Sindh Univ. Res. Jour. (Sci. Ser.) Vol.44 (3) 463-468 (2012)

SINDH UNIVERSITY RESEARCH JOURNAL (SCIENCE SERIES)

Complete Nucleotide Sequencing and Molecular Characterization of the pColE9-J Plasmid

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Received 07th January 2012 and Revised 12th February 2012

Abstract: The ColE9-J plasmid codes for colicin E9 (ColE9) protein, which is an antibacterial protein containing H-N-H endonuclease activity, that degrades chromosomal DNA of sensitive strains of *Escherichia coli* or closely related bacteria. The ColE9 plasmid has been used as a model system to understand various biological phenomenon such as colicin translocation and protein-protein interactions. In present study we have, for the first time, determined the complete nucleotide sequence of native ColE9-J plasmid. Computer based analysis of the complete nucleotide sequencing data of the pColE9-J plasmid consisting of 7577 bps revealed nine open reading frames (ORFs) located on the plasmid. Of these, seven ORFs including six ORFs coding for ColE9 operon and one ORF for replication region have been previously characterized. We have, thus, identified two more previously unrecognized putative OFRs, in addition to the seven previously characterized ORFs. One of these two ORFs exhibited close homology to the sequence coding for *mob*A gene of *E. coli* and it was located on positive strand; however the sequence for putative *mob*C gene was present on negative strand.

Keywords: Nucleotide sequence, *In silico* analysis, ColE9-J plasmid,

1. INTRODUCTION

Colicins are stress inducible narrow spectrum antibacterial proteins produced by strains of *E. coli* and closely related bacteria. E-group colicins are those colicins which utilize the vitamin B_{12} protein, encoded by the chromosomal gene *btuB,* as their surface receptor to get entry into the susceptible cell via their central receptor-binding domains (Di Masi *et al.,* 1973), and they include E1 to E9 colicin types classified on the basis of their specific immunity groups (Cooper and James, 1984; Watson *et al.,* 1985). The colicin immunity protein protects the E colicin-producing bacteria against the killing of colicin, specifically. With exception of ColE1, which forms channels into the cytoplasm of susceptible cell; all the members of E colicin group are nuclease colicins whose killing activity is attributed to degradation of the nucleic acids (DNA or RNA) of sensitive bacteria. Among nuclease colicins, E2, E7, E8, E9 are DNase (Kleanthous *et al.,* 1999; Schaller & Nomura, 1976) while E3, E4, E5, E6 are RNase (Bowman *et al.,* 1971; Curtis *et al.,* 1989; Ogawa *et al.,* 1999).

A colicin operon typically contains three genes. The first one is always the colicin structural gene that encodes the colicin protein and it is designated *cxa,* where x is the specific colicin activity (for example *cea* encodes ColE) followed by a specific immunity gene (*cxi*), encoding the immunity protein, which is responsible for the resistance of the colicin-producing strain against the bactericidal activity of the colicin produced (Chak and James, 1986; Kuhar, 1999). The third gene is the lysis gene (*cel*), encoding the lysis protein, which is also called the Bacteriocin Release Protein (BRP), and facilitates export of the colicin from the producing cell (van der Wal *et al.,* 1995). The structural gene, immunity gene and lysis gene for nuclease colicins are present on same positive strand, where the lysis gene is separated from immunity gene by only few base pairs. Therefore, transcription of all three genes of nuclease colicins occurs in the same direction which is regulated by a common SOS promoter with the immunity gene, additionally controlled by its own constitutively expressed promoter (Chak & James, 1985; Chak & James, 1986; Mark *et al.,* 1984). Apart from these three closely linked usual genes, the ColE3 and ColE9 operon comprise an additional immunity gene for colicin E8 and E5, respectively (Chak and James, 1984; Chak & James, 1986). There are two transcription terminators (T1 and T2) in a colicin operon. For nuclease colicin operons T1 and T2 are located just after the immunity gene and lysis gene, respectively (James *et al.,* 1987).

