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Metabolome Analysis of Constituents in Membrane Vesicles for *Clostridium thermocellum* Growth Stimulation

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Abstract: The cultivation of the cellulolytic bacterium, *Clostridium thermocellum*, can have cost-effective cellulosic biomass utilizations, such as consolidated bioprocessing, simultaneous biological enzyme production and saccharification. However, these processes require a longer cultivation term of approximately 1 week. We demonstrate that constituents of the *C. thermocellum* membrane vesicle fraction significantly promoted the growth rate of *C. thermocellum*. Similarly, cell-free *Bacillus subtilis* broth was able to increase *C. thermocellum* growth rate, while several *B. subtilis* single-gene deletion mutants, e.g., *yxeJ*, *yxeH*, *ahpC*, *yxdK*, *iolF*, decreased the growth stimulation ability. Metabolome analysis revealed signal compounds for cell–cell communication in the *C. thermocellum* membrane vesicle fraction (ethyl 2-decenoate, ethyl 4-decenoate, and 2-dodecenoic acid) and *B. subtilis* broth (nicotinamide, indole-3-carboxaldehyde, urocanic acid, nopaline, and 6-paradol). These findings suggest that the constituents in membrane vesicles from *C. thermocellum* and *B. subtilis* could promote *C. thermocellum* growth, leading to improved efficiency of cellulosic biomass utilization.

Keywords: cellulosic biomass utilization; membrane vesicle; cell–cell communication; *Clostridium thermocellum*; *Bacillus subtilis*; metabolome analysis

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1. Introduction

Cellulose is one of the most abundant organic materials on Earth. Bacteria that can grow on cellulose have been isolated from many environments that include soil, hot springs, cow rumen, termite gut, and the human intestinal tract [1]. *Clostridium thermocellum* (*Acetivibrio thermocellus*) [2], a Gram-positive thermophilic anaerobic soil bacterium, is a candidate for cellulosic biomass utilization. *C. thermocellum* completely degrades 4.4 g/L purified cellulose in one day [3]. It also degrades 65% of 5 g/L switchgrass in five days and 70% of 10 g/L corn hull in seven days [4,5].

C. thermocellum has been shown to produce 1.3% ethanol from 10% Avicel cellulose [6]. A strain of *C. thermocellum* multiply deleted for [FeFe] hydrogenase maturase, lactate dehydrogenase, pyruvate-formate lyase, Pfl-activating enzyme, phosphotransacetylase, and acetate kinase genes, which eliminated formate, acetate, and lactate production, and reduced H_2 production, presented a titer of 2.2% ethanol from 6% Avicel cellulose [7]. The ethanol hyper-producing strain *C. thermocellum* I-1-B produced 2.4% ethanol from 8% cellulose [8]. A co-culture of a strain lacking the lactate dehydrogenase/phosphotransacetylase gene and *Thermoanaerobacterium saccharolyticum* produced 3.8% ethanol from 9.2% Avicel cellulose in 146 h [9]. These reports show that the cultivation of *C. thermocellum* can be simplified consolidated bioprocessing (CBP). This is a promising strategy because it eliminates the need to add lignocellulose-degrading enzymes that significantly increase the cost of biofuel production [10–12].

Some cellulolytic bacteria, including *C. thermocellum*, form carbohydrate-active enzyme (CAZyme) complexes that are termed cellulosomes [13–16]. The main product of enzymatic cellulose degradation is cellobiose, which leads to the feedback inhibition of cellulosomes. Supplementation with β-glucosidase (BGL) leads to the hydrolysis of cellobiose into form two glucose molecules, thereby resolving the feedback inhibition. *C. thermocellum* preferentially utilizes cellooligosaccharide, and glucose tends to accumulate in the culture broth [17]. Supplementation with purified BGL increased glucose production by *C. thermocellum* from 10% cellulose or 12% alkali pretreated rice straw by approximately 7.7% over 10 days [18]. This technology is referred to as biological simultaneous enzyme production and saccharification (BSES). BSES is similar to CBP, does not require the diverse CAZymes for the saccharification of cellulosic biomass.

We previously reported that *C. thermocellum* produces extracellular membrane vesicles (MVs) that are released into the broth [19]. MVs are produced in Gram-negative and Gram-positive bacteria. The latter possess a membrane that is overlaid by a relatively thick and resilient cell wall enriched in peptidoglycan [20,21]. MVs have been isolated from the culture supernatant of Gram-positive bacteria that include *Bacillus subtilis*, *B. anthracis*, *Streptomyces coelicolor*, *Listeria monocytogenes*, *Staphylococcus aureus*, *Streptococcus mutans*, *S. pneumoniae*, and *Clostridium perfringens* [22–28]. Klieve et al. reported the production of MVs by *Ruminococcus* spp., a cellulolytic bacterium that resides in the ovine rumen. DNA molecules ranging in size from <20 to 49 kb, and from 23 to 90 kb are attached to MVs from *Ruminococcus* sp. YE73 and *Ruminococcus albus* AR67, respectively. Thus, MVs can function as vectors for horizontal gene transfer to confer cellulolytic activity, as documented in the mutant strain *Ruminococcus* sp. YE71 [29]. MVs from cellulolytic *Bacteroides fragilis* and *B. thetaiotaomicron* are equipped with hydrolytic enzymes and are important in polysaccharide degradation [30,31]. MVs from *Fibrobacter succinogenes* are enriched with CAZymes, and intact MVs are able to degrade a broad range of hemicelluloses and pectin [32]. We have previously proposed that *C. thermocellum* may utilize MVs to deliver cellulosomes, which enhance the cellulolytic activity of *C. thermocellum* [19].

