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MASTER THESIS

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ABSTRACT

Bolle di Magadino is a natural wetland reserve, located in Southern Switzerland, with international importance due to its particular fauna and flora. Here the floodwater mosquitoes are problematic, mainly for tourism; therefore, since 1988 have been carried out treatments with the biopesticide VectoBac-G®, whose active components are endotoxins and viable spores of *Bacillus thuringiensis* var. *israelensis* (Bti), strain AM 65-52 (referred as Vec8 throughout this thesis). During a previous project, a new strain of Bti, named A19, was isolated from Bolle di Magadino natural reserve. This thesis aimed to characterize this strain through physiological, toxicological and molecular approaches; and assess the possibility to apply A19 strain as autochthonous control agent against mosquitoes. The A19 strain growth profile was obtained through a growth curve, showing to be similar to the Vec8 Bti reference strain. The A19 strain biochemical profile was obtained by using the API galleries, Vitek ID-GP cards and serotyping. Concerning the serotyping, A19 strain was classified as "un-testable". The morphology was characterized at macro and microscopic levels, and some unique features on A19 strain were detected relatively to the Bti reference strains (Vec8, Bioflash, Bti4 e IP4444), as bigger colonies and absence of flagella. During the toxicological test its toxicity against mosquito larvae was confirmed and registered as equivalent to the Bti reference strains, through a bioassay. Furthermore, A19 strain showed to produce a bigger amount of toxin per spore than the Bti reference strains, which, for a biopesticide, can be an advantage at economic and environmental levels. The spore-phase total proteins' profile of A19 strain obtained through a SDS-PAGE, was similar to the profiles obtained for Bti reference strains. Three missing proteins were detected on the A19 strain's 2-D PAGE vegetative cell total proteins' profile, when compared with the Bti reference strains. From a dendrogram, based on the vegetative cell total proteins' profile, obtained by the MALDI-ToF MS technique, Vec8 strain showed to be the Bti reference strain more similar to A19 strain. However, there are morphological and molecular evidences that support the autochthonous condition of A19 strain. The A19 strain *cry* and *cyt* genes were detected, as well as the 16S ribosomal subunit encoding region, through a PCR followed by an electrophoresis run in agarose gel. A19 strain showed to have potential to be used as biopesticide, and to have advantages at economical and environmental level, comparatively to the Bti reference strains.

Key words: *Bacillus thuringiensis* var. *israelensis*, spore-forming bacteria, endotoxins, biopesticide

SUMÁRIO

Bolle di Magadino é uma reserva natural de zonas húmidas, localizada no sul da Suíça, com importância a nível internacional devido à sua fauna e flora únicas. Aqui, os mosquitos representam um problema, principalmente para o turismo; por isso, desde 1988 têm sido realizados tratamentos com o biopesticida VectoBac-G®, cujos componentes ativos são endotoxinas e esporos viáveis de *Bacillus thuringiensis* var. *israelensis* (Bti), estirpe AM 65-52 (referida como Vec8 ao longo desta tese). Durante um projeto anterior uma nova estirpe de Bti, nomeada A19, foi isolada na reserva natural Bolle di Magadino. O objetivo deste trabalho é caraterizar esta estirpe através de abordagens fisiológicas, toxicológicas e moleculares, e avaliar a possibilidade de aplicar a estirpe A19 como agente autóctone para controlo da população de mosquitos. O perfil de crescimento da estirpe A19, conseguido através da construção de uma curva de crescimento, apresentou-se semelhante ao perfil obtido para a estirpe de referência Vec8. O perfil bioquímico da estirpe A19 foi obtido através de galerias API, do cartão Vitek ID-GP e serotipagem. Considerando a serotipagem, a estirpe A19 foi classificada como "un-testable". A caracterização morfológica foi conseguida a nível macro e microscópico, e foram detectadas características únicas da estirpe A19 relativamente às estirpes de Bti de referência (Vec8, Bioflash, Bti4 e IP4444), como colónias maiores e ausência de flagelos. Durante o teste toxicológico, a sua toxicidade contra larvas de mosquito foi confirmada, e registada como equivalente às estirpes de Bti de referência. Para além disso, a estirpe A19 mostrou produzir uma maior quantidade de toxina por esporo, relativamente às estirpes de Bti de referência, o que pode ser vantajoso a nível económico e ambiental, como biopesticida. O perfil de proteínas da fase esporulativa obtido através de SDS-PAGE foi semelhante aos perfis obtidos para as estirpes de Bti de referência. Foi detetada a ausência de 3 proteínas no perfil 2-D PAGE de proteínas totais da fase celular vegetativa, comparativamente com as estirpes de Bti de referência. A partir de um dendrograma baseado em perfis de proteínas totais da fase celular vegetativa obtidos pela técnica MALDI-ToF MS, a estirpe de referência Vec8 foi a mais próxima da estirpe A19. No entanto existem evidências a nível morfológico e molecular que suportam a condição autóctone da estirpe A19. Os genes *cry* e *cyt*, assim como a região codificante da subunidade ribossomal 16S, foram amplificados através de PCR, e detetados através de uma eletroforese em gel de agarose. A estirpe A19 mostrou ter potencial para ser utilizada como biopesticide, e apresentou vantagens a nível económico e ambiental.

Palavras-chave: *Bacillus thuringiensis* var. *israelensis*, bateria formadora de esporos, endotoxinas, biopesticida

RESUMO

Os mosquitos representam uma ameaça para a saúde humana, pois são vetores de transmissão de várias doenças tropicais, e podem também representar um grande incómodo para o Homem. A utilização de *Bacillus thuringiensis* var. *israelensis* (Bti) como biopesticida comporta diversas vantagens relativamente a inseticidas sintéticos, uma vez que este microrganismo produz uma toxina biodegradável, com elevada especificidade para o organismo alvo.

Bolle di Magadino é uma reserva natural de zonas húmidas localizada no sul da Suíça, cantão Ticino, com importância a nível nacional e internacional devido a sua fauna e flora únicas. As enchentes periódicas do plano de Bolle di Magadino, devido a chuvas intensas durante a Primavera e Verão, tornam as condições favoráveis para a reprodução de mosquitos, principalmente das espécies *Aedes vexans* e *Ochlerotatus sticticus*. A grande densidade de mosquitos nesta área representa um grande problema, especialmente para a economia turística, e para combate-lo têm sido realizados tratamentos regulares com o biopesticida VectoBac-G® (Valent BioSciences, liberty ville, IL, USA), desde 1988. Os componentes ativos deste produto são endotoxinas e esporos viáveis de *Bacillus thuringiensis* var. *israelensis* (Bti), estirpe AM 65-52 (referenciada ao longo deste trabalho como Vec8). Durante um projeto anterior realizado por Chappuis (2002), cujo objetivo era a monitorização do destino dos esporos de Bti provenientes do tratamento com VectoBac-G® no solo da reserva de Bolle di Magadino, foi isolada uma nova estirpe de *Bacillus thuringiensis* var. *israelensis*, nomeada A19. Admitindo a possibilidade de ser uma estirpe autóctone da reserva natura Bolle di Magadino, é importante um melhor conhecimento das suas características assim como possíveis vantagens relativamente a outras estirpes de Bti, nomeadamente a estirpe Vec8. Dependendo das características da estirpe A19, a sua produção a nível comercial como biopesticida, e a substituição do produto VectoBac-G® para controlo da população de mosquitos na reserva natural Bolle di Magadino, são possibilidades a considerar.

Este trabalho tem como objetivo aprofundar o conhecimento sobre a estirpe A19 através da sua caraterização morfológica, bioquímica, proteómica, genómica e toxicológica, em paralelo com estirpes Bti de referência (referenciadas ao longo deste trabalho como Vec8, Bioflash, Bti4 e IP4444), para avaliar a possibilidade de utilizar A19 como agente autóctone de controlo da população de mosquitos da reserva natural Bolle di Magadino.

O perfil de crescimento da estirpe A19 foi analisado através de uma curva de crescimento. O tempo de duplicação (T_d) da estirpe A19 (33.16 ± 0.76 min) determinado através da construção de uma curva de crescimento, utilizando como meio de cultura o meio

líquido TSB, não apresentou diferenças significativas (t-teste, $p = 0.07$) quando comparado com a estirpe Vec8 (31.07 ± 1.02 min).

O perfil bioquímico de A19 foi determinado através das galerias API 20E, API 50CHB e complementado com o cartão Vitek de identificação de batérias Gram-positivas, e serotipagem. Os testes de fermentação de D-mannose, arbutina e D-cellobiose, de deteção da enzima phosphatidylinositol phospholipase C e a capacidade de crescer em 6.5% NaCl foram considerados discriminantes. As 5 estirpes de Bti testadas apresentaram 100% de resistência a Polimixina B, Bacitracina, O/129 e Optoquina, sendo o teste à Novomicina o único no qual nenhuma das estirpes apresentou 100% resistência. O serotipo foi testado com anticorpos anti-H-14, com a estirpe A19, estirpes de Bti de referência e uma estirpe de *Bacillus thuringiensis* var. *kurstaki* (Btk). Apesar de os resultados de serotipagem obtidos não serem evidentes, a reação de aglutinação foi considerada negativa para a estirpe A19.

A mobilidade, "swimming", das estirpes de Bti foi testada e confrontada com um controlo negativo em meio MIO semi-sólido. Contrariamente às estirpes de Bti de referência, a estirpe A19 apresentou resultado negativo para a mobilidade.

A caracterização morfológica da estirpe A19 foi obtida respeitando a terminologia utilizada no "Bergey's manual of systematic bacteriology – The Firmicutes" (Vos *et al.*, 2009). A nível macroscópico, foi feita a caracterização das colónias. Em meio de sangue agarizado as colónias da estirpe A19 e das estirpes de Bti de referência apresentaram uma forma circular, com a margem ligeiramente ondulada, a superfície plana e sem brilho, consistência butirosa, de cor cinza e opaca. A área média das colónias da estirpe A19 (49.08 ± 5.80 mm^2) foi significativamente maior (Tukey's HSD, $p < 0.05$) que as restantes estirpes. Foi também feita uma análise microscópica, considerando a morfologia da célula vegetativa, a área seccional longitudinal dos esporos, a área seccional do cristal parasporal, e deteção da presença de flagelos nas 5 estirpes através do método de coloração "flagelar Leifson". As células da estirpe A19 na fase vegetativa apresentam forma de bastonete, caraterística das batérias do género *Bacillus*. Assim como nas estirpes de Bti de referência, durante a fase esporulativa da estirpe A19 foi detetada a produção de corpos cristalinos com forma predominantemente globular tipicamente verificada em estirpes de *Bacillus thuringiensis* var. *israelensis*. A área seccional média dos esporos da estirpe A19 (1 ± 0.16 μm^2) foi significativamente mais pequena (Tukey's HSD, $p < 0.05$) que a área obtida para as estirpes Vec8 e Bioflash. A estirpe A19 distinguiu-se das estirpes de Bti de referência a nível morfológico, principalmente pela grande dimensão das suas colónias e a ausência de flagelos nas células em fase vegetativa. A não-mobilidade da estirpe A19, assim como a ausência de flagelos suportam o resultado negativo obtido na serotipagem, e levaram à classificação da estirpe A19 como "un-testable" relativamente à serotipagem, de acordo com Lee *et al*. (2003).

Relativamente à análise toxicológica, foi feita a quantificação da toxina por esporo para estirpe A19 e estipes de Bti de referência; e foi realizado um bioteste. A quantificação de esporos e de proteína total de uma suspensão de esporos-toxina da estirpe A19 e das estirpes de Bti utilizadas como referência permitiu calcular e comparar a quantidade média de toxina produzida por esporo, por cada estirpe. A estirpe A19 demonstrou produzir uma maior quantidade de toxina por esporo ($1.64x10^{-4} \pm 5.84x10^{-6}$ ng de toxina por esporo) do que as 4 estirpes de referência (Tukey's HSD, $p < 0.05$). O bioteste com larvas *Aedes aegypti*, estirpe Rockefeller, permitiu confirmar a atividade tóxica da estirpe A19 contra larvas de mosquito, determinar e comparar o valor de CL_{50} da estirpe A19 e das outras estirpes de Bti através de uma análise probit. Os valores de CL_{50} das estirpes A19 (1.05 ng mL^{-1}), Vec8 (1.23 ng mL^{-1}), Bioflash (0.97 ng mL^{-1}), Bti4 (1.14 ng mL^{-1}), e IP4444 (1.43 ng mL^{-1}) não apresentaram diferenças significativas (ANOVA, $p = 0.16$). Uma vez que a atividade tóxica apresentada é influenciada pelas proporções de δ-endotoxinas constituintes do cristal parasporal, este resultado pode significar que a constituição dos cristais produzidos pela estirpe A19 é semelhante à composição dos cristais produzidos pelas estirpes de referência.

A análise proteómica da estirpe A19 envolveu a obtenção do perfil de proteínas totais da fase esporulativa, com recurso à técnica de SDS-PAGE (12%), e comparação do perfil com as estirpes de Bti de referência, uma estirpe de *Bacillus sphaericus* e uma estirpe de *Bacillus thuringiensis* var. *Kurstaki* (Btk). A coloração dos géis de proteínas foi feita com solução de nitrato de prata ou solução de Coomassie blue. O perfil proteico da fase esporulativa da estirpe A19 apresentou-se semelhante ao perfil das estirpes de Bti testadas, afastando-se muito dos perfis correspondentes à fase de esporulação da estirpe de *B. sphaericus* e de Btk, o que suporta a presença de todo o plasmídeo pBtoxis na estirpe A19. Nos perfis obtidos para as estirpes de Bti foi possível identificar as bandas correspondentes às toxinas Cry e Cyt constituintes do cristal parasporal. O facto de a banda correspondente às proteínas Cry4 se apresentar muito fina, pode dever-se a uma migração imperfeita das proteínas ou degradação rápida das mesmas. A difícil separação entre as bandas correspondentes a Cry10Aa e Cry11Aa, e Cyt1Aa e Cyt2Ba deve-se à proximidade dos seus pesos moleculares e à elevada concentração de proteína.

Os perfis de proteínas totais da fase celular vegetativa das 5 estirpes de Bti foram obtidos através de 2-D PAGE e MALDI-ToF MS. No perfil de proteínas totais obtido através de 2-D PAGE da estirpe A19, verificou-se a ausência de 3 bandas relativamente às outras estirpes. Considerando a ausência de flagelos na estirpe A19, estas bandas podem corresponder a proteínas flagelares, como FlhF (*pI* = 5.55 e Pm = 49.85; SwissProt), e proteína flagelar 2 associada ao gancho (*pI* = 5.67 e Pm = 49.90; SwissProt). A terceira proteína parece corresponder à banda marcada como B6 por Hirose e colaboradores (2000), não

identificada. A ausência de flagelos pode significar a diminuição da libertação de fatores de virulência, o que representaria uma vantagem desta estirpe como biopesticida, por isso será importante identificar as proteínas ausentes no perfil proteico da estirpe A19.

Os perfis de proteínas obtidos através da técnica espectrometria de massa MALDI-ToF possibilitaram a construção de um dendrograma, e assim o agrupamento de diferentes estirpes de *Bacillus*, realçando a semelhança da estirpe A19 com as estirpes Bti de referência (Vec8, Bioflash, Bti4 e IP4444), nomeadamente com a estirpe Vec8. Apesar das semelhanças, evidências a nível morfológico e molecular suportam a condição autóctone da estirpe A19.v

A abordagem genómica incluiu a amplificação da região codificante da subunidade ribossomal 16S e amplificação das regiões codificantes das proteínas Cry e Cyt, através de PCR e posterior deteção em gel de agarose; e tentativa de obtenção da "impressão digital" da estirpe A19 através da aplicação da técnica de RAPD-PCR. Foi feita uma extração de DNA total, à estirpe A19 e estirpes de referência. A qualidade do DNA extraído foi confirmada através da amplificação da região de DNA codificante correspondente à subunidade ribossomal 16S, apresentando nas 5 estirpes cerca de 1500 pb. Foram detetados os genes codificantes das endotoxinas Cry4Aa, Cry4Ba, Cry10Aa, Cry11Aa, Cyt1Aa e Cyt2Ba, através da realização de um PCR e eletroforese em gel de agarose. A sua deteção apoia a hipótese da presença de todo o plasmídio pBtoxis na estirpe A19, assim como nas estirpes de referência. A obtenção de "impressões digitais" de DNA através do método de RAPD-PCR não foi bem-sucedido.

Em conclusão, este estudo suporta a classificação da estirpe A19 como *Bacillus thuringiensis* var. *israelensis*, e permitiu aprofundar o conhecimento sobre esta estirpe. A condição da estirpe A19 como "microrganismo autóctone" foi apoiada a nível morfológico e molecular. A estirpe A19 mostrou ter potencial como biopesticida e ainda vantagens a nível ambiental e económico.

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ABBREVIATIONS

% (v/v)	Percentage (mL/mL)
% (w/v)	Percentage (g/mL)
x g	Relative centrifugal force
2-D	Two-dimensional
π	Pi = 3.14159265...
APS	Ammonium persulfate
ATCC	American type culture collection
ATP	Adenosine-5'-triphosphate
ATPase	Adenisine triphosphatase
Ba	*Bacillus anthracis*
Bc	*Bacillus cereus*
bp	Base pairs
BSA	Bovine serum albumine
Bt	*Bacillus thuringiensis*
Bti	*Bacillus thuringiensis* var. *israelensis*
Btk	*Bacillus thuringiensis* var. *kurstaki*
CHAPS	3 - [(3 - cholamidopropyl) dimethylammonio] propanesulfonic acid
CHCA	α-cyano-4-hydroxycinnamic acid
CI 95%	95% confidence interval
CL_{50}	Concentração letal de 50% da população
DAPI	4',6-diamidino-2-phenylindole
DNA	Deoxyribonucleic acid
dNTPs	deoxyribonucleotide
DSM	Deutsche Sammlung von Mikroorganismen
DTT	1,4–dithio–D–threitol
EDTA	Ethylenediaminetetraacetic acid
EtOH	Ethanol
Fig.	Figure
GYS	Glucose-yeast-salts
hrs	Hours
ICP	Insecticidal crystal protein
IEF	Isoelectric focusing
IntDen	Intensity/Density
IP	Institut Pasteur
IPG	Immobiline™ DryStrip gel

p*I*	Isoelectric point
k	Specific growth rate
LB	Lysogeny broth
LC_{50}	Lethal concentration of 50% of the population
LPS	Lipopolysaccharide
MALDI	Matrix-assisted laser desorption/ionization
min	Minutes
MIO	Motility indole ornithine
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry
Mw	Molecular weight
n	Number of replicates
ND	Not determined
NL	Non linear
OD	Optical density
o/n	Overnight
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PFT	Pore forming toxins
Pm	Peso molecular
PMF	Peptide mass fingerprinting
r	Radius
RAPD	Random amplified polymorphic DNA
RNA	Ribonucleic acid
rpm	Rotations per minute
RT	Room temperature
SD	Standard deviation
SDS	Sodium dodecyl sulphate
sec	Seconds
SEM	Scanning electron microscopy
sol.	Solution
TBE	Tris - borate - EDTA
T_d	Doubling time
TEMED	Tetramethylethylenediamine
TFA	Trifluoroacetic acid

ToF	Time-of-flight
TSB	Tryptone soy broth
UV	Ultra violet
VP	Voges-Proskauer

1. INTRODUCTION

1.1. *Bacillus thuringiensis* var. *israelensis* (Bti)

1.1.1. History

Mosquitoes are the main group of arthropods responsible for diseases (Cepleanu, 1993; Ciccia *et al*., 2000). They are a big threat for public health by transmitting various tropical diseases and by being a nuisance (Otieno-Ayayo *et al*., 2008; Service, 2004). Many species of the *Anopheles*, *Aedes* and *Culex* genera, are vectors of diseases as malaria (WHO, 2006), yellow fever, dengue (Braga & Valle, 1998), hemorrhagic fever (WHO, 1997) and lymphatic filariasis (Gyapong & Twum-Danso, 2006). The development of chemical insecticides was considered a simple and inexpensive tool against some serious disease vectors (Margalit & Dean, 1985). However, over the time problems began to emerge, as the environmental pollution, human health effects, insect resistance and consequently high costs for new synthetic chemical products (Margalit & Dean, 1985; Schnepf *et al*., 1998). These occurrences led to the necessity for biological agents (Bravo *et al*., 2011; Margalit & Dean, 1985).

