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Development of Real-Time PCR Primer and Probe Sets for Detecting Degenerated and Non-degenerated Forms of the Butanol-Producing Bacterium *Clostridium acetobutylicum* ATCC 824

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Abstract Degeneration is one of the limiting factors in butanol fermentation, and it must be monitored and prevented for stable butanol production. In *Clostridium acetobutylicum* ATCC 824, the most well-known butanol-producing microorganism, degeneration is caused by the loss of the pSOL1 plasmid that carries essential genes involved in solvent production. In this study, we designed two specific primer and probe sets for real-time qPCR (RT-qPCR) detection of *C. acetobutylicum* ATCC 824 (the C. aceto set) and pSOL1-possessing *C. acetobutylicum* ATCC 824 (the DGS set). Specific primer and probe sets were designed on the basis of the 16S rDNA sequence and pSOL1 sequence. The number of degenerated *C. acetobutylicum* could be quantified by subtracting the number of *C. acetobutylicum* ATCC 824 containing pSOL1 from the total number of *C. acetobutylicum* ATCC 824. The primer and probe sets permitted the specific detection and quantification of degenerated *C. acetobutylicum* and total butanol-producing *C. acetobutylicum* by RT-qPCR.

Keywords Biobutanol · Degeneration · Monitoring · pSOL1 plasmid · Real-time qPCR

Introduction

Bioenergy has attracted the attention of many researchers because it provides solutions for environmental problems and the energy crisis. Biobutanol is one of the most promising biofuels due to its gasoline-like properties and the wide variety of its feedstock. Biobutanol has advantages over bioethanol for gasoline–alcohol blending due to its high energy content, low miscibility with water, and low volatility. This makes it possible for butanol to

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replace gasoline without any modification of the current vehicle and engine technologies. In addition, biobutanol can be produced from renewable resources such as agricultural wastes and energy crops. Moreover, butanol has a broad range of applications in a variety of industries, including fuel, food, cosmetics, and chemicals [1].

Biobutanol is produced by solventogenic clostridia during acetone–butanol–ethanol (ABE) fermentation. Solventogenic clostridia such as *Clostridium acetobutylicum* utilize sugar as a substrate and produce acids that include acetic acid and butyric acid during the initial phase of exponential growth [2]. When the concentration of these acids (or the culture pH) has reached a certain threshold, clostridia switch to solvent production [1, 3]. However, solventogenic clostridia lose their solvent formation capabilities during repeated sub-culturing of batch cultures and during continuous fermentation. This phenomenon is known as "degeneration", and it is one of the most important factors for optimizing butanol production [1, 4]. Therefore, a method for detecting and quantifying degenerated cells will enhance and stabilize butanol production during continuous fermentation.

During the last decade, researchers have been studying the phenomenon of degeneration during ABE fermentation and the genes related to it [5–7]. Cornillot et al. reported that the degeneration of *C. acetobutylicum* ATCC 824, the most well-known butanol-producing bacteria, is related to the loss of the pSOL1 plasmid [8]. Because the pSOL1 plasmid has all of the genes (including *ctfA*, *ctfB*, *adc*, and *aad*) that are related to solvent formation, its loss leads to the degeneration of *C. acetobutylicum* ATCC 824 and the loss of its solvent production capabilities [4, 5, 8]. Therefore, the detection and quantification of the pSOL1 plasmid should be one of the approaches for monitoring degenerated *C. acetobutylicum*. In this sense, Sabathe et al. investigated *amyP*, a gene in the pSOL1 plasmid that encodes an extracellular α-amylase that is transcribed during solventogenesis. They showed that *amyP* could be a good marker for detecting the degeneration of butanol-producing bacteria [4]. Schuster et al. developed a method for monitoring the degeneration of solventogenic *Clostridium* strains using Fourier transform infrared spectroscopy of bacterial cells [2]. This monitoring method is relatively complicated and inadaptable for the quantification of degeneration.

Therefore, the objective of this study is to develop a rapid and simple RT-qPCR method for detecting and quantifying the degeneration of *C. acetobutylicum*. RT-qPCR can be used for microbial quantification in a variety of research areas. It offers highly reliable and sensitive results. In addition, RT-qPCR is faster and easier than conventional microbial quantification methods [9–11]. We designed primer and probe sets that are specific for the pSOL1 plasmid and *C. acetobutylicum*, respectively, to detect and quantify degenerated *C. acetobutylicum* with RT-qPCR. In addition, we developed a method for monitoring degenerated *C. acetobutylicum* with multiplex RT-qPCR.

