Poster & Publication

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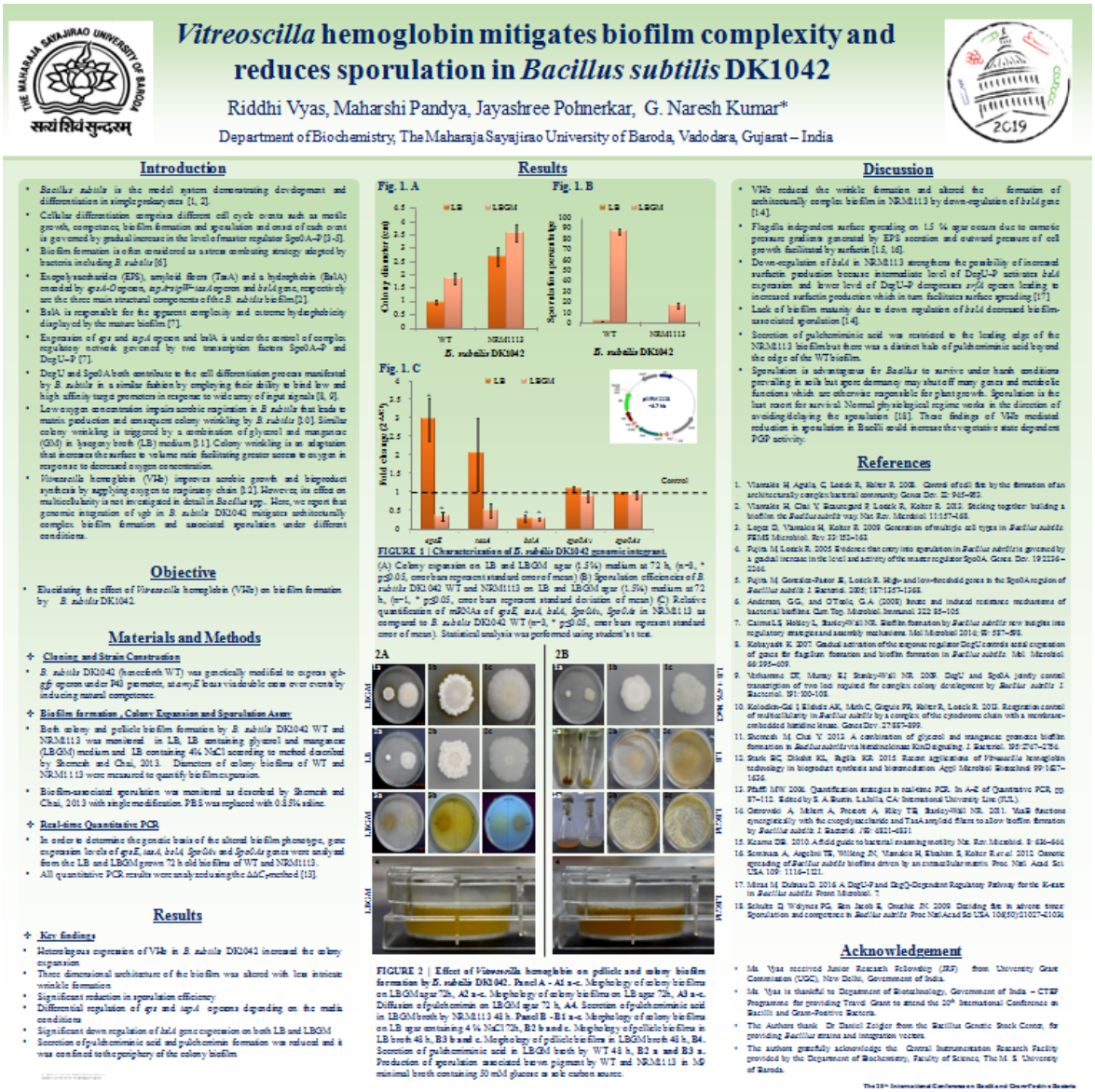

