

RESEARCH PAPER

Cryptosporulation in *Kurthia* spp. forces a rethinking of asporogenesis in Firmicutes

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Abstract

Endosporulation is a complex morphophysiological process resulting in a more resistant cellular structure that is produced within the mother cell and is called endospore. Endosporulation evolved in the common ancestor of Firmicutes, but it is lost in descendant lineages classified as *asporogenic*. While *Kurthia* spp. is considered to comprise only asporogenic species, we show here that strain 11kri321, which was isolated from an oligotrophic geothermal reservoir, produces phase-bright spore-like structures. Phylogenomics of strain 11kri321 and other *Kurthia* strains reveals little similarity to genetic determinants of sporulation known from endosporulating Bacilli. However, morphological hallmarks of endosporulation were observed in two of the four *Kurthia* strains tested, resulting in spore-like structures (cryptospores). In contrast to classic endospores, these cryptospores did not protect against heat or UV damage and successive sub-culturing led to the loss of the cryptosporulating phenotype. Our findings imply that a *cryptosporulation*

Mathilda Fatton and Sevasti Filippidou contributed equally to this study.

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phenotype may have been prevalent and subsequently lost by laboratory culturing in other Firmicutes currently considered as asporogenic. Cryptosporulation might thus represent an ancestral but unstable and adaptive developmental state in Firmicutes that is under selection under harsh environmental conditions.

INTRODUCTION

Sporulation is a morphophysiological response to unfavourable environmental conditions involving a sophisticated genetic mechanism of cellular division and differentiation. In Firmicutes, the process of sporulation is called endosporulation, because it is the result of an asymmetrical cell division that leads to the formation of a mature spore within a mother cell (Driks, 2003). Endosporulation is thought to have emerged in the last common ancestor of Firmicutes (Antunes et al., 2016) but may have been lost in many extant descendants (Galperin, 2013). Lineages that do not form spore-like cells are defined as *asporogenic*, a phenotypic classification that may be reinforced at the genetic level by genome comparisons searching for genetic determinants of endosporulation originally identified in *Bacillus subtilis*. For instance, contrary to the previous physiological knowledge, the analysis of the genomes of *Carboxydotherrmus hydrogenoformans* and *Ruminococcus bromii* suggested that these species could produce spores. This prompted researchers to experimentally demonstrate sporulation in both groups (Mukhopadhyaya et al., 2018; Wu et al., 2005).

Given the high energetic cost and genetic complexity of sporulation, bacteria might lose the ability to produce spores under constant favourable conditions for vegetative growth or alternatively only activate it sparsely and under defined environmental conditions. This has been shown experimentally in model endospore-forming species in which an asporogenic phenotype is the result of the inactivation or loss of a considerable fraction of sporulation genes (Onyenwoke et al., 2004). Norris et al. (2020) also demonstrated that laboratory strains of *Bacillus anthracis* lost their responsiveness to grow and sporulate as compared to wild strains. In addition to the different sporulation rates, the authors also demonstrated a higher abundance of proteins involved in sporulation in wild populations as compared to the laboratory ones (Norris et al., 2020). Such loss or reduction of sporulation capability after successive culturing (approximately 6000 generations) under optimal laboratory conditions was already observed in *B. subtilis* (Maughan et al., 2007). Moreover, this phenomenon seems not restricted to sporulation as *Azotobacter* cysts from soils were observed to display far more radiation-resistance than those produced by any laboratory strain (Vela & Wyss, 1965). Therefore, as a beneficial trait improving

survival and dispersal (Lennon & Jones, 2011), sporulation or other process resulting in a resistant cell, might be under selection only in highly variable environmental conditions. Accordingly, a culturing effort combined with extensive genomic analysis of the human microbiota has shown that sporulation is widely spread in bacteria inhabiting the human gut, an environment naturally subjected to high physicochemical fluctuation (Browne et al., 2016).

Poly-extreme ecosystems, such as geothermal sites, are also known to harbour a larger diversity of endospore-formers (Filippidou et al., 2016). Therefore, in order to better characterize the diversity of environmental endospore-forming Firmicutes, multiple enrichments were conducted from samples collected in geothermal environments. The resulting strains were screened for their ability to form spores, without any prior bias regarding their phylogenetic affiliation. In this way, strain 11kri321 was isolated from the geothermal spring of Krinides, Kavala, Greece. The strain was found to belong to the genus *Kurthia*. This genus is classified as asporogenic based on the characterization of a few described species (Roux et al., 2014; Ruan et al., 2014; Shaw & Keddie, 1983). In the case of *Kurthia zopfii* and *Kurthia gibsonii*, the lack of endospores was asserted in 14-day aged cultures, which were not resistant to wet heat exposure for 10 min at 80°C, resulting in the death of the strains (Shaw & Keddie, 1983). *Kurthia huakuii*, *Kurthia massiliensis* and *Kurthia senegalensis* are described as non-spore-forming, based on the absence of microscopic proof for spore-like structures (Roux et al., 2014; Ruan et al., 2014). Given the microscopic observations made in strain 11kri321, in this study, we re-examined the sporulation potential of members of the *Kurthia* genus using a combination of morphological, bioinformatic, and physiological approaches. First, optical and cryo-electron microscopy were used to characterize spore-like structures. In addition, we surveyed the genomes of four *Kurthia* spp. (*Kurthia* sp. str. 11kri321, *K. massiliensis*, *K. huakuii* and *K. senegalensis*) for orthologues of known sporulation genes from *B. subtilis* (Abecasis et al., 2013; Galperin et al., 2012). Finally, we assessed the survival of aged cultures (4-week-old cultures) in response to multiple environmental stresses, in comparison with prototypical and durable endospores formed by aging *Bacillus* cultures in response to nutrient starvation (Piggot & Hilbert, 2004; Sonenshein, 2000). Based on our

findings, we discuss the implications of defining a truly asporogenic lifestyle within Firmicutes and also the role of environmental pressure to maintain a sporulation trait.

EXPERIMENTAL PROCEDURES

Sample collection and isolation

The geothermal reservoir of Krinides (N 41°00.642' E, 024°15.371'), near Philippoi, is situated in the Rhodope Massif (Kavala, Greece) (Tranos et al., 2009). At the time of sampling, the water temperature at the output of the borepipe was 29.1°C, pH was 9 and conductivity 415 μ S/cm. Water and biofilms from the outflow were collected in a sterile 1 L bottle and filtered through a 0.22 μ m nitrocellulose membrane (Millipore, USA). The membrane was transported to the laboratory on ice and stored at 4°C for bacterial enrichment into 10 mL of nutrient broth (NB) (Biolife, Italy). The enriched culture was then plated on nutrient agar (NA) and single colonies were obtained. Each colony was plated repeatedly to attain pure aerobic bacterial isolates. Colony morphology was observed after 12 h of growth. The capability to form spores was observed after starvation for 15 days using phase-contrast microscopy (Leica DM R, magnification 1000 \times). A differential staining for endospores and vegetative cells was performed using malachite green and safranin, as previously described (Schaeffer & Fulton, 1933). Gram-staining was also performed in order to determine the Gram character of the strain.

Cell growth was monitored at different temperatures (4, 15, 20, 25, 35, 45, 50, 55, and 60°C) over 4 days in NB medium. To determine the pH range in which *Kurthia* sp. str. 11kri321 grows, NB medium at pH 4–13 was prepared (intervals of 0.5 pH unit), and growth was monitored at optimum growth temperature (25°C), over 4 days. All tests were performed in triplicates.

