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Catabolic and Anabolic Enzyme Activities and Energetics of Acetone Metabolism of the Sulfate-Reducing Bacterium *Desulfococcus biacutus*

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Acetone degradation by cell suspensions of *Desulfococcus biacutus* was CO_2 dependent, indicating initiation by a carboxylation reaction, while degradation of 3-hydroxybutyrate was not CO_2 dependent. Growth on 3-hydroxybutyrate resulted in acetate accumulation in the medium at a ratio of 1 mol of acetate per mol of substrate degraded. In acetone-grown cultures no coenzyme A (CoA) transferase or CoA ligase appeared to be involved in acetone metabolism, and no acetate accumulated in the medium, suggesting that the carboxylation of acetone and activation to acetoacetyl-CoA may occur without the formation of a free intermediate. Catabolism of 3-hydroxybutyrate occurred after activation by CoA transfer from acetyl-CoA, followed by oxidation to acetoacetyl-CoA. In both acetone-grown cells and 3-hydroxybutyrate-grown cells, acetoacetyl-CoA was thiolytically cleaved to two acetyl-CoA residues and further metabolized through the carbon monoxide dehydrogenase pathway. Comparison of the growth yields on acetone and 3-hydroxybutyrate suggested an additional energy requirement in the catabolism of acetone. This is postulated to be the carboxylation reaction ($\Delta G^{\circ\prime}$ for the carboxylation of acetone to acetoacetate, $+17.1\ kJ \cdot mol^{-1}$). At the intracellular acyl-CoA concentrations measured, the net free energy change of acetone carboxylation and catabolism to two acetyl-CoA residues would be close to 0 $kJ \cdot mol$ of acetone^{-1}, if one mol of ATP was invested. In the absence of an energy-utilizing step in this catabolic pathway, the predicted intracellular acetoacetyl-CoA concentration would be 10^{13} times lower than that measured. Thus, acetone catabolism to two acetyl-CoA residues must be accompanied by the utilization of the energetic equivalent of (at least) one ATP molecule. Measurement of enzyme activities suggested that assimilation of acetyl-CoA occurred through a modified citric acid cycle in which isocitrate was cleaved to succinate and glyoxylate. Malate synthase, condensing glyoxylate and acetyl-CoA, acted as an anaplerotic enzyme. Carboxylation of pyruvate or phosphoenolpyruvate could not be detected.

The anaerobic metabolism of acetone appears to involve an initial carboxylation of acetone to acetoacetate (or acetoacetyl coenzyme A [acetoacetyl-CoA]), followed by thiolytic cleavage to two acetyl-CoA residues (3, 32–35). The carboxylation reaction has not been measured in vitro, and evidence has come from ^{14}C labelling studies (32, 33, 35). Carboxylation reactions appear to play a role in a number of anaerobic transformations (16, 44), but these reactions have not been well studied. The carboxylation of acetone to acetoacetate, $CH_3COCH_3 + HCO_3^- \rightarrow CH_3COCH_2COO^- + H_2O$ ($\Delta G^{\circ\prime}$, +17.1 kJ · mol of acetone^{-1}), is an endergonic reaction (42). Thus, the energetic equivalent of about 1/3 mol of ATP ($\Delta G^{\circ\prime}$ of ATP hydrolysis, $-50\ kJ \cdot mol^{-1}$ [42]) is required, but the mechanism of coupling is not known. In addition, attempts to measure the carboxylase in sulfate-reducing bacteria have remained unsuccessful (21).

Many genera of sulfate-reducing bacteria are able to oxidize completely (to CO_2) the organic compounds they catabolize, and presumably they also assimilate intermediates to meet requirements for assimilatory metabolism. *Desulfobacter* species have a modified citric acid cycle (6, 14, 27, 38), while *Desulfovibrio* species and *Desulfobulbus propionicus* have some citric acid cycle enzyme activities but the cycle is incomplete (23, 25, 26, 40). Most genera of completely oxidizing sulfate-reducing bacteria oxidize acetyl-CoA to CO_2 via the acetyl-CoA–carbon monoxide dehydrogenase pathway (37, 41). Except for any compounds which may be synthesized directly from the growth substrate or its metabolic intermediates, the majority of cellular carbon must be derived from acetyl-CoA (and CO_2). This would necessitate the operation of at least a partial citric acid cycle in such organisms.

In the acetone-utilizing sulfate-reducing bacterium *Desulfococcus biacutus*, acetyl-CoA is oxidized to CO_2 via the acetyl-CoA–carbon monoxide dehydrogenase pathway (35). We further investigated the catabolic activities involved in acetone metabolism in *D. biacutus*, and we measured anabolic enzyme activities, which allowed us to postulate the major assimilatory pathways of this bacterium. In addition, measurement of intracellular acyl-CoA esters, and kinetic characterization of key enzymes, allowed us to make predictions about the initial carboxylation reaction.

