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Identification of a Sequence Containing Methylated Cytidine in *Corynebacterium glutamicum* and *Brevibacterium flavum* Using Bisulfite DNA Derivatization and Sequencing

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Abstract The principal DNA modification systems of the amino-acid-producing bacteria *Corynebacterium glutamicum* AS019, *Brevibacterium flavum* BF4, and *B. lactofermentum* BL1 was investigated using two approaches; digestion of plasmid DNA isolated from these species using *Tse*I and *Fnu*4HI, and sequence analysis of the putative methyltransferase target sites following the derivatization of DNA using metabisulfite treatment. The *C. glutamicum* and *B. flavum* strains showed similar digestion patterns to the two enzymes, indicating that the target for cytidine methyltransferase recognizes 5'-GCSGC-3' (where S is either G or C). Mapping the methylated cytidine sites by bisulfite derivatization, followed by PCR amplification and sequencing, was only possible when the protocol included an additional step eliminating any underivatized DNA after PCR amplification, thereby indicating that the derivatization was not 100% efficient. This may have been due to the high G-C content of this genus. It was confirmed that *C. glutamicum* AS019 and *B. flavum* BF4 methylated the cytidine in the Gm^5CCGC sequences, yet there were no similar patterns of methylation in *B. lactofermentum*, which was consistent with the distinctive degradation pattern seen for the above enzymes. These findings demonstrate the successful application of a modified bisulfite derivatization method with the *Corynebacterium* species for determining methylation patterns, and showed that different species in the genus contain distinctive restriction and modification systems.

Key words: Bisulfite DNA modification, *Brevibacterium flavum*, *Corynebacterium glutamicum*, cytidine methyltransferase

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Nonpathogenic corynebacteria, including *Brevibacterium flavum*, *B. lactofermentum*, *B. ammoniagenes*, and *Corynebacterium glutamicum*, have long been used for the industrial production of amino acids and nucleotides [6, 9, 11, 18]. Over the last 20 years, basic genetic tools for this group of bacteria have been developed and applied to clone genes involved in biosynthetic pathways and to improve the synthesis of selected amino acids [7, 10]. Although the application of recombinant DNA techniques to *C. glutamicum* has demonstrated that this bacterium can express foreign genes and produce functional recombinant proteins, several workers have reported poor transformation efficiency when using heterologously-derived DNA [3, 6, 19], indicating the presence of restriction-modification (RM) systems [17].

Evidence of the presence of at least two methyltransferases (MTase) in *C. glutamicum* is supported by several experimental approaches. Tauch *et al.* [17] purified plasmid DNA from *C. glutamicum* and transformed this into several *Escherichia coli* strains with different RM backgrounds, including DH5α and DH5αMCR. On the basis of the transformation frequencies obtained, these authors concluded that *C. glutamicum* DNA is restricted by a modified cytosine restriction (Mcr) system of *E. coli*, thereby inferring that the incoming DNA contained methylated cytidine in GC sequences. Jang *et al.* [5] confirmed this observation and used HPLC analysis of the genomic and plasmid DNA from *C. glutamicum*, *B. flavum*, and *B. lactofermentum* strains to show that both 5-methyl-deoxycytidine (m^5dCyd) and 6-methyl-deoxyadenosine are present, and that m^5dCyd is the dominant methylated base detected. This also indicates the presence of both cytidine and adenosine MTases in all these strains. In addition, the

cutting sites of these MTases were investigated by isolating pCSL17 plasmid DNA [4] from *E. coli* and corynebacterial hosts, and then digesting them with restriction endonucleases (ENases) that either required methylated DNA for activity or were inhibited by methylation at or near their target cutting sites [5]. When the pCSL17 from *C. glutamicum* AS019 [19] and *B. flavum* BF4 [5] were digested with *Hae*III, which cuts at GGCC and is inhibited by methylation at the first C, fewer bands were produced relative to the number seen when this plasmid was purified from *B. lactofermentum* BL1 [3] or several *E. coli* strains. The two *Hae*III sites that were not cleaved were mapped and, from the known sequence data for the plasmid, it could be concluded that *C. glutamicum* methylates specific cytidines in the sequence GGCCGC. In a recent work, Schäfer *et al.* [15] demonstrated that the expression of a MTase gene from *C. glutamicum* ATCC 13032 in *E. coli*, under the control of its own promoter, conferred a *C. glutamicum*-specific methylation pattern to the co-resident shuttle plasmids, which increased the transformation efficiency into *C. glutamicum* by 260-fold. The same authors suggested that this MTase gene is responsible for the observed cytidine MTase activity, although further characterization of the target sequence in either ENase or MTase was not reported. The present paper proves that the sequence GCSGC (wherein, S is either G or C) is one of the target sequences of MTase in *C. glutamicum* AS019 and *B. flavum* BF4, yet not in *B. lactofermentum* BL1. In addition, the cytidine modified in the recognition sequence is identified using metabisulfite derivatization of the cytidine bases in the target sequence plus a subsequent sequence analysis of the PCR amplification products.

