

# Synthesis and Cloning of a Small *Bacillus* Pheromone Gene (ComX<sub>RO-B-2</sub>) by Primer-Dimer Formation with PCR

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ComX pheromone is the major extracellular signaling peptide stimulating transformation in response to high cell density in *Bacillus* species. In this study, *Bacillus mojavensis* ComX<sub>(RO-B-2)</sub> pheromone gene was cloned to pGEMT-Easy vector based on TA cloning of PCR products. The gene encoding 11 amino acid peptides of *Bacillus mojavensis* RO-B-2 strain ComX pheromone (GLQIYTNWVPS) was obtained by PCR amplification of 2 primers complementary to each other at their 3' end. This eliminated the need for the original bacterial gene as DNA template for PCR. The amplified PCR products were ligated directly without any modification by T4 DNA ligase into pGEMT-Easy vector, which has a single overhanging T residue at the 3' ends of the cloning site.

**Key Words:** *Bacillus mojavensis* RO-B-2, ComX pheromone gene, TA cloning, primer-dimer, PCR.

## Introduction

Using PCR, a single DNA molecule can be used to generate identical copies for several purposes.<sup>1,2</sup> The most frequent application of PCR is to amplify a certain portion of a specific gene with known sequences.<sup>3</sup> Generally, a template DNA with known sequences is used with 2 primers to generate the desired gene product.<sup>4,5</sup>

In a PCR technique primer-dimer formation is an undesirable situation that leads to the inhibition of target DNA amplification.<sup>6</sup> With a poorly designed primer non-specific amplification and/or primer-dimer formation occurs, which can become competitive enough to suppress the desired product formation.<sup>1,7</sup>

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In order to clone PCR products amplified by Taq DNA polymerase enzyme, T vectors that contain complementary 3'-thymidine overhangs are widely used.<sup>3,8-10</sup> Taq DNA polymerase has terminal transferase activity that preferentially adds adenine to the 3' ends of PCR products. PCR products with single 3' adenylate extension can be cloned into a vector, containing complementary 3' thymidine overhangs by using TA cloning.<sup>8,9</sup>

In the present study, we used a simple alternative method to synthesize and clone small genes without using cells as original gene sources. Here, *Bacillus mojavensis* RO-B-2 strain, an extracellular signaling peptide ComX pheromone gene,<sup>11-16</sup> is cloned by using pGEMT-Easy vector for TA cloning, which is one of the most convenient methods for cloning PCR products. *Bacillus mojavensis* RO-B-2 strain ComX pheromone gene has a post-translational modification of geranylation on the tryptophan residue<sup>17,18</sup> and stimulates natural genetic competence controlled by quorum sensing in *Bacillus* spp. ComX pheromone is obtained by amplification of 2 complementary synthetic primers without using *Bacillus mojavensis* chromosomal DNA as the original gene source.

## Experimental

### Materials

Primers were obtained from Tib MolBiol Syntheselabor, Berlin, Germany. pGEMT-Easy vector, LigaFast<sup>TM</sup> Rapid DNA Ligation System and Taq DNA polymerase enzyme were obtained from Promega Corporation, Madison, WI, USA. High Pure PCR Product Purification Kit was obtained from Roche Applied Science, Mannheim, Germany. Microfilterfuge tubes were obtained from Rainin Instrument Co., Emeryville, CA, USA. Nusieve GTG Agarose was obtained from FMC Bioproducts, Rockland, ME, USA. Molecular weight markers 100 bp DNA Ladder and 100 bp DNA Ladder Plus were obtained from MBI Fermentas, Germany;  $\phi$ X174/Hae III Digest was obtained from Sigma Chemical Company, USA. Dio-Transquick<sup>®</sup> DNA Transformation Kit was obtained from Diomed A.Ş., İstanbul, Turkey (Patent No: TR 1999 01199 B).

