Methylation by a Unique α-class N4-Cytosine Methyltransferase Is Required for DNA Transformation of *Caldicellulosiruptor bescii* DSM6725

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Abstract

Thermophilic microorganisms capable of using complex substrates offer special advantages for the conversion of lignocellulosic biomass to biofuels and bioproducts. Members of the Gram-positive bacterial genus *Caldicellulosiruptor* are anaerobic thermophiles with optimum growth temperatures between 63°C and 78°C and are the most thermophilic cellulolytic organisms known. In fact, they efficiently use biomass non-pretreated as their sole carbon source and in successive rounds of application digest 70% of total switchgrass substrate. The ability to genetically manipulate these organisms is a prerequisite to engineering them for use in conversion of these complex substrates to products of interest as well as identifying gene products critical for their ability to utilize non-pretreated biomass. Here, we report the first example of DNA transformation of a member of this genus, *C. bescii*. We show that restriction of DNA is a major barrier to transformation (in this case apparently absolute) and that methylation with an endogenous unique α-class N4-Cytosine methyltransferase is required for transformation of DNA isolated from *E. coli*. The use of modified DNA leads to the development of an efficient and reproducible method for DNA transformation and the combined frequencies of transformation and recombination allow marker replacement between non-replicating plasmids and chromosomal genes providing the basis for rapid and efficient methods of genetic manipulation.

Introduction

Current methods for the use of lignocellulosic biomass as a substrate for microbial conversion to products of interest rely on pretreatment of the biomass with acids, alkali or organic solvents, often at high temperature [1,2] and the addition of hydrolytic enzymes that partially digest the plant cell walls [3]. Enzymatic pretreatment is particularly expensive and often prohibitive for the production of low value commodity products from biomass. Thermophilic microorganisms offer special advantages for biomass conversion, in part, because they offer the potential to decrease hydrolysis times by several-fold with the same cellulase loading or to decrease cellulase loading by several fold at constant hydrolysis times. Organisms that can use complex biomass as substrate reduce the need for pretreatment and enzymatic hydrolysis and, therefore, the cost of the process. *Clostridium thermocellum* and *Thermoaerobacterium saccharolyticum* are prominent examples of thermophilic (T<sub>opt</sub> ~ 60°C and 55°C respectively) bacteria that specialize in the solubilization and fermentation of crystalline cellulose to products that include ethanol. Recent advances in the development of genetic tools that facilitate metabolic engineering of these organisms [4,5] have made them leading candidates for consolidated bioprocessing (CBP) of biomass to biofuels. *Thermoanaerobacterium saccharolyticum* has been engineered to produce ethanol at near-theoretical yield from a variety of sugars derived from biomass pretreatment [4] and efforts to identify microbes for direct conversion of biomass is an active area of investigation [6]. *Caldicellulosiruptor* species have the ability to utilize non-pretreated biomass including both low-lignin napier and Bermuda grasses as well as high-lignin switchgrass and a hardwood, popular, for growth [7]. Members of this genus are the most thermophilic of all known organisms capable of using non-pretreated cellulose biomass [8]. The sequences of eight *Caldicellulosiruptor* genomes have been published and reveal enzymes likely to be important in lignocellulose utilization [9-12]. In addition, microarray analysis of cells grown on various substrates implicates specific genes and gene clusters in biomass degradation [13,14].

There are many interesting microorganisms that do interesting and important chemistry but the ability to manipulate them genetically is essential to making them useful. Development of genetic systems for hyperthermophiles, in general, presents many challenges, some of which result from the extreme growth requirements of these organisms. One of the most significant barriers to the genetic manipulation of uncharacterized bacteria in general and hyperthermophiles in particular is the lack of selectable markers. Antibiotic selection strategies used in meso-
philic bacteria are typically ineffective in hyperthermophiles, because of the instability of either the drug or the heterologously expressed resistance protein at high temperatures [15,16]. Nutritional markers can be particularly useful for genetic selection if an organism is able to grow on a defined medium. We developed a method for selection of transformation in *Caldicellulosiruptor bescii* based on the loss of the uracil biosynthetic enzyme orotidine-5'-monophosphate (OMP) decarboxylase (*pyrF*), first described in yeast [17] and since used successfully in both bacteria and archaea [18–23].

