DESIGN OF EXPRESSION VECTORS BASED ON pCR2 REPLICON OF *Corynebacterium renale*

by

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Thesis submitted

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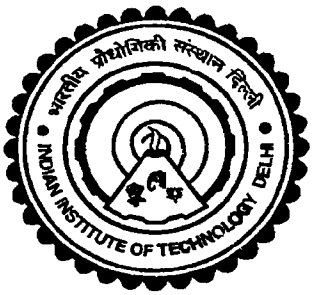

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CERTIFICATE

*This is to certify that the thesis entitled "**Design of expression vectors based on pCR2 replicon of Corynebacterium renale**" being submitted by **Rupali Walia** to the Indian Institute of Technology, Delhi, for the award of degree of **Doctor of Philosophy**, has been prepared under my supervision and guidance in conformity with the rules and regulations of Indian Institute of Technology, Delhi. The research report and the results presented in this thesis have not been submitted to any other University or Institute for the award of any other degree or diploma.*

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ABSTRACT

A 3.2 kb plasmid pCR2 was isolated from *Corynebcterium renale* which harbours four cryptic plasmids. This plasmid was used for the construction of a range of cloning and expression vectors for *E.coli.* This previously uncharacterized plasmid was first sequenced by cloning its fragments into pUC19. The complete nucleotide sequence thus obtained was annotated and submitted to GENEBANK (Accession No. EF488047). A complete restriction map was generated from the sequence obtained to identify dispensable restriction sites which are suitable for cloning.

As a first step in the construction of vectors based on this replicon, antibiotic resistance cassettes viz ampicillin and chloramphenicol were introduced into the pCR2 backbone. These constructs (pCR2amp and pCR2cat) were used to transform *E.coli* DH5α cells. These plasmids were tested for stability in *E.coli* in the absence of selection pressure and a high level stability for a large number of generations was observed with these plasmids. Since this plasmid was quite different from known *E.coli* plasmids, it was expected that pCR2 would be compatible with known *E.coli* replicons. Compatibility studies were therefore carried out with two commonly used *E.coli* plasmids viz pUC19 (pMB1 ori) and pACYC184 (p15A ori). Both the plasmids (pCR2cat and pUC19 or pCR2amp and pACYC184) were stably maintained for 60 generations in shake flask cultures in the absence of selection pressure demonstrating sustained co-existence.

Once the stability and compatibility of pCR2 had been demonstrated it was used to construct a range of expression vectors with different promoters and reporter

genes. The most commonly used promoter P_{lac} was introduced along with GFP as a reporter gene while the h-IFNγ gene was cloned along with the upstream T7 promoter onto the pCR2amp plasmid. Sustained expression was demonstrated for GFP by repeated subculturing in the absence of selection pressure. More importantly, the expression of human interferon gamma under the strong T7 promoter was checked for 60 generations in the absence of selection pressure for pCR2 replicon and no decline in the expression level was observed. In contrast, the control culture which harboured h-IFNγ cloned in the commercially used pRSET vector lost its ability to express the recombinant protein after 30 generations. These results underscore not only the extremely high level of stability but also sustained expression capacity of pCR2 based constructs while operating under stress associated with high level expression. Since this stability was observed in the absence of selection pressure this plasmid would be extremely useful for scale up studies with recombinant products. To extend the range of expression vectors, a series of corynebacterial promoters (inducible P*aceA* and P*aceB* and constitutive P-*per* and P-*45*) were also cloned into pCR2 along with GFP as the reporter gene. These constructs were found to work efficiently in *E.coli*.

We thus have a series of vector constructs which have added to the repertoire of the existing *E.coli* plasmids. The compatibility of these constructs with other well known *E.coli* vectors makes it an extremely useful tool for metabolic engineering which needs presence of multiple plasmids for the calibrated expression of a set of proteins. Simultaneously, high level stability, which is possibly due to the fact

that it is a natural isolate, would allow us to exploit it for large scale production especially when antibiotic addition needs to be avoided.

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