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High instability of a nematicidal Cry toxin plasmid in *Bacillus thuringiensis*

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ABSTRACT

In bacterial pathogens, virulence factors are often carried on plasmids and other mobile genetic elements, and as such, plasmid evolution is central in understanding pathogenicity. *Bacillus thuringiensis* is an invertebrate pathogen that uses plasmid-encoded crystal (Cry) toxins to establish infections inside the host. Our study aimed to quantify stability of two Cry toxin-encoding plasmids, BTI_23p and BTI_16p, under standard laboratory culturing conditions. These two plasmids are part of the genome of the *B. thuringiensis* strain MYBT18679, which is of particular interest because of its high pathogenicity towards nematodes. One of the plasmids, BTI_23p, was found to be highly unstable, with substantial loss occurring within a single growth cycle. Nevertheless, longer term experimental evolution in the absence of a host revealed maintenance of the plasmid at low levels in the bacterial populations. BTI_23p encodes two nematicidal Cry toxins, Cry21Aa2 and Cry14Aa1. Consistent with previous findings, loss of the plasmid abolished pathogenicity towards the nematode *Caenorhabditis elegans*, which could be rescued by addition of Cry21Aa2-expressing *Escherichia coli*. These results implicate BTI_23p as a plasmid that is required for successful infection, yet unstable when present at high frequency in the population, consistent with the role of Cry toxins as public goods.

1. Introduction

Alongside other mobile elements, plasmids play a central role in pathogen genome evolution, as they often encode factors involved in host interactions and/or survival inside a host. These include virulence factors, such as toxins or host immune resistance factors, as well as antibiotic resistance determinants (Carattoli, 2013; Guan et al., 2013; Johnson and Nolan, 2009; Li et al., 2013; Rasko et al., 2005; Trosky et al., 2008). Because the corresponding genes can confer large fitness benefits, selection often gives rise to highly dynamic plasmid populations, both at the level of variability in plasmid composition in closely related strains (Garcia et al., 2011; Grad et al., 2013; Kitchel et al., 2009; Vogel et al., 2012), as well as rapidly evolving gene content within individual plasmids (Grad et al., 2013; Vogel et al., 2012).

In addition to the capacity for horizontal transfer, plasmids have evolved various mechanisms for ensuring that they are stably transmitted to daughter cells. These include negative feedback systems that minimise fluctuations in copy number (del Solar et al., 1998), active mechanisms for partitioning plasmid copies at cell division (Gerdes et al., 2010; Salje et al., 2010), and toxin-antitoxin systems that effectively kill progeny cells lacking the plasmid (Hayes, 2003; Schuster and Bertram, 2013). Nevertheless, vertical plasmid transmission is not always completely stable, and the level of instability can be influenced by variation in any of the above factors, as well as fitness differences between plasmid-carrying and plasmid-free strains (Lau et al., 2013).

Bacillus thuringiensis is an invertebrate pathogen that infects a range of insect and nematode species, including *Caenorhabditis elegans*. Following oral uptake of pathogenic spores, infection initiates in the host gut. This process is mediated by the activity of pore-forming crystal (Cry) toxins, which are produced in conjunction with sporulation. The host range of a particular *B. thuringiensis* strain is highly specific and largely dependent on the particular Cry toxins produced (de Maagd et al., 2001; Griffitts and Aroian,

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2005). In this way, the Cry toxins are highly variable between strains, with more than 70 primary ranks (<45% amino acid identity) that have so far been identified (Crickmore et al., 2014, 1998). The toxins are encoded on plasmids, facilitating horizontal transfer. While the toxin proteins themselves are able to cause lethality in various invertebrates, as evidenced by their use in transgenic crops or as a bioinsecticide, *B. thuringiensis* has also been shown to possess a number of additional virulence factors (Bouillaut et al., 2005; Fedhila et al., 2003, 2002; Kho et al., 2011; Luo et al., 2013; Salamitou et al., 2000).

B. thuringiensis strain MYBT18679 is a nematode pathogen that expresses high virulence towards the model nematode *C. elegans* (Schulte et al., 2010; Wang et al., 2012). It is thus of particular interest as a biopesticide against nematodes and for our understanding of the molecular mechanisms of pathogenicity. Based on the whole genome sequence of this strain, two plasmids were identified to harbour genes showing high sequence similarity to previously identified Cry toxins. The size of the plasmids was found to be 22,591 bp for BTI_23p and 15,831 bp for BTI_16p (Jacqueline Hollensteiner, Heiko Liesegang, personal communication). BTI_23p contains genes encoding Cry14Aa1 and Cry21Aa2, both nematicidal toxins (Wei et al., 2003). BTI_16p contains genes encoding Cry34Aa4, Cry35Aa4, and Cry38Aa1. Cry34Aa4 and Cry35Aa4 function together as a binary toxin that has been shown to have insecticidal activity (Ellis et al., 2002; Schnepf et al., 2005), and Cry38Aa1, while sharing sequence similarity with characterised toxins, has so far not been shown to demonstrate toxic activity (Baum et al., 2004).

