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Bacteriophage Survival: Multiple Mechanisms for Avoiding the Deoxyribonucleic Acid Restriction Systems of Their Hosts

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INTRODUCTION

Bacteria in their natural environment are faced with predation by both macro- and microorganisms. Some of the more important of the predators are the bacteriophages, and bacteria have evolved means of beating off viral attackers at almost all stages of the phage life cycle. The most effective defense is to prevent any productive contact between the phage and the bacteria. This can be done by mutation of phage receptors in the cell wall or, possibly more effectively, by the secretion of a barrier that prevents the approach of the phage, such as a capsule or slime layer. In one documented case, the next stage of the viral infection is blocked; that is, the virus can absorb to its receptor on the cell wall, but is unable to inject its deoxyribonucleic acid (DNA) (32, 112, 113). Once the DNA has been injected into the cell, survival of the bacterium by at least two different mechanisms is still possible. If some component of the host is not suited to the needs of the bacteriophage the infection will be abortive at different stages of intracellular phage development; finally, DNA restriction enzymes in the host may destroy the DNA.

The phage, for its part, can adapt to such resistant or nonpermissive cells by mutation; for instance, it can overcome the cells' resistance by a host range mutation which alters its adsorption specificity. Furthermore, it is known that bacteriophages can become adapted to their host cells by host-controlled modification; depending on the cell which last served as host, the virus carries a specific modification so that the ability to replicate in cells of the same strain is improved, but the ability to replicate in cells of a different strain is restricted. Such modifications are characterized by the reversibility of the phenotypic change by one growth cycle of the virus in another host strain and are thus distinguished from mutation (82) (Fig. 1).

Authentic restriction-modification systems, according to the criteria first described by Luria (82) and outlined in Fig. 1, have been thoroughly investigated in *Escherichia coli*, *Bacillus subtilis*, *Haemophilus* spp., and *Salmonella* spp. (for recent reviews, see references 9 and 33). They have also been described in *Corynebacterium*

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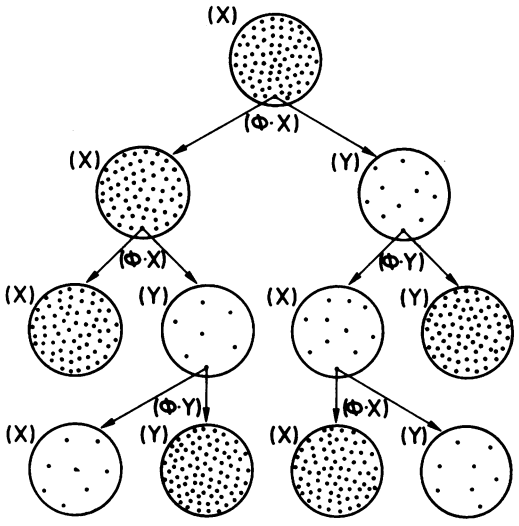

FIG. 1. Scheme for the demonstration of host-controlled modification and restriction of a virus ϕ. (I) Isolation of plaques (ϕ·X) from an X cell lawn and parallel inoculation of cell strains X and Y with the same virus population. ϕ·X is restricted on Y (less virus plaques than on strain X). (II) Transfer of the virus from the cell strains X and Y. (III) Demonstration of the reversibility of modification (proof that ϕY from the second transfer are not host range mutants, but modified viruses). When one of the cell strains (X) is restriction and modification negative, the reversibly changeable growth of the virus is only manifest on the other cell strain (Y); plating on X is not altered. In the figure the efficiency of restriction is about 10-fold; that is, the titer on the restricting host is about 10^{-1} of that on the nonrestricting host. Measured efficiencies vary with the phage and restriction system from barely detectable to about 10^{-5}.

diphtheriae, *Pseudomonas aeruginosa*, *Rhizobium leguminosum* and *Rhizobium trifolii*, *Shigella sonnei*, *Streptococcus* spp. *Staphylococcus aureus*, *Streptomyces albus*, and *Streptomyces hygroscopicus* (8, 11, 18, 33, 66, 97).

The "classical" mechanism of restriction and modification of bacteriophages consists of endonucleolytic cleavage (restriction) of phage DNA when it is not specifically methylated (modified) at certain sites (9, 11, 33, 88, 91, 92, 108, 130, 133). After one growth cycle in a particular host, the viral DNA is methylated at the recognition sites specific for the DNA host specificity system of that host and is protected in this way when infecting cells possessing the same DNA restriction-modification system. The host-controlled DNA modification is the main way to overcome DNA restriction. However, when a phage is infecting a cell with a different DNA restriction-modification system, the phage DNA is restricted because it is not methylated in the DNA sequences recognized by the new system. The restriction and modification processes affect not only phage DNA but also, as a rule, any intracellular DNA.

Meanwhile, we have learned that phenotypic restriction and modification of bacterial viruses (Fig. 1) can also occur by a mechanism that involves protein modification rather than reactions at the DNA level. In this way, the ability of a phage to adsorb to a new host cell is influenced by a modification of phage conferred to it by the previous host cell (70, 71; for a review, see reference 77).

Figure 2 represents the different mechanisms connected with the phenomenon of host-controlled restriction and modification.

Most species and strains of bacteria that have been examined contain endonucleases with the properties expected of restriction enzymes; that is, they give sequence-specific DNA cleavage, indicating the importance of restriction for bacteria. The involvement of most of these enzymes in an authentic restriction-modification system has not been demonstrated (for most, it has not even been examined); some of them may have other functions such as in recombination or repair of pathways.

Bacteriophages have learned to live with the restriction systems of their hosts by developing a wide range of different mechanisms for avoiding the worst effects of restriction. Antirestriction mechanisms of one kind or another have been found in practically every phage that has been examined, and in this review we describe the mechanisms that are used by the bacteriophages of *B. subtilis* and *E. coli*.

