DNA methyltransferase enzymes (MTases) may not enhance genomic protection against exogenous DNA invasions

by

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Abstract

DNA methylation is thought to function as a host-driven response against extracellular invaders in all three domains of life. Here I devise and test an alternative hypothesis, **the transposon-mediated immunity hypothesis**, that gene regulation via DNA methylation originated in ancestral eubacteria as a transposon-mediated countermeasure to endonuclease cleavage. I predict that transposon-coded proteins may have influenced eubacterial DNMTases into hypermethylating eubacterial restriction endonuclease sites, decreasing endonuclease enzyme's accessibility to palindromic sequences and rendering rm system II ineffective at removing transposable elements from the genome.

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Contributions:

Dr. Chris Hassel and I worked on the algorithm for R to assign species-specific, sequence probes that correspond to the endonuclease restriction and methylase sequences for the 68-eubacterial species.

Dani Fraser and I worked closely on the phylogenetic comparative method in R to correlate the total number of endonuclease restriction to the methylase sites.

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Abstract

DNA methylation is an epigenetic signal that regulates gene expression. The main hypothesis concerning its origin, the genomic autoimmunity hypothesis, claims that DNA methylation arose as a host-driven response against extracellular invaders. Here I devise and test a novel hypothesis, **the transposon-mediated immunity hypothesis**, that gene regulation via DNA methylation originated in ancestral eubacteria as a transposon-mediated countermeasure to endonuclease cleavage. I predict that transposon-coded proteins may have influenced eubacterial DNMTases into hypermethylating eubacterial restriction endonuclease sites, decreasing endonuclease enzyme's accessibility to palindromic sequences and rendering rm system II ineffective at removing transposable elements from the genome. To test this hypothesis, I compared 666 palindromic endonuclease recognition sequences to 466 methylase sequences of restriction modification system II in 262 eubacterial and archaeabacterial species. Furthermore, I analyzed 16S ribosomal RNA for 40 microbial species belonging to 23 families to quantify the number of methylated restriction sites in each microbial genome. I built a phylogeny based on 16S rRNA and tested for phylogenetic dependence using Pagel's lambda (λ). I found that endonuclease restriction sequences overlap with the corresponding methylase sequences 100% of the time. This result is consistent with my hypothesis that type II restriction enzymes experience "cognate" methylation (methylation that is conferred by the species-specific DNMTases) and result in the incomplete formation of enzyme-substrate complex between the restriction enzymes and their recognition sites. Hypermethylation of restriction sites by DNMTases seem to render rm system II ineffective at removing exogenous DNA from the genomes of eubacteria and archaeabacteria.

Introduction

DNA methylation is a chemically stable covalent modification of cytosine (Razin and Riggs 1980; Wolffe Matzke 1999; Poole *et al.* 2001) and adenine (Hattman *et al.* 1978; Vanyushin 2005), catalyzed by DNA methyltransferase (MTase) enzymes. MTases utilize S-adenosyl-L-methionine (AdoMet) methyl donors to transfer methyl groups to an array of nucleophiles (Wu and Santi 1985), such as nitrogen, oxygen, sulfur, and activated carbon atoms in DNA (Bujnicki and Radlinska 1999; Cheng and Roberts 2001). Eubacteria, archaea, protists, fungi, plants, invertebrates, and vertebrates contain DNA methylation. However, methylation is not universal to the eukaryotic kingdom because organisms such as, *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe*, and *Caenorhabditis elegans* lack detectable methylation signatures in their genomes (Colot and Rossignol 1999).

There are three main forms of DNA methylation: 5-methylcytosine (5mC), N4-methylcytosine (N4mC) and, N6-methyladenine (N6mA) (Bujnicki and Radlinska 1999) (Figure 1). 5mC is universal to all three domains of life, with few exceptions. By contrast, N6mA is found in eubacteria, the nuclear genome of ciliated protists *Tetrahymena pyriformis* and *Paramecium aurelia*, dinoflagellate *Peridinium triquetrum*, green algae, fungi, and organelle genomes of plants (Hattman *et al.* 1978; Vanyushin 2005). N4mC is only found in thermophilic and mesophilic eubacteria, archaea, and in RNA of the small ribosomal subunits of insect and mammalian mitochondria (Woese *et al.* 1975; Dubin and Hsueh 1983; Ehrlich *et al.* 1987; Noyer-Weidner and Trautner 1993; Bujnicki and Radlinska 1999; Sartori *et al.* 2002). Some flagellate protists such as kinetoplastids and dinoflagellates produce two alternative forms of DNA methylation, β -D-glucosyl-hydroxymethyluracil (β -Gluc-HOMeUra) and 5-hydroxymethyluracil respectively (Rae 1973; van Leeuwen *et al.* 1998) (Figure 2).

Figure 1. Three main types of covalently methylated nitrogenous base products.

Figure 2. Alternative forms of DNA methylation. Hexameric repeat arrays of chromosome telomeres and adjacent repetitive sub-telomeric DNA of kinetoplastids contains β -D-glucosyl-hydroxymethyluracil, also referred to as J. A majority of dinoflagellate genomes contain 5-hydroxyuracil, and, on average, nearly 40% of thymine is replaced by 5-hydroxyuracil. Thymine is merely methylated uracil.

The presumptive functions and sequence specificity of DNA methylation varies among eubacteria, archaea, and eukarya. Eukaryotic DNA methylation regulates gene expression, genomic immunity, genomic imprinting, genome-wide epigenetic resets, heterochromatin

formation, and determination of primary sex characteristics (Hall 1990; Barlow 1993; Haig 2002; Farthing *et al.* 2008; Gorelick. and Carpinone 2009; Kaneko-Ishino and Ishino 2010; Gorelick *et al.* 2011). In contrast, eubacteria and archaea use methylation as part of the restriction modification systems (rm) I, II, and III, to protect themselves from exogenous DNA insertions (Razin and Razin 1980; Zillig *et al.* 1988; Noyer-Weidner and Trautner 1993; Kobayashi *et al.* 1999; Grogan 2003).

Restriction modification (rm) systems of eubacteria and archaea can be divided into different types according to subunit composition and cofactor requirements (Wilson 1991; Wilson and Murray 1991). Type I rm system consists of three types of subunits, 'r' for restriction, 'm' for modification, and 's' for specificity and recognize bipartite recognition sequences. Methyl modification takes places within the recognition sequence, while cleavage takes place either upstream or down-stream and requires AdoMet, as well as large amounts of ATP hydrolysis (Bickle and Krüger 1993). Type III rm system consists of two subunits: 'r' and 'm'. Restriction enzymes of the rm III system require both 'r' and 'm' subgroups, as well as the presence of ATP while the methyltransferase functions in the presence of AdoMet. The modification takes place within the recognition site, however, the cleavage happens approximately 25bp downstream of the recognition site (Bickle and Krüger 1993).

Eubacterial restriction modification system II functions with two independent enzymatic units: homodimeric restriction enzymes (ENases) and monomeric DNA methyltransferases (MTases). The ENases have endonuclease activity that target the invading transposable elements and are not dependent on AdoMet or ATP to function (Pingoud and Jeltsch 1997; Nagornykh *et al.* 2008). Methyltransferases have DNA methylation capability and only require AdoMet as a cofactor to function (Razin and Riggs 1980; Razin *et al.* 1984; Nagornykh *et al.* 2008).

Endonucleases are highly sequence-specific and species-specific enzymes that target and cleave exogenous and endogenous nucleotides (Wilson 1991). For example, *EcoRI* restriction endonuclease cleaves DNA only at recognition site G/AATTC and produce 5' sticky ends in *Escherichia coli*, whereas *BamHI* only cleaves DNA at recognition sequence site G/GATCC in *Bacillus amyloliquefaciens* (Dale and Park 2010). *EcoRI* restriction endonuclease cannot function in the absence of palindromic recognition sequences nor in species other than *Escherichia coli*, specificity also found in *BamHI* and hundreds of other restriction enzymes.

I exclusively analyzed the type II rm system because it can be studied in the absence of endonuclease (restriction) activity, unlike with type I and III rm system, in which a single enzymatic unit performs the dual functions of methylation and restriction cleavage. Type II restriction enzymes typically recognize palindromic recognition sites that are 4-8bp in length, and the modification and cleavage occur within the recognition sites. In addition, type II restriction modification is the most prominent and frequently occurring genomic line of defense in a majority of eubacterial species and has the capability to produce N6-methyladenine, N4-methylcytosine and 5-methylcytosine. The role, ubiquity, diversity, and independent evolution of rm system II suggests that it plays an important role in the population structure and (co)evolution of bacteria with invading exogenous DNA elements (Levin 1993).

Exogenous DNA elements, such as viruses, repetitive elements, transposons, and retrotransposons, are capable of rapid mobilization via transposition (McClintock 1950; Kimura 1968; Bestor 1990; Levin 1993; Bheemanaik *et al.* 2006; Suzuki *et al.* 2007; Harony and Ankri 2008; Kaneko-Ishino and Ishino 2010). Transposons are present in almost all eubacteria, archaeabacteria, and eukarya, and occupy a significant portion of the genome. For example, about half of the human genome is derived from transposable elements (Boyes and Bird, 1991),

with more than a dozen hominoid functional genes originating from ancestral transposons (Lander *et al.* 2001; Allis *et al.* 2007). Transposons have a high degree of genetic and functional diversity and remarkable ability to adapt and thrive within host lineages. Some eubacterial species require exogenous DNA for the survival and spread while in other eubacterial species exogenous DNA enhance the ability of eubacterial populations to colonize and adapt to changing environments (McClintock 1950). Previous studies have shown that exogenous DNA provides (1) aid in the production of allelopathic agents such as bacteriocins (Tani and Nasu 2010), (2) protection against pathogens (Levin 1993), oxidation and fermentation of toxic carbon compounds (Liu 2006), and (3) resistance to the adverse effects of ultraviolet light, heavy metals (He and Hader 2002), and antibiotics (Courvalin 1994; Walker 1996; Tani and Nasu 2010).

Some transposable elements lack a strong target site preference, and their distribution appears more or less random. However, transposition is rarely fully random and majority of transposons are highly selective for the sites where they insert. A primary example for targeted transposition is the bacterial Tn7 transposon. Tn7 is highly specialized to insert into a single sequence motif in the *E. coli* genome (Lichtenstein and Brenner 1982). Kirchner *et al.* (1995) and Devine and Boeke (1996) showed that Ty LTR retrotransposons of the yeast *S. cerevisiae* genome exhibit strong integration bias into Ty1 and Ty3 elements that are upstream of RNA polymerase III transcribed genes. The single-celled eukaryote, *Dictyostelium discoideum* has a highly compact genome, and transposons in *D. discoideum* employ two strategies for site-directed insertion. One strategy is to integrate into previously inserted transposon clusters (Loomis *et al.* 1995). The other strategy is targeted insertion into non-protein coding regions of the genome (Craigie *et al.* 1987; Winckler *et al.* 2005). However, the question of whether

transposons preferentially insert in methylated regions of the host genome has yet to be answered.

The genomic autoimmunity hypothesis is the primary hypothesis that addresses the role of DNA methyltransferases in the restriction modification system II of eubacteria and archaeabacteria. The genomic autoimmunity hypothesis postulates that the “cognate” methyltransferases have evolved to hypermethylate endonuclease recognition sites in order to protect the host genome against its own restriction cleavage, a preventative method against genomic autoimmunity (Razin *et al.* 1984; Nagornykh *et al.* 2008; Dale and Park 2010). In other words, eubacteria and archaea use DNA methyltransferase enzymes to hypermethylate host loci to prevent endonuclease restriction cleavage of host’s genomic material. However the genomic autoimmunity hypothesis cannot explain the occurrence of the “rare cutter” restriction enzymes whose lengthy recognition sequences (approximately eight to ten base pairs) are unlikely to be present in many eubacterial viruses and/or transposable elements (Naito *et al.* 1995) and often lead to the restriction of host DNA.

Wilson and Murray (1991) showed that the genes encoding the restriction modification unit of *EcoRI* are capable of leaving the eubacterial genome via plasmids and thus participate frequently in horizontal gene transfer. In a majority of cases, the methyltransferases and endonuclease restriction enzymes do not leave the bacterial host cell in unison, but rather participate independently in horizontal gene transfer (Jeltsch and Pingoud 1996). Therefore, at times the host genome is left with endonuclease restriction enzymes without the corresponding methyltransferases, which results in the death of the bacterial host cell. Thus, Dawkins (1989) and Naito *et al.* (1995) describe the behavior of the restriction modification system II as parasitic, symbiont, and “selfish,” mobile genetic elements.

