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Two enzymes of the acetone degradation pathway of *Desulfococcus biacutus*: coenzyme B_{12}-dependent 2-hydroxyisobutyryl-CoA mutase and 3-hydroxybutyryl-CoA dehydrogenase

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Summary

Degradation of acetone by the sulfate-reducing bacterium *Desulfococcus biacutus* involves an acetone-activation reaction different from that used by aerobic or nitrate-reducing bacteria, because the small energy budget of sulfate-reducing bacteria does not allow for major expenditures into ATP-consuming carboxylation reactions. In the present study, an inducible coenzyme B_{12}-dependent conversion of 2-hydroxyisobutyryl-CoA to 3-hydroxybutyryl-CoA was demonstrated in cell-free extracts of acetone-grown *D. biacutus* cells, together with a NAD^+-dependent oxidation of 3-hydroxybutyryl-CoA to acetoacetyl-CoA. Genes encoding two mutase subunits and a dehydrogenase, which were found previously to be strongly induced during growth with acetone, were heterologously expressed in *E. coli*. The activities of the purified recombinant proteins matched with the inducible activities observed in cell-free extracts of acetone-grown *D. biacutus*: proteins (IMG locus tags) DebiaDRAFT_04573 and 04574 constituted a B_{12}-dependent 2-hydroxyisobutyryl-CoA/3-hydroxybutyryl-CoA mutase, and DebiaDRAFT_04571 was a 3-hydroxybutyryl-CoA dehydrogenase. Hence, these enzymes play key roles in the degradation of acetone and define an involvement of CoA esters in the pathway. Further, the involvement of 2-hydroxyisobutyryl-CoA strongly indicates that the carbonyl-C_2 of acetone is added, most likely, to formyl-CoA through a TDP-dependent enzyme that is co-induced in acetone-grown cells and is encoded in the same gene cluster as the identified mutase and dehydrogenase.

Introduction

Acetone (dimethylketone) can be regarded as a partially activated hydrocarbon through its oxo-group and is commonly used in numerous industrial processes, e.g., as solvent or as precursor for chemical syntheses. Furthermore, acetone is also produced by anaerobic, solventogenic bacteria like several *Clostridia* species (Dürre *et al.*, 1992; Sifniades *et al.*, 2011). Therefore, it occurs as an important pollutant in waste water and also in natural habitats. Several degradation routes in an oxic environment are known, however, its biochemical activation and utilization under anoxic conditions by sulfate-reducing bacteria (SRB) remained unclear until today. Two SRB of the *Desulfosarcina*/*Desulfococcus* clade have been isolated and described that are able to utilize acetone as growth substrate, *Desulfococcus biacutus* strain KMRActS (DSM 5651) and *Desulfosarcina cetonica* strain 480 (DSM 7267) (Platen *et al.*, 1990; Janssen and Schink, 1995b). While nitrate reducers usually activate acetone by a carboxylation reaction forming acetoacetate, no acetone carboxylase activity was detected in cell-free extracts of *D. biacutus*, and no acetone carboxylases were found in its genome and proteome (Janssen and Schink, 1995a; Gutiérrez Acosta *et al.*, 2014). Initial studies suggested an involvement of carbon monoxide, and of acetoacetaldehyde rather than acetoacetate, in the pathway (Gutiérrez Acosta *et al.*, 2013). Biochemical studies with cell-free extracts indicated the involvement of ATP and thiamine diphosphate (TDP) in acetone degradation (Gutiérrez Acosta *et al.*, 2014). However, genome sequencing and differential proteomics comparing acetone- and butyrate-grown cells (Gutiérrez Acosta *et al.*, 2014) identified several proteins that are specifically induced during growth with acetone. One of these (IMG locus tag