Previously, experimental co-evolution of *B. thuringiensis* with a *C. elegans* host revealed a loss of Cry toxin genes in some evolved clones (Schulte et al., 2010, 2013). Nevertheless, Masri et al. (2015) demonstrated that BTI_23p was selectively favoured during co-evolution of MYBT18679 with a nematode host. In this study, we specifically sought to investigate the stability of the two Cry toxin plasmids, BTI_23p and BTI_16p, in *B. thuringiensis* MYBT18679 when grown outside of a host, as well as the functional consequences of plasmid loss on *B. thuringiensis* nematicidal pathogenicity.

2. Materials and methods

2.1. Short term plasmid stability assay

MYBT18679 cells from a frozen glycerol stock were grown on 9 cm nematode growth medium (NGM) plates (Stiernagle, 2006), after diluting in appropriate volumes of liquid NGM (i.e. without agar). Plates were incubated for 3 days at 19 °C to obtain single colonies, and colony PCRs were performed to identify clones positive for both *cry21Aa2* and *cry35Aa4*.

The remaining cell suspensions for 15 positive clones were diluted in appropriate volumes of liquid NGM and spread onto 9 cm NGM plates, then incubated at 19 °C for 3 days to obtain single colonies. Colony PCRs were then performed for *cry21Aa2* and *cry35Aa4* for 45 colonies derived from each original clone to measure the stability of BTI_23p and BTI_16p, under the assumption that if the founding cell contained the plasmid, then enough copies would be present to give a PCR product. While this assumption may not be strictly true if plasmid loss occurs early in the growth of the second colony, any resulting inaccuracies in measurements would have essentially no bearing on the interpretation of our results. The remaining cell suspensions were stored at 4 °C for 3 days after which some of them were used for survival assays (see Section 2.3).

To determine whether loss of *cry21Aa2* corresponds with loss of the entire BTI_23p plasmid, PCRs were performed with four additional primer pairs spaced around the plasmid: 5′-CTAATAATGCGC GACCTACTG-3′/5′-GTACCAGCTATTGCACAACC-3′ (PCR product spans positions 5433–5931 of BTI_23p), 5′-AGAACGAAACCCAA GAATGCC-3′/5′-TATAAACCGTGGGGGTTCGTC-3′ (positions 14541–14909), 5′-CAATATGTTCCGCGTCATGTG-3′/5′-AAGAAGGCTGGTGT GTACTCG-3′ (positions 10563–10862), and 5′-CGTCGTAATGAGCTC GAAGAG-3′/5′-ACCGAAACGAACTAGTAGGGG-3′ (positions 59–388). Template for these reactions consisted of the samples used for survival assays, after the additional growth step described below.

2.2. Experimental evolution for long term plasmid stability assay

Single clones positive for both *cry21Aa2* and *cry35Aa4* were identified as described above. The remaining cell suspensions after PCR were each spread onto a single 9 cm NGM plate and incubated at 19 °C for 7 days to obtain a bacterial lawn. Plates were washed with 5 ml Phosphate Buffered Saline (PBS) and samples were diluted so that 10^{-4} of the material washed off was spread onto a new plate. These new plates were incubated at 19 °C for 7 days again, and the process was repeated for a total of 10 weeks. The 7 day cycle was chosen to allow enough time for maximum population density (sporulation) to be reached prior to transfer, so total population size at each transfer step is expected to be relatively constant. Given the dilution factor, the approximate number of generations per week is given by $\log_2(10^4) = 13$. At each transfer step, washed off material was frozen at −20 °C for subsequent analysis.

As experimental evolution in the absence of a selectable marker is potentially prone to contamination, we performed PCRs at regular intervals to confirm that only the MYBT18679 strain could be detected in the evolving populations. To achieve this, we used *codY* primers (Masri et al., 2015) and 16S ribosomal primers 27f/1495r (5′-GAGAGTTTGATCCTGGCTCAG-3′/5′-CTACGGCTACCTTGTTACGA-3′), followed by Sanger sequencing with codY-R or 27f, respectively. The *codY* primers were included to enable detection of other *B. thuringiensis* strains, and the ribosomal primers were included to enable detection of other bacterial species.

2.3. Survival assays

For each clone from the short term plasmid stability assay that gave at least one colony negative for BTI_23p, two colonies were used as starting material for survival assays – one positive and one negative for BTI_23p, resulting in a total of 13 replicates for each treatment, each representing an independent loss of BTI_23p. The entire cell suspensions remaining after colony PCR were each spread onto a single 9 cm NGM plate and incubated at 19 °C for 6 days to obtain a bacterial lawn. Plates were then washed with 3.5 ml PBS. Part of each sample was used directly for the survival assay depicted in Fig. 3, while the remainder of each sample was frozen at −20 °C for use in subsequent assays, including qPCR. Microscopy (Dialux 20 microscope, Leitz) was used to confirm that each sample consisted predominantly of spores.

Cell concentration was determined using an established calibration curve by measuring absorbance at 650 nm. The concentration of each sample was then adjusted to 2×10^9 cells ml^{-1} by diluting with an appropriate volume of PBS. Each sample was then mixed 1:10 with *Escherichia coli* grown overnight in Luria–Bertani (LB) broth at 37 °C and suspended in PBS with a final OD of 5. 100 µl of the *B. thuringiensis–E. coli* mixture was then pipetted onto the centre of a 6 cm assay plate containing peptone-free nematode growth medium (PFM). The inoculated assay plates were left overnight at 20 °C to dry. On the assay day, synchronised worms at the fourth instar larval stage were washed off the growth NGM plates with PBS solution. 30–40 worms were added to each assay plate by