Patterns of Enzymic Adaptation in Species of the Genus *Azotobacter*

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SUMMARY: Fourteen strains of the *Azotobacter agile*-*A. vinelandii* group were classified into one or the other of the above species by morphological, cultural and growth characteristics. The groups were then surveyed by a study of oxidative reactions of whole cell suspensions on some intermediates of the tricarboxylic acid (TCA) cycle: acetate, malate, succinate, α -ketoglutarate and citrate.

All strains grew readily in a nitrogen-free medium. Organisms that were morphologically large rods or ovoid cells produced a green water-soluble pigment in an iron-deficient medium, and did not utilize mannitol or nitrate in the absence of another carbon or nitrogen source. Strains composed of smaller rods also produced the green pigment, and would utilize mannitol and nitrate. The first group was classified as *Azotobacter agile*, and the second as *A. vinelandii*.

Q_{O_2} (N) values on the TCA substrates tested did not indicate group variation. However, 15-24 hr. cells of the *Azotobacter agile* group as a unit did oxidize succinate with a shorter lag period (not greater than 30 min.) than strains of the *A. vinelandii* group (not less than 50 min.). A similar response to malate was noted, but in contrast most strains of *A. vinelandii* oxidized α -ketoglutarate after a shorter lag than did strains of *A. agile*. Citrate oxidation was variable among strains; all organisms but *A. vinelandii* O were apparently inhibited by the chelation of magnesium (and possibly calcium) by citrate. The physiological and oxidative responses of freshly isolated strains did not differ markedly from those of laboratory cultures of the same species.

For many years now the role of physiological methods in establishing the taxonomic position of a bacterial strain has been recognized and accepted, but some investigators are often troubled by the exclusive use of what appears to them to be rather crude biochemical tests in determining an organism's 'physiology'. Undoubtedly at one time or another, most bacterial physiologists have wondered whether more modern techniques of enzyme specification could not be applied to taxonomic questions. The difficulties of doing so on the large scale demanded by the survey of hundreds of bacterial isolates are recognized to be formidable though certainly not insurmountable. For the time being, at least, the conventional methods represented by such a physiological description as 'acid and gas on lactose', for identification and classification, must remain the basic set for initial screening. But perhaps it is not too early to begin studies in which the more advanced quantitative methods are applied to special problems that have been defined through the conventional techniques.

As an example of such an approach we have recently examined some of the enzymic properties of representative strains of the *Azotobacter agile-vinelandii* group. ('Group' is used in this paper as a convenient term to include strains

labelled either *A. agile* or *A. vinelandii* without any implication as to whether the group comprises one or more species; see Discussion.) For two reasons this group appears to offer a reasonably significant test case. First, the results of traditional methods have led to disagreement as to whether one or two species should be recognized; secondly, a considerable amount of information concerned with the enzymic properties of a few strains exists. Extension of such studies to other strains might help to decide whether the few gross morphological and physiological differences observed between members of the two groups can be correlated with more subtle differences in specific enzyme properties.

METHODS

Organisms used. Fourteen strains were used in this study, nine of which had been classified previously as *Azotobacter agile* or *A. vinelandii*. A tenth strain was received unclassified from Kansas State College and identified as *A. vinelandii* by morphology and by the following growth characteristics: ability to grow readily in a nitrogen-free liquid medium with the formation of a green water-soluble pigment when this medium is deficient in iron. The remaining four strains were fresh isolates of *A. agile* as determined by cell size and shape and by the foregoing growth properties. Source and classification of all strains are listed in Table 1.

The organisms were maintained on Burk's mineral salts medium with 2% (w/v) sucrose (Wilson & Knight, 1952). A 2-3% (v/v) inoculum was introduced into 100 ml. medium in a 500 ml. Erlenmeyer flask and shaken 350 r.p.m. at 30° for 15-24 hr. Colonial characteristics were observed from growth on the same medium solidified with 1.5% agar.

