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Control of Diauxic Growth of *Azotobacter vinelandii* on Acetate and Glucose

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Batch cultures of *Azotobacter vinelandii* were inoculated with cells pregrown on either acetate or glucose. When they were subsequently grown on a mixture of acetate and glucose, typical diauxic growth was observed, with preferential uptake of acetate in the first and glucose in the second phase of growth. Extracts from acetate-pregrown cells exhibited high acetate kinase activity in the first phase of growth. This activity decreased and activities of the two glucose enzymes glucose 6-phosphate dehydrogenase and glyceraldehyde 3-phosphate dehydrogenase increased in the second phase. Extracts from glucose-pregrown cells exhibited high initial activities of the two glucose enzymes, which decreased while acetate kinase activity increased in the first phase of growth. Again, in the second phase, activities of the two glucose enzymes increased and acetate kinase activity decreased. In any case, isocitrate dehydrogenase activity varied only slightly and unspecifically. The differences in enzyme activity and the constancy of isocitrate dehydrogenase were confirmed by experiments with either acetate- or glucose-limited chemostats. In chemostats in which both of the substrates were limiting, all of the enzymes displayed significant activities. Glucose 6-phosphate dehydrogenase activity was inhibited by acetyl coenzyme A and acetyl phosphate but not by acetate. It is proposed that diauxic growth is based on the control of enzymes involved in acetate or glucose dissimilation by which acetate or its metabolites control the expression and activity of glucose enzymes.

Azotobacter vinelandii is an aerobic dinitrogen (N_2)-fixing organism. Oxidation of a variety of organic substrates provides energy and reducing power for biomass production, including reduction of N_2 to ammonia and its assimilation into cell material. Expression of the N_2-fixing enzyme system, the nitrogenase complex, is controlled by the availability of fixed N and an organic substrate serving as the energy and carbon source. Previously, we demonstrated that upon increase of the C/N ratio in the culture medium, cells of *A. vinelandii* increased the rates of substrate consumption and, consequently, respiration before the threshold C/N ratio for nitrogenase expression was reached (2, 3). As a result, *A. vinelandii* exhibits unusually high rates of substrate consumption and oxygen uptake when fixing N_2. However, despite the high demand for organic substrates, the organisms perform diauxic growth when supplied with acetate and glucose (6).

Diauxic growth can be observed when heterotrophic bacteria grow in batch culture on sufficiently high concentrations of more than one carbon and energy source (7). In most cases, the substrate which permits the highest growth rate is used up first. Thus, the more preferred and less preferred substrates are used up sequentially. As a result, diauxic growth is characterized by two exponential phases of growth separated by a phase of no net increase in biomass. Harder and Dijkhuizen (7) distinguished at least three levels at which utilization of mixed substrates can be controlled. These are the level of substrate uptake and the levels of expression and activation-inactivation of enzymes involved in dissimilation of a substrate.

In diauxic growth of *A. vinelandii* on acetate and glucose, the former is the preferred substrate (6). To explain the diauxic effect, it has been proposed that the system of acetate uptake is constitutively expressed (6), while formation of the glucose carrier is repressed by acetate. In addition, however, it may be presumed that significant steps of control take place at the level of enzymes catalyzing dissimilation of acetate and glucose, respectively. The results of the present investigation reveal that this is the case.

MATERIALS AND METHODS

Culture conditions. *A. vinelandii* OP (ATCC 13705) was grown in batch and continuous cultures under conditions of dinitrogen fixation. Both types of cultures were performed in a pH- and oxygen-controlled fermentor as described previously (13). The pH of the cultures was maintained at pH 7.2, the temperature was 30°C, and the dissolved oxygen concentration was 68 μM. Growth media contained 0.3% (wt/vol) sodium acetate and/or 0.3% (wt/vol) glucose. In batch cultures, the substrate concentrations were decreased by 10% after addition of the inoculum. Cultures used for inoculation were grown as described in Results. In continuous culture, *A. vinelandii* was grown at a dilution rate of $D = 0.15\ h^{-1}$.

Preparation of cell extracts. Cell samples were harvested at the times indicated in Results. Samples from continuous cultures were collected after steady states had been established. Cells were sedimented by centrifugation (10 min, 4°C, 11,950 × *g*), washed, and suspended in the following buffer: 5 mM Tris hydrochloride (pH 7.6) for assays of glucose 6-phosphate dehydrogenase and isocitrate dehydrogenase or 100 mM potassium phosphate buffer (pH 7.2) plus 10 mM mercaptoethanol for assays of glyceraldehyde 3-phosphate dehydrogenase and acetate kinase. After suspension, the cells were disrupted at 55.2 MPa by two passages through a precooled French pressure cell. Cell-free extracts were obtained by centrifugation of homogenates at 4°C for 10 min at 17,210 × *g*. To diminish background NADH oxidation in the acetate kinase assay, cell extracts were centrifuged at

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4°C for 60 min at 180,000 × g. The soluble fractions were used for assays.