The use of the entomopathogenic bacterium *Bacillus thuringiensis* var. *israelensis* (Bti) as a biopesticide was a valid alternative due to several advantages. The Bt toxins are highly specific for the target organisms, harmless to humans, to other mammals, birds, amphibians, reptiles and plants, and are biodegradable, so no residual toxic products accumulate in the environment (Bravo *et al.*, 2011; Schnepf *et al.*, 1998).

In Israel, 1975-76, during a World Health Organisation (WHO) sponsored project, a new *Bacillus thuringiensis* (Bt) strain with high toxic activity against mosquito larvae was discovered (Goldberg & Margalit, 1977; Margalit & Dean, 1985). It was isolated from cadavers of mosquitoes in the Negev desert of Israel (Goldberg & Margalit, 1977; Margalit & Dean, 1985). Latter, in 1978, its identification was performed by De Barjac and published as *Bacillus thuringiensis* var. *israelensis* (Bti), serotype H-14 (De Barjac, 1978; Margalit & Dean, 1985). It was demonstrated that Bti has a toxic specificity for some insects from the Diptera order, mainly mosquitoes (Culicidae family) and black flies (Simuliidae family), leading to the emergence of interest for this microorganism as a control agent against Diptera (Glare & O'Callaghan, 1998). Commercial products started being developed in the 1980's, and during more than three decades formulations of Bti were improved and used worldwide as biopesticide (Glare & O'Callaghan, 1998).

1.1.2. Characteristics

Bacillus thuringiensis var. *israelensis* is a Gram-positive, rod-shaped, mesophilic, aerobic or facultative anaerobic, endospore-forming, usually motile with peritrichous flagella and entomopathogenic bacterium (Vos *et al.*, 2009). It is an ubiquitous bacterium that can be

isolated from different habitats, as soil and insects cadavers (Schnepf *et al.*, 1998). During sporulation, Bti produces one or more globular shaped proteinaceous crystals per spore, with toxic activity typically against members from Diptera order (Tyrell *et al*., 1981), which are codified by genes located on the pBtoxis megaplasmid (127.92 kb) (Berry *et al.*, 2002).

1.1.3. Classification

The term "*Bacillus cereus* group", usually used to refer a group of Gram-positive, rod-shaped and endospore-forming bacteria whose taxonomy is still not very clear, has no taxonomic value (Bavykin *et al*., 2004; Drobniewski, 1993; Vilas-Bôas *et al*., 2007). *Bacillus thuringiensis* (Bt) is included in the *Bacillus cereus* group, together with four other species, *B. cereus* (Bc) (*stricto sensu*), *B. anthracis* (Ba), *B. mycoides*, *B. pseudomycoides and B. weihenstephanensis* (Vilas-Bôas *et al*., 2007). Bt, Bc (*stricto sensu*) and Ba are genetically very close, which complicates their classification (Vilas-Bôas *et al*., 2007). Initially, these organisms were classified as different species due to the big importance of some phenotypical differences (Bavykin *et al*., 2004; Vilas-Bôas *et al*., 2007). Ba can produce a capsule and a toxin responsible for a human-affecting disease, the anthrax; Bc (*stricto sensu*) can cause food poisoning; and Bt produces the parasporal crystal with toxic activity against insects from diverse orders (Vilas-Bôas *et al*., 2007). Afterward, the comparison of their 16S rRNA sequences supported a single-specie hypothesis, since these organisms showed more than 99% of sequence similarity, and as conservative criterion is recommended at least 3% of divergence to delimit species (Bavykin *et al*., 2004). The single-specie hypothesis is supported by other molecular methods, such as chromosomal DNA hybridization (Seki *et al*., 1978), amplified fragment length polymorphism (AFLP) analysis and cellular fatty acid patterns (Hill *et al*., 2004).

The classical biochemical tests, that can be very variable, and morphological methods used for bacteria classification, do not allow the distinction between Bc and Bt (Schnepf *et al*., 1998). However, they complement the information about the organism and help when other techniques are unclear. The production of a parasporal body and the presence of its coding-plasmid in Bt, allow its distinction from the other members of the *Bacillus cereus* (*lato sensu*) group (Vilas-Bôas *et al*., 2007).

Bt strains have been classified into different types, based on their flagellar antigens. The H-serotyping method, based on an immunological reaction between the H-specific anti-sera and the flagellar H-antigens, has been an useful test for Bt strains classification for many years, due to its easiness and specificity (De Barjac & Frachon, 1990). Despite its utility, this methodology presents some limitations, such as the possibility of a spontaneous autoagglutination in NaCl (0.85%) in the absence of a specific antiserum, observed in some strains; and the inability to serotype Bt strains without flagella (Lecadet *et al*., 1999; Chaves

et al., 2008). Isolates without a parasporal inclusion reacting with the Bt specific anti-sera can also be found (Lecadet *et al*., 1999). These isolates can represent Bt strains that have lost the crystal proteins codifying plasmid, or Bc with antigens in common with Bt (Lecadet *et al*., 1999).

Molecular methods such as sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) of total cell proteins (Berber, 2004), random amplified polymorphic DNA Polymerase chain reaction (RAPD-PCR) (Chaves *et al*., 2010; Kumar *et al*., 2009), and extended multiplex PCR (Ben-dov *et al*., 1997), can be very informative for the identification of those strains that cannot be serotyped through the flagellar antigen technique. The use of the SDS-PAGE technique of total cell proteins to compare the different profiles obtained between strains can be useful to group bacteria, since the most similar profiles correspond to closer strains (Berber, 2004; Black, 2004). RAPD-PCR is considered a very useful technique for the discrimination of related organisms and molecular characterization based on their DNA fingerprints (Chaves *et al*., 2010; Kumar *et al*., 2009). The extended multiplex PCR screening is considered a rapid method to detect and differentiate the different Bt strains, and predict their insecticidal activities, based on the PCR product profile (Ben-dov *et al*., 1997).

1.1.4. Parasporal Crystal

The Bti crystalline inclusions are globular-shaped, with a diameter average of 1 µm (Boisvert, 2005). They are produced adjacent to the endospore during the stationary growth phase of the bacterium (Schnepf *et al*., 1998). These parasporal inclusions are produced as one or more units per spore, and are mainly composed by one or more insecticidal proteins (δ-endotoxins; ICPs; Cry and Cyt proteins) (Hofte & Whiteley, 1989; Tyrell *et al.*, 1981; WHO, 1999). The δ-endotoxins are classified in two multigenic families, Cry and Cyt, based on the tridimensional structure and mode of action of the proteins (Chaves *et al.*, 2008). Bti produces four known Cry proteins (Cry4Aa, Cry4Ba, Cry10Aa and Cry11Aa) and two Cyt proteins (Cyt1Aa and Cyt2Ba), synthesized as protoxins, i.e. proteins that need to be modified to be toxic (Angsuthanasombat *et al.*, 2004; Berry *et al.*, 2002). The proteolytic activation of the protoxins occurs by the cleavage of fragments from both N- and C-terminals (Bravo, 1997). Cry and Cyt proteins, can act synergistically (Schnepf *et al.*, 1998).

1.1.4.1. Cry proteins

The proteins from the Cry multigenic family are all structurally similar: globular molecules composed of three domains (Bravo *et al*., 2008; Schnepf *et al*., 1998). The domain I, in the N-terminal region of the polypeptide, is composed by a seven helix bundle, a hydrophobic helix surrounded by six anphipathic helices, responsible for the membrane insertion of the toxin and pore formation. The domain II is composed by three anti-parallels β-sheets

rounding a hydrophobic nucleus, and forming a prism-like structure; its loop regions are implicated in toxin-receptor interaction and specificity. The domain III is in the C-terminal region, and is composed by two anti-parallels β-sheets (Angsuthanasombat *et al*., 2004; Bravo *et al*., 2008). Its exposed regions, as for domain II, are involved in the receptors connection (Bravo *et al*., 2008). The domain III shares some structural similarity with carbohydrate-binding proteins, what suggests that the carbohydrate presence can influence the mode of action of the three Cry toxins domains (Bravo *et al*., 2008). The Cry proteins have a range of molecular masses from 50 kDa to 140 kDa (Bravo, 1997). During the activation, Cry4Aa and Cry4Ba protoxins (130 kDa) are processed in 47 - 48 kDa and 16 - 18 kDa polypeptides (Angsuthanasombat *et al*., 1993), which have an intermediate form of approximately 60 kDa (Yamagiwa *et al*., 2004). The Cry11 protoxin (70 kDa) became active after being processed in a 34 kDa and a 32 kDa associated fragments (Bravo *et al.*, 2008; Yamagiwa *et al.*, 2004).

1.1.4.2. Cyt proteins

The Cyt proteins include two very related gene families (*cyt1* and *cyt2*) (Bravo *et al*., 2008). Cyt proteins belong to a class of bacterial toxins known as pore-forming toxins (PFT); they are secreted as water-soluble proteins that undergo conformational changes in order to be insert into the membrane (Bravo *et al*., 2008; Bravo *et al*., 2011). They have a single α-β domain, where the two outer layers of the α-helix hairpins are wrapped around the β-sheet (Bravo *et al*., 2008). Cyt proteins, also known as hemolytic factors, have an hemolytic (cytolytic) activity *in vitro* (Butko, 2003; Thomas & Ellar, 1983). These proteins are mainly associated to Bt strains that are active against some insects from the Diptera order, but are not exclusive of strains with this pathotype (Bravo *et al*., 2008; Guerchicoff *et al*., 2001). Similar to the Cry proteins, the Cyt proteins are synthesized as protoxins and became active after the cleavage of small portions in the N- and C-terminal ends (Bravo *et al.*, 2008; Cahan *et al.*, 2008). The Cyt1Aa and Cyt2Bb protoxins have a molecular weight of 27 - 28 kDa, and after their activation their molecular weights are approximately 23 - 24 kDa (Cahan *et al.*, 2008; Guerchicoff *et al.*, 1997).

1.1.4.3. Mode of action

After the ingestion by the susceptible insect larvae, the Bt crystals are dissolved in the alkaline midgut pH and the protoxins are activated by proteases in the larval gut (Angsuthanasombat *et al.*, 1993, 2004; Yamagiwa *et al.*, 1999). The activated toxic peptides are relatively more resistant to the continuous action of proteases (Angsuthanasombat *et al*., 2004). Cry active toxins bind to diverse and specific receptors in the midgut epithelial cells of the microvilli apical membrane, which leads to toxin oligomerization and membrane insertion, creating a permeable pore (Gómez *et al*., 2007). These pores cause the swelling and

osmotic lyses of the target cells, resulting in serious damages of the larval midgut, with the consequent death of the larva (Angsuthanasombat *et al*., 2004). Contrary to the Cry toxins, Cyt toxins interact directly with the unsaturated membrane phospholipids, due to their highly hydrophobic nature (Cahan *et al*., 2008; Thomas & Ellar, 1983). After this interaction, the toxins insert into the membrane and form pores in the cell membrane, or destroy it by a detergent-like interaction, what will damage the larvae midgut cells, and lead to their death (Butko, 2003; Gómez *et al*., 2007).

1.1.4.4. Synergism

Up to now, no resistance to Bti toxins has been observed in the field by the mosquito species controlled with Bti-based products (Ibarra *et al*., 2003; Pérez *et al*., 2005). The apparent lack of resistance to the Bti biopesticide products may be due to the presence of the Cyt1Aa protein in the crystal (Bravo *et al*., 2008). With its presence, resistance to Cry proteins cannot be selected (Bravo *et al*., 2008; Gómez *et al*., 2007). It was established that Cyt1Aa and Cry11Aa toxins have a synergetic effect. In fact, the Bti crystals activity, that usually involves more than one protein, is much higher compared to the toxic activity of single proteins (Bravo *et al*., 2008). Cyt1Aa seems to act as a receptor molecule, i.e. Cyt1Aa inserts into the membrane and exposes specific regions, promoting the biding of Cry11Aa and its insertion into the cell membrane, leading to an increased toxic effect (Gómez *et al*., 2007; Pérez *et al*., 2005).

1.1.5. Effects on Humans

The δ-endotoxins produced by Bt present high specificity to the target organism and showed to be safe to non-target mammals (Gómez *et al*., 2007; Margalit & Dean, 1985; Thomas & Ellar, 1983b).

In order to be accepted as the main component of a biopesticide, a Bti strain has to fulfil some stringent biosafety criteria (Boisvert, 2005). Only if it is safe for humans and vertebrates and for the environment, it can be commercialized (Boisvert, 2005). Despite the tests performed to ensure the safety of a strain, it was demonstrated that, as for *B. cereus* (*stricto sensu*), also Bti strains could produce virulence factors during the vegetative growth, including some of the strains already commercialized (Ghelardi *et al*., 2002; Yuan *et al*., 2002).

1.2. The natural reserve Bolle di Magadino

1.2.1. Importance and generalities

Bolle di Magadino (Fig. 1) is a natural wetland reserve of national importance, located in Canton Ticino, Southern of Switzerland (coordinates: 46º 09’ N 008º 52’ E; Elevation: 192.2 m - 202 m, average 197 m; 662.5 ha). This reserve belongs to the municipalities of Locarno, Gordola, Magadino, and Tenero-contra (Gonet, 2004). The plain of Magadino is a residual

part of a swamp, that includes a delta created by the outlet of Ticino and Verzasca rivers on the Lake Maggiore, with a width extension of 3'400 m (Gonet, 2004). The climate is temperate (annual average temperature: 10.8 °C) due to the thermoregulatory influence of the lake and to the presence of the Alpine chain, that protects the area from the northern winds (Gonet, 2004). The precipitations are abundant (annual average precipitations: 1904 mm), mainly during the spring and autumn seasons (Gonet, 2004).

Fig. 1. Bolle di Magadino, Verzasca Valley point of view. (Picture: I. Cabral).

Bolle di Magadino is a remarkable area for its biodiversity (fauna and flora), and its importance as a breeding and migratory site for many species of birds (Gonet, 2004; Lucchini *et al*., 1990). It is also important for its social and cultural value (forestry production, fishing, agriculture, leisure activities, education and industry), and even for its landscapes (Gonet, 2004). Its characteristics made Bolle di Magadino a protected area for the Canton Ticino since 1979 (Gonet, 2004), and led to its inclusion on the list of wetlands of international importance of the Ramsar Convention, in 1982 (Ramsar, Iran, 1971).

1.2.2. Problematic

The periodic floods of the Bolle di Magadino plain during the spring and summer months due to heavy precipitation and rising level of the Lake Maggiore, made this area a major breeding site for the floodwater mosquitoes, mainly *Aedes vexans* and *Ochlerotatus sticticus* species (Eilenberg & Hokkanen, 2006). The high density of mosquitoes developing in this area represents a big problem, especially given the impact on tourism economy. Since 1998, a research group for monitoring Bti in the ecosystem, deals with the control of mosquitoes populations using a Bti-based product (Gonet, 2004). Regular treatments with VectoBac-G® (Valent BioSciences, Libertyville, IL, USA) have been carried out since 1988 in Bolle di

Magadino (Guidi *et al*., 2010). Aerial applications by helicopter have proved to be the only method to reach efficiently the larval breeding sites (Eilenberg & Hokkanen, 2006). VectoBac-G® is a biopesticide composed by a natural and innocuous corn cop granules supporting the active component, the parasporal crystals and viable spores of Bti (strain AM 65-52) (Eilenberg & Hokkanen, 2006).

During a previous project aiming to monitor the fate of Bti spores in the soil of the Bolle di Magadino reserve, a new strain of *Bacillus thuringiensis* var. *israelensis*, named A19, was isolated in 2002 by Chappuis (Chappuis, 2002). Since this new strain is probably an autochthonous organism showing toxic activity against mosquito larvae, a better knowledge of its characteristics is important. Depending on its characteristics, the replacement of the VectoBac-G® formulation and the commercial production of A19 strain, are possibilities that can be considered in the future.

1.3. Aim of the Project

This thesis aimed to deepen the knowledge about A19 Bti strain, through physiological, toxicological and molecular approaches and the application of the MALDI-ToF MS technique as a complementary analysis, in order to access the possibility to apply A19 strain as an autochthonous control agent against mosquito larvae at the natural reserve Bolle di Magadino.

In order to achieve the scope, morphological characterizations, at macro and microscopic level, were performed. Moreover the A19 strain was tested for motility, and its biochemical profile, by using API galleries, Vitek identification (ID) Gram-positive (GP) cards and serotyping, were determined. The proteomic analysis of A19 strain was carried out by spore-phase total proteins' profile by SDS-PAGE, and vegetative cell-phase total proteins' profile obtained by 2-D PAGE, and MALDI-ToF MS techniques. The genomic analysis, by amplification and detection of 16S ribosomal encoding region and detection of toxin coding genes, was performed. The toxicity of A19 strain through a bioassay, using *Aedes aegypti*, Rockefeller strain mosquito larvae, was also evaluated. All techniques used were applied in parallel to AM 65-52 Bti strain (referred throughout the study as Vec8), the active component of the VectoBac-G® commercial product currently applied in Bolle di Magadino reserve, and three other Bti reference strains (referred throughout the study as Bioflash, Bti4 and IP4444).

2. MATERIALS AND METHODS

2.1. Bacterial strains

Five different strains of *Bacillus thuringiensis* var. *israelensis* were included in this study, as well as six non-Bti strains from ICM collection used as control strains (Table 1). All the

bacterial strains used were initially stocked in skim milk solution (10% (w/v) skim milk, 10% (v/v) bovine serum, 20% (v/v) glycerol) at -80 °C.

Table 1. Bacterial strains.

Species	**Strains***	**Reference name in this work**	**Origin of the strain**
Bacillus thuringiensis var. *israelensis*	A19	A19	Sediment sample
Bacillus thuringiensis var. *israelensis*	AM 65-52	Vec8	Commercial product (VectoBac-G®; Valent BioSciences, Libertyville, IL, USA)
Bacillus thuringiensis var. *israelensis*	DSM 5724	Bti4	Commercial product
Bacillus thuringiensis var. *israelensis*	M-H-14	Bioflash	Commercial product (Bioflash® GR (Nature Biotechnology Company, Tehran, Iran)
Bacillus thuringiensis var. *israelensis*	IP4444	IP4444	Unknown
Bacillus thuringiensis var. *kurstaki*	DSM 5725	Btk5	Commercial product
Bacillus cereus	ATCC10876a	Bce1	Contaminated bottle
Bacillus sphaericus	2362	*B. sphaericus*	Commercial product VectoMax-G (Valent BioSciences, Libertyville, IL, USA)
Klebsiella pneumoniae	ATCC13883^{T}	*Klebsiella pneumoniae* ATCC13883	Unknown

Table 1. (Continued).

