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ORIGINAL ARTICLE

Identification, phylogenetic analysis, and genome mining of the tetracycline-resistant *Bacillus thuringiensis* strain m401 reveal its potential for biotechnological and biocontrol applications

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Abstract *Bacillus thuringiensis* is an entomopathogen belonging to the *Bacillus cereus* clade. We isolated a tetracycline-resistant strain called m401, recovered it from honey, and identified it as *Bacillus thuringiensis* sv. *kumamotoensis* based on the average nucleotide identity calculations (ANIb) comparison and the analysis of the *gyrB* gene sequences of different *B. thuringiensis* serovars. Sequences with homology to virulence factors [*cytK*, *nheA*, *nheB*, *nheC*, *hblA*, *hblB*, *hblC*, *hblD*, *entFM*, and *inhA*] and tetracycline resistance genes [*tet(45)*, *tet(V)*, and *tet(M)/tet(W)/tet(O)/tet(S)* family] were identified in the bacterial chromosome. The prediction of plasmid-coding regions revealed homolog sequences to the MarR and TetR/AcrR family of transcriptional regulators, toxins, and lantipeptides. The genome mining analysis revealed 12 regions of biosynthetic gene clusters responsible for synthesizing secondary metabolites.

Abbreviations: ANI, average nucleotide identities; *Bt*, *Bacillus thuringiensis*; CFU, colony-forming units; MH, Müeller Hinton medium; MIC, minimum inhibitory concentration(s); MYPGP, Müeller Hinton broth-yeast extract-potassium phosphate-glucose-pyruvate medium; MIN, minocycline; OTC, oxytetracycline; PGPB, plant growth-promoting bacterium; RiPP-like, other unspecified ribosomally synthesized and post-translationally modified peptide product; NRPS, non-ribosomal peptide synthetase cluster; Sv, serovar; TET, tetracycline; TSB, trypticase soy broth.

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We identified biosynthetic gene clusters coding for bacteriocins, siderophores, ribosomally synthesized post-translationally modified peptide products, and non-ribosomal peptide synthetase clusters that provide evidence for the possible use of *Bt* m401 as a biocontrol agent. Furthermore, *Bt* m401 showed high inhibition against all *Paenibacillus larvae* genotypes tested *in vitro*. In conclusion, *Bt* m401 owns various genes involved in different biological processes, such as transductional regulators associated with antibiotic resistance, toxins, and antimicrobial peptides with potential biotechnological and biocontrol applications.

PALABRAS CLAVE
Bacillus thuringiensis sv. *kumamotoensis*;
Péptidos antimicrobianos;
Paenibacillus larvae;
Resistencia a tetraciclina;
tet(45)

Identificación, análisis filogenético y minería genómica de la cepa de *Bacillus thuringiensis* m401 resistente a tetraciclina, y sus posibles aplicaciones biotecnológicas y de biocontrol

Resumen *Bacillus thuringiensis* es un microorganismo entomopatógeno perteneciente al clado *Bacillus cereus*. En este trabajo, aislamos de miel una cepa resistente a tetraciclina y la identificamos como *Bacillus thuringiensis* sv. *kumamotoensis* (*Bt* m401) mediante comparación de genomas completos y cálculo de identidad promedio de nucleótidos (ANIb), utilizando la base de datos EDGAR. Este resultado fue corroborado por el análisis filogenético del gen *gyrB*, que permite diferenciar serovares de *B. thuringiensis*. Se confirmó la presencia del gen de resistencia a tetraciclina *tet(45)* en el cromosoma bacteriano y se identificaron secuencias homólogas a genes de virulencia (*cytK*, *nheA*, *nheB*, *nheC*, *hblA*, *hblB*, *hblC*, *hblD* y *entFM*). El mobiloma de *Bt* m401 está constituido por 4 plásmidos y se identificaron secuencias homólogas a reguladores transduccionales (MarR y TetR/AcR), a toxinas (*Cry*1 y zeta toxin) y a péptidos antimicrobianos (*mersacidin family lantibiotics*). La cepa *Bt* m401 demostró una muy buena actividad antagónica *in vitro* contra distintos genotipos de *Paenibacillus larvae*, agente causal de la loque americana de las abejas. El estudio de minería genómica para grupos de genes biosintéticos reveló 12 regiones de clústeres de genes responsables de la síntesis de metabolitos secundarios. Se identificaron clústeres que codifican bacteriocinas, sideróforos, RiPP-*like* y NRPS y lantipéptidos. En conclusión, *Bt* m401 presentó una amplia variedad de genes que estarían involucrados en diferentes procesos biológicos, con un potencial interesante para su empleo en aplicaciones biotecnológicas.

Introduction

Bacillus thuringiensis (*Bt*) is a ubiquitous Gram positive, spore-forming bacterium isolated worldwide from a great diversity of sources, such as soil, water, insects, dust, phyllosphere, and diverse foods[20,37]. *Bt* belongs to the Cereus clade consisting of 17 species, including *Bacillus anthracis*, *Bacillus cereus*, *Bacillus mycoides*, *Bacillus thuringiensis*, and *Bacillus toyonensis*, among others[26].

Bt is the most successful microbial insecticide against different insect pest orders in agriculture[35,37]. Its action is based on insecticidal toxins that are active during the pathogenic process by synthesizing parasporal crystalline inclusions containing Cry and Cyt proteins. Some *Bt* strains also synthesize insecticidal proteins during the vegetative growth phase, known as vegetative insecticidal proteins (Vips), and secreted insecticidal proteins (Sips) that hold insecticidal activity against lepidopteran, coleopteran, and some homopteran pests[14,37].

Bt-related studies mainly focused on insecticidal activities; nevertheless, it is also considered a plant growth-promoting bacterium (PGPB)[11,25]. Moreover, many *Bt* strains produce virulence factors and other metabolites, including a broad range of antimicrobial peptides that are active against pathogenic bacteria and fungi, *e.g.*, cyclic lipopeptides, lanthipeptides, polyketides, bacteriocins, and bacteriocin-like inhibitors (BLIS)[2,11,25,30,36,40,43]. Diverse antimicrobial peptides have been isolated from different *Bt* strains showing wide or narrow bactericidal or bacteriostatic effects. Within this group, bacthuricins, entomocins, fengycins, thuricins, thurincin H, and thumolicin, among others, were reported[2,22,25,29,30,36,49].

American foulbrood (AFB) is the most widespread and destructive disease of bacterial origin that affects the larval and pupal stages of honeybees (*Apis mellifera* L.)[24]. In North and South American honey-producing countries, beekeepers have routinely treated colonies with oxytetracycline (OTC) to control AFB[38]. The widespread use of OTC has increased

tetracycline resistance (TET^R) in *Paenibacillus larvae* populations by enhancing the transfer of TET^R-encoding plasmids containing *tet(L)* genes[4]. The *tet*L determinant found in *P. larvae* is nearly identical, in sequence (99–100% identity), to one of the resistance loci harbored by honeybee gut symbionts, suggesting past horizontal transfer between commensal gut bacteria and this pathogen[46].

Spore-forming bacteria of the genus *Bacillus*, including *Bt*, are frequently present in honey and other apiarian sources[1,7] and can act as reservoirs and environmental suppliers of antibiotic resistance enabling the spread of TET^R genes between different bacterial species[46]. *Bacillus* species are also promising sources of bioactive compounds and secondary metabolites with potential uses beyond the field of apiculture, including a broad range of antimicrobial compounds with activity against pathogenic bacteria and fungi[2,12,22,29,36,43].

