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RESEARCH ARTICLE

Discovery of a novel gene involved in autolysis of *Clostridium* cells

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ABSTRACT

Cell autolysis plays important physiological roles in the life cycle of clostridial cells. Understanding the genetic basis of the autolysis phenomenon of pathogenic *Clostridium* or solvent producing *Clostridium* cells might provide new insights into this important species. Genes that might be involved in autolysis of *Clostridium acetobutylicum*, a model clostridial species, were investigated in this study. Twelve putative autolysin genes were predicted in *C. acetobutylicum* DSM 1731 genome through bioinformatics analysis. Of these 12 genes, gene SMB_G3117 was selected for testing the intracellular autolysin activity, growth profile, viable cell numbers, and cellular morphology. We found that overexpression of SMB_G3117 gene led to earlier ceased growth, significantly increased number of dead cells, and clear electrolucent cavities, while disruption of SMB_G3117 gene exhibited remarkably reduced intracellular autolysin activity. These results indicate that SMB_G3117 is a novel gene involved in cellular autolysis of *C. acetobutylicum*.

KEYWORDS cell autolysis, autolysins, *Clostridium*, gene SMB_G3117

INTRODUCTION

Cell autolysis phenomenon is widespread among bacteria that possess peptidoglycan (Smith et al., 2000). Considering the possibility that cell autolysis is involved in selective removal of peptidoglycan, it might be involved in numerous cellular processes including cell growth, cell wall turnover, peptidoglycan maturation, cell division, cell lysis, motility, chemotaxis, genetic competence, differentiation, and pathogenicity (Foster, 1994; Blackman et al., 1998). *Clostridium* is one of the largest bacterial genera, ranking the second in size next to *Streptomyces*, and classified as Gram-positive endospore-forming obligate anaerobes (Andreesen et al., 1989; Rehner and Samuels, 1994). Many species of *Clostridium* are pathogenic, e.g. *C. botulinum*, *C. septicum*, and *C. difficile*, while some species of *Clostridium*, e.g. *C. acetobutylicum* and *C. beijerinckii*, are also of biotechnological importance for solvents production. Understanding the genetic basis of autolysis of *Clostridium* cells is thus important for obtaining insights into the life cycle of this important species.

Acetone-butanol-ethanol (ABE) fermentation by solvent-producing *Clostridium* represents one of the oldest industrial fermentation processes ever known, ranking second in scale only to ethanol fermentation by yeast (Lutke-Eversloh and Bahl, 2011). Recently, this process has been revived because of the potential application of butanol as an alternative biofuel (Lee et al., 2008). Typical batch fermentation process of *C acetobutylicum* can be divided into two phases, acidogenesis and solventogenesis. Exponentially growing cells mainly produce acids in acidogenic phase, which were then reassimilated and converted to solvents by late-exponential growing cells in solventogenic phase. In the latter stage of solventogenesis, the optical density (OD_{600}) value usually sharply decreases, the so-called autolysis (Barber et al., 1979; Allcock et al., 1981). Autolysis of *Clostridium* cells might account for the diminished and even ceased production of solvents.

It is generally accepted that autolysis is triggered by autolysins, which are a group of bacteriolytic enzymes that digest the bacterial cell-wall peptidoglycan (Shockman and Holtje, 1994; Smith et al., 2000). In Gram-positive bacteria, autolysins can be classified into four main categories according to the specificity of their hydrolytic bonds, i.e. muramidases, glucosaminindases, N-acetylmuramoyl-L-alanine amidases (amidas-

es), and endopeptidases (Smith et al., 2000). In the last decades, autolysins in *Escherichia coli* (Holtje, 1995; Heidrich et al., 2001), *Streptococcus* (Ju et al., 2012; Tamura et al., 2012), *Staphylococcus aureus* (Jayaswal et al., 1990; Foster, 1995), lactic acid bacteria (ChapotChartier, 1996), and *Bacillus subtilis* (Blackman et al., 1998; Smith et al., 2000) have been extensively investigated. The autolysis phenomenon in ABE fermentation was firstly described in 1979 (Barber et al., 1979), but the molecular basis for autolysis of *C. acetobutylicum* remains poorly understood. Currently, only four lytic enzymes in solvent-producing *clostridia* have been partially purified and characterized. These include glycoprotein (28 kDa) (Webster et al., 1981), muramidase (41 kDa) (Croux et al., 1992b), and amidase (115 kDa) (Garcia et al., 1988) in *C. acetobutylicum* ATCC 824, and N-acetylmuramidase (44 kDa) in *C. saccharoperbutylacetonicum* N l-4 (Yoshino et al., 1982). A gene CA_C0554 in *C. acetobutylicum* ATCC 824, which encodes a lysozyme consisted of 324 amino acids with a calculated molecular mass of 34.939 kDa, was expressed in *E. coli* and characterized (Croux and Garcia, 1992).

