RESEARCH ARTICLE

Enhanced Butanol Production Obtained by Reinforcing the Direct Butanol-Forming Route in *Clostridium acetobutylicum*

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ABSTRACT **Butanol is an important industrial solvent and advanced biofuel that can be produced by biphasic fermentation by *Clostridium acetobutylicum*. It has been known that acetate and butyrate first formed during the acidogenic phase are reassimilated to form acetone-butanol-ethanol (cold channel). Butanol can also be formed directly from acetyl-coenzyme A (CoA) through butyryl-CoA (hot channel). However, little is known about the relative contributions of the two butanol-forming pathways. Here we report that the direct butanol-forming pathway is a better channel to optimize for butanol production through metabolic flux and mass balance analyses. Butanol production through the hot channel was maximized by simultaneous disruption of the *pta* and *buk* genes, encoding phosphotransacetylase and butyrate kinase, while the *adhE1*^{D485G} gene, encoding a mutated aldehyde/alcohol dehydrogenase, was overexpressed. The ratio of butanol produced through the hot channel to that produced through the cold channel increased from 2.0 in the wild type to 18.8 in the engineered BEKW(pPthlAAD**) strain. By reinforcing the direct butanol-forming flux in *C. acetobutylicum*, 18.9 g/liter of butanol was produced, with a yield of 0.71 mol butanol/mol glucose by batch fermentation, levels which are 160% and 245% higher than those obtained with the wild type. By fed-batch culture of this engineered strain with *in situ* recovery, 585.3 g of butanol was produced from 1,861.9 g of glucose, with the yield of 0.76 mol butanol/mol glucose and productivity of 1.32 g/liter/h. Studies of two butanol-forming routes and their effects on butanol production in *C. acetobutylicum* described here will serve as a basis for further metabolic engineering of clostridia aimed toward developing a superior butanol producer.**

IMPORTANCE **Renewable biofuel is one of the answers to solving the energy crisis and climate change problems. Butanol produced naturally by clostridia has superior liquid fuel characteristics and thus has the potential to replace gasoline. Due to the lack of efficient genetic manipulation tools, however, strain improvement has been rather slow. Furthermore, complex metabolic characteristics of acidogenesis followed by solventogenesis in this strain have hampered development of engineered clostridia having highly efficient and selective butanol production capability. Here we report for the first time the results of systems metabolic engineering studies of two butanol-forming routes and their relative importances in butanol production. Based on these findings, a metabolically engineered *Clostridium acetobutylicum* strain capable of producing butanol to a high titer with high yield and selectivity could be developed by reinforcing the direct butanol-forming flux.**

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Acetone-butanol-ethanol (ABE) fermentation by clostridia represents one of the oldest industrial fermentation processes known (1). The industrial fermentative production of butanol continued until the 1980s and stopped due to the loss of economic competitiveness (1). There has recently been much renewed interest in fermentative production of butanol from renewable biomass as an important industrial solvent and a liquid fuel that is superior to ethanol (2–5). The key metabolic pathways and the biphasic fermentation model (Fig. 1A) for ABE production by *Clostridium acetobutylicum* have been well studied (6–11). The clostridial biphasic fermentation model consisting of acidogenic and solventogenic phases has been well accepted (6, 12–14). Acetic and butyric acids are produced together with ATPs in the acidogenic phase, during which cells grow (13, 15–17). Some parts of acids formed are subsequently taken up for solvent production during the solventogenic phase (14, 18–20). Examination of metabolic pathways suggests that butanol can also be formed through the direct route from acetyl-coenzyme A (CoA) via butyryl-CoA. However, it is still unclear whether and how much this direct butanol-forming route contributes butanol production in *C. acetobutylicum*. Furthermore, the metabolic pathways leading to the formation of acids seem to be more complex than previously thought, since knocking out the butyrate kinase (*buk*) gene and the phosphotransacetylase (*pta*) and acetate kinase (*ack*) genes in

