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Corynebacterium glutamicum harbours a molybdenum cofactor-dependent formate dehydrogenase which alleviates growth inhibition in the presence of formate

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Here, we show that *Corynebacterium glutamicum* ATCC 13032 co-metabolizes formate when it is grown with glucose as the carbon and energy source. CO_2 measurements during bioreactor cultivation and use of ^{13}C-labelled formate demonstrated that formate is almost completely oxidized to CO_2. The deletion of *fdhF* (cg0618), annotated as formate dehydrogenase (FDH) and located in a cluster of genes conserved in the family *Corynebacteriaceae*, prevented formate utilization. Similarly, deletion of *fdhD* (cg0616) resulted in the inability to metabolize formate and deletion of cg0617 markedly reduced formate utilization. These results illustrated that all three gene products are required for FDH activity. Growth studies with molybdate and tungstate indicated that the FDH from *C. glutamicum* ATCC 13032 is a molybdenum-dependent enzyme. The presence of 100 mM formate caused a 25 % lowered growth rate during cultivation of *C. glutamicum* ATCC 13032 wild-type in glucose minimal medium. This inhibitory effect was increased in the strains lacking FDH activity. Our data demonstrate that *C. glutamicum* ATCC 13032 possesses an FDH with a currently unknown electron acceptor. The presence of the FDH might help the soil bacterium *C. glutamicum* ATCC 13032 to alleviate growth retardation caused by formate, which is ubiquitously present in the environment.

INTRODUCTION

Formate plays an important role in microbial metabolism. On the one hand, many micro-organisms such as methanogens and sulfate-reducing bacteria are able to use formate as a substrate for growth (Ferry, 2011; Plugge *et al.*, 2011). On the other hand, formate is an end product of bacterial fermentations (Ferry, 2011; Lin & Iuchi, 1991). Formate can be formed by the coenzyme A-dependent cleavage of pyruvate to yield formate and acetyl-CoA (Lin & Iuchi, 1991), by formaldehyde oxidation or by hydrolysis of 10-formyltetrahydrofolate (Misset-Smits *et al.*, 1997; Nagy *et al.*, 1995). Formate is a crucial compound in many syntrophic associations, where it is an intermediate in the conversion of biological polymers to methane and carbon dioxide (Crable *et al.*, 2011; McInerney *et al.*, 2009; Stams & Plugge, 2009).

In accordance with the multiple physiological roles of formate in various bacteria and archaea different types of formate dehydrogenases (FDHs) have evolved, thereby FDHs comprise a heterogeneous group of enzymes found in both prokaryotes and eukaryotes, which catalyse the oxidation of formate to CO_2. FDHs can be found in prokaryotic micro-organisms such as aerobic methylotrophs and chemoautotrophs, anaerobic or facultative anaerobic bacteria, as well as methanogenic archaea (Friedebold & Bowien, 1993; Jormakka *et al.*, 2002; Karzanov *et al.*, 1991; Schauer *et al.*, 1986). Depending on the type of FDH, the electrons are transferred to an acceptor such as NAD^+, $NADP^+$, cytochrome, cofactor F_{420}, fumarate, nitrate, sulfate or still unknown acceptors (Chistoserdova *et al.*, 2007; Friedebold & Bowien, 1993; Kröger *et al.*, 1979; Schauer *et al.*, 1986; Yagi, 1979; Yamamoto *et al.*, 1983; Jormakka *et al.*, 2003; Kröger *et al.*, 1986; Moura *et al.*, 2004; Sebban *et al.*, 1995). NAD^+-dependent FDHs are widely used as efficient biocatalysts for NADH regeneration in industrial applications and by the finding that the FDH is capable of selectively cleaving formic acid esters to the respective alcohol, FDH has emerged as a superior deformylation catalyst compared with hydrolases (Fröhlich *et al.*, 2011; Tishkov & Popov, 2004).

The industrial workhorse *Corynebacterium glutamicum* is well known for its ability to produce amino acids (Eggeling & Bott, 2005; Kabus *et al.*, 2007; Takors *et al.*, 2007;

Abbreviation: FDH, formate dehydrogenase.

Two supplementary figures and a supplementary table are available with the online version of this paper.

Wendisch *et al.*, 2006a, b) and has also been engineered for production of amino-acid-derived products, alcohols and organic acids from glucose and other carbon sources (Blombach *et al.*, 2011; Buschke *et al.*, 2011; Gopinath *et al.*, 2011; Litsanov *et al.*, 2012a, b; Niimi *et al.*, 2011; Rittmann *et al.*, 2008; Sasaki *et al.*, 2009; Schneider *et al.*, 2011, 2012; Stäbler *et al.*, 2011). In initial experiments on the use of different carbon sources by this organism, we observed that *C. glutamicum* ATCC 13032 could consume formate. In both genome sequences of *C. glutamicum* ATCC 13032 (Ikeda & Nakagawa, 2003; Kalinowski *et al.*, 2003), an FDH gene has been annotated with homology to formate dehydrogenase from *Escherichia coli*.

E. coli harbours three FDH isoenzymes. FdnGHI (FDH-N) and FdoGHI (FDH-O) are respiratory enzymes which work under anaerobic conditions in the presence of nitrate as terminal electron acceptor and are anchored to the periplasmic side of the inner membrane (Jormakka *et al.*, 2002; Thomé *et al.*, 2012). In the absence of an exogenous electron acceptor under anaerobic conditions, FdhF (FDH-H) is expressed as part of the formate–hydrogen lyase complex and is located at the cytoplasmic side of the membrane (Axley *et al.*, 1990; Boyington *et al.*, 1997; Raaijmakers & Romão, 2006). It delivers electrons from formate to hydrogenase 3 and protons are reduced to hydrogen molecules (Ingledew & Poole, 1984). The expression of the FDH-H is induced by formate and repressed by oxygen, nitrate, nitrite and other electron acceptors (Axley *et al.*, 1990; Pecher *et al.*, 1983). X-ray structure analysis of FdhF revealed the presence of a Fe_4S_4 cluster and a molybdenum cofactor in the catalytic subunit (Boyington *et al.*, 1997; Jormakka *et al.*, 2002). Furthermore, it is a selenoprotein in which selenium is covalently bound as a selenocysteine and the UGA codon directs the co-translational insertion into the polypeptide (Zinoni *et al.*, 1987).

The predicted formate dehydrogenase from *C. glutamicum* ATCC 13032 has not been shown, to our knowledge, to be involved in formate consumption. In this study we show that *C. glutamicum* ATCC 13032 is able to oxidize formate, identify genes involved in this reaction, and propose a physiological function.

METHODS

Bacterial strains, plasmids, media and growth conditions. Strains constructed or used in this study are listed in Table 1. *E. coli* DH5α (Life Technologies) was used for cloning purposes. Plasmid pK19mobsacB was used for construction of the defined in-frame deletion mutants (Schäfer *et al.*, 1994) *C. glutamicum* ATCC 13032 Δcg0618, *C. glutamicum* ATCC 13032 Δcg0616 and *C. glutamicum* ATCC 13032 Δcg0617. For complementation studies, strains *C. glutamicum* ATCC 13032 Δcg0618/pAN6-cg0618, *C. glutamicum* ATCC 13032 Δcg0616/pAN6-cg0616, *C. glutamicum* ATCC 13032 Δcg0617/pAN6-cg0617 and the reference strain *C. glutamicum* ATCC 13032 pAN6 were constructed. Plasmid pEKEx2-cg0618-strep contains the cg0618 coding region (including a StrepTag-II coding sequence at the 3′-end) plus a 400 bp upstream region carrying the native promoter of cg0618. This plasmid was transferred into *C. glutamicum* ATCC 13032 Δcg0618 and used for production and purification of a C-terminally Strep-tagged FdhF protein, which served to determine the amino terminus of FdhF.

Several media were used for cultivation of *C. glutamicum* ATCC 13032 (at 30 °C) and *E. coli* (at 37 °C) under oxic conditions. LB medium contained (l^{-1}) 10 g NaCl (Sigma-Aldrich), 10 g tryptone (BD) and 5 g yeast extract (BD). For selection against *sacB*, LB medium was supplemented with 10 % (w/v) sucrose (Sigma-Aldrich). BHI medium contained 37 g brain heart infusion l^{-1} (BD). BHIS medium represents BHI medium supplemented with 91 g sorbitol l^{-1} (AppliChem). For agar plates, 1.8 % (w/v) agar (BD) was added. CGXII medium for growth of *C. glutamicum* ATCC 13032 strains was prepared as described by Keilhauer *et al.* (1993). If necessary, the media contained kanamycin (25 μg ml^{-1} for *C. glutamicum* ATCC 13032, 50 μg ml^{-1} for *E. coli*). Formate was added to the culture medium as a 5 M potassium formate stock solution that was neutralized with HCl. Growth was determined by measuring OD_{600}.