Enzyme assays. Glucose 6-phosphate dehydrogenase was measured by the method of Senior and Dawes (14), except that the assay buffer was Tris hydrochloride (pH 8.2) at a final concentration of 32 mM and glucose 6-phosphate and NAD^+ were each added at a final concentration of 2 mM. Isocitrate dehydrogenase was assayed on the basis of $NADP^+$ reduction as described by Jurtshuk et al. (8). Glyceraldehyde 3-phosphate dehydrogenase was measured by the method of Senior and Dawes (14) in 19 mM sodium PP_i buffer (pH 7.5) containing 4 mM cysteine and fructose 1,6-diphosphate at a concentration of 5 mM. Glyceraldehyde 3-phosphate was formed from fructose 1,6-diphosphate by the activity of aldolase. Acetate kinase was assayed as described by Suzuki et al. (15). In this coupled assay system, acetate kinase provides ADP used by pyruvate kinase to liberate pyruvate, which is reduced by lactate dehydrogenase at the expense of NADH. The final concentrations in 35.5 mM imidazole hydrochloride (pH 8.0) were as follows: ATP, 14 mM; phosphoenolpyruvate, 1.2 mM; NADH, 0.28 mM; $MgCl_2$, 12 mM; KCl, 47 mM; sodium acetate, 100 mM; pyruvate kinase, 12 μg of protein per ml; lactate dehydrogenase, 12 μg of protein per ml. To determine background NADH activity, acetate was omitted from the assay system. All of the enzyme activities were corrected for background activity.

Quantitative determinations. Protein was determined by the method of Lowry et al. (10). Acetate and glucose concentrations in culture fluids from batch and continuous cultures were determined with commercially available test combinations (Boehringer GmbH; Mannheim, Federal Republic of Germany).

RESULTS

Diauxic growth after adaptation to acetate. Previous studies with different bacterial species showed that occurrence of diauxic growth behavior critically depends on the history of the inoculum (5, 9, 11). Therefore, batch cultures were first inoculated with cells pregrown with acetate. These cultures exhibited typical diauxic growth when supplied with a mixture of acetate and glucose (Fig. 1). Acetate was consumed first, sustaining the first phase of growth at a specific growth rate of $\mu = 0.35\ h^{-1}$. At 7.5 h postinoculation, acetate was exhausted and the cultures entered the first stationary phase of growth, which lasted 4.5 h. Following this, the organisms started to utilize glucose and growth resumed at a specific rate of $\mu = 0.15\ h^{-1}$. About 7 h later, glucose was exhausted and the organisms entered the final stationary phase of diauxic growth. Growth on acetate yielded about 0.140 mg of cell protein per ml, while after the subsequent consumption of glucose a total protein yield of 0.340 mg/ml was reached.

During growth with acetate, the specific activity of acetate kinase increased and reached a maximal level at the end of the first phase of growth. Subsequently, during growth with glucose, the activity of acetate kinase decreased by a factor of about 20. Isocitrate dehydrogenase, an enzyme of central metabolism, increased in activity and reached a maximum at the end of the first stationary phase. Glucose 6-phosphate dehydrogenase and glyceraldehyde 3-phosphate dehydrogenase were assayed as enzymes representing the glucose dissimilation system. The activities of both enzymes were negligible in extracts from cultures grown with acetate. As soon as acetate was exhausted, however, the specific activities of both enzymes increased. In particular, glucose

