

Detecting dipicolinic acid production and biosynthesis pathways in Bacilli and Clostridia

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1

2 **ABSTRACT** Bacterial endospores are highly resistant structures and dipicolinic acid is a key component of
3 their resilience and stability. Due to the difficulty in controlling endospore contaminants, they are of interest
4 in clean rooms, food processing, and production industries, while beneficial endospore-formers are sought
5 for potential utility. Dipicolinic acid production has traditionally been recognized in Bacilli, Clostridia, and
6 Paenibacilli. Here, sixty-seven strains of aerobic and anaerobic endospore-forming bacteria belonging to the
7 genera *Bacillus*, *Brevibacillus*, *Clostridium*, *Fontibacillus*, *Lysinibacillus*, *Paenibacillus*, *Rummeliibacillus*, and
8 *Terribacillus* were grown axenically and sporulated biomasses were assayed for dipicolinic acid production
9 using fluorimetric detection. Strains testing positive were sequenced and the genomes analyzed to identify
10 dipicolinic acid biosynthesis genes. The well-characterized biosynthesis pathway was conserved in 59 strains
11 of Bacilli and Paenibacilli as well as two strains of Clostridia; six strains of Clostridia lacked homologs to genes
12 recognized as involved in dipicolinic acid biosynthesis. Our results confirm dipicolinic acid production across
13 different classes and families of Firmicutes. We find that members of *Clostridium* (cluster I) lack recognized
14 dipicolinic acid biosynthesis genes and propose an alternate genetic pathway in these strains. Finally, we
15 explore why the extent and mechanism of dipicolinic acid production in endospore-forming bacteria should
16 be fully understood. We believe that understanding the mechanism by which dipicolinic acid is produced can
17 expand the methods to utilize endospore-forming bacteria, such as novel bacterial strains added to products,
18 for genes to create inputs for the polymer industry and to be better equipped to control contaminating spores
19 in industrial processes.

20

KEYWORDS

Bacilli
Clostridia
Dipicolinic Acid
Endospore
Iron-sulfur flavo-
protein

1 INTRODUCTION

2 Bacterial spores are highly resistant structures in a dormant state,
3 with little, if any, detectable metabolic activity (McKenney *et al.*
4 2013) (Figure 1). Spores are formed in response to adverse environ-
5 mental conditions and spore formation, or sporulation, generally
6 occurs when bacteria are challenged by nutritional stress (Driks
7 2002; Errington 2003; Piggot and Hilbert 2004). Spores can remain
8 alive for extended periods of time and possess the ability to reac-
9 tivate if nutrients become available and conditions are favorable
10 (Moir 2003; Setlow 2003; Paredes-Sabja *et al.* 2011). Spores can
11 survive many conditions that would otherwise destroy vegetative

cells. The unique architecture of spores, such as the spore coat,
12 cortex, and core, explains in large part their resistance to stresses
13 and their ability to survive under extreme conditions (Henriques
14 and Moran 2007).
15

Firmicutes are characterized by their ability to produce en-
16 dospores (Figure 2-6), and compared to other spore-forms en-
17 dospores are many times more resistant to oxidizing agents, heat,
18 desiccation, and radiation (Setlow 1995). Pyridine-2,6-dicarboxylic
19 acid, or dipicolinic acid (DPA) is an important component of bac-
20 terial endospores (Powell 1953). It has been shown that DPA is
21 located in the spore core, and can represent 5–14% of endospore dry
22 weight (Murrell 1969). DPA is maintained within intact endospores,
23 and can be degraded under aerobic (Arima and Kobayashi 1962;
24 Taylor and Amador 1988; Amador and Taylor 1990) and anaerobic
25 (Seyfried and Schink 1990) conditions after it is released during
26 spore germination. Dipicolinic acid forms a complex with calcium
27

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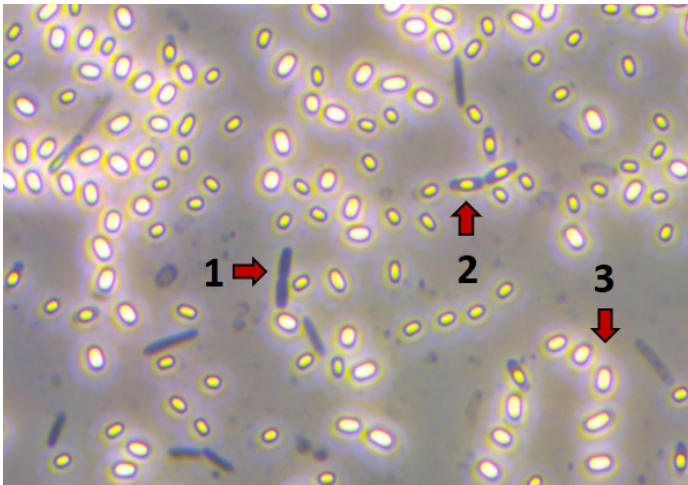


Figure 1 Phase-contrast microscopy (100X) of *Bacillus* sp. AS739 with examples of vegetative cells (1), endospores (2), and mature spores (3) visible.



Figure 2 Phase-contrast microscopy (100X) of *Bacillus marisflavi* AS47 with endospores visible throughout.

ions within the endospore core. This complex binds free water molecules causing dehydration of the spore. As a result, the heat resistance of macromolecules within the core increases (Gerhardt 1989). In addition, the calcium-dipicolinic acid complex also functions to protect DNA from heat denaturation by inserting itself between the nucleobases, thereby increasing the stability of DNA (Moeller et al. 2014).

In Bacilli, the DPA biosynthetic pathway has been well characterized (Wolska et al. 2007). In *B. subtilis*, the DPA synthetic pathway is encoded by four operons: *dapG*, *asd*, *dapA* and *dpaAB*. Aspartate kinase, encoded by *dapG*, is responsible for the first step of the biosynthesis cascade, producing L-4-aspartyl phosphate from L-aspartate. Aspartate-semialdehyde dehydrogenase, encoded by *asd*, is responsible for the second step, producing L-aspartate 4-semialdehyde. Dihydrodipicolinate synthase, encoded by *dapA* is responsible for the third step, producing L-2,3-dihydrodipicolinate (Takahashi et al. 2015). These steps are also used in lysine biosynthesis. DPA synthase which is responsible for the production of DPA, is encoded by *dpaAB*. Dipicolinate synthase subunit A (*dpaA*, otherwise known as *spoVFA*) encodes a putative dehydrogenase, and dipicolinate synthase subunit B (*dpaB*, otherwise known as *spoVFB*) appears to be a flavoprotein (Daniel and Errington 1993).