In previous studies, we evaluated several *Bacillus* strains for their *in vitro* antimicrobial activity against *P. larvae*, the causal agent of AFB[6,12,34]. These inhibitory effects correlated with BLIS production[34] and other antimicrobial peptides[12]. The present paper presents further insight into the genome of *Bacillus thuringiensis* strain m401 isolated from honey. We performed a comparative analysis with other *Bt* strains mainly focused on detecting genes encoding antibiotic resistance, antimicrobial peptides, toxins, and related regulatory gene factors. At the same time, we investigated the *in vitro* antagonism of *Bt* m401 against 52 strains of *P. larvae* belonging to different genotypes from diverse geographical areas and with different tetracycline sensitivity.

Materials and methods

Bacterial strains and culture conditions

Bt m401 was isolated from a honey sample from Pigüe, Argentina (37°36′S; 64°24′W), using polymyxin-pyruvate-egg-yolk-mannitol agar (PEMBA) as previously described[31]. The isolate was kept as frozen stocks at −80 °C in trypticase soy broth (TSB) containing 20% v/v glycerol. The *P. larvae* strains (n = 52) used were obtained from different geographical areas (Table 1). The collection had been previously characterized by rep-PCR with primers BOX and ERIC[6] and tetracycline resistance[4,5]. *P. larvae* isolates were preserved in Müeller Hinton broth-yeast extract-potassium phosphate-glucose-pyruvate (MYPGP) broth[21] containing 20% v/v glycerol. For short-term storage, the strains were kept at 4 °C in the appropriate semi-solid medium.

Species identification

To identify the isolate m401 at the species level, bacterial smears were examined for the presence and location of spores within cells and the size and shape of vegetative cells[3,31]. Unstained globules in the cytoplasm were examined by phase contrast microscopy, and parasporal crystals were single-stained with Coomassie Blue. Colony characteristics and media appearance in Bacillus *Chromoselect* agar (Sigma-Aldrich®) and PEMBA (Britania®) were evaluated as previously described[3,31]. Furthermore, the isolate was tested for catalase, production of lecithinase, Voges-Proskauer reaction, mannitol, and arabinose utilization, anaerobic utilization of glucose, hemolytic activity, and starch and gelatin hydrolysis according to standard protocols[3,31]. Additionally, the identity was confirmed by a PCR-RFLP assay, as described by López and Alippi[32].

Genome re-assembly of *Bt* m401 and confirmation of a new plasmid

The previously assembled genomic DNA of *Bt* m401[1] was reassembled using Unicycler v0.4.7. Bioinformatic evidence showed that scaffold 26 (18 160 bp) of the old assembly matches the scaffold of the new assembly (19 094 bp) but with a different relative position. Moreover, Unicycler predicts the new scaffold as a circular replicon, a new plasmid. To confirm this, we designed primers to target the outer region's ends of scaffold 26. Thus, if this amplifies the region, it would confirm the plasmid. The primers Scaff26_F/R were designed using Primer-Blast (www.ncbi.nlm.nih.gov/tools/primer-blast/). Primers Scaff26_F/R sequences were 5′-AGCAAAGTCGTGTATCCTGC-3′ and 5′-TGGTGCGCTTCTAAATGGTG-3′, respectively. The reaction mixture (25 μl) contained 2.5 μl buffer 1× (Promega®), 0.5 mM Scaff26_F/R primers; 1.25 mM $MgCl_2$ (Promega®); 1.76 mM of each dNTP (Promega®); 1 U T-plus Taq DNA polymerase (Inbio Highway®) and 2 μl of DNA (25 μg/ml) obtained as described above. The cycling program consisted of a 95 °C (5 min) step; 35 cycles of 95 °C (30 s), 58 °C for annealing (2 min), 72 °C (2 min); and a final step of 72 °C (4 min). Amplifications were performed in a thermal cycler Eppendorf Mastercycler (Eppendorf AG, Hamburg, Germany).

PCR amplicons obtained by primers Scaff26_F/R were further confirmed by digestion using restriction enzymes. After amplifying a PCR product of 1917 bp, subsamples of 4 μl were incubated with endonucleases *Hae*III and *Cfo*I according to the manufacturer's specifications (Promega®, Buenos Aires, Argentina). In each case, the expected sizes of the digested fragments were visualized. Plasmid DNA corresponding to scaffold 26 was named pBTm401d. Amplification products were separated in 1.5% agarose gel in 0.5× TBE buffer, stained with ethidium bromide, and visualized with a UV transilluminator (UVP®, Upland, USA). Photographs were digitalized using DigiDoc-It (UVP®, v.1.125, Upland, USA).

Bioinformatic analyses of the genome and prediction of biosynthetic gene clusters in the genome

Putative coding regions were determined using GLIMMER v3 and EasyGene, both trained with the *B. cereus* ATCC 10987 genome (NC_003909). We used the BLASTx program at the NCBI database (www.ncbi.nlm.nih.gov/) for identifying potential protein products, and sequence similarities and identities were analyzed using BLASTn.

Previous reports demonstrated that *gyrB* gene nucleotide sequence analyses could distinguish *Bt* serovars[44]. A phylogenetic tree was constructed using the *gyrB* nucleotide sequences from *Bt* m401 and those from *Bt* serovars aligned using ClustalW. The study involved 59 *gyrB* sequences of

Table 1 Antibacterial spectrum of *Bt* m401 against *Paenibacillus larvae* strains as determined by the well diffusion technique

P. larvae strain*	Source	Geographic origin	Year of isolation	ERIC genotype	BOX genotype	Inhibition in mm 24 h§	Inhibition in mm 48 h§
PL4 UB-CIDEFI	Larval remains	Argentina	1994	I	A	31.67 ± 1.25	31.67 ± 1.25
PL11 UB-CIDEFI	Larval remains	Argentina	1994	I	D	21.00 ± 0.82	33.67 ± 1.25
PL23 UB-CIDEFI	Scales	New Zealand	1995	I	A	28.00 ± 2.17	37.00 ± 0.80
PL38 UB-CIDEFI	Larval remains	Argentina	1995	I	C	30.67 ± 0.47	40.33 ± 0.48
PL39 UB-CIDEFI	Larval remains	Argentina	1995	I	C	17.33 ± 0.47	18.00 ± 0.82
PL42 UB-CIDEFI	Scales	Italy	1992	I	B	30.68 ± 0.47	33.33 ± 0.48
PL48 UB-CIDEFI	Scales	France	1995	I	A	29.33 ± 0.47	38.00 ± 0.82
PL56 UB-CIDEFI	Scales	Sweden	1995	I	B	27.67 ± 3.09	29.33 ± 0.47
PL62 UB-CIDEFI	Honey	Argentina	1995	I	B	24.33 ± 0.47	39.67 ± 0.47
PL72 UB-CIDEFI	Scales	Poland	1996	I	A	27.00 ± 0.82	40.67 ± 0.94
PL91 UB-CIDEFI	Scales	Germany	1994	I	A	26.39 ± 1.89	33.33 ± 0.47
PL92 UB-CIDEFI	Larval remains	Argentina	1995	I	A	31.00 ± 0.82	42.33 ± 1.70
PL94 UB-CIDEFI	Honey	Argentina	1996	I	A	27.00 ± 0.82	36.33 ± 3.09
PL96 UB-CIDEFI	Bacterial culture	UK	N/A	I	B	25.67 ± 0.47	27.67 ± 0.94
PL100 UB-CIDEFI	Honey	Tunisia	1997	I	A	32.67 ± 0.47	44.67 ± 0.47
PL213 UB-CIDEFI	Honey	Canada	1999	I	B	28.67 ± 0.42	41.00 ± 0.82
PL225 UB-CIDEFI	Honey	Argentina	1999	I	D	24.67 ± 0.47	34.33 ± 2.05
PL252 UB-CIDEFI	Honey	Spain	2000	I	A	33.00 ± 1.41	46.67 ± 0.47
PL286 UB-CIDEFI	Larval remains	Uruguay	2005	I	C	28.67 ± 0.47	33.67 ± 0.94
PL289 UB-CIDEFI	Bacterial culture	Japan	N/A	I	A	27.00 ± 0.82	37.33 ± 0.94
PL305 UB-CIDEFI	Bacterial culture	Belgium	N/A	I	A	26.67 ± 0.94	37.67 ± 0.47
PL312 UB-CIDEFI	Bacterial culture	Chile	2001	I	B	24.00 ± 0.82	39.67 ± 0.47
PL368 UB-CIDEFI	Honey	Brazil	2001	I	A	26.67 ± 0.47	41.00 ± 0.82
PL373 UB-CIDEFI	Honey	USA	2005	I	D	26.00 ± 0.82	38.67 ± 0.47
PL374 UB-CIDEFI	Honey	USA	2005	I	D	24.67 ± 0.47	39.33 ± 0,47
PL388 UB-CIDEFI	Honey	Panama	2007	I	A	31.67 ± 0.47	31.67 ± 0.47
PL391 UB-CIDEFI	Honey	USA	2008	I	D	24.33 ± 0.47	24.33 ± 0.47

