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Archaeoglobus fulgidus couples CO oxidation to sulfate reduction and acetogenesis with transient formate accumulation

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Summary

The genome sequence of *Archaeoglobus fulgidus* VC16 encodes three CO dehydrogenase genes. Here we explore the capacity of *A. fulgidus* to use CO as growth substrate. *Archaeoglobus fulgidus* VC16 was successfully adapted to growth medium that contained sulfate and CO. In the presence of CO and sulfate the culture OD_{660} increased to 0.41 and sulfide, carbon dioxide, acetate and formate were formed. Accumulation of formate was transient. Similar results, except that no sulfide was formed, were obtained when sulfate was omitted. Hydrogen was never detected. Under the conditions tested, the observed concentrations of acetate (18 mM) and formate (8.2 mM) were highest in cultures without sulfate. Proton NMR spectroscopy indicated that CO_2, and not CO, is the precursor of formate and the methyl group of acetate. Methylviologen-dependent formate dehydrogenase activity (1.4 μmol formate oxidized min^{-1} mg^{-1}) was detected in cell-free extracts and expected to have a role in formate reuptake. It is speculated that formate formation proceeds through hydrolysis of formyl-methanofuran or formyl-tetrahydromethanopterin. This study demonstrates that *A. fulgidus* can grow chemolithoautotrophically with CO as acetogen, and is not strictly dependent on the presence of sulfate, thiosulfate or other sulfur compounds as electron acceptor.

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Introduction

Archaeoglobus fulgidus is a strict anaerobic hyperthermophilic archaeon that oxidizes lactate completely to CO_2 with sulfate as electron acceptor (Stetter *et al*., 1987; Stetter, 1988). For complete oxidation of lactate it employs the acetyl-CoA pathway (Möller-Zinkhan, 1990). Presence and activity of all enzymes and cofactors of the pathway were demonstrated (Stetter *et al*., 1987; Möller-Zinkhan *et al*., 1989; 1990; Gorris *et al*., 1991; Schwörer *et al*., 1993; Kunow *et al*., 1995; Dai *et al*., 1998). Key enzyme of the pathway is the acetyl-CoA synthase/CO dehydrogenase complex (ACS/CODH). In Achaea this complex is composed of five different subunits, α, β, γ, δ, ε (Grahame and DeMoll, 1996; Lindahl and Chang, 2001). The genome of *A. fulgidus* encodes one set of β-γ-δ, and two sets of α-ε subunits, all at different locations on the genome (Klenk *et al*., 1997). CODH activity is associated with the α-ε set (Grahame and DeMoll, 1996). Notably, in lactate/sulfate grown *A. fulgidus* cells only one α-ε is expressed (Dai *et al*., 1998). A third CODH gene, besides the two α-ε sets, is present in *A. fulgidus*. This third gene is highly similar to *cooS*, that is found in *Rhodospirillum rubrum* and *Carboxydothermus hydrogenoformans* (Klenk *et al*., 1997; Gonzalez and Robb, 2000). In these bacteria CooS oxidizes CO to CO_2 and transfers electrons via the ferredoxin-like protein CooF to an energy converting hydrogenase (ECH) that reduces protons to H_2 and translocates protons or sodium ions (Hedderich *et al*., 2004; Singer *et al*., 2006). CooF is also encoded in the *A. fulgidus* genome. Based on the presence of CooS and CooF it has been suggested that *A. fulgidus* is capable of carboxydotrophic hydrogenogenic growth as displayed by *R. rubrum* and *C. hydrogenoformans* (Klenk *et al*., 1997; Gonzalez and Robb, 2000). However, the *A. fulgidus* genome sequence lacks ECH genes, thus a hydrogenogenic lifestyle seems unlikely. Instead reduction of sulfate with CO as electron donor or acetogenesis may support growth.

Acetogens reduce CO_2 to acetate via the acetyl-CoA pathway (Drake, 1994). Only recently it was shown that also the archaeal domain harbors acetogens. *Methanosarcina acetivorans* C2A grew acetogenically with carbon monoxide (Rother and Metcalf, 2004; Lessner *et al*.,

2006). Like *A. fulgidus*, the *M. acetivorans* genome encodes genes for CooS, CooF and multiple ACS/CODHs (Klenk *et al*., 1997; Galagan *et al*., 2002). Besides acetate *M. acetivorans* accumulated formate and formed methane.