The major genera identified as endospore forming bacteria include *Bacillus*, *Paenibacillus*, and *Clostridium* (Fritze 2004; Logan and Halket 2011; Galperin 2013). Since 1990, the genus *Bacillus* has been split into several families and genera of endospore-forming bacteria based on 16S rRNA analysis (Galperin 2013). The unifying characteristic of these bacteria is that they are Gram-positive, form endospores, and aerobic. An increased concentration of DPA is a biochemical hallmark for endospore-forming bacteria such as *B. subtilis* (Piggot and Hilbert 2004). We therefore hypothesized that strains capable of sporulating and previously classified as *Bacillus*, such as *Brevibacillus*, *Fontibacillus*, *Lysinibacillus*, *Rummeliibacillus*, and *Terribacillus* should produce DPA and contain the same set of genes responsible for DPA synthesis in Bacilli.

Work by others has shown that members of the *Clostridium sensu stricto* (cluster I) lack genes with significant homology to *dpaAB* (Stragier 2002; Onyenwoke et al. 2004) but nevertheless produce DPA during sporulation (Paredes-Sabja et al. 2008). This cluster is particularly important due to the presence of industrially useful

strains such as *Clostridium acetobutylicum* and *C. beijerinckii*, as well as human pathogens such as *C. perfringens*, *C. botulinum* and *C. tetani*. Osburn et al. (2010) implicated an electron transfer flavoprotein α -chain (*etfA*) that is directly involved in DPA synthesis in *C. perfringens* using a modified version of the Bach and Gilvarg assay system (Bach and Gilvarg 1966; Orsburn et al. 2010). We hypothesized that other members of the *Clostridium* (cluster I) such as *C. beijerinckii*, *C. carboxidivorans*, *C. scatologenes*, and *C. tyrobutyricum*, use the same or similar biosynthesis pathways.

DPA can be detected by a range of analytical techniques (Beverly et al. 1996; Yilmaz et al. 2010; Cowcher et al. 2013; Wang et al. 2011). The terbium dipicolinate fluorescence method (Rosen et al. 1997; Hindle and Hall 1999) is both tractable and sensitive, allowing the detection of nanomolar concentrations. This technique is therefore appropriate for testing if the genera hypothesized to produce DPA (*Brevibacillus*, *Fontibacillus*, *Lysinibacillus*, *Rummeliibacillus*, and *Terribacillus*) do in fact have the capacity for DPA-production. The terbium dipicolinate fluorescence method can also be used to establish that Clostridia such as *C. beijerinckii*, *C. carboxidivorans*, *C. scatologenes*, and *C. tyrobutyricum* produce DPA prior to sequencing and genomic evaluation to look for *dpaAB* and *etfA* DPA biosynthesis gene homologues.

In the present study, bacteria isolated from soil and a microbial product were taxonomically identified using 16S rRNA sequence analyses. Presumptive endospore-forming bacteria were then screened for DPA production *in vitro*. DPA detection was followed by whole genome-sequencing to confirm taxonomic identifications at the species-level and to examine whether known DPA biosynthesis pathways were present.

MATERIALS AND METHODS

Strain Isolation and Initial Identification

All strains used were isolated from either bulk soil or from a sample of HYT® A (Table S1) (Agrinos, <https://agrinos.com>). Strains were then repeatedly streaked onto semi-solid media until isogenic. Initial taxonomic classifications were made using 16S rRNA gene sequences. Full length 16S genes were amplified from each strain using genomic DNA and/or colonies as the PCR template (27F, 5'-AGRGTTCGATCMTGGCTCAG-3'; 1492R, 5'-GGTTACCTTGTTACGACTT-3'; following (Singer et al. 2016) with minor modifications). PCR products were sequenced directly

using the forward and reverse PCR primers. The high-quality sequence traces were then analyzed and a BLAST analysis was performed using the NCBI 16S ribosomal RNA sequence database (Madden 2013).

Terbium Dipicolinate Fluorescence Assay

Prior to testing, strains were grown as follows. Aerobic strains were cultured on Trypticase Soy Agar (i.e. TSA; Tryptone 17 g/L, Soytone 3 g/L, Dextrose 2.5 g/L, Sodium chloride 5 g/L, Dipotassium phosphate 2.5 g/L, Agar 15 g/L) in a static incubator at 30° for up to three days. Anaerobic strains were cultured on Reinforced Clostridial Medium (i.e. RCM; BD Difco™ Reinforced Clostridial Medium, Cat No. 218081) in a static incubator at 35° for up to five days.

All cultures were visualized using phase-contrast microscopy at 100X magnification to verify the presence of refractile spores (Bulla *et al.* 1969). A modified version of the protocol used by Hindle and Hall (1999) was used to detect the presence of DPA. Using a sterile disposable loop, a 1 µl loopful was taken off a plate and resuspended in 10 ml of sodium acetate buffer (0.2 M, pH 5) in screw cap glass test tubes. Samples were autoclaved for 15 minutes at 121° to heat the spores and release any DPA. Samples were then cooled for 10 min in a room temperature (22°) water bath. Following cooling, for each sample 100 µl was mixed with 100 µl of terbium chloride solution (TbCl₃, 30 µM) in wells of a 96-well flat-bottomed clear microtiter plate. Fluorescence was measured with a BioTek® Cytation 5 Multi-Mode Reader (BioTek Instruments, Inc., <https://www.biotek.com/>) using time resolved fluorescence (delay 50 µs, excitation wavelength 272 nm, emission wavelength 540 nm) (Brandes Ammann *et al.* 2011). Strains testing higher than negative controls, with a fluorescence intensity of more than 10,000 relative fluorescence units, and endospores visible with phase-contrast microscopy were considered DPA-producers.

Whole-genome sequencing and Bioinformatic Analysis

Whole-genome sequencing was performed by CoreBiome (CoreBiome, Inc., <https://www.corebiome.com>). Briefly, isolates were grown in appropriate liquid culture. A minimum of 1 x 10⁹ cells were spun down and the supernatant removed prior to freezing at -20°. Samples were then sent to CoreBiome for DNA extraction, followed by whole-genome sequencing, assembly, and annotation using their StrainView™ whole-genome shotgun sequencing service. Genome assembly quality was assessed using QUAST (Gurevich *et al.* 2013). Whole-genome sequence annotation was performed using Prokka (<https://github.com/tseemann/prokka>) (Seemann 2014). 16S sequences were identified using Barrnap (<https://github.com/tseemann/barrnap>). To assign function to CDS features, a core set of well characterized proteins are first searched using BLAST+, then a series of slower but more sensitive HMM databases are searched using HMMER3 (<http://hmmer.org>) (Finn *et al.* 2011).

