First high-quality draft genome of *Ochrobactrum haematophilum* P6BS-III, a highly glyphosate-tolerant strain isolated from agricultural soil in Argentina

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Abstract

We report here on a high-quality draft genome sequence of *Ochrobactrum haematophilum* strain P6BS-III (DSM 106071), a Gram negative, non-sporulating bacterium isolated from a pastureland (Buenos Aires province, Argentina) which had been chronically exposed to the herbicide glyphosate. The genome of 5.25 Mb with a DNA G+C content of 56.63% size was estimated to contain 5,291 protein coding genes and 57 RNA genes. Genome analysis revealed the presence of the *phn* operon, which is involved in the phosphonate degradation pathway, and a class II 5-enolpyruvylshikimate-3-phosphate synthase (EPSP) that confers tolerance to glyphosate. Genes related to plant growth promotion traits are also present, and include genes for phosphorus metabolism, calcium phosphate and phytate solubilization, siderophore production, organic acid biosynthesis and indole acetic acid (IAA) production.

Keywords Glyphosate · *Ochrobactrum haematophilum* · 5-Enolpyruvylshikimate-3-phosphate synthase · Soil contamination · Plant growth promotion

Abbreviations

EPSP 5-Enolpyruvylshikimate-3-phosphate
LB Luria–Bertani
COG Clusters of orthologous genes

Introduction

Glyphosate (*N*-phosphonomethyl glycine) is the most widely used herbicide in the world due to its broad spectrum weed control properties, and the extended use of genetically modified crops carrying the glyphosate-insensitive EPSP synthase of *Agrobacterium tumefaciens* CP4 (Green 2016). Increasing in popularity over the last 30 years, these genetically modified crops allow farmers to use glyphosate in combination with strategies such as zero tillage agriculture to increase land productivity. Increased glyphosate use has led to concerns about environmental and human health risks, illustrated by the World Health Organization's International Agency for Research on Cancer report on potential human carcinogenic effects (Guyton et al. 2015). Glyphosate removal from the environment is needed to reduce exposure to non-target organisms via contaminated soil and drinking water. In contrast to conventional dig and dump approaches, phytoremediation is a sustainable, low-cost remediation technology for removing herbicide residues from soil, while preserving soil texture and garnering public acceptance (Vangronsveld et al. 2009).

During our research, we isolated bacteria from pasturelands chronically exposed to glyphosate in the Humid

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Pampa, the main agricultural region in Argentina, with the aim of finding suitable bacterial strains to be employed as bioinoculants for plant–bacteria remediation systems. Among the isolated strains, *Ochrobactrum haematophilum* P6BS-III, a Gram-negative member of the Brucellaceae family, exhibited high tolerance to glyphosate and was able to grow using glyphosate as the sole source of phosphorus. The *Ochrobactrum* genus has been studied for its capacity to degrade a range of organic compounds including nicotine, glyphosate and chlorpyrifos (Ermakova et al. 2008; Yu et al. 2014; Abraham and Silambarasan 2016). Until now, complementary genome studies focusing both on contaminant tolerance and plant growth promoting abilities have not been conducted for this genus.

Materials and methods

Strain origin, isolation and identification

O. haematophilum P6BS-III was isolated from a grassland chronically exposed to glyphosate in Chascomús county, Argentina (35°44'39.26"S 58° 3'27.33"W). Bulk soil samples were homogenized and added at 10% (w/v) to a minimum medium amended with 500 mg L⁻¹ of glyphosate as the sole added source of phosphorus and incubated at 25 °C under agitation between 4 and 6 days. After sub-culturing at 10% (v/v) into fresh liquid medium six times, colonies were isolated on solid medium, and isolate purity was verified on rich media. P6BS-III was able to grow in Reasoner's 2A agar (R2A), Luria–Bertani (LB) and *Rhizobium* selective media (Graham 1969). Growth was tested at temperatures ranging from 25 to 37 °C, in solid and liquid cultures at pH ranging from 6.8 to 7.0. A portion of the 16S rRNA gene was amplified and sequenced to assign a species name, using the universal bacterial primers 27f (5'-AGAGTTTGATC-MTGGCTCAG-3') and 1492r (5'-TACGGYTACCTTGTTACGACTT-3') using PCR conditions previously described (Thijs et al. 2014).

