Cellular redox state affects biofilm formation by a surfactin -dependent manner in Bacillus amyloliquefaciens?

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Abstract

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Cellular redox state affects biofilm formation by a surfactin - dependent manner in *Bacillus amyloliquefaciens*?

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Running title: Redox state, surfactin and biofilm

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Abbreviation

NADH/NAD+ (Nicotinamide adenine dinucleotide)

IPTG (Isopropyl -D-Thiogalactoside)

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Abstract

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Keywords: *Bacillus amyloliquefaciens* , redox, surfactin, biofilm, Spx, PerR.

1 Introduction

Bacillus species like *B. subtilis* , *B. amyloliquefaciens* , *B. licheniformis* , are Gram-positive, spore-forming bacteria widely distributed in soil, water, air, etc. During times of environmental insult such as poor nutrient, the bacteria undergo developmental changes leading to quorum sensing responses, by which the cells form multicellular communities known as biofilm [1,2]. Now it is known that upon harsh environment, the cells produce a secondary metabolite called surfactin, which is a nonribosomal lipopeptide biosynthesized by a multienzyme system encoded by the gene cluster *srfA* [3-5], as a signal to trigger biofilm formation [6-8].

Generally, harsh environments lead to a change of cellular redox state, which is an important parameter to affect surfactin production and biofilm formation [9]. For example, *B. subtilis* can switch from an unicellular state to a multicellular state of biofilm via responding to a redox switch[1]. The $NADH/NAD^+$ ratio, or another indicator of the cellular redox potential, is sensed by regulatory pathways to control features of colony wrinkling. Also, colony development can both respond to and affect redox homeostasis [9]. For instance, isocitrate dehydrogenase is capable of regulating biofilm formation by modulating intracellular redox homeostasis [10]. Also, the cells are capable of transferring glucose to acetoin as a response to enough carbon source and reducing power, which are essential for robust biofilm growth and conserving redox balance [11,12].

Despite importance of redox, we know little about how the shifts in redox state regulates biosynthesis of surfactin for triggering biofilm formation. Surfactin production can be regulated by the cellular redox status via some regulatory proteins such as Spx and PerR. Spx interacts with RNA polymerase to control transcription in response to oxidative stress [13,14], which plays a key role in maintaining the redox homeostasis exposed to disulfide stress, ensuring an immediate response to oxidative stress [15]. The redox state of cytoplasm is the major effector driving *Spx* activation, which activity is enhanced by reversible formation of a disulfide bond and thereby directly modulated by the intracellular redox status [16]. The transcription of*spx* is negatively regulated by the repressor PerR and is positively regulated by a sigma factor of SigB [15]. SigB is a general stress response transcription factor [17], and PerR senses H_2O_2 to mediate adaptation to peroxide stress. PerR also represses oxidative stress resistance genes including the catalase *katA* , alkylhydroperoxide reductase *ahpC* , iron uptake repressor (*fur*), and *perR* itself [18,19]. After oxidation PerR is inactivated, so the oxidative response genes including*spx* are capable of being successfully expressed. Thereby, PerR plays an auxiliary role in coordinating the disulfide stress responses [19].

Previously, we reported that mutation of *srfA* resulted in a very seriously defective growth and biofilm formation but could be restored by glucose in *B. amyloliquefaciens* WH1 [20]. Glucose an its intermediate metabolites such as NADH are reductive substances, we hypothesized that these reductants might influence biofilm formation via some regulators such as Spx and PerR to regulate surfactin production as a response to the change of ceullular redox homeostasis. In this study, we characterized the interplay among redox

state, surfactin production and biofilm formation, and found the redox status influenced by oxidants and reductants could affect biofilm formation via regulation of surfactin production in *B. amyloliquefaciens* . Moreover, some reductants such as glucose could also influence biofilm formation by a surfactin-independent way in this bacterium.

