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Characterization and Whole Genome Sequencing of AR23, a Highly Toxic *Bacillus thuringiensis* Strain Isolated from Lebanese Soil

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Abstract

The demand for sustainable and eco-friendly control methods of pests and insects is increasing worldwide. From this came the interest in *Bacillus thuringiensis*, an entomopathogenic bacterium capable of replacing chemical pesticides. However, the possibility of pests developing resistance to a particular strain may impair its use, and there is a need to identify novel strains of this species as potential commercial biopesticides. *B. thuringiensis* sv. *israelensis* is one of the most successful serovars, widely commercialized for its activity against black fly and mosquito larvae. In this study, we isolated, characterized, and sequenced a new Lebanese *B. thuringiensis* sv. *israelensis* isolate, strain AR23. Compared to the commercialized reference strain AM65-52 (Vectobac®, Sumitomo), AR23 showed an increased activity against several mosquito species. The genomic analysis revealed that this strain, compared to AM65-52, possesses a simplified plasmid content and an additional functional *cry4Ba* coding gene that most likely accounts for the increased effectiveness of this strain in mosquito larvae killing.

Introduction

Disease carrying vectors, as well as phytopathogens, are commonly controlled by chemical pesticides that have a negative impact on the environment. This has led to the use of entomopathogenic bacteria such as *Bacillus thuringiensis* as biocontrol agents [1]. *B. thuringiensis* owes its insecticidal activity to the production, in parallel with spore formation, of crystalline inclusions consisting of proteins toxic to insect larvae upon ingestion [2]. The proteins accumulated in the sporange as a crystalline inclusion are released upon completion of sporulation. The insecticidal proteins in the crystalline bodies are produced during sporulation and have been shown to contain mainly two types of insecticidal proteins: pore-forming Cry toxins and cytolytic Cyt toxins [3, 4]. *B. thuringiensis* crystals are completely biodegradable. The specific basic pH of the larvae's digestive tract is necessary for their solubilization and the high specificity to their receptors makes these toxins generally safe for non-target organisms [2, 5–7]. Each *B. thuringiensis* strain is characterized by its activity spectrum, which depends on the diversity and the combination of Cry and/or Cyt toxins forming its

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crystals [8]. Cry and Cyt toxins are both membrane-perforating proteins although unrelated structurally. Genes encoding these toxins are carried by large plasmids, some of which are conjugative or mobilizable [9]. These genes can also be associated with transposable elements [10, 11].

Among the various commercialized *B. thuringiensis* strains, the serovar *israelensis*, used to control mosquito and black fly larvae, is unique for its capacity to produce Cyt1 and Cyt2 proteins that synergize with the Cry proteins. The target insects for *B. thuringiensis* sv. *israelensis* include the larvae of different dipteran species of the genus *Aedes*, *Anopheles*, and *Culex*. One of the well-studied plasmids it holds is pBtoxis, a 128-Kb plasmid carrying the genes encoding all the mosquitocidal endotoxins present in the strain, namely Cry4Aa, Cry4Ba, Cry10Aa, Cry11Aa, Cyt1Aa, and Cyt2Ba [12]. Each protein shows individual toxicity. However, their combination and synergistic interactions delays resistance evolution in the field [13, 14] and allows a more pronounced efficacy and an enhanced toxicity of the individual toxins [5, 15–18]. No other strain of *B. thuringiensis* has this property.

Improvement in *B. thuringiensis*-based biopesticides depends on combining various toxins as well as the identification of new strains [19]. To this aim, new isolates are regularly tested and with the advancement in sequencing techniques, many laboratories are conducting whole genome sequencing (WGS) on the most toxic strains. In fact, *B. thuringiensis* whole genome analysis accelerates the identification of potential genes encoding new virulence factors and toxins. *B. thuringiensis* sv. *chinensis* strain CT-43, having a full-length genome of 6.15 Mb, was the first screened for new *cry* genes using WGS [20]. Since then, many other strains have been characterized for their insecticidal gene content by WGS, such as *B. thuringiensis* sv. *kurstaki* strain HD-73, *B. thuringiensis* sv. *thuringiensis* strain IS5056, and *B. thuringiensis* sv. *israelensis* strain HD-789 [21–23]. So far, only three complete genomes are reported for *B. thuringiensis* sv. *israelensis*: HD-789 [21], HD1002 [24], and AM65-52 [25].

