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

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**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-hydroxynorleucine, 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 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 formulation 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* mc155 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* mc155. The concentration of stock solutions for the forward and reverse primers was 200 μM, which were diluted to 20 μM 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 0.2 μM, 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 pUC19 vector 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° (ºC) |
|-------|------------------|----------|---------|
| *fxbA* F | ATGTGGGGCTGCTTGAAGACCCGC | 23 | 63.9 |
| *fxbB* A | TCATCGACGACCGCAAGACGC | 22 | 62.7 |
| *fxbB* F | GTGACCGCAGGATTCGGTGC | 19 | 73.5 |
| *fxbB* R | CATGGCCGACAGTATGCTCG | 20 | 70.0 |
| *fxbC* F | GTGTCCCGGGGCTCTGCGT | 19 | 80.5 |
| *fxbC* R | GAGCTCCTCTGGCAATGCTTC | 24 | 77.0 |
| *exiT* F | GTGTGCTGACCGGCTTCTATC | 21 | 65.5 |
| *exiT* R | CTAATGTGCTGCTGACCTTAC | 21 | 65.8 |

*Table 1: Primer sequences.*

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using the standard CaCl\textsubscript{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 Bertani+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 - fxb\textsubscript{A} (1.1 kb), fxb\textsubscript{B} (4.9 kb), fxb\textsubscript{C} (6.3 kb) and exiT (3.3 kb), was successfully carried out, as shown by agarose gel electrophoresis (Figure 1).

| PCR Steps | Program | fxb\textsubscript{A} | fxb\textsubscript{B} | fxb\textsubscript{C} | 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 |
| **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 fxb\textsubscript{A}, fxb\textsubscript{B}, fxb\textsubscript{C} and exiT.

**Screening for recombinants**

Analysis of white colonies showed the presence of fxb\textsubscript{A}, fxb\textsubscript{C} and exiT genes in separate isolates. The clone with:

- fxb\textsubscript{A} gene in pUC19 was named pKGxA
- fxb\textsubscript{C} 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, fxb\textsubscript{A}, fxb\textsubscript{C} and exiT were 3.8 kb, 9.0 kb and 6.0 kb, respectively. Even though the fxb\textsubscript{B} 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 fxb\textsubscript{A}:** Single digestion of pKGxA was carried out using EcoRI restriction enzyme: pUC19 has one EcoRI site in its MCS and fxb\textsubscript{A} has one EcoRI site. If fxb\textsubscript{A} was ligated in such a way that the 5’-3’ orientation of the insert is in the same direction as that of lac\textsubscript{Z} 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 lac\textsubscript{Z} gene of pUC19, as two fragments, 1.0 kb and 2.8 kb were obtained after digestion with EcoRI.

**Orientation of fxb\textsubscript{C}:** Double digestion of pKGxC was carried out using BglII and HindIII restriction enzymes. pUC19 has one HindIII site and fxb\textsubscript{C} has one BglII site. If fxb\textsubscript{C} was ligated in such a way that 5’-3’ orientation of the insert is in the same direction as that of lac\textsubscript{Z} gene of the vector, then double digest would show 0.7 kb and 8.4 kb fragments, whereas, BglII site is close to HindIII site of the vector. If fxb\textsubscript{C} was ligated...
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.
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.

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.

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**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.

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

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Conflict of Interest

None to declare.

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