***Vitreoscilla* hemoglobin mitigates biofilm complexity and reduces sporulation in *Bacillus subtilis* DK1042**

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Introduction

- *Bacillus subtilis* is the model system demonstrating development and differentiation in simple prokaryotes [1, 2].
- Cellular differentiation comprises different cell cycle events such as motile growth, competence, biofilm formation and sporulation and onset of each event is governed by gradual increase in the level of master regulator Spo0A~P [3-5].
- Biofilm formation is often considered as a stress combating strategy adopted by bacteria including *B. subtilis* [6].
- Exopolysaccharides (EPS), amyloid fibers (TasA) and a hydrophobin (BslA) encoded by *epsA-O* operon, *ayuRABP-tasA* operon and *bslA* gene, respectively are the three main structural components of the *B. subtilis* biofilm [2].
- BslA is responsible for the apparent complexity and extreme hydrophobicity displayed by the mature biofilm [7].
- Expression of *eps* and *tapA* operon and bslA is under the control of complex regulatory network governed by two transcription factors Spo0A~P and DegU~P [7].
- DegU and Spo0A both contribute to the cell differentiation process manifested by *B. subtilis* in a similar fashion by employing their ability to bind low and high affinity target promoters in response to wide array of input signals [8, 9].
- Low oxygen concentration impairs aerobic respiration in *B. subtilis* that leads to matrix production and consequent colony wrinkling by *B. subtilis* [10]. Similar colony wrinkling is triggered by a combination of glycerol and manganese (GM) in lysogeny broth (LB) medium [11]. Colony wrinkling is an adaptation that increases the surface to volume ratio facilitating greater access to oxygen in response to decreased oxygen concentration.
- *Vitreoscilla* hemoglobin (VHb) improves aerobic growth and biosynthetic synthesis by supplying oxygen to respiratory chain [12]. However, its effect on multicellularity is not investigated in detail in *Bacillus* spp. Here, we report that genomic integration of *vgb* in *B. subtilis* DK1042 mitigates architecturally complex biofilm formation and associated sporulation under different conditions.

Objective

- Elucidating the effect of *Vitreoscilla* hemoglobin (VHb) on biofilm formation by *B. subtilis* DK1042.

Materials and Methods

Cloning and Strain Construction

- *B. subtilis* DK1042 (henceforth WT) was genetically modified to express *vgb-gfp* operon under P43 promoter, at *amyE* locus via double cross over events by inducing natural competence.

Biofilm formation , Colony Expansion and Sporulation Assay:

- Both colony and pellicle biofilm formation by *B. subtilis* DK1042 WT and NRM1113 was monitored in LB, LB containing glycerol and manganese (LBGM) medium and LB containing 4% NaCl according to method described by Shemesh and Chai, 2013. Diameters of colony biofilms of WT and NRM1113 were measured to quantify biofilm expansion.
- Biofilm-associated sporulation was monitored as described by Shemesh and Chai, 2013 with single modification: PBS was replaced with 0.85% saline.

Real-time Quantitative PCR

- In order to determine the genetic basis of the altered biofilm phenotype, gene expression levels of *epsE*, *tasA*, *bslA*, *Spo0Av* and *Spo0Ar* genes were analyzed from the LB and LBGM grown 72 h old biofilms of WT and NRM1113.
- All quantitative PCR results were analyzed using the $\Delta\Delta C_T$ method [13].

Results

Key findings

- Heterologous expression of VHb in *B. subtilis* DK1042 increased the colony expansion.
- Three dimensional architecture of the biofilm was altered with less intricate wrinkle formation.
- Significant reduction in sporulation efficiency.
- Differential regulation of *eps* and *tapA* operons depending on the media conditions.
- Significant down regulation of *bslA* gene expression on both LB and LBGM.
- Secretion of pulcherriminic acid and pulcherriminin formation was reduced and it was confined to the periphery of the colony biofilm.

Results

Fig. 1. A

Fig. 1. B

Fig. 1. C

FIGURE 1 | Characterization of *B. subtilis* DK1042 genomic integrant.

(A) Colony expansion on LB and LBGM agar (1.5%) medium at 72 h, (n=9, * $p \leq 0.05$, error bars represent standard error of mean) (B) Sporulation efficiencies of *B. subtilis* DK1042 WT and NRM1113 on LB and LBGM agar (1.5%) medium at 72 h, (n=4, * $p \leq 0.05$, error bars represent standard deviation of mean) (C) Relative quantification of mRNAs of *epsE*, *tasA*, *bslA*, *Spo0Av*, *Spo0Ar* in NRM1113 as compared to *B. subtilis* DK1042 WT (n=3, * $p \leq 0.05$, error bars represent standard error of mean). Statistical analysis was performed using student's t test.

