Development of a Sensitive Gene Expression Reporter System and an Inducible Promoter-Repressor System for Clostridium acetobutylicum

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A sensitive gene expression reporter system was developed for Clostridium acetobutylicum ATCC 824 by using a customized gusA expression cassette. In discontinuous cultures, time course profiles of β-glucuronidase specific activity reflected adequately in vivo dynamic up- and down-regulation of acidogenesis- and/or solventogenesis-associated promoter expression in C. acetobutylicum. Furthermore, a new inducible gene expression system was developed in C. acetobutylicum, based on the Staphylococcus xylosus xylose operon promoter-repressor regulatory system.

The anaerobic bacterium Clostridium acetobutylicum converts a variety of sugars into solvents. With its sequenced genome (11), C. acetobutylicum ATCC 824 is considered the type strain for the study of the physiology and the molecular biology of solventogenic clostridia. Since regulatory mechanisms controlling gene expression differ vastly from one bacterium to another, development of reporter systems adapted to Clostridium species is required. Several different genes have been used as reporters in Clostridium: (i) the chloramphenicol acetyltransferase gene (catP) cloned from the C. perfringens plasmid pIP401 (8, 18), (ii) the lacAB genes encoding the luciferase enzyme of Vibrio fischeri (12), (iii) the β-1,4-endoglucanase gene (eglA) cloned from C. acetobutylicum P262 (13), and (iv) a lacZ gene isolated from Thermoanaerobacterium thermosulfurigenes (19, 20). In this study, we report the development of a sensitive reporter gene system and an inducible promoter-repressor system for C. acetobutylicum ATCC 824.

gusA gene expression in C. acetobutylicum. The Escherichia coli β-glucuronidase (gusA) reporter system is popularly used by Jefferson and coworkers (5, 6), was selected for the construction of a reporter system in C. acetobutylicum ATCC 824 (Table 1). A customized gusA expression cassette for better expression in C. acetobutylicum was constructed by PCR amplification from E. coli MC 1061 genomic DNA (15): the artificially introduced ribosome binding site (AGGAGG) and its ten adjacent bases were from thlA, encoding thiolase, an enzyme involved in the central metabolism of C. acetobutylicum and showing high activity (21) in both acidogenesis and solventogenesis; the terminator was from adc, encoding the acetoadetate decarboxylase. Our customized gusA expression cassette was previously successfully used in C. beijerinckii (14). The gusA expression cassette was cloned into the pSOS95 vector to create pSGUSa, in which gusA expression was under the control of the thlA promoter. The pSGUsa and pIMP1 (control vector) (10) plasmids were introduced into C. acetobutylicum ATCC 824 (9), and β-glucuronidase activity in anaerobic flask cultures was assayed with a sensitive fluorimetric assay (7). The β-glucuronidase activity measured in strain 824(pSGUsa) was 1,000-fold higher than that in strain 824(pIMP1), showing that GusA was expressed, functional, and nontoxic in C. acetobutylicum and that the endogenous β-glucuronidase activity was low. A gusA gene expression reporter system, pGUSA, was then constructed by removing the thlA promoter of pSGUsa and replacing it with a polylinker containing four unique restriction sites. In strain 824(pGUSA) cultivated in a batch culture at a pH of 4.8 on synthetic medium supplemented with clarithromycin (40 μg/ml) (21), the measured β-glucuronidase activities (Fig. 1) indicate that basal and/or endogenous β-glucuronidase activities remained at very low levels throughout the batch culture experiment.

Use of gusA as a reporter gene. The effectiveness of the gusA reporter system response was tested by cloning two other clostridial promoters known for their expression in acidogenesis and/or solventogenesis in the polylinker region of pGUSA. The putative promoter of adc, encoding the acetoadetate decarboxylase, was PCR amplified from C. acetobutylicum ATCC 824 total genomic DNA and cloned into pGUSA to generate pADCgusA. The acetoadetate decarboxylase is required in solventogenesis to convert acetoadetate into acetone. The promoter of pib, encoding the phosphotransbutyrylase (the first enzyme of the butyric acid formation pathway), was PCR amplified from pPbS94 and introduced into pGUSA, leading to pPTBgusA.

The C. acetobutylicum strains 824(pSGUsa), 824(pADCgusA), and 824(pPTBgusA) were cultivated in batch cultures on glucose at a pH of 4.8. The maximal β-glucuronidase activity levels in strains 824(pSGUsa), 824(pADCgusA), and 824(pPTBgusA) (Fig. 2) were significantly higher than that in the control strain 824(pGUSA).

The β-glucuronidase activity profile for strain 824(pSGUsa) grown at a pH of 4.8 (Fig. 2A) revealed that, during the growing phase, the thlA promoter had high and almost constant activity. On the other hand, when the cells started to lyse, a sharp decrease of the activity was observed. An approximately
similar expression profile was reported for the \textit{thlA} promoter by using the \textit{lacZ} reporter gene system (20), while a different regulation pattern was described when solventogenesis was induced by letting the pH drop in a chemostat culture (1, 24). Experiments performed with strain 824(pADCgusA) (Fig. 2B) indicated that the \textit{adc} promoter is switched on at the onset of acetone production and is predominantly active during the solventogenic phase. This result correlates well with findings of previous studies of \textit{adc} promoter activity and regulation using this \textit{gusA} reporter gene system expressed in \textit{C. beijerinckii} (14) or the \textit{T. thermosulfurigenes lacZ} reporter gene system expressed in \textit{C. acetobutylicum} (20).

