



Adenine Addition Restores Cell Viability and Butanol Production in *Clostridium saccharoperbutylacetonicum* N1-4 (ATCC 13564) Cultivated at 37°C

Keiji Kiyoshi, Sohei Kawashima, Kosuke Nobuki, Toshimori Kadokura, Atsumi Nakazato, Ken-ichiro Suzuki, Shunichi Nakayama

Department of Fermentation Science and Technology, Faculty of Applied Bio-science, Tokyo University of Agriculture, Tokyo, Japan

ABSTRACT We have developed butanol-producing consolidated bioprocessing from cellulosic substrates through coculture of cellulolytic clostridia and butanol-producing *Clostridium saccharoperbutylacetonicum* strain N1-4. However, the butanol fermentation by strain N1-4 (which has an optimal growth temperature of 30°C) is sensitive to the higher cultivation temperature of 37°C; the nature of this deleterious effect remains unclear. Comparison of the intracellular metabolites of strain N1-4 cultivated at 30°C and 37°C revealed decreased levels of multiple primary metabolites (notably including nucleic acids and cofactors) during growth at the higher temperature. Supplementation of the culture medium with 250 mg/liter adenine enhanced both cell growth (with the optical density at 600 nm increasing from 4.3 to 10.2) and butanol production (increasing from 3.9 g/liter to 9.6 g/liter) at 37°C, compared to those obtained without adenine supplementation, such that the supplemented 37°C culture exhibited growth and butanol production approaching those observed at 30°C in the absence of adenine supplementation. These improved properties were based on the maintenance of cell viability. We further showed that adenine supplementation enhanced cell viability during growth at 37°C by maintaining ATP levels and inhibiting spore formation. This work represents the first demonstration (to our knowledge) of the importance of adenine-related metabolism for clostridial butanol production, suggesting a new means of enhancing target pathways based on metabolite levels.

IMPORTANCE Metabolomic analysis revealed decreased levels of multiple primary metabolites during growth at 37°C, compared to 30°C, in *C. saccharoperbutylacetonicum* strain N1-4. We found that adenine supplementation restored the cell growth and butanol production of strain N1-4 at 37°C. The effects of adenine supplementation reflected the maintenance of cell viability originating from the maintenance of ATP levels and the inhibition of spore formation. Thus, our metabolomic analysis identified the depleted metabolites that were required to maintain cell viability. Our strategy, which is expected to be applicable to a wide range of organisms, permits the identification of the limiting metabolic pathway, which can serve as a new target for molecular breeding. The other novel finding of this work is that adenine supplementation inhibits clostridial spore formation. The mechanism linking spore formation and metabolomic status in butanol-producing clostridia is expected to be the focus of further research.

KEYWORDS *Clostridium*, butanol production, metabolomic analysis, spore formation

Biobutanol produced by *Clostridium* spp. can be used as a biofuel and/or as a chemical feed stock (1). Biobutanol production from renewable resources such as cellulosic plant biomass is required from the perspective of sustainable development (2, 3). We have developed a butanol-producing consolidated bioprocess (CBP) from crys-

Received 25 October 2016 Accepted 20 January 2017

Accepted manuscript posted online 27 January 2017

Citation Kiyoshi K, Kawashima S, Nobuki K, Kadokura T, Nakazato A, Suzuki K, Nakayama S. 2017. Adenine addition restores cell viability and butanol production in *Clostridium saccharoperbutylacetonicum* N1-4 (ATCC 13564) cultivated at 37°C. Appl Environ Microbiol 83:e02960-16. <https://doi.org/10.1128/AEM.02960-16>.

Editor Robert M. Kelly, North Carolina State University

Copyright © 2017 American Society for Microbiology. All Rights Reserved.

Address correspondence to Shunichi Nakayama, s3nakaya@nodai.ac.jp.

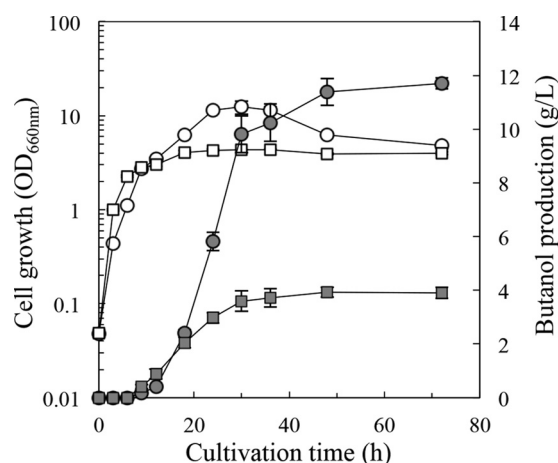


FIG 1 Effects of cultivation temperature on cell growth and butanol production of *C. saccharoperbutylacetonicum* strain N1-4. Time courses of cell growth at 30°C (white circles) and 37°C (white squares) and of butanol production at 30°C (gray circles) and 37°C (gray squares) are provided. These parameters were assayed in triplicate in each of a minimum of three independent experiments; representative data from one such experiment are shown. Values are shown as means of samples assayed in triplicate; error bars indicate standard errors.

talline cellulose and delignified rice straw through coculture of the thermophilic and cellulolytic *Clostridium thermocellum* with the mesophilic, butanol-producing, *Clostridium saccharoperbutylacetonicum* strain N1-4 (4, 5). This coculture system includes both cellulase and butanol production phases, with butanol production proceeding via simultaneous saccharification and fermentation. Thus, the cellulase produced by *C. thermocellum* cultivated at 60°C hydrolyzes cellulosic substrates; the butanol-producing *C. saccharoperbutylacetonicum* then ferments the released monosaccharides or disaccharides to butanol at 30°C.

In our previous work, we tested three different clostridia for use in our system. While *C. saccharoperbutylacetonicum* N1-4 was the best of the three strains for butanol production in this coculture system, the optimal temperature for fermentation by strain N1-4 was 30°C, and this strain was sensitive to cultivation at temperatures over 34°C (5). This temperature optimum for use in butanol fermentation represents a potential problem in large-scale butanol production; overcoming this temperature sensitivity would be essential to rendering this process commercially viable. The fact that other butanol-producing clostridia normally grow and produce butanol at 37°C (6, 7) suggests that strain N1-4 also has butanol production potential at 37°C. Indeed, as we show in the present work (Fig. 1), strain N1-4 can grow at 37°C, although growth and butanol production by this strain ceased after 36 h of cultivation. This observation suggested that the 37°C cultivation temperature is not lethal *per se*; instead, it appears that some factor becomes limiting for N1-4 growth at the elevated temperature. However, the nature of the deleterious effect of temperature remained unclear.

We hypothesized that the cessation of growth and butanol production by strain N1-4 cultivated at 37°C reflects the depletion of a particular metabolic pathway or metabolite that is essential for growth. Several recent omics analyses have revealed that various metabolic pathways are affected by high cultivation temperatures. For example, in *Escherichia coli*, the transcription of genes related to fundamental metabolic processes, such as ATP synthesis, nucleotide or amino acid biosynthesis, and proton transport, is downregulated during growth at 43°C (8). The expression of genes related to the biosynthesis of nucleic acids and aminoacyl-tRNAs in *Staphylococcus aureus* also is downregulated during growth at 43°C (9). These reports showed that the expression of genes related to the syntheses of primary metabolites tends to be decreased in cells grown at higher temperatures; decreased expression presumably leads to the depletion of primary metabolites essential for cell growth.

