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ORIGINAL ARTICLE

Bacillus subtilis EA-CB0575 genome reveals clues for plant growth promotion and potential for sustainable agriculture

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Abstract

Bacillus subtilis is a remarkably diverse bacterial species that displays many ecological functions. Given its genomic diversity, the strain *Bacillus subtilis* EA-CB0575, isolated from the rhizosphere of a banana plant, was sequenced and assembled to determine the genomic potential associated with its plant growth promotion potential. The genome was sequenced by Illumina technology and assembled using Velvet 1.2.10, resulting in a whole genome of 4.09 Mb with 4332 genes. Genes involved in the production of indoles, siderophores, lipopeptides, volatile compounds, phytase, bacilibactin, and nitrogenase were predicted by gene annotation or by metabolic pathway prediction by RAST. These potential traits were determined using in vitro biochemical tests, finding that *B. subtilis* EA-CB0575 produces two families of lipopeptides (surfactin and fengycin), solubilizes phosphate, fixes nitrogen, and produces indole and siderophores compounds. Finally, strain EA-CB0575 increased 34.60% the total dry weight (TDW) of tomato plants with respect to non-inoculated plants at greenhouse level. These results suggest that the identification of strain-specific genes and predicted metabolic pathways might explain the strain potential to promote plant growth by several mechanisms of action, accelerating the development of plant biostimulants for sustainable agricultural.

Keywords *Bacillus* · Plant growth-promoting rhizobacteria (PGPR) · Biostimulants · Genomic and biochemical potential

Introduction

Plant growth-promoting rhizobacteria (PGPR) have been used as biostimulants and biopesticides in different crops of economic relevance. They promote plant growth through diverse mechanisms like phytohormone production, nitrogen fixation, siderophore, antibiotic production, induced systemic resistance (ISR), and competition between others (Backer et al. 2018). From the commercially available PGPRs, *Bacillus subtilis* outstand for its resistance to stress conditions due to

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spore formation, broad metabolite spectrum, fast growth in liquid media, and the ability to colonize plant surfaces (Kumar et al. 2011). Therefore, different efforts have been made to elucidate its potential and mechanisms of action from different omics perspectives.

From the genome perspective, the first sequenced strain was *B. subtilis* Marbug 168, which has also become the most studied Gram-positive bacterium, revealing important information about sporulation, germination, nutrition, and development for *B. subtilis* group. Its genome is 4.2 Mb composed of 4100 genes that encode proteins from which 4% is devoted to antibiotic production and another 4% for sporulation and germination processes (Kunst et al. 1997; Moszer 1998). Since then, the whole-genome sequence of around 200 strains of *B. subtilis* group have been sequenced (https://www.ncbi.nlm.nih.gov/assembly/?term=Bacillus%20subtilis), generating information on the genomic diversity but also on the systematics of *Bacillus* genus and the adaptations of this taxon in their natural habitat (Li et al. 2016; Smith et al. 2014; Zeigler 2011). Access to whole-genome sequences and their comparative analyses with different *Bacillus subtilis* strains and close relatives is important to understand gene flow, speciation, diversity, and genomic dynamics in bacilli for physiological, ecological, and evolutive studies (Earl et al. 2012; Shaligram et al. 2016). Currently, there are studies where several genomes of *Bacillus* strains are elucidated in order to establish the safety and the efficiency for industrial microbiology (Sulthana et al. 2019), to investigate the genetics of fermenting strains and its relationship with the productivity of metabolites (Deng et al. 2019; Kamada et al. 2015), to clarify the phylogeny of *Bacillus* groups (Stevens et al. 2019), or to determine plasmid-independent species-species markers (Ivanova et al. 2003). The study of the genome of microorganisms used for biofertilizers and biostimulants production is important to bioinoculants technology because it helps identify genes that contribute to the beneficial activity and increasing knowledge of the molecular mechanisms related to plant growth potential. Also, this provides information for inoculants biosafety and the compliance with existing and developing regulations for this kind of products whose supply and consumption is growing (EPA 2019). The use of next generation sequencing (NGS) has allowed whole-genome sequencing of PGPRs (plant growth-promoting rhizobacteria) isolated from different crops such as wheat, pepper, cotton, and coconut (Gupta et al. 2014; Kai-Jium et al. 2018); and it is an opportunity to obtain information about strains used and to design strategies for development and use of such PGPRs to support sustainable agriculture.

In this research, we present the sequencing, assembling, and characterization of *B. subtilis* EA-CB0575 whole genome, isolated from a commercial crop of *Musa* AAA in Colombia; this microorganism has shown successful results for plant growth promotion of banana crops, maize, and tomato (Posada et al. 2018). We hypothesize that the genome sequence of *B. subtilis* EA-CB0575 will reveal genes involved in plant growth promotion mechanisms, which will be expressed in vitro and potentially promote growth of tomato plants. Therefore, the presence of homologous genes associated with mechanisms of plant growth promotion such as indole and siderophores production, phosphate solubilization, nitrogen fixation, LPs production, and production of volatile compounds was determined for the assembled genome. Biochemical traits, related to these characteristics, were evaluated at in vitro level, and the strain's potential for growth promotion of tomato crop was determined at greenhouse level as evidence of the relationship between the genomic potential and the capacity of growth promotion in a plant.

Methodology

Microorganisms and culture conditions

B. subtilis EA-CB0575 was isolated from roots of a banana plant cv. Valery from Urabá – Colombia (Northeastern 07° 51′ 58.6″ N, − 76° 37′ 39″ W) in 2009 (Posada et al. 2016). The microorganism was identified by analysis of 16s rRNA gene sequencing (Accession number KC170988) and characterized as a Gram-positive rod (1.85 ± 0.31 μm), forming central spores (0.72 ± 0.15 μm), and producing irregular and matt white colonies with crateriform elevation in TSA X 0.5 (105458, Merck). The strain was stored in TSB medium (TSB, 105459, Merck) with glycerol (20% *v*/*v*) at − 80 °C and registered on the bacterial collection RNC 191 (Instituto Alexander Von Humboldt). *B. subtilis* 168 (Zeigler et al. 2008) and *B. velezensis* FZB42 (Fan et al. 2018), previously *B. amyloliquefaciens* FZB42 (Fan et al. 2012), were donated by Dr. Camilo Ramírez (Universidad de Antioquia, Colombia) and Dr. Rainer Borriss (ABiTEP GmbH ABI, Germany) respectively.

Genomic DNA preparation

B. subtilis EA-CB0575 genomic DNA was obtained from cells harvested in the exponential phase of a submerged culture in TSB. Briefly, the strain was cultured 14 h at 150 rpm and 30 °C, centrifugated, and obtained pellet used for DNA extraction using Ultraclean Microbial DNA isolation (Mobio). DNA quality was determined using NanoDrop and agarose 1% gel electrophoresis.