In this study, we describe a highly toxic *B. thuringiensis* sv. *israelensis* strain (AR23), active against several dipteran larvae, isolated from a Lebanese soil sample. In order to understand its high toxicity in vivo, we investigated its genetic background and specifications by WGS.

Materials and Methods

Bacterial Strains

Bacillus thuringiensis sv. *israelensis* AM65-52, isolated from the commercial sample (Vectobac®, Sumitomo), was our reference strain. It was kindly provided by Pr. Christina Nielsen-Leroux from the GME laboratory of the National Institute for Agronomical Research (INRA−Jouy-en-Josas). The strain AR23 was selected for being the most toxic among different *B. thuringiensis* sv. *israelensis* strains isolated from a Lebanese soil sample and tested against third-instar mosquito larvae of *Aedes albopictus, Culex pipiens*, and *Anopheles gambiae*.

Crystal Proteins Extraction and SDS-PAGE Analysis

B. thuringiensis strains were grown on solid T3 medium [26] for 72 h at 30 °C. Spores and crystals were collected and washed twice with 1 M sodium chloride solution, and six times with cold sterile water. The spore-crystal mixtures were then solubilized in 50 mM sodium hydroxide buffer at 30 °C [27], and quantified using Bradford reagent (Bio-Rad Protein assay, Cat. 500-0006; [28]). The proteins were separated on SDS-PAGE for profiling [29] and stained with Coomassie Brilliant Blue [30]. For purified crystals, the washed spore-crystal mixture was loaded on a discontinuous sucrose gradient of 72 and 79% (w/v, sucrose in deionized water) and centrifuged overnight 19 h at 25,800×*g* [31].

Bioassays

Bioassays were conducted on *C. pipiens, A. albopictus*, and *A. gambiae* third-instar larvae, reared in the biology department of the American University of Beirut, at 27 °C, 80% relative humidity, and a 12:12 dark:light period. Bioassays were performed in three biological replicates, each time on 14 larvae in 15 mL of dechlorinated water. First, the most accurate range of concentrations and the perfect time to calculate the LC_{50} were determined. Afterwards, seven concentrations of the spore-crystal mixtures of each strain were tested against *Culex*, *Aedes*, and *Anopheles* larvae, with the concentrations for *Anopheles* being slightly higher. Treated larvae were housed at 27 °C and examined after 2 h 30 for *Culex*, 3 h 30 for *Anopheles* and 5 h for *Aedes*. An untreated group was included as a negative control in each bioassay.

The 50% lethal concentrations of each strain were calculated using non-linear regression, and then compared using a Student *t* test analysis at a confidence level of 95%. After calculating the standard error deviation (s(LC50)) and the degree of freedom (nu) in each case, the standard deviation on the difference of the two means (*s**) was determined. This allowed us to find the experimental *t* value (t-obs) and the bilateral critical *t* value (t-crit bi), as well as the risk of error (α bi). In order to have a significant difference in toxicity, the experimental *t* value has to be higher than the theoretical *t* value, or the risk of error has to be lower than 5%.

Sequencing and Assembly

Genomic DNA was extracted using the Puregene yeast/Bact. KitB from Qiagen following an overnight culture in LB broth [32] at 30 °C. AR23 genome was then sequenced using the Illumina MiSeq platform (570 insert size, 2 × 250). FASTX-Toolkit (https://hannonlab.cshl.edu/fastx_toolkit/, last accessed March 19, 2018) was used to filter artifacts, low-quality bases, and reads with a length inferior to 200 bp as well as the right-tail clipping using a quality threshold of 20. Sequences containing undefined nucleotides (Ns) were discarded using PRINSEQ v0.20.4 [33]. The script Velvetk (March 19, 2018) was used to estimate best k-mer value for de novo assembly using Velvet [34]. The contigs obtained were scaffolded using SSPACE v3.0 [35]. MAUVE was used for alignment and reordering [36], using as reference the genome of *B. thuringiensis* AM65-52 [25]. Finally, Gap5 was used to manually join overlapping contigs [37].

