Available online at www.sciencedirect.com
SCIENCE DIRECT®
Plasmid 51 (2004) 246–255
PLASMID
www.elsevier.com/locate/yplas

Improvement of pCVD442, a suicide plasmid for gene allele exchange in bacteria

Nadège Philippe,[a] Jean-Pierre Alcaraz,[b] Evelyne Coursange,[a] Johannes Geiselmann,[a] and Dominique Schneider[a,*]

[a] *Laboratoire Adaptation et Pathogénie des Microorganismes, CNRS UMR 5163, Université Joseph FOURIER, 460, rue de la piscine, 38400 Saint Martin d'Heres, France*
[b] *Laboratoire Génétique Moléculaire des Plantes, CNRS UMR 5575, Université Joseph FOURIER, 460, rue de la piscine, 38400 Saint Martin d'Heres, France*

Received 22 December 2003, revised 16 February 2004

Abstract

Allelic exchange experiments allow investigation of the functions of many unknown genes identified during the sequencing of entire genomes. Isogenic strains differing by only specific mutations can be constructed. Among other tools, suicide plasmids are widely used for this task. They present many advantages because they leave no scars on the chromosome, and therefore allow combining several mutations in the same genetic background. While using the previously described pCVD442 suicide plasmid [Infect. Immun. 59 (1991) 4310], we found untargeted recombination events due to the presence of an IS*1* element on this plasmid. The plasmid was therefore improved by removal of the IS*1* element. We also replaced the *bla* gene of pCVD442, conferring ampicillin resistance, by the *cat* gene conferring chloramphenicol resistance, leading to the new suicide plasmid pDS132. The plasmid was entirely sequenced. We demonstrate that this new vector can be easily used to introduce various types of mutations into different genetics backgrounds: removal of IS elements, introduction of point mutations or deletions. It can be introduced into bacterial strains by either transformation or conjugation.

Keywords: Gene inactivation; Recombination; IS elements; Isogenic strains

1. Introduction

The relationship between phenotypes and corresponding genotypes is often obtained by analyses of a mutant phenotype and subsequent characterisation of the gene. Alternatively, reverse genetic analysis consists in constructing mutations in specific target genes and investigating the phenotype of the generated mutant strains. Random mutations can be created by chemical or transposon mutagenesis, whereas specific mutations are usually generated by homologous recombination on the chromosome of many bacterial strains. This technique reduces the need for screening

* Corresponding author. Fax: +33-4-76-63-56-63.
E-mail address: dominique.schneider@ujf-grenoble.fr (D. Schneider).

0147-619X/$ - see front matter © 2004 Elsevier Inc. All rights reserved.
doi:10.1016/j.plasmid.2004.02.003

procedures, especially for genes with no selectable phenotype.

The genome of more than 100 bacterial strains is now completely sequenced. Open reading frames represent about 90% of bacterial chromosomes, but the function of 40–60% of the genes remains unknown. Reverse genetic analysis and construction of specific mutations through allelic exchange has to be used to decipher the function of these genes. Moreover, some genes present redundant functions and several mutations therefore have to be combined in the chromosome. This can be achieved by constructing "clean" mutations without any resistance markers or scars, which also avoids subsequent recombination due to new homologies in the chromosome.

A number of tools have been described for introducing mutations in bacterial chromosomes (Martinez-Morales et al., 1999; Murphy et al., 2000; Yu et al., 2000). To avoid antibiotic resistance markers or scars in the chromosome, the most useful tools are suicide vectors, which also allow combination of multiple mutations. These systems involve a two-step procedure with plasmid integration within the target sequence by homologous recombination, followed by its excision via a second cross-over event, resulting in allelic exchange. Two types of suicide plasmids have been engineered: temperature-sensitive plasmids like pSC101 (Cornet et al., 1994), pKO3 and their derivatives (Link et al., 1997), and plasmids carrying the replication origin of R6K, such as pCVD441 and its derivatives (Donnenberg and Kaper, 1991). The replication of the first type of plasmids is possible only at the permissive temperature, while the second type of plasmids are able to replicate only in strains producing the π protein, the product of the *pir* gene. Integration of the plasmids into the chromosome is selected by an antibiotic resistance marker, either at the restrictive temperature or in a *pir*$^-$ background. Excision of the integrated plasmid for allelic exchange is selected with counter-selectable markers: if the plasmid is still integrated in the chromosome, the cell will die in the presence of the counter-selective compound (Reyrat et al., 1998). There are three major counter-selectable determinants that have been used to construct mutant strains in many bacteria: (i) *The fusaric acid-sensitivity system*: the counter-selectable marker is encoded by *tetAR*, which results in tetracycline resistance but in fusaric or quinalic acids hypersensitivity. Cells that have lost *tetAR* can therefore be selected through their increased resistance to fusaric acid (Maloy and Nunn, 1981). However, this system often generates deletions with polar effects on downstream genes. (ii) *The streptomycin-sensitivity system*: the wild-type *rpsL* gene encodes the ribosomal protein S12, the target of streptomycin. Some mutations in *rpsL* provide streptomycin resistance. The wild-type *rpsL* allele is used on a suicide plasmid containing the sequence to introduce into the chromosome of an *rpsL*$^-$ streptomycin-resistant strain. Resistance being recessive in a merodiploid strain, excision of the suicide plasmid can be selected through the loss of the *rpsL*$^+$ allele and therefore by streptomycin resistance. This system therefore involves the selection of spontaneous streptomycin-resistant clones (Dean, 1981). (iii) *The sucrose-sensitivity system*: the *Bacillus subtilis sacB* gene encodes levane saccharase, which is lethal in most gram-negative bacteria in the presence of sucrose (Gay et al., 1983). Plasmid excision in the cells can therefore be selected by growth in a sucrose-containing medium. This system is widely used in most common suicide plasmids.

