Chapter 9

Host-Mimicking Strategies in DNA Methylation for Improved Bacterial Transformation

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Additional information is available at the end of the chapter

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1. Introduction

In 1928, Griffith [1] reported that soluble substances from virulent pneumococcal cells transformed non-virulent pneumococcus to virulent forms. This substance has now been demonstrated to be DNA [2-4]. This is considered to be the first report on genetic transformation of bacteria by exogenous DNA. Subsequently, natural competence of *Bacillus subtilis* was reported in 1958 by Young and Spizizen [5]. They also demonstrated genetic transformation of natural competent *B. subtilis* cells using exogenous DNA. It was in 1970 that genetic transformation of *Escherichia coli* using chemically competent cells was reported [6]. Thus, genetic transformation of common bacterial models was established at an early stage in the development of bacteriology. The alternative view is that bacterial models such as *B. subtilis* and *E. coli* have become the mainstay of this field because of high transformation ability. Genetic transformation techniques remain important for studying numerous bacteria and for the advancement of bacteriology, biochemistry, applied microbiology, and microbial biotechnology. Moreover, recent developments in the search for new bacteria and genome sequencing have provided numerous effective bacteria that are useful for biological studies and industrial applications. With these developments, there is a greater demand for establishing genetic transformation methods for more bacteria.

DNA introduction is an essential process for transforming target bacterium by exogenous DNA. Various methods for introducing DNA into bacteria have been developed to date, including chemotransformation, electroporation, sonopolation, tribos, and conjugational transfer [7]. In spite of these developments, it is often difficult to establish transformation methods for target bacterium. A possible reason is the difficulty faced while exploring suitable conditions for introducing DNA, which requires not only theoretical understanding but also a trial and error approach. Circumventing bacterial RM systems is a major challenge.

These systems defend bacteria against transformation by exogenous DNA, such as bacteriophages, and effectively hamper genetic transformation by exogenous plasmids. RM systems selectively digest exogenous DNA by differentiating them from host-endogenous DNA on the basis of host-specific DNA methylation [8]. Therefore, DNA that imitates the methylation patterns of the host bacterium (host-mimicking DNA) is incorporated into the bacterium without restriction. Thus, RM systems can be overcome by theoretical host-mimicking strategies (Figure 1), rather than exploring conditions for introducing DNA. This chapter explains host-mimicking strategies and provides tips for establishing transformation methods for new bacteria.

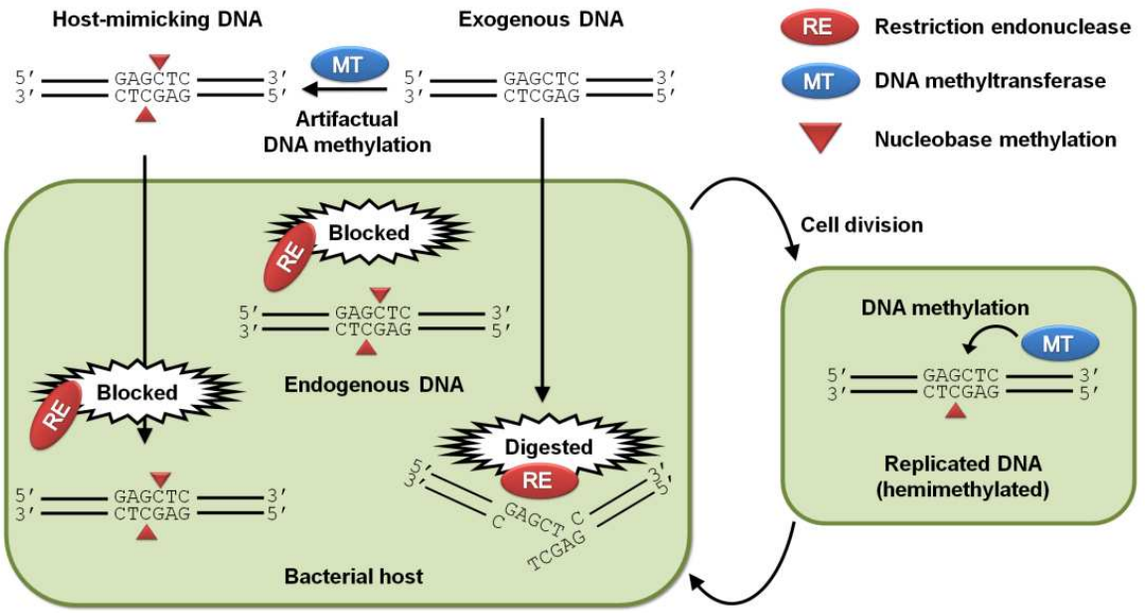

Figure 1. Host-mimicking strategies for circumventing restriction–modification (RM) systems in bacteria.RM systems serve to defend bacteria against invasion by exogenous DNA, and thereby hamper genetic transformation by exogenous plasmids. In typical RM systems, restriction endonuclease (RE) digests exogenous DNA but not endogenous DNA that has been methylated by cognate DNA methyltransferase (MT). Host-mimicking DNA that imitates the methylation patterns of the bacterial host is recognized as endogenous DNA by the host because RM systems depend on host-specific DNA methylation to distinguish between exogenous and endogenous DNA.

