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Original Article

Comparative Study of Extracellular Proteomes for *Bacillus subtilis* and *Bacillus amyloliquefaciens*

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Abstract

Bacillus subtilis and *Bacillus amyloliquefaciens* are closely related species that share a similar genomic background, and are both known to secrete large amounts of proteins directly into a medium. The extracellular proteomes of two strains of *Bacillus subtilis* and two strains of *Bacillus amyloliquefaciens* were compared by 2-D gel electrophoresis during the late exponential growth phase. The relative abundance of some minor protein spots varied among the four strains of *Bacillus*. Over 123 spots of extracellular proteins were visualized on the gel for *B. subtilis* CH 97, 68 spots for *B. subtilis* 3-5, 230 spots for *B. amyloliquefaciens* CH 51, and 60 spotsfor *B. amyloliquefaciens* 86-1. 2D gel electrophoresis images of the four *Bacillus* strains showed significantly different protein profiles. Consistent with the 2D gel electrophoretic analysis, most of the *B. subtilis* proteins differed from the proteases secreted by the *B. amyloliquefaciensstrains*. Among the proteins identified from *B. subtilis*, approximately 50% were cytoplasmic and 30% were canonically extracellular proteins. The secreted protein profiles for *B. subtilis* CH 97 and *B. subtilis* 3-5 were quite different, as were the profiles for *B. amyloliquefaciens* CH 51 and 86-1. The four proteomes also differed in the major protein composition. The *B. subtilis* CH 97 and *B. amyloliquefaciens* CH 51 proteomes both contained large amounts of secreted hydrolytic enzymes. Among the four strains, *B. subtilis* 3-5 secreted the least number of proteins. Therefore, even closely related bacteria in terms of genomic sequences can still have significant differences in their physiology and proteome layout.

Keywords : Bacillus, Secreted proteome, MALDI-TOF, 2-D gel

Introduction

Bacillus subtilisis regarded as a representative model organism of Gram positive bacteria, and its genome sequence, published in 1997 by Kunst et al. provides its “blue-print of life”. Plus, due to the availability of its genome sequence and advent of recent technologies, this organism is also regarded as a model of functional genomics (transcriptomics, proteomics, metabolomics etc.). The growing field of “omics” has also broughtthe genome sequence to cell physiology.

While about 4100 different open reading frames (ORFs) have already been predicted to express proteins from the genome of *Bacillus subtilis,* the genome sequence information has extended the proteomic analysis of *Bacillus* in various areas. Moreover, the advances and availability of technology for identifying proteomes, such as high-resolution 2-DE with a high reproducibility, high-throughput mass spectrometry with a high sensitivity, and efficient database searching techniques and software using sophisticated bioinformatics algorithms, have resulted in significant progress in functional proteomics.

The ability of *Bacillus* cells to secrete large amounts of proteins has long been of interest to the fermentation industry. Although *Bacillus amyloliquefaciens* and *Bacillus* subtilis are closely related in terms of their genome sequences, there are significant differences between these two species as regards their growth characteristics and secreted protein profiles.

Accordingly, this study compared the extracellular proteins of *Bacillus subtilis* and *Bacillus amyloliquefaciens* during the late exponential growth phase using their respective protein profile patterns. The proteomic view of these extracellular proteins can provide a comprehensive understanding of metabolism and growth processes, while the secreted enzymes offer practical industrial applications, such as fermentation monitoring and developing fermentation products. Among the 4100 proteins expressed in *B. subtilis*, the number of extracellular proteins

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is not yet known or how the secretion machinery proteins facilitate their extracellular secretion. Therefore, this study identified the secretory proteins separated on 2-D gels to demonstrate the secretory protein profiles from *B. subtilis* and *B. amyloliquefaciens* and compare the functional proteomes quantitatively and qualitatively. Hence, the secreted proteins were identified using a proteomic approach based on two-dimensional gel electrophoresis and a peptide mass fingerprinting analysis using matrix assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectrometry.

