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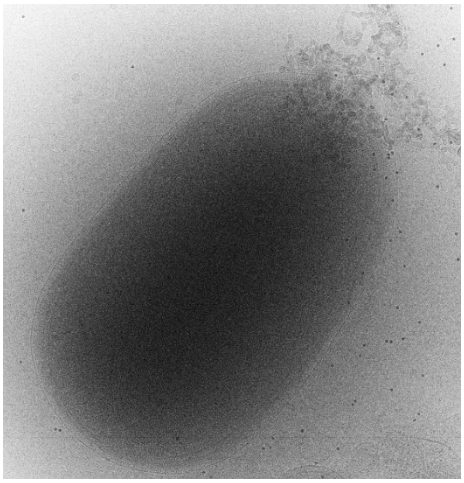
Plasticity of the stress response in *Kurthia* spp.: a study highlighting the challenges to work with non-standard bacterial model species



A dissertation submitted to the University of Neuchâtel

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« *A ma petite solution,*

Au soleil dans l'équipage,

A celui qui a fait de moi une guerrière,

A toi, mon fils, Inoé. »

Inspiré du texte de Sim's « Même pas peur »

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Summary

Natural environments represent an unstable habitat for living organisms with suitable periods for growth and reproduction alternating with suboptimal periods. Accordingly, organisms have to withstand the suboptimal periods, and for this they have developed various survival strategies. Microorganisms, including bacteria, are the most diverse and abundant groups of organisms in natural environments and they evolved really diverse survival strategies. One of the most extreme of these survival strategies is dormancy. During this process, upon the detection of unfavourable environmental conditions, microbes enter in a dormant state with a reversible interruption of growth or/and metabolism. Once conditions improve, dormant structures return to an active state and resume growth and reproduction. Several forms of dormancy exist, but the most well-known until now is sporulation. Sporulation is a complex cell differentiation process ending with the production of resistant dormant spores. Spores were observed in different bacterial phyla, and among them, endospores formed by the Firmicutes are usually considered as the reference. However, because endosporulation, as well as other dormancy strategies, are complex and costly for cells, not all bacterial cells are able to enter in these processes. Accordingly, alternative survival strategies may exist outside the well described spore-formers groups and to investigate those, the use of non-standard models is required.

The aim of this thesis was to investigate resistance strategies in the non-standard bacterial model *Kurthia* sp. str. 11kri321. Because this environmental strain was initially isolated from an extreme environment, it represents a promising model to explore survival strategies. We cultivated *Kurthia* sp. str. 11kri321 under challenging growth conditions in the laboratory and observed its response through different approaches, including light microscopy, cryo-electron microscopy, genomics, and transcriptomics. Our results show the plasticity of the stress response in *Kurthia* sp. str. 11kri321. The strain was observed under challenging conditions to produce alternative resistant structures to spores (i.e., cryptospores), but also to modify its cell morphology and to shutdown essential molecular processes such as translation and transcription. In addition to possess a variety of survival strategies, *Kurthia* sp. str. 11kri321 adapted quickly to laboratory conditions and changed at the genomic level, which might explain the observed decreased production of cryptospores and reduced heat resistance. Further investigations would be necessary to confirm that the responses displayed by *Kurthia* sp. str. 11kri321 under challenging conditions provide a survival benefit to the strain. However, this might be quite challenging regarding the instability of the strain when maintained in the laboratory.

To conclude, despite the challenges of working with a poorly known environmental species, this thesis paves the way for using non-standard bacterial models to investigate alternative survival strategies. Because bacterial survival strategies impact largely several pressing societal issues, such as the on-going antibiotics resistance crisis and global climate change, this work opens the field of research around alternative survival strategies in environmental bacteria.

Keywords: sporulation, cryptosporulation, survival, non-standard bacterial model

Résumé

Les environnements naturels représentent un habitat aux conditions instables pour les organismes qui y vivent, avec une alternance de périodes propices à la croissance et reproduction, et de périodes ne permettant pas ces processus. De ce fait, les organismes doivent faire face à ces périodes non-optimales et pour cela, ils ont développé une variété de stratégies de survie. Les microorganismes, y compris les bactéries, sont le groupe le plus divers et abondant au sein des environnements naturels et ont eux aussi évolué des stratégies de survies. L'une des plus extrêmes stratégies de survie est la dormance. Au cours de ce process, les microbes qui détectent des conditions environnementales non favorables entrent dans un stade de dormance qui implique une interruption réversible de la croissance et/ou du métabolisme. Une fois que les conditions s'améliorent, ces structures dormantes peuvent retourner à un stade de vie actif et reprendre leur croissance et reproduction. Il existe plusieurs formes de dormance, mais la mieux connue jusqu'à maintenant est la sporulation. La sporulation implique une différentiation cellulaire complexe qui permet la production de spores dormantes et résistantes. Des spores ont été observées au sein de plusieurs phyla bactériens, mais en général, les endospores associées aux Firmicutes sont considérées comme la référence. Cependant, en raison de la complexité et du coût liés à l'endosporulation, ainsi qu'aux autres formes de dormance, toutes les cellules bactériennes ne sont pas capables de ces stratégies. Ainsi, des stratégies de survie alternatives doivent exister en-dehors des groupes bien décrits de bactéries sporulantes, et des modèles non-standards sont requis afin de décrire ces stratégies alternatives.

Le but de cette thèse était d'étudier les stratégies de résistance du modèle bactérien non-standard *Kurthia* sp. str. 11kri321. Puisque cette souche a été initialement isolée d'un environnement extrême, elle représente un modèle prometteur pour l'exploration des stratégies de survie. On a cultivé *Kurthia* sp. str. 11kri321 dans des conditions stressantes en laboratoire, puis on a évalué sa réponse à l'aide de différentes méthodes, telles que la microscopie optique, la cryomicroscopie électronique et la transcriptomique. Nos résultats ont démontré la plasticité de la réponse au stress chez *Kurthia* sp. str. 11kri321. En réponse à des conditions de croissance stressantes, la souche a produit des structures de résistance alternatives aux spores (cryptospores), mais elle a également modifié sa morphologie cellulaire et ralenti certaines processus moléculaires clés, tels que traduction et transcription. En plus de posséder cette variété de réponse, *Kurthia* sp. str. 11kri321 s'est adaptée rapidement aux conditions de laboratoire et a changé au niveau génomique, ce qui pourrait expliquer l'observation d'une production moindre de cryptospores et d'une résistance à la chaleur réduite. De futures recherches seront nécessaires pour confirmer que les réponses observées chez *Kurthia* sp. str. 11kri321 lui offrent une meilleure survie à de conditions stressantes. Cependant, cela pourrait s'avérer fastidieux au vu de l'instabilité de la souche lorsque cette dernière est maintenue en laboratoire.

En conclusion, malgré des difficultés certaines à travailler avec des espèces environnementales peu connues, cette thèse ouvre la voie à l'utilisation plus fréquente de modèles bactériens non-standard afin d'étudier les stratégies de survie. En raison de l'impact important des stratégies de survie bactériennes sur plusieurs problématiques sociétales, telles

que les actuelles crises de résistance aux antibiotiques ou du changement climatique, cette thèse ouvre le champ de recherche à propos des stratégies de survie alternatives chez les bactéries environnementales.

Mots-clés : sporulation, cryptosporulation, survie, modèle bactérien non-standard

Glossary

Spore-former: organism that is able to form spore, a cellular structure considered as dormant with a reversible interruption of growth and metabolism and that is considered as more resistant than the other cells. Synonyms: **sporulating organism**, or **sporogenic organism**.

Non-spore-former: by opposition to spore-form, an organism that is not able to produce dormant resistant spores. Synonyms: **non-sporulating organism**, or **asporogenic organism**.

Sporulation: a complex cell differentiation triggered by suboptimal growth conditions and that ends with the formation of resistant dormant spores.

Endosporulation: a particular type of sporulation that is found in Firmicutes and that involves engulfment of the pre-spore by the mother cell. This process results in the formation of endospores.

Cryptospore: alternative cell structure that possesses some common features with canonical resistant spores, but that might be different in its structure, resistance, and mechanism of formation.

General introduction

1. Challenging natural environments and the need to survive

1.1 Dormancy: a survival strategy with a high biological cost

In natural habitats, the environmental conditions are not stable and therefore, represent a challenge for organisms living therein. In fact, the conditions can drastically and unpredictably change, leading to a situation that is not favourable for growth and reproduction (Harrison 1979; Lennon and Jones 2011). Thus, being able to survive during these challenging periods will represent a clear advantage from an evolutionary perspective.

Microorganisms are vital part of these changing natural environments, where they constitute the most abundant and diverse group of organisms (Van den Bergh, et al. 2018). Microorganisms are well known to adapt quickly due to a rapid evolution rate and large population size (Elena and Lenski 2003). Accordingly, they have developed various strategies to persist in fluctuating environments and dormancy is one of them. A dormant cell is considered as a cell in a resting state, with a reversible interruption of growth or metabolism. Dormancy provides several benefits: first, dormancy contributes to the persistence and fitness of microbes when environmental variables such as pH, oxygen and resources availability are suboptimal for growth and reproduction. Secondly, dormancy diminishes the strength of competition and promotes species coexistence (Lennon, et al. 2021). Last but not least, dormancy represents a refuge against predator consumption and parasite infection. For example, dormancy can protect against viral infections. Indeed, dormant cells undergo morphological changes that affect how viruses interact with their host. The protective layer that covers the exterior of dormant cells masks the surface molecules normally used by viruses for attachment. Even if a virus succeeds to enter into dormant cell, it will then be constrained by the reduced metabolism of its host (Schwartz, et al. 2022).

Dormancy is often initiated if an organism detects unfavourable changes in its environment. In fact, various environmental cues, like temperature, osmotic pressure, light or resources availability, can signal to microorganisms that it is time to enter in dormancy and to produce dormant resting structure (Lennon, et al. 2021). Even if dormancy can be viewed as beneficial as it allows survival, this strategy has a high biological cost. In fact, if an organism is to enter in dormancy, it requires energy in order to activate the machinery that leads to the production of a dormant cellular state (Callahan, et al. 2008; Van Bodegom 2007). In addition, the cell has to interpret correctly the environmental cues signalling the onset of unfavourable environmental conditions, because if it enters in dormancy when this is unnecessary, it will miss the opportunity to grow and reproduce (trade-off survival versus growth/reproduction) (Ratcliff, et al. 2013). Therefore, the initiation of dormancy is tightly controlled by a complex regulatory network (Galperin, et al. 2022; Garg and Maldener 2021; Lennon and Jones 2011).

After their entrance into dormancy, microorganisms can display resting structures with a large diversity of phenotypes, including spores (Bobek, et al. 2017; Setlow 2007), conidia (Dijksterhuis 2017), cysts (Berleman and Bauer 2004; Segura, et al. 2014) or akinetes (Garg

and Maldener 2021). Compared to active cells, resting structures often differentiate in several aspects like size, density, motility, concentration of nucleic acids, lipids, fatty acids and proteins, as well as concentrations of storage compounds or reserves (Lennon, et al. 2021; Lennon and Jones 2011). However, in many systems, active and resting cells have similar morphological features and occupy the same habitat (Lennon, et al. 2021).

Of course, dormancy can only be considered as an adaptive strategy if, at the end, the resting structures are able to exit dormancy and to reproduce. This is the last step of the process, called resuscitation. If conditions improve, dormant individuals can communicate and coordinate resuscitation with other members of the dormant population in a density-dependent manner (Lennon, et al. 2021).

To conclude, dormancy is an efficient and adaptive solution for microorganisms to persist in the environment, nevertheless with biological costs (energy requirement, missing reproduction/growth's opportunities, etc.). Therefore, entrance and exit of dormancy have to be tightly controlled and environmental cues well interpreted.

1.2 Sporulation, a form of dormancy

As mentioned above, dormant cells have different phenotypes and one particularly well known is the so-called "spore", a cell produced during a process called sporulation. Spores represent two great advantages for spore-formers. First, these highly resistant dormant structures allow survival for extended periods of time in natural environments. Second, spores increase the dissemination rate. These two advantages allow the organisms to avoid local unfavourable conditions for growth and/or reproduction (Nicholson 2002).

The sporulation process and the produced spores are well described in five bacterial phyla, with different types of spores: endospores formed by members of the Firmicutes (Nicholson, et al. 2000; Setlow 2014), exospores produced by members of the Actinobacteria (Beskrovnaya, et al. 2021; Hoskisson and van Wezel 2019), akinetes for the Cyanobacteria (Garg and Maldener 2021; Kaplan-Levy, et al. 2010), cysts produced by the Azotobacteraceae order (Segura, et al. 2014; Vela 1974), and myxospores for the Myxococcales order (Muñoz-Dorado, et al. 2016). Among these five models, only endospores are really well understood and only little is known about the other types. Therefore, endospores are often used as standard when comparing with other bacterial spores (Paul, et al. 2019). Endospores are produced during endosporulation, a process that implies a complex cell differentiation, and more precisely, an asymmetric cell division, followed by several unique steps (Paul, et al. 2019; Setlow 2014). Most of the knowledge about endospores was obtained using the well-studied bacterial model *Bacillus subtilis*.

1.3 Endosporulation in Firmicutes and the sporulating model *Bacillus* spp.

Endospore formation is a specific process found within the bacterial phylum Firmicutes and was extensively investigated in the model organism, *B. subtilis*. The spore-forming *Bacillus* has a life cycle divided in three different physiological states; (1) vegetative growth, (2) sporulation, and (3) germination. The cells change from one state to another according to living conditions, especially nutrients' availability. If resources are sufficient, vegetative

growth occurs, with a binary symmetric fission of the mother cell forming two sister cells. Vegetative cells of *Bacillus* appear phase-dark under phase-contrast microscopy. However, in the case of nutrient depletion or other stressful conditions *Bacillus* vegetative cells differentiate into resistant endospores (Sella, et al. 2014). The endosporulation is a complex process involving several steps (Fig. 1) (Tan and Ramamurthi 2014). In a nutshell, endosporulation starts with an asymmetrical cell division, leading to two morphologically and functionally distinct cells: the pre-spore and the mother cell. The pre-spore is next "embraced" by the mother cell, a characteristic step called engulfment. Engulfment results in an intermediate diderm cell (forespore). To obtain the final dormant cell (endospore), this intermediate cell is remodelled by the mother cell, notably by the formation of the cortex and coat layers, and the accumulation of calcium ions (Ca^{2+}) and dipicolinic acid (DPA), among others. The endosporulation process ends with lysis of the mother cell, releasing the mature endospore (Paul, et al. 2019; Setlow 2014; Tan and Ramamurthi 2014).

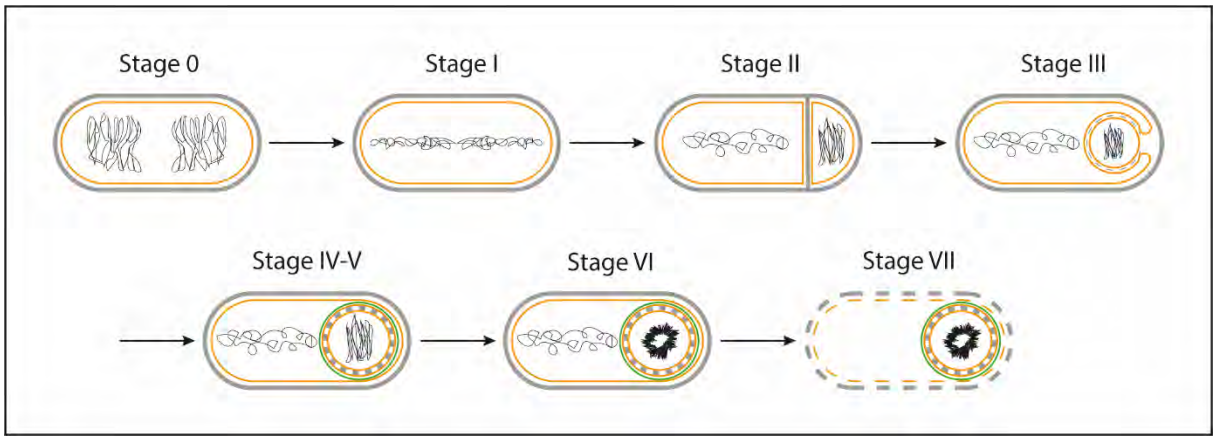

Figure 1. Schematic representation of *Bacillus subtilis* sporulation. Depiction of the different stages recognized during sporulation. Peptidoglycan layer is shown in gray, membranes in orange, DNA in black, and spore coat in green. Stage 0: chromosome replication. Stage I: chromosome condensation and anchoring of origins of replication to the extreme poles of the cell. Stage II: elaboration of polar septum. Stage III: engulfment of the forespore. Stage IV-V: cortex and coat assembly, respectively. Stage VI: spore maturation. Stage VII: lysis of mother cell and release of the mature, dormant, and highly resistant spore into the environment. Figure modified from Tan & Ramamurthi, 2014.

Endospores are different from vegetative cells at the structural level with several unique layers not found in the latter (Fig. 2): the exosporium (thin layer made of proteins, not always present in the final structure), the spore's coat (specific spore proteins), the cortex (modified peptidoglycan layer), and the core (central part with germ cell wall, membrane, cytoplasm, ribosomes, and other compounds) (Setlow 2006; Setlow 2014; Tan and Ramamurthi 2014). In addition, endospores present an accumulation of DPA and calcium ions (Ca^{2+}), a dehydrated spore core, and finally, a large quantity of small acid-soluble proteins (SASPs) (Setlow 2006; Setlow 2014).

Endospores are defined as the most resistant cellular structures on Earth (Abecasis, et al. 2013). They are highly resistant to heat, radiation, and chemical agents. Even if all the

mechanisms leading to this remarkable resistance are still not completely understood, it is commonly admitted that the dehydrated core contributes to heat resistance, whereas SASPs bind to DNA and protect it from UV damage (Setlow 2006; Setlow 2014).

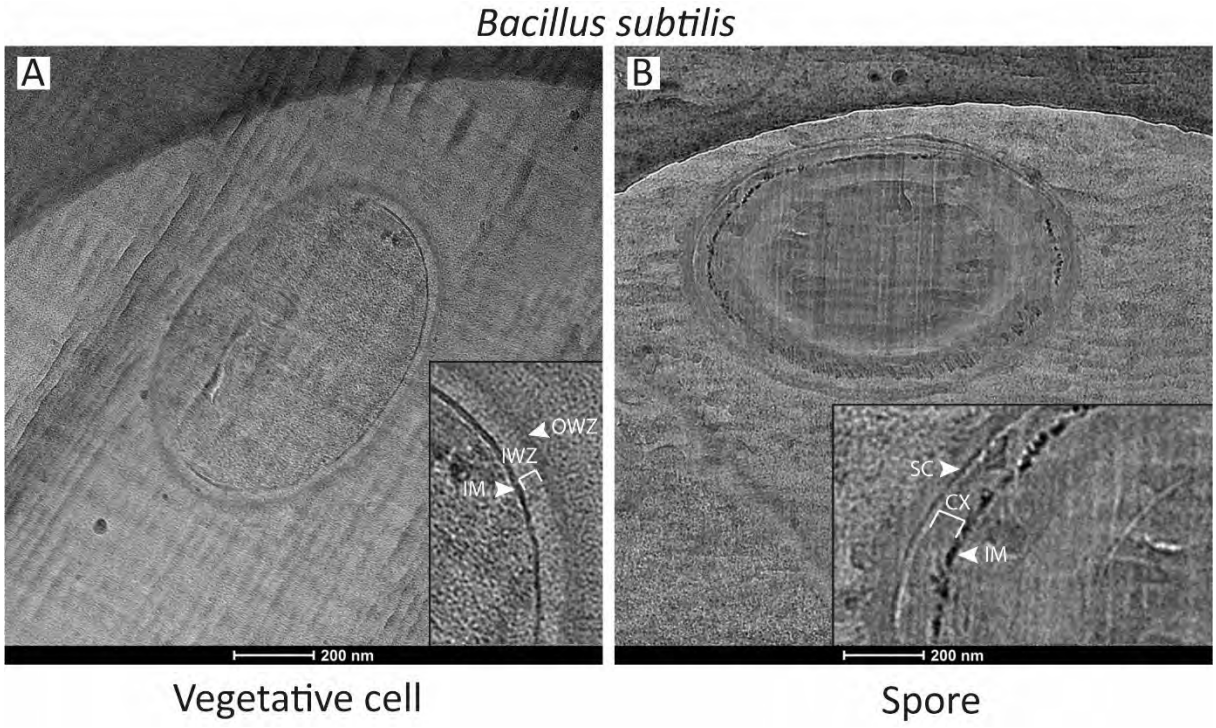

Figure 2. Cryo-electron microscopy of vitreous sections (CEMOVIS) of *Bacillus subtilis*' vegetative cell and spore. (A) Cell envelope of vegetative cells comprises inner membrane (IM), inner well zone (IWZ), and outer well zone (OWZ). **(B)** Spores are composed of unique layers with spore coat (SC) and cortex (CX). They also possess an IM. Images acquired by Andrea Corona.

Finally, endospores are not a dead end and when environmental conditions improve, germination occurs allowing a return to a metabolically active cell, able to grow and reproduce. Initially, germinant receptors located in the inner membrane of spores respond to germinants such as specific L-amino acids or D-sugars. After their stimulation, the germinants receptors trigger the opening of channels in the inner membrane, which allow the release of cations and of DPA. After this release, DPA is replaced by water, leading to a core water content changing from 35% to 45%. Germination is then continued by the activation of cortex-lytic enzymes that degrade spore cortex. This cortex hydrolysis results in two-fold expansion of the spore core via more water uptake (i.e., spore core water content increases from 45% to 80%). This ends in a fully germinated spores, with synthesis of DNA, RNA, and proteins (Christie and Setlow 2020).

1.3.1 Minimal set of sporulation-specific genes in Firmicutes

Spore-formers regulate the expression of multiple transcription factors leading to an irreversible commitment to dormancy. Entering dormancy has important implications for fitness in time-varying environments. In fact, even if production of endospores helps species

to survive under harsh conditions, those species might also miss opportunities to reproduce. Indeed, on average, *Bacillus* cell needs eight hours to create a functional spore and during that time, the same individual, with a 20 min doubling time, could give rise to 10^7 offspring (Lennon, et al. 2021). Accordingly, sporulation is tightly regulated. In the model organism *B. subtilis*, endosporulation involves more than 500 genes, with a large fraction of them acting sequentially. Among those genes, some are exclusively involved in sporulation, but many housekeeping genes are also necessary during spore germination and vegetative growth (Liu, et al. 2004).

The complete genetic pathway involved during the sporulation of *Bacillus* spp. has been extensively reported (Errington 2003; Galperin, et al. 2012). Broadly, the initiation and regulation of sporulation depend mostly upon the master regulator of sporulation, Spo0A, and sporulation-specific sigma factors σ^E and σ^K (in the mother cell) or σ^F and σ^G (in the developing spore) (Galperin, et al. 2012). First, Spo0A is activated through a phosphorylation relay system with several kinases (i.e., enzymes catalysing transfer of phosphate groups) and two intermediates (Spo0F and Spo0B). The second key positive regulator is σ^H. Thus, the vegetative cells start sporulation thanks to high levels of σ^H and Spo0A, immediately followed by expression of pro-σ^F. After the asymmetric division and the production of the mother cell and the pre-spore, σ^F is activated only in the pre-spore. Next, and only in the mother cell, a proteolytic process activates pro-σ^E in σ^E. Once engulfment is complete, σ^E triggers the production in the forespore of the transcription factor σ^G. Finally, σ^G controls the production of SpoIVB protein and this will trigger the proteolytic process leading to the activation, in the mother cell, of pro-σ^K into σ^K. The mature spore is ready and the mother cell will lyse to free it (Sella, et al. 2014).

Because of the complexity of the genetic sporulation pathway in *B. subtilis*, it has been proposed that spore formation will not be functional if all the components required in the sequential process are not present (Onyenwoke, et al. 2004). Accordingly, previous studies postulated that a conserved set of approximately 60 sporulation genes of *B. subtilis*, called the minimal set of sporulation-specific genes, is shared by spore-forming members of Bacilli and Clostridia (De Hoon, et al. 2010; Galperin, et al. 2012). This conservation of core sporulation genes suggested that sporulation was a common ancient feature of the Firmicutes, inherited from their last common spore-forming ancestor (De Hoon, et al. 2010; Galperin 2016). However, several genes from this conserved set were shown to be absent from the genomes of various spore-formers (Abecasis, et al. 2013; Galperin, et al. 2012; Ramos-Silva, et al. 2019). Hence, a recent study showed that many SASPs, spore coat proteins, and germination proteins are missing in spore-formers members of Clostridia and other classes of Firmicutes (Galperin, et al. 2022). As an example, it appeared that in *Clostridium difficile*, numerous regulators required for efficient sporulation in other spore-formers are missing. Accordingly, *C. difficile* was shown to possess novel mechanisms and distinct regulatory pathways to control sporulation initiation and sporulation-specific gene expression. One striking difference concerns the control of Spo0A activity. Indeed, as presented above, in *B. subtilis* and other *Bacillus* species, the activity of Spo0A is regulated by a phosphorylation relay system, whereas in *C. difficile* genome, those phosphorelay orthologs are absent. Instead, Spo0A activity in *C. difficile* is controlled through at least three orphan histidine kinases and other unidentified

factors. A novel regulatory pathway to mediate Spo0A phosphorylation may have evolved in *C. difficile* thanks to its highly specialized ecological niches (i.e., gastrointestinal niche and outside of its host) (Edwards and McBride 2014).

2. Ecological and societal importance of dormancy

The accumulation of bacterial spores and other dormant structures in the environment creates a seed bank (Jones and Lennon 2010; Shoemaker and Lennon 2018). Seed banks contribute to the diversity and dynamics of microbial communities in future generations. Indeed, even if composed of dormant cells, seed banks should be considered as dynamic, and once environmental conditions improve, members of this seed bank can return to an active state of life and shape the microbial communities (Jones and Lennon 2010). In addition, seed banks are crucial in the recovery of terrestrial ecosystems after environmental catastrophes, such as fire. While active cells will die during such catastrophic events, dormant cells will survive and can afterwards colonise the environment (Lennon, et al. 2021). For example, in the case of wild fires, spore-formers were shown to survive the direct heating better than the other groups and they also had an initial advantage in using dissolved organic carbon (DOC). In addition, bacteria capable of entering into dormancy have the potential to survive for long periods as dormant cells in the soil. Thus, spore-formers were shown with values more than 20 times higher in a site that had burned 32 months before, as compared to the preserved reference site (Bárcenas-Moreno, et al. 2011). Therefore, this dormant seed bank has a role in maintaining a reservoir of genetic and phenotypic microbial diversity over long, multi-annual timescales (Shoemaker and Lennon 2018; Wisnoski and Lennon 2021). In addition, seed banks alter the molecular evolution of microorganisms, by diminishing the accumulation of *de novo* genetic diversity and the fluctuations of genetic variants, as dormant cells do not reproduce and the majority of mutations are acquired during reproduction (Shoemaker, et al. 2022). Finally, as seed bank mediates microbial diversity, it also impacts other important ecological processes, like successional dynamics (Lennon and Jones 2011), community stability (Kalamees and Zobel 2002) and ecosystem processes (Wang, et al. 2014).

In the context of global climate change, a better understanding of the dormant microbial fraction would be crucial. Indeed, the seed bank represents a source of preserved dormant cells that will facilitate re-establishment of microbial communities when environmental conditions improve (Wisnoski and Lennon 2021). Therefore, knowing this dormant diversity might help to predict the future microbial communities and their response to climate change. Moreover, microbial diversity preserved in seed bank reflects the past environmental conditions (i.e., paleoecology) and have indications on how past conditions might help to understand the present environmental conditions, or even to predict the future ones (Payne, et al. 2006).

Next, from a clinical standpoint, dormancy has attracted early attention because of its role in human diseases such as anthrax, cholera, and tuberculosis (Coates 2003). Nowadays, those diseases are rare. However, as close as 2016, an outbreak of anthrax killed thousands of reindeer and infected dozens of humans in Siberia. This outbreak was due to the activation of

spores after permafrost thawing (Ezhova, et al. 2021), highlighting the importance of spores in epidemiology. In addition, many infection diseases are caused by microorganisms able to persist in their human host thanks to dormancy. Due to their metabolically inactive state, microbial pathogens can escape the host immune defences and they are also less susceptible to drugs that affect the transcriptional and translational machinery of growing cells (Lennon, et al. 2021). Accordingly, many microbial pathogens are able to tolerate high concentrations of antibiotics through the production of dormant resistant structures (Fisher, et al. 2017; Lennon, et al. 2021), which nowadays attracts considerable attention due to the antibiotic resistance crisis (Shrivastava, et al. 2018). Moreover, the study of dormancy is crucial as it is universally recognized that cancer dynamics are affected by the ability of cells to enter in dormancy. Indeed, patients can be in remission for decades until cancer cells resuscitate (Lennon, et al. 2021). Finally, it was recently shown that dormant cells have also an impact on the evolutionary history of phages. Indeed, sigma factors, key regulators required for transitioning into a dormant state, were detected in phages. It is possible that these sporulation-like sigma factors are auxiliary genes that can alter sporulation in bacteria (Schwartz, et al. 2022), which is of great interest as a way to maybe reduce human diseases in which spore-formers are involved (Schwartz, et al. 2022).

Finally, besides their impact on ecological processes or human health, dormant structures should also be of interest simply because prevalence of dormancy is really large. Dormant structures are highly represented in complex microbial communities ranging from the human gut to the world's oceans (Shoemaker and Lennon 2018). For example, >90% of microbial biomass in soils is considered to be metabolically inactive (Lennon and Jones 2011).

3. Dormancy and alternative resistance strategies in non-standard models

Due to the prevalence and impacts of the dormant resistant fraction, it is crucial to explore the diversity of dormant resistant structures also beyond the well-described (endo-)spores formed by sporulating bacteria. Alternative dormant structures have been observed in groups considered as non-sporulating (Suzina, et al. 2004). For example, cyst-like resting structures were reported for *Rhodospirillum centenum*, a photosynthetic member of the *Azospirillum* clade (Dong and Bauer 2015), whereas *Serratia ureilytica* was shown to produce resistant structures different from the vegetative cells (Filippidou, et al. 2019). In consequence, dormancy and resting resistant structures may be prevalent among more diverse bacterial phyla than initially thought. Moreover, alternative stress resistance strategies to dormancy were reported, such as for example the extreme slow growth rate observed in non-sporulating *B. subtilis* cells, which is defined as oligotrophic growth. During this oligotrophic growth, the doubling time is equal to 4 days, whether it is equal to 40 min during normal exponential growth. This dramatic reduction in growth rate might explain why oligotrophic cells are tolerant to some antibiotics and cope better with oxidative stress. Oligotrophic cells are able to rapidly resume normal growth when nutrient conditions improve, which represents an advantage compared to sporulating cells that have to go through the complex and time-

consuming process of germination (Gray, et al. 2019). In addition, modifications of cell morphology, as well as transient shutdown of the general translation and mRNA transcription, can be cited as alternative stress resistance strategies to dormancy (Grigull, et al. 2004; Paschen 2003; Yang, et al. 2016). In the light of the examples above, dormancy and alternative stress resistance strategies might be widely spread over diverse bacterial phyla. Accordingly, the study of dormancy (including sporulation) and production of resting structures, as well as of alternative strategies, in non-standard models, is needed.

3.1 Studying non-standard models in the laboratory

3.1.1 Setbacks of laboratory cultivation

The investigation of dormancy and of alternative stress resistance strategies in non-standard models can be achieved through molecular techniques. However, even if molecular methods are largely used nowadays, in order to fully describe physiology or ecological roles of uncharacterised bacteria, they need to be isolated and cultivated in the laboratory (Stewart 2012; Tanaka, et al. 2014). Accordingly, the investigation of stress resistance strategies in non-standard models would as well require cultivation in the laboratory. However, cultivation in the laboratory has important setbacks. First, not all bacteria from a given natural environment will grow on laboratory media. Those bacteria do not grow because essential aspects of their environment are not replicated in the laboratory and most of the time it is unclear which facet of the environment is yet not properly replicated (nutrients, temperature, pH, osmotic conditions, etc.) (Stewart 2012). The variety of factors that may be limiting laboratory cultivation is vast, making it a challenge to recreate a growth medium that supports cell growth (Tanaka, et al. 2014). Secondly, even if laboratory media mimic enough the natural environment to support growth, the growth conditions (including the interaction with other microorganisms) will still not be exactly similar to the ones of the original habitat. Because of this, selection for physiological traits associated to the natural environment may be relaxed under laboratory conditions and specific traits may be lost due to the accumulation of neutral mutations. Indeed, mutations are normally deleterious when the trait is under positive selection (e.g., in the original environment), but if selection is relaxed (e.g., in the laboratory), these mutations become neutral and can accumulate in bacterial genomes. Alternatively, traits may be lost because of selection; traits may be costly to maintain if selection is no longer present (e.g., in the laboratory compared to natural environments) and therefore, these traits are lost, which allows to reallocate resources in some other needed traits (Maughan, et al. 2006).

As presented above, dormancy is a costly trait not only because it requires high energetic investment (Dworkin and Losick 2005), but also because bacteria entering in dormancy might miss opportunities to grow/reproduce (Lennon, et al. 2021; Ratcliff, et al. 2013). In addition, even alternative stress resistance responses to dormancy have a direct cost, with for example the production of stress protectants, and an indirect cost as bacteria displaying a stress response have a reduced capability to compete vegetatively for nutrients (trade-off between self-reservation and nutritional competence, called the SPANC balance) (Ferenci and Spira 2007). Because of these costs, traits conferring stress resistance in natural environments might be lost in the laboratory, as the growth conditions therein will be different and therefore

selection on those traits might be relaxed. This was shown previously on laboratory strains of *B. anthracis* that lost their responsiveness to grow and sporulate as compared to wild strains (Norris, et al. 2020), as well as with *Azotobacter* cysts from soils that were far more resistant to radiation as compared to the laboratory strains (Vela and Wyss 1965).

3.1.2 Challenging growth conditions in laboratory

Despite the above-mentioned difficulties when investigating stress resistance under laboratory conditions, there are some approaches that can be used in order to recreate challenging growth conditions, and accordingly to trigger a stress resistance response in the non-standard models we want to investigate. First, in previous studies sporulation has been induced in the laboratory using specific sporulation media (Bressuire-Isoard, et al. 2018). The composition of these sporulation media varies, but one common point is the low level of available nutrients as compared to the rich nutrient media usually used to support optimal bacterial growth (Brandes Ammann, et al. 2011; Bressuire-Isoard, et al. 2018). In fact, although several stresses can play a role, the most commonly recognized stimulus for sporulation is starvation (Errington 2003), which is a stress often encountered by bacteria in natural environments (Gray, et al. 2019). Despite nutrient availability, other parameters can be modified in order to create a stress. Among them, we can cite water and iron availability, as both of these elements are crucial for bacterial growth and low-water and/or low-iron availability constitute common environmental stresses (Andrews, et al. 2003; Esbelin, et al. 2018).

One element that has been recognized for decades as a sporulation trigger is manganese (Charney, et al. 1951). Indeed, manganese is an essential element for the sporulation of Bacilli (Bosma, et al. 2021; Que and Helmann 2000; Sinnelä, et al. 2019; Weinberg 1964). Even if it is still not entirely clear why manganese is required for sporulation, it is known since a long time that manganese is a co-factor of many enzymes (Oh and Freese 1976). For example, Mn^{2+} is a cofactor for fructose-1,6-bisphosphatase and glutamine synthetase (Oh and Freese 1976), which appear to have a regulatory role in the initiation of sporulation process (Opheim and Bernlohr 1975; Pan and Coote 1979). Beyond its implication in sporulation, manganese is also involved in many other fundamental cellular processes, including oxidative stress protection (Bosma, et al. 2021; Randazzo, et al. 2020). Manganese protects bacterial cells against reactive oxygen species (ROS), either as a cofactor for manganese-dependent catalases and superoxide dismutases, or via its ability to chemically scavenge ROS (Aguirre and Culotta 2012; Anjem, et al. 2009). Moreover, under oxidative stress, sporulation might be initiate as a stress response through the interaction of proteins that are sensible to redox changes and to manganese supplementation. Indeed, it was shown that under reduced conditions, YlaD is found in a reduced form and in a complex with YlaC. However, in case of oxidative stress, YlaC is oxidized and dissociates from the YlaC-YlaD complex, which allows YlaC to attach to its specific promotor and induce transcription of genes involved in oxidative stress response and in sporulation. In addition, the redox state of YlaD (reduced or oxidized) was also suggest to impact expression of genes involved in the aforementioned functions (Kwak, et al. 2018; Ryu, et al. 2006). Despite its crucial role in central cellular processes, manganese is also highly toxic when in excess (Randazzo, et al. 2020). Accordingly, varying the availability to manganese

might represent a promising approach to trigger and study stress resistance response in non-standard models.

3.1.3 Molecular and microscopy analysis to explore stress resistance strategies in non-standard models

Once grown in the laboratory, several tools are available to investigate stress resistance strategies in non-standard models. Among them, molecular techniques with the analysis of DNA (genomics), RNA (transcriptomics), proteins (proteomics) or metabolites (metabolomics). All together these methods can be used to characterize a single organism, here for example a non-standard model. Genomics or transcriptomics are currently used to respectively described genes and functions associated with a microorganism. Proteomics accesses also the functions displayed by a microorganism under specific conditions, but with the additional indication of which proteins are actually responsible for the functional activity. Finally, metabolomics identifies the final or intermediate products produced by the microorganisms under specific conditions (Bouchez, et al. 2016).

However, in order to associate a specific molecular profile (representing a function) to a general cellular aspect or to appearance of some particular cell structures, microscopy is always informative. Several techniques have been developed. First, light microscopy can discriminate only shapes and forms, unless distinctive staining is used to differentiate fine structures. Despite of this, light microscopy continues to be a hallmark of microbiology and can give a first snapshot at low magnification of the global cell morphology under certain conditions (Reddy, et al. 2007). Next, electron microscopy (e.g., transmission (TEM) or scanning (SEM) electron microscopy) is needed to image cells and cell structures at high magnification. Electron microscopy uses electrons instead of visible light (photons), and because the wavelength of electrons is much shorter, it improves the resolution. For example, resolving power of light microscope is about 0.2 µm, whereas it is about 0.2 nm for transmission electron microscopy (Madigan, et al. 2010). However, in order to improve contrast or be able to observe particular structures both for light and electron microscopy, samples of interest have to be dehydrated and stained. In addition, in the case of electron microscopy, electron irradiation leads to damages in the observed samples (breaking of chemical bonds and creating free radicals). One way to mitigate such damages is to coat samples with reagents containing heavy atoms. However, these conventional sample preparation methods are artefact prone (e.g., sample flattening after coating with heavy atoms) (Dubochet, et al. 1983; Milne, et al. 2013).

One approach to solve the problem of artefacts is cryo-electron microscopy. Cryo-electron microscopy, abbreviated cryo-EM, performs imaging in transmission electron microscope under cryogenic conditions (frozen samples maintained at liquid nitrogen temperature). Cryo-EM encompasses several techniques such as cryo-electron tomography, single-particle cryo-electron microscopy and electron crystallography (Milne, et al. 2013). In order to perform cryo-EM, the sample of interest has to be prepared using “vitrification”. For this step, a thin film of bacterial culture is rapidly plunged into a cryogen that contains liquid ethane cooled by liquid nitrogen to temperature of -173°C. This preparation is deposited on a grid covered by a carbon film with holes, so that cells that end up in a hole are embedded in a thin layer of ice.

It results in the formation of vitreous ice, allowing the capture of cells in their native state at the instant of freezing. The frozen grid is always maintained at -173°C, including during the imaging process under the electron microscope (Milne and Subramaniam 2009). This technique allows to collect images without any adsorption artefact or background signal from the support layer. Cryo-EM provides two-dimensional (2D) images, but three-dimensional (3D) structures can also be determined. Indeed, if the sample is structurally and chemically homogenous, thousand cryo-EM images in various orientations can be averaged to reconstruct the 3D structure (single particle reconstruction). Otherwise, cryo-electron tomography, abbreviated cryo-ET, allows also 3D reconstruction. Cryo-ET involves to tilt the sample through a large angular range (e.g., -70° to +70°) and then collects series of images of a single sample area. The tilt series of images are then used to reconstruct the structure of interest in 3D (Stewart 2017).

4. Outline of this thesis

According to the importance of spores and alternative resistant structures in natural environment and human health (see above section "Ecological and societal importance of dormancy"), the production of such structures has to be studied outside the best-studied sporulating models, such as members of the Bacilli and Clostridia. Therefore, this thesis aims to explore sporulation, as well as alternative stress resistance strategies, in a non-standard model, under laboratory conditions.

One first needs to isolate and characterize such non-standard model. As already indicated, sporulation is a beneficial trait that improves survival and dispersal under unfavourable conditions (Nicholson 2002). Hence, the screening of extreme habitats might be a good strategy, as multiple limiting environmental conditions were shown to favour sporulating bacteria (Fernández-Gómez, et al. 2019; Filippidou, et al. 2016; Schimel 2018). Accordingly, geothermal environments with their steep chemical and physical gradients are potential good examples of novel environments where to search for a large diversity of spore-formers (i.e., non-standard sporulating models). Therefore, we initiated multiple enrichments to isolate aerobic and anaerobic strains originating from some geothermal environments. The strains were screened for their capability to form spores, without any prior knowledge of their phylogenetic affiliation. In this way, *Kurthia* sp. str. 11kri321 was isolated from the geothermal spring of Krinides, Kavala, Greece. The genus *Kurthia* belongs to the Planococcacea family from the Firmicutes phylum (Ruan, et al. 2014). The first species of this genus, named initially *Bacterium zopfii* and that is nowadays *Kurthia zopfii*, was isolated from intestinal contents of chicken (Kurth 1883; Trevisan 1885). The genus comprised five recognized species: *K. zopfii* (Trevisan 1885), *K. gibsonii* (Keddie and Shaw 1986), *K. sibirica* (Belikova, et al. 1986), *K. massiliensis* (Roux, et al. 2012), and *K. senegalensis* (Roux, et al. 2014). The *Kurthia* species were isolated from very diverse habitats such as stool (Roux, et al. 2012), biogas slurry (Ruan, et al. 2014), snail gastrointestinal tracts (Pawar, et al. 2012), medical samples (Roux, et al. 2012) or from natural environment, such as a geothermal spring for *Kurthia* sp. str. 11kri321 (Fatton, et al. 2022). Even if *Kurthia* was, so far, classified as a non-spore forming genus among Firmicutes, spore-like cells of the strain 11kri321 were observed to refract light under the

phase-contrast microscope and could be stained with malachite green (Fatton, et al. 2022). Thanks to these observations of cell structures sharing common features with canonical endospores, we decided to use the isolated environmental strain *Kurthia* sp. str. 11kri321 as a non-standard model. However, due to multiple culturing realized under laboratory conditions, the initial isolated strain of *Kurthia* sp. str. 11kri321 might adapt and evolve. In this context, we realized that a mix of different sub-strains evolved gradually all along the different experiments. Therefore, in the different chapters we consider that our work has involved two sub-strains of *Kurthia* sp. str. 11kri321; the original environmental isolated strain (OS strain, maintained as a cryo-preserved culture) and the strain that was maintained under laboratory conditions (LS strain). The two strains could be distinguished by genomic investigations; however, those were only performed at the end. In consequence, it is not always possible to indicate which of the sub-strain was used in the different experiments considered in this thesis. To help the reader, this information will be clearly provided at the start of the different chapters. Finally, in addition to *Kurthia* sp. str. 11kri321, we also included as well other members of the *Kurthia* genus.

First, we conducted a more detailed investigation of the sporulation capacity in the genus *Kurthia*, using diverse analytic tools including light microscopy, cryo-electron microscopy, as well as genomic analysis (Chapter 1).

After focusing on a potential sporulation in the non-standard models *Kurthia* spp., the field of research was broadened. Thus, as a second aim of this thesis, we explored the reaction of *Kurthia* sp. str. 11kri321 when grown under challenging conditions in laboratory. We went beyond sporulation and investigated the stress response of this strain (Chapter 2). In addition, as cultivation of bacterial strains in laboratory might influence several traits, including resistance capability (see above section “Studying non-standard models in the laboratory”), we compared stress resistance of a laboratory-maintained and of an original environmental strain of *Kurthia* sp. str. 11kri321 (Chapter 2). The morphological response of both strains was complemented by the comparative analysis of the full genomes of the two populations (Chapter 2).

Following the investigation of stress resistance response in our non-standard model, and because manganese was shown to be a key element in sporulation and oxidative stress protection (see above section “Challenging growth conditions in laboratory”), we decided to explore the response of *Kurthia* sp. str. 11kri321 after manganese supplementation using transcriptomics (Chapter 3). As a reference, we also obtained transcriptomic data for the classic sporulating model *B. subtilis* after its exposure to manganese (Chapter 3). As cultivation under artificial growth media may modify stress resistance response of the two bacterial species (see above section “Studying non-standard models in the laboratory”), we decided to compare the effect of manganese treatment both in liquid and solid growth medium.

Overall, all the investigations conducted on *Kurthia* sp. str. 11kri321, highlight some setbacks of working with bacterial non-standard models. This thesis provides an insight into why it is challenging to choose as a model a bacterial species that is yet not commonly considered as a model organism and for which previous characterizations usually do not exist.

4.1 "Cryptosporulation", the production of "cryptospores"

One of the key results/challenges we encountered while working with the non-standard model *Kurthia* sp. str. 11kri321 was the production of spore-like cells that are different from canonical endospores produced by other Firmicutes members and accordingly, we were confronted with the need to define these structures and name the process of their production, which is so far unique to the *Kurthia* genus. Indeed, as indicated above differentiated cells were observed in nutrient-depleted cultures and they were observed to refract light and to retain malachite green staining. These three elements led us to hypothesize that *Kurthia* sp. str. 11kri321 is able to produce spore-like cells. We propose that they are spore-like cells and not canonical endospores as numerous key genes link to endosporulation are missing in the genome of *Kurthia* sp. str. 11kri321 (results described in chapter 1). We implemented the terms "cryptospore" to name those structures and "cryptosporulation" to designate the production process itself.

Thus, though this thesis the term cryptospore is used to name the cells that are morphologically different from the reference cells associated to *Kurthia* sp. str. 11kri321. This term comprised the word "spore" because those differentiated cells shared features with canonical spores (e.g., produced under starvation, phase-brightness, stained by malachite green dye, smaller cell size). In addition, the prefix "crypto-" is used to highlight that those spore-like cells are still cryptic as 1) they are produced only under highly specific conditions that are still not completely understood, 2) their resistance compared to the vegetative cells is still unknown, and 3) their structure and formation are not described yet. Because the formation of such cryptospores by the *Kurthia* genus is not yet unravelled, we used the term cryptosporulation to name it.

4.2 Research questions

The general research question of this thesis is:

"As the non-standard environmental model *Kurthia* sp. str. 11kri321 was isolated from an extreme environment (geothermal spring), is this strain able to sporulate in case of sub-optimal growth conditions recreated in the laboratory or does it respond by an alternative stress resistance strategy?"

