

# Enzymes Involved in a Novel Anaerobic Cyclohexane Carboxylic Acid Degradation Pathway

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The anaerobic degradation of cyclohexane carboxylic acid (CHC) has so far been studied only in *Rhodopseudomonas palustris*, in which CHC is activated to cyclohexanoyl coenzyme A (cyclohexanoyl-CoA [CHCoA]) and then dehydrogenated to cyclohex-1-ene-1-carboxyl-CoA (CHeneCoA). This intermediate is further degraded by reactions of the *R. palustris*-specific benzoyl-CoA degradation pathway of aromatic compounds. However, CHeneCoA is not an intermediate in the degradation of aromatic compounds in all other known anaerobic bacteria; consequently, degradation of CHC was mostly unknown in anaerobic bacteria. We identified a previously unknown CHC degradation pathway in the Fe(III)-reducing *Geobacter metallireducens* by determining the following CHC-induced *in vitro* activities: (i) the activation of CHC to CHCoA by a succinyl-CoA:CHC CoA transferase, (ii) the 1,2-dehydrogenation of CHCoA to CHeneCoA by CHCoA dehydrogenase, and (iii) the unusual 1,4-dehydrogenation of CHeneCoA to cyclohex-1,5-diene-1-carboxyl-CoA. This last represents a previously unknown joint intermediate of the CHC and aromatic compound degradation pathway in bacteria other than *R. palustris*. The enzymes catalyzing the three reactions were purified and characterized as specific enzymes after heterologous expression of the encoding genes. Quantitative reverse transcription-PCR revealed that expression of these genes was highly induced during growth with CHC but not with benzoate. The newly identified CHC degradation pathway is suggested to be present in nearly all CHC-degrading anaerobic bacteria, including denitrifying, Fe(III)-reducing, sulfate-reducing, and fermenting bacteria. Remarkably, all three CHC degradation pathways always link CHC catabolism to the catabolic pathways of aromatic compounds. We propose that the capacity to use CHC as a carbon source evolved from already-existing aromatic compound degradation pathways.

Cyclohexane carboxylic acid (CHC) occurs in nature predominantly as a functional group in ubiquitous biological compounds. Examples are naphthenic acids in crude oil (1, 2) or polyketide antibiotics from *Streptomyces* species (3). The biosynthetic origin of CHC is the shikimate pathway, as shown for *Alicyclobacillus acidocaldarius* species and *Streptomyces collinus* (3, 4). Moreover, CHC has been reported to be a product during the fermentation of benzoate or crotonate (see Fig. 1C) (5, 6).

Aerobic and anaerobic microorganisms have developed different strategies to use CHC as a carbon and/or energy source. The aerobic degradation of CHC was investigated in *Alcaligenes* and *Arthrobacter* strains and in *Corynebacterium cyclohexanicum* and involves monooxygenase- and oxidase-dependent steps yielding the aromatic 4-hydroxybenzoate (see Fig. 1A) (7, 8, 9, 10). Further degradation proceeds via the central intermediate protocatechuate that is channeled into the  $\beta$ -ketoacid pathway.

The oxygen-independent degradation of CHC has so far been studied only in the facultatively anaerobic, photosynthetic alpha-proteobacterium *Rhodopseudomonas palustris*. Here, CHC is first activated by an AMP-forming cyclohexanoyl coenzyme A (cyclohexanoyl-CoA [CHCoA]) synthetase (AliA) (see Fig. 1B) (11, 12). In a second step, CHCoA is oxidized to cyclohex-1-ene-1-carboxyl-CoA (CHeneCoA) by a specific acyl-CoA dehydrogenase (AliB) (13). The product is subsequently hydrated to the corresponding alcohol by a specific enoyl-CoA hydratase and oxidized to 2-oxocyclohexanoyl-CoA; the ring structure is then cleaved hydrolytically, yielding the aliphatic pimeloyl-CoA (13, 14). The subsequent modified  $\beta$ -oxidation steps yield three acetyl-CoA molecules and one CO<sub>2</sub> molecule. In *R. palustris*, CHeneCoA is the characteristic joint intermediate of the degradation of CHC and aromatic compounds, such as benzoate. During the degradation of the latter, the central benzoyl-CoA intermediate is dearo-

matized by a four-electron reduction to CHeneCoA, catalyzed by an ATP-dependent class I benzoyl-CoA reductase (15, 16). This so-called *R. palustris* type of benzoyl-CoA degradation pathway via the characteristic CHeneCoA intermediate has also been suggested to be present in the archaeon *Ferroplasma acidophilum* by genomic and transcriptomic analyses (17). However, it differs from the *Thauera aromatica* type of benzoyl-CoA degradation pathway present in all other known aromatic compound-degrading anaerobic bacteria. In denitrifying, sulfate-reducing, Fe(III)-reducing, and fermenting bacteria, the class I and II benzoyl-CoA reductases reduce their substrate only by two electrons, yielding cyclohex-1,5-diene-1-carboxyl-CoA (CHdieneCoA) (18, 19). As a consequence, a different set of enzymes is involved in CHdieneCoA degradation, including specific hydratases, dehydrogenases, and ring-cleaving hydrolases that form 3-hydroxypimeloyl-CoA and not pimeloyl-CoA. Notably, the enzymes involved in these reactions do not convert the corresponding intermediates of the *R. palustris*-type pathway (18, 20).

