Nucleotide Sequence of the Gene (*ard*) Encoding the Antirestriction Protein of Plasmid ColIb-P9

EUGENE P. DELVER,[1] VERA U. KOTOVA,[2] GENNADI B. ZAVILGELSKY,[2] AND ANATOL A. BELOGUROV[1]*

Department of Biotechnology, Institute of Experimental Cardiology, USSR Cardiology Research Center, Moscow 121552,[1] and Institute of Genetics and Selection of Industrial Microorganisms, Moscow,[2] USSR

The IncI1 plasmid ColIb-P9 was found to encode an antirestriction function. The relevant gene, *ard* (alleviation of restriction of DNA), maps about 5 kb from the origin of transfer, in the region transferred early during bacterial conjugation. Ard inhibits both restriction and modification by each of the four type I systems of *Escherichia coli* tested, but it had no effect on restriction by either *Eco*RI, a type II system, or *Eco*P1, a type III system. The nucleotide sequence of the ColIb *ard* gene was determined; the predicted molecular weight of the Ard polypeptide is 19,193. The proposed polypeptide chain contains an excess of 25 negatively charged amino acids, suggesting that its overall character is very acidic. Deletion analysis of the gene revealed that the Ard protein contained a distinct functional domain located in the COOH-terminal half of the polypeptide. We suggest that the biological role of the ColIb Ard protein is associated with overcoming host-controlled restriction during bacterial conjugation.

A variety of self-transmissible plasmids are able to mediate DNA transfer between different gram-negative bacteria species (13, 14, 43). However, when foreign DNA is introduced into restriction-proficient cells, it is attacked by the restriction endonuclease encoded by the new host (1, 9, 10, 19, 45). These observations are associated with the native mechanism that may control gene flow between different populations of bacteria.

Efficient transfer of the large self-transmissible broad-host-range plasmids suggested that these plasmids may carry genes which express antirestriction functions. Such genes have been described for some bacteriophages (26, 40, 46). Previously, we identified the IncN plasmid pKM101 gene which affected the type I restriction in *Escherichia coli* (8). This gene, named *ard* (alleviation of restriction of DNA), was located near the *oriT* region of pKM101. We have also found that other plasmids of the IncN and IncI incompatibility groups conferred a marked Ard phenotype (25).

In this paper, we show that the IncI1 plasmid ColIb-P9 carries an *ard*-type gene which efficiently prevents restriction and modification by some type I systems in *E. coli*. The location of the ColIb *ard* gene, its nucleotide sequence, and some aspects of its function are reported.

MATERIALS AND METHODS

Bacterial strains. AB1157 is an F⁻ derivative of *E. coli* K-12 having the mutagenotype *thr-1 leu-6 proA2 his-4 thi-1 argE3 lacY1 galK2 ara-14 xyl-5 mtl-1 tsx-33 rpsL31 supE44* (3). AB2463 is a *recA13* derivative of AB1157. Both strains were obtained from P. Howard-Flanders. BA556 is an r_K^- m_K^+ derivative of AB1157 (8). WA2377 (obtained from W. Arber) (2), HB129 (obtained from H. W. Boyer) (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 is the r_R^+ m_{RI}^+ derivative of AB1157 (8), carrying the *Eco*RI plasmid. BA532, the r_{P1}^+ m_{P1}^+ derivative of AB1157 lysogenic for P1CMts, was obtained by chloramphenicol selection at 30°C (8). JM109, which is *recA1 endA1 gyrA96*

thi hsdR17 supE44 relA1 Δ(*lac-proAB*) [F' *traD36 proAB lacIqZΔM15*] (from J. Messing) (44), was used for maxicell analysis and as a host for the plasmids and M13 vectors. JC 8111 (obtained from J. C. Clark), which is a *recB21 recC22 sbcB15 recF148* derivative of *E. coli* K-12, was used for isolation of ColIb-P9. GY7429 (*recA441 sfiB114* Δ*lac* [λ*cI-ind⁻ sfiA::lacZ⁺*]), obtained from R. Devoret, was used for testing the Psi phenotype (6).

Plasmids and phages. Plasmid ColIb-P9 was obtained from N. Datta. Phages M13mp18 and mp19 and plasmids pUC18, pUC19 (44), and pBR322 (12) were used as vectors for cloning and DNA sequence analysis. The bacteriophage referred to as λ was λvir (obtained from R. Devoret). Unmodified phages, denoted by λ.0, were grown on *E. coli* C r_o m_o (9) which lacks restriction and modification functions. Modified phages, denoted by λ.K, were grown on the r_K^+ m_K^+ strain AB1157.

Media and reagents. H, Luria-Bertani, and 2YT media and agars were prepared according to Miller (30) with Difco reagents. If needed, supplements (from Sigma) were added at the following concentrations (in micrograms per milliliter): ampicillin, 100; chloramphenicol, 20; kanamycin, 40; 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal), 40; and isopropyl-β-D-thiogalactopyranoside (IPTG), 240.

DNA manipulation and analysis. Standard procedures were used essentially as described elsewhere (28, 37). Large-plasmid DNA was obtained according to the procedure of Hansen and Olsen (21). For isolation of small plasmid DNA, the procedure of Birnboim and Doly (11) was used. Purification of plasmid DNA was performed by cesium chloride-ethidium bromide density gradient centrifugation. Restriction endonuclease digests were carried out according to the manufacturer's recommendations (New England BioLabs). Digests were analyzed following separation of DNA fragments by electrophoresis in 0.8% agarose gels.