Sequencing and annotation of *B. subtilis* EA-CB0575 genome

B. subtilis EA-CB0575 genome was sequenced using Illumina HiSeq 2000 paired-end method and 100 bp reads with 265 bp

average insert size were obtained. The quality of the FASTQ files was verified with FastQC (Andrews 2010) and reads were trimmed to ensure high quality (Phred score > 30) using Trimmomatic version 0.35 (Bolger et al. 2014). The genome was assembled with Velvet 1.2.10 (Zerbino and Birney 2008) using k-mer = 95 and derived statistics of the assembly process were determined (genome total length, N_{50}, N_{90}, time for running (min), cluster numbers, cluster average size (pb)). Subsequently, OriFind and Rast software (Aziz et al. 2008) were used for ORI and ORF prediction, respectively, and the order of alignments with ProgressiveMauve aligner and Murasaki software (Popendorf et al. 2010) with *B. subtilis* 168 as the reference strain. Once the genome was assembled, gene prediction and annotation were performed using RAST (http://rast.nmpdr.org/), and circular genome representation was done with CGView webserver (http://stothard.afns.ualberta.ca/cgview_server/). RAST metabolic predictions were used to determine indole, siderophores, and volatile compounds production. Phosphate solubilization and nitrogen fixation capacity were determined to evaluate the presence of *phy* and *nif* genes inside the genome. This search was done using the reference strains *B. velezensis* FZB42 (GenBank Accession number: CP000560.1) for indole, specifically IAA (indole acetic acid), volatiles, and siderophores production; *B. subtilis* 168 for phytase production; and *Klebsiella pneumoniae* (Genbank accession number X13303.1) for nitrogen fixation. Local BLAST between references and *B. subtilis* EA-CB0575 was performed and subsequently, a combined procedure between the RAST metabolic annotation (KEGG tool) and the search for genes of interest was used to find related genes to the PGPR traits in the assembled genome of the *B. subtilis* strain EA-CB0575. The *nif* and *phy* cluster genes search was performed comparing the homology of these genes with sections of the evaluated strains.

Identification and characterization of NRPS genomic clusters

The presence of non-ribosomal peptide synthetases (NRPS) coding regions in *B. subtilis* EA-CB0575 genome was evaluated using homologous sequences to coding regions of *B. velezensis* FZB42 strain, which produce three families of LPs (Koumoutsi et al. 2004). BLASTn algorithm was used to determine differences in operon sequences throughout the genome, and Murasaki software 1.68.6 (Popendorf et al. 2010) was used to determine ORFs. RAST and FgenesB operon predictor (Softberry, Inc., NY, USA) was used to evaluate coding sequences. NCBI Conserved Domain Database (CDD) (Marchler-Bauer et al. 2011) was used to contrast reported information with *B. subtilis* EA-CB0575 genome information and to predict structural domains related to each ORF previously found.

Comparative genomics of *B. subtilis* EA-CB0575 and phylogenetic study

B. velezensis FZB42, *B. subtilis* 168, and *B. subtilis* EA-CB0575 genomes were compared to locate gene clusters involved in the secondary metabolites production using Murasaki 1.68.6 software (Popendorf et al. 2010) and GMV tool. A phylogenetic reconstruction analysis was performed to infer the evolutionary relationships of *B. subtilis* EA-CB0575 and others *Bacillus* spp. with whole genomes reported in GenBank NCBI. This analysis was done using five housekeeping genes (*rpoB*, *gyrA*, *purH*, *polC*, and *groEL*) for thirty-one *Bacillus* spp. strains and using *Peptoclostridium difficile* as the outgroup (Table 1). Bayesian inference (BI) and maximum likelihood (ML) were used for the phylogenetic reconstruction. Sampled genes were aligned independently in an amino acid–based fashion using RevTrans. First, nucleotide coding sequences were translated to amino acids using VirtualRibosome from RevTrans 1.4 package and using genetic bacterial code table # 11 (https://www.ncbi.nlm.nih.gov/Taxonomy/Utils/wprintgc.cgi). Amino acid sequences of each gene were aligned independently using MAFFT 7.213 in automatic mode. Then, alignments were reverse-translated from amino acids to nucleotides with RevTrans 1.4 software (Wernersson and Pedersen 2003). Aligned nucleotide sequences for each gene were concatenated into a single FASTA file and converted to NEXUS format by ReadSeq 2.1.19 (Gilbert 2003), and to Phylip using Biopython utilities. Partitionfinder 1.1 software (Lanfear et al. 2014) was used to find the best partitioning scheme for the data and the appropriate nucleotide substitution models for each partition. The BI analysis was done using the software MrBayes 3.2.2 (Ronquist et al. 2012). Four independent MCMC (Markov Chain Monte Carlo) analyses were carried out with 10 million generations and eight chains (seven hot and one cold) for each one and a relative burn-in fraction of 0.35. Consensus tree for BI was obtained using the remaining trees. ML analysis was performed with Garli HPC-MPI 2.07 (Bazinet et al. 2014). A search was carried out with eight replicates and the one with the highest probability was selected (likelihood), followed by an analysis with 1000 bootstrap replicates. SumTrees 3.3.1 (Sukumaran and Holder 2010) was used to map bootstrap replicates on the best tree from the first analysis. The visualization of the obtained trees was done with FigTree 1.4.1 (Rambaut 2014). The roots of both trees were fixed based on the external group used (*Peptoclostridium difficile*).

Phenotypic characterization of *B. subtilis* EA-CB0575 and reference strains

Production of total indoles was determined by the colorimetric technique using Salkowski's reagent as described by Patten and Glick (2002). Siderophore production was determined by

Table 1 Microorganisms used for the phylogenetic analysis

Accession number	Strain	Reference
NC_006270.3	*Bacillus licheniformis* DSM 13 ATCC 14580	Veith et al. 2004
NZ_CP010524.1	*Bacillus licheniformis* BL-09	Pengfei et al. 2015
NC_009848.1	*Bacillus pumilus* SAFR-032	Tirumalai et al. 2013
CP011150.1	*Bacillus pumilus* W3	Zheng-Bing et al. 2015
NC_012659.1	*Bacillus anthracis* str. A0248	Md. Anisur et al. 2014
NC_005957.1	*Bacillus thuringiensis* serovar *konkukian* str. 97-26	Li et al. 2015
NZ_CP009335.1	*Bacillus thuringiensis* HD1011	Li et al. 2015
NC_004722.1	*Bacillus cereus* ATCC 14579	Ivanova et al. 2003
NC_011969.1	*Bacillus cereus* Q1	Zhaohui et al. 2009
NC_012472.1	*Bacillus cereus* 03BB102	Costa et al. 2018
NZ_CP009692.1	*Bacillus mycoides* ATCC 6462	Li et al. 2018
NZ_CP007626.1	*Bacillus mycoides* 219,298	Liu et al. 2017
CP009746,1	*Bacillus weihenstephanensis* WSBC 10204	Liu et al. 2018
CP006952.1	*Bacillus amyloliquefaciens* LFB112	Cai et al. 2014
NC_022653.1	*Bacillus amyloliquefaciens* CC178	Kim et al. 2015
NC_014551.1	*Bacillus amyloliquefaciens* DSM 7	Rückert et al. 2011
CP000560.1	*Bacillus velezensis* str. FZB42	Chen et al. 2007
NC_016047.1	*Bacillus subtilis* subsp. *spizizenii* TU-B-10	Zeigler 2011
CP002183.1	*Bacillus subtilis* subsp. *spizizenii* str. W23	Zeigler 2011
NZ_CM000487.1	*Bacillus subtilis* subsp. *subtilis* str. 168	Kunst et al. 1997
NZ_CM000489.1	*Bacillus subtilis* subsp. *subtilis* str. JH642	Smith et al. 2014
NZ_CM000488.1	*Bacillus subtilis* subsp. *subtilis* str. NCIB 3610	Nye et al. 2017
NZ_CM000490.1	*Bacillus subtilis* subsp. *subtilis* str. SMY	Zeigler et al. 2008
NC_020507.1	*Bacillus subtilis* subsp. *subtilis* 6051-HGW	Khatri et al. 2016
NC_020832.1	*Bacillus subtilis* subsp. *subtilis* str. BAB-1	Guo et al. 2015
NC_017195.1	*Bacillus subtilis* subsp. *subtilis* str. RO-NN-1	Zeigler 2011
NC_020244.1	*Bacillus subtilis* XF-1	Guo et al. 2015
CP002468.1	*Bacillus subtilis* BSn5	Deng et al. 2011
CP006881.1	*Bacillus subtilis* PY79	Schroeder and Simmons 2013
KC170988	*Bacillus subtilis* EA-CB0575	This study
GQ375229	*Bacillus subtilis* EA-CB0015	Unpublished
NZ_LN614756.1	*Peptoclostridium difficile* CD630DERM	Pereira et al. 2016