2 Materials and Methods

2.1 Ethics statement

In vivo experiments with Balb/C mice were carried out according to guidelines approved by Huazhong Agricultural University.

2.2 Bacterial strains,animals and materials

Experiments were performed with the strains listed in Table S2 (Supplementary materials). Materials for DNA manipulation were purchased from Takara Bio (China). Other chemicals were of analytical grade supplied by Sinopharm Chemical Reagent (China). Mice (Balb/C, male, 6-weeks old) were purchased from the Laboratory animal center in our University, Wuhan, China.

2.3 Construction of mutant and complementary strains

The gene *spx, perR* , *aphC* , *sodA* , *sigB* and*rex* was deleted by double crossover homologous recombination, respectively [35]. The detailed method to construct mutant strains were described in the supplementary materials. The plasmids were constructed for compensation of *spx* , *perR* and *rex* , respectively. Briefly, the gene *spx* , *perR* and *rex*with their own promoters and terminators were amplified from the genomic DNA by PCR with the primers listed in Table S3 (Supplementary materials), cloned into T2(2)-ori joined by *BamH* I and *Xba*I restriction sites, then the constructed plasmids were used for transformation of Δ*spx* , Δ*perR* and Δ*rex* for construction of the complementary strain C-Δ*spx* , C- Δ*perR* and C-Δ*rex* respectively.

2.4 Determining growth

Strains were cultured in 5 ml LB medium at 37 °C and 180 rpm overnight, then 2 μl broth of different strains were used for inoculating 200 μl fresh LB medium, or LB medium added with different concentrations of glucose (0, 2, 5, 10 and 20 g/L) in 96-well microplates. The microplates were cultured at 37 °C and 180 rpm, and the growth curve of strains were recorded with an automatic growth curve analyzer (Bioscreen Cpro, Finland).

2.5 Analysis of hemolytic activity

The hemolytic activity in WH1 and Δ*srfA* were determined with agar plates containing sheep blood cells. Briefly, 1 μL fresh broth of WH1 or Δ*srfA* was used for inoculation of agar plates containing sheep blood cells, incubated at 37 °C for 48 h, then the hemolytic activity was determined to evaluate surfactin production in the strains [20].

The hemolytic activity of broth was also used to assay surfactin production in different strains [36]. The blood was collected from mice, added into 10 ml Alsever's solution (glucose 2.05 g, sodium citrate 0.8 g, NaCl 0.42 g and citric acid 0.055 g in 100 ml water, pH 6.1), then centrifuged at 300 g for 8 min. The blood cells were collected, washed once with 10 ml Alsever's Solution, then suspended with saline solution to 2% (v/v). The broth of different strains were centrifuged at 12,000 rpm for 5 min, then the supernatant was collected and mixed with the same volume of 2% red blood cells after serial dilution. After incubation at 37 °C for 1 h, the mixture was centrifuged at 300 g for 2 min, then the supernatant was collected for determining the OD_{540} value. 2% red blood cells were incubated with pure water as positive control, and incubated with the relative medium for culturing bacterial strains as negative control. The hemolytic rate was calculated to assess surfactin production in different strains as the following formula: the hemolytic rate = (OD_{540}s-OD_{540}n)/OD_{540}p x 100%. OD_{540}s: the OD_{540} value of sample; OD_{540}n: the OD_{540} value of negative control; OD_{540}p: the OD_{540}value of positive control.

2.6 Detection of biofilm

WH1, mutant strains and complementary strains were cultured on LB agar plates, then the morphology of colonies were observed by microscope. Robust pellicles (floating biofilms) were determined in multiwell (24-well) plates with 2 ml LB medium each well [37]. Briefly, 20 μL of the fresh broth of each strain was used for inoculating 2 ml LB medium, or LB medium containing glucose (0, 2, 5, 10 and 20 g/L)or H_2O_2 (0, 0.2, 0.5, 1.0, 2.0 and 4.0 mM) in each well of Multiwell (24-well) plates, then cultured at 28°C for 48 h to allow float biofilms formation.