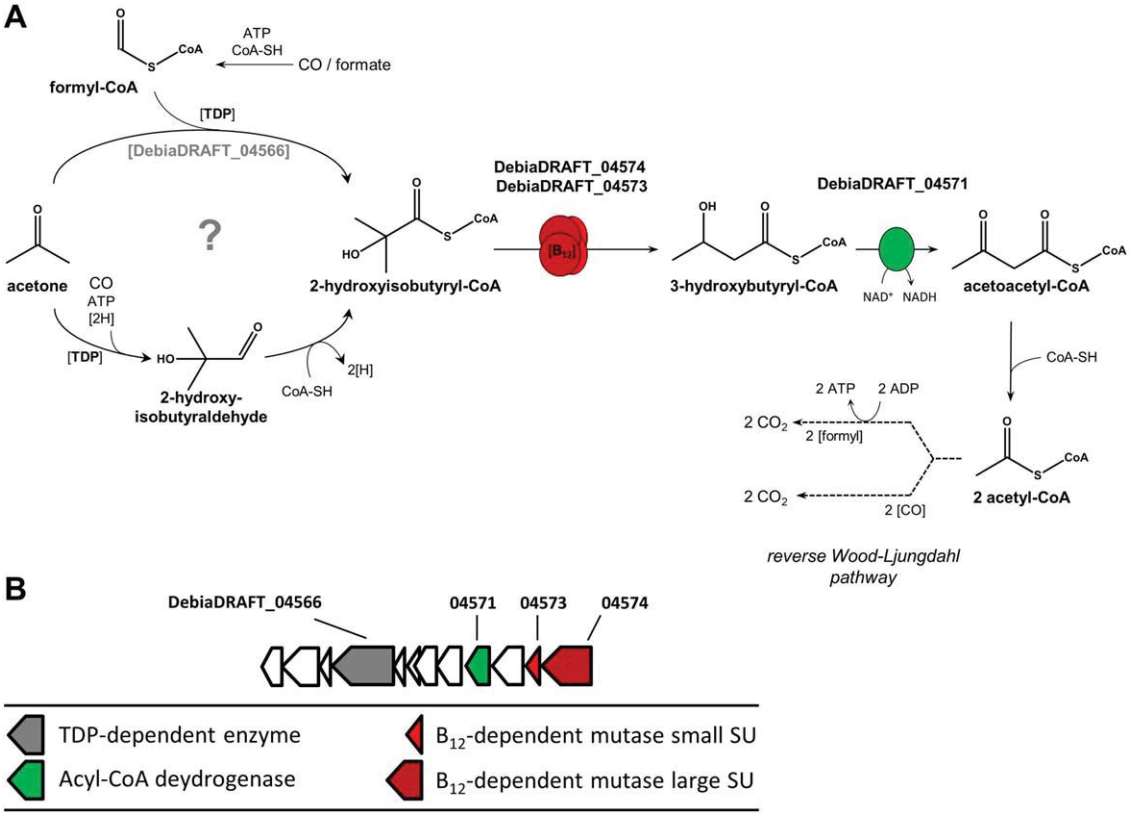

Fig. 1. Hypothetical and confirmed reactions in *D. biacutus* for an acetone degradation pathway (A) that employs a yet inaccessible carbonylation or formylation of acetone, and a 2-hydroxyisobutyryl-CoA mutase and 3-hydroxybutyryl-CoA dehydrogenase as identified in this study, and (B) illustration of the corresponding gene cluster in *D. biacutus*.
A. The enzyme reactions identified in this study are the isomerization of 2-hydroxyisobutyryl-CoA to 3-hydroxybutyryl-CoA, which is catalyzed by a cofactor-B_{12} dependent methylmalonyl-CoA type mutase (red), and the subsequent oxidation of 3-hydroxybutyryl-CoA to acetoacetyl-CoA, which is catalyzed by a NAD^+-dependent dehydrogenase (green). Subsequent reactions are cleavage of acetoacetyl-CoA into two acetyl-CoA, and the oxidation of acetyl-CoA *via* the reversed Wood-Ljungdahl pathway; the latter pathway is coupled to dissimilatory sulfate reduction (not shown) in *D. biacutus*.
B. For the identified enzymes, the corresponding gene cluster and protein/gene identifiers are indicated (color coding) (IMG locus tags prefix, DebiaDRAFT_0), as well as for the co-encoded TDP-dependent enzyme, which is co-expressed during growth with acetone and which most likely is involved in an initial acetone carbonylation or formylation reaction (see question mark in A, and the text).

DebiaDRAFT_04514) has recently been characterized as a zinc-dependent alcohol dehydrogenase capable of converting a wide range of aldehydes and alcohols (Frey *et al*., 2016). Further, in a different gene cluster, genes of four strongly acetone-inducible proteins annotated as a predicted TDP-dependent enzyme (DebiaDRAFT_04566), two subunits of a B_{12}-dependent methylmalonyl-CoA mutase-like enzyme (DebiaDRAFT_04573 and 04574), and a predicted short-chain alcohol dehydrogenase (DebiaDRAFT_04573), were also identified by proteomics (Gutiérrez Acosta *et al*., 2014). The possible involvement of a B_{12}-dependent mutase led to the speculation whether a branched-chain CoA ester may be formed during acetone activation, prior to its conversion to, e.g., acetoacetyl-CoA (Gutiérrez Acosta *et al*., 2014).

As illustrated in Fig. 1A, two routes of acetone activation leading to a branched-chain CoA ester were considered likely, based on the preliminary data described above. First, a direct carbonylation of the C_2-carbon atom of acetone (shown as the lower route in Fig. 1A) may produce a branched-chain aldehyde (2-hydroxy-2-methylpropanal), which may subsequently be processed to a CoA ester. Second, addition of a formyl residue, e.g., in form of formyl-CoA (shown in the upper route in Fig. 1A), could produce a branched-chain CoA ester intermediate, 2-hydroxyisobutyryl-CoA. The predicted B_{12}-dependent methylmalonyl-CoA mutase-like enzyme would then isomerize the branched-chain CoA ester to 3-hydroxybutyryl-CoA, which could be oxidized to acetoacetyl-CoA (Fig. 1A).

In the present study, a B_{12}-dependent isomerization of 2-hydroxyisobutyryl-CoA to 3-hydroxybutyryl-CoA was demonstrated, and an oxidation of 3-hydroxybutyryl-CoA to acetoacetyl-CoA, both in cell-free extracts of *D.*

biacutus as well as with recombinant enzymes produced from candidate genes attributed by differential proteomics. The results strongly support the involvement of these enzymes in acetone degradation, and further highlight the potential role of a highly expressed TDP-dependent enzyme for initial acetone activation. This TDP-dependent enzyme is currently under investigation in our lab, and will be addressed in a further study.

