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Plasmid pKM101 Encodes Two Nonhomologous Antirestriction Proteins (ArdA and ArdB) Whose Expression Is Controlled by Homologous Regulatory Sequences

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The IncN plasmid pKM101 (a derivative of R46) encodes the antirestriction protein ArdB (alleviation of restriction of DNA) in addition to another antirestriction protein, ArdA, described previously. The relevant gene, *ardB*, was located in the leading region of pKM101, about 7 kb from *oriT*. The nucleotide sequence of *ardB* was determined, and an appropriate polypeptide was identified in maxicells of *Escherichia coli*. Like ArdA, ArdB efficiently inhibits restriction by members of the three known families of type I systems of *E. coli* and only slightly affects the type II enzyme, *Eco*RI. However, in contrast to ArdA, ArdB is ineffective against the modification activity of the type I (*Eco*K) system. Comparison of deduced amino acid sequences of ArdA and ArdB revealed only one small region of similarity (nine residues), suggesting that this region may be somehow involved in the interaction with the type I restriction systems. We also found that the expression of both *ardA* and *ardB* genes is controlled jointly by two pKM101-encoded proteins, ArdK and ArdR, with molecular weights of about 15,000 and 20,000, respectively. The finding that the sequences immediately upstream of *ardA* and *ardB* share about 94% identity over 218 bp suggests that their expression may be controlled by ArdK and ArdR at the transcriptional level. Deletion studies and promoter probe analysis of these sequences revealed the regions responsible for the action of ArdK and ArdR as regulatory proteins. We propose that both types of antirestriction proteins may play a pivotal role in overcoming the host restriction barrier by self-transmissible broad-host-range plasmids. It seems likely that the *ardKR*-dependent regulatory system serves in this case as a genetic switch that controls the expression of plasmid-encoded antirestriction functions during mating.

DNA transfer between different gram-negative bacterial species may be mediated by self-transmissible plasmids and temperate bacteriophages (13, 16, 29, 42). Some of them, including R plasmids, encode antibiotic resistance, and their dissemination among bacterial pathogens is a major problem worldwide in infection diseases. However, gene transfer may be limited by the host-encoded restriction endonucleases that recognize the methylation pattern of guest DNA (1, 8, 9, 43). Some bacteriophages were shown to encode antirestriction functions which permit them to overcome host restriction (19, 24, 39, 46). One antirestriction function encoded by bacteriophage T7 has been studied in detail. It has been shown that the phage-encoded antirestriction 0.3 protein inhibits the host-controlled (type I) restriction-modification systems by binding directly to the enzymes (5, 20, 28, 30, 37). Recently, we have identified two homologous antirestriction proteins, Ard and ArdA, encoded by unrelated plasmids ColIb-P9 and pKM101, respectively. Like the 0.3 protein of T7 phage, these proteins are very acidic and specifically inhibit both the restriction and the modification activities of type I systems (6, 18). All three acidic antirestriction proteins are encoded by genes located in leading regions of plasmid and phage genomes, which enter the host cell early during conjugation or infection. Some aspects of regulation of plasmid-encoded Ard functions have been studied in detail (6, 32). In this study, we show that plasmid pKM101 encodes a second antirestriction protein, designated ArdB, in addition to ArdA. These proteins are functionally similar in that both specifically inhibit restriction by members of all three families of type I systems of *Escherichia coli*. However, ArdA and ArdB have no extensive homology, and unlike ArdA, ArdB is only slightly acidic and ineffective against the modification activity of the type I *Eco*K complex. We also found that the expression of the two antirestriction proteins is regulated jointly by two pKM101-encoded proteins, ArdK and ArdR.

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MATERIALS AND METHODS

Bacterial strains and media. AB1157 is an F^- derivative of *E. coli* K-12 having the genotype *thr-1 leu-6 proA2 his-4 thi-1 argE3 lacY1 galK2 ara-14 xyl-5 mtl-1 tsx-33 rpsL31 supE44*. BA556 is an r_K^- m_K^+ derivative of AB1157 (7). WA2377 (2), HB129 (33), and BZ216 (obtained from T. A. Bickle) are the r_A^+ m_A^+, r_B^+ m_B^+, and r_D^+ m_D^+ derivatives of *E. coli* K-12, respectively. BA509 and BA2357 are the r_{RI}^+ m_{RI}^+ and r_{R124}^+ m_{R124}^+ derivatives of AB1157, carrying the *Eco*RI plasmid (7) and plasmid R124 (6, 22), respectively. BA532 is the r_{P1}^+ m_{P1}^+ derivative of AB1157 lysogenic for P1CM*ts* (7). JM109 (45) was used for maxicell analysis and as the host for plasmids. Media used in this study were described previously (18).

Plasmids and phages. The IncN plasmid pKM101, which is a 35.4-kb derivative of R46 (12, 26), was obtained from G. C. Walker. Plasmids pBluescriptII (Stratagene) and pACYC184 (14) were used as vectors for cloning and DNA sequence analysis. Plasmid pKK232-8 was used as a promoter probe vector (10). The bacteriophage referred to as λ was λ*vir*. Phage λ*b*506cI857*bio*256 was used as the λ*ral* mutant.