2.7 qRT-PCR

The genes correlated with biosynthesis of surfactin, including*srfA* , *sfp* , *spx* and *perR* were analyzed by qRT-PCR. Single colony of each strain was selected for inoculating LB medium and cultured at 37 °C overnight, then the broth was transferred into fresh LB medium, or LB medium added with glucose (10 g/L) or H_2O_2 (2.0 mM) at a ratio of 1% (v/v). After incubation at 37 °C for 24 h, the broth was collected for isolating mRNA with RNeasy Mini Kit (Qiagen, German). cDNA was produced by reverse transcription with 1 μg RNA, iScript Select cDNA Synthesis Kit and random oligonucleotide primers. qRT-PCR was performed with cDNA, SsoAdvanced Universal SYBR Green Supermix (Bio-Rad) and target-specific primers in Table S4 (Supplementary materials) in CF96 Real-Time System (Bio-Rad) as following: 1 cycle of 95 °C for 5 min, 40 cycles of 95°C for 10 s, 45 °C for 20 s and 70°C for 30 s. All expression data were normalized to the copy number of 16S rRNA in each sample [38].

2.8 Western blot analysis

The *perR* gene was amplified by PCR with primers *perR* -F (CG*GGATCC* ATGGCTGCACAT -GAATTAAA) and *perR* -R (CC*CTCGAG* GTGGTTCTCTTTTTTGGAAC), subcloned into the pET28a vector, then the recombinant plasmid was transformed into *Escherichia coli* BL21. The expression of PerR was induced by IPTG (2 mM), purified by Ni-NAT column (Qiagen, German), then used for intraperitoneal immunization of mice at 50 μg/mouse for 3 dosages after emulsification with Freund's adjuvant (Sigma, USA) [38]. After one week, the sera were collected for the following Western blot assay.

The cell pellets were prepared as the method described above for isolating mRNA. The cell pellets were lyzed by ultrasonification, then the total proteins concentration of each sample was adjusted to 10 μg/μL. After being mixed with sample buffer, 10 μL of each protein sample was separated by 15% SDS-PAGE gel, transferred onto the polyvinylidene fluoride (PVDF) membrane (Millipore, USA), then incubated with the antisera at a dilution of 400 x for 1 h. After extensive wash, the membrane was incubated with goat anti-mouse IgG labeled with Horseradish Peroxidase (HRP) (Boster Biological Technology, China) at a dilution of 5,000 x for 30 min. After that, the membrane was washed, reacted with BeyoECL Star (Beyotime Biotechnology, China), then scanned by Monad QuickChemi 5100 (Zhuhai, China) [38].

2.9 Statistical analysis

All experiments were repeated in triplicates. Data between two groups were compared by a student t -test. Differences among multiple groups were analyzed by Analysis of Variance (ANOVA). * and ** mean a significant and very significant difference between two groups, respectively. Different capital letters indicate significant differences among different groups.

3 Results

3.1 Hemolytic activity of surfactin

Surfactin has strong hemolytic activity [20]. We detected the hemolytic activity of surfactin using the agar plates containing sheep blood cells, and found WH1 showed obvious hemolytic activity around the colony, but the mutant strain Δ*srfA* without surfactin production lost this ability. We further determined the hemolytic activity in broth, and found it was also disappeared in Δ*srfA* (Fig. S1 in supplementary materials). Thereby, the hemolytic activity could be used for representation of the surfactin production in different strains.

3.2 Effects of reductants on surfactin production and biofilm formation in WH1

Glucose at high concentrations (*e.g.* 10 or 20 g/L) significantly increased the biomass in 24 h, while it significantly delayed and reduced the surfactin production in a concentration-dependent effect (Fig. 1A). We detected the genes transcription related with surfactin production in different strains using qRT-PCR. With glucose , the transcription of genes including *srfAB* , *sfp* that encodes 4'-phosphopantetheinyl transferase, *spx* and *perR,* were all significantly down-regulated (Fig. 1B), consistent with the result that these reductants led to a significant decrease of surfactin production in WH1.

