J. Microbiol. Biotechnol. (2016), 26(11), 1993–2005
http://dx.doi.org/10.4014/jmb.1602.02042

jmb

Extracellular Proteome Profiling of *Bacillus pumilus* SCU11 Producing Alkaline Protease for Dehairing

Chao Wang, Shiqiang Yu, Ting Song, Tingting He, Huanhuan Shao, and Haiyan Wang*

Key Laboratory of Bio-Resources and Eco-Environment, Ministry of Education, Sichuan Key Laboratory of Molecular Biology and Biotechnology, College of Life Sciences, Sichuan University, Chengdu 610064, P.R. China

Received: February 22, 2016
Revised: July 23, 2016
Accepted: July 25, 2016

First published online
July 27, 2016

**Corresponding author*
Phone: +86-28-85412738;
Fax: +86-28-85412738;
E-mail: hayawang@scu.edu.cn

Supplementary data for this paper are available on-line only at http://jmb.or.kr.

pISSN 1017-7825, eISSN 1738-8872

Bacillus pumilus is one of the most characterized microorganisms that are used for high-level production of select industrial enzymes. A novel *B. pumilus* SCU11 strain possessing high alkaline protease activity was obtained in our previous work. The culture supernatant of this strain showed efficient dehairing capability with minimal collagen damage, indicating promising potential applications in the leather industry. In this study, the strain's extracellular proteome was identified by LC-MS/MS-based shotgun proteomic analysis, and their related secretory pathways were characterized by BLAST searches. A total of 513 proteins, including 100 actual secreted and 413 intracellular proteins, were detected in the extracellular proteome. The functions of these secreted proteins were elucidated and four complete secretory systems (Sec, Tat, Com, and ABC transporter) were proposed for *B. pumilus*. These data provide *B. pumilus* a comprehensive extracellular proteome profile, which is a valuable theoretical and applicative basis for future genetic modifications and development of industrial enzymes.

Keywords: *Bacillus pumilus*, secretome, protease, secretory pathway

Introduction

With the growing worldwide demand for clean and sustainable development, much work is focused on using ecofriendly enzymes in place of hazardous chemicals in industrial processes. The gram-positive endospore-forming genus *Bacillus* is one of the best-known industrial microorganisms for high-scale production of industrial enzymes, such as amylase, protease, and lipase. Currently, approximately 60% of industrial enzymes of the world are produced by *Bacillus* [43, 51].

Proteases are a large class of important industrial enzymes widely used in the food, detergent, and leather industries [41]. In leather processing, alkaline proteases are used as an alternative to conventional chemicals in dehairing. Alkaline conditions facilitate hair root swelling, and subsequent protease attack on hair follicle protein, which then allows easy hair removal [16]. A novel *B. pumilus* SCU11 with high alkaline protease activity has been obtained through screening and mutagenesis. Fermentation supernatant from this strain shows efficient dehairing capabilities with minimal collagen damage [19], indicating that extracellular proteases produced by this strain have good potential for future applications in the leather industry. Apart from proteases, *B. pumilus* secretes other useful enzymes that can be used in various fields, such as xylanase for paper-pulp processing [38], lipase for fatty acids production [24], and keratinases for keratin protein degradation [7]. It is noteworthy that industrial enzyme products are usually prepared directly from fermentation supernatant containing complex components, some of which might lead to unwanted consequences, for example, skin collagen damage during the biodehairing process [50]. Determining fermentation supernatant components would be helpful for improving the usefulness of enzyme reagents. In addition to being used as biotechnological workhorses for the industrial production of extracellular enzymes, *B. pumilus* is also used as an active ingredient in agricultural fungicides [2]. All of these applications are associated with this species' extracellular proteins. *Bacillus* species have a high capacity to secrete proteins into the medium, and improved fermentation technologies have also contributed to the

protein export [48]. However, the expression and secretion of heterologous proteins by *Bacillus* species remain hampered by several bottlenecks, such as poor targeting to translocase, degradation of the secretory protein, and slow or incorrect folding [44]. Understanding the mechanisms and controlling factors of the secretory pathway will help ameliorate the secretory system problems and broaden the species' application as an industrial enzyme source.

To date, 22 genomes of strains and isolates of *B. pumilus* have been sequenced, including isolate BA06 [55], the progenitor of strain SCU11. Nevertheless, studies focusing on the extracellular proteome and related secretory systems of *B. pumilus* remain inadequate. Handtke *et al.* [18] used two-dimensional electrophoresis and mass spectrometry (2-DE/MS) to identify 86 secreted proteins from *B. pumilus* Jo2 as well as some components of its Sec and Tat secretory pathways. In previous work in this group, 10 differentially expressed extracellular proteins of *B. pumilus* SCU11 under alkaline stress were identified by 2-DE/MS [54]. These results provided preliminary reference data for *B. pumilus*'s secretome and related secretory pathways. However, there remains a lack of comprehensive insight from the overall perspective of the *B. pumilus* secretome, such as detailed functional categorization and analysis of secreted proteins, a complete secretory pathway prediction, and identification of other potentially useful proteins worthy of further exploration.

In this study, extracellular proteins were extracted from *B. pumilus* SCU11 culture supernatant and identified by liquid chromatography-tandem mass spectrometry (LC-MS/MS)-based shotgun proteomic analysis. The secreted proteins were predicted based on a bioinformatics analysis, and comprehensive insight was presented into the physiological and molecular functions of these proteins. The complete range of *B. pumilus* secretory pathways were clarified for the first time. These findings provide important theoretical reference data for enzyme exploitation, strain genetic modification, and molecular mechanism studies.

Materials and Methods

Microorganisms and Growth Conditions

Bacillus pumilus strain SCU11 had been previously isolated and preserved in this laboratory [50]. A single clone of this strain was cultured overnight at 34°C in 4 ml of Luria-Bertani broth (LB) medium overnight, after which 1 ml of overnight culture was transferred into 50 ml of fresh LB medium and grown to exponential growth phase for strain activation. After two rounds of culturing and activation as described above, 10 ml of exponential growth phase culture was inoculated into 500 ml of LB medium and cultivated at 34°C with vigorous shaking (200 rpm). Cell death and lysis were unavoidable during cultivation, and this issue becomes increasingly obvious with extended culture times. To minimize this interference, the cell culture was harvested at the late-exponential growth phase (OD_{600nm} = 1.8–2.0).

Extracellular Protein Extraction

Culture supernatant was collected by centrifugation at 12,000 ×*g* at 4°C for 30 min. Supernatant residual cells and debris were removed by filtration through a 0.22 μm membrane. Protein in the cell-free filtrate was precipitated with 12.5% chilled trichloroacetic acid (TCA; w/v) at −20°C for 2 h. The precipitate was harvested by centrifugation at 15,000 ×*g* at 4°C for 20 min and washed 5 times with 80% ice-cold acetone to remove residual TCA. After centrifugation (12,000 ×*g*, 4°C) for 30 min, the resulting pellets were collected and dried at room temperature. Finally, the pellets were dissolved in 9 M urea, the protein content was adjusted to 1 mg/ml, and a total of 500 μg of protein was used for LC-MS/MS analysis.

LC-MS/MS-Based Shotgun Proteomic Analysis

Isolated extracellular proteins were subjected to LC-MS/MS analysis at the facilities of the Beijing Genomics Institute (BGI). Samples were resolved using a 12% polyacrylamide gel stained with Coomassie blue R-250. Thirteen bands were excised and destained using 50 mM ammonium bicarbonate in 50% acetonitrile (ACN). Gel pieces were incubated with 10 mM dithiothreitol in 25 mM ammonium bicarbonate at 60°C for 1 h to reduce disulfide bonds. Cysteine alkylation was performed by incubating samples with 55 mM iodoacetamide in 25 mM ammonium bicarbonate at room temperature in darkness for 45 min. Next, Trypsin Gold (Promega Corp, USA) digestion was carried out at 37°C for 16 h, and the resulting peptides were extracted from gel bands using two rounds of 0.1% formic acid (FA) in 50% ACN and then 100% ACN.