FIGURE 2 | Effect of *Vitreoscilla* hemoglobin on pellicle and colony biofilm formation by *B. subtilis* DK1042. Panel A - A1 a-c. Morphology of colony biofilms on LBGM agar 72h, A2 a-c. Morphology of colony biofilms on LB agar 72h, A3 a-c. Diffusion of pulcherriminin on LBGM agar 72 h, A4. Secretion of pulcherriminic acid in LBGM broth by NRM1113 48 h. Panel B - B1 a-c. Morphology of colony biofilms on LB agar containing 4 % NaCl 72h, B2 b and c. Morphology of pellicle biofilms in LB broth 48 h, B3 b and c. Morphology of pellicle biofilms in LBGM broth 48 h, B4. Secretion of pulcherriminic acid in LBGM broth by WT 48 h, B2 a and B3 a. Production of sporulation associated brown pigment by WT and NRM1113 in MS minimal broth containing 50 mM glucose as sole carbon source.

Discussion

- VHb reduced the wrinkle formation and altered the formation of architecturally complex biofilm in NRM1113 by down-regulation of *bslA* gene [14].
- Flagella independent surface spreading on 1.5 % agar occurs due to osmotic pressure gradients generated by EPS secretion and outward pressure of cell growth facilitated by surfactin [15, 16].
- Down-regulation of *bslA* in NRM1113 strengthens the possibility of increased surfactin production because intermediate level of DegU~P activates *bslA* expression and lower level of DegU~P dampeners *srfA* operon leading to increased surfactin production which in turn facilitates surface spreading [17].
- Lack of biofilm maturity due to down regulation of *bslA* decreased biofilm-associated sporulation [14].
- Secretion of pulcherriminic acid was restricted to the leading edge of the NRM1113 biofilm but there was a distinct halo of pulcherriminic acid beyond the edge of the WT biofilm.
- Sporulation is advantageous for *Bacillus* to survive under harsh conditions prevailing in soils but spore dormancy may shut off many genes and metabolic functions which are otherwise responsible for plant growth. Sporulation is the last resort for survival. Normal physiological regime works in the direction of avoiding/delaying the sporulation [18]. These findings of VHb mediated reduction in sporulation in Bacilli could increase the vegetative state dependent PGP activity.

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SHORT REPORTS

Vitreoscilla hemoglobin promotes biofilm expansion and mitigates sporulation in *Bacillus subtilis* DK1042

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Abstract

Biofilm formation is considered as a stress combating strategy adopted by bacteria in response to variety of cellular and environmental signals. Impaired respiration due to low oxygen concentrations is one such signal that triggers wrinkling and robust biofilm formation in *Bacillus subtilis*. *Vitreoscilla* hemoglobin (VHb) improves microaerobic growth and bioproduct synthesis in a variety of bacteria by supplying oxygen to the respiratory chain. Present study was carried out to determine the effect of VHb on multicellularity of *B. subtilis*. Thus, *B. subtilis* DK1042 (WT) was genetically modified to express *vgb* and *gfp* genes under the control of P43 promoter at *amyE* locus by double cross over events. Biofilm formation by the integrant NRM1113 and WT was monitored on Lysogeny broth (LB) and LB containing glycerol and manganese (LBGM) medium. The WT produced more wrinkled colonies than NRM1113 on LB and LBGM medium. Concomitantly, biofilm-associated sporulation and production of pulcherriminic acid was decreased in NRM1113 as compared to WT on LB as well as LBGM. Expression studies of genes encoding structural components of biofilms revealed ~ 70% down-regulation of *bslA* gene in NRM1113 on both LB and LBGM which is correlated with reduced wrinkling in NRM1113. Moreover, NRM1113 showed increased colony expansion compared to WT in LB, LBGM and high osmolarity conditions. VHb expression alters various processes in different host cells, our study represents that VHb modulates biofilm formation, sporulation and pulcherriminic acid formation in *B. subtilis* DK1042.

Keywords *Bacillus subtilis* · Biofilm · Sporulation · *Vitreoscilla* hemoglobin · Surface spreading · Pulcherriminic acid

