First Observation of an Acetate Switch in a Methanogenic Autotroph (*Methanococcus maripaludis* S2)

Microbiology Insights
Volume 13: 1–7

DOI: 10.1177/1178636120945300

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ABSTRACT: The transition from acetate production by a microorganism in its early growth phase to acetate re-uptake in its late growth phase has been termed acetate switch. It has been observed in several heterotrophic prokaryotes, but not in an autotroph. Furthermore, all reports hitherto have involved the tricarboxylic acid cycle. This study reports the first observation of acetate switch in a methanogenic autotroph *Methanococcus maripaludis* S2, which uses the Wolfe cycle for its anaerobic respiration. When grown in minimal medium with carbon dioxide as the sole carbon source, and either ammonium or dinitrogen as the sole nitrogen source, *M. maripaludis* S2 dissimilated acetate in the early growth phase and assimilated it back in the late growth phase. The acetate switch was more pronounced in the dinitrogen-grown cultures. We postulate that the acetate dissimilation in *M. maripaludis* S2 may serve as a metabolic outlet for the carbon overflow in the early growth phase, and the assimilation in the late growth phase may be due to the scarcity of the carbon source. Based on the primary and secondary protein structures, we propose that MMP0253 may function as the adenosine diphosphate (ADP)-forming acetyl-CoA synthetase to catalyse acetate formation from acetyl-CoA. To verify this, we produced MMP0253 via the ligation-independent cloning technique in *Escherichia coli* strain Rosetta (DE3) using pNIC28-Bsa4 as the vector. The recombinant protein showed catalytic activity, when added into a mixture of acetyl-CoA, ADP, and inorganic phosphate (P_i). The concentration profile of acetate, together with the enzymatic activity of MMP0253, shows that *M. maripaludis* S2 can produce acetate and exhibit an acetate switch.

KEYWORD: acetate switch, acetate dissimilation, acetate assimilation, ***Methanococcus maripaludis***, ADP-forming acetyl-CoA synthetase, Acd

RECEIVED: April 9, 2020. **ACCEPTED:** July 1, 2020.

TYPE: Original Research

FUNDING: The author(s) disclosed receipt of the following financial support for the research, authorship, and/or publication of this article: This research is supported by the National Research Foundation, Prime Minister's Office, Singapore, under its CREATE programme. Concomitantly, this work is supported by the National University of Singapore through its Graduate Research Scholarship. This work also received partial financial support under a Tier 1 grant R-279-000-541-114 from the Ministry of Education, Singapore.

DECLARATION OF CONFLICTING INTERESTS: The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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Introduction

Many microorganisms alternate their metabolic pathways to adapt to the nutrients in their immediate environments. They prioritise growth when nutrients are abundant and survival when they are scarce. One such behaviour is the 'acetate switch', which has been studied extensively in *Escherichia coli*.[1] Acetate switch involves the transition from acetate dissimilation (production and secretion) to assimilation (uptake and utilisation). In the early growth phase, *E. coli* dissimilates acetate when the carbon flux into the cells exceeds the capacity of its central pathways or when its tricarboxylic acid (TCA) cycle does not operate fully due to limited oxygen.[1] This dissimilation allows *E. coli* to generate adenosine triphosphate (ATP) and reduce acetyl-CoA (Ac-CoA) accumulation.[1] In the later growth phase, when the metabolic bottleneck allows the uptake of acetate due to respiratory capacity, *E. coli* switches to acetate assimilation. The acetate switch is defined as the point where acetate dissimilation equals assimilation. Figure 1 illustrates a typical acetate switch profile in *E. coli*. Besides *E. coli*, acetate switch has been observed in several other bacteria[2-4] and 3 halophilic archaea.[5] To date, all reported cases have involved heterotrophs and the TCA cycle.

