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Carbonylation as a Key Reaction in Anaerobic Acetone Activation by *Desulfococcus biacutus*

Olga B. Gutiérrez Acosta,[a,c] Norman Hardt,[b,c] Bernhard Schink[a,c]

Department of Biology,[a] Department of Chemistry,[b] and Konstanz Research School of Chemical Biology,[c] Universität Konstanz, Constance, Germany

Acetone is activated by aerobic and nitrate-reducing bacteria via an ATP-dependent carboxylation reaction to form acetoacetate as the first reaction product. In the activation of acetone by sulfate-reducing bacteria, acetoacetate has not been found to be an intermediate. Here, we present evidence of a carbonylation reaction as the initial step in the activation of acetone by the strictly anaerobic sulfate reducer *Desulfococcus biacutus*. In cell suspension experiments, CO was found to be a far better cosubstrate for acetone activation than CO_2. The hypothetical reaction product, acetoacetaldehyde, is extremely reactive and could not be identified as a free intermediate. However, acetoacetaldehyde dinitrophenylhydrazone was detected by mass spectrometry in cell extract experiments as a reaction product of acetone, CO, and dinitrophenylhydrazine. In a similar assay, 2-amino-4-methylpyrimidine was formed as the product of a reaction between acetoacetaldehyde and guanidine. The reaction depended on ATP as a cosubstrate. Moreover, the specific activity of aldehyde dehydrogenase (coenzyme A [CoA] acylating) tested with the putative physiological substrate was found to be 153 ± 36 mU mg^{-1} protein, and its activity was specifically induced in extracts of acetone-grown cells. Moreover, acetoacetyl-CoA was detected (by mass spectrometry) after the carbonylation reaction as the subsequent intermediate after acetoacetaldehyde was formed. These results together provide evidence that acetoacetaldehyde is an intermediate in the activation of acetone by sulfate-reducing bacteria.

Acetone is produced by bacterial fermentations, for example, by several *Clostridium* species (1). It is also produced in chemistry as a solvent and as an intermediate in the synthetic chemical industry. Aerobic degradation of methyl ketones was first observed with hydrocarbon-utilizing bacteria (2). Acetone is degraded by some aerobic bacteria (3) and mammalian liver cells via oxygenase-dependent hydroxylation to acetol (4). Carboxylation of acetone to acetoacetate as a means of acetone activation was first proposed for a methanogenic enrichment culture (5). The requirement of CO_2 as a cosubstrate for acetone degradation was also observed with the nitrate reducer *Thiosphaera pantotropha* (6) and with *Rhodobacter capsulatus* and other phototrophs (7). The reaction was studied with the nitrate-reducing strain Bun N under anoxic conditions, and it was concluded that acetoacetate was formed by the ATP-dependent carboxylation of acetone (8, 9).

Attempts to measure an *in vitro* carboxylation of acetone at that time were unsuccessful. However, exchange of radioactively labeled CO_2 with the carboxyl group of acetoacetate was catalyzed by cell extracts of strain Bun N (10). A similar CO_2- and ATP-dependent activation reaction was observed with the aerobic bacterium *Xanthobacter autotrophicus* strain Py2 (11). A comparison between the acetone carboxylase of strain Py2 and the carboxylase of the phototrophic bacterium *Rhodobacter capsulatus* showed that they are identical in subunit composition ($\alpha_2\beta_2\gamma_2$ multimers of 85-, 78-, and 20-kDa subunits) and in kinetic properties (12, 13). A similar subunit composition was recently found with the acetone carboxylase of the nitrate reducer *Aromatoleum aromaticum* (14) and with the acetone carboxylases of *Alicycliphilus denitrificans*, *Paracoccus denitrificans*, and *Paracoccus pantotrophus* (15). Thus, it appears to be well established that aerobic and nitrate-reducing bacteria activate acetone by an ATP-dependent carboxylation reaction. Because the γ and β phosphodiester bonds of ATP need to be hydrolyzed during the reaction, two ATP equivalents are invested into a reaction that theoretically would require less than one ATP (acetone + CO_2 → acetoacetate$^-$ + H^+; $\Delta G_0' = +17.1$ kJ mol^{-1}). At least one further ATP is required for acetoacetate activation to acetoacetyl coenzyme A (acetoacetyl-CoA). This energy expenditure can be afforded by aerobic and nitrate-reducing bacteria because the subsequent oxidation of the acetyl moieties releases sufficient energy.

Acetone degradation by sulfate-reducing bacteria (SRB) is energetically more difficult. Oxidation of the acetyl residue of acetyl-CoA through the CO dehydrogenase (Wood-Ljungdahl) pathway can form only about one ATP equivalent per acetyl residue. Thus, acetone degradation through the carboxylation reaction described above could not be supported through the subsequent oxidation of the acetyl residues. Therefore, a different mechanism for CO_2-dependent acetone activation has to be postulated for these bacteria.

Acetone degradation was studied with the sulfate-reducing bacteria *Desulfococcus biacutus* and *Desulfobacterium cetonicum* (16, 17). No acetone-carboxylating or acetoacetate-decarboxylating activity could be found in cell extracts of these bacteria. There was high acetoacetyl-CoA thiolase activity present in acetone-grown cells but no activity of an acetoacetate-activating CoA transferase or CoA ligase. Moreover, these bacteria excreted acetate at a 1:1 ratio during growth on butyrate or 3-hydroxybutyrate but did not accumulate acetate during growth on acetone. From these results we concluded that acetoacetate is not a free intermediate in acetone metabolism and that activation of acetone may lead directly to an activated acetoacetyl residue, e.g., acetoacetyl-CoA (17).

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Address correspondence to Bernhard Schink, Bernhard.Schink@uni-konstanz.de.

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Since both sulfate reducers oxidize acetyl residues through the Wood-Ljungdahl pathway, they have CO dehydrogenase activity. Therefore, they could convert CO_2 to CO and employ this as a cosubstrate in acetone activation to form acetoacetaldehyde rather than acetoacetate as a reaction product. In the present study, we elucidated this hypothesis with *D. biacutus* and found strong evidence for this novel type of reaction.

MATERIALS AND METHODS

Bacterial growth conditions. *Desulfococcus biacutus* strain KMRActS was grown in freshwater mineral medium as described before (17, 18). The medium was reduced with 1 mM sulfide, buffered with CO_2-bicarbonate, and adjusted to a final pH of 7.2. Cells were grown in 1-liter flasks with medium supplemented with 5 mM acetone or 5 mM butyrate as the sole carbon source and 10 mM sulfate as the electron acceptor. Cultures were incubated under a strictly anoxic N_2-CO_2 (80/20) atmosphere at 30°C in the dark.

