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INVESTIGATION OF *BACILLUS THURINGIENSIS* PLASMID INSTABILITY AND ITS EFFECT ON THE SYNTHESIS OF CRYSTALS

ABSTRACT

In order to explore plasmid instability in *Bt*, four *Bt* strains belonging to two *Bt* subspecies were cultured at 42°C for 9 days. HD1 and QBT376 belong to subspecies *kurstaki*, while H14 and QBT218 belong to subspecies *israelensis*. Results showed 100% crystal loss for H14 and QBT218, while 76% and 90% crystal loss for HD1 and QBT376, respectively, showing that *cry*-carrying plasmids are more stable in Bt *kurs*. than in Bt *isr*.. HD1, QBT376, and QBT218 cured clones showed significant protease activity compared to their non-cured counterparts. Microscopic observation revealed the delay of sporulation for high number of HD1 and QBT376 cry- clones, while the absence of spores in several H14 and QBT218 cry- clones. Spo$^-$cry$^-$ clones of *Bti* strains had irregular elongated cell shape. Kinetics/day of plasmid curing for H14 and QBT218 showed H14 to have higher pBtoxis plasmid stability. The number of vegetative cells in *Bti* strains increased with the increase of curing period. As an attempt to create hybrid *Bt* strains, *cry1Aa* gene was extracted to transform cured and non-cured strains.

OBJECTIVES

1- Study the effect of the growth at high temperature on plasmid stability in *Bacillus thuringiensis*.

2- Study of the effect of the plasmid loss on the *Bacillus thuringiensis* crystal synthesis.

LITERATURE REVIEW

Bt **is a rod-shaped, facultative aerobic, motile Gram$^+$, spore-forming bacterium (Beena *et al.*, 2019). It is the most used microbial pesticide and bio-insecticide due to its production of protein crystals responsible for its insecticidal action. It is considered among the safest and least environmentally damaging insecticidal products available (Raymond & Federici, 2017). Over the last decade, several tests showed instability of Bt formulations due to high or low temperatures (Farghal & Darwish, 1988; Moustafa et al., 2018). In addition, many cases of insect resistance to *Bt* were recorded (Kain et al., 2015; Tabashnik & Carrière, 2015). Therefore, the need to evaluate plasmid instability of *Bt* before commercial production, in addition to finding novel strains, producing stable formulations, and creating *Bt* hybrids becomes crucial more than ever.**

Materials & Methods

Bacterial strains and their derivatives

Two Qatari *Bt* strains, QBT376 & QBT218 that belong to 2 different *Bt* subspecies producing bipyramidal and spherical crystals, respectively were explored. HD1 & H14 were used as reference strains.

Plasmid curing

1. An isolated colony of the four *Bt* strains was inoculated in LB Broth & incubated at 42°C for 24h. Then, 100 µl of the culture was transferred to fresh LB broth. This step was repeated for 8 days.

2. On the 9th day, the culture was serially diluted, spread on LB agar and left overnight at 30°C.

3. Then,100 single isolated colonies from each strain were transferred to T3 agar and milk-agar plates.

Exploration of crystal formation

Light microscope (100x magnification) was used to study the crystal forms.

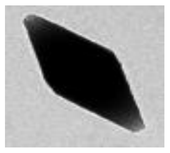
Exploration of *cry* gene content by PCR

Lep2A/2B & Dip2A/2B primers were used in PCR experiments to explore *cry*-type genes.

Extraction of cry1Aa gene

Transformation of cured and non-cured *Bt* clones using Gene Pulser XcellTM Electroporation System

RESULTS and DISCUSSION



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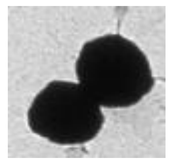
***Bacillus thuringiensis* crystals**
Kurst.* vs. *Isr.



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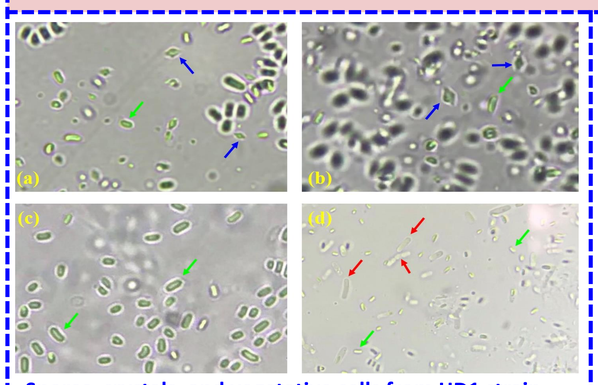
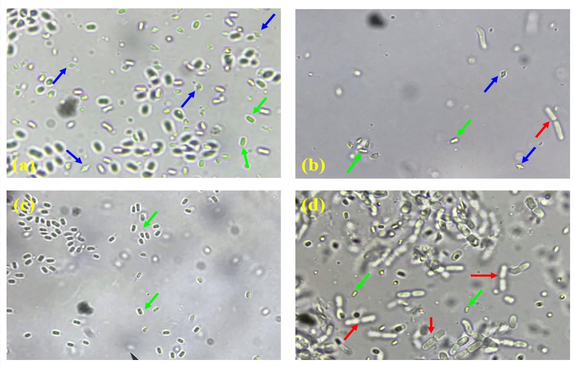
1. Plasmid instability affects *Bacillus thuringiensis* crystal formation, sporulation, and cell shape after 9 days growth at 42°C