Furthermore, Type II DNA methyltransferases do not distinguish between endogenous and exogenous DNA (Lauster *et al.* 1989). Rather, DNMTases search along a given DNA strand and covalently modify cytosine and adenine nitrogenous bases. The DNA target-recognition domains (TRD's) of the amino acid chain of type II MTases are responsible for identifying the methylase recognition sites (Lauster *et al.* 1989; Kumar 2004). However, Lauster *et al.* (1989) postulated that eubacterial type II MTases do not have an enzymatic domain that regulates the identification of self to non-self. According to Kruger and Reuter (1999) and Kumar (2004) the target recognition domain (TRD) is not conserved between different groups of eubacterial MTases, and is completely devoid of self-recognition motifs. A significant characteristic of eubacterial DNA methylation is that eubacterial MTases will methylate all available target sites without discrimination as long as the sites are found within the methylase recognition sequences (Kumar 2004).

Is there a fundamental difference between exogenous and endogenous DNA in eubacteria and archaeabacteria? If there is no, how can the host genome protect itself against extracellular invaders? Even though, type II methyltransferases cannot distinguish between exogenous and endogenous DNA, transposons and retrotransposons have unique structural characteristics that differ from the host's genomic material. All DNA transposons belonging to eubacteria and archaeabacteria contain two sets of flanking repeat sequences referred to as IS (insertion sequence) elements and/or IS-like elements. The IS elements are either direct or inverted repeats ranging from 20-40bp in length (Starlinger 1980; Calos and Miller 1980). All DNA transposons also carry the transposase gene that is essential for transposition. Transposase is a transposon-coded DNA binding protein that is essential for the formation of the transposome, a nucleoprotein synaptic complex. Transposomes contain the DNA of the transposable element,

the transposon-encoded transposase enzyme, targeted host DNA, and host-encoded proteins factors (Surret *et al.* 1987; Agrawal *et al.* 1998; Hiom *et al.* 1998; Izsvaik *et al.* 2002; Kapitonov and Jurka 2005; Walisko *et al.* 2006).

Calos and Miller (1980) postulated that the insertion sequences and/or transposase gene can be used as molecular markers to locate transposon-derived genes in eubacteria and eukarya because the insertion sequences and/or the transposase gene remain as part of the host genome post-transposition. Rice and Marshall (1992) discovered the presence of conserved 121bp-long inverted repeats belonging to Tn552 and Tn4002 transposons in the transposon-derived, beta-lactamase gene in *Enterococcus faecalis* strains CH19 and CX19. Tamura *et al.* (2000) used the conserved, inverted terminal repeats of piggyBac transposon in the germline of the silkworm, *Bombyx mori* to confirm if piggyBac transposition is transgenerational. Furthermore, Youngson *et al.* (2005) showed that the sushi-ichi transposase gene is highly conserved and remains intact in nine Ty3/gypsy retrotransposon-derived mammalian-genes. Kapitonov and Jurka (2004) identified a widely expressed HARBI1 gene encoding a 350 amino acid protein that is entirely derived from Harbinger transposase belonging to the Harbinger superfamily of DNA transposons. The HARBI1 gene is believed to have transposed 450-500 million years ago and is conserved in humans, rats, mice, cows, pigs, chickens, frogs, and bony fish. Therefore, even though host-encoded, type II methyltransferases cannot distinguish between exogenous and endogenous DNA, transposons and retrotransposons have unique structural characteristics such as flanking insertion sequences and transposase genes. Insertion sequences and/or transposase genes are conserved in transposon-derived genes in eubacteria and eukarya and thus can be used to set apart transposon-derived elements from host genomic material.

Based on the notion that transposable elements are structurally unique pre and post transposition, I present a modified version of the genomic autoimmunity hypothesis termed the “transposon-mediated immunity hypothesis”, which emphasizes the possible role of transposable elements in regulating the function of type II DNA methyltransferases. **I hypothesize that gene regulation via DNA methylation originated in ancestral eubacteria as a transposon-mediated countermeasure to endonuclease cleavage.** Transposon-coded proteins influenced eubacterial DNMTases into hypermethylating eubacterial restriction endonuclease sites. This decreases restriction enzyme’s accessibility to palindromic sequences and may prevent restriction system II from removing transposable elements from the genome (Figure 3). Therefore, I predict that “cognate” methyltransferase sites (methylation conferred by species-specific MTases) present in a majority of microbial genomes are similar in nucleotide identity and frequency of occurrence to restriction endonuclease sites.

Figure 3. Methylation of palindromic sites prevents systematic cleavage of transposons by endonuclease restriction enzyme, *EcoRI*. 1) *EcoRI* is part of the restriction endonuclease system and uses palindromic sequence GAATTC to attach and initiate exogenous DNA cleavage. 2) GGACC is the target sequence for *Dcm* methylase enzyme. Due to specificity of *Dcm* methylase, only nucleotide sequences that are identical to its methylation pattern will undergo covalent modification. 3) Post-methylation of CCAGG sequence by *Dcm* methylase. Black lines represent the added methyl groups to the cytosine nitrogenous bases and the green colored cytosine represent the overlapping region between methylase and restriction sites. 4) *EcoRI* restriction enzyme can no longer gain access to the restriction site to initiate cleavage due to molecular hindrance created by the added methyl groups.

Materials and methods:

Sequence comparison

I extracted 666 palindromic restriction sequences of 344 endonuclease enzymes and 466 corresponding methylase sequences of 212 “cognate” type II methyltransferases for 261 microbial species belonging to archaea and eubacteria. In order to avoid sampling bias, I did not

pick the 261 species, but rather chose the 344 most prominent endonuclease restriction enzymes that are exclusive to the restriction modification system II. These 344 enzymes are not present in species that use type I or type III restriction modification systems. I used the online restriction enzyme database REBASE at <http://rebase.neb.com/rebase/rebase.html>, NCBI GenBank at <http://www.ncbi.nlm.nih.gov/genbank/>, volume 5 of *Gene Amplification and Analysis* (Roberts 1987), and Blakesley *et al.* (1981) to collect the sequence information. Then I used ClustalX2 (Larkin *et al.* 2007) to align each endonuclease sequence with the corresponding methylase sequence to calculate the percent nucleotide identity.

Next, I quantified the total number of methylated restriction sites for each of the 68 species (66 eubacterial and 2 archaeabacterial) in 27 families. The sample size reduced from 261 to 68 species due to lack of sequence data available on GenBank, i.e. a majority of the eubacterial species that I used in the analysis were not sequenced at these loci.

I extracted fully and partially sequenced 16S ribosomal RNA sequences in FASTA format and obtained the corresponding accession numbers from NCBI GenBank. I used the 16S rRNA gene because it is one of the few gene loci that are conserved amongst all eubacterial species. I attempted to use 12S rRNA and RNA polymerase β as secondary and tertiary molecular markers to validate the results. However, the amount of nucleotide data available from GenBank for 12S rRNA and RNA polymerase β was insufficient.

I used two types of species-specific reference probes: probes exclusively targeting the methylase sequences and probes exclusively targeting the endonuclease restriction sequences. I quantified the hybridization between the probes and the target sequences, as well as the

overlapping regions between methylase and endonuclease sites using seqinr (Charif and Lobry 2007) and ape (Paradis *et al.* 2004) packages in R.

Phylogenetic construction

I used the 16S rRNA sequences to create a phylogeny for the 38 eubacterial and two archaeobacterial species in 23 families that showed positive hybridization between the probes and the target sequences. I used MEGA version 5.05 (Tamura *et al.* 2011) for the phylogenetic and molecular evolutionary analyses and used ClustalX2 for the pairwise and multiple sequence alignments. I used the alignment scores and three published eubacterial and archaeobacterial supertrees (Ludwig and Schleifer 1994; Daubin *et al.* 2001; Case *et al.* 2007) to construct a consensus tree based on maximum likelihood in MEGA 5.05. I calculated bootstrap confidence intervals for each node of the phylogeny with 500 bootstrap replicates. I constructed a phylogeny based on maximum likelihood, as opposed to maximum parsimony, because maximum likelihood is computationally more accurate than maximum parsimony.

Phylogenetic signal

I computed Pagel's lambda (λ) correlation using Phytools and Geiger packages in R to test for phylogenetic dependence between the total number of endonuclease and methylase sites in 68 microbial species.

Pagel's lambda (λ) is the most common way to estimate phylogenetic signal. The test assumes a constant-variance, random effects model of evolution and is based on maximum likelihood (Pagel 1999). Pagel's λ varies between 0 (phylogenetic independence) and 1 (phylogenetic dependence). I calculated Pagel's lambda for the total number of endonuclease and methylase sites corresponding to the original, 16S rRNA tree of the 40 taxa (Figure 4, Appendix

II). Then I transformed the original 16S rRNA tree with 40 taxa into a null tree with uniform branch lengths, portraying zero phylogenetic relatedness between the 40 species, and again calculated Pagel's λ . If the lambda value for the 16S rRNA tree is less or equal to the lambda value for the null tree, then I considered the data set to be independent and lacking phylogenetic covariance. Annotated R codes for all the above analyses are in Appendix I.

Results

ClustalX2 alignment of the 666 palindromic endonuclease restriction sequences with the 466 species-specific, methylase sequences produced 100% nucleotide identity.

The total number of methylated restriction sites in the 16S rRNA gene locus of 40 microbial taxa yielded 100% overlap between endonuclease restriction sites and the "cognate" methylase sites ($R^2=1.00$) (Table 1). The number of endonuclease restriction sequences for each species was identical to the number of methylase sequences for that species. In other words, every species contained an equal number of restriction sites and methylase sites at the 16S rRNA locus.

However, I saw something unexpected in 28 out of the 68 microbial species, in that they contained neither endonuclease restriction nor methylase sites (Table 2).

Table 1. Total number of type II endonuclease restriction sites, number of methylase sites, and number of overlapping sites in 16S rRNA genomes of 40 microbial species in 23 families.

Microorganisms	Number of endonuclease restriction sites*	Number of methylase sites*	Number of overlaps
Acetobacter aceti	1	1	1
Acetobacter pasteurianus	1	1	1
Anabaena flos-aquae	2	2	2
Anabaena variabilis	4	4	4
Arthrobacter protophormiae	1	1	1
Bacillus brevis	3	3	3
Bacillus firmus	2	2	2
Bacillus smithii	1	1	1
Bacillus subtilis	12	12	12
Citrobacter freundii	3	3	3
Clostridium formicoaceticum	2	2	2
Deinococcus radiophilus	2	2	2
Desulfovibrio desulfuricans, Norway strain	3	3	3
Enterobacter cloacae	1	1	1
Flavobacterium aquatile	1	1	1
Flavobacterium okeanoikoites	2	2	2
Fusobacterium nucleatum	15	15	15
Gluconobacter oxydans	1	1	1
Haemophilus aegyptius	7	7	7
Haemophilus aphrophilus	6	6	6
Haemophilus haemoglobinophilus	6	6	6
Haemophilus haemolyticus	11	11	11
Haemophilus parahaemolyticus	4	4	4
Haemophilus parainfluenzae	7	7	7
Methanothermobacter wolfei	6	6	6
Methylophilus methylotrophus	6	6	6
Micrococcus species CCGE3063	5	5	5
Moraxella bovis	9	9	9
Moraxella nonliquefaciens	10	10	10
Moraxella species 0408225	4	4	4
Neisseria lactamica	2	2	2
Providencia alcalifaciens	8	8	8
Pseudomonas fluorescens	1	1	1
Rhodopseudomonas sphaeroides	3	3	3

<i>Streptococcus cremoris</i>	7	7	7
<i>Streptomyces phaeochromogenes</i>	1	1	1
<i>Thermoplasma acidophilum</i>	9	9	9
<i>Thermus aquaticus</i>	3	3	3
<i>Thermus filiformis</i>	3	3	3
<i>Xanthomonas campestris</i>	1	1	1

* The number of restriction and methylase sites for type II endonuclease and methylase enzymes represents the total for a species. Refer to the supplementary material (S2) for a complete list of results categorized by each enzyme.