Introduction

Bacillus subtilis is the model system demonstrating development and differentiation in simple prokaryotes. Cellular differentiation comprises different cell cycle events such as motile growth, competence, biofilm formation and sporulation (López et al. 2009a, b). Onset of each event is governed by gradual increase in the level of master regulator Spo0A~P. At low level of Spo0A~P, cells remain motile, intermediate level of Spo0A~P induces matrix production genes and at high levels, cells undergo sporulation (Vlamakis et al. 2013). Biofilm formation is a protective mechanism to withstand environmental challenges (Costerton et al. 1995; Ciofu and Tolker-Nielsen 2019). Low oxygen concentration impairs aerobic respiration in *B. subtilis* that leads to matrix production and consequent colony wrinkling by *B. subtilis* (Kolodkin-Gal et al. 2013). Similar colony wrinkling is triggered by a combination of glycerol and manganese (GM) in lysogeny broth (LB) medium (Shemesh and Chai 2013). Colony wrinkling is an adaptation that increases the surface to volume ratio facilitating greater access to oxygen in response to decreased oxygen concentration. *Vitreoscilla* hemoglobin (VHb) improves aerobic growth and bioproduct synthesis by supplying oxygen to respiratory chain (Stark et al. 2015). Heterologous expression of VHb improved cell growth and total protein secretion, insecticidal crystal protein yields, γ-PGA production and biodegradation of phenol and *p*-nitrophenol in *B. subtilis*, *B. thuringiensis*, *B. amyloliquefaciens* and *B. cereus*, respectively (Kallio and Bailey 1996; Feng et al. 2007; Zhang et al. 2013; Velez-Lee et al. 2015). However, its effect on multicellularity is not

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investigated in detail in *Bacillus* spp. Here, we report that genomic integration of *vgb* in *B. subtilis* DK1042 mitigates architectural complexity of biofilm formation and associated sporulation under different conditions.

In *B. subtilis*, biofilm formation is a very well orchestrated process involving expression of multicistronic *epsA-O* and the *tapA–sipW–tasA* operons coding for exopolysaccharide (EPS) and amyloid fiber (TasA) components, respectively, of the biofilm matrix (Cairns et al. 2014). Expression of these operons is under negative control by SinR and AbrB. Low to intermediate levels of Spo0A~P represses expression of *abrB* and turns on the synthesis of two anti-repressor proteins SinI and AbbA which derepress the genes under the control of SinR and AbrB, respectively (Banse et al. 2008). Spo0A is activated by phosphorylation via a multicomponent phosphorelay involving five sensory histidine kinases (KinA–E), Spo0F and Spo0B in response to various environmental signals (Jiang et al. 2000). In addition to EPS and TasA, bacterial hydrophobin encoded by *bslA* gene is yet another extracellular component of *B. subtilis* biofilm that acts synergistically with EPS and TasA to form robust biofilm (Cairns et al. 2014; Arnaouteli et al. 2016). BslA is responsible for the apparent complexity and extreme hydrophobicity displayed by the mature biofilm. Production of BslA is directly repressed by AbrB (Verhamme et al. 2009) and indirectly activated by Rok (Kovacs and Kuipers 2011) as well as intermediate level of DegU~P. DegU has different regulatory activities in its phosphorylated (DegU~P) and non-phosphorylated (DegU) forms. DegU and low level of DegU~P activate genetic competence and motility, respectively (Hamoen et al. 2000; Kobayashi 2007). Intermediate level of DegU~P shuts off motility genes and activates biofilm formation while very high amount of DegU~P inhibits biofilm formation and leads the cells towards terminal developmental stage of sporulation by affecting the level of Spo0A~P (Marlow et al. 2014). Thus, DegU and Spo0A both contribute to the cell differentiation process manifested by *B. subtilis* in a similar fashion by employing their ability to bind low and high-affinity target promoters in response to wide array of input signals (Murray et al. 2009; Verhamme et al. 2009).

Materials and methods

Cloning and strain construction

The *Bacillus* strains, plasmids and primers used and constructed in this study are listed in Table S1. The *B. subtilis* DK1042 (henceforth WT) and the integrant were propagated in Lysogeny broth medium (HiMedia) at 37 °C for routine use. Biofilm formation was studied in four different media, Lysogeny broth (LB), LB medium containing 4% NaCl, Biofilm-promoting LBGM medium (Shemesh and Chai 2013) and Mineral salt glycerol glutamate (Msgg) medium (Branda et al. 2004) at 30 °C. Neomycin was added at 6 µg/ml during the selection of *B. subtilis* integrant. For the growth of *Escherichia coli*, antibiotics were added at the following concentrations 100 µg/ml of ampicillin, 100 µg/ml of streptomycin and 50 µg/ml of kanamycin.

Cloning experiments were carried out using *E. coli* DH10B. Integration vector pNRM1113 containing *vgb-gfp* operon under P43 promoter was constructed in pDK plasmid (Supplemental material and methods). P43 promoter is widely used as a strong constitutive promoter for the overexpression of genes in *B. subtilis* (Wang and Dio 1984). *B. subtilis* DK1042 was genetically modified to develop genomic integrant NRM1113 by transformation with pNRM1113 via double cross over recombination at *amyE* locus using natural competence (Juhas and Ajioka 2016). *amyE* is a widely used and neutral integration site for the integration of genes (Shimotsu and Henner 1986). Double cross-over homologous recombination events were confirmed by screening neomycin resistant colonies on starch agar plate for the absence of amylase activity.