Materials and Methods

Design of Primer and Probe Sets We designed the *C. acetobutylicum* ATCC 824-specific primer and probe set (C. aceto set) based on the 16S rDNA sequence of *C. acetobutylicum* ATCC 824. The pSOL1-specific primer and probe set (DGS set) was designed with the sequence of the pSOL1 plasmid using PRIMER EXPRESS (Applied Biosystems). Among possible candidate primer and probe sets, the C. aceto and DGS sets were selected by following the guidelines provided by the manufacturer of the RT-qPCR equipment (Applied Biosystems). The specificity of the C. aceto set was tested using the PROBE MATCH program on the RDP-II.

The T_m of the TaqMan probes were designed to be about 10 °C higher than the T_m of the two primers in the corresponding sets. The G+C contents were between 40% and 70%. TaqMan probes with a G residue on the 5′ end were avoided to prevent quenching.

Microorganisms and Media All of the strains except M5 were purchased from the Korean Collection for Type Cultures (KCTC); M5, a degenerated mutant of *C. acetobutylicum* originated from *C. acetobutylicum* ATCC 824, was purchased from the Delaware Biotechnology Institute. The strains used in this work to test the specificity of the primer and probe sets were *C. acetobutylicum* ATCC 824, degenerated *C. acetobutylicum* (M5), *Clostridium tyrobutylicum* ATCC 25755, *Clostridium saccharoperbutylacetonicum* N-14, *Clostridium beijerinckii* NCIMB 8052, *Clostridium butyricum* DSM 2477, *Clostridium pasteurianum* DSM 525, and *Clostridium diolis* DSM 15410. Each strain was inoculated into appropriate media according to KCTC instructions or into modified CAB media [1].

Extraction of Genomic DNA and Construction of Plasmid DNA for Standards After overnight incubation, cells from 2 ml of culture medium were harvested by centrifugation at 13,000 rpm for 5 min followed by decantation of the supernatant. The pellet was resuspended in 1 ml of sterilized distilled water and was centrifuged again. This washing step was done two times to ensure removal of residual medium. The Power soil kit (Mobio) was used to extract DNA from harvested cells. DNA was extracted as per the manufacturer's instruction. RT-qPCR standards were prepared as plasmid DNA to decrease the impact of complex genomic DNA on RT-qPCR efficiency [10]. Purified DNA of *C. acetobutylicum* was amplified using 16S rDNA PCR primers and AmyS primers: 27f (5′-AGAGTTTGATCMTGGCTCAG-3′) and 1492r (5′-GGTTACCTTTGTTACGACTT-3′) for the C. aceto set and AmyS1 (5′-GGTGTTATGCTTCATGCTTTTGACTGG-3′) and AmyS2 (5′-ATCAAAACGAAAACCGGTTGCACC-3′) for the DGS set. PCR conditions were as follows: 1 μl of template DNA, 20 pmol of each PCR primer, and 27 μl of Platinum Blue PCR Supermix (Invitrogen). The amplification was performed with one denaturation at 94 °C for 2 min, followed by 30 cycles of denaturation at 94 °C for 1 min, annealing at 58 °C for 1 min, and extension at 72 °C for 1 min with a final extension of 10 min. Amplified PCR products were cloned into a TOPO vector (Invitrogen) according to the procedure recommended by the manufacturer and then used as a standard for RT-qPCR. The concentration of the standard plasmid was measured with a fluorimeter (BioRad) with PicoGreen dsDNA quantification reagent (Molecular Probes).

RT-qPCR To evaluate the specificity of the designed primer and probe sets, the DNA of the clostridia strains mentioned above was amplified using RT-qPCR. TaqMan probes were labeled with a fluorescent reporter dye (FAM or VIC) and a fluorescent quencher dye (TAMRA) at the 5′ end and 3′ ends, respectively. RT-qPCR was performed using a 7300 Real-Time qPCR System (Applied Biosystems). PCR conditions were as follows: 1 μl of template DNA, 9 pmol of each PCR primer, 2.5 pmol of each probe, and 12.5 μl of TaqMan universal PCR master mix (Applied Biosystems). The amplification was done with one pre-amplification step at 50 °C for 2 min, one denaturation step at 95 °C for 5 min, followed by 40 cycles of denaturation at 95 °C for 15 s, annealing and extension at 60 °C for 1 min. Different initial 16S rDNA and *amyP* gene copy numbers in the range of eight orders of magnitude were amplified by RT-qPCR with the C. aceto and DGS sets, respectively. Logarithmic values of the different DNA amounts were plotted against the threshold cycle (C_t) from the RT-qPCR assay. The qPCR efficiency (E) of the sets was calculated using the equation $E=[10^{(-1/\text{slope})}-1]$. An efficiency of 1 means a doubling of product in each cycle [12].