Strain identification

gDNA extraction and sequencing

Genomic DNA was extracted from an overnight culture using the Genomic-tip 20/G kit (Qiagen GmbH, Germany). Sequencing was performed with the PacBio RS II system based on single molecule, real-time (SMRT) technology (Pacific Biosciences, California). The draft genome of *Kurthia* sp. str. 11kri321 presents a unique contig of 2,964,527 bases and a G + C content of 36.7%. Genome annotation was performed using an Ergatis-based (Hemmerich et al., 2010) workflow with minor manual curation and visualized with the Artemis Genome Browser and Annotation Tool (Carver

et al., 2005). A total of 2893 coding sequences (CDSs), 82 tRNAs, and 27 rRNAs (nine copies of 16S, 23S and 5S rRNA genes) were predicted. This whole-genome project has been deposited at GenBank under the Bio-project PRJNA301103 and the Biosample ID SAMN04235798.

Induction of sporulation

Five sporulation media were prepared (SM1 with and without carbon source, Donnellan et al., 1964; SM2 with and without carbon source, Brandes Ammann et al., 2011; Angle, Angle et al., 1991; with 10% glycerol). Pre-cultures of the four *Kurthia* strains (*K. massiliensis* str. JC30, *K. huakuii* str. LAM0618, *K. senegalensis* str. JC8E, and *Kurthia* sp. strain 11kri321) were inoculated overnight under optimal conditions. Biomass was retrieved with centrifugation at 6000 g for 3 min and transferred in the sterile sporulation media. The new cultures were incubated at optimal conditions for 7, 14, and 28 days. The presence of spores, vegetative, and dead cells was verified in the contrast-phase microscope and the three cell types were quantified in a Neubauer chamber. The same procedure was applied for cells of the four *Kurthia* strains that were previously regularly subcultured on NA.

Resistance test

Exhaustion of nutrients is a known factor to trigger sporulation in *B. subtilis*. Therefore, aged *Kurthia* spp. (*Kurthia* sp. str. 11kri321, *K. massiliensis*, *K. huakuii*, *K. senegalensis*) cells, as well as *B. subtilis* cells (control strain), were prepared by leaving the cultures on NA at room temperature for 4 weeks. The 4-week-old cells were collected and resuspended in physiological water (0.9% NaCl) in order to have samples with an optical density (OD₆₀₀) at 0.1. In parallel, cells from a fresh overnight culture grown in NB of the same species were centrifuged (3000 g, 10 min), resuspended in physiological water and diluted to the same OD₆₀₀. For each strain, 1 mL of fresh and aged cell suspension was exposed in duplicate to three different stresses applied at four exposure times (1, 5, 10, and 20 min). The stresses were 70°C wet heat, 70°C dry heat, and UV exposure (30 W/30 cm). After wet and dry heat, the samples were directly put on ice for at least 5 min. All the treated samples were diluted 10 \times with fresh NB and incubated at 30°C for 30 min (heat activation). In order to check whether cells were able to regrow after the treatment, 25 μ l of the heat activated suspensions was spread on a quarter of NA plate. After 24 h of incubation at 30°C, pictures of all the plates were taken and cell morphology was verified in selected colonies under the microscope. All the treatments were compared to a

control culture (*Kurthia* sp. str. 11kri321 aged and fresh cells).

Fluorescence and time-lapse microscopy

Cultures were grown either overnight (fresh cultures) or for 15 days (old cultures). The membrane dye FM4-64 (Invitrogen) was used at a final concentration of 500 ng/mL (diluted in dimethyl sulfoxide) and incubated for 5 min before imaging. For time-lapse microscopy, a spore preparation (see below) was immobilized using a thin layer of tryptic soy agar. Phase-contrast microscopy images were taken at a sample frequency of one frame per 2 min. In both cases, images were acquired with an alpha Plan-Apochromatic * 100/1.46 ph 3 (Zeiss) on an Axio Imager M2 microscope (Zeiss) and a CoolSNAPHQ2 camera (Photometrics) controlled through Metamorph V7.5 (Universal imaging). Images were processed using ImageJ (NIH, Bethesda, MD).

Electron cryotomography sample preparation and imaging

Cells grown on plates for 2 months were re-suspended in growth medium and frozen immediately for cryotomography studies. Mature spores and vegetative cells were collected from agar plates by resuspending them in growth medium and imaged with light microscopy at room temperature before and after freezing. In both cases, phase-bright objects were observed in the resuspension. Samples were then mixed with 20 nm colloidal gold particles, loaded onto glow-discharged carbon grids (R2/2, Quantifoil) and plunge-frozen into liquid ethane-propane mix cooled at liquid nitrogen temperatures with a FEI Mark IV Vitrobot maintained at room temperature and 70% humidity. The grids were imaged with a FEI Titan Krios TEM at 300 keV with a GIF2002 Imaging Filter (Gatan) and images were recorded on a 2Kx2K CCD Megascan model 795 camera (Gatan). Targets were picked randomly ($n = 35$) and imaged. Images were acquired under low-dose conditions (final dose of $100 \text{ e}^-/\text{\AA}^2$), $10 \mu\text{m}$ underfocus at $11,500\times$ magnification, such that each pixel represented 7.9 \AA . Tilt series were collected from -60° to $+60^\circ$ with 1° oscillation using EPU tomography software. Three-dimensional reconstructions were generated using the IMOD programme (Mastronarde, 2008).

Samples of cultures grown in liquid medium were treated as follows. The $3.5 \mu\text{l}$ of vegetative cells or mature spores was mixed with $1 \mu\text{l}$ of 10 nm Protein A-Gold (Department of Cell Biology of the University Medical Center, Utrecht, The Netherlands). The $3.5 \mu\text{l}$ of that mixture was transferred to a 3 mm freshly glow-discharged Quantifoil R2/2 Cu 300 mesh holey carbon grid (Quantifoil Micro Tools GmbH, Jena, Germany).

Excess liquid was blotted away using a Leica EM GP plunge-freezer at room temperature and 80% humidity (blot time 2–3 s) and the grid was immediately plunge frozen in liquid ethane cooled by liquid nitrogen. Data were collected on a FEI Titan Krios TEM at 300 keV with a Quantum-LS energy filter (20 eV slit width) and a K2 Summit electron counting direct detection camera (Gatan).

Images of the cells and spores were recorded at magnifications of $1285\times$, $7252\times$ and $11,927\times$, resulting in calibrated physical pixel size of 38.9, 6.9 and 4.2 \AA . The underfocus was changed between 100 and $7 \mu\text{m}$ with a total dose between 22 and $0.7 \text{ e}^-/\text{\AA}^2$. The images were recorded using the programme SerialEM (Mastronarde, 2005).

Phylogenetic analysis

16S rRNA gene sequences ($>1200 \text{ bp}$) of Firmicutes were retrieved from RDP (<http://rdp.cme.msu.edu>) (Cole et al., 2005) and aligned using the default parameters of MAFFT (Katoh et al., 2002). A maximum likelihood phylogenetic tree was built using PhyML (Guindon et al., 2010) and then graphics using the Newick utilities (Junier & Zdobnov, 2010). Exhaustive Hidden Markov Model (HMM)-based homology searches were carried out on a local genome databank of 253 Firmicute species by using the HMMER package (Johnson et al., 2010) and as queries the HMM profiles of the complete set of 54 bacterial ribosomal proteins from the Pfam 29.0 database (<http://pfam.xfam.org>; Finn et al., 2016). Twelve ribosomal proteins were discarded due to their absence from $>50\%$ of the considered genomes and because they contained paralogous copies making identification of orthologues difficult.

The remaining 42 single protein data sets were aligned with MAFFT v7.222 (Katoh et al., 2002) with the L-INS-I algorithm and default parameters, and unambiguously aligned positions were selected with BMGE 1.1 (Criscuolo & Gribaldo, 2010) and the BLO-SUM30 substitution matrix. Single protein datasets were concatenated by allowing a maximum of four missing taxa, resulting in a character supermatrix containing 5254 amino acid positions. A Bayesian tree was calculated from the ribosomal protein concatenate with PhyloBayes v3.3 (Lartillot et al., 2009) and the evolutionary model GTR + CAT + G4 (Lartillot & Philippe, 2004). Two independent chains were run until convergence, assessed by evaluating the discrepancy of bipartition frequencies between independent runs. The first 25% of trees were discarded as burn-in and the posterior consensus was computed by selecting one tree out of every two. The tree was rooted based on previous evidence (Antunes et al., 2016). The tree was visualized and metadata was added to it using iTOL (Letunic & Bork, 2011).

