Journal of Biochemistry and Molecular Biology, Vol. 34, No. 6, November 2001, pp. 584-589

JOURNAL OF
Biochemistry and Molecular Biology

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Short communication

Characterization of the Small Cryptic Plasmid, pGD2, of *Klebsiellia* sp. KCL-2.

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Received 13 June 2001, Accepted 9 July 2001

One of the cryptic plasmids from the oil degrading bacterium *Klebsiella* sp. KCL-2, the small plasmid pGD2, has been identified and characterized. This plasmid has a size of 3.6 kb with unknown functions. We constructed the recombinant plasmid pMGD2. The nucleotide sequences of the plasmid were determined and two open reading frames were detected. ORF1 encodes a replication initiator protein (RepA), which has a high degree of homology with the protein of ColE2 plasmid. The product encoded by ORF2 showed a high similarity with the transposase protein of IS5. IS5 is 1195 bp long and contains an inverted terminal repetition of 16 bp with one mismatch. Stem-loop structures in the 5'untranslated region of the *repA* suggest that a putative gene, *incA*, is located in a complementary strand to the leader region of the *repA* mRNA.

Keywords: *Klebsiella* sp., Cryptic plasmid, Replication initiator protein, IS5.

Introduction

Several bacterial strains that utilize crude oil as their sole carbon and energy sources were isolated from marine (Cha *et al.*, 1999; Cha *et al.*, 2000). One of the strains, *Klebsiella* sp. KCL-2, contains several cryptic plasmids (Cha *et al.*, 2000). These plasmids may encode a large number of genes. Thus, many of the oil-degrading bacteria that had plasmids and certain genes for oil utilization were located on the plasmid of *Pseudomonas maltophilia* N246 (Choi *et al.*, 1991; Kim *et al.*, 1993). However, the function of these plasmids is unknown.

Plasmids ColE2-P9 and ColE3-CA38 require plasmid-coded Rep proteins for plasmid replication (Itoh & Horii, 1989). The Rep protein of some replicons, such as *Pseudomonas* pPS10 and *Enterobacterial* pSC101, F and R6K, have an important second function they recognize inversely repeated sequences as an operator, which overlaps the promoter of their own coding genes, acting as self-repressors. These plasmids are useful model systems for the analysis of the initiation mechanism of DNA replication (Jeong *et al.*, 1995).

Insertion sequences are a class of short DNA segments with special properties. They are found in multiple copies in the chromosomes of prokaryotes and in their episomes (Kroger *et al.*, 1982; Naas *et al.*, 1999). At low frequency these elements can transpose to other locations in the chromosome. In recent years, a number of cryptic plasmids were isolated and analyzed from several strains (Mibas *et al.*, 1993; Vagars *et al.*, 1995; Mikiewicz *et al.*, 1997; Dery *et al.*, 1997; Vagars *et al.*, 1999). However, the functions of most of these plasmids remain unknown. In this study, we report the isolation of cryptic plasmid from *Klebsiella* sp. KCL-2. A small plasmid, pGD2 has been selected for further molecular characterization.

Materials and Methods

Bacterial strains, plasmids and culture conditions The *Klebsiella* sp. KCL-2 strain was isolated from polluted seawater (Cha *et al.*, 2000). The *Klebsiella* strain was routinely grown in a carbon minimal medium, which contained 1% crude oil (Cha *et al.*, 1999). *E. coli* strains were grown aerobically in a LB medium. pBluescript KS (+) and pKK223-4 were used for cloning vectors (Park *et al.*, 1999; Cha *et al.*, 2000; Kim, K. J., 2000). All of the cultures were incubated at 37°C. The used antibiotic concentration was 50 μg/ml ampicillin.

DNA isolation and manipulation Rapid, small-scale plasmid DNA isolation was performed by the method of Birnbaum and Doly (1979). Plasmid preparation for sequencing purposes was performed by using the Wizard kit of Promega Biotech. All of the restriction enzymes, T4 DNA ligation kit ver. 2, and polynucleotide kinase were purchased from the Takara Shuzo Co. and used according to the recommendation of the suppliers. General recombinant DNA manipulation was carried out according to the