More specifically, this thesis was conducted in order to answer the following questions:

- Is the environmental non-standard model *Kurthia* sp. str. 11kri321 able to produce spore-like cells that would confer stress resistance to the strain? (Chapter 1)
- How does the environmental non-standard model *Kurthia* sp. str. 11kri321 respond when grown under challenging conditions, such as nutrient depletion, low-water availability, low-iron availability or presence of a sporulation trigger? (Chapter 2)
- Does stress resistance differ between the strain of *Kurthia* sp. str. 11kri321 that was maintained under optimal laboratory growth conditions and the original isolated environmental strain? (Chapter 2)

- Can the different morphological responses to challenging growth conditions of the laboratory-maintained strain and of the original environmental strain of *Kurthia* sp. str. 11kri321 be assigned to modifications at the genomic level? (Chapter 2)
- Will gene expression of the non-standard *Kurthia* sp. str. 11kri321 show that sporulation, or an alternative stress resistance response, is initiated after manganese supplementation? (Chapter 3)
- Will gene expression of *B. subtilis* show that sporulation is initiated after a manganese supplementation? (Chapter 3)

4.3 Research hypotheses

Linked to the above questions, we formulate the following hypotheses:

- We hypothesize that *Kurthia* sp. str. 11kri321 is able to produce spore-like cells and that those differentiated cells will be more resistant than the vegetative cells based on the observation of phase-bright structures in nutrient-depleted cultures (Chapter 1)
- We hypothesize that when *Kurthia* sp. str. 11kri321 is grown under challenging conditions in laboratory, it will be able to enter in a stress resistance response with a cell differentiation leading to the production of spore-like cells more resistant than the vegetative ones. (Chapter 2)
- We hypothesize that the strain of *Kurthia* sp. str. 11kri321 maintained under optimal laboratory conditions will be less resistant to stress as compared to the original environmental strain. (Chapter 2)
- We hypothesize that the different morphological responses to challenging growth conditions of the laboratory-maintained strain and of the original environmental strain of *Kurthia* sp. str. 11kri321 can be assigned to modifications at the genomic level. (Chapter 2)
- We hypothesize that the gene expression of *Kurthia* sp. str. 11kri321 will be modified under manganese supplementation, with the up-regulation of genes involved in cell differentiation in order to produce spore-like cells more resistant than the vegetative cells. (Chapter 3)
- We hypothesize that the gene expression of *B. subtilis* will be modified under manganese supplementation, with the up-regulation of sporulation-specific genes, indicating that the strain enters in sporulation. (Chapter 3)

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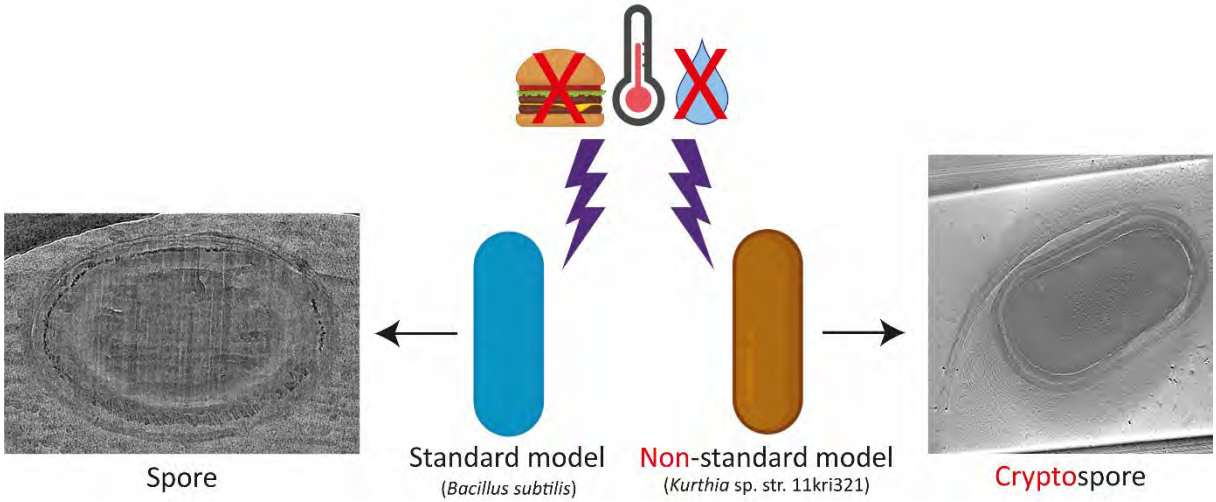
Chapter 1 – Cryptosporulation in *Kurthia* spp. forces a rethinking of asporogenesis in Firmicutes



In a nutshell: Sporulation is a survival strategy that has been extensively studied in Firmicutes, in which this process is unique (i.e., endosporulation) and result of highly resistant endospores. While investigating sporulation in the environmental non-standard model *Kurthia* sp. str. 11kri321, we propose to consider an intermediate lifestyle, with the production of "cryptospores". The discovery of spore-like cells outside from the well-known spore-former groups paves the way to investigate sporulation in non-standard bacterial models. Thus, "cryptosporulation" might represent an ancestral but unstable and adaptive developmental state in Firmicutes that is under selection only under challenging environmental conditions.

Disclaimer: The results of this chapter were likely produced by a combination of sub-strains that could not be differentiated.

Statement of contribution

The study presented in this chapter was started by Dr. Sevasti Filippidou (SF) during her PhD and I took over the characterization of the stress response on *Kurthia* spp. during my PhD thesis.

SF isolated, identified and characterized the environmental strain of this study; *Kurthia* sp. str. 11kri321. SF observed spore-like structures in nutrient-deprived cultures of *Kurthia* sp. str. 11kri321 and characterized those structures, in particular using cryo-electron tomography. Based on the characterization of the spore-like structures, SF concluded that they were spores. Accordingly, SF investigated the presence of sporulation genes in the genome of *Kurthia* sp. str. 11kri321, as well as in three other *Kurthia* representatives (*K. huakuii*, *K. massiliensis* and *K. senegalensis*). SF investigated the process of sporulation in the four *Kurthia* spp. and produced the material used for cryo-electron microscopy, that I utilized later on to establish engulfment in some species. Finally, SF measured dipicolinic acid (DPA) content in the spore-like cells of *Kurthia* sp. str. 11kri321 and show absence of this chemical compound. I confirmed this result using Raman spectroscopy. Based on the results collected by SF and despite the lack of multiple sporulation genes and of DPA, we discussed the cryptosporulation process and production of cryptospores in *Kurthia* genus. However, after submission for publication, the reviewers pointed out that to be considered as spores, the observed structures should be more resistant than the vegetative cells.

Accordingly, I tried to obtain some of those spore-like structures previously characterized by SF in order to compare their resistance with vegetative cells. However, I was unable to produce again those phase-bright spore-like cells. Thus, as aged bacterial cultures face nutrient-depletion, a known stress to trigger sporulation or at least resistant structures, I compared the resistance to heat and UV of aged and fresh cultures of *Kurthia* spp. The results showed that aged cultures did not contain any phase-bright structures and were not more resistant than the fresh cultures. Based on these additional results, I modified our interpretation of the data and re-wrote the results and discussion sections of the resulting manuscript. As the sub-culturing of *Kurthia* spp. under laboratory conditions clearly reduced the production of phase-bright structures in all species, we hypothesized that specific conditions are required to trigger the differentiation of more resistant spore-like structures. We suggested that genetic drift or epigenetic mechanism lead to the loss of unstable spore-like structures in absence of environmental pressure. We proposed 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.

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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.

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 *Carboxydothermus hydrogenoformans* and *Ruminococcus bromii* suggested that these species could produce spores. This prompted researchers to experimentally demonstrate sporulation in both groups (Mukhopadhya 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 dead of the strains (Shaw & Keddie, 1983). *Kurthia huakuii*, *Kurthia massilensis* 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 μS/cm. Water and biofilms from the outflow were collected in a sterile 1 L bottle and filtered through a 0.22 μm 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×). A differential staining for endospores and vegetative cells was performed using malachite green and safranine, 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 Bioproject 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 (OD_{600}) 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 OD_{600}. 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× 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 μl 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 $e^{-}/Å^2$), 10 μm underfocus at 11,500× magnification, such that each pixel represented 7.9 Å. Tilt series were collected from −60° to +60° 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 μl of vegetative cells or mature spores was mixed with 1 μl of 10 nm Protein A-Gold (Department of Cell Biology of the University Medical Center, Utrecht, The Netherlands). The 3.5 μl 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×, 7252× and 11,927×, resulting in calibrated physical pixel size of 38.9, 6.9 and 4.2 Å. The underfocus was changed between 100 and 7 μm with a total dose between 22 and 0.7 $e^{-}/Å^2$. The images were recorded using the programme SerialEM (Mastronarde, 2005).

Phylogenetic analysis

16S rRNA gene sequences (>1200 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 BLOSUM30 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 AYTB00000000.1, *K. massiliensis* str. JC30: NZ CAEU00000000.1, and *K. senegalensis* str. JC8E: NZ CAEW00000000.1. 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 μs. 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 μm.

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),

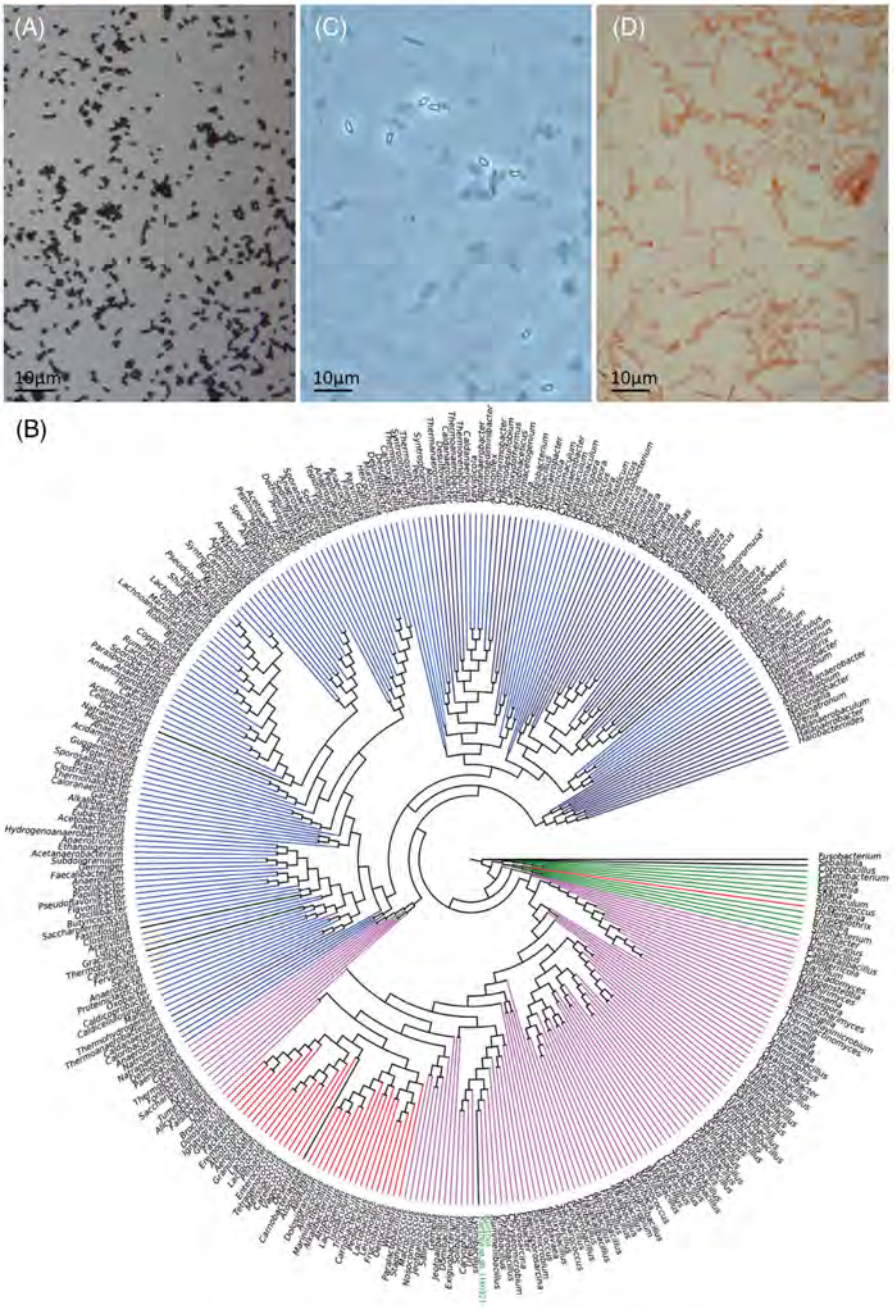

FIGURE 1 Morphological characterization and phylogenetic placement of *Kurthia* sp. str. 11kri32. (A). Strain 11kri321 is a Gram-positive bacterium. (B) Maximum-likelihood tree showing the positioning of the *Kurthia* genus among Firmicutes. Branches are coloured by class (magenta: Bacillales; blue: Clostridiales; green: Erysipelotrichales; navy: Halanaerobiales; crimson: Lactobacillales; mediumblue: Natranaerobiales; darkblue: Selenomonadales; midnightblue: Thermoanaerobacterales; dodgerblue: 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

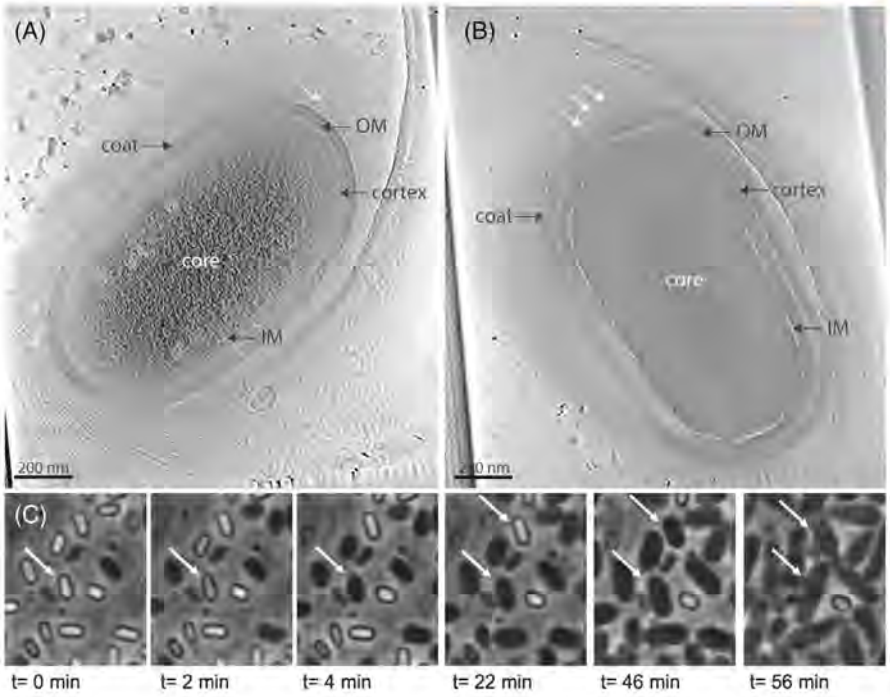

FIGURE 2 Detailed morphological characterization of the spore-like structures of *Kurthia* sp. str. 11kri321 by cryo-electron tomography (cryo-ET) and monitoring of their transition from phase-bright to phase-dark by light microscopy. (A,B) Cryo-ET images of the spore-like structures of *Kurthia* sp. 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

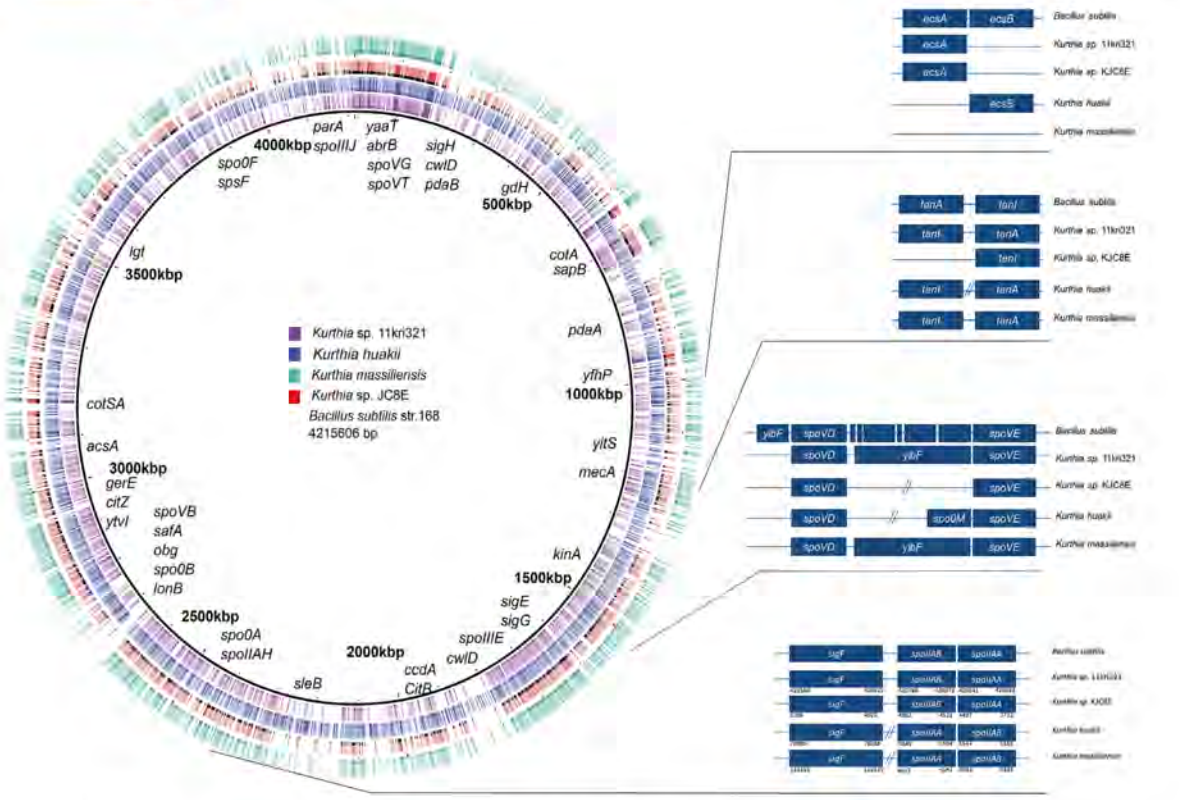

FIGURE 3 Comparison of the four *Kurthia* genomes publicly available to *Bacillus subtilis* subsp. *subtilis* str. 168. Genes related to sporulation are shown on the circular representation of the genomes. Four operons related to sporulation are also presented showing differences among the four *Kurthia* 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 bi-directional 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 (SpoIIAB) 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

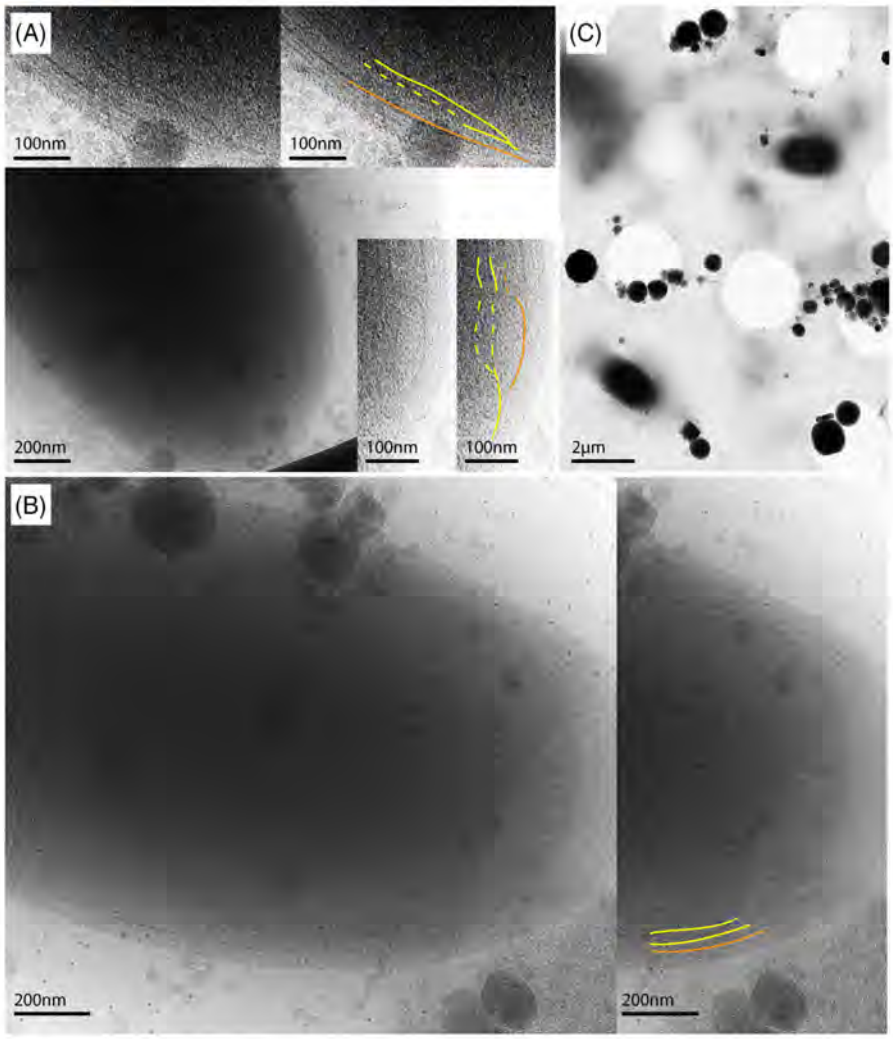

FIGURE 4 Cryo-electron microscopy (cryo-EM) imaging of putative engulfment in *Kurthia* spp. (A) Cryo-EM projection images reveal the steps of engulfment in *K. huakuii* str. LAM0618. The areas in which engulfment leads to the production of a putative pre-spore-like cells with two membranes (yellow) 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 endosporulation. A similar gene content was detected in all *Kurthia* spp, which was consistently below the suggested minimal

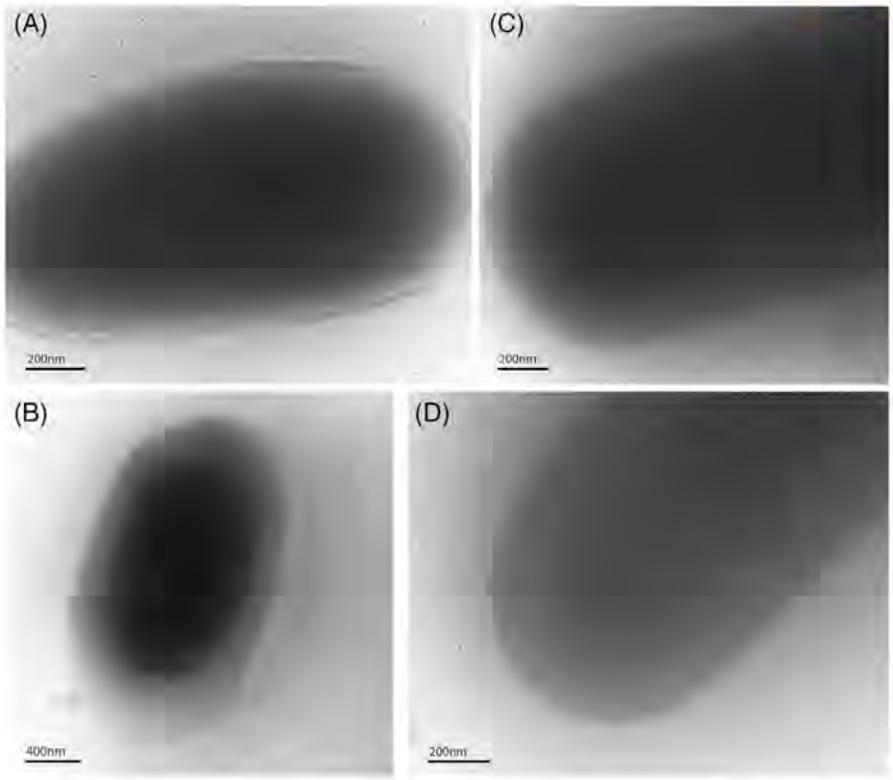

FIGURE 5 Cryo-electron microscopy (cryo-EM) imaging of spore-like structures and vegetative cells of *Kurthia* spp. Spore-like structures of *Kurthia* sp. str. 11kri321 (A) and *K. massiliensis* str. JC30 (B) showed a similar structure and shape; however, size is different: *Kurthia* sp. str. 11kri321 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 concentrically arranged 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 endosporulation. 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

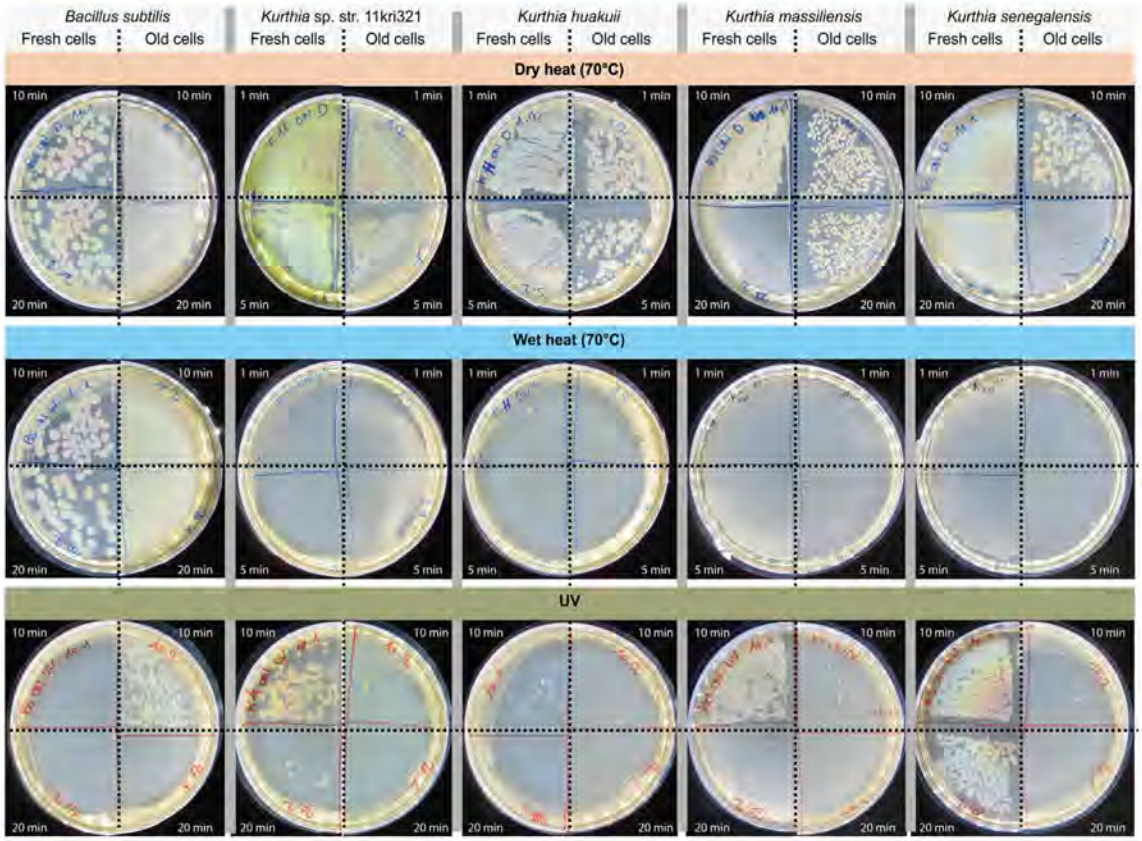

FIGURE 6 Resistance tests on fresh and 4-week aged cells of *Kurthia* spp. and *Bacillus subtilis*. The results shown correspond to a subset of the results to illustrate the effect of three stress factors: dry heat at 70°C (top panels), wet heat at 70°C (middle panels), and UV radiation (lower panels). 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 endosporulation (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 endosporulation) 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 endosporulation 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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Chapter 2 – Out of place: challenges of investigating resistance in environmental bacteria under laboratory conditions

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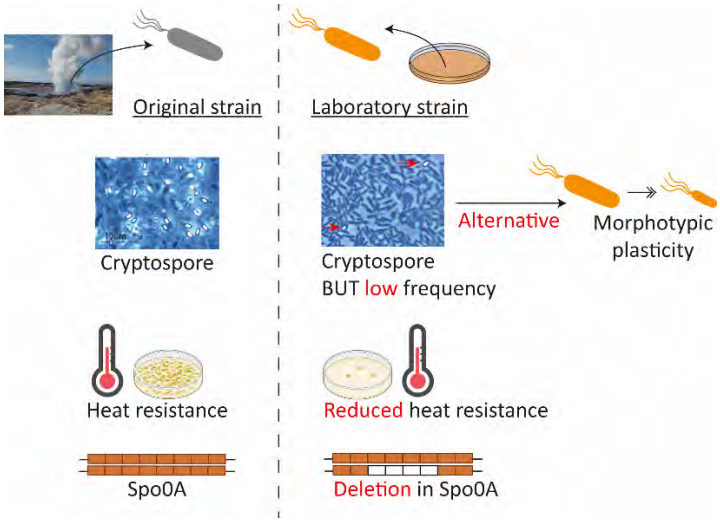

In a nutshell: Resistance strategies are costly for bacteria and accordingly, such strategies are often reduced or even completely lost when selection pressure for survival is relaxed. Accordingly, the laboratory strains maintained under optimal growth conditions are usually less resistant than "wild" strains. While investigating stress resistance strategies in the laboratory strain of the non-standard model *Kurthia* sp. str. 11kri321, we showed that this strain suffers from a reduced heat resistance as compared to the original environmental strain. This reduced resistance might be linked to a decreased ability to form cryptospores caused by a deletional mutation in the master regulator of sporulation, Spo0A. Despite this reduced resistance, the laboratory strain was still able to respond to stress through morphotypic plasticity as an alternative strategy.

Disclaimer: The experiments realized in the first part of this chapter (morphological response under challenging growth conditions) were realized only with the sub-strain that was maintained under laboratory conditions (LS strain). For the other parts of this chapter, we used the two different sub-strains as the aim was to compare the resistance (second part) and the genomes (third part) of the laboratory strain (LS strain) and of the original environmental strain (OS strain).

Abstract

Natural environments are reservoirs of an extraordinary bacterial diversity. However, not all the bacteria present in the environment are in the same metabolic state. When environmental conditions are suboptimal, active growth and multiplication are not possible and microorganisms are mostly found in a survival resting state. This has been postulated to be case for sporulating Firmicutes, which are able to form highly resistant endospores. However, the production of simpler resistant cell structures was also demonstrated in non-sporulating Firmicutes, and accordingly, the resistant fraction of non-sporulating Firmicutes in natural environments might be highly diverse. However, this diversity is poorly appreciated and investigating non-standard models producing these alternative survival structures is needed to better characterize survival strategies in this group. Therefore, in this study, we investigated the resistance strategies of *Kurthia* sp. str. 11kri321, a non-standard model recently isolated from a geothermal spring. In addition, we evaluated the persistence of resistance strategies in the laboratory. To do so, we grew the strain under multiple growth conditions and evaluated its morphological response. We also compared the resistance to UV and heat exposure and changes in the gene content of cell populations preserved shortly after isolation and cells maintained under optimal laboratory growth conditions. Our results demonstrated that *Kurthia* sp. str. 11kri321 displays a stress response characterized by cell size reduction and the production of phase-bright cells. In addition, we observed that the strain maintained under laboratory conditions was different from the original environmental strain, with a decreased resistance to heat and a modified cell morphology after UV exposure. Moreover, the genome of the two strains was not identical, with a very significant deletion in the gene coding the sporulation master regulator Spo0A. Our study illustrates the diversity of stress resistance strategies in a non-standard model and highlights some of the challenges to study resting cells of environmental bacteria under laboratory conditions, more specifically, the maintenance of resistance-associated traits under optimal growth conditions.

1. Introduction

Each one of the habitable environments on Earth is characterized by a unique set of particular environmental conditions. Thanks to their remarkable ability to evolve and adapt to these multitude of factors (Elena & Lenski, 2003), microorganisms are the most abundant and diverse life forms on the planet (Van den Bergh et al., 2018). Although some environments, for instance soils, seem highly appropriate to support microbial life, they also display specific conditions, such as a heterogeneous distribution of nutrients (Hodge, 2004) or the presence of competitors (Kaye & Hart, 1997), which can be challenging and select the diversity of microorganisms actively growing. Therefore, in a given environment, not all microbial cells will be found in the same metabolic state. In fact, natural fluctuations result in conditions that are suboptimal for active growth and multiplication, and a large fraction of the cells might be present in a survival resting state (Suzina et al., 2006).

Members of the phylum Firmicutes are an excellent model group to investigate survival strategies. For instance, some clades within this phylum are capable of endosporulation,

which is considered the most extreme survival strategy in terms of the modification of the cellular structure conferring resistance (Filippidou et al., 2016). Endosporulation leads to the production of endospores, which are defined as the most resistant cellular structures on Earth (Abecasis et al., 2013). However, despite the undeniable role of endospores in survival, their formation demands a very important energetic investment. Endospore formation requires the maintenance and replication of large genomes to accommodate the extra genes (or even extra chromosomes) involved in the process, representing a significant energetic cost (Barton, 2005). In addition, sporulation itself is also costly (Ratcliff et al., 2013; Siebring et al., 2014). Accordingly, the majority of bacterial species do not form spores, but are nevertheless able to survive under harsh environmental conditions (Gray et al., 2019). This might be achieved through the production of less complex survival structures. In this context, *Kurthia* sp. str. 11kri321, an environmental strain of the poorly studied Firmicutes genus *Kurthia*, was isolated from an extreme environment (a geothermal spring in Greece). This strain was observed to produce cells structurally different to the vegetative cells. As those cells shared some, but not all features of well-described endospores, they were called cryptospores and were expected to allow the strain to persist in its original environment. Due to this ability to produce cryptospores, a previous study tested the resistance of nutrient-deprived cells of *Kurthia* sp. str. 11kri321 based on resistant tests performed on endospores. The results show that the nutrient-deprived cells were not more resistant than cells grown under optimal nutrient availability (Fatton et al., 2022). However, cryptospores were observed at such a low frequency, that conditions to increase cryptospore production are required to test directly their resistance properties.

Therefore, in order to better understand the effect of growth conditions on the production of cryptospores or other alternative cellular modifications, we grew a strain maintained in laboratory conditions during approximately three months (Laboratory strain or LS strain) under several challenging conditions considering multiple stresses and nutrient deprivation. Those challenging conditions were recreated in laboratory by combining two factors; the type of growth medium and chemical supplementation (Fig. 1). In this way, several combinations of low nutrient availability, desiccation, and iron bioavailability could be simulated in the laboratory. All of these factors are known to be common stresses encountered by bacteria in natural environments (Andrews et al., 2003; Esbelin et al., 2018; Gray et al., 2019). In addition, the presence of a sporulation trigger (manganese) was used to reinforce the stress (Charney et al., 1951). We observed an altered cell morphology in response to challenging growth conditions. Cryptospores were also produced albeit still at a very low frequency. Given the low frequency of observation of cryptospores, we hypothesized that resistance of the LS strain was reduced due to its maintenance under optimal growth conditions. To test this, we compared the resistance to heat and UV of the LS and a strain cryopreserved shortly after its isolation (“Original” environmental strain or OS). Finally, the morphological response of both strains was complemented by re-sequencing and comparative analysis of the full genomes of the two populations, in order to determine if the morphological changes can be ascribed to modifications at the genomic level.

2. Material and Methods

2.1 Bacterial species and pre-culture conditions

The bacterial strain *Kurthia* sp. str. 11kri321 was initially isolated from the geothermal spring of Krinides (Greece). This Gram-positive Firmicutes bacterium belongs to a genus described as encompassing non-sporulating species. Physiological tests revealed that the optimal growth temperature for *Kurthia* sp. str. 11kri321 is 25°C, but the strain was able to grow at temperatures between 20°C to 45°C and pH between 5.5 to 11.5 (Fatton et al., 2022). We considered two different cryo-cultures of *Kurthia* sp. str. 11kri321. The first cryo-culture contained cells frozen shortly after their isolation from the geothermal spring (Fatton et al., 2022) and it is hereafter referred to as the original environmental strain (OS strain). The second cryo-culture contained cells that, prior to their cryo-preservation, were maintained under optimal laboratory conditions for approximately three months, and it is hereafter referred to as the laboratory strain (LS strain). During these three months, the cells were repetitively replated on nutrient agar plates (NA; 8 g/L nutrient broth, Carl Roth and 15 g/L technical agar, Biolife), followed by incubation at 30°C for 24 to 48 h. As the maintenance of cells under optimal laboratory conditions was initially not intended as a controlled experiment, the precise duration of this maintenance is unknown and three months is only an approximation. The OS and LS strains were reactivated from frozen stocks by culturing overnight (approximately 18 h) into 10 mL liquid nutrient broth (NB; Carl Roth, Germany) at 30°C under agitation at 115 rpm; this constituted the pre-cultures used for further experiments.

2.2 Growth of the laboratory strain (LS strain) under challenging conditions

In order to investigate the reaction of the LS strain when confronted to sub-optimal growth conditions, several different conditions were tested. Those were mimicked by combining two factors: type of growth medium and chemical supplementation (Fig. 1). Three different growth media were used: regular NA, NA with double the amount of agar (NA 3%), and Sporulation Medium 2 (SM2) supplemented with acetate as carbon source. The first medium (regular NA) is a nutrient rich medium supporting optimal bacterial growth and served as control. Then, we used NA 3% as a dryer and firmer growth surface with lower water availability (Shi & Zusman, 1993). Indeed, variations in the availability of water constitute a widespread environmental stress that is challenging for microorganisms, and especially bacteria (Esbelin et al., 2018). Finally, we used SM2 as a low-nutrient containing medium (Brandes Ammann et al., 2011) to induce nutrient depletion, which is a common stress encountered by bacteria in natural environments (Gray et al., 2019). To reinforce stress in the SM2 medium, acetate was added as a carbon source of lower quality as compared to glucose (Liu et al., 2005). Compared to glucose, acetate is not the primary chosen carbon source for a vast range of microorganisms (Oh et al., 2006; Sun et al., 2020). The reason for this is the energetic balance between uptake and the energetic yield after oxidation (2 and 9 ATPs, respectively for acetate versus 2 and 24 ATPs for glucose) (Kutscha & Pflügl, 2020). For the chemical supplementation, manganese sulphate (solution of $MnSO_4$ at 10 mg/L, Honeywell Fluka, USA) was chosen as a stress factor, as this chemical is a known sporulation trigger (Charney et al., 1951). Secondly, the iron

	Name	Description	Composition		Treatment effect	Visual code
Challenging factor 1 - Growth medium	**NA**	Nutrient Agar: non-selective rich nutritive medium with 1.5% of agar	Beef extract	3 g/L	Control (no stress)	
			Peptones	5 g/L		
			Agar	15 g/L		
	SM2 acetate	Sporulation medium 2 acetate: low-nutrient containing medium, with acetate as a carbon source	NH_4Cl	1 g/L	Low-nutrient availability + presence of acetate, a carbon source with a low energy content, offers just enough energy for cell growth	
			$MgSO_4$	200 mg/L		
			$CaCl_2$	70 mg/L		
			$FeSO_4$	10 mg/L		
			EDTA	10 mg/L		
			KH_2PO_4	900 mg/L		
			$C_2H_3NaO_2$	1 g/L		
	NA 3%	Nutrient Agar 3%: non-selective rich nutritive medium with 3% of agar	Beef extract	3 g	Low-water availability due to double quantity of agar compared to the control	
			Peptones	5 g		
			Agar	30 g		
Challenging factor 2 - Chemical supplementation	**$MnSO_4$**	Manganese sulfate (CAS n° 10034-96-5)	$MnSO_4$	10 mg /L	Known as a sporulation trigger (Charney, et al. 1951)	
	2,2'-dipyridyl	Lipid-soluble ferrous iron chelator (CAS n° 32190-42-4)	$C_{10}D_8N_2$	200 µM	Chelates free iron and therefore mimicks low-iron availability (Granato, et al. 2018), which represents a stress for bacteria (i.e. iron participated in many major biological processes such gene regulation, DNA biosynthesis (Andrews, et al. 2003))	

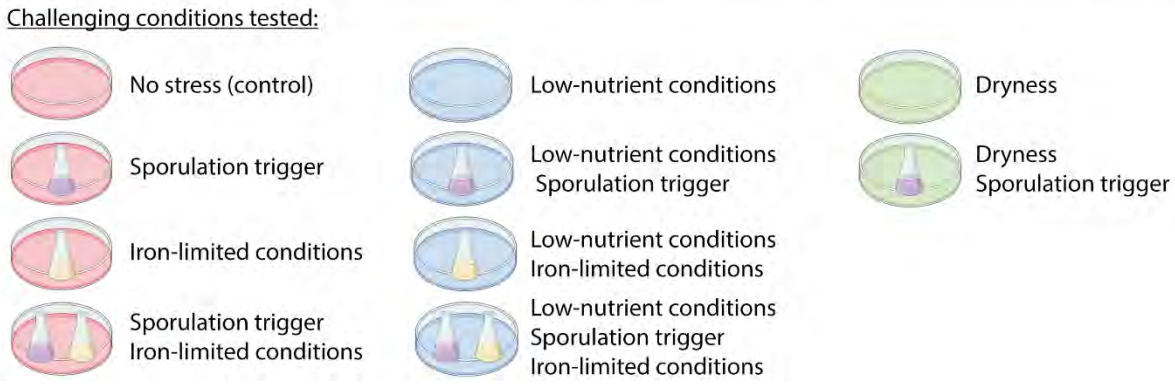

Figure 1. Challenging growth conditions recreated in the laboratory. In order to mimic challenging growth conditions in laboratory, two factors were combined: type of growth medium (factor 1) and chemical supplementation (factor 2). The top table gives for each growth medium and chemical supplementation a brief description, as well as their composition and expected effect on the bacterial strains used in this study. The last column attributes a visual code to each element. The below part of the figure summarizes the 10 challenging growth conditions created through multiple combinations of challenging factor 1 and challenging factor 2. (Illustration partially made with BioRender).

chelator 2,2'-dipyridyl (solution at 200 µM, Carl Roth, DE) was used to create iron-limited growth condition (Granato et al., 2018). Low-iron availability represents a stress for bacteria as iron is key for many major biologic processes, such as gene regulation and DNA biosynthesis (Andrews et al., 2003). We combined these factors in order to create 10 challenging growth media. A LS pre-culture (50 µL) was homogenously spread onto those 10 media (Fig. 2B and C). All the inoculated plates were incubated at 30°C for 24, 48, or 72 h. Afterwards, microscopy

was performed on the cultures. A loop was used to collect cells from the growth media. Cells were resuspended in 1 mL of physiological water. From this suspension, 3 µL were deposited onto an agar pad and a cover slip was added on top. Agar pads were prepared by depositing 500 µL of boiling 1.2% agar onto a glass slide for microscopy. The surface was homogenously flattened using a cover slip. Imaging acquisition was performed using a light-transmitted optical microscope (Leica DM4B) equipped with a camera (Leica DFC7000 T) (Fig. 2D).

2.2.1 Statistical analysis

Quantitative data was extracted from the microscopic images using ImageJ (NIH, Bethesda, MD, USA). For each challenging growth condition (Fig. 2C), we measured both the length and width of 43 to 50 cells on the corresponding image. Single and two factor ANOVA analyses were employed to determine statistical significance of the results. Standard deviation, the statistical significance calculations and graphs were produced using RStudio software (RStudio Team (2022), Integrated Development Environment for R, RStudio, PBC, Boston, MA, USA).

2.3 Resistance tests

2.3.1 Sample preparation

The OS and LS pre-cultures were centrifugated at 3000 *g* for 10 min, the supernatants were discarded and the pellets were resuspended in 1 mL physiological water (NaCl 9 g/L) (washing step). A second centrifugation was done and the pellets were diluted in physiological water in order to have an optical density (OD) at 600 nm at 0.1 (approximately $1*10^8$ cells/mL). From these bacterial suspensions, 1 mL was transferred in tubes in order to be exposed to the stress treatments (Fig. 3B).

2.3.2 Stress treatments

Three different stresses were applied at three to four different time durations in duplicates (Fig. 3C). For the wet temperature stress, we used a water bath to expose cells to 60°C wet heat for 1, 5, 10, and 20 min. After the exposure, the cells were directly cooled down on ice for at least 5 min (Beaman et al., 1982). The second stress was 70°C dry heat, which was applied using a heat block. Preliminary data showed that after 10 min of dry heat all the cells were systematically dead, so we implemented a shorter exposure as compared to the two other stresses. Therefore, dry heat was applied for 5, 7, and 9 min. The cells were also directly cooled down on ice. For the last stress, germicidal ultra-violet (UV) light (254 nm) was applied for 1, 5, 10, and 20 min. Finally, OS and LS cultures not exposed to stress treatments were used as controls.

2.3.3 Resistance assessment

After exposure to treatments, 1 mL of bacterial suspension was diluted 10x using NB and incubated at 30°C under agitation (125 rpm) for 30 min. This step represented a heat activation to trigger the regrowth of the cells that survived (Beaman et al., 1982). From these heat-activated solutions, we homogeneously spread 25 µL on one quarter of NA plate. The plates were incubated for 24 h at 30°C and the cell growth was accessed visually. In addition,

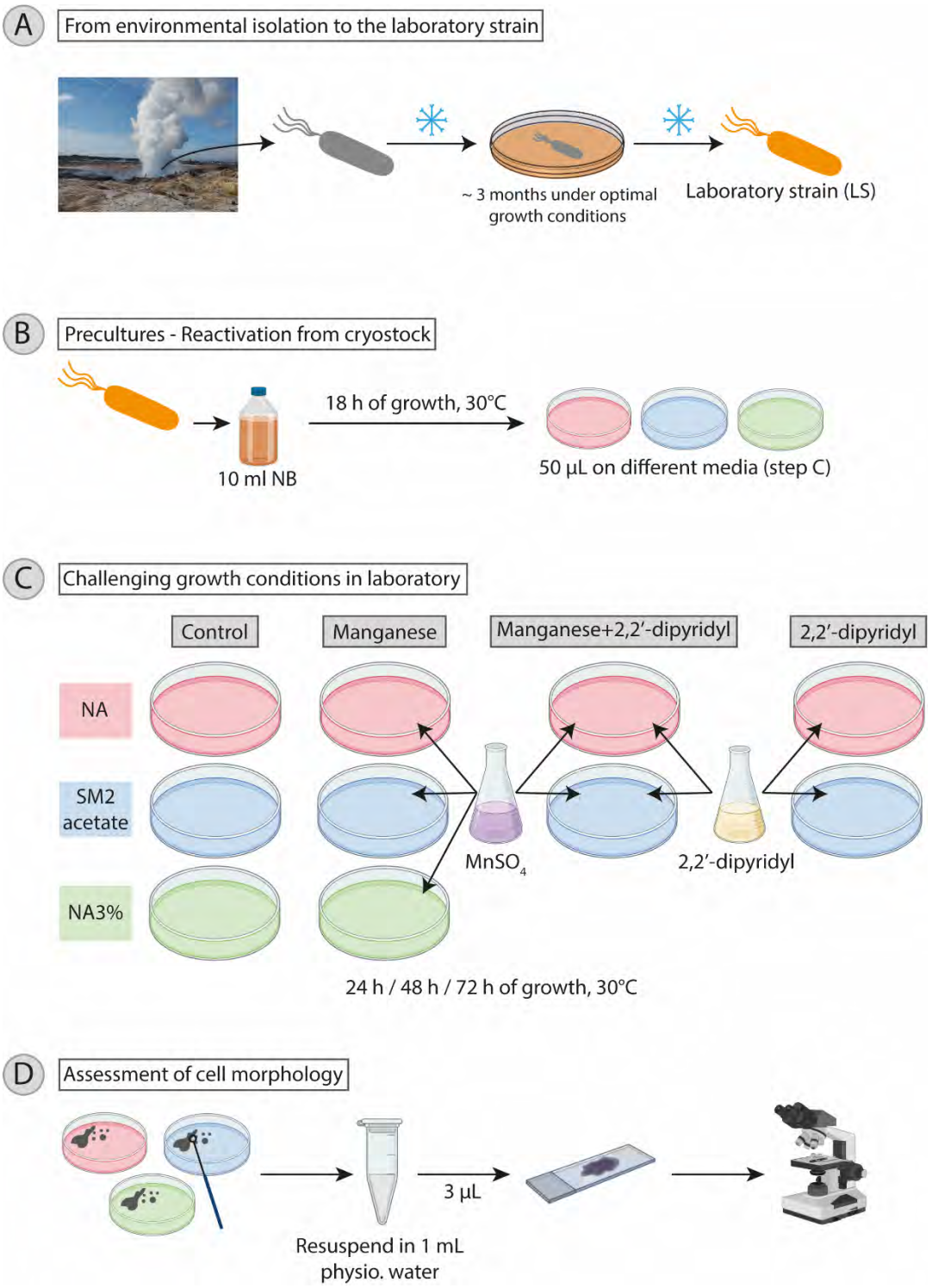

Figure 2. Method to explore the reaction of the laboratory strain (LS strain) of *Kurthia* sp. str. 11kri321 when confronted to challenging growth conditions. (A) After its isolation, *Kurthia* sp. str. 11kri321 was maintained under optimal laboratory growth conditions for approximately three months. This resulted in the laboratory-adapted strain (LS strain). **(B)** The LS strain was reactivated from frozen stock by culturing overnight (18 h, 30°, 115 rpm agitation) into 10 mL liquid nutrient broth (NB). From this pre-culture, 50 µL was homogenously spread onto the different media. **(C)** Ten growth media were inoculated in order to test different challenging growth conditions (Fig. 1). All the plates were incubated for 24 h, 48 h and 72 h at 30°C. **(D)** After the incubation, a loop was used to collect cells from the growth media and to resuspended them into 1 mL of physiological water. From this suspension, 3 µL were deposited onto a microscopic slide and microscopy analysis was conducted. (Geothermal spring picture acquired by Pilar Junier. Illustration partially made with BioRender).