MATERIALS AND METHODS

Organism and culture conditions. *D. biacutus* strain KMRActS (35) had been maintained in liquid culture since its isolation. Stock cultures were grown in completely filled 50-ml screw-cap glass bottles by using a sulfide-reduced bicarbonate-buffered defined mineral medium supplemented with a seven-vitamin mixture (11), with the addition of 10 mM acetone and 20 mM Na_2SO_4. Larger culture volumes were grown in 250-ml glass bottles containing 200 ml of the same medium under a headspace of N_2 plus CO_2 (80:20, vol/vol) and sealed with black rubber stoppers. All cultures were incubated in the dark at 30°C.

Preparation of cell suspensions and extracts. Cells were harvested in the late logarithmic phase (25 days for acetone-grown cultures and 15 to 18 days for 3-hydroxybutyrate-grown cultures) by centrifugation under strictly anoxic conditions as previously described (20), and then they were washed and resuspended in anoxic 125 mM triethanolamine · HCl (pH 7.4) containing 2.5 mM dithioerythritol. Cell extracts were prepared by French press treatment as described elsewhere (20).

Cell suspensions (0.3 to 0.5 mg of protein · ml^{-1}) were prepared by centrifu-

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gation under strictly anoxic conditions, and then the cells were washed and resuspended in anoxic 50 mM potassium phosphate buffer (pH 7.2) supplemented with 20 mM Na_2SO_4 and 2.5 mM dithioerythritol and containing 1.0 g of NaCl per liter and 0.6 g $MgCl_2 \cdot 6H_2O$ per liter. The suspensions were incubated at 30°C sealed in glass vials (30 ml) under a headspace either of N_2 plus CO_2 (80:20, vol/vol) with the addition of 30 mM $NaHCO_3$ to the buffer or of N_2 (without addition of $NaHCO_3$). An appropriate substrate was then added (the cells had been grown on the same substrate), and degradation was monitored by taking samples by syringe for analysis.

Enzyme assays. Assays were carried out at 30°C by using a 100-40 photometer (Hitachi, Tokyo, Japan) with 1-ml volumes in 1.5-ml semimicrocuvettes under N_2 unless noted otherwise. Linearity with added crude extract was tested, and dependence on key additions in the test (e.g., substrate, CoA, and ADP) was checked. All enzyme assays were carried out under strictly anoxic conditions (7). Enzyme activities are expressed as micromoles of substrate transformed per milligram of crude cell extract protein, calculated from published extinction coefficients (28). The reduction of 2 μmol of methylviologen was defined as the oxidation of 1 μmol of substrate. The Michaelis constant (K_m) and maximum velocity were calculated from best fits by using linear regression fitted to Lineweaver-Burk double reciprocal plots of the data and the specific activities at specified substrate concentrations derived from these fit equations, all with a computer graphics package (Origin version 2.94; MicroCal Software, Northampton, Md.).

Acetyl-CoA:3-hydroxybutyrate CoA transferase (EC 2.8.3.-) and acetyl-CoA: acetoacetate CoA transferase (EC 2.8.3.-) were assayed as described by Janssen and Schink (20), by using 10 mM sodium DL-3-hydroxybutyrate plus 2 mM NAD^+ and 10 mM lithium acetoacetate plus 2 mM NADH, respectively. 3-Hydroxybutyrate CoA ligase (EC 6.2.1.-) and acetoacetate CoA ligase (EC 6.2.1.16) were assayed by the acetate thiokinase assay of Oberlies et al. (30), the reaction being started with 1 mM sodium DL-3-hydroxybutyrate and 1 mM lithium acetoacetate, respectively, and by assay *b* of Fukui et al. (13), by using 0.5 mM sodium DL-3-hydroxybutyrate plus 2 mM NAD^+ and 0.5 mM lithium acetoacetate plus 0.2 mM NADH, respectively. 3-Hydroxybutyrate dehydrogenase (EC 1.1.1.30), 3-hydroxybutyryl-CoA dehydrogenase (EC 1.1.1.35/36/157), and isopropanol dehydrogenase (EC 1.1.1.80) were all tested with NADH or NADPH as described by Platen et al. (35). 3-Enoyl-CoA hydratase (EC 4.2.1.17/55), with bovine serum albumin omitted and the NAD^+ concentration increased to 2 mM, and acetyl-CoA acetyltransferase (EC 2.3.1.9) were assayed as described by Wofford et al. (46). Carbon monoxide dehydrogenase (EC 1.2.99.2) was measured as described by Diekert and Thauer (9), and formate dehydrogenase (EC 1.2.1.2) was assayed in the same way except under N_2 with the addition of 10 mM sodium formate.