In parallel, our paired-end reads were mapped against *B. thuringiensis* sv. *israelensis* strain AM65-52 (CP013275-84), using BWA [38]. Chromosome variant calling was performed with Genome Analysis Toolkit v3.4.0 [39], and filtered by the following criteria: alternate allele frequency, > 90%; read depth (DP), > = 10; quality by depth (QD), > 2.0; Fisher strand bias (FS), < 60.0; mapping quality (MQ), > 40.0; mapping quality rank sum test (MQRankSum), greater than − 12.5; read position rank sum test (ReadPosRankSum), greater than − 8.0 [35].

Annotation

The assembled genome was annotated by Prokka [40]. Insertion sequences and RNA genes were identified and annotated with BLASTn searches against the ISFinder database and RFAM, respectively [41–43]. Toxin genes were annotated through BLASTp searches of the complete identified proteome against a database constructed with the sequences listed in the *B. thuringiensis* toxin nomenclature database (https://www.btnomenclature.info/; [44]). BLASTx searches of Intergenic spacers (IGS) were carried to identify possible traces of toxins corresponding to unannotated or pseudogeneized strains.

Phylogenetic Relationships

SNPs among 36 different strains belonging to the *Bacillus cereus* sensu lato group were identified with the tool kSNP2, using a k-mer size of 21. This group comprises nine ecologically diverse but phylogenetically close species such as *B. anthracis, B. cereus* sensu stricto*, B. thuringiensis,* and the divergent *B. cytotoxicus,* whose strains were removed to obtain a proper value of core SNPs [45]. kSNP uses FastTree to obtain a quick ML phylogeny using core SNPs [46]. Core genome alignments of were obtained with Parsnp [47], and the recombinant SNPs were identified with Gubbins [48]. A Maximum likelihood tree was built from the non-recombinant core SNP alignment using RaxML [49], using a General Time Reversible (GTR) substitution model with 1000 bootstrap replicates.

Results

Bacterial Strains and Bioassays

AR23 was isolated from a Lebanese soil sample and its plasmid and crystal protein profiles were established (Supplementary Fig. S1). In order to determine the optimal time for the calculation of the 50% lethal concentration (LC50) of AR23 and AM65-52 toxins, a preliminary screening was conducted, which allowed us to fix 2.5, 3.5, and 5 h and a tight range of toxin concentrations for the in vivo tests and LC50 determination against *A. albopictus, C. pipiens,* and *A. gambiae* larvae. As shown in Fig. 1, AR23 was more toxic to the larvae than the reference *B. thuringiensis* sv. *israelensis* AM65-52. The LC50 on the different larvae was calculated using non-linear regression at the significance level of 5%. For AM65-52 and AR23, respectively, the LC50 are $(74.0 \pm 1.1) \times 10^{-4}$ and $(68.0 \pm 1.5) \times 10^{-4}$ µg/mL for *Culex* $(83.9 \pm 0.9) \times 10^{-4}$ and $(71.0 \pm 1.4) \times 10^{-4}$ µg/mL for *Aedes* and $(336.3 \pm 5.0) \times 10^{-4}$ and $(236.0 \pm 7.1) \times 10^{-4}$ µg/mL for *Anopheles*. Using a Student *t* test analysis at a confidence level of 95% (Table 1), we calculated the experimental (t-obs) and theoretical or critical t value. In order to have a significant difference between the 50% lethal concentrations of the two strains, the former must be higher than the latter. This is the case for all tested larvae, meaning that AR23 is significantly more toxic than AMA65-52 with a risk of error lower than 5%. In order to exclude the spore effects on the observed toxicity in each test, the survival experiments were also performed using purified crystals of each bacterial strain obtained with a sucrose gradient. The survival curves confirm the previous results against the different larvae (data not shown).