Each fraction was resuspended in buffer A (2% ACN, 0.1% FA) and 10 μl of supernatant was loaded by an autosampler onto an LC-20AD nano high-performance liquid chromatograph (HPLC; Shimadzu Corp, Japan) equipped with a 2 cm C18 trap column. Then, peptides were eluted onto a 10 cm analytical C18 column (inner diameter, 75 μm) packed in-house. Samples were loaded at 8 μl/min for 4 min, then a 44 min gradient driven at 300 nl/min ranging from 2% to 35% buffer B (98% ACN and 0.1% FA), followed by a linear gradient to 80% over 2 min, then maintenance at 80% B for 4 min, and finally a return to 5% in 1 min. Peptides were subjected to nanoelectrospray ionization followed by tandem mass spectrometry (MS/MS) in a linear trap quadrupole LTQ Orbitrap Velos (Thermo) coupled to the HPLC. Peptides were selected for MS/MS using a collision-induced dissociation operating mode with a normalized collision energy setting of 35%. Ion fragments were detected in the LTQ, and a data-dependent procedure alternated between one MS scan followed by 10 MS/MS scans.

All MS/MS data were searched against the *Bacillus-pumilus*_txid1048 database of NCBI using the Mascot search engine (Matrix ScienceLtd, UK; ver. 2.3.02). For protein identification, one missed cleavage in trypsin digests was allowed. Gln→pyro-Glu (N-term Q), oxidation (M), and deaminated (NQ) were considered potential variable modifications, and carbamidomethyl (C) as a fixed modification. The charge peptide states were set to +2 and +3. The probability of false peptide identification was reduced by counting as identified only peptides with significance scores (≥20) at the 99% confidence interval according to a Mascot probability analysis greater than "identity." Each confident protein identification involved at least one unique peptide. All LC-MS/MS experiments were tested and analyzed in triplicates. For proteins identified between replicate experiments, proteins with the highest coverage and at least one unique peptide were chosen for subsequent analysis.

Protein Annotation and Gene Ontology Categorization

Proteins indentified from the extracellular proteome were automatically functionally annotated using BLASTp against the Nr database using the Blast2GO program. After achieving Gene Ontology (GO) annotation for every unigene, the compiled text outputs were subjected to GO category analysis using the Web Gene Ontology Annotation Plot (WEGO, http://wego.genomics.org.cn/cgi-bin/wego/index.pl). Protein function classifications were processed according to cluster orthologous groups (COG) using the WebMGA server [52].

N-Terminal Signal-Peptide Prediction

Actual secreted proteins among these extracellular proteins were determined using different bioinformatics analysis programs. SignalP 4.1 (http://www.cbs.dtu.dk/services/SignalP/) was used for signal peptide detection. TMHMM 2.0 (http://www.cbs.dtu.dk/services/TMHMM/) was used to predict protein transmembrane helices. TatP1.0 (http://www.cbs.dtu.dk/services/TatP/) and LipoP 1.0 (http://www.cbs.dtu.dk/services/LipoP/) were used for lipoprotein predictions.

Results and Discussion

B. pumilus Secretome Prediction by Genome Analysis

In previous studies, *B. pumilus* BA06, the parent strain of SCU11, was isolated from living waste in Chengdu, China. Based on this strain's genome sequence, a bioinformatics analysis was carried out to predict its secretome. Of 3,680 protein coding sequences, SignalP, TatP, and TMHMM analyses predicted 220 proteins to be secreted proteins, which is more than the 177 proteins predicted in the *B. pumilus* SAFR-032 genome [18] and less than the 300 proteins predicted in the *B. subtilis* genome [1]. These discrepancies might have derived from differences in strain genomes, genome annotation, and the software and methods used for prediction.

Secretory System of *B. pumilus*

In *B. subtilis*, four distinct pathways for protein export from the cytoplasm to the extracellular space have been indentified, including general secretory (Sec), twin-arginine (Tat), pseudophilin (Com), and ATP-binding cassette (ABC) transporter pathways [8]. In *B. subtilis*, proteins involved in these pathways have been systematically studied and many relevant proteins identified [8, 47, 48]. Here, these secretory system-related proteins were BLAST searched against the genome and transcriptome database of *B. pumilus* BA06 (unpublished). On the basis of identified homologous components (Table S1), *B. pumilus* was deduced to possess a complete array of Sec, Tat, Com, and ABC transporter secretory systems. A simple schematic representation of the four secretory pathways in *B. pumilus* is shown in Fig. 1.

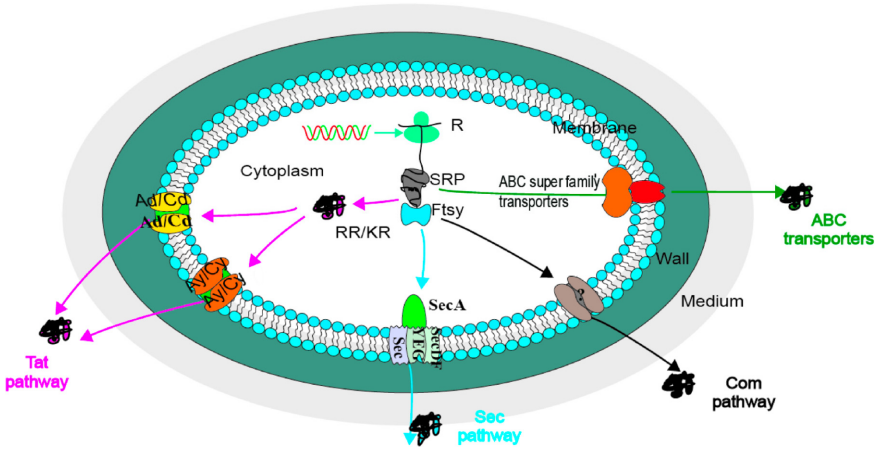

Fig. 1. Schematic representation of the network of four putative protein secretion pathways in *B. pumilus*.

The present results showed that the *B. pumilus* secretory pathways were generally similar to *B. subtilis*. Compared with *B. subtilis*, three SPase (SipS/U/V) and two folding catalyst proteins (TepA and BdbA) were absent from the *B. pumilus* genome. SipP, which is located on a *B. subtilis* plasmid and can perform the functions of SipS and SipT [48], was indentified in *B. pumilus* (Table S1). These results implied that the secretory systems of *B. pumilus* and *B. subtilis* might be somewhat different.

Protein secretion rates can be improved through overexpression rate-limiting factors (SipT and PrsA) for protein secretion or fusion of efficient-processing signal peptides to heterologous proteins [8]. Hence, optimizing the secretory machinery would be an important objective for boosting protein secretion, especially for heterologous protein secretion.

LC-MS/MS Analysis and Cellular Localization of the Proteome

Late-exponential phase culture supernatants of *B. pumilus* SCU11 were collected and prepared for LC-MS/MS analysis. Combined with a BLAST search against the *B. pumilus*_txid1048 database, a total of 513 proteins were indentified. Although the cell culture was collected at the late-exponential phase and residual cells and debris in the supernatant were removed by filtration, it was inevitable that some intracellular proteins appeared in the supernatant, due to cell death and lysis during cultivation. In this context, the term "extracellular proteome" refers to all proteins present in the extracellular milieu; the term "secretome" refers to proteins that are secreted from the cell or parts of surface proteins but excludes integral membrane and membrane-inner surface proteins [13, 56].