We describe here the construction and use of a derivative of the well-described suicide plasmid pCVD442 (Donnenberg and Kaper, 1991), which carries *R6K ori*, the replication origin of plasmid R6K, the *mob* (plasmid mobilisation region) and *bla* (ampicillin resistance conferring gene) regions of the suicide vector pGP704 (Miller and Mekalanos, 1988), the *sacB* gene of *B. subtilis*, and five unique restriction endonuclease sites for cloning. While using pCVD442 we were confronted with problems of untargeted integration in the chromosome and discovered that they were due to the presence of part of an IS*1* element on the plasmid. This element therefore constitutes a good substrate for homologous recombination in the chromosome in a wide variety of bacteria. We have modified pCVD442 by removal of this insertion sequence. We also replaced *bla* by the *cat* gene conferring

chloramphenicol resistance. The new suicide plasmid, called pDS132, was sequenced.

2. Materials and methods

2.1. Strains, plasmids, and media

The strains and plasmids used in this study are listed in Table 1.

Strains were grown routinely in rich liquid or solid (12 g/L agar) Luria Broth medium (LB)[1] (Sambrook et al., 1989). The media can be supplemented with kanamycin (50 μg/mL), chloramphenicol (30 μg/mL) or streptomycin (25 μg/mL). Solid LB medium with 5% sucrose and without NaCl was used to select plasmid excision from the chromosome, during the gene allelic exchange experiments. Omission of NaCl from this medium was shown previously to improve the sucrose counterselection in *Escherichia coli* (Blomfield et al., 1991).

2.2. Electrotransformation

Cells were grown in LB until mid-log phase ($OD_{600\,nm} = 0.5$). After collection by centrifugation, cells were washed twice with cold sterile water and once with cold sterile 10% glycerol. Electrocompetent cells were then resuspended into 10% glycerol at 3.5×10^{10} CFU/mL, and aliquoted in 40 μL samples, which were stored at −80 °C. For electrotransformation, 50 ng of the desired plasmid were mixed with a sample of electrocompetent cells. The mixture was submitted to 2500 V for 4.5 ms, with a resistance of 200 Ω and a capacitance of 25 μF using a Biorad electroporator. Cells were immediately resuspended in 1 mL LB and grown for 1 h at 37 °C, before plating on selective growth medium.

[1] *Abbreviations used:* Amp^R, ampicillin resistant; Cam^R, chloramphenicol resistant; Cam^S, chloramphenicol sensitive; CFU, colony forming unit; IPTG, isopropyl-thio-β-D-galactoside; LB, Luria Broth medium; PCR, polymerase chain reaction; RFLP, restriction fragment length polymorphism; Str^R, streptomycin resistant; Suc^R, sucrose resistant; Xgal, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside.

2.3. Conjugation

Donor and recipient strains were grown in LB until late log phase ($OD_{600\,nm} = 0.8$). Cells were then mixed at an equal ratio and spotted onto a nylon filter on a LB plate. After 6 h of conjugation at 37 °C, cells were recovered by washing the filters with 10 mM $MgSO_4$ and plated on the appropriate selective medium to eliminate the donor strain and to select for plasmid integration into the chromosome. SM10 *λpir* was used as the donor strain to allow efficient replication of the *R6K ori*-based suicide plasmid. The recipient strain does not contain the *pir* gene, therefore preventing relication of the plasmid.

2.4. Construction of pDS132

The construction of pDS132 is summarised in Fig. 1. pDS132 is derived from pCVD442 plasmid (Donnenberg and Kaper, 1991).

pCVD442 contains 500 bp of an IS*1* element between the *sacB* and *bla* genes, which can generate undesired recombination events. Hence, there are, for example, 20 copies of IS*1* in the *E. coli* B chromosome (Papadopoulos et al., 1999) and six in the *E. coli* W3110 chromosome (Naas et al., 1994). This may therefore interfere in knockout experiments of specific genes using pCVD442 as a suicide plasmid. To overcome this problem, this IS*1* sequence together with the *bla* gene were removed by digesting pCVD442 with *Nde*I and *Asp*I (Fig. 1). The large fragment was blunt-ended with the Klenow fragment of DNA polymerase I and ligated to the *cat* gene, obtained after a *Sma*I digestion of pCamI, leading to pDS132, which was subsequently sequenced. The *cat* gene confers chloramphenicol resistance and replaces *bla* in the selection of the plasmid integration event. The pDS132 nucleotide sequence has been deposited in the GenBank database and has been assigned the Accession No. AY489048.

