Molecular Biology: Open Access
ISSN: 2168-9547

Gokarn et al., Mol Biol 2016, 5:4
DOI: 10.4172/2168-9547.1000176

Research Article Open Access

Cloning of *Mycobacterium smegmatis* Exochelin MS genes *fxbA, fxbC* and *exiT* in *Escherichia coli*

Karuna Gokarn[1,2]*, Ramprasad B Pal[1] and Vishwas Sarangdhar[2]

[1]*Department of Microbiology, Sir H.N Medical Research Society, Mumbai, 400002, India*
[2]*Caius Research Laboratory, St. Xavier's College, Mumbai 400 001, India*

Abstract

Siderophores are low molecular weight compounds with a strong affinity for iron. For instance, exochelin MS is a siderophore produced by *M. smegmatis*. In the present study, we have cloned exochelin MS biosynthesis genes of *Mycobacterium smegmatis*, namely, *fxbA*, *fxbC* and *exiT* in *E. coli* DH5α. These genes were amplified by PCR and cloned in plasmid vector pUC19. The clones harbouring the recombinant plasmids were isolated, purified and sequenced. The sequencing and the BLAST results using NCBI database confirmed the success of cloning *fxbA*, *fxbC* and *exiT* genes.

Keywords: Exochelin MS; PCR; Cloning; NCBI; GenBank

Introduction

Iron chelating agents (siderophores) are small molecules (<1000 Da) produced by bacteria, fungi and plants [1]. Exochelin MS (Exo-MS), a water-soluble siderophore produced by *M. smegmatis* is a pentapeptide comprising of one residue of N-hydroxyformylornithine, two residues of N-hydroxyornithine, one residue of β-alanine and one residue of D-threonine [2].

The Exo-MS biosynthesis genes *fxbA*, *fxbB* and *fxbC* (ferri exochelin biosynthesis) and the transport gene *exiT* (exochelin in transport) are involved in the synthesis, assembly and transport of Exo-MS in *M. smegmatis*. The *fxbB* and *fxbC* genes produce large proteins of 257 and 497 kDa, respectively, which catalyze the non-ribosomal peptide synthesis of Exo-MS [3]. The *exiT* gene encoding a protein for the export of Exo-MS is a member of the ATP-binding cassette superfamily of transport proteins and *fxbA* is responsible for the formylation of the pentapeptide, a step necessary for Exo-MS production [4].

All the genes were amplified using gene-specific forward and reverse primers and genomic DNA (gDNA) of *M. smegmatis* mc^{2}155 as the template. This gDNA was isolated by a simple procedure which uses only ethanol [5]. Cloning of these amplicons was then carried out in *E. coli* DH5α.

Materials and Methods

Exo-MS cloning

Primer design: The primers for *fxbA*, *fxbB*, *fxbC* and *exiT* genes of *M. smegmatis* were designed based on the DNA sequences (*fxbA* - Accession number: U10425 and *fxbB, fxbC, exiT* - Accession number: AF034152) available at the National Centre for Biotechnology Information NCBI site. The primers (Table 1) were synthesized commercially.

Gene amplification: Amplification of each gene was performed in a gradient thermal cycler (Eppendorf) using the above primers in separate reactions using Phusion Flash PCR master mix (Finnzyme) and the gDNA of *M. smegmatis* mc^{2}155. The concentration of stock solutions for the forward and reverse primers was 200 µM, which were diluted to 20µM working stock solution. The final concentration in the amplification reaction was 2 µM of each primer. However, at these primer concentrations, *fxbB* and *exiT* were not amplified. Hence, standardization using different primer concentration and combinations were carried out. It was found that the *fxbB* could be amplified with forward primer at 2 µM and the reverse primer at 0.2µM, while *exiT* could be amplified with forward primer at 0.2 µM and the reverse primer at 2 µM concentrations. Further standardization was also carried out to determine the optimum temperature and time for annealing and extension. For *fxbC* and *exiT* genes, final concentration of 2% DMSO was necessary in the amplification mixture. PCR programs used for successful amplification of each gene are listed in Table 2.