Construction of deletions in the *ard* gene. Plasmid pED1 was made by ligating a 2.3-kb *Eco*RI-*Pvu*II fragment of pVK8 to pUC19 DNA cut with *Eco*RI and *Sma*I. Deletions were made with *Bal* 31 nuclease (New England BioLabs); degradation was initiated from either the *Eco*RI or *Hin*dIII site of pED1. For the construction of deletions from the

---

* Corresponding author.

TABLE 1. Effect of *ard* locus of CoIIb on *Eco*K restriction

Restricting strains	Efficiency of test phage plating[a]	Relief of restriction[b]
AB1157	1×10^{-4}	1
AB1157(CoIIb)	5×10^{-3}	50
AB1157(pVK4)	3×10^{-1}	3,000
AB1157(pVK8)	1.0	10,000
AB1157(pED1)	1.0	10,000
AB1157(pED3)	1×10^{-4}	1
AB1157(pED4)	1×10^{-4}	1

[a] The efficiency of plating of unmodified test phage λ.0 was determined by measuring the ratio of phage titer on the restricting strains to the titer on the nonrestricting strain BA556; in all cases, the plating efficiency of modified phage λ.K was equal to 1.

[b] The relief of *Eco*K restriction is the efficiency of plating of λ.0 on the strains carrying plasmids relative to that on the parent strain AB1157.
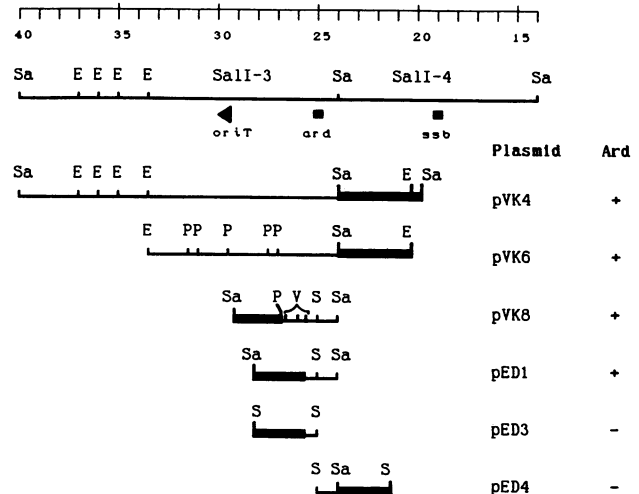

FIG. 1. Restriction map of recombinant plasmids carrying the CoIIb *ard* locus. Line 1 (top) indicates kilobase coordinates on the CoIIb map (22, 32). Line 2 shows restriction sites in the *Sal*I-3 and *Sal*I-4 fragments of CoIIb and the location of the origin of transfer (*oriT*; arrowhead), *ssb* gene (22, 23), and *ard* gene. Vector plasmids are represented by thick horizontal line (pBR322 for pVK4 and pVK6; pUC19 for pVK8, pED1, and pED3; and pUC18 for pED4). Transcription of *ard* and *ssb* is from right to left. Restriction sites: E, *Eco*RI; P, *Pst*I; S, *Sma*I; Sa, *Sal*I; V, *Pvu*II. There are more restriction sites in the vectors than are represented.

*Eco*RI site, pED1 was linearized with *Eco*RI, treated with *Bal* 31 nuclease, and the fragments generated by *Hin*dIII digestion were purified and ligated with *Hin*dIII-*Sma*I cleaved pUC19 DNA. The deletion series from the *Hin*dIII site was generated by *Bal* 31 nuclease treatment of pED80. The endpoints of these deletions were determined by DNA sequence analysis. Plasmid pED3 was constructed by deleting a 1.0-kb *Sma*I fragment of pED1. For the construction of plasmid pED4, the *Sal*I-*Sma*I fragment of pED1 was subcloned into *Sal*I-*Sma*I cleaved pUC18.

Plasmid protein identification. The maxicell procedure of Sancar et al. (34) was used. The [³⁵S]methionine-labeled proteins that were produced were resolved by electrophoresis in a 15% polyacrylamide gel containing sodium dodecyl sulfate (SDS) (27) and visualized by autoradiography.

DNA sequence analysis. Restriction fragments of the *ard* gene region and its deletion derivatives were subcloned into M13mp18 and mp19. The nucleotide sequence was determined by the dideoxy chain termination method (35) with M13 sequence primers and the Klenow fragment of DNA polymerase (Pharmacia).

Measurement of progeny phage modification. 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 the phage titer on the restricting indicator strain (r_K^+) to the phage titer on the nonrestricting indicator strain (r_K^-) (17, 46). The strains were grown in Luria-Bertani medium to about 2×10^8 cells/ml, harvested, and resuspended at a concentration of 1×10^9 cells/ml. Phages were adsorbed at a ratio of 0.01 phages/cell for 20 min at 37°C. The infected cells were diluted 1,000-fold in fresh Luria-Bertani medium and incubated with aeration for 90 min at 37°C. The progeny phages obtained were plated on isogenic r_K^+ and r_K^- strains (AB1157 and BA556).

Nucleotide sequence accession number. The nucleotide sequence of the *ard* gene has been deposited in GenBank.

