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DNA Mimicry by Antirestriction and Pentapeptide Repeat (PPR) Proteins

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1. Introduction

Protein mimicry of DNA is a recently discovered direct mechanism of regulation of DNA-dependent enzyme activity by means of proteins that mimic DNA structure and interact with a target enzyme and completely inhibit (or modulate) its activity. DNA-mimicking inhibitor proteins bind directly to the enzyme and thus blocks or alters the activity of the latter. Protein mimicry of DNA was first described in Ugi derived from PBS2 bacteriophage of *Bacillus subtilis* (Mol et al, 1995). This protein of 84 amino acid residues with a total charge of (–12) inhibits uracil-DNA glycosylase (UDG), an enzyme involved in DNA repair (Mol et al, 1995; Putnam & Tainer, 2005). Subsequently, this type of protein mimicry was found in the ribosomal elongation factor EF-G (tRNA-like motif), and in the dTAFII 230 component of eukaryotic transcription factor TFIID (DNA-like domain) (Liu et al., 1998). The family of DNA mimetics further includes DinI, a negative SOS response regulator in *E. coli* (Ramirez et al., 2000), and a nucleosome forming protein HI1450 of *Haemophilus influenzae* (Parsons et al., 2004). However, in most of these cases, only a part of the protein molecule is DNA-like, in contrast to antirestriction and pentapeptide repeat (PPR) proteins, whose entire structure mimics the B-form of DNA. For instance, the X-ray structure of Ugi reveals a domain similar to the B-form of DNA, but the molecule as a whole is globular. Note that, in Ugi, the crucial negative charges are those of E20, E28, and E31 in the N domain (Mol et al.,1995).

Horizontal gene transfer is a fundamental mechanism for driving diversity and evolution. Transmission of DNA to bacterial cells that are not direct descendants of the donor is often achieved via mobile genetic elements such as plasmids, conjugative transposons and bacteriophages. Mobilization of these elements can lead in the spread of antimicrobial resistance in clinical environments and in the wider community.

Over 50% of eubacteria and archaea contain the genes for one or more of the four classes of known DNA restriction and restriction-modification (RM) systems (Roberts et al., 2005). RM systems work by recognizing specific DNA sequences and triggering an endonuclease activity which rapidly cleaves the foreign DNA allowing facile destruction by exonucleases (Bickle & Kruger,1993; Murray, 2000; Loenen, 2003).

Mobile genetic elements such as plasmids, transposons and bacteriophage contain the specific genes encoding anti-RM systems. Activation of anti-RM system weakens or negates

the RM defence system allowing further horizontal gene transfer (Wilkins, 1995; Zavilgelsky, 2000; Murray, 2002; Tock & Dryden, 2005).

The genes encoding antirestriction proteins are situated on conjugational plasmids (*ard*A gene) and some bacteriophages (*ocr* and *dar*A genes). Antirestriction proteins inhibit the type I restriction-modification enzymes and thus protect unmodified DNA of plasmids and bacteriophages from degradation. Genes *ard* (*a*lleviation of *r*estriction of DNA) facilitate the natural DNA transfer between various types of bacteria ensuring overcoming intercellular restriction barriers (horizontal genes transfer). Genes *ocr* (bacteriophage T7) and *dar*A (bacteriophage P1) significantly increase the infection efficiency by phages of the bacterial cells.

Antirestriction proteins ArdA and Ocr belong to the group of very acidic proteins and contain a characteristic sequence of negative charges (Asp and Glu). X-ray diffraction study of proteins ArdA and Ocr carried out demonstrated that these proteins were like the B-form of DNA (Walkinshaw et al., 2002; McMahon et al., 2009). Therefore the antirestriction proteins operate on the principle of concurrent inhibition replacing DNA in the complex with the enzyme (DNA mimicry).

DNA-mimetic antirestriction proteins ArdA and Ocr can be electroporated into cells along with transforming DNA and protect unmodified DNA from degradation. As a result the antirestriction proteins improve transformation efficiency. The highly charged, very acidic proteins Ocr and ArdA can be used as a purification handle similar to other fusion tags. A monomeric mutant of the Ocr protein was used as a novel fusion tag which displayed solubilizing activity with a variety of different passenger proteins (DelProposto et al., 2009).

The pentapeptide repeat is a recently discovered protein fold. MfpA and Qnr (A,B,C,D,S) are two newly characterized pentapeptide repeat proteins (PPRs) that interact with type II topoisomerase (DNA gyrase) and confer bacterial resistance to the drugs quinolone and fluoroquinolone [Hegde et al., 2005; Hedge et al., 2011). The *mfp*A gene is chromosome borne in *Mycobacterium tuberculosis (*Hegde et al., 2005; Montero et al., 2001), while *qnr* genes are plasmid borne in Gram-negative enterobacteria (Martinez-Martinez, L. et al.,1998; Tran et al., 2005; Cattoin & Nordmann, 2009; Rodriguez-Martinez et al. 2011). The size, shape, and surface potential of MfpA and Qnr proteins mimics duplex DNA (Hegde et al., 2005; Vetting et al., 2009; Hegde et al., 2011).

2. Type I restriction-modification systems

Restriction–modification (RM) systems form a barrier protecting a cell from the penetration by foreign DNA (Murray, 2000; Loenen, 2003). In the modern understanding, RM enzymes are a part of the "immigration control system", which discriminates between its own and foreign DNA entering the cell (Murray, 2002). The system is based on two conjugated enzymatic activities: those of restriction endonucleases and DNA methyltransferases. RM enzymes recognize a specific nucleotide sequence in the DNA, and the restriction endonuclease cleaves the double strand of unmodified DNA. The host DNA is protected from enzymatic cleavage by specific methylation of the recognition sites produced by DNA methyltransferases. RM enzymes are classified in four types. We shall now discuss the features of type I RM systems, since it is these systems that are efficiently inhibited by antirestriction proteins. Figure 1 schematically represents the activity of a type I enzyme, e.g., EcoKI. EcoKI comprises five subunits (R_2M_2S): two R subunits are restriction

endonucleases that cleave the double helix of unmodified DNA, two M subunits are methyltransferases that methylate adenine residues at the recognition site, and an S subunit recognizes a specific DNA site (sK) and forms a stable complex with it.

