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Construction of shuttle vectors for cloning in *Vibrio cholerae* and *Escherichia coli*

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Abstract. Starting from a naturally occurring cryptic plasmid pVC540 of *Vibrio cholerae* non-OI. strain 1095, a number of plasmid vectors have been constructed for cloning genes in *Vibrio cholerae* by introducing antibiotic resistance markers containing a set of unique cloning sites. The constructs pVC810 and pVE920 have the origins of both *Vibrio cholerae* and *Escherichia coli* replicons and are stable in both organisms in the absence of selective pressure. These plasmids can serve as shuttle vectors between *Escherichia coli* and *Vibrio cholerae.* The plasmid vectors reported here along with the demonstration of transformation in *Vibrio cholerae* by plasmid DNA will facilitate genetic analysis of this important human pathogen.

Keywords. *Vibrio cholerae*; cryptic plasmid; shuttle vector; origin of replication; plasmid stability.

1. Introduction

Vibrio cholerae, a non-invasive Gram-negative bacterium and the causative agent of the diarrhoeal disease cholera, is limited in its ability to accept and maintain plasmids from most incompatibility groups (Davey and Pittard 1975; Rahal *et al* 1975; Kuwahara *et al* 1983). The only *V. cholerae* plasmid which has been studied in some detail is the P plasmid, a fertility factor (Bhaskaran 1964; Bhaskaran *et al* 1974). The incidence of naturally occurring plasmids in clinical and environmental isolates of strains belonging to serovar O1 is rare. Most of the naturally occurring plasmids of *V. cholerae* reported so far are from strains of non-O1 serovar (Newland *et al* 1984) and were found to be cryptic. The occurrence of R plasmids in *V. cholerae* is much lower compared to other Gram-negative enteric pathogens (Prescott *et al* 1968; Hedges and Jacob 1975; O'Grady *et al* 1976; Hedges *et al* 1977).

The importance of extending recombinant DNA technology to new and different bacteria with novel properties has been realized. The use of *V. cholerae* as a host for cloning genes might have an added advantage over other hosts because of the leakiness of its cell surface (Lohia *et al* 1984; Panda *et al* 1991; Paul *et al* 1992), whereby cloned gene products might be excreted and accumulate in the culture supernatant. With the recent demonstration of transformation of V. *cholerae* by plasmid DNA (Panda *et al* 1991) it would now be possible to clone genes in

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V. cholerae provided a suitable vector and a *rec*A mutant strain become available. Since the identified *V. cholerae* plasmids are all cryptic, it is necessary to modify these plasmids by incorporating antibiotic resistance markers containing suitable cloning sites. The present report describes the construction of plasmid vectors for cloning genes in *V. cholerae* which can shuttle between *E. coli* and *V. chalerae,* starting from a naturally occurring cryptic plasmid isolated from a non-OI strain.

2. Materials and methods

2.1 *Bacterial strain, plasmids and growth condition*

The bacterial strains and the plasmids used in this study are listed in table 1.

Table 1. Bacterial strains and plasmid used.