This study reports the first observation of an acetate switch in a methanogenic autotroph, specifically *Methanococcus maripaludis* S2. This archaeon is mesophilic, hydrogenotrophic,[6] and can thrive on minimal nutrients, carbon dioxide (CO_2) as the sole carbon source, and either ammonium or dinitrogen (N_2) as the sole nitrogen source. Acetate assimilation by adenosine monophosphate (AMP)-forming acetate-CoA ligase (Acs) is a known phenomenon[7] in *M. maripaludis* S2. However, the only record of acetate dissimilation by *M. maripaludis* S2 is the work of Abdel Azim et al,[8] who reported an acetate concentration of about 1 mmol/L after incubating *M. maripaludis* for 150 hours in a minimal medium supplemented with propionate. Other methanogens such as *Methanosphaera stadtmanae*,[9] *Methanococcus voltae*,[10] *Methanospirillum hungatei*,[11] and *Methanothrix* sp.[12] are known to assimilate acetate, but have not been reported to *produce* acetate. On the other hand, *Methanococcus jannaschii*[13] can

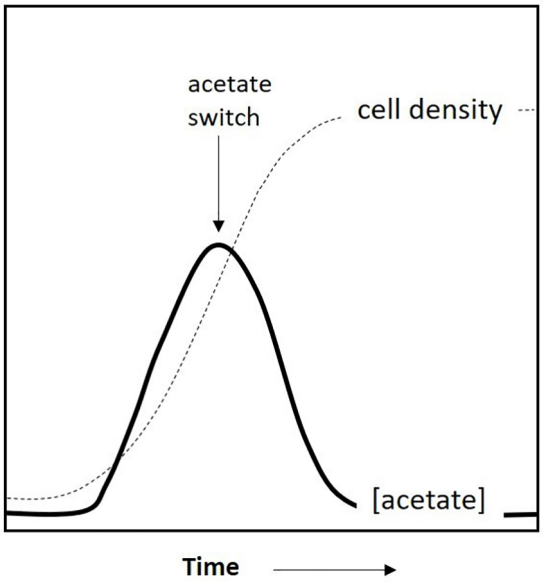

Figure 1. A schematic of acetate switch profile. Acetate concentration increases to a maximum in the early growth phase and decreases thereafter. Acetate concentration reaches zero as the cells enter the stationary phase.

dissimilate acetate, but is unable to assimilate it. Notably, *Methanosarcina acetivorans*[14] and the non-methanogenic *Pyrobaculum islandicum*[15] can both dissimilate and assimilate acetate, but under different growth conditions. No methanogen or autotroph has been reported to display an acetate switch.

In this study, batch culture experiments of *M. maripaludis* S2 were performed in a minimal medium (void of propionate) to investigate its acetate switch. CO_2 and either ammonium or N_2 were used as the sole carbon and nitrogen sources, respectively. As adenosine diphosphate (ADP)-forming Ac-CoA synthetase (Acd) is known[16] to catalyse reversible formation of acetate from Ac-CoA in some other archaea, we propose MMP0253 (GenBank accession no. CAF29809) as the putative Acd in *M. maripaludis*. We produced it and confirmed its enzymatic activity.

Materials and Methods

Gases and chemicals

Pure H_2, CO_2, N_2, and CH_4 gases were purchased from AIR Liquide, Singapore. Mass flow controllers (Red-Y Compact 2 Series, Vögtlin Instruments, Basel-Landschaft, Switzerland) were used to prepare gas mixtures (H_2/CO_2 and $H_2/CO_2/N_2$). K_2HPO_4, $MnSO_4{\cdot}H_2O$, and NH_4Cl were obtained from VWR, Singapore. Nitrilotriacetic acid and $Na_2S{\cdot}9H_2O$ were purchased from Thermo Fisher Scientific, New Jersey, United States. Other chemicals, if not stated otherwise, were purchased from Sigma-Aldrich, Singapore.

Strain and medium preparation

Methanococcus maripaludis S2 (*DSM* 14266) was purchased from Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures. Ammonium samples were prepared as reported.[17] No carbon source was added into the medium and vitamins were also omitted. In N_2 samples, NH_4Cl was omitted in the minimal medium and $Fe(NH_4)_2(SO_4)_2{\cdot}6H_2O$ was replaced by 140 µL of $FeSO_4{\cdot}7H_2O$ solution (0.01% w/v). About 460 mL of the minimal medium was dispensed into 1 L serum bottles (Chemglass Life Sciences LLC, New Jersey, USA). To create an anaerobic environment, the bottles were flushed with 80/20 v/v H_2/CO_2 gas mixture at 2 bars for 30 minutes. Subsequently, the bottles were sterilised by autoclaving at 121°C for 21 minutes. After the bottles had cooled down to room temperature, 2.0 mL of Na_2S stock solution (12.5% w/v) was injected into each bottle.

