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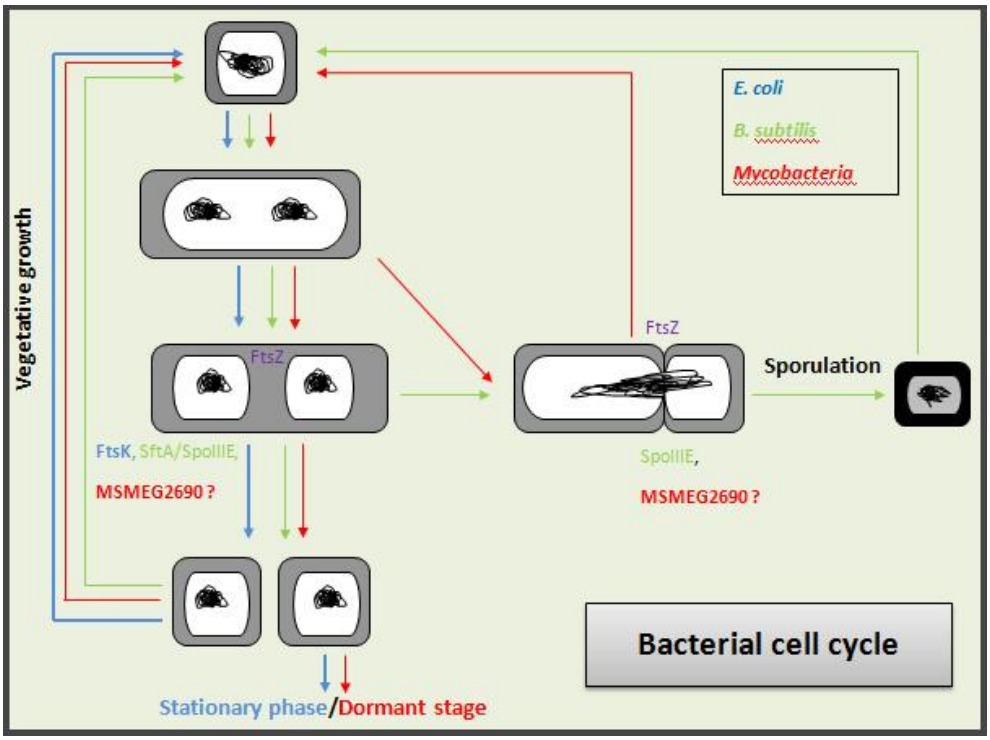
Cloning of mycobacterial putative cell division gene (MSMEG2690) in shuttle vector and understanding its function



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Cloning of a putative mycobacterial putative cell division gene (MSMEG2690) in a shuttle vector and understanding its function

Abstract

Bacteria divide by binary fission forming a septum at a precise mid-cell location. Recently, Mycobacteria have been shown to divide randomly rather than at a fixed mid-cell position. This study was focused on finding out whether Mycobacterium have different DNA translocases specialized for division at different cellular locations. *Mycobacterium smegmatis* ORF MSMEG2690 has been annotated as a putative DNA translocase. Using an Antisense technique, we tried to identify the biological function of MSMEG2690 as a true DNA translocase, and its role during cell division and chromosome segregation.

Key terms: Binary fission, Mycobacteria, M. smegmatis, septum, DNA translocase, antisense, MSMEG2690

Contents

Introduction

Mycobacteria are gram-positive bacteria which belong to the family *Mycobacteriaceae*. These acid-fast and non-motile bacteria have a rod like filamentous morphology. Most mycobacterial species are commonly found in ground and tap water, soil and plants (Web references 1, 2); but some pathogenic species like *M. tuberculosis, M. bovis, M. leprae* are the causative organisms of deadly diseases in humans and animals. *M. tuberculosis* causes tuberculosis (TB) in humans and kills approximately two million people each year (*Singh et al.,* 2010).

