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Utilization of Acetate by *Beggiatoa*[1]

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ABSTRACT

BURTON, SHERIL D. (Institute of Marine Science, University of Alaska, College), RICHARD Y. MORITA, AND WAYNE MILLER. Utilization of acetate by *Beggiatoa*. J. Bacteriol. 91:1192–1200. 1966.—A proposed system which would permit acetate incorporation into four-carbon compounds without the presence of key enzymes of the citric acid cycle or glyoxylate cycle is described. In this system, acetyl-coenzyme A (CoA) is condensed with glyoxylate to form malate, which, in turn, is converted to oxaloacetate. Oxaloacetate then reacts with glutamate to produce α-ketoglutarate, which is subsequently converted to isocitrate. Cleavage of isocitrate produces glyoxylate and succinate. Thus, the proposed system is similar to the glyoxylate bypass in that malate is produced from glyoxylate and acetyl-CoA, but differs from both the citric acid cycle and the glyoxylate bypass, since citrate and fumarate are not involved. Fumarase, aconitase, catalase, citritase, pyruvate kinase, enolase, phosphoenolpyruvate carboxylase, lactic dehydrogenase, α-ketoglutarate dehydrogenase, and condensing enzyme were not detectable in crude extracts of *Beggiatoa*. Succinate was oxidized by a soluble enzyme not associated with an electron-transport particle. Isocitrate was identified as the sole compound labeled when $C^{14}O_2$ was added to a reduced nicotinamide adenine dinucleotide, CO_2 generating system (crystalline glucose-6-phosphate dehydrogenase and glucose-6-phosphate) in the presence of α-ketoglutarate.

The metabolism of *Beggiatoa* has remained obscure, despite its widespread occurrence and early recognition. The observation that catalase permits abundant growth when added to culture media (6) has provided a means to grow these microorganisms in mass culture.

Several investigators have noted increased growth of *Beggiatoa* in the presence of acetate (9, 14). Winogradsky (32) included a small amount of acetate in the medium he used for propagation of his cultures. Kiel (12) is the only investigator who obtained pure cultures of *Beggiatoa* which did not require organic matter.

Scotten and Stokes (25) observed reduction of methylene blue by intact filaments when placed with numerous organic compounds. Formate, lactate, and succinate provided the most rapid reduction of methylene blue. Oxygen uptake occurred with lactate, glutamate, α-ketoglutarate, succinate, pyruvate, and acetate, whereas glucose and glycerol were inactive. Their studies were hampered by uncontrollable lysis and subsequent loss of activity.

Faust and Wolfe (9) isolated several strains of *Beggiatoa* which were unable to utilize a variety of organic compounds for growth, but were stimulated by acetate and low levels of sulfide. Their investigations into the metabolism of *Beggiatoa* were limited by low cell yields.

MATERIALS AND METHODS

Organism. The *Beggiatoa* strain used in these studies was isolated by Maiers (Ph.D. Thesis, Ohio State Univ., Columbus, 1963). This culture has been deposited with the American Type Culture Collection (accession number 15551). The methods of cell maintenance and production of mass cultures and cell-free extracts were as described by Burton and Morita (6). Protein and nucleic acids were estimated by the method of Warburg and Christian (31).

Chemicals and biological reagents. The following items were obtained from Sigma Chemical Co., St. Louis, Mo.: catalase, stock no. C-3, crude sterile; catalase, stock no. C-100, twice crystallized; lactic dehydrogenase, type II (free from pyruvate kinase); glucose-6-phosphate dehydrogenase, type V; coen-

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zyme A (CoA); acetylphosphate (lithium salt); thiamine pyrophosphate chloride (TPP); tris(hydroxymethyl)aminomethane (Tris); glucose-6-phosphate (disodium salt); and *Clostridium kluyveri* (dried cells, used as source of phosphotransacetylase).

Crystalline fumarase, DL-isocitrate (disodium salt), phosphoenolpyruvic acid (trisodium salt, A grade), nicotinamide adenine dinucleotide (NAD), reduced nicotinamide adenine dinucleotide ($NADH_2$), and nicotinamide adenine dinucleotide phosphate (NADP) were obtained from Calbiochem.

Glyoxylic acid and phenazine methosulfate (PMS) were obtained from Nutritional Biochemicals Corp., Cleveland, Ohio. All other reagents and compounds were of reagent grade and were used without further purification.

Spectrophotometric analysis. Absorption spectra were determined in a Cary model II recording spectrophotometer. Routine spectrophotometric assays were performed with a Beckman DU spectrophotometer.

Enzyme assays. The determination of malate synthetase activity was performed by a coupled reaction with phosphotransacetylase as described by Ajl (1). Phosphotransacetylase was prepared from *C. kluyveri* as described by Novelli (16) and assayed as described by Stadtman (29).

