UNIVERSITY OF CALIFORNIA, SAN DIEGO

A Study of the Impact of Secondary Organisms on Physiology of *Moorella thermoacetica*

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by

Dongyeon Kim

Committee in charge:

Bernhard Palsson, Chair
Milton Saier, Co-chair
Karsten Zengler
Moselio Schaechter

2012

The thesis of Dongyeon Kim is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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Co-chair

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Chair

University of California, San Diego

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I dedicate this thesis to my parents for their love and support.

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ABSTRACT OF THESIS

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by

Dongyeon Kim

Master of Science in Biology

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Professor Bernhard Palsson, Chair

Professor Milton Saier, Co-chair

The isolation and cultivation method of microorganisms has been explored to study morphology, physiology, genetics, and pathogenicity of organisms in detail. Pure cultured microorganisms provide us informative knowledge on the physiology of organisms but it has some limits in the examination of interactions of microorganisms with their microbial community. Coculture study has strong merits in that it helps us understand interactions between microorganisms and predict the phenotypic changes that might occur during the cultivation.

The physiology of *Moorella thermoacetica* was examined when it was cocultured with six secondary organisms: *Geobacter sulfurreducens, Thermotoga maritima, Clostridium ljungdhalii, Sporomusa ovata, Methanosarcina barkeri,* and *Methanococcus maripaludis*. *M. thermoacetica* growth was enhanced only with supplementation of *G. sulfurreducens* cells. To interpret the cause of the amplified growth of *M. thermoacetica*, different components of *G. sulfurreducens* were examined. The metabolites were analyzed by mass spectrometer in order to find the compound responsible for the growth advantage of *M. thermoacetica*. From LCMS analysis, total 138 compounds were detected as metabolites of *M. thermoacetica*. The genetic profiles of *M. thermoacetica* and *M. thermoacetica* with filtered *G. sulfurreducens* media were analyzed. Detection of plasmid pJIR418 in Cm^r transformants of *M. thermoacetica* was also performed by PCR after the extraction of plasmid.

Single compound or genes responsible for the stimulation of growth of *M. thermoacetica* were unable to be identified. When *M. thermoacetica* was supplemented with *G. sulfurreducens*, the biomass was doubled but the hydrogen production was decreased. RNA sequencing result shows no significant change in hydrogenase activity between two samples, which indicates *M. thermoacetica* produces hydrogen and consumes hydrogen for respiration as well. The genes identified for the respiration, however, were not up-regulated with the supplementation of *G. sulfurreducens*. The results indicate that the secondary organisms have an impact on the proteomics or fluxomics level of *M. thermoacetica*.

1. Background

1.1 Cells in microbial ecology

The isolation and cultivation method of microorganisms has been explored to study morphology, physiology, genetics, and pathogenicity of organisms in detail. Pure cultured microorganisms provide us informative knowledge on the physiology of organisms such as nutrient cycling, formation and degradation of organic and inorganic molecules, and energy production. They also allow us to access more comprehensive understanding of microbial ecology, (Figure 1.1) [1]. In spite of many advantages, monoculture cultivation has some limits in the examination of interactions of microorganisms with their microbial community within the natural environment. Isolated monoculture microorganisms are most likely to lack the interspecies communications that are common between microorganisms and fail to represent a community as a whole. Coculture study has strong merits in that it helps us understand interactions between microorganisms and predict the phenotypic changes that might occur during the cultivation.

Culturing microorganisms in the laboratory is still a difficult task. It is estimated that only 1% of bacteria on Earth can be cultivated in laboratory settings [2], [3], and majority of published microbiological researches from 1991 to 1997 were dedicated to only eight bacteria genera [4]. This implies how difficult it is to culture microorganisms and to study the interactions of microbial community in the laboratory in the context of natural environment. In 1970s, George Fox and Carl Woese first proposed rRNA gene sequence as an evolutionary marker to study phylogeny and evolution [5], [6], [7]. Drancourt used 16S rRNA to analyze 177 environmental samples, which were not

identifiable with traditional phenotypic characterization methods [8]. It seemed this approach would give better resolution to the study of community dynamics without cultivating individual microorganisms. Furthermore, 16S rRNA analysis was commonly used when there was lack of resolution at the species level [9], [10]. The rRNA can provide phylogenic context but it is helpful only to predict the physiology, since physiology is not necessarily compatible with phylogeny [1]. However, the reports from metagenomics surveyed by Baker *et al*. and Huber *et al*. proclaim that using 16S rRNA gene primers is not ubiquitous and it is possible to miss some organisms by targeting genes directly [11], [12]. In spite of the advantages of analyzing community samples or metagenomics data, which can provide a big picture of community interactions, the broad approach can not capture every microorganism present in the environment and might overlook the physiology and interspecies interactions [13].

Microorganisms can interact with each other using various signals and these signals allow the organisms to communicate not only within the species but also between different bacterial species and between organisms from different kingdom [14]. Some of the signaling molecules are involved in growth [15] and Matsuhashi *et al*. suggested that microorganisms are able to communicate using physical signals, i.e. the growth of *Bacillus carboniphilus* are stimulated by single sine sound waves produced by neighboring cells [16]. Physical signals are based on faster cell signaling mechanism and they propagate faster than chemical signals [17]. These various communications cannot be found in monoculture settings. Thomas *et al*. showed coculture of *C. thermocellum* and *C. thermohydrosulfuricum* could produce ethanol in two times higher than in *C.*

thermocellum monoculture fermentations. Potentially, they can use this coculture as a direct method of producing ethanol from biomass [18].

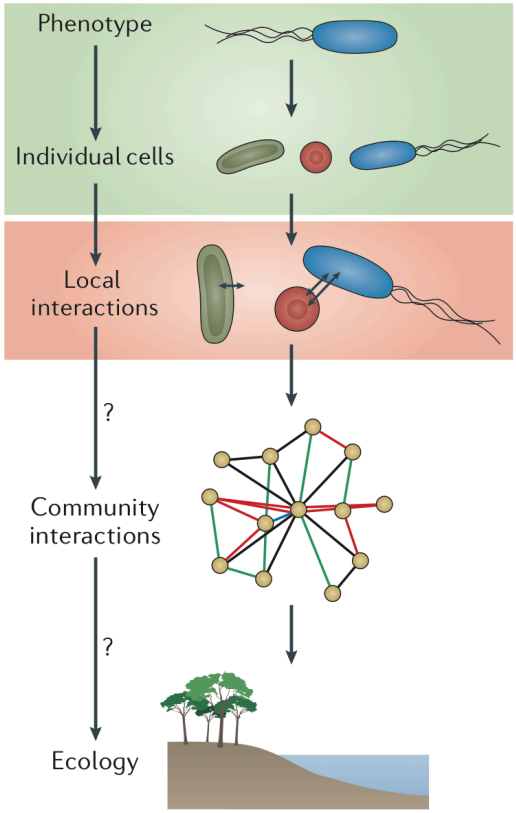


Figure 1.1 Pure culture to community sample diagram in community systems (CoSy) biology [13].

Recently, Summers *et al*. found coculture of *G.matallireducens* and *G. sulfurreducens* formed aggregates that were electrically conductive during metabolizing ethanol. It implied *G. metallireducens* consumed hydrogen produced by *G. sulfurreducens*, while *G. sulfurreducens* could consume acetate that *G. metallireducens* produced from ethanol [19]. Interspecies formate transfer is not possible since *G. sulfurreducens* is unable to use formate as an electron donor [20]. As a result, they suggested that direct electron transfer gives growth advantages to two *Geobacter* species when interspecies hydrogen transfer is no longer possible [19].

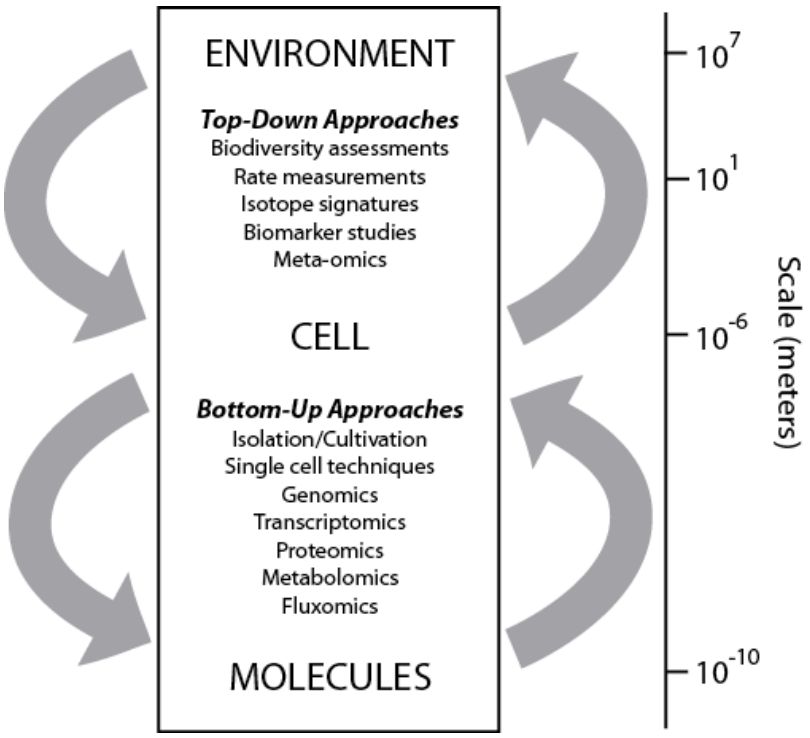


Figure 1.2 Top-down and bottom-up approaches in microbial ecology, spanning orders of magnitude in spatial resolution [13].

Defined media in the laboratory setting is not a representative of natural environment. One of the most challenging issues in the research area is to define the right media condition [15]. Any results from the laboratory setting would not represent the microbial activities in nature, due to the lack of other microorganisms or nutrients which are present in natural environment. Thus studying microbes in pure culture is not informative enough to give full understanding of their roles in the environment. On the other hand, mixed microbial community generally contains so many organisms that it is hard to decipher inter microbial interactions. Contrary to both approaches, coculture can show the interactions that cannot be detectable in monoculture as well as provide more direct observation of the interactions than mixed microbial community, resulting in better

elucidation of bacterial communication.

1.2 Acetogens and *M. thermoacetica*

Acetogens are anaerobic bacteria that use the acetyl-CoA pathway for energy production and for fixing CO_2 into cellular carbon. In the world of bacteria, acetogens are found in more than one phylum, but most of them are associated with the gram-positive, low DNA G+C content [21]. The first acetogen, *Clostridium aceticum*, was isolated by Dutch microbiologist K. T. Wieringa in 1936. He performed the growth of this spore-forming, mesophilic bacteria under the expense of H_2-CO_2 according to the following stoichiometry, which illustrates the acetate production from fixation of CO_2 [22], [23]:

$$4H_2 + 2CO_2 \rightarrow CH_3COOH + 2H_2O \quad (1.1)$$

C. aceticum was lost after one study of defining its nutritional requirements [24], but it was isolated again in the early 1980s [25], [26], [27]. In 1942, F. E. Fontaine and his colleagues isolated *Clostridium thermoaceticum* as the second acetogen. This spore-forming, thermophilic bacterium, which was isolated from the horse manure, catalyzed the conversion of glucose to acetate (Figure 1.3) [28]:

$$C_6H_{12}O_6 \rightarrow 3CH_3COOH \quad (1.2)$$

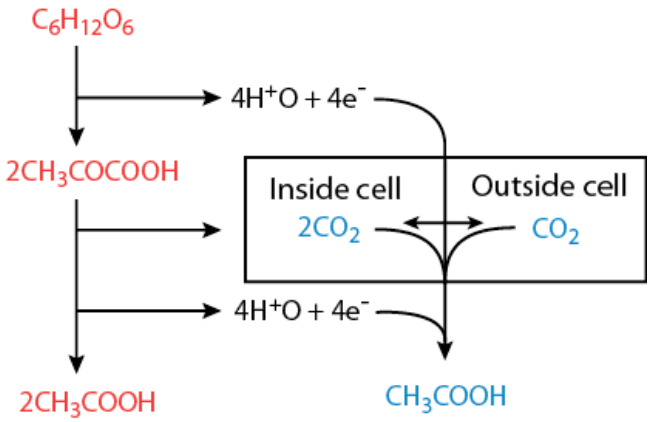


Figure 1.3 Homoacetate fermentation by *M. thermoacetica*. The diagram depicts the two molecules of acetate from glucose and one molecule synthesized from CO_2 [29].

In 1952, H. G. Wood performed synthesis of acetate from two molecules of CO_2 with *C. thermoaceticum* using $^{13}CO_2$ and mass spectrometry [30]. It played a major role in understanding of the acetyl-CoA pathway, the reduction of CO_2 to acetate [29]. In 1994, as a taxonomy of genus *Clostridium* was reorganized, *C. thermoaceticum* was reclassified as *M. thermoacetica* [31]. *M. thermoacetica* became the most important acetogen, because it was the only acetogen available for a laboratory study [21].

M. thermoacetica is one of the most metabolically diverse acetogens and it can use wide range of electron donors and electron acceptors. This spore-forming, thermophilic bacterium can utilize many substrates and grow both autotrophically and heterotrophically [32]. As for electron donors, it can utilize sugars, gases, alcohols, organic acids, and methoxylated aromatic compounds: glucose, fructose, xylose for sugars; H_2, CO for gases; methanol, ethanol, *n*-propanol, *n*-butanol for alcohols; formate, oxalate, glyoxylate, glycolate, pyruvate, lacate for organic acids, etc. [29]. As electron acceptors, it can utilize CO_2, nitrate, nitrite, thiosulfate, dimethylsulfoxide [29]. This

bacterium has only one organic nutritional requirement, nicotinic acid [33]. *M. thermoacetica* is a acetogen that guided us to understand physiological diversities and biochemistry of acetogens and it played a major role in understanding of the acetyl-CoA pathway, the reduction of CO_2 to acetate [29].

The acetyl-CoA pathway is a linear process of fixing CO_2 into cellular carbons (Figure 1.4) [29]. There are other metabolic pathways that fix CO_2, such as the Calvin cycle, the reductive tricarboxylic acid cycle, and the hydroxypropionate cycle. These cycles require recycled intermediates; rebulosebiophosphate, oxaloacetate, and acetyl-CoA, respectively [34], [35]. The acetate synthesis occurs by substrate-level phosphorylation and produces four molecules adenosine triphosphate (ATP), and three acetates are produced for each hexose [36]. However, during the autotrophic condition, growth under H_2 and CO_2, there is no net gain of ATP during production of formate, while one ATP is produced during the acetate synthesis from acetylphosphate by acetate kinase [37], [38].