Materials and Methods

Strains and culture conditions

Four strains of *Bacillus*, namely *Bacillus subtilis* CH97, *Bacillus subtilis* 3-5, *Bacillus amyloliquefaciens* CH51, and *Bacillus amyloliquefaciens* 86-1, were each grown in 500 mL of a Luria-Bertani (LB) broth in 2 L Erlenmeyer flasks agitated at 200 rpm at 42℃, and taken after 12 and 24 h.

Extracellular sample preparation for proteome analysis

The supernatant fractions from each strain were collected after high-speed centrifugation of the cultures at 10000 rpm for 20 min. The supernatants were then dialyzed at 4℃ with 4 buffer changes over 36 h, lyophilized to powders, dissolved in 0.1X PBS with protease inhibitors, and desalted using a PD-10 column (GE Healthcare, Uppsala, Switzerland). The proteins in the void volume from the desalting column were precipitated using 10% tri-chloroacetic acid. Thereafter, the precipitated proteins were washed with 100% ice-cold ethanol, washed with 70% ethanol, and dried under a vacuum. Finally, the dried proteins were dissolved in a rehydration buffer containing 7 M urea, 2 M thiourea, 4% CHAPS, 0.1% dithiothreitol, and 0.2% ampholyte with a pH range of 3 to 10.

2D protein gel electrophoresis

Two-dimensional (2-D) sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was conducted to separate the proteins. First, dimensional isoelectric focusing was carried out using the passive-loading method with an immobilized pH gradient (IPG) strip at pH 4-7 (Amersham Biosciences, Freiburg, Germany). The IPG strips were initially equilibrated in an equilibration buffer containing 6M urea, 2% SDS, 0.375 M Tris-HCl, pH 8.8, 20% glycerol, and 130 mM dithiothreitol for the first equilibration and then in an equilibration buffer containing 135 mM iodoacetamide. Next, the strips were overlaid on a 12% separating gel for the2-D SDS-PAGE. The resulting 2-D gels were fixed with 40% v/v ethanol and 10% v/v acidic acid for 1-2 h and then stained with colloidal CBB (Amersham Biosciences, Freiburg, USA),

Preparation of peptide mixtures for MALDI-MS

All the visible spots were excised from the gel, destained by washing three times in deionized water and two times in 50 mM NH_4HCO_3 and acetonitrile (60:40) with gentle shaking, and freeze-dried. The gel pieces were then digested for 18 h using 10 ng trypsin/μL of 50 mM NH_4HCO_3 and the peptides extracted using 0.1% trifluoroacetic acid in 50 mM NH_4HCO_3.

Analysis of peptides by MALDI-TOF MS and identification of proteins

The MALDI-TOF measurement of the spotted peptide solutions was carried out using a Voyager DE-STR MALDI Biospectrometry Workstation Proteome-Analyzer 4700 (Applied Biosystems, USA). The spectra were recorded in the reflector mode within a mass range from 800 to 3500 Da. The peak lists were created using the "peak to mascot" script of the 4700 Explorer™ Software and calibrated using *M-over-Z* (Genomic Solutions, USA). The database searches used the search engines Ms-Fit (http://prospector.ucsf.edu) and MASCOT (Matrix Science).

Results and Discussion

Comparison of extracellular proteomes for *B. subtilis* CH97 and *B. subtilis* 3-5

Several studies have already shown that the highest levels of protein secretion are usually during the late exponential phase until the onset of the stationary phase. Therefore, in this set of experiments, the *B. subtilis* cells were grown in an LB medium and harvested during the late exponential phase. The extra-cellular proteins were precipitated, subjected to 2D gel electrophoresis at pH 4-7, and visualized with colloidal CBB staining. Based on the theoretical proteome map of all *B. subtilis* proteins (Buttner et al. 2001), the proteins were separated using a single 2-DE gel with a pH range of 4–7. Thereafter, an analysis of the 2-DE images and peptide mass fingerprinting allowed the identification of 101 proteins from approximately 123 spots that were visualized on the gel of *B. subtilis* CH97 (Figure 2A) and about 100 spots visualized on the gel of *B. subtilis* 3-5 (Figure 2B).

