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Metaproteomic analysis of bacterial communities in marine mudflat aquaculture sediment

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Abstract Bacteria living in marine sediment play crucial roles in the benthic–pelagic interface coupling process. However, the complexity of the marine environment and the abundance of interfering materials hamper metaproteomic research of the marine mudflat environment. In this study, a modified sequential protein extraction method was used for marine mudflat sediment metaproteomic investigation. For marine sediment samples in cultured clam mudflat, more than 1000 protein spots were visualized in a two-dimensional gel electrophoresis map and 78 % of 194 randomly selected spots were successfully identified by mass spectrometry. We further applied this method to compare long-term clam aquaculture and natural mudflat sediment and identified 53 altered proteins from different microbe resources, which belonged to different functional categories or metabolic pathways. We found that proteins involved in stress/defense response process, ATP regeneration and protein folding more inclined to increase abundance while arginine biosynthesis and signal transduction process related proteins preferred to decrease in clam cultured mudflat sediment. Meanwhile, proteins were abundant in pathogens of bivalves, such as *Vibrio* and *Photobacterium*, and decreased in *Acinetobacter,* after about 8 months clam cultured. Furthermore, the terminal restriction fragment length polymorphism assay was performed to compare microbial community composition between sediments mentioned above. Results showed that the top three enrich genera in natural sediment were *Cytophaga*, *Butyrivibrio* and *Spirochaeta*, while *Cytophaga*, *Spirochaeta* and *Azoarcus* were found enrichment in long-term mudflat aquaculture sediment.

Keywords Marine mudflat sediment · Metaproteomics · Mudflat aquaculture · Bacterial community

Introduction

Mudflat accounts for a certain proportion of marine sediment and is considered ideal for the development of aquaculture (Karthik et al. 2005). It is well known that

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mudflat aquaculture is developing rapidly and becoming a major mainstay in many coastal cities. However, owing to the extreme physical and chemical characteristics of marine sediment, the biota of this vast habitat is largely unknown, especially for microbes which play crucial roles in the benthic–pelagic interface coupling process and fuel material cycling (Findlay and Watling 1998). To date, the extensive aquaculture has resulted in decreased productivity of food and environmental deterioration of natural resource, which contribute to economic loss and marine pollution (Gray 2002; Wang and Wall 2010; Wang et al. 2010). The stability of the mudflat sediment microbial community likely has a large effect on the balance between the marine ecosystem and aquaculture benefits. Therefore, a thorough understanding of the microbial community in marine mudflat aquaculture is necessary (Lomstein et al. 2012).

Recently, metaproteomic approaches have been recognized as a promising strategy to investigate the composition and function of the environmental microbial community in situ, such as in soil, freshwater, acid mine drainage, marine water, wastewater and marine cold seep sediment (Siggins et al. 2012; Stokke et al. 2012; VerBerkmoes et al. 2009). Among these researches, sample preparation is a key procedure for further microbial community analysis. Thus, many proteins extraction methods were developed such as SDS alone, SDS–TCA, SDS–phenol, NaOH–phenol and citrate–phenol extraction (Abram et al. 2009; Chen et al. 2009; Chourey et al. 2010; Erickson et al. 2010; Pierre-Alain et al. 2007; Wang et al. 2011a). All these researches largely improved our understanding of the microbial community in marine sediment. However, due to the complexity of the marine environment and the abundance of interfering materials, the structure and function of environmental microbial communities in marine sediment, especially in mudflat aquaculture sediment, is poorly understood.

In this study, we introduced a protein extraction method for mudflat sediment proteomics analysis, which was modified from our previous crop soil metaproteomic study (Wang et al. 2011a). Our results showed that this method was suitable for obtaining the extra- and intracellular proteins from marine sediment too. Then, altered proteins between natural mudflat and long-term mudflat aquaculture marine sediments were identified and compared by two-dimensional gel electrophoresis (2-DE) combined with MALDI TOF–TOF. Furthermore, the composition and structure of the whole microbial community in mudflat aquaculture marine sediments was analyzed by using the terminal restriction fragment length polymorphism (T-RFLP).

