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Accumulation of Single-Stranded DNA in *Escherichia coli* Carrying the Colicin Plasmid pColE3-CA38

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Abstract

We sequenced the complete 7118 bp circular plasmid pColE3-CA38 (pColE3) from *Escherichia coli*, located the previously identified colicin components together with 2 new ORFs that have homology to mobilization and transfer proteins, and found that pColE3 is highly similar to a plasmid present in enterohemorrhagic *E. coli* O111. We also found unusual aspects of the plasmid include the inability to be completely digested with restriction endonucleases and asymmetric Phred DNA sequencing quality scores, with significantly lower scores in the forward direction relative to the colicin and immunity proteins consistent with plus (+) strand DNA. Comparing the A₂₆₀ with picogreen double-stranded DNA (dsDNA) fluorescence and oligreen single-stranded DNA (ssDNA) fluorescence as well as metachromatic staining by acridine orange, we found that the undigested pColE3 DNA stains preferentially as ssDNA and that it coexists with dsDNA. We also identified ssDNA in pColE5 and pColE9 but not in pColE1. Colicin plasmids producing ssDNA may represent a new subclass of rolling-circle replication plasmids and adds to the known similarities between colicins and filamentous phage.

Keywords

Colicin; single-stranded DNA; rolling-circle replication; acridine orange metachromatic staining; Phred quality scores; picogreen; oligreen

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1. Introduction

Colicins are a class of bacterially produced bacteriocidal proteins generally known as bacteriocins. Colicins are produced by *Escherichia coli* strains and primarily inhibit other strains of *E. coli* while the strains that produce them are themselves protected by an immunity protein that keeps the colicin inactive during translation and export or reentry (Cascales *et al.*, 2007). Colicin E3 (ColE3) cleaves the 16S rRNA of susceptible *E. coli* (Senior and Holland, 1967; Bowman *et al.*, 1971) at a specific site 49 nucleotides from the 3'-OH terminus (Lasater *et al.*, 1989). Colicin plasmids share a common genetic organization consisting of the colicin gene that is expressed under control of the SOS regulon, an immunity protein as well as a lysis protein that facilitates the release of the colicin from the periplasm (Riley & Wertz, 2002).

Although colicins are generally narrow spectrum antibiotics, the ColE3 RNA cleavage recognition sequence is also present in eukaryotic 18S subunit, and ColE3 has been shown to be cytotoxic to some cancer cells (Fuska *et al.*, 1979; Lancaster *et al.*, 2006; Cornut *et al.*, 2008). Several bacterial strains with tumor-targeting capability including *Salmonella sp.* are currently being investigated for their potential use in cancer therapeutics (Pawelek *et al.*, 1997; Low *et al.*, 1999; Forbes 2010; Hoffman & Zhao 2014). Colicins including ColE3 may have utility in augmenting antitumor bacterial strains such of *Salmonella* (Clairmont *et al.*, 2000; Lancaster *et al.* 2007; Leschner & Weiss 2010) by engineering them to directly express the colicin within the tumor in order to achieve a localized antitumor effect.

E. coli are usually found as members of highly complex communities of bacteria within the guts of humans, cattle and other vertebrates, and can also be recovered from environmental samples of water and soil from locations inhabited by the species that carry them as part of their gut microbiota. Because colicins result in a phenotype that kills other *E. coli*, there has been interest in the ability of colicins to influence the biological distribution of *E. coli* strains (Riley & Wertz, 2002; Cascales *et al.*, 2007; Dobson *et al.*, 2012). Colicins could be involved in competitive exclusion of a preexisting *E. coli* strain against colonization by other strains of *E. coli*, or it may be that colicins facilitate colonization of an *E. coli* strain into the gut or an environment already inhabited by another *E. coli* strain, or a combination of these effects. Petkovšek *et al.* (2012) correlated colicin insensitivity with pathogenicity, where resistance appeared to contribute to overcoming competitive exclusion at extraintestinal locations such as the skin. Based upon the elevated incidence of their occurrence in pathogenic strains, Rijavec *et al.* (2007) and Šmajš *et al.* (2010) correlated bacteriocins with the virulence potential in uropathogenic *E. coli*. In these two cases, colicins apparently act as colonization factors by helping to eliminate preexisting microbes that might otherwise prevent colonization. Colicins are also prevalent among diarrheagenic clones, including those associated with Shiga toxin production (O111, O15, O26, and O157:H7; Murinda *et al.*, 1998). Colicins have been shown to influence the production of Shiga toxin and can either increase or suppress its production (Toshima *et al.*, 2007). Conversely, it is also known that preexisting bacterial population can suppress Shiga toxin producing *E. coli* (STEC) O157:H7 (Gamage *et al.*, 2006) and that some STECs are sensitive to colicins (Murinda *et al.*, 1996; Schamberger *et al.*, 2004; Jordi *et al.*, 2006).

The colicin E1 origin of replication from the ColE1-encoding plasmid is widely used in conventional plasmid cloning vectors, such as pUC series of plasmids (Vieira & Messing, 1982). The ColE1 type replicon does not encode a replication (Rep) protein, and therefore replication of plasmids with this type of origin continues in the presence of chloramphenicol (Clewell 1972; Clewell & Helinski 1972), which allows for plasmid amplification. A 580 base pair region containing the colicin E1 *ori* and an RNA transcription site is essential for replication (Ohmori & Tomizawa 1979; Oka *et al.*, 1979; Tomizawa *et al.*, 1981). pColE2-P9 and pColE3-CA38 plasmids have previously been shown to be sensitive to the presence of chloramphenicol (Watson and Visentin, 1982; Horii & Itoh, 1989; Yasueda *et al.*, 1989; Kido *et al.*, 1991), which is due to the requirement of the Rep protein encoded by these plasmids (Yagura *et al.*, 2006; Aoki *et al.*, 2007; Han *et al.*, 2007). Their Rep protein origin of replication consists of 32–37 base pairs with three subregions for 1) stable binding the Rep protein, 2) Rep protein binding and initiation of DNA replication and 3) initiation of DNA replication without binding the Rep protein.

The genes encoding the colicin E3 toxin, immunity and lysis proteins, the origin of replication and its cognate replication protein have been previously mapped by restriction endonuclease analysis as well as having been partially cloned and sequenced (Watson & Visentin, 1980; 1982; Masaki & Ohta 1985; Horii & Itoh, 1989; Yasueda *et al.*, 1989; 1994), but the complete DNA sequence of the plasmid has not been reported. Plasmids existing as single stranded DNA were first isolated from the Gram positive bacteria *Bacillus subtilis* and *Staphylococcus aureus* (te Riele *et al.*, 1986) and subsequently found in gram negative bacteria (del Solar *et al.*, 1993), although none have been found in *E. coli*. These plasmids replicate by the rolling circle mechanism of DNA synthesis (Espinosa *et al.*, 1995; Khan 2005), which is also used by some bacteriophage such as M13. In this report we present the entire sequence of the pColE3-CA38 plasmid and show that it exists as both ssDNA and dsDNA in *E. coli*, and that differences in the methods of DNA preparation accounts for earlier studies that did not reveal the presence of ssDNA.