Only little is known about CHC degradation in anaerobic bacteria other than *R. palustris*. The complete degradation of CHC was reported for the sulfate-reducing *Desulfococcus multivorans* (21) and the fermenting *Syntrophus aciditrophicus* (22) (both are

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deltaproteobacteria) and in the denitrifying betaproteobacterium *T. aromatica*. These organisms degrade aromatic compounds via the *T. aromatica* type of benzoyl-CoA degradation pathway, where CHeneCoA is not a true intermediate. This finding suggests that CHC degradation in most anaerobic bacteria must be different from that reported for *R. palustris*.

In this work, we report on a novel CHC degradation pathway in *Geobacter metallireducens* that is likely to be present in all CHC-degrading anaerobic bacteria other than *R. palustris*. The specific enzymes involved were characterized as a CoA transferase and two acyl-CoA dehydrogenases that convert CHC to CHdieneCoA, a previously unknown joint intermediate of the anaerobic aromatic compound and CHC degradation pathways.

## MATERIALS AND METHODS

**Materials.** Chemicals and biochemicals were obtained from Roche Diagnostics (Mannheim, Germany), Fluka (Neu-Ulm, Germany), Merck (Darmstadt, Germany), Roth (Karlsruhe, Germany), Sigma-Aldrich (Deisenhofen, Germany), Applichem (Darmstadt, Germany), and Bio-Rad (Munich, Germany). Coenzyme A was obtained from Applichem. CoA esters were synthesized as described earlier (23, 24). Materials and equipment for protein purification were obtained from GE Healthcare (Munich, Germany), Millipore (Bedford, MA), and Sigma-Aldrich (Deisenhofen, Germany). The Champion pET101 directional Topo expression kit and chemically competent *Escherichia coli* OneShot-TOP10 and BL21Star (DE3) OneShot were purchased from Life Technologies (Grand Island, NY).

**Computational analysis.** Plots were prepared and regression coefficients were calculated using the GraphPad Prism 6 software package. BLAST searches were performed using the NCBI BLAST server (<http://www.ncbi.nlm.nih.gov/BLAST/>). Phylogenetic alignments were performed using the Mega5 software package ([www.megasoftware.net](http://www.megasoftware.net)).

**Cultivation of *Geobacter metallireducens*.** *G. metallireducens* was obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ 7210) and was cultivated at 25°C in 400-ml or 2-liter bottles under strictly anoxic conditions in a mineral salt medium (25) with nitrate (10 mM) as the electron acceptor. Either benzoate or CHC was added from sterile, pH-adjusted stock solutions (5 mM final concentrations) as a carbon and energy source. Cells were harvested at an optical density (578 nm) of approximately 0.5 under anaerobic conditions and stored at -20°C until further use.

**Determination of CHC and nitrate via ion chromatography.** Medium samples were taken during growth of *G. metallireducens*. After centrifugation, the supernatants were analyzed by ion chromatography (system, Dionex ICS 2100; column, Dionex IonPac AG11-HC RFIC, 2 by 250 mm; conductivity monitor; Thermo Scientific). Concentrations of ions in the medium were calculated based on calibration series with 5  $\mu$ M to 5 mM CHC, nitrite, and nitrate. The ions eluted after a 3-min isocratic start in a 24-min gradient from 1 to 60 mM potassium hydroxide.

**Heterologous gene expression and enzyme production in *Escherichia coli*.** The genes encoding the putative CHCoA dehydrogenase (*Gmet\_3307*), CHeneCoA dehydrogenase (*Gmet\_3306*), and the CHC-activating CoA transferase (*Gmet\_3304*) were amplified from *G. metallireducens* chromosomal DNA by PCR using primers suitable for the Champion pET101 directional Topo expression kit (Life Technologies) and for introducing a C-terminal 6 $\times$ His tag according to the manufacturer's instructions. For the oligonucleotide sequences used, see the supplemental material (Table S1). The following PCR conditions were used: 35 cycles of 10 s of denaturation at 98°C, 30 s of primer annealing at 63°C, and 50 s of elongation at 72°C; Q5 DNA polymerase (New England Biolabs) was used. Gel elution, ligation, cloning, and expression were performed according to the manufacturer's protocol. Chemically competent *Escherichia coli* BL21 Star(DE3) OneShot cells were transformed with the purified vector and grown at 37°C in LB medium containing 100  $\mu$ g ml<sup>-1</sup>

ampicillin and 50 mg liter<sup>-1</sup> riboflavin. Cells were induced with 1.0 mM isopropyl- $\beta$ -D-thiogalactopyranoside (IPTG) at an optical density of 0.4 to 0.6. After growth overnight at 20°C, the cells were harvested by centrifugation and stored at -20°C until use.

