Dual functionality of the amyloid protein TasA in *Bacillus* physiology and fitness on the phylloplane

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Bacteria can form biofilms that consist of multicellular communities embedded in an extracellular matrix (ECM). In *Bacillus subtilis*, the main protein component of the ECM is the functional amyloid TasA. Here, we study further the roles played by TasA in *B. subtilis* physiology and biofilm formation on plant leaves and in vitro. We show that ΔtasA cells exhibit a range of cytological symptoms indicative of excessive cellular stress leading to increased cell death. TasA associates to the detergent-resistant fraction of the cell membrane, and the distribution of the fliotillin-like protein FloT is altered in ΔtasA cells. We propose that, in addition to a structural function during ECM assembly and interactions with plants, TasA contributes to the stabilization of membrane dynamics as cells enter stationary phase.
In response to a wide range of environmental factors\(^{1,2}\), some bacterial species establish complex communities called biofilms\(^3\). To do so, planktonic cells initiate a transition into a sedentary lifestyle and trigger a cell differentiation program that leads to: (1) a division of labor, in which different subpopulations of cells are dedicated to covering different processes needed to maintain the viability of the community\(^4,5\), and (2) the secretion of a battery of molecules that assemble the extracellular matrix (ECM)\(^6,7\).

Studies of *Bacillus subtilis* biofilms have contributed to our understanding of the intricate developmental program that underlies biofilm formation\(^7-10\) that ends with the secretion of ECM components. It is known that the genetic pathways involved in biofilm formation are active during the interaction of several microbial species with plants\(^1,11,12\). In *B. subtilis*, the lipopeptide surfactin acts as a self-trigger of biofilm formation on the melon phylloplane, which is connected with the suppressive activity of this bacterial species against phytopathogenic fungi\(^13\). Currently, the *B. subtilis* ECM is known to consist mainly of exopolysaccharide (EPS) and the TasA and BslA proteins\(^7\). The EPS acts as the adhesive element of the biofilm cells at the cell-to-surface interface, which is important for biofilm attachment\(^14\) and BslA is a hydrophobin that forms a thin external hydrophobic layer and is the main factor that confers hydrophobic properties to biofilms\(^15\). Both structural factors contribute to maintain the defense function performed by the ECM\(^11,15\). TasA is a functional amyloid protein that forms fibers resistant to adverse physicochemical conditions that confer biofilms with structural stability\(^16,17\). Additional proteins are needed for the polymerization of these fibers: TapA appears to favor the transition of TasA into the fiber state, and the signal peptide SipW processes both proteins into their mature forms\(^18,19\). The ability of amyloids to transition from monomers into fibers represents a structural, biochemical, and functional versatility that microbes exploit in different contexts and for different purposes\(^20\).

Like in eukaryotic tissues, the bacterial ECM is a dynamic structure that supports cellular adhesion, regulates the flux of signals to ensure cell differentiation\(^21,22\), provides stability and serves as an interface with the external environment, working as a formidable physicochemical barrier against external assaults\(^23-25\). In eukaryotic cells, the ECM plays an important role in signaling\(^26,27\) and has been described as a reservoir for the localization and concentration of growth factors, which in turn influence cell fate, e.g., by activating the apoptotic program\(^31,32\). In both eukaryotes and prokaryotes, senescence involves global changes in cellular physiology, and in some microbes, this process begins with the entry of the cells into stationary phase\(^33-35\). This process triggers a response typified by molecular mechanisms evolved to overcome environmental adversities and to ensure survival, including the activation of general stress response genes\(^36,37\), a shift to anaerobic respiration\(^38\), enhanced DNA repair\(^39\), and induction of pathways for the metabolism of alternative nutrient sources or sub-products of primary metabolism\(^40\).

Based on previous works\(^13\), we hypothesize that the ECM makes a major contribution to the ecology of *B. subtilis* in the poorly explored phyllosphere. Our study of the ecology of *B. subtilis* NCIB3610-derived strains carrying single mutations in different ECM components in the phyllosphere highlights the role of TasA in bacteria-plant interactions. Moreover, we demonstrate a complementary role for TasA in the stabilization of the bacteria’s physiology. In *ΔtasA* cells, gene expression changes and dynamic cytological alterations eventually lead to a premature increase in cell death within the colony. Complementary evidences prove that these alterations are independent of the structural role of TasA in ECM assembly. All these results indicate that these two complementary roles of TasA, both as part of the ECM and in contributing to the regulation of cell membrane dynamics, are important to preserve cell viability within the colony and for the ecological fitness of *B. subtilis* in the phylloplane.

**Results**

**TasA contributes to the fitness of *Bacillus* on the phylloplane.**

Surfactin, a member of a subfamily of lipopeptides produced by *B. subtilis* and related species, contributes to multicellularity in *B. subtilis* biofilms\(^41\). We previously reported how a mutant strain defective for lipopeptide production showed impaired biofilm assembly on the phylloplane\(^13\). These observations led us to evaluate the specific contributions made by the ECM structural components TasA and the EPS to *B. subtilis* fitness on melon leaves. Although not directly linked to the surfactin-activated regulatory pathway, we also studied the gene encoding the hydrophobin protein BslA (another important ECM component). A *ΔtasA* mutant strain (*ΔtasA*) is defective in the initial cell attachment to plant surfaces (4 h and 2 days post-inoculation) (Fig. 1A). As expected, based on their structural functions, all of the matrix mutants showed reduced adhesion and survival (Supplementary Figs. 1A and 1B); however, the population of *ΔtasA* cells continuously and steadily decreased over time compared to the populations of *eps* or *bslA* mutant cells (Fig. 1B and Supplementary Fig. 1B). Examination of plants inoculated with the wild-type strain (WT) or with the *ΔtasA* strain via scanning electron microscopy (SEM) revealed variability in the colonization patterns of the strains. WT cells assembled in ordered and compact colonies, with the cells embedded in a network of extracellular material (Fig. 1C, top). In contrast, the *ΔtasA* cells were prone to irregular distribution as large masses of cells on the leaves, which also showed collapsed surfaces or lack of surface integrity, suggesting alterations in cellular structures (Fig. 1C, center). Finally, *eps* and *bslA* mutant cells formed flat colonies (Supplementary Fig. 2A) with the same colonization defects observed in the *ΔtasA* mutant cells (Supplementary Fig. 1C).

Based on the reduced fitness exhibited by the single ECM component mutant strains and their deficiencies in biofilm formation, we hypothesized that these strains may also be defective in their antagonistic interactions with *Podosphaera xanthi* (an important fungal biotrophic phytopathogen of crops\(^42\)) on plant leaves. Strains with mutations in *eps* and *bslA* partially ameliorated the disease symptoms, although their phenotypes were not significantly different from those of the WT strain (Supplementary Fig. 1D). However, contrary to our expectations, the *ΔtasA* strain retained similar antagonistic activity to that of the WT strain (Fig. 1D). The simplest explanation for this finding is that the antifungal activity exhibited by the *ΔtasA* cells is due to higher production of antifungal compounds. In situ mass spectrometry analysis revealed a consistently higher relative amount of the antifungal compound plipastatin (also known as fengycin, the primary antifungal compound produced by *B. subtilis*) on leaves treated with *ΔtasA* cells compared to those treated with WT cells (Fig. 1E). These observations argue in favor of the relevance of the ECM and specifically TasA in the colonization, survival, and antagonistic activity of *B. subtilis* on the phylloplane.

**Loss of TasA causes a global change in bacterial cell physiology.**

The increased fengycin production and the previously reported deregulation of the expression pattern of the *tapA* operon in a *ΔtasA* mutant strain\(^23\) led us to explore whether loss of *tasA*
Fig. 1 TasA is essential for the fitness of Bacillus on the melon phylloplane. 

a Adhesion of the WT, ΔtasA and JC81 (TasA Lys68Ala, Asp69Ala) strains to melon leaves at 4 h (hpi) and 2 days post-inoculation (dpi). Statistically significant differences between WT and ΔtasA were found at 2 dpi. At 4 hpi, N = 3 for all the strains. At 2 dpi N = 6 for the WT strain, N = 6 for the ΔtasA strain and N = 3 for the JC81 strain. N refers to the number independent experiments. In each experiment, 10 leaves were analyzed. Average values are shown. Error bars represent the SEM. Statistical significance was assessed via two-tailed independent t-tests at each time-point (*p value = 0.0262).

b The persistence of the ΔtasA cells at 21 days was significantly reduced compared with that of the WT cells. The persistence of JC81 cells on melon leaves was reduced compared to that of the WT cells. The first point is taken at 4 hpi. Average values of five biological replicates are shown with error bars representing the SEM. Statistical significance was assessed by two-tailed independent t-test at each time-point (*p value = 0.0329).

c Representative scanning electron microscopy micrographs of inoculated plants taken 20 days post-inoculation show the WT cells (top) distributed in small groups covered by extracellular material and the ΔtasA cells (bottom) in randomly distributed plasters of cells with no visible extracellular matrix. JC81 (TasA Lys68Ala, Asp69Ala) strain shows an intermediate colonization pattern between those of the WT and ΔtasA null mutant strains. Scale bars = 25 µm (left panels) and 5 µm (right panels). Experiments have been repeated at least three times with similar results.

d The WT and ΔtasA strains showed comparable biocontrol activity against the fungal phytopathogen Podosphaera xanthii. However, JC81 (TasA Lys68Ala, Asp69Ala) strain failed to control the disease. Biocontrol activity was measured after 15 days post-inoculation of the pathogen. For the WT strain, N = 22. For the ΔtasA strain and JC81 strain, N = 12. For the Δpps strain, N = 9. N refers to number of plants analyzed over three independent assays. Three leaves per plant were infected and inoculated. Average values are shown with error bars indicating the SEM. The Δpps is a mutant strain in fengycin production and it is used as a negative control. Statistical significance was assessed by two-tailed independent Mann–Whitney tests between each strain and the Δpps mutant (****p < 0.0001).

