Bacillus thuringiensis serovar kurstaki (Btk) Strains for Industrial Production in wheat bran based medium: Insights from Genomic Exploration and Nutritional Optimization

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This project has received funding from the European Union's Horizon 2020 Research and Innovation program under Grant Agreement No 734921.

Scientific context

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1.1. IPM-4-Citrus (MSCA RISE. No. 734921. 2017-2023)

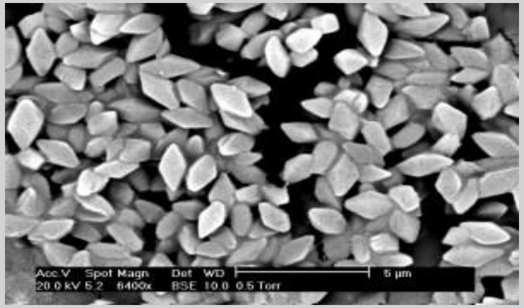
HORIZON 2020 FUNDED

Marie Slodowska Curie Action

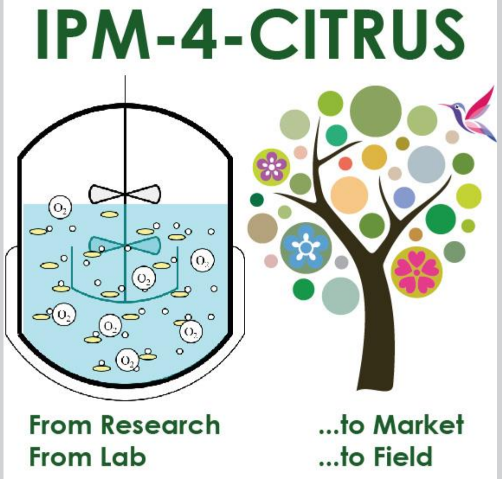
Research & Innovation Staff Exchange

INTEGRATED PEST MANAGEMENT

- ✓ **Understanding & sensitising stakeholders** about the health risks related to citrus pests
- ✓ **Developing an alternative IPM approach**
- ✓ based on biological control



HD1
Lip
BLB1



CITRUS

11 PARTNERS

6 COUNTRIES

4 → 6 YEARS DURATION

TARGETED PESTS:
insect larvae : Phyllocnistis citrella & Prays citri

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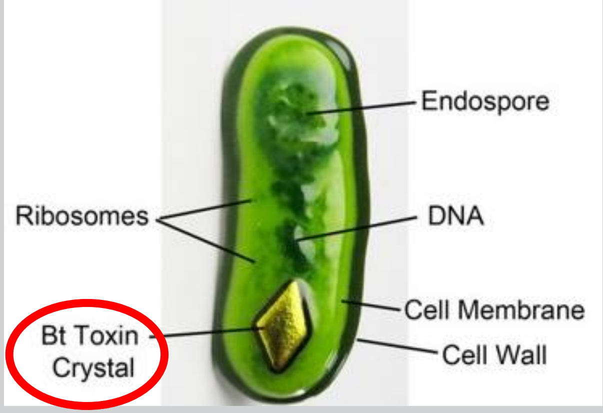
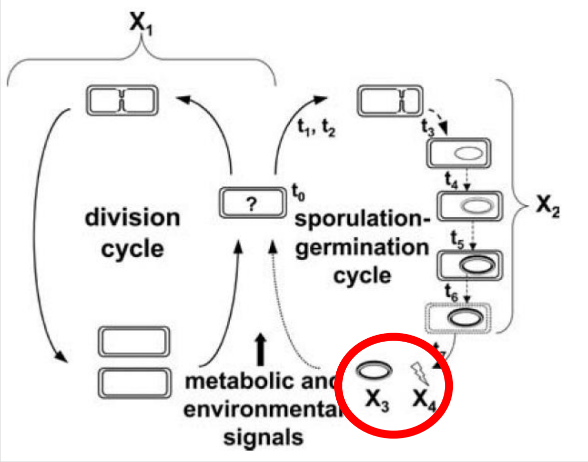
1.2. *Bacillus thuringiensis* subsp. *kurstaki* (Btk)

Bacillus thuringiensis kurstaki **(LIP, HD1)**

- **Bacilli**
- **Gram +**
- **Facultative aerobic**
- **Sporulant**
- **δ-Endotoxin producer**

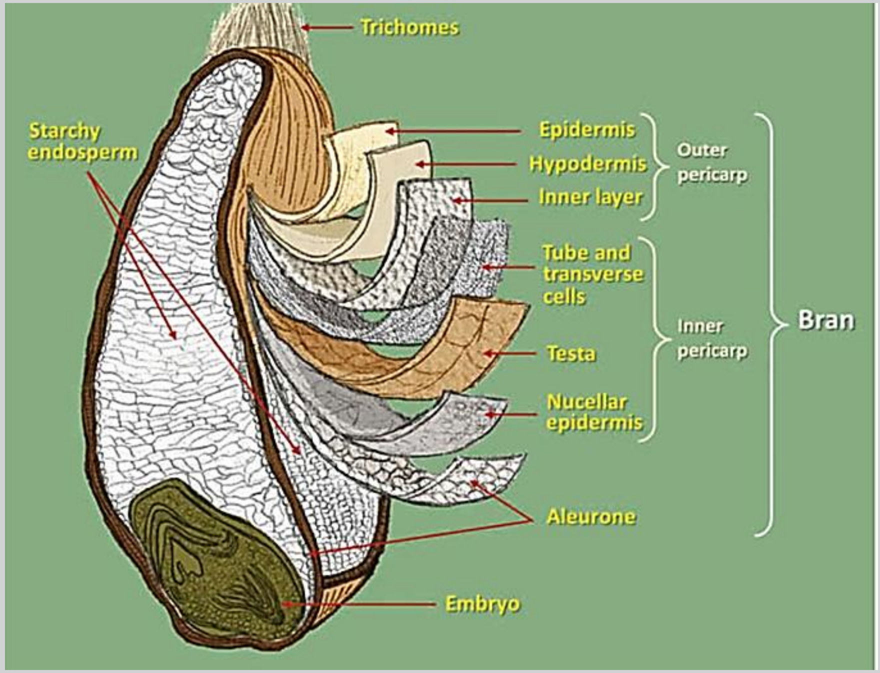
Bacillus thuringiensis (Bt) life cycle and endotoxins production (https://www.bpprc.org/)





