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Comparative Analysis of Anti-restriction Activities of ArdA (ColIb-P9) and Ocr (T7) Proteins

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Abstract—Anti-restriction proteins ArdA and Ocr are specific inhibitors of type I restriction–modification enzymes. The IncI1 transmissible plasmid ColIb-P9 *ard*A and bacteriophage T7 *0.3*(*ocr*) genes were cloned in pUC18 vector. Both ArdA (ColIb-P9) and Ocr (T7) proteins inhibit both restriction and modification activities of the type I restriction–modification enzyme (*Eco*KI) in *Escherichia coli* K12 cells. ColIb-P9 *ard*A, T7 *0.3*(*ocr*), and the *Photorhabdus luminescens lux*CDABE genes were cloned in pZ-series vectors with the $P_{ltetO-1}$ promoter, which is tightly repressible by the TetR repressor. Controlling the expression of the *lux*-genes encoding bacterial luciferase demonstrates that the $P_{ltetO-1}$ promoter can be regulated over an up to 5000-fold range by supplying anhydrotetracycline to the *E. coli* MG1655Z1 *tet*R^+ cells. Effectiveness of the anti-restriction activity of the ArdA and Ocr proteins depended on the intracellular concentration. It is shown that the dissociation constants K_d for ArdA and Ocr proteins with *Eco*KI enzyme differ 1700-fold: K_d (Ocr) = 10^{-10} M, K_d (ArdA) = $1.7 \cdot 10^{-7}$ M.

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The *ard* (*a*lleviation of *r*estriction of *D*NA) genes are responsible for synthesis of small (140-180 amino acid residues) acidic proteins (the net charge of a molecule is from −10 to −30), which are specific inhibitors of type I restriction endonucleases [1-3]. The *ard* genes are localized in a leading area of conjugative plasmids and are among the first enter into the recipient cell upon conjugative DNA transfer [4, 5]. The *ard* genes play an important role in DNA transfer between bacterial cells of different species and genera: they assist plasmids to overcome restriction barriers. The bacteriophage T7 genome contains the gene *0.3*(*ocr*) encoding the anti-restriction protein Ocr, which is not homologous to the Ard family proteins. It is significantly shorter (116 amino acid residues), but, like the Ard proteins, has a large negative charge [6].

Anti-restriction proteins belong to the DNA mimic protein family (protein mimicry of DNA), whose spatial structure imitates the B-structure of double-stranded DNA [7, 8]. This protein mimicry allows an anti-restriction protein to compete with DNA for the binding site of a restriction–modification enzyme and thus to inhibit DNA degradation (restriction) and methylation (modification). From the position of classic enzymatic catalysis, the anti-restriction mechanism is an example of competitive inhibition by an inhibitor molecule due to a structural likeness with the natural substrate.

The ArdA and Ocr proteins are similar in their functional activity, because they specifically inhibit only enzymes of ATP-dependent restriction–modification system type I. However, since the life cycles of conjugative plasmids (symbiosis with bacterial cell) and bacteriophages (infection and lysis of bacteria) are significantly different, it is of interest to compare the inhibitory activities of these proteins.

To perform this task, we have cloned the *ard*A and *0.3*(*ocr*) genes under the strictly controlled promoter and measured the anti-restriction activities of their products depending on the protein inhibitor concentration in the bacterial cell.

MATERIALS AND METHODS

Bacterial strains, bacteriophages, and plasmids. The *Escherichia coli* K12 MG1655 Z1 strain used in this work contains in its chromosome the gene encoding TetR, a

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protein repressor of P_{tetA} promoter. This strain was prepared by transduction using phage P1; the strain *E. coli* DH5αZ1 *tet*R, in which in the genome the gene *tet*R is tightly (90% co-transduction) linked with a gene determining the resistance of the bacterium to spectinomycin, was used as a donor.