DNA techniques and plasmid constructions. Standard procedures were used essentially as described elsewhere (27). Restriction endonuclease digestions were carried out as recommended by the manufacturer (New England Biolabs). Deletions were prepared by exonuclease III and mung bean nuclease treatment as instructed by the supplier (Stratagene). DNA was sequenced on both strands by the dideoxy-chain termination method (35), using *Taq* polymerase, unidirectional deletions, and oligodeoxynucleotides. To locate Tn*5* insertions, primers derived from the sequence of Tn*5* terminal inverted repeats were used.

Plasmids pAB1 and pAB7 were described previously (6). pAB36, pAB37, and pAB38 were made by subcloning the appropriate *Xho*I-*Bam*HI fragments of pKM101::Tn*5*Ω130, pKM101::Tn*5*Ω246, and pKM101::Tn*5*Ω64 to pBluescriptII KS DNA cut with *Xho*I and *Bam*HI. Note that pKM101 has no *Xho*I sites; all of the *Xho*I sites of pKM101::Tn*5* derivatives used are generated by Tn*5* insertions. In this case, the cloned fragments contain both pKM101 DNA and a 489-bp end of Tn*5* resulting from *Xho*I digestion of Tn*5* insertions (3).

For construction of unidirectional deletions of the *ardB* region (the pAB66 series), plasmid pAB38 was linearized by *Bgl*II and *Sac*I, treated with exonuclease III and mung bean nucleases, recircularized with T4 DNA ligase, and transformed in AB1157 harboring the compatible $ardK^+$ plasmid pOR81. For the expression of *ardB* under the control of the *lac* promoter, pAB40 and pAB41 were made by subcloning the appropriate *Xho*I-*Nde*I fragments of pAB36 and pAB38, respectively, to pBluescriptII SK DNA cut with *Xho*I and *Eco*RV. To construct pAB81 and pAB85, plasmid pAB69 (6) was linearized by *Eco*RI and *Pst*I, treated with exonuclease III and mung bean nuclease, and recircularized with T4 DNA ligase.

To construct the compatible Km^r Cm^s plasmids pOR39, pOR42, and pOR81, pACYC184 was used as a vector. The $ArdR^+$ plasmid pOR81 was made by subcloning the insert of pAB81 in *Hin*dIII-*Bam*HI-cleaved pACYC184. The $ArdR^+$ plasmid pOR39 was made by deleting a 5.7-kb *Nde*I fragment of a derivative of pACYC184, pOR36, described previously (6). The $ArdK^+$ $ArdR^+$ plasmid pOR42 was made by ligating a 1.0-kb *Sal*I-*Bam*HI fragment of pAB81 to pOR39 DNA cut with *Sal*I and *Bam*HI. Finally, all three plasmids were digested with *Eco*RI, and the Tn*903* Km^r gene cassette of pUC-4K (40) was inserted into their *Eco*RI sites located in the *cat* gene.

To construct the pCAT66 plasmids, the appropriate pAB66 plasmids were digested with *Pvu*II, and their inserts were subcloned into *Sma*I-cleaved promoter probe vector pKK232-8, which lacks the *Nde*I site. Plasmids with fragments in the appropriate orientation were chosen. The resulting plasmids were digested with *Nde*I and *Hin*dIII, filled in, and recircularized with T4 ligase. The reference plasmid pCAT1 was made by subcloning a 0.45-kb *Pvu*II fragment of pBluescript II KS containing the *lac* promoter in the *Sma*I site of pKK232-8.

Measurement of antirestriction activity. The antirestriction activity of plasmids was defined as the efficiency of plating (EOP) of unmodified phage λ.O on the experimental (plasmid-bearing) strain divided by the EOP on the plasmidless restricting strain (6). EOP was expressed as (phage titer on the restricting strain)/(titer on a nonrestricting strain [either BA556 or WA802]). In all experiments testing restriction activity in the cell, the modified reference phage was plated on the experimental (plasmidless and plasmid-bearing) strains in parallel with the unmodified test phage. In all cases, no influence of plasmids on the EOP of modified reference phage was detected, suggesting that the measurement of EOP of unmodified test phage really reflects the level of restriction activity in the bacterial cells. Unmodified phages, designated λ.O, were grown on *E. coli* C r_0 m_0 (8), which lacks restriction and modification functions. Modified phages, designated λ.K, were grown on the r_K^+ m_K^+ strain AB1157. McrA and McrBC restriction were tested as described previously (6).

Assay of chloramphenicol resistance. Strain AB1157 carrying plasmids was grown overnight, diluted 1,000-fold, and spread on LB agar containing ampicillin (100 μg/ml), kanamycin (40 μg/ml), and concentrations of chloramphenicol ranging from 0 to 80 μg/ml. Colonies were scored after incubation for 24 h at 37°C, and chloramphenicol resistance was taken as the highest concentration that caused no significant reduction in colony number relative to the control. Promoter activity was defined as the ratio of chloramphenicol resistance conferred by the pCAT66 plasmids to chloramphenicol resistance (50 μg/ml) conferred by the reference plasmid pCAT1 expressing *cat* activity under the control of *lac* promoter in the presence of its inducer, isopropylthiogalactopyranoside (IPTG). Since promoter probe vector pKK232-8 confers a resistance to chloramphenicol at a concentration of 2 μg/ml, and the same chloramphenicol resistance was found in the plasmidless strain AB1157, we defined the appropriate level of promoter activity (<0.04) as 0. No significant variation in copy number between the pCAT66 plasmids tested for promoter activity was observed.

Nucleotide sequence accession number. The nucleotide sequence of the *ardB* gene has been deposited in GenBank and assigned accession number L09114.