the colorimetric CAS method (Schwyn and Neilands 1987); phosphate solubilization by growing on NBRIP medium (NaCl 1 g; $CaCl_2$*$2H_2O$ 0.2 g; $MgSO_4$*$7H_2O$ 0.4 g; NH_4NO_3 1 g; glucose 10 g, benomyl 300 ppm, bactoagar 7 g, and phosphoric rock 3.5 g, using phosphoric rock instead tricalcium phosphate) (Bashan et al. 2013; Kim et al. 1997; Murphy and Riley 1962) and nitrogen fixation were determined by growth on NFb medium (Eckert et al. 2001). Antagonistic potential against banana pathogens as *Fusarium oxysporum* (strain EAHP-0015, donated by Banana Growers Association from Colombia, AUGURA) and *Ralstonia solanacearum* (EA-EP009, isolated of banana plant at Universidad EAFIT, Medellin) was evaluated by using the dual-culture plate method previously described by Lemessa and Zeller (2007). Antagonistic potential against *Pseudocercospora fijiensis* (isolated of banana leaves plant using necrotic tissue by ascospore discharge method) was evaluated using the varnish technique modified by Talavera et al. (1998) and percent inhibition of germ tube was determined by Gutierrez-Monsalve et al. (2015). Finally, production and purification of lipopeptides was carried out by a methodology previously described (Villegas-Escobar et al. 2013). Briefly, 20 mL of an overnight culture of *B. subtilis* EA-CB0575 (12 h, 30 °C) was transferred to 500-mL Erlenmeyer flask containing 180 mL of MOLP medium (Jacques et al. 1999) and incubated for 3 days at 30 °C and 150 rpm. After 12 h of incubation, 4% (*w*/*v*) of amberlite resin XAD-16 (Alfa Aesar®) was added to the culture. After

incubation, the resin was recovered, washed with 400 mL of sterile distilled water, and adsorbed metabolites were eluted with 200 mL of methanol. The methanolic extract was evaporated under reduced pressure in a rotary evaporator (50 °C, − 50 psig) obtaining 99 mg of solid residue from 200 mL total culture. The solid residue was suspended in distilled water (37 mg/mL) and applied to a solid-phase extraction (SPE C18) cartridge (Baker®, 10 g). The cartridge was then rinsed successively with 80 mL of distilled water, 80 mL of 50% methanol, and 160 mL of 100% methanol. The methanolic fraction was evaporated and the solid residue dissolved in methanol (50 mg/mL) for RP-HPLC analysis. The methanolic fraction was purified by RP-HPLC using an Eclipse XDB C18 column (250 × 4.6 mm, 5 μm) connected to an Agilent G1311A quaternary pump and with a solvent system consisting of 0.1% in water (solvent A) and 0.1% TFA in acetonitrile (solvent B). Forty microliters of the sample was injected into the column and the compounds were eluted by a gradient program (30/100/100% B in 0/25/35 min) at a flow rate of 1 mL/min and UV detection at 214 nm. Peaks with different retention times were collected, vacuum evaporated (Eppendorf Concentrator Plus™, Harburg, Germany), and stored at 4 °C. Purified HPLC fractions and surfactin standard (Sigma Aldrich, S3523) were analyzed by high-resolution mass spectrometry (Iowa State University's Protein Facility, Ames, IO, USA) using an HPLC (Agilent 1260) coupled to Thermo Scientific Hybrid Q-Exactive ™ quadrupole-orbitrap tandem mass spectrometer equipped with a NanoSpray Flex ionization source and cyano cinnamic acid as matrix. Solvent A (water + 0.1% formic acid) and B (acetonitrile with 0.1% formic acid) with a gradient of 20–100% were used for 13 min. Data were acquired in a range between 200 and 2000 m/z in positive ionization mode. MS/MS analysis was performed using an HCD ion collision cell and the software Xcalibur (Thermo Scientific, version 2.2). Proteowizard 3.0.10.200 (Chambers et al. 2012) was used for the analysis of the results.

Plant growth promotion of *B. subtilis* EA-CB0575 in tomato

Plant growth promotion effect of *B. subtilis* EA-CB0575 and two reference strains (*B. velezensis* FZB42 and *B. subtilis* 168) were evaluated in tomato variety Chonto-Santa Cruz at greenhouse level using a completely randomized design (CRD). Tomato seeds were disinfected successively with 70% ethanol for 15 min, 5% sodium hypochlorite for 5 min, and three washes with sterile distilled water. After scarification at 4 °C for 24 h, seeds were inoculated by immersing the roots in a bacterial suspension (1×10^8 CFU/mL) or in sterile distilled water (control) for 1 h. The seeds were sowed in pots with 500 g of commercial soil (1 sand: 0.5 black soil: 0.5 rice husk) and kept at greenhouse conditions (22–30 °C, 12 h light/12 h dark, 60% of maximum water-holding capacity) for 1 month. Plants were fertilized after 15 days of inoculation with 15-15-15 NPK. The growth-promoting effect of the different strains was performed by measuring shoot length (SL) and dry weights (shoot dry weight (SDW), root dry weight (RDW), and total dry weight (TDW)). This experiment was conducted twice with 8 replicates per treatment and two plants per pot.

Statistical analysis

Analysis of variance (ANOVA) was used to analyze each experiment in RStudio. The assumptions of normality (Shapiro-Wilks test), homoscedasticity (Levenne's test and Barlett's test), and independence (graphic residues vs. run order and Durbin-Watson statistics) were determined. In the case of significant P value ($P < 0.05$), means were compared by using LSD or Dunnett multiple comparison test.