2. RM systems

In 1962, Arber and Dussoix [9] found that bacteriophage λ carried specificity for *E. coli* strains in which they were produced. For examples, bacteriophage λ from *E. coli* K-12 was efficiently transfected into K-12 and C strains, but not strains B and K(P1) (efficiencies were $<10^{-4}$ fold). Meanwhile, bacteriophage λ produced by *E. coli* C efficiently transformed strain C, but not strains K-12, B, and K(P1) (efficiencies were $<4\times10^{-4}$ fold). Thus, bacteriophage λ vectors readily infected *E. coli* strains that produced them but the infection to other strains was "restricted". This occurrence, termed restriction, is explained by *E. coli* RM systems that act as host defense against exogenous DNA.

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RM systems selectively digest exogenous DNA and greatly influence the efficiency of genetic transformation by exogenous DNA. Numerous RM systems have been found in bacteria and archaea, and are classified into four main types (type I–IV) [10]. Their general properties are summarized in Table 1. The REBASE database (rebase.neb.com) [11] has accumulated large amounts of information about RM systems, including types, gene sequences, recognition sites, and origin organisms. Among the four types of RM systems, type II consists of RE and MT. In this type, RE cuts exogenous DNA at specific sites but not endogenous DNA that has been methylated by MT (Figure 1). Type I and III systems also cut exogenous DNA by similar mechanisms, but comprise protein complexes of some subunits. Type IV is known as a modification-specific restriction system to cut DNA with heterologous modifications. Intriguingly, RM systems behave as selfish elements like viruses and transposons [12], implying that RM systems have been irreversibly distributed in bacteria. The following sections describe more details of RM systems.

2.1. DNA modifications involved in DNA restriction

Several DNA modifications have been elucidated and in almost all cases the modifications that are involved in restriction are nucleobase methylations. The main forms of methylated nucleobases are *N* ^{6}-methyladenine (^{6m}A; Figure 2), 5-methylcytosine (^{5m}C), and *N* ^{4}-methylcytosine (^{4m}C). These modifications are performed for double-stranded DNA using methyltransferases or other methyltransfer machinery. In addition, some bacteriophage DNA contain 5-hydroxymethylcytosine (^{5hm}C) instead of cytosine. This modification is incorporated during phage DNA replication using 5-hydroxymethyldeoxycytidine triphosphate as the substrate [13]. The hydroxyl group is further glucosylated to produce β-glucosyl-5-hydroxymethylcytosine (^{ghm}C) in a phage-specific pattern by glucosyltransferase [14]. This modification has various biological functions, including circumvention of restriction barriers of RM systems in phage hosts [13, 14]. The modified cytosine ^{5hm}C is also found in mammalians [15-17]. Unlike phages, it is produced by oxidation of ^{5m}C in double-stranded DNA [18]. In bacteria, there are no known type I–III RM systems involving ^{5hm}C or ^{ghm}C. However, several type IV systems that restrict DNA containing ^{5hm}C and/or ^{ghm}C have been reported [19-21].

RM system	Restriction		Methylation	
	Machinery	Cleavage site	Machinery	Nucleobase
Type I	R_2M_2S	Variable	M_2S	^{6m}A
Type II	RE	Fixed	MT	^{6m}A, ^{5m}C, ^{4m}C
Type III	R_2M_2	Variable	M_2	^{6m}A
Type IV	RE	Variable		

Table 1. General properties of four types of RM systems.Type I comprises R, M, and S subunits. Methylation is performed by subunits M and S. Type III comprises R and M subunits. The M subunit alone catalyzes methylation. Type II comprises two independent proteins, RE and MT. Type IV comprises only RE and restricts DNA with heterologous modifications. Methylation produces ^{6m}A, ^{5m}C, or ^{4m}C.

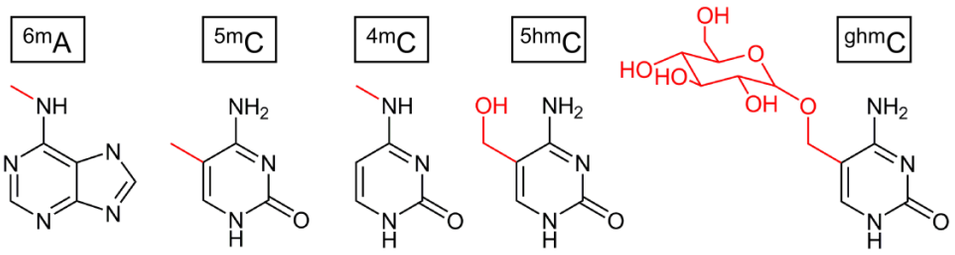

Figure 2. Chemical structures of modified nucleobases (modification moieties are indicated in red).