Ligation: pUC19 was digested with *SmaI* restriction enzyme, the amplicons and the *SmaI* cut pUC19 were purified using Nucleopore Sure Extract-PCR Clean-up/Gel Extraction Kit.

Blunt-end ligation of the *SmaI* cut pUC19 vector, and the amplicons was carried out at a vector:insert molar ratio of 1:3 and 1:5 using the T4 DNA ligase kit. The ligation mixtures were incubated at 22°C for 1 hr and then at 16°C overnight.

Bacterial transformation: From each of the ligation mixes of the four amplicons, 5.0 µL was used to separately transform *E. coli* DH5α

Genes	Primer sequences	Size (nt)	T_m (°C)
***fxbA* F**	ATGTGGGGCTCGTTGAAACCGCC	23	63.9
***fxbA* R**	TCATCGACGACCCGCAAGACCG	22	62.7
***fxbB* F**	GTGACCGCGGATTCGCTGG	19	73.5
***fxbB* R**	CATGGCGCAGCATGTACTCG	20	70.0
***fxbC* F**	GTGTCCGGGGCCCTGCCTG	19	80.5
***fxbC* R**	GAGCTCCTCTGCGGCAATGCTTTC	24	77.0
***exiT* F**	GTGTCGGTACCCGTTCTGATC	21	65.5
***exiT* R**	CTAGTTGTGCCTGATCCAGGC	21	65.8

Table 1: Primer sequences.

***Corresponding author:** Karuna Gokarn, Sir HN Hospital and Medical Research Society,Microbiology, L.T Marg, Dhobi Talao, Mumbai, Maharashtra 400 002, India, Tel: +91 9820590072; E-mail: karuna.gokarn@xaviers.edu

Received October 19, 2016; **Accepted** October 27, 2016; **Published** October 31, 2016

Citation: Gokarn K, Pal RB, Sarangdhar V (2016) Cloning of *Mycobacterium smegmatis* Exochelin MS genes *fxbA, fxbC* and *exiT* in *Escherichia coli*. Mol Biol 5: 176. doi:10.4172/2168-9547.1000176

using the standard $CaCl_2$-mediated transformation protocol [6]. The recombinants were detected by blue-white colony selection using the isopropyl β-d-1-thiogalactopyranoside+5-bromo-4-chloro-3-indolyl-β-D-galacto-pyranoside (IPTG+X-Gal) system on Luria Bertanii+Ampicillin (LB+Amp) agar plates.

Screening for recombinants: From the plates white colonies were isolated on fresh LB+Amp plates to obtain single clones. Each colony was then inoculated into LB+Amp broth and allowed to grow at 37°C for 18 hr with aeration. Plasmids were isolated and their sizes were determined by agarose gel electrophoresis with 0.7% agarose gel and NEX-GEN DNA ladder 1kb, (Genetix Biotech Asia, India).

Restriction digestion and DNA sequencing: The presence of the expected gene was confirmed by restriction digestion with specific enzymes. Sequencing of the clones was done commercially and online program - Basic Local Alignment Sequence Tool (BLAST) was used to compare the sequences with the NCBI database.

Results

Gene amplification

The amplification of all the four Exo-MS biosynthesis genes - *fxbA* (1.1 kb), *fxbB* (4.9 kb), *fxbC* (6.3 kb) and *exiT* (3.3 kb), was successfully carried out, as shown by agarose gel electrophoresis (Figure 1).

PCR Steps	Program	*fxbA*	*fxbB*	*fxbC*	*exiT*
1	Initial denaturation	98°C 4 min	98°C 4 min	98°C 4 min	98°C 4 min
2	Denaturation	98°C 5 s	98°C 10 s	98°C 10 s	98°C 10 s
3	Annealing	72°C 30 s	72°C 1 min	72°C 1 min	55°C 15 s
4	Extension	----------	75°C 3 min	75°C 3 min	72°C 1 min
Number of cycles		40	40	40	40
5	Final extension	72°C 5 min	75°C 10 min	75°C 10 min	72°C 5 min

Table 2: Thermal cycling programs for *fxbA*, *fxbB*, *fxbC* and *exiT*.