**Purification of recombinant proteins from *G. metallireducens*.** (i) **Preparation of cell extracts.** Frozen *E. coli* cells (*Gmet\_3306* and *Gmet\_3307*, 2 to 3 g wet cell mass per 5 ml; *Gmet\_3304*, 10 g per 10 ml) were suspended in 20 mM potassium phosphate, 200 mM sodium chloride, and 20 mM imidazole, pH 7.9 (buffer A), containing 0.1 mg of DNase I and 0.5 mg of lysozyme per ml. The cell suspension was passed through a French pressure cell at 138 MPa (4°C). The cell lysate was centrifuged at 100,000  $\times$  g for 60 min.

(ii) **Affinity chromatography.** For nickel affinity chromatography, the supernatant obtained after ultracentrifugation was applied onto a 1-ml Ni-chelating affinity column (HisTrap HP; GE Healthcare) that had been equilibrated with buffer A. After washing with buffer A, the His-tagged proteins eluted in a gradient from 0.02 to 0.5 M imidazole in buffer A over 10 ml at a flow rate of 1.0 ml min<sup>-1</sup>. Active fractions (2.5 ml) were pooled. Imidazole was removed by passing the enzyme over a desalting PD10 column (GE Healthcare). It eluted in 3.5 ml of 20 mM potassium phosphate, pH 7.9 (buffer B). Fifty percent glycerol in buffer B was added to a final concentration of 25% (vol/vol) glycerol. The enzymes were stored at -20°C.

**Acyl-CoA dehydrogenase assays.** CHCoA dehydrogenase and CHeneCoA dehydrogenase activities were measured in a discontinuous assay at 30°C via reverse-phase ultrahigh-performance liquid chromatography (RP-UPLC). The 200- $\mu$ l assay volumes contained 200 mM morpholinepropanesulfonic acid (MOPS), 15 mM MgCl<sub>2</sub>, 0.2 mM ferrocenium hexafluorophosphate, and 1 to 10  $\mu$ g enzyme, and the reaction was started by addition of 200  $\mu$ M substrate. Twenty-microliter samples were taken at different time points and stopped by addition of 20  $\mu$ l methanol. After centrifugation, the supernatants were applied to RP-UPLC (Waters) using an acetonitrile gradient (2% to 10% in 90 s, step to 20% in 1 s, and 20% to 30% in 139 s) in 10 mM phosphate buffer, pH 6.8. CHCoA, CHeneCoA, cyclohex-2-ene-1-carboxyl-CoA, cyclohex-3-ene-1-carboxyl-CoA, cyclohexa-1,5-diene-1-carboxyl-CoA (CHdieneCoA), 6-hydroxy-cyclohex-1-ene-1-carboxyl-CoA, butyryl-CoA, crotonyl-CoA, and isobutyryl-CoA were tested as substrates (0.2 mM [each]). For determination of  $K_m$  values, substrate concentrations were varied from 6.25 to 200  $\mu$ M. Spectrophotometric assays were discarded as cell extracts of *G. metallireducens* exhibited high unspecific activities with ferrocenium hexafluorophosphate.

**CoA transferase assay.** CoA transferase activity was determined in 100- $\mu$ l assay volumes with 10 to 100  $\mu$ g enzyme in buffer (100 mM Tris and 10 mM MgCl<sub>2</sub>, pH 7.8) with 0.25 mM acyl-CoA as a CoA donor and 5 mM free acid as a CoA acceptor. For determination of  $K_m$  values, the initial concentrations were varied from 0.125 mM to 5 mM for the free organic acid and from 7.8  $\mu$ M to 250  $\mu$ M for the CoA donor. The reaction was started by addition of the CoA acceptor. Twenty-microliter samples were taken at different time points and stopped by addition of 20  $\mu$ l 1 M HCl. After centrifugation, the supernatants were applied to RP-UPLC using an acetonitrile gradient (2% to 10% in 90 s, step to 20% in 1 s, and 20% to 30% in 139 s) in 10 mM phosphate buffer, pH 6.8.

**Determination of molecular mass.** Native masses were determined by analytical gel filtration via Superdex 200 (10/300 GL column [GE Healthcare], 25-ml volume, 0.5 ml min<sup>-1</sup>) using 20 mM triethanolamine-HCl, pH 7.8, 4 mM MgCl<sub>2</sub>, and 150 mM KCl. For calibration, ferritin (440 kDa), catalase (245 kDa), aldolase (158 kDa), bovine serum albumin (BSA) (67 kDa), ovalbumin (43 kDa), and carbonic anhydrase (29 kDa) were used.