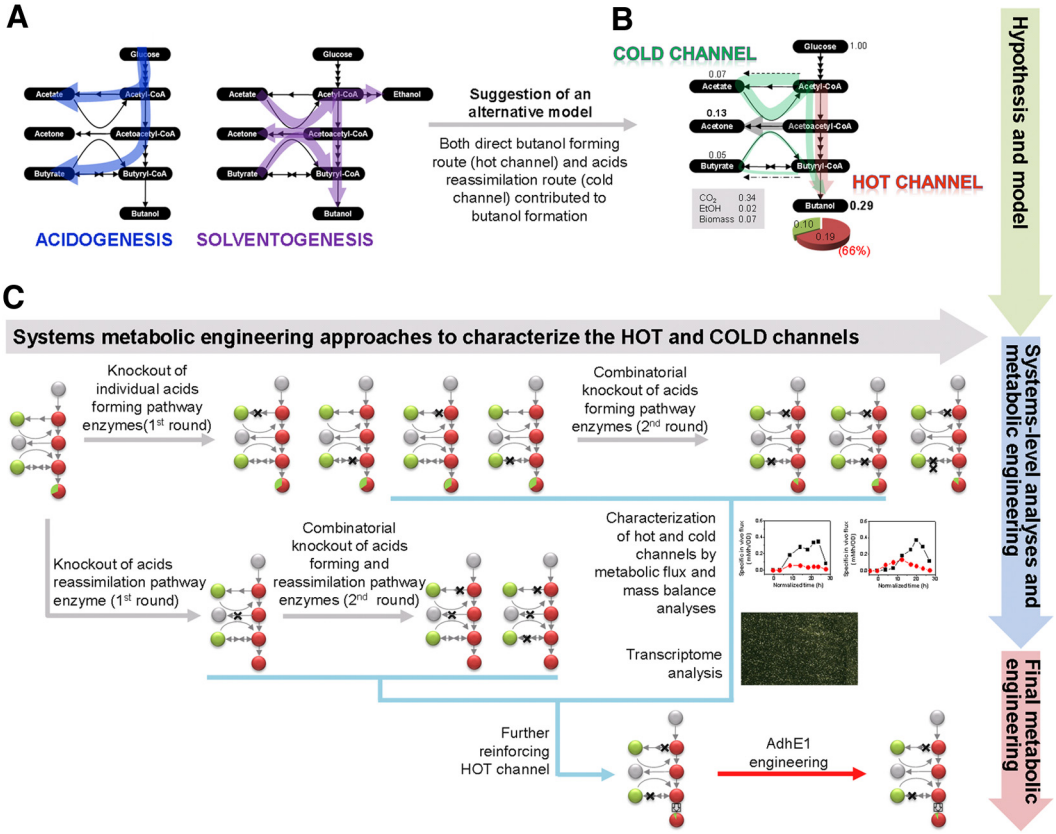

FIG 1 Strategies for characterizing the complex butanol-forming routes by metabolic engineering coupled with system-level metabolic flux and mass balance analyses. (A) The biphasic fermentation model of clostridia can explain both acidogenesis and solventogenesis relatively well. Acetic and butyric acids are produced together with ATPs in the acidogenic phase during which cells grow. Some parts of acids are reassimilated for solvent production during the solventogenic phase. However, butanol can also be formed from butyryl-CoA through the direct forming route from acetyl-CoA without acid reassimilation. (B) Thus, an alternative model for butanol formation is suggested in this article: dual operation of a direct butanol-forming route (hot channel) and an acid reassimilation route (cold channel). Metabolic flux and mass balance analyses predicted that the hot channel (red) that forms butanol directly from butyryl-CoA without acid reassimilation is decoupled from a cold channel (green) that involves a detour route accompanied by acid reassimilation through CoA transferase. (C) Experimental design to examine the relative importances of hot and cold channels. To control the hot-to-cold-channel ratio, the genes encoding enzymes responsible for acid formation and uptake, *pta* or *ack* and *ptb* or *buk* (or *bukII*) and *ctfB*, were individually and combinatorially knocked out. After it was verified that the hot channel plays a more important role in butanol production, the *adhE1* gene was overexpressed to further reinforce the hot channel flux. In the simplified metabolic pathway shown, hot (red) and cold (green) channels together with their relative contributions to butanol formation are indicated. The "x" represents the knockout of the corresponding gene, while the boxed arrow represents the overexpression of the *adhE1* gene.

C. acetobutylicum did not completely eliminate the formation of butyrate and acetate, respectively (13, 21, 22). Thus, a better understanding on the metabolic pathways leading to the formation of acids and solvents is needed to perform metabolic engineering of clostridia toward enhanced butanol production.

Here we describe an alternative model (Fig. 1B) for the production of butanol in *C. acetobutylicum* to better understand the metabolic pathways leading to the formation of acids and solvents, which is needed to perform metabolic engineering of clostridia toward enhanced butanol production. We report the relative contributions of the two butanol-forming pathways, the direct forming route (hot channel) and the acid reassimilation route (cold channel); the former is a better channel to optimize for butanol production by *C. acetobutylicum*. Based on this finding, metabolic engineering of *C. acetobutylicum* was performed to reinforce the hot channel flux, which resulted in enhanced production of butanol.

RESULTS

Alternative model for production of butanol in *C. acetobutylicum*. Metabolic flux and mass balance analyses suggested that the hot channel (red in Fig. 1B), which forms butanol directly from butyryl-CoA without acid reassimilation, is decoupled from the cold channel (green), which involves acid reassimilation through CoA transferase (Fig. 1B). The amount of butanol formed through the cold channel was calculated based on the acetone molar equivalent, as follows (Table 1; see also Table S1 in the supplemental material). Acetone is the end product in the fermentation of *C. acetobutylicum* and is formed from acetoacetate through an irreversible decarboxylation reaction (12). If CoA transferase mainly catalyzes the reactions of reassimilating acetate and butyrate to form acetyl-CoA and butyryl-CoA, respectively, then the molar equivalent of acetone formation would be equal to that of total acid reassimilation. Reassimilation of 1 mol of acetate as an equivalent of acetone would result in the formation of 0.5 mol of butanol, whereas reassimilation of 1 mol of butyrate would result

TABLE 1 Calculation of butanol formed through the hot channel by using the reassimilation ratio of acetate to butyrate

Strain	Growth rate[c] (h^{-1})	Metabolite produced				$\int rACUP_{VOL}/\int rBUUP_{VOL}$[e]	Molar equivalent of butanol formed through hot and cold channels (mmol)		% of butanol formed through hot and cold channels		Ratio of hot- to cold-channel butanol (HotBuOH/ ColdBuOH; mol/mol)
		Metabolite	Conc (g/liter)	Molar equivalent (mmol)	Yield[d] (mol/mol)		ColdBuOH[f]	HotBuOH[g]	ColdBuOH	HotBuOH	
ATCC 824	0.33	Acetone	5.4	93.0							
		Butanol	11.8	159.2	0.43	5.5	53.6	105.6	34	66	2.0
EKW	0.28	Acetone	7.7	132.6							
		Butanol	17.2	232.1	0.52	3.3	81.7	150.4	35	65	1.8
BKW	0.38	Acetone	8.0	137.7							
		Butanol	15.2	205.1	0.48	6.5	78.1	127.0	38	62	1.6
BEKW	0.36	Acetone	2.4	41.3							
		Butanol	16.0	215.9	0.54	1.4	29.3	186.6	14	86	6.4
PEKW	0.19	Acetone	2.2	37.9							
		Butanol	5.9	79.6	0.24	2.1	25.0	54.6	31	69	2.2
BEKW*[a]	0.29	Acetone	2.1	36.2							
		Butanol	18.4	248.2	0.67	1.1	26.6	221.7	11	89	8.3
BEKW**[b]	0.27	Acetone	1.5	25.8							
		Butanol	18.9	255.0	0.71	1.81×10^5	12.9	242.1	5	95	18.8

[a] BEKW*, BEKW(pPptbAAD).
[b] BEKW**, BEKW(pPthlAAD**).
[c] Growth rate was calculated only for those cells in log phase.
[d] Butanol yield on glucose.
[e] Reassimilation ratio of acetate to butyrate ($\int rACUP_{VOL}/\int rBUUP_{VOL}$). Integration of the flux was carried out using a function of the calculus/integrate in the Origin 7.0 software program (OriginLab Corp., Northampton, MA). See Table S1 in the supplemental material for details on the calculation of the $\int rACUP_{VOL}/\int rBUUP_{VOL}$ value.
[f] Butanol formed through the cold channel: ColdBuOH (mmol) = [molar equivalent of butanol produced (mmol) × $\int rACUP_{VOL}/(\int rACUP_{VOL} + \int rBUUP_{VOL})$]/2 + [molar equivalent of butanol produced (mmol) × $\int rBUUP_{VOL}/(\int rACUP_{VOL} + \int rBUUP_{VOL})$].
[g] Butanol formed through the hot channel: HotBuOH (mmol) = molar equivalent of butanol (mmol) − ColdBuOH (mmol).

in the formation of 1 mol of butanol (23). The reassimilation ratio of acetate to butyrate during the solventogenesis was determined as the ratio of integrated volumetric acetate uptake flux ($\int rACUP_{VOL}$) to integrated volumetric butyrate uptake flux ($\int rBUUP_{VOL}$) (see Table S1); the acetate ($rACUP_{VOL}$) and butyrate ($rBUUP_{VOL}$) uptake fluxes were estimated by metabolic flux analysis (Fig. 2; see Fig. S1 and Table S2). Then, butanol formed through the cold channel was calculated from the acetone molar equivalent, as seen in Table 1. It was found that the hot channel is a better route to optimize for butanol production in *C. acetobutylicum.*