Species	Strains*	Reference name in this work	Origin of the strain
Pseudomonas aeruginosa	ATCC27853	*Pseudomonas aeruginosa* ATCC27853	Blood culture
Escherichia coli	K12 (GM48 genotype) ATCC 39099	*E. coli* K12	large intestine, colon

* ATCC: American Type Culture Collection; DSM: Deutsche Sammlung von Mikroorganismen, Braunschweig, Germany; IP: Institut Pasteur, Paris, France
[T] Type strain

2.2 Growth conditions

Before each test, bacteria were grown overnight (o/n) on blood agar solid medium (Nutrient Columbia Agar Base supplemented with 5% of sheep erythrocytes; Becton Dickinson AG, Basel, Switzerland) to obtain the fresh cultures.

For the bacterial growth curve, bacteria were grown in TSB medium (3% (w/v) tryptone soy broth (TSB-broth; Oxoid, Switzerland), distilled water) with agitation (approximately 150 rpm) (Unimax 1010 shaker, Heidolph, Germany).

To obtain the spores-toxins suspensions for bioassays, Bti strains (A19, Vec8, Bioflash, Bti4 and IP4444) from blood agar cultures were inoculated in sporulation solid GYS medium (0.1% (w/v) glucose, 0.2% (w/v) yeast extract, 0.2% (w/v) $(NH_4)_2SO_4$, 0.05% (w/v) K_2HPO_4, 0.02% (w/v) $MgSO_4.7H_2O$, 0.005% (w/v) $MnSO_4.H_2O$, 0.008% (w/v) $CaCl_2.H_2O$ and 2% (w/v) of agar) (Rogoff & Yousten, 1969) and incubated at 30°C during 3 - 4 days, until sporulation was completed. Each culture was then washed two times in 1 mL of sterile water by centrifugation (13'100 *x g*, 5 min, at 4 °C). The spores-toxins suspension was obtained by re-suspension of the remaining pellet (spores and toxins) in 1 mL of sterile water. The absence of vegetative cells was confirmed by microscopic observation (AxioLab Microscope; Carl Zeiss AG, Feldbach, Switzerland) of the spores-toxins suspensions stained with Coomassie blue staining solution (0.13% (w/v) Coomassie blue in 50% (v/v) acetic acid) (Rampersad *et al.*, 2002).

Bti spores-crystals suspension, used for morphological characterization and proteomic analysis (SDS-PAGE), was obtained in liquid sporulation GYS medium (0.1% (w/v) glucose, 0.2% (w/v) yeast extract, 0.2% (w/v) $(NH_4)_2SO_4$, 0.05% (w/v) K_2HPO_4, 0.02% (w/v) $MgSO_4.7H_2O$, 0.005% (w/v) $MnSO_4.H_2O$, 0.008% (w/v) $CaCl_2.H_2O$) (Rogoff & Yousten, 1969). Cultures were grown in 50 mL of liquid GYS medium at 30 °C, with agitation (150 rpm) until sporulation was complete (48 h). The spores and crystals were harvested by

centrifugation (10'620 *x g*, 10 min, at 4 °C). The supernatant was discarded; the pellet was washed two times with sterile distilled water and the Bti spores-crystals suspensions were stored at -20 °C.

The MIO semi-solid medium (BD BBL™ Motility Indole Ornithine; Becton Dickinson AG) was used to test the motility of the strains (Ederer & Clark, 1970).

The NaCl (0.45%) solution was used as medium for the Vitek biochemical analysis.

The incubation temperature of the bacterial cultures was always 30 °C, except for Vitek biochemical tests, for which was 37 °C.

2.3. Growth curve

The growth curves for A19 and Vec8 strains were performed. For both strains, a pre-culture was prepared in 20 mL of TSB medium and incubated o/n at 30 °C with agitation (150 rpm) (Unimax 1010 shaker, Heidolph). The OD (optical density) of each suspension was measured with a wavelength of 600 nm, using a spectrophotometer (UV/VIS Spectrometer Lambda 2S, Perkin Elmer, Schwerzenbach, Switzerland). The volumes of the inocula were adjusted to have an initial culture OD of 0.024. Fifty mL of cultures were incubated at 30 °C with agitation (150 rpm). Two replicates were performed at different times for both strains. The OD measurements were performed at time intervals of 29.02 to 55.80 min for the first replicate and 27 to 67.2 min for the second replicate, until the stationary phase was reached. The OD was registered in triplicate at every measurement time, 0.93, 1.58, 2.07, 2.63, 3.17, 3.72, 4.28, 5.03, 5.67, 6.28 and 6.88 hours for the first replicate; and at 0.75, 1.47, 2.07, 2.62, 3.37, 3.87, 4.42, 5.03, 5.63, 6.20, 6.78, 7.28, 8.40 and 8.85 hours for the second one. The doubling time (T_d) (min) and the specific growth rate (*k*) (OD min^{-1}) were determined for both replicates, considering the OD's average obtained in the exponential phase of the curves, instead the number of cells, with the following formula:

$$N = N_0\, 2^{n}$$

$$\log N = \log N_0 + n \log 2$$

$$n = (\log N - \log N_0) / \log 2$$

$$T_d\ (\text{min}) = (t - t_0) / n$$

$$\boxed{T_d\ (\text{min}) = ((t - t_0) / (\log N - \log N_0)) / \log 2}$$

$$k\ (\text{OD min}^{-1}) = \log 2\, n / (t - t_0)$$

$$\boxed{k\ (\text{OD min}^{-1}) = (\log 2 \times (3.3\ (\log N - \log N_0))) / (t - t_0)}$$

In this case, N is the OD corresponding to the absorbance value measured at time t, N_0 is the OD corresponding to the absorbance value measured at time t_0, *n* is the number of generations occurred during the time interval of exponential growth, *k* is the specific growth rate, $(t - t_0)$ is the time interval between t_0 and t times, corresponding to the exponential phase of the growth curve (Madigan & Martinko, 2006). For the first replicate, t_0 = 124.02 min

and t = 302.04 min, and for the second replicate, t_0 = 124.00 min and t = 370.02 min, in both A19 and Vec8 strains.

The *k* and T_d ± SD were determined using the Excel software (Microsoft® Excel®, version 12.0, 2008 for mac). An unpaired t-test (t-test, α set to 0.05) was performed to check if there were significant differences between the doubling time of A19 and Vec8 Bti strains, using the GraphPad Prism software (version 5 for windows, GraphPad software, San Diego California USA, www.graphpad.com).

2.4. Biochemical tests

2.4.1. API galleries

The biochemical characterization was performed with the micromethods API20E and API50CHB standard tests according to the manufacturer's instructions (API®, bioMérieux, Geneva, Switzerland). The tests included in the API galleries are specified in the results.

2.4.1.1. API 20E

For every Bti strain, a cell suspension was prepared in an ampoule of NaCl 0.85% medium (5 mL) to reach a cell density of 2 McFarland. The tubes of the API gallery were filled with the cell suspension by using a Pasteur pipette. For the CIT, VP and GEL tests, the tubes and the cupules were completely filled. An anoxic environment was created for the tests ADH, LDC, ODC, H_2S and URE, by adding a mineral oil drop above the cell suspension on the cupules of the gallery. The galleries already inoculated with the Bti strains were incubated at 30 °C. After 24 hrs of incubation, the first registration of the results was done, except for the TDA, IND and VP tests. After 48 hrs of incubation, a drop of TDA reagent was added to the TDA test, a drop of JAMES reagent was added to IND test, and a drop of each VP1 and VP2 reagents was added to VP test, and the results of all reactions were recorded. All the reactions were evaluated by direct observation, according to the manufacturer's instructions.

2.4.1.2. API 50CHB

The initial cell suspension was prepared in an ampoule of API 50CHB medium (5 mL) to obtain a cell density of 2 McFarland. The tubes of the API galleries were filled with the suspension and incubated at 30 °C, during 48 h. Results were recorded after 24 hrs and 48 hrs of incubation, according to the manufacturer's instructions.

2.4.2. Vitek identification (ID) Gram-positive (GP) cards

A bacterial suspension with an optical density of 0.5 - 0.6 McFarland was prepared in 0.45% NaCl solution using a Densicheck (bioMérieux). The Vitek ID-GP card (ID-GP card, bioMérieux) was used for biochemical analysis. This card includes tests for: reactions catalyzed by 18 enzymes (phosphatidylinositol phospholipase C, arginine dihydrolase (two

tests), β-galactosidase, α-galactosidase, alanine-phenylalanine-proline arylamidase, L-aspartic acid arylamidase, β-galactosidase, α-mannosidase, alkaline phosphatise, L-leucine arylamidase, proline arylamidase, β-glucuronidase (two tests), α-galactosidase, L-pyroglutamic acid arylamidase, alanine arylamidase, tyrosine arylamidase and urease), for the catabolism of 18 different substrates (amygdalin, xylose, α-cyclodextrin, sorbitol, galactose, ribose, lactate, lactose, N-acetyl-glucosamine, maltose, mannitol, mannose, β-methyl-D-glucopyranoside, pullulan, raffinose, salicin, sucrose, and trehalose) and for 5 different types of resistance (polymyxin B, bacitracin, novobiocin, O129, and optochin). The cell suspension was automatically inoculated in the wells of the ID-GP card, and incubated in the VITEK® 2 equipment (bioMérieux), at 37 °C. The biochemical profile was automatically read after some hours of incubation. Three replicates were made for Vec8, Bioflash, Bti4 and IP4444 Bti strains, and 4 replicates were made for A19 Bti strain.

2.4.3. Voges-Proskauer (VP) tube test

The VP test, to detect the production of acetoin (acetyl methyl carbinol: $C_4H_8O_2$), was carried out according to the classical method (Barritt, 1936). A loop of bacteria from the blood agar Bti culture was re-suspended in 1 mL of sterile distilled water supplemented by 5 mg mL^{-1} peptone (Oxoid, Pratteln, Switzerland) and 2.5 mg mL^{-1} glucose (Merck, Dietikon, Switzerland). After 48 hrs of incubation at 30 °C, 240 µL of Barritt reagent A (3.75% of α-naphtol in absolute EtOH) and 80 µL of Barritt reagent B (10% of potassium hydroxide (KOH) in sterile distilled water) were added to the culture. After 1 min of incubation at RT the results were recorded. A red colour shift of the suspension means that a positive reaction occurred.

2.4.4. Oxidase test

The oxidase test was performed according Kovacs (1956). It allows the detection of the enzyme cytochrome oxidase in a bacterial strain. A small loop of N,N-dimethyl-p-phenyl-enediamine hemioxalate salt powder (Sigma Aldrich, Buchs, Switzerland) was diluted in 10 mL of sterile distilled water. A piece of filter paper was moistened with the solution, and a loop of Bti culture was smeared above. A shift of the colour to pink and successively to brown meant a positive result, and a colourless result meant a negative reaction. The result of the reaction for each strain was immediately recorded. A strain of *Pseudomonas aeruginosa* (ATCC27853) was used as positive control.

2.4.5. Serotyping

For the serotyping classification of the Bti strains, a serotyping micromethod, using a 96-U-shaped wells microplate was performed (Laurent *et al.,* 1996).

Three mL of TSB medium were inoculated with the Bti strains, directly from the stocks preserved in skim milk (-80 °C). After 5 hrs of incubation at 30 °C, the cell suspensions were used to inoculate plates of 10 mL semi-solid GARD medium (0.4% (w/v) agar (Oxoid, L11),

3% (w/v) thyoglicollat (Oxoid, 415), distilled water). After o/n growth at 30 °C, bacteria were transferred to new GARD medium plates, and incubated again in the same conditions. A new culture was grown in 3 mL of TSB medium with 3 - 6 hrs of incubation, at 30 °C, followed by the fixation of bacteria in 15 µL of 37% formaldehyde (Fluka, Buchs, Switzerland). Ten µL of NaCl 0.15 M were added to all the wells of a 96-U-shaped wells microplate (Nunc MicroWell 96 well polystyrene plates, round bottom, Sigma, Buchs, Switzerland). The stock anti-flagellum serum (anti-H-14) was diluted ten times in NaCl 0.15 M. On the first wells' column, 10 µL of the already diluted anti-flagellum serum were added. Dilutions were made by transferring 10 µL of the solution from the first wells' column into the second wells' column, and were repeated until four different dilutions (1:200; 1:400; 1:800; 1:1600) were obtained. Ninety µL of the fixed cells' suspensions were added to each well, one row of the plate for each strain. As negative control organism, was used a *Bacillus thuringiensis* var. *kurstaki* strain (not serotyped as H-14). Moreover, negative control tests without microorganisms and a test with the fixed cells' suspension in water with no NaCl were performed. To check the auto-agglutination reaction, a test with the fixed cells' suspension in NaCl (0.85%) and without the anti-flagellum serum, was also carried out. The serotyping was performed two times, and the result observed with a magnifying glass for 96-wells microplates (Sigma) and recorded through photography (Lumix DMC-TZ7, Panasonic).

2.5. Motility test

2.5.1. MIO semi-solid medium motility test

The motility test was performed using the MIO semi-solid medium method (Ederer & Clark, 1970). A volume of 300 µL of a NaCl solution (0.85%) was inoculated with Bti vegetative cells grown on blood agar plates. With a small syringe (1 mL) the cell suspension was transferred to the MIO semi-solid medium (BD BBL™ Motility Indole Ornithine; Becton Dickinson AG). The culture was incubated o/n at 30 °C. A *Klebsiella pneumoniae* (ATCC13883) was used as negative control (Cowan *et al.*, 1960). The motility, indicated by a generalized turbidity, was checked by direct observation of the tube culture. This test was performed two times for the five Bti strains.

2.6. Morphological tests

The morphologic characterization was based on the terminology according to the "Bergey's manual of systematic bacteriology - The Firmicutes" (Vos *et al.*, 2009).

2.6.1. Colonies description and measurements

Three dilutions (1:100; 1:1000; 1:10000) were made from the initial spores-crystals suspension. Fifty µL of each dilution were used to inoculate blood agar Petri dishes. After 24 hrs of incubation at 30 °C, the colonies were characterized, considering their shape, colour, opacity and β-hemolysis. The diameters of 43 colonies were measured with a calibre. This

procedure was carried out three different times for each Bti strain. The average area and standard deviation (average ± SD) of the colonies were calculated with the Excel software. One-way analysis of variance (one-way ANOVA, α set to 0.05) was performed to check for significant differences between the average colonies' sizes of Bti strains. The Tukey's multiple comparison test (Tukey's HSD, α set to 0.05) was further performed to determine which groups presented a significant difference (SPSS statistics for Windows, ver. 17.0).

2.6.2. Vegetative cells description

A smear of Bti vegetative cells, previously grown on blood agar plates, was made on a microscope slide. After air-drying and fixing with the flame, the smear was stained with a drop of Coomassie blue solution (Rampersad *et al.*, 2002), for 5 min. After the staining the slide was washed with distilled water and air-dried. The Bti vegetative cells were observed by optical microscopy (100-fold oil immersion objective) (Carl Zeiss AG) and described.

2.6.3. Spores and parasporal crystals description and measurments

A drop of the spores-crystals suspension was added to a microscope slide. After air-drying, it was fixed with a flame and stained with Coomassie blue solution, as described above (see section 2.6.2). Spores and parasporal crystals were observed by optical microscopy using a 100-fold magnification objective (Carl Zeiss AG). To compare the dimension of spores from the different Bti strains, were performed measurements of lengths and correspondent widths of 60 Bti spores, for each strain. The area of a longitudinal section (rectangle-shaped) was calculated (length x width) for each spore measured. The diameter of the parasporal crystals was also measured and the average of maximum sectional areas was calculated for a total of 20 crystals for each strain.

The areas of the maximum longitudinal section of spores and crystals were measured using the AxioVision Image software, ver. 4.6 (Carl Zeiss AG). The average areas and standard deviations of spores and crystals were determined for each strain using the Excel software. One-way analysis of variance (one-way ANOVA, α set to 0.05) and the Tukey's multiple comparison test (Tukey's HSD, α set to 0.05) were performed to check for significant differences between the average of spore's longitudinal section areas of the different Bti strains, as well as differences between the crystal's sectional area of the strains.

Scanning electron microscope (SEM) images of Bti and Bc strains spore-phase were kindly provided by Dr. F. Barja (University of Geneva, Switzerland).

2.6.5. Leifson flagellar staining

The Leifson flagellar staining was carried out according to Clark (1976). The microscope slides were placed in glass racks inside staining jars and submerged in a cleaning solution (3% HCl, in 95% ethanol). After four days, the slides were washed 10 times with tap water and 2 times with distilled water. After air-drying, the slides were handled with forceps.

The stain was made by preparing 3 separate solutions: (a) 1.5% NaCl in distilled water; (b) 3% tannic acid in distilled water; (c) 69.2% of *para*-rosaniline acetate and 23.1% of *para*-rosalinine hydrochloride, in 7.7% of ethyl alcohol (95%). The alcoholic dye solution, (c), was left o/n at RT to ensure complete solution. Equal volumes of the 3 solutions were well mixed and left 2 hrs at RT, without disturbing, to have a clear supernatant stain solution.

The cell cultures were initially grown o/n on GARD semi-solid medium at 30 °C. A loop of cells from the periphery of the colonies was transferred to 4 mL of TSB medium, and incubated o/n at 30 °C with agitation (150 rpm). After the growth of the cultures, were added 0.25 mL of 37% (v/v) formaldehyde solution (Fluka) to 5 mL of cell suspension, to fix the cells. After 15 min, the cells were centrifuged (4'100 *x g*, 10 min, RT), washed with sterile distilled water, centrifuged again (4'100 *x g*, 10 min, RT) and the pellet was gently re-suspended in sterile distilled water, until the suspension was barely turbid. A loop (10 µL) of bacterial suspension was placed on a tilt washed microscope slide, to let the liquid flow lengthwise, and it was left to air-dry. One mL of the clear supernatant stain solution (at RT) was applied above the smear on the slide. After the formation of a precipitate (5 - 15 min), the slide was carefully washed with tap water and air-dried. The stained flagella were observed with an optical microscope (Carl Zeiss AG) using the 100-fold magnification objective.

2.7. Toxicological test

2.7.1. Spores quantification

The mechanism of a weakly staining of the dormant spores using 4',6'-diamino-2-phenylindole (DAPI) staining, a primarily nucleic acid-specific, is not well known, but it must occur at the cortex level (Magge *et al.*, 2009). Based on this information spores quantification was carried out by fluorescence microscopy, using the DAPI dye. The filtration manifold equipment (Millipore AG, Volketswil, Switzerland) was first cleaned. A 3.0 µm white subfilter (type SSWP, mixed cellulose esters, diameter 13 mm; Millipore AG, Volketswil, Switzerland) was added on the filtration manifold and humidified with sterile distilled water. Above the 3.0 µm subfilter a 0.22 µm brown polycarbonate filter (type GTBP, diameter 13 mm; Millipore AG) was added. Two mL of sterile water, 10 µL of spores-crystals suspension, and 30 µL of concentrated DAPI stain were introduced on the filtration manifold, in contact with the brown filter. The sample was well mixed and incubated during 10 min at room temperature, protected from the light. After the incubation, the vacuum pump was turned on and the spores were filtered, remaining on the brown filter surface. In order to wash all the spores remaining on the borders of the filtration manifold's cylinder, 2 mL of sterile distilled water were added and filtered. The brown filter was removed and placed on a microscope slide, with a Citifluor AF1 (glycerol/PBS; Citifluor Ltd., London, UK) drop. Above the filter, was

placed a lamella with a Citifluor AF1 drop on the under surface. The observation of spores was made by epifluorescence microscopy using the UV filter (AxioLab Microscope; Carl Zeiss AG, Feldbach, Switzerland). Spores' counting was carried out using a 100-fold magnification objective and an eyepiece with a 5 x 5 square grid. For each filter, bacterial cells present in 20 fields (10 on the vertical direction of the filter and 10 on the horizontal direction), covering an area of 0.01 mm^2 each, were counted. Three filters were prepared for each spores-crystals suspension. The number of spores per mL of suspension was calculated with the Excel software.

