Identification of Restriction-Modification Systems of Bifidobacterium animalis subsp. lactis CNCM I-2494 by SMRT Sequencing and Associated Methylome Analysis

Mary O’Connell Motherway¹, Debbie Watson¹, Francesca Bottacini¹, Tyson A. Clark², Richard J. Roberts³, Jonas Korlach², Peggy Garault⁴, Christian Chervaux⁴, Johan E. T. van Hylckama Vlieg⁴, Tamara Smokvina⁴, Douwe van Sinderen¹*

1 Alimentary Pharmabiotic Centre and School of Microbiology, National University of Ireland, Cork, Ireland, 2 Pacific Biosciences, Menlo Park, California, United States of America, 3 New England Biolabs, Ipswich, Massachusetts, United States of America, 4 Danone Nutricia Research, Palaiseau, France

Abstract

Bifidobacterium animalis subsp. lactis CNCM I-2494 is a component of a commercialized fermented dairy product for which beneficial effects on health have been studied by clinical and preclinical trials. To date little is known about the molecular mechanisms that could explain the beneficial effects that bifidobacteria impart to the host. Restriction-modification (R-M) systems have been identified as key obstacles in the genetic accessibility of bifidobacteria, and circumventing these is a prerequisite to attaining a fundamental understanding of bifidobacterial attributes, including the genes that are responsible for health-promoting properties of this clinically and industrially important group of bacteria. The complete genome sequence of B. animalis subsp. lactis CNCM I-2494 is predicted to harbour the genetic determinants for two type II R-M systems, designated BanLI and BanLII. In order to investigate the functionality and specificity of these two putative R-M systems in B. animalis subsp. lactis CNCM I-2494, we employed PacBio SMRT sequencing with associated methylome analysis. In addition, the contribution of the identified R-M systems to the genetic accessibility of this strain was assessed.

Introduction

Bifidobacteria are a common component of the microbiota of the gastrointestinal tract (GIT) of a broad range of hosts, and their presence is associated with a positive health status of the gut [1,2]. Compared to other micro-organisms of industrial and pharmaceutical relevance, relatively little is known about the precise molecular mechanisms that explain how bifidobacteria confer beneficial effects, although scientific progress has been made in recent times [3,2].

A typical example of this is Bifidobacterium animalis subsp. lactis CNCM I-2494, which is a component of a fermented dairy product studied in several placebo-controlled clinical trials [4–6]. Veiga et al. [7] recently reported on the marked reduction in intestinal inflammation observed in a murine model of inflammatory bowel disease (IBD) upon administration of fermented milk containing B. animalis subsp. lactis CNCM I-2494, while Agostini et al [8] demonstrated that a dairy product containing this strain reduces visceral hypersensitivity associated with acute stress in a murine model. However, the molecular mechanisms of these observations remain unclear.

Insights into bacterial modes of action may be obtained by comparative and functional genomic approaches. The last decade has seen a vast expansion of complete genome sequences obtained from various human, animal or insect-derived bifidobacterial species [9]. While these have contributed very significantly to advancing our knowledge on bifidobacterial genomics, genetics and metabolism, the availability of a genome sequence is merely a first step towards a better understanding of the beneficial attributes of a specific strain. The complete genome sequence of CNCM I-2494 has been reported [10] and genetic determinants potentially involved in host interaction have been identified. However, demonstration of the role and functionality of these determinants demands the availability of suitable molecular tools. In this respect, the genus Bifidobacterium is renowned for being genetically recalcitrant and while numerous E. coli-bifidobacterial shuttle vectors have been created [11], the successful application of these vectors can be strain-dependent. At present the ability to access the chromosome to create mutants via site-specific homologous

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* E-mail: d.vansinderen@ucc.ie

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recombination approaches remains feasible for relatively few bifidobacterial strains [12,13], while transposon mutagenesis has only very recently been described [14]. Merely 3 years ago we published the first method for the creation of site-specific homologous recombination 

Nucleotide sequence analysis

Sequence data were obtained from the Artemis-mediated [39] genome annotations of the B. animalis subsp. lactis CNCM I-2494 [10]. Database searches were performed using non-redundant sequences accessible at the National Centre for Biotechnology Information internet site (http://www.ncbi.nlm.nih.gov) using BLAST [40,41] and the REBASE [42] site accessible at the New England Biolabs internet site (www.neb.com/rebase). Sequence assembly, verification and analysis were performed using the Seqman and Seqbuilder programs of the DNASTAR software package (DNASTAR, Madison, WI, USA).

DNA manipulations

Chromosomal DNA was isolated from bifidobacteria as previously described [43]. Minipreparation of plasmid DNA from E. coli or B. animalis subsp. lactis CNCM I-2494 was achieved using the Qiaprep spin plasmid miniprep kit (Qiagen GmBH, Hildén, Germany). For bifidobacteria an initial lysis step was incorporated into the plasmid isolation procedure, cells were resuspended in lysis buffer supplemented with lysozyme (30 mg ml\(^{-1}\)) and incubated at 37°C for 30 min. Procedures for DNA manipulations were performed essentially as described by Sambrook et al. [38]. Restriction enzymes, shrimp alkaline phosphatase and T4 DNA ligase were used according to the supplier's instructions (Roche Diagnostics, Bell Lane, East Sussex, UK). Synthetic single stranded oligonucleotide primers used in this study were synthesized by MWG Biotech AG (Ebersberg, Germany). Standard PCRs were performed using TaqPCR mastermix (Qiagen), while high fidelity PCR was achieved using PfuII Ultra polymerase (Agilent, Limerick, Ireland). PCR fragments were purified using the Qiagen PCR purification kit (Qiagen). Electroporation of plasmid DNA into E. coli was performed as described by Sambrook et al. [39], and into L. lactis as described by Wells et al. [44]. The correct orientation and integrity of all constructs was verified by DNA sequencing, performed at MWG Biotech (Ebersberg, Germany).

PacBio SMRT sequencing and methylome analysis

DNA isolation, purification, SMRT bell library preparation were performed as previously described [35,36]. SMRT sequencing was performed on a PacBio RS instrument (Pacific Biosciences, Menlo Park CA, USA). Sequencing reads were processed and mapped to the sequence of B. animalis subsp. lactis CNCM I-2494 (Genbank accession number CP002915). Interpulse durations were measured as previously described [35]. To identify methylated positions the Pacific Biosciences SMRTPortal analysis platform (version 1.4) was adopted, this employs an in silico kinetic reference and a t-test based kinetic score detection of modified base positions.