MVs contain various compounds that include DNA and RNA. These cargos are delivered to neighboring cells. MVs have several important functions related to cell–cell interactions. In *Pseudomonas aeruginosa*, a hydrophobic cell–cell communication signal termed *Pseudomonas* quinolone signal is released from the bacteria via MVs [33,34]. MVs can also serve as organic carbon sources for heterotrophs. For example, MVs derived from cyanobacteria support the growth of *Alteromonas* and *Halomonas* as the sole carbon source, indicating that MVs should be considered in the marine food web and may have important roles in the carbon flux of the ocean [35]. In *Mycobacterium tuberculosis*, the causative agent of tuberculosis, increased MV production in response to iron restriction has been observed [36]. These MVs contain a siderophore called mycobactin. Mycobactin can serve as an iron donor to support the growth of iron-starved *M. tuberculosis*.

In this study, we demonstrated that the MV fractions collected from *C. thermocellum* and *B. subtilis* can promote *C. thermocellum* growth. Metabolome analysis was also performed to identify the candidate compounds with the growth stimulation.

2. Materials and Methods

2.1. Strains and Culture Conditions of C. thermocellum and B. subtilis

One hundred microliters of *C. thermocellum* DSM 1313 (DSMZ, Braunschweig, Germany) culture was inoculated in 5 mL of CTFUD medium (3 g/L sodium citrate tribasic dehydrate, 1.3 g/L $(NH_4)_2SO_4$, 1.5 g/L KH_2PO_4, 130 mg/L $CaCl_2$ $2H_2O$, 500 mg/L L-cysteine-HCl, 11.56 g/L 3-morpholinopropanesulfonic acid, 2.6 g/L $MgCl_2$ $6H_2O$, 1 mg/L $FeSO_4$ $7H_2O$, 4.5 g/L Bacto yeast extract, 1 mg/L resazurin, pH 7.0) containing 0.5% cellobiose (Tokyo Chemical Industry, Tokyo, Japan) with 16 × 125 mm Hungate tubes (Chemiglass Life Sciences, Vineland, NJ, USA), and cultured at 60 °C under anaerobic conditions with nitrogen gas [37].

B. subtilis KAO/NAIST chromosomal deletion mutants [38] and BKE genome-scale deletion mutants [39] were obtained from the National BioResource Project *B. subtilis* (National Institute of Genetics, Shizuoka, Japan). *B. subtilis* strains were aerobically cultured in Luria Bertani broth at 37 °C.

2.2. Preparation of MV Fraction of *C. thermocellum*

Five milliliters of *C. thermocellum* and *B. subtilis* culture was centrifuged at 10,000× *g* for 2 min at 4 °C, and the supernatant was filtered through a 0.22-µm syringe filter to remove cells. The filtrate was centrifuged at 179,000× *g* for 1 h at 4 °C and the pellet was washed twice with 2 mL of sterile phosphate-buffered saline (PBS). The pellet was resuspended in PBS and used as the MV fraction. The MV fraction was kept on ice before use.

MVs were visualized using transmission electron microscopy. Six microliter aliquots of the MV fraction was added to 300-mesh carbon and formvar-coated copper grids and incubated for 1 min. After removing the extra solution with filter paper, each specimen was stained with 2% phosphotungstic acid. The sample was observed with a JEM-1011 microscope (JEOL, Tokyo, Japan) at an accelerating voltage of 80 kV.

2.3. Growth Evaluation of *C. thermocellum* with MV Supplementation

One hundred microliters of *C. thermocellum* DSM 1313 culture was inoculated in 5 mL of CTFUD medium containing 0.5% cellobiose with the supplementation of the collected MV fraction. *C. thermocellum* was cultured at 60 °C under anaerobic conditions with nitrogen gas. The *C. thermocellum* growth was evaluated with optical density of the broth at 600 nm.

2.4. Liquid Chromatography-Tandem Mass Spectrometry (LC-MS/MS) Analysis of *C. thermocellum* MV and *B. subtilis* Broth

The *C. thermocellum* MV fraction was treated with 10 mg/L surfactin, and the filtrate obtained after ultrafiltration with Vivaspin 2-100 K (Cytiva, Marlborough, MA, USA) was used to obtain the constituents in MVs. Cell-free supernatants of *B. subtilis trpC2* and *trpC2 yxeJ* broth were prepared by centrifugation and filtration with a 0.22-µm syringe filter. These specimens were homogenized with zirconia beads in 75% methanol, and the supernatants were collected after centrifugation at 15,000× *g* rpm for 10 min. The supernatants were applied to a MonoSpin C18 column (GL Science, Tokyo, Japan) and were filtered through a 0.22-µm syringe filter.