The growth of *B. subtilis* 3-5 was faster than that of *B. subtilis* CH97, as the *B. subtilis* 3-5 strain reached the stationary phase after 21 h, whereas the *B. subtilis* CH97 strain was still in the late exponential phase after 24 h (Figure 1A), which may

explain the higher number of detected spots for the latter strain. Notwithstanding, both strains produced extracellular proteins that were identified as cytosolic proteins.

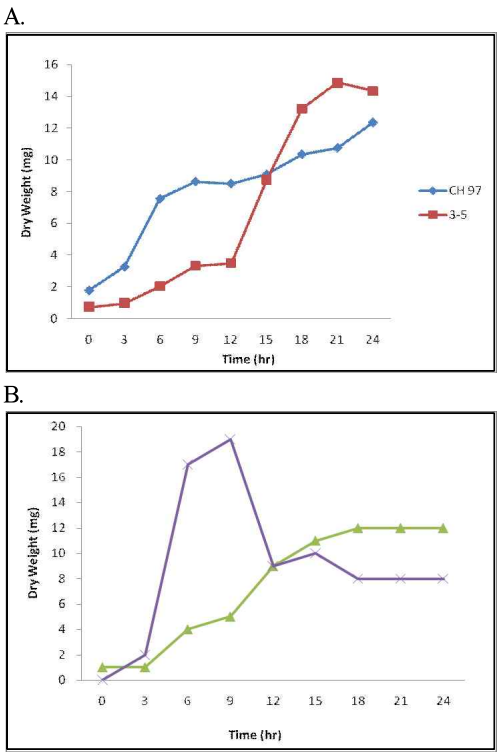

Figure 1. Growth curves for *B. subtilis* (A) and *B. amyloliquefaciens* (B). *B. subtilis* CH 97 (■), *B. subtilis* 3-5 (■), *B. amyloliquefaciens* CH 51 (▲), and *B. amyloliquefaciens* 86-1 (x) were grown in LB medium at 42℃.

Comparison of extracellular proteomes for *B. amyloliquefaciens* CH51 and *B. amyloliquefaciens* 86-1

An analysis of the 2-DE images and peptide mass fingerprinting allowed the identification of 35 different proteins from the approximately 100 spots that were visualized on the gel of *B. amyloliquefaciens* 86-1 (Figure 3B) and about 200 spots visualized on the gel of *B. amyloliquefaciens* CH51 (Figure 3A). The growth of *B. amyloliquefaciens* 86-1 was faster than that of *B. amyloliquefaciens* CH51, as the *B. amyloliquefaciens* 86-1 strain reached the stationary phase after 12 h, whereas it took 18 h for the *B. amyloliquefaciens* CH51 strain (Figure 1B), which may explain the fragility of the first strain and hence the smaller number of detected spots. Overall, both strains secreted enzymes related to carbohydrates and their metabolism, transferases, sporulation-specific proteins, and transcription regulators (Figure 4B). The functions of the other identified proteins remain unknown.

Comparison of extracellular proteomes for *B. subtilis* and *B. amyloliquefaciens*

The availability of the genome sequence for *B. subtilis* in 1997 and *B. amyloliquefaciens* in 2007 has enabled the prediction of all proteins containing signals for known systems of protein secretion. From the genome data, 4,107 ORFs have been identified for *B. subtilis* and 5,224 ORFs for *B. amyloliquefaciens* (Kunst et al. 1997; Chen et al. 2007). Based on the sesequences, 297 proteins have the potential to be translocated extracellularly from the cytoplasm of *B. subtilis* (Tjalsma et al. 2000; van Dijl et al. 2002).

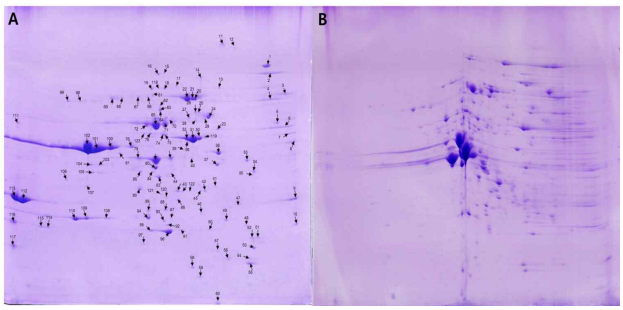

Figure 2. Extracellular proteins from B. subtilis CH 97 (A) and B. subtilis 3-5 (B) profiled on 2D gels. Cells were grown in LB broth at 42℃ and sampled during lateexponential growth phase.