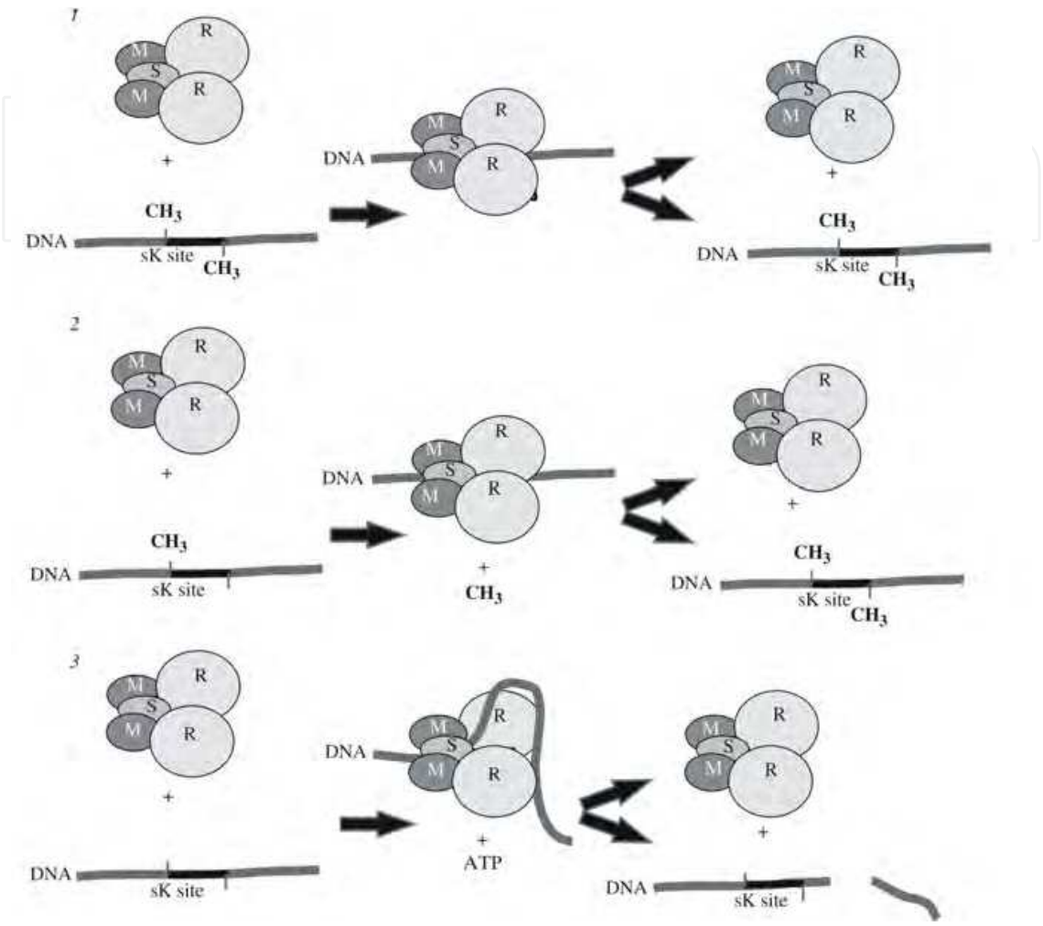

Fig. 1. Activity of a type I restriction–modification enzyme. 1, Both DNA strands at the sK site are methylated. The enzyme–DNA complex dissociates. 2, One of DNA strands at the sK site is methylated. The methylase (M) methylates the adenyl residue of the other strand, and the complex dissociates. 3, Both DNA strands at the sK site are unmethylated. The enzyme initiates DNA translocation through the R subunits accompanied by the formation of a supercoiled loop and subsequent double-stranded DNA break.

The sK site is "hyphenated", i.e., only seven outmost nucleotides of the 13 bp long recognition sequence are conserved (e.g. EcoKI recognizes 5'-AACNNNNNNGTGC-3'). According to the footprinting data, EcoKI covers 66 bp of the DNA sequence. Further events depend on the sK status. If both DNA strands at the site are methylated, the complex dissociates.

If only one strand is methylated, the methylase M methylates the respective adenyl residue, and the complex dissociates. If both DNA strands are unmethylated, the DNA helix is translocated through the R subunits, while the S subunit remains bound to the sK site. The endonuclease R randomly cleaves the DNA strands at a considerable distance from the sK site. This is the principal difference between the type I RM enzymes and type II restriction

endonucleases, which introduce a double-strand DNA break directly at the recognition site or at a specific distance from it. The translocation process itself is associated with considerable energy expenditure in the form of ATP. As a result, type I RM enzymes are ATP-dependent, whereas type II enzymes are not. Another characterizing feature of the EcoKI–sK complex is that the S subunit binds only to the outmost conserved nucleotides of the site. As a result, the double stranded DNA undergoes significant deformation, acquiring a kink of approximately 34°, which sets additional energy demands. Nucleotide sequences of the recognition sites vary and are specific for each type I enzyme (EcoK, EcoB, EcoA, EcoD, Eco124, StyLT, StySP, CfrAI, and many others). Based on their homology and the possibility of subunit exchange, type I RM systems are classified into four families: IA, IB, IC, ID. Restriction is efficient against foreign DNA irrespective of the way it is introduced into the cell: by injection from a phage, transformation, or conjugative transmission. Thus, type I RM systems constitute a socalled restriction barrier that prevents interspecies horizontal gene transfer.

3. Conjugative plasmids and transposons, bacteriophages, and antirestriction

Natural horizontal gene transfer between bacteria is mediated primarily by transmissible plasmids, conjugative transposones, and bacteriophages (Wilkins, 1995). Evolution of all transmissible plasmids, conjugative transposones and some bacteriophages gave rise to systems enabling them to overcome restriction barriers. This phenomenon has been termed antirestriction (Zavilgelsky, 2000; Tock & Dryden, 2005). An investigation of antirestriction mechanisms employed by transmissible plasmids showed that the process involves a specialized antirestriction protein encoded by the *ard*A gene (*a*lleviation of *r*estriction of *D*NA). *ard*A genes were first discovered in plasmids of the incompatibility group N in 1984–1985 (Belogurov et al.,1985), and later in other types of plasmids (Kotova et al., 1988; Delver et al., 1991). In 1991–1995, *ard*A genes were sequenced and the primary structure of ArdA proteins was determined (Delver et al., 1991; Chilley & Wilkins, 1995). Genes *ard*A are located in the leader region of the plasmid sequence, which lies next to *ori*T and is the first to enter the host cell in the course of conjugative transfer. The *ori*T site, the origin of plasmid conjugative replication, is located at the boundary of the *tra* operon with the rest of plasmid.

The conjugative transposon Tn916 of the bacterial pathogen *Enterococcus faecalis* contains *orf*18 gene, which is located within position region and encodes an ArdA antirestriction protein (Serfiotis-Mitsa et al., 2008). Genes of the *ard*A family encode small, very acidic proteins comprised of 160–170 amino acid residues and bearing a characteristic total negative charge of (–20 to –30) which act as specific highly efficient inhibitors of cellular type I RM enzymes. ArdA proteins inhibit restriction endonucleases of different families (IA, IB, IC, and ID) and with different recognition site sequences with nearly the same efficiency. Thanks to this property of ArdA, transmissible plasmids can overcome the restriction barriers through horizontal transmission from the donor cell into bacteria of various species and genera.

Some bacteriophages also possess genes encoding antirestriction proteins, such as *0.3(ocr)* (phage T7) and *dar*A (phage P1) (Dunn et al., 1981; Kruger et al., 1983; Iida et al., 1988). These genes increase the efficiency of phage infection.

Antirestriction proteins, both of plasmid (ArdA) and phage origin (Ocr), inhibit only type I RM enzymes, whose genes (*hsd*RMS) are usually located on the bacterial chromosome, but not type II restriction endonucleases, the genesof which are normally located on plasmids.

4. DNA mimicry by antirestriction proteins

It has been supposed that antirestriction proteins of the ArdA family, as well as Ocr are modulator proteins with a structure similar to that of the B-form DNA, and the characteristic surface distribution of negatively charged D and E residues (aspartic and glutamic acids) imitates the distribution of negatively charged phosphate groups along the DNA double helix (Zavilgelsky, 2000). That is, antirestriction proteins imitate the DNA structure, which is currently termed "protein mimicry of DNA". The spatial structure of the smallest antirestriction protein, Ocr of phage T7 (116 amino acids), was published in 2002 (Walkinshaw et al., 2002). As shown by X-ray crystallography, the spatial structure of Ocr was similar to the B-form of DNA (Fig. 2). The major stem of the Ocr monomer is constituted by three α-helices: A (residues 7–24), B (residues 34– 44), and a long, somewhat bent one, D (residues 73–106); the helices form a tightly packed bunch with strictly regularly positioned negatively charged D and E carboxyls along the stem axis, nearly reproducing the distribution of negatively charged phosphate groups along DNA double helix. The short α-helix C (residues 49–57) is a part of the interface determining the contact of monomers and stable dimer formation.

The structure of the Ocr dimer, both in solution and in crystal form, is similar in length and charge distribution to 24 bp of DNA double helix. The contact of monomers is established by a Van der Waals interaction between hydrophobic clusters within the C α-helices in the middle of the polypeptide: A50, F53, S54, M56, A57, and V77.

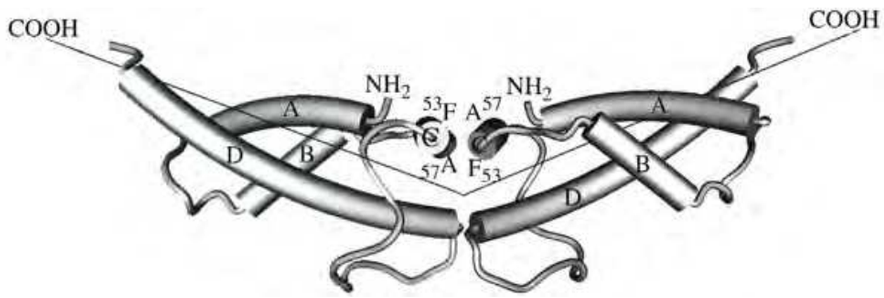

Fig. 2. Spatial structure of the $(Ocr)_2$ protein dimer. Shown is the positioning of α-helices A, B, C, D, and amino acid residues 53F and 57A in the hydrophobic cluster 52IFSVMAS, which determines the Van der Waals attraction of the monomers.