To verify whether the hot channel is a better channel to optimize for butanol production, experiments were designed to control the hot-to-cold-channel ratio. The genes encoding enzymes responsible for acid formation and uptake, including the *pta* or *ack* gene and the *ptb* (encoding phosphotransbutyrylase) or *buk* gene and the *ctfB* (encoding acetoacetyl-CoA transferase) gene, were individually and combinatorially knocked out (Fig. 1C). The percentage of butanol produced in the wild-type ATCC 824 strain through the hot channel was 66%, resulting in a ratio of hot- to cold-channel butanol of 2.0 (Fig. 1B; Table 1). Thus, it was found, in a difference from the well-known biphasic fermentation model, that the hot channel plays a more important role in butanol production by *C. acetobutylicum.*

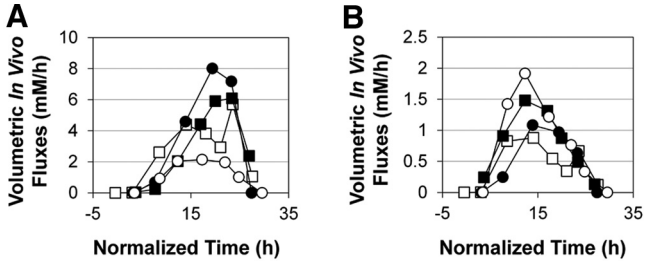

FIG 2 Metabolic flux analysis of *C. acetobutylicum* ATCC 824 (□) and engineered EKW (■), BKW (●), and BEKW (○) strains: (A) Volumetric acetate uptake flux; (B) Volumetric butyrate uptake flux. See Fig. S1A and Table S2 in the supplemental material for a schematic diagram of the model and reactions for metabolic flux analyses used in this study, respectively.

Effect of blocking the cold channel on production of butanol. To examine the effects of possibly enhancing the hot channel flux by blocking the cold channel on the production of butanol, the *ctfB* gene was disrupted to construct the CKW strain (Fig. 3F and M). Acetoacetyl-CoA transferase is directly involved in the reassimilation of both acetate and butyrate, with concomitant production of acetoacetate, which is further converted to acetone (Fig. 1B). Anaerobic fermentation of the CKW strain showed that butanol was produced only through the hot channel, although at a lower butanol yield of 0.21 $C_{butanol}$ mol per mol of $C_{glucose}$ (Fig. 4B; see Table S3 in the supplemental material) compared with the ATCC 824 strain (0.29 $C_{butanol}$ mol per mol of $C_{glucose}$). Batch fermentation of the CKW strain showed continuous accumulation of acetate (up to 9.3 g/liter), with a yield of 0.24 $C_{acetate}$ mol per mol of $C_{glucose}$ (Fig. 4B; see Table S3); these values are much higher than those obtained with the wild-type ATCC 824 strain, which produced 4.8 g/liter acetate with a yield of 0.07 $C_{acetate}$ mol per mol of $C_{glucose}$ (Fig. 4A; see also Fig. S3A and Table S3). The final butyrate concentration was 4.3 g/liter after reaching the peak

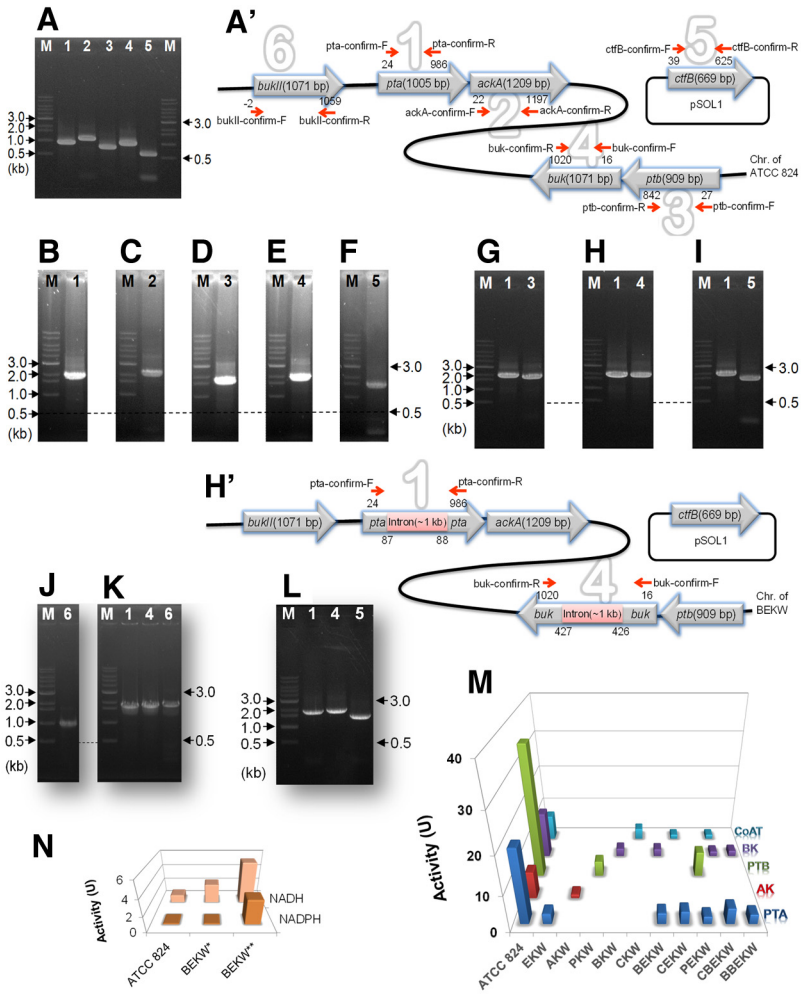

FIG 3 Confirmation of gene knockout by PCR and assay of enzyme activity: ATCC 824 as a control (A and J), EKW (B), AKW (C), PKW (D), BKW (E), CKW (F), PEKW (G), BEKW (H), CEKW (I), BBEKW (K), or CBEKW (L) strain; chromosome of ATCC 824 (A′) or of BEKW (H′); or specific enzyme activities of the wild type and its mutants (M) or alcohol dehydrogenase activities coupled with NADH and NADPH (N). See Fig. S4D in the supplemental material for chromosomes of strains EKW, AKW, PKW, BKW, CKW, PEKW, CEKW, BBEKW, and CBEKW. Lanes in agarose gels: M, 1-kb ladder; 1, PCR product amplified using primers pta-confirm-F and pta-confirm-R; 2, PCR product amplified using primers ackA-confirm-F and ackA-confirm-R; 3, PCR product amplified using primers ptb-confirm-F and ptb-confirm-R; 4, PCR product amplified using primers buk-confirm-F and buk-confirm-R; 5, PCR product amplified using primers ctfB-confirm-F and ctfB-confirm-R; 6, PCR product amplified using primers bukII-confirm-F and bukII-confirm-R. Primer sequences used in this study are listed in Table S4. Abbreviations for enzymes are as follows: PTA, phosphotransacetylase; AK, acetate kinase; PTB, phosphotransbutyrylase; BK, butyrate kinase; and CoAT, CoA transferase. BEKW* and BEKW** represent the BEKW(pPptbAAD) and BEKW(pPthlAAD**) strains, respectively. The enzyme activity of 1 U is defined as the amount of enzyme required for converting 1 μmol of substrate to product at 30°C per min per milligram of protein. Each assay was performed in duplicate, and the results shown are average values.

