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Elucidation of the roles of adhE1 and adhE2 in the primary metabolism of Clostridium acetobutylicum by combining in-frame gene deletion and a quantitative system-scale approach

Minyeong Yoo1,2,3, Christian Croux1,2,3, Isabelle Meynial-Salles1,2,3 and Philippe Soucaille1,2,3,4*

Abstract

Background: Clostridium acetobutylicum possesses two homologous adhE genes, adhE1 and adhE2, which have been proposed to be responsible for butanol production in solventogenic and alcohologenic cultures, respectively. To investigate their contributions in detail, in-frame deletion mutants of each gene were constructed and subjected to quantitative transcriptomic (mRNA molecules/cell) and fluxomic analyses in acidogenic, solventogenic, and alcohologenic chemostat cultures.

Results: Under solventogenesis, compared to the control strain, only ΔadhE1 mutant exhibited significant changes showing decreased butanol production and transcriptional expression changes in numerous genes. In particular, adhE2 was over expressed (126-fold); thus, AdhE2 can partially replace AdhE1 for butanol production (more than 30% of the in vivo butanol flux) under solventogenesis. Under alcohologenesis, only ΔadhE2 mutant exhibited striking changes in gene expression and metabolic fluxes, and butanol production was completely lost. Therefore, it was demonstrated that AdhE2 is essential for butanol production and thus metabolic fluxes were redirected toward butyrate formation. Under acidogenesis, metabolic fluxes were not significantly changed in both mutants except the complete loss of butanol formation in ΔadhE2, but numerous changes in gene expression were observed. Furthermore, most of the significantly up- or down-regulated genes under this condition showed the same pattern of change in both mutants.

Conclusions: This quantitative system-scale analysis confirms the proposed roles of AdhE1 and AdhE2 in butanol formation that AdhE1 is the key enzyme under solventogenesis, whereas AdhE2 is the key enzyme for butanol formation under acidogenesis and alcohologenesis. Our study also highlights the metabolic flexibility of C. acetobutylicum to genetic alterations of its primary metabolism.

Keywords: AdhE, Butanol, Clostridium acetobutylicum, System-scale analysis

Background

Clostridium acetobutylicum is now considered as the model organism for the study of solventogenic Clostridia [1, 2]. The superiority of butanol over ethanol as an alternative biofuel has attracted research interest into C. acetobutylicum and other recombinant bacteria producing butanol as major products [3].

In phosphate-limited chemostat cultures, C. acetobutylicum can be maintained in three different stable metabolic states [4–8] without cellular differentiation [9]: acidogenic (producing acetate and butyrate) when grown at neutral pH with glucose; solventogenic (producing
acetone, butanol, and ethanol) when grown at low pH with glucose; and alcohologenic (forming butanol and ethanol but not acetone) when grown at neutral pH under conditions of high NAD(P)H availability [5, 6, 10].

AdhE1 (CA_P0162 gene product, also referred to as Aad) has long been considered as an NADH-dependent bifunctional alcohol/aldehyde dehydrogenase responsible for alcohol formation in solventogenic C. acetobutylicum cultures [1, 2, 11]. Recently, however, AdhE1 was purified and shown to have lost most of its alcohol dehydrogenase activity despite its NADH-dependent aldehyde dehydrogenase activity [12].

Prior to the identification of adhE2 (CA_P0035), the existence of alcohologenesis-specific gene(s) responsible for alcohol formation was predicted because (i) there was high NADH-dependent butanol dehydrogenase activity in alcohologenesis versus high NADPH-dependent butanol dehydrogenase activity in solventogenesis [5, 7] and (ii) previously identified genes related to butanol production (bdhA, bdhB, adhE1) were not induced in alcohologenic cultures [13]. The adhE2 gene is the second aldehyde/alcohol dehydrogenase-encoding gene and is carried by the pSol1 megaplasmid, as is adhE1 [14]. The two genes are not clustered, in contrast to the observations for C. ljungdahlii [15] and their expression patterns differ [9, 12]. adhE1, ctfA, and ctfB (CA_P0163 and CA_P0164) form the sol operon [1, 11]; ctfA and ctfB encode the CoA-transferase responsible for the first step of acetone formation, while the second step, catalyzed by acetoacetate decarboxylase, is encoded by adc (CA_P0165), located downstream of the sol operon. However, adc is transcribed under the control of its own promoter, which is oriented in the opposite direction of the sol operon [11].

In the three metabolic states, the contributions of the different enzymes responsible for the butyraldehyde dehydrogenase and butanol dehydrogenase activities to butanol flux have recently been characterized [12]. Under acidogenesis, the low butanol flux is catalyzed by AdhE2 (100 %) for butyraldehyde dehydrogenase activity, while BdhB and BdhA are responsible for butanol dehydrogenase activity. Under solventogenesis, AdhE1 (95 %; the other 5 % is contributed by AdhE2) is the key player responsible for butyraldehyde dehydrogenase activity, while BdhB, BdhA, and BdhC are responsible for butanol dehydrogenase activity. Under alcohologenesis, AdhE2 plays a major role in both butyraldehyde dehydrogenase (100 %) and butanol dehydrogenase activities. In the study of Cooksley et al. [16], adhE1 and adhE2 knockout mutants were (i) constructed using the Clostron method [17] and (ii) phenotypically characterized in batch culture using Clostridium basal medium (CBMS) without pH adjustment. The adhE1 knockout mutant obtained in their study exhibited low ethanol and no butanol formation along with scant acetone production; these findings were consistent with the polar effect of the intron on ctfAB transcription [16]. Using the adhE2 knockout mutant, no alteration of solvent production was observed; however, the adhE2 knockout mutant has not been evaluated under alcohologenic conditions, under which it is normally thought to play a major role [14].

The aim of this study was to perform clean individual in-frame deletions of adhE1 and adhE2 to characterize their roles in butanol formation in the three different metabolic states in more detail. Furthermore, to study the metabolic flexibility of C. acetobutylicum in response to each of these gene deletions, a complete fluxomic and quantitative transcriptomic analysis was also performed in the three conditions known for the wild-type strains: acidogenic, solventogenic, and alcohologenic states. The results presented here not only support our previous studies [12, 14] on the roles of AdhE1 and AdhE2 in butanol formation in different metabolic states but also highlight the metabolic flexibility of C. acetobutylicum to genetically alter its primary metabolism.

