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Short communication

Molecular characterization of a DNA fragment carrying the basic replicon of pTUC100, the natural plasmid encoding the peptide antibiotic microcin J25 system

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Abstract

The *Escherichia coli* plasmid pTUC100 encodes production of, and immunity to, the peptide antibiotic microcin J25. In the present study, an approximately 8-kb fragment immediately adjacent to the previously sequenced microcin region was isolated and its DNA sequence was determined. The main features of the newly characterized region are: (i) a basic replicon which is almost identical to that of the RepFIIA plasmid R100; (ii) two ORFs with 96% identity to two ORFs of unknown function on pO157, a large plasmid harbored by enterohemorragic *E. coli*, and a large ORF which does not show significant homology to any other reported nucleotide or protein sequence; and (iii) two intact insertion sequences, IS*1294* and IS*1*. Sequence analysis, as well as that of the G + C content of both the 8-kb fragment and the previously sequenced microcin locus, lead us to propose that plasmid pTUC100 is a composite structure assembled from DNA elements from various sources.

Keywords: Plasmid pTUC100; Microcin J25; Replicon; Mosaic structure

1. Introduction

Microcin J25 (MccJ25) is a 21-amino-acid-residue peptide antibiotic active against the DNA-dependent RNA polymerase of Gram-negative bacteria (Delgado et al., 2001; Salomón and Farías, 1992). Production of, and immunity to, MccJ25 is specified by the low-copy-number plasmid pTUC100, harbored by a fecal isolate of *Escherichia coli* (Salomón and Farías, 1992). We have previously cloned the region of pTUC100 encod-

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ing the genetic determinants for MccJ25. Genetic studies and nucleotide-sequence analysis localized three genes (*mcjA*, *mcjB*, and *mcjC*) essential to MccJ25 production and one immunity gene, *mcjD*, to a continuous 4.8-kb region of pTUC100 plasmid DNA (Solbiati et al., 1996, 1999; GenBank Accession No. AF061787). Herein, genetic characterization of an approximately 8-kb plasmid fragment located immediately downstream from the microcin genes is presented. The fragment was shown to contain, among other features, a replicon region almost identical to the R100 replicon.

Bacteria were grown aerobically in Luria broth (LB) at 37 °C, or on plates with LB supplemented with 1.5% agar. When required, ampicillin (50 μg ml^{-1}) or kanamycin (30 μg ml^{-1}) was added for plasmid maintenance. *E. coli* K-12 strains RYC1000 (*araD139 gyrA* Δ*lacU169 rbs*Δ7 *recA56 relA rpsL thiA*) and DH5α [*endA1*, *hsdR17* (r_k^- m_k^+) *supE44 thi*-1 *recA*1 *gyrA relA*1 Δ(*lacI-ZYA-argF*)*U169deoR*(ϕ 80d*lac*Δ(*lacZ*)*M15*)] were used as hosts for transformations. Plasmid DNA was isolated with the Wizard miniprep DNA purification system (Promega). Digestions with restriction endonucleases, ligation with T4 DNA ligase, transformation of competent cells by the $CaCl_2$ procedure, and agarose gel electrophoresis were done as described previously (Sambrook et al., 1989). Automated DNA sequencing was carried out by dideoxy-termination (Sanger et al., 1977) using a custom primer walking strategy. Analysis of the DNA sequence was performed with programs available on the webpage of the National Center for Biotechnology Information (www.ncbi.nlm.nih.gov). The sequence described here has been assigned GenBank Accession No. AY091607.

Earlier estimates of the size of pTUC100, based on a comparison with other plasmids of known size, suggested that it was composed of 60 kb (Salomón and Farías, 1992). In the present study, the size of pTUC100 was more precisely estimated to be 50 kb by restriction fragment analysis.