Among the four strains used in this study, *B. subtilis* CH 97 and *B. amyloliquefaciens* CH51 produced the most visible spots on the 2D gel (Figures 2 and 3). Some of the secreted proteins occurred as multiple spots, like serine protease, dihydrolipoamide, and flagellin. Altogether, 100 different proteins were identified from *B. subtilis* and 35 from *B. amyloliquefaciens*. However, not all the spots were successfully identified. In this study, the PMF identification success rate was relatively higher for the larger proteins than for the small proteins. Due to the lower number of peptides after trypsiniztion, identifying low-molecular-weight proteins is difficult using PMF. Thus, increasing the peptide numbers by efficient trypsinization and/or analyzing the proteins using LC-MS/MS is recommended.

As shown in Figures 2 and 3, the protein profiles differed significantly among the four strains. In the case of *B. subtilis*, flagellin (Hag) was the main protein, whereas it was proteases in the case of *B. amyloliquefaciens*. Another major difference in the overall composition of the respective extracellular proteomes was the proteins involved in carbohydrate metabolism, such as chitosanase, xylanase, glucanase, amylase,

Table 1. Identified extracellular proteins from *B. subtilis* CH97

Spot #	MOWSE Score	% cov	MW (Da)/pI	Access #	Protein Name	Location
2	1.07E+10	25.2	68135/5.2	Q52R76	Extracellular protease vpr	Secreted
3	6.88E+06	12.1	95077/6.1	P39143	Transcription activator gutR	Cytoplasm
4	216811	22.4	40817/6.8	P31102	3-dehydroquinate synthase	cytoplasm
5	243535	17	56705/6.7	O31671	Sensor protein	membrane
6	508768	21	35593/6.0	Q45454	Putative uncharacterized protein orf4.60	unknown
7	1.97E+06	19.6	41947/5.1	P42094	3-phytase precursor	secreted
8	8.22E+06	18.2	56522/7.2	P68735	Bacillolysin precursor	secreted
9	1.01E+06	11.9	79279/6.9	P38050	Penicillin-binding protein 1F	membrane
10	2.49E+05	37.1	22575/5.3	P54375	Superoxide dismutase [Mn]	cytoplasm
11	6.18E+06	21.5	43361/6.1	P71007	bacteriocinsubtilosin biosynthesis protein	unknown
12	1.13E+08	25.6	87750/7.9	O31522	YesS protein	cytoplasm
13	8.37E+08	20.4	53658/4.8	O32106	Probable cytosol aminopeptidase	cytoplasm
14	4.29E+06	22.9	48789/5.2	P23630	Diaminopimelate decarboxylase	cytoplasm
15	233078	12.3	69560/6.0	P54523	1-deoxy-D-xylulose-5-phosphate synthase	cytoplasm
16	1.85E+06	11.8	85609/5.9	P29141	Minor extracellular protease vpr precursor	secreted
17	8.08E+09	26.4	77464/5.1	P50849	Polyribonucleotide nucleotidyltransferase	cytoplasm
18	8.53E+05	11	89184/5.2	O34481	YrrC protein	cytoplasm
19	5.55E+07	33.7	56309/5.3	P39773	phosphoglyceratemutase	cytoplasm
20	1.67E+10	33	49733/5.0	P21880	Dihydrolipoyl dehydrogenase	cytoplasm
21	2.79E+16	40.9	49733/5.0	P21880	Dihydrolipoyl dehydrogenase	cytoplasm
22	3.15E+12	44.7	49733/5.0	P21880	Dihydrolipoyl dehydrogenase	cytoplasm
23	5.44E+08	18.8	45510/4.7	P55179	Peptidase T	cytoplasm
24	405264	20.7	45635/5.2	P94415	Response regulator aspartate phosphatase	cytoplasm
25	754702	16.2	51053/4.9	O34944	Putative peptidase	cytoplasm
27	3.47E+06	20.2	45635/5.2	P94415	Response regulator aspartate phosphatase	cytoplasm
28	514942	16	51871/5.0	O32133	YunD protein	unknown
29	1.14E+06	35.6	26239/9.1	O34315	permease protein tcyL	membrane
30	6.52E+10	45.5	41947/5.1	P42094	3-phytase precursor	secreted
31	1.38E+12	35.6	41947/5.1	P42094	3-phytase precursor	secreted
32	1.58E+15	47.4	41947/5.1	P42094	3-phytase precursor	secreted
33	2.36E+05	28.3	35833/6.3	O31589	YhbB protein	unknown
34	511952	23.5	32130/5.3	O31570	Uncharacterized isomeraseyfhB	unknown
35	2.54E+08	13.3	124622/6.3	O31875	Ribonucleoside-diphosphatereductase	cytoplasm
36	78205	19.2	29199/7.7	P46342	Phosphate import ATP-binding protein	membrane
37	1.09E+10	47.4	35475/4.7	P21882	Pyruvate dehydrogenase subunit beta	cytoplasm
38	1.83E+09	50.5	34791/4.8	P39646	Phosphate acetyltransferase	cytoplasm
39	321412	23.2	32031/7.7	P14383	Pyrroline-5-carboxylate reductase 1	cytoplasm
41	5.15E+06	19.6	42969/5.9	O34559	Putative glycosyl hydrolase yteR	cytoplasm

