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Cloning and Characterization of the Inc A/C Plasmid RA1 Replicon

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The Inc A/C plasmids, like Inc P and Inc Q plasmids, have a broad host range. However, their maintenance functions remain to be studied. An autoreplicative region of 2.79 kb named RepA/C, able to replicate both in the family *Enterobacteriaceae* and in *Pseudomonas* spp., was isolated and sequenced. The stability, copy number, and incompatibility expression of this replicon were determined. RepA/C and a nonautoreplicative fragment of 1.6 kb of this replicon were used as probes and showed specific hybridizations with the Inc P3-A/C plasmids from *Pseudomonas* spp. and members of the *Enterobacteriaceae*. These probes could be used as tools for identification of the plasmids of this epidemiologically important Inc group.

Replication systems encoded by broad-host-range plasmids are generally more complex than those of narrow-host-range plasmids. In some cases the genes are scattered across several regions of the plasmid genome (35; see reference 15 for a review). Because of their large host range, these plasmids are of considerable interest both as natural vectors for disseminating genetic information among diverse species and as cloning vehicles (20).

The maintenance of plasmids within bacterial populations requires both accurate replication and consistent distribution of plasmids to viable progeny (see reference 21 for a review). The replication and maintenance functions of the broad-host-range plasmids Inc P (7, 24, 34) and Inc Q (9, 20) have been studied in detail. However, the maintenance functions of the Inc A/C plasmids have not been investigated. These plasmids are stably maintained in a variety of different hosts and are equivalent to the Inc P3 group in *Pseudomonas* spp. (12).

We present herein the results of the isolation and the first molecular analysis of the RepA/C replicon specific to the Inc A/C group of members of the family *Enterobacteriaceae* and the Inc P3 group of *Pseudomonas* spp.

Cloning of the replicon of RA1. DNA of the Inc A/C plasmid RA1 (130 kb, Su, Tc) (1, 3) was isolated with caution because of its size, according to the method described by Tourneur (36) derived from the method of Casse (4).

The autoreplicative region was isolated in two steps: a DNA bank of the RA1 parental plasmid was constructed in the pKIL19 vector (derived from pUC19 [2.9 kb, Ap, *ccdB*] [2]); in a second step, this bank was used to transform an *Escherichia coli polA*$_1$ strain (HMS50, *polA*, *thyA*, *thi*, *endA*; a gift from Molineux) to select autoreplicative fragments.

(i) pKIL19 contains a polylinker sequence fused to the *ccdB* gene of the mini-F plasmid which encodes a cytotoxic protein under the control of the *Plac* promoter. If no insert is cloned in the polylinker of this vector, induction of *Plac* results in synthesis of the cytotoxic CcdB protein, which kills the host; if