We recently discovered that *Caldicellulosiruptor bescii* has a potent restriction endonuclease, CbeI, an isochizomer of HaeIII that cleaves unmethylated sequences at 5’-GG/CC-3’ [24]. Type II restriction endonucleases like CbeI have been shown to be a barrier to DNA transformation of several bacterial strains and overcoming restriction by the hosts was a key to successful transformation. Approaches include engineering the transforming DNA to contain fewer restriction sites [25–27], in vitro methylation by purified methyltransferases [28] or cell extracts [29,30], or in vivo methylation by *E. coli* [26,31]. We were unable to transform *C. bescii* in many attempts using a variety of transformation procedures and speculated that restriction by CbeI might be a barrier. As CbeI (Cbes 2438) recognizes and cleaves the same sequence as HaeIII, we anticipated that methylation by HaeIII methyltransferase (M.HaeIII) might protect DNA from *E. coli* from cleavage by CbeI and eliminate it as a barrier to DNA transformation. M.HaeIII (NEB) partially protected DNA from cleavage by both HaeIII and CbeI in vitro, but no transformants were detected when this DNA was used in a variety of transformation protocols. DNA isolated from *E. coli* strains containing combinations of methyltransferases that facilitated transformation of the thermophiles *Bacillus* *methanolicus* [26] and *Clostridium thermocellum* [32] also failed to transform *C. bescii* using the same protocols. A gene for an apparent cognate methyltransferase, M.CbeI (Cbes 2437) is present adjacent to CbeI in the genome as HaeIII, we anticipated that methylation by HaeIII was a key to successful transformation. A non-replicating plasmid was constructed with engineered restriction site within the pyrC locus but containing an *pyrBCF* open reading frame, diagrammed in Fig 1A, and was used for further analysis. The extent of the deletion was defined by PCR amplification of the pyrBCF region in the mutant (Fig 1C) and subsequent sequencing of the PCR product. Since mutations in *pyrE* also lead to uracil auxotrophy and 5FOA resistance, the region around the *pyrE* locus (Cbes1302) was amplified from this strain and sequenced to ensure that it was wild type. *pyrE* is located 3 ORFs and 2.4 kb downstream of *pyrF* and 5.6 kb downstream of *pyrF* and is unlikely to be transcriptionally coupled to *pyrBCF* or *pyrA*. While the deletion would be expected to affect only the *pyrBCF* genes, qPCR analysis was performed to monitor expression of the *pyrA* gene (Cbes1370) as well as the Cbes1374 open reading frame predicted to encode a uracil xanthine permease. Expression of *pyrA* and Cbes1374 in the deletion mutant was indistinguishable from the wild type, suggesting that the deletion within the *pyrBCF* locus did not affect expression of surrounding genes. The Δ*pyrBCF* strain, JWC8002, (Table 1) was a tight uracil auxotroph and because it contained a deletion, reversion to uracil prototrophy was not a concern making protoplastic selection possible no matter how low the frequency of transformation. Growth of this mutant supplemented with uracil (20 μM) was indistinguishable from that of the wild type, reaching a cell density of ~2×10^8 in 20 hours. To assay transformation, a non-replicating plasmid was constructed with the wild type copy of the *pyrBCF* locus but containing an engineered restriction site within the *pyrC* open reading frame to distinguish it from the chromosomal wild type allele. This plasmid was used to transform the *pyrBCF* deletion strain selecting marker replacement events that repaired the deletion (strategy diagrammed in Fig 1A).

We were unable to transform *C. bescii* in many attempts using this strategy with DNA isolated from *E. coli*. We used and modified methods known to work well for other Gram-positive bacteria including electroporation [36,39,40], artificially induced competence [41], natural competence [19,42], and methods that altered membrane permeability [43]. Mating with *E. coli*, a method of DNA transfer that works well for similar bacteria [15,44], did not work for *C. bescii* or the other *Caldicellulosiruptor* species we tested using the same approach.