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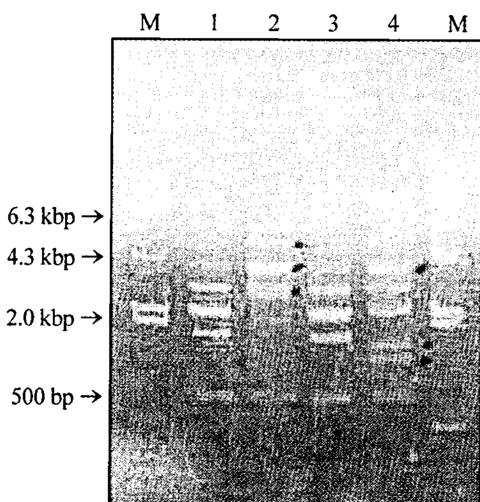

Fig. 1. Agarose gel electrophoresis pattern of the plasmid DNA isolated from *Klebsiella* sp. KCL-2 M; size marker, 1; KCL-2, 2; KCL-2/ *Eco*RI, 3; KCL-2/*Bam*HI, 4; KCL-2/ *Hind* III.

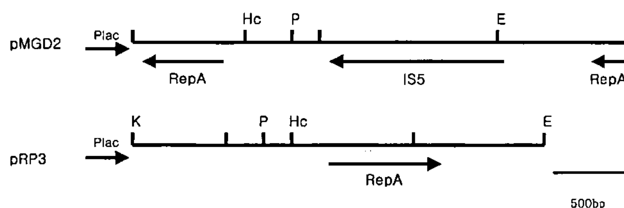

Fig. 2. Physical maps of pMGD2 and pRP3 carrying the *repA* and *IS5* genes. The thick arrows show the transcriptional directions of the *repA* and *IS5* genes. Abbreviations: H; *Hind* III, Hc; *Hinc* II, P; *Pst*I, E; *Eco*RI, K; *Kpn* I, S; *Sac* II, C; *Cla* I, Plac; *lac* promoter.

protocol suggested by Sambrook *et al.* (1989).

Cloning and sequencing of the pGD2 plasmid Plasmid pMGD2 was constructed by digestion of pGD2 with *Hin*dIII and ligation to *Hin*dIII-digested pBluescript (pKS+) (Fig. 2). Various restriction fragments, derived from the pMGD2 plasmid DNA, were subcloned into the same restriction enzyme site of pKS (+). Double-stranded DNA was used as templates for the sequencing reactions. Both strands of the DNA were sequenced by the dideoxy-chain termination method, using an AutoRead DNA sequencing kit and A.L.F. DNA sequencer (Pharmacia) (Sanger *et al.*, 1977). The DNA sequences were analyzed with the BLAST program of the National Center for Biotechnology Information (Bethesda, USA).

Nucleotide sequence accession number The nucleotide sequence data reported in this paper was deposited in the GenBank Library under Accession No. AY033498.

Results and Discussion

Isolation of cryptic plasmid from oil degrading strain In a previous paper (Cha *et al.*, 2000), we reported the isolation of an oil degrading strain, *Klebsiella* sp KCL-2. In this study the strain was screened in order to identify the presence of plasmid DNA. When the Eckhardt method was utilized, no megaplasmid was detected in the strain (Eckhardt, 1978). When the alkaline-lysis method was used, however, four plasmids were detected. A preliminary restriction analysis of the plasmids indicated that the plasmids possessed sites for *Hind* III (Fig. 1). In order to analyze and construct a detailed restriction map of the plasmid, the 3.6 kb *Hind* III-digested plasmid DNA, pGD2, was subcloned into pBluescript KS (+), resulting in plasmid pMGD2. The inability of pMGD2 to replicate in *Klebsiella* sp. suggested that the restriction site *Hind* III lay in an essential replication region of pGD2. To identify the region of pGD2 required for replication, we constructed recombinant plasmid pRP3. For the construction of pRP3, the 3 kb *Kpn* I- *Eco*R I region of pGD2 was inserted into the same restriction sites of pKS(+) (Fig. 2). It was found that pRP3 was able to replicate in both *E. coli* and *Klebsiella* sp. KCL-2.