Pyruvate kinase (EC 2.7.1.40) was assayed as described by Bergmeyer et al. (1), and the assay was started with 5 mM ADP. Pyruvate P_i dikinase (EC 2.7.9.1) and pyruvate water dikinase (EC 2.7.9.2) were assayed by using the same method, but starting with 5 mM AMP plus 20 mM Na_4 PP_i and 5 mM AMP plus 20 mM KH_2PO_4, respectively. Pyruvate synthase (EC 1.2.7.1) was measured as described by Diekert and Thauer (9). ATP:citrate lyase (EC 4.1.3.8) was assayed by the methods of Brune and Schink (7) and Schauder et al. (38). Assays for isocitrate lyase (EC 4.1.3.1), the reaction being started with 1.2 mM DL-isocitrate, and malate synthase (EC 4.1.3.2) were as described by Dixon and Kornberg (10).

The following enzymes were assayed as described by Brune and Schink (7): phosphoenolpyruvate (PEP) carboxylase (EC 4.1.1.31), PEP carboxykinase (EC 4.1.1.32), pyruvate carboxylase (EC 6.4.1.1), citrate synthase (EC 4.1.3.7), isocitrate dehydrogenase (EC 1.1.1.42) with either NAD^+ or $NADP^+$, malate dehydrogenase (EC 1.1.1.37) with either NADH or NADPH, fumarase (EC 4.2.1.2), succinate dehydrogenase (EC 1.3.99.1) with $K_3Fe(CN)_6$, 2-oxoglutarate synthase (EC 1.2.7.3), 2-oxoglutarate dehydrogenase complex (EC 1.2.4.1, etc.) with either NAD^+ or $NADP^+$, succinyl-CoA:acetate CoA transferase (EC 2.8.3.-), succinyl-CoA:acetoacetate CoA transferase (EC 2.8.3.5), and succinate CoA ligase (EC 6.2.1.4/5).

Aconitase (EC 4.2.1.3) was assayed with the following reaction mixture: 100 mM Tris-HCl (pH 8.3), 10 mM $MgCl_2$, 2.5 mM dithioerythritol, 2.5 mM $NADP^+$, 0.5 U of isocitrate dehydrogenase, 5 mM Na_3 citrate.

Acyl-CoA ester determination. Dense suspensions (1 ml) of acetone-grown cells (3.5 to 5.5 mg of protein $\cdot$ ml^{-1}) were incubated in 2-ml vials at 30°C in anoxic 50 mM potassium phosphate buffer (pH 7.2) supplemented with 20 mM Na_2SO_4, 30 mM $NaHCO_3$, and 2.5 mM dithioerythritol, and containing 1.0 g of NaCl per liter and 0.6 g of $MgCl_2 \cdot 6H_2O$ per liter, under a headspace of N_2 plus CO_2 (80:20, vol/vol). Acetone (5 mM) was added, and then 200-μl samples were taken at various intervals by using accurate Unimetrics syringes (Machery-Nagel, Düren, Germany) and added to 25 μl of perchloric acid. The acidified samples were allowed to stand for 30 min and then centrifuged in a benchtop microcentrifuge at 5,000 × *g*. The acyl-CoA esters were separated by reversed-phase high-performance liquid chromatography (7, 15) and quantified by comparison with standards of acyl-CoA esters also treated with perchloric acid. The acyl-CoA esters were stable under the experimental conditions used. Intracellular acyl-CoA ester concentrations remained constant throughout the 5-min experiment. To calculate intracellular concentrations, we used the cell volume (3.2 μl $\cdot$ mg of $protein^{-1}$) for *D. propionicus*, a sulfate-reducing bacterium similar in size (35, 45), as determined by Kreke and Cypionka (22).

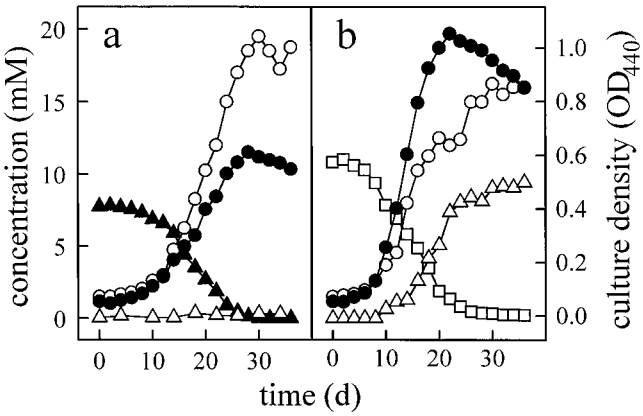

FIG. 1. Growth of *D. biacutus* on acetone plus sulfate (a) and 3-hydroxybutyrate plus sulfate (b). Symbols: ▲, acetone; □, 3-hydroxybutyrate; △, acetate; ○, sulfide; ●, culture density measured at 440 nm. OD_{440}, optical density at 440 nm.