MATERIALS AND METHODS

Bacterial Strains, Plasmid, and Growth Conditions

Corynebacteria strains used were: *C. glutamicum* AS019 [19]; *B. flavum* [5]; *B. lactofermentum* BL1 [3]. *E. coli* strain LE392 was used in this work. The plasmid used was pCSL17, a 7.2-kb *E. coli*-corynebacteria shuttle vector [4, 5]. Corynebacteria strains were grown routinely in Luria-Bertani broth (1% tryptone, 0.5% yeast extract, 0.5% NaCl) supplemented with 0.5% (w/v) glucose (LBG) [5]. *E. coli* LE392 was grown in LB at 37°C, and corynebacteria in LBG at 30°C .

DNA Isolation, Methylation, and Restriction Enzyme Analysis

Plasmid DNA was isolated using an alkaline lysis method [3], and further purified using CsCl-EtBr density gradient centrifugation [14]. Restriction endonucleases were purchased from Boehringer Mannheim GmbH Biochemica (Germany), and NEB (Beverly, MA, U.S.A.). For DNA methylation, pCSL17 DNA was methylated using *Hae*III MTase (NEB, U.S.A.). The reaction mixture (60 µl) contained 15 µg of pCSL17 DNA derived from *E. coli* LE392, 80 µM S-adenosylmethionine, 6 µl of a 10× *Hae*III MTase buffer (500 mM NaCl, 50 mM Tris, pH 8.5, 10 mM dithiothreitol) and 50 U of *Hae*III MTase (10 U/µl), and was incubated at 37°C for 3 h. Restriction enzyme digests of DNA were resolved using 0.8% or 1.5% (w/v) agarose gels as described previously [14].

Electroporation Procedures

Cells of *C. glutamicum* AS019 or ATCC 13032 were grown in LBG-GI (LBG medium supplemented with 2% glycine and 4 mg/ml isonicontinic acid hydrazide) at 30°C and harvested at an A_{600} of 0.35–0.45. Transformation by electroporation was based on the protocol described by Haynes and Britz [3] and this was carried out using a Gene-Pulser system (Bio-Rad Laboratories, Richimond, U.S.A.). pCSL17 DNA (0.5 µg) obtained from either *E. coli* LE392, or *Hae*III methylated *E. coli* LE392, or *C. glutamicum* AS019 was added to 40 µl of cells (approximately 10^9 cells) in 15% glycerol prior to pulsing at 2.5 kV and 25 µF (time constant: 4.5–4.9). Following exposure to a single pulse, the cells were immediately removed from the electrodes and stored on ice for 5 min. Subsequently, 1 ml of recovery medium (LBG medium containing 10 mM $CaCl_2$ and 10 mM $MgCl_2$) was added to the cells. Following incubation without shaking for 1 h, at 30°C, 10-fold serial dilutions of cells were made in SMMC [19] and appropriate dilutions spread onto ET plates [3] and ET containing 50 µg/ml of kanamycin (ET-Km) to enumerate transformants. Cell counts were performed in triplicate and average numbers were taken.