Growth curve experiment

WT and NRM1113 were grown in LB at 30 °C to mid-log phase and then cells were inoculated at 1% v/v in LB and LBGM broth. Cells were inoculated at 5% v/v in LB growth containing 6% NaCl. Growth curve was monitored at different shaking conditions (200 rpm and 100 rpm) at 30 °C and the cell optical density was measured at every hour till the slow-down phase. The experiment was performed with three replicates.

Biofilm formation, colony expansion and sporulation assay

Biofilm formation and sporulation by WT and NRM1113 were monitored according to the method described by Shemesh and Chai (2013). *B. subtilis* cells were grown in LB broth till mid-log phase (OD_{600} = 0.4–0.6) at 37 °C. Two microliter of culture was spot inoculated on LB, LBGM and Msgg medium with 1.5% agar concentration and plates were incubated at 30 °C for 72 h before examination of colony morphology and sporulation efficiency. Diameters of colony biofilms of WT and NRM1113 were measured to quantify biofilm expansion. Pellicle formation was monitored in LB, LBGM and Msgg broth. Images were taken using Nikon D3400 digital camera. Biofilm-associated sporulation was monitored as described by Shemesh and Chai (2013). Briefly, biofilms were scraped from the agar surface and suspended in 0.85% saline. Biofilms were subjected to mild sonication (three rounds of 12 one-second pulses at 20%

power) to free single cells. Cells were serially diluted and heat kill was performed at 80 °C for 20 min in a water bath. Total cell numbers before and after heat kill were quantified by the plating method. Sporulation efficiency was calculated by dividing the total number of viable spores after heat kill by the total number of cells before heat kill.

Gene expression analysis

To determine the genetic basis of the altered biofilm phenotype, gene expression analyses of *epsE*, *tasA* and *bslA* genes coding the structural components of biofilm was carried out. Total RNA was isolated from 72 h old colony biofilms of WT and NRM1113 using the Mascherey-Nagel Nucleospin mini RNA isolation kit. Three microgram of total RNA was applied to the reverse transcription reaction using the Quantinova Reverse Transcription kit (Qiagen), as described in the provided protocol. Real-time PCR was performed to compare the level of *epsE*, *tasA*, *bslA, spo0Av and spo0As* gene expression in WT and NRM1113 colony biofilms grown on LB and LBGM medium with the Evagreen PCR master mix (Bio-Rad). Gene-specific primers are listed in Table S2. One µl of cDNA samples were used as the template for real-time PCR. PCR was performed in the CFX connect (Bio-Rad) using the following program: one cycle of 95 °C for 30 s and 40 cycles of 95 °C for 5 s, 58 °C for 10 s, and 72 °C for 15 s and the melting curves were determined from 65.0 to 95.0 °C for 6.5 min. *rpoB* and *gyrB* genes were used as internal controls and the geometric mean of cycle threshold (C_T) values of these two transcripts were used to normalize the C_T values of target gene transcripts (Ho et al. 2011; Vargas-Bautista et al. 2014). All quantitative PCR results were analyzed using the $\Delta\Delta C_T$ method (Pfaffl 2004). qRT-PCR analysis was performed as technical triplicate. Experiments were also performed with three biological replicates.

Results and discussion

VHb enhanced biofilm expansion in *B. subtilis* DK1042

We wished to check that how does constitutive expression of VHb affect wrinkle formation in *B. subtilis* DK1042 on LB and LBGM agar. Our assumption was that VHb should reduce wrinkle formation by increasing oxygen availability because wrinkle formation is an adaptation to increase surface to volume ratio to allow greater excess to oxygen (Koldkin-Gal et al. 2013). Incorporation of VHb reduced complexity and increased the diameter of colony biofilm in NRM1113 as compared to WT on both LB and LBGM (Figs. 1a, 2a Panels 1 and 2). Colony diameter of NRM1113