2. Materials and Methods

2.1. Bacterial strains and plasmids

The colicin-containing bacteria used in this study, BZB2106 containing the plasmid pColE3-CA38 (pColE3), BZB2108 containing pColE5-099 (pColE5), and PAP1407 containing the plasmid pColE9-J (pColE9; Pugsley & Oudega, 1987), were obtained from Margret Riley, University of Massachusetts, Amherst, MA, and the strain W3110 ColE1 containing the ColE1 plasmid was obtained from the Coli Genetic Stock Center (New Haven, CT). pUC19, and DH5 α were obtained from InVitrogen (Carlsbad, CA) and EC100 was obtained from Epicentre (Madison, WI). The bacteria were maintained on Luria Bertani (LB) media containing 10 g tryptone, 5 g yeast extract, 10 g NaCl per liter for broth, and with 1.5% agar for petri plates, and incubated at 37°C. pUC19 was transformed into chemically competent DH5 α or EC100, selected for and maintained on LB agar with 100 μ g/ml ampicillin (LB-amp). M13mp18 (M13) double-stranded (ds) and single stranded (ss) DNA were obtained from Bayou Biolabs (Metairie, LA). Colicin-containing supernatants were generated by inducing a freshly initiated culture of BZB2106 using ultraviolet light, allowing it to grow to

stationary phase, pelleting the bacteria and passing the supernatant through a 0.22 μm low-binding polyethersulfone (PES) filter (Millex, Cork, Ire). The colicin-containing supernatant was tested for *E. coli* killing capacity by placing 3 μl drops of a serial dilution onto a plate containing an *E. coli* B (CGSC # 5713) soft agar overlay. A strain of DH5 α containing pColE3 was generated by transforming chemically competent DH5 α with the pColE3 plasmid and plating the transformation to colicin-selective plates containing LB agar to which a 1.0 ml overlay of an undiluted colicin-containing supernatant had been added and allowed to be absorbed.

2.2. DNA preparation and ssDNA and dsDNA staining

Alkaline lysis mini-plasmid preps were performed using the Fermentas Gene Jet plasmid purification kit (Vilnius, Lithuania), which includes RNase A treatment to remove RNA. In order to concentrate the DNA, two, 1.5 ml lysates were passed through the same binding column. DNA produced by the Fermentas alkaline lysis miniprep kit was also compared with the alkaline protease miniprep kit (Wizard Plus SV, Promega, Madison, WI) and the ZR Plasmid Miniprep kit (Zymo Research, Irvine, CA). DNA was separated by agarose gel electrophoresis isolated from the gel using 0.65 μm polyvinyl difluoride (PVDF) centrifugal filter unit (Millipore, Billerica, MA). Cesium chloride (CsCl) density gradients were performed according to Sambrook *et al.* (1989), with CsCl density adjusted to a final concentration of 1.05 g per ml, sealed in a Sorvall ultracrip centrifuge tube, loaded into a Sorvall T865.1 fixed angle rotor and subjected to 45,000 rpm for 20 hours. DNA quantification based upon A_{260} was performed using a NanoDrop 2000s (ThermoFisher Scientific, NanoDrop Products, Wilmington, DE), assuming dsDNA and an extinction coefficient of $\text{OD}_{260} = 50 \mu\text{g/ml}$ or $\text{OD}_{260} = 33 \mu\text{g/ml}$ for ssDNA. Quantitative assays were also performed using picogreen (double stranded DNA stain) and oligreen (single-stranded DNA stain) fluorescent dye assays according to the manufacturer's instructions (Invitrogen, Carlsbad, CA). The presence of ss- and dsDNA alone and in mixed solutions was inferred by the relative fluorescence of A_{260} -normalized DNA concentrations using either picogreen or oligreen compared to M13 ssDNA and dsDNA, alone and mixed, as standards. A one-way ANOVA was performed to test for significance among the groups using Prism (Graphpad; San Diego, CA) followed by posthoc 2-tailed unpaired t-tests.

2.3. DNA restriction endonuclease analysis and gel electrophoresis

pUC19, M13 single- and double-stranded DNA, PCR products and pColE3 preparations were subjected to restriction endonuclease digestion according to the manufacturer's instructions using *AlwNI*, *BamHI*, *EcoRI*, *HindIII*, and *PvuII* endonucleases (Fermentas). DNA was separated by agarose gel electrophoresis using 0.9% agarose in TAE buffer containing 40mM Tris, 20mM acetic acid, and 1mM EDTA using 60 mA constant current. Linearized DNA fragment molecular weight markers were used for size analysis (GeneRuler 1 kb Plus, Fermentas). For gels containing ethidium bromide (Thermo Fisher Scientific, Waltham, MA), 1 $\mu\text{g/ml}$ was incorporated into the gel. Duplicate gels lacking dye were subjected to submersion in DNA staining baths consisting of either 1) ethidium bromide (1 $\mu\text{g/ml}$ in TAE), 2) acridine orange (Sigma Aldrich, St. Louis, MO; 7.5 $\mu\text{g/ml}$ for 30 min in 10 mM sodium phosphate buffer, pH 7.0 with 2 – 16 hrs destaining using three washes dH_2O (McMaster & Carmichael, 1977), 3) SYBR gold (1:10,000 dilution of manufacturer's stock

solution in TAE used without destaining; InVitrogen), 4) picogreen (1:10,000 dilution of manufacturer's stock solution in TAE and destained with three washes in TAE; InVitrogen) or 5) oligreen (1:1000 dilution of manufacturer's stock solution in TAE and destained with three washes in TAE; InVitrogen) and then visualized by 302 nm and/or 365nm ultraviolet light using either an ethidium bromide (UVP, Upland, CA) or SYBR Gold/acridine orange Wratten No. 15 (Kodak, Rochester, NY) optical filters.