Results

Sequence comparison and functional prediction

The amino acid sequence of DebiaDRAFT_04574 of *D. biacutus* contains a conserved catalytic N-terminal domain of methylmalonyl-CoA mutase-like enzymes, which belong to a small family of coenzyme B_{12}-dependent isomerases (protein family pfam01642) (large subunit). The amino acid sequence of the neighboring locus tag, DebiaDRAFT_04573, is predicted to represent the corresponding coenzyme B_{12}-binding small subunit of methylmalonyl-CoA mutases (pfam02310) (Banerjee and Ragsdale, 2003). A sequence comparison across all currently available bacterial genomes (as per November 2017; NCBI BLAST) revealed for both subunits highest identities to a predicted enzyme in *Desulfosarcina cetonica* (WP_054693862 and WP_054693859; 86% and 86% identity respectively), which is the only other known bacterium that is able to couple acetone degradation to sulfate reduction (Janssen and Schink, 1995b). The genes in *D. cetonica* are encoded in a similar gene cluster, e.g., together with a predicted TDP-dependent enzyme gene as in *D. biacutus*. Moreover, the subunit sequences exhibited 62% and 62% identity respectively, to the subunits of characterized 2-hydroxyisobutyryl-CoA mutase (HcmAB) of *Kyrpidia tusciae* (WP_013074530 and WP_013074531) (Weichler *et al*., 2015), and 42% and 45% identity respectively, to the subunits of characterized 2-hydroxyisobutyryl-CoA mutase of *Aquincola tertiaricarbonis* (AFK_77668 and AFK_77665) (Yaneva *et al*., 2012). An alignment of the sequences of the confirmed 2-hydroxyisobutyryl-CoA mutases of *K. tusciae* and *A. tertiaricarbonis* together with the candidate HcmAB sequences of *D. biacutus* and *D. cetonica* is shown in the Supporting Information file (Fig. S1A and B); for comparison, sequences of characterized isobutyryl-CoA mutase of *Streptomyces cinnamonensis* (37% and 46% identity) and methylmalonyl-CoA mutase of *Pyrococcus horikoshii* (46% and 48% identity) (Ratnatilleke *et al*., 1999; Yabuta *et al*., 2015) are also included in the alignment. Hence, based on the sequence analysis we questioned whether the *D. biacutus* DebiaDRAFT_04574 and 04573 proteins, which are highly induced during growth with acetone (Gutiérrez Acosta *et al*., 2014), might also constitute a mutase enzyme that isomerizes 2-hydroxyisobutyryl-CoA to 3-hydroxybutyryl-CoA as part of an acetone-activating pathway in *D. biacutus*.

DebiaDRAFT_04571, which is strongly induced in acetone-grown cells (Gutiérrez Acosta *et al*., 2014) and encoded in the gene cluster as well (Fig. 1B), was annotated (IMG pipeline) as 3-oxoacyl-[acyl-carrier protein] reductase family of $NAD(P)^+$-binding short-chain alcohol dehydrogenases (pfam00106) (Marchler-Bauer *et al*., 2014). Its amino acid sequence exhibited around 30% sequence identity to characterized 3-hydroxybutyryl-CoA dehydrogenases/acetoacetyl-CoA reductases, e.g., of *Ralstonia solanacearum* (PhaB, D8NBJ9), and the highest sequence identity (82%) was found for a dehydrogenase candidate encoded in the gene cluster of *D. cetonica* described above (Kim *et al*., 2014). Hence, DebiaDRAFT_04571 represents a good candidate for a conversion of 3-hydroxybutyryl-CoA to acetoacetyl-CoA, which is postulated to be formed during acetone activation.

2-Hydroxyisobutyryl-CoA mutase activity detected in cell-free extract of acetone-grown cells

We synthesized 2-hydroxyisobutyryl-CoA and 3-hydroxybutyryl-CoA (and acetoacetyl-CoA, see Supporting Information) for enzyme tests, and as analytical standards for establishing appropriate HPLC and MS/MS conditions for detecting these compounds, e.g., in cell-free extracts (CFE) or with recombinant protein (see below). Indeed, a B_{12}-dependent isomerization of 2-hydroxyisobutyryl-CoA to 3-hydroxybutyryl-CoA was detectable in CFE of acetone-grown *D. biacutus* cells. As illustrated in Fig. 2, anoxically prepared CFE at a total protein concentration of 944 $\mu g\ ml^{-1}$ in the presence of 50 μM adenosylcobalamin showed almost complete disappearance of 0.5 mM 2-hydroxyisobutyryl-CoA within 120 min of incubation, as followed by HPLC-MS (Fig. 2A). The initial substrate disappearance as followed by HPLC-UV (Fig. 2B) was estimated to represent a specific activity of approximately 8 $mU\ mg^{-1}$ of protein. Concomitant with the disappearance of the peak of 2-hydroxyisobutyryl-CoA, the chromatograms (Fig. 2A) showed an appearance of another peak that exhibited a UV absorption spectrum typical of a CoA-ester as well, and which co-chromatographed with the authentic 3-hydroxybutyryl-CoA standard. HPLC-MS/MS analysis of the novel compound showed a fragmentation pattern identical to that of the 3-hydroxybutyryl-CoA reference compound, but different from that of the substrate 2-hydroxyisobutyryl-CoA (Fig. 2C). Hence, the product of the reaction was 3-hydroxybutyryl-CoA.