2.7.2. Toxin quantification

The toxin quantification was performed following the Bradford Microassay protocol (Bio-Rad Laboratoires AG, Reinach, Switzerland), after the parasporal crystals solubilisation. Triplicates of spores-crystals suspensions ($8x10^7$ cfu mL^{-1}) were mixed (50% (v/v)) with an alkaline solubilisation buffer 1 (0.01 M Na_2HPO_4, pH 11 (NaOH)) (Robinson & Stokes, 1968) and incubated o/n at 37 °C, with agitation (300 rpm). For bioassay experiment, 500 µL of each sample were mixed with 500 µL of Bradford Reagent dye (Quick Start Bradford 1x Dye Reagent, Bio-Rad) in a spectrophotometer cuvette, and incubated during 5 min at room temperature. To subtract the spores influence on the toxin quantification, was used a strain of *Bacillus cereus* spores suspension, since it doesn't produce the toxin. Samples with 250 µL of *B. cereus* spore suspension, 250 µL of buffer and 500 µL of Bradford reagent were used as blank. For protein quantification in 2-D PAGE, the samples were centrifuged (10'620 *x g*, 10 min, at 4°C), after the o/n toxin solubilisation; 500 µL of their supernatant were used for the toxin quantification. In this case, was used water as blank, instead the *B. cereus* spores' suspension. Measurements were made with a wavelength of 595 nm, in a spectrophotometer (UV/VIS Spectrometer Lambda 2S; Perkin Elmer). Quantifications of the toxin were carried out using a standard curve.

The standard curve for toxin quantification was created using BSA (BSA standard, 2 mg mL^{-1}, Bio-Rad), following the Bradford microassay protocol (Bio-Rad Laboratoires AG). The volumes were calculated for a 1 mL cuvette volume (Table 2). The alkaline solubilisation buffer 1 was used as blank.

Table 2. Bradford microassay dilutions for standard curve

Tube #	BSA Standard (µL)	Source of Standard	Diluent* Volume (µL)	Final Protein Concentration (µg/mL)
1	20	BSA Stock (2 mg/mL)	1580	25
2	32.5	BSA Stock (2 mg/mL)	3217.5	20
3	15	BSA Stock (2 mg/mL)	1985	15
4	1625	tube 2	1625	10
5	1625	tube 4	1625	5
6	1625	tube 5	1625	2.5
7	1500	tube 6	1500	1.25
8 (Blank)	-	-	1600	0

*The alkaline solubilisation buffer 1 used to solubilise the Bti toxin was used as diluent. # = number.

2.7.3. Amount of toxin per spore

The quantity of toxin per spore produced by each strain was calculated. The spores-crystals suspension was produced in solid GYS medium. For each Bti strain, the number of spores in the spores-crystals suspension was counted using the DAPI staining technique by epifluorescence microscopy (see section 2.7.1.). The correspondent amount of toxin was quantified following the Bradford microassay method (see section 2.7.2.).

The amount of toxin (ng) per spore (average ± SD) was calculated using the Excel software. One-way analysis of variance (one-way ANOVA, α set to 0.05) and a Tukey's HSD post-hoc test (Tukey's HSD, α set to 0.05) were performed to check if there were significant differences among the amounts of toxin per spore produced by the different Bti strains (SPSS statistics for Windows, ver. 17.0).

To confirm the relative amounts of toxin per spore between the Bti strains, new spores-crystals' suspensions were prepared in liquid GYS medium. The spores of each suspension were counted (see section 2.7.1.) and the toxins quantified (see section 2.7.2.).

A 12% SDS-PAGE, with a stacker of 5% polyacrylamide, was also performed. For each sample, a spores-toxins suspension volume corresponding to $3.5x10^6$ spores was dried on the Speed Vac (Savant SpeedVac® concentrator; Thermo Scientific, USA). After dried, the pellet was re-suspended in 10 µL of sterile distilled water. Was added an equal volume of alkaline solubilisation buffer 2 (0.05 M sodium carbonated, 0.01 M DTT, pH 10) (Attathom *et al.*, 1995) to the suspension and incubated o/n at 37 °C, with agitation (250 rpm). After o/n

incubation, the samples were centrifuged (10'620 *x g*, 10 min, at 4 °C) and the supernatants containing the solubilised toxins suspension, correspondent to $3.5x10^6$ spores, were kept. The samples for the gel were then prepared to have a final volume of 25 µL, correspondent to 80% of the toxin suspension and 20% of loading buffer (0.5 M Tris-HCl pH 6.8, 10% SDS, 4% β-mercaptoethanol, 8% glycerol, 0.1% bromophenol blue (1% Bromophenol blue, 0.05 M Tris, distilled water)) (Chaves *et al*., 2008). The marker sample consisted on 8% of molecular weight marker (SDS-PAGE Molecular Weight Standards, Broad Range, Bio-Rad) and 92% of loading buffer. A sample of 2% BSA standard (Bio-Rad), in 78% of lysis buffer (0.03 M Tris-HCl pH 8.5, 7 M urea; 2 M thiourea, 4% (w/v) 3-(3-cholamidopropyl-dimethyl-ammonio)-1-propanesulfonate (CHAPS), 1% (w/v) bromophenol blue) and 20% of loading buffer, was also included to avoid doubts concerning the bands' weight. All the samples were warmed at 95 °C during 5 - 10 min, and loaded directly in the wells of the 12% polyacrylamide gel (16 cm). Samples were let migrate for 30 min at 100 V, followed by 6 hrs of migration at 120 - 200 V. After the migration, the gel was washed with distilled water and kept o/n at RT submerged in a fixing solution (50% (v/v) methanol, 12% (v/v) acetic acid, distilled water) at 150 rpm (Heidolph). Protein profiles were stained with the silver nitrate staining.

2.7.4. Silver nitrate staining

This procedure was performed always with gloves to avoid proteins contamination. The gels were washed with distilled water and incubated three times in the washing solution (50% (v/v) EtOH, distilled water) for 10 min. Then, the gels were rehydrated in distilled water during 15 min and incubated in thiosulfate solution (0.02% (w/v) $Na_2S_2O_3$, distilled water) for 1 min. Gels were washed again three times with distilled water for 20 sec, and incubated in silver nitrate solution (0.2% (w/v) $AgNO_3$, 0.075% (v/v) formaldehyde, distilled water) for 20 min. After being washed three consecutive times with distilled water, gels were incubated in the development solution (6% (w/v) Na_2CO_3, 0.05% (v/v) formaldehyde, distilled water) until the bands appeared. The development solution was eliminated and the reaction was stopped with fixing solution for 2 h, under agitation (Heidolph) (150 rpm, at RT).

The picture of the gel was taken using a printer (RICOH Aficio MP C2800 PCL 5c). The gel analysis was performed with the ImageJ software (version 1.44o for mac, ImageJ software, National Institutes of Health, USA, http://imagej.nih.gov.ij), by measuring the intensity (IntDen) of the bands corresponding to the amount of toxin in the gel for each strain's profile. The graphic representation was created with the Excel software.

2.7.5. Rearing of mosquito larvae

The rearing of the larvae was carried out in a temperature-controlled environment (27 °C), with 12 hrs photoperiod (WHO, 2005). A piece of paper disc covered by mosquito eggs was submerged in a small recipient with tap water to induce the hatching of larvae. The tap water

used for the rearing of larvae and for further bioassays was treated with AquaSafe® (Tetra GmbH, Melle, Germany), to remove chlorine and neutralize heavy metals. After the hatching, (day 0), approximately 300 larvae were transferred to a tray containing about 600 mL of water (about 3 cm of height), and fed with 0.2 g of milled fish food (TetraMin®; Tetra GmbH). The amount of fish food necessary to feed larvae, increased with their growth; L1-instars was achieved on day 1 (0.4 g of fish food), L2-instars was achieved on day 2 (1.2 g of food), and during the day 3 larvae reached the early L3-instars (2.0 g of food).

2.7.6. Bioassay

The Bioassay was carried out to test the toxic effect of A19 strain, comparing with Vec8, Bioflash, Bti4 and IP4444 Bti strains, against L3-early instar's larvae of the biological model *Aedes aegypti* Rockefeller. For that, volumes of the spores-toxins suspensions were adjusted to have stock solutions with identical starting concentrations of Bti toxins. This bioassay was based on the "Guidelines for laboratory and field testing of mosquito larvicides" (WHO, 2005), with some modifications.

The Bioassay was performed at Swiss Tropical and Public Health Institute (Swiss TPH, Basel, Switzerland).

Larvae were exposed to different concentrations of Bti crystal toxins. A set of Bti toxins dilutions were prepared starting from the stock solutions. Every bioassay series involved ten 0.6-fold serial dilutions of Bti toxin, from 10 to 0.1 ng mL^{-1} (0.10; 0.17; 0.28; 0.46; 0.77; 1.29; 2.15; 3.59; 5.99; 10 ng mL^{-1}). Twenty-five L3-instar's larvae were tested with each concentration in plastic cups, with a final volume of 100 mL (WHO, 2005). Each concentration was tested in duplicate, and every series of bioassay was repeated three times at different days. Larvae added to tap water, treated with AquaSafe®, were used as negative control. After 24 hrs of exposure to the Bti toxin (27 °C), the living mosquito larvae were counted to access to the mortality of the larvae.

The percentages of mortalities were calculated and plotted against the corresponding log transformed toxin concentration, using the Excel software. The LC_{50} (lethal concentration for 50% of the treated larvae) value of each Bti strain was extrapolated from the middle region of the exponential phase of their mortality curves, where the most reproducible response occurs (Yu, 2008). It was determined through a probit analysis (LC_{50}, 95% CI), using the BioStat software (AnalystSoft Inc., BioStat v2009 - statistical analysis program; www.analystsoft.com). The LC_{50} value for each replicate was calculated and averaged. One-way ANOVA was performed to check significant differences between the five strains LC_{50} (GraphPad Prism software). The mortality curve of A19 strain was compared with the curves of the other reference Bti strains.

2.8. Proteomics

2.8.1. Spore-phase protein's profile

2.8.1.1. Sodium dodecyl sulfate polyacrylamyde gel electrophoresis (SDS-PAGE)

The spore-phase total protein profiles of Bti were obtained in a SDS-PAGE 12% polyacrylamide gel (Sterile distilled water, 12% acrylamide bis (29:1), 0.375 M Tris-HCl pH 8.8, 10% (v/v) SDS, 10% (v/v) ammonium persulphate (APS), 0.05% (v/v) tetramethylethylenediamine (TEMED)) with a stacker of 5% polyacrylamide (Sterile distilled water, 5% acrylamide bis (29:1), 0.125 M Tris-HCl pH 6 - 8, 10% SDS, 10% APS, 0.1% TEMED) (Chaves *et al*., 2008; Laemmli, 1970).

The bacterial cultures were grown in liquid GYS medium at 30 °C with agitation (150 rpm) until the spore-phase was achieved (48 h). The spores-crystals suspensions were centrifuged (10'620 *x g*, 20 min, 4 °C) and re-suspended in 1 mL of sterile distilled water. For each strain, was prepared a sample with a final volume of 25 µL, consisting in 25% loading buffer and 75% of concentrated Bti spores-crystals suspension. The marker sample consisted in 8% of stock molecular weight marker (SDS-PAGE Molecular Weight Standards, Broad Range, Bio-Rad) and 92% of loading buffer. The samples were boiled (100 °C) during 10 min and loaded directly in the wells of the gel. A sample of 2 µg of BSA (BSA standard, 2 mg mL^{-1}, Bio-Rad), a sample of *Bacillus sphaericus,* and a sample of a *Bacillus thuringiensis* var. *kurstaki*, were used as controls. The samples were let to migrate for about 15 min at 100 V and 6 hrs at 120 - 200 V. After this period gels were washed with distilled water and kept o/n at RT, submerged in fixing solution (50% (v/v) methanol, 12% (v/v) acetic acid, distilled water) at 150 rpm (Heidolph). The protein profiles were stained with Coomassie Brilliant Blue G-250 stain.

2.8.1.2 Coomassie blue staining

After the incubation in the fixing solution, the gels were rehydrated during 15 min in distilled water and incubated in a freshly prepared Coomassie blue solution for gel staining (Stock dye solution: 2% (v/v) phosphoric acid, 10% (w/v) ammonium sulphate, 5% (w/v) Coomassie brilliant blue G-250; Final dye: 80% of stock dye solution, 20% methanol), during 16 h. The gels were then washed with distilled water, and scanned with a printer (RICOH Aficio MP C2800 PCL 5c).

2.8.2. Vegetative cell total protein's profile

2.8.2.1. Protein Extraction

Vegetative cultures were grown o/n in 20 mL of TSB medium at 30 °C, with agitation (150 rpm). Suspensions were centrifuged (2'250 *x g*, 15 min, 4 °C) and the pellets re-suspended in 2 mL of PBS 1x buffer (pH 7.2). Three washings with 1 mL of PBS 1x buffer, and two

washings with 0.5 M Tris-HCl, pH 6.8 (centrifugation speed 6'800 *x g*, for 30 sec, at 4 °C) were performed. The supernatant was discarded and another centrifugation (speed 6'800 *x g*, for 1 min, at 4 °C) was performed to remove completely the remaining liquid. The pellets were re-suspended in 1 mL of lysis buffer and stored o/n in the freezer at -20 °C. The cells were then lysed by 5 sonication cycles (SONOPLUS HD 2070, Bandelin electronics, Berlin, Germany) of 15 sec each at about 20% of the maximum power. Between every sonication cycles, samples were placed on ice for 2 - 3 min. After cell lysis, the samples were centrifuged (13'100 *x g*, 15 min, 4 °C). The protein concentration in the supernatant was measured using the Bradford methodology (Bradford, 1976), according to the manufacturer's instructions (Bio-Rad). To check the presence and the quality of proteins in each sample, 4 µg of total protein extract were resolved on a 12% polyacrylamyde SDS-PAGE.

2.8.2.2. Two-dimensional polyacrylamide gel electrophoresis (2-D PAGE)

The first dimensional separation according to the proteins isoelectric point (p*I*), was conducted using the precast strips Immobiline™ DryStrip gel (IPG) (7 cm long, pH 3 - 10 NL (Non-Linear); GE Healthcare, Glattbrugg, Switzerland). The IPG were rehydrated in a specific support (GE Healthcare) containing 150 µL of a rehydratation buffer. The rehydratation buffer was composed by 3 µg of Bti total protein sample, lysis buffer and 1% IPG buffer (pH 3 to 10 NL) (GE Healthcare). The IPG were covered with 3 mL of IPG cover fluid oil (GE Healthcare) to prevent the evaporation and incubated o/n at RT. After rehydrated, the IPG were moved to the assembled Multiphor II (GE Healthcare) with the gel face up, and aligned. Two pieces of paper strips (IEF electrode strip, PKG/100; GE Healthcare) (11 cm long) were humidified and placed in contact with the gel ends of the IPG. The IPG were then covered with 20 - 25 mL of IPG cover fluid oil (GE Healthcare) and the Multiphor II equipment closed. The temperature was set to 18 °C. The isoelectric focusing (IEF) was performed in 4 steps: 200 V for 0.01 h; 300 V for 2 h; 3'500 V for 2 h; and 3'500 V for 1 h.

The 2nd dimension of the proteins' separation was performed according to their molecular weight. Previously, was prepared the A solution (6 M urea, 0.5 M Tris-HCl pH 6.8, 30% (v/v) glycerol, 2% (w/v) SDS, 1% (w/v) bromophenol blue). The IPGs were incubated for 12 min with agitation (Heidolph) in contact with the A1 solution (2 mL sol. A, 2% (w/v) dithiothreitol (DTT)) (165 rpm, RT), followed by a second incubation, for 5 min in contact with A2 solution (2 mL sol. A, 2.5% (w/v) iodoacetamide) (165 rpm, RT). The equilibrated IPGs were then placed on top of the SDS-PAGE 12% polyacrylamide gels and covered with 0.5% (w/v) hot agarose solution (0.5% (w/v) agarose, 0.025 M Tris, 0.19 M glycine, 0.1% (w/v) SDS, 1% bromophenol blue). A well was created near the '+' side of the IPG for the molecular weight marker (SDS-PAGE Molecular Weight Standards, Broad Range, Bio-Rad) sample. After the

solidification of the 0.5% agarose, the gels were placed into the migration equipment (Bio Rad MINI PROTEAN™, Bio-Rad) and covered with the running buffer (0.026 M Tris-HCl pH 7, 0.193 M glycine, 0.0035 M SDS). Twenty µL of the molecular marker diluted in the loading buffer (1:100) were added to the first well of the gel. The proteins' migration was carried out at 80 V for 15 min, followed by 2 hrs at 120 V. After the migration, the gels were washed with distilled water, and incubated o/n at RT in fixing solution (50% (v/v) methanol, 12% (v/v) acetic acid, distilled water), with agitation (150 rpm) (Heidolph). The proteins were stained with silver nitrate staining solution (see section 2.7.4.).

To take the picture, the gels were rehydrated in distilled water for 30 min and scanned with a printer (RICOH Aficio MP C2800 PCL 5c).

2.8.2.4. Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-ToF MS)

To analyse the protein profiles from vegetative cell-phase of the different Bti, Btk and Bc strains, a protein mass fingerprint was performed for each strain using MALDI-ToF MS technique. A small amount of a bacteria, grown o/n in blood agar medium, was transferred to a target spot of a FlexiMass™ MALDI target plate (Schimatzu - Biotech Corp., Kyoto, Japan), using a disposable loop. Four wells were prepared for each strain. One µL of the α-cyano-4-hydroxyeinnamic acid (CHCA) matrix (2.94% (w/v) of 97% CHCA, 32.35% (v/v) acetonitrile, 32.35% (v/v) of 100% EtOH, sterile distilled water, 2.94% (v/v) concentrated trifluoroacetic acid (TFA)) was added on each sample and air-dehydrated within 1 - 2 min at RT. The protein mass profiles of Bti strains were obtained using a MALDI-ToF MS Axima™ Confidence machine (Shimadzu - Biotech Corp.), with detection in the linear positive mode, at a laser frequency of 50 Hz and within a mass range of 2'000 - 20'000 Da. The acceleration voltage was 20 kV and the extraction delay 200 ns. Were used 20 laser shots per sample to generate each spectrum. For each bacterial sample, a total of 50 protein mass fingerprints were averaged and processed, using the LaunchpadTM v. 2.8 software (Shimadzu-Biotech Corp.). This software automatically performed smoothing, normalization, baseline subtraction, and peak calling. For each sample was generated a list of the significant spectrum peaks, that included the mass-to-charge (*m/z*) values for each peak, and signal intensity. Calibration was conducted for each target plate using spectra of the reference strain *E. coli* K12 (GM48 genotype), grown o/n on LB medium (LB broth, Miller (Luria-Bertani), Difco BD Switzerland) at 30 °C. Generated protein mass fingerprints were analyzed with SARAMIS™ (Spectral ARchive and Microbial Identification System, AnagnosTec GmbH) software (AnagnosTec, Potsdam, Germany) with default settings. For each strain, identification was also performed by comparing the generated peak lists against the current reference library in SARAMIS™. The resulting peak lists of individual samples were

processed using the SARAMIS™ software package (AnagnosTec) and submitted to cluster analysis (single-linkage clustering using the Dice coefficient) to produce a taxonomic tree (0.08% error, range from *m/z* 2'000 to 20'000).

