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Physiological and Biochemical Role of the Butanediol Pathway in *Aerobacter (Enterobacter) aerogenes*

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Aerobacter (Enterobacter) aerogenes wild type and three mutants deficient in the formation of acetoin and 2,3-butanediol were grown in a glucose minimal medium. Culture densities, pH, and diacetyl, acetoin, and 2,3-butanediol levels were recorded. The pH in wild-type cultures dropped from 7.0 to 5.8, remained constant while acetoin and 2,3-butanediol were formed, and increased to pH 6.5 after exhaustion of the carbon source. More 2,3-butanediol than acetoin was formed initially, but after glucose exhaustion reoxidation to acetoin occurred. The three mutants differed from the wild type in yielding acid cultures (pH below 4.5). The wild type and one of the mutants were grown exponentially under aerobic and anaerobic conditions with the pH fixed at 7.0, 5.8, and 5.0, respectively. Growth rates decreased with decreasing pH values. Aerobically, this effect was weak, and the two strains were affected to the same degree. Under anaerobic conditions, the growth rates were markedly inhibited at a low pH, and the mutant was slightly more affected than the wild type. Levels of alcohol dehydrogenase were low under all conditions, indicating that the enzyme plays no role during exponential growth. The levels of diacetyl (acetoin) reductase, lactate dehydrogenase, and phosphotransacetylase were independent of the pH during aerobic growth of the two strains. Under anaerobic conditions, the formation of diacetyl (acetoin) reductase was pH dependent, with much higher levels of the enzyme at pH 5.0 than at pH 7.0. Lactate dehydrogenase and phosphotransacetylase revealed the same pattern of pH-dependent formation in the mutant, but not in the wild type.

When *Aerobacter (Enterobacter) aerogenes* is grown with glucose as the carbon source, the main fermentation products are acetoin, 2,3-butanediol, ethanol, lactic acid, formic acid, and acetic acid. The proportion of these compounds varies with oxygen supply and the pH (12).

2,3-Butanediol is formed from pyruvate with acetolactate and acetoin as intermediates (see Fig. 5), and by this pathway the organism is able to divert metabolism from acid production to the formation of the neutral compounds. The three enzymes involved, the pH 6 acetolactate-forming enzyme, acetolactate decarboxylase (EC 4.1.1.5), and diacetyl (acetoin) reductase, have recently been purified (5, 18, 23, 26).

Diacetyl (acetoin) reductase from *A. aerogenes* combines the catalytic properties of two other enzymes found in other organisms. (5, 14). One is diacetyl reductase or acetoin dehydrogenase (acetoin: nicotinamide adenine dinucleotide [NAD^+] oxidoreductase, EC 1.1.1.5), which irreversibly reduces diacetyl to acetoin (3, 7, 21), and the other is butylene-glycol dehydrogenase (2,3-butyleneglycol: NAD^+ oxidoreductase, EC 1.1.1.4), which catalyzes the reversible reduction of acetoin to 2,3-butanediol (9).

Acetate plays a key role in the operation of the butanediol pathway. It induces the three enzymes (4, 5, 24), activates the pH 6 acetolactate-forming enzyme (25), and regulates the balance between acetoin and 2,3-butanediol (16) (see Fig. 5).

We have compared batch cultures of *A. aerogenes* wild type, grown in a glucose minimal medium, with those of three mutants deficient in the formation of acetoin and 2,3-butanediol. Neutral metabolites, pH, and culture densities were recorded.

In addition, the wild type and one of the mutants were grown aerobically and anaerobically at constant pH (pH 7.0, 5.8, and 5.0). Their growth rates and the levels of alcohol

dehydrogenase (EC 1.1.1.1), lactate hydrogenase (EC 1.1.1.27), phosphotransacetylase (EC 2.3.1.8), and diacetyl (acetoin) reductase, were recorded in extracts from cells growing exponentially.

