

SPM43.1 Contributes to Acid-Resistance of Non-Sytoplasmata-Forming Cells in *Pantoea agglomerans* YS19

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Abstract *Pantoea agglomerans* YS19 is a rice endophytic bacterium characterized to form multicellular biofilm-like structures called sytoplasmata. Phenotypic distinctions between sytoplasmata-forming cells and planktonic cells are crucial for understanding YS19's survival strategies. In this study, a 43.1 kDa protein SPM43.1 was identified to show significant resistance to the aggregation effect caused by denaturing acidic conditions. MALDI-TOF analysis data indicated that it is a maltose-binding protein homolog while contains sequence homologous to the chaperone protein, ClpB. The purified SPM43.1 protein was detected to exhibit chaperone-like activities at acidic conditions, where its conformation transformed from an ordered to a globally less ordered structure as revealed by circular dichroism spectroscopy, showing a similar property to most chaperone proteins. The expression of SPM43.1 in YS19 is initiated when bacterial cells begin to aggregate, yet its amount in planktonic cells greatly exceeds that in sytoplasmata-forming cells, suggesting its crucial role to the survival of planktonic cells in experiencing environmental fluctuations. However, the bacterium prefers to form sytoplasmata, while not to express SPM43.1 proteins, for surviving the artificially set fluctuant (acid here) environments. This study provides valuable information on the life styles and survival strategies of microorganisms that forms multicellular aggregates at specific growth stages.

Introduction

Microbial multicellular aggregations have been shown to be the dominant mechanism by which most microorganisms position themselves in their niches [6]. Within such cellular organizations, known as biofilms or biofilm-like structures, multiple cell–cell interactions are enhanced (i.e., communication and metabolic association) [19]. Consequently, assembled bacterial cells may undergo profound changes, ranging from gene regulation to metabolic status [7]. These changes are significant, because not only are a number of protective genes activated [1] but also the bacteria generate phenotypic versatility to provide “insurance effects” for the microbial colony [2]. Thus, understanding these physiological modifications between the planktonic and aggregated cells is crucial for deciphering the survival strategies of the microorganisms.

Originally isolated from rice (*Oryza sativa*) cv. Yuefu, *Pantoea agglomerans* YS19 is an endophytic bacterium [10] that shows remarkable nitrogen-fixing activity and is capable of promoting host plant growth [10, 20]. YS19 and other *P. agglomerans* strains are characterized by the formation of biofilm-like multicellular structures called sytoplasmata, structurally maintained by uniquely tight cell–cell bindings [9]. Our previous work revealed that YS19 initiates sytoplasmata formation in the early stationary growth phase after a single cell growth stage [9]. Unlike biofilm structures formed by other bacteria, a sytoplasmatum can form by cellular aggregation whether there are surfaces to adhere or not [8]. More strikingly, although it has revealed that the sytoplasmata formation promotes bacterial adaptability, there are still a large number (of the same order of magnitude) of planktonic cells that co-exist with sytoplasmata-forming bacteria throughout the cultivation [8]. This observation seems to

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imply that these isolated cells have also evolved special mechanisms to protect themselves from environmental adversity. Elucidating the different survival strategies between symplasmata-forming cells and planktonic cells will be help to understand the ecological significance of different life styles in microbes.

To identify different physiological responses between symplasmata-forming and planktonic cells, we chose to investigate the differences in expression patterns of proteins that are self-stable and/or possess chaperone-like activity in hostile acidic environments. We identified a protein that was produced in a large amount in the planktonic cells when YS19 cells began to aggregate. This protein is homologous both to MalE and ClpB, and exhibited chaperon-like activity. These results provide clues to the genetic events underlying the phenotype changes and adaptability differences between planktonic and aggregative bacterial cells.

Materials and Methods

Strains and Growth Conditions

Pantoea agglomerans YS19 was isolated in our lab as a diazotrophic endophyte from rice cv. Yuefu [3]. An inoculum of the bacterium was prepared by inoculating a colony into 10 ml of LB medium. Cultures of *P. agglomerans* were grown in LB at 30°C on a rotary shaker at a rate of 3 rev s⁻¹.

Aggregation Assay of Cellular Extracts Under Various pH Conditions

Whole cellular extracts of *P. agglomerans* YS19 grown for 18 h (the time point at which the co-existence of symplasmata-forming cells and planktonic cells is apparent) were obtained by sonication in buffer A (50 mM Tris-HCl at pH 7.3, 0.1 M NaCl) containing 1 mM PMSF (Boehringer Mannheim). The lysates were centrifuged at 20,000 g, and the protein concentration was determined by the Bradford assay [3]. Aggregation assays of the cell extracts under denaturing acidic conditions were carried out following the methods of Liu et al. [16], and the proteins that remained in the supernatants were examined by SDS-PAGE. Coomassie staining is acquiescent in the text; otherwise, silver staining will be stated.