Phylogenetics

For each sequence, a BLAST analysis was performed using the NCBI 16S ribosomal RNA sequence database (Madden 2013). To make species-level identifications, representative genomes were retrieved from NCBI and the whole-genome similarity metric Average Nucleotide Identity (ANI) was calculated for top 16S BLAST hits. Whole-genome comparisons with ANI values of greater than 95% were considered to be the same species (Konstantinidis and Tiedje 2005; Goris *et al.* 2007). For whole-genome comparisons with ANI values less than 95%, strains were labeled as *Genus sp. ()*, with

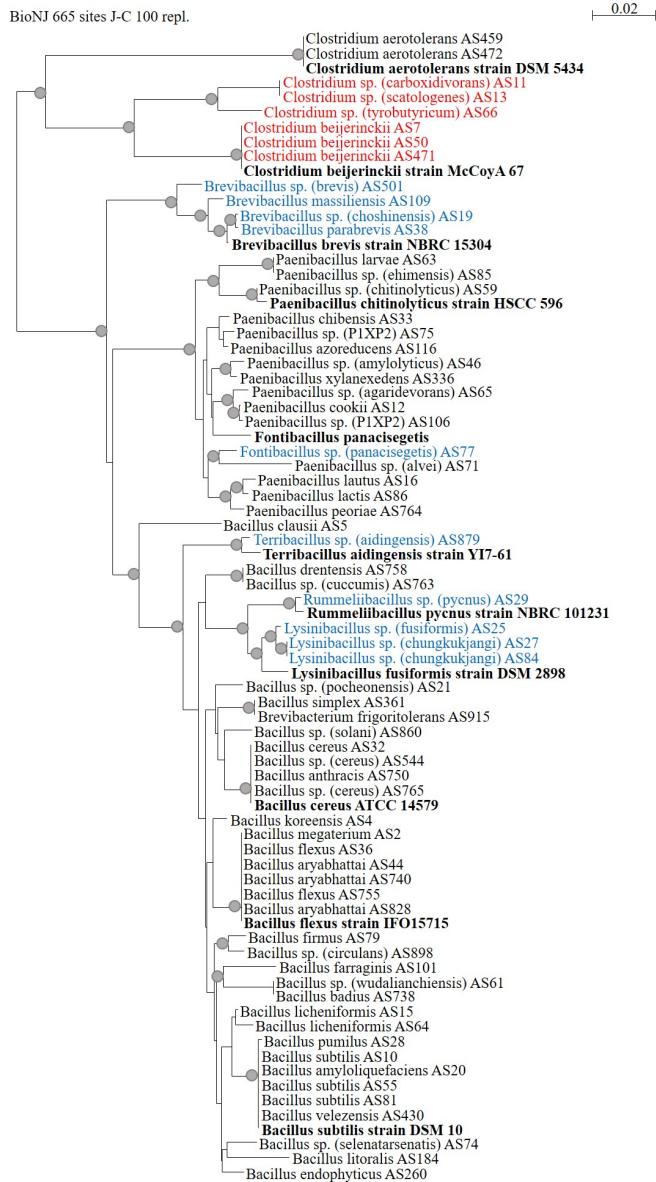


Figure 3 Neighbor-joining tree representing 16S rRNA-based phylogenetic distribution of strains assayed herein. Reference strains are highlighted in bold. Recently re-classified *Bacilli* and *Paenibacilli* experimentally demonstrated as producing DPA are highlighted in blue. Strains where known DPA synthase genes were not detectable are highlighted in red. Bootstrap support of >70% is noted by grey circles at branch points (Hillis and Bull 1993).

1 the closest known relative indicated. Using Seaview Version 4.6.1
2 (Gouy *et al.* 2009; Galtier *et al.* 1996), 16S sequences were aligned
3 using MUSCLE (Edgar 2004) and BioNJ trees (Gascuel 1997) were
4 computed using Jukes & Cantor (Jukes and Cantor 1969) pairwise
5 phylogenetic distances with all gap containing sites excluded from
6 the analysis.

7 Data Availability

8 Table S1 contains a list of all strains used in this study, RFUs
9 measured using the terbium-DPA fluorescence assay, and DPA
10 biosynthesis gene copy-number for each strain. File S1 contains an
11 alignment of 16S rRNA sequences. File S2 contains an alignment
12 of *dpaA* protein sequences. File S3 contains an alignment of *dpaB*
13 protein sequences. File S4 contains an alignment of *Isf* protein
14 sequences.

15 RESULTS

16 Of the 67 strains included in this study (Table S1), all were Firmi-
17 cutes representing the classes of Bacilli, Clostridia and Paenibacilli.
18 As expected, all 49 strains of *Bacillus* and *Paenibacillus* (Figure 3,
19 in black) were found to form endospores via phase-contrast mi-
20 croscopy (see Figures 1 and 2). DPA biosynthesis was detected *in*
21 *vitro* (Table S1), and genome sequences revealed that all of these
22 strains possessed known DPA synthase genes (Files S2 & S3).

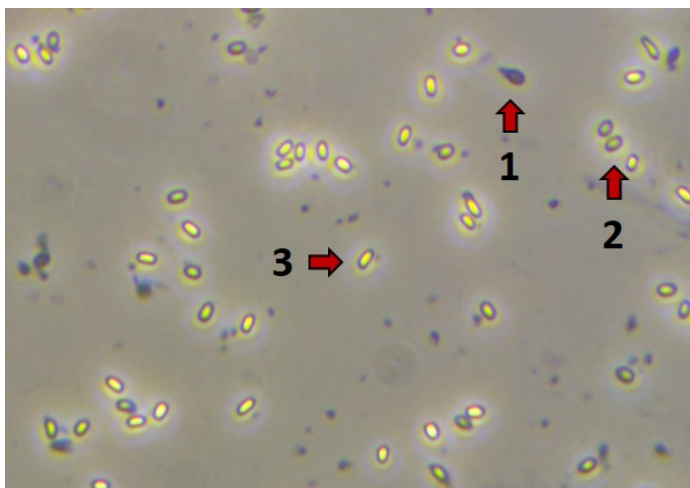


Figure 4 Phase-contrast microscopy (100X) of *Terribacillus* sp. (*aidingensis*) AS879 with examples of vegetative cells (1), endospores (2), and mature spores (3) visible.