### Synthesis of *Bacillus mojavensis* ComX Pheromone Gene

Desired PCR products for the 11 amino acid peptide of *Bacillus mojavensis* RO-B-2 strain ComX pheromone (GLQIYTNWVPS; nucleotide sequence: 5'- GGG CTG CAA ATT TAT ACT AAT GGT AAT TGG GTT CCT TCG ) were designed and amplified by Taq DNA polymerase enzyme by using 2 complementary primers CXB2 Forward: 5'- TAA **AGC TTA** TCG AAG GTC GCG GGC TGC AAA TTT ATA CTA ATG GTA A and CXB2 Reverse: 5'- **GGA TCC** TAC GAA GGA ACC CAA TTA CCA TTA GT without any template DNA for the formation of primer-dimers. CXB2 Forward primer has Hind III and CXB2 Reverse primer has BamHI restriction enzyme site, shown in black in the nucleotide sequence. The GenBank accession number of the *Bacillus mojavensis* RO-B-2 strain ComX sequence used in this study is AY003905.<sup>14</sup>The PCR reaction was carried out in a total volume of 50  $\mu$ L containing 5  $\mu$ L of each primer (100 pmol), 5  $\mu$ L of Mg<sup>++</sup> (25 mM), 4  $\mu$ L of deoxyribonucleotide triphosphate mix (2.5 mM each), 10  $\times$  amplification buffer, and 0.25  $\mu$ L of Taq DNA polymerase enzyme. Fifty cycles were performed, with each cycle consisting of the following steps: 94 °C for 20 s, 50 °C for 20 s, and 72 °C for 20 s. After amplification, agarose gel electrophoresis was performed and 67 bp amplification product bands were identified.

## Purification of PCR Products

Prior to cloning studies, PCR products were purified either by using a high pure PCR product purification kit or by extracting from agarose gel by centrifugation. High pure PCR product purification was done with 100  $\mu\text{L}$  of PCR product according to the instruction manual. To purify by centrifugation, amplified product bands were cut from the agarose gel, slices were placed in a sterile 1.5 mL microcentrifuge tube, which was incubated for 1 h at room temperature with 1 mL of Tris-EDTA buffer (pH 8.0) to remove the ethidium bromide. After incubation, gel slices were placed in a microfilterfuge tube with 10,000 NMWL cut off and centrifuged at 14,000 rpm for 2 h. Flow-through solution was collected and run on agarose gel to check the presence and integrity of purified PCR products.

## Cloning of *Bacillus mojavensis* ComX Pheromone Gene

### Ligation of Vector and PCR Fragments

Taq polymerase amplified 67 bp ComX PCR products were ligated by T4 DNA ligase into pGEMT-Easy vector. The LigaFast<sup>TM</sup> Rapid DNA Ligation System was used for ligation of vector and PCR products. The reaction was set up by preparing standard and control reaction tubes. Standards contained either purified PCR product ComX inserts or unpurified PCR products. Ligation reaction mixture was incubated for 5 min at room temperature prior to transformation.

### Transformation of the Ligation Reaction Mixture

Ligation reaction mixtures were transformed into *Escherichia coli* JM 109 and XL-1 Blue cells and clones were determined by ampicillin selection. Then 200  $\mu\text{L}$  of JM 109 and XL-1 Blue cells were transformed by using a Dio-Transquick<sup>®</sup> DNA Transformation Kit; 200  $\mu\text{L}$  of the cells were spread over the Luria-Bertani agar containing ampicillin. After overnight incubation at 37 °C, transformed colonies were counted and the presence of plasmids was checked by miniprep plasmid purification.

### Selection of the Clones Containing Inserts Positioned in the Right Orientation

Since clones that have inserts in the frame with a LacZ promoter site are important for the synthesis of fusion proteins the orientation of the ligated PCR fragments was determined. Three methods, namely amplification, gel electrophoresis of restriction enzyme fragments, and sequence analysis, were used for this purpose.

In the amplification method, recombinant plasmids were isolated from the clones and PCR analysis was performed by using 3 primers specific for different sites on the plasmid (Pg-rev: 5'- AGG AAA CAG CTA TGA C; Pg-for: 5'- TTT TCC CAG TCA CGA C; Pg-for2: 5'- CTC ACT ATA GGG CGA ATT GG). Experiments were performed according to the design given in the Table by randomly selected clones. Amplification occurred by CXB2F and Pg-rev primers if inserts were in the wrong orientation and by CXB2F and Pg-for primers if inserts were in the right orientation. Pg-for and Pg-rev primers were used to indicate the presence of inserts in the clones.