DNA RESTRICTION-MODIFICATION ENZYMES

Three broad groups of restriction-modification systems can be discerned that differ from each other in the complexity of the enzyme structures and reaction mechanisms and in the kinds of DNA sequences that are recognized. The major characteristics of these three groups are described in Table 1. The vast majority of the known enzymes are of type II. This is because these are the enzymes that recognize simple, generally symmetrical, sequences and that cut the DNA in a fixed position relative to their recognition sequence. They are the enzymes that have revolutionized biology during the last decade by the possibilities that they offer for gene cloning and DNA analysis; in consequence, many genera and species of bacteria have been systematically screened for their presence. These screening procedures are relatively easy; the enzymes give well-defined and characteristic DNA fragments that can be recognized after agarose or polyacrylamide gel elec-

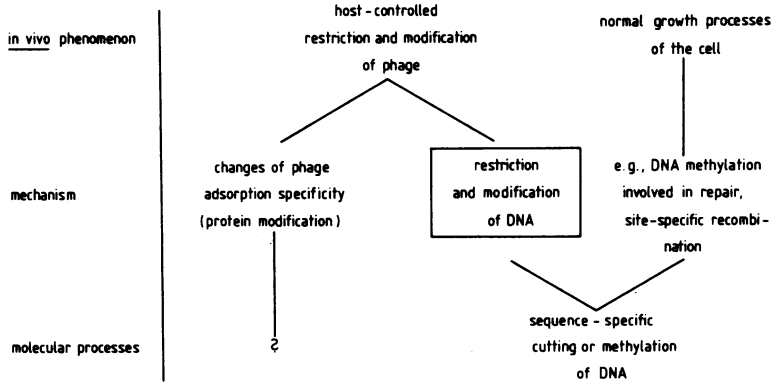

FIG. 2. Restriction and modification of DNA in the hierarchy of biological and molecular processes.

trophoresis, and the reaction conditions are simple, Mg^{2+} being the only cofactor required. The enzymes are oligomers (generally dimers) of a single subunit, and the modification methylases, in the few cases that have been examined, are separate monomeric proteins (reviewed recently in references 91, 92, and 130).

The only examples that are known of the types I and III restriction-modification systems are found in the *Enterobacteriaceae* and in the genus *Haemophilus*. They are probably more widely distributed than this; very few workers have ever looked for them, and they are not detected by the screening procedures used for the type II enzymes because of their requirement for ATP and, for the type I enzymes, *S*-adenosylmethionine (SAM).

The type I enzymes of the *Enterobacteriaceae* are, with one exception, closely related to each other. Two of the subunits show strong immunological cross-reactions among the different members of the group, and the genes for these subunits cross-hybridize with each other (93). The gene for the third subunit, that which recognizes the DNA sequences specific for the particular system, shows much less cross-hybridization (93). Three of these recognition genes have now been sequenced (38); apart from two short conserved regions, which may be involved in interactions with the other subunits, the sequences are completely different. The enzymes that are physically unrelated to the other type I systems are those of the *Eco*A system of *E. coli* 15 T^- (3, 93). We have evidence (including some to be discussed below) that these enzymes are also very different from the other type I enzymes in their subunit structure and reaction mechanism. They should probably be considered as the first examples of a new class of restriction system.

The two type III restriction-modification systems that are found in *E. coli* are also closely

TABLE 1. General properties of restriction endonucleases[a]

Type of enzyme	Protein structure or function	Mol wt (approximate)	Cofactor requirements for restriction[b]	DNA recognition sequence (5′–3′)	DNA cleavage site
I	Complex molecules with 3 different subunits (endonuclease, methylase, recognition)	400,000	SAM, ATP, Mg^{2+}	*Eco*B, TGA-N_8-TGCT; *Eco*K, AAC-N_6-GTGC	More than 1,000 base pairs from recognition site (random?)
II	Endonuclease and methylase are different molecules	20,000 to 80,000	Mg^{2+}	Usually palindromic (4 to 7 base pairs long); e.g., *Eco*RI, GAATTC	Usually at recognition site
III	Complex molecules with 2 subunits (endonuclease and methylase-recognition)	200,000 to 300,000	ATP, Mg^{2+} (stimulated by SAM)	*Eco*P1, AGACC; *Eco*P15, CAGCAG	24 to 27 base pairs to 3′ side of recognition site

[a] For details see reference 9, 33, 91, 92, 108, 130, and 133.
[b] For all types, SAM is the methyl donor for modification.

related to each other. Again, the major region of nonhomology between the two systems is found in the gene coding the subunit that recognizes the specific sequences (43, 56). (For recent reviews on type I and III restriction systems, see references 9, 33, and 133).

The extraordinary diversity of restriction enzymes documented above means that it is unlikely that a bacteriophage can develop equally effective mechanisms for combatting all of the enzymes that it is likely to encounter in its hosts. Furthermore, many bacterial strains produce more than one enzyme, and the kinds of enzymes produced may change with time as some systems are lost by mutation and new ones are acquired through processes such as conjugation or transduction.

INHIBITION OF RESTRICTION ENZYMES BY VIRAL PROTEINS

ocr Gene Product of Phages T3 and T7

The most thoroughly investigated example of active antirestriction is that exerted by the closely related phages T3 and T7. It has long been known that T3 and T7 can be passaged through *E. coli* strains with different DNA host specificities (for example, *E. coli* B and K-12) without being phenotypically restricted (34).

The phage gene responsible for the antirestriction has been variously called 0.3 (118, 119), from its map position (gene 0.3 is the viral gene that maps closest to the left end of the DNA), and *sam* and *ocr*, derived from the phenotypes associated with the genes (77, 78). In this review we will use the name *ocr*, which stands for "overcome classical restriction." Since the basic discovery that ocr^- mutants are unable to replicate when they are passaged between *E. coli* K-12 and B hosts (118, 119), the protection mechanism has been extensively studied. It was shown that the *ocr* gene product of T3 has two different phenotypes: Ocr itself, and Sam, the ability to hydrolyze intracellular SAM (77, 78). The T7 gene product only shows the Ocr phenotype and is nevertheless as resistant to restriction as T3. Furthermore, Sam^- mutants with mutations in the T3 *ocr* gene can be isolated that can no longer hydrolyze SAM, but that still retain the Ocr phenotype. Mutants with mutations in the *ocr* gene of both T3 and T7 also exist that are phenotypically Ocr^-. Phages carrying these mutations are subject to classical restriction and modification of DNA (78).