Spot #	MOWSE Score	% cov	MW (Da)/pI	Access #	Protein Name	Location
43	1.05E+06	24.4	47737/5.4	P22326	Tyrosyl-tRNAsynthetase 1	cytoplasm
44	135513	20.1	31811/5.2	P54504	Uncharacterized protein yqhA	cytoplasm
45	95514	22.3	40136/5.6	P80862	Phosphoserine aminotransferase	cytoplasm
46	261640	17	25975/5.7	Q9F4F7	4'-phosphopantetheinyl transferaseffp	cytoplasm
47	97033	29.8	33851/6.6	O06973	UPF0042 protein yvcJ	unknown
48	1.66E+07	15.7	79279/6.9	P38050	Penicillin-binding protein 1F	membrane
49	27662	19	25844/5.7	Q06755	cytidylyltransferase	cytoplasm
50	2.49E+06	40.4	22748/5.6	P54480	Putative nucleotidaseyqfW	cytoplasm
55	9.74E+06	15.5	106033/5.9	P54394	helicase dinG homolog	nucleus
57	8.53E+06	23.2	55473/5.3	P42176	Nitrate reductase beta chain	membrane
58	166917	34.7	16355/4.8	P54332	Phage-like element PBSX protein	unknown
59	4.26E+07	40.7	21824/5.5	P81100	Stress response protein SCP2	cytoplasm
61	56590	20.5	49084/5.3	P39772	Asparaginyl-tRNAsynthetase	cytoplasm
62	812995	22.5	56224/5.7	P37966	Lipoprotein lplA precursor	membrane
63	1.22E+06	30.2	45592/8.3	O32267	glycosyltransferasetuaH	cytoplasm
65	2.52E+06	24.7	72800/5.9	P00691	Alpha-amylase precursor	secreted
66	319835	21.1	91335/6.3	Q45066	DNA topoisomerase 4 subunit A	cytoplasm
67	5.12E+06	22.1	119469/5.8	O08394	P-450/NADPH-P450 reductase	cytoplasm
68	1.06E+07	24.1	99563/4.9	P39793	Penicillin-binding protein 1A/1B	membrane
69	619649	26	101747/5.0	Q05873	Valyl-tRNAsynthetase	cytoplasm
70	5.61E+06	30.4	57061/5.5	P94531	Alpha-N-arabinofuranosidase 1	cytoplasm
71	3.62E+06	26.4	73316/6.0	Q797B3	lipoteichoic acid synthase 1	membrane; secreted
72	429968	24.9	72258/4.9	P46208	Chaperone protein htpG	cytoplasm
73	1.62E+06	35.8	37847/5.1	Q04797	Aspartate-semialdehyde dehydrogenase	cytoplasm
74	1.13E+08	35.2	39992/5.1	P54531	Leucine dehydrogenase	cytoplasm
75	4.42E+06	41.3	38411/5.3	O34499	Uncharacterized protein ykgB	unknown
76	2.54E+07	29.2	56522/7.2	P68735	Bacillolysin precursor	secreted
78	124809	25.6	47212/5.7	P50735	NAD-specific glutamate dehydrogenase	cytoplasm
79	278216	29.3	32627/5.0	P02968	Flagellin	secreted
80	5.10E+06	21.5	34615/5.4	P54327	Phage-like element PBSX protein xkdG	unknown
82	50761	20.5	61838/5.3	P54551	Uncharacterized protein yqjN	cytoplasm
83	1.05E+06	25	59301/4.6	P23447	Flagellar M-ring protein	membrane; flagella
84	45607	23.8	45635/5.2	P94415	Response regulator aspartate phosphatase	cytoplasm
87	20988	34.2	21824/5.5	P81100	Stress response protein SCP2	cytoplasm
90	102427	25.3	24791/5.8	P54601	Uncharacterized protein yhcQ	spore wall
91	3.87E+07	53.5	22575/5.3	P54375	Superoxide dismutase [Mn]	cytoplasm
92	3.87E+07	53.5	22575/5.3	P54375	Superoxide dismutase [Mn]	cytoplasm
93	2.23E+07	45.9	25378/5.1	O34925	Purine nucleoside phosphorylase	cytoplasm
95	137011	33.5	21364/5.9	P54390	UPF0302 protein ypiB	unknown