Screening for recombinants

Analysis of white colonies showed the presence of *fxbA*, *fxbC* and *exiT* genes in separate isolates. The clone with:

- *fxbA* gene in pUC19 was named pKGxA
- *fxbC* gene in pUC19 was named pKGxC
- *exiT* gene in pUC19 was named pKGxT

Since none of the genes were expected to contain *HindIII* site, all the clones were linearized with *HindIII* to confirm their sizes. As expected, *fxbA, fxbC* and *exiT* were 3.8 kb, 9.0 kb and 6.0 kb, respectively. Even though the *fxbB* amplicon was obtained, its ligation in pUC19 could not be achieved despite several attempts.

Orientation of recombinant plasmids was determined by digesting the clones with appropriate restriction enzymes as follows

Refer to Figure 2 for the map of pUC19 vector.

Orientation of *fxbA*: Single digestion of pKGxA was carried out using *EcoRI* restriction enzyme: pUC19 has one *EcoRI* site in its MCS and *fxbA* has one *EcoRI* site. If *fxbA* was ligated in such a way that the 5'-3' orientation of the insert is in the same direction as that of *lacZ* gene of the vector, then the *EcoRI* sites on the vector and the insert would be close together. Therefore, the digest would show 0.1 kb and 3.7 kb fragments. In the opposite orientation, the *EcoRI* site is away from that of the vector, then 1.0 kb fragment and 2.8 kb fragment would be obtained from the digest.

As seen from Figure 3, the orientation of the insert in pKGxA is 3'-5' with respect to that of the *lacZ* gene of pUC19, as two fragments, 1.0 kb and 2.8 kb were obtained after digestion with *EcoRI*.

Orientation of *fxbC*: Double digestion of pKGxC was carried out using *BglII* and *HindIII* restriction enzymes. pUC19 has one *HindIII* site and *fxbC* has one *BglII* site. If *fxbC* was ligated in such a way that 5'-3' orientation of the insert is in the same direction as that of *lacZ* gene of the vector, then double digest would show 0.7 kb and 8.4 kb fragments, where, *BglII* site is close to *HindIII* site of the vector. If *fxbC* was ligated