The phenomenon of growth inhibition in the absence of primary metabolites is recognized as “Liebig’s law of the minimum,” which states that growth is controlled by the scarcest resource (the limiting factor) (10). For instance, based on genome-scale proteomic simulations, Chang et al. suggested that cofactor synthesis was the bottleneck for *E. coli* growth at 42°C, and they confirmed this hypothesis by showing that the addition of selected cofactors was sufficient to yield improved growth rates at the elevated temperature (11). If the levels of primary metabolites required for cell growth are depleted by decreases in transcription or enzyme activity under high-temperature conditions, then metabolomic analysis may permit identification of the metabolites that create the bottleneck. Indeed, metabolomic analysis of *E. coli* cultivated at 43°C showed decreases in the levels of metabolites involved in processes such as glycolysis, the pentose phosphate pathway, the tricarboxylic acid cycle, and the biosynthesis of selected nucleotides and amino acids (8).

Alternatively, it is possible that the cessation of growth and butanol production by strain N1-4 cultivated at 37°C reflects spore formation. Specifically, clostridia are known to generate spores (a dormant cell type) in response to stress and nutrition limitations (12); cells that have entered sporulation do not exhibit butanol-producing activity (13). In the model spore-forming bacterium *Bacillus subtilis*, entry into sporulation reflects the intracellular level of metabolites. One *Bacillus* sensor of nutritional state is the CodY protein, a transcriptional repressor of sporulation genes that controls its regulon by sensing the intracellular concentrations of GDP, GTP, and branched-chain amino acids (14, 15). This triggering mechanism, which is coordinated by CodY sensing of intracellular nutritional status, also has been reported to apply in *Clostridium difficile* (12). Thus, cultivation temperature, which can act as a stressor, may be inducing spore formation by strain N1-4. As suggested above, evaluation of the metabolite profile would provide insights into the sporulation and intracellular nutritional status of *C. saccharoperbutylacetonicum* under these conditions.

Therefore, we compared the metabolite profiles of strain N1-4 cultivated at 30°C (the optimal temperature for butanol fermentation) and at 37°C (a temperature at which cell growth and butanol production are known to cease) (Fig. 1). We extended this metabolomic analysis by screening among depleted metabolites for compounds that, when added back into the culture, supported growth and butanol production at 37°C. We found that the addition of adenine restored the growth and butanol production of strain N1-4 cultivated at 37°C to levels comparable to those obtained at 30°C. We further consider the potential mechanism whereby adenine supplementation aids cell growth and fermentation at 37°C.

RESULTS

Comparison of cell growth and butanol production of strain N1-4 cultivated at 30°C and 37°C. Increases in the optical density at 600 nm (OD_{600}) of strain N1-4 cultivated at 30°C were observed until 30 h; the maximum cell density (OD_{600}) was 12.4 ± 2.0 (Fig. 1). Although strain N1-4 grew faster at 37°C than at 30°C for the first 6 h, the increase in OD_{600} at 37°C stopped after 18 h; the peak cell density at 37°C (OD_{600} of 4.4 ± 0.0) was 42% of that of the strain cultivated at 30°C. The strain cultivated at 30°C completely consumed 40 g/liter of glucose and produced 11.7 ± 0.2 g/liter of butanol at 72 h (Fig. 1). On the other hand, glucose consumption by the strain cultivated at 37°C stopped after 36 h, when butanol production also ceased. Similarly, at 72 h, the level of butanol produced at 37°C (3.9 ± 0.2 g/liter) was lower than that generated at 30°C.

Metabolomic analysis. Cells cultivated at 30°C and 37°C were harvested at multiple time points with respect to butanol production, including prior to the start of production (6 h) and at the early (12 h), intermediate (24 h), and late (36 h) phases of production (Fig. 1). The samples were then assayed for the levels of intracellular metabolites (see Table S1 in the supplemental material). The principal-component analysis (PCA) estimated from the metabolites showed that the samples extracted from cells cultivated at 30°C and 37°C for 6, 12, and 36 h were located relatively close to each

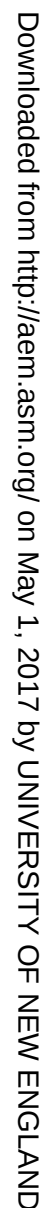
other, while the samples from the 24-h time points were located relatively far from each other (Fig. S1A). This result suggested that the metabolites extracted from the 24-h cultivations would be the most different from each other among the four time points. Consistent with the PCA, butanol levels at 6 h (not detectable) and 12 h (~1 g/liter) were similar when levels of production at the two temperatures at a given time point were compared (Fig. 1). At 24 h, strain N1-4 cultivated at 30°C actively produced butanol (0.57 g/liter/h); however, the rate of butanol production by strain N1-4 cultivated at 37°C was significantly lower (0.16 g/liter/h). Considering these results, we focused on the metabolites extracted from the 24-h cultivations.

Hierarchical cluster analysis (HCA) was performed based on the metabolites listed in Table S1; the corresponding heat map is shown in Fig. 2A. Since the PCA suggested that the difference in metabolites at 24 h was most significant, we focused on the metabolites that were increased only in the 30°C cultivation, which were expected to be decreased in the 37°C cultivation (Fig. 2A [cluster B] and B, i.e., 64 metabolites), and on those that were increased only in the 37°C cultivation (Fig. 2A [cluster A] and C, i.e., 16 metabolites). Virtually all glycolysis metabolites were included in cluster B. Although these glycolysis metabolite levels were mapped in a metabolic context to identify the bottleneck pathway (Fig. S1B), none of the accumulated metabolites corresponded to the intermediates that would be expected to accumulate if a glycolytic pathway were the rate-limiting step. This observation suggested that a specific metabolic pathway in glycolysis was not inhibited at the elevated temperature. The levels of nucleic acid-related metabolites (such as ATP, dADP, dGTP, guanosine, and so on) were decreased at 24 h at the elevated temperature. The levels of almost all purine- and pyrimidine-related nucleic acid compounds were decreased at 37°C, and none of the obviously accumulated metabolites corresponded to intermediates for either pathway (Fig. S1C and D). The level of NADPH, which is used as a cofactor in many metabolic pathways, also was decreased (Fig. 2B; also see Fig. S1B). Based on these results, overall metabolic activity is apparently decreased in strain N1-4 cultivated at 37°C.

Cluster A included CDP-choline (a compound related to cell membrane biosynthesis) and tryptophan and ornithine (known amino acid precursors). Although these accumulated metabolites might act as inhibitors of cell growth, there have been (to date, to our knowledge) no reports that these metabolites show inhibitory effects in clostridia. We hypothesized that depletion of primary metabolites caused decreased cell growth and glucose consumption. Therefore, we investigated whether supplementation with any of the depleted metabolites would be sufficient to restore cell growth and butanol production to strain N1-4 cultivated at 37°C.