Primary structure analysis of pGD2 plasmid originated *Klebsiella* sp. To confirm whether the pGD2 plasmid carries the gene that is related to oil degrading, the DNA region of the plasmid that harbors *Klebsiella* sp. was analyzed at the molecular level. The pGD2 was found to be 3564 bp long (Fig. 3, GenBank Accession No. AY033498). Analysis of the sequence revealed the presence of two complete ORFs, orf1 and orf2, which were oriented in the same direction. However, we observed no significant homology to proteins that are known to be related to oil-degradation. Orf1 is 867 nucleotides in length and encodes 289 amino acids with a deduced molecular mass of 32,900 Da. The putative ribosomal binding sites (GTAGG, AAGGG) of the orf1 and orf2 were located 6 and 5 bp upstream of the ATG start codons, respectively. Orf2 is 1195 nucleotides in length and encodes 338 amino acids with a deduced molecular mass of 39,400 Da. Upon insertion into a DNA segment, IS5, the insertion sequence causes a duplication of 4 bp (CTAG). The inverted terminal repeat of 16 bp with one mismatch (5'-GGAAGGTGCGAACAANAAG-3') was located at the left and right junction of IS5 (Jeffrey and Mark, 1981). A putative promoter with -35 (CTGAGA) and -10 (GCTAAA) boxes that were separated by canonical 18 nucleotides was located at approximately 180 nucleotides upstream of the orf1. The predicted products of the two ORFs of pGD2 were analyzed for structural similarity. The orf1 showed the highest similarity (71%) to the RepA protein of the *Plesionomonas shigelloides* plasmid pUB6060 (Avison *et al.*, 2001). We designated the product as Rep A. Homology in the amino acid sequence between RepA and other the Rep proteins of the ColE2-related plasmid ColE2-K317 of *E. coli* (Hiraga *et al.*, 1994. Accession No. D30062), pColE6-CT14 of *Shigella sonnei* (Hiraga *et al.*, 1994. Accession BAA06299), pE12 of *Edwordssiella ictaluri* (Accession No. NP 061811), and pHE1 of *Halomonas elongator* (Vargas *et al.*, 1999. Accession No. CAB42632) was 59, 58, 54, and 45%, respectively (Fig. 4). In