**Determination of cofactor content.** For flavin content determination, 100  $\mu$ l of purified enzyme was precipitated with 200  $\mu$ l methanol. The supernatants obtained after centrifugation were analyzed by RP-UPLC (see enzyme assays) and compared with a calibration curve from 0.1 to 10  $\mu$ M flavin adenine dinucleotide (FAD). For reconstitution ex-



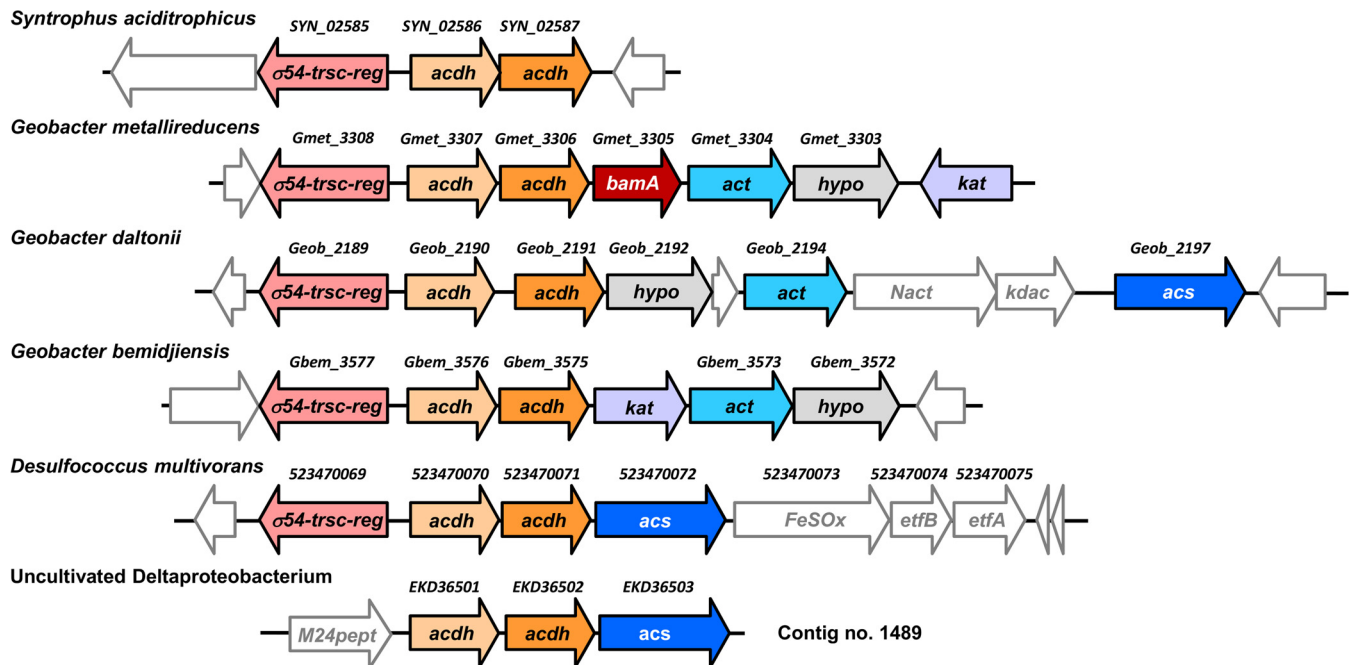


FIG 2 Gene clusters putatively involved in CHC degradation in deltaproteobacteria. Genes encoding proteins with significant sequence similarities are displayed in the same colors: CHCoA and CHeneCoA dehydrogenases (light and dark orange), proteins annotated as  $\sigma$ 54-dependent regulators (pink), CoA transferases (light blue), carboxylic acid CoA ligases (dark blue), and similar hypothetical proteins (gray). The gene cluster in *G. metallireducens* contains an additional *bamA* gene encoding a ring-opening hydrolase (red). Abbreviations:  $\sigma$ 54-trsc-reg, sigma54-dependent transcription regulator; acdh, acyl-CoA dehydrogenase; act, acyl-CoA transferase; hypo, hypothetical protein; kat, ketoacyl-CoA thiolase; Nact, *N*-acetyltransferase; kdac, zinc-dependent lysine deacetylase; acs, acyl-CoA synthetase; FeSO<sub>x</sub>, FeS oxidoreductase; etf, electron-transferring flavoprotein; M24pept, Met24 peptidase.

ond copy of a *bamA* gene putatively encoding the ring cleaving 6-oxocyclohex-1-ene-1-carboxyl-CoA hydrolase. It has >90% amino acid sequence identity to the product of the experimentally verified *bamA* gene present in the benzoic acid metabolism gene cluster (20). Finally, a protein with high similarities to a transcriptional regulator of the DNA-binding FIS family (factor for *in*-version stimulation) is part of the gene cluster (Fig. 2).

**Growth of *G. metallireducens* with CHC.** Although growth with CHC has not been reported before, *G. metallireducens* was chosen as a well-characterized model organism to study the hypothesized alternative CHC degradation pathway. *G. metallireducens* indeed grew exponentially with CHC (5 mM) as the sole carbon source and nitrate (10 mM) as a terminal electron acceptor (ammonification conditions). Per mol CHC, 7.5 mol nitrate was consumed, suggesting a complete oxidation of CHC to CO<sub>2</sub>. The doubling time with CHC was similar to that with benzoate as a growth substrate ( $7.8 \pm 0.5$  h at 25°C). With 5 mM CHC, the optical density reached 0.47 (Fig. 3). At 30°C, the specific substrate consumption rate of exponentially growing cells was determined to be maximally  $29 \text{ nmol min}^{-1} [\text{mg protein}]^{-1}$ .

