JOURNAL OF BACTERIOLOGY, Mar., 1966
Vol. 91, No. 3

Synthesis of the Enzymes of the Mandelate Pathway by *Pseudomonas putida*

III. Isolation and Properties of Constitutive Mutants

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Received for publication 26 October 1965

ABSTRACT

HEGEMAN, G. D. (University of California, Berkeley). Synthesis of the enzymes of the mandelate pathway by *Pseudomonas putida*. III. Isolation and properties of constitutive mutants. J. Bacteriol. **91**:1161–1167. 1966.—Mutants of *Pseudomonas putida* constitutive for the synthesis of L(+)-mandelate dehydrogenase were obtained after mandelate- or benzoylformate-limited growth in a chemostat. When grown in media noninducing for the wild type, the mutants are capable of coordinate, constitutive synthesis of the first five enzymes of the mandelate pathway. Later enzymes of the pathway that were examined are normally repressed. The constitutive mutants have two other noteworthy properties: they are superinducible by some compounds which induce the mandelate group enzymes in the wild type, or as a result of exhaustion of the carbon and energy source of the medium in which they are grown; and they exhibit a decreased specificity of induction, being inducible by a wide range of compounds devoid of inductive function for the wild type. These results, together with other evidence indicating that the five mandelate group enzymes comprise a regulatory unit, are discussed and evaluated in the context of the general problem of the regulation of complex dissimilatory pathways.

Mutants with lesions in the control apparatus are valuable sources of information for the resolution of an inducible pathway into its component regulatory subunits. The existence of constitutive mutants, which have been found for several inducible systems, is an important part of the evidence cited for the existence of regulatory genes (4). Such mutations are pleiotropic; the effect of a single mutation is exerted simultaneously upon all enzymes of a regulatory unit.

Previous experiments with *Pseudomonas putida* (1, 2), in which gratuitous synthesis of the early enzymes of the mandelate pathway was obtained, showed that the first five enzymes (the mandelate group) are synthesized coordinately. This behavior is a necessary condition for recognition of the mandelate group as a regulatory unit, and implies that the structural genes specifying the five enzymes of the mandelate group share a repressor in common. If this is true, mutants selected for constitutive synthesis of any one of the member-enzymes of the mandelate group should also synthesize the other four members in the absence of inducer; and the synthesis of all five enzymes should be coordinate.

MATERIALS AND METHODS

Organism and methods of cultivation. P. putida A.3.12 and mutants derived from it were used. The media and general cultivation methods have been previously described (1).

Enzymological methods and extraction procedure. The assay and extraction procedures described in the first paper of this series (1) were used.

Recognition of constitutive mutants. Before the isolation of mutants constitutive for the early enzymes of the mandelate pathway could be attempted, it was essential to develop a simple qualitative test to screen colonies grown in the absence of inducer for the presence of these enzymes. L(+)-Mandelate dehydrogenase, second enzyme of the pathway, is well suited for such a test, since its activity can be detected visually by coupled dye reduction. Accordingly, the search for constitutive mutants was conducted by testing for constitutive synthesis of this particular enzyme.

A mass of cells from a colony or a patch was picked with a sterile toothpick and emulsified in a tube containing 1 ml of a mixture of: 5×10^{-3} M DL-mandelate, 10^{-5} M disodium 2,6-dichlorophenol-indophenol, 0.05 M phosphate buffer (*p*H 6.8), and 0.1% (v/v) Tween 80 (Atlas Powder Co., Wilmington, Del.). When the cells were completely suspended, one drop

of a mixture of equal volumes of toluene and carbon tetrachloride was added to destroy the permeability barriers of the cells and prevent induction during performance of the test. The tubes were examined after incubation for 1 hr at 30 C. Cells which contain L(+)-mandelate dehydrogenase quickly decolorize the dye; uninduced cells of the wild type do not substantially decolorize the dye under these conditions. Mutant *md*$^-$, which is incapable of synthesizing active L(+)-mandelate dehydrogenase (2), did not give a positive test even after growth in the presence of mandelate, and was used in each experiment as a control. Experiments with artificial mixtures of induced and uninduced cells showed that the enzymatic activity of a mixture containing a ratio of 49 uninduced cells to 1 induced cell could be distinguished from that of uninduced cells. Approximately 200 colonies or patches may be screened in 1 hr for L(+)-mandelate dehydrogenase by this method.