The *ocr* protein synthesized in virus-infected cells counteracts the type I systems *Eco*K and *Eco*B (67, 78, 118, 119), the related *Salmonella* systems SA and SB (69), and type III (*Eco*P1) systems (73–75). It is also active in vivo against the unclassified *Eco*RIII enzyme (D. H. Krüger and L. S. Chernin, unpublished data) coded by the plasmid R124 (5, 53, 115). The inhibition does not depend on SAM hydrolysis, but intervenes at a later step in the interaction between the restriction endonuclease and the modification methylase preventing both DNA cleavage and methylation (73, 77). The type II restriction enzymes such as *Eco*RV are not blocked by the *ocr* gene product: T3 DNA has five *Eco*RV sites and is restricted both in vivo and in vitro (T7 DNA has no *Eco*RV sites) (76). Using T3 mutants that had acquired an *Eco*RI site, Miyazaki et al. showed that *ocr* is also ineffective against this type II enzyme (90).

The effect of the *ocr* protein is to actively inhibit the cellular restriction enzyme, thus protecting the unmodified recognition sites in the phage DNA against cleavage. This protection is, moreover, extended to any other foreign DNA simultaneously introduced into the cells. When T3 or T7 are heavily irradiated with UV light so that they are unable to replicate and lyse the cells, but are still able to express the *ocr* gene, it is possible experimentally to introduce plasmid DNA into the cells by conjugation (67) or transformation (71, 104) without it being restricted. The recipient cells survive and replicate their own as well as the newly acquired plasmid DNA (for a review, see reference 77).

Completely independently of SAM hydrolysis, the *ocr* protein of T7 and sam^- T3 mutants also prevents host-controlled modification (68, 75; I. G. Bogdarina, M. Reuter, Y. I. Buryanov, and D. H. Krüger, submitted for publication).

In vitro studies with the purified *ocr* protein from both T3 and T7 have confirmed the distinction between the Ocr and Sam phenotypes of the T3 protein (117), the lack of activity against type II restriction enzymes (76, 85) and the blocking of both the endonucleolytic (85, 117; D. H. Krüger, M. Reuter, and T. A. Bickle, unpublished data) and the methylase activity of *Eco*B and *Eco*K (Bogdarina et al., submitted for publication). The *ocr* protein functions by binding directly to the restriction enzyme (85, 117), offering an interesting example of a protein-protein interaction where the enzymatic properties of a protein that interacts with DNA are changed. The *ocr*-mediated protection of DNA against restriction by the type III enzyme *Eco*P1 which was demonstrated in vivo (74, 75) could not be simulated under simple in vitro conditions, indicating that a more complex interaction exists in cells harboring the *Eco*P1 system (D. H. Krüger, C. Levy, and T. A. Bickle, unpublished data). It should be noted that even though the *ocr* function prevents *Eco*P1-mediated cleavage of infecting T7 DNA, phage growth is nonetheless severely inhibited on P1 lysogens. This inhibition is not due to DNA restriction (75, 77).

Restriction enzymes normally cleave foreign DNA immediately after its entry into the cell (30). How, then, can the *ocr* gene product appear early enough during the infection to block the restriction enzymes, especially since it has been shown that protection of the DNA depends on de novo protein synthesis in the infected cell (111; D. H. Krüger, unpublished data)? A delayed injection of phage DNA into the cell may be the "trick" by which the virus prevents exposure of DNA recognition sites before the appearance of *ocr* protein and the blockage of the cellular restriction enzyme (77). The phage first injects the left end of its genome, which contains the *ocr* gene; transcription of this region of the genome is a prerequisite for the injection of the rest of the DNA (Fig. 3). Support for this idea comes from experiments that showed that injection of T7 DNA into *E. coli* is blocked by the transcription inhibitors rifampin and streptolydigin (136) and from sequencing data that have shown that the first *Eco*B site lies at the position 11.9% and that no *Eco*K site is found in the sequence of the left 30% of the T7 genome (29). The *ocr* gene itself is situated between positions 2.3% and 3.2% from the left end of the T7 genome (29). The development of the antirestriction function or functions of phage T5 seems to follow a similar schedule (see below).

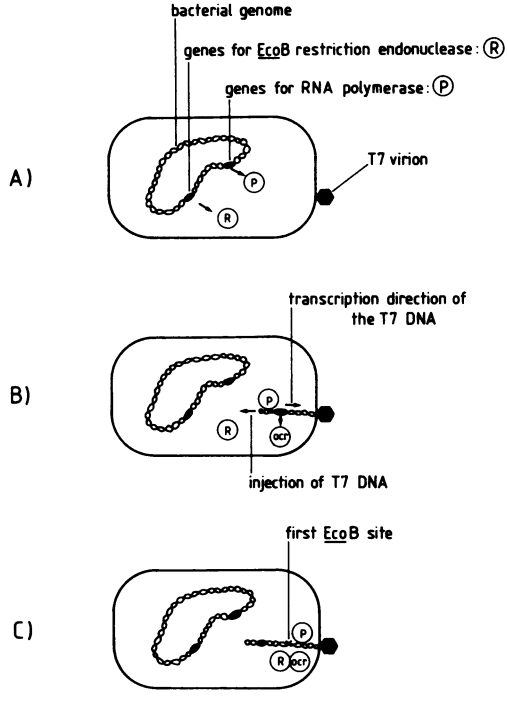

FIG. 3. Schedule of *ocr*$^+$-mediated T7 DNA protection. (A) T7 adsorbs to an *E. coli* B cell. (B) The cellular RNA polymerase transcribes the early region of T7 genome as a precondition for injection of the rest of the DNA into the cell. The first virus gene expressed in the cell is the *ocr* gene (located between positions 2.3 and 3.2 of the T7 genome). (C) The *ocr* protein binds to the *Eco*B restriction endonuclease and blocks its activity. When the first *Eco*B recognition site on the T7 DNA (located at position 11.9) appears in the cell, the enzyme is already inhibited.

Inhibitor Produced by *Bacillus* Phages ϕNR2rH and ϕ1rH

In *Bacillus amyloliquefaciens* producing the *Bam*Nx type II restriction enzyme, the phages ϕNR2rH and ϕ1rH are not restricted despite the fact that their DNA contains sites for this enzyme (83, 84). Both phages produce a protein that blocks the *Bam*Nx enzyme. The protein coded by ϕ1rH has been isolated; it has a molecular weight of 20,000 and is an inhibitor of *Bam*Nx, but not of any other enzyme tested, including even *Ava*II, which recognizes the same DNA sequence as *Bam*Nx (84).

