Complementation of the mutant strain with the wild copy of the eccD3 gene

3.1   Introduction

The solution to the persistence of the problem of TB caused by *M. tuberculosis* lies in the complete understanding of the molecular mechanisms responsible for its remarkable pathogenicity. An approach from the perspectives of contemporary molecular genetics appears to hold the key to this long lasting problem. Understanding of the mycobacterial physiology and metabolism can be better understood with the help of modern molecular genetics method.

A new era began with the complete sequencing of the whole genome of *M. tuberculosis* [1]. In order to study the functional role of genes in *Mycobacterium*, mutants of *Mycobacterium* species are generated through homologous recombination or transposon mutagenesis [2-5]. The resulting mutations have led to the generation of highly attenuated strains or strain with elevated virulence [6-8]. However, the phenotypes exhibited by the mutant strains are to be confirmed by the generation of strains in which the wild type copy of the gene is introduced in the mutant strain. The complementation test helps to understand whether the concerned phenotype is the result of a particular gene mutation and not because of any secondary mutation that may have happened during the generation of the mutant. With the help of complementation test, one can also confirm that the phenotype exhibited by the mutant is not because of the polar effect of the gene in which deletion or insertion of a gene present in an operon may interfere in the transcription or translation of the downstream genes. The introduction of a gene and its subsequent expression in the mutant mycobacterial strains requires cloning strategies. The cloning vector used in complementation might be a replicating plasmid that contains the Origin of replication for *Mycobacterium* or an integrative vector which has the ability to get integrated in the mycobacterial genome. Earlier the first generation vectors, *E. coli*-mycobacterial shuttle vectors in which *E. coli* cosmid incorporated in mycobacteriophage e.g. pYUB12 was used [9,10]. However, the large size of the vector, absence of restriction sites and selectable marker discouraged the use of such vectors in the genetic study of *Mycobacterium* [11]. The second generation vectors were developed that consisted of a 1.8kb segment of plasmid from pAL5000 encoding the Mycobacterial Origin of replication (*oriM*). The vector also comprised of three additional components- (i) *E. coli* Origin of replication from pUC19 (*oriE*), (ii) the *aph* gene coding for kanamycin resistance derived from Tn903 as a selectable marker,

(iii) an expression cassette containing a mycobacterial promoter, multiple cloning site and a transcription terminator. Later on, an integration plasmid was constructed by replacing the *oriM* in the earlier plasmid which was named as pMV361 by the attachment site (*attB*) and the integrase gene of the mycobacteriophage L5 [12]. One disadvantage of using an integrative vector like pMV361 or pMV306 is that when native promoters are used for transcription of the integrated gene, the integration locus might interfere. This disadvantage is even more pronounced when the gene being expressed is present in an operon and it is not the first gene [13-15]. A large number of *E. coli* and mycobacterial shuttle vectors have been developed thereafter. The episomal plasmids like the pMV261 vector have the advantage of high copy number but these plasmids are associated with instability of the recombinant vectors. However these instabilities might be attributed to the promoter or protein toxicity [15-18].

In this study the episomal plasmid pMV261hyg, a derivative of the pMV261 vector was used to introduce the wild-type copy of the *eccD3* gene in the mutant strain of *M. smegmatis*. The plasmid consisted of a hygromycin resistance gene cassette as a selectable marker.

3.2   Materials and methods

3.2.1   *Bacteria strain, plasmid, and growth conditions.*

The wild-type *M. smegmatis* mc^{2}155 and the mutant bacteria were grown in Middlebrook 7H9 (MB-7H9, Himedia) medium supplemented with 0.05% Tween80 and 0.2% glycerol or Middlebrook 7H10 (MB-7H10, Himedia) plates supplemented with 0.5% glycerol. Luria-Bertani (LB, Himedia) medium was used for the of *E. coli* strains. The antibiotics were added in the following concentrations, Ampicillin (100μg/ml for *E. coli*), Kanamycin (25μg/ml for *E. coli* and *M. smegmatis*) and Hygromycin (10μg/ml for *E. coli* and *M. smegmatis*) were used as appropriate. Growth conditions for *E. coli* were fixed at 37^{0}C for 12 hours, whereas, growth conditions for *M. smegmatis* were fixed at 37^{0}C for 24hours.