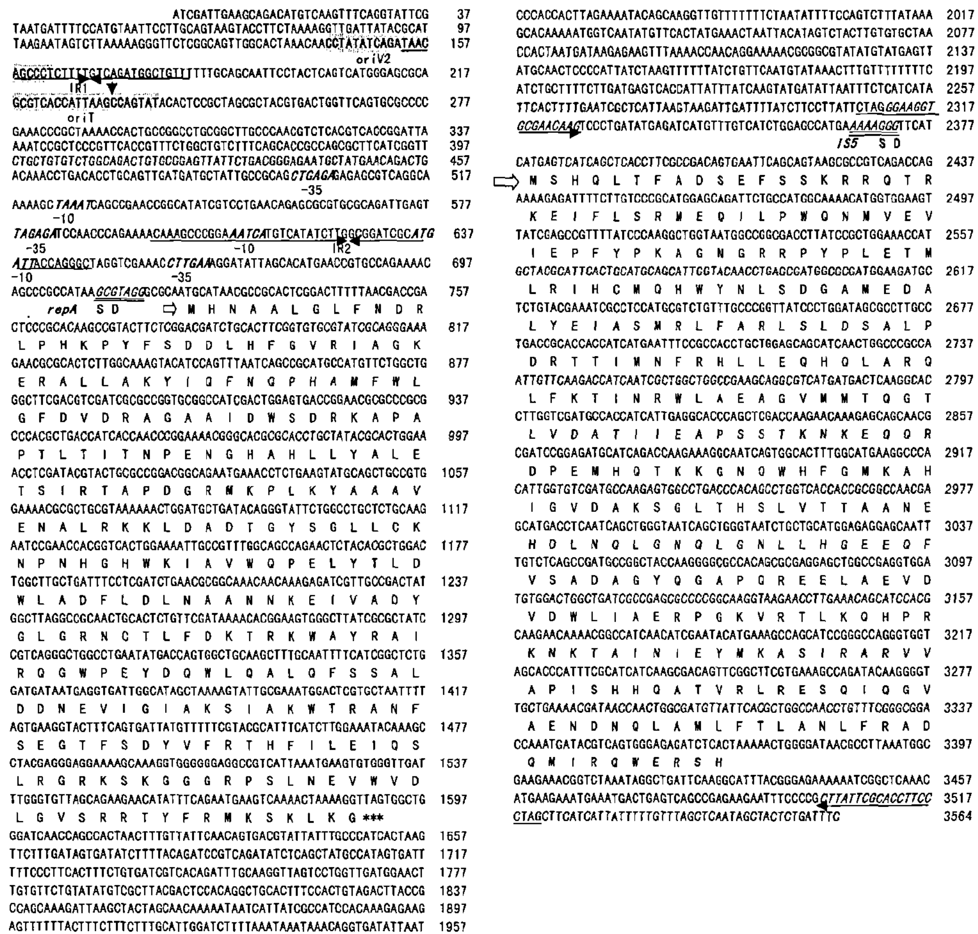

Fig. 3. Nucleotide and deduced amino acid sequences of the *repA* and *IS5* genes region from pMGD2 plasmid. The peptide sequence that corresponds to the ORFs is shown under the nucleotide sequences. The putative promoter sites (–10 and –35) are indicated by bold italic characters. The double underlines indicate the putative Shine-Dalgarno sequence. The initiation sites of the translation are indicated by broad arrows. A stick arrow indicates the inverted repeat sequences. Shadow characters show the OriV and OriT sites.

a comparative analysis of the replicons of eleven ColE2-related plasmids (Hiraga *et al.*, 1994), a highly conserved region (common to all these Rep proteins) was proposed to be involved in primase activity. As shown in Fig. 4, this region is highly homologous in all of the replication proteins. An analysis of the nucleotide sequence of orf2 showed that homology in the amino acid sequence between transposase protein (IS5) and *E. coli* (Jeffrey and Mark 1981), *Bacteriophage* (Kroger *et al.*, 1982), *Vibrio cholerae* (Heidelberg *et al.*, 2000), *Pseudomonas putida* (Chablain *et al.*, 2001), and *Pseudomonas aeruginosa* (Naas *et al.*, 1999) was 95, 90, 73, 53, and 51% respectively (data not shown). *IS5* was originally isolated as an insertion (KH100) of about 1200 bp in the in *cI* gene of bacteriophage λ, and identified as IS5 by Szybalski (Szybalski, 1977; Jeffrey and Mark, 1981). These sequence results might suggest that the IS5 of the plasmid pGD2 originated from the bacteriophage.

Replication system of pGD2 The origins of replication, *oriV* of ColE2 like plasmids show a marked degree of sequence conservation (Del Solar *et al.*, 1998). A sequence analysis of the plasmid pGD2 revealed that the coding information for one protein, RepA, is involved in the initiation of replication. Therefore, we looked for the origins of replication as well as the controlling elements that are involved in the initiation of replication. Plasmid pGD2 contained a sequence homologous to the partial *bom* region, putative *oriT* nick site, and *oriV* site of ColE1 (Fig. 3). The extent of the sequence homology of the *bom* regions in the ColE1-related plasmids is about 95% (Fu *et al.*, 1995, 1998; Fig. 5). However, pGD2 has no *mob* genes in the downstream of the *bom* region. We also found that pGD2 and pUB6060 (Avison *et al.*, 2001) have a very well conserved nick site region. The putative nick site of pMGD2 is between nucleotides G and C at plasmid coordinates 231 and 232, upstream of the translation start site of RepA.

The RepA protein of plasmid ColE2-P9 binds to double-stranded DNA, containing the ColE2 origin (Vargas *et al.*, 1999). In a computer-assisted search for the helix-turn-helix motifs, we were able to identify a possible DNA-binding

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a MHNAALGLFNDRLPHKPYFSDDLHFGVRIAGKERALLAKYIQFNQPHAMFWLGFDVDRAGAAIDWSDRNAPAPTLTITNP  80
b MANQALTLFNDRLPHKPYFSDDLQFGVRIAGKERALLAKYIQFNQPHAMYWLCFDVDRAGAAIDWADLGAPAPTLTIKNP  80
c   SAALQYFDENLPHRPYHTDDLAFGLRISGKGRALLARYIQQNQPHAQFWLVFDVDREGAAIDWSDRNAPAPNITVKNP  79
d   SAALQYFEENLPHRPYHTDDLAFGLRISGKGRALLAGYIQQNQPHAQFWLVFDVDREGAAIDWSDRNAPAPNITVKNP  79
e MTSPALSLFCEHLPRKPYHTNELLHGVRIGAASRAMLARYIQHNQPHAMYWLVFDVDRLGAAIDWSDVNAPTPNLTVKNP  80