Whole Cellular Protein Analysis at Different Growth Stages

Pantoea agglomerans YS19 cells were grown in LB for 12, 24, 36, and 48 h, respectively. Each culture was filtered twice so that the symplasmata and planktonic cells could be separated. During the filtration, a G-2 filter (pore diameter:

30–50 µm) followed by a G-4 sintered-glass filter (pore diameter: 4–7 µm, Changchun Glass Instrument Co.) were used. The resuspended cake of the G-2 filter, which was mainly comprised of symplasmata, and the planktonic cells in the final filtrate were examined under a light microscope to confirm the separation efficiency. Both the unseparated bacterial cells grown for different times (from 0 to 11 h) and the separated symplasmata and planktonic cells of the four cultures (grown to 12, 24, 36, and 48 h) were centrifuged for 3 min at 12,000 rpm. The collected cells were washed three times with distilled water, resuspended in 1 ml of SDS sample buffer (0.06 M Tris, 2.5% glycerol, 0.5% SDS, 1.25% β-mercaptoethanol), denatured in boiling water for 10 min, and finally analyzed by SDS-PAGE. The amounts of symplasmata and the planktonic cells supernatant loaded were adjusted to an equivalent biomass.

Protein Purification

Whole cellular extract of YS19 were obtained as described above. After centrifugation (4°C, 20,000×g, 45 min) two times, the supernatant was diluted with 30 mM Tris-HCl (pH 7.0) containing 1 mM EDTA and 4 M ammonium sulfate was added (30% saturation). Insoluble fraction was removed by centrifugation and the supernatant was applied to a DEAE-Sepharose FF 16/25 column (Amersham Pharmacia Biotech). After being eluted with a linear gradient ranging from 0.1 to 1 M NaCl (pH 7.0), the SPM43.1-containing fractions were pooled and dialyzed against buffer B (30 mM Tris-HCl at pH 8.0), and then applied to a Source Q15 10/40 column (Amersham Pharmacia Biotech) containing strong ion-exchange resin pre-equilibrated with buffer B. The column was eluted using a linear gradient ranging from 0.01 to 1 M NaCl (pH 8.0). The target protein-containing fractions were pooled and dialyzed against deionized water. The purity of the target protein was estimated by densitometry analysis of Coomassie brilliant blue-stained bands on SDS-PAGE.

Protein Identification

The purified protein was subjected to an in-gel tryptic (Amresco0458) digestion to obtain a variety of polypeptide fragments for analysis. The mass fingerprints of the peptides were obtained by MALDI-TOF mass spectrometry (Ultraflex TOF/TOF, Bruker Daltonics Co.). The data were analyzed by MASCOT, and matching proteins were searched in the SWISS-Prot database.

Chaperone-Like Activity

Chaperone-like activity assay was performed by testing the protection effect of the target protein under various stress

conditions as described by Hong et al. [13]. In our research, alcohol dehydrogenase (ADH) (Sigma) was used as the substrate, where 1 mol l^{-1} HCl was added to adjust the pH values to pH 2 to achieve effective aggregation of the substrate proteins both in the absence and presence of target chaperon proteins with various concentrations. After 30 min incubation at 25°C and centrifugation at $10,000\times g$ for 10 min, the sample supernatant was neutralized to pH 7 by adding 0.02 mol l^{-1} Tris (pH 10), and the presence of substrate protein in the supernatant was examined by SDS-PAGE. So if the protein sample had chaperone-like activity, the substrate proteins could be protected and would remain in the supernatants.

Circular Dichroism Spectrometry

The concentration of purified target protein was determined by the Bradford assay. Samples ($200 \mu\text{g ml}^{-1}$) in 50 mM sodium phosphate buffer (pH 7.0) were adjusted to various pHs with 1 M HCl and incubated for 1 h at 25°C . Far-UV circular dichroism (CD) spectra of the treated and untreated (control) samples were recorded on a J-715-150L spectropolarimeter (JASCO) using a quartz cuvette of 0.1-cm path length. Spectra were the average of five scans from 200 to 250 nm, and the buffer base lines were subtracted. Shorter wavelengths were not analyzed because of increased noise.