e MALDI-TOF/TOF MS analysis revealed higher fengycin levels on melon leaves treated with ΔtasA (right) cells compared with that on leaves treated with WT cells (left) after 20 days post-inoculation. Source data are provided as a Source Data file.
disrupts the genetic circuity of the cells. We sequenced and analyzed the whole transcriptomes of ΔtasA and WT cells grown in vitro on MSgg agar plates, a chemically defined medium specifically formulated to support biofilm. We observed that deletion of tasA resulted in pleiotropic effects on the overall gene expression profile of this mutant (Fig. 2A and Supplementary Fig. 3A), with 601, 688, and 333 induced genes and 755, 1077, and 499 repressed genes at 24, 48, and 72 h, respectively (Supplementary Fig. 3A). A closer look at the data allowed us to cluster the expression of different genes into groups with similar expression profiles over the time course (Fig. 2A). In general, four different expression profiles were found in which genes show positive (profiles 1 and 2) or negative (profiles 3 and 4) variations from 24 h to 48 h, and genes with expression levels that remain either stable (profiles 1 and 3) or altered (profiles 2 and 4) from 48 h to 72 h (Fig. 2A). Profiles 1 and 2 included genes related to the SOS response (profile 1), transcription and replication (profile 1 and 2), purine biosynthetic process (profile 2), and toxin-antitoxin systems (profile 2). Profiles 3 and 4 included genes related to sporulation (profiles 3 and 4), cellular metabolism in general (profile 3) and lipids (profile 3), carbohydrates (profile 3 and 4), monosaccharides (profile 3), polysaccharides (profile 4), or peptidoglycans (profile 3) in particular. These gene expression profiles reflect a general picture that suggests: (i) the existence of cellular stress and DNA damage, in which the cells need to fully activate different sets of genes to cope with and compensate for the damage and maintain viability, (ii) a decrease in the overall cellular energy metabolism, and (iii) strong repression of the sporulation pathway. To study the observed alterations in gene expression in ΔtasA cells, the differentially expressed genes at all the time points were classified into their different regulons. Indeed, the sigK, sigG, gerR, and gerE regulators (Supplementary Data 2 and 3), which control the expression of many of the genes related to sporulation, were repressed in the ΔtasA cells from 48 h (Fig. 2B and Supplementary Fig. 3B), consistent with the delayed sporulation defect previously reported in ECM mutants10,23 (Supplementary Fig. 4). In contrast, the expression levels of biofilm-related genes, including the epsA-O, and tapA operons, were higher in the ΔtasA cells at all times compared to their expression levels in WT cells (Fig. 2B and Supplementary Fig. 3B) (Supplementary Data 1–3). We found repression of sinR at 24 h (Supplementary Data 1), induction of the slrR transcriptional regulator at all times (Supplementary Data 1–3), and repression of the transition state genes transcriptional regulator abrB at 24 h and 48 h (Supplementary Data 1 and Supplementary Data 2), which could explain the induction of the ECM-related genes.

The analysis of the transcriptional changes in the tasA mutant cells highlighted the broad metabolic rearrangements that take place in ΔtasA colonies from 24 h to 72 h, including the expression alteration of genes implicated in energy metabolism, secondary metabolism and general stress, among other categories (Supplementary Data 1–3, Fig. 2B and Supplementary Fig. 3B). First, the alsS and alsD genes, which encode acetolactate synthase and acetolactate decarboxylase, respectively, were clearly induced at all times (Supplementary Data 1–3). This pathway feeds pyruvate into acetoin synthesis, a small four-carbon molecule that is produced in B. subtilis during fermentative and overflow metabolism. Additionally, we found induction of several regulators and genes that are involved in anaerobic respiration and fermentative metabolism. The two-component regulatory system resD and resE, which senses oxygen limitation, and their target genes, were induced in ΔtasA cells at 24 h and 48 h (Supplementary Data 1 and 2 and Supplementary Fig. 3B). Consistently, induction of the transcriptional regulator fnr and the anaerobic respiration related genes narGHIJK, which encode the nitrate reductase complex, as well as all of the proteins required for nitrate respiration were induced at 24 h and 48 h (Supplementary Data 1 and 2 and Supplementary Fig. 3B). Second, we observed induction at all times of the genes involved in fengycin biosynthesis (Supplementary Data 1–3), consistent with the overproduction of this antifungal lipopeptide in planta (Fig. 1E), genes involved in the biosynthesis of surfactin, subtilosin, bacilysin, and bacillaene (all secondary metabolites with antimicrobial activities45–48) (Fig. 2B, Supplementary Fig. 3B and Supplementary Data 1–3), as well as the operon encoding the iron-chelating protein bacillibactin (dbhACEBF) (Supplementary Data 1–3). The induction of all of these genes is possibly due to the repression of transcriptional regulators of transition state genes that occurs at 24 h and 48 h, e.g., abrB (which controls activation of the genes involved in the synthesis of fengycin, bacilysin, subtilosin, and bacillaene) and abh (which contributes to the transcriptional control of the genes involved in surfactin production) (Supplementary Data 1 and 2). The transcriptional changes of other regulators, such as resD (for subtilosin) or comA (for surfactin), both upregulated at 24 h and 48 h (Supplementary Data 1 and 2), also contribute to the induction of the genes that participate in the synthesis of all of these secondary metabolites and might explain their overall activation at 72 h (Fig. 2B). Finally, the gene encoding the regulator AscR was induced at 48 h and 72 h. AscR controls transcription of the snaA (snaA-

Additional signs of excess cellular stress in the ΔtasA cells were: (i) the strong overexpression of the sigma factor SigB (β) at 24 h and 72 h (Fig. 2B, Supplementary Fig. 3B and Supplementary Data 1 and 3, which controls the transcription of genes related to the general stress response36, and (ii) the repression at 24 h of lecA (Supplementary Data 1), a transcriptional repressor of the SOS response regulon, as well as the induction of other genes that confer resistance to different types of stress, i.e., ahpC and ahpF (induced at all times, Supplementary Data 1–3) against peroxide stress or liaH and liaI (induced at 48 h, Supplementary Data 2), which confer resistance to cell wall antibiotics. Indeed, ~41% of the SigB-regulated genes are induced at 24 h (Supplementary Fig. 3B and Supplementary Data 1), and these genes are involved in multiple and different functions, including protease and chaperone activity, DNA repair or resistance against oxidative stress. At 72 h, ~10% of the genes of the SigB regulon were still upregulated, suggesting the existence of cellular stress during colony development (Fig. 2B and Supplementary Data 3). Furthermore, the activation of the SOS response points toward the existence of DNA damage in ΔtasA cells, another sign of stress, with induction of uvrA (at 24 and 72 h, Supplementary Data 1 and 3) and uvrB (at 24 h, Supplementary Data 1), both of which are involved in DNA repair. The presence of DNA damage in ΔtasA cells is further indicated by the induction of almost all of the genes belonging to the lysogenic bacteriophage PBSX at 72 h, a feature that has been reported to occur in response to mutations as well as to DNA or peptidoglycan damage51,52 (Fig. 2B and Supplementary Data 3).

In general, the transcriptional changes observed in the ΔtasA cells illustrate an intrinsic major physiological change that
Fig. 2 The tasA mutant displays major gene expression changes. a Heatmap and gene profiles (1–4) of genes with similar expression patterns. Genes in the same profile are categorized (right) according to their gene ontology (GO) terms. Each gene profile represents statistically significant genes. Statistical significance was assessed by a $\chi^2$ test comparing the number of clustered genes with the expected theoretical number of genes if gene distribution were random. In the heatmap, induced genes are colored in dark gray and repressed genes in light gray. b Differentially expressed genes at 72 h clustered into different regulons. The bigger circles indicate the main regulator of that regulon, which is surrounded by arrows pointing to smaller circles that are the differentially expressed genes. The thickness of the arrows indicates expression levels. The color in the arrows indicates induction (red) or repression (green).
progresses over time and suggest the accumulation of excessive cellular stress. These changes result in the early entry of the cells into stationary phase, indicated by the state of the ΔtasA colony at 72 h compared to the WT (Fig. 2B) and supported by increased expression levels of genes related to: (i) biofilm formation (ii) synthesis of secondary metabolites (siderophores, antimicrobials, etc.); (iii) anaerobic respiration, fermentative metabolic pathways, and overflow metabolism; (iv) paralogous metabolism and assimilation of modified or toxic metabolic intermediates; (v) general stress and DNA damage; and (vi) induction of the lysogenic bacteriophage PBSX.

ΔtasA cells exhibit impaired respiration and metabolic activity. Our transcriptomic analysis suggested that ΔtasA cells exhibit a shift from aerobic respiration to fermentation and anaerobic respiration as well as activation of secondary metabolism, physiological features typical of stationary phase cells38,53. Based on the higher abundance of fengycin on leaves treated with ΔtasA cells and its key role in the antagonistic interaction between B. subtilis and fungal pathogens, we further investigated the kinetics of fengycin production in vitro. Flow cytometry analysis of cells expressing YFP under the control of the fengycin operon promoter demonstrated the induction of fengycin production in a subpopulation of cells (26.5%) at 48 h in the WT strain. However, more than half of the ΔtasA population (67.3%) actively expressed YFP from the fengycin operon promoter at this time point (Fig. 3A, top). At later stages of growth (72 h), the promoter was still active in the ΔtasA cells, and the population of positive cells was consistently higher than that in the WT strain (Fig. 3A, bottom). Mass spectrometry analysis of cell-free supernatants from WT or ΔtasA MOLP (a medium optimized for lipopeptide production) liquid cultures demonstrated that this expression level was sufficient for the tasA mutant cells to produce nearly an order of magnitude more fengycin (Fig. 3B, bottom spectrum) consistent with our findings in plants (Fig. 1E). Additionally, relatively higher levels of fengycin were detected in cells or agar fractions of ΔtasA colonies compared to WT colonies grown on solid MSgg, the medium used in all of our experimental settings (Supplementary Fig. 5, top and bottom spectra respectively). Similar results were obtained for the lipopeptide surfactin in these fractions (Fig. 3B, top spectrum, and Supplementary Fig. 5), consistent with our RNA-seq analysis (Supplementary Data 1–3). In agreement to these observations, in vitro experiments showed that the cell-free supernatants from ΔtasA cells exhibited antifungal activity against P. xanthii conidia equivalent to that of WT cells, even in highly diluted spent medium (Fig. 3C). These results confirm the robust antimicrobial potency of ΔtasA cells and imply that primary metabolic intermediates are diverted to different pathways to support the higher secondary metabolite production in the ΔtasA mutant cells.

