Genome-wide transcriptomic analysis of *n*-caproic acid production in *Ruminococcaceae* bacterium CPB6 with lactate supplementation

Yong Tao (taoyong@cib.ac.cn)
CIB CAS: Chengdu Institute of Biology
Shaowen Lu
Chengdu Institute of Biology
Yi Wang
Auburn University
Cuicui Wei
Chengdu Institute of Biology
Hong Jin
Chengdu Medical College
Xiaohong He
Chengdu Institute of Biology

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Abstract

Background

n-Caproic acid (CA) is gaining increased attention due to its high value as a chemical feedstock. *Ruminococcaceae* bacterium strain CPB6 is an anaerobic mesophilic bacterium that is highly prolific in its ability to perform chain elongation of lactate to CA. However, little is known about the genome-wide transcriptional analysis of strain CPB6 for CA production triggered by the supplementation of exogenous lactate.

Results

In this study, 0.5% lactate was supplemented into the fermentation with *Ruminococcaceae* bacterium CPB6 for CA production. Compared to the control (without lactate supplementation), lactate supplementation led to earlier CA production and higher final CA titer and productivity. Transcriptional analysis was carried out using RNA-Seq for the culture with and without lactate supplementation (two groups) at growth and stationary phases, respectively. It has been indicated that 295 genes whose changes in expression patterns were substrate and/or growth dependent. These genes cover crucial functional categories. Specifically, 5 genes responsible for the reverse β-oxidation pathway, 11 genes encoding ATP-binding cassette (ABC) transporters, 6 genes encoding substrate-binding protein (SBP) and 4 genes encoding phosphotransferase system (PTS) transporters were strikingly upregulated in response to the addition of lactate. These genes would be candidates for future studies aiming at understanding the regulatory mechanism of lactate conversion into CA, as well as for the improvement of CA production in strain CPB6.

Conclusions

This study suggested that lactate supplementation can promote CA production by altering the expression patterns of genes involved in the essential metabolic pathways, such as central pyruvate metabolism, the reverse β-oxidation pathway, ABC and PTS transports. The findings presented herein reveal unique insights into the biomolecular effects of lactate on CA production at the gene transcriptional level.

Background

The increasing demand for fuels and chemicals and the scarcity of fossil resources necessitate the development of sustainable and innovative strategies for the industrial production. Organic residual streams (e.g., food waste and brewery wastewater) have great potential to be employed as feedstock for the production of biofuels and biochemicals [1, 2].

n-Caproic acid (CA) is a medium chain fatty acid of 6-carbon, which has recently gained considerable attention due to its high value as a fuel and chemical feedstock [3]. Biosynthesis of CA can be achieved in some anaerobic bacteria (e.g., *Clostridium kluyveri*) via the chain elongation pathway with ethanol as reduced substrate (electron donor) [4, 5], in which the oxidation of ethanol provides energy and acetyl-CoA for the reverse β-oxidation pathway [6]. Many studies show that the addition of ethanol during the acidification of wastes can promote chain elongation, and lead to higher volumetric production rates and a high CA selectivity [7, 8], indicating that ethanol is an efficient reduced substrate for CA production. In addition, researchers have explored other chemicals used as electron donors for CA production, including hydrogen [9], methanol [10], propanol [11], and *D*-galactitol [12].

Recently, lactate was showed to be a potential alternative to ethanol for the production of CA by reactor microbiome systems [13, 14]. More recently, our study demonstrates that CA is efficiently produced from lactate by the pure culture of a *Ruminococcaceae* bacterium CPB6 isolated from a fed-batch reactor microbiome [15]. Strain CPB6 is an anaerobic mesophilic bacterium that is highly prolific in its ability to perform chain elongation of lactate to caproate, and can produce CA (C6) from lactate (as electron donor) with C2-C4 carboxylic acids as electron acceptors, and heptoic acid (C7) with C3-C5 carboxylic acids [16]. The phylogenic analysis based on 16S rRNA sequences and the whole genome showed that strain CPB6 might belong to a new clade (genus) of the family Ruminococcaceae, it is thus tentatively christened with the name Ruminococcaceae bacterium CPB6 [15, 17]. Furthermore, we identified sets of genes correlated with the chain elongation pathway by sequencing and annotating the whole genome of the CPB6 strain. However, to date, very little information is available on genome-wide transcriptomic analysis of the CPB6 strain for CA production using lactate as the electron donor, which is essential to understand the effect of lactate on the metabolic pathway shift for CA production at the molecular level and thus elucidate proper strategies for further strain improvement.

RNA-Seq is a powerful method to elucidate the metabolism and identify specific genes/enzymes for the particular interesting metabolic pathways [18, 19]. In the present study, RNA-Seq for the transcriptomic analysis of strain CPB6 was carried out to investigate the effect of lactate supplementation on cell growth, CA production and other essential metabolisms. The identified key genes herein provide candidates for the metabolic engineering in the future in order to develop more robust strains based on CPB6 for enhanced production of CA and relevant biochemicals.

Results And Discussion

Growth and the production of CA

As shown in Fig. 1a, cells took approximately 18 h to grow to the stationary phase. Although the maximum OD_{600} of the culture with lactate supplementation was slightly higher than that of the control (without lactate supplementation) at the stationary phase (1.25 vs 1.16), both cultures showed similar growth kinetics, indicating that lactate supplementation had little impact on cell growth. The CA production was started to be observed in the culture with lactate

supplementation after 6 h of cultivation, and the CA titer continued to increase and reached 1717.2 mg L^{-1} at 21 h (Fig. 1b), while CA production was not detected until 15 h of cultivation in the control, the CA titer of which only reached 618 mg L^{-1} at 21 h. In both cultures, while more significantly in the culture with lactate supplementation, the main endproduct was CA together with small amount of butyrate, suggesting that the carbon flux of acetyl-CoA may be inclined to be channelled to CA synthesis, especially in the presence of lactate. This is consistent with our previous studies [15].

In sum, compared to the control without lactate supplementation, the lactate supplementation had little effect on the cell growth, but led to earlier initiation for CA production (6 vs 15 h), higher final CA titer (1717 vs 618 mg L^{-1}) and higher CA productivity (81.8 vs 29.4 mg L^{-1} h^{-1}).

RNA-Seq statistics

Samples were taken for RNA-Seq analysis from both the growth (12 h) and stationary (18 h) phases for both the culture with lactate supplementation and the control. For each culture, independent biological triplicates (a, b, and c) were included (Table 1). Therefore, a total of twelve samples were taken for cDNA libraries construction and sequencing on the Illumina HiSeq platform (Illumina, San Diego, USA). The number of raw reads generated from the sequencing for each library was from 15.7 to 23.5 million (Table S1 in Additional files). A total of 224 Mb sequence reads from 12 cDNA libraries were mapped to the genome of strain CPB6. Only those reads that mapped unambiguously to the CPB6 genome were used for further analysis.

Table 1
Summary of RNA-Seq sequencing and data analysis results

Sample Name	L1			L2			C1			C2	
	a	b	c	a	b	c	a	b	c	a	b
Total reads	22982792	18614536	18087962	16881648	16844058	15343814	18684112	19108148	19099082	19909258	23032
No. of read mapped	22684858	18390709	17865180	16713319	16606726	15098988	18437261	18893873	18905208	19631686	22572
Ratio of reads mapped (%)	98.7	98.8	98.77	99	98.59	98.4	98.68	98.88	98.98	98.61	98.01
No. of unique reads mapped	22339068	17915954	17559600	16540060	16324768	14862898	18050316	18505697	18508661	19247256	22187
No. of genes with detectable expression	1969	1968	1968	1968	1969	1968	1968	1968	1968	1968	1969
Range in expression levels (TPM)	8.3–2.7 × 10^4	8.0-1.7 × 10^4	11.5–1.7 × 10^4	3.2–5.3 × 10^4	26.4–3.7 × 10^4	6.6–7.3 × 10^4	4.3-2.0 × 10^4	3.8–1.8 × 10^4	4.1–1.9 × 10^4	5.2–2.4 × 10^4	10.8– × 10^4
L1: cell culture with lactate supplementation from the growth phase;											
L2: cell culture with lactate supplementation from the stationary phase;											
C1: Control culture without lactate supplementation from the growth phase;											
C2: Control culture without lactate supplementation from the stationary phase.											
a, b and c represented the biological triplicate samples.											

Overall, out of reads derived from all the samples, 15.1 to 22.7 million reads were unambiguously mapped to the CPB6 genome, and over 98% reads were mapped (Table 1). A total of 1968/1969 out of 2045 protein-coding genes had detectable expression in both conditions (with or without lactate supplementation) (Table 1), indicating that the RNA-Seq analysis in this study achieved comprehensive coverage of the CPB6 transcriptome. The transcription levels (the number of transcripts per million, TPM) of most active protein-coding genes were in the range of 3.2×10^4–7.3×10^4.