Genome Sequencing

In order to investigate what causes the difference in toxicity between our strain AR23 and the reference AM65-52, we resorted to a whole genome sequencing approach. AR23 genome was sequenced using Illumina MiSeq. The assembled contigs were sorted with Mauve using the complete genome of the *B. thuringiensis* AM65-52 as reference. This analysis indicated that AR23 shared five plasmids with AM65-52: pAM65-52-1-360 K (CP13276.1), pAM65-52-4-128 K (CP13279.1), pAM65-52-6-16 K (CP13281.1),

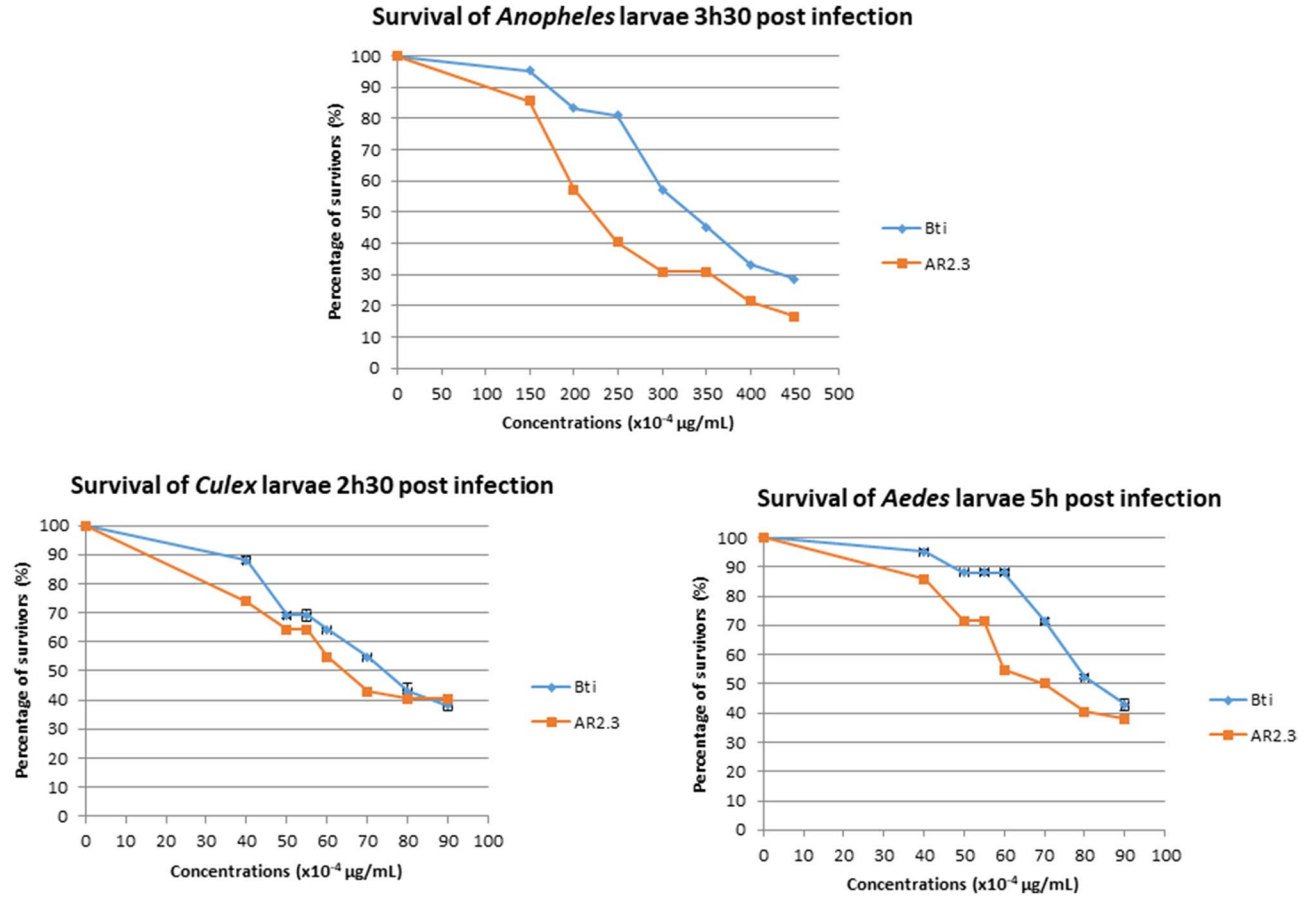

Fig. 1 In vivo toxicity tests of the selected Lebanese strain AR23 and the *B. thuringiensis* sv. *israelensis* reference strain AM65-52. The graphs show the mean percentage of survivors issued from biological triplicates for different concentrations of spore-crystal mixtures, for 2 h 30, 5 h, and 3 h 30 incubation times for *Culex, Aedes,* and *Anopheles* larvae, respectively