Consistent with these findings, we observed two complementary results that indicate less efficient metabolic activity in ΔtasA cells compared to that in WT cells: first, the induction at 24 h and 48 h of the genes responsible for the synthesis of the anaerobic respiration machinery (Supplementary Data 1 and 2, and Supplementary Fig. 3B) mentioned above, and second, the differential expression at 72 h of the nasD and nasF genes (parts of the anaerobic respiration machinery) and the differential expression of genes at all times encoding several terminal oxidases found in the electron transport chain (Supplementary Data 1–3). The analysis of the respiration rates of these strains using the tetrazolium-derived dye 5-cyano-2,3-ditolyl tetrazolium chloride (CTC) and flow cytometry revealed a higher proportion of ΔtasA cells with lower respiration rates at 24 h and 72 h compared to the WT proportions (69.10% vs. 43.07% at 24 h and 74.56 vs. 65.11% at 72 h, respectively) (Supplementary Table 1 and Fig. 4A). Second, the expression levels of the alsSD genes, which are responsible for the synthesis of acetoin (a metabolite produced by fermentative pathways) were higher in the ΔtasA strain than in the WT strain at all times (Supplementary Data 1–3). Indeed, all of the factors required for acetoin synthesis from
pyruvate were overexpressed at 72 h, whereas some key factors involved in the divergent or gluconeogenetic pathways were repressed (Supplementary Data 1–3 and Supplementary Fig. 6). Expression of alsS and alsD is induced by acetate, low pH and anaerobiosis. Acetoin, in contrast to acetate, is a neutral metabolite produced to manage the intracellular pH and to ameliorate over-acidification caused by the accumulation of toxic concentrations of acetate or lactate, and its production is favored during bacterial growth under aerobic conditions. Reduced respiration rates typically result in the accumulation of higher cellular proton concentrations, which leads to cytoplasmic acidification. These observations led us to postulate that the activation of the alsSD genes and the lower respiration rates observed in ΔtasA colonies might also reflect acidification of the intracellular environment, a potential cause of stationary phase-related stress. Measurements of the intracellular pH levels using the fluorescent probe 5-(6)carboxyfluorescein diacetate succinimidyl ester confirmed a significant decrease in the intracellular pH of nearly one unit (−0.92 ± 0.33) in ΔtasA cells at 72 h (Fig. 4B) compared to that in WT cells.

Loss of TasA increases membrane fluidity and cell death. The reduction in metabolic activity of ΔtasA cells, along with their acidification of the intracellular environment, might be expected to result in reduced bacterial viability. Measurements of the dynamics of viable bacterial cell density, expressed as CFU counts, showed that after 48 h, ΔtasA colonies possessed nearly an order of magnitude fewer CFUs than did WT colonies (Fig. 4C). These results suggest the hypothesis that ΔtasA colonies might exhibit higher rates of cell death than WT colonies. To test this possibility, we analyzed the live and dead sub-populations using the BacLight LIVE/DEAD viability stain and confocal microscopy (Fig. 4D right). The proportion of dead cells in ΔtasA colonies ranged from between 16.80% (16.80 ± 1.17) and 20.06% (20.06 ± 0.79) compared to 4.45% (4.45 ± 0.67) and 3.24% (3.24 ± 0.51) found in WT colonies at 48 h and 72 h, respectively (Fig. 4D, left). The significantly higher rate of cell death in ΔtasA compared to WT is consistent with the drastically lower bacterial counts found in the ΔtasA mutant colonies after 48 h. To rule out the influence of media composition on the observed phenotype, we performed the same experiments on solid LB medium, on which B. subtilis can still form a biofilm, as reflected by the wrinkly phenotype of the colonies (Supplementary Fig. 7A). We found that ΔtasA colonies exhibited a significantly higher proportion of dead cells at 48 h (17.86 ± 0.92) than did WT colonies (3.88 ± 0.33) (Supplementary Fig. 7B). Interestingly, the higher rate of cell death exhibited by the tasA mutant was not reproducible when both strains were grown in liquid MSgg with shaking, conditions that promote planktonic growth. WT and ΔtasA cultures showed similar growth rates under these conditions (Supplementary Fig. 7C), and the proportion of cell death was measured in exponential (ΔtasA 0.32 ± 0.03 vs WT 0.78 ± 0.30) or stationary phase cultures (ΔtasA 2.31 ± 0.44 vs WT 0.56 ± 0.08) (Supplementary Fig. 7D), indicating that the lower viability of ΔtasA cells is observable when biofilms form on solid media.

The impaired respiration rates and the acidification of the cellular environment found in the ΔtasA cells are causes of cellular stress that can lead to ROS generation, a well-known trigger of stress-induced cell death. To determine if ΔtasA cells possess abnormal ROS levels, we monitored ROS generation using hydroxyphenyl fluorescein (HPF), a fluorescent indicator of the presence of highly reactive hydroxyl radicals. Flow cytometry analysis revealed a larger proportion of HPF-positive cells (which have increased ROS levels) in the ΔtasA strain at 24 h compared to the WT proportion (42.38% vs. 28.61%, respectively) (Fig. 4A and Supplementary Table 1). To test whether this higher ROS production has negative effects on cellular components and functions, we first performed TUNEL assays to fluorescently stain bacterial cells containing DNA strand breaks, a known hallmark of the cell death induced by cellular damage and a frequent outcome of ROS production. At 24 h and 48 h, we found a significantly higher number of fluorescently stained ΔtasA cells compared with the number of fluorescently stained WT cells (Fig. 5A, left and 5B, left). These results indicated that DNA damage appears to occur not only earlier, but also with a higher frequency, in ΔtasA cells than in WT cells. A sizeable number of stained cells was also found at 72 h in the ΔtasA colonies, the same time-point at which the TUNEL signal started to increase in the WT colonies (Fig. 5A left). The TUNEL signal in the ΔtasA cells at this time-point was not significantly different from that of the WT cells (Fig. 5B left), probably due to the increased cell death in the ΔtasA cells.

Next, we examined the cellular membrane potential, another phenotype related to cell death, using the fluorescent indicator tetramethylrhodamine, methyl ester (TMRM). Consistent with all previous analysis, the alterations in the membrane potential of the ΔtasA cells were significantly different at all time points compared with the corresponding values for the WT cells (Fig. 6A left panel and 6B left). As a control, ΔtasA cells after 72 h of growth and treated with carbonyl cyanide m-chlorophenyl hydrazine (CCCP), a chemical ionophore that uncouples the proton gradient and can depolarize the membrane, showed a strong decrease in the fluorescence signal (Supplementary Figs. 8A, B). These results indicate that after 48 h (the same time point at which the cell death rate increases and the cell population plateaus in ΔtasA colonies) ΔtasA cells also exhibit increased membrane hyperpolarization compared with that in the WT cells, a feature that has been linked to mitochondrial-triggered cell death in eukaryotic cells.

The differences in ROS production, DNA damage level and membrane hyperpolarization between the WT and ΔtasA cell populations are consistent with increased cellular stress being the cause of the higher cell death rate observed in ΔtasA colonies after 24 h. To test the idea that loss of tasA results in increased cellular stress that leads to abnormal cellular physiology and increased cell death, we investigated the level of membrane lipid peroxidation, a chemical modification derived from oxidative stress that subsequently affects cell viability by inducing toxicity and apoptosis in eukaryotic cells. Staining with BODIPY 581/591 C11, a fluorescent compound that is sensitive to the lipid oxidation state and localizes to the cell membrane, showed no significant detectable differences in the levels of lipid peroxidation at any time point (Supplementary Fig. 16). However, treatment
with cumene hydroperoxide (CuHpx), a known inducer of lipid peroxide formation, resulted in different responses in the two strains. WT cells showed high reduced/oxidized ratios at 48 and 72 h and, thus, a low level of lipid peroxidation (Fig. 6A, center panel and Fig. 6B, center). In contrast, the comparatively lower reduced/oxidized ratios in ΔtasA cells at 48 and 72 h indicated increased lipid peroxidation. These results demonstrate that the ΔtasA strain is less tolerant to oxidative stress than is the WT strain, and, therefore, is more susceptible to ROS-induced damage. This finding along with the increased ROS production in ΔtasA cells, led us to study the integrity and functionality of the plasma membrane. First, no clear differences in the integrity, shape, or thickness of the cell membrane or cell wall were observed via transmission electron microscopy (TEM) of negatively stained thin sections of embedded ΔtasA or WT cells at 24 and 72 h under our experimental conditions (Supplementary Fig. 9). Next, we examined membrane fluidity, an important functional feature of biological membranes that affects their

**Fig. 4 Respiration rates and cell viability are compromised in ΔtasA cells.** a Flow cytometry density plots of cells double stained with the HPF (Y axis) and CTC (X axis) dyes show that ΔtasA cells were metabolically less active (lower proportion of cells reducing CTC) and were under oxidative stress as early as 24 h (higher proportion of HPF-stained cells). b Measurements of intracellular pH show significant cytoplasmic acidification in the ΔtasA cells at 72 h. Average values of four biological replicates are shown. Error bars indicate the SEM. Statistical significance was assessed by one-way ANOVA with Tukey multiple comparison test (*p < 0.05). c The population dynamics in ΔtasA (dashed line) and WT colonies (solid line) grown on MSgg agar at 30 °C showed a difference of nearly one order of magnitude in the ΔtasA colony from 48 h. Average values of three biological replicates are shown. Error bars represent the SEM. Statistical significance was assessed by two-sided independent t-tests at each time point (****p value = 0.0084 *p value = 0.0410). d Left, Quantification of the proportion of dead cells treated with the BacLight LIVE/DEAD viability stain in WT and ΔtasA colonies at different time-points revealed a significantly higher population of dead cells at 48 h and 72 h in ΔtasA colonies compared to that found in the WT colonies. N = 5 colonies of the corresponding strains examined over three independent experiments. Average values are shown. Error bars represent the SEM. For each experiment and sample, at least three fields-of-view were measured. Statistical significance was assessed via two-tailed independent t-tests at each time-point (****p < 0.0001). Right, Representative confocal microscopy images of fields corresponding to LIVE/DEAD-stained WT or ΔtasA samples at 72 h. Scale bars = 10 μm. Source data are provided as a Source Data file.
were measured. Error bars indicate the SEM. Statistical significance was assessed via two-tailed independent t-tests at each time-point (** p ≤ 0.002). Source data are provided as a Source Data file.