RESULTS

Cloning of the *ard* gene. Previously, we found that *Eco*K restriction of unmodified phage λ was 50-fold alleviated in *E. coli* K-12 strains harboring plasmid CoIIb-P9 (25) (Table 1). A locus encoding the Ard function (alleviation of restriction of DNA) was identified by subcloning various *Sal*I restriction fragments of CoIIb in ColE1-type vectors (Fig. 1). Recombinant plasmid pVK4, carrying the 16-kb *Sal*I-3 fragment, efficiently prevented *Eco*K restriction of unmodified phage λ in *E. coli* K-12 (Table 1). This drastic increase in Ard

activity presumably reflects the increased copy number of the *ard* locus after subcloning in the multicopy vector. Further subcloning positioned the *ard* locus in a 1.5-kb fragment delimited by *Sal*I and *Pvu*II sites. These studies also showed that the DNA spanning the *Sma*I site was needed for expression of the Ard function (Fig. 1). The *ard* coding region was more precisely located by *Bal* 31 deletions (Fig. 2). In the deletion series from the *Rsa*I site, the Ard⁺ phenotype was lost when the deletion extended to 220 bp (pED220). In the other deletion series, a 255-bp deletion from the *Pvu*II site (pED707) inactivated Ard function.

Identification of the *ard* gene product. The gene product associated with Ard function was identified by labeling proteins encoded by each of the recombinant plasmids in *E. coli* maxicells. Examination of the fluorogram presented in Fig. 3 reveals that the Ard⁺ plasmids pED80, pED199 (lanes 2 and 3), and pED760 (data not shown) encode a protein of about 22 kDa which is absent in cells carrying vector plasmid (lane 1). Neither of the Ard⁻ plasmids pED220 and pED605 (Fig. 2) direct the synthesis of the 22-kDa protein. This polypeptide is absent from maxicells harboring the deletion derivative pED220, which lacks the 220-bp region rightward from *Rsa*I (Fig. 3, lane 3). In contrast, pED605, which was obtained by deleting the *ard* insert from the *Pvu*II site, specifies a markedly smaller polypeptide (about 20 kDa) (Fig. 3, lane 4). One interpretation of these observations is that deleting the *ard* gene region from the *Rsa*I site (pED220) inactivates the *ard* promoter, while deletion from the *Pvu*II site (pED605) removes the 3' portion of *ard* and induces the formation of a fusion polypeptide.

Support for this conclusion was obtained by inverting the orientation of the *ard* insert with respect to the *lac* promoter of vector. Fig. 2 shows that in contrast to pED220, plasmid pED220R, which contains the same insert as pED220 but in the opposite orientation with respect to the *lac* promoter,
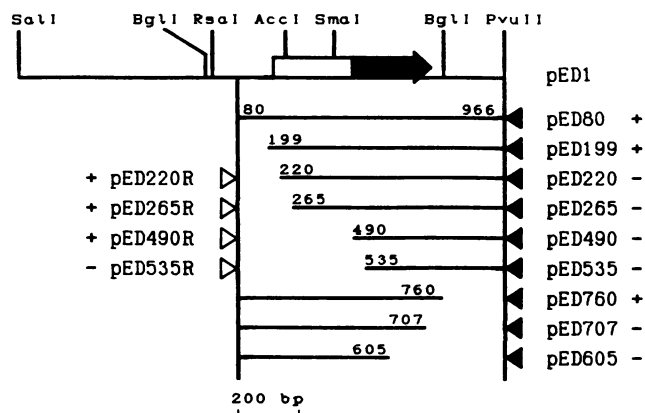
FIG. 2. Location of the *ard* gene and its 3'-portion encoding a functional domain of the Ard protein. The top of the figure shows the position of restriction enzyme sites in the Collb insert on pED1 (this map is inverted relative to Fig. 1). Deletions extending from the *Sal*I and *Pvu*II sites of the *ard* insert were obtained by *Bal* 31 treatment, and the truncated fragments were cloned into pUC18 and pUC19 in two different orientations with respect to the *lac* promoter, as designated by triangles. Deletion endpoints were determined by DNA sequence analysis and indicated by the sequence numbers used in Fig. 4. Individual transformants of AB1157 or AB2463 by these recombinant plasmids were tested for Ard phenotype with (▷) or without (◀) IPTG. + and − signs represent the presence or absence of Ard activity, respectively. The arrow indicates the extent and direction of the *ard* coding region. The solid region of the arrow represents the 3' portion of *ard* that codes for a functional domain of the Ard protein.

FIG. 3. Polypeptide synthesis directed by *ard*-derivative plasmids in maxicells. Maxicells were labeled with [^{35}S]methionine and analyzed in SDS-15% polyacrylamide gels. Lanes: 1, vector pUC19; 2, pED80; 3, pED199; 4, pED220; 5, pED605. Arrowheads indicate the presumptive products of the normal and mutant *ard* genes. The other identifiable polypeptide is β-lactamase (30 kDa), specified by the vector. Positions of size standards (in kilodaltons) are indicated on the left.

express Ard activity in the presence of IPTG, an inducer of the *lac* operon. This observation strongly indicates that pED220R contains the *ard* gene in the correct direction for *lac* promoter activity and provides confirmation that transcription of the *ard* gene is from left to right, as suggested above (Fig. 2). Thus, from deletion studies and maxicell analysis of protein patterns, we located the *ard* gene within the *Rsa*I-*Bgl*I fragment and identified the *ard* gene product.