Addition of nucleobases restores butanol production at 37°C. Metabolomic analysis revealed that N1-4 cells cultured for 24 h at 37°C exhibited decreased levels of glycolytic metabolites and other primary metabolites, such as nucleic acids and NADPH. We assumed that these primary metabolites became limiting for the growth of strain N1-4 cultivated at 37°C, and we postulated that supplementation with these depleted metabolites might be sufficient to restore growth and butanol production at the higher temperature. The amino acids also were added to the culture, because the addition of amino acids is known to increase tolerance to butanol stress (7, 16), although our metabolomic analysis did not suggest that the amino acids were significantly affected at 24 h. In general, nucleotides are degraded to nucleobases, and the resulting nucleobases are used to regenerate nucleotides by reaction with phosphoribosyl pyrophosphate (PRPP) (17). Therefore, strain N1-4 was cultivated with the addition of either all 20 naturally occurring amino acids (to 100 mg/liter each), pyridine nucleotides (NAD⁺, NADH, NADP⁺, and NADPH), or five nucleobases (adenine, guanine, thymine, cytosine, and uracil) instead of nucleotides (Fig. 3).

The maximum cell densities (OD₆₀₀ values) of strain N1-4 cultivated at 30°C and 37°C were 6.4 ± 0.4 and 2.6 ± 0.1 , respectively. The levels of butanol production of strain N1-4 cultivated at 30°C and 37°C were 8.1 ± 1.3 and 3.5 ± 0.7 g/liter, respectively. The



aem.asm.org 5

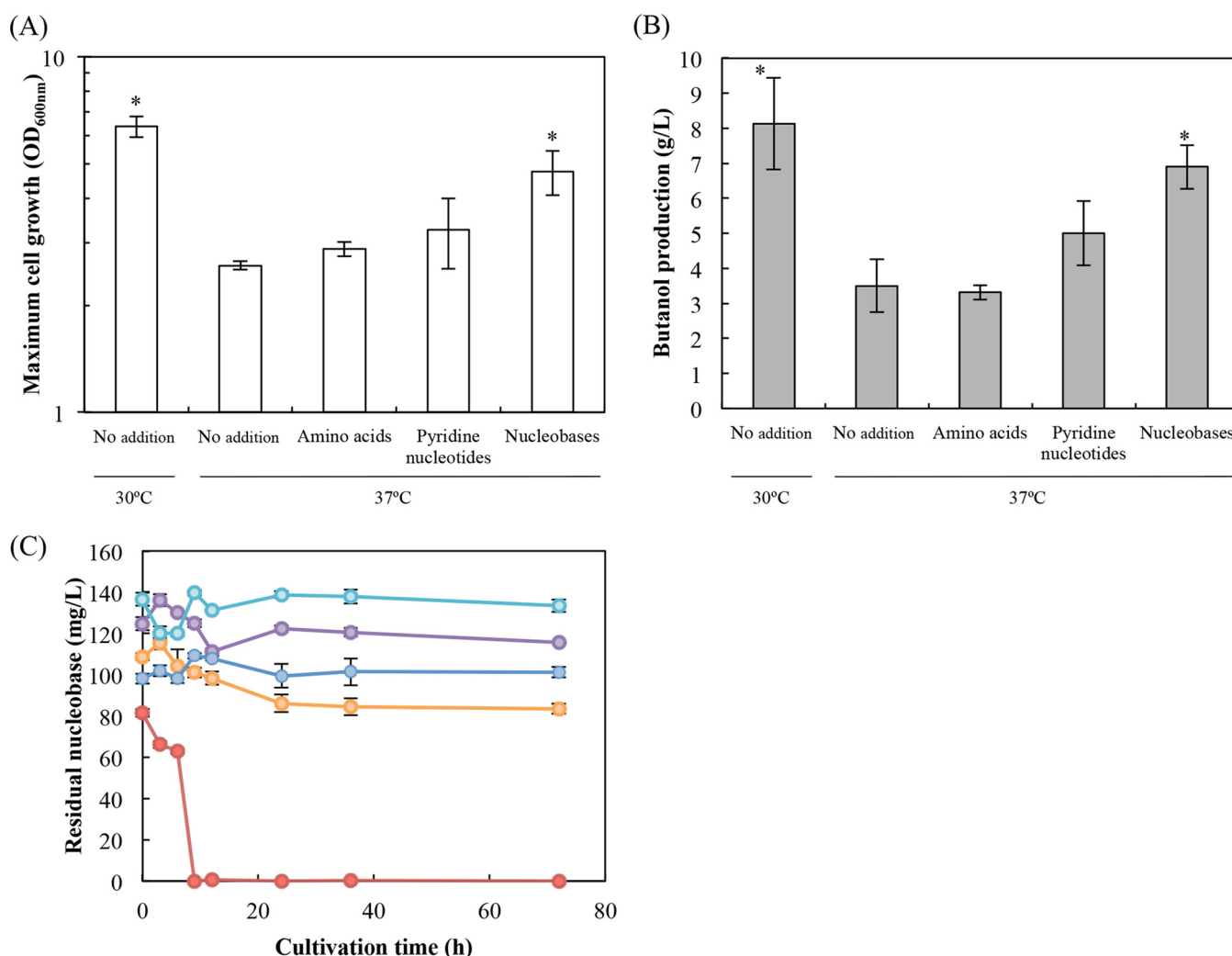


FIG 3 Maximum cell growth (A) and butanol production (B) of strain N1-4 cultivated at 37°C for 72 h with the addition of various primary metabolites and the levels of residual nucleobases in the supernatants of strain N1-4 cultivated in medium supplemented with nucleobases (C). The supernatant levels of adenine (red), guanine (orange), thymine (purple), cytosine (aqua), and uracil (blue) are indicated. These parameters were assayed in triplicate in each of a minimum of three independent experiments; representative data from one such experiment are shown. Values are shown as means of samples assayed in triplicate; error bars indicate standard errors. *, $P < 0.05$ for cell growth and butanol production versus control cultures grown at 37°C in unsupplemented medium.

addition of amino acids did not appear to significantly restore cell growth and butanol production at 37°C ($P > 0.05$). The addition of pyridine nucleotides also did not yield significant increases in cell growth or in butanol production, compared to the controls, although the failure to achieve significance may have reflected the larger variance in the values obtained for the pyridine-supplemented cultures ($P > 0.05$). On the other hand, the addition of nucleobases yielded significant ($P < 0.01$) increases in both cell growth (OD_{600} of 4.8 ± 0.1) and butanol production (6.9 ± 0.6 g/liter), compared to those of the cultures grown at 37°C without supplementation. Notably, cell growth and butanol production by the nucleobase-supplemented cultures grown at 37°C were not statistically distinguishable ($P > 0.05$) from the respective values for the cultures grown at 30°C without supplementation.

Adenine enhances cell growth and butanol production in cultures grown at 37°C. To identify which nucleobases contributed to the restoration of cell growth and butanol production at 37°C, the levels of the residual nucleobases in the supernatant of the supplemented cultures grown at 37°C were measured (Fig. 3C). The concentrations of cytosine, thymine, uracil, and guanine remained effectively unchanged through 72 h. In contrast, the concentration of adenine in the culture supernatant decreased

rapidly after 6 h of cultivation and fell below the limit of detection by 9 h. This finding suggested that supplementation of adenine levels might serve to enhance cell growth and butanol production by strain N1-4 at 37°C.

In order to determine whether depletion of adenine alone was the source of impaired growth, we repeated the cultivation of strain N1-4 at 37°C using medium supplemented with each combination of four of the five nucleobases (i.e., excluding each candidate nucleobase, one by one, from the supplementing mixture) (Fig. S2). Notably, when adenine alone was omitted (i.e., in medium supplemented with guanine, thymine, cytosine, and uracil), cell growth and butanol production at 37°C both remained low, with both parameters resembling those seen at this temperature in unsupplemented medium. To further demonstrate the effect of adenine, strain N1-4 was cultivated in medium supplemented with various initial concentrations of adenine (Fig. S3). The supplementation with adenine at ≥ 100 mg/liter stably restored butanol production to levels resembling those obtained for strain N1-4 cultivated at 30°C in unsupplemented medium. These results indicated that the addition of adenine alone was sufficient to restore the growth and butanol production of strain N1-4 at 37°C.

