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Improvement of Cellulolytic Properties of *Clostridium cellulolyticum* by Metabolic Engineering

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Cellulolytic clostridia have evolved to catabolize lignocellulosic materials at a seasonal biorhythm, so their biotechnological exploitation requires genetic improvements. As high carbon flux leads to pyruvate accumulation, which is responsible for the cessation of growth of *Clostridium cellulolyticum*, this accumulation is decreased by heterologous expression of pyruvate decarboxylase and alcohol dehydrogenase from *Zymomonas mobilis*. In comparison with that of the wild strain, growth of the recombinant strain at the same specific rate but for 145 h instead of 80 h led to a 150% increase in cellulose consumption and a 180% increase in cell dry weight. The fermentation pattern was shifted significantly: lactate production decreased by 48%, whereas the concentrations of acetate and ethanol increased by 93 and 53%, respectively. This study demonstrates that the fermentation of cellulose, the most abundant and renewable polymer on earth, can be greatly improved by using genetically engineered *C. cellulolyticum*.

Cellulolytic microorganisms play an important role in the biosphere by recycling cellulose, and cellulolytic clostridia are of cardinal importance in anaerobic environments rich in plant materials (24). They are also important in some industrial fermentation processes, particularly in anaerobic digesters fed with municipal solid waste or agricultural raw materials containing a high percentage of lignocellulosic compounds (5). The most widely used methods of waste matter treatment are dumping and incineration, both of which contribute to the greenhouse gas effect. As an alternative, anaerobic conversion into methane by bacterial consortia is one of the most promising methods (12). The production of methane requires a trophic chain of at least three interacting metabolic groups of strictly anaerobic microbes (9): (i) the fermentative group decomposes cellulose and other complex molecules into volatile carboxylic acids (mainly acetate), CO_2, and H_2; (ii) syntrophic bacteria further degrade the alcohols and fatty acids into acetate, H_2, and CO_2; and (iii) acetate, H_2, and CO_2 finally serve as substrates for the methanoarchaea. At present, the process relies on naturally occurring bacteria, but the efficiency conceivably could be improved by using better strains, particularly for cellulolysis, the limiting step of the process. Indeed, cellulolytic clostridia show analogous growth properties: they use small quantities of carbohydrates due to early inhibition of metabolism and growth (10).

Recent metabolic investigations with *Clostridium cellulolyticum* ATCC 35319, the best-understood cellulolytic mesophilic bacterium, indicated better control of carbohydrate catabolism on mineral salt media than on complex media (13, 14, 16, 24, 26). Indeed, natural ecosystems, where cellulolytic microbes proliferate, rarely contain all nutrients in saturating quantities, and complex media with high concentrations of substrates are unfavorable to *C. cellulolyticum*, which is unable to deal with a surfeit of substrates. Under these conditions, the nutrients or products of its metabolism accumulate intracellularly to toxic levels, such as has been demonstrated for NADH and pyruvate (13–16). It is reasonable to suppose that during the course of evolution, these bacteria have evolved to optimize the catabolism of few available carbon sources over a time scale of months. In natural lignocellulosic compounds, cell walls can be regarded as a giant macromolecular composite of cellulose fibers embedded in a covalently joined matrix of pectin, lignin, and hemicellulose, which may hinder cellulolysis and hence reduce carbon availability. Thus, there is no need for cells to regulate the entry of high carbon flow. When *C. cellulolyticum* was grown on easily metabolizable substrates, such as cellobiose (a disaccharide of two β-1,4 linked D-glucose units) or even pure cellulose, high carbon flux was attained, leading to pyruvate excretion (6, 7, 13, 14, 16). The pyruvate overflow suggested that the carbon flux through glycolysis was higher than the rate of processing of pyruvate-ferredoxin oxidoreductase (PFO) and lactate dehydrogenase (LDH) (the acetyl coenzyme A and the lactate branches, respectively) (Fig. 1). As a result, such catabolic overflow leads to an accumulation of intracellular inhibitory compounds that are directly responsible for the early cessation of growth of *C. cellulolyticum* (7, 13, 14).