Analytical procedures. Protein was quantified by the microassay of Bradford (5) by using bovine serum albumin as a standard. Sulfide was measured as described by Trüper and Schlegel (43). Acetate and 3-hydroxybutyrate were measured by high-performance liquid chromatography (12). Acetone was measured colorimetrically in 1.5-ml plastic reaction vessels as follows. Samples (100 μl) were added to 100 μl of 0.1% 2,4-dinitrophenylhydrazine in 2 M HCl, care being taken to avoid loss of acetone by slowly introducing the sample below the reagent surface. After the addition of 500 μl of water and 100 μl of 10 M NaOH, the test solutions were thoroughly mixed and then centrifuged (5 min) in a benchtop microcentrifuge at 5,000 × *g*. The A_{540} was measured, and the concentration was calculated from a standard curve after correction for a medium or buffer blank.

RESULTS

Growth on acetone and 3-hydroxybutyrate. *D. biacutus* grew on acetone (Fig. 1a), with sulfate as the terminal electron acceptor, with a doubling time of 136 h ($\mu = 0.122$ day^{-1}). With 3-hydroxybutyrate plus sulfate (Fig. 1b), the doubling time was 72 h ($\mu = 0.230$ day^{-1}). Sulfide was produced concomitantly with growth and substrate utilization. Acetate accumulated in cultures growing on 3-hydroxybutyrate at a little less than 1 mol $\cdot$ mol of substrate $metabolized^{-1}$. Cultures

TABLE 1. Growth yields and substrate degradation balances of *D. biacutus*

Growth substrate	Amt of substrate degraded (μmol)	Amt of product (μmol)		Sulfide recovery (%)[a]	Cell yield (mg)	Y_s (g $\cdot$ mol of $substrate^{-1}$)[b]	$Y_{sulfate}$ (g $\cdot$ mol of $sulfate^{-1}$)[c]
		Acetate	Sulfide				
Acetone	1,566	0	3,100	99	16.7	10.7	5.39
3-Hydroxybutyrate	1,928	1,254	2,880	93	28.6	14.8	9.93

[a] Expressed as a percentage of the theoretical sulfide production (see equations in Discussion).
[b] Y_s, specific dry mass growth yield in batch culture.
[c] $Y_{sulfate}$, growth yield normalized on the basis of sulfate reduced. The amount of sulfate reduced was considered equivalent to the amount of sulfide produced.

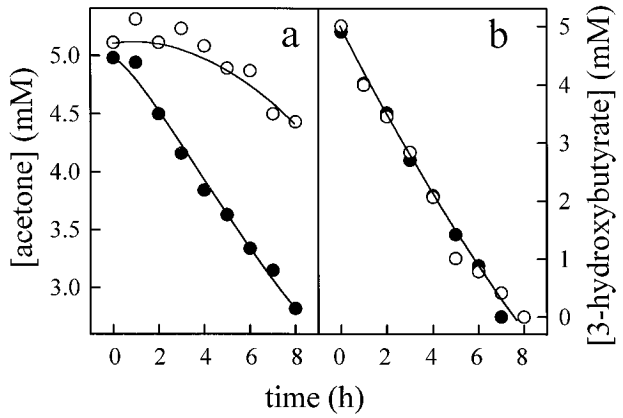

FIG. 2. Degradation of acetone (a) and 3-hydroxybutyrate (b) by cell suspensions of *D. biacutus* in the presence (●) and absence (○) of CO_2 and HCO_3^-.

growing on acetone accumulated only traces (less than 0.1 mM) of acetate. The specific dry mass growth yields in batch culture were 10.7 g · mol of acetone^{-1} and 14.8 g · mol of 3-hydroxybutyrate^{-1} (Table 1). If the growth yields are normalized on the basis of sulfate reduced, the yields were 5.39 g · mol of sulfate^{-1} on acetone and 9.93 g · mol of sulfate^{-1} on 3-hydroxybutyrate. From the specific growth rates and specific growth yields, the in vivo substrate turnover rates in growing cultures were calculated to be 0.019 μmol · min^{-1} · mg of protein^{-1} on acetone and 0.025 μmol · min^{-1} · mg of protein^{-1} on 3-hydroxybutyrate.

Substrate degradation in cell suspensions. Acetone was degraded by cell suspensions of *D. biacutus* with an initial specific activity of 7.0 nmol · min^{-1} · mg of protein^{-1} in the presence of CO_2 (Fig. 2a). In the absence of added CO_2, the acetone degradation rate was only 0.2 nmol · min^{-1} · mg of protein^{-1}. 3-Hydroxybutyrate degradation was not dependent on the presence of CO_2 (9.5 and 9.7 nmol · min^{-1} · mg protein^{-1} in the presence and absence of CO_2, respectively; Fig. 2b).