The plasmids encoding colicins are usually referred as pCol and these range from low molecular weight to high molecular weight replicons in size depending on the colicin type (Hardy, 1975). The

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plasmid pColE9-J was first isolated from a strain of *E. coli* from chicken caeca in 1984 (Cooper and James, 1984). Since then, the genes for ColE9, Im9, LysE9, DNase domain of ColE5, Im5, LysE5 and Rep proteins on ColE9-J plasmid have been cloned and sequenced (Curtis *et al.*, 1989; Eaton and James, 1989; James *et al.*, 1987; Lau and Condie, 1989). The complete nucleotide sequence of the plasmid pColE9-J and its bioinformatics analysis remained to be carried out. In this study we show the genetic map of pColE9-J plasmid and kinetics of the colicin production from the *E. coli* strain carrying this plasmid.

2. MATERIALS AND METHODS

2.1 Isolation and maintenance of the plasmid pColE9-J

The pColE9-J plasmid was maintained in host strain *E. coli* JM83 (*F⁻araΔ(lac-proAB) rpsL (Str^r)[φ80 dlacΔ(lacZ)M15] thi*). *E. coli* JM83 cells were routinely grown on LB Agar or in LB broth and incubated at 37°C. *E. coli* JM83 containing pColE9-J plasmid were selected on the basis of colicin activity, which can be determined by stab test. Large scale circular plasmid DNA isolation was performed using spin columns by QIAGEN midi prep kit (Qiagen, UK) according to the manufacturer's guidelines. The concentration of purified plasmid DNA was quantified using a NanoDrop (ND-1000) spectrophotometer (Thermo Scientific). The purified DNA samples were stored at -20°C for subsequent analysis.

2.2 Endonuclease digestion of plasmid DNA

Endonuclease digestion of plasmid DNA was accomplished according to Sambrook (1989). Restriction endonuclease enzymes were purchased from New England BioLabs, UK.

2.3 DNA sequencing

Nucleotide sequencing was carried out by primer walking method using specific primers designed from the sequenced DNA fragments (Table 1). DNA sequencing reaction was performed (into 0.2 ml tube) using a Big Dye Terminator kit (PE Applied Biosystems) in Eppendorff Mastercycler Personal. The reaction consisted of 25 cycles of 96°C for 10 sec, 50°C for 5 sec and 60°C for four min. Ethanol precipitation was performed prior to running the reactions on Prism 310 Genetic Analyzer (ABI) at the School of Molecular Medical Sciences, University of Nottingham, UK.

2.4 Computer based analysis

Nucleotide sequence analysis and bioinformatics analysis based on homologous DNA sequences available at EMBL and GenBank database libraries were performed using the Vector NTI programme (Invitrogen). The prediction of open reading frames was made by use of the Gene Mark software (Besemer and Borodovsky, 1999) available at http://exon.gatech.edu/heuristic_hmm2.cgi.

2.5 Determination of colicin activity of *E. coli* containing native pColE9-J plasmid

Single colony from overnight culture of *E. coli* pColE9-J was picked and stabbed into LB agar plate and incubated at 37°C for at least 16 hr. Then the cells on this plate were killed by exposure to chloroform vapours for 30 min allowing diffusion of colicin from cells. After allowing for evaporation of the chloroform, 6 ml of molten 0.7% (w/v) agar containing *ca.* 100 µl of appropriate colicin sensitive indicator cells (*E. coli* JM83) were overlaid on to the plate and incubated at 37°C overnight. Colicin activity was estimated by observing a clear zone of growth inhibition around the stabbed culture.

2.6 Preparation of extracellular and intracellular colicin extracts

Colicin produced from *E. coli* containing pColE9-J was obtained essentially as described previously (Chak & James, 1984) with slight modifications. Briefly, an overnight culture of *E. coli* JM83 containing pColE9-J was diluted 1:100 with fresh LB and grown at 37°C to an OD_{600} of ~0.2-0.4. An aliquot of 1 ml culture at defined time point was collected and centrifuged at 6000 × *g* for 2 min. The supernatant was collected into a sterile eppendorf tube and it was used as extracellular colicin fraction. The culture pellet was re-suspended into 50 µl of sterile phosphate buffered saline ([PBS] Sigma, UK) and equal amount of chloroform was added followed by incubation at 4°C for at least 15 min. The sample was then centrifuged at 10,000 × *g* for 15 min. The aqueous phase was removed and used immediately as intracellular colicin.

