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Biphenyl- and carvone-induced protein expression patterns in *Rhodococcus* sp. ACS

Jong-Shik Kim

Gyeongbuk Institute for Marine Bioindustry, Uljin, Republic of Korea; jskim@gimb.or.kr

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ABSTRACT

Protein expression patterns in the polychlorinated biphenyl (PCB)-degrading *Rhodococcus* sp. strain ACS were examined following growth on two substrates capable of inducing the enantioselective biotransformation of PCBs via different degradation pathways. Eleven inducible proteins were identified by SDS-PAGE and characterized by LC-MS/MS. Four of the peptides, a spore coat protein, an extracellular serine protease, a spoVP, and a molecular chaperonin from *Bacillus subtilis*, were identified as being unique to biphenyl-induced cells, whereas an extracellular serine protease from *B. subtilis* was identified as being unique to carvone-induced cells. None of the peptides identified had sequences that corresponded to known dioxygenases or other PCB-degrading enzymes of this Gram-positive bacterium, suggesting that the identified induced proteins may be involved in either PCB degradation or adaptive responses that protect cells from toxicity.

Keywords: Polychlorinated Biphenyl (PCB); Carvone; *Rhodococcus*; Protein Expression

1. INTRODUCTION

Polychlorinated biphenyls (PCBs) were once widely used as coolants and lubricants in transformers, capacitors, and dielectric insulators; however, the production and use of PCBs have since been outlawed due to severe toxicity and environmental contamination. It is estimated that approximately 1.5 million tons of PCBs were produced worldwide before 1988 [1]. Although the production of these compounds was halted due to their long-term persistence, PCBs continue to threaten human health in contaminated areas, as well as through metabolic products that persist in the environment [2,3].

PCBs are commonly degraded by co-metabolic processes involving dioxygenases, which are induced by growth on medium containing biphenyl or other inducing substrates as the sole carbon source [4,5]. Carvone, a plant-derived monoterpene and the principal component of spearmint oil, has several properties that promote PCB biodegradation [6]. Recent evidence suggests that Gram-positive and -negative bacteria respond differently to various inducing substrates, and that Gram-positive bacteria are strongly induced to degrade PCBs following exposure to plant-derived monoterpenes [4,6-10]. A Gram-positive bacterium, *Rhodococcus* sp. ACS, was isolated by Andrew Singer from PCB-contaminated soil obtained from a site in Staten Island, New York, in 1997. The bacterium is similar in morphology to *Arthrobacter* sp. strain B1B [11] and has the ability to co-metabolize PCBs when grown in the presence of carvone, as well as other terpenes, including citral and cineole [12]. An analysis of the enantioselectivity of *Rhodococcus* sp. ACS further suggested that multiple degradation pathways are induced depending on the inducing substrate [12]. The key enzymes involved in PCB degradation are dioxygenases, which are polymeric enzymes. To date, most dioxygenase enzymes have been studied in Gram-negative bacteria [13-15]; relatively few dioxygenases have been characterized from Gram-positive bacteria [16-19]. Previously characterized dioxygenase enzymes contain a large subunit that ranges in size from 51,000 to 65,000 Da [13-15] and multiple small subunits that range in size from 22,000 to 27,300 Da [15]. The large and small subunits assemble into holoenzymes that are approximately 200,000 to 250,000 Da in size [13,15].

2. MATERIALS AND METHODS

As a first step toward identifying PCB-degrading enzymes, we compared the protein expression patterns of *Rhodococcus* sp. strain ACS grown on two different inducing substrates. The bacteria were cultured on either 1000 mg/L biphenyl or 250 mg/L carvone in a mineral

salt medium containing 1% fructose. The concentration of carvone used was the maximum concentration that could be tolerated without negatively affecting growth. Soluble proteins were separated by SDS-PAGE and the protein expression patterns analyzed to identify inducible proteins as compared to control cells. The proteins were separated by 15% Laemmli SDS-PAGE, and then stained with Coomassie blue. Proteins that were differentially expressed were excised from the gel. The gel slices were dried in a vacuum and then hydrated in 40 mL of 20 mg/mL trypsin (Promega, Madison, WI) [20]. In-gel digestion was performed for 16 h at 37˚C. Peptides were eluted from the gel slices with 50% (v/v) acetonitrile/5% (v/v) trifluoroacetic acid, and the eluent was dried in a vacuum [20]. A mass spectrometric analysis was performed by LC-MS/MS (Waters, Milford, MA) at the Analytical Chemistry Instrumentation Facility of the University of California, Riverside (Riverside, CA) to determine similarities to other previously characterized proteins in the NCBI protein database. The nucleotide sequence data for *Rhodococcus* sp. ACS will appear in the GenBank/EMBL/DDBJ nucleotide sequence databases under accession number DQ286393.