Enzymology of acetone degradation. Acetyl-CoA acetyltransferase (3-ketothiolase) activity in crude cell extracts of acetone-grown cells of *D. biacutus* (harvested in the late logarithmic phase) was measured (Table 2). This enzyme displayed an apparent K_m for acetoacetyl-CoA of 77 μM, and it displayed classic Michaelis-Menten kinetics. Formate dehydrogenase and carbon monoxide dehydrogenase activities, both measured with methylviologen as an artificial acceptor, were present (Table 2). 3-Hydroxybutyryl-CoA dehydrogenase (with NAD^+) and 3-enoyl-CoA hydratase (tested with crotonyl-CoA) activities were also detected (Table 2). The 3-hydroxybutyryl-CoA dehydrogenase activity in crude cell extracts of *D. biacutus* had an apparent K_m for acetoacetyl-CoA of 18 μM, and it also displayed classic Michaelis-Menten kinetics.

Enzymology of 3-hydroxybutyrate degradation. Cells grown on 3-hydroxybutyrate contained a CoA transferase able to activate 3-hydroxybutyrate and a 3-hydroxybutyryl-CoA dehydrogenase coupled to NAD^+ reduction. Acetyl-CoA acetyltransferase and enzymes of the carbon monoxide dehydrogenase pathway were also present (Table 2).

Anabolic enzyme activities. In *D. biacutus*, pyruvate synthase activity, utilizing methylviologen as an artificial electron acceptor and dependent on the addition of CoA in the direction tested (pyruvate to acetyl-CoA), was present. In addition, pyruvate kinase was also present, but no activities resulting in oxaloacetate formation from either pyruvate or PEP were detected (Table 3).

TABLE 2. Catabolic enzyme activities in crude cell extracts of acetone-grown and 3-hydroxybutyrate-grown *D. biacutus*[a]

Enzyme activity[b]	Sp act (nmol · min^{-1} · mg of protein^{-1})[c]	
	Acetone-grown cells	3-Hydroxybutyrate-grown cells
1 Acetyl-CoA:3-hydroxybutyrate CoA transferase	7–9	20–24
2 3-Hydroxybutyryl-CoA dehydrogenase[d]	418–471	272–283
3 3-Enoyl-CoA hydratase (crotonase)	69,204–72,088	53,676–70,956
4 Acetyl-CoA acetyltransferase (3-ketothiolase)	2,416–3,019	8,177–8,919
5 Formate dehydrogenase	2,474–3,013	7,539–8,123
6 Carbon monoxide dehydrogenase	233–299	604–689

[a] The following activities were not detected (<1 nmol · min^{-1} · mg of protein^{-1}): isopropanol dehydrogenase (NAD^+ or $NADP^+$), 3-hydroxybutyrate dehydrogenase (NAD^+ or $NADP^+$), 3-hydroxybutyryl-CoA dehydrogenase ($NADP^+$), acetyl-CoA:acetoacetate CoA transferase, 3-hydroxybutyrate CoA ligase, and acetoacetate CoA ligase.
[b] The numbers of the enzyme activities are those of Fig. 3.
[c] Ranges of activities obtained from three to five determinations are presented.
[d] NAD^+ was the electron acceptor.

Citrate synthase activity was detected, but ATP:citrate lyase was not (Table 3). Aconitase, isocitrate dehydrogenase (with $NADP^+$ but not with NAD^+), malate dehydrogenase (with NAD^+ but not with $NADP^+$), fumarase, and succinate dehydrogenase (using $Fe(CN)_6^{3-}$ as an artificial acceptor) were all present (Table 3). Enzymes oxidatively decarboxylating 2-oxoglutarate were not detected. Isocitrate lyase and malate synthase activities were also present. The isocitrate lyase activity was at a very low level, but it was still easily measurable, since the assay is very sensitive (10).

Intracellular acyl-CoA esters. The major acyl-CoA esters measured in dense cell suspensions catabolizing acetone in the presence of sulfate were acetoacetyl-CoA (1.58 nmol · mg of protein^{-1}), acetyl-CoA (0.89 nmol · mg of protein^{-1}), 3-hydroxybutyryl-CoA (1.08 nmol · mg of protein^{-1}), and crotonyl-CoA (0.14 nmol · mg of protein^{-1}), while coenzyme A was

TABLE 3. Anabolic enzyme activities in crude cell extracts of acetone-grown *D. biacutus*[a]

Enzyme activity (acceptor)[b]	Sp act (nmol · min^{-1} · mg of protein^{-1})[c]
7 Citrate synthase	35–51
8 Aconitase	11–14
9 Isocitrate dehydrogenase ($NADP^+$)	31–47
10 Isocitrate lyase	1–3
11 Succinate dehydrogenase	149–212
12 Fumarase	1,852–1,901
13 Malate synthase	55–63
14 Malate dehydrogenase (NAD^+)	6,231–6,565
15 Pyruvate synthase	336–363
16 Pyruvate kinase	10–14

[a] The following activities were not detected (<1 nmol · min^{-1} · mg of protein^{-1}): 2-oxoglutarate synthase, 2-oxoglutarate dehydrogenase complex (NAD^+ or $NADP^+$), ATP:citrate lyase, malate dehydrogenase ($NADP^+$), isocitrate dehydrogenase (NAD^+), PEP carboxylase, PEP carboxykinase, pyruvate carboxylase, pyruvate P_i dikinase, pyruvate water kinase, succinate CoA ligase, succinyl-CoA:acetate CoA transferase, and succinyl-CoA:acetoacetate CoA transferase.
[b] The numbers of the enzyme activities are those of Fig. 3.
[c] Ranges of activities obtained from three to six determinations are shown.

detected at approximately 0.3 nmol · mg of protein^{-1}. By using a cell volume of 3.2 μl · mg of protein^{-1}, the intracellular concentrations were calculated to be 494 μM acetoacetyl-CoA, 278 μM acetyl-CoA, 336 μM 3-hydroxybutyryl-CoA, 43 μM crotonyl-CoA, and 94 μM CoASH.