23 Ten strains from five genera that were re-classified from *Bacillus*
24 to *Brevibacillus*, *Fontibacillus*, *Lysinibacillus*, *Rummeliibacillus*, and
25 *Terribacillus* which were hypothesized to produce DPA (Figure 3,
26 highlighted in blue) were found to form endospores via phase-
27 contrast microscopy (see Figure 4). DPA biosynthesis was detected
28 *in vitro* (Table S1), and genome sequences revealed that all of these
29 strains possessed known DPA synthase genes, *dpaAB* (Files S2 &
30 S3).

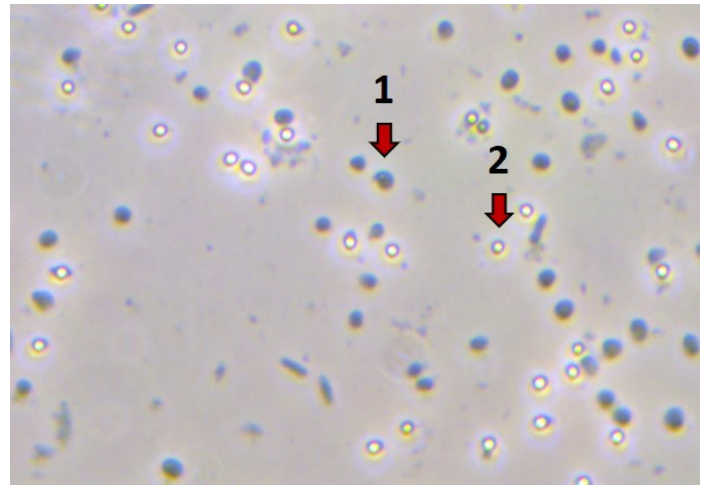


Figure 5 Phase-contrast microscopy (100X) of *Clostridium aerotolerans* AS472 with examples of vegetative cells (1) and mature spores (2) visible.

31 Eight strains of Clostridia which were expected to produce DPA
32 were found to form endospores via phase-contrast microscopy
33 (see Figures 5 and 6). DPA biosynthesis was detected *in vitro*
34 (Table S1), and genome sequences revealed that two strains of
35 *Clostridium aerotolerans* possessed homologs to known DPA sythase
36 genes, *dpaAB* (Files S2 & S3). Genome sequences from the remain-
37 ing six Clostridia (Figure 3, highlighted in red) did not have
38 detectable known *dpaAB* or *etfA* genes (Table 1). *etfA* contains a
39 flavin mononucleotide (FMN), therefore a search was performed
40 for other flavoproteins that contained a FMN and were common
41 among the six Clostridia that lacked homologs to known DPA
42 synthesis genes.

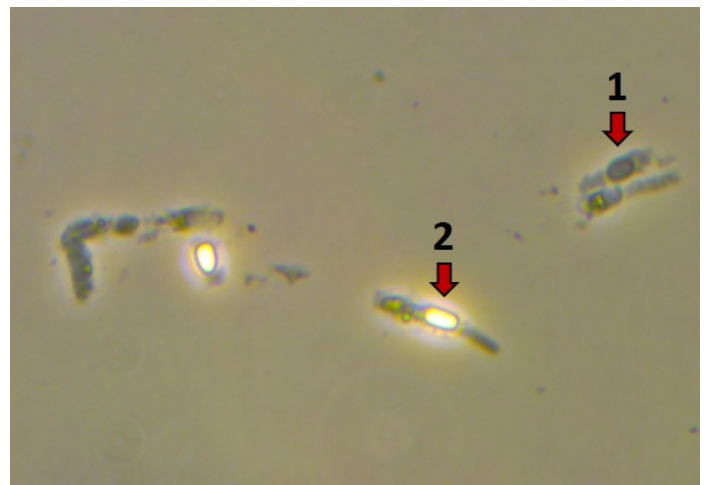


Figure 6 Phase-contrast microscopy (100X) of *Clostridium scatologenes* AS13 with examples of vegetative cells (1) and mature spores (2) visible.

43 While the *Clostridium* (cluster I) strains lack the recognized DPA
44 biosynthesis genes, they all possess at least one copy of an iron-
45 sulfur flavoprotein (*Isf*) (File S4), and strains with more *Isf* copies
46 produced more DPA (Table 1). Both *etfA* and *Isf* are thought to be
47 involved in electron transport (Tsai and Saier Jr 1995; Becker *et al.*
48 1998). It is possible that these two genes have analogous activity,

Table 1 DPA biosynthesis gene copy count and levels of DPA detected in strains of *Clostridium*

Taxonomy ^a	<i>dpaA</i> ^b	<i>dpaB</i> ^c	<i>etfA</i> ^d	<i>Isf</i> ^e	RFU ^f
<i>Clostridium beijerinckii</i> AS471	0	0	0	10	103,839
<i>Clostridium beijerinckii</i> AS50	0	0	0	11	386,574
<i>Clostridium beijerinckii</i> AS7	0	0	0	6	65,340
<i>Clostridium sp. (carboxidivorans)</i> AS11	0	0	0	1	48,558
<i>Clostridium sp. (scatologenes)</i> AS13	0	0	0	1	45,462
<i>Clostridium sp. (tyrobutyricum)</i> AS66	0	0	0	1	47,567

^a For strains denoted as sp., the species in parenthesis is the closest recognized species

^b Dipicolinic acid synthase subunit A

^c Dipicolinic acid subunit B

^d Electron transfer flavoprotein α -chain

^e Iron-sulfur flavoprotein

^f Relative fluorescence units

1 catalyzing the formation of DPA from L-2,3-dihydrodipicolinate
 2 (Figure 7). Future work including *Isf* gene knockout mutants
 3 that lack DPA production and/or observing upregulation of *Isf*
 4 expression during sporulation would be consistent with *Isf* having
 5 a role in DPA formation.

6 DISCUSSION

7 Here we have found that genera was a good predictor of DPA
 8 production, and added experimental evidence to support the
 9 production of DPA by additional Bacillaceae. This result, which is
 10 consistent with their previous classification as *Bacillus*, is yet an
 11 important observation given the functional diversity that these
 12 genera possess. In addition, we found the *Clostridium* (cluster I)
 13 lack recognized biosynthesis pathways for DPA production. This
 14 result, while similar to previous work (Orsburn *et al.* 2010), fails to
 15 confirm their findings and we propose an alternate genetic path-
 16 way in these strains.