**Results**

**A spontaneous deletion of the *C. bescii* pyrBCF locus allows nutritional selection of transformants**

Attempts to use a thermostable kanamycin resistance gene previously used for selection of transformants in *Thermoanaerobacterium* species at 60°C [36,37] to select transformants in *C. bescii* was complicated by the fact that *C. bescii* (that grows optimally at 75°C) grows very poorly at or below 70°C. In fact, growth at 60°C increased the spontaneous mutation frequency significantly, from 10^{-7} to 10^{-5}, making the detection of transformants over this background of spontaneous drug resistance problematic. Attempts to use a hygromycin phosphotransferase (*hph*) gene from *E. coli*, that had been selected for function at 85°C in *Sulfolobus solfataricus* [38] were compromised by the level of natural resistance to hygromycin in *C. bescii* and the fact that, in our hands, this hygromycin resistance gene is not reliable for selection above 70°C. To generate a mutant strain for nutritional selection of transformants, *C. bescii* cells were plated on 5-fluoroorotic acid (5-FOA). OMP decarboxylase, encoded by the *pyrF* gene in bacteria (ura3 in yeast), converts the pyrimidine analog 5-fluorooroticidine monophosphate to 5-fluorouridine monophosphate which is ultimately converted to fluorodeoxyuridine by the uracil biosynthetic pathway, a toxic product that kills growing cells that are auxotrophic for uracil. Mutants of *pyrF* are, therefore, uracil auxotrophs resistant to 5-FOA. Spontaneous resistance to 5-FOA (8 mM) was observed at a frequency of approximately 10^{-5} at 60°C. One such mutant contained a deletion that included part of the carboxy terminus of *pyrF* (Cbes1377) open reading frame, the entire *pyrC* (Cbes1376) open reading frame and the amino terminus of *pyrB* (Cbes1375) open reading frame, diagrammed in Fig 1A, and was used for further analysis. The extent of the deletion was defined by PCR amplification of the *pyrBCF* region in the mutant (Fig 1C) and subsequent sequencing of the PCR product. Since mutations in *pyrE* also lead to uracil auxotrophy and 5FOA resistance, the region around the *pyrE* locus (Cbes1302) was amplified from this strain and sequenced to ensure that it was wild type. *pyrE* is located 3 ORFs and 2.4 kb downstream of *pyrF* and 5.6 kb downstream of *pyrF* and is unlikely to be transcriptionally coupled to *pyrBCF* or *pyrA*. While the deletion would be expected to affect only the *pyrBCF* genes, qPCR analysis was performed to monitor expression of the *pyrA* gene (Cbes1370) as well as the Cbes1374 open reading frame predicted to encode a uracil xanthine permease. Expression of *pyrA* and Cbes1374 in the deletion mutant was indistinguishable from the wild type, suggesting that the deletion within the *pyrBCF* locus did not affect expression of surrounding genes. The Δ*pyrBCF* strain, JWC8002, (Table 1) was a tight uracil auxotroph and because it contained a deletion, reversion to uracil prototrophy was not a concern making protoplastic selection possible no matter how low the frequency of transformation. Growth of this mutant supplemented with uracil (20 μM) was indistinguishable from that of the wild type, reaching a cell density of ~2×10^8 in 20 hours. To assay transformation, a non-replicating plasmid was constructed with the wild type copy of the *pyrBCF* locus but containing an engineered restriction site within the *pyrC* open reading frame to distinguish it from the chromosomal wild type allele. This plasmid was used to transform the *pyrBCF* deletion strain selecting marker replacement events that repaired the deletion (strategy diagrammed in Fig 1A).

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Overcoming Restriction for DNA Transformation

CbeI, a potent restriction endonuclease in \textit{C. bescii}, recognizes and cleaves the same sequence as HaeIII, unmethylated DNA at the sequence 5'-GG/CC-3' [24]. We anticipated that methylation by HaeIII methyltransferase (M.HaeIII) might protect DNA from \textit{E. coli} from cleavage by CbeI and overcome its potential to barrier DNA transformation. This, in fact, was shown to be true for transformation of the archaean \textit{Sulfolobus acidocaldarius} using M.HaeIII to avoid restriction by SuI [45]. Plasmid DNA treated with M.HaeIII, \textit{in vitro}, was partially protected from cleavage by both HaeIII and CbeI \textit{in vitro} (Fig 2), but no transformants were obtained when this DNA was used in electroporation experiments or added to cells that had been subjected to a procedure to induce natural competence in \textit{Mycobacterium} and \textit{Thermoanaerobacterium} species [41,42]. In addition, various strains of \textit{E. coli} containing combinations of methyltransferases were used to prepare DNA for transformation (Table 2), a method that was successful for transforming \textit{Clostridium thermocellum} using a \textit{dam$^{\text{dcm}}$} \textit{E. coli} strain [32]. No transformants of \textit{C. bescii} were detected using DNA from these strains. In total we performed more than 1000 electroporation experiments varying conditions for cell growth, transformation conditions, and assay conditions as well as using DNA from different strains of \textit{E. coli} or methylated with M.HaeIII.