Mandelate racemase, the first enzyme of the mandelate group, can also be detected qualitatively by a slight modification of the procedure described above. D(−)-Mandelate replaced DL-mandelate in the modification, and the otherwise similar mixture was supplemented by addition of 0.5 mg/ml of washed particle fraction from induced cells [L(+)-mandelate dehydrogenase in excess]. Both screening procedures were essentially qualitative adaptations of the quantitative assays for the respective enzymes (1).

Design and operation of the chemostat. A simple chemostat was used to select constitutive mutants. The device consisted of a Mariotte bottle reservoir which contained the medium, a screw clamp to regulate, and a calibrated drip-tube to monitor the rate of flow. The growth vessel consisted of a wide-mouth 1-liter Erlenmeyer flask fitted with a rubber stopper. Inlet and outlet tubes were fitted into the stopper as well as a cotton-plugged glass port which served for inoculation, sampling, and as an air inlet. The outflow tube extended nearly to the bottom of the growth vessel, and was connected through a trap to a vacuum line. A pressure difference, provided by opening the vacuum line, served to draw air through the growth vessel and to remove excess culture from it by suction, thereby maintaining a constant volume in the growth vessel. The growth flask was agitated in a constant-temperature water bath (30 C) during operation.

It was established that the carbon source, at usual concentrations, was the first factor that limited growth in the basal medium. By regulating the rate of supply of fresh medium, it was possible to hold the rate of growth within narrow limits. Return to the steady state after transient disturbances caused by additions or removal of samples was rapid and without overshoot effects. Problems with contamination were infrequent.

RESULTS

It has been shown that mutants of *Escherichia coli* constitutive for the synthesis of β-galactosidase may be selected from the inducible wild-type population by continuous culture under conditions where the growth rate is limited by the inducer-substrate, lactose (3). Accordingly, an attempt to isolate mutants constitutive for the first enzymes of the mandelate pathway was undertaken by analogous methods.

The wild type was grown in the chemostat with either mandelate or benzoylformate as carbon source and rate-limiting nutrient. During operation of the chemostat, growth rates were maintained at 20 to 30% of the maximal rates obtainable with these substrates. The growth of *P. putida* in the chemostat invariably resulted after 48 to 72 hr in the development of patches of cells adhering to the walls of the growth chamber. Material from these patches and from the fluid contents of the growth chamber was periodically streaked on plates of asparagine-basal medium. After growth on the plates had occurred, individual colonies were screened for the presence of L(+)-mandelate dehydrogenase activity, indicative of the constitutive synthesis of this enzyme. Colonies which appeared constitutive on the basis of this test began to appear on plates prepared from samples removed after the chemostat had been in operation for 3 days. They were purified by restreaking, checked for their biological identity with *P. putida*, and retested for constitutive synthesis of L(+)-mandelate dehydrogenase after growth in liquid succinate-basal medium.

All the colonies which gave positive tests for the presence of L(+)-mandelate dehydrogenase after growth on asparagine-basal medium differed markedly from the wild type in colony morphology, being smaller, denser, and more self-adherent. These colonies at first occurred almost exclusively on plates prepared from patches of growth adhering to the walls of the culture vessel, although they also arose on plates streaked with the culture fluid in later samples from the chemostat. When subcultured on maintenance medium (solid yeast extract-basal medium), this morphological variant was quickly overgrown by the wild type. Cultivation on solid asparagine, succinate, or mandelate-basal medium apparently favors the variant (Fig. 1). The variant clumps badly when grown in liquid medium. It occurs in stocks of the wild type maintained on solid medium, but becomes a significant fraction of the total population only when cultures are grown in the chemostat. Although the constitutives isolated all possessed this colony form, the morphological variation is independent of constitutivity, as shown by the fact that revertants to wild-type colony form remain constitutive.