Construction of the E.coli-bifidobacterial shuttle vectors pDM1 and pDM2

The spectinomycin resistance gene, including its presumed promoter region, was amplified from pMG30S using primer combinations SpecF and SpecR, which harbor SacI sites at their 5’ end, or SpecF1 and SpecR1, which contain EcoRI and HindIII sites, respectively. In each case the 1117-bp ampiclon was digested with either SacI, or a combination of EcoRI and HindIII, and ligated to similarly digested pAM5 [43] or pDG7 [46], respectively. The ligations were transformed into E. coli EC101 with selection on LB agar containing kanamycin and spectinomycin. A number of transformants were selected and screened for plasmid content by restriction analysis and DNA sequencing. In one of the
recombinant plasmids, which was termed pDM1, the tet\textsuperscript{R} cassette of pAM5 had been replaced with the spectinomycin resistance cassette, while in the second recombinant plasmid, designated pDM2, the spectinomycin cassette had been cloned in the unique EcoRI and HindIII sites of pDG7.

**Transformation of B. animalis subsp. lactis CNCM I-2494**

48 ml of mMRS including the supplemented (0.05%) cysteine (1%) maltose was inoculated with 2 ml of an overnight culture of B. animalis subsp. lactis CNCM I-2494 and incubated anaerobically at 42\textdegree C. At an optical density (OD\textsubscript{600 nm}) of approximately 0.8, bacterial cells were collected by centrifugation at 4,700 \texttimes g for 10 min at 4\textdegree C, and the resulting cell pellet was then washed twice with chilled sucrose citrate buffer (1 mM citrate [pH 5.8], 0.5 M sucrose). The cells were subsequently suspended in 300 \textmu l of chilled sucrose citrate buffer. Fifty microliters of the cell suspension was used for each electrotransformation, and cells and plasmid DNA were mixed and held on ice prior to the pulse at 25 \textmu F, 200 Ohms and 2.5 kV. After transformation, cells were suspended in 1 ml of mMRS supplemented with cysteine and maltose, and incubated for 3 hours at 42\textdegree C. Serial dilutions were plated on RCA supplemented with maltose and containing the appropriate antibiotic and incubated at 42\textdegree C for 24–36 h to allow visible colonies to form.

**Cloning of the BanLI R-M system and bacteriophage plaque assays**

For the construction of plasmid pWSK29-RM.BanLI, DNA fragments encompassing the coding sequences of ban\textsubscript{LI}RM (corresponding to locus tags BALAC2494_1402 and BALAC2494_1401) were generated by PCR amplification employing chromosomal DNA of B. animalis subsp. lactis CNCM I-2494 as a template, and using PFU Ultra DNA polymerase plus primer combinations RMBanLIF and RMBanLIR (Table S1). The forward primer contained the sequence of a ribosome binding site to facilitate translation of corresponding mRNA following transcriptional fusion to the lac promoter on pWSK29. The amplified fragment was restricted with NotI and PstI, and ligated to similarly digested pWSK29. The ligation was then used to transform E. coli Xl1Blue. The plasmid content of a number of Amp\textsuperscript{R} transformants was screened by restriction analysis and the integrity of positively identified clones was verified by sequencing, and one of the thus identified plasmids was designated pWSK29-RM.BanLI. This construct was transferred to the phage sensitive host E. coli DSMZ5911 by electroporation to facilitate plaque assays. Plaque assays were performed in accordance with standard procedures [38] adopting serial dilutions of two P\textsubscript{1}vir phage lysates generated following propagation of P\textsubscript{1}vir on E. coli strains MG1655 or EC100. Agar plates were incubated at 37\textdegree C for 24 hours after

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**Table 1. Bacterial strains, bacteriophage and plasmids used in this study.**

| Strain or plasmid | Relevant characteristics | Reference or Source |
|-------------------|--------------------------|---------------------|
| **Strains**       |                          |                     |
| B. animalis subsp. lactis CNCM I-2494 | Danone |
| E. coli strains   |                          |                     |
| EC101             | Cloning host, km\textsuperscript{R} | [15] |
| X11Blue           | Cloning host, tet\textsuperscript{R} | Stratagene |
| MG1655            | Host for plaque assays   | (62) |
| EC100             | Host for plaque assays   | EPICENTRE Biotechnologies, Inc. |
| Lactococcus lactis NZ9000 | MG1363, pepN:nisRK, nisin-inducible overexpression host | [63] |
| **Bacteriophage** |                          |                     |
| P\textsubscript{1}vir-MG1655 | P\textsubscript{1}vir phage propagated on E. coli MG1655 for plaque assays | UCC, School of Microbiology bacteriophage collection |
| P\textsubscript{1}vir-EC100 | P\textsubscript{1}vir phage propagated on E. coli EC100 for plaque assays | UCC, School of Microbiology bacteriophage collection |
| **Plasmids**      |                          |                     |
| pAM5              | pBC1-puc19-Tc\textsuperscript{R} | [45] |
| pDG7              | pMB1-Cm\textsuperscript{R}-Amp\textsuperscript{R} | [46] |
| pMG365            | Spectinomycin resistance plasmid (Spec\textsuperscript{R}) | Molgen plasmid collection, Groningen, The Netherlands |
| pDM1              | pAM5 derivative containing spectinomycin resistance cassette | This study |
| pDM2              | pDG7 derivative containing spectinomycin resistance cassette | This study |
| pNZ44             | Cm\textsuperscript{R}, expression vector | [47] |
| pWSK29            | Amp\textsuperscript{R}, low copy number E. coli cloning plasmid | [48] |
| pWSK29-M.BanLI    | pWSK29 derivative containing \(M\text{.banLI}\) | This study |
| pWSK29-M.BanLI    | pWSK29 derivative containing \(M\text{.banII}\) | This study |
| pWSK29-RM.BanLI   | pWSK29 derivative containing \(RM\text{.banLI}\) | This study |
| pWSK29-M.BanLI- M.BanLI | pWSK29 derivative containing \(M\text{.banLI}\) and \(M\text{.banII}\) | This study |

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Characterisation of Bifidobacterial Restriction Modification Systems

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which the number of plaque forming units for each phage and host combination was enumerated.