As illustrated in Fig. 2, the gene expression could be classified into four levels: low (TPM < 30), moderate (TPM: 30–150), high (TPM: 150–1000), and very high (TPM > 1000). The number of genes at some specific expression levels was significantly different for the two cultures (with or without lactate supplementation). For the growth phase, there were slightly more genes in the moderate and high and very high expression level in the culture with lactate supplementation than in the control, but lowly expressed genes were significantly decreased. While for the stationary phase, the culture with lactate supplementation had more genes in the moderate expression level, but fewer genes in the high and very high expression level. These results suggested that more changes in gene expression are triggered by the addition of lactate at the stationary phases.

Functional annotation and classification

In the transcriptome of strain CPB6, a total of 1122 expressed genes were allocated into three primary Gene Ontology (GO) categories (Fig. 3), including the category of biological process (601 genes), cellular component (524 genes), and molecular function (916 genes). In each category, the genes were further

assigned into 28 functional groups, such as metabolic process (478 genes), cellular process (440 genes), cell part (307 genes), membrane part (297 genes), catalytic activity (654 genes), binding (561genes), and etc. The analysis of the genes based on the KEGG annotation identified a total of 1046 unigenes allocated into six primary KEGG categories including 35 subcategories (Figure. S1 in Additional files). The top 5 categories of genes were: carbohydrate metabolism, amino acid metabolism, membrane transport, translation, and metabolism of cofactors and vitamins, respectively. The top 10 enriched pathways included ABC transporters (58 genes), Amino sugar and nucleotide sugar metabolism (27 genes), Starch and sucrose metabolism (26 genes), Glycolysis / Gluconeogenesis (24 genes), Purine metabolism (40 genes), Pyrimidine metabolism (37 genes), Peptidoglycan biosynthesis, Aminoacyl-tRNA biosynthesis (28 genes), Ribosome (52 genes), and Quorum sensing (26 genes). The analysis based on the Clusters of Orthologous Groups (COGs) showed that 1785 unigenes were allocated to four primary COG categories containing 20 COG functional clusters. The top 5 annotated genes corresponding to the KEGG pathways were Replication recombination and repair (155 genes), Translation, ribosomal structure and biogenesis (142 genes), Amino acid transport and metabolism (126 genes), Carbohydrate transport and metabolism (110 genes), and Inorganic ion transport and metabolism (Figure S2 in Additional files).

Differential expression of global genes

The correct identification of differentially expressed genes (DEGs) between specific conditions is a key in the understanding phenotypic variation of organisms under environmental stress. As shown in Table 2, there were only 34 differentially expressed genes (DEGs, FC ≥ 2 or ≤ 0.5 with P-value < 0.05) identified during the growth phase between two culture conditions, of which 15 genes were upregulated, and 19 genes were downregulated more than two-fold. In addition, a total of 245 DEGs were identified at the stationary phase, of which 123 genes were significantly upregulated and 122 genes were downregulated (Table S2 in Additional files). It suggested that the addition of lactate led to differences in gene expression between the two cultures (with and without lactate supplementation) during different growth phases.

Table 2
The differentially expressed genes in culture with/without lactate supplementation during the growth phase

No.	Gene_ID	Gene name	Gene description	TPM		FC (L1/C1)	P-value
				C1	L1		
15 upregulated genes (FC ≥ 2.0); all statistically significant (P < 0.05)							
1	B6259_RS06365	atoB	acetyl-CoA C-acetyltransferase	1224	5204	3.45	7.9E-39
2	B6259_RS06360	crt	enoyl-CoA hydratase	795	3434	3.46	2.4E-33
3	B6259_RS06355	hbd	3-hydroxybutyryl-CoA dehydrogenase	1418	6306	3.49	2.3E-27
4	B6259_RS07830	pta	phosphate acetyltransferase	271	666	2.09	4.3E-24
5	B6259_RS00440	-	methionine ABC transporter ATP-binding protein	51	504	5.25	6.2E-18
6	B6259_RS00450	-	metal ABC transporter substrate-binding protein	30	699	5.69	5.9E-15
7	B6259_RS00445	-	ABC transporter permease	27	446	5.17	6.9E-14
8	B6259_RS08190	cysK	cysteine synthase A	390	7426	4.07	6.4E-10
9	B6259_RS08440	-	unknown function	1048	3598	2.33	5.2E-06
10	B6259_RS06010	-	hypothetical protein	21	89	2.52	8.1E-06
11	B6259_RS07140	-	hypothetical protein	154	470	2.17	3.0E-05
12	B6259_RS01720	cadA	cadmium-translocating P-type ATPase	22	66	2.16	3.9E-05
13	B6259_RS06870	-	Hsp20/alpha crystallin family protein	315	1102	2.21	1.6E-04
14	B6259_RS00455	pepT	peptidase T	37	576	2.26	2.0E-04
15	B6259_RS02585	bdh	butanol dehydrogenase	82	242	2.04	3.1E-04
19 downregulated genes (FC ≤ 0.5); all statistically significant (P < 0.05)							
1	B6259_RS08515	-	peptide ABC transporter substrate-binding protein	98	53	0.48	1.7E-23
2	B6259_RS09280	-	PTS glucose transporter subunit IIA	1200	484	0.37	5.5E-23
3	B6259_RS09735	ilvH	acetolactate synthase small subunit	564	302	0.48	9.4E-19
4	B6259_RS06995	-	hypothetical protein	276	43	0.20	2.7E-18
5	B6259_RS08565	-	hypothetical protein	143	79	0.50	2.3E-13
6	B6259_RS07000	-	sugar ABC transporter permease	113	31	0.30	9.2E-13
7	B6259_RS01525	-	unknown function	2683	1010	0.37	8.4E-12
8	B6259_RS03200	-	unknown function	2683	1010	0.37	8.4E-12
9	B6259_RS07010	tag	glycosylase	144	46	0.34	9.9E-11
10	B6259_RS07005	-	carbohydrate ABC transporter permease	90	33	0.37	1.1E-09
11	B6259_RS01865	-	DUF2520 domain-containing protein	260	85	0.36	6.7E-09
12	B6259_RS01880	panD	aspartate 1-decarboxylase	444	156	0.37	9.5E-09
13	B6259_RS01870	panB	3-methyl-2-oxobutanoate hydroxymethyltransferase	314	105	0.37	1.1E-08
14	B6259_RS01875	panc	pantoate-beta-alanine ligase	350	115	0.37	1.2E-08
15	B6259_RS01760	-	hypothetical protein	820	369	0.44	8.9E-08
16	B6259_RS02315	-	basic amino acid ABC transporter substrate-binding protein	147	79	0.50	1.8E-07
17	B6259_RS00100	fruK	1-phosphofructokinase	1256	276	0.35	1.7E-06
18	B6259_RS00095	-	PTS fructose transporter subunit IIC	1273	372	0.37	1.8E-06
19	B6259_RS00105	-	DeoR/GlpR transcriptional regulator	1304	278	0.36	3.6E-06
L1: lactate-supplemented cells at growth phase							
C1: no-lactate-supplemented cells (controls) at growth phase							

The COG distribution of the DEGs at both the growth phase and the stationary phase was illustrated in Fig. 4. It revealed the potential genes related to the pathways and bioprocesses for the utilization of lactate for CA production. At the growth phase, predominant number of DEGs belong to the 'inorganic ion transport and metabolism, [P]' group and the 'carbohydrate transport and metabolism, [G]' group. While at stationary phase, most upregulated genes belong to

the ‘carbohydrate transport and metabolism, [G]’ group, which play important roles for the degradation and utilization of carbohydrate substrates [20, 21]. It was worth noting that a large number of DEGs (for both upregulated and downregulated ones) fall into the ‘function unknown, [S]’ group.), meaning that their functions are unknown. This may be because CPB6 belongs to a novel species or clade (*Clostridium* cluster IV) of the family *Ruminococcaceae*, sharing low 16S rRNA sequence similarity (< 92.6%) with the other organisms in GenBank and RDP [15, 17].

Cluster analysis of the DEGs between the culture with lactate supplementation and the control was showed in Fig. 5. The results showed that the gene expression of the triplicate (a, b and c) of each sample demonstrated very similar expression patterns. According to Fig. 5, L1 (with lactate supplementation at the growth phase) cluster was most closely with C1 (without lactate supplementation at the growth phase) cluster, indicating the presence of a small amount of DEGs caused by the addition of lactate at this phase. However, the distinct difference of gene expression was observed between the two cultures at the stationary phase (L2 vs C2), Further, venn diagram showed that 295 genes whose changes in expression patterns were substrate and/or growth dependent, of which 31 genes were substrate (lactate) dependent, 228 genes were growth dependent, and 36 genes were substrate-growth dependent (Fig. 6a). Specifically, 11 and 20 lactate-dependent genes were significantly upregulated and downregulated, as well as 98 and 130 growth-dependent genes were significantly upregulated and downregulated, respectively (Fig. 6b). It was suggested that the differences in gene expression are stronger for stationary phase vs growth phase than for plus/minus lactate. Similar results was observed for *Clostridium thermocellum*, in which growth rate had stronger effects on gene expression than substrate type (insoluble cellulose vs soluble cellobiose) [22]. These DEGs were described in detail in later section.