MATERIALS AND METHODS

Bacterial strains. Wild-type *A. aerogenes* 1033 and mutant P 14-43 were obtained from H. E. Umbarger, and the mutants III-45 and IV-2 from Y. S. Halpern. The three mutants are defective in the formation of acetoin and butanediol. Mutant III-45 has less than 1% of the wild-type levels of the three enzymes of the butanediol pathway (24): pH 6 acetolactate-forming enzyme, acetolactate decarboxylase, and diacetyl (acetoin) reductase. Mutant IV-2 has less than 5% of the first and the last of these enzymes (24). During this work the mutant P 14-43 was shown to be deficient in the formation of acetoin and 2,3-butanediol. The three mutants require valine and isoleucine for growth (11), and P 14-43 has an additional requirement for guanine (27).

Media and growth conditions. In the experiments with batch cultures, the bacteria were grown in a 2-liter flask with gyrotory shaking (130 rpm and 37 C). One liter of minimal medium (6) was supplemented with trace elements (15) and 10 g of dextrose (Difco). The media of the mutants contained L-leucine (12.5 mg), DL-valine (25 mg), and DL-isoleucine (25 mg), and 10 mg of guanosine/liter was added to P 14-43. Two milliliters of a glucose broth culture grown overnight at 37 C was used as inoculum.

In the experiments with constant pH the cells were cultivated in minimal medium (1) containing 10 g of dextrose (Difco) per liter in a 1-liter fermentor using a titrator TTr2 connected to an Autoburette ABU 13 (Radiometer Ltd., Copenhagen, Denmark). NaOH (2.0 M) was used to keep pH at the desired value during growth. The media of the mutant III-45 contained amino acids as described above.

As inocula bacteria growing exponentially under the same conditions (i.e., pH and oxygen supply) as were used in the experiments were used. Inoculum volumes were adjusted (between 1 and 25 ml) to initiate growth with approximately the same number of organisms corresponding to a final absorbancy measured at 680 nm (A_{680}) of 0.01. At indicated A_{680}, samples varying from 50 to 100 ml were removed. The bacteria were washed once in 50 mM potassium phosphate, pH 7.0, and sonicated for 3 × 30 s with 60-s intervals with a Branson Sonifier. Cell debris was removed by centrifugation at 4 C in a Sorvall RC 2B at 27,000 × *g* for 30 min, and cell-free extracts were tested for enzyme activities.

When the cells were grown aerobically, air (3 liters/min) was sparged through the flask, and for anaerobic growth, 95% N_2 + 5% CO_2 of the purest grade available was used.

Determination of pH, glucose, acetate, diacetyl, acetoin, and 2,3-butanediol. Five-milliliter samples were harvested at intervals (Fig. 1), pH was measured at room temperature, and the samples were cooled in ice water. Culture density was determined at 610 nm, and centrifugation was performed at 20,000 × *g* for 20 min in a Sorvall RC 2B centrifuge at 4 C. Culture supernatants were analyzed immediately or stored at −20 C.