Fermentation profile of strain N1-4 cultivated at 37°C with added adenine. Fig. 4A and E provide time courses of cell growth and fermentation production for strain N1-4 cultivated at 37°C in medium supplemented with 250 mg/liter of adenine. The maximum OD₆₀₀ and level of butanol production for strain N1-4 cultivated at 37°C with adenine (4.5 ± 0.1 and 8.7 ± 1.0 g/liter of butanol, respectively) were higher than those of the strain grown at 37°C without adenine addition (3.3 ± 0.1 and 3.4 ± 0.1 g/liter of butanol, respectively), while the butanol production yield of strain N1-4 cultivated at 37°C with adenine (0.22 g/g) was lower than that of the strain cultivated at 30°C in the absence of adenine supplementation (0.29 g/g) (Fig. 4). The nominal decrease in the butanol yield with adenine supplementation at 37°C correlated with a nominal increase in the acetone yield (0.09 g/g and 0.06 g/g for the respective culture conditions). With the exception of the acetone yield, the amounts of fermentation products generated by the strain cultivated at 37°C with additional adenine were very similar to those generated by the strain cultivated at 30°C in the absence of adenine supplementation. Thus, supplementation with adenine enhanced both cell growth and butanol production in strain N1-4 grown at 37°C, while yielding nominal decreases in the butanol yield and nominal increases in the acetone yield.

Interestingly, supplementation with adenine at 30°C yielded an increased maximum OD₆₀₀ (OD₆₀₀ of 9.8 ± 0.5) along with elevated glucose consumption and butanol production rates, compared to cultures grown at 30°C without additional adenine (Fig. 4C). Thus, adenine supplementation reinforced cell growth and butanol production even during cultivation at 30°C, without significant differences in butanol yields (0.29 g/g).

Adenine addition restores cell viability and inhibits spore formation. The cell viabilities of strain N1-4 cultivated at 30°C or 37°C with or without the addition of 250 mg/liter of adenine were estimated by counting CFU (Fig. 4F); the goal was to elucidate why cell growth and butanol production were inhibited at 37°C and restored by supplementation with adenine at the elevated temperature. For cells grown in standard (unsupplemented) medium, CFU numbers in 30°C cultures were maintained through 48 h, while CFU numbers in 37°C cultures decreased drastically after 16 h of cultivation. Notably, supplementation with adenine provided restoration of CFU numbers through 36 h in the 37°C cultures. These results indicated that the decreased cell growth in cultures grown at 37°C in standard medium reflects a loss of cell viability, an effect that is counteracted by the addition of adenine to the medium.

Why does the addition of adenine restore cell viability? One possible reason is that this compound contributes to the maintenance of cellular homeostasis, because adenine can be converted to ATP in the cells (17). Therefore, intracellular ATP concentrations were compared in cells grown in the presence and absence of adenine supplementation (Fig. 4G). As expected, ATP concentrations were maintained through 36 h in

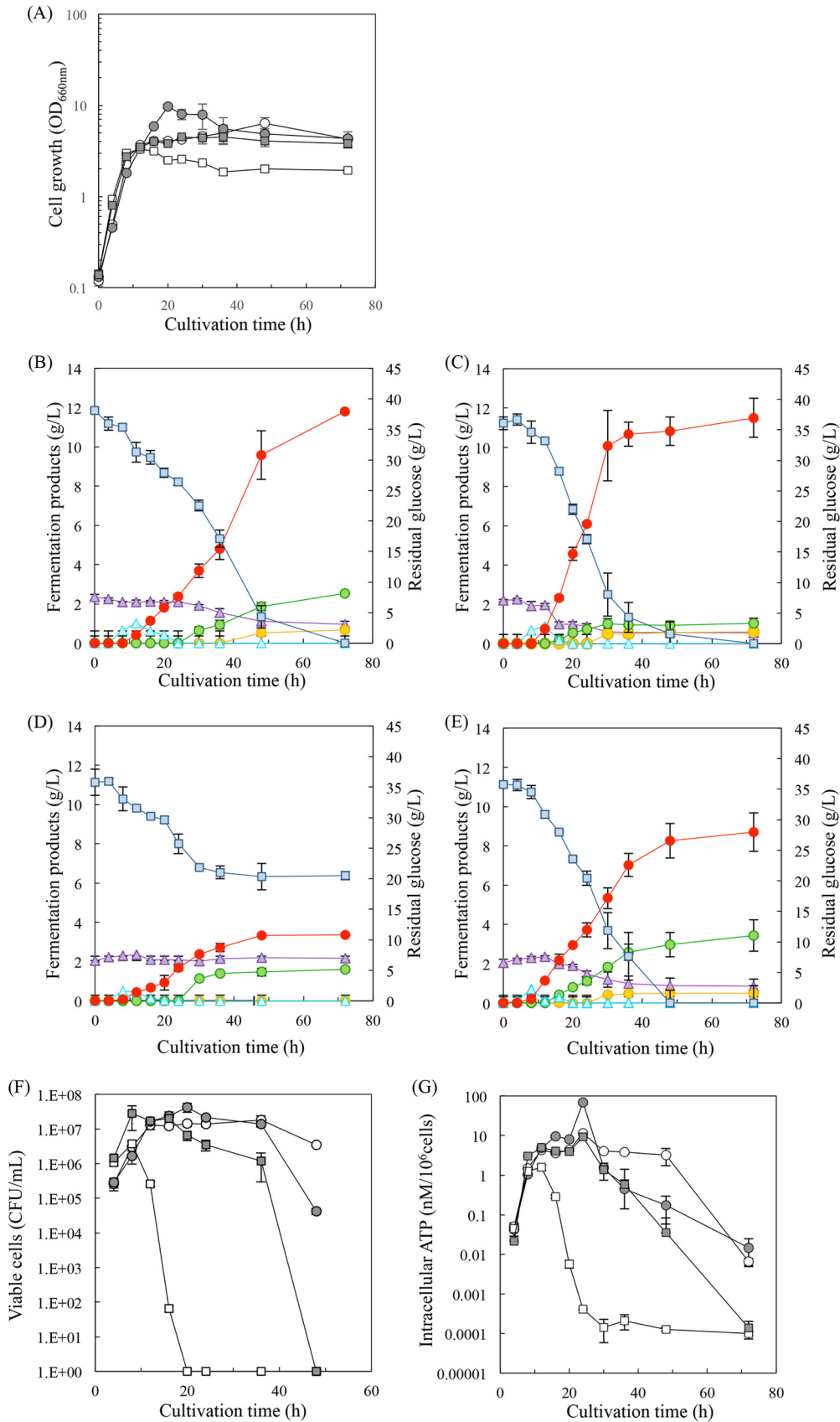


FIG 4 Effects of cultivation temperature and adenine supplementation on cell growth (A), fermentation profiles (B, C, D, and E), CFU (F), and intracellular ATP levels (G) of *C. saccharoperbutylacetonicum* strain N1-4. Time courses of cell
(Continued on next page)