Strain	Description	Source or reference
E. coli		
HB101	F⁻, Pro, Leu, Thi, lacY, Str^r endA, recA13, hsdR hsdM	Boyer and Roulland-Dossoix (1969)
JM101	Thi, (lac-ProAB), [F′-traD36 ProAB] lacI^{q}Z M15	Yanish-Perron *et al* (1985)
S17-1	Rec⁻ hsdR Pro RP4-2TC :: Mu KM :: Tn7	Priefer *et al* (1985)
CSR603	thr-1 ara14 leuB6 (gpt-ProA)62 lacY1 tsx 33 supE44 phr-1 galK2 λ⁻ rac gyrA98 recA1 rpsL31 KdgK51 xyl-5 mtl-1 uvrA6 HB101/F′lac	Sancar *et al* (1979)
D1210	HB101/F′lac	Storz *et al* (1990)
V. cholerae		
569B	Prototroph, hypertoxinogenic, classical biotype; Inaba, Serotype	Finkelstein (1973)
1095	non-O1, plasmid bearing natural isolate	Cholera Res. Center, Calcutta, India
Plasmids		
pBR322	Ap^r Tc^r	Boliver *et al* (1977)
pBR325	Ap^r Tc^r Cm^r	Prentki *et al* (1981)
pUC19	Ap^r	Yanish-Perron *et al* (1985)
pRK290	Broad host range conjugative plasmid Tc^r	Ditta *et al* (1980)
pVC800 pVC540 pVC400	Naturally occurring plasmids isolated from the *V. cholerae* non-O1 strain 1095	Present study
pJD810	Plasmid cointegrate of pVC540 and pUC19, 8·1 kb, Ap^r	Present study
pUD650	Derivative of pVC540 containing Tc^r gene from pBR322, 6·5 kb, Tc^r	Present study
pVE970	Plasmid cointegrate of pVC540 and pBR322, Ap^r Tc^r	Present study
pVE920	pVE970 derivative, 9·2 kb, Ap^r Tc^r	Present study
pJD620	pJD810 derivative containing Cm^r gene from pBR325, 6·2 kb, Cm^r	Present study

V. cholerae cells were grown and maintained as described previously (Roy *et al* 1982). *E. coli* cells were grown in Luria broth (LB) at 37°C. Cell growth was assayed as colony forming units (CFU) in LB agar plates.

2.2 *Plasmid preparation*

E. coli plasmids were isolated following the method of Birnboim and DoIy (1979). For isolation of plasmids from *V. cholerae* cells, EDTA and lysozyme were omitted from the cell suspension buffer because of sensitivity of *V. cholerae* cells to these agents.

2.3 *Transformation*

Transformation of *E. coli* was performed by using standard methods (Maniatis et *al* 1982). The transformation of *V. cholerae* cells by plasmid DNA was carried out using the method described by Panda *et al* (1991).

2.4 *Protein labeling In maxicells*

Plasmids were introduced into the maxicell strain CSR 603 of *E. coli* (Sancar *et al* 1979). The transformed cells were grown in minimal media containing 1% Casamino acids, irradiated with UV light (10–50 J/m^2) and incubated at 37°C. After 1 h of incubation, 200 µg D-cycloserine was added per ml and the incubation was continued for another 16 h at 37°C. The labelled cells were then harvested, suspended in fresh sulphur depleted medium containing [^{35}S]methionine (5 µCi/mI) and incubated at 37°C for 1 h. The labelled cells were harvested, washed and whole cell lysates were analysed by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS–PAGE) followed by autoradiography of dried gels.

2.5 *Stability of plasmids*

Stability of plasmids was determined following the method of Chiang and Bremer (1988). Plasmid bearing cells were grown in presence of the appropriate antibiotic at 37°C for 12–14 h. The culture was diluted 10^{-5}-fold into 10 ml of fresh medium in absence of antibiotic and was grown at 37°C for about 100 h which is equivalent to about 130 generation time. At 18 h intervals, samples were diluted (10^{-5}- to $l0^{-6}$-fold and colony forming units were assayed. The percentage of colony forming cells on antibiotic plates compared to those on nutrient agar plates was plotted as a function of time.

2.6 *Plasmid copy number*

The copy numbers of pVC810 in *E. coli* and *V. cholerae* cells were determined as described by Chiang and Bremer (1988) with some modifications. Plasmids isolated from a fixed number of cells were electrophoresed; the gels were stained with EtBr and scanned using a microdensitometer (LKB 2202 Ultroscan, Sweden).

The amount of plasmid DNA was estimated from the ratio of the peak areas of standard and plasmid pVC810. In the estimation of the copy number, the fact that linear DNA takes up 10% more stain was taken into account.

