

# Plasmid-encoded Antirestriction Protein ArdA Can Discriminate between Type I Methyltransferase and Complete Restriction–Modification System

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Many promiscuous plasmids encode the antirestriction proteins ArdA (alleviation of restriction of DNA) that specifically affect the restriction activity of heterooligomeric type I restriction-modification (R-M) systems in *Escherichia coli* cells. In addition, a lot of the putative *ardA* genes encoded by plasmids and bacterial chromosomes are found as a result of sequencing of complete genomic sequences, suggesting that ArdA proteins and type I R-M systems that seem to be widespread among bacteria may be involved in the regulation of gene transfer among bacterial genomes. Here, the mechanism of antirestriction action of ArdA encoded by IncI plasmid Collb-P9 has been investigated in comparison with that of well-studied T7 phage-encoded antirestriction protein Ocr using the mutational analysis, retardation assay and His-tag affinity chromatography. Like Ocr, ArdA protein was shown to be able to efficiently interact with EcoKI R-M complex and affect its *in vivo* and *in vitro* restriction activity by preventing its interaction with specific DNA. However, unlike Ocr, ArdA protein has a low binding affinity to EcoKI Mtase and the additional C-terminal tail region (VF-motif) is needed for ArdA to efficiently interact with the type I R-M enzymes. It seems likely that this ArdA feature is a basis for its ability to discriminate between activities of EcoKI Mtase (modification) and complete R-M system (restriction) which may interact with unmodified DNA in the cells independently. These findings suggest that ArdA may provide a very effective and delicate control for the restriction and modification activities of type I systems and its ability to discriminate against DNA restriction in favour of the specific modification of DNA may give some advantage for efficient transmission of the *ardA*-encoding promiscuous plasmids among different bacterial populations.

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## Introduction

Bacterial plasmids are typically transmissible between cells by the process of bacterial conjugation and may play an important role in the dissemination of a variety of genes including those practically

significant (for instance, antibiotic resistance and virulence determinants) among different bacterial populations.<sup>1,2</sup> It seems likely that some of them may be involved in the process of modification of bacterial genome and function as accessory mini-chromosomes. In addition, the functionally essential genes may be acquired by the host chromosome from these “visitors” as a result of natural genetic engineering processes and *vice versa*. These mechanisms permit gene flux among virtually all eubacteria, and even among the domains of life, i.e. between Bacteria and Eucarya (yeast, plant and even mammalian cells).<sup>3–5</sup> It seems possible, therefore, that the prokaryotic community may be viewed as a

Abbreviations used: R-M, restriction and modification; Mtase, methyltransferase; IPTG, isopropyl- $\beta$ , D-thiogalactopyranoside.

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single, heterogenetic multicellular organism containing replicons that are in continuous movement from one group of cells to another.<sup>6,7</sup> However, DNA transfer mediated by these natural vectors can be essentially limited by a host “immigration control” system such as restriction endonucleases that recognize the “molecular passport”, the methylation pattern of guest DNA, and seem to be widespread among eubacteria and even Archaea.<sup>8–11</sup> Most of these restriction and modification (R-M) systems are traditionally divided into four classes, designated types I, II, III and IV, on the basis of enzyme subunit composition, co-factor requirements, DNA specificity characteristics and reaction products. However, among these R-M systems, the type I enzymes seem to be most sophisticated and adapted to play the pivotal role in the gene flow control. The type I R-M systems are hetero-oligomeric complexes that commonly contain one HsdS, two HsdM and two HsdR subunits conferring DNA sequence specificity (S), modification (M) and restriction endonuclease (R) activities, respectively. Unlike type II and III R-M systems, type I enzymes are able not only to restrict the foreign DNA transfer into bacterial cells but provide the possibility for any gene acquisition by a recipient genome as a result of their ability to cleave DNA at variable positions remote from their recognition sites.<sup>12,13</sup> Besides, it seems likely that the type I restriction barrier to the

acquisition of foreign DNA is inconstant and may be modulated.<sup>8,11</sup> Some phages were shown to specify the antirestriction functions that interfere with the type I restriction and improve the chance of phage survival in the restriction-proficient host.<sup>8,14–16</sup>

To overcome the host restriction barrier, many self-transmissible plasmids encode antirestriction proteins Ard (alleviation of restriction of DNA) that specifically repress the type I restriction in cells.<sup>17–24</sup> All antirestriction proteins (Ard) studied are encoded by self-transmissible IncI, IncFV, IncN and IncW plasmids and fall into three distinct groups, ArdA, ArdB and ArdC, on the basis of amino acid sequence homology.<sup>23–25</sup> In addition, a lot of the putative *ardA* genes encoded by plasmids and bacterial chromosomes are found as a result of sequencing of complete genomic sequences. ArdA proteins seem to be prevalent among Ard proteins and the Pfam database<sup>26</sup> contains about 50 ArdA-like sequences today, some of which are presented in Figure 1. Antirestriction proteins belonging to the ArdA family are strongly acidic<sup>24,25</sup> and look like, therefore, a well-studied acidic antirestriction Ocr (0.3 gene) protein of T7 bacteriophage that mimics DNA and inhibits type I R-M systems by direct binding to the enzymes.<sup>28–33</sup> Comparison of the amino acid sequences of all the known Ard proteins and Ocr that has no considerable sequence similarity with Ard proteins revealed only one small region of



**Figure 1.** Sequence alignment of the ArdA family proteins. The alignment was done for the C-terminal portions of ArdA proteins designated as the mini-ArdA region (coordinates, 90–166; see the text for details) using ClustalX<sup>27</sup> and the Pfam database.<sup>26</sup> The coloring scheme represents similar (A, S, T; V, I, L, M+ F, W, Y; D, E, N, Q; K, R, H; H, Y) and identical amino acid residues and their conservation in proteins used in comparative studies. The stars shown above sequences indicate the residues essential for ArdA activity. The antirestriction motif<sup>24</sup> (conserved for all known plasmid-encoded Ard proteins and the phage-encoded antirestriction protein Ocr) and conserved residues that are located in the C-terminal tail region of ArdA and essential for the ArdA activity (VF-motif) are underlined. The bacterial species and sequence accession numbers are referred to as follows: IncI1 (IncI1 plasmid Collb-P9, Q51598); IncN (IncN plasmid R46, Q52021); IncFV (IncFV plasmid *F<sub>0</sub>lac*, Z34467); YERPE (plasmid pMT1, *Yersinia pestis*, O68792); STAAU (*Staphylococcus aureus*, Q93211); LISMO (*Listeria sp.*, Q8Y811); ENTFA (the conjugative transposon Tn916, *Enterococcus faecalis*, Q47730 and Q833Q8); CLODI (the conjugative transposon Tn5397, *Clostridium difficile*, Q84F51); T7 Ocr (the “antirestriction” motif of Ocr (0.3 gene) protein, bacteriophage T7, P03775), End, the end of Ocr protein; BACTH (*Bacteroides thetaiotaomicron*, Q8ABN1); PSEPU (*Pseudomonas putida*, Q88KJ5); XANAX (*Xanthomonas axonopodis*, Q8PNK0); NITEU (*Nitrosomonas europaea*, Q82XK5); SALRU (*Salinibacter rubber*, Q2S3K6); LEGPH (*Legionella pneumophila*, strain Philadelphia, Q5ZWV9); LEGPA (*Legionella pneumophila*, strain Paris, Q5X211); FRATU (*Francisella tularensis*, Q5NFZ2); AGRTU (*Agrobacterium tumefaciens*, Q8UG51); SYNSP (plasmid pSYSX, cyanobacterium *Synechocystis sp.*, Q6YRS0); ANASP (plasmid pCC7120gamma, cyanobacterium *Anabaena sp.* Q8YK88).

similarity including 14 residues designated as an "antirestriction" motif.<sup>24,25</sup> The observation that this amino acid sequence shares a similarity with well-known conserved repeats found in the DNA sequence specificity (HsdS) subunits of type I systems raises the possibility that this conservation of motifs within restriction and antirestriction proteins may give a structural basis for their specific interaction. However, the exact mechanism by which the Ard proteins inhibit type I restriction systems remains unclear.

Here, we have studied the *in vivo* and *in vitro* interactions between antirestriction protein ArdA and type I EcoKI enzymes to derive the insight into the mechanism of their interplay. It was shown that like Ocr protein, ArdA is able to interact with both EcoKI methyltransferase (Mtase) and the complete R-M system and protect them from the interaction with specific DNA. However, unlike Ocr, ArdA can discriminate between type I Mtase and the R-M complex. We also mapped the ArdA region involved in this interaction.