Table 1. *Source of strains of azotobacter*

<i>Azotobacter agile</i>		<i>Azotobacter vinelandii</i>	
Strain	Source	Strain	Source
9040	American Type Culture Collection; R. L. Starkey, donor	7492	American Type Culture Collection; D. Burk, donor
7494	ATCC; N. R. Smith, donor	7496	ATCC; N. R. Smith, donor
4-4	Stock culture of the University of Wisconsin; originally from Dr H. A. Barker, University of California	7484	ATCC; N. R. Smith, donor
S-1	Isolated from Lake Mendota, Madison, Wisconsin by J. Schutter	O	Original strain from the University of Wisconsin
S-2	Lake Mendota, Madison, Wisconsin	3	Stock culture of the University of Wisconsin; originally from R. L. Starkey
S-3	Lake Mendota, Madison, Wisconsin	K	From T. G. G. Wilson; originally from Rothamsted Experimental Station, England
S-4	Lake Mendota, Madison, Wisconsin	K-S4	From J. O. Harris, Kansas State College

Nitrate utilization. Because Green & Wilson (1953) observed that *Azotobacter vinelandii* utilizes potassium nitrate in the absence of molecular nitrogen, while *A. agile* 4.4 does not, all strains were tested. A modification of the method of Horner & Allison (1943) was used; 125 ml. flasks each containing 15 ml. Burk's media with 2% (w/v) sucrose and 300 $\mu\text{g./ml.}$ nitrate as KNO_3 were inoculated with 0.1 ml. active culture from a nitrogen-free medium. The flasks in 200 cm. vacuum desiccators were maintained under 0.2 atm. of oxygen and 0.8 atm. of hydrogen, the latter to prevent any fixation of atmospheric nitrogen. The desiccators were shaken 100 strokes/min. on a reciprocal shaker at 30°. After 4 days the pH value was determined. Evidence of visible growth was accompanied by rise in pH value owing to disappearance of nitrate ion. This simple test was occasionally checked by actual measurement of the amount of nitrate that disappeared by the method of Nelson, Kurtz & Bray (1954).

Manometric experiments. Organisms to be tested for oxidative activity were removed at 15–24 hr. from liquid cultures by centrifugation, washed once by suspending in 10 times the cell volume of cold 0.2% (w/v) KCl solution, recentrifuged and resuspended in a fresh portion of KCl. Total nitrogen was determined by a semimicro-Kjeldahl method.

Standard manometric procedures were used to measure rate of oxygen uptake on sucrose and the tricarboxylic acid (TCA) intermediates: acetate, malate, succinate, α -ketoglutarate, and citrate. $Q_{O_2}(\text{N})$ values ($\mu\text{l.O}_2/\text{hr./mg. N}$), corrected for the low endogenous respiration, were calculated after the end of the lag period from the rate of oxygen uptake on the linear part of the curve. Unless otherwise stated, the contents of Warburg vessels were: a volume of cell suspension containing 60–100 $\mu\text{g. N}$; 80 μmole potassium phosphate buffer at pH 7.0; 2 μmole MgSO_4 ; water to make a total volume of 3.2 ml.; 40 μmole substrate in the side arm; 0.1 ml. 20% KOH in the centre well. The atmosphere was 50% O_2 plus 50% He, and the temperature was 36°. For general characteristics of oxidations by the azotobacter under these conditions see Williams & Wilson (1954).

RESULTS

General morphological, cultural and physiological characteristics

A primary separation of *Azotobacter agile* strains from those of *A. vinelandii* could be made on the basis of the morphology of young cells. The *A. agile* organisms were usually ovoid or spherical, measuring 2.0×2.0 – 4.5μ . The *A. vinelandii* organisms were smaller rods not exceeding 1.5μ . in width and 3.0μ . in length (see Jensen, 1954). The colonies of the strains of *A. vinelandii* were gummy in contrast with the drier, smaller colonies of *A. agile*. After an incubation period of 1 week no strains of *A. agile* had grown visibly in liquid Burk's medium in which 2% mannitol had been substituted for sucrose; all the *A. vinelandii* strains grew within 24 hr. Likewise all strains of *A. vinelandii*, but none of *A. agile*, grew when KNO_3 was the sole source of nitrogen.