Amplified clones by Pg-for2 and Pg-rev primers (Clone 1, Clone 5, and Clone 136) were digested with Hind III restriction enzyme and fragments were run on 2% (w/v) agarose gel. 72 bp + 197 bp fragments would be seen for the wrong orientation and 134 bp + 135 bp fragments would be seen for the right orientation.

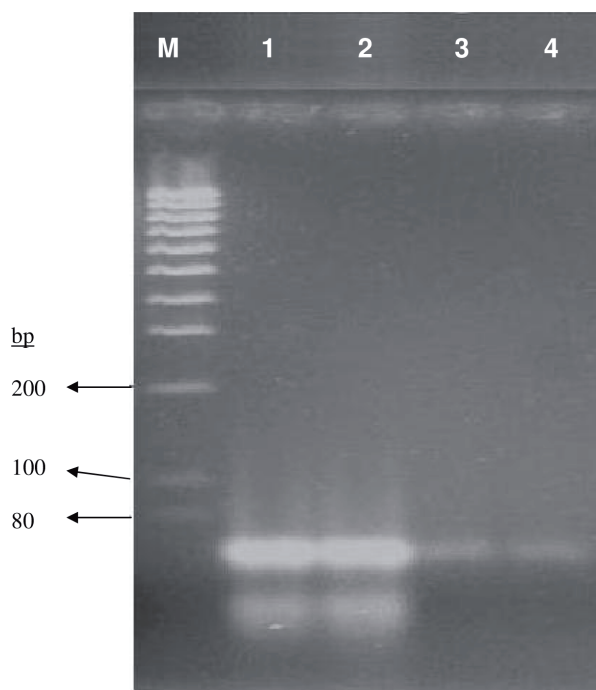
Insert orientations in Clone 68 and Clone 136 were confirmed by DNA sequencing.

**Table.** Experimental design to determine the insert orientation.

Experiment	I	II		III	IV
Primer 1	CXB2F	CXB2F		Pg-for	Pg-for2
Primer 2	Pg-rev	Pg-for		Pg-rev	Pg-rev
Template DNAs	Clone 1 Clone 18 Clone 29 Clone 105 Clone 136	Clone 1 Clone 18 Clone 29 Clone 105 Clone 136	Clone 5 Clone 17 Clone 68 Clone 83 Clone 134	Clone 5 Clone 68 Clone 134 Clone 136	Clone 1 Clone 5 Clone 136
In the right orientation	Amplification (-)	Amplification (+) (168 bp)		Amplification (+) (319 bp)	Amplification (+) (269 bp)
In the wrong orientation	Amplification (+) (200 bp)	Amplification (-)		Amplification (+) 319 bp	Amplification (+) 269 bp

## Results and Discussion

*Bacillus mojavensis* RO-B-2 strain ComX pheromone gene was generated by using novel primer-dimer formation with the amplification of 2 synthetic primers complementary to their 3' ends and amplified PCR products were purified prior to cloning studies. Agarose gel images of 67 bp ComX PCR product and purified ComX PCR products are seen in Figure 1. ComX PCR products could be purified by using either by a

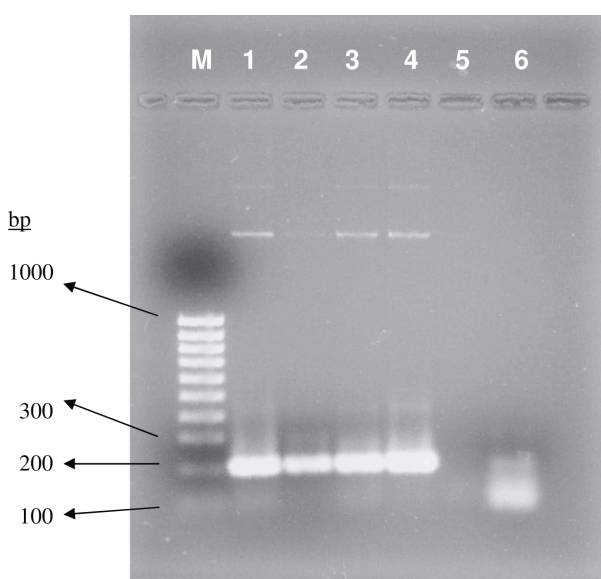


**Figure 1.** Gel (3% w/v Nusieve agarose + 1% w/v agarose) electrophoresis photograph of ComX PCR product. M: DNA Molecular weight marker (100 bp DNA Ladder), 1: ComX PCR product, 2: ComX PCR product unpurified, 3: ComX PCR product purified using kit, 4: ComX PCR product purified using centrifugation.