Signal peptides of the 513 identified extracellular proteins were analyzed to determine actual secreted proteins using four different signal peptide forecast software programs; SignalP 4.1, TMHMM 2.0, LipoP 1.0, and TargetP 1.0. The results predicted 100 proteins to be secreted to the extracellular space, representing 45% (100/220) of the predicted *B. pumilus* secretome. These 100 proteins were also manually checked in the 220 bioinformatic-predicted database using BLAST homolog alignments to confirm they were actual secreted proteins of *B. pumilus*.

A traditional 2-DE-based proteomic method has been widely applied in *Bacillus* proteome analyses. Using this method, Handtke *et al*. [18] have identified 86 secreted proteins from *B. pumilus* Jo2 growing exponentially in complex medium. Tjalsma *et al.* [47] identified 90 extracellular proteins from *B. subtilis* 168 at the early-stationary phase in rich medium (LB), and an additional 23 extracellular proteins from the same strain in a very rich (2× SNB) medium or under phosphate-starvation conditions [1]. Further signal peptide analysis of 113 extracellular proteins indicated 54 proteins were predicted to be secreted [1]. All of these results indicated that intracellular proteins exist extensively in the extracellular proteome and that shotgun proteomic analysis can detect more proteins, most of which belong to low-abundance intracellular proteins (discussed later) that are invisible in the master gel.

Protein Annotation and GO Category

The physiological and functional differences of the 100 secreted proteins were explored by analyzing their sequences with BLASTp using Blast2Go software and GO categories [6]. Thus, of the 100 proteins, 65 were functionally annotated (Table 1), whereas the remaining proteins were "unclassified" or "unclear classification" (Table S2). The complied text outputs of Blast2Go were subjected to GO categories using WEGO online analysis tools. Three groups of databases were simultaneously subjected to online analysis and the results are shown in Fig. 2. Analyses of the proteins' putative functions revealed they were involved in 6 GO terms of cellular components, 4 GO terms of molecular functions and 13 terms of biological processes.

The 220-protein predicted secretome was also annotated and categorized as shown in Fig. 2. In both proteome databases, most of these proteins were involved in catalytic and metabolic processes. For proteins predicted only from the genome, they were mainly related to antioxidant, electron carrier, and structural entities. For predicted but undetected proteins in the extracellular proteome, it was possible that they were not expressed or had low expression rates at the late-exponential growth phase.

Functional Category and Analysis of Secreted Proteins

The 65 detected secreted proteins with function classifications were subjected to further analyses. On the basis of the predicted GO terms and COG results, they were categorized into eight groups (Fig. 3). Detailed information is listed in Table 1 with some manual adjustments.

Secreted Proteases of *B. pumilus*

As a model organism for *Bacillus*, *B. subtilis* extracellular proteases have been extensively studied. SubtiList, the reference database for the *B. subtilis* genome, revealed that there were eight major extracellular proteases; namely, AprE, Vpr, WprA, Epr, Bpr, Mpr NprE, and NprB [28, 30]. Searching gene sequences for these eight proteases in the

Table 1. Identification and functional category of secreted proteins of *B. pumilus*.

No.	Protein name	Protein ID	Description	Reference organism	Protein MW	pI	Coverage	Unique peptides
Extracellular proteases								
1	AprE1[b]	7415642	Alkaline serine proteinase	Bpum_0972	39,496.94	9.21	17.49%	4
2	Epr[b]	194010703	Minor extracellular protease Epr	Bpum_0233	58,759.1	10.68	16.94%	8
3	WprA[b]	194010894	Cell wall-associated protease	Bpum_0220	96,503.83	10.2	17.10%	14
4	Vpr[a]	194012134	Minor extracellular protease Vpr	Bpum_3455	86,545.34	7.4	13.00%	7
Protein metabolism-related proteins								
5	Mpr[c e]	74473705	S1 family glutamyl endopeptidase	Bpum_1681	32,367.98	8.83	29.14%	6
6	Peptidase M14[c e]	157680254	M14 family carboxypeptidase	Bpum_0711	60,344.64	8.9	7.73%	3
7	OppA[b]	194013365	Oligopeptide-binding protein OppA	Bsu11430	61,208.01	7.69	41.83%	17
8	GGT[a]	194013944	Gamma-glutamyltransferase	Bpum_1164	63,301.18	4.73	6.69%	2
Carbohydrate metabolism-related proteins								
9	YbbD[e]	157679709	Glycoside hydrolase	Bpum_0154	70,340.23	10.12	1.41%	1
10	BglS[a]	157680014	Licheninase	Bpum_0463	27,500.15	8.16	36.63%	6
11	YheN1[d]	157680455	Possible polysaccharide deacetylase	Bpum_0915	32,345.82	9.77	23.24%	5
12	YjeA[d]	157681842	Possible polysaccharide deacetylase	Bpum_2320	53,547.69	9.45	9.42%	3
13	LytD[a]	157682742	Beta-*N*-acetylhexosaminidase	Bpum_3233	95,476.68	10.31	8.68%	4
14	PelB[b]	157683019	Pectate lyase	Bpum_3515	37,395.82	9.94	11.11%	3
15	Pme[d]	157681335	Pectinesterase	Bpum_1808	36,069.23	9.65	4.60%	1
Nucleotide metabolism-related proteins								
16	-[c e]	532528	Ribonuclease precursor	Bpum_3110	17,903.24	9.91	12.96%	2
17	YfkN[b]	194012268	2′,3′-Cyclic-nucleotide 2′-phosphodiesterase	Bpum_0732	156,659.24	6.66	0.84%	1
18	YhcR[a]	194013720	Endonuclease YhcR	Bpum_0870	106,502.91	6.64	4.14%	2
Cell wall /envelope metabolism-related proteins								
19	DacA[a]	157680056	Serine-type D-Ala-D-Ala carboxypeptidase	Bpum_0505	48,452.96	8.84	9.98%	3
20	LytE[d]	157680437	Vegetative cell wall hydrolase	Bpum_0897	29,996.31	11.01	9.03%	2
21	YocH[b]	157681378	Cell wall protein	Bpum_1851	27,842.59	10.17	3.42%	1
22	CtpA[c d]	157681406	S41 family carboxy-terminal processing peptidase	Bpum_1880	50,417.35	9.23	4.99%	2
23	YwtD[b]	157682764	C40 family endopeptidase	Bpum_3255	45,310.29	9.68	9.09%	2
24	YqgS[d]	194010614	Glycerol phosphate lipoteichoic acid synthase 2 (LTA synthase 2) (Polyglycerol phosphate synthase 2)	Bpum_2214	74,150.75	5.35	18.62%	9
25	CwlS[d]	194011390	Excreted arabinogalactan oligomer endo-hydrolase	Bsu19410	47,600.34	10.71	1.56%	1
26	PgdS[d]	194012831	Gamma-DL-glutamyl hydrolase (Poly-gamma-glutamate depolymerase) (PGA depolymerase)	Bsu35860	45,259.33	9.7	3.69%	1
27	LytC[b]	194012865	*N*-Acetylmuramoyl-L-alanine amidase CwlB (Cellwall hydrolase) (Autolysin)	Bpum_3214	52,440.91	10.72	35.61%	14
Proteins associated with inorganic ion transport								
28	YckB[d]	157679858	Amino acid ABC superfamily ATP binding cassette transporter, binding protein	Bpum_0305	32,470.6	8.97	3.45%	1
29	YcKK[d]	157679892	L-cystine ABC superfamily ATP binding cassette transporter, binding protein YckK	Bpum_0340	29,774.25	9.52	19.93%	4
30	YycQ[c d]	157679898	Amino acid ABC superfamily ATP binding cassette transporter, binding protein	Bpum_0347	29,269.43	9.42	12.50%	2

Table 1. Continued.