The aim of this study was to identify and characterize genes that might be involved in cell autolysis of *C. acetobutylicum* DSM 1731. Autolysin relevant features including intracellular autolysin activity, growth profile, viable cell counts, and cellular morphology were performed. Finally, a novel gene, SMB_G3117, was found to play a role in the autolysis of *C. acetobutylicum* cells.

RESULTS

Identification of putative autolysin genes in *C. acetobutylicum* through bioinformatics analysis

There are comprehensive descriptions concerning autolysins in *Bacillus subtilis* (Smith et al., 2000), which is moderately related to *C. acetobutylicum* (Nolling et al., 2001), thus providing a theoretical reference for our research. In *B. subtilis* 168, eight genes representing conserved elements of four autolysin families (Table 1) were extracted from Kyoto Encyclopedia of Genes and Genomes (KEGG, http://www.genome.jp/kegg/). To identify genes encoding putative autolysin in *C. acetobutylicum* DSM 1731 (Bao et al., 2011), the sequences of 8 autolysin genes in *B. subtilis* 168 were individually used as query sequences to blast against *C. acetobutylicum* DSM 1731 genome with *E*-value < 10^{-5}. Nine homologous genes were identified in the genome of *C. acetobutylicum* DSM 1731. To avoid missing target genes relevant to autolysin in DSM 1731, the sequences of these 9 genes were used to identify other possible autolysin genes through function domains search, resulting in 3 more candidate genes identified (Table 1), in which SMB_G0566 and SMB_G1901 are paralog genes of SMB_G1126, while SMB_G3128 is paralog gene of SMB_G3117. In total, 12 putative autolysin genes were identified in the *C. acetobutylicum* DSM 1731 genome (Fig. 1). These genes can be divided into four categories on the basis of sequence similarities, i.e., muramidases (SMB_G0566, SMB_G1126, SMB_G1901), glucosaminindases (SMB_G2359), N-acetylmuramoyl-L-alanine amidases (SMB_G0700, SMB_G3117, SMB_G3128), and endopeptidases (SMB_G0078, SMB_G0429, SMB_G0493, SMB_G2513, SMB_G3064).

Preliminary evaluation of the putative autolysin genes in *C. acetobutylicum*

All 12 putative autolysin genes were overexpressed in *C. acetobutylicum* SMB009 which is capable of accepting unmethylated DNA (Dong et al., 2010) to evaluate whether these genes are involved in autolysis. Considering that constitutive expression of autolysin genes may lead to cellular toxicity, the inducible expression vector pGusA2-2tetO1 that we previously developed (Dong et al., 2012) was used.

Twelve isolated transformants were cultivated in CGM medium supplemented with 50 μg/mL erythromycin (Em), and

Table 1. Identification of putative autolysin genes in *C. acetobutylicum* through bioinformatics analysis

Autolysin families	*B. subtilis* 168	*C. acetobutylicum* DSM 1731		
	8 query genes	9 subject genes	Maximal similarity[a]	3 paralog genes
Endopeptidases (KO7052)	BSU06010 BSU23010 BSU40830	SMB_G2513 SMB_G0078 SMB_G0493 SMB_G0429 SMB_G3064	24.4% 24.2% 22.2% 23.6% 24.9%	
Glucosaminindases (KO1227)	BSU35780	SMB_G2359	39.3%	
N-acetylmuramoyl-L-alanine amidases (KO1449)	BSU02600 BSU13820 BSU22930	SMB_G0700 SMB_G3117	34.2% 22.6%	 SMB_G3128
Muramidases	BSU14071	SMB_G1126	23.7%	SMB_G0566 SMB_G1901

[a]Similarity of amino acid sequence.