Batch cultivation

The inoculum was pre-cultured for 2 days in the respective minimal medium. At the start of the experiment, 40 mL of the pre-cultured inoculum was transferred to each serum bottle containing fresh medium. H_2/CO_2 gas mixture (74%-85% of H_2 by volume, balance was CO_2) was used to pressurise the ammonium-grown cultures to 259-265 kPa. $H_2/CO_2/N_2$ gas mixture (68%-73% H_2% and 10%-17% CO_2 by volume, balance was N_2) was used to pressurise the N_2-grown cultures to 304-313 kPa. The cultures were incubated at 37°C and 180 rpm (Orbital Shaking Incubator LM-570RD, Yihder, China). Minimal medium without inoculum was used as a negative control. At the start of each day, the bottles were de-pressurised, flushed, and re-pressurised with H_2/CO_2 and $H_2/CO_2/N_2$, respectively. At the end of each day, the cultures were kept at 20°C without shaking, until the following morning. All growth experiments were performed in duplicates.

Analytical procedures

About 2 mL of liquid sample was withdrawn hourly using a disposable hypodermic needle (25G × 1", Terumo Corporation, USA) attached to a sterile disposable syringe (3 mL, Nipro Medical Corporation, USA) and deposited in a quartz cell (Agilent Technologies, USA). The optical densities of the samples were measured at 600 nm using a double-beam UV/Vis spectrophotometer (Agilent Technologies, Carry, USA). The concentration of acetate in the liquid sample was determined by a high-performance liquid chromatography (HPLC) (1260 Infinity I, Agilent Technologies, USA) equipped with Aminex HPX-87 H column and G7165A multiple wavelength detector (190-240 nm). About 5 mM of H_2SO_4 solution at 0.5 mL/min was used as the mobile phase. The column oven was set at 60°C. The liquid samples were centrifuged at 20 g for 5 minutes before analysis. Acetate was calibrated using standard solutions at 0.01, 0.05, 0.1, 0.25, 0.5, and 0.75 mM. Two additional methods were used to confirm the presence of acetate: gas chromatography (GC) (PerkinElmer Clarus 580, USA) and 500 MHz proton nuclear magnetic resonance (NMR) spectroscopy (Bruker, USA). A control sample with minimal medium and no inoculum was