In view of these considerations, it can be argued that *C. cellulolyticum* is not adapted to use a carbon source in excess and that the biotechnological exploitation of this microorganism requires genetic improvements. Since the efficiency of cellulose hydrolysis correlates with the population of cellulolytic clostridia, we focus on the reduction of the accumulation of pyruvate identified previously as one of the inhibitory compounds of bacterial growth (14). Using a metabolic engineering strategy, we demonstrate in this report that the removal of pyruvate accumulation can be successfully achieved and, as a consequence, the fermentation of cellulose by *C. cellulolyticum*

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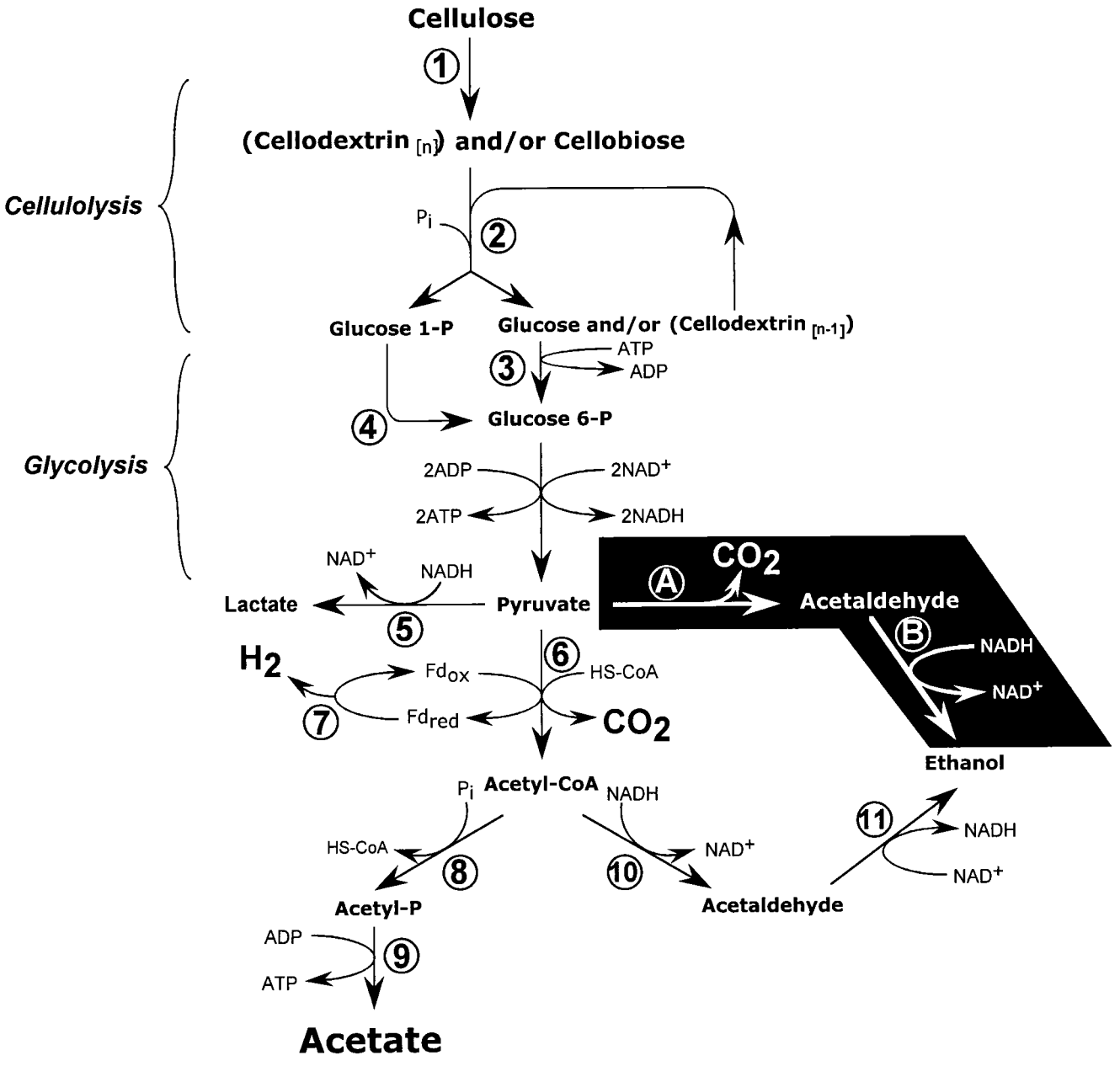