\textbf{M.CbeI is a novel \alpha-class N4-Cytosine methyltransferase}

As shown in Fig 3A, the region of the chromosome that contains CbeI also contains an open reading frame, Cbes2437, predicted to encode an adenine specific methyltransferase [46]. This open reading frame was cloned into an \textit{E. coli} expression vector, pDCW73 (Fig 3B) that placed a His-Tag at the carboxy terminus of the protein allowing purification on a Ni-NTA column. \textit{E. coli} cells containing this plasmid were viable at 23\degree C but not 37\degree C suggesting that expression of M.CbeI was toxic to growing cells. Expression of this methyltransferase was, therefore, performed at 23\degree C to avoid problems related to toxicity and in \textit{E. coli} BL21-CodonPlus(DE3)-RIPL to alleviate problems arising from the significant differences in codon usage between \textit{M.CbeI} and \textit{E. coli}. Purified M.CbeI from \textit{E. coli} was the size predicted from the open reading frame, 33 kDa (Fig 3C). No cleavage of DNA was detected by purified CbeI at 75\degree C when DNA from \textit{E. coli} was methylated \textit{in vitro} by the purified methyltransferase (Fig 3D) and we named this enzyme M.CbeI. To determine the optimal temperature for \textit{M.CbeI} methyltransferase activity, we performed the \textit{in vitro} methylation reactions with purified M.CbeI at temperatures ranging from 25\degree C to 100\degree C and tested the modified DNA for restriction by CbeI. Reactions performed between 65\degree C and 85\degree C, the growth temperature range of \textit{C. bescii}, resulted in the best protection against cleavage by CbeI.

Even though CbeI is an isoschizomer of HaeIII and M.CbeI would be expected to methylate the same sequence as M.HaeIII, methylation varies in the sites of methylation and specific or cognate methylation may be required for full protection. The pattern of DNA methylation by M.CbeI was compared to that by M.HaeIII using a method [47,48] that relies on the fact that the extent of incorporation of fluorescently labeled dideoxynucleotides during DNA sequencing is influenced by methylated bases in the template DNA. pUC18 DNA was methylated \textit{in vitro} by either M.CbeI or M.HaeIII and direct sequencing of the DNA revealed that DNA methylated with M.CbeI showed a higher degree of incorporation of dideoxyguanosine in the 5’-GG/CC-3’ recognition sequence than DNA methylated with M.HaeIII. N4-
methylycytosine results in an increase in the complementary G (G GCC) signal and this signature (Fig 3E) indicates that M.CbeI methylated DNA contains N4-methylcytosine (m4C). M.HaeIII methylates the C5 position of cytosine (m5C).

Methylation of *E. coli* DNA, in *vitro*, with purified M.CbeI protein allows transformation of *C. bescii*

Plasmid DNA from *E. coli* (dam^+dcm^+) methylated by M.CbeI *in vitro* readily transformed the *C. bescii* ΔpyrBCF strain resulting in marker replacement of the deletion with the wild type allele containing the engineered KpnI site (Fig 1). Amplification of the pyrBCF region from wild type *C. bescii* resulted in a 3.2 kb product while the product generated from the deletion strain was 1.63 kb. Amplification of this region in the transformant generated a wild type size product. Digestion with KpnI resulted in no cleavage of the product generated from the wild type or the ΔpyrBCF mutant. The product generated from transformant was digested with KpnI showing that the transformant contained the allele from the plasmid and its presence in the *C. bescii* chromosome resulted from marker replacement (Fig 1C). Transformation efficiencies were routinely on the order of 50 transformants per microgram of non-replicating plasmid DNA (Table 2, Fig 1 B). This extremely low transformation efficiency may be an underestimate of the actual efficiency as the plating efficiency of *C. bescii* on selective solid medium is less than 10^-4 (plating 10^8 cells as determined by cell count resulted in fewer than 100 colonies).