**Results and discussion**

**Construction of ΔadhE1 and ΔadhE2 mutant strains**

Construction of the ΔadhE2 mutant was relatively straightforward, as adhE2 is expressed in a monocistronic operon [14] (Fig. 1a). However, the position of adhE1 as the first gene of the sol operon made the construction of ΔadhE1 more complicated because the transcription of downstream ctfAB genes could be affected. Figure 1b–d shows different configurations of the sol operon promoter, ctfAB genes, and either catP cassette with two FRT (Flippase Recognition Target) sites or a single FRT site remaining after Flippase (Flp)-FRT recombination of the three different types of ΔadhE1 mutants generated in this study. The first constructed ΔadhE1 mutant, ΔCA_C1502ΔuppΔadhE1::catP (Fig. 1b), was unable to form acetone as predicted because a transcriptional terminator was included in the catP cassette, which is located upstream of ctfAB encoding the acetoacetyl coenzyme A:acetate/butyrate:coenzyme A transferase that is responsible for the first specific step of acetone formation [11]. However, after removing the catP cassette from ΔCA_C1502ΔuppΔadhE1::catP, acetone production was unexpectedly not recovered in ΔCA_C1502ΔuppΔadhE1::catP (Fig. 1c). The presence of the megaplasmid pSOL1 was confirmed by the production of ethanol and butanol under alcohologenic conditions and was attributed to adhE2 expression. By sequencing the pSOL1 region around the adhE1 deletion, we confirmed that there was no mutation in the sol promoter, ctfAB and adc (encoding acetoacetate decarboxylase, which is responsible for the last step of acetone production). Based on these results, the possibility of unsuspected
early transcriptional termination by the FRT site remaining after catP removal was deduced. To confirm the early termination of transcription by an FRT site and to eliminate this polar effect on acetone production, a new plasmid was constructed to position both of the FRT sites carried by the catP cassette upstream of the sol operon promoter and was used to construct the ΔadhE1 mutant ΔCA_C1502ΔuppΔadhE1::catP-A1A4 mutant (Fig. 1d). Consistent with our hypothesis, this last ΔadhE1 mutant recovered acetone production (Fig. 2, Additional file 1: Fig. S3). To the best of our knowledge, the potential role of an FRT site as a transcriptional terminator was reported once in Salmonella [18] and twice in yeast [19, 20], although the FRT site is not generally recognized as possessing this additional activity. However, the high score of the FRT site hit from the “Dimers and Hairpin Loops analysis” in Vector NTI software (Invitrogen) and the detection of this activity upon deleting adhE1 in C. acetobutylicum unambiguously demonstrate that the FRT site can function as a transcriptional terminator.

Hereafter, C. acetobutylicum ΔCA_C1502ΔuppΔadhE1::catP-A1A4 (Fig. 1d) is referred to as ΔadhE1 in all the chemostat culture experiments.

**Carbon and electron fluxes of ΔadhE1 and ΔadhE2 mutants under different physiological conditions**

The ΔadhE1 and ΔadhE2 mutants were first evaluated under acidogenic conditions and compared to previously published data for the control strain [12]. All the strains behaved the same, and no significant changes in the metabolic fluxes were recorded (Additional file 1: Fig. S3), except that butanol production was completely abolished in the ΔadhE2 mutant strain (Fig. 2, Additional file 1: Fig. S3).

The two mutant strains were then evaluated under solventogenic conditions and compared to previously published data for the control strain [12]. The control and ΔadhE2 strains behaved the same, with no significant change in metabolic fluxes (Additional file 1: Fig. S3). However, the ΔadhE1 mutant exhibited a completely different behavior. In the first phase, before the “pseudo steady state” was reached, this mutant exhibited considerable fluctuations in growth, glucose consumption, and metabolite profiles. Under “pseudo steady state conditions,” the butanol and acetone fluxes were stable, while the butyrate flux showed fluctuations between 2.2 and 2.9 mmol g⁻¹ h⁻¹. In ΔadhE1, the butanol, ethanol,
acetone fluxes decreased by 60, 49, and 46 %, respectively (Additional file 1: Fig. S3), compared to the control strain; thus, the acetone and ethanol fluxes were not reduced as greatly as the butanol fluxes. These results support the previously proposed [1, 11, 12, 14] key role of AdhE1 in butanol production under solventogenic conditions and demonstrate that an \textit{adhE1} knockout strain with no polar effect on \textit{ctfAB} transcription can still produce acetone. The level of \textit{ctfAB} expression was 3-fold higher in the \textit{adhE1} knockout compared to the control strain. This indicates that the lower flux of acetone production is the result of a control at the enzyme level due to a lower acetoyl-CoA concentration and/or higher acetyl-CoA/butyryl-CoA concentrations. The remaining ability of the \textit{ΔadhE1} strain to produce butanol under solventogenesis is explained by the higher \textit{adhE2} expression (~127-fold higher than the control strain, but only 25 mRNA molecules/cell) (Table 1, Additional file 2: Dataset S1). For the \textit{ΔadhE1} mutant, the butyrate flux increased by 5-fold compared to the control strain (Additional file 1: Fig. S3), although neither \textit{ptb-buk} (CA_C3076–CA_C3075) nor \textit{buk2} (CA_C1660) experienced a significant transcriptional increase (Additional file 2: Dataset S1). Thus, flux is controlled at the enzyme level via an increase in the butyryl-CoA pool due to the lower flux in the butanol pathway. However, as the AdhE2 level in the mutant is the same as the AdhE1 level in the control (6.31 \times 10^4 versus 5.99 \times 10^4 protein molecules/cell), the lower flux of butanol production can be explained by (i) a lower catalytic efficiency of AdhE2 for butyryl-CoA and/or NADH or (ii) a lower intracellular pH under solventogenic conditions that would be less optimal for AdhE2 that is normally expressed under alcohologenic conditions at neutral pH. The second hypothesis can be eliminated as the previously measured intracellular pH [4, 21] in solventogenic and alcohologenic cells are relatively close (5.5 and 5.95, respectively) as the ΔpH is inverted (more acidic inside) under alcohologenic conditions [6]. Finally, as we will see below, the fact that ethanol flux is less affected than the butanol flux might be explained by the existence of an ethanol flux through the Pdc (pyruvate decarboxylase, encoded by CA_P0025) and bdhA/BdhB.
The two mutant strains were also evaluated under alcohologenic conditions and compared to previously published data for the control strain [12]. The control and ΔadhE1 strains behaved the same, with no significant changes in metabolic fluxes (Additional file 1: Fig. S3). However, the ΔadhE2 mutant exhibited a completely different behavior; no flux toward butanol was detected, whereas fluxes toward butyrate became the primary fluxes, as opposed to butanol in the control strain (Additional file 1: Fig. S3). In addition, acetate levels increased by ~3-fold, and such changes were accompanied by changes in electron fluxes (Fig. 3), which are described in detail below. These phenomena were not observed by Cooksley et al. [16] with their adhE2 knockout mutant, as they performed batch fermentation without promoting alcohologenic conditions. As adhE1 was not expressed under the “alcohologenic conditions” of the ΔadhE2 mutant, the physiological function of adhE2 does not appear to be compensated by adhE1 (Table 1). To verify that loss of the butanol-producing ability under alcohologenic conditions did not result from loss of the pSOL1 mega-plasmid [22, 23] but rather from the deletion of adhE2, the culture was switched to solventogenic conditions before the experiment was ended; under solventogenic conditions, high butanol and acetone production fluxes were recovered (data not shown).

The butanol pathway was analyzed for three different conditions in the respective mutants (Additional file 1: Fig. S2) by calculating the contribution of each of the five enzymes potentially involved in each of the two steps to the fluxes (see methods for the calculation).