Recombinant DNA work. The enzymes for recombinant DNA work were obtained from Roche Diagnostics or Fermentas. Chromosomal DNA from *C. glutamicum* ATCC 13032 was prepared as described by Eikmanns *et al.* (1994). Plasmids were isolated by using the QIAprep spin miniprep kit (Qiagen). *E. coli* was transformed by using the RbCl method (Hanahan, 1983), *C. glutamicum* ATCC 13032 by electroporation (van der Rest *et al.*, 1999). Routine methods such as PCR,

Table 1. Strains used or constructed in this study

Strain	Purpose	Reference or source
E. coli DH5α	Cloning	Life Technologies
C. glutamicum ATCC 13032	Wild-type reference	Abe *et al.* (1967)
C. glutamicum Δcg0618	In-frame deletion of *fdhF*	This study
C. glutamicum Δcg0618/pAN6-cg0618	Plasmid-based complementation of *fdhF*	This study
C. glutamicum Δcg0616	In-frame deletion of *fdhD*	This study
C. glutamicum Δcg0616/pAN6-cg0616	Plasmid-based complementation of *fdhD*	This study
C. glutamicum Δcg0617	In-frame deletion of cg0617	This study
C. glutamicum Δcg0617/pAN6-cg0617	Plasmid-based complementation of cg0617	This study
C. glutamicum pAN6	Vector control	Frunzke *et al.* (2008)
C. glutamicum Δcg0618/pEKEx2-cg0618-strep	Plasmid-based expression of FdhF for determination of the N-terminus	This study

restriction or ligation were carried out according to standard protocols (Sambrook *et al.*, 1989).

The oligonucleotides used for cloning were obtained from Operon and are listed in Table 2. The in-frame deletion mutants of *C. glutamicum* ATCC 13032 were constructed via a two-step homologous recombination procedure, as described previously (Niebisch & Bott, 2001). According to this procedure, for deletion of *cg0618*, the primers PΔcg0618_1, PΔcg0618_2, PΔcg0618_3 and PΔcg0618_4 were used. For deletion of *cg0616*, the primers PΔcg0616_1, PΔcg0616_2, PΔcg0616_3 and PΔcg0616_4 were used. For deletion of *cg0617*, the primers PΔcg0617_1, PΔcg0617_2, PΔcg0617_3 and PΔcg0617_4 were used. The resulting deletion mutants were verified by DNA sequencing using oligonucleotides binding outside the homologous regions used for deletion with pK19mobsacB (P_fdhF_left and P_fdhF_right for verification of *C. glutamicum* ATCC 13032 Δ*fdhF*; P_fdhD_left and P_fdhD_right for verification of *C. glutamicum* ATCC 13032 Δ*fdhD*; P_cg0617_left and P_cg0617_right for verification of *C. glutamicum* ATCC 13032 Δcg0617; Table 2).

For complementation studies, plasmid pAN6-cg0618 was constructed using primers Pcg0618_fw and Pcg0618_rv (plasmid provided by Boris Litsanov, from our institute). Plasmid pAN6-cg0616 was constructed using primers Pcg0616_fw and Pcg0616_rev. Plasmid pAN6-cg0617 was constructed using primers Pcg0617_fw and Pcg0617_rev. For expression of C-terminally Strep-tagged protein Cg0618, the plasmid pEKEx2-cg0618-strep was constructed using primers Pcg0618_strep_fw and Pcg0618_strep_rev. The constructed plasmids were verified by DNA sequencing using plasmid-specific primers.

Determination of glucose and formate concentration. To determine glucose and formate concentration, HPLC analysis was performed using an Agilent 1100 HPLC system (Agilent Technologies) with a cation exchange column (Organic acid Refill-column, 300 × 8 mm, CS-Chromatographie Service). Supernatants of *C. glutamicum* ATCC 13032 cultures were obtained by centrifugation of cell suspensions in 1.5 ml reaction tubes. If necessary, cell-free samples were diluted to the linear range of detection. The substances were eluted within 38 min with 100 mM sulfuric acid at a constant flow rate of 0.4 ml min^{-1} at 40 °C. The eluted organic acids were detected by a diode array detector (DAD G1315B) at a wavelength between 190 and 400 nm. Glucose was detected by a refractive index detector (RID G1362A). Concentrations were calculated from peak areas using calibration with external standards. Uptake rates were calculated in nmol min^{-1} (mg dry weight)$^{-1}$ (Frunzke *et al.*, 2008).

Determination of $^{13}CO_2$ and $^{12}CO_2$. To check and monitor the conversion of formate to CO_2 by *C. glutamicum* ATCC 13032 strains in ^{13}C labelling experiments, cells were grown in CGXII medium in a DASGIP Parallel Bioreactor System (DASGIP). During cultivation, pH, dissolved oxygen concentration and off-gas (CO_2 and O_2) were monitored online via the DASGIP Monitoring System (DASGIP Control 4.0). The pH of 7.0 was controlled by PID control loops and regulated by addition of 3 M KOH or 20 % (v/v) phosphoric acid via peristaltic pumps. O_2 saturation of >30 % in the medium was

Table 2. Primers used for cloning

Underlined nucleotides mark the restriction site.

Primer name	DNA sequence (5′–3′)	Restriction enzyme
PΔcg0618_1	GACCTGCAGAATTTGAAGATGCAAGC	*Pst*I
PΔcg0618_2	CCCATCCACTAAACTTAAACATGGTTTATTCATGGTGAGCAACGG	
PΔcg0618_3	TGTTTAAGTTTAG TGGATGGGCCAGTGTCCAAGTCAGTTGTG	
PΔcg0618_4	CAGGTCGACG AGCATTAACCAACGTGGAC	*Sal*I
P_fdhF_left	CTGTAATGTGCATTAGAGCGC	
P_fdhF_right	ACACAACGCGTGGGACCTG	
PΔcg0616_1	CAGTTGTGGTTCGCCTTGAAGCTTGAC	*Hin*dIII
PΔcg0616_2	CCCATCCACTAAACTTAAACACCGACCCATTTTTATTTAGCCTCC	
PΔcg0616_3	TGTTTAAGTTTAGTGGATGGGCTTGCTGGTTTTGTTCGGGGC	
PΔcg0616_4	CTGTAGATCAGCGAAAGTATGAGGGATCCGTC	*Bam*HI
P_fdhD_left	CTGAGGCCAACGTATTGGTTC	
P_fdhD_right	TGAAGGTTAGGCCTCGAGGAGG	
PΔcg0617_1	GACAAGCTTAACCAATGTGATTGAGCTACCC	*Hin*dIII
PΔcg0617_2	CCCATCCACTAAACTTAAACACGTTTCTGATGATGGCTGCAATTG	
PΔcg0617_3	TGTTTAAGTTTAGTGGATGGGAATGCTCTGCATCTTCAAGAAATC	
PΔcg0617_4	CAGGTCGACTCAACTG CTCAATAGACGTCG	*Sal*I
P_cg0617_left	GAGCGCATCTTCAACACATC	
P_cg0617_right	ATCCGAGGATCCGGTGTAATC	
Pcg0618_fw	TATACATATGACAACCCCTCCAACTGAG	*Nde*I
Pcg0618_rv	TATAGCTAGCCTAAGAAGCAGTACGTCCTGTTG	*Nhe*I
Pcg0616_fw	GACCATATGGGTCGG ATTACCCAAAAC	*Nde*I
Pcg0616_rev	GTCGCTAGCTTATCCGAGCTCGCC CGCATAG	*Nhe*I
Pcg0617_fw	GACCATATGTTGCAGCCATCATCAGAAACGAG	*Nde*I
Pcg0617_rev	GTCGCTAGCTTATTTAGCCTCCGGGTAATTTTTAG	*Nhe*I
Pcg0618_strep_fw	GACCCTGCAGGTTGAACCTTT CATTTTCAATTCTG	*Sbf*I
Pcg0618_strep_rev	GACGGTACCCTACTTCTCGAACTGTGGGTGGGACCAAGAAGCAGTACGTCCTGTTG	*Kpn*I

obtained by a cascaded dissolved oxygen control with agitation speeds of 400–1200 r.p.m. and a constant gas flow rate of 6 (standard litre) h^{-1}. If required, antifoam 204 (Sigma Life Science) was added. Growth was monitored offline by OD_{600} measurements. ^{13}C-Labelled sodium formate (Sigma-Aldrich) was added to the culture medium as stock solution. $^{13}CO_2$ and $^{12}CO_2$ off-gas analysis and quantification was performed with an FT-IR gas analyser (GASMET CR-2000i, Ansyco) between 900 and 4200 cm^{-1}, and the Calcmet software (Version 10).