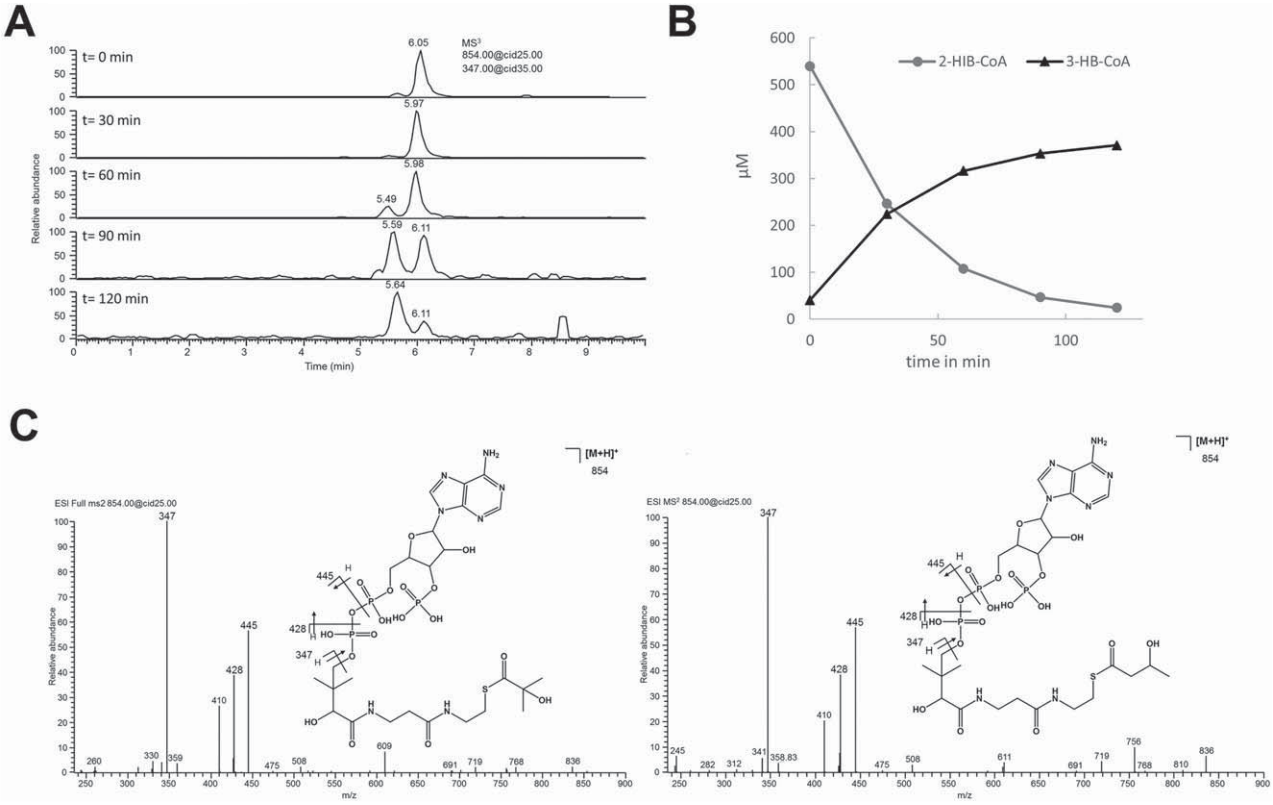

Fig. 2. Isomerization of 2-hydroxyisobutyryl-CoA to 3-hydroxybutyryl-CoA as observed in strict anoxically prepared cell-free extract (944 µg in a 1 ml reaction) of acetone-grown *D. biacutus* cells.
A. HPLC-MS chromatograms of samples taken during the reaction showing the formation of a product, which was identified as 3-hydroxybutyryl-CoA, concomitant with disappearance of the substrate 2-hydroxyisobutyryl-CoA, which eluted after product 3-hydroxybutyryl-CoA under the chromatographic conditions we used (see *Experimental procedures*).
B. Illustration of the kinetics of substrate disappearance and product formation, as determined by HPLC-UV under the same chromatographic conditions.
C. MS^3-fragmentation pattern of 2-hydroxyisobutyryl-CoA (*1*) and of 3-hydroxybutyryl-CoA (*2*).

Control reactions without addition of adenosylcobalamin showed no significant activity, supporting that the enzyme reaction depends on coenzyme B_{12}. The 2-hydroxyisobutyryl-CoA mutase reaction was very sensitive to oxygen, similar to most coenzyme B_{12}-dependent radical reactions (Buckel and Golding, 2006), and the enzyme was unstable in CFE: more than half of the activity was lost within a day when CFE was prepared and stored anoxically on ice. However, freshly and anoxically prepared CFE of butyrate-grown *D. biacutus* cells exhibited only app. 10% of the 2-hydroxyisobutyryl-CoA mutase activity observed in CFE of acetone-grown cells, thus confirming that the enzyme is specifically induced during growth with acetone.

Production and activity of recombinant 2-hydroxyisobutyryl-CoA mutase and 3-hydroxybutyryl-CoA dehydrogenase

The DebiaDRAFT_04574 and 04573 genes were cloned each into His_6-tag expression vectors, and recombinant proteins were produced in *E. coli* Rosetta 2 cells grown aerobically at 37°C in LB medium. The cells were induced at OD_{600} of 0.5–0.8 with 0.5 mM isopropyl-β-D-thiogalactopyranosid (IPTG) in the presence of 3% (v/v) ethanol (Frey *et al*., 2016), and incubated for additional 5 h at 18°C. Afterwards, cells were harvested by centrifugation and the preparation of the cell pellets proceeded under anoxic conditions. Figure S2 (Supporting Information) shows denaturing SDS-PAGE gels of representative protein preparations after the Ni-NTA purification step, for the large subunit (Fig. S2A) and for the small subunit (Fig. S2B), as well as of a native PAGE of a mixture of both preparations (Fig. S2C). The prominent bands on the denaturing gels showed the expected molecular mass (67 kDa for the large and 18 kDa for the small subunit, each including the His_6-tag with ~3 kDa); their identities were confirmed also by proteomics. The native PAGE of a mixture of both subunits showed a single band at a molecular mass of approximately 170 kDa (Fig. S2C), thus suggesting a heterotetrameric

