3 Biotech (2019) 9:370
https://doi.org/10.1007/s13205-019-1911-5

GENOME REPORTS

Draft genome analysis of the endophyte, *Bacillus toyonensis* COPE52, a blueberry (*Vaccinium* spp. var. Biloxi) growth-promoting bacterium

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Received: 7 June 2019 / Accepted: 19 September 2019 / Published online: 25 September 2019

Abstract

In this work, we report an analysis of the draft genome of the blueberry (*Vaccinium* spp. var. Biloxi) growth-promoting endophyte *Bacillus toyonensis*, strain COPE52. The genome of COPE52 consists of a single 5,806,513 bp replicon, with a 35.1% G + C content. Strain COPE52 was strongly affiliated to *B. toyonensis* species, based on species delimitation cut-off values established for average nucleotide identity (> 95–96%), genome-to genome distance calculator (> 70%) and phylogenomic analysis. The RAST genomic annotation of the COPE52 strain revealed a total of 5979 total genes, including 5631 protein-coding genes, 11 rRNA genes, 5 ncRNAs, 81 tRNA genes, and 251 pseudogenes. To further validate the in silico analysis results, experiments were carried out to detect the production of indoleacetic acid, protease activity, and the emission of volatiles like acetoin, 2,3-butanediol and dimethyl disulphide as potential plant growth-promoting mechanisms. COPE52 also showed antifungal action against the grey mould phytopathogen, *Botrytis cinerea*, during in vitro bioassays. In addition, inoculation with strain COPE52 promoted growth biomass and chlorophyll content in blueberry plants (*Vaccinium* spp. var. Biloxi) under greenhouse conditions. To our knowledge, this is the first study showing genomic and experimental evidence of *B. toyonensis* as plant growth-promoting bacteria (PGPB).

Keywords Plant growth-promoting bacteria · Endophytic bacteria · Antifungal activity · Volatiles · Greenhouse

Introduction

Species of the genus *Bacillus* are widely used as biocontrol agents, biofertilizers, and biostimulants in agriculture (Ruzzi and Aroca 2015; Villarreal-Delgado et al. 2018). *B. thuringiensis* was among the first biocides to be used in agricultural crops for its insecticidal properties (Bravo et al. 2011). Likewise, *B. subtilis* is another species that has been characterized as the main agent of commercial biofungicides and biostimulants of plant development (Pérez-García et al. 2011; Xie et al. 2009). The mechanisms of plant protection or stimulation used by the Bacilli can be diverse (direct and indirect), and include the production of bacteriocins, Cry toxins, chitinases, lipases, lipopeptides, siderophores, and other multiple diffusible and volatile compounds (Santoyo et al. 2012; Ryu et al. 2003). Additionally, Bacilli commonly produce the enzyme 1-aminocyclopropane-1-carboxylate (ACC) deaminase, which induce plant growth under different types of stress, by reducing ethylene levels (Gamalero and Glick 2015).

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s13205-019-1911-5) contains supplementary material, which is available to authorized users.

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Bacillus toyonensis was recently proposed as a new species belonging to the group of *B. cereus*, although it had previously been studied as a probiotic due to its production of toyocerin, which improves intestinal microbiota and nutrition in animals (Jadamus et al. 2002; Jiménez et al. 2013). However, studies on the putative beneficial roles of *B. toyonesis* in plants are few as far as we know. For example, strain BAC3151 (previously characterized as *B. thuringiensis*), isolated from leaves of common bean plants (*Phaseolus vulgaris*), showed antagonistic activity against phytopathogenic bacteria during a pre-screening test (Lopes et al. 2015). Also, in silico analysis of *B. toyonensis* BAC315 revealed genes associated to the synthesis of antimicrobial compounds, including bacteriocins, non-ribosomal peptides, and chitinases, among others (Lopes et al. 2017). In another recent work, bacterial endophytes were isolated from the medicinal plant *Lavandula dentata* L., among which, several strains were identified as *B. cereus* biovar *Toyoi*, which could belong to the *B. toyonensis* species, but this was not corroborated. These strains showed production of siderophores, indoleacetic acid (IAA), NH3, and hydrocyanic acid (HCN) and lytic activities (Pereira et al. 2016). However, these previous studies only show the potential benefits of the strains and lack experimental evidence of biocontrol and induction of plant growth.