Bisulfite Derivatization Reaction

The pCSL17 (2 µg) DNA derived from *C. glutamicum* AS019 or *B. lactofermentum* BL1 was digested with 20 µl of 10 U of *Bgl*II ENase at 37°C for 3 h, and denatured by adding freshly prepared 3 M NaOH to a final concentration of 0.3 M, and then incubated for 15 min at 37°C. At this stage, solutions of 10 mM hydroquinone and 2 M sodium metabisulfite were prepared and adjusted to pH 5.0 with 10 M NaOH. The solution of bisulfite was achieved by gently inverting the reagent/water mixture, and the pH adjusted before all solids were dissolved. Aliquots of 208 µl of sodium metabisulfite plus 12 µl hydroquinone were added to each reaction tube containing denatured DNA to give a final concentration of 0.5 mM metabisulfite. The reaction mixtures were then overlayed with 40 µl of mineral oil and incubated at 55°C, in the dark, for 16 h. The bisulfite-treated DNA was recovered from under the oil layer by pipetting, and the free bisulfite removed by passing the sample through a desalting column (Wizard DNA Clean-Up System, Promega, Madison, WI,

U.S.A.) and eluting with 50 µl of deionized water. Freshly prepared NaOH (3 M) was added to a final concentration of 0.3 M and the sample incubated at 37°C for 15 min after vortexing. The solution was neutralized by the addition of 33 µl of 5 M ammonium acetate (pH 7.0) and the modified DNA was collected by ethanol precipitation (330 µl). The DNA was dried, resuspended in 100 µl of a TE buffer (10 mM Tris·Cl, 1 mM EDTA, pH 8.0), and stored at -20°C.

PCR Amplification of the Target DNA and Sequencing
The 100 µl PCR reaction mixtures contained 5 µl of the bisulfite-treated DNA, 200 µM deoxynucleoside triphosphates, 0.5 µM oligonucleotide primer, 3 mM $MgCl_2$, 50 mM KCl, 10 mM Tris·Cl, pH 8.3, and 0.5 µl (2.5 U) *Taq* DNA polymerase. The following conditions were used: 94°C for 2 min, 1 cycle; 94°C for 1 min, 50°C for 2 min, 72°C for 3 min, 5 cycles; 94°C for 0.5 min, 50°C for 2 min, 72°C for 1.5 min, 25 cycles; 72°C for 6 min, 1 cycle, in a Perkin-Elmer DNA Thermal Cycler 480 (Norwalk, CT, U.S.A.). The sequencing reactions were prepared using an ABI PRISM™ Dye Terminator Cycle Sequencing Ready Reaction Kit (Perkin-Elmer, Norwalk, CT, U.S.A.). Sequence analysis was performed using an ABI 373-Automated DNA Sequencer (Perkin-Elmer, Norwalk, CT, U.S.A.).

RESULTS AND DISCUSSION

Effect of *Hae*III Methyltransferase Treatment of pCSL17 DNA on the Frequency of Transformation
The impact of cytidine methylation in GGCCGC sequences of pCSL17 DNA was investigated by initially extracting pCSL17 DNA from *E. coli* LE392 and methylating this *in vitro* at the first cytidine base in the GGCC sequences by incubating with *Hae*III MTase. The two sources of pCSL17 DNA, the originally-derived one from *E. coli* and its *Hae*III MTase-treated counterpart, were subsequently digested using *Hae*III ENase: the former was cleaved and the latter was not (data not shown), thereby indicating that the *Hae*III MTase methylation was successful. This DNA was then transformed into two strains of *C. glutamicum*, AS019 and ATCC 13032, by electroporation, using pCSL17 from AS019 as the control. When the *E. coli*-derived pCSL17 DNA was methylated, the electrotransformation frequencies for the two *C. glutamicum* strains were approximately four times higher than those observed for the unmethylated *E. coli*-derived plasmid (Table 1), yet not as high as that seen for the homologous DNA. This indicates that the prior methylation of heterologous DNA using *Hae*III MTase methylation was insufficient to protect it fully from the *C. glutamicum* restriction system. This also suggests that the methylation systems involved in protecting the DNA in *C. glutamicum* were not equivalent to the *Hae*III MTase, and that activity with a different, yet overlapping, specificity was present.

Table 1. Effect of *Hae*III methylation of *E. coli* LE392-derived plasmid DNA on transformation frequency of *C. glutamicum.*

Host strain	Transformation frequencies for pCSL17 DNA from[a]		
	E. coli LE392	*E. coli* LE392, *Hae*III MTase	*C. glutamicum* AS019
AS019	1.9	8.0	200
ATCC13032	0.8	3.4	1,100

[a](Transformants/survivors/µg DNA)×10^6. Data are from the average of five independent experiments.