Cell suspension experiments. Cells were harvested in the late exponential growth phase at an optical density at 600 nm (OD_{600}) of 0.3. All experiments with cell extracts and cell suspensions were done under strictly anoxic conditions inside an anoxic glove box. Cells were centrifuged at 6,000 × *g* at 10°C. The pellet was washed at least twice with 50 mM potassium phosphate (KP) buffer, pH 7.2, supplemented with 3 mM dithioerythritol as the reducing agent. Cells were resuspended in the same buffer with the addition of NaCl (1.0 g · liter^{-1}) plus $MgCl_2 \cdot 6H_2O$ (0.6 g · liter^{-1}). Cell suspensions with a final OD_{600} of 12 were prepared in 5-ml flasks containing KP buffer with 5 mM acetone and 10 mM sulfate. The sulfate-reducing activity was measured at different time intervals for several hours. The gas phase was either N_2-CO (90/10), N_2-CO_2 (80/20), or N_2.

Preparation of cell extracts. Cells were harvested as described above; however, a temperature of 4°C was used. The cell pellet was resuspended in the KP buffer described above containing 0.5 mg DNase ml^{-1} and 1 mg ml^{-1} of complete protease inhibitor cocktail (Complete, Mini, EDTA-free protease inhibitor cocktail tablets; Roche Diagnostics GmbH, Mannheim, Germany). Cells were disrupted by passing them two times through a cooled French pressure cell at 100 MPa. Cell debris and unopened cells were removed by centrifugation at 27,000 × *g* for 20 min at 4°C.

CODH assay. The activity of carbon monoxide dehydrogenase (CODH) was measured at 30°C with a photometer (100-40; Hitachi, Tokyo, Japan). Cell extracts of acetone-grown cells were used for enzyme assays.

Enzyme activity was tested in the already described KP buffer with the addition of 2 mM benzyl viologen (BV) as the electron acceptor. The activity was tested in cuvettes previously flushed with CO or by addition of CO to the complete reaction mixture. The effect of CODH inhibition by potassium cyanide (KCN) was checked with final concentrations of 3 and 5 mM KCN. Reduction of BV was followed at 578 nm (ε_{578} = 8.65 mM$^{-1} \cdot$ cm^{-1}). One unit was defined as 1 μmol of BV reduced per min.

Aldehyde dehydrogenase (CoA-acylating) assay. The activity of aldehyde dehydrogenase was measured in anoxic cuvettes in the same Hitachi photometer. Cell extracts of acetone-grown cells were used for enzyme assays; control experiments with extracts prepared from butyrate-grown cells were run under the same conditions. Enzyme activity was followed in 50 mM KP buffer, pH 7.2, supplemented with 3 mM dithioerythritol as described before, with the addition of 2 mM CoA and 5 mM NAD^+ as the electron acceptor. The reaction was started by addition of 2 mM acetaldehyde or by addition of 20 μl of acetoacetaldehyde-containing solution (see below). NADH formation was followed at 340 nm (ε_{340} = 6.292 mM$^{-1} \cdot$ cm^{-1}). Control assays were run with boiled cell extracts. One unit was defined as 1 μmol of NAD reduced per min. Preparation of acetoacetaldehyde solution was done as follows: 20 μl (9.96 mg) of acetylacetaldehyde dimethyl acetal (4,4-dimethoxy-2-butanone; Aldrich Chemistry, Sigma-Aldrich) was mixed with 40 μl of 37% HCl in 2 ml KP buffer, and the mixture was stirred for 20 min. The reaction mix was diluted with 4 volumes of the same KP buffer (200 μl in 1 ml), and 20 to 25 μl from this final mixture was added to the cuvette for assay of acetoacetaldehyde dehydrogenase.

Activation of acetone in cell extract. Extracts of *D. biacutus* cells grown with acetone were used for enzyme assays; control experiments were run with extracts of butyrate-grown cells. All assays were carried out under strictly anoxic conditions at 30°C. Activation of acetone was tested in a total volume of 4 ml with 5 mM acetone, 5 mM ATP, and CO (10% in the headspace) as a cosubstrate. The reaction mix was incubated under mild stirring for at least 3 h, and samples were taken at different time intervals with syringes that had previously been flushed with N_2. The increment of carbonyl groups was quantified with 2,4-dinitrophenyl hydrazine (DNPH) or by derivatization of the reaction product with guanidine hydrochloride to form 2-amino-4-metylpyrimidine (see "Analytical methods" below). The acetone activation reaction was also tested in the presence of 5 mM KCN, an inhibitor of CO oxidation by CO dehydrogenase (19).

In a further reaction setup, the same reaction mix received, in addition, 2 mM CoA and 5 mM NAD^+. Samples of 250 μl were taken at different time intervals and acidified with 50 μl of 3 M HCl, followed by centrifugation at 10,000 × *g* for 10 min. The supernatant was mixed with acetonitrile (50:50) and used for the assay of acetoacetyl-CoA by electrospray ionization (ESI)-mass spectrometry (MS). Authentic acetoacetyl-CoA (Sigma) was used as a reference.

Preparation of acetoacetaldehyde for derivatization with DNPH. Acetoacetaldehyde was prepared by chemical deprotection of acetylacetaldehyde dimethyl acetal. The protected compound (9.96 mg) was mixed with 100 μl of 1.25 M HCl in methanol and 800 μl of acetonitrile. The reaction mix was stirred under an N_2 atmosphere at room temperature and was monitored by thin-layer chromatography (TLC).

Chemical synthesis of DNPH derivatives. DNPH (10 mg) was dissolved in 10 ml acetonitrile or ethyl acetate, and the mixture was stirred for approximately 30 min under an N_2 atmosphere until a clear red solution was obtained. The product of the acetoacetaldehyde dimethylacetal deprotection reaction was immediately transferred into the DNPH solution and kept at room temperature while stirring for 60 min. The reaction was followed by TLC. The product of the derivatization was purified by column chromatography using a mixture of 95% dichloromethane and 5% methanol as the eluent. The product was analyzed with ESI-MS and proton nuclear magnetic resonance (^{1}H NMR) spectroscopy.

Isolation and characterization of DNPH derivatives from the enzyme reaction. The product of acetone activation was derivatized with DNPH. For that purpose, 300 μl of each sample taken from the reaction mix was slowly introduced into 300 μl of freshly prepared DNPH solution and the components were mixed for 1 h. DNPH derivatives were extracted by mixing a defined volume of the derivatization reaction mix with ethyl acetate. Derivatives were detected by high-pressure liquid chromatography (HPLC), UV spectrophotometry, and TLC. The main spot observed was scraped from the TLC plate and dissolved in dichloromethane. Further analysis using ESI-MS and ^{1}H NMR spectroscopy was performed to characterize the derivatization product.