FIG. 1. Conversion of cellulose into fermentation products by an engineered strain of *C. cellulolyticum* (strain CC-pMG8). 1, Cellulosome hydrolysis; 2, cellodextrin phosphorylase (EC 2.4.1.49) and cellobiose phosphorylase (EC 2.4.1.20); 3, glucokinase (EC 2.7.1.2); 4, phosphoglucomutase (EC 5.4.2.2); 5, L-LDH (EC 1.1.1.27); 6, PFO (EC 1.2.7.1); 7, hydrogenase (EC 1.18.99.1); 8, phosphotransacetylase (EC 2.3.1.8); 9, acetate kinase (EC 2.7.2.1); 10, acetaldehyde dehydrogenase (EC 1.2.1.10); 11, ADH (EC 1.1.1.1). A, PDC (EC 4.1.1.1); B, ADH (EC 1.1.1.1). P, phosphate; Fd, ferredoxin; ox, oxidation; red, reduction; HS-CoA, coenzyme A.

can be improved significantly. Such an engineered microorganism offers potential biotechnological applications, one of them being the improvement of biogas production.

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture conditions. Table 1 lists all the bacterial strains and plasmids used in this study. *Escherichia coli* XL1Blue was used for plasmid construction, and *C. cellulolyticum* strain H10 (ATCC 35319) (27) was used as the recipient strain. Recombinant *E. coli* strains were grown at 37°C in Luria-Bertani medium (28) supplemented with ampicillin (100 mg per liter). *C. cellulolyticum* was grown in a synthetic medium with cellulose as the sole carbon and energy source (7, 14) and supplemented with erythromycin (20 mg per liter) only for the recombinant strains.

Vector construction. The expression shuttle vector (pMG8) harboring the prototype operon (pyruvate decarboxylase [PDC]-alcohol dehydrogenase [ADH]) was constructed by cloning into plasmid pMTL500F (25) (expression shuttle vector) two products obtained by PCR-mediated overlap extension (18) by using plasmid pLOI295 (carrying the *pdc* and *adhII* genes from *Zymomonas mobilis* CP4; GenBank accession no. M15393 and M15394, respectively) (20) as the template. The *pdc* gene was amplified by using primers GCTCTAGAAGGAGGTTAAGCAATGAAGTTATACTGCTGC (forward) and TTTAGATCTC

TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Relevant characteristics[a]	Source or reference
Strains		
E. coli XL1Blue	*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* [F′ *proAB lacI*^{q}*ZΔM15* Tn*10* (Tet^r)]	Stratagene
C. cellulolyticum ATCC 35319	Wild type; strain H10	22
CC-500F	*C. cellulolyticum* carrying plasmid pMTL500F; MLS^r	This work
CC-pMG8	*C. cellulolyticum* carrying plasmid pMG8; MLS^r	This work
Plasmids		
pLOI295	pUC18 pMB1 ori Ap^r *pdc adh*	15
pMTL500F	pMB1 ori Ap^r pAMβ1 ori MLS^r	18
pMG8	pMTL500F pMB1 ori Ap^r pAMβ1 ori MLS^r *pdc adh*	This work

[a] Tet^r, tetracycline resistance; MLS^r, macrolide-lincosamide-streptogramin resistance; pMB1 ori, origin of replication (*E. coli*); Ap^r, ampicillin resistance; *pdc*, PDC gene; *adh*, ADH gene; pAMβ1 ori, gram-positive origin of replication.

