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Comparative Proteome Analysis of Two Antagonist *Bacillus subtilis* Strains

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Natural wild-type strains of *Bacillus subtilis* are extensively used in agriculture as biocontrol agents for plants. This study examined two antagonist *B. subtilis* strains, KB-1111 and KB-1122, and the results illustrated that KB-1122 was a more potent inhibitor of the indicator pathogen than KB-1111. Thus, to investigate the intrinsic differences between the two antagonist strains under normal culture conditions, samples of KB-1111 and KB-1122 were analyzed using MALDI-TOF-MS. The main differences were related to 20 abundant intracellular and 17 extracellular proteins. When searching the NCBI database, a number of the differentially expressed proteins were identified, including 11 cellular proteins and 10 secretory proteins. Among these proteins, class III stress-response-related ATPase, aconitate hydratase, alpha-amylase precursor, and a secretory protein, endo-1, 4-beta-glucanase, were differentially expressed by the two strains. These results are useful to comprehend the intrinsic differences between the antagonism of KB-1111 and KB-1122.

Keywords: *Bacillus subtilis*, *Magnaporthe grisea* P131, *in vitro* antifungal activity, proteomics, 2DE-PAGE

The species of *Bacillus subtilis* is the best-characterized member of the *Bacillus* genus, and has become a paradigm organism of Gram-positive bacteria [15]. *B. subtilis* has many characteristics as an excellent biocontrol agent, including the production of structurally diverse antibiotics [19], formation of viable spores [5], promotion of plant growth [24], and a ubiquitous presence in soil [3]. In recent years, many researchers have focused on the antifungal mechanism of antagonist bacteria or fungi, resulting in the characterization of numerous antifungal compounds [9, 10, 19, 22, 26]. Among these antagonistic compounds, big-molecule hydrolytic enzymes identified as cellular or secretory proteins, such as alpha-amylase [17, 30], antifungal protein (AFP) [9], glucanase [13, 18], and neutral protease [12, 29], would appear to play a major role in the growth inhibition process of antagonist bacteria against fungal pathogens. However, most previous studies have mainly focused on the purification and characterization of such single antifungal proteins, while relatively few have analyzed the antifungal proteins expressed in the *B. subtilis* species, particularly the differentially expressed proteins in some antagonist *B. subtilis* strains.

As a powerful analytical tool for studying full protein expression patterns, proteomics can be used to bring the virtual life of genes to the real life of proteins, and proteome information can also provide clues to a comprehensive understanding of the metabolism and growth processes of bacteria [11]. The recent publication of the *B. subtilis* genome sequence was a real breakthrough in proteomics, since it facilitated the routine identification of proteins on 2D gels by mass spectrometry (MS). In addition to the *B. subtilis* genome sequence information and application of MS, proteomic analysis has also proven to be a powerful method for studying the protein expression profiles of *B. subtilis* in response to various stresses [8, 15, 20, 27, 31].

Until now, there has been no report focused on a proteome analysis of antagonistic proteins in antagonist *B. subtilis*. Previously, the current authors characterized two *B. subtilis* strains, KB-1111 and KB-1122, and demonstrated their excellent antifungal activities against several pathogenic fungi that cause diseases in crops and horticultural plants; particularly, *Magnaporthe grisea* P131 [32]. Accordingly, this report compares the antagonistic ability of KB-1111 and KB-1122 against *M. grisea* P131, and uses a proteomic approach to compare the cellular and extracellular proteomes of the two *B. subtilis* strains cultured under normal conditions in an effort to identify the proteins associated with the antagonistic ability of *B. subtilis*.

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Materials and Methods

Strains and Culture Conditions

The two strains of *Bacillus subtilis*, KB-1111 and KB-1122, were provided by Kureha Chemical Industry Co., Ltd., Japan. The strains were cultured as described by Mäder *et al* [20] with slight modifications. All the *B. subtilis* strains were grown aerobically in 100-ml flasks containing 30 ml of a Luria-Bertani (LB) broth in a shaker at 160 rpm and 28°C. *Magnaporthe grisea* P131 as the indicator pathogen was provided by Peng Youliang, Department of Plant Pathology, China Agriculture University, China. This strain was incubated on a potato dextrose agar (PDA) medium at 28°C.