Results

The SPM43.1 Protein, a Highly Aggregation Resistant Protein, is Differentially Expressed at the Symplasmata-Forming Stage

In our previous researches, we discovered that *P. agglomerans* YS19 could acidify the culture to very low pH [10]. This observation implies that YS19 cells employ certain components to tolerate adverse acidic conditions. Thus, we tried to determine these components by identifying cellular proteins that remain soluble in low pH solutions. When the whole cellular extracts of YS19 cultivated to the symplasmata-forming stage were subjected to denaturing acidic conditions (pHs 2 or 1), we noticed that four proteins (43, 29, 28, and 19 kDa) are still present in the supernatant, remained about 80% soluble, while other proteins mostly, if not completely, aggregated and disappeared from the supernatant (Fig. 1a, cf. lanes 2–4).

Previously, it was shown that symplasmata formation could remarkably enhance the viability of *P. agglomerans* YS19 when suffering from various environmental stresses (e.g., dehydration, heavy metal toxicity, and osmotic shock) [17]. However, under acidic conditions, although

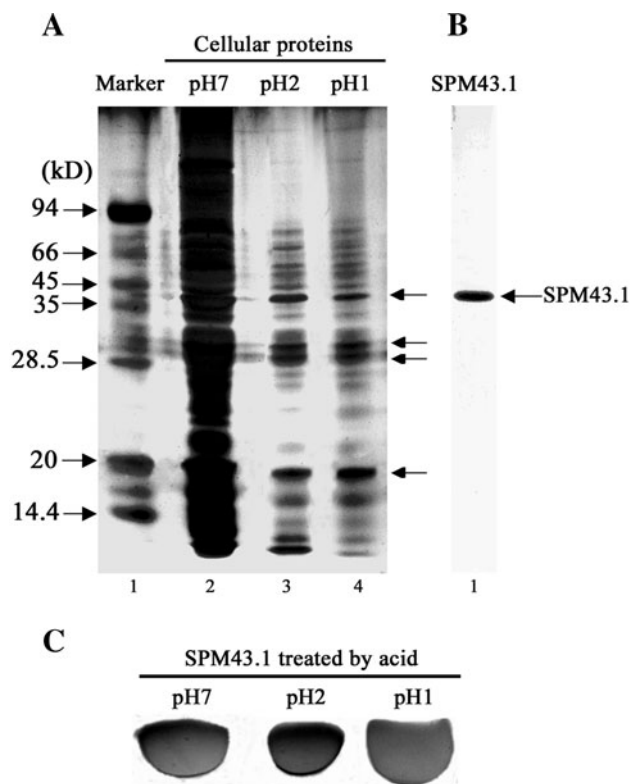


Fig. 1 SDS-PAGE suggesting the SPM43.1 protein is resistant to aggregation caused by acidic inducement: **a** the whole cellular proteins of YS19 grown for 18 h were subjected to pH 2 (lane 3) and pH 1 (lane 4) treatments. Untreated samples were used as a control (lane 2); **b** the SPM43.1 protein was purified to homogeneity; **c** the purified SPM43.1 was subjected to various low pH treatments. Each treatment was performed with a final protein concentration of 0.5 mg/ml, and the proteins remaining in the supernatants were examined

the planktonic cells harvested at the non-symplasmata-forming stage (e.g., before 6 h of culturing [9]) mostly cannot survive, the viability of the planktonic and also the aggregative cells are both enhanced when symplasmata begin to form (e.g., after 6 h of culturing [9]). Given that multicellular aggregates and planktonic cells represent different life styles with distinct protein expression profiles, we next sought to determine if any of those aggregation resistant proteins expressed differentially between the symplasmata-forming and planktonic cells.

The whole cellular protein separation profile on SDS-PAGE gel indicates that the 43 kDa protein, which was absent at the beginning of the growth (Fig. 2, lanes 1–6), began to be produced after 6 h of culture and accumulated as the incubation time increased (Fig. 2, lanes 7–11). YS19 cells initiate symplasmata formation when the bacterium are cultivated to about 6 h [9], which corresponds to the time course of the expression of the 43 kDa protein. Therefore, the 43 kDa protein is related to symplasmata-forming and it seems more significant than the other three