Cloning of genes encoding putative methyltransferases

For construction of plasmids pWSK29-M.BanLI and pWSK29-M.BanLII, DNA fragments encompassing banLLM (BALAC2494_1402) and banLII (BALAC2494_0032) were generated by PCR amplification employing chromosomal DNA of B. animalis subsp. lactis CNCM I-2494 as a template, and using PFU Ultra DNA polymerase plus primer combinations BanLIF and BanLIR, or BanLIIF and BanLIIIR, respectively (Table S1). Each forward primer contained the sequence of a ribosome binding site to facilitate translation of corresponding mRNA. For the banLLM, encompassing fragment PstI and HindIII sites were incorporated into the forward and reverse primers, respectively, to facilitate ligation to similarly digested pNZ44 [47]. Ligation was introduced into L. lactis NZ9000 by electroporation and transformants were selected on chloramphenicol resistance. The presence and integrity of the cloned insert in one of the recombinant plasmids, designated pNZ44-BanLI, was confirmed by restriction and sequence analysis. The region encompassing the banLLM coding sequence and the constitutive p44 lactococcal promoter, specified by pNZ44, was amplified by PCR from a representative pNZ44-BanLI plasmid using primer pair BanLIF1 and BanLIR. The resultant fragment was restricted with BglII and HindIII and ligated to BamHI and HindIII-restricted pWSK29 [48]. The ligation mixture was introduced into E. coli Xl1Blue by electro- poration and several of the resulting Amp’ transformants were then checked for plasmid content and, where relevant, sequence integrity.

Transcriptional fusion of the gene to the lac promoter on pWSK29 was achieved by cloning a banLII-M-encompassing amplicon, which had been restricted with XbaI and PstI (sites included in the primers), into similarly digested pWSK29. Each ligation was transformed into E. coli Xl1Blue. Plasmid content of a number of Amp’ transformants was screened by restriction analysis. The integrity of positively identified plasmids was verified by sequencing, of which one was designated pWSK29-M.BanLII.

To construct plasmid pWSK29-M.BanLI-M.BanLII, which expresses both of the identified B. animalis subsp. lactis CNCM I-2494 methylases, the DNA fragment encompassing p44-M.BanLI was amplified from pWSK29-M.BanLI by PCR using primer combinations BanLIF2 and BanLIR2 (Table S1), which had SalI restriction sites incorporated at their 5’ ends. The resulting 1347 bp amplicon was digested with SalI, ligated into similarly digested pWSK29-M.BanLI, followed by introduction into E. coli Xl1Blue by electroporation and selection on ampicillin resistance. Plasmid content of a number of Amp’ transformants was screened by restriction analysis and the expected sequence integrity of positively identified clones was verified by sequencing.

Results

Genetic organization and amino acid analysis of the BanLI, BanLII and BanLIII R-M systems from B. animalis subsp. lactis CNCM I-2494

Three loci, predicted to encode two apparently complete R-M systems, designated BanLII and BanLIII, and a single incomplete R-M system, termed BanLIII and represented by an apparent orphan methylase, were identified on the genome sequence of B. animalis subsp. lactis CNCM I-2494. The G+C content of the genes encoding each of the complete R-M systems is 50%, which is lower than the overall G+C content for bifidobacteria (60%) and also lower than the G+C content for this strain (60.49%), while the G+C content of the gene specifying the predicted orphan methylase is 67.00%, higher than that of the genome. The first gene of the BanLI system (BALAC2494_1403) encodes a protein that shows homology to putative phage integrases from B. animalis subsp. lactis and B. longum subsp. infantis ATCC15697. The second gene, designated banLLM, encodes a protein (M.BanLI; 41.68 kDa) with homology to adenosine-specific methyltransferases. This gene is conserved in all available genome sequences of B. animalis subsp. lactis [48] (Fig. S1). In addition, the protein product shares 62% and 53% identity with predicted methyltransferases from B. bifidum strains LMG13195 and NCIMB41171, respectively. Two of the four conserved motifs characteristic of the N12 class of N6-adenosine-methyltransferase, CMI and CMII [49] can be identified in M.BanLI (Fig. S2A). The third gene of the BanLI gene cluster, banLII.R, is conserved in all B. animalis subsp. lactis genome sequences, and encodes a 43.5 kDa protein, which exhibits similarity to various type II R-M system restriction subunits and for this reason is presumed to represent the restriction component of the BanLI R-M system.

The second identified R-M system encoded by the genome of B. animalis subsp. lactis CNCM I-2494 (and other sequenced B. animalis subsp. lactis strains), BanLII, shares 52% identity with predicted R-M system methylases encoded by B. dentium strains Bsl1 and ATCC27678. The BanLII R-M system is specified by two genes with a convergent orientation and is predicted to represent an isoschizomer of AvaII. The first gene, banLII.M, encodes a 502 amino acid protein (56.68 kDa), which shares 42% identity with MAVaII, a 5’-methylcytosine methyltransferases [50,51]. Consistent with this designation, the six highly conserved motifs of cytosine-specific MTases are all present in M.BanLII (Fig. S2B). The second gene banLII.R encodes a 317 amino acid protein (36.21 kDa), and, based on its location and corresponding GC content, is predicted to encode a restriction endonuclease, although no significant similarity to any known restriction endonucleases was observed on performing rebase searches.

The third RM system, designated BanLIII is predicted not to be functional due to the first gene of the cluster, represented by BALAC2494_259, being a suspected pseudogene. The protein remnant encoded by BALAC2494_259 shows similarity to the helicase domain of the Restriction-subunit of Type III R-M systems. The methyltransferase (191 amino acids; 20.75 KDa) predicted to be encoded by BALAC2494_260, and designated MBanLIII, shows 61% identity to predicted adenosine-methyltransferases from B. bifidum strains NCIMB41171 and BGN4. Interestingly, this gene appears to be absent in the majority of B. animalis subsp. lactis genome sequences, being only identifiable in the genome of B. animalis subsp. lactis ADO11, while a homolog with 94% identity was present on the genome of B. animalis subsp. animalis ATCC25527.