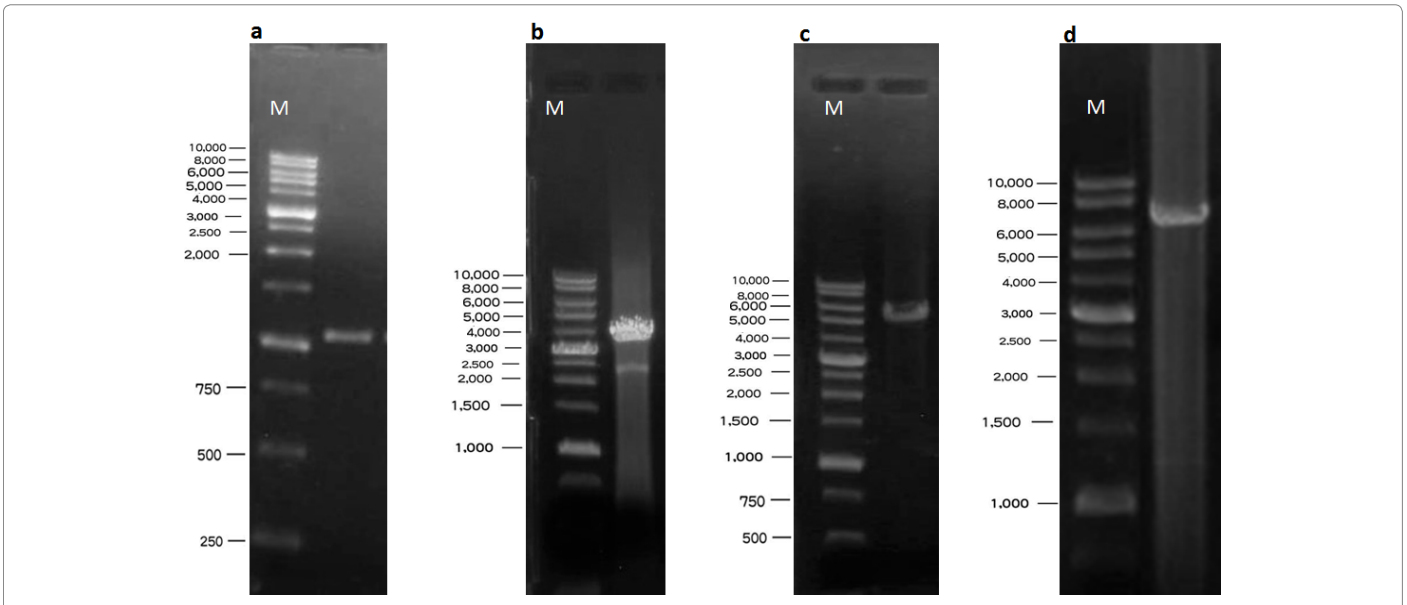

Figure 1: Amplicons of various Exo-MS biosynthesis genes of *M. smegmatis*. The amplicons were electrophoresed on 0.7% agarose gel. (a) *fxbA* gene–1.1 kb b) *exiT* gene–3.3 kb (c) *fxbB* gene–4.9 kb (d) *fxbC* gene–6.3 kb 1.0 kb DNA ladder.

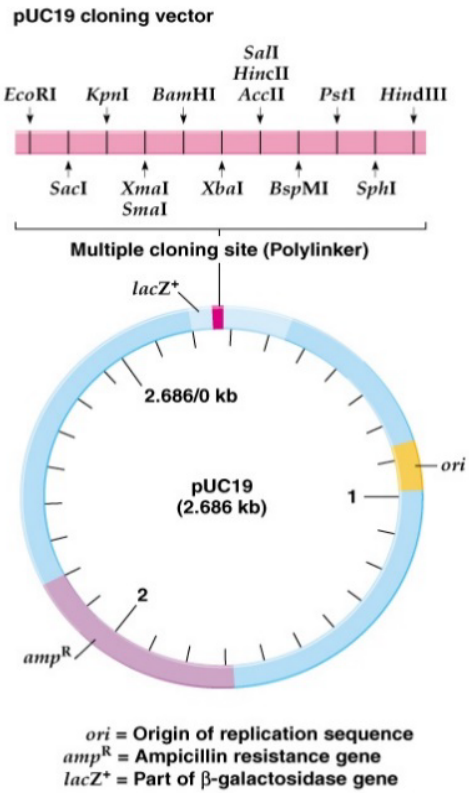

Figure 2: Map of pUC19 with multiple cloning sites. Source: Peter Russel [7].

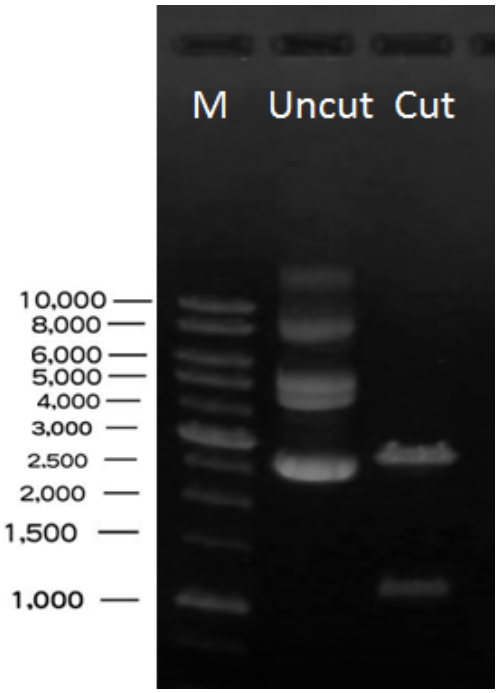

Figure 3: Orientation of *fxbA* gene in pKGxA. pKGxA was digested with *EcoRI* and *HindIII* and electrophoresed on 0.7% agarose gel. It showed two fragments, 1.0 kb and 2.8 kb. M: 1.0 kb DNA ladder.