In a previous study, we had cloned a 13-kb *Bam*HI fragment from pTUC100 in the vector pBR325 (Salomón and Farías, 1992). From this construction, a 4.8-kb *Hin*dIII–*Eco*RI fragment containing the MccJ25 genetic system (Fig. 1) was subcloned and sequenced (Solbiati et al., 1999; GenBank Accession No. AF061787). In the current work, the entire *Bam*HI fragment was recloned in pBR322 and the recombinant plasmid named pTUC200 (Fig. 1). This plasmid was stably maintained in a *polA1* host, indicating that the 13-kb segment cloned harbored the basic replicon of pTUC100 (since the vector pBR322 cannot replicate in *polA* cells). To assess the functionality of this origin we constructed a miniplasmid by ligating the *Bam*HI fragment to a kanamycin resistance cassette derived from transposon Tn*5*. The ligation mixture was used to transform strain RYC1000,

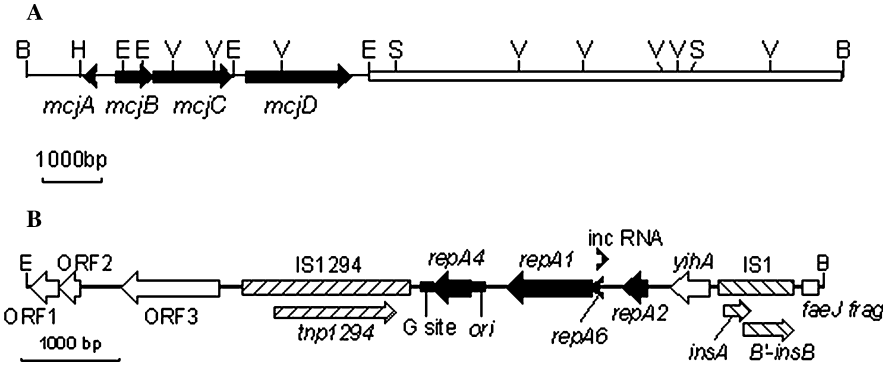

Fig. 1. (A) Physical map of the *Bam*HI pTUC100 fragment cloned in pTUC200, showing the location and arrangement of the MccJ25 genes previously sequenced. The open bar represents the extent of the *Eco*RI–*Bam*HI region subjected to DNA sequence analysis in this study. (B) Genetic organization of the *Eco*RI–*Bam*HI fragment. The deduced ORFs are shown by arrows indicating the direction of transcription. The positions of insertion sequences IS*1294* and IS*1* are shown by hatched boxes. Replication elements are in black. *faeJ frag*, fragment of gene *faeJ* (see text). Abbreviations for restriction sites: H, *Hin*dIII; B, *Bam*HI; E, *Eco*RI; V, *Eco*RV; and S, *Sal*I.

selecting for kanamycin-resistant clones. One of these clones was characterized and shown to contain the expected miniplasmid, designated pOD103. As expected, pOD103 could stably transform PolA$^-$ strains and directed microcin production at levels similar to those from wild-type pTUC100.

To determine the relative copy number of plasmid pOD103, it was transformed into strain RYC1000 harboring compatible multicopy plasmids pACYC184 and pBR322 as internal references. Estimation of the ratio of pOD103 DNA–pACYC184 DNA and pOD103 DNA–pBR322 DNA showed that pOD103 is maintained in the cell at a level of approximately four copies. In contrast, pTUC200 showed a copy number similar to that of pBR322, indicating that the hybrid plasmid is replicating under pBR322 control. Plasmid pOD103 was found to be unstable when the host cells were grown in the absence of antibiotic selection. After approximately 20 generations, 18% of 200 colonies tested had lost the kanamycin resistance marker. This suggests that the plasmid lacks genes of pTUC100 which are required for proper partitioning.

To localize the replication region, we sequenced the *Eco*RI–*Bam*HI fragment from pTUC200 extending just to the right of the previously sequenced 4.8-kb *Hin*dIII–*Eco*RI DNA fragment encompassing the microcin J25 system (Fig. 1). To this end, the *Eco*RI–*Bam*HI fragment was subcloned into pUC18 and sequencing was carried out using the universal priming sites and a primer walking strategy. The entire sequence is 7904 bp in length, including the *Eco*RI–*Bam*HI termini. Fig. 1B presents the most salient features derived from sequence analysis, which are discussed below.