3.2.2   *Primers used in the study*

The primers used in the study are listed in Table 3.1

Table 3.1 List of primers used in the complementation study

Primers	Sequences
rrsA	CTTTCAGCACAGACGAAGCG
rrsB	TCACGAACAACGCGACAAAC
eccD3For	**GGTCAT**CTTCTCCGAGGCAC
eccD3Rev	**GACGAT**GAACTGTCCGAGCA
mycP3For	CGGTCTGGTACTGCTGATCG
mycP3Rev	CACAGAGGTCGTGATGCACT

3.2.3   Mutant complementation

To complement the *eccD3* mutant, the wild-type *eccD3* gene was PCR amplified from the *M. smegmatis* genome using the primers in Table 3.1. The PCR product was cloned at the *PvuII-HindIII* sites in pMV261hyg, an episomal plasmid containing a Hygromycin (hyg^r) resistance cassette. The resulting plasmid was electroporated into *eccD3* deleted mutant strain, and transformants were selected on MB-7H10 containing hygromycin and kanamycin. The complemented strain was named, Ms0622/pEccD3.

3.2.4   Isolation of RNA

RNA was isolated from the *M. smegmatis* strains as described [19]. Briefly, *M. smegmatis* cells were grown to the early to mid-log phase (OD$_{600}$ = 0.1 to 0.2) at 37^{0}C. Cells were pelleted at 3000g for 10 mins. The cells were resuspended in 1ml Trizol (Sigma). The cells were then bead beaten for 3 seconds with intermittent cooling. The glass beads were separated by centrifugation at 12,000g for 1 min. The homogenized samples were incubated at room temperature for 5 mins. 300µl of chloroform: isoamyl alcohol (24:1) was added and the tubes were vigorously shaken for 15 seconds. The samples were then subjected to centrifugation at 12,000g for 15 mins at room temperature. The aqueous phase was transferred to a fresh tube and 0.8 volumes of isopropanol were added. The samples were incubated at room temperature for 10 mins at 4^{0}C and centrifuged at 12,000 x g for 10 mins at 4 ^{0}C. The supernatant was removed and the RNA pellet was washed with75% ethanol. The pellet was air dried and resuspended in 50µl of RNase free water.

3.2.5   *Preparation of cDNA*

cDNA was prepared using the Verso cDNA synthesis kit (Thermo Scientific). Briefly, equal amount of RNA was taken from mc^{2}155, Ms0622 and Ms0622/pEccD3 as a template. Random hexamers were used as the RNA primer. The cDNA was synthesized for 60 mins at 42^{0}C followed by the inactivation cycle for 2mins at 95^{0}C.

3.2.6   *Semi-quantitative RT-PCR*

Semi-quantitative RT-PCR was performed as described [20] using the primers listed in Tale 3.1. The absence of DNA contamination from the RNA samples was confirmed by PCR using the RNA samples as the template. For semi-quantitative results, *eccD3* was amplified along with *rrsA* as the internal control. PCR was carried for 31 cycles. The semi-quantitative RT-PCR bands were quantified on 1% agarose gel using Gel QuantNet software.