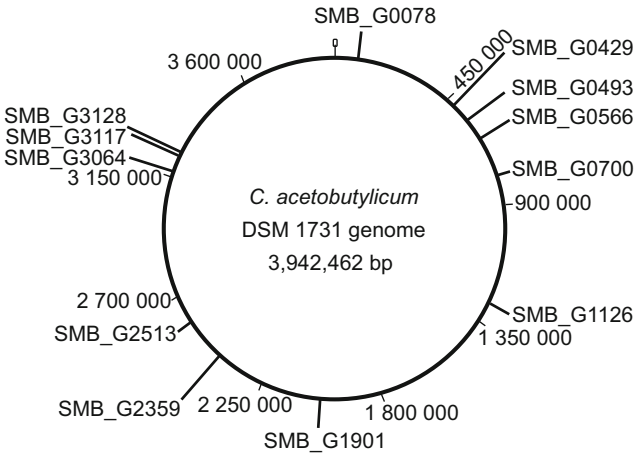

Figure 1. The distribution of 12 putative autolysin genes in *C. acetobutylicum* DSM 1731 genome.

100 ng/mL anhydrotetracycline (aTc) for inducible expression. The results (Fig. 2A) showed that only inducible expression of gene SMB_G2359 (Fig. 2A-a) and SMB_G3117 (Fig. 2A-b) led to a 53.4% and 72.3% decrease of OD_{600}, respectively, as compared to that of the control. We also observed that inducible expression of SMB_G2359 and SMB_G3117 at OD_{600} = 0.5 (around 6 h) (Fig. 2B) led to a 13% and 10% decrease of OD_{600}, while the non-induced strain (Fig. 2C) showed the same OD_{600}, as compared to the control. This suggests that gene SMB_G2359 and SMB_G3117 might play a role in cell lysis.

To investigate the specific function of the selected gene SMB_G2359 and SMB_G3117, attempts to disrupt these genes in *C. acetobutylicum* DSM1731 were made using ClosTron pMTL008 vector (Dong et al., 2010; Heap et al., 2010). To disrupt gene SMB_G2359, three targeted intron insertion sites (732/733s, 867/868s, and 1292/1293s) were tested, respectively. For each target site, more than 200 integrants were screened through colony PCR (Table S1). Unexpectedly, no positive integrants were obtained, suggesting that gene SMB_G2359 might be essential for cell growth. Gene SMB_G3117 was successfully disrupted, generating mutant strain KO3117. Therefore, gene SMB_G3117 was selected for further investigation.

Functional analysis of gene SMB_G3117 in *C. acetobutylicum* DSM 1731

To further investigate the function of gene SMB_G3117, we compared the autolysis related features of the SMB_G3117 disrupted strain KO3117, the SMB_G3117 inducibly expressed strain DSM 1731 (p3117), with their control strains wild type strain DSM 1731 and strain DSM 1731 (p2tetO1), respectively. The tested features include intracellular autolysin activity characterized as the decrease of OD_{600} in sodium phosphate buffer, growth profile (increase of OD_{600}), viable cell counts (spreading plates and colony counting), and cellular morphology (TEM observation).

Compared to wild type strain DSM 1731, the intracellular autolysin activity of the mutant strain KO3117 decreased by 37.7% (Fig. 3C). Nevertheless, no significant difference was observed in growth profile, viable cell numbers (Fig. 3A and 3B), and cellular morphology (Fig. 4C and 4D) between these two strains. We examined the dynamic transcriptional profile of *C. acetobutylicum* ATCC 824 published in a previous study (Jones et al., 2008) and calculated the expression level of relevant genes. The expression level of the gene CA_C3081 in strain ATCC 824 (same to SMB_G3117 in strain DSM 1731; the genome of strain ATCC 824 and DSM 1731 are highly similar (Bao et al., 2011)) was 5-fold lower than the average transcription level of all genes during vegetative growth (Fig. 5). This suggests that the expression level of SMB_G3117 might be quite low, so that the disruption of this gene in wild type did not exhibit clear phenotype.

Clear autolysis related phenotypes were observed in strain DSM 1731 (p3117). Growth profile assay also revealed that the cease of growth of strain DSM 1731 (p3117) occurred at 12 h, which was 12 h ahead of the control strain (Fig. 3D). In addition, the viable cell numbers of strain DSM 1731 (p3117) were 15-fold less than its control strain (Fig. 3E). Moreover, TEM analysis (Fig. 4A and 4B) showed that electrolucent cavities, which were termed as “nuclear vacuoles” previously (Eltsov and Zuber, 2006), were clearly observed in strain DSM 1731 (p3117) at 12 h, in sharp contrast with that of the control strain. We also observed that the intracellular autolysin activity of strain DSM 1731 (p3117) was increased by 14.0% (Fig. 3F), not as high as expected though. All these observations conceive that SMB_G3117 overexpression led to significant cellular autolysis during vegetative growth.

