Phenotype sequencing uncovers mutations in candidate genes that mediate tectivirus-resistance in *Bacillus thuringiensis*

Annika Gillis[1], Marc Harper[2], Christopher Lee[2, 3, 4] and Jacques Mahillon[1]

[1] Laboratory of Food and Environmental Microbiology, Université catholique de Louvain, B-1348 Louvain-la-Neuve, Belgium; [2] Institute for Genomics and Proteomics, University of California Los Angeles; [3] Dept. of Chemistry & Biochemistry, University of California Los Angeles; [4] Molecular Biology Institute, University of California Los Angeles, Los Angeles, CA, USA.

Email: annika.gillis@uclouvain.be

Background

Tectiviridae **is one peculiar phage family preying on members of the *Bacillus cereus sensu lato* (*s.l.*) group. For instance, GIL01, Bam35 and GIL16 are tectiviruses infecting *Bacillus thuringiensis*, while AP50 and Wip1 have been reported to only infect *Bacillus anthracis*. Additionally, these phages exhibit a strong similarity to the *B. cereus* linear plasmid pBClin15 (Fig. 1) [1-4].**

Tectiviruses in the *B. cereus* group are capable to reside as temperate phages that do not integrate into the host genome upon infection and remain as autonomous linear plasmids in the cell [1]. Therefore, it is important to understand the selective pressure undergone by the bacteria when facing this type of phages.

The aim of this work was to study phage-resistant phenotypes that are triggered when *B. thuringiensis* is challenged by the presence of lytic variants of phages GIL01 and GIL16.

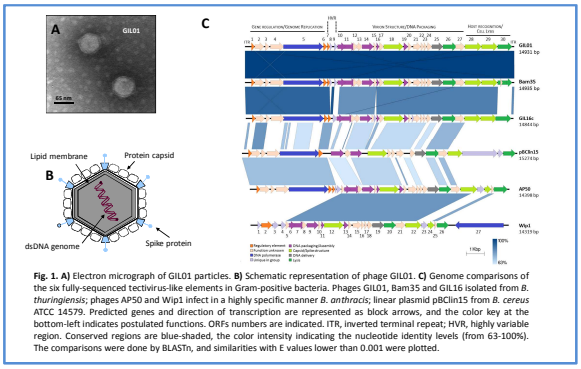

Fig. 1. A) Electron micrograph of GIL01 particles. **B)** Schematic representation of phage GIL01. **C)** Genome comparisons of the six fully-sequenced tectivirus-like elements in Gram-positive bacteria. Phages GIL01, Bam35 and GIL16 isolated from *B. thuringiensis*; phages AP50 and Wip1 infect in a highly specific manner *B. anthracis*; linear plasmid pBClin15 from *B. cereus* ATCC 14579. Predicted genes and direction of transcription are represented as block arrows, and the color key at the bottom-left indicates postulated functions. ORFs numbers are indicated. ITR, inverted terminal repeat; HVR, highly variable region. Conserved regions are blue-shaded, the color intensity indicating the nucleotide identity levels (from 63-100%). The comparisons were done by BLASTn, and similarities with E values lower than 0.001 were plotted.

B. thuringiensis tectiviruses-resistant mutants isolation and phenotypic characterization

B. thuringiensis **sv. *israelensis* strain GBJ002 was subjected to a selective pressure after repetitive propagation with clear plaque (*cp*) mutants of phages GIL01 and GIL16. The *cp* mutants showed an elevated efficiency of killing mainly because they propagate exclusively lytically. Twenty completely tectivirus-resistant bacterial mutants were isolated (Table I and Fig. 2).**

Table I. Lytic (clear plaque) variants of phages GIL01 and GIL16 used to generate and select twenty fully phage-resistant mutants of *B. thuringiensis* GBJ002. The lytic phage variant used to generate the phage-resistant mutant is indicated, but all the bacterial mutants are resistant to the six lytic phages and to the parental phages GIL01 and GIL16.