Genomic DNA preparation, genome sequencing and assembly

RNA-free genomic DNA was isolated using the Invitrogen PureLink Genomic DNA Mini Kit (Life Technologies Inc., Burlington, ON) from stationary phase cells grown in LB broth prior to enzymatic digestion, and ligation of sequencing adapters and barcodes using an Ion Xpress Plus Fragment Library Kit (Life Technologies Inc., Burlington, ON). Adapter-ligated DNA was size selected to 480 bp on a 2% E-Gel SizeSelect agarose gel, and Agencourt MAPure XP beads (Beckman Coulter, Mississauga, ON) were used for purification. The library dilution factor was determined

using an Ion Library Quantitation Kit prior to amplification and enrichment with an Ion PGM Template Hi-Q OT2 400 kit on an Ion OneTouch 2 system. The enriched ion sphere particles were quantified using an Ion Sphere Quality Control Kit prior to sequencing.

Sequencing was performed on a 316v2 chip with an Ion PGM Hi-Q View Sequencing Kit on an Ion Torrent PGM (Life Technologies Inc., Carlsbad, CA). A total of 1.1 million reads (mean length 308 bases) generated 345 Mb of data (> 324 M Q20 bases) in Torrent Suite 5.0.4. These were assembled using SPAdes 3.8.2 (uniform coverage mode; kmers 21, 33, 55, 77, 99) into 65 contigs greater than 1000 bp, giving a consensus length of 5,253,115 bp at 61.7 coverage (GC content 56.63%; largest contig 445 701 bp; N50 = 242 240 bp) (Bankevich et al. 2012).

Genome annotation and pangenome analysis

Genome annotation was performed using the RAST annotation system (Overbeek et al. 2014), NCBI Prokaryotic Genome Automatic Annotation Pipeline (PGAP) (Tatusova et al. 2016), and the MicroScope platform using the Magnifying Genomes, MaGe tool (Vallenet et al. 2006). Clusters of orthologous genes (COG) and metabolic pathway reconstruction were carried out using the MaGe tools KEGG, MetaCyc and BioCyc. Homologs of genes of interest were defined based on an *e* value cutoff < 0.0001, > 20% query coverage and > 50% sequence similarity (Pearson 2013). Genes in internal clusters were assigned using CD-HIT Suite v4.6.8 (Huang et al. 2010), genes with functional predictions assigned were found using EggNOG 4.5.1 (Huerta-Cepas et al. 2016), genes with a Pfam domain were assigned using the HMMER server (Finn et al. 2015), genes with signal peptides were assigned using the SignalP 4.1 server (Petersen et al. 2011), and genes with transmembrane helices were predicted using the TMHMM Server v. 2.0 (Krogh et al. 2001). No CRISPR repeats were found according to the RAST annotation system. The PHASTER software (Arndt et al. 2016) was used for phage and prophage searches. To find the closest genomes to P6BS-III, whole genome sequence comparisons were performed using the JSpecies Web server (Richter et al. 2015) and the Genome-to-Genome Distance Calculator Web server (GGDC) (Meier-Kolthoff et al. 2013). Tetra Correlation Search was used to get a list of the closest reference genomes to P6BS-III, and then Average Nucleic Identity based on Blast + (ANiB) (Goris et al. 2007), Average Nucleic Identity based on MUMmer (ANIm) (Kurtz et al. 2004), and GGDC 2.1 (Meier-Kolthoff et al. 2013) were run with the 16 closest candidates. Pan-genome analysis of all 24 species within the genus *Ochrobactrum* available on the NCBI Microbial Genome Database (up to 06-10-2017) was performed by using the Bacterial Pan Genome Analysis (BPGA) pipeline (Chaudhari et al. 2016),

employing the Usearch (Edgar 2010) clustering algorithm with a sequence identity clustering cutoff of 50%. Plots were made with gnuplot 4.6.6 (Williams and Kelley 2011).

Traits related to glyphosate tolerance and plant growth promotion

Tolerance to glyphosate was assessed in *Ochrobactrum haematophilum* P6BS-III with a method proposed by The Clinical and Laboratory Standards Institute, which was originally designed to test response to antibiotics (Cockerill et al. 2012). For this, P6BS-III was grown at glyphosate concentrations of 0, 100, 500, 1000, 2000, 3000, 5000, 7500 and 10,000 mg kg⁻¹ in M9 minimal salts medium to cell densities between 1×10^8 and 2×10^8 CFU mL⁻¹. Then, 2 µL of the cell suspensions, with approximately 1×10^4 CFU, was spotted onto the surface of M9 solid medium. Five replicates were prepared for each glyphosate concentration. Plates were incubated at 25 °C for 6 days, and end points were defined as the glyphosate concentration at which colony growth was inhibited by more than 50% compared to the colonies on control plates (M9 without glyphosate). As a positive control, *Escherichia coli*, which is naturally sensitive to glyphosate, was used.