3. RESULTS AND DISCUSSION

One-dimensional separation will allow the detection of differentially expressed proteins only if they are not at the same molecular weight as a highly expressed constitutive protein and as long as the bands that are excised are likely to contain multiple proteins, not just one. Coomassie staining will only detect those proteins that are highly expressed in at least one of the treatments. Proteins with lower expression levels may be functionally important but undetectable by these techniques. Thus, the lack of dioxygenases among the identified peptides may reflect these limitations.

SDS-PAGE revealed differences in the expression levels of at least eleven peptides; these peptides were in the approximate size range of previously described large and small subunits of known dioxygenases [13-15]. The eleven peptides selected for analysis included one that was down-regulated in cells grown on biphenyl and carvone, four peptides that were unique to biphenyl-grown cells, and one peptide that was unique to carvone-grown cells; the remaining four peptides were induced by both biphenyl and carvone (**Figure 1**).

The eleven proteins were analyzed by LC-MS/MS and compared against the NCBI database using the Mascot algorithm [21] for identification. Most of the peptide sequences exhibited significant homology with previously identified proteins in both Gram-positive and -negative bacteria; however, most of these proteins were either hypothetical or poorly characterized [22]. None of the eleven major inducible proteins corresponded to

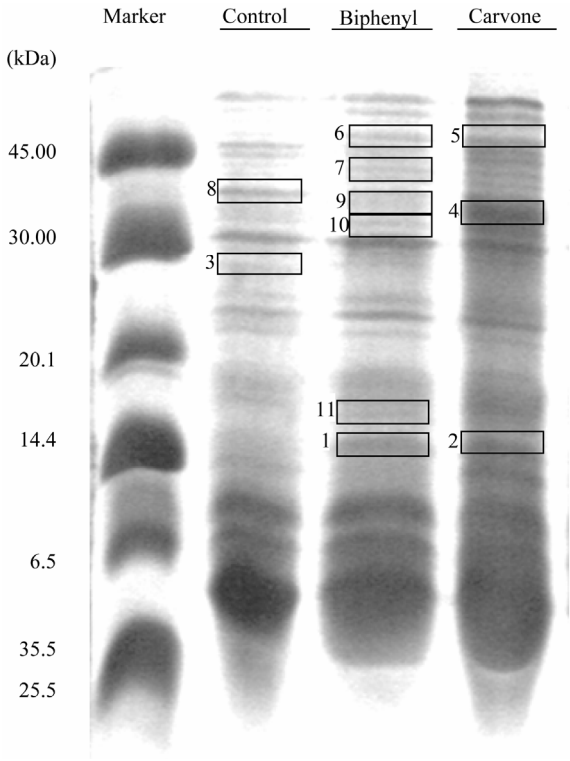

Figure 1. SDS-PAGE analysis of extracellular proteins from *Rhodococcus* sp. strain ACS induced by biphenyl and carvone.

amino acid sequences of dioxygenases previously associated with PCB degradation. The two peptides (**Figure 1**, peptide bands 3 and 8) expressed by cells during growth on carvone were a strong match to a known extracellular protease. The protein down-regulated by growth on biphenyl and carvone had matches that corresponded to a variety of very different enzymes, including an alcohol dehydrogenase, a serine protease from *Bacillus subtilis*, and Omp C from *Escherichia coli* (band 8, **Supplementary Table S1**). Proteins induced by both biphenyl and carvone (**Figure 1**, peptide bands 1, 2, 5, and 6) included a small acid-soluble protein from *B. subtilis* (bands 1 and 2) and an uncharacterized hypothetical protein from *B. subtilis* (bands 5 and 6).

