A novel *Bacillus thuringiensis* Cry-like protein from a rare filamentous strain promotes crystal localization within the exosporium

Running title: A novel cry-like gene promotes crystal localization

**Contributing Authors**

1David R. Ammons, 1Antonio Reyna, 3Jose C. Granados, 2Antonio Ventura-Suárez, 2Luz I. Rojas-Avelizapa, 3John D. Short, and 1Joanne N. Rampersad.

**Institutional Affiliation**

1The University of Texas- Pan American, Dept. of Chemistry, Edinburg, TX U.S.A
2Departamento de Microbiología, Escuela Nacional de Ciencias Biológicas del I.P.N, Mexico DF.
3University of Texas Health Science Center at San Antonio-Regional Academic Health Center
1214 W. Schunior, Edinburg, TX 78541
4University of Texas Health Science Center at San Antonio, Dept. of Pharmacology, 7703 Floyd Curl Drive, San Antonio, TX 78229

#Correspondence should be sent to Dr. Joanne Rampersad, The University of Texas- Pan American, Dept. of Chemistry, 1201 W. University Drive Edinburg, TX U.S.A, 78539
E-mail: jrampersad@utpa.edu, Fax: +1 956 665-5006, Phone#: +1 956 665-2097
Abstract

Mutation of a novel cry-like gene (cry256) from Bacillus thuringiensis resulted in a protein crystal, normally located within the spore’s exosporium, to be found predominately outside the exosporium. Gene cry256 codes for a 3-domain Cry-like protein that does not correspond to any of the known Cry protein holotypes.

During sporulation, the bacterium Bacillus thuringiensis (Bt) produces a protein crystal comprised of one or more members of the Cry family of protein toxins. The crystal is normally located outside the exosporium that surrounds the spore (1), however a relatively few strains of Bt have been shown to localize their crystals within the exosporium (2, 3). With the aim of understanding how some crystals are localized within the exosporium, Debro et al., showed that in Bt ssp. finitimus, the genes necessary for localizing crystals within the exosporium where located on two plasmids (4). Zhu et al. later identified these genes (5), and showed that not all Bt used these genes to localize crystals within their exosporium. Building on this previous work, we undertook a study to identify genes responsible for crystal localization in a novel filamentous strain of Bt called Bt2-56 (Fig. 1A/A’), (6).

Inactivation of gene cry256 causes loss of crystals from the exosporium. Genes in Bt2-56 were mutated by random transposon insertion using plasmid pMarA (7). Mutants with crystals located outside the exosporium were identified by viewing stained preparations of sporulated mutants (8). Approximately 84% of the crystals from a mutant called M1-11 were observed outside the exosporium (Fig. 1B/B’).
Inverse PCR using primers left-tn1 and oIPCR2a (Table 1), was used to amplify M1-11 genomic DNA flanking the transposable element, indicating that transposons had integrated into both a histidine kinase gene and a cry-like gene we call cry256. The DNA sequence of both strands of the entire cry256 gene and its immediate flanking regions was then determined by ‘walking’ the gene using primers designed from previously sequenced DNA regions (GenBank # JQ670887). The DNA sequence of cry256 was also determined from Bt2-56 (the un-mutated parent strain of M1-11), using an Illumina GAIIx sequencer at the University of Houston’s IMD Sequencing Center. The 36 bp sequencing reads were assembled using Velvet (9) and visualized with the software program Tablet (10). DNA translations were performed using a web-based DNA-to-protein tool (11). The transposon was found to have inserted into a region that separated the last 49 amino acids of the protein encoded by cry256, from the rest of protein (Supplemental file 1, insertion site highlighted in red at position 1528). Aside from the transposon, the DNA sequence of cry256 in mutant M1-11 was identical in sequence obtained for the wild type cry256 gene in Bt2-56.