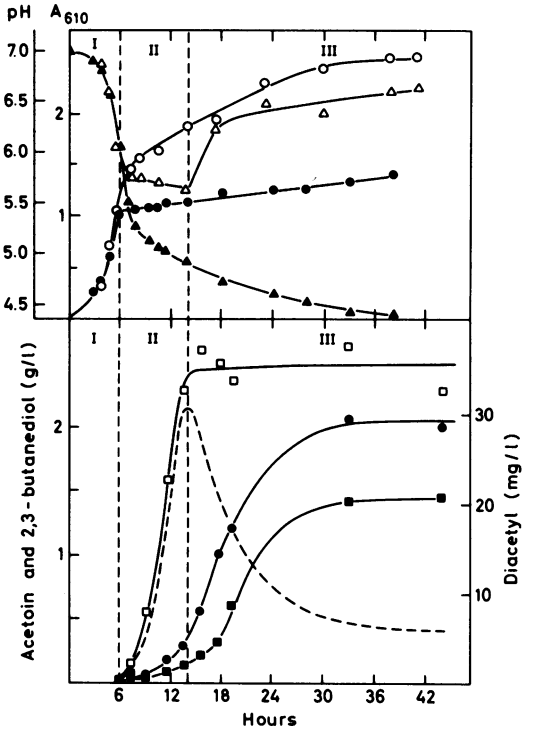

FIG. 1. *Batch cultures of A. aerogenes wild type and mutant IV-2, deficient in the butanediol-forming enzymes. The cells were grown in a New Brunswick metabolic shaker at 130 rpm and 37 C. I, II, and III, and vertical stipled lines indicate phases I, II, and III (for explanation, see discussion). Upper part: culture densities, wild type* (○), *and mutant IV-2* (●), *pH, wild type* (△), *mutant IV-2* (▲). *Lower part: wild-type culture levels if diacetyl* (■), *acetoin* (●), *2,3-butanediol plus acetoin* (□), *and 2,3-butanediol* (-----).

Glucose was determined with phenol-sulfuric reagent (8); acetate was measured as the hydroxamate of acetylphosphate after treatment with acetate kinase (20); diacetyl was determined by gas chromatography using a head space technique (13); acetoin was estimated by the method of Westerfeld (28); and 2,3-butanediol was measured after oxidation to acetoin with bromine (22).

Enzyme assays. Lactate dehydrogenase activity was determined by following the pyruvate-dependent oxidation of NAD, reduced form (NADH). The assay system contained, in 1.0 ml: potassium phosphate (pH 6.2), 100 μmol; sodium pyruvate, 5 μmol; NADH, 0.1 μmol (29). Alcohol dehydrogenase activity was determined by following the ethanol-dependent reduction of NAD. The assay system contained in

1.0 ml: sodium carbonate (pH 9.0), 300 μmol; ethanol, 1.6 mmol and NAD, 0.6 μmol (19). Diacetyl (acetoin) reductase activity was determined by following the 2,3-butanediol-dependent reduction of NAD. The assay system contained in 1.0 ml: potassium phosphate (pH 7.0), 50 μmol; 2,3-butanediol, 100 μmol; and NAD, 2 μmol (26). Phosphotransacetylase activity was determined by following the formation of acetyl coenzyme A (CoA) at 233 nm. The assay system contained in 1.0 ml: potassium phosphate (pH 7.0), 100 μmol; acetyl phosphate (dilithium salt), 0.25 μmol; CoA, 0.05 μmol; and 0.5 mg of albumin (2).

Reactions were initiated by the addition of 10 to 100 μl of crude extract, and measurements were made at room temperature with a Shimadzu multipurpose recording spectrophotometer model MPS-50L. Initial reaction rates were measured by observing the rate of change in the optical density at 340 nm for lactate dehydrogenase, alcohol dehydrogenase, and diacetyl (acetoin) reductase, and at 233 nm for phosphotransacetylase.

Specific activities of the respective enzymes are expressed as follows: phosphotransacetylase, micromoles of acetyl CoA per milligram of protein per minute; lactate dehydrogenase, micromoles of NAD per milligram of protein per minute; and diacetyl (acetoin) reductase, micromoles of NADH per milligram of protein per minute. Protein was estimated by the method of Lowry et al. (17).

Chemicals. Acetoin, 2,3-butanediol, α-naphthol, and creatine were obtained from Fluka AG, Germany; diacetyl, acetate kinase, sodium pyruvate, CoA, acetylphosphate dilithium salt, NAD, and NADH were from Sigma Chemical Co. Other chemicals were of analytical grade and commercially available.

RESULTS

pH profiles, culture densities, and formation of metabolites in batch cultures. *A. aerogenes* wild type and three mutants deficient in the formation of acetoin and 2,3-butanediol were grown in batch cultures in a glucose minimal medium. pH profiles, growth curves, and formation of 2,3-butanediol, acetoin, and diacetyl are presented in Fig. 1. In the wild type, the pH decreased from 7.0, stabilized at about 5.6 at the end of the exponential growth, and remained constant for approximately 8 h. Then pH increased to about 6.5. In the mutants, pH decreased throughout the time studied, and pH values of 4.5 or lower were observed. Culture density curves also differed between wild type and mutants. In the latter, growth was arrested at about 6 h, whereas wild-type culture density increased at a reduced rate for more than 30 h. The data shown are for mutant IV-2, but the two mutants III-45 and P 14-43 behaved identically.