In addition to the methyl-based modifications described above, sulfur modification (phosphorothioation) of DNA backbones has been observed [22, 23]. Notably, it is suggested that this modification is involved in DNA restriction in *Salmonella enterica* [24]. The gene cluster for this RM system consists of eight genes, of which four are involved in phosphorothioation, while seven genes are essential for restricting unphosphorothioated DNA. Similar gene clusters, along with DNA containing phosphorothiol bonds, are found in many bacteria, implying that phosphorothioation is a widespread DNA modification [24, 25]. A type IV system that restricts phosphorothioated DNA has been also reported [26].

2.2. Type I RM systems

The *E. coli* strain K-12 harbors one type I RM system (EcoKI) encoded by the genes *hsdR* (R subunit), *hsdM* (M subunit), and *hsdS* (S subunit). This system constructs a multi-subunit complex that comprises two R subunits, two M subunits, and one S subunit (R_2M_2S), and scans double-stranded DNA after replication [27, 28]. When the complex recognizes DNA that is unmethylated at recognition sites, it acts as an ATP dependent endonuclease to digest DNA. The sequences of recognition sites are asymmetric but not palindromic. Examples include 5′-AACN_6GTGC-3′ and 5′-GCACN_6GTT-3′ for EcoKI, 5′-TGAN_8TGCT-3′ and 5′-AGCAN_8TCA-3′ for EcoBI, and 5′-TTAN_7GTCY-3′ and 5′-RGACN_7TAA-3′ for EcoDI (methylated adenine is underlined; R: A/G, Y: C/T, N: A/C/G/T) [29, 30].

The cleavage positions are distal from recognition sites and are variable. It is believed that the complex of type I RM system, while binding to recognition sites, translocates (or pulls) the DNA along in an ATP dependent fashion, and cleaves DNA when the translocation is impended by collision and/or by stalling with another translocating complex [27, 31, 32]. Electron microscopy analysis has been used to detect ATP-dependent formation of loop DNA during DNA cleavage by a type I RM complex [32]. The R subunit is responsible for ATP hydrolysis, translocation, and endonuclease activity, but not DNA binding. The binding depends on subunits M and S, which are therefore essential for both endonuclease and methyltransferase activities.

The complex of type I RM system acts similar to methyltransferase when it recognizes DNA that is hemimethylated (methylated on one strand) at recognition sites [27]. The methylation is performed by M and S subunits using *S*-adenosyl-L-methionine as the methyl donor [8]. The M subunit has the binding site for *S*-adenosyl-L-methionine, while the S subunit is essential for determining recognition sites. The R subunit is unnecessary for methyltransferase

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activity. In all cases reported so far, methylation by type I RM systems occurs in adenine to produce ^{6m}A.

2.3. Type II RM systems

Type II RM systems are extremely diverse and are currently classified into 11 subfamilies [8]. Generally, these comprise two enzymes, RE and MT. Cleavages of exogenous DNA at unmethylated recognition sites is carried out by RE, which spares endogenous DNA that has been methylated by the cognate MT. Most REs require Mg^{2+} ions as a cofactor for cleavage. Although RE may form monomers, dimers, or tetramers, it functions without forming complexes with the cognate MT. The recognition of cleavage sites is highly precise and recognition sequences are often palindromic. Such sites include 5′-GAATTC-3′ for EcoRI and 5′-GGATCC-3′ for BamHI, which are cleaved symmetrically within the sites. Because of these useful properties, more than 3,500 REs have been characterized, and many are widely utilized in recombinant DNA technology [8]. The enzyme MT catalyzes methylation at recognition sites using *S*-adenosyl-L-methionine as the substrate and generally acts as a monomer. The nucleobases produced are ^{6m}A, ^{5m}C, or ^{4m}C. For example, EcoRI and BamHI methyltransferases produce 5′-GA^{6m}ATTC-3′ and 5′-GGAT^{4m}CC-3′, respectively.

2.4. Type III RM systems

Type III RM systems operate with multi-subunit machinery comprising two R subunits and two M subunits (R_2M_2) [33]. Subunit M contains recognition domain for binding to specific sites and also a methyltransferase domain. It can thereby bind at recognition sites independently, and methylate DNA using *S*-adenosyl-L-methionine as a substrate. The nucleobase produced is ^{6m}A in all cases reported so far. Unlike type I and II RM systems, full modification is actually hemimethylation (methylation on one strand). The recognition sequences are asymmetric, such as 5′-CAGCAG-3′ for EcoP15I (methylated adenine is underlined).