Expression of glycolysis genes

An overview of the metabolic pathway in strain CPB6 and the expression levels of genes involved in key metabolic processes with their fold change (FC) were shown in Fig. 6 and Table 3. Generally, in clostridia, glucose is converted into pyruvate via glycolysis, and the produced pyruvate is further converted into acetyl-CoA for the production of acetate and butyrate at acidogenic phase [23–25]. In the present study, the expression of genes for glycolysis was detected in the CPB6 transcriptome, which reinforced its genome annotation [17]. Most glycolytic genes were expressed at a relatively high level (TPM > 150) between the culture with lactate supplementation and the control, but there was no significant difference between them at the growth phase. Three glycolytic genes exhibited different expression patterns at the stationary phase. Gene encoding phosphofructokinase (PFK, B6259_RS06095) was significantly downregulated, while genes encoding glucose-1-phosphate adenylyltransferase (GlgC, B6259_RS09035) and 1, 4-alpha-glucan branching enzyme (GlgB, B6259_RS09040) were upregulated by 4.58 and 3.42-fold in the culture with lactate supplementation than in the control, respectively. GlgB and GlgC are typically associated with glycogen synthesis, why expression of these genes be affected by lactate supplementation remains unclear. Overall, the addition of lactate has little impact on the expression of glycolytic genes.

Table 3
The differentially expressed genes within the important metabolic pathways in culture with/without lactate supplementation

Functional description	Gene_ID	TPM of genes from culture with lactate supplementation[a]		TPM of genes from the Control[a]		RNA relative fold change (Treatment/Control)	
		12 h	18 h	12 h	18 h	12 h	18 h
Glycolysis							
PTS-Glc-EIIA, PTS glucose transporter subunit IIA	B6259_RS09280	484	260	1200	517	**0.37**[b]	0.62
GlgC, glucose-1-phosphate adenylyltransferase	B6259_RS09035	153	1323	236	241	0.57	**4.58**[c]
GlgB, 1,4-alpha-glucan branching enzyme	B6259_RS09040	194	745	236	201	0.72	**3.42** [c]
sugar phosphate isomerase/epimerase	B6259_RS06500	181	175	150	233	1.05	0.88
PGM, phosphoglucomutase	B6259_RS09200	95	189	127	113	0.66	1.80
GPI, glucose-6-phosphate isomerase	B6259_RS04825	2015	1789	1833	1818	0.96	1.12
PFK, phosphofructokinase	B6259_RS06095	426	97	580	516	0.66	**0.23** [b]
ALDO, fructose-bisphosphate aldolase	B6259_RS00415	749	402	800	891	0.83	0.57
TPI, triose-phosphate isomerase	B6259_RS09105	224	229	315	493	0.65	0.58
GapA, glyceraldehyde phosphate dehydrogenase	B6259_RS09050	5322	4284	4790	7732	0.98	0.70
PGK, phosphoglycerate kinase	B6259_RS09100	523	524	705	1029	0.67	0.65
gpmI, 2,3-bisphosphoglycerate-independent phosphoglycerate mutase	B6259_RS09110	203	200	284	469	0.66	0.55
ENO, phosphopyruvate hydratase	B6259_RS04810	41	65	30	52	1.14	1.46
PK, pyruvate kinase	B6259_RS02335	254	102	293	228	0.77	1.46
Central pyruvate metabolism							
PpdK, pyruvate phosphate dikinase	B6259_RS00120	1301	823	1163	1535	0.99	0.69
Pfor, pyruvate: ferredoxin (flavodoxin) oxidoreductase	B6259_RS09135	4329	4382	2044	1225	1.83	**3.26** [c]
PCK, phosphoenolpyruvate carboxykinase	B6259_RS09255	368	159	554	1031	0.62	**0.23** [b]
PflD, formate C-acetyltransferase	B6259_RS09900	98	188	107	471	0.83	0.54
ADH, alcohol dehydrogenase	B6259_RS03100	200	116	163	159	1.07	0.84
Incomplete TCA cycle							
CS, citrate synthase, citrate lyase	B6259_RS03360	936	187	543	642	1.42	**0.39** [b]
ACO, aconitate hydratase	B6259_RS05795	227	162	153	201	1.27	0.94
IDH, isocitrate dehydrogenase	B6259_RS05805	237	232	197	291	1.04	0.93
FUM, fumarate hydratase	B6259_RS07270	310	186	260	437	1.04	**0.49** [b]
PCK, phosphoenolpyruvate carboxykinase	B6259_RS09255	368	159	554	1031	0.62	**0.23** [b]
Hydrogen production							
HydE, [FeFe] hydrogenase H-cluster	B6259_RS02550	113	73	174	44	1.44	**2.24** [c]
HydF, [FeFe] hydrogenase H-cluster	B6259_RS09690	67	40	50	24	1.43	1.17
Lactate fermentation pathway							
D-ldh, D-lactate dehydrogenase	B6259_RS06770	76	88	58	108	1.14	0.95
L-ldh, L-lactate dehydrogenase	B6259_RS09845	79	111	119	295	0.59	**0.44** [b]

a, Data presented as mean of independent triplicates

b, Significantly upregulated (FC ≥ 2.0, $p < 0.05$)

c, Significantly downregulated (FC ≤ 0.5, $p < 0.05$)

Functional description	Gene_ID	TPM of genes from culture with lactate supplementation[a]		TPM of genes from the Control[a]		RNA relative fold change (Treatment/Control)	
		12 h	18 h	12 h	18 h	12 h	18 h
Acetate fermentation pathway							
PTA, phosphate acetyltransferase	B6259_RS07830	666	697	271	321	**2.09** [c]	**2.23** [c]
ACK, acetate kinase	B6259_RS03430	290	297	288	233	0.88	1.41
The reverse β-oxidation pathway							
AtoB, acetyl-CoA C-acetyltransferase	B6259_RS06365	5204	9909	1224	1077	**3.45** [c]	**6.31** [c]
HBD, 3-hydroxybutyryl-CoA dehydrogenase	B6259_RS06355	6306	13975	1418	1022	**3.49** [c]	**8.59** [c]
CRT, enoyl-CoA hydratase	B6259_RS06360	3434	7348	795	647	**3.46** [c]	**7.34** [c]
BCD1, butyryl-CoA dehydrogenase	B6259_RS01790	3278	3104	3787	3014	0.76	1.19
BCD2, butyryl-CoA dehydrogenase	B6259_RS02600	42	313	41	66	0.90	**4.49**[c]
EtfA, electron transfer flavoprotein subunit alpha	B6259_RS01785	2657	2968	3175	2572	0.73	1.31
EtfB, electron transfer flavoprotein subunit beta	B6259_RS01780	3996	4830	4357	4169	0.71	1.31
CAT, butyryl-CoA: acetate CoA-transferase	B6259_RS06345	521	1497	283	330	1.55	**4.01**[c]
Fructose fermentation pathway							
PPF, 1-phosphofructokinase	B6259_RS00100	276	2174	1256	239	0.35	**7.33**
Starch and sucrose metabolism							
PYG, glycogen phosphorylase	B6259_RS00300	90	163	121	103	0.66	1.71
MalQ, 4-alpha-glucanotransferase	B6259_RS07805	53	270	55	61	0.85	**4.34**
PGM, Phosphoglucomutase	B6259_RS09200	95	189	127	113	0.66	1.80
Energy conservation							
energy-coupling factor transporter ATPase	B6259_RS02790	141	104	117	159	1.04	0.76
electron transport complex protein RnfA	B6259_RS06245	230	162	357	362	0.58	0.52
Sporulation							
stage 0 sporulation protein	B6259_RS00205	379	279	233	252	0.97	0.82
stage II sporulation protein D	B6259_RS09065	98	59	96	53	0.97	1.29
stage III sporulation protein AD	B6259_RS03910	126	54	87	26	1.67	1.27
stage IV sporulation protein A	B6259_RS04975	65	30	58	16	1.42	1.58
stage V sporulation protein AC	B6259_RS09190	89	46	77	40	0.99	1.42
stage V sporulation protein AD	B6259_RS09195	69	41	66	34	1.05	1.57
stage V sporulation protein AE	B6259_RS00500	292	226	200	167	1.15	1.02
sporulation transcription factor Spo0A	B6259_RS05505	127	115	83	106	0.94	0.94
sporulation transcriptional regulator SpoIIID	B6259_RS01550	213	188	140	207	0.79	1.01
sporulation protein YtfJ	B6259_RS04885	291	183	145	159	1.00	0.65
Transporter genes							
ABC transporter permease	B6259_RS00445	446	274	27	235	**5.17** [c]	1.27
metal ABC transporter	B6259_RS00450	699	628	30	457	**5.69** [c]	1.52

a, Data presented as mean of independent triplicates

b, Significantly upregulated (FC ≥ 2.0, $p < 0.05$)

c, Significantly downregulated (FC ≤ 0.5, $p < 0.05$)