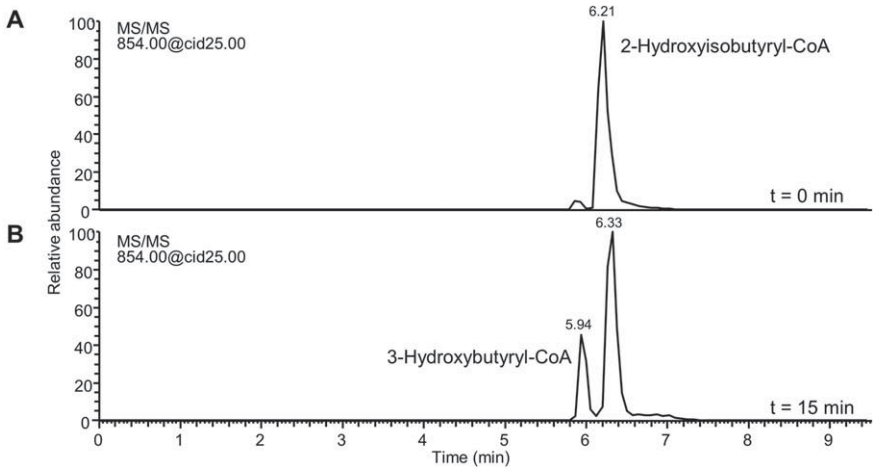

Fig. 3. Isomerization of 2-hydroxyisobutyryl-CoA to 3-hydroxybutyryl-CoA as catalyzed by purified recombinant 2-hydroxyisobutyryl-CoA mutase (HcmAB; 22 μg in a 1 ml reaction). HPLC-MS chromatograms of samples taken before (A) and during (15 min) a reaction (B) illustrate the formation of product 3-hydroxybutyryl-CoA, as shown here as total-ion extract chromatograms of the fragmentation of molecular ions (TIC of $m/z = 854$) of substrate and product.

($\alpha_2\beta_2$) structure of the enzyme, which resembles other described mutases (Ratnatilleke *et al.*, 1999; Kurteva-Yaneva *et al.*, 2015; Yabuta *et al.*, 2015).

Activity of the recombinant enzyme with 2-hydroxyisobutyryl-CoA in the presence of adenosylcobalamin was measurable by HPLC as described for the reaction in CFE (see above), i.e., both as disappearance of the substrate 2-hydroxyisobutyryl-CoA and as formation of the product 3-hydroxybutyryl-CoA (Fig. 3). The specific activity of the recombinant mutase was estimated at approximately 140 mU mg^{-1} protein. No substrate turnover was observed with recombinant enzyme in the absence of adenosylcobalamin, nor in assays that contained adenosylcobalamin but only one of its subunits. Hence, the DebiaDRAFT_04574 and 04573 subunit genes were confirmed to encode a 2-hydroxyisobutyryl-CoA mutase as predicted. However, the enzyme activity of the recombinant enzyme appeared to be extremely labile (complete loss of activity after one day of anoxic storage on ice), and removal of residual imidazole from Ni-NTA purification by gel filtration was essential for obtaining an active enzyme. Further, strictly anoxic conditions during cell opening, Ni-NTA purification and gel filtration (and during the enzyme assays) were essential for obtaining active enzyme.

The predicted 3-hydroxybutyryl-CoA dehydrogenase DebiaDRAFT_04571 was cloned as well, and recombinant protein was produced and purified. Denaturing SDS-PAGE after the Ni-NTA purification (Supporting Information Fig. S2D) showed a prominent band of the expected molecular mass (28 kDa predicted; 31 kDa observed, including the His_6-tag). The protein exhibited a specific activity of 24.3 ± 3.7 mU mg^{-1} protein for oxidation of 50 μM 3-hydroxybutyryl-CoA in the presence of 5 mM NAD^+. For the reverse reaction, the recombinant dehydrogenase showed a specific activity of 14.1 ± 4.4 mU mg^{-1} protein for the reduction of 250 μM acetoacetyl-CoA in the presence of 0.5 mM NADH. When the preparation was done under anoxic conditions, the recombinant protein exhibited up to threefold higher activity (76.7 ± 20.7 mU mg^{-1} with 3-hydroxybutyryl-CoA). The reaction product was acetoacetyl-CoA, as confirmed by HPLC-MS (see Fig. S3 in Supporting Information). No reaction was observed with $NADP^+$, hence, the dehydrogenase appeared to be specific for NAD^+ as electron acceptor.

Discussion

For the acetone degradation pathway in *D. biacutus*, our initial observations and experiments with cell suspensions and cell-free extracts suggested a direct carbonylation of acetone leading to the unstable intermediate acetoacetaldehyde (Gutiérrez Acosta *et al.*, 2013), while biochemical studies (Gutiérrez Acosta *et al.*, 2014) and genome sequencing and differential proteomics (Gutiérrez Acosta *et al.*, 2014) suggested an involvement of a predicted carbon–carbon bond forming TDP-dependent enzyme and of an branched-chain CoA-ester intermediate. The results of our present work now clearly assigned two enzyme functions of the pathway, 2-hydroxyisobutyryl-CoA mutase and 3-hydroxybutyryl-CoA dehydrogenase (Fig. 1A), as well as identified three of the genes that are co-located in the same gene cluster with the TDP-dependent enzyme gene (Fig. 1B), all four of which are specifically and highly induced during growth with acetone (Gutiérrez Acosta *et al.*, 2014).