Retrieval of sporulation genes sequences

Complete and draft sequences of spore-forming Firmicutes were downloaded from Comprehensive Microbial Resource (CMR) and Integrated Microbial Genome (IMG) websites. Search for spore-related genes was based on gene function category sporulation (CMR; sporulating category in IMG). The CMR version was 24.0 data release and the IMG version was 3.0. In addition to the protein sequence, nucleotide sequences including a 50-bp flanking region at both 5'- and 3'- ends were downloaded. Additional information on all retrieved genomes was obtained from the GenBank database.

Sequence data analysis

The *Kurthia* sp. str. 11kri321 genome sequence was scanned for orthologs of the Firmicute core sporulation genes (as protein sequences) with TBLASTN (Altschul et al., 1990), using default parameters and an e-value cut-off of $1e^{-11}$. A TBLASTN run on the shuffled protein sequences as a negative control set showed no hit with an e-value lower than $4e^{-4}$. The hits were ordered by position on the *Kurthia* sp. str.11kri321 genome and inspected manually. The above procedure did not detect orthologues of SpoVFA and SpoVFB; therefore, we attempted to detect those by pairwise dynamic-programming alignment. The protein sequences of SpoVFA and SpoVFB were each compared to all sequences of the *Kurthia* proteome using Needleman and Wunsch's algorithm (Needleman & Wunsch, 1970), as implemented by EMBOSS's 'needle' programme (Rice et al., 2000). No hits were found. The publicly available genomes of *K. huakuii* str. LAM0618, *K. massiliensis* str. JC30, and *K. senegalensis* str. JC8E were scanned for sporulation gene orthologs as described above. The comparison of the four *Kurthia* genomes to *B. subtilis* subsp. *subtilis* str. 168 was plotted using BRIG (Alikhan et al., 2011), followed by further manually curation.

The sequences of the *Kurthia* genomes analysed herein were retrieved from GenBank under accession numbers: *K. huakuii* str. LAM0618: NZ AYT000000000.1, *K. massiliensis* str. JC30: NZ CAEU000000000.1, and *K. senegalensis* str. JC8E: NZ CAEW000000000.1. The genome of *B. subtilis* subsp. *subtilis* str. 168: NC_000964.3 was also retrieved. In addition, we repeated two comparative genomics approaches published previously for assessing the minimal set of sporulation genes in Firmicutes (Abecasis et al., 2013; Galperin et al., 2012).

Dipicolinic acid measurement

The presence of dipicolinic acid (DPA) in the spores was assessed, according to a previously published

method (Brandes Ammann et al., 2011). Fluorescence was measured with a Perkin-Elmer LS50B fluorometer. The excitation wavelength was set at 272 nm with a slit width of 2.5 nm. Emission was measured at 545 nm (slit width 2.5 nm). The device was set in the phosphorescence mode (equivalent to time-resolved fluorescence). The delay between emission and measurement was set at 50 μ s. Measurements were performed every 20 ms. The integration of the signal was performed over a duration of 1.2 ms. Values recovered for each measurement corresponded to the mean of the relative fluorescence unit (RFU) values given by the instrument within the 30 s following sample introduction in the device. Finally, to transform RFU units into DPA concentrations, a 10-point standard curve was established using increasing concentrations of DPA from 0.5 up to 10 μ m.

RESULTS

Characterization of the isolate

Strain 11kri321 was isolated from a geothermal spring (Krinides, Kavala, Greece) in a mixed biofilm and was characterized as a Gram-positive bacterium based on Gram staining (Figure 1A). Affiliation of the strain to the genus *Kurthia* was supported by 16S rDNA sequencing and average amino acid identity (AAI) pairwise genomic relatedness analysis of its genome as compared to other genomes from the genus. The AAI values for the comparison between strain 11kri321 are 68.88% with *K. massiliensis*, 68.50% with *K. huakuii* and 68.58% with *K. senegalensis*. The phylogenetic placement of strain 11kri321 was further verified by a phylogenomic analysis showing its position relative to other members of the Firmicutes (Figure 1B).

Strain 11kri321 grew at a pH between 5.5 and 11.5. Its optimal growth temperature was 25°C; however, it could grow at temperatures between 20 and 45°C. The *in situ* pH of the site was 9 and the temperature was 29°C, both of which are within the limits of tolerance for all previously described *Kurthia* spp. (Hutchison et al., 2014), and within the values established for the vegetative growth of strain 11kri321. These environmental conditions should thus support vegetative growth of 11kri321 in the borehole, provided nutrients are available.

Production of spore-like structures by *Kurthia* sp. str. 11kri321

Although the genus *Kurthia* is reported to comprise only asporogenic species (Britton et al., 2002; Roux et al., 2014; Ruan et al., 2014; Shaw & Keddie, 1983),