At the 5′ end of the sequence are two open reading frames, designated ORF1 and ORF2, which show 96% identity with two ORFs of unknown function on pO157, a large plasmid harbored by enterohemorragic *E. coli*. (GenBank Accession Nos. NP_052676 and NP_052677, respectively.) They are followed by a 1.4-kb, low-G + C-content block (35%) containing a large open reading frame, designated ORF3 (Fig. 1), which would encode a hydrophilic protein of 325 amino acids with a molecular mass of 38,632 Da. Neither ORF3 nor the predicted protein show significant homology with any other reported gene or protein sequence.

Downstream from ORF3 there is an intact copy of the insertion sequence IS*1294* (Fig. 1), which is 1714-bp in length, whereas that described by Tavakoli et al. (2000) in *E. coli* has 1687 bp. Within IS*1294* we have found an open reading frame potentially encoding a 402-amino acid transposase (*tnp1294* in Fig. 1), which shares 95% identity with the 351-amino-acid transposase of *E. coli* IS*1294* (GenBank Accession No. AJ008006) and 97.7% identity with the 402-amino acid transposase of *Shigella flexneri* IS*1294* (GenBank Accession No. NP_085219). IS*1294* belongs to a family of atypical insertion sequences, of which IS*91* is the type element (Diaz-Arroca et al., 1984), which transpose using rolling-circle replication. So far, two preferred target sites, CTTG and GTTC, have been reported for IS*1294* (Tavakoli et al., 2000). After insertion, the right end of IS*1294* (*ori*IS) lies immediately adjacent to the 3′ end of the target sequences. Interestingly, in our sequence the *ori*IS was preceded by the previously undescribed tetranucleotide CTCG, which could represent an alternative target site for IS*1294*. Since both *oriS* and the flanking tetranucleotide are required for IS*1294* transposition (Tavakoli et al., 2000), it was interesting to test whether the different target site found in our sequence was functional for transposition. To this end, we undertook to entrap IS*1294* in the cosmid pUCD800 (Gay et al., 1985). pUCD800 contains the *sacB* gene of *Bacillus subtilis*, expression of which is lethal to *E. coli* when cultured on medium containing sucrose. Thus, cells containing pUCD800 do not grow on medium containing 5% sucrose unless *sacB* is inactivated by insertion. Briefly, the procedure involved cotransforming plasmids pTUC200 and pUCD800 into *E. coli* DH5α, growing of the double plasmid strain for approximately 100 generations, and plating on LB agar plus sucrose. Four putative pUCD800::IS*1294* recombinant plasmids were isolated from sucrose-tolerant colonies. Nucleotide sequences at the junctions of IS*1294* *ori*IS and pUCD800 in two of them indicated that there had been precise insertions of IS*1294* into different sites in each derivative (Table 1). The retrieval of IS*1294* present in pTUC200 by the use of pUCD800 dem-

Table 1
Nucleotide sequences adjacent to the junction points of the right end of IS*1294* and *sacB* in two pUCD800::IS*1294* derivatives

Derivative	IS*1294*	*sacB*
pUCD800–1	AAAGGATATGCA	**CTTG**ACGACATGTAAAACTAT
pUCD800–3	AAAGGATATGCA	**GTTC**CTACGACAGAAACTGTT

IS*1294* target sites on *sacB* are shown in boldface.

onstrated that this IS element is indeed capable of transposition.