The qualitative production of IAA was tested according to Patten and Glick (2002). Inorganic phosphate, Ca₃(PO₄)₂, solubilization was tested following the protocol described by Nautiyal (1999). Phytate mineralization was determined as per Jorquera and collaborators (2008). The qualitative assessment of siderophore production was based on the method described by Schwyn and Neilands (1987).

Biochemical and chemotaxonomic information

The metabolic profile of *Ochrobactrum haematophilum* P6BS-III was carried out using GN2 MicroPlate™ (Biolog, Hayward, USA) plates. Each plate provides 95 different biochemical tests for Gram-negative microorganisms. Gas chromatographic analysis of total fatty acid methyl esters, FAME (EMSL, Cinnaminson, NJ), was used to characterize the fatty acid profile of P6BS-III.

Results and discussion

Genome properties

The 5,253,115 bp genome of *Ochrobactrum haematophilum* P6BS-III has a GC-content of 56.63%, with 5526 genes, 5291 protein coding genes (5204 with function prediction), 4 rRNAs, 49 tRNAs, and 4 ncRNAs (Fig. 2a). Other metadata for P6BS-III is shown in Table 1. To determine its closest relative, a phylogenetic tree was constructed based on the

Table 1 MixS data

Item name	Description
Investigation type	Bacteria
Project name	<i>Ochrobactrum</i> sp. P6BS-III
Geographic location (latitude and longitude)	35°44'39.26"S, 58° 3'27.33"W
Geographic location (country and/or sea, region)	Argentina: Buenos Aires
Collection date	19/08/2014
Environment (biome)	Area of pastureland (ENVO:01000891)
Environment (feature)	Agricultural feature (ENVO:00000077)
Environment (material)	Contaminated soil (ENVO:00002116)
Soil depth	0.33 m
Sequencing method	Ion Torrent PGM

complete 16S rRNA gene sequence of P6BS-III and type strains of the genus *Ochrobactrum* (Fig. 1).

Strain P6BS-III has 231 pseudo genes, 81 genes in internal clusters, 4587 genes assigned to COGs (Fig. 2b), 4973 genes with Pfam domains, 443 genes with signal peptides, and 1,201 genes with transmembrane helices. The PHASTER software identified one intact prophage region (58 kb) and a second putative prophage region. This finding is in accordance with Jäckel and collaborators (2017), who reported a low number of prophages for some *Ochrobactrum* species.

Genes related to glyphosate tolerance and degradation

Genome analysis revealed a single copy of the class II enzyme EPSP synthase sequence (EC 2.5.1.19), previously proposed to confer high glyphosate tolerance in microorganisms. Phylogenetic analysis showed that the P6BS-III EPSP synthase is closely related to the one carried by *Agrobacterium* sp. CP4 (Fig. 3). Two regions well conserved in the class II EPSPS enzymes, RXHTE and NPTR (Yi et al. 2016), are present in EPSP synthase of P6BS-III. Multiple sequence alignment indicates that the amino acid substitution P101L, which causes structural changes of the enzyme active site, impacting the spatial orientation of amino acids G96 and T97 toward glyphosate (Healy-Fried et al. 2007), is present in the P6BS-III genome. This result is consistent with tolerance evaluation assays carried out on this strain, in which no growth inhibition was observed until 10,000 mg L⁻¹.

The glycine oxidase (ThiO) gene (EC 1.4.3.19) was also found in the P6BS-III genome. ThiO can catalyze glyphosate oxidation, although its affinity for this substrate is low in comparison with normal physiological substrates, mainly primary and secondary amines (Pollegioni et al. 2011).

Fig. 1 Phylogenetic tree based on 16S rRNA gene sequences showing *Ochrobactrum haematophilum* P6BS-III (indicated with a red dot) in relation to a close strain set, calculated with *Campylobacter jejuni* NCTC 11,351 (AF372091) as outgroup (excluded from the figure). Sequences were aligned using INFERNAL (v1.1rc4) in the

Ribosomal Database Project, and phylogenetic analysis based on the maximum likelihood method was performed in MEGA7. Confidence levels for individual branches were determined by bootstrap analysis with 1000 replicates. (T) indicates type strain. In the scale-bar, 1 cm is equal to 0.01 nucleotide substitutions per site