The following *E. coli* K-12 strains were also used in the study: JM109 *rec*A1 *end*A1 *gyr*A96 *thi sup*E44 *rel*A1 *hsd*R17 Δ*lac-pro*AB) [F′*tra*D36 *pro*AB *lac*I^{q}Z ΔM15]; TG-1 *thi rel*A *sup*E44 *hsd*R17 *hsd*M Δ*lac-pro*AB) [F′*tra*D36 *pro*AB *lac*I^{q}Z ΔM15]; AB1157 F$^-$ *thr-1*, *leu-6*, *pro*A2, *his-4*, *thi-1*, *arg*E3, *lac*Y1, *gal*K2, *ara*14, *xyl-5*, *mtl-1*, *tsx*-33, *rpsL*31, *sup*E44, $r^+ m^+$; as well as *E. coli* C_s $r_o m_o$ (prototroph). All the strains were obtained from the collection of the State Research Institute of Genetics.

Bacteriophages P1 and T7 were obtained from the collection of the State Research Institute of Genetics. Bacteriophage λ_{vir} was kindly provided by Prof. R. Devoret, France. The unmodified phages λ.0 and modified phages λ.K, grown in *E. coli* C_s (or TG-1) and *E. coli* K12 AB1157, respectively, were used in the study.

The pZ series vectors with replicons ColE1 (pZE21 Kn^r) and pSC101 (pZS33 Cm^r) were used as vectors containing a strictly regulated promoter: the copy numbers were 60-70 and 8-10 per cell, respectively [9]. A DNA fragment containing $P_{ltetO-1}$ as a strictly regulated promoter-operator area was inserted into these vectors.

The $P_{ltetO-1}$ promoter is composed of a promoter part P_l from the phage λ genome and an operator part *tetO2* from the regulator area of the tetracycline resistance operon of the transposon Tn10 [9]. The vectors pZE21 Kn^r and pZS33 Cm^r also contain the Shine–Dalgarno sequence (RBS) and a polylinker, which serves for insertion of the DNA fragment carrying the studied gene.

Vectors of the pUC series were also used in the study. Multicopy plasmids were isolated by the alkaline method according to [10].

Media, enzymes, and chemicals. Bacteria were grown on L-broth and L-agar. The following antibiotic concentrations in the media were used: ampicillin (100 μg/ml), kanamycin (40 μg/ml), and chloramphenicol (15 μg/ml). Cleavage and ligation were conducted using enzymes purchased from Fermentas (Lithuania). *Escherichia coli* cells were transformed using the calcium method according to [10]. Anhydrotetracycline (Sigma, USA) was used as inducer of gene expression in the strain *E. coli* MG1655Z1 with hybrid pZ series plasmids.

Plasmid design. The plasmid pVK6 with inserted gene ColIb-P9 *ard*A was used as a source of the *ard*A gene [11]. Bacteriophage T7 DNA was used as a source of the *0.3*(*ocr*) gene. Cleavage with endonucleases, ligation of DNA fragments, electrophoresis in agarose gel, and isolation of DNA fragments from the agarose gel were performed as described in [10]. The following oligonucleotides were used as primers for cloning of the ColIb-P9 *ard*A gene with following amplification of PCR products:

*Bam*HI
ardA-N_{dir}, 5′-CTGGGATCCGGGAATGTCTGTTGTTGC-3′;

*Pst*I
ardA-N_{rev}, 5′-CATTCTGCAGCGGGGGAGTAAATCACCG-3′.

The following ocr-N_{dir} and ocr-N_{rev} oligonucleotides were used as primers for cloning of the T7 *0.3*(*ocr*) gene:

*Bam*HI
ocr-N_{dir}, 5′-TTATGGGATCCACTAATAACTGCAC-3′;

*Hind*III
ocr-N_{rev}, 5′-CCGTATTGAAGCTTGGTAGTAGAC-3′.

In the second step, *ard*A and *0.3*(*ocr*) were recloned into vectors pZE21 and pZS33 under the stringent control of the $P_{ltetO-1}$ promoter. The DNA fragments were inserted into the vectors at the *Kpn*I and *Hind*III sites.

The pXen7 plasmid was used as the source of *lux*-genes, which are contained the *Photorhabdus luminescens* ZM1 *lux*CDABE genes [12]. The DNA fragment carrying these genes was inserted into vectors pZE21 and pZS33 at the *Kpn*I-*Hind*III sites.