Downstream from the IS*1294* element, there is a region 95% identical at the nucleotide level to the replicon of plasmid R100 (Fig. 1). This 95-kb self-transmissible antibiotic resistance plasmid, which is also referred to as NR1 or 222 (Womble and Rownd, 1988) belongs to the RepFIIA family of replicons, and is present at approximately two copies per chromosome in *E. coli* (Womble and Rownd, 1988). The pTUC100 replicon contains the essential replication elements present in R100 (Fig. 1), including a replication origin (*ori*) which is 97% identical to the *ori* of R100 and a priming signal G site (Tanaka et al., 1994). Between them lies the *repA4* ORF, of unknown function, which is conserved in IncFII plasmids (Ryder et al., 1982) (Fig. 1). As in R100, a DnaA box (TTATCCACA) is located immediately upstream from pTUC100 *ori*. Replicons are classified mainly on the basis of sequence similarity between RepA1 proteins. pTUC100 *repA1* encodes a 285-amino-acid protein, which shares 97% identity with the 285-amino-acid RepA1 of R100 (GenBank Accession No. NP_052990). The *trans*-acting elements that regulate expression of *repA1* in R100 are also present in pTUC100 (Fig. 1): (i) an antisense RNA, *inc* RNA, which exerts negative control on the *repA1* transcript (Nordstrom and Wagner, 1994); (ii) RepA2, a *trans*-acting repressor of the *repA1* promoter; and (iii) *repA6*, whose expression impedes the binding of *inc* RNA to the *repA1* transcript (Wu et al., 1992). It is of note that the replication and stability regions are not linked together in pTUC100. This is also the case of R100, in which the *repA* and *stb* regions are separated by about 30 kb (Womble and Rownd, 1988).

Upstream from *repA2* of R100 lies *yihA*, which specifies a 196-residue protein of unknown function (GenBank Accession No. NP_052987). Similarly, in pTUC100 there is a 127-residue ORF at this position, which is 98% identical to residues 70–196 of R100 YihA. Thus, the YihA ORF in our sequence is truncated by 69 amino acids at the amino terminus compared with its R100 homolog. This partial allele of *yihA* signals the end of pTUC100 homology to R100. Eighty six base pairs downstream of the partial *yihA* ORF, resides an intact copy of the 768-bp-long insertion sequence IS*1* (Ohtsubo and Sekine, 1996) (Fig. 1), which shares 98% identity with the *E. coli* IS*1* (GenBank Accession No. V00609). This element includes two intact consecutive partly overlapping ORFs, *insA* and B′–*insB*, which have been implicated in transposition (Matsutani, 1994), suggesting that it is functional.

Interestingly, the 3′ end of the pTUC100 sequence has been found to contain a region of 180 bp showing 86% identity to the last 180 bp of gene *faeJ* (774 bp) (GenBank Accession No. Z11700), designated *faeJ frag* (for *frag*ment) in Fig. 1. *faeJ* forms part of a gene cluster (*faeC–faeJ*) involved in the biosynthesis of K88 fimbriae in porcine enterotoxigenic *E. coli* (Bakker et al., 1992). The K88 gene cluster is located on large, usually nonconjugative plasmids (Shipley et al., 1978).

The natural isolate producing MccJ25 is an *E. coli* strain. In the current study we have determined the G + C content of the microcin genes and found that it is in the range of 33–36%, much lower than that found for the *E. coli* genome (51%). It is therefore likely that the microcin cluster evolved in a bacterium that does not belong to the family Enterobacteriaceae (which have a base composition much closer to 50%). In this connection, it is interesting that a comparison of the deduced amino acid sequences of McjC and McjD with those of other known proteins showed that the highest degree of similarity was with polypeptides from Gram-positive organisms (Salomón, 2003).

The mosaic nature of the sequence analyzed herein suggests that the development of pTUC100

occurred through the acquisition of DNA elements from various sources. The striking similarity between the pTUC100 and R100 replicons and the presence on pTUC100 of an IS*1294* insertion sequence more closely related to the *S. flexneri* element than to that described in *E. coli* suggest that pTUC100 could have originated in *Shigella* [of note, R100 was initially isolated from a *S. flexneri* 2a strain (Nakaya et al., 1960)] and was then transmitted to *E. coli*. However, the current-day pTUC100 is not capable of self-transfer by conjugation. One possibility is that it was mobilized by a conjugative plasmid. Indeed, we have previously demonstrated that pTUC100 can be mobilized by an F plasmid (Salomón and Farías, 1992). Alternatively, it is possible that a historical precursor of pTUC100 was conjugation proficient, and that transfer functions were lost subsequent to acquisition of the plasmid by *E. coli*.

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References

Bakker, D., Willemsen, P.T.J., Willems, R.H., Huisman, T.T., Mooi, F.R., Oudega, B., Stegehuis, F., de Graaf, F.K., 1992. Identification of minor fimbrial subunits involved in biosynthesis of K88 fimbriae. J. Bacteriol. 174, 6350–6358.