Results

Genome analysis of *B. subtilis* subsp. *subtilis* EA-CB0575 reveals the potential for plant growth promotion

16S rRNA gene sequencing was performed to define the strain taxonomy, finding that it belongs to *Bacillus subtilis* species with a query cover of 100%, an identity of 99%, and an E-value of 0.0. The complete genome of *B. subtilis* EA-CB0575 was assembled in 16 contigs, reaching an average size of 255,665 bp. For this assembly, an average G-C content of 43.7% and a total genome size of 4.09 Mb was determined (Fig 1). The average coverage of the cluster was 110× and half of the genome bases were found in 2 contigs (N50), 90% in 6 (N90), and 95% in 7 contigs (N95). The maximum coverage of the genome was 2000×. Table 2 shows the number of genes annotated and involved in *B. subtilis* EA-CB0575 metabolic and cellular processes. There is a high similarity among the number of genes in each category between the strain EA-CB0575 and *B. subtilis* 168 type strain; but the former has more genes in several metabolism-related functions (fatty acids, lipids and isoprenoids, sulfur, carbohydrates, aromatic compounds, and phosphorus) and in cellular processes (division and cellular cycle, cell wall formation and capsule formation, motility and chemostasis, regulation and cellular signaling, and genes related to phages, prophages, transposable elements, plasmids). The genome contains 4332 genes, 4237 genes encoding proteins, 95 RNA genes (including 10 copies of the 16S rRNA), 2917 genes encoding proteins with predicted function, and 1320 genes encoding hypothetical proteins. The presence of related genes to PGPR mechanisms or the metabolic pathway prediction of RAST was found from the gene annotation. The production of enzymes involved in the

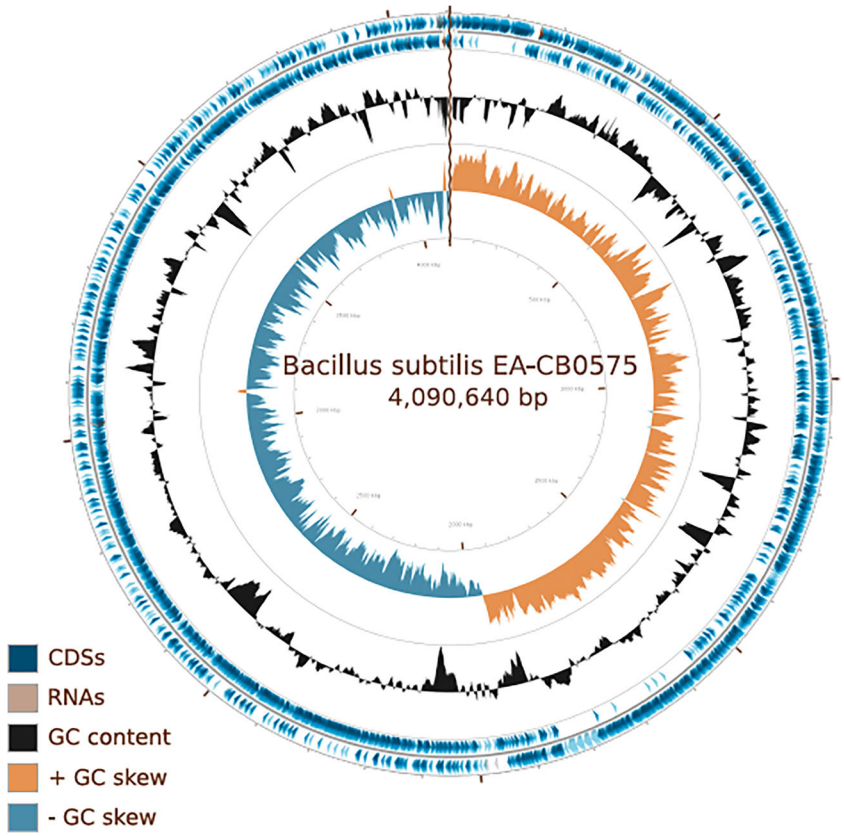


Fig. 1 Whole-genome distribution of *B. subtilis* EA-CB0575

metabolism of indole via the tryptophan pathway was predicted, suggesting that *B. subtilis* EA-CB0575 has the potential to produce these compounds. Indole, indole acetate, and indoleacetamide production was predicted (Fig. S1). The potential for production of siderophores as bacillibactin type, enterochelins (enterobacins), vibriobacins, and related ones synthesized by NPRSs was found. Some enzymes related to the production of compounds such as salicylate, isocorismate, and dihydroxybenzoate would likely be produced by *B. subtilis* EA-CB0575 (Fig. S2). The gene cluster that encodes to produce bacilibactin (*dhb*ACEBF) was found in the strain genome when compared with the reference genomes (Table 3)**.** Volatile compounds as 2,3-butanediol and acetoin might be produced by this strain (Fig. S3) given that it has the potential to produce the enzymes α−acetolactate synthetase, α−acetolactate decarboxylase, acetoin-reductase, acetylacetoin-synthase, and others reported (Qi et al. 2014). The phyC genes, coding for enzymes that sequentially remove phosphates of phytate, were found in the genome of the strain *B. subtilis* EA-CB0575, suggesting the capacity of this strain for organic phosphate solubilization. Also, some genes related to enzyme activity to fully perform citric acid pathway (TCA cycle) were found in the strain 575 genome, indicating a possible inorganic phosphate solubilization by organic acid production (Vyas and Gulati 2009).

Finally, the environmental nitrogen fixation capacity of strain EA-CB0575 was evaluated. The genes *nif*U, *nif*S, *nif*V, and *nif*F were present in the strain genome which are involved in nitrogenase enzymatic activity responsible for the biological fixation of nitrogen. However, the presence of all the genes of the nitrogenase cluster which are essential for the nitrogenase activity was not found. The assembled and annotated genome was saved on Universidad EAFIT server, operating under Ubuntu 14.04 and accessed through the GBrowse2 genome browser (Stein et al. 2002). An analysis of the cluster ends was performed, finding that 50% of the genes in this section correspond to rRNA, 26.67% to non-ribosomal peptide synthases, followed by 6.67% for tRNAs, and the rest corresponds to phages and repeated GXT proteins.

The genome of *B. subtilis* EA-CB0575 harbors an array of gene clusters involved in synthesis of secondary metabolites

The presence of NRPS coding sequences involved in the synthesis of surfactins, fengycins, and iturins in *B. subtilis* EA-CB0575 genome was evaluated using *B. velezensis* FZB42 as the genome reference. Coding regions for surfactins and fengycins production were found; however, no coding regions were found for iturins production (Fig. 2). The coding regions for NRPSs that produce surfactins were found in clusters 5, 6, and 7 and for fengycins production in clusters 9, 10, and 11. *B. subtilis* EA-CB0575 loci for NRPS was compared respect to the same loci for references