During the constant pH period, glucose was rapidly consumed, and acetoin and 2,3-butanediol were formed. Some eight times more diol than acetoin was excreted at this stage. When pH started to increase, more than 97% of the glucose had disappeared from the medium, and the sum of acetoin and 2,3-butanediol had reached a constant level. A continued increase in acetoin was compensated by a simultaneous decrease in 2,3-butanediol concentration.

Parallel with the increase in acetoin concentration, the level of diacetyl increased, but the amount of diacetyl was 50 to 100 times lower.

In the mutant cultures, negligible amounts of acetoin, 2,3-butanediol, and diacetyl accumulated. After 20 h the levels of each of these compounds were less than 1% as compared to concentrations in the wild-type culture.

Acetate concentrations were the same (10 mM) in cultures of the wild type and mutant IV-2 at the end of exponential growth. In the former, this level was maintained for more than 20 h, but in the mutant acetate accumulation continued, and more than 25 mM was found at 20 h.

Growth properties and enzyme levels at constant pH. Growth experiments with *A. aerogenes* wild type and mutant III-45 were performed under aerobic and anaerobic conditions at three constant pH values, pH 7.0, 5.8, and 5.0 (Table 1). A reduced growth rate was observed for decreasing pH in all instances. This influence of low pH upon growth rate was greater under anaerobic conditions. For corresponding conditions, only minor differences between the micrometer values (doublings per hour) of the wild type and the mutant were observed, with the exception of anaerobic growth at pH 5.0, where a somewhat larger impairment of growth rate is observed in the latter strain.

The influence of pH and aeration upon the pattern of some fermentative enzymes in *A.*

TABLE 1. *Doublings per hour (μm) determined for A. aerogenes wild type and the mutant III-45 deficient in the butanediol-forming system*[a]

Growth conditions	pH	Wild type (μm)	Mutant (μm)
Aerobic	7.0	1.9	2.0
	5.8	1.3	1.4
	5.0	1.3	1.3
Anaerobic	7.0	1.1	0.9
	5.8	0.9	0.6
	5.0	0.4	0.2

[a] The cells were grown aerobically or anaerobically at several constant pH values.

aerogenes wild type and the mutant III-45 were studied. The strains were grown as above, and the cells were harvested in the exponential phase at A_{680} values indicated in Fig. 2–4. Cell-free extracts were prepared, and levels of alcohol dehydrogenase, lactate dehydrogenase, phosphotransacetylase, and diacetyl (acetoin) reductase were determined.

Phosphotransacetylase activities are regarded as representative of the acetate pathway, since acetate kinase is formed in coordination with this enzyme under various conditions (4). Diacetyl (acetoin) reductase should be representative of the butanediol pathway for similar reasons (24).

Under all conditions, very low specific activity of alcohol dehydrogenase was observed, and reliable quantitative data could not be obtained. This was also the case for diacetyl (acetoin) reductase activity in the mutant.

During aerobic growth (Fig. 2) pH did not influence the enzyme levels, and only minor differences in enzyme patterns between the wild type and the mutant were observed. The level of phosphotransacetylase decreased slightly in the wild type, but increased in the mutant. For lactate dehydrogenase, the opposite effect was observed. Low specific activity of diacetyl (acetoin) reductase was observed during aerobic growth.

Anaerobically grown cells exhibited distinctly different enzyme patterns (Fig. 3 and 4) and several differences between the wild type and the mutant became apparent. In the wild type the level of diacetyl (acetoin) reductase was clearly pH dependent, with increased activity for decreasing pH. The other two enzymes were, as for aerobic conditions, unaffected by the pH, but a somewhat higher level of phosphotransacetylase was obtained anaerobically. In the mutant, the levels of phosphotransacetylase and lactate dehydrogenase were strongly pH dependent, and very high activities were found at pH 5.0.