The spatial structure of the ArdA protein from the conjugative transposon Tn916 (166 amino acids), was published in 2009 (McMahon et al., 2009). As was shown by X-ray crystallography, ArdA protein has a extremely elongated curved cylindrical structure witn defined helical groowes. The high density of Asp and Glu residues on the surface follow a helical pattern and the whole protein mimics a 42-base pair stretch of B-form DNA making ArdA dimer by far the largest DNA mimic known (Fig. 3). Each monomer of this dimeric structure can be decomposed into three domains: the N-terminal domain 1 (residues 3-61), the central domain 2 (residues 62-103) and the C-terminal domain 3 (residues 104-165). The N-terminal domain 1 consists of a three-stranded anti-parallel β-sheet and one short α -helix interspersed with three large loops of 10 or more residues. The central domain 2 of ArdA is a four α–helix bundle. The C-terminal domain 3 has a three-stranded β -sheet and three α-helices packed together in a manner that creates a groove in the structure 11 angstrem wide. Analysis of the electrostatic surface of ArdA shows that 2 and 3 domains have a profoundly negative potential (the pI of ArdA is 4). The ArdA dimer, like the monomer, is highly

elongated and curved (Fig. 3). The chord that connects the extreme ends has the length of 140 angstrem. The pattern of negative charge even extends across the dimer interface through the conserved residues D109, D111, D112, D115, E122, E123 and E129.

This distribution and conservation of charged residues is evidence for the necessity of dimer formation for protein function and suggests that ArdA across all species will have similar structural requirements. The dimer interface contains the anti-restriction motif (amino acids 126-140 in the Tn916 ArdA protein) identified previously (Belogurov & Delver, 1995) conserved as well.

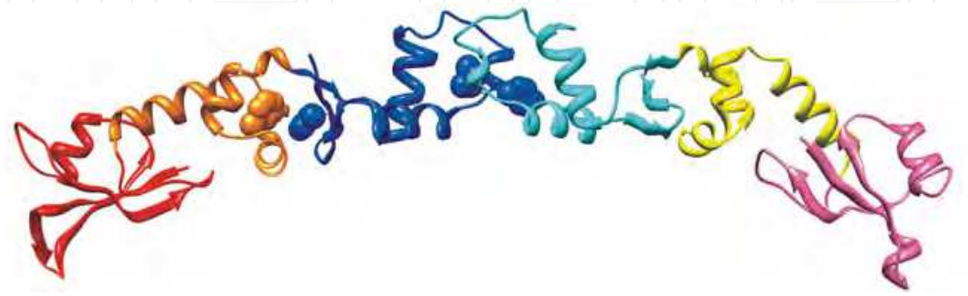

Fig. 3. Spatial structure of the $(ArdA)_2$ protein dimer.

The ArdA dimer appears to mimic about 42 bp of bent B-form DNA. This is comparable in length to the footprint of the EcoKI Type IA RM enzyme, without its cofactors, on DNA. In comparison, the Ocr dimer from phage T7 mimics only about 24 bp., similar in length to the 30 bp footprint of the Type I RM enzyme in the presence of its cofactors and to the footprint of the MTase core, M.EcoKI, of the Type I RM enzyme. The typical DNA target for a Type I RM enzyme is 14 bp long and bipartite, e.g. EcoKI recognizes 5'-AACNNNNNNGTGC-3', and lies centrally in the experimental DNA footprint. It was built the M.EcoKI-ArdA model: domain 3 overlaps the EcoKI target sequence, domain 2 contacts the extremites of the DNA-binding groove in M.EcoKI and domain 3 projects beyond the M.EcoKI structure. Domain 1 is not essential for antirestriction as it can be deleted (Delver et al., 1991) indicating that the key aspect of antirestriction by ArdA is the binding to the MTase core using domains 2 and 3.

The mimicry of DNA enables antirestriction proteins to compete with DNA for binding with the RM enzyme and thus to inhibit DNA degradation (restriction) and methylation (modification). From the point of view of classical enzymatic catalysis, antirestriction is a case of competitive inhibition based on structural similarity between the enzyme substrate and the inhibitor molecule. The relative positioning of monomers in the $(Ocr)_2$ dimer is typical: the angle between their longitudinal axes is approximately 34° (Fig. 2). This dimer structure is nearly equivalent to the kinked DNA double helix structure that is formed at the recognition site of the type I RM enzyme–DNA complex (Murray, 2000). Consequently, Ocr does not require additional energy to bind to EcoKI, and efficiently displaces double-stranded DNA from the complex (the complex formation constant for Ocr–EcoKI is approximately 100 times higher than for DNA–EcoKI) (Atanasiu et al., 2002).

5. Antirestriction and antimodification activities of ArdA and Ocr proteins

Both ArdA and Ocr inhibit ATP-dependent type I RM enzymes. However, the great difference between the life cycles of transmissible plasmids (symbiosis with a bacterial cell) and

bacteriophages (infection and lysis of bacteria) makes it interesting to compare the inhibition efficiencies of these proteins. For this purpose, we cloned *ard*A and *ocr* under a strictly regulated promoter. To quantify the intracellular concentration of the antirestriction proteins, we developed a bioluminescence method that utilizes the *Photorhabdus luminescens luxCDABE* genes as reporters. The *luxCDABE* genes were cloned in the pZE21 and pZS33 vectors under the control of the PltetO_1 promoter. The hybrid plasmids were introduced in MG1655Z1 cells. Expression of the *lux* genes was induced by adding anhydrotetracycline in the medium, and the bioluminescence intensity was measured. Since the bioluminescence intensity is directly proportional to the luciferase concentration and the sensitivity of the bioluminescence method is high, it is possible to estimate the enzyme concentration in the cell within a broad range, starting with extremely low concentrations. A calibration plot was constructed to characterize the intracellular content of the enzyme (in relative units (RU)) as a function of the inductor (anhydrotetracycline) concentration (Fig. 4). The luciferase content in MG1655Z1 cells varied from 1 (in the absence of anhydrotetracycline) to 5000 (20 ng/ml anhydrotetracycline or more) RU. It is natural to assume that the relative contents of the proteins synthesized from the *ardA* and *0.3(ocr)* genes cloned in the pZE21 and pZS33 vectors vary within the same range as the luciferase content under the same expression conditions.

To measure the antirestriction activities of the ArdA and Ocr proteins, titration with phage λ.0 was performed for MG1655Z1 cells carrying a hybrid plasmid with the *ardA* or *0.3(ocr)* gene; cells without the hybrid plasmid were used as a control. Since the genome of strain MG1655Z1 contains the *hsdRMS* genes, which code for the *Eco*KI restriction–modification enzyme, the phage λ.0 seeding efficiency was approximately four orders of magnitude lower than in the case of control strain TG_1. However, when MG1655Z1 cells contained a plasmid with the cloned *ardA* or *0.3(ocr)* gene, the phage seeding efficiency changed depending on the production of the antirestriction protein. As the protein production increased, the phage seeding efficiency grew from 10^{-4} (no inhibition) to 1 (complete inhibition of restriction–modification enzymes).

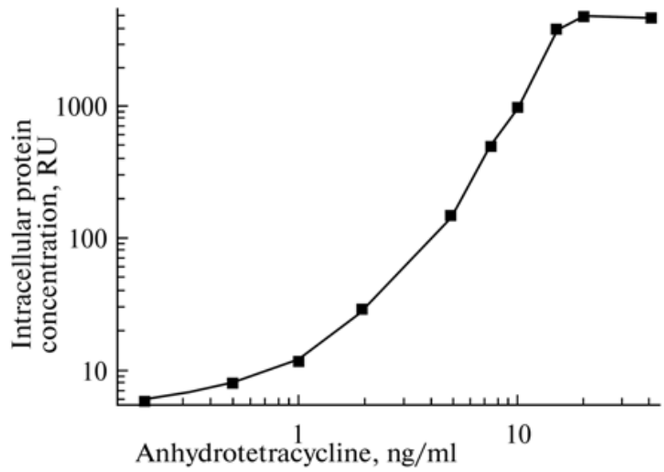

Fig. 4. Luciferase content (relative units, RU) in *E. coli* MG1655Z1 cells containing the pZS33_*lux* or pZE21_*lux* plasmid as a function of anhydrotetracycline content. The *P. luminescens luxCDABE* genes were cloned in the pZS33 and pZE21 vectors under the control of the P1tetO_1 promoter. The luciferase content in the presence of the pZS33_*lux* plasmid and the absence of the inductor anhydrotetracycline was taken as unity.