TAAGAGGAGCTTGTTAACAG (reverse) so that the 1,731-bp product contained a 5′ *Xba*I site (underlining in the forward primer) followed by a ribosome binding site (RBS) (double underlining) from the *C. cellulolyticum* scaffolding gene (*cipC*; GenBank accession no. U40345) located six nucleotides upstream from the initiation codon and a 3′ *Bgl*II site (underlining in the reverse primer). The *adhII* gene was amplified by using primers TTTAGATCTAGGAGGATA GCTATGGCTTCTTCAACTT (forward) and AATCTGCAGTGACGGTAGG CTTAATAGCCTGT (reverse) so that the 1,327-bp amplifica contained a 5′ *Bgl*II site (underlining in the forward primer) followed by an RBS (double underlining) from the *C. cellulolyticum cipC* gene located six nucleotides upstream from the initiation codon and a 3′ *Pst*I site (underlining in the reverse primer). After double restriction digestion with *Xba*I and *Bgl*II and with *Bgl*II and *Pst*I, the two amplifica were successively ligated into similarly digested vector pMTL500F, resulting in plasmid pMG8 (9.76 kb). The *Z. mobilis pdc* and *adhII* genes were cloned under the control of a strong constitutive *Clostridium pasteurianum* ferredoxin promoter (11) carried by expression shuttle vector pMTL500F (25).

Transformation of *C. cellulolyticum*. Transformation of *C. cellulolyticum* was carried out as recently published (21) with some modifications. In brief, cells were grown in a synthetic medium (40 ml) until mid-log phase, washed with ice-cold electrotransformation buffer (270 mM sucrose, 5 mM K_2HPO_4 [pH 6.5]), and resuspended in 1.5 ml of the same buffer. A 0.5-ml sample of the cell suspension was transferred to a 0.4-cm electroporation cuvette containing 5 μl (0.5 to 1.0 μg) of DNA methylated with *Msp*I methylase (1 U per μg, 37°C, 3 h). After a pulse with a Bio-Rad gene pulser apparatus (2.0 kV, 1,000 Ω, 25 μF), the cell suspension was immediately incubated anaerobically in 5 ml of prewarmed (34°C) synthetic medium for 6 h. Then, the cells were spread on selective plates (synthetic medium supplemented with 15 g of agar per liter and 20 mg of erythromycin per liter) and incubated anaerobically for 3 to 5 days at 34°C.

Bioreactor experiments. Bacteria were cultured aseptically in a 2-liter bioreactor (LSL Biolafitte, St. Germain en Laye, France) with a 1.5-liter working volume. The temperature was maintained at 34°C, and the pH was controlled at 7.2 by the automatic addition of 3 M NaOH. Agitation was kept constant at 50 rpm. The inoculum, from an exponentially growing culture, was 10% by volume. The cellulose bioreactor was connected to a gasometer filled with a saturated NaCl-water solution and acidified to pH 1.0 with H_2SO_4 to prevent gas dissolution as described previously (7). All tubing was made of Viton to preserve the anoxic conditions of the cell culture, and anaerobiosis was controlled in the presence of resazurin as described previously (16).

Analytical procedures. Biomass was estimated by bacterial protein measurement by use of the Bradford dye method (4) as modified by Desvaux et al. (7). The cellulose concentration was determined as described by Huang and Forsberg (19). Residual cellulose was washed with acetic acid-nitric acid reagent and water to achieve the removal of noncellulosic materials as described by Updegraff (30). Cellulose was then quantified by use of the phenol-sulfuric acid method (8) with glucose as the standard. Anhydroglucose equivalents were used for the calculation.

Culture supernatants (10,000 × *g*, 15 min, 4°C) were stored at −80°C until they were analyzed. Acetate, ethanol, and lactate levels were determined by using the appropriate enzyme kits (Boehringer Mannheim, Meylan, France). Extracellular pyruvate was assayed enzymatically by fluorimetric detection of NADH as previously described (14). Y_{ATP} values (energetic yield of biomass) were calculated as previously established (7).

Preparation of cell extracts and enzyme assays. Cell extracts were obtained as described previously (6). PDC was assayed essentially as described by Hoppner and Doelle (17) by monitoring of the pyruvate-dependent reduction of NAD^+ with ADH as a coupling enzyme. ADH activity was determined by monitoring of NADH-dependent acetaldehyde reduction at 340 nm as described previously (23). One unit of enzyme activity represents the conversion of 1 μmol of substrate per min into specific products.

