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RESEARCH ARTICLE

Biochemical, physiological, and molecular characterisation of a large collection of aerobic endospore-forming bacteria isolated from Brazilian soils

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Abstract

The aerobic endospore-forming bacteria (AEFB) comprise species of *Bacillus* and related genera and have long been regarded as prominent constituents of the soil bacterial community. The wide diversity of AEFB renders appropriate categorisation and generalisations a challenging task. We previously isolated 312 AEFB strains from Brazilian soils that we designated SDF (*Solo do Distrito Federal*) strains. To better understand the SDF diversity and explore their biotechnological potential, we addressed the biochemical and physiological profiles of these 312 environmental strains by performing 30 tests in this work. Of these, the 16S rRNA gene sequences segregated 238 SDF strains into four genera in the family Bacillaceae and two in the Paenibacillaceae. *Bacillus* spp. were the most prevalent, followed by species of *Paenibacillus*. We summarised the phenotypic test relationships among selected SDF strains using a Pearson correlation-based clustering represented in heatmaps. In practice, biochemical and physiological profiles are often less discriminatory

than molecular data and may be unstable because of the loss of traits. Although these test reactions are not universally positive or negative within species, they may define biotypes and be efficient strain markers, enhancing the accuracy of unknown sample identification. It can also help select the most representative phenotypes of samples. Along with the other phenotypic and genotypic data, the present results are of great importance for the robust classification of the SDF strains within the scope of the polyphasic approach.

Keywords
Bacillales, bacterial identification, bacterial metabolism, endosporulation, Firmicutes, phenotyping, taxonomy

Introduction

Aerobic endospore-forming bacteria (**AEFB**) are widely distributed in nature, and soil is recognised as their main reservoir (De Vos 2011; Mandic-Mulec and Prosser 2011). AEFB encompass species from the genus *Bacillus* and related genera and harbour species of significant importance in health, environment, and biotechnology (Logan et al. 2009; Alina et al. 2015; Ehling-Schulz et al. 2019). These bacteria produce dormant and highly resistant cells called spores that can germinate within seconds when external conditions become favourable (Driks and Eichenberger 2016; Christie and Setlow 2020).

AEFB exhibits high levels of genetic, biochemical, and physiological diversity and appreciable resistance to adverse environments (Galperin 2013; Driks and Eichenberger 2016; Christie and Setlow 2020). The high heterogeneity in the phenotypic and genotypic characteristics has been hampering the taxonomy of these species (Logan et al. 2009; Galperin 2013). The first identification and classification schemes of AEFB were based on the morphology of the colonies, vegetative cells, sporangia, spores, and Gram-staining response, besides biochemical, physiological, and chemotaxonomic properties (Logan et al. 2009). Today's polyphasic taxonomy distinguishes and classifies strains based on these classical phenotypic data, supplemented with genotypic and other phenotypic results obtained at the molecular level (Das et al. 2014). Combining classical and molecular data, notably 16S rRNA gene sequencing, has revolutionised our understanding of the domain *Bacteria* and led to a rapid increase in the number of descriptions of novel AEFB taxa, especially at genus and species levels (Maughan and Van der Auwera 2011).

AEFB are allocated in the phylum Firmicutes, within the class Bacilli, order Bacillales, where seven families harbour aerobic spore-forming genera: Bacillaceae, Alicyclobacillaceae, Paenibacillaceae, Planococcaceae, Pasteuriaceae, Sporolactobacillaceae, and Thermoactinomycetaceae (De Vos et al. 2009; Logan and Halket 2011; Galperin 2013; Parte 2018). Phylogenetic studies based on the 16S rRNA gene sequences suggest clusters of closed-related AEFB species, designated groups (Ash et al. 1991; De Vos et al. 2009; Alina et al. 2015). The early rRNA groups 1 to 5 of *Bacillus* species proposed by Ash et al. (1991) were expanded to house alkaliphilic and alkalitolerant species, while other groups of species, such as those allocated in genera *Paenibacillus* (group 3), *Brevibacillus* (group 4), and other distinct taxa have been reclassified (Stackebrandt and Swiderski 2002).

Since observable features from growth conditions and enzymatic reactions are related to the genome expression, the resulting profiles detect phenotypic patterns for the evaluated species. Thus, investigating these intrinsic metabolic activities is still essential for identifying and classifying new AEFB isolates. These assays are highly recommended in characterising AEFB strains (Logan et al. 2009). To help understand AEFB diversity and explore their biotechnological potential, we isolated 312 strains from soil samples collected at random areas of the Federal District, Midwest region of Brazil (Cavalcante et al. 2019; Orem et al. 2019; Martins et al. 2020). These strains, designated SDF0001-SDF0312 (*Solo do Distrito Federal* or SDF) are deposited at the *Coleção de Bactérias Aeróbias Formadoras de Endósporos* (AEFB Collection-AEFBC), hosted at the University of Brasilia, Federal District. A polyphasic strategy is being used to analyse the SDF strains for taxonomic purposes. In the present work, 30 biochemical and physiological tests were performed to investigate substrate utilisation and transformation, in addition to the growth-condition capabilities of 312 SDF strains. Among them, 246 were classified by 16S rRNA sequences, and a Pearson correlation based on a clustering method (Gu et al. 2016) was used to construct heatmaps to summarise the relationships of selected SDF strains to these phenotypic tests.

Methods

Bacterial strains

The 312 SDF strains evaluated in this study were isolated, as described in Cavalcante et al. (2019) and Orem et al. (2019). The reference strains used as positive and negative controls for the physiological and biochemical tests (Table 1) are deposited at Coleção de Culturas do Gênero Bacillus e Gêneros Correlatos (**CCGB**), of the Instituto Oswaldo Cruz (LFB-Fiocruz-RJ, Brazil).

Ethics statement

Specific permissions required to collect bacterial strains used in this study were endorsed by the Federal Brazilian Authority (CNPq; Authorisation of Access and Sample of Genetic Patrimony n° 010439/2015-3). Sampling did not involve endangered or protected species.