Fig. 2 **a** Circular genome of *Ochrobactrum haematophilum* P6BS-III. The circles show (from the outside to the inside): percentage of GC deviation; clockwise predicted coding sequences; counterclockwise

predicted coding sequences; GC skew; and in the inner circle, rRNA (blue), tRNA (green), miscRNA (orange), transposon-related elements (pink) and pseudogenes (grey). **b** COG functional categories

Fig. 3 Phylogenetic tree of the EPSP synthase protein sequences. Class I sequences were obtained from: *Arabidopsis thaliana* (P05466), *Brassica napus* (P17688), *Petunia hybrida* (P11043), *Solanum lycopersicum* (P10748), *Bordetella pertussis* (P12421), *Aeromonas salmonicida* (Q03321), *Rahnella aquatilis* (C9EGX2), *Escherichia coli* (P0A6D3), *Salmonella typhimurium* (P07637), *Pseudomonas fluorescens* (A1Z0H6), *Pseudomonas putida* (Q683S1), *Janibacter limosus* (L7PA93). Class II sequences were obtained from: *Bacillus subtilis* (P20691), *Streptococcus pneumoniae*

(Q9S400), *Staphylococcus aureus* (Q05615), *Dichelobacter nodosus* (Q46550), *Halovibrio variabilis* (Q1H624), *Agrobacterium sp.* CP4 (Q9R4E4), *Achromobacter sp.* LBAA (P0A2Y5), *Ochrobactrum anthropi* (A6WUV8). P6BS-III is indicated with a red dot. The multiple alignment of amino acid sequences was performed using Muscle, and the phylogenetic tree was obtained using the maximum likelihood method with 1000 bootstrap resamples in MEGA 7. Bar, 2 cm is equal to 0.2 substitutions per site

Homologs of the *phn* operon associated with the breakdown of a wide range of phosphonates, including glyphosate (via the C–P lyase pathway), revealed the putative presence of the *phnGHIJKLMNCDEE* transcriptional unit, as well as the presence of a *phnM*-domain of unknown function. Overall, glyphosate metabolism in P6BS-III is suggested to occur via the *phn* operon and glyphosate oxidase pathways, since no additional phosphorus source was added in seven successive sub-cultures.

Phosphate acquisition and plant growth promoting traits

Analysis of the *Ochrobactrum haematophilum* P6BS-III genome unveiled several genes involved in phosphorus metabolism, including genes for nine high-affinity phosphate transporters, PHO regulon control genes, 26 genes related to phosphate metabolism, 3 related to polyphosphate utilization, and 17 to alkylphosphonate use (including the *phn*

operon). Strain P6BS-III possesses genes related to calcium phosphate and phytate solubilization, including 4-phytase, and the *pqqABCDE* operon that is involved in gluconic acid synthesis (An and Moe 2016).

Iron uptake by P6BS-III is probably carried out through transporters coded by the ferric iron ABC transporter genes *pitA*, *pitD*, *pitC*, and a low pH-inducible system. Genes involved in the synthesis of the siderophore aerobactin were found, although genes for L-lysine N-(6)-monooxygenase (NADPH) and N6-hydroxylysine O-acetyltransferase were missing. IAA production is probably carried out via tryptophan-dependent biosynthesis, since homologs involved in amino acid synthesis have been found along with homologs of the aldehyde dehydrogenase, *aldA*, responsible for the oxidation of indole-3-acetaldehyde to indole-3-acetic acid.

Whole genome sequence comparison

To find the closest genomes to P6BS-III, a whole genome sequence comparison was performed (Table 2). The genome for P6BS-III showed a digital DNA–DNA hybridization, ANIb and ANIm estimate of 39.2%, 89.05% and 90.27%, respectively, when compared to the other *Ochrobactrum* species (highest score, *O. haematophilum* FI11154), indicating a poor correlation at this taxonomic level.

The pan-genome of 24 *Ochrobactrum* species is composed of 13,194 orthologous gene families, while the core genome is composed of 429 orthologous gene families. The trend line of the pan-genome Core-Pan plot does not reach a plateau, indicating that future *Ochrobactrum* genomes will contribute to the genomic characterization of the *Ochrobactrum* genus (Fig. 4). The *O. haematophilum* P6BS-III genome itself contributes 744 unique genes.

