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Development of a Shuttle Vector for Nocardioforms

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Abstract: Construction of *Escherichia coli* – nocardioforms shuttle vector pAR1 was achieved using the recombinant DNA technology. Developmental strategy included following cloning and fusion steps. Firstly, insertion of kanamycin resistance cassette into the suicide vector pEcoR251 was performed. This was followed by ligations of a nocardial replicon from the pCY104 and *Eco*RI endonuclease from the pEcoR251, lacking ampicillin resistant determinant. New recombinant plasmid was engineered with a positive (suicide) function and a single selectable marker expressed in both Gram-positive and Gram-negative bacteria. Plasmid pAR1 was relatively small ~5.0 kb in size.

Key words: shuttle vector, recombinant DNA technology, suicide gene.

Introduction

The genus *Nocardia* was named after French bacteriologist and veterinary pathologist Edmond Nocard and the common name nocardioforms was given to representative bacterial species. In 1888, Nocard first observed the pathogenic potential of these actinomycetes but since then they have been a major source of interest for the production of novel antimicrobial agents (McNeil and Brown 1994, Prescott *et al.* 1999). Nocardioforms are described in Section 17 of Volume 2 of Bergey's Manual of Systematic Bacteriology and include the following genera: *Nocardia, Rhodococcus, Gordonia, Corynebacteria* and *Tsukamurella* (Lechevalier 1986, Steingrube *et al.* 1997).

Nocardioforms are 0.5-1.2 mm in diameter and they have rudimentary to extensive vegetative hyphae, which can fragment into rod-shaped and coccoid forms. They form aerial hyphae and may produce conidia, catalase positive and all genera have a high 64-72% G+C content. In addition, they have type IV cell wall that contains a carbohydrate composed of arabinose and galactose, mycolic acids, no

peptide interbridge and most species have peptidoglycan with *meso*-diaminopimelic acid. In general, all are strict aerobes (Lechevalier 1986, Prescott *et al.* 1999).

The cloning vector system for *Nocardia* species developed by Yao *et al*. (1994) includes the *Nocardia – E. coli* shuttle vector, named pCY104 and the author was particularly interested in this vector because of its broad host range. Development of the pCY104 was based on the ligation of a 2.5 kb cryptic *N. asteroides* plasmid into the *E. coli* vector pIJ4625. A new recombinant shuttle vector, 8.9 kb in size, contains the resistance genes for kanamycin, chloramphenicol and thiostrepton that are expressed in *N. asteroides* and *N. corallina* while kanamycin and chloramphenicol are expressed in *E. coli*. In addition, pCY104 has several useful cloning sites, the unique *Eco*RI site in the chloramphenicol gene, the unique *Eco*RV site in the thiostrepton gene and the two *Pst*I sites in the kanamycin determinant. The expression of all these genes in *Nocardia* species is very important as it enables inactivation cloning studies of this genus.

The objective of the present work was to construct a shuttle vector for *E. coli* and nocardioforms having a positive (suicide) function, a single selectable marker for both Gram-positive and Gram-negative bacteria, additional unique restriction sites and a broad host range.

Materials and Methods

Bacterial strains, culture conditions and recombinant DNA molecules

For the growth of *Escherichia coli* MM294-4 and *E. coli* GM2929, a single bacterial colony was inoculated into 5 ml of LB and the cultures were incubated on a wheel at 37°C overnight. LB medium was solidified by addition of 1.5% agar (LA). *E. coli* MM294 was grown at temperature not higher than 35°C. In addition, the growth medium for MM294-4 was supplemented with 50 mg/ml nalidixic acid and for GM2929 with 50 mg/ml rifampicin.

The *Rhodococcus* strains were grown in LB at 28°C for 2-4 days. *R. erythropolis* SQ1 and *R. rhodochrous* Ri8-R were grown in the medium supplemented with 50 mg/ml rifampicin while in Ri8 and 011 media 20 mg/ml streptomycin was added. For electroporation rhodococcal strains were grown in LBSG containing glycine: *R. equi* 1%, *R. erythropolis* 2%, and *R. rhodochrous* 3% glycine.

Tab. 1. Plasmids and vectors used in this study.