**Enzyme activities in extracts from *G. metallireducens*.** Amino acid sequence similarities of known enzymes and the putative proteins involved in CHC degradation suggested the activation of CHC by a succinyl-CoA-dependent CoA transferase followed by the oxidation to CHdieneCoA by CHCoA and CHeneCoA dehydrogenase activities. To verify this assumption, *in vitro* enzyme activities were determined, in which substrate consumption and product formation were monitored by RP-UPLC analysis. The succinyl-CoA-dependent formation of CHCoA from CHC was observed with a specific activity of  $16 \text{ nmol min}^{-1} \text{ mg}^{-1}$

in extracts from cells grown with CHC. This value is slightly below the specific substrate consumption rate of exponentially growing cells, which might be caused by unspecific thioesterases or suboptimal assay conditions. Such extracts also catalyzed the time-, protein-, and ferrocenium hexafluorophosphate-dependent oxida-

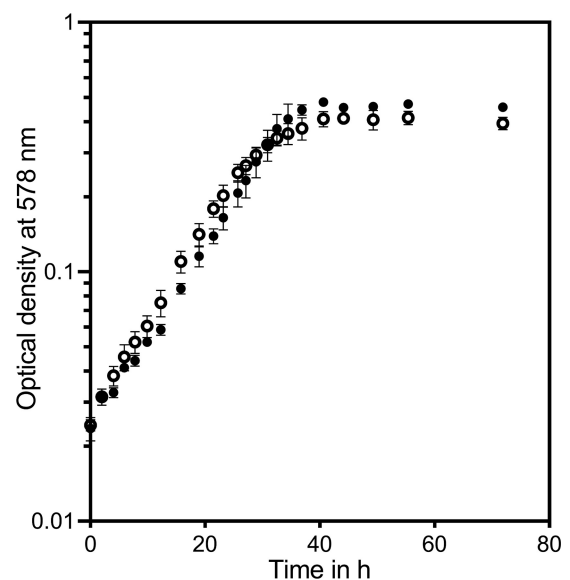
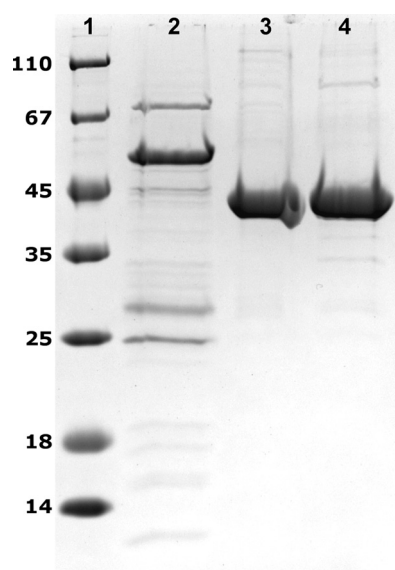


FIG 3 Growth of *G. metallireducens* with cyclohexane carboxylate (black circles) and benzoate (white circles). For each substrate, four independent cultures were measured. Error bars represent standard deviations of the means.



**FIG 4** SDS-polyacrylamide gels of heterologously expressed and purified proteins involved in the degradation of CHC. Lane 1, molecular mass standard; lane 2, succinyl-CoA:CHC CoA transferase; lane 3, CHeneCoA dehydrogenase; lane 4, CHCoA dehydrogenase (7  $\mu\text{g}$  each).

tion of CHCoA to CHeneCoA (61  $\text{nmol min}^{-1} \text{mg}^{-1}$ ) and CHeneCoA to CHdieneCoA (64  $\text{nmol min}^{-1} \text{mg}^{-1}$ ). The latter was further oxidized to benzoyl-CoA, a side reaction that has also been observed for CHeneCoA dehydrogenase from *S. aciditrophicus* (31). The dehydrogenase activities were 4- to 6-fold higher than in cells grown on benzoate (CHCoA, 17  $\text{nmol min}^{-1} \text{mg}^{-1}$ ; CH1eneCoA, 11  $\text{nmol min}^{-1} \text{mg}^{-1}$ ).

**Heterologous expression of putative genes involved in CHC degradation.** The genes *Gmet\_3304*, *Gmet\_3307*, and *Gmet\_3306*, (Fig. 2), putatively coding for a CoA transferase and two acyl-CoA dehydrogenases, were individually expressed in *E. coli* BL21 Star with a C-terminal His tag. After purification by Ni-chelating affinity chromatography, in each case highly enriched protein bands were obtained, as evidenced by SDS-polyacrylamide gel analysis (Fig. 4). According to the presence of 6-fold His tags, each protein migrated at a slightly higher molecular mass than calculated from the amino acid sequence (47.8 kDa, 41.2 kDa, and 40.9 kDa for *Gmet\_3304*, *Gmet\_3307*, and *Gmet\_3306*, respectively).