2.9. Genomics

2.9.1. DNA extraction

Total DNA from Bti vegetative cells was extracted using the QIAamp DNA Mini Kit (QIAGEN, Basel, Switzerland) according to the manufacturer's instructions. For each Bti strain, a loop of bacteria was taken from the Bti culture after o/n growth on blood agar plate, and re-suspended in 1 mL of nanopure distilled water. The suspension was centrifuged (5'000 x *g*, 5 min) and the supernatant discarded. To the remaining pellet, 20 µL of proteinase K and 200 µL of AL lysis buffer were added and mixed by vortexing (15 sec). After briefly centrifuged, the suspension was firstly incubated for 10 min at 56 °C, then during 10 min at 95 °C. Two-hundred µL of ethanol 96 - 100% were added and mixed by vortexing (15 sec). After briefly centrifuged, the whole suspension was transferred to a Quiagen spin column, and centrifuged (4'300 x *g*, 1 min). For the first washing, the column was transferred to a new collecting tube and 500 µL of AW1 wash buffer were added. Tube was centrifuged for 1 min at 4'300 x *g*. The second washing was made using 500 µL of AW2 and centrifuged for 3 min at 13'100 x *g*. The collection tube with the filtrate was discarded and the column was placed in a new tube and subjected to a second centrifugation (13'100 x *g*, 1 min). Finally the column was placed in a 1.5 mL *eppendorf* tube, and 80 µL of AE elution buffer were added to the column. After 5 min incubation at RT, the tube was centrifuged (4'300 x *g*, 1 min). The DNA extracted was kept at 4 °C.

2.9.2. 16S ribosomal coding region

To test the quality of the DNA extracted with the Qiagen DNA extraction kit, the 16S ribosomal subunit coding region was amplified by PCR, with the universal primers UniL (forward primer 5' - AGAGTTTGATCATGGCTCA - 3', amplified region 8 - 26 *E. coli*) and UniR (reverse primer 5' - GTGTGACGGGCGGTGTGTA - 3', amplified region 1411 - 1391 *E. coli*) (Microsynth, Balgach, Switzerland) (Demarta *et al.*, 1999). Each PCR reaction consisted of 25 µL of a solution containing 71.5% of sterile distilled water, 10% of 10x PCR Buffer (QIAGEN PCR buffer), 6% of 25 mM $MgCl_2$, 2% of dNTPs mix (QIAGEN), 2% of both primers, 0.005% HotStarTaq DNA Polymerase (250U, QIAGEN) and 6% of genomic DNA. DNA amplifications were performed in a Veriti® 96-well Thermal Cycler (Applied Biosystems, Rotkreuz, Switzerland). The thermal cycling conditions included an initial HotStarTaq DNA Polymerase activation at 95 °C (15 min), 35 cycles of 30 sec at 94 °C (denaturation), 1 min at 52 °C (primers annealing) and 1.5 min at 72 °C (extension); and after the 35 cycles, 7 min at 72 °C. The amplified DNA was checked in a 1.5% (w/v) agarose gel electrophoresis

(Agarose standard, Eurobio, Courtaboeuf, France), containing 0.001% (v/v) of red fluorescent nucleic acid dye Gel RED (Biotium Inc., Hayward, USA), in 1x TBE buffer (1.08% (w/v) Tris base, 0.55% (w/v) boric acid, 0.4% (v/v) 0.05 M EDTA (18.61% (w/v) $Na_2EDTA.2H2O$, pH 8.0 (NaOH)), pH 8.0). The DNA segments' molecular weights were confirmed by comparison with a 100 - 1500 bp marker (DNA Molecular Weight Marker XIV (100 – 1500 bp), Roche Diagnostics GmbH, Mannheim, Germany).

2.9.3. *cry* and *cyt* genes' detection

The DNA extracted from pure vegetative Bti cultures was subjected to end-point PCR, by targeting and amplification of the *cry4Aa*, *cry4Ba*, *cry10Aa*, *cry11Aa*, *cyt1Aa* and *cyt2Ba* genes of each strain.

Each reaction mix consisted in 20 µL of PCR water, 1.5 µL of both forward (d) and reverse (r) primers (Table 3), 25 µL of MasterMix (Taq PCR Master Mix Kit, QIAGEN), and 2 µL of the bacterial DNA, in a total volume of 50 µL. The genes amplification was performed with the Veriti® 96-well Thermal Cycler (Applied Biosystems). The cycler conditions began with an initial denaturation and Taq polymerase activation step at 95 °C for 1 min, followed by 35 cycles of 2 min at 95 °C (denaturation), 1 min at 50 °C for *cry4Aa*, *cry4Ba*, *cry11Aa*, *cyt2Ba* / 1 min at 51 °C for *cry10Aa* / 1 min at 52 °C for *cyt1Aa* (annealing), and 1 min at 72 °C (extension), followed by 5 min at 72 °C. The samples were stored at 4 °C.

Table 3. Primers (Microsynth AG) for *cry* and *cyt* genes.

Primers*	**Oligonucleotide sequence**	**Oligonucleotide position**	**Reference**
cry4Aspe(d)	5' – TCA AAG ATC ATT TCA AAA TTA CAT G – 3'	1706 – 2165	(Ibarra *et al*., 2003)
cry4Bspe(d)	5' – CGT TTT CAA GAC CTA ATA ATA TAA TAC C – 3'	1706 – 2165	(Ibarra *et al*., 2003)
cry4ABspe(r) ‡	5' – CGG CTT GAT CTA TGT CAT AAT CTG T – 3'		
cry10spe(d)	5' – TCA ATG CTC CAT CCA ATG – 3'	978 – 1326	(Ibarra *et al*., 2003)
cry10spe(r)	5' – CTT GTA TAG GCC TTC CTC CG – 3'		
cry11spe(d)	5' – CGC TTA CAG GAT GGA TAG G – 3'	990 – 1332	(Ibarra *et al*., 2003)
cry11spe(r)	5' – GCT GAA ACG GCA CGA ATA TAA TA – 3'	1025 – 1368 1048 – 1400	

Table 3. (Continued).

Primers*	Oligonucleotide sequence	Oligonucleotide position	Reference
cyt1gral(d)	5'– CCT CAA TCA ACA GCA AGG GTT ATT – 3'	197 – 674	(Ibarra *et al*., 2003)
		85 – 565	
cyt1gral(r)	5' – TGC AAA CAG GAC ATT GTA TGT GTA ATT – 3'	97 – 574	
cyt2gral(d)	5' – ATT ACA AAT TGC AAA TGG TAT TCC – 3'	509 – 865	(Ibarra *et al*., 2003)
		529 – 884	
cyt2gral(r)	5' – TTT CAA CAT CCA CAG TAA TTT CAA ATG C – 3'	649 – 1004	
		196 – 551	

* (d) Forward primers; (r) reverse primers.
‡ cry4ABspe(r) is the reverse primer for both *cry4Aa* and *cry4Ba* genes.

After PCR, the amplification of *cry* and *cyt* genes was checked by electrophoresis on a 1.5% agarose gel (see section 2.9.2.). The molecular weights of the DNA segments were confirmed by comparison with a 50 - 750 bp marker (DNA Molecular Weight Marker XIII (50 – 750 bp), Roche Diagnostics GmbH). The detection was performed under UV light using the Quantum ST4 system and the Quantum Capt software (Witec AG, Littau, Switzerland).

2.9.4. Random amplified polymorphic DNA polymerase chain reaction (RAPD-PCR)

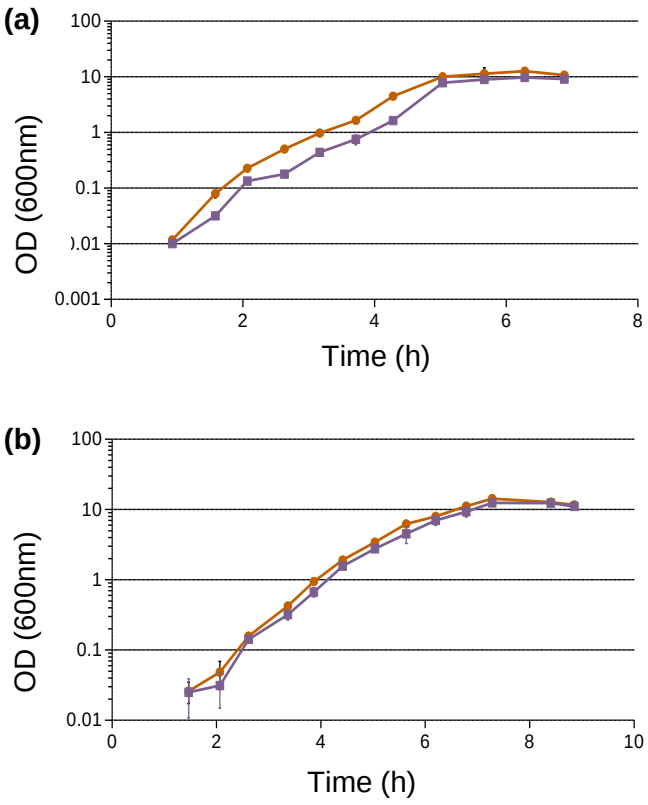
The RAPD-PCR analysis was performed according to the procedure of Kumar *et al.*, (2009), with some modifications. Three different primers were used for this experiment: OPA08: GTCCACACGG; OPD14: CTTCCCCAAG; and OPE17: CTACTGCCGT (Microsynth) (Kumar *et al.*, 2009). Two different PCR reactions were tested: (1) 1x low stringency PCR buffer (0.01 M Tris-HCl pH 8.3, 0.05 M KCl, 0.1% triton x100), 2.5 mM $MgCl_2$, 250 µM of each nucleotide (Mix dNTPs), 25 pmol of primer, 50 ng of genomic DNA, and 1 U of HotStarTaq DNA Polymerase; (2) MasterMix (Taq PCR Master Mix Kit, QIAGEN) with addiction of 0.1% Triton x100, 25 pmol of primer and 50 ng of genomic DNA. Due to the unavailability of a different DNA polymerase when the experiment was performed, a HotStarTaq DNA Polymerase (250U, QIAGEN) was used for the amplification reactions. This enzyme needs to be activated at 95 °C for 15 min, therefore the cycler conditions began with an initial denaturation and DNA polymerase activation step at 95 °C for 15 min, followed by 35 cycles of 1 min at 94 °C (denaturation), 1 min at 40 °C (annealing), and 3 min at 72 °C (extension), followed by a final extension of 15 min at 72 °C.

3. RESULTS

3.1. Growth curve

The A19 and Vec8 strains growth curves had a similar profile in both replicates (Fig. 2). In the 1st replicate (Fig. 2a), the stationary phase was reached after approximately 5.03 h, and in the 2nd replicate (Fig. 2b) it was reached after 6.20 h. The maximum absorbance verified was approximately 10 in all growth curves.

The specific growth rate (*k*) and doubling times (T_d) for each strain and replicate, are specified on the figure 2. The T_d average and SD for A19 and Vec8 strains were 33.16 ± 0.76 min and 31.07 ± 1.02 min, respectively. No significant differences between the doubling times were observed (t-test, p = 0.07).



	k (OD min^{-1})	T_d (min)
A19	9.17x10^{-3}	32.62
Vec8	9.85x10^{-3}	30.35

	k (OD min^{-1})	T_d (min)
A19	8.88x10^{-3}	33.69
Vec8	9.41x10^{-3}	31.79

Fig. 2. Growth curves of A19 (orange line) and Vec8 (purple line) Bti strains. (a) and (b) correspond to the first and the second replicates, respectively.

3.2. Biochemical Characterization

The biochemical characterization of A19 strain was carried out by 2 different methods, the API galleries micromethod (Table 4) and the Vitek ID-GP cards (Table 5 and 6).

3.2.1. API 20E and API 50CHB

According to the API galleries results, A19 strain presented small differences in its biochemical characteristics when compared to the other Bti strains (Table 4).

API galleries tests revealed that glucose, fructose, N-acetylglucosamine, esculin, maltose, trehalose, amidon and glycogen were metabolized by all the tested strains at their optimal temperature of growth (30 °C). In contrast to the other strains, A19 strain showed a clear positive result in the ribose fermentation test. Mannose was fermented by all strains, with the exception of Bioflash strain. However, Bioflash fermented cellobiose, which wasn't verified for the other strains. The arbutin is fermented by Bioflash and IP4444 strains.

The VP test of API galleries didn't provide clear results.

Table 4. API 50CHB and API 20E biochemical tests.

Test	Amount (mg)	Reactions/ Enzymes	Results									
			A19		Vec8		Bioflash		Bti4		IP4444	
			24h	48h	24h	48h	24h	48h	24h	48h	24h	48h
Control (-)	-	-	-	-	-	-	-	-	-	-	-	-
RIB	1.4	fermentation	+	+	-	-	?	?	-	-	?	?
GLU	1.56	fermentation	+	+	+	+	+	+	+	+	+	+
FRU	1.4	fermentation	+	+	+	+	+	+	+	+	+	+
MNE	1.4	fermentation	+	+	+	+	-	-	+	+	+	+
NAG	1.28	fermentation	+	+	+	+	+	-	+	-	+	+
ARB	1.08	fermentation	-	-	-	-	-	+	-	-	?	+
ESC	1.16	fermentation	-	+	?	+	?	+	-	+	?	+
CEL	1.32	fermentation	-	-	-	-	-	+	-	-	-	?
MAL	1.4	fermentation	+	+	+	+	+	+	+	+	+	+
TRE	1.32	fermentation	+	+	+	+	+	+	+	+	+	+
AMD	1.28	fermentation	+	+	+	+	+	+	+	+	+	+
GLYG	1.28	fermentation	+	+	+	+	+	-	+	-	+	?
ADH	1.9	arginine dihydrolase	+	+	+	+	+	+	+	+	+	+
CIT	0.76	citrate utilization	-	+	-	+	-	+	-	+	?	+

Table 4. (Continued)

Test	Amount (mg)	Reactions/ Enzymes	Results									
			A19		Vec8		Bioflash		Bti4		IP4444	
			24h	48h	24h	48h	24h	48h	24h	48h	24h	48h
VP*	1.9	acetoin production	ND	?	ND	?	ND	?	ND	?	ND	?
GEL	0.6	gelatinase	+	+	+	+	+	+	+	+	+	+

(+) Positive test; (-) Negative test; (?) Doubtful test; (ND) Not Determined. The reactions whose outcome is variable among strains are highlighted.
* The VP test from API galleries didn't provide clear results; therefore this test was confirmed with the classical tube method.
Tests: Negative control (Control (-)), glycerol (GLY), erythritol (ERY), D-arabinose (DARA), L-arabinose (LARA), D-ribose (RIB), D-xylose (DXYL), L-xylose (LXYL), D-adonitol (ADO), β-methyl-D-xylopyranoside (MDX), D-galactose (GAL), D-glucose (GLU), D-fructose (FRU), D-mannose (MNE), L-sorbose (SBE), L-rhamnose (RHA), dulcitol (DUL), inositol (INO), D-mannitol (MAN), D-sorbitol (SOR), α-methyl-D-mannopyranoside (MDM), α-methyl-D-glucopyranoside (MDG), N-acetylglucosamine (NAG), amygdalin (AMY), arbutin (ARB), esculin (ESC), salicin (SAL), D-celiobiose (CEL), D-maltose (MAL), D-lactose (bovin origin) (LAC), D-melibiose (MEL), D-saccharose (SAC), D-trehalose (TRE), inulin (INU), D-melezitose (MLZ), D-raffinose (RAF), amidon (AMD), glycogen (GLYG), xylitol (XLT), gentiobiose (GEN), D-turanose (TUR), D-lyxose (LYX), D-tagatose (TAG), D-fucose (DFUC), L-fucose (LFUC), D-arabitol (DARL), L-arabitol (LARL), potassium gluconate (GNT), potassium 2-ketogluconate (2kg), potassium 5-ketogluconate (5kg), 2-nitrophenyl-β-D-galactopyranoside (ONPG), L-arginine (ADH), L-lysine (LDC), L-ornithine (ODC), trisodium citrate (CIT), sodium thiosulfate (H_2S), urea (URE), L-tryptophane (TDA), L-tryptophane (IND), sodium pyruvate (VP), gelatin (GEL).

3.2.2. Vitek ID-GP card

The Bti4 and IP4444 strains presented similar biochemical profiles when tested with the Vitek ID-GP card (Table 5). Vec8 and Bioflash strains biochemical profiles were similar, and almost equal to Bti4 and IP4444 strains, just differing in the Phosphatidylinositol phospholipase C reaction. Bti4, IP4444 and A19 strains showed positivity in the Phosphatidylinositol phospholipase C reaction (33.3%, 66.7% and 50%, respectively). Only A19 strain was able to grow in 6.5% NaCl, with 75% of positivity (Table 5).

Table 5. Vitek biochemical test.

Test	Amount (mg)	Reactions/ Enzymes	Results				
			A19	Vec8	Bioflash	Bti4	IP4444
dRIB	0.3	fermentation	100%	100%	100%	100%	100%
NAG	0.3	fermentation	100%	100%	100%	100%	100%
dMAL	0.3	fermentation	100%	100%	100%	100%	100%
dTRE	0.3	fermentation	100%	100%	100%	100%	100%
PIPLC	$1.50x10^{-02}$	phosphatidylinositol phospholipase C	50%	-	-	33.3%	66.7%

Table 5. (Continued).

Test	Amount (mg)	Reactions/ Enzymes	Results				
			A19	Vec8	Bioflash	Bti4	IP4444
ADH1	$1.11x10^{-01}$	arginine dihydrolase 1	100%	100%	100%	100%	100%
AGLU	$3.60x10^{-02}$	α-glucosidase	100%	100%	100%	100%	100%
PHOS	$5.04x10^{-02}$	phosphatase	25%	100%	100%	100%	100%
PyrA	$1.80x10^{-02}$	L-pyrrolidonyl-arylamidase	100%	100%	100%	100%	100%
NC6.5	1.68	growth in 6.5% NaCl	75%	-	-	-	-

(-) Negative test. For positive reactions, the percentage of positivity is shown (A19: n = 4, other strains: n = 3). The reactions whose outcome is variable among strains are highlighted. The negative reaction for all the strains tested are not represented on the table.
Tests: catabolism of the substrates D-amygdalin (AMY), D-xylose (dXYL), α-cyclodextrin (CDEX), D-sorbitol (dSOR), D-galactose (dGAL), D-ribose (dRIB), L-lactate alkalinization (lLATk), lactose (LAC), N-acetyl-D-glucosamine (NAG), D-maltose (dMAL), D-mannitol (dMAN), D-mannose (dMNE), β-methyl-D-glucopyranoside (MBdG), pullulan (PUL), D-raffinose (dRAF), salicin (SAL), sucrose (SAC), and D-trehalose (dTRE); reactions catalyzed by the enzymes phosphatidylinositol phospholipase C (PIPLC), arginine dihydrolase 1 (ADH1), arginine dihydrolase 2 (ADH2s), β-galactosidase (BGAL), α-glucosidase (AGLU), alanine-phenylalanine-proline arylamidase (APPA), L-aspartate arylamidase (AspA), β-galactopyranosidase (BGAR), α-mannosidase (AMAN), phosphatase (PHOS), Leucine arylamidase (LeuA), L-proline arylamidase (ProA), β-glucuronidase (BGURr), α-galactosidase (AGAL), L-pyrrolidonyl-arylamidase (PyrA), β-glucuronidase (BGUR), alanine arylamidase (AlaA), tyrosine arylamidase (TyrA) and urease (URE); and growth in 6.5% NaCl (NC6.5).