PacBio SMRT Genome sequencing and Methylobe analysis

In order to identify methylated bases on the genome of B. animalis subsp. lactis CNCM I-2494 [10] and to determine if the identified methylases were responsible for such methylation, the genome of this strain was subjected to SMRT sequencing coupled to a subsequent methylene analysis. Two SMRTbell libraries of power-cleaned genomic DNA were prepared, five SMRT Cells were run using a 10 kb library and adopting the XL polymerase enzyme during which a 120 minute movie was captured. Using this approach the kinetic signature of 5-mC was shown to be weak, making detection during SMRT sequencing challenging. For this reason a conversion of 5-mC to 5-caC (5-carboxyl cytosine) using the Tet1 enzyme (WiseGene), as a means of enhancing the
detection of 5-mC, was performed on genomic DNA randomly sheared to 1.5 kb and subsequently converted into a SMRTbell library [52,53]. Four SMRT cells were run using the C2 polymerase enzyme and two 45 minute movies were captured. For each library the methylome was determined using SMRT Analysis software version 1.4. Genome wide motif analysis resulted in the identification of 5′-RTC6 mAGG-3′ and 5′-GGW5 mCC-3′ as the 6mA and 5mC recognition sequences, respectively (Fig. 1).

The extent of methylation across the genome at 5′-RTC6 mAGG-3′ and 5′-GGW5 mCC-3′ was expected to be 1576 positions, all of which were indeed detected by the methylome analysis, while for 5′-GGW5 mCC-3′, the AvaII recognition sequence, 4420 positions were present on the genome, of which 4124 (93.3%) were detected as being methylated based on our methylome analysis.

Assessment of restriction activity in *B. animalis* subsp. *lactis* CNCM I-2494

To establish if the identified R-M-systems are functional in *B. animalis* subsp. *lactis* CNCM I-2494 and whether or not they affect plasmid-mediated transformation efficiency of this strain, the transformation frequency of two *E. coli*-bifidobacterial shuttle vectors, pDM1 and pDM2 (Fig. 2), was determined when these plasmids had been isolated either from *B. animalis* subsp. *lactis* CNCM I-2494 (in which case the plasmid DNA is assumed to be protected from R-M) or from *E. coli* (such DNA would be expected to be sensitive to the bifidobacterial R-M systems). 200 ng quantities of each of these plasmid DNA preparations, isolated from these different host vectors, were used to transform *B. animalis* subsp. *lactis* CNCM I-2494 by electroporation. Transformants were selected on RGA supplemented with 1% maltose and spectinomycin, and enumerated following anaerobic incubation at 42°C for 36 hours. There was an approximately 27 and 20 fold higher transformation efficiency for the pDM1 and pDM2 plasmid DNAs, respectively, isolated from *B. animalis* subsp. *lactis* CNCM I-2494 as compared to corresponding plasmid DNA isolated from *E. coli* (Fig. 3), thus clearly indicating that one or more of the identified R-M systems encoded by *B. animalis* subsp. *lactis* CNCM I-2494 is functional and affects the efficiency at which plasmids can be introduced in this strain.

M.BanLI and M.BanLII represent two functional R-M methyltransferases in *B. animalis* subsp. *lactis* CNCM I-2494

To determine the precise MTase activity of M.BanLI, the coding sequence of banLII together with a sequence specifying a synthetic ribosome binding site (ATAAGAGGAGCCTCACC) were firstly cloned downstream of the p44 promoter on pNZ44, to generate pNZ44-banLLM. This banLLM-encoding sequence together with the p44 promoter was subsequently amplified from pNZ44-banLLM by PCR and cloned in pWSK29 to generate pWSK29-M.BanLI (see Experimental Procedures for details). Plasmid DNA preparations of pWSK29 (control) or pWSK29-M.BanLI, each isolated from *E. coli* XL1Blue, were analysed by PacBio SMRT sequencing and subsequent methylome analysis. The native R-M systems in *E. coli* XL1Blue, M.EcoKDam and M.EcoKl, methylated the plasmid complement of pWSK29 (control) or pWSK29-M.BanLI at 5′-G6 mATC-3′ and 5′-A6 mACNNNNNGTGC-3′, respectively (data not shown). An additional single stranded 6 methyl adenine sequence, 5′-RTC6 mAGG-3′, was detected at nine positions across the sequence of pWSK29-M.BanLI, thereby attributing M.BanLI to this novel methylation activity encoded by *B. animalis* subsp. *lactis* CNCM I-2494.

In order to verify that *B. animalis* subsp. *lactis* also encodes a distinct MTase that protects, based on sequence similarity to characterised R-Ms and on the obtained methylome data, DNA sequences restricted by AvaII, genomic DNA of *B. animalis* subsp. *lactis* CNCM I-2494 was restricted by AvaII and analysed by agarose gel electrophoresis. The results obtained indeed demonstrated that the *B. animalis* subsp. *lactis* CNCM I-2494 genomic DNA is protected from restriction with AvaII (Fig. 4A), thereby suggesting that the predicted cytosine methyltransferase M.BanLII is responsible for methylation at this recognition sequence. To verify this prediction we constructed pWSK29-M.BanLII, in which the coding sequence of banLII:M.BanLII was transcriptionally fused to the lac promoter on pWSK29. Plasmid DNA preparations of pWSK29-M.BanLII isolated from *E. coli* XL1Blue, were indeed insensitive to digestion with AvaII (Fig. 4B). Collectively, these results demonstrate that the genomic methylation patterns of the *B. animalis* subsp. *lactis* CNCM I-2494 genomic DNA can be attributed to two R-M methyltransferases, namely M.BanLI and M.BanLII, that methylate 5′-RTC6 mAGG-3′ and 5′-GGW5 mCC-3′, respectively and that can be successfully expressed in the heterologous host *E. coli*.

Expression of M.BanLI and M.BanLII in *E. coli* and methylation of plasmid DNA

In order to assess the impact of each identified methylase on the transformation efficiency of *B. animalis* subsp. *lactis* CNCM I-2494 by prior methylation of plasmid DNA, we adopted *E. coli* XL1Blue-pWSK29-M.BanLI and *E. coli* XL1Blue-pWSK29-M.BanLII (expressing M.BanLI and M.BanLII, respectively, and as described above). To evaluate the effect of methylation of plasmid DNA on transformation efficiency, pDM1 was introduced into *E. coli* XL1Blue that already harbored pWSK29-M.BanLI or pWSK29-M.BanLII. Plasmid preparations of *E. coli* pWSK29-M.BanLI + pDM1 or *E. coli* pWSK29-M.BanLII + pDM1 were

![Figure 1. Methylome determination for *B. animalis* subsp. *lactis* CNCM I-2494.](image-url)

Expression of M.BanLII and M.BanLII in *E. coli* and methylation of plasmid DNA
then used for *B. animalis* subsp. *lactis* CNCM I-2494 transformation experiments. pDM1 DNA isolated from *E. coli* harboring pWSK29-M.BanLI gave approximately a 1.8-fold higher transformation frequency as compared to pDM1 from *E. coli* pWSK29, while plasmid DNA isolated from *E. coli* pWSK29-M.BanLII gave a 16-fold higher transformation frequency, thereby demonstrating that each individual R-M system does contribute to a different and in one case very modest degree to the genetic accessibility of *B. animalis* subsp. *lactis* CNCM I-2494.