Constitutive mutants were obtained by limiting growth in the described manner with D(−)-mandelate, with DL-mandelate, and with benzoylformate. The mutant strain selected with D(−)-

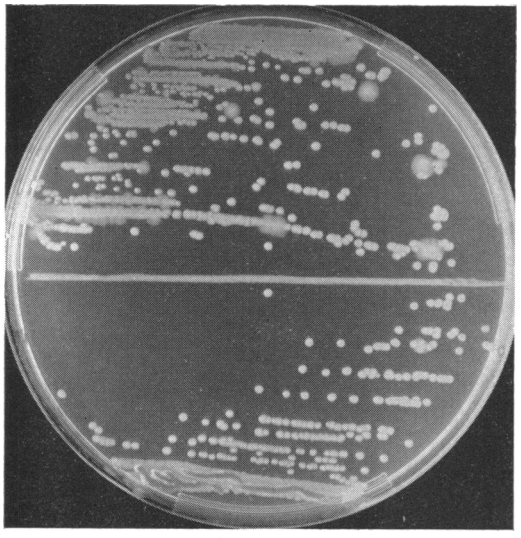

FIG. 1. *Portions of a single colony of the morphological variant were maintained through one transfer on yeast agar-basal medium and* DL-*mandelate-basal medium. Growth from each slant was resuspended and streaked on one-half of the depicted yeast agar-basal medium plate. Top: From yeast-extract-basal medium; bottom: from* DL-*mandelate-basal medium. The larger colonies have the typical wild-type morphology.*

mandelate was used in experiments described below, unless otherwise stated.

Synthesis of mandelate pathway enzymes by the constitutive mutant in the absence of inducer. The mutant was grown in basal medium with asparagine and with succinate. The cells were harvested in the exponential phase of growth, and extracts were prepared and assayed for enzymes of the mandelate pathway (Table 1). All the enzymes of the mandelate group are present in these extracts, whereas pyrocatechase and lactonizing enzyme are undetectable. All the enzymes of the mandelate group are induced to comparable levels, some 10 to 20% of their levels in fully induced wild-type cells. This represents an increase of at least 100-fold over the basal (uninduced) level characteristic of the wild type (1). Considerably higher levels occur in cells harvested from liquid cultures after entry into the stationary phase as a consequence of exhaustion of the carbon source, or in cells grown on plates of the homologous solid media. This indicates that the constitutive mutants become even further derepressed under conditions of starvation.

Specificity of induction of constitutive mutants. During the screening of aromatic compounds as inducers for the wild type (1), constitutives were sometimes included to provide a positive control. This led to the discovery that constitutives show a dramatic inductive response to many aromatic compounds, both metabolizable and nonmetabolizable, which do not detectably induce the wild type. To examine these effects more precisely, the D(−)-mandelate-derived constitutive mutant was grown in liquid asparagine-basal medium supplemented with a series of aromatic compounds which had given indications of acting as inducers in the screening tests. The cultures were harvested during exponential growth, and cell-free extracts were prepared and assayed for mandelate racemase, benzoylformate decarboxylase, and lactonizing enzyme (Table 2).

This experiment revealed several remarkable properties of the constitutive mutant. The mutant is hyperinduced by DL-mandelate: the levels of both mandelate racemase and benzoylformate decarboxylase are approximately double those characteristic of fully induced wild-type cells. This effect is confined to the enzymes of the mandelate group, as shown by the fact that in the same cells the level of lactonizing enzyme is identical with that found in mandelate-induced cells of the wild-type. Secondly, phenoxyacetate, which is a relatively weak inducer of the enzymes of the mandelate group in the wild type (1), is a considerably more powerful inducer than mandelate for the constitutive mutant. Lastly, the remarkably narrow specificity of inductive response characteristic of the wild type (1) is greatly broadened in the constitutive mutant: a number

TABLE 1. *Specific activities* of mandelate pathway enzymes in extracts of the constitutive mutant grown with noninducing substrates and in extracts of the wild type grown with* DL-*mandelate*

Enzyme	Constitutive mutant		Wild type
	Succinate	Asparagine	DL-Mandelate†
Mandelate racemase	44	50	400
L(+)-Mandelate dehydrogenase	49	73	522
Benzoylformate decarboxylase	102	78	603
NAD-benzaldehyde dehydrogenase	45	50	265
NADP-benzaldehyde dehydrogenase	24	28	152
Pyrocatechase	≦0.05	≦0.05	288
Lactonizing enzyme	≦0.05	≦0.1	485

* Expressed as millimicromoles of substrate converted per minute per milligram of extract protein.

† Values taken from Hegeman (1), Table 1.