Table 1 (*Continued*)

P. larvae strain*	Source	Geographic origin	Year of isolation	ERIC genotype	BOX genotype	Inhibition in mm 24 h§	Inhibition in mm 48 h§
PL394 UB-CIDEFI	Honey	USA	2008	I	A	21.33 ± 1.25	36.00 ± 2.16
PL395 UB-CIDEFI	Honey	USA	2008	I	D	23.33 ± 0.47	37.67 ± 1.25
PL426 UB-CIDEFI	Larval remains	South Africa	2010	I	A	24.67 ± 0.47	39.67 ± 0.47
PL442 UB-CIDEFI	Honey	Aruba	2011	I	A	24.67 ± 0.47	38.57 ± 0.47
PL443 UB-CIDEFI	Honey	USA	2011	I	A	21.33 ± 0.47	21.67 ± 0.47
PL448 UB-CIDEFI	Honey	Austria	2012	I	D	26.00 ± 0.82	37.00 ± 0.82
PL454 UB-CIDEFI	Honey	Cuba	2013	I	A	26.00 ± 0.82	44.00 ± 0.82
PL455 UB-CIDEFI	Scales	Bolivia	2014	I	A	23.00 ± 2.16	44.67 ± 0.47
PL468 UB-CIDEFI	Honey	Turkey	2019	I	A	27.00 ± 0.82	27.00 ± 0.82
ATCC 9545	Bacterial culture	USA	N/A	I	A	25.67 ± 1.25	36.67 ± 1.25
ATCC 25367	Bacterial culture	Unknown	N/A	IV	E	14.00 ± 0.82	20.00 ± 0.82
ATCC 25368	Bacterial culture	Unknown	N/A	IV	E	14.67 ± 0.47	19.67 ± 0.47
NRRL B-3555	Bacterial culture	USA	N/A	I	A	30.33 ± 0.47	41.00 ± 0.82
NRRL B-3688	Bacterial culture	USA	N/A	IV	E	21.67 ± 0.47	21.67 ± 0.47
NRRL B-14152	Bacterial culture	Denmark	N/A	IV	E	17.67 ± 0.47	22.33 ± 1.25
NRRL B-14154	Bacterial culture	France	N/A	IV	E	20.67 ± 0.47	21.99 ± 0
CCM 38	Bacterial culture	Canada	N/A	IV	E	21.67 ± 0.47	21.67 ± 0.47
CCM 4483	Bacterial culture	Czech Republic	N/A	I	A	30.33 ± 1.25	31.67 ± 0.47
SAG 289	Bacterial culture	USA	N/A	II	B	21.67 ± 0.47	21.67 ± 0.47
SAG 290	Bacterial culture	USA	N/A	II	B	24.33 ± 0.47	24.67 ± 0.47
SAG 9712	Bacterial culture	Unknown	N/A	II	E	20.00 ± 0	20.00 ± 0.82
SAG 10230	Bacterial culture	Unknown	N/A	II	B	19.33 ± 0.47	19.33 ± 0.47
SAG 10236	Bacterial culture	Unknown	N/A	II	B	18.67 ± 0.47	19.67 ± 0.47
SAG 10367	Bacterial culture	Unknown	N/A	II	E	14.67 ± 0.47	15.67 ± 0.47
SAG 10754	Bacterial culture	Unknown	N/A	II	E	23.50 ± 0.71	23.50 ± 0.71

* UB-CIDEFI: Unidad de Bacteriología-Centro de Investigaciones de Fitopatología, Facultad de Ciencias Agrarias y Forestales, Universidad Nacional de La Plata, La Plata, Argentina; ATCC: American Type Culture Collection, Rockville, Maryland, USA; CCM: Czech Collection of Microorganisms, Brno, Czech Republic; NRRL: Northern Utilization Research and Development Division, Peoria, Illinois, USA; SAG: Servicio Agrícola Ganadero, Chile.

§ Data are from triplicate trials.

Bt serovars retrieved from GenBank and a sequence of *Escherichia coli* (NC_000913.3) as an outgroup. The evolutionary history was inferred using the Maximum Likelihood (ML) method based on the General Time Reversible model. A bootstrap resampling analysis employing 1000 pseudoreplicates was used to assess the reliability of the phylogenetic trees, all using the MEGA 7 program.

Moreover, the *Bt* m401 genome was uploaded to EDGAR Database for comparative analysis. We selected 40 strains of the Cereus clade, including different *Bt* serovars. A phylogenetic tree on core genome sequences was constructed using EDGAR 2.0. First, EDGAR calculates the core genes of the selected genomes; then, it aligns each core gene and concatenates them[13]. Afterward, FastTree software (http://www.microbesonline.org/fasttree/) was used to generate maximum-likelihood phylogenetic trees. EDGAR was also used to calculate an average nucleotide identity (ANI) matrix for the selected genomes. The complete genome sequences of 34 *Bt* serovars and 6 type strains belonging to the Cereus clade were compared, and the ANI matrix for a selected set of the closest genomes (n = 13) was made.

Additionally, BLASTn sequences comparisons of *Bt* m401 plasmids with plasmids of related species were visualized using Kablammo (Kablammo.wasmuthlab.org). GC viewer server (gcviewer download|SourceForge.net) was used to calculate plasmid G+C content.

Biosynthetic gene clusters (BCGs) in the genome and their corresponding secondary metabolites were predicted using the bacterial version of antiSMASH 6.0 (https://antismash.secondarymetabolites.org/). The gene clusters found were cross-referred in the MIBiG repository (https://mibig.secondarymetabolites.org/).

In vitro antagonism against *Paenibacillus larvae*

The antibacterial spectrum of *Bt* m401 against 52 *P. larvae* strains from diverse geographical origins was determined using a well diffusion technique described by Alippi and Reynaldi[6].