concentration of 6.8 g/liter in the fermentation of the CKW strain (see Table S3). These results suggest that acetate reassimilation depends mainly on the cold channel by the CoA transferase reaction, while butyrate reassimilation occurs through the reverse reactions of PTB-BK (22, 24), as well as the cold channel by the CoA transferase reaction in *C. acetobutylicum* (see Text S1).

The *pta* gene was subsequently inactivated in the CKW strain to see the effects of simultaneously removing the acetate-forming pathway together with the acid reassimilation pathway on hot channel flux (Fig. 3I and M). In order to knock out multiple genes, a gene knockout strategy based on the Targetron system (25), employing alternating antibiotic markers, was developed (see Fig. S2 and Text S1 in the supplemental material). The *pta-ctfB*-deficient CEKW strain showed a significantly decreased yield of butanol (0.02 $C_{butanol}$ mol per mol of $C_{glucose}$) compared with that of the *ctfB* mutant (Fig. 4B and 4C). On the other hand, the CEKW strain showed increased butyrate production (9.6 g/liter with a yield of 0.38 $C_{butyrate}$ mol per mol of $C_{glucose}$) compared with that of the ATCC 824 strain (2.6 g/liter, with a yield of 0.05 $C_{butyrate}$ mol per mol of $C_{glucose}$) and the CKW strain (4.3 g/liter with a yield of 0.15 $C_{butyrate}$ mol per mol of $C_{glucose}$) (Fig. 4; see also Table S3). Thus, the surplus carbon flux available by knocking out the *pta* gene in the *ctfB* mutant was channeled mainly toward butyrate rather than the hot channel at the butyryl-CoA node.

The *buk* gene was subsequently inactivated in the CEKW strain to see the effects of simultaneously removing both acid-forming pathways together with the CoA transferase reaction on the hot channel flux (Fig. 3L and M). The *pta-buk-ctfB*-deficient CBEKW strain showed a butanol yield of 0.23 $C_{butanol}$ mol per mol of $C_{glucose}$, with an increased butyrate yield of 0.34 $C_{butyrate}$ mol per mol of $C_{glucose}$ (see also Fig. S4A and C in the supplemental material). These results suggest that knocking out the *ctfB* gene without complete blockage of acid production cannot improve butanol yield due to the stress caused by the high-level accumulation of acids.

Effects of removing acid-forming pathways on hot channel flux. To examine the effects of removing the acid-producing pathways on hot channel flux in more detail, the genes responsible for the formation of either acetate (*pta* and *ack*) or butyrate (*ptb* and *buk*) were individually deleted in *C. acetobutylicum* ATCC 824 to construct mutant strains, EKW (Δ*pta*), AKW (Δ*ack*), PKW (Δ*ptb*), and BKW (Δ*buk*) (Fig. 3B to E and M). Among these single-gene knockout mutants, the *pta* and *buk* mutant strains were selected as the representative strains lacking the acetate and butyrate pathways, respectively, based on fermentation results showing higher butanol production (see Fig. S3C to S3F and Table S3 in the supplemental material; see also Text S1 for details on butanol production by these mutant strains). The single-gene knockout mutant strains EKW and BKW showed increased butanol yields of 0.34 and 0.32 $C_{butanol}$ mol per mol of $C_{glucose}$ (Fig. 4D and E; see also Table S3), respectively, compared with strain ATCC 824 (0.29 $C_{butanol}$ mol per mol of $C_{glucose}$). However, the percentages of butanol produced in the EKW and BKW strains through the hot channel were 65% and 62%, giving ratios of hot- to cold-channel butanol of 1.8 and 1.6, respectively, which are