LC-MS analysis was performed on an Ultimate 3000 rapid separation LC (RSLC) and the Q Exactive system (Thermo Fisher Scientific, Waltham, MA, USA). Ultimate 3000 RSLC analysis was performed with the following parameters: column, InertSustain AQ-C18 (GL Science); column temperature, 40 °C; injection volume, 2 µL; solvent flow rate, 200 µL/min. The eluting solution was 0.1% formic acid containing 2% acetonitrile. The Q Exactive system had the following parameters: measurement time, 3–30 min; ionization method, electrospray ionization; measurement mass range, *m*/*z*: 80–1200; full scan resolution, 70,000; and MS/MS scan resolution, 17,500. The obtained data were analyzed with PowerGetBatch and MFSearcher [40]. The LC-MS analysis was performed in triplicate.

3. Results and Discussion

3.1. MV Constituents Promote *C. thermocellum* Growth

A previous study reported that the co-culture of the engineered *C. thermocellum* and *T. saccharolyticum* strains produced 3.8% ethanol from cellulose for 6 days [9]. *C. thermocellum* cultivation with BGL supplementation for 10 days reportedly produced 76.7 g/L glucose from alkali pretreated rice straw [18]. It seems that the growth rate of *C. thermocellum* is an important factor in improving the efficiency of CBP and BSES. In this study, we collected MVs from *C. thermocellum* broth (Figure S1). MVs contain various compounds, such as DNA and RNA, which function in cell–cell communication. When *C. thermocellum* was grown in the presence of the MV fraction, the growth rate did not change.

However, when the MVs were lysed using the lipopeptide surfactin [41] the cell density of *C. thermocellum* had significantly increased at 24 h after the inoculation (Figure 1). The surfaction supplementation alone did not affect the *C. thermocellum* growth rate. The final growth yield in each sample had not changed significantly. These results suggest that the constituents in the MV fraction could promote the growth rate of *C. thermocellum*.

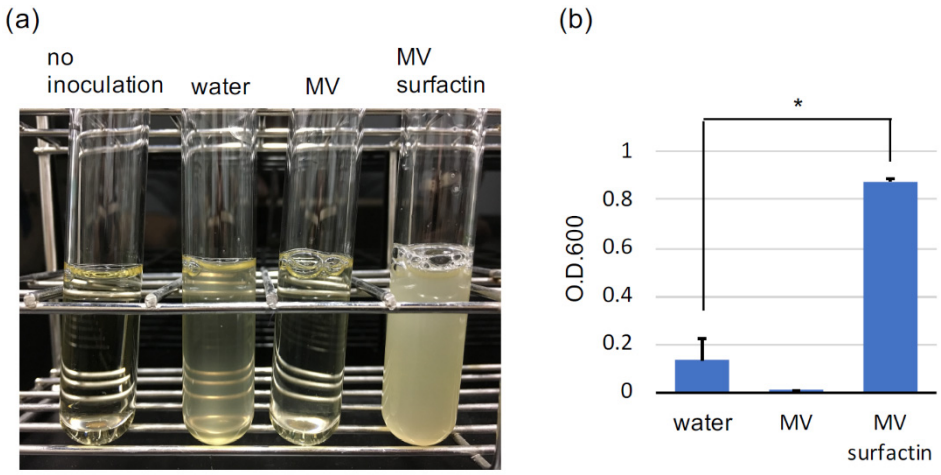

Figure 1. *C. thermocellum* growth stimulation by the MV constituents. *C. thermcellum* was cultured in CTFUD medium for 24 h with the supplementation of water, the MV fraction, or the surfactin-treated MV fraction. The cultures (**a**) and their optical densities (**b**) are shown. The experiment was duplicated. Error bars show standard error. * Student's *t*-test $p < 0.01$.

3.2. *B. subtilis* Broth Promotes *C. thermocellum* Growth Rate

Cell-free *B. subtilis* broth containing MVs also promoted the *C. thermocellum* growth rate, similar to the *C. thermocellum* MV fraction (Figure S1 and Figure 2a). Again, the surfaction supplementation alone did not affect the *C. thermocellum* growth rate (Figure 2a). Mukamolova et al. purified the resuscitation promoting factor (Rpf) from the broth of the Gram-positive bacterium, *Micrococcus luteus*. The purified Rpf promoted the growth of this bacterium as well as *Mycobacterium avium*, *M. bovis*, *M. kansasii*, *M. smegmatis*, and *M. tuberculosis* [42]. Genes homologous to the *rpf* gene were found to be widespread in a number of *Mycobacterium* species, as well as in Gram-positive bacteria with a high GC content, such as *Corynebacterium gultamicum* and *Streptomyces rimosus*. The Rpf protein shows peptidoglycan degradation activity [43]. Shah et al. reported that muropeptide fragments released from the peptidoglycan of the Gram-positive bacterium, *B. subtilis*, stimulate the germination of bacterial spores. Staurosporine, which inhibits related eukaryotic kinases in bacteria, blocks muropeptide-dependent bacterial spore germination [44]. We evaluated the effect of staurosporine on *C. thermocellum* growth with cell-free *B. subtilis* broth, however no significant inhibition was observed.

We further evaluated the *C. thermocellum* growth promotion effect of the broth of *B. subtilis* genome deletion mutants [38]. All the mutants, especially six mutants in which the *pdp-rocR* genomic region, were deleted (MGB723, MGB773, MGB822, MGB834, MGB860, MGB874) promoted *C. thermocellum* growth by accelerating the growth rate (Figure 2b, Table S1). Subsequently, we evaluated the *C. thermocellum* growth promotion effect of 100 *B. subtilis* mutants in which single genes within the *pdp-rocR* genomic region were deleted under a *trpC2* gene deletion background (Table S2) [39]. We did not find *B. subtilis* mutants that promoted *C. thermocellum* growth more than *trpC2* strain as the parent strain. Contrary to our expectation, the effect of 23 *B. subtilis* mutants was significantly lower than that of the parent strain (Figure 2c).