Production, purification and MS analysis of C-terminally Strep-tagged FdhF. *C. glutamicum* ATCC 13032 Δcg0618 carrying plasmid pEKEx2-cg0618-strep was grown in 2000 ml baffled Erlenmeyer flasks filled with 300 ml CGXII medium and 25 μg kanamycin ml^{-1}. The culture was inoculated to OD_{600} 1 and induced with 0.7 mM IPTG (AppliChem) at OD_{600} 1.5. After 4 h of induction, the cells were harvested by centrifugation at 5000 ***g*** (30 min, 4 °C) and washed with TE buffer (100 mM Tris, 1 mM EDTA, pH 8.0). The cell pellet was resuspended in 8 ml TE buffer containing one protease inhibitor tablet (complete Mini-EDTA-free, Roche Diagnostics) and cells were disrupted via five passages through a French press (AMINCO Spectronic Instruments) at a pressure of 172 MPa. The protein-containing soluble fraction was separated from cell debris by a first centrifugation at 6000 ***g*** (20 min, 4 °C) and a second at 50 000 ***g*** (60 min, 4 °C). The supernatant was used for Strep-Tactin affinity chromatography and was incubated with 10 μl tetrameric avidin solution (5 μg $μl^{-1}$; Sigma-Aldrich) for 30 min on ice for saturation of biotinylated proteins. Afterwards, the protein solution was added to a 5 ml polypropylene column (Qiagen) containing 2 ml Strep-Tactin Sepharose (Strep-Tactin Superflow, Qiagen). After column equilibration with 4 ml buffer W (100 mM Tris, 1 mM EDTA, 100 mM NaCl, pH 8.0) the supernatant was applied onto the column for binding of the tagged protein to Strep-Tactin. The matrix was washed with 20 ml buffer W to remove non-specifically bound proteins. The elution of specifically bound proteins was performed with 8 × 1 ml buffer E (100 mM Tris, 1 mM EDTA, 400 mM NaCl, 2.5 mM desthiobiotin, pH 8.0). The elution fractions were separated by SDS-PAGE and visualized by Coomassie staining. The protein band at the expected size of tagged FdhF was subjected to in-gel digestion with trypsin. The peptides were eluted and subjected to MALDI-TOF-MS as described by Schultz *et al.* (2009).

RESULTS

Influence of formate on growth of *C. glutamicum* ATCC 13032 and consumption of formate

To test the influence and the fate of formate in cultures of *C. glutamicum* ATCC 13032 under oxic conditions, potassium formate was added, in concentrations up to 100 mM, to minimal medium containing 111 mM glucose (2 % w/v). The supplementation with formate resulted in a concentration-dependent decrease of the growth rate, whereas the final cell density was unaffected. The growth rate without formate was 0.43 h^{-1}, with 10 mM formate 0.41 h^{-1}, with 40 mM 0.37 h^{-1}, with 70 mM 0.36 h^{-1} and with 100 mM 0.32 h^{-1}. The concentration of formate leading to half-maximal growth inhibition of *C. glutamicum* ATCC 13032 wild-type was determined to be about 290 mM. When 100 mM KCl was added instead of potassium formate, the growth rate was 0.43 h^{-1}, showing that the inhibitory effect was caused by formate. Within 24 h of cultivation, formate was completely consumed by *C. glutamicum* ATCC 13032 (Fig. 1), as was glucose (data not shown). As shown below, formate consumption was dependent on the presence of the cells. The specific formate consumption rates were 28–30 nmol min^{-1} (mg dry weight)$^{-1}$, independent of the initial formate concentration. Interestingly, formate consumption was independent of growth, as it continued after the cells had reached the stationary phase. The observation that the final OD_{600} was not influenced by the presence of formate suggested that it is not assimilated in significant amounts.

FDH gene cluster and computational analysis of cg0618

The fact that *C. glutamicum* ATCC 13032 is able to metabolize formate gave rise to questions about the genes and proteins responsible for formate consumption. In the genome of *C. glutamicum* ATCC 13022 the ORF cg0618 has been annotated as a putative FDH and the gene was named *fdhF* (Kalinowski *et al.*, 2003). Immediately downstream of *fdhF*, two further genes related to FDH are located, cg0617 and cg0616 (*fdhD*), annotated as a putative molybdopterin guanine dinucleotide biosynthesis protein and an FDH accessory protein, respectively. A similar genetic organization is also found in other species of the suborder *Corynebacterineae*, e. g. in *Corynebacterium efficiens*, *Mycobacterium tuberculosis*, *Mycobacterium bovis* and *Rhodococcus erythropolis* (Fig. 2). In the genomes of the latter three species, however, there are no genes homologous to cg0617 based on sequence similarity, but genes homologous to molybdopterin guanine dinucleotide synthase MobA from *E. coli* can be found elsewhere in these genomes (Rv2453c, Mb2480c and RER_22150). It should be noted that there is no significant homology of

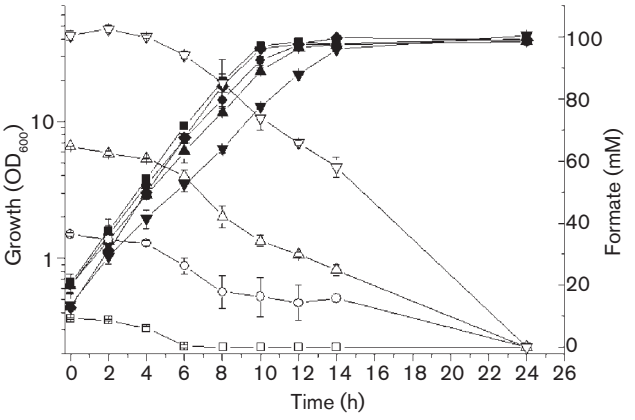

Fig. 1. Influence of formate on growth of *C. glutamicum* ATCC 13032 wild-type (OD_{600}, left axis, closed symbols) under oxic conditions in 60 ml CGXII medium containing 111 mM glucose and 0 mM (◆), 10 mM (■), 40 mM (●), 70 mM (▲) or 100 mM (▼) formate. Open symbols represent the formate concentration in the supernatant of the cultures (right axis). Mean ± SD values from two replicate experiments are shown.

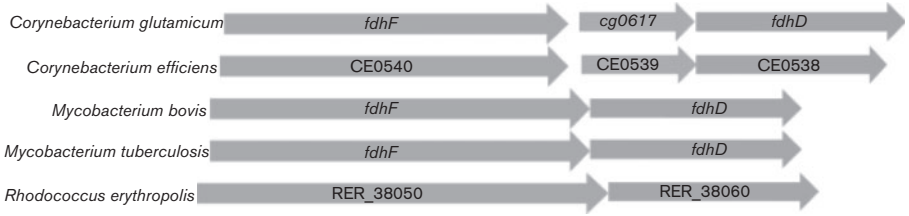

Fig. 2. Genetic organization of *fdhF* (cg0618) and *fdhD* (cg0616) in *C. glutamicum* ATCC 13032 and homologues in other species of the suborder *Corynebacterineae*. The genes *fdhF*, CE0540 and RER_38050 encode a putative formate dehydrogenase. The genes *fdhD*, CE0538 and RER_38060 encode a putative formate dehydrogenase accessory protein. The genes cg0617 and CE0539 encode a hypothetical protein annotated as a putative molybdopterin guanine dinucleotide biosynthesis protein.

the cg0617 protein from *C. glutamicum* ATCC 13032 to the MobA protein from *E. coli*, but cg1350 located elsewhere in the genome shows significant homology to MobA.

We used the amino acid sequence of the putative formate dehydrogenase encoded by *fdhF* in similarity searches using the NCBI BLAST tool. Relatively low sequence identities (22–29 %, BLAST tool, NCBI) were found to experimentally characterized FDHs participating in methylotrophic (Chistoserdova *et al.*, 2004, 2007), chemoautotrophic (Bowien & Kusian, 2002; Müller *et al.*, 1978), respiratory (Jormakka *et al.*, 2002) or fermentative (Raaijmakers & Romão, 2006) metabolism. Higher sequence identities were found with non-characterized FDHs from different *Streptomycetaceae* (e.g. 52 % with FDH from *Streptomyces coelicolor* and *S. lividans*) and from different mycobacteria including the human pathogen *M. tuberculosis* (48 %). However, a high sequence identity of 46 % also exists with FDH4 from *Methylobacterium extorquens*, which has been characterized as a molybdenum-dependent FDH with an important role in methanol metabolism (Chistoserdova *et al.*, 2007).

Using the NCBI conserved domain (CD) search tool (Marchler-Bauer *et al.*, 2011), the amino acid sequence of FdhF from *C. glutamicum* ATCC 13032 was shown to possess similarity to CDs of the FDH-alpha-like multidomain family and molybdopterin-binding superfamily, respectively. The molybdenum cofactor is required for functioning of many bacterial molybdoenzymes, including FDHs. Crystal structures of molybdoenzymes revealed amino acid residues involved in the binding of molybdopterin guanine dinucleotide (MGD) cofactor. In FDH-H from *E. coli* 35 residues coordinate the MGD cofactor, of which 23 are well-conserved in known MGD-containing FDHs (Boyington *et al.*, 1997). To identify conserved amino acids in FdhF from *C. glutamicum* ATCC 13032 that possibly bind the molybdenum cofactor, we compared the protein sequences of FdhF from *C. glutamicum* ATCC 13032, the well-characterized FDH-H from *E. coli*, and further FDH enzymes of the molybdopterin-binding superfamily. The protein sequence alignment shown in Fig. S1 (available with the online version of this paper) revealed at least 11 conserved or related amino acids in FdhF from *C. glutamicum* ATCC 13032 that could be involved in the coordinated binding of the molybdenum cofactor. Furthermore, the sequence alignment revealed five conserved cysteine residues in FdhF, four of which could be involved in the coordination of an Fe_4S_4 cluster (Cys-63, Cys-66, Cys-82, Cys-195; Fig. S1). The fifth conserved cysteine found in FdhF from *C. glutamicum* ATCC 13032 (Cys-199; Fig. S1) could be essential for catalytic activity, similar to a conserved cysteine or serine in other molybdenum cofactor-dependent enzymes or selenocysteine, as in the FDH-H from *E. coli* (Boyington *et al.*, 1997).