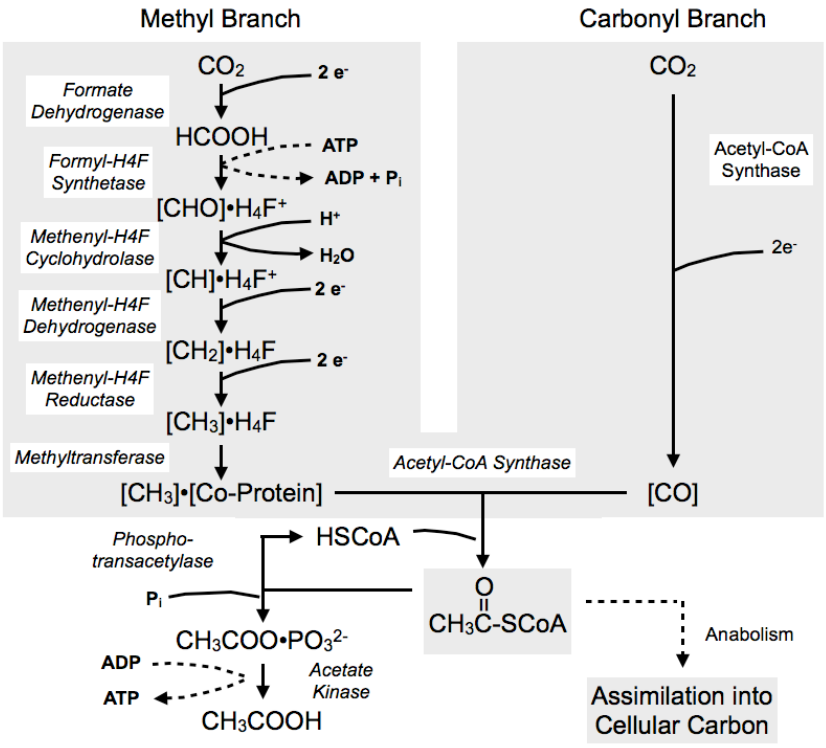


Figure 1.4 The acetyl-CoA pathway as resolved from *M. thermoacetica*. Abbreviations: THF, tetrahydrofolate; CoA, coenzyme A; P1, inorganic phosphate; [e-], reducing equivalent; Co-Protein, corrinoid enzyme [21].

There are more than 100 acetogens which use the acetyl-CoA pathway or its variations and these bacteria can fix or recycle 10^{12} kg of CO_2 per year. The fixation of CO_2 producing acetate is the most direct pathway for forming acetyl-coA, and this autotrophic fixation may be the primordial biochemical pathway [38]. On Earth's crust, H_2, CO_2, CO, and metals such as cobalt, nickel, iron, tungsten, molybdenum, and selenium are present. These gaseous molecules and metallic elements are involved in the catalysis of the acetyl-CoA pathway. Moreover, this pathway is frequently found in thermophilic and anaerobic microbes, which indicates that this pathway developed before

the emergence of O_2 on earth. Taking everything into account, the acetyl-CoA pathway may be one of the earliest biochemical pathways [34], [39].

M. thermoacetica was an important organism which guided us to understand physiological diversities and biochemistry of acetogens. It also played a major role in the study of the reduction of CO_2 to acetate. In order to get more understanding on the metabolic behavior of *M. thermoacetica*, it is needed to study what interaction it can have with other organisms. Coculturing allows *M. thermoaceticaM. thermoacetica* to interact with secondary organisms, which exhibits closer representation of microbial interactions in a community. It will help us to access deeper understanding of microbial ecology. The focus of this thesis is to decipher the relationship of *M. thermoacetica* with the secondary organisms and understand how the growth of *M. thermoacetica* is affected by neighboring cells.

2. Materials and Methods

2.1 *M. thermoacetica* culturing conditions

2.1.1 Growth conditions for *M. thermoacetica*

M. thermoacetica strain ATCC 39073 was obtained from the American Type Culture Collection Center. The standard growth conditions for *M. thermoacetica* were under anaerobic conditions in triplicate in 100 mL of ATCC medium 1203 within 125 mL serum bottles at 65 °C. The yeast extract medium, undefined media for *M. thermoacetica*, contained (in grams per liter): glucose, 18.0 (solution A); KH_2PO_4, 150.0 and K_2HPO_4, 290.0 (solution B); yeast extract, 5.0, $(NH_4)_2SO_4$ 1.3, $MgCl_2$, 0.75, $CaCl_2.\cdot 2H_2O$, 0.013, $FeSO_4\cdot 7H_2O$, 0.125% solution 1.0mL (collectively as solution C). Solution A, B, and C were sparged with CO_2 and N_2 gas mixture, and autoclaved in liquid cycle separately. The pH of all media (before inoculation) was 6.6-6.8. The minimal medium contained same salt as undefined medium except yeast extract and supplemented with mineral and vitamin solution. All medium components were of analytical grade. During inoculation, 100 mM cysteine, solution A, and solution B were injected to the media to obtain 10 mM of cysteine, 10 mM of phosphate buffer solution, and 10 mM glucose, respectively. Transfer and inoculations were performed anaerobically using syringes and needles flushed with N_2.

Final concentration of 10 mM glucose was used as electron donor, and carbon dioxide was used as electron acceptor. Samples were collected every 4 to 12 hours from lag phase to stationary phase. At each time point, the optical density, gas chromatography

(GC), and high performance liquid 1 chromatography(HPLC) samples were collected for growth curve.

2.1.2 Culturing conditions: *M. thermoacetica* with secondary organisms

M. thermoacetica was cultured, with various organisms such as *Geobacter sulfurreducens, Thermotoga maritima, Clostridium ljungdhalii, Sporomusa ovata, Methanosarcina barkeri,* and *Methanococcus maripaludis* to examine the effect of the secondary organism. All the supplementary organisms were grown to stationary phase and 5 mL of these organisms were injected into *M. thermoacetica* culture during inoculation. Pure culture of *M. thermoacetica* was also cultured as a negative control. The cultures were grown in triplicates at 65 °C and collected every 4 to 12 hours from lag phase to stationary phase. At each time point, the optical density, GC, and HPLC samples were collected for growth curve.

2.1.3 Culturing conditions: *M. thermoacetica* with *G. sulfurreducens*

Various components were separated from *G. sulfurreducens* and were injected into *M. thermoacetica* individually during inoculation to determine the effect of *G. sulfurreducens*. The whole cell conditions included following four components. The first component was 5 mL of *G. sulfurreducens* culture including media with stationery phase cells. The second component was pellet: 5 mL of *G. sulfurreducens* culture was centrifuged down at 10,000 xg for 5 minutes and the pellet was washed twice with phosphate buffered solution (PBS). The third component was the filtered *G. sulfurreducens* media: 5 mL of *G. sulfurreducens* culture was filtered through steriled

PVDF membrane filter with 0.22 μm pore size to eliminate any intact cells. The last component was fresh water media: no cells were cultured in the fresh water media and 5 mL of the media was injected to *M. thermoacetica* during inoculation. The fresh media contained(in grams per liter): NH_4Cl, 0.25; KCl, 0.1; $NaH_2PO_4 \cdot H_2O$, 0.575; $NaHCO_3$, 2.5; 1 μM Na_2SeO_4, and were supplemented with trace mineral and vitamin solution. The trace mineral contained (in milligram per liter): nitrilotriacetate, 15; $MgSO_4$, 30; $MnSO_4 \cdot H_2O$, 5; NaCl 10; $FeSO_4 \cdot 7H_2O$, 1; $CaCl_2 \cdot 2H_2O$, 1; $ZnCl_2$, 1.3; $CuSO_4 \cdot 5H_2O$, 0.1; $AlK(SO_4) \cdot 12H_2O$, 0.1; H_3BO_3, 0.1; $Na_2MoO_4 \cdot 2H_2O$, 0.25; $NiCl_2 \cdot 6H_2O$, 0.25; $Na_2WO_4 \cdot 2H_2O$, 0.25. Vitamin solution of 1.0 mL (1,000x) was supplemented to every liter of medium. The 1,000x vitamin solution contained (in milligram per liter): Pyridoxine·HCl, 0.1; p-Aminobenzoic acid, 0.050; Nicotinic acid, 0.050; dl-Ca-pantothenate, 0.050; Thiamine·HCl, 0.050; dl-6,8-thiotic acid (lipoic acid), 0.050; Riboflavin, 0.040; Biotin, 0.020; Folic acid, 0.020; Vitamin B12, 0.001. All medium components were of analytical grade.

As for the lysed *G. sulfurreducens* samples, stationary phase *G. sulfurreducens* was boiled at 95 °C for 15 minutes, centrifuged down at 10,000 xg for 5 minutes, and the pellet and supernatant were separated. The pellets were resuspended in fresh water media and injected into *M. thermoacetica* media bottle right before inoculation. Each *M. thermoacetica* bottle was supplemented with pellet and supernatant with the volume equivalent to 5 mL of *G. sulfurreducens* culture. Transfer and inoculation were performed anaerobically using syringes and needles flushed with N_2. Cultures were grown in triplicate at 65 °C and collected every 4 to 12 hours from lag phase to stationary phase.

At each time point, the optical density, GC, and HPLC samples were collected for growth curves.

2.1.4 Culturing conditions: *M. thermoacetica* with cytochrome C

A stock solution of cytochrome C, 20 mg/L, was prepared under anaerobic condition and filtered into a sterile, anoxic serum bottle. Cytochrome C stock solution of 1.0 mL was injected anaerobically with needle and syringe to 100 mL of *M. thermoacetica* media before inoculation. Growth was initiated by injecting 5.0 mL of inoculum per serum bottle. Cultures were grown in triplicates at 65 °C and collected every 4 to 12 hours from lag phase to stationary phase. At each time point, the optical density, GC, and HPLC samples were collected for growth curves.

2.2 Physiological screening

2.2.1 Growth curves, HPLC and GC

Optical density (OD) was measured with Thermo Spectromic Biomate 3 at 600 nm. Glucose and acetate were measured using refractive index detection by high-performance liquid chromatography (HPLC) (Waters, Milford, MA) with Bio Rad Aminex HPX-87H Ion exclusion column (300 mm x 7.8 mm) as HPLC organic acid analysis column. The injection volume was 10 μL and 5 mM H_2SO_4 as the mobile phase with a flow rate of 0.5 mL/min. Hydrogen and methane were measured using gas chromatography (GC) (GC-2014 Shimadzu).

2.2.2 Dry cell weight

Dry cell weight was obtained by cultures grown until mid-log phase and harvested at three different points during mid-log phase of *M. thermoacetica*, which were determined by previously established growth curves versus time. *M. thermoacetica* cultures were grown in duplicates at 65 °C and three different samples were collected every 4 to 6 hours during mid-log phase (Figure 2.1). Cultures were filtered through cellulose nitrate membrane filters with pore size of 0.22 μm by Whatman. Weight of each membrane was measured before the filtration to determine cell weight density to OD. After filtrations, the membranes were dried for 48 hours at 81 °C and the weight was measured again after drying.

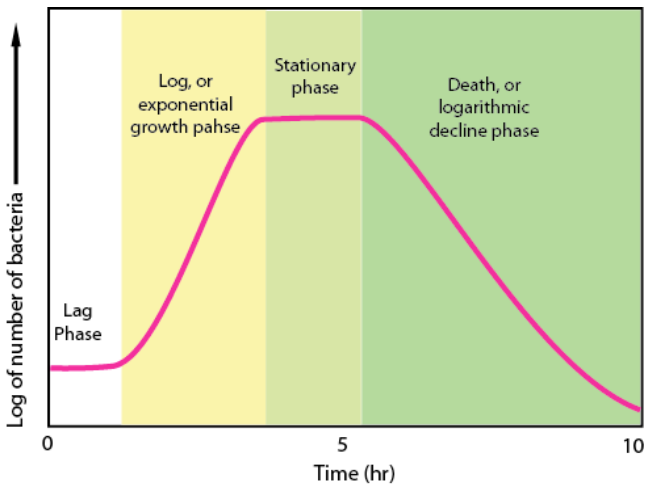


Figure 2.1 General bacterial growth curves with indication of lag, log, and stationary phase.

2.3 Metabolomics analysis

2.3.1 Internal standard samples

In order to circumvent the cost of purchasing label standards, isotopic standards were generated metabolically by growing *E. coli* on a uniformly labeled carbon source (U13C-Glucose). This protocol was based on the "Miracle principle" and isotope dilution mass spectroscopy [40], [41]. *E. coli* was cultured in M9 minimal media supplemented with 2 g/L U13C-Glucose. M9 minimal media contained (in grams per liter): Na_2HPO_4, 6.8; KH_2PO_4, 3.0; NaCl, 0.5; NH_4Cl, 10, and supplemented with trace mineral solution and 10 mL of 400 g/L glucose solution. The trace mineral solution contained (in grams per liter): $FeCl_3 \cdot 6H_2O$, 27.0; $ZnCl_4 \cdot 4H_2O$, 2.0; $CoCl_2 \cdot 6H_2O$, 2.0; $Na_2MoO_4 \cdot 2H_2O$, 2.0; $CaCl_2 \cdot 2H_2O$, 1.0; $CuCl_2 \cdot 6H_2O$, 1.3; H_3BO_3, 0.5 was dissolved in 1.0 L milli-Q water with 10.0 mL of concentrated HCl. All medium components were of analytical grade and autoclaved on liquid cycle.

Pre-culture and cultures were prepared with M9 minimal media with uniformly labeled U13C-Glucose. *E. coli* K-12 MG1655 strain was inoculated in 25 mL culture flask with 5 mL of U13C M9 minimal media and cultured overnight at 37 °C. The overnight culture of 1.0 mL was passed to three 100 mL of U13C M9 minimal media and incubated at 37 °C until the cells reached OD_{600} of 1.0. The cells were collected in 50 mL conical tube using a pippettemanat OD_{600} of 1.0. This generated biomass of 22.5 mg DCW to be aliquoted as internal standards. The limits of detection were estimated to be 50-0.5 mg dry cell biomass [42]. Collected cells were cooled in a bucket of crushed ice for 5 minutes and all cells were kept at 4 °C for the following procedures. The medium

was separated by centrifugation at 4 °C at 8,000 g for 5 minutes. After pouring off the medium, the pellets were suspended with residual liquid and flash frozen in liquid nitrogen. The 80/20 methanol/water (%v/v) was pre-cooled to -80 °C for the extraction of intracellular metabolites. The 600 μL of pre-cooled 80/20 methanol/water (%v/v) were added to each cell pellet and mixed by vortexing. The mixture was transferred to 1.5 mL eppendorf tube and incubated on dry ice for 15 minutes. The cells were centrifuged at 4 °C at 14,000 rpm for 5 minutes. The supernatant was retrieved and placed into a new 1.5 mL eppendorf tube. The secondary extraction was mixed with 400 μL of pre-cooled 80/20 methanol/water (%v/v), mixed by vortexing and incubated for 15 minutes on dry ice. The solution was centrifuged at 4 °C at 14,000 rpm for 5 minutes to remove the pellet and combine with the first supernatant. Extraction was repeated with another 400 μL of pre-cooled methanol-water and final extracted supernatant gave a total volume of 1,400 μL. All uniformly labeled biomass extracts were combined into a single pool, vortexed, and 500 μL was aliquoted into new eppendorf tubes. The samples were stored at - 80 °C until they were ready for MS analysis [43].