to the vector in 3'-5' orientation such that its *BglII* site would be away from that of the vector, then 5.7 kb and 3.4 kb fragments would be obtained.

As shown in Figure 4, the orientation of the insert in pKGxC is 5'-3' with respect to that of the *lacZ* gene of pUC19, as two fragments, 8.4 kb and 0.7 kb, were visible after double digestion with *BglII* and *HindIII*.

Orientation of *exiT*: Double digestion of pKGxT was carried out with *SmaI* and *HindIII* restriction enzymes. *exiT* has *SmaI* site, but pUC19 clone has no *SmaI* but has *HindIII* site. If *exiT* was ligated in such a way that 5'-3' orientation of the insert is in the same direction as that of *lacZ* gene of the vector, then double digest would show 0.8 kb and 5.2 kb fragments, where, *SmaI* site would be close to *HindIII* site of the vector. In the opposite orientation, its *SmaI* site would be away from that of the vector, then 2.5 kb fragment and 3.5 kb fragment would be obtained.

As shown in Figure 5, the orientation of the insert in pKGxT is 5'-3' with respect to that of the *lacZ* gene of pUC19, as two fragments were obtained, one at 5.2 kb, although the other at 0.8 kb was very faint after double digestion with *SmaI* and *HindIII*.

Restriction digestion results established the presence of *fxbA*, *fxbC* and *exiT* inserts in all three clones, which were further confirmed by DNA sequencing and the BLAST results of these sequences.

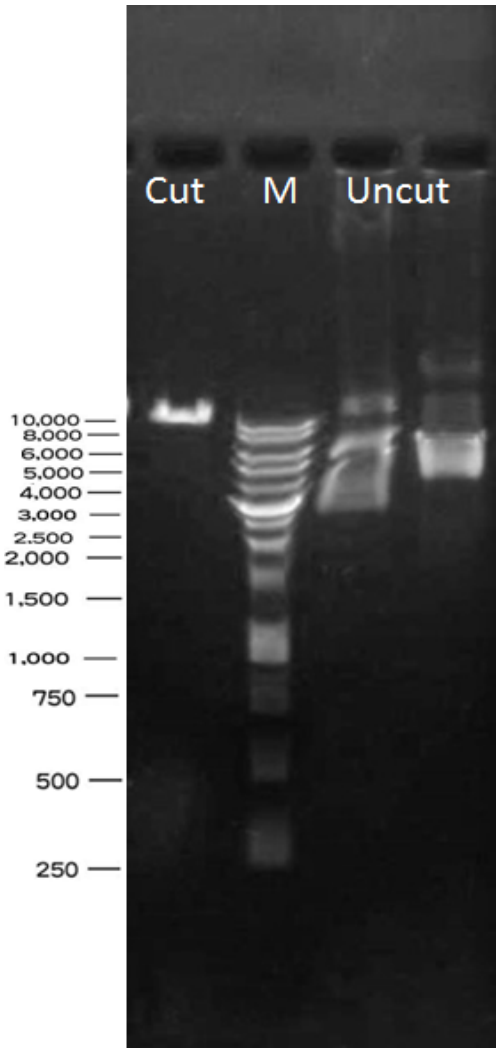

Figure 4: Orientation of *fxbC* gene in pKGxC. pKGxC was digested with *BglII* and *HindIII* and electrophoresed on 0.7% agarose gel. It showed two fragments, 8.4 kb and a faint 0.7 kb. M: 1.0 kb DNA ladder.