**Characterization of the CHCoA-forming enzyme.** The gene *Gmet\_3304* was predicted to code for a class I CoA transferase that operates in a ping-pong mechanism (34). The heterologously produced product had a homodimeric composition with a native molecular mass of  $95 \pm 7$  kDa as determined by gel filtration analysis. CoA transferase activity was tested by RP-UPLC analysis using various CoA donors (0.25 mM) and carboxylic acids (5 mM). Activation of CHC to CHCoA showed highest activities with succinyl-CoA (2.0  $\mu\text{mol min}^{-1} \text{mg}^{-1}$ ) and butyryl-CoA (2.7  $\mu\text{mol min}^{-1} \text{mg}^{-1}$ ) as a CoA donor; benzoate and cyclohex-1-ene-1-carboxylate were converted with lower specific activities (Table 1). The equilibrium constants ( $K_{\text{eq}}$ ) were extracted from the equilibrium substrate/product concentrations reached from the forward and reverse directions and gave highly similar numbers. The values obtained revealed that the formation of CHCoA from succinyl-CoA or butyryl-CoA as donors was favored over the reverse reaction, supporting that CHCoA formation is the *in vivo* role of

the CoA transferase. Interestingly, the  $K_m$  values and specific activities with CHC, benzoate, and cyclohex-1-ene-1-carboxylate were apparently affected by the choice of the CoA donor (Table 1) and were always lower with butyryl-CoA than with succinyl-CoA or other CoA donors. Such observations were not expected for a true ping-pong mechanism, and the mechanism of the CoA transferase needs to be studied in future detailed kinetic analyses. The results obtained suggest, on the first view, that butyryl-CoA serves as a natural CoA donor. However, a mass spectrometry-based CoA-ester pool analysis in exponentially grown cells revealed that succinyl-CoA is the most abundant CoA ester, whereas butyryl-CoA was hardly detectable, with a ratio of succinyl-CoA to butyryl-CoA of  $\gg 1,000$  (P. Kiefer, ETH Zürich, personal communication). For this reason, succinyl-CoA is considered a natural CoA donor for CHC activation.

**Characterization of CHCoA and CHeneCoA dehydrogenases.** Gel filtration analysis revealed a native molecular mass of  $140 \pm 10$  kDa for both *Gmet\_3306* and *Gmet\_3307*, suggesting a homotetrameric composition, respectively, that is typical for acyl-CoA dehydrogenases. The enzymes contained 0.80 (*Gmet\_3307*) and 0.45 (*Gmet\_3306*) FAD per subunit after incubation with free FAD followed by a desalting step.

The *Gmet\_3307* gene product catalyzed the ferrocenium hexafluorophosphate-dependent oxidation of CHCoA to CHeneCoA with a specific activity of 29  $\mu\text{mol min}^{-1} \text{mg}^{-1}$ ; the product formed was not further dehydrogenated to CHdieneCoA. The reaction followed a Michaelis-Menten kinetic, with a  $K_m$  value of  $< 5 \mu\text{M}$ . As an alternative substrate, cyclohex-3-ene-1-carboxyl-CoA was converted to the corresponding cyclohexadiene-1-carboxyl-CoA isomers (30% rate compared to CHC). No activity was observed ( $< 0.1\%$ ) with 6-hydroxycyclohex-1-ene-1-carboxyl-CoA, cyclohex-2-ene-1-carboxyl-CoA, butyryl-CoA, crotonyl-CoA, isobutyryl-CoA, glutaryl-CoA, or succinyl-CoA. Due to the low  $K_m$  and high specificity, *Gmet\_3307* is referred to as a CHCoA dehydrogenase.

The protein derived from *Gmet\_3306* converted CHeneCoA to CHdieneCoA at a rate of 2.3  $\mu\text{mol min}^{-1} \text{mg}^{-1}$  and CHdieneCoA to benzoyl-CoA at a rate of 1.0  $\mu\text{mol min}^{-1} \text{mg}^{-1}$  with a  $K_m$  of  $31 \pm 16 \mu\text{M}$  for CHeneCoA and a  $K_m$  of  $85 \pm 9 \mu\text{M}$  for

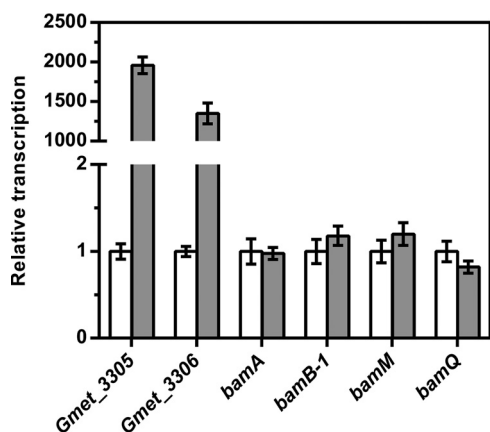
**TABLE 1** Kinetic properties of the succinyl-CoA:CHC CoA transferase<sup>a</sup>