ArdA ColIb-P9, Ocr T7 antirestriction and antimodification activities were avaluated as a function of the inhibitor concentration, that enabled us to estimate the relative difference in dissociation constants (K_d) that describe the interaction efficiency for ArdA or Ocr and EcoKI (Fig. 5) (Zavilgelsky et al., 2008).

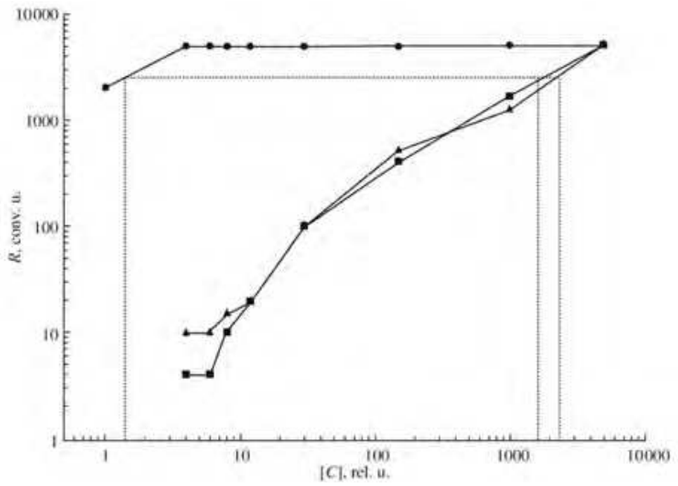

Fig. 5. Antirestriction activity of ArdA ColIb-P9, Ocr T7, and Ocr mutant F53D A57E as a function of their intracellular levels. X-axis: intracellular antirestriction protein concentration (relative units). Y-axis: Antirestriction activity (unmodified λ DNA was used as an EcoKI target). Dotted lines indicate the K_d points. Circles, native Ocr; squares, Ocr F53D A57E; triangles, ArdA.

The antimodification activity of the ArdA and Ocr proteins was inferred from the seeding efficiency of phage $\lambda_{MG1655Z1}$ (phage λ.0 propagated for one cycle in MG1655Z1 cells carrying a plasmid with the *ardA* or *0.3(ocr)* gene) on strains AB1167 r+m+ and TG1 r–m–. The ratio between the phage titers on these strains reflected the extent of phage DNA modification (methylation). The *ardA* and *0.3(ocr)* genes were cloned in the pZE21 and pZS33 vectors with the strongly regulated $P_{ltetO-1}$ promoter; the results are summarized in Tables 1 and 2. The intracellular concentrations of the ArdA and Ocr proteins were estimated from the calibration plot constructed by the bioluminescence method (Fig. 1). The ArdA and Ocr proteins substantially differed in the capability of inhibiting the *Eco*KI enzyme. The Ocr protein almost completely inhibited the *Eco*KI restriction–modification system, affecting both restriction and modification activities of the enzyme in a broad Ocr concentration range. The effect was already detectable when Ocr was present at several tens of molecules per cell (1 RU corresponds approximately to ten molecules of the inhibitor protein per cell) (Table 1).

In the case of the ColIb_P9 ArdA protein, the efficiency of inhibition of the restriction activity of the *Eco*KI enzymes started to decrease when the protein concentration was approximately half its threshold value (which corresponded to complete inhibition of *Eco*KI activity), that is, when ArdA occurred at 10000– 15000 molecules per cell. Inhibition of modification activity of the *Eco*KI enzyme started at higher intracellular ArdA concentrations, at approximately 45000–50000 ArdA molecules per cell (Table 2).

The antirestriction and antimodification activities of the ArdA and Ocr proteins as functions of their intracellular concentrations (in RU) are shown in Fig. 6. While the Ocr protein

inhibited both activities of the *Eco*KI enzyme with similar efficiencies and acted already at extremely low concentrations in the cell, the antirestriction and antimodification activity curves substantially differed in the case of the ArdA protein. As estimations showed, the dissociation constant *K*d(met) characteristic of ArdA_dependent inhibition of methylase activity of the *Eco*KI enzyme was tenfold higher than Kd(rest).

The difference in inhibitory properties of the Ocr and ArdA proteins toward type I restriction–modification enzymes is probably determined by the difference in life cycle between phages and transmissible plasmids; i.e., a phage kills the cell, while a plasmid becomes part of cell genetic material.

The ArdA proteins lose their capability of inhibiting modification activity of *Eco*KI_like proteins relatively easy. For instance, the ArdA antirestriction proteins encoded by the R16 (incB) and R64 (incI1) transmissible plasmids inhibit restriction activity of the *Eco*KI enzyme, but do not affect its modification activity [25, 26]. Yet the proteins are highly homologous to the ColIb_P9 ArdA protein. In the 166 amino acid residues, differences are observed only in four positions with R64 ArdA and in nine positions with R16 ArdA. We have earlier found that certain single or double substitutions of hydrophobic amino acid resdues for negatively charged residues (D and E) in the region of the antirestriction motif abolish antimodification activity of ArdA encoded by the pKM101(incN) transmissible plasmid, while its its antirestriction activity is still preserved [17].

In this work, we used site_directed mutagenesis and constructed the ColIb_P9 ArdA mutant that contained three amino acid substitutions in the C_terminal domain; hydrophobic residues were replaced with a more hydrophobic one: F156I, F158I, and V163I.Activities of the mutant protein are characterized in Table 3. As is seen, the mutant protein inhibited antirestriction activity of the *Eco*KI enzyme, but lost the inhibitory effect on its modification activity.

Likewise, certain amino acid substitutions transform the Ocr protein into an antirestriction protein that inhibits only antirestriction activity of the *Eco*KI enzyme. X_ray analysis of the Ocr protein in crystal demonstrates that a contact of the monomers in the (Ocr)2 homodimer is due to hydrophobic interactions between F53 and A57, which are in the hydrophobic fragment 52_IFSVMAS_ in a short α_helix [11]. We constructed an Ocr mutant with two substitutions, F53D and A57E, assuming that repulsion of negative charges (D…E) would lead to dissociation of the dimer. The *0.3(ocr)* gene with a single or double mutation was cloned in the pUC18 vector. The Ocr F53D A 57E double mutant was tested for functional activity and proved to efficiently inhibit only *Eco*KI restriction activity without affecting methylase activity of the enzyme (Table 4, data on the antirestriction activity of the proteins are omitted). Note that the single amino acid substitutions of the interface region did not affect the antimodification activity of the Ocr protein (Table 4). Like the Ocr protein, the ArdA proteins are active in a homodimeric form. This is true for both the native ColIb_P9 ArdA protein and the R64 ArdA mutant, which is incapable of inhibiting methylase activity of the enzymes.

Based on the data obtained for the Ocr and ArdA mutant proteins, we assume that the antirestriction proteins form complexes of two types with a type I restriction–modification type, which consists of five subunits (R2M2S) [27]. When an antirestriction protein interacts with the S subunit, which recognizes a specific site in DNA, the DNA strand is displaced, and both restriction and modification activities of the enzyme are inhibited. When an antirestriction protein interacts with the R subunit, which is responsible for ATP_dependent translocation and endonucleolytic cleavage of nonmethylated DNA, only restriction activity of

the enzyme is inhibited. To check this hypothesis, it was important to construct the Ocr mutants that were incapable of inhibiting methylase activity of the enzymes and preserved the effect on their restriction activity. Such properties were observed for the Ocr F53D A57E mutant, which was constructed in this work and had two substitutions of negatively charged amino acid residues for hydrophobic residues in the interface region of the (Ocr)2 homodimer. Thus, the model of type I restriction– modification enzymes with two different binding sites for antirestriction proteins is applicable not only to the ArdA proteins, whose genes are in transmissive plasmids, but also to the Ocr proteins, whose genes are in bacteriophage genomes.