No.	Protein name	Protein ID	Description	Reference organism	Protein MW	pI	Coverage	Unique peptides
Proteins associated with inorganic ion transport								
31	YclQ[b]	157680527	ABC superfamily ATP binding cassette transporter, binding protein	Bpum_0987	35,108.11	6.55	16.24%	4
32	AppA[c d]	157680602	Oligopeptide ABC superfamily ATP binding cassette transporter, binding protein	Bpum_1062	61,712.27	4.75	9.39%	4
33	YtcQ[d]	157682179	ABC superfamily ATP binding cassette transporter, binding protein	Bpum_2665	56,755.98	5.74	13.83%	5
34	YcdH[d]	157682222	ABC superfamily ATP binding cassette transporter, binding protein	Bpum_2708	36,519.1	5.52	6.08%	1
35	YfiY[b]	157682422	Ferric siderophore ABC superfamily ATP binding cassette transporter, binding protein	Bpum_2911	35,745.5	5.54	32.49%	9
36	(YvrC)[d]	157682491	Iron (Fe) ABC superfamily ATP binding cassette transporter, binding protein	Bpum_2981	34,336.98	8.38	6.33%	1
37	FhuD[b]	157682504	Ferrichrome ABC superfamily ATP binding cassette transporter, binding protein	Bpum_2994	34,514.08	9.58	21.47%	5
38	AppAB[d]	157682580	Oligopeptide ABC superfamily ATP binding cassette transporter, binding protein	Bpum_3070	60,998.26	9.13	7.59%	4
39	FeuA[b]	157682596	Ferrichrome ABC superfamily ATP binding cassette transporter, binding protein	Bpum_3086	33,456.82	9.48	4.00%	1
40	-[d]	157682747	Sugar ABC superfamily ATP binding cassette transporter, binding protein	Bpum_3238	45,958.02	4.68	17.81%	6
41	-[e]	157683030	Iron (Fe) ABC superfamily ATP binding cassette transporter, binding protein	Bpum_3526	32,779.78	5.75	9.83%	2
42	(YxeB)[b]	194011083	Putative iron-binding protein	Bpum_2591	34,413.99	9.71	28.43%	6
43	-[e]	194012405	ABC superfamily ATP binding cassette transporter, binding protein	Bpum_0316	39,491.22	8.96	17.45%	4
Proteins related to general function								
44	Lip[a]	130750925	Lipase	Bpum_2613	22,390	10.38	9.52%	1
45	YacD[c d]	157679612	Possible peptidylprolyl isomerase	Bpum_0056	34,913.01	5.4	6.95%	2
46	YddG[d]	157680124	Hypothetical membrane protein	Bpum_0575	87,681.7	7.17	1.26%	1
47	YerH[d]	157680175	Hypothetical secreted protein	Bpum_0627	44,596.68	5.26	4.05%	1
48	YvgO[a]	194010286	Stress response protein YvgO	Bpum_0751	18,411.29	10.16	7.65%	1
49	PrsA[a]	157680478	Peptidylprolyl isomerase PrsA	Bpum_0938	32,896.19	9	7.88%	2
50	Med[c d]	157680594	Positive regulator of *comK*	Bpum_1054	35,729.2	5.27	5.31%	1
51	(YmaC)[d]	157681155	Hypothetical bacteriophage protein	Bpum_1617	27,290.38	4.74	4.10%	1
52	YodJ[a]	157681409	Serine-type D-Ala-D-Ala carboxypeptidase	Bpum_1883	31,025.84	8.31	25.72%	5
53	YufN[c d]	157682334	ABC superfamily ATP binding cassette transporter, binding protein	Bpum_2823	38,775.19	9.41	25.83%	9
54	TdK[d]	157682955	Possible S9 family peptidase	Bpum_3349	40,196.63	9.92	12.01%	3
55	BlyA[d]	194011256	*N*-Acetylmuramoyl-L-alanine amidase	Bpum_2399	56,686.56	9.88	15.97%	5
56	-[e]	194011934	Cell surface protein	BCE_4671	66,449.42	4.78	16.53%	8
57	(Ina)[c e]	194012002	Immune inhibitor A	Bpum_3580	87,325.14	5.67	11.73%	4
Proteins with other functions								
58	GlpQ[d]	157679736	Glycerophosphodiester phosphodiesterase	Bpum_0181	33,139.46	9.48	8.87%	2
59	FliL[d]	157681068	Flagellar protein FliL	Bpum_1529	15,663.45	5.07	20.42%	2

Table 1. Continued.

No.	Protein name	Protein ID	Description	Reference organism	Protein MW	pI	Coverage	Unique peptides
Proteins with other functions								
60	SdhA [b]	157682020	Succinate dehydrogenase flavoprotein subunit	Bpum_2502	65,152.42	5.67	23.04%	11
61	YurI [a]	157682418	Deoxyribonuclease I	Bpum_2907	32,694	6.52	7.88%	1
62	LytR [d]	157682726	Transcriptional regulator LytR	Bpum_3217	34,679.62	9.53	21.57%	7
63	YbbE [d]	194010688	Beta-lactamase	Bpum_0155	50,771.11	9.82	17.74%	6
64	LytB [b]	194012797	Amidase enhancer (Modifier protein of major autolysin)	Bpum_3215	76,503.53	10.52	2.42%	1
65	YvfO [d]	157683116	Arabinogalactan endo-1,4-beta-galactosidase	Bpum_3616	44382.15	8.96	24.13%	8

Proteins were classified according to their predicted functional categories as described in Materials and Methods. Table 1 only lists 65 proteins with function annotated; the other 35 proteins with function "unclassified" or "unclear classification" are listed in Table S2.

Coverage: the degree of coverage of the protein by the identified peptides.

Unique peptides: the peptide that is peculiar to a uniprotein.

[a]Indicating the protein was identified among *B. subtilis* 168, and *B. pumilus* SCU11 and Jo2.

[b]Indicating the protein was identified both in *B. subtilis* 168 and *B. pumilus* SCU11.

[c]Indicating the protein was identified both in *B. pumilus* Jo2 and *B. pumilus* SCU11.

[d]Indicating the protein was identified in the extracellular proteome of *B. pumilus*, and it has a higher homology in the *B. subtilis* genome.

[e]Indicating the protein was identified in the extracellular proteome of *B. pumilus*, and it has a low homology (<30%) in the *B. subtilis* genome.

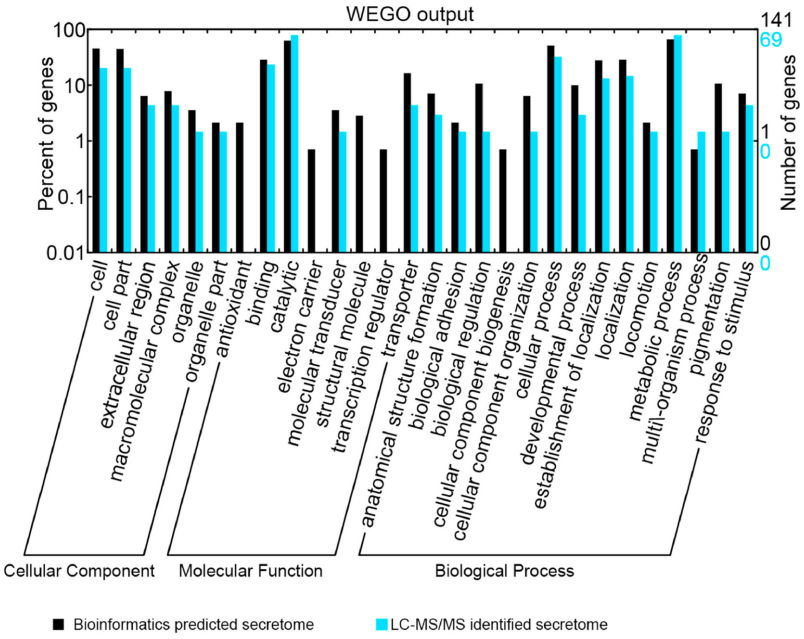

Fig. 2. GO categories for the bioinfomatics-predicted secretome and LC-MS/MS-identified secretome using Web Gene Ontology Annotation Plot (WEGO).