Table 1 Primer and probe sets designed for RT-qPCR assays targeting *C. acetobutylicum* (C. aceto set) and the pSOL1 plasmid (DGS set).

Name	Function	Target DNA	Sequence (5′→3′)	T_m(°C)	GC(%)	Amplicon size (bp)
C. acetof	F primer	*C. acetobutylicum*	GCCAAAGGATTTATTCGCTATGA	58	39	
C. aceto	Probe		ACCCGCGGCGCATTAGCTTGT	68	62	74
C. acetor	R primer		GCCTTGGTGAGCCGTTACC	59	63	
pSOL1 125f	F primer	pSOL1 plasmid	CGAATTCTTCTGACTGGTGGCTAT	60	46	
DGS	Probe		CAGCCAACCAATCAAGCTATAGGAAATGCTC	68	45	86
pSOL1 210r	R primer		TTTGAAATCATCGTAACTCCCAAGT	59	36	

Results and Discussion

Design of Specific Primer and Probe Sets C. acetobutylicum ATCC 824 has a 210-kb plasmid (pSOL1) encoding several solventogenic genes, and the loss of this plasmid causes degeneration. Therefore, it is possible to evaluate the portion of degenerated *C. acetobutylicum* by measuring the respective numbers of *C. acetobutylicum* ATCC 824 and pSOL1 plasmid.

We designed primer sets (C. aceto and DGS) to detect and quantify *C. acetobutylicum* and non-degenerated *C. acetobutylicum* by RT-qPCR. The C. aceto set was designed from the 16S rDNA sequence of *C. acetobutylicum* ATCC 824. The DGS set was designed from the sequence of the *amyP* gene. The *amyP* gene, which encodes α-amylase, is located in pSOL1 and is transcribed during solventogenesis [4].

Table 1 shows the characteristics of the C. aceto set and the DGS set. The T_m of the forward and reverse primers ranged from 58 to 60 °C, and the T_m of both probes was 68 °C, which meets the design criteria. It is usually recommended that the difference in T_m between the primers and probe for RT-qPCR should be approximately 10 °C. The G+C contents were in the range of 36–63%, which is in the recommended range of 30–80%. The amplicon sizes for the C. aceto set and the DGS set were 74 and 86 bp, respectively. According to the manufacturer's instructions, effective RT-qPCR amplification can be conducted with amplicons ranging from 50 to 150 bp.

To verify the specificity of the C. aceto and DGS primer sets, RT-qPCR amplification was conducted using clostridia DNA as a template. Figures 1 and 2 show the changes in the intensity of the fluorescence signals during RT-qPCR amplification of clostridia DNA using the C. aceto and DGS primers. The strains used for the specificity tests were *C. acetobutylicum* ATCC 824, M5, *C. tyrobutylicum* ATCC 25755, *C. saccharoperbutylacetonicum* N-14, *C. beijerinckii* NCIMB 8052, *C. butyricum* DSM 2477, *C. pasteurianum* DSM 525, and *C. diolis* DSM 15410.

In the RT-qPCR test of the C. aceto set, 16S rDNA from *C. acetobutylicum* ATCC 824 and M5 was specifically detected (Fig. 1). Some strains, including *C. tyrobutylicum* and *C. butyricum*, were detected as false-positives at late stages of amplification. However, the signal intensities were too low to impact the quantification of *C. acetobutylicum*. The levels of the false-positive signals were less than 0.0001% of the *C. acetobutylicum* signal.