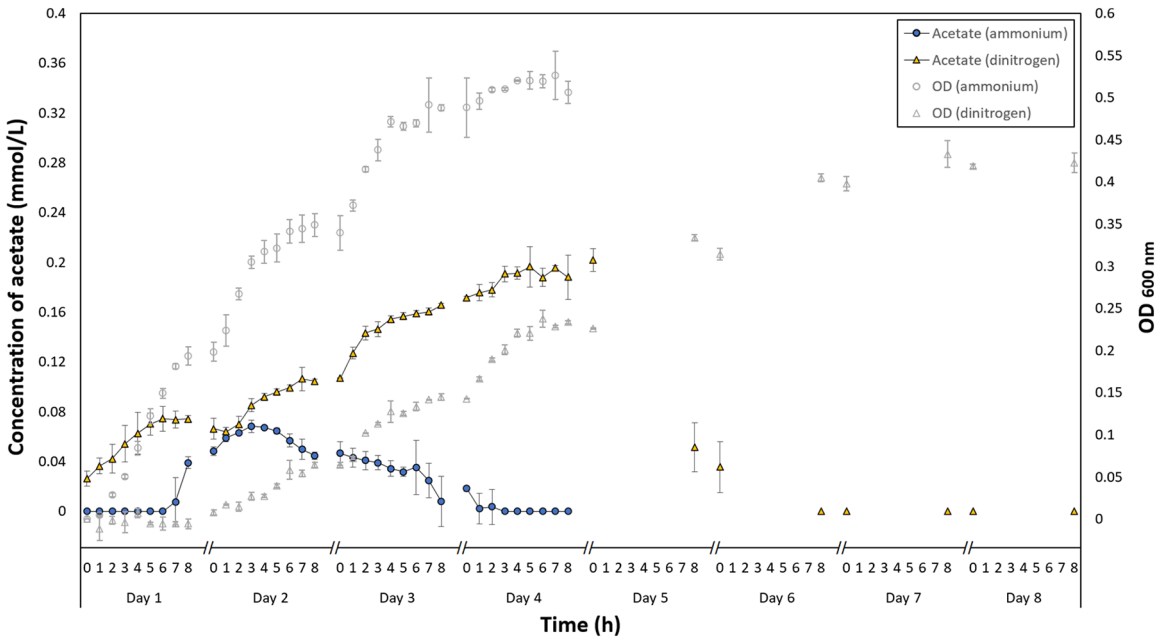

Figure 2. Concentration of acetate (in mmol/L) in ammonium-grown cultures (filled circle, n=2) and dinitrogen-grown cultures (filled triangle, n=2). The optical density (OD) measured at 600 nm of ammonium (empty circle) and dinitrogen (empty triangle) grown cultures were also shown. The data were collected hourly over an 8-hour period in the first 4 days. For subsequent days, only dinitrogen-grown cultures were monitored, and the data were collected at time t=0 and t=8 h each day. The symbol // indicates a break in data collection, during which the cultures were kept at 20°C without any shaking. Error bars indicate one standard deviation of the sample data. Some error bars are smaller than the symbols. OD indicates optical density.

also tested with all 3 methods to confirm our detection and measurement of acetate.

Identification of Acd in M. maripaludis S2 and comparison against other known Acd

The genome[18] of *M. maripaludis* S2 was studied using Basic Local Alignment Search Tool (BLAST)[19] with Acd in *M. jannaschii* (GenBank accession no. WP_010870094) as a query. MMP0253 was identified as a potential Acd. Its primary and secondary structures were compared against the known[16,20] Acd enzymes in *M. jannaschii, Haloarcula marismortui* (GenBank accession no. AAV45866) and *Archaeoglobus fulgidus* (GenBank accession no. WP_048095590) using BLAST and HHpred.[21]

Production of recombinant MMP0253

MMP0253 was cloned using the ligation-independent cloning (LIC) technique with pNIC28-Bsa4 as the vector.[22] Briefly, the vector was linearised by BsaI and overhangs were created by treating the linear vector with T4 DNA polymerase and deoxyguanosine triphosphate (dGTP). The *acd* insert was amplified by the polymerase chain reaction (PCR) technique with Phusion DNA polymerase and primers. Subsequently, the purified PCR product was treated with T4 DNA polymerase and deoxycytidine triphosphate (dCTP) to create overhangs. The vector and insert were then annealed, and *E. coli* strain Rosetta (DE3) was transformed. The *E. coli* with the recombinant plasmid was cultivated in Terrific Broth (TB) medium at 37°C and 200 rpm. The protein expression was induced by isopropyl β-D-1-thiogalactopyranoside (IPTG) at 18°C. The recombinant protein was purified by immobilised metal ion affinity chromatography (IMAC) followed by gel filtration.

Test for enzymatic activity

The activity of recombinant MMP0253 was tested at 37°C under oxic conditions. Each assay mixture (0.1 mL) contained 200 mM Tris-HCl (pH 7.7), 5 mM $MgCl_2$, 0.1 mM 5,5'-dithiobis-(2-nitrobenzoic acid) (Ellman's reagent), 0.1 mM Ac-CoA, 0.5 mM ADP, and 5 mM K_2HPO_4. The absorbance at 412 nm was measured by Infinite 200 PRO (Tecan, Switzerland) before the addition of MMP0253 and 2 min after its addition. Each assay condition was tested in triplicates.