RESULTS

Recently, we found that the IncN plasmid pKM101 encodes the *ardA* gene, which efficiently prevents restriction by members of the three known families of type I systems of *E. coli*. This gene maps at coordinate 25 kb, and its activity is controlled by the *ardK* and *ardR* genes (6). Data presented in Fig. 1 show that both plasmids pAB1 and pAB7 carrying *ardA* exhibit the strong antirestriction phenotype. In contrast, a deletion derivative of pAB1, pAB36, that harbors pKM101 DNA between the Tn*5*Ω130 insertion and the *Bam*HI site, coordinates 27.2 and 34.2 kb, respectively, and lacks the *ardA* region failed to express any antirestriction activity.

However, we were surprised to find that a 0.5-kb deletion from the Tn*5*Ω130 insertion (pAB38) that inactivated the *ardR* gene caused complete inhibition of *Eco*K restriction. The region spanning the Tn*5*Ω246 insertion site seems to be essential for expression of this new antirestriction function because the deletion derivative pAB37, which lacks sequence to the left of this insertion, retains no antirestriction activity. Thus, these data suggest that the site of Tn*5*Ω246 insertion lies within a gene that encodes a second antirestriction function in addition to ArdA. This gene has been designated *ardB*. Note that the observation that the deletion of *ardR* gene induces the *ardB*-encoded antirestriction function suggests that like ArdA, ArdB is controlled by the *ardR* gene product.

However, it seems likely that the control of expression of ArdA function is relaxed relative to that of ArdB. This suggestion is based on the observation that unlike the $ArdB^+$ $ArdR^+$ plasmid pAB36, the $ArdA^+$ $ArdB^+$ $ArdR^+$ plasmid

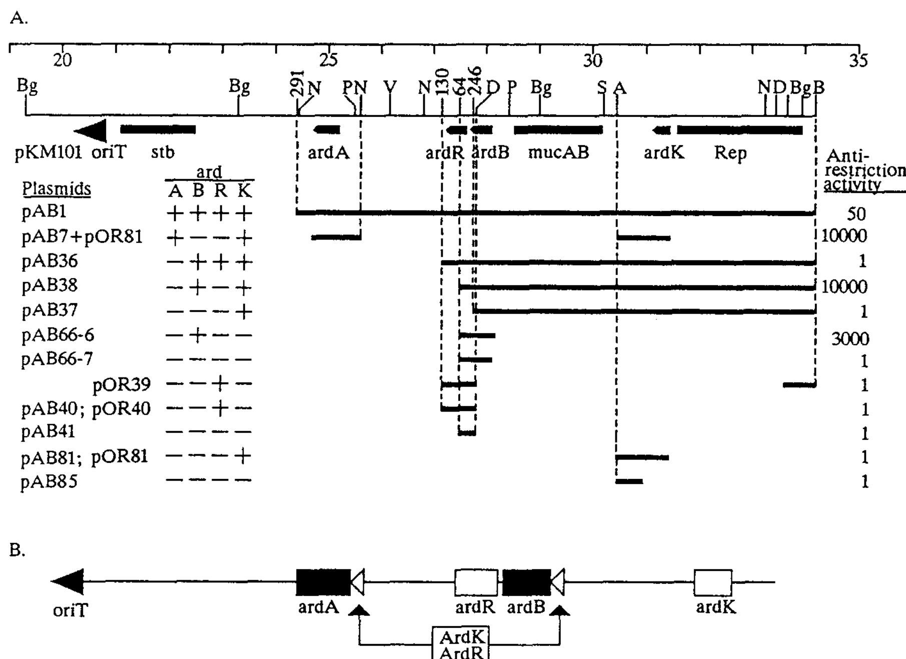

FIG. 1. Locations and regulation of *ard* genes encoded by the leading region of pKM101. (A) The top line indicates kilobase coordinates on the pKM101 map (44). The next line shows restriction sites and locations of *oriT* (origin of transfer) (15, 44), Rep (origin of replication) (12, 44), *stb* (required for stability in recombination-proficient hosts) (44), *mucAB* (mutagenesis enhancement) (31, 44), *ardA*, *ardR*, and *ardK* (6), and *ardB*. Transcription of all *ard* genes presented is from right to left. Numbers in boldface refer to the positions of Tn*5* insertions used for introducing the *Xho*I sites into pKM101 DNA. Only relevant *Acc*I, *Nde*I, and *Pvu*II sites of pKM101 are indicated. The lines below the map indicate the regions of pKM101 subcloned into either vector pBluescriptII KS (pAB plasmids) or vector pACYC184 (pOR plasmids). pAB7+pOR81 indicates the combination of two compatible plasmids encoding *ardA* and *ardK*, respectively. Antirestriction activity, expressed by these recombinant plasmids and indicated on the right, was measured in restricting strain *E. coli* K-12 AB1157 as described in Materials and Methods. None of the pOR plasmids represented express any antirestriction activity. Abbreviations: A, *Acc*I; B, *Bam*HI; Bg, *Bgl*II; D, *Nde*I; N, *Not*I; P, *Pvu*II; S, *Sma*I; V, *Eco*RV. (B) Model for the negative regulation of *ardA* and *ardB* gene expression. The regulatory proteins ArdK and ArdR (boxed) specified by the respective genes (closed boxes) may bind to 218-bp CUP sequences (open triangles) of the *ardA* and *ardB* genes (closed boxes) and inhibit the expression of ArdA and ArdB antirestriction proteins. The position of the *ardR* gene suggests that the ArdR protein may be autoregulatory and repress its own synthesis by binding to the *ardB* promoter region (CUP sequence).

pAB1 exhibits a detectable antirestriction activity (Fig. 1A). Note that the same level of antirestriction activity is observed in the presence of the parent plasmid pKM101 in the type I restriction-proficient host (7), suggesting that *ardA* may be responsible for this restriction alleviation.