## Results and Discussion

### Mutational analysis of ArdA protein

Deletion studies of *ardA* revealed that the N-terminal half of ArdA protein plays a minor role in its antirestriction action and seems to be inessential for its activity.<sup>18,19</sup> To map more precisely the ArdA region responsible for antirestriction function we generated the 5' deletions of *ardA* as PCR fragments encoding the 3' portion of the T7 tag of the pET21 vector and the 3' deletions as PCR fragments encoding the 5' portion of *ardA* terminated by the TGA codon in the appropriate positions. Figure 2 shows that the C-terminal portion of ArdA containing 77 residues (coordinates 90 to 166) is able to function against EcoKI restriction as a native ArdA. We designated it as the mini-ArdA region, since the deletion derivative of ArdA that lacks two next residues (A90 and Y91) is absolutely functionally inactive.

Notably, unlike the N terminus of ArdA, its C-terminal tail region seems to be very sensitive to deletions. We found that deletion of the final four amino acid residues of the C terminus of ArdA (VFRR-166) abolished the Ard activity *in vivo*, suggesting that this region may be essential for ArdA function. To verify this proposal, each of pairs VF and RR were mutated to alanine. Figure 2 shows that unlike the R165A+R166A substitution, the double mutation V163A+F164A severely affected the antirestriction activity of ArdA protein, suggesting that these residues may be essential for antirestriction activity of ArdA. Residue F164 seems to play a major role in this pair, since unlike V163A, the single mutation F164A retains its knock-out effect on antirestriction activity of ArdA. In contrast, alteration of F164 to valine only moderately reduced the ArdA activity. This finding

Mutations	Antirestriction activity	
MSVVAPAVYVGTWHKYNCGSIAGRWFDLT	wild type	2000
TFDDEERDFFAACRALHQDEADPELMFQDY		
EGFPGNMASECHINWAWVEGFRQARDEGC		
EEAYRLWVEDTGETDFDTRDAWGEADS		
ΔE89->	E89	2000
ΔA90 (mini-ArdA region)->	A90	1000
ΔR92->	R92	1
ΔL93->	L93	1
Antirestriction motif	Y91A	10
	R92A	1000
	L93A	600
EEAFAVEFASDTGLLADVPETVALYFD		
VF-motif		
YEAARDLFLDSFTFIDGHVFR-166		
<-H(tga163)	TGA163	1
AA	RR->AA	1000
AA	VF->AA	2
A	F164A	10
A	V163A	600
A	H162A	600
A	G161A	1000
A	D160A	1600
V	F164V	200
I	F164I	200
L	F164L	400
W	F164W	1000
Y	F164Y	1400
	vector	1

**Figure 2.** Mapping the functional regions of the ArdA protein. The amino acid sequence of ArdA and positions of deletions and substitutions used for its mutational analysis are indicated on the left. The mini-ArdA region identified as a result of deletion analysis of ArdA is underlined. Stars indicate the residues essential for ArdA activity. The vertical bars above and letters below the amino acid sequence of ArdA indicate the positions of mutated residues. Antirestriction activities of ArdA and its derivatives were measured as described in Materials and Methods. The antirestriction value of one indicates no detectable antirestriction activity. RR->AA, R165A+R166A; VF->AA, V163A+F164A.

suggests that the high hydrophobicity of F164 and probably V163 may play an essential role in the antirestriction activity of the ArdA protein. This suggestion is supported by the observation that the ArdA activity is relatively tolerant of alterations of F164 to hydrophobic and aromatic residues and increased in a row V, I, L, W and Y (Figure 2). Since this pair of hydrophobic residues, V163 and F164, is essential for ArdA activity and is conserved among 20 members of the ArdA family represented in Figure 1 we designated it as a VF-motif. The role of other residues located in the C-terminal tail region of ArdA (D160, G161, H162, R165 and R166) is not so essential for its antirestriction function, since their alanine substitutions only slightly influence the *in vivo* activity of ArdA (Figure 2).

We also mutated each of the five residues (91-YRLWV) adjoining the N-terminal edge-point of the mini-ArdA region to alanine and found that unlike the other four alanine mutations, only substitution Y91A affects the ArdA activity sufficiently *in vivo*

(Figure 2 and Table 1). Since this finding is associated with the idea that the aromatic residues may play an essential role in the interaction between ArdA and type I R-M systems we studied the effect of alanine substitutions on all other aromatic residues located in the mini-ArdA region. Analysis of this series of alanine substitutions (14 aromatic residues) shown in Table 1 reveals only two mutations, Y141A and F142A, that significantly affect the antirestriction activity of ArdA. It is possible that the effect of these substitutions is a result of their involvement in the organization of the region designated as the “antirestriction” motif that is conserved for all known plasmid-encoded Ard proteins, the phage-encoded antirestriction protein Ocr and the specificity subunits of type I R-M systems<sup>24,25</sup> and seems to play a role in the interaction between Ard proteins and type I R-M systems. Notably, all four aromatic residues (Y91, Y141, F142 and F164) found in positions that are sensitive to alanine substitution are conserved among members of the ArdA family (Figure 1).

Since like Ocr, ArdA has an excess of acidic residues<sup>24,25</sup> and some of them may be significant for its antirestriction action, we mutated six conserved negatively charged residues (D127, D133, E136, D143, D150, D154 and D160) located in the C-terminal tail and antirestriction domain regions of ArdA. However, we failed to detect the significant delay in the antirestriction activities of ArdA pro-

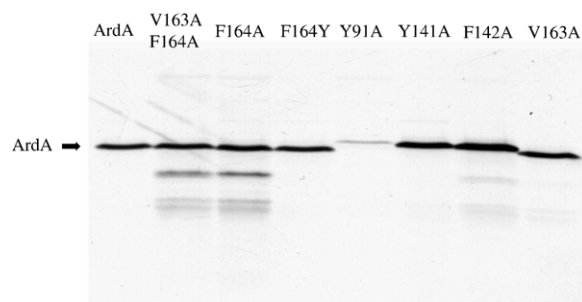
**Table 1.** Effect of amino acid substitutions in ArdA on its antirestriction activity *in vivo*

Mutations	Efficiency of test phage plating <sup>a</sup>	Antirestriction activity <sup>a</sup>
Wild-type	1.0	2000
Vector <sup>b</sup>	$5 \times 10^{-4}$	1
His-ArdA <sup>c</sup>	1.0	2000
W94A	$1 \times 10^{-1}$	200
V95A	$8 \times 10^{-1}$	1600
F103A	$3 \times 10^{-1}$	600
F106A	$1 \times 10^{-1}$	200
W110A+W111A	$2 \times 10^{-1}$	400
F120A	$5 \times 10^{-1}$	1000
F124A	$5 \times 10^{-1}$	1000
Y141A	$4 \times 10^{-2}$	80
F142A	$5 \times 10^{-3}$	10
Y144A	$5 \times 10^{-1}$	1000
Y147A	$3 \times 10^{-1}$	600
F152A	$1 \times 10^{-1}$	200
F156A	$3 \times 10^{-1}$	600
F158A	$2 \times 10^{-1}$	400
D127V	$7 \times 10^{-1}$	1400
D133V	$5 \times 10^{-1}$	1000
E136A	$8 \times 10^{-1}$	1600
D143A	$7 \times 10^{-1}$	1400
D150V	$7 \times 10^{-1}$	1400
D154V	$8 \times 10^{-1}$	1600
D160A	$8 \times 10^{-1}$	1600

<sup>a</sup> Efficiency of test phage plating (EOP) and antirestriction activity expressed by plasmids of the pAB763 series encoding the *ardA* gene and its mutants were measured as described in Materials and Methods. An antirestriction value of 1 indicates no detectable antirestriction activity.

<sup>b</sup> pBluescriptII KS(+).

<sup>c</sup> ArdA encoded by pAB2016.



**Figure 3.** Mutant derivatives of ArdA protein (soluble fractions). Polypeptides encoded by the pAB763 plasmids were specifically labeled with [<sup>35</sup>S]methionine by using the T7 expression system, analyzed by SDS-PAGE and visualized by autoradiography. The arrow indicates the position of the product of the wild-type ArdA protein.

teins carrying alanine or valine substitutions in these positions (Table 1).