**Discussion**

While there are many challenges in the development of transformation protocols, restriction of DNA from *E. coli* by host bacteria is often an issue. Restriction/modification of DNA, first recognized as a mechanism of protection against phage infection [49], varies in effectiveness depending on the activity of restriction endonuclease and the methylation state of the DNA substrate. Methylation of DNA may either facilitate or limit the activity of endonucleases and plays a major role in transformation of heterologous DNA no matter what the source of the DNA or the host for transformation. Transformation of DNA from *E. coli* to *Caldicellulosiruptor bescii* is apparently especially sensitive to restriction/modification and here we show that the use of a novel endogenous methyltransferase provided specific modification of DNA from *E. coli* that allowed efficient transformation.

M.CbeI was annotated as a D12 class N6 adenine-specific DNA methyltransferase in GenBank [46], but our analysis clearly shows that it functions as a cytosine specific methyltransferase. Like all known methyltransferases it contains a conserved F_G_G amino acid motif that facilitates interaction with S-adenosylmethionine,

### Table 1. Strains/plasmids used and constructed in this study.

| Strains/Plasmids | Description | Source |
|------------------|-------------|--------|
| DSM 675          | *Caldicellulosiruptor bescii* wild type | DSMZ^1 |
| JWCB002          | *C. bescii* ΔpyrBCF | This study |
| JWCB003          | JWCB002 with *pyrBCF* restored by marker replacement | This study |
| JW 284           | *E. coli* DH5s containing pDCW73 | This study |
| pDCW 70          | Contains the *pyrBCF* locus with an engineered KpnI site | This study |
| pDCW 73          | M.CbeI Expression vector | This study |

^1German Collection of Microorganisms and Cell Cultures. doi:10.1371/journal.pone.0043844.t001

### Table 2. Influence of methylation on transformation efficiency of pDCW70.

| *E. coli* strain used to prepare pDCW70 | Transformation efficiency (Transformants/μg of DNA)^* |
|---------------------------------------|--------------------------------------------------|
| DHS5x (dam^+dcm^-)                    | ND^5                                              |
| BL21 (dam^-dcm^-)                     | ND^5                                              |
| ET12567 (dam^-dcm^-)                  | ND^5                                              |
| DHS5x (dam^+dcm^-)/M.HaeII            | ND^5                                              |
| DHS5x (dam^-dcm^-)/M.CbeI             | ~50^5                                             |

^*Each transformation experiment used approximately 10^6 cells and 600 ng of transforming DNA.

^5ND (Not detected) : based on at least 30 independent transformation experiments.

^6Average of the results of five independent transformation experiments.

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Figure 2. Protection of DNA by M.CbeI or M.HaeIII from digestion *in vitro*. In each panel, lane 1 is unmethylated plasmid DNA (pUC18) isolated from *E. coli* (dam^+dcm^+), lane 2 is plasmid DNA methylated in *vitro* with M.HaeIII (NEB) at 37°C. lane 3 is plasmid methylated with purified M.CbeI at 78°C. (A) undigested, (B) digested with HaellI (C) digested with purified CbeI. DNAs were subjected to electrophoresis in a 1.2% TAE-agarose gel, and then stained with ethidium bromide. M: 1kb DNA ladder (NEB). doi:10.1371/journal.pone.0043844.g002
the source of the methyl group in these reactions. M.CbeI also contains a DPPY motif typical of N6-adenine methyltransferases, all of which contain a (D/N)PP(Y/F) motif [50,51]. Its SPP(Y/F) motif is the hallmark of N4 cytosine methyltransferases active site [52], making M.CbeI unusual in that it contains a DPPY motif in the active site (Fig 4). Furthermore, the M.CbeI protein has no reported significant sequence similarity to any characterized N4 cytosine methyltransferase. Our own search revealed similarity to DmtB from Anabaena variabilis ATCC 29413 (Fig 4), which has been shown to have m4C methyltransferase activity specific to the inner cytosine in the 5'-GGCC-3' recognition sequence [53]. These proteins, which show 57% amino acid identity, represent a new α-class methyltransferase specific for the GGCC sequence, different from the previously characterized β-class of N4-methyltransferases in hyperthermophiles, M.SuaI [45] and M.PhoI [54], isolated from the archaea Sulfolobus acidocaldarius and Pyrococcus horikoshii OT3, respectively. These data suggest that these proteins may have evolved from different common ancestors with other β-class N4-methyltransferases specific for 5'-GGCC-3'. M.CbeI is the first characterized α-class m4C methyltransferase from a hyperthermophile and homologs exist in two other Caldicellulosiruptor species, Calhy0409 (88% of protein sequence identity) from C. hydrothermalis 108 and Calkr2088 (85% of protein sequence identity) from C. kristjanssonii 177R1B (Fig 4).

