Draft genome sequence of *Bacillus subtilis* strain S2794, an isolate useful for microbial control

Projeto de sequência genómica de *Bacillus subtilis* strain S2794, um isolado útil para o controlo microbiano

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ABSTRACT
The pathogenic microorganisms affecting agriculture and chronic threats to sustainable food production and ecosystem stability worldwide. One of the promising microorganisms for sustainable agriculture is Bacillus subtilis, which has been reported as a growth promoter and as antagonistic to a variety of pathogens in vitro and greenhouse and field studies. This work describes the draft genome sequence of the B. subtilis S2794 which contains several specific singletons including genes showing inhibitory activity against numerous plant fungal pathogens. The selective bioassays indicated that the species has pathogenic potential for insects of the order Lepidoptera, such as H. armigera and C. includens, in addition to the phytopathogen S. sclerotiorum. The S2794 strain was resistant to the antibiotics ampicillin, penicillin, sulfamethoxazole, and polymyxin B.

Keywords: surfactin, fungal plant pathogen, growth and biofilm formation.

RESUMO
Os microrganismos patogénicos que afectam a agricultura e as ameaças crónicas à produção alimentar sustentável e à estabilidade do ecossistema em todo o mundo. Um dos microrganismos promissores para a agricultura sustentável é o Bacillus subtilis, que tem sido relatado como promotor de crescimento e como antagonista de uma variedade de patogénicos in vitro e estudos de estufa e de campo. Este trabalho descreve o projecto de sequência genómica do B. subtilis S2794 que contém vários singletons específicos, incluindo genes que mostram actividade inibitória contra numerosos agentes patogénicos fúngicos vegetais. Os bioensaios selectivos indicaram que a espécie tem potencial patogénico para insectos da ordem Lepidoptera, tais como H. armigera e C. incluindo, para além do fitopatógeno S. sclerotiorum. A estirpe S2794 era resistente aos antibióticos ampicilina, penicilina, sulfametoxazol, e polimixina B.

Palavras-chave: surfactina, patogénico de plantas fúngicas, crescimento e formação de biofilme.

1 INTRODUCTION

Bacillus subtilis is the most studied biocontrol agent that is widely used in sustainable agriculture. Different *B. subtilis* strains have demonstrated antagonistic activities were rhizosphere or endophytic bacteria or rhizobacteria or *in vitro* assays (1). Many other strains have the potential to control different diseases caused by rhizobacterial or fungal pathogens in greenhouses and fields (2).

Antibiotic production has been postulated to play a principal role in disease suppression by microorganisms including *B. subtilis*. More than two dozen antibiotics with wide spectra and diverse structures have been reported from *B. subtilis* (3).

The species of *B. subtilis* also can produce biofilm, a self-produced extracellular matrix that encapsulates cells and facilitates their attachment to surfaces. This mechanism is widely used for numerous biotechnological or biomedical purposes (4).

2 METHODOLOGY

2.1 ISOLATION AND MORPHOLOGICAL CHARACTERIZATION OF THE STRAIN

The bacterial species were isolated from soil in the Minas Gerais, Unaí in 2019. The strain isolation process was carried out according to the methodology established by Praça (5). For the analysis of colony morphology and bacterial strain cytomorphology, the principles of Bergey's Manual of Systematic Bacteriology were followed (6). The morphological properties were observed in a scanning electron microscope, model DSM 962-Zeiss, and transmission electron microscope, model JEM-2100-Jeol.

2.2 GENOME SEQUENCING

Purified genomic and plasmid DNA`s from strain S2794 were extracted by Masterpure Gram-positive DNA purification kit (Epicentre) and QIAGEN Plasmid Maxi Kit (QiAGEN). Afterward, these DNA`s were sequenced at Macrogen, Inc. (Seoul, Korea) using high-throughput HiSeq2000 and GS-FLX Plus platforms getting one lane of 100 bp and 1/8 region plate, respectively. Unicycler pipeline (7) was used for reads assemble, which were annotated with prokka (8). Average nucleotide identity (ANI) was applied for species taxonomic confirmation (9).