Comparison of Susceptibility of Corynebacterial DNAs to Digestion by Restriction Enzymes
The recognition sequences of MTases are often 5-base pairs as well as 4- or 6-base pairs [13], yet the ENases used to date to analyze the methylation of pCSL17 DNA only recognize 4- or 6-bp [5]. To determine whether pCSL17 DNA purified from *C. glutamicum* contained m^5dCyd in specific sequences recognized by 5-base-pair cutting ENases, plasmid DNA was prepared from three corynebacteria strains (AS019, BL1, and BF4) and *E. coli* LE392 and then digested with the *Tse*I or *Fnu*4HI (NEB, Beverly, MA, U.S.A.) (Fig. 1). The target sequence for *Tse*I is 5'-GCWGC-3' (where W is either A or T) and it

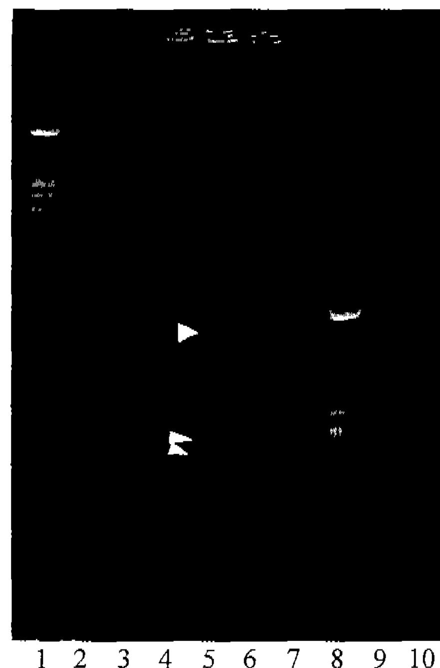

Fig. 1. Agarose gel electrophoresis of corynebacteria- and *E. coli*-derived pCSL17 DNA treated with *Tse*I and *Fnu*4H1.
Lanes: 1, λDNA digested with *Eco*R1 and *Hin*dIII; 2, PCR size markers (50, 150, 300, 500, 750, 1,000 bp); 3–6, pCSL17 DNA from *C. glutamicum* AS019, *B. flavum* BF4, *B. lactofermentum* BL1, and *E. coli* LE392 digested with *Tse*I; 7–10, digests in the same strain order for *Fnu*4H1. Arrows indicate the fragments generated from the digestion of an endogenous *B. lactofermentum* plasmid, pBL1 [10], purified with pCSL17 DNA.

cleaves at this site, yet not at GCSGC (where S is C or G). *Fnu*4HI recognizes 5'-GCNGC-3', where N is A, T, G, or C and it cleaves at GCSGC when the cytidine is not methylated and at 5'-GCWGC-3'. Figure 1 shows that both enzymes exhibited multiple cutting sites in the pCSL17 DNA and the digestion patterns seen for *Fnu*4HI in the pCSL17 derived from *B. lactofermentum* BL1 and *E. coli* LE392 produced more fragments than those seen for *Tse*I. This was anticipated due to the overlapping cutting sites of these enzymes (GCWGC) and the additional capacity of *Fnu*4HI to cleave at GCSGC. For the pCSL17 DNA derived from *C. glutamicum* AS019 and *B. flavum* BF4, both restriction enzymes exhibited identical band patterns, however, there were fewer fragments than seen for the above strains. This is consistent with *Fnu*4HI failing to cut in Gm^{5}CSGC sequences. From this result, when put together with the known *Hae*III methylated sites, it was concluded that all GCSGC sequences in *C. glutamicum* AS019 and *B. flavum* BF4-derived DNA were methylated, since the Gm^{5}CSGC sequences were protected from *Fnu*4HI digestion. However, the possibility that more than one cytidine in this sequence was methylated could not be eliminated.