Anhydrotetracyclin, ng/ml	Ocr concentration in the cell, RU	EcoKI modification alleviation factor (R) for Ocr**	EcoKI restriction alleviation factor (R) for Ocr
0.0 (vector pZS33)	1	2000	2000
0.0 (vector pZE21)	4	5000	5000
0.2	6	5000	5000
0.5	8	5000	5000
1.0	12	5000	5000
2.0	30	5000	5000
5.0	150	5000	5000
10.0	1000	5000	5000
20.0	5000	5000	5000
40.0	5000	5000	5000

Notes: * The *0.3* (*ocr*) gene was cloned either in the pZE33 vector (row 1) or in the pZE21 vector (other rows) under the control of the P1tetO_1 promoter.
** Here and in Table 3: Restriction or modification alleviation factor $R = K+ / K-$, where $K-$ is the coefficient of restriction for MG1655Z1 cells without the plasmid containing the *0.3* (*ocr*) gene and $K+$ is the coefficient of restriction for MG1655Z1 cells carrying the plasmid.

Table 1. Antimodification and antirestriction activities of the Ocr protein as dependent on its intracellular concentration*

Anhydrotetracyclin, ng/ml	Ard concentration in the cell, RU	EcoKI modification alleviation factor (R) for ArdA	EcoKI restriction alleviation factor (R) for ArdA**
0.0 (vector pZS33)	1	Not determined	Not determined
0.0 (vector pZE21)	4	1	5
0.2	6	1	6
0.5	8	1	10
1.0	12	1	20
2.0	30	1	120
5.0	150	4	400
7.5	500	10	1000
10.0	1000	100	2500
15.0	4000	400	5000
20.0	5000	1000	5000

Table 2. Antimodification and antirestriction activities of the ColIb_P9 ArdA protein as dependent on its intracellular concentration*

We conclude that the dimeric form of an antirestriction protein is essential for inhibiting both activities of a type I restriction–modification system, while the monomeric form is sufficient for inhibition of its restriction activity.

- The *ardA* gene was cloned in the pZE21 vector under the control of the P1 tetO_1 promoter.

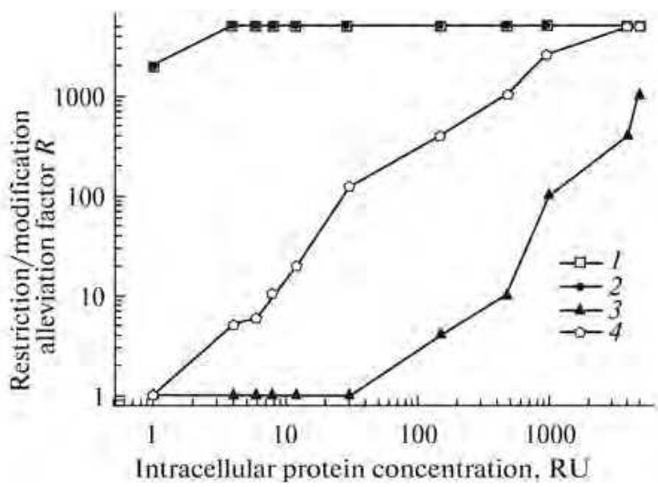

Fig. 6. Antirestriction and antimodification activities of the ColIb_P9 ArdA and T7 Ocr proteins as functions of their intracellular concentrations. Curves: *1*, antimodifi cation activity of Ocr; *2*, antirestriction activity of Ocr;*3*, antimodification activity of ArdA; *4*, antirestriction activity of ArdA.

ArdA and Ocr differ considerably in their ability to inhibit the methylase (modification) activity of EcoKI-like enzymes. As a rule, if *ard*A and *0.3(ocr)* genes are governed by a strong promoter, antirestriction and antimodification activities of ArdA and Ocr are established simultaneously (Delver et al., 1991; Chilley & Wilkins, 1995;Atanasiu et al., 2002). Some data suggest, however, that the inhibition of endonuclease and methylase activities depends on different interactions of ArdA proteins with type I RM enzymes. For instance, some natural ArdA proteins inhibit only the endonuclease activity of EcoKI. The respective genes are located in transmissible plasmids R16 (incB) (Thomas et al., 2003) and R64 (incI1) (Zavilgelsky et al., 2004). Furthermore, *in vitro* quantification of the ArdA– EcoKI complex showed that ArdA interacts more efficiently with the complete enzyme R_2M_2S than with its methylase form M_2S, which can only modify DNA (Nekrasov et al., 2007). In contrast to ArdA proteins, Ocr from phage T7 binds to the entire EcoKI enzyme and to its methylase form with nearly equal affinities (Atanasiu et al., 2002), and, therefore, even in very low concentrations it inhibits both the endonuclease and the methylase activities of the enzyme (Fig. 5). This property of Ocr is probably related to the difference between the life cycles of a phage and of a transmissible plasmid: a phage kills the host cell, whereas a plasmid becomes part of its genetic material. However, a double amino acid substitution in the 52IFSVMAS hydrophobic cluster of the Ocr interface (an Ocr homodimer is formed by a Van der Waals interaction between these clusters), that is, a substitution of acidic 53D and 57E for hydrophobic 53F and 57A (Fig. 6), causes the mutant protein Ocr F53D A57E to lose the antimethylation while retaining the antirestriction activity against EcoKI. In addition, the mutant protein Ocr F53D A57E has a K_d of 10^{-7}M, which is 1000 times higher than the K_d of the native Ocr form (Fig. 6) (Zavilgelsky et al., 2009).

Plasmid	Protein	Coefficient of restriction (K) of phage λ.0 on AB1157 $r^+m^{+(**)}$	Coefficient of restriction (K) of phage λ_{jm109} on AB1157 r^+m^+
pUC18	Absent	2.0×10^{-4}	1
pVB2(pUC18)	ArdA F1561 F1581 V1631	1	1
pSR3(pUC18)	ArdA native	1	2.0×10^{-4}

Notes: * Phage λ.0 was used to infect *E. coli* JM109 r–m+ cells. A phage lysate obtained after one reproduction cycle (λjm109) was titrated on strains TG_1 and AB1157.
** The coefficient of restriction *K* (column 3), which was used to estimate the antirestriction activity of the ArdA proteins, was determined as the ratio of the titer of phage λ.0 on strain AB1157 to the titer of the same phage on strain TG_1 r–m–.

Table 3. Effects of the ArdA (ColIb_P9) protein and its F156I F158I V163I mutant on *Eco*KI restriction and *Eco*KI modification in *E. coli* K_12 AB1157 r+ m+ and MJ109 r–m+* cells upon the cloning of the corresponding genes in the pUC18 vector

Plasmid	Protein	Coefficient of restriction (K) of phage λ_{jm109} on TG1 r^-m^-	Coefficient of restriction (K) of phage λ_{jm109} on AB1157 r^+m^+
pUC18	Absent	1	1
pSR8	Ocr native	1	2.0×10^{-4}
pSR9	Ocr F53D	1	2.0×10^{-4}
pSR10	Ocr A57E	1	2.0×10^{-4}
pSR11	Ocr F53D A57E	1	1

* Phage λ.0 was used to infect *E. coli* JM109 r– m+ cells. A phage lysate obtained after one reproduction cycle (λjm109) was titrated on strains TG_1 and AB1157. The results were averaged over five replicate experiments.

Table 4. Effects of the native and mutant T7 Ocr proteins on *Eco*KI_dependent modification in *E. coli* K_12 JM109 r– m+ cells*

K_d is determined by intracellular protein concentration characterized with a 50% decrease in the inhibition of EcoKI endonuclease activity. For Ocr, this level was approximately 1700 times lower than for ArdA. According to in vitro data, the Ocr–EcoKI complex formation had a K_d of 10^{-10} M (Atanasiu et al., 2002). Therefore, the K_d for ArdA–EcoKI complex formation is 1.7×10^{-7}M.