Biochemical and physiological assays

Strains were grown in nutrient agar (33 °C, 24 h) under atmospheric aerobic conditions. Cells used in the tests were obtained from a single colony and transferred to a tube containing nutrient broth, incubated at 33 °C, under constant stirring (200 rpm), for about 16 h. The 30 biochemical and physiological tests (Table 1) were performed according to Bergey's Manual of Systematic Bacteriology (Smith et al. 1952; Gordon et al. 1973; Claus and Berkeley 1986; Oliveira et al. 1998; De Vos et al. 2009). All tests were carried out in duplicate in two independent experiments.

Taxonomic assignments of SDF strains

DNA preparation, PCR amplification, sequencing, and sequence analyses were performed as described in Orem et al. (2019). Briefly, the nearly full length of both strands of 16S rRNA genes was amplified using total DNA and primers 27F (5' AGA GTT TGA TCM TGG CTC AG 3') and 1492R (5' GGY TAC CTT GTT ACG ACT T 3'). PCR products were bi-directionally sequenced employing the Sanger method. These sequences were filtered for Q≥20 in Phred scores and taxonomically assigned using BLAST and Classifier.

Heatmaps

The biochemical and physiological assays results were arranged in heatmaps (Gu et al. 2016) to enhance the potential of visually revealing patterns and correlations among them. We took the dichotomous values 0 (for Negative) and 1 (for Positive) as binary variables representing the association between the species and their results of biochemical and physiological assays. Using Pearson's correlation, the species were clustered, taking similar biochemical and physiological results (Hummel et al. 2017). R scripts are available at https://github.com/waldeyr/bafes_figures.

Results and discussion

Due to the importance of metabolism for the identification and classification of AEFB new isolates (Logan et al. 2009), we applied 30 biochemical and physiological tests (Table 1) to 312 AEFB strains isolated from Brazilian soils, designated SDF strains (Cavalcante et al. 2019; Orem et al. 2019; Martins et al. 2020). The profiles obtained from enzymatic reactions and growth conditions are described in Suppl. material 1: table S1, available in the online Supplementary Material. All 312 SDF strains studied are aerobic or facultative anaerobic endospore-formers and Gram-positive or Gram-variable cells (Cavalcante et al. 2019; Orem et al. 2019; Martins et al. 2020). The latter characteristics are common to taxa found in Bacillales (Logan et al. 2009; Galperin 2013), where these environmental AEFB strains are allocated.

Of these 312 SDF strains, the taxonomic assignments of 246 were addressed using 16S rRNA gene sequences, as described in Orem et al. (2019). The lowest and highest inter-species pairwise 16S rRNA gene sequence similarities spanned from 90% to 100% (Suppl. material 1: table S1). Considering the similarity thresholds for genera 96%, and ≥97% for species (Stackebrandt and Goebel 1994), the classification obtained segregated 238 SDF strains into 6 genera, being 4 part of the family Bacillaceae and 2 of Paenibacillaceae (Fig. 1A). Among the SDF strains described in the present work, *Bacillus* spp., belonging to the family Bacillaceae, are the most prevalent (207 strains; 84.14%), followed by species of genera *Paenibacillus* (14; 5.69%; family Paenibacillaceae), *Lysinibacillus* (7; 2.84%; Bacillaceae), *Brevibacillus* (6; 2.43%; Paenibacillaceae), *Terribacillus* (1; 0.40%; Bacillaceae), and

Table 1. Biochemical and physiological profiles analysed in this work and the respective controls.

	Test	Control	
		Positive	**Negative**
Growth condition	Citrate utilization	*Bacillus cereus CCGB406*	*Paenibacillus macerans CCGB126*
	Propionate utilization	*Bacillus licheniformis CCGB407*	*Bacillus subtilis CCGB1249*
	7% NaCl	*Bacillus amyloliquefaciens CCGB452*	*Paenibacillus macerans CCGB126*
	10% NaCl	*Bacillus amyloliquefaciens CCGB452*	*Paenibacillus macerans CCGB126*
	0.001% lysozyme	*Bacillus cereus CCGB406*	*Bacillus pumilus CCGB124*
	45 °C	*Geobacillus stearothermophilus CCGB412*	*ND**
	65 °C	*Geobacillus stearothermophilus CCGB412*	*Bacillus thuringiensis CCGB1163*
	pH 5.7	*Bacillus cereus CCGB406*	Paenibacillus alvei CCGB414
	Anaerobiosis	*Bacillus cereus CCG406*	*Bacillus megaterium CCGB408*
Enzyme	Catalase	*Bacillus cereus CCGB406*	*ND**
	Oxidase	*Lysinibacillus sphaericus CCGB745*	*Bacillus cereus CCGB406*
	Hemolysin	*Bacillus thuringiensis CCGB1163*	*Lysinibacillus sphaericus CCGB745*
	Nitrate reductatase	*Bacillus cereus CCGB406*	*Bacillus megaterium CCGB408*
Hydrolysis	Casein	*Bacillus megaterium CCGB408*	*Paenibacillus macerans CCGB126*
	Gelatin	*Bacillus cereus CCGB406*	*Geobacillus stearothermophilus CCGB412*
	Esculin	*Bacillus subtilis CCGB1249*	*Lysinibacillus fusiformis CCGB743*
	Starch	*Bacillus cereus CCGB406*	*Lysinibacillus sphaericus CCGB745*
Amino acid decomposition	Phenylalanine degradation	*Bacillus megaterium CCGB408*	*Bacillus cereus CCGB406*
	Tyrosine degradation	*Bacillus cereus CCGB406*	*L. sphaericus CCGB745*
	Arginine dihydrolase	*Bacillus licheniformis CCGB407*	*Bacillus megaterium CCGB408*
	Lysine decarboxylase	*Bacillus thuringiensis CCGB1163*	*Bacillus megaterium CCGB408*
	Ornithine decarboxylase	*Bacillus thuringiensis CCGB1163*	*Bacillus megaterium CCGB408*
Indole production		*Paenibacillus alvei CCGB414*	*Bacillus cereus CCGB406*
Production of acid from	D-Glucose	*Bacillus megaterium CCGB408*	*Lysinibacillus fusiformis CCGB743*
	L-Arabinose	*Bacillus megaterium CCGB408*	*Brevibacillus brevis CCGB052*
	Lactose	*Bacillus megaterium CCGB408*	*Lysinibacillus fusiformis CCGB743*
	Mannitol	*Bacillus megaterium CCGB408*	*Lysinibacillus fusiformis CCGB743*
	Sucrose	*Bacillus amyloliquefaciens CCGB452*	*Lysinibacillus sphaericus CCGB745*
	D-Xylose	*Bacillus megaterium CCGB408*	*Brevibacillus brevis CCGB052*
Voges-Proskauer test		*Bacillus cereus CCGB406*	*Bacillus megaterium CCGB408*

*not determined. **CCGB**: *Coleção de Culturas do Gênero Bacillus e Gêneros Correlatos*. CCGB is an integrant of the World Federation for Culture Collec6ons WFCC (#574).