Thus, the mutational analysis of ArdA protein reveals that its antirestriction activity is dependent on at least four aromatic residues (Y91, Y141, F142 and F164) that are conserved for ArdA proteins (Figure 1) and located in the mini-ArdA region. However, the marked decrease in mobility of mutant proteins Y91A, Y141A and F142A in SDS-PAGE and the low solubility of the Y91A derivative (Figure 3) compared with the wild-type and F164A proteins raise the possibility that the effect of these mutations on the ArdA activity may be at least partially a result of their influence on the folding of ArdA. Figure 3 also shows that both mutant proteins containing the substitution F164A have tendency to slight proteolysis. However, its level is too low to explain the drastic decrease (more than 100-fold) in the activity of these ArdA mutants.

### Like Ocr, the antirestriction protein ArdA specifically binds to EcoKI Mtase

Previous studies raised the possibility that like T7 phage-encoded Ocr protein, plasmid-encoded ArdA that has also the significant excess of negatively charged amino acid residues may inhibit the activity of type I R-M systems by direct protein-protein interaction.<sup>16,24,25</sup> The Ocr protein was found to have extremely strong affinity ( $K_d \approx 10^{-10}$  M) to both the EcoKI R-M complex and Mtase.<sup>32,34</sup> Unlike the R-M complex, EcoKI Mtase has no HsdR subunits, confers only modification activity and consists of one specificity (HsdS) and two modification (HsdM) subunits.<sup>35,36</sup> To detect the interplay between ArdA and EcoKI enzymes we examined the interaction between the <sup>35</sup>S-labeled native EcoKI Mtase and *in vivo* active His-ArdA (Table 2) using His-tag affinity chromatography.

Figure 4(a) shows that His-ArdA previously exposed on a Ni-NTA column (lane 2) is able to form a complex with the native EcoKI Mtase (lane 3) from the *E. coli* extract prepared after induction of the EcoKI Mtase synthesis. In the inverse case, the native

**Table 2.** Effect of the level of *ardA* and *ocr* expression on EcoKI restriction and modification

Genes under the control of repressed T7 promoter <sup>a</sup>	T7 promoter activity (in <i>lac</i> units) controlled by pLysS <sup>b</sup>	Efficiency of test phage plating <sup>c</sup>	Antirestriction activity <sup>c</sup>	Efficiency of DNA modification <sup>d</sup> (%)
<i>ardA</i>	0.03 (pLysS)	1.0	2000	70
	0.5 (vector)	1.0	2000	0.5
<i>ocr</i>	0.03 (pLysS)	1.0	2000	0.1
	0.5 (vector)	1.0	2000	0.1
<i>his-ocr</i>	0.03 (pLysS)	1.0	2000	0.1
None (vector)	0.5 (vector)	$5 \times 10^{-4}$	1	NT
<i>ardA tga143</i>	0.03 (pLysS)	$5 \times 10^{-4}$	1	NT
	0.5 (vector)	$4 \times 10^{-2}$	80	NT

<sup>a</sup> Plasmids pAB763, pAB763tga143, pAB1736 and pAB1781 encoding the *ardA*, *ardAtga143*, *ocr* and *his-ocr* genes under the control of repressed T7 promoter (in the presence of pLysS encoding the inhibitor of T7 RNA polymerase and/or in the absence of inducer, IPTG), and vector pBluescriptII KS (+) as a reference plasmid were used.

<sup>b</sup> The activity of repressed T7 promoter used for the *ardA* and *ocr* gene expression was estimated as described in Materials and Methods and related to that of the *lac* promoter; vector, p15A plasmid pACYC184.

<sup>c</sup> Efficiency of test phage plating and antirestriction activity expressed by plasmids encoding the *ardA* and *ocr* genes were measured as described in Materials and Methods. An antirestriction value of 1 indicates no detectable antirestriction activity.

<sup>d</sup> Unmodified test-phages  $\lambda$  were grown for three to four cycles on the plating strain AB2463 (DE3) that is proficient in EcoKI restriction and modification and carries pAB763, pAB1736 and pAB1781, encoding *ardA*, *ocr* and *his-ocr*, respectively, under the control of repressed T7 promoter (in the presence of pLysS encoding the inhibitor of T7 RNA polymerase and/or in the absence of inducer, IPTG). The efficiency of progeny test phage modification was determined as the ratio of phage titer on the EcoKI-restricting strain AB1157 to the phage titer on the non-restricting isogenic strain BA556. NT, not tested.

ArdA also binds to the *in vivo* and *in vitro* active His-tagged version of EcoKI Mtase containing the short His-tags on the N-terminal ends of its HsdM subunits (Figure 4(b), lane 3). As expected, the N-terminal TrxA derivative of ArdA (lane 4) and ArdA resulted from thrombin cleavage of its His (TrxA) derivative encoded by pAB2016 (data not shown) are also able to bind to His-Mtase. Such a type of experiment easily reveals the interaction between EcoKI Mtase and the well-studied antirestriction protein Ocr. Figure 4 demonstrates the binding between the *in vivo* active His-Ocr (Table 2) and native EcoKI Mtase (Figure 4(a), lane 7) and between the native Ocr and EcoKI His-Mtase (Figure 4(b), lane 5). These findings also suggest that the modifications of the N termini of ArdA, Ocr and HsdM subunits of EcoKI Mtase used in these experiments do not seem to impede their interactions.

To examine the state of aggregation of ArdA in solution, <sup>35</sup>S-labeled wild-type ArdA was analyzed by gel filtration chromatography and SDS-PAGE of column fractions followed by Coomassie staining and autoradiography. Figure 5 shows that ArdA eluted from the column with the single peak at approximately 44 kDa corresponding to the molecular mass of the ArdA dimer. These findings suggest that like Ocr, ArdA protein even at low concentrations (50 nM–100 nM) exists in solution mainly as a dimer.

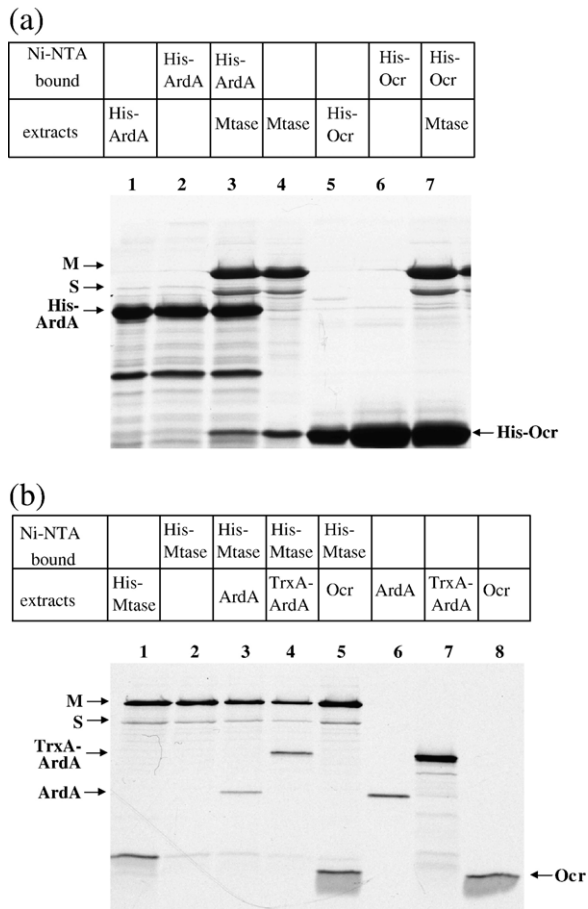
### Unlike Ocr, ArdA protein is able to distinguish between type I Mtase and complete R-M system

It was shown that the classical antirestriction protein Ocr encoded by T7 bacteriophage can displace DNA from the Mtase core of type I R-M systems and efficiently abolish both their restriction and modification activities.<sup>29,32,34</sup> Data presented in Table 2 show that like Ocr, ArdA demonstrates *in*

*in vivo* the high efficiency of action against the restriction activity of the EcoKI R-M system. However, in contrast to Ocr, ArdA is ineffective against EcoKI modification when its concentration in the cell is low as a result of low *ardA* expression. In these experiments the promoters of *ardA* and *ocr* were very weak and their activities were estimated as 0.03 of that of the *lac* promoter. Sufficient increase (at least 10–20-fold) in the activity of the *ardA* promoter is needed to affect both the restriction and modification activities of the EcoKI system. These findings are consistent with the observations that the ArdA homologues encoded by other transmissible plasmids also showed specificity in targeting the restriction function of the EcoKI R-M system, leaving a significant level of Mtase activity under the condition in which restriction was almost completely prevented.<sup>38,39</sup>

To study this phenomenon in detail we tried to find the difference between the effects of Ocr and ArdA proteins on the binding of type I Mtase and the R-M system to specific DNA using a gel retardation assay. The concentration of the EcoKI system in *Escherichia coli* cells when EcoKI genes are chromosomally encoded was estimated to be approximately 100 molecules per cell or 100 nM, respectively.<sup>36</sup> In cells EcoKI HsdM and HsdS subunits were commonly presented with the stoichiometry of 2:1 and in an excess (at least twofold) over HsdR subunits, suggesting that EcoKI Mtase may be assembled and function in cells independently.<sup>35–37</sup> This observation arises the possibility that the difference in the *in vivo* effects of ArdA and Ocr proteins on the methylation in *E. coli* cells may reflect the ability of ArdA in contrast to Ocr to bind to type I Mtase and the R-M complex with different efficiency.

