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# Analysis of replication region of the cryptic plasmid pAG20 from *Acetobacter aceti* 3620

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**Abstract**

The DNA sequence of small cryptic plasmid pAG20 in *Acetobacter aceti* was determined at 3064 bp with 51.6% GC pairs. The plasmid encoded a 186 amino acid protein which is important for plasmid replication in Gram-negative bacteria except *Escherichia coli*. Two 21 bp large direct repeat sequence 1 and two 13 bp direct repeat sequence 2 were determined in the regulation region upstream from gene encoded Rep protein. Vector pAG24 with kanamycin gene and two deletion derivatives pAG25 and pAG26 without *rep* gene from plasmid pAG20 were constructed. Plasmid pAG24 was replicated in a broad host range like *E. coli*, *Acetobacter pasteurianus*, *A. aceti*, *Comanomonas* spp., *Serratia marcescens*, and *Shigella* spp.


*Keywords:* *Acetobactor aceti*; Plasmid; Replicon region

Bacteria of the genus *Acetobacter* are Gram-negative obligatory aerobic bacteria characterised by strong tendencies to oxidise alcohols and a wide range of sugars. They are non-pathogenic, commonly available, easily cultivable in laboratory condition, grow at low pH, and able to oxidize different carbon substrates into organic acids.

Plasmids are autonomously replicating DNA molecules that are common vectors in the horizontal transfer of genes between bacteria. In Gram-negative bacteria, most plasmids encode two elements for replication [1]. The first is a *cis*-acting DNA sequence (the origin of replication *ori*) and the second is a *trans*-acting protein (Rep) that binds to *ori*, establishing a nucleoprotein pre-initiation assembly [2].

Numerous recent studies have demonstrated that the new plasmids isolated from environmental bacteria are often not closely related to well-characterised plasmids from acetic acid important bacteria. However, despite such diversity, the majority of plasmids can be assigned only to a limited number of evolutionary related groups, as judged by the comparative analysis of plasmid-encoded Rep proteins [1]. Rep proteins are encoded by a majority of plasmids and are essential for initiation of their replication. A limited number of plasmids that do not encode replication proteins include the well-known ColE1 plasmids [1].

Previously, a number of plasmids were characterised in *Acetobacter* strains [3–8]. In *Acetobacter pasteurianus* plasmid pAC1 (19 kb) [9–12], group of four plasmid series pAP1, pAP2, pAP3, and pAP4 [13], were identified and in *Gluconoacetobacter europaeus* JK2 the plasmid pJK2-1 [14] was described. All of the plasmid encoded Rep proteins were clearly related to previously described θ-type plasmids from Gram-negative bacteria [14,15].

Here, we have isolated and characterised the minimal replicon derived from *Acetobactor aceti* 3620 plasmid DNA pool, most likely belonging to a low copy number plasmid. The replicon is capable of autonomous replication in a range of Gram-negative bacterial genera and encodes replication protein.

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## Materials and methods

*Bacterial strains and growth conditions.* Table 1 lists the bacterial strains used in the present study. *Escherichia coli*, *Comanomonas*, *Serratia marcescens*, and *Shigella* spp. strains served as a host for plasmid DNA manipulation and were grown at 37 °C in Luria–Bertani (LB) medium [16]. Bacteria *Acetobacter* genera were cultivated at 28 °C in YPG medium [9]. The following antibiotics were added to media at the indicated concentrations (μg/ml): 100, ampicillin; 50, kanamycin.

*Isolation of plasmid DNA.* The plasmids used in this work are listed in Table 1. Plasmid DNA from *E. coli* cells was isolated by the standard Birnboim and Doly [17] method, and from *Acetobacter*, *Comanomonas*, *S. marcescens*, and *Shigella* spp. cells by the modified Grones et al. [9] method. Plasmids for sequence analyses were purified by using the Wizard Plus Midipreps DNA Purification System (Promega).

*Molecular genetic techniques.* Restriction enzyme digestion, isolation of DNA fragments from agarose gels, and molecular cloning were performed by using standard techniques [16]. *E. coli*, *Comanomonas*, *S. marcescens*, and *Shigella* spp. cells were transformed according to the methods of Mandel and Higa [18], and transformation of plasmid DNA to *Acetobacter* used the methods of Bilská and Grones [19]. Plasmid DNA was separated on 0.7–1% agarose gel in TAE buffer medium [16]. DNA bands were visualised by trans illumination with an UV light source and photographed.