B. pumilus BA06 genome indicated that five highly homologous extracellular protease genes existed, including *aprE*, *vpr*, *wprA*, *epr*, and *bpr*. The other three genes (*mpr*, *nprE*, and *nprB*) were not found in the BA06 genome. Among the 100 secreted proteins of *B. pumilus* SCU11, four proteases were detected; namely, AprE, Epr, Vpr, and WprA.

AprE is a member of the subtilisins, a family of alkaline serine endoproteases secreted by a wide variety of *Bacillus* species. Some well-known proteases, such as subtilisin E from *B. subtilis*, BPN′ from *B. amyloliquefaciens*, and Carlsberg from *B. licheniformis*, also belong to this family [36]. In view

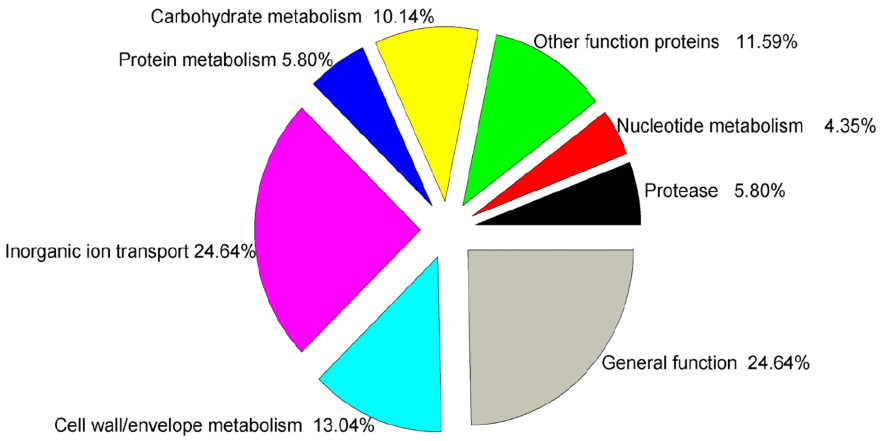

Fig. 3. Functional classification of the 65 secreted proteins.

of industrial applications, subtilisin E from *B. subtilis* represents the world's largest commercial enzyme market, mainly as detergent additives and for food and leather processing. Our previous study showed that *B. pumilus* BA06 culture supernatant possesses efficient dehairing capability with minimal collagen damage. Native and SDS-PAGE protein analyses demonstrated that alkaline protease AprE is the most abundant protease secreted by *B. pumilus* BA06 at the late-decline phase [50]. Thus, much work has been done on this enzyme over the past decade to promote its application in the leather industry. Huang *et al*. [19] purified AprE from BA06 and showed that the purified protease as well as the fermentation broth exhibited high dehairing activity. A protein engineering strategy has also been employed on AprE to ameliorate the enzyme's properties to meet the needs of industrial applications. AprE mutants with altered thermostability, cold adaptability, substrate specificity, or improved activity have been obtained and characterized [20]. Moreover, researchers from other groups have also studied alkaline proteases from different strains of *B. pumilus* and revealed their potential uses for dehairing, laundry detergent, and animal feed processing [17, 21, 22, 53].

Proteases Epr and Vpr, encoded by the *epr* and *vpr* genes, respectively, are minor extracellular proteases. Epr is involved in *Bacillus* swarming motility by providing essential signals required for swarming [15]. Vpr has been confirmed to have the activity of a fibrinolytic enzyme [23], as well as activities that degrade raw feathers and proteins [5, 11]. Vpr secretion is 5.3-fold greater under alkaline stress [54]. A *B. subtilis* mutant lacking three proteases (AprE, Epr, and Vpr) shows a significant defect in competence and sporulation factor production, suggesting that these three proteins are involved in this production [29]. Protease WprA, a cell wall-associated protease, is thought to act as an important quality control factor in the protein secretory pathway by degrading misfolded secretory proteins in the cell wall [45]. It has been reported that *wprA* gene disruption in *B. subtilis* strongly enhances the production and extracellular stability of a foreign protein, staphylokinase [31].

In general, "extracellular proteases" of *Bacillus* refers mainly to the eight proteases mentioned above. However, according to protease nomenclature, another eight peptidases in the secretome could also be classified as proteases in a sense, although they were functionally categorized into different groups in Table 1.

As the protein sample for LC-MS/MS analysis was first digested by trypsin to generate varying peptide lengths, proteins having high protein content will occupy a larger proportion of the peptides and generate a more unique spectrum in the following LC-MS/MS analysis. Thus, the protein content could be roughly represented by its unique spectrum number. Based on this view, the relative contents of the 100 secreted proteins were analyzed. Six proteins had unique spectrum numbers of >100, 13 proteins at 50–100, 30 at 10–50, and the remaining 51 proteins at <10. The relative contents of 12 proteases/peptidases are shown in Fig. 4. The C40 family peptidases, WprA and Vpr, represented the most extensively secreted proteases. In addition, the top 10 most abundant proteins in the extracellular samples are also shown in Fig. 4, which will be discussed below.

Protein Metabolism-Related Proteins

Four proteins related to protein metabolic processes were identified, and their functions mainly involved protein absorption, synthesis, and degradation, which were different from the protease functions mentioned above. Two peptidases, the S1 family glutamyl endopeptidase and

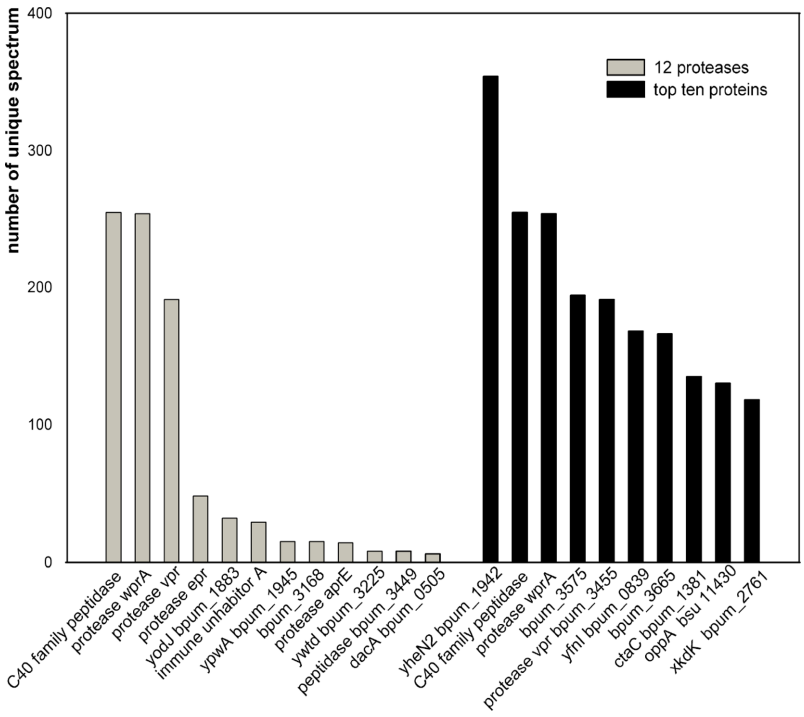

Fig. 4. Relative content of proteases and the top ten extracellular proteins.

M14 family carboxypeptidase, have exopeptidase activity. Gamma-glutamyltransferase plays an important role in metabolism of extracellular reduced glutathione. In addition, gamma-glutamyltransferase might be related to the generation of reactive oxygen species in the presence of iron or other transition metals [37].