Under acidogenesis, adhE1 was not expressed, and thus AdhE1 could not replace AdhE2 for the conversion of butyryl-CoA to butyraldehyde in the ΔadhE2 mutant (Additional file 1: Fig. S2). This failure of AdhE1 to replace AdhE2 led to the absence of butanol production in the ΔadhE1 mutant, which behaved the same as the control strain, leaving AdhE2 responsible for all the conversion. The ΔadhE1 mutant behaved the same as the control strain with respect to the conversion of butyraldehyde to butanol under these conditions, and AdhE2 (45 % of the flux), BdhB (34 % of the flux), and BdhA (14 % of the flux) were the main contributors (Additional file 1: Fig. S2). The ΔadhE2 mutant was not analyzed because it does not produce butanol.

Under solventogenesis, AdhE2 replaced AdhE1 for the conversion of butyryl-CoA to butyraldehyde in the ΔadhE1 mutant, while in the ΔadhE2 mutant, which behaved the same as the control strain, AdhE1 was responsible for all the conversion. The two main contributors to the conversion of butyraldehyde to butanol under these conditions were AdhE2 (67 % of the flux) and BdhB (30 % of the flux) in the ΔadhE1 mutant, while in the ΔadhE2 mutant, which behaved the same as the control strain, BdhB (75 % of the flux) and BdhA (16 % of the flux) were the main contributors (Additional file 1: Fig. S2).

Under alcohologenesis, adhE1 was not expressed (Table 1, Additional file 2: Dataset S1), and thus, AdhE1 could not replace AdhE2 for the conversion of butyryl-CoA to butyraldehyde in the ΔadhE2 mutant. This failure of AdhE1 to replace AdhE2 led to the absence of butanol production, while in the ΔadhE1 mutant, which behaved the same as the control strain, AdhE2 was responsible for all the conversion. The ΔadhE1 mutant behaved the same as the control strain with respect to the conversion of butyraldehyde to butanol under these conditions, and AdhE2 was the main contributor (Additional file 1: Fig. S2). The ΔadhE2 mutant was not analyzed because it does not produce butanol.

Two possible routes are known for the conversion of pyruvate to acetaldehyde in C. acetobutylicum: (i) a two-step reaction by pyruvate:ferredoxin oxidoreductase (PFOR) and acetaldehyde dehydrogenase via acetyl-CoA production or (ii) a one-step reaction by pyruvate decarboxylase (Pdc, encoded by CA_P0025) [24]. In the wild-type strain, the former route is considered as the primary pathway [2, 25]. Under acidogenic and alcohologenic

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Table 1 Transcriptional changes of genes coding for the six key enzymes for alcohol production

| Metabolic state/gene | Control | ΔadhE1 | ΔadhE2 |
|---------------------|---------|--------|--------|
| **Acidogenesis**    |         |        |        |
| adhE1 (CA_P0162)    | 0.09 ± 0.01 | 0 ± 0   | 0.2 ± 0.01 |
| adhE2 (CA_P0035)    | 0.42 ± 0.02 | 2.31 ± 0.6 | 0 ± 0 |
| bdhA (CA_C3299)     | 8.15 ± 0.32 | 4.33 ± 1.03 | 5.76 ± 0.2 |
| bdhB (CA_C3298)     | 16.31 ± 0.45 | 5.13 ± 2.48 | 1.52 ± 0.11 |
| bdhC (CA_C3392)     | 8.63 ± 0.94 | 7.55 ± 0.28 | 17.65 ± 0.44 |
| pdc (CA_P0025)      | 5.6 ± 0.81 | 1.74 ± 0.1 | 3.23 ± 0.24 |
| **Solventogenesis** |         |        |        |
| adhE1 (CA_P0162)    | 7.09 ± 0.73 | 0 ± 0   | 11.4 ± 4.71 |
| adhE2 (CA_P0035)    | 0.21 ± 0.02 | 26.6 ± 0.26 | 0 ± 0 |
| bdhA (CA_C3299)     | 8.22 ± 1.33 | 4.62 ± 0.06 | 7.55 ± 0.75 |
| bdhB (CA_C3298)     | 28.1 ± 5.07 | 34.78 ± 1.55 | 17.76 ± 2.83 |
| bdhC (CA_C3392)     | 11.28 ± 1.68 | 12.52 ± 0.36 | 9.16 ± 0.67 |
| pdc (CA_P0025)      | 5.17 ± 2.78 | 6.59 ± 0.3 | 6.23 ± 1.03 |
| **Alcohologenesis** |         |        |        |
| adhE1 (CA_P0162)    | 0.13 ± 0.01 | 0 ± 0   | 0.18 ± 0.01 |
| adhE2 (CA_P0035)    | 68.6 ± 12.95 | 62.56 ± 7.58 | 0 ± 0 |
| bdhA (CA_C3299)     | 6.08 ± 0.37 | 48.2 ± 0.13 | 7.39 ± 0.21 |
| bdhB (CA_C3298)     | 14.33 ± 2.65 | 16.96 ± 0.25 | 15.16 ± 0.46 |
| bdhC (CA_C3392)     | 10.73 ± 0.94 | 11.05 ± 0.25 | 8.95 ± 0.32 |
| pdc (CA_P0025)      | 1.23 ± 0.51 | 0.83 ± 0.03 | 1.86 ± 0.07 |

The numbers of mRNA molecules per cell are shown as mean values ± SD from three biological replicates.
conditions of the ΔadhE2 mutant, ethanol production was observed, but no butanol production was detected (Fig. 2, Additional file 1: Fig. S3). As previously reported [12], AdhE1 retains only aldehyde dehydrogenase activity, whereas AdhE2 possesses both aldehyde and alcohol dehydrogenase activities. Thus, the ethanol production of the ΔadhE2 mutant suggests that the latter route is active. In other words, Pdc could be functional, and the ethanol dehydrogenase activity in acidogenesis could be due to BdhA, BdhB, or BdhC (Table 1). The same pathway might also be functional in solventogenesis and explains why in the ΔadhE1 mutant the ethanol flux was less affected than the butanol flux.

Because the predominant use of reduced ferredoxin is for hydrogen production [12], no significant effects were observed under acidogenesis in both the ΔadhE1 and ΔadhE2 mutants with respect to electron flux (Fig. 3). In addition, solventogenesis of the ΔadhE2 mutant exhibited similar flux levels to the control strain due to the small contribution of AdhE2 (5 % for butyraldehyde dehydrogenase function and 9 % for butanol dehydrogenase function) under these conditions in the control strain. However, under the same conditions as for ΔadhE1, both the fluxes for NADH, known as the partner of AdhE1 and AdhE2, and for NADPH, known as the partner of BdhA, BdhB, and BdhC, were reduced (by ~2.7-fold and 1.8-fold, respectively) due to decreased carbon fluxes toward alcohols (Fig. 3, Additional file 1: Fig. S3). The most striking changes were observed in the ΔadhE2 mutant under alcohologenesis, in which the primary use of reduced ferredoxin was switched from NADH to hydrogen production. The absence of butanol formation resulted in a ~3.6-fold decreased flux toward NADH production and a 1.7-fold increased flux toward hydrogen production (Fig. 3).