2.3.2 Metabolic labeling for *M. thermoacetica*

To compare metabolites between pure *M. thermoacetica* culture and *Moorella* with *G. sulfurreducens* filtered media, samples from five different time points were harvested: the first point from lag phase, three points from exponential phases, and the last one from early stationary phase. In addition, cells were cultured both in yeast extract and in minimal media. Conical tubes of 50 mL were prepared with predetermined amount

of quenching solution (80/20 methanol/water (%v/v)) with 25 μL of internal standard samples prepared in the earlier steps. The amount of quenching solution was 5 times the volume of sample collected. The conical tube was weighed and stored in the - 80 °C. When the cell reached each phase, predetermined amount of culture volume was transferred into the 50 mL conical tube. In addition, another sample was collected and predetermined amount of cultures was filtered through 0.22 μm PVDF membrane as extracellular metabolite samples. For the analysis, 5.0 mL of culture volume was collected for OD_{600} less than 1.0, 3.0 mL of culture volume for OD_{600} between 0.2 to 0.3, 2.0 mL of culture volume for OD_{600} between 0.3 to 0.4, 1.5 mL of culture volume for OD_{600} between 0.5 to 0.7, and 1.0 mL of culture volume for OD_{600} of 0.8 or above. The limits of detection were estimated to be 50 to 0.5 mg dry cell biomass [42]. After all the samples were collected, the conical tubes were cooled for 5 minutes in a bucket of crushed ice. All samples were kept at 4 °C during the extraction procedure. Each of the 50 mL conical tube's weight was measured to determine the exact amount of volume sampled. To separate supernatant from cell pellets, 50 mL conical tubes were centrifuged at 4 °C with the speed of 13,200 rpm for 5 minutes. Each supernatant was saved into a new eppendorf tube and 200 μL of pre-cooled 80/20 methanol/water (%v/v) was added to the pellet, mixed with vortexing. The mixture was transferred to 1.5 mL eppendorf tube and incubated for 15 minutes on dry ice. The solution was centrifuged at 13,200 rpm for 5 minutes at 4 °C to separate supernatant, and the supernatant was saved with the first extraction. The 200 μL of pre-cooled 80/20 methanol/water (%v/v) was added to the pellet, mixed with vortexing, and incubated on dry ice for 15 minutes. The solution was

centrifuged at 13,200 rpm for 5 minutes at 4 °C to separate supernatant and the supernatant was saved with the pervious extraction. This process was repeated and final extraction gave a total volume of quenching solution volume (5 times cultured cells) plus 600 μL from the extraction. The extracted solutions were stored at - 80 °C until further procedures. Rotorvap or Speedvac were used to concentrate all the samples. The temperature was set to 37 to 40 °C and vacuum was used to decrease concentration time. For the Speedvac, all samples were aliquoted into 1.5 mL eppendorf tubes to decrease concentration time. Once all samples were completely dried, 200 μL of HPLC-MS grade water was added to one of the aliquots of the samples and vortexed. The next aliquot of dry mass was resolubilized using the same solution. This process was repeated until all aliquots of samples were reconstituted into a single aliquot. The previous steps were repeated for all samples to collect the dry mass and samples were store at - 80 °C for MS analysis. Targeted metabolomics analysis was performed with AB SCIEX QTRAP 5500 LC/MS/MS system.

2.4 Transcriptomic profiling of *M. thermoacetica* using RNA-seq

Total RNA was isolated from *M. thermoacetica* and *M. thermoacetica* with filtered *G. sulfurreducens* cells using phenol chloroform. Residual DNA was removed with a 30 minute DNAse I digestion at 37 °C (Qiagen) followed by purification with the RNeasy Mini kit (Qiagen). A total 2.5 μg of RNA was treated with the Gram-Positive RiboZero kit (Epicentre). Paired end, strand specific RNA sequencing was performed

using a variation of the dUTP method outlined in [44], [45] with the following changes. The 100 ng of rRNA subtracted from RNA was fragmented with RNA fragmentation reagents (Ambion) for 3 minutes at 70 °C. First strand synthesis was primed using random hexamers (Invitrogen). Downstream library construction was performed as previously described [46]. Final libraries were quantified using High Sensitivity DNA chips for the Bioanalyzer (Agilent). A total of 8 pM of library was loaded onto the Miseq (Illumina) for sequencing as a paired-end 31 run.

The obtained RNA-seq reads were mapped into the *M. thermoacetica* genome sequence using the short-read aligner Bowtie (http://bowtie-bio.sourceforge.net) [47] with 2 mismatches allowed per read alignment. To estimate transcript abundances, FPKM values were calculated using the tool Cufflinks (http://cufflinks.cbcb.umd.edu/) [48] with appropriate parameters set for the strand-specific library type and upper-quartile normalization. Differential expression analysis was performed between *M. thermoacetica* alone and *M. thermoacetica* cultured in the presence of *G. sulfurreducens* using the Cuffdiff feature of the cufflinks package. Genes with greater than 2 times of change in expression value and false discovery rate (FDR) of < 0.05 were considered to be differentially expressed.

2.5 Transformation

2.5.1 Media, strains, and cultivation conditions

M. thermoacetica cells were grown in the yeast extract media described above. The media for *M. thermoacetica* contained (in grams per liter): glucose, 18.0; yeast extract, 5.0; $(NH_4)_2SO_4$ 1.3; $MgCl_2$, 0.75; $CaCl_2.\cdot 2H_2O$, 0.013; $FeSO_4\cdot 7H_2O$, 0.125% solution 1.0 mL, trace mineral, and vitamin solution. The 1g of gelrite (Gelzan, CAS Number 71010-52-1) was dissolved per 100 mL media for 1% gelrite agar [49]. The medium was autoclaved at liquid cycle and glucose was supplemented after the autoclave cycle. For selection of transformants, the media additionally contained 25 μg/mL chloramphenicol for a selection marker. Agar was poured inside the anoxic chamber right after the autoclave; a big media bottle held 30 mL of agar and a small media bottle held 12 mL. Agar plates were prepared within a day of plating to prevent degradation of antibiotics as much as possible.

Plasmid pJIR418 was used as a test plasmid for the transformation of *M. thermoacetica*. The plasmid was introduced by Sloan *et al*. as a new construction of *C. perfringens-E. coli* shuttle vector [50]. pJIR418 contained chloramphenicol resistance and the chloramphenicol was fairly stable at high temperature such as 65 °C. Degradation constant for chloramphenicol in anaerobic medium was – 0.59 at 72 °C and +0.22 at 50 °C when the pH was 7.3 [51]. The plasmid contained the origin of replication of the *C. perfringens* plasmid pIP404. It contained the following restriction site (position in bp): EcoRI, 1; HindIII, 57; Pvul, 175; FspI, 195; NarI, 216; Tth111I, 397; SpeI, 3,235; NheI,

3549; NaeI, 3,653; HpaI, 42,06; EcoRV, 4,723; SmaII, 4,984; AvaII, 5,059; ScaI, 5,850 (Figure 2.2) [52].

2.5.2 Transformation protocol of *M. thermoacetica* with pJIR 418

All procedures, from the preparation of cultures to electro-transformation, were carried out inside an anaerobic chamber. *M. thermoacetica* cells were grown in serum bottle with 100 mL yeast extract medium at 65 °C without supplementation of chloramphenicol. Final concentration of 10 mM glucose was added as a carbon source during inoculation. After inoculation, cultures were allowed to grow for 30 to 50 hours until they reached mid-log phase (OD_{600} 0.2-0.4). Cells were harvested by centrifugation in 50 mL falcon tubes at 10,000 xg for 10 min at 4 °C, washed twice in an anaerobic chamber with 10 mL ice-cold electroporation buffer containing 10% (v/v) glycerol, and resuspended in the same buffer to a final volume of 200 μL. Plasmid DNA (1.273 ng) was added to 100 μL cell suspension pre-chilled in an electroporation cuvette (Bio-Rad, 2 mm electrode gap). Bacteria were pulsed once at 25 kV using a Bio-Rad Gene Pulser and the resulting pulse duration was 5-6 ms. Immediately after electroporation, the cell suspension was transferred to pre-warmed fresh medium (5 mL) in a small serum bottle and incubated at 65 °C for 6-8 hours. Plates harboring pJIR418 were incubated at 65 °C for 5-10 days in anaerobic media bottles [53], [54].

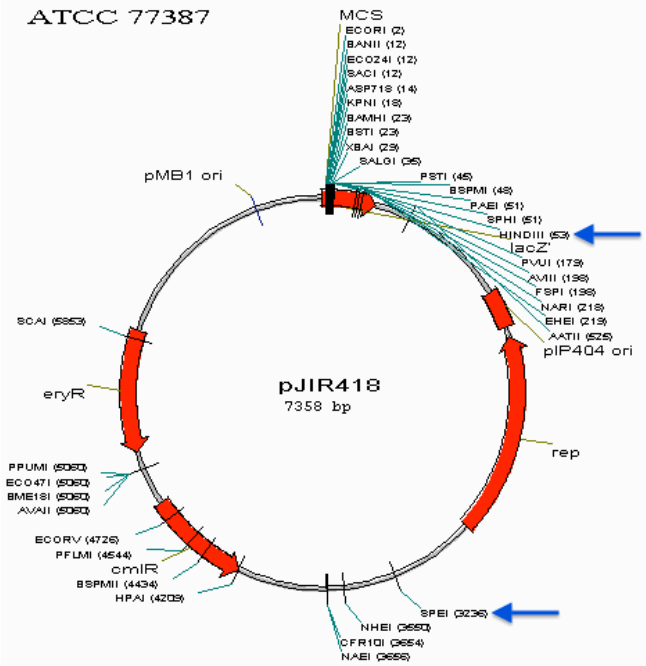


Figure 2.2 Construction of *C. perfringens-E. coli* shuttle plasmid pJIR418 (ATCC 77387). Two blue arrows indicate the restriction site.

2.5.3. Verification of plasmid

Transformation of *M. thermoacetica* was carried out by modifying *E. coli* electro-transformation protocol and with plasmid pJIR418. Transformants election was based on chloramphenicole resistance. The cells of *M. thermoacetica* were transformed with plasmid pJIR418 and Cm resistant colonies were obtained on the plates containing Cm, while no colony was formed on the same plates from the cells electroporated without plasmid. Single colonies of transformed cells were picked and transferred into 5 mL of yeast extract media with 5 μg/mL Cm [53], [54]. Transformants from 5 mL culture were transferred again to new media without antibiotics to obtain dense culture. The presence of pJIR418 in Cm^r transformants of *M. thermoacetica* was verified with plasmid isolation. Plasmid DNA was extracted via QIAprep Spin Miniprep Kit and the liquid culture

inoculated with single colony of transformed cell was used as conformation of electro-transformation. Restriction digestions of transformants were performed using HindIII and SpeI after plasmid isolation. For the restriction digestion reaction, 150-300 ng of isolated plasmid DNA, NEBuffer 2, and 100 μg/mL Bovine Serum Albumin were used. Reaction with only HindIII was expected to have 7,358 bp fragment and reaction with both HindIII and SpeI was expected to have two fragments with the size of 4,220 and 3,138 bp. Restriction digest of stock plasmid solution with concentration of 136.3 ng/μL was carried out as a positive control.

Detection of plasmid in Cm^r transformants of *M. thermoacetica* was performed by PCR using isolated plasmid as templates and 5'-TTGAGTGTGCAAGAGCAACC–3' (primer 1) and 5'-GCAGAGCGAGGTATGTAGGC-3' (primer 2) with the expected PCR product size of 1,118 bp. Primers were designed using Primer 3 software (http://frodo.wi.mit.edu/cgi-bin/primer3-web-cgi-bin-0.4.0/primer3_results.cgi) [55]. *M. thermoacetica* specific primers 5'- TGGTCGGCCACACTGGGACT-3' (primer 1) and 5'-AGGCCTTCATCGCTCACGCG-3' (primer 2) were used to validate all transformants *M. thermoacetica*. The expected size of *M. thermoacetica* specific PCR product was 117 bp.

3. Results

3.1 *M. thermoacetica* with secondary organisms

The physiology of *M. thermoacetica* was examined when it was cocultured with other organisms such as *Geobacter sulfurreducens, Thermotoga maritima, Clostridium ljungdhalii, Sporomusa ovata, Methanosarcina barkeri,* and *Methanococcus maripaludis*. In the first stage, 6 organisms were added as a secondary organism to *M. thermoacetica* during its growth. The growth curves were established by plotting optical density versus time. From previous experiments, no growth of monocultures of these secondary organisms was observed in *M. thermoacetica* medium at 65 °C. From these results, the assumption was made that the supplemented secondary organisms might not grow with *M. thermoacetica*.

Among the added secondary organisms, only the stationary phase *G. sulfurreducens* cells showed a positive effect in both growth and growth rate of *M. thermoacetica*. The growth curve comparison showed significant growth advantages when *M. thermoacetica* was supplemented with *G. sulfurreducens* (Figure 3.1). There was some positive effect observed with the supplementation of *T. maritima* to *M. thermoacetica*, but the effect was not reproducible. Further examination was conducted to discover the interaction and effect of *G. sulfurreducens* on the growth of *M. thermoacetica*.

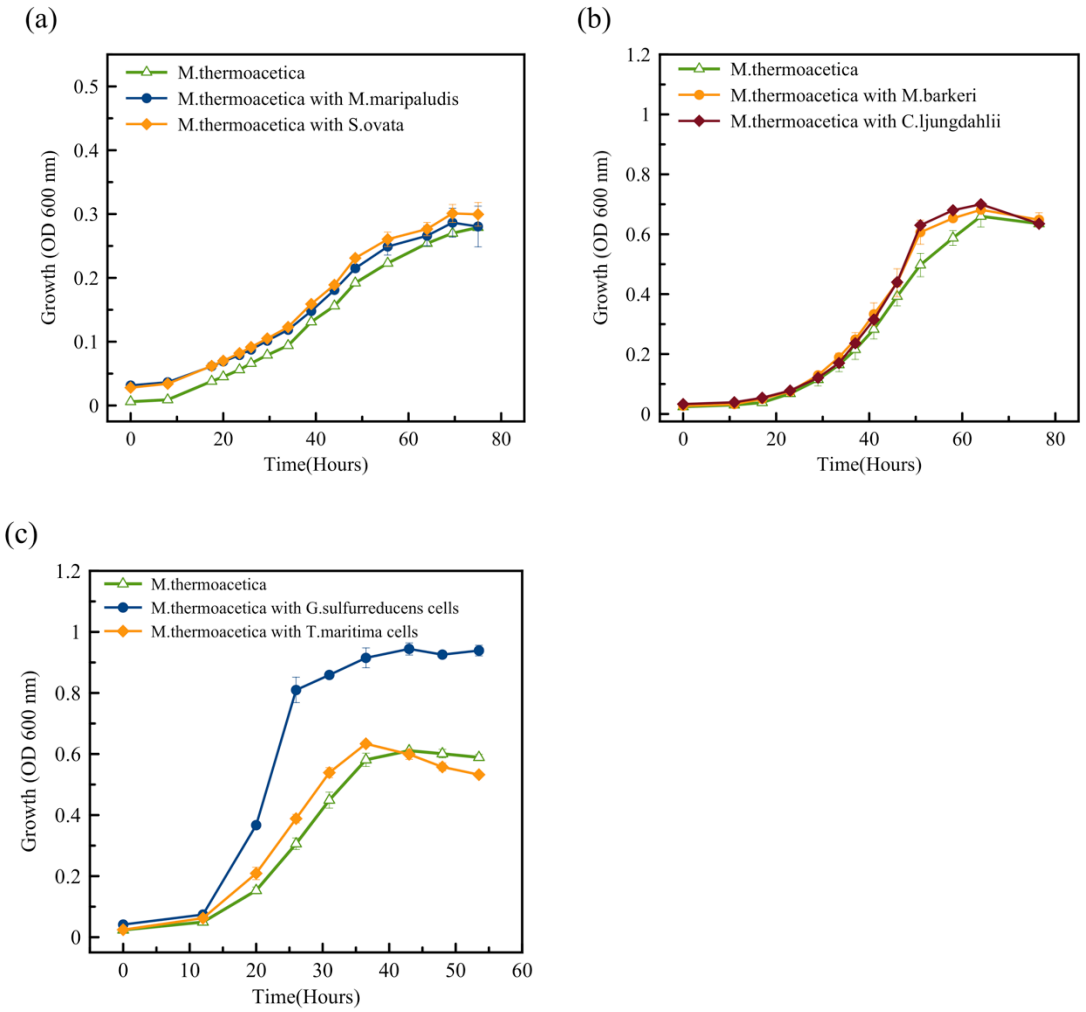


Figure 3.1 Effect of secondary organism on *M. thermoacetica* grown in the yeast extract medium. (a) Supplemented secondary organisms were *M. maripaludis* and *S. ovata*. (b) Supplemented secondary organisms were *M. barkeri* and *C. ljungdhalii*. (c) Supplemented secondary organisms were *G. sulfurreducens* and *T. maritima*. Values are the means of triplicate cultures.