The above data also revealed a disparity between the transformation results and the number of R.BanLI and R.BanLII recognition sites on pDM1. pDM1 harbours 13 R.BanLI recognition sequences (5'-RTCAGG-3') and 3 R.BanLII recognition sequences (5'-GGWCC-3'), and despite this a higher

Figure 2. Schematic representation of the *E. coli*-bifidobacterial shuttle vectors pDM1 and pDM2. pDM1 is a derivative of pAM5 where the tetracycline resistance gene has been replaced with the spectinomycin resistance cassette. pDM2 is a derivative of the plasmid pDG7 where the spectinomycin resistance cassette was cloned in the unique EcoR1 and HindIII sites of pDG7.

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Figure 3. Transformation of *B. animalis* subsp. *lactis* CNCM I-2494. pDM1 or pDM2 DNA was isolated from *E. coli* (grey bars) or *B. animalis* subsp. *lactis* CNCM I-2494 (black bars). Data presented are averages of triplicate experiments.

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transformation efficiency was observed upon methylation of pDM1 with M.BanLI. To address the unexpectedly low impact of M.BanLI-mediated pDM1 methylation on transformation efficiency and to establish if R.BanLI was indeed active, the complete BanLI R-M system was cloned downstream of the lac promoter on pWSK29 (see experimental procedures) to generate pWSK29-RMBanLI. This construct was introduced into E. coli DSMZ5911 to generate E. coli DSMZ5911-pWSK29-RMBanLI. This strain and E. coli DSMZ5911 or E. coli DSMZ5911-pWSK29 (controls) were used for plaque assays with E. coli P1vir bacteriophage that was propagated on E. coli MG1655 or E. coli EC100. The efficiency of plaquing (EOP) for each phage preparation on E. coli DSMZ5911-pWSK29-RMBanLI relative to the control strains (EOP = 1) was approximately 10^3 for each phage, thereby demonstrating that R.BanLI is active but apparently has very limited restriction activity under the conditions used (Fig. S3).

In order to examine if the combined methylation of pDM1 by M.BanLI and M.BanLI would further increase the transformation efficiency of B. animalis subsp. lactis CNCM I-2494 an additional E. coli strain harbouring pWSK29-M.BanLI-M.BanLI was constructed (see Experimental Procedures). pDM1 was introduced into E. coli X11Blue harbouring pWSK29-M.BanLI-M.BanLI by electroporation. As expected the plasmid complement of pWSK29-M.BanLI-M.BanLI+pDM1 was resistant to restriction with AvaiI, demonstrating that methylation did occur (Fig. 5). Transformation of pDM1 isolated from X11Blue pWSK29-M.BanLI-M.BanLI+pDM1 into B. animalis subsp. lactis CNCM I-2494 gave an approximately 22-fold higher transformation efficiency as compared to unmethylated DNA (Fig. 6). This transformation efficiency is approaching that obtained with plasmid DNA isolated from B. animalis subsp. lactis.

**Discussion**

It is now well established that the ingestion of functional foods or pharmabiotics containing prebiotics and/or probiotic bifidobacteria and lactobacilli can beneficially impact on the microbiota and (thereby) positively influence host physiology [2]. These beneficial effects are thought to be imparted through an array of structural and metabolic microbial features that contribute to restoring and/or maintaining gastrointestinal homeostasis, and include the ability to alter the intestinal microbiota structure or functioning, support colonization resistance against pathogens and influence host immune responses [2,3]. The consumption of a fermented milk product containing B. animalis subsp. lactis CNCM I-2494 has been assessed in several clinical trials and found to be effective in improving digestive comfort in (healthy) adults [4], as well as in an IBS cohort study [5].

Defining how B. animalis subsp. lactis CNCM I-2494 positively contributes to host health requires an in depth molecular understanding of the probiotic attributes of this strain and this can be facilitated through comparative and functional genomics approaches coupled to host response analysis using in vitro or in vivo models. As for all bifidobacteria a major barrier to genetic manipulation of B. animalis subsp. lactis CNCM I-2494 is the lack of reliable molecular tools and procedures for members of this genetically recalcitrant genus. In addition, it is now appreciated that strain-specific R-M barriers are a contributing factor in the inability to genetically access several bifidobacterial strains.
However, these R-M barriers have recently been overcome in several cases by appropriate methylation of plasmid DNA in heterologous hosts [17–19].

The main function of R-M systems is to protect the host against invading foreign DNA, as the unmodified incoming DNA is targeted by the restriction endonuclease component of the R-M. The host DNA is resistant to cleavage as the recognition sites of the endonuclease are modified by the cognate methyltransferase at adenosyl or cytosyl residues. In silico analysis of the genome of *B. animalis* subsp. *lactis* CNCM I-2494 coupled with SMRT sequencing and methylome analysis established that genomic DNA of CNCM I-2494 is methylated at two recognition sequences; the first representing a novel single stranded asymmetric adenosyl-methylation at 5′-RTC\(^{\text{m6m}}\)AGG-3′, and the second a cytosyl-methylation at 5′-GGW\(^{\text{m6}}\)CC-3′. The latter indicates that CNCM I-2494 harbours an isochizomer of the type IIP R-M system AvaII which was subsequently assigned to be BanLI by restriction analysis of methylated plasmid DNA. In addition, the protection afforded to genomic DNA isolated from *B. animalis* subsp. *lactis* CNCM I-2494 from restriction with commercially available AvaII was ascribed to M. BanLI. The novel single stranded adenosyl methylation at 5′-RTC\(^{\text{m6m}}\)AGG-3′ was attributed to M.BanLI and suggests that the BanLI R-M systems may represent an incomplete type IIS R-M system owing to the absence of a second methyltransferase, or methyltransferase domain. The presence of two methyltransferases responsible for sequence recognition and strand-specific methylation is characteristic of type IIS R-M systems, however, for *B. animalis* subsp. *lactis* CNCM I-2494 just one methyltransferase-encoding gene was present in the BanLI R-M system, and it may just be that the second methylase-encoding gene was lost due to an integration event upstream of *banLI*. It is thought that recognition and methylation of strand-specific sequences by type IIS R-M systems have also been found to be encoded on conjugative plasmids in *Lactococcus lactis*, the LlaJI R-M system was identified on a 46.2 kb plasmid, pTR2030 [60,61], while the LlaJI R-M system was identified as one of three phage resistance systems present on the 65 kb plasmid, pNP10 [55–57]. The impact of the absence of the second methyltransferase for the BanLI system of *B. animalis* subsp. *lactis* CNCM I-2494 was further evident from the bacteriophage plaque assays conducted with *E. coli* DSMZ5911-pWSK29-RMBanLI where R.BanLI conferred only a very minor protection against phage infection as compared to infection of *E. coli* DSMZ5911-pWSK29 thereby suggesting that R.BanLI is not fully active. This likely represents an example of specific gene loss within the *B. animalis* subsp. *lactis* CNCM I-2494 (and apparently all known *B. animalis* subsp. *lactis* strains) genome with consequent loss of functionality of the BanLI Type IIS R-M system.