Mycobacterium smegmatis is a non-pathogenic, saprophytic species of mycobacteria, which is relatively safe and generally does not cause diseases. It is an obligate aerobe with a high GC content in its genome. In comparison with other pathogenic mycobacterial species, *M. smegmatis* is a fast growing bacterium, and is transformable at high frequencies (web reference 2).This particular bacterium is easy to handle and work with in laboratories.

Growth and cell division in bacteria

Bacterial cells reproduce asexually. The process of cell division in most of the rod-shaped bacteria occurs by a simple binary fission, which means that the reproduction of a cell occurs by division into two parts, each of which has identical genetic material and capacity to grow to the size of the parent cell. As shown in Figure 1, *E. coli* and *B. subtilis* divide at mid-cell during vegetative growth, whereas in contrast *B. subtilis* divides asymmetrically when undergoing sporulation *(Levin and Grossman,* 1998; *Errington* 2001). Recent experimental evidence (*Singh et al.,* 2010) shows that cell division in *M. marinum* and *M. smegmatis* occurs in two different manners: either symmetrically from the mid-cell septum or asymmetrically from polar septum.

Septum formation in bacteria is initiated by FtsZ localization at the mid-cell position, a process called Z- ring formation. Formation of the septum is carried out by a number of proteins (**FtsA, ZipA, FtsEX, FtsK, FtsQ, FtsL, FtsW, Ftsl, FtsN and AmiC**) assembling together, that localize to the division site and form a multiprotein complex (*Weiss,* 2004***)***. The protein **Ftsk** in *E. coli* and the Ftsk orthologue, **SftA** and **SpoIIIE** in . *subtilis* are DNA translocases that are involved in coordinating the late stages of cytokinesis and chromosome segregation (*Sherratt et al*., 2010; *Biller and Burkholder*, 2009), which are the important processes in cell division. It has been reported that SftA plays a role similar to FtsK but cannot replace SpoIIIE for chromosome segregation (*Biller and Burkholder,* 2009). SpoIIIE is an essential DNA translocase during sporulation in *B. subtillis* (*Errington,* 2001). Regarding mycobacterial cell division it has been reported that FtsZ is an essential cell division protein in *M. smegmatis* and that the cell division process in mycobacteria is sensitive to the intracellular levels of FtsZ (*Dziadek et al.,* 2003). Since mycobacteria possess alternate division sites during their vegetative growth (*Singh* et al., 2010), it is unclear whether they utilize two different DNA translocases. MSMEG2690 is the only ORF annotated for a putative DNA translocase function. In the current work, we used an antisense RNA technique to test whether expression of MSMEG2690 was essential for cell survival and if it was the only

DNA translocase available in these cells. The present report shows a cloning strategy for an antisense MSMEG2690 in a shuttle vector.