Table 2. 28 microbial species with zero endonuclease and methylase sites in the 16S rRNA gene locus

<i>Acinetobacter calcoaceticus</i>	<i>Neisseria mucosa</i>
<i>Agrobacterium gelatinovorum</i>	<i>Plesiomonas shigelloides</i> 319-73
<i>Agrobacterium tumefaciens</i> C58	<i>Proteus vulgaris</i>
<i>Arthrobacter</i> ON14	<i>Providencia stuartii</i> 164
<i>Bacillus amyloliquefaciens</i>	<i>Pseudomonas aeruginosa</i>
<i>Bacillus caldolyticus</i>	<i>Saccharopolyspora hirsute</i> ATCC 2787
<i>Bacillus coagulans</i>	<i>Serratia marcescens</i>
<i>Bacteroides caccae</i> ATCC43185T	<i>Sphaerotilus natans</i>
<i>Caryophanon latum</i> L	<i>Staphylococcus warneri</i>
<i>Erwinia toletana</i>	<i>Streptomyces caespitosus</i>
<i>Haemophilus influenza</i>	<i>Streptomyces albus</i>
<i>Micrococcus luteus</i>	<i>Streptomyces fimbriatus</i>
<i>Mycoplasma fermentans</i>	<i>Streptomyces griseus</i>
<i>Neisseria denitrificans</i>	<i>Xanthomonas oryzae</i>

The molecular phylogeny of the 40 microbial species in 23 families generated a consensus tree (Figure 4, Appendix II). My estimated phylogeny matches previously published, molecular phylogenies based supertrees (Ludwig and Schleifer 1994; Daubin *et al.* 2001; Case *et al.* 2007).

Phylogenetic signal analysis using Pagel's lambda (λ) correlation produced a lambda value of 1.0×10^{-5} for the original, 16S rRNA tree of the 40 microbial species. The transformed, null, tree with uniform branch lengths produced a lambda value of 0.5.

Discussion

I tested whether hypermethylation of protein-coding genes in eubacteria and archaea might be a host-driven mechanism against exogenous DNA invasions. Alternatively, do exogenous DNA mediate eubacterial DNA methyltransferase enzymes into hypermethylating the eubacterial palindromic recognition sites to avoid restriction cleavage? Genomic autoimmunity hypothesis, postulated that the host genomic machinery regulates the enzymatic dynamics of endonuclease and methylase enzymes of rm system II to minimize the spread of exogenous DNA elements. However, I hypothesized that gene regulation via DNA methylation may have originated in ancestral eubacteria as a transposon-mediated countermeasure to endonuclease cleavage. I predicted that transposon-coded proteins may have influenced eubacterial DNMTases into hypermethylating eubacterial restriction endonuclease sites. This decreases endonuclease enzyme's accessibility to palindromic sequences and may render rm system II ineffective at removing transposable elements from the genome. The fundamental message of the transposon-mediated immunity hypothesis is that endonuclease restriction enzymes and methyltransferases of rm system II function as independent enzymatic units and that type II DNA methyltransferases may not necessarily improve the effectiveness of rm system II against transposon invasions.

I showed that there is a significant correlation between the palindromic restriction endonuclease sequences and the "cognate" methylase sequences in eubacteria and archaea. This

corroborates the findings of Noyer-Weidner and Trautner (1993) and Geier and Modrich (1979) by showing that the addition of methyl moieties to restriction recognition sequences seems to prevent endonucleases from binding to and forming a stable enzyme-substrate complex with palindromic recognition sites.

The sequence alignment of the 666 palindromic, endonuclease restriction sequences with the 466, “cognate” methylase sequences produced 100% nucleotide identity. However, I did not calculate the percent identity of 200 endonuclease restriction sequences due to the lack of published sequence data available for the corresponding methylase sequences. Nevertheless, I predict that the remaining 200 restriction sequences will yield similar results.

I used 16S rRNA to build the phylogeny for the 66 eubacterial and 2 archaeal species because 16S rRNA is ubiquitous and one of the few housekeeping genes, along with RNA polymerase β subunit gene (*rpoB*), that is highly conserved among eubacteria and archaea. Since 1990, the use of 16S rRNA as a genomic marker has revolutionized microbiology in understanding phylogenetic diversity (Amann *et al.* 1990; García-Martínez *et al.* 1999; Case *et al.* 2007).

The reason why the endonuclease sequence probes and the methylase sequence probes failed to hybridize with the 16S rRNA sequences for 28 out of 68 microbial species (Table 2) is probably because, according to Case *et al.* (2007), multiple copies of 16S rRNA gene are often present in a given bacterium and intragenomic copies can differ in sequence identity. These heterogeneity hot spots were found in a majority of gene fragments that are commonly used in molecular phylogenetic analysis and the hot spots can influence hybridization of primers and probes, 16S rRNA gene tree topology, and phylogenetic resolution at the species level (Case *et*

al. 2007). Lack of hybridization between endonuclease and methylase probes and the target sequences in the 28 microbial taxa does not mean that these species completely lack endonuclease and methylase sites. I did not probe the complete genomes of the 68 microbial species to quantify the total number of endonuclease and methylase sites. For each species, I probed the 16S rRNA locus, which represents a small portion of each microbial genome. Lack of hybridization simply means that the 16S rRNA genomic regions in which I probed for hybridization, in fact, lack the targeted endonuclease and methylase sites. In addition, the 16S rRNA loci that I extracted from NCBI GenBank for the 28 eubacterial species with zero hybridization were all partial sequences that ranged from 241bps to 721 bps. This is approximately one quarter the size of the remaining 16S rRNA loci that I used in the analysis, which are well over 1561bps.

Phylogenetic comparative methods are commonly used in interspecific comparative studies (Martins and Hansen 1997; Price 1997; Freckleton *et al.* 2002; Blomberg *et al.* 2003) because species that are closely related tend to share similar trait values compared to species that are distantly related. Statistical non-independence that arises due to phylogenetic distance can be resolved using phylogenetic comparative methods, such as independent contrasts and phylogenetic generalized least squares regression (Blomberg *et al.* 2003; Felsenstein 1985; Grafen 1989). However, it is not necessary to control for phylogenetic bias in all comparative studies (Bjorklund 1994; Fitter 1995; Losos 1999). I did not use the phylogenetic comparative method in this study because Pagel's lambda (λ) indicated that my data on total number of endonuclease and methylase sites lacked a phylogenetic signal.

Currently the most widely accepted hypothesis regarding the origin of eubacterial and archaeabacterial rm system II is the genomic autoimmunity hypothesis that describes rm II as the

main line of genomic defense against exogenous DNA. The genomic autoimmunity hypothesis emphasizes that DNA methyltransferase enzymes protect the host genome from exogenous DNA insertions and the host genomic machinery is solely responsible for regulating the function and maintenance of restriction modification and methyltransferase enzymes.

In this thesis, I presented a modified version of the genomic autoimmunity hypothesis, namely the transposon-mediated immunity hypothesis, which postulates that the presence of DNA methylation as part of the restriction modification system II of eubacteria and archaea is not a host-driven mechanism against transposition, but may be a transposon-mediated countermeasure to gain access to the host genome via bypassing endonuclease cleavage. I found that palindromic endonuclease restriction sequences and “cognate” methylase sequences share 100% nucleotide identity in 210 eubacterial and 2 archaeabacterial species. Furthermore, the total number of restriction and methyltransferase sequences in the 16S rRNA of 40 microbial species showed a perfect correlation ($R^2=1.00$). This result is consistent with the transposon-mediated immunity hypothesis in that hypermethylation of endonuclease restriction sites by “cognate” DNMTases seem to render restriction modification system II ineffective at removing transposable elements from the genomes of eubacteria and archaeabacteria.

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Appendix I

S1. Restriction sequences of 344 type II endonuclease enzymes and 466 methylase sequences of 212 type II methyltransferases, belonging to 261 microbial species.

Microorganism	Restriction enzyme	Recognition sequence	Methylase enzyme	Methylase sequence
Acetobacter aceti	AatII	GACGT/C	M.AatII	GACGTC ^b
Acetobacter pasteurianus	Apal	GGGCC/C	M.Apai	GGG _{m5} CCC
Acetobacter pasteurianus	ApalI	G/TGCAC	M.ApalI	G/TGCAC ^d
Acinetobacter calcoaceticus	AccI	GT/(A,C)(G,T)AC	M.AccI	GTAGA _{m6} C
Acinetobacter calcoaceticus M4	AcII	AA/CGTT	M.AcII	AA _{m5} CGTT
Agrobacterium gelatinovorum	AgeI	A/CCGGT	M.AgeI	A/CCGGT ^d
Agrobacterium tumefaciens C58	AtuCI ^a (BclI)	GANTC	M.Atucl	G ^{m6} ANTC
Anabaena flos-aquae	AflII	C/TTAAG	M.AflII	C/TTAAG ^b
Anabaena flos-aquae	AflIII	A/C(G,A)(C,T)GT	M.AflIII	A ^{m4} C(G,A)(C,T)GT
Anabaena variabilis	AvaI	C/(C,T)CG(G,A)G	M.AvaI	C/(C,T)CG(G,A)G ^c
Anabaena variabilis	AvaII	G/G(A,T)CC	M.AvaII	G/G(A,T)CC ^d
Anabaena variabilis	AvaIII	ATGCAT	M.AvaIII	ATGCAT ^b
Anabaena variabilis uw	AvrI ^a (AvaI)	C(C,T)CG(G,A)G	M.AvrI	C(C,T)CG(G,A)G ^b
Anabaena variabilis uw	AvrII	C/CTAGG	M.AvrII	CCTAGG ^b
Arthrobacter citreus	Acil	CCGC (-3/-1)	M.Acil	_{m5} CCGC
Arthrobacter luteus	AluI	AG/CT	M.AluI	AG/ _{m5} CT
Arthrobacter	ApoI	(G,A)AATT(C,T)	M.ApoI	(G,A)AATT(C,T) ^c

protophormiae

Arthrobacter species	AscI	GG/CGCGCC	M.AscI	GGCGCGCC ^d
Bacillus amyloliquefaciens F	BamFI ^a (BamHI)	GGATCC	M.BamFI	GGAT _{m5} CC
Bacillus amyloliquefaciens H	BamHI	G/GATCC	M.BamHI	G/GAT _{m5} CC
Bacillus amyloliquefaciens K	BamKI ^a (BamHI)	GGATCC	M.BamKI	GGAT _{m5} CC
Bacillus amyloliquefaciens N	BamNI ^a (Bam HI)	GGATCC	M.BamNI	GGAT _{m5} CC
Bacillus brevis	BbvI	GCAGC (8/12)	M.BbvI	G _{m5} CAGC
Bacillus brevis S	BbvSI	GC(A,T)GC	M.BbvSI	G _{m5} C(A,T)GC
Bacillus caldolyticus	BclI	T/GATCA	M.BclI	TG _{m6} ATCA
Bacillus coagulans	BcgI	CGANNNNNNTGC (12/10)	M.BcgI	CG _{m6} ANNNNNNTGC
Bacillus firmus	BfiI	ACTGGGNNNNN (6 or 7)	M1.BfiI	A _{m4} CTGGG
Bacillus globigii	BglI	GCCNNNN/NGGC	M.BglI	GC _{m4} CNNNNNGGC
Bacillus globigii	BglII	A/GATCT	M.BglII	AGAT _{m4} CT
Bacillus smithii	BscGI	CCCGT	M1.BscGI	CCCGT ^d
Bacillus smithii	BscGI	CCCGT	M2.BscGI	CCCGT ^d
Bacillus species H	BspHI	T/CATGA	M.BspHI	TCATGA ^e
Bacillus species LU11	BspLU11III	GGGAC (10/14)	M1.BspLU11III	GGG _{m6} AC
Bacillus species LU11	BspLU11III	GGGAC (10/14)	M2.BspLU11III	3-CC _{m5} CTG-5
Bacillus species M	BspMI	ACCTGCNNNN/NNNN	M1.BspMI	ACCTGC ^d
Bacillus species M	BspMI	ACCTGCNNNN/NNNN	M2.BspMI	ACCTGC ^d
Bacillus species M	BspMII	T/CCGGA	M.BspMII	T/CCGGA ^d
Bacillus species R	BseRI	GAGGAG (10/8)	M.BseRI	GAGGAG ^b