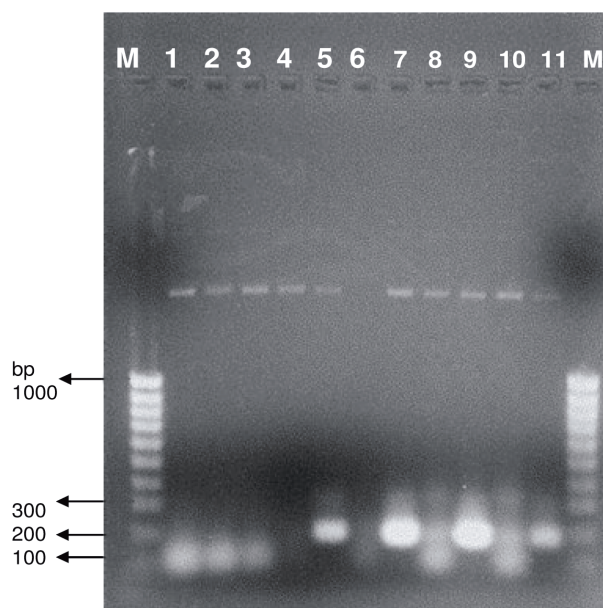
gel purification kit or by centrifugation. Since it is difficult to sediment DNA, during purification using a gel extraction kit when DNA size is small, we developed a centrifugation method as an alternative for purification of small size DNA prior to cloning.

Amplified clones by CXB2F and pg-rev primers are seen in Figure 2 (Lanes 1-4). ComX insert is in the right orientation in Clone 136 (Lane 5).

In Figure 3, amplification occurred by CXB2F and pg-for primers. The clones that have inserts in the right orientation are clone 136, Clone 5, Clone 68, and Clone 134 (Lane 5, Lane 7, Lane 9, and Lane 11, respectively).



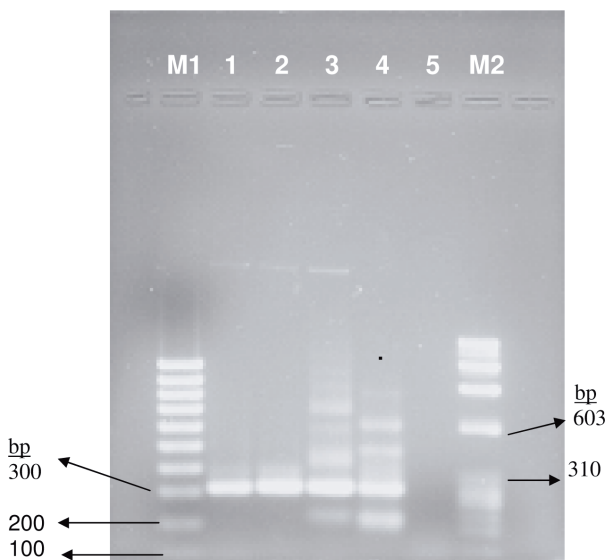
**Figure 2.** Agarose gel (1.7% w/v) electrophoresis photograph of clones amplified by CXB2F + pg-rev primers. M: DNA Molecular weight marker (100 bp DNA Ladder), 1: Clone 1 (Amplification occurred, wrong orientation), 2: Clone 18 (Amplification occurred, wrong orientation), 3: Clone 29 (Amplification occurred, wrong orientation), 4: Clone 105 (Amplification occurred, wrong orientation), 5: Clone 136 (Amplification did not occur, right orientation), 6: Blank (Control).