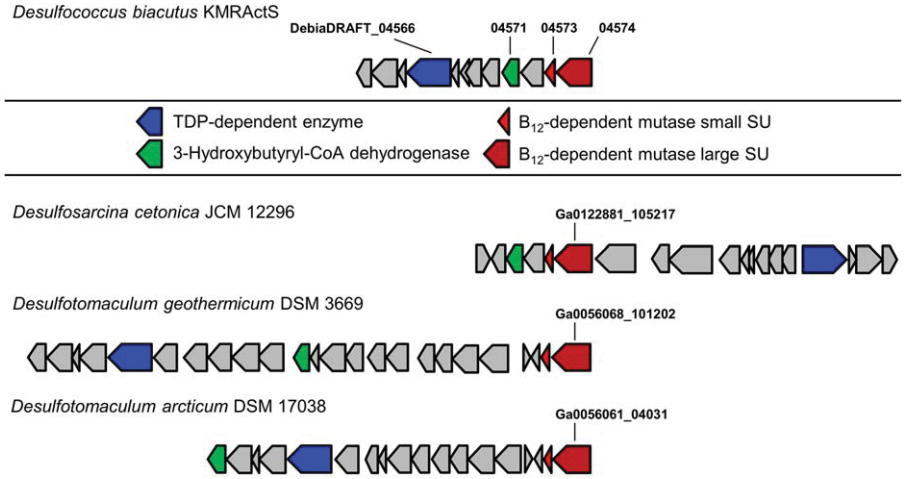

Fig. 4. Illustration of the acetone-activation gene cluster of *Desulfococcus biacutus* and of homologous gene clusters found in genomes of other SRB. Shown are gene clusters containing gene homologs (indicated by color-coding) for at least three of the four (predicted) core enzymes of the acetone-activation pathway, that is, of *Desulfosarcina cetonica* strain 480, which is known to degrade acetone and *Desulfotomaculum arcticum* strain 15 (DSM 17038) and *Desulfotomaculum geothermicum* strain BSD (DSM 3669). Homologous gene clusters were retrieved from the IMG database *via* the Gene Cassette Search tool and the Gene Neighborhood viewer.

In order to define the function of the above-mentioned candidate enzymes in the assumed pathway, the predicted CoA ester substrates and products had to be made available through chemical syntheses (Supporting Information). The inferred 2-hydroxyisobutyryl-CoA mutase activity was confirmed to be active in freshly and strictly anoxically prepared CFE of acetone-grown cells (Fig. 2), but the activity was unstable under the conditions we used. Further, an NAD^+-dependent oxidation of 3-hydroxybutyryl-CoA to acetoacetyl-CoA, which was detected in CFE before (Janssen and Schink, 1995a), was confirmed to be present.

For the recombinant 2-hydroxyisobutyryl-CoA mutase, strictly anoxic conditions throughout preparation and purification were indispensable to obtain active enzyme, which is not a common observation for mutases catalyzing B_{12}-dependent radical reactions (Ratnatilleke *et al*., 1999; Erb *et al*., 2008; Yabuta *et al*., 2015). In the literature, some B_{12}-dependent isomerization reactions are described to be oxygen-sensitive (Banerjee and Ragsdale, 2003), but the preparation of the holoenzyme is commonly done under oxic conditions. However, with the enzyme described here, preparation under oxic conditions did not yield active enzyme. Fast inactivation of anoxically prepared recombinant enzyme may further be due to a lack of a (re)activating compound or chaperone, as described for other B_{12}-dependent enzymes (Kurteva-Yaneva *et al*., 2015; Weichler *et al*., 2015). Indeed, the *D. biacutus* gene cluster encodes also an 'Hcm-associated gene' (DebiaDRAFT_04572), though this protein was not detectable by our differential proteomics analyses, and was not further considered in this study. More details on the mutase enzyme (e.g., kinetic parameters and substrate range), are to be explored in a future study.

The specific activity of 3-hydroxybutyryl-CoA oxidation measured in CFE (app. 450 mU mg^{-1} protein) (Janssen and Schink, 1995a) was much higher than the activity measured with the recombinant dehydrogenase (76.7 ± 20.7 mU mg^{-1} protein). This can be explained by the presence of several 3-hydroxyacyl-CoA dehydrogenases in *D. biacutus* that are expressed during growth with acetone (e.g., also DebiaDRAFT_04512) (Gutiérrez Acosta *et al*., 2014). Further, we cannot exclude yet that the recombinant dehydrogenase may require an additional activating factor, or a thiolase as an accessory enzyme activity for pulling the reaction by removal of the product acetoacetyl-CoA.

As illustrated in Fig. 4, the genes in the described cluster coding for the predicted TDP-dependent enzyme, as well as for the confirmed 2-hydroxyisobutyryl-CoA mutase and the 3-hydroxybutyryl-CoA dehydrogenase in *D. biacutus* (Fig. 1B), are found each with very high sequence identity ($>$ 80% throughout) in a homologous cluster in *Desulfosarcina cetonica*, the only other known acetone-utilizing sulfate reducer. Homologous gene clusters were found also in *D. arcticum* DSM17038 and *D. geothermicum* DSM 3669 (Fig. 4), and therefore these organisms are predicted to be acetone-utilizing SRB.

The identification of a 2-hydroxyisobutyryl-CoA-isomerizing enzyme suggests 2-hydroxyisobutyryl-CoA as an intermediate formed through the initial acetone-activating reaction. Furthermore, this indicates that the carbonyl-C_2 of acetone is added to either (activated)

carbon monoxide or, most likely, to formyl-CoA, through an involvement of the highly induced TDP-dependent enzyme in *D. biacutus* (Fig. 1A); the exact function and activity of this TDP-dependent enzyme is still under investigation. As long as this enzyme has not been characterized, it remains unclear whether activated carbon monoxide (i.e., a formyl residue) is added to acetone directly to form a branched-chain aldehyde, which could then be converted by a CoA-acylating dehydrogenase to 2-hydroxyisobutyryl-CoA, or whether the formyl residue is added in the form of formyl-CoA (Fig. 1A). Formyl-CoA could be bound and coordinated by the TDP-dependent enzyme for a nucleophilic attack on the C_2-carbon of acetone to form 2-hydroxyisobutyryl-CoA. This reaction may indeed reflect a reversal of the α-oxidation of branched-chain CoA esters, in which an aldehyde and formyl-CoA are released by a TDP-dependent enzyme (2-hydroxyacyl-CoA lyase) (Casteels *et al*., 2007). However, the TDP-dependent enzyme in *D. biacutus* was observed to be associated (but not tightly bound) to the membrane, and it may be speculated whether the acetone-activating reaction may be coupled to, e.g., the reversed Wood-Ljungdahl pathway for a direct formyl transfer, or to a Rnf complex (Gutiérrez Acosta *et al*., 2014). Nonetheless, activation of acetone either *via* carbonylation or the use of formyl-CoA would require only one ATP, which is less than the expense for an activation *via* carboxylation; for a carboxylation, at least two ATP equivalents have to be invested to form acetoacetate plus at least one further ATP for the activation to acetoacetyl-CoA.