Since the cause and effect relationship of mutating cry256 and crystal localization in M1-11 was complicated by the insertion of a second transposon into a histidine kinase gene, we confirmed the role of cry256 in crystal localization by independently inactivating cry256 via homologous recombination. Primers 256e-F and 256e-R were used to amplify a sub-region of the cry256 that was cloned into a unique EcoRI site in pKO1 (a derivative of pMarA that lacks the transposon), creating pKO1-256. The cry256 gene in Bt2-56 was inactivated by inserting pKO1-256 into cry256 via a single crossover event, creating mutant 15-54 (Fig. 2), which had a phenotype similar to mutant M1-11 (Fig. 1C/C’). Disruption of cry256 in mutant 15-54 was
confirmed by PCR using primers m1-11rc6 and Ltest, which amplify a specific sized fragment only if pK01-256 crosses into cry256 (Fig. 2).

**Gene cry256 codes for a Cry-like protein.** Gene cry256 codes for P256, a 1098 amino acid protein of approximately 127 kD, and differs from genes involved with crystal localization in Bt ssp. finitimus (5). NCBI’s homology search tool blastp (12), indicated that the *B. thuringiensis* Cry protein Cry21Ba1 (GenBank # BAC06484), a nematicidal protein, had the highest amino acid homology to P256, followed by three other Cry proteins also associated with nematicidal activity (Cry5, Cry12 and Cry14). An amino acid comparison of P256 with these other nematicidal genes (Supplemental file 1), revealed a strongly conserved carboxyl region corresponding to the Cry protein crystallization domain, the highly divergent “linker” region commonly located between Domain III of a 3-domain toxin and the crystallization domain, noticeable conservation of the 5 amino acid blocks that broadly show conservation among Cry proteins exhibiting the 3-domain structure (13, 14), other conserved protein blocks showing conservation among nematicidal proteins and P256 (blocks 7-9), and a highly truncated Domain II region. Domain II of P256 is 83 amino acids in length while those of the other nematicidal proteins are 189, 213, 215, 204 and 205 amino acids in length.

**Cry256 is probably not found in the exosporium-localized crystal.** Loss of cry256 did not result in an observable loss in crystal size or shape (data not shown). Furthermore, SDS PAGE analysis of gradient purified crystals from the un-mutated strain Bt2-56 showed only one band in the 100 kD range. Automated Edman-based N-terminal protein sequencing of this protein (Protein Analysis Facility, Institute for Cellular and Molecular Biology, University of Texas, Austin, TX, USA) showed that its amino terminal sequence (MVQLDDLPLLNPYNNVLANp), differs from that calculated for cry256 (MDNSSNSSISNNVLITP).
Cry256 appears to promote, but is not required, for crystal localization. Both mutants M1-11 and 15-54 showed that even with a disrupted cry256 gene, some bacteria were still able to localize their crystals within the exosporium (Fig. 1 B & C). This result indicates that cry256 may act to increase the efficiency of the cell’s crystal localization machinery, as opposed to being an indispensible component. We are presently working to determine the identity of other genes involved with crystal localization in Bt2-56, and to determine how P256 promotes crystal localization.

Acknowledgements

AVS was supported by a scholarship from CONACYT. JNR thanks the Faculty Research Council at the University of Texas- Pan American for financial support of work described in this Report and Welch Foundation Grant BG-0017. We would like to acknowledge the technical assistance of Dr. Klaus Linse, University of Texas, Austin, in performing N-terminal protein sequencing.

References


Figures

Fig. 1: Bt strains Bt2-56 A) stained coommasie blue, A’) Negative-Stained, Transmission Electron Micrograph; M1-11 B) stained coommasie blue, B’) Scanning Electron Micrograph; 15-54 C) stained coommasie blue, C’) Scanning Electron Micrograph. Physical structures are: e) exosporium, s) spore, c) crystal, f) filament bundle, and nm) not mutated.