2.3 ANTIMICROBIAL RESISTANCE PROFILE

The susceptibility of the strain S2794 to following antibiotics penicillin G, clindamycin, erythromycin, vancomycin, rifampicin, cefazolin, ceftriaxone, chloramphenicol, ampicillin, oxacillin, tetracycline, lincomycin, clarithromycin, polymyxin B, chlortetracycline, sulfamethoxazole, amikacin, clotrimazole, cloxacillin monohydrate, kanamycin, levofloxacin, and streptomycin were tested. The antimicrobial resistance profile was determined by the agar dilution test following the procedures described by the Clinical and Laboratory Standards Institute. A log-phase culture of the

strain was diluted to a concentration of approximately 1×10^8 to 2×10^8 CFU/ml (McFarland standard 0.5). The concentrations determined for the assays were 30, 70, 110, 150, 200, 250, and 300 µg/ml. The solutions were individually added to 50 ml of Embrapa-agar medium in the liquefied state, at a temperature below 60 °C, to avoid antibiotic degradation. The experimental design was completely randomized, with three replications per concentration. The evaluation was performed after 24 hours of incubation at 30 °C. The evaluation was done verifying whether there was an inhibition of bacterial growth or not, due to the action of the imposed antibiotic.

2.4 INSECT AND NEMATODE SELECTIVE BIOASSAYS

All insects used in the tests came from the Plant and Insect Breeding Platform (PCPI) of Embrapa Genetic Resources and Biotechnology. In all cases, individuals were raised on an artificial diet and under controlled temperature and photoperiod conditions, following the methodologies described in the literature (10-11).

Selective bioassays for *Spodoptera frugiperda*, *Helicoverpa armigera*, *Chrysodeixis includens*, and *Aedes aegypti* were performed as described in published works (12-13). Bioassays for *Anthonomus grandis* were performed according to the methodology described by Figueras (14). For *Caenorhabditis elegans*, bioassays were performed as described by Montalvão (15).

The correction of mortality observed in larvae of *S. frugiperda*, *H. armigera*, *C. includens*, *A. grandis*, *A. aegypti*, and in the nematode species *C. elegans* by an isolate of S2794 was performed through the equation below proposed by Henderson (16):

$$\text{Corrected \%} = \left(1 - \frac{\text{n in Co before treatment} * \text{n in T after treatment}}{\text{n in Co after treatment} * \text{n in Co after treatment}}\right) * 100$$

Where: n = Insect population, T- treated, Co = control

2.5 ANTAGONISM IN *F. OXYSPORUM* F. SP. *VASINFECTUM* AND *SCLEROTINIA SCLEROTIORUM*

The evaluation of the antagonism of the S2794 strain to *F. oxysporum* f. sp. *vasinfectum* and *Sclerotinia sclerotiorum* was performed by confrontation, with the adoption of the method of pairing cultures in Petri dishes, according to previous published

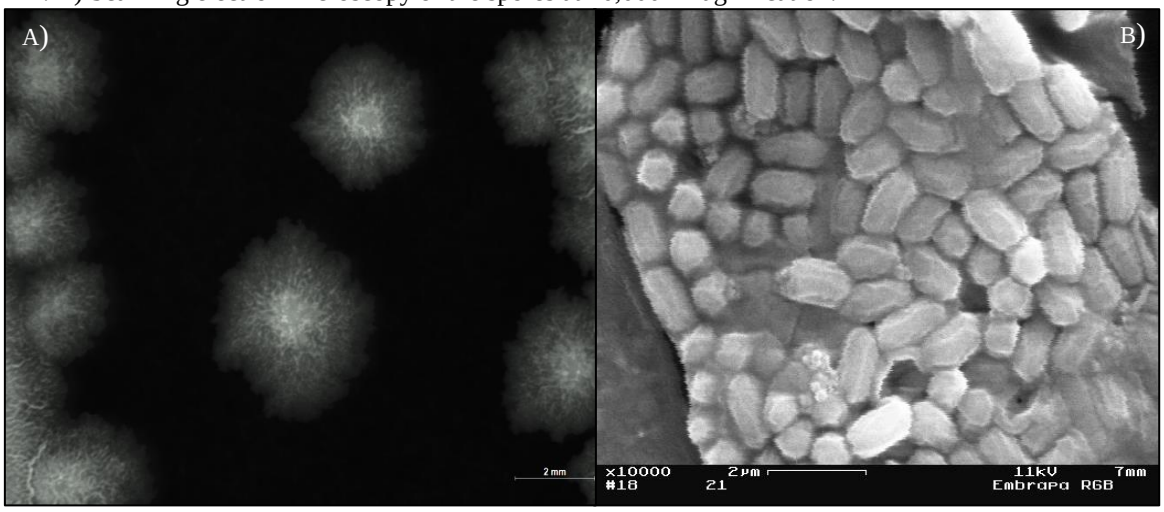
works (17-18), with adaptations, in which the selected culture medium was selected only the PDA 50% + EM 50% culture medium.