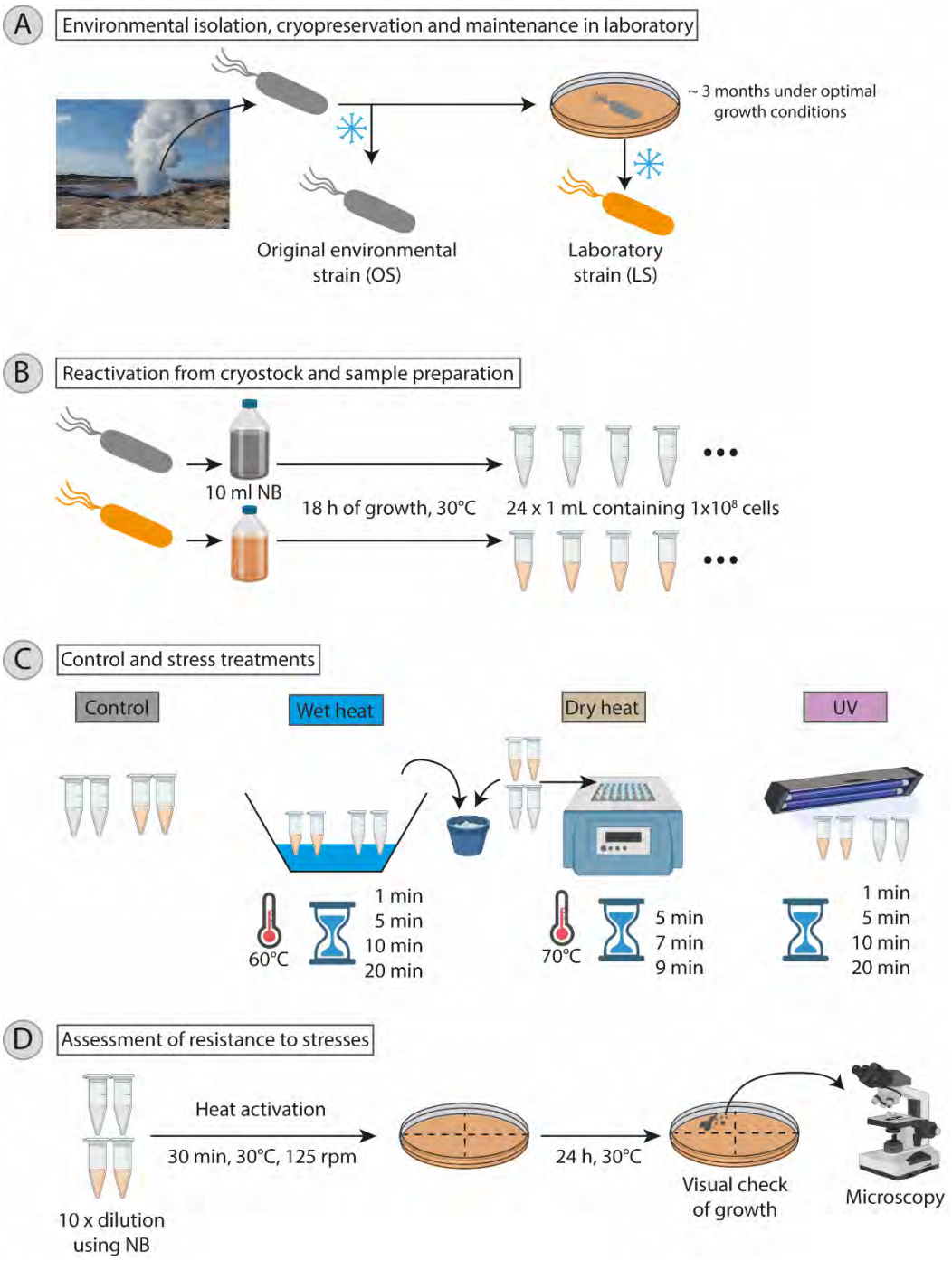

Figure 3. Method to compare stress resistance of the original environmental strain (OS) and of the laboratory strain (LS) of *Kurthia* sp. str. 11kri321. (A) After its isolation, *Kurthia* sp. str. 11kri321 was either directly cryo-preserved, which corresponded to the original environmental strain (OS strain), or maintained under optimal laboratory growth conditions for approximately three months, which corresponded to the laboratory strain (LS strain). **(B)** The OS and LS strains were reactivated from frozen stocks by culturing overnight (18 h, 30°, 115 rpm agitation) into 10 mL liquid nutrient broth (NB). From these pre-cultures, 24 x 1 mL, containing approximately 1 x 10^8 cells, were prepared in Eppendorf tubes. **(C)** For each strain, two Eppendorf tubes (duplicates) were either used as control, or exposed to 60°C wet heat (for 1, 5, 10, and 20 min), 70°C dry heat (for 5, 7, and 9 min) and UV radiation (for 1, 5, 10, and 20 min). Tubes exposed to heat treatment were directly cooled down in ice for 5 min. **(D)** After stress treatments, samples were diluted 10 x using NB and incubated 30 min, at 30°C, under agitation 125 rpm (heat activation). 25 µL were spread on one quarter of nutrient agar (NA) plate. After incubation (24 h, 30°C), cell growth was assessed visually and microscopic observations were performed. (Geothermal spring picture acquired by Pilar Junier. Illustration partially made with BioRender).

microscopic observations were performed as described above in order to compare cell morphology before and after the stress treatments (Fig. 3D).

2.3.4 Characterization of two different colony types observed for the OS strain after exposure to dry heat

During the resistance tests, after 7 min of dry heat, two different forms of colonies were observed for the OS strain. Using a sterile loop, we collected cells and repicked them onto fresh NA growth media. After 24 h of growth at 30°C, we assessed visually the aspect of colonies. In addition, cell morphology was checked by microscopy, as described above. Quantitative data was extracted from the microscopic images using the software ImageJ as indicated above. A t-test analysis was used to determine statistical significance of the results using the RStudio software. Finally, molecular identification through sequencing of the 16S rRNA gene was performed on amplicons obtained after a colony PCR amplification. For the colony PCR, we collected a single colony, resuspend it in 10 µL RNAse free water and boiled it 10 min at 98°C. We mixed 1 µL of the boiled sample with ALLin^{TM} Red Taq Mastermix and the universal primers Gm3f (5'- AGA GTT TGA TC(AC) TGG C -3') and Gm4r (5'- TAC CTT GTT ACG ACT T -3'). The amplicons were sent to Fasteris (Geneva, CH) for Sanger sequencing. The read quality control and alignment were performed Using BioEdit software version 7.2.6.1 (Hall et al., 2011). Aligned reads were compared to the NCBI database using BLAST (https://www.ncbi.nlm.nih.gov/).

2.4 Genomic analysis

In order to compare the full genome sequence of the OS and LS strains of *Kurthia* sp. str. 11kri321, pre-cultures in triplicate were prepared as described above, except that the incubation time was shorter (13 h).

2.4.1 Genomic extraction and sequencing

Genomic extraction was performed using the Wizard Genomic DNA Purification Kit (Promega, MD, USA). We applied the procedure of the kit for isolation of HMW DNA from Gram-Positive and Gram-Negative Bacteria with the following modifications. In step 2, samples were centrifugated at 16,000 *g* for 3 min and we used 100 µL physiological water (0.9% NaCl) instead of PBS to resuspend the pelleted cells. For step 3, we added 60 µL of lysozyme (10 mg/mL). In step 7, 60 µL of RNase A Solution (10 mg/mL) was used and a 30 min incubation was performed. After adding Proteinase K solution, samples were incubated for 30 min instead of 15 min. In step 10, samples were centrifugated at 20,000 *g* for 20 min at 4°C, and in step 13, at 16,000 *g* for 5 min at 4°C. In step 14, we added 1 mL of ice-cold 70% ethanol. At the end, gDNA was resuspended in 50 µL of DNA Rehydration Solution and kept at 4°C. gDNA quantification was performed using the Qubit® dsDNA HS Assay Kit on a Qubit® 2.0 Fluorometer (Invitrogen, Carlsbad, CA, USA) and purity of the gDNA extracts was assessed using a NanoDrop spectrophotometer (Witech AG, Sürsee, CH). gDNA extracts in DNA Rehydration buffer (volume of 43 µL with concentration ranking from 74 to 156 ng/µL depending the strain and replicate) were send to Lausanne Genomic Technologies Facility (Lausanne, CH) for PacBio SMRT sequencing.

2.4.2 Comparative genome analysis

In order to perform an Average Amino Acid Identity assessment, we used an online tool provided at http://enve-omics.ce.gatech.edu/aai/ (Rodriguez-R & Konstantinidis, 2016). Protein sequences for the genomes of OS, LS, and *Kurthia* sp. str. 11kri321 were provided as input. Our genomes were annotated using Rastk (Brettin et al., 2015; Overbeek et al., 2014), a web-service available in PATRICbrc. Default parameters were kept. Subsequent nucleotide and amino-acid sequences were retrieved. A whole genome 1-to-1 alignment was performed with Nucmer4, a Mummer4 utility (Marçais, et al. 2018), with default parameters. Nucmer's ".delta" output was then filtered out with show-coords (for alignments coordinates and percentage identity of the alignments) and dnadiff (for breakpoints characteristics). In addition, Nucmer's ".delta" output was fed to NucDiff script (Khelik, et al. 2017) to obtain detailed SNPs description. Genomes comparison display was obtained using the package gggenomes (Hackl and Ankenbrand 2022) with R (Team 2013). Genes present in forward and reverse strands were colour coded according to the percentage identity of the alignments provided by nucmer's “show-coords” utility. To improve readability, the breakpoint ranges were manually increased of 1 kb, except for the first and third breakpoint in OS (starting from 0), and the last breakpoint of LS. The ratio of the number of nonsynonymous substitutions per nonsynonymous site (Ka) to the number of synonymous substitutions per synonymous site (Ks), or Ka/Ks ratio, was calculated following Aylward’s 2018 pipeline (Aylward 2018). For this, we used Clustal omega (Sievers, et al. 2011) in protein sequence alignments. Then a codon-based nucleic acid alignment was performed using PAL2NAL script (Suyama, et al. 2006). The script codeml was run in order to get an estimation of synonymous substitutions (Ks) and nonsynonymous substitutions (Ka) rates (Yang 2007) and to calculate the Ka/Ks ratio. After filtering, only relevant ratios were displayed on the global genome comparison’s figure. Concerning Spo0A, sequences for this protein from our genomes (OS, LS, *Kurthia* sp. str. 11kri321, accession number: PRJNA301103), two representative genomes (*Kurthia* sp. JC30, accession number: CAEU00000000; *Kurthia* sp. LAM0618, accession number: AYTB00000000) and for the reference *B. subtilis* strain 168 (accession number0: PRJNA57675) were retrieved from PATRICbrc.org website (Davis et al., 2020). Next, multiple alignment sequences (msa) was performed using DECIPHER's R package (Wright, 2016) and the resulting alignment was displayed using ggmsa' R package (Lang & Guangchuang, 2021). In order to assess the changes in Spo0A, the sequences from the Spo0A N- and C- domains were retrieved from UniProt Consortium (https://www.uniprot.org/uniprotkb/P06534/entry) (Bateman A., 2021). Protein modelling was computed using the SWISS-MODEL service, which provides a fully automated protein structure homology-modelling capability. It allows also template search for 3D models. Based on literature (Lewis et al., 1999; Lewis, Muchová, et al., 2000), we chose the 1qmp.2 template (SMTL ID) and 3D-modeled all previously mentioned Spo0A sequences, accordingly. The 3-D model for Spo0A was available in all the searched templates performed with SWISS-MODEL. Exported as pbd files, they were then displayed in the rcsb 3dviewer for 3D superposition in order to highlight the differences between the different strains and species (Berman et al., 2000; Burley et al., 2021; Sehnal et al., 2021). Seqres, the reference sequence (Lewis, Muchová, et al., 2000), was included in the 3D comparison to follow the AA-location nomenclature used for the discussion.

3. Results

3.1 Effect of challenging growth conditions on the morphology of the laboratory strain (LS strain)

Kurthia sp. str. 11kri321 has been shown to produce modified cells (i.e., cryptospores) in response to suboptimal growth conditions (Fatton et al., 2022). To better understand the effect of growth conditions on the production of cryptospores or other alternative cellular modifications, we grew a laboratory strain (LS strain) under several challenging conditions and visualized the response of the different cell populations using light microscopy (Fig. 1 and Fig. 2). First of all, after 48 h of growth, the cell size was not homogenous for the tested growth conditions (Fig. 4). Quantitative analyses of cell size were performed to compare the different treatments. In the control condition (NA medium), the averaged cell length was 2.22 µm and the width was 0.75 µm (average over 50 cells). In all other conditions, the averaged cell length varied from 3.18 to 2.03 µm, whereas the average width ranged from 0.84 to 1.08 µm. To assess the significance of this difference, we compared both the length and the width of cells grown under challenging conditions. Both the chemical supplementation factor and the growth medium factor significantly affected cell length (*p*-value= <2e-16 and *p*-value= 0.0165, respectively). Moreover, the interaction between the two factors was also significant (*p*-value= 5.87e-15). Concerning the growth medium factor, in absence of chemical supplementation, the cell length was the smallest on SM2, followed by on NA 3% (Fig. 4K). However, as chemical supplementation was the most significant factor (*p*-value= <2e-16), the most significant difference in length was observed between cells grown on NA medium without any chemical supplementation (control) and cells grown on NA with manganese supplementation (*p*-value= 0.0000) (Fig. 4K). Cell length was also reduced in presence of 2,2'-dipyridyl alone or in combination with manganese, but overall, regardless of the growth medium, cell length was the smallest in case of manganese supplementation (Fig. 4B). Concerning cell width, chemical supplementation, type of medium and the interaction between these two factors had also a significant effect (*p*-value= 6.13e-09, *p*-value= <2e-16, and *p*-value= 2.9e-06, respectively). However, contrary to length, the medium type was the most influencing factor on cell width. Overall, the cell width was more homogenous than length and there was no clear evidence of reduced or increased cell width associated to a specific growth condition (Fig. 4L).

The second key observation made in response to growth under challenging growth conditions was the presence of modified cells that refracted light (cryptospores; Fig. 4, red arrows). Those phase-bright cells were observed at low frequency (<5%) and not under all growth conditions. Indeed, phase-bright cells of the LS strain were observed with a frequency of 0.52% on the growth medium NA supplemented with both manganese and 2,2'-dipyridyl (Fig. 4I), 2.27% on SM2 acetate without any chemical supplementation (Fig. 4B), 4.65% on SM2 acetate supplemented with 2,2'-dipyridyl (Fig. 4H) and 2.21% on SM2 acetate supplemented with both chemicals (Fig. 4J).

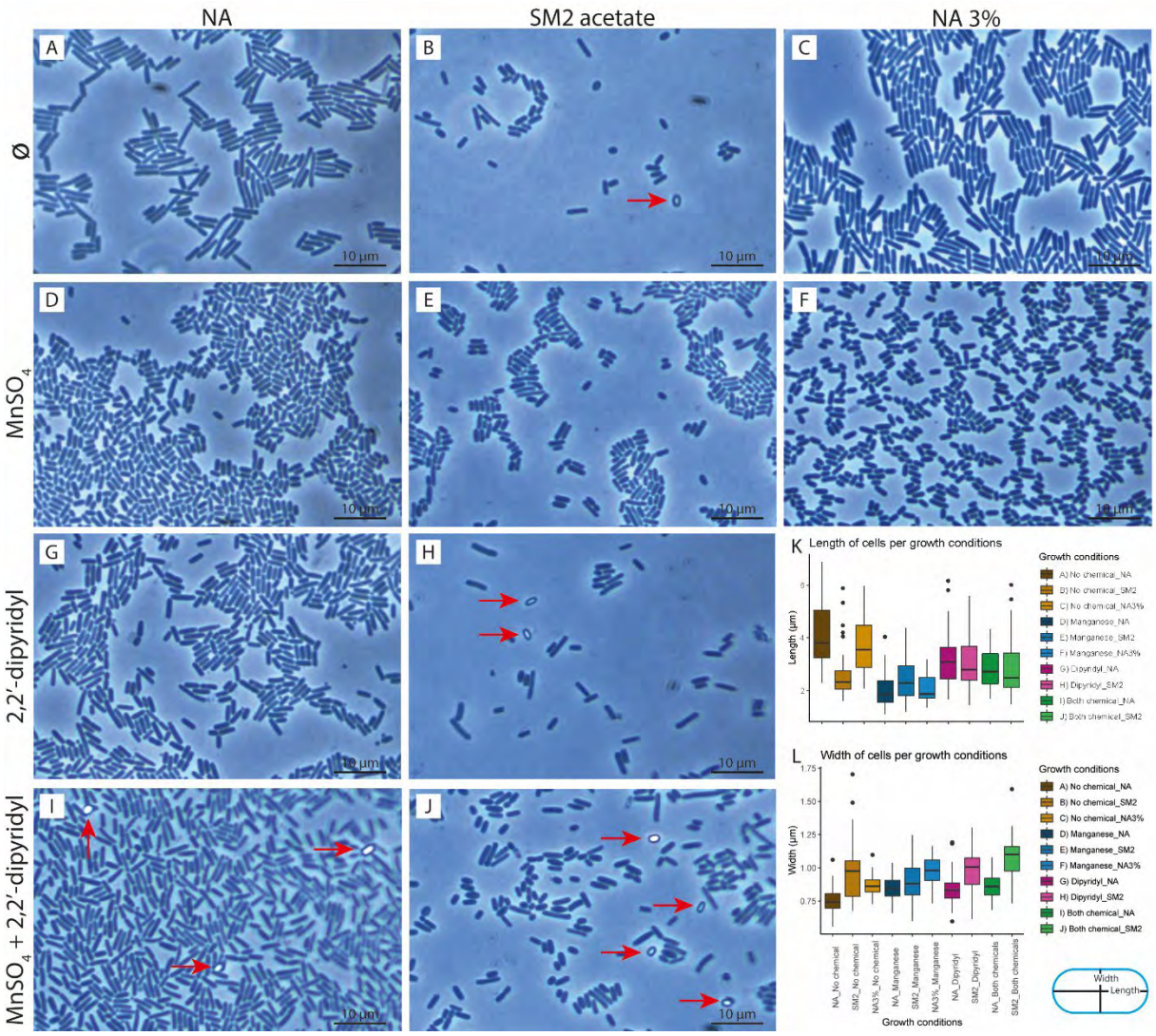

Figure 4. Response of the laboratory strain (LS) of *Kurthia* sp. str. 11kri321 grown under challenging conditions. We grew the LS strain under optimal growth conditions (control) and under nine different challenging growth conditions created by the combination of different growth media and chemical supplementation (details are described in the "Material and Methods section" and Fig. 1). **(A)** Nutrient agar (NA) without any chemical was used as control. **(B to J)** SM2 acetate and NA 3% supplemented or not with $MnSO_4$ or/and 2,2'-dipyridyl as challenging growth media. Microscopic observation was conducted after 48 h of growth, and phase-bright cells were observed in low frequency and not under all challenging growth conditions (red arrows). For each growth condition, we measured on the corresponding image both length and width of 50 cells, when possible (i.e., for the growth medium SM2 acetate with no chemical and with 2,2'-dipyridyl supplementation we could measure only 43 cells), using the ImageJ software (NIH, Bethesda, MD). Average length and width are compared according to growth conditions in bar plots **(K and L, respectively)**, carried out using RStudio software (RStudio Team (2022), Integrated Development Environment for R, RStudio, PBC, Boston, MA).

3.2 Resistance of the original environmental (OS) and laboratory (LS) strains to heat and UV treatments

The low frequency of cryptospores (<5%) in the LS strain and their total absence under most of the tested challenging growth conditions, suggested this survival resistance strategy (and other potential strategies) might have been lost during growth under optimal laboratory conditions. Therefore, in order to compare stress resistance of the OS strain and of the LS

strain of *Kurthia* sp. str. 11kri321, we exposed liquid cultures (approximately 1 x 10^8 cells/mL) to heat (wet and dry) and UV, for different time durations. After the stress treatments, the cultures were transferred onto solid growth media to establish if the cells can regrow (withstand the stress). Cell morphology was also investigated using optical microscopy (Fig. 5).

Parameters	Original strain	Laboratory strain
Wet heat 60°C		
1min	4	4
5min	3	0
10min	1	0
20min	0	0
Dry heat 70°C		
0min (control)	4	4
5min	4	4
7min	2	1
9min	0	0
UV		
1min	4	4
5min	4	4
10min	4	4
20min	3	4

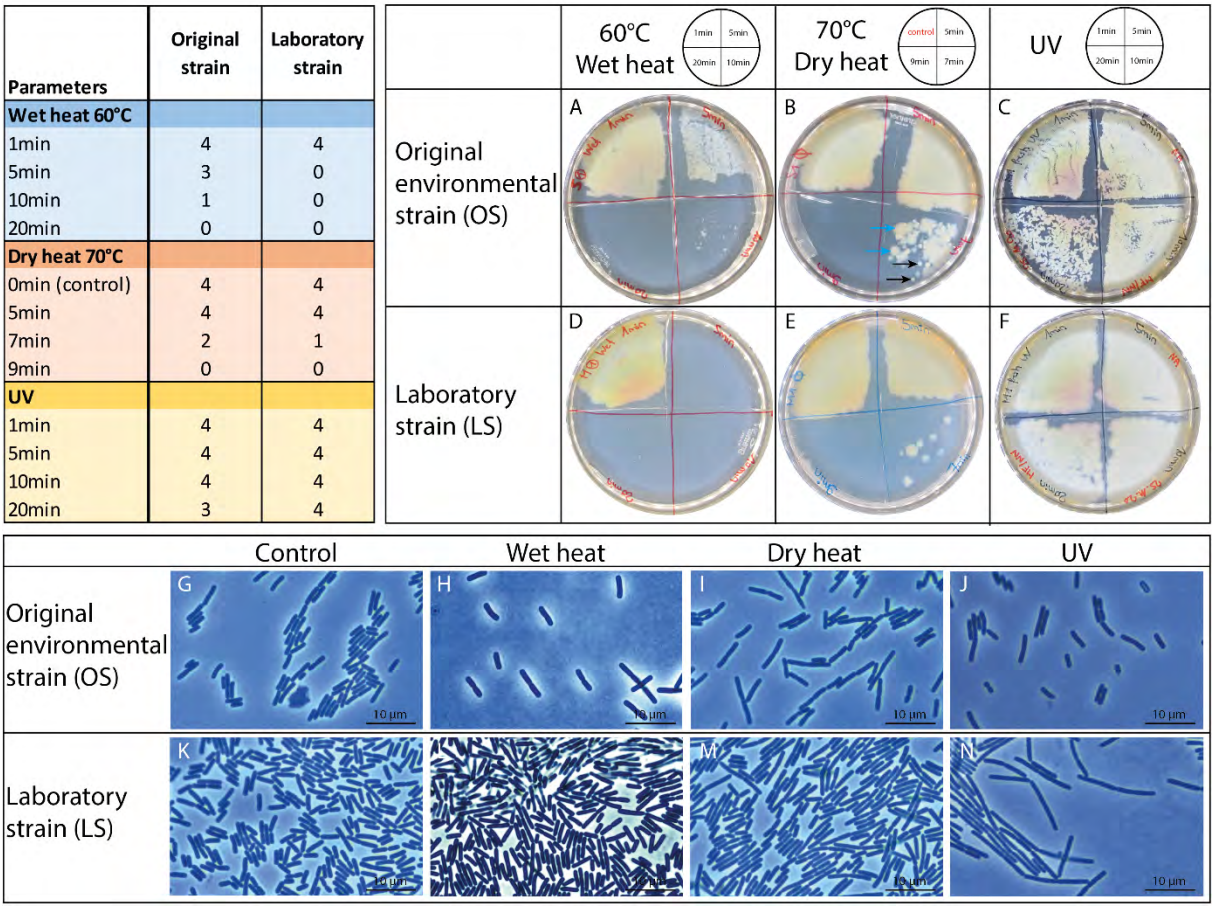

Figure 5. Resistance of the original environmental (OS) and laboratory (LS) strains of *Kurthia* 11kri321 to heat and UV treatments. The table on the left presents as a score the cell growth on nutrient agar (NA) plates (24 h of growth) after different durations of exposure to wet heat (60°C), dry heat (70°C) and UV radiation, with score 4 associated to a dense and high number of cells and score 0 to no cell growth. Pictures on the right illustrate cell growth on NA for the OS strain after exposure to wet heat **(A)**, dry heat **(B)** and UV **(C)**, as well as for the LS strain **(D to F)**. Cells confronted to each duration of stress were plated in a different quarter of the plates (white circles on top). Cells of OS and LS strains not exposed to any treatment (control) were inoculated in the top left quarter of the plates in **B** and **E,** respectively. Blue and black arrows highlight two different types of colonies. Cells of the OS and LS strains not exposed to heat nor UV (control) were collected and observed using optical microscopy **(G and K, respectively)**. The same procedure was applied for the OS and LS cells exposed to stress treatments **(H to J and L to N, respectively)**.

After the stress treatments, we observed that the OS cells were able to grow after a longer exposure to both wet and dry heat as compared to LS cells (Fig. 5, respectively A-B and D-E). After being exposed for 5 min to 60°C wet heat, the OS cells transferred onto solid growth medium grew in high density after 24 h of incubation (Fig. 5A, top right quarter), whereas LS cells did not grow (Fig. 5D, top right quarter). For the dry heat, after 7 min exposure, both OS and LS strains were able to grow, but in lower density for the LS strain (Fig. 5B and 5E, bottom right quarters). In addition, after 7 min of dry heat, two different forms of colonies were

observed for the OS strain: small, round, well-delimited colonies and large, diffuse colonies (Fig. 5B, black and blue arrows respectively). Despite, these morphological differences, when we compared the 16S rRNA sequences obtained from cells of these two colony types to the NCBI database, the best result corresponded to *Kurthia* sp. str. 11kri321 for both (for the small colonies: 100% query cover and 99.89% identity; for the large colony 99% query cover and 97.75% identity). Overall, considering the two types of heat, dry heat had a lower impact on cells, as both the OS and LS strains grew better after a longer exposure to this stress (Fig. 5, respectively B and E), as compared to wet heat (Fig. 5, respectively A and D). After exposure to UV, both the OS and LS strains were able to grow, regardless of the duration of the stress application (i.e., 1 min to 20 min). However, the LS strain grew always better in comparison to the OS strain (Fig. 5 respectively F and C). Finally, the negative controls (no heat or UV exposure) showed that in absence of any stress, our experimental design did not affect the capability of the two strains to grow on NA (Fig. 5B and E, top left quarters).

To evaluate the response of the strains to stress at the cellular level, we collected individual colonies (when possible) in order to compared cell morphology after stress treatments (Fig. 5 G to N). The cell morphology of the OS and LS strains was identical before and after the stress treatments, as well as identical to the morphology of the controls (no stress applied) (Fig. 5 G and K); the cells had a bacilli shape, as expected for *Kurthia* sp. str. 11kri321. The only exception was for the LS strain after UV exposure. In this case, the cells were elongated (Fig. 5N).

Concerning the two types of colonies noticed for the OS strain after 7 min exposure to dry heat (Fig. 5B, black and blue arrows), after we repicked them onto new nutrient growth media, the colony's aspect was still different with large, diffuse colonies and small, round, well-delimited colonies for each morphology, respectively (Supp. Fig. 1A and B). Moreover, at the cell level, the morphology was also different for the two colony types (Supp. Fig. 1C and D). After their transfer on fresh nutrient growth medium, cells forming large colonies (Supp. Fig. 1A) and cells forming small colonies (Supp. Fig. 1B) were composed of cells with a statistically significant different average length (*p*-value= 2.129e-15), with an average length of 3.34 and 2.01 µm respectively (Supp. Fig. 1C and D).

3.3 Genomic comparison:

In addition to culturing and microscopic observations, we performed a comparison of the OS and LS genomes. We also included in the comparison the genome of *Kurthia* sp. str. 11kri321 that was previously published (referred here as *Kurthia* sp. str. 11kri321 strain). First, the genome size was different between the strains; the genome of OS strain was larger than the genome of the LS strain with a difference of 27'671 bp. The published genome is more similar in size to the genome of the LS strain with only 2'945 bp of difference between them (Table 1). This decrease in size for the LS genome was associated with a decrease in the number of proteins with a functional assignment (2935 versus 2896 proteins with PATRIC genus-assignments for OS and LS strains, respectively) (Table 1). Next, based on average amino-acid identity (AAI) (Kim et al., 2021), we calculated the distance between the three genomes. This resulted in Figure 6, indicating that the genome of OS strain is the most distinct one, whereas the LS genome and the published genome are more similar to each other. Then, we performed

additional analyses only comparing the genomes of the OS and LS strains to obtain more information concerning the nature of the differences between these two genomes. The results are presented in Figure 7, as well as in Table 2 and Supplementary Table 1.

Table 1. Summary of the quantitative data retrieved after genome comparison.

	OS strain	LS strain	Kurthia sp. str. 11kri321 (published genome)
Lenght	2989253	2961582	2964527
GC %	36.67	36.68	36.67
CDS	2982	2934	2938
tRNA	82	82	82
repeat_region	63	45	45
crispr_repeat	30	30	30
crispr_spacer	29	29	29
rRNA	27	27	27
Hypothetical proteins	994	954	957
Proteins with functional assignments	1988	1980	1981
Proteins with GO assignments	605	608	608
Proteins with PATRIC genus-specific family (PLfam) assignments	2935	2896	2899
Proteins with PATRIC cross-genus family (PGfam) assignments	2941	2901	2904

While comparing the genomes of LS strain, OS strain and of *Kurthia* sp. str. 11kri321 (published genome), we reported all the relevant information in this table.

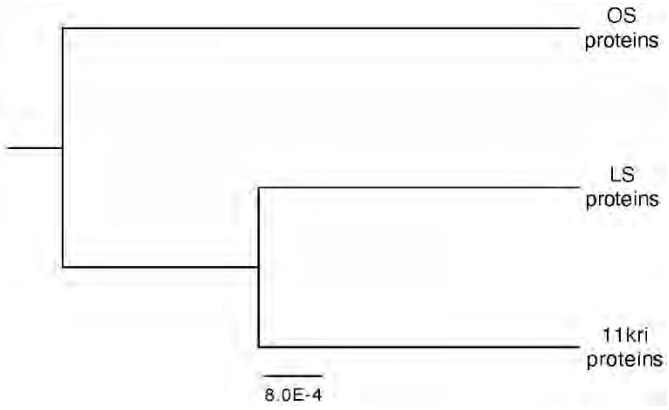

Figure 6. Phylogenetic tree based on average amino-acid identity (AAI) to show the relationship between the three strains of *Kurthia* sp. str. 11kri321. Based on average amino acid identity (AAI), we compute a tree showing that LS strain and *Kurthia* sp. str. 11kri321 are grouped together, whereas the OS strain is at the root.

First, an important gap in the sequence alignment of the master regulator of sporulation *spo0A* gene was detected with a 66 bp deletion in LS sequence, even if this gap is not presented in SNPs' table (filtered out by the default parameters; Supp. Table 1). After whole genome alignment, there are several breakpoints when comparing the OS (reference) and LS (query) genomes (Fig. 7 and Table 2). These breakpoints were of different types and concerned various ranges. The last breakpoint in the LS genome is the most important breakpoint, located around 1.7 Mbp and concerned ca. 22.5 kbp. This breakpoint occurred at the main rearrangement of LS genome. The joining location of the rearrangement (LS genome) occurred near one of the regions with the lowest percentage identity of the alignments sequence (area with genes coloured in yellow). In addition, synonymous versus nonsynonymous substitutions ratio (Ka/Ks) presented a congruent pattern. Indeed, after filtering, the only two Ka/Ks >1 were present near this joining point (green bars). This pattern was again visible by the important density of SNPs near 1.7 Mbp on the LS genome. Overall, this area appeared to be a clear unstable portion of the LS genome with several genomic modifications. This region of genomic instability is also the location of *spo0A* gene. Accordingly, we investigated more in detail the impact of the genome

modifications in the resulting Spo0A protein in the LS and OS strains. We aligned all the Spo0A protein sequences using as reference *B. subtilis* (Fig. 8) and we detected a deletion of 22 amino acids (66 nucleotides) in the Spo0A sequence of the LS strain as compared to the OS strain and the reference *B. subtilis*. For this portion of 22 amino acids, 16 out of the 22 amino acids were identical between the OS strain and the reference *B. subtilis* (Fig. 8, black dots). The deletion of 22 amino acids in LS and *Kurthia* sp. str. 11kri321 genomes is located in the N-terminal domain of Spo0A (Fig. 8). Based on 3D protein modelling, we were able to highlight the differences in Spo0A for the OS and LS strain and the reference *B. subtilis* (Fig. 9). The amino acid Asp55, which is known to be the site of phosphorylation in Spo0A (Lewis, Muchová, et al., 2000), is well preserved in all the proteins (Fig. 9, black area). However, we observed that among the 22 amino acids absent from the Spo0A sequence of LS strain, one of them was Phe103 (Fig. 9, highlighted in red), is a residue shown to be critical in relaying the phosphorylation signal associated with other response regulator, such as CheY in *Escherichia coli* (Lewis, Muchová, et al., 2000; Zhu et al., 1996).

Table 2. Breakpoints retrieved after whole genome alignment when comparing *Kurthia* sp. str. 11kri321 genomes of OS and LS strains.

Genome	Breakpoint type	Start point	End point	Gap length in OS	Gap length in LS	Gap difference
OS	Break	1	1881	1881	NA	NA
OS	Gap	74552	75599	48	-18	66
OS	Gap	250229	253209	2981	0	2981
OS	Jump	1722788	1723787	0	NA	NA
LS	Gap	1469569	1470568	0	2981	-2981
LS	Gap	1645198	1646179	-18	48	-66
LS	Jump	1717772	1695105	-22666	NA	NA

While comparing the genomes of LS strain and OS strain, we reported all the breakpoints occurring in the reference genome (OS) or in the query genome (LS) in this table.

4. Discussion

Kurthia sp. str. 11kri321, a strain initially isolated from an extreme environment (geothermal spring), was previously shown to produce cryptospores when cultivated under nutrient-depleted conditions. However, these cryptospores were produced in low frequency and did not provide an apparent increase in survival in aged cultures (Fatton et al., 2022). Accordingly, the first goal of the present study was to test other challenging growth conditions that may promote production of cryptospores in the LS strain of *Kurthia* sp. str. 11kri321. An important point to consider when investigating survival strategies in the laboratory is that those strategies are frequently lost in laboratory strains as the growth conditions are less challenging than for the wild strains (Norris et al., 2020; Vela & Wyss, 1965). Accordingly, as a second goal, we tested if stress resistance is reduced in the LS strain as compared to a strain cryopreserved shortly after isolation (OS strain), because of the three-months maintenance under optimal growth conditions for the former. Finally, linked to the hypothesized difference in resistance between the OS and LS strains, our third goal consisted in comparing the genome of the two

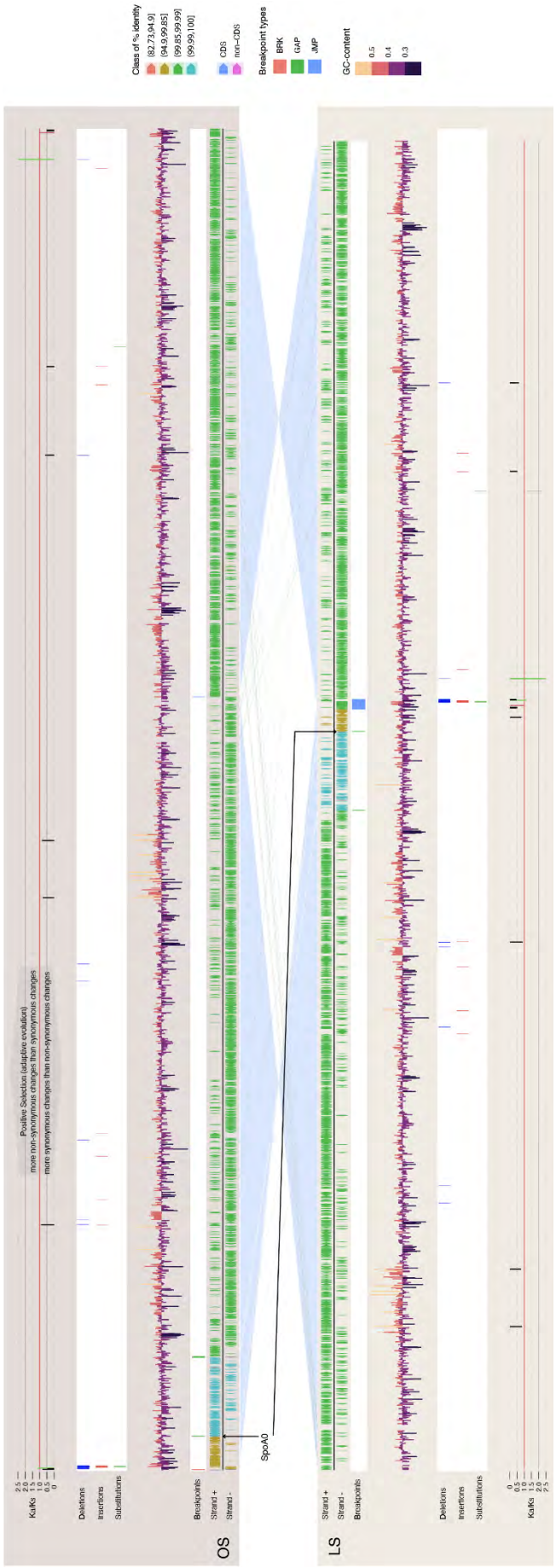

Figure 7. Genome rearrangement in the LS strain of *Kurthia* sp. str. 11kri321. After whole genome alignment using MUMmer4, when comparing the *Kurthia* sp. str. 11kri321 genomes of the OS (reference) and LS (query) strains, several difference in genome structure were detected. Starting from the middle, this figure shows the major rearrangements of coding sequences (CDS, blue triangles), as well as for the non-coding sequences (non-CDS, pink lines). Then the percentage of identity of the alignments (genes colour coded by class of percentage identity) is displayed on both forward and reverse strands. The breakpoints and their type are also presented (BRK= break, GAP= gap and JMP= jump). GC-content is represented by a continuous colour scale. Finally, single nucleotide polymorphisms (SNPs) are denoted according to their type (deletions, insertions and substitutions) and the SNPs with a relevant synonymous versus nonsynonymous ratios (Ka/Ks) are also reported above/below (OS/LS). The location of the master sporulation regulator Spo0A is highlighted with the black arrows.

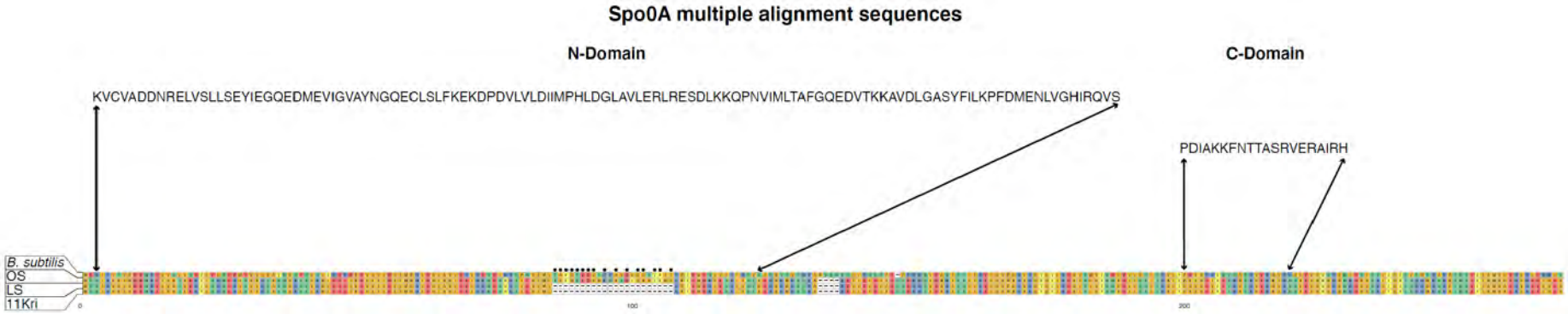

Figure 8. Multiple sequence alignment for Spo0A of the different *Kurthia* sp. str. 11kri321 strains. Using *B. subtilis* as a reference, we aligned the proteins' sequences of Spo0A for the OS strain, LS strain and *Kurthia* sp. str. 11kri321 (published genome). The two domains of Spo0A are annotated; N-domain and C-domain. We identified a deletion of 22 amino acids in the N-domain for LS strain and *Kurthia* sp. str. 11kri321, in comparison to OS strain and *B. subtilis*. For this portion, 16 out of the 22 amino acids were identical between OS strain and the reference *B. subtilis* (black dots).

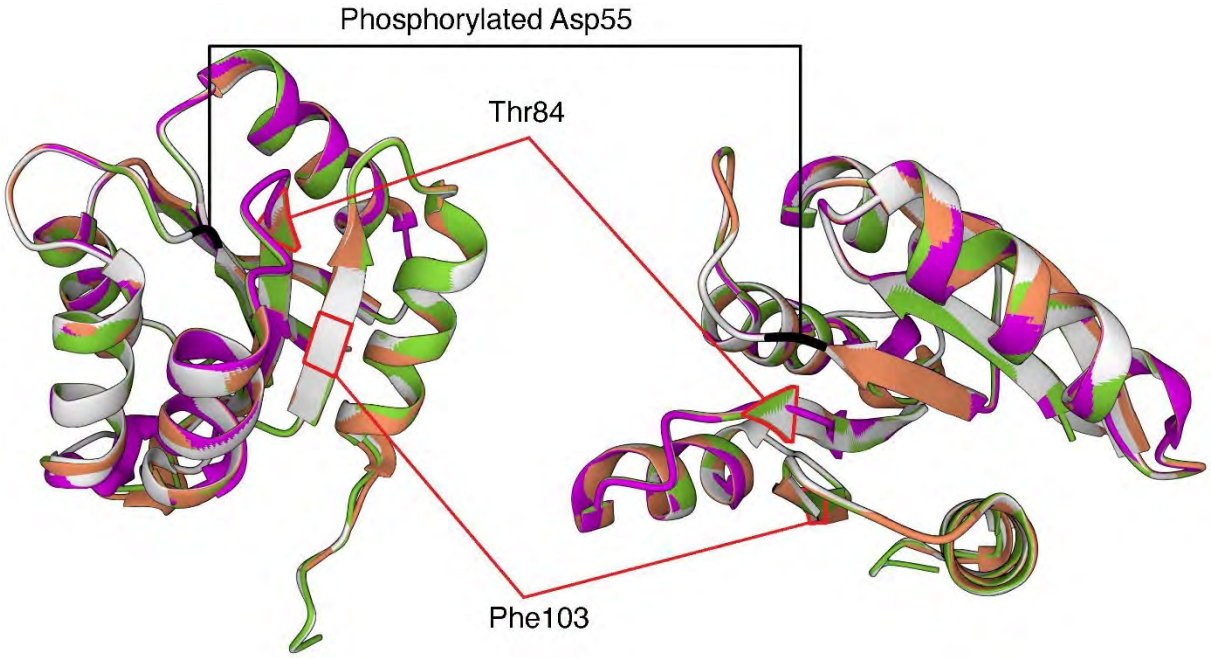

Figure 9. 3D protein modelling of Spo0A to highlight differences between the different strains of *Kurthia* sp. str. 11kri321. On this 3D model of the Spo0A protein, the LS strain is depicted in purple, the OS strain in orange, *B. subtilis* in green and the reference sequence (Lewis et al., 1999) in white. Moreover, the residue Asp55 (known as phosphorylation site (Lewis et al., 1999; Lewis, Muchová, et al., 2000)) corresponds to the black area. Two other residues, Thr84 and Phe103, shown to be key in the phosphorelay cascade of Spo0A (Muchova et al., 2004), are highlighted in red.

strains. After growing the LS strain of *Kurthia* sp. str. 11kri321 under challenging conditions, we observed two types of response: reduction of cell size and production of phase-bright cryptospores in low frequency (Fig. 4, red arrows). The significant reduction of cell length was most remarkable in the case of manganese supplementation (Fig. 4). Manganese supplementation was shown to be a sporulation trigger (Charney et al., 1951). However, production of endospores is costly and favoured in extreme environments (Filippidou et al., 2016). Accordingly, size reduction might represent an alternative stress response with a lower biological cost (Yang et al., 2016). Cell size is tightly controlled in bacteria as it is a fundamental aspect of cell cycle. During their cell cycle, bacteria have to decide whether they continue to grow or they divide in order to have the appropriate size for the current environmental conditions (Weart et al., 2007). Therefore, it was shown that a correlation exists between growth rate and cell size, which was defined as the “growth law” (Schaechter et al., 1958). A slow growth rate was shown to be an alternative survival strategy to sporulation in *B. subtilis* and was termed oligotrophic growth (Gray et al., 2019). Accordingly, *Kurthia* sp. str. 11kri321 might exhibit a slower growth rate as an alternative stress response when grown under challenging conditions, which would in turn impacts the cell size. Regarding the impact of challenging conditions on cell size, nutrient availability is usually considered as the strongest influencing factor for many microorganisms (Weart et al., 2007). For example, when culturing *Salmonella typhimurium* in nutrient rich medium, the cells are up to 3 times larger as compared to those in nutrient poor medium, and *B. subtilis* cells grown in rich medium are approximately twice the length of cells cultured under nutrient-poor conditions (Sargent, 1975). However, nutrient availability is not the only challenging factor that influences bacterial

cell size. For example, in *E. coli*, the cells changed from a rod to shorter more rounded shape under the control of *bolA* and overexpression of this gene was shown to occur in response not only to starvation, but also after osmotic shock, and oxidative stress (Westfall & Levin, 2017). Finally, a smaller size represents a survival strategy often found within pathogens. For example, minimization of bacterial size is a mechanism used by *Streptococcus pneumoniae* to escape killing by the host (Dalia & Weiser, 2011).

Next, phase-brightness is a well-known feature associated to the resistance of *B. subtilis* endospores. Dehydration of the spore's coat and the high solid/water ratio leads to a reflection of the light and explains the phase-bright aspect of endospores (Kong et al., 2011). Therefore, phase-brightness of cryptospores might be an indication of a higher solid/water ratio that provides additional resistance. Cryptospores were produced in response to challenging conditions created through the use of the low-nutrient SM2 acetate medium and/or the supplementation in 2,2'-dipyridyl and/or manganese. One hypothesis is that cryptospores were observed in a low frequency as not all cells have enough energy to differentiate into resistant structures (Pope et al., 2010). In fact, even in *B. subtilis* populations not all the cells undergo sporulation (only 50-70% of cells make the commitment to sporulate) (Schultz et al., 2009), and complex behaviours such as cannibalism are put in place to support the completion of sporulation (González-Pastor, 2011).