Materials and methods

Marine mudflat sediment samples collection

Marine sediment samples were collected from long-term mudflat aquaculture areas which cultivated clam (*Meretrix meretrix*) from January to August 2012 using a five-point sampling strategy that includes the four corners and the center of an aquatic farm. The site was located in Putian, a city located in the southeastern Chinese province of Fujian (25.18N, 119.08E). The samples were collected from the top sediment (0–10 cm) and mixed thoroughly. The samples were sealed and immediately stored at 4 °C. The natural marine sediment (25.19N, 119.09E) was collected using the same method at a nearby mudflat aquaculture area that was never used for mudflat aquaculture.

Sample preparation

A extraction method was used to extract proteins from fresh marine sediment sample as previously described, which was modified by adding a second phenol extraction and two water-wash steps (Wang et al. 2011a). Briefly, about 2 g of marine sediment sample was homogenized and extracted by shaking for 1 h with 5 mL SDS extracting buffer (1.25 % w/v SDS, 0.1 M Tris–HCl, pH 6.8, 20 mM DTT) at room temperature (RT) before being centrifuged at 12,000 rpm for 30 min at 4 °C. The supernatants were filtered through nylon mesh (0.45 mm) and shaken for 15 min with 3 mL buffered phenol (pH 8.0) at RT. After centrifugation (12,000 rpm, 4 °C) for 30 min, the supernatants were collected and shaken with 3 mL buffered phenol (pH 8.0) again. The phenol phases were combined and washed (by shaking) for 15 min with 3 mL Milli-Q water. This treatment was repeated twice. Afterwards, these phases were separated by centrifugation (12,000 rpm, 4 °C) for 30 min. The proteins in the lower phenol phase were precipitated with a fivefold volume of 0.1 M ammonium acetate dissolved in methanol at −20 °C overnight. Proteins were recovered by centrifugation (12,000 rpm, 4 °C) for 30 min. The pellet was washed once with cold methanol and twice with cold acetone. The washed pellet was air-dried and then stored at −80 °C or solubilized in sample buffer for further use.

2-DE separation of sediment proteins

2-DE PAGE was performed as described previously (Wu et al. 2011). Briefly, 150 μg sediment proteins were dissolved in 7 M urea, 2 M thiourea, 65 mM DTT and 4 % CHAPS and rehydrated in 24 cm IPG strips (pH 4–7). The samples were separated by IEF in the first dimension, and

then 12 % SDS-PAGE in the second dimension. The preparative gels were stained with CBB R-250 and scanned with ImagescanIII (Amersham Bioscience, Uppsala, Sweden). All data were collected from three independent replicates and analyzed with the ImageMaster™ 2D Platinum software 5.0 (Amersham Bioscience, Uppsala, Sweden). Significantly changed spots were selected by rate increased/decreased 1.5 fold or complete appearance and disappearance.

Protein identification by mass spectrometry

Protein spots of interest were excised manually from gels for mass spectrometric analysis as described previously (Lin et al. 2012a). Briefly, each selected spot was digested by 12.5 μg/mL trypsin (Promega, Madison, WI, USA) and incubated at 37 °C overnight. A volume of 1 μL of the solution was spotted onto stainless steel sample target plates and analyzed by a Bruker UltraFlex III MALDI TOF/TOF mass spectrometer (Bruker Daltonics, Bremen, Germany) (Lin et al. 2012b). Mass spectra were obtained for each sampled spot by accumulation of 600–800 laser shots in the 800–5000 Da mass range. Both MS and MS/MS data were interpreted and processed using Flexanalysis 3.0 (Bruker Daltonics, Bremen, Germany) and searched by MASCOT search engine (v2.3, Matrix Science, London, UK). Parameters selected included: the NCBInr database in SwissProt, taxonomy of all entries, trypsin as the digestion enzyme, at less two missed cleavage site, mass tolerance of 100 ppm, MS/MS mass tolerance of 0.6 Da, carbamidomethylation of cysteine for fixed modification, and methionine oxidation for variable modification. The MASCOT score (confidence $\geq$ 95 %) higher 80 was used as a highly conservative threshold for correct identification. Proteins that matched at least two MS/MS peptides or three peptide mass finger printings (PMFs) were subjected to further identification. The biological functions of identified proteins were classified according to the published reports.