Pigment formation varies according to the mineral content of the medium

particularly that of iron and molybdenum (Wilson & Knight, 1952). For test we have used Burk's medium without Fe+Mo solution. Under these circumstances the *Azotobacter vinelandii* strains turned the medium a bright green, whereas the colour formed by *A. agile* was less intense. On ageing in the refrigerator the agar slopes of *A. agile* became reddish to purple, whereas those of *A. vinelandii*, which were originally greenish yellow, faded.

Oxidative properties and enzymatic adaptation

Table 2 summarizes the rates of respiration of the fourteen strains on typical TCA intermediates. Figs. 1 and 2 present the adaptative patterns for typical representatives of each type (similar figures for *Azotobacter vinelandii* O and for *A. agile* 4.4 are included in previous publications (e.g. Repaske & Wilson, 1953; Williams & Wilson, 1954). No consistent differences among the rates

Table 2. *Rates of respiration by azotobacter strains with various substrates*

Strain	Substrate					
	Sucrose	Acetate	Malate	Succinate	α -keto-glutarate	Citrate*
	$Q_{O_2}(N): \mu l. O_2/hr./mg. N$					
	<i>Azotobacter vinelandii</i>					
O	7,900	13,600	12,300	11,600	11,800	5,000
K	8,800	16,800	16,200	20,200	14,900	3,300
3	3,800	15,600	13,500	17,600	21,000	1,500
7492	5,700	11,500	8,100	15,500	11,200	0
7484	6,000	8,200	9,400	8,100	8,700	Not tested
7496	8,700	10,700	14,600	14,300	11,200	0
K-S4	7,000	9,300	8,900	10,900	11,700	0
	<i>Azotobacter agile</i>					
4.4	10,700	13,500	16,500	17,600	12,600	3,600
9040	13,100	22,200	17,500	18,400	11,300	1,900
7494	12,300	13,900	13,900	13,200	10,400	2,800
S-1	7,800	12,400	9,600	13,600	9,500	2,200
S-2	13,400	14,500	20,700	17,900	6,600	1,100
S-3	13,200	16,800	15,300	13,600	4,300	2,600
S-4	14,800	16,500	17,100	16,700	3,500	Not tested

* Values obtained under special conditions cited in text.

of respiration could be correlated with the type of organism, but a study of results similar to those shown in Figs. 1 and 2 for the entire 14 strains suggested certain differences in the pattern of adaptation to a few of the substrates.

Over a period of 2 years experiments have been made to determine whether such patterns were consistent enough to allow any helpful differentiation between the two types. From the results we have concluded that the patterns of adaptation, at least with these 14 strains, are reasonably stable so that behaviour on succinate and to a lesser extent on α -ketoglutarate and malate can differentiate between *Azotobacter agile* and *A. vinelandii*. Fig. 3 illustrates typical findings with 6 strains; the curves for the others showed similar clustering about the proper group of lines in this figure. Analogous though

Fig. 1

Fig. 2

Fig. 1. Respiratory activity of sucrose-grown *Azotobacter vinelandii* 7492 on sucrose and five substrates of the tricarboxylic acid cycle.

Fig. 2. Respiratory activity of sucrose-grown cells of *Azotobacter agile* S-1 on sucrose and five substrates of the tricarboxylic acid cycle.

Fig. 3. Respiratory activity of sucrose-grown cells of *Azotobacter vinelandii* O, K, and 7496 and *Azotobacter agile* 9040, 4-4, and S-2 on sodium succinate.

not so clear-cut differences were obtained with α -ketoglutarate and malate; with the strains we have used, however, succinate certainly would be the substrate of choice for screening.

The results with citrate call for special mention, particularly, since observations made in this laboratory with citrate first suggested this study. Extended experiments with the 14 strains, however, did not lend support to our original view that it might be used as a differentiating substrate. While these experiments were in progress, we learned through private communication with Dr N. Levy of Imperial Chemical Industries Ltd. that an important factor determining whether citrate (and probably other TCA intermediates) is utilized by *Azotobacter* spp., and probably other species, is the ratio of citrate:Mg⁺⁺. Dr Levy kindly furnished us with a summary of his results* prior to their publication so that we were able to test the influence of this factor on 12 of the 14 strains. Three distinct responses, which were independent of the type of organism, were observed when the ratio citrate:Mg⁺⁺ was varied: (a) one strain (*A. vinelandii* O) adapted to citrate independently of the citrate:Mg⁺⁺ ratio; (b) three strains (*A. vinelandii* 7492, 7496, K-S 4) did not adapt during these experiments (210 min.) even with the lowest citrate:Mg⁺⁺ ratio; (c) the remaining strains did or did not adapt, depending on the citrate: Mg⁺⁺ ratio (see Tables 2 and 3).