Bacillus sphaericus	Bsp1286I	G (A,G,T)GC(A,C,T)/C	M.Bsp1286I	G (A,G,T)GC(A,C,T)/C ^d
Bacillus sphaericus GC subgroup	BsgI	GTGCAC (16/14)	M.BsgI	GTGCAG ^b
Bacillus sphaericus R	BspRI ^a (HaeIII)	GG/CC	M.BspRI	GG/m ₅ CC
Bacillus stearothermophilus	BsaAI	(C,T)AC/GT(G,A)	M.BsaAI	(C,T)A _{m5} C/GT(G,A)
Bacillus stearothermophilus	BsrBI	CCG/CTC	M1.BsrBI	CCGCTC ^c
Bacillus stearothermophilus	BsrBI	CCG/CTC	M2.BsrBI	CCGCTC ^c
Bacillus stearothermophilus	BsrI	ACTGGN/	M1.BsrI	ACTGGN/ ^c
Bacillus stearothermophilus	BsrI	ACTGGN/	M2.BsrI	ACTGGN/ ^c
Bacillus stearothermophilus	BstNI ^a (EcoRII)	CC/(A,T)GG	M.BstNI	C _{m4} C(A,T)GG
Bacillus stearothermophilus	BstXI	CCANNNNN/NTGG	M.BstXI	CCANNNNN/NTGG
Bacillus stearothermophilus 1503-4R	BstI ^a (BamHI)	G/GATCC	M.BstI	G/G _{m6} ATCC
Bacillus stearothermophilus A664	BsmAI	GTCTCN/NNNN	M.BsmAI	GTCTC ^d
Bacillus stearothermophilus D70	BsrDI	GCAATGNN/	M1.BsrDI	GCAATG ^b
Bacillus stearothermophilus D70	BsrDI	GCAATGNN/	M2.BsrDI	GCAATG ^b
Bacillus stearothermophilus ET	BstEII	G/GTNACC	M.BstEII	GGTNACC ^b

Bacillus stearothermophilus ET	BstEIII	GATC	M.BstEIII	GATC ^b
Bacillus stearothermophilus NUB36	BsmI	GAATGCN/	M1.BsmI	GAATGC ^b
Bacillus stearothermophilus NUB36	BsmI	GAATGCN/	M2.BsmI	GAATGC ^b
Bacillus subtilis	Bsu1076I ^a (HaeIII)	GGCC	M.Bsu1076	GG/m ₅ CC
Bacillus subtilis	Bsu1114I ^a (HaeIII)	GGCC	M.Bsu1114	GG/m ₅ CC
Bacillus subtilis Marburg 168	BsuMI	CTCGAG	M.BsuM	CTm ₅ CGAG
Bacillus subtilis strain X5	BsuRI ^a (HaeIII)	GG/CC	M.BsuRI	GG/m ₅ CC
Bacteroides caccae	BccI	CCATCNNNN/N	M1.BccI	CCATC ^b
Brevibacterium albidum	BalI	TGG/CCA	M.BalI	TGGCCA ^c
Brevibacterium albidum	BalI	TGG/CCA	M.Bal I	TGG/m ₄ CCA
Brevibacterium luteum	BluII ^a (HaeIII)	GGCC	M.BluII	GG/m ₅ CC
caryophanon latum	ClfI ^a (HaeIII)	GG/CC	M.Clf I	GG/m ₅ CC
Caryophanon latum L	ClaI	AT/CGAT	M.Cla I	AT/CG _{m6} AT
Chlorella strain NC64A	CviJI	(G,A)G/C(C,T)	M.CviJI	(G,A)G/m ₅ C(C,T)
Chlorella strain NC64A	CviRI	TG/CA	M.CviRI	TGC _{m6} A
Chlorella strain NC64A	CviRII	G/TAC	M.CviRII	GT _{m6} AC
Citrobacter freundii RFL10	Cfr10I	(G,A)/CCGG(C,T)	M.Cfr10I	(G,A)/m ₅ CCGG(C,T)
Citrobacter freundii RFL2	CfrI	(C,T)GGCC(G,A)	M.CfrI	(C,T)GG _{m5} CC(G,A)

Clostridium acetobutylicum ABKn8	Cac8I	GCN/NGC	M.Cac8I	GCN/NGC ^b
Clostridium formicoaceticum	CfoI ^a (HhaI)	GCG/C	M.CfoI	Gm5CG/C
Deinococcus radiophilus	DraII	(G,A)G/GNCC(C,T)	M.DraII	(G,A)G/GNCC(C,T) ^b
Deinococcus radiophilus	DraIII	CACNNN/GTG	M.DraIII	Cm6ACNNN/GTG
Desulfovibrio desulfuricans Norway strain	DdeI	C/TNAG	M.DdeI	m5CTNAG
Diplococcus pneumoniae	DpnI	GA/TC	M.Dpn I	Gm6ATC
Diplococcus pneumoniae	DpnII ^a (MboI)	/GATC	M1.Dpn II	Gm6ATC
Diplococcus pneumoniae	DpnII ^a (MboI)	/GATC	M2.DpnII	Gm6ATC
Enterobacter cloacae	EcaI ^a (BstEII)	G/GTNACC	M.EcaI	GGTNm6ACC
Erwinia species RFL3	Esp3I	CGTCTCN/NNNN	M.Esp3I	CGTm5CTC
Escherichia coli	EcoNI	CCTNN/NNNAGG	M.EcoNI	CCTNN/NNNAGG ^b
Escherichia coli (PI)	EcoPI	AGACC (25/27)	M.EcoPI	AGm6ACC
Escherichia coli B	EcoBI	TGANNNNNNNTGCT	M.EcoBI	TGm6ANNNNNNNTGCT
Escherichia coli J62	EcoRV	GAT/ATC	M.EcoRV	Gm6ATATC
Escherichia coli K MG1655	EcoKI	AACNNNNNNGTGC	M.EcoKI	Am6ACNNNNNNGTGC
Escherichia coli P15	EcoP15I	CAGCAG (25/27)	M.EcoP15I	CAGCm6A
Escherichia coli R245	EcoRII	CC(A,T)GG	M.EcoRII	Cm5C(A,T)GG
Escherichia coli RFL31	Eco31I	GGTCTCN/NNNN	M1.Eco31I	3'-CCm6AGAG-5'
Escherichia coli RFL31	Eco31I	GGTCTCN/NNNN	M2.Eco31I	GGTm5CTC
Escherichia coli RFL47	Eco47III	AGC/GCT	M.Eco47III	AGC/GCT ^b
Escherichia coli RFL57	Eco57I	CTGAAGNNNNNNNNNNNNNNNNNN	M.Eco57I	CTGA _{m6} AG

<i>Escherichia coli</i> RY13	EcoRI	G/AATTC	M.EcoRI	G/Am6ATTC
<i>Flavobacterium aquatile</i>	FauI	CCCGCNNNN/NN	M1.FauI	Cm5CCGC
<i>Flavobacterium okeanokoites</i>	FokI	GGATGNNNNNNNNN/NNNN	M.FokI	GGm6ATG
<i>Frankia</i> species Eu11b	FseI	GGCCGG/CC	M.FseI	GGCCGG/CC ^d
<i>Fusobacterium nucleatum</i> 4H	Fnu4HI	GC/NGC	M.Fnu4HI	GCNGC ^d
<i>Fusobacterium nucleatum</i> 4H	Fnu4HI	GC/NGC	M.Fnu4HI	GC/NGC ^d
<i>Fusobacterium nucleatum</i> D	FnuDII	CG/CG	M.FnuDII	m5CGCG
<i>Fusobacterium nucleatum</i> D	FnuDIII	GCG/C	M.FnuDIII	GCGC ^b
<i>Fusobacterium nucleatum</i> D	FnuDI ^a (HaeIII)	GG/CC	M.FnuDI	GG/m5CC
<i>Fusobacterium nucleatum</i> D	FnuDII	CG/CG	M.FnuDII	m5CGCG
<i>Fusobacterium nucleatum</i> D	FnuDIII ^a (HhaI)	GCG/C	M.FnuDIII	Gm5CG/C
<i>Gluconobacter suboxydans</i> H-15T	GsuI	CTGGAG (16/14)	M.GsuI	CTGGAG ^b
<i>Haemophilus aegyptius</i>	HaeII	(G,A)GCGC(C,T)	M.HaeII	(G,A)GCGC(C,T) ^d
<i>Haemophilus aegyptius</i>	HaeIII	GG/CC	M.HaeIII	GGm5CC
<i>Haemophilus aphrophilus</i>	HapII ^a (HpaII)	C/CGG	M.HapII	Cm5CGG
<i>Haemophilus gallinarum</i>	HgaI	GACGC (5/10)	M1.HgaI	3'-CTGm5CG-5'
<i>Haemophilus gallinarum</i>	HgaI	GACGC (5/10)	M2.HgaI	GAm5CGC
<i>Haemophilus haemoglobinophilus</i>	Hhgl ^a (HaeIII)	GGCC	M.Hhgl	GG/m5CC

Haemophilus haemolyticus	HhaII ^a (HinfI)	GANTC	M.HhaII	G/m ₆ ANTC
Haemophilus haemolyticus	HhaI	GCG/C	M.HhaI	Gm ₅ CGC
Haemophilus influenza serotype c, 1160	HincII ^a (HindII)	GT(C,T)(G,A)AC	M.HincII	GT(C,T)(G,A) _{m6} AC
Haemophilus influenzae Rd	HindII	GT(C,T)(G,A)AC	M.HindII	GT(C,T)(G,A) _{m6} AC
Haemophilus influenzae Rd	HindIII	A/AGCTT	M.HindIII	_{m6} AAGCTT
Haemophilus influenzae Rf	HinfI	G/ANTC	M.HinfI	Gm ₆ ANTC
Haemophilus influenzae Rf	HinfIII	CGAAT	M.HinfIII	CGAm ₆ AT
Haemophilus parahaemolyticus	HphI	GGTGA (8/7)	M1.HphI	3'-CCAm ₅ CT-5'
Haemophilus parahaemolyticus	HphI	GGTGA (8/7)	M2.HphI	GGTGm ₆ A
Haemophilus parainfluenzae	HpaI	GTT/AAC	M.HpaI	GTTA _{m6} AC
Haemophilus parainfluenzae	HpaII	C/CGG	M.HpaII	Cm ₅ CGG
Herpetosiphon giganteus HP1023	HgiAI	G(A,T)GC(A,T)C	M.HgiAI	G(A,T)GC(A,T)C ^b
Herpetosiphon giganteus Hpa2	HgiDI	G(G,A)CG(C,T)C	M.HgiDI	G(G,A)CG(C,T)C ^d
Herpetosiphon giganteus Hpg 24	HgiEI ^a (AvaII)	G/G(A,T)CC	M.HgiEI	G/G(A,T)CC ^d
Herpetosiphon giganteus Hpg 5	HgiBI ^a (AvaII)	G/G(A,T)CC	M.HgiBI	G/G(A,T)CC ^d
Herpetosiphon giganteus Hpg 9	HgiCII ^a (AvaII)	G/G(A,T)CC	M.HgiCII	G/G(A,T)CC ^d