TABLE 2. *Inductive response of a constitutive mutant to several compounds**

Compound added	Activity		
	Mandelate racemase	Benzoylformate decarboxylase	Lactonizing enzyme
None (asparagine as carbon and energy source)	17	19	0†
DL-Benzilate	23	19	0
DL-Atrolactate	91	112	0
Benzenesulfonate	285	256	0
Phenoxyacetate	365	350	0
Oxanilate	44	37	0
DL-Phenylalanine‡	—	33	0
L(−)-Tyrosine‡	—	45	0
Phenylacetate‡	—	103	0
Benzoate‡	100	75	65
DL-Mandelate‡	200	225	99

* The cells were grown in asparagine-basal medium supplemented with the compound at a concentration of 0.01 M. Enzyme activities are expressed as per cent of the specific activities found for fully induced wild-type extracts. See Table 1 for values.

† A value of zero denotes a specific activity less than or equal to the sensitivity of spectrophotometric assay.

‡ Compounds metabolized by the wild type and the mutant.

of aromatic compounds with little structural resemblance either to one another or to the natural inducers of the enzymes of the mandelate group can induce the constitutive mutant to varying degrees. The most active of these compounds is benzenesulfonate, which is a more effective inducer of the constitutive mutant than mandelate. DL-Atrolactate, phenylacetate, and benzoate are approximately half as effective as mandelate; and oxanilate, DL-phenylalanine and L(−)-tyrosine all possess slight but significant inductive capacity. The induction by benzoate is particularly surprising, since Mandelstam and Jacoby (6) showed that benzoate is a highly effective repressor of the enzymes of the mandelate group in the wild type. Less detailed studies on the benzoylformate-derived constitutive mutant indicate that it has also undergone substantial changes in inductive specificity. Accordingly, the phenomenon seems to be a characteristic feature of this particular kind of mutation to constitutivity.

Coordinateness of synthesis of enzymes of the mandelate group in the constitutive mutant. Although the data in Tables 1 and 2 show that there is a fairly constant ratio of activity between mandelate racemase and benzoylformate decarboxylase in the constitutive mutant under many different conditions of induction, a more critical test of coordinateness seemed desirable. To this end, the differential rates of synthesis of the two enzymes were determined during growth of the constitutive mutant in asparagine-basal medium alone, and in the same medium supplemented with two different inducers (Table 3). Although the differential rates of synthesis of the enzymes under the three growth conditions examined varied over a range of more than 20-fold, their relative differential rates of synthesis were almost identical in the three cultures, and corresponded closely to the relative specific activities which had been determined in other experiments (Tables 1 and 2).

DISCUSSION

Possible genetic interpretations of the constitutive mutants. In the first description of these constitutive mutants (10), their constitutivity was tentatively interpreted as the consequence of a mutation affecting the repressor substance (an *i* gene mutation, in the terminology developed to describe the β-galactosidase system; *see* 4). The partial nature of constitutivity could in these terms be interpreted as the result of an endogenous derepression by aromatic metabolites (e.g., aromatic amino acids), brought about by the broadened specificity of the repressor. It was recognized, however, that the mandelate constitutives did not have the phenotypic traits of classical i^- constitutives of the lactose operon in *E. coli* (4). Subsequently, C. D. Willson (*personal communication*) pointed out that in certain phenotypic respects they resemble much more closely the other principal class of mutants constitutive

TABLE 3. *Differential rates of synthesis* of two enzymes of the mandelate group by a constitutive mutant*

Conditions of cultivation	Enzyme	
	Mandelate racemase	Benzoylformate decarboxylase
Unsupplemented asparagine-basal medium (no inducer)	8.0	7.4
Asparagine-basal medium with 0.01 M DL-mandelate	200	193
Asparagine-basal medium with 0.01 M DL-atrolactate†	96	104

* Rate of change of activity in a culture × (growth rate)$^{-1}$. Arbitrary units.

† A compound neither active as an inducer nor metabolized by the wild type.

for the lactose operon in *E. coli*, namely, operator constitutives (o^c). Both are partially constitutive, the rates of enzyme synthesis being increased many-fold by saturating quantities of inducer (12); and both show an increase in the rate of enzyme synthesis under conditions of starvation. Unfortunately, no decision between these possible interpretations can be made until a system permitting genetic recombination in *P. putida* is developed.