a ENGHAHLLYALETSIRTAPDGRMKPLKYAAAVENALRKKLDADTGYSGLLCKNPNHGHWKIAVWQPELYTLDWLADFLDL 160
b DNGHAHLLYALNIAVRTAPDGRGRLLKYAAAIENALRKKLGADAGYSGLICKNPNHLHWQITVWQPELYTLDWLADYLDL 160
c VNGHAHLLYALNIAVRTAPDSSVKALKYAAAIERSLCEKLGADVNYSGLICKNPFHLEWQVMEWREEAYTLDELADYLDL 159
d VNGHAHLLYALNIAVRTAPDASVKALKYAAAIERALCEKLGADVNYSGLICKNPFHLEWLVMEWREEAYTLDELADYLDL 159
e ANGHAHLLYALDTAVRTAPDNSLKALRYAAAIERGLRDKLRADLGYSSLLCKNPLHDYWGVTEWRSEPYTLDELADYVDL 161

a NAANNKEIVADYGLGRNCTLFDKTRKWAYRAIRQGWPEYDQWLQA---------LQFSSALDDNEVIGIAKSIAKWTRAN 231
b GAANDREILPDYGLGRNCTLFDKTRKWAYRAIRQGWPEYSQWLQACIERAKAYNLQFSAPLDENEVMGIAKSISKWTMVT 240
c SASARRSIDKHYGMGRNCHLFEMTRKWAYRAMRQGWPEFSQWLDAVIQRVEMYNASLPVPLSPPECRAIGRSIAKYTHRN 239
d SASARRSIDKHYGMGRNCHLFEMTRKWAYRAIRQGWPVFSQWLDAVIQRVEMYNVSLPVPLSPAECRAIGKSIAKYTHRN 239
e SASDPRREAQAYGLGRNCQLFEKTRTWSYRAIRQGWPEYDQWLSAVIQRVEAYNAQLTVPLSLAECKAIGKSIAKWTHQR 241

                                                           •• •  •  •• •• •• •••
a FSEGTFSDYVFRTHFILEIQSLRGRKSKGGGRP---------------SLNEVWVDLGVSRRTYFRMKSKLKG   289
b YRSLGFDEYVKLTH-SPEVQAYRGRRSKGGGRP---------------SIGEPWLALGISRRSYFRWK        292
c FTPETFAQYVADTH-TPEIQAARGR--KGGSKSKRSTVATSA------RTLKPWEALGISRAWYYQLK       298
d FTPETFAQYVADTH-TPEIQAARGRKGGKIGGAKSKRGAVATSA----RTLKPWETLGISRAWYYQ         300
e ITEQGFAQYVADTH-TPEIQAARGGRNTHESQAAKGRKSKRGAVEDSARSLKPWEALGIS              300
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Fig. 4. Comparison of the primary structures among RepA proteins from different organisms. a; *Klebsiellia* sp., b; *Pleisiomonas shigelloides* (pUB6060), c; *E. coli* (pColE2-K317), d; *Shigella sonnei* (pColE6-CT14), e; *Edword ssiellaictaluri* (pE12). The primerase activity region is overlined and the DNA binding region that postulated for the ColE2 protein is marked by dots.

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pGD2     227  TTAAGCCAGTATACACTCCGCTAGCGC-TACGTGACTGGTTCAG----GCGCCCCGAAACCCGCTAAAACCA-CTGCCG-
pSW200  1155  TTAAGCCAGTATACACTCCGCTAGCGC-TACGTGACTGGTTCAGGGCTGCGCCCCGAAACCCGCTAAAACCA-CTGACGC
pSW100   882  TTAAGCCAGTATACACTCCGCTATCGC-TACGTGACTGGGTCAGGGCTGCGCCCCGACACCCGCTAAAACCA-CTGACAC
ColE    1461  TTAAGCCAGTATACACTCCGCTATCGC-TACGTGACTGGGTCAGGGCTGCGCCCCGACACCCGCTAAAACCTGCTGACGC
pIMVS1        -TAAGCCAGTATACACTCCGCTAGCGCCTGCGTGACTGGTTCACGGCTGCGCCCCGAAACCCGCTAA---CACCCCTAGC