Herpetosiphon giganteus Hpg9	HgiCI	G/G(C,T)(C,T)CC	M.HgiCI	G/G(C,T)(C,T) _{m5} CC
Klebsiella pneumoniae OK8	KpnI	GGTAC/C	M.KpnI	GGT _{m6} ACC
Maraxella asloensis	MslI	CA(C,T)NN/NN(G,A)TG	M.MslI	CA(C,T)NN/NN(G,A)TG (B.U)
Methanobacterium wolfei	MwoI	GCNNNNN/NNGC	M.MwoI	GCNNNNN/NNGC ^c
Methylophilus methylophilus	MmeI	TCC(G,A)AC	M..MmeI	TCC(G,A) _{m6} AC
Methylophilus methylophilus	MmeII	GATC	M.MmeII	GATC ^b
Micrococcus luteus	MluI	A/CGCGT	M.MluI	ACGCGT ^c
Micrococcus species	MseI	T/TAA	M.MseI	TTA _{m6} A
Moraxella bovis	MboI	GATC	M1.MboI	G _{m6} ATC
Moraxella bovis	MboI	GATC	M2.MboI	G _{m6} ATC
Moraxella bovis	MboII	GAAGA (8/7)	M1.MboII	GAAG _{m6} A
Moraxella bovis	MboII	GAAGA (8/7)	M2.MboII	GAAGA
	MnnI ^a			
Moraxella nonliquefaciens	(HindII)	GT(C,T)(G,A)AC	M.MnnI	GT(C,T)(G,A) _{m6} AC
	MnnII ^a			
Moraxella nonliquefaciens	(HaeIII)	GGCC	M.MnnII	GG/ _{m5} CC
	MnnIV ^a			
Moraxella nonliquefaciens	(HhaI)	GCGC	M.MnnIV	G _{m5} CG/C
	MspI ^a			
Moraxella species	(HpaII)	C/CGG	M.MspI	_{m5} C/CGG
Mycoplasma fermentans	MfeI	C/AATTG	M.MfeI	CAATTG ^c
Neisseria denitrificans	NdeI	Ca/TATG	M.NdeI	CATATG ^c
Neisseria lactamica	NlaIII	CATG/	M.NlaIII	C _{m6} ATG
Neisseria lactamica	NlaIV	GGN/NCC	M.NlaIV	GGN/NCC ^d
Neisseria mucosa	NheI	G/CTAGC	M.NheI	GCTAGC ^b

<i>Nocardia aerocolonigenes</i>	NaeI	GCC/GGC	M.NaeI	GCCGGC ^d
<i>Nocardia corallina</i>	NcoI	C/CATGG	M.NcoI	C/CATGG ^c
<i>Nocardia ottitidis-caviarum</i>	NotI	GC/GGCCGC	M.NotI	GC/GGCCGC ^c
<i>Nocardia rubra</i>	NruI	TCG/CGA	M.NruI	TCG/CGA
<i>Nostoc species C</i>	Nspl	(G,A)CATG/(C,T)	M.Nspl	(G,A) _{m5} \CATG/(C,T)
<i>Plesiomonas shigelloides</i> 319-73	PshAI	GACNN/NGTC	M.PshAI	G _{m6} ACNN/NGTC
<i>Proteus vulgaris</i>	PvuI	CGAT/CG	M.PuvI	CGAT/CG ^b
<i>Proteus vulgaris</i>	PvuII	CAG/CTG	M.PvuII	CAG _{m5} CTG
<i>Providencia alcalifaciens</i>	PalI	GG/CC	M.PalI	GG/ _{m5} CC
<i>Providencia stuartii</i> 164	PstI	CTGCA/G	M.PstI	CTGC _{m6} A
<i>Providencia stuartii</i> 164	PstI	CTGCA/G	M.PstI	CTGC _{m6} A/G
<i>Pseudomonas aeruginosa</i>	PaeR7I	C/TCGAG	M.PaeR7	C/TCG _{m6} AG
<i>Pseudomonas fluorescens</i>	PflMI	CCANNN/NTGG	M.PflMI	CCANNN/NTGG ^b
<i>Pseudomonas lemoignei</i>	PleI	GAGTCNNNN/N	M.PleI	GAGTCNNNN/N ^b
<i>Pseudomonas putida</i> M	PpuMI	(G,A)G/G(A,T)CC(C,T)	M.PpuMI	(G,A)G/G(A,T)CC(C,T) ^d
<i>Rhodopseudomonas sphaeroides</i>	RsaI	GT/AC	M.RsaI	GT _{A_{m5}} C
<i>Rhodopseudomonas sphaeroides</i>	RshI ^a (PvuI)	CGAT/CG	M.RshI	CGAT/CG ^d
<i>Rhodopseudomonas sphaeroides</i>	RsrI ^a (EcoRI)	G/AATTC	M.RsrI	GA _{m6} ATTC
<i>Rhodopseudomonas sphaeroides</i>	RsrII	CG/G(A,T)CCG	M.RsrII	CG/G(A,T)CCG ^d
<i>Saccharopolyspora species</i>	SapI	GCTCTTCN/NNN	M.SapI	GCTCTTCN/NNN ^b
<i>Salmonella typhi</i> 27	StyI	C/C(A,T)(A,T)GG	M.StyI	C/C(A,T)(A,T)GG ^b
<i>Serratia marcescens</i> Sb	SmaI	CCC/GGG	M.SmaI	C _{m4} CCGGG
<i>Serratia species</i> SAI	SspI	AAT/ATT	M.SspI	AATATT ^c

Sphaerotilus natans	SpeI	A/CTAGT	M.SpeI	A/CTAGT ^b
Sphaerotilus natans	SnaBI	TAC/GTA	M.SnaBI	TAm ₄ CGTA
	Sau3A ^a			
Staphylococcus aureus 3A	(MboI)	GATC	M.Sau3A	GATm ₅ C
Staphylococcus aureus PS96	Sau96I ^a			
	(AsuI)	G/GNCC	M.Sau96I	GGNm ₅ CC
Staphylococcus warneri	SwaI	ATTT/AAAT	M.SwaI	ATTT/AAAT ^c
Streptomyces caespitosus	ScaI	AGT/ACT	M.ScaI	AGTAm ₄ CT
Streptomyces exfoliatus	SexAI	A/CC(A,T)GGT	M.SexAI	A/CC(A,T)GGT ^d
Streptococcus cremoris	ScrFI	CC/NGG	M.ScrFI	CC/NGG ^d
Streptococcus durans RFL3	SduI	G(A,G,T)GC(A,C,T)/C	M.SduI	G(A,G,T)GC(A,C,T)/C ^b
Streptococcus faecalis	SfeI	C/T(G,A)(C,T)AG	M.SfeI	C/T(G,A)(C,T)AG ^b
Streptococcus faecalis ND547	SfaNI	GCATCNNNNN/NNNN	M.SfaNI	GCATC ^b
Streptococcus faecalis var. Zymogenes	SfaI ^a			
	(HaeIII)	GGCC	M.SfaI	GG/m ₅ CC
Streptomyces achromogenes	Sac I	GAGCT/C	M.SacI	GAGm ₅ CTC
Streptomyces achromogenes	SacII	CCGC/GG	M.SacII	CCGCGG
Streptomyces albus	SalI	G/TCGAC	M.SalI	GTCGm ₆ AC
Streptomyces fimbriatus	SfiI	GGCCNNNN/NGGCC	M.SfiI	GGCCNNNN/NGGCC ^c
Streptomyces griseus	SgrAI	C(G,A)/CCGG(G,A)G	M.SgrAI	C(G,A)/CCGG(G,A)G ^d
Streptomyces phaeochromogenes	SphI	GCATG/C	M.SphI	GCATG/C ^b
	SstI ^a			
Streptomyces stanford	(SacI)	GAGCT/C	M.SstI	GAGCT/C ^b
Streptomyces tubercidicus	StuI	AGG/CCT	M.StuI	AGG/CCT ^b

Thermoplasma acidophilum	ThaI ^a (FnuDII)	CG/CG	M.ThaI	CG/CG ^c
Thermus aquaticus YTI	TaqI	T/CGA	M.TaqI	T/CG _{m6} A
Thermus filiformis	TfiI	G/A(A,T)TC	M.TfiI	G _{m6} A(A,T)TC
Thermus species 93170	TseI	G/C(A,T)GC	M.TseI	G/C(A,T)GC ^d
Thermus species strain R	TspRI	NNCA(G,C)TGNN	M.TspRI	m ₅ CA(G,C)TG
Thermus species strain YS45	Tsp45I	GT(G,C)AC	M.Tsp45I	GT(G,C) _{m6} AC
Thermus thermophilus strain 111	Tth111I	GACN/NNGTC	M.Tth111I	G _{m6} ACNNNGTC
Vibrio species 343	VspI	AT/TAAT	M.VspI	ATTAm ₆ AT
Xanthomonas amaranthicola	XamI ^a (SalI)	GTCGAC	M.XamI	GTCGAC ^b
Xanthomonas badrii	XbaI	T/CTAGA	M.XbaI	TCTAG _{m6} A
Xanthomonas campestris	XcmI	CCANNNNN/NNNNTGG	M.XcmI	CC _{m6} ANNNNNNNNNNTGG
Xanthomonas holcicola	XhoI	C/TCGAG	M.XhoI	CTCG _{m6} AG
Xanthomonas holcicola	XhoII	(G,A)GATC(C,T)	M.XhoII	(G,A)GAT _{m4} C(C,T)
Xanthomonas malvacearum	XmaI	C/CCGGG	M.XmaI	C/CCGGG ^c
Xanthomonas malvacearum	XmaIII	C/GGCcG	M.XmaIII	CGG _{m5} CCG
Xanthomonas manihotis 7AS1	XmnI	GAANN/NTTC	M.XmnI	GAANN/NTTC ^b
Xanthomonas oryzae	XorII ^a (PvuI)	CGATC/G	M.XorII	CGATC/G ^d

^a Restriction enzymes with isoschizomers

^c The sequence is methylated by N4-methyl cytosine at an unknown location

^b The site and the type of methylation of this sequence is unknown

^d The sequence is methylated by 5-methyl cytosine at an unknown location

^e The sequence is methylated by 6-methyl adenine at an known location

S2. NCBI GenBank accession numbers for 16s rRNA, type II restriction endonuclease restriction enzymes and respective palindrome restriction sites, methylase enzymes and respective methylase sites for all 68 species.

Microorganism	Accession numbers	Endonuclease restriction enzymes	Endonuclease restriction sites	Methylase enzymes ^a	Methylase sites
Acetobacter aceti	D30768.1	AatII	gacgtc	M.AatII	gacgtc
Acetobacter pasteurianus	AY883035.1	ApaI	gggccc	M.Apai	gggccc
		ApaLI	gtgcac	M.ApaLI	gtgcac
Acinetobacter calcoaceticus	EU330417.1	AccI	gtagac	M.AccI	gtagac
		AccI	gtatac	M.AccI	gtatac
		AccI	gtcgac	M.AccI	gtcgac
		AccI	gtctac	M.AccI	gtctac
		AccII	aacgtt	M.AccII	aacgtt
		AccIII	tccgga	M.AccIII	tccgga
Agrobacterium gelatinovorum	D88523.1	AgeI	accggt	M.AgeI	accggt
Agrobacterium tumefaciens C58		AtuCI	gaatc	M.AtuCI	gaatc
		AtuCI	gactc	M.AtuCI	gactc
		AtuCI	gagtc	M.AtuCI	gagtc
		AtuCI	gattc	M.AtuCI	gattc
Anabaena flos-aquae	AB042858.1	AflII	cttaag	M.AflII	cttaag
		AflIII	acgcgt	M.AflIII	acgcgt
		AflIII	acgtgt	M.AflIII	acgtgt
		AflIII	acacgt	M.AflIII	acacgt
		AflIII	acatgt	M.AflIII	acatgt
Anabaena variabilis	AB016520.1	AvaI	cccggg	M.AvaI	cccggg
		AvaI	cccgag	M.AvaI	cccgag
		AvaI	ctcggg	M.AvaI	ctcggg
		AvaI	ctcgag	M.AvaI	ctcgag
		AvaII	ggacc	M.AvaII	ggacc

		AvaII	gggcc	M.AvaII	gggcc
		AvaIII	atgcat	M.AvaIII	atgcat
		AvaIVP	gctaagc	M.AvaIVP	gctaagc
		AvaIVP	gctcagc	M.AvaIVP	gctcagc
		AvaIVP	gctgagc	M.AvaIVP	gctgagc
		AvaIVP	gcttagc	M.AvaIVP	gcttagc
		AvaIX	accggc	M.AvaIX	accggc
		AvaIX	gccggc	M.AvaIX	gccggc
		AvaIX	accggt	M.AvaIX	accggt
		AvaIX	gccggt	M.AvaIX	gccggt
Arthrobacter ON14	AJ810894.1	AscI	ggcgcgcc	M.AscI	ggcgcgcc
Arthrobacter protophormiae	AY577525.1	ApoI	gaattc	M.ApoI	gaattc
		ApoI	gaattt	M.ApoI	gaattt
		ApoI	aaattc	M.ApoI	aaattc
		ApoI	aaattt	M.ApoI	aaattt
Bacillus amyloliquefaciens	HM136792.1	BamFI	ggatcc	M.BamFI	ggatcc
Bacillus brevis	X60612.1	BbvI	gcagc	M.BbvI	gcagc
		BbvII	gaagac	M.BbvII	gaagac
Bacillus caldolyticus	Z26924.1	BclI	tgatca	M.BclI	tgatca
Bacillus coagulans	D16267.1	BcgI	cgannnnnntgc	M.BcgI	cgannnnnntgc
Bacillus firmus	DQ118015.1	BfiI	actggg	M1.BfiI	actggg
Bacillus smithii	Z26935.1	BscGI	cccgt	M1.BscGI	cccgt
Bacillus subtilis	AB598736.2	BsuRI	ggcc	M.BsuRI	ggcc
		BsuMI	ctcgag	M.BsuMI	ctcgag
		Bsu1076I	ggcc	M.Bsu1076	ggcc
		Bsu1114I	ggcc	M.Bsu1114	ggcc
Bacteroides caccae					
ATCC43185T	X83951.1	BccI	ccatc	M1.BccI	ccatc
Caryophanon latum L	X70314.1	ClaI	atcgat	M.ClaI	atcgat