A coupled reaction with malic dehydrogenase was employed to assay citritase. The reaction mixture contained (in micromoles): $NADH_2$, 0.2; citrate, 1.0; Tris-HCl buffer (*p*H 7.4), 200. Enzyme (containing a minimum of 500 units of malic dehydrogenase) and distilled water were added to bring the volume to 3 ml. The change in absorption was observed at 340 mμ for 5 min. Malic dehydrogenase activity was determined by following $NADH_2$ oxidation as described by Ochoa (18), with the exception that the 0.25 M glycylglycine buffer was replaced with 0.2 M Tris-HCl (*p*H 7.4) buffer.

The chemical transfer of the acetyl group of acetyl-CoA to hydroxylamine and the colorimetric estimation of acetohydroxamate was used to determine acetyl thiokinase activity (10). A similar process (22) was used to assay for acetokinase.

The reduction of 2,6-dichlorophenol-indophenol (DCIP) in the presence of PMS was used to measure succinate oxidation by extracts of *Beggiatoa*. The reaction mixture contained (in micromoles): Tris-HCl (*p*H 8.7), 200; succinate, 150; DCIP, 0.2; sodium cyanide, 10.0. The volume was adjusted to 2.9 ml after addition of enzyme. The reaction was begun by adding 0.1 ml of 0.1% PMS, and the change in absorption was observed for 3 min. The nonspecific reduction of DCIP was determined in reaction mixtures from which succinate was eliminated.

The change in absorption at 252 mμ in the presence of semicarbazide and isocitrate (19) and the spectrophotometric determination of the 2,4-dinitrophenylhydrazone of glyoxylate after incubation of extracts with isocitrate (28) were used to determine isocitritase activity.

Aconitase and fumarase were assayed by spectrophotometric procedures (21).

The reduction of α-lipoic acid by $NADH_2$ (24) was used to determine lipoic dehydrogenase activity. The reaction rate was followed by the decrease in absorption at 340 mμ.

DCIP-linked $NADH_2$ diaphorase was determined by following the decrease in absorption at 605 mμ as described by Mahler (15).

A coupled reaction with malic dehydrogenase (2) was used to determine phosphoenolpyruvate carboxylase activity.

Enolase activity was determined as described by Bücher (4). The coupled reaction with lactic dehydrogenase (5) was used to assay pyruvate kinase. The procedure described by Kornberg (13) was used to assay lactic dehydrogenase activity.

The spectrophotometric determination of DCIP reduction by α-ketoglutarate dehydrogenase (24) and the reduction of NADP by isocitrate dehydrogenase (20) were used to determine these enzymes.

The procedure described by Cohen (7) for glutamic-aspartic transaminase was modified by using malic dehydrogenase to measure oxaloacetate formation.

A Tri-Carb 3000 series automatic liquid scintillation spectrometer was used to determine radioactivity. All counts are corrected for background. Procedures described by Bray (3) for using aqueous solutions in a liquid scintillator were employed. Carrier-free $C^{14}O_2$ was prepared from $BaC^{14}O_3$ (Oak Ridge National Laboratory) and sealed in glass ampoules until used. Fixation of $C^{14}O_2$ by isocitrate dehydrogenase was demonstrated in a system coupled to glucose-6-phosphate dehydrogenase as described by Ochoa (17). Citric acid cycle carboxylic acids were isolated by chromatographic procedures described by Varner (30).

RESULTS

The components required for acetyl thiokinase activity are listed in Table 1. Sodium salts were inhibitory to the reaction, whereas CoA and ATP were required.

TABLE 1. *Acetyl thiokinase activity of Beggiatoa extracts*

Assay system	Units of enzyme activity*
Complete system†	0.70
− Enzyme	0.00
− CoA	0.20
− ATP	0.00
− EDTA (1 μmole)	0.60
+ NaCl (200 μmoles)	0.00
− KF	0.70

* A 1-unit amount of enzyme = 0.4 μmole of acetylhydroxamic acid produced per 20 min.

† Complete system contained in 1.0 ml: 25 units of CoA, 10.0 μmoles of ATP, 20 μmoles of potassium acetate, 100 μmoles of Tris-HCl buffer (*p*H 8.2), 200 μmoles of hydroxylamine, 50 μmoles of KF, 10.0 μmoles of $MgCl_2$, 10.0 μmoles of glutathione, and 0.2 mg of enzyme.

The assay system employing semicarbazide to determine isocitritase detected activity with both dialyzed and undialyzed extracts. When glyoxylate was measured after the enzyme reaction, activity was demonstrable only with dialyzed extracts. Undialyzed extracts removed added glyoxylate.