Fig. 5 The ΔtasA cells exhibit higher levels of DNA damage. a CLSM analysis of TUNEL assays revealed significant DNA damage in the ΔtasA cells (bottom panels) compared to that in the WT cells (top panels). Cells were counterstained with DAPI DNA stain (top images). Scale bars = 5 μm. Experiments have been repeated at least three times with similar results. b Quantification of the TUNEL signals in WT and ΔtasA colonies. The results showed significant differences in the DNA damage levels between ΔtasA and WT cells after 24 and 48 h of growth. For the WT strain, N = 5 at 24 h, N = 8 at 48 h and N = 7 at 72 h. For the ΔtasA strain N = 5 at all times. N refers to the number of colonies examined over three independent experiments. For each experiment and sample, at least three fields-of-view were measured. Error bars indicate the SEM. Statistical significance was assessed via two-tailed independent t-tests at each time-point (*** p = 0.061 *** p = 0.002). Source data are provided as a Source Data file.

permeability and binding of membrane-associated proteins, by measuring the Laurdan generalized polarization (Laurdan GP) 

Our results show that the Laurdan GP values were significantly lower at 48 h and 72 h in ΔtasA cells compared to the values in WT cells (0.65 ± 0.03 or 0.82 ± 0.03 respectively, at 48 h, and 0.87 ± 0.006 or 0.73 ± 0.007 respectively, at 72 h) (Fig. 6A, right panel and Fig. 6B, right). These results indicate incremental changes in membrane fluidity, comparable to that resulting from treatment of cells with benzyl alcohol, a known membrane fluidifier (Supplementary Fig. 10A, top and center panels, B). Membrane fluidity has been associated with higher ion, small molecule, and proton permeability, which might contribute to the higher concentration of fengycin found in the in cell-free supernatants of ΔtasA cultures (Fig. 3B). These effects could also explain why ΔtasA cells are impaired in energy homeostasis as well as the subsequent effects on the intracellular pH and membrane potential that eventually contribute to cell death.

TasA associates to detergent-resistant fractions of the membrane. The negative effects on membrane potential and fluidity observed in the ΔtasA cells suggest alterations in membrane dynamics, which in bacterial cells are directly related to functional membrane microdomains (FMM); FMMs are specialized membrane domains that also regulate multiple important cellular functions. The bacterial flotillins FloT and FloA are localized in FMMs and are directly involved in the regulation of membrane fluidity. This line of evidence led us to propose a connection between the membrane fluidity and permeability of ΔtasA cells and changes in the FMMs. We initially studied the membrane distribution of FloT as a marker for FMMs in WT and ΔtasA cells using a FloT-YFP translational fusion construct and confocal microscopy (Fig. 7A). The WT strain showed the typical FloT distribution pattern, in which the protein is located within the bacterial membrane in the form of discrete foci (Fig. 7A, top). However, in the ΔtasA cells, the fluorescent signal was visible only in a subset of the population, and the normal distribution pattern was completely lost (Fig. 7A bottom). In agreement with these findings, quantification of the fluorescent signal in WT and ΔtasA samples showed significant decreases in the signal in the ΔtasA mutant cells at 48 and 72 h (Fig. 7B). Consistently, our RNA-seq data showed fluctuations in the FloT expression levels at all times (Supplementary Data 1–3).

The alterations in FloT expression and the loss of the normal FloT distribution pattern in the cell membrane that occurs in the ΔtasA mutant cells led us to consider the presence of TasA in FMMs. Membranes from both prokaryotes and eukaryotes can be separated into detergent-resistant (DRM) and detergent-sensitive fractions (DSM) based on their solubility in detergent solutions. Although it is important to point out that the DRM and FMMs (or lipid rafts in eukaryotes) are not equivalent, the DRM fraction has a differential lipid composition and is enriched with proteins, rendering it more resistant to detergents; furthermore, many of the proteins present in FMMs are also present in the DRM. Immunodetection assays of the DRM, DSM, and cytosolic fractions of each strain using an anti-TasA antibody showed the presence of anti-TasA reactive bands of the expected size primarily in the DRM fraction and in the cytosol (Fig. 7C, top). As controls, the fractions from the tasA mutant showed no signal (Fig. 7C, top). Western blots of the same fractions isolated from WT and ΔtasA strains carrying a FloT-YFP translational fusion with an anti-YFP antibody (Fig. 7C, bottom) confirmed that FloT was mainly present in the DRM of WT cells (Fig. 7C, bottom, lane 1). The signal was barely noticeable in the same fraction from ΔtasA cells (Fig. 7C, bottom, lane 4), mirroring the reduced fluorescence levels observed via microscopy (Fig. 7A), and consistent with the RNA-seq data. These results confirm that TasA is indeed associated with the DRM fraction of the cell membrane. Furthermore, we asked whether the loss of FloT foci is somehow related to the increased cell death observed in the absence of TasA. We used a LIVE/DEAD viability stain in a ΔfloT colony and in a ΔfloTΔfloA colony, a double mutant for the two flotillin-like proteins in the B. subtilis genome. The results show no significant differences in the proportion of cell death compared to the WT strain at 48 h and 72 h (Supplementary Fig. 11). These experiments demonstrate that the increased cell death is not caused by loss of the FloT distribution pattern that occurs in the tasA mutant.
**Fig. 6 ΔtasA cell membrane exhibit cytological anomalies.** **a** Left panel. A TMRM assay of WT and ΔtasA cells, located at the top or bottom respectively in each set, showed a decrease in membrane potential in the WT cells, whereas the ΔtasA cells exhibited hyperpolarization at 48 and 72 h. Center panel. Assessment of the lipid peroxidation levels using BODIPY 581/591 C11 reagent in WT and ΔtasA cells after treatment with 5 mM CuHpx and analysis by CLSM. The ratio images represent the ratio between the two states of the lipid peroxidation sensor: reduced channel(590–613 nm emission)/oxidized channel(509–561 nm emission). The ratio images were pseudo-colored depending on the reduced/oxidized ratio values. A calibration bar (from 0 to 50) is located at the bottom of the panel. Confocal microscopy images show that CuHpx treatment was ineffective in the WT strain at 72 h, whereas the mutant strain showed symptoms of lipid peroxidation. Right panel. Laurdan GP analyzed via fluorescence microscopy. The images were taken at two different emission wavelengths (gel phase, 432–482 nm and liquid phase, 509–547 nm) that correspond to the two possible states of the Laurdan reagent depending on the lipid environment. The Laurdan GP images were pseudo-colored depending on the lipid environment. The Laurdan GP images were pseudo-colored depending on the laurdan GP values. A calibration bar (from 0 to 1) is located at the bottom of the set. The Laurdan GP images show an increase in membrane fluidity (lower Laurdan GP values) in the ΔtasA mutant cells at 48 and 72 h. All scale bars are equal to 5 µm. **b** Left. Quantification of the TMRM signal. N = 3. Center. Quantification of lipid peroxidation. N = 3. For the WT at 48 h, N = 4. Right. Quantification of laurdan GP values. For the WT strain, N = 6 at 48 h and N = 4 at 72 h. For the ΔtasA strain, N = 5 at all times. N refers to the number of colonies examined over three independent experiments. Average values are shown. Error bars represent the SEM. For each experiment and sample, at least three fields-of-view were measured. Statistical significance in the TMRM experiments was assessed via two-tailed independent t-tests at each time-point (**p < 0.0001). Statistical significance in the lipid peroxidation experiments was assessed via two-tailed independent t-tests at each time-point (**p < 0.0001, *p value = 0.0115). Statistical significance in the laurdan GP experiments was assessed via two-tailed independent Mann-Whitney tests at each time-point (**p value = 0.0087 at 48 h and **p value = 0.0095 at 72 h respectively). Source data are provided as a Source Data file.
All together, these results allow us to conclude that TasA is located in the DRM fraction of the cell membrane where it contributes to membrane stability and fluidity, and that its absence leads to alterations in membrane dynamics and functionality, eventually leading to cell death.

Mature TasA is required to maintain viable bacterial physiology. TasA is a secreted protein located in the ECM and additionally found associated to the DRM fraction of the cell membrane (Fig. 7C). Reaching these sites requires the aid of secretion-dedicated chaperones, the translocase machinery and membrane-bound signal peptidase SipW75. It is known that TasA processing is required for assembly of the amyloid fibrils and biofilm formation18,76. However, formation of the mature amyloid fibril requires the accessory protein TapA, which is also secreted via the same pathway19, is present in the mature amyloid fibers and is found on the cell surface76. Considering these points, we first wondered whether TapA is involved in the increased cell death observed in the ΔtasA mutant. By applying the BacLight LIVE/DEAD viability stain to a ΔtapA colony, we found a similar proportion of live to dead cells as those found in the WT colony at 72 h (Fig. 8A), suggesting that the tapA mutant does not exhibit the cytological alterations and cellular damage that occurs in ΔtasA cells. TapA mutant cells produce a much lower number of TasA fibers but still expose TasA in their surfaces76; thus we reasoned that mature TasA is necessary for preserving the cell viability levels observed in the WT strain. To test this possibility, we constructed a strain bearing a mutation in the part of the tasA gene that encodes the TasA signal peptide27. To avoid confounding effects due to expression of the mutated tasA gene in the presence of the endogenous operon, we performed this analysis in a strain in which the entire tapA operon was deleted and in which the modified operon encoding the mutated tasA allele was inserted into the neutral lacA locus. The strain carrying this construct was designated as "ΔtasA Sipmutant" (for Signal Peptide mutant) and included three amino acid substitutions from the initial lysines of the signal peptide. Specifically, the introduced mutations were Lys4Ala, Lys5Ala, and Lys6Ala. The endogenous version of TasA successfully restored biofilm formation (Supplementary Fig. 12A), while the phenotype of SiP mutant on MSgg medium at 72 h was different from those of both the WT and ΔtasA mutant strains (Fig. 8B and Supplementary Fig. 2B). Immunodetection analysis of TasA in fractionated biofilms confirmed the presence of TasA in the cells and ECM fractions from the WT strain and the strain expressing the endogenous version of tasA (Fig. 8C). However, a faint anti-TasA reactive signal was observed in both fractions of the SiP mutant (Fig. 8C). This result indicates that TasA is not efficiently processed in the SiP mutant, and thus, the protein levels in the ECM were drastically lower. The faint signal detected in the cell fraction might be due to the fact that the pre-processed protein is unstable in the cytoplasm and is eventually degraded over time27. Consistent with our hypothesis, the levels of cell death in the SiP mutant were significantly different from those of the WT strain (Fig. 8A, D). Taken together, these results rest relevance to TapA to the increase cell death observed in the absence of TasA and indicate that TasA must be processed to preserve the level of cell viability found in WT colonies.