The oligopeptide transporter system is a complex consisting of five components (OppA, B, C, D, and F) of which only OppA was identified in this study. OppA, located periplasmically, binds and delivers substrate to the transmembrane core of the transporter, which in turn determines the specificity of this system [9]. Furthermore, OppA plays an important role in solute and nutrient uptake, biofilm formation, sporulation, cell wall muropeptide recycling, and a variety of other physiological functions [9].

Carbohydrate Metabolism-Related Proteins

Seven proteins related to carbohydrate metabolic processes were identified in the present secreted proteome. Glycoside hydrolase YbbD is related to nutrient absorption both intra- and extracellularly. The protein PdaC possesses GlcNAc and MurNAc deacetylase activites and is involved in peptidoglycan deacetylation metabolism, which is a major cell wall modification necessary for bacteria to adapt to various environmental conditions [26]. Pectate lyase has been applied in removing noncellulosic "impurities" from raw cotton to make the surface more hydrophilic. Klug-Santner *et al.* [25] purified a pectate lyase from *B. pumilus* BK2 that can be used for bioscouring cotton fabric. Protein Pme (pectinesterase) is a carboxylic acid esterase and belongs to the hydrolase enzyme group. De-esterification processes of the galacturonan backbone of pectic substances are catalyzed by Pme, rendering this enzyme useful in various industrial applications [27].

LytD plays an important role in cell wall recycling and provides bacterial resistance to a wide variety of β-lactam antibiotics. In addition, LytD catalyzes in vitro the formation of new glycosidic bonds, an ability that has been used for synthetic purposes. These findings implied that the pectate lyase, Pme, as well as LytD of *B. pumilus* SCU11 are worthy of further study to broaden the industrial applications of this strain.

Nucleotide Metabolism-Related Proteins

Three proteins related to nucleotide metabolism processes were identified, including ribonuclease precursor, endonuclease (YhcR), and nucleotide phosphodiesterase (YfkN). YhcR degrades nucleic acid, which would be useful in the food industry [3]. YfkN plays a role in cellular reprocessing of nucleotide phosphates in the medium. A

study has demonstrated that the sorting sequence of YhcR is specifically used to display heterologous proteins on the *Bacillus* cell wall [35].

Cell Wall/Envelope Metabolism-Related Proteins

Ten proteins related to cell wall metabolism were detected, with five of them involved in digesting or degrading cell wall components. YocH is used by *B. subtilis* to digest peptidoglycan released by other cells in the milieu [42]. The *ywtD* gene codes for an enzyme that degrades polyglutamic acid [46]. The bacterial C-terminal processing peptidase CtpA has been shown to be important for degradation of incorrectly synthesized proteins [40]. CtpA of *Staphylococcus aureus* increases bacterial sensitivity to heat shock and also plays a critical role during infection [4].

The other five proteins participate in cell wall formation or regulation-related processes. In gram-positive bacteria, cell wall peptidoglycan is essential for maintenance of cellular viability and shape determination. DacA is a bacterial enzyme that crosslinks peptidoglycan chains to form mature cell wall peptidoglycan, and DacA secretion was slightly increased under alkaline stress [54]. Lipoteichoic acid is a major cell wall constituent in gram-positive bacteria and required for bacterial growth and cell division [14]. *Bacillus* secretes several murein hydrolases during vegetative growth, and three of them, LytC, LytE, and LytF, were detected in the present proteome. These enzymes participate in many cell wall-related processes, such as regulation of cell wall growth, peptidoglycan turnover during growth, daughter cells separation during cell division, and autolysis [49].

Proteins Associated with Inorganic Ion Transport

The present data also demonstrated that 16 proteins are associated with inorganic ion transport. The ATP-binding Cassette Family, also known as the ABC superfamily, is the largest transporter family with hundreds of different transmembrane transport proteins, which can utilize ATP to transport a specific substrate or group of substrates across cell membranes. Bacterial ABC transporters are not only essential for many cellular processes but also play an important role in drug resistance [10, 40]. Transporters identified in the *B. pumilus* secretome were involved in the import and export of sugars, L-cystine, oligopeptide, amino acids, and iron.

Proteins Related to General Functions

Fourteen proteins were found to be related to general functions, mainly in general metabolic processes. Two of them, YvgO and immune inhibitor A, were notable for their antibacterial activity. YvgO is a recently characterized antifungal protein that exhibits an enduring antifungal activity under wide pH and temperature ranges [32]. With further study, YvgO might have potential uses as a prophylaxis for food safety and preservation, or even as a medicament against opportunistic fungal pathogens [33].

M6 family metalloendopeptidase, particularly immune inhibitor A from *B. thuringiensis*, infects insects by cutting antibacterial humoral proteins that are produced by their immune system [39]. Molina *et al*. [34] have screened *B. pumilus* and obtained a strain that is highly toxic to Mediterranean fruit fly (medfly) larvae. Whether *B. pumilus* SCU11 has the potential to be applied in biological control applications requires further study.

Secretome Differences between *B. pumilus* and *B. subtilis*

B. subtilis and *B. pumilus* share the same genetic background and occupy similar ecological niches [12] but differences between the two species still remain unclear in numerous areas. As a model organism, the proteome and extracellular proteome of *B. subtilis* have been studied comprehensively. In this study, the extracellular proteomes of *B. subtilis* 168 [47] and of *B. pumilus* Jo2 and SCU11 were compared to determine differences between *B. pumilus* and *B. subtilis*. Each strain was cultivated under similar conditions, grown in rich medium, and harvested at the late-exponential or early-stationary phase. The extracellular proteomes of *B. subtilis* 168 and *B. pumilus* Jo2 were analyzed by 2-DE/MS, whereas an LC-MS/MS-based shotgun analysis was applied to *B. pumilus* SCU11.

There were 90, 86, and 100 secreted proteins experimentally identified in *B. subtilis* 168, and *B. pumilus* Jo2 and SCU11, respectively. Comparison of these proteins showed that 14 were identified in all three strains (labeled [a] in Tables 1 and S2), 18 were (labeled [b]) detected only in *B. subtilis* and *B. pumilus* SCU11, 3 were confined to *B. subtilis* 168 and *B. pumilus* Jo2, and 21 proteins (labeled [c]) were restricted to *B. pumilus* SCU11 and Jo2. Further analysis was carried out on the 68 proteins indentified in *B. pumilus* SCU11 but absent from *B. subtilis* 168. The results showed that 47 possessed high homology with the *B. subtilis* genome (labeled [d]), whereas the other 21 proteins showed low homology (<30%, labeled [e]), which implied these latter proteins might be peculiar to *B. pumilus*. These differences, whether between *Bacillus* species or between strains of the same species, might have derived from the different methods used for sample collection, preparation, and protein identification, as well as the differences between

their genomes. Further investigation of these proteins will be very helpful for understanding the differences between *B. subtilis* and *B. pumilus* in their adaption to environment changes, such as in nutrient absorption and stress responses [12].

Intracellular Proteins Detected in Extracellular Medium

In addition to secreted proteins, it is also important to note intracellular proteins appearing in the culture supernatant. Of the 513 identified extracellular proteins, 413 were classified as intracellular because of their lack of signal peptides. Even so, it is also possible that some of these proteins were secreted through an atypical signal peptide or an alternative, yet unrecognized pathway.

The GO category of the 413 intracellular proteins showed that they were mainly related to cell, cell part, binding, catalytic, general cellular process, and metabolic processes, which was similar to the 220 predicted and 100 experimentally identified secretory proteins. However, several functions, including envelope, extracellular region parts, transcription regulator, and multicellular organismal processes, were only detected in the 413 intracellular proteins, suggesting that the proteins involved in these metabolic processes/pathways were mainly originally located intracellularly.