The fact that endonuclease and methylase EcoKI activities are inhibited by ArdA or Ocr separately suggests that antirestriction proteins can bind type I enzymes in two ways: the complex formation of the first type inhibits both endonuclease and methylase activity of the enzyme, whereas in the complex of the second type, endonuclease activity is blocked while methylase activity is retained. As a working hypothesis, we propose the following model of interaction between antirestriction proteins (ArdA and Ocr) and type I RM enzymes (Fig. 7). ArdA and Ocr can form a complex both with the S-subunit that contacts with the *s*K site on

DNA, and with the R-subunit responsible for the translocation and cleavage of unmodified DNA. The binding of ArdA or Ocr to the S-subunit simultaneously inhibits both endonuclease and methylase activity by displacing DNA from its complex with the R_2M_2S enzyme (Fig. 7, *1*). However, the binding can be easily disrupted if, as a result of amino acid substitutions, the protein is not in the dimeric form, or if the angle between the longitudinal axes of the monomers differs from the critical 34°. As a consequence, it becomes energetically unfavorable for a DNA-mimic protein to displace kinked DNA from its complex with the S-subunit. On the other hand, the interaction of ArdA or Ocr with the R-subunit probably does not depend on the particular dimer structure, since the R-subunit is responsible for DNA strand translocation and the respective complex is not site-specific. Thus ArdA and Ocr inhibit only the endonuclease activity of the enzyme, while its methylase activity is preserved: DNA can still bind to the S-subunit, and the M-subunit specifically methylates adenyl residues at the sK site (Fig. 7, *2*).

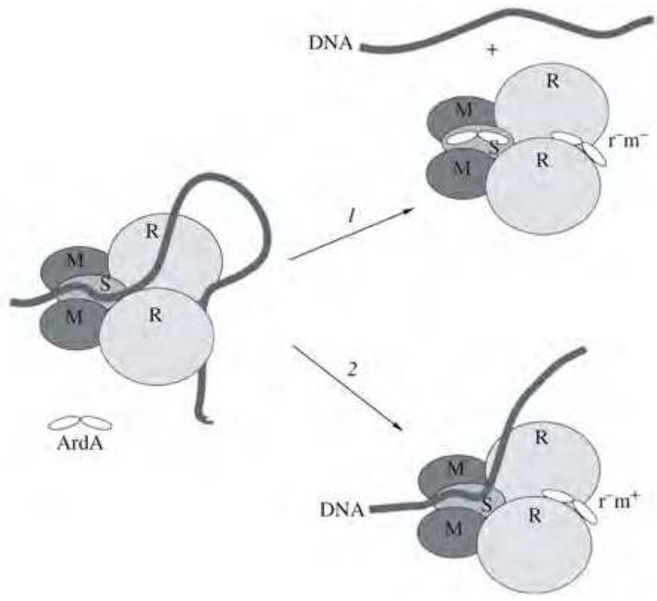

Fig. 7. Putative scheme of ArdA or Ocr interaction with a type I RM enzyme (R_2M_2S). *1*, An ArdA/Ocr complex with the S subunit: unmodified DNA is entirely displaced. Both endonuclease and methylase activities are inhibited ($r^- m^-$ - phenotype). 2, An ArdA/Ocr complex with the R subunit: a DNA strand is displaced from the translocation center. Only endonuclease activity is inhibited (r– m+ phenotype). Endonuclease and methylase activities of a type I RM enzyme are designated as "r" and "m" respectively.

In vitro experiments showed that the R_2M_2S form of EcoKI binds two $(Ocr)_2$ dimers, while the methylase form M_2S binds only one (Atanasiu et al., 2002). This result fits well into the above model of inhibition by antirestriction proteins. As the ArdA binding constant is higher for M_2S than for R_2M_2S, moderate levels of ArdA synthesized under natural conditions inhibit only the endonuclease activity of type I RM enzymes so as to protect the plasmid DNA in transmission, but do not affect the methylase activity which is crucial for maintaining the integrity of the plasmid and the host chromosome. The native Ocr form from phage T7 binds to RM enzymes, simultaneously inhibiting both the endonuclease and the methylase activity, and, therefore, interacts with the S-subunit. There is an obvious

reason for the Ocr activity being so high (*K*d = 10^{-10} M): in the course of infection, the phage DNA is immediately attacked by cellular endonucleases.

6. Pentapeptide repeat proteins (ppr proteins)

6.1 Inhibitors of DNA gyrase

Quinolones and also fluoroquinolones are synthetic derivatives of nalidixic acid; they belong to a group of antibiotics with wide spectrum of action and high activity and inhibit DNA gyrase. Quinolones bind to the gyrase–DNA complex. This results in stabilization of the covalent enzyme tyrosyl-DNA phosphate ester (a transient reaction intermediate) and causes death of bacteria. Quinolones have been successfully used for inactivation of *Mycobacterium tuberculosis* cells. During the first years of clinical use of quinolones, findings of *M. tuberculosis* strains resistant to quinolones were rather rare events. Studies of the nature of resistance to quinolones in the laboratory strains of *M. tuberculosis* and the related strain *M. smegmotis* have shown that this effect is determined by missense mutations (amino acid substitutions) in A-chain of DNA gyrase, or it represents the result of regulatory mutation potentiating expression of a protein pump responsible for the extracellular efflux of toxic compounds. However, the wide use of quinolones in medical practice resulted in the discovery of a new type of quinolone resistance. It was shown that the gene determining such type of resistance in *M. smegmotis* and *M. tuberculosis* encodes the MfpA protein, a specific inhibitor of DNA gyrase (Hegde et al., 2005; Montero et al., 2001). The MfpA proteins of *M. tuberculosis* and *M. smegmotis* consist of 183 and 192 residues correspondently; they share 67% identity. In 1998, the resistance to quinolones found in *Klebsiella pneumoniae* was shown to be encoded by the *qnr*A gene and transferred by the conjugated plasmid (Martinez-Martinez et al., 1998). Subsequent investigations have established that *qnr* genes have a worldwide distribution in a range of bacterial pathogens, mainly Gram-negative opportunist (particularly *Enterobacteriaceae*)(Robicsek et al., 2006). Sequence comparison of plasmids isolated from clinical Gram-negative strains differentiates five distinct *qnr* subfamilies *qnr*A, *qnr*B, *qnr*S (Jacoby et al., 2008), and most recently *qnr*C and *qnr*D (Wang et al., 2009; Cavaco et al., 2009). The proteins encoded by these genes exhibit the same function of DNA gyrase inhibition.

MfpA and QnrABCDS proteins belong to the pentapeptide repeat protein (PRP) family. Amino acid sequences of these proteins contain a repeated pentapeptide with the consensus [S, T, A, V][D, N][L, F][S, T, R][G]. MfpA consists of 183 amino acid residues and in these pentapeptides each second amino acid is D or N and each third amino acid is L or F. Table1 shows that MfpA protein consists of 30 pentapeptides, which determine characteristic features of its spatial structure. Figure 6a (taken from (Vetting et al., 2006) shows the spatial structure of the MfpA protein; it consists of a righ-handed β-helix, which corresponds to B-form DNA in size, shape, and electrostatics. In solutions, MfpA forms a dimer due to hydrophobic contact of several amino acids located at the C-end of an α-helical site. The monomeric MfpA consists of eight coils, and four repeated pentapeptides form four sides of a quadrant (1-4) (Table 5). Such spatial structure was named RHQBH (right-handed quadrilateral beta-helix) or "Rfr" (Repeated five-residues). The dimer $(MfpA)_2$ has a rod-like shape 100 angstrem in length and 27 angstrem in diameter. The total charge of the dimer is (–10), but the negative charges are distributed non-randomly. This results in $(MfpA)_2$ dimer, which mimicks a 30 bp segment of B-form duplex DNA. Docking analysis revealed the

existence of tight contact between $(MfpA)_2$ dimer and A_2 dimer of the DNA gyrase A subunit (Fig. 8) due to electrostatic complementation between strongly cationic "seat" of the A_2 dimer interface and a strongly anionic surface of the $(MfpA)_2$ dimer.

Structural analysis of the *Aeromonas hydrophila,* AhQnr protein is shown that it contain two prominent loops (1 and 2) that project from the PRP structure (Xiong et al., 2011). Deletion mutagenesis demonstrates that both contribute to the protection of *Escherichia coli* DNA gyrase from quinolones. A model for the Qnr:DNA gyrase interaction was suggested, where loop1 interacts with the gyrase A "tower" and loop2 with the gyrase B TOPRIM domains.