In Vitro Antifungal Activity of Antagonist Strains KB-1111 and KB-1122 Against *M. grisea* P131

The *in vitro* antagonism of the antagonist strains KB-1111 and KB-1122 towards *M. grisea* P131 was carried out in a hyphal diffusion inhibition assay [16, 19] with some modifications. A small piece of a fungal disk from a freshly grown culture was inoculated onto the center of a 100×15 mm Petri-dish plate containing 25 ml of PDA and incubated at 28°C for 48 h. Two sterile filter paper disks (6 mm in diameter) were then placed on both sides of the disk, 2 cm away from the center. An aliquot (6 µl) of the *B. subtilis* strain culture grown to the log phase was added to each disk. The plates were further incubated at 28°C for 72 h to allow development of the fungal mycelium and *B. subtilis* strains, and the appearance of a transparent zone of fungal inhibition on the plates. The experiments with the two *B. subtilis* strains were conducted twice, with three replicates.

Extraction of Intracellular and Extracellular Proteins

The *B. subtilis* cells were routinely cultured and harvested in the initial stationary phase (1.5×10^8 cells/ml), and then washed thoroughly with a potassium phosphate buffer (pH 7.4) to remove any bound extracellular proteins and other contaminants. Next, the cells were lysed by sonication (with cooling on ice) with 1 ml of a precooled homogenization buffer containing 20 mM Tris/HCl (pH 7.5), 250 mM sucrose, 10 mM EGTA, 1 mM PMSF, 1 mM DTT, and 1% Triton X-100. After homogenization, the homogenate was centrifuged at 15,000×*g* for 15 min at 4°C. The supernatant was then mixed with a 1/4 volume of 50% cold trichloroacetic acid (TCA) and kept in an ice bath for 30 min. Thereafter, it was centrifuged at 15,000×*g* for 15 min at 4°C, and the supernatant discarded. The pellet was washed with cold acetone three times, centrifuged, and vacuum-dried. The dried powder was then solubilized in a sample buffer containing 7 M urea, 2 M thiourea, 4% CHAPS, 2% ampholine (pH 3.5–10), and 20 mM DTT. The resulting protein solution was used for the 2-DE or stored at −80°C until use.

To extract the proteins from the culture supernatant, the *B. subtilis* strains were routinely cultured with an initial concentration of 1.5×10^8 cells/ml. After incubation, the cells in the suspension were removed by filtration through a 0.22-µm-pore-size membrane. The extracellular proteins in the cell-free filtrate were precipitated for 30 min with ice-cold 10% (w/v) TCA [23]. The precipitates were then harvested by centrifugation at 16,000×*g* for 45 min at 4°C. Thereafter, the supernatant was discarded, and the protein pellet washed three times with cold acetone. After centrifugation, the pellet was vacuum-dried and then solubilized in a lysis buffer, as described for the intracellular protein fraction.

Two-Dimensional Gel Electrophoresis

The 2-DE was carried out according to Abbasi and Komatsu [1] with minor modifications. The first-dimensional IEF was performed in a 13-cm-long glass tube with a 3-mm diameter. The gel mixture contained 4% acrylamide, 5% carrier ampholytes (pH 3.5–10.0:pH 5.0–8.0=1:1), and 2% NP-40. The IEF was performed at 200 V for 30 min, 400 V for 15 h, and then 800 V for 1 h. Generally, about 500 µg of proteins was loaded. After the first-dimensional run, the gels were incubated in an equilibration buffer (0.05 M Tris-HCl, pH 6.8, 2.5% SDS, 10% [v/v] glycerol, and 5% β-mercaptoethanol) for 15 min, twice. The second-dimensional electrophoresis was performed on vertical slab gels and using 15% polyacrylamide gels with 5% stacking gels. After the electrophoresis, the gels were stained with 0.1% Coomassie Brilliant Blue (CBB) R-250.

Image Scanning and Analysis

The CBB-stained gels were scanned at a 300 dots per inch (dpi) resolution using a UMAX Power Look 2100XL scanner (Maxium Tech, Taipei, China). The transparency mode was used to obtain a grayscale image. The image analysis was performed with ImageMaster 2D Platinum software. The optimized parameters were as follows: saliency 2.5, partial threshold 4, and minimum area 50. To correct the experimental variation, the protein spots that presented on at least two gels for one species (3 gels for each species) based on the image analysis were identified as expressed protein spots. The identified spots were manually rechecked. The normalized values of the protein spots on three replicate 2D gels for each species were exported to SPSS Version 13.0 (Lead Technologies, Chicago, IL, U.S.A.) for statistical analysis. Only those spots with significant and consistent changes were considered to be differentially accumulated proteins (>1.5-fold, $p<0.05$).