Deletion of genes in the FDH gene cluster and influence on formate consumption

To study the relevance of cg0618 (*fdhF*), cg0617 and cg0616 (*fdhD*) for formate consumption, the in-frame deletion mutants *C. glutamicum* ATCC 13032 Δcg0618, *C. glutamicum* ATCC 13032 Δcg0617 and *C. glutamicum* ATCC 13032 Δcg0616 were created as described in Methods. The deletions were confirmed by PCR analysis of the corresponding genomic regions and sequencing of the PCR products.

The deletion of cg0618 led to the inability of *C. glutamicum* ATCC 13032 to consume formate in defined medium with glucose (Fig. 3a). Moreover, during the course of the cultivation, *C. glutamicum* ATCC 13032 Δcg0618 showed slower growth compared with the wild-type, which could be attributed to the constantly high level of formate in the medium causing growth inhibition. In short-term (10 h) growth experiments we did not detect growth differences between *C. glutamicum* ATCC 13032 Δcg0618 and the wild-type in the absence of formate. To check whether there is a difference in growth in the long run, we performed long-term fitness experiments with two different initial mixtures of *C. glutamicum* ATCC 13032 wild-type and Δcg0618 mutant cells (40/60 % and 60/40 %) for direct comparison. The mixed cells were exponentially grown under oxic conditions for 6 days (144 h) by repeated inoculation into new shake flasks containing fresh

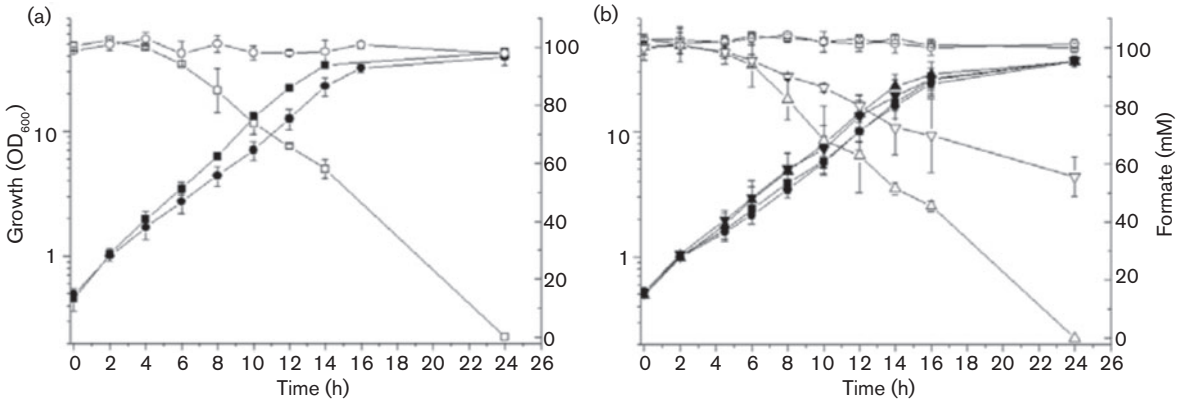

Fig. 3. Growth (left axes) and formate consumption (right axes) of different *C. glutamicum* ATCC 13032 strains under oxic conditions in minimal medium with 111 mM glucose and 100 mM formate. (a) OD_{600} (filled symbols) and formate concentration (open symbols) of the wild-type (■) and the deletion mutant Δcg0618 (●). (b) OD_{600} and formate concentration of strain Δcg0618/pAN6-cg0618 cultivated without (▲) or with (▼) 20 μM IPTG, and of the reference strain Δcg0618/pAN6 without (●) or with (■) IPTG. Mean ± SD values from two replicate experiments are shown.

medium with 111 mM glucose. Total genomic DNA from the initial (0 h) and the resulting (144 h) cell mixtures were analysed by quantitative real-time PCR to determine the relative content of the Δcg0618 locus compared with the *recF* locus which is present in both strains (data not shown). In both mixtures, we found a similar decrease in the relative Δcg0618 content after 144 h in both conditions, in the absence and in the presence of formate. The decrease corresponds to a growth rate of the Δcg0618 mutant which is about 3 % lower than the growth rate of the wild-type when grown in mixtures.

To exclude the possibility that the phenotype of *C. glutamicum* ATCC 13032 Δcg0618 was due to a secondary mutation, the mutant was transformed with the *fdhF* expression plasmid pAN6-cg0618 or, as a control, with pAN6. *C. glutamicum* ATCC 13032 Δcg0618/pAN6-cg0618, but not the control Δcg0618/pAN6, regained the ability to utilize formate (Fig. 3b). Again, the strain able to utilize formate showed faster growth over time than the strain unable to utilize it.

Strain Δcg0616 showed the same phenotype as Δcg0618, as it was completely unable to utilize formate (Fig. 4). The deficiency could be reversed by plasmid-borne expression of cg0616 using plasmid pAN6-cg0616, whereas plasmid pAN6 did not restore the ability to utilize formate. This shows that besides cg0618 cg0616 is also essential for formate utilization. Deletion of cg0617 did not cause a complete inability to utilize formate, but led to a decelerated formate consumption rate of about 6 nmol min^{-1} (mg dry weight)$^{-1}$, with a remaining formate concentration of about 60 mM after 29 h of cultivation (Fig. 4). Complementation of mutant Δcg0617 with plasmid pAN6-cg0617 restored the wild-type formate consumption rate of 30 nmol min^{-1} (mg dry weight)$^{-1}$. Again, the complemented strain showed better growth than the reference strain Δcg0617/pAN6.

Functional studies on the FDH from *C. glutamicum* ATCC 13032

To prove the conversion of formate to CO_2 by *C. glutamicum* ATCC 13032, ^{13}C-labelled sodium formate was used. *C. glutamicum* ATCC 13032 Δcg0618 and the wild-type were cultivated under controlled conditions in bioreactors using glucose minimal medium with and without formate. In a preliminary test, the $^{12}CO_2$ production rates of the two strains in the presence of ^{12}C-labelled formate were calculated from the monitored $^{12}CO_2$ concentrations in the off-gas. The wild-type showed a higher $^{12}CO_2$ production rate [137 nmol min^{-1} (mg dry weight)$^{-1}$] than the mutant strain Δcg0618 [105 nmol min^{-1} (mg dry weight)$^{-1}$], supporting the participation of FdhF in formate-dependent CO_2 production. In a second series of experiments, both strains were grown in glucose minimal medium supplemented with 70 mM sodium ^{13}C-labelled formate, and $^{13}CO_2$ production was monitored with an FT-IR gas analyser able to differentiate between $^{13}CO_2$ and $^{12}CO_2$. *C. glutamicum* ATCC 13032 Δcg0618 did not consume formate under controlled conditions, whereas formate was fully consumed by the wild-type within 12 h of cultivation (Fig. 5a). The calculated formate consumption rate was about 43 nmol min^{-1} (mg dry weight)$^{-1}$. The wild-type generated $^{13}CO_2$ whereas *C. glutamicum* ATCC 13032 Δcg0618 did not and the formation of $^{13}CO_2$ ceased with the depletion of ^{13}C-labelled formate after 12 h of cultivation (Fig. 5b). Overall, the calculated amount of $^{13}CO_2$ formed correlated with the total consumption of ^{13}C-labelled formate. In 12 h of cultivation, 70 mM ^{13}C-labelled formate was consumed and 67.3 mM $^{13}CO_2$ was produced. A gap in the $^{13}CO_2$ balance is expected since some $^{13}CO_2$ should be fixed in the biomass via the anaplerotic carboxylases. However, the result confirms that ^{13}C-labelled formate is directly oxidized to $^{13}CO_2$. The production of $^{12}CO_2$ was very similar in both cultures (Fig. 5b).