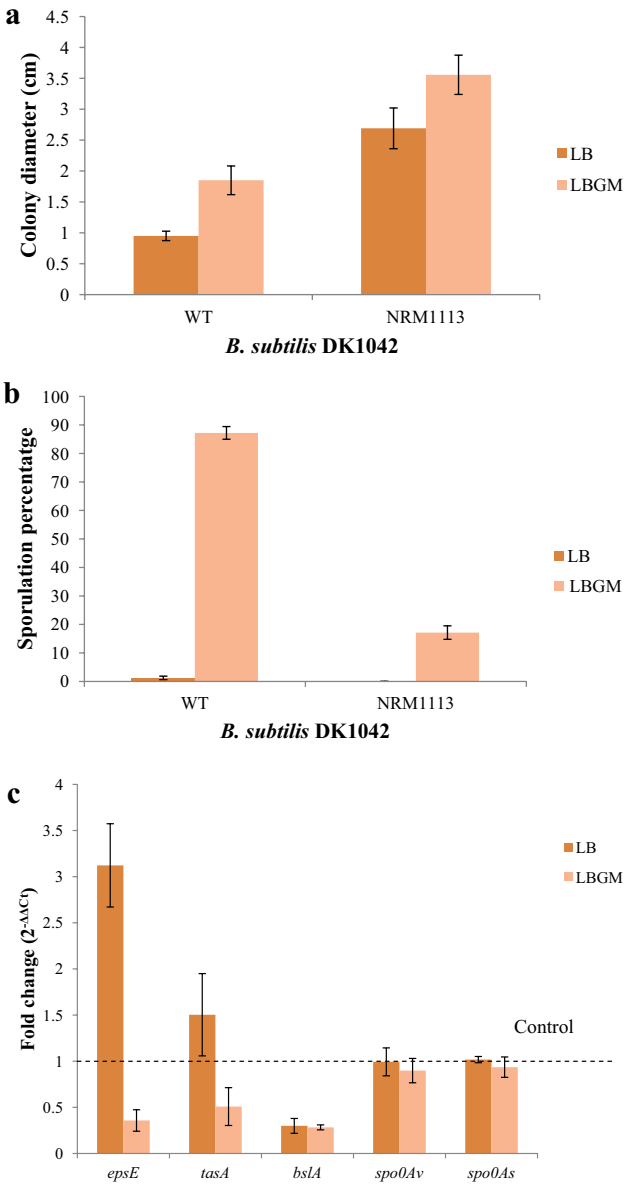

Fig. 1 Characterization of *B. subtilis* DK1042 genomic integrant. **a** Colony expansion on LB and LBGM agar (1.5%) medium at 72 h, ($n=3$, $P<0.05$, error bars represent standard error of mean). **b** Sporulation efficiencies of *B. subtilis* DK1042 WT and NRM1113 on LB and LBGM agar (1.5%) medium at 72 h, ($n=1$, $P\leq0.05$, error bars represent the standard deviation of mean). **c** Relative quantification of mRNAs of *epsE*, *tasA*, *bslA*, *Spo0Av*, *Spo0As* in NRM1113 as compared to *B. subtilis* DK1042 WT ($n=3$, $P\leq0.05$, error bars represent standard error of mean). Statistical analysis was performed using student's *t* test

increased 2.83- and 1.95-fold with respect to WT on LB and LBGM, respectively ($n=3$, $P<0.05$). Strikingly, surface spreading was also observed on LB containing 4% NaCl (Fig. 2a, Panel 4). To determine the reason for this surface spreading, WT and NRM1113 were assayed for growth in LB, LBGM and 6% NaCl LB broth. Growth curve