Parental phage	Clear plaque (*cp*) phage variant	GBJ002 phage-resistant mutant
GIL01	GIL01^{cp1}	GBJ002^{Mut01}
		GBJ002^{Mut13}
		GBJ002^{Mut16}
		GBJ002^{Mut90}
	GIL01^{cp2}	GBJ002^{Mut17}
		GBJ002^{Mut18}
		GBJ002^{Mut33}
		GBJ002^{Mut210}
	GIL01^{cp3}	GBJ002^{Mut15}
		GBJ002^{Mut30}
GIL16	GIL16^{cp1}	GBJ002^{Mut21}
		GBJ002^{Mut42}
		GBJ002^{Mut43}
		GBJ002^{Mut46}
		GBJ002^{Mut91}
		GBJ002^{Mut250}
	GIL16^{cp2}	GBJ002^{Mut24}
		GBJ002^{Mut25}
		GBJ002^{Mut39}
	GIL16^{cp3}	GBJ002^{Mut306}

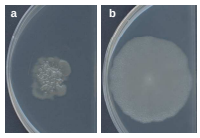

Fig. 2. Evaluation of bacterial resistance to a lytic tectivirus by an inverted spotting assay. Bacteria were inoculated on top of LB-agar containing the lytic phage variant GIL01^{cp2}. Phage resistance/susceptibility was evaluated after incubation for 24 h at 30 °C. (**a**) Parental strain *B. thuringiensis* GBJ002 is susceptible to the lytic action of the phage. (**b**) Derivative mutant GBJ002^{Mut01} displaying a completely phage-resistant phenotype.

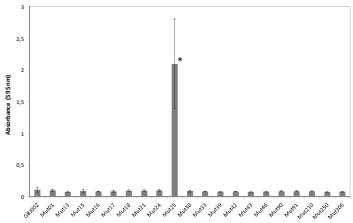

Fig. 4. Biofilm formation of *B. thuringiensis* GBJ002 and the twenty phage-resistant derivative mutants. Biofilm was quantified by the crystal violet staining method using absorbance at 595 nm. Each plotted data represents the average of five replicate wells. The error bars indicate the standard deviation values. The asterisk indicates significant differences between the parental strain GBJ002 and the phage-resistant mutant (Tukey's test, $p < 0.05$).

One of the mechanisms of phage-resistance is to block the phage receptors on the host cell surface by production of extracellular matrix. Mutant GBJ002^{Mut25} seems to have taken advantage of this type of mechanism as indicated by the large mass of biofilm that it is able to produce (Fig. 4).

Some of the twenty phage-resistant bacteria showed differences in cell and colony morphotypes (Fig. 3) and displayed distinct adaptation features, such as biofilm formation (Fig. 4), growth kinetics (Fig. 5), swarming motility (Fig. 6) and some differences in metabolic profiles and antibiotic(s) susceptibilities (data not shown).

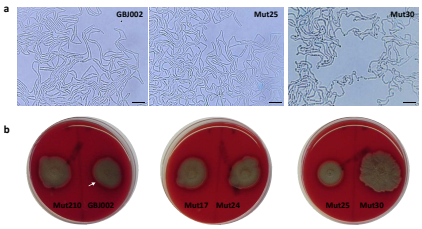

Fig. 3. Cell and colony morphologies of *B. thuringiensis* GBJ002 and some representative phage-resistant mutants. (**a**) Bright-field micrographs of overnight bacterial cultures in LB medium. Scale bars, 10 µm. All the mutants presented the same cell dimensions and cell-chain formation than that of the parental strain GBJ002, with one single exception: GBJ002^{Mut30}. The bacilli of this particular phage-resistant mutant formed long chains of twisted cells that seem to be impaired in cell division (**b**) Colonies growing on Columbia agar plates supplemented with 5% Sheep Blood (CSB). Images were acquired after 48 h of incubation at 30 °C. Mutant GBJ002^{Mut30} showed a different colony morphotype than that of the parental strain GBJ002. The white arrow indicates the weak hemolysis activity of parental strain GBJ002. Similar hemolysis activities were observed in twenty phage-resistant mutants.

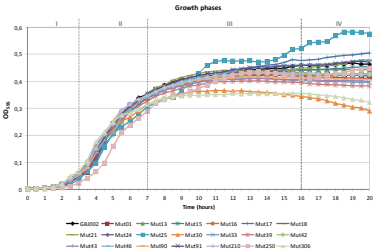

Fig. 5. Growth kinetic of *B. thuringiensis* GBJ002 and the twenty phage-resistant mutants. Bacterial growth was measured by optical density at 595 nm (OD_{595}) during 20 h at 30 °C. Grey dashed lines separate bacterial growth phases I-IV.