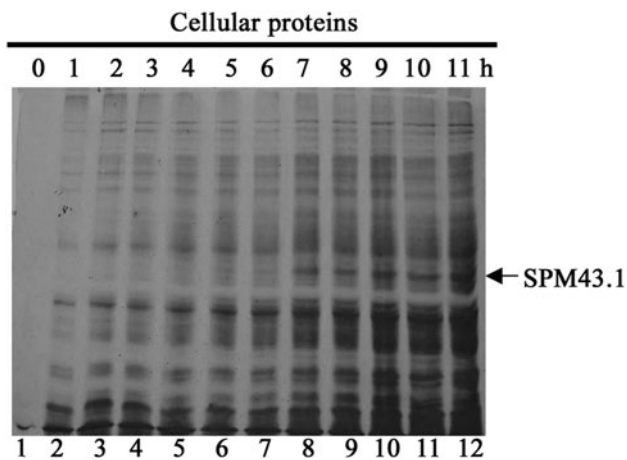


Fig. 2 Time course analysis on the protein expression of YS19 by SDS-PAGE. The whole cellular protein samples of the YS19 cultivated for 0–11 h (corresponding to lanes 1–12, respectively) were analyzed on SDS-PAGE with an equal amount of loaded proteins (about 10 μ g) and visualized by silver staining

proteins. In contrast, the expression profile in the other three proteins (29, 28, and 19 kDa) was not altered.

Since the presence of cellular chaperones (e.g., HdeA [12]) might contribute to the anti-aggregation effect of a protein, we purified the 43 kDa protein to homogeneity (Fig. 1b) and treated it in a similar fashion to eliminate alternative explanations. Consistent with the above results, the purified protein was also highly resistant to the aggregation effect caused by low pH (Fig. 1c): almost all of the target protein remained soluble when it was subjected to an extremely low pH (e.g., pH 1). These phenomena indicate that the 43 kDa protein exhibited a remarkable tolerance to acidic conditions in comparison with almost all the other cellular proteins. We named this protein SPM43.1.

SPM43.1 Contains Homologous Sequence with Maltose-Binding Protein and Chaperone ClpB

To obtain more information about the function of this protein, the purified SPM43.1 protein was digested by trypsin and the obtained peptides were analyzed by MALDI-TOF mass spectrometry. The peptide mass fingerprint of this protein was successfully obtained and database searching with Mascot in the SWISS-Prot database was performed.

It is indicated that the best matched protein is the maltose-binding protein (MBP), MalE, from *Enterobacter aerogenes*. Among 53 peptides of SPM43.1, there are 14 peptide that match with it. It also bears nine peptides similar to the chaperone ClpB. These data seem to suggest that SPM43.1 maintains a chimeric structure composed of both an MBP-like and a chaperone ClpB-like region. Peptide mass fingerprint matching also suggested that

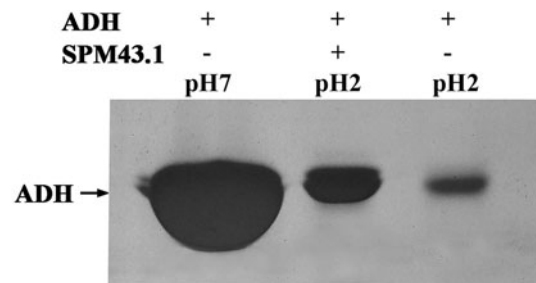


Fig. 3 SDS-PAGE examination on the protective effects of SPM43.1 on substrate protein ADH: under native (lane 1) or denaturing acidic condition (lanes 2, 3), in the presence (lane 2) or absence (lane 3) of the SPM43.1, the quantity of the substrate ADH protein in the supernatant was examined

SPM43.1 contains a signal peptide, indicating it to be a periplasmic protein.

SPM43.1 Shows Chaperone-Like Activity Under Stress Conditions

Considering the remarkable homology between SPM43.1 and multiple well-known chaperone-like proteins, we next set to determine if SPM43.1 also exhibit chaperone or chaperone-like activities. This assay was achieved by comparing the quantity of the soluble substrate proteins in the supernatant under different denaturing conditions. SDS-PAGE profile clearly shows that under denaturing acidic treatment, the aggregation of the substrate protein was effectively suppressed in the presence of SPM43.1, while the majority of the substrate protein was missing in the supernatant in the absence of SPM43.1 (Fig. 3), indicating that SPM43.1 might play a significant role in the cells by helping other crucial components to resist the aggregation effect caused by stress conditions.

SPM43.1 Transforms into a Globally Less Ordered Conformation in the Acidic Stress Condition

Since SPM43.1 resists the aggregation effect brought about by low pH, it is reasonable to speculate that such an aggregation resistance might be attributed to the structural stability. The purified SPM43.1 samples were adjusted to a designated pH value and incubated for 1 h and then the secondary structures of the protein were examined by Far-UV CD spectroscopy. The CD spectrum of the SPM43.1 recorded under different pHs (Fig. 4) reveal that (i) in a neutral environment, the secondary structure of SPM43.1 mainly consists of several α -helices; and (ii) when the pH was decreased below pH 2, the global secondary structure of SPM43.1 exhibited a sudden transition from a highly ordered conformation to a less ordered one. However,