Determination of tetracycline, oxytetracycline, and minocycline resistance

Minimal inhibitory concentration (MIC) values of tetracycline (TET) (Sigma®, USA), oxytetracycline (OTC) (Oxoid®, USA), and minocycline (MIN) (Wyeth-Whitehall®, Brazil) were determined by the broth macrodilution method, according to the recommendations of the Clinical and Laboratory Standards Institute[17,18]. Increasing concentrations between 0.0312 and 256 μg/ml TET, OTC, and MIN were tested. Tubes containing Müller-Hinton (MH) broth without antibiotics were used for controls. Vegetative cells of *Bt* m401 growing on MH agar for 24 h at 30 ± 1 °C were suspended in sterile distilled water and adjusted to a concentration of 0.5 Mc Farland[17,18]. Appropriate dilutions of the inoculum were prepared to deliver a final concentration of bacteria of about 5×10^5 CFU/ml. Two replications were tested for each antibiotic concentration. Inoculated tubes were examined after 24–48 h incubation at 37 ± 1 °C in constant agitation. *Staphylococcus aureus* ATCC 29213 and *Escherichia coli* ATCC 25922 were used for quality control.

Visualization of plasmid DNA

Bacterial cells were screened for extrachromosomal DNA using the technique described by Fagundes and co-workers[23]. *Bt* m401 was grown in 50 ml Spizizen broth[23], supplemented with 16 μg/ml TET, and incubated at 30 ± 1 °C overnight under constant agitation. *P. larvae* strain PL373, containing two plasmids of approximately 5000 bp and 8000 bp[4], was used as a control and was grown in MYPGP broth supplemented with 16 μg/ml TET and incubated at 37 ± 1 °C. Gels were visualized with a UV transilluminator, as described before.

Plasmid and total DNA extraction

A rapid procedure using whole cells from plates was employed for total genomic DNA preparations[7], while plasmid DNA was prepared by alkaline lysis with the SDS procedure described by Sambrook and Russell[41].

PCR analysis of tetracycline-resistant genes

The presence of *tet(K)*, *tet(L)*, *tet(M)*, *tet(O)*, *tet(W)*, *otr(A)*, and *otr(B)* genes was assessed by PCR as described elsewhere[33,45,47]. In the case of the tetracycline-resistant *tet(45)* gene, the primers used were Tet45-F and Tet45-R as described by You and co-workers[48] but using 1 mM of primers and a final volume of 30 μl for the premix. This selection included the most commonly detected *tet* genes in *Bacillus* (http://faculty.washington.edu/marilynr/tetweb3.pdf). Both genomic and plasmid DNA were used as templates, and the amplification products were separated and visualized in 1.5% agarose gels in TBE buffer as previously described.

Phylogenetic analysis of *tet(45)* gene

The *tet(45)* gene found in *Bt* m401 and other *tet(45)* and *tet(L)* tetracycline-resistant genes retrieved from GenBank were aligned using Clustal W. The analysis involved 14 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 743 positions in the final dataset. The amino acid sequence of the *tet(M)* gene from *Clostridium difficile* CD2386 (JN846696.1) was used as an outgroup. The tree was constructed using the ML method based on the General Time Reversible model. A bootstrap resampling analysis employing 1000 pseudoreplicates was used to assess the reliability of the phylogenetic trees, all using MEGA 7.

Results

Species identification

Bt m401 stained Gram positive and was facultative anaerobic and catalase-positive. The strain showed ellipsoidal spores not distending the sporangia in a central position. As seen by phase contrast microscopy, the cytoplasm was

filled with unstained globules. Moreover, parasporal crystals were detected in stained preparations. Bacterial colonies in Bacillus *Chromoselect* agar were light blue over a pinkish medium, while in PEMBA, *Bt* m401 produced crenated colonies of turquoise blue surrounded by egg-yolk precipitation haloes as a result of lecithinase activity. The strain hydrolyzed gelatin and starch and was positive for Voges-Proskauer, and reduction of nitrates to nitrites and negative for mannitol and arabinose utilization. It also showed hemolytic activity in blood agar plates. Additionally, the isolate showed the expected restriction patterns for *Bacillus thuringiensis* when using a combination of *Alu*I and *Cfo*I enzymes[32].

Bioinformatic analyses of the genome

Previously, we sequenced and assembled the *Bt* m401 genome containing one chromosome and three plasmids[1]. However, experimental assays suggested the presence of a fourth plasmid (data not shown). In the present work, we reassembled the genome using Unicycler v0.4.7 to uncover potential genes related to tetracycline resistance, virulence factors, and antimicrobial peptides. The final draft genome assembly consists of 45 scaffolds generating a 6 005 135 bp genome size with an average GC content of 34.72% (Table S1). The data set comprises a chromosome and four circular plasmids, which vary from 8307 to 69 591 bp (Table S2). Plasmids pBTm401a, pBTm401b, and pBTm401c coincided with the first assembly, and a new plasmid of 19 094 bp, pBTm401d, was detected and assembled. PCR and enzymatic digestions confirmed the circular DNA of pBTm401d (Table S2).

The phylogenetic relationships based on *gyrB* sequences between *Bt* m401 and different serovars of *Bt* are shown in Figure 1. The 52 *Bt* serovars were separated into 5 clusters (I, II, III, IV, and V). *Bt* m401 was located in cluster I, revealed at 93% nucleotide sequence identities, together with 21 serovars, including *kumamotoensis*, *yunnanensis*, *indiana*, and *londrina*, among others.

A phylogenetic tree based on core genome sequences was constructed using EDGAR 2.0. and is shown in Figure 2A, where *Bt* m401 was located in the same cluster with *Bt* sv. *kumamotoensis*. From the results obtained in the core genome tree, we selected the closest serovars (n = 13) to compare the average nucleotide identity calculations (ANIb) (Fig. 2B). The analysis reinforces the high similarity between *Bt* m401 and *Bt sv. kumamotoensis*, with an ANIb value of 98.11%, followed by *Bt* sv. *londrina* (97.66%), *Bt* sv. *indiana* (97.51%), *Bt* sv. *japonensis* (97.44%), *Bt* sv. *kurstaki* (97.26%), *Bt* sv. *coreanensis* (97.24%), *Bt* sv. *yunnanensis* (97.2%), and *Bt* sv. *galleriae* (97.11%) (Fig. 2B). Based on both phylogenetic trees, we conclude that *Bt* m401 belongs to serovar *kumamotoensis*.

The complete sequences of plasmids pBTm401a, pBTm401b, pBTm401c, and pBTm401d were analyzed using different bioinformatics tools. A graphical representation is shown in Fig. S1, and their main characteristics are listed in Tables S2 and S3.

Plasmid pBTm401a (8307 bp) presented genes coding a multiple antibiotic resistance regulator (MarR), an antibiotic biosynthesis monooxygenase, and a plasmid recombination protein (Table S3). The remaining genes were predicted as hypothetical proteins. Furthermore, pBTm401a contains genetic elements typical of plasmids replicating *via* the rolling-circle mechanism (RCR)[10] (Fig. S1A). The single-strand origin (*sso*) is located upstream of the mobilization gene (4123–4378 bp), and the double-strand origin (*dso*) is located downstream from the MarR and recombinase genes (6828–7179 bp). Both *dso* and *sso* were identified by homology sequence with corresponding regions of other plasmids, such as pDx14.2 from *B. mycoides* (AJ871638) (82% identity for *sso* and 91% for *dso*) and pFR12 from *Bt* INTA-FR7-4 (EU362917) (83% identity for *sso* and 92% identity for *sso*). The origin of the transfer site (*oriT*) is located between the *sso* and the recombinase genes (4401–4430 bp) and showed 90% homology with the *oriT* of plasmid pTX14-3 (X56204.1) from *Bt* sv. *israelensis*. The best hits obtained after BLASTn analysis of pBTm401a were selected for the individual comparison of the plasmids; we found partial similarities between pBTm401a and plasmid pFR12 from *Bt* strain INTA-FR7-4 (EU362917) (Fig. S2A), plasmid unnamed 32 from *B. cereus* NJ-W (CP012485), and plasmid pDx14.2 from *B. mycoides* (AJ871638), respectively. The conserved region between them corresponds to the predicted *marR* transcriptional regulator, the recombinase gene, and the genetic elements *sso* and *dso*.