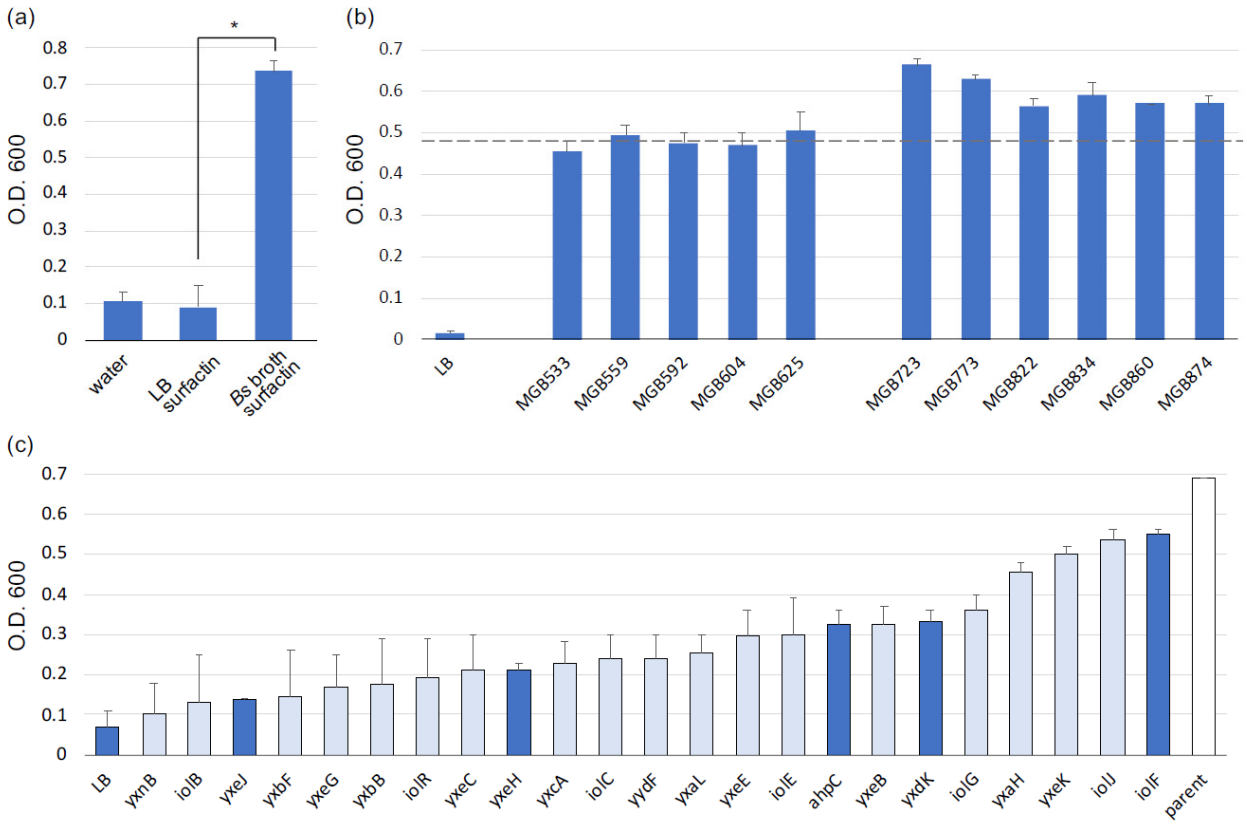

Figure 2. (**a**) *C. thermocellum* growth stimulation using cell-free *B. subtilis* broth. *C. thermcellum* was cultured in CTFUD medium with surfactin-treated cell-free *B. subtilis* broth for 24 h. The experiment was performed in triplicate. * Student's *t*-test $p < 0.01$. (**b**) *C. thermocellum* growth promotion effect of the broth of *B. subtilis* genome deletion mutants evaluated. The genotypes of the genome deletion mutants are listed in Table S1. The experiment was duplicated. (**c**) Evaluation of the *C. thermocellum* growth promotion effect of the broth of *B. subtilis* single-gene deletion mutants. Dark and light blue indicate significant differences compared with the effect of the parent strain (*trpC2*) with Student's *t*-test at $p < 0.01$ and < 0.05, respectively. The experiment was duplicated. Error bars indicate the standard error.