Acceptor	CoA donor (0.25 mM)	$K_m^b$ ( $\mu\text{M}$ )	Activity (%)	Equilibrium $K_{\text{eq}}$
Cyclohexane carboxylic acid	Succinyl-CoA	$56 \pm 15$	100	1.7
	Butyryl-CoA	$14 \pm 6$	135	5
	Acetyl-CoA	$> 500$	31	ND <sup>c</sup>
	Propionyl-CoA	ND <sup>c</sup>	9	ND
	Acetoacetyl-CoA	ND	5	ND
	Crotonyl-CoA	ND	50	ND
	Glutaryl-CoA	ND	28	ND
Benzoic acid	CH1eneCoA	$> 200$	22	ND
	Succinyl-CoA	$416 \pm 50$	15	0.001
Cyclohex-1-ene-1-carboxylic acid	Butyryl-CoA	$125 \pm 48$	42	0.003
	Succinyl-CoA	$158 \pm 56$	40	0.007
	Butyryl-CoA	$52 \pm 12$	140	0.03

<sup>a</sup> "100%" refers to 2.0  $\mu\text{mol min}^{-1} \text{mg}^{-1}$ .  $K_m$  values are given for the free acids.

<sup>b</sup> Apparent  $K_m$  values as calculated using the GraphPad software package Prism 6.

<sup>c</sup> ND, not determined.



**FIG 5** Transcriptional analysis of genes involved in the anaerobic degradation of CHC and benzoate. Genes involved in the benzoyl-CoA degradation pathway (*bamA*, *bamB-1*, *bamM*, and *bamQ*) and genes of the CHC degradation cluster (*Gmet\_3305* and *Gmet\_3306*) are shown. For each gene, relative transcription in cells grown on benzoate (white) was compared to that in cells grown on CHC (gray). *Gmet\_3305* and *Gmet\_3306* ( $P \leq 0.001$ ) but none of the other tested genes ( $P > 0.05$ ) exhibited a statistically significant differential transcription. Error bars represent standard errors of the means.

CHdieneCoA. Neither CHCoA, cyclohex-2-ene-1-carboxyl-CoA, cyclohex-3-ene-1-carboxyl-CoA, butyryl-CoA, nor crotonyl-CoA was converted ( $<0.1\%$  compared to result for CHeneCoA). The kinetic properties strongly suggest that *Gmet\_3306* encodes a specific CHdieneCoA-forming CHeneCoA dehydrogenase catalyzing a 1,4-dehydrogenation reaction with the capacity to oxidize its product further to benzoyl-CoA.

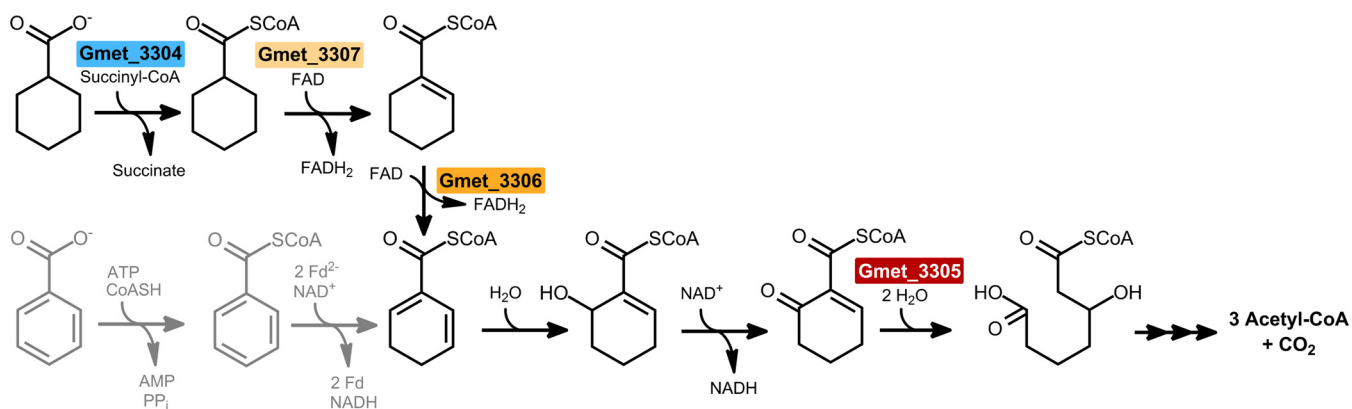
**Transcriptional regulation of genes involved in benzoate and cyclohexane carboxylate degradation.** The differential transcription of selected genes during growth with benzoate and CHC was investigated by quantitative reversed transcription-PCR. The targeted genes included the two genes of the newly identified putative CHC degradation-related gene cluster (*Gmet\_3305* [encoding a putative 6-oxocyclohex-1-ene-1-carboxyl-CoA hydrolase] and *Gmet\_3306* [encoding CHeneCoA dehydrogenase]) and genes involved in the benzoyl-CoA degradation pathway (previously shown to be differentially translated during growth on benzoate [35]): *bamA* (encoding a 6-oxocyclohex-1-ene-1-carboxyl-CoA

hydratase), *bamB* (encoding a class II benzoyl-CoA reductase active site-containing subunit), *bamM* (encoding glutaryl-CoA dehydrogenase), and *bamQ* (encoding 6-hydroxycyclohex-1-ene-1-carboxyl-CoA dehydrogenase). Analysis of transcripts revealed a 3-orders-of-magnitude-higher transcription of *Gmet\_3305* and *Gmet\_3306* during growth with CHC than during growth with benzoate. Genes involved in the benzoyl-CoA degradation pathways were not differentially transcribed (Fig. 5).