Common criteria used for quantitative transcriptomic analysis
To filter the data from only significant results, the same criteria used to compare the wild-type strain under different physiological conditions [12] were used to compare the mutants to the control strain. The first criterion was >4.0-fold higher expression or >4.0-fold lower expression in ΔadhE1 or ΔadhE2 than in the control strain under the same physiological condition, and the second criterion was >0.2 mRNA molecules per cell in at least one of the two strains being compared.

Genes affected by adhE1 or adhE2 deletion under acidogenesis
As alcohols are minor products under acidogenesis, the deletion of adhE1 or adhE2 did not significantly alter the metabolic flux map (Additional file 1: Fig. S3). However, a surprisingly large number of genes (100 genes increased in ΔadhE1, 108 genes decreased in ΔadhE1, 119 genes increased in ΔadhE2, 170 genes decreased in ΔadhE2) showed significant changes in mRNA molecules/cell in response to the deletion of each gene (Table 2). Furthermore, 50 genes (>4-fold increase) and 87 genes (>4-fold
decrease) revealed the same patterns of change in both the \( \Delta \text{adhE1} \) and \( \Delta \text{adhE2} \) mutants (Table 2). The primary metabolism-related genes that influence metabolic fluxes did not exhibit significant changes, whereas mostly subordinaterelated metabolism-related genes were affected (Additional file 1: Table S2, Additional file 1: S3, and Fig. 4).

Interestingly, a large portion (18 genes showed >a 4-fold increase, and 2 genes showed a >2.8-fold increase out of 30 genes proposed by Wang et al. [26]) of the cysteine metabolism regulator (CymR) regulon showed significantly increased expression in both mutants under acidogenesis (CymR regulons are indicated in Table 3). In particular, an operon involved in cysteine and sulfur metabolism (CA_C0102–CA_C0110) showed a >10-fold increase in both mutants. This operon was reported to respond to butyrate/butanol stresses and to be up-regulated under alcohologenesis in wild-type strains [12, 26, 27] and under solventogenesis in the \( \Delta \text{adhE1} \) mutant [28]. In addition, the expression of two putative cysteine ABC transporter operons belonging to the CymR regulon [26, 27], namely CA_C0878–CA_C0880 and CA_C3325–CA_C3327, was also up-regulated.

A long gene cluster linked to iron/sulfur/molybdenum metabolism (CA_C1988–CA_C2019) exhibited significantly decreased expression (except for CA_C1988, CA_C1990, CA_C1992 and CA_C1995, for which some values were below the significance criterion of 4-fold but were higher than 3-fold) (Table 3, Additional file 2: Dataset S1). A part of this cluster, CA_C1988–CA_C1996, was previously reported to be down-regulated under oxygen-exposed conditions [29]. Moreover, this cluster was shown by Schwarz et al. [30] to be repressed by butanol stress in an acidogenic chemostat.

**Transcriptional changes due to \( \Delta \text{adhE1} \) or \( \Delta \text{adhE2} \) deletion under solventogenesis**

Under solventogenesis, a drastic change in fluxes was observed in the \( \Delta \text{adhE1} \) mutant, while the fluxes remained unchanged in the \( \Delta \text{adhE2} \) mutant; additionally, as expected, more genes showed significant changes in \( \Delta \text{adhE1} \) than in \( \Delta \text{adhE2} \) (Table 2, Additional file 1: Table S4, Additional file 1: S5). Specifically, in \( \Delta \text{adhE1} \), 55 genes were up-regulated, and 127 genes were down-regulated (Table 2). In \( \Delta \text{adhE2} \), 22 genes were up-regulated, and 17 genes were down-regulated (Table 2). In contrast to the observations previously made under acidogenesis, no gene was significantly increased in both the \( \Delta \text{adhE1} \) and \( \Delta \text{adhE2} \) mutants, and only 1 gene (CA_C3612, encoding a hypothetical protein) was significantly decreased in both mutants.

In \( \Delta \text{adhE1} \), the CA_C0102–CA_C0110 operon which was shown to be up-regulated in acidogenesis and belongs to the CymR regulon, was also up-regulated by >18-fold under solventogenesis (Additional file 1: Table S4). However, the up-regulation of this operon (under alcohologenesis in the control strain, acidogenesis and solventogenesis in \( \Delta \text{adhE1} \), or acidogenesis in \( \Delta \text{adhE2} \)) did not have striking shared features with the main product profile.

Interestingly, expression of the natAB operon (CA_C3551–CA_C3550) (>10-fold), encoding a potential Na\(^+\)-ABC transporter, and the kdp gene cluster (CA_C3678–CA_C3682), encoding a potential K\(^+\) transporter (>20-fold), was highly up-regulated under solventogenesis (Additional file 1: Table S4, Additional file 2: Dataset S1) in \( \Delta \text{adhE1} \). The natAB operon and the kdp gene cluster have previously been reported to be up-regulated by both acetate and butyrate stress [27]. As the ability of the \( \Delta \text{adhE1} \) mutant to produce butanol was highly affected and as butyrate and acetate were the primary fermentation products (Fig. 2), this strain struggled to survive under acidic conditions (i.e., under the pH of 4.4 for solventogenesis); consequently, genes involved in ion transport were up-regulated.

The operon CA_P0029–CA_P0030, which potentially encodes a transporter and an isochorismatase, was up-regulated under acidogenesis in both mutants as well as

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**Table 2** Numbers of significantly changed genes by each gene deletion and genes exhibiting the same pattern of change for both deletions under three different metabolic states (the genes exhibiting the same pattern for both deletions under acidogenesis are listed in Table 3)

|                        | \( \Delta \text{adhE1} \) | \( \Delta \text{adhE2} \) | Same pattern in \( \Delta \text{adhE1} \) and \( \Delta \text{adhE2} \) | Note* |
|------------------------|---------------------------|---------------------------|---------------------------------|-------|
| Up-regulation under acidogenesis | 100                        | 119                       | 50                              | Most CymR regulons are included |
| Down-regulation under acidogenesis | 108                        | 170                       | 89                              | Most butanol response genes are included |
| Up-regulation under solventogenesis | 55                         | 22                        | 0                               | CA_C3612 |
| Down-regulation under solventogenesis | 127                        | 17                        | 1                               |       |
| Up-regulation under alcohologenesis | 1                          | 35                        | 0                               | CA_C3274 |
| Down-regulation under alcohologenesis | 14                         | 38                        | 1                               |       |

*Representative features or locus number of the sole gene showing same pattern under certain condition are shown*
under solventogenesis in ΔadhE2 (>20-fold) (Table 2, Additional file 1: Table S5). Two neighboring genes, CA_C3604 (ilvD), encoding dihydroxyacid dehydratase linked to valine/leucine/isoleucine biosynthesis, and CA_C3605 (gntP), encoding high affinity gluconate/L-idonate permease, exhibited striking increases (>120-fold) (Additional file 1: Table S5) in ΔadhE2.

