Identification of the endolysin encoded by Deep-Blue phage infecting *Bacillus weihenstephanensis*

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ENDOLYSINS: PROMISING ANTIBACTERIAL AGENTS

Endolysins are **phage encoded enzymes** that break down **peptidoglycan.** They are produced in the **last step of phage life cycle** to release newly formed virons [1]

- Endolysins are organized in two domains:
  - ✓ A N-terminal **Enzymatically Active Domain (EAD)**
  - ✓ A C-terminal **Cell wall Binding Domain (CBD)**
- Most endolysins pass through the inner membrane using **holins**
- Endolysin **applications**: Biocontrol and detection of pathogenic bacteria

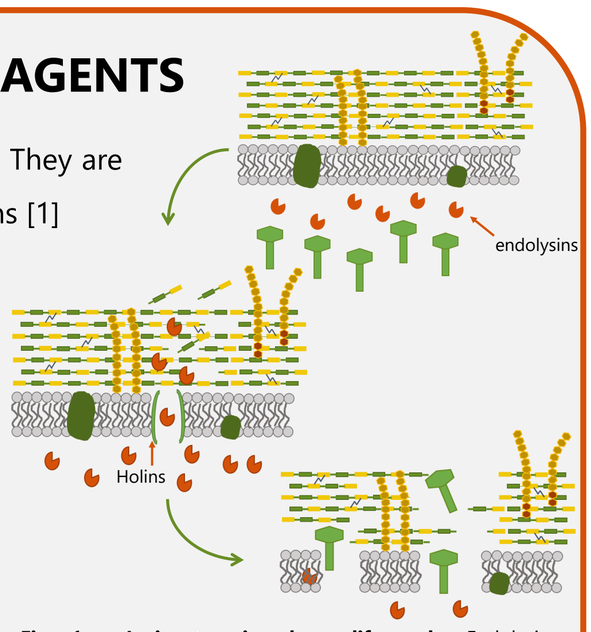

Fig. 1 – Lysis step in phage life cycle. Endolysins accumulate in the cytoplasm waiting for holins to form holes in the inner membrane to allow endolysin to access the peptidoglycan layer (endolysin-holin canonical lysis system)

GP221: DEEP-BLUE PHAGE ENDOLYSIN

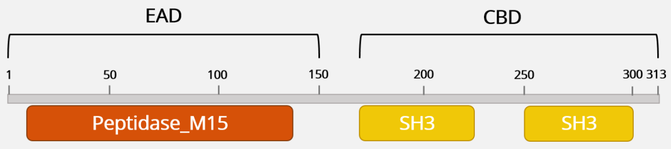

Fig. 2 – Domain architecture of Deep-Blue putative endolysin gp221.

Gp221 presents **the two domain organization** commonly encountered in endolysins (Fig. 2):

- **EAD**: Peptidase_M15 → L-Ala-D-Glu peptidase
- **CBD**: 2xSH3 → Binds proline rich region

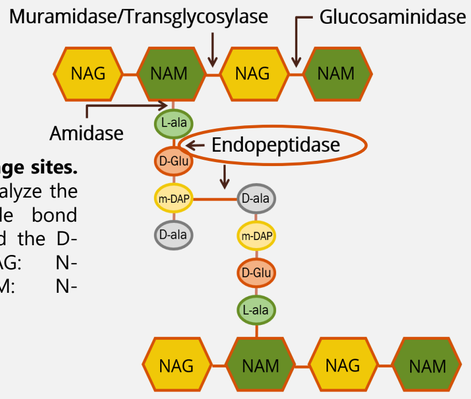

Fig. 3 – Endolysin cleavage sites. L-Ala-D-Glu peptidases catalyze the hydrolysis of the peptide bond between the L-alanine and the D-glutamine (circle). NAG: N-acetylglucosamine; NAM: N-acetylmuramic acid.

Gp221 EAD displays **sequence homology** with confirmed endolysins from *B. cereus* phages:

- LysB4 (Phage B4) – 88% identity
- PlyP56 (Phage Phrodo) – 85% identidy

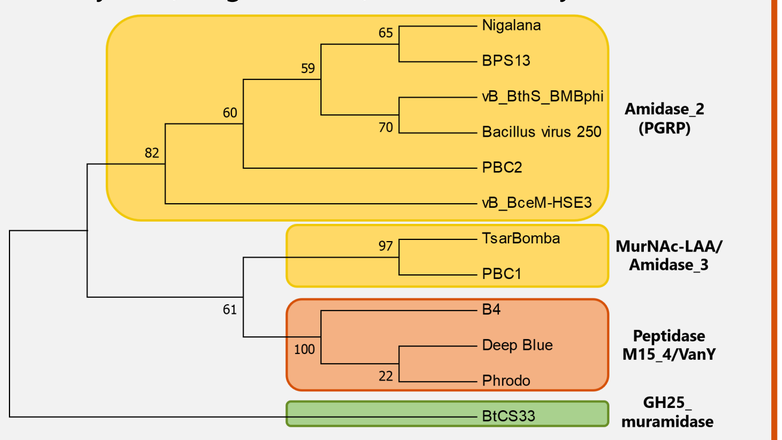

Fig. 4 – Phylogenetic tree of identified endolysins from phages infecting the *B. cereus* group. The tree was built using the maximum likelihood method. Color clustering indicates enzymatic activity: Yellow= Amidase; Orange= Peptidase; Green= Muramidase. The conserved domains of the EAD are indicated on the right.