Plasmids / Vectors	Relevant features	Source
pEcoR251	*E. coli* cloning vector with Amp-R, *Eco*RI gene, 3.3 kb	Dabbs, E.
pEcoR251-Kan-R	*Bam*HI fragment carrying Tn903 from pUC4K inserted into *Bam*HI site of pEcoR251	This work
pACYC184	*E. coli* cloning vector with Cm-R and Tet-R, 4.2 kb	Dabbs, E.
pACYC184-Kan-R	*Bam*HI fragment carrying Tn903 from pUC4K inserted into *Bam*HI site of pACYC184	This work
pCY104	Shuttle vector for *Nocardia* (*cat*, *neo* and *tsr*) and *E. coli* (*cat* and *neo*), 8.9 kb	Fernandes, P.
pAR1	*E. coli* – nocardioform shuttle vector, Kan-R, ~5.0 kb	This work

Plasmids and shuttle vectors used in this work were selected for and maintained in their host strains. For *E. coli* plasmids this was achieved by the addition of Amp at 200 mg/ml. Plasmid carrying an intact suicide gene, pEcoR251 was propagated in λMM294. It had a positive selection function due to the *Eco*RI endonuclease gene with expression turned off by the bacteriophage λ repressor, so this was maintained in a λ lysogen of *E. coli* at -70°C. Purified vectors were stored in sterile dH_2O (plasmids with inserts), 4mM Tris-HCl (plasmids with the suicide gene) or in aqueous CsCl at -20°C.

Molecular techniques

Standard protocols were used for manipulation of DNA according to Burden and Whitney (1995), Ausubel *et al.* (2001) and Sambrook *et al.* (1989). Plasmid DNA was isolated using the GFX™ *Micro* Plasmid Prep Kit (Amersham Pharmacia Biotech). *E. coli* was transformed using standard $CaCl_2$ transformation (Sambrook *et al.*1989) and *Rhodococcus* either by PEG-mediated transformation or by electroporation. DNA fragments were purified from an agarose gels using QIAquick® Gel Extraction Kit.

Results and Discussion

Initially a few bacterial strains were transformed with pCY104 to test the host range of the potential shuttle vector (Table 2). Then, the first step in the vector development was to insert the Kan-R cassette into the *Bam*HI site of the pEcoR251. This cassette was purified from low gelling agarose and ligated into the vector. Selection in a λ lysogen of MM294-4 was simultaneous for ampicillin and kanamycin. This resulted in a construct that was approximately 4.6 kb in size and it was designated pEcoR-Kan-R (Figure 1).

Tab. 2. Transformation of bacterial strains using the pCY104 vector

Number	Organism	Strain	pCY104
1	*Cellulomonas fermentans*	232/B	no
2	*Gordonia rubropertincta*	ATCC25593	yes c
3	*Nocardioides simplex*	FJ21-A	no
4	*Nocardioides* sp.	4P1-A	no
5	*Rhodococcus equi*	ATCC14887	yes
6	*Rhodococcus fascians*	ATCC20131	yes c
7	*Rhodococcus erythropolis*	SQ1	yes c
8	*Rhodococcus erythropolis*	I2	yes c
9	*Rhodococcus opacus*	CB24-1	yes c
10	*Rhodococcus opacus*	HC24-1	no
11	*Rhodococcus opacus*	DNP14-5	yes c
12	*Rhodococcus opacus*	PN1	yes c
13	*Rhodococcus opacus*	RB1	yes c
14	*Rhodococcus opacus*	PKSM	yes c
15	*Rhodococcus rhodochrous*	CW21	yes c
16	*Rhodococcus* sp.	DSM1069	yes c

c: Extracts of these were shown to retransform *E. coli* confirming that an intact plasmid was present in these bacteria.

The presence of the *Bsp*HI site within the Kan-R cassette made future work on the vector development difficult and it was necessary to remove this site. The aim was for the vector to contain the unique *Bsp*HI within the suicide gene for a library construction. The site removal was achieved using the vector pACYC184. The *E. coli* plasmid pACYC184 is a small, low copy number cloning vector that carries chloramphenicol (Cm-R) and tetracycline (Tet-R)

resistance genes. Within the Tet-R determinant there was a unique *Bsp*HI site that was filled with the Klenow enzyme. The plasmid was then transformed into *E. coli* MM294-4 using 20 μg/ml Cm as a selectable marker. After transformation, 136 colonies were patched on 20 μg/ml Cm and 20 μg/ml Tet containing plates and 117 colonies were sensitive to Tet. These were then used for mini plasmid preparation and insertion of the *Bam*HI Kan-R cassette from pUC4K.