(M. Sarrafzadeh et al 2005)

1.3. Wheat bran (WB) structure & chemical composition



Wheat Kernel structure
(Balandran et al. 2015)

Mean composition of Wheat Bran (WB)
(Hell et al.. 2016. Sapirstein et al. 2016. Stoffel et al.. 2019)

Component		Content in WB (%w/w)
Polysaccharides	Reserve sugars - Oligosaccharides - Starch	15-45 3.7 13-40
	Fibres (structure) - Cellulose - Hemicellulose - Lignin	35.7-62.3 6.5-11 20.8-33 9.8-16
Proteins		13.2-21.1
Ashes/Minerals		2.2-8
Water		9-12

1.4. Background

WB: good medium for Bt crystals production

(*Devi et al. 2005*)

→

Effective low cost Btk crystal production in cheap WB based medium compared to semi synthetic medium (*Mounsef et al. 2014*)

→

Sieving of WB into 4 classes:
class 1 (>850 µm),
class 2 (500-850 µm),
class 3 (250-500 µm) &
class 4 (<250 µm).
Optimal Btk growth & crystal production in class 2. ([WB]=73.6 g/L), 248 rpm (*Abboud et al. 2017*)

1.5. Main Scientific Questions

1. Does the substrate elemental composition differ between one class and another?

2. Do the mass balance and the elemental composition inform us about the progress of the culture, the limiting nutrients and the fermentable fraction?

3. In terms of scale-up approach, are the limiting components in bioreactor culture the same as in flask?

4. Can the elemental composition help us interpreting the 3 phases of culture & the biokinetics in bioreactor?

Materials & Methods

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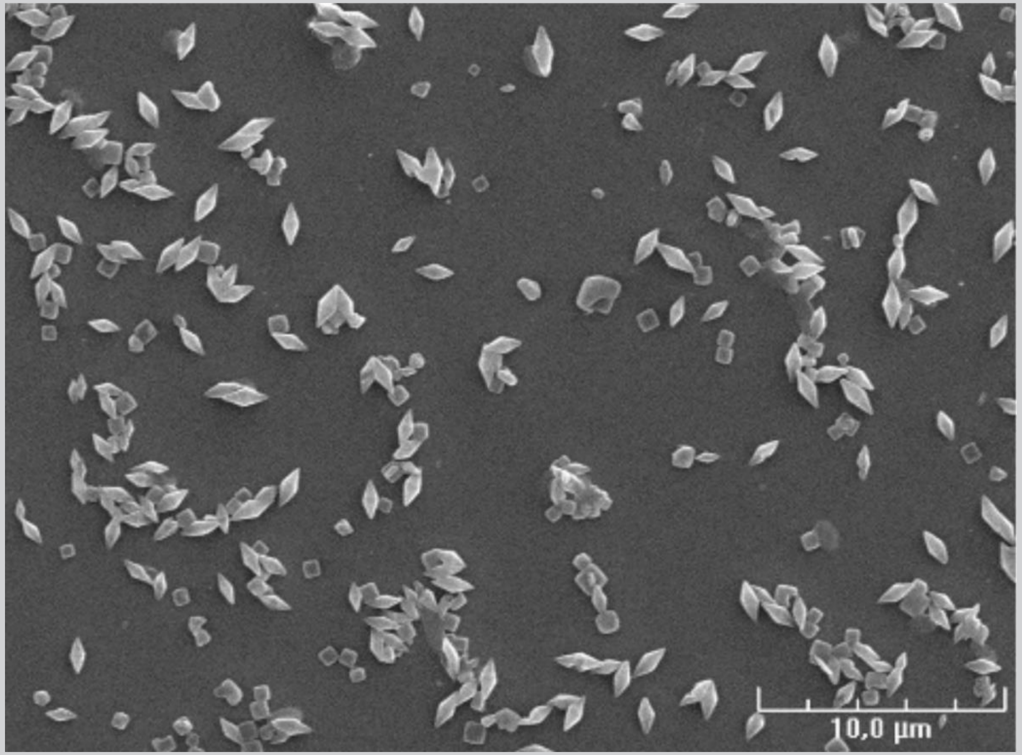
2. Materials and Methods

Bacillus thuringienis Lip strain



Crystals from Btk strain Lip

Bioproject reference on NCBI: PRJNA924104
Assembly: GCA_028356555.1
Source: Fayad N, 2023 Microbiol Resour Announc.
El Khoury M, 2014 Arch Microbiol.