3.2 *M. thermoacetica* with *G. sulfurreducens*

M. thermoacetica growth was enhanced with supplementation of *G. sulfurreducens* cells. To interpret the cause of the amplified growth of *M. thermoacetica*, different components of *G. sulfurreducens* were examined: whole cell, pellet, filtered *G. sulfurreducens* media, and fresh water media. The experiment was performed in both yeast extract media and minimal media for *M. thermoacetica*. Fresh water media without the *G. sulfurreducens* cells did not change the growth of *M. thermoacetica*, which indicates that the growth advantage comes from the cell itself but not from minerals or vitamins transferred from the media (Fig. 3.3 c).

When *M. thermoacetica* was grown in yeast extract media, the pellet and the filtered media had similar effect (Fig. 3.3 a and b). On the other hand, the filtered media showed more significant effect when the cultures were grown in the minimal media (Fig 3.3 c). Supplementation of *G. sulfurreducens* filtered media without the cells resulted the most significant advantage on *M. thermoacetica* growth.

Glucose consumption and acetate production were observed by HPLC analysis. The supplementation of *Geobacter* helped to utilize glucose twice faster than pure culture of *M. thermoacetica*. In the undefined media, the pure *M. thermoacetica* culture produced maximum 20.09 mM of acetate from 6.81 mM glucose. *M. thermoacetica* supplemented with filtered media and with *G. sulfurreducens* cells produced maximum of 26.52 and 26.58 mM of acetate, respectively, from 9.0 mM of glucose. The conversion ratio from glucose to acetate was 2.9. However, *M. thermoacetica* with *G. sulfurreducens* pellet and filtered media utilized 10 mM of glucose within 31 hours, while monoculture of *M.*

thermoacetica required more than 53.5 hours to consume all the glucose. The acetate production rate was 7.71 when *M. thermoacetica* was cultured alone, while the rate increased to 10.88 with *G. sulfurreducens* cells and 9.52 with filtered media of *Geobacter*. Culturing *M. thermoacetica* with cells or filtered media of *G. sulfurreducens* showed faster utilization of glucose and twice higher production of biomass, while the production of hydrogen decreased in half.

In order to find out whether the positive effect of *G. sulfurreducens* relied on the viability of the cells, *G. sulfurreducens* precultures were boiled at 95 °C for 15 minutes before inoculation. The entire *G. sulfurreducens* cell lysate was separated into pellet and supernatant. The separated pellet and supernatant were added to the *M. thermoacetica* culture. The boiling of the *G. sulfurreducens* still had positive effect on *M. thermoacetica* growth but the advantage was much less compared to live cells (Figure 3.2). It is possible that the heat destroyed proteins or amino acids that were part of compounds which allowed the boost to the growth of *M. thermoacetica*.

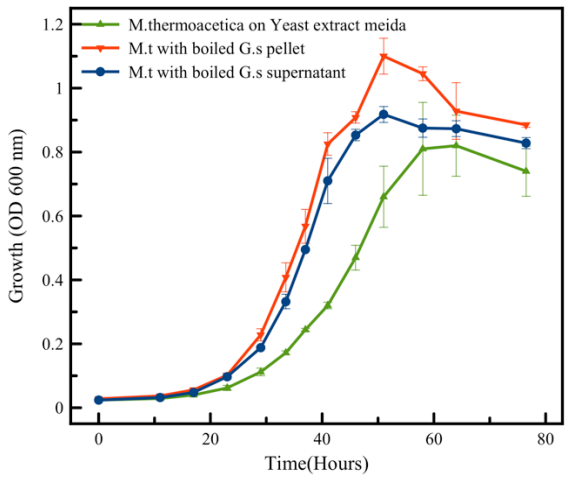


Figure 3.2 Growth curves of *M. thermoacetica* with boiled *G. sulfurreducens* pellet and supernatant. Values are the means of triplicate cultures.

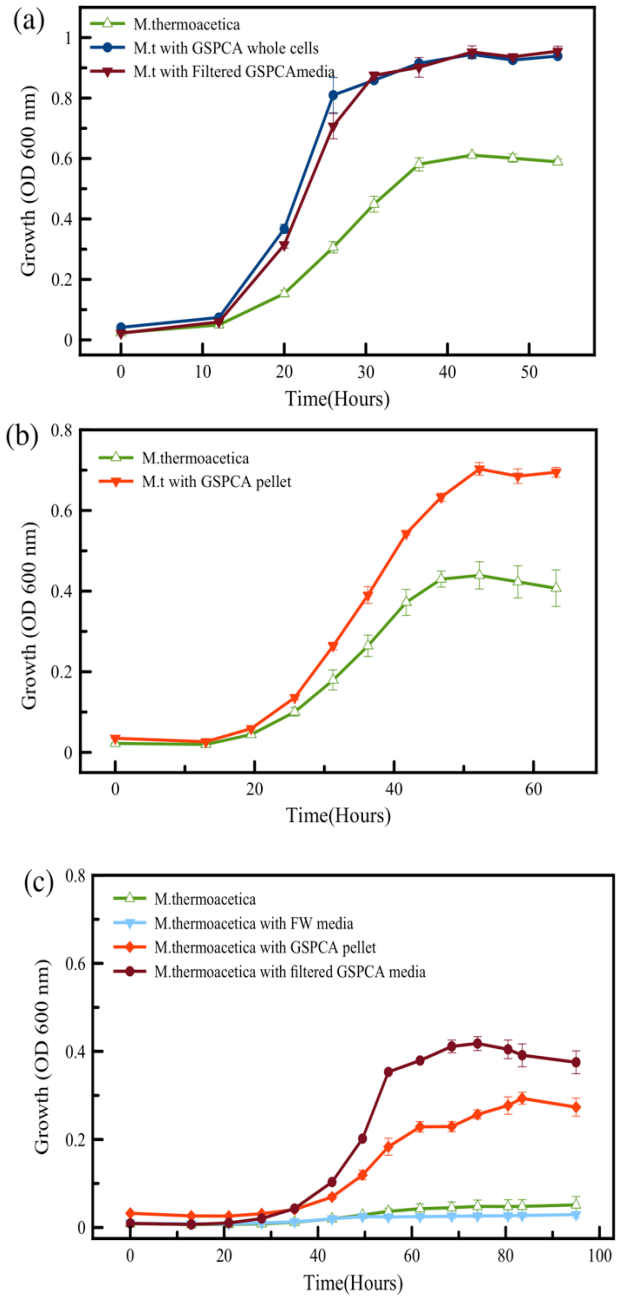


Figure 3.3 Effect of *G. sulfurreducens* on *M. thermoacetica* grown in the yeast extract medium. (a) Supplemented components of *G. sulfurreducens* were whole cell, filtered medium. (b) Pellet. (c) Effect of *G. sulfurreducens* on *M. thermoacetica* grown in the minimal medium. Supplemented components of *G. sulfurreducens* were pellet, filtered medium and fresh water media. Values are the means of triplicate cultures.

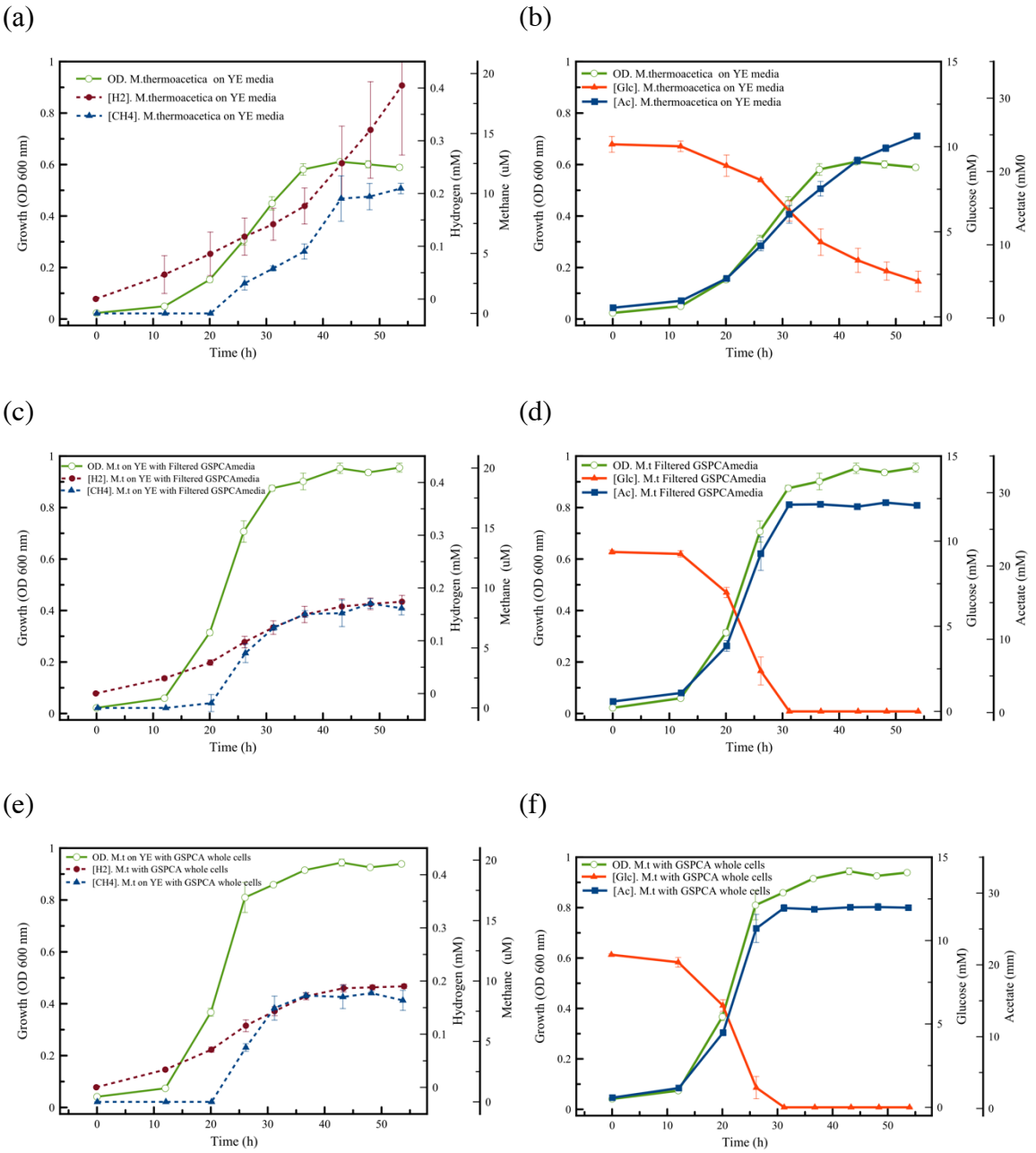


Figure 3.4 GC and HPLC analysis of *M. thermoacetica* in yeast extract media with different *G. sulfurreducens* components. (a) GC analysis of *M. thermoacetica* in yeast extract media, (b) HPLC analysis of *M. thermoacetica* in yeast extract media, (c) GC analysis of *M. thermoacetica* with *G. sulfurreducens* filtered media, (d) HPLC analysis of *M. thermoacetica* with *G. sulfurreducens* filtered media, (e) GC analysis of *M. thermoacetica* with *G. sulfurreducens* whole cells, (f) HPLC analysis of *M. thermoacetica* with *G. sulfurreducens* whole cells. Values are the means of triplicate cultures.

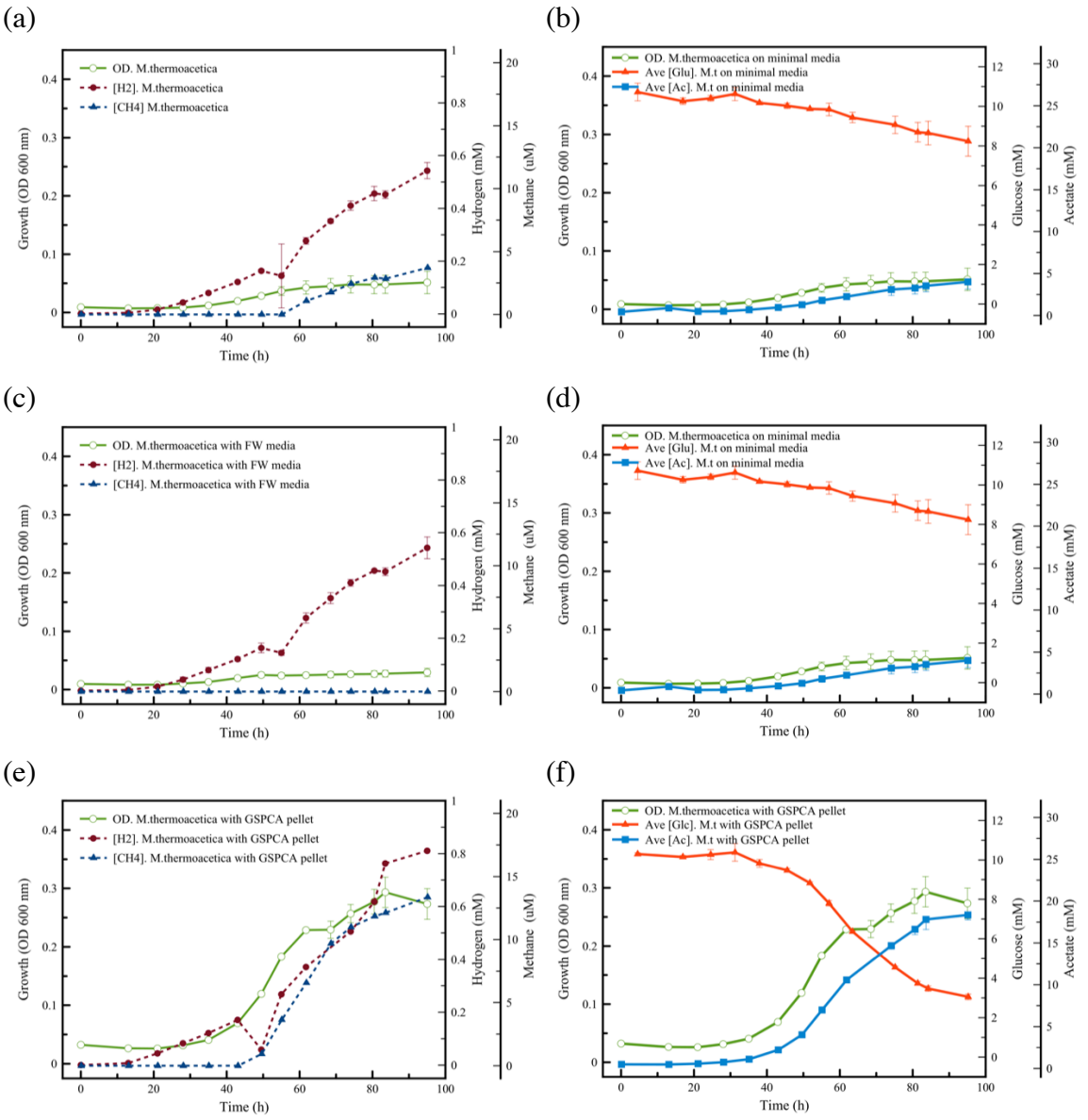
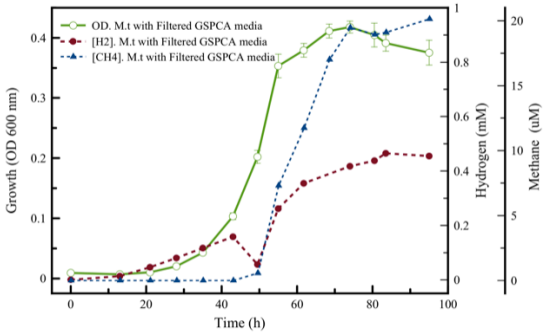


Figure 3.5 GC and HPLC analysis of *M. thermoacetica* in minimal media with different *G. sulfurreducens* components. (a) GC analysis of *M. thermoacetica* in minimal media (b) HPLC analysis of *M. thermoacetica*, (c) GC analysis of *M. thermoacetica* with FW media, (d) HPLC analysis of *M. thermoacetica* with FW media, (e) GC analysis of *M. thermoacetica* with *G. sulfurreducens* pellet, (f) HPLC analysis of *M. thermoacetica* with *G. sulfurreducens* pellet, (g) GC analysis of *M. thermoacetica* with *G. sulfurreducens* filtered media, (h) HPLC analysis of *M. thermoacetica* with *G. sulfurreducens* filtered media. Values are the means of triplicate cultures.