Complex microbial community analysis by terminal restriction fragment length polymorphism (T-RFLP)

Total DNA from collected sediment samples was extracted by using the high salt/SDS method with the following modifications (Fang et al. 2013). Bacterial 16S rRNA gene sequences were amplified with a 6-carboxyfluorescein-labeled primer 8F-FAM (5′-AGAGTTTGATCCTGGCTCAG-3′) and 926R (5′-CCGTCAATTCCTTTRAGTTT-3′). The reaction mixture consisted of 12.5 μL Taq PCR Master Mix (2X) (Sangon, Shanghai, China), 0.8 μL of each primer (10 μM) and 20 ng template DNA in a final volume of 25 μL. Cycling conditions were: initial denaturation and enzyme activation at 94 °C for 5 min, followed by 35 cycles of denaturation at 94 °C for 1 min, annealing at 49.5 °C for 45 s, extension at 72 °C for 1 min, followed by a final 5 min extension at 72 °C. Then, the purified PCR products were digested with restriction endonucleases *Msp*I and *Hae*III at 37 °C for 5 h, respectively. The length of terminal restriction fragments (T-RFs) were determined by the ABI 3730xl DNA sequencer (Applied Biosystems, Foster City, CA, USA) in the GeneScan mode.

Results

2DE profile of marine sediment

Acquiring high quality protein is the first important step for metaproteomics analysis. We firstly tested the C–S–P–M method (citrate buffer, SDS, and phenol extraction) in marine sediment as our previously article used in crop soil, while only showed about 300 spots with many horizontal streaking in gel (data not show). Thus, a modified protein extraction method was established with an additional phenol extraction procedure for further protein extraction in this study (Fig. 1). Approximately 1000 separated protein spots from marine long-term mudflat aquaculture sediment were visualized (Fig. 2a). Proteins of both high and low molecular weights were obtained, which confirmed that our method showed no discrimination in protein extraction. Mass spectrometric analysis was conducted for further protein identification. It should be mentioned that one of the purpose of this study is to validate an extraction method for mudflat sediment research, we did not identify all of the visualized 2DE spots by MALDI TOF–TOF MS/MS. A total of 98 spots were identified by MALDI PMF combined with MS/MS, 32 spots by MS/MS alone, and 21 spots by MALDI PMF alone (Tables S1, S2, S3). Thus, a total 151 of 194 randomly selected protein spots were successfully identified with high resolution and repeatability in Coomassie blue stained 2-DE gels.

Further functional analysis revealed that these proteins could be sorted into eight categories based on their biological functions (Fig. 2b). The categories are stress and defense responses (20 %), TCA pathway (12 %), protein synthesis (11 %), amino acid metabolism (9 %), signal transduction (4 %), ATP regeneration (4 %), unknown functions (18 %) and others (22 %). Identified proteins also showed various origins. They could be classified into nine groups according to the best matching microbial sources (Fig. 2c), that is, *Vibrio* (35 %), *Acinetobacter* (16 %), *Shewanella* (10 %), *Photobacterium* (10 %), *Pseudomonas* (5 %), *Lysinibacillus* (3 %), *Bacillus* (2 %), *Nitrosomonas* (2 %) and others (1.3 %). The various biological functions