DISCUSSION

On the basis of the pH profile of batch cultures of *A. aerogenes* wild type (Fig. 1), one can divide the cultural events into three distinct periods, phases I, II, and III, respectively. Phase I is characterized by a rapid drop in the pH to about 5.8, phase II exhibits nearly constant pH at this level, and in phase III the pH increases to about 6.5. Other events in wild-type cultures correspond with these phases, or the transitions between them. In contrast, similar division into three phases was not possible for the mutant cultures.

In phase I the wild type and the three mutants grow exponentially. No differences

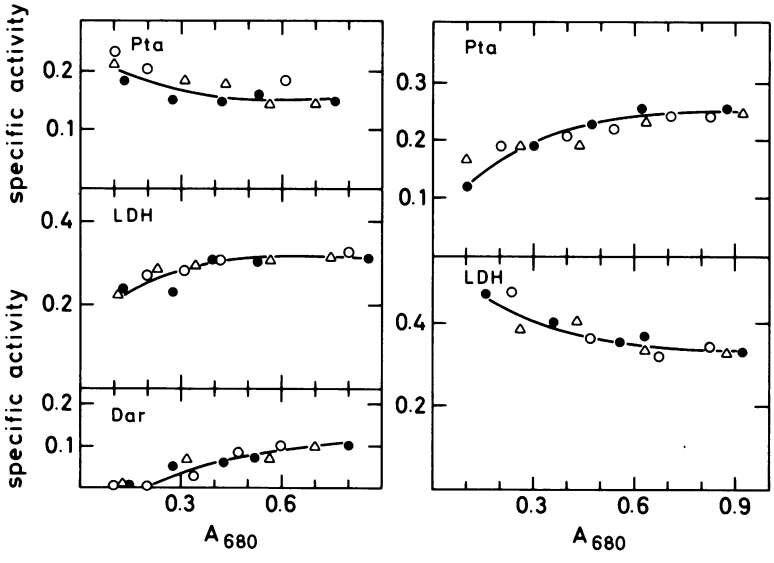

FIG. 2. *Formation of lactate dehydrogenase (LDH), phosphotransacetylase (Pta), and diacetyl (acetoin) reductase (Dar) during aerobic growth of A. aerogenes wild type (left), and the mutant III-45, deficient in the butanediol-forming enzymes (right). Cultures were grown in a fermentor at constant pH under aerobic conditions as described. Cells were harvested at indicated A_{680}. Cell-free extracts were prepared, and enzyme assays were carried out as described. Symbols: (△) pH 5.0, (●) pH 5.8, (○) pH 7.0.*

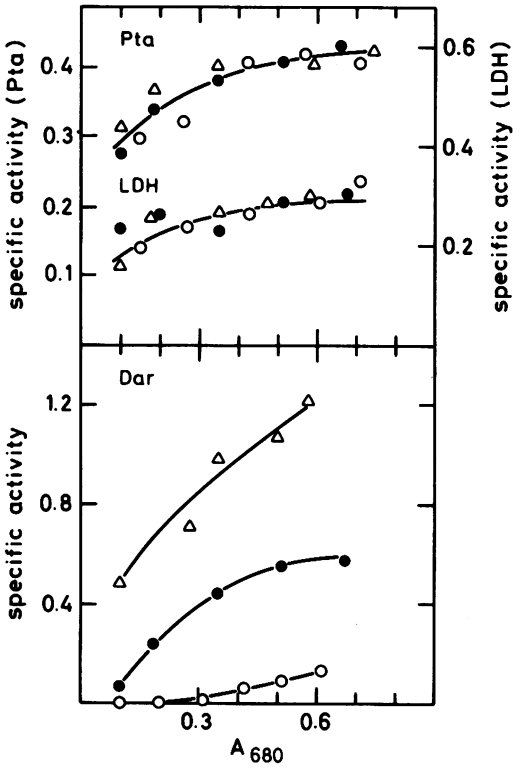

FIG. 3. *Formation of LDH, Pta, and Dar during anaerobic growth of A. aerogenes wild type. For symbols and experimental details see Fig. 2 and Materials and Methods.*

between the strains could be observed in growth or pH profiles (Fig. 1), and acetate accumulated to the same degree. Thus, no physiological advantage for the wild type could be observed in phase I. In accordance with this the butanediol pathway is not operative; neither acetoin nor 2,3-butanediol was formed at this stage (Fig. 1).