Measurements of anti-restriction and anti-modifying activities of ArdA and Ocr proteins. The strains *E. coli* K-12 AB1157 and JM109 (with hybrid plasmids based on the pUC18 vector) and MG1655Z1 (with hybrid plasmids based on the pZ series vectors) were used for measurements of anti-restriction and anti-modifying activities of ArdA and Ocr proteins. The cells of AB1157 and MG1655Z1 strains contain an active restriction–modification type I system *Eco*KI (R_2M_2S), whereas the cells of JM109 strain contain a methylase form M_2S.

The protocol for measurements of anti-restriction and anti-modifying activities of Ard family proteins is described in [4, 11].

Measurement of bioluminescence intensity. Bioluminescence intensity in cell suspensions was measured using a luminometer composed of a FEU-85 photomultiplier and B2-15 microvoltmeter. The luminescence was measured at room temperature in cells with suspension volume of 200 μl.

RESULTS AND DISCUSSION

Designing of hybrid plasmids. In the first stage, the ColIb-P9 *ard*A and T7 *0.3*(*ocr*) genes were cloned in pUC18 vector at *Bam*HI-*Pst*I sites. A DNA fragment of approximately 500 bp containing the ColIb-P9 *ard*A gene was produced by PCR using ardA-N_{dir} and ardA-N_{rev} primers; pVK6 plasmid was used as the template. A DNA fragment of approximately 400 bp containing the *0.3*(*ocr*)

gene was produced by PCR as well, using ocr-N_{dir} and ocr-N_{rev} primers, T7 phage DNA being used as the template. The resulting pSR5 and pSR8 plasmids were constructed containing ColIb-P9 *ard*A and T7 *0.3*(*ocr*) genes, respectively, under the P_{lac} promoter.

In the second stage, the *ard*A and *0.3*(*ocr*) genes were recloned in pZE21 and pZS33 vectors under the control of strictly regulated $P_{ltetO-1}$ promoter. DNA fragments were incorporated into the vectors at *Kpn*I-*Hind*III sites.

Escherichia coli MG1655Z1 cells were transformed by the constructed hybrid plasmids pZE21-ardA, pZS33-ardA, pZE21-ocr, and pZS33-ocr. $P_{ltetO-1}$ promoter transcription was induced by a derivate of tetracycline, anhydrotetracycline; both substances interact with protein-repressor TetR and inactivate its repressor activity. However, anhydrotetracycline causes decreased cell lethality compared to tetracycline, and, in addition, it displays several orders of magnitude higher binding constant with the protein-repressor: the effect of promoter opening (transcription initiation and, respectively, gene product synthesis) can be observed at anhydrotetracycline concentrations below 0.2 ng/ml.

For quantitative estimation of the intracellular concentrations of anti-restriction proteins, we developed a bioluminescence method using *P. luminescens lux*CDABE reporter genes encoding luciferase (*lux*AB genes) and reductase (*lux*CDE genes) synthesizing tetradecanal, the substrate for the luciferase reaction.

A DNA fragment containing *P. luminescens lux*CDABE genes was incorporated into pZE21 and pZS33 vectors under $P_{ltetO-1}$ promoter. As a result, two hybrid plasmids pZE-lux and pZS-lux were constructed.

Measurement of anti-restriction and anti-modification activities of ArdA and Ocr proteins by cloning of the respective genes in pUC18 vector under Plac promoter. *Escherichia coli* K-12 AB1157 r^+m^+ strain with an active system of restriction–modification type I *Eco*KI (R_2M_2S) was used for measurement of anti-restriction activity of the ArdA and Ocr proteins, whose genes were cloned in the pUC18 vector. The results of this experiment are presented in Table 1. Since the efficacy of unmodified phage λ.0 inoculation (the phage DNA contains five *Eco*KI-sites) on the AB1157 strain with hybrid plasmids pSR5 and pSR8 is equal to that on the control strain TG-1 r^-m^-, which does not contain *Eco*KI enzyme, we conclude that virtually total inhibition of restriction (endonuclease) activity of *Eco*KI enzyme takes place for the AB1157 strain containing hybrid plasmids both with *ard*A ColIb-P9 and *0.3*(*ocr*) T7 genes (Table 1).