A TasA variant restores cellular physiological status but not biofilm. The fact that the ΔtapA strain forms altered and fewer TasA fibers but does have normal cell death rates, as well as the
increased membrane fluidity and the changes in expression and loss of the normal distribution pattern of the flotillin-like protein FloT in the ΔtasA strain led us to hypothesize that the TasA in the DRM, and not that in the ECM, is responsible for maintaining the normal viability levels within the WT colonies. To explore this hypothesis, we performed an alanine scanning experiment with TasA to obtain an allele encoding a stable version of the protein that could support biofilm formation. To produce these constructs, we used the same genetic background described in the above section. The strain JC81, which expresses the TasA (Lys68Ala, Asp69Ala) variant protein, failed to fully restore the WT biofilm formation phenotype (Fig. 9A, Supplementary Figs. 2B and 12A). Immunodetection analysis of TasA in fractionated biofilms confirmed the presence of the mutated protein in the cells and in the ECM (Fig. 9B, left and Supplementary Fig. 12B). Tandem mass spectrometry analysis revealed that the mutated protein found in the ECM corresponded to the mature form of TasA (Supplementary Fig. 13A, left and Supplementary Fig. 13A, right), indicating exclusively a malfunction in the protein’s structural role in proper ECM assembly. Electron microscopy coupled to immunodetection with anti-TasA and immunogold-conjugated secondary antibodies showed the presence of a dense mass of extracellular material in JC81 cells with an absence of well-defined TasA fibers, as opposed to WT cells, in which we also observed a higher number of gold particles, indicative of the higher reactivity of the sample (Supplementary Fig. 13B, left and center panels). The cell membrane fractionation analysis revealed, however, the presence of mutated TasA in the DRM, DSM and cytosolic fractions (Fig. 9B right). Accordingly, JC81 was reverted to a physiological status comparable to that of the WT strain. This feature was demonstrated by similar expression levels of genes encoding factors involved in the production of secondary metabolites (i.e., ppsD, albE, bacB, and srfAA) or acetoin (alsS), indicating comparable metabolic activities between the two strains (Fig. 9C). Further evidence confirmed the restoration of the metabolic status in JC81. First, similar proportions of WT and JC81 cells expressing YFP from the fengycin operon promoter were detected after 72 h of growth via flow cytometry analysis (Fig. 9D, green curve). In agreement with these findings, there were no differences in the proportions of cells respiring or accumulating ROS or in the intracellular pH values between the JC81 and WT strains (Figs. 9E and 10A). Consistently, the population dynamics of JC81 resembled that of the WT strain (Fig. 10B), and, as expected, its level of cell death was comparable to that of the WT strain (Fig. 10C). Finally, there were no differences in any of the examined parameters related to
oxidative damage and stress-induced cell death (i.e., DNA damage, membrane potential, susceptibility to lipid peroxidation, and membrane fluidity) between JC81 and WT cells (Supplementary Figs. 14 and 15 respectively), and the mutated allele complemented the sporulation defect observed in the ECM mutants (Supplementary Fig. 4). To further confirm these results, we performed a viability assay in a mixed ΔtasA and Δeps colony co-inoculated at a 1:1 ratio, and we found that, despite the ability of the mixed colony to rescue the wrinkly phenotype typical of a WT colony (Supplementary Fig. 17A, top), the proportion of cell death is significantly higher than that observed in WT cells at 48 h (10.39 ± 1.20) and 72 h (14.04 ± 0.72) (Supplementary Fig. 17B). In addition, exogenous TasA did not revert the colony morphology phenotype of ΔtasA cells on solid MSgg (Supplementary Fig. 18A) or the increased cell death rate observed in the ΔtasA strain (Supplementary Fig. 18B). These results show that the extracellular TasA provided by the Δeps strain is sufficient to complement the ECM assembly and biofilm formation defects but not to prevent cell death, similar to the effects of exogenous TasA supplementation. Thus, TasA must be produced by the cells to reach the cell membrane and exert this function. Interestingly, a ΔsinI strain, which is mutant for the sinR anti-repressor that inhibits sinR, and therefore, has strong repression of ECM genes and is unable to assemble biofilms (Supplementary Fig. 17A, bottom), showed similar levels of cell death as the WT strain at all times (1.53 ± 0.25 at 48 h and 2.51 ± 0.50 at 72 h) (Supplementary Fig. 17B).
In agreement with our prediction, the reduced infection (Fig. 1D). Thus, we conclude that the ECM, by means of intermediate between those of the WT and ΔtasA mutant on the melon phylloplane versus its ability to assemble a proper ECM, confirming that indeed, cells lacking a structured ECM do not exhibit the physiological changes observed in cells lacking TasA.

Taken together, these findings assign TasA complementary functions, via its localization to the DRM fraction of the cell membrane, contributing to cell membrane dynamics and cellular physiology during normal colony development that prevent premature cell death, a role beyond the well-known structural function of amyloid proteins in biofilm ECMs.

The TasA variant impairs B. subtilis fitness on the phylloplane.

Our analysis of the intrinsic physiological changes in ΔtasA cells showed how the absence of TasA leads to the accumulation of canonical signs of cellular damage and stress-induced cell death, a physiological condition typical of stationary phase cells. These observations help to reconcile two a priori contradictory features of B. subtilis ecology on plant leaves: the reduced persistence of the ΔtasA mutant on the melon phylloplane versus its ability to efficiently exert biocontrol against the fungus P. xanthii, which occurs via overproduction of fengycin and other antimicrobial molecules. Following this line of thought, we predicted that the JC81 strain, which expresses a version of TasA that is unable to restore biofilm formation but preserves the physiological status of the cells, would show overall signs of reduced fitness on melon leaves. The JC81 cells retained their initial ability to adhere to melon leaves (Fig. 1A); however, their persistence decreased (Fig. 1B) and their colonization showed a pattern somewhat intermediate between those of the WT and ΔtasA strains (Fig. 1C, bottom images). In agreement with our prediction, the reduced fitness of this strain resulted in a failure to manage P. xanthii infection (Fig. 1D). Thus, we conclude that the ECM, by means of the amyloid protein TasA, is required for normal colonization and persistence of B. subtilis on the phyllosphere. These ecological features depend on at least two complementary roles of TasA: one role related to ECM assembly and a new proposed role in the preservation of the physiological status of cells via stabilization of membrane dynamics and the prevention of premature cell death.

Discussion

The ECM provides cells with a myriad of advantages, such as robustness, colony cohesiveness, and protection against external environmental stressors. Studies of B. subtilis biofilms have revealed that the ECM is mainly composed of polysaccharides and the proteins TasA and BslA. TasA is a confirmed functional amyloid that provides structural support to the biofilm in the form of robust fibers. A recent study demonstrated that there is heterogeneity in the secondary structure of TasA; however, in biofilms, its predominant conformation is in the form of stable fibers enriched in β-sheets. In this study, we demonstrate that in addition to its structural role in ECM assembly, TasA is also required for normal colony development – both of which are functions that contribute to the full fitness of Bacillus cells on the phylloplane.

The physiological alterations observed in ΔtasA null strain reflect a process of progressive cellular deterioration characteristic of senescence, including early activation of secondary metabolism, low energy metabolic activity, and accumulation of damaged molecular machinery that is required for vital functions. Indeed, it has been previously demonstrated that such metabolic changes can trigger cell death in other bacterial species, in which over-acidification of the cytoplasm eventually leads to the activation of cell death pathways. Interestingly, cytoplasmic acidification due to the production of acetic acid has been linked to...
higher ROS generation and accelerated aging in eukaryotes. As mentioned throughout this study, ROS generation leads to ongoing DNA damage accumulation, phospholipid oxidation, and changes in cell membrane potential and functionality, all of which are major physiological changes that eventually lead to declines in cellular fitness and, ultimately, to cell death. The fact that we could restore the physiological status of tasA null mutant cells by ectopically expressing a mutated TasA protein incapable of rescuing biofilm formation permitted us to separate two roles of TasA: (i) its structural function, necessary for ECM assembly; and (ii) its cytological functions involved in regulating membrane dynamics and stability and preventing premature cell death. Our data indicate that this previously unreported function does not involve TasA amyloid fibers or its role in ECM assembly, and that it is more likely related to the TasA found in the DRM of the cell membrane, where the FMMs (like the lipid rafts of eukaryotic cells) are located. It is not unprecedented that amyloid proteins interact with functional domains within the cell membrane. In eukaryotic cells, for instance, it has been reported that lipid rafts participate in the interaction between the amyloid precursor protein and the secretone required for the production of the amyloid-β peptide, which is responsible for Alzheimer’s disease. Indeed, our results are supported by evidence that TasA can preferentially interact with model bacterial membranes, which affects fiber assembly, and that TasA fibers are located and attached to the cell surface via a proposed interaction with TapA, which forms foci that seem to be present on the cell wall. Interestingly, TapA has been recently characterized as a two-domain, partially disordered protein. Disordered domains can be flexible enough to interact with multiple partners, suggesting a similar mechanism for TapA: the N-terminal domain might be involved in the interaction with other protein partners, whereas the C-terminal disordered domain might anchor the protein to the cell surface. All of these observations led us to propose that TasA may drive the stabilization of the FMMs in the cell membrane either directly via interactions with certain phospholipids or indirectly via interactions with other proteins. This model is further supported by the fact that the ΔtasA cells show alteration of FloT levels and loss of the typical FloT distribution pattern (Fig. 7A), which is typically present in the FMM, and induction of many genes that encode ECM components or other factors that interact with FloT alone or with FloT and FloA (Supplementary Data 1–3). Considering these novel findings, it is tempting to speculate that cells can regulate membrane dynamics by, among other processes, tuning the amount of TasA present in the membrane at a given moment, which would permit a better physiological response to different environmental cues. In particular, our results suggest that the membrane instability caused by the absence of TasA triggers a cascade of malfunctions in biological processes that eventually lead to cell death in a subset of the population. This alteration in membrane stability might cause, given the differences in the expression levels of many genes involved in the respiratory process (Supplementary Data 1–3), the impaired respiration observed in ΔtasA cells, which could lead to increased ROS generation, leading to the full range of transcriptional and cytological alterations found in the tasA mutant over the course of time.