Results

Acetate concentration profiles

All 3 methods (HPLC, GC, and proton NMR spectroscopy) indicated the presence of acetate in our samples. Thus, in spite of its low concentration, there was a high confidence in the acetate detection. Figure 2 shows the acetate concentration profiles in both ammonium and N_2-grown cultures over multiple days. The 500 MHz proton NMR spectroscopy showed a signal at 1.839 ppm (see Supplementary Material 1), which matches the signal of pure acetate. No acetate was detected in the control sample.

In the ammonium-grown cultures, acetate reached a peak concentration of 0.069 ± 0.005 mmol/L midway through the growth phase on the second day and declined thereafter. In the N_2-grown cultures, the acetate concentration was non-zero at

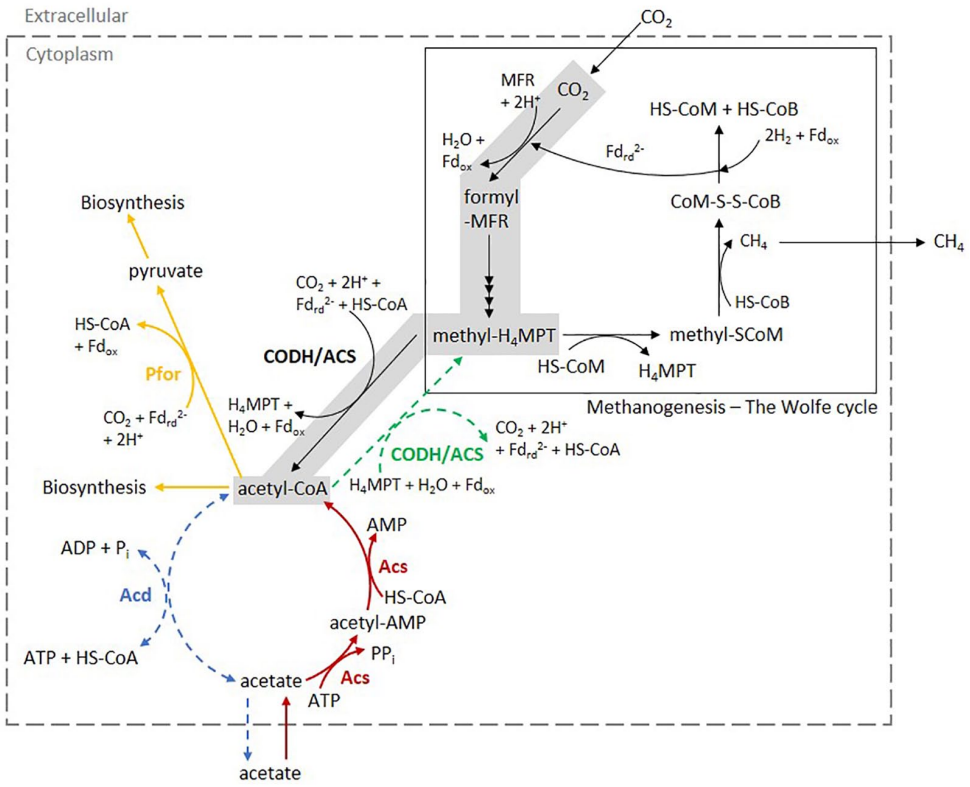

Figure 3. Simplistic view of methanogenesis and biochemical reactions involved acetate and acetyl-CoA. Methanogenesis is shown in the rectangle with solid line border. Grey background indicates the reductive acetyl-CoA pathway. Acetate dissimilation and assimilation were shown. Hypothetical pathways were indicated as dotted lines. Enzymes involved in the Wolfe cycle were not included. Metabolites: acetyl-CoA indicates acetyl coenzyme A; acetyl-AMP, acetyladenylate; ADP, adenosine diphosphate; AMP, adenosine monophosphate; ATP, adenosine triphosphate; Fd_{rd}^{2-}, reduced ferredoxin; Fd_{ox}, oxidised ferredoxin; MFR, methanofuran; H_4MPT, tetrahydromethanopterin; HS-CoA, coenzyme A; HS-CoB, coenzyme B; HS-CoM, coenzyme M; P_i, inorganic phosphate; PP_i, inorganic pyrophosphate. Enzymes (bolded): Acd, ADP-forming acetyl-CoA synthetase; Acs, AMP-forming acetate-CoA ligase; CODH/ACS, carbon monoxide dehydrogenase/acetyl-CoA synthase complex; Pfor, pyruvate: ferredoxin oxidoreductase.

