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Inactivation of an Aldehyde/Alcohol Dehydrogenase Gene from *Clostridium acetobutylicum* ATCC 824

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ABSTRACT

A nonreplicative plasmid containing an internal *aad* gene fragment has been integrated into the chromosome of *Clostridium acetobutylicum* ATCC 824. Transformation was accomplished by electroporation with relatively high concentrations of methylated plasmid DNA. Southern hybridization experiments revealed that integration occurred by single crossover homologous recombination inactivating the *aad* gene. Integrants were relatively stable after 25 generations. Inactivation of the *aad* gene drastically reduced solvent production. This result suggests that aldehyde/alcohol dehydrogenase (AAD) plays a important role in butanol production.

Index Entries: *Clostridium acetobutylicum*; aldehyde/alcohol dehydrogenase; solvent production; gene inactivation; homologous recombination.

INTRODUCTION

Clostridium acetobutylicum is a gram-positive, spore-forming, saccharolytic bacterium capable of fermenting a wide variety of sugars to acids (acetate and butyrate) and solvents (ethanol, acetone, and butanol). The production of acetone and butanol by *C. acetobutylicum* was a thriving industrial process, but the fermentation can no longer compete with the chemical synthesis of solvents from petroleum. However, renewed interest in using butanol as a fuel extender or chemical feed stock has focused research on the genetic manipulation of *C. acetobutylicum* with the aim of developing a more competitive industrial process.

The biochemical pathways for the conversion of carbohydrates to acids and solvents by *C. acetobutylicum* have been well reviewed *(1)*. Acetyl-CoA, acetoacetyl-CoA, and butyryl-CoA function as key intermediates for ethanol, acetone, and butanol production, respectively. Acetyl-CoA is first reduced to acetaldehyde and then to ethanol in reactions catalyzed by acetaldehyde dehydrogenase (ACDH) and ethanol dehydrogenase (EDH), respectively. Likewise, butyryl-CoA is reduced to butyraldehyde and then butanol in reactions catalyzed by butyraldehyde dehydrogenase (BYDH) and butanol dehydrogenase (BDH). The reassimilation of acetate and butyrate is coupled to the irreversible production of acetoacetate from acetoacetyl-CoA by acetoacetyl-CoA:acetate/butyrate:coenzyme transferase

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(CoAT). Acetone and carbon dioxide are produced from the decarboxylation of acetoacetate by acetoacetate decarboxylase (AADC).

Genes encoding AADC (*adc*), the two subunits of CoAT (*ctf*A and *ctf*B), two BDH isozymes (*bdh*A and *bdh*B) and aldehyde/alcohol dehydrogenase (AAD) (*aad*) have been cloned and sequenced from *C. acetobutylicum* ATCC 824. The *aad* gene resides immediately upstream of the previously cloned *ctf* genes (*2*), and the three genes form an operon (*3,4*). The *adc* gene lies downstream of the *ctf* genes in a separate operon (*5*). The *bdh* genes are linked in adjacent monocistronic operons, but are not in close proximity to other solventogenic genes (*6*). Complementation studies suggest that BYDH activity resides largely in AAD (*7*), but it is unclear what role AAD and the BDH isozymes play, if any, in ethanol and butanol formation. We hope to finally resolve the role of AAD in solvent production by inactivating *aad* on the chromosome.

Nonreplicative integrational plasmids have been used to advance genetic studies in gram-positive bacteria (*8,9*). Such plasmids need a DNA fragment from the host and a genetic marker for which selection can be made. After transfer, plasmids may become established by integrating into the homologous region on the host replicon in a Campbell-like fashion (*10*). Plasmid integration may result in two direct repeats of the homologous region flanking the plasmid DNA. Insertion can be mutagenic if the homologous DNA fragment on the integrational plasmid lies entirely within a gene or transcription unit (*11*). Insertion may also result in stable gene amplification if the homologous DNA fragment on the plasmid contains the entire gene (*12*).

Gene integration involving nonreplicative plasmids is usually limited to bacteria that can only be transformed at a high frequency. Since clostridia are notoriously difficult to transform, the use of this type of integration system has been limited to *Clostridium beijerinckii* (formerly known as *C. acetobutylicum* NCIMB 8052), for which conjugative transfer systems have been developed (*13,14*). Several integrational plasmids have been established in the chromosome of *C. beijerinckii* by homologous recombination (*15*).