17 Members of the Bacillaceae family found to produce DPA have
 18 recently been shown to possess many functions and utilities, and
 19 the survivability that DPA production adds, makes these strains
 20 ripe for use in novel products. For example, strains of *Brevibacil-
 21 lus* have been found to have larvicidal activity (Zubasheva *et al.*
 22 2010), biological control of plant pathogens (Chandel *et al.* 2010;
 23 Liu *et al.* 2010), as well as improving shelf-life of fruits (Che *et al.*
 24 2011). *Fontibacillus* strains have been shown to produce a novel
 25 and highly reusable pullanase (Alagöz *et al.* 2016) which is used as
 26 a processing aid for the production of ethanol or sweeteners from
 27 grain. *Lysinibacillus* species have been shown to degrade harmful
 28 insecticides (Singh *et al.* 2012), selectively desulfurize dibenzoth-
 29 iophene (Bahuguna *et al.* 2011), and produce mosquito larvicidal
 30 toxins (Lozano *et al.* 2011). In addition, strains can produce 10-
 31 hydroxystearic acid from olive oil (Kim *et al.* 2012). *Rummeliibacil-
 32 lus* species have been shown to transform palm oil mill effluent
 33 to polyhydroxyalkanoate and biodiesel (Junpadit *et al.* 2017), en-
 34 hance growth and health in tilapia (Yih *et al.* 2019), and has the
 35 potential for biomineralization (Mudgil *et al.* 2018). Finally, *Ter-
 36 ribacillus* species have been found to produce a novel chitinase as
 37 well as antifungal enzymes that repress plant diseases (Essghaier
 38 *et al.* 2014). Given the wealth of functionalities that this family pos-
 39 sesses, coupled with a long shelf-life and potential to survive in a
 40 variety of manufacturing processes due to DPA production, makes
 41 Bacillaceae well suited for industrial processes and an untapped
 42 well of biotechnology potential.

We have confirmed here that some *Clostridia* possess a DPA
 synthase homolog. We have also confirmed that *Clostridium* (clus-
 ter I) do not possess a DPA synthase homolog. Unlike previous
 findings, we were unable to identify *etfA* in our assayed *Clostridium*
 (cluster I) strains. Despite the lack of *dpaAB* and *etfA* genes, these
 strains were still able to produce detectable quantities of DPA.

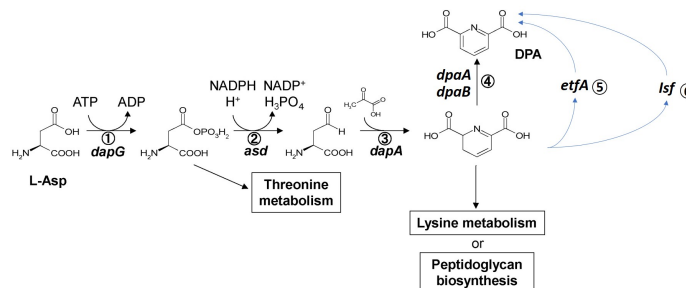


Figure 7 Dipicolinic acid biosynthesis pathways. ¹*dapG*: aspar-
 tokinase; ²*asd*: aspartate-semialdehyde dehydrogenase; ³*dapA*:
 dihydrodipicolinate synthase; ⁴*dpaAB*: dipicolinate synthase. ⁵*etfA*:
 electron transfer flavoprotein subunit A; ⁶*Isf*: iron-sulfur flavoprotein.
 Modified from (Takahashi *et al.* 2015).

We propose the *Isf* gene product as an alternate pathway for
 DPA-production in *Clostridium* (cluster I). This alternate biosynthe-
 sis pathway for DPA synthesis represents a new potential route for
 bioengineering the production of DPA in other strains of bacteria
 via the addition of a single gene to the lysine pathway, particu-
 larly in anaerobes. Dipicolinic acid production has been engi-
 neered in bacterial strains using the traditional *dpaAB* genes (Zelder
et al. 2009; McClintock *et al.* 2018). DPA has numerous industrial
 uses, for example, the monomer can be used as a replacement for
 petroleum derived isophthalic acid in the synthesis of polyesters
 or polyamide copolymers (McClintock *et al.* 2018) resulting in a
 more biodegradable compound. The scalable synthesis of DPA
 using a variety of bacteria could increase its use as a replacement
 for other aromatic dicarboxylic acids.

Probiotics have been developed for human use as well as for
 use in animal feeds, and a large number of these formulations
 utilize spore-forming *Bacillus* (Hong *et al.* 2005). Effective probi-
 otics need to thrive in anaerobic conditions and retain viability in
 aerobic conditions on the shelf, as well as cross the gastric barrier

1 and enter the upper GI tract in a viable state following consump-
2 tion. Probiotics utilizing DPA-producing endospores are proven
3 to have better survivability during passage through the acidic
4 stomach environment, show better survival during manufacturing,
5 and have a longer shelf-life (Bader *et al.* 2012). The potential of
6 Clostridia as probiotics has only recently been discussed (Cartman
7 2011). DPA-containing Clostridial endospores are ideal vehicles
8 for overcoming the challenges probiotic bacteria encounter, as they
9 can survive aerobic storage and remain viable while crossing the
10 host gastric barrier, followed by germination in the anaerobic en-
11 vironment of the upper GI tract where they are well adapted to
12 survive. *Clostridium* have been documented as members of healthy
13 GI tracts in humans (Arumugam *et al.* 2011; Qin *et al.* 2010; Tap
14 *et al.* 2009), have been shown to increase resistance against Irritable
15 Bowel Disease (Atarashi *et al.* 2011), and can protect against *C. dif-*
16 *ficile* infection (Merrigan *et al.* 2009; Sambol *et al.* 2002). Given the
17 survivability of DPA-producing Clostridia, and the recently high-
18 lighted benefits of *Clostridium* as members of the gut microbiota,
19 they may prove to be an ideal probiotic.

20 In addition, an alternate biosynthesis pathway for DPA rep-
21 represents a new target for designing drugs to prevent contamina-
22 tion, as inhibiting the production of DPA would make endospores
23 more susceptible to traditional manufacturing procedures. Many
24 of the endospore-forming bacteria can survive food processing,
25 decreasing the shelf-life of processed foods (e.g. *Clostridium ty-*
26 *robotyricum*), and potentially making them unsafe to consume (e.g.
27 *Clostridium botulinum*) (Daelman *et al.* 2013; Soni *et al.* 2016; André
28 *et al.* 2017). Endospore-forming *Clostridium* also contain members
29 that are known for their pathogenic potential, such as *Clostridium*
30 (e.g. *C. perfringens*, *C. novyi*, *C. septicum*, *C. tetani*, and *C. difficile*)
31 (Ehling-Schulz *et al.* 2019; Wells and Wilkins 1996).

32 Here we have added experimental evidence to support the pro-
33 duction of DPA by additional Bacillaceae and we have proposed an
34 alternate pathway in *Clostridium* (cluster I) strains that lack recog-
35 nized dipicolinic acid biosynthesis genes. By fully understanding
36 the breadth and mechanisms by which DPA is produced, we can
37 utilize endospore-forming bacteria in new ways: as novel bacterial
38 strains added to products, for genes to create inputs for the poly-
39 mer industry, and to be better equipped to control contaminating
40 spores in industrial processes.