As described above, the solventogenic culture of ΔadhE1 has a lower glucose consumption rate than the control strain (Fig. 2) and consequently more glucose remained unconsumed in the medium. Accordingly, numerous genes related to sugar metabolism were down-regulated under this metabolic state. For instance, all the structural genes on the mannitol phosphotransferase system (PTS)-related operon mtlARFD (CA_C0154–CA_C0157) and the mannose PTS-related operon (CA_P0066–CA_P0068) were decreased by >10-fold (Additional file 1: Table S4).

Interestingly, one of two operons encoding a quorum-sensing system and putatively involved in sporulation, CA_C0078–CA_C0079 (agrBD) [31], was strongly down-regulated (infinity-fold for CA_C0078 and 667-fold for CA_C0079) in ΔadhE2 relative to the control strain (Additional file 1: Table S5). However, the other operon, CA_C0080–CA_C0081 (agrCA), did not significantly change (<3-fold decreases) (Additional file 2: Dataset S1). Quantitatively, less than 1 agrCA mRNA molecule was found per cell, whereas more than 1 agrBD mRNA molecule was found per cell under all conditions in the control strain [12]. These different expression levels are not surprising because agrBD and agrCA are independently transcribed [31–33]. In addition, agrBD was repressed under all conditions in ΔadhE2, although the sporulation of this mutant was not affected (Additional file 2: Dataset S1).

Transcriptional changes due to adhE1 or adhE2 deletion under alcohologenesis

Under alcohologenesis, a drastic change in fluxes was observed in the ΔadhE2 mutant, while in the ΔadhE1 mutant, the fluxes remained unchanged. As expected, more genes showed significant changes in the ΔadhE2 mutant than in the ΔadhE1 mutant (Table 2). Specifically, in ΔadhE1, only 1 gene was up-regulated (agrB), and 14 genes were down-regulated, while in ΔadhE2, 35 genes were up-regulated, and 38 genes were down-regulated.

The most dynamic changes in the ΔadhE2 mutant were observed in CA_C3604 (ilvD, 297-fold) and CA_C3605 (gntP, 301-fold) (Additional file 1: Table S7). As mentioned previously, these genes were highly up-regulated (>84-fold) under all the conditions in the ΔadhE2 mutant (Additional file 2: Dataset S1). Interestingly, two genes located immediately downstream of adhE2, CA_P0036, which encodes a cytosolic protein of unknown function, and CA_P0037, which encodes a putative transcriptional regulator, exhibited a ~ 9-fold increase under alcohologenesis (Additional file 1: Table S7) in ΔadhE2.

A sucrose metabolism operon comprising scrAKB (CA_C0423–CA_C0425), encoding a PTS II BCA domain on a single gene, fructokinase and sucrose-6-P hydrolase [35, 36], was strikingly down-regulated (>47-fold) (Additional file 1: Table S6). Moreover, the gene immediately upstream, scrT (CA_C0422) (encoding a putative transcriptional antiterminator), and the gene downstream, CA_C0426, encoding a putative AraC-type of regulator, were also decreased, by 9.3-fold and 8-fold, respectively (Additional file 1: Table S6). The similar expression patterns of CA_C0422, CA_C0426, and scrAKB support the hypotheses of previous studies regarding their roles in regulating scrAKB [35, 36].
Table 3  Genes exhibiting the same pattern of change for both deletions under acidogenesis