DISCUSSION

In this study, two genes SMB_G2359 and SMB_G3117 that might contribute to autolysis of *C. acetobutylicum* were identified from twelve putative autolysin genes under the available experimental conditions. Gene SMB_G2359, annotated as N-acetylglucosaminidase domain- and ChW repeat-containing cell wall hydrolase (encoded by *lytD* gene), is the only predicted autolysin gene in *LytD* glucosaminidase family. In *B. subtilis*, only one *lytD* gene was identified and proved to be one of the major autolysins. However, the *lytD* mutant of *B. subtilis* still exhibits glucosaminidase activity, suggesting alternative glucosaminidase genes may exist in the genome (Rashid et al., 1993). The failure to disrupt SMB_G2359 in this work may indicate that this gene is the only one responsible for glucosaminidase activity in *C. acetobutylicum*.

Inducible expression of gene SMB_G3117 led to severely weakened cell viability and integrity, and caused cell autolysis. However, disruption of this gene in *C. acetobutylicum* DSM1731 did not exhibit growth difference compared to wild type strain. This suggests that the expression of gene SMB_

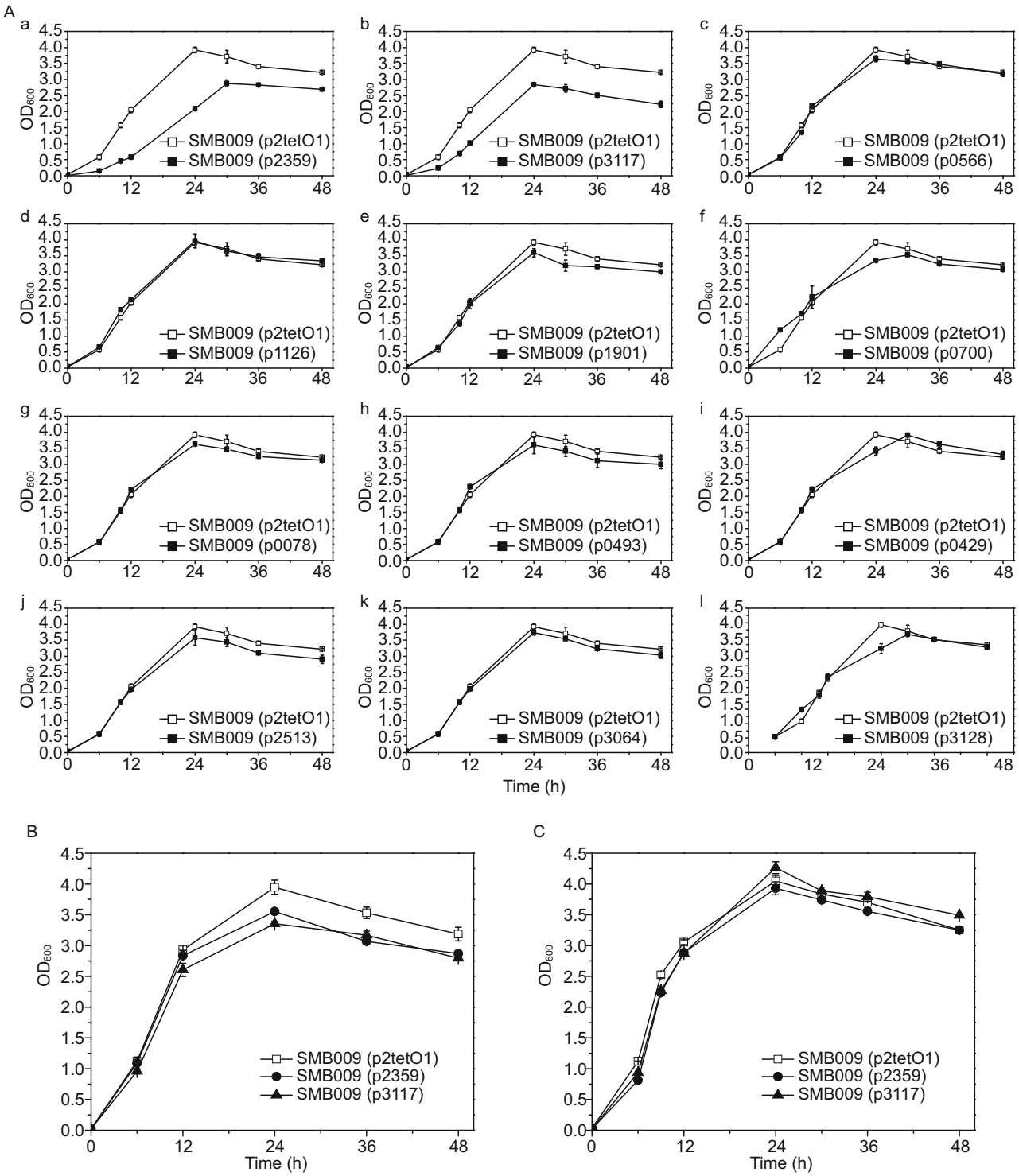

Figure 2. Growth profiles of aTc-inducible expression of predicted autolysin relevant genes in CGM medium. (A) Twelve predicted autolysin relevant genes with addition of aTc at 0 h. (B) Two critical autolysin relevant genes with addition of aTc at OD_{600} = 0.5. (C) Two critical autolysin relevant genes without addition of aTc. Values are mean of triplicates and error bars represent standard deviations (n = 3).

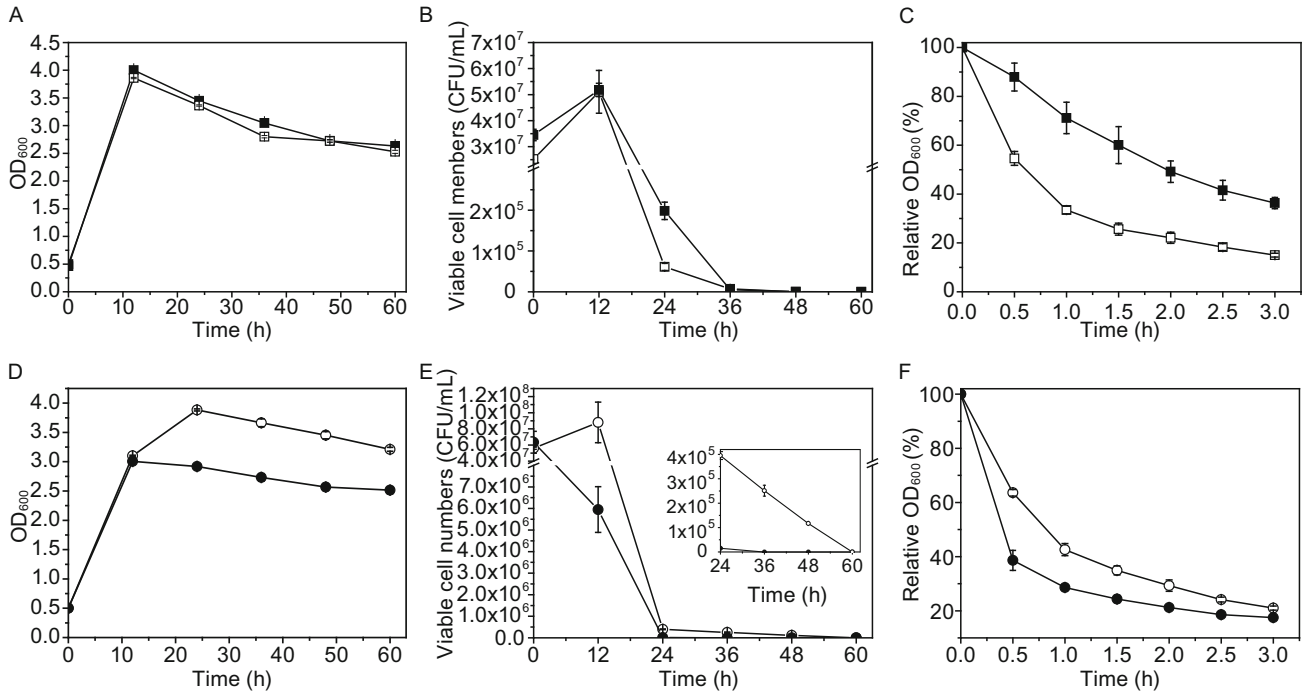

Figure 3. Functional analysis of gene SMB_G3117 in *C. acetobutylicum* DSM 1731. Effect of disruption of gene SMB_G3117 on growth (A), viable cell numbers (B), and intracellular autolysin activities (C); Effect of inducible expression of gene SMB_G3117 on growth (D), viable cell numbers (E), and intracellular autolysin activities (F). Symbols: ■, KO3117; □, DSM 1731; ●, DSM 1731 (p3117); ○, DSM 1731 (p2tetO1). Values are mean of triplicates and error bars represent standard deviations (n = 3).