The *perR* gene was amplified from WH1, then ligated into pET28a for expression. After induction with IPTG, the recombinant PerR in*E. coli* was purified by Ni-NTA column (Fig. S2 in supplementary materials). The purified rPerR was used for immunization of mice for producing antibodies, then the antisera were collected for Western blotting assay. The cells were collected after being cultured for 24 h, lyzed by ultrasonication, then the total cell proteins were adjusted to the same concentration for Western blotting assay. The result showed the content of PerR was decreased in the group added with 10 g/L glucose compared to WH1, consistent with the *perR* transcription described above (Fig. 1B). rPerR was used as control, which showed a higher molecular weight because it includes an extra sequence containing His-tag from the vector.

Glucose was capable of enhancing biofilm formation in a concentration-dependent manner (Fig. 1C). Interestingly, glucose could also restore and enhance biofilm formation in the strain Δ*srfA*(Fig. 1D), which had no surfactin production at all as described above.

3.3 Effects of oxidants on surfactin production and biofilm formation in WH1

H_2O_2 at high concentrations had negative influences on the cell growth. Consistently, H_2O_2 led to a decrease of surfactin production in a dose-dependent manner (Fig. 2A). Accompanying with reduced surfactin production, biofilm formation was also delayed and weakened. At 24 h, float biofilm was unobserved in all groups added with H_2O_2. At 36 h, the group added with 0.2 or 0.5 mM H_2O_2 was observed with growing biofilm. At 48 h, float pellicle was still unobserved in the group added with 2.0 or 4.0 mM H_2O_2 (Fig. 2B). However, H_2O_2 had no obvious influence on biofilm formation in Δ*srfA* because this strain lost the ability to produce biofilm in a surfactin-dependent manner (Fig. 2C).

With H_2O_2, the transcription of*srfAB* , *sfp* , *spx* and *perR* were all significantly down-regulated (Fig. 2D), consistent with the result that H_2O_2 led to a significant decrease of surfactin production in WH1. Western blot analysis showed that the content of PerR was decreased in the group added with 2 mM H_2O_2 (Fig. 2E), also consistent with the result of *perR* transcription level described above.

3.4 Effects of reductants on surfactin production and biofilm formation in Δspx

The *spx* knockout strain (Δ*spx*) was constructed (Fig. S3 in supplementary materials). Δ*spx* showed a colony morphology with less wrinkles, and displayed a weaker floating pellicle with less wrinkles than WH1 (Fig. 3A). Δ*spx* showed a slightly weaker growth but a significant decrease of surfactin production compared to WH1 (Fig. 3B). The weakened ability to form biofilm in Δ*spx* could be partially restored by compensation of *spx* , and the reduced surfactin production was also restored in the complementary strain (C-Δ*spx*) (Fig. 3A&B). The transcription of *srfAB* was significantly decreased in Δ*spx* compared to WH1. The PerR protein was slightly decreased in Δ*spx* compared to WH1, consistent with the transcription level of *perR* in Δ*spx* (Fig. 3C).

Just like that in WH1, glucose at high concentrations (*e.g.* 10 or 20 g/L) significantly improved the cell growth but significantly inhibited the surfactin production in Δ*spx* (Fig. 3D). qRT-PCR showed that glucose resulted in a significant down-regulation of genes transcription like *srfAB* , *sfp* and *perR* . Western blot analysis showed that glucose could slightly increase the content of PerR in Δ*spx* (Fig. 3G).

Like that in WH1, glucose was favorable for forming a robuster floating pellicle with more wrinkles in Δ*spx* (Fig. 3F). Thereby, glucose affected the biofilm formation by a surfactin-independent manner.