Structural similarity between MfpA and Qnr proteins and DNA duplex of the gyrase substrate determines the effectiveness of competitive inhibition of the gyrase; this represents the molecular basis of bacterial resistance to quinolone antibiotics. It should be noted that in contrast to gyrase inhibition by quinolones, the inhibition of gyrase by MfpA and Qnr proteins is not accompanied by cell chromosome degradation. Consequently, the presence of the genes *mfp*A or *qnr* in the bacterial genome is very important because the "fee" for the rescue from the inactivating effect of antibiotics is delayed development of the cell. It is possible that the main function of DNA mimic inhibitors of gyrase consists in modulation of DNA supercoiling, which may potentiate supercoiling at the stage of DNA replication and decrease the rate of supercoiling when the level of chromosome compactness becomes optimal in a particular cell.

6.2 Another PRP family proteins

The first protein of the PRP family was originally found in *Anabaena* cyanobacteria (Black et al., 1995). The HglK protein (encoded by the *hgl*K gene and consisting of 727 residues) contains a series of 36 tandem pentapeptides with the consensus sequence ADLSG. Using methods of bioinfor matics, a group of proteins belonging to PRP family has been identified in *Synechocystis cyanobacteria*; there are 15 proteins with series of tandem pentapeptide repeats varying from 13 to 44 (Bateman et al., 1998). By now the proteins of the PRP family have been found in almost all living organisms excluding yeasts. According to data analysis (Vetting et al., 2006), 525 proteins (484 prokaryotic and 41 eukaryotic) with the pentapeptide motif have been identified. Sequencing of the genome of the cyanobacterium *Cyanothece sp.* PCC 51142 revealed 35 pentapeptide- containing proteins. It was determined (Buchko et al., 2006a) the spatial structure of the Rfr32 protein, which consists of 167 residues. The authors demonstrated that the 21 tandem pentapeptide repeats (with the consensus motif A(N/D)LXX) fold into a right-handed quadrilateral β- helix, or Rfr-fold (as in the case of the MfpA protein); this structure imitates the rod-like structure of B-form DNA. The Rfr structure is also typical for another protein, Rfr23, encoded by a gene that has also been found in the genome of *Cyanothece sp.* PCC 51142 (Buchko et al., 2006b). The real functions of the pentapeptide-containing proteins found in cyanobacteria remain unknown. Some proteins determining immunity of bacteria to their own synthesized antibiotics also belong to the PRP family. These include the McbG protein (encoded by a *mcb*G gene located in the operon responsible for biosynthesis of microcin B17 (Pierrat & Maxwell, 2005) and the OxrA protein, which determines the resistance of *Bacillus megatherium* to oxetanocin A (Morita et al., 1999). In contrast to quinolones, microcin B17 interacts with B-subunit of DNA gyrase. A significant group of pentapeptide repeat family proteins has complex structure and contains

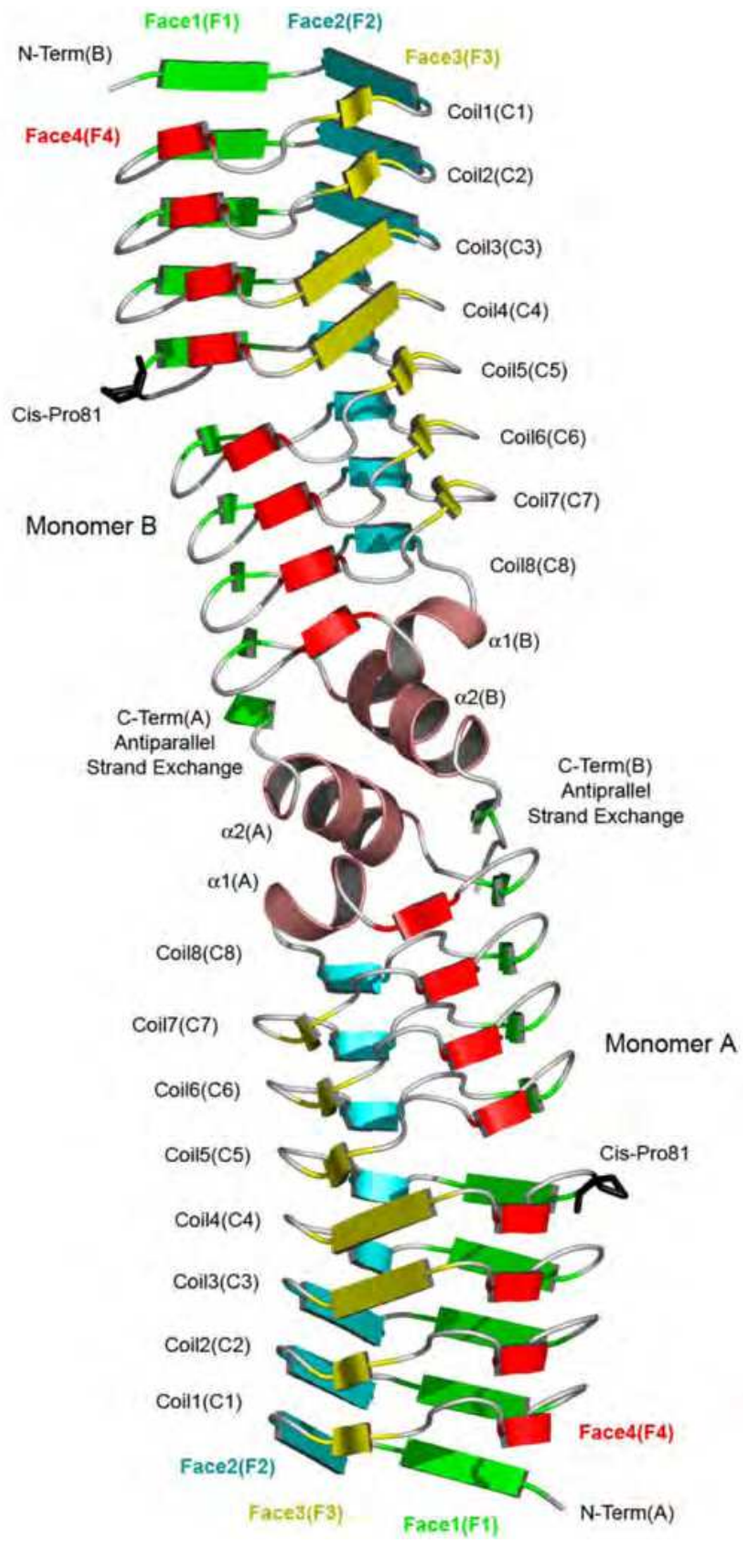

Fig. 8. Ribbon diagram of the *Mycobacterium tuberculosis* MfpA dimer. The four faced of the quadrilateral β-helix are colored green (face 1), blue (face 2), yellow (face 3) and red (face 4).

several domains, including those with catalytic functions. However, the functional role of the pentapeptide repeats in this group remains unknown. But if the putative catalytic function of such protein consists of posttranslational modification of some DNA-binding protein (e.g. histone acetylation), one can suggest that binding of the target protein to the pentapeptide domain would significantly increase selectivity of such a modification reaction.

Coil	Quadrant sides				Amino acid position in the protein chain
	1	2	3	4	
1	QQWVD	CEFTG	RDFRD	EDLSR	21
2	LHTER	AMFSE	CDFSG	VNLAE	41
3	SQHRG	SAFRN	CTFER	TTLWH	61
4	STFAQ	CSMLG	SVFVA	CRLRP	81
5	LTLDD	VDFTL	AVLGG	NDLRG	101
6	LNLTC	CRLRE	TSLVD	TDLRK	121
7	CVLRG	ADLSG	ARTTG	ARLDD	141
8	ADLRG	ATVDP	VLWRT	ASLVG	161
	ARVDV	*DQAVA*	*FAAAH*	*GLCLA*	181

Table 5. Position of pentapeptides along the axis of the MfpA protein molecule.

7. Application of new DNA mimetics

Since genes encoding DNA mimics (e.g. *ard*A and *qnr*ABCDS) are located on transmission elements, transposons, and plasmids, this promotes their wide distribution among bacteria of various species and genera.