The BLASTx analysis of plasmid pBTm401b (9934 bp) showed only three putative protein products that codify a uracil permease, a MarR family transcriptional regulator, and an integrase (Fig. S1B and Table S3). The BLASTn analysis showed partial similarities with plasmids reported in *Bt* strains. The region comprising approximately from 2 kb to 7 kb in pBTm401b presented high homology with plasmid pBT1850012 from *B. thuringiensis* strain Bt185 (GenBank, CP014288) (Fig. S2B), plasmid p5 from *B. thuringiensis* strain QZL38 (GenBank, CP032611) and plasmid unnamed2 from *B. cereus* strain 09 (GenBank, CP042876). The sequence between 1.5 kb and 6 kb of pBTm401b presented high similarity with plasmid unnamed 8 from *B. mycoides* strain Gnyt1 (GenBank, CP020751).

Plasmid pBTm401c (69 591 bp) encodes two genes of mersacidin family lantibiotics, a lanthipeptide synthetase LanM, and an ABC transporter consecutively located (Fig. S1C and Table S3). Other relevant genes are summarized in Table S3, including a *cry1* gene that showed 99% identity with *cry1* described in megaplasmid poh1 of *Bt* ATCC 10792[16]. The best BLASTn hits for pBTm401c correspond to plasmids from strains of the Cereus clade: plasmid pBTm401c showed several small areas similar to those detected in plasmid p1 from *B. thuringiensis* strain HM-311 (CP040783) (97.3% identity), plasmid pBT1850636 from *B. thuringiensis* strain Bt185 (CP014283) (98.9% identity), plasmid pBb from *Bacillus bombyseptic*us (CP007513) (98.6% identity), and plasmid unnamed1 from *B. cereus* strain 09 (GenBank, CP042875) (97.6% identity) (Fig. S2C). We found many similar regions in plasmid unnamed1 from *B. cereus* strain 09 (97.6% identity), including the 40–50 kb region, which codifies lantibiotic genes.

Finally, pBTm401d (19 094 bp) analysis showed three putative protein products, a non-ribosomal peptide synthetase, and two amino acid adenylation domain-containing proteins (Fig. S1D and Table S3).

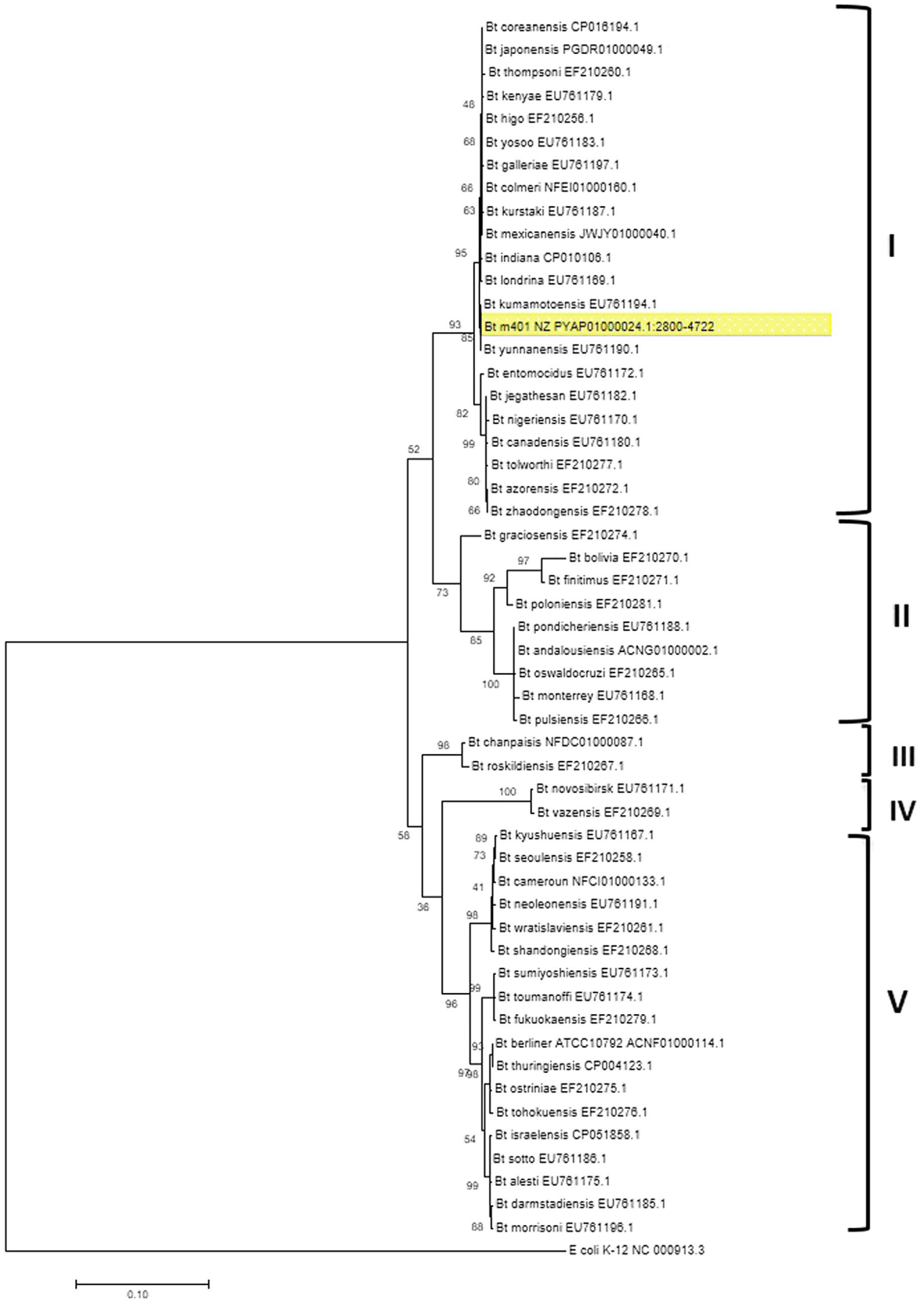

Figure 1 Bootstrapped Maximum Likelihood tree of different *Bacillus thuringiensis* serovars generated from the alignment of *gyrB* nucleotide sequences. The analysis involved 53 nucleotide sequences. Bootstrap values (1000 replicates) are shown next to the branches. All positions containing gaps and missing data were eliminated. *E. coli* K12 (NC_000913.3) was used as an outgroup. Evolutionary analyses were conducted in MEGA7.