3.3   Results

3.3.1   *Cloning of the eccD3 into pMV261hyg vector*

The amplification of the *eccD3* was successfully performed. The amplification was performed with Phusion high fidelity DNA polymerase (ThermoFisher Scientific) which is specialized for GC-rich templates and long range PCR. Moreover its proof-reading capacity ensures minimal incorporation of wrong bases in the amplicon. The PCR conditions were standardized using a gradient thermal cycler and the annealing temperature was fixed at 59^{0}C (Figure.3.1). The amplicon was then successfully digested with *PvuII and HindIII* and ligated into *PvuII-HindIII* digested pMV261hyg (Figure 3.2 [A]). Plasmids isolated from the clones showed a distinct shift in size on 0.8% agarose gels (Figure 3.2 [B]).
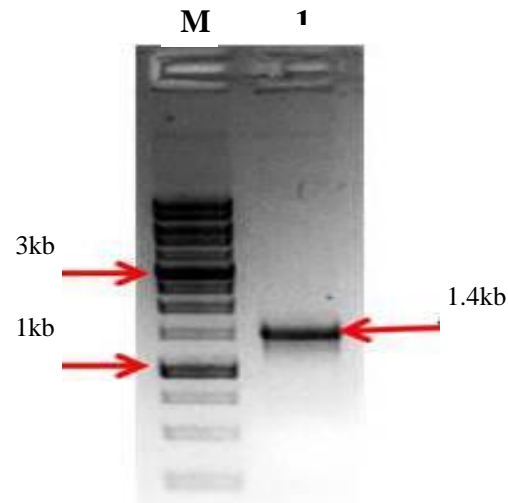
Figure 3.1: 0.8% agarose gel showing the results of PCR amplification of 1428bp region of *eccD3* in a thermal cycler with annealing temperatures of 59˚C. Lane 1: Amplified 1.4kb product, Lane M: 1 kb DNA ladder (Fermentus).
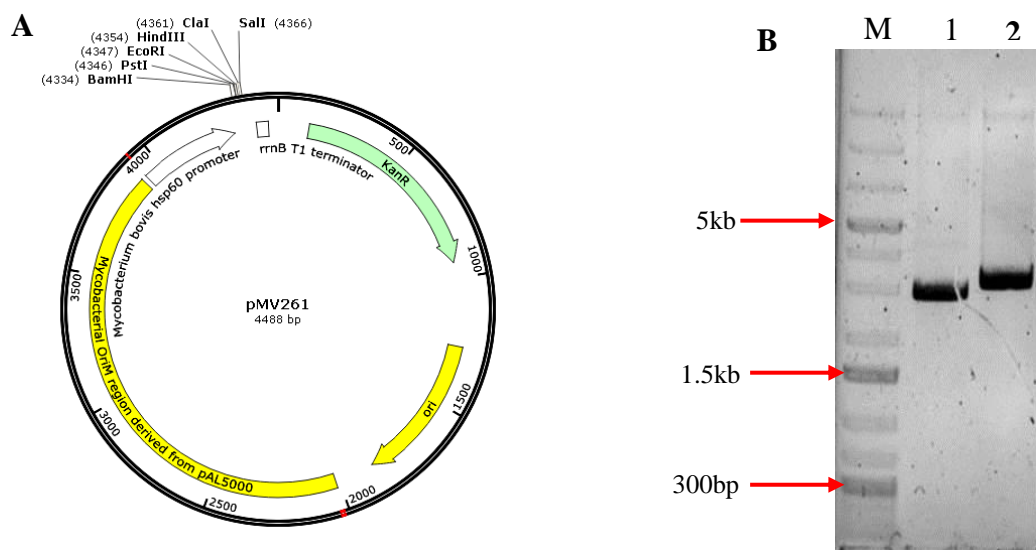
Figure 3.2: [A] Vector map of pMV261; [B]) 0.8 % agarose gel showing the band shift of the rpMV261hyg plasmid. Lane1: pMV261hyg plasmid, Lane2: Colony1, Lane M: 1kbPlus DNA Ladder (Fermentus).