An efficient and reliable plasmid transformation protocol, involving in vivo methylation, now exists for *C. acetobutylicum* ATCC 824 (*16*). In addition, most of the genes involved in acid and solvent formation pathways have recently been identified (*17*). Since cloned genes provide possible sites for integration studies, it should now be possible to exploit integrational plasmid technology fully in this strain. We report on use of a nonreplicative integrational plasmid containing an internal *aad* fragment to inactivate *aad* on the chromosome. This article provides valuable information about gene function and demonstrates the potential of using integrational plasmids to modify *C. acetobutylicum* ATCC 824 genetically.

METHODS

Bacterial Strains and Plasmids

Bacterial strains and plasmids are listed in Table 1.

Growth Conditions and Maintenance

Escherichia coli was grown aerobically at 37°C in Luria-Bertani (LB) medium. *C. acetobutylicum* was grown anaerobically at 37°C in 10-mL tube cultures of Clostridium growth medium (CGM; *18*). Colonies were obtained on agar-solidified rein-

Table 1
Bacterial Strains and Plasmids

Strain/plasmid	Relevant characteristics[a]	Source of gram-positive replicon	Source/reference
Strain			
C. acetobutylicum			
ATCC 824			ATCC
PJC4AAD	*aad*$^-$Em^r		Insertion of pJC4aad into ATCC 824
Escherichia coli			
ER2275	*rec*A$^-$*mcr*BC$^-$		New England Biolabs
Plasmid			
pAN1	Cm^{r}ϕ3T I		*(16)*
pJC4	Em^r Tc^r		*(24)*
pCAAD	Ap^r Em^r *aad*	*B. subtilis* pIM13	*(3)*
pSYL2	Ap^r Em^r	*C. butyricum* pCBU2	*(24)*
pJC4aad[b]	Em^r		This study

[a]Abbreviations: *aad*, aldehyde/alcohol dehydrogenase; Ap^r, ampicillin resistant; Cm^r, chloramphenicol resistant; Em^r, erythromycin resistant; *mcr*BC$^-$, lacking methylcytosine-specific restriction system; *rec*A$^-$, homologous recombination abolished; Tc^r, tetracycline resistant; ϕ3T I, ϕ3T methylase.

[b]pJC4aad was constructed by subcloning a 1.3-kb *Bam*HI-*Hin*dIII *aad* fragment from pCAAD into pJC4.

forced clostridial medium (RCM; Difco, Detroit, MI). LB medium was supplemented, as required, with erythromycin (200 μg/mL), tetracycline (15 μg/mL), and chloramphenicol (32 μg/mL). RCM and CGM media were supplemented with 20 μg/mL and 40 μg/mL of erythromycin, respectively.

DNA Isolation and Manipulation

The manipulation, transformation, and isolation of plasmid DNA from *E. coli* ER2275 were performed using standard procedures *(19)*. Large-scale plasmid isolation was undertaken with a plasmid purification kit (Qiagen, Studio City, CA). The restriction enzymes and T4 DNA ligase were used in accordance with the supplier's instructions (Promega, Madison, WI). Prior to transformation of *C. acetobutylicum*, pJC4aad was methylated in *E. coli* ER2275 (pAN1) by the *Bacillus subtilis* phage ϕ3T methyltransferase, which protects the plasmid DNA from restriction by the clostridial endonuclease *Cac*824I. The plasmid DNA was desalted and concentrated using MICROCON 100 microconcentrators (Amicon, Beverly, MA). Approximately 15 μg of methylated plasmid DNA were used to transform *C. acetobutylicum* by electroporation using a previously described method *(20)*. Chromosomal DNA was prepared from *C. acetobutylicum* using a PUREGENE DNA isolation kit (Gentra Systems, Research Triangle Park, NC). Cells were harvested from a 10-mL CGM tube culture during the exponential phase (A_{600} ~ 0.8).

Enzyme Assays

Cell extracts were prepared from 50 mL of solventogenic *C. acetobutylicum* culture. The cells were harvested by centrifugation (20,000*g* for 20 min at 4°C). The

cell pellet was resuspended in 10 mL of 15 m*M* potassium phosphate buffer (pH 7.0) containing 1 m*M* dithiothreitol and 0.1 m*M* $ZnSO_4$. The cells were lysed by two passes through a precooled French pressure cell (SLM model FA-078) at an operating pressure of 10,000 psi, and the cell debris was removed by centrifugation (35,000*g* for 20 min at 4°C). BDH assays were performed in the physiological direction as previously described *(21)* with butyraldehyde at a final assay concentration of 25 m*M*. One unit (U) is defined as the amount of enzyme necessary to oxidize 1 μmol of NADH/min. The protein concentration was determined using a dye reagent in accordance with the manufacturer's instructions (Bio-Rad, Hercules, CA).