In this work, we present the draft genome of the endophyte *Bacillus toyonensis* strain COPE52, as well as in vitro antifungal bioassays against *Botrytis cinerea* and greenhouse experiments that demonstrate its capacity as a blueberry plant (*Vaccinium* spp., *Var*. Biloxi) growth-promoting bacterium. The COPE 52 strain was previously selected from a screening searching for plant growth-promoting endophytic bacteria isolated from *Rubus fruticosus* roots (Contreras et al. 2016). Additionally, the production of diffusible and volatile compounds was also corroborated experimentally (Santoyo et al. 2019). As far as we know, this is the first work confirming the *B. toyonensis* species as a plant growth-promoting bacterium (PGPB).

A single colony of the strain *B. toyonensis* COPE52 was inoculated on 50 ml of Nutrient Broth (NB), and grown overnight at 28 °C with agitation at 250 rpm. From the culture, the genomic DNA was extracted using the Wizard® Genomic DNA Purification Kit according to the manufacturer's (Promega) instructions. The quality and quantity of the final DNA sample were evaluated via agarose–TAE gel (1.5%) electrophoresis, and using a NanoDrop 1000 Spectrophotometer (Thermo Scientific), respectively. Genomic samples were sequenced, using the Illumina MiSeq platform (Mr. DNA, Texas, USA) with a coverage of 64.0×. The quality control of the reads was conducted with a FastQC analysis version 0.11.5 (Andrews 2010). Trimmomatic version 0.32 (Bolger et al. 2014) was used to remove adapter sequences and low-quality bases. De novo assembly was performed using SPAdes version 3.10.1 (Bankevich et al. 2012) and the "-careful" parameter for reads error correction. The assembled contigs were aligned by Mauve contig Mover (MCM) (Rissman et al. 2009), using the reference genome *Bacillus toyonensis* BCT-7112 (NCBI project accession: CP006863.1). Additionally, PLACNETw revealed the absence of plasmids in the COPE52 genome (Vielva et al. 2017). The draft genome of COPE52 consists of a single 5,806,513-bp chromosome, with 35.1% G + C content, 5979 total genes, 5631 protein-coding genes, 5 ncRNAs, 11 rRNA genes, 81 tRNA genes, and 251 pseudogenes.

For taxonomic assignment of strain COPE52, its 16S rRNA gene sequence was first used as a blast homology search template in the GenBank databases, revealing high identity with the *B. thuringiensis* strain ATCC 10792 (99.93%), *B. mobilis* AH1271 (99.93%), *B. toyonensis* BCT-7112 (99.86%), and *B. pacificus* EB422 (99.80%), among others. Then, these Bacilli species [based on the cut-off values on species delimitation established for 16S rRNA (> 98.7%) (Chun et al. 2018)] were compared to COPE52 at the genome level by employing the average nucleotide identity (ANI), using the OrthoANI algorithm (Yoon et al. 2017) and the genome-to-genome distance calculator (GGDC) 2.1 via BLAST (Meier et al. 2013). Based on pre-stablished cut-off values on species delimitation for ANI > 95–96% (Varghese et al. 2015) and GGDC > 70% (Espariz et al. 2016), COPE52 was found to be affiliated to *Bacillus toyonensis* (Table 1).

Table 1 ANI and GGDC values obtained from the comparison of strain COPE52 and closely related Bacilli species (16S rRNA > 98.7%) and genome-to genome distance calculator (> 70%)

Bacillus toyonensis COPE52 vs	Ortho ANI (%)	GGDC (%)
Bacillus toyonensis BCT-7112^T	98.55	87.4
Bacillus thuringiensis ATCC 10792^T	91.48	44.6
Bacillus cereus ATCC 14579^T	91.41	44.3
Bacillus wiedmannii FSL W8-0169^T	91.18	43.3
Bacillus luti TD41^T	90.95	43
Bacillus tropicus N24	90.93	42.7
Bacillus mobilis AH1271	90.92	42.6
Bacillus paranthracis Mn5	90.83	42.2
Bacillus anthracis Ames	90.81	42.5
Bacillus albus N35-10-2	90.8	42.3
Bacillus pacificus EB422	90.72	41.9
Bacillus proteolyticus TD42	90.61	40.1
Bacillus nitratireducens 4049	90.41	41.4
Bacillus mycoides ATCC 6462	90.23	40.9
Bacillus paramycoides NH24A2	89.65	38.9
Bacillus gaemokensis KCTC 13318	82.84	26.6
Bacillus bingmayongensis FJAT-13831	82.81	27.3
Bacillus pseudomycoides DSM 12442	82.54	27.2