Alternatively, the low frequency of cryptospores might be the reflection of a reduced capability of the strain that was maintained for three months under optimal laboratory growth conditions to mount an effective stress response. Natural environments harbour multispecies communities and are constituted of complex multifactorial changing niches. This "real world" contrasts greatly with standardized and highly controlled laboratory growing conditions (Fux et al., 2005). This striking difference explains why environmental strains maintained in the laboratory rapidly mutate and can drastically change compared to their wild homologs. Among those changes, resistance to stress conditions was shown to be rapidly decreased, or even completely lost, when environmental bacterial are cultivated under optimal laboratory conditions. (Maughan et al., 2007; Norris et al., 2020; Vela & Wyss, 1965). In fact, stress resistance is not only subject to the direct cost linked to resistance mechanisms and production of stress protectants, but also to an indirect cost. This indirect cost is associated to the reduced ability to grow vegetatively and therefore, a reduced capacity to compete for nutrients at low concentrations. Due to this trade-off between self-preservation and nutritional competence, different environments with particular stress levels and nutrient concentrations impose different selective pressures on resistance strategies. This explains why evolution has not optimized a universal stress resistance in all bacteria to all stresses. Even within a species like *E. coli*, stress resistance is not constant between strains, which supports that stress resistance is under positive or negative selection depending on the environment (Ferenci & Spira, 2007). Thus, as laboratory strains are maintained in a completely artificial environment in term of stress levels and nutrients concentrations, stress resistance might be modified. Our comparison of heat resistance between the OS and the LS strains of *Kurthia* sp. str. 11kri321 supports this theory: cells maintained under controlled laboratory conditions tolerated less heat exposure than cells maintained for shorter time under laboratory conditions (Fig. 5). Similar results were already shown with laboratory strains

of *Bacillus anthracis* that lost their responsiveness to grow and sporulate as compared to wild strains (Norris et al., 2020), as well as *Azotobacter* cysts from soils that were observed to be far more radiation-resistant as compared than those produced by laboratory strains (Vela & Wyss, 1965).

In addition to the modified heat resistance, the OS and LS strains of *Kurthia* sp. str. 11kri321 differ also concerning the morphotypic variation of cell colonies. There is an enormous diversity among colonies of the same bacterial species (Pipe & Grimson, 2008) and colony patterns were shown to change drastically according to environmental conditions (Ohgiwari et al., 1992). Colonial morphology is determined by many parameters, like colony size, cell arrangement, and colony form, and it varies according to biotic and abiotic surrounding factors, including temperature (Xiao et al., 2018). For example, at a temperature of 15°C, *Microcystis* cells form larger colonies (i.e., up to 100 µm in diameter) compared to higher temperatures (Li et al., 2013). In contrast, an increase in colony diameter was observed for *Serratia marcescens* in response to rising incubation temperature (Pipe & Grimson, 2008). In the case of the OS strain of *Kurthia* sp. str. 11kri321, the dry heat treatment triggered a morphotypic colony variation with two different colony types (large-diffuse or small-round-well delimited; Fig. 5B, blue and black arrows respectively). Even if we did not compare the resistance of these two colonial morphotype, they might be more resistant/sensible to heat. Indeed, small colony variants (SCVs) were reported for various bacterial species (e.g. *Pseudomonas aeruginosa* (Häußler et al., 1999), *Enterococcus faecalis* (Wellinghausen et al., 2009) and *E. coli* (Roggenkamp et al., 1998)) and most of these SCVs were isolated under harmful environmental conditions, such as physical stress, starvation, antibiotic therapy, thermal stress, or exposure to disinfectants (Kahl, 2014). Accordingly, *Kurthia* sp. str. 11kri321 may have switch to SCVs in response to heat, which constitutes challenging conditions. In addition, the individual cells composing the different colony morphotypes were also different in size (Supp. Fig. 1C and D). As mentioned above, a smaller cell size is often observed under environmental stress and therefore could also represent a response to suboptimal growth conditions due to heat. A last hypothesis to explain the two cell-size morphotypes observed after heat treatment is linked to lipids composing the inner membrane of bacterial cells. Indeed, adjustment of membrane lipid composition was shown to occur in response to temperature change. Such adjustment ensures that fluidity of the membrane is kept stable, even under fluctuating growth temperatures (Aguilar et al., 2001; Zhang & Rock, 2008). For example, in *B. subtilis*, a molecular thermosensor allows regulation of membrane fluidity (Aguilar et al., 2001). Because lipid composition and transmembrane lipid dynamics in the inner membrane of *E. coli* was shown to impact cell size and cell shape (Bogdanov et al., 2020), we might consider that *Kurthia* sp. str. 11kri321 responded to the heat treatment by a modification of lipids in its inner membrane, which impacted its cell shape. However, additional experiments would be necessary to confirm any difference in heat resistance/sensibility between the two morphotypes. Meanwhile, this morphotypic colony variation was never observed in the LS strain (i.e., always only one type of colony), suggesting that this trait was lost under optimal growth conditions.

Further, we showed that the OS and LS strains react differently to UV radiation. Even if, after UV treatment, both strains were able to grow on the fresh nutrient growth medium, in the LS

strain we observed longer cells (Fig. 5N) compared to control condition (Fig. 5K), whereas this was not the case for the OS strain (Fig. 5G and J). Inhibition of cell division leading to filamentous cells was shown to be part of the SOS-response in *E. coli* (Hill et al., 1997). Moreover, production of such filamentous cells was shown to occur in *Pseudomonas aeruginosa* when exposed to UV-C (Elasri & Miller, 1999). Therefore, production of filamentous cells might be the response of LS strain to UV exposure. Such response was not observed in the OS strain, which confirms that the two strains are different and accordingly, respond differently due to their respective evolution trajectories in the original natural environment or in the laboratory.

Finally, the genomic analysis confirmed that considerable changes at the genome level occurred between the OS and LS strains. The first change corresponded to a decrease in genome size in the LS strain (27'671 bp of difference) (Table 1). Variation in genome size is mainly due to two evolutionary forces: genetic drift and selection. It was shown that bacteria have larger genome when selection is more effective, whereas eukaryotes have more streamlined genomes when selection is more effective. Therefore, the same force, selection, has an opposite effect on genomes of bacteria and eukaryotes. This could be explained by deletional bias, a mechanism occurring only in bacteria. In bacteria the mutational processes are different from those occurring in eukaryotes, with a strong mutational bias toward deleting superfluous sequences observed in bacteria (Andersson and Andersson, 2001; Mira et al., 2001). Thus, mutations will be eliminated or kept in genome due to the balance between genetic drift and selection. If selection is strong, mutations that provide a benefit will be retain, thus counteracting the deletional bias, and a genome will be larger. On the contrary, if selection is less effective, mutations will be more easily eliminate due to deletional bias, which leads to smaller genome. Accordingly, due to a relaxed survival pressure under optimal laboratory growth conditions, mutations could be more easily eliminated from the genome of the LS strain (deletional bias), which leads to a smaller genome as compared to the OS strain. In addition to genome size reduction, we also observed several breakpoints when comparing the genomes of OS and LS strains (Table 2), leading to an important genome rearrangement (Fig. 7). The joining location of the rearrangement in the LS genome is characterized by a region of clear genomic instability (i.e., important density of SNPs, lowest % identity of the alignments sequence and Ka/Ks >1). Interestingly, the master sporulation regulator gene *spo0A* is located in this region of genomic instability. Accordingly, a striking difference between the two strains was the deletion of 22 amino acids in the N-terminal domain of Spo0A, which were deleted in the LS strain as compared to in OS strain. Because this part of the sequence in the OS strain has 16 out of 22 identical amino acids to the reference *B. subtilis* (Fig. 8, black dots), and following the principle of parsimony (i.e., the most acceptable explanation of an occurrence, phenomenon, or event is the simplest, involving the fewest entities, assumptions, or changes (Martin & Hine, 2015)), it is more reasonable to consider that a deletion of 22 amino acids occurred in the Spo0A sequence of LS strain, rather than an insertion of 22 amino acids in the OS strain. Accordingly, we might be confident that the OS strain carried the more ancestral version of Spo0A sequence, whereas the LS strain and *Kurthia* sp. str. 11kri321 evolved a different version while maintained under laboratory conditions. Spo0A is the master transcriptional regulator of sporulation and is responsible for

sporulation initiation (Errington, 2003; Tan & Ramamurthi, 2014). The initiation of sporulation involved both the downregulation of transcription of genes associated to stationary phase and the activation of genes associated to sporulation. These changes in gene expression depend on the concentration threshold of phosphorylated Spo0A (Spo0A~P) (Lewis, Krzywda, et al., 2000). The phosphorylation cascade of Spo0A starts by the autophosphorylation of two kinases, KinA and KinB, in response to appropriate stimulus. Those kinases transfer the phosphate to Spo0F and next, phosphorylated Spo0F (Spo0F~P) transfers the phosphate to Spo0B. Finally, Spo0B~P phosphorylates Spo0A (Al-Hinai et al., 2015). Moreover, Spo0A is additionally controlled by a positive feedback loop involving sigma-factor H (σ^H). σ^H will in turn stimulates transcription of *spo0A* and of *kinA* (Fujita & Losick, 2005). Once activated, Spo0A~P binds to its operator site, a consensus DNA-sequence of 7 bp (5'-TGNCGAA-3'), called "0A-box" (Castilla-Llorente et al., 2006; Zhou et al., 2020). Through this binding, Spo0A~P will lead to the downregulation of stationary phase genes through the repression of *abrB* transcription and activation of the expression of multiple sporulation-specific genes (Lewis, Krzywda, et al., 2000).

Concerning its structure, Spo0A is a single polypeptide chain composed by two domains, an N-terminal regulatory domain (N-Spo0A) and a C-terminal trans-activation domain (C-Spo0A). These two domains are separated by a linker region. The N-Spo0A contains all signatures residues characteristics of response regulators and is a substrate for the phosphorelay. On the other hand, the C-Spo0A contains a helix-turn-helix (HTH) DNA-binding motif, that is conserved only among Spo0A homologues. In response to phosphorylation of the N-Spo0A, the C-Spo0A binds to DNA and activate transcription of several sporulation-specific genes (Lewis, Krzywda, et al., 2000; Shukla et al., 2020).

Under continuous culturing, asporogenic mutants were observed for several spore-formers, such as *B. sphaericus* (Idachaba & Rogers, 2001), *B. thuringiensis* (Sachidanandham & Jayaraman, 1993), and *B. subtilis* (Maughan et al., 2007). Mutations in Spo0A, both in N-Spo0A or C-Spo0A, were often retrieved in those asporogenic mutants (Sastalla et al., 2010). In the case of *Kurthia* sp. str. 11kri321, we showed that N-Spo0A suffered from a deletion of 22 amino acids in the LS strain. Due to the role of N-Spo0A in the phosphorelay activation cascade, a modification occurring in this domain might create an issue in sporulation initiation. Even if the deletion did not concern directly the phosphorylation site (Asp55), it concerns Phe103 (Fig. 9, Asp55 highlighted in black and Phe103 in red), a key residue involved in the phosphorylation signal of multiple response regulators (Lewis, Muchová, et al., 2000; Zhu et al., 1996), including the N-domain of Spo0A. In *B. subtilis*, when the phosphoryl group is transferred to Spo0A, Asp56 is phosphorylated, and Spo0A dimerizes. Due to this dimerization, Thr84 is pulled toward Asp56, and the repositioning of Thr84 also provokes a structural rearrangement of Phe105 (Muchova et al., 2004). This complex rearrangement is a conserved aspect of signalling in diverse response regulators (Gardino et al., 2003; Park et al., 2002). To evaluate the importance of Phe105 for sporulation in *B. subtilis*, a previous study introduced an alanine substitution at this position. The results show that after substitution, Spo0A was still phosphorylated but did not form a dimer, which could severely impact the phosphorelay signalling cascade (Muchova et al., 2004). Concerning *Kurthia* sp. str. 11kri321, we hypothesize that due to deletion of Phe103 in Spo0A of the LS strain (Fig. 9, highlighted in red),

phosphorylation of Spo0A might still be possible, whereas the incapability to form a correct dimer might severely reduce the production of cryptospores, something confirmed by our microscopic investigation.

5. Conclusion

To conclude, we showed in our study that manganese, a well-known sporulation inducer (Charney et al., 1951), triggers a morphological change (i.e., cell length reduction) in the LS strain of *Kurthia* sp. str. 11kri321, which might represent an alternative stress response. In addition, we showed that combination of known environmental stresses triggers the production of cryptospores in the LS strain, but at a very low frequency. Even if we did not test the higher resistance of those modified cells, we suggest that the low frequency of production is due to a modified stress response in the LS strain of *Kurthia* sp. str. 11kri321 after its maintenance under optimal laboratory growth conditions. The low frequency of cryptospores in the LS strain might be due to a deletion in the N-terminal regulatory domain of Spo0A, which is responsible to initiate sporulation. Such deletion did not occur in the OS strain, supporting that the two strains evolved independently. All our observations suggest that the LS and OS strains are different from each other due to their respective evolution in laboratory or in a natural extreme environment. The Spo0A sequence, resistance to heat, colonial morphotypic variation, and cell size plasticity, are traits that were all expressed differently between OS and LS strains. Therefore, the study of environmental bacterial strains in laboratory is quite challenging, as they rapidly adapt to their new environment and become different not only in their resistance capability, but also morphologically and genetically, as compared to the original isolated strain.

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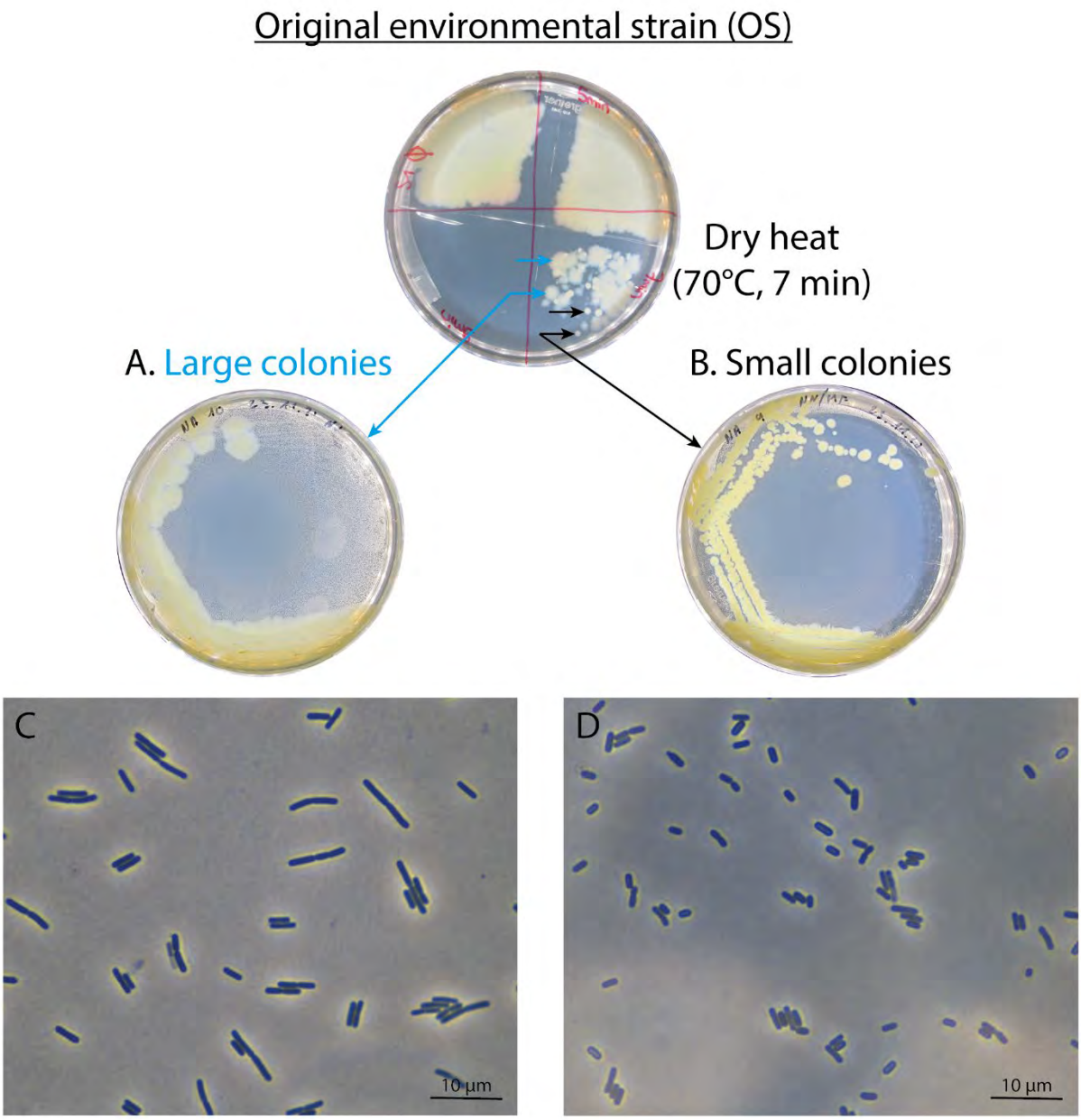
Supplementary material



Supplementary Figure 1. Morphotypic variation observed for the original environmental strain (OS) of *Kurthia* sp. str. 11kri321 after exposure to dry heat. After 7 min exposure to dry heat (70°C), the OS strain was observed to produce two different types of colonies when inoculated for 24 h on fresh nutrient agar (NA) medium (blue and black arrows). After those two colonies were repicked separately on fresh NA medium, they still had a different morphology after 24 h of growth with either large, diffuse colonies **(A)** or small, round, well-limited colonies **(B)**. Using optical microscopic, cells composing the larger colonies were observed to be longer in length **(C)**, as compared to cells composing the smaller colonies **(D)**.

Supplementary Table 1. Single nucleotide polymorphisms (SNPs) detected after whole genome alignment to compare the *Kurthia* sp. str. 11kri321 genomes of OS and LS strains.

SNPs in CDS of OS genome with OS genome as a reference based on reference coordinates										
Reference sequence	**Start position in reference**	**End position in reference**	**SNP identifier**	**SNP type in query**	**Query sequence**	**Start position in query**	**End position in query**	**Bases in query**	**Bases in reference**	**Function**
OS	2011	2011	SNP_87	deletion	LS	1717642	1717642	-	A	hypothetical protein
OS	2556	2556	SNP_86	deletion	LS	1717098	1717098	-	C	hypothetical protein
OS	6656	6656	SNP_69	deletion	LS	1713006	1713006	-	G	hypothetical protein
OS	7444	7444	SNP_58	deletion	LS	1712226	1712226	-	G	Phage antirepressor protein
OS	545670	545670	SNP_10	insertion	LS	1177107	1177107	T	-	hypothetical protein
OS	546948	546948	SNP_9	deletion	LS	1175829	1175829	-	T	hypothetical protein
OS	546953	546953	SNP_8	deletion	LS	1175825	1175825	-	G	hypothetical protein
OS	601886	601886	SNP_6	insertion	LS	1120893	1120893	A	-	Multi antimicrobial extrusion protein (Na(+)/drug antiporter), MATE family of MDR efflux pumps
OS	750623	750623	SNP_3	insertion	LS	972155	972155	A	-	Polyketide synthase modules and related proteins
OS	1089229	1089237	SNP_2	deletion	LS	633549	633549	-	GACCAAACA	Ribonuclease J1 (endonuclease and 5' exonuclease)
OS	1127836	1127836	SNP_1	deletion	LS	594951	594951	-	A	Sulfate and thiosulfate import ATP-binding protein CysA (EC 3.6.3.25)
OS	2261563	2261563	SNP_93	deletion	LS	2422807	2422807	-	A	Flagellar motor rotation protein MotB
OS	2459465	2459465	SNP_91	insertion	LS	2224904	2224904	G	-	3-hydroxyacyl-CoA dehydrogenase [fadN-fadA-fadE operon] (EC 1.1.1.35) / Enoyl-CoA hydratase [fadN-fadA-fadE operon] (EC 4.2.1.17)
OS	2900932	2900932	SNP_89	insertion	LS	1783436	1783435	TT	-	Chaperone protein DnaJ
OS	2920954	2920954	SNP_88	deletion	LS	1763413	1763413	-	A	Cytidine deaminase (EC 3.5.4.5)

SNPs in CDS of LS genome with OS genome as a reference based on query coordinates										
Query sequence	**Start position in query**	**End position in query**	**SNP identity**	**SNP type in query**	**Reference sequence**	**Start position in reference**	**End position in reference**	**Bases in query**	**Bases in reference**	**Function**
LS	1165568	1165568	SNP_7	deletion	OS	557211	557211	-	A	hypothetical protein
LS	1713366	1713366	SNP_72	deletion	OS	6294	6294	-	T	Repressor (CI-like) [Bacteriophage A118]
LS	1713451	1713451	SNP_73	deletion	OS	6208	6208	-	A	Repressor (CI-like) [Bacteriophage A118]
LS	1713543	1713543	SNP_74	insertion	OS	6116	6116	T	-	Repressor (CI-like) [Bacteriophage A118]
LS	1713610	1713610	SNP_75	deletion	OS	6049	6049	-	G	Repressor (CI-like) [Bacteriophage A118]
LS	1713866	1713866	SNP_76	deletion	OS	5792	5792	-	A	Chromosome segregation ATPase
LS	1713888	1713888	SNP_77	insertion	OS	5770	5770	T	-	Chromosome segregation ATPase
LS	1714069	1714069	SNP_78	insertion	OS	5590	5590	T	-	Chromosome segregation ATPase
LS	1714122	1714122	SNP_79	deletion	OS	5537	5537	-	G	Chromosome segregation ATPase
LS	1714186	1714186	SNP_80	insertion	OS	5473	5473	A	-	Chromosome segregation ATPase
LS	1714268	1714268	SNP_81	deletion	OS	5391	5391	-	A	Chromosome segregation ATPase
LS	1714526	1714526	SNP_82	deletion	OS	5132	5132	-	A	Chromosome segregation ATPase
LS	1715673	1715673	SNP_84	deletion	OS	3983	3983	-	A	hypothetical protein
LS	1716368	1716368	SNP_85	deletion	OS	3287	3287	-	G	hypothetical protein
LS	1717098	1717098	SNP_86	deletion	OS	2556	2556	-	G	hypothetical protein
LS	1717642	1717642	SNP_87	deletion	OS	2011	2011	-	T	hypothetical protein

Chapter 3 - A transcriptomics study of the effect of manganese in the model sporulating bacterium *Bacillus subtilis* and the non-model environmental *Kurthia* spp.

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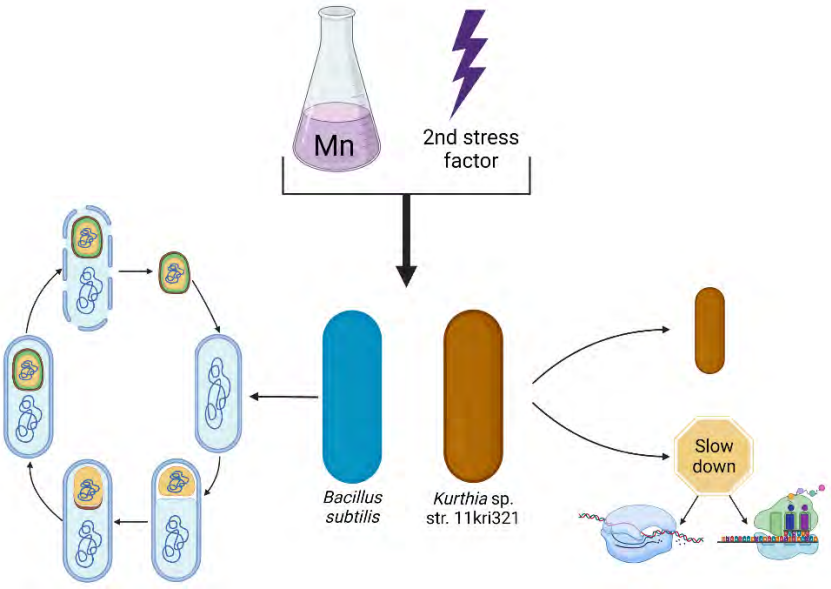

In a nutshell: Manganese is a well-known trigger of sporulation. In this chapter, we provide transcriptomics data to show that the sporulating model *Bacillus subtilis* initiates sporulation under manganese supplementation, whereas the non-standard model *Kurthia* sp. str. 11kri321 responds through an alternative stress resistance strategy resulting in cell size reduction and transient shutdown of translation/transcription. However, manganese supplementation alone did not trigger sporulation or an alternative stress response; a second stress factor was necessary for the organisms to engage their energetic resources in these costly processes.

Disclaimer: The results of this chapter were likely produced by a combination of sub-strains that cannot be differentiated.

Abstract

Manganese (Mn^{2+}) is an essential trace element that impacts numerous bacterial processes. Manganese has been shown to be important for both growth and sporulation in multiple species of *Bacillus*. Sporulation is a complex morphophysiological response used by members of the Firmicutes to survive unfavourable environmental conditions through the production of highly resistant and dormant spores. Because the overall importance of spores produced by both pathogenic and beneficial species, these structures are intensively studied and for this, empirical supplementation of the growth medium with manganese is still widely used to trigger sporulation under laboratory conditions. However, the role of manganese on sporulation is far from understood. Therefore, in this study, we used a transcriptomic approach to investigate the role of manganese in sporulation of the model organism *B. subtilis*, comparing the effect of supplementation with this element in cells growing in solid and liquid media. In addition, we conducted an equivalent transcriptomic study to investigate the response to manganese supplementation in environmental representatives of the non-model Firmicutes genus *Kurthia*. Gene expression, both in *B. subtilis* and *Kurthia* spp., was affected by the exposure to manganese, but the effect depended on the growth media. Multiple sporulation genes involved in late sporulation stages in *B. subtilis* were up-regulated upon exposure to manganese. However, sporulation was only observed in solid medium, indicating that manganese alone is not sufficient to trigger sporulation. Concerning the non-model *Kurthia* spp., the response was unique to each one of the species investigated, and for instance in one of the species, *Kurthia* sp. str. 11kri321, a morphological change of the cells and a shutdown of translation/transcription processes were detected after supplementation with manganese in solid medium. Both modifications represented alternative stress responses to manganese exposure. Our results highlight the contrasting behaviour of model and non-model species in response to environmental stressors. The transcriptomics data also showed that manganese alone is not sufficient to trigger sporulation (or an alternative stress response), but that the cellular reaction is part of a multi-stress response dependent on growth conditions (solid versus liquid media).

1. Introduction

Manganese (Mn^{2+}) is an essential trace element for bacteria (Madigan et al., 2010). Manganese is often considered as a key enzymatic co-factor, but only a relatively few Mn-dependent proteins are known, and those are often co-factored by other cations (Kehres & Maguire, 2003). Instead, manganese homeostasis is not only important for enzymatic function, but it also impacts transcriptional control and gene expression in numerous bacterial processes including pathogenicity, oxidative stress, and sporulation (Kehres & Maguire, 2003; Rosch et al., 2009). In the case of oxidative stress and sporulation, the two might be connected, as manganese has been shown to alleviate oxidative stress during vegetative growth and during sporulation in the model sporulating bacterium *Bacillus subtilis* (Inaoka et al., 1999).

Sporulation in Firmicutes is a complex morphophysiological response used to survive unfavourable environmental conditions. The sporulation process involves a sophisticated program of cellular division and differentiation that leads to the production of highly resistant and dormant spores (Tan & Ramamurthi, 2014). As sporulation allows persistence under unfavourable growth conditions, environmental conditions play a non-negligible role in the entry into this survival strategy. Among those environmental conditions, it is known that mineral composition of growth media may affect sporulation (Sinnelä et al., 2019), and manganese has been shown to be essential for both growth and sporulation of *Bacillus* spp. (Charney et al., 1951; Sinnelä et al., 2019). In 1951 Charney *et al*. showed that manganese, in the form of a $MnCl_2$ solution, strongly stimulated sporulation in several species of bacilli (Charney et al., 1951). Manganese triggered a faster sporulation rate when sporulating bacteria were cultivated in both solid and in liquid media. However, in liquid medium, a longer incubation time was necessary to observe sporulation in *B. subtilis* (sporulation percentage: 5% after 24 h incubation, 50% after 72 h), as compared to culturing in solid medium (sporulation percentage: 25% after 24 h, 50% after 48 h) (Charney et al., 1951). Since then, several other studies have investigated the effect of manganese on sporulation (Inaoka et al., 1999; Weinberg, 1964). Even if those studies confirmed the initial observations, the mechanisms by which manganese is an efficient sporulation trigger remains unclear. One of the potential explanations is the need of manganese as a co-factor for the phosphoglycerate phosphomutase (Pgm). This enzyme catalyses the degradation of 3-phosphoglyceric acid, which acts as a growth inhibitor and a suppressor of normal sporulation (Oh & Freese, 1976). This enzyme is the only manganese II-dependent enzyme known to date to be required for optimal sporulation (Vasantha & Freese, 1979).

Since these early studies, the investigation of the role of manganese on sporulation has been neglected, despite the fact that manganese supplementation remains a widely used method to induce sporulation. In this study we used a transcriptomic approach to investigate the role of manganese in sporulation of the model organisms *B. subtilis*, comparing the effect of supplementation with this element in cells growing in solid and liquid media. In addition, we conducted a comparable transcriptomic study to investigate the response to manganese supplementation in environmental representatives of the non-model Firmicutes genus *Kurthia*. Multiple sporulation genes involved in late sporulation stages in *B. subtilis* were up-regulated upon exposure to manganese. However, this response was only observed in solid medium. Concerning the non-model *Kurthia* spp., the response was unique to each one of the species investigated. These results highlight the contrasting behaviour of model and non-model species in response to environmental stressors.

2. Results

2.1 Results for *B. subtilis*

2.1.1 Comparison of manganese versus no-manganese treatment

Vegetative cells with a bacilli shape were observed in most growth conditions (Fig. 1A, C and D). However, after 24 h of growth, a morphological change was observed in *B. subtilis* when the cells were cultivated on solid medium in presence of manganese. Under these conditions, phase-bright engulfed pre-spores were identified inside 30% of mother cells (Fig. 1B; counts for approximately 500 mother cells with 147 of them having an endospore). To understand the effect of manganese on sporulation in *B. subtilis*, we performed transcriptomics (RNA sequencing) in cells cultivated with or without added manganese, both in liquid and solid culture conditions. When the effect of manganese was assessed regardless of the media, only 12 genes changed significantly in their expression. Nine genes were up-regulated and three genes were down-regulated in presence of manganese (Table 1 and Supp. Table 1). All up-regulated genes with a known function (*cotY*, *cotX*, *cotV*, *cotG*, *yxeE*, *cgeA*, and *cgeB*), had a role in sporulation and are involved in spore coat formation, which corresponds to the late sporulation stage V (Fig. 2A-B, Table 2 and Supp. Table 1).

2.1.2 Comparison of the manganese treatment in solid versus liquid growth conditions

Several parameters differ drastically when cultivation in the laboratory is done in liquid or on solid medium. Those include the distribution of nutrients and metabolites (Koutsoumanis et al., 2004; Wilson et al., 2002), oxygen and osmotic pressures, and salt and oxidative stress (Cuny et al., 2007). All these differences are known to impact microbial behaviour (Koutsoumanis et al., 2004). Accordingly, when comparing the effect of manganese in liquid and solid growth medium, the number of down- and up-regulated genes in manganese-treated cells of *B. subtilis* was much higher than the effect of manganese alone, and corresponded to 319 and 74 genes, respectively (Table 1 and Supp. Table 2). Excluding genes coding for hypothetical or uncharacterized proteins, almost 40% of the up-regulated genes identified in cells growing in solid medium are associated to sporulation, including those coding for the sporulation delaying protein C and its immunity protein SdpI, as well as the gene coding for subtilosin-A. This result is in agreement with the microscopic data, which showed the presence of vegetative cells at 24 h when cells were exposed to manganese in the liquid medium, but not of endospores (or sporulating cells) (Fig. 1D). In addition, several genes involved in general stress response were also up-regulated in response to exposure to manganese in solid medium (Table 1 and Supp. Table 2). Excluding again the genes coding for hypothetical or uncharacterized proteins, 50% of the genes observed with a modified expression are known to be transcribed during common cellular processes, such as general metabolism, DNA replication, transcription, translation (with a large fraction of tRNAs (16 tRNAs) down-regulated in cells growing in liquid medium), and energy (Table 1 and Supp. Table 2). Those key processes were required in both conditions, but are reported to involve dramatically different genes depending on the growth medium (Kuwana et al., 2002).

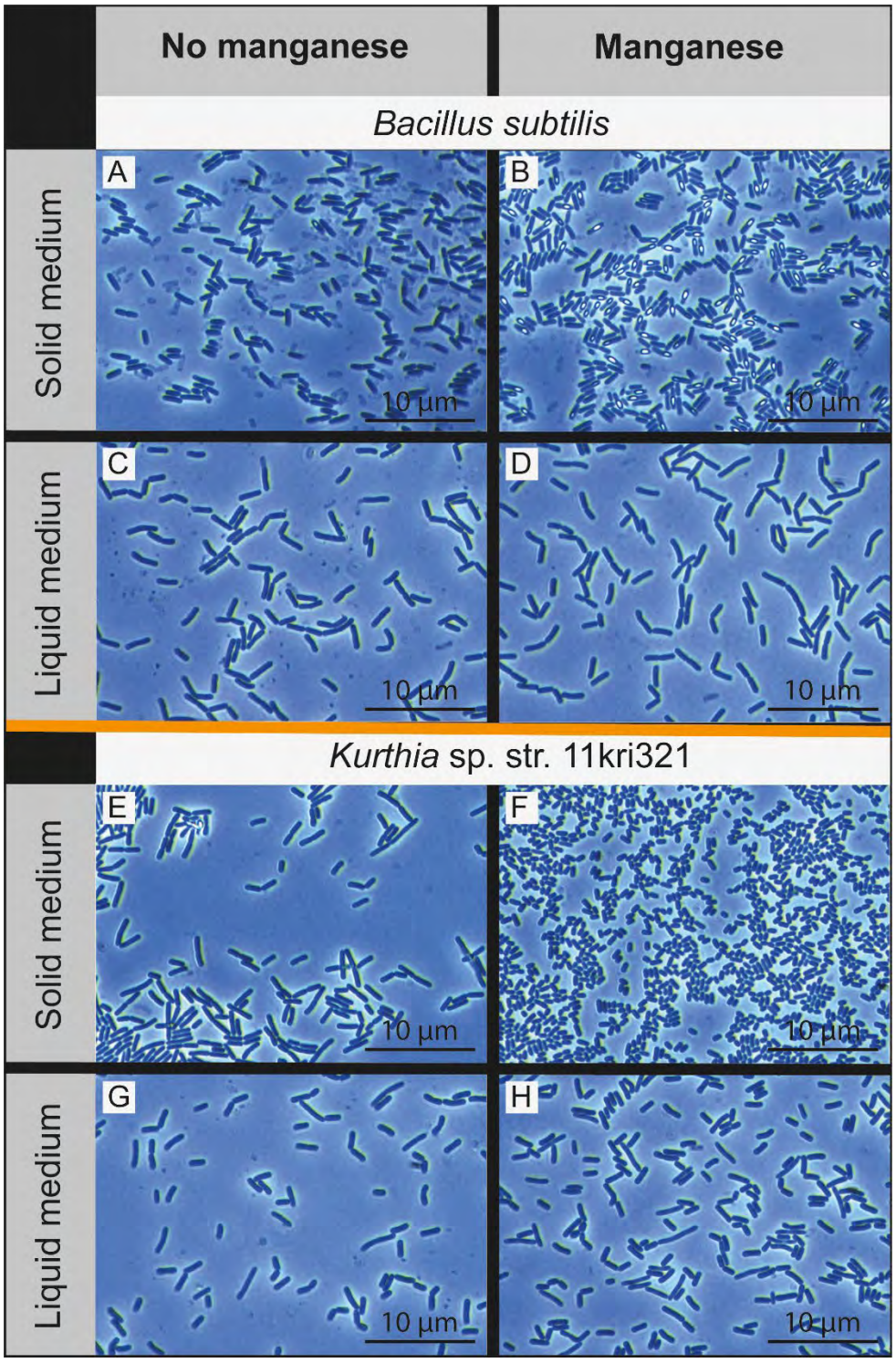

Figure 1: Microscopic observation of *B. subtilis* and *Kurthia* sp. str. 11kri321 cells after 24 h incubation at 30°C, comparing manganese supplementation and no supplementation and type of growth medium. On solid medium (NA plate) and in absence of manganese, only vegetative cells of *B. subtilis* cells were observed **(A)**, whereas spores inside mother cells were present upon exposure to manganese **(B)**. When *B. subtilis* was grown in liquid medium (NB), we observed vegetative cells both in absence **(C)** and in presence of manganese **(D)**. For *Kurthia*, the cells had a bacilli shape on solid medium in absence of manganese **(E)**, whereas the cells shrunk and looked rounder in presence of manganese **(F)**. In liquid medium, both in presence **(G)** and in absence of manganese **(H)**, *Kurthia*'s cells had a bacilli shape.

Table 1. Summary of the impact of manganese and of the growth medium on the gene expression of *Bacillus subtilis* and *Kurthia* sp. str. 11kri321.

	Expression with manganese treatement				Expression with manganese in liquid medium			
	B.subtilis		*Kurthia* sp. str. 11kri321		*B. subtilis*		*Kurthia* sp. str. 11kri321	
	Up-regulated	Down-regulated	Up-regulated	Down-regulated	Up-regulated	Down-regulated	Up-regulated	Down-regulated
Sporulation	7					80		
General metabolism		1		8	15	19	7	
Energy production				10	10	5	8	
DNA replication						5	7	
Transcription				7	5	3	16	
Translation				40	16	57	25	
Membrane		1	1	16	7	18	10	
Stress response		1		7	12	14	5	
Motility					1		2	
Biofilm				1		1		
Others				5	2			
Hypothetical/unknown	2			82	6	117	17	
TOTAL	9 genes	3 genes	1 gene	176 genes	74 genes	319 genes	97 genes	0 gene

The two bacteria up- or down-regulated the transcription of some genes when the cells were exposed to manganese compared to not exposed (grey) and when manganese-treated cells were grown in liquid compared to solid medium (blue). Among the total number of genes with a differential expression (padj <0.05; log2FoldChange >1 or <-1) (line highlighted in orange), we classified how many of them are known to be involved in the biological cellular processes defined here. The "hypothetical/unknown category" contains the genes coding for a hypothetical or unidentified protein.

Table 2. Overview of the individual bacterial species response to manganese supplementation in solid growth conditions at the level of gene expression.

Bacillus subtilis	
Category	Genes
Sporulation	cotY cotX cotV cgeA cgeB cotG yxeE
Translation	Genes coding tRNA
Stress response	ymzB yplP cspD sdpC sdpI sboA yjbI ymaD

***Kurthia* sp. str. 11kri321**	
Category	Genes
Cell enveloppe / Membrane	ftsH glmU Gene coding acyltransferase Gene coding SH3-domain protein Gene coding transglycosylase Genes coding transembrane proteins
Motility	fliG
Translation	Several genes
Transcription	Several genes

K. massiliensis	
Category	Genes
Cell enveloppe / Membrane	ftsA

K. senegalensis
No change in gene expression

After manganese supplementation in solid growth conditions, gene expression of the model sporulating *B. subtilis* and of three environmental representatives of the non-model Firmicutes genus *Kurthia* (*Kurthia* sp. str. 11kri321, *K. massiliensis* and *K.senegalensis*) was modified. As a large number of genes were concerned, we only discussed the implication of genes with known function, and those are summarized in this table. The genes are classified according to biological cellular processes in which they are known to be involved.

2.2 Results for *Kurthia* spp.

Exposure to manganese did not result in a common response in the three *Kurthia* species investigated here: *Kurthia* sp. str. 11kri321, *K. massiliensis* and *K. senegalensis* (Fig. 3). In *K. senegalensis*, no effect on cell morphology was observed (Supp. Fig. 1), and accordingly,

manganese only affected the expression of three genes (Supp. Table 3). Concerning *K. massiliensis*, the microscopic pictures clearly showed a division septum in almost all the cells for the manganese-treated cells on solid medium, whereas this was not the case for all the other conditions (Supp. Fig. 1). Accordingly, in *K. massiliensis*, the expression of *ftsA*, a key gene involved in cell division (Errington et al., 2003), was modified when manganese was applied on solid medium (Table 2).

The most striking morphological changes were observed in the case of *Kurthia* sp. str. 11kri321. After 24 h of growth on solid medium and in presence of manganese, a drastic modification in cell morphology was observed for *Kurthia* sp. str. 11kri321, as observed for *B. subtilis*. While cells with a characteristic bacilli shape were observed in most of the culture conditions (Fig. 1E, G and H), the cells shrunk and had a rounder shape when grown on solid medium with manganese (Fig. 1F). This modification was not observed for the two other *Kurthia* species analysed (*K. massiliensis* and *K. senegalensis*) (Supp. Fig. 1). The transcriptomics analysis performed to evaluate the impact of manganese regardless of the media, showed that in response to manganese, 176 genes were down-regulated and only one gene was up-regulated in *Kurthia* sp. str. 11kri321 (Table 1 and Supp. Table 4). If we exclude the genes coding for hypothetical/unknown proteins, more than 70% of the down-regulated genes play a role in important cellular processes such as transcription, translation and energy production (Table 1).

3. Discussion

3.1 Sporulation and additional stress responses in *B. subtilis* under manganese supplementation and solid growth conditions

The clear evidence for sporulation after 24 h on solid medium and in the presence of manganese (Fig. 1B) confirmed previous published results (Charney et al., 1951) indicating that supplementation with manganese in solid medium triggers fast sporulation in *B. subtilis*. Even if the sporulation program is a continuum, it is classically divided into seven major stages (Tan & Ramamurthi, 2014) (Fig. 2A). The sporulation genes with an up-regulated expression in presence of manganese and regardless of the growth medium (*cotY*, *cotX*, *cotV*, *cotG*, *yxeE*, *cgeA*, and *cgeB;* Table 2 and Supp. Table 1) are all known to be involved in the late stage V (Tan & Ramamurthi, 2014). Therefore, our transcriptomic data show that at the time the cells were sampled (24 h of growth), the sporulating cells were already forming the spore's coat, a complex multi-layered structure (Liu et al., 2016; McKenney et al., 2010) (Fig.2B). The expression of the genes *cotX*, *cotY*, *cotV* (which are part of the *cotVWXYZ* cluster), *cotG*, *yxeE*, as well as the expression of the operon *cgeAB* (formed by the two genes *cgeA* and *cgeB*) are jointly controlled by σ^K and GerE (Ducros et al., 2001; Kuwana et al., 2007; Roels & Losick, 1995; Sacco et al., 1995). The genes *cotX*, *cotY*, *cotV* and *cotG* are involved in the formation of the crust (outermost layer of the spore) (Shuster et al., 2019; Zilhao et al., 2004), whereas the gene *yxeE* participates to the formation of the inner spore coat (Kuwana et al., 2007). Finally, the operon *cgeAB* controls the glycosylation of the crust, which may protect this layer against biological degradation and scavenging (Bartels et al., 2019) (Fig. 2B). It was proposed that late

in sporulation the rising level of GerE and the falling level of SpoIIID, together, dictates the timing and level of spore coat protein synthesis, ensuring optimal assembly of the spore's coat (Ichikawa & Kroos, 2000). Therefore, the coordinated gene expression of all above cited genes makes sense at the stage of coat formation, and it is likely under the control of two transcription factors: SpoIIID and GerE, and the RNA polymerase σ^K.

In response to manganese treatment on solid medium, our transcriptomic data highlighted cannibalism, a social behavior known to be important during sporulation (González-Pastor, 2011). Sporulating bacterial populations are heterogenous with two types of cells: sporulating and nonsporulating cells. Sporulating cells are reported to excrete antimicrobial components but also immunity proteins to be protected against these toxic molecules. On the contrary, nonsporulating cells do not express this immunity system and are therefore killed by sporulating siblings. Cell lysis releases nutrients that sporulating cells can use in order to survive until completion of sporulation. The killing of the nonsporulating cells is mediated by the gene cluster *sdp*, for sporulation delaying protein. This *sdp* cluster is formed by the operons *sdpABC* and *sdpRI* and it is transcribed if the master regulator of sporulation Spo0A is expressed. We observed here that exposure to manganese applied in solid growth conditions resulted in the upregulation of two genes coding respectively for the sporulation delaying protein C (SdpC) and the immunity protein SdpI (Fig. 2C, Table 2 and Supp. Table 2) (González-Pastor, 2011). Therefore, the mix population observed (Fig. 1B) appears to have adopted this cannibalistic behaviour with the sporulating cells expressed the killing protein SdpC (and its immunity counterpart SdpI) in order to cannibalise on their nonsporulating siblings. Such cannibalism is also supported by the up-regulation of *sboA,* which codes subtilosin-A, a protein with a bacteriocidal activity against some Gram-positive bacteria, including *Bacillus* species (Babasaki et al., 1985; Huang et al., 2009) (Fig. 2C, Table 2 and Supp. Table 2).

The microscopic observation of endospores (Fig. 1B) and the up-regulation of sporulation-related genes on solid medium but not in liquid medium (Fig. 1A), indicates that manganese alone is not sufficient to induce sporulation. Therefore, other changes associated to growth on solid medium must play also a significant role on sporulation. In liquid culture medium, nutrients and metabolites are more uniformly distributed and growth of microorganisms is typically planktonic. In solid medium, nutrients and metabolites are distributed as patches and microorganisms are immobilized and constrained to form colonies (Koutsoumanis et al., 2004; Wilson et al., 2002). In addition, other factors such as the pressure of oxygen and osmotic pressure, salt and oxidative stress, as well as distance between cells, are not identical in liquid or solid medium (Cuny et al., 2007). All these differences impact microbial behaviour and, for example, it has been shown that bacteria forced to grow as colonies had a different metabolic activity and grow slowly as compared to planktonic cells (Koutsoumanis et al., 2004). Thus, manganese applied in solid medium would act as one of multiple stressor factors triggering sporulation. This hypothesis is supported by the fact that genes involved in a general stress response were also up-regulated only when exposure to manganese happened during growth on solid medium (Fig. 2C, Table 2 and Supp. Table 2). Among those up-regulated stress response genes, two are involved in protection against oxidative stress: *yjbI* and *ymaD* (uniprot.org) (Imai et al., 2021; Prestel et al., 2015). The products of these genes are proteins with peroxidase-like (Imai et al., 2021) and peroxiredoxin-related activity (Prestel et al., 2015),

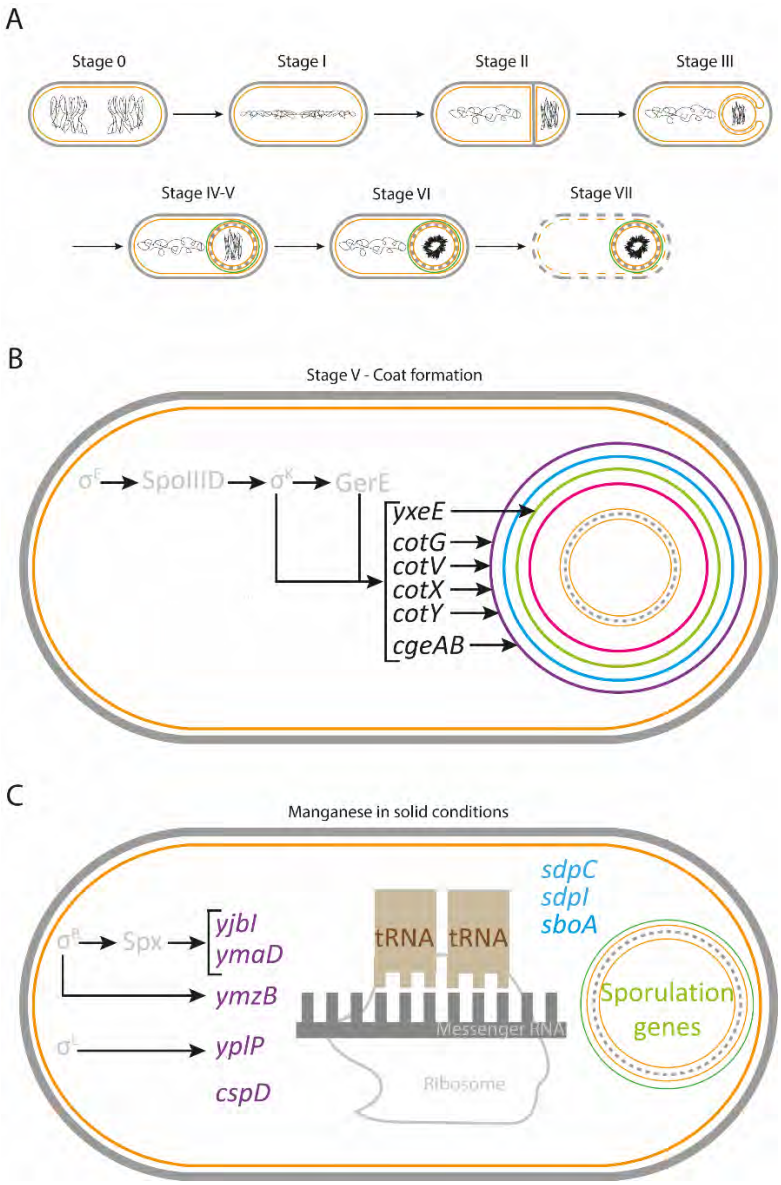

Figure 2: Schematic representation of *Bacillus subtilis* sporulation, of the activation of genes involved in spore coat formation, and of the effect of manganese in solid growth conditions on gene expression. (A) Depiction of the different stages recognized during sporulation. Peptidoglycan layer is shown in gray, membranes in orange, DNA in black, and spore coat in green. Stage 0: chromosome replication. Stage I: chromosome condensation and anchoring of origins of replication to the extreme poles of the cell. Stage II: elaboration of polar septum. Stage III: engulfment of the forespore. Stage IV-V: cortex and coat assembly, respectively. Stage VI: spore maturation. Stage VII: lysis of mother cell and release of the mature, dormant, and highly resistant spore into the environment. Figure modified from Tan & Ramamurthi, 2014. **(B)** Regulatory cascade of some of the genes involved in spore coat formation (stage V). The spore's coat is organized into four distinct layers: basement layer (pink), inner coat (green), outer coat (blue) and crust (purple) (Liu et al., 2016; McKenney et al., 2010). Genes *cotX*, *cotY*, *cotV, cotG* and operon *cgeAB* are involved in crust formation (purple) (Bartels et al., 2019; Shuster et al., 2019; Zilhao et al., 2004). The gene *yxeE* is involved in inner coat formation (green) (Kuwana et al., 2007). **(C)** Other genes up-regulated when manganese was applied in solid growth conditions. Genes highlighted in purple are known to be involved in general stress response; *yjbI* and *ymaD* are involved in protection against oxidative stress, whereas *ymzb* is involved in case of salt stress. *yplP* and *cspD* encode cold shock proteins important in the response to salt stress and during translation process. Sporulation genes (in green), as well as transfer RNA (tRNA; in brown), were also up-regulated. Genes in blue are involved in a cannibalistic behaviour of the sporulating cells toward the nonsporulating cells; *sdpI*, *sdpC* and *sboA*, which code respectively the immunity protein SdpI, the killing protein SdpC and the bactericidal protein Subtilosin-A.

respectively. The upregulation of these genes on solid agar-based medium could be the result of the accumulation of growth inhibitors in agar media such as peroxide and others radicals (Tanaka et al., 2014). Another gene upregulated in solid conditions is *ymzB*, a gene involved in resistance to salt stress (Höper et al., 2005), suggesting a higher osmotic stress under solid growth conditions. This is supported by a previous study showing that genes involved in salt stress response have a higher expression level in cells grown on agar plates as compared to cells grown in liquid medium (Kunst & Rapoport, 1995).