pAM65-52-8-7 K (CP13283.1), and pAM65-52-9-5 K (CP13278.1). The sequence of the 128 kb plasmid was completed by PCR. Its organization is similar to that of pBtoxis (AL731825.1; [12]), since it does not contain the inversion described in pAM65-52-4-128 K, located around the origin and the end of the replication origin (Fig. 2). The sequence data are available on the European Nucleotide archive with the project number PRJEB28570. SNP-based phylogenetic analysis (Fig. 3) shows that AR23 is closely related to the clade grouping the strains HD-771, G9842, and HD-789.

Thirty-one genetic differences were identified between AR23 and AM65-52 genomes, taking into account both SNP and small Indels. These variations are summarized in Supplementary Table S1. Fifteen out of the thirty-one differences were detected in the 128-kb plasmid that carried most of the toxin genes. From these variants, five SNPs and one insertion were found within the sequence of the *cry4Aa* pseudogene (Ψ*cry4Aa*) annotated in pAM65-52-4-128 K (CP013279). Interestingly, these differences pointed out a likely functional *cry4Aa* gene in AR23. The other mutations in this plasmid affect other pseudogenes and mobile genetic elements. As for the 13 mutations located on the chromosome, none of them seems related to the insecticidal activity of AR23.

Other than on the 128-kb plasmid and the chromosome, two mutations affected genes potentially involved in the pathogenesis. A mutation located in a chitinase gene, homologous to that located on pAM65-52-1-360 K (ATN07_29645), was inactivated in strain AR23, and a deletion of three amino acids was observed in a collagen-like protein homologous to ATN07_34715 encoded by pAM65-52-8-7 K.

Toxin Annotation

In total, five *cry* toxin genes were identified on the 128-kb pBtoxis-like plasmid, namely one *cry10Aa4*, one *cry11Aa3*, and two *cry4* toxin-coding genes (one *cry4Ba5* toxins and one *cry4Aa4*), and finally there is a pseudogeneized, *cry4Ba* toxin gene. All *cry4* toxin genes were associated with insertion sequences, such as IS*240* and elements belonging to the IS*6* family. As shown in Fig. 2, the inversion of a central

Table 1 Larvicidal activity of *B. thuringiensis* strains AM65-52 and AR23 on third-instar larvae of *A. albopictus*, *A. gambiae*, and *C. pipiens*

	AM65-52	AR23	*s**	t-obs	t-crit bi (5%)	*α* bi
Culex						
LC50 ($\times 10^{-4}$ µg/mL)	74.0	68.0				
CV % (LC50)	1.5	2.3				
LC50MIN (2.5) ($\times 10^{-4}$ µg/mL)	71.6	65.0				
LC50MAX (97.5) ($\times 10^{-4}$ µg/mL)	76.2	71.0				
s(LC50) ($\times 10^{-4}$ µg/mL)	1.1	1.5	1.9	3.26	2.6	0.99
nu	5	5	10			
AR23/AM65-52	0.92					
Aedes						
LC50 ($\times 10^{-4}$ µg/mL)	83.9	71.0				
CV % (LC50)	1.1	1.9				
LC50MIN (2.5) ($\times 10^{-4}$ µg/mL)	82.1	68.2				
LC50MAX (97.5) ($\times 10^{-4}$ µg/mL)	85.7	73.8				
s(LC50) ($\times 10^{-4}$ µg/mL)	0.9	1.4	1.7	7.72	2.3	0.00
nu	5	5	9			
AR23/AM65-52	0.70					
Anopheles						
LC50 ($\times 10^{-4}$ µg/mL)	336.3	236.0				
CV % (LC50)	1.5	3				
LC50MIN (2.5) ($\times 10^{-4}$ µg/mL)	326.3	221.8				
LC50MAX (97.5) ($\times 10^{-4}$ µg/mL)	346.3	250.2				
s(LC50) ($\times 10^{-4}$ µg/mL)	5.0	7.1	8.7	11.56	2.26	0.00
nu	5	5	9			
AR23/ AM65-52	0.85					