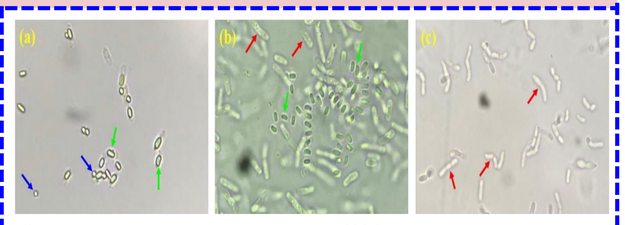
Spores, crystals, and vegetative cells from HD1 strain
(a): Spores and crystals of non-cured HD1; (b): spores and crystals of cured HD1; (c): spores only of cured HD1; (d): spores and vegetative cells of cured HD1].
*Green arrows indicate spores; *Blue arrows indicate crystals; *Red arrows indicate vegetative cells



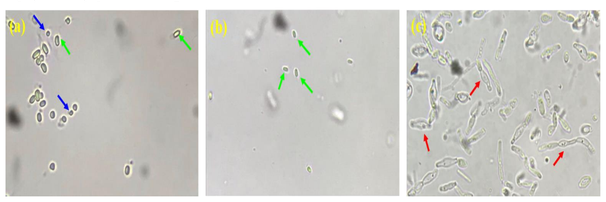
Spores, crystals, and vegetative cells from QBT376 strain
(a): spores and crystals of non-cured QBT376; (b): spores and crystals of cured QBT376; (c): spores only of cured QBT376; (d): spores and vegetative cells of cured QBT376].

***Btk*. Plasmid instability was recorded as the absence of bipyramidal and cuboidal crystals under light microscope**

% Plasmid instability of the plasmids carrying *cry* genes of *Btk*		
Strain	HD1 (control)	QBT376
% instability	76%	90%



Spores and vegetative cells from H14 strain
(a): Spores and spherical crystals of non-cured H14; (b): spores and vegetative cells of cured H14 that have inclusion bodies inside them; (c): cured vegetative cells (spo$^-$cry$^-$) of H14].

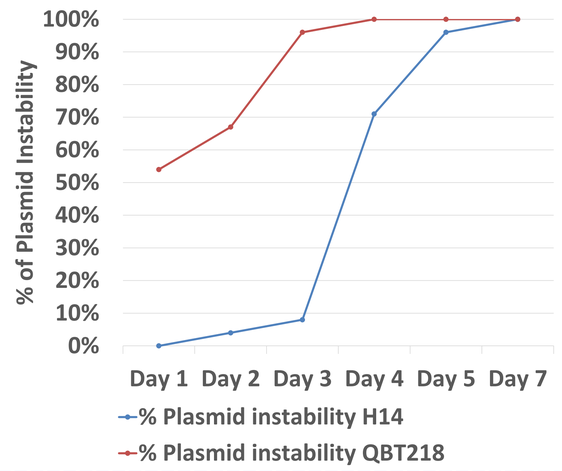


Spores and vegetative cells from QBT218 strain
(a): spores and spherical crystals of non-cured QBT218; (b): spores of cured QBT218; (c): cured vegetative cells (spo$^-$cry$^-$) of QBT218 with abnormal shape

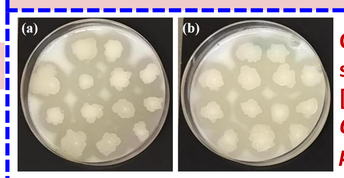
100% plasmid instability was observed in *Bti* strains

2. Kinetics of *Bti* loss of crystal formation, sporulation, and plasmid instability during 7 days growth at 42°C

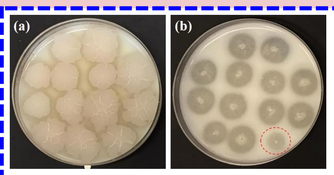
In *Bti*, pBtoxis plasmid is more unstable is QBT218 than is in the reference strain H14



3. Plasmid instability affects the production of proteases in *Bt*



Cured clones of *Btk* strains on milk agar dish [a: HD1, b: QBT376] *Overproducing proteases*



Cured colonies of *Bti* strains [a: H14, b: QBT218]. *Overproducing proteases*

Depending on the *Bt* sub-species/strain, plasmid stability affects positively or negatively the secretion of proteases. Proteases are highly secreted in HD1, QBT376, and QBT218.