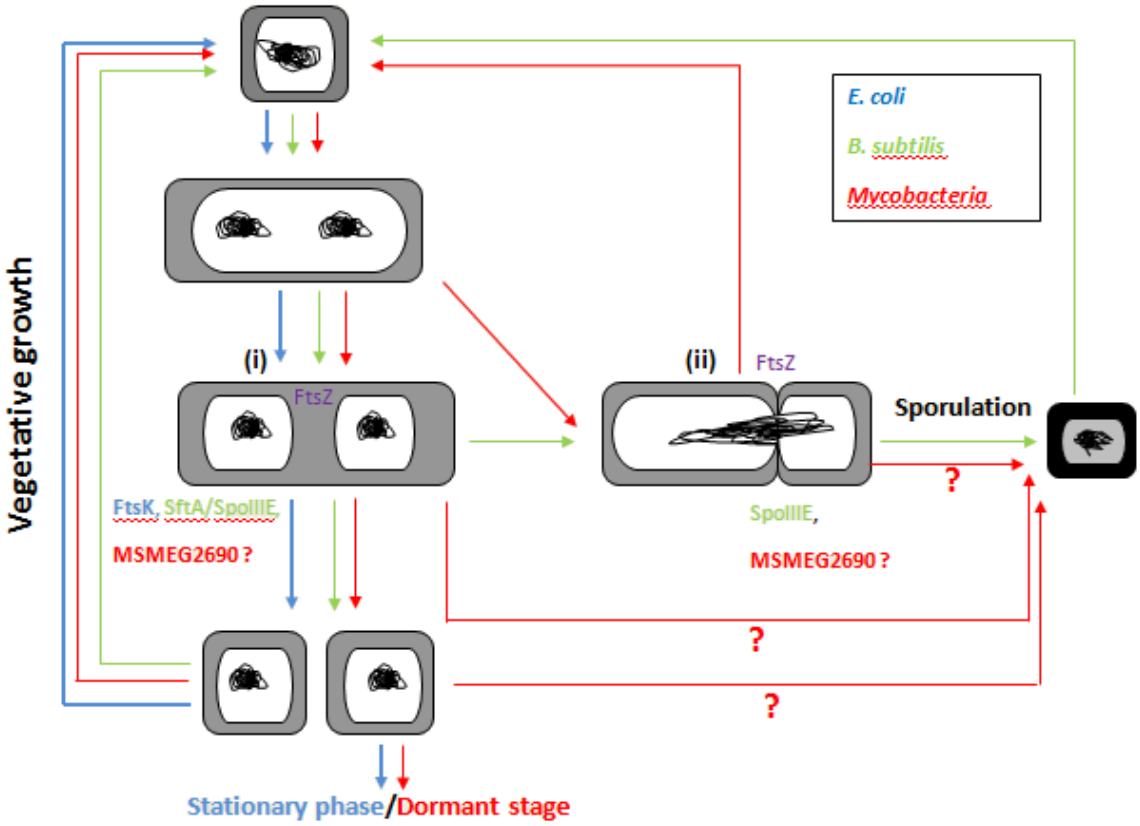

Figure 1. Symmetric (i) and asymmetric (ii) cell division in bacteria. Mycobacteria exhibit both types of division during their growth. Arrows and different bacteria (E. coli, B. subtillis and Mycobacteria) are color coded. (Modified from (Levin and Grossman, 1998)).

Materials and methods

I. Bacterial strain, media and growth conditions

Top 10 *E. coli* cells were streaked in Luria Agar plates with Kanamycin (50µg/ml) and incubated at 37ºC overnight. *Mycobacterium Smegmatis* MC2 155 wild-type cells were grown at 37ºC on Middlebrook agar 7H10 plates supplemented with Middlebrook OADC enrichment and 25µg/ml kanamycin (Ghosh *et al.* 2009).

II. Construction of plasmid pBS305

Construction of plasmid $P^{BS305}$was done by inserting PCR-amplified MSMEG2690 (from *M. smegmatis*) into shuttle vector P^{MIND} (Blokpoel *et al*, 2005)) at BamHI and SpeI cloning sites.

a) PCR amplification of MSMEG2690 (from M. smegmatis)

MSMEG2690 was PCR amplified using primers 27 (forward) and 28 (reverse) that contained flanking SpeI and BamHI restriction sites, respectively (Table 1). Ready-to-go-PCR beads (illustra^{TM} GE Healthcare) were used and PCR was done using the manufacturer's recommendations. In brief, 0.4µM of each primers were used to amplify MSMEG2690 from 3ng of purified *M. smegmatis* genomic DNA, in presence of 1X Q-solution (Qiagen), in a total reaction volume of 25µl. PCR reaction was set as follows: pre-denaturation at 98ºC for 5 min followed by 40 cycles of PCR amplification (denaturation at 98ºC for 30 sec; annealing at 53ºC for 45 seconds; extension at 72º C for 3 min; final extension at 72ºC for 7 min). At completion the PCR programme was set to stay at 4ºC indefinitely. Gel electrophoresis was performed, using 1% agarose (prepared in 1% TAE buffer), to check the size of the desired PCR product. After the completion of the PCR reaction, 15 µl of the PCR product was mixed with 2.5 µl of DNA loading dye ((6X)Fermentas), vortexed and loaded onto the gel. An electric field was applied with 100 volts and the gel was run for 45 minutes. The gel was scanned under UV light, and a positive PCR product was confirmed with a size of approximately 2.8 Kb, using a 1Kb DNA ladder (Fermentas) as a size standard.