the very beginning, which is probably due to the dissimilated acetate in the N_2-acclimated inoculum. The daily concentration profile exhibited a repeated trend during the first 4 days. It increased at a slowing rate and plateaued near the day's end. The acetate concentration reached a peak of 0.202 ± 0.009 mmol/L on the fourth day, midway through the growth phase. In both ammonium and N_2-grown cultures, the acetate concentration reached zero as the cells entered the stationary phase.

The overall profiles in both cultures follow an initial rising and subsequent falling trend with a distinct peak characteristic of the acetate switch. In other words, the growth of *M. maripaludis* S2 exhibited 2 phases: an initial acetogenic phase followed by an acetotrophic phase. Acetate assimilation and dissimilation are likely to be competitive and balance each other at the acetate peaks. Both profiles of ammonium and N_2-grown cultures are markedly similar to the acetate switch profile in *E. coli*.[1] There have been no reports of this for an autotroph in the literature thus far.

MMP0253 showed similarities to other known Acd

Our amino acid sequence analysis via BLAST shows that MMP0253 is 35% (e-value: 5.6e-116) similar to Acd in *H. marismortui*, 44% (e-value: 2.4e-110) similar to *A. fulgidus*, and 52% (e-value: 9.5e-120) similar to *M. jannaschii*. These seemingly low percentages do not discredit the claim of MMP0253 as an Acd, because the characterised Acd in *H. marismortui, A. fulgidus*, and *M. jannaschii* are also 36% to 42% similar to each other. Our analysis of the 4 secondary protein structures via HHpred showed marked resemblance (see Supplementary Material 2). All 4 have 2 conserved histidine residues (His-242 and His-538 in *M. maripaludis*), which may be analogous to the active sites for the transient phosphorylation in acetate formation.[23] Residues associated with the formation of Ac-CoA binding site and signature motifs of Acd[24] can also be found in MMP0253. In essence, the structural evidence at both levels suggests that MMP0253 in *M. maripaludis* is homologous to Acd in the other 3 archaea. Abdel Azim et al,[8] Goyal et al,[25] and Richards et al[26] have also annotated MMP0253 as Acd.

MMP0253 catalysed acetate formation from Ac-CoA

After recombinant MMP0253 was added into the reagents mixture, a rapid yellow colourisation was observed. Acetate formation from Ac-CoA released coenzyme A (HS-CoA), which reacted with Ellman's reagent to give yellow-coloured

Table 1. Test for the enzymatic activity of MMP0253 at 37°C under oxic conditions.

REAGENTS AND ENZYMES				CHANGE IN ABSORBANCE AT 412 NM
ACETYL-COA	ADP	K_2HPO_4	MMP0253	
X	X	X	X	0.3695 ± 0.0278
	X	X	X	0.1164 ± 0.0081
X		X	X	0.1155 ± 0.0139
X	X		X	0.0772 ± 0.0028
			X	0.0899 ± 0.0255
X	X	X		0.0038 ± 0.0004

Abbreviations: ADP, adenosine diphosphate; X, present in the assay mixture.
Unless indicated otherwise, each assay mixture (0.1 mL) consisted of 200 mM Tris-HCl (pH 7.7), 5 mM $MgCl_2$, 0.1 mM 5,5'-dithiobis-(2-nitrobenzoic acid) (Ellman's reagent), 0.1 mM acetyl-CoA, 0.5 mM ADP, and 5 mM K_2HPO_4. Absorbance readings at 412 nm were obtained 2 minutes after the addition of MMP0253. For the tests in which MMP0253 was not added, absorbance readings were obtained after 30 minutes. Each assay was done in triplicates.