Phylogenomic analysis of *B. toyonensis* and other Bacilli also corroborated a close relationship within a single clade with *B. toyonensis* BCT-7112^T, *B. toyonensis* Rock1-3, and *B. toyonensis* Rock3-28, which were grouped apart from *B. cereus* ATCC 14579^T, *B. cereus* B4264, *B. thuringiensis* ATCC 10792^T, *B. thuringiensis* BMB171, *B. luti* AFS058404, *B. luti* TD41^T, *B. wiedmannii* BAG30-1, and *B. wiedmannii* FSL W8-0169^T. Genomic sequences were aligned by the reference sequence alignment-based phylogeny builder method, REALPHY (Bertels et al. 2014,) and the genome-based phylogenetic tree was constructed by MEGA 7 (Kumar et al. 2016), using the Neighbor-Joining method, with a bootstrap support of 1000 replications (Fig. 1).

Gene annotation of strain COPE52 was carried out using the Rapid Annotation using Subsystem Technology (RAST) server (http://rast.nmpdr.org) (Aziz et al. 2008), by the RAS-Ttk pipeline. A total of 5631 protein-coding DNA sequences (CDSs) were assigned to 358 subsystems, the most abundant being Amino Acids and Derivates (434), Carbohydrates (341), Cofactors, Vitamins, Prosthetic Groups, Pigments (198), Protein Metabolism (180), Nucleosides and Nucleotides (124), and Dormancy and Sporulation (110). Among those relevant for antagonism, rhizo- and endospheric colonization, the genes involved in virulence, disease and defence (74), stress response (41), motility and chemotaxis (7), iron (52), phosphorous (42) and sulphur (7) metabolism are worth mentioning (Fig. 2).

The strain COPE52 was experimentally evaluated to detect direct and indirect (antifungal effects) plant growth-promoting traits (Santoyo et al. 2019). COPE52 was able to produce IAA (24.08 ± 0.50 µg/mL) and protease activity (28.6 ± 1.6, measured as a halo diameter in skim milk medium), but siderophore production, ACC deaminase activity, and phosphate solubilization were not detected. However, COPE52 restricted mycelial growth of *Botrytis cinerea* by diffusible (11.49 ± 0.84% decrease in mycelium diameter) and volatile (36.41 ± 2.3% decrease in mycelium diameter) organic compound (VOC) emission (Supplementary Table 1). Since *B. toyonensis* COPE52 exhibited better antagonism through VOCs against the phytopathogen, compared with *B. cinerea*, the VOCs produced by the studied endophyte were analysed using SPME–GC–MS, as previously reported (Hernández-León et al. 2015). Supplementary Table 2 shows the cocktail of VOCs produced by COPE52, highlighting the production of acetoin and 2,3-butanediol, which induce systemic resistance and growth in *Arabidopsis* (Ryu et al. 2003; Rudrappa et al. 2010), and dimethyl disulphide, which has been associated with antifungal action in PGPB (Rojas-Solís et al. 2018). It is relevant to mention that the *B. cinerea* commonly affects berries around