Biochemical and chemotaxonomic information

As determined using the BIOLOG GN2 assay, strain P6BS-III can oxidize or utilize a variety of carbohydrates, esters, alcohols and amino acids, such as *N*-acetyl-D-galactosamine, *N*-acetyl-D-glucosamine, adonitol, L-arabinose, D-arabitol, D-cellobiose, D-fructose, L-fucose, D-galactose, gentiobiose, α-D-glucose, m-inositol, maltose, D-mannitol, D-mannose, β-methyl-D-glucoside, D-psicose, L-rhamnose, D-sorbitol, sucrose, D-trehalose, turanose, xylitol, methyl pyruvate, 2,3-butanediol, glycerol, D-alanine, L-alanine, L-asparagine, L-aspartic acid, L-glutamic acid, glycyl-L-aspartic acid, glycyl-L-glutamic acid, L-histidine, hydroxy-L-proline, L-leucine, L-ornithine, L-proline, L-serine, L-threonine, D, L-carnitine and γ-aminobutyric acid.

GC analysis of total fatty acid methyl esters from P6BS-III consisted of the following saturated fatty acids (%): C14:0 (0.29), C16:0 (9.09); C17:0 (1.41), C18:0 (9.32) and C20:0 (0.23); unsaturated fatty acids: C15:1 ω5c (0.19); C16:1 ω7c/C16:1 ω6c (2.32); C17:1 ω8c (0.19); C17:1 ω6c (0.30); C18:2 ω6,9c (0.23); C18:1 ω7c (62.99); C18:1 ω5c (0.17), C20:1 ω7c (0.21); and cyclopropanoid fatty acids: C19:0 cyclo ω8c (13.06). The FAME profile matched, with 62.8% similarity, to *Ochrobactrum anthropi* in the ITSA1 1.00 database. Comparative analysis revealed it was not significantly different from previously described fatty acid profiles for other *Ochrobactrum* species (Kampfer et al. 2007).

Table 2 Genome-to-genome comparison of *Ochrobactrum haematophilum* P6BS-II with known related strains or type strains

Strain ID	BioProject	Size (Mb)	GC %	ANIb (%)	ANIm (%)	GGDC (%)
<i>O. haematophilum</i> L1Suc	PRJNA329823	4.91	59.1	84.49	86.91	29.8
<i>O. haematophilum</i> FI11154	PRJEB23545	5.48	57.0	89.05	90.27	39.2
<i>O. anthropi</i> OAB	PRJNA244571	4.90	56.1	80.05	85.90	24.7
<i>O. anthropi</i> ATCC 49,188	PRJNA19485	5.21	56.2	80.03	85.94	24.7
<i>O. sp.</i> UNC390CL2T	PRJNA234825	4.62	56	80.18	85.82	24.6
<i>O. anthropi</i> ML7	PRJNA274439	4.90	56	80.19	85.81	24.7
<i>O. anthropi</i> CTS-325	PRJNA85133	4.73	56	80.17	85.82	24.7
<i>O. anthropi</i> 60a	PRJNA169004	4.59	56.1	80.46	86.15	25.7
<i>O. anthropi</i> W13P3	PRJNA238403	5.28	56.3	80.05	85.86	24.7
<i>O. sp.</i> EGD-AQ16	PRJNA215965	4.83	57.6	80.39	85.75	25.0
<i>O. intermedium</i> 2745-2	PRJNA233556	4.80	57.6	80.31	85.55	24.8
<i>O. intermedium</i> LMG 3301	PRJNA37725	4.73	57.7	80.27	85.58	24.8
<i>O. intermedium</i> M86	PRJNA178498	5.19	57.9	80.17	85.58	24.7
<i>B. inopinata</i> BO1	PRJNA41855	3.37	57.1	80.16	85.03	24.3
<i>B. suis</i> bv. 4 str.40	PRJNA34745	3.31	57.2	80.27	85.04	24.0
<i>Brucella</i> sp. BO2	PRJNA41857	3.31	57.2	80.21	84.99	24.1

Fig. 4 Pan-genome plot of *Ochrobactrum* species

Conclusions

We described the whole genome sequence of *Ochrobactrum haematophilum* P6BS-III. The outstanding tolerance to glyphosate, combined with expression of plant growth promotion traits, makes this strain a potential inoculant of agronomically valuable plant species, and in phytoremediation experiments to enhance glyphosate removal from agricultural and other polluted soils. Pan-genome analysis of P6BS-III has contributed to a better understanding of the genus *Ochrobactrum*.

Culture collection numbers and sequence data accession numbers

Ochrobactrum haematophilum strain P6BS-III has been deposited in the Leibniz Institute culture collection under culture collection number DSM 106071, in the UHAS culture collection under UHS000020 and in the Laboratorio de Malezas y Herbicidas under LMH00001.

The genome sequencing project has been deposited at DDBJ/EMBL/GenBank under Accession Number MPPJ00000000 and BioProject PRJNA354074. The version described in this paper is the first version, MPPJ00000000.1.

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