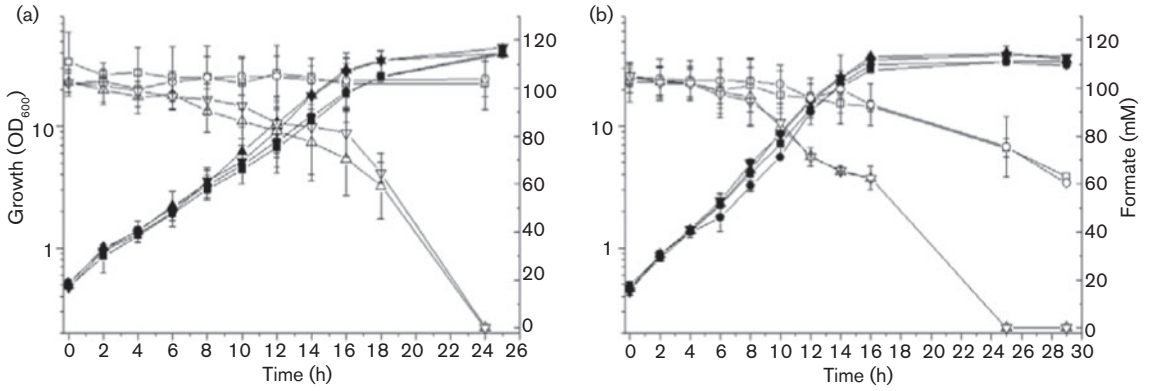

Fig. 4. Growth (left axes) and formate consumption (right axes) of different *C. glutamicum* ATCC 13032 strains under oxic conditions in minimal medium containing 111 mM glucose and 100 mM formate. (a) OD_{600} (filled symbols) and formate concentration (open symbols) of strain Δcg0616/pAN6-cg0616 without (▲) or with (▼) 20 μM IPTG and of the reference strain Δcg0616/pAN6 without (■) or with (●) 20 μM IPTG. (b) OD_{600} and formate concentration of strain Δcg0617/pAN6-cg0617 cultivated without (▲) or with (▼) 20 μM IPTG and of the reference strain Δcg0617/pAN6 without (■) or with (●) 20 μM IPTG. Mean ± SD values from two replicate experiments are shown.

Cofactor dependency and translational start of FdhF

The amino acid sequence analyses described above indicated that the *C. glutamicum* ATCC 13032 FdhF protein encoded by cg0618 contains a molybdopterin cofactor. To get further evidence for this assumption, the influence of sodium molybdate and sodium tungstate on formate consumption by the wild-type was tested (Fig. 6). It is known that the pterin can, in principle, bind either molybdenum or tungsten to form the biologically active molybdenum or tungsten cofactor and that the exchange of molybdenum and tungsten leads to inactive enzymes (McMaster & Enemark, 1998). As shown in Fig. 6, the presence of 2 μM sodium tungstate strongly inhibited formate consumption and this inhibition could be prevented by simultaneous addition of 2 μM sodium molybdate. Thus, the FDH from *C. glutamicum* ATCC 13032 appears to be a molybdenum-dependent enzyme.

In the two genome sequences published for *C. glutamicum* ATCC 13032, two different lengths were predicted for *fdhF*. The annotation by Ikeda & Nakagawa (2003) predicted a protein of 711 amino acids (cgl0529, NCgl0507), whereas the annotation by Kalinowski *et al.* (2003) predicted a protein of 762 amino acids (cg0618). To determine the

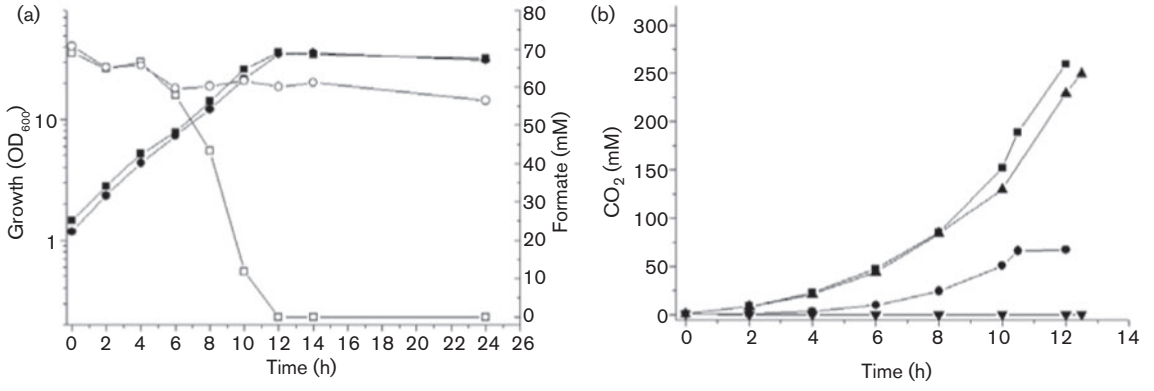

Fig. 5. Growth (left axis) and formate consumption (right axis) (a) and formation of $^{13}CO_2$ and $^{12}CO_2$ (b) of *C. glutamicum* ATCC 13032 wild-type and the deletion mutant Δcg0618 during cultivation under oxic conditions in DASGIP bioreactors containing minimal medium with 111 mM glucose and 70 mM ^{13}C-labelled sodium formate. (a) OD_{600} (closed symbols) and formate concentration (open symbols) for the wild-type (squares) and mutant Δcg0618 (circles). (b) The amount of $^{13}CO_2$ (●, ▼) and $^{12}CO_2$ (■, ▲) generated by *C. glutamicum* ATCC 13032 wild-type (●, ■) and mutant strain Δcg0618 (▼, ▲) as calculated from the off-gas analysis.

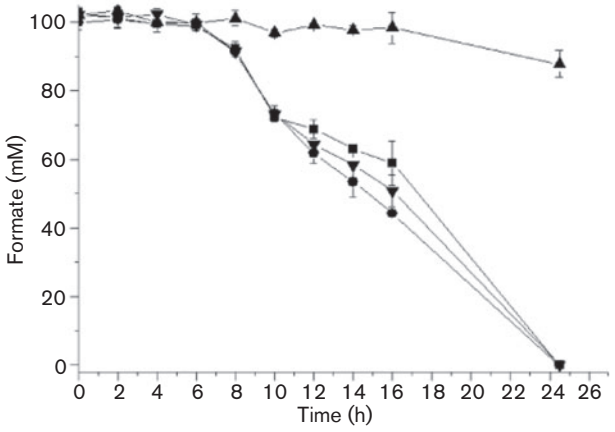

Fig. 6. Formate consumption of *C. glutamicum* ATCC 13032 wild-type under oxic conditions in minimal medium with 111 mM glucose and 100 mM formate without supplements (■), or supplemented with either 2 μM sodium tungstate (▲), 2 μM sodium molybdate (●) or 2 μM of each of the salts (▼). Mean ± SD values from two replicate experiments are shown.

amino terminus of native FdhF, C-terminally Strep-tagged FdhF was produced in *C. glutamicum* ATCC 13032 Δcg0618 using plasmid pEKEx2-cg0618-strep, which contains besides the *fdhF* coding region 400 bp upstream of the start codon of the 762 amino acid protein. After purification by StrepTactin affinity chromatography, the desthiobiotin eluate contained a dominant protein band of about 80 kDa as shown by SDS-PAGE and Coomassie staining (data not shown). The band was excised and after tryptic digestion was used for peptide mass fingerprint analysis by MALDI-TOF MS. The protein on hand was identified as FdhF by 31 matched mass peaks, corresponding to 43 % sequence coverage (Table S1 and Fig. S2). The mass with an *m*/*z* ratio of 2742.3 corresponded to the N-terminal peptide of the 762 amino acid FdhF protein with the N-terminal methionine cleaved off. The mass with an *m*/*z* ratio of 2108.2 corresponded to the peptide covering amino acids 27–47 of the 762 amino acid FdhF protein. These results show that native FdhF is a protein composed of 761 amino acids with the N-terminal sequence TTPPTEI.

DISCUSSION

From the central position of formate in the microbial world and its ubiquitous presence in the environment, it is plausible that soil bacteria harbour FDHs to be able to use formate as an energy source, and possibly for detoxification to alleviate growth retardation. Carboxylic acids such as formate, acetate and propionate can inhibit bacterial cell growth resulting in cellular responses including expression changes (Lee *et al.*, 2006; Litsanov *et al.*, 2012a; Polen *et al.*, 2003). *C. glutamicum* ATCC 13032 also showed reduced growth rates with increasing formate concentrations (Fig. 1). A possible explanation for the inhibitory effect is that formic acid (pK_a 3.77), but not its anion, can diffuse across the cytoplasmic membrane and thereby dissipate ion gradients and, dependent on the pH gradient, increase the internal anion concentration (Russell, 1992; Zaldivar & Ingram, 1999). However, with a pK_a of 3.77, formate exists predominantly in the deprotonated anionic form at physiological pH, necessitating a transport system to pass formate through the cell membrane. In *E. coli* the integral membrane protein FocA, which is impermeable to water but allows the passage of formate, was identified (Suppmann & Sawers, 1994; Wang *et al.*, 2009). In *C. glutamicum* ATCC 13032 no FocA-like sequence was found by BLAST analysis and the formate transport in *C. glutamicum* remains to be studied.

Our results show that an FDH is present in *C. glutamicum* ATCC 13032 and probably other related micro-organisms including *M. tuberculosis*. The FDH somewhat alleviated growth inhibition of *C. glutamicum* ATCC 13032 in the presence of formate, and *fdhF* and *fdhD* were essential for formate metabolism. No other formate-converting enzyme appeared to be present in *C. glutamicum* ATCC 13032 under the conditions tested. Also, BLAST analysis did not reveal other promising candidate formate dehydrogenase genes in the genome of *C. glutamicum* ATCC 13032.