2.4. DNA sequencing

Oligonucleotides used in this study shown in Table 1 were purchased from Integrated DNA Technologies (San Diego, CA). DNA was sequenced by Sanger thermocycle dye termination (Sequetech, Mountain View, CA and Genewiz, South Plainfield, NJ) using 0.04 to 0.5 µg/µl DNA and 6.0 pmols primer per reaction. DNA sequencing of the entire plasmid was initiated based on the published colicin and immunity genes and subsequently completely sequenced by overlapping primer extension sequencing reactions. Raw DNA sequence ends were cropped using the automatic cropping tool in 4PEAKS (Nucleobytes, Aalsmeer, Netherlands). DNA sequence analysis also utilized Geneious (Biomatters LTD, Auckland, NZ) and BLAST (NCBI, Bethesda, MD). Plasmid maps were generated with Geneious and PowerPoint (Microsoft, Redmond, WA). DNA sequencing quality scores (Phred) were analyzed in association with known and putative single- and double-stranded DNA. Phred quality scores were analyzed for bases 100 to 500 where sequence quality was normally the highest and averaged. Bar graphs were generated using Prizm. Statistical analyses were performed using Prizm 2-tailed unpaired t-tests.

2.5. Generation of pUC19 *ori*:ColE3 Rep *ori* fusions

Due to the presence of multiple origins of replication in the pColE3 plasmid, we sought to assess their effect on plasmid DNA preparations alone and in combination, with or without the pUC19 origin. Based on the complete DNA sequence and annotation that was produced, PCR was used to amplify the Rep protein from a site upstream beginning at bp 2792 using the E3RepF1 primer which contains an *Sfi*I site compatible with *Alw*NI as well as *Alw*NI and a *Hind*III site, and downstream to either the replication *ori* (E3RepR1 primer) or the *ssi ori* (E3ssiR1 primer), each of which contain *Bam*HI sites, and cloned into pUC19 into either the *Bam*HI and *Alw*NI sites, which resulted in deletion of most of the pUC19 *ori*, or cloned into the *Bam*HI and *Hind*III sites, which preserved the pUC19 *ori*. PCR was performed using an MJ Research PCT200 thermocycler (Waltham, MA). The polymerases used were Phusion (Fermentas), and Taq polymerase (PCR Master Mix 2X; Fermentas). The Taq-containing PCR consisted of one cycle of 95° C for 5 min followed by 30 cycles of 95° C for 30 sec, 58° C for 30 sec and 72° C for 2 min, with a final extension of 72° C for 5 min. The Phusion-containing PCR consisted of one cycle of 98° C for 3 min followed by 33 cycles of 98° C for 10 sec, 58° C for 30 sec and 72° C for 1 min, with a final extension of 72° C for 5 min. Genes were ligated into the pUC19 vectors using T4 rapid DNA ligation kit (Fermentas). Clones were transformed into chemically competent DH5α or EC100 and grown on LB-Amp plates. The DNA sequence of the Phusion PCR products was determined by DNA sequencing (Sequetech) with DNA representing all four constructs confirmed to be complete and accurate. Relative amounts of plasmid produced by the different clones was

determined by 1) using A₂₆₀ and 2) by resolving equivalent volumes (10 µl of standard 50 µl plasmid minipreps derived from 1.5 ml of fresh overnight cultures) on agarose gels.

3. Results

3.1. Complete sequence of pColE3-CA38

Single primer extension DNA sequencing revealed that the plasmid is a 7118 pb circular molecule. The complete nucleotide sequence was deposited in GenBank as accession number KM287568. The plasmid consists of 7 open reading frames (ORFs) and two sites for DNA replication, the E3 replication origin (*Rep ori*) and a single stranded initiation (*ssi*) origin (Figure 1A), with the DNA sequence
GGTAGCGCTCGCCGCAGTCTCATGACCGAGCGTAGCGAGCGAATGAGCGAGGA
AGCGCAAAGGCGTCCGGTGGTGC. The ORFs include the colicin E3 toxin (ColE3), ColE3 immunity, ColE8 immunity, ColE3 lysis, the replication protein (Rep), a mobilization protein (Mob) and a conjugal transfer protein. The Mob and conjugal transfer gene were not previously known to be associated with the pColE3 plasmid. A BLAST search revealed that this plasmid is most highly similar to the 8140 bp plasmid pO111_4 of the enterohemorrhagic *Escherichia coli* O111:H- str. 11128 (Genbank Accession AP010964; Nakayama *et al.*, 2009), with a BLAST coverage of 95% and having 98% identity (Figure 1B). Present on the pO111_4 plasmid with similar organization is a colicin gene with sequence similarity to ColE3 (dark green shading) and to DNase colicins ColE2, ColE8 and ColE9 (lighter green shading), the ColE8 immunity gene (which is also present on the pColE3 plasmid; also highly similar to ColE2 immunity), a lysis protein, and an additional hypothetical protein with sequence similarity to the pColE3 plasmid but lacking a start codon in pColE3. Also present with a high degree of similarity in both organization and DNA sequence are the loci for the Rep protein, the pColE3 Rep origin (*Rep ori*), the *ssi*, Mob and conjugal transfer proteins. In addition, pO111_4 encodes for ColE6 immunity (also highly similar to cloacin immunity and to ColE8 immunity) and ColE2 immunity (also highly similar to other colicin immunity proteins), and an additional hypothetical protein.

3.2. Restriction endonuclease analysis

We compared the DNA isolated from *E. coli* carrying pColE3-CA38 and our DH5α strain containing the pColE3 plasmid (not shown) with the published restriction endonuclease digestions of pColE3 by Watson & Visentin, (1980; Figure 2). Our restriction digests resulted in DNA bands consistent with the predicted sizes of 597, 3143 and 3378 for *EcoRI* and 2392 and 4723 for *PvuII*, and were indistinguishable from those of Watson and Visentin (1980) except that a faster-migrating, diffuse band was present and apparently resistant to restriction endonuclease digestion (Figure 2A; arrows). We found that purification of the plasmid by CsCl density gradients resulted in DNA that lacked the faster-migrating band and resulted in complete digestion with *EcoRI* and *PvuII* (Figure 2B). We subsequently made a similar comparison with M13 single- and double-stranded DNA, which gave the expected restriction patterns in agarose gels for one *EcoRI* and three *PvuII* sites in the M13 dsDNA, (note: a small band predicted to be a 93 bp fragment in lane C3 is not visible), but no restriction digestion of the single-stranded M13 DNA (Figure 2C & D).