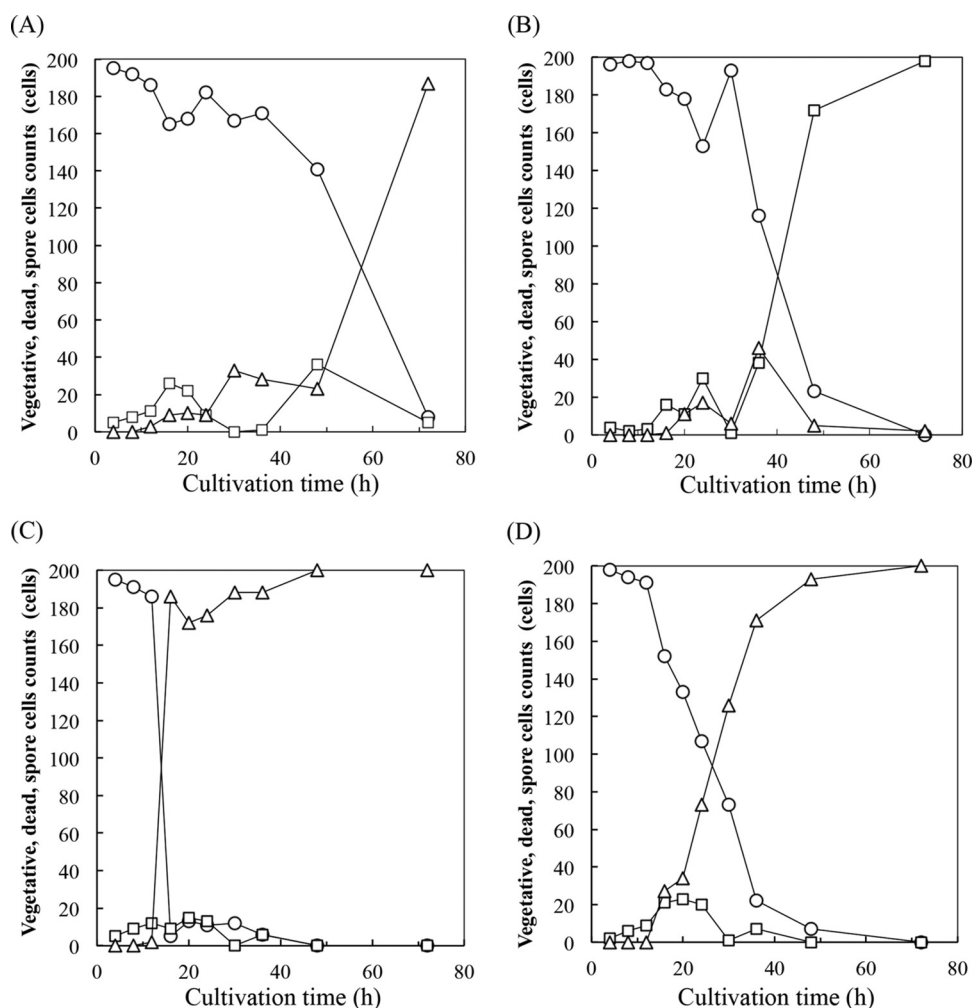


FIG 5 Comparison of proportions of vegetative (circles), dead (squares), and spore-forming (triangles) cells of strain N1-4 cultivated at 30°C (A), at 30°C with the addition of adenine (B), at 37°C (C), and at 37°C with the addition of adenine (D). These parameters were assayed in triplicate in each of a minimum of four independent experiments; representative data from one such experiment are shown.

strain N1-4 cultivated at 30°C with or without added adenine and in cells cultivated at 37°C with added adenine; in contrast, ATP levels decreased rapidly after 20 h for cells cultivated at 37°C without added adenine.

The other possible process affecting cell viability is that of spore formation, a process of which clostridia are capable. The ratios of vegetative, dead, and sporulating cells were calculated by counting the cells distinguished by fluorescence microscopy following double staining with Syto9 and propidium iodide (PI) (Fig. 5; also see Fig. S4). For cells grown at 30°C, the culture consisted primarily (>85%) of vegetative cells through 36 h, with sporulating cells appearing after 72 h of cultivation (Fig. 5A). For cells grown at 30°C with adenine supplementation, the predominance of vegetative cells persisted through 30 h, with the majority of the

FIG 4 Legend (Continued)

growth (A), CFU (F), and intracellular ATP levels (G) are indicated for cultures grown at 30°C (white circles), at 30°C with the addition of adenine (gray circles), at 37°C (white squares), and at 37°C with the addition of adenine (gray squares). Time courses of the levels of residual glucose (blue) and the fermentation products acetate (purple), butyrate (aqua), ethanol (orange), acetone (green), and butanol (red) are provided for cells cultivated at 30°C (B), at 30°C with the addition of adenine (C), at 37°C (D), and at 37°C with the addition of adenine (E). These parameters were assayed in triplicate in each of a minimum of four independent experiments; representative data from one such experiment are shown. Values are shown as means of samples assayed in triplicate; error bars indicate standard errors.

population converting to dead cells by 48 h (Fig. 5B). For cells grown at 37°C (without supplementation), the culture consisted primarily of vegetative cells only for the first 12 h; the vegetative cells were quantitatively converted to sporulating cells by 16 h (Fig. 5C). In contrast, cells grown at 37°C with added adenine consisted primarily of vegetative cells and sporulation was inhibited through 20 h, after which the vegetative cells were quantitatively converted to sporulating cells (Fig. 5D). Thus, supplementation of the medium with adenine delayed the onset of sporulation by 20 h in cultures grown at the elevated temperature, a result that correlates well with the effects on cell viability shown in Fig. 4F. Based on the present results, we cannot distinguish whether the depletion of ATP induces spore formation or spore formation leads to ATP depletion. Nonetheless, this work demonstrated that adenine supplementation enhances the maintenance of cell viability and inhibits spore formation, both processes that suffice to explain continued growth and butanol production by cells growing in the supplemented medium at the elevated temperature.

DISCUSSION

As demonstrated in an earlier work, *C. saccharoperbutylacetonicum* strain N1-4 is highly suitable for butanol production from cellulosic substrates through coculturing with *C. thermocellum* (4, 5). However, as shown in that earlier work and as confirmed in the present work, growth and butanol production by strain N1-4 cease at 37°C, in contrast to several other butanol-producing clostridia that grow normally and produce butanol at higher temperatures (6, 7). We hypothesized that strain N1-4 grown at a higher temperature (a known stressor) becomes depleted in some metabolite(s) required for cell growth and butanol production. Therefore, we compared the metabolite profiles of strain N1-4 cultivated at 30°C and 37°C, in an attempt to identify the putatively depleted compound(s), and then we tested the ability of these candidates to restore growth and butanol production to cells growing at an elevated temperature.