3.5 Effects of oxidants on surfactin production and biofilm formation in Δspx

H_2O_2 had no significant influence on the cell growth, but significantly improved the surfactin production in Δ*spx* (Fig. 4A), very different from that in WH1. qRT-PCR showed that H_2O_2 could up-regulate the transcription of *sfp* and *perR. C* onsistent with the result that these two oxidants were able to improve the surfactin production in Δ*spx* . Western blot assay also showed that H_2O_2 could slightly increase the PerR protein in Δ*spx* (Fig. 4B&D).

The biofilm formation in Δ*spx* was inhibited by H_2O_2 in a dose-dependent manner, just like that in WH1 (Fig. 4C).

*3.6 Effects of reductants on surfactin production and biofilm formation in Δ*perR*

The *perR* gene knockout strain (Δ*perR*) was constructed (Fig. S3 in supplementary materials). Δ*perR* displayed a flat colony morphology and defective floating pellicle without wrinkles (Fig. 5A). Δ*perR* showed a weaker growth than WH1, and surfactin production was significantly decreased in Δ*perR* but could be partially restored by compensation of *perR* (Fig. 5B). However, the colony morphology and float pellicle could not be well restored in the complementary strain (C-Δ*perR*) (Fig. 5A). Consistently, qRT-PCR showed that knockout of *perR* led to a significant decrease of *srfAB* transcription (Fig. 5B).

Glucose had obvious influence on the cell growth in Δ*perR* . In 12 h, glucose promoted the cell growth at all concentrations used here. At 24 h, only 10 or 20 g/L glucose improved the cell growth. Similar to WH1, glucose reduced the surfactin production by a dose-dependent manner in Δ*perR* (Fig. 5C).Just like that in WH1 and Δ*spx* . Unexpectedly, the transcription of *srfAB* was significantly up-regulated in the group added with glucose(Fig. 5D). Glucose was favorable for forming a robuster floating pellicle with more wrinkles in Δ*perR* (Fig. 5E), consistent with that in WH1 and Δ*spx* .

*3.7 Effects of oxidants on surfactin production and biofilm formation in Δ*perR*

H_2O_2 at 2 mM or 4 mM significantly inhibited the cell growth in Δ*perR* , non-consistent with that in WH1 or Δ*spx* . Similar to WH1 but different from Δ*spx* , H_2O_2 at 2 mM or 4 mM significantly reduced the surfactin production in Δ*perR* (Fig. 6A). H_2O_2 significantly up-regulated the transcription of *srfAB* , *sfp* and *spx* , while $FeCl_3$ mainly increased the transcription of *srfAB*in Δ*perR* (Fig. 6B). H_2O_2obviously inhibited the biofilm formation (Fig. 6C).

3.8 Roles of aphC and sodA in surfactin production and biofilm formation

The *aphC* and *sodA* gene knockout strains were constructed, respectively (Fig. S3 in supplementary materials). Deletion of*aphC* and *sodA* had no significant influence on the cell growth, as well as the surfactin production at most of time points except for 48 h. At this time point, Δ*sodA* showed a significant decrease of surfactin production compared to WH1. The colony morphology and biofilm formation in Δ*aphC* and Δ*sodA* were both similar to WH1 (Fig. S4 in supplementary materials).

3.9 Influence of rex and sigB on surfactin production and biofilm formation

We further deleted the genes for globally regulating anti-oxidation reactions such as *rex* and *sigB* in WH1 (Fig. S3 in supplementary materials). The colony morphology and biofilm in Δ*rex* and Δ*sigB* were both similar to WH1 (Fig. 7A). The growth of Δ*sigB* was also similar to WH1, but the growth of Δ*rex* was obviously weaker than WH1 (Fig. 7B).