Western blot analysis. The protein extracts prepared for the enzyme activity assays were used in a Western blot analysis performed by standard procedures (28). PDC was probed with a goat antiserum directed against *Z. mobilis* PDC protein, and ADH was detected with a rabbit antiserum raised against *Z. mobilis* ADH II protein (1). Binding of the primary antibody was carried out by incubating the preparation with a peroxidase-conjugated secondary immunoglobulin G antibody and detected by using an enhanced chemiluminescence detection system (Amersham Pharmacia Biotech). Relative quantifications of blot signals were carried out by using the Gel Doc system (Bio-Rad).

RESULTS AND DISCUSSION

Construction of recombinant strains of *C. cellulolyticum*. To achieve the cessation of excessive pyruvate production, metabolic engineering constitutes the most promising and efficient approach, particularly through the expression of a new heterologous pathway branched directly on the target (i.e., pyruvate) (Fig. 1). Hence, we chose to express in *C. cellulolyticum* the *Z. mobilis* (strain CP4) genes coding for active PDC and ADH through an artificial operon (20). Although genetic transfer in clostridia is largely recognized as a difficult process,

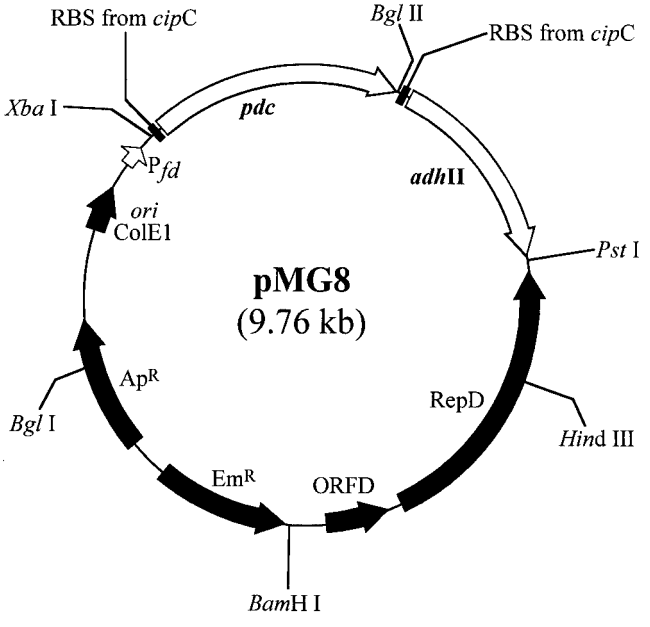

FIG. 2. Map of the expression shuttle vector pMG8, which carries the genes encoding PDC and ADH II from *Z. mobilis* strain CP4. pMG8 was constructed in shuttle vector pMTL500F (25). The *pdc* and *adhII* genes were cloned under the control of the promoter of the ferredoxin gene (P_{fd}) from *C. pasteurianum* (11). The restriction sites used in cloning were *Xba*I, *Bgl*II, and *Pst*I. ORFD, open reading frame D.