Figure 3. Cloning, expression, purification and partial characterization of M.CbeI. (A) The region of the C. bescii chromosome containing CbeI and M.CbeI. (B) Diagram of pDCW73 used to produce a His-tagged version of M.CbeI in E. coli. (C) Purified M.CbeI displayed on a 10–20% Tris-HCl gradient gel (CriterionTM Precast Gel, Bio-Rad Laboratories, Hercules, CA) stained with coomassie blue. lane 1: protein molecular weight standards (BioRad); lane 2: 15 ng of purified M.CbeI protein. (D) lane 1, undigested unmethylated pDCW70; lane 2, undigested M.CbeI methylated pDCW70; lane 3, unmethylated pDCW70 digested with purified CbeI; lane 4, M.CbeI methylated pDCW70 digested with purified CbeI, MW: 1 kb DNA ladder (NEB). (E) DNA sequence traces of DNA methylated with M.CbeI (top panel) or M.HaeIII (bottom panel). Differences in the G residue signals between M.HaeIII and M.CbeI pUC18 are shown in the middle panel.

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One reason for the fact that even though M.HaeIII partially protects DNA from cleavage by CbeI, it is not sufficient to allow transformation of C. bescii is the activity of CbeI itself. In a previous study [45], M.HaeIII-modified DNA (m5C) was cleaved at reasonable efficiency by purified SuaI, a GGCC specific restriction enzyme completely blocked by m4C methylation at the inner cytosine residue in high concentrations. M.HaeIII is also known to have a significant level of promiscuous methylation activity at non-canonical sites [55] and may actually increase restriction activity in vector DNA by methyl-directed restriction enzymes.

Efforts to optimize the transformation procedure for C. bescii have included adding cell wall weakening agents (isoniacin or glycine) during cell growth, altering temperature during the preparation of electro-competent cells, changing the composition of the washing and electroporation buffers, altering incubation times and temperatures of the cells with DNA prior to electric-pulse, varying the electrical settings during the electric pulse, and altering the composition of the recovery medium and incubation period before plating onto selective medium. None of these modifications have improved the efficiency but as optimization of these procedures is largely empirical we continue to investigate the possibilities. Attempts to generalize the use of M.CbeI to facilitate transformation of other Caldicellulosiruptor species are also in progress.

Methods and Materials

Strains and growth conditions

All strains used in this study are listed in Table 1. All growth of Caldicellulosiruptor species was performed under anaerobic...
Overcoming Restriction for DNA Transformation

C. bescii DSM 6725 (Cbes2437)

\[ \text{AdoMet-binding region (Motif I)} \]

\[ \text{Target recognition region} \]

\[ \text{Catalytic Region (Motif IV)} \]

\[ \text{C. bescii DSM 6725} \]

\[ \text{C. kristjanssonii 177R1B} \]

\[ \text{C. hydrothermalis 108} \]

\[ \text{Anabaena variabilis} \]

\[ \text{(ATCC 29413)} \]

Figure 4. Domain structure of M.CbeI. Predicted functional domains of M.CbeI and sequence alignments of conserved motifs of three M.CbeI homologues from members of *Caldicellulosiruptor* species as well as DmtB from *Anabaena variabilis*, which also contains an M.CbeI homologue showing unique features of this methyltransferase.

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Plasmid Construction and DNA manipulation

Primers used in these constructions are listed in Table 3. All PCR amplifications were performed using *Pfu* Turbo DNA polymerase (Agilent Technologies). A 1.858 kb fragment containing the pSgt101 replication origin was amplified from pDCW68 [24] using primers DC081 and DC230, which contain KpnI and AatII sites at each 5' end, respectively. A 4.343 kb fragment containing the apramycin resistance and pyrBCF cassettes was amplified from pDCW68 using primers DC084 and DC232 to which an AatII and KpnI site had been added at each 5' end. An additional fragment (1.801 kb) containing DNA sequences used in other work not relevant to the experiments described here was amplified using primers DC212 and DC231. These three DNA fragments were cut by restriction enzymes, KpnI and AatII, and then ligated to yield pDCW69 (8.014 kb). pDCW70 was constructed by introducing a single nucleotide change (an A to C transversion resulting in a silent mutation) in the +978 amino acid of *pyrC* (Cbes 1376) ORF using “PCR based Site Directed Mutagenesis”, using DC 214 and DC 215 primers, to create the KpnI site (GGTAC/C), in pDCW 69. To construct pDCW73, the 0.837 kb M.CbeI (Cbes 2437) open reading frame was amplified by PCR using primers DC238 and DC239 using *Pfu* Turbo DNA polymerase and ligated to pET24d [56], which had also been digested with BamHI and XhoI. This vector contains a His-tag cDNA as template. The PCR product was digested with BamHI and XhoI and ligated to pET24d [56], which had also been digested with BamHI and XhoI. This vector contains a His-tag sequence that is added to the C-terminus of the expressed protein. All plasmids used in this study were sequenced to confirm their structure.