Rummeliibacillus (1; 0.40%; Bacillaceae). These findings are not surprising since the selective procedure we used to isolate SDF strains intended to favour non-fastidious AEFB species, excluding strict anaerobic endospore-forming and Gram-negative cells (Cavalcante et al. 2019; Orem et al. 2019; Martins et al. 2020).

Included in the 224 SDF strains classified at the species level (Suppl. material 1: table S1), members of the *B. pumilus* subgroup were predominant (83 strains; 37.05%), followed by *B. cereus* group species (48; 21.42%), *B. megaterium* group (35; 15.62%), other members of *B. subtilis* complex (12; 5.35%), *B. simplex* (7; 3.12%), *B. clausii* (3; 1.33%), *B. subterraneus* (2; 0.89%), besides one (0.44%) of each for *B. australimaris*; *B. arbutinovorans*; *B. circulans*; *B. kochii*; *B. luciferensis*; *B. oleronius*; *B. siamensis*, and *B. senegalensis*. Outside the genus *Bacillus*, other species belonging to the family Bacillaceae were *Lysinibacillus sphaericus* (3; 1.33%); *L. xylanilyticus* (2; 0.89%); *L. fusiformis* (2; 0.89%), and *Terribacillus goriensis* (1; 0.44%). *Paenibacillus* spp. (12

strains; 5.35%) and *Brevibacillus* spp. (5 strains; 2.23%), allocated in the family Paenibacillaceae, complete the list of SDF strains classified at the species level (see below). The diversity of the SDF strains is represented in Fig. 1B.

It is worth mentioning that members of the *B. cereus* group or *sensu lato* (*s.l.*) and *B. subtilis* complex or subgroups are composed of very related members (>99% similarity), restricting species delimitation when considering only the 16S rRNA gene analyses. Conversely, *B. megaterium* and *B. aryabhattai* share 99.7% of identity in the 16S rRNA gene sequences, even though the genomes are less than 70% identical (Shivaji et al. 2009). Therefore, distinguishing between these two species using only this technique is also challenging. Thus, our taxonomic assignments based on 16S rRNA gene sequences are a preliminary inference of genera or species. Accordingly, when 16S rRNA gene profiling placed these strains within these AEFB taxa, an analysed sample could belong to two or even more species alternatives within the same affiliation cluster. In these instances, this approach can find groups of bacteria but cannot assign them accurately to a species due to its low discrimination ability. Since 10 SDF strains exhibited similarity rates spanning from 90 to 95% (Suppl. material 1: table S1), the 16S rRNA gene-sequencing tool failed to classify these environmental strains even at the genus level.

Bacillus is the type genus of the order Bacillales, and *Bacillus* spp. have been isolated from a wide range of environments. Soil, along with freshwater, is considered one of the least restrictive environments for these species (Logan and Halket 2011; Mandic-Mulec and Prosser 2011). It is worth noting that certain species found in

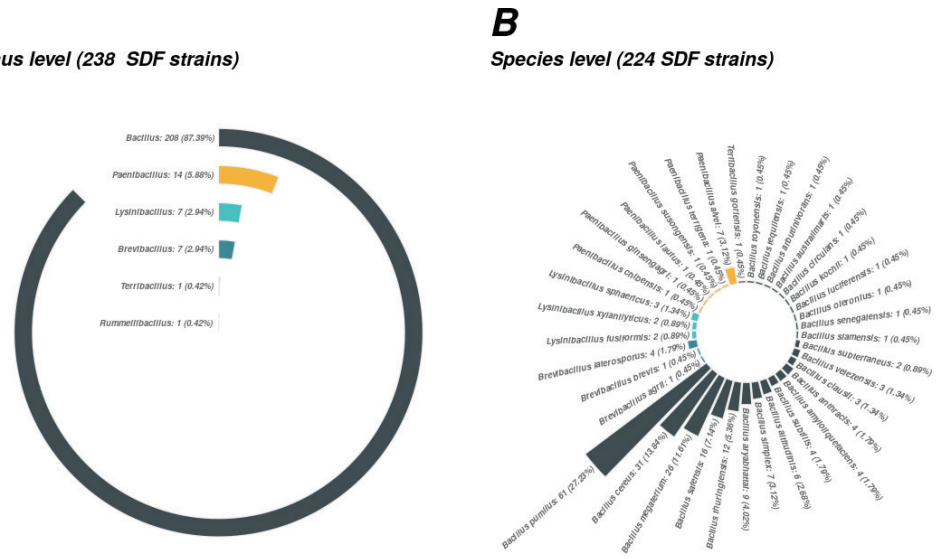

Figure 1. Overall repartition of SDF strains according to 16S rRNA gene sequencing classification. **(A)** Distribution of 238 SDF strains in six genera belonging to families Bacillaceae (*Bacillus*, *Lysinibacillus*, *Terribacillus*, and *Rummeliibacillus*) and Paenibacillaceae (*Paenibacillus* and *Brevibacillus*). **(B)** Species assignments of 224 SDF strains.

soils are inactive in these environments. It could be the case for some SDF strains isolated from Brazilian soils. The method of isolation based on heat shock allowed the dormant spores to germinate and grow *in vitro*.