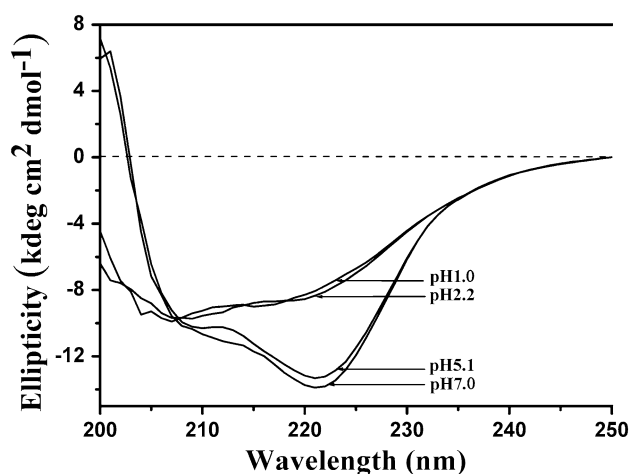


Fig. 4 Far-UV CD spectra of the native (pH 7.0) or acid-treated (pHs 5.3, 2.2, and 1.0) SPM43.1 protein at a final protein concentration of $200 \mu\text{g ml}^{-1}$

SPM43.1 is still capable of maintaining most of its secondary structure under these extreme acidic conditions.

SPM43.1 is Mainly Expressed in the Non-Aggregate Planktonic Cells When Sympasmata Begin to Form

Given that the SPM43.1 protein is differentially expressed at the sympasmata-forming stage when a large number of planktonic cells still co-exist with the sympasmata-forming bacteria in cultures (Fig. 5a), we then tried to explore which type of cells primarily expresses the SPM43.1 protein. Sympasmata and planktonic cells harvested at different cultivation time points (e.g., 12, 24, 36, and 48 h) were separated by two rounds of filtrations. The whole cellular proteins of both types of cells were then compared by using SDS-PAGE separation. The results indicated that even though SPM43.1 was expressed in both the sympasmata-forming and planktonic cells; the amount in the latter exceeded that in the former to a dramatic extent (Fig. 5b). Moreover, with extended cultivation time, the amount of SPM43.1 did not seem to increase in the sympasmata (Fig. 5b, lanes 2, 4, 6, 8), but significantly accumulated in the planktonic cells (Fig. 5b, lanes 1, 3, 5, 7), suggesting that the temporal variation in the SPM43.1 expression stems from the increased expression in non-aggregated planktonic cells at the sympasmata-forming stage.

Sympasmata Formation, Which Lacks SPM43.1 Proteins, is the Preferential Life Style for Surviving Acid Environments

In view of the fact that SPM43.1 protein is related to sympasmata formation yet significantly accumulated in