Purification of His-tagged M.CbeI and *in vitro* methylation of DNA

Purification of M.CbeI was similar to the method described by Chung et al. [24]. BL21-CodonPlus(DE3)-RILP cells (Agilent Technologies), containing pDCW73, were used for M.CbeI protein expression. Cells were grown at 23°C in L broth supplemented with kanamycin (25 µg/ml) and chloramphenicol (50 µg/ml) to OD_{600} 0.7 and induced by addition of 0.5 mM isopropyl-β-D-1-thiogalactopyranoside (IPTG) at 23°C overnight.
Table 3. Primers used in this study.

| Primers   | Sequences (5’ to 3’) |
|-----------|----------------------|
| DC081 reverse | ACCAGCTTACATTCTAGCTTGGA |
| DC084 forward | TCTGACGCTGATGGCAAGGA |
| DC156 forward | TCTAAGAGTTGCTGCCTTGATA |
| DC163 forward | TCTGACAATTAAAACACAAATAACC |
| DC188 reverse | TGGAACATGTTGGTTGCTCTAGT |
| DC212 reverse | ACCTCTAAATAAAAACACAAATAACC |
| DC213 forward | GTATTTATCTTGTTGATAAGTC |
| DC214 forward | AGGCGTACATGGGGTGAGAC |
| DC215 reverse | TGC AGCAAGGTAAATACCTGACATT |
| DC230 forward | TCACTGTTGATGTTGACAG |
| DC232 reverse | TAAGAAGATTCTGCTGGTTGATA |
| DC238 forward | AGAGAATGACATCACAACACGCTTCC |
| DC239 reverse | TCTCCTGAGACACCAAGTCGTTTTC |
| DC236 forward | TCGAGTCTGCTGATTAAGCCAA |
| DC239 reverse | AGGTGGTTTGAAGATTTCCAAGG |
| M13F(−20) forward | GTAAAGACGACGCTGAC |
| M13R(−20) reverse | CGGATAAACATTTCAACACAG |
| MD003C forward | ATCTTGAAATATCGAGCGAGAGG |
| MD004C reverse | CAAGAGGATACCTCAACACGCTTCC |
| MD005C forward | TTATAGCCCCAACAGGTAAAACCT |
| MD006C reverse | TACCGATATTTACTGCTTATTG |
| MD007C reverse | TATATCGTGTCTGTTGACAG |
| MD009C reverse | GAACATGAGTTAGGGTTGAGAGG |
| MD010C reverse | TGGCTCTGGTTTGAATTGATTG |
| MD011C reverse | CAGAGCAGGATTATACTCAGTG |
| MD012C reverse | TGATGTGTTGTTGCTAGCATAAACG |
| MD015C forward | AATATCAACAATCTTCCTTCGAC |
| MD016C reverse | GAGAAAAGCTTAACTCCAACATCG |

His-tagged (carboxy terminus) M.CbeI was purified as described previously [24] except for the use of a His-Spin Protein Miniprep™ (Zymo Research). Protein concentration was determined by the Bio-Rad protein assay using bovine serum albumin (BSA) as the standard. Purified protein was displayed using sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE) and stained with Coomassie brilliant blue G-250 as described [57]. Protein purity was determined to be >98%.