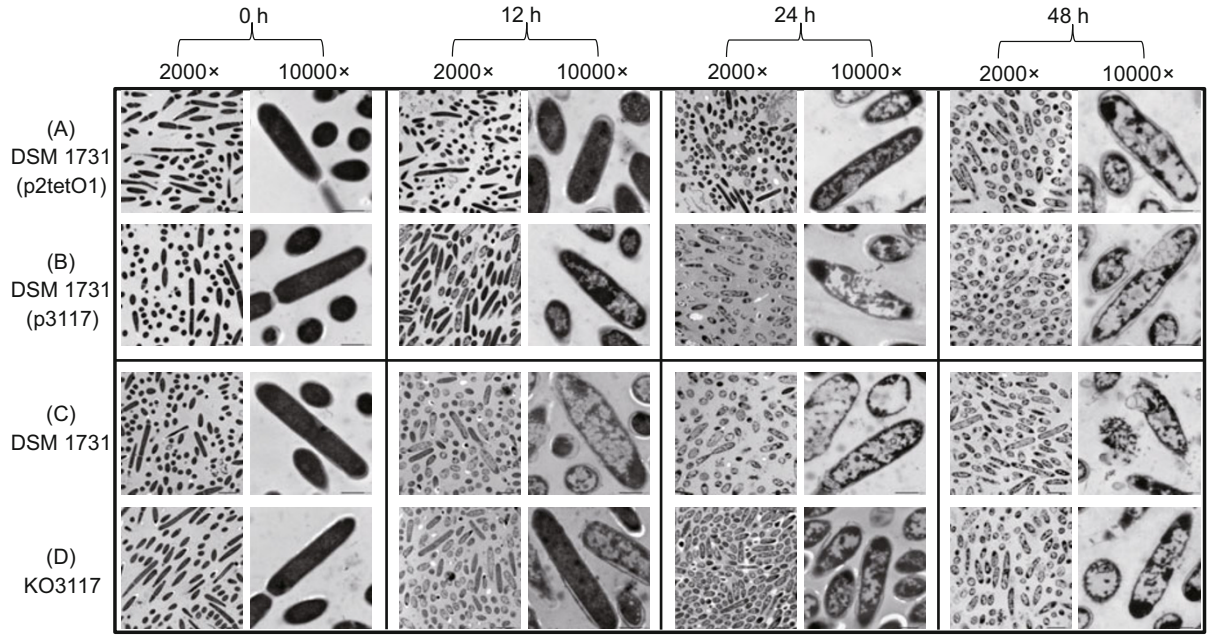

Figure 4. Effect of disruption or overexpression of gene SMB_G3117 on cellular morphology. The dark particles (0–12 h) are ribosomes and the ribosome-free spaces contain chromatin (Levin-Zaidman et al., 2000). Electrolucent cavities (Eltsov and Zuber, 2006), the so-called "nuclear vacuoles", were present at the post-exponential phase of growth (12–24 h). Typical endospore was formed with a developing endospore and electron translucent granulose was still visible at the end phase of growth (24–48 h). Scale bars for 2000× panels, 2.0 μm; scale bars for 10,000× panels, 500.0 nm.