*Sequencing procedure.* Plasmid DNA cloned into *E. coli* was isolated from cells by using the Wizard Plus Midipreps DNA Purification System (Promega). Nucleotide sequences of both strains were determined by the subcloning of deletion plasmid's derivatives into pUC19 [20]. Cloned products were sequenced by using M13 universal and reverse primers. Sequences were analysed in sequencing machine ABI PRISM 310 and compared with the information deposited in the GenBank/EMBL Database.

## Results and discussion

In the bacterial strains of *A. aceti* CCM 3620 was identified by a standard procedure using purified plasmid pAG20. Cryptic plasmid was linearised by Sal1 nuclease, cloned into the same position in plasmid pUC19, and transformed into *E. coli* JM105. The plasmid pAG100 was constructed this way and the primary nucleotide sequence of pAG20 was determined by the sequence analysis. The plasmid pAG20 consists of 3064 bp with 51.6% GC pairs. The analysis of nucleotide sequence of the plasmid pAG20 detected potential protein encoding genes. Fifteen ORFs encoding for more than 50 amino-acid residues were detected. The largest identified reading frame ORF1 is localised between 719 and 1262 bp, the second ORF2 is localised (1663–1391 bp), and ORF3 is localised in position 1892–1629 bp.

From the cryptic plasmid pAG20 was constructed the vector added to the kanamycin resistance gene. Linearised plasmid pAG20 by *Sal*I nuclease was ligated with *Pst*I kanaymcin gene from plasmid pUC4-KAPA [22]. After blending the cohesive ends of both fragments by S1 nuclease, pAG24 was constructed (Fig. 1).

From the analysis of the nucleotide sequence of plasmid pAG20, we detected three ORFs encoding proteins larger than 90 amino-acid residues. The largest identified reading frame ORF1 (719–1262 bp) encodes the protein composed of 181 amino-acid residues. This protein has very low identity and similarity to a group of putative replication proteins determined in other bacterial plasmids. The analysed protein that was compared with a similar *Rep* protein of pBHRK18 plasmid from *E. coli* [23] exhibited 50% identity and 65% similarity over 165 amino-acid residue segment (Fig. 2). The *Rep* protein encoded by pBHRK18 plasmid is larger by about 224 amino-acid residues.

The region upstream of initiation codon has been searched for incidence of −10 promoter region (5′-TAC ATT-3′) in position 641–646 bp and −35 promoter region (5′-TTGTCC-3′) in position 615–621 bp. Both detected

Table 1
Bacterial strains and plasmids DNA

| | | References |
|---|---|---|
| Strains | | |
| *A. pasteurianus* 3614 | | CCM |
| *A. aceti* 3620 | | CCM |
| *Comanomonas* spp. | | CCM |
| *E. coli* DH1 | F$^-$ *recA1 endA1 gyrA96* (Nal$^r$) *thi-1 rsdR17 supE44 relA1* | [21] |
| *E. coli* JM105 | (F′ tra D36 lacI$^q$ Δ(lacZ)M15 proAB/recA1 endA1 gyrA96 (Nal$^r$) thi hsdR17 ($r_{K^-}m_{K^+}$) sup E44 relA1 Δ(lac-proAB) | [20] |
| *E. coli* RR1 | F$^-$ recA$^+$ *hsdS20* ($r_{B^-}m_{B^-}$) *leu supE44 ara14 galK2 lacY1*, *proA2 rpsL20* (Str$^r$) *xyl5 mtl1 recA13 mctB* | [15] |
| *Shigella* spp. | | CCM |
| *S. marcescens* | | CCM |
| Plasmids | | |
| pUC19 | Ap$^r$, *ColE1* replicon | [20] |
| pAG20 | *AG20* replicon | This study |
| pAG24 | Kn$^r$, *AG20* replicon | This study |
| pAG25 | Kn$^r$, *AG20* replicon | This study |
| pAG26 | Kn$^r$, *AG20* replicon | This study |

All bacterial strains and plasmids were from Collection of Department of Molecular Biology in Bratislava.