Among these 23 genes, the functions of several genes have been experimentally evaluated. The *asnH* operon, which comprises *yxbB*, *yxbA*, *yxnB*, *asnH*, and *yxaM*, might be involved in the biosynthesis of asparagine [45]. The *iolJ*, *iolG*, *iolF*, *iolE*, *iolC*, *iolB*, and *iolR* genes in the *iolABCDEFGHIJ* and *iolRS* operon are responsible for *myo*-inositol catabolism involving multiple and stepwise reactions [46–48]. We observed a slight growth inhibition of *C. thermocellum* in the presence of *myo*-inositol, however this required a high concentration (1 mg/mL) of *myo*-inositol (Figure S2). YydF is predicted to be an exported and modified peptide that has antimicrobial and/or signaling properties [49,50]. YxaL, which contains a repeated pyrrolo-quinoline quinone (PQQ) domain that forms a beta-propeller structure, interacts with the DNA helicase PcrA in *B. subtilis* [51]. Kim et al. reported that treatment of *Arabidopsis thaliana* and *Oryza sativa* L. seeds with 1 mg/L purified YxaL was effective in improving root growth [52]. PQQ, which was first recognized as an enzyme cofactor in bacteria, displays bioactivities for various eukaryotes and prokaryotes. For many bacterial species, PQQ has growth stimulation effect and serves as a cofactor for a special class of dehydrogenases/oxidoreductases [53]. PQQ has been described as an essential growth factor for various microbes [54–56]. We observed a slight *C. thermocellum* growth promotion effect by PQQ. This effect was not enough to explain the effect of *B. subtilis* broth (Figure S3). More than 50 proteins are involved in *B. subtilis* spore coat assembly. Of these, YxeE is an inner spore coat protein [57,58]. *ahpC* encodes thiol-specific peroxidase that plays a role in protecting cells against oxidative stress by detoxifying peroxides [59]. Utilization of a hydroxamate siderophore, ferrioxamine, requires the FhuBGC ABC transporter together with a ferrioxamine-binding protein, YxeB [60]. A range of siderophores can act

as growth factors for various previously uncultured bacteria [61]. YxdK is assumed to be a subunit of the two-component sensor histidine kinase, with its potential cognate response regulator, YxdJ [62]. Co-cultivation with *B. subtilis* allows the growth of *Synechococcus leopoliensis* CCAP1405/1 on solid media. However, the *yxdK* deletion mutant reportedly loses this ability [63]. The *yxeK* gene, which encodes FAD-dependent monooxygenase, contributes to the metabolism of S-(2-succino)cysteine to cysteine [64].

3.3. Metabolome Analysis of the Constituents in C. thermocellum MV and B. subtilis Broth

We collected the constituents in *C. thermocellum* MVs and analyzed them using LC-MS/MS. Among the 534 detected peaks, the intensities of seven peaks were significantly higher in the fraction where MVs had been disrupted by surfactin compared to MVs not disrupted using surfactin (Table S3). The structure of five significantly detected compounds in surfactin-treated *C. thermocellum* MVs specimen can be estimated by MS/MS analysis (Table 1 and Table S5).

Table 1. The constituents in *C. thermocellum* MVs detected by LC-MS/MS analysis.

No.	Formula	Exact Mass	Name	Database	Database ID
3203	$C_{16}H_{31}O_1N_1$	253.241		EX-HR2	
3013	$C_{12}H_{22}O_2$	198.162	Ethyl 2-decenoate	UC2	HMDB0037329
	$C_{12}H_{22}O_2$	198.162	Ethyl 4-decenoate	UC2	HMDB0039220
	$C_{12}H_{22}O_2$	198.162	Methyl 9-undecenoate	UC2	HMDB0037305
	$C_{12}H_{22}O_2$	198.162	Methyl 10-undecenoate	UC2	HMDB0029585
	$C_{12}H_{22}O_2$	198.162	Allyl nonanoate	UC2	HMDB0029763
	$C_{12}H_{22}O_2$	198.162	cis-3-Hexenyl hexanoate	UC2	HMDB0033378
	$C_{12}H_{22}O_2$	198.162	2-Hexenyl hexanoate	UC2	HMDB0038924
	$C_{12}H_{22}O_2$	198.162	Hexyl 2E-hexenoate	UC2	HMDB0038269
	$C_{12}H_{22}O_2$	198.162	Hexyl 2-methyl-3-pentenoate	UC2	HMDB0040158
	$C_{12}H_{22}O_2$	198.162	Hexyl 2-methyl-4-pentenoate	UC2	HMDB0040163
	$C_{12}H_{22}O_2$	198.162	1-Ethenylhexyl butanoate	UC2	HMDB0037498
	$C_{12}H_{22}O_2$	198.162	2-Octenyl butyrate	UC2	HMDB0038081
	$C_{12}H_{22}O_2$	198.162	cis-4-Decenyl acetate	UC2	HMDB0032214
	$C_{12}H_{22}O_2$	198.162	Menthyl acetate	UC2	C00036314
	$C_{12}H_{22}O_2$	198.162	Rhodinyl acetate	UC2	HMDB0037186
	$C_{12}H_{22}O_2$	198.162	Citronellyl acetate	UC2	C00035564
	$C_{12}H_{22}O_2$	198.162	2-Dodecenoic acid	UC2	HMDB0010729
	$C_{12}H_{22}O_2$	198.162	4-dodecenoic acid	UC2	C00051284
	$C_{12}H_{22}O_2$	198.162	5-dodecenoic acid	UC2	HMDB0000529
	$C_{12}H_{22}O_2$	198.162	11-Dodecenoic acid	UC2	HMDB0032248
	$C_{12}H_{22}O_2$	198.162	5-dodecalactone	UC2	HMDB0037742
	$C_{12}H_{22}O_2$	198.162	gamma-Dodecalactone	UC2	C00030347
	$C_{12}H_{22}O_2$	198.162	epsilon-Dodecalactone	UC2	HMDB0038895
	$C_{12}H_{22}O_2$	198.162	alpha-Heptyl-gamma-valerolactone	UC2	HMDB0037813
	$C_{12}H_{22}O_2$	198.162	4-butyl-4-hydroxyoctanoic acid lactone	UC2	HMDB0036182
	$C_{12}H_{22}O_2$	198.162	2,6-Dimethyl-5-heptenal propyleneglycol acetal	UC2	HMDB0032235
	$C_{12}H_{22}O_2$	198.162	citral dimethyl acetal	UC2	HMDB0040361