Table 1: List of Primer sequences used in the experiment

Primer ID	Primer Name	Primer Sequences
01	pMIND_01	CATCGATAACTTTATCTTAGATAAAGTGACTGCT
02	pMIND_02	TTCCTGGTCGTTCCGCAGGCT
27	Ms Ak-27	GGACTAGT TTGCTCATACAACGATCACTGGAC
28	Ms Ak-28	CGGGATCCTCAGAACTCCTCGCCGTCCTC

b) PCR purification

PCR purification was performed using QIAquick PCR Purification kit (Qiagen) according to the manufacturer's recommendation.

c) Restriction digestion of purified* PCR *MSMEG2690 and plasmid P^{MIND}

Restriction digestion of purified PCR MSMEG2690 and plasmid P^{MIND} was performed to cleave DNA molecules at specific BamH1 and Spe1 sites using fast digest restriction enzymes BamH1 and Spe1 (Fermentas). One Restriction digestion reaction contained 2µl fast-digest buffer (10X), a total of 1µg of PCR-Pure MSMEG2690, 2ul fast-digest enzyme BamH1 and 2ul fast-digest enzyme Spe1. Sterile water was added to make a final volume of 20µl. In the same way, Restriction digestion of the plasmid P^{MIND} was also done by mixing 1µg of plasmid P^{MIND} , along with the same amount and concentration of sterile water, Fast digest buffer (10X), enzymes BamH1 and SpeI as before in order to make a final reaction volume of 20µl. The contents were vortexed for 2 sec; spun for 2-3 sec in a table top micro centrifuge, and then were kept at 37ºC incubator for 30 min.

d) PCR purification of restriction digested PCR MSMEG2690 and restriction digested plasmid P^{MIND}

After incubation for 30 min, PCR purification of restriction-digested Anti-MSMEG2690 and restriction-digested plasmid P^{MIND} was done using a QIAquick PCR Purification kit (Qiagen) according to the manufacturer's recommendation.

e) Ligation of purified, Restriction digested PCR MSMEG2690 (BamHI/SpeI) and purified Restriction digested P^{MIND} (BamHI/SpeI): Construction of plasmid P^{BS305}

For inserting the Anti-MSMEG2690 gene in the P^{MIND} vector, 20µl ligation reaction was prepared by mixing 3µl restriction-digested P^{MIND}, 12 µl restriction-digested Anti-MSMEG2690, 2µl T4 DNA ligase buffer (10X) and 1µl T4 DNA ligase. The contents were briefly vortexed and spun for 2 sec each and kept at room temperature over-night. In this way, the new plasmid P^{BS305} was constructed.