The relative proportions of the 513 identified proteins were also represented by their unique spectrum numbers in LC-MS/MS analysis. Among the top 10 most abundant extracellular proteins (Fig. 4), six belonged to secretory proteins. Consequently, although the numbers of intracellular proteins were greater than secreted proteins in the supernatant, the amount of secreted proteins still represented the major proportion. The most abundant protein identified in the supernatant was protein YheN2, an intracellular polysaccharide deacetylase that catalyzes the hydrolysis of *N*-acetyl-D-galactosaminoglycan. Three other highly abundant intracellular proteins, YfnI, CtaC, and Xkdk, might also have been involved in some vital processes. Further investigations focusing on these proteins are needed.

In conclusion, the results presented here provided a comprehensive picture of the *B. pumilus* SCU11 extracellular proteome. The strain's secretory systems were predicted by homologous alignment. A total of 513 proteins were identified by LC-MS/MS in its extracellular proteome at the late-exponential phase supernatant, and 100 of them were actual secreted proteins. A comprehensive illumination of the composition and function of the secreted proteome was provided, which revealed that some proteins, in addition to protease AprE, have potential application in different industrial fields. These results provide valuable theoretical data for the exploitation of enzyme products, comparative proteomics study, and genetic modification of the strain.

The results also revealed that some secreted proteins with no assigned functions might be peculiar to *B. pumilus*. Further studies are needed to investigate the functions of these proteins and to elucidate proteomic differences between *B. pumilus* and other *Bacillus* spp. As the extracellular proteome can be considered as an essential component of the bacterial cells, the study of these proteins will also help to clarify distinctive aspects of *B. pumilus* and its reciprocity with the environment, such as colonization of the host, cell survival, and protection against stresses.

Acknowledgments

This project was supported by the National Natural Science Foundation of China (31070071) and the National High Technology Research and Development Program of China ("863" Program) (2012AA022204).

References

1. Antelmann H, Van Dijl JM, Bron S, Hecker M. 2006. Proteomic survey through secretome of *Bacillus subtilis*. *Methods Biochem. Anal.* **49:** 179.
2. Bottone EJ. 2003. Production by *Bacillus pumilus* (MSH) of an antifungal compound that is active against Mucoraceae and *Aspergillus* species: preliminary report. *J. Med. Microbiol.* **52:** 69-74.
3. Britton RA, Eichenberger P, Gonzalez-Pastor JE, Fawcett P, Monson R, Losick R, Grossman AD. 2002. Genome-wide analysis of the stationary-phase sigma factor (sigma-H) regulon of *Bacillus subtilis*. *J. Bacteriol.* **184:** 4881-4890.
4. Carroll RK, Rivera FE, Cavaco CK, Johnson GM, Martin D, Shaw LN. 2014. The lone S41 family C-terminal processing protease in *Staphylococcus aureus* is localized to the cell wall and contributes to virulence. *Microbiology* **160:** 1737-1748.
5. Choi N-S, Chung D-M, Park C-S, Ahn K-H, Kim JS, Song JJ, *et al.* 2010. Expression and identification of a minor extracellular fibrinolytic enzyme (Vpr) from *Bacillus subtilis* KCTC 3014. *Biotechnol. Bioprocess Eng.* **15:** 446-452.
6. Conesa A, Gotz S, Garcia-Gomez JM, Terol J, Talon M, Robles M. 2005. Blast2GO: a universal tool for annotation, visualization and analysis in functional genomics research. *Bioinformatics* **21:** 3674-3676.
7. El-Refai H, Abdel-Naby M, Gaballa A, El-Araby M, Fattah AA. 2005. Improvement of the newly isolated *Bacillus pumilus* FH9 keratinolytic activity. *Process Biochem.* **40:** 2325-2332.

8. Fu LL, Xu ZR, Li WF, Shuai JB, Lu P, Hu CX. 2007. Protein secretion pathways in *Bacillus subtilis*: implication for optimization of heterologous protein secretion. *Biotechnol. Adv.* **25:** 1-12.
9. Gao J, Li X, Feng Y, Zhang B, Miao S, Wang L, Wang N. 2012. Purification and crystallization of the ABC-type transport substrate-binding protein OppA from *Thermoanaerobacter tengcongensis*. *Biochem. Biophys. Res. Commun.* **423:** 45-49.
10. Garmory HS, Titball RW. 2004. ATP-binding cassette transporters are targets for the development of antibacterial vaccines and therapies. *Infect. Immun.* **72:** 6757-6763.
11. Ghosh A, Chakrabarti K, Chattopadhyay D. 2008. Degradation of raw feather by a novel high molecular weight extracellular protease from newly isolated *Bacillus cereus* DCUW. *J. Ind. Microbiol. Biotechnol.* **35:** 825-834.
12. Gohar M, Gilois N, Graveline R, Garreau C, Sanchis V, Lereclus D. 2005. A comparative study of *Bacillus cereus, Bacillus thuringiensis* and *Bacillus anthracis* extracellular proteomes. *Proteomics* **5:** 3696-3711.
13. Greenbaum D, Luscombe NM, Jansen R, Qian J, Gerstein M. 2001. Interrelating different types of genomic data, from proteome to secretome: 'oming in on function. *Genome Res.* **11:** 1463-1468.
14. Grundling A, Schneewind O. 2007. Synthesis of glycerol phosphate lipoteichoic acid in *Staphylococcus aureus*. *Proc. Natl. Acad. Sci. USA* **104:** 8478-8483.
15. Gupta M, Rao KK. 2009. Epr plays a key role in DegU-mediated swarming motility of *Bacillus subtilis*. *FEMS Microbiol. Lett.* **295:** 187-194.
16. Gupta R, Beg QK, Lorenz P. 2002. Bacterial alkaline proteases: molecular approaches and industrial applications. *Appl. Microbiol. Biotechnol.* **59:** 15-32.
17. Gupta RK, Prasad D, Sathesh J, Naidu RB, Kamini NR, Palanivel S, Gowthaman MK. 2012. Scale-up of an alkaline protease from *Bacillus pumilus* MTCC 7514 utilizing fish meal as a sole source of nutrients. *J. Microbiol. Biotechnol.* **22:** 1230-1236.
18. Handtke S, Volland S, Methling K, Albrecht D, Becher D, Nehls J, *et al.* 2014. Cell physiology of the biotechnological relevant bacterium *Bacillus pumilus* — an omics-based approach. *J. Biotechnol.* **192:** 204-214.
19. Huang Q, Peng Y, Li X, Wang H, Zhang Y. 2003. Purification and characterization of an extracellular alkaline serine protease with dehairing function from *Bacillus pumilus*. *Curr. Microbiol.* **46:** 169-173.
20. Huang R, Yang Q, Feng H. 2015. Single amino acid mutation alters thermostability of the alkaline protease from *Bacillus pumilus*: thermodynamics and temperature dependence. *Acta Biochim. Biophs. Sin.* **47:** 98-105.
21. Jaouadi NZ, Jaouadi B, Hlima HB, Rekik H, Belhoul M, Hmidi M, *et al.* 2014. Probing the crucial role of Leu31 and Thr33 of the *Bacillus pumilus* CBS alkaline protease in substrate recognition and enzymatic depilation of animal hide. *PLoS One* **9:** e108367.
22. Jayakumar R, Jayashree S, Annapurna B, Seshadri S. 2012. Characterization of thermostable serine alkaline protease from an alkaliphilic strain *Bacillus pumilus* MCAS8 and its applications. *Appl. Biochem. Biotechnol.* **168:** 1849-1866.
23. Kho CW, Park SG, Cho S, Lee DH, Myung PK, Park BC. 2005. Confirmation of Vpr as a fibrinolytic enzyme present in extracellular proteins of *Bacillus subtilis*. *Protein Expr. Purif.* **39:** 1-7.
24. Kim HK, Choi HJ, Kim MH, Sohn CB, Oh TK. 2002. Expression and characterization of Ca^{2+}-independent lipase from *Bacillus pumilus* B26. *Biochim. Biophys. Acta* **1583:** 205-212.
25. Klug-Santner BG, Schnitzhofer W, Vrsanska M, Weber J, Agrawal PB, Nierstrasz VA, Guebitz GM. 2006. Purification and characterization of a new bioscouring pectate lyase from *Bacillus pumilus* BK2. *J. Biotechnol.* **121:** 390-401.
26. Kobayashi K, Sudiarta IP, Kodama T, Fukushima T, Ara K, Ozaki K, Sekiguchi J. 2012. Identification and characterization of a novel polysaccharide deacetylase C (PdaC) from *Bacillus subtilis*. *J. Biol. Chem.* **287:** 9765-9776.
27. Kuhad RC, Singh A. 2013. *Biotechnology for Environmental Management and Resource Recovery*. Springer, Berlin/Heidberg.
28. Kunst F, Ogasawara N, Moszer I, Albertini A, Alloni G, Azevedo V, *et al.* 1997. The complete genome sequence of the gram-positive bacterium *Bacillus subtilis*. *Nature* **390:** 249-256.
29. Lanigan-Gerdes S, Dooley AN, Faull KF, Lazazzera BA. 2007. Identification of subtilisin, Epr and Vpr as enzymes that produce CSF, an extracellular signalling peptide of *Bacillus subtilis*. *Mol. Microbiol.* **65:** 1321-1333.
30. Lechat P, Hummel L, Rousseau S, Moszer I. 2007. GenoList: an integrated environment for comparative analysis of microbial genomes. *Nucleic Acids Res.* **36:** D469-D474.
31. Lee SJ, Kim DM, Bae KH, Byun SM, Chung JH. 2000. Enhancement of secretion and extracellular stability of staphylokinase in *Bacillus subtilis* by *wprA* gene disruption. *Appl. Environ. Microbiol.* **66:** 476-480.
32. Manns DC, Churey JJ, Worobo RW. 2012. Functional assignment of YvgO, a novel set of purified and chemically characterized proteinaceous antifungal variants produced by *Bacillus thuringiensis* SF361. *Appl. Environ. Microbiol.* **78:** 2543-2552.
33. Manns DC, Churey JJ, Worobo RW. 2014. Nutrient-dependent efficacy of the antifungal protein YvgO correlates to cellular proliferation rate in *Candida albicans* 3153A and *Byssochlamys fulva* H25. *Probiotics Antimicrob. Proteins* **6:** 198-207.
34. Molina CA, Cana-Roca JF, Osuna A, Vilchez S. 2010. Selection of a *Bacillus pumilus* strain highly active against *Ceratitis capitata* (Wiedemann) larvae. *Appl. Environ. Microbiol.* **76:** 1320-1327.
35. Nguyen HD, Phan T, Schumann W. 2011. Analysis and application of *Bacillus subtilis* sortases to anchor recombinant proteins on the cell wall. *AMB Express* **1:** 22.
36. Page MJ, Di Cera E. 2008. Serine peptidases: classification,