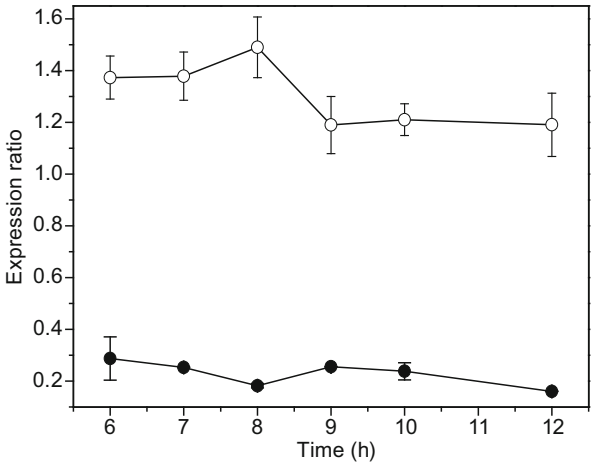

Figure 5. Transcriptional profiles for CA_C3081 in *C. acetobutylicum* ATCC 824 (same to SMB_G3117 of *C. acetobutylicum* DSM 1731) based on time-series microarray analysis of a published paper (Jones et al., 2008). Data from this paper was used to interrogate the transcript abundance of CA_C3081, with the average abundance of all transcripts calculated as a reference. Symbols: ●, the expression ratios of CA_C3081; ○, the average of expression ratios of all genes.

G3117 is tightly regulated to avoid overexpression to a lethal level. Although gene SMB_G3117 is annotated as spore-cortex-lytic enzyme, a type of N-acetylmuramoyl-L-alanine amidases, our results showed that overexpression of gene SMB_G3117 triggered autolysis of *C. acetobutylicum* during vegetative growth. In another recent study, a novel *Streptococcus* suis gene *atl* containing one N-acetyl-muramoyl-L-alanine amidase domain, also exhibited autolysis activity in conditions consistent with our study, and was proved to take part in cell autolysis and separation of daughter cells (Ju et al., 2012). This suggests that the autolysis function of SMB_G3117, as revealed in this study, might be an important part of the life cycle of *C. acetobutylicum* cells that is worthy of further investigation.

MATERIALS AND METHODS

Strains, plasmids and culture conditions

The bacterial strains and plasmids used in the present study are listed in Table 2. *Escherichia coli* strains were routinely grown aerobically at 37°C and 200 r/min in liquid LB medium or solid LB with agar (1.5%) medium supplemented, when necessary, with ampicillin (100 mg/mL) and/or chloramphenicol (Cm, 30 mg/mL). All *C. acetobutylicum* strains were grown anaerobically at 37°C in liquid RCM medium (Hirsch and Grinsted, 1954) or solid RCM with agar (1.5%) medium supplemented,

Table 2. Strains and plasmids used in this study

Strain or plasmid	Relevant characteristics[a]	Source or reference[b]
Strains		
C. acetobutylicum		
DSM 1731	Wild type	DSMZ
SMB009	CAC1502::intron	Dong et al., 2010
DSM 1731 (p2tetO1)	Plasmid control strain of DSM 1731, containing control vector p2tetO1	This study
DSM 1731 (p3117)	Inducible expression strain of DSM 1731, containing gene SMB_G3117 expression vector p3117	This study
KO3117	SMB_G3117::intron	This study
E. coli		
DH5α	General cloning host strain	TaKaRa
ER2275	*hsdR mcr recA1 endA1*	NEB
TOP10	*mcrA Δ(mrr-hsdRMS-mcrBC) recA1*	Invitrogen
Plasmids		
pAN1	*Φ3tI* gene, *p15a ori*, Cm^r	Mermelstein et al., 1992
pGusA2-2tetO1	Amp^r, MLS^r, aTc-inducible gene expression vector containing *gusA* gene	Dong et al., 2012
p2tetO1	Control vector by removing the *gusA* gene and self-ligation	This study
p3117	pGusA2-2tetO1 containing SMB_G3117 gene (replacing *gusA*)	This study
pAN2	*Φ3t I* gene, *p15a ori*, Tet^r	Heap et al., 2007
pMTL008	ClosTron vector	Dong et al., 2010
pMTL008-3117	For SMB_G3117 gene disruption by group II intron method	This study

[a] Cm^r, chloramphenicol/thiamphenicol resistance; Amp^r, ampicillin resistance; MLS^r, macrolide-lincosamide-streptogramin resistance; *Φ3t I* gene, Φ3T I methyltransferase gene of *Bacillus subtilis* phage Φ3T I.

[b] DSMZ, Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany; NEB, New England Biolabs, Beverly, MA.

when necessary, with chloramphenicol (Cm, 30 mg/mL), erythromycin (Em, 50 mg/mL) and anhydrotetracycline (aTc, 100 ng/mL). The CGM medium (Hartmanis and Gatenbeck, 1984) was also used for *C. acetobutylicum* growth. All *C. acetobutylicum* and *E. coli* strains were maintained frozen in 15% (*v/v*) glycerol at –80°C.