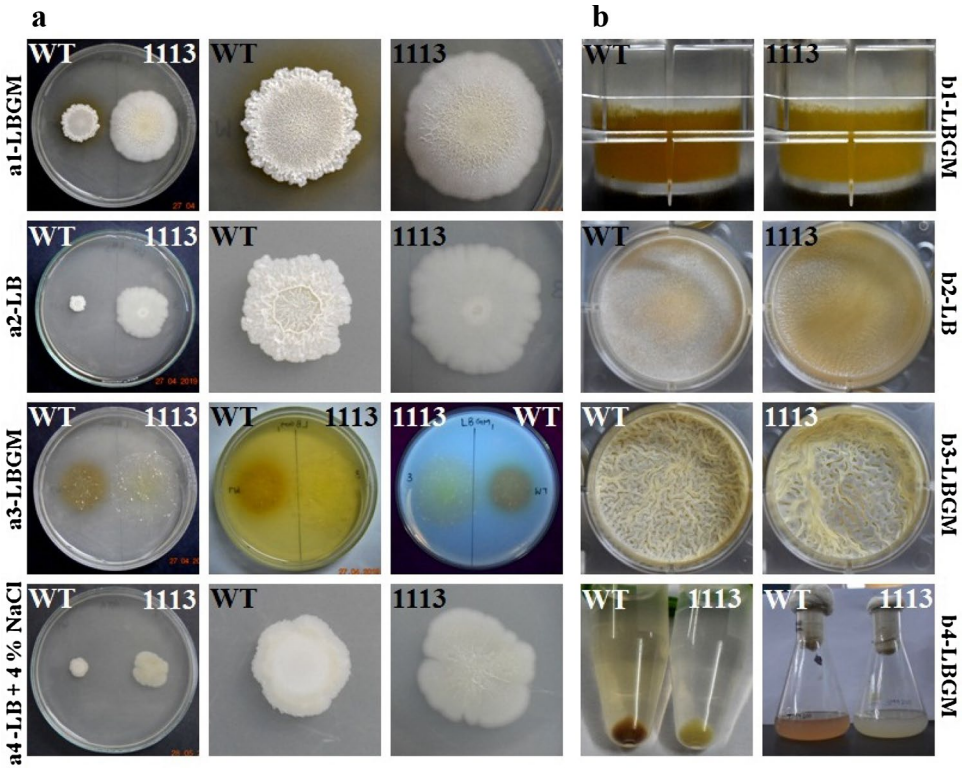

Fig. 2 Effect of *Vitreoscilla* hemoglobin on pellicle and colony biofilm formation and pulcherriminic acid secretion by *B. subtilis* DK1042. Panel **a**—**a1** Morphology of colony biofilms on LBGM agar 72 h, **a2** morphology of colony biofilms on LB agar 72 h, **a3** diffusion of pulcherrimin on LBGM agar 72 h, **a4** morphology of colony biofilms on LB agar containing 4% NaCl 72 h. Panel **b**—**b1** Secretion of pulcherriminic acid in LBGM broth by WT and NRM1113 48 h. **b2** Morphology of pellicle biofilms in LB broth 48 h, **b3** Morphology of pellicle biofilms in LBGM broth 48 h, **b4** Production of sporulation associated brown pigment by WT and NRM1113 in M9 minimal broth containing 50 mM glucose as sole carbon source

experiments showed that both WT and NRM1113 followed similar growth trajectories but specific growth rate (μ) of NRM1113 was slightly higher than that of WT under all the experimental conditions which may contribute to the increased fitness of the integrant (Fig. S2). Doubling time of NRM1113 was 9 min shorter than that of WT under osmostic stress. Biofilm phenotype was also monitored on Msgg medium where WT produced more wrinkled colonies as compared to NRM1113 after 24 h incubation. Morphological difference between these two was reduced with further incubation. Similar trend appeared in pellicles formed in Msgg broth (Fig. S1). Morphological difference was less significant between the colony biofilms of WT and NRM1113 grown on Msgg medium in comparison to colonies grown on LB and LBGM possibly because Msgg is a minimal medium containing glycerol and glutamate as the carbon source and glycerol is an energy poor carbon source in comparison to complex LB and LBGM media (Martinez-Gomez et al. 2012; Chubukov et al. 2013; Wang et al. 2015).

VHb altered matrix gene expression in *B. subtilis* DK1042

Flagella independent surface spreading on 1.5% agar occurs due to osmotic pressure gradients generated by EPS secretion and outward pressure of cell growth facilitated by surfactin (Kearns 2010; Seminara et al. 2012; Mhatre et al. 2017). We wished to elucidate the role of matrix producing genes in surface spreading in the integrant. Relative gene expression analysis in comparison to WT showed 64.17% (± 11.6) downregulation of *epsE* gene in LBGM grown biofilm of NRM1113 while it was upregulated 3.12 (± 0.45) fold in LB grown biofilm of NRM1113 ($n = 3$, $P \leq 0.05$). Differential regulation of *epsE* in NRM1113 depending upon different media conditions justifies the extent of colony expansion on LB and LBGM medium. Colony expansion on LB could be due to the upregulation of *eps* operon as well as surfactin production while on LBGM it could chiefly be attributed to surfactin mediated sliding motility. *bslA* gene was downregulated 70.02% (± 8.0) and 71.65% (± 2.7) in LB and LBGM grown biofilm of NRM1113, respectively ($n = 3$, $P \leq 0.05$) (Fig. 1c). This significant down-regulation of *bslA* in NRM1113 further strengthens the possibility of increased surfactin production because intermediate level of DegU~P activates *bslA* expression and lower level of DegU~P derepresses *srfA* operon leading to increased surfactin production which in turn facilitates surface spreading (Miras and Dubnau 2016). Expression of *tasA* in NRM1113 was statistically insignificant with respect to WT in both the media conditions. Expression of *spo0A* transcripts from vegetatitve (Spo0Av) as well as sporulation (Spo0As) promoters was also similar in NRM1113 on LB and LBGM indicating that VHb does not alter the transcription of *spo0A* and alterations in biofilm phenotype of integrant could mainly be due to change in the levels of Spo0A~P.