The 2,4-dinitrophenylhydrazine derivatives were prepared from the end products of enzymatic action on isocitrate. The absorption spectra of these dinitrophenylhydrazones are shown in Fig. 1. The dinitrophenylhydrazones of pyruvate, α-ketoglutarate, and glyoxylate are given in Fig. 2. Comparison of these figures indicates that glyoxylate accumulates when dialyzed extracts are used and that α-ketoglutarate accumulates when NADP is added to the reaction mixture.

The *p*H for maximal isocitritase activity was between 7.8 and 8.2 (Fig. 3).

The oxidation of succinate by whole cells and crude extracts is shown in Fig. 4 and 5. The long lag observed in Fig. 4 for crude extracts to reduce DCIP has been eliminated in Fig. 5 by addition of cyanide to the reaction mixture. The components required for maximal activity and evidence for the inhibitory action of malonate and ferricyanide are presented in Table 2. The optimal pH was 8.7 (Fig. 6). Preliminary attempts to assay succinate oxidation with use of methylene blue or ferricyanide as hydrogen acceptors were

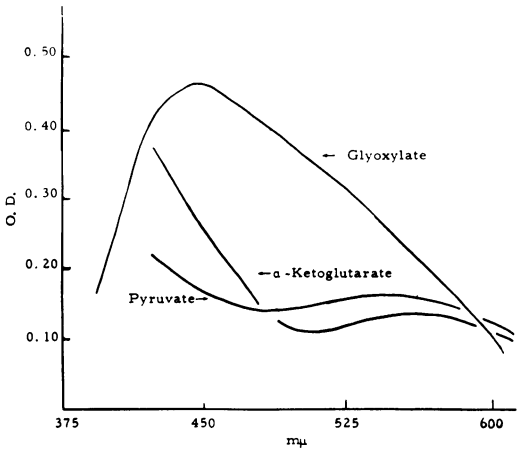

FIG. 2. *Absorption spectra of glyoxylate, pyruvate, and α-ketoglutarate phenylhydrazones. Samples (0.1 μmole in 1 ml of water) of each keto acid were treated with 1 ml of 2,4-dinitrophenylhydrazine (0.1% in 2 N HCl) for five min, after which 2.0 ml of 95% ethyl alcohol, 1.0 ml of water, and 5.0 ml of 1.5 N NaOH were added. Spectra were determined 3 min after addition of NaOH.*

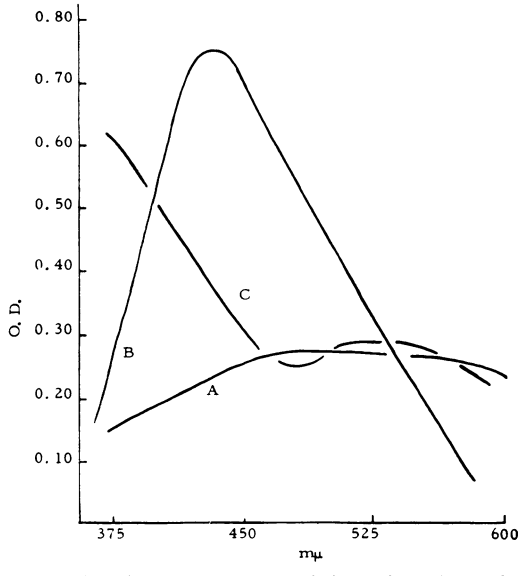

FIG. 1. *Absorption spectra of the end products obtained from the action of cell-free extracts of Beggiatoa on isocitrate during various incubation procedures. All reaction mixtures contained, in 1.5 ml: 20 μmoles of DL-isocitrate, 100 μmoles of Tris-HCl (pH 8.0), 3 μmoles of $MgCl_2$, and 2 μmoles of cysteine. The following additions were made: A, 0.2 mg of undialyzed extract; B, 0.2 mg of dialyzed extract; and C, 0.2 mg of dialyzed extract plus 0.2 μmoles of NADP. Samples (1.0 ml) were assayed for keto acids, as described in Fig. 2.*

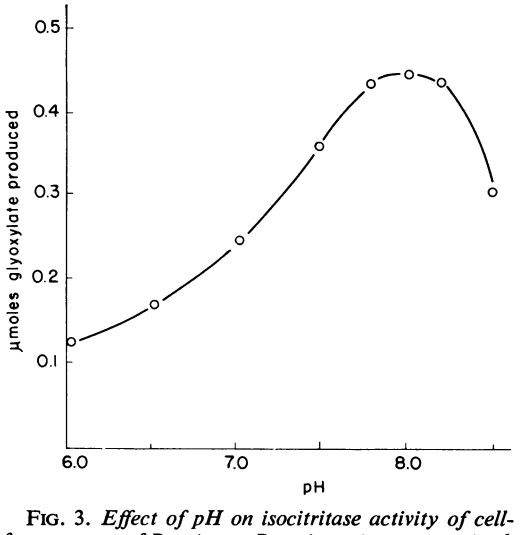

FIG. 3. *Effect of pH on isocitritase activity of cell-free extracts of Beggiatoa. Reaction mixture contained, in 1.5 ml: 100 μmoles of Tris-HCl_2 (at stated pH), 3 μmoles of MgCl, 2 μmoles of cysteine, 5 μmoles of DL-isocitrate, and 0.1 mg of protein. Glyoxylate was determined after 10 min of incubation at 30 C.*