Four peptides were identified as being unique to the biphenyl-grown cells. The first peptide (band 11, 17 kDa) had a match of 120 (Mascot probability score) with spoVP and a match of 60 with peptidyl-prolyl isomerase from *B. subtilis*; there was also a weak match for a serine protease from *B. subtilis* (**Supplementary Table S1**). The second peptide (band 10, 32 kDa) had a probability score of 172 for a molecular chaperonin from *B. subtilis*, and lower scores for several other peptides, including a spore coat-associated protein from *B. subtilis*, outer membrane protein II from *Shigella dysenteriae*, and glucose dehydrogenase (EC 1.1.1.47) from *B. subtilis* (**Sup-**

Table 1. List of identified peptide of bands.

Band no.	Protein description	MW/pI	Size (kDa)	Mascot score	Coverage (%)	Accession no.	Organism	Mass (m/z)	Identified peptide
1	Small acid-soluble spore protein (gamma-type SASP)	9262/8.20	15	252	94	16077932	*Bacillus subtilis*	815.42	TNAQQVR
2	Small acid-soluble spore protein (gamma-type SASP)	9262/8.20	15	179	80	16077932	*Bacillus subtilis*	815.43	TNAQQVR
3	Uridine phosphorylase	27142/5.81	27	180	26	16131680	*Escherichia coli*	898.48	QTESHAVK
4	Extracellular serine protease	85555/5.87	37	337	14	16080860	*Bacillus subtilis*	881.59	VPTLLIVK
5	Similar to hypothetical proteins	48607/5.72	48	207	15	16077084	*Bacillus subtilis*	897.53	LYIPPAPK
6	Similar to hypothetical proteins	48607/5.72	48	198	15	16077084	*Bacillus subtilis*	897.53	LYIPPAPK
7	Similar to spore coat protein	41219/5.16	41	72	5	16080144	*Bacillus subtilis*	914.48	APLTEEQK
8	Protein I, membrane	37197/4.43	37	146	11	223138	*Escherichia coli*	821.48	VGGVATYR
9	Extracellular serine protease	85555/5.87	37	82	4	16080860	*Bacillus subtilis*	1147.64	AAIMNTAVTLK + Oxidation (M)
10	Molecular chaperonin	32490/8.77	32	172	21	16078059	*Bacillus subtilis*	930.49	TGEVSDPVK
11	Alternate gene name: spoVP	55140/4.74	17	120	9	16079337	*Bacillus subtilis*	1045.56	LSLMPENAR + Oxidation (M)

plementary Table S1). The third peptide (band 9, 37 kDa) had a match of 82 with extracellular serine protease from *B. subtilis*, and outer-membrane protein A (outer membrane protein) from *E. coli* (**Supplementary Table S1**). The forth peptide (band 7, 41 kDa) had a probability score of 72 for spore coat protein from *B. subtilis* (**Supplementary Table S1**). The peptide unique to the carvone-grown cells (band 4) had strong match to an extracellular serine protease from *B. subtilis* (337) (**Table 1**).

Generally, a Mascot probability score of >30 indicates similarity to a known enzyme [9]. The results of this study, however, suggest that the proteins from *Rhodococcus* sp. ACS induced by growth on either carvone or biphenyl have functions unrelated to those from other microorganisms exhibiting high probability scores. The lack of dioxygenases in the protein matches may be due to methodological limitations; moreover, many other proteins may also be important for the response of the bacterium to biphenyl and carvone. Functional analyses, through methods such as transposon mutagenesis, will be necessary to determine the activity of these proteins and their role in PCB degradation. In addition to dioxygenases, PCB degradation pathways contain numerous enzymes involved in stepwise transformations of PCBs and their intermediates. Monoterpenes, including carvone, are toxic at high concentrations and cause cell lysis above 500 ppm [6]. There are minimal estimates of several hundred million genes present in environmental samples, the majority of which code for unknown proteins. It is therefore not surprising that protein sequence alignments are currently unable to provide insight into the degradative enzymes of poorly characterized microorganisms. The identification of major inducible proteins is nonetheless a first step in identifying important degradative enzymes, which can now be targeted for further study.

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Supplementary Table S1. List of the identified peptide bands.