Substrate

Raw WB
Class 1 (>850 µm): <1%
Class 2 (500-850 µm): 19.8%
Class 3 (250-500 µm): 51.4%
Class 4 (<250 µm) : 28.2%

Suspension

HT without culture: Flask, Bioreactor
HT+ culture:
- Flask (48h, 30°C, [WB]= 73.6 g/L, 250 rpm)
- Bioreactor (48h, 30°C, [WB]= 73.6 g/L, pH7.5, pO_2> 25%)

Sample

Insoluble fraction (substrate)

Permeate (cells, spores, δ-endotoxins)

Bioperformances	Chemical analysis
CFU (not shown) Spores (not shown) δ-Endotoxins	Dry matter (gravimetry), Ashes (gravimetry) Starch: Colorimetry (I_2/KI), Nitrogen (Kjeldahl) CHONS (Combustion Flash + GC)

Determination of mass balance (permeate/insoluble fraction).
Studying the elemental composition of WB before and after culture

Results & Discussion

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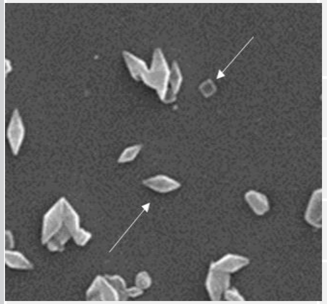
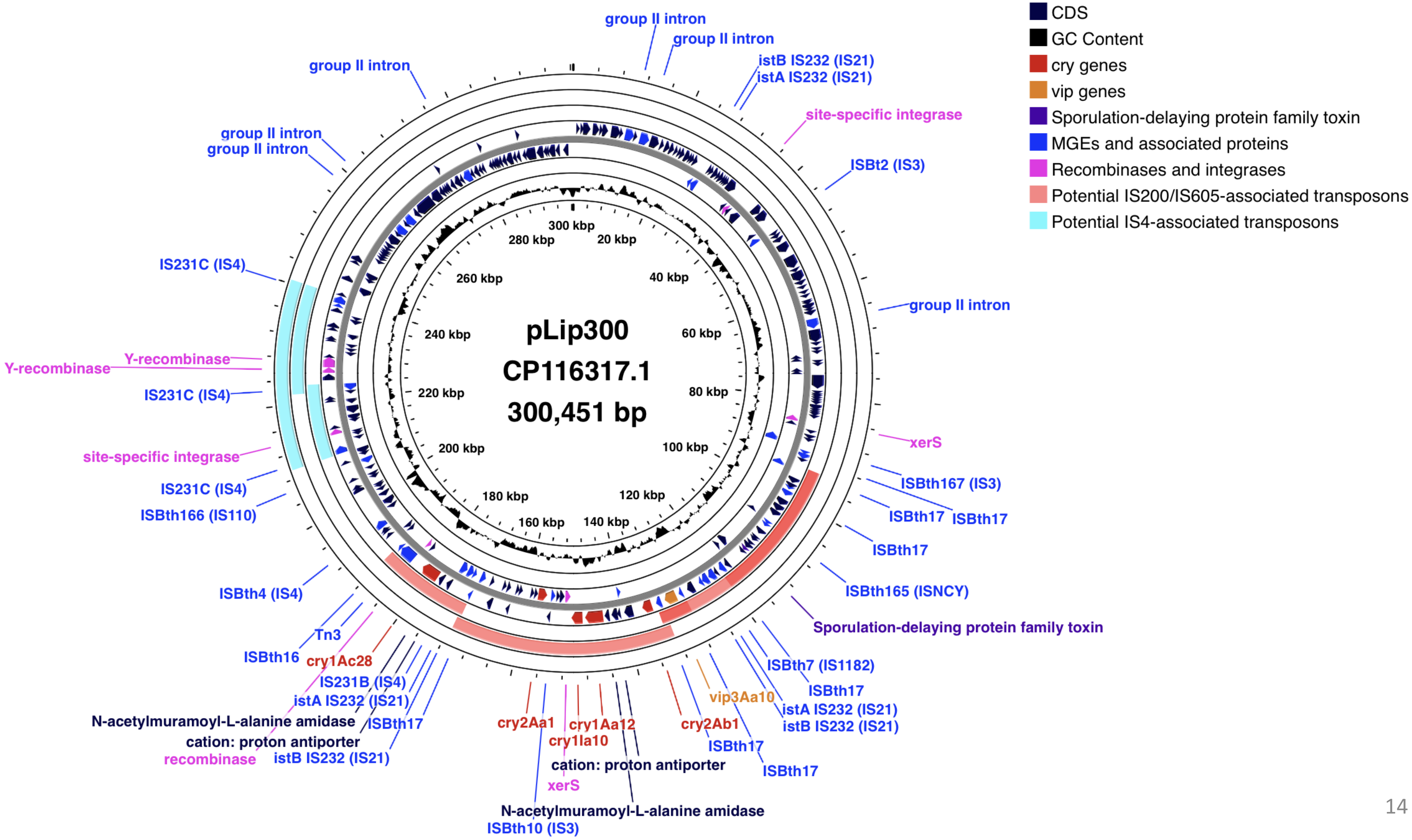
3.1 Lip Genomic features

	Replicon	Bioproject / Biosample / NCBI SRA accession	Accession number	Length (bp)	CDS # (total)	GC content (%)
Lip	Chromosome	PRJNA924104 / SAMN32746259 / SRX20261752	CP116313	5,293,947	5,632	35.2
	pLip2189		CP116316	2,189	4	35.3
	pLip7635		CP116320	7,635	8	32.2
	pLip7911		CP116321	7,911	9	32.3
	pLip8513		CP116322	8,513	9	30.8
	pLip12		CP116314	12,276	21	31.1
	pLip15		CP116315	15,008	28	34.9
	pLip69		CP116319	69,004	76	32.2
	pLip91		CP116323	91,357	102	31.2
	pLip97		CP116324	97,437	85	34.5
	pLip300		CP116317	300,451	267	33.1
	pLip457		CP116318	457,481	408	32.7