Protein Identification

The Coomassie Blue-stained protein spots were manually excised from the gels and cut into small pieces. The protein digestion and MALDI-TOF-MS analysis were performed as described by Shen *et al.* [25] with slight modifications. Briefly, each small gel piece with a protein was destained with 50 mM NH_4HCO_3 in 50% (v/v) methanol for 1 h at 40°C, and the destaining step was repeated until the gel became colorless. The protein in the gel piece was then reduced with 10 mM DTT in 100 mM NH_4HCO_3 for 1 h at 60°C and incubated with 40 mM iodoacetamide in 100 mM NH_4HCO_3 for 30 min at room temperature. Next, the gel pieces were minced and lyophilized, and then rehydrated in 25 mM NH_4HCO_3 with 10 ng of sequencing-grade modified trypsin (Promega, Madison, WI, U.S.A.) at 37°C overnight. After digestion, the protein peptides were collected and the gels washed with 0.1% TFA in 50% ACN three times to collect the remaining peptides. The tryptic peptide masses were measured using a MALDI-TOF mass spectrometer (Shimadzu Biotech, Kyoto, Japan).

The National Center for Biotechnology Information (NCBI) database was searched for the acquired peptide mass fingerprinting (PMF) data using the MASCOT software available at http://www.matrixscience.com. *B. subtilis* was chosen as the taxonomic category. To determine the confidence of the identification results, the following criteria were used: in addition to a minimum MOWSE score of 51, the sequence coverage of the protein could not be less than 10% by the matching peptides. Only the best matches with high confidence levels were selected, and the protein spots with a low level of confidence are not shown in this paper, even though these protein spots exhibited differential expression profiles.

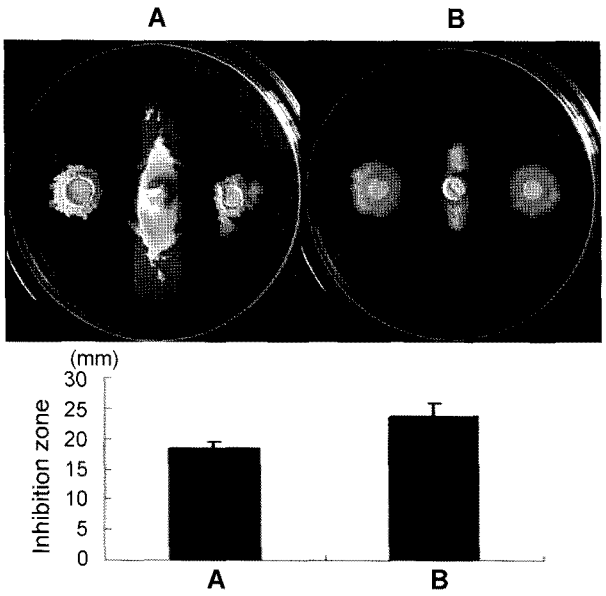

Fig. 1. Antifungal activity of two *B. subtilis* strains (KB-1111 and KB-1122) against indicator pathogen *M. grisea* P131.
Aliquots (6 μl) of the KB-1111 and KB-1122 cultures in the log phase were added to the disks on plates A and B, respectively. Average change in width is expressed as inhibition zone. Values are means ± SD (n=3). (**A**) KB-1111; (**B**) KB-1122.

RESULTS

In Vitro Antifungal Activities of KB-1111 and KB-1122 Towards *M. grisea* P131

Previous research by the current authors showed that the two *B. subtilis* strains KB-1111 and KB-1122 exhibited antifungal activities against the pathogenic fungus *Magnaporthe grisea* P131 [32]. Thus, to investigate the differences in their antifungal activities, a hyphal diffusion inhibition assay was conducted, as described in the Materials and Methods section. As shown in Fig. 1, the inhibition zone of KB-1122 was significantly larger than that of KB-1111, and the growth of the pathogen mycelium exhibited distinct suppression when challenged by KB-1122. In addition, the colony forms of KB-1111 exhibited mucous wrinkles and an irregular growth mode without any glisten, whereas KB-1122 showed a smooth surface and well-regulated growth pattern with glisten (Fig. 1). This observation indicated that KB-1122 has a much stronger antifungal activity against *M. grisea* P131 than KB-1111.