Derivatization with guanidine. The product of acetone activation was also derivatized with guanidine hydrochloride. The reaction conditions were set according to a procedure proposed before (20). Three hundred microliters of each sample taken from the reaction mix was slowly introduced into 500 μl of an aqueous 0.5 M guanidine hydrochloride solution (pH 9.0). The reaction was stirred for at least 24 h at 30°C. The reaction product was analyzed with reverse-phase (RP)-HPLC and compared with a 2-amino-4-methylpyrimidine reference compound (Aldrich Chemistry, Sigma-Aldrich).

Chemicals. Most chemicals were of analytical grade, were purchased from Acros, Fluka, Sigma, Merck, or Aldrich, and were used without any further purification. Dry solvents were purchased from Fluka; solvents for column chromatography were either distilled from technical grade (dichloromethane) or purchased as for chromatography grade (ethyl acetate and methanol).

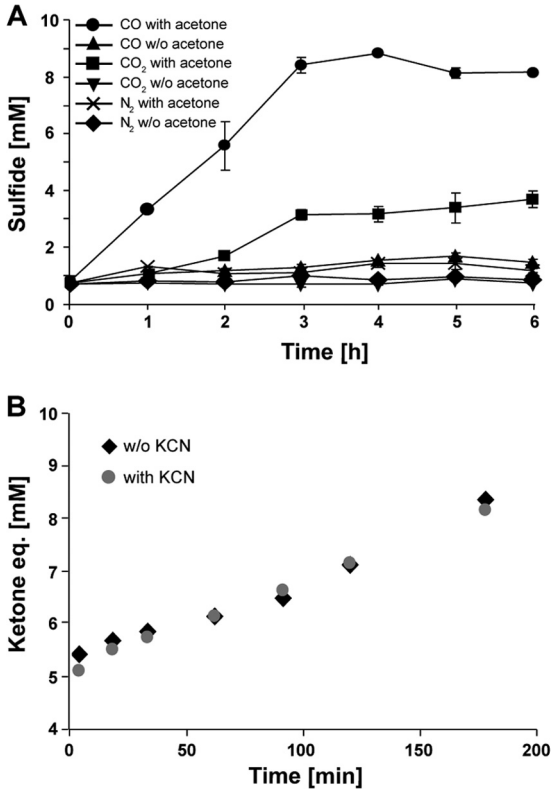

FIG 1 Acetone degradation in cell suspensions and in cell extracts of *Desulfococcus biacutus*. (A) Sulfide production in cell suspension experiments. Acetone and sulfate were added at concentrations of 5 and 10 mM, respectively, and CO and CO_2 were present at initial concentrations of 10% and 20% (vol/vol), respectively. (B) Formation of ketone equivalents (eq.) measured with DNPH during acetone activation in cell extracts. Acetone and ATP were added at an initial concentration of 5 mM each, and CO was present at an initial concentration of 10% in the headspace. Inhibition of CO dehydrogenase was performed using 5 mM KCN. Before the addition of acetone, both samples were preincubated for 20 min with or without (w/o) KCN.

Analytical methods. DNPH solution for quantification of carbonyl groups was prepared as follows: 0.1 g of DNPH (60%, wt/wt) was slowly introduced into a solution of 2 M HCl. The solution was stirred for 2 h at room temperature and passed through a cellulose acetate membrane filter (Whatman OE 66; pore size, 0.2 μm). This solution was prepared fresh every time that it was required, as were the standards for calibration curves. For determination of carbonyl compounds, samples from the acetone activation assay mix were slowly introduced into 100 μl of DNPH solution with further addition of 500 μl water and 100 μl 10 M NaOH. Samples were mixed for approximately 1 h, and subsequently, the absorbance of the hydrazone derivative was measured spectrophotometrically at 540 nm and 360 nm. Standards of acetone dinitrophenylhydrazone were prepared in the same way and used for quantification of carbonyl groups. The sulfide formed in the cell suspension experiments was quantified with the methylene blue method (21). The protein content of cell extracts was determined with the bicinchoninic acid (BCA) assay (BCA protein assay kit; Thermo Scientific). TLC was done on silica plates (silica gel 60; Merck). After the samples were dried under air for 2 min, the run started with a mobile phase of 95% dichloromethane plus 5% methanol. Spots were visualized by UV light and I_2 vapor.

Mass spectrometric analysis was performed with an ESI source (ESI-ion trap [IT], Bruker Esquire 3000 Plus) in the positive and negative ion mode under the following fixed instrument settings: spray ion voltage, 1,000 V; nebulizer, 13 lb/in^2; gas flow, 7 liters min^{-1}; capillary temperature, 300°C. For NMR analysis, Bruker Avance III 400- and 600-MHz spectrometers were used. ^{1}H chemical shifts are reported relative to the residual solvent peak and are given in ppm (δ). Spectra were measured at approximately 17°C and processed using the MestReNOVA (v5.3.1) program. Analysis of 2-amino-4-methyl pyrimidine was performed by reversed-phase HPLC. A Shimadzu HPLC system equipped with a UV-visible diode array detector was used. For analysis, 50-μl samples were injected on a C_{18} reverse-phase column (5 μm, 150 by 4.6 mm; Grom-Sil 120 octyldecyl silane; Grom). Eluents contained 10 mM Na_2HPO_4-KH_2PO_4 buffer, pH 7.0 (buffer A), and acetonitrile (buffer B), and a flow rate of 0.8 ml min^{-1} was used. The elution cycle proceeded as follows: 20% buffer B for 2 min and then a linear increase to 90% buffer B within 9 min and a return to 20% buffer B within 1 min, followed by an equilibration step at 20% buffer B for 6 min. The DNPH derivative was detected with the same HPLC system using the following elution cycle: 10% buffer B in the first minute and then a linear increase to 90% buffer B within 55 min and a final 5-min equilibration at 10% buffer B.

RESULTS

Acetone degradation in cell suspensions. As a first approach to examine the hypothesis of possible acetone carbonylation, we checked for acetone-dependent sulfate reduction in suspensions of intact cells of *D. biacutus* with CO, CO_2, or N_2 in the gas phase (Fig. 1A). Sulfide formation was measured as an indicator of acetone degradation. Figure 1A shows that the highest activity and the highest extent of sulfide formation were detected with CO in the gas phase. With CO as a cosubstrate, sulfide was formed to a concentration of 8.5 mM after 3 h of reaction. With CO_2 only about one-fourth of this activity was observed, and nearly no sulfide was produced in the absence of either CO, CO_2, or acetone (Fig. 1A).