Delgado, M.A., Rintoul, M.R., Farías, R.N., Salomón, R.A., 2001. *Escherichia coli* RNA polymerase is the target of the cyclopeptide antibiotic microcin J25. J. Bacteriol. 183, 4543–4550.

Diaz-Aroca, E., de la Cruz, F., Zabala, J.C., Ortiz, J.M., 1984. Characterization of the new insertion-sequence IS*91* from an alpha-hemolysin plasmid of *Escherichia coli*. Mol. Gen. Genet. 193, 493–499.

Gay, P., Le Coq, D., Steinmetz, M., Berkelman, T., Kado, C.I., 1985. Positive selection procedure for entrapment of insertion sequence elements in Gram-negative bacteria. J. Bacteriol. 164, 918–921.

Matsutani, S., 1994. Genetic evidence for IS*1* transposition regulated by InsA and the δInsA–B′–InsB species, which is generated by translation from two alternative internal initiation sites and frameshifting. J. Mol. Biol. 240, 52–65.

Nakaya, R., Nakamura, A., Murata, Y., 1960. Resistance transfer agents in *Shigella*. Biochem. Biophys. Res. Commun. 3, 654–659.

Nordstrom, K., Wagner, E.G., 1994. Kinetic aspects of control of plasmid replication by antisense RNA. Trends Biochem. Sci. 19, 294–300.

Ohtsubo, E., Sekine, Y., 1996. Bacterial insertion sequences. Curr. Top. Microbiol. Immunol. 204, 1–26.

Ryder, T.B., Davison, D.B., Rosen, J.I., Ohtsubo, E., Ohtsubo, H., 1982. Analysis of plasmid genome evolution based on nucleotide-sequence comparison of two related plasmids of *Escherichia coli*. Gene 17, 299–310.

Salomón, R.A., 2003. The biology of microcin J25, a cyclopeptide antibiotic from *Escherichia coli*. In: Pandalai, S.G. (Ed.), Recent Research Developments in Bacteriology, vol. 1. Transworld Research Network, Kerala, India, pp. 151–177.

Salomón, R.A., Farías, R.N., 1992. Microcin 25, a novel antimicrobial peptide produced by *Escherichia coli*. J. Bacteriol. 174, 7428–7435.

Sambrook, J., Fritsch, E.F., Maniatis, T., 1989. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Sanger, F., Nicklen, S., Coulson, A.R., 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA 74, 5463–5467.

Shipley, P.L., Gyles, C.L., Falkow, S., 1978. Characterization of plasmids that encode for the K88 colonization factor. Infect. Immun. 20, 559–566.

Solbiati, J.O., Ciaccio, M., Farías, R.N., Salomón, R.A., 1996. Genetic analysis of plasmid determinants for microcin 25 production and immunity. J. Bacteriol. 178, 3661–3663.

Solbiati, J.O., Ciaccio, M., Farías, R.N., González-Pastor, E., Moreno, F., Salomón, R.A., 1999. Sequence analysis of the four plasmid genes required to produce the circular peptide antibiotic microcin J25. J. Bacteriol. 181, 2659–2662.

Tanaka, K., Rogi, T., Hiasa, H., Miao, D.-M., Honda, Y., Nomura, N., Sakai, H., Komano, T., 1994. Comparative analysis of functional and structural features in the primase-dependent priming signals, G sites, from phages and plasmids. J. Bacteriol. 176, 3606–3613.

Tavakoli, N., Comanducci, A., Dodd, H.M., Lett, M.-C., Albiger, B., Bennett, P., 2000. IS*1294*, a DNA element that transposes by RC transposition. Plasmid 44, 66–84.

Womble, D.D., Rownd, R.H., 1988. Genetic and physical map of plasmid NR1: comparison with other IncFII antibiotic resistance plasmids. Microbiol. Rev. 52, 433–451.

Wu, R., Wang, X., Womble, D.D., Rownd, R.H., 1992. Expression of the *repA1* gene of IncFII plasmid NR1 is translationally coupled to expression of an overlapping leader peptide. J. Bacteriol. 174, 7620–7628.