Reduction of sulfate with CO as electron donor is perhaps the most likely metabolism of *A. fulgidus*. However, CO inhibits growth of many strict anaerobic microorganisms (Davidova *et al*., 1994; Sipma *et al*., 2006). Especially sulfate reducing bacteria appear sensitive towards CO, although this strongly depends on the species or even strain. Some species do not grow with CO levels (P_{CO}) above 2 kPa while others tolerate a P_{CO} up to 20 kPa. Only *Desulfotomaculum carboxydivorans* was reported to grow with CO levels up to 200 kPa without noticeable inhibition. Under these conditions it converts CO to $H_2 + CO_2$ instead of reducing sulfate (Parshina *et al*., 2005a). *Desulfotomaculum kuznetsovi* and *Desulfotomaculum thermobenzoicum* ssp. *thermosyntrophicum* grow with CO up to 70 kPa but reverted to acetogenesis instead of sulfate reduction (Parshina *et al*., 2005b). Methanogenic Achaea have been described to grow at low levels of CO, although growth is often poor (Daniels *et al*., 1977). Growth of *M. acetivorans* C2A with CO was achieved by multiple transfers with increasing CO levels (Rother and Metcalf, 2004).

We aimed to test the hypothesis that *A. fulgidus* is capable of growth with CO as a substrate, as was expected based on genomic data. Adaptation of *A. fulgidus* was successful. Sulfide, CO_2, acetate and formate were formed during growth of *A. fulgidus* in the presence of CO and sulfate. Chemolithoautotrophic formation of acetate and formate by an archaeon is unusual. Therefore, ^{1}H-NMR spectroscopy was used to identify the pathway of acetate formation and evaluate previously raised hypotheses that explain formate formation (Rother and Metcalf, 2004).

Results and discussion

Inoculation of *A. fulgidus* VC16 in serum vials with lactate (30 mM), sulfate (15 mM) and a 170-kPa CO:CO_2 (80:20) gas phase resulted in a 14-day lag phase. Thereafter, the strain could be subcultured on CO with a lag phase comparable to lactate/sulfate grown cultures (data not shown). Lag phases of CO adapted and unadapted *A. fulgidus* cultures were compared in an experiment where inoculations were made in serum vials with lactate (30 mM) and sulfate (15 mM) and a P_{CO} of 0, 5, 10, 20, 40, 80 and 136 kPa. In addition, serum vials with a P_{CO} of 80 kPa but without lactate were inoculated. CO levels up to 40 kPa did not or only slightly affect the lag phase in cultures inoculated with unadapted cells. CO levels of 40 and 80 kPa resulted in a noticeable lag phase increase of 2–13 days. Interestingly, cultures with a P_{CO} of 80 kPa without lactate showed a 4-day shorter lag phase than similar cultures with lactate (Table 1).

Table 1. Lag phase of *A. fulgidus* cultures inoculated with CO adapted and unadapted cells in the presence of sulfate.

%CO	Lactate (30 mM)	Unadapted cells lag phase (d)[a]	CO adapted cells lag phase (d)[a]
0	+	2	2
3.5	+	2	2
10	+	2	2
20	+	3	2
40	+	4	2
80	+	15	3
80	–	10	3

a. lag phase expressed as days before $OD_{660} > 0.1$.

Growth of *A. fulgidus* with CO and sulfate (15 mM) was studied in more detail. Results are plotted in Fig. 1A. CO (102 mmol l^{-1} medium) and sulfate (9.1 mM) were consumed by the cultures and this led to formation of CO_2 (68 mmol l^{-1} medium) and sulfide (8.6 mM). Organic acid analysis revealed the transient formation of formate (6.4 mM maximum) and the accumulation of acetate (11 mM). H_2 and methane were not detected. The OD_{660} of the cultures increased to 0.41 indicating growth (Fig. 1C) and the final pH was 6.3. The formation of acetate indicates that *A. fulgidus* can grow homoacetogenically with CO possibly even in the absence of sulfate. Therefore, a similar growth experiment was performed simultaneously with medium where $MgSO_4$ was replaced by $MgCl_2$ (Fig. 1B). In these cultures CO (98 mmol l^{-1} medium) was consumed completely with formation of acetate (18 mM) and CO_2 (53 mmol l^{-1} medium). Formate (8.2 mM) was formed transiently. The formation of acetate and CO_2 led to a final pH of 5.2. Cultures without sulfate obtained lower optical densities (0.27 vs. 0.41 at 660 nm) (Fig. 1C). Besides acetate and formate small amounts (< 0.5 mM) of propionate and butyrate were detected by HPLC (data not shown). Although final concentrations of 2 mM formate were reported by HPLC analysis, in ^{1}H-NMR experiments described below no formate was detected in final culture samples. Detection of formate by ^{1}H-NMR is more sensitive than by HPLC. Instead of 2 mM formate a compound with similar HPLC retention time as formate must have been formed by *A. fulgidus* cultures. This compound could not be identified.