Discussion

Even though the sequences of Exo-MS biosynthesis genes of *M. smegmatis* are known, they have been reported as part of the genomic DNA in cosmid libraries. The size of complete *fxbB* and *fxbC* genes are 8 kb and 15 kb, respectively [8]. However, partial genomic sequences of these two have shown the ability to complement null mutations of these genes in *M. smegmatis* [3]. The size of these truncated genes are 4.9 kb and 6.3 kb, respectively. We used the truncated sequences for primer design and amplified *fxbB* and *fxbC* sequences successfully. Cloning of *fxbB* was not achieved, though it was amplified successfully. That the *fxbA* gene (though responsible for formylation of Exo-MS) is required to restore Exo-MS biosynthesis in mutants was demonstrated using deletion analysis [4]. So also the transport gene *exiT*, null mutants of which neither excrete nor accumulate Exo-MS, suggesting that the synthesis may be tightly coupled with export [3]. The ORFs of *fxbA*, *fxbC* and *exiT* under a controllable promoter in a cloning vector has not been published so far.

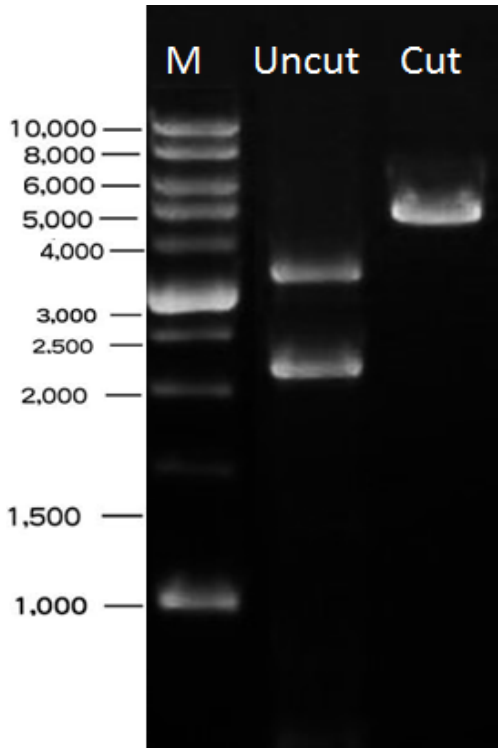

Figure 5: Orientation of *exiT* gene in pKGxT. pKGxT was digested with *SmaI* and *HindIII* and electrophoresed on 0.7% agarose gel. It showed two fragments, 5.2 kb and a very faint 0.8 kb. M: 1.0 kb DNA ladder.

However, none of genes in pKGxA, pKGxC and pKGxT were in frame with respect to the *lacZ* gene of pUC19 to be expressed. Therefore, these genes need to be precisely excised out of the pUC19 vector with restriction enzymes and ligated individually into expression vectors such as pQE30, pQE31 and pQE32 (Figure 6) and transformed into *E. coli* BL21 host. Upon induction with IPTG, the protein can be detected by SDS-PAGE.

Based on the sequence of pKGxA clone, the *fxbA* ORF should be excised with *BamHI* and *HindIII*. It should then be ligated to expression vector pQE31 digested with the same enzymes for it to be expressed.

Similarly, based on the sequence of pKGxT clone, the *exiT* ORF should be excised with *SacI* and *PstI*. It should then be ligated to expression vector pQE32 digested with the same enzymes for it to be expressed.

For pKGxC clone, the *fxbC* ORF should be excised with *NdeI* and *HindIII*. It should then be ligated to expression vector pQE32 digested with the same enzymes for it to be expressed. In this splicing reaction, a part of pUC19 will also be transferred to the resultant plasmid. However, this part lies beyond the 3' end of *fxbC* ORF and therefore, will not interfere with the expression of the *fxbC* gene.

An extension of this work would lead to all the genes being arranged sequentially in a single vector, which would yield sufficient quantities of Exo-MS in a heterologous expression system.