3. Results and discussions

3.1 *Plasmids of V. cholerae*

Several *V. cholerae* strains belonging to serovars O1 and non-O1 (obtained from the National Institute of Cholera and Enteric Diseases, Calcutta) were examined for the presence of plasmids. No plasmid bearing strain was found among the 100 strains of O1 serovar examined. This is in agreement with the previous reports (Baron and Falkow 1961; Davey und Pittard 1975; Rahal *et al* 1975) that plasmids occurring naturally in *V. cholerae* O1 Strains are rare. On the other hand, 7 out of 16 non-O1 strains examined carried one or more plasmids of sizes ranging between 3 and 15 kilobases (kb). None of the plasmid bearing strains of *V. cholerae* showed resistance to ampicillin. (15 μg/ml), tetracycline (3 μg/ml), kanamycin (20 μg/ml), chloramphenicol (5 μg/ml), spectinomycin (50 μg/ml), trimethoprim (10 μg/ml) or streptomycin (50 μg/ml).

The non-Ol strain 1095 carried several plasmids of which three plasmids of sizes 40, 5·4 and 8·0 kb were designated pVC400, pVC540 and pVC800 respectively (figure 1). The plasmids pVC400 and pVC54O showed extensive nucleotide sequence homology in Southern blot analysis between themselves and with all the plasmids isolated from other non-Ol strains. The plasmid pVC800 holds little or no homology with either pVC400 or pVC540. None of the *V. cholerae* plasmids showed DNA sequence homology with the pBR or pUC vectors. The plasmid pVC540 was selected for the development of a *V. cholerae* vector which can be stably maintained in both *V. cholerae* and *E. coli.*

3.2 *Construction and stability of pVC650*

A physical map of the plasmid pVC540 was constructed using several restriction enzymes (figure 2). The enzymes *Hind*III , *Eco*RI, *Cla*I and *Ava*I have single sites while the enzymes *Bam*HI, *Pst*I and *Sal*I have no sites on the plasmid. Since the plasmid pVC540 does not have any antibiotic resistance marker, the 1·4 kb *Eco*RI –*Bal*I fragment of the plasmid pBR322 carrying the Tc^R marker was ligated to the 5·1 kb *Eco*Rl-*Bal*I fragment of pVC540. The recombinant Tc^R plasmid pVC650 (figure 2) was examined for its stability in *V. cholerae* and *E. coli* cells.

E. coli HB 10l and *V. cholerae* strain 569B were transformed by either pVC650 or pBR322 and transformants were selected by screening colonies on agar plates containing the desired amount of Tc (10 μg/ml for *E. coli* and 3 μg/ml for *V. cholerae*). The stability of the plasmids in the absence of Tc was determined. While the plasmid pVC650 was highly stable in *V. cholerae* cells, it could not be stably maintained in *E. coli* cells. In the absence of selective pressure more than 90% of the *E. coli* cells lost their plasmids within 18 h of growth (figure 3). Thus for stable maintenance and propagation in *E. coli, V. cholerae* plasmids need Col E1 or some other *E. coli* origins of replication. The *E. coli* plasmids, on the other

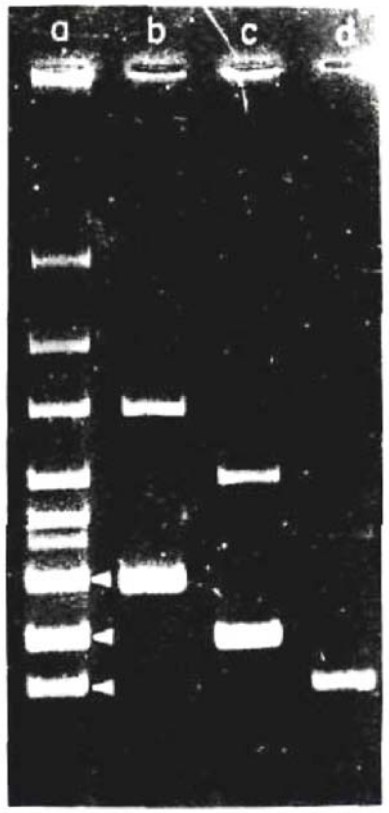

Figure 1. Plasmids pVC540 and pVC800 from the non-OI strain 1095 of *V. cholerae.* Plasmids were isolated as described in the text. The total plasmid DNA was analysed in a 0·8% agarose gel (lane a). The three lowermost bands (arrows) were separately eluted from low melting agarose gels and rerun on 0·8% agarose gels. These bands corresponded to the supercoiled from of pVC800 (lane b). pVC540 (lane c) and pVC400 (lane d). (lane d).