Table 1. *Cont.*

No.	Formula	Exact Mass	Name	Database	Database ID
	$C_{12}H_{22}O_2$	198.162	citronelloxyacetaldehyde	UC2	HMDB0041449
	$C_{12}H_{22}O_2$	198.162	Chokol A	UC2	C00011518
	$C_{12}H_{22}O_2$	198.162	Cybullol	UC2	C00013221
42	$C_{12}H_4O_3S_1$	227.988		EX-HR2	
3000	$C_{18}H_{35}O_2N$	297.267	Lepadin D	UC2	C00026353
	$C_{18}H_{35}O_2N$	297.267	Cassine	UC2	C00002027
2834	$C_8H_{13}ON$	139.100	5-Pentyloxazole	UC2	HMDB0038792
	$C_8H_{13}ON$	139.100	4,5-Dimethyl-2-propyloxazole	UC2	HMDB0037869
	$C_8H_{13}ON$	139.100	4,5-Dimethyl-2-isopropyloxazole	UC2	HMDB0037871
	$C_8H_{13}ON$	139.100	4-Butyl-2-methyloxazole	UC2	HMDB0037855
	$C_8H_{13}ON$	139.100	2,4-Dimethyl-5-propyloxazole	UC2	HMDB0037868
	$C_8H_{13}ON$	139.100	4,5-Diethyl-2-methyloxazole	UC2	HMDB0037870
	$C_8H_{13}ON$	139.100	2-Pentyloxazole	UC2	HMDB0037818
	$C_8H_{13}ON$	139.100	7beta-Hydroxy-1-methylene-8alpha-pyrrolizidine	UC2	C00026172
	$C_8H_{13}ON$	139.100	2-propionyltetrahydropyridine	UC2	HMDB0034884
	$C_8H_{13}ON$	139.100	alpha-Phosphinylbenzyl alcohol	UC2	HMDB0029613
	$C_8H_{13}ON$	139.100	Supinidine	UC2	C00002120
	$C_8H_{13}ON$	139.100	Tropinone	UC2	C00037960

An aliphatic compound with the chemical formula $C_{12}H_{22}O_2$ was specifically detected in surfactin-treated *C. thermocellum* MVs (Table 1). Cis-2-decenoic acid was reported to decrease persister formation and revert dormant cells to a metabolically active state. Wang et al. demonstrated that three medium-chain unsaturated fatty acid ethyl esters (ethyl trans-2-decenoate, ethyl trans-2-octenoate, and ethyl cis-4-decenoate) decreased persister formation in *Escherichia coli*, *P. aeruginosa*, and *Serratia marcescens*, suggesting that fatty acid ethyl esters disrupt bacterial dormancy [65].

Some aliphatic acids function as diffusible signal factors (DSFs). These include cis-11-methyl-2-dodecenoic acid from *Xanthomonas campestris* and cis-2-dodecenoic acid from *Burkholderia cenocepacia*, among others [66]. DSFs are synthesized by and interact with a diverse group of microbes, including fungi, suggesting a broad conservation of cell-cell communication among these organisms [67–70]. Mutation of the DSF biosynthesis gene in *B. cenocepacia* results in substantially impaired growth in minimal medium [71]. Dean et al. demonstrated that *Burkholderia* DSF inhibits the formation and disperses *Francisella* biofilms. Furthermore, *Burkholderia* DSF was reported to upregulate the genes involved in iron acquisition in *F. novicida*, which increased siderophore production [72].

Subsequently, we compared the metabolites in the broth of *B. subtilis trpC2* and *trpC2 yxeJ* (Figure 2). Among the 3150 detected peaks, the intensities of 40 peaks were significantly higher in the broth of *B. subtilis trpC2* compared to that of *trpC2 yxeJ* (Table S4). The structures of 32 significantly detected compounds in *B. subtilis trpC2* broth were estimated by MS/MS analysis (Table 2 and Table S5). Diverse peptides were detected in *B. subtilis trpC2* broth. Nicotinamide reportedly enhances growth of both Gram-negative and Gram-positive bacteria, such as *M. avium*, *Propionibacterium acnes*, *S. aureus*, and *B. macerans* [73–76]. Indole-3-carboxaldehyde was shown to efficiently inhibit biofilm formation by *Vibrio cholerae* O1 [77]. The utilization of urocanic acid by *Pseudomonas* and *Aeromonas* strains has been reported [78,79]. Nopaline is a carbon and nitrogen source

metabolized by *Agrobacterium*. 6-Paradol was reported to have significant anti-adhesive activity against *S. aureus* [80].

Table 2. The constituents in *B. subtilis trpC2* broth detected by LC-MS/MS analysis.