LC50 is the mean 50% lethal concentration (LC50) estimated by Probit analysis using statistical parameters at the significance level of 5%. s(LC50) is the standard deviation error, CV % (LC50) is the coefficient of variation, and LC50min and LC50max delimitate the 95% confidence limits. Student t test analysis comparing the mean 50% lethal concentrations (LC50) of the two strains AM65-52 and AR23. *s** is the standard deviation on the difference between the two means, t-obs is the experimental *t* value, t-crit bi (5%) is the critical t value for a bilateral test at a 5% significance level, and α bi is the risk of error. Nu is the degree of freedom. ($\times 10^{-4}$ µg/mL)

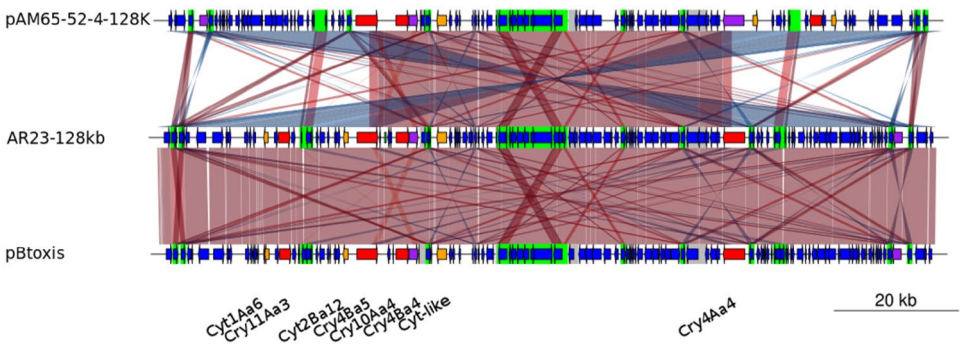

Fig. 2 Comparison between the different crystal toxin encoding plasmids. Arrows represent CDS, *cry* genes in red, in orange genes encoding Cyt toxins, and in purple pseudogeneized toxin genes. Green blocks represent complete IS elements, while gray blocks represent interrupted IS elements. Red blocks between sequences represent homologous regions in the same orientations, while when they are in blue they are homologous sequences in opposite orientation (Color figure online)