hand, could not be stably maintained in *V. cholerae* cells. Within 72 h of growth in the absence of antibiotics, 90% of *V. cholerae* cells lost the plasmid pBR322 (figure 3). Plasmids having only Col El origin of replication apparently are not capable of replication and maintenance in *V. cholerae* cells. To construct vectors which can be maintained in both *V. cholerae* and *E. coli* cells, it is, therefore, necessary to introduce into the *V. cholerae* plasmid an *E. coli* origin of replication. However, the plasmid pVC650 containing the Tc^R marker and a set of unique cloning sites can be used for cloning in *V. cholerae* cells.

3.3 *Construction and characterization of pVC810 and pVE920*

To incorporate the Col E1 origin of replication along with an antibiotic resistance marker, the plasmid pVC540 was digested with the enzyme *Hind*III and was ligated to *Hind*III digested *E. coli* plasmid pUC 19. The recombinant plasmid pVC810 (figure 2) was used to transform the *E. coli* strain JM101 and *V. cholerae* strain

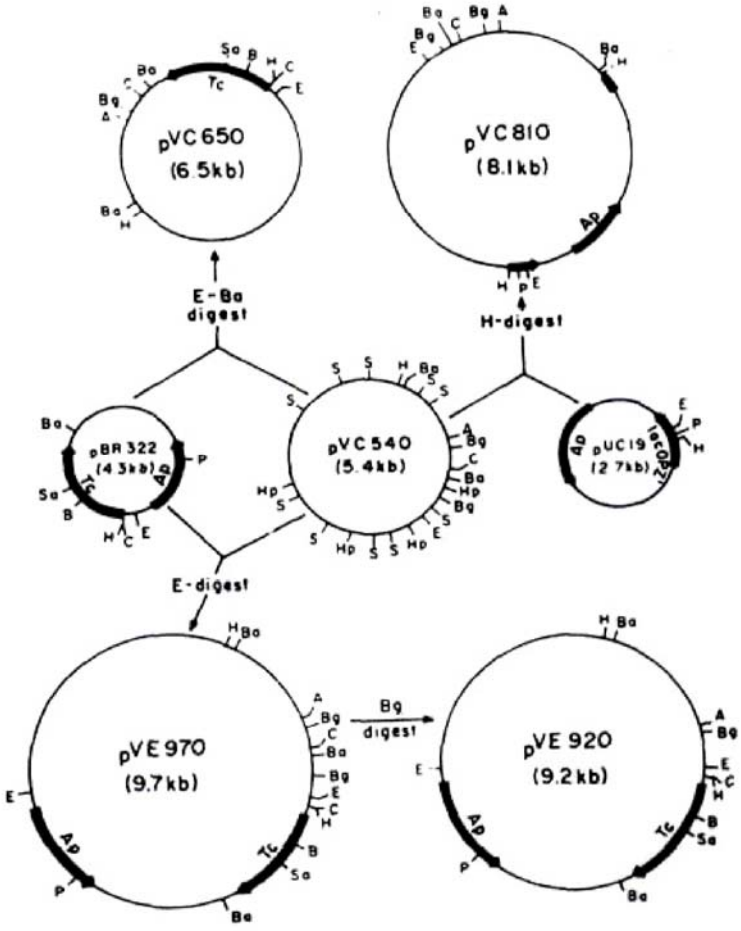

Figure 2. Physical map of pVC540 and construction of pVC650, pVC810 and pVE920. In case of the construction of pVC650 the Ball digest of pVC540 was partial. Tc and Ap indicate tetracycline and ampicillin resistance markers, the thick lines with arrow heads show the extent and direction of transcription. A, *Ava*I; Bg. *Bgl*II, Ba, *Bal*I, C, *Cla*I, H. *Hind*III; S. Sau3A; Hp, *Hpa*II, Sa, *Sal*I; P, *Pst*I; B, *Bam*HI