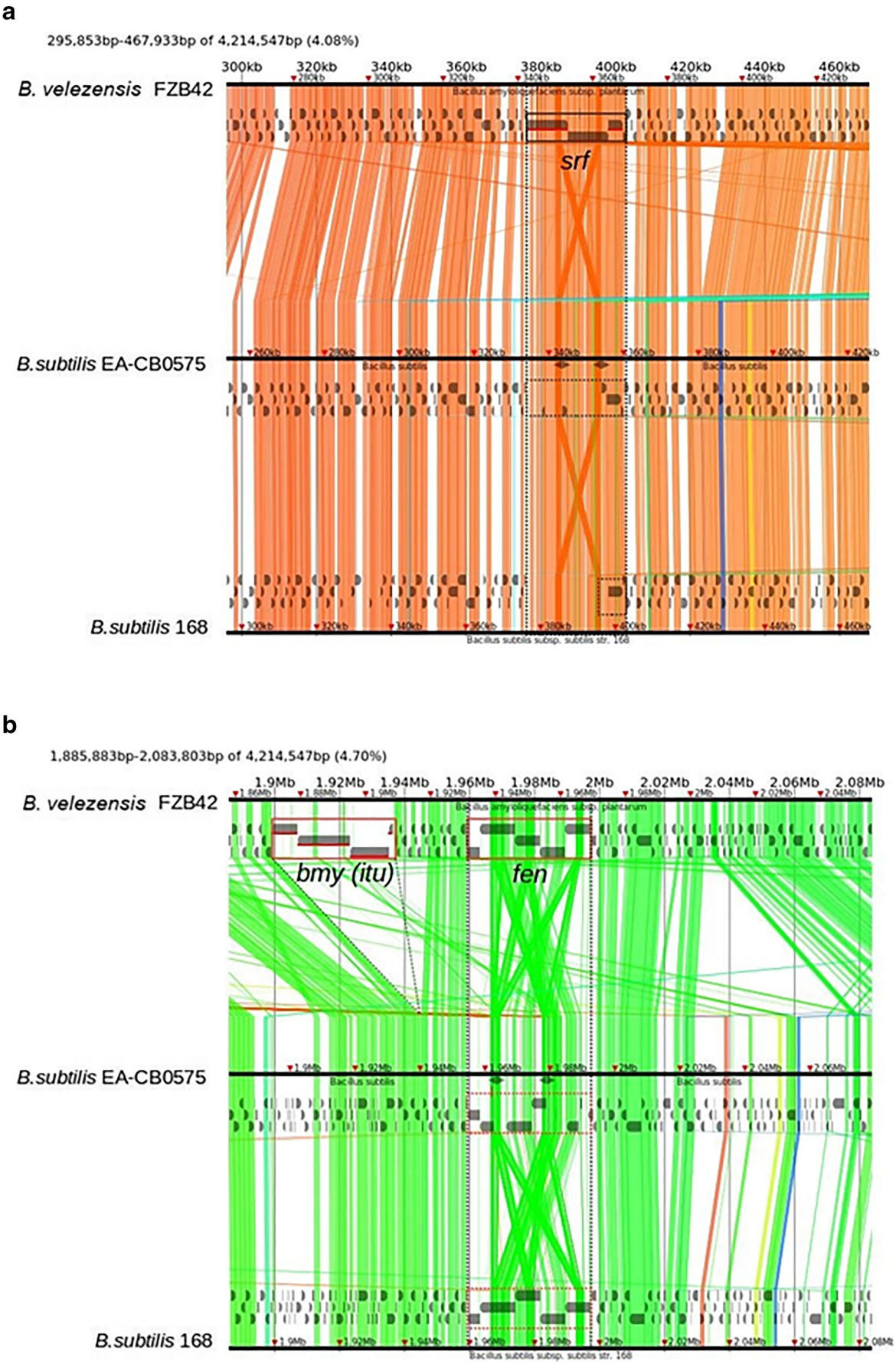
Table 2 Annotation of genes involved in the metabolism and other cellular processes of *B. subtilis* EA-CB0575 and strains reference *B. subtilis* 168 and *B. velezensis* FZB42

Genes function		*B. subtilis* EA-CB0575	*B. subtilis* 168
Genes related to metabolism	Fatty acids, lipids, and isoprenoids	119	102
	Amino acids derivatives	428	432
	Sulfur	47	44
	Carbohydrates	514	504
	Cofactors, vitamins, prosthetic groups, pigments	218	265
	Aromatic compounds	13	10
	DNA	112	136
	Phosphorus	30	25
	Iron	28	32
	Secondary metabolism	4	6
	Nitrogen- proteins	242	269
	Nucleosides and nucleotides	122	139
	Potassium	15	26
	RNA	163	194
Genes related to cellular processes	Division and cellular cycle	55	48
	Dormancy and sporulation	122	141
	Cellular wall and capsule formation	151	130
	Photosynthesis	0	0
	Miscellaneous	49	64
	Motility and chemostasis	89	66
	Regulation and cellular signaling	61	50
	Related to phages, prophages, transposable elements, plasmids	13	0
	Respiration	69	82
	Response to stress	109	111
	Membrane transport	73	75
	Virulence, disease, and defense	71	77

B. velezensis FZB42 and *B. subtilis* 168 and 4 ORFs were reconstructed for the surfactin operon, with two gaps of a few nucleotides between assembly clusters 5 and 6, and 6 and 7 in the *srf*AA and *srf*AC genes, respectively. Seven

Table 3 Comparative genomics of *B. subtilis* EA-CB0575 with the reference strains

Compound	Type		Number of genes	Genes	Gen (kpb)	*B. subtilis* EA-CB0575	*B. subtilis 168*	*B. velezensis* FZB42
Bacilibactin	Siderophore	NRPS	5	*dhbACEBF*	11.7	+	+	+
Iturin Bacillomycin	LP	NRPS/PKS	4	*bmyDABC*	37.2	–	–	+
Fengycin		NRPS	5	*fenABCDE*	37.6	+	+	+
Surfactin		NRPS	4	*srfABCD*	26.2	+	–	+
Bacilysin	Antibiotic	NRPS	5	*bacABCDE*	4.7	+	+	+
Macrolactin		PKS	9	*mlnABCDEFGHI*	53.9	–	–	+
Bacillaene		PKS	14	*baeBCDEGHIJLMNRS. acpK*	72.4	+	+	+
Difficidine		PKS	15	*dfnAYXBCDEFGHIJKLM*	70.0	–	–	+
Subtilosin		Sactipeptide	8	n/d	6.9	+	+	–
Dedicated portion of the genome for secondary metabolite production (%)					3.90		3.79	8.02
Genome length (Mpb)					4.09		4.21	3.91


a
295,853bp-467,933bp of 4,214,547bp (4.08%)
300kb
320kb
340kb
360kb
380kb
400kb
420kb
440kb
460kb
B. velezensis FZB42
srf
B.subtilis EA-CB0575
B.subtilis 168
b
1,885,883bp-2,083,803bp of 4,214,547bp (4.70%)
1.9Mb
1.92Mb
1.94Mb
1.96Mb
1.98Mb
2Mb
2.02Mb
2.04Mb
2.06Mb
2.08Mb
B. velezensis FZB42
bmy (itu)
fen
B.subtilis EA-CB0575
B.subtilis 168

◀ **Fig. 2** Determination of the loci to produce the NRPSs of surfactins, fengycins, and iturins in *B. velezensis* FZB42, *B. subtilis* EA-CB0575, and *B. subtilis* 168 genomes. **a** Diagram of location for responsible loci to the synthesis of NRPS related to surfactin in *B. subtilis* EA-CB0575 strain in contrast to the reference strains *B. subtilis* 168 and *B. velezensis* FZB42 **b** Diagram of location for responsible loci for genes involved in the production of the iturin bacillomycin and fengycin B in *B. subtilis* EA-CB0575 strain in contrast to the reference strains *B. subtilis* 168 and *B. velezensis* FZB42

domains of adenylation and a thioesterase/acyltransferase were obtained from the amino acid sequences of the ORFs. Three of 5 ORFs of the operon for fengycin production were obtained, corresponding to *fen*A, *fen*C, and *fen*E genes. Two domains of adenylation were obtained for the first, two for the second, and one for the adenylation and one thioesterase for the third. For the reference strains, the coding regions for the synthases related to the production of surfactins and fengycins were found; however, the region of iturin was determined for FZB42 strain and not for 168 strain. Comparative genomics of *B. subtilis* EA-CB0575 was performed with the two reference strains *B. subtilis* 168 and *B. velezensis* FZB42 to produce some secondary metabolites (Table 3). Similarities were observed in genome sizes and genes. There is a greater similarity between the percentages of the genome dedicated to the production of secondary metabolites in *B. subtilis* strains than *B. velezensis*, this latter with the highest percentage of the genome dedicated to the production of these compounds (8.02%). Some important clusters of genes, such as those related to the production of antibiotics, siderophore bacilibactin, and some LPs, are shared among the evaluated species.