The genus *Bacillus* remains the largest AEFB taxon, accommodating 614 species, as registered in the List of Prokaryotic Names with Standing in Nomenclature (LPSN: https://www.bacterio.net/Bacillus.html; accessed on February 01, 2022). Taxonomy within the genus *Bacillus* is hampered by high heterogeneity at phenotypic and genotypic levels (Logan et al. 2009; Galperin 2013; Ehling-Schulz et al. 2019). Further, these divergencies restrict the distinction between *Bacillus* spp. and those allocated in other genera inside Bacillaceae. *B. cereus* and *B. anthracis* are human pathogens causing food-borne illness and anthrax, respectively (Ehling-Schulz et al. 2019). On the other hand, the metabolic breadth of *Bacillus* spp. has been explored by the industry for producing a vast range of antibiotics, molecules for the promotion of plant growth, hydrolyses, toxins against plants, fungi, insects, and nematodes, in addition to other bioproducts (Alina et al. 2015; Ehling-Schulz et al. 2019). Due to their significant relevance to economy and health issues, the *B. cereus* group and *B. subtilis* complex have received considerable attention (Maughan and Van der Auwera 2011; Ehling-Schulz et al. 2019).

B. cereus group

The *B. cereus* group hosts *B. cereus sensu stricto* (*s.s.*), *B. anthracis*, *B. thuringiensis*, *B. mycoides*, *B. pseudomycoides*, *B. weihenstephanensis*, *B. toyonensis*, and *B. cytotoxicus* (Ehling-Schulz et al. 2019). Organisms placed in this group belong to 16S rRNA/DNA group 1. Traditionally, these bacteria have been differentiated based on phenotypic characteristics, especially pathogenic potential (Ehling-Schulz et al. 2019). Nonetheless, this group is a highly homogeneous subdivision inside the genus *Bacillus*. Furthermore, they are hardly distinguishable with standard biochemical and chemotaxonomic methods or phylogenetically relevant target genes (Bavykin et al. 2004; Arnesen et al. 2008). However, specific biochemical and physiological characteristics of the *B. cereus* group are advantageous to differentiate these taxa from the other aerobic endospore-forming species.

Out of 224 SDF strains classified at the species level (Suppl. material 1: table S1), 48 (21.42%) were members of the *B. cereus* group. Using a Pearson correlation-based clustering method (Gu et al. 2016), we constructed a heatmap (Fig. 2) to summarise the relationships of these 48 environmental strains to the 30 biochemical and physiological tests performed (Table 1). It is feasible to distinguish which strains respond similarly to the tests when they are in the same clade. For example, those strains in distant clades respond differently. It is also possible to discern which SDF strains respond similarly to each test and discriminate them by correlating rows and columns. The 30 tests formed two clusters, one with 17 columns and one with 13 tests, in which most of the 48 SDF strains responded positively and negatively, respectively.

Although many AEFB may not respond positively to the catalase test, most rod-shaped species, either Gram-positive or Gram-positive only in the initial stages of growth, are catalase-positive, especially members of the genus *Bacillus* (Logan and De Vos 2009). However, in most cases, respiratory metabolism occurs at low O_2 levels. Here, all the 48 SDF members of the *B. cereus* group responded positively to this enzyme linked to respiration in the presence of atmospheric O_2 (Fig. 2). This positivity seems to be a characteristic of this group of sporulating prokaryotes. As assessed in this work, it is worth noting that *B. cereus s.s.* can grow under certain anaerobiosis conditions (Logan and De Vos 2009). The cytochrome C oxidase is especially useful for discriminating Gram-negative pathogens *Vibrio* spp. (oxidase positive) from the oxidase-negative enteric bacteria (Vila et al. 1992). This enzyme catalyses the oxidation of cytochrome C while reducing oxygen to form water. The oxidation test in vitro employs colourless artificial acceptors such as dimethyl or tetramethyl p-phenylenediamine, resulting in purple colour when positive. This essay distinguishes *Neisseria* and *Moraxella* (oxidase positive) from *Acinetobacter* spp. (oxidase negative) (Henriksen 1976; Powell and Marcon 2012). Of the 48 SDF strains allocated in the *B. cereus* group, 25 (52.08%) were oxidase-positive. Logan and De Vos (2009) point out this variability for this genus and related genera, demonstrating the apparent inactivity of this enzyme or even that the traditional method failed to detect the oxidase activity in almost half of these samples. Anaerobiosis assays, performed in tubes containing aldehyde-reduced agar medium inoculated with a needle revealed growth of a few centimetres below the interface of the culture medium with atmospheric air in 24 (50%) of the SDF strains belonging to the *B. cereus* group. This effect denotes anaerobic growth, a property conserved among AEFB.

Some *Bacillus* species do not appear to utilise carbohydrates (Logan and De Vos 2009). Nevertheless, the acid production profiles from monosaccharides and disaccharides are of great value in characterising and identifying these species. Most SDF strains allocated to the *B. cereus* group used D-glucose, L-arabinose, D-xylose, and other fermentable carbohydrates as sole sources of carbon and energy (Suppl. material 1: table S1; fig. 2). Probably, they have the genetic information to conduct the pathway of Embden-Meyerhof-Parnas, coupled with the Krebs cycle, verified by acid production (Logan and De Vos 2009). Regarding glucose consumption, five SDF strains, one classified as *B. thuringiensis* (SDF0225) and four as *B. cereus s.s.* (SDF0124; SDF0229; SDF0237, and SDF248), responded negatively to the use of this monosaccharide, which is rare among rod-shaped AEFB, as they usually assimilate and degrade D-glucose. Although the formation of acid from D-mannitol is frequently negative for members of the *B.* cereus group and positive for strains of other groups (Fritze 2002), three SDF strains classified as *B. cereus s.s.* (SDF0219; SDF0124, and SDF0022) and *B. anthracis* SDF0199 were able to ferment this sugar (Fig. 2). Interestingly, these strains were gathered in the uppermost and lowermost rows of the strains' list. Indeed, clustering heatmaps can group samples based on the similarity of their phenotypic patterns, allowing us to identify eventual atypical responses (Zhao et al. 2014), as observed for these four SDF strains able to ferment mannitol.