The pCamI plasmid, from which the *cat* gene was excised, was obtained by PCR amplification from plasmid pBC with the two oligonucleotides Cm top (CGG GAT CCC GCC CGG GAA TTA CGC CCC GCC C) and Cm bottom (CGG GAT CCC GCC CGG GCA GGA GCT AAG GAA

Table 1
Strains and plasmids used in this study

	Description	Reference
Strains		
E. coli DH5α *λpir*	*supE4 ΔlacU169* (Φ80 *lacZ*ΔM15) *hsdR17 recA1 endA1 gyrA96 thi-1 relA1 λpir*	Simon et al. (1983)
E. coli SM10 *λpir*	*thi thr leu tonA lacY supE recA*::RP4-2-Tc::Mu Km *λpir*	Donnenberg and Kaper (1991)
E. coli REL606	B Bc251 $T6^R$ Str^R rm_{III} Ara^-	Lederberg (1966) and Schneider et al. (2002)
E. coli S1	A small-cell clone isolated from REL606 during a laboratory evolution experiment. *b2875*::IS*150*, *menC*::IS*186*	Rozen and Lenski (2000)
Plasmid		
pCVD442	*R6K ori*, *mobRP4*, *bla*, *sacB*	Donnenberg and Kaper (1991)
pCR-II-TOPO	*ColE1 ori*, *Plac* MCS *lacZα*, *f1 ori*, Kan^R, Amp^R	Invitrogen
pCamI	*cat* gene cloned into pCR-II-TOPO	This work
pBC	*pUC ori*, *Plac* MCS *lacZα*, *f1 ori*, Cam^R	Stratagene
pDS132	Derived from pCVD442, without IS*1* sequences. *bla* gene replaced by the *cat* gene	This work
pDS101	*menC* cloned into pDS132	This work
pDS102	*ΔmenC* cloned into pDS132	This work
pDS107	*b2875* cloned into pDS132	This work
pDS109	*Δb2875* cloned into pDS132	This work

GCT A), each containing a *Sma*I site. The PCR product was then cloned in pCRII-TOPO, using the pCRII-TOPO kit (Invitrogen), resulting in pCamI. The *cat* gene can subsequently be excised as a 600-bp*Sma*I fragment.

2.5. Allelic exchange

The cloning steps of the desired gene alleles into pDS132 were performed in *E. coli* DH5α *λpir* or SM10 *λpir* strains to allow replication of the plasmid. The recombinant plasmids were then purified and introduced in the appropriate strains in either of two ways: either by transformation of electrocompetent cells of the chosen strain, or by conjugative transfer from *E. coli* SM10 *λpir*, which carries the *tra* genes of the RP4 plasmid, into the Str^R target strain.

The first step of allelic exchange was selection of plasmid integration into the recipient chromosome (which does not carry the *pir* gene) by plating cells on chloramphenicol-LB plates. For conjugation experiments, the donor strain SM10 *λpir* was eliminated by adding streptomycin in the medium. After overnight growth at 37 °C, one colony was picked, diluted in 10 mM $MgSO_4$, and serial dilutions were plated on LB agar plates with 5% sucrose and without NaCl. This plating step allowed selection of plasmid excision from the chromosome by a second cross-over. After overnight incubation at 37 °C, 100 clones were streaked on chloramphenicol-containing LB agar plates and on LB agar with 5% sucrose and without NaCl. Suc^R Cam^S clones were stored in a glycerol suspension at −80 °C. Since allelic exchange depends on the localisation of the second cross-over event, we screened several clones by PCR or Southern blot (Southern, 1975) in order to identify those carrying the desired allele. Labelling of the probes and hybridisation experiments were performed using the DIG labelling kit of Roche, according to their recommendations.

3. Results and discussion

3.1. pCVD442 carries a fragment of an IS1 element

Untargeted chromosomal insertions, using pCVD442 as a suicide plasmid, were detected while trying to inactivate the *lac* operon in the REL606 *E. coli* strain. An internal fragment of the