Determination of Methylated Cytidine on the DNA Sequence GCCGC in *C. glutamicum* AS019

Subsequently, one of the GCCGC sites was studied using the method of Clark *et al.* [1] to determine which cytidine in the putative recognition sequence was methylated. This method, based on a chemical modification of cytidine residues in the presence of sodium metabisulfite [1, 2], has been used extensively in determining methylation patterns in eukaryotic systems, yet rarely been applied in bacterial systems, with the exception of Schumann *et al.* [16], where it was used to determine the methylation target of a multi-specific m^{5}dCyd MTase in *Bacillus stearothermophilus* H3. In the first step of the bisulfite reaction, 2-deoxycytidines (dCyd) are sulfonated and deaminated, converting them to uridine sulfonate. A subsequent desulfonation at a high pH completes the conversion from cytidines to uridines, whereas the m^{5}dCyd are not modified under the conditions used. After the bisulfite treatment, the DNA is amplified in PCR reactions and the PCR products sequenced. The polymerase recognizes m^{5}dCyd as dCyd whereas all unmethylated dCyd appear as thymidine following bisulfite derivatization, which is reflected in the sequences obtained subsequently. Following bisulfite derivatization reaction, the resulting DNA was analyzed by PCR amplification and sequencing. However, because the bisulfite treatment altered the unmethylated dCyd in the DNA strands, the resulting strands were no longer complementary. Therefore, primers were designed to amplify and produce double-stranded products from only one of the two original strands (Fig. 2), taking into account the anticipated changes in the template DNA arising from bisulfite derivatization. Initially, the outer

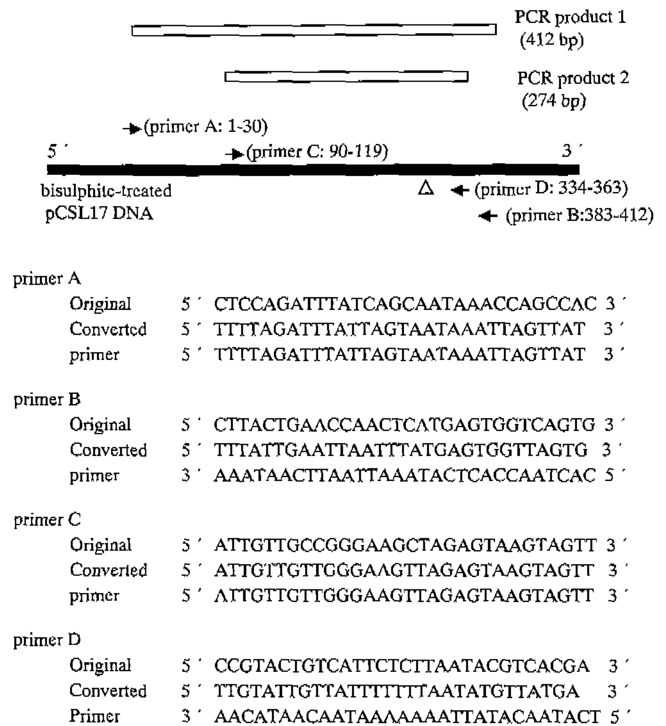

Fig. 2. Schematic diagram of the nested primer set designed for amplification of segment of pCSL17 DNA derived from *C. glutamicum* AS019 or *B. lactofermentum* BL1.

Numbers in parentheses give the nucleotide positions of each primer. Numbering is started from the beginning of primer A. The target site (GCCGC) is indicated by the symbol (Δ) and located between 305 and 309. Primers A-D were; original, DNA sequence before bisulfite treatment; converted, DNA sequence after bisulfite treatment.

primer sets (primer A and primer B in Fig. 2) were used to amplify the target DNA using a PCR reaction. Once completed, the target DNA was again amplified using the inner primer sets, to generate sequences in the order of the original DNA.

When the products (274 bp) from the second-round PCR reactions were sequenced without further steps, signals from the bisulfite-modified PCR products were contaminated with signals from the PCR products from the unchanged templates, which made an analysis of the DNA sequencing information impossible (data not shown). This indicated that the bisulfite treatment was not 100% efficient so that many dCyd in the original DNA were not deaminated, which was reflected in the sequence of the PCR products. To determine which dCyd bases were methylated previously, published methods have included a step involving cloning the PCR products of the DNA derived from a bisulfite treatment and sequencing several (typically 10–20) clones. This is done to ensure that any cytidines in the amplified products are due to the presence of m^{5}dCyd in the original template DNA, and not simply present due to a failure of the bisulfite treatment to deaminate any unmethylated dCyd. The current study used a different approach involving the removal of any underivatized DNA prior to sequencing. The DNA fragment