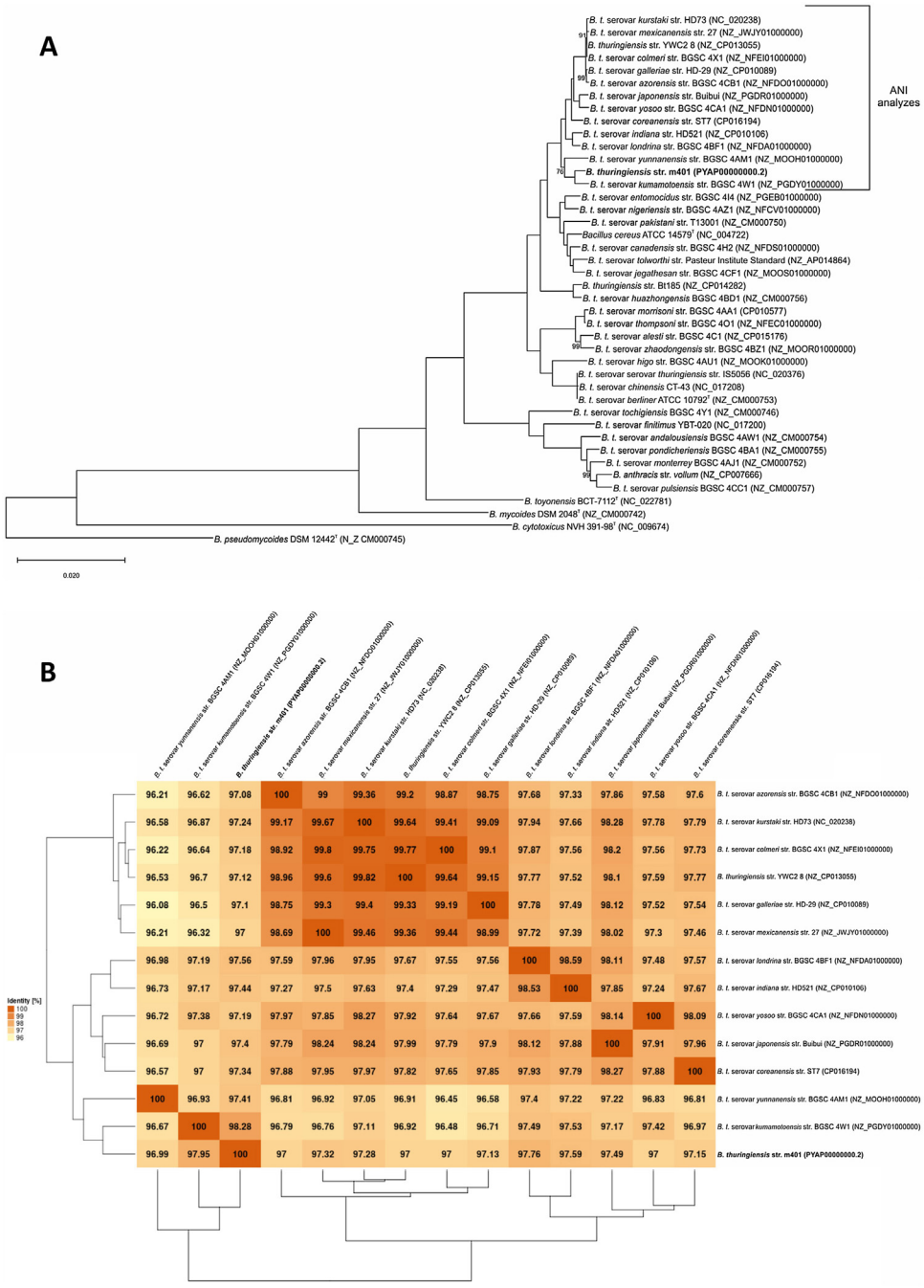

Figure 2 (A) Phylogenetic analyses based on bacterial core genomes of strains belonging to the Cereus clade. The tree for 42 genomes was built out of a core of 2034 genes per genome, 85 428 genes in total. The numbers at the branching points are local support values computed by FastTree software using the Shimodaira-Hasegawa test (×100). Only values other than 100 are shown. (B) ANIb matrix for comparison of selected genomes retrieved from GenBank (accession numbers in parentheses). ANIb value calculations, clustering, and visualization as heat maps were done through EDGAR 2.0 web server.

In addition, a set of genes associated with virulence factors were detected in *Bt* m401. In the chromosome, we found the operon *hbl*ABCD that encodes the hemolytic enterotoxin HBL. This feature correlates with a positive hemolytic phenotype in a blood agar test. These proteins are commonly found in strains of the Cereus clade, including *Bt* strains[27]. Other virulence factors found in the chromosome were the non-hemolytic enterotoxin complex, cytotoxin K, and enterotoxin FM (Table S4). The presence of sequences associated with virulence genes *cytK*, *nheA*, *nheB*, *nheC*, *hblA*, *hblB*, *hblC*, and *hblD* was confirmed by PCR (data not shown).

Prediction of biosynthetic gene clusters in the genome

Biosynthetic gene clusters (BCGs) in the genome and their corresponding secondary metabolites were predicted using the bacterial version of antiSMASH 6.0. The server predicted 12 regions of BGCs in the genome related to secondary metabolism, including bacteriocins, siderophores, ribosomally synthesized post-translationally modified peptide product clusters (RiPP-like), and non-ribosomal peptide synthetase clusters (NRPS) (Table 2).

In vitro antagonism against *Paenibacillus larvae*

We investigated the antagonistic potential of *Bt* m401 against 52 strains of *P. larvae* using a well diffusion technique. Clear inhibition zones were observed when testing *Bt* m401 against all the strains tested, ranging from 17.33 ± 0.47 to 32.67 ± 0.47 mm for genotypes ERIC I; from 14.67 ± 0.47 to 24.33 ± 0.47 mm for genotypes ERIC II, and from 14 ± 0.82 to 21.67 ± 0.47 mm for genotypes ERIC IV, respectively at 24 h (Table 1). Interestingly, all the tetracycline-resistant strains of *P. larvae* (*i.e.*, PL373, PL374, PL391, PL394, PL395, PL442, and PL443) were inhibited by *Bt* m401.

Tetracycline resistance genes

Tetracycline-resistance genes and relatives detected in *Bt* m401 are summarized in Table 3. *Bt* m401 owns two TET^R genes corresponding to *tet(45)* and *tet(M)/tet(O)/tet(S)* family, both located in the chromosome. The best hits of the BLASTn analysis revealed high identity homology (>95%) with megaplasmids from *Bt* strains, *i.e.*, plasmid p1 from HM-311 (CP040783.1), plasmid pBT62A from BT62 (CP044979.1), plasmid pBT1850636 from *Bt*185 (CP014283.1), and the megaplasmid poh1 from *Bt* ATCC 10792[16]. The presence of these genes in the *Bt* m401 chromosome led us to suspect that this genome region could be part of an integrated plasmid. Additionally, we found a putative tetracycline resistance determinant *tet(V)* located in the chromosome with high identity homology (99%) with *B. cereus* VD140 (GCA_000399545).

Determination of tetracycline, oxytetracycline, and minocycline resistance

MIC values for TET, OTC, and minocycline (MIN) were determined for *Bt* m401. The results obtained were MIC TET=128 (R), MIC OTC>128 (R), and MIC MIN=2 (S), respectively. Interestingly, in *Bt* m401, cross-resistance was found between TET and OTC but not with MIN. Values of MIC obtained for reference strains *S. aureus* ATCC 29213 and *E. coli* ATCC 25922 were within the tolerance range acceptable for quality control, according to CLSI standards[17,18].

Visualization of plasmid DNA

The plasmid profile of *Bt* m401 showed two plasmids between 5000 bp and about 8000 bp and two larger plasmids of more than 19 000 bp (Fig. S3). These observations correlated with genome assembly data showing four plasmids of 8307 bp, 9934 bp, 19 094 bp, and 69 591 bp, respectively (Table S2).

PCR analysis of tetracycline-resistant genes and phylogenetic analysis of *tet(45)* and *tet(L)* genes

Results of the PCR assays revealed that *Bt* m401 yielded the expected *tet(45)* product of about 107 bp and also the *tet(L)* product of approximately 788 bp, respectively, when using genomic DNA as a template (Fig. S4). However, no amplification was obtained when using plasmid DNA as a template. In contrast, no PCR products were detected using the *tet(K)*, *tet(M)*, *tet(O)*, *tet(W)*, *otr(A)*, or *otr(B)* primers, neither with genomic nor plasmid DNA as a template. The phylogenetic analysis of the *tet(45)* gene of *Bt* m401 is shown in Fig. S5, confirming that it was more closely related to the *tet(45)* gene from *Bacillus* sp. 6f (KX091845.1).