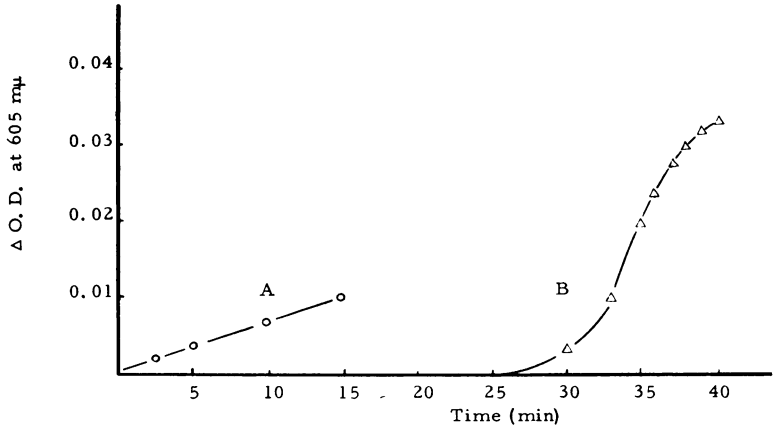

FIG. 4. *Oxidation of succinate by whole cells and crude extracts of Beggiatoa. Reaction mixture contained, in 3.0 ml: 150 μmoles of succinate, 200 μmoles of tris-HCl buffer (pH 7.4), and 0.2 μmole of DCIP. The following additions were made: A, 0.5 ml of whole cells (OD 0.5 at 625 mμ); B, crude extract (0.5 mg of protein). Temperature was maintained at 30 C.*

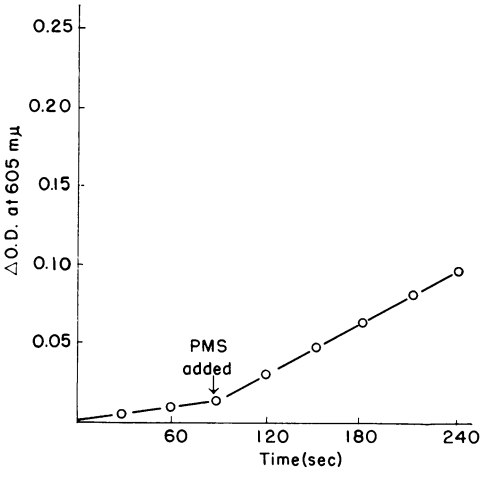

FIG. 5. *Effect of PMS on succinate oxidation by cell-free extracts of Beggiatoa. Assay mixture contained, in 3.00 ml: 200 μmoles of Tris-HCl buffer (pH 7.4), 150 μmoles of succinate, 0.2 μmole of DCIP, 10 μmoles of sodium cyanide, 0.2 mg of protein, and 0.1 ml of 0.1% PMS (added at 90 sec). Values were corrected for nonspecific reduction of DCIP in absence of succinate.*

TABLE 2. *Oxidation of succinate by Beggiatoa extracts*

Assay system	Units of enzyme activity
Complete system*	23
− CN	0
− Succinate	2
− PMS	2
− Enzyme	0
+ Malonate (150 μmoles)	4
+ Ferricyanide (1 μmole)	0
+ EDTA (2 μmoles)	23

* Complete system contained in μmoles: Tris-HCl buffer (*p*H 8.7), 200; succinate, 150; DCIP, 0.2; sodium cyanide, 10. Water and enzyme (0.2 mg) was added to bring volume to 3.0 ml. Reaction was begun by adding 0.1 ml of 0.1% PMS. Enzyme rate was followed by observing the reduction in absorption at 605 mμ.

unsuccessful. The inhibition of DCIP reduction by ferricyanide would, however, indicate that ferricyanide can combine with the enzyme.

Both dialyzed and undialyzed extracts reduced the DCIP-PMS mixture with isocitrate as substrate. Glyoxylate, pyruvate, and α-ketoglutarate did not reduce DCIP or the DCIP-PMS mixture. The addition of lipoic acid, NAD, NADP, TPP, or CoA failed to activate any of the above substrates. Fumarase, citritase, aconitase, catalase, pyruvate kinase, enolase, phosphoenolpyruvate carboxylase, and NAD-linked lactic dehydrogenase were also absent from crude extracts.