Acetone activation in cell extracts. Activation of acetone with CO was tested in cell extracts. Since we expected the formation of acetoacetaldehyde as the reaction product, the activity was measured by quantifying keto and aldehyde groups with DNPH. In the presence of acetone, CO, and ATP, cell extracts of *D. biacutus* catalyzed the formation of ketone equivalents, as shown in Fig. 1B. Inhibition of CODH with KCN was checked before testing the carbonylation reaction. According to the results in Table 1, CODH was strongly inhibited after 20 min of incubation with 5 mM KCN. Therefore, to prevent a possible oxidation of CO by CODH, KCN was added to the acetone activation reaction mix to a final concentration of 5 mM, and this mix was preincubated for 20 min before addition of acetone. Figure 1B shows that the presence of KCN did not affect the acetone activation reaction that was measured with quantification of carbonyl groups. The reaction was stimulated by the presence of NH_4^+ ions or, less efficiently, by K^+ ions.

Carbonylation of acetone was also tested in the same reaction system containing acetone, CO, and ATP with the addition of CoA and NAD^+. The formation of acetoacetyl-CoA was analyzed by

TABLE 1 Activity of CO dehydrogenase measured in acetone-grown cell extracts of *Desulfococcus biacutus*

Growth condition	Sp act (mU/mg protein)	Approx. % activity
Without KCN	882 ± 191	100
With KCN (3 mM)	121 ± 16	14
With KCN (5 mM), 10-min preincubation	24 ± 2	3
With KCN (5 mM), 20-min preincubation	13 ± 2	1

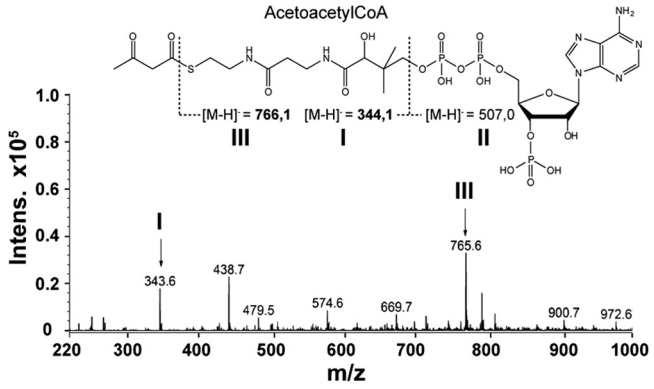

FIG 2 ESI-MS of acetoacetyl-CoA that was formed after the acetone activation reaction. Activation of acetone was tested in a total volume of 4 ml with 5 mM acetone, 5 mM ATP, and CO (10% in the headspace) as a cosubstrate supplemented with 2 mM CoA and 5 mM NAD^+. The CoA derivative was detected in the negative mode. The signal at *m/z* 343.6 (indicated as compound I) was assigned to a fraction of acetoacetyl-CoA, after the loss of a fractal of 507.0 Da, corresponding to 3′-phospho-ADP (indicated as compound II). The signal at *m/z* 765.6 belongs to CoA (indicated as compound III). Intens., intensity.

ESI-MS. In the mass spectrum shown in Fig. 2, a specific peak signal at *m/z* 343.6 (compound I) was assigned to a fraction of acetoacetyl-CoA, after the loss of of a 507.0-Da (compound II) fraction. Compared to an acetoacetyl-CoA standard, the same loss of the 507.0-Da (compound II) fraction was observed. The minor deviation of the mass analysis (0.5 mass unit) is due to a calibration error of the ESI-MS system. The cleavage at one of the phosphorus-oxygen bonds produces the lost fraction, which corresponds to 3′-phospho-ADP. This loss has been observed to be a common phenomenon of acyl-CoA compounds (22–24).

Identification of products from deprotection of acetoacetaldehyde dimethylacetal. The hypothetical reaction intermediate of acetone carbonylation, acetoacetaldehyde, is known to be highly reactive (25, 26) and is not commercially available. The commercially available acetoacetaldehyde dimethylacetal could easily be deprotected in acidic solution and converted to acetoacetaldehyde. While TLC analysis indicated that only one compound was formed, in the ESI mass spectrum of this deprotection reaction (Fig. 3A), a specific peak at *m/z* 205.2 (compound IV) was observed and was attributed to the trimer of acetoacetaldehyde; a second peak at *m/z* 273.2 (compound V) was attributed to the tetramer of acetoacetaldehyde, and a third one at *m/z* 291.3 (com-

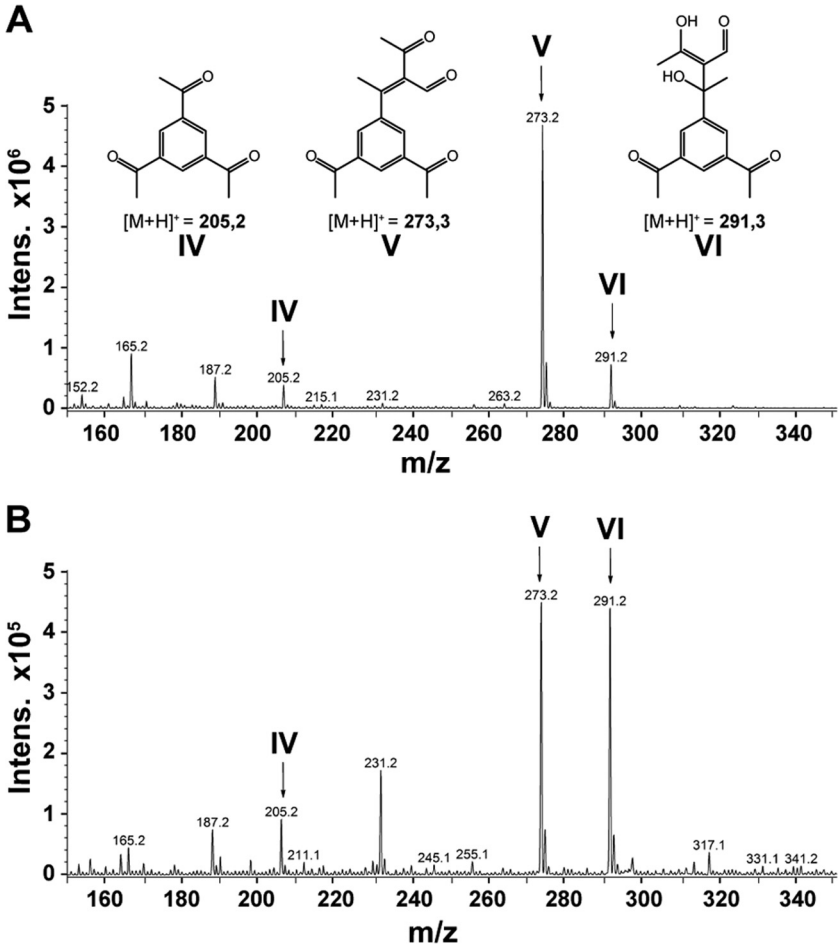

FIG 3 Identification of products of the acetoacetaldehyde dimethyl acetal deprotection reaction. (A) ESI-MS spectrum of the chemically deprotected acetoacetaldehyde dimethyl acetal; (B) ESI-MS spectrum after derivatization of the reaction products with DNPH. Products of acetoacetaldehyde cyclization were identified as follows: compound IV corresponds to triacetylbenzene (trimer of acetoacetaldehyde), compound V corresponds to the tetramer of acetoacetaldehyde, and compound VI represents compound V plus water. Intens., intensity.