Nucleotide sequence of the *ard* gene. The sequence of *ard* region was determined for both strands from overlapping DNA fragments. Nucleotide sequence of the coding strand, presented in Fig. 4, revealed (Fig. 2) only one long open reading frame (ORF) (residues 214 to 727) that could code for a polypeptide of about 22 kDa. Figure 2 shows that the 5' end of the *ard* gene is located between nucleotides 200 and 221. On the basis of these data, two potential initiator codons were identified (positions 212 and 227). However, only the ATG codon at nucleotide 227 was preceded by a strong Shine-Dalgarno sequence, TAtGGAGG (lowercase letters represent divergence from the consensus sequence) (36). The spacing between the 3' end of this sequence and the first nucleotide of the initiation codon is 7 nucleotides, which is common for *E. coli* ribosome-binding sites (39). These data suggest that the *ard* coding region begins with an ATG at bp 227 and extends 498 nucleotides to a TGA stop codon at bp 725. This open reading frame would code for a polypeptide of 166 amino acids, with a predicted molecular weight of 19,193. Although the Ard protein migrates on SDS gels with an apparent molecular weight of about 22,000 (Fig. 3), we suggest that this ORF must be the *ard* gene. We have no obvious explanation for the disparity between the estimated and calculated molecular weights of the Ard protein.
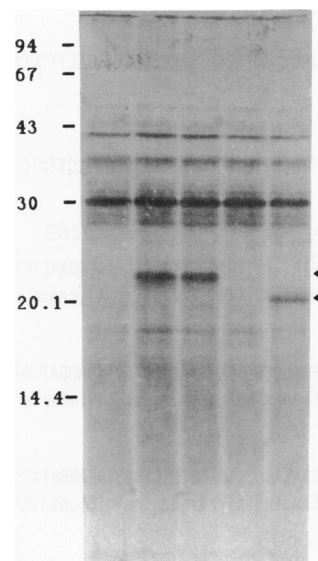
Upstream of this ORF, a poor −10 hexamer (gAacAT) at

nucleotides 200 to 205 was found. There was no hexamer at the appropriate distance away that showed significant homology to the consensus −35 sequence. This finding is consistent with the observation that a 199-bp deletion from the *Rsa*I site (pED199) which removed a potential −35 consensus region did not affect Ard function or the expression of the Ard protein in maxicells (Fig. 2 and 3).

Codon usage. The codon usage for the *ard* gene is consistent with the nonrandom codon usage identified for a number of *E. coli* genes (24). The frequency of rare codons such as ATA (Ile), TCG (Ser), CCT and CCC (Pro), ACG (Thr), CAA (Gln), AAT (Asn), and AGG (Arg) was 3.6%. This value is around the average for genes that are moderately expressed in *E. coli* (24).

It should be noted that the proposed polypeptide chain contains an excess of 25 negatively charged amino acids (Asp + Glu), suggesting that its overall character is very acidic.

The Ard protein contains the functional antirestriction domain. We have found that deletion of 13 codons from the 5' end of the putative *ard* gene in pED265R, making the remaining part of *ard* fused in frame with *lacZ'*, did not affect Ard function (Fig. 2). To determine which part of the protein is essential for Ard activity, the *ard* coding region was sequentially deleted from the 5' end by using *Bal* 31 exonuclease. The remaining part of the gene was religated to the 5' portion of *lacZ* contained in pUC19.

Figure 2 shows the Ard phenotypes of the deletion derivatives pED490R and pED535R, in which the *ard* gene region was fused in frame with *lacZ'*. Deleting up to nucleotide 535, as in pED535R, resulted in an Ard$^-$ phenotype, while deleting up to position 490 (pED490R) did not affect Ard activity. This result suggests that the 5' endpoint of the coding sequence for the Ard functional domain must occur between nucleotides 490 and 535. All of the deletions at the
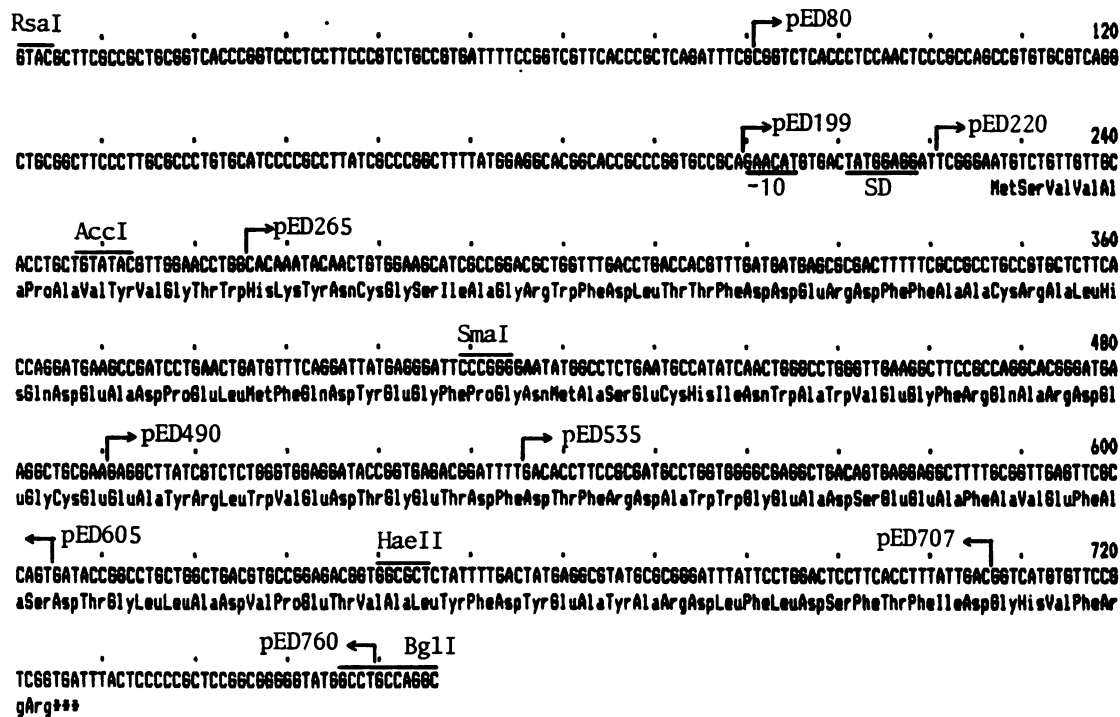
RsaI · · · · · · · · ┌→pED80 · · 120
GTACGCTTCGCCGCTGCGGTCACCCGGTCCCTCCTTCCCGTCTGCCGTGATTTTCCGGTCGTTCACCCGCTCAGATTTCGCGGTCTCACCCTCCAACTCCCGCCAGCCGTGTGCGTCAGG