To localize *ardB* more precisely, we attempted to delimit the pKM101 region sufficient for ArdB function. Deletion studies indicated that the *ardB*$^+$ derivatives of pAB38 which lacked the *ardK* region were stably maintained in the host cells only in the presence of the compatible *ardK*$^+$ plasmid pOR81 (Fig. 1A), suggesting that like *ardA*, the cloned *ardB* locus requires *ardK* for its stability in cells. Taken together, these results and observations suggest that the activities of the *ardA* and *ardB* genes are controlled by the *ardK* and *ardR* gene products.

Nucleotide sequence of *ardB*. Deletion studies positioned the *ardB* gene in a 0.7-kb insert of pAB66-6 (Fig. 1A). A deletion derivative of pAB66-6, pAB66-7, showed no antirestriction activity, suggesting that the deleted region is essential for *ardB*. In addition, we found that unlike most of the deletion derivatives constructed, pAB66-6 and pAB66-7 are stably maintained in the host cells even in the absence of *ardK*.

The nucleotide sequence of the *ardB* coding region was determined for both strands from overlapping DNA fragments and is shown in Fig. 2. Sequence analysis revealed only one long open reading frame (ORF) between positions 319 and 744 to encode *ardB*. Note that the Tn*5*Ω246 insertion that affects ArdB function is located in this ORF. The predicted ArdB protein has a molecular weight of 16,481. The potential initiation codon ATG at nucleotide 319 is preceded by a possible ribosome-binding sequence, 5'-GAGG, which appears seven nucleotides upstream of the ATG. There are two additional potential ATG codons in the same ORF (positions 367 and 376). However, only the ATG codon at nucleotide 319 was preceded by a typical Shine-Dalgarno sequence (36).

This ORF is followed by the *ardR* coding region (Fig. 2), suggesting that *ardB* and *ardR* may be transcribed in one operon. We also found that the *ardR* ORF is terminated at a site 67 bp upstream from the Tn*5*Ω130 insertion. The *mucA* ORF is located 413 bp upstream of *ardB* and oriented in the opposite direction to that of the *ardB* gene. No other long ORFs (>230 bp) were detected between the *ardB* and *mucA* coding regions (17).

Identification of the *ardB*, *ardK*, and *ardR* gene products. The *ardB* gene product was identified by labeling plasmid-encoded proteins in *E. coli* maxicells (Fig. 3). Analysis of protein patterns expressed by the *ardB* plasmid pAB66-6 revealed the 15.8-kDa polypeptide (lane 1), whose size agreed with that predicted from the nucleotide sequence of *ardB*. This polypeptide was absent from maxicells carrying

FIG. 2. Nucleotide and deduced amino acid sequences of the *ardB* gene. Sequence numbering begins at the *Pvu*II site. The proposed Shine-Dalgarno sites (SD) and initiation ATG codons of *ardB* and *ardR* are underlined. The bent arrows indicate the endpoints in the deleted clones. "Instable" means that the plasmid was unable to produce viable transformants of *E. coli*. The arrowhead indicates the location of Tn*5* insertion. (+) and (−) represent the presence and absence, respectively, of ArdB activity. Solid arrows indicate the inverted repeat. The ArdB region conserved for pKM101 ArdA, ColIb Ard proteins, and the 0.3 gene protein of phage T7 is shown in boldface and underlined. The result of alignment of these proteins is presented below. Similar (in one-letter notation, A, S, T; D, E; N, Q; R, K; I, L, M, V; F, Y, W) and identical amino acids are shown in boldface if they are conserved for at least three of the proteins tested.

either vector pBluescriptII (lane 7) or the *ardB* mutant pAB66-7 (lane 2), suggesting that it is in fact the *ardB* gene product.

We also were able to identify the proteins encoded by the *ardR* and *ardK* genes. The $ardR^+$ plasmid pAB40 directed the synthesis of the 19.8-kDa polypeptide (lane 3) that is absent in maxicells carrying either vector (lane 7) or the *ardR* mutant pAB41 (lane 4). Since the ORF corresponding to *ardR* would encode a protein of about 16 kDa (17), we concluded that this 19.8-kDa polypeptide may be the product of the *ardR* gene. This conclusion is supported by the observation that the *ardR* mutant pAB41, which was made by deleting the 3′ portion of *ardR* (17), specifies a markedly smaller polypeptide (about 12 kDa). Note that the *ardR* gene in pAB40 was expressed under the control of the *lac* promoter because its own promoter is very weak and supports a detectable but low-level synthesis of ArdR (data not shown).

