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SHORT COMMUNICATION

Sequence analysis and characterization of rolling-circle replicating plasmid pVCM01 from *Salmonella enterica*

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ABSTRACT

Aims: Characterization of cryptic plasmid pVCM01 (accession number JX133088) isolated from *Salmonella enterica* Enteritidis.
Methodology and results: The complete sequence of pVCM01 was obtained. This plasmid possesses 1981 bp, with G+C content of 57% in agreement of the range of *Salmonella* genomic DNA. pVCM01 has a high degree of similarity to pB and pJ plasmids. It possesses six main open reading frames, only one have a very high degree of amino acid identity with protein involved in the rolling-circle-like replication (RCR). Based on the sequence similarities, pVCM01 plasmid belonged to the pC194/pUB110 rolling-circle replicating plasmid family. The Rep pVCM01 possesses the motifs: FLTLTVRN, HPHFHTL, SGDGYVKHERW, which were present in all Rep proteins.
Conclusion, significance and impact of study: The small size of pVCM01 plasmid and its stability in *E. coli* cells, make it an attractive candidate to develop new vectors, such as cloning and/or expression vector.

Keywords: Salmonella enterica, rolling-circle replicating plasmid, Rep protein

INTRODUCTION

Salmonella enterica subsp. enterica serovar Enteritidis (S. Enteritidis) is the most frequent causative agent of human salmonellosis in the world (Rabsch *et al.*, 2001), and therefore its epidemiology is subject of particular interest. *S. enterica* is a Gram negative and facultative intracellular bacterium that causes Salmonellosis. *Salmonella* are widespread and important causes of foodborne throughout the world (Haneda *et al*., 2004). The vehicles usually involved in salmonellosis outbreaks are meat, eggs, poultry and milk (Abdelwaheb *et al*., 2008).

Several strains of *Salmonella* carried different types of mobile genetics elements including bacteriophages, transposons and plasmids. The presence of plasmid in *Salmonella* is very common. Plasmid profile analisys has been used for epidemiological studies (Helmuth *et al*., 1985).

According Rychlik *et al*. (2006) serovars of *S. enterica* frequently associated with infections of humans and farm animals (Enteritidis, Typhimurium, Dublin, Cholerae-suis, Gallinarum, Pullorum and Abortus-ovis) normally harbouring plasmids. More than 1000 field strains of S. *enterica* Enteritidis were initially characterized by plasmid profiling analysis (Rychlik *et al*., 1998). From these strains, Gregorova *et al*. (2004) select 15 strains representing the most frequent plasmids observed. Thirty low molecular mass plasmids were identified, and classified as belonging to three distinct groups: ColE1, ColE2, and rolling-circle-like replicating (RCR) plasmids.

Taskale and Akcelik (2012) isolated 41 strains of *Salmonella* from food samples, 10 strains harboured at least one plasmid, their sizes ranging from 30 to 400 kb. The presence of more than one plasmid in strains of *Salmonella* is common (Rychlik *et al.*, 1998; Gregorova *et al.*, 2004).

Prokaryotic plasmids have a dual importance in the microbial world: first they have a great impact on the metabolic functions of the host cell, providing additional traits that can be accumulated in the cell without altering the gene content of the bacterial chromosome. Second, plasmids can provide a basis for genomic rearrangements *via* homologous recombination, they can facilitate the loss or acquisition of genes (Fondi *et al*., 2010).

In previous work we isolate several strains of S. *enterica* Enteritidis from poultry carcasses obtained from abattoirs in the region of Rio Verde – Goias – Brazil. Plasmidial profile analysis showed the existence of

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different plasmids. One of the isolates showed the presence of at least two plasmids. The larger plasmid (pVCM04) is a theta type replication with 3583 bp (accession number HM231165). The aim of the present work was the characterization of the smaller cryptic plasmid (pVCM01).

MATERIALS AND METHODS

The plasmidial DNA was obtained by alkaline phenol procedure. In order to obtain the complete sequence of pVCM01, plasmidial DNA was digested with *EcoR*I and subcloned into pUC18. The DNA sequence was obtained by diodeoxy chain termination method, using ABI 377 DNA sequencer. The complete sequence was determined by a combination of subclones and primer walking. To confirm that the obtained sequence is complete, the wild plasmid was sequenced with primers flanking *EcoR*I site.