B. subtilis EA-CB0575 belongs to the clade of *B. subtilis* subsp. *subtilis*

The phylogenetic relationship of *B. subtilis* EA-CB0575 with other *Bacillus* sp. strains was performed by comparing a set of housekeeping genes (Fig. 3). The best partitioning scheme of the alignment for the concatenated genes was composed of 6 subsets, with the 15 preliminary partitions given, one for each position of codons (1, 2, 3) in each of the 5 genes analyzed. Two major clades were observed: the first containing *B. thuringiensis*, *B. anthracis*, *B. cereus*, *B. mycoides*, and *B. weihenstephanensis*, which are *Bacillus* spp. pathogens of various hosts. The second clade was composed of *B. pumilus*, *B. licheniformis*, *B. subtilis*, *B. amyloliquefaciens*, and *B. velezensis.*

PGPR traits found in *B. subtilis* EA-CB0575 genome are expressed in vitro

To determine if genes related to PGPR traits were functional, different in vitro evaluations were performed (Table 4). Among evaluated strains, *B. subtilis* EA-CB0575 produced IAA (15.0 ± 2.0 μg/mL) and siderophores (15.4 ± 1.1 μM), solubilized phosphate, and had the ability to fix environmental nitrogen while *B. subtilis* 168 produced 2.0-fold lower concentrations of IAA (6.9 ± 1.4 μg/mL) and siderophores (7.6 ± 0.9 μM), and no capacity to solubilized phosphate or fix nitrogen. On the other hand, *B. velezensis* FZB42 produced IAA and siderophores, solubilized phosphate, but has no potential to fix nitrogen in vitro. *B. subtilis* EA-CB0575 inhibited the growth of the phytopathogens *R. solanacearum (*halo 1.2 ± 0.3 cm on BGA medium*)*, *F. oxysporum* (reduction of growth of 21 ± 0.1% on PDA medium), and *P. fijiensis* (reduction of 33.1 ± 4.3% on germinative tubes of ascospores) when *Bacillus* was evaluated in coculture with the fungi tested in vitro (Table 4). Finally, to determine if gene clusters related to NRPS were expressed in *B. subtilis* EA-CB0575, different purified fractions obtained by HPLC were analyzed by mass spectrometry analysis. Two families of lipopeptides were identified: surfactins and fengycins (Table 4; Fig. 4 and supplementary material Tables S1 and S2 and, Fig. S5, S6, S7, S8). In particular, the precursor ions observed at m/z $[M + H]^+$ 1008.66, 1022.67, 1036.69 and at m/z $[M + Na]^+$ 1030.64, 1044.66, 1058.67, and 1072.69 $[M + Na]^+$ (Fig. S5) were used for further MS/MS analysis, and results showed product ions characteristic of surfactins with different lengths of fatty acid chains (C12, C13, C14, C15, and C16) and therefore different molecular weights. For the other purified fraction, the precursor ions at m/z 1408, 1435.77, 1463.81, 1477.82, 1491.83, and 1505.85 corresponding to protonated molecules $[M + H]^+$, and m/z 1444, 1460 to sodiated $[M + Na]^+$, and potassium $[M + K]^+$ adducts respectively (Fig. S7), were also used for further MS/MS analysis. In general, product ions obtained were characteristic of fengycins A (m/z 1080.50 and 966.45) and B (m/z 1108.58 and 994.49). These results all together suggest that genes found in the genome of *B. subtilis* EA-CB0575 are functional in vitro and may confer potential for promoting plant growth.

B. subtilis is a potential PGPR for tomato crops

Tomato seeds variety Chonto-Santa Cruz were inoculated with suspensions of *B. subtilis* EA-CB0575 as spores or vegetative cells and the reference strains *B. subtilis* 168 and *B. velezensis* FZB42 as vegetative cells. We determined an average increase of 29% for the variable TDW of the plants with application of *B. subtilis* strains respect to the control. *B. subtilis* EA-CB0575 was the strain with a higher increase of this variable (34.60%). SDW and RDW shown differences between *B. subtilis* EA-CB0575 and reference strains and the control, respectively; with percentage