To examine the interaction of purified antirestriction proteins ArdA and Ocr with EcoKI Mtase and



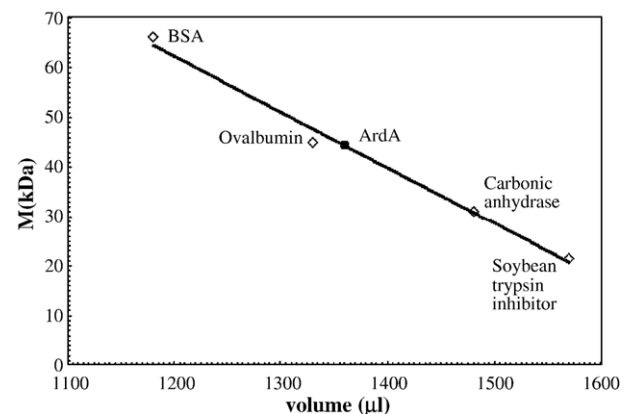
**Figure 4.** Interaction of antirestriction proteins ArdA and Ocr with EcoKI Mtase *in vitro*. (a) Binding of His-tagged ArdA and Ocr with the native EcoKI Mtase. The Ni-NTA columns with the immobilized  $^{35}\text{S}$ -labeled His-ArdA (lane 2) and His-Ocr (lane 6) encoded by pAB2016 and pAB1781, respectively, were loaded with the extract with the native  $^{35}\text{S}$ -labeled EcoKI Mtase (lane 4) and washed with 5 mM imidazole. Retained proteins were eluted with 1 M imidazole, analyzed by SDS-PAGE and visualized by autoradiography (lanes 3 and 7, respectively) to reveal the complex of EcoKI Mtase with ArdA or Ocr. (b) Binding of His-tagged EcoKI Mtase with ArdA and Ocr protein. The Ni-NTA columns with the immobilized  $^{35}\text{S}$ -labeled His-tagged EcoKI Mtase (lane 2) were loaded with the extracts with the  $^{35}\text{S}$ -labeled native ArdA (lane 6), its TrxA derivative (lane 7) and native Ocr (lane 8) and washed with 5 mM imidazole. Retained proteins were eluted with 1 M imidazole, analyzed by SDS-PAGE and visualized by autoradiography (lanes 3, 4 and 5, respectively). Arrows indicate the positions of ArdA, His-ArdA, TrxA-ArdA, Ocr proteins and subunits M (HsdM) and S (HsdS) of EcoKI Mtase.

the R-M complex in the retardation assay in the *in vivo* range of their concentrations (50–100 nM) the experiments were carried out with *E. coli* extracts containing EcoKI Mtase and the R-M complex to escape the problem with instability of purified EcoKI Mtase at concentrations below 100 nM.<sup>34,36</sup> Since native EcoKI Mtase and His-Mtase demonstrated very similar activities in the gel-retardation

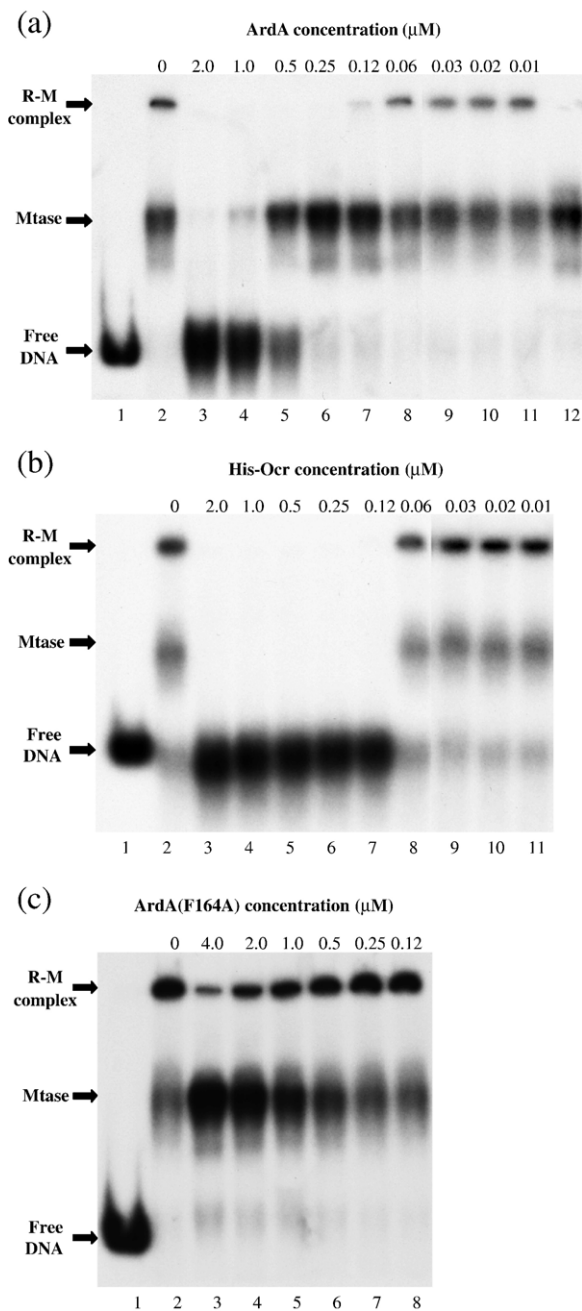
and *in vitro* EcoKI restriction assays we used both of them in these experiments.

Our dilution experiments show that native and His-tagged Mtases in cell extracts are stable and able to bind to specific DNA up to a concentration of 20–25 nM (data not shown) and this observation is consistent with the estimation of  $K_d$  for the functionally active Mtase complex ( $M_2S$ ) as 15 nM.<sup>36</sup> In retardation experiments the radioactively labeled 257 bp fragment containing the EcoKI recognition site was incubated with a mixture of cell extracts prepared after induction of the EcoKI Mtase (100 nM) and HsdR subunits (100 nM) in the presence of a different concentration of previously purified ArdA and His-Ocr. It is clear that such a type of experiment is the most informative in the following concentration ranges:  $[\text{ArdA dimer}]$  or  $[\text{Ocr dimer}] \gg [\text{Mtase}] + [\text{R-M complex}] \gg [\text{specific unmodified DNA}]$ .

Figure 6(a) shows that in the absence of ArdA at these concentrations of EcoKI proteins specific DNA fragment (lane 1) is retarded as two bands that represent its complex with EcoKI Mtase and the R-M complex (lane 2). In the absence of HsdR subunits only the band corresponding to Mtase was observed (lane 12) and no retardation was found in the presence of HsdR subunits alone (data not shown). Since the assembly of HsdR subunits with Mtase is irreversible and stoichiometry of the R-M complex is two HsdR subunits per one molecule of Mtase,<sup>36</sup> the mixing Mtase and HsdR subunits at a concentration of 100 nM may produce the R-M complex at a concentration about 50 nM and an excess of free Mtase (about 50 nM). The pattern of retardation shown in Figure 6(a) (lane 2) is in accordance with this estimation. A twofold decrease in EcoKI Mtase concentration did not influence the intensity of the band of the R-M complex but markedly reduced that of EcoKI Mtase, suggesting that in this case most of the Mtase assembled with HsdR subunits and



**Figure 5.** Analysis of the state of aggregation of ArdA in solution by gel-filtration. Gel filtration was carried out using a Sephacryl S-100 column (Pharmacia). Samples containing  $^{35}\text{S}$ -labeled ArdA and molecular mass standard proteins were eluted at a flow rate of 13 cm/h and the column fractions were assayed by SDS-PAGE followed by Coomassie staining and autoradiography.