Regulation of the mandelate pathway: mechanisms. The data presented here and in the two preceding papers of this series (1, 2) have shown that synthesis by *P. putida* of the first five enzymes of the mandelate pathway (the enzymes of the mandelate group) is strictly coordinate. This conclusion rests on several independent kinds of evidence: the response of the wild-type to a nonmetabolizable inducer; the responses of mutants to normal inducer-substrates which they can no longer metabolize as a result of the genetic deletion of specific enzymes of the mandelate pathway; and the properties of mutants constitutive for the synthesis of the enzymes of the mandelate group (Table 4). It seems likely that the enzymes of the mandelate group are under the genetic control of a complex operon, but a formal proof of this cannot yet be offered.

The hypothesis of strictly sequential induction first proposed to account for synthesis of the enzymes of the mandelate pathway (8) is evidently incorrect. However, the primary event of coordinate induction in the mandelate pathway equips the cell to perform only the first four reactions of the eleven required to convert D(−)-mandelate to succinate and acetyl-coenzyme A, and sequential inductions do intervene to control later events of specific enzyme synthesis. The first sequential step takes place at the level of benzoate, the product formed by successive action of the five enzymes of the mandelate group on the primary substrate. Hence, inductive control of the mandelate *pathway* is neither purely sequential nor purely coordinate, but an intricate blend of the two types of regulatory control. An analogous situation has been discovered in the convergent metabolic pathway originating with L-tryptophan (7); but here induction by a metabolite, not by the primary substrate, introduces an additional element of complexity.

The position of some sequential inductive steps in a complex, inducible metabolic pathway can be inferred with great precision from the study of constitutive mutants, and from an examination of the response of the wild type to nonmetabolizable inducers. In the present work, both kinds of evidence pinpoint benzoate as the first component of the pathway which is a sequential inducer in addition to being an intermediate. It has been found that kinetic analysis is also useful in determining the existence of sequential inductive steps, although it does not necessarily pinpoint

TABLE 4. *Patterns of induction in the mandelate pathway: wild-type, blocked mutants and constitutive mutants*

Organism and inducer*	Enzyme					
	Mandelate group				Below benzoate	
	Mandelate racemase	L(+)-Mandelate dehydrogenase	Benzoylformate decarboxylase	NAD + NADP benzaldehyde dehydrogenases	Pyrocatechase	Lactonizing enzyme
Wild-type induced with:						
DL-mandelate or benzoylformate	+	+	+	+	+	+
Benzoate	−	−	−	−	+	+
Phenoxyacetate†	+	+	+	+	−	−
rac⁻ induced with:						
L(+)-mandelate	Deleted	+	+	+	+	+
D(−)-mandelate†	Deleted	+	+	+	−	−
md⁻ induced with:						
Benzoylformate	+	Deleted	+	+	+	+
DL-Mandelate†	+	Deleted	+	+	−	−
Constitutive mutant with:						
No inducer	+	+	+	+	−	−
DL-Mandelate	+	+	+	+	+	+

* Inducers were added to asparagine-basal medium at a concentration of 0.01 M.

† Nonmetabolizable inducers; gratuitous conditions of mandelate group enzyme synthesis.

the specific site of sequential induction. When a growing population of cells is furnished with the primary inducer-substrate of the mandelate pathway, there is an immediate synthesis of enzymes directly derepressed by this compound, but a marked and readily measurable temporal lag in the synthesis of enzymes that lie below the first sequential inductive step (1). The utility of kinetic analyses in this context has also been shown by Palleroni and Stanier (7).

Mandelstam and Jacoby (6) and Stevenson and Mandelstam (11) recently described complex end-product repressions of enzymes of the mandelate pathway in our strain of *P. putida*. Although the study of this aspect of the regulatory problem was not a primary goal of the present work, it is worth noting that when they have been specifically looked for, phenomena of end-product repression have not been detected (1). The levels of the enzymes of the mandelate group are not measurably reduced in the wild type under conditions of exponential growth when either asparagine or succinate is added as an accessory carbon and energy source to a mandelate-basal medium. However, the growth conditions under which Mandelstam and his collaborators observed end-product repressions are so different from those employed here that there is not necessarily a contradiction between the two sets of findings. It should also be noted that an attempt to demonstrate feedback inhibition of several enzymes of the mandelate group by later metabolites of the mandelate pathway (Hegeman, *unpublished data*) did not reveal any substantial effects; accordingly, it is probable that feedback inhibition plays little, if any, role in the physiological regulation of the mandelate pathway.