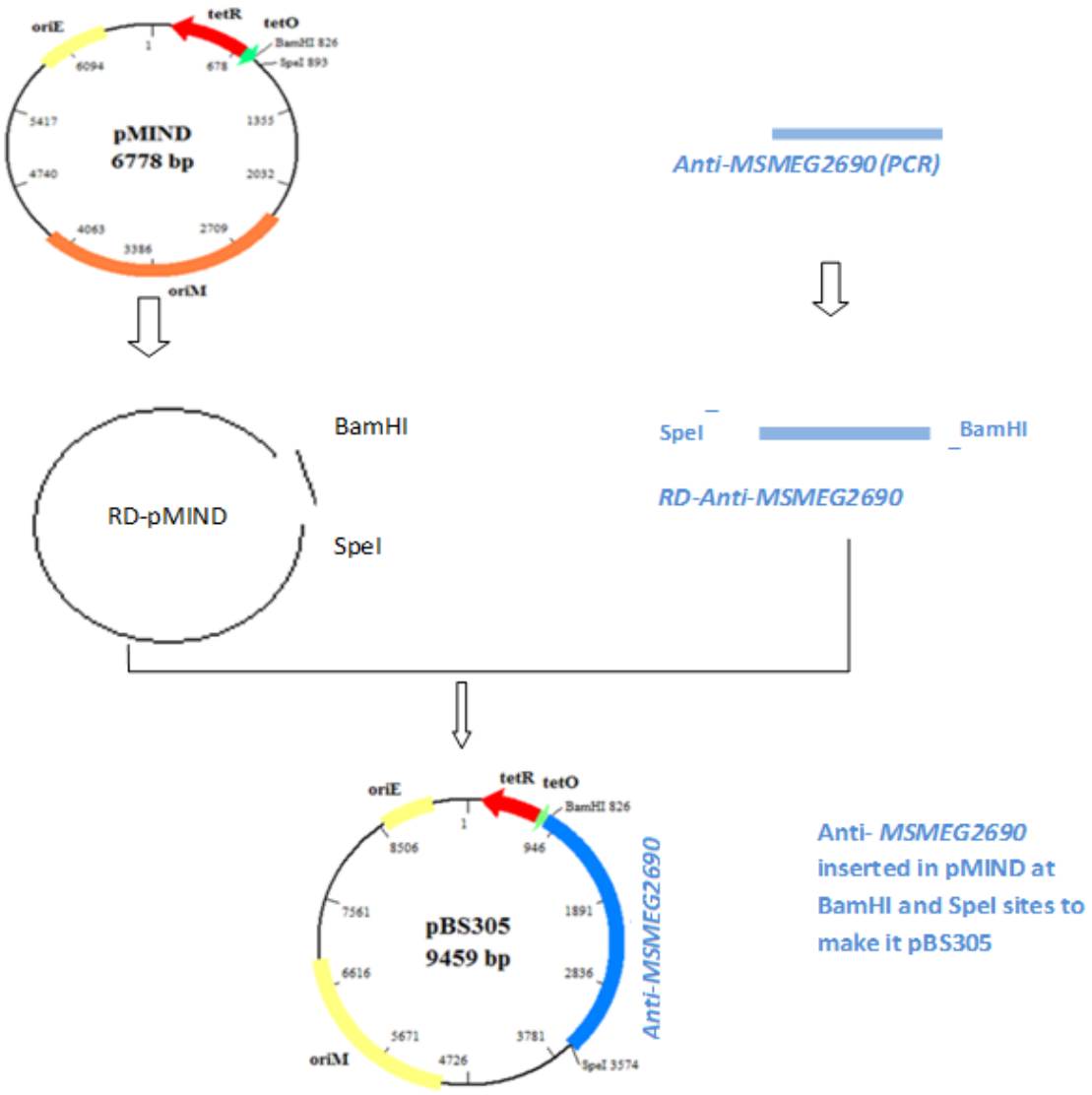

Figure2. Schematic diagram of construction of plasmid pBS305 with PCR amplified Anti-*MSMEG2690* (from *Mycobacterium smegmatis) cloned* in plasmid pMIND at BamHI and SpeI cloning sites

III. Transformation of the plasmid P^{BS305} *into chemically competent Top10 E. coli cells*

After construction of the desired plasmid P^{BS305}, transformation was performed with the chemically competent *E. coli*. Competent cells were thawed from -70ºC on ice, 5µl of ligation mixture was added to the cells and they were held on ice for 5 minutes. Cells were then heat-shocked for 1 min at 42ºC and immediately returned to ice and kept for 2 minutes. 400µl LB was added. Cells were incubated for 60 min at 37ºC with 200 rpm shaking. Cells were then plated onto Luria Agar plates supplemented with 50µg/ml kanamycin, spread with glass spreader, and grown overnight at 37ºC to select for successfully transformed cells.