After transition to phase II the importance of the butanediol pathway becomes apparent. Marked differences in pH profiles of the wild type and the mutant are evident from Fig. 1. Due to the inability of the mutants to convert pyruvate into the neutral metabolites, acetoin and 2,3-butanediol, these cultures become increasingly more acid. In the wild type, however, more than 2.5 g/liter of acetoin and 2,3-butanediol is formed, corresponding to a channelling of some 55% of the pyruvate in this direction. The high activity of the butanediol pathway is also evident from the large proportion of the three enzymes involved. They constitute approximately 2.5% of total protein in the cell (5).

When the carbon source is exhausted, transition to phase III occurs. In the absence of

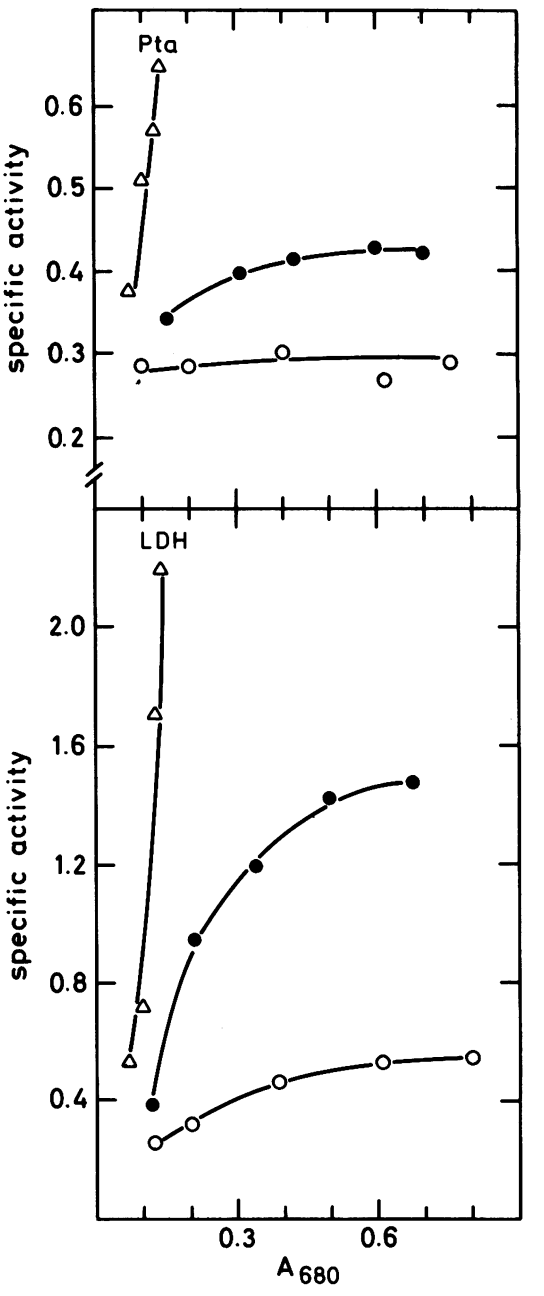

FIG. 4. *Formation of LDH and Pta during anaerobic exponential growth of A. aerogenes mutant III-45. For symbols and experimental details see Fig. 2 and Materials and Methods.*

glucose, no more pyruvic acid is formed which can be converted to acetoin and 2,3-butanediol, and hence the net synthesis of these compounds ceases. The ratio between them changes as 2,3-butanediol in the medium is reoxidized to

acetoin in phase III. This must reflect a change in the intracellular balance of the two neutral compounds.

The parallel increase in acetoin and diacetyl concentrations in phases II and III indicates that the formation of these compounds are connected. The absence of diacetyl in the three mutants also supports this. It is still not known how diacetyl is formed in *A. aerogenes*, since diacetyl (acetoin) reductase cannot oxidize acetoin to diacetyl (14).