Band (Size)		Protein description	MW/pI	Mascot score	Coverage (%)	Accession no.	Organism	Mass (m/z)	Identified peptide
1 (15 kDa)	1	Small acid-soluble spore protein (gamma-type SASP)	9262/8.20	252	94	16077932	*Bacillus subtilis*	815.42	TNAQQVR
								880.4	ANSNNFSK
								1173.57	QQNQSAEQNK
								2781.3	QNQQSAAGQGQFGTEFASET-NAQQVR
								2839.3	QNQQSAGQQGQFGTEFASET-DAQQVR
								2909.39	KQNQQSAAGQGQFGTEFASE-TNAQQVR
								2967.4	KQNQQSAGQQGQFGTEFASE-TDAQQVR
	2	Small acid-soluble protein gamma-type	9333/8.20	169	92	13676638	*Bacillus subtilis*	815.42	TNAQQVR
								880.43	ANSNNFSK
								1173.57	QQNQSAEQNK
								2781.3	QNQQSAAGQGQFGTEFASE-TNAQQVR
								2909.39	KQNQQSAAGQGQFGTEFASE-TNAQQVR
								2910.39	QNQQSAAGQQGQFGTEFASE-TDAQQVR
	3	Small, acid-soluble spore protein gamma-type (SASP)	9015/8.20	87	53	134246	*Geobacillus stearothermophilus*	815.42	TNAQQVR
								1173.57	QQNQSAEQNK
								971.14	KQNQQSAAGQGQFGTEFASE-TDAQQVR
	4	Similar to hypothetical proteins	19245/ 9.61	74	13	6080160	*Bacillus subtilis*	1196.63	TLPAAGTYTFR
								1441.85	VPLALDLLGAGEFK
2 (15 kDa)	1	Small acid-soluble spore protein (gamma-type SASP)	9262/8.20	179	80	16077932	*Bacillus subtilis*	815.43	TNAQQVR
								880.4	ANSNNFSK
								2781.3	QNQQSAAGQGQFGTEFASE-TNAQQVR
								2839.3	QNQQSAGQQGQFGTEFASE-TDAQQVR
								2967.38	KQNQQSAGQQGQFGTEFASE-TDAQQVR
	2	Small acid-soluble protein gamma-type	9333/8.20	136	80	13676638	*Bacillus subtilis*	815.43	TNAQQVR
								880.4	ANSNNFSK
								2781.3	QNQQSAAGQGQFGTEFASE-TNAQQVR
								2910.38	QNQQSAAGQQGQFGTEFASE-TDAQQVR
	3	Lysozyme C precursor (1,4-beta-N-acetylmuramidase C) (Allergen Gal d 4) (Gal d IV)	16228/ 9.37	123	23	126608	*Gallus gallus* (chicken)	873.43	HGLDNYR
								1427.67	FESNFNTQATNR
								1752.86	NTDGSTDYGILQINSR
	4	Lysozyme (E.C.3.2.1.17) mutant	14290/ 9.46	123	27	809243	*Gallus gallus* (chicken)	873.43	HGLDNYR
								1427.67	FESNFNTQATNR
								1752.86	NTDGSTDYGLLQINSR

Continued

3 (27 kDa)	1	Uridine phosphorylase	27142/ 5.81	180	26	16131680	*Escherichia coli* K12	898.48	QTESHAVK
								1100.63	IAALMDKPVK + Oxidation (M)
								1101.6	AGMVAGVIVNR + Oxidation (M)
								1115.61	SDVFHLGLTK
								1404.66	TQQEIPNAETMK + Oxidation (M)
								1750.91	NDLQGATLAIVPGDPDR
	2	Major outer membrane lipoprotein precursor	8234/8.93	110	33	127525	*Serratia marcescens*	1376.64	VDQLSNDVNAMR + Oxidation (M)
		(Murein-lipoprotein)						1530.81	IDQLSSDVQTLNAK
	3	Chain A, purine nucleoside phosphorylase	25802/ 5.42	108	9	1633413	*Escherichia coli*	1285.72	IALESVLLGDKE
								1326.66	YIAETFLEDAR
	4	Outer membrane protein 3a (II*;G;d)	37178/ 5.99	105	23	15800816	*Escherichia coli* O157	914.52	AQGVQLTAK
							H7 EDL933]	1082.55	SDVLFNFNK
								1653.84	LGYPITDDLDIYTR
								2231.17	FGQGEAAPVVAPAPAPAPE-VQTK
								2600.31	NHDTGVSPVFAGGVEYAIT-PEIATR
4 (37 kDa)	1	Extracellular serine protease	85555/ 5.87	337	14	16080860	*Bacillus subtilis*	881.59	VPTLLIVK
								1048.61	VVIPAHQTGK
								1147.62	AAIMNTAVTLK + Oxidation (M)
								1164.62	AGTYEGTVIVR
								1278.63	ALGEQVADFSSR
								1464.71	SSQVLTEEPFTVE
								1655.87	LPAGEYYLLAYAANK
								1790.89	ADSLVSPGSYSYGTFLK
								1829.8	DSDGEVYPHNAQGAGSAR
	2	Albumin	66088/ 5.76	131	6	229552	*Bos aurus* (cow)	921.48	AEFVEVTK
								926.49	YLYEIAR
								1162.63	LVNELTEFAK
								1566.78	DAFLGSFLYEYS
	3	Protein I,membrane	37197/ 4.43	93	11	223138	*Escherichia coli*	821.44	VGGVATYR
								1020.54	AVGLHYFSK
								1085.52	FTNTSGFANK
								1248.55	YADVGSFDYGR
	4	Similar to alcohol dehydrogenase	30826/ 7.01	90	13	16078104	*Bacillus subtilis*	1216.64	TAIITGGDSGIGR
								1314.73	SLSQSLVQQGIR
								1424.75	GSSIINTASITAYK