The activity of the NADP-linked isocitrate dehydrogenase is illustrated in Fig. 7. NAD could not replace NADP. This reaction was reversible and could be followed in the reverse direction by determining the rate of reduced NADP ($NADPH_2$) oxidation in the presence of α-ketoglutarate and CO_2.

The fixation of CO_2 into α-ketoglutarate to form labeled isocitrate is demonstrated in Table 3.

Crude extracts of *Beggiatoa* had an extremely high rate of CO_2 fixation in the absence of added

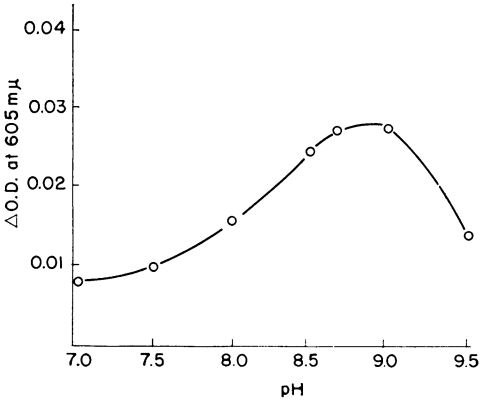

FIG. 6. *Effect of pH on succinate oxidation by Beggiatoa extracts. Assay mixture contained, in 3.0 ml: 400 μmoles of Tris-HCl buffer (at pH stated), 150 μmoles of succinate, 10 μmoles of cyanide, 0.2 mg of protein, and 0.1 ml of 0.1% PMS.*

TABLE 3. *Fixation of CO_2 by isocitrate dehydrogenase*

Assay system	Counts per 10 min
Complete system*	63,010
− $NADPH_2$	10,500
− α-Ketoglutarate	3,570
Heat-denatured extract	15,158

* Complete system contained in 3.0 ml: 5 μc of $C^{14}O_2$, 100 μmoles of Tris-HCl (*p*H 7.4), 3 μmoles of EDTA, 4 μmoles of $MnSO_4$, 0.4 μmole of $NADPH_2$, 80 μmoles of α-ketoglutarate, and *Beggiatoa* extract containing 0.170 mg of protein. All reaction mixtures were incubated for 30 min at 24 C, after which the solutions were placed in a boiling-water bath for 10 min, cooled to room temperature, and flushed for 10 min with N_2. A 100-μmole amount of carrier isocitrate was then added to all reaction mixtures. Samples (0.10 ml) were added to standard scintillation vials, and 10 ml of liquid scintillator solution was added. Radioactivity was determined with window settings of 50 to 1,000 at a gain setting of 20%.

substrates or cofactors. This activity was completely removed by passing the extract through a G-25 Sephadex column and collecting only the first portion of the protein eluate. Full activity was regained only after addition of α-ketoglutarate and $NADPH_2$. This indicates that crude extracts contain considerable amounts of α-ketoglutarate and $NADPH_2$. The radioactivity of the various fractions which were isolated by paper chromatography is presented in Table 4. Isocitrate was the only compound labeled.

The activity of malic dehydrogenase was greater than the activity of the other enzymes

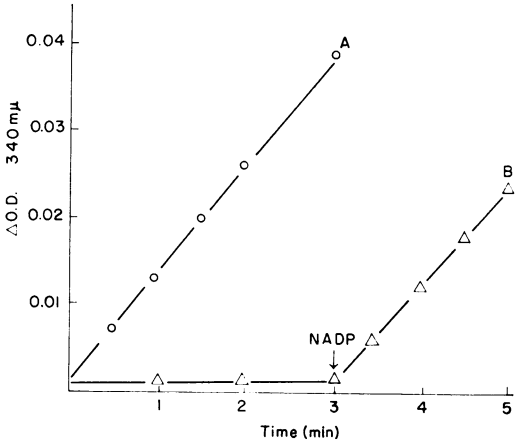

FIG. 7. *Isocitrate dehydrogenase activity of Beggiatoa extracts. Assay mixture contained, in 3.0 ml: 200 μmoles of Tris-HCl buffer (pH 7.4), 0.3 μmole of ethylenediaminetetraacetate, 2.0 μmoles of $MnSO_4$, 0.2 mg of protein, and 80 μmoles of DL-isocitrate. Curve A had 0.2 μmole of NADP added at zero time. Curve B had 0.2 μmole of NAD added at zero time, and 0.2 μmole of NADP added at 3 min.*

TABLE 4. *Radioactivity in various chromatogram spots after $C^{14}O_2$ fixation*