· · · · · · · · ┌→pED199 · ┌→pED220 240
CTGCGGCTTCCCTTGCGCCCTGTGCATCCCCGCCTTATCGCCCGGCTTTTATGGAGGCACGGCACCGCCCGGTGCCGCAGAACATGTGACTATGGAGGATTCGGGAATGTCTGTTGTTGC
                                                                        -10   SD   MetSerValValAl

AccI · ┌→pED265 · · · · · · · 360
ACCTGCTGTATACGTTGGAACCTGGCACAAATACAACTGTGGAAGCATCGCCGGACGCTGGTTTGACCTGACCACGTTTGATGATGAGCGCGACTTTTTCGCCGCCTGCCGTGCTCTTCA
aProAlaValTyrValGlyThrTrpHisLysTyrAsnCysGlySerIleAlaGlyArgTrpPheAspLeuThrThrPheAspAspGluArgAspPhePheAlaAlaCysArgAlaLeuHi

· · · · Sma I · · · · · 480
CCAGGATGAAGCCGATCCTGAACTGATGTTTCAGGATTATGAGGGATTCCCGGGGAATATGGCCTCTGAATGCCATATCAACTGGGCCTGGGTTGAAGGCTTCCGCCAGGCACGGGATGA
sGlnAspGluAlaAspProGluLeuMetPheGlnAspTyrGluGlyPheProGlyAsnMetAlaSerGluCysHisIleAsnTrpAlaTrpValGluGlyPheArgGlnAlaArgAspGl

┌→pED490 · · · ┌→pED535 · · · · 600
AGGCTGCGAAGAGGCTTATCGTCTCTGGGTGGAGGATACCGGTGAGACGGATTTTGACACCTTCCGCGATGCCTGGTGGGGCGAGGCTGACAGTGAGGAGGCTTTTGCGGTTGAGTTCGC
uGlyCysGluGluAlaTyrArgLeuTrpValGluAspThrGlyGluThrAspPheAspThrPheArgAspAlaTrpTrpGlyGluAlaAspSerGluGluAlaPheAlaValGluPheAl

←┐pED605 · HaeII · · · pED707 ←┐ 720
CAGTGATACCGGCCTGCTGGCTGACGTGCCGGAGACGGTGGCGCTCTATTTTGACTATGAGGCGTATGCGCGGGATTTATTCCTGGACTCCTTCACCTTTATTGACGGTCATGTGTTCCG
aSerAspThrGlyLeuLeuAlaAspValProGluThrValAlaLeuTyrPheAspTyrGluAlaTyrAlaArgAspLeuPheLeuAspSerPheThrPheIleAspGlyHisValPheAr

· · pED760 ←┐ BglI
TCGGTGATTTACTCCCCCGCTCCGGCGGGGGTATGGCCTGCCAGGC
gArg***

FIG. 4. Nucleotide sequence of the *ard* gene. The sequence numbering begins at the *Rsa*I site located upstream of *ard* and extends to the *Bgl*I site. The deduced amino acid sequence is also shown. The proposed −10 sequence and the proposed Shine-Dalgarno site are underlined and labeled. Arrows in the coding sequence indicate the endpoints in the deleted clones.

3' end of the gene resulted in the loss of Ard activity (Fig. 2). Thus, these data indicate that the Ard protein contains a distinct functional domain located in the COOH-terminal half of the polypeptide (Fig. 2). No data are available on the function of the N-terminal half of the protein.

Specificity of Ard function. We examined the specificity of Ard function toward various restriction systems of *E. coli*. Data in Table 2 show that plasmid pED80, carrying the cloned *ard* gene, affects all of the type I restriction systems tested (*Eco*A, *Eco*B, *Eco*D, and *Eco*K). No influence of *ard* on either the type II (*Eco*RI) or the type III (*Eco*P1) restriction system was detected. These results suggest that *ard* specifically interferes with type I restriction systems.

***ard* affects *Eco*K modification.** To determine whether Ard function influences host-controlled modification of the type I restriction system, we measured *Eco*K modification of progeny phage derived from the unmodified parent phage during a single growth cycle in a r⁻ m⁺ host carrying an *ard*⁺ or *ard*⁻ plasmid. The data presented in Table 3 show that host-controlled *Eco*K modification of unmodified phage λ is abolished by the presence of pED80, harboring the cloned CoIIb *ard* gene. This result strongly indicates that the CoIIb *ard* gene affects *Eco*K modification. We have also found that *ard* is effective against *Eco*A and *Eco*D modification (data not shown). We conclude that both activities of the type I restriction systems (restriction and modification) are affected by the CoIIb *ard* gene.