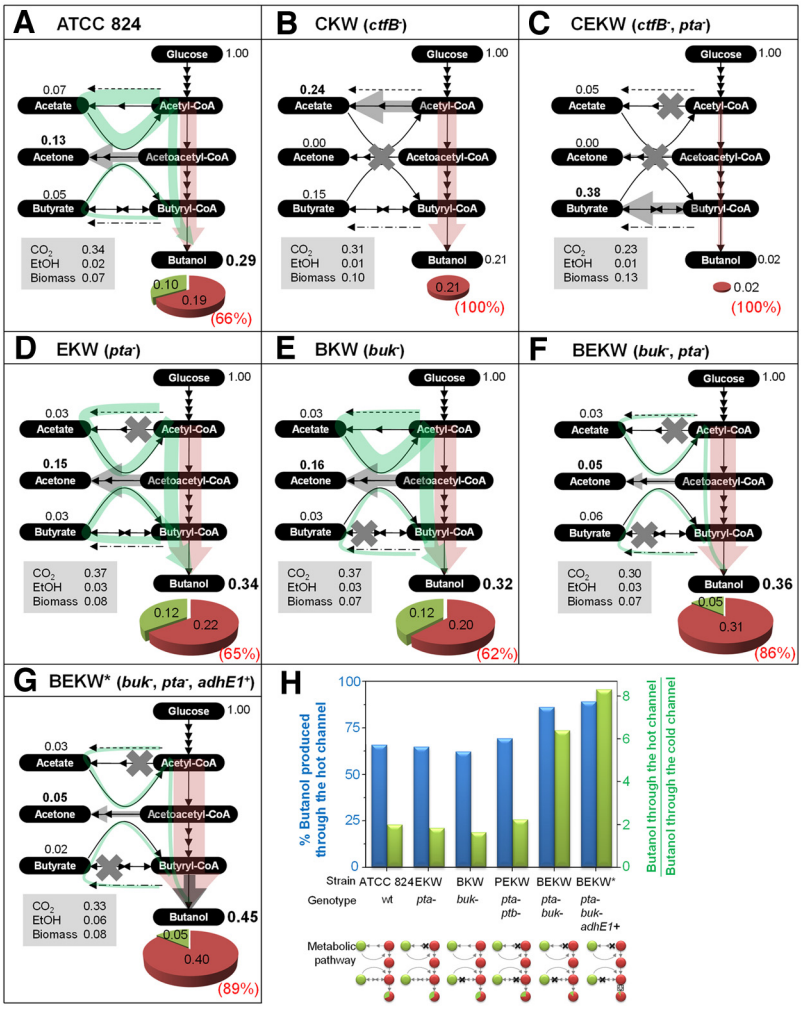

FIG 4 Mass balance analysis of *C. acetobutylicum* ATCC 824 (A) or engineered strain CKW (B), CEKW (C), EKW (D), BKW (E), BEKW (F), or BEKW(pPptbAAD) (G). BEKW* represents the BEKW(pPptbAAD) strain. The numbers next to the metabolites represent carbon molar yields. A green arrow represents the cold channel, and a red arrow represents the direct butanol-forming hot channel. Dashed and dashed-dotted lines represent the unknown alternative pathways for forming acetate and butyrate, respectively. The numbers in pie graphs represent carbon molar yields for butanol produced through the cold (green) or hot (red) channel. The percentage shown next to the pie chart represents the percentage of butanol produced through the hot channel. The reassimilation ratios of acetate to butyrate ($\int rACUP_{VOL}/\int rBUUP_{VOL}$) were 5.5, 3.3, 6.5, 1.4, and 1.1 for ATCC 824, EKW, BKW, BEKW, and BEKW*, respectively (Table 1; see also Table S1 in the supplemental material). (H) Hot- and cold-channel butanol production in ATCC 824 and the engineered EKW, BKW, PEKW, BEKW, and BEKW(pPptbAAD) strains. The percentage of butanol produced through the hot channel (blue; left axis) and the ratio of butanol produced through the hot and cold channels (green; right axis) are shown. In the simplified metabolic pathway shown below, hot (red) and cold (green) channels, together with their relative contributions to butanol formation, are indicated. The "x" represents the knockout of the corresponding gene, while the boxed arrow represents the overexpression of the *adhE1* gene.

lower than the values (66% and 2.0) obtained with ATCC 824 (Fig. 4A, D, E, and H). This result suggests that the selected disruption of either the acetic or butyric acid pathway results in an increased butanol yield by enhancing the cold channel flux through the remaining (opposite) acid-forming pathway rather than increasing the hot channel flux.

Since the *pta* and *buk* single-knockout mutants produced more butanol than the wild-type strain, the combined deletion of the *pta* and *buk* genes was attempted to see if butanol production could be further increased. The *buk* gene was additionally knocked out in the EKW strain to construct the BEKW strain (Fig. 3H, H′, and M). The *pta-buk*-deficient BEKW strain produced butanol with a higher yield of 0.36 $C_{butanol}$ mol per mol of $C_{glucose}$ (Fig. 4F; see also Fig. S3B in the supplemental material). Interestingly, butanol was mainly produced through the hot channel in the BEKW strain (Fig. 4F and H), which is different from what was observed in the EKW and BKW strains. The percentage of butanol produced in strain BEKW through the hot channel was 86%, resulting in a ratio of hot- to cold-channel butanol of 6.4 (Fig. 4H), values which are much higher than those (66% and a ratio of 2.0) obtained with ATCC 824.

It was also notable that the butanol selectivity obtained with the BEKW strain was 0.80 g butanol/g of total solvents, which is significantly higher than those, 0.65, 0.60, and 0.64 g butanol/g of total solvents, obtained with the ATCC 824, BKW, and EKW strains, respectively (see Table S3 in the supplemental material). The high butanol selectivity obtained with BEKW was due mainly to the enhancement of the hot channel flux together with less acetone (2.4 g/liter) production (Fig. 4F; see also Table S3). The other double-knockout strain, the *pta-ptb*-deficient PEKW strain, also showed increased (69%) butanol formation through the hot channel (Fig. 3G and M and Table 1; see also Fig. S3G), which is higher than that in ATCC 824 but lower than that obtained with the BEKW strain.

Further reinforcement of hot channel flux by *adhE1* overexpression. During metabolic flux analysis together with mass balance analysis, interesting results were found with respect to the solventogenesis triggering point (see Text S1 in the supplemental material). The specific butanol formation rate in the BEKW strain increased at 3.5 h, while that in the ATCC 824 strain increased at 8.6 h, which suggested a rapid induction of phase transition from acidogenesis to solventogenesis by enhancing the hot channel flux (see Fig. S1 and Table S3; see also Text S1). Transcriptome analysis showed that the expression level of the solventogenic *adhE1* gene, encoding aldehyde/alcohol dehydrogenase, increased by 43.3 times in the BEKW strain during the late acidogenic phase compared with that in the wild-type strain, ATCC 824. Based on this finding, further increasing butanol formation through the hot channel during the acidogenic phase was attempted by overexpressing the *adhE1* gene under control of the *ptb* promoter (acidogenic phase promoter).

Plasmid pPptbAAD (see Table S4 in the supplemental material), expressing the *adhE1* gene, was transformed into the BKEW strain to construct BEKW(pPptbAAD). Expression of the *adhE1* gene in the BEKW strain indeed enhanced butanol production through the hot channel, resulting in the ratio of hot- to cold-

channel butanol of 8.3 (Fig. 4G and H). Anaerobic batch fermentation of the BEKW(pPptbAAD) strain resulted in the production of 18.4 g/liter butanol with a yield of 0.45 $C_{butanol}$ mol per mol of $C_{glucose}$ (Fig. 4G; see also Fig. S3H), values which are much higher than those obtained with other strains (Fig. 4). These results suggest that the hot channel plays a more important role than the cold channel for the enhanced production of butanol by *C. acetobutylicum*.

High-level production of butanol by hot-channel-reinforced strain. To examine if this hot-channel-reinforced strain can be employed for industrial butanol production, further strain optimization was performed, followed by bioprocess development. In order to ensure enough reducing power for butanol production, the modified aldehyde/alcohol dehydrogenase that possesses cofactor affinities for both NADH and NADPH was employed (the 485th amino acid residue Asp was replaced with Gly) (Fig. 3N) (see Materials and Methods; see also Fig. S5 in the supplemental material). Also, it was aimed to overexpress the *adhE1* gene during both acidogenic and solventogenic phases by employing the thiolase promoter instead of the *ptb* promoter (26). Anaerobic batch fermentation of the resulting BEKW(pPthlAAD**) strain was able to produce 18.9 g/liter butanol (ratio of hot- to cold-channel butanol of 18.8), with a yield of 0.71 mol/mol and a selectivity of 0.88 g butanol/g total solvents from 64.8 g/liter glucose consumed (Fig. 5A; Table 1), which are the highest values obtained with *C. acetobutylicum* to date.