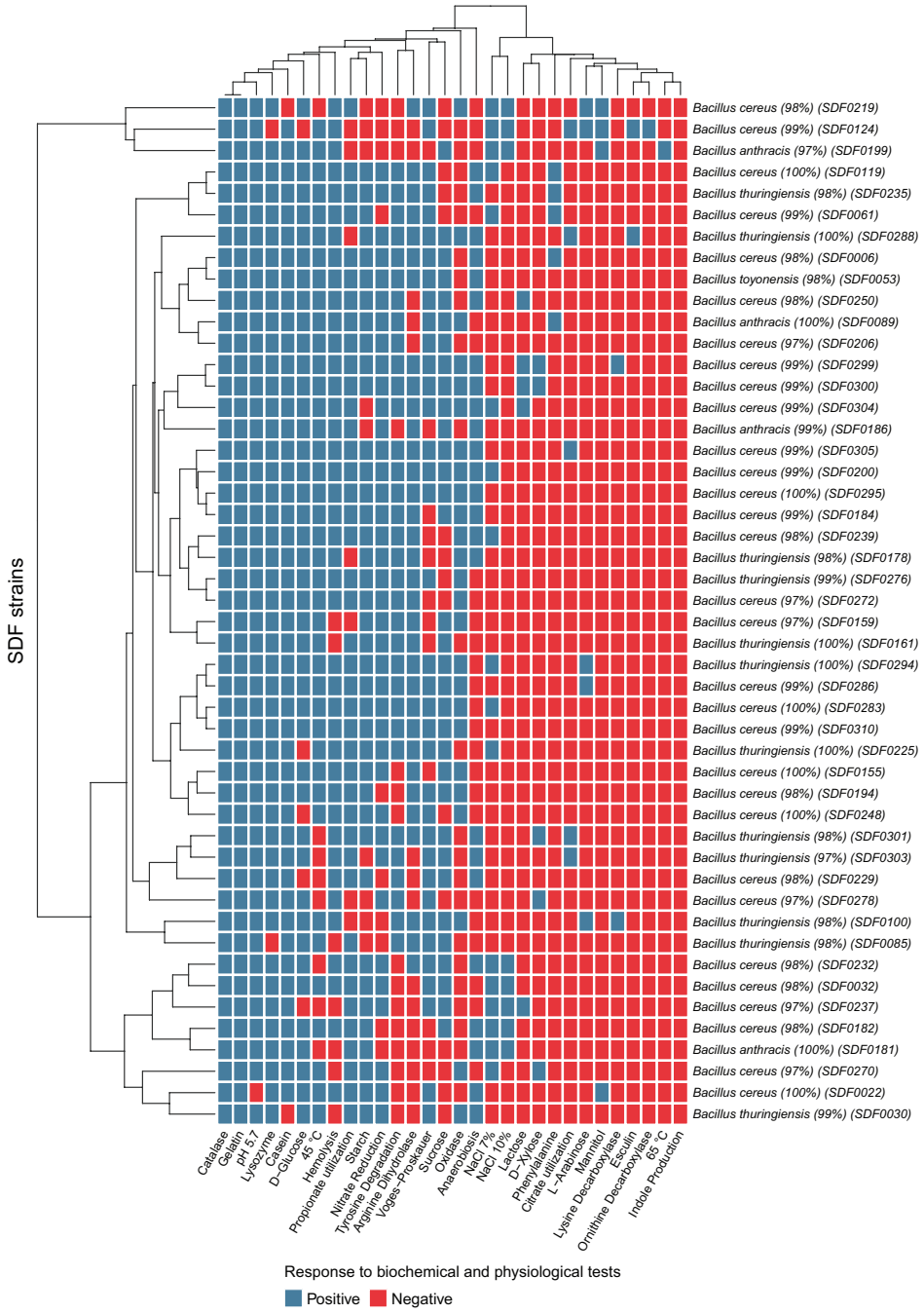

Figure 2. Correlation between SDF strains belonging to the *B. cereus* group and growth conditions or enzyme activities. A Person correlation-based clustering method was employed to construct a heatmap associating 48 SDF strains allocated in the *B. cereus* group (right) and 30 phenotypical features (bottom) contributing to AEFB identification and classification. The top dendrogram clustered the SDF strains into two parts based on the prevalence of positive responses (blue) to 30 growth conditions and enzyme reactions described at the bottom of the graphic. Negative responses are shown in red.

The Voges-Proskauer test showed that some species, such as two *B. thuringiensis* strains (SDF0161 and SDF0178), three *B. anthracis* (SDF181; SDF0186, and SDF0199) and seven *B. cereus s.s.* (SDF0155; SDF0159; SDF0182; SDF0184; SDF0239; SDF0270, and SDF0272) responded negatively to the acetyl-methylcarbinol production assay. These assays made us suspect that these strains may not produce enzymes that decarboxylate lactic acid from the glycolytic pathway or do not have an enzyme capable of bonding two molecules originating from the production of acetate ions. Oliveira et al. (1998) established a standardised protocol for detecting gelatin hydrolysis by *Lysinibacillus sphaericus*-former *B. sphaericus* (Seldin et al. 1984; Ash et al. 1994)–, showing that 93.3% of strains belonging to this species hydrolyses this incomplete protein after four days of incubation. Here, bulk 48 SDF strains accommodated in the *B. cereus* group were able to use gelatin. Considering the relatively high number of strains submitted to this type of biochemical test, we deemed it a very valid verification. The development in the presence of lysozyme is another characteristic of the *B. cereus* group and hardly occurs in the other species of other groups (Fritze 2002). However, *B. cereus* SDF0124 and *B. thuringiensis* SDF085 did not grow in this condition, indicating that this enzyme can hydrolyse the cell wall of these two strains.

The production of haemolysin and cell morphology in a few strains of the *B. cereus* group are also relevant phenotypes for taxonomic studies (Fritze 2002, 2004; Logan and De Vos 2009; Logan et al. 2009). *B. cereus s.s.*, in general mobile, is heavily haemolytic but does not produce a rhizoid growth pattern, a characteristic that can be used to differentiate it from colonies of *B. mycoides* strains (Fritze 2002). Most *B. anthracis* strains are neither mobile nor haemolytic (Fritze 2004; Maughan and Van der Auwera 2011). However, non-mobile *B. cereus* strains and hemolytic *B. anthracis* may hinder the differentiation between these two species. In addition, the latter species can be differentiated by parasporal crystal formation, typically described for *B. thuringiensis* (Fritze 2002). Of the 48 SDF strains allocated in the *B. cereus* group, three samples classified as *B. cereus* (SDF0159, SDF0237, and SDF0270), one *B. anthracis* SDF0181, and three *B. thuringiensis* (SDF0161; SDF0085, and SDF0030) presented no haemolysin activity. It is significant to mention that *B. thuringiensis* SDF0030 produces a typical parasporal crystal (Cavalcante et al. 2014), a classical feature distinguishing *B. thuringiensis* strains from *B. cereus s.s*. (Ehling-Schulz et al. 2019). Conversely, three strains classified as *B. anthracis* (SDF0199, SDF089, and SDF0186) were positive for haemolysin activity. The main phenotypical properties frequently used to distinguish *B. cereus*, *B. thuringiensis*, and *B. anthracis* are related to the presence or absence of large plasmids, where the replicons are localised (Ehling-Schulz et al. 2019). Future investigation on the extrachromosomal profiles of these SDF strains will help to understand the evolutionary relatedness of these species.