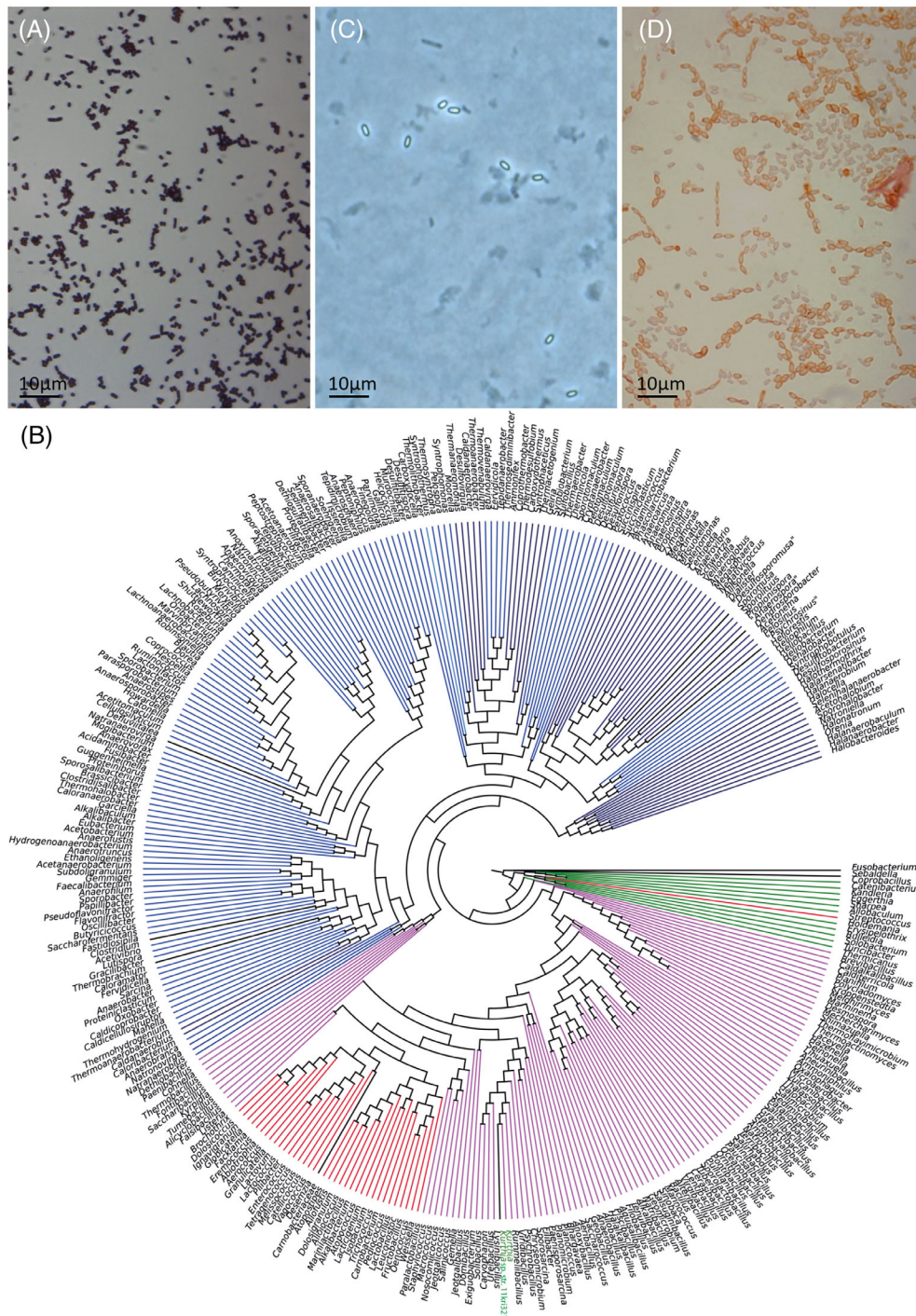


FIGURE 1 Morphological characterization and phylogenetic placement of *Kurthia* sp. str. 11kri32. (A) Strain 11kri321 is a Gram-positive bacterium. (B) Maximum-likelihood tree showing the positioning of the *Kurthia* genus among Firmicutes. Branches are coloured by class (magenta: Bacillales; blue: Clostridiales; green: Erysipelotrichales; navy: Halanaerobiales; crimson: Lactobacillales; medium blue: Natranaerobiales; dark blue: Selenomonadales; midnight blue: Thermoanaerobacterales; dodger blue: Thermolithobacterales; *Kurthia* (including *Kurthia* sp. str. 11kri321) is highlighted in green lettering. (C) Spore-like structures observed in *Kurthia* sp. str. 11kri321 appeared phase bright. (D) The spore-like cells retained the malachite green staining; (C) and (D) are two characteristics that are always observed for endospores of *Bacillus subtilis*.

spore-like structures were observed in a nutrient-deprived culture of *Kurthia* sp. str. 11kri321 that also contained vegetative cells. Those spore-like structures refracted light and appeared phase-bright by optical

microscopy (Figure 1C). We therefore speculated that nutrient starvation induces the differentiation of vegetative cells into spore-like cells. In order to better characterize these structures, we performed staining with

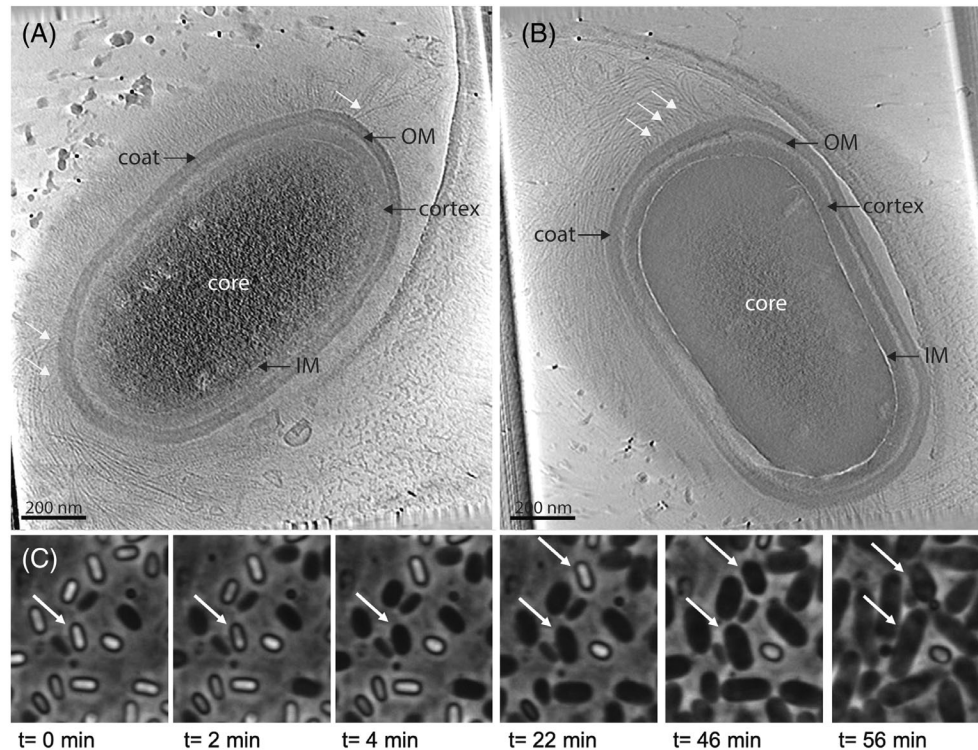


FIGURE 2 Detailed morphological characterization of the spore-like structures of *Kurthia* sp. str. 11kri321 by cryo-electron tomography (cryo-ET) and monitoring of their transition from phase-bright to phase-dark by light microscopy. (A,B) Cryo-ET images of the spore-like structures of *Kurthia* sp. str. 11kri321 show the inner (IM) and outer (OM) membranes, cortex, coat, and filamentous appendages (white arrows). (C) Pictures from snapshots of the time-lapse movie in Movie S1 showing key stages of the transition from phase-bright to phase-dark of *Kurthia* sp. str. 11kri321 spore-like structures.

malachite green (Schaeffer-Foulton stain). This chemical was retained by the *Kurthia* spore-like structures but not by vegetative cells (Figure 1D).

A more detailed morphological characterization of these spore-like structures was conducted using cryo-electron tomography (cryo-ET). The average spore-like structure measured 700×1200 nm. Each spore-like structure possessed features similar to those of endospores (Tocheva et al., 2011; Tocheva et al., 2013) such as a core, thick cortex, inner and outer membranes (IM and OM, respectively), and a coat (Figure 2A,B). Due to the thickness of the spore-like structures, the bilayer lipid of the IM and OM could not be resolved. Filamentous proteinaceous appendages analogous to an exosporium emerged from both poles and connected to the coat (Figure 2A,B, white arrows).

To investigate whether the observed phase-bright bodies produced during aging behave as spores, we monitored germination by time-lapse microscopy after exposure to conditions favouring growth and division (Figure 2C; Movie S1). We observed a shift from bright to dark phase, a swelling of the cells, and elongation, resulting in the characteristic short-rod-shaped of dividing vegetative cells. Only 6% of the phase-bright bodies did not undergo this morphological change, a rate comparable to *B. subtilis* (Pandey et al., 2013).

Presence of DPA in the spore-like structures of strain 11kri321

An important molecule produced in the mother cell of endospore-forming Firmicutes and introduced to the spore at later stages of sporulation is DPA. DPA is responsible for accumulation of minerals (especially calcium ions) in the spore core, to create a more stable core and to guarantee resistance to wet heat (Daniel & Errington, 1993). The two genes that encode the DPA synthetase subunits A and B (*spoVFA*, *spoVFB*) were not detected in the genome of *Kurthia* sp. str. 11kri321 and accordingly, we were unable to detect any DPA from aged preparations (Figure S1). *Kurthia* sp. str. 11kri321 also lacks the gene *ger3*, as it is the case of *B. subtilis* *ger3* mutants that produce stable DPA-free spores, which display a decreased resistance to wet heat (Paidhungat et al., 2000; Setlow et al., 2006).