Discussion

We characterized the strain of *B. thuringiensis* m401 isolated from honey using several approaches. We performed an in-silico analysis of the *Bt* m401 genome, mainly focused on detecting genes encoding antibiotic resistance, antimicrobial peptides, toxins, and related regulatory gene factors. We also tested its capacity as a biocontrol agent against *P. larvae*, the causal agent of AFB disease of honeybees.

The bacterial colonies and media appearance in Bacillus *Chromoselect* agar and PEMBA correspond to those described for *B. thuringiensis*[3,31]. In addition, the results of morphological and biochemical tests and PCR/RFLP analysis of genes encoding 16S rRNA match those reported for *Bacillus thuringiensis*[3,32]. The identity of the strain was confirmed as *Bacillus thuringiensis* sv. *kumamotoensis* based on the average nucleotide identity calculations (ANIb) comparison and the phylogenetic analysis of the *gyrB* nucleotide sequences.

The genome of *Bt* m401 contained four plasmids of 8307 bp, 9934 bp, 19 094 bp, and 69 591 bp, respectively. Other authors also reported various plasmids and megaplasmids in *Bt* strains[9,16,23,27]. The analysis of plasmid sequences showed that pBTm401a and pBTm401b contain coding one-component signal transduction regulators such as MarR, while pBTm401c contains a transduction regulator TetR/AcrR. Plasmids containing signal transduction regulators have been reported in *Bt*, *e.g.*, plasmids pFR12.5 and pFR55 from *Bt* INTA-FR7-4 that codify a putative transcriptional regulator MerR[9] and *Bt* ATCC 10792 that possess a megaplasmid poh1 containing a TetR/AcrR coding gene[16]. The target genes of these transcriptional regulators involve various critical functions, including osmotic stress, efflux pumps, multi-drug resistance, and virulence[19].

The organization of replication elements in pBTm401a is similar to corresponding regions of pTX14-3 and other plasmids belonging to RCR group VII[10]. The mob gene (mob14-3) sequence from plasmid pTX14-3 showed 86% homology with the recombinase gene of pBTm401a, and the replication

Table 2 Predicted biosynthetic gene clusters (BGCs) characterization in *Bacillus thuringiensis* m401 with antiSMASH 6.01 database and MIBiG repository

Type	Location	Accession	Length (bp)	Description	Most similar known clusters Blast	Similarity >30%	MIBiG closest match	Compound/ similarity score
Lanthipeptide	pBTm401c	PYAP02000003.1	23,075	Lanthipeptide class II	–	–	BGC0000504	Cytolisin/0.32
NRPS	pBTm401d	PYAP02000004.1	19,094	NRPS cluster	Bacitracin	33%	BGC0000426	Sevadicin/0.37
RiPP-like	Chromosomal	PYAP02000006.1	23,507	LAP, RiPP-like cluster	–	–	BGC0000567	Trifolitoxin/0.63
Siderophore	Chromosomal	PYAP02000007.1	13,708	Siderophore	Petrobactin	100%	BGC0000942.1	Petrobactin/0.69
NRPS	Chromosomal	PYAP02000007.1	46,315	NRPS	Bacillibactin	46%	BGC0001185.1	Bacillibactin/0.52
NRPS	Chromosomal	PYAP02000007.1	49,656	NRPS	–	–	BGC0000389.1	Masetolide A/0.42
Lanthipeptide	Chromosomal	PYAP02000011.1	114,937	Lanthipeptide class II	Zwittermicin A	100%	BGC000410591	Zwittermicin A/1.60
Sactipeptide	Chromosomal	PYAP02000011.1	21,389	Sactipeptide cluster	Thurincin H	70%	BGC0000600.1	Thurincin H/0.50
NRPS-like	Chromosomal	PYAP02000012.1	43,582	NRPS-like cluster	–	–	BGC0000427.1	Shinorine/0.34
Betalactone	Chromosomal	PYAP02000013.1	25,239	Betalactone	Fengycin	40%	BGC0000234	Jadomycin/0.21
RiPP-like	Chromosomal	PYAP02000013.1	9,542	RiPP-like cluster	–	–	BGC000896.1	Cycloserine/0.13
RiPP-like	Chromosomal	PYAP02000013.1	10,267	RiPP-like cluster	–	–	BGC0001994.1	B-ethylserine/0.14

Abbreviations: LAP: linear azol(in)e containing peptide; RiPP-like: other unspecified ribosomally synthesized and post-translationally modified peptide product cluster; NRPS: non-ribosomal peptide synthetase cluster.

Table 3 Tetracycline-resistance genes in *Bacillus thuringiensis* m401

Gene	Location	Accession	Locus tag	Genome position
tet(45)	Chromosomal	PYAP02000014.1	C5676_019470	171,803–173,179
tet(M)/tet(W)/tet(O)/tet(S) family	Chromosomal	PYAP02000022.1	C5676_028210	68,473–70,416
Tetracycline resistance efflux system leader peptide gene	Chromosomal	PYAP02000011.1	C5676_019475	173,213–173,275
TetR/AcrR family transcriptional regulator gene	pBTm401c	PYAP02000003.1	C5676_000190	17,213–17,782
TetR/AcrR family transcriptional regulator gene	Chromosomal	PYAP02000007.1	C5676_011065	80,204–80,794
MFS transporter – putative tetracycline resistance determinant *tetV*	Chromosomal	PYAP02000007.1	C5676_010775	15,785–17,047

p14-3 gene showed 76% homology with *marR* transcriptional regulator of pBTm401a.

Plasmid pBTm401c encodes two genes of mersacidin family lantibiotics and a lanthipeptide synthetase (Tables 2 and S3). Lanthipeptides are a class of ribosomally synthesized and post-translationally modified peptides (RiPP) containing characteristic thioether cross-links imperative for bioactivity and stability, and those that possess antibacterial activity are called lantibiotics[28]. Lantibiotics have been extensively studied for their broad-range activity against clinically relevant pathogens. Several *Bacillus* species, including *Bt*, produce lantibiotics, *e.g.*, clausin in *B. clausii*, formicin in *B. paralicheniformis* APC 1576, ericins in various strains of *B. subtilis*, haloduracin in *B. halodurans* strain C-125; thusin in *B. thuringiensis* strain BGSC 4BT1; lichenicidins in *B. licheniformis* VK21 and I89 strains and mersacidin in *B. amyloliquefaciens* FZB42 and *B. subtilis* HIL Y-85,54728[2,28,29]. Another lipopeptide biosynthetic gene cluster followed by a transposase gene was detected in the 312 kb plasmid from *B. thuringiensis* BMB171[49]. The presence of lantibiotic genes encoded on plasmids and flanked by transposases allows to infer a possible mobilization of these elements among bacterial strains. Other relevant genes found in pBTm401c codify for a zeta toxin, two azaleucine resistance proteins AzlC/AzlD, a type VII secretion system, and a *cry*1 gene (Tables S3 and S4). The presence of a putative *cry* gene that codifies parasporal crystals correlates with the crystals observed by optical microscopy. The parasporal crystal protein *Cry* is the primary insecticidal toxin of *B. thuringiensis*[35]. It has been reported that some *Bt* strains produce Vip and Sip insecticidal proteins[37]; however, we did not find any homologous sequence to *sip* or *vip* genes that codify, respectively, to Vip and Sip protein families.