(g)



(h)

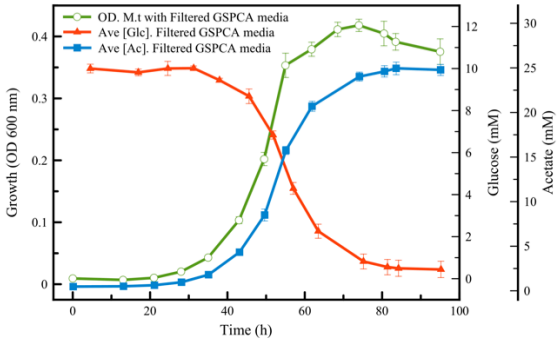


Figure 3.5 Continued.

The GC analysis shows the supplementation of the *G. sulfurreducens* cells resulted in a low hydrogen production from *M. thermoacetica* (Figure 3.4 and 3.5). Specific hydrogen production rate at early exponential phase was compared between samples and media in Table 1. With the presence of the yeast extract in media, the hydrogen production rate of was decreased in half when it was supplemented with *G. sulfurreducens*. In the minimal media, the hydrogen production rate was significantly decreased with the supplementation. The change of hydrogen production rate shows the inverse proportionality to the growth advantage of *M. thermoacetica*.

Table 1 Specific hydrogen production rate (mM/gDCW/h) of *M. thermoacetica* with *G. sulfurreducens*. In parentheses, OD_{600} values are presented.

Hydrogen production rate	***M. thermoacetica***	***M. thermoacetica* with *G.s* cells**	***M. thermoacetica* with filtered *G.s* media**
Undefined media (YE)	0.0655 (0.611)	0.0399 (0.957)	0.0346 (0.955)
Defined media (minimal)	0.6712 (0.027)	0.3340 (0.293)	0.0661 (0.391)

3.3 *M. thermoacetica* with electron acceptors

The reduced hydrogen production with supplementation of *G. sulfurreducens* cells leads to two potential hypotheses: one is that some electrons are deposited to the secondary reservoir instead of being used for the production of hydrogen; the other is that *M. thermoacetica* uses the hydrogen produced during fermentation for respiration. Cytochrome C, an abundant electron acceptor in *G. sulfurreducens,* was supplemented to observe whether *M. thermoacetica* could deposit electrons to extracellular electron acceptors. Horse cytochrome C with final concentration of 20 μg/L was supplemented to *Moorella* media for this experiment. Supplementation of cytochrome C did not affect *M. thermoacetica* growth in both yeast extract and minimal media conditions (Figure 3.6).

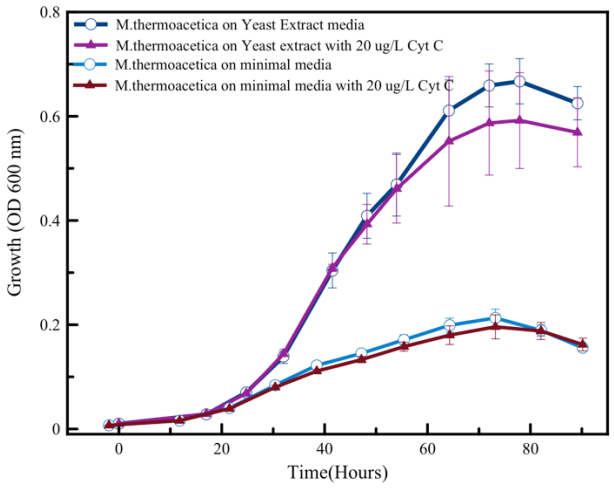


Figure 3.6 Growth curves of *M. thermoacetica* grown in yeast extract medium and minimal media with supplementation of cytochrome C (20 μg/L).

3.4 Metabolomics results

Metabolites were analyzed by mass spectrometer in order to find the compound responsible for the growth advantage of *M. thermoacetica*. From LCMS analysis, total 138 compounds were detected as metabolites of *M. thermoacetica*. Each compound was plotted with bar chart and heatmap to observe the change of metabolites in samples MetATT [56]. Bar charts represent peak height ratios of the compounds to compare the changes of metabolites over time. The first four bar sets represent intracellular metabolites and the last four ones extracellular metabolites. Each set consists of four samples: monoculture of *M. thermoacetica* in undefined medium (yeast extract medium), *M. thermoacetica* with *G. sulfurreducens* filtered media in undefined medium, monoculture of *M. thermoacetica* in defined medium (minimal medium), and *M. thermoacetica* with *G. sulfurreducens* filtered media in defined medium. Each sample was collected at five different time points: the first point was right after inoculation, three points during exponential growth, and the last point at early stationary phase. The consumption of extracellular metabolites and the production of intracellular metabolites at each time point would provide insight into the metabolic activity of the cells during their growth.

The cells were filtered out for extracellular metabolite samples, and intracellular metabolites were calculated based on the difference between broth and filtered samples. The intracellular metabolite was represented as zero in case the endogenous components was not detected as a peak or in case the internal peak height ratios and filtered peak height ratio were equal or greater than the broth height peak ratio. The metabolites were

categorized into 13 classes of molecules (Figure 8) and majority of metabolites were amino acids, nucleosides, and organic acids.

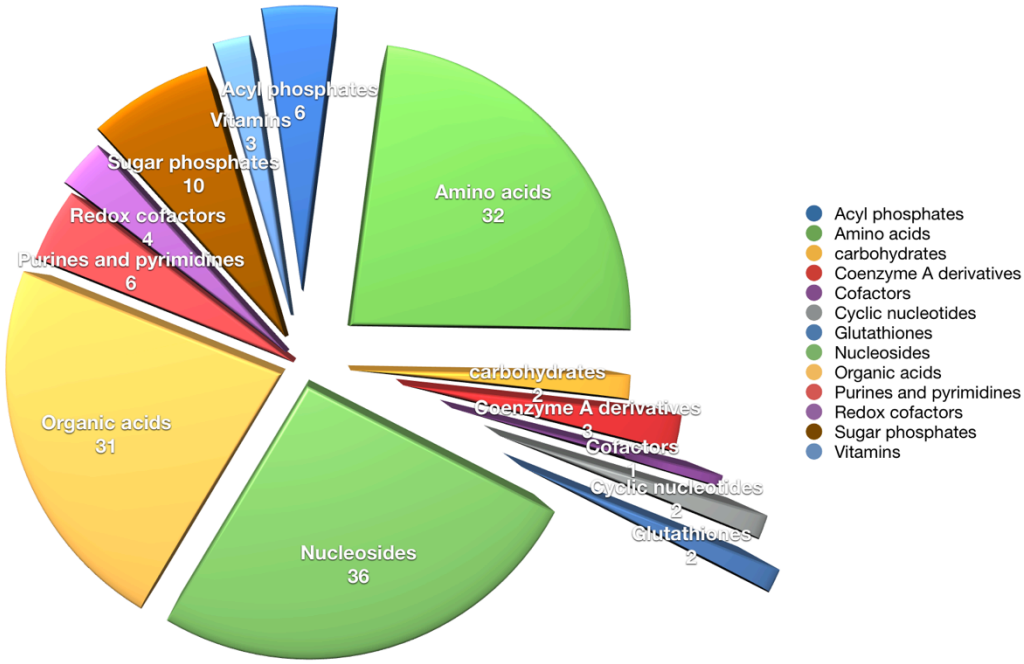


Figure 3.7 Different classes of metabolites according to their chemical properties. The numbers indicate numbers of metabolites found in *M. thermoacetica*, both intracellular and extracellular metabolites.

Visualization of two-way heatmap was acquired with the program MetATT (Metabolomics tool for Analyzing Two-factor and Time-series data) from the webpage of http://metatt.metabolomics.ca/MetATT/faces/Home.jsp [56]. Pearson distance mapping was used to generate the heatmaps (Figure 3.8). Extra- and intracellular metabolites were arranged to compare different samples with each same time point. Five time points were generated from lag phase to stationary phase.

(a)

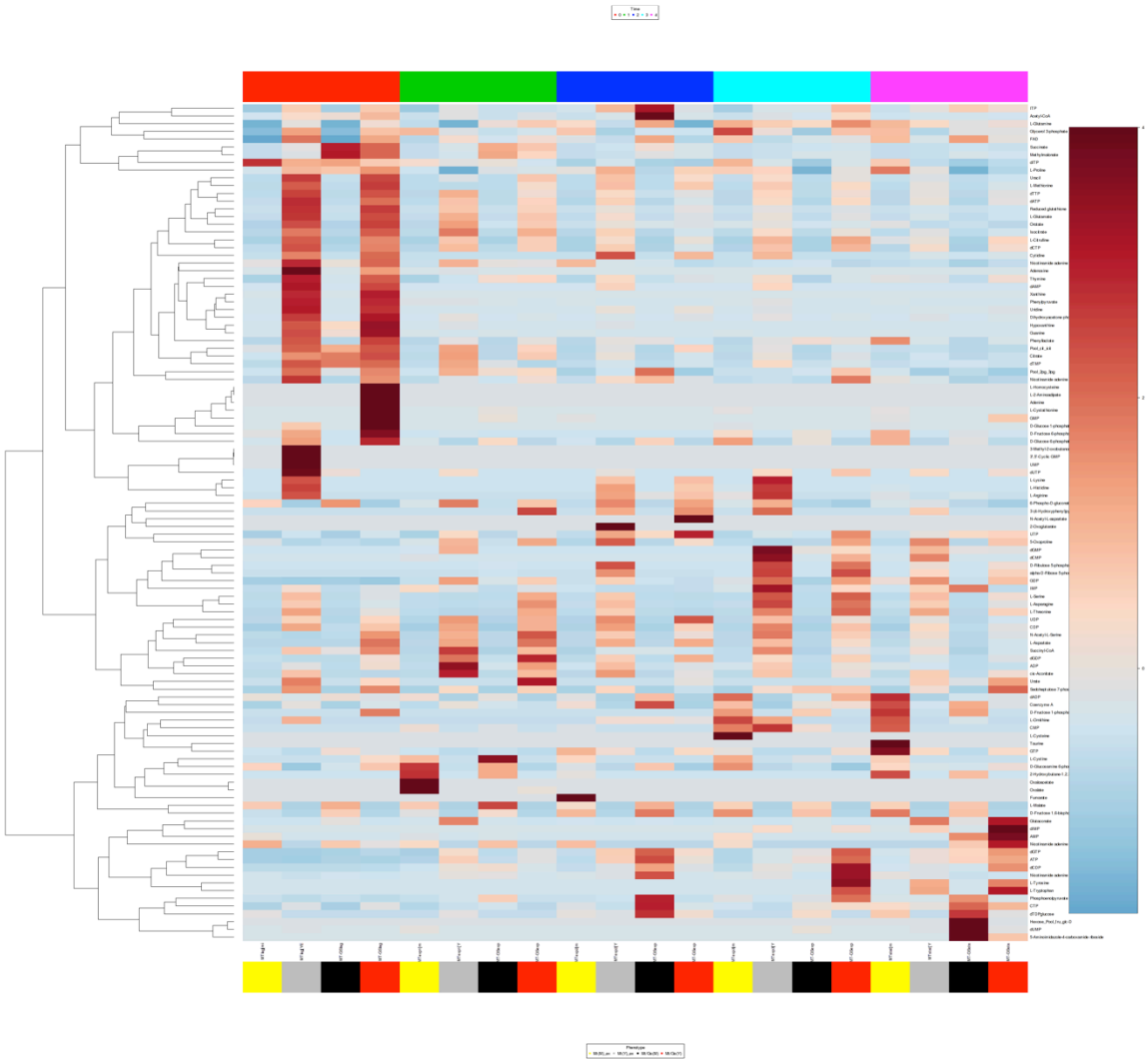


Figure 3.8 Heatmaps of metabolites of *M. thermoacetica* generated by MetATT. Pearson's correlation coefficient (PCC) was used for distance measurement. (a) extracellular metabolites, (b) intracellular metabolites.

(b)

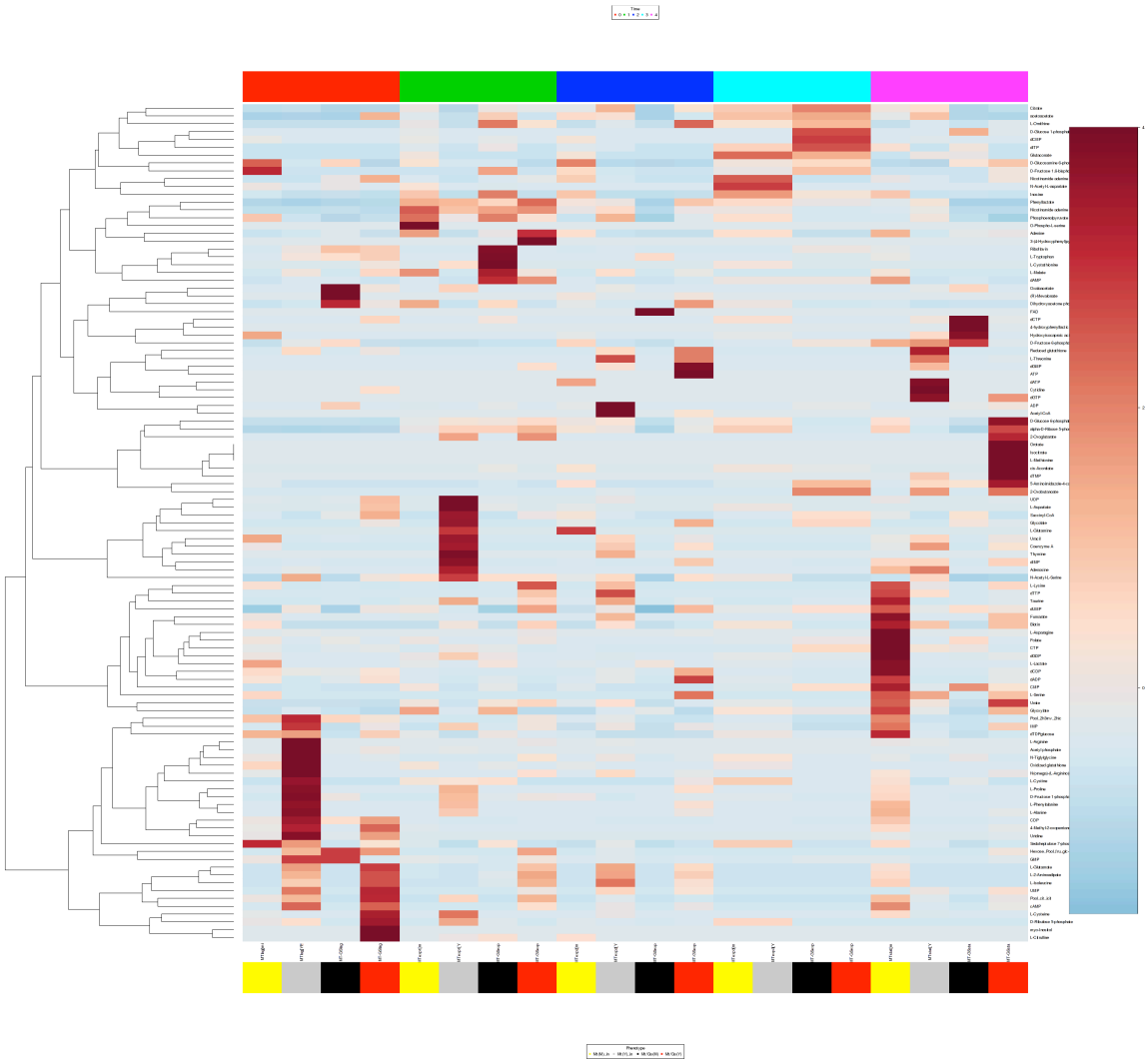


Figure 3.8 continued.