         302   GCC-TGCGG--CTTGCCCAA
        1235   GCC-TGCGG--CTTGTCCAA
         962   GCC-TGCG---CTTGTCCAA
        1541   GCCCTGACGGGCTTGTCAGC
               TAC-TGG    ATATCACAA
```

Fig. 5. Nucleotide sequence similarities of the *bom* region with ColE1-related plasmids. The upward-pointing arrow indicates the CilE1 nick site.

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Rep Kebsiella            264 SLNEVWVDLGVSRRTYFRMKSKLKG
Rep ColE2-P9             271 SLKPWEA-LGISRATYYR---KLK
Rep Shig                 280 SLKPWEA-LGISRATYYR---KLK
DctD R. leguminosarum    416 VRRTIEA-LGIPRKIFYD---KLQ
NtrC K.pneumoniae        445 KQEAARL-LGWGRNTLTR---KLK
```

Fig. 6. Positions of the putative Rep proteins of ColE2-related plasmids show homology to the DNA-binding domains (helix-turn-helix). Identical amino acids present in at least four of the five proteins are boxed.

domain in Rep A of a ColE2-type pGD2. This is homologous to the putative sequence-specific DNA binding domains of *Rhizobium leguminosarum DctD* (Roson *et al.*, 1987) and *K. pneumoniae NtrC* (Drummond *et al.*, 1986) proteins (Fig. 6). These sequence results suggest that the C-terminal region of Rep proteins contain the plasmid-specific DNA-binding domains.

Del Solar *et al.* (1998) reported that the plasmid replicon encodes a small diffusible RNA molecule, which acts as an antisense transcript that negatively regulates the replication of the plasmid. We looked for inverted repeat sequences that could form stable stem-loop structures of antisense RNAs. In the sequenced region, there are two imperfect inverted repeats of IR1 (nt. no. 155 to 182) and IR2 (nt. no. 597 to 653) sequences (Vagars *et al.*, 1999). This region might produce short antisense RNA nucleotides that could regulate the synthesis of Rep A (Rosenberg *et al.*, 1979). We designated this RNA as RNA1, and its corresponding putative gene *IncA*. Figure 7 predicts a putative secondary structure for RNA1 from the nucleotide sequence. Within this region, there are candidates for promoter that would direct transcription of an antisense RNA, but none is wholly convincing. This would generate a transcript with a sequence of approximately 150 nucleotides that are complementary to the leader sequences on the *repA* transcript. Jeong *et al.* (1995) found a potential stem-loop structure in the *ssi* (single strand initiation) of plasmid. The stem-loop structures are essential for the SSi function. Lee *et al.* (2000) reported that the stem-loop structure in the regulatory region is involved in the regulation of the *E. coli*

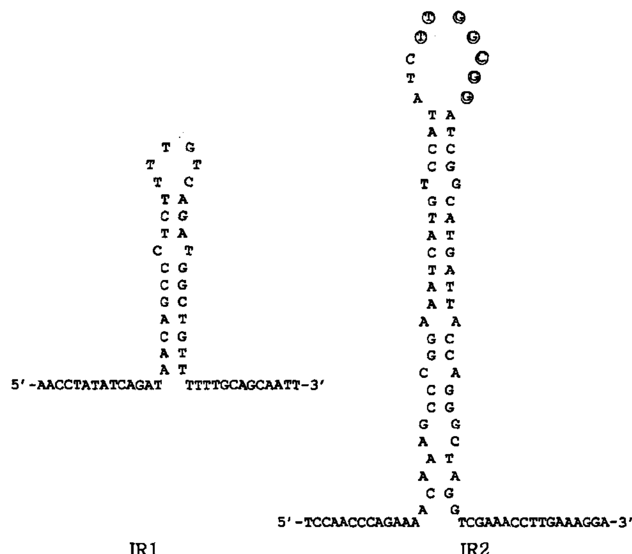

Fig. 7. Secondary structure proposed for the putative RNAI molecule of plasmid pGD2 of the IncA specificity type. Open circles indicate the highly conserved sequence of the IncA loop region.

trxA gene. For the ColE2-related plasmids IncA specificity group, the nine-nucleotide sequences (5'-UCUUGGCGG-3') around the loop regions are well conserved in all of the sequenced plasmids of the different IncA specificity groups (Hiraga *et al*., 1994). The sequence is well conserved around the loop portion of RNA1.

Acknowledgments This work was supported by a research grant from the Korea Science and Engineering Foundation through the CIIPMS at Dong A University.

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