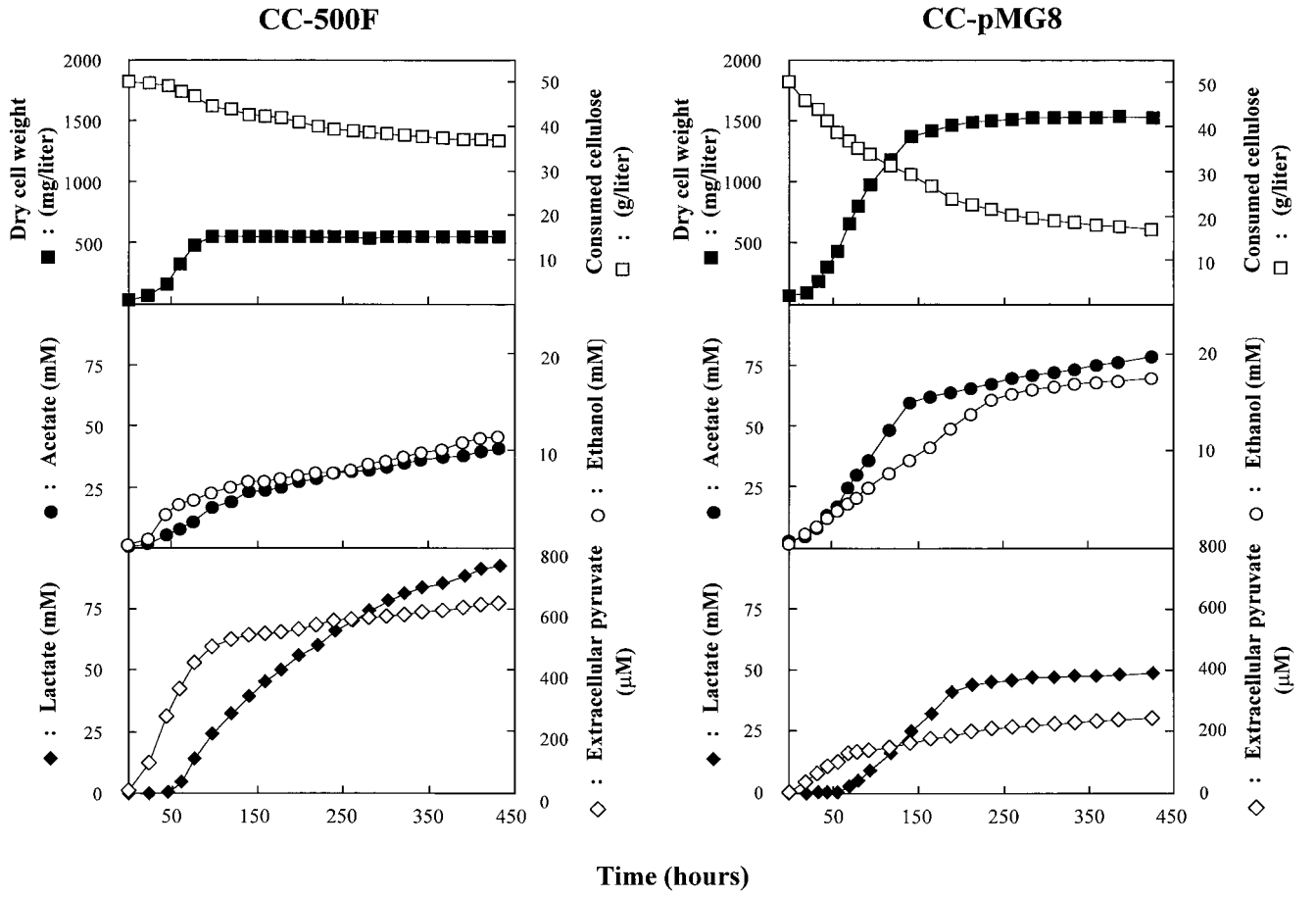

FIG. 3. Comparative fermentation patterns of *C. cellulolyticum* CC-500F and *C. cellulolyticum* CC-pMG8. *C. cellulolyticum* was grown in batch cultures with cellulose as the carbon source. Growth conditions and analytical procedures were previously described (7, 14).

this strategy was clearly realistic, since the propagation of replicons was recently achieved in *C. cellulolyticum* (21).

During the construction of the artificial operon as described in Materials and Methods, we introduced a new RBS upstream of the *pdc* and *adh* genes and based on that of the *cipC* gene (GenBank accession no. U40345), encoding the scaffolding protein of *C. cellulolyticum*. The resulting expression vector (pMG8) (Fig. 2) was introduced into *C. cellulolyticum* ATCC 35319 by electrotransformation, and tranformants appeared after 3 to 4 days on agar synthetic medium supplemented with erythromycin.