B. subtilis complex

B. subtilis s.s., the type species of the genus *Bacillus*, is prominent in microbial history and plays a distinct role as a model for Gram-positive bacteria and in the understanding of stress-resistance of bacterial spores (Galperin 2013; Driks and Eichenberger 2016; Ehling-Schulz et al. 2019). Besides being recognised as a model, this species, along with other closely related species accommodated in the *B. subtilis* complex, is extensively employed in industry and agriculture (Fan et al. 2017). As a taxonomic unit above the species level, the *B. subtilis* species complex can be split into four clades. These recognisable monophyletic groups comprise clade I, consisting of three subspecies of *B. subtilis* (*subtilis*, *spizenii*, and *inaquosorum*), besides *B. tequilensis*, *B. vallismortis*, *B. mojavensis*, and *B. atrophaeus*; clade II, containing species *B. amyloliquefaciens*, *B. siamensis*, and a conspecific complex embracing *B. methylotrophicus*, *B. velezensis*, and *B. amyloliquefaciens* subsp. *plantarum*; clade III encompasses *B. licheniformis*, *B. sonorensis*, and related species; and clade IV includes *B. pumilus*, *B. safensis*, *B. xiamenensis*, and a conspecific group involving the type strains of *B. altitudinis*, *B. stratosphericus*, and *B. aerophilus*. Like strains from the *B. cereus* group, these taxa are placed in 16S rRNA/DNA group 1 and are remarkably similar both phylogenetically and physiologically (Fritze 2004).

Of the 224 SDF strains classified at the species level employing 16S rRNA gene sequences, 95 (42.41%) were allocated in the *B. subtilis* complex (Suppl. material 1: table S1). Among them, the *B. pumilus* subgroup represented 83 (37.05%), 61 (27.23%) of which were classified as *B. pumilus*, 16 (7.14%) as *B. safensis*, and 6 (2.67%) as *B. altitudinis*. Seven strains (3.12%) belonged to the *B. amyloliquefaciens* subgroup, including 4 (1.78%) *B. amyloliquefaciens* strains and 3 (1.33%) *B. velezensis*. The remaining 4 (1.78%) SDF strains were placed in the *B. subtilis* subgroup, 3 (1.33%) of which were *B. subtilis s.s.* and 1 (0.44%) *B. tequilensis*. The relationships of these 95 strains to the 30 biochemical and physiological tests described in Table 1 were also analysed. The resulting heatmap (Gu et al. 2016) shown in Fig. 3 revealed two clusters encompassing 13 and 17 tests, where these SDF strains responded positively and negatively, respectively. In general, the SDF strains in this group corroborate the traits described in Bergeys' Firmicutes (Logan and De Vos 2009). Furthermore, according to Logan and Forsyth, and unpublished observations cited in this manual, *B. pumilus* strains isolated from Antarctic soils and penguin rookeries present phenotypic peculiarities, such as producing a diffusible yellow pigment.

Family *Paenibacillaceae*

Outside Bacillaceae, 17 (7.58%) of the 224 SDF strains were placed in two genera of the family Paenibacillaceae. *Paenibacillus* spp. accounted for 12 (5.35%) strains, 7 (3.12%) of *P. alvei* and 1 (0.44%) of each for *P. chibensis*; *P. ginsengagri*; *P. lautus*; *P. susongensis*, and *P. terrigena* (Suppl. material 1: table S1). Five (2.23%) strains of the