Comparative Analysis of KB-1111 and KB-1122 Intracellular Proteomes

To obtain a better understanding of the intrinsic differences between the antagonism of KB-1111 and KB-1122, a comparative analysis of their intracellular proteomes was conducted. The intracellular protein expression profiles of KB-1111 and KB-1122 under normal conditions were analyzed using 2D-PAGE (Fig. 2), and a comparative analysis of the 2-DE maps of the *B. subtilis* strains (KB-1111 and KB-1122) was performed using ImageMaster 2D Platinum software. More than 600 reproducible protein spots were detected on the individual CBB R-250-stained 2D gels for each *B. subtilis* strain within a range of pH 3.5–10 and molecular mass of 20–100 kDa. Most of the protein spots were located near the center of the gels (Fig. 2). Although a total of 47 differentially expressed protein spots were detected for strains KB-1111 and KB-1122, the present study focused on 20 protein spots with a relatively high abundance. According to the identification criteria, among the 20 tested protein spots, 11 spots (Fig. 2) had a higher Mowse score than the threshold (Fig. 4a; Table 1), and among these 11 spots, spots 1 through 8 were upregulated in KB-1122 when compared with KB-1111. It was also found that aconitate hydratase (No. 1), a factor regulating bacterial motility [28], was solely expressed in KB-1122. Meanwhile, three (Nos. 6–8) spots were identified as the same protein, class III stress response-related ATPase, involved in stress response [14]. Furthermore, the alpha-amylase precursor (No .3) is an enzyme involved in the regulation of protein secretion

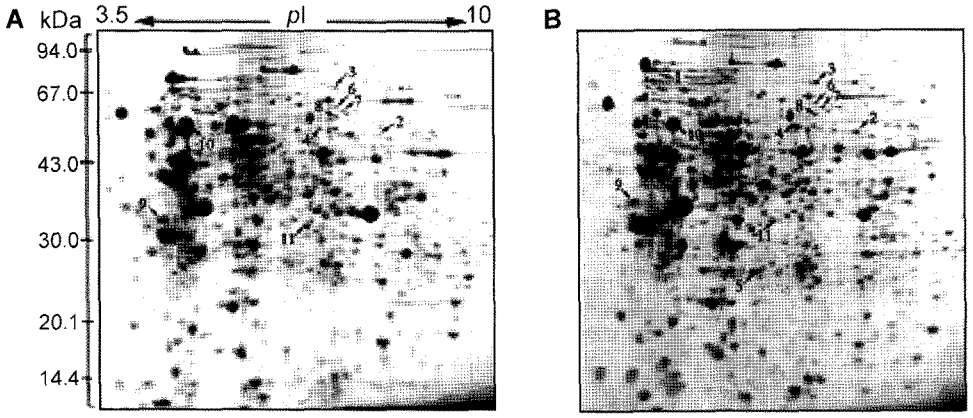

Fig. 2. 2-DE profiles of intracellular proteins from two *B. subtilis strain* cultured under normal conditions.
Arrows indicate the changed proteins. The protein spots are numbered and correspond to those in Table 1. **A**. KB-1111, **B**. KB-1122.

Table 1. Identification of intracellular proteins extracted from *B. subtilis* KB-1111 and KB-1122.

Protein No.	Accession No.	Protein name	Score	Queries matched	No. of queries	SC (%)	Theoretical Mr(kDa)/pI
1 [U2]	NP_389683	Aconitate hydratase	52	6	22	10%	99.6/5.09
2 [U2]	NP_389336	Hypothetical protein BSU14530	122	9	26	22%	61.8/5.93
3 [U2]	BAA31528	Alpha-amylase precursor	110	9	21	19%	72.2/5.63
4 [U2]	NP_390335	Glycine dehydrogenase subunit2	59	5	21	12%	54.6/5.4
5 [U2]	AAC37016	Dihydroxynapthoic acid (DHNA) synthetase	51	4	27	19%	28.8/5.44
6 [U2]	NP_387967	Class III stress response-related ATPase	83	7	24	13%	90.1/5.82
7 [U2]	NP_387967	Class III stress response-related ATPase	101	6	9	11%	90.1/5.82
8 [U2]	NP_387967	Class III stress response-related ATPase	94	7	16	13%	90.1/5.82
9 [U1]	NP_388688	Acetoin dehydrogenase E1 component (TPP-dependent beta subunit)	67	6	31	17%	36.9/4.59
10 [U1]	NP_389344	Dihydrolipoamide dehydrogenase	77	5	14	17%	49.9/4.95
11 [U1]	NP_389111	Hypothetical protein BSU12290	128	8	11	25%	41.9/6.28

Protein scores greater than 51 are significant ($p<0.05$).
[U1] Upregulated protein in *B. subtilis* KB-1111.
[U2] Upregulated protein in *B. subtilis* KB-1122.
SC (%), amino acid sequence coverage for the identified proteins.