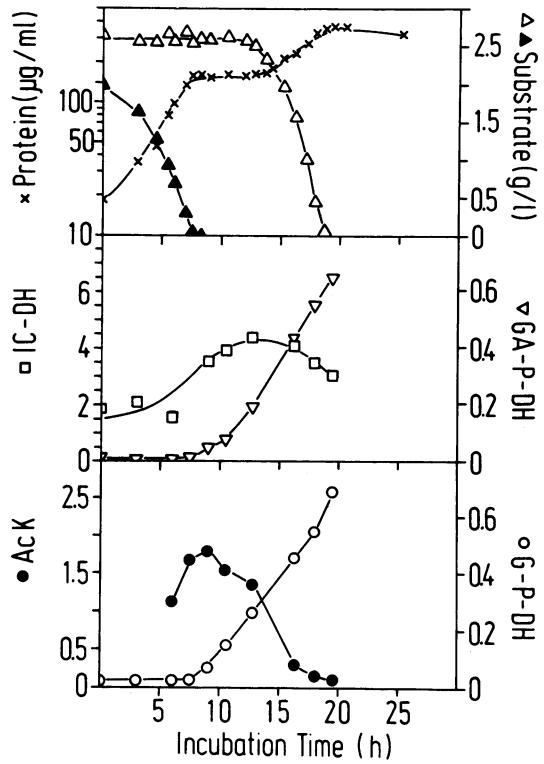

FIG. 1. Growth of *A. vinelandii* in batch culture on a mixture of acetate (0.27% [wt/vol] = 33 mM sodium acetate) and glucose (0.27% [wt/vol] = 15 mM glucose). The inoculum was grown on acetate. The dissolved oxygen concentration and pH were kept constant at 68 μM and 7.2, respectively. Cell protein (×), acetate (▲), and glucose (△) concentrations in the culture fluid and specific activities (micromoles per minute per milligram of protein) of glucose 6-phosphate dehydrogenase (G-P-DH), glyceraldehyde 3-phosphate dehydrogenase (GA-P-DH), isocitrate dehydrogenase (IC-DH), and acetate kinase (AcK) in extracts from *A. vinelandii* are shown.

6-phosphate dehydrogenase and glyceraldehyde 3-phosphate dehydrogenase activities increased by factors of about 20 and 60, respectively.

Diauxic growth after adaptation to glucose. When batch cultures of *A. vinelandii* were inoculated with cells pregrown on glucose, the first phase of growth lasted 8 h, followed by a stationary phase of 4 h and a second phase of growth lasting 4 h (Fig. 2). Protein yields of 0.170 and 0.300 mg/ml were obtained after the first and the second phases of growth, and the corresponding specific growth rates were $\mu = 0.34$ and $0.14\ h^{-1}$, respectively. Determination of acetate and glucose concentrations in the culture medium showed that, again, both substrates were consumed largely sequentially. However, in contrast to the previous experiment using an acetate-grown inoculum, acetate utilization was delayed and the concentration of glucose slightly decreased during the first phase, which was characterized by preferential acetate consumption.

Isocitrate dehydrogenase activity varied by a factor of about 3 and reached its maximum by the end of the first stationary phase. Acetate kinase activity, on the other hand, increased by a factor of 15 during the first phase of growth with acetate and subsequently decreased by a factor of 30. As expected, the activities of the two glucose enzymes were already relatively high at the beginning of the experiments.

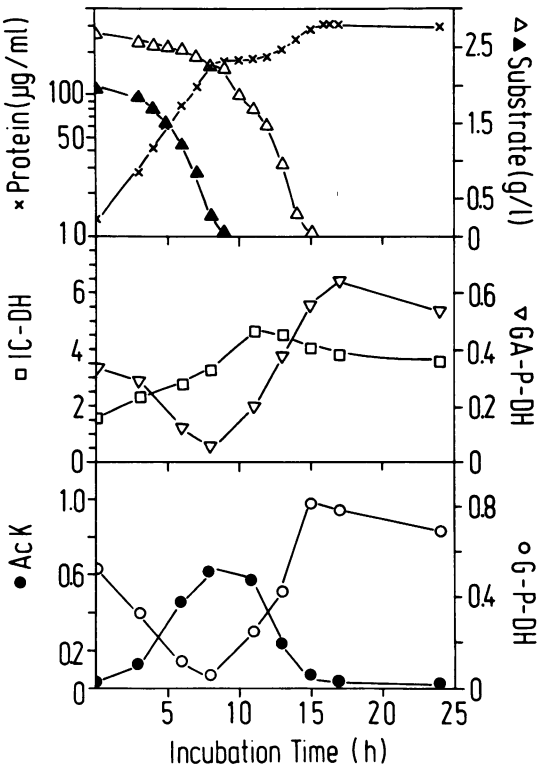

FIG. 2. Growth of *A. vinelandii* in batch culture on a mixture of acetate and glucose as described in the legend to Fig. 1. The inoculum was grown on glucose. For definitions, see the legend to Fig. 1.