Southern Hybridization

Chromosomal DNA was digested to completion with *Sca*I, transferred from an agarose gel to a Hybond-N^+nylon membrane (Amersham, Arlington Heights, IL) by capillary blotting *(22)*, and then probed with a radiolabeled 1.3-kb *Bam*HI-*Hin*dIII *aad* fragment, isolated from pCAAD (Table 1). The gene fragment was labeled with [α-^{32}P]dATP using a random primer DNA labeling system (Gibco BRL, Gaithersburg, MD), and unincorporated radionucleotides were removed by exclusion chromatography on Sephadex G-50. The prehybridization, hybridization, and washing steps were performed in accordance with the membrane manufacturer's instructions, and the radioactivity was detected by autoradiography.

Analytical Methods

Cell growth was monitored at 600 nm using a Beckman DU 64 spectrophotometer with cuvets of 1-cm light path. Samples were diluted in distilled water, if necessary, so that the absorbance at 600 nm ($A_{600\,nm}$) did not exceed 0.8. Fermentation products (acetate, butyrate, ethanol acetone, and butanol) were determined by gas chromatography using a Hewlett-Packard 5890 Series II Gas Chromatograph with a flame ionization detector and reporting integrator. Samples of culture supernatant and a standard solution (10 m*M* ethanol, 20 m*M* acetone, 40 m*M* butan-1-ol, 30 m*M* acetate, and 30 m*M* butyrate) were acidified with an equal volume of 20% (v/v) phosphoric acid containing 12.5 m*M* pentan-3-one (internal standard). Acidified samples (5 μL) were injected onto a glass column (4 ft × 4 mm internal diameter) containing Porapak Q (80–100 mesh; Hewlett-Packard, Wilmington, DE). Separation of sample components was achieved using a temperature program. The column temperature was held at 135°C for 8 min, increased by 30°C/min for 2 min, and then held at 195°C for 10 min. The injector and detector temperatures were 245° and 215°C, respectively. The carrier gas (N_2) flow rate was 25 mL/min.

Measurement of the Recombination Frequency

The integrational strain was grown overnight in CGM with erythromycin. The culture was diluted approx 1000-fold in 50 mL of prewarmed CGM lacking erythromycin and incubated until the A_{600} reached 1.0. The culture was serially diluted in CGM, and plated on nonselective and selective CGM medium. The proportion of antibiotic-resistant cells was determined by dividing the number of colonies found on selective medium by the number of colonies found on nonselective medium. Subculturing steps and colony counts were repeated until the bacterial population had undergone about 25 generations of growth. The segregation fre-

Table 2
Electrotransformation Efficiencies of Replicative and Nonreplicative Plasmid DNA

Plasmid	Insert size, kb	Ability to replicate in *C. acetobutylicum*	DNA concentration, μg	Number of transformants
pAN1	0	–	5	0
pAN1 and pSYL2	0	+	0.05	500
pAN1 and pJC4	0	–	15	0
pAN1 and pJC4aad	1.3	–	15	13

quency was calculated by dividing the proportion of antibiotic-sensitive colonies by the number of generations of growth *(23)*.

RESULTS

Electrotransformation

C. acetobutylicum ATCC 824 was transformed by electroporation with the methylation plasmid (pAN1) and plasmid mixtures containing pAN1 and either pSYL2, pJC4, or pJC4aad. The DNA in these mixtures was methylated, in vivo, by the pAN1-encoded methylase, which protects vector DNA from *Cac*824I restriction *(16)*. The number of transformants observed after electroporation is shown in Table 2. The methylation plasmid (pAN1) did not transform *C. acetobuylicum*. This plasmid lacks a suitable origin of replication and cannot therefore propagate in *C. acetobutylicum*. The plasmid also lacks an erythromycin marker and cannot be maintained in selective media. Plasmid pSYL2 is an efficient shuttle vector *(16)*, and transformation with this vector serves as a positive control. The plasmid contains a gram-positive replicon *(24)*, and the results show that this vector transformed ATCC 824 at a relatively high frequency (1×10^4 colonies/μg DNA). Plasmid pJC4 and its derivative pJC4aad lack suitable replicons that would enable them to replicate in *C. acetobutylicum*. They do, however, contain erythromycin markers, and selective pressure may force them to integrate into the chromosome. Plasmid pJC4 did not transform ATCC 824, whereas pJC4aad transformed at a low frequency (0.9 colony/μg DNA). This result suggests that a small proportion of the incoming pJC4aad molecules integrated into the chromosome by homologous integration.