3.3. Relationship of DNA sequencing quality to single-stranded DNA

Primer extension sequencing resulted in Phred quality scores that were considerably lower in the forward direction relative to the colicin and immunity genes as compared to the reverse direction, and the electropherograms contained a notable amount of noise for all of the forward primer-generated sequences despite using the same DNA template derived from alkaline lysis minipreps (Figure 3). This was especially true when we reduced the amount of DNA template in the sequencing reactions. We found that when DNA prepared by CsCl density gradients was used, DNA sequencing was improved. Because of these asymmetric sequencing results we questioned whether it could be due to the presence of a single stranded template. We compared Phred quality scores in the forward and reverse direction for M13 single- and double-stranded templates using forward and reverse primers (M13seqF1 and M13seqR1; Table 1) with pColE3 using forward and reverse primers (SeqF2 and E35'R1; Table 1) and found a similar degree of low quality and high quality for M13 forward or reverse primers, respectively, used on a single-stranded (plus strand DNA) template as compared with the double-stranded template. These data were consistent with the presence of ssDNA in the pColE3 minipreps, but not in the CsCl preparations. When we isolated the ColE3-resistant putative ssDNA band from an agarose gel, it too resulted in forward sequencing reactions with low Phred scores (Figure 3). Because the isolated restriction endonuclease-resistant diffuse band resulted in sequence in the reverse direction identical to pColE3, the possibility that this band was due to the presence of another plasmid was ruled out.

3.4. DNA dye binding patterns

We normalized our DNA samples using absorbance at 260 nm and determined the picogreen fluorescence and oligreen fluorescence of M13 double- and single-stranded DNA alone and mixed. We found that picogreen showed a highly significant difference in fluorescence between the ds- and ssDNA, consistent with its preference for dsDNA, with an intermediate level of fluorescence for the mixture of the two forms (Figure 4). Analysis of the ColE3 miniprep also showed a value that was intermediate between ss- and dsDNA, and was significantly different from either ss- or dsDNA standards alone. Results performed using oligreen for this test were also consistent with the colicin containing a mixture of ss- and dsDNA. However, we found that oligreen only has a slight increase in binding for ssDNA and the assay was only performed a single time (data not shown). We also separately stained agarose gels of pColE3 minipreps with either picogreen or oligreen and compared them with SYBR gold-stained gels and found that the restriction endonuclease resistant band preferentially bound to the oligreen relative to the picogreen (data not shown).

3.5. Metachromatic staining with acridine orange and effects of ColE3 Rep *ori* and pUC19 *ori* combinations on plasmid copy number

We analyzed the pColE3 minipreps using acridine orange metachromatic staining (McMaster & Carmichael, 1977; Figure 5A). We found that the restriction endonuclease-resistant band observed in agarose gels of pColE3 stained as a distinct red band (single-stranded staining), whereas the other DNA in the gel stained green (double-stranded staining). In the gels shown, both the M13 and ColE3 single stranded DNA (arrows) migrate

more slowly in the gel without incorporated ethidium bromide (arcidine orange staining was performed after running the gel; right panel) compared to the gel with ethidium bromide pre-incorporated into the gel (left panel). Thus, the presence of ethidium bromide in the gel significantly altered the mobility of ssDNA relative to dsDNA. Uncut DNA prepared using the Fermentas, Promega and Zymo Research kits all produced diffuse bands that stained red with acridine orange (data not shown). The presence of ssDNA was also apparent in our ColE3 DH5 α strain (data not shown). In addition, acridine orange-stained agarose gels also showed that minipreps of pColE5 and pColE9, but not pColE1, contain significant amounts of single-stranded DNA (data not shown).

We subsequently tested four constructs with different combinations of replication origins (Figure 5B), 1) the ColE3 Rep *ori* alone (truncated pUC *ori*), 2) the ColE3 Rep *ori* with the *ssi ori* (truncated pUC *ori*), 3) the pUC19 *ori* with the ColE3 Rep *ori*, and 4) the pUC19 *ori* with the ColE3 Rep *ori* and the *ssi ori*, by running them on agarose gels either with either ethidium bromide pre-incorporated into the gel or staining with acridine orange after electrophoresis (Figure 5C). Observation of these gels did not reveal any distinctly red-staining bands, or bands with significant shift in mobility due to the presence of ethidium bromide; only a minor shift in one band was noted (arrows). However, we did note distinct differences in the relative amounts of plasmid obtained in minipreps of these different constructs. As compared with pUC19 in DH5 α and the wild type pColE3 that produced relatively little plasmid, the cloned ColE3 Rep *ori* in the pUC19 with a truncated origin produced the least amount of plasmid, whereas the combination of the pUC19 *ori* together with either the Rep *ori* or the Rep *ori* plus the *ssi ori* produced very large amounts of plasmid. A quantitative comparison of the minipreps is shown in Figure 5D, with a highly significant ($p < 0.0001$) increase in DNA production when the pUC *ori* and the Rep *ori* were combined.

4. Discussion

Our study has resulted in a complete DNA sequence and map of pColE3-CA38 from strain BZB2106. Several components of the pColE3 have long been known, but the complete sequence has not been previously reported. pColE3 is very similar to the 8140 bp pO111_4 plasmid found in the enterohemorrhagic *Escherichia coli* O111:H-. The colicin protein that plasmid encodes for is highly homologous to both ColE3 in the N-terminus, and to the DNase colicins ColE2, ColE8 and ColE9 in the C-terminus, and is likely to be a functional DNase colicin based on the high amino acid sequence conservation. Since pO111_4 and pColE3 contain multiple colicin resistance genes including those for ColE2, ColE6 and ColE8 in pO111_4, the data underscores the potential role for colicin resistance as a key feature in the ability to colonize an environment already occupied by strains producing colicins, as well as the potential for the colicins themselves to aid in the process.

Our methods included two new approaches to detecting ssDNA; A₂₆₀:picogreen fluorescence ratios and the use of Phred DNA sequencing quality scores. The A₂₆₀:picogreen fluorescence ratios were deliberately assessed because of their specificity for dsDNA whereas the finding that the Phred scores were indicative of ssDNA was

serendipitous. Other methods such as preferential degradation of ssDNA by S1 and mung bean nucleases could also have been employed.