Thus it is important to investigate in detail the structure of such proteins and the mechanisms of their action. The most illustrative example is the distribution of *qnr*ABCDS genes responsible for the resistance to quinolone antibiotics among clinical bacterial strains. The search for and analysis of genes encoding DNA mimics and representing constituents of transmission elements are important tasks. Below we consider some putative variants of use of DNA-mimicking proteins. The DNA mimics may be successfully used for substitution of DNA during elucidation of spatial structure of the DNA-dependent enzymes by means of X-ray analysis (Dryden, 2006). In some cases, it is difficult to obtain crystals of the complexes of the DNA dependent enzymes and DNA and it is possible that substitution of DNA by the DNA mimics may solve this problem. There are examples illustrating successful use of such substitutions: Ugi–UDG (Putnam &Tainer, 2005). It is suggested that substitution of DNA by the Ocr protein might be used for crystallization of Ocr in its complex with an S-subunit of EcoKI. Therefore, it should be noted that spatial structure of S-subunit of two type I restriction–modification enzymes has been determined (Kim et al., 2005; Calisto et al., 2005). The DNA mimics can be used in affinity chromatography. Affinity columns with a DNA mimics can be used with high effectiveness for detection and purification of various types of DNA-dependent enzymes. Use of radioactive or fluorescent labels will increase the

sensitivity of such method. The perspectives of *in vitro* construction of new types of DNA mimics (i.e. generation of proteins with different "design" and new functions) may be quite wide. These include potential tasks of constructing of DNA mimics, inhibiting or modulating activity of specific groups of DNA-dependent enzymes and tasks related to site directed changes in the structure of already known DNA mimics. Such works are rather successful. For example, using site-directed mutagenesis we have modified the structure of ArdA and Ocr; the modified proteins selectively inhibit the endonuclease (restriction) activity of type I restriction–modification enzymes without any influence on their methylase (modification) activity (Zavilgelsky et al., 2011). The use of such type of antirestriction proteins in gene engineering gives an opportunity to develop stable strains with hybrid plasmids because the process of specific modification of chromosome DNA remains unimpaired. The protein Ocr has already been used as an effective factor promoting significant increase of bacterial transformation by plasmids. Adding a small amount of the Ocr protein to solution with plasmid DNA causes significant (by several orders of magnitude) increase in effectiveness of cell transformation during electroporation. In this case unmethylated DNA and host bacteria with active type I restriction–modification system are used. The Ocr protein (as well as plasmid DNA) easily penetrates inside cells and immediately protects unmethylated DNA against degradation (EPICENTRE Forum 9, 8, htpp//www.epibio.com/forum.asp).

The highly charged, very acidic proteins Ocr and ArdA may be used as a purification handle similar to other fusion tags. A monomeric mutant of the Ocr protein (13.8 kDa, very acidic, pI = 3.8) was used as a novel fusion tag whith displays solubilizing activity with a variety of different passenger proteins (DelProposto et al., 2009).

In general, perspectives of the use of the DNA mimics might be related diagnostics and therapy of various diseases (e.g. for inhibition of specific enzymes and corresponding biochemical processes in cells).

8. Conclusion

Modern data on the mechanisms of the modulation of the DNA - binding enzymes by protein mimicry of DNA are reviewed. It has recently been demonstrated that DNA-binding enzymes can be controlled by the direct binding of a control protein to the DNA-binding site on the enzyme. The structures of these control proteins have been discovered to mimic the structure and electrostatics of DNA. Such DNA-mimics might be able to target bacterial restriction systems (Ocr, ArdA), drug resistance systems (MfpA, QnrABCS), as well as replication, recombination, and repair. It puts forward a range of potential uses of new DNA mimics in applied biotechnology.

Figure 9 shows structures of Ocr and MfpA monomers and B-form DNA. Their comparison emphasizes the extraordinary capacities of living nature to develop unique forms crucial for adaptation. The most surprising thing is that nature has chosen different ways for design of proteins mimicking the DNA duplex. In one case (e.g. Ocr) these are tightly packed α-helices, in the other it is a right-handed β-helix (MfpA). Existence of significant negative charge (of the whole macromolecule or particular domain) required for similarity with the DNA polyanion is a common feature of DNA mimics. However, this is a necessary but not sufficient precondition. At the moment the only reliable method for detection of DNA mimics is X-ray analysis.

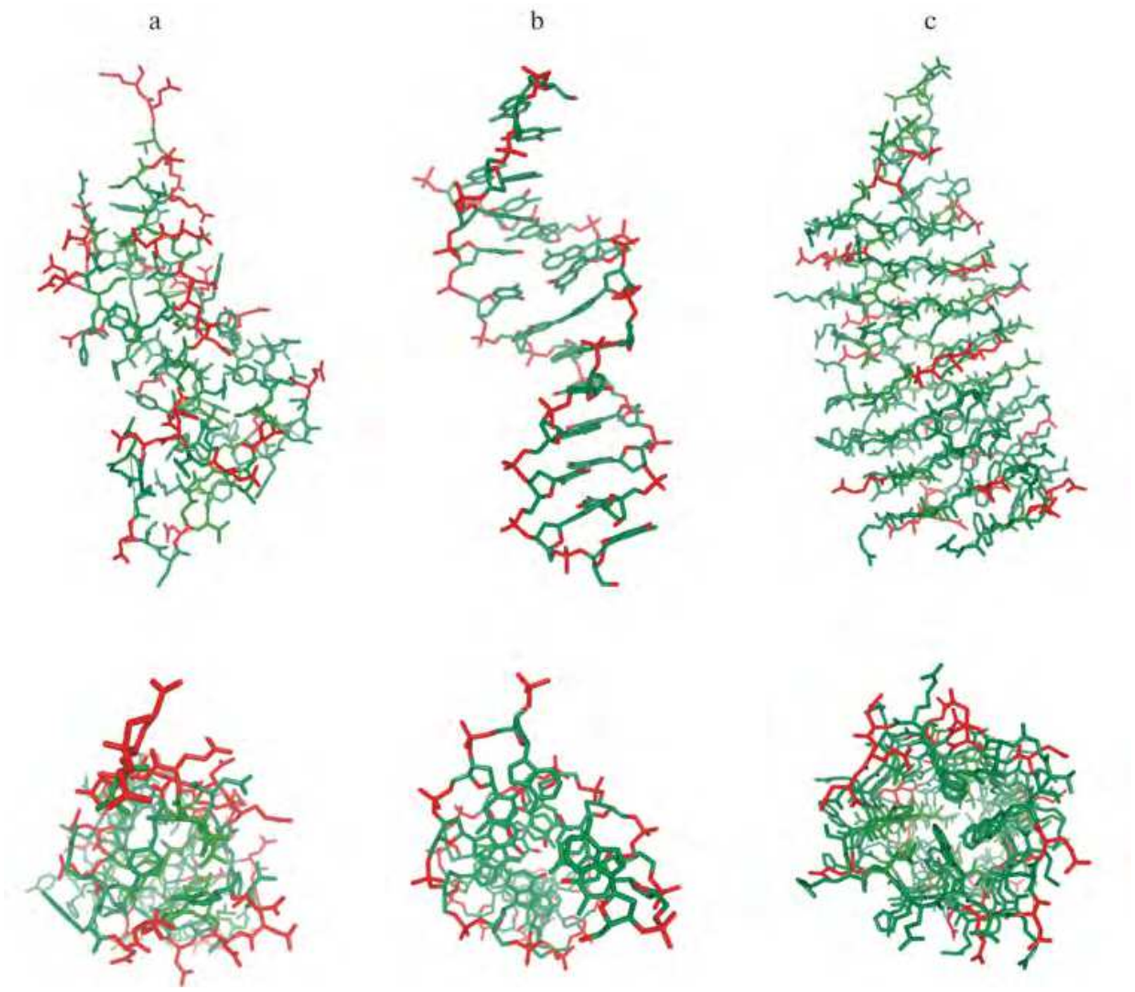

Fig. 9. The structures of Ocr(a) and MfpA(c) monomers and B-form of DNA(b)

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Innovations in Biotechnology

Edited by Dr. Eddy C. Agbo

ISBN 978-953-51-0096-6
Hard cover, 474 pages
Publisher InTech
Published online 17, February, 2012
Published in print edition February, 2012

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