Because a lowering of the citrate:Mg⁺⁺ ratio permitted citrate oxidation by 10 of the 14 strains, it was reasoned that an inhibitory factor in previous experiments had been the chelation of ions such as Mg⁺⁺ and Ca⁺⁺ by an excess citrate, rendering these unavailable for TCA cycle oxidations. Harris (1954) suggested the same type of inhibition of citrate oxidation by *Azotobacter chroococcum*; since calcium is required by the organism for maximum respira-

* We thank Dr Levy and his associates not only for supplying us with this information in time for testing on the collection we used but also for the courtesy of allowing us to quote from a letter:

'For *A. vinelandii* (Rothamsted strain 5921) response to citrate is governed by (i) citrate: magnesium ratio in the medium and (ii) concentration of Mg⁺⁺ and Ca⁺⁺ in the cells, in close conformity with a membrane equilibrium model for Mg(Ca) transfer, based on the 1:1 citrate:kation complex and citric acid dissociation. In growth experiments using 1 per cent mannitol medium with 5-40 p.p.m. Mg and c. 1 p.p.m. soluble Ca, citrate stimulates fixation when the citrate:Mg ratio is not much above 1, inhibits completely above 5 and extends the lag (with ultimate stimulation) at intermediate ratios. Raising soluble Ca to 10 p.p.m. roughly doubles these thresholds, i.e. in given cases adequate Ca will convert inhibition to normal or stimulated growth. For a range of organic anions (tartrate, malate, citrate, acetyl acetate, E.D.T.A.) inhibitory power rises with the stability constant of the 1:1 complex.

Oxidation of citrate by resting cells can also be varied from complete to slight according to (i) and (ii) above. The oxygen-uptake curves for acetate, malate, succinate, and citrate under equivalent conditions show "lags" rising in that order from zero to 4-5 hr. This "lag" could be explained, not by enzyme adaptation or anion permeability change, but by initial rapid membrane equilibrium (extraction or other immobilization of Mg⁺⁺ and Ca⁺⁺) followed by re-mobilization and accelerating oxidation as the anion is consumed. Acetate has virtually no chelating power and is attacked without delay; citrate has the highest stability constant in this series and the longest "lags". It is thus possible to set up with a single strain parallels to the behaviour of the different groups you mention.'

Obviously, concentrations and/or ratios of Mg⁺⁺ and citrate other than those used by ourselves (Table 3) might lead to variations in the results, for example, the recalcitrant strains of *A. vinelandii* might be induced to use citrate.

Table 3. *Influence of citrate:Mg⁺⁺ ratio on respiratory activity of whole azotobacter cells*

Strain	Citrate:Mg ⁺⁺ ratio	Citrate (μ mole)	Mg ⁺⁺ (μ mole)	Lag period (min.)	Q _{O₂} (N) (μ l. O ₂ /hr./mg.N)
<i>Azotobacter vinelandii</i>					
O	20:1	40	2	10	5000
	10:1	20	2	10	5900
	5:1	10	2	10	4500
	2:1	8	4	10	4500
	1:1	6	6	10	3800
	5:0	5	0	10	3100
K	20:1	40	2	180-210	—*
	2:1	8	4	180-210	—
	1:1	10	10	160	3200
	1:1	14	14	170	865
	1:2	10	20	170	1800
	1:1	6	6	180-210	—
3	20:1	40	2	210	—
	1:1	10	10	210	—
	1:1	6	6	200	1470
<i>Azotobacter agile</i>					
9040	20:1	40	2	180-210	—
	10:1	20	2	180-210	—
	5:1	10	2	180-210	—
	3:1	60	20	180-210	—
	2:1	8	4	150	1800
	1:1	6	6	150	2000
	1:1	40	40	180-210	—
	5:0	5	0	180-210	—
4-4	20:1	40	2	180-210	—
	1:1	10	10	150	3400
	1:1	18	18	170	3800
7494	20:1	40	2	210	—
	1:1	10	10	160	2800
S-1	20:1	40	2	210	—
	1:1	10	10	210	—
	1:1	6	6	190	2180
S-2	20:1	40	2	210	—
	1:1	10	10	160	850
	1:1	6	6	200	1100
S-3	20:1	40	2	210	—
	1:1	6	6	200	2550