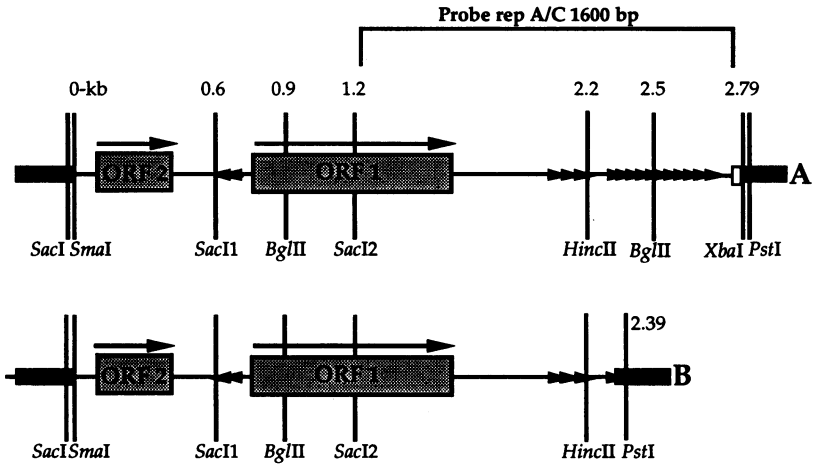

FIG. 1. Scheme of the autoreplicative fragment RepA/C (2.79 kb) of pKA/C1, deduced from its sequence (A). ORF1 may be a rep gene. Subclones S1, S6, and B3 contained fragments of 0 to 0.6 kb, 0.6 to 1.2 kb, and 0.9 to 2.5 kb, respectively. The fragment used as a probe for hybridizations is indicated; the two ORFs are indicated with their directions of transcription; the DnaA box is indicated as a white rectangle; direct repeats are indicated by arrows (13), and inverted repeats are indicated by inverted arrows (2). A clone deleted of 400 bp in the A-T-rich region and of part of the origin of replication is represented; this fragment is not autoreplicative (B).

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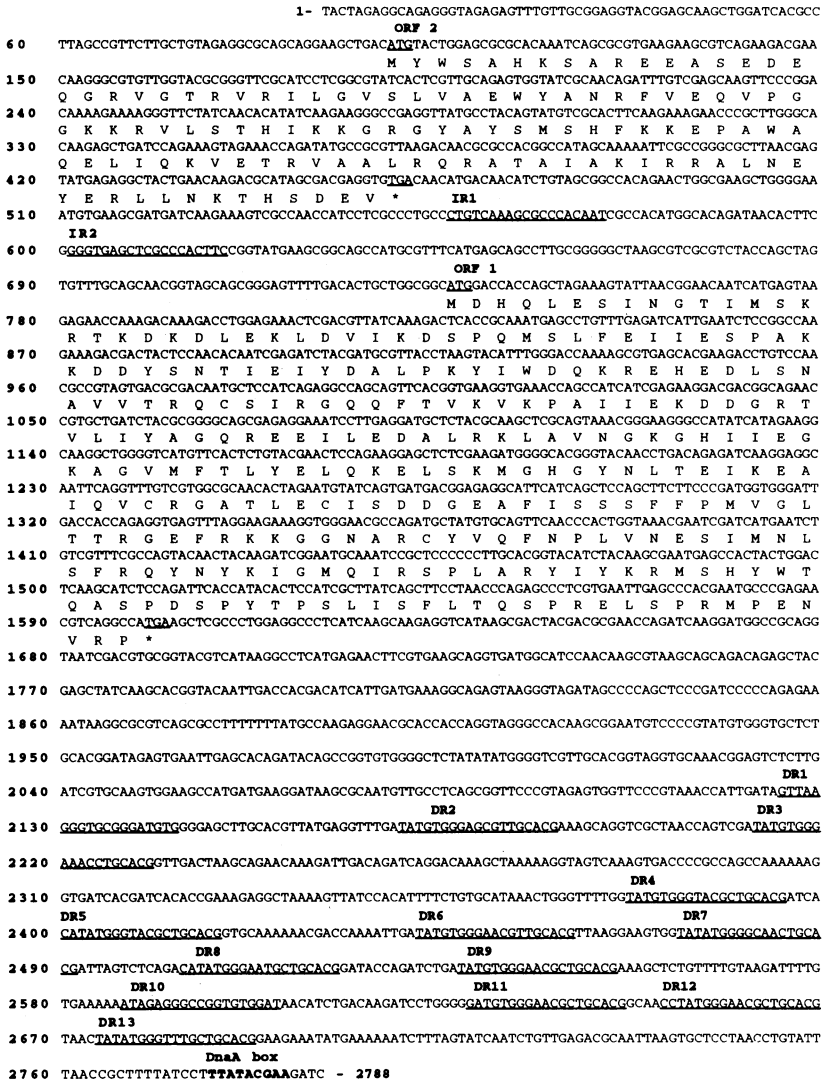

FIG. 2. Nucleotide sequence of the 2.79-kb RepA/C replicon. The two ORFs are indicated, the 13 direct repeats (DR) and the 2 inverted repeats (IR) are underlined, and the DnaA box is in boldface type.