Our investigation has led to the conclusion that pColE3 produces ssDNA in *E. coli*, in addition to dsDNA, based upon incomplete restriction endonuclease digestion, asymmetric DNA sequencing which included direct sequencing of the restriction endonuclease-resistant band, the relative fluorescence of picogreen compared to A_{260} , the preferential binding of the single-stranded DNA dye oligreen and metachromatic staining with acridine orange. When we used restriction endonucleases on alkaline lysis minipreps we encountered incomplete digestion of the DNA, with a prominent diffuse band that was endonuclease resistant which we hypothesized might be ssDNA, and showed a similar result is obtained using restriction endonucleases with single-stranded M13 DNA. The lack of complete digestion did not coincide with the original report on mapping of pColE3 that showed complete restriction by endonucleases (Watson & Visentin, 1980). However, when DNA was prepared by CsCl density gradient purification as it was in that report, the DNA was completely digested and lacked the diffuse faster running single-stranded DNA band. These results clearly indicate that different methods of DNA preparation are responsible for the absence or presence of the diffuse single-stranded DNA band, and explains why it was not observed earlier, although we did not examine exactly at which step the ssDNA is lost. We found that several different commercially available miniprep kits resulted in the isolation of both ss- and dsDNA from ColE3, and since ssDNA was produced by the ColE3 DH5 α strain, production of ssDNA is not limited to the original ColE3 strain. When we sequenced the plasmid DNA outward from the previously described colicin and immunity proteins in both directions, DNA sequence quality was lower in the forward direction relative to the colicin and its immunity proteins (Figure 3), consistent with (+) DNA (relative to the colicin and immunity genes) and we also showed that the same result that is obtained using forward and reverse primers to sequence single-stranded (+) M13, but not double-stranded M13. Better sequencing of pColE3 was obtained in both directions with the CsCl purified DNA that lacked the diffuse, restriction endonuclease-resistant band. When the diffuse band was isolated by gel electrophoresis and sequenced, the DNA also only sequenced efficiently in the reverse direction. Since the gel-purified restriction endonuclease-resistant band of DNA resulted in DNA sequence that was identical to that of the pColE3 plasmid, this result ruled out possibility that this band was due to the presence of another plasmid. We now interpret these results as having been due to the miniprep sample containing a large amount of single stranded (+) DNA which generated high-quality sequence in the reverse direction, but contained a relatively smaller portion of double stranded DNA that resulted in a limiting quantity of template that had the consequence of a lower DNA sequence quality in the forward direction. Metachromatic staining of miniprep DNA with acridine orange revealed presence of both a red-stained band indicative of single-stranded DNA as well as green-stained double-stranded DNA that is present in the same preparations. Our results also show that minipreps of pColE5 and pColE9, but not those of pColE1, contain sufficient amounts of single-stranded DNA to stain positive (red) with acridine orange.

When different origins of replication were combined in pUC19, large quantities of DNA were produced due to the coexistence of two origins, the pUC19 *ori* and the Rep *ori*. One

possibility is that the two origins are not only compatible, but that they are synergistic since the resulting amount of DNA produced is greater than the sum of the two individual plasmids. It is possible that the replicative intermediates produced by one of the origins acts as a better template for the replication initiated from the other origin. It is also possible that compared to the pColE3, the dual replication constructs lack cop negative regulatory elements (yet to be identified) which would therefore increase the relative amount of plasmid produced, although this is not true in the absence of the pUC19 *ori*.

The rolling-circle mechanism of DNA replication used in some phage and plasmids involves continuous synthesis of the leading strand of DNA, and due to asymmetry, results in accumulation of ssDNA (del Solar *et al.*, 1993). In our hands, the ColE3 single stranded DNA intermediate was highly stable, and our results showing the accumulation of ssDNA by colicins suggests that they may be closed circular molecules produced by a replication mechanism with a link to rolling-circle plasmids. Indeed, in addition to the production of single-stranded DNA we describe here, several of the features that are associated with rolling-circle plasmids are also known to occur in colicin plasmids (Table 2). For example, antibiotic resistance is a feature associated with many (but not all) rolling circle plasmids. In colicin plasmids, their immunity proteins are essentially antibiotic resistance proteins, although they are not usually discussed in that context. The mobilization and transfer genes that we identified on pColE3 are also common components of many rolling-circle plasmids. Based on these and other similarities shown in Table 2, we suggest that these colicins likely represent a category of rolling-circle plasmids. Additional investigation of the replicative intermediates will be required to determine the actual mechanism of replication.

Several authors have also suggested a possible relationship between colicins and phage (e.g., Jabrane *et al.*, 2002; Cascales *et al.*, 2007) pointing out overlap in the import/translocation machinery between colicins and filamentous phages that includes TolAQR of the bacterial cell envelope. Mutations in these genes confer resistance to both phage and colicins by interfering with their internalization. As single-stranded (+) DNA production represents the virion conformation for the genome of filamentous phage such as M13, the present study extends the overlap of these two systems to include the production of single stranded (+) DNA by colicins, and may occur by a rolling-circle mechanism of replication also used by filamentous phage.

Our agarose gels showed variation in the apparent molecular weight of the ssDNA band, with the ssDNA running either faster or slower relative to the dsDNA molecular weight markers. M13 ssDNA has previously been shown to adopt two different conformations with different sedimentation coefficients in sucrose density gradient centrifugation (Forsheit & Ray, 1970), with a faster-sedimenting form occurring under low ionic conditions. We found that the incorporation of ethidium bromide into the gel resulted in a faster-migrating form of the ssDNA. Ethidium bromide has been shown to enhance the condensation of DNA loops (Belyaev *et al.*, 1999) and thus may result in shifting the ssDNA to a more tightly packed faster-migrating form with a lower apparent molecular weight. We also noted shifts in the migration of the ssDNA relative to the dsDNA molecular weight marker under different electrophoresis current conditions, and subsequently used the same constant current throughout our experiments.