## DISCUSSION

In this work, we identified a third CHC degradation pathway via the characteristic intermediate CHdieneCoA, formed from CHeneCoA by CHeneCoA dehydrogenase, the key enzyme of the novel CHC degradation pathway. Notably, this reaction proceeds by an unusual 1,4-dehydrogenation and not by the 1,2-dehydrogenation catalyzed by conventional acyl-CoA dehydrogenases (36). The observed further dehydrogenation of CHdieneCoA to benzoyl-CoA by CHeneCoA dehydrogenase is not considered to play a physiological role, since the CHdieneCoA intermediate is readily converted to 6-hydroxycyclohex-1-ene-1-carboxyl-CoA by a highly active CHdieneCoA hydratase (18) and other enzymes of the benzoyl-CoA degradation pathway.

It is striking that all three CHC degradation pathways convert CHC to intermediates of aromatic compound degradation pathways: (i) the oxygenase- and oxidase-dependent aromatization to 4-hydroxybenzoate in aerobic bacteria (8), (ii) the oxidation to CHeneCoA in *R. palustris* (14), and (iii) the oxidation to CHdieneCoA in *G. metallireducens* (Fig. 6). We propose that with the exception of *R. palustris* and probably *F. placidus* (17), the pathway identified in *G. metallireducens* is present in all CHC-degrading anaerobic microorganisms. This proposal is based on the finding that except for *R. palustris* (and *F. placidus*), all aromatic compound-degrading anaerobes employ the *T. aromatica* type of benzoyl-CoA degradation pathway, where CHdieneCoA but not CHeneCoA is the intermediate formed by class I or II benzoyl-CoA reductases (37). The former was identified as a joint intermediate involved in anaerobic aromatic compound and CHC degradation. For the initial activation of CHC to CHCoA, two alternatives appear to exist: while a CoA transferase is present in *Geobacter* species, other deltaproteobacteria, such as *D. multivorans*, lack such a transferase in the predicted CHC degradation



**FIG 6** The newly identified cyclohexane carboxylic acid degradation pathway in *G. metallireducens*. The involved gene products are depicted in the colors used in Fig. 3. The intermediate CHdieneCoA interfaces the CHC and aromatics degradation pathways (shown in gray). The cyclohexa-1,5-diene-1-carboxyl-CoA dehydrogenase (*Gmet\_3306*) represents a key enzyme that catalyzes a 1,4-dehydrogenation and is uniquely employed in the new CHC degradation pathway.

gene cluster. In these organisms, a gene putatively coding for a CoA ligase is proposed to be involved in CHCoA formation. *G. daltonii* seems to contain both alternatives for CHC activation (Fig. 2).

The genes encoding the CoA transferase and the two acyl-CoA dehydrogenases involved in the *G. metallireducens* type of CHC degradation pathway are always located in a gene cluster that are likely to form a transcriptional unit. The upregulation of gene transcription and the induction of the corresponding enzyme activities during growth on CHC support this assumption. The presence of the cluster in the genomes of the CHC-degrading *D. multivorans* and *S. aciditrophicus* confirms the predicted abundance, and its presence in the genomes of *G. bemidjensis* and *G. daltonii* strongly suggests that these organisms are able to utilize CHC as carbon source.

All studied and predicted CHC-degrading anaerobes have the capacity to degrade aromatic compounds, but not necessarily *vice versa*. In agreement, at least in *G. metallireducens*, CHC induces the expression of genes involved in both CHC and aromatic compound degradation, whereas benzoate did not induce the genes involved in CHC degradation. Among the 26 known aromatic compound-degrading anaerobes with available genomes (37), only a few contain genes similar to that encoding CHeneCoA dehydrogenase. This observation suggests that the enzymatic equipment for anaerobic CHC degradation expanded the existing carbon source utilization capacity among certain aromatic compound-degrading anaerobes. This suggestion is in line with the much higher abundance of aromatic functionalities in nature than of the CHC moiety. A likely scenario for the evolution of the CHC degradation pathway discovered in this work comprises a duplication of a gene encoding an acyl-CoA dehydrogenase (e.g., butyryl-CoA dehydrogenase). Notably, CHCoA and CHeneCoA dehydrogenases are always located adjacent to each other in the known/predicted CHC degradation gene clusters. The succinyl-CoA:CHC CoA transferase might have evolved from the succinyl-CoA:benzoate CoA transferase that has been described for *G. metallireducens* (*Gmet\_2054*) (33). Based on these considerations, we postulate that organisms with the capability of degrading CHC very likely also utilize aromatic compounds.

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