Identified Lip cry genes

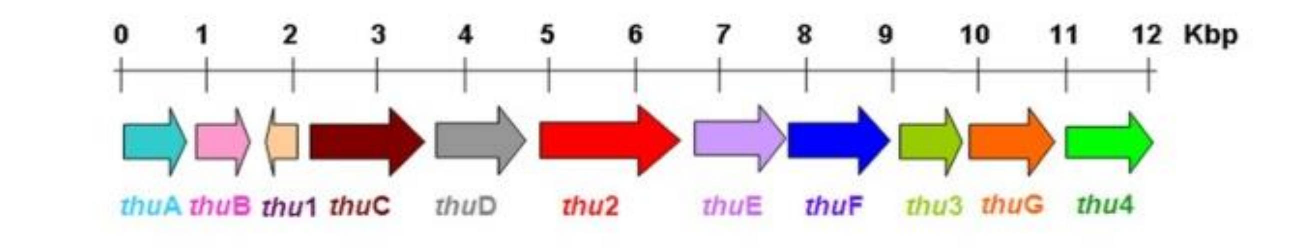
Strain	Plasmids	ID	Toxins	Length
Lip	pLip97	CP116324.1	Cry1Ab1	1155
			Cry1Ac11	1178
	pLip300	CP116317.1	Cry1Ac28	1176
			Cry1Aa12	719
			Cry1Ia10	1052
			Cry2Aa1	633
			Cry2Ab1	633
HD-1	pBMB65	CP004873.1	Cry1Ab1	1155
	pBMB95	CP004875.1	Cry1Ac5	1177
	pBMB299	CP004876.1	Cry1Aa6	620
			Cry1Ia10	719
			Cry2Aa1	633
			Cry2Ab1	633

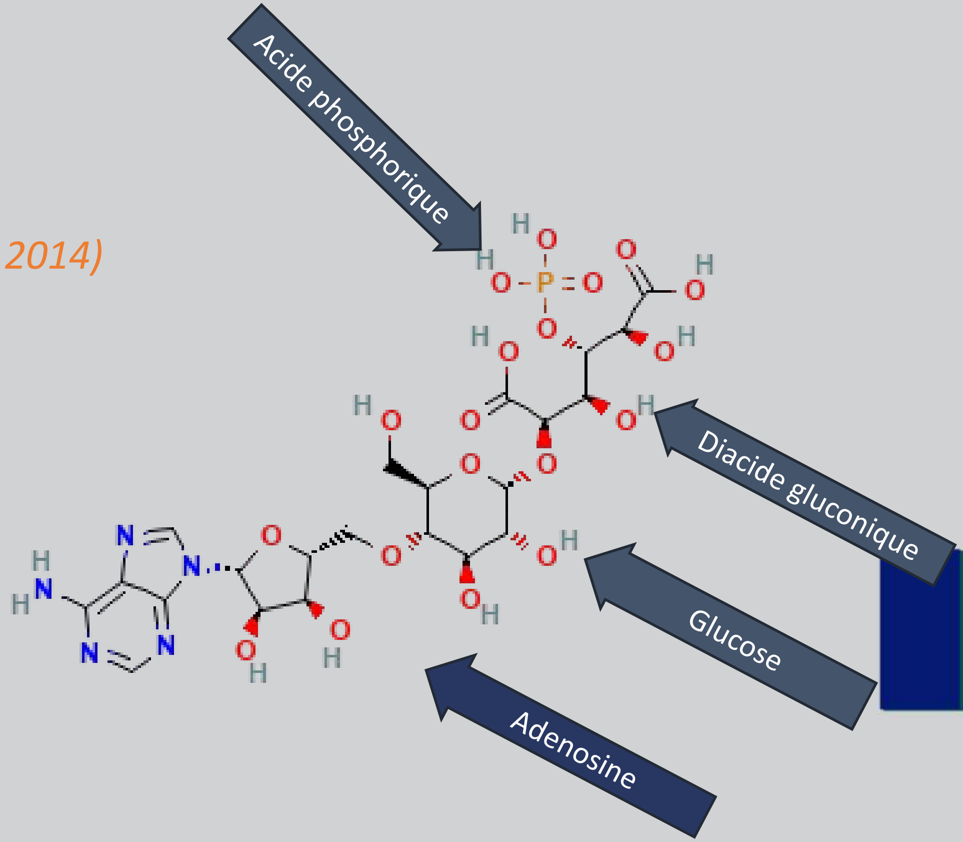
The main toxin carrying plasmid pLip300



Beta-exotoxin (Thuringiensin)

- Inhibits RNA synthesis
- Encoded by a cluster of 11 open-reading frames (ORFs) *(Liu et al., 2014)*
- The proteins work together to assemble Thuringiensin (700 Da)





By advice of the World Health organization (WHO), any bacterial strain possessing the complete *thu* cluster, especially the kinase-coding *thuE* gene, is deemed banned from public use, so they cannot be used as biopesticides.

- *BerA* and *berB* were found in all the strains.

	THUA	THUB	THU1	THUC	TGUD	THU2	THUE	THUF	THU3	THUG	THU4
LIP	XX	X									
HD-1	X	X									

Screening of Thu proteins in the Btk strains of interest. The "X" symbol represents the presence of the Thu protein. "XX" represents the presence of two proteins in the genome (in Lip's case, the second is a pseudogene).