However, during the first 6 h, glucose 6-phosphate dehydrogenase activity decreased by a factor of 5 and cell protein increased by a factor of 6. This suggests repression and thus dilution of the enzyme when the cultures grew with acetate. Largely the same behavior was observed with respect to glyceraldehyde 3-phosphate dehydrogenase. Upon acetate exhaustion, both activities increased by factors of about 10 and stayed largely constant within the final stationary phase. It should be noted that glucose enzymes passed comparable activities while decreasing and increasing during growth on acetate and glucose, respectively. For example, the enzyme activities determined after about 5 h of diauxic growth on acetate were reached again 5 h later. At that time, the culture had attained the second, i.e., glucose, phase of diauxic growth.

Regulation of glucose 6-phosphate dehydrogenase activity. After transfer to diauxic conditions, cells pregrown on glucose exhibited activities of glucose enzymes comparable to those of cells taken from the second, i.e., glucose, phase of diauxic growth (Fig. 1 and 2). Nevertheless, already during the initial period of about 5 h, acetate rather than glucose was the preferred substrate when cultures adapted to glucose were supplied with a mixture of acetate and glucose. This suggests that acetate or a metabolite thereof might inhibit the activity of glucose utilization. In fact, the activity of glucose 6-phosphate dehydrogenase in extracts from *A. vinelandii* were inhibited by acetyl coenzyme A (acetyl-CoA) and, to a lesser extent, acetyl phosphate (Fig. 3). The specificity of this inhibitory reaction was supported by the insensitivity of the dehydrogenase toward acetate at concentrations of up to 37 mM. Fifty percent inhibition of

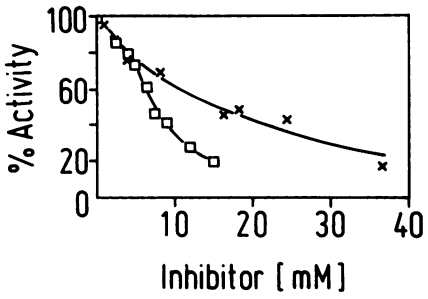

FIG. 3. Inhibition of glucose 6-phosphate dehydrogenase by acetyl-CoA (□) or acetyl phosphate (×). *A. vinelandii* was grown on glucose.

activity was attained in the presence of about 8 mM acetyl-CoA and about 20 mM acetyl phosphate.

Chemostat experiments. The experiments reported so far, done with batch cultures, revealed that enzyme activities did not reach steady-state levels in actively growing cultures. Consequently, comparison of enzyme activities becomes questionable. This problem can be largely excluded if the organisms are grown in chemostat cultures. Therefore, in the following experiments, *A. vinelandii* was grown substrate limited at a constant dilution rate of $D = 0.15\ h^{-1}$ and a constant ambient dissolved oxygen concentration of 68 µM.

Cultures grown acetate limited reached a steady-state biomass level of 0.135 ± 0.006 mg of protein per ml of culture, corresponding to 3.7 g of protein formed per mol of acetate consumed (this is the molar yield coefficient, *Y*). Extracts from these cells displayed significant acetate kinase activities (Table 1). However, glucose 6-phosphate dehydrogenase and glyceraldehyde 3-phosphate dehydrogenase activities were hardly detected. Conversely, glucose-limited cultures were characterized by a steady-state biomass level of 0.191 ± 0.008 mg of protein per ml, $Y = 11.5$ g/mol, and high activities of the two enzymes of glucose metabolism but rather low acetate kinase activity. Isocitrate dehydrogenase activity reached largely identical levels under all of the sets of conditions shown in Table 1.

When limited by both acetate and glucose, the cultures reached steady-state protein levels predictable on the basis of the levels attained with either acetate or glucose, i.e., 0.317 ± 0.019 mg of protein per ml of culture. All of the enzymes tested exhibited activities which, except for isocitrate dehydrogenase, were lower than those reported above for cultures limited by either one of the two substrates.

TABLE 1. Steady-state specific activities of enzymes involved in acetate and glucose metabolism in *A. vinelandii* grown in acetate- and/or glucose-limited chemostat cultures[a]

Enzyme	Mean ± SEM enzyme sp act[b]		
	Acetate	Glucose	Glucose-acetate
G-P-DH[c]	0.01	3.18 ± 0.26	2.72 ± 0.22
GA-P-DH[d]	0.004	1.12 ± 0.14	0.73 ± 0.03
IC-DH[e]	3.64 ± 0.35	4.04 ± 0.42	4.54 ± 0.09
AcK[f]	2.06 ± 0.27	0.06 ± 0.01	0.66 ± 0.05

[a] Dilution rate, 0.15 h^{-1}.
[b] In micromoles per minute per milligram of protein. Concentrations in the feed medium, 36.6 mM acetate and/or 16 mM glucose.
[c] G-P-DH, Glucose 6-phosphate dehydrogenase.
[d] GA-P-DH, Glyceraldehyde 3-phosphate dehydrogenase.
[e] IC-DH, Isocitrate dehydrogenase.
[f] AcK, Acetate kinase.