Continued

5 (48 kDa)	1	Similar to hypothetical proteins	48607/ 5.72	207	15	16077084	*Bacillus subtilis*	897.53	LYIPPAPK
								1039.52	EAYNQFLR
								1227.68	EGLEISTALAPK
								1305.64	DIESNAYLEPR
								1413.72	GNQVSENLQQAAR
								1608.8	DVIEYALTEMPANK + Oxidation
	2	Elongation factor Tu	43566/ 4.92	129	15	16077181	*Bacillus subtilis*	1155.58	TTVTGVEMFR + Oxidation (M)
								1702.92	LLDYAEAGDNIGALLR
								1738.78	GTAMAYDQIDGAPEER + Oxidation (M)
								2230.06	DTEKPFMMPVEDVFSITGR + 2 Oxidation (M)
	3	Prolidase	50449/ 5.41	124	11	1314852	*Pseudoalteromonas haloplanktis*	996.58	LPAAEIVER
								1219.68	VEAFKPFGGIR
								1360.77	IAQLLSDFDIVK
								2042.97	IEDNIIVHEDSLENMTR + Oxidation
	4	Elongation factor Tu	30668/ 4.45	88	13	11612428	*Enterococcus pseudoavium*	1155.58	TTVTGVEMFR + Oxidation (M)
								1194.56	ALEGDPSYSEK
								1702.92	LLDYAEAGDNIGALLR
6 (48 kDa)	1	Similar to hypothetical proteins	48607/ 5.72	198	15	16077084	*Bacillus subtilis*	897.53	LYIPPAPK
								1039.53	EAYNQFLR
								1227.69	EGLEISTALAPK
								1305.65	DIESNAYLEPR
								1413.73	GNQVSENLQQAAR
								1608.75	DVIEYALTEMPANK + Oxidation (M)
	2	Prolidase	50449/ 5.41	143	14	1314852	*Pseudoalteromonas haloplanktis*	996.57	LPAAEIVER
								1219.69	VEAFKPFGGIR
								1360.78	IAQLLSDFDIVK
								1724.98	LAVLYAEHIATLQQR
								2042.99	IEDNIIVHEDSLENMTR + Oxidation (M)
	3	Elongation factor Tu	43566/ 4.92	93	10	16077181	*Bacillus subtilis*	1155.58	TTVTGVEMFR + Oxidation (M)
								1702.93	LLDYAEAGDNIGALLR
								1738.82	GTAMAYDQIDGAPEER + Oxidation (M)
	4	Similar to carboxy-terminal processing protease	52765/ 8.22	44	5	16080577	*Bacillus subtilis*	1273.7	GSASASEILAGALK
								1456.73	AYELISNEYVEK
7 (41 kDa)	1	Similar to spore coat protein	41219/ 5.16	72	5	16080144	*Bacillus subtilis*	914.48	APLTEEQK
								1400.63	EELESAFEYER
	2	Spore coat protein	41058/ 6.61	54	9	16080142	*Bacillus subtilis*	1274.71	LTEIEGEPFLK
								1429.76	ELHSITYDLPSR
								1451.62	EMIYYDAEQMK + 2 Oxidation (M)
	3	Plasminogen	90526/ 6.89	35	2	39593458	*Homo sapiens*	1045.6	LSSPADITDK
	4	Hypothetical protein CBG05704	36853/ 5.79	33	1	49529146	*Caenorhabditis briggsae*	855.53	LATVPDLK