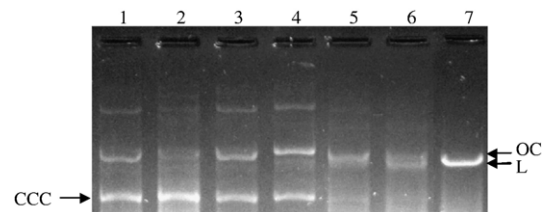


**Figure 6.** Effect of ArdA, its F164A mutant and Ocr on the interaction of the EcoK R-M complex and Mtase with the unmodified DNA. The  $^{32}\text{P}$ -labeled fragment (257 bp) of unmodified DNA (lane 1) was incubated with the *E. coli* extracts containing EcoKI His-Mtase and R-M complex in the presence of (a) purified ArdA, (b) His-Ocr and (c) the F164A mutant of ArdA in the range of their dimer concentrations from 0 to 2  $\mu\text{M}$  (lanes 2–11). Lane 12 represents specific DNA in the presence of EcoKI Mtase and in the absence of ArdA (see the text for details). The mixtures were analyzed by electrophoresis in 5% polyacrylamide gel followed by autoradiography. Arrows indicate the positions of the specific DNA fragment bound with EcoKI Mtase and the R-M complex.

formed the R-M complex (data not shown). Such an approach allowed us to estimate the concentration of HsdR subunits in *E. coli* extracts using the concentration of His-Mtase as a reference probe.

We also found that this EcoKI R-M complex assembled *in vitro* was active and gave efficient cleavage of unmodified circular pBR322 DNA when present at a concentration of two to three molecules per EcoKI target site (pBR322 contains two EcoKI sites). Figure 7 shows that the cleavage was rapid and initially produced linear forms of pBR322 DNA after 1 min incubation (lane 6) followed by progressive DNA degradation (data not shown) as predicted by the Studier and Bandyopadhyay model.<sup>40</sup> No cleavage of modified pBR322 DNA was observed under these conditions after 10 min incubation (data not shown). No cleavage of unmodified DNA was also observed after 10 min in the absence of cofactors (SAM, ATP and  $\text{Mg}^{2+}$ ), the EcoKI R-M complex, Mtase, and the HsdR subunit (lanes 1, 2, 3 and 4, respectively).

Titration of EcoKI Mtase and the R-M complex by ArdA reveals that ArdA protein strongly prevents the DNA retardation by Mtase at a concentration of ArdA dimer up to 0.5  $\mu\text{M}$  (Figure 6(a), lane 5). This finding suggests that the affinity of ArdA to EcoKI Mtase is not so high as that of Ocr ( $K_d \cong 10^{-10}$  M) and the value of the apparent  $K_d$  for the ArdA–Mtase complex may be in the range of  $10^{-7}$ – $10^{-6}$  M. However, it seems likely that ArdA is more effective in preventing DNA retardation by the EcoKI R-M complex. The band corresponding to the R-M complex was seen only when the ArdA concentration was approximately equal or less than that of the R-M complex (about 50 nM) (lanes 8 and 9). These findings suggest that the binding affinity of ArdA protein to the R-M complex is higher than that to Mtase. Unfortunately, we were unable in this type of experiment to estimate more exactly the value of  $K_d$  for binding of ArdA to the R-M complex, since the measurements were performed at concentrations higher than the dissociation constant for their assembly, and further work will be needed for a more careful estimation of dissociation constants for this interaction. It is apparent that in the same type of



**Figure 7.** Digestion of unmodified pBR322 DNA by the *E. coli* extract containing the EcoKI R-M complex and cofactors (SAM, ATP and  $\text{Mg}^{2+}$ ) for 20 s (lane 5) and 1 min (lane 6) and under conditions of the absence of cofactors, EcoKI R-M complex, Mtase, and HsdR subunits (lanes 1, 2, 3 and 4, respectively). Lane 7, pBR322 DNA linearized with HindIII. Arrows indicate covalently closed circular pBR322 (CCC), open circular (nicked) (OC) and linear (L) forms.

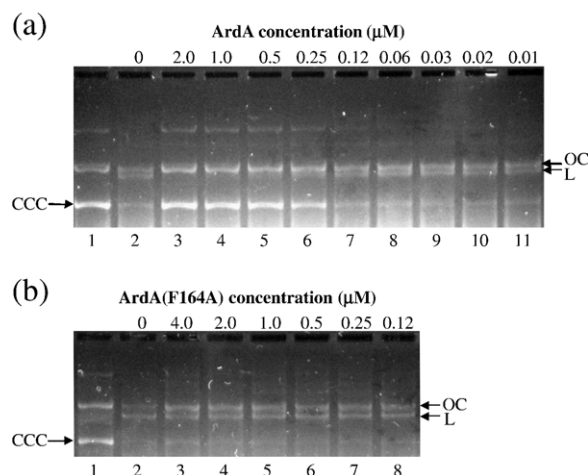
experiments with Ocr protein no retardation of specific DNA fragment by Mtase and the R-M complex was observed in the range of concentrations 0.12  $\mu\text{M}$ –2.0  $\mu\text{M}$  of His-Ocr (Figure 6(b), lanes 3–7) or the wild-type Ocr (data not shown). These observations are consistent with the findings that Ocr protein binds very efficiently to both EcoKI Mtase and the R-M complex ( $K_d$  is about 100 pM) and is unable to differentiate between these EcoKI enzymes.<sup>34</sup>

Thus, it seems possible that unlike Ocr, the ArdA protein is able to discriminate between EcoKI Mtase and the R-M complex and this ArdA feature is based on its different binding affinity to these EcoKI enzymes. The additional support for this suggestion comes from the retardation experiment with the F164A mutant of ArdA protein that is ineffective in antirestriction activity *in vivo*. Figure 6(c) shows that the mutation F164A considerably reduces the ArdA ability to prevent the binding of the EcoKI R-M complex to specific DNA and the DNA retardation by the R-M complex is observed even at high concentrations of ArdA (1  $\mu\text{M}$ –4  $\mu\text{M}$ ). However, no effect on the wild-type of retardation pattern was observed for the F164Y mutant (data not shown) that is *in vivo* so effective in antirestriction as the wild-type ArdA protein (Figure 2).

It seems likely, therefore, that the antirestriction effect of ArdA *in vivo* is correlated with its ability to prevent binding of the EcoKI complex to the target DNA. This conclusion is further supported by the finding that ArdA is able to block the cleavage of unmodified pBR322 by the EcoKI R-M complex *in vitro* in the range of concentrations 0.25  $\mu\text{M}$ –2.0  $\mu\text{M}$  (Figure 8(a), lanes 3–6), suggesting that both the restriction and retardation activities of the EcoKI system are inhibited by ArdA in a similar mode. As expected, the F164A mutant of ArdA that is unable to prevent the retardation of a specific DNA fragment by the EcoKI complex is also ineffective in inhibition of unmodified DNA cleavage specified by the EcoKI R-M system (Figure 8(b), lanes 3–8).

These findings suggest that the antirestriction action of ArdA protein resulted from its ability to specifically interact with the EcoKI R-M complex and protect it from interaction with specific DNA. However, unlike Ocr, ArdA protein has a low binding affinity to EcoKI Mtase ( $K_d \approx 10^{-7}$ – $10^{-6}$  and  $10^{-10}$  M for ArdA and Ocr, respectively) and the additional C-terminal tail region (VF-motif) is needed for ArdA to efficiently interact with EcoKI enzymes. It is possible this ArdA feature may be a basis for its ability to discriminate between EcoKI Mtase and complete the R-M complex at a low concentration of ArdA (<0.1  $\mu\text{M}$ ). At a high concentration of ArdA ( $\geq 1$   $\mu\text{M}$ ) both EcoKI Mtase and the R-M complex are unable to bind to specific DNA and their activities are strongly inhibited.