Since butanol is very toxic to microbes, including *C. acetobutylicum*, fermentation coupled with an *in situ* solvent recovery process (27) was performed (Fig. 5B). Butanol adsorption polymer resin (Sephabeads SP850; Shimadzu, Tokyo, Japan) was employed to recover butanol *in situ* during the fed-batch fermentation. The BEKW(pPthlAAD**) strain developed by reinforcing the hot channel was able to produce 585.3 g of butanol from 1,861.9 g of glucose in 111 h of fed-batch fermentation with an *in situ* recovery process (Fig. 5C). A high yield of 0.76 mol butanol/mol glucose (0.31 g/g; 87% of the theoretical maximum) and a high butanol productivity of 1.32 g/liter/h were achieved (Fig. 5C), suggesting that the engineered strain possessing reinforced flux through the hot channel can significantly reduce the overall butanol production cost.

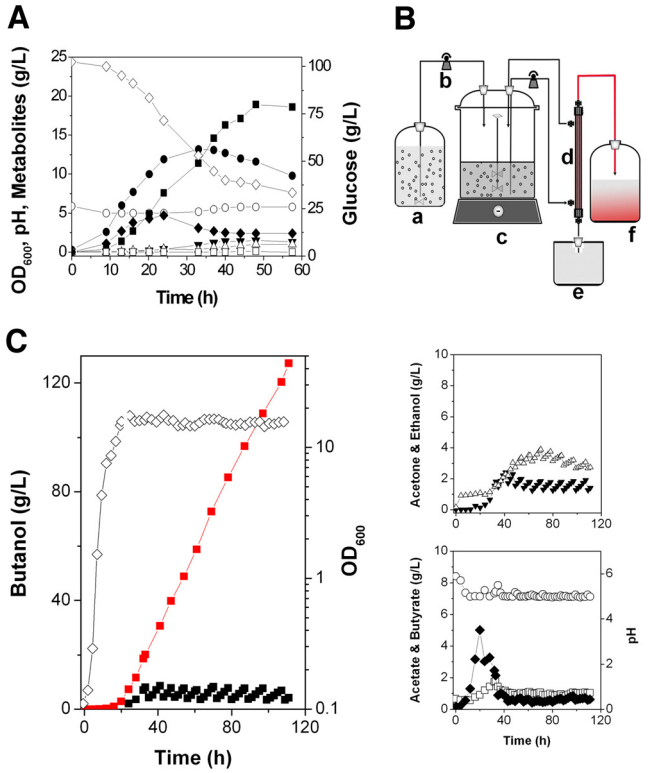

FIG 5 Fermentation profiles of the *C. acetobutylicum* BEKW (pPthlAAD**) strain. (A) Batch fermentation in CGM supplemented with 100 g/liter glucose at 37°C. (B) Schematic of fed-batch fermentation coupled with *in situ* recovery process. Symbols are as follows: a, feed tank; b, pump; c, bioreactor; d, column containing Sephabeads; e, steam generator; f, collection tank. (C) Fed-batch fermentation equipped with *in situ* recovery process. Symbols are as follows: ◇, OD_{600}; ○, pH; □, acetate; ♦, butyrate; ■, butanol; △, ethanol; ▼, acetone; red square, total BuOH produced.

DISCUSSION

It has been well accepted that *C. acetobutylicum* produces ABE through its characteristic biphasic fermentation (Fig. 1A). Butanol can also be produced by the direct forming route, named hot channel here, but its contribution to butanol production has not been well studied. Here, we report the important role of the direct butanol-forming hot channel in butanol production by *C. acetobutylicum* through combined metabolic flux and mass balance analyses (Fig. 1B). Based on these results, strategies for enhancing butanol production by reinforcing the hot channel flux were designed and implemented.

The key enzyme for the cold channel flux, CoA transferase encoded by the *ctfAB* genes, is induced during the solventogenic phase and is responsible for the uptake of produced acids during the acidogenic phase (12, 20, 22). It was found that reducing the cold channel flux without the complete blockage of acid production could not improve butanol production due to the high-level accumulation of acids. Anaerobic cultivation of the mutant strains CKW, CEKW, and CBEKW, which lack the cold channel fluxes, showed butanol production only through the hot channel, but the butanol yields obtained were rather low. On the other hand, these strains produced much more (3- to 8-fold higher) acetate or butyrate than the wild-type strain. High-level accumulation of acids is due to the lack of cold channel fluxes in these strains. Thus, the metabolic pathways leading to the formation of acids seem to be more complex than previously thought in *C. acetobutylicum*.

The ratio of hot- to cold-channel butanol is enhanced by engineering acid-forming pathways without the modification of the key enzyme of the cold channel, CoA transferase. The BEKW strain produced butanol with a high yield of 0.36 $C_{butanol}$ mol per mol of $C_{glucose}$ by enhancing the hot channel (86% of butanol formed through the hot channel and a ratio of hot- to cold-channel butanol of 6.4). However, the selected disruption of either the acetic or butyric acid pathway resulted in an increased butanol yield by enhancing the cold channel flux through the remaining (opposite) acid pathway rather than increasing the hot channel flux. At the acetyl-CoA node during acidogenic phase, acetyl-CoA can be converted into acetate or butyryl-CoA (as a precursor for butyrate) through condensation into acetoacetyl-CoA (12, 20). The other double-knockout combination in the acetate and butyrate pathways, the *pta-ptb*-deficient PEKW strain, also showed increased butanol formation through the hot channel (69% of butanol formed through the hot channel), which was higher than

that obtained with ATCC 824 but lower than that obtained with the BEKW strain. This can be explained by the role of butyryl-P as a signal metabolite. It has been reported that butyryl-P plays a regulatory role and its accumulation might trigger solventogenesis in *C. acetobutylicum* (12).

It has been reported that the overexpression of the *adhE1* gene increased butanol formation in clostridia (28). In our study, it was more interesting to find that the expression of the *adhE1*^{D485G} gene in the BEKW strain increased the ratio of hot- to cold-channel butanol to 18.8. Batch fermentation of the BEKW(pPthlAAD**) strain resulted in production of 18.9 g/liter butanol with a yield of 0.71 mol/mol of glucose. These results also suggest that enhancing the hot channel flux by overexpression of the *adhE1*^{D485G} gene led to the production of butanol to the highest level reported for *C. acetobutylicum* under the same culture condition. Indeed, fed-batch culture of this engineered strain coupled with the *in situ* recovery process allowed production of 585.3 g butanol from 1,861.9 g glucose, with a high yield of 0.76 mol butanol/mol glucose (87% of the theoretical maximum) and a high productivity of 1.32 g/liter/h.