DISCUSSION

Substrate degradation. The substrate transformation stoichiometries of *D. biacutus* on acetone and 3-hydroxybutyrate, with sulfate as the terminal electron acceptor, agreed with the following equations ($\Delta G^{\circ\prime}$ values were calculated as described by Thauer et al. [42]): $CH_3COCH_3 + SO_4^{2-} \rightarrow HCO_3^- + HS^- + H^+$ ($\Delta G^{\circ\prime}$, −125.6 kJ · mol of acetone^{-1}) and $CH_3CHO\ HCH_2COO^- + 1^1/_4 SO_4^{2-} \rightarrow CH_3COO^- + 2HCO_3^- + 1^1/_4\ HS^- + {}^3/_4 H^+$ ($\Delta G^{\circ\prime}$, −120.4 kJ · mol of 3-hydroxybutyrate^{-1}). Since the acetate formed is the product of a CoA transferase reaction (see below), ATP formation can presumably only be coupled to reactions of the carbon monoxide dehydrogenase pathway and to sulfate reduction. The growth yields, normalized to sulfate to take this into account (Table 1), suggest that the net ATP yield on acetone is lower than that on 3-hydroxybutyrate. *D. biacutus* also displayed a lower specific growth rate on acetone compared with growth on 3-hydroxybutyrate, a substrate not requiring carboxylation. These results indicate that energy has to be invested in the initiation of acetone degradation. The lower growth rate and yield on acetone are not likely to be due to transport, since acetone will readily traverse the membrane, but they could be the result of the endergonic carboxylation of acetone to acetoacetate (+17.1 kJ · mol^{-1} [42]). The requirement for energy in the form of ATP for the carboxylation has been postulated previously (35). A requirement for CO_2 for the catabolism of acetone supports the hypothesis that a carboxylation step is involved in this catabolism. It appears that carboxylation is generally the first step in anaerobic acetone degradation (3, 32, 33, 35).

In cultures growing on 3-hydroxybutyrate, acetate accumulated in the growth medium at approximately 1 mol of acetate per mol of substrate utilized. In addition, CoA transferase activities were detected in 3-hydroxybutyrate-grown cells, but no ligase activities were detected. In acetone-grown cultures, in contrast, there was no acetate accumulation, and there were also no CoA ligase activities activating either acetoacetate or 3-hydroxybutyrate. This suggests that the activation of the product of acetone carboxylation may be catalyzed by the same enzyme (complex) catalyzing the carboxylation.

Catabolic pathways. The enzyme activities detected in cell extracts of acetone-grown cells of *D. biacutus* were consistent with a pathway in which acetoacetyl-CoA was thiolytically cleaved to two acetyl-CoA moieties, which were then completely oxidized through the carbon monoxide dehydrogenase pathway (Fig. 3). These activities were also detected in an earlier study of *D. biacutus* (35).

We attempted to measure the incorporation of $^{14}CO_2$ into intracellular acyl-CoA esters in dense cell suspensions metabolizing acetone (21). A rapid labelling of the acyl-CoA esters followed the addition of the radioactive CO_2, but control experiments with 3-hydroxybutyrate produced similar results, suggesting that the incorporation of label was due to the rapid exchange catalyzed by enzymes of the carbon monoxide dehydrogenase pathway. An exchange between CO_2 and the carboxyl group of acetyl-CoA has been shown to occur in a number of sulfate-reducing bacteria which oxidize acetyl-CoA via this pathway (19, 37, 39).

Acyl-CoA esters in acetone-metabolizing cells of acetone-grown *D. biacutus* could be measured. The intracellular acyl-CoA ester concentrations were in the same range as those measured for *Clostridium kluyveri* (36) and *Clostridium acetobutylicum* (4, 17), and the esters were those postulated (Fig. 3) to be involved in the oxidation of acetone and the formation of poly-β-hydroxybutyrate. The concentration of acetoacetyl-CoA was around 500 μM, about six times higher than the K_m of the acetyl-CoA acetyltransferase (77 μM), meaning that this activity is not substrate limited in vivo.