The resistance to five different antibiotics was also tested with Vitek ID-GP card. The resistance occurs when the bacteria exposed to the antibiotic can grow. The five Bti strains showed 100% resistance against polymyxin B, an antibiotic produced by *Bacillus polymyxa,* that binds specifically to the lipopolysaccharides (LPS) constituents of the bacterial membrane; 100% resistance against bacitracin, an antibiotic produced by *Bacillus subtillis,* which interferes with the dephosphorylation of the C_{55}-isoprenyl pyrophosphate, carrier of the building blocks of the peptidoglycan bacterial cell wall outside the inner membrane; 100% resistance against O/129, a vibriostatic compound; and 100% resistance against optochin, a chemical that specific inhibits the $F_0F_1H^+$ - ATPase usually associated to *Streptococcus pneumonia* (Muñoz *et al.*, 1996). A19 strain, as the Bti reference strains, showed sensitivity only to Novobiocin, a coumarin antibiotic which inhibits the super coiling activity of gyrase by binding to the gyrase B (GyrB) subunit, and prevents the binding of ATP molecule (Sugino *et al*., 1978) (Table 6).

Table 6. Vitek resistance test.

Test	Amount (mg)	Reactions/ Enzymes	A19	Vec8	Bioflash	Bti4	IP4444
POLYB	$9.30x10^{-04}$	resistance	100%	100%	100%	100%	100%
BACI	$6.00x10^{-04}$	resistance	100%	100%	100%	100%	100%
NOVO	$7.50x10^{-05}$	resistance	25%	-	-	66.7%	66.7%
O129R	$8.40x10^{-03}$	resistance	100%	100%	100%	100%	100%
OPTO	$3.99x10^{-04}$	resistance	100%	100%	100%	100%	100%

(-) Negative test. For positive reactions, the percentage of positivity is shown (A19: n = 4, other strains: n = 3). The reactions whose outcome is variable among strains are highlighted.
Tests: polymyxin B resistance (POLYB), bacitracin resistance (BACI), novobiocin resistance (NOVO), O/129 resistance (O129R), and optochin resistance (OPTO).

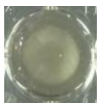
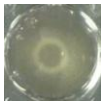
3.2.3. Oxidase tests

The oxidase test result was negative for A19 strain, as for the Bti reference strains.

3.2.4. Serotyping

The results obtained with the serotyping were not very clear. The A19 strain showed different reactions comparing with the other strains (Table 7). A19 strain's cells deposit at the bottom of the U-wells, forming a halo, while the other Bti strains showed a floccular deposition, although not clear (Table 7, Bioflash). The negative control Btk5 presented a non-floccular and more homogeneous deposition than the ones verified for Bti reference strains (Table 7, Btk5). Since the A19 strain's result differed from the positive results verified in the reference strains, its reaction was considered negative. The auto-agglutination test and the controls without NaCl and anti-H-14 serum were negative for all the strains tested.

Table 7. Serotyping reactions.

Reaction	Bt strains		
	A19	Bioflash‡	Btk5
With NaCl (0.15 M) and anti - H-14 serum			
With NaCl (0.15 M) without anti - H-14 serum*			
Without NaCl (0.15 M) without anti - H-14 serum			

*Auto-agglutination test. For the interpretation was considered the Laurent and collaborators (1996) description of a positive agglutination reaction: a floccular deposit on the well bottom.
‡ Bioflash strain was shown as example for Bti strains.

3.3. Motility

The swimming motility was tested for the five Bti strains. As the negative control *Klebsiella pneumoniae* (ATCC13883), a negative result was verified for A19 strain, contrary to the Bti reference strains, Vec8, Bioflash, Bti4 and IP4444, which had a positive result. The positive control strains mediums were turbid, due to the displacement of the cells from the initial inoculum area. Differently, the medium of the negative control and A19 strain, remained translucent.

3.4. Morphological Characterization

3.4.1. Macroscopic

The colonies of A19 strain, as well as the colonies of Vec8, Bioflash, Bti4 and IP4444 strains, were round with undulate edges; their surfaces were flat and dull. The colonies showed a matt texture, with a butyrous consistency, creamy-grey-colored and opaque. The β-hemolysis of Vec8, Bioflash and Bti4 strains, was marked and complete, in contrary, the β-hemolysis of A19 and IP4444 strains were partial and showed a thin area. The five Bti strains' colonies presented average areas with significant differences between them (ANOVA, $p < 0.0001$). The A19 strain's colonies (49.08 ± 5.80 mm^2) were significantly bigger than the Bti reference strains' colonies (Tukey's HSD, $p < 0.05$) (Table 8). The IP4444 strain's colonies (39.61 ± 4.87 mm^2) showed to be significantly bigger than Vec8 (33.93 ± 4.53 mm^2), Bioflash (32.90 ± 6.35 mm^2) and Bti4 (32.89 ± 4.78 mm^2) strains' colonies, but significantly smaller than A19 strain's colonies (Tukey's HSD, $p < 0.05$). The area of Vec8, Bioflash and Bti4 strains' colonies were similar (Tukey's HSD, Vec8 and Bioflash strains $p = 0.85$; Vec8 and Bti4 strains $p = 0.89$; Bti4 and Bioflash strains $p = 1.00$).

3.4.2. Microscopic

The vegetative cells of A19 Bti strain were rod-shaped, as well as the other Bti strains analyzed (Vec8, Bioflash, Bti4 and IP4444).

The A19 strain spores were subterminal placed on the mother-cell, and had an ellipsoidal shape. Its longitudinal section area of the Bti spores of each strain was significantly different (ANOVA, $p < 0.0001$). The A19 strain spores' average area (1.00 ± 0.16 μm^2) (Table 8) was significantly smaller than Vec8 (1.20 ± 0.24 μm^2) and Bioflash (1.17 ± 0.20 μm^2) Bti strains spores' average areas (Tukey'HSD, $p < 0.05$). There was no significant difference between A19, Bti4 (0.98 ± 0.15 μm^2) and IP4444 (0.95 ± 0.19 μm^2) strains spores' average areas (Tukey's HSD, A19 and Bti4 strains $p = 1.00$; A19 and IP4444 strains $p = 0.63$; Bti4 and IP4444 strains $p = 0.85$).

The parasporal crystals of A19 strain had predominantly a globular shape, as well as Vec8, Bioflash, Bti4 and IP4444 Bti strains crystals (Fig. 3). As expected, Bce1 didn't

produce crystals (Fig. 3f). The average of the maximum area section of A19 strain's crystals was 5.17 ± 0.78 μm^2 (Table 8). No significant difference between the crystals' size of A19 strain and the other Bti strains was observed (ANOVA, p = 0.79).

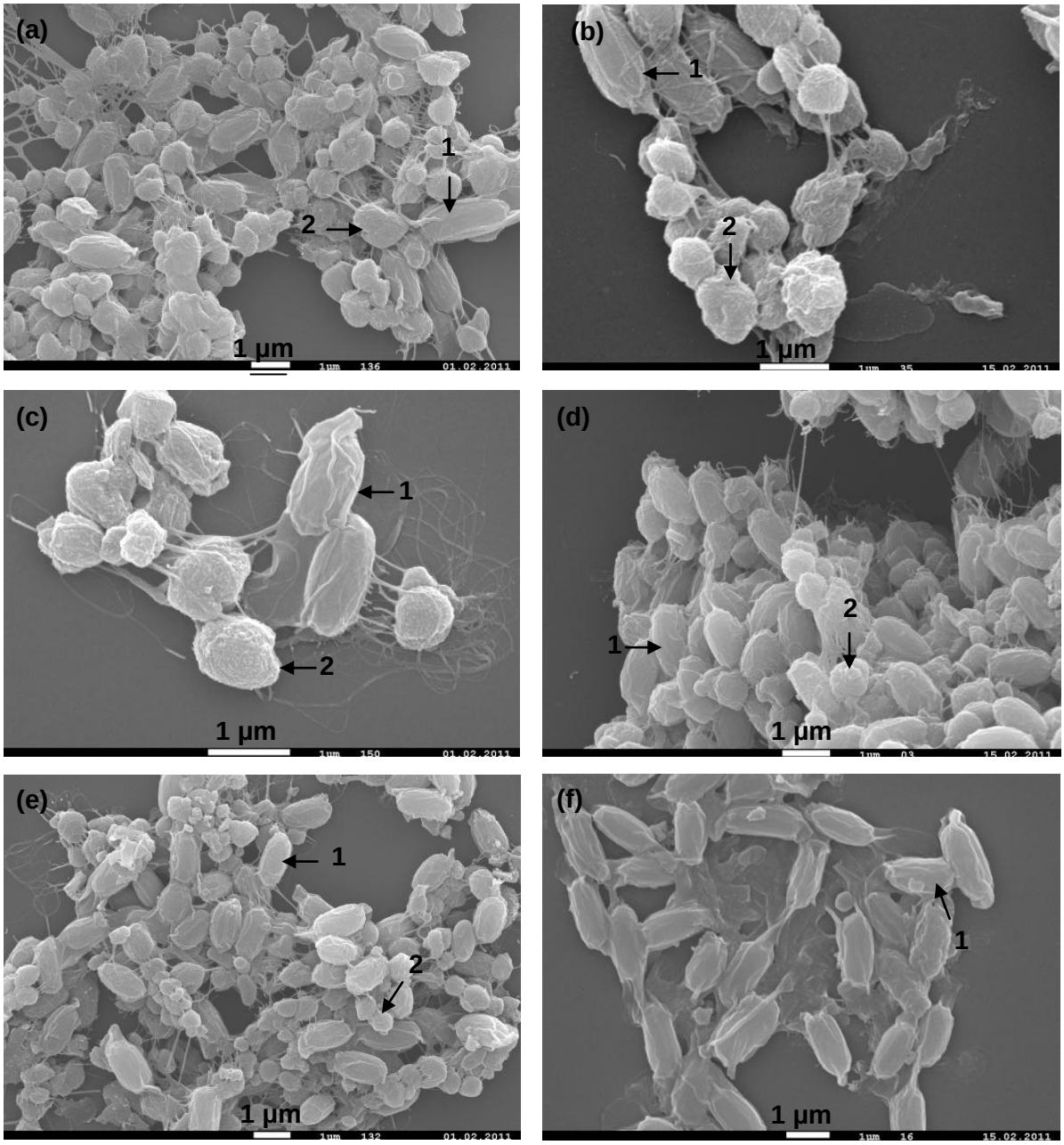


Fig. 3. SEM images of Bti and Bc strains spore-phase. (a) A19 (9'000x magnification); (b) Vec8 (16'000x magnification); (c) Bioflash (19'000x magnification); (d) Bti4 (11'000x magnification); (e) IP4444 (8'500x magnification); (f) Bce1 (10'000x magnification). 1. spores; 2. crystals. (Picture: Dr. F. Barja, University of Geneva). The bar correspondent to 1 µm is in white.

Table 8. Morphological measurements.

Bti Strains	Colony area (mm^2)	Spore longitudinal section area (μm^2)	Crystal maximum sectional area (μm^2)
A19	49.08 ± 5.80	1.00 ± 0.16	5.17 ± 0.78
Vec8	33.93 ± 4.53	1.20 ± 0.24	4.85 ± 1.35
Bioflash	32.90 ± 6.35	1.17 ± 0.20	4.92 ± 1.01
Bti4	32.89 ± 4.78	0.98 ± 0.15	5.04 ± 1.03
IP4444	39.61 ± 4.87	0.95 ± 0.20	ND

(ND) Not Determined. Values are expressed as average ± SD. Colonies measurements: n = 43; spores measurements: n = 60; crystal measurements: n = 20.

Among the five Bti strains tested, A19 strain was the only one without flagella (Fig. 4a). Vec8, Bioflash and Bti4 strains showed peritrichous flagella (Fig. 4b, 4c and 4d). The strain IP4444 seemed to be monotrichous or lophotrichous (Fig. 4e).

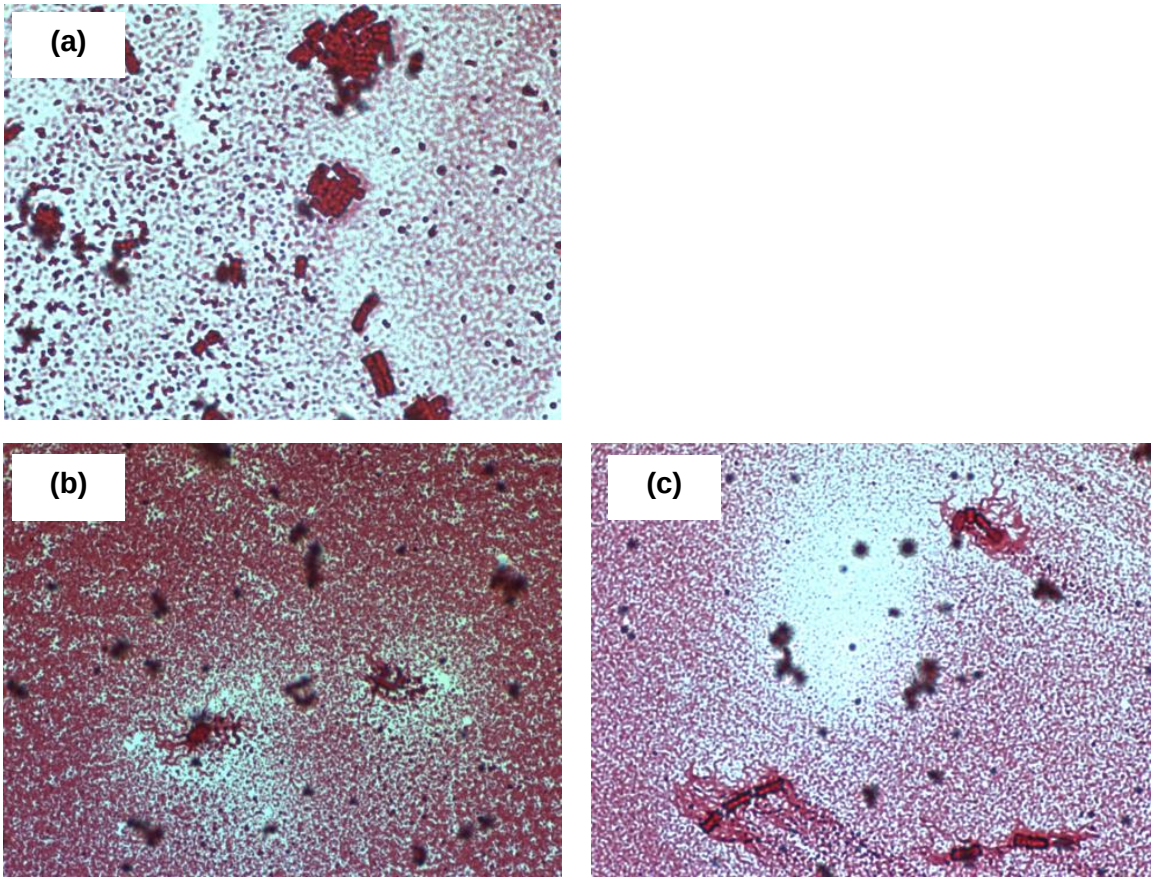


Fig. 4. Highlighted flagella by the Leifson flagellar staining. (a) A19 strain; (b) Vec8 strain; (c) Bioflash strain (100x objective).

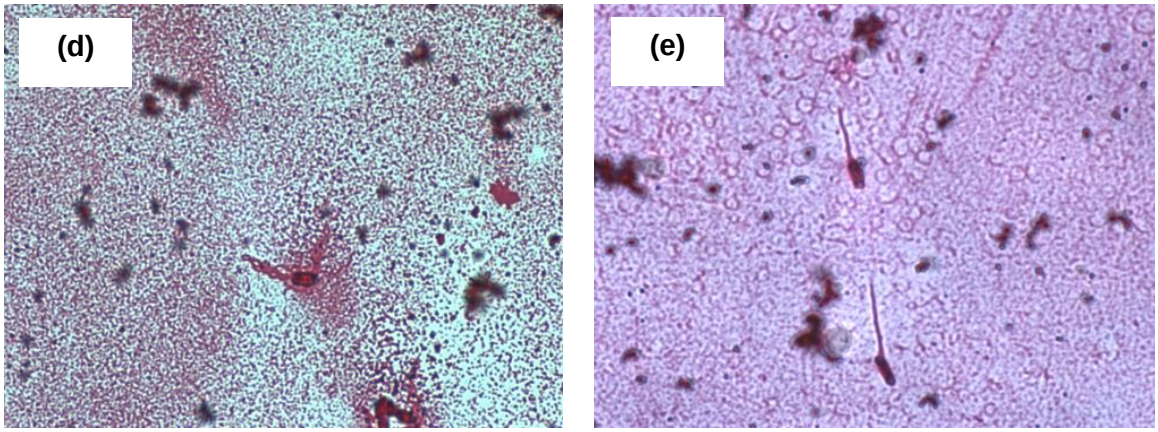


Fig. 4. (Continued). (d) Bti4 strain; and (e) IP4444 strain; (100x objective).

3.4. Toxicological test

3.4.1. Amount of toxin per spore

The amount of toxin per spore was determined, to compare the productivity of the strains. The A19 strain produced on average ± SD, $1.64x10^{-4} \pm 5.84x10^{-6}$ ng of toxin per spore, Vec8 strain $1.34x10^{-4} \pm 4.99x10^{-6}$ ng, Bioflash strain $1.32x10^{-4} \pm 9.38x10^{-6}$ ng, Bti4 strain $9.91x10^{-5} \pm 4.99x10^{-6}$ ng and IP4444 strain $6.89x10^{-5} \pm 7.60x10^{-6}$ ng of toxin per spore (Fig. 5).

Significant differences were obtained in the amount of toxin produced by the Bti strains tested (ANOVA, $p < 0.0001$). A19 strain produced significantly more toxin per spore than the other Bti strains (Tukey's HSD, $p < 0.05$). The amount of toxin per spore produced by the Bti IP4444 strain was significantly smaller than the other strains (Tukey's HSD, $p < 0.05$). Vec8 and Bioflash Bti strains produced a similar quantity of toxin per spore (Tukey's HSD, $p = 0.97$), and both produced significantly more toxin per spore than Bti4 (Tukey's HSD, $p < 0.05$).

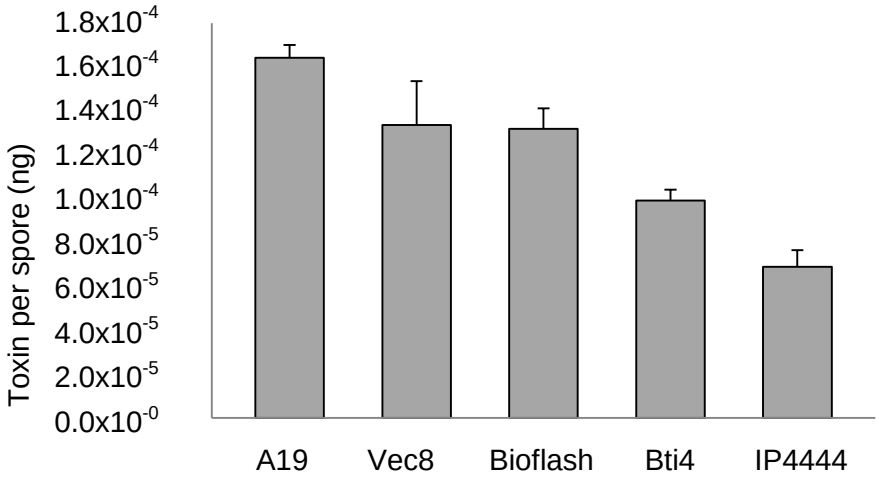


Fig. 5. Graphical representation of the amounts of toxin per spore of Bti strains tested. Bars represent standard deviations (n = 3).