Colony PCR to verify positive clone on transformants and plasmid purification

Plates incubated on the previous day were observed for colonies. Different colonies were chosen at a time for verification by colony PCR. Falcon tubes were prepared by labeling them and adding 5ml LB with 5µl of 50 mg/ml kanamycin to each of them. A PCR sample was prepared using 0.4µM of each primers 01 and 02 (Table 1), 1X Q-solution (QIAGEN) and Ready-to-go-PCR beads (illustra^{TM} GE Healthcare), and sterile water was added in order to make a total reaction volume of 25µl. For setting up liquid cultures, a sterile pipette tip was used to touch the selected colony. Furthermore the pipette tip was plunged into PCR tube and then the pipette tube was released in the respective falcon tube. This procedure was repeated for rest of the colonies. Those falcon tubes with cultures were incubated at 37ºC overnight with shaking. The PCR reaction was set as follows: pre-denaturation at 98ºC for 5minutes, followed by 40 cycles of PCR amplification (denaturation at 98ºC for 30 seconds, annealing at 53ºC for 45 seconds, and extension at 72º C for 3 minutes), with a final extension at 72ºC for 7 min, followed by 4ºC indefinitely. PCR tubes were centrifuged for 10 seconds in a table top PCR-Tube centrifuge. Agarose gel electrophoresis was performed in the same way as done before to confirm whether our cloning was successful. A positive PCR was confirmed with a size of approximately 3 Kb, by comparison with a 1Kb DNA ladder. Plasmid P^{BS305} was purified, from the positive clone, using a QIAprep plasmid preparation kit (QIAGEN) according to the manufacturer's recommendation. Insertion of Anti-MSMEG2690 into shuttle vector P^{MIND} and the orientation of the insert were confirmed by PCR using the different primer combinations: 01/02; 02/27; 01/28; 27/28; 01/27 and 02/28. The primer pairs 01/02 and 27/28 confirmed the identity of the insertion, whereas primers pairs 01/27 and 02/28 were used to identify the correct orientation (Figure 6)

Preparation of competent wild-type Mycobacterium smegmatis cells

M. smegmatis cells were grown in sterile 15ml 7H9+OADC media by inoculating single colony from 7H10 plate and grown at 37ºC with rotary shaking overnight. The overnight culture was chilled on ice for 1 hour and then transferred to a pre-chilled 15 ml Falcon tube. Cells were spun down at 3000 g in a centrifuge (Skafte Medlab) at 4ºC for 10 minutes. Supernatants were discarded and pellets gently re-suspended in 7 ml 10% ice-cold glycerol. Centrifugation was repeated with the same settings as before and cells were re-suspended in 700 µl 10% ice-cold glycerol. Cells were transferred to a pre-chilled and sterile microcentrifuge tube and spun at 17,900g at 4ºC for 5 minutes. The supernatant was discarded and the pellet was gently re-suspended in 200 µl 10% ice-cold glycerol to give a final volume of about 1 to 1.5 ml depending on the yield. In this way competent cells were made ready to use for transformation.

IV. Transformation of mycobacteria

A 15 ml culture tube was labeled and 5 ml (4.5 ml 7H9 mixed with 500 µl OADC) media was prepared using sterile conditions. A 0.2 cm electroporation cuvette was kept on ice. 6µl of 196.34 ng/µl (1µg) plasmid DNA was mixed with 100 µl electrocompetent *M. smegmatis* wild-type cells and placed in an electroporation cuvette. The Gene Pulser apparatus and pulse controller accessory were set with resistance of 1000 Ohms,

capacitance of 25 µFD and 2.50 KV. The cells were pulsed once and then the voltage display blinked. After the gene pulser beeped once, 1 ml of the (7H9+OADC) media was quickly transferred to the cuvette, mixed well and then the entire sample was transferred to the 15 ml culture tube. Then the tube was incubated at 37ºC for overnight. Next day, the culture was spread on two 7H10 plates supplemented with OADC, 25µg/ml kanamycin and 100µg/ml hygromycin, and incubated at 37ºC for 3 to 4 days.