Several cold shock proteins are also known to be induced by salt stress or heat shock (Graumann et al., 1996). Two genes coding for cold shock proteins were up-regulated when manganese treatment was applied in solid growth conditions; *yplP* and *cspD* genes (Fig. 2C, Table 2 and Supp. Table 2). The gene *yplP* is activated by the sigma factor σ^L, which is involved in the regulation of at least two cold shock adaptation pathways in *B. subtilis*. One of them is the σ^L/YplP-dependent pathways that has a probable role in cold shock adaptation (Wiegeshoff et al., 2006). Concerning CspD, it belongs to the CSPD protein family, which has a well-known role in cold adaptation (Graumann et al., 1997). Therefore, we hypothesize that the up-regulation of genes coding cold shock proteins in solid growth conditions is not the result of exposure to cold temperatures, but to salt stress due to growth on agar plates (Kunst & Rapoport, 1995). Moreover, CspD proteins, as well as other cold shock proteins, have been suggested to function as initiator of translation, both at low and optimal growth temperatures (Graumann et al., 1997), and indeed, sporulation requires an important investment of the translation machinery. This is supported by the large fraction of tRNAs that was up-regulated during growth in solid medium. Several studies have reported an increase of the ratio tRNA/rRNA during sporulation (Doi, 1965; Okamoto & Vold, 1992). Moreover, Narasimhan showed that this increase in tRNA synthesis happens in the late stages of sporulation (Narasimhan, 1987), which is coherent with our microscopic observation of an advanced stage of sporulation (endospores inside the mother cells; Fig. 1B) and with the up-regulation of sporulation genes involved in the late sporulation stages (Fig. 2B, Table 2 and Supp. Table 1 and 2).

In addition to the salt and radical stress response suggested by the transcriptomic results, another non-negligible source of stress is the transfer of cells from an overnight liquid culture onto solid medium. Indeed, several relevant parameters change drastically when cells are switched from liquid to solid media, such as (i) an increase in oxygen pressure, (ii) the presence of hydrogen peroxide on the plate, and (iii) an increased distance between cells, forcing bacteria to fight stress as individual cells and not as a population (Cuny et al., 2007). In summary, the transcriptomic response in *B. subtilis* suggests that growth on solid medium generated additional stress conditions, which combined with manganese exposure triggered sporulation. *B. subtilis* is ubiquitous in natural environments and to withstand the encountered challenging conditions, it has developed multiple survival mechanisms such as motility, uptake of exogenous DNA, and biofilm formation (Tan & Ramamurthi, 2014). These responses usually precede sporulation, because of the energetic costs of spore-formation (Ratcliff et al., 2013; Siebring et al., 2014). Also, as commitment to sporulation is irreversible, cells also risk losing opportunities for growth if conditions improve (Lennon & Jones, 2011). Accordingly, sporulating bacteria have been shown to be favoured in habitats with multiple

limiting environmental factors (Fernández-Gómez et al., 2019; Filippidou et al., 2016; Schimel, 2018). No single gene or protein protects against multiple stress factors, and therefore, bacteria have to combined different mechanisms in order to survive (Esbelin et al., 2018). Our results indicate that upregulation of stress response genes might represent a multiple protective strategy triggering sporulation by *B. subtilis* cells grown onto agar plates in presence of manganese.

3.2 Variety of stress responses in *Kurthia* spp. under manganese supplementation and solid growth conditions

In contrast to the common effect of manganese on sporulation reported for multiple *Bacillus* species (Charney et al., 1951; Inaoka et al., 1999; Weinberg, 1964), the morphological observation and transcriptomic response observed in three *Kurthia* spp. investigated here (Fig. 1E to H, Table 2, Supp. Fig. 1 and Supp. Table 3, 4 and 5) indicated the absence of a generalized response in this genus. The three *Kurthia* species are aerobic and have an optimal growth between 30°C and 50°C (Fatton et al., 2022; Roux et al., 2012; Roux et al., 2014). However, the ecology of the three environmental *Kurthia* spp. is expected to be unique and allow survival in their own natural habitat. Both *K. massiliensis* and *K. senegalenesis* were isolated from the faecal flora of a healthy patient (Roux et al., 2012; Roux et al., 2014), whereas *Kurthia* sp. str. 11kri321 is an environmental strain isolated from a geothermal reservoir in Greece (Fatton et al., 2022). Because of their initial ecology, the three *Kurthia* spp. responded differently to the stress encountered in response to manganese. Illustrating the absence of a generalized response, we did not observe any effect of manganese on cell morphology or gene expression in *K. senegalensi*s (Fig. 3A, Table 2, Supp. Fig. 1), whereas manganese had an effect on both cell aspect and gene expression in the two other *Kurthia* species.

Concerning *K. massiliensis*, the microscopic images clearly showed a division septum in almost all the cells for the manganese-treated cells on solid medium, whereas this was not the case for the other conditions (Supp. Fig. 1). During cell division, the Z ring (or septal ring) is located at the division site in the middle of the cell. Later, during septation, this ring contracts and new cytoplasmic membranes and cell wall material are synthesized, which form the division septum. Although formation of the Z ring starts by polymerization of the tubulin-like protein FtsZ, other proteins are also recruited and play various functions in order to complete cell division. Among those proteins, FtsA modulates the assembly state of FtsZ (Ishikawa et al., 2006; Jensen et al., 2005; Weiss, 2004). In presence of manganese in solid conditions, the expression of *ftsA* gene in *K. massiliensis* was downregulated, suggesting a blockage of cell division (Fig. 3B and Table 2). If cell division stopped, the cells could not continue their division beyond the formation of division septum and accordingly, this structure was observable in all cells (Supp. Fig. 1). In *B. subtilis* and *E.coli*, cell division was shown to be inhibited under nutrient-limiting conditions or in case of DNA damage (Adams & Errington, 2009). Thus, in a similar way to *B. subtilis* and *E. coli*, the combination of multiple stress factors generated by the exposure to manganese in solid growth medium blocked cell division in *K. massiliensis*, which might represent an alternative stress response to sporulation as observed in *B. subtilis*.

A very significant shrinking of the cells was observed in *Kurthia* sp. str. 11kri321 in response to manganese addition in solid medium (Fig. 1F). Cell shape modification has been shown to help cells to persist under stressful conditions (Yang et al., 2016), and a smaller cell size is usually adopted under suboptimal conditions of growth (Weart et al., 2007). In addition to size reduction, a transient shutdown of the general translation and mRNA transcription often happen as part of a general stress response (Grigull et al., 2004; Paschen, 2003). Such shutdown was observed here for *Kurthia* sp. str. 11kri321 in response to manganese in solid growth conditions, and not in liquid conditions (Fig. 3C, Table 1 and Supp. Table 5). Same as for sporulation in *B. subtilis*, a stress response appears to be costly for *Kurthia* sp. str. 11kri321 and was therefore only triggered in the case of multiple stress factors (here represented by the combination of manganese and growth in solid medium).

When comparing solid and liquid conditions, multiple genes coding proteins involved in membrane formation or anchored to the membrane had a modified expression. This is not surprising as the membrane is tightly in contact with the cell wall, which is a key feature to determine cell shape (Silhavy et al., 2010), as was observed here in solid conditions (i.e., cells changing from bacilli to a round cell shape; Fig. 1F). For example, a *ftsH* homolog was down regulated in cells of *Kurthia* sp. str. 11kri321 that changed their shape upon manganese exposure in solid growth conditions (Fig. 3C, Table 2 and Supp. Table 5). The gene *ftsH* was shown to be required in *Escherichia coli* for the presence of FtsZ ring at mid-cell site, which is essential during cell division process and cell shape modification (Bhatt, 2013). In addition, *ftsH* gene produces an essential membrane bound ATPase of *E. coli*, which is involved in regulating the biosynthesis of lipopolysaccharides found in the membrane (Katz & Ron, 2008). In addition to conferring shape to bacterial cells, the membrane is also the first site of contact with the surrounding environment (Šajbidor, 1997). Accordingly, external factors affect the order, packing, and membrane phospholipid composition, notably by the activity of acyltransferases that are involved in phospholipid formation (Sastre et al., 2020). In response to the combination of multiple stressors (manganese and solid growth conditions), *glmU* and the predicted gene ASO14_219, both coding for acyltransferase proteins, had a modified expression, which might have impacted the membrane composition of *Kurthia* sp. str. 11kri321 (Zhang et al., 2009) (Fig. 3C, Table 2 and Supp. Table 5). Concerning the impact of external living conditions, proteins with an SH3 domain have a predominant role as this domain mediates protein binding to cell wall (Desvaux et al., 2006). Here, one protein containing an SH3 domain was differentially expressed in *Kurthia* sp. str. 11kri321 in response to manganese applied in solid medium (Fig. 3C, Table 2 and Supp. Table 5). Also, the expression of the predicted gene ASO14_783, coding for a transglycosylase, was modified in response to manganese applied onto agar plates, supporting a modification at the cell envelope level (Fig. 3C, Table 2 and Supp. Table 5). Transglycosylases are multidomain membrane-bound proteins important for cell envelope synthesis by the polymerization of biochemical intermediates into peptidoglycan (Goldman & Gange, 2000). Finally, membrane curvature was shown to modulate distribution of many transmembrane proteins, which function as gateways to allow transport of molecules across the cell membrane (Aimon et al., 2014). Accordingly, the cell shape modification observed in solid conditions for *Kurthia* sp. str. 11kri321 (Fig. 1F) impacted the expression of several transmembrane proteins (AMA61987.1,

AMA62050.1, AMA63703.1, AMA62887.1 and AMA63901.1; Fig. 3C, Table 2 and Supp. Table 5).

One well-known difference among liquid and solid growth conditions is bacterial motility. In liquid environment, individual cells swim, whereas migration onto solid surfaces is generally achieved through swarming, a multicellular movement of bacteria (Calvio et al., 2005; Kearns, 2010). Therefore, it is not surprising that in *Kurthia* sp. str. 11kri321, the predicted gene responsible for the production of flagellin protein (ASO14_2420) and *fliG* (coding for flagellar motor switch protein; Fig. 3C, Table 2 and Supp. Table 5), were both up-regulated in liquid condition. In fact, the two mentioned proteins are involved in flagellum formation and thus in motility (uniprot.org).

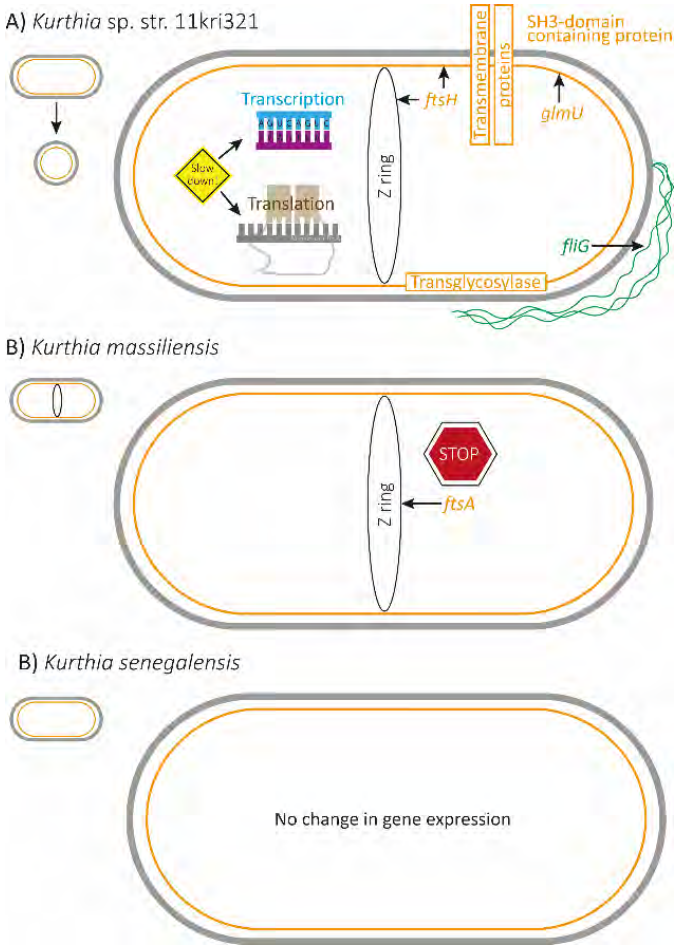

Figure 3: Schematic representation of the effect of manganese in solid growth conditions on the gene expression of *Kurthia* spp. (A) In presence of manganese in solid growth conditions, *Kurthia* sp. str. 11kri321 responded by a modification of cell shape, switching from bacilli to round cells. Accordingly, expression of genes impacting the membrane (*glmU* and *ftsH*), the cell division (*ftsH*) or coding proteins that affect the cell envelope (transmembrane proteins, transglycosylase, and SH3-domain containing protein) (in orange), was modified. In addition, this strain responded by a transient shutdown of transcription (in blue) and translation (in brown) processes. Finally, due to solid growth conditions, *Kurthia* sp. str. 111kri321 down-regulated the expression of *fliG* gene that is involved in flagellar motility. **(B)** *K. massiliensis* responded by a blockage of cell division. Accordingly, a division septum was observed in almost all cells, whereas it was not the case in the other growth conditions. In addition, *ftsA*, which is involved in cell division process, had a down-regulated expression. **(C)** *K. senegalensis* neither changed cell shape nor its gene expression in response to manganese supplementation in solid growth conditions.

4. Conclusion

Our transcriptomic study of the response of *B. subtilis* to manganese exposure highlights that the combination of different factors is necessary to make manganese a sporulation inducer. Manganese alone was not sufficient as a fast trigger of sporulation. Only when manganese was applied on the correct media (here agar-based solid medium), we observed the production of endospores and accordingly, the up-regulation of multiple sporulation genes, but also genes related to a general stress response and cannibalism to support sporulation. Therefore, when the appropriate combination of factors is present, manganese is a sporulation inducer and accordingly, this metal can be considered as a stress factor that is part of a multi-faceted stress response in bacteria. We also show that this response differed between model and non-model bacteria. *Kurthia* sp. str. 11kri321, an environmental representative of the non-model Firmicutes genus *Kurthia*, responded to manganese exposure in solid medium through a morphological change and a shutdown of translation/transcription processes. These two changes are known to occur under stress conditions (Grigull et al., 2004; Paschen, 2003; Weart et al., 2007; Yang et al., 2016). Likewise, *K. massiliensis* responded to manganese exposure by a blockage of cell division, which is also known as a stress response (Adams & Errington, 2009). Finally, both gene expression and cell morphology were unmodified in *K. senegalensis*. Overall, our study provides transcriptomic data to support that manganese can be a trigger of sporulation as part of a multi-stress response in bacteria. We highlight the contrasting behaviour of model and non-model species in response to environmental stressors. Finally, we confirmed that the numerous parameters differing in liquid or solid growth medium (Cuny et al., 2007; Koutsoumanis et al., 2004; Wilson et al., 2002) severely impacting key physiological processes, both in *B. subtilis* and *Kurthia* spp.

5. Material and methods

5.1 Bacterial species and pre-culture conditions

The following species were used in this study. The sporulating model *Bacillus subtilis* (NEU16), was obtained from our culture collection. *Kurthia* sp. str. 11kri321 (NEU1325), is an environmental strain that was initially isolated from an oligotrophic geothermal reservoir in Krinides, Greece (Fatton et al., 2022). In addition, two other representatives of the genus *Kurthia* were purchased from the DSMZ: *K. massiliensis* (DSM 24639) and *K. senegalensis* (DSM 24641). All the species were reactivated from frozen stocks in our culture collection by culturing overnight (approximately 18 h) into 5 mL liquid nutrient broth (NB; Carl Roth, Germany) at 30°C under 115 rpm agitation; this constituted the pre-cultures used for further experiments (Fig. 4A)

5.2 Manganese treatment

In order to investigate the effect of manganese supplementation and of the type of growth medium, pre-cultures (see above) were used to prepare triplicates for each experimental condition (Fig. 4B). After bacterial inoculation, nutrient agar plates (NA; NB supplemented

with 1.5 g/L technical agar; Biolife, Italy) and nutrient broth samples (representing solid and liquid growth conditions, respectively) were either directly incubated (control) or supplemented with manganese (treatment). For the manganese supplementation in solid medium, drops of 20 µL were deposited onto the agar plates (solid conditions) using a $MnSO_4$ solution at 1000 mg/L. For supplementation of the liquid medium, 50 µL were added in the liquid cultures (final concentration at 10 mg/L). All the cultures were incubated 24 h at 30°C, and under 115 rpm agitation for the liquid ones (Fig. 4B).

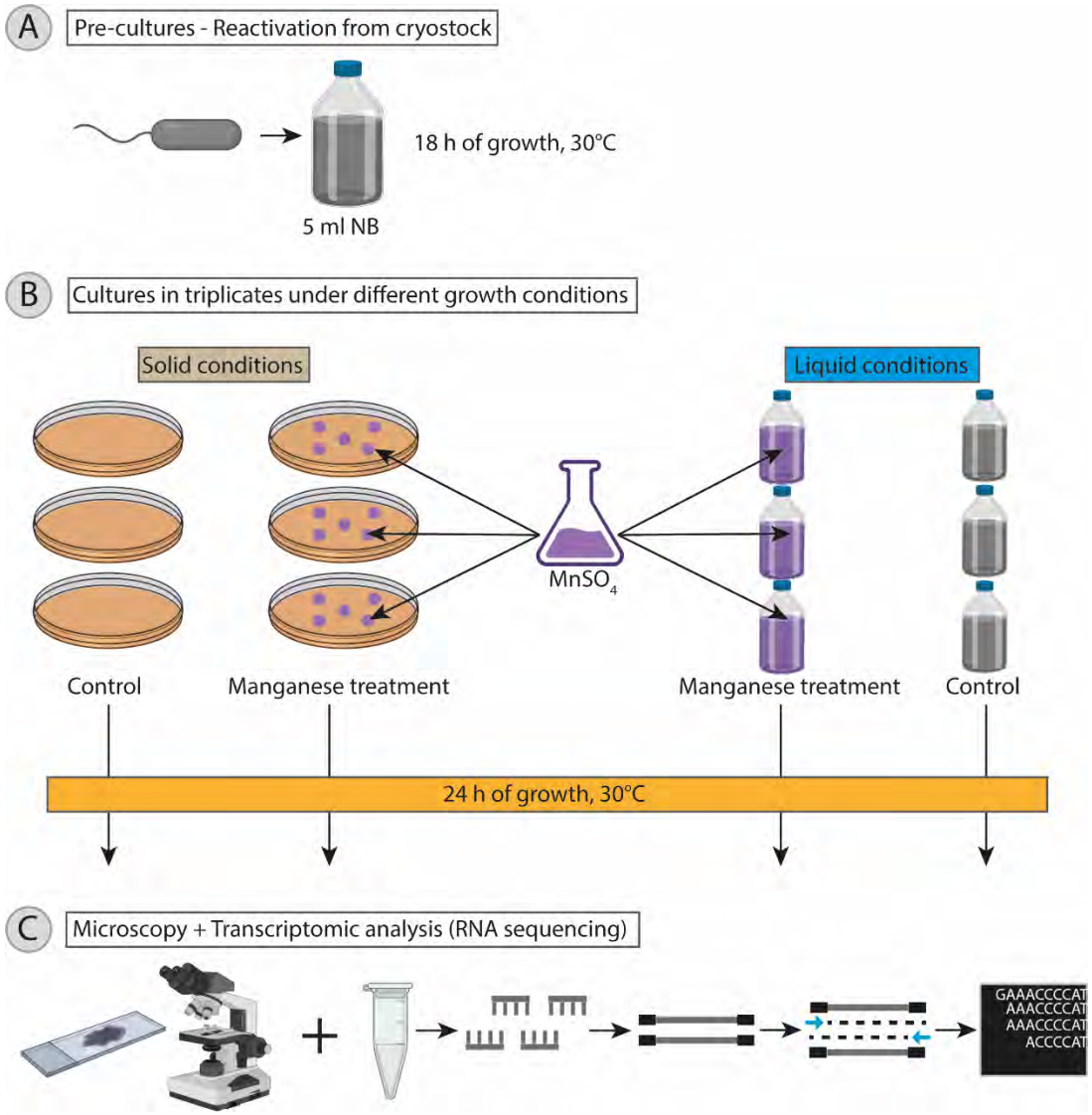

Figure 4: Experimental procedure to investigate the effect of manganese supplementation and type of growth medium on morphology and gene expression of bacterial species. (1) Starting with frozen stocks, bacterial species were reactivated in 5 mL NB for 18 h at 30°C. **(2)** Using the precultures, triplicates for each experimental condition were prepared. After bacterial inoculation, nutrient agar plates and nutrient broths (representing solid and liquid growth conditions, respectively) were either directly incubated (control) or supplemented with manganese (treatment). All the cultures were incubated 24 h at 30°C. **(3)** Cells collected from each culture were observed using optical microscopy (morphology investigation). In addition, RNA extraction was performed in order to do then RNA sequencing (gene expression investigation). Illustration made with BioRender and Adobe Illustrator.

5.3 Microscopy analysis

From the liquid cultures, 3 µL were pipetted and deposited onto an agar pad (1.2% agar; Biolife, Italy) moulded on a slide for microscopy. The samples were covered with a cover slip. For the solid cultures, we used a plastic loop to scratch cells onto the agar surface and resuspend them into 1 mL physiological water (0.9% NaCl); concerning the manganese treatment, the cells were carefully scratched in the zones where drops of manganese were deposited at the time of inoculation. From these bacterial suspensions, 3 µL were pipetted and deposited onto an agar pad. Observation and image acquisition were performed using a light-transmitted optical microscope (Leica DM4B) equipped with a camera (Leica DFC7000 T).

5.4 Transcriptomic analysis

5.4.1 Samples preparation

Liquid cultures were centrifugated at max speed (6461 g) for 1 min and the supernatant was removed. The pellet was resuspended in 1 mL physiological water (0.9% NaCl) and centrifugated a second time (washing step). After removal of the supernatant, the pellet was frozen at -80°C until we performed the RNA extraction. For the solid cultures, cells were scratched onto the agar surface after 24 h of growth and RNA extraction was directly performed; concerning the manganese treatment, the cells were carefully scratched in the zones where drops of manganese were deposited at the time of inoculation.

5.4.2 RNA extraction

RNA extraction was performed using the Quick-RNA Fungal/Bacterial Microprep™ kit (ZymoResearch, USA). Frozen pellets (corresponding to liquid cultures) and scratched cells (corresponding to solid cultures) were resuspended in 800 µL of RNA lysis buffer from the kit. At the end of the extraction, RNA extracts were resuspended in PCR-grade water. RNA quantification was performed using the Qubit® dsRNA HS Assay Kit on a Qubit® 2.0 Fluorometer (Invitrogen, Carlsbad, CA, USA) and purity of the RNA extracts was assessed using a NanoDrop spectrophotometer (Witech AG, Sürsee, CH).

5.4.3 RNA sequencing

After ribosomal RNA depletion, total RNA sequencing was performed by Fasteris SA (Geneva, Switzerland), using the Illumina NovaSeq platform (Illumina, San Diego, USA). Between 25 and 40 million 50 bp pair-ended reads were obtained per sample. Reads were filtered, trimmed of the first, low-quality, 5 bps and searched for residual adapter sequences using *fastp* (v. 0.20.0) (Chen et al., 2018). Remaining reads were mapped onto the *B. subtilis* (GCF_000009045.1), *Kurthia* sp. str. 11kri321 (GCA_001534765.1), *K. massiliensis* (GCF_000285555.1) or *K. senegalensis* (GCF_000285595.1) genomes using *bowtie2* (v. 2.3.4.3) (Langmead & Salzberg, 2012). Reads were counted using *featureCounts* (v. 2.0.0) (10.1093/bioinformatics/btt656) with default count parameters. Analysis was performed in R (v. 4.0.0) (Team, 2013) using the *DESeq2* (v. 1.28.1) package (Love et al., 2014) after removing highly expressed ribosomal RNAs and low-expression genes (genes retained had at least 12 reads in five or more samples out

of six); the statistical model used for analysis included both media (liquid vs solid) and manganese (with vs without) as independent variables.

5.4.4 Analysis of RNA data

Genes with an adjusted *p*-value < 0.05 and log2 fold-difference > 1 or <-1 were considered as significantly modified. The proteins encoded by those genes and the biological processes in which they are known to be involved were identified using the UniProt plateform (uniprot.org). The genes coding for hypothetical proteins or for which the function is unknown were classified together in a category called “others”. We complied all the information in tables and supplementary tables. All the tables and supplementary tables present transcriptomic data for (1) cultures supplemented or not with manganese, regardless the growth medium, and (2) for supplemented cultures in liquid and solid growth medium.

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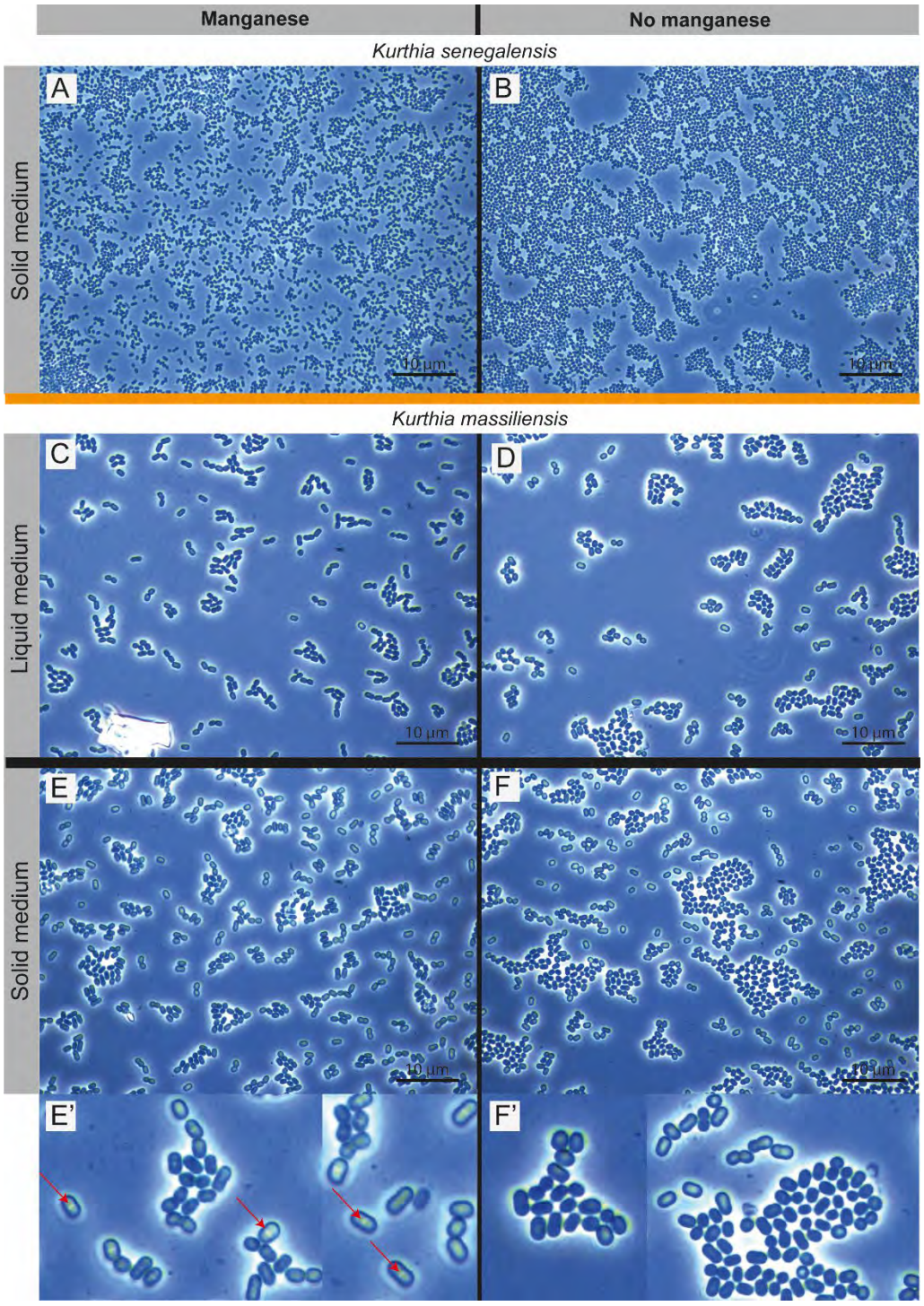
Supplementary material



Supplementary Figure 1: Microscopic observation of *K. senegalensis* and *K. massiliensis* cells after 24 h incubation at 30°C, comparing manganese supplementation and no supplementation and type of growth medium. The cell morphology of *K. senegalensis* did not change according to presence/absence of manganese (**A and B** respectively). For *K. massiliensis*, the cell morphology looked similar in all conditions, but we observed a division septum in almost all manganese-treated cells in solid medium (**E and E'** with red arrows highlighting some septum), whereas this was not the case for all the other conditions (**C, D, F and F'**).

Supplementary Table 1. Genes identified in *Bacillus subtilis* with a significant modified expression according to manganese supplementation

Upregulated in presence of manganese							
Category	**Sub-category**	**Tag**	**Gene's name**	**Protein's name**	**Role**	**Adjusted *p* -value**	**Log2 (fold-change)**
Sporulation							
Sporulation		BSU_11750	cotY	Spore coat protein Y		0.0416	2.7081
		BSU_11760	cotX	Spore coat protein X		0.0313	3.2942
		BSU_11780	cotV	Spore coat protein V	Spore coat formation	0.0416	3.0034
		BSU_36070	cotG	Spore coat protein G		0.0313	3.808
		BSU_39580	yxeE	Protein YxeE		0.0416	2.7647
		BSU_19780	cgeA	Protein CgeA	Maturation of the outermost spore layer	0.0416	3.0626
		BSU_19790	cgeB	Protein CgeB		0.0416	2.8287
Others (including genes coding for hypothetical or unknown protein)							
Others				2 proteins			
TOTAL							**9 upregulated genes**

Downregulated in presence of manganese					
Category	**Tag**		**Name**	**Role**	
General metabolism					
General metabolism	BSU_36000	alsD	Alpha-acetolactate decarboxylase	Converts acetolactate into acetoin, may be a mechanism to control internal pH of cells	0.0416	-2.0944
Membrane					
Membrane	BSU_26650	czcD	Cadmium, cobalt and zinc/H(+)-K(+) antiporter	Ion transmembrane transporter	0.0313	-2.1752
Stress response					
Stress response	BSU_13040	hmp	Flavohemoprotein	Flavohemoglobin protein (induced in presence of reactive nitrogen and oxygen species)	0.0313	-2.6366
TOTAL					**3 downregulated genes**

Using the UniProt plateform (uniprot.org), we classified the genes with a significant up- or down-regulated expression (padj <0.05; log2FoldChange >1 or <-1) in category corresponding to the biological process they are known to be involved. The genes coding for hypothetical proteins or for which the function is still unknown were classified together in a category called "others". Gene expression was analysed by comparing simply the impact of manganese supplementation compared to no supplementation regardless the growth medium (liquid or solid).

Supplementary Table 2. Genes identified in *Bacillus subtilis* with a significant modified expression according to manganese supplementation in liquid or solid growth medium.

Upregulated in liquid conditions							
Category	**Sub-category**	**Tag**	**Gene's name**	**Protein's name**	**Role**	**Adjusted *p* -value**	**Log2 (fold-change)**
Growth (sporulation)							
Growth (sporulation)		BSU_33910	gpmI	2,3-bisphosphoglycerate-independent phosphoglycerate mutase	Interconversion of 2-phosphoglycerate and 3-phosphoglycerate (essential for sporulation and rapid growth)	0.0019	2.9673
		BSU_38170	qoxA	Quinol oxidase subunit 2	ATP synthesis coupled electron transport (major component for energy conversion during vegetative growth)	1.11E-06	3.4847
General metabolism							
General metabolism		BSU_02140	glpT	Glycerol-3-phosphate transporter	Glycerol metabolic process (important for lipids)	0.0029	2.0984
		BSU_04050	pxpA	5-oxoprolinase subunit A	Role in catabolism of glutamate and carbohydrates (both are essential to many metabolic processes)	0.0013	1.4649
		BSU_04070	ycsI	Hydro-lyase YcsI	Catalysis the cleavage of C-C, C-O, C-N and other bonds by other means than by hydrolysis or oxidation, or conversely adding a group to a double bond	0.0001	2.2226
		BSU_04340	ydaP	Putative thiamine pyrophosphate-containing protein YdaP	Catalytic activity, magnesium ion binding and thiamine pyrophosphate binding	0.0027	1.4468
		BSU_07350	yfmT	Benzaldehyde dehydrogenase YfmT	Converts vanillin to vanillic acid	4.05E-04	2.9830
		BSU_36710	fdhD	Sulfur carrier protein FdhD	Mo-molybdopterin cofactor biosynthetic process (molybdenum forms the catalytic centre of various enzymes)	5.22E-05	1.8180
		BSU_37150	pyrG	CTP synthase	CTP biosynthetic process (involved in pyrimidine metabolism)	0.0021	1.8389
		BSU_37490	speB	Agmatinase	Catalyses formation of putrescines from agmatine (amine and polyamine biosynthesis)	8.66E-05	2.0976
		BSU_37960	ywdH	Putative aldehyde dehydrogenase YwdH	Aldehyde dehydrogenase	0.0032	1.8162
		BSU_38340	ywbF	Transporter YwbF	Lactose symporter activity (organic substance transport)	0.0010	1.2269

General metabolism		BSU_40080	gntZ	6-phosphogluconate dehydrogenase, NAD(+)-dependent, decarboxylating	Gluconate utilization (functional role remains obscure)	1.53E-05	2.0138
		BSU_40320	rocF	Arginase	Arginine metabolic process	0.0020	1.5244
	Co-factor biosynthesis	BSU_38290	thiE	Thiamine-phosphate synthase	Thiamine diphosphate biosynthetic process (co-factor biosynthesis)	0.0010	1.9821
		BSU_38300	thiM	Hydroxyethylthiazole kinase		0.0011	2.0866
	Purine metabolism (purines= metabolic signal, provide energy and others processes)	BSU_06420	purE	N5-carboxyaminoimidazole ribonucleotide mutase	IMP biosynthetic process	0.0035	1.3529
Energy production							
Energy production		BSU_02840	ycdG	Probable oligo-1,6-glucosidase 2	Oligosaccharides catabolic process (source of carbohydrates)	0.0007	1.4614
		BSU_02860	znuC	High-affinity zinc uptake system ATP-binding protein ZnuC	ABC-type zinc transporter activity and ATPase-coupled transmembrane transporter	0.0006	1.3628
		BSU_03050	ldh	L-lactate dehydrogenase	Conversion of lactate to pyruvate (part of fermentation and glycolytic process)	1.87E-05	4.1324
		BSU_14590	pdhB	Pyruvate dehydrogenase E1 component subunit beta	Conversion of pyruvate to acetyl-CoA (glycolytic process)	0.0042	2.3755
		BSU_33920	tpiA	Triosephosphate isomerase	Involved in gluconeogenesis	0.0035	2.7592
		BSU_33940	gapA	Glyceraldehyde-3-phosphate dehydrogenase 1	Oxidative phosphorylation (involved in glycolysis)	0.0022	3.1472
		BSU_35010	nagA	N-acetylglucosamine-6-phosphate deacetylase	Biosynthesis of amino-sugar-nucleotides (carbohydrates metabolic process)	0.0034	1.1683
		BSU_39670	iolJ	6-phospho-5-dehydro-2-deoxy-D-gluconate aldolase	Fructose 1,6-bisphosphate metabolic process, glycolytic process	0.0016	2.0505
		BSU_40060	gntK	Gluconokinase	D-gluconate catabolic process (carbohydrate acid metabolism)	0.0008	1.9523
		BSU_40070	gntP	Gluconate permease		0.0045	1.9535
Transcription							
Transcription		BSU_01060	ybxB	Uncharacterized protein YbxB	Methylation, post-transcriptional RNA modification	8.78E-05	2.1443
		BSU_38310	ywbI	Uncharacterized HTH-type transcriptional regulator YwbI	DNA-binding transcription factor activity	0.0015	1.5970
		BSU_38910	yxjL	Uncharacterized transcriptional regulatory protein YxjL	Regulation of transcription	0.0027	1.4576
		BSU_40350	rocR	Transcriptional activator RocR	Transcriptional regulator of operons rocABC and rocDEF (arginine)	5.88E-05	1.2246
	Nucleotides synthesis	BSU_00510	prs	Ribose-phosphate pyrophosphokinase	Formation of nucleotides	5.19E-05	2.0506
Translation							
Translation		BSU_01389	ybzG	Putative ribosome-binding protein YbzG	Initiation of translation (binds mRNA molecules to the ribosomes)	0.0003	2.6851
		BSU_01390	infA	Translation initiation factor IF-1	One of the essential components for the initiation of protein synthesis	0.0027	2.6843
		BSU_03490	srfAB	Surfactin synthase subunit 2	Multifunctional enzyme (polymerise amino acids Leu, Glu, Asp and Val and antibiotic biosynthesis)	0.0016	2.2564
		BSU_04610	ydcA	Putative rhomboid protease YdcA	Serine-type endopeptidase (breaks peptides in smaller units)	0.0040	1.2466
		BSU_34860	hisI	Histidine biosynthesis bifunctional protein HisIE	Histidine biosynthetic process (amino acids synthesis)	0.0043	1.8072
		BSU_34910	hisD	Histidinol dehydrogenase		0.0001	1.7739
		BSU_39480	yxeO	Probable amino-acid import ATP-binding protein YxeO	ABC transporter involved in amino acids import	0.0020	2.1796
		BSU_39510	scmL	S-(2-succino)cysteine N-acetyltransferase	Cysteine biosynthetic process	3.67E-05	2.0213
	Ribosomal proteins	BSU_01050	rplL	50S ribosomal protein L7/L12		0.0036	2.4579
		BSU_01220	rpsC	30S ribosomal protein S3		0.0047	2.2177
		BSU_01230	rplP	50S ribosomal protein L16		0.0018	2.7730
		BSU_01240	rpmC	50S ribosomal protein L29		0.0021	3.0410
		BSU_01300	rpsH	30S ribosomal protein S8		0.0004	2.4451
		BSU_01310	rplF	50S ribosomal protein L6		0.0015	2.3183
		BSU_01340	rpmD	50S ribosomal protein L30		0.0045	2.3611
	tRNA (adapters	BSU_37330	argS	Arginine--tRNA ligase	Arginyl-tRNA aminoacylation (protein biosynthesis)	0.0019	1.3408
Membrane							
Membrane		BSU_38530	dltD	Protein DltD	D-alanylation of lipoteichoic acid (lipoteichoic acid biosynthesis, part of cell wall biogenesis)	0.0005	2.2005
		BSU_38540	dltE	Uncharacterized oxidoreductase DltE	D-alanine transfer (biosynthesis of techoic acid)	0.0022	1.6972

Membrane	Transmembrane transport	BSU_02400	ybgF	Uncharacterized amino acid permease YbgF	Amino acid transmembrane transport	0.0027	1.5218
		BSU_37230	ywjA	Uncharacterized ABC transporter ATP-binding protein YwjA	Transmembrane transporter (for lipids)	0.0027	1.5218
		BSU_38260	efeN	Deferrochelatase/peroxidase EfeN	Iron import into cells	0.0002	2.2115
		BSU_38280	efeU	Ferrous iron permease EfeU	Uptake of Fe2+ across membrane, iron homeostasis	7.15E-06	1.9383
		BSU_39710	iolF	Minor myo-inositol transporter IolF	Transmembrane transporter activity	0.0007	1.8821
Stress response							
Stress response		BSU_02260	ybfK	Carboxylesterase YbfK	Carboxylesterase activity (detoxify xenobiotics)	0.0032	1.2633
		BSU_02670	lmrB	Lincomycin resistance protein LmrB	Transmembrane transporter activity and response to antibiotics	0.0004	1.4890
		BSU_02890	yceC	Stress response protein SCP2	Salt and cold stress induced protein	0.0006	2.2856
		BSU_02910	yceE	Uncharacterized protein YceE	Survival of ethanol stress and low temperature	0.0008	2.9273
		BSU_02920	yceF	Uncharacterized membrane protein YceF	Resistance to Mn^{2+} intoxication	0.0048	2.3702
		BSU_04600	ydbT	UPF0699 transmembrane protein YdbT	Resistance against antmicrobial compounds	0.0010	2.0419
		BSU_36930	ywlE	Protein-arginine-phosphatase	Protein tyrosine phosphatase activity (general stress response)	0.0002	1.9832
		BSU_37410	albE	Antilisterial bacteriocin subtilosin biosynthesis protein AlbE	Involved in the production of the bacteriocin subtilosin (bacteriocin biosynthetic process)	0.0015	1.6454
		BSU_39650	yxdK	Sensor histidine kinase YxdK	Member of the two-component regulatory system YxdK/YxdJ (may activate YxdJ in response to the antibacterial protein)	3.94E-05	1.0601
	Environmental changes	BSU_31260	mcpB	Methyl-accepting chemotaxis protein McpB	Respond to changes in the concentration of attractants and repellents in the environment (chemotaxis, signal transduction)	0.0008	3.0913
	Oxidative stress	BSU_00710	hslO	33 kDa chaperonin	Protect both thermally unfolding and oxidatively damaged proteins from irreversible aggregation (defense against oxidative stress)	0.0037	1.3545
		BSU_36130	ywrA	Un characterized transporter YwrA	Chromate transmembrane transporter activity (chromate detoxification)	0.0003	2.1555
Motility							
Motility		BSU_35360	hag	Flagellin	Subunit protein which polymerizes to form the filaments of bacterial flagella	2.31E-10	5.2800
Others (including genes coding for hypothetical or unknown protein)							
Others				6 proteins			
TOTAL							**74 upregulated genes**

Downregulated in liquid conditions

Category	Sub-category	Tag	Gene's name	Protein's name	Role	Adjusted *p* -value	Log2 (fold-change)
Sporulation							
Sporulation		BSU_00160	sleL	Cortical fragment-lytic enzyme	N-acetylglucosaminidase (cortex peptidoglycan degradation during germination)	0.0036	-3.2219
		BSU_00240	csfB	Anti-sigma-G factor Gin	Prevents premature activation of Sigma G factor in forespore	7.26E-07	-5.0998
		BSU_01570	pdaB	Probable polysaccharide deacetylase PdaB	Maintain spores and involved in cortex formation	0.0007	-2.6837
		BSU_03789	yczN	Uncharacterized membrane protein YczN	Putative spore germination protein)	6.90E-07	-5.9568
		BSU_06300	cotA	Laccase, spore coat protein A	Involved in brown pigmentation during sporogenesis	0.0003	-4.2131
		BSU_08779	ygzC	Spore coat protein F-like protein YgzC	Spore coat protein (sporulation resulting in formation of a cellular spore)	2.05E-06	-1.9642
		BSU_08980	yhbH	Stress response protein YhbH	Sporulation (stress response)	0.0021	-2.9965
		BSU_09150	yhcN	Probable spore germination lipoprotein YhcN	Contributes to early event in germination and role in spore outgrowth	8.96E-06	-4.7507
		BSU_09400	spoVR	Stage V sporulation protein R	Spore cortex formation	0.0009	-2.7177
		BSU_09750	sspB	Small acid-soluble spore protein B	Protects spore DNA (binds to it) and confers high resistance to spores	1.59E-05	-5.2706
		BSU_09770	yheD	Endospore coat-associated protein YheD	Spore protection (ATP-binding spore coat protein)	0.0015	-3.1687
		BSU_09940	yhaL	Sporulation protein YhaL	Required for efficient sporulation	7.08E-07	-4.1175
		BSU_09958	sscA	Small spore coat assembly protein A	Assembly of several components of the spore coat and spore germination	0.0001	-5.6325
		BSU_09959	sscB	Small spore coat assembly protein B		4.07E-05	-3.3844
		BSU_10730	yisI	Aspartyl-phosphate phosphatase YisI	Dephosphorylates Spo0A-P and negatively regulates the sporulation initiation (control proper timing of sporulation)	3.36E-05	-3.8313
		BSU_17920	ynzD	Aspartyl-phosphate phosphatase YnzD		0.0006	-2.2367