Subunit R has an ATP dependent DNA helicase and endonuclease domains that are responsible for DNA cleavage. This subunit is unable to bind to DNA and therefore requires subunit M to cleave DNA. Two unmethylated sites that are inversely oriented (head-to-head orientation) serve as the target for DNA cleavage. Cleavage occurs at 25–27 bp downstream of one of the recognition sites, which is chosen randomly from the two sites. Even DNA with 3.5 kb between the two sites is cleaved. The cleavage requires ATP similar to type I RM systems. However, the amount of ATP consumed is only ~1% of that required for cleavage by type I RM systems. This fact makes it difficult to transpose the translocation model that is proposed in type I RM systems to type III RM systems. Thus, some alternative models have now been proposed [33].

2.5. Type IV restriction systems

Several enzymes specifically restrict modified DNA [10, 19-21, 26, 34-36]. These systems offer very efficient to defense from bacteriophages with highly-modified DNA. Among these, the enzymes for which cleavage sites are very specific and precise are classified into the M

subfamily of type II RM systems [21]. Examples include DpnI (recognition sequence: 5′-G^{6m}ATC-3′), GlaI (5′-G^{5m}CG^{5m}C-3′), BisI (5′-G^{5m}CNGC-3′; N: A/G/C/T), and MspJI (5′-^{5m}CNNR-3′; R: G/A). The enzymes with non-specific and variable cleavage sites are classified as type IV restriction systems. The *E. coli* strain K-12 harbors three type IV systems encoded by *mcrA*, *mcrB-mcrC*, and *mrr*. The enzyme McrA recognizes 5′-Y^{5m}CGR-3′ site (Y: C/T; R: G/A) [37], whereas McrBC recognizes pairs of 5′-R^{m}C-3′ (^{m}C: ^{5m}C or ^{4m}C) separated by 40–3000 bp, and cleaves DNA ~30 bp distal from one of the sites [36]. The Mrr system recognizes DNA containing ^{6m}A, ^{5m}C, or ^{4m}C, but its recognition sites have not been well defined [36]. The enzyme SauUSI of *Staphylococcus aureus* recognizes 5′-S^{5m}CNGS-3′ and 5′-S^{5hm}CNGS-3′ (S: C/G; N: A/G/C/T) and cleaves at position 2–18 bp downstream of the recognition site [21]. The enzyme GmrSD restriction system of *E. coli* CT596 cuts DNA containing ^{ghm}C [20]. Thus, all species of modified nucleobases are potentially restricted by type IV restriction systems.

Microbe	Host-mimicking DNA		Reference
	Production	Introduction	
Bacillus anthracis	*In vivo* (MF)	Electroporation	[50]
Bacillus cereus	*In vitro* (EX)	Electroporation	[51]
Bacillus weihenstephanensis	*In vitro* (EX)	Electroporation	[51]
Bifidobacterium adolescentis	*In vivo* (GM)	Electroporation	[52]
Bifidobacterium longum	*In vitro* (MT/SD)	Electroporation	[53]
Borrelia burgdorferi	*In vitro* (MT)	Electroporation	[54]
Clostridium acetobutylicum	*In vivo* (HG)	Electroporation	[55]
Clostridium difficile	*In vivo* (HG/SD)	Conjugation	[56]
Clostridium thermocellum	*In vivo* (IG)	Electroporation	[57]
Geobacillus kaustophilus	*In vivo* (GM/IG)	Conjugation	[41]
Helicobacter pylori	*In vitro* (EX)	Competency	[58]
Salmonella typhimurium	*In vivo* (DS)	Competency	[59]
Staphylococcus aureus	*In vivo* (DS)	Electroporation	[60]
Streptomyces avermitilis	*In vivo* (MF)	Protoplast	[61]
Streptomyces bambergiensis	*In vivo* (MF)	Conjugation	[62]
Streptomyces coelicolor	*In vivo* (MF)	Protoplast	[35]
Streptomyces griseus IFO 13350	*In vivo* (GM/HG)	Protoplast	[40]
	In vivo (DS)	Protoplast	[63]
Streptomyces griseus NRRL B-2682	*In vitro* (MT)	Protoplast	[64]
Streptomyces natalensis	*In vivo* (MF)	Conjugation	[65]
Sulfolobus acidocaldarius	*In vivo* (HG)	Electroporation	[38]
Thermoanaerobacter sp. X514	*In vivo* (IG)	Sonoporation	[66]

Table 2. Microbial transformation using host-mimicking DNA.DS: *in vivo* methylation in a strain that is related to the target bacterium and is deficient in restriction and proficient in methylation; EX: *in vitro* methylation using a crude extract of the target bacterium; GM: *in vivo* methylation using methyltransferase genes in the target bacterial genome; HG: *in vivo* methylation using heterologous genes; IG: *in vivo* methylation using *E. coli* intrinsic genes; MF: methyl-free DNA; MT: *in vitro* methylation using commercially available methyltransferases; and SD: transformation using DNA with specifically abolished recognition sites.