These two phages provide the first examples of an active viral block of a type II restriction enzyme. They were originally described as mutants since their parental phages are sensitive to *Bam*Nx (83, 84). However, the high frequency with which *Bam*Nx-resistant mutants appeared in the population (10^{-4}) makes it more likely that they arise by processes other than mutation, perhaps by DNA rearrangements similar to those that control the expression of different host range genes in phage Mu (19) or that they represent the original virus type which was "overgrown" in the laboratory under nonselective conditions by mutants that had lost the antirestriction property.

VIRUS-CODED DNA MODIFICATIONS

Site-Directed DNA Methylation by Some *Bacillus* Phages

The restriction endonuclease R.*Bsu*RI of *B. subtilis* cuts the sequence 5'-GGCC-3' (13) if the central cytosine is not methylated by the corresponding methylase, M.*Bsu*RI (42). However, in certain restriction- and modification-negative *B. subtilis* strains a DNA methyltransferase with the same sequence specificity as M.*Bsu*RI is inducible (41). This enzyme is prophage coded and is not expressed in stable lysogens (60). Such specific methylase genes were found to be carried by the phages SPβ (129) and ϕ3T (20, 99). It is important to note that these genes are expressed not only after induction of prophage but also during lytic infection, so that the DNA in phage particles produced by either route is

modified. In this way the phage protect their own DNA, and also heterologous DNA, against restriction in *Bsu*RI-coding cells (60, 99, 127). Since restriction enzymes recognizing the sequence GGCC are demonstrable in several strains of *B. subtilis* and other *Bacillus* species (see also Table 3), this methylation could allow the spread of phage to many different potential hosts.

Recently it was shown that the SPβ used in the studies cited above (60, 127) differs from the original SPβ phage, and it has now been renamed SPR (98). A comparative study demonstrated that the phages SPR, φ3T, SPβ, and ρ11 are all insensitive to restriction in *Bsu*RI-coding cells because of their DNA self-methylation. The methyltransferase genes of the different phages are interchangeable. No homology was found between these phage genes and *B. subtilis* chromosomal DNA which codes for M.*Bsu*RI (98). The methyltransferases M.*Bsu*RI of cell chromosomal origin (39, 40) and M.SPR of phage origin (U. Günthert, personal communication) were purified and shown to be unrelated. Interestingly, it seems that the phage and chromosomal genes encoding DNA methylases with the same sequence specificity are sufficiently different from each other that they must have evolved independently. This provides an excellent example of convergent evolution.

In addition to the central cytosine of the GGCC sequence, the phage methyltransferases are able to methylate other sequences (the central cytosine of 5′-CCGG-3′ in the case of M.SPR; the central cytosine of 5′-CCNGG-3′ in the case of M.SPβ, M.φ3T, and M.ρ11); the activity methylating GGCC and that active on the second sequence copurify (U. Günthert, personal communication).

Unique DNA Modification Directed by Phage Mu

Bacteriophage Mu is relatively insensitive towards the *E. coli* restriction systems *Eco*K, *Eco*B, *Eco*P1, and *Eco*A, due to the viral gene function *mom* (standing for modification of Mu). Phages with mutations in the *mom* gene are much more severely restricted. The in vivo sensitivity of Mu to *Eco*RI is not influenced by *mom* (124, 125). The *mom* gene was located on the β segment of the Mu genome (126), and it is expressed at a higher level after prophage induction than before infection (124, 125). Protection results from a *mom*-specific modification of DNA which also acts in *trans* so that not only the Mu DNA itself, but also any other DNA present in the cell (i.e., superinfecting λ phage), is modified (124, 125). However, the *mom*-directed DNA modification does not consist of a methylation, but is an acetimidation of the N^6 position of about 15% of the adenine residues in DNA (46–48; S. Hattman, personal communication; R. Kahmann, personal communication).

It is still unknown how the Mom function manages the conversion of adenine to the modified base N^6-(1-acetamido)adenine. The *mom* protein has not been characterized or purified. Recent in vitro studies have shown that the Mu *mom*$^+$ DNA is at least partially resistant to the action of several type II restriction endonucleases, whereas Mu *mom*$^-$ DNA is sensitive. By comparing and aligning the recognition sequences of restriction enzymes whose cleavage is affected by the Mom function, we deduced a consensus recognition sequence for Mom of 5′-C/G-A-G/C-N-Py-3′ (63). By checking the recognition sequences of *E. coli* restriction enzymes (Table 1), one can see that the sequences for *Eco*P1, *Eco*P15, *Eco*B, and *Eco*K all overlap with the Mom recognition sequence, but that the *Eco*RI sequence differs completely, so that the in vivo protection against the first group of restriction endonucleases, but not against *Eco*RI, is understandable.

For the self-modification of phage not only the *mom* gene function, but also the cellular adenine (*dam*) methylase, is necessary (65, 125). However, the sole function of *dam* appears to be that of stimulating transcription of *mom* by methylating specific DNA sequences upstream of the *mom* gene (49, 101).

The first indication of a positive correlation between DNA methylation and gene expression in procaryotes was the finding that nonmethylated T3 genomes are not expressed in starved cells (72, 77). The regulation of *mom* gene expression is a more specific example of this. The situation in procaryotes therefore seems to be the opposite of that obtained in higher eucaryotes where methylation (formation of 5-methylcytosine) at specific sites in the genome can inhibit gene expression (for reviews, see references 28, 31, and 102).

DNA Adenine Methylase of Phage T2

The T-even phages T2, T4, and T6 are naturally resistant to all of the restriction systems that they encounter in their hosts because their DNA contains an unusual base and is, in addition, post-synthetically modified by glucosylation (see below). Phages T2 and T4 (but not T6) also code for a DNA-adenine methyltransferase that methylates a fraction of the adenine residues in the phage DNA to 6-methylaminopurine (37). Perfectly viable mutant phages can be found in which the postsynthetic glucosylation does not occur. These mutant phages are still protected against most of the restriction systems of their

hosts, the exception being the type III *Eco*P1 system; phages containing non-glucosylated DNA are restricted in P1 lysogens expressing *Eco*P1 (106).