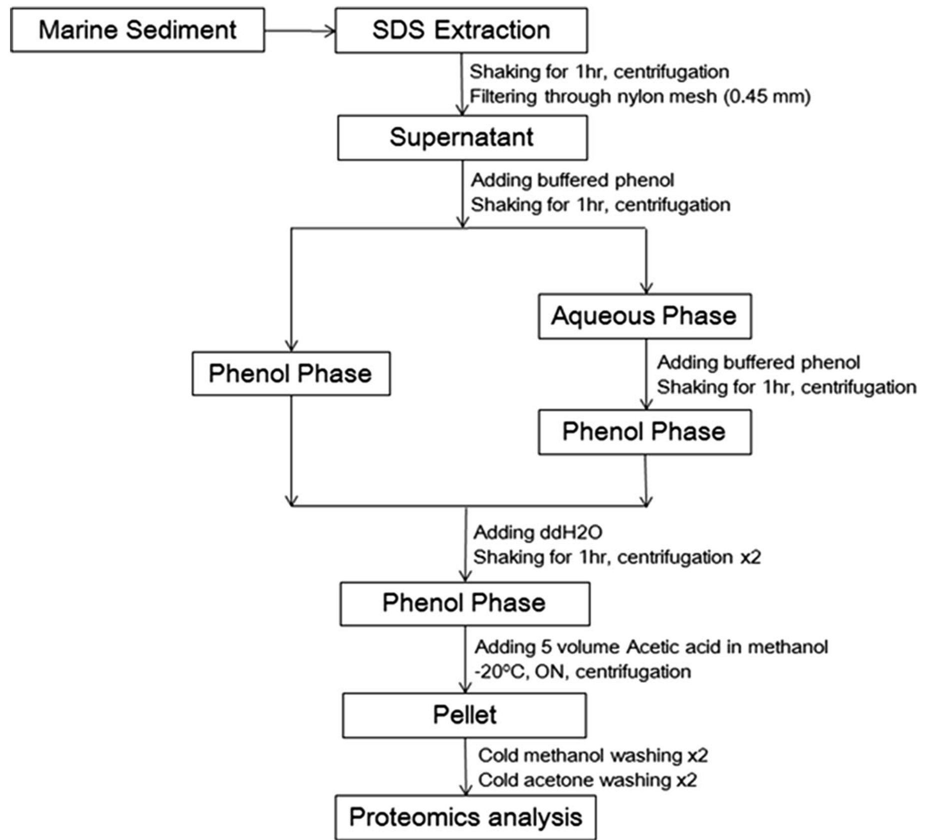

Fig. 1 Schematic representation of the protein extraction procedure from marine sediment