NMR spectroscopy was used to identify the carbon fixing pathway that is used by *A. fulgidus* during chemolithoautotrophic growth. *Archaeoglobus fulgidus* was grown in batch culture with an initial gas phase composed of 80:20 $^{13}CO/^{12}CO_2$. Liquid samples were collected over time and analysed by HPLC and ^{1}H-NMR spectroscopy. After 6 h of cultivation 68% of the C_1 (carboxyl) and 30% of C_2 (methyl) of acetate was labelled (Table 2, Fig. 2).

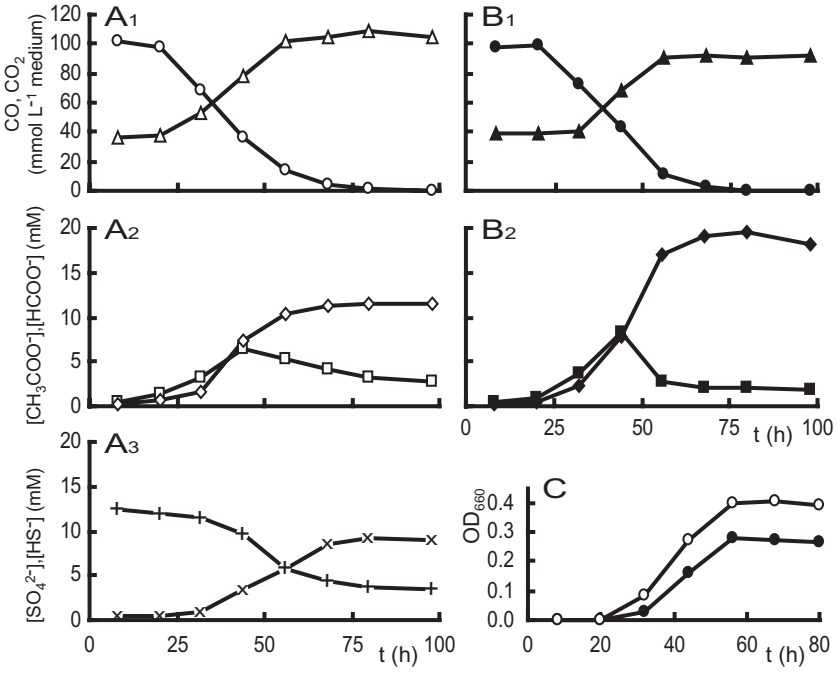

Fig. 1. A and B. CO conversion by *A. fulgidus* in the presence (A) and the absence (B) of sulfate. Symbols: CO (circles); CO_2 (triangles); acetate (diamonds); formate (squares); sulfate (+); sulfide (x).
C. Growth of these cultures indicated by the OD_{660}, with open symbols representing cultures that contained sulfate.

These results indicate a preferential incorporation of CO as the carboxyl moiety and of CO_2 as the methyl moiety of acetate. Of the four known CO_2 fixing pathways, the Calvin cycle, reductive tricarboxylic acid cycle, 3-hydroxypropionate cycle and acetyl-CoA pathway, only the acetyl-CoA pathway allows direct incorporation of CO through ACS/CODH.

It can be questioned whether CO or CO_2 is the precursor of formate in the metabolism of *A. fulgidus*. In the NMR experiment described above, the concentration of unlabelled formate showed only a decrease from the first sample at 6 h (5.7 mM) to the final sample, while the concentration of labelled formate first increased from the 6 h sample (4.3 mM) to a maximum at 10 h (5.6 mM) and then decreased (Table 2, Fig. 2). Additionally, the fraction of labelled formate of the total formate pool increased over all successive samples with detectable formate concentrations. Thus, first mainly unlabelled formate is formed, while later mainly labelled formate is formed. This is explained by the increase of the fraction-labelled CO_2 in the pool of total CO_2 over time, by oxidation of ^{13}CO to $^{13}CO_2$ by *A. fulgidus*. It is concluded that CO_2 is the precursor of formate and not CO.

