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In-well cell lysis technique reveals two new megaplasmids of 103·0 and 212·6 MDa in the multiple plasmid-containing strain V517 of *Escherichia coli*

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Aims: Identification of two new plasmids in the multiple plasmid-containing strain V517 of *Escherichia coli*.

Method and Results: By using an in-well mild cell lysis technique suitable for megaplasmids observation, two plasmids of 103·0 and 212·6 MDa were detected in the multiplasmid-containing *E. coli* V517.

Conclusions: The two new megaplasmids that were completely overlooked when standard disruptive procedures were used, can now be added to the list of eight plasmids with molecular size from 1·36 to 35·84 MDa reported earlier.

Significance and Impact of the Study: This finding allows to use the strain V517 not only as a size reference of small and moderately large plasmids but as a size reference of megaplasmids as well.

INTRODUCTION

Since Macrina *et al*. (1978) characterized the strain V517 of *Escherichia coli* and reported that this strain carried eight cryptic plasmids (e.g. pVA517A–H) of different molecular size, it has been widely used as a convenient single source of covalently closed circular plasmids reference of small and moderately large molecular weight (e.g. 1·36–35·8 MDa). But, when larger plasmids were needed, other bacteria than *E. coli*, sometimes fastidious micro-organisms were used as the source of megaplasmid size reference. Usually the detection of megaplasmids is not easy because most plasmid manipulations are carried out with extraction procedures based on disruptive techniques that permits a reliable extraction of small or moderately large plasmids ranging from 1·5 to 20 MDa (Meyers *et al*. 1976; Birnoim and Doly 1979; Ortlepp 1989) but eliminate vestiges of megaplasmids, thereby its existence is completely overlooked. When techniques to observe megaplasmids were developed (Eckhardt 1978; Kado and Liu 1981), they were successfully used for the analysis and characterization of plasmids ranging from 30 to 200 MDa, carried by bacteria of the genus *Pseudomonas*, *Rhizobium, Azospirillum* and other water or soil micro-organisms (Nuti *et al*. 1977; Michiels *et al*. 1989; Onyeocha *et al*. 1990). However, these techniques were seldom used on already characterized micro-organisms.

In this communication we report the presence in the strain V517 of *E. coli* of two megaplasmids (103·0 and 212·6 MDa), in addition to those already reported (Macrina *et al*. 1978), that can be used as a source of megaplasmid size markers.

MATERIALS AND METHODS

The strain V517 of *E. coli* was grown from a single colony overnight in LB medium (Sambrook *et al*. 1989) at 37°C (100 r.p.m.). The presence of high molecular weight plasmids was detected using the in-well lysis method described by Eckhardt (1978) and modified by Hynes and McGregor (1990). Cells of a 0·1-ml overnight culture (O.D. 600 = 1) were washed twice with cold 0·3% (v/v) sarkosyl in Tris-borate buffer and centrifuged (16 000 *g*, 7 min). The cell pellet was gently suspended to avoid a premature cell disruption. After washing, the pellet was suspended in