\textit{ptb} expression in strain 824(pPTBgusA) (Fig. 2C) shows a profile similar to that of \textit{ptb} expression in \textit{C. beijerinckii} (14): expression was the highest during exponential phase and declined rapidly thereafter in agreement with the butyric acid concentration. With the use of \textit{T. thermosulfurigenes lacZ} as a reporter system (20), the decrease of \textit{ptb} activity after the late exponential phase was slow compared to our results. This may be because the stability of \textit{T. thermosulfurigenes} \textit{β}-galactosidase is higher than that of the \textit{E. coli} \textit{β}-glucuronidase. If this possibility is confirmed and, as in bacteria, the mRNA half-life is very short, \textit{gusA} would be a better reporter system than \textit{T. thermosulfurigenes lacZ}.

\textbf{Transcriptional regulation of \textit{C. acetobutylicum} \textit{[Fe]hydrogenase in discontinuous culture.} Gorwa and coworkers (3) have cloned the putative \textit{hydA} gene encoding the \textit{[Fe]hydrogenase} of \textit{C. acetobutylicum} ATCC 824, the key enzyme of the electron metabolism (2, 21). They showed that \textit{hydA} was regulated at the transcriptional level with lower \textit{hydA} mRNA levels in solvent-producing continuous cultures than in acid-producing ones. To monitor the dynamic transcriptional regulation of \textit{hydA} during the shift between acidogenesis and solventogenesis, the putative \textit{hydA} promoter was PCR amplified from pMFH1 (3) and cloned into pGUSA to create pHGusA. \textit{β}-glucuronidase activity in strain 824(pHGusA) grown in batch culture (Fig. 3) showed that the \textit{hydA} promoter was indeed associated with early acidogenic growth phase, with very strong expression (the strongest of the four used promoters), and then shut down in the middle of the exponential phase, though not completely since a residual expression level was still measured in solventogenesis, consistent with the fact that hydrogen production is still present in solventogenesis. A similar 10-fold decrease was observed in vitro hydrogenase activity between acid- and solvent-producing continuous cultures (3).

In addition, the high \textit{β}-glucuronidase activity in \textit{C. acetobu-
The product profiles (data not shown).

tylicum recombinant cells affected neither the growth rate nor density (OD) at 620 nm; —, optical density (OD) at 620 nm; —, optical density; ▲, β-glucuronidase specific activity; †, β-glucuronidase specific activity. The arrow indicates the time at which the butyric acid concentration reached its maximum value, indicating the shift between acidogenesis and solventogenesis.

In conclusion, we demonstrated that our customized gusA expression cassette is functional and adapted to promoter analysis in C. acetobutylicum. We hope that the pGUSA promoterless reporter gene vector will be a useful tool for the study of the Clostridium community.

Nucleotide sequence accession numbers. The pSOS94, pSOS95, and pGUSA plasmids have been submitted to the GenBank database and assigned accession numbers AY187685, AY187686, and AY292368, respectively.

We thank Sophie Mondeil for her technical assistance in the construction of the pSgusA plasmid.

REFERENCES

FIG. 1. Time course profiles of β-glucuronidase specific activity in C. acetobutylicum ATCC 824(pPHgusA) in discontinuous cultures at a constant pH of 4.8. Symbols: —, optical density (OD) at 620 nm; —, glucose concentration; ▲, β-glucuronidase specific activity. The arrow indicates the time at which the butyric acid concentration reached its maximum value, indicating the shift between acidogenesis and solventogenesis.

Sequence analyses of the four promoters used in this study showed that only the adc promoter contains Spo0A-binding motifs (4, 14). Regulation of the two acidogenic phase-associated promoters ptb and hydA in C. acetobutylicum may involve other, as yet unidentified, regulatory proteins. An alignment of the two promoters revealed a conserved sequence containing an inverted repeat sequence (consensus sequence CGTTAAT nTTTAAC [n, nonconserved nucleotide]) located, respectively, 4 and 24 bp downstream of ptb and hydA transcription start sites (3, 22). It is thus tempting to suggest that this sequence may be a recognition sequence for a common regulatory factor of ptb and hydA gene expression, and according to its position in the promoter regions, this regulator would act as a transcriptional repressor probably involved in the gene expression shutdown.

Inducible β-glucuronidase expression in C. acetobutylicum. To develop the first inducible gene expression system in C. acetobutylicum, we have tested the xylose-inducible system from Staphylococcus xylosus. The S. xylosus xylose operon encodes a xylose isomerase (xylA) and a xylulokinase (xylB) (16). In the absence of xylose, the transcription of the xylose operon is repressed by XylR, which binds to a xylA operator palindrome (17). In the presence of xylose, which functions as an inducer, the XylR repressor is inactive and transcription of the operon occurs. The XylR gene and xylA promoter-operator sequence were PCR amplified by using S. xylosus DSM 20267 chromosomal DNA and cloned into pGUSA to generate pXYLgusA. β-glucuronidase activities in strain 824(pXYLgusA) grown in anaerobic flask cultures were $71 \pm 26 \text{ pmol min}^{-1} \text{ mg}^{-1}$ under noninducing conditions (glucose present in 2YT medium) and $1260 \pm 180 \text{ pmol min}^{-1} \text{ mg}^{-1}$ under inducing conditions (xylose instead of glucose present in 2YT medium at 10 g of xylose/liter). Thus, the utilization of xylose as the sole carbon source led to 17-fold higher β-glucuronidase expression. The combination of xylA induction in the presence of xylose with its potential glucose-mediated catabolite repression by CcpA (17, 23), which function still has to be confirmed in C. acetobutylicum, will make the pXYLgusA a powerful cloning vector for tight and modulated expression of cloned genes in C. acetobutylicum.

We thank Sophie Mondeil for her technical assistance in the construction of the pSgusA plasmid.
Clostridium acetobutylicum is inverted (more acidic inside) when the in vivo activity of hydrogenase is decreased. J. Bacteriol. 176:6146–6147.


