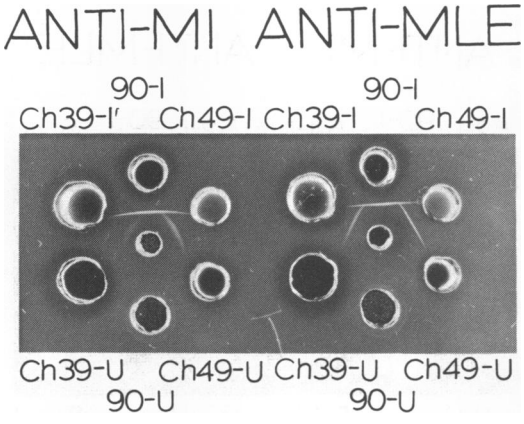

FIG. 11. *Comparative immunodiffusion experiments with extracts of two strains of P. stutzeri; for key, see Fig. 6. The immunodiffusion patterns are very similar to those of P. fluorescens and P. putida biotype B.*

minate strains that do not liquefy gelatin are also irregular. A few of these strains (e.g., 91, Ch 17, PJ 795) show indices of dissimilarity low enough to suggest that they might be aberrant members of *P. putida* biotype A, but others are immunologically remote from it. These findings accordingly provide independent support for the view that the fluorescent group (exclusive of *P. aeruginosa*) is taxonomically complex.

DISCUSSION

Evolutionary implications. Immunodiffusion experiments did not provide evidence for an immunological relationship between MLE and CMLE, or between MI and CMD, in *P. putida* strain 90. Immunological analysis, therefore, does not support the hypothesis that the enzymes of these two pairs are homologous proteins. The

TABLE 2. *Indices of dissimilarity of muconate lactonizing enzyme (MLE) and muconolactone isomerase (MI) from different Pseudomonas species and biotypes, determined by microcomplement fixation*

Taxon	Strain	Indices of dissimilarity[a]		Taxon	Strain	Indices of dissimilarity[a]	
		MLE	MI			MLE	MI
P. putida biotype A	90	1.0	1.0	*P. fluorescens* biotype C	213	60	ND
	49	0.95	1.0		214	60	ND
	PJ 395	1.0	0.98		217	34	ND
	76	—	1.15				
	130	—	1.01	*P. fluorescens* biotype D	30	10.5	>240
	43	1.45	1.20		31	11	>240
	7	1.50	1.60		32	20.5	>240
	5	1.56	2.75				
				P. fluorescens biotype E	36	10.5	>240
					38	16	>240
P. putida biotype B	96	15	ND		39	10.5	>240
	98	13	>240				
	107	17	ND				
	110	14	ND	*P. stutzeri*	319	10	100
	PJ 991	15	ND		Ch 44	10.5	ND
					Ch 49	11	120
P. aeruginosa	45	11.5	3.0				
	52	11	2.8	Taxonomically indeterminate gelatinase-positive strains ("biotype G" of *P. fluorescens*)	1	19.5	
	54	12	3.0		99	13	
					124	15	
					164	6.0	6.5
P. fluorescens biotype A	126	20	ND		171	6.3	3.1
	188	19.5	ND				
	189	12	ND				
				Taxonomically indeterminate gelatinase-negative fluorescent strains	91	1.24	1.3
					59	2.5	2.74
P. fluorescens biotype B	93	22	ND		92	7.0	4.0
	406	48	ND		322	9.4	3.0
	408	44	ND		95	16.5	ND
	413	21.5	ND		101	20	ND
					PJ 764	10	150
					PJ 943	21	ND
					Ch 17	2.25	1.28
					PJ 795	2.25	1.24

[a] The MLE and MI of *P. putida* biotype A, strain 90, were used as the immunological reference points. ND indicates that complement fixation could not be detected.

results do not, of course, disprove this hypothesis, since evolutionary divergence might have caused a complete loss of antigenic determinants shared by MLE and CMLE, and by MI and CMD.

Members of the genus *Pseudomonas* that contain MLE and MI immunologically related to those of *P. putida* include two other major fluorescent species, *P. aeruginosa* and *P. fluorescens*, as well as one nonfluorescent species, *P. stutzeri*. These findings are concordant with our unpublished data on DNA hybridization in vitro, which show a considerable degree of genetic homology among all fluorescent pseudomonads, and a much weaker homology between some fluorescent species and *P. stutzeri*. The MLE and MI of two other nonfluorescent species, *P. multivorans* and *P. acidovorans*, do not cross-react with the antisera against enzymes of *P. putida* biotype A. This finding is likewise concordant with our unpublished data on DNA hybridization, which show that *P. multivorans* and *P. acidovorans* belong to different genetic homology groups, the DNA of which does not hybridize detectably with DNA from species of the fluorescent group.