Although a metabolically engineered *C. acetobutylicum* strain capable of producing butanol to a high titer with high yield and selectivity could be developed by reinforcing the direct butanol-forming flux in this work, acid formation was not completely blocked. Individual knockout of the *pta*, *ack*, *ptb*, and *buk* genes of *C. acetobutylicum* did not completely eliminate the formation of acetate and butyrate in this study. To prevent the possibility of acid formation through a known metabolic pathway, the *pta-buk-bukII*-deficient *C. acetobutylicum* strain BBEKW was constructed, as was the PEKW strain. Even in these knockout mutants, acids were still produced during batch fermentation (see Text S1 in the supplemental material), and the activities of enzymes that were knocked out still remained at the level of 16% of those in the wild-type strain (Fig. 3M). In the previous studies, the knockout of the *pta*, *ack*, *ptb*, and *buk* genes in *C. acetobutylicum* ATCC 824 also did not completely abolish the corresponding enzyme activities and resulted in reduced enzyme activities, 1.9% to 19% of those in the wild-type strain (13, 21, 29, 30). Our results obtained with the *pta*, *ack*, and *buk* mutants are consistent with those reported in the previous studies (13, 21, 29). However, the results obtained with the *ptb* knockout mutant were different from those reported in the previous study even though the same insertion site was targeted (30); the *ptb* mutant did not produce butyrate in the previous work (30). Although this is not clear, there might be uncharacterized enzymes performing phosphotransacetylase-, acetate kinase-, phosphotransbutyrylase-, and butyrate kinase-catalyzed reactions. For example, acids can be produced from aldehydes by aldehyde ferredoxin oxidoreductase (AFOR). It has been reported that this enzyme, from some thermophile archaea, can convert short-chain aldehydes into their corresponding carboxylic acids (31, 32). The enzyme encoded by the CAC2018 gene has an amino acid identity of 30% with the AFOR enzyme encoded by PF0346 from *Pyrococcus furiosus* DSM 3638. Thus, further studies are needed to elucidate the mechanisms of acid formation in the mutant strains constructed in this study.

In summary, system-level analysis of butanol-forming routes in *C. acetobutylicum* suggested new metabolic characteristics of butanol production in this bacterium. The direct butanol-forming hot channel and acid-reassimilating cold channel cooperate in the production of butanol, while the former plays a more important role in enhanced butanol production. Butanol production by *C. acetobutylicum* could be enhanced with respect to all important bioprocess objectives, including product concentration, yield, selectivity, and productivity, by reinforcing the direct butanol-forming hot channel rather than the traditionally well-known acid reassimilation (cold channel) pathways. The new butanol-forming metabolic characteristics described here, together with the metabolic engineering strategies based on these findings, would be valuable for the development of a superior *C. acetobutylicum* strain capable of highly efficient butanol production. Further metabolic engineering will focus on achieving higher butanol tolerance, increased carbon flux toward butanol, and further reduction of by-product formation.

MATERIALS AND METHODS

Strains, plasmids, oligonucleotides, and culture conditions. All bacterial strains, plasmids, and oligonucleotides used in this study are listed in Table S4 in the supplemental material. *C. acetobutylicum* strains were cultivated anaerobically in an anaerobic chamber (Forma Scientific, Marietta, OH) containing 96% nitrogen and 4% hydrogen at 37°C. See Text S1 for details of culture conditions.

Gene knockout. Knocking out the chromosomal genes in clostridia is not trivial. The detailed procedure for knocking out the genes is described in Text S1 and Fig. S2 in the supplemental material. Briefly, the plasmids pCACYS3-PtaKO, pCACYS3-AckAKO, pCACYS3-PtbKO, pCACYS3-BukKO, and pCACYS3-CtfBKO were used to disrupt the *pta*, *ack*, *ptb*, *buk*, and *ctfB* genes, respectively. For the construction of double gene knockout strains, the plasmids pJY2-BukKO, pJY2-CtfBKO, and pJY2-PtbKO were used to disrupt the *buk*, *ctfB*, and *ptb* genes, respectively, as the second gene to be knocked out. Plasmid pCACYS3-BukIIKO was used to disrupt the *bukII* gene as the third target to be knocked out. Each knockout plasmid was transformed into *C. acetobutylicum*. A transformant was subcultured several times in Clostridial Growth Medium (CGM; see Text S1 in the supplemental material) supplemented with an appropriate antibiotic to induce integration of the intron. The knockout mutant was screened in a medium containing the same antibiotic.

Construction of *adhE1*-expressing plasmid pPptbAAD. The *adhE1* gene was cloned between SalI and EcoRI sites of the *C. acetobutylicum* expression vector pIMP1exter (see Table S4 in the supplemental material), in which the target gene to be expressed can be inserted at multiple cloning sites between the *ptb* promoter (P_{ptb}) and the thiolase terminator (TT_{thl}). The *adhE1* gene was amplified with the total DNA of *C. acetobutylicum* ATCC 824 using the primers AdhE1-F and AdhE1-R (see Table S4), containing SalI and EcoRI recognition sequences. pIMP1exter was constructed by cloning the P_{ptb} and the TT_{thl} at the PstI/SalI sites and EcoRI/NdeI sites of pIMP1, respectively. Both P_{ptb} and TT_{thl} were amplified with total DNA of *C. acetobutylicum* ATCC 824 using the Pptb-F and Pptb-R primer pair and the TTthl-F and TTthl-R primer pair (see Table S4).

Construction of *adhE1*^{D485G}-expressing plasmid pPthlAAD**. The 485th amino acid residue, Asp, of *C. acetobutylicum* AAD was determined by the alignment of *C. acetobutylicum* AAD and *Zymomonas mobilis* ADH2 (see Fig. S5 in the supplemental material). In a previous report, the D38G variant of ADH2 of *Z. mobilis* showed an increased affinity for NADPH (26). To construct the *adhE1*^{D485G} mutant, two DNA fragments of the *adhE1* gene were amplified with the total DNA of *C. acetobutylicum* ATCC 824 using the primer pairs AdhE1-F0/D485G-R and D485G-F/AdhE1-R0 (see Table S4). Both resulting PCR products were used as the template to amplify the mutant *adhE1*^{D485G} gene with the primers AdhE1-F0 and AdhE1-R0, containing PstI and AvaI recognition sequences. Finally, the *adhE1*^{D485G} gene was cloned between PstI and AvaI sites of the *C. acetobutylicum* expression vector pTHL1-Cm (see Table S4), in which the target gene to be expressed can be inserted in multiple cloning sites downstream of the *thl* promoter.