Mutations of phage T2 have been found that are partially protected against P1 restriction even though their DNA is still not glucosylated (106). These mutants were found to have an altered DNA-adenine methyltransferase which methylated about two to three times more of the adenines in the phage DNA than did the wild-type enzyme (45). There is some disagreement in the literature regarding the sequences recognized by these enzymes (14, 51). However, it is most likely that the wild-type methylase methylates the adenosine residue in the sequence 5'-G-A-T-3', whereas the mutant that provides protection against *Eco*P1 recognizes both 5'-G-A-T-3' and 5'-G-A-C-3' (for a discussion of this, see reference 48). This relaxed specificity of the mutant enzyme immediately explains why it affords protection against *Eco*P1, because the recognition sequence for *Eco*P1 (5'-A-G-A-C-C-3', see Table 1) contains the 5'-G-A-C-3' sequence and *Eco*P1 modification methylates the central A residue (4, 50).

This example of antirestriction may seem rather artificial, because the phage must first carry mutations in the genes for the glucosylases to make them sensitive to *Eco*P1, and then a further mutation in the methylase is needed to generate the antirestriction activity. However, this scheme, even though it was carried out in the laboratory, affords a fascinating glimpse of the way in which antirestriction processes may have evolved in nature and the flexibility of the bacteriophage response to challenge by a restriction system to which it was not previously susceptible.

DDVI-Coded Guanine Methylase

In cells infected by phage DDVI, an enzyme that methylates the 7 position of guanine and modifies about 0.25% of the guanine bases in the phage DNA, was detected (96). This is a very unusual result, since 7-methylguanine is known to be a very unstable constituent of DNA (48, 87). Even when phage DDVI is grown in *E. coli* B cells, DDVI DNA lacks 6-methylaminopurine (the product of the *Eco*B host specificity methylation), but it is not restricted in these cells (96). Nikolskaya et al. concluded that the 7-methylguanine residues could protect the phage DNA against *Eco*B restriction (96). On the other hand, it was earlier shown that DDVI DNA contains the unusual base 5-hydroxymethylcytosine (HMC), which is further modified by glucosylation (95), and DNA with this structure is resistant to *Eco*B restriction (see below).

STIMULATION OF HOST MODIFICATION FUNCTIONS

Bacteriophage λ and a few other lambdoid phage encode a most unusual antirestriction function, specific for type I systems, that operates not by inhibiting restriction, but by stimulating the modification reaction (135). The function is coded by the *ral* gene, which maps in the major leftward early operon of the phage between the genes *c*III and *N* (135). The *ral* gene product has never been identified; however, the DNA sequence of this region of the λ genome predicts that *ral* should be a small protein of molecular weight 7,600 (58, 110). The *ral* function is active only against type I restriction systems, and its effects are seen not on the primary infection of a restricting host where restriction is normal, but upon reinfection of the same host strain with the progeny from the first infection; ral^+ phages are fully modified, whereas ral^- phages are only partially modified and plate with low efficiency. The *ral* effect is readily understandable in terms of what we know about the reaction mechanism of type I modification enzymes. These enzymes methylate fully unmodified DNA very slowly, their best template being DNA that already contains a methyl group in one of the two strands of the recognition sequence (16, 128). Such hemimethylated DNA is, of course, generated in cells by the normal processes of DNA replication and repair. The effect of *ral* would be to stimulate the enzymes to methylate nonmodified DNA faster. Unfortunately, it has not been possible to isolate the *ral* protein so that this hypothesis could be tested in vitro (K. Ineichen and T. Bickle, unpublished results).

The *ral* gene product has also been reported to interact with other ATP-dependent nucleic acid enzymes of *E. coli*, including the transcription termination factor rho. It inhibits the activity of this factor leading to a phenotypic antitermination activity which, unlike the action of the λ *N* gene product, is not promoter specific (23).

UNUSUAL BASES IN PHAGE DNA

We define as unusual DNA bases all those bases which are not adenine, cytosine, guanine, or thymine and which are synthesized at the level of nucleotide metabolism. These are to be contrasted with the modified bases, which are made by the addition of adducts to bases after DNA replication (postsynthetic DNA modification).

B. subtilis Phages

Many *B. subtilis* phages contain unusual bases in their DNA; e.g., in phages SPO1, SP8, SP82G, φ25, φe, and 2C, thymine is completely

replaced by 5-hydroxymethyluracil, and in phages PBS1 and PBS2 thymine is completely replaced by uracil. Other cases of the occurrence of unusual bases are also known (for reviews see references 54 and 129). Using φe and PBS2 DNA, Berkner and Folk (7) have undertaken studies on the influence of unusual bases in phage DNA on the cleavage activity of different restriction endonucleases. They found that the influence of the base exchange depended in part on the sequence specificity of the restriction enzymes and in part on the particular enzyme: different enzymes recognizing the same sequence were affected to different extents. Huang et al. (55) have done similar, but more extensive, studies with 30 different restriction enzymes and the following highly substituted DNA molecules: *B. subtilis* phages PBS1 and SPO1 DNAs (described above); DNA from the phage SP15, in which some 60% of the thymine residues are replaced by 5-dihydroxypentyluracil, which is also glucosylated and phosphoglucuronated post-synthetically; HMC-containing DNA from the *Xanthomonas oryzae* phage XP12; and DNA from the coliphage T4, which contains glucosylated HMC (see below). All of these DNAs were refractory to cleavage by most of the enzymes, although slow cleavage was reported for some. An exceptional enzyme was *Taq*I from *Thermus aquaticus*, which could cleave all of the DNAs to some extent.

It can be concluded that the occurrence of unusual bases in the DNA of *B. subtilis* phages prevents the action of certain restriction endonucleases and thus protects the phage DNA against cleavage in host cells carrying the appropriate restriction systems. In addition, the unusual bases are expected to fulfill other biological functions, for instance, transcription and replication regulation or DNA conformation and packaging (129).

Uracil is occasionally produced in normal DNA by spontaneous deamination of cytosine. This potentially mutagenic lesion is normally removed by an enzyme called uracil-*N*-glycosylase, which cleaves the *N*-glycosidic bond (81). Phages like PBS2, whose DNA normally contains uracil instead of thymine, direct the synthesis of a protein that inhibits the host uracil *N*-glycosylase (35, 62, 63, 123). The evolution of such an inhibitor demonstrates the importance of the unusual base uracil for the phage.

Hydroxymethylcytosine in T-Even Phage DNA

The T-even phages of *E. coli* (and also phage DDVI of *Shigella sonnei* [95]) contain DNA in which cytosine is completely replaced by the unusual base HMC (132). The HMC residues are, in addition, glucosylated. As with the *B. subtilis* phages discussed above, the unusual base is made as a precursor and is incorporated into DNA during replication or repair.