The *fdhD* gene is annotated as an FDH accessory protein and is conserved in the *Corynebacterineae* and can also be found in other genomes. In *E. coli*, FdhD was found to be essential for activity of the two respiratory FDHs FDH-N and FDH-O as well as for the fermentative FDH-H (Abaibou *et al.*, 1995; Schlindwein *et al.*, 1990; Stewart *et al.*, 1991). Recently, FdhD from *E. coli* was shown to interact with IscS, one of the three *E. coli* L-cysteine desulfurases (Thomé *et al.*, 2012). The interaction of IscS with FdhD results in a sulfur transfer between IscS and FdhD in the form of persulfides. Substitution studies on the strictly conserved residues Cys-121 and Cys-124 of FdhD from *E. coli* showed that both are essential for the function of FdhD which was assessed through the FdhF activity (Thomé *et al.*, 2012). Corresponding cysteine residues are also present within the conserved motif Cys-Gly-Val-Cys-Gly in the FdhD proteins from *C. glutamicum* ATCC 13032 (Cys-151, Cys-154), *C. efficiens* YS-314, *M. tuberculosis* H37Rv, *M. bovis* AF2122/97, *Rhodococcus erythropolis* PR4 and others (data not shown). It is very likely that FdhD from *C. glutamicum* ATCC 13032 also acts as a sulfur transferase between a desulfurase and FdhF and therefore is essential for the formation of an active FDH. Several homologues of IscS from *E. coli* can also be found in the genomes mentioned above and have also been described in *C. glutamicum* ATCC 13032 (cg1214, cg1388 and cg1761) (see Schaffer *et al.*, 2001; Marienhagen *et al.*, 2005; Teramoto *et al.*, 2010).

Our experimental results and computational sequence analysis of FdhF indicate that it is a molybdenum-dependent

formate dehydrogenase which presumably contains an iron–sulfur cluster. Conserved cysteine residues were identified that are possibly involved in the coordination of an Fe_4S_4 cluster (Fig. S1). These residues are also conserved in FDH2 from *Methylobacterium extorquens* and FDH-S from *Ralstonia eutropha* (Fig. S1). The latter is known to contain Fe_4S_4 clusters (Chistoserdova *et al.*, 2004; Friedebold & Bowien, 1993). However, the presence of the iron–sulfur cluster in FdhF from *C. glutamicum* ATCC 13032 has still to be verified experimentally. The sequence analyses also revealed conserved residues for binding of the molybdopterin cofactor which can either bind molybdenum or tungsten to create the biologically active form. Tungsten-dependent enzymes were mainly found in anaerobic, thermophilic bacteria and archaea present in deep-sea hydrothermal vents, where tungsten is enriched. In most other habitats, tungsten is usually 100-fold less abundant than molybdenum (Hille, 2002; Schwarz *et al.*, 2007). Recently, tungsten-dependent enzymes were also found in aerobic bacteria, e.g. in *Methylobacterium extorquens* (Laukel *et al.*, 2003) or in *R. eutropha* (Cramm, 2009). In various studies it was shown that the interchange of molybdenum and tungsten led to inactive enzymes which can be exploited to identify whether the enzyme of interest is molybdenum- or tungsten-dependent (May *et al.*, 1988; McMaster & Enemark, 1998). However, for the N-formylmethanofuran dehydrogenases from *Methanobacterium thermoautotrophicum* and *Methanobacterium wolfeii* the interchange of molybdenum and tungsten yields enzymes with comparable activities (Johnson *et al.*, 1996). Our results show that the FDH from *C. glutamicum* ATCC 13032 is a molybdenum-dependent enzyme since the presence of tungstate almost completely inhibited formate consumption by *C. glutamicum* ATCC 13032 (Fig. 6).

In contrast with the deletion of cg0618 or cg0616, the deletion of cg0617 did not lead to the inability to consume formate, yet the consumption was strongly diminished. No putative conserved protein domain could be identified for Cg0617. However, although there is no significant homology to MobA from *E. coli*, cg0617 is annotated to encode a molybdopterin guanine dinucleotide biosynthesis protein which could be functionally identical to MobA of *E. coli* and *M. tuberculosis*. In contrast, the predicted protein encoded by cg1350 (*mob*) exhibits significant homology to MobA (27 % sequence identity) and it is possible that it partially complements *C. glutamicum* ATCC 13032 Δcg0617, explaining the lowered consumption of formate by reduced FDH activity. However, the detailed function of Cg0617 remains to be studied.

The ^{13}C-labelled formate experiment showed that formate is completely converted to $^{13}CO_2$ by *C. glutamicum* ATCC 13032 wild-type. The oxidation of formate to CO_2 generates two electrons transferred to an electron acceptor. We did not find evidence for FdhF being an integral membrane protein since protein sequence analysis tools did not find indications for signal peptides or transmembrane helices. Thus, FdhF from *C. glutamicum* ATCC 13032 appears to be a soluble protein in the cytosol or associated to the membrane. In enzyme assays with cell-free extracts, we could not detect formate dehydrogenase activity using NAD^+ or $NADP^+$ as cofactors (data not shown). In whole cell biotransformation processes, FDHs are frequently used for cofactor regeneration, like the NAD^+-dependent FDH from *Mycobacterium vaccae* (Bäumchen *et al.*, 2007; Litsanov *et al.*, 2012a). However, FDH from *C. glutamicum* ATCC 13032 is most likely not applicable for such processes and the oxidation of formate led to the generation of reducing equivalents, which are transferred to a currently unknown electron acceptor.

Well-characterized FDHs from various organisms participate in methylotrophic and chemoautrotrophic metabolism or are involved in anaerobic fermentative or respiratory processes. *C. glutamicum* ATCC 13032 does not contain the ability for either methylotrophic or chemoautotrophic growth. Furthermore, the FDH from *C. glutamicum* ATCC 13032 is active under oxic conditions. Also, oxidation of formate to CO_2 seemingly does not generate additional energy that could be used for growth. We speculate that the FDH in *C. glutamicum* ATCC 13032 is involved in the stress response. The growth of *C. glutamicum* ATCC 13032 is inhibited by the presence of formate, which is a natural component of soil, the habitat of *C. glutamicum* ATCC 13032. However, formate is mostly found only in micromolar amounts in the soil (Ahumada *et al.*, 2001). Possibly, the ability to inactivate (toxic) formate may confer a little growth advantage in the natural habitat. Putative FDHs with high sequence similarity to FdhF were found in a wide range of other soil bacteria, including *R. eutropha* and *Methylobacterium extorquens*, as well as in various corynebacteria, streptomyces, mycobacteria and rhodococci. These FDHs might fulfil functions in the stress response of the host and contribute to C1 turnover in syntrophic associations (Crable *et al.*, 2011).

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REFERENCES

Abaibou, H., Pommier, J., Benoit, S., Giordano, G. & Mandrand-Berthelot, M. A. (1995). Expression and characterization of the *Escherichia coli fdo* locus and a possible physiological role for aerobic formate dehydrogenase. *J Bacteriol* **177**, 7141–7149.

Abe, S., Takayama, K. & Kinoshita, S. (1967). Taxonomical studies on glutamic acid producing bacteria. *J Gen Appl Microbiol* **13**, 279–301.

Ahumada, I., Mendoza, J., Escudero, P., Mossert, K. & Ascar, L. (2001). Determination of organic acids of low molecular weight and phosphate in soil by capillary electrophoresis. *J AOAC Int* **84**, 1057–1064.

Axley, M. J., Grahame, D. A. & Stadtman, T. C. (1990). *Escherichia coli* formate-hydrogen lyase. Purification and properties of the selenium-dependent formate dehydrogenase component. *J Biol Chem* **265**, 18213–18218.

Bäumchen, C., Roth, A. H., Biedendieck, R., Malten, M., Follmann, M., Sahm, H., Bringer-Meyer, S. & Jahn, D. (2007). D-mannitol production by resting state whole cell biotransformation of D-fructose by heterologous mannitol and formate dehydrogenase gene expression in *Bacillus megaterium*. *Biotechnol J* **2**, 1408–1416.

Blombach, B., Riester, T., Wieschalka, S., Ziert, C., Youn, J. W., Wendisch, V. F. & Eikmanns, B. J. (2011). *Corynebacterium glutamicum* tailored for efficient isobutanol production. *Appl Environ Microbiol* **77**, 3300–3310.

Bowien, B. & Kusian, B. (2002). Genetics and control of CO_2 assimilation in the chemoautotroph *Ralstonia eutropha*. *Arch Microbiol* **178**, 85–93.

Boyington, J. C., Gladyshev, V. N., Khangulov, S. V., Stadtman, T. C. & Sun, P. D. (1997). Crystal structure of formate dehydrogenase H: catalysis involving Mo, molybdopterin, selenocysteine, and an Fe_4S_4 cluster. *Science* **275**, 1305–1308.

Buschke, N., Schröder, H. & Wittmann, C. (2011). Metabolic engineering of *Corynebacterium glutamicum* for production of 1,5-diaminopentane from hemicellulose. *Biotechnol J* **6**, 306–317.