* Oxygen uptake less than 50 μ l./hr.

tory activity, the lowering of the rate of oxygen uptake may arise from the formation of 'insoluble calcium citrate salts'. If a magnesium (calcium) deficiency were a factor in the low rate of citrate oxidation, it was reasoned that the presence of excess citrate would lower respiratory rate on other substrates oxidized through the TCA cycle. This was substantiated in part by the addition of acetate to Warburg vessels containing cells that had been preincubated with 40 μ mole of citrate and 2 μ mole of MgSO₄ for approximately 1 hr. (Table 4).

DISCUSSION

Before summarizing conclusions to be drawn from this study, two side observations of the research should be mentioned:

(1) Adaptive patterns of the strains have been tested over a 2-year period and found to be constant. Even more important, freshly isolated strains of the agile group possessed the same adaptive patterns as did stock strains which had been kept on laboratory media for many years. These data suggest that the patterns found are stable and representative of the organism in nature and not a laboratory artefact.

Table 4. *Influence of preincubation with citrate on the respiratory activity of azotobacter cells on acetate*

Strain	Q _{O₂} (N) values on acetate	
	Preincubation without citrate	Preincubation with 40 μmole citrate
	(μl. O ₂ /hr./mg. N)	
	<i>Azotobacter agile</i>	
4-4	18,900	7,400
S-1	12,400	5,400
S-2	17,600	11,700
7494	11,200	6,900
<i>Azotobacter vinelandii</i>		
O	14,600	4,500
K	16,500	5,800
3	13,200	7,100
K-S4	16,500	4,400

(2) The difficulty of comparing lag times without rigid control of the age of the organisms under observation can be ascertained from the data of Williams & Wilson (1954), in which lag times for *Azotobacter vinelandii* O on succinate and α -ketoglutarate were shown to vary widely with the age of the organisms from 6 to 60 hr. However, since the present study was in part to evolve a simple technique that would facilitate identification of a questionable strain of *Azotobacter* an age variation of 15–24 hr. was allowed in the experiments. Within this range only succinate emerged as a clear ‘indicator’ substrate: *A. agile* organisms 15–24 hr. old had a lag period of 30 min. or less, whereas *A. vinelandii* strains 15–24 hr. old had lag periods of at least 50 min. Also within this range, a trend of lag differences between species on oxidation of malate and α -ketoglutarate was noted; but a few strains of one species have lag times overlapping those of strains of the other species when comparisons are made between organisms that may vary as much as the allowed 10 hr. in age.

Independent of the particular mechanism concerned with adaptation, be it physical or biological, certain major conclusions appear permissible from these studies. First, ability to adapt to succinate and to a lesser extent to malate and α -ketoglutarate might be useful criteria for differentiating between

members of these two groups of *Azotobacter* spp. Secondly, the observations encourage the belief that similar and additional studies of this type (e.g. examination of cell-free extracts for characteristics, including concentration of specific enzymes) might be a powerful tool for more precise definition of an organism's physiology. Such definition should be particularly useful in classification of doubtful cases found in taxonomic studies of this and other micro-organisms. Whether the observed differences are sufficient for division into species in the present example is not a decision for the physiologist but for the expert in taxonomy. The physiologist can only ask that, if his field is to be used in making such decisions, recognition be taken of the advances in this area. Thus more modern methods will be increasingly utilized, methods that he believes might be more powerful and selective than those at present represented in *Bergey's Manual of Determinative Bacteriology* and other taxonomic manuals.

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(Received 8 November 1954)