HMC-containing DNA is resistant to the action of many restriction enzymes in vitro. It is completely resistant to *Hha*I and *Hpa*II and almost completely resistant (less than 10% cleavage) to the enzymes *Bam*HI, *Hin*dII, and *Hin*dIII (7, 55). The susceptibility to the enzymes normally found in *E. coli* will be discussed later.

If phages that replace normal bases by unusual ones do so to protect themselves from host restriction enzymes (see above), the host may respond by evolving nucleases specific for the unusual bases. Such an enzyme can be considered as an "anti-antirestriction mechanism." One such enzyme is found in *E. coli*, which produces an enzyme that is specific for non-glucosylated, HMC-containing DNA (105). This enzyme, the product of the *rgl* genes (*rgl* for restriction of non-glucosylated DNA), is completely independent of the classical DNA restriction system and cannot in itself be considered a restriction enzyme, because there is no evidence for sequence specificity and no cognate methylation enzyme was found. The classical restriction enzymes and the *rgl* enzyme coexist in most strains of *E. coli*, unfortunately, some publications have not sufficiently discriminated between them (e.g., reference 25), a situation that can lead to confusion.

The T-even phages have, in turn, evolved a gene called *arn* (for anti-restriction endonucleases, again a confusion between restriction enzymes and *rgl*), which produces an inhibitor of the *rgl* enzymes. This inhibitor is not effective in preventing *rgl* destroying the DNA of an infecting T-even phage, probably because it is not made fast enough, but does prevent the action of *rgl* on a superinfecting phage (26). The gene has been mapped and cloned in a small plasmid (27).

These multiple defensive interactions between T-even phages and *E. coli* indicate that phage and host have had a long evolutionary association. One entirely hypothetical scenario for their evolution is that the phage developed the replacement of cytosine by HMC in their DNA as a response to the presence of restriction enzymes in their host. The host responded by evolving the *rgl* systems as a specific protection against T-even phages. The phages then evolved two separate means of avoiding the *rgl* effect: *arn*, which is relatively inefficient because it will only protect superinfecting phages, and glucosylation. The development of glucosylation would have been the latest evolutionary event. Most laboratory strains of *E. coli* have no defense against HMC-containing, fully glucosylated DNA.

There is not necessarily any correlation be-

tween the ability of a restriction enzyme to cleave HMC-containing DNA in vitro and its effectiveness in restricting T-even phage in vivo. The effects of the major *E. coli* restriction enzymes on different forms of T4 DNA in vitro and in vivo are compared in Table 2. The different kinds of DNA are as follows: wild type (HMC containing and glucosylated); HMC containing, but non-glucosylated; and finally cytosine containing and non-glucosylated (this can be obtained by using a phage carrying multiple mutations [122]). These differences in restriction in vivo and in vitro suggest that the T-even phages may have additional, as yet undetected, antirestriction functions.

COINJECTION OF PHAGE INTERNAL PROTEINS AND DNA

Phage P1, like all temperate phages, has no unusual bases in its DNA, but it is nevertheless remarkably insensitive to the type I (but not types II and III) restriction enzymes of *E. coli*. The reason for this is that phage heads contain two different proteins, the products of the nonessential phage genes *darA* and *darB* (*dar* for defense against restriction [S. Iida and T. A. Bickle, unpublished data]). When the *darA* gene is mutated by deletion or transposon insertion, phage particles are produced that contain neither *darA* nor *darB* gene products, and these phages are efficiently restricted by the *E. coli* K-12 B and A restriction systems. When *darB* is mutated, on the other hand, the phage particles lack the *darB* gene product, but contain the *darA* protein. These phages are restricted by the classical type I *Eco*K and *Eco*B systems, but not by the aberrant *Eco*A system. It thus appears that the *darA* gene protects against *Eco*A, whereas the *darB* gene protects against the classical type I systems. The *dar* systems will protect any DNA from restriction so long as it is packaged within a P1 head. For example, when phage λ is transduced inside a P1 head, it is protected from restriction. If, however, cells are simultaneously infected with both phage P1 and phage λ, the P1 DNA is protected, whereas the λ DNA is not. Even *dar*$^-$ phages can be protected against restriction if they are first grown on strains carrying the cloned *dar* genes; phage particles are produced that contain the *dar* gene products even though the genome remains *dar*$^-$, and these particles are protected against restriction in the next round of infection (M. Streiff, S. Iida, and T. Bickle, unpublished data).

The precise mechanism of antirestriction by the *dar* proteins remains unknown. It seems clear that the proteins are injected into host cells along with the DNA and remain attached to the DNA during the process. The *darA* gene product is the only one of the two to have been purified, but studies on its mode of action must await the purification of its target enzyme, *Eco*A.

DESTRUCTION OF RESTRICTION ENDONUCLEASE COFACTORS

The type I and III restriction enzymes require SAM as an essential (type I) or stimulating (type III) effector for stable binding to substrate DNA (Table 1). This tight binding is a prerequisite for enzymatic activity. Thus, a depletion of the intracellular pool of SAM, at least for the type I enzymes, should prevent restriction. This has been experimentally demonstrated for the type I enzyme *Eco*K: cells in which SAM biosynthesis was blocked (by starvation for the precursor, methionine) were defective in restriction (79). In similar experiments with cells containing type III (or type II) restriction systems, the chromosomal DNA was restricted after long periods of starvation (79).