structure and function. *Cell. Mol. Life Sci.* **65:** 1220-1236.
37. Paolicchi A, Dominici S, Pieri L, Maellaro E, Pompella A. 2002. Glutathione catabolism as a signaling mechanism. *Biochem. Pharmacol.* **64:** 1027-1035.
38. Poorna CA, Prema P. 2006. Production and partial characterization of endoxylanase by *Bacillus pumilus* using agro industrial residues. *Biochem. Eng. J.* **32:** 106-112.
39. Rawlings ND, Barrett AJ. 1993. Evolutionary families of peptidases. *Biochem. J.* **290:** 205-218.
40. Rawlings ND, Waller M, Barrett AJ, Bateman A. 2014. MEROPS: the database of proteolytic enzymes, their substrates and inhibitors. *Nucleic Acids Res.* **42:** D503-D509.
41. Schallmey M, Singh A, Ward OP. 2004. Developments in the use of *Bacillus* species for industrial production. *Can. J. Microbiol.* **50:** 1-17.
42. Shah IM, Dworkin J. 2010. Induction and regulation of a secreted peptidoglycan hydrolase by a membrane Ser/Thr kinase that detects muropeptides. *Mol. Microbiol.* **75:** 1232-1243.
43. Shao H, Cao Q, Zhao H, Tan X, Feng H. 2015. Construction of novel shuttle expression vectors for gene expression in *Bacillus subtilis* and *Bacillus pumilus*. *J. Gen. Appl. Microbiol.* **61:** 124-131.
44. Sibbald MJ, Ziebandt AK, Engelmann S, Hecker M, de Jong A, Harmsen HJ, *et al.* 2006. Mapping the pathways to staphylococcal pathogenesis by comparative secretomics. *Microbiol. Mol. Biol. Rev.* **70:** 755-788.
45. Stephenson K, Jensen C, Jørgensen S, Harwood C. 2002. Simultaneous inactivation of the *wprA* and *dltB* genes of *Bacillus subtilis* reduces the yield of α-amylase. *Lett. Appl. Microbiol.* **34:** 394-397.
46. Suzuki T, Tahara Y. 2003. Characterization of the *Bacillus subtilis ywtD* gene, whose product is involved in gamma-polyglutamic acid degradation. *J. Bacteriol.* **185:** 2379-2382.
47. Tjalsma H, Antelmann H, Jongbloed JD, Braun PG, Darmon E, Dorenbos R, *et al.* 2004. Proteomics of protein secretion by *Bacillus subtilis*: separating the "secrets" of the secretome. *Microbiol. Mol. Biol. Rev.* **68:** 207-233.
48. Tjalsma H, Bolhuis A, Jongbloed JD, Bron S, van Dijl JM. 2000. Signal peptide-dependent protein transport in *Bacillus subtilis*: a genome-based survey of the secretome. *Microbiol. Mol. Biol. Rev.* **64:** 515-547.
49. Vollmer W, Joris B, Charlier P, Foster S. 2008. Bacterial peptidoglycan (murein) hydrolases. *FEMS Microbiol. Rev.* **32:** 259-286.
50. Wang HY, Liu DM, Liu Y, Cheng CF, Ma QY, Huang Q, Zhang YZ. 2007. Screening and mutagenesis of a novel *Bacillus pumilus* strain producing alkaline protease for dehairing. *Lett. Appl. Microbiol.* **44:** 1-6.
51. Westers L, Westers H, Quax WJ. 2004. *Bacillus subtilis* as cell factory for pharmaceutical proteins: a biotechnological approach to optimize the host organism. *Biochim. Biophys. Acta* **1694:** 299-310.
52. Wu S, Zhu Z, Fu L, Niu B, Li W. 2011. WebMGA: a customizable Web server for fast metagenomic sequence analysis. *BMC Genomics* **12:** 444.
53. Yao D, Qu J, Chang P, Tao Y, Yang D. 2012. Production and characterization of alkaline protease from hemoglobin-degrading *Bacillus pumilus* NJM4 to produce fermented blood meal. *J. Food Sci. Technol.* **49:** 626-631.
54. Yu SQ, Gui JH, Wang HY. 2014. Proteome analysis of *Bacillus pumilus* extracellular proteins by two dimensional electrophoresis and identification of differential proteins under alkaline stress. *Chin. J. Appl. Environ. Biol.* **2014:** 217-222.
55. Zhao C-W, Wang H-Y, Zhang Y-Z, Feng H. 2012. Draft genome sequence of *Bacillus pumilus* BA06, a producer of alkaline serine protease with leather-dehairing function. *J. Bacteriol.* **194:** 6668-6669.
56. Zhou M, Theunissen D, Wels M, Siezen RJ. 2010. LAB-secretome: a genome-scale comparative analysis of the predicted extracellular and surface-associated proteins of lactic acid bacteria. *BMC Genomics* **11:** 651.