Functional description	Gene_ID	TPM of genes from culture with lactate supplementation[a]		TPM of genes from the Control[a]		RNA relative fold change (Treatment/Control)	
		12 h	18 h	12 h	18 h	12 h	18 h
ABC transporter permease	B6259_RS02670	296	130	441	387	0.60	**0.40** [b]
ABC transporter permease	B6259_RS02665	180	96	258	231	0.62	**0.48** [b]
carbohydrate ABC transporter permease	B6259_RS07005	33	124	90	41	**0.37** [b]	**3.51** [c]
carbohydrate ABC transporter permease	B6259_RS07905	71	744	71	40	0.90	**12.71** [c]
carbohydrate ABC transporter permease	B6259_RS07810	39	229	40	45	0.85	**5.48** [c]
carbohydrate ABC transporter permease	B6259_RS02030	26	71	16	39	1.35	**2.14** [c]
sugar ABC transporter permease	B6259_RS07910	82	1175	88	50	0.86	**14.74** [c]
sugar ABC transporter permease	B6259_RS03335	39	401	26	61	1.30	**5.61** [c]
sugar ABC transporter permease	B6259_RS07815	36	197	37	49	0.85	**4.34** [c]
sugar ABC transporter permease	B6259_RS07000	31	135	113	38	**0.30** [b]	**3.48** [c]
iron ABC transporter permease	B6259_RS00320	53	1278	77	89	0.62	**10.05** [c]
ABC transporter ATP-binding protein	B6259_RS00440	504	277	51	239	**5.25** [c]	1.39
ABC transporter ATP-binding protein	B6259_RS00325	60	2032	94	100	0.58	**11.14** [c]
ABC transporter ATP-binding protein	B6259_RS08900	153	682	233	214	0.58	**3.13**
ABC transporter ATP-binding protein	B6259_RS07940	190	40	259	94	0.66	0.42
carbohydrate ABC transporter substrate-binding protein	B6259_RS07915	216	3434	203	103	0.93	**14.51**
maltose ABC transporter substrate-binding protein	B6259_RS03345	30	501	22	37	1.15	**7.65**
ABC transporter substrate-binding protein	B6259_RS07820	372	1913	451	344	0.73	**4.63**
sugar ABC transporter substrate-binding protein	B6259_RS02005	30	93	29	48	0.92	**2.29**
peptide ABC transporter substrate-binding protein	B6259_RS08515	53	78	98	369	**0.48**	**0.28**
peptide ABC transporter substrate-binding protein	B6259_RS02685	1385	819	1442	2222	0.85	**0.50**
ABC transporter ATP-binding protein	B6259_RS02660	238	119	369	320	0.58	**0.45**
ABC transporter ATP-binding protein	B6259_RS07940	190	58	259	166	0.66	**0.42**
PTS fructose transporter subunit IIC	B6259_RS00095	372	2117	1273	485	**0.37**	**3.87**
PTS glucose transporter subunit IIA	B6259_RS09280	484	260	1200	517	**0.37**	0.62
PTS β-glucoside transporter subunit IIABC	B6259_RS01415	81	760	134	141	0.54	**4.70**
PTS mannitol transporter subunit IICBA	B6259_RS00370	29	89	19	44	1.26	**2.34**
ferrous iron transport protein B	B6259_RS03880	471	389	531	150	0.81	**2.72**
a, Data presented as mean of independent triplicates							
b, Significantly upregulated (FC ≥ 2.0, $p < 0.05$)							
c, Significantly downregulated (FC ≤ 0.5, $p < 0.05$)							

Expression of butyrate- and CA-producing genes

The bioproduction of CA is a well-known chain elongation process from acetate (C2) to caproate (C6) via the reverse β-oxidation pathway, in which an acetyl-CoA (from ethanol) unit is combined with another acetyl-CoA (from acetate), and consequently C2 is elongated to C4, and further C4 is elongated to C6 [4]. Thus, acetyl-CoA is a key intermediate of flux distribution for the chain elongation. The conversion of pyruvate into acetyl-CoA is mainly catalyzed by the pyruvate: ferredoxin (flavodoxin) oxidoreductase (Pfor) that is a flavodoxin- and NADPH-dependent enzyme [6, 26]. Here, the Pfor gene (B6259_RS09135) maintained at very high expression level (TPM > 4000) in the culture with lactate supplementation (Table 3), which were upregulated by 1.83- and 3.26-fold

than that in the control in the growth and stationary phase, respectively (Fig. 7). High-level expression of the Pfor gene would result in increased acetyl-CoA, which provides the high amount of acetyl-CoA for chain elongation from acetate to butyrate and CA.

Key enzymes involved in the butyrate formation include acetyl-CoA C-acetyltransferase (AtoB), 3-hydroxybutyryl-CoA dehydrogenase (HBD), enoyl-CoA hydratase (CRT), NAD-dependent butyryl-CoA dehydrogenase (BCD), Electron transfer flavoprotein (Etf) and butyryl-CoA: acetate CoA transferase (CAT) [6, 17]. Here, genes encoding AtoB (B6259_RS06365), CRT (B6259_RS06360) and HBD (B6259_RS06355) were identified from the transcriptomes of strain CPB6, whose expression levels maintained at very high levels (TPM > 3000) in the culture with lactate supplementation throughout the growth and stationary phases, and were upregulated by 3.5–8.6 folds compared to the control. It suggested that the high-level expression of the three genes can be induced by supplemented lactate. In addition, two BCD genes (B6259_RS01790 and _RS02600) and EtfAB (B6259_RS01785 and _RS01780) responsible for the conversion of crotonyl-CoA to butyryl-CoA showed different expression profiles. B6259_RS01790 was expressed at a very high level (TPM > 3000) throughout the fermentation phases, but showing no change in expression in the two cultures. B6259_RS02600 was expressed at relatively low level at the growth phase, but its expression was induced to high levels in the culture with lactate supplementation at the stationary phase, which was > 4.4-fold higher than that in the control. More research is needed to determine which BCD gene plays the key role in acidogenesis in the CPB6. EtfAB showed no significant change in the two cultures during the growth and stationary phases. A CAT gene (B6259_RS06345) was detected in the CPB6 transcriptome, and its expression was markedly upregulated by 4-fold in the culture with lactate supplementation than that in the control in the stationary phase, and kept at very high expression levels. CAT is key enzyme responsible for catalysing the last step of the butyrate formation [26]. High-level expression of CAT gene should theoretically result in a high concentration of butyric acid in the culture with lactate supplementation. Nevertheless, significant accumulation of CA instead of butyric acid was observed in the CPB6 culture with lactate supplementation, suggesting that the CAT is likely more intent to transforming caproy-CoA to caproate instead of converting butyryl-CoA to butyrate. Up to now, little is known about the key functional genes responsible for CA synthesis from butyryl-CoA. Genes involved in butyrate synthesis via the reverse β-oxidation (e.g., AtoB, CRT, HBD, BCD and CAT) are assumed to have the function in the caproyl-CoA and CA formation [6]. However, *Clostridium* sp. BPY5 and *C. tyrobutyricum*, which contains these genes, only produce butyric acid instead of CA [26, 27], while *C. kluyveri* and *Ruminococcaceae* bacterium CPB6, which contain these genes, can further elongate butyric acid to CA [6, 16]. It suggested that there may be differences in structure and function between these genes from different organisms. Therefore, the further study needs to be performed to explore the functions of these genes in strain CPB6.

Lactate is a major endproduct of glycolysis in the absence of oxygen [28]. Its formation or conversion requires lactate dehydrogenase (LDH) with the regeneration of NADH to NAD+. There are two LDH genes in the CPB6 genome [17], encoding L-lactate dehydrogenase (L-LDH) and D-lactate dehydrogenase (D-LDH), respectively. In this study, the expression of LDH genes (L-LDH, B6259_RS09845; D-LDH, B6259_RS06770) were also detected in the transcripts of strain CPB6. However, the two genes showed relatively low expression levels in both the culture with lactate supplementation and the control, except that slightly higher expression level was observed in the control at the stationary phase (Table 3). LDH catalyzes the reaction converts pyruvate to lactate or the reverse reaction that converts lactate to pyruvate [29]. This conversion is essential in hypoxic and anaerobic conditions when ATP production by oxidative phosphorylation is disrupted. The recent study showed that lactate can be transformed into CA in either mixed microbiome [2, 13, 14], or in the pure culture where the conversion of lactate to acetyl-CoA coupling with the reverse β-oxidation is speculated to result into chain elongation [15]. Interestingly, the lactate supplementation in this study did not lead to increased expression levels of LDHs in either growth or stationary phases, indicating that the expression of LDHs might be uncoupled from the utilization of lactate. It warrants further investigation concerning the function of LDH in the conversion of lactate to CA in the CPB6 strain.