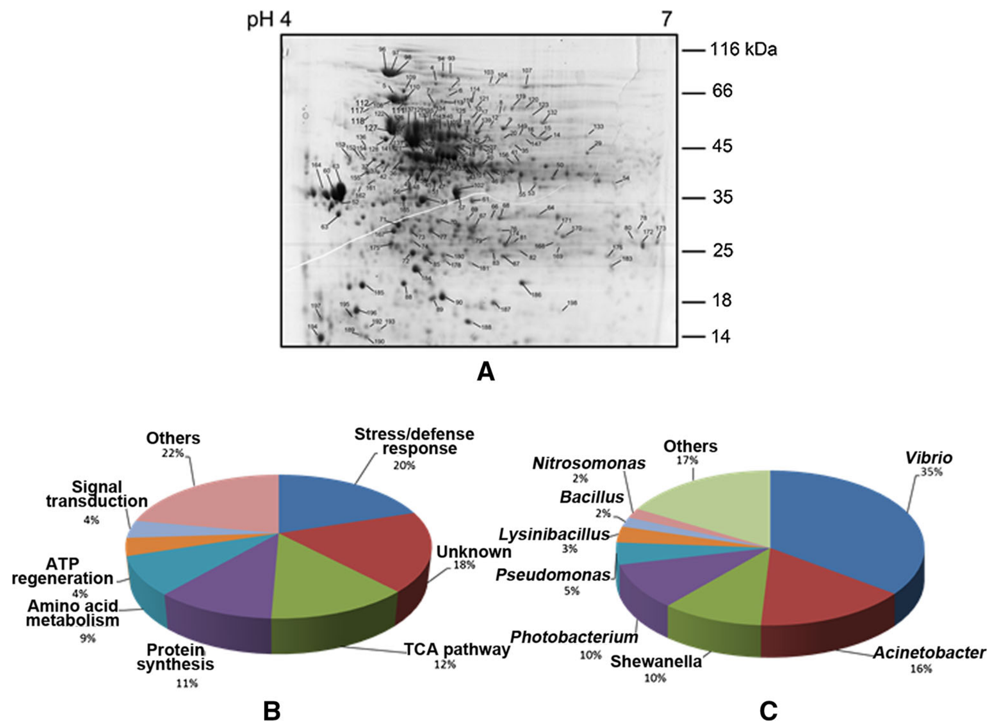

Fig. 2 2-DE profile of proteins extracted from marine long-term mudflat aquaculture sediment and analysis. **a** 150 µg of protein was loaded on a 24 cm IPG strip with a linear gradient of in the range pH 4–7 and separated by SDS PAGE in the second dimension; **b** biological functional categories of marine long-term mudflat aquaculture sediment according to mass spectrometry identification result; **c** they could be classified into nine groups according to the best matching microbial species

and species of identified proteins further confirmed that this protein extraction method is suitable for metaproteomic research from complex marine sediment.

Differential expression of proteins between long-term mudflat aquaculture and natural sediments

Using the modified protein extraction method described above, we further compared the differential expression of proteins from the microbial community between long-term mudflat aquaculture and natural mudflat marine sediment. A total of 53 altered proteins including 35 increase and 18 decrease abundance were identified between the two groups (Table 1; Fig. 3a, b). Compared to the natural mudflat sediment, up to 29.4 % increasing abundance of best matching proteins were belong to *Photobacterium*, including *P. damselae* (6 %), *P. profundum* (17 %) and *Photobacterium* sp. (16 %), up to 23.5 % belong to *Vibrio.*, including *V. cholera* (6 %), *V. coralliilyticus* (6 %), *V. furnissii* (9 %) and *V. vulnificus* (2.9 %), about 17.6 % were *Shewanella* sp. which included *S. amazonensis* (6 %), *S. denitrificans* (2.9 %), *S. oneidensis* (2.9 %) and *Shewanella* sp. (6 %, Fig. 3c). Most of them are fish pathogen and indicate the survival environment for *M. meretrix* getting worse after 8 months cultivated. Meanwhile, major decreasing abundance of proteins were belong to *Acinetobacter* (up to 60 %) including *A. baumannii* (53 %) and *Acinetobacter* sp. (7 %), which are human pathogens and cause serious multidrug resistant problem in hospital while looks harmless to most of fish (Fig. 3d; Qureshi et al. 2015). Interesting, two protein enzymes (Deoxyribose phosphate aldolase and Ornithine carbamoyltransferase) were decreased while GroEL, OmpU and ABC transporter PotD were increased in *Vibrio*. That suggests the stress response or nutrition transport proteins are more susceptible to over-express and intrinsic metabolic level of *Vibrio* may prefer to decrease in long-term aquaculture mudflat. The former concept was proofed by the biological functions analysis of altered proteins (Fig. 3e). Total 10 unique proteins (OmpU, OmpA, thiol specific antioxidant, putative superoxide dismutase, DnaK, Hsp90, Hsp60, GroEL, porin, putative immunogenic protein) related with stress/defense response process increased in long-term mudflat while only 2 decreased (OmpA and superoxide dismutase), which account for 30.3 and 13.3 % in total altered proteins, respectively. Besides this, we also found ATP regeneration (ATP synthase F1, ATP synthase subunit B, F0F1 ATP synthase subunit beta and ATP synthase beta chain) and protein folding related proteins more inclined to increase and arginine biosynthesis related proteins preferred to decrease. In general, activate the stress/defense response system, make sure the correct protein folding, supply plentiful ATP source and maintain the intrinsic metabolic homeostasis, are essential characteristics for surviving in the competitive ecology of microbial community in long-term mudflat aquaculture.