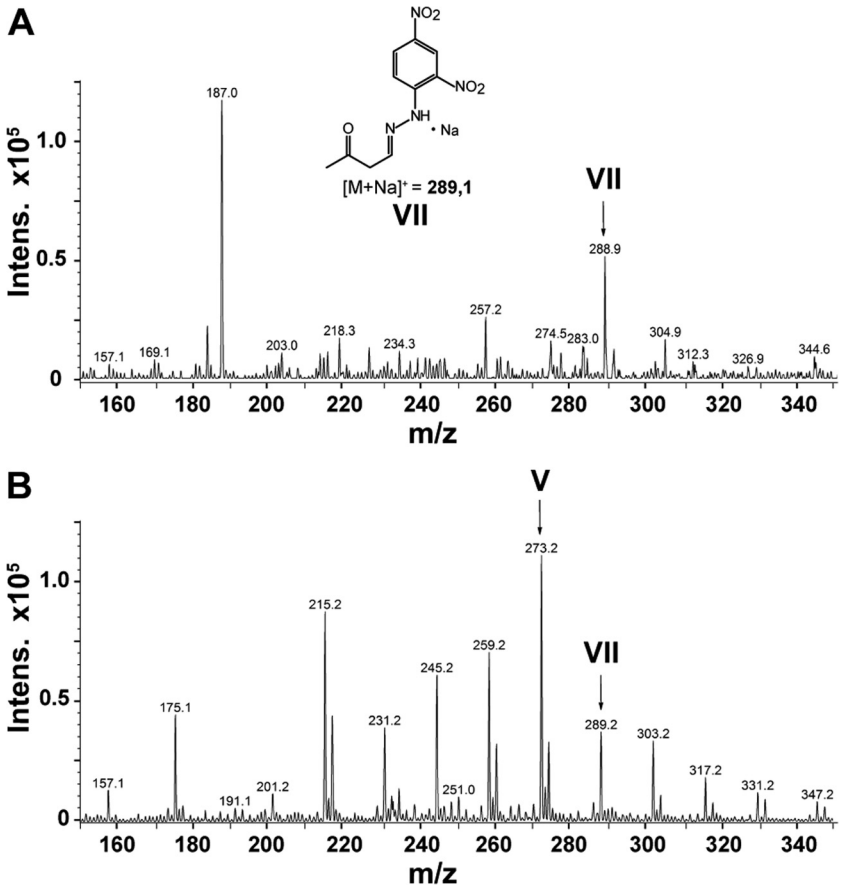

FIG 4 Identification of reaction products of acetone activation by cell extracts. (A) ESI-MS spectrum of the DNPH derivative from the enzyme reaction product. Compound VII was attributed to the acetoacetaldehyde-DNPH. (B) ESI-MS spectrum of the DNPH derivative after column chromatography. Compound V represents the tetramer of acetoacetaldehyde, and compound VII was assigned to the acetoacetaldehyde-DNPH. Spectra were measured in the positive mode. Intens., intensity.

pound VI) belonging to the tetramer of acetoacetaldehyde plus water was also observed. With ^{1}H NMR analysis, we confirmed that one of the produced compounds was triacetylbenzene (see ^{1}H NMR analysis data). Trimerization of acetoacetaldehyde to triacetylbenzene has been observed before (25, 26). Therefore, we concluded that acetoacetaldehyde was produced during the deprotection reaction and reacted with itself to form triacetylbenzene and also the tetrameric derivative. Attempts to derivatize acetoacetaldehyde with DNPH in a two-step process including deprotection and derivatization or, alternatively, in a continuous reaction led to compounds with the specific signals of the trimer and tetramer of acetoacetaldehyde and presented a mass spectrum pattern that was highly similar to that produced without addition of DNPH (Fig. 3B), indicating that the DNPH adduct was not formed under these conditions.

Identification of the DNPH derivative formed during the acetone activation reaction. In order to identify the hypothetical intermediate acetoacetaldehyde, the product of acetone metabolism in cell extracts was derivatized with DNPH as described above. Samples taken from the reaction mix for acetone activation in cell extracts were reacted with DNPH. In the ESI-MS analysis, the acetoacetaldehyde-DNPH derivative ion $[M + Na]^+$ could be observed at *m/z* 288.9 (compound VII) after 180 min of enzyme reaction (Fig. 4A). All attempts to purify the acetoacetaldehyde-DNPH derivative failed because the compound proved to be unstable during the isolation process. Interestingly, after column chromatography (silica column; see Materials and Methods) we observed in the mass spectrum the presence of the tetramer of the acetoacetaldehyde peak signal at *m/z* 273.2 and the remains of acetoacetaldehyde-DNPH at *m/z* 289.2 (Fig. 4B). Unfortunately, the acetoacetaldehyde-DNPH derivative that was formed after the acetone activation reaction could not be isolated for analysis by ^{1}H NMR spectroscopy.

NMR analysis. The products formed during deprotection of acetoacetaldehyde dimethylacetal were analyzed by ^{1}H NMR and resulted in the following chemical shifts: ^{1}H NMR (400 MHz, dimethyl sulfoxide [DMSO]) δ 8.62 (s, 3H), 2.72 (s, 9H). This analysis showed the presence of triacetylbenzene. The spectrum of commercial triacetylbenzene (TCI Europe) was checked for comparison and resulted in the following chemical shifts: ^{1}H NMR (400 MHz, DMSO) δ 8.63 (s, 3H), 2.72 (s, 9H).