an insert is cloned in the polylinker, the CcdB protein is not synthesized and the cell remains viable. This system can be used for the direct selection of recombinants (2). A bank of total DNA of RA1 was cloned by ligating 2- to 5-kb fragments, obtained by partial *Sau*3A hydrolysis and purified by centrifugation through a sucrose density gradient (5 to 25% [wt/vol]) (26) into the *Bam*HI site of pKIL19. *E. coli* D1210 (25) was transformed and plated on Mueller-Hinton agar (BioMerieux, Charbonnières-les-Bains, France) supplemented with 100 mg of ampicillin liter^{-1} (Beecham-Sévigné, Paris, France) to select transformants and with 0.3 mM IPTG (isopropyl-thio-β-D-galactopyranoside; Boehringer-Mannheim Biochemicals, Meylan, France) to induce the *Plac* promoter. Seven hundred viable transformants were obtained and pooled, and plasmid DNA was isolated from the pool by the alkaline lysis method (23).

(ii) To select autoreplicative regions, the pooled plasmid DNA was used to transform *E. coli polA$_1$* HMS50. This polymerase I-deficient strain allows RA1 replication but not replication of pKIL19 (a pUC19 derivative). One transformant

repeat 1:	GTTAAGGGTGCGGGATGTG	(2129-2147 bp)
repeat 2:	TATGTGGGAGCGTTGCACG	(2174-2192 bp)
repeat 3:	TATGTGGGAAACCTGCACG	(2216-2134 bp)
repeat 4:	TATGTGGGTACGCTGCACG	(2381-2399 bp)
repeat 5:	CATATGGGTACGCTGCACG	(2404-2422 bp)
repeat 6:	TATGTGGGAACGTTGCACG	(2446-2464 bp)
repeat 7:	TATATGGGGCAACTGCACG	(2477-2495 bp)
repeat 8:	CATATGGGAATGCTGCACG	(2508-2526 bp)
repeat 9:	TATGTGGGAACGCTGCACG	(2541-2559 bp)
repeat 10:	ATAGAGGGCCGGTGTGGAT	(2591-2609 bp)
repeat 11:	GATGTGGGAACGCTGCACG	(2632-2650 bp)
repeat 12:	CCTATGGGAACGCTGCACG	(2655-2673 bp)
repeat 13:	TATATGGGTTTGCTGCACG	(2678-2696 bp)
consensus:	-AT-TGGG---G-TGCACG	
inverted repeat 1:	ATTGTGGGCGCTTTGACAG	(624-642 bp)
inverted repeat 2:	GAAGTGGGCGAGCTCACCC	(669-687 bp)
inverted consensus:	---GTGGGCG---T-AC--	

FIG. 3. Nucleotide sequence of the 13 repeats located between bp 2129 and bp 2696 in the RepA/C origin of replication. The consensus sequence is indicated. The nucleotide sequences of the two degenerated inverted repeats located between bp 624 and bp 687 upstream of ORF1 are also indicated.

called pKA/C1, selected in HMS50 on Mueller-Hinton agar supplemented with thymine (100 mg liter^{-1}; Merck-Clevenot, Nogent-sur-Marne, France) and 30 mg of ampicillin liter^{-1} was obtained; this clone contains a 2.79-kb autoreplicative fragment.

Hybridization of immobilized DNA with cloned probes. The specificity of the cloned replicon RepA/C was checked by testing its hybridization with a set of other cloned replicons and reference plasmids, by the replicon typing method described by Couturier et al. (6). Hybridization was performed with two different radiolabeled probes: the entire autoreplicative fragment RepA/C (2.79 kb) and the 1.6-kb *Sac*I-*Xba*I nonautoreplicative fragment of RepA/C. Two hundred and seventy plasmids from the National Collection of Type Cultures (London) belonging to 35 incompatibility groups were tested. Hybridizations were performed at 68°C for 18 h and were followed by three washings at 68°C (the first with 3× SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate]–0.1% SDS; the second with 1× SSC–0.1% SDS; and the third with 0.5× SSC–0.1% SDS).