VHb mitigates sporulation in *B. subtilis* DK1042

Biofilm associated sporulation was less in NRM1113 than in WT on LB and LBGM agar (Fig. 1b). The sporulation percentage for WT and NRM1113 was 0.91% and 0%, respectively, on LB at 72 h. On the other hand, the efficiency of sporulation was 87.2% in WT and 17.14% in NRM1113 on LBGM at 72 h. Precise mechanisms responsible for VHb driven alterations in biofilm formation and sporulation in NRM1113 remains to be determined but significant downregulation of *bslA* is responsible for the decrease in the complexity of colony biofilms and lack of biofilm maturity in turn reduced biofilm-associated sporulation in NRM1113 possibly via a checkpoint protein, KinD until mature biofilm is formed (Aguilar et al. 2010; Ostrowski et al. 2011).

Reduction in brown pigmentation in VHb harboring *B. subtilis* DK1042

Secretion of pulcherriminic acid was restricted to the leading edge of the NRM1113 biofilm but there was a distinct halo of pulcherriminic acid beyond the edge of the WT biofilm (Fig. 2a, Panel 3). These results are well in accordance with the recent study linking the diffusion of pulcherriminic acid and pulcherrimin formation with the growth arrest of the biofilm in *B. subtilis* (Arnaouteli et al. 2019). The integrant started accumulating pulcherriminic acid in growth-limiting quantities similar to WT only after 96 h and that was limited to the center of the colony (Fig. S1). This raises the possibility of yet another unidentified stress signal that triggers the secretion and accumulation of pulcherriminic acid at growth-limiting quantities in the integrant as the center of the colony faces depletion of nutrients and oxygen and accumulation of toxic products (Stewart and Franklin 2008). WT and NRM1113 produced similar pellicle morphology in LB and LBGM broth (Fig. 2b, Panels 2 and 3). However, pulcherriminic acid secretion was less in NRM1113 with reference to WT in LBGM broth (Fig. 2b, Panel 1). Sporulation defective mutants do not produce brown pigment in *B. subtilis* (Piggot and Coote 1976). Reduced brown pigmentation in M9 minimal medium (Kleijn et al. 2010) shake flask cultures corroborates that sporulation might be delayed/reduced in the integrant as compared to WT. VHb increased the levels of antioxidants such as ascorbate in *Arabidopsis* plants (Wang et al. 2009) and it also possesses peroxidase activity (Kvist et al. 2007). Antioxidant enzymes such as catalase and peroxidase contribute to tackle ROS mediated stress (Liao et al. 2015; Khan et al. 2016). Increasing accessibility of oxygen to respiratory chain by VHb stimulates electron transport thereby increasing NAD^+/NADH ratio and ATP production in the host cell (Zhang et al. 2007; Stark et al. 2015). Upregulation of energy generating and biosynthetic pathways is associated with the early stages of biofilm formation (Pisithkul et al. 2019). Whether the observed effects in the integrant NRM1113 are due to improved energy and antioxidants status by VHb needs further investigation. The future challenges include elucidation of downstream signaling process with the multi-omics approach involving metabolomic, transcriptomic, and proteomic analyses.

Conclusion

Constitutive expression of VHb has resulted in the enhanced expansion of biofilm and reduced sporulation in *B. subtilis* DK1042. Sporulation is advantageous for *Bacillus* to survive under harsh conditions prevailing in soils but spore dormancy may shut off many genes and metabolic functions which are otherwise advantageous for promoting plant growth. Sporulation is the last resort for survival. Normal physiological regime works in the direction of avoiding/delaying the sporulation (Schultz et al. 2009; López et al. 2009; González-Pastor 2011). In addition to this, biofilm formation is positively correlated with the biocontrol property and colonization ability of various strains of *Bacillus subtilis* on plant surfaces (Chen et al. 2012; Molina-Santiago et al. 2019). It would be interesting to see how do altered biofilm formation and sporulation influence root colonization and biocontrol property of VHb containing *B. subtilis* DK1042.

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Compliance with ethical standards

Conflict of interest The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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