569B. In the presence of IPTG, X-gal and Ap (l00 µg/ml for *E. coli* and 15 µg/ml for *V. cholerae*), white colonies were picked up as transformants. The presence of the plasmid pVC810 was confirmed by isolating plasmids from transformed cells. The plasmid pVC810, containing both *E. coli* and *V. cholerae* origins of replication, was highly stable in both *E. coli* and *V. cholerae* cells (figure 3). The copy numbers of pVC810 in *E, coli* and *V, cholerae* cells were 145 and 78 respectively.

The proteins coded by the plasmid pVC810 were examined by the maxicell method (Sancar *et al* 1979). Besides the proteins coded by the plasmid pUC19, two other proteins of sizes 25 and 32 kDa could be resolved in the gel (Figure 4). These two proteins are coded by the naturally occurring *V. cholerae* plasmid

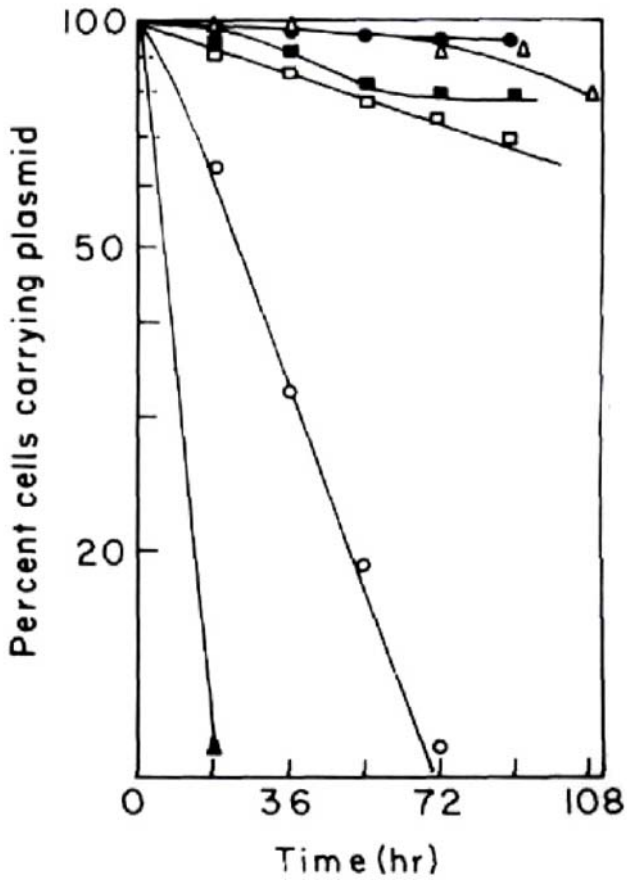

Figure 3. Stability of pVC6·50, pVC810 and pBR322 in *E. coli* HB 101 and *V. cholerae* 569B during growth in the absence of antibiotics. Each point on the graph represents an average of at least three sets of independent experiments. (●)HB101 :: pBR322; (O) 569B :: pBR322; (▲) HB101 :: pVC650; (Δ) 569B:: pVC650; (■) HB101 :: pVC810: (□) 569B. :569B.:: pVC810.

pVC540, constituting part of the recombinant plasmid pVC810. The functions of these proteins are not known.

The *Pst*I-*Cla*I digest of the plasmid pVC810 produces two fragments of sizes 3·8 and 4·3 kb. Both these fragments were ligated to *Pst*I-*Cla*I digested plasmid pBR325 and the recombinant plasmids were used to transform *E. coli* strain D1210. Cm^R, Tc^s, Ap^s colonies were selected. The recombinant plasmid pVC620 containing the 3·8 kb pVC810 fragment and 2·4 kb pBR325 fragment carrying the Cm^R marker can be maintained in *E. coli* only in the presence of selective pressure (30 μg/ml for *E. coli* and 5 μg/ml for *V. cholerae*), but can be stably maintained in *V. cholerae* cells. Thus, the *V. cholerae* origin of replication in the plasmid pVC810 is located in the 3·8 kb fragment which comes from the plasmid pVC540. Maxicell analysis showed that this fragment codes for both 25 and 32 kDa proteins coded by pVC810.