The Ard polypeptide has no Psi-like activity. Since some CoIIb mutants were found to confer a marked Psi phenotype (plasmid-mediated SOS inhibition) (22), and since *psi*-type genes seemed to be located in the leading region of the IncI1 plasmids (20), we tested whether the Ard protein was functionally related to the Psi protein. Expression of Psi

interferes with induction of the SOS regulon, which includes the *sfiA* gene (4), and Psi function is monitored conveniently by measuring the β-galactosidase activity specified by *sfiA::lacZ*⁺ fusion in a *recA441* background (GY7429) at 42°C (20).

In *recA441 sfiA::lacZ*⁺ cells, the level of β-galactosidase produced at 42°C for 2 h was about 2,800 U/mg, and this level did not decrease when pVK8 carrying the cloned CoIIb

TABLE 2. Effect of *ard* on different *E. coli* restriction systems

Restriction system[a]	Efficiency of test phage plating on the strains[b]		Relief of restriction[c]
	Without pED80*ard*⁺	With pED80*ard*⁺	
*Eco*A (Type I)	4 × 10⁻³	1.0	250
*Eco*B (Type I)	2 × 10⁻⁴	1.0	5,000
*Eco*D (Type I)	4 × 10⁻⁴	1.0	2,500
*Eco*K (Type I)	1 × 10⁻⁴	1.0	10,000
*Eco*RI (Type II)	2 × 10⁻⁴	2 × 10⁻⁴[d]	1
*Eco*P1 (Type III)	5 × 10⁻⁵	5 × 10⁻⁵	1

[a] The restriction strains used were: *Eco*A, WA2377; *Eco*B, HB129; *Eco*D, BZ216; *Eco*K, AB1157; *Eco*RI, BA509; and *Eco*P1, BA532. Since strains BA509 and BA532 contain two restriction systems, we used λ.K test phages in order to subject them to only *Eco*RI and *Eco*P1 restriction. In other cases, we used the λ.0 test phage.

[b] The efficiency of plating of unmodified test phage was determined as described in footnote *a* to Table 1.

[c] The relief of restriction is the efficiency of plating of unmodified test phages on the strain carrying pED80 relative to that on the same strain without plasmid (or with pED220*ard*⁻).

[d] A kanamycin-resistant derivative of pED80 constructed by inserting the Tn*903* Km^r gene cassette of pUC-4K (42) into the *Bam*HI site of pED80 was used.

TABLE 3. Effect of *ard* on progeny phage modification

Strains	*ard*	Efficiency of *EcoK* modification of progeny phages	Amd[a]
JM109	−	1.0	−
JM109(pED80)	+	1×10^{-4}	+
JM109(pED220)	−	1.0	−

[a] Amd phenotype (alleviation of modification of DNA).

ard gene was introduced into the cells (2,500 U/mg). In contrast, plasmid R100, which is known to confer the Psi phenotype (5, 20), reduced β-galactosidase expression in the *recA441 sfiA::lacZ*⁺ host by a factor of 10. These results clearly indicate that the Ard protein has no Psi-like function.

DISCUSSION

We have described a plasmid gene, designated *ard* (alleviation of restriction of DNA), whose expression specifically affected members of two families of type I restriction and modification systems in *E. coli*. The position of the *ard* gene within the ColIb plasmid was located in the region of the transfer origin *oriT* (Fig. 1) that is known to be transferred early during bacterial conjugation (23). This observation is consistent with our suggestion that Ard function permits self-transmissible plasmids to overcome the host-controlled restriction barrier during conjugation. Further support for this idea came from the findings that the IncN plasmid pKM101, a derivative of R46, carries near the *oriT* region an *ard*-type gene which affects type I restriction (8), and that other IncN (N3 and pJA4733), IncI (R64, R144, R621a, and R648), and IncFII (R6-5 and R100) plasmids exhibit a marked Ard phenotype (unpublished data) (25).

The nucleotide sequence of the *ard* coding region revealed an ORF of 498 bp that would code for a 19.2-kDa protein containing 166 amino acids. Although the Ard polypeptide migrates on SDS gels with an apparent molecular mass of about 22 kDa, the deletion studies and maxicell analysis of protein patterns indicate that this ORF is in fact the *ard* gene. A search through 1991 versions of GenBank sequence and NBRF protein sequence data bases revealed no major similarities with *ard* and provided no obvious clues to the mechanism of Ard function.

We suggest that the antirestriction activity of the Ard protein might result from either of two types of interaction. Ard protein (i) may interact directly with the restriction-modification complex or (ii) may interfere or compete with the restriction-modification system binding to its recognition sites.

Binding to DNA seems unlikely, given the strongly acidic nature of the Ard protein. On the other hand, the large number of acidic residues in the Ard protein suggests that it may act as a polyanion that can inactivate the host restriction-modification complex by binding to its DNA-binding site. A similar mechanism of antirestriction has been proposed for the acidic 0.3 protein of phage T7 (16). This protein inhibits the host-controlled restriction and modification systems by direct binding (7, 16, 38) and may serve as a model for Ard protein. It is interesting to note that although no homologies between the nucleotide and deduced amino acid sequences of ColIb *ard* and T7 *0.3* genes are found, these systems share a number of features. Both are acidic proteins encoded by genes located in regions of the phage or plasmid which enter the host cell early. They both inhibit both the

restriction and modification activities of the type I systems in *E. coli* and do not affect the type II restriction enzymes (7, 29). Our future studies will be directed toward a purification of the Ard protein and an examination of its interaction with the type I restriction-modification complex.

It should be noted that Ard function is probably unrelated to the antirestriction function encoded by the *ral* gene of bacteriophage λ (46). This conclusion is based on the observation that in contrast to the Ard and 0.3 gene proteins, the *ral* gene product seems to operate not by inhibiting restriction, but by stimulating the modification activity of type I systems (46).