Deletion of *rex* resulted in a significant decrease of surfactin production when compared to WH1 at all time points. At 12 h, the biomass of Δ*rex* was similar to WH1, but the surfactin production was significantly lower than WH1 (Fig. 7C). Knockout of *sigB* also led to a significant decrease of surfactin production at 36 and 48 h when compared to WH1 (Fig. 7C). The transcription of *perR* was significantly increased in Δ*rex* , while the transcription of*srfAB* and *perR* were both significantly increased and the transcription of *spx* was significantly decreased in Δ*sigB*compared to WH1 (Fig. 7D). Western blot analysis also showed that the protein PerR was slightly increased in Δ*rex* and Δ*sigB* when compared to WH1 (Fig. 7D).

We constructed the *rex* complementary strain C-Δ*rex* (Fig. S3 in supplementary materials), and found it

could form a robuster biofilm than Δ*rex* and WH1 (Fig. 7A), but unfortunately the surfactin production was not well restored in C-Δ*rex* when compared to WH1 (Fig. 7C). This result also confirmed that the signal to trigger biofilm formation is not always dependent on surfactin in*B. amyloliquefaciens* .

4 Discussion

In the environment, the cells undergo constant shifts in redox state [12]. It has been revealed that the redox balance influences the community structural development in biofilms [10,21]. Glucose is not only a carbon source but also a reductant for microorganisms. Here, we found that glucose significantly increased the biomass but reduced the surfactin production in *B. amyloliquefaciens* WH1 and its mutant strains like Δ*spx* and Δ*perR* , consistent with previous report [22]. If glucose is enough, the metabolism of glucose to produce NADH is vigorous to keep the cytoplasm in a reductive state [23,24]. In this study, although glucose was negatively correlated with surfactin production, it was positively correlated with biofilm formation by a dose-effect manner for WH1, Δ*spx* and Δ*perR* , suggesting that glucose can affect biofilm formation by a surfactin-independent pathway in *B. amyloliquefaciens* . This could also be verified by the result that Δ*srfA* was also able to form a robuster float pellicle in the presence of glucose although it was unable to produce surfactin. In the presence of enough glucose, the cells grew vigorously, and a substantial increase in biomass robustness was correlated with an increase of genes expression for producing matrix [2,9,25,26]. As a result, the biofilms exhibited a strikingly wrinkled appearance, which is thought to maximize access to oxygen by increasing the surface to volume ratio [12,27].

H_2O_2 is an oxidant containing reactive oxygen, which can derepress the *perR* regulon including*srfA* to promote surfactin production [19,28]. However, H_2O_2 led to a significant decrease of surfactin accompanying with a decrease of genes transcription including*srfAB* , *sfp* , *spx* and *perR* in WH1. Consistently, the biofilm formation was delayed and weakened in the presence of H_2O_2 [1].

Spx controls the genes transcription responded to oxidative stress, playing a key role in maintaining the cellular redox homeostasis exposed to disulfide stress [14-16]. Also, Spx has been reported to repress the transcription of *srfA* in *B. subtilis* [4,15]. Here, knockout of *spx* resulted in a significant decrease of surfactin accompanying with a decrease of *srfAB* transcription. This result suggested that *spx* plays a positive role in biosynthesis of surfactin, different from previous report [15]. Δ*spx* showed a similar response to reductants, but displayed a very different response to oxidants from WH1, implying that Spx mainly responses to oxidative rather than reductive stress [16]. Δ*spx* was more resistant to H_2O_2 stress than WH1. Perhaps, Spx repressed some anti-oxidation genes for antagonizing H_2O_2 [4,15], so deletion of*spx* was favorable for expression of the anti-oxidation enzymes in*B. amyloliquefaciens* . Moreover, H_2O_2 led to a significant decrease of surfactin accompanying with down-regulation of the transcription of*srfAB* , *sfp* , *spx* and *perR* in WH1, but resulted in a significant increase of surfactin accompanying with up-regulation of the transcription of *sfp* and *perR* in Δ*spx* . This could be explained that the principal regulator for biosynthesis of surfactin is ComA, and Spx plays a role in fine-tuning[3,4,28]. For this reason, although deletion of*spx* reduced the *srfA* transcription, H_2O_2 could improve ComA or other regulators to increase the surfactin production in Δ*spx.*