Escherichia coli K12 JM109 r^-m^+ strain carrying the mutated *hsd*R gene and *Eco*KI enzyme in the form of M_2S possessing methyltransferase activity was used for the measurement of anti-modification activity of the ArdA and Ocr proteins. The anti-modification activity was estimated by the following method.

Table 1. Effect of ColIb-P9 ArdA and T7 Ocr proteins on *Eco*KI-restriction in *Escherichia coli* K12 AB1157 r^+m^+ cells*

Plasmid	Protein	Restriction coefficient (*K*) of phage λ.0 on AB1157**	Decrease factor (*R*) for *Eco*KI-restriction***
pUC18	absent	$4.0 \cdot 10^{-4}$	1
pSR5	ArdA ColIb-P9	1	2500
pSR8	Ocr T7	1	2500

* ColIb-P9 *ard*A and T7 *0.3*(*ocr*) genes were cloned in the pUC18 vector under the Plac promoter. Mean data of five independent experiments are given.

** Restriction coefficient (*K*) is equal to the ratio of the phage λ titer on AB1157 r^+m^+ to the titer of phage λ.0 on TG-1 r^-m^-.

*** The restriction decrease factor $R = K^+/K^-$ (K^- was determined on AB1157 without plasmid, K^+ – on AB1157 with the plasmid).

Table 2. Effect of ColIb-P9 ArdA and T7 Ocr proteins on *Eco*KI-modification in *Escherichia coli* K12 JM109 r^-m^+ cells*

Plasmid	Protein	Restriction coefficient (*K*) of phage $\lambda.0_{jm109}$ on TG-1 r^-m^- strain	Restriction coefficient (*K*) of phage $\lambda.0_{jm109}$ on AB1157 r^+m^+ strain
pUC18	absent	1	1
pSR5	ArdA ColIb-P9	1	$4.0 \cdot 10^{-4}$
pSR8	Ocr T7	1	$4.0 \cdot 10^{-4}$

* *Escherichia coli* JM109 r^-m^+ cells were infected by the phage λ.0 and, after a single multiplication cycle, the resulting phagolysate (marked as $\lambda.0_{jm109}$ in the table) was titrated on TG-1 and AB1157 strains. Mean data of five independent experiments are given in the table.

Escherichia coli JM109 r^-m^+ strain containing hybrid plasmid with *ard*A or *0.3*(*ocr*) gene was inoculated with the phage λ.0 (the vector deficient in these genes was used for the control), and, after the phage was yielded in one multiplication cycle, the phage was used for inoculation on TG-1 r^-m^- strains (to estimate the phage titer) and on AB1157 r^+m^+ (to estimate the phage DNA modification extent). The results of this experiment are given in Table 2. The table shows that when *ard*A and *0.3*(*ocr*) are absent from the cells of JM109 strain (control, upper row of Table 2), the phage DNA is totally modified, and hence the phage has equal inoculation efficacy on TG-1 and AB1157 strains. However, when JM109 cells bear the plasmid with ColIb-P9 *ard*A gene or T7 *0.3*(*ocr*) gene

(the second and third rows of Table 2), the ArdA and Ocr proteins inhibit the modifying activity of *Eco*KI enzyme; as a result, the phage DNA is unmodified, and, hence, the efficacy of inoculation of this phage on AB1157 is almost four orders of magnitude lower than on TG-1. Hence, the modifying (methylase) activity of *Eco*KI enzyme causes virtually complete inhibition in the JM109 bacteria containing hybrid plasmids with ColIb-P9 *ard*A gene or T7 *0.3*(*ocr*) gene (Table 2).

Measurement of anti-restriction activity of ArdA and Ocr proteins by cloning of corresponding genes in pZ series vectors with strictly regulated promoter $P_{ltetO-1}$. The anti-restriction activity in ArdA and Ocr was measured by determining phage λ.0 inoculation efficacy on MG1655Z1 cells containing hybrid plasmid with the *ard*A or *0.3*(*ocr*) gene. Since the genome of MG1655Z1 strain contains *hsd*RMS genes encoding restriction–modification type I enzyme *Eco*KI, the efficacy of the phage λ.0 inoculation is approximately four orders of magnitude lower than that on the control TG-1 strain devoid of restriction–modification type I system (Table 3, lower row). However, when the cells of MG1655Z1 strain contain a plasmid with cloned *ard*A or 0.*3*(*ocr*) gene, the efficacy of the phage λ inoculation changes depending on the anhydrotetracycline inducer concentration and, correspondingly, on the amount of the synthesized anti-restriction protein.