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3.3.2 Sequencing of the eccD3 gene cloned in pMV261hyg

Score	Expect	Identities	Gaps
1666 bits(902)	0.0	904/905(99%)	0/905(0%)

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Query  59    TCACCTGTCGAGCACGAGGCTGAACAGGCCGACCAGCAGCGCCATCACCGGGATCAGCGA
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1442  TCACCTGTCGAGCACGAGGCTGAACAGGCCGACCAGCAGCGCCATCACCGGGATCAGCGA

Query  119   CGCGTCGAGGCCGGTGGCGAGGAAGCCGACCATGCGGCGCATCGGCAGCGAATAGGTGTC
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1382  CGCGTCGAGGCCGGTGGCGAGGAAGCCGACCATGCGGCGCATCGGCAGCGAATAGGTGTC

Query  179   CGGCGAGGCGATCTTCGGGTTGAGCGCCGCGACGATCCAGACCAGCACGAGCACGGCGAG
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1322  CGGCGAGGCGATCTTCGGGTTGAGCGCCGCGACGATCCAGACCAGCACGAGCACGGCGAG

Query  239   CGCGGCCAGCGCCCACAGCGCGGCCTGGTACCGATCGCCGATCACGAACGCCACCAGCAG
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1262  CGCGGCCAGCGCCCACAGCGCGGCCTGGTACCGATCGCCGATCACGAACGCCACCAGCAG

Query  299   TGCCACGGCCAGCAGATAGGAGTGCCCCAGCAGCCACGCCTTGCAGGCCGCGGAGTCCCA
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1202  TGCCACGGCCAGCAGATAGGAGTGCCCCAGCAGCCACGCCTTGCAGGCCGCGGAGTCCCA

Query  359   GACCCGGGCGCGCAGCGCCGCACCGGCCGCCGCGGCGACCACGATGTACCAGGCCCACGG
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1142  GACCCGGGCGCGCAGCGCCGCACCGGCCGCCGCGGCGACCACGATGTACCAGGCCCACGG

Query  419   CGAGGCGTTCGCCGAGCTCACCAGGGCCACCGAACCGGCGACGCCCAACAGCACACCCGC
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1082  CGAGGCGTTCGCCGAGCTCACCAGGGCCACCGAACCGGCGACGCCCAACAGCACACCCGC

Query  479   GGCGATCACACCGGTCTGGTGCGCCTGGCTCACCCGCACGCGGCGCGGCAGATCGGCGAG
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1022  GGCGATCACACCGGTCTGGTGCGCCTGGCTCACCCGCACGCGGCGCGGCAGATCGGCGAG

Query  539   TACCGAAAGGGGCCGCGCGGCAGGCGTCGGATCGCCGGGCGCCGGGATCACCGGCAGCGG
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  962   TACCGAAAGGGGCCGCGCGGCAGGCGTCGGATCGCCGGGCGCCGGGATCACCGGCAGCGG

Query  599   AAACCGGGCCCACATCGCCGACAGTTGTGCGGCCTGCACCGTGACGATCAGACCGAGGAG
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  902   AAACCGGGCCCACATCGCCGACAGTTGTGCGGCCTGCACCGTGACGATCAGACCGAGGAG

Query  659   CACCAGCGCGCAGCCGATGACGTCCGACGAGATGACCCACAGCGACGCCGCGCCCGCGAC
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  842   CACCAGCGCGCAGCCGATGACGTCCGACGAGATGACCCACAGCGACGCCGCGCCCGCGAC

Query  719   CAGCAGGACGCCGACGCCGGTCACGGCGGTCGCGGTGAACACCGCGATACCGCGGTCGTC
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  782   CAGCAGGACGCCGACGCCGGTCACGGCGGTCGCGGTGAACACCGCGATACCGCGGTCGTC

Query  779   GGGCGAGCCGGCCATGCTGATCAACGACCACGCGGCGACACCGGCCGCGGCGAGCAGCAC
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  722   GGGCGAGCCGGCCATGCTGATCAACGACCACGCGGCGACACCGGCCGCGGCGAGCAGCAC

Query  839   GTTCGGCGCGCCGAAATCACCTGGCACACCCAGCGCGAACGCCGCGGCCACTGGGACGAG
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  662   GTTCGGCGCGCCGAAATCACCTGGCACACCCAGCGCGAACGCCGCGGCCACCGGGACGAG