Since pVCM01 does not present a gene encoding antibiotic resistance, in order to access the plasmidial stability, the pVCM01 was fused with pUC18. As control, cells transformed with pUC18 were used. *E. coli* cells carrying pUC18 or pVCM01/pUC18 were cultured in the presence of ampicillin and incubated at 37 °C for 15 h. After initial incubation, 100 µL of the cultures were transferred to another incubation medium without selection pressure and incubated under the same conditions. This procedure was repeated daily until elapsed more than 200 generations. The presence of plasmids was shown by comparison of cells growth on plates with and without selective pressure.

RESULTS AND DISCUSSIONS

The pVCM01 is a 1981 base pairs (bp) plasmid, with G+C content of 57%, which agree with the range of G+C content of *Salmonella* DNA (Wang and Reeves, 2000). The entire sequence of pVCM01 was used to search GenBank for homologous sequences. The pVCM01 was high similar to the plasmids pB (1093 bp) and pJ (2096 bp) which were isolated from *S. enterica* Enteritidis (Gregorava *et al*., 2004). RCR plasmids have a wide host range, and are known to replicate in both Gram negative and Gram positive bacteria, generally are small in size and showing high copy-number (Khan, 2005; Biswas *et al.*, 2008). We speculate that the pVCM01 presents a high copy-number, like as the majority of RCR plasmids.

RCR plasmids contain three important elements, a gene encoding the initiator protein (Rep), the double strand origin (dso), and the single strand origin (sso). More than 200 RCR plasmids have been identified and can be divided into more than a dozen families. Based on the sequence similarities, pVCM01 plasmid belonged to the pC194/pUB110 rolling-circle replicating plasmid family (Yasukawa and Masamune, 1997; Khan, 2005) (Figure 1). Six ORFs with more than 50 amino acids were found using ORFinder program. Only one ORF showed high degree of similarity to initiator (Rep) protein. The ORF 2, with 329 amino acids, is high homologous to rolling-circle-replication (RCR) initiator protein. The pVCM01 Rep protein was 100% similar to the Rep protein of the pB plasmid and 97% similar to the pJ Rep protein (Table 1).

The Rep protein have three characteristic domains: one for DNA binding, one for plasmid nick-closing and one for binding to double strand origin of replication. The conserved nick-closing domain showed the residue of tirosyne 187. According Khan (1997; 2005) the nicked strand becomes covalently attached to the 5' phosphate through tyrosine residue present in the active site of the Rep protein. Rep proteins also have three conserved motifs, pVCM01 Rep protein possesses the following motifs: FLTLTVRN 48-50 HPHFHTL 55-60 SGDGYVKHERW. The conserved tyrosine 187 is found in the third motif.

ORFs 03 and 06 showed homology with relaxossome component of *E. coli* (Table 1). These two ORFs began in the 84 bp long inverted repeats. Only initial 20 amino acids of each ORF are similar to NickA protein of *E. coli* ED1a. The product of both ORFs are smaller than NickA, which possesses 139 amino acids (Furuya and Komano, 1995).

The two small rolling-circle-like replicating plasmids studied by Gregora *et al.* (2004) are essentially identical, plasmids pJ (2096 bp) and pB (1983 bp). They encoded only a single ORF showing extensive homology to Rep proteins of plasmids replicating by rolling-circle mechanism. Repeats sequences are the most important diferences between them. Eight direct repeats sequence (TGTGGG) were identified, around position 1330-1442 of plasmid pJ. This direct repeat region was absent in plasmid pB. In the plasmid pB a 84 bp long inverted repeat sequence was found.

pVCM01	1788 TCTCGCGCGCGATCCTTGTATTTATACTTAAGGGATAA	1825
pJ	1903 TCTCGCGCGCGATCCTTGTATTTATACTTAAGGGATAA	1940
pB	1790 TCTCGCGCGCGATCCTTGTATTTATACTTAAGGGATAA	1827
pBS512	417 TCTCGCGCGTGATCCTTGTATTTATACTTAAGGGATAA	454
pSSO46	417 TCTCGCGCGTGATCCTTGTATTTATACTTAAGGGATAA	454
pKYM	413 TCTCGCGCGTGATCCTTGTATTTATACTTAAGGGATAA	450

Figure 1: A stretch similar to dso replication origin of pC194/pUB110-family plasmids.