Investigation of spore-like structures in other *Kurthia* species

In order to determine whether production of spore-like structures may be characteristic of the entire genus, we attempted chemical induction of sporulation in three

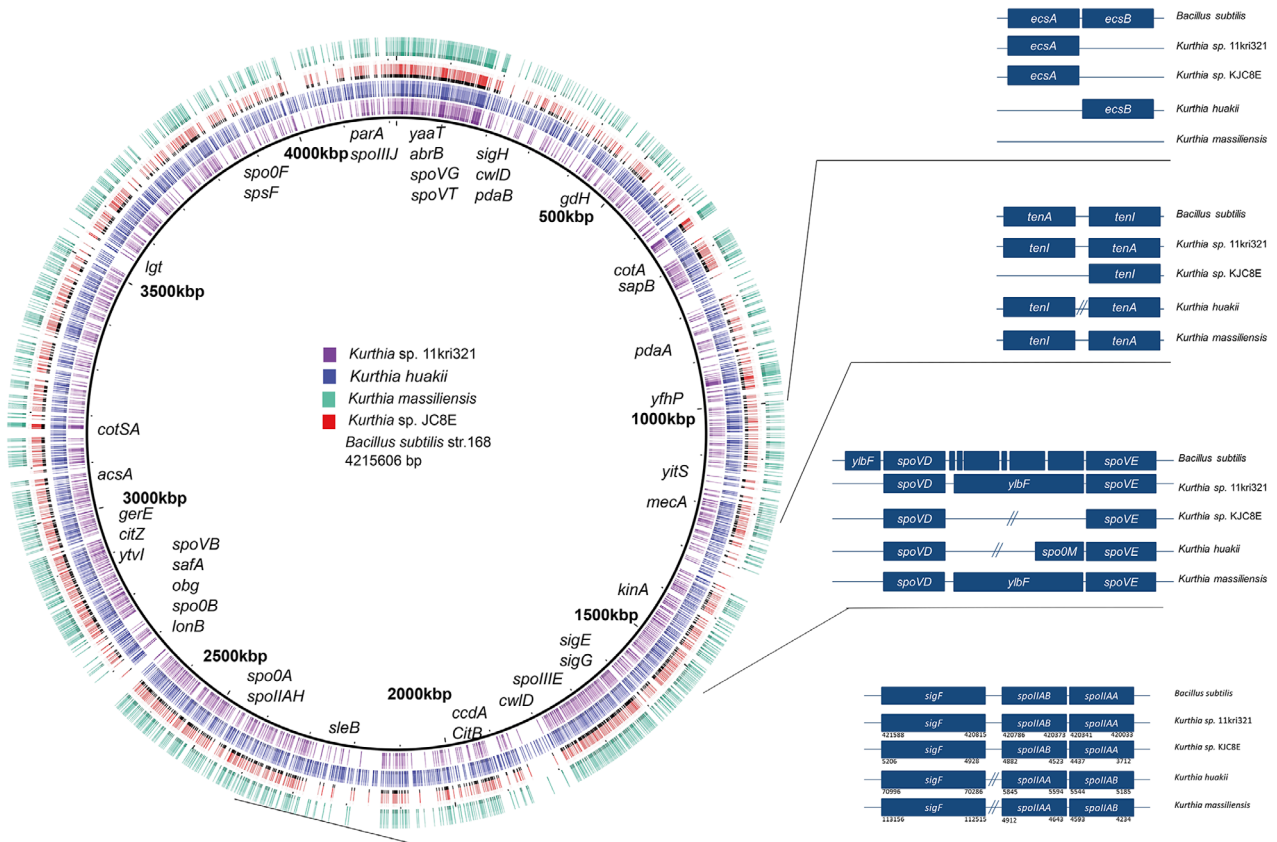


FIGURE 3 Comparison of the four *Kurthia* genomes publicly available to *Bacillus subtilis* subsp. *subtilis* str. 168. Genes related to sporulation are shown on the circular representation of the genomes. Four operons related to sporulation are also presented showing differences among the four *Kurthia* strains and *Bacillus subtilis* subsp. *subtilis* str. 168. The parallel lines in the operon representations show that in the respective strains, there is not an operon organization of these genes and that in contrary are distantly located in the chromosomes

additional *Kurthia* species (*K. huakuii* str. LAM0618, *K. senegalensis* str. JC8E, and *K. massiliensis* str. JC30), using 10% glycerol or nutrient deprivation in two sporulation media, with and without a carbon source (Brandes Ammann et al., 2011; Donnellan et al., 1964). All published *Kurthia* strains we used were able to produce in some replicates phase-bright bodies on sporulation media, albeit at a frequency never exceeding 10% (Figure S2).

Genomic imprints of sporulation

The different genomes of the four strains of *Kurthia* were screened for sporulation-related genes using multiple approaches. First, the presence of 213 sporulation gene homologues from 34 endospore-forming Firmicutes (Table S1) was assessed by bi-directional BLAST. In total, between 37 and 53 sporulation-related genes were detected and assigned to the different stages of the endospore-formation pathway (Table S2). Among the genes with a clear function, a striking difference between the genes identified in *K. massiliensis*

and the other strains of *Kurthia* is found at the stage of decision-making to commit to sporulation by regulators controlling the activation of early sporulation genes. While homologous to SigH, Spo0A and Spo0F were found in all *Kurthia* genomes, Spo0B, which has a role in signal transduction on the *yaaT* gene, was only found in *K. massiliensis*. The *spoIIIAH* gene was detected in the genome of *K. massiliensis* but was absent from the other screened genomes. The other sporulation genes with a known function appear to be conserved within *Kurthia* spp. A comparative representation of the four *Kurthia* genomes to *B. subtilis* subsp. *subtilis* str. 168 is shown in Figure 3, including potential sporulation operons deduced from the genomic analysis.

Compared to other endospore-forming Firmicutes, this screening revealed that only a few sporulation genes are present in *Kurthia* spp. genomes. For instance, in *Kurthia* sp. str. 11kri321, only a small fraction of the genes (18 genes) corresponded to those reported as conserved in all spore-forming Bacilli and Clostridia, and even a smaller number (14 genes), corresponded to the 44 genes considered to be essential for sporulation in *B. subtilis* (Galperin et al., 2012). To

confirm this result, two additional genomic surveys were conducted. The first one, consisted in searching into the genomes for cluster of orthologue genes (COGs) defined previously to investigate the minimal core of sporulation genes (Galperin et al., 2012). Based on this analysis, the complement of genes involved in sporulation is minimal as compared to a model endospore-forming organism such as *B. subtilis* (Table S3). Finally, a comprehensive database of genes including a more extensive set of conserved genes that appear in multiple endospore-forming bacteria (Abecasis et al., 2013) was also tested using a bidirectional BLAST approach starting with the proteome of *B. subtilis* (Table S4). This analysis revealed that other than genes controlling the entry into sporulation, the potential genomic complement for sporulation in *Kurthia* spp. is minimal.

Genes homologous to *sigH* and *spo0A*, encoding two of the main transcriptional regulators responsible for controlling the onset of sporulation (Britton et al., 2002; Hilbert & Piggot, 2004), were consistently detected in all the approaches used. However, the evidence for the presence of other sigma factors directing the differential gene expression after asymmetrical cell division varied. Four sigma factors (SigF, SigE, SigG and SigK) were detected (with the exception of SigK in *K. senegalensis*) when the bidirectional BLAST approach was applied onto an extended database of multiple endospore-forming Firmicutes (Tables S1 and S2). However, SigE was not detected in any of the *Kurthia* spp. genomes when the bidirectional BLAST was performed using as query the homologue of *B. subtilis*. In contrast, SigG and the anti-sigma factor for SigF (SpollAB) were detected in all the genomes (Table S4). In conclusion, with the exception of the transcriptional regulators controlling the initiation of sporulation (or other stress responses) in Bacilli and Clostridia, no compelling genomic evidence surfaced to support the existence of a prototypical endosporulation pathway in *Kurthia* spp., suggesting that the spore-like cells are not the result of a canonical endosporulation process.

Microscopic observations

The process of sporulation in *Kurthia* spp. was also investigated using 2D cryo-electron microscopy (cryo-EM). The results clearly show evidence of an engulfment step and the production of a forespore within the mother cell in two of the strains (*K. huakuii* and *K. senegalensis*) (Figure 4). Finally, spore-like structures in *K. massiliensis* showed a similar structure as those of *Kurthia* sp. str. 11kri321 (Figure 5A,B), but the layer, that could correspond to an exosporium in endospores, appeared to be more complex in *K. massiliensis* (Figure S3).

Resistance test in *Kurthia* spp.