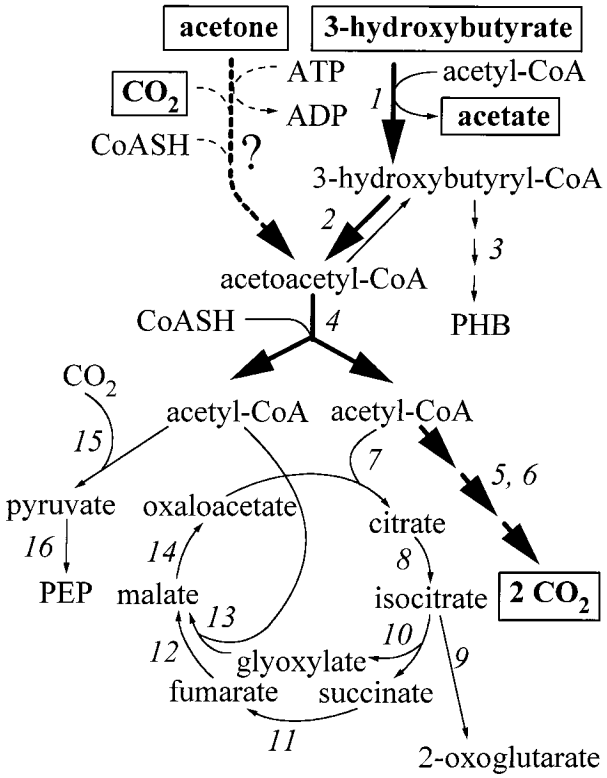

FIG. 3. Catabolic and anabolic enzyme activities detected in *D. biacutus* grown on acetone and 3-hydroxybutyrate. The thick arrows indicate catabolic enzyme activities, and the thin arrows indicate other activities. The specific activities are given in Tables 2 and 3. The carboxylation of acetone could not be measured in vitro.

The K_m for acetoacetyl-CoA determined for the 3-hydroxybutyryl-CoA dehydrogenase of *D. biacutus* was 18 μM, similar to those found for the same enzyme in other PHB-forming bacteria (8, 18, 29, 31). This enzyme, together with the detected 3-enoyl-CoA hydratase (crotonase), is presumably involved in PHB synthesis. In vivo, this enzyme is not substrate limited, since the intracellular acetoacetyl-CoA concentration was about 500 μM. This means that PHB synthesis is favored. However, the 3-hydroxybutyryl-CoA dehydrogenase from *Clostridium beijerinckii* is inhibited by acetoacetyl-CoA at low NADH concentrations (8), meaning that only if NADH cannot be reoxidized will acetoacetyl-CoA be channeled to PHB formation rather than being cleaved to acetyl-CoA. Allosteric regulation of this enzyme in *D. biacutus* was not investigated.

Anabolic pathways. Cell matter in acetone-grown cultures has to be synthesized from acetone, acetoacetyl-CoA, 3-hydroxybutyryl-CoA, crotonyl-CoA, and acetyl-CoA. In *D. biacutus*, the enzymes of an incomplete citric acid cycle were present (Fig. 3), with generally low activities. These activities probably produce the precursors for biosynthetic pathways for cell growth. Acetyl-CoA from catabolic metabolism can condense with oxaloacetate to form citrate by using a citrate syn-

thase. ATP:citrate lyase activity was not detected. Aconitase activity indicated the transformation of citrate to isocitrate. Isocitrate could be oxidatively decarboxylated to 2-oxoglutarate by an $NADP^+$-dependent isocitrate dehydrogenase (NAD^+ did not act as an electron acceptor). 2-Oxoglutarate was not oxidatively decarboxylated to succinyl-CoA, either with pyridine nucleotides (NAD^+ or $NADP^+$) or with methylviologen (as a substitute for ferredoxin). Instead, isocitrate can be cleaved to succinate and glyoxylate by an isocitrate lyase activity. Succinate dehydrogenase [with $Fe(CN)_6^{3-}$ as an artificial electron acceptor], fumarase, and malate dehydrogenase with NAD^+ (but not $NADP^+$) as an electron acceptor are able to regenerate oxaloacetate for condensation with further acetyl-CoA.

Activities of a possible anaplerotic sequence were also detected, in the form of malate synthase, condensing acetyl-CoA with glyoxylate formed by the activity of isocitrate lyase (Fig. 3). The pathway allows one four-carbon dicarboxylic acid molecule to be assimilated into cell carbon per two acetyl-CoA molecules entering the pathway. No glyoxylate-forming or -utilizing activities and no sequence converting isocitrate to succinate were detected in *Desulfovibrio* spp. (26). Thus, in *Desulfovibrio* spp., the citric acid cycle enzymes detected do not act to form a cycle, but rather they form sequences to yield metabolic intermediates for biosynthetic purposes. In *Desulfobacter* spp., the citric acid cycle serves to oxidize acetyl-CoA to CO_2, or to reduce CO_2 to acetyl-CoA (6, 14, 38). In *D. biacutus*, the modified citric acid cycle appears to form biosynthetic precursors from acetyl-CoA.