DEEP-BLUE MYOVIRUS

- Isolated from an agricultural soil
- Genome 156 kb [2]
- Specific to the *B. cereus* group
- Strictly lytic
- Biocontrol potential for emetic strains of *B. weihenstephanensis* in food and biofilms [3]

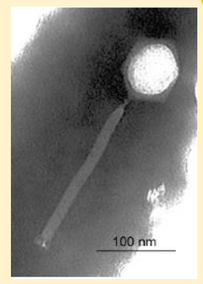

Fig. 5 – Deep-Blue TEM image

GLOBAL STRATEGY

Expression system: pET30 - *E. coli* BL21(DE3)
Purification: Ni-NTA column

Deep-Blue gp221

Whole endolysin → Activity test in 96-well plate
Conditions: 50 mM Tris-HCl pH 8, 30°C

N-ter GFP fusion - CBD → Cell wall binding assay
Conditions: PBS pH 7.4, RT

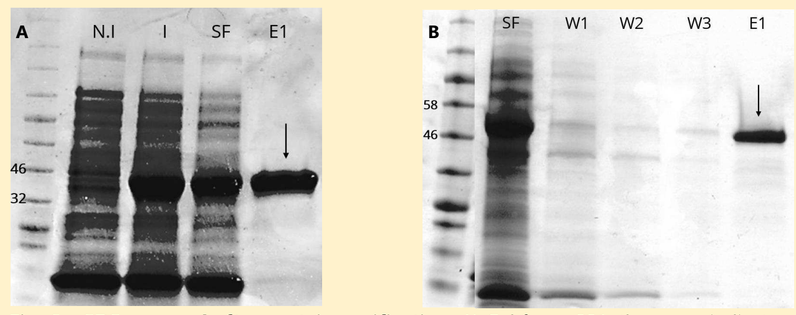

Fig. 6 – SDS page gel after protein purification. A) Gel for gp221, the arrow indicates the purified protein with a predicted MW of 38.9 kDa B) Gel for GFP::gp221_CBD, the arrow indicates the purified protein with a predicted MW of 50.5 kDa. N.I: Non-induced fraction; I: Induced fraction; SF: Soluble fraction; W: Wash fraction; E1: First elution fraction.

ACTIVITY AND BINDING SPECTRUM

Table 1 - Deep-Blue host-range, activity spectrum of gp221 and binding activity of gp221_CBD. [a]Deep-Blue host spectrum via Spot-on-plate ; S: Sensitive strain (Plaques); L: Lyse but no plaque; R: unsensitive. [b]Lytic activity was obtained by OD_{595nm} reduction at 30°C during 30 min with 100 µg/ml of protein: - <10%; + 10 – 30%; ++ 30 – 60 % ; +++ > 60%.

Species	Strain	Phage[a]	gp221[b]	CBD
B. weihenstephanensis	Si0239	S	+++	+
	BtB2-4	S	+	+
	WSBC10204	L	+	+
	WSBC10202	S	+++	+
	MC67	L	++	+
B. cereus	F4810/72	L	+	+
	H3081.97	L	+++	+
	ATCC14579	L	++	+
	ATCC10987	R	+++	+
B. cytotoxicus	BcA20 086	L	+	+
	NVH391-98	L	++	+
B. mycoides	KBS 2-12	L	++	+
	KNC2-18	R	+	+
B. thuringensis	AW73	S	++	+
	GBJ001	L	+++	+
E. coli	ATCC8739	R	-	-

***B. cereus* group:** gp221 binds and lyses all the tested strains including those that cannot be infected by Deep-Blue.

Outside the *B. cereus* group: With the exception of *B. megaterium*, gp221_CBD cannot bind strain of *B. subtilis*, *B. amyloliquefaciens*, *B. licheninformis*, *B. pumilus*, *L. ivanovii*, *S. epidermidis*, *L. brevis*, *E. coli* and *P. aeruginosa*.