2-nitro-5-thiobenzoate dianions. Therefore, the observed colourisation (absorbance change of approximately 0.4) confirms the catalytic activity of MMP0253 (Table 1). In the absence of MMP0253, the absorbance change was negligible even after 30 minutes. When Ac-CoA, ADP, or K_2HPO_4 was not added into the reagents mixture, the absorbance change was about 0.1. However, this is due to the interaction between MMP0253 and Ellman's reagent as verified by adding MMP0253 into a mixture of Tris-HCl (pH 7.7), $MgCl_2$, and Ellman's reagent only. This suggests that the acetate formation from Ac-CoA catalysed by MMP0253 requires all 3 reagents, Ac-CoA, ADP, and inorganic phosphate (P_i).

Discussion

While acetate assimilation by *M. maripaludis* S2 has been reported in the literature,[7] its dissimilation has not been conclusively established prior to this study. Although one record[8] of acetate dissimilation does exist for this methanogen, it was limited to the growth with propionate. Our experimental results confirm that *M. maripaludis* S2 can produce acetate when grown in a minimal medium (void of propionate) with either ammonium or N_2 as the nitrogen source. Our preliminary enzymatic study also shows that MMP0253 may function as Acd, which is known[16] to catalyse the reversible formation of acetate from Ac-CoA and generate ATP via substrate-level phosphorylation: Ac-CoA + ADP + Pi ↔ Acetate + ATP + HS-CoA.

Having established acetate dissimilation in *M. maripaludis* S2, we observe that our acetate concentration profiles have a marked resemblance to the acetate switch profile in *E. coli*. Acetate switch, a phenomenon that involves the transition from an acetogenic phase to an acetotrophic phase, has only been observed in heterotrophs (Table 2).[1] Furthermore, all reported microorganisms use the TCA cycle.[1] In contrast, *M. maripaludis* S2 is an autotroph, which uses the Wolfe cycle for methanogenesis (Figure 3).

While we do not have a solid evidence to explain the above acetate profile in this archaeon, we put forth a hypothesis based on the literature on other microorganisms. It is established[1] that acetate dissimilation in *E. coli* and other heterotrophs serves as a metabolic outlet for carbon overflow, which occurs when their TCA cycles are saturated or inhibited. The dissimilation also helps to reduce Ac-CoA accumulation and recycle HS-CoA. In the late growth phase when the carbon source depletes, these microorganisms assimilate acetate as an additional carbon source. In addition, as acetate accumulates in the growth medium, it diffuses back across the cell membrane. This acidifies the cytoplasm and interferes with other biosynthetic processes.[3] The cell removes acetate by converting it into Ac-CoA via a single-step reaction.

Based on the above explanation of the acetate switch in other microorganisms, we can postulate that a similar hypothesis may hold for *M. maripaludis* S2. When the carbon flux into the Wolfe cycle exceeds its capacity, *M. maripaludis* must find a metabolic outlet for the overflow. While the Wolfe cycle has various exits leading to the formations of purines, thymidylate, and methionine, the formation of Ac-CoA from methyl-tetrahydromethanopterin (methyl-H_4MPT) is the largest.[37] This may lead to an accumulation of Ac-CoA and diminution of HS-CoA pools, which the cell may avoid via biosynthesis or acetate dissimilation. However, biosynthesis needs ATP, and its primary supply (Wolfe cycle) is already saturated. Hence, acetate dissimilation seems to be the only metabolic outlet to relieve the carbon overflow. While no concrete evidence exists for this hypothesis at the present, we observed that it is consistent with the daily acetate profiles (Figure 2). As our study involved batch culture and CO_2 was replenished daily, the CO_2 supply (flux or overflow) in the liquid medium gradually decreased during each day. As carbon supply decreased over time, carbon overflow decreased, and hence dissimilation decreased and eventually plateaued off. When CO_2 was replenished the following morning, carbon overflow occurred at the start and dissimilation continued at a higher rate than the previous evening.

Table 2. A comparison of the characteristics of the bacteria and archaea with acetate switch.