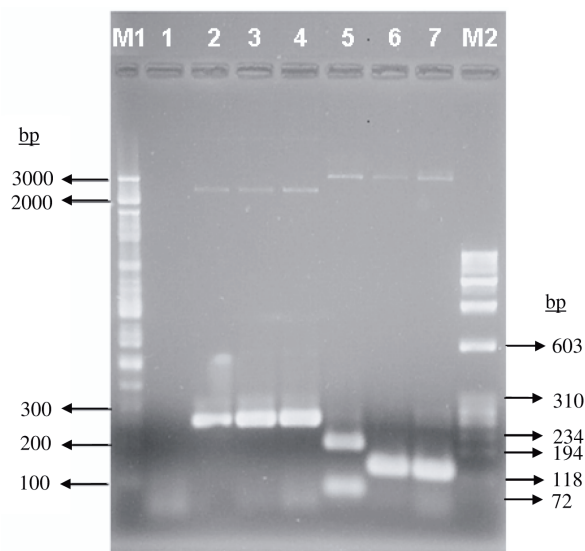
**Figure 3.** Agarose gel (1.7% w/v) electrophoresis photograph of clones amplified by CXB2F + pg-for primers. M: DNA molecular weight marker (100 bp DNA Ladder), 1: Clone 1 (Amplification did not occur, wrong orientation), 2: Clone 18 (Amplification did not occur, wrong orientation), 3: Clone 29 (Amplification did not occur, wrong orientation), 4: Clone 105 (Amplification did not occur, wrong orientation), 5: Clone 136 (Amplification occurred, right orientation), 6: Blank (Control), 7: Clone 5 (Amplification occurred, right orientation), 8: Clone 17 (Amplification did not occur, wrong orientation), 9: Clone 68 (Amplification occurred, right orientation), 10: Clone 83 (Amplification did not occur, wrong orientation), 11: Clone 134 (Amplification occurred, right orientation).

Presence of 319 bp fragments after amplification of the clones by pg-rev and pg-for primers indicated that clone 5, Clone 68, Clone 134, and Clone 136 included ComX insert (Figure 4).

After amplification of Clone 1, Clone 5, and Clone 136 by pg-rev and pg-for2 primers, 269 bp amplification products were digested by Hind III restriction enzyme. Restriction enzyme analysis showed that the expected 72 bp and 197 bp fragments were obtained from Clone 1, which had an insert in the wrong orientation (Figure 5, Lane 5). 134 bp and 135 bp fragments yielded by Clone 5 and Clone 136 had inserts in the right orientation (Figure 5, Lane 6 and Lane 7; fragment sizes are similar).



**Figure 4.** Agarose gel (1.7% w/v) electrophoresis photograph of clones amplified by pg-rev + pg-for primers. M1: DNA molecular weight marker (100 bp DNA Ladder), 1: Clone 5, 2: Clone 68, 3: Clone 134, 4: Clone 136, 5: Blank (Control), M2: DNA molecular weight marker ( $\phi$ X174/Hae III Digest).



**Figure 5.** Agarose gel (2% w/v) electrophoresis photograph of clones amplified by pg-rev + pg-for2 primers. M1: DNA molecular weight marker (100 bp DNA Ladder Plus), 1: Blank (Control), 2: Clone 1, 3: Clone 5, 4: Clone 136, 5: Clone 1 + Hind III (Wrong orientation: 72 bp + 197 bp fragments), 6: Clone 5 + Hind III (Right orientation: 134 bp + 135 bp fragments), 7: Clone 136 + Hind III (Right orientation: 134 bp + 135 bp fragments), M2: DNA molecular weight marker ( $\phi$ X174/Hae III Digest).

It was confirmed by DNA sequencing that the inserts were in the right orientation in Clone 68 and Clone 136.

These results demonstrate that the ComX insert was in the right orientation in Clone 5, 68, 134, and 136 and in the wrong orientation in Clone 1, 17, 18, 29, 83, and 105.

We tried an alternative method to clone small genes by using the primer-dimer approach with the PCR technique. We recommend this as a practical method that eliminates the need for cells that may be hard to obtain as the gene source and gives the opportunity to easily create experimental genes that do not exist in nature. Small genes with known nucleotide sequences can be designed and cloned to express the desired protein by using 2 synthetic primers complementary at their 3' ends.

By using primer-dimer formation; the collection, extraction, and DNA sample preparation steps from the biological materials can be eliminated, which may be very limiting in some cases.

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