Results and discussion

1. **PCR-amplification of Anti-MSMEG2690 gene** (from *M. smegmatis)* was done using primers 27 and 28.A positive PCR was confirmed with a size of approximately 2.8 Kb, when compared with 1Kb DNA ladder (fig. 3).

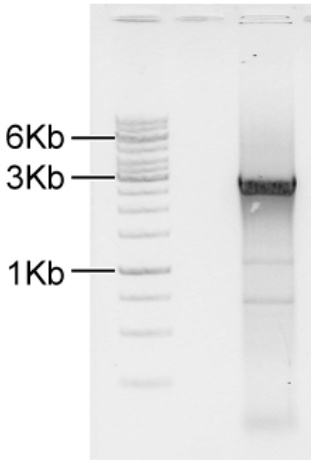

Figure 3 Anti MSMEG2690 PCR (2.8Kb)

2. Purified PCR and plasmid P^{MIND} were digested using BamHI and SpeI restriction sites. Restriction digestion was confirmed by running the digested products on gel (Figure 4)

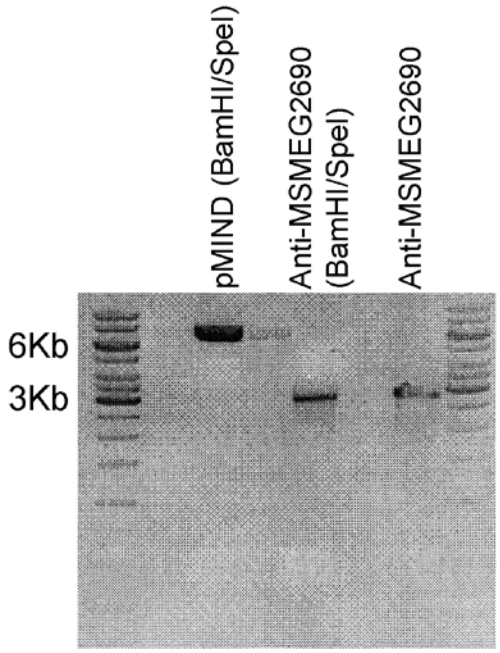

Figure 4. Restriction digestion of Plasmid pMIND (BamHI/SpeI) and Anti - MSMEG2690 (BamHI/SpeI). Restricted digested plasmid showed a single ~6Kb band. Small second band was less than 250bp (Not visible in scanned picture). Restriction digested PCR showed right size of around 2.8Kb.

3. Restriction digested products were ligated using T4 DNA ligase and were transformed in chemically competent Top10 *E. coli* cells. After transformation of the newly constructed plasmid P^{BS305} into Top 10 *E. coli* cells, verification of the clone was done by colony PCR using primers 01 and 02. A total of 130 colonies were screened in order to figure out the positive clone. Clone number 124 showed right size (fig. 5). Plasmid P^{BS305} was extracted from this clone and further verified by PCR using different combinations of primers (Figure 5, Table 1)

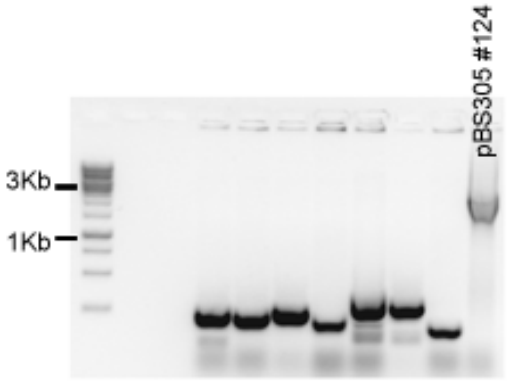

Figure 5: Colony PCR using primers 01 and 02. Positive clone P^{BS305} # 124 (Approx 3Kb).