2.7 Luminescence *(lux)* reporter assay

E. coli DPD1718, containing a fusion of the promoter region of *recA* of *E. coli* to the *Photorhabdus luminescens luxCDABE* integrated into *lacZ* locus of *E. coli* DPD1692, was used as a luminescent reporter strain. The assay was performed as described previously (Vankemmelbeke *et al.*, 2005). Briefly, an overnight culture of *E. coli* DPD1718 was diluted to 1:100 with LB supplemented with 30 µg ml^{-1} chloramphenicol, and incubated at 37°C with shaking until OD_{600} reached to ~ 0.4. The cells were then diluted 1:2 with LB in total volume of 100 µl in a 96-well microtiter plate (Nunc, UK), and 4 µl of defined colicin extract was added to

each well. The luminescence was determined over time at 37°C using a microtiter plate luminometer (Lucy1, Anthos Labtech, Salzburg, Austria). Prior to undertaking the analysis, the Luminometer, microtiter plate and medium were pre-warmed to 37°C in an effort to prevent unwanted stress to the cells.

Table 1: List of primers used in this study for sequencing pColE9-J plasmid

Primers	Sequence (5´- 3´)	Location
SBM9R	GTTACCACTTGTGCTATGCG	2112-2094
SBM10F	TGTCGTTGGGGGTAGTGTAAG	5473-93
SBM17R	TTACCGCCTGTTCCCGAGCCAC	2294-74
SBM18R	CTCACGGCGATGTTGAGTGCATAG	5879-57
SBM19F	TGCCACCTGTTCGAAATTACG	6125-45
SBM20F	CGCCATTCGGAACGGTCG	5257-74
SBM25R	CAACAGCAGATTCACCGTCC	1428-47
SBM29R	TGCATTATGCCAGGTCAGT	7063-81
SBM30F	AGAGTGTTCATGACCCGGTG	1083-1102
SBM31R	TTCCAGAAGCGGAGTTGTC	693-75

3. RESULTS AND DISCUSSION

3.1 Complete nucleotide sequencing of pColE9-J plasmid

The pColE9-J plasmid was identified around 30 years ago (Cooper & James, 1984). The authors further localized and characterized the ColE9 operon using recombinant plasmids and sub cloning techniques, Consequently, the DNA region corresponding to ColE9 operon on the plasmid was sequenced previously (James *et al.*, 1987). However, in order to understand the nucleotide sequences of the rest of the plasmid backbone to facilitate subsequent characterization studies, we undertook to determine the complete nucleotide sequence of the pColE9-J plasmid by primer walking method which revealed that the ColE9-J plasmid comprises 7577 bps. The complete sequence has been deposited at the EMBL/GenBank Data libraries under accession number FJ573246 (http://www.ncbi.nlm.nih.gov/nuccore/FJ573246). The sequence contains 26.3% A; 22.5% C; 27.2% G; 24.0% T with molecular weight 4671.2 kDa.

3.2*In silico* analysis of sequence of gene of ColE9-J plasmid

The gene prediction data obtained by using Gene Mark software revealed that one ORF was located on the negative strand whereas seven ORFs were on positive strand. Six of the ORFs present on positive strand have previously been characterized as colicin operon consisting of *ceaI*, *ceiI*, *celI*, *ceaE*, *ceiE*, and *celI* genes encoding ColE9 activity, Im9, atypical ColE9 lysis, ColE5 DNase domain of ColE5 activity, Im5 and ColE5 lysis proteins, respectively. Schematic map of pColE9-J plasmid is shown in Fig.1. One more ORF on positive strand has also been suggested for encoding Rep protein (Hiraga *et al.*, 1994). The single-strand initiation sequence; ssi of pColE9-J plasmid (gtagcgctcgccgcagtctcatgaccgagcgtagcgagcgaatgagcgag gaagcgcaaaggcgtccggtg) was present between 6631-6701bp **(Fig. 1)** and demonstrated 100% sequence homology with ssiA sequence of ColE2 plasmids (Nomura *et al.*, 1991).