Table 3. Anti-restriction activity of T7 Ocr protein depending on the concentration of anhydrotetracycline in *Escherichia coli* K12 MG1655Z1 r^+m^+ cells*

Anhydrotetracycline concentration, ng/ml	Restriction coefficient (*K*) of phage λ.0 on MG1655Z1**	Decrease factor (*R*) for *Eco*KI-restriction***
0.0 (pZS33 vector)	$5.0 \cdot 10^{-4}$	2000
20.0 (– « –)	1	5000
0.0 (pZE21 vector)	1	5000
0.2 (– « –)	1	5000
0.5 (– « –)	1	5000
1.0 (– « –)	1	5000
2.0 (– « –)	1	5000
5.0 (– « –)	1	5000
10.0 (– « –)	1	5000
20.0 (– « –)	1	5000
Control (the strain without plasmid)	$2.0 \cdot 10^{-4}$	1

* Gene *0.3*(*ocr*) is cloned in pZS33 and pZE21 vectors under $P_{ltetO-1}$ promoter. The mean data of five independent experiments are given.
** Restriction coefficient (*K*) is equal to the ratio of phage λ.0 titer on MG1655Z1 r^+m^+ to the phage λ.0 titer on TG-1 r^-m^-.
*** The restriction decrease factor $R = K^+/K^-$ (K^- was determined on MG1655Z1 without plasmid, K^+ – on MG1655Z1 with the plasmid).

Table 4. Anti-restriction activity of ColIb-P9 ArdA protein depending on the concentration of anhydrotetracycline in *Escherichia coli* K12 MG1655Z1 r^+m^+ cells*

Anhydrotetracycline concentration, ng/ml	Restriction coefficient (*K*) of phage λ.0 on MG1655Z1**	Decrease factor (*R*) for *Eco*KI-restriction***
0.0	$2.0 \cdot 10^{-3}$	10
0.2	$3.0 \cdot 10^{-3}$	15
0.5	$4.0 \cdot 10^{-3}$	20
1.0	$2.0 \cdot 10^{-2}$	100
2.0	$1.0 \cdot 10^{-1}$	500
5.0	$2.5 \cdot 10^{-1}$	1250
10.0	1	5000
20.0	1	5000
Control (strain without plasmid)	$2.0 \cdot 10^{-4}$	1

* ColIb-P9 *ard*A was cloned in pZE21 vector under $P_{ltetO-1}$ promoter. The mean data of five independent experiments are given.
** Restriction coefficient (*K*) is equal to the ratio of phage λ.0 titer on MG1655Z1 r^+m^+ to the phage λ.0 titer on TG-1 r^-m^-.
*** The restriction decrease factor $R = K^+/K^-$ (K^- was determined on MG1655Z1 without plasmid, K^+ – on MG1655Z1 with plasmid).

As a result, the following dependences of anti-restriction activity of Ocr and ArdA proteins on the anhydrotetracycline concentration were obtained (Tables 3 and 4). Virtually complete inhibition of the *Eco*KI restriction–modification system over a broad concentration range of the inducer takes place for the Ocr T7 protein (Table 3). About 2.5-fold decrease in the inhibition efficacy was observed when only the *0.3*(*ocr*) gene was cloned in the low-copy vector pZS33 and in absence of anhydrotetracycline (Table 3, upper row).

The experimental data for ColIb-P9 ArdA protein is given in Table 4. The decrease in the inhibition efficacy of restriction activity of *Eco*KI enzymes is exhibited already at anhydrotetracycline concentration about 5 ng/ml (when *ard*A gene is cloned in the multicopy vector pZE21).