The physiological alterations observed in the ΔtasA strain have ecological implications. The intrinsic stress affecting the mutant cells reduced their ability to survive in natural environments; however, paradoxically, their higher induction of secondary metabolism seemed to indirectly and efficiently target fungal pathogens. This could explain why ΔtasA cells, which show clear signs of stress, display efficient biocontrol properties against P. xanthii. However, the sharp time-dependent decrease in the ΔtasA population on leaves suggests that its antifungal production could be beneficial during short-term interactions, but insufficient to support long-term antagonism unless there is efficient colonization and persistence on the plant surface. In this scenario, the deletion of tasA has a strong negative effect on bacterial cells, as we have demonstrated how a ΔtasA strain is more susceptible to ROS-induced damage (Fig. 6A, center panel, 6B, center graph), especially in the phyllosphere, where microbial cells are continuously subjected to different types of stresses, including oxidative stress. We previously speculated that biofilm formation and antifungal production were two complementary tools used by Bacillus cells to efficiently combat fungi. Our current study supports this concept, but also enhances our understanding of the roles of the different ECM components. More specifically, we demonstrated that the amyloid protein TasA is the most important bacterial factor during the initial attachment and further colonization of the plant host, as it is necessary for the establishment of the bacterial cells over the plant leaves and for the maintenance of the normal cellular structure. The fact that the naturally occurring overexpression of the eps genes in the ΔtasA is unable to revert the adhesion defect of this strain downplays the importance of the EPS during the early establishment of physical contact. These observations are more consistent with a role for the EPS, along with BslA, in providing biofilms with protection against external stressors.

A similar role for a functional amyloid protein in bacterial attachment to plant surfaces was found for the Escherichia coli curli protein. Transcriptomic studies showed induction of curli expression during the earlier stages of attachment after the cells came into contact with the plant surface, and a curli mutant strain was defective in this interaction. The distinct morphological and biochemical variations typical of amyloids make them perfect candidates for modulating cellular ecology. The observation that ΔtasA cells are incapable of colonization in the rhizosphere clearly indicates the need for more in-depth investigation into these two distinctive ecological niches to understand the true roles of specific bacterial components. In addition to demonstrating enhanced production of antifungal compounds, our study revealed additional features that might contribute to the potency of stressed Bacillus cells in arresting fungal growth, in particular the overproduction of acetoin via increased expression of the alsS and alsD genes. Acetoin is a volatile compound produced via fermentative and overflow metabolism, and it has been demonstrated to mediate communication between beneficial bacteria and plants by activating plant defense mechanisms either locally or over long distances in a phenomenon known as induced systemic resistance (ISR).

In summary, we have proven that the amyloid protein TasA participates in the proper maturation of Bacillus colonies, a function that, along with its previously reported role in ECM assembly, contributes to long-term survival, efficient colonization of the phylloplane, and a competitive advantage mediated by antifungal production. The absence of TasA leads to a series of physiological changes, likely triggered by alterations in membrane stability and dynamics, and effects on the FMMs, including an arrest of cell differentiation that paradoxically increases the competitiveness of the mutant cells during short-term interactions via their ability to adapt to stress and their cellular response to early maturation. However, lack of TasA reduces cell fitness during mid- to long-term interactions via increased intrinsic cellular stress and the absence of a structured ECM, both of which limit the adaptability of the cells to the stressful phylloplane.

Methods
Bacterial strains and culture conditions. The bacterial strains used in this study are listed in Supplementary Table 2. Bacterial cultures were grown at 37 °C from frozen stocks on Luria-Bertani (LB: 1% tryptone (Oxoid), 0.5% yeast extract...
(Oxoid) and 0.5% NaCl plates. Isolated bacteria were inoculated in the appropriate medium. The biotrophic fungus *Podosphaera xanthii* was grown at 25°C from a frozen stock on cucumber cotyledons and maintained on them until inoculum preparation. Biofilm assays were performed on Mssg medium: 100 mM morpholinepropanesulfonic acid (MOPS) (pH 7), 0.5% glycerol, 0.5% glutamate, 5 mM potassium phosphate (pH 7), 50 µg/ml tryptophan, 50 µg/ml phenylalanine, 50 µg/ml threonine, 2 mM MgCl₂, 700 µM CaCl₂, 30 µM FeCl₃, 50 µM MnCl₂, 2 µM thiamine, 1 µM ZnCl₂. For the in vitro lipopeptide detection and assays with cell-free supernatants, medium optimized for lipopeptide production (MOLP) was used: 30 µl/giter peptone, 20 g/liter saccharose, 7 g/liter yeast extract, 1.9 g/liter KH₂PO₄, 0.001 mg/liter CaSO₄, 0.005 mg/liter FeCl₃·6H₂O, 0.004 mg/liter Na₂MoO₄·2H₂O, 1.36 g/liter MgCl₂·6H₂O, 0.45 g/liter MgSO₄·7H₂O, 0.014 mg/liter ZnSO₄·7H₂O, 0.01 mg/liter H₂BO₃, and 10 mM lidoceric acid. The pH was adjusted to 7.5 with NaOH prior to sterilization. For cloning and plasmid replication, *Escherichia coli* DH5α was used. *Escherichia coli* BL21(DE3) was used for protein purification. *Bacillus subtilis* 168 is a domesticated strain used to transform the different constructs into *Bacillus subtilis* NCIB3610. The antibiotic final concentrations for *B. subtilis* were: MLS (1 µg/ml erythromycin, 25 µg/ml lincomycin); spectinomycin (100 µg/ml); tetracycline (10 µg/ml); chloramphenicol (5 µg/ml); and kanamycin (10 µg/ml).

**Strain construction.** All of the primers used to generate the different strains are listed in Supplementary Table 3. To build the strain YNG001, the promoter of the fengycin operon was amplified with the Pps–ecorR FL and Ppps–HindIII R primer pair. The PCR product was digested with EcoRI and HindIII and cloned into the pKM003 vector cut with the same enzymes. The resulting plasmid was transformed by natural competence into *B. subtilis* 168 replacing the amylE neutral locus. Transformants were selected via spectinomycin resistance. The same protocol was used to build the strain YNG002 by transforming a ΔtasA strain of *B. subtilis* 168. Strains were constructed using the primers amyEUP-Fw, amyc-DOWN-Rv, Ppps-Fw, Ppps-Rv, Yfp-Fw, Yfp-Rv, Cat-Fw, Cat-Rv, amyEDOWN-Fw, and amyEDOWN-Rv to separately amplify the relevant fragments. The fragments were then joined using the NEL builder HiFi DNA Assembly Master Mix (New England Biolabs). The construct was made using pUC19 digested with BamHI as the vector backbone. The final plasmid was then transformed into *B. subtilis* 168 replacing amyE, and transformants were selected via chloramphenicol resistance. Strain JC97 was generated by using the primers bsaUP-Fw, bsaDOWN-Rv, Spc-Fw, Spc-Rv, bsaUP-Fw and bsaDOWN-Rv, and XbaI-digested pUC19 as the vector backbone. The fragments were assembled using NEL Builder HiFi DNA Assembly Master Mix.

Strains JC70, JC81, and JC149 were constructed via site-directed mutagenesis (QuickChange Lightning Site Directed Mutagenesis Kit – Agilent Technologies). Briefly, the tapA operon (tapA–sipW–tasA), including its promoter, was amplified using the primers TasA_1 mutb and YSRI_2, and the resulting product was digested with BamHI and SalI and cloned into the pDR183 vector. Next, the corresponding primers (Supplementary Table 3) were used to introduce the alanine substitution mutations into the desired positions of the TasA amino acid sequence. The entire plasmid was amplified from the position of the primers using Pfu DNA polymerase. The native plasmid, which was methylated and lacked the mutations, was digested with DpnI. The plasmid containing the plasmid that replaced the TasA (IC70) or the mutated versions (IC81 and IC149) were transformed into the *B. subtilis* 168 Δ(tapA–sipW–tasA) strain replacing the lacA neutral locus. Genetic complementation was observed in strain IC70 as a control. Transformants were selected via MLS resistance. Plasmid pDR6 (pDR183–tasA), which contains the open reading frame of the *tasA* gene from *B. subtilis* NCIB3610 without the signal peptide or the stop codon, was constructed as previously described.

Primers used in the analysis of gene expression by qRT–PCR are listed in Supplementary Table 4. All of the *B. subtilis* strains generated were constructed by transforming *B. subtilis* 168 via its natural competence and then using the positive clones as donors for transferring the constructs into *B. subtilis* NCIB3610 via generalized SPP1 phage transduction.

**Biofilm assays.** To analyze colony morphology under biofilm-inducing conditions, the bacterial strains were grown on LB plates overnight at 37°C, and the resulting colonies were resuspended in sterile distilled water at an OD₆₀₀ of 1. Next, 2-µl drops of the different bacterial suspensions were spotted on Mssg or LB agar (depending on the assay) agar plates and incubated at 30°C. Colonies were removed at the appropriate time points (24, 48, and 72 h) for the different analyses. For the Δeps-ΔtasA co-inoculation assay, colonies were resuspended in sterile distilled water and mixed at a final OD₆₀₀ of 1. Next, the bacterial suspension was inoculated onto Mssg agar plates and incubated as described above. For the external complementation assay using purified TasA, a drop containing 80 µg of protein was spotted onto Mssg agar plates and allowed to dry. Next, ΔtasA cells were inoculated on top of the dried drop and incubated as described above. For the CFU counts of the colonies from the different strains, 24, 48- and 72-h-old colonies were grown on LB plates and the colony forming units (CFUs) were counted after 15 min incubation on a plate reader. The fluorescence was recorded in a HiPep 26/10 desalting column (GE Healthcare), and the buffer was exchanged to Tris 20 mM, NaCl 0.5 mM to perform the corresponding experiments.