The gene encoding phosphate acetyltransferase (PTA, B6259_RS07830), one important enzyme involved in acetate formation, was remarkably upregulated in the culture with lactate supplementation than in the control. However, the expression of acetate kinase (ACK, B6259_RS03430) showed no change in response to the addition of lactate. By including the production of H_2 and CO_2 into the loop, it could provide a whole picture for carbon balance for the substrate utilization and cell biomass production. Unfortunately, the production of H_2 and CO_2 was not monitored in this study. In the future studies, this should be taken into consideration for improvement.

Expression of putative ABC transporter and sporulation genes

Strain CPB6, affiliated with *Clostridium* cluster IV of the family *Ruminococcaceae* in the order of Clostridiales, is a spore-forming, obligate anaerobic bacterium that can produce CA from lactate (Zhu et al. 2017). As shown in Table 3, sporulation genes showed similar expression patterns in both groups, e.g., *spo*0, *spo*IID, *spo*V, *spo*YtfJ, were induced to high expression under both conditions (with or without lactate supplementation) at the growth and stationary phases, while *spo*IID, *spo*IIAD, *spo*IVA, *spo*VAC, *spo*VAD *spo*VAE were expressed at low or moderate levels. Some bacteria, such as bacilli and clostridia, develop into highly resistant spores to protect their genome and cell from certain doom when living conditions become intolerable [30]. It ensures bacterial survival under adverse environmental conditions. Sporulation in *Clostridium* spp. is ordinarily not triggered by starvation but by cessation of growth in the presence of excess carbon source or exposure to oxygen [31]. The two most critical factors involved in the shift to solventogenesis, a decrease in external pH and accumulation of acidic fermentation products, are generally assumed to be associated with the initiation of sporulation in *Clostridium* spp., to some extent [32]. But recent studies showed that the sporulation events were uncoupled from the induction of solventogenesis in *C. beijerinckii* [23]. In this study, the sporulation genes showed no significant difference between the culture with lactate supplementation and the control, indicating that the sporulation events are not associated with the production of CA in the CPB6 until the stationary phase. This may be because low concentrations of CA (1717 mg/L) are not sufficient to initiate sporulation for the CPB6. It will be investigated in the future whether high concentrations of CA trigger sporulation.

In the transcriptome of strain CPB6, most genes encoding ATP-binding cassette (ABC) transporters and substrate-bind proteins (SBP) maintained at low expression levels in the control, but were induced to relatively high expression levels (particularly upregulated by 2–14 folds at the stationary phase) in the culture with lactate supplementation. ABC transporters are ubiquitous membrane proteins that couple the transport of diverse substrates across cellular membranes to the hydrolysis of ATP [33]. ABC transporters are generally divided into importers and exporters on the basis of the polarity of solute movement. ABC importers are found mostly in bacteria and are crucial in mediating the uptake of solutes including sugar, metal ions and vitamins [34]. In the present

study, most genes for ABC transporter and substrate-binding protein (SBP) were no significant change in the two cultures, except two ABC transporter genes (B6259_RS00445, B6259_RS00450), and one SBP gene (B6259_RS00440) which were upregulated by more than 2-fold in the culture with lactate supplementation than in the control. It was suggested that the three genes might be related to the intake and use of lactic acid. In addition, nine ABC transporter genes and six SBP genes were markedly upregulated at the stationary phase. Specially, B6259_RS07905, _RS07910, _RS00320, _RS00325 and B6259_RS07915 were increased over 10-fold in the culture with lactate supplementation than in the control, demonstrating that these genes are associated with the extrusion of CA from the cell and the maintenance of osmotic homeostasis in cytoplasm [35].

In addition, two phosphotransferase system (PTS) transporter genes (B6259_RS01415 and B6259_RS00370) and one ferrous iron transporter gene (B6259_RS03880) were upregulated by 2- to 4-fold in the culture with lactate supplementation than in the control. PTS is a multiple-component carbohydrate uptake system that drives specific saccharides across the bacterial inner membrane while simultaneously catalyzing sugar phosphorylation [36]. Five distinct subfamilies of proteins related to PTS have been identified within the glucose superfamily: the lactose family, the glucose family, the β-glucoside family, the mannitol family, and the fructose family [37]. In this study, four PTS transporter genes were detected in the transcriptome of strain CPB6, including PTS fructose transporter subunit IIC (B6259_RS00095), PTS glucose transporter subunit IIA (B6259_RS09280), PTS β-glucoside transporter subunit IIABC (B6259_RS01415), and PTS mannitol transporter subunit IICBA (B6259_RS00370). Genes encoding PTS fructose and glucose transporters were highly expressed under both conditions, but the two genes were significantly downregulated at the growth phase in the culture with lactate supplementation than in the control, indicating that PTS transporter-mediated sugar transport in membrane vesicles in CPB6 is inhibited by the lactate supplementation. However, at the stationary phase, genes encoding PTS fructose, β-glucoside and mannitol transporters were all strikingly upregulated.

Overall, the addition of lactate caused distinct changes in gene expression of the CPB6 strain, especially at stationary phase. The detailed mechanism remains to be further studied.

Conclussions

This study showed that lactate supplementation induced earlier CA production, higher CA titer and productivity, but had little impact on cell growth, suggesting that the supplemented lactate is more involved in secondary metabolism (e.g., the production of CA) than the carbon assimilation. The gene transcriptional profiles based on RNA-Seq demonstrated that supplemented lactate promoted CA production by altering the expression patterns of genes involved in crucial metabolic pathways. Specifically, the Pfor gene involved in central pyruvate metabolism, 5 genes (AtoB, HBD, CRT, BCD and CAT) involved in the reverse β-oxidation pathway, 11 genes encoding ABC transporter, 6 SBP genes, and 4 PTS transporter genes showed sharp upregulation in response to the addition of lactate. The findings presented herein provide unique insights into the metabolic effects of lactate on CA production at the gene regulation level.

Methods

Microorganisms, media and fermentation experiment

Ruminococcaceae bacterium CPB6 (GDMCC No.60133) was routinely cultured at 37 ^{o}C anaerobically in a modified tryptone-glucose-yeast extract (mTGY) medium containing the following compounds (g L^{-1}, pH 6.0): tryptone, 5.0; glucose, 2.0; yeast extract, 5.0; sodium acetate, 3.5; $K_2HPO_4{\cdot}3H_2O$, 0.41; KH_2PO_4, 0.23; NH_4Cl, 0.25; $MgSO_4{\cdot}7H_2O$, 0.20; $NaHCO_3$, 2.5; L-cysteine, 0.25; $Na_2S{\cdot}9H_2O$, 0.25; resazurin, 0.0005; and 1 ml of trace element solution SL-10 and 1 ml of vitamin solution [14]. The suspension of activated strain CPB6 was inoculated with a 5% ratio into the same medium as described above and incubated for 12–15 h until the OD_{600} of the culture reached 0.8-1.0. Then the culture would be used as the seed inoculum (5% ratio, v/v) for batch experiments. To investigate the effect of lactate on cell growth and CA production, 5 g L^{-1} sodium lactate was supplemented into the modified TGY medium (mTGYL). Batch experiments were performed in 250 mL serum bottles containing 100 mL of modified mTGY or mTGYL media. The headspace of the bottle was filled with highly pure N_2. Each fermentation was performed in triplicate. The fermentation was carried out at 37 ^{o}C for 24 h in an E500 anaerobic workstation (Gene Science, USA) under N_2: CO_2: H_2 (volume ratio of 80:10:10) atmosphere.

Samples were taken at specific times and processed for cell concentration determination and HPLC analysis. Samples for RNA isolation were taken at the growth and stationary phases of the cell growth.

Analytical methods

Culture growth was monitored by measuring the optical density at 600 nm (OD_{600}) using a TU-1810 UV/Vis Spectrophotometer (Puxi Instrument Co. Ltd., Beijing). Lactic acid, acetic acid, butyric acid, and caproic acid were quantified using an HPLC system (Agilent 1260 Infinity, USA) equipped with a differential refraction detector (RID) and a Agilent Hi-Plex H column (300 × 6.5 mm) following the procedure as previously described [16].

RNA isolation, library construction, and sequencing

In preparation for RNA isolation, 10 ml cell culture was harvested at each time point, and centrifuged at 8,000 × g for 10 min at 4 °C. Cells were then frozen in liquid nitrogen prior to storage at -80 °C. The RNA was extracted and purified using a RNA extraction kit (DP430, Tiangen Biotech, Beijing, China) following the manufacturer's protocol. RNA quality and quantity were characterized using a NanoDrop2000 (NanoDrop Technologies, Wilmington, DE), agarose gel electrophoresis (RNA integrity detection) and Agilent 2100 (RIN value measurement). Only the RNA samples with high-quality (≥ 5 μg; ≥200 ng/μL; OD260/280 = 1.8 ~ 2.2) were used for the cDNA library construction and sequencing. Before library construction, rRNAs were removed with the Ribo-Zero rRNA Removal Kit (Epicentre, San Diego, CA) following the manufacturer's protocol. The enriched mRNA was fragmented into short fragments (approximately 200 bp) randomly using metal ions. Then the first strand cDNA was synthesized using the random hexamer-primer with the mRNA fragment as the template. After synthesizing the second strand cDNA using DNA polymerase I and RNase H, double-stranded cDNA was further end repaired, A-tailed, and indexed

adapters ligated. The final cDNA library was constructed using the mRNA-Seq library construction kit (Illumina Inc., San Diego, CA), and then sequenced by Illumina platform (Illumina Inc., San Diego, CA) with 2 × 150 bp.