Characterization of recombinant strains of *C. cellulolyticum*. Enzymatic analyses of strain CC-pMG8 grown in a synthetic medium with cellulose as the sole carbon and energy source demonstrated the presence of PDC (0.58 U/mg) and ADH II (0.77 U/mg) activities. PDC activity was undetectable in the parent strain and in the control strain, CC-500F, which contained the shuttle vector alone, whereas 0.45 U of endogenous ADH/mg was found in the parent and control strains. The heterologous expression of PDC and ADH II was further confirmed by Western blot analysis (data not shown). *E. coli* transformed with pLOI295 was used as a positive control, whereas *E. coli*(pUC18) and CC-500F were used as negative controls. In the protein extracts obtained from CC-pMG8, analyses revealed two bands at 60 and 40 kDa, corresponding to the molecular masses expected for PDC and ADH II, respectively. No immunologically cross-reacting materials were detected in extracts from negative controls. Despite the presence of one rarely used codon in the *pdc* gene in *C. cellulolyticum* (<0.01%), i.e., the CGG arginine codon (frequencies of codon utilization are available at http:www.kazuka.or.jp/codon), PDC and ADH II were efficiently expressed in CC-pMG8 cells, as generally found with foreign genes cloned in *Clostridium* species (29) and in contrast to clostridial proteins poorly produced in *E. coli* (31). The results were confirmed by the quantification of Western blot signals, which revealed only 3- and 1.5-fold better production of PDC and ADH II in *E. coli* than in *C. cellulolyticum*.

Growth and fermentation pattern of *C. cellulolyticum* strain CC-pMG8. Recombinant strain CC-pMG8 was capable of growth in cellulose-containing synthetic medium, with a specific growth rate (0.049 h^{-1}) comparable to that of strain CC-500F and to that of the parent strain (0.044 h^{-1}); however, the growth period was prolonged: 145 h for the recombinant strain instead of 80 h for the parent strain (Fig. 3). The result was an increased biomass of CC-pMG8, which reached 1,500 mg per liter, compared to 500 mg per liter for CC-500F. This growth improvement for CC-pMG8 was the direct result of the ex-

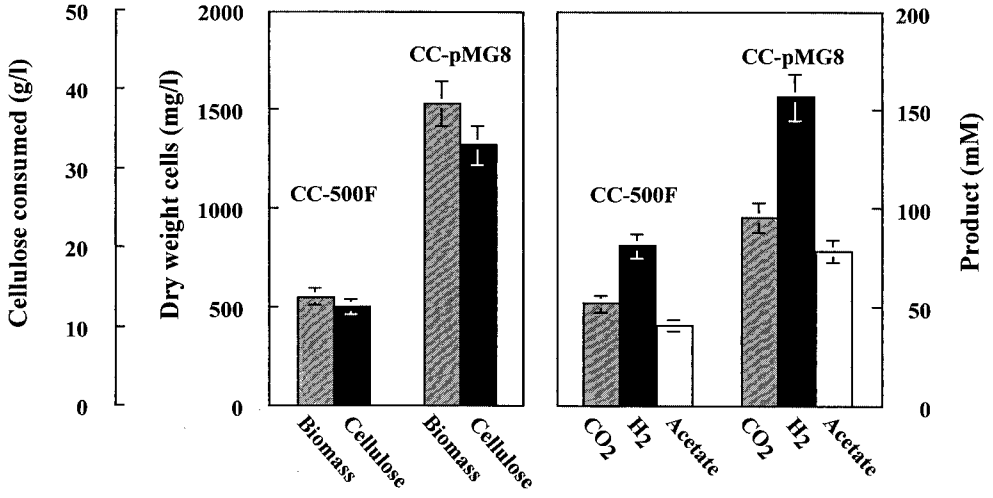

FIG. 4. Summary of cellulose consumption and biomass, CO_2, H_2, and acetate formation by strain CC-500F containing shuttle vector pMTL500F alone and strain CC-pMG8 harboring plasmid pMG8. Error bars show standard deviations.

pression of the *pdc-adh* operon decreasing pyruvic acid production by 60%, as shown in Fig. 3. These results indicated that the introduction of *Z. mobilis* PDC and ADH II activities may significantly relieve this overflow and have a great impact on cell growth and on the fermentation pattern (Fig. 3).