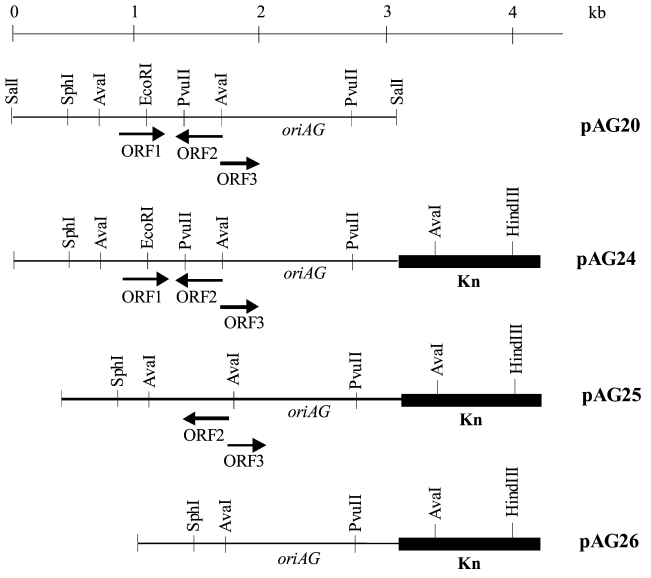


Fig. 1. Physical map and construction of derivatives of cryptic plasmid pAG20 from *A. aceti* 3620.

promoter boxes displayed considerable homology to consensus box sequences. The −10 sequence was found 72 bp upstream of initiation codon.

By analysis of the nucleotide sequence we determined several specific sequences in a segment between 550 and 690 bp. At 5′ end of the analysed region the segment in the presence of an unusually high number of repetitive sequences was detected. This segment contained two copies of 21 bp direct repeat 1 (5′-TAGCCCACCAGCTATGG GCTA-3′) in position 555–575 and 580–600 bp; two copies of 13 bp direct repeat 2 (5′-TCCAAATTTGGAT-3′) in position 618–630 and 673–685 bp; and 9 bp inverted repeat 2 in position 578–586 bp (5′-CCTAGCCCA-3′) and 594–602 (5′-TGGGCTAGG-3′). The first copy of direct repeat 2 overlaps with the identified −35 sequence (615–620 bp) of putative *Rep* protein ORF1 (Fig. 3).

The second ORF2 (1663–1391 bp) encodes 100 amino-acid residue protein, exhibiting a high degree of similarity to DUF322 (domain of unknown function) [24] family of hypothetical proteins that were found among many bacteria. The highest level of similarity was detected to a conserved hypothetical protein XF2080 that was identified in *Xylella fastidiosa* with 63% identity of 93 amino-acid fragment. The examination of initiation codon of this ORF revealed the presence of −10 sequence (5′-GAGC GT-3′) in position 1742–1747 bp with high degree of homology and a −35 sequence (5′-TTGGCG-3′) in position 1721–1726 bp with low homology separated by a

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pAG20    14 GTWVQVERAAMERWSKLAVSNPRAAAVMMLMTSQMGRNNALVASQATLAKMAGCGLNTLK
               G WVQ RR A E W+ L    P AA ++  + +QMG  NA+V SQ TL+K+ G  L T++
pBHRK18  21 GHWVQTERKAHEAWAGLIARKPTAAMLLHHLVAQMGHQNAVVVSQKTLSKLIGRSLRTVQ

pAG20    74 RALSVLREGNWIEVRQIGPTGTACAYIVNDRVAWSGNRDGIRYSLFSAAVLLSDDEQPDK
                A+  L    WI V ++   GT  AY+VNDRVAW   RD +R S+FSAAV++  D+Q D+
pBHRK18  81 YAVKDLVAERWISVVKLNGPGTVSAYVVNDRVAWGQPRDQLRLSVFSAAVVVDHDDQ-DE

pAG20   134 TEIGAQPPLQAIPDLYPGEKQLPTGPGLPPPSQPSFDGMEPDLPA  178
               + +G    L+ IP LYPGE+QLPTGPG  PPSQP   GMEPDLPA
pBHRK18 140 SLLG-HGDLRRIPTLYPGEQQLPTGPGEEPPSQPGIPGMEPDLPA  183
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Fig. 2. Comparison of amino-acid sequence alignment of pAG20 and pBHRK18 putative replication proteins (identities 84/165 (50%), positives 108/165 (65%), and gaps 2/165 (1%).