For in vitro methylation, DNA isolated from E. coli DH5α (dam ‑ dam ‑) was treated with either M.CbeI or M.HaeIII methyltransferase (NEB). 5 microgram of purified M.CbeI was incubated with 50 mM Tris-HCl, 50 mM NaCl, 80 μM S-adenosylmethionine (SAM), 10 mM Dithiothreitol (DTT) at pH 8.5 and 20 μg of DNA substrate in 400 μl reaction, and incubate for 2 hours at 78°C. The M.HaeIII methyltransferase reaction was performed according supplier’s instructions. To allow complete methylation, an additional 10 units of M.HaeIII and 80 μM SAM was added to the reaction every 4 h of incubation at 37°C for a total of 12 h. Methylated DNAs were purified and concentrated by Phenol/Chloroform extraction and ethanol precipitation. The extent of protection was determined by cleavage using HaeIII and NotI (NEB) restriction enzymes according to the supplier’s instructions.

Analysis of methylation by M.CbeI

To identify the site of methylation by M.CbeI, unmodified DNA was compared to that after methylation and the changes were determined by direct visualization in automated DNA sequencing chromatograms [47,48]. In vitro methylation of pUC18 DNA isolated from E. coli DH5α (dam ‑ dam ‑) was carried out using M.HaeIII (NEB) and purified M.CbeI. The efficiency of methylation was determined by cleavage of the methylated and unmethylated DNA with HaeIII (NEB), purified CbeI, and C. bescii cell free extracts (CFE). Digested DNA was displayed by agarose gel electrophoresis and visualization using ethidium bromide staining. Automatic sequencing was performed using primers M13F(−20) and M13R(−20) in an ABI automated PRISM big-dye-terminator system (Macrogen, Inc, Maryland). Sequences were analyzed using the Chromas Lite v2.01 (Technelysium Pty Ltd.) and ABI chromatograms were compared by aligning the Sequencing traces and using SeqDoc [58].

Transformation of C. bescii

To prepare cells for transformation, 2.5 milliliter of a freshly grown JWCB002 [ΔpyrBCF] culture was inoculated into 500 ml of fresh medium, and incubated at 78°C to mid-log phase (OD600 = 0.1, approximately 10⁸ cells/ml). The cultures were cooled to room temperature for 1 hour, harvested by centrifugation (5000 × g, 15 min) at 25°C and washed twice with 250 ml of pre-chilled 10% sucrose. After the final wash, the cell pellets were resuspended in a total volume of 1 ml of pre-chilled 10% sucrose and frozen in microcentrifuge tubes in a dry ice/ethanol bath in 50 μl aliquots for storage at −80°C. Plasmid DNA (0.5–1.0 μg) was added to cells, gently mixed and incubated at 10% sucrose for 15 minutes at room temperature. Electrottransformation of the cell/DNA mixture was performed via single electric pulse (1.8 kV, 600Ω, and 25 microF) in a pre-chilled 1 mm cuvette using a Bio-Rad gene Pulser. After pulsing, cells were incubated overnight at 75°C in 10 ml modified DSMZ 516 medium supplemented with 20 μM of uracil, harvested by centrifugation (at 5000 × g for 20min) and resuspended in 1 ml of 1 x base salts. A cell suspension (100 microliters) was plated onto defined medium without uracil. Plates were incubated in anaerobic jars at 75°C for three to four days. To confirm marker replacement of the pyrBCF region in the transformants, DNA from uracil prototrophic transformants was used to amplify the chromosomal region using primers DC163 and DC188 which anneal outside the regions of the pyrBCF fragment contained on pDCW70 (Fig 1). PCR products of this locus amplified from the wild type, the deletion mutant and the transformants were digested with KpnI and sequenced.

RNA extraction and RT-qPCR analyses. Total RNA was extracted using an RNaseasy Mini kit (Qiagen) and stored at −80°C. RNA was treated with RNase-free DNase (Qiagen) according to manufacturer’s instructions. cDNA was then prepared using the AffinityScript quantitative PCR (qPCR) cDNA synthesis kit (Agilent Technologies). All quantitative reverse transcription-PCR (RT–qPCR) experiments were carried out with an Mx3000P instrument (Stratagene) with the Brilliant SYBR green qPCR master mix (Agilent Technologies). The gene encoding pyruvate ferredoxin oxidoreductase (Cbes 0877) was used as an internal control for RNA. The primers used in RT-qPCR experiments are listed in Table 3.
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Author Contributions

Conceived and designed the experiments: DC JF JW. Performed the experiments: DC JF JRH EO. Analyzed the data: DC JF JW. Contributed reagents/materials/analysis tools: DC JF JRH EO. Wrote the paper: DC JF JW.

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