| Locus number | Function                                      | ΔadhE1/Control strain | ΔadhE2/Control strain | Note<sup>a</sup> |
|--------------|-----------------------------------------------|-----------------------|-----------------------|-------------------|
| CA_C0102     | O-acetylhomoserine sulfhydrylase              | 28.70                 | 20.49                 | CymR              |
| CA_C0103     | Adenylylsulfate kinase                        | 32.55                 | 22.06                 | CymR              |
| CA_C0104     | Adenylylsulfate reductase, subunit A          | 48.44                 | 28.89                 | CymR              |
| CA_C0105     | Ferredoxin                                    | 30.78                 | 21.84                 | CymR              |
| CA_C0106     | ABC-type probable sulfate transporter, periplasmic binding protein | 26.09                 | 14.54                 | CymR              |
| CA_C0107     | ABC-type sulfate transporter, ATPase component | 22.86                 | 13.03                 | CymR              |
| CA_C0108     | ABC-type probable sulfate transporter, permease protein | 35.38                 | 19.05                 | CymR              |
| CA_C0109     | Sulfate adenylyltransferase, CysD subfamily   | 42.53                 | 26.82                 | CymR              |
| CA_C0110     | GTPase, sulfate adenylyltransferase subunit 1 | 54.78                 | 42.48                 | CymR              |
| CA_C0117     | Chemotaxis protein cheY homolog               | 8.34                  | 6.69                  |                   |
| CA_C0118     | Chemotaxis protein cheA                       | 11.00                 | 8.24                  |                   |
| CA_C0119     | Chemotaxis protein cheW                       | 13.83                 | 9.52                  |                   |
| CA_C0120     | Membrane-associated methyl-accepting chemotaxis protein with HAMP domain | 6.93                  | 5.29                  |                   |
| CA_C0878     | Amino acid ABC transporter permease component | 5.61                  | 4.04                  | CymR              |
| CA_C0879     | ABC-type polar amino acid transport system, ATPase component | 8.29                  | 5.60                  | CymR              |
| CA_C0880     | Periplasmic amino acid binding protein         | 9.50                  | 6.50                  | CymR              |
| CA_C0930     | Cystathionine gamma-synthase                  | 4.58                  | 4.72                  | CymR              |
| CA_C1394     | Glutamine phosphoribosylpyrophosphate amidotransferase | 4.20                  | 4.47                  |                   |
| CA_C1394     | Folate-dependent phosphoribosylglcinamide formyltransferase | 4.11                  | 4.57                  |                   |
| CA_C2072     | Stage IV sporulation protein B, SpoIVB        | ∞                     | ∞                     |                   |
| CA_C2235     | Cysteine synthase/cystathionine beta-synthase, CysK | 8.27                  | 7.17                  | CymR              |
| CA_C2236     | Uncharacterized conserved protein of YjeB/RRF2 family | 4.29                  | 4.06                  | CymR encoding gene |
| CA_C2241     | Cation transport P-type ATPase               | 7.92                  | 7.62                  |                   |
| CA_C2242     | Predicted transcriptional regulator, arsE family | 5.01                  | 5.22                  |                   |
| CA_C2521     | Hypothetical protein, CF-41 family            | 4.33                  | 5.70                  |                   |
| CA_C2533     | Protein containing ChW-repeats                | ∞                     | ∞                     |                   |
| CA_C2816     | Hypothetical protein, CF-17 family            | 6.00                  | 11.20                 |                   |
| CA_C3049     | Glycosyltransferase                           | 4.79                  | 7.42                  |                   |
| CA_C3050     | AMSJ/WSAK-related protein, possibly involved in exopolysaccharide biosynthesis | 4.70                  | 8.25                  |                   |
| CA_C3051     | Glycosyltransferase                           | 5.16                  | 9.60                  |                   |
| CA_C3052     | Glycosyltransferase                           | 5.59                  | 9.91                  |                   |
| CA_C3053     | Histidinol phosphatase-related enzyme          | 7.03                  | 10.94                 |                   |
| CA_C3054     | Phosphoheptose isomerase                      | 6.69                  | 11.37                 |                   |
| CA_C3055     | Sugar kinase                                  | 5.90                  | 10.87                 |                   |
| CA_C3056     | Nucleoside-diphosphate-sugar pyrophosphorylase | 6.37                  | 11.28                 |                   |
| CA_C3057     | Glycosyltransferase                           | 12.36                 | 11.92                 |                   |
| CA_C3058     | Mannose-1-phosphate guanylyltransferase       | 9.94                  | 11.59                 |                   |
| Locus number | Function                                                                 | \( \Delta \text{adhE1}/\text{Control strain} \) | \( \Delta \text{adhE2}/\text{Control strain} \) | Note* |
|--------------|--------------------------------------------------------------------------|----------------------------------|----------------------------------|-------|
| CA_C3059    | Sugar transferases                                                      | 13.47                            | 12.63                            |       |
| CA_C3325    | Periplasmic amino acid binding protein                                  | 18.24                            | 10.68                            | CymR  |
| CA_C3326    | Amino acid ABC-type transporter, permease component                     | 19.82                            | 11.79                            | CymR  |
| CA_C3327    | Amino acid ABC-type transporter, ATPase component                       | 28.33                            | 16.73                            | CymR  |
| CA_C3461    | Hypothetical protein                                                    | 4.52                             | 16.79                            |       |
| CA_C3556    | Probable S-layer protein;                                               | 4.18                             | 10.41                            |       |
| CA_C3636    | Oligopeptide ABC transporter, ATPase component                          | 4.23                             | 4.68                             |       |
| CA_P0029    | Permease MDR-related                                                    | ∞                                | ∞                                |       |
| CA_P0030    | Isochorismatase                                                         | 385.91                           | 81.89                            |       |
| CA_P0031    | Transcriptional activator HLYU, HTH of ArsR family                     | 46.17                            | 10.93                            |       |
| CA_P0117    | Possible beta-xilosidase diverged, family 5/39                          | 56.53                            | 4.94                             |       |
| CA_P0118    | Possible xylan degradation enzyme (glycosyl hydrolase family 30-like domain) | 54.97                            | 5.22                             |       |
| CA_P0119    | Possible xylan degradation enzyme (glycosyl hydrolase family 30-like domain) | 46.44                            | 4.23                             |       |
| CA_C0078    | Accessory gene regulator protein B                                      | 0.04                             | 0.00                             |       |
| CA_C0079    | Hypothetical protein                                                    | 0.00                             | 0.00                             |       |
| CA_C0082    | Predicted membrane protein                                              | 0.02                             | 0.00                             |       |
| CA_C0310    | Regulators of stationary/sporulation gene expression, abrB B.subtilis ortholog | 0.15                             | 0.23                             |       |
| CA_C0381    | Methyl-accepting chemotaxis protein                                     | 0.18                             | 0.13                             |       |
| CA_C0437    | Sensory transduction histidine kinase                                   | 0.15                             | 0.23                             |       |
| CA_C0537    | Acetlyxylan esterase, acyl-CoA esterase or GDSL lipase family, strong similarity to C-terminal region of endoglucanase E precursor | 0.15                             | 0.10                             |       |
| CA_C0542    | Methyl-accepting chemotaxis protein                                     | 0.21                             | 0.08                             |       |
| CA_C0558    | Fe-S oxidoreductase                                                     | 0.24                             | 0.00                             |       |
| CA_C0660    | Hypothetical protein, CF-26 family                                      | 0.17                             | 0.08                             | BuOH  |
| CA_C0814    | 3-oxoacyl-[acyl-carrier-protein] synthase III                           | 0.11                             | 0.02                             | BuOH  |
| CA_C0815    | Methyl-accepting chemotaxis protein                                     | 0.13                             | 0.04                             | BuOH  |
| CA_C0816    | Lipase-esterase-related protein                                         | 0.17                             | 0.04                             | BuOH  |
| CA_C1010    | Predicted phosphohydrolase, lcc family                                  | 0.21                             | 0.04                             | BuOH  |
| CA_C1022    | Thioesterase II of alpha/beta hydrolase superfamily                    | 0.22                             | 0.11                             |       |
| CA_C1078    | Predicted phosphohydrolase, lcc family                                  | 0.17                             | 0.04                             | BuOH  |
| CA_C1079    | Uncharacterized protein, related to enterotoxins of other Clostridiales  | 0.15                             | 0.05                             |       |
| CA_C1080    | Uncharacterized protein, probably surface-located                       | 0.11                             | 0.01                             |       |
| CA_C1081    | Uncharacterized protein, probably surface-located                       | 0.13                             | 0.01                             |       |
| CA_C1532    | Protein containing ChW-repeats                                          | 0.22                             | 0.08                             |       |
| CA_C1766    | Predicted sigma factor                                                  | 0.19                             | 0.00                             |       |
| CA_C1775    | Predicted membrane protein                                              | 0.16                             | 0.05                             |       |
| Locus number | Function                                                                 | ΔadhE1/Control strain | ΔadhE2/Control strain | Note  |
|--------------|--------------------------------------------------------------------------|-----------------------|-----------------------|-------|
| CA_C1868     | Uncharacterized secreted protein, homolog YXK01 Bacillus subtilis        | 0.22                  | 0.18                  |       |
| CA_C1989     | ABC-type iron (III) transport system, ATPase component                  | 0.18                  | 0.11                  | BuOH  |
| CA_C1991     | Uncharacterized protein, YIM family                                      | 0.23                  | 0.10                  | BuOH  |
| CA_C1993     | Molybdenum cofactor biosynthesis enzyme, MoaA, Fe-S oxidoreductase      | 0.23                  | 0.18                  | BuOH  |
| CA_C1994     | Molybdopterin biosynthesis enzyme, MoaB                                   | 0.22                  | 0.11                  | BuOH  |
| CA_C1996     | Hypothetical protein                                                      | 0.19                  | 0.08                  | BuOH  |
| CA_C1997     | Predicted glycosyltransferase                                            | 0.19                  | 0.07                  | BuOH  |
| CA_C1998     | ABC-type transport system, ATPase component                              | 0.19                  | 0.07                  | BuOH  |
| CA_C1999     | Uncharacterized protein related to hypothetical protein Cj507c from Campylobacter jejuni | 0.20                  | 0.07                  | BuOH  |
| CA_C2000     | Indolepyruvate ferredoxin oxidoreductase, subunit beta                   | 0.19                  | 0.06                  | BuOH  |
| CA_C2001     | Indolepyruvate ferredoxin oxidoreductase, subunit alpha                  | 0.13                  | 0.04                  | BuOH  |
| CA_C2002     | Predicted iron-sulfur flavoprotein                                       | 0.16                  | 0.05                  | BuOH  |
| CA_C2003     | Predicted permease                                                        | 0.16                  | 0.08                  | BuOH  |
| CA_C2004     | Siderophore/Surfactin synthetase-related protein                          | 0.10                  | 0.04                  | BuOH  |
| CA_C2005     | Siderophore/Surfactin synthetase-related protein                         | 0.12                  | 0.05                  | BuOH  |
| CA_C2006     | Enzyme of siderophore/surfactin biosynthesis                             | 0.15                  | 0.07                  | BuOH  |
| CA_C2007     | Predicted glycosyltransferase                                            | 0.09                  | 0.03                  | BuOH  |
| CA_C2008     | 3-oxoacyl-(acyl-carrier-protein) synthase                                | 0.11                  | 0.04                  | BuOH  |
| CA_C2009     | 3-Hydroxyacyl-CoA dehydrogenase                                          | 0.10                  | 0.03                  | BuOH  |
| CA_C2010     | Predicted Fe-S oxidoreductase                                            | 0.09                  | 0.03                  | BuOH  |
| CA_C2011     | Possible 3-oxoacyl-[acyl-carrier-protein] synthase III                    | 0.12                  | 0.03                  | BuOH  |
| CA_C2012     | Enoyl-CoA hydrase                                                        | 0.12                  | 0.04                  | BuOH  |
| CA_C2013     | Hypothetical protein                                                      | 0.12                  | 0.03                  | BuOH  |
| CA_C2014     | Predicted esterase                                                        | 0.12                  | 0.02                  | BuOH  |
| CA_C2015     | Hypothetical protein                                                      | 0.15                  | 0.04                  | BuOH  |
| CA_C2016     | Enoyl-CoA hydrase                                                        | 0.12                  | 0.02                  | BuOH  |
| CA_C2017     | Acyl carrier protein                                                      | 0.15                  | 0.03                  | BuOH  |
| CA_C2018     | Aldehyde:ferredoxin oxidoreductase                                       | 0.12                  | 0.03                  | BuOH  |
| CA_C2019     | Malonyl CoA-acyl carrier protein transacylase                            | 0.12                  | 0.02                  | BuOH  |
| CA_C2020     | Molybdopterin biosynthesis enzyme, MoaA, fused to molybdopterin-binding domain | 0.20                  | 0.07                  |       |
| CA_C2021     | Molybdopterin biosynthesis enzyme, MoaA (short form)                     | 0.24                  | 0.06                  |       |
| CA_C2023     | Membrane protein, related to copy number protein COP from Clostridium perfringens plasmid pIP404 (G1:116,928) | 0.22                  | 0.12                  |       |
| CA_C2026     | Predicted flavodoxin                                                      | 0.20                  | 0.09                  |       |
| CA_C2107     | Contains cell adhesion domain                                             | 0.20                  | 0.08                  |       |
| CA_C2293     | Hypothetical secreted protein                                             | 0.13                  | 0.10                  |       |
| CA_C2581     | 6-pyruvoyl-tetrahydropterin synthase-related domain, conserved membrane protein | 0.24                  | 0.11                  | BuOH  |
| CA_C2663     | Protein containing cell wall hydrolase domain                             | 0.23                  | 0.09                  |       |
Table 3 continued