Query  899   TGCGGTGACGGCCAGCGACGTCGCGAGCACCGCGGACCGGTTGCGCACGGCCAGCGCCGC
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  602   TGCGGTGACGGCCAGCGACGTCGCGAGCACCGCGGACCGGTTGCGCACGGCCAGCGCCGC

Query  959   GATCA  963
             |||||
Sbjct  542   GATCA  538
```

Figure 3.3: Sequence alignment of the *eccD3* gene cloned in pMV261hyg and *M. smegmatis* MSMEG_0623. The *eccD3* gene sequence cloned in the pMV261hyg showed 99% sequence similarity over a query cover of 93% with MSMEG_0622, the *eccD3* gene in *M. smegmatis*.

3.3.3   *Screening of clones with the wild copy of eccD3 gene*

The use of pMV261hyg as a complement vector provided the advantage of screening the putative clones with the wild copy of the gene as it comprises of a hygromycin cassette in its vector backbone. The *eccD3* mutants that were successfully transformed with the recombinant pMV261hyg episomal vector exhibited resistance to hygromycin antibiotic. One colony out of the four colonies was selected as the putative clone that consists of the wild copy of the *eccD3* gene. The complemented strain was named as Ms0622/pEccD3.

3.3.4   **Isolation of RNA**

The RNA isolated from the wild type, mc^{2}155, mutant, Ms0622 and the complemented strain Ms0622/pEccD3 showed the distinct bands corresponding to 23S, 16S and 5S (Figure 3.4). The purity yield of the RNA isolated from the three strains was 1.8.
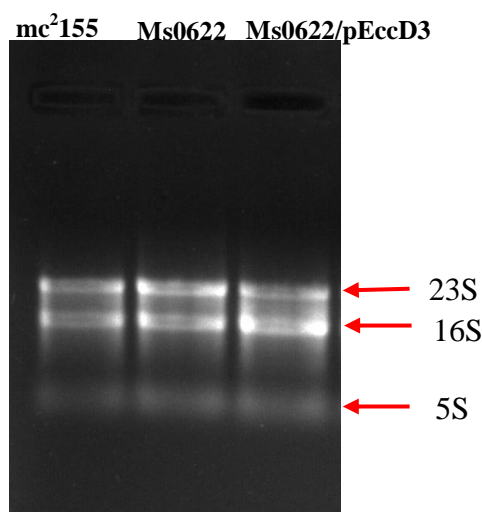

Figure 3.4: 1% agarose gel showing RNA isolated from the wild type mc2155, mutant, Ms0622 and the complemented strain Ms0622/pEccD3.

3.3.5   *Semi quantitative PCR of eccD3 gene*

The transcription of the *eccD3* gene in the parental *M. smegmatis*, mc^{2}155; *eccD3* deleted mutant, Ms0622 and the *eccD3* complemented strain, Ms0622/pEccD3 was assessed by reverse transcription polymerase chain reaction. The 16s rRNA, rrsA of *M. smegmatis* was used as the internal control. Expression of *eccD3* was absent in the knockout, Ms0622 strain whereas expression of the *eccD3* gene could be observed in the complemented strain, Ms0622/pEccD3 (Figure 3.5 [A], [B]). However, the expression of the *eccD3* gene was less than that of in wild type *M. smegmatis* ($P \leq 0.05$).
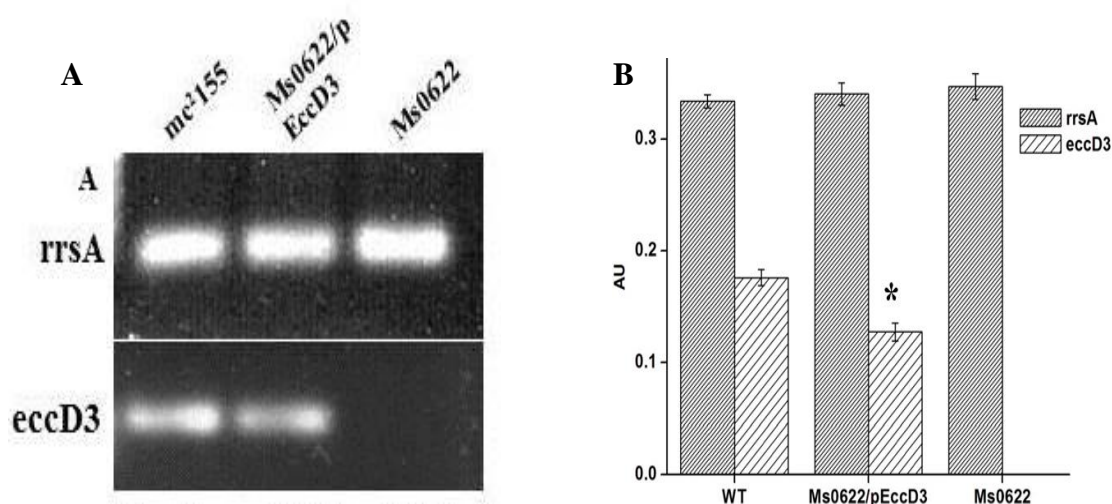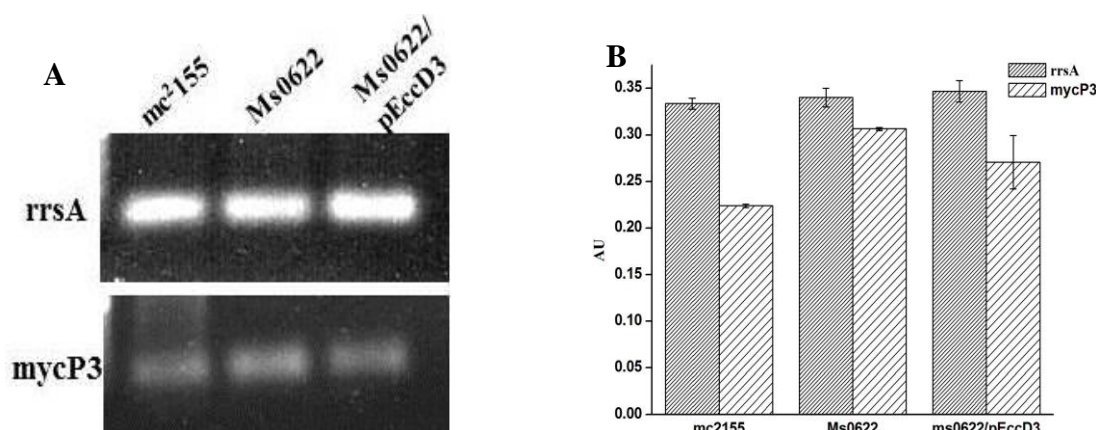
Figure 3.5: Expression profile of *eccD3* in Wild type (WT), Mutant (Ms0622), and Complement (Ms0622/pEccD3) detected by semi-quantitative RT-PCR. *rrsA* was taken as the internal control. A) Representative 1% agarose gel of RT-PCR products corresponding to each gene. B) The bar diagram represents average values of three independent quantitation derived from three independent RT-PCR experiments using different RNA samples of *M. smegmatis* strains. The expression of *rrsA* gene, internal control was not significantly different in all the three strains while the expression of the *eccD3* gene in the Ms0622/pEccD3 was different ($P \leq 0.05$) (2B). Error bars depict mean ± standard deviation and values that are significantly different ($P \leq 0.05$) are indicated by a single asterisk.