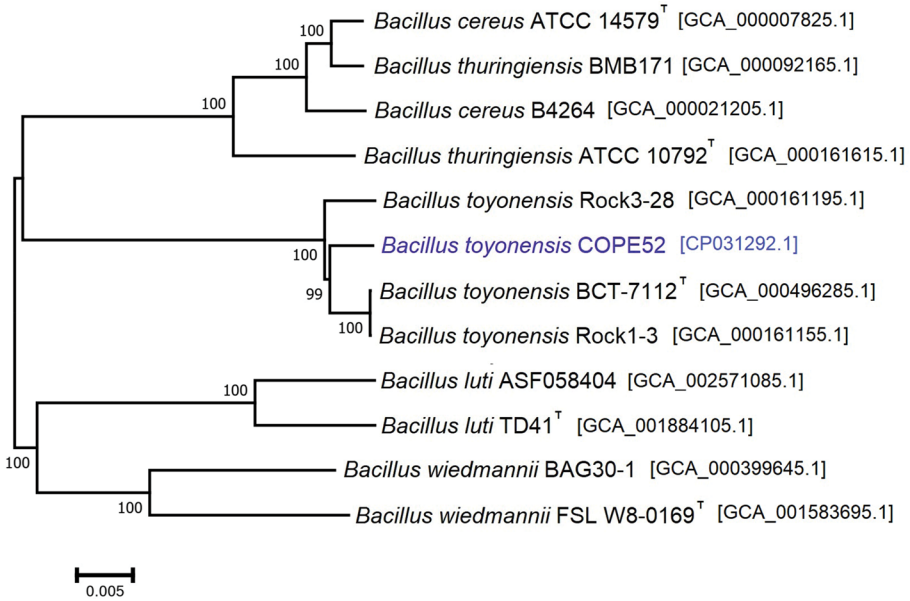

Fig. 1 Phylogenetic relationships among strain COPE52 and its closely related *Bacillus* species: *B. toyonensis* BCT-7112^T (GCA_000496285.1), *B. toyonensis* Rock1-3 (GCA_000161155.1), *B. toyonensis* Rock3-28 (GCA_000161195.1), *B. cereus* ATCC 14579^T (GCA_000007825.1), *B. cereus* B4264 (GCA_000021205.1), *B. thuringiensis* ATCC 10792^T (GCA_000161615.1), *B. thuringiensis* BMB171 (GCA_000092165.1), *B. luti* AFS058404 (GCA_002571085.1), *B. luti* TD41^T (GCA_001884105.1), *B. wiedmannii* BAG30-1 (GCA_000399645.1) and *B. wiedmannii* FSL W8-0169^T (GCA_001583695.1). The *Bacillus* genomic sequences were aligned using REALPHY (Bertels et al. 2014) and the genome-based phylogenetic tree was constructed by MEGA 7 (Kumar et al. 2016), using the Neighbor-Joining method, with bootstrap support of 1000 replications