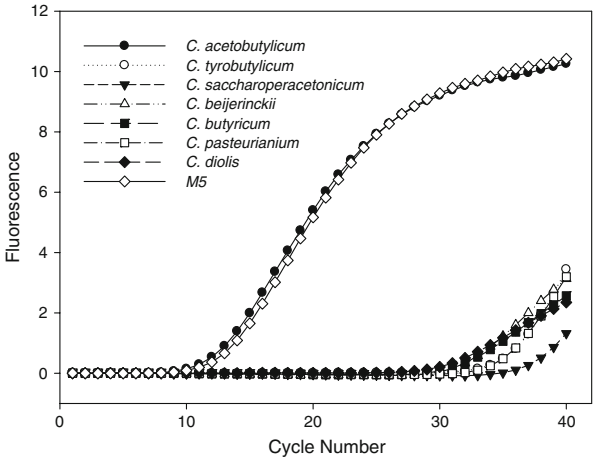

Fig. 1 Specific detection of *C. acetobutylicum* by RT-qPCR with the C. aceto set

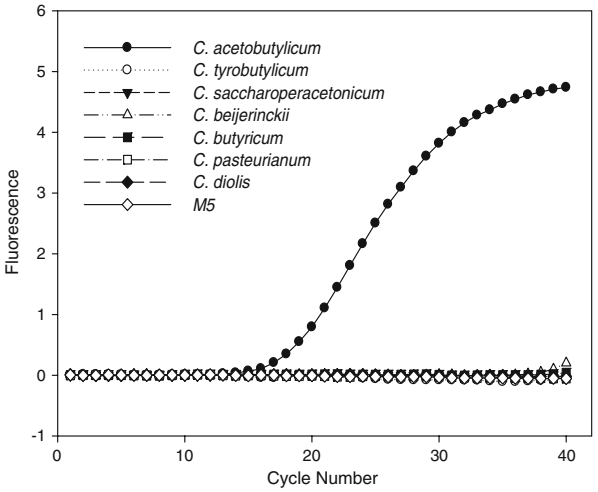

Fig. 2 Specific detection of *C. acetobutylicum* by RT-qPCR with the DGS set

In the specificity test of the DGS primers, *C. acetobutylicum* was the only strain amplified by RT-qPCR. The DGS primers did not detect any of the non-targeting strains. Degenerated *C. acetobutylicum* (M5) was not detected because of the absence of the pSOL1 plasmid (Fig. 2). Based on these results, we concluded that the newly designed primer and probe set successfully detects non-degenerated cells that possess the pSOL1 plasmid.

RT-qPCR Assay with the C. aceto and DGS Primers RT-qPCR was conducted with the C. aceto and DGS primers and 10-fold serially diluted standard DNA samples to quantify *C. acetobutylicum* and pSOL1 (Table 2). Logarithmic values of the different DNA amounts were plotted against the threshold cycle (C_t).

In the RT-qPCR test with the C. aceto primer set, the linear ranges of the standard curves were from 3.6 to 3.6×10^8 copies/μl, and the r^2 of the slope was greater than 0.997. The slope was −3.28 with a standard deviation of 0.079. The efficiency of the RT-qPCR reaction (E) with the C. aceto primers was 1.01, which is nearly a perfect score. In general, the efficiency of perfect RT-qPCR amplification is 1.0 [13]. The coefficient of variation (CV) was 2.4%, indicating that the C. aceto primers adequately detected and amplified 16S rDNA of *C. acetobutylicum* ATCC 824 with high reliability [11].

Table 2 Standard curves from RT-qPCR assays with the C. aceto and DGS primer sets.

Parameter	C. aceto set	DGS set
Linear range (rRNA gene copies)	$3.6–3.6\times10^8$	$3.6–3.6\times10^8$
Slope (average)	−3.28	−3.63
Slope (standard deviation)	0.079	0.010
Slope (r^2)	0.997	0.998
CV(%)	2.4	0.3
Intercept	38.28	42.59
Efficiency	1.01	0.89
Target	*C. acetobutylicum*	pSOL1 plasmid

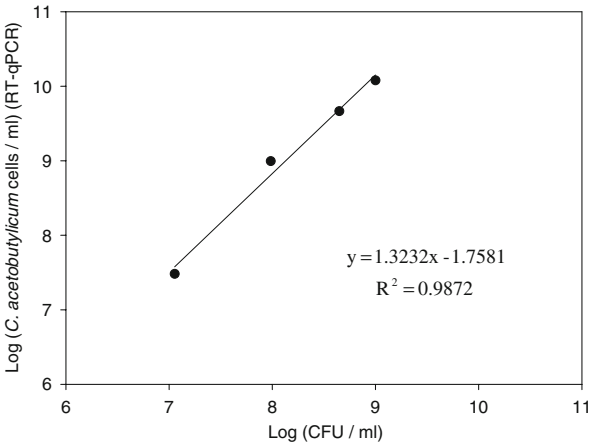

Fig. 3 Relationship between the results of quantification of *C. acetobutylicum* by RT-qPCR and plate counting