3.3.6   Absence of polar effect

The expression of the gene present downstream of the *eccD3* gene was studied to demonstrate if the in frame deletion of the *eccD3* gene had any polar effect on the downstream genes. The expression of the *mycP3* gene in the *eccD3* deleted strain, Ms0622 showed 1.5 fold higher expressions than the wild type whereas the *eccD3* complemented strain, Ms0622/pEccD3 showed 1.2 fold more expression than in the wild type *M. smegmatis* (Figure 3.6 [A], [B]).

Figure 3.6: Expression profile of *mycP3* in Wild type (mc²155), Mutant (Ms0622), and

Complement (Ms0622/pEccD3) detected by semi-quantitative RT-PCR. *rrsA* was taken as the internal control. [A] 1% agarose gel of RT-PCR products corresponding to each gene. [B] The bar diagram represents average values of three independent quantifications derived from three independent RT-PCR experiments using different RNA samples of *M. smegmatis* strains.

3.4   Discussion

The wild copy of the *eccD3* gene was successfully cloned into the episomal plasmid pMV261hyg and transformed into the *M. smegmatis* mutant strain lacking complete copy of the *eccD3* gene. The pMV261hyg vector provided the advantage of an antibiotic selection marker, mycobacterial *Origin of replication*, an hsp60 promoter, a multiple cloning site and a transcription termination site [11]. The wild copy of the *eccD3* gene was cloned downstream of the hsp60 promoter and the resultant complemented strain was termed as Ms0622/pEccD3. The semi-quantitative expression of the *eccD3* gene in the three strains revealed that there was a complete lack of expression of the gene in the mutant strain as expected. However, the *eccD3* gene in the complemented strain showed less expression in comparison to the wild type *M. smegmatis* strain. The comparatively less expression of the *eccD3* gene in the complemented strain might be attributed to the instability of the expression plasmids. The structural instability of an expression plasmid is common in many Gram positive microorganisms. The instability of the plasmids in these organisms might be attributed to the genetic rearrangements occurring in the plasmid because of the homologous or non- homologous recombination events [21]. In a report, the expression of the *eccD3* gene cloned in an episomal vector pDMNI yielded