Derivatization with guanidine. Another strategy to trap the hypothetical acetoacetaldehyde was an N—C—N condensation reaction to form 2-amino-4-methylpyrimidine (20). We employed this type of condensation reaction as well to derivatize the acetoacetaldehyde hypothetically formed in the enzymatic acetone activation reaction. Samples taken from the reaction mix were reacted with guanidine hydrochloride, and the products

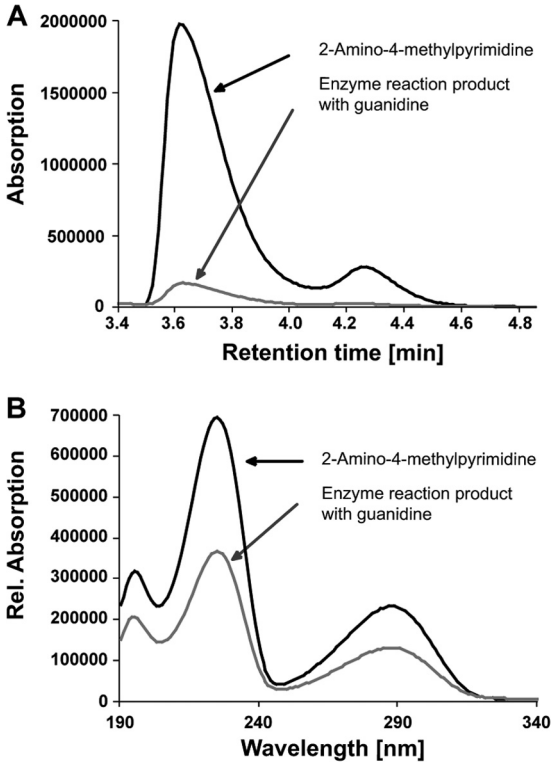

FIG 5 Identification of the reaction product of acetone activation after derivatization with guanidine. (A) HPLC analysis of the 2-amino-4-methylpyrimidine reference compound and of the product formed during the reaction between the intermediate (acetoacetaldehyde) and guanidine; (B) absorption spectra of the commercial 2-amino-4-methylpyrimidine and the product formed after the reaction between acetoacetaldehyde and guanidine. Rel., relative.

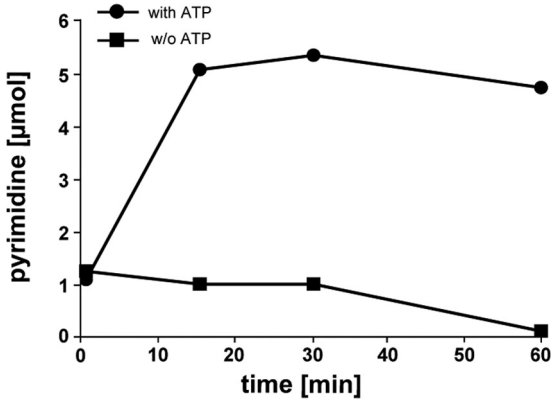

FIG 6 Dependence of acetoacetaldehyde formation from acetone and CO on the presence of ATP as a cosubstrate. The formation of 2-amino-4-methylpyrimidine was quantified as the product of the reaction between acetoacetaldehyde and guanidine. Acetone and ATP were added at an initial concentration of 5 mM each. CO was present at an initial concentration of 10% in the headspace. Quantification of 2-amino-4-methylpyrimidine was done by measuring the absorption at 290 nm.

were analyzed by RP-HPLC. The analysis showed that this product appeared at exactly the same retention time (3.65 min) as a commercial reference of 2-amino-4-methylpyrimidine and that it also presented the same UV absorption spectra, thus indicating that the expected reaction between acetoacetaldehyde and guanidine had occurred (Fig. 5A and B). The pyrimidine that formed could be detected only if guanidine was present at high excess. Due to the high background of excess guanidine, it was not possible to follow this reaction by ESI-MS and NMR spectroscopy. However, since we detected 2-amino-4-methylpyrimidine, it is highly probable that acetoacetaldehyde was produced during the reaction of acetone with CO, and this conclusion is also supported by the detection of the acetoacetaldehyde-DNPH derivative by mass spectrometry and by the detection of acetoacetyl-CoA after the carbonylation reaction.

Formation of 2-amino-4-methylpyrimidine in the assay system described above required ATP as a cosubstrate. In the absence of ATP, no such product was formed (Fig. 6). Dependence on ATP was also confirmed in the test system using DNPH as the trapping agent. The described reactions were observed only with extracts of acetone-grown cells. Control experiments with extracts of butyrate-grown cells did not produce DNPH or guanidine-reactive products.

Aldehyde dehydrogenase (CoA-acylating) activity. The activity of aldehyde dehydrogenase was measured in cell extracts of *D. biacutus* with acetaldehyde and with acetoacetaldehyde that was prepared by deprotection of acetoacetaldehyde dimethylacetal. Previous experiments showed the instability of acetoacetaldehyde; nevertheless, this compound must be an intermediate before the trimer and tetramer are formed. Therefore, this acidic solution was also added as a substrate for aldehyde dehydrogenase with the addition of CoA and NAD^+ as the electron acceptor. The activity was detected in extracts of acetone-grown cells at 18 ± 3 mU mg^{-1} protein with acetaldehyde and 153 ± 36 mU mg^{-1} protein with the acetoacetaldehyde preparation (Table 2). Addition of CoA caused an increase of the activity from 30 to 100%. A control assay with extracts of butyrate-grown cells indicated that aldehyde dehydrogenase is specifically induced during the metabolism of acetone. The activity with acetaldehyde increased 5-fold after addition of 20 mM NH_4^+ to the reaction mix.

DISCUSSION

In the present study, degradation of acetone under strictly anoxic conditions was investigated with the sulfate-reducing bacterium *Desulfococcus biacutus*. Based on our experimental results, we propose that acetone is activated by carbonylation with CO to form acetoacetaldehyde rather than by carboxylation to acetoacetate, as described for aerobic or nitrate-reducing bacteria. CO proved to be a far better cosubstrate for acetone degradation than CO_2 in cell suspension experiments. Since *D. biacutus* can reduce CO_2 to CO

TABLE 2 Aldehyde dehydrogenase (CoA-acylating) activity measured in cell extracts of *Desulfococcus biacutus*

	Sp act (mU/mg protein)		
	With acetaldehyde		
Cell extract	Without NH_4^+	With NH_4^+	With acetoacetaldehyde[a]
Acetone grown	5 ± 0.5	18 ± 3	153 ± 36
Butyrate grown	ND[b]	1 ± 0.2	20 ± 7

[a] Ammonium addition did not stimulate the reaction with acetoacetaldehyde.
[b] ND, not detected.

by its carbon monoxide dehydrogenase enzyme (17), CO is available as a cosubstrate for this activation reaction.

Derivatization of carbonyl compounds with DNPH has been used in the quantification of aldehydes and ketones (27–30). In our study, the increase of ketone equivalents measured with DNPH in cell extracts indicated that CO and acetone were condensed to an aldehyde molecule.

A simultaneous experiment in which CO dehydrogenase was inhibited by KCN gave a similar increase of the ketone equivalents, suggesting that CO is the real cosubstrate for the acetone-activating reaction, rather than being oxidized to CO_2 by CO dehydrogenase. This result is supported by cell suspension experiments. In control assays with chemically prepared acetoacetaldehyde, no reaction with DNPH was observed. Obviously, the formed acetoacetaldehyde had undergone a cyclization reaction to form a trimer, as observed before, (25, 26). The formed 1,3,5-triacetylbenzene, which was identified by ESI-MS and ^{1}H NMR spectroscopy, further reacted to form the tetrameric compound. Interestingly, after the enzymatic acetone activation reaction, the DNPH-acetoacetaldehyde derivative was detected by mass spectrometry with a peak at *m/z* 288.9, which strongly suggests that acetoacetaldehyde was indeed produced in the enzymatic activating reaction. While trying to purify this derivative, we could detect the tetramer of acetoacetaldehyde (*m/z* 273.2) and minor amounts of the DNPH-acetoacetaldehyde derivative (*m/z* 289.2), indicating that the derivative might have disintegrated, perhaps due to the acidic conditions that were used during the chromatographic separation process.