Spot #	MOWSE Score	% cov	MW (Da)/pI	Access #	Protein Name	Location
96	129609	28.1	25132/7.7	P38491	Uncharacterized protein ypfA	unknown
101	4.09E+07	27.1	56522/7.2	P68735	Bacillolysin precursor	secreted
102	3.77E+07	41.1	56522/7.2	P68735	Bacillolysin precursor	secreted
104	4.32E+08	36.2	79079/9.0	P39814	DNA topoisomerase 1	cytoplasm
105	1.84E+07	27	119469/5.8	O08394	P-450/NADPH-P450 reductase 1	cytoplasm
107	1.24E+07	51.6	33418/6.2	O34385	lipoprotein mntA precursor	membrane
108	4.28E+06	30.1	99528/5.2	Q798L9	YwqA protein	
110	1.44E+06	43	27464/6.7	O31614	Bis(5'-nucleosyl)-tetraphosphatase	Spore
111	1.25E+08	35.7	92100/5.4	P05653	DNA gyrase subunit A	
112	1.68E+06	27.3	39468/9.0	P00783	Subtilisinamylosaccharitic precursor	secreted
113	7.48E+07	36.5	31497/8.7	O07921	Chitosanase precursor	secreted
114	1.54E+06	26.8	77464/5.1	P50849	Polyribonucleotide nucleotidyltransferase	cytoplasm
115	4.13E+06	47.5	17515/5.0	O05396	Uncharacterized protein yrhD	Unknown
116	6.44E+06	35.3	24876/9.6	O05411	Uncharacterized protein yrpD	Unknown
117	6.44E+06	35.3	24876/9.6	O05411	Uncharacterized protein yrpD	Unknown
118	9.13E+06	30.1	45635/5.2	P94415	Response regulator aspartate phosphatase	Cytoplasm
119	4.63E+07	30.9	41947/5.1	P42094	3-phytase precursor	Secreted
120	4.80E+09	59.7	32417/4.9	P70999	Agmatinase	Cytoplasm
123	3.92E+07	11.4	120530/5.9	P39774	Subtilinbiosynthesis protein spaB	Membrane

and enolase. *B. subtilis* secreted a higher number of these carbohydrate-degrading enzymes than *B. amyloliquefaciens*. Conversely, *B. amyloliquefaciens* secreted a higher number of peptidases and proteases.