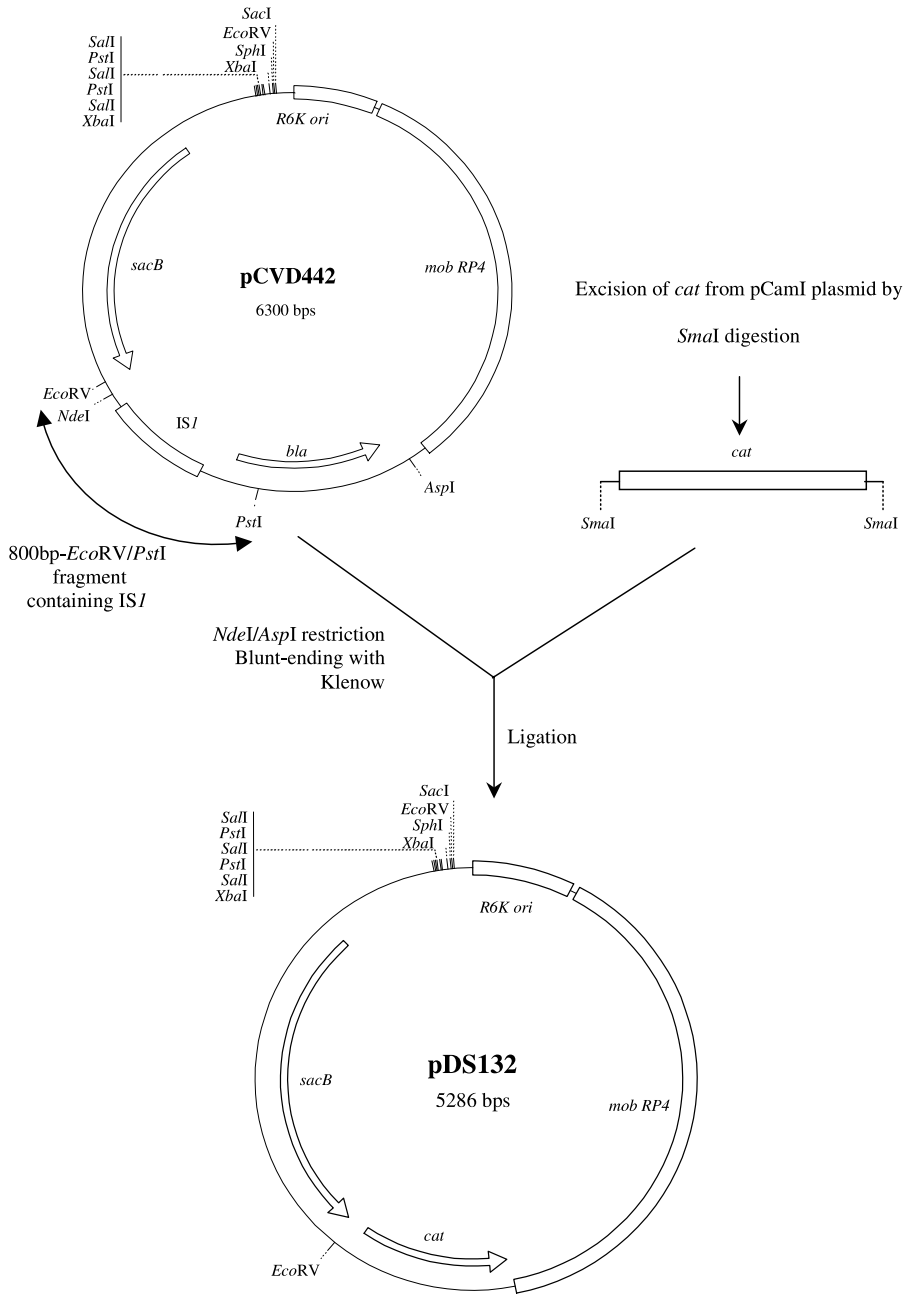

Fig. 1. Construction of pDS132. The pDS132 plasmid is derived from pCVD442 (Donnenberg and Kaper, 1991), which contains the *bla* gene conferring ampicillin resistance, the *mob* region of the RP4 plasmid (mobilisation), the *sacB* gene encoding levane saccharase, a multiple cloning site, and the *R6K ori* region from plasmid R6K. pCVD442 also carries 500 bp of an IS*1* element between the *sacB* and *bla* genes, which may generate undesired recombination events with resident IS*1* elements in the recipient strains. To overcome this problem, this IS*1* sequence, together with the *bla* gene, was replaced by the *cat* gene obtained from plasmid pCamI after digestion with *Sma*I (see Section 2.4). The *cat* gene confers chloramphenicol resistance. This new vector was called pDS132. The details of the construction are given in Section 2.4. The size of the *Eco*RV/*Pst*I fragment of pCVD442 carrying the fragment of IS*1* is shown.

lac operon was cloned into pCVD442 and the recombinant plasmid was used to transform REL606. The chromosomal insertion of the plasmid by homologous recombination in the *lac* operon was selected on LB plates with ampicillin. One hundred ampicillin-resistant individual colonies were further streaked onto LB plates containing ampicillin, Xgal and IPTG. We expected almost all of the clones to be Amp^R and white (i.e., Lac^-). This was, however, not the case: 90% of the Amp^R clones remained blue (Lac^+). The presence of the *lac* wild-type allele was confirmed in these clones by Southern blot of their genomic DNA and hybridisation using a fragment of the *lac* operon as a probe (data not shown). To confirm the presence of pCVD442 in the chromosome of these clones, we used the suicide plasmid as a probe against the genomic DNA of these clones cut with *Eco*RV: about 20 fragments were detected. The hybridisation patterns were similar to the ones obtained with IS*1* as a probe against genomic DNA of REL606 (Papadopoulos et al., 1999). Hybridisation experiments using IS*1* as a probe indeed revealed the presence of an IS*1* element within the 800-bp *Eco*RV/*Pst*I fragment of pCVD442 (data not shown). Sequencing of the *Eco*RV/*Pst*I fragment revealed the presence of a part (500 bp) of an IS*1* (not shown). In order to conserve the use of pCVD442 as a suicide plasmid for gene allelic exchange experiments, we removed this IS*1* fragment. We also replaced the *bla* gene, conferring ampicillin resistance by *cat*, conferring chloramphenicol resistance (Fig. 1, see Section 2.4). The new plasmid was called pDS132 and was subsequently tested for different kinds of allelic exchanges.

3.2. Removal of IS elements

As a first type of allelic exchange, we removed IS elements from their insertion sites within defined genes. Two IS-containing genes were chosen: *menC*, encoding *o*-succinyl benzoate synthase involved in menaquinone biosynthesis (Meganathan, 1996), and *b2875*, a gene with unknown function (Blattner et al., 1997). An IS*150* insertion within *b2875* and an IS*186* insertion within *menC* (unpublished data) had been characterised in clones called S, isolated from a long-term experimental evolution using *E. coli* (Cooper and Lenski, 2000; Lenski et al., 1991; Rozen and Lenski, 2000).