RNA-Seq data analysis

High quality reads in each sample were aligned to the CPB6 genome using Bowtie2, and those that did not align uniquely to the genome were discarded using the default quality parameters. Each base was assigned a value based on the number of mapped sequence coverage. Gene expression levels were defined using the number of transcripts per million (TPM), which is proportional to the quantity of cDNA fragments derived from the gene transcripts. The quantitative gene expression values between samples were identified by calculating the number of unambiguous tags for each gene and then normalizing this to TPM, which was calculated following the method reported by Parto et al., [38]. The gene expression results were visualized using heatmap plots.

Statistical analysis

Significant differences of the gene expression between the culture from fermentation with lactate supplementation vs. the control were determined using ANOVA in R software (version 3.5.2). TPM values were first transformed to log10-scale. The log10-transformed TPM values were then properly centered for better representation of the data using the heatmap plots. Fold changes (FCs) as the ratio of the TPM values were calculated following the method reported by Love et al. [39], and were used to compare the differentially expressed genes (DEGs) between the culture from fermentation with lactate supplementation and the control.

Declarations

Availability of data and materials

The RNA-Seq sequencing data have been deposited in the NCBI Sequence Read Archive (SRA) under the accession number PRJNA564589

Ethics approval and consent to participate

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Authors’ contributions

YT planned and analyzed the data, and wrote the manuscript. SL performed the experiments. YW participated in the planning and revision of manuscript, HJ participated in analysis of the data. CW and XH participated in the experiments. All authors discussed the results, read and approved the final manuscript.

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Figures

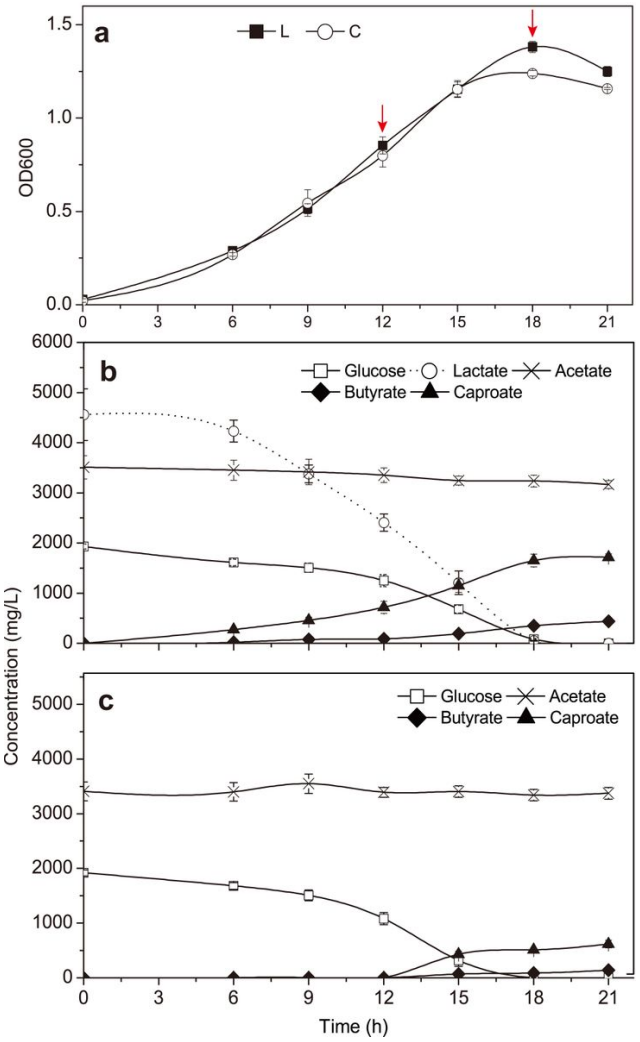

Figure 1

Fermentation kinetics of Ruminococcaceae bacterium CPB6. (a) Cell growth profiles. Time points for taking samples subjected to RNA-Seq are indicated with red vertical arrows. L: fermentation with lactate supplementation; C: control fermentation without lactate supplementation; (b) Sugar consumption and metabolites production during the fermentation with the supplementation of lactate; (c) Sugar consumption and metabolites production during the control fermentation (without the supplementation of lactate). Values represent the mean of the biological triplicates and error bars represent the standard deviations.

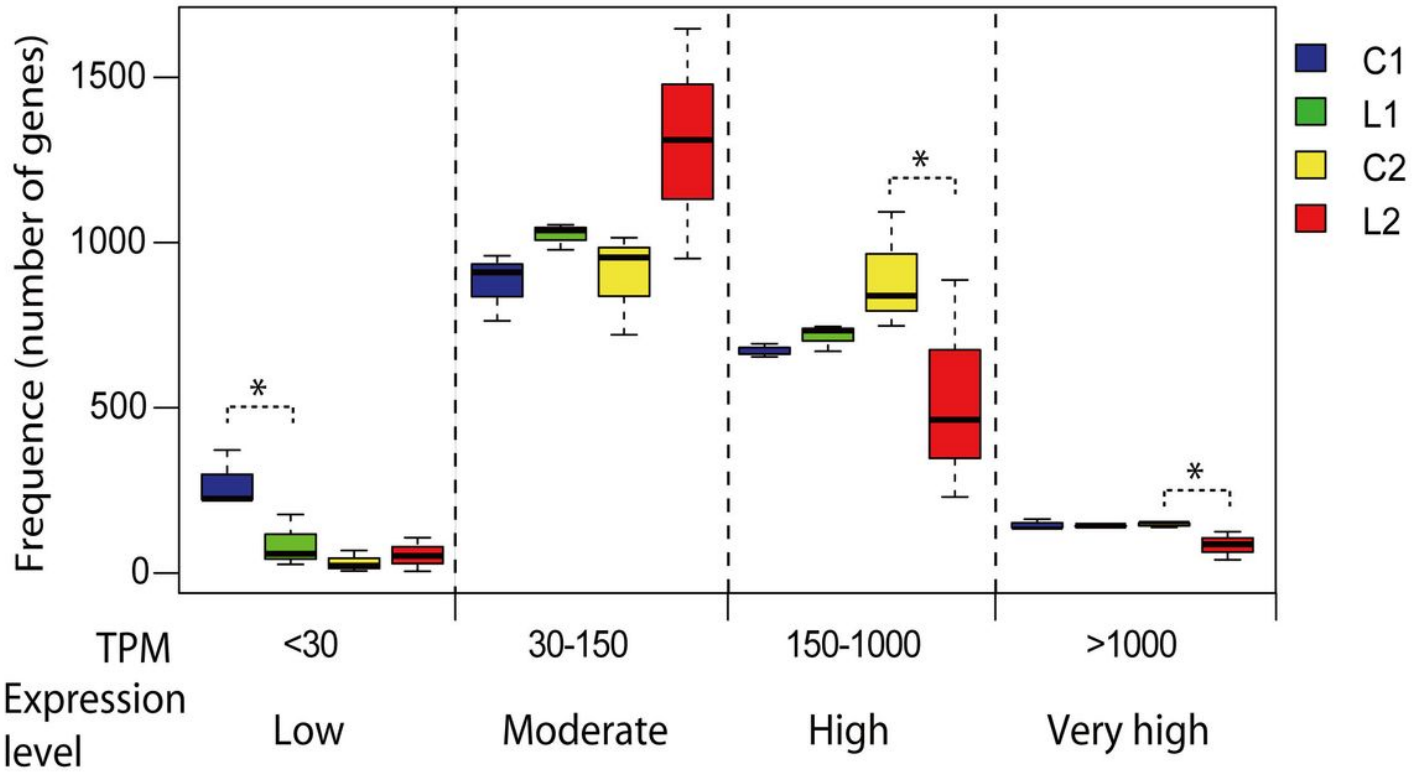

Figure 2

Frequency histogram of transcripts from the RNA-Seq results. L: cell culture with lactate supplementation; C: control culture without lactate supplementation; Number 1 and 2 represented the growth phase and stationary phase, respectively. The diagram shows the distribution of the number of genes expressed at different transcripts per million (TPM) levels. The percentage value above each bar indicates the genes at the specific expression level accounting for the proportion of the total number of genes. The '∗' mark indicates that significantly different frequencies (i.e., numbers of genes) were observed between the two RNA-Seq data sets from lactate-supplemented fermentation (L) vs. the control (C), respectively.