The *Bam*HI Kan-R cassette from pUC4K was purified using low gelling agarose. Following ligation of this fragment into *Bam*HI of pACYC184, the new recombinant plasmid was retransformed into MM294-4 using 20 μg/ml Cm and 100 μg/ml Kan as selectable markers. Plasmids were prepared and once again the *Bsp*HI site was filled using the Klenow enzyme. Twelve transformants were randomly selected to check the presence/absence of the *Bsp*HI site and 5/12 plasmids tested lost the site. This desired cassette was inserted back into the pEcoR251 intermediate construct. Throughout experimental procedure it was always important to check the suicide function to avoid spontaneous mutations. In the case of pEcoR251, the *Eco*RI endonuclease gene is under the control of the P_R promoter of phage λ. The plasmid may be maintained in λ lysogen using the *bla* gene for ampicillin resistance (Cheng and Modrich 1983). Further tests focused on the positive (suicide) gene function of the pEcoR-Kan-R-Amp-S construct by transformation into a nonlysogen *E. coli* MM294-4, which resulted in expression of the endonuclease, digestion of the DNA and death of the cell. In addition, insertional inactivation of the construct included ligation of the *Bgl*II restricted *R. erythropolis* genomic DNA with the *Bgl*II digested vector. If DNA is cloned into the unique *Bgl*II site in the *Eco*RI gene, endonuclease activity is abolished and transformation no longer leads to death of the transformed cell.

Restriction analysis of the vector pCY104 revealed that there were two *Cla*I sites bracketing the nocardial replicon of the plasmid. This enzyme was then used to separate the Gram-positive replicon from that of *E. coli*. After the *Cla*I digestion, the larger of the two bands was partially restricted with *Sau*3A, which produced the cohesive compatible ends for the ligations with remaining *Bam*HI site of the construct. It was necessary to dephosphorylate the vector DNA with alkaline phosphatase in order to prevent its religation. The insertional inactivation of the suicide was performed and the resulting recombinant plasmid was transformed into *R. rhodochrous* using kanamycin as a selectable marker. Furthermore, rhodococcal cellular extracts were shown to transform non-lysogenic *E. coli*. The plasmids were prepared from the Gram-negative host and insert was taken out using the low gelling agarose. The shuttle vector (pAR1) was then re-ligated and both lysogenic and non-lysogenic *E. coli* strains were transformed with pAR1. Strain MM294-4 resulted in no transformants indicating that the suicide function was still present. The recombinant plasmid intermediates and the stages in the development of the pAR1 shuttle vector are shown below in Figure 1.

Presently, our understanding of the genetics of a particular genus is limited by the dearth of suitable genetic tools, including cloning vectors (Quan and Dabbs 1993). Scientists have conducted extensive research in order to identify vectors for use in the genus *Rhodococcus* as a mean of manipulating their metabolic activities (Dabbs and Sole 1988, Dabbs 1998). Therefore identifying vectors that can be

shuttled in and out of these organisms would provide a useful method of manipulating their metabolic activities. Most available vectors have a narrow host range and are developed by combining indigenous cryptic rhodococcal plasmids with resistance determinants from streptomycetes (Dabbs *et al.,* 1990, De Mot *et al.* 1997).