Cluster incomplete → Synthesis of Thuringiensin is not possible by Lip

3.2 Biochemical limitation of Biopesticide production on WB based medium

3.2.1 Endotoxins production in WB vs Semi-synthetic medium (SSM)

***Btk δ-* endotoxins yield in mg/mL**

	Culture in bioreactor		Culture in flasks		
***Btk* Strains**	**SSM** Sarrafzadeh et al. 2005	**WB Raw** Rahbani et al. 2015	**WB Class 2**	**WB Class 3**	**WB Class 4**
HD1	**0.7**	**0.43**	**/**	**/**	**/**
BLB1	**0.73**	**0.63**	**/**	**/**	**/**
Lip	**0.19**	**0.43**	**0.549 ±0.117**	**0.547 ±0.071**	**0.432 ±0.062**

- *Btk* Lip: **Higher yield** in WB vs SSM
- *Btk* Lip bioperformances ↑ on class 2 & 3

3.2.2 Raw and sieved WB chemical composition

			Mass	Water content	Starch	Protein Kjeldahl	Elemental Composition [%w/w]					
Class	Size (µm)	D [4.3] (µm)	[%w/w]	[g water /gdm]	[g/gdm]	[g eq.protein /gdm]	C	H	O	N	S	Ash
Raw		598.5	100	0.119 ±0.07	0.207 ±0.008	0.138 ±0.012	44.79 ±0.44	6.74 ±0.20	36.53 ±0.37	2.50 ±0.24	0.00	4.43 ±0.12
2	500-850	865.4	19.80	0.116 ±0.04	0.173 ±0.35	0.145 ±0.012	44.21 ±0.19	6.50 ±0.12	35.62 ±0.45	2.60 ±0.23	0.00	3.70 ±0.12
3	250-500	531.1	51.41	0.116 ±0.04	0.144 ±1.35	0.127 ±0.014	45.34 ±0.08	6.46 ±0.04	37.89 ±0.44	2.48 ±0.04	0.00	4.28 ±0.12
4	<250	275.3	28.20	0.116 ±0.04	0.347 ±0.47	0.155 ±0.016	42.40 ±0.04	6.61 ±0.12	34.60 ±0.37	2.61 ±0.24	0.00	3.23 ±0.12

- WB granulometry ➔ ∅ effect on elemental composition
- Class 4: highest starch [ ]
- WB molar formula (mol/C_mol): $CH_{1.74}O_{0.53}N_{0.04}$
- WB massic formula (g/g_C): $CH_{0.14}O_{0.71}N_{0.05}Ash_{0.1}$

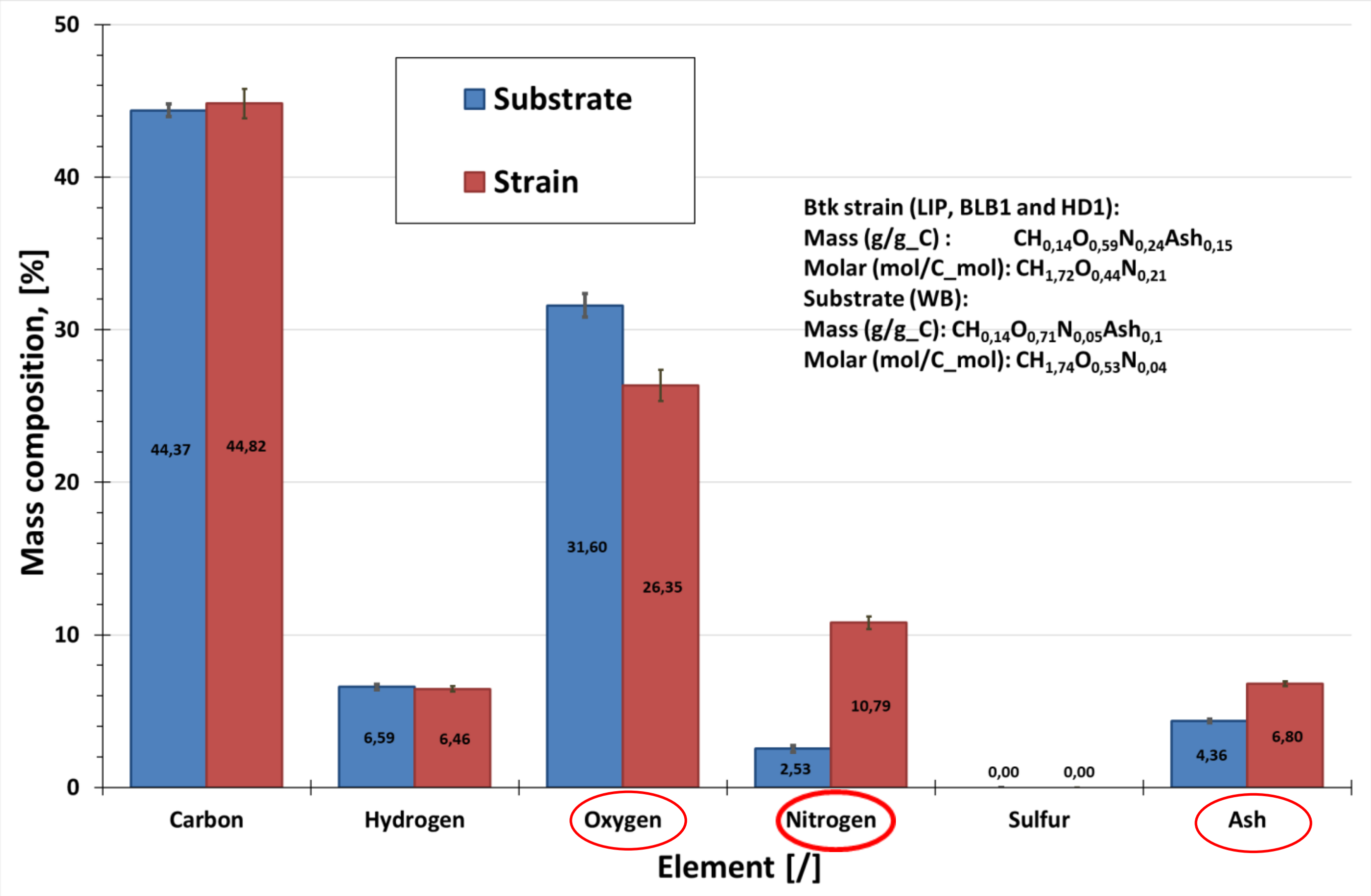
3.3. *Btk* elemental composition

Btk Strains	C (%w/w)	H (%w/w)	O (%w/w)	N (%w/w)	S (%w/w)	Ashes (%w/w)
Lip	43.37	6.22	24.84	10.48	0. 00	7
BLB1	45.7 ±0.067	6.61 ±0.09	26.89 ±0.87	10.51 ±0.011	0. 00	6.7
HD1	44.65 ±0.3	6.42 ±0.072	26.56 ±0.68	11.23 ±0.068	0. 00	NA
Mean value	44.82 ±0.98	6.46 ±0.17	26.35 ±1	10.79 ±0.4	0. 00	6.8 ±0.17