Interestingly, at the high level of *ardA* expression the deletion derivative ArdA(tga143) that lacks the C-terminal tail region as a result of insertion of the TGA stop codon in the *ardA* sequence in position 143 demonstrates the detectable antirestriction activity (Table 2) and may be regarded as a low-efficiency



**Figure 8.** Effect of ArdA and its F164A mutant on the digestion of unmodified pBR322 DNA by the EcoKI R-M complex. Unmodified supercoiled pBR322 (lane 1) was incubated for 1 min with a reaction mixture containing the EcoKI R-M complex and cofactors in the presence of (a) purified ArdA, and (b) its F164A mutant in the range of their dimer concentrations from 0 to 2  $\mu\text{M}$  (lanes 2–11). Reactions were stopped by the addition of an equal volume of phenol/chloroform and after the extraction with phenol/chloroform, samples were analyzed by 0.8% agarose gel electrophoresis in the presence of ethidium bromide (0.5  $\mu\text{g}/\text{ml}$ ).

prototype of the Ocr protein. This observation is associated with the idea that ArdA protein consists of two parts: the N-terminal Ocr-like region terminated by the conserved antirestriction motif and the C-terminal tail region containing the VF-motif that is typical only for ArdA proteins. The observation that the amino acid sequence of Ocr protein is terminated by the region that has a similarity with the antirestriction domain of Ard proteins is consistent with this suggestion (Figure 1). Although the N-terminal portion of ArdA flanked by the antirestriction motif shows no homology to Ocr, secondary structure predictions suggest similar structural features including marked elongation and a series of negatively charged amphipathic helices that might mimic the DNA substrate.<sup>16,25</sup> It seems likely that the high and approximately equal efficiency of Ocr protein against both type I Mtase and the R-M system answers its mission to overcome the host restriction in bacterial cells infected by virulent phage and makes the C-terminal tail region typical for ArdA proteins unnecessary for Ocr. However, the more complex configuration of ArdA protein that is able to discriminate between the activities of type I Mtase (modification) and the complete R-M enzyme (restriction) and allows to modify plasmid DNA in the new host cell may give some advantage for efficient adaptation and maintenance of the *ardA*-encoding transmissible plasmids in the new restriction-proficient host.

This suggestion is associated with the idea that such an interaction between antirestriction and type

I restriction proteins may provide the effective mechanism for controlling the transfer of foreign genes into cells and make bacterial cells more adaptable and evolutionary flexible. This hypothesis is consistent with the observations that despite the presence of two promoters, there is no evidence for transcriptional regulation of gene expression for type I R-M systems.<sup>41–43</sup> In contrast, the *ardA* genes encoded by promiscuous plasmids may be involved in the complex regulatory circuits containing, for instance, SOS boxes and other regulatory elements<sup>44</sup> and may provide, therefore, the efficient and adequate regulation of type I restriction activity. It is shown that the *ardA* genes encoded by promiscuous plasmids are highly expressed during entry into the new cell as a result of de-repression of efficient promoters regulated by plasmid-encoded repressors.<sup>44–47</sup> At the first stage of plasmid DNA transfer at the high concentration of ArdA protein in cells the activities of both host-encoded type I Mtase and R-M systems are strongly inhibited. However, the *ardA*-encoded promiscuous plasmids have a good chance to be modified by host type I Mtase when the level of *ardA* expression in bacterial cell is drastically decreased as a result of the increase of concentration of plasmid-encoded repressors in the cell and the renewal of the *ardA* repression state. Notably, the protease ClpXP has been recently shown to be involved in the modulation of type I restriction activity in *E. coli*<sup>11,48</sup> in response to the cell's DNA damage by UV light<sup>49–51</sup> or the base analog 2-aminopurine.<sup>52</sup> It seems possible that this antirestriction function based on the specific degradation of the HsdR subunit of type I R-M complex by ClpXP is able not only to prevent the restriction of unmodified regions generated as a result of DNA repair of the bacterial chromosome<sup>8,11</sup> but like ArdA proteins, may also facilitate the gene flux into the bacterial cell under these unfavorable conditions.

Thus, in addition to the advantageous abilities of type I enzymes (i) to cleave DNA at variable positions remote from their recognition sites and, therefore, provide the possibility for any gene to be available for acquisition by recipient genome,<sup>12,13</sup> and (ii) to change their sequence specificity as a result of some recombination events within the *hds* genes,<sup>53–55</sup> the heterooligomeric structure of these R-M systems seems also to provide the possibility for the gene flow regulation resulting from their ability to interact with different antirestriction functions induced in bacterial cells under certain and most probably unfavorable circumstances. The findings that type I R-M systems are widespread among bacteria and that a lot of the putative *ardA* genes encoded by plasmids and bacterial chromosomes are found as a result of sequencing of complete genomic sequences are consistent with this idea. It seems likely, therefore, that in combination with antirestriction functions the type I R-M systems appear to be more developed “modulators of the frequency of genetic variation”<sup>56</sup> than in the cases when they function alone.

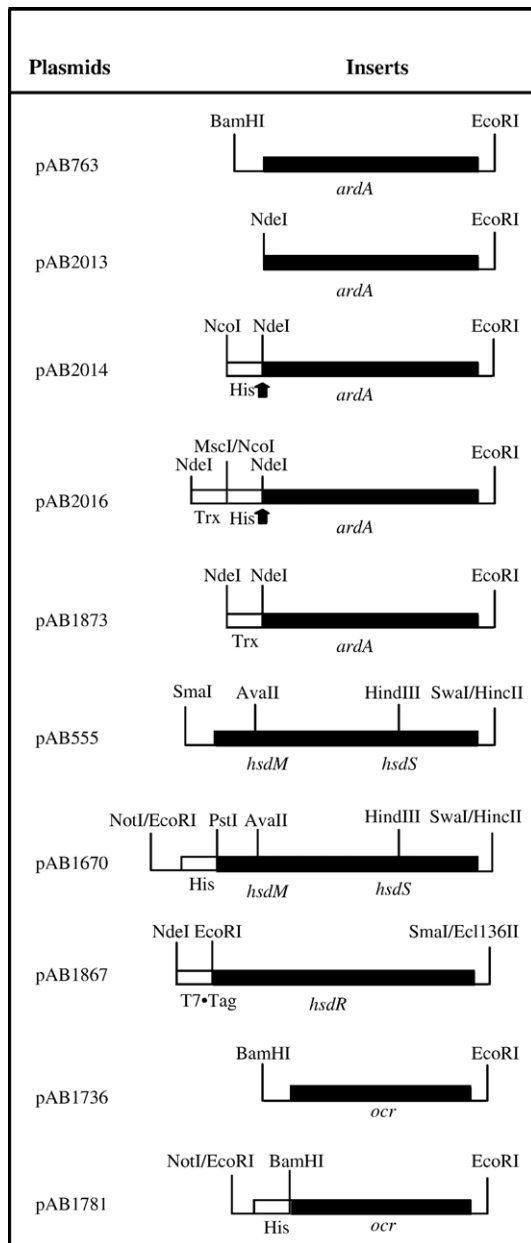
## Materials and Methods

### Bacterial strains, phages and plasmids

*E. coli* K-12 strain JM109 ( $r_{\text{KM}}^+$ )<sup>57</sup> was used as host for plasmids. *E. coli* strain BL21(DE3) ( $r_{\text{MB}}^-$ )<sup>58</sup> harboring the compatible p15A plasmid pREP4 (Qiagen) that specifies the LacI repressor was used for protein production. EcoKI-restricting strains AB2463 and AB2463 (DE3) ( $r_{\text{KM}}^+$ ) that is a *recA* derivative of *E. coli* AB1157<sup>59</sup> and the non-restricting derivative of AB1157, BA556, ( $r_{\text{KM}}^-$ )<sup>17</sup> were used for measuring the antirestriction activity of ArdA proteins and its mutants. *E. coli* strain WA834(DE3)- $\Delta$ *hdsRMS* used for production of EcoKI Mtase and HsdR subunits was constructed by transduction of  $\Delta$ (*mcrC-hsdRMS-mmr*)::Tn10 from ER1647 (New England Biolabs) to WA834 (obtained from W. Arber) and lysogenization by  $\lambda$ DE3 phage (Novagen). Plasmids pBluescriptII (Stratagene), pET21a(+), pET28a(+), pET32a(+), (Novagen) and pALTER-1 (Promega) were used as vectors for cloning, DNA sequence analysis and oligonucleotide-directed mutagenesis. Plasmid pKK232-8 containing the promoterless *cat* gene cassette was used as the promoter probe vector.<sup>60</sup> Plasmids pBg3 and pRH3<sup>61</sup> that served as the source of the *hds* genes encoding the EcoKI R-M system were obtained from N. Murray. pAR2993 encoding *ocr* (0.3 gene) was obtained from A. H. Rosenberg and F. W. Studier. The bacteriophage referred as  $\lambda$  test phage was  $\lambda$ vir (*ral*<sup>+</sup>) (obtained from R. Devoret).