3.2.1. Cloning of b2875 and menC wt and deletion alleles into pDS132

The *b2875* gene was isolated from the genomic DNA of the reference strain REL606 as an *Eco*RI/*Hin*dIII fragment of 7221 bp, which was cloned into the pBC vector resulting in plasmid pDS103 (Fig. 2A). (The IS*150* element is located at position 4405 in this fragment in the S clones). This plasmid was then digested by *Asp*700 and *Srf*I, generating three fragments: one 4270-bp *Asp*700/*Srf*I fragment containing the 5′ side of the 7221-bp *Eco*RI/*Hin*dIII fragment, one 4821-bp *Asp*700 fragment containing the 3′ side of that same fragment, and a 1529-bp *Srf*I/*Asp*700 fragment localised in the middle of the fragment. (The IS*150* insertion site in the S clones is located within this last 1529-bp *Asp*700/*Srf*I fragment.) The first two fragments were then religated, resulting in plasmid pDS106. This plasmid therefore carries an allele of *b2875* containing a deletion including the site where the IS*150* element is inserted in the S clones. An *Acc*I digestion of pDS106 allowed the isolation of a 2391-bp *Acc*I fragment containing the *b2875*-deletion allele with 896 and 1495 bp of adjacent sequences at the 5′ and the 3′ sides of the deletion, respectively. The 2391-bp *Acc*I fragment was purified and the cohesive ends were filled-in with the Klenow fragment of DNA polymerase. The resulting fragment was subsequently cloned into pDS132 cut with *Sal*I and blunt-ended, resulting in plasmid pDS109 (Fig. 2C). A similar strategy was used to clone the wt allele of *b2875* into pDS132: a 3920-bp *Acc*I fragment containing the wt *b2875* allele, was obtained from plasmid pDS103. After filling-in the cohesive ends, this fragment was cloned into pDS132 cut with *Sal*I and blunt-ended, resulting in plasmid pDS107 (Fig. 2B).

A similar strategy, described in Fig. 3, was used to clone the *menC* alleles into pDS132.

3.2.2. Gene allele exchange experiments for b2875 and menC

The four plasmids, pDS107, pDS109, pDS101, and pDS102 were used to replace the *b2875*::IS*150*