The contribution of each R-M system in impeding plasmid transformation of *B. animalis* subsp. *lactis* CNCM I-2494 was determined and it was established that both BanLI and BanLII impact on the transformation efficiency, albeit to different degrees. In agreement with our bacteriophage plaque assays and in silico analysis we observed only a modest 1.8 fold increase in transformation efficiency of pDM1 plasmid DNA methylated with M.BanLI despite the presence of 13 BanLI sites on this 65 kb plasmid, pNP10 [55–57]. However, these R-M barriers have recently been overcome in several cases by appropriate methylation of plasmid DNA in heterologous hosts [17–19].
efficiency of M.BanLII methylated pDM1 DNA was observed as compared to unmethylated DNA. To facilitate complete methylation of plasmid DNA by both M.BanLI and M.BanLII, and thereby enhancing the transformation efficiency of B. animalis subsp. lactis CNCM I-2494, an additional E. coli strain was constructed expressing both M.BanLI and M.BanLII. pDM1 isolated from this strain had a 22 fold higher transformation efficiency as compared to unmethylated DNA. Methylation of plasmid DNA with M.BanLI and M.BanLII heterologously expressed in E. coli allows the restriction barrier imposed by B. animalis subsp. lactis CNCM I-2494 to be overcome. The ability to overcome this restriction barrier combined with the development of molecular tools will advance research on the molecular biology, physiology and understanding of the molecular mechanisms by which B. animalis subsp. lactis CNCM I-2494 cross-talks with the human host.

Supporting Information

Figure S1 Comparison of the predicted banLII and banLI genetic loci of B. animalis subsp. lactis CNCM I-2494 with corresponding R-M encoding loci from other sequenced B. animalis subsp. lactis strains and other bifidobacteria. Each solid arrow indicates an open reading frame. The lengths of the arrows are proportional to the length of the predicted open reading frame. The colour coding which is indicative of its putative function, is indicated within the arrow. Orthologs are marked with the same colour while the amino acid identity of each predicted protein is indicated as a percentage relative to its equivalent protein encoded by B. animalis subsp. lactis CNCM I-2494. (TIF)

Figure S2 (A) Sequence alignment of M.BanLI and M.EcoRI. Amino acids are shaded if both of the depicted proteins contain identical residues at corresponding positions. The boxed sequences CMI and CMII represent two of the conserved regions of N6-adenine MTases. (B) Alignment of the six highly conserved motifs of the predicted cysteine methyltransferase M.BanLI with annotated cysteine methyltransferases from B. breve UCC2003 and B. adolescentis ATCC 15703 and MAHl and M.EcoRII. Highly conserved amino acids are marked with an asterisk above the sequence and indicated in bold. Amino acids are shaded in grey if at least three of the depicted protein sequences contain an identical residue at a particular position. (TIF)

Table S1 Oligonucleotide primers used in this study (DOCX)

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Author Contributions

Conceived and designed the experiments: MOCM TS DeS. Performed the experiments: MOCM DW TG. Analyzed the data: MOCM DW FB TC. Contributed reagents/materials/analysis experiments: MOCM DW TC. Made the first draft of the paper: MOCM DvS. Contributed reagents/materials/analysis experiments: MOCM DW TC. Analyzed the data: MOCM DW FB TC JRJ JK JvHV PG CC TS DVS. Wrote the paper: MOCM DeS.