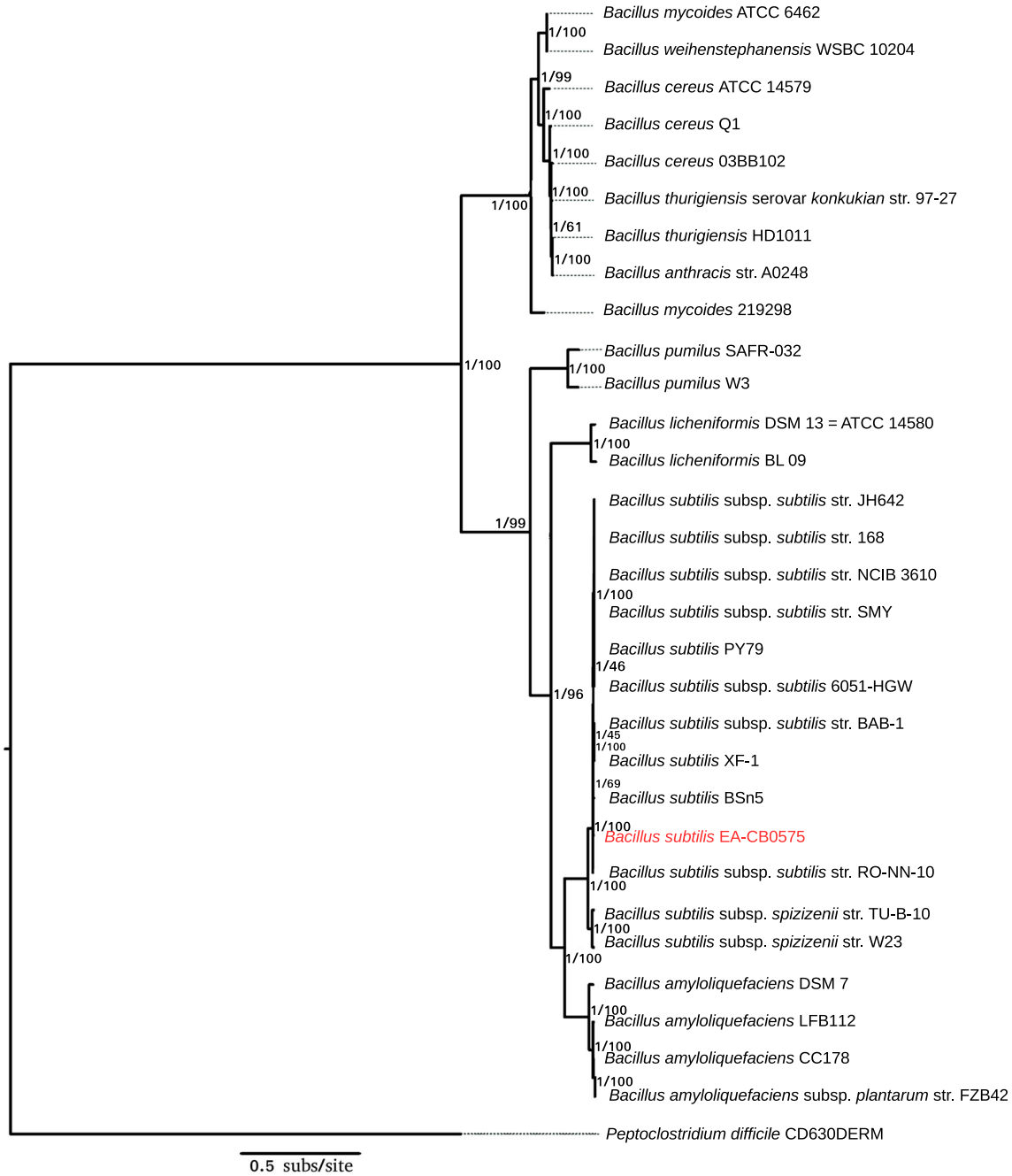


Fig. 3 Phylogeny of *Bacillus* spp. from housekeeping genes (*rpo*B, *gyr*A, *pur*H, *pol*C, and *gro*EL) obtained by Bayesian inference and maximum likelihood methods. The support values are shown for the internal nodes: posterior probability (PP) for BI and percentage of bootstrap (BP) for ML. The study strains are highlighted in red

increase related to control of 48.4% for SDW, when vegetative cells of *B. subtilis* EA-CB0575 were applied; and 54.2% for RDW when spores were inoculated. *B. velezensis* FZB42 did not show increase with respect to control samples without application of the microorganism (Table 5). These results are consistent with assays previously reported, indicating a possible relation between *B. subtilis* EA-CB0575 colonization root potential and plant growth potential for tomato (Posada et al. 2018).

Discussion

The results of the genomic assembly of *B. subtilis* EA-CB0575 are similar to those reported for other genomes of *B. subtilis* strains (Guo et al. 2015; Liu et al. 2018; Rahimi et al. 2018). The genome presented some breaks in the corresponding sections to genes encoding rRNA, tRNAs, non-ribosomal peptide synthases, inserts of phages, and transposases; it could be due to the difficulty for the assembler

Table 4 PGPR biochemical traits at in vitro level for *Bacillus* EA-CB0575 and reference strains *Bacillus* sp.

PGPR trait		*B. subtilis* EA-CB0575	*B. subtilis* 168	*B. velezensis* FZB42
IAA (μg/mL)		15.0 ± 2.0 a	6.9 ± 1.4 b	17.7 ± 1.7 a
Siderophores (μM)		15.4 ± 1.1 a	7.6 ± 0.9 b	9.8 ± 0.3 b
Phosphate solubilization (mg/L)		0.1 ± 0.0 a	0.0 ± 0.0 b	0.1 ± 0.0 a
Nitrogen fixation		+	–	–
LP's production		Surfactins. fengycins	Not reported	Surfactins. fengycins (Fan et al. 2012)
In vitro antagonism	*Ralstonia solanacearum* (cm)	+ (1.2 ± 0.3)	ND	ND
	Fusarium oxysporum	+ (32.6 ± 5.2%)	ND	ND
	Pseudocercospora fijiensis	+ (33.1 ± 4.3%)	ND	ND

Different letters denote significant differences ($P < 0.05$) according to multiple ranges of LSD. + denotes growth in NFb medium indicative of possible nitrogen fixation. − denotes absence of growth in NFb medium. + denotes positive antagonism. − denotes negative results for antagonism test. *Halo of inhibition and percentage of inhibition determined by dual plate assay **Percentage of inhibition determined by ascospores germinative tube inhibition methodology. ***Indoles by Salkowski's colorimetric methodology, siderophores production evaluation by CAS methodology, phosphate solubilization by molybdate blue method using NBRIP medium with phosphoric rock, nitrogen fixation by growth on Nfb medium, and lipopeptides production by HPLC, and mass spectrometry evaluation. *ND*, not determined

to work with repeating sequences (Nederbragt et al. 2010), because the algorithm of graphs does not solve copies separately (Compeau et al. 2011). In addition, regions with modular structure preserved at the sequence level, such as NRPSs, are another cut-off point of the assembly, because of the ambiguity at the ends that is not computationally solved with the available information. Fragmentation of the genome obtained (16 clusters, with an average length of 255,665 bp) is good for genomic-derived analyses. When the obtained number of contigs is compared with the number for other similar projects, in our case, there is a smaller division than for other *Bacillus* (205 partitions for the type strain, and 390 for *B. natto* BEST195, among others) (Kunst et al. 1997; Ulyanova et al. 2016).

When some genes related to PGPR mechanisms were annotated and metabolic predictions were performed, we determined that *B. subtilis* EA-CB0575 genome has genes or enzymes related to the conversion pathway of tryptophan to indole, which is consistent with the determined indole production (15 μg/mL), concentration reported as not deleterious to the plant (Barazani and Friedman 1999). Metabolic prediction was evaluated for siderophores production, finding its metabolic machinery inside the *B. subtilis* EA-CB0575 genome, which was biochemically corroborated with an in vitro production of 15.4 μM. These compounds give to the plant and microorganisms possibilities to improve iron assimilation and are elicitors of systemic resistance in the plant (Aznar and Dellagi 2015). The presence of *nif*U, *nif*S, *nif*V, and *nif*F, all essential for encoding the components of the enzymatic modules of nitrogenase (Li et al. 2016), was determined. It suggests the possibility of the strain to fix environmental nitrogen, which was determined experimentally by *B. subtilis* EA-CB0575 growth on NFb medium. The *phy*C genes, coding for enzymes that sequentially remove phosphates of phytate, were found in the genome of the strain *B. subtilis* EA-CB0575, indicating a possibility for organic phosphate solubilization. It has been reported, about their presence in *B. subtilis* 168 (Idriss et al. 2002). Several genes with potential enzyme activity to fully perform citric acid pathway (TCA cycle) were found in the strain 575 genome, such intermediate acids (e.g., oxalic acid, malic acid, citric acid) could be potentially involved in inorganic phosphate solubilization exhibited by *B. subtilis* EA-CB0575. This was corroborated in biochemical evaluations for strain EA-CB0575, which could grow in medium NBRIP with phosphoric rock and showed positive results using molybdate blue test (Murphy and Riley 1962). The

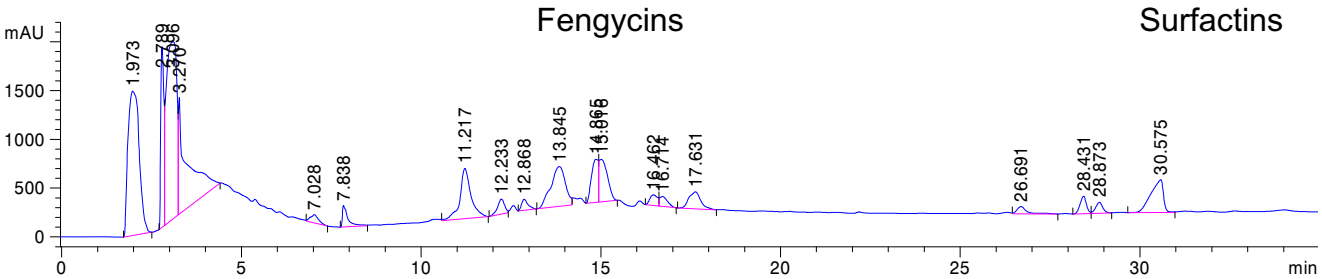


Fig. 4 Lipopeptides analysis production by HPLC chromatogram for SPE-100% methanol extract from *B. subtilis* EA-CB0575 culture in MOLP medium. HPLC operation conditions: 214 nm, injection of 40 μL (concentration 10 mg/mL), flow 1 mL/min, 30 °C, column eclipse XDB C18 250 × 4.6 mm 5 um. A: water HPLC + 0, 1% TFA. B: CH_3CN + 0.1% TFA. Gradient 30/100/100% B in 0/25/35 min