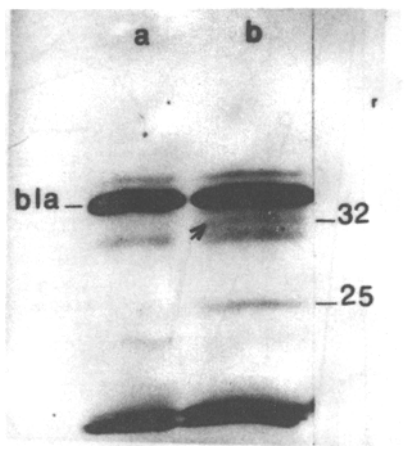

Figure 4. UV irradiated cells carrying the plasmid or cells carrying plasmid with the insert were labelled with [^{35}S] methionine for 1 h and soluble extracts of proteins were analysed by SDS-PAGE followed by autoradiography as described in the text. CSR603 cells carrying the plasmids pUC19 (lane a) and pVC810 (lane b). bla, beta-lactamase resistance gene product. The numbers indicate molecular sizes in kDa.

When the plasmid pVC810, isolated from *V. cholerae* 569B cells, was used to transform *E. coli* strain CSR603, the transformation efficiency was 1000-fold less than when pVC810 isolated from CSR603 was used for transformation of the same cells. One possible explanation of this observation might be that the restriction-modification systems of *E. coli* CSR603 and *V. cholerae* 569B are different. While the plasmid pVC810 can be stably maintained in *V. cholerae* and *E. coli* cells, the major difficulty with this vector is that it has only one antibiotic resistance marker. A more useful vector should have two such markers. This was achieved by ligating the *Eco*RI digested pBR322 and pVC540 plasmids. The recombinant vector pVE970 has Tc and Ap resistant markers (figure 2). The size of the vector was reduced by deleting the 0.5 kb *Bgl*II-*Bgl*II fragment coming from pVC540. This new construct pVE920 (figure 2) is as stable as pVC810 in *E. coli* and *V. cholerae* and the *Bam*HI, *Pst*I and *Sal*I sites are convenient for cloning genes in this vector.

3.4 *Cloning genes in pVE920*

To determine the efficacy of pVE920 as a shuttle vector, the stability and expression of the cloned *mutS* gene of *V. cholerae* in *E. coli* and *V. cholerae* were examined. The 4.7 kb *V. cholerae* DNA fragment coding for the 92 kDa MutS protein (Bera *et al* 1989) was cloned at the *Pst*I site of pVE920 and the recombinant vector was used to transform *mut*S mutants of *E. coli* and *V. cholerae.* The transformed cells were seeded on agar plates containing 100 or 10 μg of rifampicin per ml for *E. coli* and *V. cholerae* respectively. *mutS* mutants exhibit high rate of spontaneous mutation. Transformants that reduced the spontaneous mutation frequency by more

than 10-fold compared to the mutant cells grown in rifampicin containing plates were selected. Plasmids were isolated from the recombinant cells and the presence of the 4·7 kb insert was confirmed. Deletion in the coding region of the *mutS* failed to complement the mutator phenotype showing that the observed reduction in the spontaneous mutation frequency is indeed due to the expression of *mutS* gene in the mutant cells. The recombinant plasmid was found to be stably maintained after repeated subculturing.

The construction of these vectors along with the demonstration of reliable transformation in *V. cholerae* by plasmid DNA (Panda *et al* 1991) increases the range of genetic tools which can be used with this human pathogen. Once *rec*A mutants become available, the usefulness of the *V. cholerae* host-vector system in cloning genes can be assessed.

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