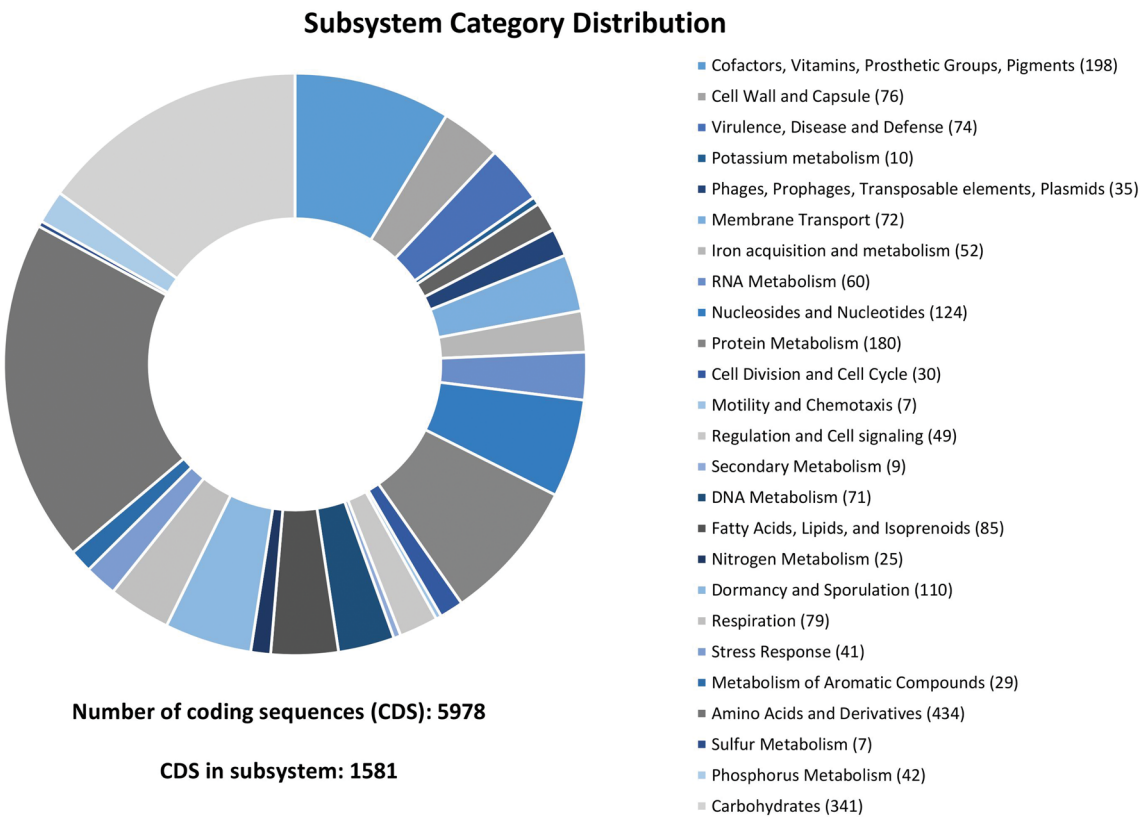

Fig. 2 Subsystem category distribution of CDC from the *Bacillus toyonensis* strain COPE52

the world, including blueberry plants (Rosslenbroich and Stuebler 2000).

To further confirm the role of *B. toyonensis* COPE52 as a PGPB, greenhouse experiments were performed in sterile peat moss to evaluate growth promotion in blueberry (var. Biloxi) plants, exerted by COPE52. The experimental design included two treatments: control ($n=88$) plants without inoculant and plants inoculated with COPE52 ($n=88$), with a total of 166 experimental units. The first bacterial inoculum was applied after 1 week of pot transplantation, in total four inoculations were applied to plants. Control plants were irrigated with sterile deionized water. Bacterial inoculum was adjusted to 1×10^8 Colony Forming Units (CFU)/mL. Throughout the experiment, the plants were irrigated every third day with sterile deionized water. The effect of adding COPE52 on the root length, aerial parts, fresh and dry weight, and chlorophyll concentration was evaluated after 20 weeks of plant growth. The chlorophyll concentration was measured in at least three leaves from each plant as previously reported (Rojas-Solís et al. 2018). Thus, the inoculation of *B. toyonensis* COPE52 resulted in a growth stimulation of blueberry plants, compared to un-inoculated plants. Except for the measurement of root length, COPE52 induced a significant ($p=0.05$) shoot length, fresh and dry weight of shoots and roots, and chlorophyll content (Table 2).

Table 2 Blueberry plants (var. Biloxi) growth promotion by the inoculation of *Bacillus toyonensis* COPE52 in greenhouse assay

Variables	Control	*Bacillus toyonensis* COPE52
Shoot length (cm)	23.8 ± 0.97	29.4 ± 0.50*
Root lenght (cm)	12.0 ± 0.52	13.4 ± 0.35
Chlorophil content (U)	13.3 ± 1.81	26.0 ± 2.21*
Fresh weight of shoot (g)	2.5 ± 0.28	3.8 ± 0.14*
Dry weight of shoot (g)	0.7 ± 0.07	1.1 ± 0.03*
Fresh weight of root (g)	0.3 ± 0.07	2.1 ± 0.18*
Dry weight of root (g)	0.2 ± 0.03	0.5 ± 0.03*

Values shown are the mean of ten independent replicates with ± standard errors values. Asterisks indicate significant differences according to *t* Student test ($p\leq0.05$)

In conclusion, our results confirm the function of predicted genes in the genome of COPE52, as well as the diverse plant growth-promoting traits exhibited by this strain. To our knowledge, this is the first work that demonstrates genomic and experimental evidences of *Bacillus toyonensis* as antifungal and plant growth-promoting bacterium.

Acknowledgements We thank Dr. Lourdes Macías for her help with the VOCs analysis. We thank to Coordinación de la Investigación

Científica-UMSNH for supporting our research projects (2018–2019) and to CONACYT-México (Project: A1-S-15956). M.C.-P. received a PhD scholarship from CONACYT-México.

Accession numbers This Whole Genome Shotgun sequence project has been deposited to GenBank under the accession number, CP031292.1. The BioSample (SAMN09462204) and BioProject (PRJNA476959) numbers are also available.

Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

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