The production of a bigger amount of toxin per spore by A19 strain, relatively to the Bti reference strains, was supported by the SDS-PAGE result. Through the analysis of the gel picture was possible to measure the higher intensity of pigments, corresponding to a higher concentration of protein, in the A19 strain proteins' profile relatively to the other strains, considering the same amount of spores ($3.5x10^6$ spores) (Fig. 6). Despite the result obtained by SDS-PAGE was not statistically proven, it also supports that A19 strain produces a bigger amount of protein per spore during the spore phase in sporulation GYS medium, than the Bti reference strains, Vec8, Bioflash, Bti4 and IP4444.

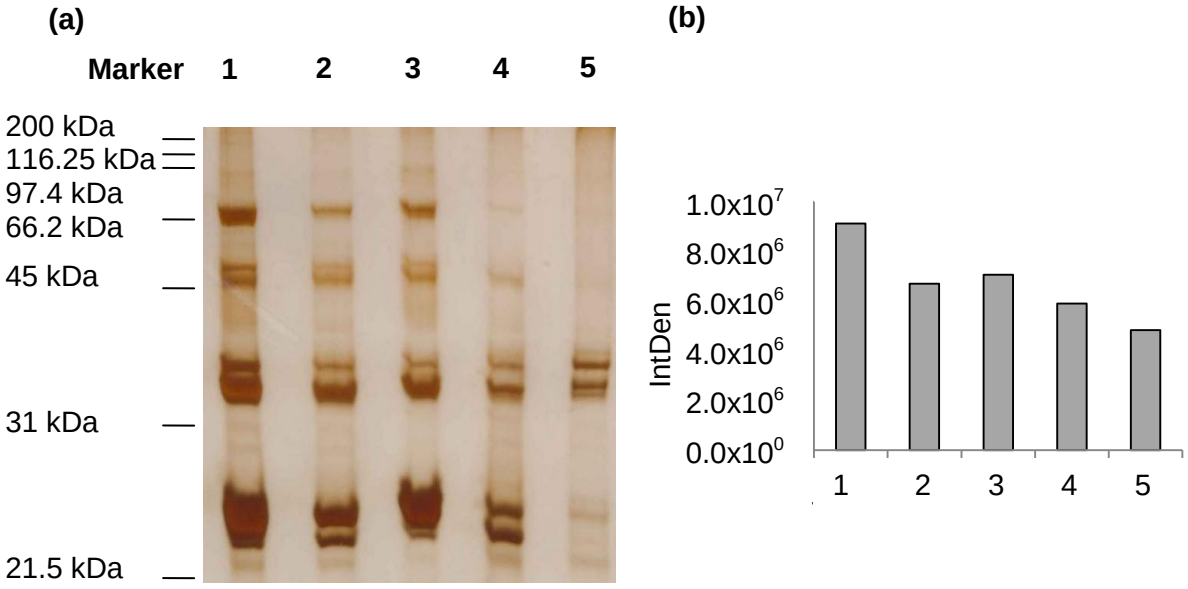


Fig. 6. Amount of protein for $3.5x10^6$ spores (SDS-PAGE): (a) SDS-PAGE (12%) with total proteins of Bti spore-phase. Marker: SDS-PAGE Molecular Weights Standards, Broad Range: 200 kDa; 116.25 kDa; 97.4 kDa; 66.2 kDa; 45 kDa; 31 kDa; 21.5 kDa; 14.4 kDa; 6.5 kDa (Bio-Rad). 1. A19; 2. Vec8; 3. Bioflash; 4. Bti4; 5. IP4444. (b) Graphical representation of SDS-PAGE band intensity.

3.4.2. Bioassay

The toxic activity of A19 strain parasporal crystals against *Aedes aegypti*, Rockerfeller strain mosquito larvae was confirmed after 24 hrs, as well as the toxicity of the reference Bti strains. The mortality curves obtained for each Bti strain were sigmoid-shaped (Fig. 7), since the larval mortality is affected by the toxin concentration.

The average of the Bti LC_{50} values and 95% CI were 1.05 ng mL^{-1} (0.86 - 1.30) for A19 strain, 1.23 ng mL^{-1} (0.87 - 1.49) for Vec8 strain, 0.97 ng mL^{-1} (0.68 - 1.57) for Bioflash strain, 1.14 ng mL^{-1} (0.94 - 1.39) for Bti4 strain, and 1.43 ng mL^{-1} (1.06 - 2.02) for IP4444 strain. There was no significant differences between the five strains LC_{50} values (ANOVA, $p = 0.16$).

The Abbott's formula, which corrects the mortality caused by the toxin excluding the natural death, was not applied, since there was less than 5% of controls mortality (WHO, 2005).

It was observed that, after 24 hrs in contact with the A19 strain toxin maximum concentration (10 ng mL^{-1}), one of the 150 tested larvae was alive. This was not observed in the other Bti strain tested.

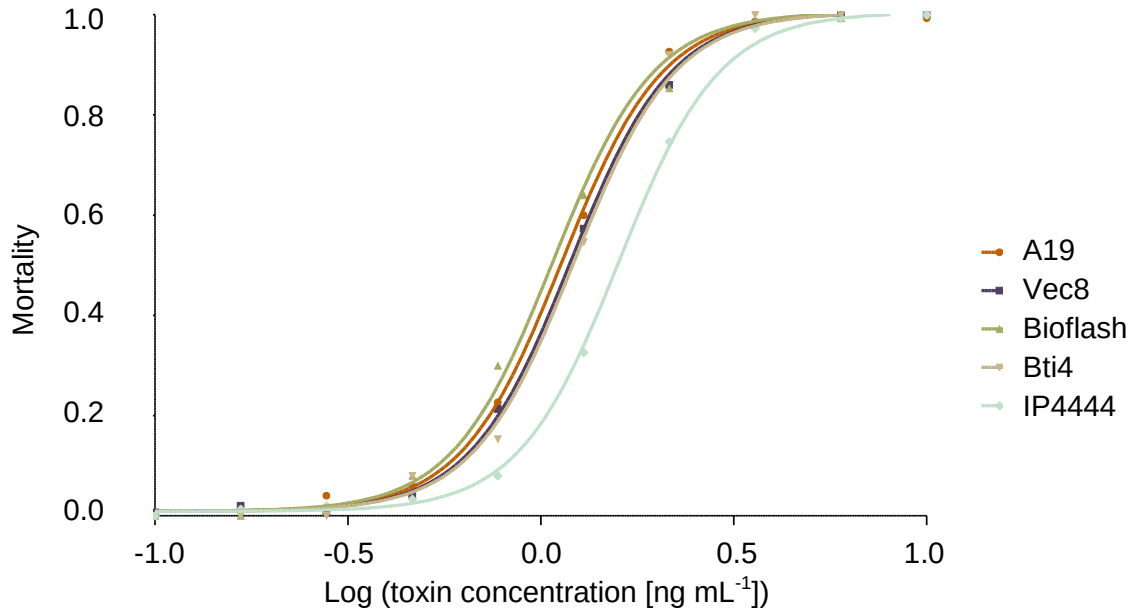


Fig. 7. Mortality curves of L3-early instars larvae of the biological model *Ae. aegypti* Rockefeller exposed to different Bti strains.

3.5. Proteomics

3.5.1. Total Bti spore-phase protein profile (SDS-PAGE)

From the spore-phase protein profiles obtained through SDS-PAGE, the distinction between the Bti strains and the 2 non-Bti strains (*B. sphaericus* strain and *B. thuringiensis* var. *kurstaki*) is clear. The A19 strain spore-phase protein profile was very similar to the ones of the Bti reference strains (Fig. 8).

The bands corresponding to Cry4Aa, Cry4Ba, Cry10Aa, Cry11Aa, Cyt1Aa and Cyt2Ba toxins were identified, despite the bands corresponding to the Cry4 proteins were very thin (Fig. 8).

Were also observed protein bands with molecular weights that don't correspond to any of the 6 toxic proteins referred above, which constitute the parasporal crystal. These unknown bands were not identified.

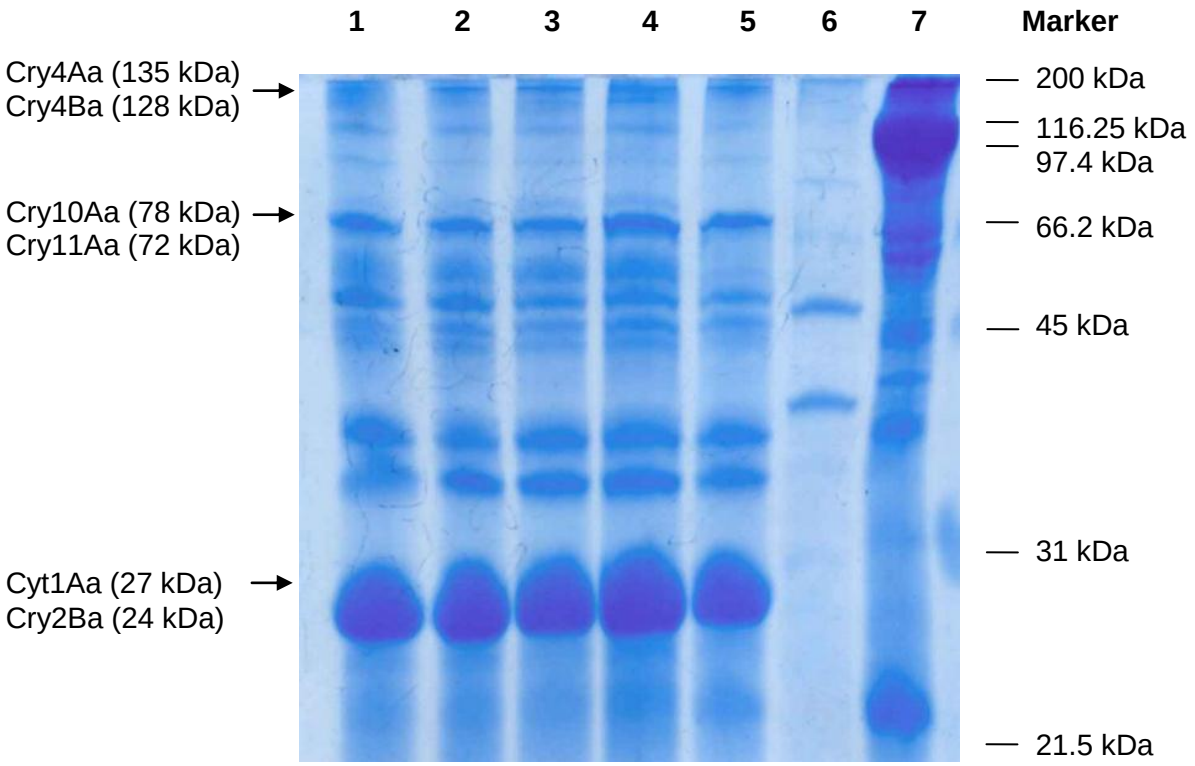


Fig. 8. SDS-PAGE (12%) of total proteins from the Bti spore-phase. 1. A19 strain; 2. Vec8 strain; 3. Bioflash strain; 4. Bti4 strain; 5. IP4444 strain; 6. *B. sphaericus* strain; 7. *B. thuringiensis* var. *kurstaki* strain. Marker: SDS-PAGE Molecular Weights Standards, Broad Range: 200 kDa; 116.25 kDa; 97.4 kDa; 66.2 kDa; 45 kDa; 31 kDa; 21.5 kDa; 14.4 kDa; 6.5 kDa (Bio-Rad).

3.5.2. Total Bti vegetative cell protein profile (2-D PAGE)

The 2-D PAGE vegetative cell-phase total protein profiles showed a low p*I* (isoelectric point) for the major part of the proteins (Fig. 9).

The absence of three protein correspondent spots was noticed in the A19 strain vegetative cell-phase, when compared with the Bti reference strains' protein profiles (Fig. 9). Despite the A19 strain gel protein migration was not perfect, as is possible to see on the gel picture (Fig. 9a), when compared with the protein profiles obtained for the Bti reference strains, is possible to infer their molecular weights. The absent spots in A19 strain's protein profile, correspondent to acidic proteins, showed a molecular weight of about 50 kDa, and the 3rd spot, correspondent to a basic protein, has about 45 kDa. Despite these differences of A19 strain protein profile, comparing with the Bti reference strains, their vegetative total protein's profiles obtained by 2-D PAGE, were very similar.

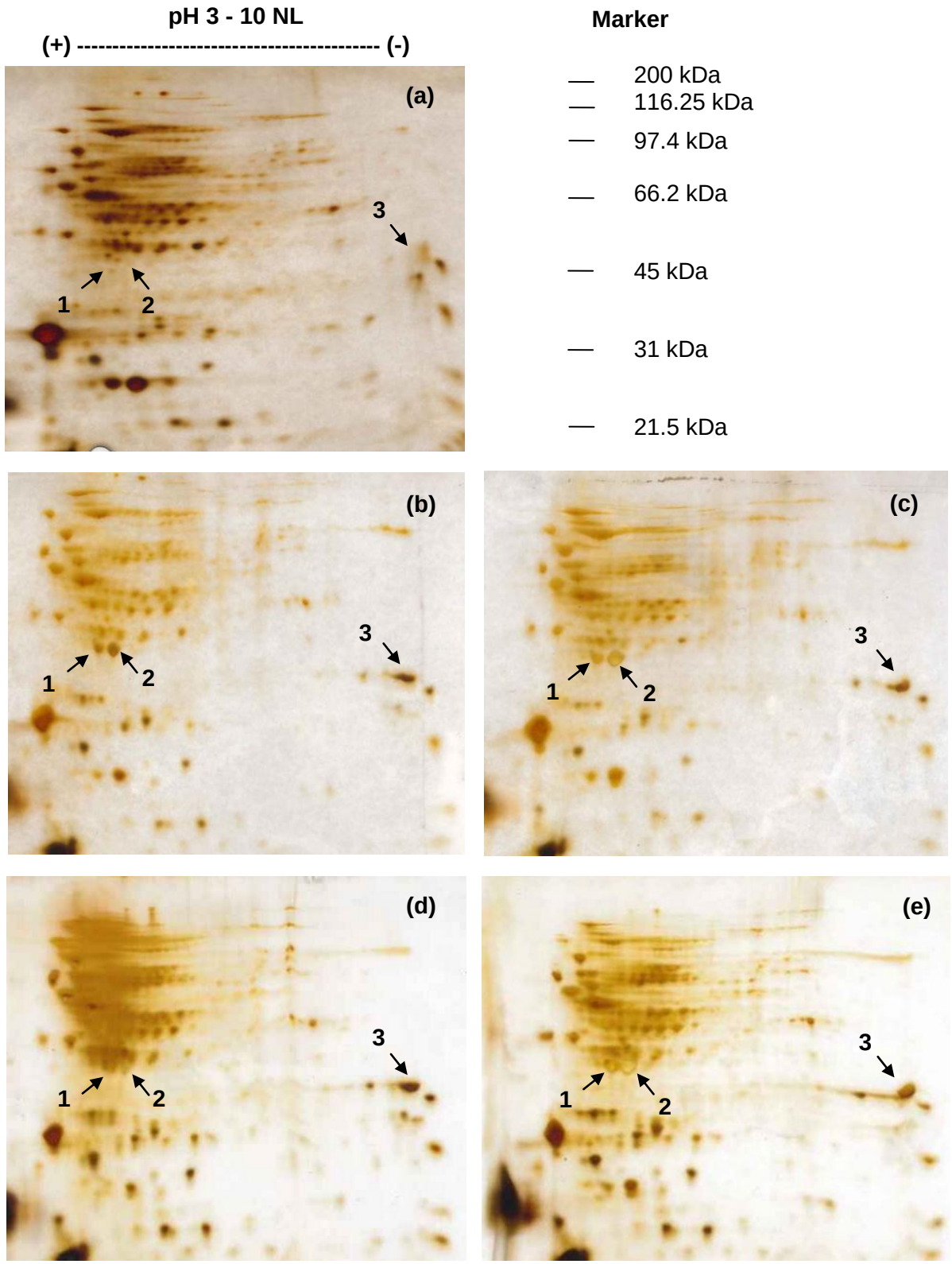


Fig. 9. 2-D PAGE (12%) of total protein profile from the vegetative cell-phase. (a) A19 strain, (b) Vec8 strain, (c) Bioflash strain, (d) Bti4 strain, and (e) IP4444 strain. The three proteins spots (1 to 3) missing in the A19 Bti strain profile (black arrows). Marker: SDS-PAGE Molecular Weights Standards, Broad Range: 200 kDa; 116.25 kDa; 97.4 kDa; 66.2 kDa; 45 kDa; 31 kDa; 21.5 kDa; 14.4 kDa; 6.5 kDa (Bio-Rad).

3.5.3. Total Bti vegetative cell protein profile (MALDI–ToF MS)

The analysis of the vegetative cell-phase total protein's profiles of Bti, Bc and Btk strains, achieved by MALDI-ToF MS technique, allowed the construction of a single linkage dendrogram. In the dendrogram obtained, A19 strain is included in a cluster with the other Bti strains, which excludes the Bce1 and Btk5 strains. The percentage of similarity between A19 strain and the other Bti strains was: 74% shared with Vec8, 67% with IP4444 and 65.5% with Bti4 and Bioflash strains (Fig. 10).

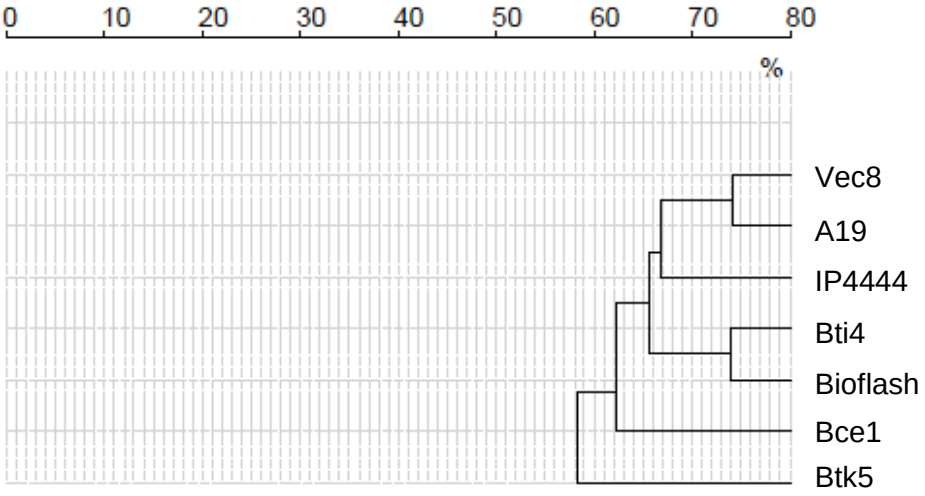


Fig. 10. Phyloproteomics relationship between A19 and other *Bacillus* strains, based on profiles of total proteins from vegetative cells, obtained by MALDI-ToF MS. (SARAMIS database software, error 0.08%; mass range from *m/z* 2'000 to 20'000).

3.6. Genomic analysis

3.6.1. 16S ribosomal coding region

After the DNA extraction from the Bti cells, its quality was checked by amplification of the 16S ribosomal subunit coding region. The amplification was successfully achieved for all strains, with an amplicon size of about 1500 bp (Fig. 11).