Both the entire autoreplicative fragment and the nonautoreplicative fragment used as probes hybridized specifically with RA1 and with a set of reference plasmids of the Inc A/C (pHH1350, pHH1465, pIP16a, pIP40a, pIP55, pIP55-1, pIP216, pIP218, P-lac, R16a, R57b, R666, R667, R668, R669, R670, R671, R686, R692, R699-a, R707, R714-b, R719, R807, R808, R810, R812, R816, R817, R936, R1184, RA1, and RA2) and of the C-like (pJA8001 and pJANG8310) incompatibility groups.

Inc A/C plasmids are multiresistant plasmids commonly found in many members of the *Enterobacteriaceae* (8, 10, 11). Their rapid identification by hybridization with the probe isolated in this work may be useful for epidemiological studies (17). Hybridizations with repP and repQ probes have shown that this technique can be used for typing *Pseudomonas aeruginosa* plasmids of the Inc P1 and Inc P4 groups, respectively (unpublished work). Thus, this RepA/C replicon can be used as an epidemiological tool, to probe for plasmids of the Inc A/C group of the *Enterobacteriaceae* and for those of the *Pseudomonas* Inc P3 group.

Stability assay of the RepA/C replicon. The maintenance of RepA/C in *E. coli* HMS50 was assessed by comparing the colony viability of HMS50 containing the parental plasmid RA1 with that of HMS50 containing pKA/C1, which replicates under the control of RepA/C in this strain. After exponentially growing on liquid media without selective pressure, the cultures were plated in selective media to determine the percentage of plasmid-containing cells. After about 60 generations, pKA/C1 was lost from 60% of the cells, whereas RA1 was stably maintained. Therefore, it seems that the 2.79-kb RA1 autoreplicative region cloned into pKIL19 does not possess all the functions required for its stable inheritance.

Comparison of the copy numbers of RepA/C and the parental plasmid RA1. The relative plasmid copy number of RepA/C was estimated by transforming HMS50 *polA* with pKA/C1, to determine whether the cloned replicon and the RA1 replicon in *E. coli* HMS50 had similar properties. The *polA* mutation in the host strain HMS50 ensured that the copy number of the pKA/C1 hybrid was dependent only on the RA1 replicon it carried.

The relative copy number was determined by densitometry (38). HMS50(pKA/C1) and HMS50(RA1) were grown overnight in brain heart infusion (Diagnostic Pasteur, Marne-la-Coquette, France) and were diluted to an optical density at 600 nm of 0.7 (type DU 650 apparatus; Beckman). DNA was extracted by the lysis method of Ish-Horowicz (as described in reference 27). After denaturation at 100°C, various dilutions of the DNA preparations were dot blotted onto a nylon membrane (Hybond N$^+$; Amersham). The labeled *Xba*I-*Sac*I probe (1.6 kb) was hybridized under the conditions described above and was autoradiographed. The autoradiograph was scanned with an XL Ultroscan (LKB Products). The signals proportional to the copy number were similar for plasmids pKA/C1 and RA1, suggesting that they share the same copy number.

```
          2720
RepA/C    GTATCAATCTGTTGAGACGCAATT--AAGTGCTCCTAACCTGTATTTAACCGC
          :::::::    ::: : :::  ::    ::    :::::: :  :::::::
R751      GTATCAACGGCTTGCG-CGCGTTTTCCGGTCAAGCTAACCGGCTTTTAACCCT

                              2790
          TTTTATCCTTTATACGAAGA
          :  :: ::::::::  ::
          TCATAACCTTTATATTAAAC
```

FIG. 4. DNA sequence comparison of the putative replication origin of RepA/C (from bp 2720 to bp 2790) and the replication origin of R751 (Inc P).