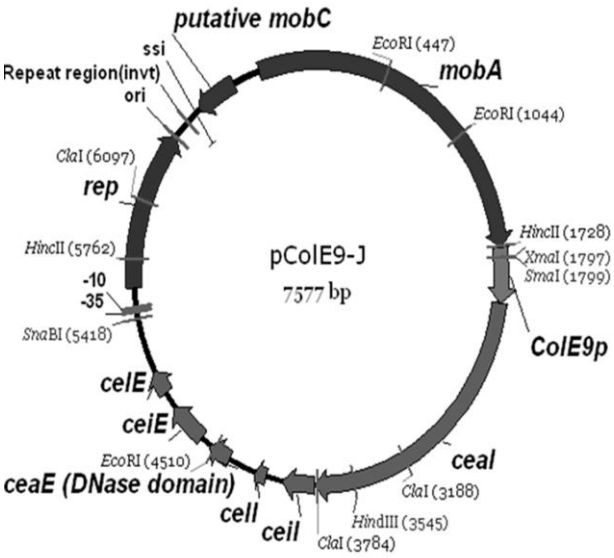


Fig1. Physical and genetic map of plasmid pColE9-J.The arrows indicate the positions and orientations of functional genes on the basis of sequence comparisons. Restriction sites for several restriction endonucleases are indicated. The promoter of the colicin operon is indicated as *ColE9p*. The ORFs for genes encoding colicin activity, immunity and lysis proteins of ColE9, DNase domain of colicin activity, immunity and lysis proteins of ColE5 are shown as *ceaI, ceiI, celI, ceaE*(DNase domain), *ceiE, and celE*, respectively. The sequences -35 (consensus sequence TTGACA),-10 (consensus sequence TATAAT) regions of promoter upstream of the gene encoding Rep protein are indicated.

3.3 Characterization of the native pColE9-J plasmid

Following DNA sequencing, the ColE9-J plasmid was digested using *Sna*BI restriction enzyme to confirm its predicted molecular weight. *Sna*BI cuts once to plasmid DNA, therefore, as expected one linear DNA band corresponding to ColE9-J was observed. Plasmid gel electrophoresis pattern **(Fig 2)** shows the linear and circular ColE9-J plasmid based on the size of the native plasmid. Furthermore, antibacterial activity of *E. coli* cells containing native pColE9-J plasmid is shown in **(Fig. 3).**

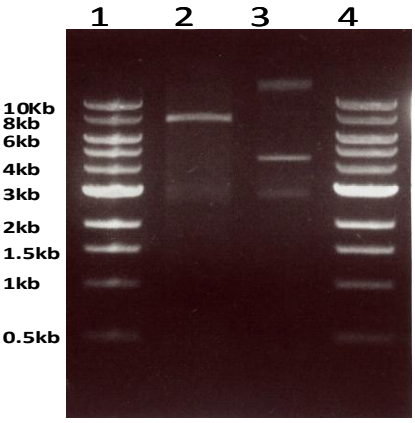


Fig. 2. Agarose gel electrophoresis analysis of pColE9-J. Lanes 1 and 4, DNA markers, Lane 2, *Sna*BI digested linear pColE9-J and Lane 3, uncut plasmid.

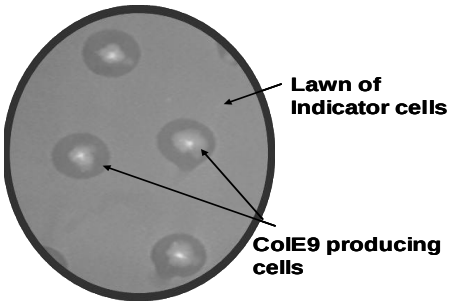


Fig 3. Stab test plate showing colicin production from*E. coli* containing native ColE9-J plasmid.