Extracellular metabolites in lag phase were analyzed to identify the compounds transferred from *G. sulfurreducens* to *M. thermoacetica*. By comparing the lag phase samples of yeast extract medium and minimal medium samples, 24 compounds were detected to be transferred from yeast extract as extracellular metabolites. As for the compounds, coenzyme A derivatives, nucleosides, organic acids, sugar phosphates, acyl

phosphates, purines and pyrimidines, and amino acids were supplemented from yeast extract: acetyl-CoA as coenzyme A derivatives; adenosine, CDP, dTMP, dTTP, GDP as nucleosides; citrate, isicitrate, orotate, phenyllacetate, and phenylpyruvate as organic acids; D-fructose 6-phosphate, D-glucose 1-phosphate, D-glucose 6-phosphate, and sedoheptulose 7-phosphate as sugar phosphates; dihydroxyacetone phosphate as acyl phosphates; guanine, hypoxanthine, uracil, and xanthine as purines and pyrimidines; and L-citrulline, L-glutamate (Figure 3.9 a), L-methionine, and L-proline as amino acids.

In addition, 13 compounds were suspected to be transferred from *G. sulfurreducens* filtered media with comparison of lag phase samples between monoculture and cultures supplemented with the filtered media. Majority of these compounds were also supplemented from both yeast extract media and *Geobacter* filtered media. They contained components from cell debris, for example, acetyl-CoA, citrate, isocitrate, dTMP, guanine, hypoxanthine, L-proline, uracil, and xanthine. Following four compounds were supplemented to *M. thermoacetica* from *Geobacter* filtered media: GTP, methylmalonate (Figure 3.9 b), succinate, and succinyl-CoA. As time goes from lag to stationary phase, the extracellular metabolites decrease, which indicates consumption of the metabolites by cells.

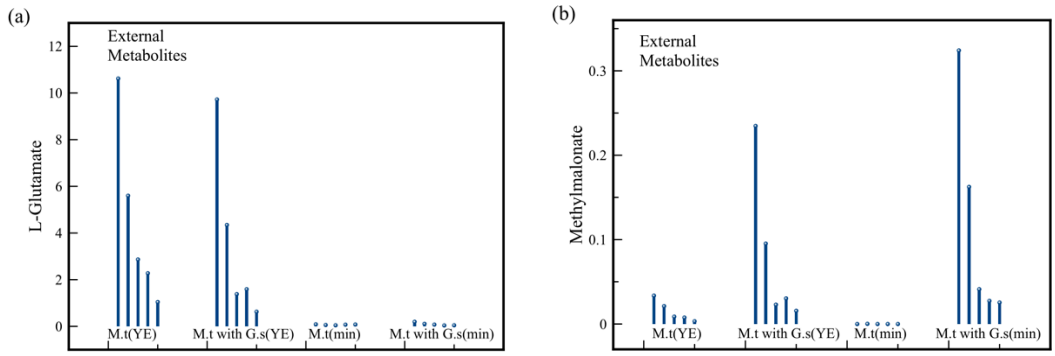


Figure 3.9 Metabolomics data for L-glutamate and methylmalonate. The y-axis is the peak height ratios of between the succinate in the samples and the internal standard from *E. coli*. Each sample has five time points of samples collection.

The previous HPLC and MS analysis show that succinate and acetate were transferred from *G. sulfurreducens* preculture to *M. thermoacetica* during inoculation. The succinate was detected by MS analysis. The extra cellular metabolite peak intensity shows that the succinate was consumed by *Moorella (*Figure 3.10 a). The succinate and acetate with final concentration of 1.6 mM and 0.5 mM, respectively, were supplemented to *M. thermoacetica* culture, but no growth advantage was observed (Figure 3.10 b). The concentration of succinate and acetate were determined by HPLC analysis. Even though the succinate was consumed by cells, which depicted from the decrease of the concentration over time, the result of growth curve showed no growth advantages to *M. thermoacetica*. The succinate alone was not responsible for the two times production of the biomass that we observed from *G. sulfurreducens* supplementation.

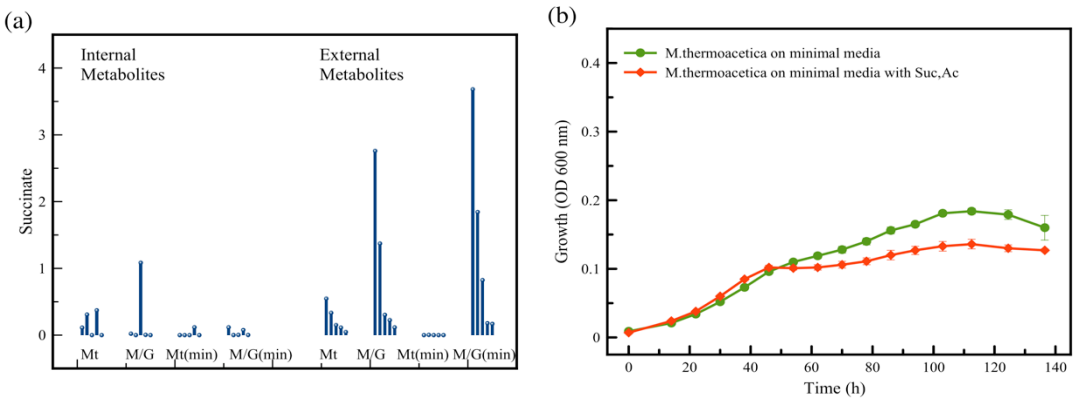


Figure 3.10 (a)Metabolomics data for succinate. The y-axes are the peak height ratios of between the succinate in the samples and the internal standard from *E. coli*. Each sample has five points which were collected at different time course and it is divided as intracellular and extracellular metabolites. Intracellular metabolites were calculated from the difference between the broth and the filtered samples. (b) Growth curves of *M. thermoacetica* with supplementation of 1.6 mM succinate and 0.5 mM acetate.

3.5 RNA sequencing data

The Genetic profiles of *M. thermoacetica* and *M. thermoacetica* with filtered *G. sulfurreducens* media were analyzed. Total 2,464 genes were isolated. The gene expressions were compared using log 2 fold change between monoculture and coculture samples. The supplementation of the filtered media resulted in down-regulation of 64 genes and up-regulation of 31 genes. Among them, some of genes were hypothetical proteins, 17 genes from down-regulated and 6 from up-regulated (Table A1). Large numbers of down-regulated genes were related to sporulation (Moth_1530-1535) and NADH dehydrogenase operon contained genes encoding subunits E, F, and G of the NADH dehydrogenase complex (Moth_1883-88) [57]. The down-regulation of sporulation shows that cells were actively growing and suppressing dormant phase. The three major regions that were up-regulated with *G. sulfurreducens* were genes related to molybdopterin biosynthesis (Moth_0721-0724), purine biosynthesis (Moth_2044-2048), and Arginine and proline metabolism as well as ornithine biosynthesis

(Moth_2281-2290). In order to process amino acids and secondary metabolites transferred from *Geobacter*, *M. thermoacetica* up-regulated these genes to support its growth.

3.6 Transformation of *M. thermoacetica* and verification of transformants

The development of a genetic transformation system is needed to examine the gene function and regulation of *M. thermoacetica*. Antibiotic-resistant colonies were observed after 6 days of plating. The incidence of such colonies was rarely detected. The transformant was recovered twice in 5 mL media without antibiotics. Each recovery phase was incubated overnight. As a control, cells were washed and plated on 1% gelrite plate with chloramphenicol before electroporating with the plasmid. While antibiotic-resistant colonies were observed after 6 days of plating, no colonies were observed on the chloramphenicol plate that had cells without plasmid.

Detection of plasmid pJIR418 in Cm^r transformants of *M. thermoacetica* was performed by PCR after the extraction of plasmid. As one of the verifications of the transformants, the plasmid extracted from the kit had the concentration of 37.3 ng/μL for the first liquid culture with Cm and the concentration of 76.3 ng/μL for the second one without chloramphenicol. NanoDrop was used to determine the concentrations of the isolated plasmid. The restriction digestion with stock plasmid with concentration of 136.3 ng/μL was also carried out as a positive control (Figure 3.11).

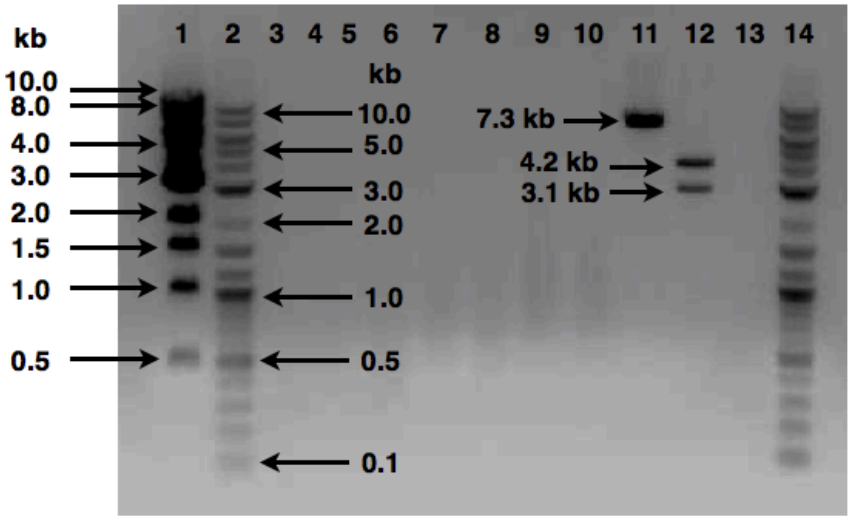


Figure 3.11 Agarose gel electrophoresis of restriction digestion of purified plasmid from transformants with HindIII and SpeI. Lane 1: 2-log DNA ladder, Lane 2 : 2-log DNA ladder, Lane 3; WT *M. thermoacetica* from 9/24/12 liquid culture with with HindIII , Lane 4; WT *M. thermoacetica* isolated from 9/24/12 liquid culture HindIII and SpeI, Lane 5; Purified transformant isolated from 9/24/12 with HindIII, Lane 6; Purified transformant isolated from 9/24/12 with HindIII and SpeI, Lane 7; WT *M. thermoacetica* washed with electroporation buffer from 9/29/12 with HindIII, Lane 8; WT *M. thermoacetica* washed with electroporation buffer from 9/29/12 with HindIII and SpeI, Lane 9; WT *M. thermoacetica* from 9/27/12 liquid culture with with HindIIILane 10; WT *M. thermoacetica* from 9/27/12 liquid culture with with HindIII and SpeI. Lane 11; pJIR 418 stock plasmid with with HindIII, Lane 12; pJIR 418 stock plasmid with pJIR418 specific primers, Lane 13; Empty lane,Lane 14; 2-log DNA ladder.

After restriction digest of plasmid, to further confirm transformation, PCR was carried out using primers specific to pJIR418 and fragments with the expected size of 1,118 bp (Figure 3.13). The primers were confirmed with stock plasmid solution with concentration of 136.3 ng/μL (lane 10 of Figure 3.12).

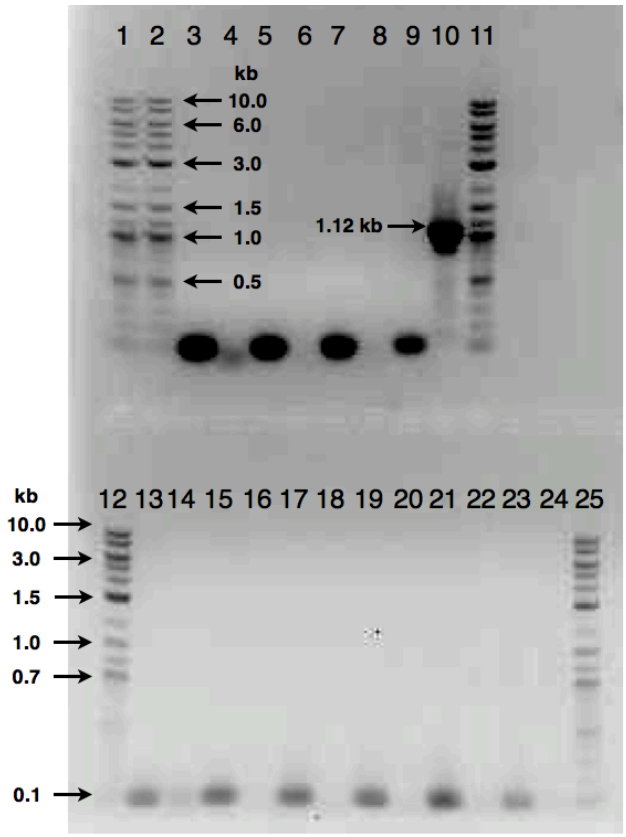


Figure 3.12 Agarose gel electrophoresis of PCR product. Band size of 1,100 bp is pJIR 418 specific PCR products and band size of 100 bp is M. thermoacetica specific PCR products. Lane 1: 2-log DNA ladder, Lane 2 : 2-log DNA ladder, Lane 3; WT *M. thermoacetica* from 9/24/12 liquid culture with *M. thermoacetica* specific primers, Lane 4; WT *M. thermoacetica* isolated from 9/24/12 liquid culture with pJIR418 specific primers, Lane 5; Purified transformant isolated from 9/24/12 *M. thermoacetica* specific primers, Lane 6; Purified transformant isolated from 9/24/12 with pJIR418 specific primers, Lane 7; WT *M. thermoacetica* washed with electroporation buffer from 9/29/12 *M. thermoacetica* specific primers, Lane 8; WT *M. thermoacetica* washed with electroporation buffer from 9/29/12 with pJIR418 specific primers, Lane 9; pJIR 418 stock plasmid with *M. thermoacetica* specific primers, Lane 10; pJIR 418 stock plasmid with pJIR418 specific primers, Lane 11; 2-log DNA ladder, Lane 12; 2-log DNA ladder, Lane 13; Transformants from isolated from 9/6/12 liquid culture with *M. thermoacetica* specific primers, Lane 14; Transformants from isolated from 9/6/12 liquid culture with pJIR418 specific primers, Lane 15; Purified transformants from isolated from 9/6/12 with *M. thermoacetica* specific primers, Lane 16; Purified transformants from isolated from 9/6/12 with pJIR418 specific primers, Lane 17; Transformants from isolated from 9/27/12 liquid culture #7 with *M. thermoacetica* specific primers, Lane 18; Transformants from isolated from 9/27/12 liquid culture #7 with pJIR418 specific primers, Lane19; Purified transformants from isolated from 9/27/12 liquid culture #7 with *M. thermoacetica* specific primers, Lane 20; Purified transformants from isolated from 9/27/12 liquid culture #7 with pJIR418 specific primers, Lane 21; Transformants from isolated from 9/27/12 liquid culture #8 with *M. thermoacetica* specific primers, Lane 22; Transformants from isolated from 9/27/12 liquid culture #8 with pJIR418 specific primers, Lane 23; Purified transformants from isolated from 9/27/12 liquid culture #8 with *M. thermoacetica* specific primers, Lane 24; Purified transformants from isolated from 9/27/12 liquid culture #8 with pJIR418 specific primers, Lane 25; 2-log DNA ladder.