The composition and structure of microbial community in marine mudflat sediments

The T-RFLP phylogenetic assignment tool (PAT) was used to analyze the bacterial community between long-term mudflat aquaculture and natural sediments (Fig. 4). In addition to unclassified bacterial phyla, 15 and 30 genera of bacteria were identified in natural and long-term mudflat aquaculture sediments, respectively. Several novel genera of bacteria, such as *Enterococcus*, *Sorangium*, *Frankia*, *Thauera*, *Nitrosomonas* and others appeared in long-term mudflat aquaculture sediment but were absent from the natural sediment. The largest genera proportion in natural sediment was the bacteria belonging to *Cytophaga* (26.9 %), followed by *Butyrivibrio* (7.69 %) and *Spirochaeta* (7.69 %), while those belonging to *Cytophaga* (12.5 %), followed by *Spirochaeta* (7.14 %), *Azoarcus* (5.36 %) and *Alicyclobacillus* (5.36 %) were found in highest proportions in long-term mudflat aquaculture sediment. Several bacterial groups which determined by proteomic analysis, such as *Acinetobacter* and *Shewanella*, were not found in the genomic counterpart T-RFLP analysis, which might be attributed to complete genomic information available to date on environmental microbes. Therefore, it is necessary to combine results from different approaches in order to get as much information as possible.

Discussion

To date, there is an increasing interest to understand the function of the ecosystem and the structure of microbial communities directly within their respective environments (Georges et al. 2014; Narayanasamy et al. 2015). A variety of powerful molecular analysis tools of environmental samples, mostly based on the analysis of 16S rRNA genes, have greatly improved our knowledge of the vast array of microbial compositions and functions (Bowen et al. 2011; Du et al. 2011; Vandieken and Thamdrup 2013). Recent studies have shown that the protein expression level of microorganisms even with similar genomes could cause significant ecological differences (Kleiner et al. 2012). Thus, extended marine mudflat aquaculture sediment protein identification is essential to understand sediment ecological processes and the environmental factors that affect the function of the marine aquaculture mudflat ecosystem. Therefore, metaproteomics has become a powerful complement of functional genomics for high throughput

Table 1 Statistically significant differentially expressed proteins between marine long-term mudflat aquaculture and natural mudflat sediment