Strain	Elements (%m/m)					Chemical formula	Molar Mass
	Ashes	C	H	O	N	(Cmol)	(g/mol)
Bt spp. kurstaki (MSc)	6.80	44.82	6.46	26.35	10.7 9	$CH_{1.72}O_{0.44}N_{0.21}$	25.52
Lactobacillus helveticus	9.03	47.54	6.25	24.39	12.7 9	$CH_{1.58}O_{0.39}N_{0.23}$	25.26
Flavobacterium Dehydrogenans	13.5	45.16	6.15	24.29	10.8 7	$CH_{1.63}O_{0.40}N_{0.21}$	26.60
Escherichia coli	11.3	47.83	6.95	21.65	12.3	$CH_{1.74}O_{0.34}N_{0.22}$	25.11
Bacillus cereus	9.98	46.05	5.73	26.26	11.9 8	$CH_{1.49}O_{0.43}N_{0.22}$	26.08

- *Btk* strains: identical elemental composition
- **Molar formula of the strain (mol/C_mol): CH1.72O0.44N0.21**
- **Massic formula of the strain (g/g_C): CH0.14O0.59N0.24Ash0.15**
- *Btk*: **elemental composition close to *Bacillus cereus*** *(Popovic et al. 2019)*

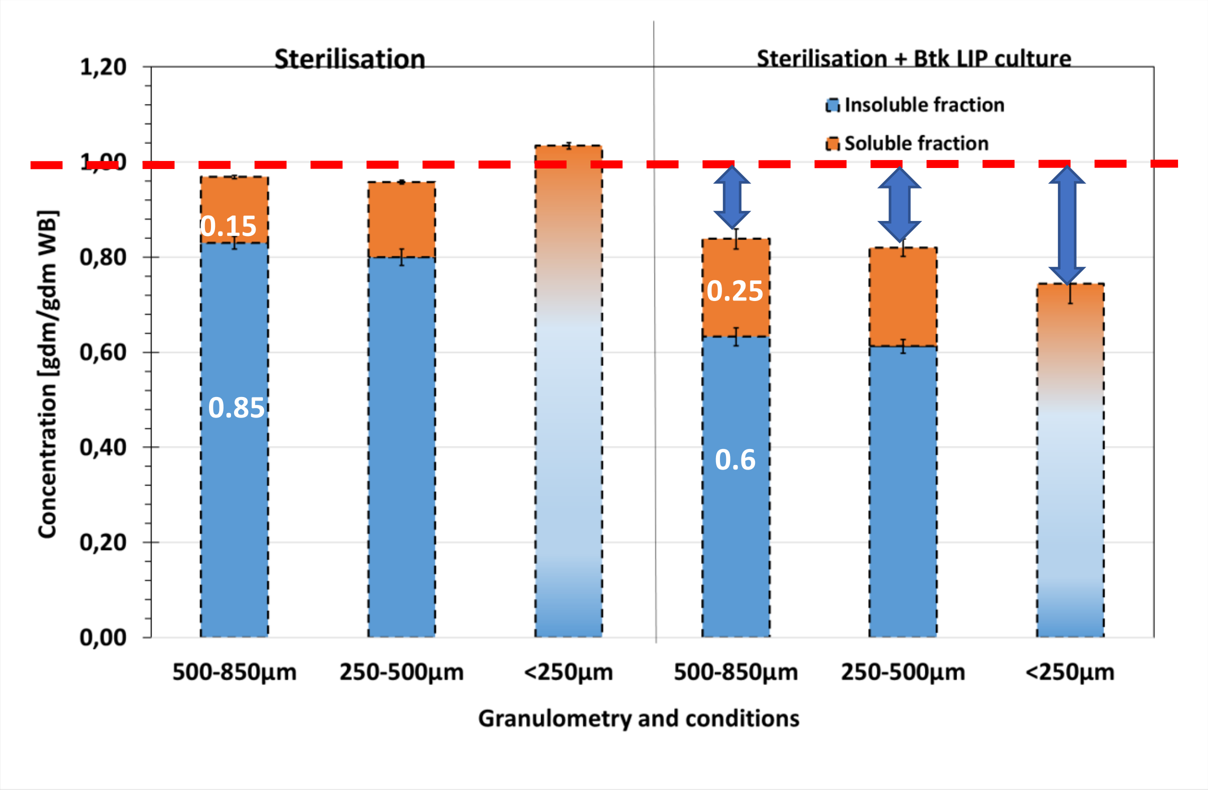
3.4. *Btk* vs WB. Elemental composition



- Strains are more rich in Nitrogen & minerals and less in Oxygen compared to substrate (WB)
- WB is the **only source of Nitrogen** during *Btk* culture

3.5. Evaluation of the fermentable fraction over classes

Substrat + Oxygène + Azote + Oligo + ... ➔ Biomasse + CO2 + produits (Spore, endotoxine) + eau + énergie
1/6 $C_6H_{12}O_6$ + α O_2 + β NH_3 + Sels +... ➔ Y_{sx} X ($CH_{1,8}O_{0,3}N_{0,2}$) + δ CO_2 + λ H_2O + ε Spores + ξ Endotoxine + Energie...