In conclusion, this novel type of formylation or carbonylation reaction on alkanones yielding a branched-chain CoA-ester such as 2-hydroxyisobutyryl-CoA from acetone, and the subsequent rearrangement of the carbon skeleton to yield a straight-chain CoA-ester such as 3-hydroxybutyryl-CoA, may play a role also in other strictly anaerobic bacteria (see above) degrading acetone or other methylketones. For example, the pathway depicted in Fig. 1A may also allow *D. biacutus* to utilize 2-butanone, and preliminary results from a proteomic analysis indicated a strong induction of the same set of enzymes during growth of *D. biacutus* with this substrate. The previously identified zinc-dependent dehydrogenase (strongly induced during growth with acetone, and encoded in a different gene cluster), which catalyzes NAD^+/NADH-dependent oxidations/reductions of a wide range of short-chain alcohols/aldehydes, e.g., reduction of 3-hydroxybutanal, may help to sequester side products formed during acetone activation, for example during CoA cofactor limitation or due to unspecific reactions forming reactive aldehydes (Fig. 1A) (Gutiérrez Acosta *et al*., 2014; Frey *et al*., 2016).

Experimental procedures

Chemicals

All purchased chemicals were from Sigma-Aldrich (Germany), Carl Roth GmbH (Germany) or Merck KGaA (Germany) and were at least of analytical grade. Biochemicals were purchased from Sigma-Aldrich (Germany), Apollo Scientific (UK) or AppliChem (Germany).

Chemical syntheses

Synthesis of the CoA esters was performed using thiophenyl esters, which were also not commercially available and were also synthesized by ourselves. The syntheses are described in detail in the Supplementary Information; NMR spectra for all products are also shown (Supporting Information Fig. S4).

Bacterial growth conditions

Desulfococcus biacutus strain KMRActS (DSM 5651) was grown as described previously (Janssen and Schink, 1995a; Gutiérrez Acosta *et al*., 2013). The medium was supplemented with 5 mM acetone or 5 mM butyrate as sole carbon source, and with 10 mM sodium sulfate as electron acceptor. *Escherichia coli* strains TOP10 (Invitrogen) and Rosetta 2 (Merck) were grown aerobically at 37°C in LB medium (10 g peptone, 5 g yeast extract and 10 g NaCl per liter) supplemented with 100 μg ml^{-1} ampicillin (TOP10) and additional 35 μg ml^{-1} chloramphenicol (Rosetta 2).

Plasmid construction

The genes of interest were amplified by PCR using the following primer sequences (5′ → 3′; directional-cloning overhang underlined): for Debia-ACPR (locus tag 04571) forward primer CACCATGCTATCTATTAAAGGATCGGTTGC and reverse primer CCCCTTGGATCCCAAACGAA; for Debia-HcmB (locus tag 04573) forward primer CACCATGGATAGGAAAGTCAGAGTGGTAA and reverse primer CACGATGCTTATGCCTGAGTAG; for Debia-HcmA (locus tag 04574) forward primer CACCGTGGAGAAAAGCAAAGCG and reverse primer CAGAAGCGGAAAAGCCCACAAG. Genomic DNA of *D. biacutus* as PCR template was purified usingthe Qiagen Genomic DNA Kit (Qiagen, Germany). The polymerase used was Phusion High-Fidelity DNA Polymerase (New England Biolabs), and the PCR conditions were 35 cycles of 45 s denaturation at 98°C, 45 s annealing at 60°C (65°C for 04573 and 04574) and elongation at 72°C depending on product length (1 min per 1000 bp). The PCR products were inserted either into the expression vector pET100 (N-terminal His_6-tag; for Debia-HcmB) or pET101 (C-terminal His_6-tag; for Debia-ACPR and Debia-HcmA) of the Champion pET Directional TOPO Expression Kit (Invitrogen)

(Frey *et al.*, 2016). Plasmid DNA was purified from *E. coli* using the Zymo MiniPrep Kit (Zymo Research), and used for sequencing (GATC Biotech, Germany). Transformation of chemically competent *E. coli*-Rosetta 2 (DE3) cells (Merck KGaA, Germany) was performed, as described previously (Frey *et al.*, 2016). Primer design and sequence data analysis was accomplished using the DNASTAR Lasergene software package.

Heterologous overexpression, preparation of cell-free extracts and purification of His-tagged proteins

Recombinant proteins were overexpressed as described previously (Frey *et al.*, 2016). When anoxic conditions were used, all preparation steps were performed in an anoxic tent. Cell-free extract of *D. biacutus* was prepared as described previously (Gutiérrez Acosta *et al.*, 2013). *E. coli* cells were suspended in binding buffer containing 50 mM KH_2PO_4/K_2HPO_4 (pH 7.2), 100 mM NaCl, 40 mM imidazole, 3 mM DTT and 30% (v/v) glycerol, were opened in a cooled French pressure cell (140 MPa), as described previously (Frey *et al.*, 2016). Cell debris and intact cells were removed by centrifugation (14 100 $\times$ *g*, 10 min). The supernatants were either loaded on Protino Ni-NTA columns (Macherey-Nagel, Germany) and purified under air (initial experiments), or under anoxic conditions on HisSpinTrap colums (GE Healthcare, Germany) in an anoxic tent. Non-specifically bound proteins were washed off three times with binding buffer containing 60 mM imidazole, and the bound His-tagged proteins were eluted with the same buffer containing 400–500 mM imidazole. Eluted proteins were transferred into the same buffer without imidazole through Illustra NAP-10 columns (GE Healthcare, Germany). Protein concentration was determined with Bradford reagent and bovine serum albumin (BSA) as standard (Bradford, 1976).