Sporulation	BSU_11730	cotO	Spore coat protein O	Assembly of coat proteins, required for appearance of a morphologically normal outer coat	3.27E-06	-4.5904
	BSU_11740	cotZ	Spore coat protein Z		2.68E-05	-6.3353
	BSU_11750	cotY	Spore coat protein Y		2.82E-14	-8.2621
	BSU_11760	cotX	Spore coat protein X	Spore crust assembly (spore resistance)	2.60E-21	-10.0036
	BSU_11770	cotW	Spore coat protein W		1.81E-19	-9.6261
	BSU_11780	cotV	Spore coat protein V		1.81E-19	-9.6078
	BSU_11790	yjcA	Sporulation protein YjcA	Involved in sporulation	7.65E-06	-4.6812
	BSU_11809	yjcZ	Sporulation protein YjcZ		6.53E-13	-7.0613
	BSU_11810	spoVIF	Sporulation-specific transcription factor SpoVIF	Transcription factor involved in spore coat assembly and spore resistance	8.20E-06	-6.2902
	BSU_12090	cotT	Spore coat protein T	Inner spore coat protein, plays a role in germination	2.30E-14	-8.6422
	BSU_12430	rapA	Response regulator aspartate phosphatase A	Prevents sporulation by dephosphorylating Spo0F	8.27E-11	-8.2259
	BSU_12440	phrA	Phosphatase RapA inhibitor	Controls sporulation initiation (inhibits activity of phosphatase RapA)	6.09E-05	-4.0243
	BSU_13980	pbpH	Penicillin-binding protein H	Polymerization of peptidoglycan, determines rod-shape of spore and growing cells!	0.0043	-1.9345
	BSU_14120	ykzF	Uncharacterized protein YkzF	Inhibits AbrB (controls expression of genes involved in starvation-induced processes)	0.0035	-2.5750
	BSU_14250	yknT	Sporulation protein YknT	Protection of spore (spore coat protein)	2.10E-10	-5.9930
	BSU_14800	ylaJ	Probable spore germination lipoprotein YlaJ	Contributes to early event in germination	0.0007	-2.9626
	BSU_14970	ylbD	Uncharacterized protein YlbD	Protection of spore (spore coat protein)	0.0004	-3.6976
	BSU_15810	spoVM	Stage V sporulation protein M	Coordinates coat and cortex spore assembly	0.0036	-2.5806
	BSU_16740	dpaB	Dipicolinate synthase subunit B	Conversion of dihydrodipicolinate to dipicolinate (DPA), which constitutes up to 10% of the dry weight of the spore	0.0016	-3.0358
	BSU_16980	spoVS	Stage V sporulation protein S	Induced early in sporulation under the control of sigma-H. Play a positive role in allowing cells to progress beyond stage V of sporulation.	0.0046	-2.9208
	BSU_17310	ymaG	Uncharacterized protein YmaG	Protection of spore (spore coat protein)	0.0033	-3.6649
	BSU_17410	cwlC	Sporulation-specific N-acetylmuramoyl-L-alanine amidase	Involved in sporulation (lyze the mother cell wall peptidoglycan) but also in general processes (cell separation, cell-wall turnover)	0.0001	-3.9897
	BSU_17700	cotC	Spore coat protein C	Protection of spore (spore coat protein)	3.44E-06	-7.1645
	BSU_17740	ynzB	Uncharacterized protein YnzB	Involved in spore germination	7.69E-06	-2.8814
	BSU_17900	sirA	Sporulation inhibitor of replication protein SirA	Inhibits DNA replication in growing cells and therfore promotes sporulation	0.0037	-1.8488
	BSU_17930	ccdA	Cytochrome c-type biogenesis protein CcdA	Required for cytochrome c synthesis and stage V of sporulation	5.79E-09	-2.3743
	BSU_17970	cotM	Spore coat protein M	Involved in spore outer coat assembly (stability to final structure)	1.55E-09	-6.3453
	BSU_19110	yobW	Protein YobW	Sporulation membrane protein	0.0026	-2.7826
	BSU_19490	gerT	Spore germination protein GerT	Involved in early stages of spore germination	0.0019	-4.7658
	BSU_19750	cgeE	Uncharacterized N-acetyltransferase CgeE		0.0004	-1.5212
	BSU_19760	cgeD	Protein CgeD	Maturation of outermost spore layer	0.0022	-1.5362
	BSU_19770	cgeC	Protein CgeC		0.0026	-2.1762
	BSU_19780	cgeA	Protein CgeA		1.62E-08	-7.2465
	BSU_19790	cgeB	Protein CgeB		1.13E-11	-6.8458
	BSU_22000	sspL	Small acid-soluble spore protein L	Protects spore DNA (binds to it) and confers high resistance to spores	0.0001	-4.0939
	BSU_22200	cotD	Spore coat protein D	Resistance of spore	2.06E-17	-9.8953
	BSU_22250	yppG	Uncharacterized protein YppG	Resistance of spore	0.0002	-3.2103
	BSU_22290	sspM	Small acid-soluble spore protein M	Protects spore DNA (binds to it) and confers high resistance to spores	2.32E-06	-3.3990
	BSU_22520	ypjB	Uncharacterized protein YpjB	Sporulation protein (part of sigma-E regulon, expression regulation in mother cell)	0.0048	-1.0660
	BSU_22800	spoIVA	Stage IV sporulation protein A	Role at an early stage in the morphogenesis of the spore coat outer layers (proper assembly of the spore coat)	5.94E-05	-4.3828
	BSU_23460	spoIIAB	Anti-sigma F factor	Regulation of sporulation	0.0016	-2.0938
	BSU_23470	spoIIAA	Anti-sigma F factor antagonist	Counteracts SpoIIAB and thus releases sigma f from inhibition (timing of sporulation)	0.0003	-2.4378
	BSU_24230	spoIVB	SpoIVB peptidase	Central role in sigma-K checkpoint (coordinates genes expression during later stages of sporulation)	0.0007	-3.3257
	BSU_24370	spoIIIAG	Stage III sporulation protein AG		0.0002	-2.5218
	BSU_24380	spoIIIAF	Stage III sporulation protein AF	Activation of SigG	0.0008	-2.6119
	BSU_24660	ykzE	Uncharacterized protein YqzE		0.0005	-2.5047
	BSU_24870	gluP	Rhomboid protease GluP	Serine-type endopeptidase (intramembrane proteolysis= important for normal cell division and sporulation)	2.53E-05	-1.2782
	BSU_25840	phrE	Phosphatase RapE inhibitor	Involved in sporulation initiation	0.0005	-3.9979

Sporulation	BSU_27840	safA	SpoIVD-associated factor A	Early stage of spore coat assembly (assembly of CotG). Both lysozyme resistance and germination.	6.53E-06	-5.2916
	BSU_28110	spoVID	Stage VI sporulation protein D	Assembly of spore coat (component of the innermost layer)	2.63E-05	-4.4491
	BSU_28380	gerM	Spore germination protein GerM	Affects both sporulation and germination	0.0035	-2.1430
	BSU_28410	gerE	Spore germination protein GerE	Directs the transcription of genes that encode structural components of the protein coat that encases the mature spore (regulation of spore formation)	7.70E-16	-9.3194
	BSU_29500	ytfJ	Uncharacterized spore protein YtfJ	Sporulation protein	0.0007	-3.4711
	BSU_30900	cotS	Spore coat protein S	Assembly of the CotSA protein in spores	3.72E-08	-4.8503
	BSU_31470	kapD	Probable 3'-5' exonuclease KapD	Inhibits KinA pathway to sporulation	0.0013	-2.5838
	BSU_31730	yuzC	Uncharacterized spore coat protein YuzC	Resistance of spore	0.0040	-3.0375
	BSU_32070	yuiC	Uncharacterized protein YuiC	Stationary phase survival protein (sporulation protein)	0.0001	-3.7890
	BSU_32640	sspG	Small acid-soluble spore protein G	Protects spore DNA (binds to it) and confers high resistance to spores	4.83E-09	-6.3088
	BSU_33340	sspJ	Small acid-soluble spore protein J	Protects spore DNA (binds to it) and confers high resistance to spores	1.62E-08	-6.8689
	BSU_36070	cotG	Spore coat protein G	Morphogenetic protein, incorporation of protein CotB into the spore coat	1.07E-18	-10.2358
	BSU_36430	usd	Protein usd	Required for translation of SpoIIID	3.85E-05	-6.3324
	BSU_36550	spoIIQ	Stage II sporulation protein Q	Forespore engulfment and anchoring of membrane proteins on the forespore side	0.0040	-2.4973
	BSU_38130	ywcE	Spore morphogenesis and germination protein YwcE	Required for proper spore morphogenesis. Important for spore germination	2.99E-07	-5.7534
	BSU_39580	yxeE	Uncharacterized spore coat protein YxeE	Resistance of spore	1.24E-10	-7.6402
General metabolism						
General metabolism	BSU_08970	prkA	Protein PrkA	Kinase activity (widely distributed in microbes and with multiple functions)	0.0047	-2.7058
	BSU_10920	yitA	Probable sulfate adenylyltransferase	Synthesis of sulfites from sulfates (sulfur metabolism)	0.0008	-3.8963
	BSU_10930	cysH1	Adenosine 5'-phosphosulfate reductase 2		0.0039	-3.5960
	BSU_13520	ykrP	Putative membrane-bound acyltransferase YkrP	Acyltransferase activity	0.0023	-2.0322
	BSU_13940	ykwB	Uncharacterized N-acetyltransferase YkwB		0.0010	-1.7919
	BSU_14670	suhB	Inositol-1-monophosphatase	Hydrolyzes myo-inositol monophosphate (inositol metabolic process, signal transduction)	0.0024	-1.0803
	BSU_17940	ccdB	Protein CcdB	Phosphorelay signal transduction system (respond to stimuli through modification of gene expression)	0.0001	-2.2214
	BSU_17820	yndL	Protein YndL	Hydrolase activity, degradation of poly-γ-glutamic acid, which is produced by few Gram-positive bacteria	1.14E-05	-3.3758
	BSU_18130	eglS	Endoglucanase	Role in cellulose degradation	0.0014	-2.1215
	BSU_18239	yngHB	Biotin/lipoyl attachment protein	Binds biotin and lipoic acid (catalytic role in some carboxyl transfer reactions)	0.0037	-2.3130
	BSU_18260	yngJ	Probable Acyl-CoA dehydrogenase YngJ	Acyl-CoA dehydrogenase activity (catalyses first reaction in fatty acid metabolism)	0.0046	-2.5584
	BSU_18410	ggt	Glutathione hydrolase proenzyme	Glutathione metabolism and sulfur metabolism	0.0013	-2.0052
	BSU_18620	yoaI	Probable 4-hydroxyphenylacetate 3-monooxygenase	Production of DHPA (phenylacetate catabolic process)	0.0018	-2.1608
	BSU_18670	oxdD	Oxalate decarboxylase OxdD	Converts oxalate to formate and CO2 (oxalate metabolic process)	2.77E-05	-4.5354
	BSU_19310	dhaS	Putative aldehyde dehydrogenase DhaS	Cellular aldehyde metabolic process	3.66E-06	-3.9856
	BSU_19810	ypqP	Protein YpqP	Legionaminic acid synthesis (analogs of sialic acid that occur in cell surface)	2.54E-05	-4.1079
	BSU_25160	ispH	4-hydroxy-3-methylbut-2-enyl diphosphate reductase	Isoprenoids biosynthesis (isoprenoids are essential for cell survival and involved in vital biological functions)	0.0024	-1.5127
	BSU_25600	comER	ComE operon protein 4	L-proline biosynthesis (L-proline can be used as a carbon or nitrogen source)	0.0001	-3.8926
	BSU_30870	ytcB	Uncharacterized UDP-glucose epimerase YtcB	Degradation of galactose	0.0022	-3.4892
Energy production						
Energy production	BSU_14900	ctaD	Cytochrome c oxidase subunit 1	Component of respiratory chain, which allows oxidative phosphorylation and energy production	0.0013	-1.6042
	BSU_14920	ctaF	Cytochrome c oxidase subunit 4B		0.0007	-2.4208
	BSU_21750	ypmQ	SCO1 protein homolog	Insertion of copper into the active site of cytochrome c oxidase and copper homoestasis	0.0024	-1.8301
	BSU_14930	ctaG	Protein CtaG	Formation of functional cytochrome C-oxidase (caa3)	0.0025	-1.9419
	BSU_14629	argB	Acetylglutamate kinase	Control of arginine metabolism and glycolysis	4.00E-08	-6.3339

DNA replication							
DNA replication		BSU_07840	yfkN	Trifunctional nucleotide phosphoesterase protein YfkN	Cellular reprocessing of nucleotides present in the medium, under conditions of phosphate shortage	0.0042	-2.7068
		BSU_17660	yncF	Deoxyuridine 5'-triphosphate nucleotidohydrolase YncF	Nucleotide metabolism (necessary so that uracil cannot be incorporated into DNA)	0.0026	-2.0459
		BSU_23020	recS	ATP-dependent DNA helicase RecS	Required in synaptic and/or post-synaptic stages of recombination (DNA recombination and DNA repair)	0.0015	-2.9026
		BSU_24670	comGG	ComG operon protein 7	Required for transformation and DNA binding	3.84E-05	-1.8328
		BSU_24690	comGE	ComG operon protein 5		3.84E-05	-1.8328
Transcription							
Transcription		BSU_19200	desR	Transcriptional regulatory protein DesR	Member of regulatory system DesR/DesK (regulation of transcription)	0.0019	-1.6679
		BSU_19600	yodH	Uncharacterized methyltrans	Methylation	1.62E-07	-5.2005
		BSU_24600	sinI	Protein SinI	Antagonist to SinR, prevents SinR from binding to its target sequence on the gene for AprE (regulation of transcription)	1.82E-05	-2.4354
Translation							
Translation		BSU_10300	aprE	Subtilisin E	Serine alkaline protease that catalyzes the hydrolysis of proteins and peptide amides	8.27E-11	-4.6841
		BSU_14560	defB	Peptide deformylase 2	Removes the formyl group from the N-terminal Met of newly synthesized proteins	0.0014	-2.1966
		BSU_17260	aprX	Serine protease AprX	Serine-type endopeptidase activity	0.0010	-3.4803
		BSU_17640	alr2	Alanine racemase 2	Acts on alanine interconversion and may also acts on other amino acids	0.0037	-2.7034
		BSU_19690	kamA	L-lysine 2,3-aminomutase	Interconversion of lysine (lysine catabolic process, amino-acid degradation)	0.0015	-2.4587
		BSU_21850	ypiP	Uncharacterized protein YpiP	rRNA methyltransferase activity	0.0039	-1.7561
		BSU_21910	metAA	Homoserine O-acetyltransferase	Formation of acetyl-L-homoserine (L-methionine biosynthesis, amino-acid biosynthesis)	0.0024	-1.2550
		BSU_21970	ypeP	Uncharacterized protein YpeP	Nucleic acid binding and RNA-DNA hybrid ribonuclease activity	3.21E-07	-1.9361
		BSU_21980	ypdP	Probable queuosine precursor transporter	Import of queuosine (modification of tRNA)	0.0032	-2.2169
		BSU_24570	gcvT	Aminomethyltransferase	Catalyses degradation of glycine (glycine cleavage system)	0.0022	-1.8411
		BSU_27270	mtnN	5'-methylthioadenosine/S-adenosylhomocysteine nucleosidase	L-methionine biosynthesis (amino acids biosynthesis)	0.0040	-1.1187
		BSU_27440	glnH	ABC transporter glutamine-binding protein GlnH	Glutamine transport (amino acid transport)	0.0032	-3.4972
		BSU_misc_RNA_70			Small regulatory RNA and messenger RNA (arginine metabolism)	4.20E-06	-4.9880
	tRNA (adapters allowing synthesis of proteins from mRNAs)	BSU_tRNA_9				0.0007	-4.0406
		BSU_tRNA_20		tRNA-Ala		0.0012	-3.4455
		BSU_tRNA_28				0.0022	-3.5375
		BSU_tRNA_60				0.0016	-4.0140
		BSU_tRNA_58		tRNA-Arg		0.0007	-3.4786
		BSU_tRNA_23				1.77E-05	-4.7966
		BSU_tRNA_29		tRNA-Asn		3.69E-06	-5.3428
		BSU_tRNA_70				0.0005	-4.8541
		BSU_tRNA_22				0.0013	-3.9619
		BSU_tRNA_34		tRNA-Asp		0.0001	-5.6982
		BSU_tRNA_65				0.0003	-5.4791
		BSU_tRNA_42		tRNA-Cys		0.0002	-4.6993
		BSU_tRNA_31		tRNA-Glu		0.0001	-4.7755
		BSU_tRNA_25				0.0008	-3.7794
		BSU_tRNA_41		tRNA-Gly		0.0018	-4.1215
		BSU_tRNA_68				0.0011	-4.8976
		BSU_tRNA_39		tRNA-His		0.0001	-5.2136
		BSU_tRNA_67				0.0013	-4.0376
		BSU_tRNA_5		tRNA-Ile		0.0004	-4.0692
		BSU_tRNA_8			Adapters allowing synthesis of proteins from mRNAs	0.0005	-3.9668
		BSU_tRNA_15				0.0041	-3.5898
		BSU_tRNA_55		tRNA-Leu		0.0042	-3.0541
		BSU_tRNA_57				0.0032	-3.5491
		BSU_tRNA_21				0.0004	-3.6097
		BSU_tRNA_33		tRNA-Met		0.0002	-4.4620
		BSU_tRNA_64				0.0002	-4.3305
		BSU_tRNA_35		tRNA-Phe		0.0001	-5.3338
		BSU_tRNA_19				3.67E-05	-4.4850
		BSU_tRNA_27		tRNA-Pro		7.04E-05	-4.8437
		BSU_tRNA_59				9.08E-05	-5.1779
		BSU_tRNA_13				0.0032	-3.5342
		BSU_tRNA_24		tRNA-Thr		0.0015	-3.6626
		BSU_tRNA_36				0.0003	-4.9906
		BSU_tRNA_53				0.0017	-3.6067
		BSU_tRNA_37		tRNA-Tyr		0.0003	-4.6284
		BSU_tRNA_12				3.36E-05	-5.2195
		BSU_tRNA_32		tRNA-Val		3.85E-05	-5.6299
		BSU_tRNA_52				6.53E-06	-6.3988
		BSU_tRNA_78				0.0009	-4.9667

Translation	Protein transport	BSU_17710	tatAc	Sec-independent protein translocase protein TatAc	Transport large folded proteins across membrane (twin-arginine translocation (Tat) system)	1.54E-05	-2.1488
	Breakdown of peptides/proteins	BSU_08780	ygaJ	Uncharacterized peptidase YgaJ		0.0013	-1.6008
		BSU_13190	isp	Major intracellular serine protease	Serine-type peptidase activity	5.49E-10	-6.6040
		BSU_19170	yocD	Putative carboxypeptidase YocD		0.0019	-1.9214
		BSU_14700	nprE	Bacillolysin	Extracellular Zn metalloprotease that break peptides by involving Zn (metalloendopeptidase activity)	5.70E-05	-3.4533
Membrane							
Membrane		BSU_08140	yfjD	Uncharacterized lipoprotein YfjD		0.0046	-2.3544
		BSU_10180	yhfC	Uncharacterized membrane protein YhfC		3.75E-05	-5.4074
		BSU_13430	ykoX	Uncharacterized membrane protein YkoX		0.0013	-1.7138
		BSU_17800	yndJ	Uncharacterized membrane protein YndJ	Located at the membrane (but function unknown)	0.0006	-1.6971
		BSU_17830	yndM	Uncharacterized membrane protein YndM		0.0025	-2.3001
		BSU_17950	yneJ	Uncharacterized membrane protein YneJ		2.68E-05	-1.7834
		BSU_19749	yoyG	Uncharacterized membrane protein YoyG		2.30E-14	-8.0867
		BSU_14620	slp	Pal-related lipoprotein	Structural protein (homolog to peptidoglycan-associated lipoproteins (PAL) essential for outer membrane invagination in Gram-negative bacteria)	0.0002	-2.8189
		BSU_19190	desK	Sensor histidine kinase DesK	Sensor of membrane fluidity (member of the two-component regulatory system DesR/DesK)	0.0014	-2.0914
		BSU_21920	ugtP	Processive diacylglycerol beta-glucosyltransferase	Involved in biosynthesis of both the bilayer and non-bilayer-forming membrane glucolipids (glycolipid biosynthetic process and lipotechoic acid biosynthetic process)	2.91E-05	-2.9551
		BSU_23940	yqjB	Putative L,D-transpeptidase YqjB	Cell wall organization, peptidoglycan-protein cross-linking and regulation of cell shape	0.0006	-1.9075
		BSU_35530	tagO	Probable undecaprenyl-phosphate N-acetylglucosaminyl 1-phosphate transferase	Involved in the synthesis of anionic cell-wall polymers (techoic acid biosynthesis, lipopolysaccharide biosynthesis and cell wall organization)	0.0012	-3.5728
	Transmembrane transport	BSU_11381	appA	Oligopeptide-binding protein AppA	Component of an oligopeptide permease, which has a peptide transmembrane transporter activity (role in sporulation)	0.0002	-4.1086
		BSU_12930	dppB	Dipeptide transport system permease protein DppB	Part of ABC membrane transporter involved in dipeptide transport across membrane	0.0047	-2.6848
		BSU_25010	yqgE	Uncharacterized protein YqgE		0.0047	-1.9149
		BSU_28570	yshE	Transmembrane protein YshE	Transmembrane transporter activity (but uncharacterized)	0.0031	-1.4126
		BSU_31480	yuxJ	Uncharacterized MFS-type transporter YuxJ		0.0033	-3.1501
		BSU_30990	thiT	Thiamine transporter ThiT	Thiamine transmembrane transporter activity (provides energy to transport different substrates)	0.0014	-2.8162
Stress response							
Stress response		BSU_13440	ykoY	Uncharacterized membrane protein YkoY	Resistance to Mn^{2+} intoxication	0.0025	-1.3588
		BSU_13460	rsgI	Anti-sigma-I factor RsgI	Negatively regulates SigI activity, which is involved in heat-shock response	7.07E-05	-1.4582
		BSU_17240	ymzB	Uncharacterized protein YmzB	Survival of ethanol and salt stresses	0.0038	-2.1729
		BSU_17730	yndB	Uncharacterized protein YndB	May be involved in stress response to chalcone-like flavonoids (emitted by plants in case of pathogens infection)	0.0033	-2.2701
		BSU_21710	ypnP	Probable multidrug resistance protein YpnP	Na^+ driven multidrug efflux pump (damage inducible)	0.0003	-1.7136
		BSU_21780	yplP	Putative sigma L-dependent transcriptional regulator YplP	Survival at low temperatures	0.0005	-1.9356
		BSU_21790	yplQ	Hemolysin-3 homolog	Homolog of virulence factor, involved in cytolysis	2.77E-05	-1.8565
		BSU_21930	cspD	Cold shock protein CspD	Regulation of gene expression in case of stress	0.0035	-2.5756
		BSU_27130	rsiV	Anti-sigma-V factor RsiV	Negatively regulates SigV, which is involved in resistance to lytic enzymes	0.0035	-1.3435
		BSU_33770	sdpC	Sporulation delaying protein C	Lysis of cells that have not entered the sporulation pathway, inducing cannibalism to provide a source of nutrients to support sporulation (cell killing, cytolysis, defense response to bacterium and pathogenesis)	0.0030	-2.8464
		BSU_33780	sdpI	Immunity protein SdpI	Protection against toxic effects of SDP	0.0031	-2.6389

Stress response		BSU_37350	sboA	Subtilosin-A	Bacteriocidal activity against some Gram-positive bacteria (cytolysis, defense response to bacterium)	0.0020	-3.6099
	Oxidative stress	BSU_11560	yjbI	Group 2 truncated hemoglobin YjbI	Low peroxidase activity (oxidative stress protection, oxygen transport and cell redox homeostasis)	0.0001	-1.7555
		BSU_17280	ymaD	Uncharacterized protein YmaD	Oxidative stress protection	0.0022	-1.2988
Biofilm formation							
Biofilm formation		BSU_24610	sinR	HTH-type transcriptional regulator SinR	Govern the transition between a state in which bacteria swim or swarm and a state in which bacteria assemble into multicellular communities (biofilm formation) and play also a role in sporulation!	0.0014	-1.7742
Others (including genes coding for hypothetical or unknown protein)							
Others				117 genes			
TOTAL							**319 downregulated genes**

Using the UniProt plateform (uniprot.org), we classified the genes with a significant up- or down-regulated expression (padj <0.05; log2FoldChange >1 or <-1) in category corresponding to the biological process they are known to be involved. The genes coding for hypothetical proteins or for which the function is still unknown were classified together in a category called "others". Gene expression was analysed by comparing simply the impact of manganese supplementation in liquid growth medium, compared to solid medium.

Supplementary Table 3. Number of genes with a dysregulated expression according to manganese treatment and to type of growth medium for *three Kurthia* species.

	Expression with manganese treatement		Expression with manganese in liquid medium	
	Up-regulated	Down-regulated	Up-regulated	Down-regulated
Kurthia sp. str. 11kri321	1	177	97	0
K. massiliensis	176	10	483	215
K. senegalensis	3	0	213	575

Kurthia sp. str. 11kri321, *K. massiliensis* and *K. senegalensis* changed the expression of some genes when the cells were exposed to manganese supplementation compared to no supplementation (grey) and when manganese-treated cells were grown in liquid compared to solid medium (blue). The table summarizes the total number of genes with a significant up- or down-regulated expression (padj <0.05; log2FoldChange >1 or <-1) for each experimental condition and each species.

Supplementary Table 4. Genes identified in *Kurthia* sp. str. 11kri321 with a significant modified expression according to manganese supplementation.

Upregulated in presence of manganese							
Category	**Sub-category**	**Tag**	**Gene's name**	**Protein's name**	**Role**	**Adjusted *p* -value**	**Log2 (fold-change)**
Membrane							
Membrane	Transmembrane transport	AMA63703.1	ASO14_664	GRAM domain protein	Transport of messengers into and out of cells, messengers invovled into signalling pathways	0.0025	1.0650
TOTAL					**1 upregulated gene**		
Downregulated in presence of manganese							
Category	**Sub-category**	**Tag**	**Gene's name**	**Protein's name**	**Role**	**Adjusted *p* -value**	**Log2 (fold-change)**
General metabolism							
General metabolism		AMA62287.1	pruA	Putative delta-1-pyrroline-5-carboxylate dehydrogenase	Dehydrogenase (acts on NAD or NADP, central cofactor for bacterial metabolism)	0.0329	-1.4431
		AMA62518.1	ASO14_261	Acetyltransferase family protein	Catalysis of the transfer of an acetyl group to a nitrogen atom on the acceptor molecule.	0.0128	-1.1582
		AMA62655.1	ispH	4-hydroxy-3-methylbut-2-enyl diphosphate reductase	Isoprenoid precursor biosynthesis (essential for vital biological functions and survival)	0.0053	-1.8627
		AMA62742.1	sufC	FeS assembly ATPase SufC	ATPase activity (use ATP to achieve several metabolic processes)	0.0195	-1.1225
		AMA63689.1	ASO14_1389	Dienelactone hydrolase family protein	Catalyzes hydrolysis of ester bonds (ubiquitous in life)	0.0132	-1.0477
	Co-factor synthesis	AMA63552.1	thiE	Thiamine-phosphate synthase	Thiamine biosynthesis,essential cofactor	0.0019	-2.0082
	Purine metabolism (purines= metabolic signals,	AMA62600.1	prs	Ribose-phosphate pyrophosphokinase	Synthesis of 5-phospho-alpha-D-ribose 1-diphosphate (involved in purine metabolism)	0.0329	-1.1392
		AMA63129.1	apt	Adenine phosphoribosyltransferase	Formation of AMP, involves in purines metabolism --> purines act as metabolic signals, provide energy and others processes	0.0019	-1.3408
Energy production							
Energy production		AMA61744.1	sdhA	Succinate dehydrogenase/fumarate reductase, flavoprotein subunit		0.0128	-2.0069
		AMA62641.1	ASO14_942	Succinate dehydrogenase and fumarate reductase iron-sulfur family protein	Metal ion binding involved in energy production (electron transport chains)	0.0068	-1.3471
		AMA63748.1	sdhC	Succinate dehydrogenase and fumarate reductase, cytochrome b subunit		0.0255	-1.3494

Energy production		AMA61992.1	sucC	Succinate--CoA ligase [ADP-forming] subunit beta	Role in citric acid cycle (TCA), provides ATP or GTP	0.0058	-1.6123
		AMA62672.1	sucD	Succinate--CoA ligase [ADP-forming] subunit alpha		0.0105	-1.1104
		AMA62759.1	ASO14_2658	Polysaccharide deacetylase family protein	Carbohydrate metabolic process	0.0259	-1.2954
		AMA62898.1	gap	Glyceraldehyde-3-phosphate dehydrogenase	Glucose metabolic process	0.0025	-1.9358
		AMA63045.1	guaA	GMP synthase	Catalyzes the synthesis of GMP from XMP	0.0021	-2.2482
		AMA64071.1	guaB	Inosine-5'-monophosphate dehydrogenase	GMP biosynthetic process	0.0029	-3.0700
		AMA64385.1	pckA	Phosphoenolpyruvate carboxykinase (ATP)	Gluconeogenesis (formation of glucose)	0.0014	-2.3448
Transcription							
Transcription		AMA62454.1	ASO14_829	HTH deoR-type domain-containing protein	DNA binding transcription factor activity	0.0063	-1.3267
		AMA62495.1	ASO14_1566	MerR regulatory family protein	DNA binding protein, regulation of transcription	0.0273	-1.8259
		AMA62533.1	recF	DNA replication and repair protein RecF	DNA metabolism (replication and normal SOS inducibility)	0.0307	-1.1436
		AMA63381.1	ASO14_282	Response regulator	DNA binding portein	0.0103	-1.0851
		AMA64036.1	dnaX	DNA polymerase III subunit gamma/tau	Replicative synthesis	0.0009	-1.6198
		AMA64171.1	ASO14_2042	TrkA-C domain protein		0.0029	-1.1733
		AMA64482.1	ASO14_2633	Winged helix DNA-binding domain protein	DNA-binding transcription factor activity	0.0025	-1.0453
Translation							
Translation		AMA61802.1	ASO14_302	Rhomboid family protein	Serine-type endopeptidase (breaks peptides in smaller units)	0.0371	-1.3767
		AMA62342.1	ASO14_2443	Trypsin family protein		0.0391	-1.7627
		AMA62621.1	pepF	Oligoendopeptidase F	Metalloendopeptidase activity	0.0426	-1.0238
		AMA62736.1	pepF			0.0110	-2.0204
		AMA63070.1	tig	Trigger factor	Protein export and chaperone (prevent missfolding of proteins)	0.0249	-1.3698
		AMA63088.1	khpa	RNA binding protein KhpA	RNA binding protein (post-transcriptional processes)	0.0455	-1.3622
		AMA63232.1	ASO14_2895	S4 domain protein		0.0193	-1.0845
		AMA63315.1	ASO14_2640	Peptidase M20/M25/M40 family protein	Hydrolase activity (breaks peptides in smaller units)	0.0017	-1.9307
		AMA64025.1	infC	Translation initiation factor IF-3	Initiate proteins synthesis (30S subunits)	0.0368	-1.6878
		AMA64086.1	rsgA	Small ribosomal subunit biogenesis GTPase RsgA	Role in assembly of ribosomal proteins	0.0219	-1.6902
		AMA64384.1	tatAy	Sec-independent protein translocase protein TatA	Protein transport by Tat complex	0.0068	-1.8732
		AMA64558.1	ASO14_2030	X-Pro dipeptidyl-peptidase family protein	Peptidase activity	0.0485	-1.2287
	Amino acids synthesis	AMA61753.1	dapA	4-hydroxy-tetrahydrodipicolinate synthase	L-lysine biosynthesis	0.0274	-1.1226
		AMA62719.1	glyA	Serine hydroxymethyltransferase	Serine and glycine biosynthetic processes	0.0025	-1.8861
		AMA63736.1	sufS	Cysteine desulfurase	Cystein metabolic process	0.0012	-1.7946
		AMA64145.1	gcvPA	Probable glycine dehydrogenase (decarboxylating) subunit 1	Catalyse degradation of glycine	0.0101	-1.6744
	Ribosomal proteins (in conjunction with rRNA make up the ribosomal subunits)	AMA62275.1	rpmE	50S ribosomal protein L31		0.0025	-1.8590
		AMA62871.1	rpsU	30S ribosomal protein S21		0.0102	-1.2231
		AMA63562.1	rplU	50S ribosomal protein L21	Structural constituent of ribosome	0.0212	-1.0876
		AMA64102.1	rplM	50S ribosomal protein L13		9.99E-06	-3.2761
		AMA64570.1	rplS	50S ribosomal protein L19		0.0323	-1.6023
					16S ribosomal RNA 5S ribosomal RNA		
	tRNA	tRNA-Ala tRNA-Arg tRNA-Asn tRNA-Asp tRNA-Cys tRNA-Gln tRNA-Glu tRNA-His tRNA-Ile tRNA-Leu tRNA-Lys tRNA-Met tRNA-Phe tRNA-Ser tRNA-Trp tRNA-Val			Adapters allowing synthesis of proteins from mRNAs		
		AMA63607.1	ASO14_842	Putative tRNA binding domain protein		0.0138	-1.7273
Membrane							
		AMA62763.1	ASO14_1431	Putative Membrane protein	Membrane protein	0.0021	-1.0508
		AMA64200.1	ASO14_1896			0.0102	-1.4653

Membrane		AMA62811.1	ASO14_381		Involved in peptidoglycan binding (and this domain is also found in proteins involved in other biological processes)	0.0007	-1.9477
		AMA63888.1	ASO14_1428	LysM domain protein		2.18E-06	-3.2645
		AMA64399.1	ASO14_1861			0.0001	-2.2204
	Membrane formation	AMA61920.1	ASO14_2348	Transglycosylase SLT domain protein	Peptidoglycan metabolic process	0.0404	-1.0544
		AMA63076.1	ASO14_219	1-acyl-sn-glycerol-3-phosphate acyltransferase	Phospholipid biosynthetic process	0.0016	-1.6503
		AMA63335.1	glmU	Bifunctional protein GlmU	Part of bacterial outer membrane biogenesis	0.0331	-1.0217
		AMA64009.1	ASO14_2442	NlpC/P60 family protein	Peptidoglycan hydrolase	0.0314	-1.0794
	Transmembrane transport	AMA62380.1	ASO14_1360	Major facilitator superfamily protein	Transmembrane transporter activity	0.0143	-1.3763
		AMA62395.1	ASO14_465	Copper-translocating P-type ATPase	Ion transmembrane transporter	0.0244	-1.6269
		AMA62713.1	ASO14_1456	Sodium symporter family protein	Transmembrane transporter activity	0.0014	-2.1907
		AMA62841.1	ASO14_964	ABC 3 transport family protein	ATPase-coupled transmembrane transporter activity	2.51E-06	-2.1083
		AMA63677.1	ASO14_924	Bacterial extracellular solute-binding s, 5 Middle family protein	Transmembrane transporter activity	0.0021	-1.8609
		AMA63686.1	czcD	Cadmium, cobalt and zinc/H(+)-K(+) antiporter	Cation transmembrane transporter activity	0.0008	-2.6345
		AMA64394.1	ASO14_965	ABC transporter family protein	ATPase-coupled transmembrane transporter activity	5.30E-11	-3.6799
Stress response							
Stress response	General response	AMA62099.1	spxA	Regulatory protein spx	Regulation of transcription and prevention of protein aggregation during severe heat stress	0.0004	-2.6293
		AMA62368.1	ASO14_606	Bacterial regulatory s, tetR family protein	Widely associated with antibiotic resistance	0.0025	-1.0525
		AMA62635.1	spxA	Global transcriptional regulator Spx	Regulation of genes (transcription regulation) in response to stress	0.0128	-1.1920
		AMA62693.1	groL	Chaperonin GroEL	Ensure proper folding of polypeptides generated under stress	0.0132	-1.0036
	Oxidative stress	AMA62727.1	msrA	Peptide methionine sulfoxide reductase MsrA	Repair enzyme for proteins that have been inactivated by oxidation	0.0025	-1.2970
		AMA63156.1	ahpC	Alkyl hydroperoxide reductase C	Protection against oxidative stress (detoxify peroxides)	7.57E-05	-3.4627
		AMA63164.1	ASO14_2745	Catalase family protein	Response to oxidative stress	0.0038	-2.5573
Biofilm							
Biofilm		AMA61811.1	ssaB	Manganese ABC transporter substrate-binding lipoprotein	Metal ion binding protein (transport of Mn) and role in cell adhesion to other cells or to surface	3.80E-05	-1.4337
Related to ions/metals							
Related to ions/metals		AMA61853.1	ASO14_2727	Zinc-binding dehydrogenase family protein	Zinc-binding dehydrogenase family protein (oxidoreductase activity and Zn-binding protein)	0.0468	-1.4751
		AMA62573.1	ASO14_686	SUF system FeS assembly protein, NifU family		0.0204	-1.2080
		AMA62920.1	sufD	FeS assembly protein SufD	Iron-sulphur cluster assembly	0.0255	-1.1569
		AMA62954.1	sufB	FeS assembly protein SufB		0.0098	-1.2879
		AMA63980.1	ASO14_225	SEC-C motif family protein	Chelate Zn ions	0.0249	-1.2161
Others (including genes coding for hypothetical or unknown protein)							
Others				82 genes			
TOTAL							**177 downregulated genes**

Using the UniProt plateform (uniprot.org), we classified the genes with a significant up- or down-regulated expression (padj <0.05; log2FoldChange >1 or <-1) in category corresponding to the biological process they are known to be involved. The genes coding for hypothetical proteins or for which the function is still unknown were classified together in a category called "others". Gene expression was analysed by comparing simply the impact of manganese supplementation compared to no supplementation regardless the growth medium (liquid or solid).

Supplementary Table 5. Genes identified in *Kurthia* sp. str. 11kri321 with a significant modified expression according to manganese supplementation in liquid or solid growth medium.

Upregulated in liquid conditions							
Category	**Sub-category**	**Tag**	**Gene's name**	**Protein's name**	**Role**	**Adjusted *p* -value**	**Log2 (fold-change)**
General metabolism							
General metabolism		AMA63982.1	ASO14_2950	3-beta hydroxysteroid dehydrogenase/isomerase family protein	Isomerase activity (Structural rearrangment of molecules)	1.12E-05	1.5685
		AMA64150.1	ASO14_1466	Cyclase family protein	Multiple role in metabolism	0.0004	1.7366
		AMA64302.1	ASO14_1884	HAD hydrolase, IA, variant 1 family protein	Hydrolase activity	0.0106	1.3870
		AMA64554.1	ASO14_2490	Glycosyl transferases group 1 family protein	Initiation and elongation of glycan chains (which serve structure, energy storage, etc.)	0.0385	1.2577
	Co-factor biosynthesis	AMA63211.1	ASO14_2592	Phos_pyr_kin domain-containing protein	Thiamine biosynthetic process	0.0110	1.9409
	Purine metabolism (purines= metabolic	AMA63018.1	hpt	Hypoxanthine phosphoribosyltransferase	Synthesizes IMP from hypoxanthine	0.0011	1.9479
		AMA63045.1	guaA	GMP synthase	Catalyzes the synthesis of GMP from XMP	0.0158	2.3577
Energy production							

Energy production		AMA61744.1	sdhA	Succinate dehydrogenase/fumarate reductase, flavoprotein subunit	Metal ion binding involved in energy production (electron transport chains)	0.0106	2.8463
		AMA63748.1	sdhA	Succinate dehydrogenase/fumarate reductase, cytochrome b subunit, b558 family protein		0.0337	1.8248
		AMA61995.1	cls	Cardiolipin synthase	Cardiolipin is a major phospholipid in mitochondria and is involved in the generation of cellular energy in the form of ATP	0.0237	1.3849
		AMA62072.1	glmS	Glutamine--fructose-6-phosphate aminotransferase	carbohydrate metabolic process Source: UniProtKB-UniRule glutamine metabolic process	0.0025	1.9315
		AMA62759.1	ASO14_2658	Polysaccharide deacetylase family protein	Hydrolase activity on carbon-nitrogen bonds (carbohydrate metabolic process)	0.0084	2.0033
		AMA62915.1	atpD	ATP synthase subunit beta	Produces ATP in presence of a proton gradient across the membrane	0.0247	1.3537
		AMA63631.1	eno	Enolase	Degradation of carbohydrates via glycolysis	0.0373	1.5184
		AMA64385.1	pckA	Phosphoenolpyruvate carboxykinase (ATP)	Involved in the gluconeogenesis	0.0068	2.5660
DNA replication							
DNA replication		AMA62533.1	recF	DNA replication and repair protein RecF	DNA metabolism (replication and normal SOS inducibility)	0.0021	2.0741
		AMA62569.1	gyrB	DNA gyrase subunit B	DNA binding, favors all processes involved in DNA strand separation	0.0004	1.0006
		AMA62680.1	dnaB	Replicative DNA helicase	Initiation and elongation during chromosomes replication	1.30E-05	1.0780
		AMA63128.1	ASO14_2892	DNA topoisomerase (ATP-hydrolyzing)	DNA binding, favors all processes involved in DNA strand separation	0.0013	1.0495
		AMA63352.1	gyrA			3.75E-05	1.3196
		AMA64036.1	dnaX	DNA polymerase III subunit gamma/tau	Replicative DNA synthesis	0.0034	1.9560
		AMA64209.1	ssb	Single-stranded DNA-binding protein	DNA replication, recombination and repair	1.82E-07	1.3838
Transcription							
Transcription		AMA62044.1	rpoA	DNA-directed RNA polymerase subunit alpha	Catalyses transcription of DNA into RNA	0.0092	1.2544
		AMA64164.1	rpoC	DNA-directed RNA polymerase subunit beta		0.0409	1.5279
		AMA64231.1	rpoB			0.0114	2.2091
		AMA62248.1	ASO14_2832	S1 RNA binding protein	Nucleic acid binding	5.31E-06	1.1124
		AMA62617.1	ASO14_2900	R3H domain protein		0.0049	1.0294
		AMA62796.1	ASO14_1647	Double zinc ribbon family protein	Essential for recruitment of RNA polymerase (DNA to RNA, regulation of gene expression)	0.0011	3.1140
		AMA62950.1	ASO14_1648			0.0269	2.0938
		AMA62970.1	nusA	Transcription termination/antitermination protein NusA	Participates in both transcription termination and antitermination	0.0426	1.8147
		AMA63153.1	sigW	RNA polymerase sigma factor	Initiation of transcription	2.24E-06	3.4656
		AMA63505.1	ASO14_551	Winged helix DNA-binding domain protein	DNA-binding transcription factor activity	0.0173	1.7226
		AMA63629.1	ASO14_732	Helix-turn-helix family protein		0.0010	3.2366
		AMA63651.1	ASO14_1642			0.0020	2.3748
		AMA64131.1	ASO14_2052	MerR regulatory family protein	Regulation of transcription	2.56E-05	2.9956
	Nucleotides synthesis	AMA62210.1	glnA	Glutamine synthetase I alpha	Glutamine biosynthetic process	7.99E-05	3.0609
		AMA62600.1	prs	Ribose-phosphate pyrophosphokinase	Formation of nucleotides	0.0005	2.3666
		AMA64071.1	guaB	Inosine-5'-monophosphate dehydrogenase	Conversion of IMP to XMP, synthesis of guanine nucleotides (regulation of cell growth/purine metabolism)	2.38E-06	4.6727
Translation							
Translation		AMA61802.1	ASO14_302	Rhomboid family protein	Serine-type endopeptidase (breaks peptides in smaller units)	0.0106	2.0063
		AMA61880.1	tuf	Elongation factor Tu	Promotes the GTP-dependent binding of aminoacyl-tRNA to the A-site of ribosomes during protein biosynthesis.	0.0003	2.5924
		AMA62736.1	pepF	Oligoendopeptidase F	Metalloendopeptidase activity (breaks proteins in smaller units)	0.0006	3.5097
		AMA62828.1	rlmH	Ribosomal RNA large subunit methyltransferase H	Methlyation of rRNA	2.96E-16	1.0806
		AMA63020.1	ASO14_1122	Putative S49 family serine peptidase domain protein	Serine peptidase protein (breaks proteins in smaller units)	0.0003	3.7430
		AMA63232.1	ASO14_2895	S4 domain protein	RNA-binding protein	0.0018	1.8634
		AMA63442.1	asnB	Asparagine synthase	Amino acids synthesis (asparagine and glutamine)	0.0013	2.7029
		AMA63609.1	ASO14_1971	Neutral zinc metallopeptidase family protein	Metallopeptidase (breaks proteins in smaller units)	0.0149	1.5387
		AMA64374.1	fusA	Elongation factor G	Catalyzes the coordinated movement of the two tRNA molecules, the mRNA and conformational changes in the ribosome	2.90E-05	2.4460
		AMA61713.1	rplY	50S ribsomal protein L25		4.36E-06	1.0062

Translation	Ribosomal proteins	AMA61766.1	rpsJ	30S ribosomal protein S10		0.0108	1.0297
		AMA61828.1	rplO	50S ribosomal proteinL15		6.97E-06	1.2351
		AMA62097.1	rplV	50S ribosomal protein L22		0.0234	1.4490
		AMA62220.1	rplQ	50S ribosomal protein L17		1.59E-06	1.4189
		AMA62260.1	rpsM	30S ribosomal protein S13		0.0030	1.3408
		AMA62896.1	rpsK	30S ribosomal protein S11	In conjunction with rRNA make up the ribosomal subunits (translation)	0.0069	1.4559
		AMA63191.1	rplB	50S ribosomal protein L2		0.0020	1.5824
		AMA63540.1	rpsL	30S ribosomal protein S12		3.44E-06	1.6654
		AMA63915.1	rpsG	30S ribosomal protein S7		0.0008	1.3338
		AMA64102.1	rplM	50S ribosomal protein L13		0.0292	2.6736
		AMA64141.1	rplI	50S ribosomal protein L9		0.0002	1.1035
		AMA64211.1	rplC	50S ribosomal protein L3		0.0027	1.4348
		AMA64379.1	rplD	50S ribosomal protein L4		0.0006	2.0321
		AMA64589.1	rpsC	30S ribosomal protein S3		0.0081	1.2499
	tRNA (adapters allowing synthesis of proteins from mRNAs)			tRNA-Ala (2 times)	Protein synthesis		
Membrane							
Membrane		AMA62148.1	ftsH	ATP-dependent zinc metalloprotease FtsH	Quality control of integral membrane proteins	0.0012	2.4295
		AMA63076.1	ASO14_219	1-acyl-sn-glycerol-3-phosphate acyltransferase	Phospholipid biosynthetic process	0.0184	1.7811
		AMA63335.1	glmU	Bifunctional protein GlmU	Involved in LPS lipid A biosynthesis (outer membrane biogenesis, cell shape)	0.0084	1.7268
		AMA63950.1	ASO14_783	Transglycosylase associated family protein	Polymerises glycan chains (essential for peptidoglycans of membrane)	0.0055	1.4069
		AMA63976.1	ASO14_1705	Bacterial SH3 domain protein	Significant roles in substrate recognition, membrane localization and regulation of the kinase activity	0.0020	2.7674
	Transmembrane proteins	AMA61987.1	ASO14_450	Tic20-like family protein	Transmembrane helix protein	0.0002	2.0711
		AMA62050.1	ASO14_819	Putative membrane protein	Transmembrane helix protein	0.0006	2.2110
		AMA62887.1	ASO14_1714	5-bromo-4-chloroindolyl phosphate hydrolysis family protein	Transmembrane proteine (hydrolase located at the membrane)	4.24E-05	3.8103
		AMA63703.1	ASO14_664	GRAM domain protein	Transport of messengers into and out of cells (signalling pathways)	0.0013	1.5042
		AMA63901.1	ASO14_421	Cytochrome oxidase subunit II family protein	Transport of protons and electrons across membrane	0.0099	1.7919
Stress response							
Stress response		AMA62164.1	ASO14_1715	Toxic anion resistance family protein	Tellurite resistance	9.67E-06	3.5890
		AMA63180.1	floA	Flotillin-like protein FloA	SigmaW regulon antibacterial protein (protection against agents that impair cell wall biosynthesis)	2.56E-05	4.1619
	Oxidative stress	AMA62365.1	tpx	Thiol peroxidase	Detoxify peroxides	0.0068	1.8517
		AMA63156.1	ahpC	Alkyl hydroperoxide reductase C		0.0179	3.1284
	Temperature stress	AMA64122.1	ASO14_1455	Cold-shock' DNA-binding domain protein	DNA binding protein (cold-shock)	0.0004	3.0440
Motility							
Motility		AMA64510.1	ASO14_2420	Flagellin	Subunit protein which polymerizes to form the filaments of bacterial flagella	0.0010	3.5113
		AMA64597.1	fliG	Flagellar motor switch protein FliG	Bacterial-type flagellum-dependent cell motility and chemotaxis	0.0498	1.2830
Others (including genes coding for hypothetical or unknown protein)							
Others				17 genes			
TOTAL							**97 upregulated genes**

Downregulated in liquid conditions							
Category	**Sub-category**	**Tag**	**Gene's name**	**Protein's name**	**Role**	**Adjusted *p* -value**	**Log2 (fold-change)**
TOTAL							**0 downregulated gene**

Using the UniProt plateform (uniprot.org), we classified the genes with a significant up- or down-regulated expression (padj <0.05; log2FoldChange >1 or <-1) in category corresponding to the biological process they are known to be involved. The genes coding for hypothetical proteins or for which the function is still unknown were classified together in a category called "others". Gene expression was analysed by comparing simply the impact of manganese supplementation in liquid growth medium, compared to solid medium.