The production of an endospore is expected to confer resistance to a number of chemical and physical factors that would otherwise kill the vegetative cell. In laboratory, production of (endo)spores can be induced by nutrient limitation (i.e. reduction of the carbon, nitrogen or phosphorus sources), which is induced by culturing cells on a nutrient-poor sporulation medium (Piggot & Hilbert, 2004; Sonenshein, 2000). Alternatively, it is also possible to allow cells to exhaust nutrients from the growth medium and reach stationary phase directly on the culture plates (aging process) (Sonenshein, 1999). Therefore, to confirm that the spore-like cells observed in the cultures from *Kurthia* also provide such enhanced resistance, the resistance of cells from aged cultures was compared with those from fresh ones.

Aged cultures of endospore-forming *B. subtilis* (used as reference) produced phase-bright spores. Pre-spores inside mother cells were observed already after 24 h of growth, and free spores were observed after 48 h (data not shown). The spores of *B. subtilis* were more resistant to heat (dry and wet) and to UV radiation, as compared to the fresh vegetative cells (Figure 6). In contrast, 4-week aged cultures of *Kurthia* spp. did not show any phase-bright bodies and instead the cells appear simply in a stationary phase or already dying (Figure S4D–F–H–J). In contrast to *B. subtilis*, 4-week-old cells of *Kurthia* spp. were less resistant to dry heat and to UV than the fresh cells (Figure 6). Such observation was true for all the *Kurthia* sp. strains tested (*Kurthia* sp. str. 11kri321, *K. massiliensis*, *K. huakuii* and *K. senegalensis*). Concerning wet heat, none of the *Kurthia* spp., both fresh and old cells, tolerated this stress, as no regrowth was observed after the treatment even for as short as 1 min exposure (Figure 6).

Although we did not expect resistance to some of the stressors tested (e.g. wet heat given the lack of DPA), the microscopic observations of the aged cultures suggested that the production of spore-like cells becomes less consistent with time, something that was accelerated by the sub-culturing of the strains under optimal laboratory conditions. Indeed, after sub-culturing on optimal growth medium (NA), we were unable to observe phase-bright cells for the different *Kurthia* species (Figure S4A–H). On the contrary, *B. subtilis* produced phase-bright cells after aging process (4-week-old cells) consistently even after sub-culturing (Figure S4J).

DISCUSSION

The overall results presented in this study question the validity of describing organisms as asporogenic on the

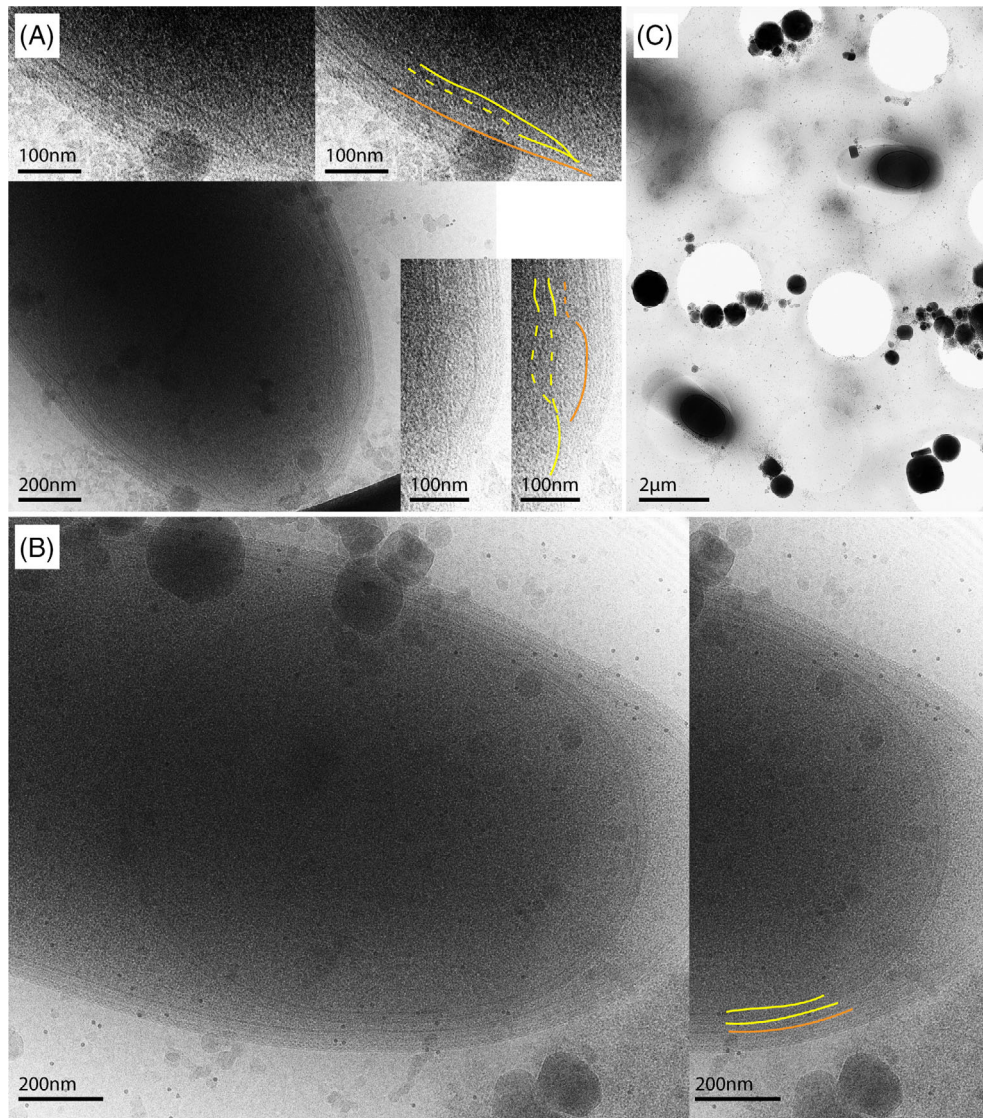


FIGURE 4 Cryo-electron microscopy (cryo-EM) imaging of putative engulfment in *Kurthia* spp. (A) Cryo-EM projection images reveal the steps of engulfment in *K. huakuii* str. LAM0618. The areas in which engulfment leads to the production of a putative pre-spore-like cells with two membranes (yellow) by the engulfment of the membrane from the mother cell (orange) are shown enlarged in the inserts. (B) A projection image of *K. huakuii* str. LAM0618 at the end of the engulfment step with the spore-like cell (two membranes highlighted in yellow) inside the mother cell (inner membrane highlighted in orange). (C) A projection image showing spore-like cells inside two mother cells of *K. senegalensis* str. JC8E.

basis of the fact that sporulation has not been observed. Indeed, we showed that after nutrient starvation, freshly isolated cultures of *Kurthia* spp. were capable to produce structures with some similar morphological characteristics to spores (spore-like structures), including the ability to refract light (phase-brightness) (Figure 1C) and to retain malachite green staining (Figure 1D). In addition, we observed evidence for a form of engulfment in two *Kurthia* spp. (*K. huakuii* and *K. senegalensis*) (Figure 4). However, aged cultures were not resistant to environmental stressors as compared to fresh cells (Figure 6), in contrast to truly endospore-forming species in which aging cultures (presumably composed of spores mostly) are more resistant to heat and UV radiation, as compared to

fresh cells (Camilleri et al., 2019). Indeed, aged cultures are expected to have depleted the nutrients available in the growth medium and confronted to starvation (for carbon, nitrogen and/or phosphorus sources), trigger sporulation and production of resistant spores (Piggot & Hilbert, 2004; Sonenshein, 2000). Yet, aged cells of *Kurthia* spp. were not more resistant than the fresh cells (Figure 6), questioning therefore the ability of this genus to produce mature spores with similar properties to other Firmicutes endospores.