MICROORGANISM	DOMAIN	TYPE	ACETATE DISSIMILATION ENZYMES	ACETATE ASSIMILATION ENZYMES	REFERENCES
Escherichia coli	Bacteria	Heterotroph	Pta-Ack	Acs Reverse Pta-Ack	Brown et al,[27] Kumari et al[28]
Salmonella enterica	Bacteria	Heterotroph	EutD-ACK	Acs Reverse Pta-Ack Reverse EutD-Ack	Starai et al[4,29]
Bacillus subtilis	Bacteria	Heterotroph	Pta-Ack	Acs	Grundy et al,[2] Presecan-Siedel et al[30]
Corynebacterium glutamicum	Bacteria	Heterotroph	No data	Reverse Pta-Ack	Dominguez et al,[31] Reinscheid et al,[32] Gerstmeir et al[33]
Vibrio fischeri	Bacteria	Heterotroph	No data	Acs	Studer et al[34]
Staphylococcus aureus	Bacteria	Heterotroph	Pta-Ack	Acs	DeMars and Bose[35]
Campylobacter jejuni	Bacteria	Heterotroph	Pta-Ack	Acs	Wright et al[36]
Mycobacterium tuberculosis	Bacteria	Heterotroph	Pta-Ack	Acs Reverse Pta-Ack	Rucker et al[3]
Haloarcula marismortui *Haloferax volcanii* *Halobacterium saccharovorum*	Archaea	Heterotroph	Acd	Acs	Brasen and Schonheit[5]
Methanococcus maripaludis	Archaea	Autotroph	Acd	Acs	This study

Abbreviations: Acd, ADP-forming acetyl-CoA synthetase; Ack, acetate kinase; Acs, AMP-forming acetate-CoA ligase; EutD, *eut* operon-encoded phosphotransacetylase; Pta, phosphotransacetylase.

The hypothesis can also explain the greater acetate dissimilation in N_2-grown cultures. Diazotrophy is an ATP-demanding process.[38] Hence, the Wolfe cycle in the N_2 samples will saturate earlier for the same flux of CO_2 (mmol/gDCW/h). Furthermore, the biosynthesis level with N_2 is known[39] to be lower. Therefore, the N_2-grown cultures will experience a higher carbon overflow and hence greater acetate dissimilation. Abdel Azim et al[8] reported an acetate dissimilation when incubating *M. maripaludis* in a minimal medium supplemented with propionate. While they hypothesised that propionate might have reduced the electrochemical proton gradient across the cell membrane, forcing the cell to restore it via the ATP-consuming proton extrusion process, they did not explain why acetate was produced. This ATP-consuming process may have an effect similar to diazotrophy and thus caused acetate dissimilation to occur.

In the late growth phase, *M. maripaludis* transitioned to acetate assimilation. This is possibly due to carbon deficiency, similar to the phenomenon in other microorganisms as discussed earlier. The acidification of the cytoplasm by acetic acid may play a role in inducing *M. maripaludis* to convert it to Ac-CoA; however, further study is needed to verify this.

Acknowledgements

We thank Professors Zhou Zhi (Purdue), Zhou Kang (NUS), Zhao Dan (NUS), Yan Ning (NUS), He Jianzhong (NUS), and Dr Karthika Nagarajan (NUS) for their invaluable help, guidance, and suggestions during the course of this work. Similarly, we thank Matthew James Rogers (NUS), Prerna Goyal (NUS), Lim Ee Yang (NUS), and Dr Hugo Gerald Schmidt (CARES Ltd). We are indebted to Professor Tong Yen Wah (NUS) and Energy and Environmental Sustainability for Megacities (E2S2) for lending us equipment, which enhanced and expedited our work. We thank Dr Dai Chencheng (CARES Ltd) for his assistance with high-performance liquid chromatography. We thank the NTU Protein Production Platform (www.proteins.sg) for the cloning, expression tests, and purification of protein construct Acd.

Author Contributions

All authors designed the experiment. CHV performed the experiment. All authors wrote and edited the manuscript. All authors read and approved the final manuscript.

Availability of Data and Material

The main datasets generated and/or analysed during the current study are attached as supplementary materials. Any other datasets are available from the corresponding author on reasonable request.

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Supplemental Material

Supplemental material for this article is available online.

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