The formation of acetoacetaldehyde from acetone and CO is also supported by the formation of 2-amino-4-methylpyrimidine with guanidine as a cosubstrate, a reaction which is very specific for the detection of 1,3-dioxo aliphatic compounds.

A further strong indication of the formation of an aldehyde as a first reaction product in acetone activation is the presence of aldehyde dehydrogenase activity. This activity was found only in extracts of acetone-grown cells and was substantially higher when the putative physiological substrate was added, thus indicating that an aldehyde is formed specifically during degradation of acetone, and it is highly probable that this aldehyde is our hypothetical acetoacetaldehyde. Moreover, the detection of acetoacetyl-CoA after activation of acetone in the presence of CO, ATP, CoA, and NAD^+ again supports the formation of acetoacetaldehyde as an intermediate.

The acetone-carbonylating activity was stimulated by monovalent cations, such as NH_4^+ or K^+. The activating enzyme differs from the ketone carboxylases employed by aerobic and nitrate-reducing bacteria, which depend on the presence of divalent cations, such as Mg^{2+} and Mn^{2+} (31, 32). However, the acetone-carbonylating enzyme activity of *D. biacutus* was stimulated by NH_4^+ ions, similar to the acetone carboxylases of *Cupriavidus metallidurans* strain CH34 and *Xanthobacter autotrophicus* strain Py2 (13, 33). Neither genomic nor proteomic analysis of acetone-grown cells of *D. biacutus* provided any indication of acetone carboxylases similar to those described for aerobic or nitrate-reducing acetone oxidizers (unpublished results from our labs).

The observed conversion of acetone with CO to acetoacetaldehyde required ATP as a cosubstrate. This ATP is perhaps needed to stabilize the enol tautomer of acetone in the form of acetone enolphosphate. This compound is the real substrate of carboxylation by the acetone carboxylases described in the past (14, 34) and may

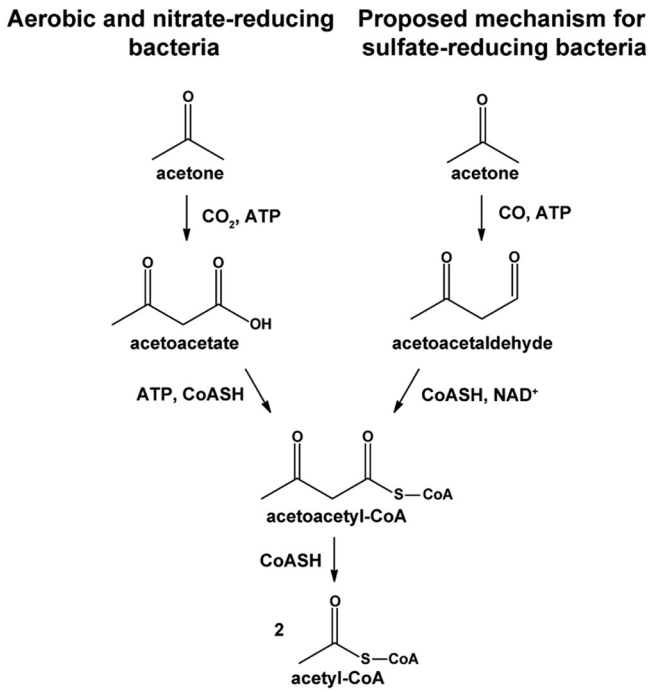

FIG 7 Acetone activation mechanism by aerobic and nitrate-reducing bacteria and the proposed novel activation by carbonylation in sulfate-reducing bacteria. CoASH, coenzyme A.

as well be the real acceptor of CO in the carbonylation reaction proposed here. Since the reaction product, acetoacetaldehyde, is extremely reactive, it appears to be plausible that it is not released free into the cytoplasm but is immediately oxidized further to acetoacetyl-CoA, perhaps in a multienzyme complex. After all, acetone activation and conversion to acetoacetyl-CoA through this new carbonylation pathway (Fig. 7) would require a minimum of only one ATP equivalent rather than three, as in the well-described carboxylation pathway, and would therefore be much better suited for bacteria operating at a small energy budget, such as sulfate-reducing bacteria. Thus, acetone activation is another example to demonstrate that strict anaerobes, such as sulfate reducers, use strategies in the degradation of comparably stable compounds that are basically different from those employed by nitrate reducers, as studies with various aromatic compounds have shown in the past (35). The biochemistry of the novel acetone carbonylation reaction will be subject to further studies in our lab.

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REFERENCES

1. **Han B, Gopalan V, Chukwuemeka ET.** 2011. Acetone production in solventogenic Clostridium species: new insights from non-enzymatic decarboxylation of acetoacetate. Appl. Microbiol. Biotechnol. **91**:565–576.