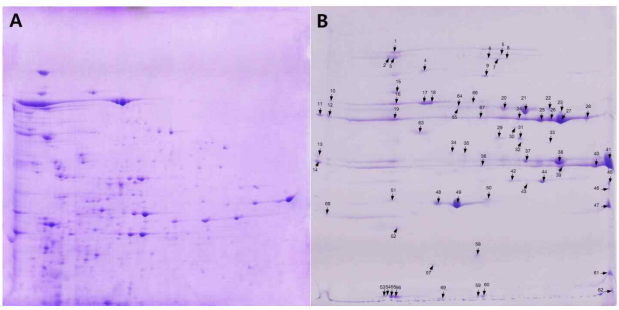

Figure 3. Extracellular proteins from *B. amyloliquefaciens* CH 51 (A) and *B. amyloliquefaciens* 86-1 (B) profiled on 2D gel. Cells were grown in LB Broth at 42℃ and sampled during late exponential growth phase.

Overall, the extracellular proteomesfor *B. subtilis* and *B. amyloliquefaciens* included numerous unpredicted secreted proteins (cytoplasmic, membrane, and flagellar). Hag migrated as the largest spot on the 2D gel of extracellular protein preparations. The secretion of Hag is not seemingly assisted by SRP and Sec translocase in *E. coli* (Hueck, 1998; Namba et al. 1989). The appearance of cytosolic proteins in the extracellular preparations was probably due to breakage of the cells or the leakage of cytoplasmic proteins by an unknown function. The cells of *B. subtilis* 3-5 and *B. amyloliquefaciens* 86-1 were easily lysed, as shown by their shorter growth curves compared to the other two strains (Figures 1 and 3).

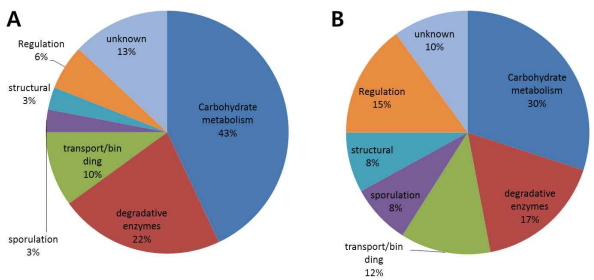

Figure 4. Functional classifications of identified extracellular proteins from *B. subtilis* (A) and *B. amyloliquefaciens* (B).

The 101 identified extracellular proteins from *B. subtilis* included 16 proteins with unknown functions (Table 1).