With the pH regulated externally, the butanediol pathway seems to be of minor importance under aerobic conditions, as indicated by the identical growth rates of the wild type and the mutant (Table 1). Anaerobically, the mutant was more seriously affected by low pH than the wild type, and the butanediol pathway thus has its main function when oxygen supply is limited. The strong effect of decreasing pH upon growth rates of both strains shows that low pH values are critical for growth, and demonstrates the importance of the butanediol pathway in preventing the occurrence of a low pH level in batch cultures.

Important information about regulation of the fermentative pathways in *A. aerogenes* was obtained by comparing enzyme levels in the wild type and the mutant III-45 grown exponentially under various conditions. Thus, some of the enzymes were unaffected by aeration and by pH, whereas the specific activities of others were strongly dependent on these parameters.

The low levels of alcohol dehydrogenase, independent of aeration and pH, is an indication that this enzyme does not play any important role during exponential growth. Even when the butanediol pathway is blocked, the amounts of pyruvate metabolized via the ethanol pathway must be negligible.

Since the aerobic levels of diacetyl (acetoin) reductase were low, this would be another indication that the butanediol pathway is unimportant in *A. aerogenes* when sufficient oxygen is present. This is further confirmed by the observation that a block in this pathway does not influence the enzyme levels of the other pathways studied, in contrast to the findings in the anaerobic experiments (see below).

In the wild type, anaerobic formation of diacetyl (acetoin) reductase was pH dependent, in contrast to the formation of lactate dehydrogenase and phosphotransacetylase, but in the mutant the formation of the two latter enzymes becomes pH dependent anaerobically. The reason for the high levels of lactate dehydrogenase and phosphotransacetylase in the mutant at low pH may be an intracellular accumulation of pyruvate. This indicates a control mechanism for the formation of these enzymes, different from the effect by acetate on the butanediol pathway (Fig. 5). Pyruvate is a possible candidate as an inducer of phosphotransacetylase and lactate dehydrogenase. It has been reported that the latter enzyme is induced by pyruvate in *Staphylococcus aureus* (10). Phosphotransacetylase and acetate kinase have four times higher specific activities when pyruvate replaces glucose, glycerol, or acetate as the carbon source during aerobic growth of *A. aerogenes* (4).

In addition to controlling the pH, the butanediol pathway participates in the regulation of the NADH-NAD ratio. Large amounts of NAD are regenerated from NADH during glucose degradation, as acetoin is reduced to 2,3-butanediol. After glucose exhaustion, when

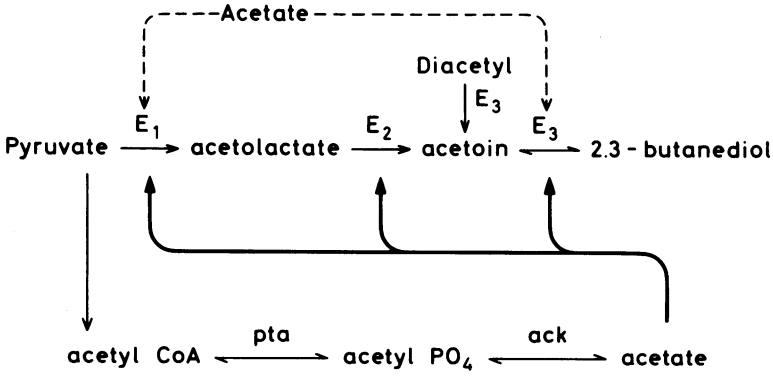

FIG. 5. *Regulation by acetate of the 2,3-butanediol-forming system in A. aerogenes. E_1, pH 6 acetolactate-forming enzyme; E_2, acetolactate decarboxylase; E_3, diacetyl (acetoin) reductase; Pta, phosphotransacetylase; and ack, acetate kinase. Heavy lines indicate the induction of the butanediol-forming enzymes by acetate, and stipled lines the acetate activation of E_1 and inhibition of E_3 in the direction of acetoin from 2,3-butanediol (favoring the formation of 2,3-butanediol).*

NADH is not formed, this reaction is reversed, and extracellular 2,3-butanediol acts as a reservoir of reducing equivalents.

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