To obtain higher concentration of plasmid from plasmid extraction, transformants were recovered in liquid media. PCR with *M. thermoacetica* specific primers and plasmid specific primers were performed to confirm the electro-transformation. *M. thermoacetica* specific PCR products were observed in all lanes at 100 bp, which confirms all the recovered cells were *M. thermoacetica*. But no plasmid was detected from the transformants.

4. Discussion

One of major goals in microbial ecology and microbial systems biology is to understand the relationships between microorganisms and other neighboring organisms in a community, and the individual species' role in their environment or their community [13]. *M. thermoacetica* is metabolically diverse acetogen which helps us to understand the physiology of acetogens. The diverse metabolic behavior of *M. thermoacetica* can be studied by coculturing. Coculturing allows us to study the metabolic behavior of *M. thermoacetica*.

Table 2 Overall experiments and the results

Aim	Setting	Results
What kind of interactions *M. thermoacetica* will have with other organisms?	*M. thermoacetica* was supplemented with 6 different organisms.	*G. sulfurreducens* had positive effect on the growth of *M. thermoacetica*.
What part of *G. sulfurreducens* can result this?	*M. thermoacetica* was supplemented with viable cells, lysated cells, pellet, supernatant and filtered media of *G. sulfurreducens*.	*G. sulfurreducens* media without cells showed most significant growth advantage to *M. thermoacetica*.
What compounds in *G. sulfurreducens* can cause this phenomenon?	Mass spec analysis of *M. thermoacetica* with filtered media of *G. sulfurreducens*.	13 compounds were found. Succinate, Succinyl-CoA, GTP and methylmalonate are specific to *G. sulfurreducens*.
What genes are involved in this phenomenon?	RNA-seq analysis of *M. thermoacetica* with filtered media of *G. sulfurreducens*.	Biosynthesis of secondary metabolites, arginine and proline metabolism, purine synthesis, and molybdopterin biosynthesis and TCA cycle

4.1 Culturing *M. thermoacetica* with secondary organisms

In this study, the effect of secondary organisms on the growth of *M. thermoacetica* was examined. The coculturing of *M. thermoacetica* with other organisms in defined media could give more concrete representation of microbial interaction in nature by pin-pointing mechanisms, even though it would be simpler than the study of the whole community. The supplemented secondary organisms were both gram-positive and gram-negative bacteria. The gram-positive bacteria *C. ljungdahlii, M. barkeri,* and *M. maripaludis* and the gram-negative bacteria *G. sulfurreducens, T. maritima, S. ovata* were supplemented. The interactions of *M. thermoacetica* with the supplemented organisms were examined. The growth curves suggest that the increase of biomass of *M. thermoacetica* with *G. sulfurreducens* is not from the mineral or vitamins supplied from the media. The results suggest that *M. thermoacetica* has growth advantage with either the *G. sulfurreducens* cells or the filtered media used by *G. sulfurreducens* (Figure 4.1). The growth advantages of *M. thermoacetica* would be resulted from the cellular products or molecules secreted during the growth of *G. sulfurreducens*.

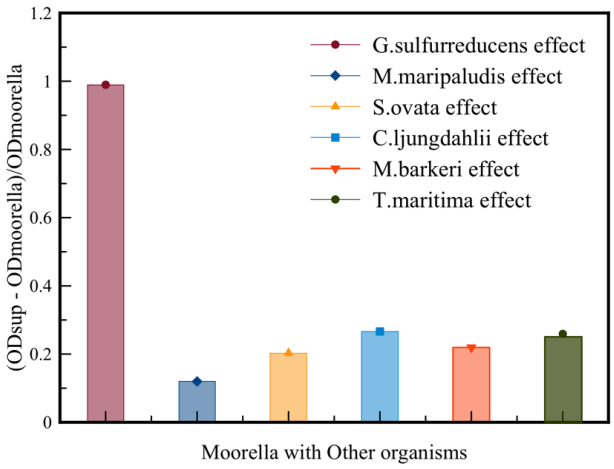


Figure 4.1 Effect of secondary organisms on *M. thermoacetica* growth

4.2 Effect of *G. sulfurreducens* on *M. thermoacetica*

The positive growth effect of *G. sulfurreducens* on *M. thermoacetica* was further analyzed by modifying the growth condition of *M. thermoacetica*. The *G. sulfurreducens* cells were separated into pellet, filtered media, and supernatant. *M. thermoacetica* were cultured in yeast extract and minimal media with the various components of *G. sulfurreducens* to narrow down the key factors that might promote the growth. The most substantial change in *M.thermoacetica*'s growth was observed with the filtered media (Figure 4.2).

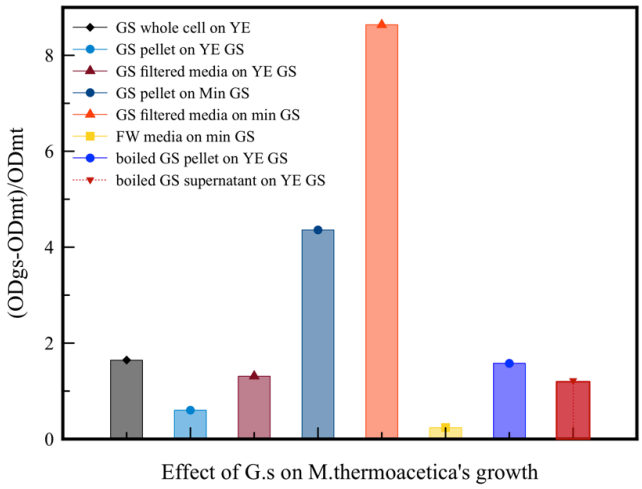


Figure 4.2 Effect of *G. sulfurreducens* components on *M. thermoacetica* growth

M. thermoacetica undergoes fermentation according to following equation:

$$C_6H_{12}O_6 + NH_4^+ + CO_2 + H^+ + e^- \rightarrow CH_3COOH + H_2 + CH_4 + H_2O \quad (4.1)$$

Regardless of the presence of *Geobacter* during the growth of *M. thermoacetica* in the yeast extract media, the acetate production from 10 mM glucose did not change (they

would produce 2.8 mM of acetate from 1 mM glucose). However, in the minimal media, the monoculture could only produce 2.0 mM, while *G. sulfurreducens* supplemented *Moorella* produced 2.5 mM acetate from 1.0 mM glucose. As we observed from metabolomics data, the filtered *Geobacter* media contain amino acids, organic acids, purines, and pyrimidines, etc. that can assist growth of *M. thermoacetica* as yeast extract promotes the growth of *Moorella*. Samples of three different time points along with the growth of *M. thermoacetica* were examined to decipher the role of filtered media of *G. sulfurreducens* at each growth phase (Table 2). It showed differences in hydrogen production by *M. thermoacetica* with and without *G. sulfurreducens*. Regardless of the presence of the yeast extract in *M. thermoacetica* media, the hydrogen production rate of monoculture *M. thermoacetica* was significantly higher than the *M. thermoacetica* supplemented with *G. sulfurreducens* (Figure 4.3), which indicates that there was considerably less hydrogen production with the supplementation. This change shows that electrons used to produce hydrogen ($2H^+ + 2e^- \rightarrow H_2$) were used to produce more biomass of *M. thermoacetica*.

When *M. thermoacetica* was supplemented with *G. sulfurreducens*, the biomass was increased but the hydrogen production decreased. RNA sequencing results show no significant change in hydrogenase activity between two samples, which indicates *M. thermoacetica* produces hydrogen and consumes hydrogen for respiration as well. The genes identified for the respiration, however, are not up-regulated with the supplementation of *G. sulfurreducens*. Our hypothesis is that molecules or cofactor from

Geobacter cells or filtered media enable *M. thermoacetica* to grow heterotrophically and autotrophically at the same time.

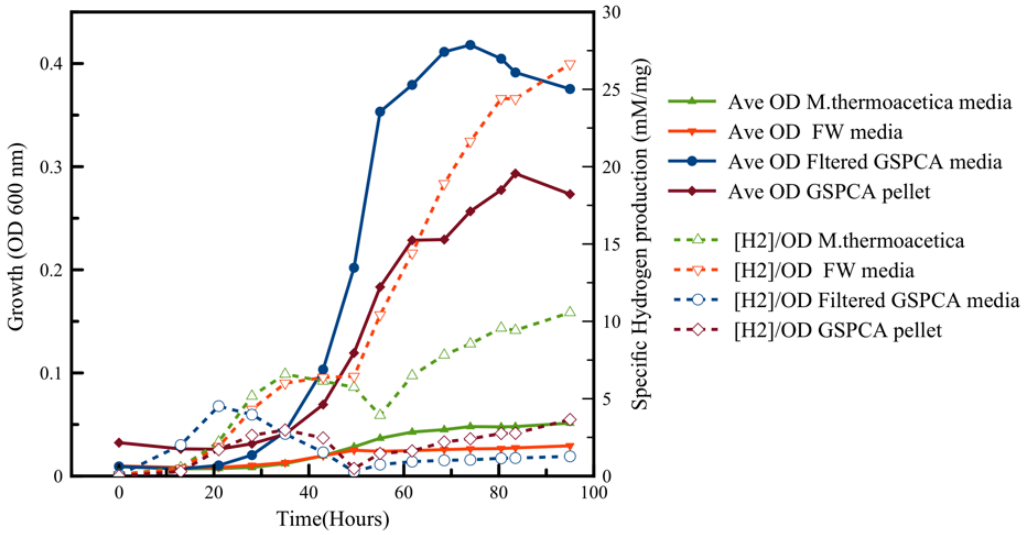


Figure 4.3 OD_{600} and specific hydrogen production per gDCW

Table 3 Balanced equation of glucose fermentation to acetate

Samples	IN						OUT							
in uM	Gluco	NH4+	CO2	H2O	H+	e-	Aceta	Cell ((	CH4	H2	H2O	CO2	H+	e-
MT(YE) lag	0.24	0.44	3.31	0	11.42	11.86	1.92	0.44	0	0.05	3.31	0	0	0
MT(YE) exp	0.69	0.44	2.29	0	6.48	6.93	2.76	0.44	0	0.03	2.3	0	0	0
MT(YE) Sta	1.116	0.44	0.77	0	0	0.44	3.29	0.44	0	0.05	0.77	0	0.000	0.000
MT/GS (YE) Lag	0.518	0.443	0.857	0.000	1.967	2.410	1.539	0.443	0.000	0.038	0.857	0.000	0.000	0.000
MT/GS (YE) Exp	0.969	0.443	0.804	0.000	0.381	0.824	2.865	0.443	0.001	0.014	0.804	0.000	0.000	0.000
MT/GS (YE) Stat	0.958	0.443	0.692	0.000	0.031	0.474	2.778	0.443	0.001	0.020	0.692	0.000	0.000	0.000
MT/GS media (YE) Lag	0.228	0.443	2.774	0.000	9.551	9.994	1.627	0.443	0.000	0.040	2.774	0.000	0.000	0.000
MT/GS media (YE) Exp	0.821	0.443	1.746	0.000	4.122	4.565	2.893	0.443	0.001	0.014	1.747	0.000	0.000	0.000
MT/GS media (YE) Stat	0.879	0.443	1.167	0.000	1.929	2.372	2.777	0.443	0.001	0.017	1.168	0.000	0.000	0.000
MT(min) lag	4.545	0.443	0.000	21.79	0.000	0.000	2.296	0.443	0.000	0.651	0.000	21.79	88.16	87.72
MT(min)exp	2.396	0.443	0.000	4.810	0.000	0.000	4.339	0.443	0.000	0.390	0.000	4.810	22.8	22.36
MT(min)Sta	4.310	0.443	0.000	8.486	0.000	0.000	8.237	0.443	0.006	0.940	0.000	8.492	40.3	39.86
MT/GS (min) Lag	1.153	0.443	0.000	3.004	0.000	0.000	1.514	0.443	0.000	0.297	0.000	3.004	12.94	12.5
MT/GS (min) Exp	1.364	0.443	0.000	0.207	0.000	0.000	3.544	0.443	0.002	0.146	0.000	0.209	4.083	3.639
MT/GS (min) Stat	2.327	0.443	0.000	1.257	0.000	0.000	5.905	0.443	0.004	0.276	0.000	1.261	10.38	9.936
MT/GS media (min) Lag	1.293	0.443	0.000	0.666	0.000	0.000	3.104	0.443	0.000	0.271	0.000	0.666	5.224	4.781
MT/GS media (min) Exp	1.616	0.443	0.000	0.154	0.000	0.000	4.325	0.443	0.002	0.074	0.000	0.156	4.794	4.351
MT/GS media (min) Stat	2.430	0.443	0.000	1.169	0.000	0.000	6.256	0.443	0.005	0.116	0.000	1.174	10.70	10.26

4.3 Metabolomics and RNA sequencing analysis

Metabolomics and transcriptomics of *M. thermoacetica* and *M. thermoacetica* with filtered media were analyzed to figure out the key factors which promote the growth of *M. thermoacetica*. Four compounds were identified, which would be supplemented by the filtered media: GTP, methylmalonate, succinate, and succinyl-CoA. In addition, metabolomics data provided more information of the molecules supplemented from yeast extract. Genes highly expressed with filtered media were considered to be responsible for the following processes in *M. thermoacetica*: TCA cycle, carbon fixation pathways, biosynthesis of secondary metabolites, arginine and proline metabolism, purine synthesis, and molybdopterin biosynthesis.

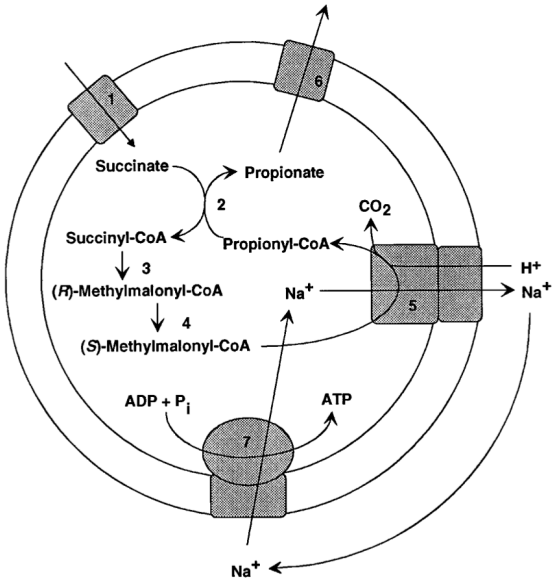


Figure 4.4 Energy metabolism of *P. modestum* [58].