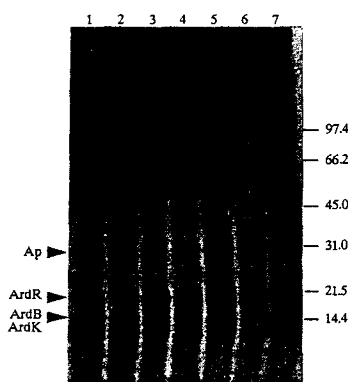

FIG. 3. Proteins produced by *ard* derivative plasmids. The polypeptides encoded by plasmids were specifically labeled with [^{35}S]methionine by using the maxicell system (34), analyzed by electrophoresis on a 5 to 25% gradient sodium dodecyl sulfate-polyacrylamide gel, and visualized by autoradiography. Lanes: 1, pAB66-6 ($ardB^+$); 2, pAB66-7 ($\Delta ardB$); 3, pAB40 ($ardR^+$); 4, pAB41 ($\Delta ardR$); 5, pAB81 ($ardK^+$); 6, pAB85 ($\Delta ardK$); 7, vector pBluescriptII KS. Arrowheads indicate the presumptive products of *ardB*, *ardR*, and *ardK* genes and vector-encoded polypeptide β-lactamase (Ap). Positions of size standards (in kilodaltons) are indicated on the right.

Comparison of protein patterns expressed by the $ArdK^+$ plasmid pAB81 (Fig. 3, lane 5) and the $ArdK^-$ derivative pAB85 (lane 6) revealed that the *ardK* gene encodes the protein of 15-kDa, corresponding to the size expected from the reading frame (about 13 kDa) (17).

ArdA and ArdB are nonhomologous, but their expression is controlled by homologous regulatory sequences. A search of different data banks (GenBank, EMBL Nucleotide Sequence Database, and SWISS-PROT Protein Sequence Data Bank) failed to detect any extensive similarities with nucleotide and deduced amino acid sequences of *ardB*. Comparison of ArdB with ArdA revealed only one small region of similarity (Fig. 2).

However, data presented above indicate that *ardA* and *ardB* are controlled jointly by the *ardK* and *ardR* gene products, suggesting possible similarities in regulatory regions. Indeed, comparison of the sequences upstream of *ardA* and *ardB* revealed a strong homology (about 94%) over 218 bp (Fig. 4, nucleotides 85 to 302). To define more precisely the regions responsible for gene regulation, the *Pvu*II-*Nde*I fragments of the pAB66 plasmids harboring the upstream sequence and 5′ portion of *ardB* were inserted into the promoter probe vector pKK232-8*cat*, yielding the pCAT66 plasmids (Fig. 5). Promoter activities of these fragments were examined in the presence of compatible plasmids encoding the regulatory proteins ArdK and ArdR.

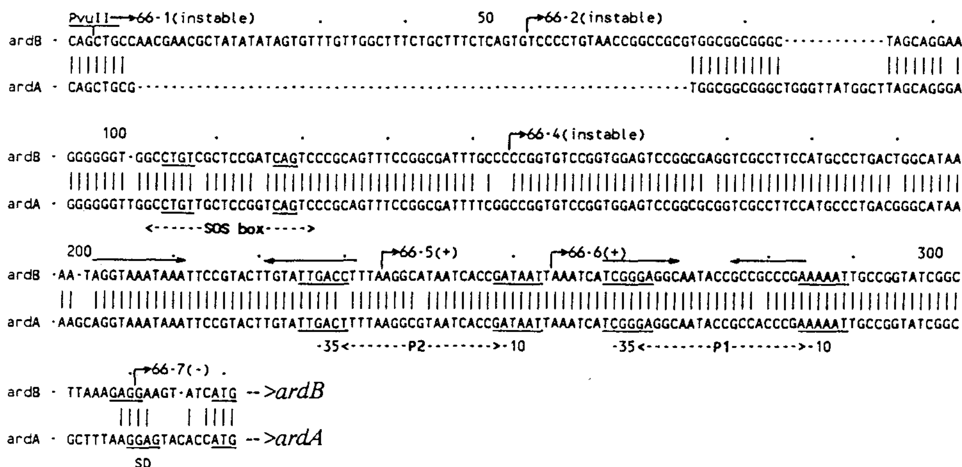

FIG. 4. Comparison of nucleotide sequences immediately upstream of the *ardB* and *ardA* genes. Exact matches are shown by vertical bars, and gaps inserted to optimize homology are indicated by dots. The proposed P1 and P2 promoter sites and LexA-binding sequence (SOS box) are indicated by dotted arrows. The proposed −10 and −35 sequences are underlined. Other symbols are defined in the legend to Fig. 2.

In addition, we tested the effects of ArdK and ArdR on ArdB activity directed by the pAB66 deletion derivatives.

Data presented in Fig. 5 show that in contrast to pCAT66-7, pCAT66-6 exhibits a detectable *cat* activity, suggesting that the upstream sequence of *ardB* encoded by pCAT66-6 contains a weak promoter. This promoter has been designated P1, and its proposed −10 and −35 hexamers are shown in Fig. 4. The presence of ArdR abolished *cat* activity expressed by pCAT66-6, while ArdK was ineffective in this respect (Fig. 5). These results suggest that the P1 promoter is controlled by the ArdR but not by the ArdK protein. Similar results were obtained for the pCAT66-5 deletion.