For protein precipitation, 2 ml of the cell or ECM fractions were used. The cell fraction was treated with 0.1 mg/ml lysozyme for 30 min at 37°C. Next, both fractions were treated with a 10% final concentration of trichloroacetic acid and incubated in ice for 1 h. Proteins were collected by centrifugation at 13,000 g for 20 min, washed twice with ice-cold acetone, and dried in an Eppendorf Concentrator Plus 5305 (Eppendorf).

**Cell membrane fractionation.** Crude membrane extracts were purified from 50 ml Mssg liquid cultures (with shaking) of the different *B. subtilis* strains. Cultures were centrifuged at 7000 g for 10 min at 4°C and then resuspended in 10 ml of PBS. Lysozyme was added at a final concentration of 20 µg/ml and the cell suspensions were incubated at 37°C for 30 min. After incubation, the lysates were centrifuged at 7000 g for 10 min with a Branson 450 digital sonifier using 40 µs pulses at 20% amplitude to ensure homogenous lysis suspension. The bacterially derived suspensions were centrifuged at 9000 × g for 20 min to separate the cells from the extracellular matrix. The cell fraction was resuspended in 10 ml of MS medium and stored at 4°C until further processing. The ECM fraction was filtered through a 0.22-µm filter and stored at 4°C. For protein precipitation, 2 ml of the cell or ECM fractions were used. The cell fraction was treated with 0.1 mg/ml lysozyme for 30 min at 37°C. Next, both fractions were treated with a 10% final concentration of trichloroacetic acid and incubated in ice for 1 h. Proteins were collected by centrifugation at 13,000 g for 20 min, washed twice with ice-cold acetone, and dried in an Eppendorf Concentrator Plus 5305 (Eppendorf).

**Protein expression and purification.** Protein was expressed and purified as previously described with some changes. Briefly, freshly transformed BL21(DE3) E. coli colonies were picked, resuspended in 10 ml of liquid LB with 100 µg/ml of ampicillin and incubated O/N at 37°C with shaking. The next day, the pre-inoculum was used to inoculate 500 ml of LB supplemented with ampicillin, and the culture was incubated at 37°C until an OD₆₀₀ of 0.7–0.8 was reached. Next, the culture was induced with 1-mM isopropyl-β-D-1-thiogalactopyranoside (IPTG) and incubated O/N at 30°C with shaking to induce the formation of inclusion bodies. The next day, cells were harvested via centrifugation (5000 × g, x 15 min, 4°C) resuspended in buffer A (Tris 50 mM, 150 mM NaCl, pH 8), and then centrifuged again. The pellets were kept at –80°C until purification or processed after 15 min. All of the cell pellets were resuspended in buffer A (Tris 50 mM, NaCl 150 mM, EDTA 0.4% w/v, the membrane fractions were centrifuged at 7000 g for 15 min to 4°C). The supernatant, which contained the cytosolic proteins, was separated and kept at –20°C. The pellet, which contained the crude membrane extract, was washed three times with PBS and processed using the Celllytic MEM protein extraction kit from Sigma. Briefly, the membrane fractions were resuspended in 600 µl of lysis and separation working solution (lysis and separation buffer + protease inhibitor cocktail) until a homogenous suspension was achieved. Next, the suspension was incubated overnight at 4°C on a stirring wheel. After incubation, the suspension is incubated at 37°C for 30 min and then centrifuged at 3000 × g for 3 min. The DSM (upper phase) was separated and kept at –20°C, and the DRM (lower phase) was washed three times with 400 µl of wash buffer by repeating the process from the 37°C incubation step. Three washes were performed to ensure the removal of all hydrophilic proteins. The isolated DRM was kept at –20°C until use. The DRM, DSF, and cytosolic fractions were used directly for immunodetection.
SDS-PAGE and immunodetection. Precipitated proteins were resuspended in 1x Laemmli sample buffer (BioRad) and heated at 100 °C for 5 min. Proteins were separated by electrophoresis on SDS-PAGE (12% acrylamide gels) and then transferred to PVDF membranes using the Trans-Blot Turbo Transfer System (BioRad) and PVDF transfer packs (BioRad). For immunodetection of TasA, the membranes were probed with anti-TasA antibody (rabbit) used at a 1:20,000 dilution in Pierce Protein-Free (TBS) blocking buffer (ThermoFisher). For immunodetection of Flot-YPF, a commercial anti-GFP primary antibody (Chontec living colors full-length polyclonal antibody) developed in rabbit were used at a 1:1000 or dilution in the buffer mentioned above. A secondary anti-rabbit IgG antibody conjugated to HRP (BioRad) was used at a 1:1000 dilution in the same buffer. The membranes were developed using the Pierce ECL Western Blotting Substrate (ThermoFisher).

Mass spectrometry analysis of protein bands. The sequence corresponding to the band of the ECM fraction of JCR1 (Supplementary Fig. 13A) was identified via tandem mass spectrometry using a “nano” ion trap system (HPLC-ESI-MS/MS). Briefly, the bands obtained after electrophoresis were cut out, washed, and destained. Subsequently, the disulfide bridges were reduced with DTT, cysteines were alkylated via the use of iodoacetamide, and in-gel trypsin digestion was performed to extract the peptides corresponding to the protein samples. This entire process was carried out automatically using an automatic digester (DigestPro, Intavis Bioanalytical Instruments). The peptides were then concentrated and desalted using a capture column C18 ZORBAX 300SB-C18 (Agilent Technologies, Germany), 5 x 0.3 mm, with 5-µm particle diameter and 300-Å pore size, using a flow rate of 9 µL/min for 6 min. The capture column was connected in line to a ZORBAX 300SB-C18 analytical column (Agilent Technologies), 150 x 0.075 mm, with a 3.5-µm particle diameter, and 300-Å pore size, through a 6-π port valve. Elution of the samples from the capture column was performed over a gradient using FA 0.1% in water as the mobile phase A and FA 0.1% in ACN 80%/water 20% as the mobile phase B. The LC system was coupled through a nanospray source (CaptiveSpray, Bruker Daltonics) to a 3D ion trap mass spectrometer (a mzQuant speed ETD, Bruker Daltonics) operating in positive mode with a capillary voltage set to 1500 V and a sweep range m/z 300–1500. “Data-dependent” acquisition was carried out in automatic mode, which allowed the sequential collection of an MS spectrum in “full scan” (m/z 300_1400) followed by an MS spectrum in tandem via CID of the eight most abundant ions. For identification, the software ProteinScape 3 (Bruker Daltonics) coupled to the search engine Mascot 3.1 (Matrix Science) was used, matching the MS/MS data against the Swiss-Prot and NCBI rRNA databases.

Bioassays on melon leaves. Bacterial strains were grown in liquid LB at 30 °C overnight. The cells in the cultures were washed twice with sterile distilled water. The bacterial cell suspensions were adjusted to the same OD600 and sprayed onto leaves of 4- to 5-week-old melon plants. Two hours later, a suspension of P. xanthii conidia was sprayed onto each leaf at a concentration of 4–10 x 10^6 spores/mL. The plants were placed in a greenhouse or in a growth chamber at 25 °C with a 16-h photoperiod, 3800 lux, and 85% RH. The severity of the symptoms in melon leaves was evaluated by the estimation of disease severity106. Disease severity was calculated by quantifying the leaf area covered by powdery mildew using Fiji107 image software analysis and pictures of infected leaves. Briefly, the channels of the image were split and the area covered by powdery mildew was measured in 8-bit images by selecting the powdery mildew damage area (white powdery stains that cover the leaf) through image thresholding, given that the stains caused by the disease have higher pixel intensity values. Total leaf area was determined by manually calculating the leaf outline using the polygon selection tool the ratio of infection was calculated using the formula (eq 1):

\[
\text{Ratio of infection} = \frac{\text{damaged area}}{\text{total leaf area}} \times 100
\]

The persistence of bacterial strains on plant leaves was calculated via CFU counts performed over the twenty-one days following inoculation. Three different leaves from three different plants were individually placed into sterile plastic stomacher bags and homogenized in a lab blender (Colworth Stomacher-400, Seward, London, UK) for 3 min in 10 ml of sterile distilled water. The leaf extracts were serially diluted and plated to calculate the CFUs at each time point. The plates were incubated at 37 °C for 24 h before counting.

The adhesion of bacterial cells to melon leaves was estimated by comparing the number of cells released from the leaf versus the cells attached to the surface. The surfaces of individual leaves were placed in contact with 100 ml of sterile distilled water at room temperature and after 10 min of shaking (300 rpm), the water was filtered through a 255/8 G needle in BirnBoim A107 buffer (20% sucrose, 10 mM Tris-HCl pH 8, 10 mM EDTA and 50 mM NaCl). Lysozyme (10 mg/mL) was added and the samples were grown on MSgg plates for 72 h at 30 °C. For the cell fractions, whole colonies were resuspended as described above in 1 ml of sterile distilled water and stained with 5000 x g for 5 min. The colonies were then resuspended in 1 ml of methanol and sonicated in a bath for 10 min. Cells were harvested via centrifugation at 5000 x g for 5 min, and the supernatant containing the solubilized lipopolysides was filtered through a 0.22-µm filter and stored at -4 °C prior to analysis. For the agar fraction, after the colonies were removed, a piece of agar of approximately the same surface was sliced out and introduced into 1 mL of 2% Eppendorf tube containing glass beads. In all, 1 mL of methanol was added and then the tube was vigorously vortexed until the agar was broken down. Finally, the mixture was sonicated in a bath for 10 min and centrifuged at 5000 x g for 5 min. The supernatant was filtered through a 0.22-µm filter and stored at 4 °C prior to and following MALDI-TOF analysis.

For in situ lipopolyside detection on inoculated leaves, leaf disks were taken 21 days post-inoculation with a sterile cork borer and then placed directly on an UltraflexTrixe MALDI plate. A matrix consisting of a mixture of CHCA (α-cyano-4-hydroxycinnamic acid) and DHB (2,5-dihydroxybenzoic acid) was spotted over the disks or the supernatants (for the colonies’ analysis), and the plates were inserted into an UltraflexTrixe MALDI-TOF/TOF mass spectrometer. The mass spectra were acquired using the Bruker Daltonics FlexControl software and were processed using Bruker Daltonics FlexAnalysis.