DISCUSSION

It is known that diauxic growth is largely restricted to cultures inoculated with cells pregrown with the preferred substrate. In such cases, enzymes involved in the metabolism of the less preferred substrate occur after exhaustion of the preferred substrate (1, 4, 5, 7, 9, 11). However, if the inoculum is grown on the less favored substrate, the lag between the two phases of diauxic growth becomes obscured. In such cultures, the corresponding enzymes are present from the beginning of growth on mixed substrates, so that both the more preferred and the less preferred substrates can be consumed simultaneously (5, 9, 11).

In accordance with the observations mentioned above, *A. vinelandii* pregrown on acetate took up glucose and expressed the enzymes necessary for its metabolism only after exhaustion of the acetate supply. In contrast to previous results, however, typical diauxic growth was possible, although the organisms were pregrown on glucose and the enzymes of glucose dissimilation were already present at the beginning of the experiments. To explain sequential consumption of acetate and glucose by cultures preadapted to glucose dissimilation, a variety of mechanisms, discussed below, may be envisaged.

Since glucose 6-phosphate dehydrogenase specific activity decreased and cell protein increased by essentially the same factor, it may be concluded that the enzyme was repressed and therefore diluted out of the cells in the course of the first phase of growth on acetate. This, however, should not be the only reason for largely delayed glucose consumption, because similar decreases in enzyme activities were reported for organisms lacking typical diauxic growth under comparable regimens (5, 11). In addition, activities of glucose enzymes attained during growth on acetate could be determined, as well, with cells from the second, i.e., glucose, phase of diauxic growth. The possibility that inducer exclusion inhibits glucose uptake by acetate was ruled out by Melton and coworkers (6, 12). A third mechanism might be proposed to take place at an energetic level, namely, that the high cost of energy required for N_2 fixation and acetate phosphorylation should limit the energy-consuming transport of glucose. This possibility is excluded by the results obtained with acetate- plus glucose-limited continuous cultures, which revealed that limitation of the carbon and energy source led to the occurrence of acetate and glucose enzymes, as well as to disappearance of diauxic growth. Therefore, comparison with chemostat cultures suggests that in batch cultures acetate or metabolites of it have to be present at measurable concentrations to inhibit glucose consumption in the first phase of diauxic growth. This is supported by the inhibitory action of acetyl-CoA and acetyl phosphate on glucose 6-phosphate dehydrogenase. The concentrations of acetyl-CoA and, in particular, acetyl phosphate required for inhibition are quite high. However, this may warrant preferred utilization, on the one hand, of acetate at high acetate concentrations and, on the other, glucose dissimilation in the presence of low acetate concentrations, i.e., under conditions of acetate limitation. In addition, it should be remembered that the specificity of the inhibitory reactions became apparent when it was shown that acetate did not affect enzyme activity. In conclusion, we propose that in the presence of sufficiently high, i.e., nonlimiting, concentrations of acetate, glucose dissimilation is delayed by both inactivation and repression of glucose enzymes.

The activity of isocitrate dehydrogenase participating in acetate and glucose metabolism varied by a factor of up to about 3 in batch cultures. The physiological significance of this variation is not known, but since this variation was not observed in chemostat cultures, it may be assumed that the variation was a result of undefined conditions in the batch system rather than a specific response to substrate consumption. Conversely, differences in activities of glucose 6-phosphate and glyceraldehyde 3-phosphate dehydrogenases, as well as the activity of acetate kinase observed with cells from batch cultures, are enforced by similar results obtained with cells from chemostats.

The activities of the enzymes tested in this investigation were slightly lower with extracts from chemostat cultures grown acetate plus glucose limited than with extracts from cultures limited by either one of the two substrates. This leads to the popular question concerning the homogeneity of populations in chemostat cultures. In particular, it might be possible that the chemostat culture housed two populations, one adapted to acetate and the second adapted to glucose. In the present case, this possibility can be rejected simply on the grounds that all of the organisms present in the culture were fed at the same time with both substrates and even cells which might have been adapted to glucose took up acetate as the preferred substrate.

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