- ➚ fermentable fraction when granulometry➘
- **If Yx/s = 0.5 fermentable fraction ~ Starch**

➔ Class 2 : 32.3%
➔ Class 3 : 36.1%
➔ Class 4 : 51.1%

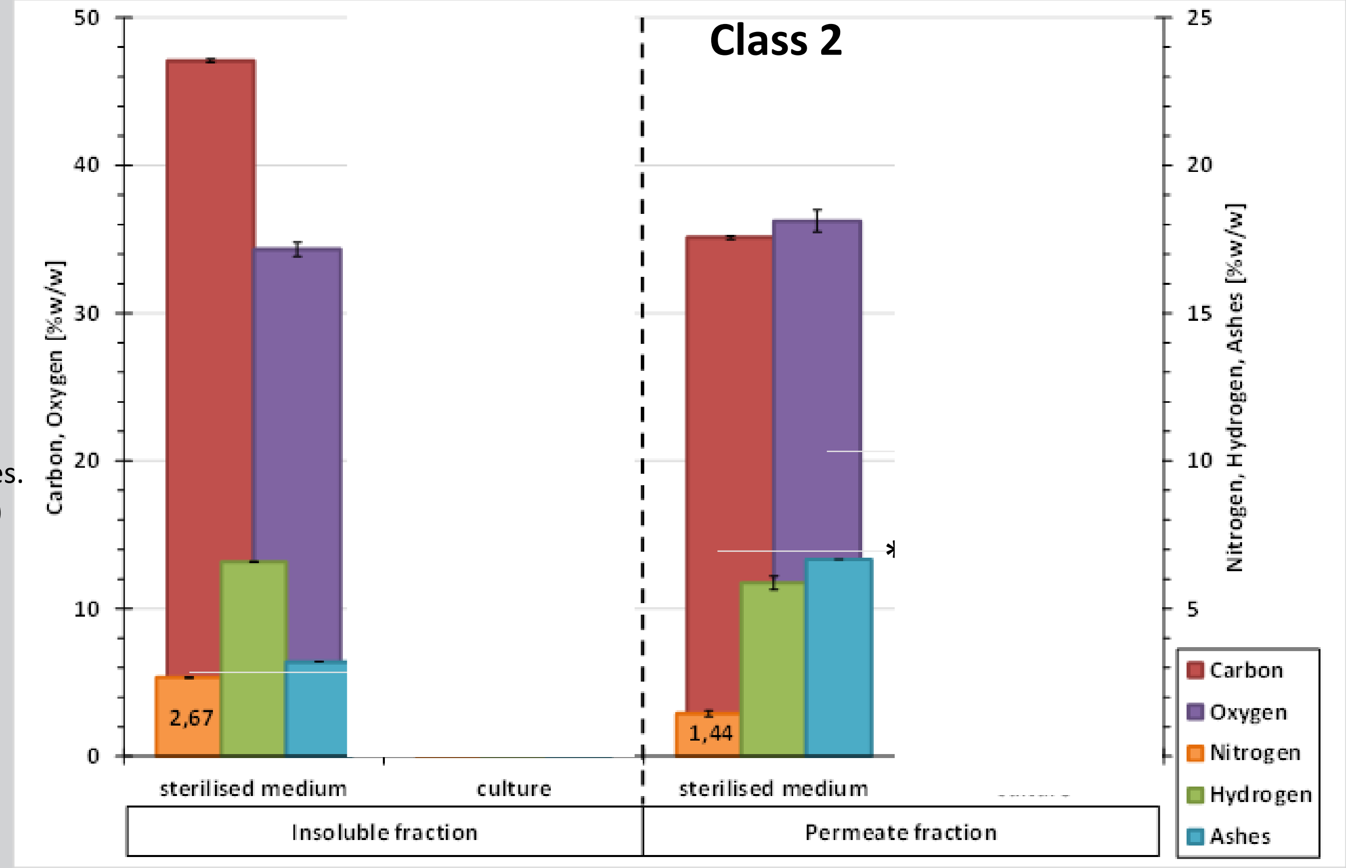
[Starch]= 0.173, 0.144 & 0.347 gdm/gdmWB for classes 2, 3 & 4 respectively