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3. Host-mimicking strategies

Circumvention of RM systems is critical for establishing transformation methods for target bacteria (Table 2). This is true not only for bacteria but also archaea [38]. This section describes some strategies for circumventing RM systems and focuses on host-mimicking. Because all types of RM systems digest exogenous DNA after distinguishing it from endogenous DNA on the basis of host-specific methylation patterns, DNA modification that mimic these patterns evade digestion. A general flowchart for producing host-mimicking DNA is shown in Figure 3. The details are described in the following sections.

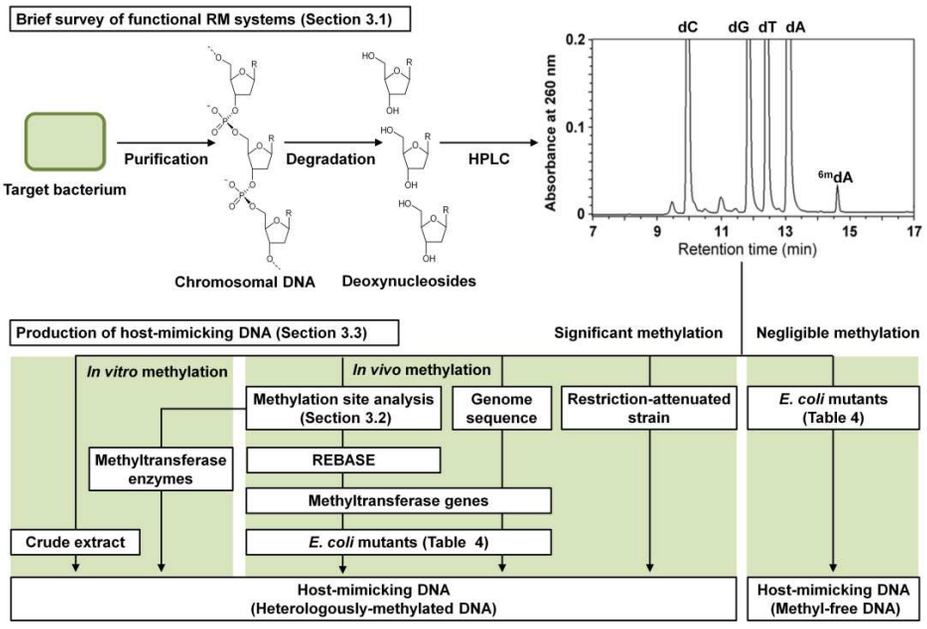

Figure 3. General flowchart for producing host-mimicking DNA to target bacteria.The chromatogram is the data from HPLC analysis of deoxynucleosides prepared from *G. kaustophilus* chromosomes [41]. It includes 2′-deoxyadenosine (dA), 2′-deoxycytidine (dC), 2′-deoxyguanosine (dG), 2′-deoxythymidine (dT), and *N* ^{6}-methyl-2′-deoxyadenosine (^{6m}dA) but not 5-methyl-2′-deoxycytidine (^{5m}dC) or *N* ^{4}-methyl-2′-deoxycytidine (^{4m}dC).

3.1. A brief survey of functional RM systems

As mentioned earlier, type I–III RM systems involve DNA methylation in their functions. Therefore, the presence of methylated DNA in bacterial chromosomes indicates a type I–III RM system in the bacterium. Hence analysis of methylated DNA is an effective survey method to identify functional RM systems in target bacterium. Methylated DNA in chromosomes can be analyzed using high-performance liquid chromatography (HPLC) [15, 18, 39-45]. This method determines the presence of deoxynucleosides and methylated deoxynucleosides. Deoxynucleosides are prepared by hydrolyzing chromosomal DNA with nuclease P1 and alkaline phosphatase [39-41], are separated using reverse mode C_{18}-based silica columns, and are detected by ultraviolet absorption at 260 and/or 280 nm. Authentic ^{6m}dA, ^{5m}dC, and ^{4m}dC are commercially available or can be prepared from methylated 2′-deoxynu-

cleoside-5′-triphosphate by dephosphorylation using *E. coli* alkaline phosphatase [40]. Note that this analysis requires large amounts of DNA (>10 μg). Contamination with RNA, proteins, and chemicals that absorb ultraviolet radiation may also complicate accurate analysis. In some reports [18, 42, 43], deoxynucleosides are more accurately identified by combining HPLC and mass spectrometry analysis. In addition, immunochemical methods are available for analyzing methylated DNA [46-49]. Several anti-^{5m}C antibodies have been developed and are commercially available. Although commercially available antibodies against other methylated DNA are limited, Kong *et al*. [49] have reported successful production of rabbit polyclonal antibodies against ^{6m}A and ^{4m}C.