Table 5 Effect of *B. subtilis* EA-CB0575 and reference strains inoculation on the promotion of tomato growth at the greenhouse level in commercial substrate

Treatment	SL (cm)	LN	SDW (g)	RDW (g)	TDW (g)
B. subtilis EA-CB0575 (VC)	13.5 ± 0.5	6.5 ± 0.2	0.38 ± 0.03 a*	0.13 ± 0.01 ab	0.50 ± 0.03 a*
B. subtilis EA-CB0575 (S)	14.1 ± 1.1	5.9 ± 0.4	0.28 ± 0.01 b	0.18 ± 0.01 a*	0.47 ± 0.02 a*
B. subtilis 168 (VC)	14.2 ± 0.6	5 ± 0.8	0.33 ± 0.03 ab	0.14 ± 0.03 ab	0.47 ± 0.02 a*
B. velezensis FZB42 (VC)	12.1 ± 0.6	6.4 ± 0.4	0.26 ± 0.02 b	0.12 ± 0.01 ab	0.37 ± 0.02 b
Control	9.4 ± 1.9	4.8 ± 0.2	0.25 ± 0.02 b	0.11 ± 0.01 b	0.36 ± 0.02 b
LSD test (*P* value)			0.002	0.020	0.001

Data are averages of two independent evaluations in different times each with $n = 8$ per treatment. Different letters correspond to treatments with significant differences according to the LSD test. The asterisks correspond to the treatments with significant differences with the water control by the Dunnett test, both with 95% confidence. *VC*, vegetative cells; *SC*, spore cells; *SL*, shoot length (cm); *LN*, leaves number; *SDW*, shoot dry weight (g); *RDW*, root dry weight (g); *TDW*, total dry weight (g)

type strain 168 did not grow in the medium and did not show positive results for the test, indicating that phosphate solubilization for this strain could be related to organic solubilization processes, using phytates (Ahmad et al. 2017). In addition, it was found that *B. subtilis* EA-CB0575 possesses the machinery to produce 2,3-butanediol and acetoin, but this was not corroborated at in vitro level.

The characterization of regions for coding to NPRSs of the LPs was carried out. *B. velezensis* FZB42 and *B. subtilis* 168 strains were used as the reference. It was determined that *B. subtilis* EA-CB0575 genome has homologous regions to regions that codify for surfactins and fengycins NRPSs. No coding regions were found for iturin production, consistent with experimental results (Villegas-Escobar et al. 2013). LPs production in MOLP medium was evaluated using HPLC and mass spectrometry. We determined the production of surfactins C12 to C16, as well as fengycins A (C14, C15, C16, C17) and B (C16 and C17). These compounds could be responsible for antagonistic activity against evaluated phytopathogens, as reported by several authors (Fan et al. 2018; Villegas-Escobar et al. 2013). We determined the presence of loci for surfactins and fengycins codification for the reference strains, but their in vitro evaluations were not done. Different reports indicate lack of detection for these that compounds at in vitro cultures of the *B. subtilis* 168 as being due to a mutation in the *sfp* gene, which affects the production of surfactins (Chen et al. 2007). Loci to encode NRPS production of iturins was found for FBZ42 strain; and experimental results for reported researches shown in vitro production of the three LP families announced (Koumoutsi et al. 2004).

B. subtilis EA-CB0575 has more elements inserted in the genome than the reference strains, possibly because of their wild character and the domestication of the reference strain 168 (Veening et al. 2006). In addition, 3.9% of its genome is associated with secondary metabolism. However, its potential is related not only to the number of genes involved but also to the levels being expressed by these genes. We suggest that transcriptomic analyses should be performed in order to know this. It was determined that many of the gene clusters that are present in the reference strain *B. amyloliquefaciens* FZB42, active principle of Biomex® and Rhizovital®42, are present in *B. subtilis* EA-CB0575; this makes this strain promissory for bioproducts development. The topologies of the phylogenetic trees obtained were coincident and have high values of support in most branches (BP, > 75; PP, > 0.95). It was determined that the offered resolution by trees based on the 16S gene is not enough to differentiate the strains in the *B. amyloliquefaciens*, *B. subtilis*, and *B. licheniformis* groups, probably because of the high similarity in 16s rDNA sequences (Rooney et al. 2009). Two clades were found: one for *B. thuringiensis*, *B. anthracis*, *B. cereus*, *B. mycoides*, and *B. weihenstephanensis*, considered pathogens of several hosts; and a second clade formed by *B. pumilus*, *B. licheniformis*, *B. subtilis*, *B. velezensis*, and *B. amyloliquefaciens*, all present in the soil. *B. amyloliquefaciens*, *B. velezensis*, and *B. subtilis* differ in well-resolved siblings clades and independent of *B. licheniformis* clade, which did not happen in phylogeny inferred with information from the 16S ribosomal gene (Fig. S4).

We could determine the PGPR potential of *B. subtilis* EA-CB0575 and *B. subtilis* 168 strains in tomato plants. We evaluated this potential for *B. subtilis* EA-CB075 in others crops as banana (Posada et al. 2016), and we established a potential colonization capacity on tomato and banana roots using FISH and CARD-FISH methods (Posada et al. 2018). These results are related with bacterial genomic and biochemical potential evaluated in this research. Better performance of *B. subtilis* microorganisms than *B. velezensis* to promote plant TDW was found with percentages from 25.40 to 34.60%. *B. velezensis* did not show differences with control; however, this strain has been reported as a growth promoter through indirect promotion mechanisms such as biocontrol (Chowdhury et al. 2015).

Conclusion

B. subtilis EA-CB0575 is a potential PGPR due to its genomic and phenotypic capacity related to the mechanisms of plant growth promotion. Its genome was assembled in this project in 16 contigs, and genes related to plant growth promotion mechanisms were annotated and analyzed. It was found genes related to IAA, siderophores, acetoin, 2,3-butanediol and LPs production, nitrogen fixation, phosphate solubilization and antagonistic activity against phytopathogens. The biochemical test allowed to determine the possibility of the microorganism for the production of LPs of surfactin families (surfacins C12, C13, C14, C15, and C16), fengycins A (C14, C15, C16, C17), and fengycins B (C16 and C17). Also, we corroborate its possibility for IAA and siderophores production, to solubilize phosphates and fix environmental nitrogen. It was determined that the microorganism has 3.9% of its genome dedicated to the production of secondary metabolites and belongs to *B. subtilis subtilis* clade. In addition, *B. subtilis* EA-CB0575 and its type strains promote TDW of the tomato plant.

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