In this context, we screened the genomes of *Kurthia* sp. str. 11kri321 and of other representatives of the genus for known genes involved in endospore formation. A similar gene content was detected in all *Kurthia* spp., which was consistently below the suggested minimal

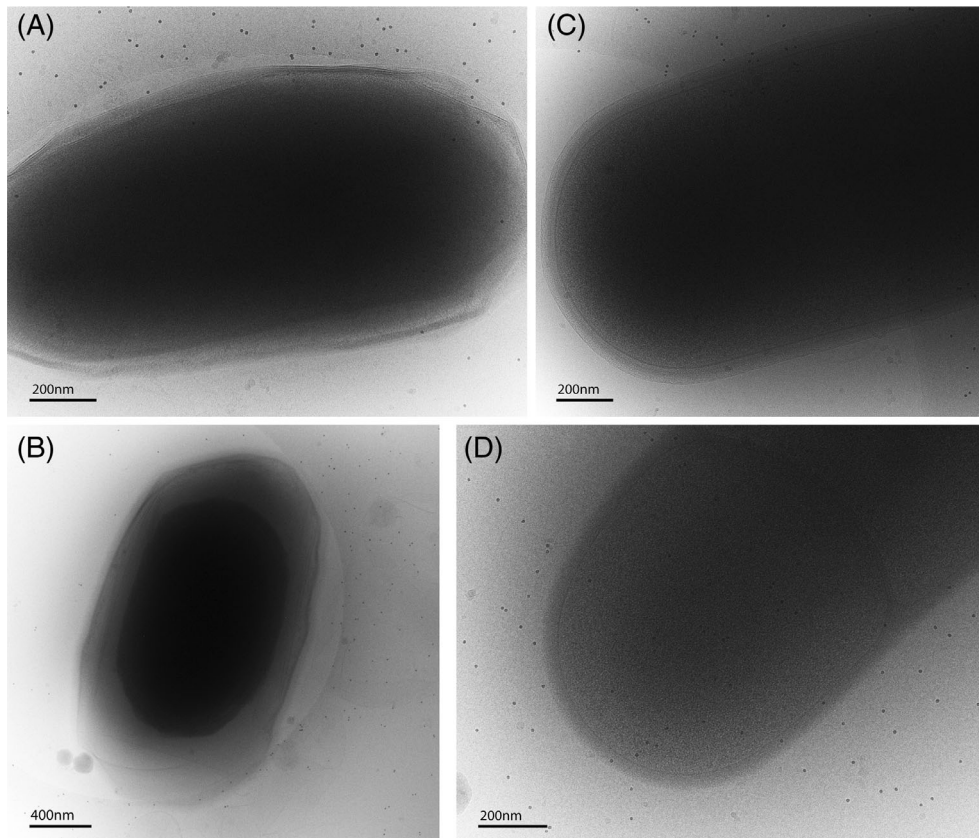


FIGURE 5 Cryo-electron microscopy (cryo-EM) imaging of spore-like structures and vegetative cells of *Kurthia* spp. Spore-like structures of *Kurthia* sp. str. 11kri321 (A) and *K. massiliensis* str. JC30 (B) showed a similar structure and shape; however, size is different: *Kurthia* sp. str. 11kri321 spore-like cells seem to be approximately double the size than those of *K. massiliensis* str. JC30. Because spore-like cells appear to be constituted of concentric structures (Driks, 2004), the external edges of spore-like cells look blurred compared to vegetative cells of *Kurthia* sp. str. 11kri321 (C) and *K. massiliensis* str. JC30 (D) in which the cytoplasmic membrane and cellular content are visible.

core of sporulation genes in other Firmicutes (Galperin et al., 2012). Known sporulation proteins present in *Kurthia* spp. included the Spo0A response regulator, as well as alternative RNA polymerase sigma factors. Those represent the main regulators of the pathway responsible to enter in the energy-demanding process of sporulation and appear to be highly conserved among endospore-forming Firmicutes (Britton et al., 2002; de Hoon et al., 2010; Hilbert & Piggot, 2004; Traag et al., 2013). The master transcriptional regulator of sporulation, Spo0A, and some of the sporulation-specific sigma factors were detected by at least two bioinformatic approaches in *Kurthia* spp. (Tables S2 and S4). This suggests a conservation in the directing elements responsible for starting the process of spore-like structures production in *Kurthia* spp. This hypothesis is also supported by detection of homologues to SpoIIAA and SpoIIAB, two regulators of the expression of the sigma factors mentioned above (Duncan et al., 1996; Illing & Errington, 1991).

One of the most striking differences between the sporulation gene content in *Kurthia* spp. compared to other Firmicutes concerns the stage of engulfment, the

hallmark of endospore formation. Engulfment is a remarkable example of a very rare phenomenon occurring in bacteria that allows the internalization of the cell membrane within the cytoplasm by a phagocytosis-like phenomenon (Broder & Pogliano, 2006). The so-called zipping process is a key mechanism operating during engulfment, but none of the genetic components identified so far in *Bacillus* spp. were detected in *Kurthia* spp. The process of engulfment normally requires three essential proteins: SpoIID, SpoIIM, and SpoIIP (DMP zipper system). The DMP complex plays a crucial role during septal thinning, which is the first step of the process of the degradation of septal peptidoglycan (Abanes-De Mello et al., 2002; Aung et al., 2007; Broder & Pogliano, 2006). From these three proteins, solely a gene encoding the peptidoglycan hydrolase SpoIID (Abanes-De Mello et al., 2002) was detected in the genome of *K. massiliensis*. A second mechanism for membrane migration mediated by SpoIIQ and SpoIIAH (Q-AH zipper system) was also discovered in cells of *B. subtilis* in which peptidoglycan was removed enzymatically (Aung et al., 2007; Broder & Pogliano, 2006). This suggests that multiple