Figure 5 also shows that deletion derivative pCAT66-4 exhibits the increased level of promoter activity that is abolished only in the presence of both ArdK and ArdR proteins. Unlike pCAT66-5 and pCAT66-6, this plasmid is stably maintained in the host cells only in the presence of the regulatory protein ArdK, and ArdR is unable to prevent this plasmid instability. Analysis of DNA sequence between the 66-4 and 66-5 deletions revealed a potentially strong promoter (designated P2) which has −35 (TTGACc) and −10 (gATAAT) regions (lowercase letters represent divergence from the consensus sequence) separated by 17 bp (Fig. 4). This finding suggests that P2 promoter activity may be at least partly responsible for the instability of pCAT66-4 in the absence of the regulatory protein ArdK that may function as a repressor of P2 promoter. We also suggest that the increased promoter activity exhibited by pCAT66-4 in the presence of ArdK is a result of the leakage of the strong P2 promoter in a partially repressed state. Data presented in Fig. 5 also show that all promoter activities directed by pCAT66-4 in the presence of ArdK are efficiently controlled by the other regulatory protein, ArdR. Note that similar results were obtained when ArdB activity (instead of *cat* activity) was monitored for the appropriate deletions of the pAB66 series (Fig. 5, symbols in parentheses). This finding is consistent with the foregoing suggestion that the expression

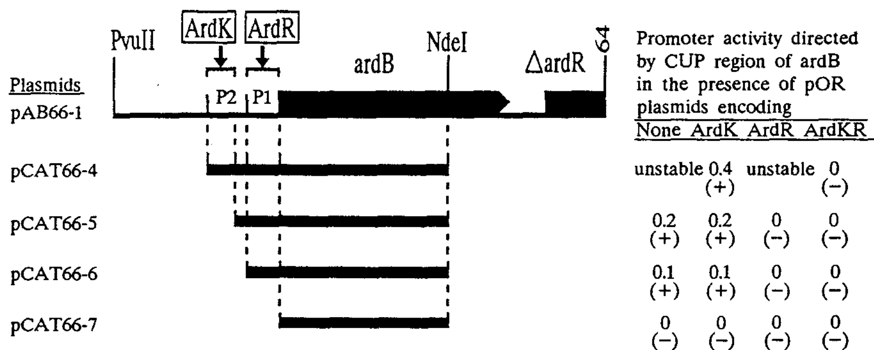

FIG. 5. Effects of ArdK and ArdR proteins on stability and promoter activity of the CUP region of the *ardB* gene. The top line shows the positions of restriction sites and Tn5Ω64 insertion in the pKM101 insert on pAB66-1. Upstream of *ardB*, the proposed P1 and P2 promoters that may be regulated by the ArdK and ArdR proteins (boxed) are indicated. The lines below indicate the other pAB66 deletions of the CUP region created and subcloned into the promoter probe vector pKK232-8*cat*. Promoter activities of these deleted fragments assayed as resistance of plasmid-bearing strains to chloramphenicol and related to the activity of the *lac* promoter are indicated on the right. The Km^r Cm^s derivatives of pACYC184 (pOR39, pOR81, and pOR42) were used as carriers of *ardR*, *ardK*, and *ardKR*, respectively. For comparison, *ardB*-mediated antirestriction activity expressed by the appropriate pAB66 plasmids is shown in parentheses as follows: (+), >3,000; (−), about 1.0 (no detectable activity). "Unstable" means that plasmid was unable to produce viable transformants of *E. coli*. Data are averages of at least five independent experiments.

TABLE 1. Effects of *ardB* on different *E. coli* restriction systems

Restriction system[a] (type)	EOP of test phage		Relief of restriction[b]	Antirestriction phenotype
	Strains without pAB66-1	Strains with pAB66-1 (*ardB*$^+$)[c]		
*Eco*A (Ib)	4×10^{-3}	4×10^{-1}	100	+
*Eco*B (Ia)	2×10^{-4}	4×10^{-1}	2,000	+
*Eco*D (Ia)	2×10^{-4}	1.0	5,000	+
*Eco*K (Ia)	1×10^{-4}	1.0	10,000	+
*Eco*R124 (Ic)	4×10^{-4}	1.0	2,500	+
*Eco*RI (II)	1×10^{-4}	3×10^{-3}[d]	30	+/−
*Eco*P1 (III)	6×10^{-5}	6×10^{-5}[d]	1	−
McrA	1×10^{-1}	1×10^{-1}	1	−
McrBC	1×10^{-1}	1×10^{-1}	1	−

[a] The restriction strains used were as follows: *Eco*A, WA2377; *Eco*B, HB129; *Eco*D, BZ216; *Eco*K, AB1157; *Eco*R124, BA2357; *Eco*RI, BA509; *Eco*P1, BA532; and McrA and McrB, AB1157. Since strains BA509, BA532, and BA2357 contain two restriction systems, we used λ.K test phages in order to subject them only to *Eco*RI, *Eco*P1, and *Eco*R124 restriction, respectively. To test McrA and McrBC restriction, λ.K phages modified by M.*Hpa*II and M.*Alu*I methylases, respectively, were used (6). In other cases, we used λ.0 test phage.

[b] EOP of unmodified test phages on the strain carrying pAB66-1 (*ardB*$^+$) relative to that on the same strain without plasmid (or with the ArdB$^-$ plasmid pAB66-7). All strains tested carried the compatible plasmid pOR81 (*ardK*$^+$).

[c] Similar results were obtained with pAB66-6 (*ardB*$^+$).