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49 LITERATURE CITED

50 Alagöz, D., D. Yildirim, H. K. Güvenmez, D. Sihay, and S. S. Tükel,
51 2016 Covalent immobilization and characterization of a novel
52 pullulanase from fontibacillus sp. strain dshk 107 onto florasil®
53 and nano-silica for pullulan hydrolysis. *Applied Biochemistry*
54 *and Biotechnology* **179**: 1262–1274.
55 Amador, J. A. and B. F. Taylor, 1990 Coupled metabolic and pho-
56 tolytic pathway for degradation of pyridinedicarboxylic acids,
57 especially dipicolinic acid. *Applied and Environmental Microbi-*
58 *ology* **56**: 1352–1356.

André, S., T. Vallaey, and S. Planchon, 2017 Spore-forming bacte-
ria responsible for food spoilage. *Research in Microbiology* **168**:
379–387.
Arima, K. and Y. Kobayashi, 1962 Bacterial oxidation of dipicolinic
acid. *Journal of Bacteriology* **84**: 759–764.
Arumugam, M., J. Raes, E. Pelletier, D. Le Paslier, T. Yamada, *et al.*,
2011 Enterotypes of the human gut microbiome. *Nature* **473**:
174.
Atarashi, K., T. Tanoue, T. Shima, A. Imaoka, T. Kuwahara,
et al., 2011 Induction of colonic regulatory t cells by indigenous
clostridium species. *Science* **331**: 337–341.
Bach, M. L. and C. Gilvarg, 1966 Biosynthesis of dipicolinic acid
in sporulating bacillus megaterium. *Journal of Biological Chem-*
istry **241**: 4563–4564.
Bader, J., A. Albin, and U. Stahl, 2012 Spore-forming bacteria and
their utilisation as probiotics. *Beneficial Microbes* **3**: 67–75.
Bahuguna, A., M. K. Lily, A. Munjal, R. N. Singh, and K. Dang-
wal, 2011 Desulfurization of dibenzothiophene (dbt) by a novel
strain lysinibacillus sphaericus dmt-7 isolated from diesel con-
taminated soil. *Journal of Environmental Sciences* **23**: 975–982.
Becker, D. F., U. Leartsakulpanich, K. K. Surerus, J. G. Ferry, and
S. W. Ragsdale, 1998 Electrochemical and spectroscopic prop-
erties of the iron-sulfur flavoprotein from methanosarcina ther-
mophila. *Journal of Biological Chemistry* **273**: 26462–26469.
Beverly, M. B., F. Basile, K. J. Voorhees, and T. L. Hadfield, 1996 A
rapid approach for the detection of dipicolinic acid in bacterial
spores using pyrolysis/mass spectrometry. *Rapid Communica-*
tions in Mass Spectrometry **10**: 455–458.
Brandes Ammann, A., L. Kölle, and H. Brandl, 2011 Detection of
bacterial endospores in soil by terbium fluorescence. *Internat-*
ional Journal of Microbiology **2011**: 1–5.
Bulla, L., G. S. Julian, R. Rhodes, and C. Hesselstine, 1969 Scan-
ning electron and phase-contrast microscopy of bacterial spores.
Applied and Environmental Microbiology **18**: 490–495.
Cartman, S. T., 2011 Time to consider clostridium probiotics? *Fu-*
ture Microbiology **6**: 969–971.
Chandel, S., E. J. Allan, and S. Woodward, 2010 Biological control of
fusarium oxysporum f. sp. lycopersici on tomato by brevibacillus
brevis. *Journal of Phytopathology* **158**: 470–478.
Che, J.-m., X.-f. Zheng, B. Liu, M.-x. Su, and Y.-j. Zhu, 2011 Prepa-
ration of brevibacillus brevis fjat-0809-glx antigen and study on
its effect on loquats (eriobotrya japonica). *Storage & Process* **5**.
Cowcher, D. P., Y. Xu, and R. Goodacre, 2013 Portable, quantitative
detection of bacillus bacterial spores using surface-enhanced
raman scattering. *Analytical Chemistry* **85**: 3297–3302.
Daelman, J., A. Vermeulen, T. Willems, R. Ongenaert, L. Jacxsens,
et al., 2013 Growth/no growth models for heat-treated psy-
chrotrophic bacillus cereus spores under cold storage. *Internat-*
ional Journal of Food Microbiology **161**: 7–15.
Daniel, R. and J. Errington, 1993 Cloning, dna sequence, functional
analysis and transcriptional regulation of the genes encoding
dipicolinic acid synthetase required for sporulation in bacillus
subtilis. *Journal of Molecular Biology* **232**: 468–483.
Driks, A., 2002 Overview: development in bacteria: spore forma-
tion in bacillus subtilis. *Cellular and Molecular Life Sciences* **59**:
389–391.
Edgar, R. C., 2004 MUSCLE: multiple sequence alignment with
high accuracy and high throughput. *Nucleic Acids Research* **32**:
1792–1797.
Ehling-Schulz, M., D. Lereclus, and T. M. Koehler, 2019 The bacil-
lus cereus group: Bacillus species with pathogenic potential.
Microbiology Spectrum **7**: 1–59.