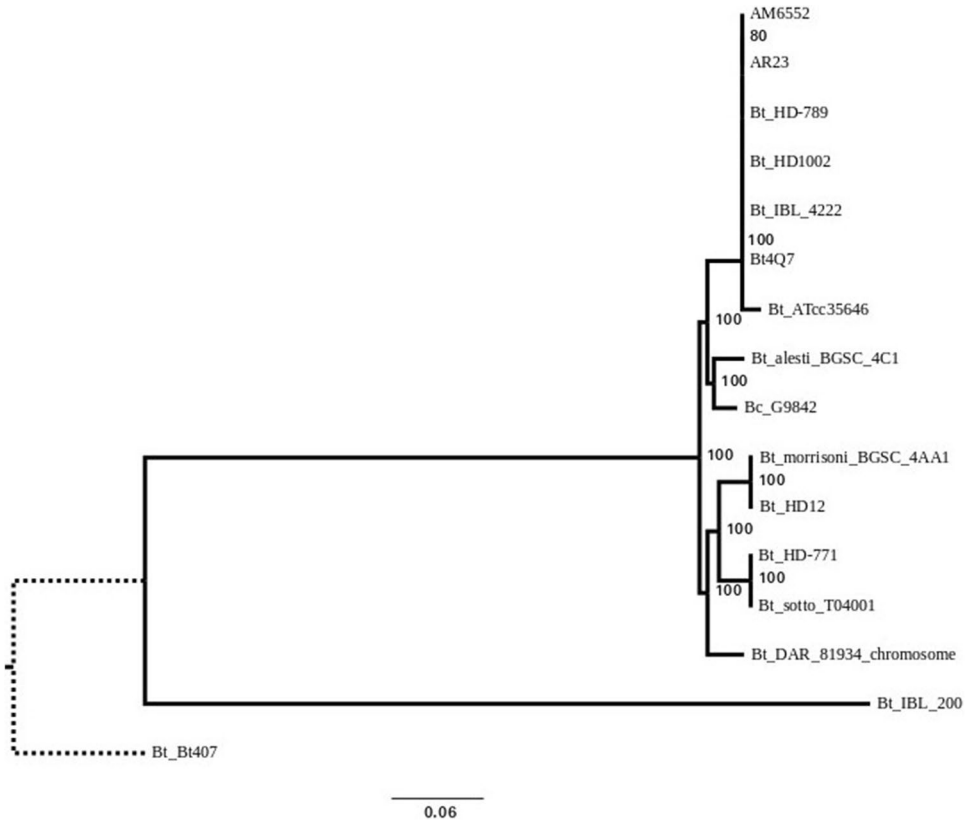

Fig. 3 Maximum likelihood tree from close related *B. thuringiensis* strains obtained with core non-recombinant SNPs, using *B. thuringiensis* 407 as outgroup

region of pAM65-52-4-128 K described earlier affects one of the genes encoding a Cry4Aa toxin that is pseudogeneized in strain AM65-52, but functional in AR23, as well as in the pBtoxis plasmid.

Besides the five *cry* genes, three *cyt* loci were located on the same plasmid: *cyt2Ba*, *cyt1Aa,* and *cyt1Ca*. Two additional potential virulence factors were detected in the contigs related to the 360-kb and 16-kb plasmids. The former is homologous to the mosquitocidal toxin protein (locus tag ATN07_29875) of *B. thuringiensis* sv. *israelensis* strain AM65-52 and contains a NPP1 domain (Necrosis Inducing protein, PF05630). The latter holds a calcineurin-like phosphoesterase domain (PF00149). Both proteins contain a Ricin-type beta-trefoil lectin domain-like domains (PF14200) and have been annotated by Prokka as hemagglutinin-related proteins.

Discussion

Vector-borne diseases cause major public health problems and the search for new efficient bacterial strains with activity against dipteran species could have a positive impact on the control of mosquitoes worldwide. Here, we have characterized a new highly active *B. thuringiensis* mosquitocidal strain, designated AR23, isolated from Lebanese soil. Its characterization included the identification of its mosquitocidal LC50 and *cry* and *cyt* genes content, as well as the determination of its complete sequence and comparison with other known *B. thuringiensis* mosquitocidal strains.

AR23 and *B. thuringiensis* sv. *israelensis* AM65-52′s highly similar crystal protein profile (Supplementary Fig. S1) suggests that this combination of insecticidal proteins is probably one of the most effective against dipteran species. Indeed, calculation of their LC50s indicated that both strains were highly toxic with concentrations in the range of 7 to 8 ng of crystal proteins per ml for *Aedes* and *Culex* and of 30 ng/ml for *Anopheles*. The higher concentration required to kill *Anopheles* is mainly related to the larvae's specific feeding at the air–water interface, thus limiting the ingestion of the crystal-spore suspensions that quickly settle at the bottom of the water after application [50]. However, the toxicity of strain AR23 was slightly, but significantly, higher than that of the reference strain *B. thuringiensis* sv. *israelensis* AM65-52, on the three tested dipteran larvae. By comparing their LC50s, it can be assumed that the toxicity of the reference strain AM65-52 could be reached by lower AR23 toxin concentrations (15% for *Aedes*, 8% for *Culex*, and 30% for *Anopheles*).