No.	Formula	Exact Mass	Name	Database	Database ID
1938	$C_{12}H_{23}O_8N$	309.142	4-O-beta-D-Glucopyranosylfagomine	UC2	C00049954
1980	$C_{15}H_{39}O_2N_7S_3$	445.233		EX-HR2	
453	$C_{16}H_{30}O_6N_6$	402.223		EX-HR2	
2242	$C_{17}H_{31}O_4N_3$	341.231	Diprotin A	UC2	C00018579
1607	$C_{25}H_{40}O_7$	452.277	Briarellin P	UC2	C00044586
799	$C_{10}H_{20}O_3N_2S$	248.119	Valyl-Methionine	UC2	HMDB0029133
	$C_{10}H_{20}O_3N_2S$	248.119	Methionyl-Valine	UC2	HMDB0028986
510	$C_{11}H_{22}O_4N_4$	274.164	Glutaminyllysine	UC2	HMDB0028802
	$C_{11}H_{22}O_4N_4$	274.164	Lysyl-Gamma-glutamate	UC2	HMDB0028965
	$C_{11}H_{22}O_4N_4$	274.164	Lysyl-Glutamine	UC2	HMDB0028949
960	$C_{21}H_{40}O_1N_3P_3$	443.238		EX-HR2	
2575	$C_8H_{13}N_3P_2$	213.058		EX-HR2	
2345	$C_{19}H_{29}N_3O_4S_1$	395.188	V1M1F1	Pep1000	
2536	$C_{29}H_{36}O_5N_4$	520.269	Lotusine F	UC2	C00027221
	$C_{29}H_{36}O_5N_4$	520.269	Nummularine S	UC2	C00029150
2237	$C_{33}H_{44}O_{11}$	616.288	Neoazedarachin A	UC2	C00039833
	$C_{33}H_{44}O_{11}$	616.288	YM 47524	UC2	C00016365
2633	$C_{23}H_{55}O_{12}N_1P_2$	599.320		EX-HR2	
2673	$C_{46}H_{67}O_2N_{10}P_1S_1$	854.491		EX-HR2	
1271	$C_{27}H_{44}O_9$	512.299	Butyrolactol B	UC2	C00016754
	$C_{27}H_{44}O_9$	512.299	Integristerone B	UC2	C00048431
	$C_{27}H_{44}O_9$	512.299	Platenolide B mycarose	UC2	C00018288
162	$C_6H_6ON_2$	122.048	Nicotinamide	UC2	C00000209
	$C_6H_6ON_2$	122.048	2-Acetylpyrazine	UC2	HMDB0031861
1710	$C_{15}H_{24}O_4N_4$	324.180		EX-HR2	
211	$C_6H_9O_3N$	143.058	SQ 26517	UC2	C00018434
	$C_6H_9O_3N$	143.058	Trimethadione	UC2	HMDB0014491
	$C_6H_9O_3N$	143.058	6-Oxopiperidine-2-carboxylic acid	UC2	HMDB0061705
	$C_6H_9O_3N$	143.058	5-ethyl-5-methyl-2,4-oxazolidinedione	UC2	HMDB0061082
	$C_6H_9O_3N$	143.058	Vinylacetylglycine	UC2	HMDB0000894
	$C_6H_9O_3N$	143.058	Methyl pyroglutamate	UC2	C00051578
1258	$C_{22}H_{66}N_2P_2S_6$	612.303		EX-HR2	
994	$C_{20}H_{33}N_5O_8$	471.233	G2[L|I]1E1P1, G1A1V1E1P1, G1A1[L|I]1D1P1, G1T2P2, A2V1D1P1, A1S1T1P2, V1E1Q1P1, [L|I]1D1Q1P1, [L|I]1E1N1P1	Pep1000	
655	$C_{16}H_{27}N_5O_6$	385.196	G3V1P1, G1A3P1, G1V1N1P1, A2Q1P1	Pep1000	