- 1 Errington, J., 2003 Regulation of endospore formation in *Bacillus*
2 *subtilis*. *Nature Reviews Microbiology* **1**: 117–126.
- 3 Essghaier, B., C. Dhieb, H. Rebib, S. Ayari, A. R. A. Boudabous,
4 *et al.*, 2014 Antimicrobial behavior of intracellular proteins from
5 two moderately halophilic bacteria: strain j31 of *Terribacillus*
6 *halophilus* and strain m3-23 of *Virgibacillus marismortui*. *Journal*
7 *of Plant Pathology & Microbiology* **5**: 1–7.
- 8 Finn, R. D., J. Clements, and S. R. Eddy, 2011 Hmmer web server:
9 interactive sequence similarity searching. *Nucleic acids research*
10 **39**: W29–W37.
- 11 Fritze, D., 2004 Taxonomy of the genus *Bacillus* and related gen-
12 era: the aerobic endospore-forming bacteria. *Phytopathology* **94**:
13 1245–1248.
- 14 Galperin, M. Y., 2013 Genome diversity of spore-forming firmi-
15 cutes. *Microbiology Spectrum* **1**: 1–27.
- 16 Galtier, N., M. Gouy, and C. Gautier, 1996 SEAVIEW and
17 PHYLO_WIN: two graphic tools for sequence alignment and
18 molecular phylogeny. *Bioinformatics* **12**: 543–548.
- 19 Gascuel, O., 1997 BIONJ: an improved version of the NJ algorithm
20 based on a simple model of sequence data. *Molecular Biology*
21 *and Evolution* **14**: 685–695.
- 22 Gerhardt, P., 1989 Spore thermoresistance mechanisms. In *Regula-*
23 *tion of Prokaryotic Development*, edited by I. Smith, R. Slepecky,
24 and P. Setlow, pp. 17–37, American Society for Microbiology,
25 Washington, D.C.
- 26 Goris, J., K. T. Konstantinidis, J. A. Klappenbach, T. Coenye, P. Van-
27 damme, *et al.*, 2007 Dna–dna hybridization values and their rela-
28 tionship to whole-genome sequence similarities. *International*
29 *Journal of Systematic and Evolutionary Microbiology* **57**: 81–91.
- 30 Gouy, M., S. Guindon, and O. Gascuel, 2009 SeaView Version 4:
31 A Multiplatform Graphical User Interface for Sequence Align-
32 ment and Phylogenetic Tree Building. *Molecular Biology and*
33 *Evolution* **27**: 221–224.
- 34 Gurevich, A., V. Saveliev, N. Vyahhi, and G. Tesler, 2013 Quast:
35 quality assessment tool for genome assemblies. *Bioinformatics*
36 **29**: 1072–1075.
- 37 Henriques, A. O. and C. P. Moran, Jr., 2007 Structure, assembly,
38 and function of the spore surface layers. *Annual Review of*
39 *Microbiology* **61**: 555–588.
- 40 Hillis, D. M. and J. J. Bull, 1993 An Empirical Test of Bootstrapping
41 as a Method for Assessing Confidence in Phylogenetic Analysis.
42 *Systematic Biology* **42**: 182–192.
- 43 Hindle, A. and E. H. Hall, 1999 Dipicolinic acid (dpa) assay revis-
44 ited and appraised for spore detection. *Analyst* **124**: 1599–1604.
- 45 Hong, H. A., L. H. Duc, and S. M. Cutting, 2005 The use of bacterial
46 spore formers as probiotics. *FEMS Microbiology Reviews* **29**:
47 813–835.
- 48 Jukes, T. H. and C. R. Cantor, 1969 Evolution of protein molecules.
49 In *Mammalian Protein Metabolism*, edited by H. N. Munro, vol-
50 ume 3, pp. 21–132, Academic Press, New York.
- 51 Junpadit, P., T. T. Suksaroj, and P. Boonsawang, 2017 Transforma-
52 tion of palm oil mill effluent to terpolymer polyhydroxyalka-
53 noate and biodiesel using *Rummeliibacillus pycnus* strain ts 8.
54 *Waste and Biomass Valorization* **8**: 1247–1256.
- 55 Kim, B.-N., Y.-C. Joo, Y.-S. Kim, K.-R. Kim, and D.-K. Oh, 2012 Pro-
56 duction of 10-hydroxystearic acid from oleic acid and olive oil hy-
57 drolyzate by an oleate hydratase from *Lysinibacillus fusiformis*.
58 *Applied Microbiology and Biotechnology* **95**: 929–937.
- 59 Konstantinidis, K. T. and J. M. Tiedje, 2005 Genomic insights that
60 advance the species definition for prokaryotes. *Proceedings of*
61 *the National Academy of Sciences* **102**: 2567–2572.
- 62 Liu, E., L. Bo, Q. Xiao, G. Feng, W. Yan, *et al.*, 2010 Effects of the
mixed inoculation of different biocontrol strains on colonization
in tobacco and control of tobacco bacterial wilt. *Chinese Journal*
of *Biological Control* **26**: 200–205.
- Logan, N. A. and G. Halket, 2011 Developments in the taxonomy
of aerobic, endospore-forming bacteria. In *Endospore-forming Soil*
Bacteria, edited by N. A. Logan and P. Vos, volume 3, pp. 1–29,
Springer, New York.
- Lozano, L. C., J. A. Ayala, and J. Dussán, 2011 *Lysinibacillus*
sphaericus s-layer protein toxicity against *Culex quinquefascia-*
tus. *Biotechnology Letters* **33**: 2037–2041.
- Madden, T., 2013 The blast sequence analysis tool. In *The NCBI*
Handbook [Internet]. 2nd edition, National Center for Biotechnol-
ogy Information (US).
- McClintock, M. K., G. W. Fahnhorst, T. R. Hoye, and K. Zhang,
2018 Engineering the production of dipicolinic acid in *E. coli*.
Metabolic Engineering **48**: 208–217.
- McKenney, P. T., A. Driks, and P. Eichenberger, 2013 The *Bacillus*
subtilis endospore: assembly and functions of the multilayered
coat. *Nature Reviews Microbiology* **11**: 33.
- Merrigan, M. M., S. P. Sambol, S. Johnson, and D. N. Gerding,
2009 New approach to the management of *Clostridium difficile*
infection: colonisation with non-toxicogenic *C. difficile* during daily
ampicillin or ceftriaxone administration. *International Journal*
of *Antimicrobial Agents* **33**: S46–S50.
- Moeller, R., M. Raguse, G. Reitz, R. Okayasu, Z. Li, *et al.*, 2014
Resistance of *Bacillus subtilis* spore DNA to lethal ionizing radi-
ation damage relies primarily on spore core components and
DNA repair, with minor effects of oxygen radical detoxification.
Applied and Environmental Microbiology **80**: 104–109.
- Moir, A., 2003 Bacterial spore germination and protein mobility.
Trends in Microbiology **11**: 452–454.
- Mudgil, D., S. Baskar, R. Baskar, D. Paul, and Y. S. Shouche, 2018
Biomineralization potential of *Bacillus subtilis*, *Rummeliibacil-*
lus stabekisii and *Staphylococcus epidermidis* strains in vitro
isolated from speleothems, Khasi hill caves, Meghalaya, India.
Geomicrobiology Journal **35**: 675–694.
- Murrell, W., 1969 Chemical composition of spores and spore struc-
tures. In *Bacillus subtilis and its closest relatives*, edited by G. Gould
and A. Hurst, pp. 215–273, Academic Press, New York, NY.
- Onyenwoke, R. U., J. A. Brill, K. Farahi, and J. Wiegand, 2004 Sporu-
lation genes in members of the low g+c gram-type-positive phy-
logenetic branch (Firmicutes). *Archives of Microbiology* **182**: 182–
192.
- Orsburn, B. C., S. B. Melville, and D. L. Popham, 2010 Etfa cataly-
ses the formation of dipicolinic acid in *Clostridium perfringens*.
Molecular Microbiology **75**: 178–186.
- Paredes-Sabja, D., B. Setlow, P. Setlow, and M. R. Sarker, 2008 Char-
acterization of *Clostridium perfringens* spores that lack spore
proteins and dipicolinic acid. *Journal of Bacteriology* **190**: 4648–
4659.
- Paredes-Sabja, D., P. Setlow, and M. R. Sarker, 2011 Germination
of spores of bacillales and clostridiales species: mechanisms and
proteins involved. *Trends in Microbiology* **19**: 85–94.
- Piggot, P. J. and D. W. Hilbert, 2004 Sporulation of *Bacillus subtilis*.
Current Opinion in Microbiology **7**: 579–586.
- Powell, J. F., 1953 Isolation of dipicolinic acid (pyridine-2: 6-
dicarboxylic acid) from spores of *Bacillus megatherium*. *Bio-*
chemical Journal **54**: 210–211.
- Qin, J., R. Li, J. Raes, M. Arumugam, K. S. Burgdorf, *et al.*, 2010
A human gut microbial gene catalogue established by metage-
nomic sequencing. *Nature* **464**: 59–65.
- Rosen, D. L., C. Sharpless, and L. B. McGown, 1997 Bacterial spore