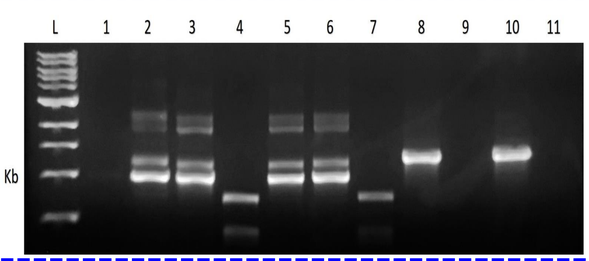
Distance between the edge of the bacterial colony (mm)		
Strains	Non-cured (control)	Cured (Mean± SD)
HD1	3.00 ± 0.00	4.60 ± 0.94
QBT376	4.00 ± 0.00	4.11 ± 1.19

Estimation of the Protease activity of *Btk* strains after 9 days of plasmid curing at 42°C

Distance between the edge of the bacterial colony (mm)		
Strains	Non-cured (control)	Cured (Mean± SD)
H14	6.00 ± 0.00	3.85 ± 1.41
QBT218	1.75 ± 0.00	4.85 ± 1.60

Estimation of the Protease activity of *Bti* strains after 9 days of plasmid curing at 42°C

4. During plasmid loss, some mutants lose *cry* gene carrying plasmids, others still retain the plasmid and its *cry* gene

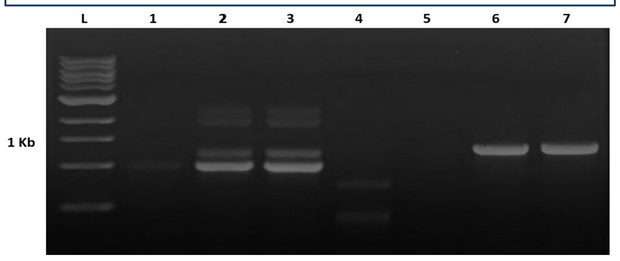


(PCR) of *cry* genes from non-cured and cured *Bt* strains [L: 1 Kb DNA ladder, 1: negative control, 2: Reference strain HD1 *Btk* non-cured, 3: HD1 *Btk* cured (cry+)*, 4: HD1 *Btk* cured (cry-)*, 5: QBT376 *Btk* non-cured, 6: QBT376 *Btk* cured (cry+), 7: QBT376 *Btk* cured (cry-), 8: Reference strain H14 *Bti* non-cured, 9: H14 *Bti* cured (cry-), 10: QBT218 *Bti* non-cured, 11: QBT218 *Bti* cured (cry-)]. Lep2A/2B primers amplified Lanes 2-7. Dip2A/2B primers amplified Lanes 8-11.

5. Transfer of *cry* genes by electroporation to plasmid cured *Bacillus thuringiensis* mutants

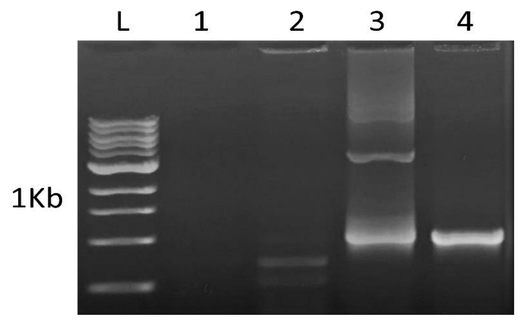
A proposed solution to fight insect resistance to *Bt* is the creation of hybrid *Bt* strains. We attempted to do so using plasmid pHT:*cry1Aa* via electroporation.

PCR confirmation of the clones used in transformation



PCR reults of clones used in transformation [L: 1 Kb DNA ladder, 1: negative control of HD1, 2: HD1 non-cured, 3: HD1 cured (cry+), 4: HD1 cured (cry-), 5:negative control of H14, 6: H14 non-cured, 7: H14 cured (cry+)]. Lanes 1-4 were amplified using primers Lep2A/2B. Lanes 5-7 were amplified using primers Dip2A/2B.

Successful extraction of plasmid pHT:*cry1Aa* from *Bt* and *E.coli*



PCR amplification of *cry1Aa* gene extracted from transformed *Bt* and *Escherichia coli* (*E.coli*) bacteria as a first step to transform cured and non-cured *Bt* strains [L: 1 Kb DNA ladder, 1: negative control, 2: pHTBlue plasmid, 3: *cry1Aa* gene extracted from *E.coli* 4: *cry1Aa* gene extracted from *Bt*. The 0.98 Kb *cry1Aa* gene was amplified using Lep2A/2B primers.

Conclusion

In the present work, we clearly demonstrated that:

1- During cell growth, *Bti* strains lose the *cry* genes carrying plasmid pBtoxis much faster than *Btk*-corresponding plasmid.

2- Among *Btk* strains, QBT376 cry- plasmid is almost as stable as that of HD1 reference strain.

3- Among *Bti* strains, QBT218 pBtoxis plasmid is less stable than the same plasmid in H14 reference strain.

4- The loss of plasmids directly affects other genes expression such as protease coding genes evidenced in the present work.

5- Sporulation genes are not expressed upon exposing *Bt* strains to high temperature.

6- Cell morphology of *Bti* strains is changed upon exposure to high temperature.

These results will be directing us to explore the δ-endotoxins content in the cured cry$^+$ mutants to give conclusive ideas about the effect of plasmid instability on δ-endotoxin synthesis.

Acknowledgments

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