Citrobacter freundii	HQ399663.1	CfrI	cggccg	M.CfrI	cggccg
		CfrI	cggcca	M.CfrI	cggcca
		CfrI	tggccg	M.CfrI	tggccg
		CfrI	tggcca	M.CfrI	tggcca
		Cfr10I	gccggc	M.Cfr10I	gccggc
		Cfr10I	gccggt	M.Cfr10I	gccggt
		Cfr10I	accggc	M.Cfr10I	accggc
		Cfr10I	accggt	M.Cfr10I	accggt
Clostridium formicoaceticum	NR_029267.1	CfoI	gcgc	M.CfoI	gcgc
Deinococcus radiophilus	NR_026402.1	DraII	gggnccc	M.DraII	gggnccc
		DraII	gggncct	M.DraII	gggncct
		DraII	aggnccc	M.DraII	aggnccc
		DraII	aggncct	M.DraII	aggncct
		DraIII	cacnnngtg	M.DraIII	cacnnngtg
Desulfovibrio desulfuricans Norway strain	HQ591418.1	DdeI	ctaag	M.DdeI	ctaag
		DdeI	ctcag	M.DdeI	ctcag
		DdeI	ctgag	M.DdeI	ctgag
		DdeI	cttag	M.DdeI	cttag
Enterobacter cloacae	HM585374.1	EcaI	ggtaacc	M.EcaI	ggtaacc
		EcaI	ggtcacc	M.EcaI	ggtcacc
		EcaI	ggtgacc	M.EcaI	ggtgacc
		EcaI	ggttacc	M.EcaI	ggttacc
Erwinia toletana	FR870447.1	Esp3I	cgtctc	M.Esp3I	cgtctc
Flavobacterium aquatile	AB517711.1	FauI	cccgc	M1.FauI	cccgc
Flavobacterium okeanoikoites	D55729.1	FokI	ggatg	M.FokI	ggatg
Fusobacterium nucleatum	AF543300.1	Fnu4HI	gcagc	M.Fnu4HI	gcagc
		Fnu4HI	gccgc	M.Fnu4HI	gccgc
		Fnu4HI	gcggc	M.Fnu4HI	gcggc

		Fnu4HI	gctgc	M.Fnu4HI	gctgc
		FnuDII	cgcg	M.FnuDII	cgcg
		FnuDIII	gcgc	M.FnuDIII	gcgc
Gluconobacter oxydans	AB308275.1	GsuI	ctggag	M.GsuI	ctggag
Haemophilus aegyptius	M75044.1	HaeII	ggcgcc	M.HaeII	ggcgcc
		HaeII	ggcgct	M.HaeII	ggcgct
		HaeII	agcgcc	M.HaeII	agcgcc
		HaeII	agcgct	M.HaeII	agcgct
		HaeIII	ggcc	M.HaeIII	ggcc
Haemophilus aphrophilus	M75041	HapII	ccgg	M.HapII	ccgg
Haemophilus					
haemoglobinophilus	M75064.1	HhgI	ggcc	M.HhgI	ggcc
Haemophilus haemolyticus	M75045	HhaI	gcgc	M.HhaI	gcgc
		HhaII	gaatc	M.HhaII	gaatc
		HhaII	gactc	M.HhaII	gactc
		HhaII	gagtc	M.HhaII	gagtc
		HhaII	gattc	M.HhaII	gattc
Haemophilus influenzae	AY360336.1	HincII	gtcgac	M.HincII	gtcgac
		HincII	gtcaac	M.HincII	gtcaac
		HincII	gttgac	M.HincII	gttgac
		HincII	gttaac	M.HincII	gttaac
Haemophilus parahaemolyticus	M75073.1	HphI	ggtga	M1.HphI	ccact
Haemophilus parainfluenzae	M75081.1	HpaI	gttaac	M.HpaI	gttaac
		HpaII	ccgg	M.HpaII	ccgg
Methanothermobacter wolfei	AB104858.1	MwoI	gcnnnnnnnngc	M.MwoI	gcnnnnnnnngc
Methylophilus methylotrophus	AB193724.1	MmeI	tccgac	M..MmeI	tccgac
		MmeI	tccaac	M..MmeI	tccaac
		MmeII	gatc	M.MmeII	gatc
Micrococcus luteus	EF154262.1	MluI	acgcgt	M.MluI	acgcgt

Micrococcus species CCGE3063	EU867326.1	MseI	ttaa	M.MseI	ttaa
Moraxella bovis	AF005183.1	MboI	gatc	M1.MboI	gatc
		MboII	gaaga	M1.MboII	gaaga
Moraxella nonliquefaciens	AF005181.1	MnnI	gtcgc	M.MnnI	gtcgc
		MnnI	gtcaac	M.MnnI	gtcaac
		MnnI	gttgac	M.MnnI	gttgac
		MnnI	gttaac	M.MnnI	gttaac
		MnnII	ggcc	M.MnnII	ggcc
		MnnIV	gcgc	M.MnnIV	gcgc
Moraxella species 0408225	HM037355.1	MspI	ccgg	M.MspI	ccgg
Mycoplasma fermentans	AB069812.1	MfeI	caattg	M.MfeI	caattg
Neisseria denitrificans	L06173.1	NdeI	catatg	M.NdeI	catatg
Neisseria lactamica	AJ247242.2	NlaIV	ggnncc	M.NlaIV	ggnncc
		NlaIII	catg	M.NlaIII	catg
Neisseria mucosa	AJ247260.2	NheI	gctagc	M.NheI	gctagc
Plesiomonas shigelloides 319-73	DQ822762.1	PshAI	gacnnnngtc	M.PshAI	gacnnnngtc
Proteus vulgaris	HM446044.1	PvuI	cgatcg	M.PvuI	cgatcg
		PvuII	cagctg	M.PvuII	cagctg
Providencia alcalifaciens	AY994312.1	PalI	ggcc	M.PalI	ggcc
Providencia stuartii 164	HM216181.1	PstI	ctgcag	M.PstI	ctgca
Pseudomonas aeruginosa	JF827020.1	PaeR7I	ctcgag	M.PaeR7	ctcgag
Pseudomonas fluorescens	HQ141340.1	PflMI	ccannnttg	M.PflMI	ccannnttg
Rhodopseudomonas sphaeroides	DQ915852.1	RsaI	gtac	M.RsaI	gtac
		RshI	cgatcg	M.RshI	cgatcg
		RsrI	gaattc	M.RsrI	gaattc
		RsrII	cggaccg	M.RsrII	cggaccg
		RsrII	cgggccg	M.RsrII	cgggccg
Saccharopolyspora Hirsutastrain ATCC 2787	U93341.1	SapI	gctcttc	M.SapI	gctcttc

<i>Serratia marcescens</i>	FN563489.2	SmaI	cccggg	M.SmaI	cccggg
<i>Sphaerotilus natans</i>	AB072236.2	SnaBI	tacgta	M.SnaBI	tacgta
		SpeI	actagt	M.SpeI	actagt
<i>Staphylococcus warneri</i>	DQ007519.1	SwaI	atttaa	M.SwaI	atttaa
<i>Streptomyces caespitosus</i>	AB184320.2	ScaI	agtact	M.ScaI	agtact
<i>Streptococcus cremoris</i>	M58836.1	ScrFI	ccagg	M.ScrFI	ccagg
		ScrFI	cccgg	M.ScrFI	cccgg
		ScrFI	ccggg	M.ScrFI	ccggg
		ScrFI	cctgg	M.ScrFI	cctgg
<i>Streptomyces albus</i>	EF059751.1	SalI	gtcgac	M.SalI	gtcgac
<i>Streptomyces fimbriatus</i>	AB045868.1	SfiI	ggccnnnnnggcc	M.SfiI	ggccnnnnnggcc
<i>Streptomyces griseus</i>	EF154278.1	SgrAI	cgccgggg	M.SgrAI	cgccgggg
		SgrAI	cgccggag	M.SgrAI	cgccggag
		SgrAI	caccgggg	M.SgrAI	caccgggg
		SgrAI	caccggag	M.SgrAI	caccggag
<i>Streptomyces phaeochromogenes</i>	AF500071.1	SphI	gcatgc	M.SphI	gcatgc
<i>Thermoplasma acidophilum</i>	M38637.1	ThaI	cgcg	M.Thal	cgcg
<i>Thermus aquaticus</i>	EU682501.1	TaqI	tcga	M.TaqI	tcga
<i>Thermus filiformis</i>	L09667.1	TfiI	gaatc	M.TfiI	gaatc
		TfiI	gattc	M.TfiI	gattc
<i>Xanthomonas campestris</i>	EF059753.1	XcmI	ccannnnnnnnntgg	M.XcmI	ccannnnnnnnntgg
<i>Xanthomonas oryzae</i>	X95921.1	XorII	cgatcg	M.XorII	cgatcg

a The methylase enzymes used in the analysis are specific to the species as well as the endonuclease restriction enzymes (“cognate”).

S3. Number of endonuclease restriction sequences, number of methylase sequences, and number of overlapping sequences of 96 type II restriction endonuclease enzymes and 96 corresponding methylase enzymes in 68 microbial species.

Microorganisms	Endonuclease restriction enzymes	Endonuclease restriction sites	Number of endonuclease restriction sites	Methylase enzymes	Methylase sites	Number of methylase sites	Number of overlaps
Acetobacter aceti	AatII	gacgtc	1	M.AatII	Gacgtc	1	1
Acetobacter pasteurianus	ApaI	gggccc	1	M.Apai	Gggccc	1	1
	ApaLI	gtgcac	0	M.ApaLI	Gtgcac	0	0
Acinetobacter calcoaceticus	AccI	gtagac	0	M.AccI	Gtagac	0	0
	AccI	gtatac	0	M.AccI	Gtatac	0	0
	AccI	gtcgac	0	M.AccI	Gtcgac	0	0
	AccI	gtctac	0	M.AccI	Gtctac	0	0
	AccII	aacgtt	0	M.AccII	Aacgtt	0	0
	AccIII	tccgga	0	M.AccIII	Tccgga	0	0
Agrobacterium gelatinovorum	AgeI	accggt	0	M.AgeI	Accggt	0	0
Agrobacterium tumefaciens C58	AtuCI	gaatc	0	M.AtuCI	Gaatc	0	0
	AtuCI	gactc	0	M.AtuCI	Gactc	0	0
	AtuCI	gagtc	0	M.AtuCI	Gagtc	0	0
	AtuCI	gattc	0	M.AtuCI	Gattc	0	0
Anabaena flos-aquae	AfIII	cttaag	0	M.AfIII	Cttaag	0	0
	AfIII	acgcgt	1	M.AfIII	Acgcgt	1	1
	AfIII	acgtgt	0	M.AfIII	Acgtgt	0	0
	AfIII	acacgt	1	M.AfIII	Acacgt	1	1
	AfIII	acatgt	0	M.AfIII	Acatgt	0	0
Anabaena variabilis	AvaI	cccggg	1	M.AvaI	Cccggg	1	1
	AvaI	cccgag	1	M.AvaI	Cccgag	1	1
	AvaI	ctcggg	0	M.AvaI	Ctcggg	0	0