Among the cross-reacting species, the quantita-

tive immunological divergences are relatively large. Even within *P. putida* biotype A, from a strain of which the immunizing antigens were isolated, immunological homology is not complete: indices of dissimilarity can be as high as 2.75. The taxon with the closest immunological relationship to biotype A of *P. putida* is *P. aeruginosa*. These two taxa differ considerably in mean DNA base composition. Using several strains of each taxon, Mandel (14) obtained values of 62.5 ± 0.9 moles per cent guanine plus cytosine for *P. putida* biotype A, and 67.2 ± 1.1 moles per cent for *P. aeruginosa*. Hence, a significant evolutionary divergence between two bacterial species with respect to overall DNA base composition is not necessarily accompanied by a loss of immunological cross-reactivity between specific homologous proteins. This is also evident from the immunological data on the phosphatases (4, 27) and tryptophan synthetases (16) of coliform bacteria, and on the nicotinamide adenine dinucleotide-dependent lactic dehydrogenases of *Lactobacillus* spp. (F. Gasser, *personal communication*). It is possible that the small number of bacterial enzymes so far examined from the comparative immunological standpoint all happen to be proteins of which the primary structure is very strongly conserved; in this event, further comparative studies should reveal that most enzymes in these taxa do not show such striking retentions of immunological homology. However, a possible alternative explanation should not be overlooked: namely, that a large fraction of the base substitutions which have produced systematic evolutionary divergences of mean DNA base composition within a bacterial group of common descent are "silent" ones (11), and hence do not affect the structures of the corresponding proteins.

It is of some interest to compare our comparative immunological data with those for certain vertebrate enzymes. Wilson et al. (28) have made a wide-ranging study of the immunological relationships of four glycolytic enzymes in vertebrates, using the chicken as the immunological reference point. The indices of dissimilarity generally did not exceed 7 for any of these enzymes in representatives of the classes Aves and Reptilia. Indices in the general range from 10 to 100 were found in representatives of the classes Amphibia and Osteicthyes. Yet, as the present work shows, indices greater than 100 can be obtained for enzymes in different species of the bacterial genus *Pseudomonas*, and most of these species show many gross phenotypic similarities. Furthermore, the enzymes from certain *Pseudomonas* species do not cross-react at all with antisera prepared against enzymes of *P. putida*. These observations are suggestive of the enormous disparity, in genetic and evolutionary terms, between taxa of equivalent rank among vertebrates and bacteria. Microcomplement-fixation studies of alkaline phosphatase (4) and tryptophan synthetase (16) in coliform bacteria and of lactic dehydrogenase in *Lactobacillus* species (F. Gasser, *personal communication*) are consistent with the *Pseudomonas* results. Thus, there is ample support for the suggestion that the vertebrate subphylum is equivalent in terms of genetic dispersion to no more than a genus or family of bacteria.

MLE and MI are very closely associated with one another, both in function and in synthesis. They catalyze successive steps in one reaction sequence; they are synthesized coordinately (20); and, in *P. aeruginosa* (10), genetic evidence indicates that they fall under control of the same operon. Yet, they have diverged immunologically at markedly different rates in the genus *Pseudomonas*. Furthermore, the relative rates of immunological change are not uniform: in *P. aeruginosa*, MI has preserved a closer immunological relationship than MLE to the homologous enzyme of *P. putida* biotype A, whereas the converse is true of the other species and biotypes examined. These observations suggest that prudence should be used in attempting to deduce detailed evolutionary relationships among bacteria from comparative immunological evidence. The presence in several species of an isofunctional enzyme which also cross-reacts immunologically constitutes strong evidence that the species in question are related, but there is not necessarily a close correspondence between the magnitude of immunological homology for any given enzyme and interspecific evolutionary distance.

The available evidence suggests that there is in general a satisfactory correlation between degree of sequence resemblance and strength of immunological cross-reaction within a series of homologous proteins (1). Consequently, immunological methods do appear to provide reasonably reliable estimates of the degree of homology between amino acid sequences of proteins. However, the rates of evolution of the individual proteins of an organism are not necessarily identical: although there is a general tendency for amino acid sequences to evolve at a steady rate, marked fluctuations of rate can occur (11, 17).

Taxonomic implications. The serotaxonomy of bacteria has so far been based largely on comparisons of the antigenic properties of cellular surface structures: walls, capsules, and flagella. With rare exceptions, the information obtained

has not been helpful for the differentiation of specific and supraspecific taxa; its principal value has been in making infraspecific differentiations. The present work suggests that enzymes may prove to be more useful antigens for taxonomic purposes. With antisera directed against two enzymes prepared from a single strain of *P. putida* biotype A, immunodiffusion experiments differentiate three relatively large cross-reacting groups. One is the homologous (or near-homologous) group, comprising only strains of *P. putida* biotype A. Two heterologous groups are readily distinguishable: *P. aeruginosa* makes up one group, and the other is a large cluster which includes all biotypes of *P. fluorescens*, *P. stutzeri*, and *P. putida* biotype B. The members of this complex heterologous group contain MLE and MI that are very distant from the immunological reference point, and could probably be divided into several distinct groups if immunological reference points within the cluster were available. A detailed comparative study of the immunological properties of lactic dehydrogenases in the genus *Lactobacillus* (F. Gasser, *personal communication*), conducted with several immunological reference points, permitted clear-cut differentiation of many of the constituent species of this large bacterial genus.

Taxonomic circumscription of P. putida. Although biotypes A and B of *P. putida* are difficult to distinguish in terms of their nutritional properties, they can be sharply distinguished in terms of the immunological properties of their MLE and MI. Our reference strain, a representative of biotype A, is the proposed neotype of this species (24). All other strains of biotype A that we have examined contain MLE and MI that are closely related immunologically to the enzymes of the reference strain. However, the MLE and MI of strains assigned to biotype B are far more distant immunologically from the enzymes of the reference strain than are the MLE and MI of *P. aeruginosa*. Hence, we believe that the circumscription of *P. putida* proposed by Stanier et al. (24) was incorrect. Biotype B should be excluded from this species, of which the proposed neotype is a representative of biotype A. For the time being, the strains of biotype B should be placed in the limbo (already well populated!) of taxonomically indeterminate fluorescent strains.

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