Expression of incompatibility by RepA/C. Plasmid incompatibility is usually determined by reciprocal transformations, in this case, with *E. coli* HMS50(RA1) and *E. coli* HMS50 (pKA/C1). However, because of the natural instability of pKA/C1 and the stability of RA1, incompatibility was evaluated only with pKA/C1, as the incoming plasmid to strain HMS50(RA1). Transformed colonies, selected for the incoming plasmid, were picked on two different selective media (30 mg of ampicillin liter^{-1} and 30 mg of ampicillin liter^{-1}-40 mg of tetracycline liter^{-1}) to test the retention or the exclusion of the resident plasmid (16). We found that under selective pressure, pKA/C1 exhibited strong incompatibility with RA1 in the *polA* strain (95% of cases). The loss of RA1 was confirmed by agarose gel electrophoresis of total plasmid DNA (13).

Restriction map and sequence determination of RepA/C. The restriction map of the autoreplicative 2.79-kb fragment was determined by single and double restrictions (Boehringer Mannheim Biochemicals) (Fig. 1). This map was used for the construction of various subclones of the RepA/C replicon. S1 (0.6-kb *Sac*I-*Sac*I1 fragment), S6 (0.6-kb *Sac*I1-*Sac*I2 fragment), and B3 (1.6-kb *Bgl*II-*Bgl*II fragment) were inserted into pKIL19 and their sequences were determined. The Rep A/C replicon contains 20 *Sau*3A restriction sites, which could explain why only one clone containing the whole replicon was obtained from a set of 700 recombinant clones in the screening strain HMS50.

Overlapping DNA fragments of the 2.79-kb *Xba*I-*Sma*I insert of pKA/C1 were generated by unidirectional nested deletions using exonuclease III and S1 nuclease, according to the manufacturer's recommendations (Pharmacia, Saint Quentin-en-Yvelines, France).

The DNA sequences of double-stranded templates were determined by the dideoxy-chain termination method (28), using T7 DNA polymerase (Pharmacia). DNA sequences were compiled and analyzed with version 7.2 of the UNIX GCG software package.

The 2.79-kb RepA/C replicon sequence (Fig. 2) contains 13 direct repeats of 19 bp, located between bp 2130 and bp 2700 (Fig. 3). Near this region, there is a sequence (bp 2720 to bp 2790) which shares significant similarities (63.4% identity) with the replication origin of the Inc P plasmid R751 (Fig. 4). This region is A-T rich, with an A+T content of 70% (like the R751 plasmid, which has an A+T content of 76%) (31), compared with 46% (34% for R751) for the whole sequence. Similar A-T regions are often described as essential parts of replication origins (15, 34). Consistent with this region containing the

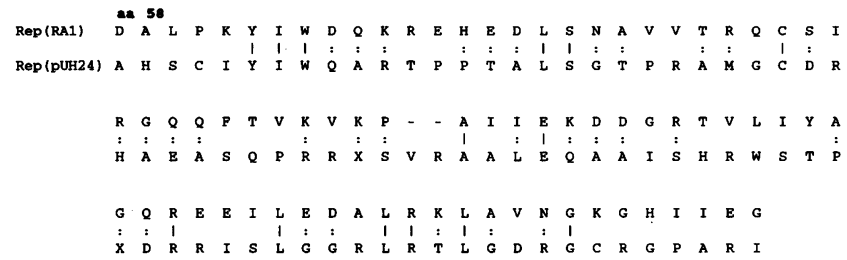

FIG. 5. Amino acid sequence comparison between ORF1 and the *repA* gene of pUH24 from *Synechococcus* spp. |, identity between residues; :, physical or chemical similarity between residues.

origin of replication, a 400-bp deletion (one of the nested deletions used for sequencing) in this region abolished the autoreplicative function (Fig. 1). Like RA1, R751 is a broad-host-range plasmid, with polymerase I-independent replication, regulated by iterons (11 repeats) (15, 31–33). These similarities, observed between RepA/C and R751, suggest that these replicons have common strategies for initiation of replication in hosts as different as members of the *Enterobacteriaceae* and *Pseudomonas* spp.