[d] A kanamycin-resistant derivative of pAB66-1 constructed by inserting the Tn*903* Km^r gene cassette of pUC-4K (40) into the *Sal*I site of pAB66-1 was used.

of *ardB* is regulated by ArdK and ArdR at the transcriptional level. We also found that the promoter activity directed by the upstream sequence of *ardA* (pAB7) was similarly regulated by ArdK and ArdR (data not shown). However, ArdK and ArdR inhibited the activity of *ardA* (in pAB7) less strongly than that of *ardB* (in pAB66-4). This finding is consistent with the suggestion made above that *ardA* is partially derepressed and responsible for low basal level of antirestriction activity expressed by pKM101 in *E. coli* (7).

Figure 4 shows that two imperfect 9- and 11-bp inverted repeats are within the P1 and P2 promoter regions, respectively. It is possible that these repeats may be required for binding the regulatory proteins ArdK and ArdR. We also found that the sequence located between positions 102 and 121 presents a high degree of homology with the consensus sequence of LexA-binding sites (SOS boxes) (41).

ArdB specifically inhibits type I restriction, but unlike ArdA, it does not influence the type I modification. We examined the specificity of ArdB function toward various restriction systems of *E. coli*. Data in Table 1 show that the *ardB*$^+$ plasmid pAB66-1 affects all five of the type I restriction systems tested (*Eco*A, *Eco*B, *Eco*D, *Eco*K, and *Eco*R124). In addition, ArdB slightly affects type II (*Eco*RI) restriction. However, ArdB seems to be ineffective against the type III (*Eco*P1) restriction and the 5-methylcytosine-specific restriction systems McrA and McrBC. These results suggest that like ArdA, ArdB efficiently inhibits only the type I restriction in *E. coli*.

To determine whether antirestriction functions (ArdA and ArdB) influence host-controlled modification of a type I restriction system, we measured *Eco*K modification of progeny phage derived from the unmodified parent phage during a single growth cycle in $r^- m^+$ host carrying the *ardA*$^+$ or *ardB*$^+$ plasmid. The data presented in Table 2 show that host-controlled *Eco*K modification of unmodified phage λ is abolished by the *ardA*$^+$ plasmid pAB7 (6). However, the

TABLE 2. Effects of *ardA* and *ardB* on progeny phage modification

Strain[a]	*ard* gene	Efficiency of *Eco*K modification of progeny phage[b]		Antimodification phenotype
		λ*ral*$^+$.0	λΔ*ral*.0	
BA556		1.0	10^{-2}	−
BA556(pAB7)	*ardA*	10^{-4}	NT	+
BA556(pAB66-1)	*ardB*	1.0	10^{-2}	−

[a] All strains tested carried the compatible plasmid pOR81 (*ardK*$^+$).

[b] Unmodified phages λ.0 were grown for one cycle in $r_K^- m_K^+$ strains, and the efficiency of progeny phage modification was determined as the ratio of phage titer on the restricting indicator strain AB1157 to phage titer on the nonrestricting indicator strain BA556 (18); the bacteriophages referred to as λ*ral*$^+$.0 and λΔ*ral*.0 were λ*vir* and λ*b*506*c*I857*bio*256, respectively. NT, not tested.

presence of the *ardB*$^+$ plasmid pAB66-1 does not influence *Eco*K modification of either λ*ral*$^+$.0 or λΔ*ral*.0 phage. These findings suggest that the ArdB function neither inhibits (like ArdA function) nor stimulates (like Ral function of bacteriophage λ) (46) the modification activity of the *Eco*K system.

DISCUSSION

We show that the IncN plasmid pKM101 encodes the antirestriction protein ArdB in addition to another antirestriction protein, ArdA, described previously (6). These proteins are nonhomologous but functionally similar in that both efficiently inhibit restriction by members of all three families of type I systems of *E. coli*. In addition, they slightly affect the type II (*Eco*RI) restriction and do not influence either the type III (*Eco*P1) restriction or the 5-methylcytosine-specific restriction systems McrA and McrBC. However, unlike ArdA, ArdB is ineffective against the modification activity of type I (*Eco*K) complex.

Analysis of the deduced amino acid sequence of ArdB revealed that, in contrast to the strongly acidic ArdA (an excess of 27 negatively charged amino acids), ArdB is only slightly acidic and contains an excess of only seven acidic residues (Asp and Glu). These antirestriction proteins share only one small region of homology containing nine residues (Fig. 2). Interestingly, this region is also conserved in the 0.3 antirestriction protein of T7 phage, which has no other extensive homology with either ArdA and ArdB (data not shown). It is possible that this protein region is responsible for binding to the restriction-modification complex. These findings suggest that like the 0.3 protein of phage T7 (5), ArdB may inactivate the type I systems by direct binding to a restriction enzyme. On the other hand, the essential difference in sequence between these antirestriction proteins raises the possibility that ArdB has other modes of action.

Data presented here suggest that the expression of antirestriction proteins ArdA and ArdB is regulated jointly by the diffusible *ardK* and *ardR* gene products. Maxicell analysis of protein patterns indicate that these regulatory factors are proteins with molecular weights of about 15,000 and 20,000, respectively. The finding that the sequences immediately upstream of both *ardA* and *ardB* share about 94% homology over 218 bp suggests that their expression may be controlled by ArdK and ArdR at the transcriptional level. To test whether ArdK and ArdR participate in the regulation of ArdB expression, deletion derivatives of the conserved upstream (CUP) sequence of *ardB* were made and cloned into the promoter probe vector pKK232-8*cat*.