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References

- Aoki K, Shinohara M, Itoh T. Distinct functions of the two specificity determinants in replication initiation of plasmids ColE-P9 and ColE3-CA38. *J Bacteriol.* 2007; 189:2392–2400. [PubMed: 17237180]
- Belyaev IY, Eriksson S, Nygren J, Torudd J, Harms-Ringdahl M. Effect of ethidium bromide on DNA loop organization in human lymphocytes measured by anomalous viscosity time dependence and single cell gel electrophoresis. *Biochimica et Biophysica Acta.* 1999; 1428:348–356. [PubMed: 10434054]
- Bowman CM, Dahlberg JE, Ikemura T, Konisky J, Nomura M. Specific inactivation of the 16S ribosomal RNA induced by colicin E3 *in vivo*. *Proc Natl Acad Sci USA.* 1971; 68:964–968. [PubMed: 4930244]
- Cascales E, Buchanan SK, Duché D, Kleanthous C, Llobès R, Postle K, Riley M, Slatin S, Cavard D. Colicin Biology. *Microbiol Molecular Biol Rev.* 2007; 71:158–229.
- Clairmont C, Troy L, Luo X, Li Z, Zheng L-M, King I, Bermudes D. Expression of colicin E3 by tumor-targeted *Salmonella* enhances anti-tumor efficacy. *Proc Amer Assoc Cancer Res Ann Meeting.* 2000; 41:466.
- Clewell DB. Nature of Col E1 plasmid replication in *Escherichia coli* in the presence of the chloramphenicol. *J Bacteriol.* 1972; 110:667–676. [PubMed: 4336693]
- Clewell DB, Helinski DR. Effect of growth conditions on the formation of the relaxation complex of supercoiled ColE1 deoxyribonucleic acid and protein in *Escherichia coli*. *J Bacteriol.* 1972; 110:1135–1146. [PubMed: 4555406]
- Cornut G, Fortin C, Soulières D. Antineoplastic properties of bacteriocins: Revisiting potential active agents. *Amer J Clin Oncol.* 2008; 31:399–404. [PubMed: 18846002]
- del Solar G, Moscoso M, Espinosa M. Rolling circle-replicating plasmids from gram-positive and gram-negative bacteria: A wall falls. *Mol Microbiol.* 1993; 8:789–796. [PubMed: 8355606]
- Dobson A, Cotter PD, Ross RP, Hill C. Bacteriocin production: a probiotic trait? *Appl Environ Microbiol.* 2012; 78:1–6.10.1128/AEM.05576-11 [PubMed: 22038602]
- Espinosa M, del Solar G, Rojo F, Alonso JC. Plasmid rolling circle replication and its control. *FEMS Microbiol Lett.* 1995; 130:111–120. [PubMed: 7649431]
- Forbes N. Engineering the perfect (bacterial) cancer therapy. *Nat Rev Cancer.* 2010; 10:785–794. [PubMed: 20944664]
- Forsheit AB, Ray DS. Conformations of the single-stranded DNA of bacteriophage M13. *Proc Natl Acad Sci USA.* 1970; 67:1534–1541. [PubMed: 4922292]
- Fuska J, Fusková A, Smarda J, Mach J. Effect of colicin E3 on leukemia cells P388 *in vitro*. *Experimentia.* 1979; 35:406–407.
- Gamage SD, Patton AK, Strasser JE, Chalk CL, Weiss AA. Commensal bacteria influence *Escherichia coli* O157:H7 persistence and Shiga toxin production in the mouse intestine. *Infect Immun.* 2006; 74:1977–1983. [PubMed: 16495578]
- Han M, Gagura M, Itoh T. Specific interaction between the initiator protein (Rep) and origin of plasmid ColE2-P9. *J Bacteriol.* 2007; 189:1061–1071. [PubMed: 17098894]
- Hoffman RM, Zhao M. Methods for the development of tumor-targeting bacteria. *Expert Opin Drug Discov.* 2014; 9:741–750. [PubMed: 24949888]
- Horii T, Itoh T. Replication of ColE2 and ColE3 plasmids: The regions sufficient for autonomous replication. *Mol Gen Genet.* 1989; 212:225–231. [PubMed: 2841566]

- Jabrane A, Sabri A, Compere P, Jacques P, Vandenberghe I, Van Beeumen J, Thonart P. Characterization of serracin P, a phage- tail-like bacteriocin, and its activity against *Erwinia amylovora*, the fire blight pathogen. *Appl Environ Microbiol*. 2002; 68:5704–5710. [PubMed: 12406768]
- Jordi BJ, Boutaga K, van Heeswijk CM, van Knapen F, Lipman LJ. Sensitivity of Shiga toxin-producing *Escherichia coli* (STEC) strains for colicins under different experimental conditions. *FEMS Microbiol Lett*. 2001; 204:329–334. [PubMed: 11731144]
- Khan SA. Plasmid rolling-circle replication: highlights of two decades of research. *Plasmid*. 2005; 53:126–136. [PubMed: 15737400]
- Kido M, Yasueda H, Itoh T. Identification of a plasmid-encoded protein required for initiation of ColE2 DNA synthesis replication. *Nucleic Acids Res*. 1991; 19:2875–2880. [PubMed: 1829159]
- Lancaster LE, Wintermeyer W, Rodnina MV. Colicins and their potential in cancer treatment. *Blood Cells Mol Dis*. 2007; 38:15–18. [PubMed: 17134920]
- Lasater LS, Cann PA, Glitz DG. Localization of the site of cleavage of ribosomal RNA by Colicin E3. *J Biol Chem*. 1989; 264:21798–21805. [PubMed: 2689447]
- Leschner S, Weiss S. *Salmonella*-allies in the fight against cancer. *J Mol Med*. 2010; 88:763–773. [PubMed: 20526574]
- Low KB, Ittensohn M, Le T, Platt J, Sodi S, Amoss M, Ash O, Carmichael E, Chakraborty A, Fischer J, Lin SL, Luo X, Miller SI, Zheng L-m, King I, Pawelek JM, Bermudes D. Lipid A mutant *Salmonella* with suppressed virulence and TNF α induction retain tumor-targeting *in vivo*. *Nat Biotechnol*. 1999; 17:37–41. [PubMed: 9920266]
- Masaki H, Ohta T. Colicin E3 and its immunity genes. *J Mol Biol*. 1985; 182:217–227. [PubMed: 3889348]
- McMaster GK, Carmichael GG. Analysis of single- and double-stranded nucleic acids on polyacrylamide and agarose gels by using glyoxal and acridine orange. *Proc Natl Acad Sci USA*. 1977; 74:4835–4838. [PubMed: 73185]
- Murinda SE, Liu S-M, Roberts RF, Wilson RA. Colicinogeny among *Escherichia coli* serotypes, including O157:H7, representing four closely related diarrheagenic clones. *J Food Protect*. 1998; 61:1431–1438.
- Murinda SE, Roberts RF, Wilson RA. Evaluation of colicins for inhibitory activity against diarrheagenic *Escherichia coli* strains, including serotype O157:H7. *Appl Environ Microbiol*. 1996; 62:3196–3202. [PubMed: 8795210]
- Nakayama K, Kurokawa K, Tobe T, Hattori M, Hayashi T. Comparative genomics reveal the mechanism of the parallel evolution of O157 and non-O157 enterohemorrhagic *Escherichia coli*. *Proc Natl Acad Sci USA*. 2009; 106:17939–17944. [PubMed: 19815525]
- Nomura N, Masi H, Inuzuka M, Miyazaki C, Ohtsubo E, Itoh T, Sasamot S, Matsui M, Ishizaki R, Arai K-i. Identification of eleven single-strand initiation sequences (*ssi*) for priming of DNA replication in the F, R6K, R100 and ColE2 plasmids. *Gene*. 1991; 108:15–22. [PubMed: 1761225]
- Ohmori H, Tomizawa J-i. Nucleotide sequence of the region required for maintenance of Colicin E1 plasmid. *Mol Gen Genet*. 1979; 176:161–170. [PubMed: 393952]
- Oka A, Nomura N, Morita M, Sugisaki H, Sugimoto K, Takanami M. *Mol Gen Genet*. 1979; 172:151–159. [PubMed: 384144]
- Pawelek JM, Low KB, Bermudes D. Tumor-targeted *Salmonella* as a novel anti-cancer agent. *Cancer Res*. 1997; 57:4537–4544. [PubMed: 9377566]
- Petkovšek Z, žgur-Bertok D, Erjavec MS. Colicin insensitivity correlates with a higher prevalence of extraintestinal virulence factors among *Escherichia coli* isolates from skin and soft-tissue infections. *J Med Microbiol*. 2012; 61:762–765. [PubMed: 22403139]
- Pugsley, AP.; Oudega, B. Methods for studying colicins and their plasmids. In: Hardy, KG., editor. *Plasmids, A Practical Approach*. Vol. Chapter 5. IRL Press; Oxford: 1987. p. 105-161.
- Rijavec M, Budi M, Mrak P, Müller-Premru M, Podlesek Z, žgur-Bertok D. Prevalence of colE1-like plasmids and colicin K production among uropathogenic *Escherichia coli* strains and quantification of inhibitory activity of colicin K. *Appl Environ Microbiol*. 2007; 73:1029–1032. [PubMed: 17122402]