Protein gel electrophoresis and identification

Analysis of protein expression and protein purification was done by polyacrylamide gel electrophoresis (1D-PAGE) or native PAGE (Frey *et al.*, 2016); proteins were stained with colloidal Coomassie Brilliant Blue (Neuhoff *et al.*, 1988). Proteins were identified by peptide fingerprinting mass spectrometry at the Proteomics facility of University of Konstanz (Gutiérrez Acosta *et al.*, 2014).

Enzyme assays

Enzyme assays were performed under strictly anoxic conditions (in N_2-flushed 4-ml glass vials sealed with butyl rubber stoppers) in either 25 mM MOPS buffer or 50 mM potassium phosphate buffer, pH 7.2 (mutase) or pH 8.0 (dehydrogenase), each containing 1 g l^{-1} NaCl, 0.6 g l^{-1} $MgCl_2 \times 6\ H_2O$ and 3 mM DTT. Cofactor B_{12}-dependent reactions contained additional 50 μM adenosylcobalamin, and were incubated in the dark at 30°C. Reduction of acetoacetyl-CoA was assayed with 0.1 mM NADH (or NADPH), and oxidation of 3-hydroxybutyryl-CoA with 5 mM NAD^+ (or $NADP^+$), as co-substrates. Reactions were started by addition of substrate and were followed either discontinuously by HPLC-UV or HPLC-MS (see below), or by spectrophotometrical measurement (in N_2-flushed, rubber-stoppered 1.4 ml quartz cuvettes) of absorption (increase or decrease) of NADH at 340 nm ($\varepsilon_{NADH} = 6.292$ mM cm^{-1}) (Ziegenhorn *et al.*, 1976). All enzyme assays had a total volume of 1 ml. Anoxic substrate addition and sampling (150 μl per sample) was performed with gastight syringes (Hamilton AG, Switzerland).

HPLC analysis

Samples taken from the enzyme reactions for HPLC and LC-MS analysis were thoroughly mixed with dichloromethane in order to stop the reactions. The water phase obtained after centrifugation (16 000 $\times$ *g*, 5 min) was used for analysis. For routine analysis of 2-hydroxyisobutyryl-CoA and 3-hydroxybutyryl-CoA, a Kinetex PFP column (5 μm, 100 Å, 250 $\times$ 4.6 mm; Phenomenex, USA) was used on a Shimadzu Prominence system with PDA detector (SPD-M20A). The eluents were 100 mM ammonium acetate (eluent B) and acetonitrile (eluent A). The method started with 5% eluent A for 5 min, followed by a gradient from 5% to 25% eluent A between 5 and 19 min; the flow rate was 0.75 ml min^{-1} and the column was maintained at a temperature of 40°C. The injection volume was 5 μl.

LC-MS measurements

For HPLC-electrospray ionization (ESI)-MS/MS, an Agilent 1100 HPLC system and the Kinetex PFP column (see above), or a Synergi polar RP column (250 mm $\times$ 2 mm, 4 μm; Phenomenex, USA), connected to an LCQ ion trap mass spectrometer (Thermo Fisher Scientific) was used. For separation of 2-hydroxyisobutyryl-CoA and 3-hydroxybutyryl-CoA, the Kinetex PFP column was run isocratically with 95% 100 mM ammonium acetate and 5% acetonitrile for 10 min at a flow rate of 0.75 ml/min at 40°C; 5 μl were injected. For separation of the CoA esters (e.g., acetoacetyl-CoA), a Synergi polar RP column was used with a gradient from 2% to 100% eluent B between 2 and 18 min at a flow rate of 0.25 ml/min; eluent A was 30 mM ammonium acetate, and eluent B acetonitrile containing 0.1% acetic acid.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Fig. S1. Amino acid sequence alignment of the large (A) and small (B) subunits of B_{12}-dependent mutase enzymes. The sequences of the candidate 2-hydroxyisobutyryl-CoA mutases (HcmAB) of *D. biacutus* and *D. cetonica*, of confirmed HcmAB mutases of *K. tusciae* and *A. tertiaricarbonis*, and of characterized isobutyryl-CoA mutase of *Streptomyces cinnamonensis* and methylmalonyl-CoA mutase of *Pyrococcus horikoshii* for comparison, are included in the alignment (see also main text). *Asterisks* indicate residues conserved in all sequences; grey indicates conserved residues in Hcm sequences; black indicates the conserved B_{12}-binding domain. The alignment was done using MEGA6 software.

Fig. S2. Evaluation of the purity of recombinantly produced 2-hydroxyisobutyryl-CoA mutase subunits HcmA (A; 0.6 μg) and HcmB (B; 1.7 μg) by denaturing PAGE, estimation of the molecular weight of the native HcmAB complex by native PAGE (C; 1.7 μg), and (D; 2.7 μg) evaluation of the purity of recombinantly produced 3-hydroxybutyryl-CoA dehydrogenase. M, molecular weight marker (kDa).

Fig. S3. LC-MS/MS ion traces following the educt decrease (3-hydroxybutyryl-CoA) and product formation (acetoacetyl-CoA) in an enzyme assay performed with recombinant dehydrogenase (DebiaDRAFT_04571).

A. Time course of educt decrease (quasimolecular ion *m/z* 854) and product formation (quasimolecular ion *m/z* 852) monitoring the specific ion traces *m/z* 347 and 344 of the MS/MS fragmentation of 3-hydroxybutyryl-CoA and acetoacetyl-CoA. B. ESI-MS/MS $[M+H]^+$ of the quasimolecular ion *m/z* 852 of acetoacetyl-CoA.

Fig. S4. NMR spectra of CoA ester synthesis and precursors.