- Partial consumption of lignocellulosic fraction

3.6. How does the medium elemental composition evolve?

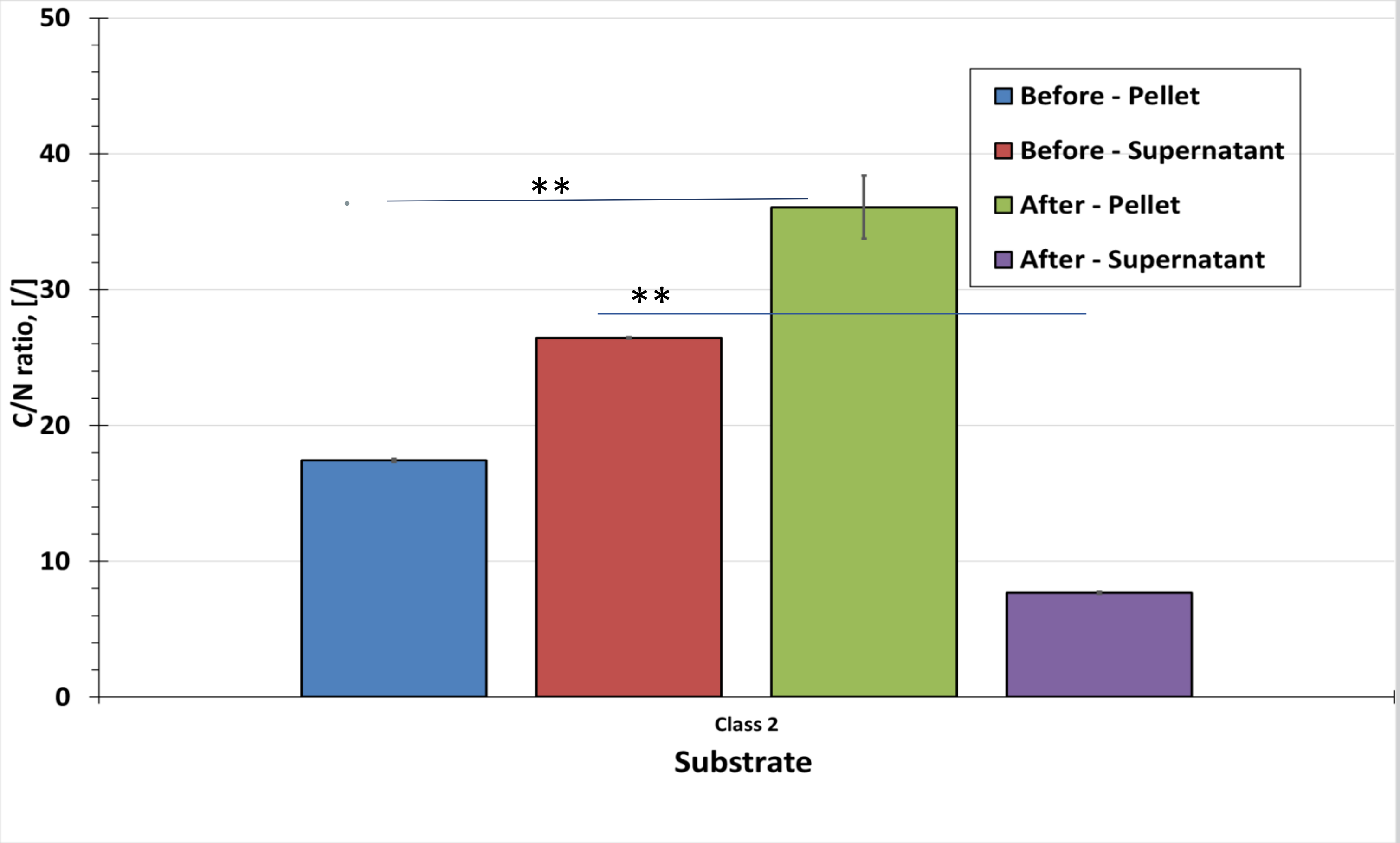
Insoluble fraction (substrate)

Permeate (cells. spores. endotoxins)



- Permeate: Biomass production
- Insoluble fraction: substrate proteins consumption
- **N: limiting nutrient**
- N disponible ~ 45% N accessible
- Class 3: same behaviour
- Class 4: No separation insoluble fraction/permeate

3.7. C/N ratio: indicator of the process evolution in flask?

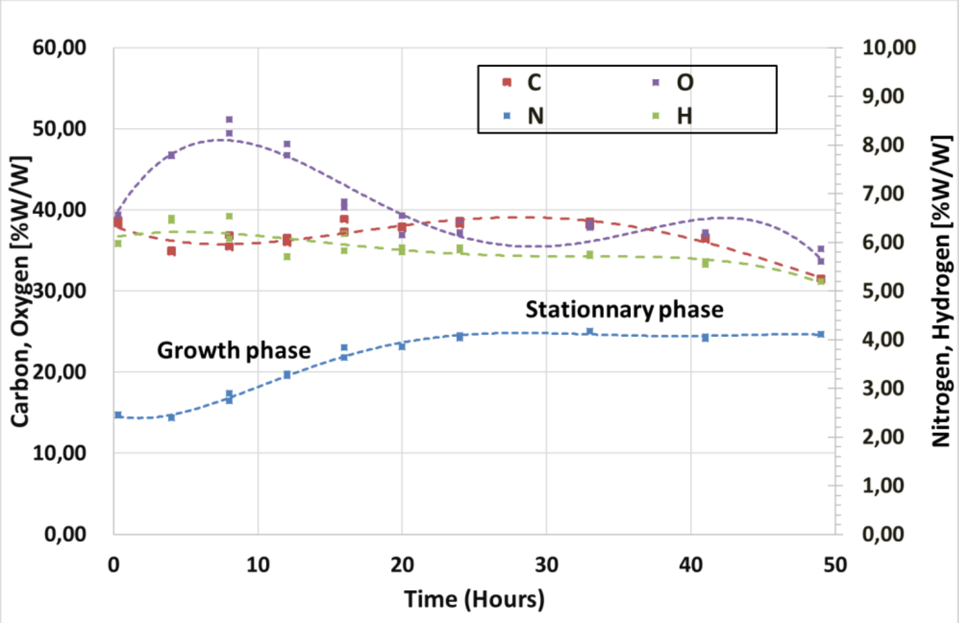


- C/N is a reliable indicator to interpret the process evolution & phases of culture

3.8. Monitoring of culture biokinetics through elemental composition analysis in bioreactor

Permeate

Insoluble fraction



- Same behaviour between flask and bioreactor.
- Biokinetics: 0-12h: growth phase then 20/25- 48h: stationnary phase

4. Conclusion and perspectives

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- **WB elemental composition: no evolution between classes**
- **Fermentable fraction: starch & lignocellulosic fraction**
- **N: culture limiting nutrient**
- **Residual N: Physically non accessible**
- **C/N ratio: reliable indicator of culture phases & bioperformances (flask & bioreactor scale)**

Determination of chemical limitations → optimisation of biopesticide production in a low cost WB medium at large scale

5. Highlights & perspectives

01 Study WB physical characteristics (morpho-granulometry, rheometry, settling speed)

02 Define an appropriate mathemetical model in order to better describe bioperformances & optimise δ- endotoxin production

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IPM-4-Citrus (MSCA RISE. No. 734921. 2017-2023)

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Thank you for your attention

Roman ruins: BAALBECK, Lebanon

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