Formate dehydrogenase genes have not been annotated in the *A. fulgidus* genome, neither are biochemical data available to indicate the presence of FDH in *A. fulgidus*. However, the observation that *A. fulgidus* reduces sulfate with formate as electron donor suggests the presence of FDH (Zellner *et al*., 1989). We detected formate-dependent methylviologen (MV) reducing activity in cell-free extracts (1.4 μmol formate oxidized min^{-1} mg^{-1}). Based on these results it was expected that a formate dehydrogenase is encoded by the *A. fulgidus* genome. Initial BLAST (Altschul *et al*., 1997) and PSI-BLAST

Table 2. Concentrations and relative ^{13}C-labelling of formate and acetate isotopomers over time in ^{13}CO fed *A. fulgidus* cultures.

		Acetate							Formate		
					Acetate isotopomers (%)						
Time (h)[a]	CO[b] (%)	(mM)	Total $^{13}C_2$-Ac (%)	Total $^{13}C_1$-Ac (%)	$^{13}C_1$-$^{13}C_2$	$^{12}C_1$-$^{13}C_2$	$^{13}C_1$-$^{12}C_2$	$^{12}C_1$-$^{12}C_2$	(mM)	Total ^{13}C-Fo (%)	Total ^{12}C-Fo (%)
6	55	4.8	30	68	22	8	46	24	10	43	57
10	31	7.1	37	71	28	9	43	20	11	51	49
14	14	11	44	72	33	11	39	16	7.0	61	39
25	1.8	14	49	72	36	13	36	15	2.6	n.d.	n.d.
240	n.a.	n.a.	50	73	37	13	36	14	n.a.	n.d.	n.d.

a. Time of incubation while shaking
b. Remaining percentage of start amount of CO.
n.a., not analysed; n.d., not detected.

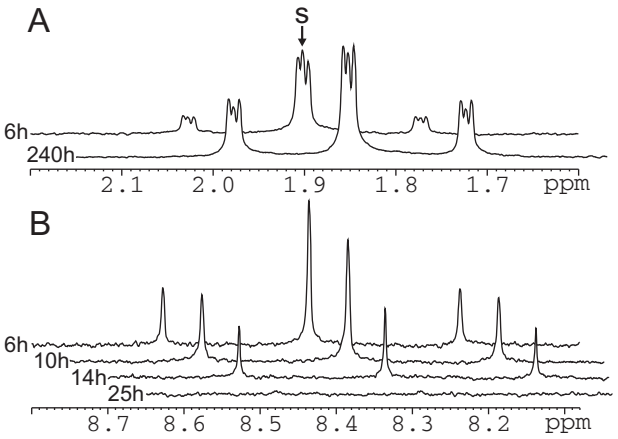

Fig. 2. ^{1}H-NMR spectra of acetate (A) and formate (B) present in samples obtained from *A. fulgidus* cultures grown with ^{13}CO as substrate. Respective concentrations of acetate and formate are listed in Table 2. Singlet acetate, marked by S, served as reference and was set at 1.9 p.p.m. in accordance with its known relative position towards tetramethylsilan. *X*-axes correspond to the topmost spectra; each successive spectrum is plotted offset by 0.05 p.p.m.

(Schäffer *et al*., 2001) searches indicated AF1203 as possible FDH alpha subunit (data not shown). AF1203 shared highest amino acid sequence identity with uncharacterized molybdopterin-binding oxidoreductases of bacteria (data not shown). The putative FDH alpha subunit of *Pyrococcus abyssi*, PAB1389, was the archaeal gene that shared highest identity (31%) with AF1203. PAB1389 shared 37% identity with the characterized formate dehydrogenase A of *Methanobacterium formicicum* (GenBank Accession number J02581) (Schauer and Ferry, 1982; Shuber *et al*., 1986), while AF1203 only shared 27% of identity with the *M. formicicum* FDH. If AF1203 is an FDH it might represent a novel type.