4. Insertion of Anti-MSMEG2690 into the shuttle vector P^{MIND} and its orientation of insertion were confirmed by PCR using different primer combinations 01/02, 02/27, 01/28, 27/28, 01/27 and 02-28. The primer pairs 01/02 and 27/28 confirms the insertion, primers pairs 01/27 and 02/28 showed the correct orientation (Figure 6)

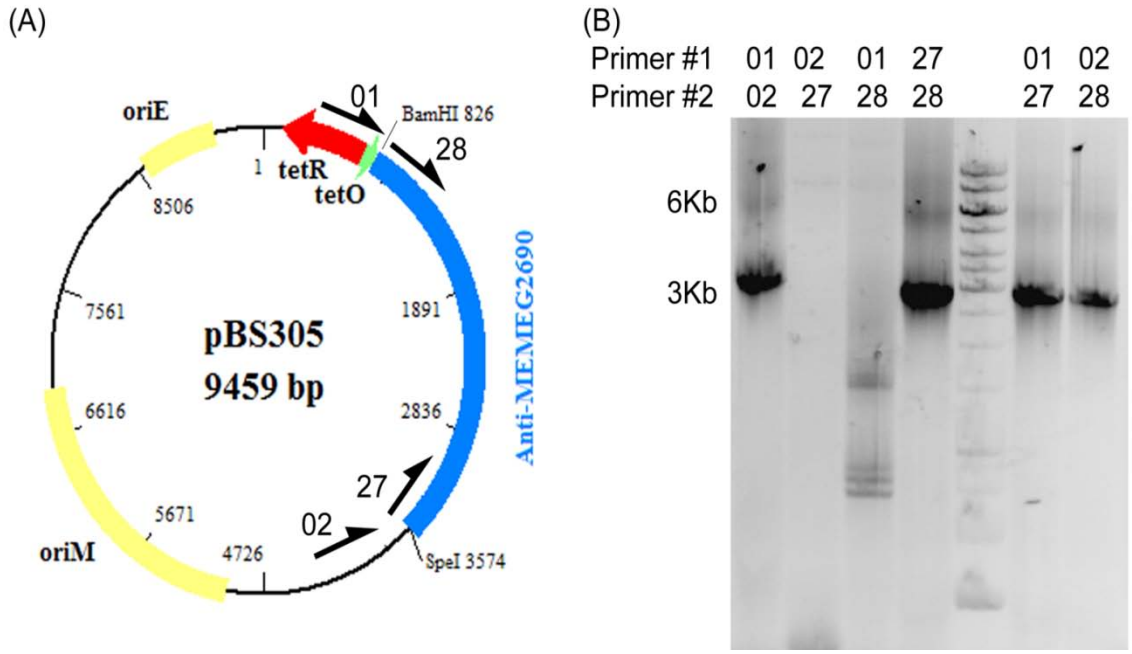

Figure 6 (A)Plasmid P^{BS305} showing oriE, oriM, tetR, tetO, and the orientation of the primers 01, 02, 27 and 28 including the cloning sites BamHI and SpeI. (B) Insertion of Anti-MSMEG2690 in P^{MIND} and its orientation of insertion were confirmed by PCR using primer combinations 01-02 (+), 02-27 (-), 01-28 (-), 27-28 (+), 01-27 (+), 02-28 (+).

Conclusions

I have successfully cloned Anti-MSMEG2690 from *M. smegmatis* into an appropriate expression system (P^{BS305}) and transformed it into *E. coli*. Transformation of the plasmid into mycobacteria has been done but the result is yet to be verified. This clone will be used to determine the cellular function of MSMEG2690 in mycobacterial cell division.

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