### DNA techniques and plasmid constructions

Standard procedures were used essentially as described.<sup>62</sup> Plasmids of the pAB series used here are represented in Figure 9. Plasmid pAB763 carrying the native *ardA* under the control of the T7 promoter was made by cloning a 529 bp PCR BamHI-EcoRI fragment that contained the *ardA* coding region including its ribosome-binding sequence GGAGG and the ATG initiation and TGA stop codons (coordinates 214–727; Genbank accession no. M74923) to pBluescriptII KS(+) cut with BamHI and EcoRI (PCR primers 5'-gggatccaggaggattcgg-gaattgtctg and 5'-gggaattcaccgacggaacacatgac). pAB2013 and pAB2014 carrying *ardA* under the control of the T7 promoter were made by cloning a 501 bp NdeI-EcoRI PCR fragment that contained the *ardA* coding region including the ATG initiation and TGA stop codons to pET21a(+) and pET28a(+) vectors, respectively, cut with NdeI and EcoRI (PCR primers 5'-atggaggattcgcacatgtctgtgttgca and 5'-gggaattcaccgacggaacacatgac). pAB2016 was made by ligating a 557 bp NcoI-EcoRI fragment of pAB2014 to pET32a(+) cut with MscI and EcoRI. The NcoI cohesive end was previously filled in to protrude a blunt end for efficient ligation with the blunt end generated by MscI. This plasmid contains a 340 bp NdeI-MscI fragment of pET32 encoding TrxA·Tag (thioredoxin protein), a 56 bp NcoI-NdeI fragment of pET28a(+) encoding His·Tag/thrombin/ and a 501 bp NdeI-EcoRI PCR fragment containing the *ardA* coding region of pAB2013. The frame was confirmed by DNA sequencing. This N-terminal combination of standard fusion sequences His·Tag and TrxA·Tag that can be removed with thrombin was used for preparation of ArdA protein from its His(TrxA) derivative purified by Ni-NTA resin as described below and markedly increased its recovery. pAB1873 was made by inserting a 345 bp NdeI-NdeI fragment of pET32a(+) containing TrxA·Tag to pAB2013 cut with NdeI. The 5'-deletion of *ardA* were



**Figure 9.** Schematic presentation of plasmid inserts containing the *ardA*, EcoKI *hsdMS*, EcoKI *hsdR* and *ocr* (*0.3*) genes and their coding regions (filled boxes) fused to the standard T7-Tag, His-Tag and Trx-Tag sequences (open boxes) that were sub-cloned in multi-copy vectors (pBluescriptII and pET series) under the control of the T7 promoter and used for production of appropriate proteins and analysis of their *in vivo* and *in vitro* interactions (see the text for details). Only relevant restriction sites are indicated. Arrowheads indicate the thrombin cleavage sites.

prepared by cloning BamHI-EcoRI PCR fragments containing the deleted *ardA* region to pET21a(+) cut with BamHI and EcoRI. Resulting plasmids contained unidirectional deletions of *ardA* flanked by a BamHI site at their 5' ends and fused in-frame to T7-Tag of the PET21a(+) vector. PCR fragments were amplified from pAB763 using the pair of primers. The 3' deletions of *ardA* were made by introducing the TGA stop codon in the 3' portion of the

*ardA* region using the Altered Sites II *in vitro* Mutagenesis System (Promega) and oligonucleotides as described below. pAB555 was made by ligating a 2.16 kb SmaI-HindIII fragment of pBg3 and a 1.02 kb HindIII-SwaI fragment of pRH3 into pBluescriptII KS(+) cut with SmaI and HincII. The resulting plasmid contained the *hsdM* and *hsdS* genes encoding EcoKI Mtase under the control of the T7 promoter. pAB1670 encoding the *in vivo* and *in vitro* active EcoKI His-Mtase was made by ligating a 528 bp PCR fragment (containing 5' portion of *hsdM* except for the first ATG codon) flanked by PstI and AvaII and a 2.54 kb AvaII-XhoI fragment of pAB555 into pBluescriptII-6His cut with PstI and XhoI (PCR primers 5'-ttctgcagaacaataac-gatctgtgctg and 5'-ccgggtctgcaccacttcac). pBluescriptII-6His was made by sub-cloning a 63 bp EcoRI-BamHI fragment of pQE9 (Qiagen) encoding 6xHis-Tag to pBluescriptII KS(+) cut with NotI and BamHI. In these experiments, the EcoRI and NotI cohesive ends of plasmids were previously filled in to protrude blunt ends for efficient ligation. pAB1867 encoding the *in vivo* and *in vitro* active modified EcoKI HsdR subunits carries the *hsdR* coding region except the first seven codons fused to the N-terminal T7-Tag in-frame was made by sub-cloning a 3.57 kb EcoRI-SmaI fragment of pBg3 to modified pET21a(+) vector cut with EcoRI and Ecl136II that recognizes the SacI site and generates blunt ends. The modified pET21a(+) vector was made by inserting the sequence ggattccccgggctgagggaattc in the polylinker region between its BamHI and EcoRI sites. Note that our attempts to overexpress the native EcoKI *hsdR* from T7 and other inducible promoters have been unsuccessful. This finding is consistent with observations of other investigators<sup>36,63</sup> and suggests that the several first codons of *hsdR* (or the appropriate amino acid residues of HsdR) may be involved in the regulation of *hsdR* expression. pAB1736 encoding the native *ocr* (*0.3*) gene<sup>28</sup> of T7 phage under the control of T7 promoter was made by cloning a 551 bp PCR fragment of the T7 genome (coordinates 837–1379) flanked by BamHI and EcoRI sites and amplified from pAR2993 to pBluescriptII KS(+) cut with BamHI and EcoRI (PCR primers 5'-ataggatcctcttgagtgcattgactagc and 5'-ataagaattc-tgtgccattgagtgacc). pAB1781 encoding *his-ocr* under the control of the T7 promoter was made by inserting a 469 bp BamHI-EcoRI fragment containing the coding region of *ocr* except the ATG initiation codon to pBluescriptII-6His cut BamHI and EcoRI (PCR primers 5'-ataggatccgctatgtctaactgacttac and 5'-ataagaattcgtgc-cattgagtgacc). Plasmid pCAT-T7 used for the estimation of T7 promoter activity was constructed by insertion of a 56 bp BglII-XbaI fragment of pET21a(+) containing the T7 promoter region in the multiple cloning site of the promoter probe vector pKK232-8 in the appropriate orientation. DNA was sequenced on both strands by dideoxy chain termination methods.<sup>64</sup> Mutations were created in the wild-type *ardA* region (a 541 bp BamHI-HindIII fragment of pAB763 subcloned in pALTER1) using the Altered Sites II *in vitro* Mutagenesis System (Promega) and oligonucleotides. The following codons were used for substitutions: Ala, gcc, gcg and gct; Val, gtc; Ile, atc; Leu, ctt; Trp, tgg; Tyr, tac. Mutations and the *ardA* region sequence were confirmed by DNA sequencing and their effect on the ArdA activity *in vivo* was analyzed after the re-cloning of the *ardA* region to pBluescriptII KS(+) cut BamHI and HindIII.

#### Preparation of protein extracts

BL21(DE3)/pREP4 and WA834(DE3) $\Delta$ *hsdRMS*/pREP4 strains harboring plasmids that encode ArdA, EcoKI

Mtase, HsdR subunits and their His derivatives, were grown in M9 medium supplemented with 1% (w/v) Casamino acids at 37 °C until  $A_{600}$  of 0.6 was reached. IPTG (1 mM) was added to induce the T7 RNA polymerase synthesis in cells and after 30 min rifampicin was added to inhibit the activity of the host RNA polymerase. The cells were additionally grown for 90 min and after pulse-labeling with [ $^{35}$ S]methionine for 10 min harvested, washed, quickly frozen in liquid nitrogen and stored at -80 °C. For protein extracts preparation samples were thawed at 4 °C, resuspended in buffer (50 mM Tris-HCl, 100 mM NaCl, 1 mM PMSF (pH 7.6)), treated with lysozyme (0.1 mg/ml) for 10 min at 4 °C, sonicated and clarified by centrifugation for 15 min at 15,000g.  $^{35}$ S-labeled proteins were analyzed by SDS-PAGE and visualized by staining with Coomassie blue R250 and autoradiography. Concentration of proteins bound to the Ni-NTA column were estimated by densitometry of these gels using as the reference probes the concentrations of samples purified as described below. Concentration of HsdR subunits in extracts was estimated by titration with His-Mtase in the gel-retardation experiments with  $^{32}$ P-labeled specific DNA fragment (see Results and Discussion for details).