The host DnaA protein is essential for the initiation of replication of the chromosome oriC, and it has been shown to be essential for replication of several plasmid replicons (see reference 14 for a review). It binds to a specific recognition site (DnaA box). A recognition site conforming to the DnaA box consensus sequence 5′-(T/C) (T/C) (A/T/C) T (A/C) C (A/G) (A/C/T) (A/C)-3′ (30) is present in the replication origin of RepA/C between bp 2844 and bp 2852 (5′-TTATACGAA-3′) (Fig. 2).

The direct repeats were downstream from two open reading frames (ORFs), ORF1 (867 bp, from bp 736 to bp 1602) and ORF2 (363 bp, from bp 99 to bp 461) (Fig. 1 and 2). ORF1 has the potential to encode a polypeptide of 33 kDa, a size similar to the mean molecular mass of most Rep proteins from broad-host-range replicons (9, 22). Two DNA sequences in ORF1 (from bp 910 to bp 1090 and from bp 1135 to bp 1250) shared significant similarities (63% and 71% identity, respectively), with the *repA* gene of a narrow-host-range pUH24 *Synechococcus* sp. plasmid (Fig. 5). Moreover, the encoded amino acid sequence of this ORF (corresponding to bp 830 to bp 1365) is 20.3% identical to the RepA protein of pUH24, in which the replicon is almost entirely occupied by two overlapping genes coding for essential replication proteins (*repA* and *repB*) and by an A-T-rich region containing an inverted repeat and two 20-bp directly repeated nucleotide sequences (37).

We can note that, unlike most of the broad- and narrow-host-range replicons (for example, R1162 [16, 20], RSF1010 [15, 20], and pSC101 [18], the positioning of the iterons is downstream of the putative *rep* gene of RepA/C. Only the Inc P plasmids seem to have this type of organization (22).

Upstream of ORF1, we found two inverted repeats located between positions 624 and 687 (Fig. 3). These repeats are not well conserved compared with the 13 direct repeats, but they may nevertheless participate in the autoregulation of ORF1.

Host range of the replicon RepA/C. The parental plasmid RA1 is able to replicate in members of the *Enterobacteriaceae* and in *Pseudomonas* spp. To test whether the replicon isolated in this work can replicate in these hosts, we used pKA/C1 to transform *E. coli* and *P. aeruginosa*. pKA/C1, like RA1 (and unlike pKIL19), was able to replicate in both these hosts, proving that all the necessary information for replicating in the different hosts was contained in the 2.79-kb RepA/C fragment.

In conclusion, sequence analysis suggests that the general organization of the RepA/C replicon resembles that of the replicons controlled by the iteron mechanism, i.e., an origin of replication (*ori*) and an ORF coding for an essential replication protein (Rep) (for a review, see reference 15). In these replicons, replication is initiated or blocked by the rate-limiting Rep protein, which binds to specific DNA repeats (iterons) and is able to autoregulate the *rep* gene (5, 18, 39). Interestingly, this iteron mechanism seems to be particularly adapted to plasmid replication and to be distributed among many plasmids belonging to different incompatibility groups.

The RepA/C replicon displays features of several plasmids of different incompatibility groups, suggesting (i) genetic transfer between cells of the same or different species and genera (29) and (ii) the capacity of a pool of genetic information to interchange, assemble, dissociate, and undergo molecular rearrangements (19). We intend to analyze, from the genetic and biochemical points of view, these different features which allow the maintenance of the RepA/C replicon in its broad host range.

Nucleotide sequence accession number. The 2.79-kb RepA/C replicon sequence has been submitted to the EMBL nucleotide sequence data base (accession number: X 73674 ECRA1).

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