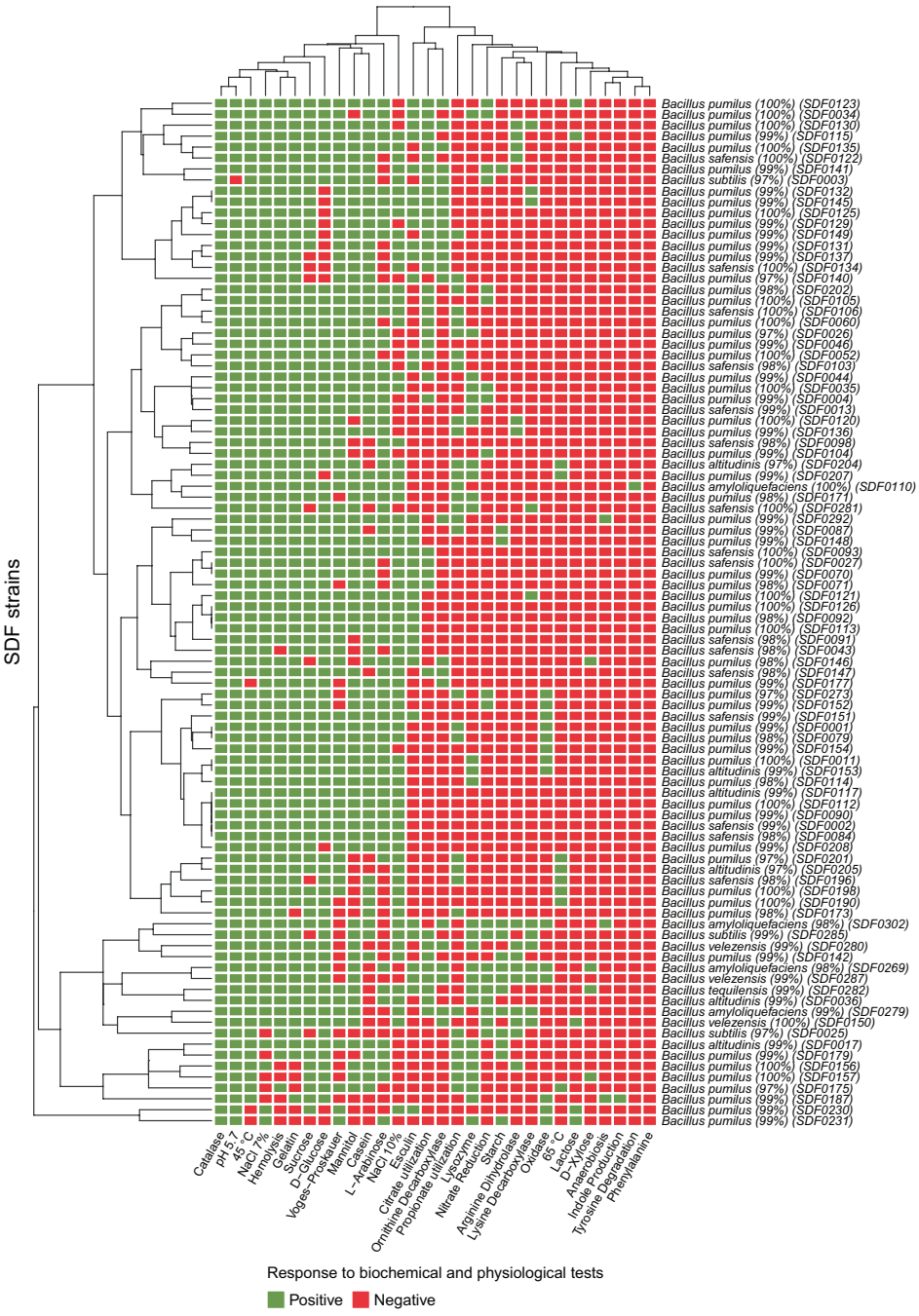

Figure 3. Correlation between SDF strains belonging to *B. subtilis* complex and growth conditions or enzyme activities. A Person correlation-based clustering method was employed to construct a heatmap associating 95 SDF strains allocated in the *B. subtilis* complex (right) and 30 phenotypical features (bottom) that contribute to AEFB identification and classification. The top dendrogram clustered the SDF strains into two parts based on the prevalence of positive responses (green) to 30 growth conditions and enzyme reactions described at the bottom of the graphic. Negative responses are shown in red.

genus *Brevibacillus* (quoted here as *Br.*), 4 (1.78%) of *Br. laterosporus* and 1 (0.44%) of *Br. agrii*, completed the SDF strains allocated into the family Paenibacillaceae (Suppl. material 1: table S1). The mutual connection between these 18 strains and the 30 biochemical and physiological tests (Table 1) is represented in Fig. 4. The two clusters distinguishable by this heatmap (Gu et al. 2016) comprehend 11 and 19 tests, embracing most of these SDF strains responding positively and negatively, respectively.

The genus *Paenibacillus* was created to reallocate species previously placed in the RNA group 3 of the genus *Bacillus* (Priest 2009). Paenibacillaceae was proposed to house the genus *Paenibacillus* and closely related genera (Ash et al. 1993; Shida et al. 1997). This family encloses two monophyletic clusters, the first consisting of the genera *Paenibacillus*, *Brevibacillus*, *Cohnella*, and *Thermobacillus*, and the second of genera *Aneurinibacillus*, *Ammoniphilus*, and *Oxalophagus* (De Vos et al. 2009). The type genus is *Paenibacillus*, which, accommodates the second largest number of AEFB species known (342) after *Bacillus*, as registered at the LPSN (https://www.bacterio.net/; accessed on February 01, 2022). *Paenibacillus* harbours aerobic or facultative rod-shaped species (Priest 2009; Galperin 2013; Parte 2018) with a typical Gram-positive cell wall structure (Shida et al. 1996). Nevertheless, even young cells react weakly or even negatively to Gram staining. It should be noted that the 12 SDF strains classified as *Paenibacillus* spp. in this work stained weakly or even Gram-negative (not shown).

Brevibacillus spp. are used as a factory for the expression of biotechnologically-important enzymes (e.g., alpha-amylase, sphingomyelinase, xylanase, CGTase, and chitosanase), as well as heterologous proteins including cytokines (EGF, IL-2, NGF, IFN-c, TNF-a, and GM-CSF), antigens, and adjuvants (Mizukami et al. 2010). Besides, *Brevibacillus* spp. are considered a valuable tool for structural and functional biology studies (Panda et al. 2014). *Br. brevis*, *Br. choshinensis*, and *Br. laterosporus* have attracted considerable interest owing to the production or transformation of valuable compounds and the biocontrol proprieties (De Vos et al. 2009). Broad entomopathogenic activity includes species from the insect orders Coleoptera, Lepidoptera, Diptera, and phyla Nematoda and Mollusca (Ruiu et al. 2013).

Conclusions

The recent improvements in analytical tools have been helping to uncover the vast physiological and genetic diversity within the AEFB, resulting in more appropriate taxonomic arrangements (Galperin 2013; Ehling-Schulz et al. 2019). As a result, many new descriptions of genera and species and reclassifications have occurred. Molecular methods, especially 16S rRNA gene sequencing, have become the prevailing technique in procaryotic identification, but significant restrictions in our ability to identify environmental bacteria to the genus and species levels remain.

Here, the performance of the 16S rRNA sequence analysis was adequate. This tool resolved 238 (96.74%) out of 246 SDF strains at the genus level, revealing 4 and 2 genera within Bacillaceae and Paenibacillaceae, respectively. Among the 246 SDF samples, 224 (91.05%) were classified at the species level. As mentioned above, using this technique, closely related strains, such as those belonging to the *B. cereus*