3.4 Kinetics of colicin production by *E. coli* containing native pColE9-J

In order to determine the earliest time point at which increased production of ColE9 occurs *E. coli* pColE9-J cells were grown and an extracellular and intracellular colicin extracts were obtained from un-induced cultures. The extracts were then examined for colicin activity using a *lux* reporter assay that detects DNA damage induced by the ColE9 DNase activity on *E. coli* cells (Vankemmelbeke *et al.*, 2005). The results shown in **(Fig. 4A)** demonstrate that the increased colicin production happened when the *E. coli* cells were at the very early stationary phase (6 hr) of growth. However, colicin extract obtained at time point 4 hr of growth showed colicin activity, while no colicin activity was observed from sample taken after 2 hr of growth indicating that the colicin production has actually started when the cells were in late log-phase of growth. These finding suggest that the ColE9 induction is growth phase dependent which is in agreement with published data on other colicin such as ColK (Mulec *et al.*, 2003). Additionally, the time point for appearance of colicin into the medium was determined using same assay with extracellular colicin extracts obtained from supernatants of the *E. coli* pColE9-J at various phases of the growth. Figure 4B demonstrates the result of *lux* reporter assay performed using the supernatants. It is apparent from the graph that the colicin activity is present only in supernant obtained at time point 8 hr and 24 hr of growth. However, a slight increase in relative luminescence units RLU was observed from the supernant obtained at time point 6 hr of growth. This data indicated that the ColE9 production actually happens earlier than its release into the outer environment. It is, therefore, speculated that this 2 hr lag period for presence of colicin into medium was due to the late expression of the lysis gene, since lysis protein is required for the export of ColE9 from producing cells.

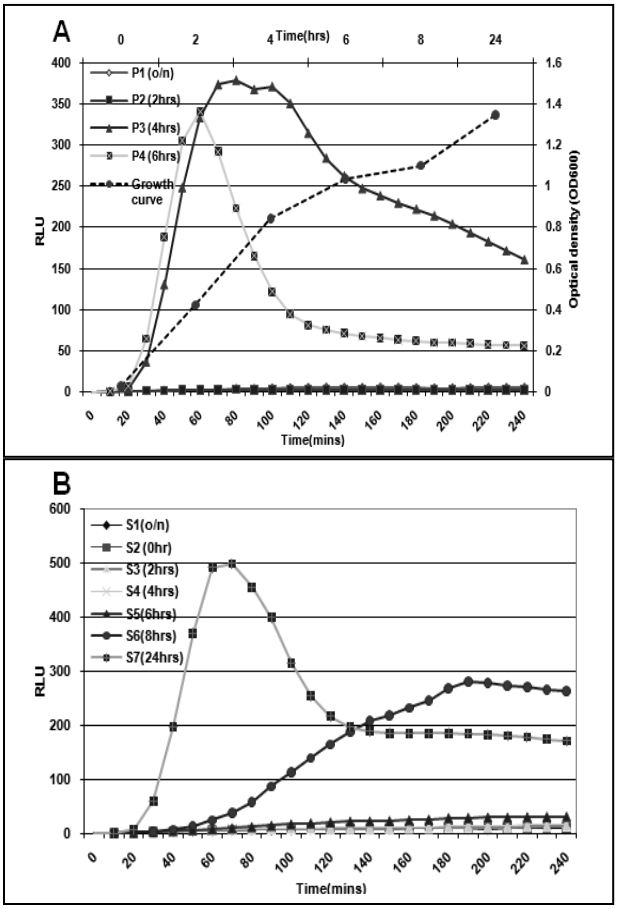


Fig. 4.*Lux* reporter assay representing the time course of spontaneous production 0f ColE9 by *E. coli* cells containing the native ColE9-J plasmid. The *lux* reporter assay was performed using intracellular colicin extracted from cell pellets (A) obtained from an overnight culture (P1), 2 hr (P2), 4 hr (P3), or 6 hr (P4)of the growth of *E. coli* JM83 pColE9-J cells, and extracellular colicin in supernatants (B) taken from overnight culture (S1), and after 0 hr (S2), 2 hr (S3), 4 hr (S4), 6 hr (S5)8 hr (S6) and 24 hr (S7) of growth *E. coli* JM83 pColE9-J cells. The dotted curve (A) represents the growth pattern of *E. coli* cells containing the native ColE9-J plasmid. Values are the average of triplicates within a representative experiment.

4. CONCLUSION

Despite the cost of production of colicin, approximately 30% of the natural population of *E. coli* has been reported to produce colicin with a high diversity of colicin types among them, (James *et al.*, 1996; Pagie&Hogeweg, 1999) and over 25 types of colicin have been characterized so far (Riley & Gordon, 1999). However, the complete nucleotide sequences of only a small number of colicin-encoding plasmids have been studied (Morlon *et al.*, 1988; Rijavec *et al.*, 2007). In the present study, we therefore determined and analyzed the complete nucleotide sequence of the ColE9 -encoding plasmid pColE9-J (Cooper & James, 1984) which will facilitate the future genetic studies and characterisation of the plasmid.

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