Deoxynucleoside	Relative coefficient	
	Detection at 260 nm	Detection at 280 nm
dC	2.34	1.74
^{5m}dC	3.51	1.77
^{4m}dC	1.77	1.57
dA	1.08	6.42
^{6m}dA	1.15	1.93

Table 3. Relative coefficients for determining deoxynucleoside molar ratios by HPLC analysis; [Relative amount of deoxynucleoside] = [Coefficient] × [Peak area on HPLC chromatogram].

Analysis of DNA using HPLC allows determination of deoxynucleoside composition and methylation frequency in chromosomes. The coefficients in Table 3 may be used for estimation. For example, HPLC analysis of *S. griseus* chromosomes revealed ^{5m}dC but not ^{6m}dA or ^{4m}dC [40]. The composition ratio of ^{5m}dC to dC was 0.7 mol%, suggesting that *S. griseus* possesses approximately one ^{5m}C per 0.5 kb of chromosomal DNA with 67% GC content. The chromosome of *G. kaustophilus* contains ^{6m}dA and the composition ratio to dA is 2.0 mol% [41]. This suggests that *G. kaustophilus* possesses approximately one ^{6m}A per 0.1 kb of chromosomal DNA with 52% GC content. Although DNA methylation unrelated to RM systems have been observed, such as *E. coli* Dam and Dcm methylation [67, 68], high frequency methylations imply that the bacteria may harbor a considerable type I–III RM system. Meanwhile, the chromosomes of *S. avermitilis*, *S. coelicolor*, and *S. lividans* contain no methylated DNA (my unpublished data). This observation suggests that these bacteria harbor no type I–III RM systems. However, the possibility remains that a functional type IV system exists in these species. Potent methyl-specific restrictions have been observed in *S. avermitilis* [61], *S. coelicolor* [35], *S. bambergiensis* [62], and *S. natalensis* [65].

3.2. Methylation site analysis

When significant DNA methylation is observed in the target bacterium, preliminary determination of DNA methylation sites is generally required to produce host-mimicking DNA. Recent epigenetic studies have developed many methods to analyze DNA methylation [29, 30, 69-76]. Although most of these studies aimed to analyze 5-methylation of cytosine at spe-

cific sites, or differential DNA methylation in chromosomes, there are a few methods that exhaustively determine methylated consensus sites in chromosomes as follows.

In *S. griseus*, bisulfite-based analysis of a plasmid library isolated from this bacterium (Figure 4A) was used to determine the two consensus sites 5′-GAG^{5m}CTC-3′ and 5′-GC^{5m}CGGC-3′ [40]. Note that this method is not employed for methylation analysis in bacteria that cannot be transformed with exogenous plasmids, and in bacteria that have methylated nucleobases other than ^{5m}C. For determining ^{6m}A consensus sites in *G. kaustophilus*, chromosome digestion using methyl-sensitive restriction enzymes (Figure 4B) was used to reveal 5′-GG^{6m}ATC-3′ and 5′-G^{6m}ATCC-3′ site [41]. Methods using methyl-sensitive restriction enzymes help identify several methylation species, including ^{6m}A, ^{5m}C, and ^{4m}C. However, methylation sites that can be identified by these methods are limited due to the lack of commercially available restriction enzymes. Recently, direct detection using real-time DNA sequencing has been reported [74, 75]. This method potentially enables the exhaustive determination of all methylation sites in chromosomal DNA. Although this method requires special equipments, which has limited availability, it may become one of the most promising methods for methylation analysis in the future. Also, the author has now developed a versatile immunological method for determining consensus sequences with methylated nucleobases.

3.3. Production of host-mimicking DNA

If the target bacterium contains no or negligible methylated DNA, methyl-free DNA should be used as host-mimicking DNA because the bacterium may harbor type IV restriction systems, as exemplified by transformation of *S. avermitilis* [61], *S. coelicolor* [35], and *S. natalensis* [65]. Methyl-free DNA can be readily produced using *E. coli* strains deficient in DNA methyltransferase genes (*dam* $^-$ *dcm* $^-$ *hsd* $^-$), such as *E. coli* IR27, ET12567, IBEC58, and HST04 (Table 4). Methyl-restrained DNA from *E. coli* GM2929 and SCS110 (*dam* $^-$ *dcm* $^-$ *hsd* $^+$) were also used for efficient transformation of *S. bambergiensis* [62] and *B. anthracis* [50], respectively. Because Hsd mediated methylation is of low frequency in *E. coli*, deficiency of this methyltion may not be essential for circumventing type IV systems.