On the other hand, plasmid pBTm401d contains a non-ribosomal peptide synthetase cluster (NRPS) (Tables 2 and S3). The presence of non-ribosomally peptide synthetases is a relevant characteristic; several *Bacillus* species produce antimicrobial peptides mediated by these synthetases; typical examples are surfactins, iturins, fengycines, and pliplastatins[43]. Surprisingly, pBTm401d showed high similarities with chromosomal regions of *Bt* strains HM-311 (CP040782) (99.2% identity), QZL38 (CP032608), and *Bt*185 (CP014282) (99.2% identity). This particular sequence exhibits repetitive regions that may interfere with assembling programs, suggesting an incorrect or incomplete genome assembly in those *Bt* strains. Besides, the whole sequence of pBTm401d showed similarity with a partial region of plasmid p1 from *B. cereus* strain CTMA 1571 (CP053657) (Fig. S2D), where this region is located between IS110 and IS3 family transposases. The presence of transposases suggests a possible horizontal transmission of the target region among different bacterial strains. The similarities among plasmid sequences of several strains of the Cereus clade, including *B. thuringiensis*, imply an active horizontal transfer of extrachromosomal elements in nature.

The genome mining analysis revealed 12 regions of biosynthetic gene clusters responsible for synthesizing secondary metabolites. We identified biosynthetic gene clusters coding for bacteriocins, siderophores, ribosomally synthesized post-translationally modified peptide products, and non-ribosomal peptide synthetase clusters. We found a BGC coding for a lantipeptide class II in plasmid pBTm401c and a BGC coding for an NRPS with 33% similarity to bacitracin in plasmid pBTm401d. Bacitracin is a cyclic cationic, non-ribosomally synthesized dodecapeptide reported in different *Bacillus* species, with activity against Gram positive bacteria and fungi[43]. Within the Cereus clade, bacitracin genes have been identified in *Bt* and *B. weihenstephanensis*[27]. As previously described, plasmid pBTm401c encodes two consecutive genes of mersacidin family lantibiotics, a lantipeptide synthetase LanM, and an ABC transporter. Mersacidin belongs to Type-B lantibiotics, and targets cell wall precursor lipid II and inhibits cell wall synthesis.

In the chromosome, we detected BCG coding for petrobactin (100%), zwittermicin A (100%), thurincin H (70%), bacillibactin (46%), and fengycin (40%). Zwittermicin A is a linear cationic lipopeptide with potent antibiotic and antifungal activity reported in *B. thuringiensis*[20,27] and *B. cereus*[42]. Thurincin H is a bacteriocin belonging to class II (non-modified peptides), subclass II.2 (thuricin-like peptides) according to Abriouel and co-workers[2], and recently reclassified as a sactipeptide[8]. Thurincin H has been reported in *Bt* serovars with antimicrobial activity against a wide range of Gram positive bacteria[15,30]. Fengycin is a cyclic non-cationic lipopeptide non-ribosomally synthesized

reported in *Bt* strain SM1 with antifungal activity against *Candida albicans*[40]. Other *Bacillus* species also produce fengycins with potent inhibition against pathogenic filamentous fungi[36]. The presence of catecholate siderophores, such as petrobactin and bacillibactin, in *Bt* m401 suggests iron acquisition abilities. This siderophore can potentially promote plant growth[11]. Other authors reported gene clusters assigned to the production of petrobactin and bacillibactin in different *Bt* strains[20,27]. These gene clusters are not exclusive to *Bt* since they are also found in other species belonging to the Cereus clade[11,20].

Clear inhibition zones ranging from 14 ± 0.82 to 33.00 ± 1.41 mm at 24 h (Table 1) were observed when testing *Bt*m401 against all *P. larvae* strains evaluated. Previous studies indicated that inhibition of *P. larvae* was strain-dependent[6,12,34]. In contrast, *Bt* m401 showed inhibition against all *P. larvae* strains tested *in vitro* (n = 52) belonging to different genotypes and geographical origins.

Antibiotic resistance determinants are also a common part of the genomes of environmental bacteria. In most bacteria, tetracycline resistance is due to acquiring new genes, often associated with mobile elements[39]. The PCR assays revealed that *Bt* m401 yielded both *tet(45)* and *tet(L)* products when using genomic DNA as a template (Fig. S4). Due to the high similarity between the *tet(45)* and *tet(L)* sequences selected to design primers for specific PCRs[47,48], we obtained the expected amplicons in both cases. Nevertheless, BLASTn analysis revealed that the nucleotide sequence presented here and located in the chromosome (PYAP02000014.1) showed higher similarities to *tet(45)* gene from *Bacillus* sp. 6f (96% identity) (KX091845.1), which was later identified as *B. cereus* strain 6f[50]; *tet(45)* gene from *Escherichia coli* strain DH5alpha (94%) (GU584222.2), and *tet(45)* gene from *Bhargavaea cecembensis* strain DMV42A (93% identity) (GU584217.2) than to the sequence of *tet(L)* from plasmid pBHS24 from *Bacillus* sp. 24 (80% identity) (HM235948.1); *tet(L)* from plasmid pERGB of *Staphylococcus aureus* strain 69371 (80% identity) (JN970906.1); and *tet(L)* gene f *Bacillus* sp. DMV3A (78% identity) (JN232537.1) (Fig. S5). As far as we know, within *Bacillus* species, the *tet(45)* determinant was only detected in seven strains of *B. cereus* and one strain of *Bt*, respectively[50]. Based on these results, we concluded that *Bt* m401 harbored the *tet(45)* resistant gene in the chromosome.

Conclusions

In this work, we identified a tetracycline-resistant strain of *Bacillus thuringiensis* sv. *kumamotoensis* (*Bt* m401) isolated from honey based on the average nucleotide identity calculations (ANIb) comparison and the analysis of the *gyrB* gene sequences of different *B. thuringiensis* serovars. The insights on the genome analysis revealed sequences with homology to virulence factors [*cytK*, *nheA*, *nheB*, *nheC*, *hblA*, *hblB*, *hblC*, *hblD*, *entFM*, and *inhA*]; tetracycline resistance genes [*tet(45)*, *tet(V)*, and *tet(M)*/*tet(W)*/*tet(O)*/*tet(S)* family], and regions of biosynthetic gene clusters coding for bacteriocins, siderophores, ribosomally synthesized post-translationally modified peptide products, and non-ribosomal peptide synthetase clusters that provide evidence for the possible use of *Bt* m401 as a biocontrol agent. Moreover, *Bt* m401 displays antagonistic activities against different genotypes of *P. larvae*, the causal agent of AFB of honeybees. The production of antagonistic compounds and resistance to tetracycline and oxytetracycline contributes to extending its host range and virulence. These findings provide a valuable background for developing biotechnological and biocontrol applications for *Bt* m401, which should be addressed in future experiments.

Data availability

This Whole Genome Shotgun project has been deposited at DDBJ/ENA/GenBank under the accession PYAP00000000. The version described in this paper is version PYAP00000000. 2. Plasmids sequences have been deposited at DDBJ/EMBL/GenBank under accession numbers PYAP02000001.1 for pBTm401a; PYAP02000002.1 for pBTm401b, PYAP02000003.1 for pBTm401c, and PYAP02000004.1 for pBTm401d, respectively. The data in this article are available in the paper and its online supplementary material.

Authors' contributions

AMA designed the experimental work, contributed to microbiological procedures and data analysis, wrote and reviewed the manuscript, and provided funding. FL completed molecular biology experiments and data analysis. EA and ACL performed experimental work. GATT executed data analysis and graphic constructions using EDGAR. All authors have read and approved the final version of the manuscript.

Conflict of interest

The authors declare that they have no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ram.2023.05.002.

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