PCA based on the extracted metabolites revealed that the metabolites in cultures grown at the distinct temperatures for 24 h showed the largest differences among the four tested time points. Interestingly, this difference coincided well with butanol productivity; specifically, while butanol was actively produced during the growth of strain N1-4 at 30°C, butanol production by this strain ceased after 24 h of growth at 37°C. Notably, the concentrations of almost all glycolytic metabolites and of most nucleic acid-related metabolites were decreased in strain N1-4 cultivated at 37°C for 24 h. The levels of most amino acids (except tryptophan and arginine) were decreased in cells cultured at 37°C, compared to cells cultured at 30°C, but the differences were most prominent at an earlier time point of 12 h (Table S1). Only in the case of *E. coli* is growth actively inhibited by the excess of an amino acid intermediate (homocysteine) (18, 19). We instead hypothesized that impaired growth of strain N1-4 at 37°C reflected depletion of one or more primary metabolites that became limiting factors for cell growth and butanol production at the elevated temperature. Supplementation experiments revealed that the addition of 20 pooled amino acids or four pyridine nucleotides did not restore cell growth or butanol production by strain N1-4 at 37°C; however, the addition of a mixture of five nucleobases, especially adenine, did restore these functions at the elevated temperature. The fact that the levels of amino acids in cells cultivated at 37°C were decreased earlier than the levels of glycolytic and nucleic acid-related metabolites initially suggested that amino acids were more important than metabolites depleted at 24 h. However, the addition of amino acids had no impact on cell growth and butanol production at 37°C. We postulate that the intracellular levels of amino acids in strain N1-4 cultivated at 37°C, although low, were sufficient for cell growth, perhaps because the TYA medium (see Materials and Methods) already contained high levels of amino acids provided by the tryptone peptone and yeast extract medium components. The addition of NAD⁺, NADH, NADP⁺, and NADPH also did not have a significant effect on cell growth or butanol production at the elevated temperature, although the levels of these pyridine nucleotides also were decreased in cells

cultured at 37°C for 24 h. In lactic acid bacteria, nicotinic acid (NA), nicotinamide mononucleotide (NMN), and nicotinamide riboside (NR) are imported into the cells, where these intermediates are converted to NAD⁺ via the NAD salvage pathway (17). Therefore, we also (in work not included here) tested the efficacy of supplementing the culture medium to 100 mg/liter each with NA, NMN, and NR. However, the addition of these three compounds did not restore butanol production in cultures growing at 37°C (data not shown). These results confirmed that the availability of pyridine nucleotide intermediates or cofactors is not the limiting factor for butanol production by strain N1-4 growing at 37°C, despite the apparent depletion of this class of metabolites in cells grown at the elevated temperature. However, we did find that adenine supplementation also restored butanol production during cocultivation at 37°C with cellulolytic *C. thermocellum*, using delignified rice straw as the substrate (Fig. S5). In the absence of adenine supplementation, butanol production was 1.9 ± 1.5 g/liter and below the limit of detection for cocultures grown at 34°C and 37°C, respectively. On the other hand, supplementation with adenine yielded increased butanol production, which reached 6.1 ± 0.6 g/liter and 1.0 ± 0.7 g/liter in cocultures grown at 34°C and 37°C, respectively. These results indicated that adenine addition allowed butanol production at elevated temperatures even during cocultivation with *C. thermocellum*.

Here we discuss the effects of adenine on cell growth and butanol production in strain N1-4 cultivated at 37°C. When N1-4 was cultivated at 37°C, CFU decreased dramatically after 12 h; in contrast, growth of the same strain at 30°C or 37°C in adenine-supplemented medium provided maintenance of CFU to 48 h or 36 h, respectively. These results indicated a correlation (for 37°C cultures) among the cessation of cell growth, the cessation of butanol production, and the loss of cell viability. Our study further revealed that cell viability correlated with the maintenance of intracellular ATP levels and the inhibition of spore formation. The major metabolite derived from adenine is ATP. Most bacteria can convert adenine to ADP and ATP via AMP synthesis, which requires the adenine phosphoribosyltransferase-catalyzed reaction of adenine with PRPP (17). Although this enzyme has not been studied in butanol-producing clostridia, a putative adenine phosphoribosyltransferase-encoding gene is present in the genome sequence of *C. saccharoperbutylacetonicum* (20). In fact, in our experiments, the ATP level was maintained until 36 h in cells cultivated at 37°C in adenine-supplemented medium, while the ATP level fell after 16 h in cells cultivated at 37°C in unsupplemented medium. ATP is a fundamental primary metabolite that is widely used to fuel metabolism. For example, ATP phosphorylates glucose and is used to generate glucose 6-phosphate in the initial steps of the glycolysis pathway. ATP is also used in transcription and protein synthesis, and thermally stressed cells consume large quantities of ATP to repair and to degrade DNA and proteins (9). In the nucleic acid synthesis pathways, ATP generates other (deoxy)nucleoside triphosphates by phosphorylation; other nucleoside triphosphates cannot serve as the phosphate donors for these reactions (17). The addition of nucleobases lacking adenine would not permit the synthesis of ATP, consistent with the observation that unsupplemented medium did not restore cell growth or butanol production to strain N1-4 at 37°C. In separate work (data not shown), we demonstrated that supplementation with adenosine also was sufficient to restore cell growth (maximum OD₆₆₀ of 4.6 ± 0.3) and butanol production (5.7 ± 0.5 g/liter) in cultures growing at 37°C. Based on these facts, we hypothesize that the addition of adenine permits the generation of ATP, thereby facilitating continued cell viability and butanol production in cells growing at 37°C.

We also observed that adenine supplementation inhibited sporulation. The relationship between adenine levels in clostridia and spore formation has not, to our knowledge, been reported previously. However, the relationship between GTP levels and spore formation has been reported in the context of the analysis of *codY* gene function. In *Clostridium perfringens*, as in *B. subtilis*, CodY has been shown to sense intracellular levels of GDP and GTP, such that this protein represses the expression of sporulation genes when the intracellular levels of GDP and GTP are high (12, 14, 15). Adenine can be converted to hypoxanthine and then to IMP by the combined activities of adenine

deaminase and hypoxanthine phosphoribosyltransferase (17). Since genes encoding these activities can be detected in the genome sequence of *C. saccharoperbutylacetonicum* (20), adenine imported into the cells is expected to be converted to GDP and GTP, which would in turn inhibit sporulation via CodY activity. If the levels of GDP and GTP are important for the inhibition of spore formation, then the addition of guanine, guanosine, xanthine, or hypoxanthine should restore cell growth through the inhibition of spore formation. In fact, supplementation with any of those compounds did not inhibit sporulation by strain N1-4 (data not shown). The failure of those nucleobases to have an effect could reflect a lack of transport of the compounds; alternatively, ATP levels could be more critical than GDP and GTP levels for the regulation of spore formation in this organism. Metabolite and global gene expression analysis in cultures grown with adenine supplementation are expected to permit the identification of metabolites and gene expression patterns associated with entry into the sporulation pathway, notably including elucidation regarding whether ATP maintenance or inhibition of spore formation is important for cell viability when strain N1-4 is cultivated at 37°C.

Metabolomic analysis proved to be a powerful tool for identifying the metabolic pathways required for cell growth and butanol production at the elevated culture temperature. We demonstrated that supplementation with adenine restored cell growth and butanol production in N1-4 monocultures and in cocultures with *C. thermocellum*. We note, however, that the need to add adenine would lead to higher costs, both in monocultures and in cocultures with *C. thermocellum*, for large-scale butanol production. Therefore, elucidation of the mechanism of adenine's effect on cell growth and butanol production and the breeding of improved strains that phenocopy the effects of adenine addition will be the subjects of our future work.