Spot[a]	GI no.[b]	Protein name	MW/pI[c]	Score (PMF)[d]	PMF/converage[e]	Score (MS–MS)[f]	Pept[g]	Best matching species	Function
Up-regulation									
28	gi|27363917	Isocitrate lyase	48,345/5.08	–	–	246	3	*Vibrio vulnificus*	TCA pathway
36	gi|269102225	Isocitrate dehydrogenase	45,885/4.71	124	22/37 %	298	4	*Photobacterium damselae*	TCA pathway
45	gi|15642085	Succinyl-CoA synthetase subunit beta	41,658/5.35	112	17/53 %	305	3	*Vibrio cholerae*	TCA pathway
58	gi|89075547	Malate dehydrogenase	32,531/4.73	83	11/33 %	318	3	*Photobacterium* sp.	TCA pathway
52	gi|297242387	Outer membrane protein U	35,898/4.49	61	11/41 %	65	1	*Vibrio furnissii*	Stress/defense response
56	gi|119774051	OmpA/MotB	40,275/5.10	87	15/45 %	170	2	*Shewanella amazonensis*	Stress/defense response
72	gi|91793445	Alkyl hydroperoxide reductase/thiol specific antioxidant/mal allergen	22,156/5.21	–	–	347	3	*Shewanella denitrificans*	Stress/defense response
88	gi|54309739	Putative superoxide dismutase	21,480/4.95	–	–	252	3	*Photobacterium profundum*	Stress/defense response
108	gi|260776585	Chaperone protein DnaK	69,020/4.59	135	22/35 %	525	4	*Vibrio coralliilyticus*	Stress/defense response
109	gi|253997105	Chaperone protein DnaK	69,212/4.87	–	–	298	2	*Methylotenera mobilis*	Stress/defense response
111	gi|117620206	Molecular chaperone DnaK	69,444/4.79	–	–	350	3	*Aeromonas hydrophila*	Stress/defense response
114	gi|119774450	Heat shock protein 90	71,649/5.15	92	19/40 %	280	4	*Shewanella amazonensis*	Stress/defense response
126	gi|260770852	Heat shock protein 60 family chaperone GroEL	57,194/4.70	108	20/53 %	649	5	*Vibrio furnissii*	Stress/defense response
154	gi|146306292	Outer membrane porin	46,729/4.83	–	–	108	2	*Pseudomonas mendocina*	Stress/defense response
164	gi|297242387	Outer membrane protein U	35,898/4.49	95	12/46 %	220	2	*Vibrio furnissii*	Stress/defense response
172	gi|54307603	Putative immunogenic protein	35,037/8.18	–	–	245	2	*Photobacterium profundum*	Stress/defense response
43	gi|270341195	Elongation factor Tu	28,618/4.69	209	24/88 %	462	4	*Alcaligenes faecalis*	Singnal transduction
167	gi|260768425	ABC transporter periplasmic spermidine putrescine-binding protein PotD	38268/4.87	152	20/50 %	117	2	*Vibrio furnissii*	Protein transporter
110	gi|269102376	SSU ribosomal protein S1p	60688/4.82	165	25/53 %	315	4	*Photobacterium damselae*	Protein synthesis

Table 1 continued

Spot[a]	GI no.[b]	Protein name	MW/pI[c]	Score (PMF)[d]	PMF/converage[e]	Score (MS–MS)[f]	Pept[g]	Best matching species	Function
137	gil113971727	Chaperonin GroEL	57075/4.82	174	26/58 %	537	4	*Shewanella* sp.	Protein location
140	gil26988095	Chaperonin GroEL	56765/4.97	111	22/44 %	340	3	*Pseudomonas putida*	Protein location
11	gil30248059	Chaperonin GroEL	57637/5.15	–	–	193	2	*Nitrosomonas europaea*	Protein location
127	gil54310454	Chaperonin GroEL	57584/4.77	–	–	392	3	*Photobacterium profundum*	Protein location
71	gil90413859	Putative spermidine/putrescine-binding periplasmic protein	38721/4.81	99	19/36 %	122	2	*Photobacterium profundum*	Protein folding
112	gil299535905	Oligopeptide-binding protein oppA precursor	67399/4.66	126	24/56 %	138	2	*Lysinibacillus fusiformis*	Protein folding
49	gil24371854	DNA-directed RNA polymerase subunit alpha	36309/4.77	117	19/55 %	173	3	*Shewanella oneidensis*	Nucleotide Metabolism
158	gil229525168	Phosphoglycerate kinase	41698/4.94	97	17/47 %	455	3	*Vibrio cholerae*	Glycolysis
135	gil89074483	Phosphoenolpyruvate carboxykinase	60262/5.14	101	19/42 %	260	2	*Photobacterium* sp.	Gluconeogenesis
47	gil54303497	Acetyl-CoA acetyltransferase	37969/4.76	–	–	202	2	*Photobacterium profundum*	Fatty acid metabolism
21	gil299130942	ATP synthase F1, beta subunit	50886/5.30	94	17/40 %	395	4	*Alicycliphilus denitrificans*	ATP regenetation
22	gil260774962	ATP synthase beta chain	50938/4.67	87	19/55 %	229	4	*Vibrio coralliilyticus*	ATP regenetation
128	gil90413754	ATP synthase subunit B	50991/4.72	109	20/51 %	453	4	*Photobacterium profundum*	ATP regenetation
150	gil119776776	F0F1 ATP synthase subunit beta	49817/4.86	124	20/59 %	202	2	*Shewanella* sp.	ATP regenetation
68	gil33591762	Amino acid-binding periplasmic protein	36346/6.63	–	–	213	2	*Bordetella pertussis*	Amino acid metabolism
30	gil226329474	Hypothetical protein PROPEN_03383	43281/5.14	78	13/29 %	236	4	*Proteus penneri*	Hypothetical protein
Down-regulation									
26	gil126642747	Dihydrolipoamide succinyltransferase component of 2-oxoglutarate dehydrogenase complex (E2)	39899/5.14	131	20/56 %	334	3	*Acinetobacter baumannii*	TCA pathway
29	gil169632626	Dihydrolipoamide dehydrogenase	51337/5.81	234	30/65 %	439	3	*Acinetobacter baumannii*	TCA pathway
39	gil299535429	NAD-dependent methanol dehydrogenase	43134/5.10	76	14/46 %	201	2	*Lysinibacillus fusiformis*	TCA pathway