In comparison with the characteristics of strain CC-500 F, in strain CC-pMG8 cell density was increased by 180% and cellulose consumption was increased by 150% (Fig. 3 and 4). The fermentation pattern of strain CC-pMG8 was shifted significantly with respect to that of strain CC-500 F and the parent strain, indicating a strong influence of PDC and ADH II activities on general carbon catabolism. Thus, the concentrations of acetate and ethanol increased by 93 and 53%, respectively, while that of lactate decreased by 48%. These data show that the partitioning of the carbon flux at the pyruvate node through the PFO is the dominant branch, compared to the flux channeled by LDH and PDC, despite the fact that PFO and PDC have similar K_ms for pyruvate (0.5 and 0.4 mM for PFO and PDC, respectively, compared to 4.5 mM for LDH) (14, 20). Hence, the excess pyruvate was redirected toward ethanol production by the PDC and ADH II activities, to the detriment of lactate production and intracellular pyruvate accumulation; nevertheless, the PDC-ADH II pathway cannot process pyruvate as rapidly as it is formed. The PDC and ADH II activities also have an effect on partitioning at the acetyl coenzyme A node, since strain CC-pMG8 produced more acetate than ethanol, whereas strain CC-500F produced less acetate than ethanol. From an NAD^+-NADH balance viewpoint, it is speculated that increased NAD^+ cycling through the new ethanol branch (PDC-ADH) may favor the acetate pathway (extra ATP-producing pathway) and, in return, contribute to increasing the biomass. This notion is confirmed by the Y_{ATP} values for both strains (CC-500F and CC-pGM8), for which no significant difference was found (13.7 g of cells/mol of ATP and 13.5 g of cells/mol of ATP, respectively), meaning that the energetic cost of the engineered metabolic pathway is largely compensated for by the benefit obtained from higher acetate production.

Figure 4 shows the advantages of strain CC-pMG8 with respect to strain CC-500 F: (i) the removal of growth inhibition leads to an increase in the population of cellulolytic bacteria and hence the efficiency of cellulose hydrolysis, since cellulasic activities are cell associated via the cellulosome; and (ii) efficient fermentation of cellulose increases CO_2, H_2, and acetate production, these compounds being the substrates of the methanoarchaea, whereas increased production of ethanol will be reduced by degradation by syntrophic bacteria into acetate, H_2, and CO_2. Hence, strain CC-pMG8 is well adapted for improving cellulose degradation and biogas production.

Improvement of strains has traditionally relied on random mutagenesis followed by screening for mutants exhibiting enhanced properties of interest. Now, the opportunity to introduce heterologous genes and regulatory elements distinguishes metabolic engineering from traditional genetic approaches (3, 22). In the present work, the use of a straightforward metabolic engineering strategy consisting of the expression of an artificial operon (PDC-ADH) to eliminate excess intracellular pyruvate led to the conversion of *C. cellulolyticum* from a weakly to a highly active fermentative bacterium. We can attribute the success of this strategy to previous detailed studies of carbon and electron flux (6, 7, 13–15) that revealed the catabolic behavior of *C. cellulolyticum* with cellulosic substrates. Cellulolytic microorganisms are regarded as having one of the most promising biotechnological potentials, yet to our knowledge, this is the first report of such an engineered cellulolytic microorganism (both procarya and eucarya). Thus, our laboratory has focused on the genetic engineering of *C. cellulolyticum*, which has led to improving the efficiency of cellulose fermentation into acetate, CO_2, and H_2 instead of ethanol.

According to the results reported here, an efficient redirection of glycolytic flux to ethanol requires deletion of the acetate kinase-phosphotransacetylase pathway, which affects the ATP yield and hence the growth rate and the final dry cell weight. Practically, the fermentation of cellulose into acetate, CO_2, and H_2 has several advantages. (i) The feasibility of producing ethanol from molasses, sugar cane, or grains has been demon-

strated, whereas many biotechnological bottlenecks must be solved for ethanol production from lignocellulosic materials. (ii) Over the past decade, the total cost of ethanol production by fermentation (determined principally by the cost of sugar) has dropped from more than $1.00 per liter to ~$0.30 to 0.5 0 per liter, with a projected cost of less than $0.25 per liter in the near future (2), so the production of ethanol from lignocellulose is thus becoming less and less competitive. (iii) Methanogenesis is also an important process, and strain CC-pMG8, which has a higher biomass, uses a high level of cellulose (Fig. 4) and provides a safe and economic alternative for eliminating lignocellulosic materials in sewage digesters, anaerobic digesters, and waste streams from paper mills.

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