MATERIALS AND METHODS

Bacterial strain and cultivation conditions for metabolomic analysis. *Clostridium thermocellum* NBRC103400 (ATCC 27405) was cultured anaerobically at 60°C on National Institute of Technology and Evaluation (NITE) Biological Resource Center (NBRC) medium 979, as described previously (5). The butanol-producing *Clostridium saccharoperbutylacetonicum* strain N1-4 (ATCC 13564) was cultured anaerobically in TYA medium (tryptone peptone, 6 g/liter; yeast extract, 2 g/liter; glucose, 40 g/liter; $\text{CH}_3\text{COONH}_4$, 3 g/liter; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.3 g/liter; KH_2PO_4 , 0.5 g/liter; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.01 g/liter [pH 6.5]) in a test tube capped with a butyl rubber stopper, after substitution of the headspace with nitrogen gas. *C. saccharoperbutylacetonicum* strain N1-4 was cultivated for 12 h and harvested by centrifugation ($5,200 \times g$ for 3 min at 4°C). The cells were suspended in fresh TYA medium to an OD_{600} of 2.0. An aliquot (10 ml) of the resulting cell suspension was used to inoculate 190 ml of prewarmed TYA medium in a serum bottle (yielding a suspension with an OD_{600} of 0.1). The bottle was topped with a butyl rubber stopper equipped with a check valve that permitted the release of evolved gas. Duplicate bottles were prepared in this manner and cultivated at 30°C or 37°C.

Measurement of metabolites. At 6, 12, 24, and 36 h after inoculation, aliquots were transferred to a collection tube and immediately chilled on ice for 90 s. Cells (10^6 cells/sample) were collected by centrifugation ($5,200 \times g$ for 5 min at 4°C) and washed twice with Milli-Q water. The cells were then resuspended in 1,600 μl of methanol and ultrasonicated for 30 s, thereby completely resuspending the cell pellets and inactivating intracellular enzymes. Next, the cell extract was combined with 640 μl of Milli-Q water containing internal standards (H3304-1002; Human Metabolome Technologies, Inc., Tsuruoka, Japan); after mixing, the extract was allowed to stand for another 30 s. The mixture was centrifuged at $2,300 \times g$ for 5 min at 4°C, and then 1,600 μl of the aqueous (upper) layer was centrifugally filtered through a Millipore 5-kDa-cutoff filter at $9,100 \times g$ for 120 min at 4°C, to remove proteins. The filtrate was centrifugally concentrated and resuspended in 50 μl Milli-Q water for capillary electrophoresis-mass spectrometry (CE-MS) analysis. Metabolomic measurements were carried out through a facility service at Human Metabolome Technologies, Inc. Hierarchical cluster analysis (HCA) and principal-component analysis (PCA) were performed using Human Metabolome Technologies proprietary software packages (PeakStat and SampleStat, respectively).

Addition of amino acids, pyridine nucleotides, or nucleobases to the culture medium. Strain N1-4 was cultivated at 37°C in TYA medium supplemented with 100 mg/liter of amino acids, pyridine nucleotides, or nucleobases. The various media were generated as follows. For amino acid-supplemented medium, a 200-mg/liter amino acid stock solution was formulated by dissolving 18 amino acids (alanine, arginine, asparagine, aspartic acid, glutamic acid, glycine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine, and valine) in boiling water. This stock was then mixed 1:1 with $2 \times$ TYA medium. The resulting mixture was autoclaved, kept under anaerobic conditions through N_2 substitution, and then further supplemented by the addition of a 1:100 volume of a filter-sterilized solution containing 10 g/liter each of glutamine and cysteine. For pyridine nucleotide-supplemented medium, a filter-sterilized stock solution of NAD^+ , NADH, NADP^+ , and NADPH was added to $1 \times$ TYA medium after autoclaving (at the same stage as for glutamine and cysteine

addition). For nucleobase-supplemented medium, adenine, thymine, guanine, cytosine, and uracil were added to the medium prior to autoclaving. For medium supplemented with adenine alone, a solution of adenine (dissolved at 10 g/liter in 0.5 M HCl) was added to the medium, and the acidification was neutralized by adjusting the pH to 6.5 with NaOH prior to autoclaving. To generate a control medium (with the same salt concentration), a separate portion of TYA medium also was acidified by the addition of an equivalent volume (compared to the adenine solution) of 0.5 M HCl, followed by neutralization to pH 6.5 using NaOH.

Analytical procedures. Cell densities were determined by using a spectrophotometer (U-2910 spectrophotometer; Hitachi Instruments Inc., Tokyo, Japan) to measure the absorbance of the samples at 600 nm. Levels of fermentation products and residual glucose were measured by high-performance liquid chromatography (HPLC), as described previously (4, 5). The concentrations of nucleic acids in the culture supernatant were measured at 260 nm with an HPLC system equipped with a UV-visible detector (LC10-ADVP pump and SPD-10AVp detector; Shimadzu, Kyoto, Japan), using an RSpak DE-413 column (150 mm by 4.6 mm by 4 μ m; Showa Denko, Tokyo, Japan). The chromatographic conditions were as follows: mobile phase, 50 mM KH₂PO₄; flow rate, 1.0 ml/min; column temperature, 30°C. The two-tailed, nonpaired, Student's *t* test was used to estimate the statistical significance of differences; *P* values of <0.05 were considered significant. Unless otherwise stated, values are presented as means $\pm$ standard errors.

Estimation of cell viability by enumeration of CFU. Cell viability was determined by measurement of CFU. Strain N1-4 was cultured at 30°C or 37°C in TYA medium with or without adenine. Cells were collected by centrifugation (5,200 $\times$ *g* for 5 min at 4°C). The cells were resuspended in an equivalent volume of sterile distilled water and subjected to 10-fold serial dilutions in sterile distilled water. Aliquots were plated onto TYA agar and incubated at 30°C for 48 h prior to counting of colony numbers.

Determination of ATP concentrations. Intracellular ATP concentrations were determined as follows. The cells (2 $\times$ 10⁶ cells/ml) were collected, immediately cooled on ice, and washed once with cold sterilized purified water. The cells were used immediately to determine intracellular ATP levels with the BacTiter-Glo microbial cell viability assay (Promega, Madison, WI, USA), following the manufacturer's instructions. Luminescence was measured using a Luminescencer PSN AB-2000 luminometer (Atto, Tokyo, Japan).

Measurement of the degree of spore formation. The degree of spore formation was estimated through counting of vegetative, dead, and sporulating cells after staining, with a modification of previously reported methods (21, 22). In brief, strain N1-4 was cultivated at 30°C or 37°C in TYA medium with or without adenine. One milliliter of each culture was collected, and the cell suspensions were washed twice with 1 ml of 1% NaCl solution. Following pelleting by centrifugation (5,200 $\times$ *g* for 5 min at 4°C), the cells were resuspended in 100 μ l of 1% NaCl. The resulting suspension was stained by the addition of 2 μ l of a mixture of 0.89 mM Syto9 and 5.0 mM PI, followed by 10 min of incubation in the dark. Stained cells were then washed with 1% NaCl and observed by fluorescence microscopy using a Biozero BZ-8000 microscope (Keyence, Tokyo, Japan), at wavelengths of 483 nm (excitation) and 503 nm (emission) for Syto9 and 535 nm (excitation) and 617 nm (emission) for PI. Vegetative, dead, and spore-forming cells were counted.