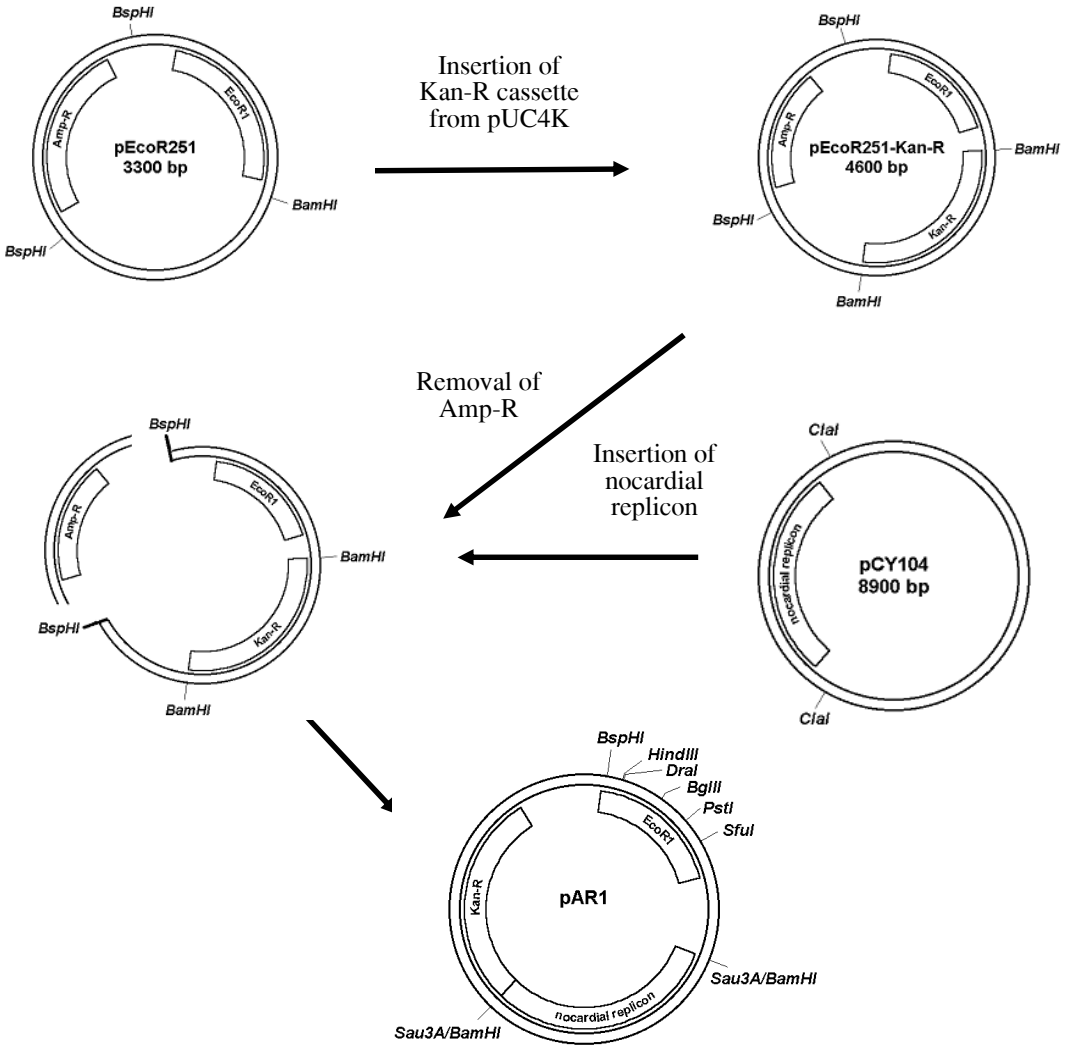

Figure 1. Schematic representation of the shuttle vector pAR1 development

One of the major complications during the development of this shuttle vector arose with the *Bsp*HI isoschizomer, called *Rca*I. Isoschizomers (iso = same, schizo = cut) are enzymes that recognize the same sequence but cleave differently and

therefore do not generate compatible cohesive ends. Restriction enzyme *Bsp*HI is blocked by the overlapping *dam* methylation within the *Eco*RI suicide gene. Unfortunately, nothing is known about *Rca*I methylation sensitivity and every single DNA manipulation using the isoschizomer resulted in interruption of the suicide gene. Moreover, during removal of the *bla* determinant from the pEcoR251, despite the fact that the *Bsp*HI enzyme was used, additional interruption of the suicide gene was observed with some constructs. This was most probably due to the partial methylation of the *Bsp*HI sequence within the *Eco*RI gene.

The future work on the shuttle vector developed in this work may include the molecular characterization of the respective rhodococcal DNA fragments responsible for stable maintenance of this cloning vehicle. One of the objectives of the present work was to develop a vector with a broad host range but a part of the nocardial replicon was lost. The electroporation technique was used to transform the shuttle vector pAR1 into various rhodococcal strains and spontaneous deletions of the nocardial replicon may have occurred. Therefore, the identification of the replicative origins and replication proteins during the rolling circle replication of the plasmid may be investigated in the future work.

Conclusion

In conclusion, pAR1 is the cloning vector constructed to be used in nocardioform bacteria. For example, due to its positive selection and small size it could be very useful in the genomic library construction. A lack of genetic tools for the versatile Gram-positive nocardioforms during scientific investigations creates many obstacles. Heterologous gene expression, gene and genome sequencing are difficult to achieve under laboratory conditions without appropriate tools. Continuous experimental use of the pAR1 could certainly facilitate future research.

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RAZVIJANJE ŠATL VEKTORA ZA NOKARDIOFORMNE AKTINOMICETE

- originalni naučni rad -

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Rezime

Konstruisanje *Escherichia coli* – nokardioform šatl vektora postignuto je korišćenjem rekombinovane DNK tehnologije. Razvojna strategija uključuje sledeće korake u kloniranju i spajanju. Najpre je usledilo ubacivanje kanamicin rezistantne kasete u samoubijajući vektor pEcoR251. Posle ovoga sledila je ligacija nokardioformnog replikona iz pCY104 vektora i *Eco*RI endonukleaze iz pEcoR251, koji nema ampicilin rezistentnu determinantu. Novi rekombinovan plazmid je konstruisan sa pozitivnom (samoubijajućom) funkcijom i sa jednim selekcionim markerom ekspresovanim u obe vrste bakterija, Gram-pozitivne i Gram-negativne. Plazmid pAR1 je relativno mali ~5.0 kb.