Fig. 2: Inactivation of gene cry256 in Bt2-56 via homologous recombination. (I) Plasmid pKO1-256, (II) gene cry256 in Bt2-56 (III) strain 15-54. Checkered area denotes cry256 DNA; thin black solid line denotes pKO1-256 plasmid backbone; thick black solid line denotes genomic DNA flanking cry256. Region IIIA denotes cry256 5’ flanking region and a partial cry256 gene; region IIB: plasmid pKO1-256 backbone, and region IIC: the entire cry256 gene along with 16 bp of its 5’ flanking sequence and 3’ prime flanking sequence. (IV) PCR confirmation of mutant 15-54 using primers 1 (m1-11rc6) & 2 (Ltest) with different templates. Lane 1: DNA markers (* denotes 1kb and 1.5kb bands), lane 2: Bt2-56 genomic DNA, lane 3: M1-11 genomic DNA, lane 4: 15-54 genomic DNA, lane 5: a equal molar mix of Bt2-56 genomic DNA and plasmid pKO1-256. Only 15-54 template (lane 4), produced the 1055 bp amplicon indicative of recombination (III). Lanes 6 through 8 contained the same templates as lanes 1 through 4 respectively, but were amplified with primers 256e-F and 256e-R, which amplified a 994 bp region of cry256, and indicated that all the templates were amplifiable.
Table 1: Primers used in this study

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
<th>Use</th>
</tr>
</thead>
<tbody>
<tr>
<td>left-tn1</td>
<td>cgcaactgctccatactctgat</td>
<td>Transposon-specific primer</td>
</tr>
<tr>
<td>olPCR2a</td>
<td>gggatctatttgaaggttgg</td>
<td>Transposon-specific primer</td>
</tr>
<tr>
<td>left-tn2</td>
<td>agttagcttagataggggt</td>
<td>Transposon-specific primer</td>
</tr>
<tr>
<td>olPCR3 (10)</td>
<td></td>
<td>Transposon-specific primer</td>
</tr>
<tr>
<td>*256e-F</td>
<td>aaaagaattccaatgaggatagtctg</td>
<td>Cry256 amplification</td>
</tr>
<tr>
<td>*256e-R</td>
<td>tegagatctaatccactggcttgctctctcg</td>
<td>Cry256 amplification</td>
</tr>
<tr>
<td>m1-11rc6</td>
<td>aaaaagctatgcagctatgtt</td>
<td>Confirm cry256 disruption</td>
</tr>
<tr>
<td>Ltest</td>
<td>acgctttctatcgaccttcagggagc</td>
<td>Confirm cry256 disruption</td>
</tr>
</tbody>
</table>

* Underlined sequence identifies the EcoRI recognition sequence in this primer

Supplemental files

Supplemental file 1: Alignment of P256 amino acid sequence with other nematicidal Cry proteins.
Table 1: Primers used in this study

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
<th>Use</th>
</tr>
</thead>
<tbody>
<tr>
<td>left-t(n)1</td>
<td>e(g)caactgc(a)ctcatac(t)gat</td>
<td>Transposon-specific primer</td>
</tr>
<tr>
<td>o(l)PCR2a</td>
<td>g(g)gaactcattgaag(g)t(g)g</td>
<td>Transposon-specific primer</td>
</tr>
<tr>
<td>left-t(n)2</td>
<td>agt(t)c(t)gtagatatgg</td>
<td>Transposon-specific primer</td>
</tr>
<tr>
<td>o(l)PCR3 (10)</td>
<td></td>
<td>Transposon-specific primer</td>
</tr>
<tr>
<td>*256e-F</td>
<td>aaa(a)agaattcaat(c)caat(c)gag(g)gattatatgt(g)g</td>
<td>Cry256 amplification</td>
</tr>
<tr>
<td>*256e-R</td>
<td>t(g)g(a)g(a)aatc(t)aat(c)ccag(c)g(t)t(t)tc(t)gc</td>
<td>Cry256 amplification</td>
</tr>
<tr>
<td>m1-11rc6</td>
<td>aaaa(a)ag(t)c(c)cat(a)ggag(a)catt(g)t</td>
<td>Confirm cry256 disruption</td>
</tr>
<tr>
<td>Ltest</td>
<td>ag(c)c(t)ttc(t)c(t)atc(c)g(a)c(t)t(t)g(a)</td>
<td>Confirm cry256 disruption</td>
</tr>
</tbody>
</table>

* Underlined sequence identifies the *Eco*RI recognition sequence in this primer