- Riley MA, Wertz JE. Bacteriocins: evolution, ecology and applications. *Ann Rev Microbiol.* 2002; 56:117–137. [PubMed: 12142491]
- Sambrook, JF.; Fritsch, EF.; Maniatis, T. *Molecular Cloning: A Laboratory Manual.* 2. Cold Spring Harbor Laboratory Press; 1989.
- Schamberger GP, Diez-Gonzalez F. Characterization of colicinogenic *Escherichia coli* strains inhibitory to enterohemorrhagic *Escherichia coli*. *J Food Prot.* 2004; 67:486–492. [PubMed: 15035362]
- Senior BW, Holland IB. Effect of colicin E3 upon the 30S ribosomal subunit of *Escherichia coli*. *Proc Natl Acad Sci USA.* 1971; 68:959–963. [PubMed: 4930243]
- Šmajš D, Micenková L, Šmarda J, Vrba M, Ševíková A, Vališová Z, Woznicová V. Bacteriocin synthesis in uropathogenic and commensal *Escherichia coli*: Colicin E1 is a potential virulence factor. *BMC Microbiol.* 2010; 10:288. [PubMed: 21078157]
- Tajima Y, Horii T, Itoh T. Replication of ColE2 and ColE3 plasmids: Two colE2 incompatibility functions. *Mol Gen Genet.* 1988; 214:451–455. [PubMed: 3063947]
- te Riele MB, Ehrlich SD. Single-stranded plasmid DNA in *Bacillus subtilis* and *Staphylococcus aureus*. *Proc Natl Acad Sci USA.* 1986; 83:2541–2545. [PubMed: 3085097]
- Tomizawa J, Itoh T, Selzer G, Som T. Inhibition of ColE1 RNA primer formation by a plasmid-specified small RNA. *Proc Natl Acad Sci USA.* 1981; 78:1421–1425. [PubMed: 6165011]
- Toshima H, Yohimura A, Arikawa K, Hidaka A, Ogasawara J, Hase A, Masaki H, Nishikawa Y. Enhancement of shiga toxin production in enterohemorrhagic *Escherichia coli* Serotype O157:H7 by DNase Colicins. *Applied Environ Microbiol.* 2007; 73:7582–7588.
- Vieira J, Messing J. The pUC plasmids, an M13mp7-derived system for insertion mutagenesis and sequencing with synthetic universal primers. *Gene.* 1982; 19:259–268. [PubMed: 6295879]
- Watson RJ, Visentin LP. Restriction endonuclease mapping of ColE2-P9 and ColE3-CA38 plasmids. *Gene.* 1980; 10:307–318. [PubMed: 6253352]
- Watson RJ, Visentin LP. Cloning of the ColE3-CA38 colicin and immunity genes and identification of a plasmid region which enhances colicin production. *Gene.* 1982; 19:191–200. [PubMed: 6293928]
- Yagura M, Nishio S-y, Kuzozumi H, Wang C-f, Itoh T. Anatomy of the replication origin of plasmid ColE2-P9. *J Bacteriol.* 2006; 188:999–1010. [PubMed: 16428404]
- Yasueda H, Horii T, Itoh T. Structural and functional organization of ColE2 and ColE3 replicons. *Mol Gen Genet.* 1989; 215:209–216. [PubMed: 2651878]
- Yasueda H, Takechi S, Sugiyama T, Itoh T. Control of ColE2 plasmid replication: negative regulation of the expression of the plasmid-specified initiator protein, Rep, at a posttranscriptional step. *Mol Gen Genet.* 1994; 244:41–48. [PubMed: 8041360]

Highlights

- We present the complete sequence of pColE3.
- pColE3 is most closely related to a plasmid from enterohemorrhagic *E. coli*.
- We used Phred DNA quality scores to infer the presence of single-stranded DNA.
- We used DNA staining dyes to demonstrate the presence of single-stranded DNA that coexists with double-stranded DNA.
- We demonstrate single-stranded DNA in other colicins but not in pColE1.
- Single-stranded DNA colicins may represent a new subclass of rolling-circle replication plasmids.

Figure 1. Genetic organization of the pColE3-CA38 plasmid

A). A circular map of the pColE3-CA38 plasmid and its features is shown. Colicin E3, gene encoding the colicin E3 toxin; Imm E3, the colicin E3 immunity gene; Imm E8, the colicin E8 immunity gene; Lysis, gene encoding the ColE3 lysis protein; Rep, the replication protein gene; Rep *ori*, the replication origin; *ssi*, the single strand initiation origin; Mob, the gene encoding the mobilization element; Conjugal transfer, the gene encoding the conjugal transfer protein. **B).** pColE3-CA38 plasmid comparison with pO111_4 (Genbank Accession# AP010964) from a Shiga-like toxin producing strain. The lighter-shaded portion of the colicin gene corresponds to the region with strong similarity to DNase colicins E2, E8 and E9. I8, immunity to ColE8; L, lysis gene; H, hypothetical proteins; I6, immunity to ColE6; I2, Immunity to ColE2.

Figure 2. Restriction digest analysis of pColE3 compared with M13 ss- and dsDNA

The DNA is either uncut or cut with the restriction endonucleases indicated; molecular weights are shown on the left. **A)** pColE3 DNA isolated using a standard alkaline lysis plasmid minipreps. Lane 1, uncut; lane 2, *EcoRI*; lane 3, *PvuII*. In each of the three lanes, an arrow indicates the diffuse band that is unaffected by the restriction endonucleases. **B)** pColE3 DNA isolated using a CsCl density gradient. Lane 1, uncut; lane 2, *EcoRI*; lane 3, *PvuII*. **C)** M13 dsDNA. Lane 1, uncut; lane 2, *EcoRI*; lane 3, *PvuII*. **D)** M13 ssDNA. Lane 1, uncut; lane 2, *EcoRI*; lane 3, *PvuII*.