References

1. Bermudez-Brito M, Plaza-Diaz J, Mateos-Quezada S, Gomez-Lorente C, Gil A. (2012) Probiotic mechanisms of action. Annu Nutr Metab 61: 160–174.
2. Turroni F, Ventura M, Butto LF, Duranti S, O’Toole PW, O’Connell Motherway M, et al. (2014) Molecular dialogue between the human gut microbiota and the host: a Lactobacillus and Bifidobacterium perspective. Cell Mol Life Sci 71: 183–203.
3. Ventura M, Turroni F, O’Connell Motherway M, MacSharry J, van Sinderen D (2012) Overcoming the restriction barrier to plasmid transformation and targeted mutagenesis in bifidobacteria. Trends Microbiol 20: 467–476.
4. Alvarez A, Houghlon LA, Morris J, Reilly R, Guyonnet D, et al. (2009) Clinical trial: the effects of a fermented milk product containing Bifidobacterium lactis DN-173 010 on abdominal distension and gastrointestinal transit in irritable bowel syndrome with constipation. Aliment Pharmacol Ther 29: 104–114.
5. Guyonnet D, Schlunberger A, Mhamdi L, Jakob S, Chasaney O (2009) Fermented milk containing Bifidobacterium lactis DN-173 010 improves gastrointestinal well-being and digestive symptoms in women reporting minor digestive symptoms: a randomised, double-blind, parallel, controlled study. Br J Nutr 102: 173 010 on abdominal distension and gastrointestinal transit in irritable bowel syndrome with constipation. Aliment Pharmacol Ther 29: 104–114.
6. Tillisch K, Labus J, Kilpatrick L, Jiang Z, Stains J, et al. (2013) Consumption of fermented milk product with probiotic modulates brain activity. Gastroenterology 144: 1394–1401.
7. Veiga P, Gallina CA, Beal C, Michaud M, Delaney ML, DuBois A, et al. (2010) Bifidobacterium animalis subsp. lactis fermented milk product reduces inflammation by altering a niche for colitogenic microbes. Proc Natl Acad Sci U S A 107: 1654–1662.
8. Tillisch K, Labus J, Kilpatrick L, Jiang Z, Stains J, et al. (2013) Consumption of fermented milk product with probiotic modulates brain activity. Gastroenterology 144: 1394–1401.
9. Veiga P, Gallina CA, Beal C, Michaud M, Delaney ML, DuBois A, et al. (2010) Bifidobacterium animalis subsp. lactis fermented milk product reduces inflammation by altering a niche for colitogenic microbes. Proc Natl Acad Sci U S A 107: 1654–1662.
10. Tillisch K, Labus J, Kilpatrick L, Jiang Z, Stains J, et al. (2013) Consumption of fermented milk product with probiotic modulates brain activity. Gastroenterology 144: 1394–1401.
11. Guglielmetti S, Mayo B, Alvarez-Martin P (2013) Mobilone and genetic modification of bifidobacteria. Benef Microbes 4: 160–166.
12. Bronaccio VF, Zhurina DS, Riedel CU (2013) Tough nuts to crack: site-directed mutagenesis of bifidobacteria remains a challenge. Bioengineered 4: 197–202.
13. Sun Z, Baur A, Zhurina D, Yuan J, Riedel CU. (2012) Accessing the inacessible: molecular tools for bifidobacteria. Appl Environ Microbiol 78: 5093–5092.
14. Ruiz L, O’Connell-Motherway M, Lanigan N, van Sinderen D (2013) Transposon mutagenesis in Bifidobacterium breve: construction and characterization of a Tn5 transposon mutant library for Bifidobacterium breve UCC2003. PLoS One 8(5):e64699.
15. Law J, Buist G, Haandriksen A, Kok J, Venema G, Leenhouts K (1995) A system to generate chromosomal mutations in Lactococcus lactis which allows fast analysis of targeted genes. J Bacteriol 177: 7011–7018.
16. O’Connell Motherway M, Fitzgerald GF, Neirynck S, Ryan S, Steidler L, et al. (2008) Characterization of ApdB, an extracellular type II amylolysin adhancer from Bifidobacterium breve UCC2003. Appl Environ Microbiol 74: 6271–6279.
17. O’Connell Motherway M, O’Driscoll J, Fitzgerald GF, and van Sinderen D (2009) Overcoming the restriction barrier to plasmid transformation and targeted mutagenesis in Bifidobacterium breve UCC2003. Microbiol Biotechnol 2: 321–332.
18. Yasui K, Kano Y, Tanaka K, Watanabe K, Shimizu-Kadota M, et al. (2009) Improvement of bacterial transformation efficiency using plasmid artificial modification. Nucleic Acids Res 37(1):e3
19. Kim JY, Wang Y, Park MS, Ji GE. (2010) Improvement of transformation efficiency through in vitro methylation and SacII site mutation of plasmid vector in Bifidobacterium longum MG1. J Microbiol Biotechnol 20: 1022–1026.
20. Pokusaeva K, Neves AR, Zomer A, O’Connell Motherway M, MacSharry J, et al. (2010) Ribose utilization by the human commensal Bifidobacterium breve UCC2003. Microbiol Biotechnol 3(3): 311–321.
21. Pokusaeva K, O’Connell-Motherway M, Zomer A, MacSharry J, Fitzgerald GF, et al. (2011) Cellodextrin utilization by Bifidobacterium breve UCC2003. Appl Environ Microbiol 7: 1681–1690.
22. O’Connell Motherway M, Kinsella M, Fitzgerald GF, van Sinderen D (2013) Transcriptional and functional characterization of genetic elements involved in.
galacto-oligosaccharide utilization by \textit{Bifidobacterium breve} UCC2003. Microb Biotechnol 6: 67–79.

23. O’Connell Motherway M, Fitzgerald GF, van Sinderen D (2011) Metabolism of a plant derived galactose-containing polysaccharide by \textit{Bifidobacterium breve} UCC2003. Microb Biotechnol 4: 403–416.

24. O’Connell Motherway M, Zomer A, Leahy SC, Reunanen J, Bottacini F, et al. (2011) Functional genome analysis of \textit{Bifidobacterium breve} UCC2003 reveals type IVb tight adherence (Tad) pil as an essential and conserved host-colonization factor. Proc Natl Acad Sci U S A 108: 11217–11222.

25. O’Connell KJ, O’Connell Motherway M, Hennessy AA, Brodhun F, Ross RP, et al. (2013a) Identification and characterization of an oleate hydrolase-encoding gene from \textit{Bifidobacterium breve}. Bioengineered 3: 313–321.

26. O’Connell KJ, O’Connell Motherway M, O’Callaghan J, Fitzgerald GF, Ross RP, et al. (2013b) Metabolism of four \(\pi\)-glycosidic linkage-containing oligosaccharides by \textit{Bifidobacterium breve} UCC2003. Appl Environ Microbiol 79: 6280–6292.

27. Fanning S, Hall LJ, Cronin M, Zomer A, MacSharry J, et al. (2012) Bifidobacterial surface-exopolysaccharide facilitates commensal-host interaction through immune modulation and pathogen protection. Proc Natl Acad Sci U S A 109: 2108–2113.

28. Ruiz L, O’Connell Motherway M, Zomer A, de los Reyes-Gavilán CG, Margolles A, et al. (2012) A bile-inducible membrane protein mediates bifidobacterial biofilm resistance. Microb Biotechnol 5: 525–535.

29. Alvarez-Martin P, Fernández M, O’Connell-Motherway M, O’Connell KJ, Sauvaudet N, et al. (2012) A conserved two-component signal transduction system controls the response to phosphate starvation in \textit{Bifidobacterium breve} UCC2003. Appl Environ Microbiol 78: 5258–5269.

30. Alvarez-Martín P, O’Connell Motherway M, Turroni F, Foroni E, Ventura M, et al. (2012) A two-component regulatory system controls autoregulated sorbit expression in \textit{Bifidobacterium breve} UCC2003. Appl Environ Microbiol 78: 7032–7041.