Effect of anhydrotetracycline on bioluminescence of MG1655Z1 cells containing hybrid pZ-series plasmids with *P. luminescens lux*CDABE genes. We used the bioluminescence method for quantitative estimation of relatively or very low intracellular concentrations of the synthesized proteins. The bioluminescence intensity in suspensions of MG1655Z1 bacterial cells containing hybrid plasmids of pZ series with cloned *lux*CDABE genes is directly proportional to intracellular concentration of the synthesized luciferase over a broad range (more than six orders of magnitude). The high sensitivity of the biolumi-

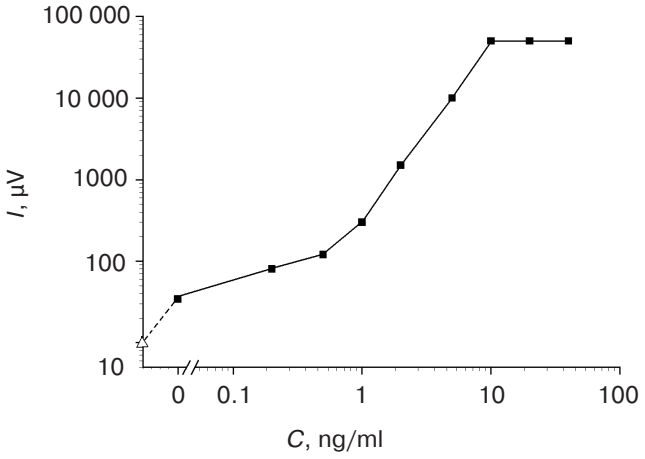

Fig. 1. Effect of anhydrotetracycline on the bioluminescence intensity in *Escherichia coli* K12 MG1655Z1 cells containing the hybrid pZE-lux plasmid. The bioluminescence intensity, I (μV), is given on the ordinate axis; the anhydrotetracycline concentration (in ng/ml) is given on the abscissa axis. The triangle on the ordinate axis marks the bioluminescence intensity of MG1655Z1 cells containing the low-copy pZS-lux plasmid (in the absence of inducer).

nescence method enables evaluation of extremely low intracellular concentrations of the enzyme. The results obtained after *lux*CDABE gene cloning in pZE21 and pZS33 vectors with strictly regulated $P_{ltetO-1}$ promoter are given in Fig. 1.

The figure shows that the intensity of bioluminescence in *E. coli* MG1655Z1 cells with hybrid plasmids containing *lux*-genes varies from 10 (in absence of anhydrotetracycline) to 50,000 (20 ng/ml and more anhydrotetracycline under the condition of complete promoter opening), i.e. approximately 5000 times. Correspondingly, the intracellular amount of the luciferase enzyme increases 5000 times. The resulting calibration curve was obtained determining the amount of luciferase protein in the cell depending on the inducer concentration (in the range from 0.1 to 40 ng/ml). It is reasonable to suppose that the relative amount of the protein varies in the same ranges as luciferase does, when *ard*A and *ocr* genes are cloned in pZE21 and pZS33 plasmids under the same conditions.

Determination of relative values of dissociation constants for ArdA and Ocr proteins. The presented results enabled evaluation of the relative difference in dissociation constants characterizing efficacy of interaction between the anti-restriction ArdA and Ocr proteins and *Eco*KI enzyme.

The dissociation constant characterizing the inhibitor–enzyme complex formation is equal:

$$K_d = [E][I]/[EI],$$

where [E] is the free enzyme concentration, [I] is inhibitor concentration, and [EI] is the concentration of the enzyme–inhibitor complex. The K_d value is equal to the inhibitor concentration at which the amounts of free enzyme and inhibitor-bound enzyme are equal.

In the given case, this condition is met when the *Eco*KI enzyme inhibition efficacy is two-fold decreased in the cell. As this takes place, half of the enzyme pool is free, and the other half is inhibited because it forms complexes with the inhibitor molecules.

The dependence of anti-restriction activity of Ocr and ArdA proteins on their intracellular concentration (determined in arbitrary units according to the calibration curve, Fig. 1) is presented in Fig. 2. The figure shows that 50% decrease in the *Eco*KI inhibition efficacy by Ocr protein is observed when its intracellular concentration is about 1700-fold lower than that of ArdA.