	AvaI	ctcgag	0	M.AvaI	Ctcgag	0	0
	AvaII	ggacc	0	M.AvaII	Ggacc	0	0
	AvaII	ggtcc	0	M.AvaII	Ggtcc	0	0
	AvaIII	atgcat	0	M.AvaIII	Atgcat	0	0
	AvaIVP	gctaagc	0	M.AvaIVP	Gctaagc	0	0
	AvaIVP	gctcagc	0	M.AvaIVP	Gctcagc	0	0
	AvaIVP	gctgagc	0	M.AvaIVP	Gctgagc	0	0
	AvaIVP	gcttagc	0	M.AvaIVP	Gcttagc	0	0
	AvaIX	accggc	0	M.AvaIX	Accggc	0	0
	AvaIX	gccggc	0	M.AvaIX	Gccggc	0	0
	AvaIX	accggt	1	M.AvaIX	Accggt	1	1
	AvaIX	gccggt	1	M.AvaIX	Gccggt	1	1
Arthrobacter ON14	AscI	ggcgcgcc	0	M.AscI	Ggcgcgcc	0	0
Arthrobacter protophormiae	ApoI	gaattc	1	M.ApoI	Gaattc	1	1
	ApoI	gaattt	0	M.ApoI	Gaattt	0	0
	ApoI	aaattc	0	M.ApoI	Aaattc	0	0
	ApoI	aaattt	0	M.ApoI	Aaattt	0	0
Bacillus amyloliquefaciens	BamFI	ggatcc	0	M.BamFI	Ggatcc	0	0
Bacillus brevis	BbvI	gcagc	3	M.BbvI	Gcagc	3	3
	BbvII	gaagac	0	M.BbvII	Gaagac	0	0
Bacillus caldolyticus	BclI	tgatca	0	M.BclI	Tgatca	0	0
Bacillus coagulans	BcgI	cgannnnnntgc	0	M.BcgI	cgannnnnntgc	0	0
Bacillus firmus	BfiI	actggg	2	M1.BfiI	Actggg	2	2
Bacillus smithii	BscGI	cccgt	1	M1.BscGI	Cccgt	1	1
Bacillus subtilis	BsuRI	ggcc	4	M.BsuRI	Ggcc	4	4
	BsuMI	ctcgag	0	M.BsuM	Ctcgag	0	0
				M.Bsu107			
	Bsu1076I	ggcc	4	6	Ggcc	4	4
				M.Bsu111			
	Bsu1114I	ggcc	4	4	Ggcc	4	4
Bacteroides caccae	BccI	ccatc	0	M1.BccI	ccatc	0	0

ATCC43185T							
Caryophanon latum							
L	Clal	atcgat	0	M.Clal	Atcgat	0	0
Citrobacter freundii	CfrI	cggccg	1	M.CfrI	Cggccg	1	1
	CfrI	cggcca	0	M.CfrI	Cggcca	0	0
	CfrI	tggccg	0	M.CfrI	Tggccg	0	0
	CfrI	tggccca	0	M.CfrI	Tggcca	0	0
	Cfr10I	gccggc	0	M.Cfr10I	Gccggc	0	0
	Cfr10I	gccggt	0	M.Cfr10I	Gccggt	0	0
	Cfr10I	accggc	1	M.Cfr10I	Accggc	1	1
	Cfr10I	accggt	1	M.Cfr10I	Accggt	1	1
Clostridium							
formicoaceticum	CfoI	gcgc	2	M.CfoI	Gcgc	2	2
Deinococcus							
radiophilus	DraII	gggnccc	1	M.DraII	Gggnccc	1	1
	DraII	gggncct	0	M.DraII	Gggncct	0	0
	DraII	aggncccc	0	M.DraII	Aggncccc	0	0
	DraII	aggncct	1	M.DraII	Aggncct	1	1
	DraIII	cacnnngtg	0	M.DraIII	Cacnnngtg	0	0
Desulfovibrio							
desulfuricans							
Norway strain	DdeI	ctaag	0	M.DdeI	Ctaag	0	0
	DdeI	ctcag	1	M.DdeI	Ctcag	1	1
	DdeI	ctgag	2	M.DdeI	Ctgag	2	2
	DdeI	cttag	0	M.DdeI	Cttag	0	0
Enterobacter cloacae	EcaI	ggtaacc	1	M.EcaI	Ggtaacc	1	1
	EcaI	ggtcacc	0	M.EcaI	Ggtcacc	0	0
	EcaI	ggtgacc	0	M.EcaI	Ggtgacc	0	0
	EcaI	ggttacc	0	M.EcaI	Ggttacc	0	0
Erwinia toletana	Esp3I	cgtctc	0	M.Esp3I	Cgtctc	0	0
Flavobacterium							
aquatile	FauI	cccgc	1	M1.FauI	Cccgc	1	1
Flavobacterium	FokI	ggatg	2	M.FokI	ggatg	2	2

okeanokoites							
Fusobacterium							
nucleatum	Fnu4HI	gcagc	2	M.Fnu4HI	Gcagc	2	2
	Fnu4HI	gccgc	1	M.Fnu4HI	Gccgc	1	1
	Fnu4HI	gcggc	0	M.Fnu4HI	Gcggc	0	0
	Fnu4HI	gctgc	0	M.Fnu4HI	Gctgc	0	0
	FnuDII	cgcg	8	M.FnuDII	Cgcg	8	8
	FnuDIII	gcgc	4	M.FnuDIII	Gcgc	4	4
Gluconobacter							
suboxydan H15T	GsuI	ctggag	1	M.GsuI	Ctggag	1	1
Haemophilus							
aegyptius	HaeII	ggcgcc	1	M.HaeII	Ggcgcc	1	1
	HaeII	ggcgct	0	M.HaeII	Ggcgct	0	0
	HaeII	agcgcc	0	M.HaeII	Agcgcc	0	0
	HaeII	agcgct	0	M.HaeII	Agcgct	0	0
	HaeIII	ggcc	6	M.HaeIII	Ggcc	6	6
Haemophilus							
aphrophilus	HapII	ccgg	6	M.HapII	Ccgg	6	6
Haemophilus							
haemoglobinophilus	Hhgl	ggcc	6	M.Hhgl	Ggcc	6	6
Haemophilus							
haemolyticus	HhaI	gcgc	2	M.HhaI	Gcgc	2	2
	HhaII	gaatc	4	M.HhaII	Gaatc	4	4
	HhaII	gactc	2	M.HhaII	Gactc	2	2
	HhaII	gagtc	1	M.HhaII	Gagtc	1	1
	HhaII	gattc	2	M.HhaII	Gattc	2	2
Haemophilus							
influenza	HincII	gtcgac	0	M.HincII	Gtcgac	0	0
	HincII	gtcaac	0	M.HincII	Gtcaac	0	0
	HincII	gttgac	0	M.HincII	Gttgac	0	0
	HincII	gttaac	0	M.HincII	Gttaac	0	0
Haemophilus							
parahaemolyticus	HphI	ggtga	4	M1.HphI	Ccact	4	4

Haemophilus							
parainfluenzae	HpaI	gttaac	1	M.HpaI	Gttaac	1	1
	HpaII	ccgg	6	M.HpaII	Ccgg	6	6
Methanothermobacter							
wolfei	MwoI	gcnnnnnnngc	6	M.MwoI	gcnnnnnnngc	6	6
Methylophilus							
methylophilus	MmeI	tccgac	0	M..MmeI	Tccgac	0	0
	MmeI	tccaac	0	M..MmeI	Tccaac	0	0
	MmeII	gatc	6	M.MmeII	Gatc	6	6
Micrococcus luteus	MluI	acgcgt	0	M.MluI	Acgcgt	0	0
Micrococcus species							
CCGE3063	MseI	ttaa	5	M.MseI	Ttaa	5	5
Moraxella bovis	MboI	gatc	7	Ml.MboI	Gatc	7	7
Moraxella bovis	MboII	gaaga	2	Ml.MboII	Gaga	2	2
Moraxella							
nonliquefaciens	MnnI	gtcgac	0	M.MnnI	Gtcgac	0	0
	MnnI	gtcaac	0	M.MnnI	Gtcaac	0	0
	MnnI	gttgac	0	M.MnnI	Gttgac	0	0
	MnnI	gttaac	1	M.MnnI	Gttaac	1	1
	MnnII	ggcc	7	M.MnnII	Ggcc	7	7
	MnnIV	gcgc	2	M.MnnIV	Gcgc	2	2
Moraxella species							
0408225	MspI	ccgg	4	M.MspI	Ccgg	4	4
Mycoplasma							
fermentans	MfeI	caattg	0	M.MfeI	Caattg	0	0
Neisseria							
denitrificans	NdeI	catatg	0	M.NdeI	Catatg	0	0
Neisseria lactamica	NlaIV	ggnncc	0	M.NlaIV	Ggnncc	0	0
	NlaIII	catg	2	M.NlaIII	Catg	2	2
Neisseria mucosa	NheI	gctagc	0	M.NheI	Gctagc	0	0
Plesiomonas							
shigelloides 319-73	PshAI	gacnnnngtc	0	M.PshAI	Gacnnnngtc	0	0
Proteus vulgaris	PvuI	cgatcg	0	M.PvuI	Cgatcg	0	0

Providencia	PvuII	cagctg	0	M.PvuII	Cagctg	0	0
alcalifaciens	Pall	ggcc	8	M.PalI	Ggcc	8	8
Providencia stuartii	PstI	ctgcag	0	M.PstI	Ctgca	0	0
164							
Pseudomonas	PaeR7I	ctcgag	0	M.PaeR7	Ctcgag	0	0
aeruginosa							
Pseudomonas	PflMI	ccannntgg	1	M.PflMI	Ccannntgg	1	1
fluorescens							
Rhodopseudomonas	RsaI	gtac	2	M.RsaI	Gtac	2	2
sphaeroides	RshI	cgatcg	1	M.RshI	Cgatcg	1	1
	RsrI	gaattc	0	M.RsrI	Gaattc	0	0
	RsrII	cggaccg	0	M.RsrII	Cggaccg	0	0
	RsrII	cgggccg	0	M.RsrII	Cgggccg	0	0
Saccharopolyspora							
hirsuta strain ATCC							
27875	SapI	gctcttc	0	M.SapI	Gctcttc	0	0
Serratia marcescens							
Sb	SmaI	cccggg	0	M.SmaI	Cccggg	0	0
Sphaerotilus natans	SnaBI	tacgta	0	M.SnaBI	Tacgta	0	0
Sphaerotilus natans	SpeI	actagt	0	M.SpeI	Actagt	0	0
Staphylococcus							
warneri	Swal	atttaa	0	M.Swal	Atttaa	0	0
Streptomyces							
caespitosus	ScaI	agtact	0	M.ScaI	Agtact	0	0
Streptococcus							
cremoris	ScrFI	ccagg	1	M.ScrFI	Ccagg	1	1
	ScrFI	cccgg	1	M.ScrFI	Cccgg	1	1
	ScrFI	ccggg	1	M.ScrFI	Ccggg	1	1
	ScrFI	cctgg	4	M.ScrFI	Cctgg	4	4
Streptomyces albus	Sall	gtcgac	0	M.Sall	Gtcgac	0	0
Streptomyces	Sfil	ggccnnnnnggcc	0	M.Sfil	Ggccnnnnngg	0	0

fimbriatus					cc		
Streptomyces							
griseus	SgrAI	cgccggggg	0	M.SgrAI	Cgccggggg	0	0
	SgrAI	cgccgggag	0	M.SgrAI	Cgccgggag	0	0
	SgrAI	caccggggg	0	M.SgrAI	Caccggggg	0	0
	SgrAI	caccgggag	0	M.SgrAI	Caccgggag	0	0
Streptomyces							
phaeochromogenes	SphI	gcatgc	1	M.SphI	Gcatgc	1	1
Thermoplasma							
acidophilum	ThaI	cgcg	9	M.ThaI	Cgcg	9	9
Thermus aquaticus	TaqI	tcga	3	M.TaqI	Tcga	3	3
Thermus filiformis	TfiI	gaatc	3	M.TfiI	Gaatc	3	3
	TfiI	gattc	0	M.TfiI	Gattc	0	0
Xanthomonas		ccannnnnnnnnt			Ccannnnnnnnn		
campestris	XcmI	gg	1	M.XcmI	ntgg	1	1
Xanthomonas oryzae	XorII	cgatcg	0	M.XorII	Cgatcg	0	0

*16S rRNA was used for the sequence analysis for the 68 microbial species.