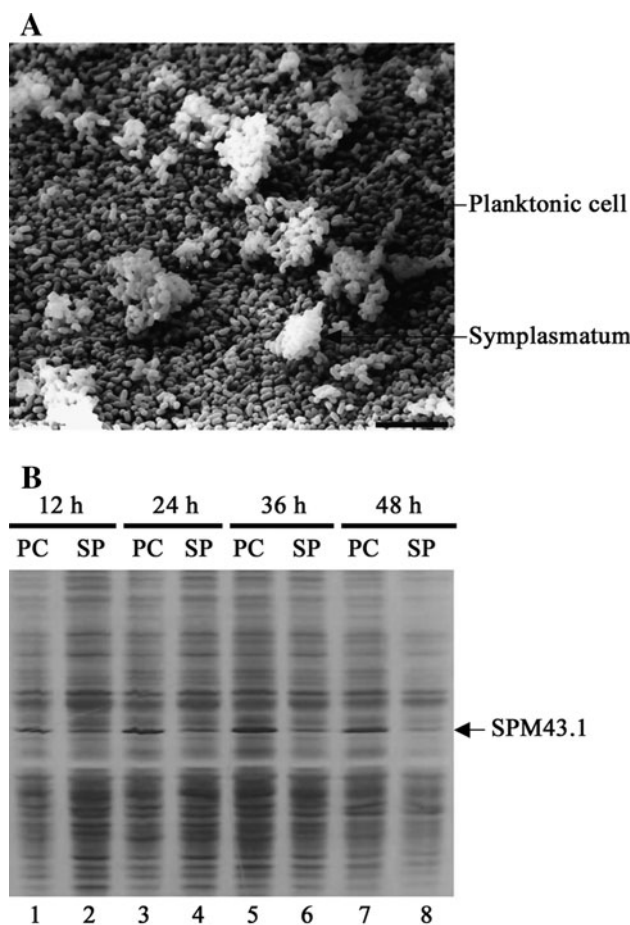


Fig. 5 Analysis on the expression pattern of SPM43.1 proteins: **a** scanning electron micrograph of YS19 cultivated for 20 h ($\times 3K$, bar = $5.0 \mu\text{m}$) indicating the coexistence of sympasmata and also planktonic cells; **b** differential expression patterns of SPM43.1 in planktonic cells and sympasmata. The whole cellular proteins of both planktonic (lanes 1, 3, 5, 7) and sympasmata-forming cells (lanes 2, 4, 6, 8) cultivated for 12 h (lanes 1, 2), 24 h (lanes 3, 4), 36 h (lanes 5, 6), and 48 h (lanes 7, 8) were analyzed by SDS-PAGE and visualized by silver staining

the planktonic cells rather than in the sympasmata, it is intriguing to investigate that whether the protein will be induced when the bacterial cells face more severe stresses in their living environment. Here, when YS19 had been grown for 2.5 h in LB medium, the broth was then adjusted to pHs 4.0, 3.5 or 3.0, respectively, by adding 1 mol l^{-1} HCl. It was detected that the growth of the bacterium was subject to the inhibition of acidification: the growth rate stopped when the pH value was lowered to 3.0, it obviously decreased at pH 3.5, and seemed not to be affected at pH 4. Therefore, pH 3.5 was selected for testing the acid-induction effect on the SPM43.1 protein expression in YS19. As examined by SDS-PAGE every 2 h (from 2.5 to 12.5 h during the acidifying cultivation), no increase of the protein was found to be induced in the bacterial cells [Fig. 6a, where samples at 2.5 h (non-acidified, lane 2) and at 12.5 h

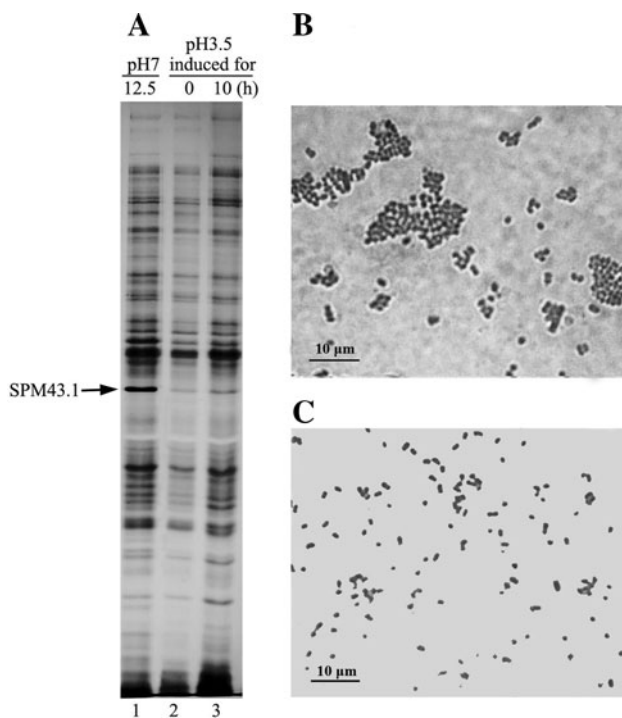


Fig. 6 SPM43.1 protein overexpression in YS19 was not induced by acid as examined by SDS-PAGE (a), but symplasmata formation was significantly promoted as detected by microscopic observation (b, c), when YS19 broth was suffered from an acid-inducement. YS19 was grown in neutral LB for 2.5 h, the broth was then adjusted to pH 3.5 by adding 1 mol l^{-1} HCl. For a, the whole cellular protein samples at 2.5 h (non-acidified, lane 2) and at 12.5 h (i.e., 10 h of acidification, lane 3), and a control at 12.5 h (non-acidified, lane 1) were treated with SDS-PAGE loading buffer (100°C , 10 min) and loaded onto SDS-PAGE (20 mA, 2 h) at equal protein concentrations and visualized by silver staining. For b and c, YS19 was cultivated for 6 h at different pH conditions (pH 7 for 2.5 h followed by pH 3.5 for another 3.5 h of acidification in b, and pH 7 for 6 h in c). Oil immersion objective lens was used for all observations. Original magnifications, $\times 1\text{K}$; bar = 10 μm

(i.e., 10 h of acidification, lane 3) and a control at 12.5 h (non-acidified, lane 1) are shown]. Microscopic observation indicates that symplasmata formation was significantly promoted in the HCl acidified-LB broth (Fig. 6b and c). These data suggest that under stress conditions, the planktonic cells of YS19 prefer to form symplasmata, while not to express SPM43.1 proteins, for surviving the artificially set fluctuant (acid here) environments.