| Locus number | Function                                                                 | ΔadhE1/Control strain | ΔadhE2/Control strain | Note |
|--------------|--------------------------------------------------------------------------|-----------------------|-----------------------|------|
| CA_C2695     | Diverged Metallo-dependent hydrolase(Zn) of DD-Peptidase family; peptodoglycan-binding domain | 0.17                  | 0.12                  | BuOH |
| CA_C2807     | Endo-1,3(4)-beta-glucanase family 16                                     | 0.21                  | 0.02                  |      |
| CA_C2808     | Beta-lactamase class C domain (PBPX family) containing protein           | 0.20                  | 0.04                  |      |
| CA_C2809     | Predicted HD superfamily hydrolase                                        | 0.14                  | 0.02                  |      |
| CA_C2810     | Possible glucamylase (diverged), 1S family                               | 0.14                  | 0.01                  |      |
| CA_C2944     | N-terminal domain intergin-like repeats and c-terminal cell wall-associated hydrolase domain | 0.23                  | 0.06                  | BuOH |
| CA_C3070     | Glycosyltransferase                                                       | 0.21                  | 0.04                  |      |
| CA_C3071     | Glycosyltransferase                                                       | 0.21                  | 0.03                  |      |
| CA_C3072     | Mannose-1-phosphate guanylyltransferase                                   | 0.18                  | 0.02                  |      |
| CA_C3073     | Sugar transferase involved in lipopolysaccharide synthesis                | 0.23                  | 0.03                  |      |
| CA_C3085     | TPR-repeat-containing protein; Cell adhesion domain                       | 0.25                  | 0.12                  |      |
| CA_C3086     | Protein containing cell adhesion domain                                   | 0.20                  | 0.11                  |      |
| CA_C3251     | Sensory transduction protein containing HD_GYP domain                    | 0.20                  | 0.11                  |      |
| CA_C3264     | Uncharacterized conserved protein, YTFJ B.subtilis ortholog              | 0.19                  | 0.15                  | BuOH |
| CA_C3265     | Predicted membrane protein                                                | 0.08                  | 0.11                  |      |
| CA_C3266     | Hypothetical protein                                                      | 0.07                  | 0.07                  |      |
| CA_C3267     | Specialized sigma subunit of RNA polymerase                              | 0.15                  | 0.16                  |      |
| CA_C3280     | Possible surface protein, responsible for cell interaction; contains cell adhesion domain and ChW-repeats | 0.23                  | 0.14                  |      |
| CA_C3408     | NADH oxidase (two distinct flavin oxidoreductase domains)                 | 0.03                  | 0.02                  |      |
| CA_C3409     | Transcriptional regulators, LysR family                                   | 0.02                  | 0.01                  |      |
| CA_C3412     | Predicted protein-S-soprenylcysteine methyltransferase                    | 0.22                  | 0.06                  |      |
| CA_C3422     | Sugar proton symporter (possible xylulose)                                | 0.05                  | 0.03                  |      |
| CA_C3423     | Acetyltranferase (ribosomal protein N-acetylase subfamily)               | 0.04                  | 0.03                  |      |
| CA_C3612     | Hypothetical protein                                                      | 0.18                  | 0.00                  | BuOH |
| CA_P0053     | Xylanase, glycosyl hydrolase family 10                                    | 0.24                  | 0.09                  | BuOH |
| CA_P0054     | Xylanase/chitin deacetylase family enzyme                                 | 0.24                  | 0.07                  | BuOH |
| CA_P0057     | Putative glycoprotein or S-layer protein                                  | 0.21                  | 0.13                  | BuOH |
| CA_P0135     | Oxidoreductase                                                            | 0.25                  | 0.21                  |      |
| CA_P0136     | AstB/chuR/nirj-related protein                                            | 0.25                  | 0.23                  |      |
| CA_P0174     | Membrane protein                                                          | 0.25                  | 0.14                  |      |

* CymR indicates CymR regulon, BuOH indicates the genes to be down-regulated by butanol stress in an acidogenic chemostat in the study by Schwarz et al. [30]
As expected based on the reduced consumption of glycerol (approximately one-fourth of the control strain) (Fig. 2) in ΔadhE2, the gene cluster for glycerol transport and utilization (CA_C1319-CA_C1322) was downregulated (>4-fold decrease) under these conditions (Additional file 1: Table S7).