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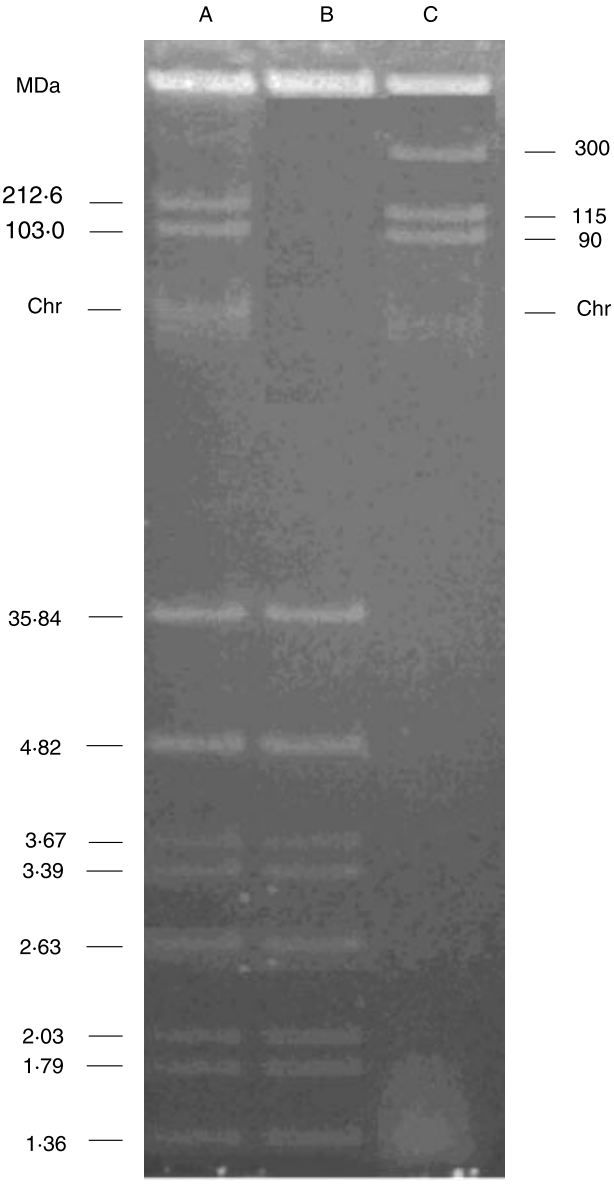

Fig. 1 Plasmid profiles observed in the strain V517 of *E. coli* when using the in-well cell lysis technique (A) or an alkaline extraction method (B). The plasmid containing strain Sp7 of *A. brasilense* was used as the megaplasmid size reference (C); Chr: denotes chromosomal DNA fragments

0·02 ml of lysis solution (sucrose 10%, RNase 10 μg ml^{-1} in TBE and lysozyme 1 μg ml^{-1}) and mixed with 0·03 ml of loading buffer (Sambrook *et al.* 1989) just before loading the gel.

Agarose gels (0·7%) supplemented with SDS (1%) were prepared and run in TBE buffer (in g l^{-1}: Tris, 10·8; EDTA, 0·93; boric acid, 5·5, pH 8·0) according to Sambrook *et al.* (1989). Gels were run at 0·7 Vcm^{-1} during 1 h, followed by 1·40 Vcm^{-1} for 2 h and 2·81 Vcm^{-1} for further 3 h. DNA was stained for 30 min in ethidium bromide (0·5 μg ml^{-1}), washed in distilled water for 50 min, and photographed with Polaroid 665 film at 320 nm. Migration distance and amount of plasmids were evaluated by densitometry directly from negatives. Ten independent experiments were carried out to determine a mean relative mobility of plasmids and plasmid sizes were determined by linear regression analysis (Statistix Ver. 1·0; Analytical Software, Tallahassee, FL, USA; 1996). High molecular weight markers were obtained from the strain Sp7 of *Azospirillum brasilense* (ATCC 29145) that contains three CCC megaplasmids (Croes *et al.* 1991).

To rule out any possible artifact, experiments to test whether the bands observed in the gel corresponded actually to plasmid DNA were carried out. Strips of agarose gel containing DNA bands corresponding to plasmids of 4·82 (pV517B), 35·84 (pV517A) and the new 103·0 and 212·6 MDa of the strain V517 (Fig. 1) were subjected to ribonuclease and deoxyribonuclease digestion. DNAase free Ribonuclease A (10 U ml^{-1}, Sigma) and deoxyribonuclease I (10 U ml^{-1}, Sigma) digestions were carried out at 37°C during 12 h in a sealed test with 2 ml of water. The agarose strips were then washed, stained and photographed as mentioned above.