Our sequencing results confirm that *fxbA*, *fxbC* and *exiT* genes are successfully cloned. The nBLAST results using the Genbank nucleotide database showed 98% to 99% similarity with sequences of Exo-MS biosynthesis genes.

The partial nucleotide sequences of *fxbA*, *fxbC* and *exiT* reported in this study have been submitted to the GenBank database under accession numbers: KX944485, KX944484 and KX944486, respectively.

Acknowledgement

We thank the Principal, St. Xavier's College, Mumbai, for permitting the use of the Caius Research Laboratory facilities.

Funding

We would like to thank University Grants Commission, India for their kind funding towards this project (UGC Minor Research Grant No. 47-581/13 WRO).

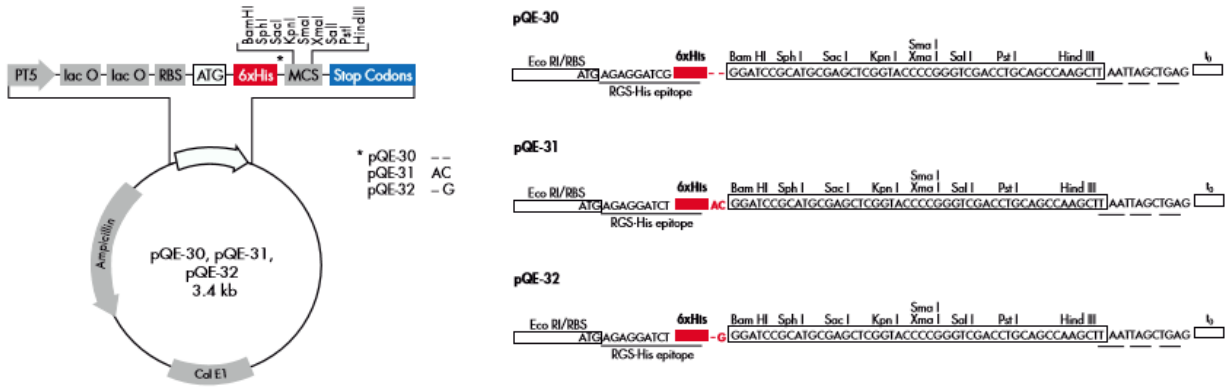

Figure 6: Map of pQE30, 31, 32 with multiple cloning sites. Source: Qiagen [9].

Conflict of Interest

None to declare.

References

1. Neilands JB (1981) Iron absorption and transport in microorganisms. Ann Rev Nutri 1: 27-46.
2. Sharman GJ, Williams DH, Ewing DF, Ratledge C (1995) Isolation, purification and structure of exochelin MS, the extracellular siderophore from *Mycobacterium smegmatis*. Biochem J 305: 187-196.
3. Zhu W, Arceneaux JEL, Beggs ML, Byers BR, Eisenach KD, et al. (1998) Exochelin genes in *M. smegmatis*: Identification of an ABC transporter and two non-ribosomal peptide synthetase genes. Mol Microbiol 29: 629-639.
4. Fiss EH, Yu S, Jacobs WR (1994) Identification of genes involved in the sequestration of iron in mycobacteria: The ferric exochelin biosynthetic and uptake pathways. Mol. Microbiol 14: 557-569.
5. Gokarn K, Sarangdhar V, Pal R.B (2016) Ethanol extraction method for DNA isolation from *Mycobacterium smegmatis*. Int J Curr Res 8: 39013-39015.
6. Sambrook J Fritsch EF, Maniatis T (1989) Molecular cloning: A Laboratory Manual. 2nd edition. Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press.
7. Russell PJ (2009) IGenetics: A Molecular Approach, Pearson International Edition.
8. Yu S, Fiss E, Jacobs WR (1998) Analysis of the exochelin locus in *Mycobacterium smegmatis*: Biosynthesis genes have homology with genes of the peptide synthetase family. J Bacteriol 180: 4676-4685.
9. http://www.helmholtz-muenchen.de/fileadmin/PEPF/pQE_vectors/pQE-30_map.pdf