The data presented in Fig. 2 a precise determination of the relative difference in dissociation constants for ArdA and Ocr proteins. Additional data is necessary to determine absolute dissociation constants. However, the dissociation constant for Ocr protein and *Eco*KI enzyme was determined in absolute units according to experiments *in vitro*: $K_d = 10^{-10}$ M [13]. Hence, for ArdA protein $K_d = 1.7 \cdot 10^{-7}$ M. Taking into account that the number of protein molecules synthesized under open promoter is approximately 30,000-40,000 per cell, the synthesis of 30-40 Ocr molecules is enough for complete inhibition of intracellular restriction–modification type I enzymes under infection by bacteriophage T7. When it is considered that a cell contains approximately 100 *Eco*KI mole-

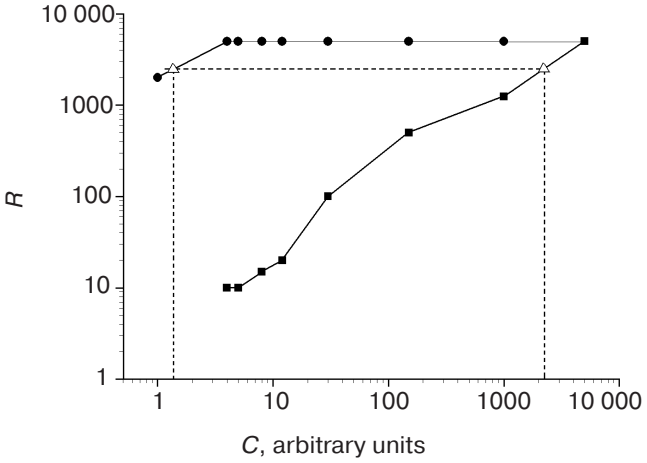

Fig. 2. Anti-restriction activity of ColIb-P9 ArdA and T7 Ocr proteins depending on their intracellular concentration. The *Eco*KI-restriction decrease factor R is given on the ordinate axis, and the intracellular concentration of anti-restriction protein is given on the abscissa axis (according to the calibration curve, Fig. 1, in arbitrary units). Minimal elevation of bioluminescence intensity of the cells over background (10 μV) observed in the variant with pZS-lux plasmid in absence of anhydrotetracycline inducer is taken as one unit. Circles designate T7 Ocr, squares designate ColIb-P9 ArdA, and triangles mark the points on the curves corresponding to the dissociation constants.

cules [14], in the light of the data obtained in our work, we conclude that only part of *Eco*KI molecules directly participates in degradation of unmodified phage λ DNA (because all the data on the ArdA- and Ocr-mediated protection from *Eco*KI-induced degradation are obtained using the non-methylated phage DNA in our experiments).

The 1700-fold decrease in Ocr K_d in comparison with ArdA K_d is apparently due to the necessity of rapid and effective inactivation of cellular restriction–modification systems, because the process of phage DNA injection into the cell takes only a few minutes, a time insufficient for synthesis of a substantial amount of protein. Another pattern takes place upon the transfer of transmissive plasmid from donor cell to recipient cell. First, plasmid DNA enters the recipient cell as single strand (DNA replication occurs according the model of "rolling circle replication") resistant to cellular restrictases, and, the second, transcription of *ard*A genes, is realized via special "single-stranded" promoters providing the synthesis of a sufficient amount of ArdA protein during the conjugation transfer.

It should be noted that ArdA and Ocr proteins, although virtually identical in their functional activity (they inhibit exclusively restriction–modification enzymes of type I), are not homologs. A characteristic feature of both proteins is a high net negative charge (from −25 to −30), providing their mimetic property of double-stranded DNA. However, the composition and distribution of non-polar amino acids are substantially different. So, ArdA protein (166 a.a.) contains fifteen F, seven W, and only one I, whereas Ocr protein (116 a.a.) contains nine I, three F, and only one W. This suggests that the presence and distribution of non-polar amino acid residues determine the specificity of the anti-restriction proteins and the efficacy of interaction with S-subunit of type I restriction–modification enzymes via hydrophobic contacts.

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