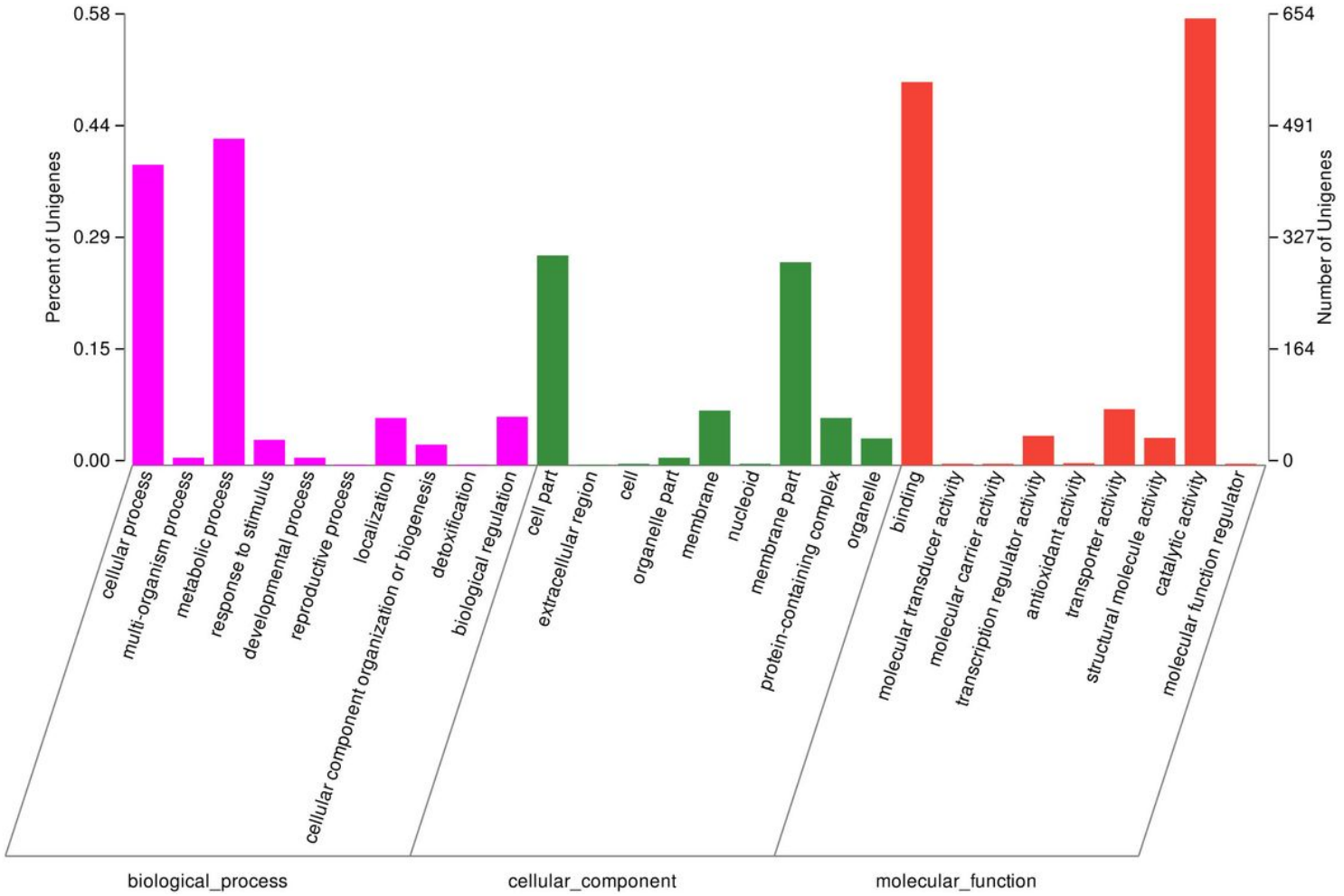

Figure 3

Annotation of genes using Gene Ontology (GO) in the transcriptome of strain CPB6. Left axis: the proportion of genes falling into each GO category; right axis: the number of genes falling into each GO category.

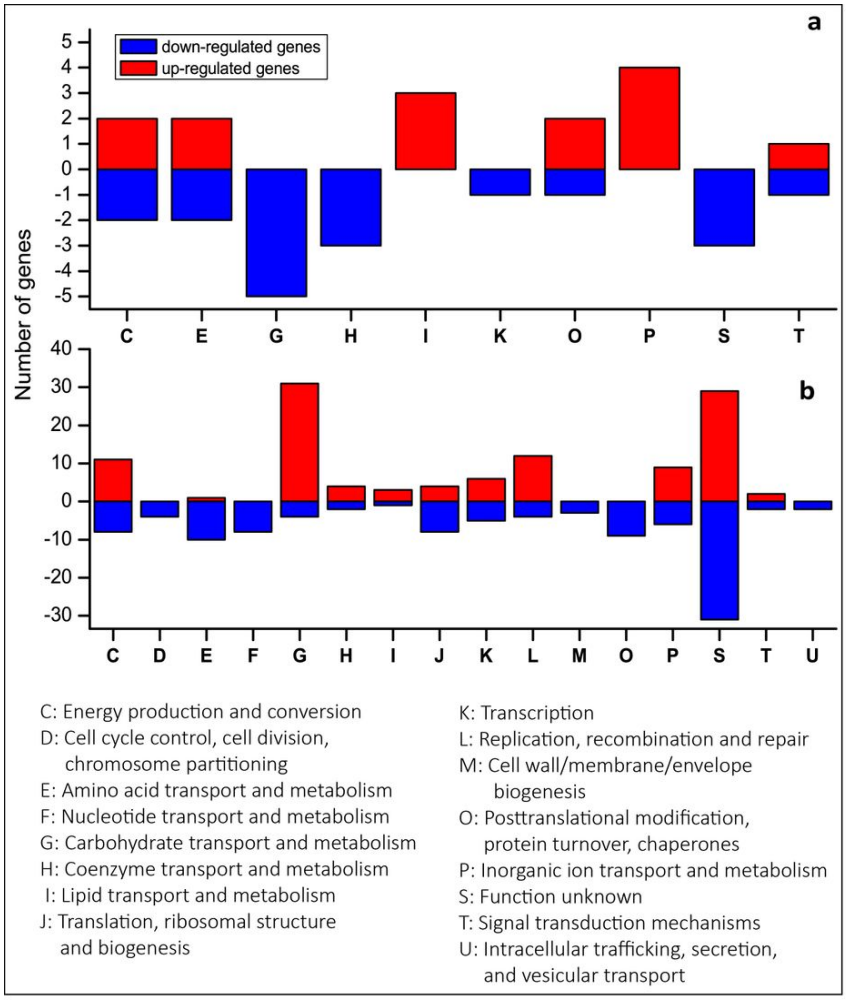

Figure 4

The number of differentially expressed genes (DEGs) between the culture with lactate supplementation and the control falling into each Clusters of Orthologous Groups (COG) categories during the growth phase (a) and the stationary phase (b), respectively. Note that since COG categories overlap, the sum of all COG annotated genes are larger than the total number of all up- and down-regulated genes analyzed.