Construction of gene SMB_G3117 inducible expression strains

The primers used to amplify these 12 genes are listed in Table S1. Twelve inducible expression vectors were constructed by replacing *gusA* gene with corresponding putative autolysin genes in pGusA2-2tetO1 using restriction enzyme *Spe*I (or *Nco*I) and *Xho*I. The control plasmid p2tetO1 was generated by removing *gusA* gene in pGusA2-2tetO1 using the same restriction enzyme digestion, end-filling using high fidelity DNA polymerase, and then self-ligation. Inducible-autolysin-expression strains were obtained by electrotransforming the respective 12 plasmids into *C. acetobutylicum* SMB009 according to the protocol developed previously (Mermelstein et al., 1992).

Construction of gene SMB_G3117 disrupted mutant

Retargeted gene ClosTron plasmids were constructed as previously described using plasmid pMTL008 (Dong et al., 2010). The primers used for the construction of these plasmids are listed in Table S1. The resulted plasmid, designated as pMTL008-3117, was confirmed by DNA sequencing using primer 14-007-R1. pMTL008-3117, methylated by co-transforming pAN2 into *E. coli* TOP10, was then electrotransformed into the strain DSM1731, resulting in strain KO3117. Integrants were selected on the basis of acquisition of chloramphenicol resistance, and then reselected again through erythromycin resistance. The positive integrant was screened through a number of diagnostic PCR and further confirmed by three pairs of primers (Fig. S1). Finally, the fragments generated were subject to nucleotide sequence analysis to definitively confirm that insertion had occurred at the desired position.

Growth measurement

Cell optical densities at 600 nm were measured dynamically using a UV-visible spectrophotometer (UV-2802PC, Unico, Shanghai, China). Samples were diluted in the appropriate medium to ensure an absorbance below 0.50.

Viable cell counts assay

A CFU (colony-forming unit) assay is employed to count the viable cell numbers (Zingaro and Terry Papoutsakis, 2012). The unit of measurement is CFU/mL. Strains were cultivated in CGM medium supplemented with 50 μg/mL erythromycin (Em), and 100 ng/mL aTc for inducible expression. Samples from different growth phases were serially diluted in sterile liquid media. Dilution level was determined based on A_{600} measurements of the samples. One hundred microliter diluted broth was taken from dilution series and spread onto agar solidified CGM plate containing appropriate antibiotics. Viable cell numbers were calculated after incubating anaerobically at 37°C for 24 h.

Intracellular autolysin activity of the inducible expression and disruption of gene SMB_G3117

Intracellular autolysin activity assay of the inducible-expression and disruption of gene SMB_G3117 was performed according to modified procedures (Croux et al., 1992a). Exponential phase cells (OD_{600} = 2.0) were harvested by centrifugation (12,000 *g*, 5 min, 4°C) and after the supernatant fluid had been discarded, residual growth medium was removed with cotton tips. The pellet was washed once with 0.1 mol/L sodium phosphate buffer (pH 6.3). The washed pellet was then diluted to an initial OD_{600} of 1 into preincubated (37°C) 0.1 mol/L sodium phosphate buffer (pH 6.3). Note that 100 ng/mL aTc was added in the inducible-expression group. The decreases in OD_{600} were monitored at 37°C every 0.5 h.

Cellular morphology of the inducible expression and disruption of gene SMB_G3117 through TEM observation

All strains were cultivated as mentioned in growth and viable cell counts assay. Cells were collected by centrifugation (12,000 *g*, 5 min, 4°C) at different phase of growth (0 h, 12 h, 24 h, 48 h) then fixed instantly by adding 2.5% (*v/v*) glutaraldehyde for more than 2 h, respectively. Samples for TEM were prepared as described previously (Jones et al., 2008; Zhang et al., 2010). Prepared cell thin sections were examined with a transmission electron microscope (JEM-1400; JEOL Ltd., Japan) operating at an acceleration voltage of 80 kV.

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COMPLIANCE WITH ETHICS GUIDELINES

Liejian Yang, Guanhui Bao, Yan Zhu, Hongjun Dong, Yanping Zhang and Yin Li declare that they have no conflict of interest.

This article does not contain any studies with human or animal subjects performed by the any of the authors.

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