Chistoserdova, L., Laukel, M., Portais, J. C., Vorholt, J. A. & Lidstrom, M. E. (2004). Multiple formate dehydrogenase enzymes in the facultative methylotroph *Methylobacterium extorquens* AM1 are dispensable for growth on methanol. *J Bacteriol* **186**, 22–28.

Chistoserdova, L., Crowther, G. J., Vorholt, J. A., Skovran, E., Portais, J. C. & Lidstrom, M. E. (2007). Identification of a fourth formate dehydrogenase in *Methylobacterium extorquens* AM1 and confirmation of the essential role of formate oxidation in methylotrophy. *J Bacteriol* **189**, 9076–9081.

Crable, B. R., Plugge, C. M., McInerney, M. J. & Stams, A. J. (2011). Formate formation and formate conversion in biological fuels production. *Enzyme Res* **2011**, 532–536.

Cramm, R. (2009). Genomic view of energy metabolism in *Ralstonia eutropha* H16. *J Mol Microbiol Biotechnol* **16**, 38–52.

Eggeling, L. & Bott, M. (2005). *Handbook of Corynebacterium glutamicum*. Boca Raton, FL: CRC Press, Taylor & Francis Group.

Eikmanns, B. J., Thum-Schmitz, N., Eggeling, L., Lüdtke, K. U. & Sahm, H. (1994). Nucleotide sequence, expression and transcriptional analysis of the *Corynebacterium glutamicum gltA* gene encoding citrate synthase. *Microbiology* **140**, 1817–1828.

Ferry, J. G. (2011). Fundamentals of methanogenic pathways that are key to the biomethanation of complex biomass. *Curr Opin Biotechnol* **22**, 351–357.

Friedebold, J. & Bowien, B. (1993). Physiological and biochemical characterization of the soluble formate dehydrogenase, a molybdoenzyme from *Alcaligenes eutrophus*. *J Bacteriol* **175**, 4719–4728.

Fröhlich, P., Albert, K. & Bertau, M. (2011). Formate dehydrogenase – a biocatalyst with novel applications in organic chemistry. *Org Biomol Chem* **9**, 7941–7950.

Frunzke, J., Engels, V., Hasenbein, S., Gätgens, C. & Bott, M. (2008). Co-ordinated regulation of gluconate catabolism and glucose uptake in *Corynebacterium glutamicum* by two functionally equivalent transcriptional regulators, GntR1 and GntR2. *Mol Microbiol* **67**, 305–322.

Gopinath, V., Meiswinkel, T. M., Wendisch, V. F. & Nampoothiri, K. M. (2011). Amino acid production from rice straw and wheat bran hydrolysates by recombinant pentose-utilizing *Corynebacterium glutamicum*. *Appl Microbiol Biotechnol* **92**, 985–996.

Hanahan, D. (1983). Studies on transformation of *Escherichia coli* with plasmids. *J Mol Biol* **166**, 557–580.

Hille, R. (2002). Molybdenum and tungsten in biology. *Trends Biochem Sci* **27**, 360–367.

Ikeda, M. & Nakagawa, S. (2003). The *Corynebacterium glutamicum* genome: features and impacts on biotechnological processes. *Appl Microbiol Biotechnol* **62**, 99–109.

Ingledew, W. J. & Poole, R. K. (1984). The respiratory chains of *Escherichia coli*. *Microbiol Rev* **48**, 222–271.

Johnson, M. K., Rees, D. C. & Adams, M. W. (1996). Tungstoenzymes. *Chem Rev* **96**, 2817–2840.

Jormakka, M., Törnroth, S., Abramson, J., Byrne, B. & Iwata, S. (2002). Purification and crystallization of the respiratory complex formate dehydrogenase-N from *Escherichia coli*. *Acta Crystallogr D Biol Crystallogr* **58**, 160–162.

Jormakka, M., Byrne, B. & Iwata, S. (2003). Formate dehydrogenase – a versatile enzyme in changing environments. *Curr Opin Struct Biol* **13**, 418–423.

Kabus, A., Georgi, T., Wendisch, V. F. & Bott, M. (2007). Expression of the *Escherichia coli pntAB* genes encoding a membrane-bound transhydrogenase in *Corynebacterium glutamicum* improves L-lysine formation. *Appl Microbiol Biotechnol* **75**, 47–53.

Kalinowski, J., Bathe, B., Bartels, D., Bischoff, N., Bott, M., Burkovski, A., Dusch, N., Eggeling, L., Eikmanns, B. J. & other authors (2003). The complete *Corynebacterium glutamicum* ATCC 13032 genome sequence and its impact on the production of L-aspartate-derived amino acids and vitamins. *J Biotechnol* **104**, 5–25.

Karzanov, V. V., Correa, C. M., Bogatsky, Y. G. & Netrusov, A. I. (1991). Alternative NAD^+-dependent formate dehydrogenases in the facultative methylotroph *Mycobacterium vaccae* 10. *FEMS Microbiol Lett* **81**, 95–99.

Keilhauer, C., Eggeling, L. & Sahm, H. (1993). Isoleucine synthesis in *Corynebacterium glutamicum*: molecular analysis of the *ilvB-ilvN-ilvC* operon. *J Bacteriol* **175**, 5595–5603.

Kröger, A., Winkler, E., Innerhofer, A., Hackenberg, H. & Schagger, H. (1979). The formate dehydrogenase involved in electron transport from formate to fumarate in *Vibrio succinogenes*. *Eur J Biochem* **94**, 465–475.

Kröger, A., Paulsen, J. & Schröder, I. (1986). Phosphorylative electron transport chains lacking a cytochrome *bc1* complex. *J Bioenerg Biomembr* **18**, 225–234.

Laukel, M., Chistoserdova, L., Lidstrom, M. E. & Vorholt, J. A. (2003). The tungsten-containing formate dehydrogenase from *Methylobacterium extorquens* AM1: purification and properties. *Eur J Biochem* **270**, 325–333.

Lee, S. E., Li, Q. X. & Yu, J. (2006). Proteomic examination of *Ralstonia eutropha* in cellular responses to formic acid. *Proteomics* **6**, 4259–4268.

Lin, E. C. & Iuchi, S. (1991). Regulation of gene expression in fermentative and respiratory systems in *Escherichia coli* and related bacteria. *Annu Rev Genet* **25**, 361–387.

Litsanov, B., Brocker, M. & Bott, M. (2012a). Toward homosuccinate fermentation: metabolic engineering of *Corynebacterium glutamicum* for anaerobic production of succinate from glucose and formate. *Appl Environ Microbiol* **78**, 3325–3337.

Litsanov, B., Brocker, M. & Bott, M. (2012b). Glycerol as a substrate for aerobic succinate production in minimal medium with *Corynebacterium glutamicum*. *Microb Biotechnol* [Epub ahead of print].

Marchler-Bauer, A., Lu, S., Anderson, J. B., Chitsaz, F., Derbyshire, M. K., DeWeese-Scott, C., Fong, J. H., Geer, L. Y., Geer, R. C. & other authors (2011). CDD: a Conserved Domain Database for the functional annotation of proteins. *Nucleic Acids Res* **39** (Database issue), D225–D229.

Marienhagen, J., Kennerknecht, N., Sahm, H. & Eggeling, L. (2005). Functional analysis of all aminotransferase proteins inferred from the

genome sequence of *Corynebacterium glutamicum*. *J Bacteriol* **187**, 7639–7646.

May, H. D., Patel, P. S. & Ferry, J. G. (1988). Effect of molybdenum and tungsten on synthesis and composition of formate dehydrogenase in *Methanobacterium formicicum*. *J Bacteriol* **170**, 3384–3389.

McInerney, M. J., Sieber, J. R. & Gunsalus, R. P. (2009). Syntrophy in anaerobic global carbon cycles. *Curr Opin Biotechnol* **20**, 623–632.

McMaster, J. & Enemark, J. H. (1998). The active sites of molybdenum- and tungsten-containing enzymes. *Curr Opin Chem Biol* **2**, 201–207.

Misset-Smits, M., van Ophem, P. W., Sakuda, S. & Duine, J. A. (1997). Mycothiol, 1-*O*-(2′-[*N*-acetyl-L-cysteinyl]amido-2′-deoxy-alpha-D-glucopyranosyl)-D-*myo*-inositol, is the factor of NAD/factor-dependent formaldehyde dehydrogenase. *FEBS Lett* **409**, 221–222.

Moura, J. J. G., Brondino, C. D., Trincão, J. & Romão, M. J. (2004). Mo and W *bis*-MGD enzymes: nitrate reductases and formate dehydrogenases. *J Biol Inorg Chem* **9**, 791–799.

Müller, U., Willnow, P., Ruschig, U. & Höpner, T. (1978). Formate dehydrogenase from *Pseudomonas oxalaticus*. *Eur J Biochem* **83**, 485–498.

Nagy, P. L., Marolewski, A., Benkovic, S. J. & Zalkin, H. (1995). Formyltetrahydrofolate hydrolase, a regulatory enzyme that functions to balance pools of tetrahydrofolate and one-carbon tetrahydrofolate adducts in *Escherichia coli*. *J Bacteriol* **177**, 1292–1298.