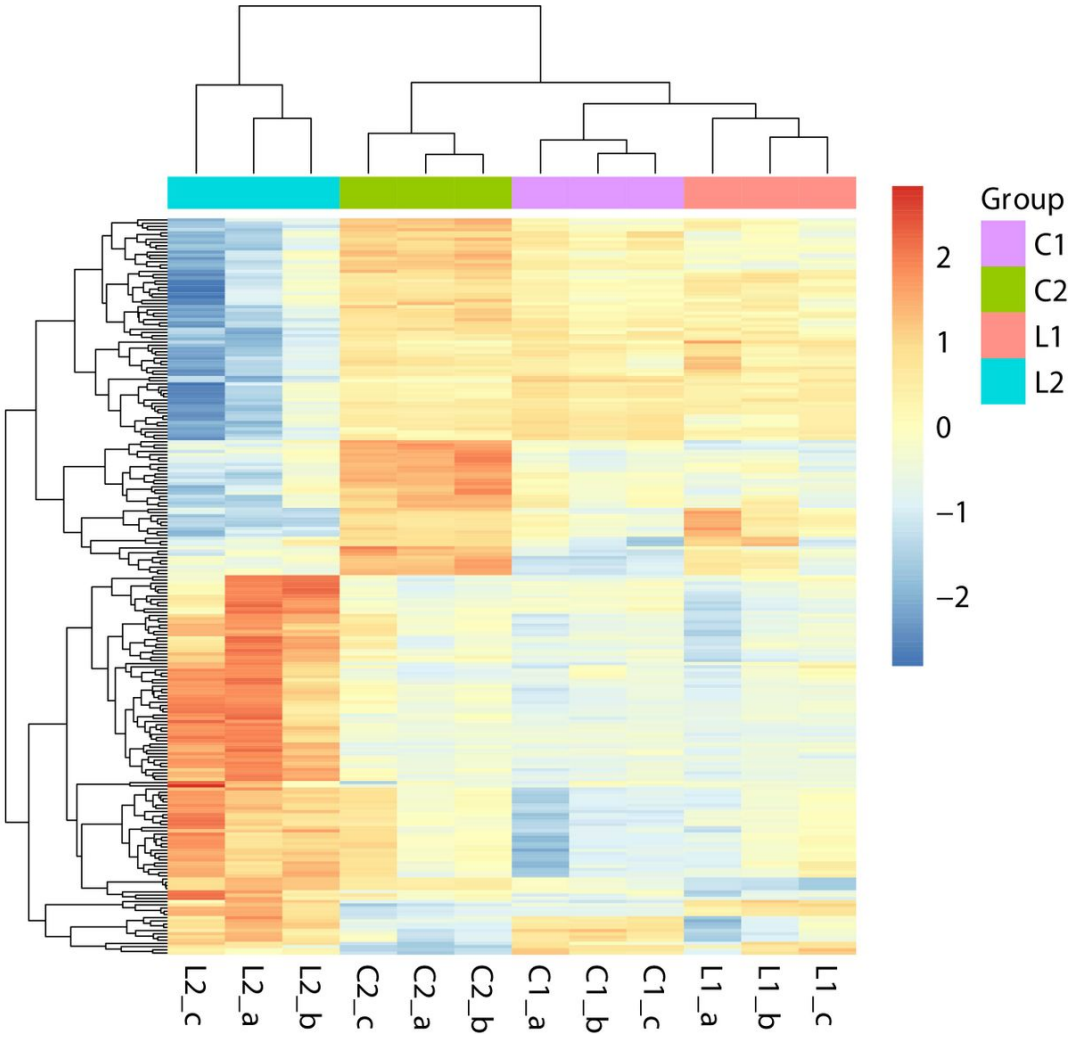

Figure 5

Clustering of differentially expressed genes (DEGs) between the culture with lactate supplementation (L) and the control (C) at the growth and stationary phases. Values of log10TPM were conducted at normalized transformation before clustering. Red indicates genes with high expression, and blue indicates genes with low expression. Each column represented a sample, and each row represented a gene. The left was the tree diagram of clustering gene, and the upper part was the tree diagram of sample clustering, and the bottom was the name of each sample (L: the culture with lactate supplementation, C: the control; Number 1 and 2 represented growth phase and stationary phase, respectively; a, b and c represented the biological triplicate samples).

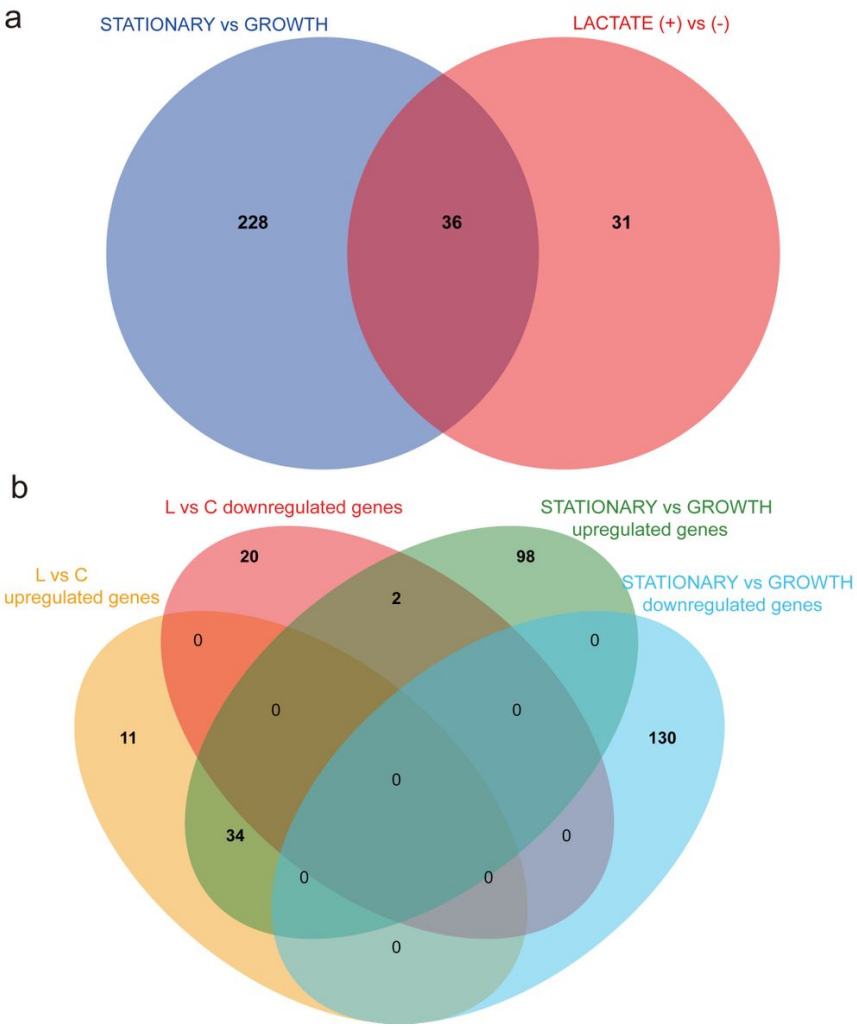

Figure 6

Venn diagram of the numbers of differentially expressed genes (DEGs) trigger by substrate type (plus/minus lactate) vs growth stage (stationary phase vs growth phase) (a), the numbers of DEGs trigger by substrate type vs growth stage (b). The overlap of circles was defined as genes affected by both substrate type and growth stage.

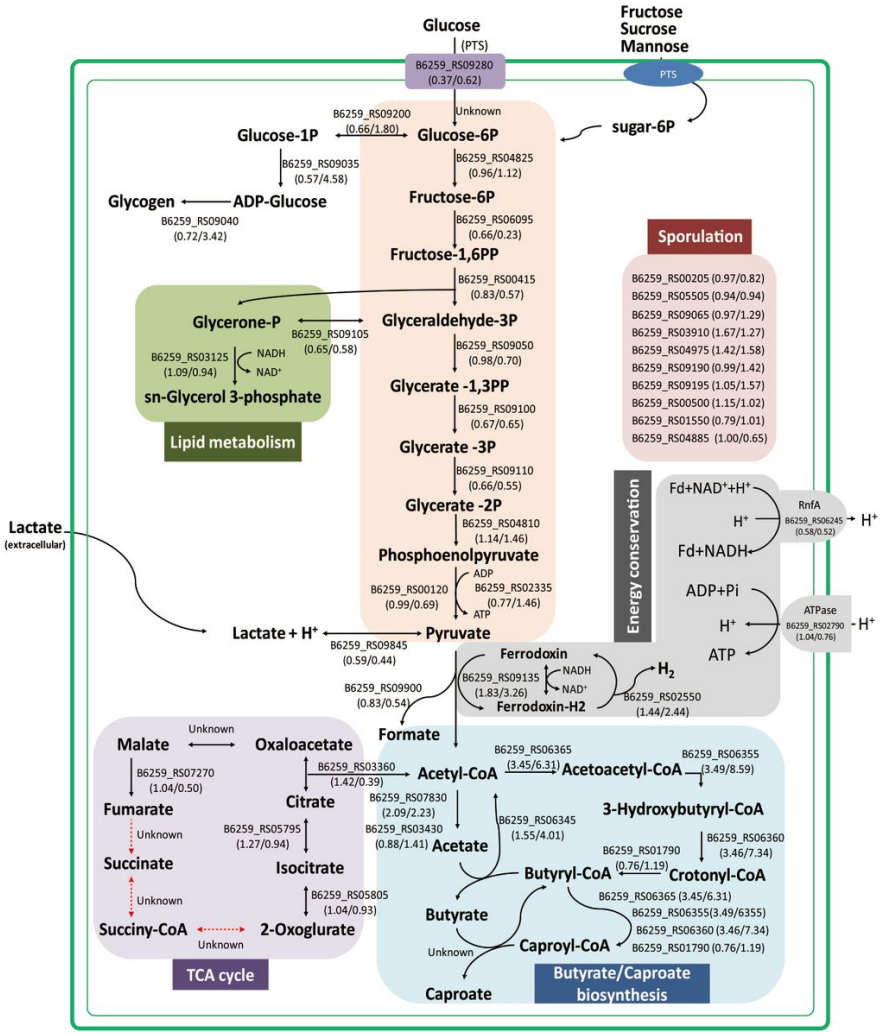

Figure 7

The overview of the central metabolic pathway in strain CPB6 with fold changes (FCs) of the expression of genes based on the RNA-Seq results. The number in parentheses represents the FC of TPM between the culture with lactate supplementation vs. the control; the first and the second numbers in the same parentheses represent the gene expression FC in the growth and stationary phases, respectively. Black solid lines symbolize enzymatic reactions. Red dashed lines mark enzymatic reactions for which corresponding enzymes are probably not encoded in strain CPB6.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- TableS2.xlsx
- TableS1.xlsx
- FigS2.jpg
- FigS1.jpg