3 RESULTS

3.1 MORPHOLOGY

The visualized cells of the strain 2794 in the electron microscopy were Gram-positive, anaerobic, motile, rod-shaped, 0.7 to 0.9 µm wide, and 1 to 2 µm long. Spores were cylindrical, central in sporangia that were not swollen. Colonies were white, irregular, with lobulated margins, with a dry, grainy consistency, and a rough, opaque surface (Figure 1). The ideal temperature for growth was 30 °C.

Figure 1. A) Colony morphology of the strain S2794 by electron transmission microscopy at a scale of 2 mm. B) Scanning electron microscopy of the spores at 10,000x magnification.

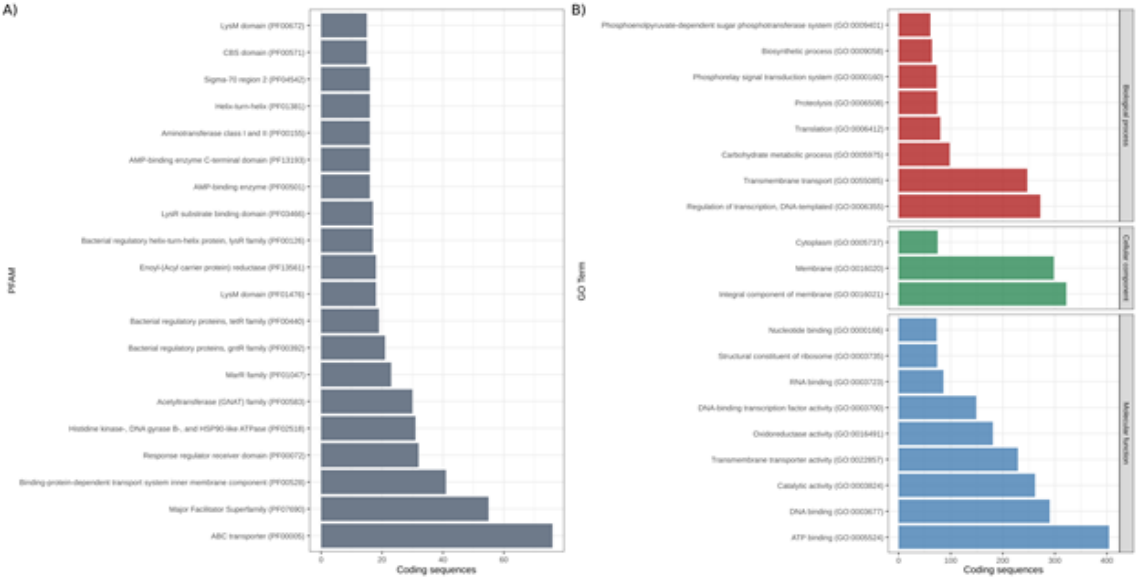


Here we present the draft genome of *Bacillus subtilis* strain S2794 with 4,051,450 bp size organized into 32 scaffolds, maximum scaffold size of 1,557,753 bp, average sequence size of 126,607 pb, N_{50} length of 1,039,968 pb and 43,52% of GC content. A total of 4,209 CDSs, 71 tRNAs, 3 rRNAs, 1 tmRNA and 89 lnRNAs were found by prokka. S2794 presents an ANI value of 98,47% with *B. subtilis* strain 168.