These observations are relevant in connection with the SAM hydrolase activity of the phage T3 *ocr* gene product, which is a very early function in the viral life cycle and destroys the intracellular SAM very soon after infection (37, 52, 77, 117). Since the same protein also inhibits type I enzymes directly by binding to them (the Ocr phenotype, see above), it is not clear whether the SAM hydrolase is necessary for antirestriction. The SAM-activated form of the *Eco*K restriction enzyme has SAM tightly bound to it; in the presence of DNA, it is very stable, with a half-life of about 6 min, and it is active in the absence of free SAM (134). Enough of this active enzyme species should survive until the phage DNA has been completely injected, whether or not free SAM is still present in the cell, to ensure efficient restriction. In support of this, one tem-

TABLE 2. Cleavage of T4 DNA by *E. coli* restriction endonucleases in vivo and in vitro[a]

T4 DNA	*Eco*B in vivo	*Eco*P1 in vivo	*Eco*RI		*Eco*RV	
			In vivo	In vitro	In vivo	In vitro
HMC containing, glucosylated	No	No	No	No	No	Yes
HMC containing, non-glucosylated	No	Yes	No	Yes	No	Yes
Cytosine containing	Yes	Yes	Yes	Yes	Yes	Yes

[a] Data are compiled from references 80, 106, and 122 and V. I. Tanyashin, personal communication.

perature-sensitive *ocr* mutant has been described that is phenotypically Ocr$^-$ and efficiently restricted at the nonpermissive temperature while remaining Sam$^+$ (117). The SAM hydrolase activity of the T3 *ocr* gene product does improve the chances of phage survival in cells containing the type III *Eco*P1 system. Here, the Ocr phenotype inhibits the endonucleolytic activity of the enzyme, but does not block the repressor-like action of *Eco*P1 on gene expression. The SAM hydrolase activity helps to alleviate this repression (75).

A second case of a phage-coded SAM hydrolase is found for the phage S_D infecting *E. coli* SK cells, Nikolskaya et al. proposed that this enzyme serves an antirestriction function (94). However, it is not known whether the restriction system of *E. coli* SK is of type I, II, or III, and it has not been determined whether the phage S_D DNA contains sites for this enzyme. No S_D mutants defective in the SAM hydrolase function have been reported; these would be very useful for elucidating the phenomenon.

COUNTERSELECTION AGAINST RESTRICTION SITES IN PHAGE DNA

Chromosomal DNA sequences, especially in coding regions, are a long way from being random (1, 114). Perhaps the best-known departure from randomness is seen in the dinucleotide frequencies of the DNA from higher eucaryotes where the sequence 5'-C-G-3' is only found at 1/20 of the expected frequency (61, 120). One consequence of this is that eucaryotic DNA has remarkably few sites for restriction enzymes that have 5'-C-G-3' in their recognition sequence. In this section we describe a number of cases of bacteriophage that have very few recognition sites in their DNA for restriction enzymes that they encounter in their hosts. If only one phage with this property had been described, it might be due to some constraint on the DNA sequence unconnected with restriction. However, so many different examples are known that we believe that it is due to a counterselection against recognition sites in the phage DNA. This kind of selection can also operate in the laboratory. In one example, coliphage fd mutants were made that had lost both of their recognition sites for *Eco*B simply by repeated cycles of growth on restricting and nonrestricting hosts (2).

The *B. subtilis* virus φ1 possesses double-stranded DNA with a molecular weight of 105 × 10^6; the DNA does not contain unusual bases (54, 64). The φ1 DNA carries a much lower number of recognition sites for certain restriction endonucleases than would be expected statistically. For instance, the sequence 5'-GGCC-3' (recognized by the *B. subtilis* restriction endonuclease *Bsu*RI), which would be predicted to occur about 400 times on a purely statistical basis, does not occur at all in the φ1 genome (64). Table 3 shows the expected number and the actual number of sites for some restriction enzymes in the φ1 DNA. It is apparent that most of the enzymes for which the low frequency or total absence of sites has been demonstrated can be isolated from *Bacillus* species or from *B. subtilis* itself.

In the DNA of *Bacillus* phage φ29 as well, extremely few recognition sites for several restriction endonucleases have been found. For instance, there is no site for *Bsu*RI, although it would be expected to appear 23 times in φ29 DNA. After cloning φ29 DNA in *E. coli* the number of cleavage sites does not change, defi-

TABLE 3. Restriction endonuclease cleavage sites in φ1 DNA

Enzyme used[a]	Recognition sequence	No. of cleavage sites		Enzyme isoschizomers in *Bacillus* spp.[b]
		Actual[a]	Predicted	
*Tha*I	CGCG	1	398	*Bsu*E = *Bsu*1231 from *B. subtilis*; *Bce*RI from *B. cereus* strain Rf
*Bgl*II	AGATCT	2	50	*Bgl*II is from *B. globigii*
*Eco*RI	GAATTC	1	50	None known
*Pst*I	CTGCAG	1	31	*Bsu*B = *Bsu*1247 from *B. subtilis*; *Bce*170 from *B. cereus*
*Hae*III	GGCC	0	398	*Bsu*RI, *Bsu*1076, from *B. subtilis* strains; *Bst*CI, *Bss*CI, *Bse*I from *B. stearothermophilus* strains; *Bsp*RI from *B. sphaericus* strain R
*Bam*HI	GGATCC	0	31	*Bam*FI, *Bam*KI, *Bam*NI from other *B. amyloliquefaciens* strains; *Bst*I from *B. stearothermophilus* 1503-4R
*Sla*I	CTCGAG	0	31	*Bsu*M = *Bsu*168 from *B. subtilis*; *Bst*HI and *Bss*HI from *B. stearothermophilus* strains
*Eco*RII	CC (A/T) GG	0	223	*Bst*GII and *Bst*NI from *B. stearothermophilus* strains

[a] Data are compiled from reference 64.

[b] Data are compiled from references 57 and 108 and from U. Günthert and T. A. Trantner, personal communication.

nitely excluding the presence of unusual bases as the cause of the infrequent cleavages (59).

A third example is the *B. subtilis* phage SPO1. On the basis of a random distribution of bases, it would be expected that *Bsu*RI would cleave SPO1 DNA approximately 300 times; however, only five *Bsu*RI cleavage sites were identified (103). Although the *Bsu*RI recognition sequence 5'-GGCC-3' should not be directly affected by the presence of hydroxymethyluracil instead of thymine in the SPO1 DNA (54), fragments of SPO1 DNA were cloned in *E. coli* to check this question. Indeed, the substitution of hydroxymethyluracil by the "normal" thymine does not change the low number of *Bsu*RI sites (103). In vivo, SPO1 is not subject to restriction and modification in cells of the *B. subtilis* strain R; possibly the existing five *Bsu*RI sites are protected by an additional antirestriction mechanism (103).

The DNA of the coliphage T7 also contains surprisingly few cleavage sites for a number of restriction enzymes (109). On the other hand, there are unexpectedly numerous *Eco*P1 sites (75), which might be explained by the fact that the *Eco*P1 recognition sequence (4) largely overlaps the recognition sequence for T7 primase (121), an enzyme that catalyzes the initiation of Okazaki fragments during DNA replication (for a review, see reference 77).