Electron microscopy analysis. For the scanning electron microscopy analysis, leaf disks were taken 21 days post-inoculation as previously described and fixed in 0.1 M sodium cacodylate and 2% glutaraldehyde overnight at 4 °C. Three washes were performed with 0.1 M sodium cacodylate and 0.1 M sucrose followed by ethanol dehydration in a series of ethanol solutions from 50% to 100%. A final drying with hexamethyldisilazane was performed as indicated108. The dried samples were coated with a thin layer of iodine using an Emtitech K375x turbo sputtering coater batch coater in a Helios Nanolab. Scanning Electron Microscope analysis by MALDI-TOF/TOF was performed with a UltraflexIII (Bruker Daltonics) with a Schottky-type field emission electron gun.

For the transmission electron microscopy analysis, bacterial colonies grown on MSgg agar for the appropriate times were fixed directly using a 2% paraformaldehyde-2.5% glutaraldehyde-0.2% sucrose mix in phosphate buffer 0.1 M (PB) overnight at 4 °C. After three washes in PB, portions were excised from each colony and then post-fixed with 1% osmium tetroxide solution in PB for 90 min at room temperature, followed by PB washes, and 15 min of stepwise dehydration in an ethanol series (30%, 50%, 70%, 90%, and 100% twice). Between the 50% and 70% steps, the colonies were incubated in-bloc in 2% uranyl acetate solution at 4 °C for 90 min followed by dehydration. The samples were gradually embedded in low-viscosity Spurr’s resin: resinethanol, 1:4; resinethanol, 3:1, 4h; and pure resin, overnight. The sample blocks were embedded in capsule molds containing pure resin for 72 h at 70 °C.

For the immunolabeling assays, samples from the corresponding strains were grown under biofilm-inducing conditions at 30 °C. After 48 h of incubation, carbon-coated copper grids were deposited into the wells over the pellicles formed at the interface between the medium and the air (in the case of mutants unable to form a pellicle, copper grids were deposited in the interface) and incubated with the samples at 28 °C for 2 h. After incubation, the grids were washed in PSB for 5 min, and then the samples were fixed with a solution of 2% paraformaldehyde for 10 min, washed in PBS and blocked with Pierce Protein-Free (TBS) blocking buffer (ThermoFisher) for 30 min. Anti-TasA primary antibody was used at a 1:150 dilution in blocking buffer, and grids were deposited over drops of the antibody solution and incubated for 1 h at room temperature. Samples were washed three times in PBS-T (50 mM Tris-HCl, 150 mM NaCl and 0.05% Tween 20) for 5 min and then exposed to 10-nm diameter immunogold-conjugated secondary antibody (Ted Pella) for 1 h at a 1:50 dilution. The samples were then washed twice with PBS-T and once with water for 5 min each. Finally, the grids were treated with glutaraldehyde (2%) for 10 min, washed in water for 5 min, negatively stained with uranyl acetate (1%) for 20 s and, lastly, washed once with water for 30 s.

The samples were left to dry and were visualized under a FEI Tecnai G2 20 TWIN Transmission Electron Microscope at an accelerating voltage of 80 KV. The images were taken using a side-mounted CCD Olympus Veieta with 2k x 2k Mp. Whole-transcriptome analysis and qRT-PCR. Biofilms were grown on MSgg agar as described above. 24-, 48-, and 72-h colonies of the corresponding strains (WT or ΔtasA) were recovered and stored at -80 °C. All of the assays were performed in triplicate. Whole-cell samples were resuspended and homogenized via passage through a 255/8 G needle in BornBoim A107 buffer (20% sucrose, 10 mM Tris-HCl pH 8, 10 mM EDTA and 50 mM NaCl). Lysozyme (10 mg/mL) was added, and the
mixture was incubated for 30 min at 37 °C. After disruption, the suspensions were centrifuged, and the pellets were resuspended in Trizol reagent (Invitrogen). Total RNA was purified using Qiagen’s RNeasy Mini Kit as previously described. RNA integrity was confirmed by running an aliquot on a 1% agarose gel. Oligo(dT)-primed reverse transcription was carried out via in-column treatment with the rDNAse included in the NucleoSpin RNA Plant Kit (Macherey-Nagel) following the instructions of the manufacturer. The integrity and quality of the total RNA was assessed with an Agilent 2100 Bioanalyzer (Agilent Technologies) and by gel electrophoresis.

Fluorescence in situ hybridization (FISH) experiments were performed using the Ribonuclease-Free RNAse (bacteria) Kit from ILLMINNA, and 100 bp single-end read libraries were prepared using the TruSeq Stranded Total RNA Kit (ILLMINA). The libraries were sequenced on an Illumina NextSeq550 instrument. The raw reads were processed with FASTQC, and quality control was performed by QoL Bioinformatics (qOligo). The raw reads were then processed with Bowtie2109 to map the reads against the genomes of interest. To quantify the amount of RNA present in each library, we used the Bowtie2 tool to map the reads against the genome sequences. We then used the qRT-PCR tool to calculate the number of reads that mapped to each gene. The results were then normalized to the total number of reads mapped to each gene.

To quantify the amount of DNA damage in the bacterial colonies, we used the image-iT Lipid Peroxidation Kit (Invitrogen) following the manufacturer’s instructions with some slight modifications. Briefly, colonies of the different strains grown on MSgg plates at 30 °C, isolated at different time points, and resuspended in 1 ml of liquid MSgg medium as described in the previous sections. In all, 5 ml cumene hydroperoxide (CuHpx)-treated bacterial samples were used to perform the RNA sequencing analysis. rRNA removal was performed using the RiboZero rRNA removal (bacteria) Kit from Illumina, and 100-bp single-end reads were generated using the TruSeq Stranded Total RNA Kit (ILLMINA). The libraries were sequenced on an Illumina NextSeq550 instrument. The raw reads were processed with FASTQC, and quality control was performed by QoL Bioinformatics (qOligo). The raw reads were then processed with Bowtie2109 to map the reads against the genomes of interest. To quantify the amount of RNA present in each library, we used the Bowtie2 tool to map the reads against the genome sequences. We then used the qRT-PCR tool to calculate the number of reads that mapped to each gene. The results were then normalized to the total number of reads mapped to each gene.

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visualized immediately using two-photon excitation with a Spectraphysics MaiTai Pulsed Laser tuned to 720 nm (roughly equivalent to 360 nm single photon excitation), attached to a Leica SP5 microscope. Emissions between 432 and 482 nm (gel phase) and between 509 and 547 nm (liquid phase) were recorded using the internal PMT detectors.

The localization of FloT in B. subtilis cells was evaluated using a floT-YFP translational fusion in a WT genetic background (see Supplementary Table 2 for full genotype of the strains). Colonies grown at 30°C on MSgg solid medium were isolated at different time points and resuspended as described above. Samples were mounted and visualized immediately with excitation at 514 nm and emission recorded from 518 to 596 nm.

All images were obtained by visualizing the samples using an inverted Leica SP5 system with a 63x NA 1.4 HCX PL APO oil-immersion objective. For each experiment, the laser settings, scan speed, PMT or HyD detector gain, and pinhole aperture were kept constant for all of the acquired images.

Image analysis. Image processing was performed using Leica LAS AF (LCS Lite, Leica Microsystems) and Fiji/ImageJ software. Images of live and dead bacteria from viability experiments were processed automatically, counting the number of live (green) or dead (red) bacteria in their corresponding channels. The percentage of dead cells was calculated dividing the number of dead cells by the total number of bacteria found on a field. For processing the lipid peroxidation images, images corresponding to the reduced and oxidized channels were smoothed and a value of 3 was then subtracted from the two channels to eliminate the background. The ratio image was calculated by dividing the processed reduced channel by the oxidized channel using the Fiji image calculator tool. The ratio images were pseudo-colored using a color intensity look-up table (LUT), and intensity values of min 0 and max 50 were selected. All of the images were batch processed with a custom process macro, in which the same processing options were applied to all of the acquired images. Quantification of the lipid peroxidation was performed in Imaris v7.4 (Bitplane) by quantifying the pixel intensity of the ratio images with the Imaris “spots” tool.

The Laurdan GP acquisitions were processed similarly. Images corresponding to the two channels and the liquid phase channel were smoothed and a value of 10 was subtracted to eliminate the background. The Laurdan GP image was then calculated by applying the following formula (see equation 2):

\[
\text{Laurdan GP} = \frac{\text{gel phase channel} - \text{liquid phase channel}}{\text{gel phase channel} + \text{liquid phase channel}}
\]

Statistical analysis. All of the data are representative of at least three independent experiments with at least three technical replicates. The results are expressed as the mean ± standard error of the mean (SEM). Statistical significance was assessed by performing the appropriate tests (see the figure legends). All analyses were performed using GraphPad Prism version 6. p values < 0.05 were considered significant. Asterisks indicate the level of statistical significance: *p < 0.05, **p < 0.01, ***p < 0.001, and ****p < 0.0001.

Reporting summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability. The RNA-seq data that support the findings of this study have been deposited in the GEO database with the accession code GSE124307 [https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE124307]. The source data underlying Figures 1A, B, D, 3A, C, 4A–D, 5B, 6B, 7B, 8C, D, 9B, C, D, E, and 10A–C; and Supplementary Figures S1A, C, D, S4, S7B, C, D, S8B, S10B, S11, S12B, S13A, S14B, S15B, S16, S17B, S18B, and S19A are provided as a Source Data file.

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Author contributions

D.R. conceived the study. D.R., and J.C.A. and Y.N. designed the experiments. J.C.A. and J.C.A and Y.N. performed the main experimental work. M.C.P.B. gave support to some physiological experiments and did q-RT-PCR experiments. C.M.S and L.D.M. analyzed and processed the whole transcriptome. J.C.A. and J.P. performed and designed the confocal microscopy work and data analysis. D.R., J.C.A., and Y.N. wrote the manuscript. D.R., J.C.A., C.M.S., A.V. A.P.G., and L.D.M. contributed critically to writing the final version of the manuscript.

Competing interests

The authors declare no competing interests.

Additional information

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