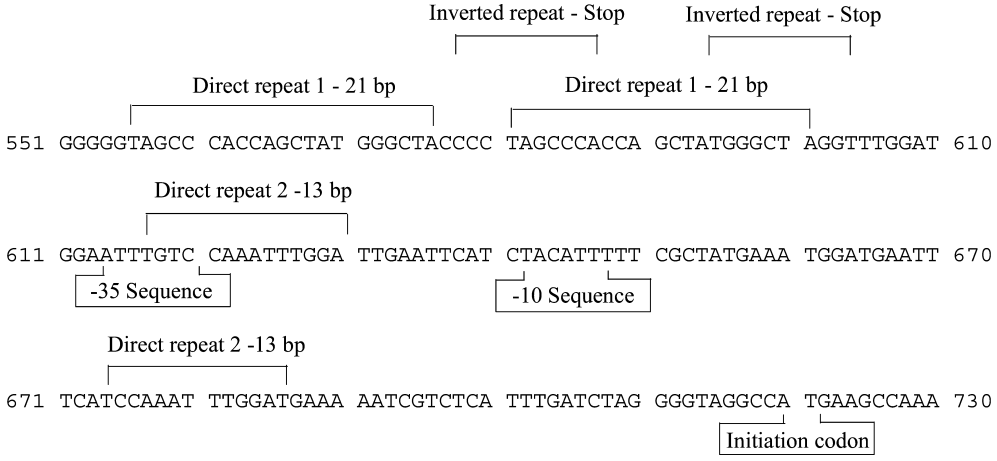


Fig. 3. Direct and inverted sequence at 550–730 segment and promoter boxes of putative replication protein of plasmid pAG20 from *A. aceti* 3620.

15 bp segment. The −10 sequence is 31 bp upstream of initiation codon.

The third largest theoretical ORF3 (1892–1629 bp) encodes 87 amino-acid residue protein which has significant homology to a DUF415 family of proteins with sizes of roughly 90 amino-acid residues. Comparison of both proteins indicated 44% identity and 63% similarity to the damage-inducible protein J identified in *Agrobacterium tumefaciens*. The expected promoter region examination revealed −10 (5′-TACACT-3′) in position 1979–1984 bp and −35 (5′-TAGACA-3′) in position 1955–1960 bp sequences with similarity to consensus sequences and 17 bp separating fragment.

Replication protein in plasmid pAG20 is no more identical with the normally described proteins in other plasmids. We decided to confirm its validity after plasmid replication. We deleted the region encoding replication protein in plasmid pAG24 by cutting *Eco*RI (in position 633 bp) and partially *Pvu*I (1411 bp) endonucleases, and we prepared the plasmid pAG25. The plasmid was smaller by about 778 bp than pAG24 with deleted putative *rep* gene and its regulation region. The second deletion derivative pAG26 was prepared after deletion of the 1451 bp large *Ava*I fragment (positions 412 and 1863). This plasmid has disrupted gene encoded replication protein, DUF322 and DUF415 family of proteins. Both plasmids pAG25 and pAG26 are able to replicate in *E. coli* DH1 ($recA^+$) and *E. coli* RR1 ($recA^-$) bacteria without the decrease in the copy number of plasmid. Results showed that plasmid pAG20 does not use any replication protein for replication in *E. coli* cells. These bacteria used for replication of plasmid pAG20 and its derivatives own replication proteins. Plasmid in *A. aceti* 3620 has about five copies per chromosome and after re-transformation in *E. coli* cells more than 30 copies.

Another situation was confirmed in other bacteria like *E. coli*. Competent cells of *A. pasteurianus* 3614, *Comanomonas*, *S. marcescens*, and *Shigella* spp. [19] by plasmids pAG24, pAG25, and pAG26 were transformed. Standard plasmid pAG24 encoding replication protein was able to replicate in all the tested bacteria. After transformation of plasmid pAG25 transformants were detected only in bacteria *Shigella* spp. Transformation cells by plasmid pAG26 did not obtain transformants and results show the necessity of plasmid encoded protein for its replication in tested bacterial strains.

The necessity of replication protein and unknown function proteins to be encoded by plasmid pAG20 for the mechanisms of replication will be the purpose of future studies.

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