31. Bottacini F, O’Connell Motherway M, Kuczynski J, O’Connell KJ, Serafini F, et al. (2014) Comparative genomics of the \textit{Bifidobacterium breve} taxon. BMC Genom In Press.

32. Fukuda S, Toh H, Hase K, Oshima K, Nakashima Y, et al. (2011) Bifidobacteria can protect from enteropathogenic infection through production of acetate. Nature 469: 543–547.

33. Hiraoka Y, Saito K, Fukuma H, Murayama H, Kano Y, et al. (2012) Development of a double-crossover markerless gene deletion system in \textit{Bifidobacterium longum}: functional analysis of the \(\sigma\)-galactosidase gene for raffinose assimilation. Appl Environ Microbiol 78: 4984–4994.

34. Sakaguchi K, He J, Tani S, Kano Y, Suzuki T. (2012) A targeted gene knockout method using a newly constructed temperature-sensitive plasmid mediated homologous recombination in \textit{Bifidobacterium breve}. J Cell Sci 115: 195–199.

35. O’Riordan K. (1998) Studies on antimicrobial activity and genetic diversity of \textit{Bifidobacterium} species: molecular characterization of a 5.75 kb plasmid and a chromosomally encoded \(m\)va gene homologue from \textit{Bifidobacterium breve}. Ph.D. National University of IrelandCork, Cork.

36. Wells JM, Wilson PW, Le Page RW (1993) Improved cloning vectors and transformation procedure for \textit{Lactococcus lactis}. J Appl Bacteriol 74: 629–636.

37. De Man JC, Rogosa A, Sharpe M E (1960) A medium for the cultivation of \textit{Lactobacillus}. J Appl Bacteriol 23: 135–158.

38. Sambrook J, Fritsch EF, Maniatis T (1989). Molecular cloning a laboratory manual. Cold Spring Harbor Laboratory Cold Spring Harbor NY.

39. Kozdon JB, Melfi MD, Luong K, Clark TA, Boitano M, et al. (2013) Global methylation state at base-pair resolution of the Caulobacter genome throughout the cell cycle. Proc Natl Acad Sci U S A 110: E6350–E6467.

40. Hill C, Miller LA, Klaenhammer TR (1991) In vivo genetic exchange of a chromosomally encoded \(m\)va gene homologue from \textit{Bifidobacterium breve} UCC2003. Appl Environ Microbiol 79: 7032–7039.

41. Alves-Martí P, O’Connell Motherway M, van Sinderen D, Mayo B. (2007). Comparative genomics of the \textit{Bifidobacterium breve} taxon. BMC Genom In Press.

42. Roberts RJ, Vincze T, Posfai J, Macelis D. (2010) REBASE—a database for DNA restriction and modification: enzymes, genes and genomes. Nucl Acids Res 38: D234–D236.

43. O’Riordan K. (1998) Studies on antimicrobial activity and genetic diversity of \textit{Bifidobacterium} species: molecular characterization of a 5.75 kb plasmid and a chromosomally encoded \(m\)va gene homologue from \textit{Bifidobacterium breve}. Ph.D. National University of IrelandCork, Cork.

44. Sambrook J, Fritsch EF, Maniatis T (1989). Molecular cloning a laboratory manual. Cold Spring Harbor Laboratory Cold Spring Harbor NY.

45. Jansson JK, Riedel A, Stahl DA (1994) Application of the \(\phi\)29 phage as a host vector for \(m\)va gene homologue from \textit{Bifidobacterium breve} UCC2003. Appl Environ Microbiol 60: 7546–7556.

46. Alvarez-Martin P, O’Connell Motherway M, van Sinderen D. (2001) Improvement and optimization of two engineered phage resistance mechanisms in \textit{Lactococcus lactis}. Appl Environ Microbiol 67: 608–616.

47. Wang RF, Keshner SR. (1991) Construction of versatile low-copy-number vectors for cloning, sequencing and gene expression in \textit{Escherichia coli}. Gen 100: 195–199.

48. Sakaguchi K, He J, Tani S, Kano Y, Suzuki T. (2012) A targeted gene knockout method using a newly constructed temperature-sensitive plasmid mediated homologous recombination in \textit{Bifidobacterium breve}. Appl Environ Microbiol 78: 4984–4994.

49. O’Riordan K. (1998) Studies on antimicrobial activity and genetic diversity of \textit{Bifidobacterium} species: molecular characterization of a 5.75 kb plasmid and a chromosomally encoded \(m\)va gene homologue from \textit{Bifidobacterium breve}. Ph.D. National University of IrelandCork, Cork.

50. Sugisaki H, Yamamoto K, Takanami M (1991) The HgaI restriction-modification system contains two cytosine methylase genes responsible for modification of different DNA strands. J Biol Chem 266: 13952–13957.

51. O’Connell KJ, O’Connell Motherway M, Fitzgerald GF, van Sinderen D, Mayo B. (2007). Comparative genomics of the \textit{Bifidobacterium breve} taxon. BMC Genom In Press.

52. Bottacini F, O’Connell Motherway M, Kuczynski J, O’Connell KJ, Serafini F, et al. (2014) Comparative genomics of the \textit{Bifidobacterium breve} taxon. BMC Genom In Press.

53. O’Connell KJ, O’Connell Motherway M, Kuczynski J, O’Connell KJ, Serafini F, et al. (2014) Comparative genomics of the \textit{Bifidobacterium breve} taxon. BMC Genom In Press.

54. Alvarez-Martin P, O’Connell Motherway M, Fitzgerald GF, et al. (2004) Lactococcal plasmid pNP0 encodes a novel, temperature-sensitive restriction-modification system. Appl Environ Microbiol 70: 5546–5556.

55. O’Connell KJ, O’Connell Motherway M, Fitzgerald GF, van Sinderen D. (2001) A dichotomous epigenetic mechanism governs expression of the \(Lla\) restriction/modification system. Mol Microbiol 37: 1532–41.

56. O’Connell KJ, Heite DF, Wilson GG, Fitzgerald GF, Roberts R, et al. (2006) A genetic digression of the \(Lla\) restriction casette reveals insights on a novel bacteriophage resistance system. BMC Microbiol 6: 40.

57. Kaczorowski T, Selsk M, Skowron P, Podhajsk A (1999) The FokI methyltransferase from \textit{Flavobacterium koreense}. J Gen Microbiol 145: 223–236.