[17, 30], while two other proteins were identified as involved in basic metabolism (No. 4 and No. 5). The upregulated proteins in KB-1111, including an acetoin dehydrogenase E1 component (TPP-dependent beta subunit) (No. 9) and dihydrolipoamide dehydrogenase (No. 10), were mainly crucial carbon-metabolism-associated proteins involved in the pathway of tricarboxylic acid (TCA) cycles. In addition, two spots were identified as hypothetical proteins, spots No. 2 and No. 11, which were upregulated in KB-1122 and KB-1111, respectively. The roles of these proteins were confirmed after further analysis.

Comparative Analysis of KB-1111 and KB-1122 Secretory Proteomes

To further investigate the different antagonism mechanisms of the two antagonist strains, a comparative analysis was conducted of the secretory protein profiles of KB-1111 and KB-1122. As shown in Figure 3, most of the extracellular protein spots on the gels were detected within a range of pH 4–9 and molecular mass between 30 and 90 kDa. About 180 protein spots were detected on the 2D gels after ignoring some faint spots and spots with undefined shapes and areas. An image analysis revealed that 23 proteins were differentially expressed in KB-1111 and KB-1122. Using MALDI-TOF-MS, 17 protein spots with a relatively high abundance were analyzed and 10 proteins successfully identified with Mowse scores greater than the threshold (Fig. 3, Fig. 4b, and Table 2). A protein spot (Ex-1), identified as an alpha-amylase precursor, shared the same identity with an intracellular protein (No. 3, Table 1) and exhibited an absolute expression level in KB-1122.

Three protein spots (Ex-7, 9, and 10) with only slight differences in the Mr and pI were identified as the same

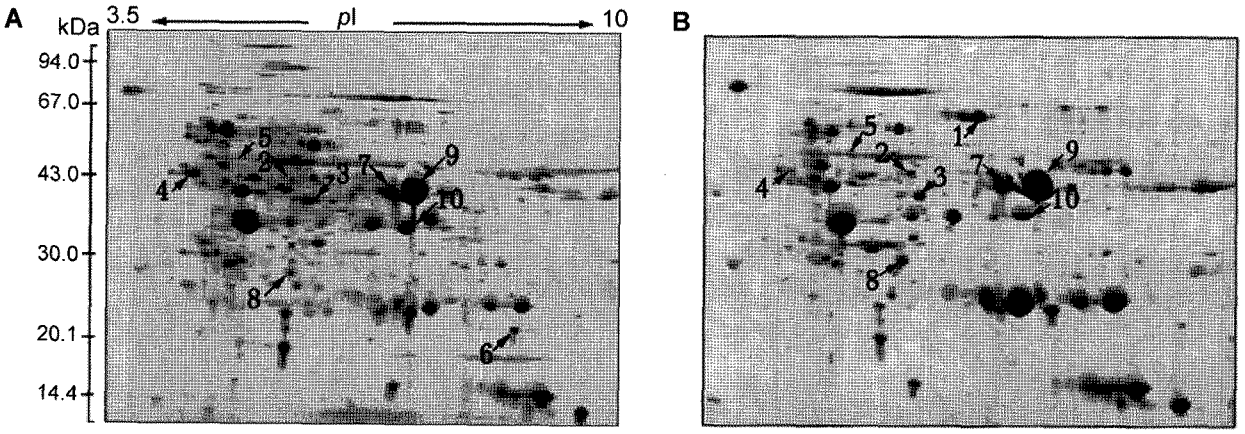

Fig. 3. 2-DE profiles of extracellular proteins from two *B. subtilis strain* cultured under normal conditions.
Arrows indicate the changed proteins. The protein spots are numbered and correspond to the numbers in Table 2. **A**. KB-1111, **B**. KB-1122.