Synthesis, general discussion, perspectives and conclusion

1. Synthesis

The general aim of this thesis was to investigate the response of the non-standard bacterial model *Kurthia* sp. str. 11kri321, an environmental strain initially isolated from an extreme environment, when confronted to stressful growth conditions in the laboratory. More specifically, we aimed to assess if this strain and other representative of *Kurthia* genus were able to produce resistant spore-like cells or if they displayed an alternative stress resistance strategy. To do so we combined several methods, such as bacterial culturing, light microscopy, cryo-EM microscopy, as well as transcriptomics, and genomics.

1.1 Chapter 1

In chapter 1, we focused on sporulation and attempted to respond to the **question**:

"Is the environmental non-standard model Kurthia sp. str. 11kri321 able to produce spore-like cells that would confer stress resistance to the strain?".

Therefore, the aim was to check the ability of *Kurthia* sp. str. 11kri321 and of three other *Kurthia* representatives (*K. huakuii*, *K. massiliensis* and *K. senegalensis*) to produce spore-like cells that would be more resistant than the cells usually produced by *Kurthia* spp. The production of phase-bright cells in *Kurthia* spp. grown in nutrient-depleted media was previously observed, albeit always at low frequency. In addition, cryo-EM investigation revealed steps of engulfment in two *Kurthia* species (Fatton et al., 2022). Regarding these results and the extreme conditions of the original environments from which all *Kurthia* species have been isolated, we hypothesized that the phase-bright cells observed in *Kurthia* spp. cultures could be spore-like cells. Accordingly, in this thesis, we conducted two additional investigations to explore sporulation in *Kurthia* spp.: a resistance test on aged cell cultures and a genome analysis searching for sporulation-specific gene orthologs. In the resistance test, we showed that 4-week old cells were not more resistant than fresh cells, which was not expected in the case of sporulation as the old cultures faced nutrient exhaustion, a well-known trigger of sporulation (Gray et al., 2019; Sella et al., 2014). Concerning the genome analysis, we showed that important sporulation-specific genetic elements such as the master transcriptional regulator of sporulation, Spo0A, and sporulation-specific sigma factors were present in the *Kurthia* spp. genomes. However, key genes for engulfment, genes coding for SASPs proteins and genes involved in spore cortex formation were not detected. We concluded that *Kurthia* spp. is able to produce differentiated cell resembling spores, but regarding the absence of numerous sporulation-specific genes, these cells might be quite different from canonical endospores in their structure, formation and resistance. We named those structures cryptospores. In addition, due to the low frequency of phase-bright cells in the initial cultures and their absence in cultures maintained under optimal laboratory growth conditions, we suggested that specific conditions are required to trigger cell differentiation and that the *Kurthia* species maintained in laboratory might have lost their ability to easily

form cryptospores due to a genetic drift or an epigenetic mechanism that could appear in absence of environmental selective pressure.

Accordingly, we only partially confirmed our initial **hypothesis**: regarding the presence of some sporulation-specific genes in its genome, the observation of engulfment step, and of phase-bright structures, *Kurthia* sp. str. 11kri321 produced differentiated cells, crysptospores, but we could not confirm that those cells are more resistant than the vegetative cells. Indeed, we did not succeed to recreate in the laboratory the specific environmental conditions required to trigger production of cryptospores and in addition, our strain might have mutated and stopped producing cryptospores.

1.2 Chapter 2

In chapter 2, we broadened our investigation about the non-standard model *Kurthia* sp. str. 11kri321 and looked beyond sporulation by exploring alternative stress resistance strategies. We had as first **question**:

How does the environmental non-standard model Kurthia sp. str. 11kri321 respond when grown under challenging conditions, such as nutrient depletion, low-water availability, low-iron availability or presence of a sporulation trigger?

Therefore, we grew the strain under challenging conditions recreated in laboratory to assess the stress response not only in case of nutrient-depletion, but also in presence of other common environmental stresses. We observed a morphological change in response to challenging growth conditions, especially in presence of manganese, a well-known sporulation trigger (Bosma et al., 2021; Charney et al., 1951; Sinnelä et al., 2019; Weinberg, 1964). In addition, phase-bright cells were obtained but always in low frequency. Even if resistance of those different cells was not tested, they might be more resistant than other cells. Therefore, the low-frequency might be due to a reduced resistance when the strain is maintained under optimal laboratory growth conditions, which led to our second **question**:

Does stress resistance differ between the strain of Kurthia sp. str. 11kri321 that was maintained under optimal laboratory growth conditions and the original isolated environmental strain?

We tested the resistance of the laboratory-maintained strain and of the original environmental strain of *Kurthia* sp. str. 11kri321 to heat and UV. We showed that indeed, the two strains were different and behave differently on several points due to the fact that each strain evolved in its particular own environment; laboratory or original extreme environment.

Due to the differences among the two strains, we had a third **question**:

Can the different morphological responses to challenging growth conditions of the laboratory-maintained strain and of the original environmental strain of Kurthia sp. str. 11kri321 be assigned to modifications at the genomic level?

We performed a genomic comparison and showed that the OS and LS strains have a different genome. This shows that due to their maintenance under different environments, the two strains evolved their proper unique genome. A major difference affecting a functional gene

was a deletion of 66 nucleotides, corresponding to 22 amino acids in the N-terminal domain of Spo0A, which is key in the phosphorelay signaling leading to sporulation initiation. Accordingly, the low frequency of cryptospores in LS strain might be explained by this deletional mutation in Spo0A.

Concerning the three **hypotheses** of chapter 2, we confirmed partially the first one: when grown under challenging conditions in laboratory, *Kurthia* sp. str. 11kri321 responds by a cell differentiation with a modified cell length, and with the production of phase-bright cells in low frequency. However, we could not confirm that these differentiated cells are spore-like cells and confer a higher resistance to stress. For the second hypothesis, we confirmed that the laboratory-maintained strain was less resistant at least to one stress, which is heat. Finally, the genomic comparison confirms that at least one difference in the morphological response to challenging conditions in OS and LS strains can be assigned to modifications at the genomic level; a deletional mutation in Spo0A of the LS strain might explain the lower frequency of cryptospores in the strain as compared to the OS strain.

1.3 Chapter 3

In chapter 3, using transcriptomics, we explored the response of the non-standard model *Kurthia* sp. str. 11kri321 and of the sporulating model *B. subtilis* to manganese supplementation, a key element involved in sporulation and oxidative stress protection (Bosma et al., 2021; Charney et al., 1951; Randazzo et al., 2020; Sinnelä et al., 2019). We had the following **questions**:

Will gene expression of the non-standard Kurthia sp. str. 11kri321 show that sporulation, or an alternative stress resistance response is initiated after manganese supplementation?

Will gene expression of B. subtilis show that sporulation is initiated after a manganese supplementation?

We showed that the gene expression in *B. subtilis* reflected that manganese triggers sporulation, as expected based on previous morphological studies. This was revealed by the up-regulation of numerous sporulation-specific genes. In *Kurthia* sp. str. 11kri321, gene expression did not reflect sporulation, but rather an alternative stress response with a transient shutdown of translation/transcription processes, as well as a modified expression for genes involved in cell morphology. Accordingly, we observed a morphological cell change (i.e., size reduction) for *Kurthia* sp. str. 11kri321. However, this response was not common in other *Kurthia* species. Our results highlight that model and non-model species, but also different species from a same genus, react differently to stress. In addition, both in *B. subtilis* and *Kurthia* sp. str. 11kri321 a stress response was observed only if multiple stress factors were present (here manganese and growth on solid medium).

To conclude, we partially confirmed our first **hypothesis** as gene expression in *Kurthia* sp. str. 11kri321 was indeed modified under manganese supplementation, with an up-regulation of genes involved in cell differentiation. However, this was the case only if two stress factors were acting and we did not observe such a drastic modification of gene expression under manganese supplementation, only. In addition, gene expression reflected cell differentiation,

but not necessarily the production of spore-like cells. Next, our second hypothesis was also partially true as gene expression in *B. subtilis* was modified under manganese supplementation, with the up-regulation of sporulation-specific genes. However, again, gene expression modification occurred only when manganese supplementation was combined with a second stress factor (type of growth medium).

1.4 Back to the initial research question

The general research **question** of this thesis was:

"As the non-standard environmental model Kurthia sp. str. 11kri321 was isolated from an extreme environment (geothermal spring), is this strain able to sporulate in case of sub-optimal growth conditions recreated in the laboratory or does it respond by an alternative stress resistance strategy?"

Overall, we can answer that the non-standard model *Kurthia* sp. str. 11kri321 is able to produce cryptospores, but only if highly specific growth conditions are applied. Moreover, this ability decreases rapidly under optimal laboratory growth conditions, most probably due to deletional mutation in the master regulator of sporulation, Spo0A, that could have fixed in the genome as the survival pressure was relaxed in the laboratory. In addition, after laboratory cultivation, the LS strain lost phenotypic plasticity that might confer general stress resistance and experienced a reduced heat resistance. However, we also highlighted that this non-standard model is able to respond to stress through alternative strategies, like a simple modification of cell morphology and a transient shutdown of translation/transcription processes.

2. General discussion

In this thesis, the use of the environmental isolate *Kurthia* sp. str. 11kri321 provided novel insights about stress resistance response in a non-standard model. The first part of this general discussion is dedicated to discuss all together those results and propose an overview into diversity of stress resistance strategies and plasticity of the cell envelope in *Kurthia* sp. str. 11kri321 and other *Kurthia* spp. Next, this thesis also highlighted the challenges of choosing as a study model a bacterial species that is yet not commonly considered as a model organism and for which previous characterizations usually do not exist. This point will be discussed in the second part of this general discussion.

2.1 *Kurthia* sp. str. 11kri321, a non-standard model that adapts quickly to stress conditions

In natural environments, microorganisms evolve on fluctuating environmental conditions, with some periods that are sub-optimal for growth and reproduction (Lennon & Jones, 2011). Accordingly, they developed various survival strategies to withstand common environmental abiotic stresses, such as nutrient depletion or water availability (Esbelin et al., 2018; Gray et al., 2019). Despite this diversity of survival strategies, one of them has been much more

considered in research studies: the formation of resistant spores through sporulation. Even more, sporulation has only been extensively studied in a restricted number of bacterial phyla and accordingly, endospores produced by Firmicutes are often recognized as standard when considering resting bacterial structures (Paul et al., 2019). However, it was shown this last decade that alternative stress resistance strategies to sporulation are diverse, with for example, production of alternative dormant structures (Suzina et al., 2004), extreme slow growth rate (Gray et al., 2019), modification of cell morphology (Yang et al., 2016), and transient shutdown of translation/transcription (Grigull et al., 2004; Paschen, 2003). According to the impact of bacterial survival strategies on multitude topics that concerned humans (see the general introduction, section "Ecological and societal importance of dormancy"), the study of alternative resistance strategies outside well-described bacterial models is crucial. In this context, all along this thesis, we used a non-standard bacterial model, *Kurthia* sp. str. 11kri321, which was initially isolated from an extreme environment (geothermal spring) and for which almost nothing was known (Fatton et al., 2022).

Overall, in this thesis, we demonstrated that the non-standard model *Kurthia* sp. str. 11kri321 is able to adapt quickly to challenging growth conditions through various resistance stress strategies (Fig. 1). Among those strategies, we observed the production of cryptospores. Even if the higher resistance of cryptospores could not be assessed in this thesis, we showed that those cells share features with canonical endospores, like phase-brightness (Kong et al., 2011) and malachite green retention (Schaeffer & Fulton, 1933). The capacity to produce spore-like cells such as cryptospores might not be surprising as *Kurthia* genome possesses some sporulation-specific genes, including the master transcriptional regulator Spo0A (Errington, 2003; Tan & Ramamurthi, 2014). Nevertheless, due to a much smaller set of sporulation-specific genes than in *B. subtilis*, with for example the absence of genes involved in cortex formation or absence of SASPs responsible for DNA protection (Djouiai et al., 2018; Setlow, 2006), *Kurthia*'s cryptospores can be expected to be different from endospores in their structure, resistance, and formation. Next, *Kurthia* showed other alternative stress resistance strategies involving the modification of cell morphology (i.e., size reduction), and a transient shutdown of translation/transcription, two phenomena demonstrated to occur in case of suboptimal growth conditions (Grigull et al., 2004; Paschen, 2003; Weart et al., 2007; Yang et al., 2016).

In addition to describing the diversity of stress resistance strategies in *Kurthia* sp. str. 11ki321, we also showed that the deployed strategy depended greatly on the environmental conditions under which *Kurthia* evolved (past evolutionary history). Indeed, we observed that when *Kurthia* was directly cryopreserved after isolation from its natural extreme environment, it reacts differently to stress as compared to when *Kurthia* was maintained under optimal growth conditions in the laboratory for three months. Thus, we considered working with two different strains; the original environmental strain (OS) and the laboratory strain (LS). The most striking differences between OS and LS strains were the following: 1) cryptospores were observed in nutrient-depleted cultures for the OS strain, whereas several stresses had to be applied in addition to starvation so that LS strain produce also cryptospores, 2) a higher heat resistance was observed for the OS strain, 3) a phenotypic cell plasticity for the OS strain with two distinct colony morphologies and two cell morphologies (i.e., modification of cell size) was

revealed after heat treatment, whereas the same plasticity (i.e., modification of cell size) seemed to be reactivated only under multiple stress condition for LS strain, and 4) a deletion of 22 amino acids occurred in the N-terminal regulatory domain of the master regulator of sporulation Spo0A of the LS strain. Accordingly, we propose that the two strains evolved differently due to their respective environment, with extreme conditions in the original geothermal spring for the OS strain, and optimal growth conditions in the laboratory for the LS strain. Because survival strategies are costly for bacteria (Filippidou et al., 2016; Ratcliff et al., 2013; Siebring et al., 2014) and as in the laboratory the selection for survival might be decreased, we hypothesize that the LS strain lost some survival traits, like the ability to support heat or to form cryptospores under the only constraint of nutrient-depletion, something supported by the deletion of 22 amino acids in Spo0A. Therefore, the energy not spent in a costly survival strategy could be reallocated for growth or reproduction for example. Despite this, we showed that when multiple stress conditions are present, the LS strain is still able to adapt quickly and display the same survival strategies as the one observed for OS strain, like cryptospores production and phenotypic plasticity with cell size reduction.

Considering phenotypic plasticity, during our different investigations and while using cryo-EM and cryo-EM of vitreous sections (CEMOVIS), we observed an unexpected outer layer in some cells of *Kurthia* sp. str. 11kri321. Indeed, as expected for a Gram-positive bacterium, the cell envelope of *Kurthia* sp. str. 11kri321 appeared on cryo-EM and CEMOVIS images to be composed of an inner periplasmic membrane (IM), an inner well zone (IWZ) and a peptidoglycan layer (PG) (Fig. 2 and Fig. 3). However, we also observed the presence of an unexpected outermost layer (Fig. 2, red arrows and Fig. 3). This outer layer was also observed in three additional *Kurthia* species (*K. huakuii*, *K. massiliensis* and *K. senegalensis*) (Fig. 2). The outer layer was present all over some cells and absent from others. Density profiles performed on CEMOVIS images confirmed this observation with an additional peak present not for all cells analyzed (Fig. 3G to I, with the outer layer captioned as “S”). On cryo-EM images, the outer layer was observed to disintegrate partially sometimes at the poles of the cells (Fig. 2, orange arrows), but also in the areas of cell division (Fig. 2, blue arrows). Moreover, this outermost layer was characterized by a highly regular and crystalline structure (Fig. 2, close-up in A, B, C and D), which led us to hypothesize that this structure may be an S-layer. S-layers, for self-assembling protein surface layers, are widely spread among bacteria and other prokaryotic cells, coating the entire cell (Silhavy et al., 2010).

S-layers have been identified in almost every taxonomic group of bacteria, including both Gram-negative and Gram-positive bacteria and they are almost a universal feature of Archaea (Sleytr et al., 2014). These two-dimensional crystalline arrays are formed by one or multiple (glyco)proteins, known as S-layer proteins, that are non-covalently self-assembled (Fagan & Fairweather, 2014;Madhurantakam et al., 2014). The mode of S-layers anchoring to the cell envelope depends greatly on the organism. In Gram-negative, S-layers are attached to the LPS located in the OM, whereas in Gram-positive they are anchored to the peptidoglycan layer through the interaction of a S-layer homology (SLH) domain with peptidoglycan-linked secondary cell wall polymers (SCWPs). In Archaea, S-layers are directly associated to the IM (Fagan & Fairweather, 2014; Madhurantakam et al., 2014). S-layers have various functions, depending on the (glycol)protein that is involved (Fagan & Fairweather, 2014). The primary

function of S-layers is to protect cells and maintain their shape, but they also shield the cell from external environmental threats by functioning as a barrier reducing permeability and are involved in pathogenesis and immunity (Madhurantakam et al., 2014). Thus, it is clear that no single function can be attributed to S-layers. However, their wide occurrence in the bacterial domain, as well as the enormous metabolic load required to produce them, suggest that S-layers play an important role for bacteria (Fagan & Fairweather, 2014).

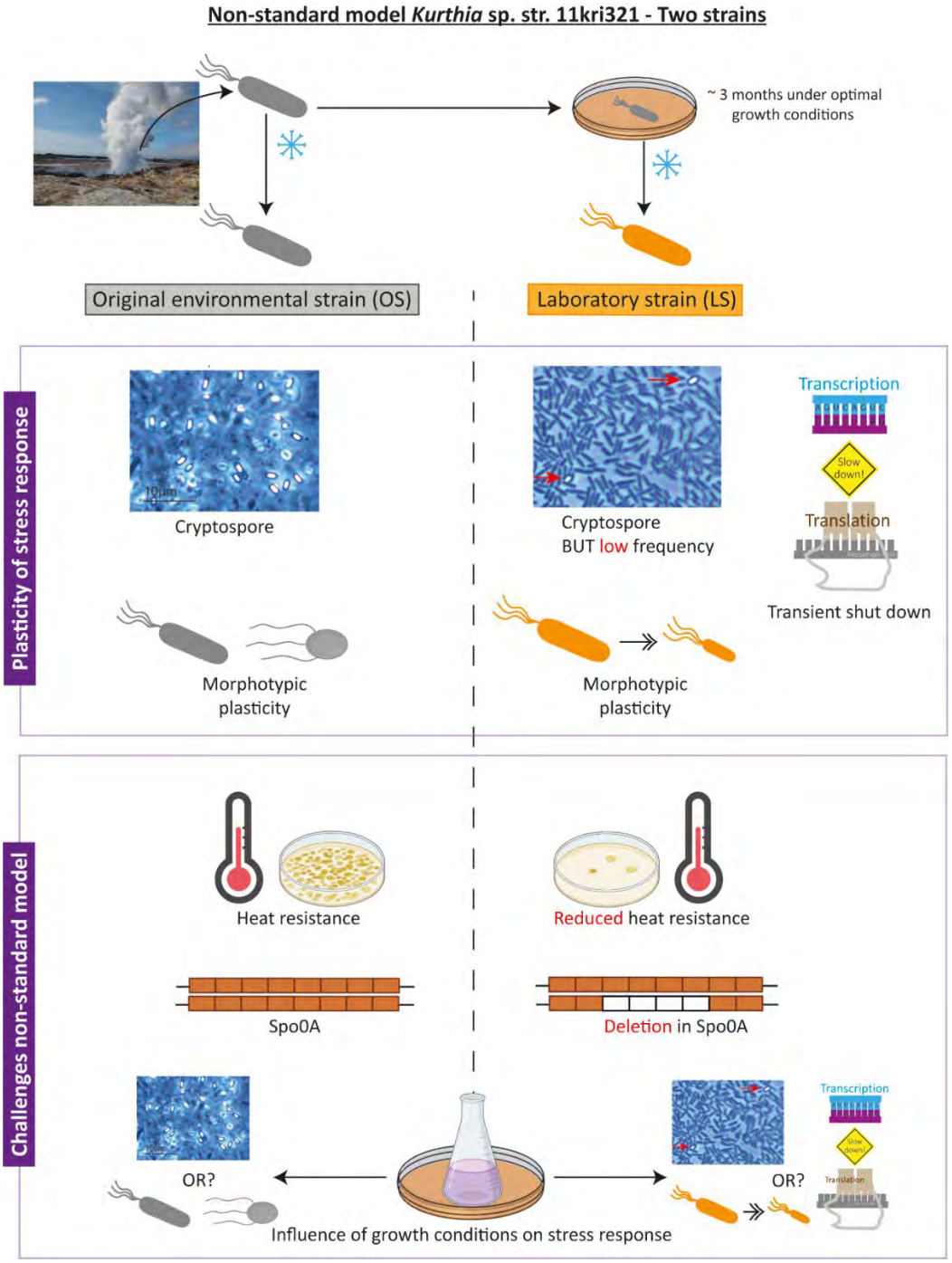


Figure 1. Visual summary of the whole study. The non-standard model *Kurthia* sp. str. 11kri321 was isolated from a geothermal spring and directly cryopreserved (OS strain). In addition, *Kurthia* sp. str. 11kri321 was maintained under laboratory conditions during three months (LS strain). While using OS and LS strain, we highlighted the plasticity of stress response in *Kurthia* sp. str. 11kri321, as well as several challenges to work with non-standard models. (Illustration made with BioRender and Illustrator).

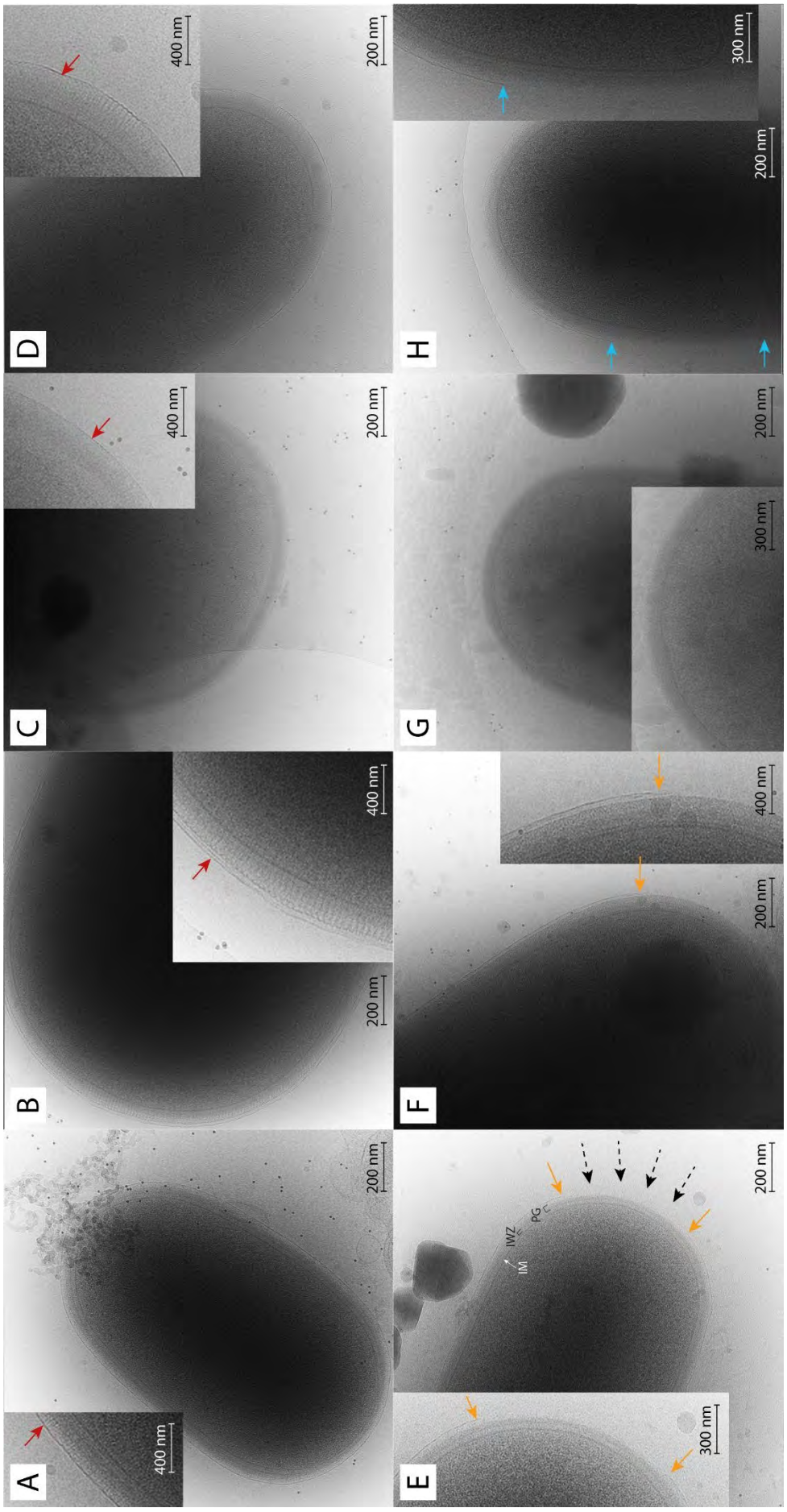


Figure 2: Cryo-electron microscopy focusing of the cell envelope and the imaging of a non-constitutive putative S-layer for four *Kurthia* species. The four top cryo-EM pictures show *Kurthia* spp. cells with a putative S-layer (red arrows, **A**= *Kurthia* sp. str. 11kri321, **B**= *K. huakuii*, **C**= *K. massiliensis* and **D**= *K. senegalensis*). The four down pictures show no putative S-layer or a discontinuous one. Indeed, under some conditions, *Kurthia* sp. str. 11kri321 **(E)** and *K. huakuii* **(F)** were observed with a discontinuous putative S-layer at cell poles (orange arrows= where the outer layer stops, dotted arrows= zone without the outer layer), *K. massiliensis* without any S-layer **(G)** and *K. senegalensis* with a discontinuous layer a cell division area **(H)**. All the cells possessed an inner membrane (IM), an inner well zone (IWZ) and a peptidoglycan layer (PG) (annotated only in **E**).

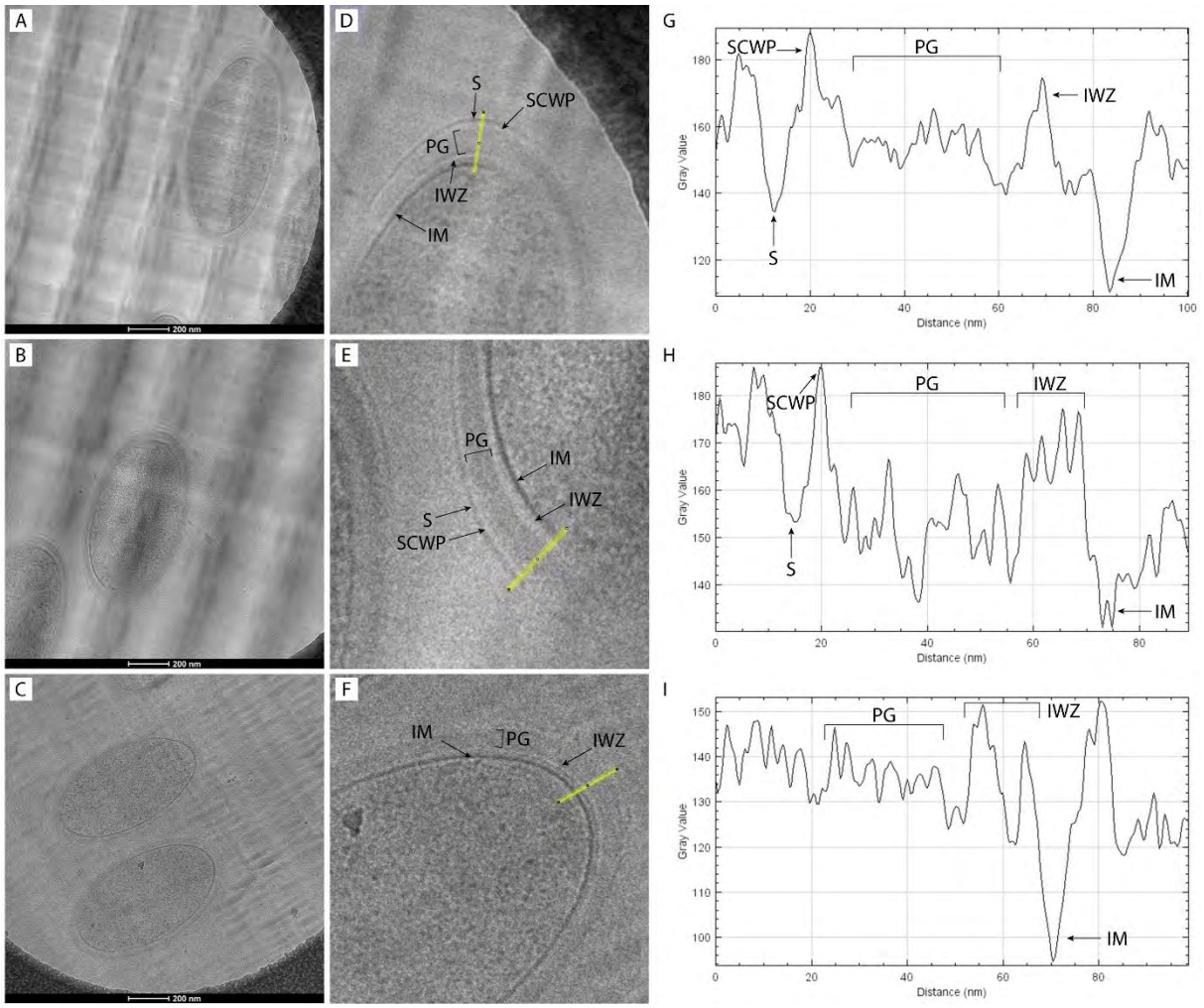


Figure 3. Cryo-electron microscopy of vitreous section (CEMOVIS) images of the cell envelope of *Kurthia* sp. str. 11kri 321 and density plots revealing a non-constitutive putative S-layer. Cryo-EM pictures of *Kurthia* sp. str. 11kri321 cells were denoised by Gaussian filtering using Adobe Photoshop (radius of 1 pixel) (**A, B, and C**. Panels **D**, **E** and **F** are a close-up showing the different layers with inner membrane (IM), the inner wall zone (IWZ) and the peptidoglycan layer (PG). A putative S-layer (S), as well as secondary cell wall polymers (SCWP) that anchored the S-layer to the peptidoglycan layer, are present in panels D and E, but absent in panel F. The density profiles in panels **G**, **H**, and **I** were obtained from denoised images corresponding to panels D, E, and F, respectively. They were averaged over a width of 20 pixels (G and H) and 60 pixels (I).

In the case of *Kurthia* sp. str. 11kri321, a dynamic S-layer might represent a protective layer to withstand suboptimal growth conditions. We therefore hypothesize that the plasticity of the cell envelope represents an additional stress resistance strategy in *Kurthia* spp. Such plasticity of the cell envelope was already shown to serve as a resistance strategy in Mycobacteria. The mycobacterial cell envelope is unique. First, at the interface of the IM and OM, some arabinogalactan are linked to the peptidoglycan layer to form the cell wall skeleton (Daffé & Zuber, 2014). Then the cell envelope of mycobacteria contains lipids that account for up to 40% of the cell dry mass, compared to less than 5% in other Gram-positive bacteria and 10% for Gram-negative. Those lipids are called mycolic acids and are organized into a Gram-negative-like OM (mycomembrane), which is highly unexpected for Gram-positive mycobacterium (Daffé & Zuber, 2014). This OM is quite different as, contrary to what it is usually observed, it is symmetrical: the lipids (i.e., mycolic acids) are distributed over both

leaflets in the mycobacterial OM and not restricted to the outer leaflet (Niederweis et al., 2010). Finally, the outermost structure is a capsule, that appears as a loose matrix of glucans and secreted proteins (Dulberger et al., 2020). To summarize, mycobacteria evolved a unique cell envelope architecture with additional layers principally made of arabinoglycans and mycolic acids. This specialized cell envelope renders mycobacteria impenetrable to many antibiotics and other toxic components (Jordan et al., 2008). In *Kurthia* sp. str. 11kri321, we propose that plasticity of cell envelope, with a dynamic "putative" S-layer as outer layer, could represent a protective strategy against antibiotics. Indeed, when we exposed a culture to penicillin (0.03 mg/L), some cells were not visible under fluorescent microscopy using the FM4-64 dye, whereas other cells appeared fluorescent (Fig. 4A). Moreover, cells from the control culture (no antibiotics treatment) were all fluorescent (Fig. 4B). As FM4-64 is a lipophilic dye staining selectively membrane, we hypothesize that penicillin treatment triggered the formation of the dynamic S-layer in some individuals, thus preventing the entrance of the dye, as well as entrance of the harmful penicillin.

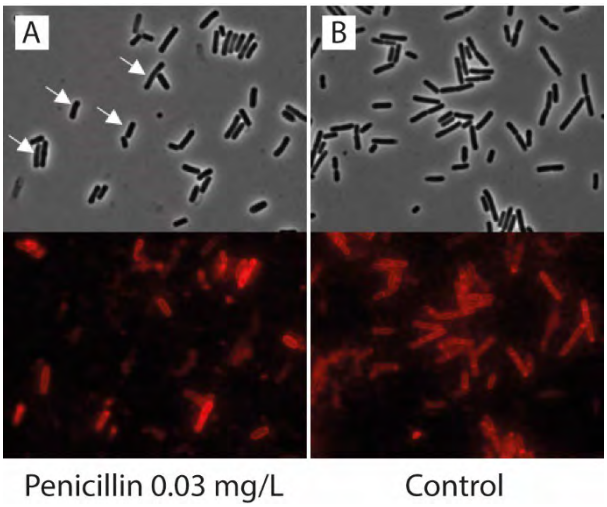


Figure 4. Putative modification of cell envelope of *Kurthia* sp. str. 11kri321 after antibiotics treatment revealed by staining with the red fluorescent FM4-64 dye. The lipophilic red fluorescent FM4-64 dye stains selectively membranes. **(A)** After exposure to penicillin (0.03 mg/L) and staining with FM4-64 dye, some cells are no longer fluorescent under the microscope (white arrows), whereas other cells were still fluorescent. **(B)** Cells from the control culture (no antibiotics treatment) were all visible under fluorescent microscopy. We propose that penicillin treatment triggered in some cells a modification of the cell envelope with formation of a putative S-layer, which prevented the entrance of the harmful antibiotic, as well as entrance of the dye.

A second example about how plasticty of the cell envelope might represent a benefit for bacteria is found in *B. anthracis*. This pathogenic bacterium, agent of anthrax, possesses a dynamic cell envelope composition with notably two switchable S-layers. *B. anthracis* cells are covered by two mutually exclusive S-layers, the Sap and EA1 S-layers. Sap S-layer forms a continuous array and is present during the exponential growth, whereas EA1 S-layer is organized as patches and expresses during the stationary growth phase (Ravi & Fioravanti, 2021). The two layers cannot cover the cell surface at the same time. Rather during transition from exponential to stationary growth phase, EA1 must displace Sap and triggers its release from cell surface because of space limitations (Mignot et al., 2002). Even if it is still unclear the advantages provide by this energetically expensive remodeling of S-layers, it may contribute to the successful pathogenicity of *B. anthracis* (Ravi & Fioravanti, 2021).

To conclude this first part of the general discussion and even if further investigations are clearly needed, we highlighted in this thesis that the plasticity of cell envelope, with an extra S-layer, might represent a stress resistance strategy in the non-standard model *Kurthia* spp.. Because, as mentioned above, no single function can be attributed to the S-layer, the use of more non-standard models such as *Kurthia sp. str. 11kri321* might permit in the future to reveal new unexpected functions of the S-layer.

2.2 The challenges to work with non-standard bacterial models

Beyond the results about stress resistance strategies, our work with *Kurthia* sp. str. 11kri321 confronted us to the challenges of working with a non-standard bacterial model. We discussed four of these challenges and proposed solutions/recommendations in this second part of the general discussion.

To begin, the use of a non-standard model showed us that nothing is really black or white when trying to characterize bacterial species and their lifestyle. For example, in the field of sporulation, bacterial species are commonly considered either as spore-formers or asporogenic (non-spore-formers), however, we showed that we need to be careful with this distinction, as the capacity to form spores depends highly on the surrounding living conditions and that an intermediate life style should be considered, such as in the case of cryptosporulation (chapter 1). Accordingly, when studying survival strategies in non-standard bacterial species, we recommend to keep an open mind and do not try to absolutely classify a bacterial species in one of the two commonly recognized categories as sporogenic or asporogenic.

Secondly, bacterial strains evolve quickly when cultivated in laboratory (Maughan et al., 2007; Norris et al., 2020; Vela & Wyss, 1965), something we confirmed with our non-standard model (i.e., reduced frequency of cryptospores, loss of phenotypic plasticity in the absence of multiple stress factors, reduced heat resistance in the laboratory-maintained strain and difference at the genome level) (chapters 1 and 2). Therefore, when non-standard models are maintained in the laboratory, we have to consider that they will probably adapt to the current growth conditions, which are usually different from the ones in the natural environment. For the known models, like *B. subtilis* or *E. coli*, the laboratory strains are already well-described and previous studies can provide information on potential differences between wild and lab strains, whereas it has to be studied for the non-standard models. In the light of this capacity to adapt to laboratory conditions, and in order to ensure that we used the same unchanged bacterial strain all along the study, two methodological processes are crucial. First, the cryopreservation of the original environmental strain has to be done as quickly as possible after the environmental isolation. Secondly, while doing laboratory experiments, it is safer to always restart from the preserved stock and do not replicate colonies coming from previous active bacterial cultures.

Thirdly, through this thesis we showed that stress resistance response varied even among a unique genus (i.e., three *Kurthia* spp. showed highly different response after manganese supplementation on solid growth medium; chapter 3). Previous studies have shown similar results when studying how mineral supplementation affects the sporulation in different

Bacillus species. Indeed, the sporulation rate was influenced by calcium and/or manganese supplementation, but levels of supplementation to maximise sporulation rate were different in *B. cereus*, *B. licheniformis*, *B. subtilis* and *B. coagulans* (Sinnelä et al., 2019). Accordingly, not surprisingly, even in a standard bacterial model, such as *B. subtilis*, stress response, as well as any type of response to surrounding living conditions, might also vary. However, with standard models, most of the time the work is already done and differences between strains are documented, whereas this is not the case for non-standard models. Accordingly, it is time-consuming to fully describe non-standard models. As survival strategies vary even at the strain's level, the resulting recommendation here is to consider several strains when studying stress response in one bacterial species. This advice applies both for standard and non-standard models and stands also for the study of any other bacterial response, not only in the context of survival.

Last but not least, the culture conditions used in the laboratory matter and influence greatly the results of an experiment. For example, this thesis showed that a stress response was observed both for *Kurthia* sp. str. 11kri321 (i.e., modification of cell morphology and transient shutdown of translation/transcription) and *B. subtilis* (i.e., sporulation) only when manganese supplementation was made in solid growth medium and not in liquid medium (chapter 3). Accordingly, it is important before any experiment to consider the effect of different growth media often used for laboratory culturing (e.g., liquid broth versus solid agar medium). Such impact is often already reported for standard models, whereas it is not the case for non-standard models. In addition to liquid/solid growth medium, many other parameters could be varied when setting up the culture conditions (e.g., time or temperature of incubation) and it is important at the beginning of any experiment to assess the impact of those parameters. This first step should always be considered when planning the work and proper time should be allocated for this task.

3. Perspectives

In this thesis, we showed that *Kurthia* sp. str. 11kri321 responds to stress, either through the production of cryptospores or through an alternative survival strategy including a simpler modification of cell morphology (i.e., size reduction) and a transient shutdown of translation/transcription. The cryptospores might be alternative resistant structures to canonical complex endospores, but this would have to be tested. However, we saw through this thesis that the main problem is their production, as it was not easy for many reasons to work with this non-standard model under laboratory conditions, and particularly, because specific and complex conditions were required to trigger a survival strategy like the production of cryptospores. Accordingly, among the possible perspectives to consider, we would need to find such specific conditions to obtain cryptospores in higher frequency. In addition to combine several common abiotic environmental stresses such as low water availability (Esbelin et al., 2018) or starvation (Gray et al., 2019), we might also consider the role of biotic factors. Indeed, even if the geothermal spring where *Kurthia* sp. str. 11kri321 was isolated represents an extreme environment, other microorganisms are certainly also present and will share this niche. Accordingly, in its natural environment *Kurthia* sp. str. 11kri321 is probably

confronted to competition with other microbes, for nutrients or space, for example. Thus, cryptospores could represent a strategy displayed by *Kurthia* sp. str. 11kri321 to withstand this competitive pressure. Indeed, if competition is strong enough, microorganisms can escape the negative impacts associated to overcrowding by switching into a dormant state (Blath & Tóbiás, 2020; Lennon et al., 2021). Moreover, the production of resistant dormant structures in response to competition was also described for organisms other than bacteria. For example, when phytoplankton compete for limiting resources, they release chemicals that inhibit other species. Due to this competition and harmful chemicals, the dinoflagellate *Scrippsiella trochoidea* was shown to produce temporary resistant cysts (Fistarol et al., 2004). However, even under the specific required challenging conditions, the production of cryptospores might still be low due to the mutation observed in the N-terminal domain of Spo0A. In this context, it would be relevant to use phage transduction to introduce DNA from (crypto-)sporogenic into asporogenic mutants (Sastalla et al., 2010). This approach may allow to overcome the deletional mutation occurring in the N-domain of Spo0A and thus allows to restore the cryptosporulant phenotype and obtain cryptospores in higher frequency. Nonetheless, due to the instability and rapid evolution of *Kurthia* sp. str. 11kri321 under laboratory conditions, such a genetic manipulation might be quite challenging. Accordingly, it might be time-saving to first control that the observed deletional mutation in Spo0A really affects the production of (crypto-)spores. To do so, we could consider the opposite approach by using phage transduction to introduce the modified Spo0A into *B. subtilis* and observe if sporulation is disrupted.

The second perspective to consider is a better characterization of the different morphotypes observed in *Kurthia* sp. str. 11kri321. For example, it would be crucial to test the resistance of those morphotypes to see if a reduction of cell size provides any survival benefit to *Kurthia* sp. str. 11kri321. As a support for this hypothesis, when well-studied bacteria, such as *Vibrio* spp. and *Pseudomonas* spp., are confronted to starvation, the first noticeable change in their cell structure is dwarfing, with a reduction in size and a rounder shape (coccus morphology) (Arias et al., 2012; Kjelleberg et al., 1983).

Finally, when exploring cells of *Kurthia* sp. str. 11kri321 using cryo-EM we observed an outer layer that was not expected for a Gram-positive bacterium. Even if further investigations are necessary, we suggest that this outer layer could be an S-layer. This suggestion is speculative as nothing is known about S-layers in the non-standard model *Kurthia* spp. In order to make a stronger case for this hypothesis, it would be key to unravel the composition of the observed outer layer. However, first we need to assess under which conditions this putative S-layer is produced by *Kurthia* spp. We could therefore consider exposing *Kurthia* spp. to antibiotics as we already shown that penicillin seems to trigger a modification in the cell envelope of *Kurthia* sp. str. 11kri321 (Fig. 4).

4. Conclusion

When exploring stress resistance strategies in bacteria, much of the work had be done around sporulation in standard models like *B. subtilis*. Therefore, this study paves the way for using non-standard bacterial models to explore alternative stress resistance response. Despite all the challenges working with a non-standard model, including a rapid modification at the genomic level, we showed that *Kurthia* spp. respond to challenging growth conditions through production of cryptospores, different cell morphotype (i.e., differing mainly in cell size), cell envelope plasticity involving a putative S-layer, and transient shutdown of translation/transcription. In further studies, it would be interesting to continue exploring cryptospores and the different cell morphotypes, mainly with the objective to test resistance of these structures. Moreover, confirming the presence of an S-layer in *Kurthia* spp. would be key as this layer was shown to have a protective role in case of suboptimal environmental conditions (Madhurantakam et al., 2014). Overall, cataloging diversity of stress resistance strategies in non-standard bacterial models might help to solve several pressing societal issues, such as the on-going antibiotics resistance crisis (Shrivastava et al., 2018) or global climate change (Abbass et al., 2022). Indeed, resistant structures are key for bacteria in order to escape current antibiotics (Fisher et al., 2017; Lennon et al., 2021) and their ability to withstand climatic change makes them a source of microbial diversity for future microbial communities (Jones & Lennon, 2010; Wisnoski & Lennon, 2021).

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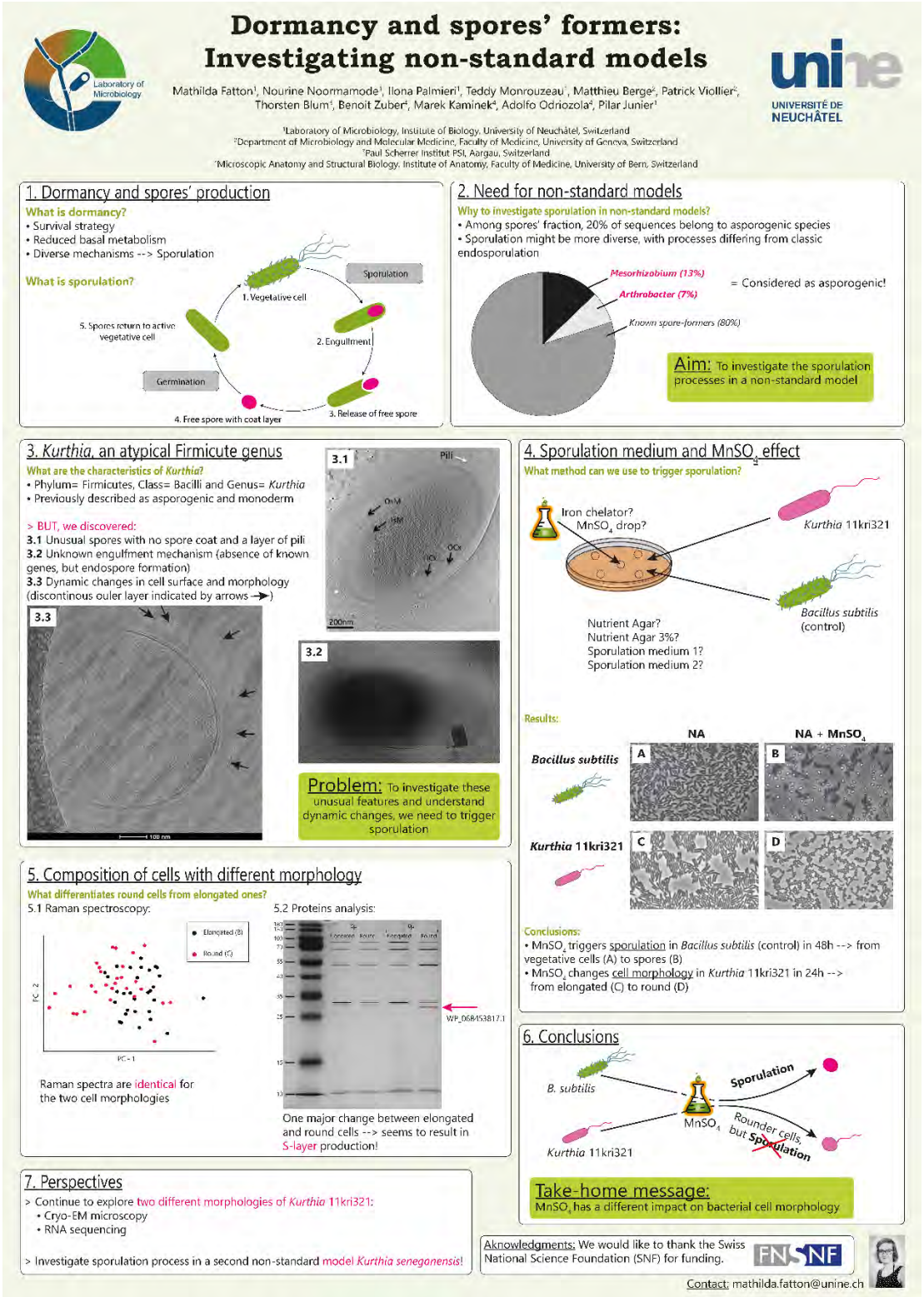
Appendices

I. Poster presentations

1. Bacterial, Morphogenesis, Survival and Virulence Conference (BMSV) 2019, Cape Town, South Africa, 24-28.11.2018



2. Annual PhD Meeting 2018, Neuchâtel, Switzerland, 06.09.2018

Dormancy and spores' formers: Investigate non-standard models

Mathilda Hayoz[1], Ilona Palmieri[1], Teddy Monrouzeau[1], Matthieu Berge[2], Patrick Viollier[2], Thorsten Blum[3], Benoit Zuber[4], Marek Kaminek[4], Adolfo Odriozola[4], Pilar Junier[1]

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[2]Department of Microbiology and Molecular Medicine, Faculty of Medicine, University of Geneva, Switzerland
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[4]Microscopic Anatomy and Structural Biology, Institute of Anatomy, Faculty of Medicine, University of Bern, Switzerland

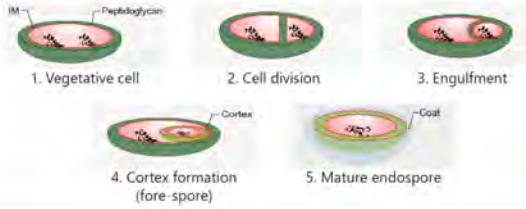
1. Dormancy and spores' production

What is dormancy?
- Strategy to respond to suboptimal environmental conditions
- Reduced basal metabolism
- Achieved through diverse mechanisms --> diverse dormant phenotypes

What are spores?
- Resting dormant cells created through morphological differentiation
- 3 stages:
  1) Initiation
  2) Resting
  3) Resuscitation
- Well-studied model = endospore-forming Firmicutes

2. Firmicutes

What are the characteristics of Firmicutes?
- Monoderm bacteria
- Spores' formation through endosporulation (ex. *Bacillus subtilis* on schema)

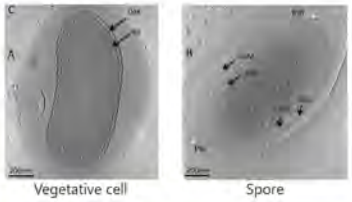


3. *Kurthia*, an atypicial Firmicute genus

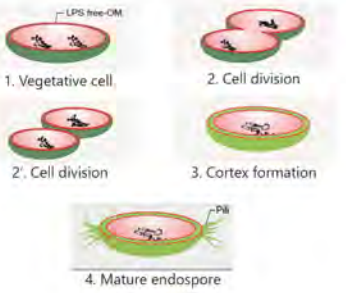
What are the characteristics of *Kurthia*?
- Phylum = Firmicutes, Class = Bacilli and Genus = *Kurthia*
- Previsouly described as asporogenic and monoderm

> BUT, we discovered:
- Diderm with unusual LPS-free outer membrane (OM)
- Spores' formation by modifying cell envelope structure

A. Morphology:

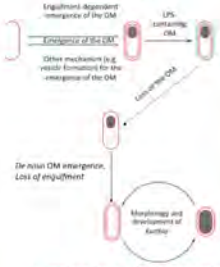


B. Hypothetical sporulation pathway:



C. Evolutionary context:

- *Kurthia* belongs to typically monoderm Bacilli
  > Presence of OM requires an explanation!