### Protein preparation

ArdA protein was prepared from BL21(DE3)/pREP4 carrying plasmid pBA2016 encoding ArdA fused to the N-terminal combination of the standard Trx-Tag and His-Tag sequence that can be removed with thrombin (His-ArdA). The 30 ml of extract with His-ArdA in binding buffer (50 mM Tris-HCl, 100 mM NaCl, 5 mM imidazole (pH 7.6)) was added to 400  $\mu$ l of nickel-nitrilotriacetic acid-agarose (Ni-NTA) (Qiagen) previously equilibrated in binding buffer (50 mM Tris-HCl, 100 mM NaCl, 5 mM imidazole (pH 7.6)) and stirred for 30 min at room temperature. Ni-NTA resin was washed twice with 2 ml of binding buffer, loaded into a column, then washed with buffer E (50 mM Tris-HCl, 100 mM NaCl (pH 7.6)) containing 5 mM imidazole followed by the same buffer containing 10 mM imidazole. His-ArdA protein was eluted with 1 ml buffer E containing 200 mM imidazole and dialyzed against buffer D (50 mM Tris-HCl, 20 mM NaCl, 10 mM MgCl<sub>2</sub> (pH 8.0)). After cleavage with thrombin (Novagen) the resulting ArdA was treated with PMSF (1 mM) to stop the cleavage reaction and after the next Ni-NTA chromatography step to remove the N-terminal tags dialyzed against buffer D containing 1 mM PMSF and stored at -80 °C after the addition of glycerol to a final concentration of 10%. The *in vivo* and *in vitro* active His-Ocr and EcoKI His-Mtase were also purified in similar way by using Ni-NTA resin (Qiagen). Concentrations of purified protein were determined spectrophotometrically using absorbance at 280 nm.

### Protein binding assay

For protein binding reactions, the 100  $\mu$ l extracts containing  $^{35}$ S-labeled His-tagged ArdA or EcoKI Mtase encoded by pAB2016 and pAB1670, respectively, were added to 50  $\mu$ l of Ni-NTA (Qiagen) previously equilibrated in binding buffer (50 mM Tris-HCl, 100 mM NaCl (pH 7.6)) and stirred 30 min at room temperature. Ni-NTA resin was washed twice with 0.5 ml of binding buffer and loaded into a column. To examine the interaction between ArdA and EcoKI Mtase, Ni-NTA columns containing immobilized His-tagged proteins with concentrations of

1–3  $\mu$ M were loaded with 50–100  $\mu$ l extracts containing  $^{35}$ S-labeled native ArdA or EcoKI Mtase and then washed with binding buffer containing 5 mM imidazole. Retained proteins were eluted with the binding buffer containing 1 M imidazole, analyzed by SDS-PAGE and visualized by both autoradiography and staining with Coomassie blue R250. All proteins used in the protein-protein binding assays were specifically labeled with [ $^{35}$ S]methionine by using the T7 expression system as described above.

### Gel retardation assay

The binding of EcoKI Mtase and the R-M complex in the presence of ArdA to the specific DNA fragment containing the EcoK recognition site 5'-AACCCACTCGTGC-3' was tested by a gel-retardation assay. A 257 bp  $^{32}$ P-labeled PCR fragment was made by amplification of the  $\beta$ -lactamase region of pBR322 in the presence of [ $\alpha$ - $^{32}$ P]dATP using the pair of primers 5'-ataggatcctgtcgccttattccc, 5'-atagaattcttgcccggcgtaacac and *Pfu* DNA polymerase according to the manufacturer's recommendations (Fermentas). Unincorporated ATP was removed using Sephadex G50 spun column chromatography.<sup>62</sup> Binding mixtures contained 2 nM of  $^{32}$ P-labelled DNA fragment, 100 nM Mtase or His-Mtase, 100 nM HsdR subunit and from 0 to 2  $\mu$ M purified ArdA (or His-Ocr) in buffer A (50 mM Tris-HCl, 100 mM NaCl, 10 mM MgCl<sub>2</sub>, 1 mM DTT, 1 mM PMSF, 100  $\mu$ M SAM, 4 mM ATP (pH 8.0)). The following order of addition of reaction components was used: after pre-incubation of EcoKI enzymes with ArdA for 10 min  $^{32}$ P-labeled DNA and then ATP were added and mixtures were incubated for 10 min at room temperature. The mixtures were analyzed by electrophoresis on 5% (w/v) PAGE followed by autoradiography.

### EcoK endonuclease activity assay

EcoKI nuclease activity was assayed at 25 °C in a 100  $\mu$ l volume containing 50 nM EcoKI Mtase or His-Mtase, 100 nM HsdR subunit (from cell extracts), from 0 to 2  $\mu$ M purified ArdA, 10 nM unmodified pBR322 DNA, containing two EcoKI sites and 100  $\mu$ M SAM. The buffer was 50 mM Tris-HCl, 100 mM NaCl, 1 mM PMSF and 10 mM MgCl<sub>2</sub> (pH 8.0). The following order of addition of reaction components was used: after pre-incubation of EcoKI enzymes with ArdA for 10 min unmodified pBR322 DNA was added and the reaction was started by the addition of ATP to a final concentration of 4 mM. The 20  $\mu$ l samples were removed at set times, reactions were stopped by the addition of an equal volume of phenol/chloroform and after the extraction with phenol/chloroform, samples were analyzed by 0.8% (w/v) agarose gel electrophoresis in the presence of ethidium bromide (0.5  $\mu$ g/ml).

### Measurement of antirestriction activities of ArdA

Antirestriction activities of ArdA protein and its mutants *in vivo* were defined as efficiency of plating (EOP) of unmodified test-phage  $\lambda$  on the EcoKI-restricting *E. coli* strain AB2463 (DE3)/pLys carrying the *ardA* plasmids (derivatives of pAB763) under the control of the repressed T7 promoter (in the absence of inducer IPTG and the presence of pLys<sup>S58</sup> encoding the inhibitor of T7 RNA polymerase) relative to that on the same restricting strain carrying vector pBluescriptII.<sup>23</sup> The EOP was determined as the ratio of phage titer on the restricting strain to phage titer on the non-restricting isogenic strain

BA556. The T7 promoter activity was estimated as described below. Unmodified test-phage  $\lambda$  was grown on *E. coli* C that lacks the host restriction and modification activities.<sup>49</sup> Modified phage  $\lambda$  was grown on the EcoKI-restricting strain AB1157. Data shown in the Figures and Tables are mean values of at least four experiments. Standard deviations from the mean in these experiments did not exceed 20%. In all cases no influence of plasmids encoding *ardA*, EcoKI *hsdSM* and *hsdR* and their mutants on the EOP of EcoKI-modified phage  $\lambda$  was detected.

### Assay of the *ardA* promoter activity

The T7 promoter was used for the *ardA* and *ocr* gene expression under the condition of its repression (in the absence of inducer, IPTG and the presence of pLysS<sup>58</sup> encoding the inhibitor of T7 polymerase) in strain AB2463 (DE3)/pLysS and its activity was estimated in the cell extract as described.<sup>44</sup> The strains AB2463 (DE3)/pLysS and AB2463 (DE3)/pACYC184 (vector) carrying plasmid pCAT-T7 were grown in LB medium containing ampicillin (100  $\mu$ g/ml) and chloramphenicol (20  $\mu$ g/ml) to a density of about  $5 \times 10^8$  cell/ml and pelleted by centrifugation, resuspended in the same volume of 50 mM Tris-HCl, 0.03 mM DTT (pH 8.0) containing 6,6'-dinitro-3,3'-dithiodinitrobenzoic acid (0.4 mg/ml) and sonicated on ice. Debris was removed by centrifugation and *cat* activity in the supernatant fraction was measured at 412 nm by the spectrophotometric assay as described.<sup>65</sup> Promoter activity was defined as the ratio of *cat* activity expressed by the pCAT-T7 plasmid under the control of the T7 promoter in the experimental strains in the absence of IPTG to the *cat* activity expressed by the reference plasmid pCAT2 under the control of the *lac* promoter in strain AB2463 in the presence of its inducer, IPTG. No significant variation in copy number between the pCAT plasmids tested for promoter activity in the presence of the compatible p15A plasmids (pLysS and vector pACYC184) was observed.

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