Fermentation and metabolite analysis. Batch fermentation of each *C. acetobutylicum* strain was repeated at least three times in a 5-liter LiFlus GX bioreactor (Biotron, Kyunggi-Do, Republic of Korea) containing 1.8 liters of CGM and 0.2 liters of seed culture under anaerobic conditions at 37°C. The pH was automatically kept above 5.0 with ammonia solution during acidogenesis and was not controlled when it became higher than the set value during the solventogenic phase. Nitrogen gas was sparged throughout the fermentation at a flow rate of 0.5 liters/min. Agitation speed was controlled at 200 rpm. Fed-batch fermentation of the *C. acetobutylicum* BEKW(pPthlAAD**) strain was performed with 4 liters of initial working volume (3.6 liters of CGM and 0.4 liters of seed culture) and equipped with a recovery column (void volume = 2 liters) packed with 500 g Sephabeads (SP850; Shimadzu, Tokyo, Japan) under the same conditions as batch cultures. A concentrated solution containing 400 g/liter glucose was fed into the fermenter automatically at feeding rate of 0.7 to 1.2 ml/min when the glucose concentration in the fermenter decreased to ca. 20 g/liter, in order to maintain the glucose concentration at 20 to 30 g/liter. The circulation of culture broth through the Sephabeads column was started using a peristaltic pump (Cole-Parmer, Vernon Hills, IL) when the butanol concentration in the fermenter reached ca. 7 g/liter to recover butanol from the reactor. The Sephabeads column was recycled by using hot-water steam (19.6 N) every 4 to 8 h.

Cell growth was monitored by measuring the optical density at 600 nm (OD_{600}) using an Ultrospec 3000 spectrophotometer (Pharmacia Biotech, Uppsala, Sweden). Concentrations of solvents, including acetone, ethanol, and butanol, were determined by gas chromatography (Agilent 7890; Agilent Technologies, California) equipped with a packed column (80/120 Carbopack B AW glass column; Supelco, Bellefonte, PA) and a flame ionization detector. Analysis of glucose and organic acids was carried out by a high-performance liquid chromatography (HPLC) system (Prostar; Varian, Palo Alto, CA) equipped with a packed column (Metacarb 87H; MetaChem Technologies, Torrance, CA) and a refractive index detector (RI-27; Shodex, Japan).

Mass balance and metabolic flux analyses. Mass balance analysis was performed for the hot channel and cold channel toward butanol formation. Mass balances for the cold-channel butanol were calculated based on the acetone molar equivalent (Table 1). Acetone is the end product in the fermentation of *C. acetobutylicum*, and acetone is formed from acetoacetate through an irreversible decarboxylation reaction (12). If CoA transferase mainly catalyzes the reactions of reassimilating acetate and butyrate to form acetyl-CoA and butyryl-CoA, respectively, then the molar equivalent of acetone formation would be equal to that of total acid reassimilation. Assuming that reassimilation of acetate and butyrate is used only for the synthesis of butanol, reassimilation of 1 mol of acetate as an equivalent of acetone would result in the formation of 0.5 mol of butanol, whereas reassimilation of 1 mol of butyrate would result in the formation of 1 mol of butanol (23). The reassimilation ratio of acetate to butyrate during solventogenesis was determined as the ratio of integrated volumetric acetate uptake flux ($\int rACUP_{VOL}$) to integrated volumetric butyrate uptake flux ($\int rBUUP_{VOL}$) (see Table S1 in the supplemental material); the acetate ($rACUP_{VOL}$) and butyrate ($rBUUP_{VOL}$) uptake fluxes were estimated by metabolic flux analysis (Fig. 2; see also Text S1). Integration of the flux was performed using the Origin 7.0 software program (OriginLab Corp., Northampton, MA). The reassimilation ratios of acetate to butyrate were 5.5, 3.3, 6.5, 1.4, 2.1, and 1.1 in strains ATCC 824, EKW, BKW, BEKW, PEKW, and BEKW(pPptbAAD), respectively (Table 1; see also Table S1).

Calculations of *in vivo* fluxes of the established strains with nonlinear constraints for acid uptake through CoA transferase were performed by following the method reported by Desai et al. (24), using GAMS (GAMS Corp., Washington, DC) for the calculations. The reaction stoichiometry is described in Table S3 in the supplemental material. Briefly, the metabolic flux analyses were a minimization of the following equation: $||W^{-1}Ar - W^{-1}x||^2 + (rBYUP[acetate]_e - 0.315rACUP[butyrate]_e)^2$, subject to $l \leq r \leq u$, where A is the stoichiometric matrix, r is the flux vector, and x is the vector which determines accumulation of each metabolite. The vectors l and u constrain the lower and upper limits of r, respectively. W is a diagonal weighting matrix. For species existing only in the intracellular space, the weighting factor determines the scale of the accumulation level. For measured extracellular species, the entries of W are the standard deviations of measurement. Further details of the nonlinear constraint are described in the previous report (24).

Calculation of distribution of substrate carbon from fermentation data. The total carbon balance was calculated as the amount of the products and biomass formed during the entire culture period as a function of the concentration of glucose consumed. The apparent catabolic balance (as a carbon molar yield) was calculated as the carbon molar ratio of the products formed, i.e., butanol, ethanol, acetone, acetate, and butyrate, to the glucose consumed at the final stage of the fermentation. The carbon balance of biomass was also calculated in the same manner, but the biomass formed was taken at the peak during fermentation. Since the exact amount of carbon dioxide formation could not be determined, the carbon balance of carbon dioxide was calculated by assuming that all carbon dioxide generated was formed by follows: 1 mol of CO_2 per mol (each) acetate and ethanol, 2 mol of CO_2 per mol (each) butyrate and butanol, and 3 mol of CO_2 per mol of acetone.

Determination of solventogenesis triggering point. The solventogenesis triggering point was defined as a minimum culture time when a detectable amount of butanol was produced during batch fermentation. See Text S1 in the supplemental material for details on determination of the solventogenesis triggering point.

Transcriptome analysis by DNA microarray. A microorganism GE microarray (3 × 20,000; *Clostridium acetobutylicum* ATCC 824; GE Healthcare, Pittsburgh, PA) was used for transcriptome analysis. See Text S1 in the supplemental material for details on the DNA microarray experiment.

Preparation of cell extracts and determination of enzyme activity. *C. acetobutylicum* cells were harvested by centrifugation at 16,000 × *g* and at 4°C at acidogenic and solventogenic phases to determine the activities of the enzymes phosphotransacetylase, acetate kinase, phosphotransbutyrylase, butyrate kinase, CoA transferase, and aldehyde/alcohol dehydrogenase. Enzyme activity was determined by using a spectrophotometer (GeneQuant 1300; GE Healthcare) under anaerobic conditions at 30°C as described in previous reports with minor modifications (14, 33, 34). The enzyme activity of 1 U was defined as the amount of enzyme required for converting 1 μmol of substrate to product at 30°C per min per milligram of protein. See Text S1 in the supplemental material for details on the determination of enzyme activities.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.00314-12/-/DCSupplemental.

Text S1, DOC file, 0.2 MB.
Figure S1, TIF file, 0.5 MB.
Figure S2, TIF file, 1.3 MB.
Figure S3, TIF file, 0.8 MB.
Figure S4, TIF file, 1.2 MB.
Figure S5, TIF file, 1.1 MB.
Table S1, DOCX file, 0.1 MB.
Table S2, DOCX file, 0.1 MB.
Table S3, DOC file, 0.1 MB.
Table S4, DOC file, 0.1 MB.

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