no expression. The *eccD3* gene was cloned upstream of the green fluorescent protein present in the vector backbone. The eccD3-GFP fusion protein was then experimented to show fluorescence but the fluorescence was absent. The null expression of the *eccD3* protein with the pDMNI vector was attributed to the toxicity of the fusion protein [22]. In 2008, Hong *et al*. demonstrated the stability of the pMV261hyg episomal vectors. They concluded that the pMV261hyg vectors are lost from the host after several generations resulting in the less expression of the cloned gene [23].

Since time immemorial the prokaryotic cells have under gone evolution and in this process, there is a high diversity in the bacterial genomes. The horizontal gene transfer among the species of a particular genus has led to the increase in the gene repertoires. The genome size of the prokaryotes determines the metabolic versatility,

regulatory complexity, effective population size, and horizontal transfer rates in that species [24]. A large number of genes in the prokaryote are present in operons such that the expression of the genes could be controlled and the circumstances like wastage of the transcription and translation products and urgent requirement of these do not arise. Prokaryotic genomes with limited mechanism of gene regulation harbor a comparatively high number of operons in comparison to the larger genomes [24]. The expression of a gene might be altered when they are present in the organization of an operon by a number of regulatory mechanisms such as translational coupling, feedback regulation and polarity effect [25-28]. The mycobacterial genome also harbors a number of operons. The ESX3 cluster of the T7SS is also present as an operon comprising of 11 gene loci [29]. The gene of interest, *eccD3* in the present study is positioned at the ninth position and two genes are present downstream of *eccD3* gene. The expression of the immediately downstream gene, *mycP3* was observed to increase by 1.5 fold in the mutant and 1.2 fold in the *eccD3* complemented *M. smegmatis* strain. In 2011, Lim *et.al*., 2011 demonstrated the relationship between the gene expression and the length of an operon. They proposed that with the increase in the distance between the first gene in an operon and the gene present at the end increases, the expression of the genes increases linearly [30]. The marginal increase in the expression of the mycP3 gene in the *eccD3* deleted strain, Ms0622 and the complemented strain, Ms0622/pEccD3 revealed that the deletion of the *eccD3* gene did not have any marked effect on the expression of the downstream genes.

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