(274 bp) of interest was contained in several sequences known to be recognized by restriction enzymes, including a *Pst*I and *Hae*III site (GGCCGC). Accordingly, following successful bisulfite treatment, if there were no methylated cytidines in the sequence, these were converted into GGTTGT; whereas if there were still any nondeaminated cytidines following the bisulfite treatment, these regenerated the original template sequence after two rounds of PCR amplification. These PCR products were then removed by digestion with these ENases, thereby leaving only bisulfite-modified products for subsequent rounds of PCR amplification or sequencing. Experimentally, the *Bgl*II-linearized pCSL17 DNA derived from *C. glutamicum* AS019 and *B. lactofermentum* BL1 were bisulfite modified, desalted, and amplified using outer primers, designed as shown in Fig. 2. Two major bands of PCR products were obtained after the agarose gel electrophoresis; the top band corresponded to 412 bp DNA and the lower band was about 30–50 bp DNA (Fig. 3a). The upper DNA band in Fig. 3a was eluted from the gel, amplified using primer C and primer D. Subsequently, the PCR products (2–3 μg) were incubated with 5 U of both *Hae*III and *Pst*I at 37°C for 3 h. Approximately 50% of the PCR product was digested by the two restriction enzymes, judging from a densitometric analysis, which indicated that not all of the DNA substrate was changed by the bisulfite treatment (data not shown). After the restriction enzyme digestion, the DNA fragments were visualized and the bands (PCR product from modified template) were excised from the agarose gel and subjected to a further round of PCR amplification using the inner primers. For DNA originating from *C. glutamicum* AS019

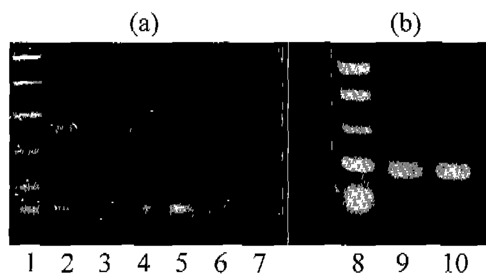

Fig. 3. PCR amplification products following restriction enzyme digestion of bisulfite-modified DNA.

*Bgl*II-linearized pCSL17 DNA derived from *C. glutamicum* AS019 and *B. lactofermentum* BL1 were bisulfite-modified as described in the text, desalted, and amplified using nested primers, designed as shown in Fig. 2. For (a), the PCR products were obtained after the first round of PCR reactions. For (b), the upper DNA band in (a) was eluted from the gel, amplified using primer C and primer D, as described in the text. Subsequently, the PCR products were incubated with *Hae*III and *Pst*I at 37°C for 3 h and amplified using primer C and primer D. (a), Lanes: 1, PCR size markers (50, 150, 300, 500, 750, 1,000 bp); (2–4)-(5–7), PCR product of strain AS019 and BL1 after first round of PCR. (b), Lanes: PCR size markers (50, 150, 300, 500, 750, 1,000 bp); 2-3, PCR product from strain AS019 and BL1 after first- and second round of PCR reaction and following *Hae*III/*Pst*I digestion.

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AS019     ATTGTTGCCG GGAAGCTAGA GTAAGTAGTT CGCCAGTTAA TAGTTTGCGC AACGTTGTTG   60
BT-AS019  ATTGTTGTTG GGAAGTTAGA GTAAGTAGTT TGTTAGTTAA TAGTTTGTGT AATGTTGTTG
BT-BL1    ATTGTTGTTG GGAAGTTAGA GTAAGTAGTT TGTTAGTTAA TAGTTTGTGT AATGTTGTTG

AS019     CCATTGCTGC AGGCATCGTG GTGTCACGCT CGTCGTTTGG TATGGCTTCA TTCAGCTCCG  120
BT-AS019  TTATTGTTGT AGGTATTGTG GTGTTATGTT TGTTGTTTGG TATGGTTTTA TTTAGTTTTG
BT-BL1    TTATTGTTGT AGGTATTGTG GTGTTATGTT TGTTGTTTGG TATGGTTTTA TTTAGTTTTG

AS019     GTTCCCAACG ATCAAGGCGA GTTACATGAT CCCCCATGTT GTGCAAAAAA GCGGTTAGCT  180
BT-AS019  GTTTTTAATG ATTAAGGTGA GTTATATGAN TTTTTATGTT GTGTAAAAAA GTGGTTAGTT
BT-BL1    GTTTTTAATG ATTAAGGTGA GTTATATGAN TTTTTATGTT GTGTAAAAAA GTGGTTAGTT