In order to test whether the new megaplasmids observed corresponded to CCC species or topoisomer species of the already known plasmids of the strain V517, the plasmid pV517A (35·84 MDa, Table 1) was isolated and purified from the gel (Sambrook *et al.* 1989) and subjected to mechanical shearing or increasing dose of UV 320 nm (Macrina *et al.* 1978). These treatments provided a good source of the three topoisomer species of the plasmid pV517A, e.g. CCC (covalently close circular), OL (open linear) and OC (open circular) that were visualized in 0·7% of agarose gels as indicated earlier, using a lambda DNA-mono cut mix (New England Biolabs) as a linear size molecular marker.

Table 1 Size estimates of cryptic plasmids found in the *E. coli* strain V517

Proposed plasmid designation	Molecular weight (MDa)	Reference
pVA517I	212·6*	This work
pVA517J	103·0*	This work
pVA517A	35·84	Macrina *et al.* (1978)
pVA517B	4·82	Macrina *et al.* (1978)
pVA517C	3·67	Macrina *et al.* (1978)
pVA517D	3·39	Macrina *et al.* (1978)
pVA517E	2·63	Macrina *et al.* (1978)
pVA517F	2·03	Macrina *et al.* (1978)
pVA517G	1·79	Macrina *et al.* (1978)
pVA517H	1·36	Macrina *et al.* (1978)

*Mean standard deviation $\sigma = 1{\cdot}5$

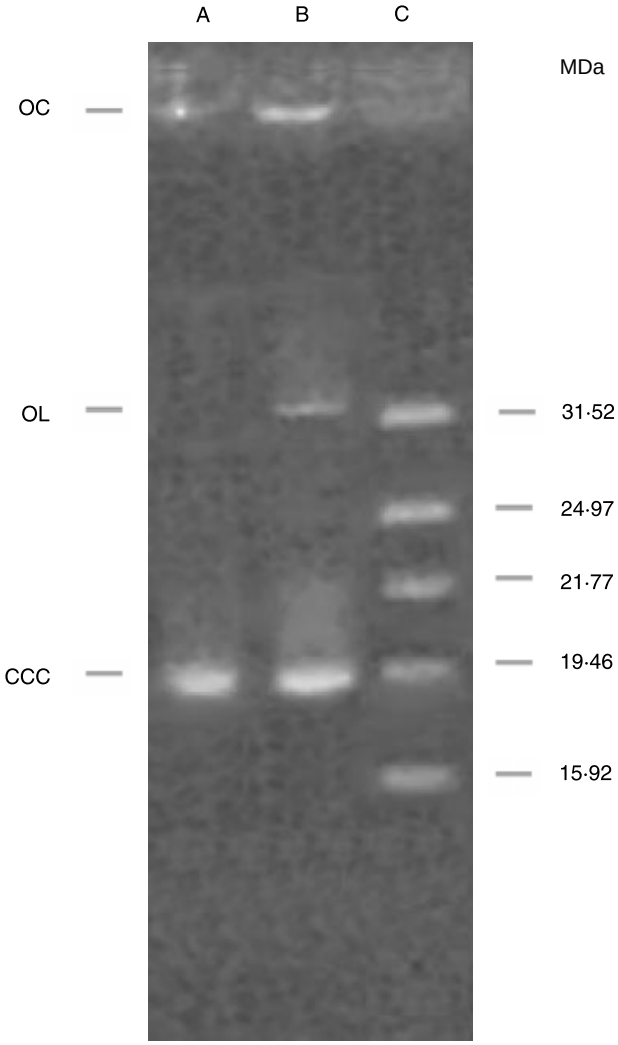

Fig. 2 Migration of the three topoisomers of the cryptic plasmid pV517A present in *E. coli* V517 in a 0·7% agarose gel. Lane A: CCC isomer of the purified pV517A plasmid (35·84 MDa). Lane B: migration of the CCC, OL and OC species of the plasmid pV517A. Lane C: linear DNA size marker (lambda DNA-mono cut mix)