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The pVCM01 was essentially identical to the pB plasmid with unique exception: pVCM01 plasmid possesses a 422 bp sequence in inverted orientation relative to the pB plasmid. Those 422 bp long region is flanking by the 84 bp long inverted repeat sequence. This 84-bp-long is found in more than 20 plasmid from *E. coli* and *Salmonella*. But only in pVCM01 and pB this sequence occurs twice (Figure 2).

From a genome perspective, plasmids can provide a basis for genomic rearrangements via homologous recombination and so they can facilitate the loss or acquisition of genes during these events, which eventually may lead to horizontal gene transfer (Fondi *et al*., 2010).

Table 1: Characteristics of the ORFs of pVCM01.

ORF	Size (aa)	Position (bp)	Homologous proteins found	E value
01	182	1109 - 1657	Hypothetic protein SNOG_12873 of *Phaeospharia nodorum* SN15	2.3
02	329	84 - 1073	Hypothetic protein Rep plasmid pB	0
03	60	1422 - 1604	Relaxossome component of *E. coli*	4E -09
04	116	587 - 237	Electron transfer flavoprotein subunit alpha of *Pseudomonas* sp.	1.6
05	129	1459 - 1070	Hypothetic protein of pMO17-54 of *Shigella* sp. MO17	5E -09
06	105	1341 - 1024	Hypothetic protein WIK_04858 of *E. coli* KTE122	6E -12

GCGGGGTGTCGGGGCGAAGCCCTGACCAGGTGGGGAATGTCTGAGCGAGCGTGCGCGGTC
CGACATTCCCACATCCTGTCCCGAAAATTCCCGCCGTTTCTCGGTTTTTTGAGCGTCGCAGAC
CGCACGACAAACGAAACGAAGTGTAGTTTGTATGAGTGCGCACTTCGTGAGATTCCCCGACT
GCCAGTTTGTCGATCGCGCGGAACAGCTGTGGCACGAAAACGGCAGAATGATTTGCTGTCCG
GTTGCCAGAACTGCGCGCTTACGTGGAGGGCGGCGCGGCGCAAAAAACTGCCGACATTGCG
CCAGTGTTCCGGTGACACAAACCCGCAGAAATCGGGACAGGATGTGGGAATGTCGGACCGC
GCACGCTCGCTCAGACATTCCCCACCTGGTCAGGGCTTCGCCCCGACACCCCGC

Figure 2: Sequence of 422-bp-long present at pVCM01 which is in inverted orientation relative to pB plasmid. The 84 bp long inverted repeats were underlined.

The existence of three essentially identical plasmids, whose main difference is related to the presence of repeated sequences, reinforces the importance of isolating and sequencing new plasmids. The difference between the pVCM01 and pB is the orientation of the fragment flanked by the inverted repeats sequence, also considering that this 84 bp long is founded in more than 20 plasmid from *E. coli* and *Salmonella* sp., we believe that these plasmids can play a role in the rearrangements with other plasmids and chromosomes.

By the plasmidial stability test, it was found that more than 90% of *E. coli* cells maintained pVCM01/pUC18 for at least 240 generations, while the pUC18 was lost, only 14% of *E. coli* cells maintained the plasmid after 24 h of incubation.

CONCLUSIONS

The small size of pVCM01 plasmid and its stability in *E. coli* cells, make it an attractive candidate to develop new vectors, such as cloning and/or expression vector. For this purpose the unique restrictions sites presents in the pVCM01 (*Apa*I, *EcoR*I, *Pvu*I) can be exploited to insert a multiple cloning sites, a promoter and/or reporter gene.

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