Spot	Disintegrations per min per ml*
Origin	87
Isocitrate	1,074
Citrate	0
Aconitate	0
Malate	0
Succinate	0
Glyoxylate	0
Fumarate	0
α-Ketoglutarate	0

* Count corrected for background and 67% counting efficiency. Reaction mixture contained in 10 ml: 1,230 μmoles of Tris-HCl buffer (*p*H 7.5), 2 μmoles of $MnCl_2$, 64 μmoles of glucose-6-phosphate, 1 μmole of NADP, 10 mg of glucose-6-phosphate dehydrogenase (10 mg will reduce 1,650 μmoles of NADP per min at *p*H 7.4), 3.2 mg of Sephadex-treated *Beggiatoa* extract, 4 μmoles of $NaHCO_2$, (16 μc of $C^{14}O_2$), and 700 moles of α-ketoglutarate. After 1 min of incubation at 25 C, the reaction was stopped by addition of an equal volume of 10% trichloroacetic acid. A 0.5-ml sample was dried on chromatographic paper, and the chromatogram was developed as described by Varner (30). The spots corresponding to the various organic acids were cut from the paper and extracted with water. Radioactivity was determined as described under Materials and Methods.

TABLE 5. *Malate synthetase activity of Beggiatoa extracts*

Assay system	Units of enzyme activity
Complete system*	0.40
2× *Beggiatoa* extract	0.75
2× *Beggiatoa* extract with oxaloacetate replacing glyoxylate	0.10
− *Beggiatoa* extract	0.00
− Phosphotransacetylase	0.00
− Acetylphosphate	0.00
− Glyoxylate	0.00

* Complete system contained in 3.0 ml: 10.0 μmoles of acetylphosphate, 2.0 μmoles of cysteine, 10.0 μmoles of $MgCl_2$, 15 μg of CoA, 25 μmoles of sodium glyoxylate, 50 units of phosphotransacetylase, 50 μmoles of KCl, 30 μmoles of Tris-HCl buffer (*p*H 8.0), and 0.2 mg of enzyme.

found in *Beggiatoa* extracts. The enzymatic reduction of oxaloacetate by $NADH_2$ is shown in Fig. 8.

The glutamic-oxaloacetate transaminase reaction coupled with malic dehydrogenase is shown in Fig. 9. The reaction was begun by adding α-ketoglutarate to the reaction mixture.

The condensation of acetyl-CoA and the glyoxylate by malate synthetase is demonstrated in Table 5. Phosphotransacetylase was used to produce acetyl-CoA from acetylphosphate, and the reaction rate was determined by following acetylphosphate disappearance.

The activity of lipoic dehydrogenase was reduced by one-half by centrifugation (Fig. 10). A DCIP-linked $NADH_2$ diaphorase was also present and was stimulated by PMS.

DISCUSSION

The inability of *Beggiatoa* to metabolize glucose is consistent with the absence of key glycolytic enzymes in cell-free extracts. Likewise, the absence of aconitase, condensing enzyme, α-ketoglutarate dehydrogenase and fumarase, as well as the lack of CO_2 production by growing cultures, rules out the operation of the citric acid cycle. It is possible, however, to devise a scheme employing the enzymes found that is consistent with the data observed. The proposed scheme is summarized in Fig. 11.

The first enzyme in this system, acetyl thiokinase, is similar to acetyl thiokinase found in other sources (10). The activity observed without the added CoA undoubtedly is due to the CoA in crude extracts. Acetyl kinase, which would catalyze the reaction ATP + acetate ⇄ acetylphosphate + ADP, could not be demonstrated with certainty.

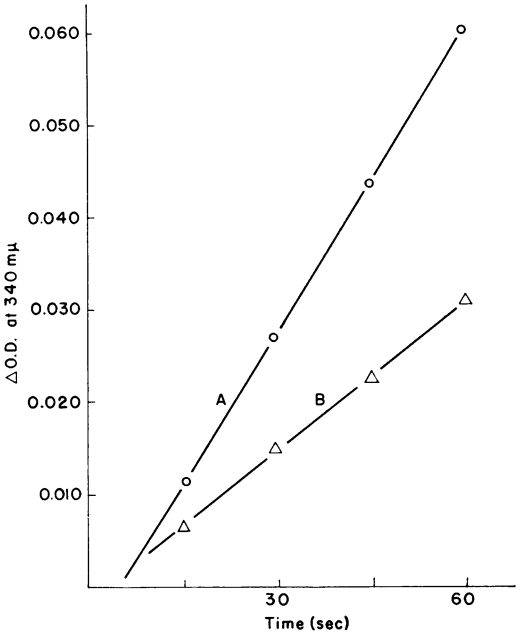

FIG. 8. *Malic dehydrogenase activity of cell-free extracts of Beggiatoa. Reaction mixture contained, in 3.0 ml: 0.2 μmole of $NADH_2$, 200 μmoles of Tris-HCl buffer (pH 7.4), and 0.2 μmole of oxaloacetate. In addition, curve A contained 0.27 mg of protein, and curve B contained 0.135 mg of protein. Temperature was maintained at 25 C.*

Acetyl-CoA may have numerous fates in *Beggiatoa*. It was of interest, however, to determine how acetyl-CoA may be used to produce four-carbon carboxylic acids without key enzymes involved in the citric acid cycle and glyoxylate bypass.