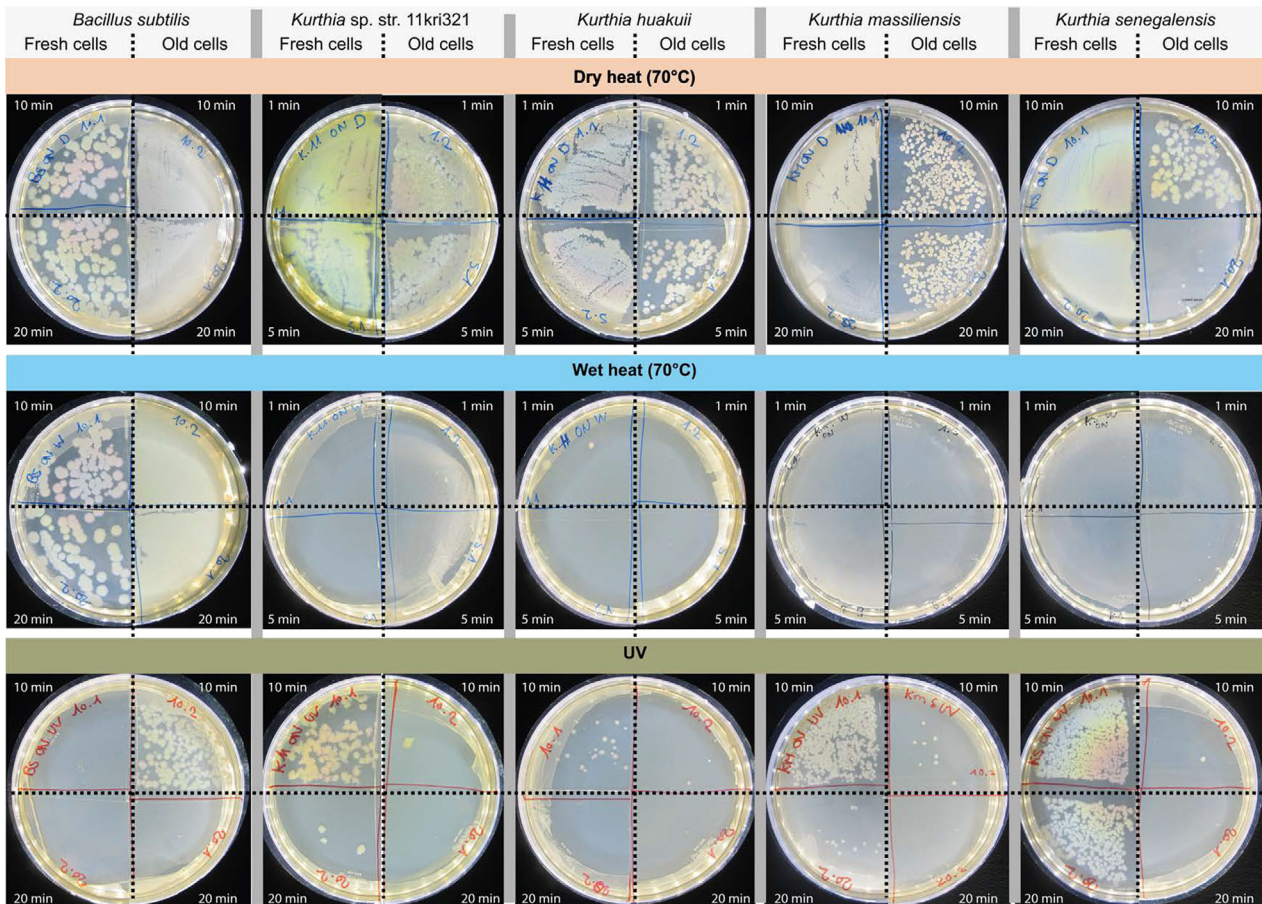


FIGURE 6 Resistance tests on fresh and 4-week aged cells of *Kurthia* spp. and *Bacillus subtilis*. The results shown correspond to a subset of the results to illustrate the effect of three stress factors: dry heat at 70°C (top panels), wet heat at 70°C (middle panels), and UV radiation (lower panels). The stress factors were all applied at four different exposure times (1, 5, 10 and 20 min), but only two of those (the one producing the most contrasting results) are presented as indicated in the figure. Fresh cells (left half part of plates) of the four *Kurthia* spp. grew denser and better after UV and dry heat treatments, compared to 4-week-old cells (right half part of plates). Neither the fresh nor the old cells of *Kurthia* spp. grew after the wet heat treatment. Spores (4-week aged cells) of *B. subtilis* were able to germinate after the three treatments, whereas fresh cells did not grow after UV exposure and grew in a lower density after heat treatments.

mechanisms might ensure that engulfment is robust. None of the genes coding the above proteins could be detected in the genomic analysis of the *Kurthia* spp. However, we clearly observed by cryo-EM stages of membrane engulfment in the case of *K. huakuii* and *K. senegalensis* (Figure 4) and at least some of the differentially expressed sigma factors generally involved in endospore formation (e.g., SigG) were detected in most of the species (Tables S2 and S4). This hypothesizes that, in the case of *Kurthia* spp., the observed spore-like structures, which appear to still have a second spore membrane, could be formed through an alternative engulfment mechanism.

Among the important sporulation genes, the ones encoding for small acid-soluble proteins (SASPs) were also absent from *Kurthia* spp. genomes. These proteins are known to bind DNA and participate in its protection against heat, UV radiation and other damaging agents and represent up to 20% of the total spore proteins found in *B. subtilis* (Driks, 2002; Setlow, 2007;

Setlow & Setlow, 1988a, 1988b). Likewise, only a small number of genes involved in cortex formation were present in the analysed genomes; therefore, *Kurthia* spp. might have a different mechanism for the formation of this layer or form an external layer quite different from the classic cortex. In the same way, even if a coat was clearly visible on the cryo-EM pictures, we identified a much smaller set of genes encoding proteins clearly related to the formation of the spore's coat (only *cotA*). Regarding the absence of SASPs and hypothetical alternative protective layers (due to the absence of genes coding classic cortex and coat), it might not be surprising that aged cells were not more resistant to heat and UV radiation, as compared to fresh cells (Figure 6). Lastly, the apparent absence of DPA in *Kurthia* spp. aged cells (Figure S1) suggests that spore-like structures observed for *Kurthia* spp. might be different in structure, physiology and resistance as compared to mature endospores of sporulating Firmicutes.

In summary, our findings suggest two potential interpretations linked to the ability of *Kurthia* spp., a genus, classified as asporogenic, to produce differentiated cell structures resembling spores. On the one hand, the analysed *Kurthia* spp. seem to be able to form spore-like structures after aging, and engulfment steps (considered as key stages during endospore formation) were observed for two of the strains (*K. huakuii* and *K. senegalensis*). In addition, even if scarce, we detected some important sporulation specific genes in the *Kurthia* spp. genomes (e.g. *spo0A*, *sigG*, and *spoIIAB*), which are crucially absent in asporogenic Firmicutes (Abecasis et al., 2013). However, on the other hand, aged cells were not more resistant to heat and UV than vegetative cells, contrary to what could be expected from spores. However, as sub-culturing clearly reduced the production of phase-bright bodies in all the species, it is also possible that specific conditions are required to trigger the differentiation of more resistant spore-like structures and to validate their resistance to stressors. This agrees with the genomic analysis. Indeed, the absence of numerous sporulation genes in *Kurthia* spp. genomes highlights that spore-like structures might differ greatly from the canonical endospores, not only structurally, but also in their formation and resistance.

Finally, the lack of spore-like phase-bright cells under optimal growth conditions suggests genetic drift or an epigenetic mechanism resulting in the loss of unstable spore-like structures in absence of environmental pressure. The genus *Kurthia* belongs to the Planococcaceae family, which is a family with a particularly complex evolutionary history. A recent phylogenetic study based on whole genome sequences suggested a re-examination of the groups included in the family. In this study, species from the genus *Kurthia* formed a monophyletic cluster related to other groups that included both spore and non-spore-forming species (Gupta & Patel, 2019). The closest relatives to *Kurthia*, the genera *Rummeliibacillus* and *Viridibacillus* contain both spore-forming species, and thus the loss of a full endospore formation pathway in this monophyletic clade appears to be recent.

To conclude, understanding the way in which endospore-formation, a seemingly ancestral characteristic, can be lost to give rise to truly asporogenic Firmicutes is essential to study the ecology and evolution of this clade. Considering that almost any natural habitat is subjected to significant variations in environmental parameters, the ability to store energy and resources through a mechanism of dormancy would be an advantage. Accordingly, our study highlights that the distinction between spore-forming and asporogenic in environmental strains might not be easy to establish. We propose to consider an intermediate life style and to use the term *cryptosporulant* to designate those groups for which some evidence for potential

sporulation emerges, but for which a detailed physiological and genomic analysis is not yet possible to reliably define the capability of a clade to produce or not (crypto-)spores.

The discovery of cryptosporulation in *Kurthia* sp. str. 11kri321, and in other species of the genus, paves the path for further investigation of cryptosporulants among asporogenic Firmicutes. The ecology of the few described species of *Kurthia* supports the cryptosporulant lifestyle of the genus. *Kurthia* strains have been isolated from diverse environments such as stool (Roux et al., 2012), biogas slurry (Ruan et al., 2014), medical samples (Roux et al., 2012), cigarettes (Rooney et al., 2005) and methanogenic bacterial complexes (Ruan et al., 2013). In metagenomic studies, the genus *Kurthia* has been found in snail gastrointestinal tracts (Pawar et al., 2012), restaurant kitchen cutting boards (Abdul-Mutalib et al., 2015) and soy sauce fermentation processes (Wei, Chao, et al., 2013; Wei, Wang, et al., 2013). In these habitats, the production of (crypto-) spores can be a trait linked to survival and therefore a process that should be under selective pressure for conservation, but only under environmental conditions.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

Any genetic information downloaded from GenBank should be considered to be part of the genetic patrimony of Greece, the country from which the sample was obtained. Users of this information agree to (1) acknowledge Greece as the country of origin in any country where the genetic information is presented and (2) contact the CBD focal point and the ABS focal point identified in the CBD website <http://www.cbd.int/information/nfp.shtml> if they intend to use the genetic information for commercial purposes.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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