Southern Hybridization

Three pJC4aad transformants were selected and designated PJC4AAD (a, b, and c). Chromosomal DNA from the integrants and parental strain was digested with *Sca*I and characterized by Southern hybridization (Fig. 1). *Sca*I was chosen because this enzyme has a single site in the backbone of the vector pJC4, but none in the clostridial *aad* insert. If a single copy of pJC4aad integrated into the chromosome, digestion with *Sca*1 should generate two fragments whose combined size equals the combined size of pJC4aad and the *Sca*I fragment on the parental chromosome that contains the aad gene. The *aad* probe hybridized to two *Sca*I fragments (approx 5.5 and 6.3 kb) from the PJC4AAD mutant strains and one *Sca*I fragment (5.6 kb) from the parental strain. Since pJC4aad is 6.2 kb and the *aad* gene resides

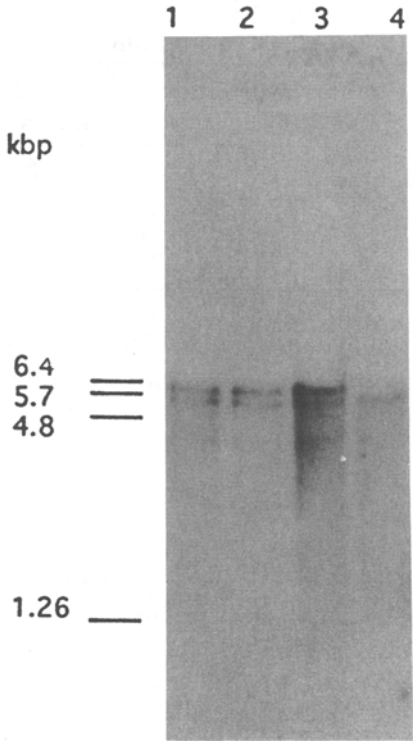

Fig. 1. Hybridization of a 1.3-kb *aad* fragment to *Sca*I-digested chromosomal DNA from three *C. acetobutylicum* PJC4AAD integrants and the parental strain ATCC 824. Lanes 1, 2, and 3 are integrants PJC4AAD(a), PJC4AAD(b), and PJC4AAD(c); lane 4 is ATCC 824. Size markers (kbp) are *Bst*EII-digested λ DNA.

within a 5.6-kb *ScaI* fragment on the chromosome *(3)*, it appears that a single copy of pJC4aad integrated into the *aad* gene on the chromosome in all three mutants tested.

Product Formation

The parental and integrational strains were grown for 48 h in 10-mL CGM tube cultures supplemented, as required, with erythromycin. The culture supernatant was analyzed for acid and solvent production by gas chromatography (Table 3). The PJC4AAD integrants (a, b, and c), previously characterized by Southern hybridization, produced on average 15% more butyrate, 9% more acetate, 38% more ethanol, and 84% less butanol than the parental strain. The integrants did not produce any acetone. The average cell biomass (A_{600}) of the mutant cultures was 11% lower than the parental strain.

Enzyme Assays

Since AAD appears to depend on either nicotinamide adenine dinucleotide (NAD) or reduced NAD (NADH) *(7)*, crude cell-free extracts from wild-type and mutant PJC4AAD(a) cultures were assayed for NADH-dependent BDH. The spe-

Table 3
Production of Acids and Solvents
by *C. acetobutylicum* ATCC 824 Wild Type and PJC4AAD[a]

C. acetobutylicum		Concentration (m*M*)				
ATCC 824	$A_{600\,nm}$	Ethanol	Acetone	Butanol	Acetate	Butyrate
PJC4AAD(a)	4.3	10.2	0	6.9	31.1	56.0
PJC4AAD(b)	4.2	10.4	0	8.1	27.4	46.8
PJC4AAD(c)	4.1	8.8	0	6.7	24	49.2
PJC4AAD average	4.2	9.8	0	7.2	27.5	50.6
Wild-type average	4.7	7.1	14.3	43.9	25.2	44.1

[a]Wild-type average values are calculated from three cultures (results not shown). PJC4AAD average values are calculated from the three mutant strains selected (PJC4AAD a, b and c).

cific activity (U/mg of protein) of BDH from PJC4AAD(a) and wild type was 0.012 and 0.018, respectively. PJC4AAD had 33% less BDH activity than the wild type.