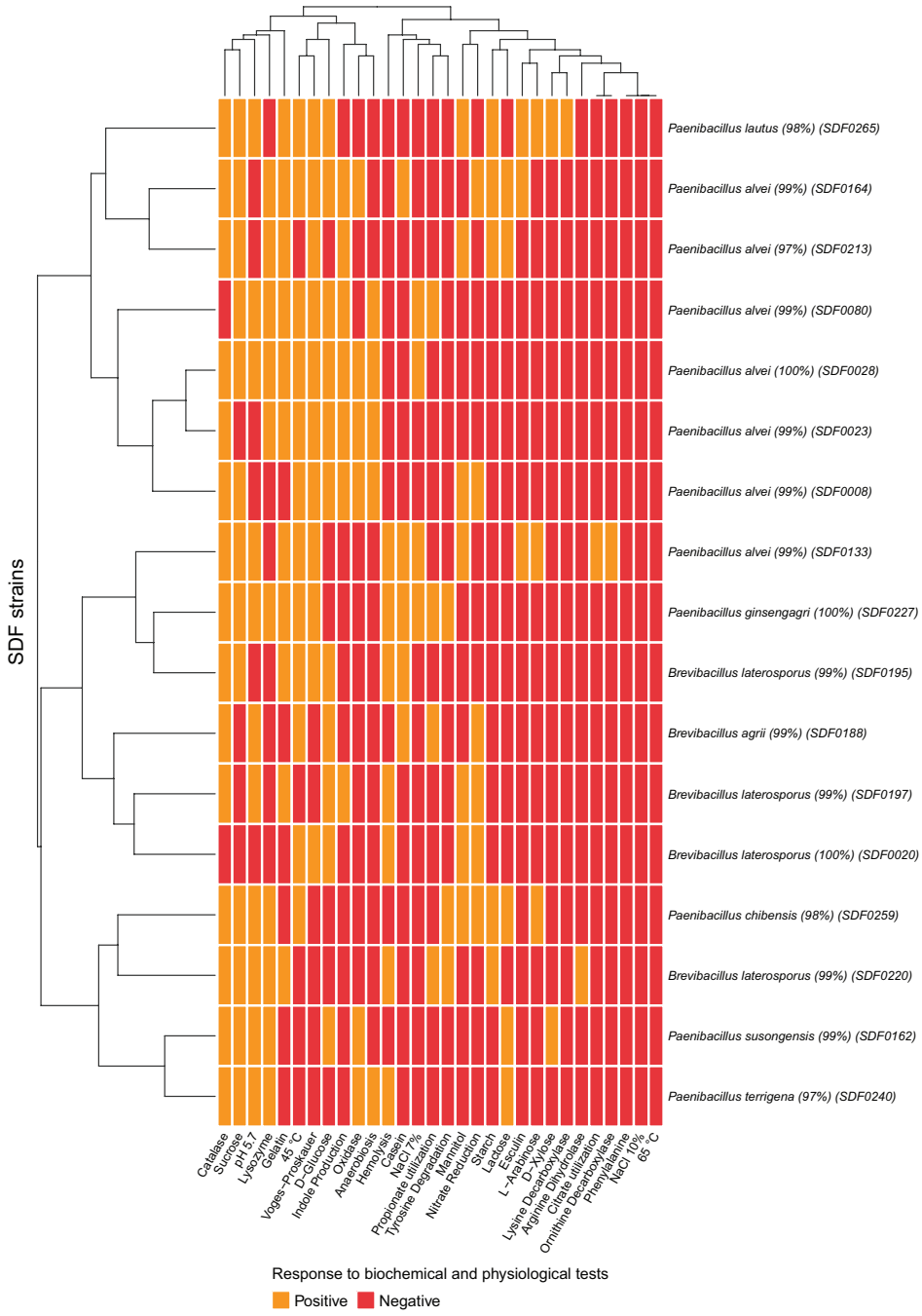

Figure 4. Correlation between SDF strains belonging to family Paenibacillaceae and growth conditions or enzyme activities. A Person correlation-based clustering method was employed to construct a heatmap associating 18 SDF strains allocated in the *B. subtilis* complex (right) and 30 phenotypical features (bottom) that contribute to AEFB identification and classification. The top dendrogram clustered the SDF strains into two parts based on the prevalence of positive responses (orange) to 30 growth conditions and enzyme reactions described at the bottom of the graphic. Negative responses are shown in red.

group, *B. subtilis* complex, and other AEFB taxa, cannot be resolved at the species level. Still, our classifications are suitable since they clearly show the genera and restrict the identity of part of these SDF strains to one or a few species in the genera. The positions of the SDF strains in this initial clustering and identification of closely related species may be more accurately determined by incorporating additional data from both genotypic and phenotypic analyses. Furthermore, our SDF strain classifications revealed well-known AEFB species and others that are scarcely described in the literature. Identifying multiple species and strains from different genera may help resolve Bacillales at the family, genus, and species levels.

In the present study, 30 biochemical and physiological tests provided profiles of all the 312 SDF strains deposited at AEFBC. From the genetic point of view, a large number of samples, such as those originating from the environment, including the SDF strains' collection, will hardly display 100% equal answers for all tests, as seen in taxonomic studies of strains isolated from non-clinical substrates (Logan and De Vos 2009; Logan and Halket 2011). In such cases, there are always taxonomically diverging strains. The ubiquitous species *B. pumilus*, isolated from Antarctic soils and penguin rookeries, corroborate this statement as a phenotypic distinction from other lineages can be observed (Logan and Forsyth, unpublished observations, apud Logan and De Vos 2009). The divergent samples need to have a separate and improved taxonomic study.

Biochemical and physiological profiles are useful for identifying these microorganisms. These assays are also part of the minimum standards proposed by Logan et al. (2009) to characterise new species of these taxa. However, the value of these tests in accurately identifying large numbers of environmental species is limited (Fritze 2004). Therefore, phenotypic similarities cannot be taken with certainty to indicate close evolutionary relatedness. However, along with other phenotypic and genotypic data (Cavalcante et al. 2019; Orem et al. 2019; Martins et al. 2020), including complete genome sequences in progress, the assays described in Suppl. material 1: table S1 will be significant for robust identification. The biochemical and physiological profiles can also help optimise the culture conditions for further characterisation and the production of bioactive metabolites by the SDF strains. Hence, the classification of AEFB at the species levels is not straightforward. Moreover, to classify and differentiate closely related SDF strains, these essays should be coupled to other classical and molecular methods involving phenotypic and genotypic types (Cavalcante et al. 2019; Orem et al. 2019; Martins et al. 2020) in a polyphasic approach. This strategy will facilitate the establishment of accurate classification of the SDF strains. It will also allow responsible exploitation of the extraordinary AEFB biotechnological potential, reliable use as insect control agents, and handling of animal pathogens.

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Supplementary material 1

Molecular, biochemical, and physiological profiles of SDF strains belonging to the AEFBC

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Data type: molecular, biochemical, and physiological data.

Link: https://doi.org/10.3897/neotropical.18.e86548.suppl1