Examination of promoter activities of these deletion derivatives showed that the CUP sequence of *ardB* contains at least two putative promoters, P1 and P2. The proposed promoter sequences of *ardA* and *ardB* have extensive similarities, suggesting that the two genes are regulated in similar fashions. We found that the P1 promoter, lying near the predicted ATG initiator codon for *ardB*, exhibits weak promoter activity and seems to be controlled by ArdR. Further upstream, the P2 promoter, with good homology to the consensus sequence, was found. The P2 promoters for *ardA* and *ardB* genes have identical −10 hexamers (GATAAT), but their −35 sites differ in one nucleotide: TTGACT and TTGACC for *ardA* and *ardB*, respectively. The distance between consensus hexamers (17 bp) of both promoters is typical for *E. coli*. Comparison of the sequences of these promoters with the known promoter sequences revealed that both consensus hexamers of the *ardA* promoter are identical to those of p_R promoter of bacteriophage λ (21). In addition, both plasmid and phage promoters have the conserved sequence element 5′-CAT located 5 bp downstream of their −10 sites. These observations suggest that like the p_R promoter of bacteriophage λ, the P2 promoters may be strong.

The finding that plasmids carrying the P2 promoter were stably maintained in *E. coli* cells only when the regulatory protein ArdK was presented in the host implies that the strong P2 promoter is at least partly responsible for the instability of these plasmids in the absence of its repressor, ArdK. This is consistent with the observations that some strong phage promoters cloned into plasmid vectors can adversely affect plasmid stability and are stably maintained only in the presence of transcriptional repressors and terminators (11, 25, 38). Note, however, that in our experiments, the rRNA T1 and T2 terminators encoded by pKK232-8 were insufficient to prevent the instability of the pCAT66 plasmids carrying the P2 promoter in the absence of ArdK and ArdR proteins (Fig. 5). This observation raises the possibility that additional factors are involved in the processes leading to the instability of the cloned upstream regions of *ardA* and *ardB*. Thus, our results suggest that both antirestriction proteins ArdA and ArdB encoded by pKM101 are not lethal to the host cell and that the regulatory upstream regions of both *ard* genes are in fact responsible for the instability of *ard*-encoding plasmids.

Data presented show that both P1 and P2 promoter activities of *ardB* were abolished by the joint action of the ArdK and ArdR proteins. We also found that ArdR, which completely inhibited the downstream P1 promoter, was unable to control P2 promoter activity and seemed only to control the residual activity of P2 promoter repressed by ArdK. ArdK, in turn, was ineffective against P1 promoter activity. One interpretation of these observations is that P1 and P2 promoters are controlled by ArdR and ArdK, respectively, and ArdR may somehow influence the transcription initiated from the upstream promoter P2 by binding to P1 promoter. We also speculate that the inverted repeats presented in both promoter regions may be required for binding of the regulatory proteins ArdK and ArdR. The basis for these interactions is currently under study.

Interestingly, the sequence located about 100 bp upstream from the P2 promoter presents a high degree of homology with the consensus sequence of LexA-binding sites (SOS boxes). This finding suggests that a *lexA*,*recA*-dependent regulatory system (SOS system) may be also involved in regulation of *ardA* and *ardB* expression. This hypothesis is consistent with our observations that the inhibition of *ardA* and *ardB* expression by ArdK and ArdR is more efficient in *recA* mutants than in *recA*$^+$ parent strains (17). Our findings also suggest that the control of expression of *ardA* seems to be relaxed relative to that of *ardB* and that partial derepression of *ardA* may be responsible for the low level of antirestriction activity observed in the presence of pKM101 in the type I restriction-proficient strains of *E. coli*.

A question arises about the biological role of this complex transcriptional regulation of antirestriction functions. Our results suggest that the *ardA* and *ardB* promoters may be induced transiently during conjugation and that the CUP sequences of both antirestriction genes thus may serve as a genetic switch that determines whether both antirestriction functions are expressed (Fig. 1B). This is consistent with the findings that some functions encoded by self-transmissible plasmids are transiently expressed during bacterial conjugation (4, 23). We also suggest that a tandem of *ardAB* promoters which differ in activity and are repressed by different proteins may provide very effective and delicate control of expression of antirestriction functions during mating. The finding that the *ardB* and *ardR* genes may be transcribed in one operon under the control of *ardR*-regulated promoter is consistent with this idea.

It is striking that at least two additional CUP-like sequences were found in the leading region of pKM101 (17). Like the CUP sequence of *ardA*, they are located near the *Not*I sites (Fig. 1A, coordinates 26.8 and 33.3 kb, respectively). Our preliminary data also indicate that these CUP sequences also may control the expression of some plasmid genes, suggesting that all CUP-controlled genes may be members of one regulatory network.

We believe that expression of two different antirestriction functions may give the self-transmissible plasmids an additional advantage in overcoming the host restriction barrier. In addition, the ability of ArdB function to inhibit only the restriction activity of type I systems may facilitate specific modification of plasmid DNA in the new host. The finding that both pKM101-encoded antirestriction functions (ArdA and ArdB) are specific for type I restriction is consistent with our earlier suggestion that unlike type II restriction enzymes, the type I systems are very susceptible to different antirestriction functions (6). We have also speculated that host-controlled (type I) restriction may be programmed to diminish in certain circumstances as result of the induction of some (phage, plasmid, and cellular) antirestriction functions. It is possible that such modulations in activity of type I restriction facilitate the transfer of a foreign gene into cells and make bacterial cells more adaptable.

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