Archaeoglobus fulgidus VC16 and *M. acetivorans* C2A both form acetate and formate with CO as substrate, with the main difference that formate accumulation is transient in *A. fulgidus* cultures, while it is not in *M. acetivorans* cultures. CO_2 was identified as precursor of formate. In bacteria CO_2 is generally reduced to formate that subsequently activated to form formyl-tetrahydrofolate. In Achaea CO_2 is generally reduced by formyl-methanofuran dehydrogenase to form formyl-methanofuran. The formyl is subsequently transferred from the methanofuran cofactor to tetrahydromethanopterin. Formyl-tetrahydromethanopterin in Achaea and formyl-tetrahydrofolate in bacteria are further reduced in the methyl branch of the acetyl-CoA pathway (Drake, 1994). It was suggested that formate is formed by hydrolysis of formyl-methanofuran or formyl-tetrahydromethanopterin in *M. acetivorans*, because its genome does not encode FDH genes and it lacks FDH activity in cell-free extracts (Rother and Metcalf, 2004). A formyl transferase/hydrolase complex (FHC) exists in the bacterium *Methylobacterium extorquens* (Pomper *et al*., 2002). It has been suggested that metabolic energy can be conserved in this step (Pomper *et al*., 2002; Rother and Metcalf, 2004). If we assume that *A. fulgidus* and *M. acetivorans* use similar metabolic pathways for formation of acetate and formate with CO as a substrate, then the FDH activity, that is present in *A. fulgidus*, is likely responsible for reuptake of formate. Oxidation of formate by FDH may be coupled to the reduction of sulfate, when present, and to reductive steps in the methyl-branch of the reductive acetyl-CoA pathway.

Here we demonstrated that CO is an excellent growth substrate for *A. fulgidus*. Sulfate is reduced with CO as electron donor. Additionally, *A. fulgidus* grows homoacetogenically with CO in the presence and absence of sulfate. Therefore, *A. fulgidus* is not strictly dependent on sulfate or thiosulfate reduction for growth. Interestingly, sulfate reduction to sulfide is not inhibited by CO. This in contrast to most sulfate reducing bacteria (Davidova *et al*., 1994; Parshina *et al*., 2005b). Acetate is formed by *A. fulgidus* through the acetyl-CoA pathway. Formate accumulates transiently in the presence and absence of sulfate. We support the hypothesis that formate is formed through hydrolysis of formyl-MFR or formyl-H_4MPT to formate by a tentative enzyme complex that has only been described for *M. extorquens* so far. This step is possibly associated with energy conservation (Pomper *et al*., 2002; Rother and Metcalf, 2004).

Experimental procedures

Organism

Archaeoglobus fulgidus strain VC-16 (DSM 4304) was obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ, Braunschweig, Germany).

Medium composition and cultivation

Archaeoglobus fulgidus was cultivated under strictly anaerobic conditions in a bicarbonate buffered medium at 80°C. The medium contained 0.14 g l^{-1} K_2HPO_4, 0.14 g l^{-1} $CaCl_2{\cdot}2H_2O$, 0.25 g l^{-1} NH_4Cl, 2.75 g l^{-1} $MgCl_2{\cdot}6H_2O$, 3.45 g l^{-1} $MgSO_4{\cdot}7H_2O$, 0.33 g l^{-1} KCl, 18 g l^{-1} NaCl, 1.5 g l^{-1} $NaHCO_3$, 0.8 mM Na_2S and 0.5 g l^{-1} yeast extract. In sulfate-free media 3.45 g l^{-1} $MgSO_4{\cdot}7H_2O$ was replaced by 2.85 g l^{-1} extra $MgCl_2{\cdot}6H_2O$. The medium used for NMR experiments contained 0.05 g l^{-1} yeast extract. Resazurin and trace elements were added from separately prepared stock solutions as described (Stams *et al*., 1993). After boiling, the medium was cooled to room temperature under a N_2 flow and dispensed in serum bottles that were closed with butyl rubber stoppers. Gas phases were changed to 170 kPa 80:20 N_2/CO_2 or CO/CO_2. Bottles of 120 ml contained 50 ml medium and were

incubated standing, unless stated otherwise. Bottles of 585 ml contained 200 ml medium and were incubated in a 1″ rotary shaker at 130 r.p.m. during cultivation. The bottles were autoclaved for 25′-at 121°C. Sodium sulfide and sodium bicarbonate were added from a separately autoclaved anaerobic stock solution prior to inoculation. In contrast to DSMZ medium 399, the medium contained Na_2WO_4 (0.1 μM), but lacked Al and Cu as trace minerals. Gas phases with 2, 5, 10, 20 and 40 kPa CO were created by addition of CO with a gastight syringe to bottles with a N_2/CO_2 gas phase prior to sterilization.