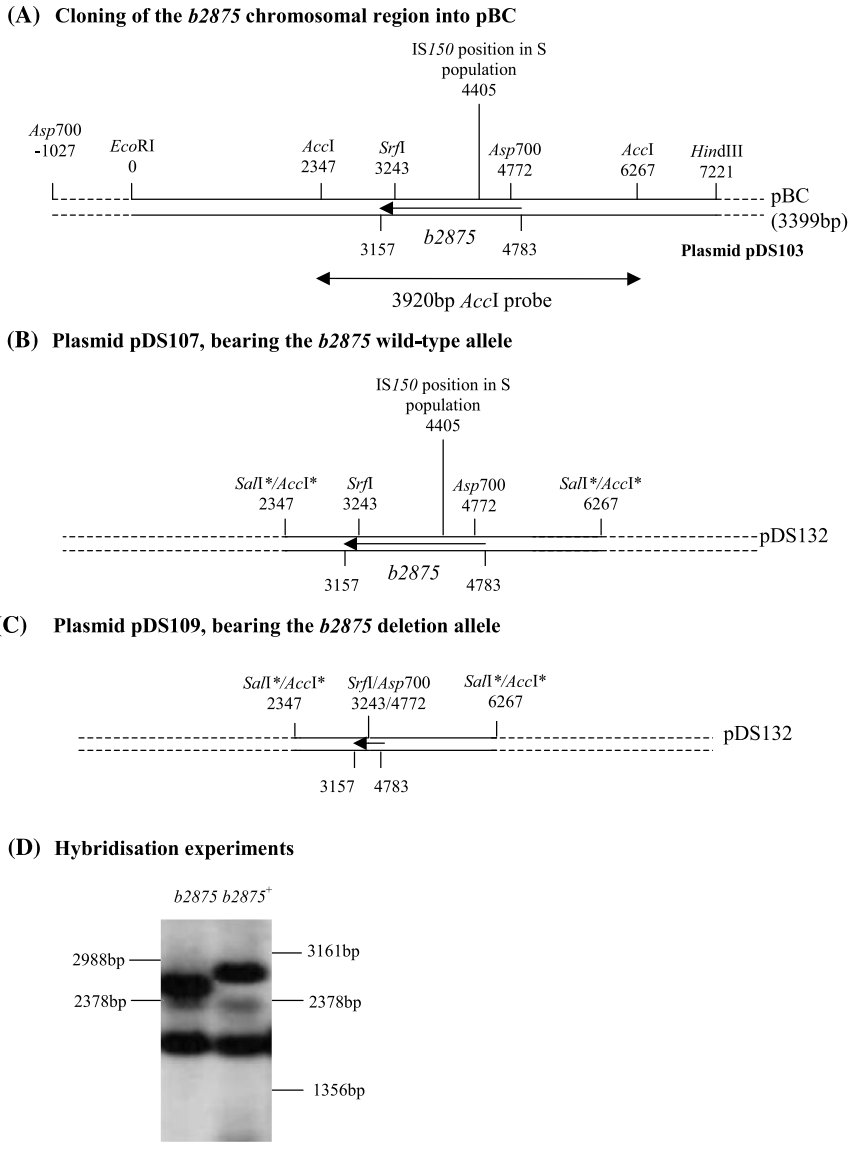

Fig. 2. Construction of suicide plasmids pDS107 and pDS109, bearing the *b2875* wt and deletion allele respectively. (A) A 7221 bp *Eco*RI/*Hin*dIII fragment containing the *b2875* gene was cloned into pBC, leading to pDS103. Relevant restriction sites are shown. Coordinates are given with respect to the *Eco*RI/*Hin*dIII fragment. The IS*150* insertion site in the S clones is shown, as well as the direction of transcription of *b2875*. The 3920-bp *Acc*I fragment used as a probe in hybridisation experiments is shown. The dashed lines represent the pBC vector. (B) Map of pDS107, the suicide plasmid carrying the *b2875* wild-type allele. It was obtained by *Acc*I digestion of pDS106 (not shown) and filling-in of the ends of the 3920-bp fragment. This fragment was cloned into pDS132, cut with *Sal*I and blunt-ended. The dashed lines represent the pDS132 vector. (The *Acc*I and *Sal*I sites with an asterisk were filled-in.) (C) Map of pDS109, the suicide plasmid carrying the *b2875* deletion allele. It was obtained by *Asp*700/*Srf*I digestion of plasmid pDS103 and subsequent ligation of a 4270-bp *Asp*700/*Srf*I fragment and a 4821-bp *Asp*700 fragment, resulting in pDS106 (not shown). pDS106 was digested by *Acc*I and the ends of the 2391-bp fragment were filled-in (shown with asterisks). This fragment was cloned into pDS132, cut with *Sal*I and blunt-ended (shown with asterisks). The dashed lines represent the pDS132 vector. (D) The presence of the expected alleles in the different constructed strains was confirmed by hybridisation experiments using a 3920 bp *Acc*I fragment of pDS103 as a probe (see A) with genomic DNA of isolated Suc^R Cam^S clones digested with *Pvu*II. The expected sizes of the fragment are 3161, 2378, and 1356 bp for the wt *b2875* allele, and 2378 and 2988 bp for the deletion allele of *b2875*.

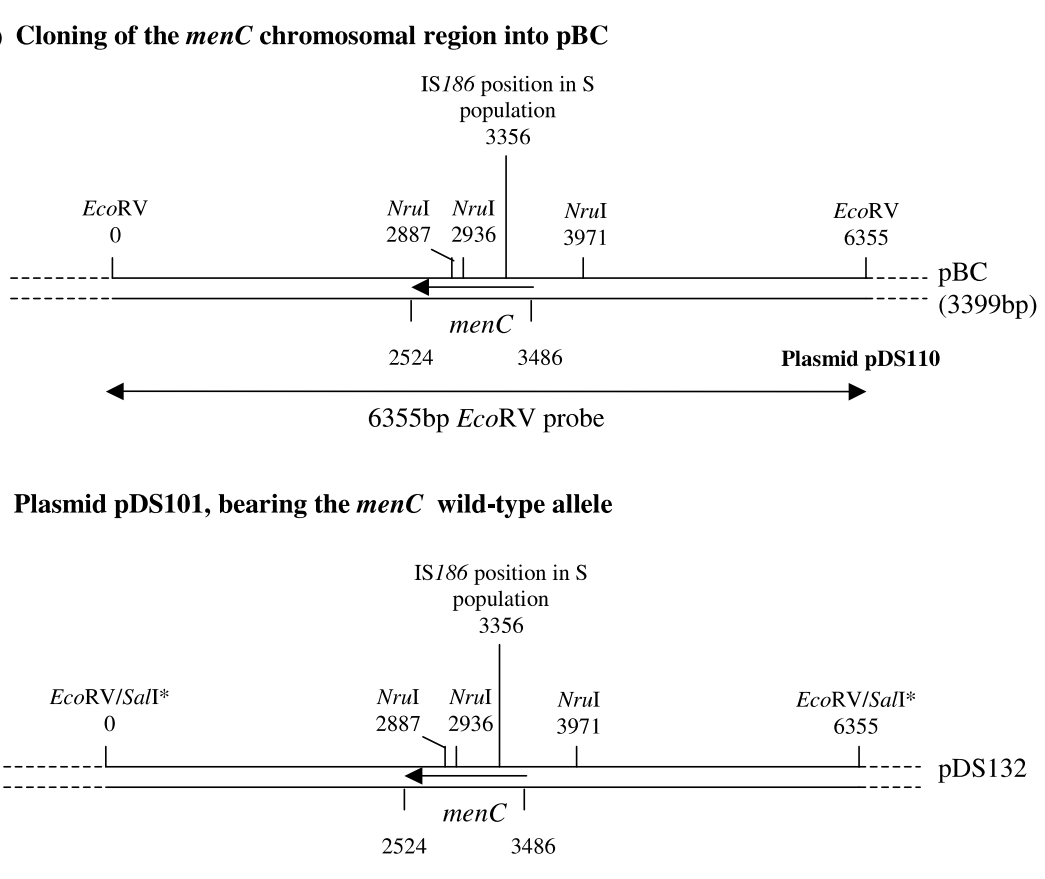

Fig. 3. Construction of suicide plasmids pDS101 and pDS102, bearing the *menC* wild-type and deletion allele respectively. (A) A 6355 bp *Eco*RV fragment containing the *menC* gene (positions 3486 to 2524) was cloned into pBC, leading to pDS110. Relevant restriction sites are shown. Coordinates are given with respect to the *Eco*RV fragment. The IS*186* insertion site in the S clones is shown, as well as the direction of transcription of *menC*. The 6355-bp *Eco*RV fragment used as a probe in hybridisation experiments is shown. (B) Map of pDS101, the suicide plasmid carrying the *menC* wild-type allele. The plasmid was obtained by *Eco*RV digestion of pDS110 and cloning of the 6355-bp fragment into pDS132 cut with *Sal*I and blunt-ended. (The *Sal*I sites with an asterisk were blunt-ended.) (C) Map of pDS102, the suicide plasmid carrying the *menC* deletion allele. This plasmid was obtained by *Nru*I digestion of plasmid pDS110 and subsequent religation of a 8700-bp fragment resulting in pDS111 (not shown). pDS111 was then digested by *Eco*RV and the 5271 bp fragment, containing a 1084 bp deletion allele of *menC*, was cloned into pDS132 cut with *Sal*I and blunt-ended (shown with asterisks), generating pDS102.