Figure 3. DNA sequence quality of pColE3

A & B) Comparison of pColE3 forward (A) and reverse (B) sequence reaction electropherograms using low concentrations of DNA (40 ng per reaction). **C)** Comparison of the Phred quality scores using forward and reverse primers for M13 dsDNA (M13 ds), M13 ssDNA, pColE3 miniprep (ColE3), pColE3 CsCl-purified DNA (ColE3 CsCl), and gel-purified putative ssDNA from a pColE3 miniprep (ColE3 ssDNA). Statistically significant differences are shown using brackets with the p values indicated above.

Figure 4. Relative fluorescence produced by A_{260} -normalized DNA samples using picogreen Comparison of M13 double-stranded (dsM13) and single-stranded (ssM13) picogreen fluorescence with a mixture of M13 ssDNA and dsDNA and pCoIE3. Statistically significant differences are shown using brackets; with highly significant p values indicated above.

Figure 5. Single-stranded DNA staining and the effect of replication origins on plasmid DNA

A) Left panel: ethidium bromide-stained DNA by pre-incorporation of the dye into the gel. Lane 1, double-stranded M13; lane 2, single-stranded M13 DNA; lane 3, pColE3 miniprep; and lane 4, CsCl-purified pColE3. In lanes 2 & 3, arrows indicate the diffuse ssDNA bands running as a lower molecular weight with ethidium bromide pre-incorporated into the gel. Right panel: acridine orange-stained DNA. Lane 1, dsM13; lane 2, ssM13; lane 3, pColE3; and lane 4, CsCl-purified pColE3. Arrows indicate the diffuse bands associated with M13 ssDNA and pColE3 that are shifted to a higher apparent molecular weight in the absence of ethidium bromide. **B)** pUC19 constructs containing pColE3 components. **C)** Left panel: ethidium bromide-stained DNA by pre-incorporation of the dye into the gel. Lane 1, pUC19; lane 2, construct 1; lane 3, construct 2; lane 4, construct 3; lane 5, construct 4. In lanes 4, an arrow indicate one bands running as a slightly lower molecular weight with ethidium bromide incorporated into the gel during electrophoresis. Right panel: acridine orange-stained DNA. Lane 1, pUC19; Lane 2, construct 1; lane 3, construct 2; lane 4, construct 3; lane 5, construct 4. In lanes 4, an arrow indicate one bands running as a slightly higher apparent molecular weight in the absence ethidium bromide incorporated into the gel during electrophoresis. **D)** Relative concentrations of DNA produced by pUC19, pColE3, construct 1 and construct 3.

Table 1

Oligonucleotide primers used in this study.

Primer	Sequence (5' – 3')
<i>Primers initially used based on the published ColE3 and immunity region</i>	
E3-5'F1	AGACCTGGCATGAGTGGAAAG
E3-5'R1	GTTGCCTGTGCTGTTCGTC
E3-3'F1	ATTCCATGTGGGAGATGGG
E3-3'R1	CGCTTTGTTTTGTCAAAGAGG
<i>ColE3-CA38 DNA sequencing primers</i>	
E3SeqF1	GGGGTGGCTTTATATGGTG
E3SeqF2	TTGCTGATGCAATAGCTGAAA
E3SeqF3	TGTTAATAACGGTTGCTTTGATG
E3SeqF4 (same as E3-3'F1)	ATTCCATGTGGGAGATGGG
E3SeqF5	TTCATCCAGCAGAACCAGC
E3SeqF6	GCGGCACAGTAGCTACATCA
E3SeqF7	TCTCCACTTCGTTTCGATTG
E3SeqF8	GAAAAACGGGAATGGAAC
E3SeqF9	TGATAACCACCGTCTGAGCC
E3SeqR1	ATCATCTGCGGTAATGACG
E3SeqR2	GCCATTGCCACATTCTGT
E3SeqR3	GAAACAAAATACTCATTATCGGAAA
E3SeqR4	AGCCTGACACGCTTTGTTTT
E3SeqR5	GTCGCTCCAGTCGATTGAT
E3SeqR6	GCAACAAACCAAGCTGCTC
E3SeqR7	TTTCTGCCGTAATGCCTTTTTGC
E3SeqR8	GTTTCCATTCCCCGTTTTTC
E3SeqR9	GTAGGTGCGGCGAAGAGAT
E3SeqR10	CAAATCCGACAAGCACTTCC
<i>Rep protein, Rep ori, and ssi ori PCR cloning and sequencing primers</i>	
E3RepF1 (<i>Hind</i> III, <i>Sfi</i> I and <i>Alw</i> NI)	GATCAAGCTTGGCCGTGGAGGCCAGTGGCTGTGAAGTGACCGATTAGCAAC
E3RepR1 (<i>Bam</i> HI)	GATCGGATCCGCAACAAACCAAGCTGCTC
E3ssiR1 (<i>Bam</i> HI)	GATCGGATCCGCACACCGGACGCCT
pUC19 F1	CTCTTCGCTATTACGCCAGC
pUC19 R1	CTTCCGGCTCGTATGTTGTG
pUC19R2	TGGTTTGTGTTGCCGGATCAAGAG
<i>Also see E3SeqF5, E3SeqF6, E3SeqR5 and E3SeqR6 listed above for DNA sequencing.</i>	
<i>M13mp18 primers</i>	
M13F1	TGCCTCGTAATCCTTTTGG
M13R1	TCATTGTGAATTACCTTATGCGA

Table 2

Comparison of rolling-circle plasmids with colicin plasmids.

Feature	Examples from rolling-circle plasmids (del Solar <i>et al.</i> , 1993; Khan 2005)	Colicin plasmids
Accumulation of single stranded DNA	A large number of examples include pMV158 and pJV1.	pColE3, pColE5, pColE9 (this report; Figure 5).
Antibiotic resistance	Tetracycline resistance in pMV158.	Colicin immunity proteins.
Mobilization element (MOB) or plasmid recombination element (PRE)	MOB/PRE in pMV158.	Mob and conjugal transfer proteins (this report; Figure 1).
Replication protein	RepC in pT181.	Rep (Yasueda <i>et al.</i> , 1989; Yagura <i>et al.</i> , 2006).
Double stranded origin of replication initiated by Rep protein	LIC (leading initiation control) in pT181 plasmid. Rep protein has a Tyr ¹⁹¹ that is involved in DNA nicking.	Replication <i>ori</i> has binding site for the Rep protein (Akoi <i>et al.</i> , 2007).
Presence of one or more single-stranded origins <i>sso</i> for lagging strand synthesis	<i>sso</i> from pT181.	Single strand initiation (<i>ssi</i> ; Namura <i>et al.</i> , 1991); the functional equivalent for lagging strand synthesis.
Copy number (Cop) repressor peptides	Cop peptides from pKMK1.	IncA in pColE2 (Taijima <i>et al.</i> , 1988)