At least three cellular functions have been found to specifically affect type I restriction in *E. coli*. They are induced when the cell's DNA is damaged by agents such as UV radiation (15) or the base analog 2-aminopurine (18) or when the DNA is unmethylated in *dam* mutants, which are deficient in adenine methylase activity (17). UV-induced alleviation of restriction seems to be an SOS function (15, 41), while others are probably not members of the SOS regulatory network (17, 18). Interestingly, one of them, the alleviation function, induced in *dam* mutants, inhibits both the restriction and modification activities of the *EcoK* (Type I) system (17) and looks like the *ard* and *0.3* gene-encoded functions in this respect. However, it is still unclear what relation is between the "guest" (phage and plasmid-encoded) and host antirestriction functions, because the genes encoding host-controlled alleviation have not yet been identified.

Note that the Ard function is active in *recA*(Def) mutants of *E. coli* K-12 (data not shown). This observation suggests that the Ard protein does not act as an inducer of SOS alleviation in the host cell.

Deletion studies and examination of the *ard* nucleotide sequence reveal potential regulatory signals for the gene. Upstream of the structural gene, a poor −10 hexamer was found, but no consensus −35 site was observed. It should be noted that adenine and thymine nucleotides in the −10 region of positively regulated *E. coli* promoters, which were shown to be the most highly conserved (31), were present in the proposed *ard* promoter (Fig. 4, positions 201 and 205, respectively). These nucleotides are believed to be the minimum requirement for *E. coli* RNA polymerase recognition. The absence of a −35 site suggests that the *ard* gene may be controlled by an activator protein (31).

Recently we have found that upstream and downstream sequences might influence *ard* expression. A subsequent paper will report details of regulation of *ard* expression in the plasmid ColIb.

REFERENCES

1. **Arber, W.** 1971. Host-controlled variation, p. 83. *In* A. D. Hershey (ed.), The bacteriophage lambda. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
2. **Arber, W., and D. Wauters-Willems.** 1970. Host specificity of DNA produced by *Escherichia coli*. XII. The two restriction and modification systems of strain 15T⁻. Mol. Gen. Genet. **108:**203–217.
3. **Bachmann, B. J.** 1972. Pedigrees of some mutant strains of *Escherichia coli* K-12. Bacteriol. Rev. **36:**525–557.
4. **Bagdasarian, M., A. Bailone, M. M. Bagdasarian, P. A. Manning, R. Lurz, K. N. Timmis, and R. Devoret.** 1986. An inhibitor

of SOS induction specified by a plasmid locus in *Escherichia coli*. Proc. Natl. Acad. Sci. USA **83**:5723–5726.

5. **Bagdasarian, M., R. D'Ari, W. Filipowicz, and J. George.** 1980. Suppression of induction of SOS functions in an *Escherichia coli tif-1* mutant by the plasmid R100.1. J. Bacteriol. **141**:464–469.

6. **Bailone, A., A. Backman, S. Sommer, J. Celerier, M. M. Bagdasarian, M. Bagdasarian, and R. Devoret.** 1988. PsiB polypeptide prevents activation of RecA protein in *Escherichia coli*. Mol. Gen. Genet. **214**:389–395.

7. **Bandyopadhyay, P. K., F. W. Studier, D. L. Hamilton, and R. Yuan.** 1985. Inhibition of the type I restriction-modification enzymes *Eco*B and *Eco*K by the gene 0.3 protein of bacteriophage T7. J. Mol. Biol. **182**:567–578.

8. **Belogurov, A. A., T. N. Yussifov, V. U. Kotova, and G. B. Zavilgelsky.** 1985. The novel gene(s) *ard* of plasmid pKM101: alleviation of *Eco*K restriction. Mol. Gen. Genet. **198**:509–513.

9. **Bertani, G., and J. J. Weigle.** 1953. Host-controlled variation in bacterial viruses. J. Bacteriol. **65**:113–121.

10. **Bickle, T. A.** 1982. The ATP-dependent restriction endonucleases, p. 85–108. *In* S. M. Linn and R. J. Roberts (ed.), Nucleases. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.

11. **Birnboim, H. C., and J. Doly.** 1979. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acids Res. **7**:1513–1523.

12. **Bolivar, F., R. L. Rodriguez, P. J. Green, M. C. Betlach, H. L. Heynecker, H. W. Boyer, J. H. Crosa, and S. Falkow.** 1977. Construction and characterization of new cloning vehicles. II. A multipurpose cloning system. Gene **2**:95–113.

13. **Bradley, D. E.** 1981. Conjugative pili of plasmids in *Escherichia coli* K-12 and *Pseudomonas* species, p. 217–226. *In* S. B. Levy, R. C. Clowes, and E. L. Koenig (ed.), Molecular biology, pathogenicity, and ecology of bacterial plasmids. Plenum Press, New York.

14. **Datta, N.** 1979. Plasmid classification: incompatibility grouping, p. 3–12. *In* K. N. Timmis and A. Puhler (ed.), Plasmids of medical, environmental and commercial importance. Elsevier/North-Holland Biomedical Press, Amsterdam.

15. **Day, S. R.** 1977. UV-induced alleviation of K-specific restriction of bacteriophage λ. J. Virol. **21**:1249–1251.

16. **Dunn, J. J., M. Elzinga, K.-K. Mark, and F. W. Studier.** 1981. Amino acid sequence of the gene 0.3 protein of bacteriophage T7 and nucleotide sequence of its mRNA. J. Biol. Chem. **256**:2579–2585.