Integrational Stability

Strain PJC4AAD(a) was grown for approx 25 generations in the absence of selective pressure. After plating serial dilutions on selective and nonselective growth medium, the segregation frequency (rate of plasmid loss per generation) was found to be about 3.5×10^{-3}.

DISCUSSION

Southern hybridization results indicate that the nonreplicative plasmid pJC4aad integrated into the homologous *aad* region on the chromosome. Integration, assumed to have taken place by homologous recombination involving a Campbell-like mechanism, resulted in two copies of the homologous region provided. Duplicated sequences are potential substrates for homologous recombination and can result in plasmid loss *(9)*. However, the integrant tested appeared to be relatively stable after 25 generations. The segregation frequency (3.5×10^{-3}) was comparable to values reported for *Clostridium beijerinckii* ($1.3–37 \times 10^{-3}$; *15*), but was one order of magnitude lower than values reported for *Lactococcus lactis* *(9)* and *B. subtilis* *(25)*. The integrants grew slower and reached slightly lower cell densities than the parental strain. Slow integrant cell growth probably increased selection for faster-growing nonintegrants, which contributed to the segregation frequency.

Analysis of the fermentation products revealed that the PJC4AAD integrants did not produce any acetone. The integrants also produced considerably less butanol than the parental strain. These results suggest that integration of pJC4aad into the *aad* gene structurally inactivates *aad* and disrupts transcription of the two genes (*ctf*A and *ctf*B) that code for CoAT, an enzyme involved in acetone production. This is no surprise, since the *ctf* genes reside immediately downstream of *aad* and are located in the same operon *(3,4)*. The three genes appear to be cotranscribed.

C. acetobutylicum AAD displays both aldehyde (ALDH) and alcohol dehydrogenase (ADH) activity *(3)*. Inactivation of the ADH domain on the *aad* gene reduced NADH-dependent BDH activity by 33% and decreased butanol production by 84%. These findings suggest that the ADH function of AAD is primarily involved in

the reduction of butyraldehyde to butanol. These results are consistent with findings from a complementation study that suggests that the ALDH function of AAD is primarily involved in the reduction of butyryl-CoA to butyraldehyde. Nair and Papoutsakis *(7)* transformed a mutant strain of *C. acetobutylicum* (M5), deficient in BYDH, with a replicative plasmid containing *aad* and found that the plasmid restored BYDH activity and butanol production. It appears that AAD is primarily responsible for most of the BYDH and BDH activity in the cell. This multifunctional AAD protein must therefore play a significant role in butanol production.

The BDH isozyme genes *bdh*A and *bdh*B encode BDHII and BDHI *(6,26)*, but their role in solvent production is unclear. Both enzymes possess EDH and BDH activity but BDHII has a higher specificity for butyraldehyde than for acetaldehyde *(6)*. The integrants produced 35% more ethanol than the parental strain, probably in an attempt by the cells to get rid of excess reducing power. The enzymes encoded by one or both *bdh* genes may be responsible for the increased ethanol production and remaining BDH activity in the AAD mutant, but their precise role in solvent production cannot be determined until the genes have been inactivated on the chromosome.

Inactivation of the *aad* gene on the chromosome greatly reduced the solvent-producing capability of the cell without drastically affecting cell growth. This result demonstrates that gene integration technology can be used to redirect cellular metabolism in *C. acetobutylicum* ATCC 824. It should be possible either to eliminate acid-producing pathways (acetate or butyrate) by gene inactivation or enhance solvent-producing pathways (acetone and butanol) by gene duplication. Not only does this technique provide valuable information about gene function, but it may be used to redirect a greater proportion of the carbon and electron flow toward solvent production, improving both the product yield and selectivity. We believe gene integration technology moves the acetone–butanol fermentation one step closer to becoming a viable alternative to the chemical synthesis of solvents.

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