If the target bacterium contains considerable methylated DNA, host-mimicking DNA that reconstitutes the methylation pattern of the target bacterium should be used for transformation. There are two main approaches to produce heterologous methylation of DNA. One is *in vitro* methylation using methyltransferases [53, 54, 64], such as Dam (catalyzing 5′-G^{6m}ATC-3′ methylation), M.TaqI (5′-TCG^{6m}A-3′), M.AluI (5′-AG^{5m}CT-3′), M.BamHI (5′-GGATC^{4m}C-3′), M.SssI (5′-^{5m}CG-3′), M.EcoRI (5′-GA^{6m}ATTC-3′), M.CviPI (5′-GC^{5m}-3′), M.HaeIII (5′-GGC^{5m}C-3′), M.HhaI (5′-G^{5m}CGC-3′), M.HpaII (5′-C^{5m}CGG-3′), and M.MspI (5′-^{5m}CCGG-3′). This approach is very simple and effective but has low cost-performance and low versatility due to the limited number of commercially available methyltransferases. In transformations of *B. cereus* [51], *B. weihenstephanensis* [51], and *H. pylori* [58], crude extracts prepared from the respective bacterium were used for DNA methylation. This method has high cost-performance and high versatility but may not be efficient because of low methyltransferase activity in crude extracts and DNA degradation by nucleases. Moreover, type I and III methylation pattern cannot be achieved by this method.

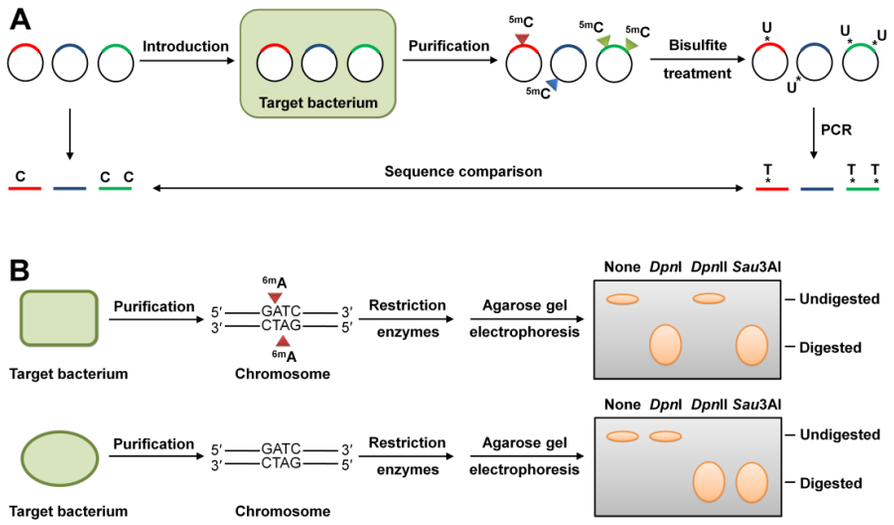

Figure 4. Methylation site analysis in target bacterium. (A) Bisulfite-based analysis to determine ^{5m}C consensus sites. Bisulfite treatment converts methyl-free cytosine to uracil without affecting ^{5m}C. Therefore, ^{5m}C positions can be determined by comparing bisulfite-treated and -untreated DNA sequences. (B) Chromosomal digestion by methyl-sensitive restriction enzymes is used to analyze 5′-G^{6m}ATC-3′ methylation. The restriction enzyme DpnI cuts 5′-G^{6m}ATC-3′ but not 5′-GATC-3′, DpnII cuts 5′-GATC-3′ but not 5′-G^{6m}ATC-3′, and Sau3AI cuts 5′-GATC-3′ and 5′-G^{6m}ATC-3′.

Another approach is *in vivo* methylation by expressing methyltransferase genes in *E. coli* cells [38, 40, 41, 52, 55, 56]. Type II and III methylation can be reconstituted by expressing MT and M subunit genes, respectively, and type I methylation can be reconstituted by simultaneous expression of M and S subunit genes. Numerous gene sequences of methyltransferases are accumulated in the REBASE database along with their methylation sites [11]. Either plasmids or chromosomal integration can be used as expression vectors for methyltransferase genes. When the genome sequence of the target bacterium has been determined, methyltransferase genes in the genome may be used for *in vivo* methylation [40, 41, 52]. Methylation site analysis is not essential for this approach; however, functional expression of methyltransferase genes requires confirmation by HPLC analysis of recombinant *E. coli* chromosomes. A methyltransferase gene of *S. griseus*, responsible for 5′-GAG^{5m}CTC-3′ methylation, was found to be nonfunctional in *E. coli*. Hence, an alternative methyltransferase gene from *S. achromogenes* (*M.SacI*) was used for DNA methylation [40]. The *E. coli* host used to produce host-mimicking DNA must be a methylation-deficient strain (Table 4) because the target bacterium may have type IV systems in addition to type I–III RM systems, as exemplified by *G. kaustophilus* transformation [41]. In addition, it is desirable that the *E. coli* host is deficient in type IV system genes (*mcrA*, *mcrBC*, and *mrr*) because these may restrict heterologous methylation in *E. coli* cells. In this regard, *E. coli* strains IR27 and HST04 are appropriate for producing host-mimicking DNA through *in vivo* methylation. Although *in vivo* methylation may be more complicated than *in vitro* methylation, this approach often has excellent cost-performance, versatility, and efficiency.