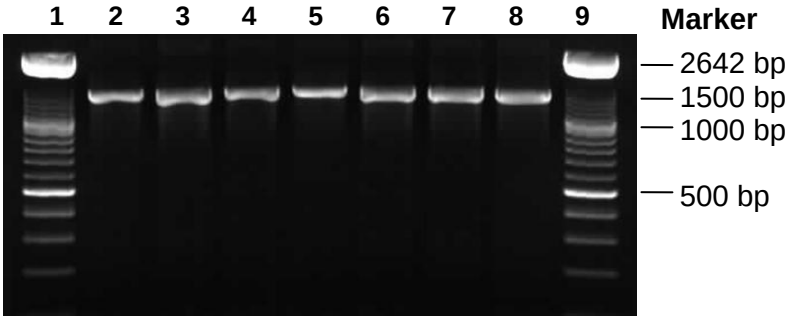


Fig. 11. 16S ribosomal subunit encoding region detection. 1. and 9. DNA Molecular Weight Marker XIV (100 - 1500 bp) (Roche); 2. A19 strain; 3. Vec8 strain; 4. Bioflash strain; 5. Bti4 strain; 6. IP4444 strain; 7. Btk5 strain; 8. *B. sphaericus* strain.

3.6.2. *cry* and *cyt* genes detection

All Cry (Cry4Aa, Cry4Ba, Cry10Aa and Cry11Aa) and Cyt (Cyt1Aa and Cyt2Ba) protein encoding genes were present in all Bti strains tested, A19, Vec8, Bioflash, Bti4 and IP4444 (Fig.12 shows the amplification products for the A19 Bti strain).

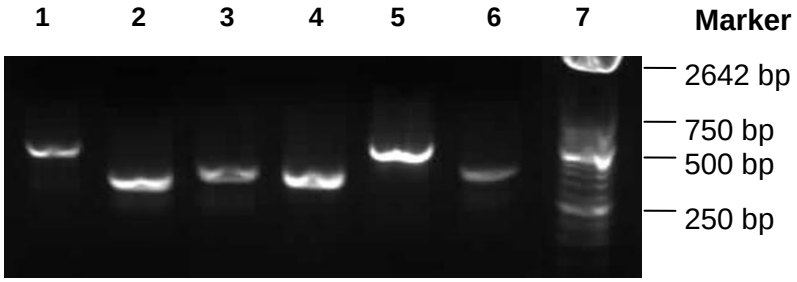


Fig. 12. A19 strain's *cry* and *cyt* genes detection. 1. *cry4Aa* (459 bp); 2. *cry4Ba* (321 bp); 3. *cry10Aa* (358 bp); 4. *cry11Aa* (342 bp); 5. *cyt1Aa* (477 bp); 6. *cyt2Aa* (355 bp); 7. DNA Molecular Weight Marker XIII (50 - 750 bp) (Roche).

3.6.3. Total Bti vegetative cell DNA profile (RAPD-PCR)

No DNA amplification occurred.

4. DISCUSSION

Throughout this thesis work the A19 Bti strain, first isolated from Bolle di Magadino by Chappuis (2002), was characterized and compared with four different Bti reference strains, among which stands out Vec8, the Bti strain currently applied at the Bolle di Magadino to control floodwater mosquitoes population. Despite the many similarities among the A19 strain and the other Bti reference strains, the differences at morphological and molecular levels suggested that this is an autochthonous strain.

The A19 strain growth curve was analysed so that the strain's growth profile and doubling time could be known, as well as its comparison with Vec8 strain under rich conditions could be performed. For both strains a typical growth profile, with evident exponential and stationary phases, was obtained, with no significant differences between their doubling times. The stationary phase, achieved as result of the depletion of the medium nutrients or the accumulation of fermentation end-products (Landwall & Holme, 1977), was attained for both strains at the same time and at the same maximum OD (approximately 10). These result showed that, in rich conditions, A19 strain needs an equivalent growth time to Vec8 strain.

It is known, by the literature, that the biochemical characterization is not enough to distinguish between Bt strains, due to the high variability of the biochemical reactions among them (De Barjac & Frachon, 1990; Ben-dov *et al.*, 1997; Pérez *et al.*, 2005). As phenotypical features, they can be influenced by environmental factors. Nevertheless, it complements the information about the microorganism. As a complementary method for API galleries Vitek ID-

GP cards were used, although this system is not specific for *Bacillus* identification. It is currently available a Vitek colorimetric reagent card (BCL card) for the specific identification of members from the genus *Bacillus*, which has 18 tests in common with the ID-GP card (BGAL, AGLU, APPA, CDEX, AspA, AMAN, LeuA, ProA, AGAL, PyrA, AlaA, dRIB, NAG, NC6.5, dMAN, dMNE, dTRE and POLYB). Anyway, neither the BCL identification card can to differentiate between *B. thuringiensis* and *B. cereus* (*stricto sensu*) species.

The discriminating biochemical features between the A19 strain and the Bti reference strains were the ability to ferment D-mannose, arbutin and D-celiobiose, the presence of phosphatidylinositol phospholipase C and the ability to grow at 6.5 NaCl. However, more replicates should have been used, and the parallel characterization of a *B. cereus* (*stricto sensu*) strain would be interesting. The ability to ferment ribose was confirmed in all strains with Vitek ID-GP cards. The positive growth of Bt at 7% NaCl was referred by Drobniewski (1993), and as expected, A19 strain grew in 6.5% NaCl; in contrast, the reference Bti strains had negative results for this test. Contrary to Vec8 and Bioflash, the Bti4, IP4444 and A19 strains showed positivity for the phosphatidylinositol phospholipase C reaction at 37 °C. The phospholipase C is considered a virulence factor, usually associated to *B. cereus* (*stricto sensu*) but also released by Bt (Carlson & Kolstø, 1993; Drobniewski, 1993). Therefore, its release can be disadvantageous in a strain used as a biopesticide. For the resistance tests, A19 strain, as well as the four Bti reference strains, showed 100% resistance against the polymyxin B antibiotic, since it acts specifically on membrane compounds lipopolysaccharides (LPS) (Galizzi *et al.*, 1975). One hundred % resistance was also observed against bacitracin, O/129 and optochin antibiotics, but none of the 5 Bti strains showed 100% resistance to novobiocin, a coumarin antibiotic, which inhibits the super coiling activity of gyrase by binding to the gyrase B (GyrB) subunit and prevents the binding of ATP molecule (Sugino *et al*., 1978). From the five resistance tests made, only the polymyxin B is present on the Vitek BCL card, for *Bacillus* identification.

The H-14 serotype is very frequently associated to the *israelensis* variety, which toxin is active against some insects from the Diptera order (Ankarloo *et al*., 2000; Guerchicoff *et al*., 1997). However, the Bti δ-endotoxins encoding genes are located mainly on pBtoxis plasmid, and don't allow the inference of the strain serotype, which is determined based on flagellar proteins encoded on the bacterial chromosome (Berry *et al.*, 2002; Carlson & Kolstø, 1993). Therefore, a serotyping of Bti and Btk (Btk5) strains was performed. Although results were not so clear, floccular deposit of the Bti reference strains, the positive controls, were identified. The A19 strain result was considered negative as the negative control Btk5 strain. Despite different results of the A19 strain anti-H-14 reaction, comparing to the negative control, its serotyping was considered negative, since it was also different from all the Bti reference strains positive controls. The negative result in the swimming motility test and the

confirmation of the absence of flagella in the A19 strain, contrary to the Bti reference strains, supported its negative result in the serotyping. According to the classification of Lee and collaborators (2003), A19 strain can be classified as an "un-testable" strain, concerning the serotyping, since it doesn't present flagella.

The morphological characteristics of the A19 strain presented similarities with the Bti reference strains both at macro and microscopic levels and respect the standards of *Bacillus thuringiensis* described in "Bergey's manual of systematic bacteriology" (Vos *et al.*, 2009). However, the bigger colonies, as well as the absence of flagella in A19 strain, supported that A19 and Vec8 are two different strains.

The presence of globular proteinaceous parasporal bodies in the A19 strain as in the Bti reference strains tested, was confirmed and supports the *Bacillus thuringiensis* nature of this strain (Angsuthanasombat *et al*., 2004). The predominant globular shape of the A19 strain parasporal crystals is a feature usually found in *israelensis* variety (Boisvert, 2005; Lecadet *et al*., 1999). The crystals' shape depends on its protoxin composition, and the globular shape detected in all Bti strains observed is usually associated to the Cry4A and Cry4B protoxins (Boisvert, 2005; Lecadet *et al*., 1999; Schnepf *et al*., 1998). The SEM images of the Bti spores-crystals suspensions showed that the crystal shape is not perfectly round, which could be associated to its diverse components, since the crystals are not only composed by Cry4, but also by other protoxins, such as Cry11, associated to a rhomboid shape (Schnepf *et al.*, 1998). The components of the culture growth medium, as well as their concentrations, can interfere with the dimension of the parasporal crystals produced (Obeta & Okafor, 1984; Rajalakshmi & Shethna, 1980; Scherrer *et al.*, 1973). Furthermore, the parasporal bodies production is not limited to one per spore (Tyrell *et al*., 1981). Thus, to obtain comparable spores-crystals suspensions, the conditions of growth used were the same for all strains (A19, Vec8, Bioflash, Bti4 and IP4444), and the amount of toxin was quantified considering the same amount of spores. A bigger amount of proteins per spore in A19 strain's spores-toxins suspension was measured, when compared to the Bti reference strains. The bigger amount of toxin produced by the A19 strain was verified in both solid and liquid GYS medium, but only the results obtained from the solid medium were statistically confirmed. Previous studies (Guidi *et al.*, 2011; De Respinis *et al.*, 2006) showed that the long-term applications of the Bti-based product VectoBac-G® increases the Bti spores' number in the soil, although a major ecological impact on Bolle di Magadino was excluded. Therefore, a bigger production of toxin could mean not only an economic advantage of A19 strain as biopesticide, but also an environmental advantage since for the same amount of toxin currently applied, fewer spores would be introduced in the environment.

The presence of Cry4A, Cry4B, Cry10Aa, Cry11Aa, Cyt1Aa and Cyt2Ba δ-endotoxins encoding genes was confirmed, which suggests the presence of whole pBtoxis plasmid,

since the toxin genes are usually all located on the same plasmid (Ben-dov *et al*., 1997; Pérez *et al*., 2005). It also supports the attribution of the *israelensis* variety to A19 strain and gives an indication about its target organism (Schnepf *et al*., 1998), since this δ-endotoxins are responsible for the toxicity of Bti against members from Diptera order (Ben-dov *et al*., 1997; Pérez *et al*., 2005).

The toxic activity of A19 strain against mosquito larvae was confirmed. A19 strain showed to produce parasporal crystals with a toxic activity similar to the Bti reference strains tested. From the literature is known that the activity of a Bti strain is influenced by the proportion of the δ-endotoxins that constitute the crystal (Aronson *et al*., 1991; Otieno-Ayayo *et al*., 2008). According to this, and considering the similar results obtained between the strains with the bioassay, it can be hypothesized that the composition of crystal of the five strains tested is similar. After 24 hrs of exposure to the A19 toxin, only a living mosquito larva was observed at the maximum toxin concentration. Since the LC_{50} values were similar for the five strains, it could be hypothesized the non-ingestion of the toxin by the larva due to the erroneous use of a larva in a more advanced stage, that had already stopped feeding itself (Boisvert, 2005).

Considering the SDS-PAGE proteomics analysis of the Bti spore-phase, there was no detectable difference between the A19 strain and the Bti reference strains. When compared to the Bti reference strains (Vec8, Bioflash, Bti4 and IP4444), a *B. sphaericus* strain and a *B. thuringiensis* var. *kurstaki* (Btk) strain profiles, A19 strain showed to be clearly more similar to the Bti reference strains than to the non-Bti reference strains. This confirmed the similar composition of the parasporal crystal produced by A19 and the other Bti strains tested, in agreement with the bioassay's results; and supports the presence of whole pBtoxis plasmid in A19 strain. The spore-phase protein profiles obtained, presented similarities with the Bti protein spore-phase profile obtained by Chaves and collaborators (2008), and Lecadet and collaborators (1999). Although quite thin, the bands corresponding to the Cry4Aa and Cry4Ba toxins were identified in all Bti strains' profiles. The thinness of Cry4 bands could be explained by imperfect migration conditions, or a rapid degradation of the Cry toxins could have influenced the visibility of the Cry4 toxins on the gel (Stotzky, 1998). The difficult separation of Cry10Aa from the Cry11Aa bands, as well as the Cyt1Aa from the Cyt2Ba bands, was due to the proximity of their molecular weights. Moreover, the Cyt toxins were present in a big concentration, making the separation difficult. The high quantity of the Cyt proteins is in accordance to the studies of Charles and collaborators (2000), indicating that the 28 kDa protein represents 54% of total crystal proteins. All the Bti protein profiles presented other bands beyond the Cry and Cyt protoxins, which could correspond to other spores-surface proteins (Tyrell *et al*., 1981).

Considering the vegetative cell-phase total proteins' profile, represented in a 2-D PAGE, A19 strain showed to be very similar when compared with the Bti reference strains. Despite

the similarities, three spots lacked in the 2-D PAGE protein profile of A19 strain. Based on the theoretical isoelectric points, molecular weight of the missing proteins (Apweiler *et al*., 2011; Gasteiger, 2003), and considering the absence of flagella in the A19 strain, it is very likely that some of the missing spots correspond to flagellum related proteins. The 1 and 2-marked missing proteins, with acidic characteristics, could be the flagellar biosynthesis protein FlhF (p*I* = 5.55 and Mw = 49.85 kDa; SwissProt: Q3EP41), a GTP-binding protein without which cells are non-motile (Carpenter *et al*., 1992), and the flagellar hook-associated protein 2 (p*I* = 5.67 and Mw = 49.90 kDa; SwissProt: Q6HKR1), that is involved in the filament formation (Homma *et al*., 1984). The third spot, corresponding to a basic protein, seems to correspond to the B6 spot labelled by Hirose and collaborators (2000) in *Bacillus subtilis,* but it was not identified. The use of Bce1 as control for the 2-D PAGE whole protein profile analysis would be helpful to identify some proteins and control some eventual differences between Bt and Bc. Despite Bt was considered safe for many of non-target organisms, including humans, due to the high specificity of its toxins (Boisvert, 2005), this bacterium can sporadically produce virulence factors that are problematic for humans health (Carlson & Kolstø, 1993; Drobniewski, 1993; Hansen & Hendriksen, 2001). From the literature it is known that flagella and motility are involved in the parasite - host interactions (Ghelardi *et al*., 2002). Moreover, it was proved that an anomaly that compromises the flagellar transport can interfere with the release of virulence factors (Ghelardi *et al*., 2002). Thus, the identification of A19 strain missing proteins would be interesting to better understand if they affect the releasing of virulence factors. Their identification could be performed, for example, through the analysis of their peptide mass fingerprinting (PMF) obtained by the MALDI-ToF MS/MS technique.

The Bti vegetative cell-phase total protein's profile was also achieved using the MALDI-ToF MS technique. This technique enables the mass determination of high molecular weight biomolecules, as the proteins (Pieles *et al*., 1993). It is a rapid and reliable technique to perform phyloproteomics (Conway *et al.*, 2001). Since it allows to obtain the whole cell proteins profile, the comparison with other organisms' protein profiles is possible. The dendrogram based on the comparison of the proteins' profiles, obtained for the different strains, allowed us to confirm the similarity of A19 strain with the Bti reference strains. The obtained low percentages of similarity between the strains is not uncommon when this technique is used (Kallow *et al.*, 2006). This can be due to the interference of different factors which create variations on each reading made, such as fluctuations on the laser power, changes on the detector answer, but mainly due to the heterogeneous incorporation of the analyte on the matrix, when the samples are prepared (Duncan *et al.*, 2008). Nevertheless, the dendrograms obtained through this technique are reliable, clustering together the related organisms (Kallow *et al.*, 2006). A19 strain was in the same cluster together with the Bti

reference strains, and showed a bigger similarity to Vec8 strain (74%) than to the other Bti strains tested. Bce1 strain was closer to the Bti strains (62% similarity) than Btk5 strain (58% similarity), but this can be due to the great taxonomic proximity between Bt and Bc species (Bavykin *et al.*, 2004; Drobniewski, 1993; Vilas-Bôas *et al.*, 2007).

Finally, to clarify the origin of A19 strain, a genomic method was employed, the random amplified polymorphic DNA – PCR (RAPD-PCR) technique, which allows to create a DNA fingerprint of each microorganism, and thus understand better their phylogenetic relationship (Chaves *et al*., 2010). The polymorphic regions on the DNA allow the discrimination among strains from the same serotype or from different serotypes (Brousseau *et al.*, 1993), thus the RAPD-PCR technique can be used to classify new strains when the serotyping is not possible (Chaves *et al.*, 2010). Since the quality of the DNA extracted from the Bti strains was confirmed by the amplification of the 16S ribosomal subunit coding region, the reason why the amplification of the Bti strains' DNA sequences with the RAPD-PCR method was not successful could be due to the unreached low stringency conditions. To promote low stringent RAPD-PCR conditions, the annealing temperature should be low, and the PCR buffer should have a high salt concentration (Kumar *et al.*, 2009). In addition, the use of HotStarTaq DNA Polymerase was inadequate, since this enzyme is very specific. Its use was due to the unavailability of a more appropriate DNA polymerase enzyme when the experiment was performed. Moreover, the MasterMix also used in a separate assay, already included a PCR buffer that doesn't promote the low stringency conditions required. It would be interesting to repeat this experiment using an adequate Taq DNA polymerase enzyme (non-high specific), since this technique would allow the acquirement of more information about the similarity between the Bti strains tested, particularly between A19 strain and the Bti strain currently applied at the Bolle di Magadino, Vec8.

5. CONCLUSION

This study supported A19 strain's classification as *Bacillus thuringiensis* var. *israelensis*, and allowed to deepen the knowledge about A19 strain, a potential biopesticide, and thus for a possible improve of the maintenance of mosquitoes' population on the Swiss natural reserve, Bolle di Magadino.

It was confirmed that A19 and Vec8 are independent strains. Differences at morphological and molecular levels between A19 strain and the Bti reference strains used in this study, including the Bti strain used at Bolle di Magadino for the control of mosquitoes' population, support the hypothesis that A19 strain is an autochthonous microorganism. Despite this, the dendrogram obtained based on total protein profiles revealed a bigger similarity between the strains A19 and Vec8. Therefore, to understand better the similarity level between these two

strains, and to confirm the origin of A19 strain, it would be important to finalize the genomic analysis through the RAPD-PCR technique, and perform a phylogeny.

The A19 strain showed to have potential as biopesticide. Its toxicity against mosquito larvae was confirmed, and showed to be equivalent to the Bti reference strains tested. As biopesticide, A19 strain could be economically more advantageous than the Bti reference strains tested, since it produces a bigger amount of toxin per spore than the other Bti strains analyzed, which means higher profitability. It could also be environmentally more advantageous, since A19 strain's higher production of toxin per spore would allow a reduction in the number of spores introduced in the environment during a treatment, without reducing the toxin concentration. The autochthonous condition of A19 strain is also an environmental advantage, since it will avoid the introduction of an external microorganism on the Bolle di Magadino natural reserve. Moreover, A19 strain showed to be a non-motile strain and the absence of flagella was confirmed. This characteristic could also be environmentally advantageous, since it can be associated to a defective release of virulence factors and consequently to a lower pathogenicity level; however, this hypothesis needs to be confirmed. The A19 strain missing proteins should be identified through MALDI-ToF MS/MS technique; and the A19 strain's safety for animals' health, should be tested.

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