and *menC*::IS*186* alleles in the S1 clone, by the wild-type and the deletion alleles. The pDS109 and pDS102 plasmids were also used to replace the wt *b2875* and *menC* alleles in the REL606 ancestor strain by the deletion alleles. The four plasmids were separately introduced into the S1 clone by conjugation with the SM10 *λpir* strain containing the corresponding suicide plasmid as a donor (see Section 2.3). (For replacement of the wt alleles, REL606 was used as a recipient.) Integration of the suicide plasmids into the chromosome was selected by plating the conjugation mixtures onto LB plates containing chloramphenicol and streptomycin. An integration frequency of about 10^{-6} was obtained in all cases. Excision of the plasmids was then selected on LB plates with 5% sucrose and without NaCl, with an observed frequency of about 10^{-2}. The presence of the expected alleles in the different constructed strains was confirmed by hybridisation experiments. A 3920-bp *Acc*I fragment of pDS103 was used as a probe (Fig. 2A) with genomic DNA of 20 isolated Suc^R Cam^S clones digested with *Pvu*II (Fig. 2D for an example) to detect *b2875* alleles. For *menC*, a 6355-bp *Eco*RV fragment of pDS110 was used as a probe with genomic DNA of 20 isolated Suc^R Cam^S clones digested with *Pst*I (data not shown). About 50% of the studied clones contained the expected alleles. Among the 20 clones analysed in each case, no significant differences were observed for the ratio of the number of clones having incorporated the new allele to the number of clones with the original allele, as a function of the length of the adjacent sequences. For example, using pDS107 (adjacent sequences of 2058 and 1495 bp, respectively), 9 out of 20 analysed clones contained the new allele. Using pDS109 (adjacent sequences of 896 and 1495 bp, respectively), 7 out of 20 analysed clones contained the expected allele.

The plasmid pDS109 was introduced by conjugation into the REL606 strain carrying the *menC* deletion allele to add the *b2875* deletion. Clones carrying the two mutations were successfully obtained using the same method. The presence of both mutations was verified by hybridisation using the same probes as the ones described above.

3.3. Construction of a deletion of the rbs operon and of a point mutation in the spoT gene

A 5563-bp deletion of the *rbs* operon, encoding genes that allow the use of ribose as a carbon source (Abou-Sabé et al., 1982), and a point mutation in *spoT*, involved in the metabolism of the signalling molecule ppGpp (Cashel et al., 1996), were introduced into different *E. coli* strains using pDS132 (Cooper et al., 2001, 2003). The flanking sequences of the *rbs* deletion allele cloned into pDS132 were very short: 648 and 662 bp, respectively on the 5′ and 3′ sides of the deletion. For the *spoT* recombination, their sizes were 624 and 598 bp, respectively. The constructed *spoT* mutant strains were checked for secondary mutations, which had been shown to be a potential problem when using suicide plasmids (Johnson et al., 2003). No secondary mutations were detected (Cooper et al., 2003).

4. Conclusions

We have constructed the plasmid pDS132, a suicide plasmid derived from the widely used pCVD442 vector. The latter can produce untargeted integration events due to the presence of part of an IS*1* element. This IS*1* DNA was removed from pCVD442. We show that pDS132 is a useful suicide vector for introducing various types of mutations (deletions, point mutations, and removal of IS elements) in different genetic backgrounds in *E. coli*, with short adjacent sequences of about 600 bp on each side. Since pDS132 leaves no scars and no markers within the chromosome, it can be used to combine mutations.

Acknowledgments

We thank Richard Lenski and members of his laboratory for sharing strains. We also thank Michael Donnenberg for the pCVD442 plasmid. This work was supported by the French CNRS, ''Université Joseph Fourier'' and ''Ministère de l'enseignement supérieur et de la recherche.''

References

Abou-Sabé, M., Pilla, J., Hazuda, D., Ninfa, A., 1982. Evolution of the D-ribose operon of *Escherichia coli* B/r. J. Bacteriol. 150, 762–769.

Blattner, F.R., Plunkett 3rd, G., Bloch, C.A., Perna, N.T., Burland, V., Riley, M., Collado-Vides, J., Glasner, J.D., Rode, C.K., Mayhew, G.F., Gregor, J., Davis, N.W., Kirkpatrick, H.A., Goeden, M.A., Rose, D.J., Mau, B., Shao, Y., 1997. The complete genome sequence of *Escherichia coli* K-12. Science 277, 1453–1474.

Blomfield, I.C., Vaughn, V., Rest, R.F., Eisenstein, B.I., 1991. Allelic exchange in *Escherichia coli* using the *Bacillus subtilis sacB* gene and a temperature-sensitive pSC101 replicon. Mol. Microbiol. 5, 1447–1457.

Cashel, M., Gentry, V.J., Hernandez, V.J., Vinella, D., 1996. The stringrent response. In: Neidhardt, F.C. (Ed.), *E. coli* and *Salmonella*: Cellular and Molecular Biology. ASM Press, Washington, pp. 1458–1496.

Cooper, V.S., Lenski, R.E., 2000. The population genetics of ecological specialisation in evolving *Escherichia coli* populations. Nature 407, 736–739.