Regulation of the mandelate pathway: evolutionary and ecological implications. In the absence of inducer, there is a very strict repression of the enzymes of the mandelate group in cells of the wild type. These enzymes are undetectable in uninduced cells by spectrophotometric assays of considerable sensitivity; and the basal levels could be measured only by the use of much more sensitive radiochemical assays. Such assays have shown that the first two enzymes of the mandelate group—mandelate racemase and L(+)-mandelate dehydrogenase—are synthesized by uninduced cells at a rate some 2,000 times less than the rate characteristic of fully induced cells. Another striking feature of the system is the high specificity of induction. Apart from the "natural" inducer-substrates (the two isomers of mandelate and the next intermediate in the pathway, benzoylformate), an extensive survey of aromatic compounds revealed only one other substance with significant inductive capacity, phenoxyacetate. Even though it is a nonmetabolizable inducer, it is far less effective than the three inducer-substrates.

These two properties of the inductive system—strict repressibility and high specificity of response—obviously have adaptive value, particularly in the control of a complex block of coordinately synthesized enzymes such as the mandelate group, which may represent a significant fraction of the total protein of the cell. [Some indication of the magnitude of protein synthesis involved is provided by the fact that benzoylformate decarboxylase, which has been crystallized (Hegeman, *unpublished data*), represents 0.4% of the total soluble protein in a fully induced wild-type cell.] Hence, it is probable that evolutionary selection has operated with considerable sharpness to maintain this system at its present level of precision. The properties of constitutive mutants are of interest in this connection. Successive mutational events could have occurred during the somewhat complex process of selection used in their isolation; and, since revertants to the wild-type phenotype have not yet been discovered, the constitutive mutants did not necessarily arise through point mutation at a single genetic locus. Nevertheless, they can be isolated by direct plating from an initially wild-type population after less than 40 generations of growth in the chemostat. Hence it is evident that in an evolutionary sense, even if not in a strictly genetic one, they are very closely related to the wild type. Yet these mutants have lost not merely the strict repressibility of the wild-type system, but also its inductive specificity.

In terms of genetic organization, the grouping of the control of enzyme synthesis into operons (4) clearly permits a reduction in operational complexity. Even in physiological terms it has significant potential advantages, provided that selection can operate on a complex regulatory unit so as to bring its component enzymes into functional balance. Once balanced, they will remain so under all environmental conditions (i.e., at all levels of induction). On the other hand, the single induction of enzymes that catalyze successive steps in a metabolic pathway introduces obvious complexities on both the genetic and the physiological levels. Yet sequential inductions evidently do have a place of some importance in the control of complex dissimilatory pathways in bacteria, and it is therefore pertinent to ask what selective value they might possess. There are two situations, one physiological and one ecological, in which the introduction of sequential control over some steps in a pathway appears to be biologically advantageous.

When two or more primary metabolic pathways converge (and many cases of this sort occur

in the bacterial metabolism of aromatic compounds), a sequential inductive control at the point of juncture provides the only means of regulation that is strictly economical in terms of protein synthesis. If coordinate control extended across the metabolic junction, this would necessarily result in synthesis of nonfunctional enzyme protein under some environmental conditions. Of course, coordinate control might nonetheless be operative in some situations of this nature, if for other reasons a cellular economy (e.g., reduction in the complexity of the control apparatus) nonfunctional protein synthesis were a biological price that the cell could afford to pay. Nevertheless, the difficulty of regulating convergent metabolic pathways with both economy and flexibility is probably one factor which has limited the extent of coordinate control.

The ecological factor is less self-evident, but may be equally important. Some (though not all) compounds which serve as metabolic intermediates in a complex dissimilatory pathway may also be able to serve as exogenous substrates (e.g., benzoate). If such compounds occur in free form in the natural environment, and are therefore on occasion available to the cell as primary substrates, it is clearly important that they possess direct inductive function. In the context of its alternate role as a metabolic intermediate, the ability of such a compound to act as an inducer would be transposed into a sequential inductive event.

ACKNOWLEDGMENTS

I am indebted to R. Y. Stanier for guidance in the course of this study, and to R. Y. Stanier and M. Doudoroff for advice in preparation of the manuscripts. Many helpful discussions with C. D. Willson are also gratefully acknowledged.

This investigation was supported by Public Health Service grant AI-1808 from the National Institute of Allergy and Infectious Diseases and by National Science Foundation grant G-11330. The experimental work was done during tenure of Public Health Service predoctoral fellowship 5-F1-GM-14,638.

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