Functional annotation was done with interproscan 5.54-87.0 (PMID: 24451626). 3,606 (85,67%) and 2,495 (59,27%) coding sequences have PFAM or GO functional terms. “ABC transporter” (PF00005), “Major facilitator superfamily” (PF07690), and “Binding-protein-dependent transport system inner membrane component (PF00528) are the most frequent PFAM IDs in S2794 coding sequences (76, 55 and 41 CDSs each). Gene ontology annotation analysis revealed that the most frequent Biological Process GO terms are “Regulation of transcription, DNA-templated” (GO:0006355),

"Transmembrane transport" (GO:0055085), and "Carbohydrate metabolic process" (GO: 0005975), with 272, 247 and 98 CDSs each. "Integral component of membrane" (GO:0016021), "Membrane" (GO:0016020), and "Cytoplasm" (GO:0005737) are the top three Cellular Component GO terms, with 322, 298 and 75 CDSs. Molecular Function top terms are "ATP binding" (GO:0005524), with 404 CDSs, "DNA binding" (GO:0003677) with 290 and "Catalytic activity" (GO:0003824), present in 262 CDSs (Figure 2).

Figure 2. Functional annotation of the genes identified of the *B. subtilis* strain S2794 by interproscan (A) and GO terms (B).



The custom genes database of the strain S2794 showed the potential biocontrol activity of these bacteria is notably due to their potential to produce multiple antimicrobial compounds that have been reported for their inhibitory activity against numerous plant pathogens such as Surfactin (srfA), Lipoteichoic acid (LTA) synthase (ltaS), Alkyl hydroperoxide reductase (ahpC), Growth and biofilm formation (tyrZ), Surfactin synthesis (tapA), Anti-sigma factor (FlgM), Polyketide production (Sfp), flagellin (Hag), Biofilm determinant (ypqP), Bacilysin (bacA) and Fengycin (fenB).

3.2 ANTIMICROBIAL RESISTANCE PROFILE

Strain S2794 was resistant to polymyxin at a minimum inhibitory concentration (MIC) of 110 µg/ml following penicillin (70 µg/ml), sulfamethoxazole (70 µg/ml) and ampicillin (30 µg/ml). The analysis of the other antibiotics, the strain 2794 was susceptible to all (Table 1).

In the genome analysis, it was possible to detect in the isolate S2794 the presence of the *tetD* resistance gene corresponding to the antibiotic chlortetracycline. However, this gene was not expressed in the strain since it was susceptible to this antimicrobial.

Table 1. Antimicrobial resistance profile of the S2794 strain.

Antimicrobial agents	**Strain S2794**	
	Resistant	**Susceptible**
Amikacin	-	+
Ampicillin	30 µg/ml	-
Azithromycin	-	+
Cefazolin	-	+
Ceftriaxone	-	+
Chloramphenicol	-	+
Chlortetracycline	-	+
Clarithromycin	-	+
Clindamycin	-	+
Clotrimazole	-	+
Cloxacillin Monohydrate	-	+
Erythromycin	-	+
Kanamycin	-	+
Levofloxacin	-	+
Lincomycin	-	+
Oxacillin	-	+
Penicillin	70 µg/ml	-
Polymyxin B	110 µg/ml	-
Rifampicin	-	+
Streptomycin	-	+
Sulfamethoxazole	70 µg/ml	-
Tetracycline	-	+
Vancomycin	-	+

3.3 SELECTIVE BIOASSAY

Through selective bioassays, it was possible to verify that the S2794 strain was pathogenic (≥ 50%) to the lepidopteran species *H. armigera,* and *C. includens*, and the phytopathogen *S. sclerotiorum*. Furthermore, it was found that the bacterial isolate was potentially toxic to the species *A. grandis*, since there was a significant mortality rate of 35% for this coleopteran.