Analytical methods

Analysis of substrates and products was performed as previously described (Henstra and Stams, 2004). Gases were analysed by GC-TCD using a molsieve 5 A column (H_2, CO) or a Poraplot Q column (CO_2). Both columns were obtained from Chrompack (Middelburg, the Netherlands). Sulfate was analysed by HPLC using an Ionpac AS9-SC column and ED 40 electrochemical detector (column and detector by Dionex, Sunnyvale, USA) (Scholten and Stams, 1995). Organic acids were analysed by HPLC using a Polyspher OA HY column (300–6.5 mm, Merck, Darmstadt, Germany) and RI SE-61 refractive index detector (Shodex, Tokyo Japan) (Scholten and Stams, 1995). Sulfide was analysed according to the colorimetric method described by Trüper and Schlegel (Trüper and Schlegel, 1964). Optical density of culture samples were measured at 660 nm (OD_{660}) on a U1500 spectrophotometer (Hitachi Instruments, San Jose, USA). Total amounts of CO, CO_2 and H_2S were expressed as mmol l^{-1} medium, representing total amounts of these gases distributed over aqueous and gaseous phase and their dissociated states, taking into account the effect of temperature, activity and pH.

Enzyme activity

All manipulations of cell material needed for enzyme activity measurements were performed anaerobically by use of anaerobic glove boxes, stoppered containers and air tight syringes. Formate-dependent MV reduction rates were analysed for cell-free extracts of CO-grown *A. fulgidus* cells. Cells were harvested by centrifugation (25 000 *g*, 30′, 20°C), washed with N_2-flushed buffer (50 mM MOPS*KOH pH 7, 1 mM dithionite) and stored at –20°C. Cells were disrupted by sonication (10 × 30″/30″) and cell-free extract was obtained after centrifugation (20′, 16 000 *g*, room temperature). Methylviologen (MV, $\varepsilon_{600} = 13.1\ mM^{-1}\ cm^{-1}$) (Graentzdoerffer *et al.*, 2003) reduction in assay buffer (50 mM MOPS*KOH pH 7, 20 mM MV, 100 mM sodium formate) was recorded using an U-2010 spectrophotometer at 80°C in N_2 flushed optical glass cuvettes.

Nuclear magnetic resonance

Incorporation of ^{13}C in formate and acetate was analysed by proton nuclear magnetic resonance (^{1}H-NMR spectroscopy) in samples obtained from *A. fulgidus* cultures incubated with a $^{13}CO/^{12}CO_2$ (80:20) gas phase. ^{13}CO 99.4% was obtained from Campro Scientific (Veenendaal, the Netherlands). The amount of yeast extract added to the medium was reduced to 0.05 g l^{-1}. Concentrations of acetate and formate were analysed by HPLC. ^{1}H-NMR spectra determined relative abundance of isotopomers of acetate ($^{12}C2$-$^{12}C_1$, $^{13}C2$-$^{12}C_1$, $^{12}C2$-$^{13}C_1$, $^{13}C2$-$^{13}C_1$) and formate (^{13}C, ^{12}C). Cultures were prepared as described and placed at 80°C without shaking for 24 h to obtain an active culture with minimal CO conversion. Subsequently, cultures were placed at 80°C and 200 r.p.m. to stimulate gas-liquid^{-1} mass transfer. Culture samples were withdrawn 6, 10, 14, 25 and 240 h after placement at 200 r.p.m. and centrifuged for 10′ at 16 000 *g*. To 450 μl of culture supernatant 50 μl of D_2O was added before ^{1}H-NMR spectra were recorded at 500.13 MHz on a Bruker AMX-500 NMR spectrometer. Each spectrum was a superposition of 2000 FIDs accumulated in 16 k data points; a 60° pulse was used and the interpulse time was 1 s.

Acknowledgements

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