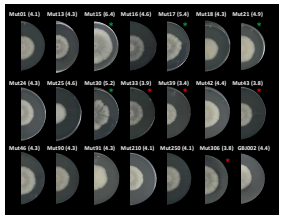

Fig. 6. Swarming motility of *B. thuringiensis* GBJ002 and the twenty phage-resistant derivative mutants. Swarming motility assays were performed in LB medium solidified with 0.6% (wt/vol) agar. Mean swarming values for each mutant are given in parentheses in cm. Green and red asterisks indicate mutants with larger and smaller swarming diameters, respectively, compared to that of parental strain GBJ002. Photographs were taken at 60 h post-inoculation.

The changes that are triggered when *B. thuringiensis* is challenged by lytic variants of tectiviruses indicated that these phages may drive life-trait changes and ecological adaptations in the *B. cereus* group members, as results of a phage-selective pressure.

B. thuringiensis tectiviruses-resistant mutants WGS and identification of candidate genes responsible for phage-resistance

To unravel the possible genetic causes for the phage-resistant phenotype of the isolated mutants, a whole-genome sequencing (WGS) approach was assessed using a pooled high-throughput sequencing method in combination with a mathematical model and bioinformatics analyses as previously described [5]. Potential genes causing the tectivirus-resistant phenotype in *B. thuringiensis* were identified (Table II).

Mutant	Position	Non-synonymous mutation	Amino acid modification[a]	Predicted function
Mut01	4872312	G → A	D625Y	Collagen adhesion protein
Mut13	4331638	A → AA	Stop codon at residue 89/417	Related to glycosyltransferase family
Mut15	4944585	G → T	Stop codon at residue 22/242	Glycosyltransferase involved in cell-wall biogenesis
Mut42	4129976	A → G	T27A	Hypothetical DNA-binding protein
Mut91	651934	G → A	C67Y	Hypothetical protein of the RraB family
Mut306	3450726	G → A	P202S	Orotate phosphoribosyltransferase

[a] Residues affected by mutations are indicated (WT-residue position-mutant). When stop codon is present, residue is indicated over the total number of residues in the WT protein.

Table II. Location of true positive variations in genomes of phage-resistant mutants of *B. thuringiensis* GBJ002 compared to the reference strain *B. thuringiensis* HD-789.

Conclusions and perspectives

In an effort to link the phage-resistant phenotypes that emerged when *B. thuringiensis* strain was challenged with *cp* variants of tectiviruses, a phenotype-sequencing analysis was performed.

The changes in different bacterial life traits, along with the mutations identified, indicated that instead of having one mutated gene responsible to mediate the primary interaction between the phage and the bacterial host (e.g., receptor recognition), the selected phage-resistant mutants displayed changes that can block different steps of the phage cycle and in different ways that do not always involved genotypic changes.

The "phenotype-sequencing" approach carried out in this work permitted to pinpoint some candidate genes responsible for the phage resistance (i.e., several genes associated with cell-wall metabolism and turn-over, as well as cell-surface proteins).

In the near future, gene complementation studies and construction of knockout mutants for the candidates genes identified in this study will confirm their role in the phage-resistant phenotypes.

Acknowledgments

This work has been supported by the National Fund for Scientific Research (FNRS-FRIA, Belgium) and the Research Department of the "*Communauté française de Belgique*" (Concerted Research Action).

References

[1]Verheust C, Fornelos N, Mahillon, J (2005) GIL16, a new Gram-positive tectiviral phage related to the *Bacillus thuringiensis* GIL01 and the *Bacillus cereus* pBClin15 elements. J. Bacteriol. 187, 1966-1973. [2]Sozhamannan, S, McKinstry, M, Lentz, SM, Jalasvuori, M, McAfee, F, Smith, A, Dabbs, J, Ackermann, HW, Bamford, JKH, Mateczun, A, Read, TD. (2008) Molecular Characterization of a variant of *Bacillus* anthracis-specific phage AP50 with improved bacteriolytic activity. Appl. Environ. Microbiol. 74, 6792-6796. [3]Kan, S, Fornelos, N, Schuch, R, Fischetti, VA. (2013) Identification of a ligand on the Wip1 bacteriophage highly specific for a receptor on *B. anthracis*. Appl. Environ. Microbiol. 195, 4355-4364. [4]Gillis A, Mahillon J. 2014. Prevalence, genetic diversity and host range of tectiviruses among members of the *Bacillus cereus* group. Appl. Environ. Microbiol. 80:4138-4152. [5]Harper MA, Chen Z, Toy T, Machado IMP, Nelson SF, Liao JC, Lee CJ. 2011. Phenotype sequencing: identifying the genes that cause a phenotype directly from pooled sequencing of independent mutants. PloS One 6:e16517.