Continued

8 (37 kDa)	1	Protein I, membrane	37197/ 4.43	146	11	223138	*Escherichia coli*	821.48	VGGVATYR
								1020.57	AVGLHYFSK
								1085.54	FTNTSGFANK
								1248.58	YADVGSFDYGR
	2	Similar to alcohol dehydrogenase	30826/ 7.01	119	13	16078104	*Bacillus subtilis*	1216.68	TAIITGGDSGIGR
								1314.76	SLSQSLVQQGIR
								1424.76	GSSIINTASITAYK
	3	Outer membrane protein OmpC	40474/ 4.55	107	6	6650193	*Escherichia coli*	1289.6	FQDVGSFDYGR
								1347.71	INLLDDNQFTR
	4	Extracellular serine protease	85555/ 5.87	91	5	16080860	*Bacillus subtilis*	881.61	VPTLLIVK
								1147.65	AAIMNTAVTLK + Oxidation (M)
								1278.66	ALGEQVADFSSR
								1464.74	SSQVLTEEPFTVE
9 (37 kDa)	1	Extracellular serine protease	85555/ 5.87	82	4	16080860	*Bacillus subtilis*	1147.64	AAIMNTAVTLK + Oxidation (M)
								1278.65	ALGEQVADFSSR
								1464.73	SSQVLTEEPFTVE
	2	Outer membrane protein A (Outer membrane protein II)	26128/ 5.14	56	9	129137	*Escherichia fergusonii*	1221.66	AQSVVDYLISK
								1408.7	IGSDAYNQGLSER
	3	CG32582-PA	5702/ 10.92	39	23	24642312	*Drosophila melanogaster*	1221.66	TGEGSSTSASCR + Phospho (ST)
	4	Unnamed protein product	81936/ 7.55	33	1	47215566	*Tetraodon nigroviridis*	1136.59	MAPSAQTLYR
10 (32 kDa)	1	Molecular chaperonin	32490/ 8.77	172	21	16078059	*Bacillus subtilis*	930.49	TGEVSDPVK
								952.54	ASHILVADK
								970.46	GELYTNMK + Oxidation (M)
								1186.5	EGQMDETFSK + Oxidation (M)
								1365.69	TQLGDQYTALEK
								1443.82	TAGASVLTQLVQEK
	2	Spore coat-associated protein	28287/ 5.58	163	20	160779518	*Bacillus subtilis*	915.5	VNVATIDGK
								955.48	DLYLMSAK + Oxidation (M)
								1127.63	NIILDDANLK
								1395.71	DATFASGTLDLSAK
								1414.71	EVLMALNYGDFK + Oxidation (M)
	3	Outer membrane protein A precursor (Outer membrane protein II)	37718/ 5.57	114	11	129143	*Shigella dysenteriae*	914.55	AQGVQLTAK
								1082.55	SDVLFNFNK
								1221.69	AQSVVDYLISK
								1279.64	DGSVVVLGYTDR
	4	Glucose dehydrogenase (EC 1.1.1.47)	27949/ 5.38	71	13	142955	*Bacillus subtilis*	1098.59	VVINYYSNK
								1142.68	VVAITGAASGLGK
								1328.7	AGGEAVVVQGDVTK

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11 (17 kDa)	1	Alternate gene name: spoVP	55140/ 4.74	120	9	16079337	*Bacillus subtilis*	1045.56	LSLMPENAR + Oxidation (M)
								1081.6	AVAPSIHMIK + Oxidation (M)
								1267.61	TEYDQVSDALK
								1632.87	VDVESEFAPIIGTEK
	2	Peptidyl-prolyl isomerase	15246/ 5.53	60	17	16079393	*Bacillus subtilis*	1255.64	TGYFLLEDGNK
								1638.79	LANEGFYDGLTFHR
	3	Extracellular serine protease	85555/ 5.87	44	1	16080860	*Bacillus subtilis*	1245.68	GVAPDATLLAYR
	4	Spore coat-associated protein	28287/ 5.58	40	8	16079518	*Bacillus subtilis*	915.52	VNVATIDGK
								1414.68	EVLMALNYGDFK