gp221 is specific to the *B. cereus* group

GP221_CBD BINDING ABILITY

***B. cereus* ATCC10987** **_E. coli_ ATCC8739**

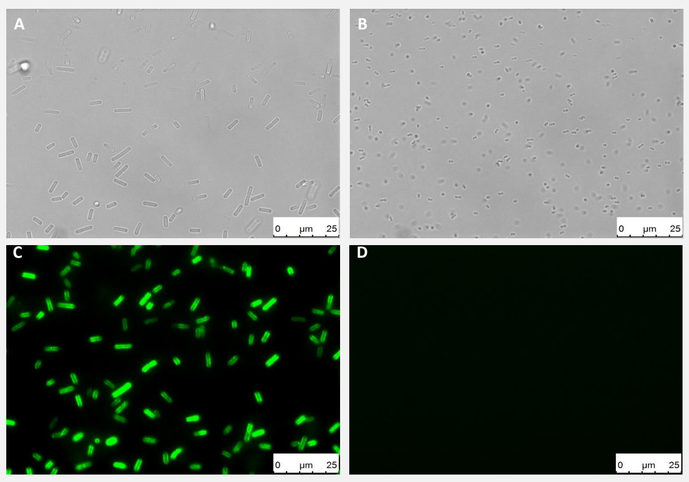

Fig 7 – Binding of GFP tagged gp221_CBD on *B. cereus* and *E. coli* cells. Phase contrast images (A-B) are shown with their corresponding fluorescent images (C-D). 10 µg of purified recombinant protein were incubated with exponentially growing cells during 15 min.

- When fused with a GFP, gp221_CBD is able to **decorate the surface** of strains from the *B. cereus* group but not G- and G+ strains outside the group (Fig. 7, Table 1).
- **After only 30 min at 30°C**, gp221 (100 µg/ml) induces a reduction of **1,1 log** for *B. weihenstephanensis* Si0239 (Sensitive) and **0,7 log** for *B. cereus* ATCC10987 (Unsensitive) whereas no significant reduction is observed for *E. coli* ATCC8739 (Fig. 8).
- The efficiency of cell lysis **depends on the protein concentration**. A 60% reduction in OD_{595} with 10, 50 and 100 µg/ml of gp221 requires 42, 13 and 11 min, respectively (Fig. 9).

GP221 LYTIC ACTIVITY

CFU REDUCTION

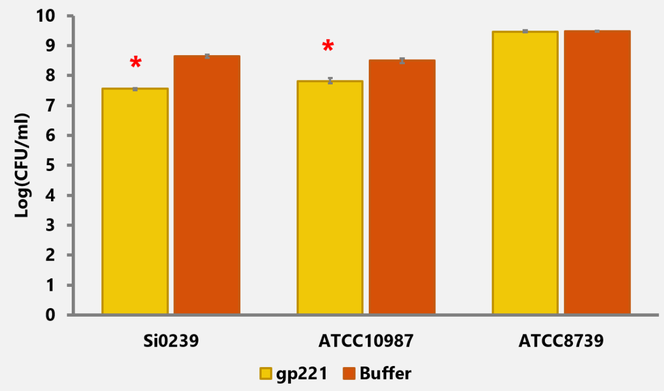

Fig. 8 – Assessment of CFU reduction of bacterial suspension treated with gp221. Stationary phase bacteria (*B. weihenstephanensis* Si0239 – sensitive strain, *B. cereus* ATCC10987 – Sensitive but no plaque formation and *E. coli* ATCC8739 – negative control) at a final OD_{595} between 0.8-0.9 were treated with 100 µg/ml of endolysin during 30 min at 30°C. The experiment was done in triplicate. Red stars indicate a significant reduction compared to the control.

Cell lysis

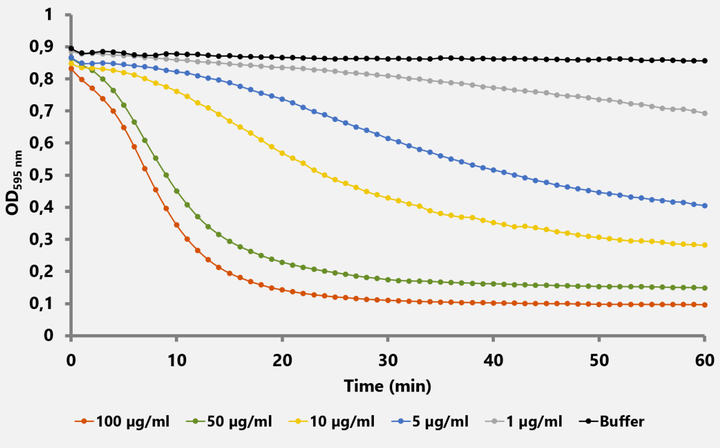

Fig. 9 - Response curve of gp221 with *B. cereus* ATCC10987. Stationary phase bacteria at a final OD_{595} between 0.8-0.9 were treated with increasing concentration of endolysin during 60 min at 30°C.

[1] Etobayeva, I., Linden, S., Alem, F., Harb, L., Rizkalla, L., Mosier, P., & Nelson, D. (2018). Discovery and biochemical characterization of PlyP56, PlyN74, and PlyTB40—*Bacillus* specific endolysins. *Viruses*, *10*(5), 276.
[2] Hock, L., Gillis, A., & Mahillon, J. (2016). Complete genome sequence of bacteriophage Deep-Blue infecting emetic *Bacillus cereus*. *Genome Announc.*, *4*(3), e00115-16.
[3] Hock, L., Leprince, A., Tournay, M., Gillis, A., & Mahillon, J. (2019). Biocontrol potential of phage Deep-Blue against psychrotolerant *Bacillus weihenstephanensis*. *Food Control*, *102*, 94-103.