Standard curves for the DGS set were also constructed in the range from 3.6 to 3.6×10^8 copies/μl. The curves were linear within this range, and the r^2 of the slope was greater than 0.998. The slope of the standard curve for the DGS set was −3.63, which corresponds to an efficiency of 0.89, and the standard deviation was 0.010. Though the efficiency of RT-qPCR with the DGS primer set was slightly lower than with the C. aceto set, the reproducibility of the reaction was very high; i.e., the variability of the slope for each reaction was negligible. In addition, the efficiency of RT-qPCR with the DGS primers could be increased by optimization of the reaction. Therefore, it is possible to use the DGS primer set to detect and quantify degenerated *C. acetobutylicum* ATCC 824 consistently and reliably.

The efficiency of newly developed quantification method was examined by comparing the numbers of cells estimated by RT-qPCR analysis and the CFU by plate counting during a batch cultivation of *C. acetobutylicum*. The copy number of cells obtained from the RT-qPCR reaction was converted to the number of cells based on the 16S rDNA copy number

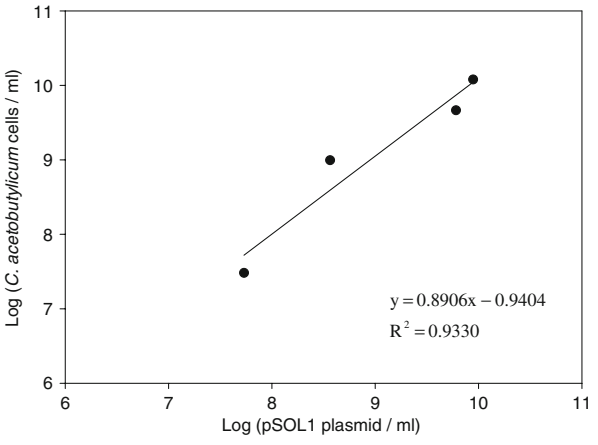

Fig. 4 Relationship between the results of quantification of non-degenerated *C. acetobutylicum* and pSOL1 plasmid by RT-qPCR

of *C. acetobutylicum* on rRNDB. The number of cells based on RT-qPCR analysis revealed a reliable correlation to the CFU by plate counting (Fig. 3). Though the number of cells by RT-qPCR was slightly higher than the CFU due to a high efficiency of RT-qPCR with C. aceto set, the quantification results using two methods were specifically correlated as shown in Fig. 3. The linear regression between two results was obtained with a slope of 1.32 (R^2= 0.987). As shown in Fig. 4., the quantification of non-degenerated *C. acetobutylicum* containing pSOL1 plasmid using RT-qPCR was also specifically correlated to the CFU by plate counting with a slope of 0.890 (R^2=0.933). To get more accurate quantification results, it could be possible to use conversion factor for the RT-qPCR analysis with C. aceto and DGS set.

In this study, we designed specific primer and probe sets for detecting and quantifying one of the most well-known biobutanol-producing microorganisms, *C. acetobutylicum* ATCC 824, and its pSOL1 plasmid. These primer and probe sets showed high specificity for the target strain, and the RT-qPCR reaction was efficiently conducted with high consistency. These newly developed RT-qPCR primer and probe sets (C. aceto and DGS) could be used to monitor the degeneration of *C. acetobutylicum* during biobutanol production. In addition, this monitoring technique could be a useful tool for developing techniques to prevent degeneration and thereby stabilize and enhance butanol production.

Conclusions

We designed RT-qPCR primer and probe sets (C. aceto and DGS) that are specific for *C. acetobutylicum* and its pSOL1 plasmid, respectively. By using RT-qPCR, we confirmed that the newly designed primer and probe sets successfully detect and quantify *C. acetobutylicum* and pSOL1. RT-qPCR assays with these primers showed a wide detection range, high efficiency, and reliable consistency. The detection ranges were eight orders of magnitude, and the r^2 of the slope was greater than 0.997. In the RT-qPCR assay with the C. aceto primer set, the PCR efficiency was 1.01, which was very close to the theoretical efficiency value, and the coefficient of variation was 2.4%. These RT-qPCR primer and probe sets could be used to specifically monitor non-degenerated and degenerated *C. acetobutylicum* ATCC 824 during butanol fermentation.

This study provides a method for the convenient, sensitive, and specific detection and quantification of *C. acetobutylicum* ATCC 824 and its degeneration. RT-qPCR assays with the C. aceto and DGS primer sets could serve as an important tool for the development of a technique for preventing degeneration during biobutanol production.

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