It is highly probable that in the course of phage evolution selective pressure can lead to a loss of DNA recognition sites for the restriction enzymes of host cells. On the other hand, the example of the abundance of *Eco*P1 sites in T7 DNA suggests that selection is also possible when such sequences have other functional importance. It is known that symmetrical DNA sequences are significant for the interaction with some regulatory proteins (for example, repressors), a fact that may also cause selection for or against specific sequences that may coincidentally also be restriction enzyme recognition sites.

PROTECTION BY UNRESOLVED MECHANISMS

Phage T5

Phage T5 DNA enters the host cell in two steps that are referred to as first-step (8% of genome) and second-step (rest of genome) transfer DNA. Intracellular expression of the first-step transfer genes is the precondition for injection of the rest of the T5 genome (for a review on phage T5, see reference 89). T5 is not restricted in host cells carrying the *Eco*K, *Eco*P1, or *Eco*RI DNA host specificity systems (21, 34). However, the T5 DNA possesses recognition sites at least for the *Eco*RI enzyme; the DNA is sensitive to *Eco*RI digestion in vitro, and six *Eco*RI sites were located in the second-step transfer DNA (36, 107).

The protection of T5 DNA against *Eco*RI restriction in vivo was studied in more detail (15, 21, 22). Indirect evidence led to the conclusion that a gene in the first-step transfer DNA of T5 determines an antirestriction mechanism (the nature of which is unknown). T5 mutants were isolated which are restricted in cells with an active *Eco*RI system. These mutants, called *ris*, were still resistant to *Eco*K and *Eco*P1. The Ris phenotype was not due to mutation in an antirestriction gene, but rather to the acquisition of new *Eco*RI sites at different positions in the first-step transfer DNA (15). When cells are infected by T5 the proposed antirestriction activity is expressed early enough to protect the six *Eco*RI sites in the second-step transfer DNA, but a newly acquired *Eco*RI site within the first-step transfer DNA of *ris* mutants cannot be protected.

Brunel and Davison believe that mutants in the protection gene itself are inviable because of other essential functions of this gene (15). This would explain why T5 can be made sensitive to *Eco*RI restriction only by the creation of new *Eco*RI sites within the nonprotected first-step transfer DNA. T5 mutants sensitive to the *Eco*P1 restriction system were also announced (22), but no further details have been published.

Male-Specific Single-Stranded DNA Phages

The filamentous, single-stranded DNA phages of *E. coli*, of which fd and M13 are the best known examples, replicate in cells as double-stranded, plasmid-like DNA molecules, but a single strand only is incorporated into phage particles (86). These phages are restricted normally in cells harboring type I and II restriction enzymes, but in vivo they are extremely resistant to the action of type III enzymes even though the double-stranded, replicative form of DNA is a good substrate for the enzymes in vitro (100). Restriction by the type III enzymes is so poor that in the first review to describe the effects of restriction on a wide variety of different phages, they were listed as being unrestricted (11). We do not know the basis for the resistance of these phages to type III restriction systems. Some possibilities can, however, be eliminated. The lack of restriction is not due to a lack of recognition sites for the enzymes; fd, for example has five sites for *Eco*P1 and two for *Eco*P15 (6) and yet is only restricted by a factor of three by *Eco*P1 and undetectably by *Eco*P15 (10). This is to be compared with the more than 1,000-fold restriction by the type I enzyme *Eco*B, which also has two sites on the fd genome (116). Again, the resistance is not due to modifi-

cation of the incoming single-stranded DNA before it is converted to the double-stranded replicative form; the type III enzymes are inactive on single-stranded DNA (B. Bächi and T. Bickle, unpublished data). The type III restriction systems are peculiar in that they methylate their recognition sequences in one strand of the DNA only; in fd DNA, three of the *Eco*P1 sites have the methylatable sequence in the incoming viral strand, but for the other two sites, the methylatable sequence is only found after DNA replication. Both of the *Eco*P15 sites are oriented such that the viral strand could not possibly be methylated (6).

The resistance of the single-stranded phages to the type III system may well be connected to a feature of the biology of these restriction systems that we still do not understand. Because a fully modified recognition site for these enzymes has a methyl group in only one of the DNA strands (4, 44, 50, 100), DNA replication gives one daughter molecule that has inherited the parental methyl group and is fully modified and a second daughter molecule that has no methyl group and is thus unmodified. If these sites remain unmodified for any length of time, they should occasionally be cleaved by the restriction enzyme; this would be lethal to the cell. One way of avoiding this would be if the modification of the DNA was very tightly coupled to replication; in the extreme case one could envisage the modification enzyme built into the replication complex. If this were the case, the single-stranded DNA of the phage could be modified during its conversion to the double-stranded form.

B. subtilis Phage SP18-Mediated Protection of Phage SP10

Preinfection of *B. subtilis* expressing the *Bsu*M restriction system with phage SP18 protects superinfecting SP10 against restriction. The mechanism of the protection is unclear; however, it operates even when RNA or protein synthesis is blocked during the preinfection with SP18 (131). So far as we know, the question of whether SP18 and SP10 have partial DNA sequence homologies has not been investigated. If they did, the protective mechanism previously described for *B. subtilis* transfection and transformation might be operating. This mechanism protects unmodified DNA entering the cell from restriction, provided that homologous DNA sequences are already present (12, 17).

CONCLUSIONS

It seems certain that bacteria have evolved DNA restriction and modification systems primarily to maintain genetic stability when foreign genetic material is introduced into the cells. This function is clearly important enough that it has evolved independently on several occasions, as attested by the several basically different classes of restriction enzymes that exist today.

To survive when infecting the different strains of their host species that they are likely to encounter, bacterial viruses have developed the antirestriction mechanisms that are the subject of this review. The most striking feature of phage antirestriction is the wide variety of different mechanisms that have been developed by different phages. Some of the antirestriction mechanisms may well play further roles in the viral life cycle.

Antirestriction is also of interest for many aspects of molecular biology, from both the theoretical and the practical point of view. Different examples provide good model systems for the study of protein-protein or protein-nucleic acid interactions. The fact of antirestriction should be borne in mind by anybody working with new hosts or with new bacteriophages in familiar hosts. It may also be encountered in the development of new host-vector systems for genetic engineering.

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