Most arginine biosynthesis-related genes known to respond negatively to butanol and butyrate stress [26] (i.e., CA_C0316 (argF/I), CA_C0973–CA_C0974 (argGH), CA_C2389–CA_C2388 (argBD), CA_C2390–CA_C2391 (argCF), CA_C2644 (carB), and CA_C2645 (carA)) were significantly down-regulated (>4-fold decrease) (Additional file 1: Table S7) in ΔadhE2. As “alcohogenic cultures” of ΔadhE2 produced 70 mM of butyrate and no butanol (Fig. 2), this down-regulation is consistent with the high butyrate stress (50 mM) response [26].

CA_C3486, which encodes a multimeric flavodoxin, was decreased by 4.4-fold in ΔadhE2 (Additional file 1: Table S7), resulting in a loss of butanol production under alcohologenesis. This finding is consistent with the proposed hypothesis [12] that under alcohologenesis, the gene product of CA_C3486 may function as a redox partner between the hydrogenase and ferredoxin-NAD⁺ reductase and may participate in the redistribution of electron fluxes in favor of butanol formation.

Conclusions
The results presented here support the hypothesis of the roles of AdhE1 and AdhE2 in butanol formation, namely that AdhE1 is the key enzyme for butanol formation in solventogenesis and that AdhE2 is the key enzyme for butanol formation in alcohologenesis. Furthermore, this study also demonstrates the metabolic flexibility of C. acetobutylicum in response to genetic alteration of its primary metabolism.

Methods
Bacterial strains and plasmid construction
All C. acetobutylicum strains used in this study and in the control study were constructed from the C. acetobutylicum ATCC 824 ΔCA_C1502 Δupp mutant strain, which was constructed for rapid gene knockout and gene knockin [38]. Detailed procedures, including all strains and primers used, are described in the online supporting information (Supplementary experimental procedures).

Culture conditions
All batch cultures were performed under strict anaerobic conditions in synthetic medium (MS), as previously described [4]. C. acetobutylicum was stored in spore form at −20 °C after sporulation in MS medium. Heat shock was performed for spore germination by immersing the 30- or 60-mL bottle into a water bath at 80 °C for 15 min.

All the phosphate-limited continuous cultivations were performed as previously described by Vasconcelos et al. [4] and Girbal et al. [21] like in the control strain study [12]. The chemostat was fed a constant total of 995 mM of carbon and maintained at a dilution rate of 0.05 h⁻¹. The maintained pH of the bioreactor and the supplied carbon sources of each metabolic state were as follows: for acidogenesis, pH 6.3, with 995 mM of carbon from glucose; for solventogenesis, pH 4.4, with 995 mM of carbon from glucose; and for alcohologenesis, pH 6.3, with 498 mM of carbon from glucose and 498 mM of carbon from glycerol.

RNA extraction and microarray
Total RNA isolation and microarray experiments were performed as previously described [12]. Briefly, 3 mL of chemostat cultures was sampled, immediately frozen in liquid nitrogen and ground with 2-mercaptoethanol. RNA was extracted by using an RNeasy Midi kit (Qiagen, Courtaboeuf, France) and RNase-Free DNase Set (Qiagen) per the manufacturer’s protocol. The RNA quantity and integrity were monitored using an Agilent 2100 Bioanalyzer (Agilent Technologies, Massy, France) and a NanoDrop ND-1000 spectrophotometer (Labtech France, Paris, France) at 260 and 280 nm. All microarray procedures were performed per the manufacturer’s protocol (Agilent One-Color Microarray-Based Exon Analysis).

Analytical methods
The optical density at 620 nm (OD620 nm) was monitored and used to calculate the biomass concentration with the correlation factor between dry cell weight and OD620 nm (path length 1 cm) of 0.28, which was experimentally determined from continuous cultures and was used in a control strain study [12]. The glucose, glycerol, acetate, butyrate, lactate, pyruvate, acetoin, acetone, ethanol, and butanol concentrations were determined using high-performance liquid chromatography (HPLC), as described by Dusséaux et al. [39]. The concentration of the eluent H₂SO₄ was changed to 0.5 mM, as this concentration was required to optimize the mobile phase for the control strain study [12].

Calculation of the cytosolic proteins concentration (protein molecules per cell)
In a previously published work [12], we quantified the amount of (i) mRNA molecules per cell for all genes and (ii) protein molecules per cell (for approximately 700 cytosolic proteins) for steady-state chemostat cultures (at a specific growth rate of 0.05 h⁻¹) of C. acetobutylicum under different physiological conditions. For 96 % of the cytosolic proteins that could be quantified, a
linear relationship was obtained, with an $R^2 > 0.9$, when the numbers of protein molecules per cell were plotted against the numbers of mRNA molecules per cell. This result indicated that for steady-state continuous cultures run at the same specific growth rate and with the same total amount of carbon supplied, the rate of protein turnover is proportional to the mRNA content for 96% of the genes. As the mutants were cultivated in chemostat culture at the same growth rate ($0.05 \, \text{h}^{-1}$), we used the absolute protein synthesis rates previously calculated [12] for each of the 700 genes to calculate the amount of protein molecule per cell for each of these 700 genes in the different mutants. (Additional file 2: Dataset S1).

Calculation of the contribution of different enzymes on the butanol flux

The contribution of the 5 proteins potentially involved in the butanol pathway, namely AdhE1, AdhE2, BdhA, BdhB, and BdhC, was made as previously described [12] by assuming that all five enzymes function at their Vmax and using the calculated amount of each protein per cell (Additional file 2: Dataset S1).

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Availability of supporting data

Microarray data can be accessed at GEO through accession number GSE69973.

Competing interests

The authors declare that they have no competing interests.

Consent for publication

Not applicable.

Ethical Approval and Consent to participate

Not applicable.

Additional files

Additional file 1. Supplementary experimental procedures and results.
Additional file 2. Dataset S1. Transcripтомic data of the total open reading frames (ORFs).

Abbreviations

Flp: flippase; FRT: flippase recognition target; catP: chloramphenicol acetyltransferase.

Authors’ contributions

CC, IMS, and PS conceived the study; MY performed all the experimental work. MY and PS performed the data analysis and drafted the manuscript. PS supervised the work. All authors read and approved the final manuscript.

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