In *D. biacutus*, PEP was formed from pyruvate by pyruvate kinase (Fig. 3). In *Desulfovibrio* spp., pyruvate carboxylase apparently acts to synthesize oxaloacetate for the citrate synthase reaction (26). In *Desulfobacter hydrogenophilus*, pyruvate synthase, pyruvate kinase, and PEP carboxylase act as an anaplerotic sequence, synthesizing oxaloacetate to compensate for intermediates of the cycle used for biosynthesis (38). It seems likely that in *D. biacutus* the production of PEP serves to provide the precursor for gluconeogenic sugar synthesis.

Energetics of acetone metabolism. Assuming that acetone diffuses freely through the membrane and that the concentrations inside and outside the cells are about equal, and using a concentration of 5 mM for acetone, 30 mM HCO_3^- for CO_2, the measured concentrations of acetoacetyl-CoA and CoASH, and a $\Delta G^{\circ\prime}$ of -35.6 kJ · mol^{-1} for the hydrolysis of an acyl-CoA ester (42), the free energy change for the carboxylation and activation to acetoacetyl-CoA can be calculated. The reaction is as follows: $CH_3COCH_3 + HCO_3^- + CoASH \rightarrow CH_3COCH_2COSCoA + 2H_2O$ ($\Delta G^{\circ\prime} = +52.7$ kJ · mol of acetone^{-1}; $\Delta G^{\prime} = +79.1$ kJ · mol of acetone^{-1} [at 30°C]). This reaction is even more endergonic under physiological conditions ($\Delta G^{\prime}$) than under standard ($\Delta G^{\circ\prime}$) conditions. The acetyl-CoA acetyltransferase reaction (3-ketothiolase) has a free energy change under standard conditions of -25.1 kJ · mol of acetoacetyl-CoA^{-1} in the direction of acetyl-CoA formation (42). At in vivo concentrations of reactants and products, this free energy change shifts only slightly (-23.8 kJ · mol of acetoacetyl-CoA^{-1} at 30°C), and it is not sufficient to pull both the carboxylation and activation reactions. This means that the initial reaction in the metabolism of acetone by *D. biacutus* must be driven directly by an energy-utilizing step. Whether this coupling is directly to ATP, or via a transmembrane gradient at the expense of, e.g., 3 H^+ or 3 Na^+ is at present unknown. If the hydrolysis of ATP is associated with a free energy change of -50 kJ · mol^{-1} (42), then the carboxylation and activation reaction(s) resulting in acetoacetyl-CoA can be postulated to be coupled to the utilization of the energetic equivalent of one ATP molecule per acetone molecule. The net free energy change in acetone metabolism to two acetyl-CoA residues will therefore be very close to 0 kJ · mol of acetone^{-1} ($+79.1$ kJ · mol^{-1} for acetone carboxylation and subsequent activation to a CoA ester, -50 kJ · mol^{-1} for ATP hydrolysis, and -23.8 kJ · mol^{-1} for the thiolytic cleavage of acetoacetyl-CoA).

If it is assumed that no energy is invested in the carboxylation step, at physiological concentrations of acetone, CO_2, and CoASH (see above), the concentration of acetoacetyl-CoA at equilibrium would be 66 aM (6.6×10^{-17} M). The actual concentration measured, around 500 μM, is thus 10^{13} times higher, and it shows that this reaction must be driven by an energy-utilizing step. At an acetoacetyl-CoA concentration of 66 aM, the activities of the acetyl-CoA acetyltransferase and 3-hydroxybutyryl-CoA dehydrogenase would be virtually zero, and acetone catabolism could not proceed at the measured specific activity of 0.019 μmol · min^{-1} · mg of $protein^{-1}$.

Previous assessment of specific growth yields of *D. biacutus* on acetone suggested that the carboxylation and subsequent activation to acetoacetyl-CoA could be coupled to the investment of the energetic equivalent of one ATP molecule (35). This idea is supported by the thermodynamic analysis of acetone catabolism in the present study. We have to date, however, been unable to develop an assay for this activity. Carboxylation of phenol results in the formation of 4-hydroxybenzoate (44) which is removed by an active CoA ligase (2, 24), perhaps to shift the equilibrium to allow the energetically unfavourable carboxylation reaction to proceed. A similar mechanism does not appear to play a role in the anaerobic acetone metabolism of *D. biacutus*, since (i) apparently no free acid intermediate is formed, (ii) the intracellular acetoacetyl-CoA ester concentration is not low enough to result in a significant shift in the equilibrium of the initial reactions, (iii) this concentration is 10^{13} times higher than that predicted for a carboxylation not coupled to an energy-utilizing step, and (iv) the kinetic properties and substrate affinities of the enzymes acting on the proposed initial product (acetoacetyl-CoA) would result in virtually no catabolic flux at acetoacetyl-CoA concentrations predicted by a carboxylation reaction without an energy-utilizing step.

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