Spx has been reported to inhibit biofilm formation [30], and inactivation of *spx* can enhance the biofilm formation in *B. subtilis* [31]. However, Δ*spx* showed a colony morphology and biofilm with less wrinkles but could be partially restored by compensation of *spx* , indicating that *spx* plays a positive role in the biofilm formation here. Unexpectedly, H_2O_2 significantly increased surfactin production but inhibited biofilm formation in Δ*spx* . H_2O_2 can be catalyzed to O_2, which suppresses production of extracellular matrix [1]. Thereby, the biofilm formation in Δ*spx* was dependent on oxygen rather than surfactin. This was also supported by the result that glucose could enhance biofilm formation although it inhibited surfactin production in Δ*spx* . The key factor was O_2, which was rich in the presence of enough $H_2O_2$and was poor with enough glucose.

PerR senses H_2O_2 by Fe-mediated histidine oxidation resulting in an inactive style [29,32]. Here, Δ*perR* showed a different colony morphology, defective floating pellicle without wrinkles, and weaker growth com-

pared to WH1, consistent with previous report [19]. Knockout of *perR* led to Spx accumulation [15], resulting in a failure to produce surfactin in*B. subtilis* [16]. Here, surfactin was decreased in Δ*perR* , but could be partially restored by compensation of*perR* . The results confirmed that *perR* was positive for surfactin production in *B. amyloliquefaciens* [28]. Interestingly, compensation of *perR* could not restore colony morphology and float biofilm.

Δ*perR s* howed a similar response to reductants, but displayed a very different response to oxidants from WH1 and Δ*spx* , suggesting that PerR mainly responded to oxidative rather than reductive stress [16]. H_2O_2 inhibited the growth of Δ*perR* , non-consistent with WH1 and Δ*spx* . WH1 was only sensitive to 4 mM H_2O_2, while Δ*spx* was not sensitive to H_2O_2 at all concentrations used here. Thereby, Δ*spx* was more resistant, while Δ*perR* was more sensitive to H_2O_2 stress. Possibly, deletion of*spx* was favorable for expression of the anti-oxidation genes like*katA* , *aphC* , *sodA* , etc, but deletion of *perR*was unfavourable for these genes transcription. However, it has been reported that the *perR* null strain of *B. subtilis* is resistant to H_2O_2 [17-19], different from our results. Also, H_2O_2 improved surfactin production in Δ*spx* , while reduced it in Δ*perR* . The float biofilm in Δ*perR* was impaired so it could not respond to H_2O_2 via a surfactin-dependent manner. All of the above results suggested that the biofilm formation in Δ*perR* was not dependent on surfactin.

Rex senses variation of NAD^+/NADH to balance the intracellular redox reactions [33]. Here, Δ*rex* grew significantly weaker, and showed a significant decrease of surfactin production than WH1. Compensation of *rex* could form a robuster biofilm, but could not efficiently restore the surfactin production compared to WH1. This result also suggested that *B.amyloliquefaciens* could form biofilm by a surfactin-independent way. The *spx* transcription is positively regulated by SigB [15,34]. Here, knockout of *sigB* also led to a significant decrease of *spx* transcription and surfactin production in*B. amyloliquefaciens* .

In *B. amyloliquefaciens* , the reductant glucose could reduce surfactin and enhance biofilm formation by a surfactin-independent way. The oxidant H_2O_2 led to a decrease of surfactin accompanying with weakened biofilm formation. H_2O_2 improved surfactin production but inhibited biofilm formation by a surfactin-independent manner in Δ*spx* . Moreover, Δ*spx* was more tolerant to H_2O_2 stress than WH1. PerR was essential for surfactin production and biofilm formation, and knockout of*perR* led to a significant decrease of surfactin and very defective biofilm thus it could not respond to H_2O_2 via a surfactin-dependent manner. Contrary to Δ*spx* , H_2O_2 reduced surfactin production, and the ability against H_2O_2 stress was weakened in Δ*perR* . Collectively, PerR is favorable for resisting oxidative stress, while Spx plays a negative role in this action. Surfactin is not a unique signal to trigger biofilm formation, and the cellular redox state can influence biofilm formation by a surfactin - dependent or - independent way in *B. amyloliquefaciens* .