The reversible NADP-linked isocitrate dehydrogenase fixes CO_2 in the direction marked in the proposed scheme. The fact that *Beggiatoa* does not produce CO_2 during growth indicates that the normal direction of this reaction is toward isocitrate production. The procedure used for separation of the components contained in the reaction mixture after $C^{14}O_2$ fixation has a high resolving power for complex mixtures (30). The absence of radioactivity in the other fractions demonstrates that isocitrate is the first compound produced.

The evidence that succinate is produced from isocitrate is provided by the ability of isocitrate to replace succinate in dialyzed extracts for reduction of DCIP. The process which occurs is the production of succinate and glyoxylate from isocitrate and the subsequent oxidation of succinate. The absorption spectra of the phenylhydra-

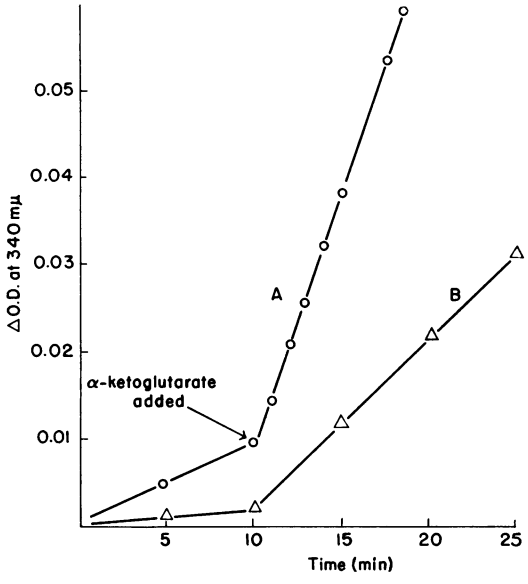

FIG. 9. *Glutamic-oxaloacetate transaminase activity of cell-free extracts of Beggiatoa. Reaction mixture contained, in 3.0 ml: 200 μmoles of Tris-HCl buffer (pH 7.4), 0.2 μmole of $NADH_2$ and 20 μmoles of α-ketoglutarate (added at 10 min). Curve A contained 0.54 mg of protein, and curve B contained 0.27 mg of protein. Temperature was maintained at 25 C.*

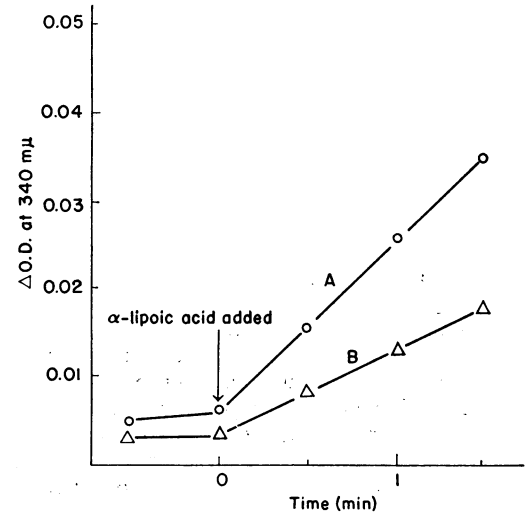

FIG. 10. *Lipoic dehydrogenase activity of cell-free extracts of Beggiatoa. Reaction mixtures contained, in 3.0 ml: 300 μmoles of Tris-HCl buffer (pH 7.4), 0.2 μmole of $NADH_2$, 2.5 μmoles α-lipoic acid, and enzyme (0.54 mg of soluble protein). A crude extract (before centrifugation at 10,000 × g) was used for curve A, and the supernant fluid (after centrifugation at 10,000 × g) was used for curve B.*

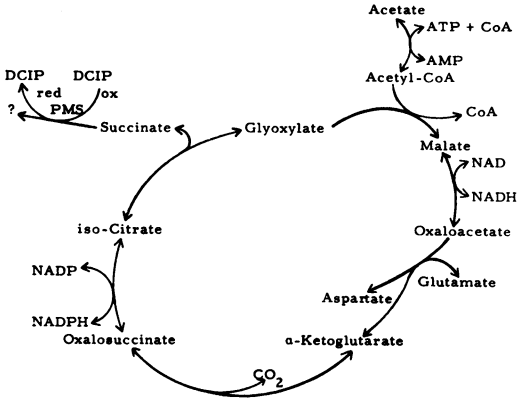

FIG. 11. *Proposed system for utilization of acetate by Beggiatoa.*

zones of the keto acids indicates that glyoxylate is the other product produced.