Butanol production from delignified rice straw through coculture of *C. thermocellum* and strain N1-4 with adenine supplementation. Coculture experiments were performed in test tubes sealed with rubber stoppers, as described previously for the optimized method (4). The initial OD₆₀₀ of *C. thermocellum* grown in NBRC979 medium was adjusted to 1.0, and cells were cultured at 60°C for 24 h. After 24 h, strain N1-4 grown in TYA medium was collected by centrifugation (6,000 $\times$ *g* for 5 min at 4°C), washed, and suspended in TYA medium. After the incubation temperature was decreased to 30°C, the N1-4 cell suspension was added to the *C. thermocellum*-cultured medium. The final OD₆₀₀ of strain N1-4 in the cocultures was adjusted to 1.0, and separate portions of the cocultures were incubated at 30°C, 34°C, or 37°C, with or without adenine (final concentration, 250 mg/liter). The adenine was added to NBRC979 medium, and the mixture was adjusted to pH 7.0 before autoclaving.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <https://doi.org/10.1128/AEM.02960-16>.

SUPPLEMENTAL FILE 1, PDF file, 0.6 MB.

ACKNOWLEDGMENT

This study was supported in part by a Grant-in-Aid for Young Scientists (A) (JSPS KAKENHI grant 25701017).

REFERENCES

1. Dürre P. 2007. Biobutanol: an attractive biofuel. *Biotechnol J* 2:1525–1534. <https://doi.org/10.1002/biot.200700168>.
2. Binod P, Sindhu R, Singhania RR, Vikram S, Devi L, Nagalakshmi S, Kurien N, Sukumaran RK, Pandey A. 2010. Bioethanol production from rice straw: an overview. *Bioresour Technol* 101:4767–4774. <https://doi.org/10.1016/j.biortech.2009.10.079>.
3. Maki M, Leung KT, Qin W. 2009. The prospects of cellulase-producing bacteria for the bioconversion of lignocellulosic biomass. *Int J Biol Sci* 5:500–516.
4. Kiyoshi K, Furukawa M, Seyama T, Kadokura T, Nakazato A, Nakayama S. 2015. Butanol production from alkali-pretreated rice straw by co-culture of *Clostridium thermocellum* and *Clostridium saccharoperbutylacetoni-*

- cum. *Bioresour Technol* 186:325–328. <https://doi.org/10.1016/j.biortech.2015.03.061>.
5. Nakayama S, Kiyoshi K, Kadokura T, Nakazato A. 2011. Butanol production from crystalline cellulose by co-cultured *Clostridium thermocellum* and *Clostridium saccharoperbutylacetonicum* N1-4. *Appl Environ Microbiol* 77:6470–6475. <https://doi.org/10.1128/AEM.00706-11>.
 6. Baer SH, Blaschek HP, Smith TL. 1987. Effect of butanol challenge and temperature on lipid composition and membrane fluidity of butanol-tolerant *Clostridium acetobutylicum*. *Appl Environ Microbiol* 53:2854–2861.
 7. Heluane H, Evans MR, Dagher SF, Bruno-Bárcena JM. 2011. Meta-analysis and functional validation of nutritional requirements of solventogenic clostridia growing under butanol stress conditions and cointilization of D-glucose and D-xylose. *Appl Environ Microbiol* 77:4473–4485. <https://doi.org/10.1128/AEM.00116-11>.
 8. Jozefczuk S, Klie S, Catchpole G, Szymanski J, Cuadros-Inostroza A, Steinhauser D, Selbig J, Willmitzer L. 2010. Metabolomic and transcriptomic stress response of *Escherichia coli*. *Mol Syst Biol* 6:364.
 9. Fleury B, Kelley WL, Lew D, Götz F, Proctor RA, Vaudaux P. 2009. Transcriptomic and metabolic responses of *Staphylococcus aureus* exposed to supra-physiological temperatures. *BMC Microbiol* 9:76. <https://doi.org/10.1186/1471-2180-9-76>.
 10. Liebig JF. 1840. Die organische Chemie in ihrer Anwendung auf Agricultur und Physiologie. F. Vieweg, Braunschweig, Germany.
 11. Chang RL, Andrews K, Kim D, Li Z, Godzik A, Pálsson BO. 2013. Structural systems biology evaluation of metabolic thermotolerance in *Escherichia coli*. *Science* 340:1220–1223. <https://doi.org/10.1126/science.1234012>.
 12. Nawrocki KL, Edwards AN, Daou N, Bouillaut L, McBride SM. 2016. CodY-dependent regulation of sporulation in *Clostridium difficile*. *J Bacteriol* 198:2113–2130. <https://doi.org/10.1128/JB.00220-16>.
 13. Al-Hinai MA, Jones SW, Papoutsakis ET. 2015. The *Clostridium* sporulation programs: diversity and preservation of endospore differentiation. *Microbiol Mol Biol Rev* 79:19–37. <https://doi.org/10.1128/MMBR.00025-14>.
 14. Ratnayake-Lecamwasam M, Serron P, Wong KW, Sonenshein AL. 2001. *Bacillus subtilis* CodY represses early-stationary-phase genes by sensing GTP levels. *Genes Dev* 15:1093–1103. <https://doi.org/10.1101/gad.874201>.
 15. Shivers RP, Sonenshein AL. 2004. Activation of the *Bacillus subtilis* global regulator CodY by direct interaction with branched-chain amino acids. *Mol Microbiol* 53:599–611. <https://doi.org/10.1111/j.1365-2958.2004.04135.x>.
 16. Masion E, Amine J, Marczak R. 1987. Influence of amino acid supplements on the metabolism of *Clostridium acetobutylicum*. *FEMS Microbiol Lett* 43:269–274. <https://doi.org/10.1111/j.1574-6968.1987.tb02156.x>.
 17. Kilstrup M, Hammer K, Ruhdal Jensen P, Martinussen J. 2005. Nucleotide metabolism and its control in lactic acid bacteria. *FEMS Microbiol Rev* 29:555–590. <https://doi.org/10.1016/j.fmrre.2005.04.006>.
 18. Mordukhova EA, Pan JG. 2013. Evolved cobalamin-independent methionine synthase (MetE) improves the acetate and thermal tolerance of *Escherichia coli*. *Appl Environ Microbiol* 79:7905–7915. <https://doi.org/10.1128/AEM.01952-13>.
 19. Roe AJ, O'Byrne C, McLaggan D, Booth IR. 2002. Inhibition of *Escherichia coli* growth by acetic acid: a problem with methionine biosynthesis and homocysteine toxicity. *Microbiology* 148:2215–2222. <https://doi.org/10.1099/00221287-148-7-2215>.
 20. Del Cerro C, Felpeto-Santero C, Rojas A, Tortajada M, Ramón D, García JL. 2013. Genome sequence of the butanol hyperproducer *Clostridium saccharoperbutylacetonicum* N1-4. *Genome Announc* 1:e0007013. <https://doi.org/10.1128/genomeA.00070-13>.
 21. Jones SW, Paredes CJ, Tracy B, Cheng N, Sillers R, Senger RS, Papoutsakis ET. 2008. The transcriptional program underlying the physiology of clostridial sporulation. *Genome Biol* 9:R114. <https://doi.org/10.1186/gb-2008-9-7-r114>.
 22. Tracy BP, Gaida SM, Papoutsakis ET. 2008. Development and application of flow-cytometric techniques for analyzing and sorting endospore-forming clostridia. *Appl Environ Microbiol* 74:7497–7506. <https://doi.org/10.1128/AEM.01626-08>.