Cooper, T.F., Rozen, D.E., Lenski, R.E., 2003. Parallel changes in gene expression after 20,000 generations of evolution in *E. coli*. Proc. Natl. Acad. Sci. USA 100, 1072–1077.

Cooper, V.S., Schneider, D., Blot, M., Lenski, R.E., 2001. Mechanisms causing rapid and parallel losses of ribose catabolism in evolving populations of *Escherichia coli* B. J. Bacteriol. 183, 2834–2841.

Cornet, F., Mortier, I., Patte, J., Louarn, J-M., 1994. Plasmid pSC101 harbors a recombination site, *psi*, which is able to resolve plasmid multimers and to substitute for the analogous chromosomal *Escherichia coli* site *dif*. J. Bacteriol. 176, 3188–3195.

Dean, D., 1981. A plasmid cloning vector for the direct selection of strains carrying recombinant plasmids. Gene 15, 99–102.

Donnenberg, M.S., Kaper, J.B., 1991. Construction of an *eae* deletion mutant of enteropathogenic *Escherichia coli* by using a positive-selection suicide vector. Infect. Immun. 59, 4310–4317.

Gay, P., Lecoq, D., Steinmetz, M., Ferrari, E., Hoch, J.A., 1983. Cloning structural gene *sacB*, which codes for exoenzyme levansucrase of *Bacillus subtilis*: expression of the gene in *Escherichia coli*. J. Bacteriol. 153, 1424–1431.

Johnson, J.R., Lockman, H.A., Owens, K., Jelacic, S., Tarr, P.I., 2003. High frequency secondary mutations after suicide-driven allelic exchange mutagenesis in extraintestinal pathogenic *Escherichia coli*. J. Bacteriol. 185, 5301–5305.

Lederberg, S., 1966. Genetics of host-controlled restriction and modification of deoxyribonucleic acid in *Escherichia coli*. J. Bacteriol. 91, 1029–1036.

Lenski, R.E., Rose, M.R., Simpson, S.C., Tadler, S.C., 1991. Long-term experimental evolution in *Escherichia coli* I. Adaptation and divergence during 2000 generations. Am. Nat. 138, 1315–1341.

Link, A.J., Phillips, D., Church, G.M., 1997. Methods for generating precise deletions and insertions in the genome of wild type *Escherichia coli*: application to open reading frame characterisation. J. Bacteriol. 179, 6228–6237.

Maloy, S.R., Nunn, W.D., 1981. Selection for loss of tetracycline resistance by *Escherichia coli*. J. Bacteriol. 145, 1110–1112.

Martinez-Morales, F., Borges, A.C., Martinez, A., Shanmugam, K.T., Ingram, L.O., 1999. Chromosomal integration of heterologous DNA in *Escherichia coli* with precise removal of markers and replicons used during construction. J. Bacteriol. 181, 7143–7148.

Meganathan, R., 1996. Biosynthesis of the isoprenoid quinines menaquinone (vitamin K2) and ubiquinone (coenzyme Q). In: Neidhardt, F.C. (Ed.), *Escherichia coli* and *Salmonella*: Cellular and Molecular Biology. ASM Press, Washington, pp. 642–656.

Miller, V.L., Mekalanos, J.J., 1988. A novel suicide vector and its use in construction of insertion mutations: osmoregulation of outer membrane proteins and virulence determinants in *Vibrio cholerae* requires ToxR. J. Bacteriol. 170, 2575–2583.

Murphy, K.C., Campellone, K.G., Poteete, A.R., 2000. PCR-mediated gene replacement in *Escherichia coli*. Gene 246, 321–330.

Naas, T., Blot, M., Fitch, W.M., Arber, W., 1994. Insertion sequence-related genetic variation in resting *Escherichia coli* K-12. Genetics 136, 721–730.

Papadopoulos, D., Schneider, D., Meier-Eiss, J., Arber, W., Lenski, R.E., Blot, M., 1999. Genomic evolution during a 10,000 generations experiment with bacteria. Proc. Natl. Acad. Sci. USA 96, 3807–3812.

Reyrat, J.M., Pelicic, V., Gicquel, B., Rappuoli, R., 1998. Counterselectable markers: untapped tools for bacterial genetics and pathogenesis. Infect. Immun 66, 4011–4017.

Rozen, D.E., Lenski, R.E., 2000. Long-term experimental evolution in *Escherichia coli* VIII. Dynamics of a balanced polymorphism. Am. Nat. 155, 24–35.

Sambrook, J., Fitsch, E.F., Maniatis, T., 1989. Molecular Cloning. Cold Spring Harbor Laboratory Press, New York.

Schneider, D., Duperchy, E., Depeyrot, J., Coursange, E., Lenski, R.E., Blot, M., 2002. Genomic comparisons among *Escherichia coli* strains B, K-12, and O157:H7 using IS elements as molecular markers. BMC Microbiol. 2, 18.

Simon, R., Priefer, U., Pülher, A., 1983. A broad host range mobilisation system for in vivo genetic engineering: transposon mutagenesis in gram-negative bacteria. Bio/Technology 1, 784–791.

Southern, E.M., 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. 98, 503–517.

Yu, D., Ellis, H.M., Lee, E.C., Jenkins, N.A., Copeland, N.G., Court, D.L., 2000. An efficient recombination system for chromosome engineering in *Escherichia coli*. Proc. Natl. Acad. Sci. USA 97, 5978–5983.

Communicated by M. Chandler