2. **Lukins HB, Foster JW.** 1963. Methyl ketone metabolism in hydrocarbon-utilizing mycobacteria. J. Bacteriol. **85:**1074–1087.
3. **Taylor DG, Trudgill PW, Gripps RE, Harris PR.** 1980. The microbial metabolism of acetone. J. Gen. Microbiol. **118:**159–170.
4. **Landau BR, Brunengraber H.** 1987. The role of acetone in the conversion of carbohydrates. Trends Biochem. Sci. **12:**113–114.
5. **Platen H, Schink B.** 1987. Methanogenic degradation of acetone by an enrichment culture. Arch. Microbiol. **149:**136–141.
6. **Bonnet-Smits EM, Robertson LA, van Dijken JP, Senior E, Kuenen JG** 1988. Carbon dioxide fixation as the initial step in the metabolism of acetone by *Thiosphaera pantotropha*. J. Gen. Microbiol. **134:**2281–2289.
7. **Birks SJ, Kelly DJ.** 1997. Assay and properties of acetone carboxylase, a novel enzyme involved in acetone-dependent growth and CO_2 fixation in *Rhodobacter capsulatus* and other photosynthetic and denitrifying bacteria. Microbiology **143:**755–766.
8. **Platen H, Schink B.** 1989. Anaerobic degradation of acetone and higher ketones via carboxylation by newly isolated denitrifying bacteria. J. Gen. Microbiol. **135:**883–891.
9. **Platen H, Schink B.** 1990. Enzymes involved in anaerobic degradation of acetone by a denitrifying bacterium. Biodegradation **1:**243–251.
10. **Janssen PH, Schink B.** 1995. $^{14}CO_2$ exchange with acetoacetate catalyzed by dialyzed cell-free extracts of the bacterial strain BunN grown with acetone and nitrate. Eur. J. Biochem. **228:**677–682.
11. **Sluis MK, Small FJ, Allen JR, Ensign SA.** 1996. Involvement of an ATP-dependent carboxylase in a CO_2-dependent pathway of acetone metabolism by *Xanthobacter* strain Py2. J. Bacteriol. **178:**4020–4026.
12. **Sluis MK, Ensign SA.** 1997. Purification and characterization of acetone carboxylase from *Xanthobacter* strain Py2. Proc. Natl. Acad. Sci. U. S. A. **94:**8456–8461.
13. **Sluis MK, Larsen RA, Krum JG, Anderson R, Metcalf WW, Ensign SA.** 2002. Biochemical, molecular, and genetic analyses of the acetone carboxylases from *Xanthobacter autotrophicus* strain Py2 and *Rhodobacter capsulatus* strain B10. J. Bacteriol. **184:**2969–2977.
14. **Schühle K, Heider J.** 2012. Acetone and butanone metabolism of the denitrifying bacterium *Aromatoleum aromaticum* demonstrates novel biochemical properties of an ATP-dependent aliphatic ketone carboxylase. J. Bacteriol. **194:**131–141.
15. **Dullius HC, Chen C, Schink B.** 2011. Nitrate-dependent degradation of acetone by *Alicycliphilus* and *Paracoccus* strains and comparison of acetone carboxylase enzymes. Appl. Environ. Microbiol. **77:**6821–6825.
16. **Janssen HP, Schink B.** 1995. Catabolic and anabolic enzyme activities and energetics of acetone metabolism of the sulfate-reducing bacterium *Desulfococcus biacutus*. J. Bacteriol. **177:**277–282.
17. **Janssen HP, Schink B.** 1995. Metabolic pathways and energetics of the acetone-oxidizing, sulfate-reducing bacterium, *Desulfobacterium cetonicum*. Arch. Microbiol. **163:**188–194.
18. **Widdel F, Pfennig N.** 1982. Studies on dissimilatory sulfate-reducing bacteria that decompose fatty acids. II. Incomplete oxidation of propionate by *Desulfobulbus propionicus* gen. nov., sp. nov. Arch. Microbiol. **131:**360–365.
19. **Wang VCC, Can Pierce M, Ragsdale ESW.** 2013. A unified electrocatalytic description of the action of inhibitors of nickel carbon monoxide dehydrogenase. J. Am. Chem. Soc. **135:**2198–2206.
20. **Haley CAC, Maitland P.** 1951. Organic reactions in aqueous solution at room temperature. Part I. The influence of pH on condensations involving the linking of carbon to nitrogen and of carbon to carbon. J. Chem. Soc. **691:**3155–3174.
21. **Cline JD.** 1969. Spectrophotometric determination of hydrogen sulfide in natural waters. Limnol. Oceanogr. **14:**454–458.
22. **Haynes CA, Allegood JC, Sims K, Wang EW, Sullards MC, Merrill AH, Jr.** 2008. Quantitation of fatty acyl-coenzyme As in mammalian cells by liquid chromatography-electrospray ionization tandem mass spectrometry. J. Lipid Res. **49:**1113–1125.
23. **Magnes C, Sinner FM, Regittnig W, Pieber TR.** 2005. LC/MS/MS method for quantitative determination of long-chain fatty acyl-CoAs. Anal. Chem. **77:**2889–2894.
24. **Norwood DL, Bus CA, Millington DS.** 1990. Combined high-performance liquid chromatographic-continuous-flow fast atom bombardment mass spectrometric analysis of acylcoenzyme A compounds. J. Chromatogr. **527:**289–301.
25. **Price EE.** 1922. Benzene and some of its derivatives, p 21–28. *In* Atomic form with special reference to the configuration of the carbon atom. Longmans, Green Company, London, United Kingdom.
26. **Dolgikh AN, Bogdanova AV, Plotnikova GI, Ushakova TM, Shostakovskii MF.** 1964. Butadiyne derivatives. Reaction of 1-buten-3-ynil sulfides with water. Communication 10. Zelinskii Institute of Organic Chemistry, Academy of Sciences, Moscow, USSR. (Translated from Seriya Khimicheskaya, no. 1, p 127-132, Izvestiya Akademii Nauk SSSR.)
27. **Cirera-Domènech E, Estrada-Tejedor R, Broto-Puig F, Teixidó J, Gassiot-Matas M, Comellas L, Lliberia JL, Méndez A, Paz-Estivill S, Delgado-Ortíz MR.** 2013. Quantitative structure-retention relationships applied to liquid chromatography gradient elution method for the determination of carbonyl-2,4-dinitropenylhydrazone compounds. J. Chromatogr. A **1276:**65–77.
28. **Uchiyama S, Inaba Y, Kunugita N.** 2011. Derivatization of carbonyl compounds with 2,4-dinitrophenylhydrazine and their subsequent determination by high-performance liquid chromatography. J. Chromatogr. B **879:**1282–1289.
29. **Huang HXH, Simon IHS, Yu, J, Z .** 2007. Determination of trace amounts of formaldehyde in acetone. Anal. Chem. Acta **604:**134–138.
30. **Van den Bergh V, Coeckelberghs H, Vankerckhoven H, Compernolle F, Vinckier C.** 2004. Study of the carbonyl products of terpene/OH radical reactions: detection of the 2,4-DNPH derivatives by HPLC-MS. Anal. Bioanal. Chem. **379:**484–494.
31. **Boyd MJ, Ellsworth H, Ensign S.** 2004. Bacterial acetone carboxylase is a manganese-dependent metalloenzyme. J. Biol. Chem. **279:**46644–46651.
32. **Jobst B, Schühle K, Linne U, Heider J.** 2010. ATP-dependent carboxylation of acetophenone by a novel type of carboxylase. J. Bacteriol. **192:**1378–1394.
33. **Rosier C, Leys N, Henoumont C, Mergeay M, Wattiez R.** 2012. Purification and characterization of the acetone carboxylase of *Cupriavidus metallidurans* strain CH34. Appl. Environ. Microbiol. **78:**4516–4518.
34. **Boyd JM, Ensign SA.** 2005. ATP-dependent enolization of acetone by acetone carboxylase from *Rhodobacter capsulatus*. Biochemistry **44:**8543–8553.
35. **Philipp B, Schink B.** 2012. Different strategies in anaerobic biodegradation of aromatic compounds: nitrate reducers versus strict anaerobes. Environ. Microbiol. Rep. **4:**469–478.