17. **Efimova, E. P., E. P. Delver, and A. A. Belogurov.** 1988. Alleviation of type I restriction in adenine methylase (*dam*) mutants of *Escherichia coli*. Mol. Gen. Genet. **214**:313–316.

18. **Efimova, E. P., E. P. Delver, and A. A. Belogurov.** 1988. 2-Aminopurine and 5-bromouracil induce alleviation of type I restriction in *Escherichia coli*: mismatches function as inducing signals? Mol. Gen. Genet. **214**:317–320.

19. **Endlich, B., and S. Linn.** 1985. The DNA restriction endonuclease of *Escherichia coli* B. II. Further studies of the structure of DNA intermediates and products. J. Biol. Chem. **260**:5729–5738.

20. **Golub, E., A. Bailone, and R. Devoret.** 1988. A gene encoding an SOS inhibitor is present in different conjugative plasmids. J. Bacteriol. **170**:4392–4394.

21. **Hansen, J. B., and R. H. Olsen.** 1978. Isolation of large plasmids and characterization of the P2 incompatibility group plasmid pMG1 and pMG5. J. Bacteriol. **135**:227–238.

22. **Howland, C. J., C. E. D. Rees, P. T. Barth, and B. M. Wilkins.** 1989. The *ssb* gene of plasmid ColIb-P9. J. Bacteriol. **171**:2466–2473.

23. **Howland, C. J., and B. M. Wilkins.** 1988. Direction of conjugative transfer of IncI1 plasmid ColIb-P9. J. Bacteriol. **170**:4958–4959.

24. **Konigsberg, W., and G. N. Godson.** 1983. Evidence for use of rare codons in the *dnaG* gene and other regulatory genes of *Escherichia coli*. Proc. Natl. Acad. Sci. USA **80**:687–691.

25. **Kotova, V. U., G. B. Zavilgelsky, and A. A. Belogurov.** 1988. Plasmid ColIb-P9 (IncI)-mediated alleviation of the type I restriction: general characterization and molecular cloning of *ard* gene. Mol. Biol. **22**:270–276. (In Russian.)

26. **Kruger, D. H., and T. A. Bickle.** 1983. Bacteriophage survival: multiple mechanisms for avoiding the deoxyribonucleic acid restriction systems of their hosts. Microbiol. Rev. **47**:345–360.

27. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.

28. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.

29. **Mark, K.-K., and F. W. Studier.** 1981. Purification of the gene 0.3 protein of bacteriophage T7, inhibitor of the DNA restriction system of *Escherichia coli*. J. Biol. Chem. **256**:2573–2578.

30. **Miller, J. H.** 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.

31. **Raibaud, O., and M. Schwartz.** 1984. Positive control of transcription initiation in bacteria. Annu. Rev. Genet. **18**:173–206.

32. **Rees, C. E. D., D. E. Bradley, and B. M. Wilkins.** 1987. Organization and regulation of the conjugation genes of IncI1 plasmid ColIb-P9. Plasmid **18**:223–236.

33. **Roulland-Dussoix, D., H. W. Boyer.** 1969. The *Escherichia coli* B restriction endonuclease. Biochim. Biophys. Acta **195**:219–229.

34. **Sancar, A., A. M. Hack, and W. D. Rupp.** 1979. Simple method for identification of plasmid-coded proteins. J. Bacteriol. **137**:692–693.

35. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74**:5463–5467.

36. **Shine, J., and C. Dalgarno.** 1974. The 3'-terminal sequence of *Escherichia coli* 16S-ribosomal RNA: complementary to nonsense triplets and ribosome binding sites. Proc. Natl. Acad. Sci. USA **71**:1342–1346.

37. **Silhavy, T. J., M. L. Berman, and L. W. Enquist.** 1984. Experiments with gene fusions. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.

38. **Spoerel, N., P. Herrlich, and T. A. Bickle.** 1979. A novel bacteriophage defence mechanism: the anti-restriction protein. Nature (London) **278**:30–34.

39. **Stormo, G. D., T. D. Schneider, and L. Gold.** 1982. Characterization of translation initiation sites in *E. coli*. Nucleic Acids Res. **10**:2971–2996.

40. **Studier, F. W.** 1975. Gene 0.3 of bacteriophage T7 acts to overcome the DNA restriction system of the host. J. Mol. Biol. **94**:283–295.

41. **Toms, B., and W. Wackernagel.** 1982. UV-induced alleviation of restriction in *E. coli* K12: kinetics of induction and specificity of this SOS function. Mol. Gen. Genet. **186**:111–117.

42. **Vieira, J., and J. Messing.** 1982. The pUC plasmids, an M13mp7-derived system for insertion mutagenesis and sequencing with synthetic universal primer. Gene **19**:259–268.

43. **Willetts, N., and B. Wilkins.** 1984. Processing of plasmid DNA during bacterial conjugation. Microbiol. Rev. **48**:24–41.

44. **Yanisch-Perron, C., J. Vieira, and J. Messing.** 1985. Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. Gene **33**:103–119.

45. **Yuan, R.** 1981. Structure and mechanism of multifunctional restriction endonucleases. Annu. Rev. Biochem. **50**:285–315.

46. **Zabeau, M., S. Friedman, M. Van Montagu, and J. Schell.** 1980. The *ral* gene of phage λ. Identification of a nonessential gene that modulates restriction and modification in *E. coli*. Mol. Gen. Genet. **179**:63–74.