A derivative of the target bacterium with methylation activity and reduced restriction activity can also be used for the production of host-mimicking DNA. In transformations of *Salmonella typhimurium* and *Staphylococcus aureus*, plasmids isolated from *E. coli* strains

were initially introduced and propagated in the restriction-deficient strains LB5000 [59] and RN4220 [60], respectively, and were then used for transformation of other strains. In *S. griseus* transformation, mutant HH1 that reduces restriction activity compared to the wild-type has been used [63]. This approach enables production of perfect host-mimicking DNA, although it is not easy to find a strain that is both deficient in restriction and proficient in methylation.

Strain	Relevant genotype	Reference
IR21	*e*14⁻(*mcrA*⁻) Δ*dam*::*metB* Δ(*mrr-hsdRMS-mcrBC*)114::*IS10 rpsL*104 (Str^R)	[41]
IR24	*e*14⁻(*mcrA*⁻) Δ*dcm*::*lacZ* Δ(*mrr-hsdRMS-mcrBC*)114::*IS10 rpsL*104 (Str^R)	[41]
IR27	*e*14⁻(*mcrA*⁻) Δ*dam*::*metB* Δ*dcm*::*lacZ* Δ(*mrr-hsdRMS-mcrBC*)114::*IS10 rpsL*104 (Str^R)	[40]
ET12567	*dam*-13::*Tn9* (Cm^R) *dcm*-6 *hsdRMzjj*-202::*Tn10* (Tet^R) *rpsL*136 (Str^R)	[77]
IBEC58	Δ*dam* Δ*dcm* Δ*hsdRMS*	[35]
HST04	Δ(*mrr-hsdRMS-mcrBC*) Δ*mcrA dam dcm rpsL* (Str^R)	TB
JM110	*dam dcm rpsL* (Str^R)	AT
SCS110	*dam dcm rpsL* (Str^R) *endA*	AT
INV110	*dam dcm* Δ(*mrr-hsdRMS-mcrBC*)102::*Tn10* (Tet^R) *rps*L (Str^R) *endA*	LT
GM48	*dam*-3 *dcm*-6	CGSC
GM272	*dam*-3 *dcm*-6 *hsdS*21	CGSC
GM2929	*dam*-13::*Tn9* (Cm^R) *dcm*-6 *hsdR*2 *mcrA mcrB rpsL*136 (Str^R)	CGSC

Table 4. *E. coli* strains deficient in genes involved in DNA methylation and/or DNA restriction.Cm^R: chloramphenicol resistance; Tet^R: tetracycline resistance; Str^R: streptomycin resistance; TB: Takara Bio Inc. (www.takara-bio.com); AT: Agilent Technologies Inc. (home.agilent.com); LT: Life Technologies Corporation (www.lifetechnologies.com); and CGSC: The Coli Genetic Stock Center (cgsc.biology.yale.edu).

3.4. Alternative methods for circumventing RM systems

In addition to host-mimicking strategies, there are some simple approaches for circumventing RM systems. One is to abolish sites recognized by RM systems. In *Clostridium difficile* transformation, five *Cdi*I sites in plasmids were abolished and used to demonstrate improved efficiency [56]. Similarly, a plasmid with three abolished *Sac*II sites was used for efficient transformation of *Bifidobacterium longum* [53]. When methyltransferase enzymes or genes are unavailable, this approach can be an effective alternative. One other approach is to reduce the restriction activity in the target bacterium temporarily by heat treatment. In *B. amyloliquefaciens* transformation, heat treatment at 46°C for 6 min increased transformation efficiency [78]. More forcible heat treatment (higher temperature and longer time) inactivates RM systems more efficiently but concurrently reduces viability of cells. Although this approach is very simple and is available for most bacteria, the heat conditions required for

inactivating RM systems are not predictable and thereby may have to be determined by repeated trials.

4. Conclusion

In this chapter, RM systems are reviewed and some approaches to produce host-mimicking DNA are described. Analysis of chromosomal DNA using HPLC is a simple method to elucidate functional RM systems in target bacterium, and is therefore highly recommended for establishing bacterial transformation methods. When negligible DNA methylation is observed, methyl-free DNA is suitable for transformation of the bacterium. On the other hand, when significant DNA methylation is observed, a host-mimicking strategy involving methylation needs to be utilized. One weak point of this strategy is that there are no methods to exhaustively, readily, and rapidly determine methylation sites in target bacterium. When this analytical method becomes more widespread, this strategy will become a crucial technique for establishing efficient bacterial transformation methods.

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