R codes used for the statistical analysis:

1) Pagel's lambda phylogenetic signal analysis in R:

```
library(geiger)
```

```
library(picante)
```

```
require(phytools)
```

```
# Insert the data file in csv format into R
```

```
data<-read.csv("C://Users//Sanoji//Desktop//Thesis Defense (MSc.)//16S_GLS_40taxa.csv",header=T)
```

```
attach(data)
```

```
data
```

```
row.names(data)<-data$Species
```

```
# Insert the maximum likelihood tree (nwk format) created in MEGA with using CLUSTALW alignments to R
```

```
tree<-
```

```
read.tree(text="((((((((((((Haemophilus_aegyptius:0.02834477,Haemophilus_haemolyticus:0.00952275)0.4940:0.00674721,Haemophilus_haemoglobinophilus:0.03542718)0.3160:0.00169733,Haemophilus_aphrophilus:0.03221469)0.5660:0.00481971,Haemophilus_parahaemolyticus:0.03345315)0.8100:0.00255476,Haemophilus_parainfluenzae:0.02651519)1.0000:0.05286736,(Providencia_alcalifaciens:0.04001225,(Citrobacter_freundii:0.00874124,Enterobacter_cloacae:0.01983125)0.9980:0.01917691)0.9820:0.02297715)0.9820:0.04481612,(Moraxella_species_0408225:0.05260118,(Moraxella_bovis:0.01587325,Moraxella_nonliquefaciens:0.01472209)0.90
```

```

20:0.01772407)1.0000:0.05090132)0.2620:0.00297452,(Neisseria_lactamica:0.10389168,(Methylophilus_methylotrophus:0.0803166
5,Rhodopseudomonas_sphaeroides:0.04893634)0.6700:0.00000000)0.6280:0.05862459)0.3200:0.01042517,Pseudomonas_fluorescen
s:0.09245554)0.2760:0.00379425,Xanthomonas_campestris:0.08262809)0.9440:0.03326924,(Gluconobacter_oxydans:0.02920671,(A
cetobacter_aceti:0.01032713,Acetobacter_pasteurianus:0.02256529)1.0000:0.01477971)0.9880:0.08186815)0.5520:0.02233481,Desu
lfovibrio_desulfuricans_Norway_strain:0.14228055)0.6660:0.00989726,(Deinococcus_radiophilus:0.11931439,Thermus_filiformis:0.
12747435)0.9980:0.07595853)0.5040:0.01470060,(((Arthrobacter_protophormiae:0.02984946,Micrococcus_species_CCGE3063:0.02
582441)1.0000:0.03548763,Streptomyces_phaeochromogenes:0.05837572)1.0000:0.07145076,((Flavobacterium_aquatile:0.2844353
3,Fusobacterium_nucleatum:0.18525005)0.4820:0.01088731,(Clostridium_formicoaceticum:0.12262417,(Streptococcus_cremoris:0.1
2663599,(Bacillus_brevis:0.05004504,(Thermus_aquaticus:0.05950836,(Bacillus_smithii:0.03143985,(Bacillus_subtilis:0.04230718,(
Bacillus_firmus:0.01828016,Flavobacterium_okeanokoites:0.04248660)0.4980:0.00061841)0.9660:0.00925016)0.8800:0.01551923)0
.9540:0.01482449)0.9580:0.02399982)0.6440:0.00887582)0.8320:0.03543660)0.3120:0.00000000)0.4820:0.00213549)0.4800:0.0590
6264,(Anabaena_flos_aquae:0.01952297,Anabaena_variabilis:0.04446831)0.9400:0.05265282,(Methanothermobacter_wolfei:0.1313
2052,Thermoplasma_acidophilum:0.21142217)1.0000:0.40041443);")

```

```
# Root the tree in order to do GLS
```

```
tree$root.edge <- 0
```

```
names(EN_Sites)<-rownames(data)
```

```
names(M_Sites)<-rownames(data)
```

```
tree2<-multi2di(tree,random=T)
```

```
plot(tree2)
```

#testing for phylogenetic signal using Blomberg's K:

```
signal<-phylosignal(EN_Sites,tree2)
```

```
signal
```

```
signal<-phylosignal(M_Sites,tree2)
```

```
signal
```

#testing for phylogenetic signal using Pagel's Lambda:

```
phylosig(tree,EN_Sites,method="lambda",test=TRUE)
```

```
phylosig(tree,M_Sites,method="lambda",test=TRUE)
```

This calculates Pagel's lambda for the actual tree

```
fitContinuous(tree2, data$EN_Sites, model= "lambda")
```

```
fitContinuous(tree2, data$M_Sites, model= "lambda")
```



```
# This creates a new, "null" tree with uninformative branch lengths
```

```
phy0 <- lambdaTree(tree, 0)
```

```
plot(phy0)
```

```
# Run the code on the new null tree
```

```
fitContinuous(phy0, data$EN_Sites,model= "lambda")
```

```
fitContinuous(phy0, data$M_Sites, model= "lambda")
```

2) Quantitative sequence comparison of endonuclease restriction sequences with the methyltransferase sequences using Seqinr and Ape packages in R:

```
# Install and load the "ape" and "seqRFLP" packages
```

```
#install.packages("ape")
```

```
library(ape)
```

```
#install.packages("seqRFLP")
```

```
library(seqRFLP)
```

```
# Read in the data from a comma-separated file
```

```
accession.numbers<-read.csv("C:\\Users\\Chris\\Documents\\Chris' Files\\Carleton Work\\Sanoji R  
stuff\\Ddatasheet.csv",header=TRUE)
```

```
# Attach this data file to the workspace
```

```

attach(accession.numbers)

# Define the column of accession numbers as "character" format
accession<-as.character(Accession)

E.results.sp<-factor(0)

E.results.enzyme<-character(0)

E.results.site<-character(0)

E.results.loc<-numeric(0)

M.results.sp<-character(0)

M.results.enzyme<-character(0)

M.results.site<-character(0)

M.results.loc<-numeric(0)

# Define the first column as the species names from the GenBank query
species<-unique(Microorganism)

# Start a loop for each sequences (each species)
for(x in 1:length(species)){
  for (y in 1:length(subset(Microorganism, Microorganism==species[x]))) {
    # reading DNA from accession numbers individually

```

```

genes<-read.GenBank(subset(Accession,Microorganism==species[x])[y], species.names = TRUE, gene.names = TRUE,
as.character = TRUE)

gene.seq<-paste(genes[[1]],sep="",collapse="")

# DNA sequence comes from the accession number, the probe is defined as the site associated with that gene and enzyme
e.locations<-findprobe(gene.seq,as.character(subset(ERS,Microorganism==species[x])[y]),tol=0)

m.locations<-findprobe(gene.seq,as.character(subset(MRS,Microorganism==species[x])[y]),tol=0)

E.results.sp<-as.character(c(E.results.sp,as.character(rep(species[x],length(e.locations)))))

E.results.enzyme<-
as.character(c(E.results.enzyme,as.character(rep(subset(E.enzymes,Microorganism==species[x])[y],length(e.locations)))))

E.results.site<-as.character(c(E.results.site,as.character(rep(subset(ERS,Microorganism==species[x])[y],length(e.locations)))))

E.results.loc<-c(E.results.loc,e.locations)

M.results.sp<-as.character(c(M.results.sp,as.character(rep(species[x],length(m.locations)))))

M.results.enzyme<-
as.character(c(M.results.enzyme,as.character(rep(subset(M.enzymes,Microorganism==species[x])[y],length(m.locations)))))

M.results.site<-as.character(c(M.results.site,as.character(rep(subset(MRS,Microorganism==species[x])[y],length(m.locations)))))

M.results.loc<-c(M.results.loc,m.locations)

}

}

E.results.sp<-E.results.sp[-1]

```

Set up two results tables with the first column as a list of species, the second column as a list of enzymes, the third column as a list of binding sites and the fourth column as a list of locations

```
E.results<-matrix(ncol=4,nrow=length(E.results.sp))
```

```
M.results<-matrix(ncol=4,nrow=length(M.results.sp))
```

Name the columns

```
colnames(E.results)<-c("Species","E.Enzyme","Sites","Locations")
```

```
colnames(M.results)<-c("Species","M.Enzyme","Sites","Locations")
```

Paste vectors into the appropriate columns

```
E.results[,1]<-as.character(E.results.sp)
```

```
E.results[,2]<-as.character(E.results.enzyme)
```

```
E.results[,3]<-as.character(E.results.site)
```

```
E.results[,4]<-as.numeric(E.results.loc)
```

```
M.results[,1]<-as.character(M.results.sp)
```

```
M.results[,2]<-as.character(M.results.enzyme)
```

```
M.results[,3]<-as.character(M.results.site)
```

```
M.results[,4]<-as.numeric(M.results.loc)
```

```
E.results<-unique(E.results)
```

```
M.results<-unique(M.results)
```

```

E.final.results<-matrix(ncol=4,nrow=nrow(unique(E.results[,c(1:3)])))

colnames(E.final.results)<-c("Species","EN enzyme","EN site","EN locations")

E.final.results[,1]<-unique(E.results[,c(1:3)]),[1]

E.final.results[,2]<-unique(E.results[,c(1:3)]),[2]

E.final.results[,3]<-unique(E.results[,c(1:3)]),[3]

for(x in 1:nrow(unique(E.results[,c(1:3)]))) {

E.final.results[x,4]<-length(subset(as.numeric(E.results[,4]),E.results[,1]==E.final.results[x,1] & E.results[,2]==E.final.results[x,2]
& E.results[,3]==E.final.results[x,3] & as.numeric(E.results[,4])>0))

}

M.final.results<-matrix(ncol=4,nrow=nrow(unique(M.results[,c(1:3)])))

colnames(M.final.results)<-c("Species","M enzyme","M site","M locations")

M.final.results[,1]<-unique(M.results[,c(1:3)]),[1]

M.final.results[,2]<-unique(M.results[,c(1:3)]),[2]

M.final.results[,3]<-unique(M.results[,c(1:3)]),[3]

for(x in 1:nrow(unique(M.results[,c(1:3)]))) {

M.final.results[x,4]<-length(subset(as.numeric(M.results[,4]),M.results[,1]==M.final.results[x,1] &
M.results[,2]==M.final.results[x,2] & M.results[,3]==M.final.results[x,3] & as.numeric(M.results[,4])>0))

}

write.table(E.final.results,"C:\\Users\\Chris\\Documents\\Chris' Files\\Carleton Work\\Sanoji R stuff\\EN final results.txt")

```

```

write.table(M.final.results,"C:\\Users\\Chris\\Documents\\Chris' Files\\Carleton Work\\Sanoji R stuff\\M final results.txt")

# Calculating the overlap between sequences.

# First, checking what the locations mean:

test.seq<-"ABCDEFGHJKLMNOP"

probe<-"FGHI"

findprobe(test.seq,probe,tol=0)

# Location=6, so

#  A B C D E F G H I J K L M N O P
#  1 2 3 4 5 F G H I

for(x in 1:2){#length(species)){
E.sites<-as.numeric(subset(E.results[,4],E.results[,1]==species[x]))
M.sites<-as.numeric(subset(M.results[,4],M.results[,1]==species[x]))
site.comparison<-matrix(ncol=3,nrow=length(E.sites)*length(M.sites))
site.comparison[,1]<-rep(E.sites,length(M.sites))
site.comparison.data<-numeric(length=0)
for(y in 1:length(M.sites)){
site.comparison.data<-c(site.comparison.data,rep(M.sites[y],length(E.sites)))
}
}

```

```
site.comparison[,1]<-site.comparison.data
}
for(y in 1:length(site.comparison[,1])){
  site.comparison[y,3]<-site.comparison[y,1]-site.comparison[y,2]
}
table(site.comparison[y,3]<nchar(
}
write.table(results,"C:\\Users\\Chris\\Documents\\Chris' Files\\Carleton Work\\Sanoji R stuff\\overlap results.txt",header=TRUE)
```

Figure 4. Molecular phylogeny of 38 eubacterial and 2 archaea species based on complete and partial 16S rRNA. The tree was constructed in MEGA 2.02 with the maximum likelihood method and 500 bootstrap intervals. The branch lengths correspond to a scale of 0.05.