Table 2. *Cont.*

No.	Formula	Exact Mass	Name	Database	Database ID
1034	$C_{10}H_{16}O_3N_2$	212.116	Butabarbital	UC2	HMDB0014382
	$C_{10}H_{16}O_3N_2$	212.116	L-prolyl-L-proline	UC2	HMDB0011180
	$C_{10}H_{16}O_3N_2$	212.116	Butethal	UC2	HMDB0015442
457	$C_{32}H_{48}O_5N_2S_1$	572.328		EX-HR2	
2755	C_9H_7ON	145.053	Indole-3-carboxaldehyde	UC2	C00000112
	C_9H_7ON	145.053	2-Quinolone	UC2	C00044432
2680	$C_{67}H_{108}O_6N_2S_5$	1196.681		EX-HR2	
115	$C_6H_6O_2N_2$	138.043	4-Methoxylonchocarpin	UC2	HMDB0031338
	$C_6H_6O_2N_2$	138.043	2-Aminonicotinic acid	UC2	HMDB0061680
	$C_6H_6O_2N_2$	138.043	Urocanic acid	UC2	HMDB0062562
	$C_6H_6O_2N_2$	138.043	Nicotinamide N-oxide	UC2	HMDB0002730
2949	$C_{11}H_{21}ON$	183.162	Tecostanin	UC2	C00001984
	$C_{11}H_{21}ON$	183.162	Incarvilline	UC2	C00050294
1600	$C_{20}H_{57}O_4N_9S_3$	583.370		EX-HR2	
1727	$C_{11}H_{20}O_6N_4$	304.138	Nopaline	UC2	C00001548
526	$C_{21}H_{56}O_{14}N_{10}P_2$	734.345		EX-HR2	
3061	$C_{17}H_{26}O_3$	278.188	1-Acetoxy-3,15-epoxygymnomitrane	UC2	C00021889
	$C_{17}H_{26}O_3$	278.188	Litsealactone B	UC2	C00044889
	$C_{17}H_{26}O_3$	278.188	9beta-Acetoxy-10(14)-aromadendren-4beta-ol	UC2	C00021235
	$C_{17}H_{26}O_3$	278.188	Furoscrobiculin C	UC2	C00021531
	$C_{17}H_{26}O_3$	278.188	[S-[R *,S *-(E)]]-6-[6-(Acetyloxy)-1,5-dimethyl-4-hexenyl]-3-methyl-2-cyclohexen-1-one	UC2	C00011679
	$C_{17}H_{26}O_3$	278.188	Panaxytriol	UC2	C00030923
	$C_{17}H_{26}O_3$	278.188	Panaxacol	UC2	HMDB0039251
	$C_{17}H_{26}O_3$	278.188	Parahigginol C	UC2	C00049252
	$C_{17}H_{26}O_3$	278.188	[1S-(1R *,2E,4R *,5R *,6E,10R *)]-3, 7, 11, 11-Tetramethylbicyclo [8.1.0]undeca-2,6-diene-4,5-diol 5-acetate	UC2	C00012427
	$C_{17}H_{26}O_3$	278.188	Isoobtusilactone	UC2	C00050966
	$C_{17}H_{26}O_3$	278.188	8beta-Acetoxy-9beta-hydroxyverboccidenten	UC2	C00020229
	$C_{17}H_{26}O_3$	278.188	Lincomolide B	UC2	C00047968
	$C_{17}H_{26}O_3$	278.188	[1S-(1R *,2E,4R *,5R *,6E,10R *)]-3, 7, 11, 11-Tetramethylbicyclo[8.1.0]undeca-2,6-diene-4,5-diol 4-acetate	UC2	C00012428
	$C_{17}H_{26}O_3$	278.188	4alpha-Hydroxygymnomitryl acetate	UC2	C00021894
	$C_{17}H_{26}O_3$	278.188	4-[(4E)-3-hydroxydec-4-en-1-yl]-2-methoxyphenol	UC2	HMDB0137260
	$C_{17}H_{26}O_3$	278.188	Ro 09-1544	UC2	C00017230

Table 2. *Cont.*

No.	Formula	Exact Mass	Name	Database	Database ID
	$C_{17}H_{26}O_3$	278.188	6-Paradol	UC2	C00002764
	$C_{17}H_{26}O_3$	278.188	Paralemnolin D	UC2	C00030924
	$C_{17}H_{26}O_3$	278.188	Fenoksan; Fenoxan; Fenozan; Fenozan acid; Irganox 1310; Phenosan; Phenoxan; Phenozan	UC2	C00016759
	$C_{17}H_{26}O_3$	278.188	[4aR-(4aalpha,5alpha,8abeta,9abeta)]-9a-Ethoxy-4a, 5, 6, 7, 8, 8a, 9, 9a-octahydro-3,4a,5-trimethyl-naphtho[2,3-b]furan-2(4H)-one	UC2	C00017405
	$C_{17}H_{26}O_3$	278.188	Petasipalin B	UC2	C00020246
	$C_{17}H_{26}O_3$	278.188	4-epi-7alpha,15-dihydroxypodocarp-8(14)-en-13-one;(-)-4-epi-7alpha,15-dihydroxypodocarp-8(14)-en-13-one	UC2	C00035020
	$C_{17}H_{26}O_3$	278.188	3-[(Acetyloxy)methyl]-6-(1,5-dimethyl-4-hexenyl)-2-cyclohexen-1-one	UC2	C00011682
	$C_{17}H_{26}O_3$	278.188	Cyclokessyl acetate	UC2	C00020354
	$C_{17}H_{26}O_3$	278.188	8-Acetoxy-4-acoren-3-one	UC2	HMDB0030974
832	$C_{12}H_{34}O_3N_6S_3$	406.185		EX-HR2	

In this study, we demonstrated that constituents in membrane vesicles significantly promoted the growth rate of *C. thermocellum*. Additionally, the MV constituents with growth stimulation were described by LC-MS/MS analysis. These findings suggest that the constituents in membrane vesicles could promote *C. thermocellum* growth, leading to improved efficiency of cellulosic biomass utilization.

Supplementary Materials: The following are available online at https://www.mdpi.com/2076-2607/9/3/593/s1, Figure S1: MVs from *C. thermocellum* and *B. subtilis*. Figure S2: Effect of *myo*-inositol on *C. thermocellum* growth. Figure S3: Effect of pyrrolo-quinoline quinone on *C. thermocellum* growth. Table S1: Genotypes of *B. subtilis* genome deletion mutants. Table S2: *B. subtilis* single gene deletion mutants used in this study. Table S3: Intensities of detected peaks in the MV fraction of *C. thermocellum* by LC-MS/MS. Table S4: Intensities of the detected peaks in cell-free *B. subtilis trpC2* broth by LC-MS/MS. Table S5: Structures of constituents detected by LC-MS/MS in this study.

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