Table 2. Identified extracellular proteins from *B. amyloliquefaciens* 86-1

Spot #	MOWSE Score	% cov	MW (Da)/pI	Access #	Protein Name	Location
3	289	11	57061/5.5	P94531	Alpha-N-arabinofuranosidase 1	Cytoplasm
6	6382	23.1	49451/6.6	P25152	Uncharacterized peptidase ywaD precursor	Secreted
7	5.55E+06	32.8	49733/5.0	P21880	Dihydrolipoyl dehydrogenase	Cytoplasm
8	235	10.6	57061/5.5	P94531	Alpha-N-arabinofuranosidase 1	Cytoplasm
9	27075	22.4	45510/4.7	P55179	Peptidase T	Secreted
11	407	13.4	22341/5.4	P81102	Putative NAD(P)H nitroreductase 12C	Cytoplasm
12	1896	14.9	50826/7.1	P27622	teichoicacid biosynthesis protein C	Cytoplasm
13	4617	18	34401/6.5	Q45462	Choline-binding protein precursor	Cytoplasm
14	242	17.1	37265/5.1	O31457	UPF0176 protein ybfQ	Unknown
15	113	10.8	33418/6.2	O34385	Manganese-binding lipoprotein	Secreted
16	5799	16.3	34401/6.5	Q45462	Choline-binding protein precursor	Secreted
17	47101	31.7	24831/5.1	Q795U4	UPF0173 metal-dependent hydrolase ytkL	Cytoplasm
18	4546	13.8	96488/9.2	P54423	Cell wall-associated protease	Secreted
19	1260	15	34401/6.5	Q45462	Choline-binding protein precursor	Secreted
20	1043	14.1	71504/5.5	P05652	DNA gyrase subunit B	Cytoplasm
23	848	6.6	71504/5.5	P05652	DNA gyrase subunit B	Cytoplasm
25	11686	19.1	69753/6.3	P25812	5-carboxymethylaminomethyl modification	Cytoplasm
26	323	10	49733/5.0	P21880	Dihydrolipoyl dehydrogenase	Cytoplasm
28	318	11.2	57061/5.5	P94531	Alpha-N-arabinofuranosidase 1	Cytoplasm
27	226	6	85609/5.9	P29141	Minor extracellular protease vpr precursor	Cytoplasm
31	1763	28.6	20948/4.6	O34384	Uncharacterized protein yceE	Cytoplasm
33	1763	28.6	20948/4.6	O34384	Uncharacterized protein yceE	Cytoplasm
35	1437	25.2	22575/5.3	P54375	Superoxide dismutase [Mn]	Cytoplasm
36	137	7.4	63305/5.2	O31755	Prolyl-tRNAsynthetase	Cytoplasm
37	1763	28.6	20948/4.6	O34384	Uncharacterized protein yceE	Cytoplasm
39	1763	28.6	20948/4.6	O34384	Uncharacterized protein yceE	Cytoplasm
40	312	20.8	37265/5.1	O31457	UPF0176 protein ybfQ	Unknown
42	1755	28.6	20948/4.6	O34384	Uncharacterized protein yceE	Cytoplasm
43	217	12.7	41372/5.0	P80886	Succinyl-CoA ligase subunit beta	Cytoplasm
44	166	10.7	78141/7.0	O34942	ATP-dependent DNA helicase recG	Cytoplasm
49	631	10.8	53658/4.8	O32106	Probable cytosol aminopeptidase	Cytoplasm
50	988	12.3	49733/5.0	P21880	Dihydrolipoyl dehydrogenase	Cytoplasm

Interestingly, the identified extracellular proteins from the Bacillus strains in this study included enzymes related to the metabolism of carbohydrates, lipid and amino acids enzymes involved in protein synthesis and folding and the synthesis and decay of nucleic acids, and enzymes involved in transcriptional regulations. This also supports the idea that the proteins in the extracellular media possibly originated from the lysis of the bacterial cells.

The metabolic enzymes identified, such as hydrolase, are known to beinvolved in several pathways for carbohydrate utilization and activated during the fermentation process (Antelmann et al. 2004). Meanwhile, some of the other enzymes play a role

in the translocation machinery of the protein secretion by *B. subtilis*. For example, one of the secreted proteins, an alpha-amylase (AmyE) precursor, is a probable presecretory protein that is recognized by a chaperone and targeted to a Sec protein translocase for transport across the membrane (Hirose et al. 2000). Furthermore, the search also identified sporulation proteins, such as lipoproteins, synthesized as precursors that function as an extracellular folding catalyst in protein folding on the membrane surface (Hecker et al. 2004; Bunai et al. 2004). The increased proteins during the growth phases at 37℃ (Figure 1A) and 42℃ (Figure 1B) showed similar changing patterns, although the growth rate at 42℃ as slightly slower than that at 37℃. The results also contradicted the previous assertion that about 75% of the secreted proteins from Bacillus have N-terminal signal peptides or specific retention signals, while the rest of the secreted proteins are transported by independent signal peptide systems, including holin-transport, flagella-transport, and cell lysis (von Heijne, 1998; Tjalsma et al. 2004). The present results indicated that cytosolic proteins were secreted from the cells throughout the growth stages by an unknown mechanism, cell lysis, or by both mechanisms. Another possibility is that the identified unchanged or increased (Figure 2) secreted proteins are so stable that they remain in the cells (Tullius et al. 2001).

As secretory proteins, such as proteases and metabolic enzymes, can affect the fermentation process, secretory protein profiles can be used to select the proper microorganisms or strains for optimizingfermentation conditions.

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