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AUTHOR CONTRIBUTIONS

F.S. and H.S. performed experiments and analysed data. X.Z. analysed data. G.Q. designed experiments, analysed data and wrote the paper.

COMPETING INTERESTS

The authors declare no competing interests.

Data availability

All relevant data are provided in the article, Supplementary information, or available from the corresponding author upon reasonable request.

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Figure legends

Fig. 1 Effects of reductants on biomass, hemolytic activity and biofilm formation in WH1. A: Effects of glucose on cell growth (left panel) and surfactin production (right panel) in WH1; **B:**Effects of glucose on genes transcription in WH1; **C:** Effects of glucose on biofilm formation in WH1; **D:** Effects of glucose on biofilm formation in Δ*srfA;* **E** :Effects of glucose on PerR expression in WH1.

Fig. 2 Effects of oxidants on biomass, hemolytic activity and biofilm formation in WH1. A: Effects of H_2O_2 on cell growth (left panel) and surfactin production (right panel) in WH1; **B:** Effects of H_2O_2 on biofilm formation in WH1;**C:** Effects of H_2O_2 on biofilm formation in $\Delta srfA$; **D:** Effects of H_2O_2 on genes transcription in WH1;

E: Effects of H_2O_2 on PerR expression in WH1.

Fig. 3 Effects of oxidants on biomass, hemolytic activity and biofilm formation in Δspx . A: Colony morphology and biofilm formation. C-Δspx : the*spx* -complementary strain. **B:** Growth (left panel) and hemolytic activity (right panel). **C:** Genes transcription (left panel) and PerR expression (right panel). **D:** Effects of glucose on cell growth (left panel) and surfactin production (right panel); **E:** Effects of glucose on genes transcription.;**F:** Effects of glucose on biofilm formation; **G** : Effects of glucose on PerR expression.

Fig. 4 Effects of oxidants on biomass, hemolytic activity and biofilm formation in Δspx . A: Effects of H_2O_2 on cell growth (left panel) and surfactin production (right panel); **B:** Effects of H_2O_2 on genes transcription ;**C:** Effects of H_2O_2 on biofilm formation; **D:** Effects of H_2O_2 on PerR expression.

Fig. 5 Effects of oxidants on biomass, hemolytic activity and biofilm formation in $\Delta perP$. A: Colony morphology and biofilm formation. C-$\Delta perR$: the*perR* -complementary strain. **B:** Growth (up-left panel), hemolytic activity (up-right panel) and genes transcription (down panel). **C:** Effects of glucose on cell growth (left panel) and surfactin production (right panel); **D:** Effects of glucose on genes transcription; **E:** Effects of glucose on biofilm formation.

Fig. 6 Effects of oxidants on biomass, hemolytic activity and biofilm formation in $\Delta perP$. A: Effects of H_2O_2 on cell growth (left panel) and surfactin production (right panel); **B:** Effects of H_2O_2 on genes transcription; **C:**Effects of H_2O_2 on biofilm formation.

Fig. 7 Phenotype and hemolytic activity in Δrex and $\Delta sigB$. A: Colony morphology and biofilm.**B:** Growth curves. **C:** Hemolytic activity. Left: knockout strains; Right: the *rex* -complementary strain.**D** : Genes transcription (left panel) and PerR expression (right panel) in Δrex and $\Delta sigB$.

Abbreviation

NADH/NAD+ (Nicotinamide adenine dinucleotide)

IPTG (Isopropyl β-D-Thiogalactoside)

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