Succinate and succinyl-CoA are intermediate compounds for TCA cycle. *M. thermoacetica*, however, can not utilize TCA cycle due to lack of enzyme to convert succinate to fumarate. The physiological screening with supplementation of succinate confirmed that there was no growth advantage to *M. thermoacetica* (Figure 3.10 b).

Another fermenting bacteria, *Propionigenium modestum,* can conserve energy by the decarboxylation of dicarboxylic acids [58]. It ATPase uses sodium and hydrogen antiporter as a driving force to convert succinate to succinyl-CoA and then to propionate as its last product. Our three compounds from MS were involved in this energy metabolism, so we observed the propionate production of *M. thermoacetica* with filtered *G. sulfurreducens* but there was no production of propionate detected by HPLC or MS analysis. Then we tested Acetogenium kivui, a thermophilic acetogenic bacterium with sodium and hydrogen antiporter [59], but there was no growth advantage to this acetogenic bacteria.

In addition, we examined the growth of *M. thermoacetica* on autotrophic growth under H_2 and CO_2. If our theory stands on the right track, the supplementation of *G. sulfurreducens* would not have any effect on *M.thermoacetica*'s growth because it underwent full respiration, not switching from fermentation to respiration anymore. The result suggests that this phenomenon is still related to the respiration of *M. thermoacetica* for the biomass production. For further study, as RNA sequencing suggested, the investigation should be focused on other pathways: biosynthesis of secondary metabolites, arginine and proline metabolism, purine synthesis, and molybdopterin biosynthesis.

4.4 Transformation

It is necessary to develop a genetic transformation system for *M. thermoacetica* to study gene function and regulation [53]. To increase the transformation efficiency, we optimized the transformation procedures as follows: (1) harvesting cell in early mid-log phase (OD_{600} 0.15-0.3), (2) increasing duration of postelectropulsing recovery hours in liquid media between 12 to 40 hours at 65 °C, (3) multiple overnight recovery in liquid media after electroporation, (4) supplementation of 5 μg/mL Cm during secondary postelectropulsing recovery period, (5) chilling cells 10 minutes before exposure to an electrical pulse, (6) plasmid concentration from 1 ng to 1μg, (7) 10% (v/v) glycerol at pH 7.4, and (8) incubation temperature of postelectropulsing media and plates (55 °C to 65 °C).

Once we have the transformation protocol, we can knockout the hydrogenase gene of *M. thermoacetica* to confirm whether it utilizes its self-produced hydrogen for the respiration with supplementation of *G. sulfurreducens*.

4.5 Conclusion and future directions

The physiology of *M. thermoacetica* was examined when it was cocultured with six secondary organisms: *Geobacter sulfurreducens, Thermotoga maritima, Clostridium ljungdhalii, Sporomusa ovata, Methanosarcina barkeri,* and *Methanococcus maripaludis*. *M. thermoacetica* growth was enhanced only with supplementation of *G. sulfurreducens* cells. To interpret the cause of the amplified growth of *M. thermoacetica*, different components of *G. sulfurreducens* were examined. The most substantial change in *M.thermoacetica*'s growth was observed with the filtered media. When *M. thermoacetica* was supplemented with *G. sulfurreducens*, the biomass was doubled but the hydrogen production was decreased. RNA sequencing result shows no significant change in hydrogenase activity between two samples, which indicates *M. thermoacetica* produces hydrogen and consumes hydrogen for respiration as well. The genes identified for the respiration, however, are not up-regulated with the supplementation of *G. sulfurreducens*. The results indicate that the secondary organisms have an impact on the proteomics or fluxomics level of *M. thermoacetica*.

Microbial community is a string of network, where different organisms interact with each other. However, it is hard to detect the relationships between microorganisms depending on monoculture study and it is also not possible to figure out the complexity of interactions between organisms from the community's 16S rRNA data. Coculturing would be an important method to investigate the physiology of microorganisms in a community. Now, it can be expected that there is higher possibility to understand various microbial interactions in natural environment with coculturing.

Appendix

Table A1 List of down-regulated genes of *Geobacter*-supplemented *M. thermoacetica* according to RNA-seq

Gene	Gene Product	M.t	M.t with G.s	log2(fold_ change)	p value	q value
Moth 1648	hypothetical protein	423.277	18.3485	-4.52787	4.96E-05	0.0040102
Moth 1888	ferredoxin	413.611	22.237	-4.21724	1.30E-09	1.76E-06
Moth 1070	hypothetical protein	77.5625	4.25993	-4.18646	0.000976999	0.0290008
Moth 1887	NADH dehydrogenase (quinone)	486.329	33.1793	-3.87358	1.50E-09	1.76E-06
Moth 2170	acetyl-coenzyme A synthetase	569.593	41.0331	-3.79507	9.84E-09	7.70E-06
Moth 1809	malto-oligosyltrehalose trehalohydrdlase	71.6093	6.23647	-3.52135	4.37E-06	0.00105567
Moth 0855	PRC-barrel	115.088	10.425	-3.46463	0.000448841	0.0190149
Moth 1886	NADH-quinone oxidoreductase E subunit	433.518	39.4951	-3.45634	4.50E-06	0.00105567
Moth 0202	cell wall hydrolase, SleB	112.977	10.3352	-3.4504	3.88E-05	0.00363596
Moth 0853	sporulation sigma factor SigG	386.554	35.5931	-3.441	1.20E-06	0.00052954
Moth 1883	ferredoxin hydrogenase	325.063	31.7165	-3.35741	9.13E-06	0.0016475
Moth 1071	ATP-dependent Clp protease proteolytic subunit ClpP	87.498	8.77945	-3.31705	3.64E-05	0.00355321
Moth 1885	4Fe-4S ferredoxin iron-sulfur binding	326.708	33.0001	-3.30746	2.13E-05	0.00259784
Moth 0799	hypothetical protein	45.2289	4.62689	-3.28913	0.000363263	0.016703
Moth 1879	rubrerythrin	12000	1249.01	-3.26418	1.80E-08	1.06E-05
Moth 1914	hypothetical protein	137.466	14.406	-3.25433	3.46E-05	0.00355321

Gene	Gene Product	M.t	M.t with G.s	log2(fold_ change)	p value	q value
Moth 1016	catalase	424.343	46.1868	-3.19968	8.39E-06	0.00163878
Moth 2098	ABC transporter related	11.7323	1.33388	-3.13679	0.000504419	0.0203942
Moth 2261	hypothetical protein	330.334	39.2288	-3.07394	0.00025118	0.0128047
Moth 0033	pyruvate flavodoxin/ferredoxin oxidoreductase-like	276.014	34.2864	-3.00904	2.14E-05	0.00259784
Moth 0926	germination protease	220.95	27.522	-3.00506	4.39E-05	0.00381104
Moth 2372	transcription regulator of sigma-E/ sigma-K-dependent gene spoIIID	180.616	22.7643	-2.98808	0.000595136	0.0219455
Moth 1915	glycoside hydrolase family protein	46.0792	5.84027	-2.98001	0.000135221	0.0079273
Moth 0854	hypothetical protein	200.703	25.489	-2.97712	0.000103756	0.00695165
Moth 1902	secretion protein HlyD	14.4529	1.88003	-2.94253	0.00149281	0.0389781
Moth 1404	manganese transport transcriptional regulator	69.842	9.21203	-2.9225	0.000667131	0.024068
Moth 0034	2-oxoglutarate synthase	198.096	26.5959	-2.89692	0.000972777	0.0290008
Moth 1826	lactate dehydrogenase	250.266	33.8582	-2.88588	7.47E-05	0.0054771
Moth 2505	heat shock protein Hsp20	96.802	13.0997	-2.8855	0.000597044	0.0219455
Moth 1284	rubredoxin-type Fe (Cys) 4 protein	1683.77	229.774	-2.87341	0.000208412	0.0108606
Moth 0032	hypothetical protein	302.987	42.3337	-2.83938	0.000184393	0.0100558
Moth 0883	orotidine-5’-phosphate decarboxylase	106.325	16.3365	-2.70231	0.000472889	0.0194548
Moth 1935	galactose-1-phosphate uridylyltransferase	104.292	16.038	-2.70106	0.000386438	0.0174269

Gene	Gene Product	M.t	M.t with G.s	log2(fold_ change)	p value	q value
Moth 1375	stage V sporulation protein AD	139.177	21.5645	-2.6902	0.000344203	0.0161431
Moth 1493	hypothetical protein	1975.32	309.444	-2.67433	0.000285309	0.0136541
Moth 1376	SpoVA protein	223.298	36.0297	-2.63171	0.000558715	0.0219455
Moth 1876	hypothetical protein	89.8197	14.6528	-2.61586	0.000573405	0.0219455
Moth 1015	hypothetical protein	264.254	44.2604	-2.57784	0.00140928	0.0379967
Moth 1536	hypothetical protein	392.788	67.486	-2.54109	0.000894265	0.0290008
Moth 1370	CoA enzyme activase	81.6005	14.0619	-2.53678	0.000961657	0.0290008
Moth 0807	hypothetical protein	599.636	103.43	-2.53543	0.000454086	0.0190149
Moth 2094	BadM/FRrf2 family transcriptional regulator	261.345	45.3055	-2.5282	0.000965217	0.0290008
Moth 2300	aldehyde ferredoxin oxidoreductase	76.3964	13.3068	-2.52134	0.000780248	0.0269071
Moth 1936	glycogen/starch synthases ADP-glucose type	95.5434	16.8048	-2.50728	0.000819094	0.0278373
Moth 0866	TraR/DksA family transcriptional regulator	79.149	14.1357	-2.48523	0.00140968	0.0379967
Moth 0116	major facilitator transporter	58.3209	10.416	-2.48521	0.00123215	0.0348121
Moth 0578	hypothetical protein	152.016	27.5691	-2.4631	0.000974161	0.0290008
Moth 0887	ATPase (E1-E2 type)	58.7504	10.7104	-2.45559	0.000946984	0.0290008
Moth 1528	amino acid permease-associated region	80.9152	14.7742	-2.45333	0.00114077	0.0326234
Moth 0077	hypothetical protein	1111.49	204.981	-2.43894	0.000167456	0.00934964

Gene	Gene Product	M.t	M.t with G.s	log2(fold_ change)	p value	q value
Moth 0083	AbrB family transcriptional regulator	2057.59	389.014	-2.40306	0.000127967	0.00769444
Moth 1649	hypothetical protein	271.693	51.71	-2.39346	0.00157037	0.0404671
Moth 0880	dihydroorotase	470.397	90.601	-2.37628	0.000271721	0.0135033
Moth 1319	stage IV sporulation protein A (spore cortex formation and coat assembly)	747.503	144.294	-2.37307	0.000121074	0.00747157
Moth 2508	hypothetical protein	272.556	52.671	-2.37147	0.00149596	0.0389781
Moth 0737	amino acid permease-associated region	73.5881	14.2771	-2.36577	0.00168667	0.0425295
Moth 0879	aspartate carbamoyltransferase catalytic subunit	427.82	83.5679	-2.35598	0.000598938	0.0219455
Moth 1210	hypothetical protein	5108.91	1037.03	-2.30055	0.00159862	0.0407475
Moth 1783	hypothetical protein	1978.89	402.563	-2.29741	0.00102108	0.0299303
Moth 1782	coat F domain-containing protein	1403.13	286.972	-2.28967	0.000957409	0.0290008
Moth 0058	electron transfer flavoprotein subunit beta	447.861	94.7921	-2.24021	0.00128491	0.0358341
Moth 0925	small acid-soluble spore protein alpha/beta type	6065.24	1293.59	-2.22919	0.000110815	0.00721836
Moth 1285	desulfoferrodoxin	1589.15	354.498	-2.1644	0.00129889	0.0358341
Moth 0882	carbamoyl-phosphate synthase large subunit	322.065	73.3673	-2.13414	0.000423011	0.0185938

Table A2 List of up-regulated genes of *Geobacter*-supplemented *M. thermoacetica* according to RNA-seq

Gene	Gene Product	M.t	M.t with G.s	log2(fold_change)	p value	q value
Moth 2286	ornithine carbamoyltransferase	689.382	3143.36	2.18893	4.66E-05	0.0039
Moth 2281	adenine-specific DNA methylase	254.301	1184.82	2.22006	0.0002	0.0106
Moth 0367	hypothetical protein	413.336	1932.6	2.22516	0.0007	0.0246
Moth 2015	hypothetical protein	161.337	779.586	2.27263	0.00096	0.029
Moth 2289	bifunctional ornithine acetyltransferase-acetylglutamate synthase protein	692.767	3352.56	2.27482	1.85E-05	0.0026
Moth 2288	acetylglutamate kinase	656.803	3216.84	2.29211	2.54E-05	0.0028
Moth 1271	peptidoglycan-binding LysM	50.6933	252.856	2.31845	0.00148	0.039
Moth 1181	propanediol utilization protein	667.114	3366.29	2.33515	4.26E-05	0.0038
Moth 2283	fumarate hydratase	298.584	1516.02	2.34408	0.00012	0.0074
Moth 2284	argininosuccinate lyase	377.444	1929.04	2.35355	1.75E-05	0.0026
Moth 2287	acetylornithine aminotransferase	520.47	2686.87	2.36804	1.17E-05	0.0018
Moth 2290	N-acetyl-gamma-glutamyl-phosphate reductase	962.088	5059.31	2.3947	7.05E-06	0.0015
Moth 2046	phosphoribosylaminoimidazole synthetase	64.5271	349.448	2.4371	0.00043	0.0186
Moth 2045	phosphoribosylglycinamide formyltransferase	43.4203	238.824	2.45951	0.00106	0.0307
Moth 1269	hypothetical protein	327.287	1820.41	2.47563	7.76E-05	0.0055
Moth 2282	tartrate/fumarate subfamily Fe-S type hydro-lyase beta subunit	193.334	1079.89	2.48172	0.00014	0.008
Moth 2285	argininosuccinate synthase	529.571	2977.86	2.49138	4.06E-06	0.0011

Gene	Gene Product	M.t	M.t with G.s	log2(fold_change)	p value	q value
Moth 2048	phosphoribosylformylglycinamidine synthase II	39.5796	230.065	2.53922	0.0006	0.0219
Moth 1982	binding-protein dependent transport system inner membrane protein	7.25541	42.5091	2.55064	0.00174	0.0435
Moth 2043	phosphoribosylamine--glycine ligase	100.207	602.304	2.58751	5.60E-05	0.0044
Moth 0721	4Fe-4S ferredoxin (iron-sulfur binding)	103.873	642.619	2.62915	0.00028	0.0135
Moth 2044	phosphoribosylaminoimidazolecarboxamide formyltransferase IMP cyclohydrolase	77.0593	485.4	2.65513	3.62E-05	0.0036
Moth 1979	ABC transporter related	9.40085	59.539	2.66297	0.00084	0.0282
Moth 0204	hypothetical protein	45.5734	305.315	2.74403	0.0007	0.0246
Moth 1659	hypothetical protein	55.1263	415.075	2.91256	0.0001	0.007
Moth 2047	amidophosphoribosyltransferase	35.023	264.593	2.9174	2.22E-05	0.0026
Moth 0722	aldehyde ferredoxin oxidoreductase	133.198	1016.25	2.93161	1.54E-06	0.0005
Moth 0724	FAD-dependent pyridine nucleotide-disulphide oxidoreductase	112.969	936.413	3.05122	1.58E-06	0.0005
Moth 0723	hypothetical protein	100.702	1013.31	3.3309	1.00E-05	0.0017
Moth 1392	manganese containing catalase	4.09193	55.0242	3.74921	5.79E-05	0.0044

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