Malic synthetase and malic dehydrogenase appear similar to enzymes isolated from other sources. The oxidation of succinate is, however, different from the classical succinic dehydrogenase. The oxidation of succinate is not associated with cytochrome *b* or an electron-transport particle, as it is in many organisms (26, 27). Methylene blue and ferricyanide fail to act as hydrogen acceptors for the oxidation of succinate by *Beggiatoa*. This correlates with the observation that purified succinic dehydrogenase fails to react with methylene blue and reacts poorly with ferricyanide (26). Slater (27) suggested that succinate acts with artificial acceptors in the following manner:

$$\text{succinate} \rightarrow \text{SD} \rightarrow \text{cyt.}b \xrightarrow{\text{factor}}$$

$$\downarrow \qquad \downarrow$$

$$K_3Fe(CN)_6 \quad \text{MB}$$

$$\text{PMS} \qquad \text{DCIP}$$

$$\text{cyt.}c_1 \rightarrow \text{cyt.}c \rightarrow \text{cyt.}a \rightarrow \text{cyt.}a_3 \rightarrow O_2$$

$$\downarrow \qquad \downarrow$$

$$\text{cyt.}c \qquad K_3Fe(CN)_6$$

The absence of cytochromes in *Beggiatoa* could explain why methylene blue would not act as a hydrogen acceptor for succinate oxidation by *Beggiatoa*.

The *p*H optimum for the oxidation of succinate (*p*H 8.7) was higher than that reported for heart (*p*H 7.5) and yeast (*p*H 7.8) succinic dehydrogenase (27). Fumarate was not detected as an end product of succinate oxidation, but this assay was hampered by the presence of PMS and DCIP

in the reaction mixture. These observations indicate that succinate oxidation differs considerably from the classical succinic dehydrogenase.

The role of the reduced coenzymes produced in the cycle (from malate and succinate oxidation) and the source of reduced NADP (for isocitrate dehydrogenase) are not known. Pyridine nucleotide transhydrogenase could generate the $NADPH_2$ required for isocitrate dehydrogenase from $NADH_2$ (8). The lipoic dehydrogenase and DCIP-linked diaphorase represent other possible methods for utilization of reduced coenzymes.

The proposed system does not yield energy to the cell. The stimulation of growth by acetate may be due to the use of this compound to build needed carbon skeletons. It is also possible that the carboxylic acids produced in this system generate energy at later steps in their metabolism. Most of the compounds could leave the proposed system and become involved in numerous side reactions. Thus, for example, aspartate is not expected to accumulate as an end product, but could be involved in the synthesis of such compounds as purines, pyrimidines, isoleucine, and lysine. It should be emphasized at this point that the proposed system is not self-sustaining, and that it can be considered only as a small portion of the entire metabolism of this organism.

In the malate synthetase system, oxaloacetate was a very poor acetyl acceptor in comparison to glyoxylate (Table 3). Whether this activity was due to impurities in oxaloacetate, the presence of condensing enzyme in the extract, or the specificity of malate synthetase was not determined. It appears unlikely that the activity was due to the presence of condensing enzyme, since aconitase and citritase were not detectable in extracts, and citrate appeared toxic to growing cultures. The toxicity of added citrate may be due to chelation of essential divalent ions. Citrate produced in vivo could be metabolized via a different pathway and not accumulate to toxic levels.

The absence of certain enzymes from *Beggiatoa* may be related to its natural habitat. In nature, *Beggiatoa* is associated with the presence of hydrogen sulfide. This compound appears to have ready access to the interior of the cell, where it is oxidized. Many of the enzymes that are absent from *Beggiatoa* are either inhibited by hydrogen sulfide or are associated with a sulfide-sensitive system.

Evolutionary patterns would eliminate strains inhibited by hydrogen sulfide and favor strains which employ metabolic systems that could tolerate or even benefit from the presence of hydrogen sulfide. It is interesting to note that many of the enzymes found in *Beggiatoa* are stimulated by, or require, reduced compounds such as cysteine, glutathione, or hydrogen sulfide.

The proposed system accounts for all of the observed information and presumably may account for a considerable portion of the acetate utilized by *Beggiatoa*. *Beggiatoa* grows in vitamin-free medium and also grows, but very poorly, in a mineral-salts medium with acetate and histidine as the only organic compounds. Histidine metabolism in mammalian tissues yields α-ketoglutarate and could thus supply this compound to the system proposed for acetate utilization.

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