Discussion

Forming multicellular biofilms or remaining in planktonic states are two main life styles of bacteria in their niches. Detecting the distinctions in the expression profiles of proteins and genes between the planktonic and aggregative cells is critical for understanding the adaptability and

developmental processes of bacteria. This investigation highlighted a chaperone-like protein, SPM43.1, whose expression increased when *P. agglomerans* YS19 began to form multicellular symplasmata, yet was mainly expressed in the non-aggregated planktonic cells. More remarkably, compared to cellular proteins, SPM43.1 exhibited an extraordinary resistance to aggregation caused by denaturing acidic conditions. MALDI-TOF analysis of the protein showed that its tryptic peptides masses were most similar to the chaperone-like protein MalE and also to the regular chaperone protein ClpB. Biochemical assay also revealed that SPM43.1 effectively increased the aggregation resistance of the substrate proteins under acidic conditions. Hence, these results lead us to propose that SPM43.1 might be a crucial protein employed by the planktonic cells to survive environmental fluctuations.

MalE was noticed because of its capability to enhance the solubility of its passenger proteins [15] and to promote the functional re-folding of denatured substrates [18]. The roles of ClpB in the stress response, such as efficiently inhibiting and reversing the aggregation of other proteins, have also been widely demonstrated in a range of bacteria [4]. This study revealed that a MalE and ClpB homologous protein, SPM43.1, the dominant cellular component extraordinarily resists the acid-induced protein aggregation in YS19 (Fig. 1), and also exhibits remarkable chaperone-like activities to effectively increase the solubility of other proteins (Fig. 3). As *P. agglomerans* YS19 has been observed to adapt to an acidic environment when it enters the symplasmata-forming stage [17], components possessing chaperone or chaperone-like capabilities must contribute to this process. In this experiment, the observation of SPM43.1 exhibiting a high stability in the hostile acidic conditions strengthened this hypothesis. A number of other chaperone-like proteins have also been shown to have similar features. For example, *hdeA* encodes a periplasmic protein that helps other proteins to resist aggregation brought by low pH [12]. Detecting its structural stability reveals that it hardly aggregates compared to other non-periplasmic proteins [13]. Interestingly, MALDI-TOF and peptide mass fingerprint matching suggested that the signal peptide of SPM43.1 could reveal a periplasmic destination. The aggregation resistance in these periplasmic proteins is crucial for bacteria to respond to stresses because they are the first line defense of the cell and must maintain their own structural integrity so that they can help other cellular components.

Although the structure of SPM43.1 becomes relatively less ordered when pH decreases further, the transition between the two conformations might be more meaningful. Like most of the chaperone or chaperone-like proteins (e.g., GroEL, HdeA), a less ordered structure usually exposes more hydrophobic surfaces allowing the protein to

better fulfill its tasks [5, 13]. When it comes to a “moonlighting” protein (e.g., MBP) that can use different domains to perform multiple functions [14], the transition of the global structure seems to be more significant. In fact, the study of the chaperone-like activity of MBP revealed that it is the global structure rather than the maltose-binding cleft that is involved in the interaction with substrates [11]. Therefore, it can be hypothesized that the presence of the acid-induced (relatively) less ordered structure of SPM43.1 enable it to carry out a chaperone-like activity and protect other proteins from environmental stresses.

This study also revealed that SPM43.1 was mainly expressed by the non-aggregated planktonic cells at the symplasmata formation stage. Previous studies on biofilms suggested that gene expression profile in biofilm-forming bacterial cells is distinct from that in planktonic cells [1]. The phenotype distinctions thus reflect the physiological and genetic differences between two life styles. Symplasmata are a kind of multicellular biofilm-like structure that allows some YS19 cells to adopt another life style. The most noticeable feature is that, even though the symplasmata-forming bacterial cells display a superior adaptability, there are still a large number of free-living YS19 planktonic cells and symplasmata formation that also do not seem to confer an advantage in the acidic environment. In light of the observation that the intracellular concentration of SPM43.1 in planktonic cells far exceeds that required for symplasmata-forming cells, and considering that SPM43.1 possesses features and homologous sequences of chaperone or chaperone-like proteins, we propose that SPM43.1 is one of the significant components responsible for protecting planktonic cells from severe damage brought by environmental stresses.

Symplasmata formation represents a much more specialized multicellular behavior. The specific mechanism that determines whether a particular cell can adopt an aggregative life style or stay as a free-living planktonic cell under stress conditions remains to be discovered. However, this study suggests that under artificially set stress conditions, YS19 planktonic cells were eager to combine into symplasmata, while not to further overexpress SPM43.1 protein, in surviving the fluctuant environments. These data indicate that the formation of symplasmata is a more effective life style in dealing with the environmental stresses for YS19. In other word, the function of SPM43.1 protein seems only to be limited to those planktonic cells that fill to form a symplasmatum, whose expression is not the first choice for the bacterium.