> Implications of this scenario:
- Explains the lack of genetic hallmarks of the canonical OM of other Gram negative Firmicutes
- Implicates that other atypical OM can be found within Firmicutes, but cannot be predicted from genetics, only

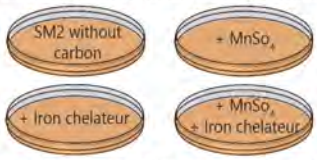
4. Aim

To investigate general morphology, composition of outer membrane, spores' resistance and sporulation process of *Kurthia* species.

IMPORTANT IMPLICATIONS = IMPORTANT MODEL

5. Experimental part

5.1 Collection of spores:
To obtain a collection of spores, we need to find a good sporulation medium:



--> Excess of $MnSo_4$ and iron chelateur are supposed to «confuse» the vegetative cells so that they sense a depletion of nutriments and respond by production of spores!

5.2 Composition of OM:
- DAPI and FM4-64 staining with exposition to different agents (like lysozyme)
- Cryo-TEM imaging

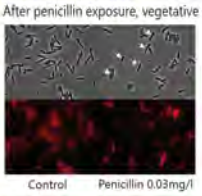
5.3 Spores' resistance:
Expose spores to:
- UV
- Dry/Wet high temperature

--> Transfer to nutrient medium to follow % of germination, because germination shows spores' survival!

5.4 Antibiotics resistance:
- DAPI and FM4-64 staining on vegetative cells and spores after exposure to penicillin

6. Primary results

Antibiotics resistance:
After penicillin exposure, vegetative cells are not stained with FM4-64 (membrane dye; red color):



Antibiotics seem to change membrane structure --> RESISTANCE through MORPHOLOGY!

7. Perspectives

> Continue experiments on *Kurthia* about:
- Morphology, including OM composition and pili presence
- Spores' resistance
- Sporulation process
- Antibiotics resistance through morphological changes

> Investigate a second non-standard model = *Arthrobacter*!

Aknowledgments: We would like to thank the Swiss National Science Foundation (SNF) for funding.

Contact: mathilda.hayoz@unine.ch

II. Other projects and collaborations

Diversity of lysis-resistant bacteria and archaea in the polyextreme environment of Salar de Huasco

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Abstract: The production of specialized resting cells is a remarkable strategy developed by several organisms to survive unfavorable environmental conditions. Spores are specialized resting cells that are characterized by low to absent metabolic activity and higher resistance. Spore-like cells are known from multiple groups of bacteria, which can form spores under suboptimal growth conditions (e.g., starvation). In contrast, little is known about the production of specialized resting cells in archaea. In this study, we applied a culture independent method that uses physical and chemical lysis, to assess the diversity of lysis resistant bacteria and archaea and compare it to the overall prokaryotic diversity (direct DNA extraction). The diversity of lysis-resistant cells was studied in the polyextreme environment of the Salar de Huasco. The Salar de Huasco is a highaltitude athalassohaline wetland in the Chilean Altiplano. Previous studies have shown a high diversity of bacteria and archaea in the Salar de Huasco, but the diversity of lysis-resistant microorganisms has never been investigated. The underlying hypothesis was that the combination of extreme abiotic conditions might favor the production of specialized resting cells. Samples were collected from sediment cores along a saline gradient and microbial mats were collected in small surrounding ponds. A significantly different diversity and composition were found in the sediment cores or microbial mats. Furthermore, our results show a high diversity of lysis-resistant cells not only in bacteria but also in archaea. The bacterial lysis-resistant fraction was distinct in comparison to the overall community. Also, the ability to survive the lysis-resistant treatment was restricted to a few groups, including known spore-forming phyla such as Firmicutes and Actinobacteria. In contrast to bacteria, lysis resistance was widely spread in archaea, hinting at a generalized resistance to lysis, which is at least comparable to the resistance of dormant cells in bacteria. The enrichment of Natrinema and Halarchaeum in the lysis-resistant fraction could hint at the production of cyst-like cells or other resistant cells. These results can guide future studies aiming to isolate and broaden the characterization of lysis-resistant archaea.

High diversity of lysis-resistant cells upon the application of targeted physical and chemical lysis to environmental samples originating from three different water bodies

Thomas Junier[1,2], Christophe Paul[1], Andrea Corona Ramirez[1], Sevasti Filippidou[1], **Mathilda Fatton**[1], Anaël Lehmann[3], Patrick S. Chain[4], Daniel Ariztegui[5], Torsten Vennemann[3], Patrick H. Viollier[6], Pilar Junier[1*]

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Abstract: In most habitats, fluctuating environmental conditions create periods of compromised survival for metabolically active organisms. In response, various survival strategies have evolved, including the formation of resilient resting cells. We assessed the biodiversity of the lysis-resistant bacteria in three different environments by applying a harsh physicochemical treatment to the samples. The bacterial diversity of the lysis-resistant fraction was compared with the bacterial diversity from the same environmental samples without the application of the enrichment method. As expected, in the lysis-resistant fraction, a significantly higher relative abundance of endospore-forming Firmicutes (for instance, *Bacillus, Clostridium* and *Paenisporosarcina*) was observed in comparison with the untreated samples. However, genera from which the existence of a resistant cell form is not yet reported were also highly enriched in comparison with the untreated samples. Our results suggest a more diversified repertoire of bacterial resistant cellular structures than previously thought.

The wild-type of flagellar filament of the Firmicute *Kurthia* at 2.8 Å resolution *in vivo*

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Abstract: Bacteria swim and swarm by rotating the micrometers long, helical filaments of their flagella. They change direction by reversing their flagellar rotation, which switches the handedness of the filament's supercoil. So far, all studied functional filaments are composed of a mixture of L- and R-state flagellin monomers. Here we show in a study of the wild type Firmicute *Kurthia* sp., that curved, functional filaments can adopt a conformation *in vivo* that is closely related to a uniform, all-L-state. This sheds additional light on transitions of the flagellar supercoil and uniquely reveals the atomic structure of a wildtype flagellar filament *in vivo*, including six residues showing clearly densities of O-linked glycosylation.

Bacterial spores, from ecology to biotechnology

Christophe Paul[1], Sevasti Filippidou[1], Isha Jamil[1], Wafa Kooli[1,2], Geoffrey L. House[2], Aislinn Estoppey[1], **Mathilda Hayoz**[1], Thomas Junier[1,3], Fabio Palmieri[1], Tina Wunderlin[1], Anaël Lehmann[4], Saskia Bindschedler[1], Torsten Vennemann[4], Patrick S.G. Chain[2], Pilar Junier[1]

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Abstract: The production of a highly specialized cell structure called a spore is a remarkable example of a survival strategy displayed by bacteria in response to challenging environmental conditions. The detailed analysis and description of the process of sporulation in selected model organisms have generated a solid background to understand the cellular processes leading to the formation of this specialized cell. However, much less is known regarding the ecology of spore-formers. This research gap needs to be filled as the feature of resistance has important implications not only on the survival of spore-formers and their ecology, but also on the use of spores for environmental prospection and biotechnological applications.

III. Lay communication

Foreword: I was always interested in science communication and I find crucial to foster sciences beyond academic circle. In this context, while doing my PhD, I had the opportunity to be part of a Service-Learning program, where I supervised a course for Bachelor students. My participation to this program resulted in the following publication that I authored.

frontiers in Education

CURRICULUM, INSTRUCTION, AND PEDAGOGY
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Microbes Go to School: Using Microbiology and Service-Learning to Increase Science Awareness and Fostering the Relationship Between Universities and the General Public

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Microbiology is an essential element of our everyday lives. This is not only the consequence of the importance of some pathogenic microorganisms, but also due to their positive and important role in processes related to human health, food production or waste recycling, to cite a few. However, the relevance of microbiology for the well-being of humankind and the planet still needs to reach beyond academic circles. Many current matters of urgency, such as environmental pollution or climate change, could partly be tackled by using green and sustainable solutions derived from microbial biotechnologies. Nevertheless, the wider public still attributes a negative connotation to microbes such as fungi and bacteria. The aim of this study was to implement service-learning as a pedagogical approach to increase scientific and microbiology literacy in society. Service-learning is a teaching and learning strategy that integrates community service and civic responsibility, fostering the dissemination of scientific knowledge. This approach is used to communicate about pressing societal issues surrounding microbes (antibiotic resistance, natural resources recycling, human microbiota, and food production among others) to an audience of pupils attending public schools. The activity of service-learning is proposed in the final year of the biology bachelor curriculum. Its purpose is to allow university students to develop and practice their lay communication skills as a key competence to be acquired during their university education. In the activity, the students developed their own concept and material for the activity and delivered it to pupils at primary and secondary school levels (pupils aged 6–15 years old). The message is also expected to be disseminated beyond the participants, when the students and pupils share their experience with family and friends. With this pedagogical approach, we intend to raise awareness regarding about the importance of microbiology and its dual roles as harmful and beneficial for humans. We also wish to increase the connection between primary, secondary and tertiary educational institutions. Finally, we want to highlight the importance of having a new generation of communication savvy professionals in biology.

Keywords: microbiology, service-learning, lay communication, antibiotic resistance, human microbiome, games

INTRODUCTION: BACKGROUND AND RATIONALE FOR THE EDUCATIONAL ACTIVITY INNOVATION

In spite of the overall positive role of microorganisms on health and well-being of humans and the environment, the general public often has a negative perception of the role of microbes in their everyday life. This is chiefly the consequence of the strong historical link made between microorganisms and diseases, which is bound to be reinforced as a consequence of the on-going pandemic. There is a clear need to change this negative perception, as microorganisms are not only essential for sustaining life on Earth, but also because of their incredible potential to be used for the benefit of humankind (e.g., pharmacologically active substances, bioremediation, soil fertility). This knowledge must reach beyond the scientific community to have an impact on society in a more direct way. Leading scientists in the field also identified this urgent need to increase awareness of microbiological science in society (Timmis et al., 2019). For this to happen, microbiology should be included in the current curriculum taught at obligatory schools, so that it becomes part of the general knowledge of future generations.

Along with the negative perception of microorganisms, an overall lack of scientific literacy can have important consequences for society. Having a scientifically literate citizenry is essential for making informed decisions ranging from an individual's daily life to developing sound public policy (Donovan and Schmitt, 2014). Moreover, our failure to create and maintain a link between education at school and academic research is sometimes considered as one of the reasons behind the dropping numbers of school pupils engaging in pursuing a career in science, technology, engineering, and mathematics (STEM) disciplines. Another emerging aspect of this barrier between academia and society is science skepticism and the rise of varied conspiracy theories (Van Der Linden, 2015). In response, many countries have developed specific initiatives to promote scientific literacy. One of the tools that can be effective at promoting the connection between science and society is service-learning (Felten and Clayton, 2011). Service-learning is a teaching and learning strategy that integrates community service with civic responsibility, fostering the dissemination of scientific knowledge (Soska et al., 2010). More specifically, service-learning can be defined as "*a course-based, credit-bearing educational experience in which students 1) participate in an organized service activity that meets identified community needs and 2) reflect on the activity in such a way as to gain further understanding of course content, a broader appreciation of the discipline and an enhanced sense of personal values and civic responsibility*" (Bringle et al., 2006; Felten and Clayton, 2011). Accordingly, as service-learning involves both students and the general community, it is an effective tool to connect science and society, in which the three concerned parties, i.e. scientific community, society, and students, all gain from the approach. Indeed, the idea behind service-learning is that the interaction between acquiring knowledge and skills and the development of a practical activity is key to learning, which is an essential aspect of academic studies (Ehrlich, 1996; Felten and Clayton, 2011).

Service-learning projects are diverse and vary depending on the local context, the targeted objectives, and the academic field in which it takes place. Such projects could integrate graduate as well as undergraduate students and the dedicated times could range from short-term modules to whole semester activities or even multiyear projects (Felten and Clayton, 2011). To maximize its effectiveness, service-learning must be used to directly communicate about societal issues in which both the scientific community and the target population have a common ground of interest. For this, microbiology offers a myriad of topics: food production or food spoilage, organic matter recycling, wastewater treatment, microbiome research and personalized medicine; all are just but a few examples of topics that can be used to this end. In this contribution, we present a case-study of undergraduate university students engaged during a whole semester activity as microbiology communicators to pupils at schools (6–15 years old) to help bridge the gap between scientific research and knowledge dissemination beyond the academic community. In our case, the activities were organized around societal issues that illustrate the two opposite sides of microbiology ("dark"-harmful side versus "bright"-beneficial side). We chose this analogy to the popular Star Wars Universe as a mean to reach the non-scientific audience (both children and adults) with easy-to-grasp examples. On the one hand, for the "dark side", the students mainly communicated about antibiotic resistance, which usually reflects the negative and problematic impacts of microbes by associating microbiology with diseases. On the other hand, the "bright side" was discussed through the discovery of bioactive compounds in nature, food production, and the role of the human microbiota on health, among others. All of the latter aimed at highlighting the positive role of microbes and their biotechnological potential.

Using antibiotic resistance as the basis for the development of teaching activities addresses one of the most pressing societal issues of our times. Indeed, antibiotic resistance has been recognized as a key research and policy area not only in Switzerland, but also worldwide (Shrivastava et al., 2018). It has been recognized that part of the problem stems from the inadequate use of antibiotics, and thus, users must be better informed on the appropriate use and limits of antibiotics. However, the measures designed to address this problem usually do not consider the pivotal role that universities can play in educating a new generation of professionals with the ability to better disseminate this message to the general population. Considering only antibiotic resistance for the service-learning activities could lead to further reinforce the association of microorganisms to diseases. Therefore, explaining the role of microorganisms for the discovery of new antimicrobial compounds also offers an opportunity to change the negative perception of the public. This allows to emphasize the role of the microbial resource not only as the origin of the problem (antibiotic resistance), but also as a potential solution (antimicrobials discovery). This shift in perception will ultimately allow us to appreciate that a large majority of microbes are beneficial to humans. The beneficial role

of microorganisms can further be strengthened by the selection of other issues in modern societies, like the challenge of food production to support the constant increasing worldwide population, natural resources recycling, soil bioremediation, or the study of human microbiota and its impact on human health.

The overall objective of our approach was to promote the dialogue between universities and society, by contributing to the development of teaching tools and materials for the dissemination at schools of scientific knowledge about pressing societal issues involving microbes. To reach this goal, we used the concept of service-learning (teaching tool) as part of the active training of new scientists at university level (bachelor level). Service-learning was used to teach school pupils (target public), which will constitute the entry gate for dissemination of scientific knowledge about antibiotic resistance and microbial diseases (the "dark side") and the roles of microbes in food production and human health (the "bright side"). At the same time, service-learning was used to teach university students how to transfer knowledge and communicate about microbiology to a non-scientific public. This represents an important soft skill for their future careers.

PEDAGOGICAL FRAMEWORK(S), PEDAGOGICAL PRINCIPLES, COMPETENCIES/STANDARDS UNDERLYING THE EDUCATIONAL ACTIVITY

Innovation in science education in Switzerland possesses unique challenges given the structure of the school system (regional responsibility at the level of each canton), as well as the multilingual nature of the country. Literacy in microbiology is rather neglected in the current curricula, as compared to other topics in biology. Therefore, in order to foster literacy in microbiology, we have developed an innovative teaching framework that includes the concept of service-learning where third-year bachelor university students deliver teaching activities at schools. This results in material and activities to discover microbiology with playful activities that are tailored for specific school levels. Service-learning allows university students to become more engaged with their local communities and to learn skills in communication and teaching. Service-learning is a modality of teaching that is considered an active learning strategy, as it requires the participants to first master the knowledge to disseminate, to then develop the class material and communicate about it. This level of engagement is highly beneficial to improve their learning capabilities and to develop their communication skills. Indeed, service-learning has been found to be positively related to students performance in STEM disciplines (Freeman et al., 2014). Moreover, this teaching approach also has a positive effect on the receiving communities (i.e. school pupils, but also their relatives), as it promotes dissemination of scientific knowledge to the society, and promotes careers in science (Donovan and Schmitt, 2014).

In order to provide the students with a sound background knowledge on the topics selected within the program and to help them to develop the tools required for the effective implementation of the service-learning activity, the preparatory lectures and practical training in microbiology of second-year bachelor students were restructured. This included partnering with the Tiny Earth Initiative, a global initiative that proposes the crowdsourcing (or even student-sourcing) of antibiotic discovery through the screening of bioactive compounds produced by soil microorganisms in the classroom (https://tinyearth.wisc.edu). The aim of this initiative is to introduce university students in biology to the field of microbiology by following the discovery journey of Fleming and other pioneers in the field of antibiotic research. In our case and given the emphasis of our research portfolio on environmental microbiology (including both bacteria and fungi), specific activities were developed for the second-year biology curriculum. These activities allowed to explore in parallel not only the issue of antibiotic resistance and antibiotic production, but also to expand the interest of our students in microbiology beyond human health. For this, we consider the beneficial use of microorganisms for plant growth promotion and protection against pests through biocontrol. In this way, third-year students participating in the service-learning activity have the knowledge and experience required to develop the scientific aspects of their activities.

METHODS: LEARNING ENVIRONMENT (SETTING, STUDENTS, FACULTY); LEARNING OBJECTIVES; PEDAGOGICAL FORMAT

Participants

The service-learning teaching module consists in 28 h of work in class + 28 h of personal work, equivalent to three credits in the European Credit Transfer and Accumulation System (ECTS). This course is offered during the last (sixth) semester of the bachelor in biology from the university of Neuchâtel (Switzerland) as an optional activity, which ensures the interest of the participants. At this crucial stage of their academic training, students are expected not only to be able to integrate the concepts learnt during their education, but they are also deciding about their future career paths, including specializing into teaching.

Pedagogical Format

The service-learning course was divided into five consecutives sections:

1. Selection of specific scientific topics within the societal issues already identified and choice of the target population (4 h): an initial presentation by the trainers is used to guide the students to narrow down the specific scientific topics to be developed.

2. Presentation of the planned activities among peers as well as to university trainers and schoolteachers (4 h): this allows discussing the complexity of the activity to be proposed and gives the participants the opportunity to share and develop their ideas together. This step helps the students to adjust their activity so that the knowledge they want to transfer is both scientifically sound and adjusted to pupil's age and background knowledge.
3. Preparation of the final activities (8 h): finalizing the teaching material.
4. Service-learning (10 h): actual work with pupils.
5. Final presentation (2 h): feedback among participants.

Learning Objectives

In terms of learning objectives, after taking the service-learning class, the students should be able to:

1) communicate a scientific message in a clear and concise way;
2) apply the knowledge acquired during the theoretical lectures in the development of the activities;
3) explain and illustrate biological phenomena in a simple way;
4) communicate with a non-academic public (e.g., avoid jargon, use of simple words and illustrative language);
5) develop a "playful" activity without missing scientific rigor;
6) evaluate knowledge transmission;
7) develop critical thinking in science;
8) work effectively in a group;
9) communicate with schoolteachers and pupils; and
10) plan and execute the activities.

Qualitative Data

To evaluate whether these objectives were attained and to grade each student individually, a combination of different types of assessments was used. Those consisted of:

1. The creation of an information leaflet (group work; 30% of the final grade); design of a 2-page leaflet summarizing the activity targeted at informing schoolteachers about the program. The evaluation was based on the ability of the students to explain the principles of service-learning in an easy-to-grasp way and to indicate the goal of their activities in a few words.
2. The implementation of an approach to evaluate the pupils following their activity (group work; 30% of the final grade); this aimed at ensuring that the activity developed and delivered by the students effectively allowed the transfer of knowledge during the service-learning. The format was free, but it had to be adapted to the age of the pupils.
3. Written statement (group work; 20% of the final grade); a short description of their activity (20 lines max.), in order to answer the following question: "How does your activity allow to achieve the transfer of knowledge to school pupils?". This assignment created a self-assessment and personal description of their activity and indicated indirectly their ability to work as a team as well as to assess whether the scientific content they wanted to transfer was clear to the students.
4. Personal written statement (individual work; 10% of the final grade); each student was asked to provide a short text about their personal experience within the service-learning activity. Two guiding questions were provided for this: "What was your main motivation to undertake this optional course?" and "How did this module contribute to your personal development?".
5. Feedback from the schoolteachers (10% of the final grade); the teachers were also asked to qualitatively evaluate the students' attitude, both overall and during their intervention in the classroom. The following questions were provided to guide them:
   - How was the communication with the students? Did the students consider your suggestions during the preparation and delivery of the activity?
   - How did the students communicate with the pupils? Were their communication efforts adequate to explain a scientific concept?
   - Were the scientific concepts presented pertinent to the age and background of the pupils?
   - How were the students organised overall (e.g., time management, readiness, preparation)?
   - Were the "playful" aspects of the activity suitable to the school system pedagogy and to the scientific objectives?

Data Analysis

To analyse the data provided by the students, pupils, and teachers, we proceeded as following:

Information leaflet (not Presented in This Study)

The quality of the leaflet was assessed by considering the following criteria:

- The description of the activity is complete, concise and accessible to a non-scientific public.
- The essential information is all present (e.g., contact information, partnership).
- The general layout is attractive.

Evaluation of the Pupils

The evaluations targeting the pupils were analysed according to pupils' age. The assessment was based on the level of information retained by the pupils, as compared to the content of the activity provided by the students.

Written Statement

The written descriptions of the activities were compiled and all identifying information removed. The first analysis of the text focused on the clarity of the descriptions provided by the students. Then, in a second stage, the category of the activity (e.g., creative, game), scientific content, and target audience (age of pupils), were determined.

Personal Written Statement

The information was also compiled and all identifying information removed. After an initial reading to assess the level of general understanding, several common themes highlighted by the students were identified. Those covered the development of personal and/or transversal skills and corresponded to "science communication", "teaching",

TABLE 1 | Service-learning activities proposed in 2020. For each activity, the category, scientific topic, target audience (standard school level according to harmonized -HarmoS- educational level defined by the « Conférence suisse des directeurs cantonaux de l'instruction publique (CDIP) »), and a brief description of the activity are provided.

Category	Scientific topic	Target audience (age bracket)	Description
Creative and experimental	Microbes in our body	2H (5–6 years old)	Crafting of "microbes" and place them in a sketch of the human body during the activities (**Figures 1A,B**). Perform a scientific experiment (grow mould on bread) to assess the impact of hand sanitation (**Figure 1C**)
Discovery and experimental	Finding microbes in the environment (our bodies and elsewhere)	7H (10–11 years old)	A cartoon was used to introduce a scientific quest to find microorganisms in different environments. This was combined with performing a "safe" at home isolation and observation experiment
Gaming	Characterizing different microbial groups (bacteria, fungi and viruses)	8H (11–12 years old)	A board game with daily activities to discover different groups of microbes and their specific features (**Figure 1D**). Field observations of microorganisms, drawings and other activities were included as part of the daily activities

"relationships and teamwork", "ability to adjust to unexpected events" and "creativity".

Feedback From the Schoolteachers

Two types of information were extracted from the feedback: 1) teachers' perception of the service-learning project in general and 2) specific feedback for the students individually. This second information was an additional criterion to grade the students individually at the end of the course. A summary of the teachers answers is presented in this study.

RESULTS TO DATE/ASSESSMENT

Academic Year 2019–2020

The service-learning course was proposed to last year bachelor students for the first-time during spring semester 2020. Ten students participated to the class. Three groups of three or four students prepared activities for three primary school classrooms (school levels 2, 7 and 8H; aged from 6 to 12 years old; **Table 1**) located in the Canton of Neuchâtel, Switzerland. The students introduced the topic of microbiology, with a highlight on its dual sides ("dark" versus "bright side"). The activities developed by the students were categorized depending on their content (**Table 1**; **Figure 1**). Given that the activities were created and conducted throughout the first lockdown in Switzerland due to the Covid-19 pandemic (March to June 2020), all the activities were adapted to an online format. For this, some students prepared informative videos accompanying their activities. "Safe" and easy-to-perform experiments, along with guiding documents, were then delivered to the pupils' homes to complement the activities.

Academic Year 2020–2021

During spring semester 2021, thirteen students selected the service-learning course; they were subsequently divided into six groups of two to three students. The groups prepared activities for pupils aged from 6 to 12 years old (school levels 3H–8H in the HarmoS system) in six primary school classrooms (**Table 2**) distributed in three schools located in the Canton of Neuchâtel, Switzerland. In 2021, the students also introduced the dual role of microbiology, through games, sports and creative activities (**Figure 2**). At this point, the sanitary conditions related to the Covid-19 pandemic allowed for frontal activities with the pupils, as well as for a visit to the university to use the classrooms and the scientific equipment available in the laboratories. In addition to this, some pupils went outside to observe microorganisms and their manifestation in the natural environment and to collect samples. In the lab, they observed these samples using stereoscopes and microscopes. Several groups also isolated microorganisms on agar-based media from environmental or daily-life samples. The approaches are all routinely used by most microbiologists. Such a practical approach had many pedagogical and teaching benefits. Indeed, most of the students noticed a positive effect on pupils' concentration and on the acquired knowledge when they were actively taking part to the activities. In addition, the observation of microorganisms in the natural environment, or from daily-life objects through isolation, helped pupils to realize that microbes are everywhere and not only linked to diseases and hospitals. Finally, visiting facilities at the university and using scientific routine material such as lab-coats, Petri dishes and pipettes, clearly created a scientific interest in these young children and may trigger their motivation to go to a higher education level such as university.

DISCUSSION ON THE PRACTICAL IMPLICATIONS, OBJECTIVES AND LESSONS LEARNED

The preparation of teaching material was a key component of the service-learning class. By offering the university students the possibility to actively participate from the design to the preparation and realization of their specific activities and material, the activity becomes their own and their investment greatly exceeds what they normally do for a traditional assessment. An important aspect is that this material requires the input and feedback of the schoolteachers, who are therefore essential for guiding the participants through the program to effectively reach the pupils and boost their interest. This reciprocal collaboration is one of the key elements in service-learning (Felten and Clayton, 2011). On the long term, the

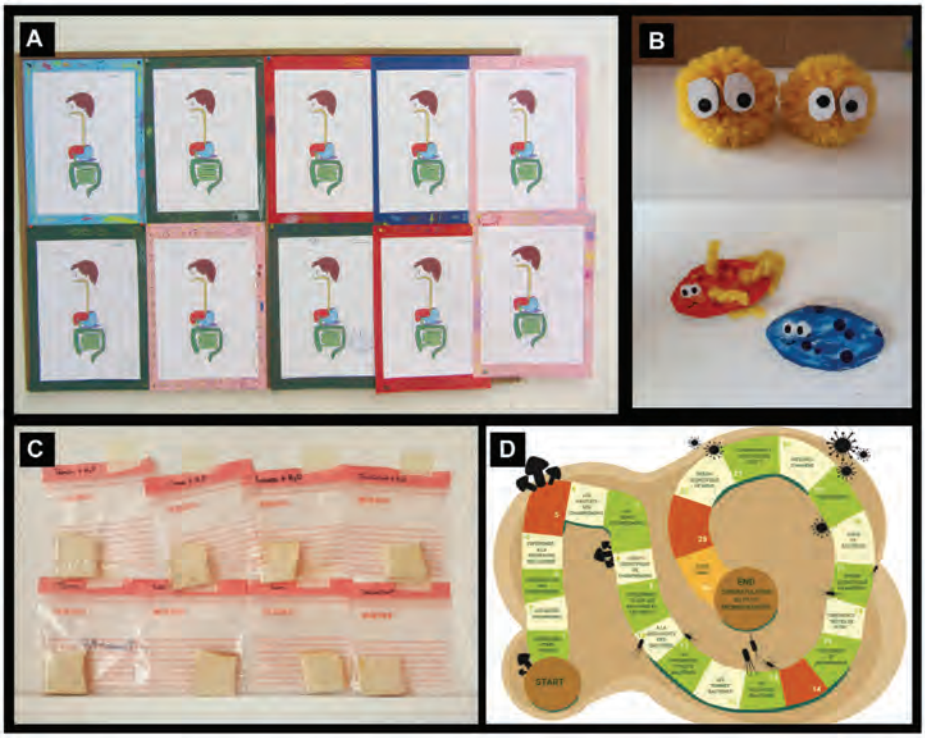

FIGURE 1 | Examples of the material created by the students in 2020 to develop their activities. Sketches of the human bodies coloured by the pupils **(A)**. In those sketches the children had to place the microbes they had crafted **(B)** to illustrate where they expected to find microorganisms in the human body. The children conducted a scientific experiment to assess the impact of mould on a piece of bread **(C)**. Illustration of the board game with daily activities around microbiology **(D)**.

TABLE 2 | Service-learning activities proposed in 2021. For each activity, the category, scientific topic, target audience (standard school level according to harmonized -HarmoS- educational level defined by the « Conférence suisse des directeurs cantonaux de l'instruction publique (CDIP) »), and a brief description of the activity are provided.

Category	Scientific topic	Target audience (age bracket)	Description
Creative and experimental	Microbes and antibiotic resistance	3H (6–7 years old)	Creation of a story about microbes, crafting of a microbe and creation of a fishing game to illustrate antibiotic resistance. Sampling in a pond and in soil to perform microscopic observations in the laboratory
Creative	Microbes, hygiene and food production	4H (7–8 years old)	Use famous story characters for children to introduce "bad" and "good" microbes (**Figure 2A**). Baking bread to illustrate the usefulness of microbes
Gaming and experimental	Environmental microbiology and link between microbiota and alimentation	6H (9–10 years old)	Board game about prevention of diseases through food habits and a healthy microbiota. Outdoors sampling and isolation of microorganisms. Microscopic observation in the laboratory
Gaming and experimental	Antibiotic resistance and development of novel treatments	7H/4H (10–11/ 7–8 years old)	Hospital board game with the aim to cure all sick patients (**Figure 2B**). Text with gaps to complete theoretical explanations. Laboratory experiment using antagonistic microorganisms and antibiotic disks
Gaming, artistic and experimental	Diversity of microorganisms	7H (10–11 years old)	Battle card game inspired from Pokemon to illustrate the diversity of microbes and their impact on humans and the environment (**Figure 2C**). Pixel art to familiarise pupils with microorganisms' shape. Microscopic observation
Sport, creative and experimental	Scientific method and environmental microbiology	8H (11–12 years old)	Board game with physical activities and orienteering race to illustrate the roles of microbes in sport (**Figure 2D**). Mind map to summarize concepts. Isolation of microbes through filtration of beverages in the laboratory

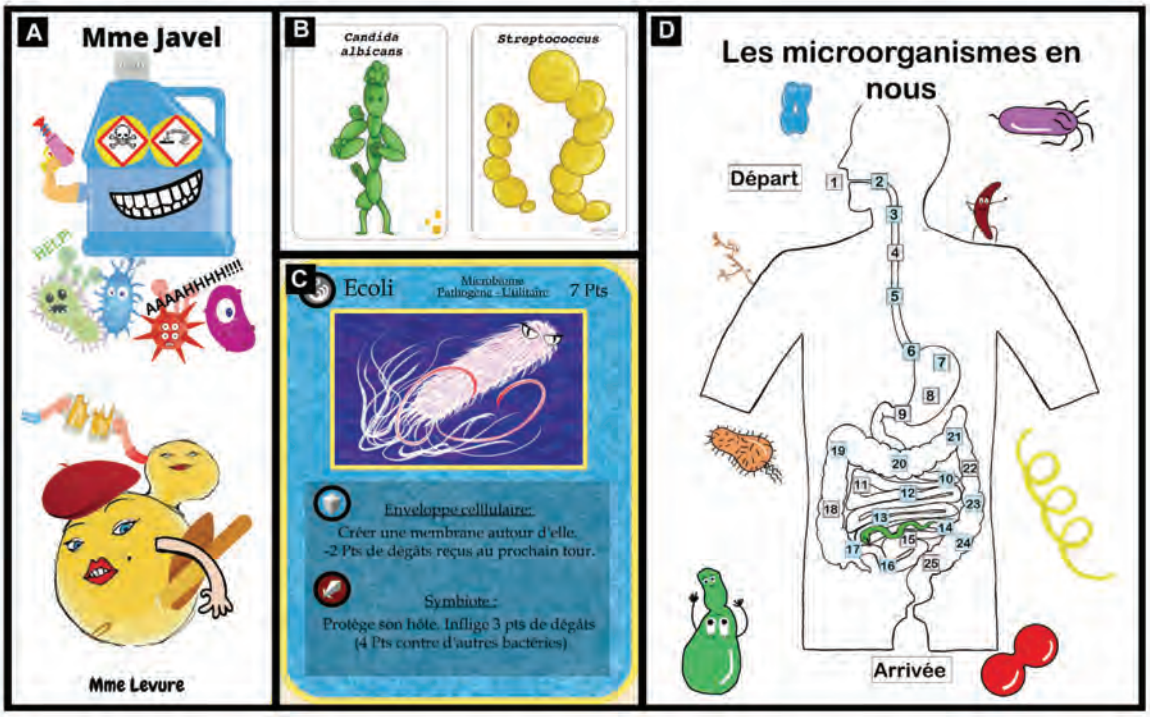

FIGURE 2 | Examples of the material created by the students in 2021 to develop their activities **(A)** Mr.-Mrs. comic characters (originally from kids books written and illustrated by Roger Hargreaves) designed here by the students to introduce the topic of microbiology **(B)** Card illustrating microorganisms that can be responsible of diseases **(C)** Card to play a battle Pokemon-like game with some "attack" and "defence" points unique to each microbe **(D)** Board game with physical challenges and questions about microbes.

material created by the students will be integrated in an electronic live repository containing presentations, document files, images, and videos. The content will constantly be updated with new examples of the different activities and some of them will be available to be used by other teachers and future participants to the program, which will allow to increase the scope of the activities beyond the target populations. Moreover, all the generated teaching material is accessible to other schoolteachers and proposed in English and three Swiss national languages (German, French, and Italian; https://unine.ch/lamun) to allow expanding this initiative beyond the Canton of Neuchâtel, in which French is the first language.

Beyond the Transfer of Knowledge–How Service-Learning Develops Personal Skills of University Students

The service-learning activity appeared as a useful tool to develop academic knowledge, civic learning and therefore, to connect academia and students with society and to spread knowledge. Beside this, the service-learning class also helped university students to develop personal and transversal skills (Eyler et al., 2001; Felten and Clayton, 2011). The latter were numerous and represented an important reason for students to sign up for this class and were identified by the participants as an asset at the end of the activity, as part of their self-assessment. These skills are presented below.

Science Communication

The students were all unanimous about the existence of a gap between scientists and society (e.g., "the scientific world tends, in my opinion, to remain compartmentalized within itself"). They also agreed that such a gap causes issues, such as "a lot of misunderstanding between the political world, the scientific world and the citizens" or promotes "skepticism from some people toward elitist and closed circles of which science is a part". In their mind, scientific popularization and transmission of knowledge is key to building a bridge between these two worlds, but at the same time, it is not an easy undertaking and requires training; "science communication is an integral part of biology and science in general as it allows to break the barrier between scientific/university community and the general public". The service-learning activity gave the students the opportunity to practice lay communication, which would both serve society ("to put science back in its place: in the hands of all"), and their personal career and projects ("the ability of making science popular is, in my eyes, useful in several circumstances, from working for a popularization journal to giving conferences about your research topic to non-specialists").

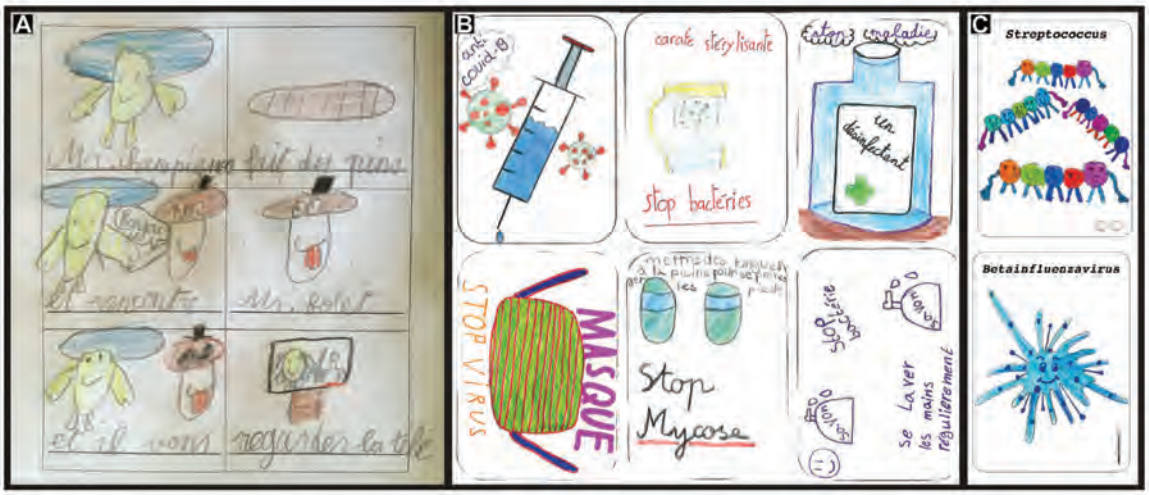

FIGURE 3 | Evaluation methods. For the 6 to 8 years old pupils, the children completed a small storyline with drawings and key words to illustrate that we use yeast to make bread **(A)**. In the evaluation method used for 9 to 11 years old pupils, the children could create their own bonus game card. Depending on the game, the pupils drew cards representing ways to be protected against microorganisms **(B)** or designed their own microorganisms **(C)**.

Teaching

Most of the enrolled students want to become teachers in the future and this was one of the main motivations to take part in the optional service-learning course; "my motivation to take this course was my ambition to work as a teacher in natural science later", "this course represents an opportunity to link teaching and science, two domains that are part of my interests and motivate me". The students largely acknowledged that participating in the service-learning activity allowed them to be confronted with challenges linked with teaching; "I was able to dive into the process behind the preparation of a lesson. It surprised me how time-consuming this task is", "I was not prepared for the need to explain biology using simplified terms, and to give simple but correct answers to questions in the interaction with kids. This definitely represents an experience that could be useful for my future career as a biology teacher.". Overall, students acquired new skills important for teaching, and more importantly, this experience empowered them; "a revelation, becoming a teacher is no more a simple objective, it is my main goal!", "step by step, I have found confidence in what I was teaching and saying", "the feedback from the children was encouraging and creates trust, making me think that I am on the right path for my future career".

Relationships and Teamwork

Beyond the objectives of teaching and scientific popularization, service-learning also aimed to connect people. Teamwork type skills (communication and patience) are crucial transversal skills useful for most careers. The service-learning activity was built to favor the exchanges between students, children, schoolteachers and all the other stakeholders involved in the project. This concept of teamwork was perceived as beneficial; "working in pairs of two was a bonus and I also appreciated the exchanges with the other students about the activity developed". The service-learning did not only benefit the children participating in the activities, but also to the students themselves as they learned and received a lot from the younger pupils; "not only a contributory act, but a reciprocal sharing I have learned a lot from the children, through their pleasantly surprising curiosity and motivation", "a pleasure to see the wonder in the eyes of all the kids when they were looking into the microscopes". At the end of the course, the importance of sharing science was even more present in the minds of the participants; "my principal interest was to share what I learned with kids in a manner that in turn will encourage them to share with their own family and friends", "I really want to keep this concept of a continuous exchange process".

Ability to Adjust to Unexpected Events

Even if the students prepared their activity ahead and planned it over the different sessions, it seems obvious that when working with young pupils, sometimes an activity can last longer than expected or could even be cancelled; "The best prepared lesson can actually happen in a way you have not intended". Therefore, the students had to be flexible and re-adapt their activities to the moment; "The main skill I acquired during this course is the ability to adapt to the situation, no matter how it evolves". The capacity to adapt is definitely an important skill in science. For example, when developing a research project where things do not develop as expected.

Creativity

According to conventional wisdom, creativity is oftentimes associated with artistic domains. Nonetheless, this skill could be valuable for scientists as well. Indeed, having well designed slides for a presentation or an attractive poster helps to convince a target audience about the importance of the research. Despite this, creativity is often neglected and traditional science courses at the university do not usually include such skills; "developing our creativity is undoubtedly beneficial as

being inventive is a considerable quality for a scientist. Unfortunately, I feel that we tend to make little use of this skill in our everyday life as undergraduate students". Creativity is particularly important when working with a young audience. Through the service-learning course, the students were able to develop their creativity by generating their own activity and material. Overall, this call for creativity was approved and praised by the students; "I immediately jumped on the occasion to link my passion for drawings", "having the opportunity to freely express my creativity was priceless".

Feedback–From the Pupils Side

All the pupils attending the activities enjoyed the activities and were eager to find out about the next session offered by the university students. Interestingly, in one class considered as difficult to handle by their schoolteacher, all pupils were able to concentrate and listen quietly for a full period of teaching by the students. This highlights that non-formal, or out-of-the-box, teaching may be a way to foster interests in otherwise non-studious pupils. Beyond this positive feeling, the most impressive and encouraging fact was the indirect feedback provided in the form of the evaluation forms that the pupils had to complete at the end of the service-learning activity. Even the teachers themselves were amazed about the quantity of complex scientific words and concepts the pupils retained all along the activity. The evaluation format was different depending on the age of the pupils. For the younger ones (6–8 years old), the evaluation was mainly based on drawings and oral discussions. For example, during the last sessions, some pupils had to draw their own microbes and a small storyline about what they have learnt during the service-learning activity (**Figure 3A**). This feedback showed that thanks to service-learning, even at a young age, pupils remembered that microorganisms are not only linked to disease, but that they can also be beneficial for humans and for the environment. The students that conducted games with pupils aged 9 to 11 years old evaluated them through the creation of bonus cards to be included in the board or card game they created. The pupils mostly drew cards representing ways to be protected against microorganisms and this highlights that the game proposed was efficient to show pupils that hygiene is important to cohabit with microorganisms (**Figures 3B,C**). Lastly, for older pupils (11 to 12 years old), the evaluation consisted in a multiple-choice quiz. Even though this quiz addressed complex scientific concepts, most of the pupils succeeded, showing that service-learning is an efficient pedagogic approach to transfer knowledge.

Feedback–From Schoolteachers

The schoolteachers that took part in the project also enjoyed this experience. They agreed that the university students did a great work by preparing activities that were well adapted to the pupils. The communication with the students was smooth and their suggestions were always considered. Overall, the feedback from the teachers supports the fact that service-learning is a beneficial teaching strategy and could be easily integrated into the classic schooling program. This is even more true for pupils with learning difficulties, as a different pedagogic method brought in by external teachers can help them to reconnect with the school system.

Conclusion: Putting the Service-Learning Activity Into Perspective

The data and information presented in this case study show that the students enrolled in the service-learning module benefited from this activity on multiple aspects. As shown by previous studies, the service-learning activity exposed students to new people and experiences that potentially lead them to consider a career path (Jones and Abes, 2004; Fitch, 2005). In our case, this is well illustrated by the fact that after the course, many of the students confirmed their desire to become schoolteacher. This desire to transfer knowledge to a younger generation is also in agreement with a previous study claiming that service-learning contributes to enhanced civic engagement (Pascarella and Terenzini, 2005). In addition, our study clearly highlighted that service-learning has a positive effect on personal development, as shown in previous studies in the field (Eyler et al., 2001). In conclusion, the results obtained demonstrate that service-learning is an efficient tool to disseminate microbiology knowledge beyond the academic world and to connect science and society. More concretely, this tool allowed the connection between university students and school pupils using microbiology as common ground (Astin et al., 2000).

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding authors.

AUTHOR CONTRIBUTIONS

MF, AS, AB, SB, and PJ contributed to the conception and design of the study. MA, LH, GH, YGF, AP, MP, and CV performed the activities. MF, AS, and SB organized the activities database. MF, and PJ wrote the first draft of the manuscript. All authors contributed to manuscript revision, read, and approved the submitted version.

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IV. Curriculum Vitae

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Born on April 20, 1993, Swiss nationality
Married, 1 child

Education:

Sep. 2018 – Oct. 2022	PhD in microbiology Laboratory of microbiology, University of Neuchâtel, 2000 Neuchâtel
Sep. 2016 – Jun. 2018	Master in biology – Grade: summa cum laude University of Neuchâtel, 2000 Neuchâtel
Sep. 2012 – Jun. 2015	Bachelor in biology – Grade: cum laude University of Neuchâtel, 2000 Neuchâtel

Academic prize:

Sep. 2018	Prix Louis-Paris, University of Neuchâtel

Academic experience:

Molecular biology and basic techniques in microbiology

Monthly sampling at wastewater treatment plant (STEP, La Chaux-de-Fonds) for microbiological analyses

Coordination for the writing of a report mandated by the canton of Neuchâtel due to the contamination of the river l'Areuse and the lake by cyanobacteria

Supervision of practical courses in microbiology and molecular biology

Supervision of a service-learning course

Academic publications:

Fatton, M., Filippidou, S., Junier, T., Cailleau, G., Berge, M., Poppleton, D., ... & Junier, P. (2022). Cryptosporulation in Kurthia spp. forces a rethinking of asporogenesis in Firmicutes. *Environmental Microbiology*.

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Work experience:

May 2017 – Nov. 2017	Internship in agri-food domain, microbiological quality control NQAC, Nestlé, 1350 Orbe
Jan. 2012 – May 2012	Laboratory work and order preparation in pharmacy domain Pharmacie de « La Grand-Rue », 2034 Peseux
May 2012 – Jun. 2012	Hospital internship (nursing school not pursued) Hôpital de La Providence, 2000 Neuchâtel

Informatic skills:

Microsoft Office: Word, Excel, Power Point
R Studio
Adobe: Illustrator, Photoshop, Creator, InDesign

Languages:

French: native language
English: academic domain
German: school level

Personal skills:

Teamwork, enthusiastic and motivated
Stress and priority management

References:

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