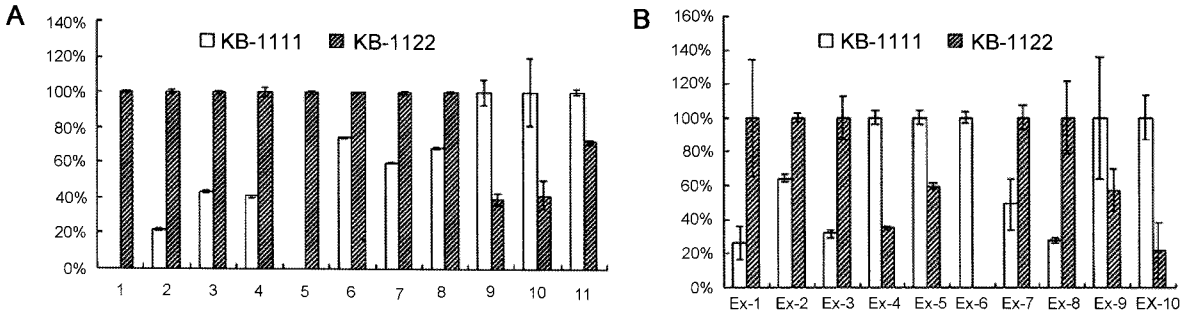

Fig. 4. Quantitative changes in identified proteins.
A. Intracellular proteins, **B**. Extracellular proteins. The average change in abundance is expressed as the intensity means ± SD (n=3).

protein, a neutral protease precursor (Table 2). Interestingly, these proteins exhibited complicated change patterns in KB-1111 and KB-1122 (Fig. 4b), where spot Ex-7 was upregulated in KB-1122, whereas the other two (Ex-9, Ex-10) were upregulated in KB-1111, indicating the possibility of different posttranslational modifications. Another three proteins were identified as enzymes involved in the basic metabolism pathway, including ATP synthase subunit A (Ex-2), L-alanine dehydrogenase (Ex-3), and enoyl-(acyl carrier protein) reductase (Ex-6). Among these proteins, Ex-2 and Ex-3 were up-regulated in KB-1122, whereas Ex-6 was upregulated in KB-1111. In addition, an elongation factor EF-2(Ex-4) and hypothetical protein BSU23820 (Ex-5) were also identified. Additionally, it is noteworthy that one protein, identified as endo-1,4-beta-glucanase (Ex-8), which is involved in fungal cell wall degradation [13, 18], was apparently upregulated in KB-1122.

DISCUSSION

B. subtilis species have been described as biocontrol agents against pathogenic fungi [6, 21, and 26]. Thus, to supplement the *in vitro* antifungal activity potential of two *B. subtilis* strains (KB-1111 and KB-1122) demonstrated in a previous study [32], this study provided a distinct comparison of the two antagonist strains against the pathogen *M. grisea* P131, which is considered as a model pathogen and causes dramatic yield losses for many crops [7], and confirmed the superior antagonism of strain KB-1122 when compared with KB-1111 (Fig. 1).

By means of a MALDI-TOF-MS analysis, a total of 11 differentially expressed protein spots (Fig. 4a) were successfully identified in the cellular proteome (Table 1). Among these protein spots, three (Nos. 6–8) with identical identities were upregulated in KB-1122 and characterized as class III stress-response-related ATPase (Fig. 3). The cell presence of this protein is crucial for the tolerance of *B. subtilis* to many types of stresses [14]. Previous research has proven that aconitate hydratase (No. 1) plays an essential role in the citric acid and glyoxylate cycles of many organisms [4]. Meanwhile, a recent report suggested the involvement of aconitase proteins in the posttranscriptional regulation of bacterial motility [28]. Interestingly, an alpha-amylase precursor protein was identified in both the cellular and secretory

Table 2. Identification of extracellular proteins extracted from *B. subtilis* KB-1111 and KB-1122.