- 1 detection and determination by use of terbium dipicolinate pho- 63
2 toluminescence. *Analytical Chemistry* **69**: 1082–1085. 64
- 3 Sambol, S. P., M. M. Merrigan, J. K. Tang, S. Johnson, and D. N. 65
4 Gerding, 2002 Colonization for the prevention of clostridium 66
5 *difficile* disease in hamsters. *The Journal of Infectious Diseases* 67
6 **186**: 1781–1789. 68
- 7 Seemann, T., 2014 Prokka: rapid prokaryotic genome annotation. 69
8 *Bioinformatics* **30**: 2068–2069. 70
- 9 Setlow, P., 1995 Mechanisms for the prevention of damage to dna
10 in spores of bacillus species. *Annual Review of Microbiology* **49**:
11 29–54.
- 12 Setlow, P., 2003 Spore germination. *Current Opinion in Microbiol-*
13 *ogy* **6**: 550–556.
- 14 Seyfried, B. and B. Schink, 1990 Fermentative degradation of dipi-
15 colinic acid (pyridine-2,6-dicarboxylic acid) by a defined cocul-
16 ture of strictly anaerobic bacteria. *Biodegradation* **1**: 1–7.
- 17 Singer, E., B. Bushnell, D. Coleman-Derr, B. Bowman, R. M. Bowers,
18 *et al.*, 2016 High-resolution phylogenetic microbial community
19 profiling. *The ISME Journal* **10**: 2020–2032.
- 20 Singh, B., J. Kaur, and K. Singh, 2012 Transformation of malathion
21 by *lysiniibacillus* sp. isolated from soil. *Biotechnology Letters* **34**:
22 863–867.
- 23 Soni, A., I. Oey, P. Silcock, and P. Bremer, 2016 *Bacillus* spores in
24 the food industry: A review on resistance and response to novel
25 inactivation technologies. *Comprehensive Reviews in Food Sci-*
26 *ence and Food Safety* **15**: 1139–1148.
- 27 Stragier, P., 2002 A gene odyssey: exploring the genomes of
28 endospore-forming bacteria. In *Bacillus subtilis and its closest*
29 *relatives*, edited by A. Sonenshein, J. Hoch, and R. Losick, pp.
30 519–525, American Society of Microbiology, Washington, D.C.
- 31 Takahashi, F., N. Sumitomo, H. Hagihara, and K. Ozaki, 2015
32 Increased dipicolinic acid production with an enhanced spovf
33 operon in *bacillus subtilis* and medium optimization. *Bioscience,*
34 *Biotechnology, and Biochemistry* **79**: 505–511.
- 35 Tap, J., S. Mondot, F. Levenez, E. Pelletier, C. Caron, *et al.*, 2009
36 Towards the human intestinal microbiota phylogenetic core. *En-*
37 *vironmental Microbiology* **11**: 2574–2584.
- 38 Taylor, B. F. and J. A. Amador, 1988 Metabolism of pyridine com-
39 pounds by phthalate-degrading bacteria. *Applied and Environ-*
40 *mental Microbiology* **54**: 2342–2344.
- 41 Tsai, M. and M. Saier Jr, 1995 Phylogenetic characterization of the
42 ubiquitous electron transfer flavoprotein families *etf- α* and *etf- β* .
43 *Research in Microbiology* **146**: 397–404.
- 44 Wang, Y., Y. Yue, H. Li, Q. Zhao, Y. Fang, *et al.*, 2011 Dye-loaded ze-
45 olite I@silica core-shell composite functionalized with europium
46 (iii) complexes for dipicolinic acid detection. *Photochemical &*
47 *Photobiological Sciences* **10**: 128–132.
- 48 Wells, C. L. and T. D. Wilkins, 1996 Clostridia: sporeforming anaer-
49 obic bacilli. In *Medical Microbiology. 4th edition*, edited by S. Baron,
50 pp. 1012–1045, University of Texas Medical Branch, Galveston,
51 TX.
- 52 Wolska, K. I., A. M. Grudniak, and A. Kraczkiewicz-Dowjat, 2007
53 Genetic and physiological regulation of bacterial endospore de-
54 velopment. *Polish Journal of Microbiology* **56**: 11–17.
- 55 Yih, T. H., S.-W. Chen, and S.-Y. Hu, 2019 Improvements in the
56 growth performance, immunity, disease resistance, and gut
57 microbiota by the probiotic *rummeliibacillus stabekisii* in nile
58 tilapia (*oreochromis niloticus*). *Fish & Shellfish Immunology* **92**:
59 265–275.
- 60 Yilmaz, M. D., S.-H. Hsu, D. N. Reinhoudt, A. H. Velders, and
61 J. Huskens, 2010 Ratiometric fluorescent detection of an anthrax
62 biomarker at molecular printboards. *Angewandte Chemie Inter-*
national Edition **49**: 5938–5941.
- Zelder, O., W. K. Jeong, C. Klopprogge, A. Herold, and
H. Schröder, 2009 Method for the production of dipicolinate.
WO2009098046A1 .
- Zubasheva, M., L. Ganushkina, T. Smirnova, and R. Azizbekyan,
2010 Larvicidal activity of crystal-forming strains of *brevibacil-*
lus laterosporus. *Applied Biochemistry and Microbiology* **46**:
755–762.