RESULTS

Figure 1 shows plasmid profiles of the strain V517 and the strain Sp7 of *Azospirillum brasilense* (ATCC 29145) that contains megaplasmids of 90, 115 and about 300 MDa (Croes *et al*. 1991). Plasmids carried by the strain V517 were used as internal standards for plasmid size and copy number evaluations and the *Azospirillum* strain was used as the reference for the calculation of megaplasmid sizes. In Fig. 1 we can see the eight plasmids previously reported by Macrina *et al*. (1978) migrating as covalently closed circular (CCC), the two megaplasmids reported in this communication and fragments of chromosomal DNA. Evidence of bands that would indicate the presence of OL or OC

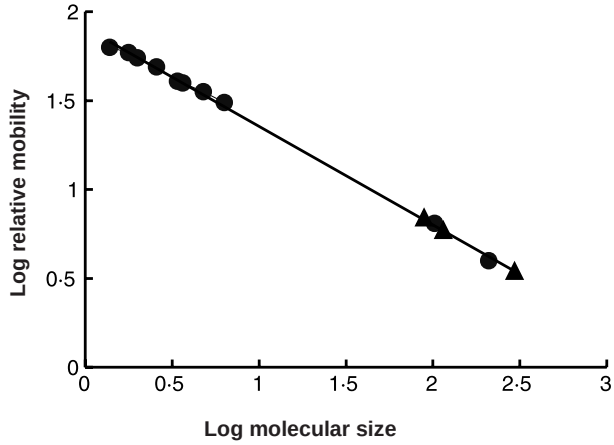

Fig. 3 Relative mobility of plasmids in a 0·7% agarose gel *vs.* size of CCC plasmids of: *Escherichia coli* V517 (●) and *Azospirillum brasilense* Sp7 (▲)

toposiomers were undetectable. The latter means that the mild-cell lysis technique used let us observe only CCC species of plasmids.

Experiments conducted to confirm whether the newly observed plasmids were not artifacts or topoisomers of the already reported plasmids (Table 1) confirmed that the two megaplasmids observed corresponded to CCC species of plasmid DNA. Deoxyribonuclease completely digested the DNA bands contained in the agarose strips whereas ribonuclease did not (results not shown). On the other hand, the plasmid pV517A mechanically or UV treated displayed the three topoisomer species but none of them migrated as the newly observed megaplasmids. In Fig. 2 we present only the UV treated pV517A and we can see that whereas the OL species (35·84 MDa) migrates close to the 31·52 MDa Lambda DNA marker (as expected) the OC species remains at the loading well or barely enters the gel matrix. Since the electrophoresis presented in Figs 1 and 2 were run under identical conditions (see Materials and Methods) we can conclude that the newly characterized megaplasmid bands migrate in different positions than the chromosomal DNA fragments (Fig. 1) and the open species of the pV517A (Fig. 2). The mobilities of the open species of smaller plasmids, e.g. pV517B-H (Table 2) were much higher than the new plasmids under study (data not shown). These results further suggest that the open species of the newly described megaplasmids remain at the loading well. Copy number estimates of the megaplasmids found indicate that they are present in a single copy per host genome.

In Fig. 3 we present the plot of the relative mobility of the plasmids as function of molecular size. In contrast to what has been observed by Rochelle *et al*. (1985), the double $\log_{10}$ plot of plasmid molecular sizes against the corresponding relative mobilities fitted reasonable well along a

straight line (Fig. 3). The latter allowed us to determine that the molecular size of the new plasmids are 103·0 and 212·6 MDa.

DISCUSSION

Our results show that the use of a suitable plasmid extraction technique, like the in-well cell lysis, permitted the identification of two new plasmids of high molecular weight which were not detected when using other techniques. Consequently, we propose that these plasmids should be added to the list of the eight plasmids previously reported for the *E. coli* strain V517 with the designations indicated in Table 1. This finding also indicates that this strain can be used as a single source of a broad range of plasmid sizes and, with the aid of a simple linear regression analysis (Fig. 3), to estimate sizes of unknown plasmids within the range of 1·36–212·6 MDa.

ACKNOWLEDGEMENTS

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