Protein No.	Accession No.	Protein name	Score	Queries matched	No. of queries	SC (%)	Theoretical Mr(kDa)/pI
Ex-1^{U2}	BAA31528	Alpha-amylase precursor	90	10	21	18%	72.2/5.63
Ex-2^{U2}	NP_391564	ATP synthase subunit A	146	14	33	33%	54.7/5.22
Ex-3^{U2}	NP_391071	L-alanine dehydrogenase	86	6	20	23%	39.7/5.28
Ex-4^{U1}	NP_387993	Elongation factor EF-2	147	11	16	22%	76.7/4.82
Ex-5^{U1}	NP_390263	Hypothetical protein BSU23820	52	5	18	15%	37.6/5.28
Ex-6^{U1}	NP_389054	Enoyl-(acyl carrier protein) reductase	91	8	22	28%	28.0/5.67
Ex-7^{U2}	ABC49679	Neutral protease precursor	72	6	19	14%	56.8/8.65
Ex-8^{U2}	AAN07019	Endo-1,4-beta-glucanase	88	6	25	24%	46.7/8.69
Ex-9^{U1}	ABU53635	Neutral protease precursor	54	6	58	16%	56.8/8.65
Ex-10^{U1}	ABC49679	Neutral protease precursor	64	6	30	14%	56.8/8.65

Protein scores greater than 51 are significant ($p<0.05$).
^{U1} Upregulated protein in *B. subtilis* KB-1111.
^{U2} Upregulated protein in *B. subtilis* KB-1122.
SC (%), amino acid sequence coverage for the identified proteins.

proteomes (Fig. 2 No. 3 and Fig. 3 Ex-1), and this result corresponds to the intrinsic property of this protein. The *B. subtilis* species vigorously secretes proteins into the extracellular environment, including alpha-amylase, and the translocation of the precursor of alpha-amylase into membrane vesicles is mainly by the Sec pathway [15]. Thus, as a secretion protein, since alpha-amylase can be secreted into the extracellular domain and involved in regulating the protein secretion pathway [17, 30], it is suggested that the differential expression of this enzyme was related to the secretion of the other secretory proteins. Interestingly, the other differentially expressed cellular proteins were universal enzymes involved in the basic metabolism pathway, including an acetoin dehydrogenase E1 component (No. 9), dihydrolipoamide dehydrogenase (No. 10), glycine dehydrogenase subunit2 (No. 4), and dihydroxynapthoic acid (DHNA) synthetase (No. 5), all of which play important roles in normal growing cells.

Various previous studies have suggested that certain extracellular proteases involved in the hydrolysis of fungal cell walls would seem to achieve the best antifungal activity [2, 12]. Thus, based on a proteomic approach, the identification of the secretory proteins, especially hydrolytic enzymes, is crucial to understand the intrinsic differences between the antagonism of antagonist strains. In this study, three protein spots with complicated changes (Ex-7, 9, and 10; Fig. 4b) in their secretory proteome maps were identified as the same hydrolytic enzyme, a neutral protease precursor. This may be explained as a result of posttranslational modifications, whereas the presence of the protein may indicate involvement in a potential defense response as a virulence factor [12, 29]. The present results indicated that the protein seemed to contribute to the antifungal activity of two strains (Fig. 1), yet it did not explain the intrinsic difference between the antagonism of KB-1111 and KB-1122. It is now well established that some extracellular hydrolytic enzymes are able to associate with the fungal cell wall surface, including endo-1,4-beta-glucanase. This protein has already been shown to play a direct role in the degradation of polysaccharides and the be involved in antifungal defense responses [13, 18]. Interestingly, this endo-1,4-beta-glucanase (Ex-8) was apparently up-regulated in KB-1122 (Fig. 3). In addition, several other extracellular proteins (Ex-2-4, Ex-6) identified in this study were also normally recognized as cellular proteins. This phenomenon may be related with the various secretome secrets of *B. subtilis*, including the apparent export of cytoplasmic proteins into the growth medium [30].

In conclusion, using a proteomic approach to compare the intrinsic differences between the antagonism of KB-1111 and KB-1122 produced the following results: first, the upregulation of the cellular protein class III stress-response-related ATPase (Nos. 6–8) indicated that the stress tolerance of KB-1122 may be better than that of strain KB-1111; second, the differential expression of aconitate hydratase (No. 1) in KB-1111 and KB-1122 may explain the different colony forms, third, the upregulation of the alpha-amylase precursor protein (No. 3; Ex-1) in KB-1122 may provide better regulation of the protein secretion pathway for this strain; and finally, the differentially expressed secretory protein, endo-1,4-beta-glucanase (Ex-8), may explain the better antifungal activity of strain KB-1122 when compared with KB-1111. Therefore, this report is an important step towards understanding the antifungal mechanisms of *B. subtilis* against pathogens. Such comparative data may also contribute to future attempts to correlate the differential antagonism with genetic characteristics.

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