Comparison between the genetic content of AR23 128 kb toxin-carrying plasmid with that of the reference

Table 2 Insecticidal factors present in the *B. thuringiensis* strains AR23 and AM65-52. (Ψ = pseudogene)

	AR23		AM65-52	
	Gene	Copies	Gene	Copies
Cry toxins	Cry11Aa3	1	Cry11Aa3	1
	Cry4Ba5	1	ΨCry4Ba5	1
	Cry10Aa4	1	Cry10Aa4	1
	ΨCry4Ba4	1	ΨCry4Ba4	1
	Cry4Aa4	1	ΨCry4Aa4	1
Cyt toxins	Cyt1Aa6	1	Cyt1Aa6	1
	Cyt2Ba12	1	Cyt2Ba12	1
	Cyt1Ca1	1	Cyt1Ca1	1
Other	Hemagglutinin related	2	Hemagglutinin related	2

strain AM65-52 (pAM65-52-4-128, CP013279; [25]) indicates the presence of the same set of anti-dipteran toxins Cry4Aa, Cry4Ba, Cry10A, Cry11A, Cyt1Aa, Cyt2Ba, and Cyt1Ca. The main difference resides in the presence of a fully functional Cry4B toxin-coding gene in AR23 *versus* a pseudogeneized version in AM65-52 (Table 2 and Fig. 2). The presence of a premature stop codon leads to the loss of 166 amino acids in the C-terminal end of the 1137 amino acids protein in AM65-52, thus leading to the deletion of three conserved sequence blocks [2]. The disruption of the C-terminal end of a toxin, most importantly, can hinder the correct folding and incorporation of a toxin into the crystal. The recovery of an intact Cry4B toxin is due to the presence of a T to C transition in strain AR23 (ATN07_33965; CP013279.1). Therefore, the presence of this functional Cry4B toxin could explain the higher efficacy of AR23, thanks to likely synergistic effects with the other components of the crystal [16, 51, 52].

Our data showed that AR23 and the reference *B. thuringiensis* sv. *israelensis* AM65-52 strain are genetically very close, and share five out of the eight plasmids found among the other *B. thuringiensis* sv. *israelensis* strains [53]. AR23 differs only by minor nucleotide changes on the chromosomic level (13 out of 31 mutations) as well as on plasmids (15 out of 31 mutations on the 128 Kb plasmid, 1 deletion of the 360 Kb plasmid, 1 deletion of the 7 Kb plasmid, and 1 SNP on the 16 Kb plasmid) (SNPs and Indels listed in Supplementary Table S1).

AR23 lacks three megaplasmids found in strain AM65-52: the conjugative plasmid pXO16 (pAM65-52-2-350 K; CP013277; [54]), the prophage like molecule pBtic235 (pAM65-52–3-235 K; CP013278; [55]), and the 100-Kb plasmid pBtic100. The 350-kb conjugative plasmid pXO16 is capable of mobilizing "non-mobilizable" DNA fragments, and thus plays a crucial role in genome dynamics, plasticity, and horizontal genetic transfers [56]. It is assumed to be the main plasmid involved in toxin-carrying plasmid mobility in natural conditions [25]. Its absence in AR23 suggests that this strain is less prone to horizontal gene transfers in the environment, therefore, a suitable candidate for use in the field. Since plasmid carriage is also assumed to present a cost for the bacteria, the absence of the three "missing" plasmids may result in a gain of fitness for AR23 as compared to AM65-52 [57]. However, it remains to be seen whether or not these plasmids harbor specific features that might be beneficial for AM65-52 lifestyle.

In conclusion, in this study, a highly toxic *B. thuringiensis* sv. *israelensis* strain, AR23, isolated from Lebanese soil was characterized. Our data showed that the increased toxicity of its crystal-spore mixture, compared to that of the reference strain AM65-52, could be due to the presence of an additional Cry4B toxin. Deletion of the *cry4B* gene followed by bioassays will allow us to confirm this conclusion. Moreover, a differential expression of the toxin-coding genes cannot be excluded and will be explored via transcriptomic and proteomic studies.

AR23 is therefore an interesting strain to explore in the field as a new potential substitute to the worldwide reference *B. thuringiensis* sv. *israelensis* strain AM65-52, since the same larvicidal activities are obtained with a lower concentration, thus reducing the release of spore-crystal mixture in the environment.

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Compliance with Ethical Standards

Conflict of interest The authors declare having no conflict of interest in the publication.

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