AS019     CCTTCGGTCC TCCGATCGTT GTCAGAAGTA AGTTGGCCGC AGTGTTATCA CTCATGGTTA  240
BT-AS019  TTTTTGGTTT TTTGATTGTT GTTAGAAGTA AGTTGGCTGT AGTGTTATTA TTTATGGTTA
BT-BL1    TTTTTGGTTT TTTGATTGTT GTTAGAAGTA AGTTGGTTGT AGTGTTATTA TTTATGGTTA

AS019     TGGCAGCACT GCATAATTCT CTTACTGTCA TGCC                              274
BT-AS019  TGGTAGTATT GTATAATTTT TTTATTGTTA TGTT
BT-BL1    TGGTAGTATT GTATAATTTT TTTATTGTTA TGTT
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Fig. 4. Multiple alignment of the sequences of the unmodified- and bisulfite-treated pCSL17 DNA of *C. glutamicum* AS019 and *B. lactofermentum* BL1.

The PCR products were directly sequenced and only results from one direction are shown here (primer C in Fig. 2 was used). The sequence of the unmodified pCSL17 DNA (AS019) is compared with those of the bisulfite-treated pCSL17 DNA of *C. glutamicum* AS019 (BT-AS019) and *B. lactofermentum* BL1 (BT-BL1). The bold type in the sequence underlined indicates the target methylation point of *C. glutamicum* methyltransferase. The target sequence (GCCGC) is underlined. The *Tse*I sequence (GCTGC and GCAGC) is shown in bold type. Several independent sequencing reactions were performed and a summary of the results is represented.

and *B. lactofermentum*, a 274-bp product was finally collected (Fig. 3b), extracted with the Wizard PCR product Clean-Up column (Promega, U.S.A.), and dissolved in 50 μl deionized water for subsequent sequencing in both directions (Fig. 4). It was found that the majority of cytidine bases in the DNAs from both strains were converted to thymidine bases. However, for the *C. glutamicum*-derived DNA, the GGCCGC sequence was amplified as GGCTGT, whereas the same sequence was changed to GGTTGT in the *B. lactofermentum*-derived pCSL17 DNA.

This sequencing data confirmed that *C. glutamicum* MTase methylated the first cytidine in the sequence of GCCGC, and also indicated that similar methylation patterns do not occur in *B. lactofermentum*, thereby demonstrating differences in the restriction and modification systems in these species of corynebacteria. Furthermore, the 274-bp PCR product contained one GCTGC (located between 66–70 bp) and one GCAGC (located between 243–247 bp) sequence, both of which are target sites of *Tse*I. From the sequencing results, none of the cytidines in these sequences were methylated in either the *C. glutamicum*- or *B. lactofermentum*-derived pCSL17 DNA. This indicated that the cytidine bases in the target sites of *Tse*I were not modified, which is consistent with the data presented in Fig. 1. Since only one strand of the bisulfite-derivatized pCSL17 DNA was analyzed, it is not known whether the cytidine-specific MTase in *C. glutamicum* methylates one or both strands in the GCCGC/GCGGC sequences.

The findings from the present work will permit the development of strategies to protect plasmid DNA introduced into *C. glutamicum* from restriction by ENase and increase the transformation efficiency of heterologously-derived DNA. Accordingly, modifying heterologous DNA *in vitro* with either a cell-free extract of *C. glutamicum* or several MTases, such as *Ngo*BVII [12] or *Lla*DII MTase [8], which recognize the DNA sequence of GCNGC (where, N is either A, C, G, or T), before introduction into *C. glutamicum*, will overcome the major restriction barriers in this species.

Although metabisulfite derivatization was successfully employed in this study to analyze the methylated sequences in *C. glutamicum* and *B. flavum* strains, it was necessary to include a modification to the previously published approaches to ensure that the amplification products obtained from the initially underivatized DNA were removed after the second round of PCR amplification. The relatively high proportion of unchanged cytidine may have arisen from the high G-C content of the *Corynebacterium* species. The successful application of this method in other genera may also require a similar approach based on prior knowledge of the putative methylated sequences and restriction enzymes that are able to cleave methylated or unmethylated sequences within the putative target of MTase activity.

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