Table 2. Mortality percentage of the strain S2784 against *H. armigera* (*H.a*), *S. frugiperda* (*S.f*), *C. includens* (*C.i*), *A. grandis* (*A.gr*), *A. aegypt* (*A.gy*), *C. elegans* (*C.e*), *F. oxysporum* f. sp. *vasinfectum* (*F.ox*), and *Sclerotinia sclerotiorum* (*S.scl*).

% Mortality in selective bioassays of the S2794 strain							
Lepidopterans			**Coleopteran**	**Dipteran**	**Nematode**	**Fungi**	
H.a	***S.f***	***C.i***	***A.gr***	***A.gy***	***C.e***	***F.ox***	***S.scl***
70%	20%	50%	35%	0%	5%	0%	50%

4 DISCUSSION

The antibiotic resistance assay showed that the S2794 isolate was susceptible to 19 types of antimicrobials, but the strain was resistant to penicillin, ampicillin, polymyxin B, and sulfamethoxazole. In contrast to this result, a study coordinated by Reva (19) found that 30 *B. subtilis* isolates showed susceptibility to several antibiotics, including penicillin and ampicillin. On the other hand, Andrews (20) observed that different six isolates of *B. subtilis* obtained divergent resistance profiles, since among the six strains, only one was resistant to penicillin, and two strains were resistant to tetracycline, while the others were not.

There are few studies related to the susceptibility of the species *B. subtilis* to antibiotics, to provide identification profiles in bacterial isolation and characterization processes. However, many studies describe the participation of the species as a producer of different antimicrobial compounds for numerous biotechnological purposes, including biocontrol.

Generally, the antimicrobial and antifungal properties of *Bacillus subtilis* strains are largely determined by the type and amount of the lipopeptides (21). In this study, genes involved in the synthesis of surfactin, bacillomycin, and fengycin were detected in the S2794 genome. Thus, it is reasonable to state that these compounds were efficiently toxic to *S. sclerotiorum*, *H. armigera* and *C. includens*, since surfactin and bacillomycin are compounds that cause cytolytic effects on yeasts and fungi (22), membrane disorder of insect larvae, causing disturbances in their metabolism (23), and alteration in the structure and permeability of the fungal cell membrane, due to the interaction of fengycin with the sterol and phospholipid molecules present in the membranes (24).

Different strains of *B. subtilis* have previously been used to control plant pests and diseases, including the cotton leaf fluke, *S. littoralis* (23), stem rot, *S. sclerotiorum* (25), the Mediterranean flour moth, *Ephestia kuehniella* Zeller (26), all wheat species related diseases (26), *Fusarium* root infection (27), and many others.

In the analysis of the genome of the S2794 strain, genes responsible for the growth and formation of biofilms (*tyrZ* and *srfA*) were also detected. Biofilms, in turn, are surface-associated microbial communities, enveloped in a self-produced extracellular matrix, which play an essential role in securing nutrients secreted by the roots, colonization, and host protection (28-29). They also participate in the treatment of effluents and are potential sources of energy in the form of microbial fuel cells (30-32).

In addition, the low pathogenicity of the strain S2794 to *C. elegans* (5%), theoretically, may be related to the extension of the life of the nematode by the strain, since studies have shown that biofilm formation by *B. subtilis* prolonged the longevity of *C. elegans*, due to resistance to different types of stress, such as heat and oxidative stress (33). However, further studies with the S2794 strain are needed to confirm this hypothesis.

The genomic analysis of promising beneficial microbes helps obtain genes encoding the biosynthetic pathways that are responsible for the production of antimicrobial compounds are valuable resources for anti-infective areas of pharmaceutical and the development of efficient, environmentally friendly biologic antimicrobial agents (34).

Nucleotide sequence accession numbers. The sequence of the *B. subtilis* strain S2794 has been deposited in GenBank with the accession number SAMN12230145.

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