Niebisch, A. & Bott, M. (2001). Molecular analysis of the cytochrome *bc1-aa3* branch of the *Corynebacterium glutamicum* respiratory chain containing an unusual diheme cytochrome *c*1. *Arch Microbiol* **175**, 282–294.

Niimi, S., Suzuki, N., Inui, M. & Yukawa, H. (2011). Metabolic engineering of 1,2-propanediol pathways in *Corynebacterium glutamicum*. *Appl Microbiol Biotechnol* **90**, 1721–1729.

Pecher, A., Zinoni, F., Jatisatienr, C., Wirth, R., Hennecke, H. & Böck, A. (1983). On the redox control of synthesis of anaerobically induced enzymes in Enterobacteriaceae. *Arch Microbiol* **136**, 131–136.

Plugge, C. M., Zhang, W., Scholten, J. C. M. & Stams, A. J. M. (2011). Metabolic flexibility of sulfate-reducing bacteria. *Front Microbiol* **2**, 81.

Polen, T., Rittmann, D., Wendisch, V. F. & Sahm, H. (2003). DNA microarray analyses of the long-term adaptive response of *Escherichia coli* to acetate and propionate. *Appl Environ Microbiol* **69**, 1759–1774.

Raaijmakers, H. C. & Romão, M. J. (2006). Formate-reduced *E. coli* formate dehydrogenase H: the reinterpretation of the crystal structure suggests a new reaction mechanism. *J Biol Inorg Chem* **11**, 849–854.

Rittmann, D., Lindner, S. N. & Wendisch, V. F. (2008). Engineering of a glycerol utilization pathway for amino acid production by *Corynebacterium glutamicum*. *Appl Environ Microbiol* **74**, 6216–6222.

Russell, J. B. (1992). Another explanation for the toxicity of fermentation acids at low pH – anion accumulation versus uncoupling. *J Appl Bacteriol* **73**, 363–370.

Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989). *Molecular Cloning: a Laboratory Manual*, 2nd Edn. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.

Sasaki, M., Jojima, T., Kawaguchi, H., Inui, M. & Yukawa, H. (2009). Engineering of pentose transport in *Corynebacterium glutamicum* to improve simultaneous utilization of mixed sugars. *Appl Microbiol Biotechnol* **85**, 105–115.

Schäfer, A., Tauch, A., Jäger, W., Kalinowski, J., Thierbach, G. & Pühler, A. (1994). Small mobilizable multi-purpose cloning vectors derived from the *Escherichia coli* plasmids pK18 and pK19: selection of defined deletions in the chromosome of *Corynebacterium glutamicum*. *Gene* **145**, 69–73.

Schaffer, S., Weil, B., Nguyen, V. D., Dongmann, G., Günther, K., Nickolaus, M., Hermann, T. & Bott, M. (2001). A high-resolution reference map for cytoplasmic and membrane-associated proteins of *Corynebacterium glutamicum*. *Electrophoresis* **22**, 4404–4422.

Schauer, N. L., Ferry, J. G., Honek, J. F., Orme-Johnson, W. H. & Walsh, C. (1986). Mechanistic studies of the coenzyme F420 reducing formate dehydrogenase from *Methanobacterium formicicum*. *Biochemistry* **25**, 7163–7168.

Schlindwein, C., Giordano, G., Santini, C. L. & Mandrand, M. A. (1990). Identification and expression of the *Escherichia coli fdhD* and *fdhE* genes, which are involved in the formation of respiratory formate dehydrogenase. *J Bacteriol* **172**, 6112–6121.

Schneider, J., Niermann, K. & Wendisch, V. F. (2011). Production of the amino acids L-glutamate, L-lysine, L-ornithine and L-arginine from arabinose by recombinant *Corynebacterium glutamicum*. *J Biotechnol* **154**, 191–198.

Schneider, J., Eberhardt, D. & Wendisch, V. F. (2012). Improving putrescine production by *Corynebacterium glutamicum* by fine-tuning ornithine transcarbamoylase activity using a plasmid addiction system. *Appl Microbiol Biotechnol* **95**, 169–178.

Schultz, C., Niebisch, A., Schwaiger, A., Viets, U., Metzger, S., Bramkamp, M. & Bott, M. (2009). Genetic and biochemical analysis of the serine/threonine protein kinases PknA, PknB, PknG and PknL of *Corynebacterium glutamicum*: evidence for non-essentiality and for phosphorylation of OdhI and FtsZ by multiple kinases. *Mol Microbiol* **74**, 724–741.

Schwarz, G., Hagedoorn, P. L. & Fischer, K. (2007). Molybdate and tungstate: uptake, homeostasis, cofactors, and enzymes. In *Molecular Microbiology of Heavy Metals*, pp. 421–452. Edited by D. H. Nies & S. Silver. Berlin, Heidelberg: Springer-Verlag.

Sebban, C., Blanchard, L., Bruschi, M. & Guerlesquin, F. (1995). Purification and characterization of the formate dehydrogenase from *Desulfovibrio vulgaris* Hildenborough. *FEMS Microbiol Lett* **133**, 143–149.

Stäbler, N., Oikawa, T., Bott, M. & Eggeling, L. (2011). *Corynebacterium glutamicum* as a host for synthesis and export of D-amino acids. *J Bacteriol* **193**, 1702–1709.

Stams, A. J. & Plugge, C. M. (2009). Electron transfer in syntrophic communities of anaerobic bacteria and archaea. *Nat Rev Microbiol* **7**, 568–577.

Stewart, V., Lin, J. T. & Berg, B. L. (1991). Genetic evidence that genes *fdhD* and *fdhE* do not control synthesis of formate dehydrogenase-N in *Escherichia coli* K-12. *J Bacteriol* **173**, 4417–4423.

Suppmann, B. & Sawers, G. (1994). Isolation and characterization of hypophosphite–resistant mutants of *Escherichia coli*: identification of the FocA protein, encoded by the *pfl* operon, as a putative formate transporter. *Mol Microbiol* **11**, 965–982.

Takors, R., Bathe, B., Rieping, M., Hans, S., Kelle, R. & Huthmacher, K. (2007). Systems biology for industrial strains and fermentation processes – example: amino acids. *J Biotechnol* **129**, 181–190.

Teramoto, H., Suda, M., Inui, M. & Yukawa, H. (2010). Regulation of the expression of genes involved in NAD de novo biosynthesis in *Corynebacterium glutamicum*. *Appl Environ Microbiol* **76**, 5488–5495.

Thomé, R., Gust, A., Toci, R., Mendel, R., Bittner, F., Magalon, A. & Walburger, A. (2012). A sulfurtransferase is essential for activity of formate dehydrogenases in *Escherichia coli*. *J Biol Chem* **287**, 4671–4678.

Tishkov, V. I. & Popov, V. O. (2004). Catalytic mechanism and application of formate dehydrogenase. *Biochemistry (Mosc)* **69**, 1252–1267.

van der Rest, M. E., Lange, C. & Molenaar, D. (1999). A heat shock following electroporation induces highly efficient transformation of *Corynebacterium glutamicum* with xenogeneic plasmid DNA. *Appl Microbiol Biotechnol* **52**, 541–545.

Wang, Y., Huang, Y., Wang, J., Cheng, C., Huang, W., Lu, P., Xu, Y. N., Wang, P., Yan, N. & Shi, Y. (2009). Structure of the formate transporter FocA reveals a pentameric aquaporin-like channel. *Nature* **462**, 467–472.

Wendisch, V. F., Bott, M. & Eikmanns, B. J. (2006a). Metabolic engineering of *Escherichia coli* and *Corynebacterium glutamicum* for biotechnological production of organic acids and amino acids. *Curr Opin Microbiol* **9**, 268–274.

Wendisch, V. F., Bott, M., Kalinowski, J., Oldiges, M. & Wiechert, W. (2006b). Emerging *Corynebacterium glutamicum* systems biology. *J Biotechnol* **124**, 74–92.

Yagi, T. (1979). Purification and properties of cytochrome c-553, an electron acceptor for formate dehydrogenase of *Desulfovibrio vulgaris*, Miyazaki. *Biochim Biophys Acta* **548**, 96–105.

Yamamoto, I., Saiki, T., Liu, S. M. & Ljungdahl, L. G. (1983). Purification and properties of NADP-dependent formate dehydrogenase from *Clostridium thermoaceticum*, a tungsten-selenium-iron protein. *J Biol Chem* **258**, 1826–1832.

Zaldivar, J. & Ingram, L. O. (1999). Effect of organic acids on the growth and fermentation of ethanologenic *Escherichia coli* LY01. *Biotechnol Bioeng* **66**, 203–210.

Zinoni, F., Birkmann, A., Leinfelder, W. & Böck, A. (1987). Cotranslational insertion of selenocysteine into formate dehydrogenase from *Escherichia coli* directed by a UGA codon. *Proc Natl Acad Sci U S A* **84**, 3156–3160.

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