The molecular details of SPM43.1 need to be further explored, e.g., to obtain the sequence of the protein and the gene. According to the finger printing data, the gene of SPM 43.1 seems to be a heterozygosis sequence which is

more different from its homologs. However, the work reported here has resolved the question of how planktonic cells cope with adverse environments and coexist with the symplasmata in *P. agglomerans* YS19. The finding that YS19 was eager to form symplasmata for surviving the fluctuant environments may provide a more convenient and effective manner than overexpression of certain proteins in bacterial adaptive growth processes under stress environments.

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References

1. Beloin C, Valle J, Lambert PL et al (2004) Global impact of mature biofilm lifestyle on *Escherichia coli* K-12 gene expression. *Mol Microbiol* 51:659–674
2. Boles BR, Thoendel M, Singh PK (2004) Self-generated diversity produces “insurance effects” in biofilm communities. *Proc Natl Acad Sci USA* 101:16630–16635
3. Bradford MM (1976) A dye binding assay for protein. *Anal Biochem* 72:248–254
4. Capestany CA, Tribble GD, Maeda K et al (2008) Role of the Clp system in stress tolerance, biofilm formation, and intracellular invasion in *Porphyromonas gingivalis*. *J Bacteriol* 190:1436–1446
5. Chen L, Sigler PB (1999) The crystal structure of a GroEL/peptide complex: plasticity as a basis for substrate diversity. *Cell* 99:757–768
6. Costerton JW, Lewandowski Z, Caldwell DE et al (1995) Microbial biofilm. *Annu Rev Microbiol* 49:711–745
7. Davey ME, O’Toole GA (2000) Microbial biofilms: from ecology to molecular genetics. *Microbiol Mol Biol Rev* 64:847–867
8. Duan JY, Shen DL, Feng YJ et al (2007) Rice endophyte *Pantoea agglomerans* YS19 forms multicellular symplasmata via cell aggregation. *FEMS Microbiol Lett* 270:220–226
9. Feng YJ, Shen DL, Song W et al (2003) In vitro symplasmata formation in the rice diazotrophic endophyte *Pantoea agglomerans* YS19. *Plant Soil* 25:5435–5444
10. Feng YJ, Shen DL, Song W (2006) Rice endophyte *Pantoea agglomerans* YS19 promotes host plant growth and affects allocations of host photosynthates. *J Appl Microbiol* 100:938–945
11. Fox JD, Kapust RB, Waugh DS (2001) Single amino acid substitutions on the surface of *Escherichia coli* maltose-binding protein can have a profound impact on the solubility of fusion proteins. *Protein Sci* 10:622–630
12. Gajiwala KS, Burley SK (2000) HDEA, a periplasmic protein that supports acid resistance in pathogenic enteric bacteria. *J Mol Biol* 295:605–612
13. Hong WZ, Jiao WW, Hu JC (2005) Periplasmic protein HdeA exhibits chaperone-like activity exclusively within stomach pH range by transforming into disordered conformation. *J Biol Chem* 280:27029–27034
14. Jeffery CJ (2003) Moonlighting proteins: old proteins learning new tricks. *Trends Genet* 19:415–417

15. Kapust RB, Waugh DS (1999) *Escherichia coli* maltose-binding protein is uncommonly effective at promoting the solubility of polypeptides to which it is fused. *Protein Sci* 8:1668–1674
16. Liu Y, Fu XM, Shen J et al (2004) Periplasmic proteins of *Escherichia coli* are highly resistant to aggregation: reappraisal for roles of molecular chaperones in periplasm. *Biochem Biophys Res Commun* 316:795–801
17. Miao YX, Zhang WB, Feng YJ (2009) Symplasmata formation in *Pantoea agglomerans* YS19 contributes to bacterial tolerance under detrimental conditions. *J Microbiol* 29:52–65 (in Chinese)
18. Richarme G, Caldas TD (1997) Chaperone properties of the bacterial periplasmic substrate binding proteins. *J Biol Chem* 272:15607–15612
19. Webb JS, Givskov M, Kjelleberg S (2003) Bacterial biofilms: prokaryotic adventures in multicellularity. *Curr Opin Microbiol* 6:578–585
20. Yang H, Sun X, Song W et al (1999) Screening, identification and distribution of endophytic associative diazotrophs isolated from rice plants. *Acta Bot Sin* 41:927–931