Table 1 continued

Spot[a]	GI no.[b]	Protein name	MW/pI[c]	Score (PMF)[d]	PMF/converage[e]	Score (MS–MS)[f]	Pept[g]	Best matching species	Function
186	gil169632935	Superoxide dismutase	22905/5.56	113	10/73 %	386	3	*Acinetobacter baumannii*	Stress/defense response
61	gil126642864	Outer membrane protein A	37342/5.13	215	22/47 %	542	6	*Acinetobacter baumannii*	Stress/defense response
102	gil126642864	Outer membrane protein A	37342/5.13	168	17/41 %	432	4	*Acinetobacter baumannii*	Stress/defense response
3	gil50084094	Elongation factor G	79089/5.05	186	40/49 %	245	3	*Acinetobacter* sp.	Singnal transduction
101	gil162286746	Elongation factor Tu	41531/4.99	123	17/53 %	273	4	*Acinetobacter baumannii*	Singnal transduction
95	gil126642698	Chaperonin GroEL	50038/4.71	187	24/69 %	426	4	*Acinetobacter baumannii*	Protein location
73	gil260771494	Deoxyribose-phosphate aldolase	28107/4.87	103	13/67 %	248	2	*Vibrio furnissii*	Pentose phosphate pathway
23	gil27365141	Glycerol kinase	55997/5.34	222	31/63 %	529	5	*Vibrio vulnificus*	Glycolysis pathway
168	gil184157662	Enoyl-CoA hydratase/carnithine racemase	29184/5.75	165	18/55 %	107	2	*Acinetobacter baumannii*	Fatty acid metabolism
54	gil146281070	Ornithine carbamoyltransferase	38034/6.07	60	18/36 %	174	2	*Pseudomonas stutzeri*	Arginine biosynthesis
55	gil260770814	Ornithine carbamoyltransferase	37864/5.60	64	12/31 %	146	3	*Vibrio furnissii*	Arginine biosynthesis
156	gil126653452	Serine hydroxymethyltransferase	44915/5.44	–	–	170	2	*Bacillus* sp.	Amino acid metabolism
31	gil169796316	Hypothetical protein ABAYE2267	46013/4.93	–	–	173	3	*Acinetobacter baumannii*	Hypothetical protein
33	gil169796316	Hypothetical protein ABAYE2267	46013/4.93	83	12/20 %	422	3	*Acinetobacter baumannii*	Hypothetical protein
34	gil169796316	Hypothetical protein ABAYE2267	46013/4.93	66	9/18 %	295	3	*Acinetobacter baumannii*	Hypothetical protein

[a] The numbering corresponds to the 2-DE gel in Fig. 2

[b] GI number in NCBI

[c] Theoretical p*I* and molecular weight

[d] MASCOT score of PMF

[e] Number of peptides identified by MS/sequence percentage coverage

[f] MASCOT score of MALDI TOF–TOF MS/MS

[g] Number of peptides identified by MS/MS

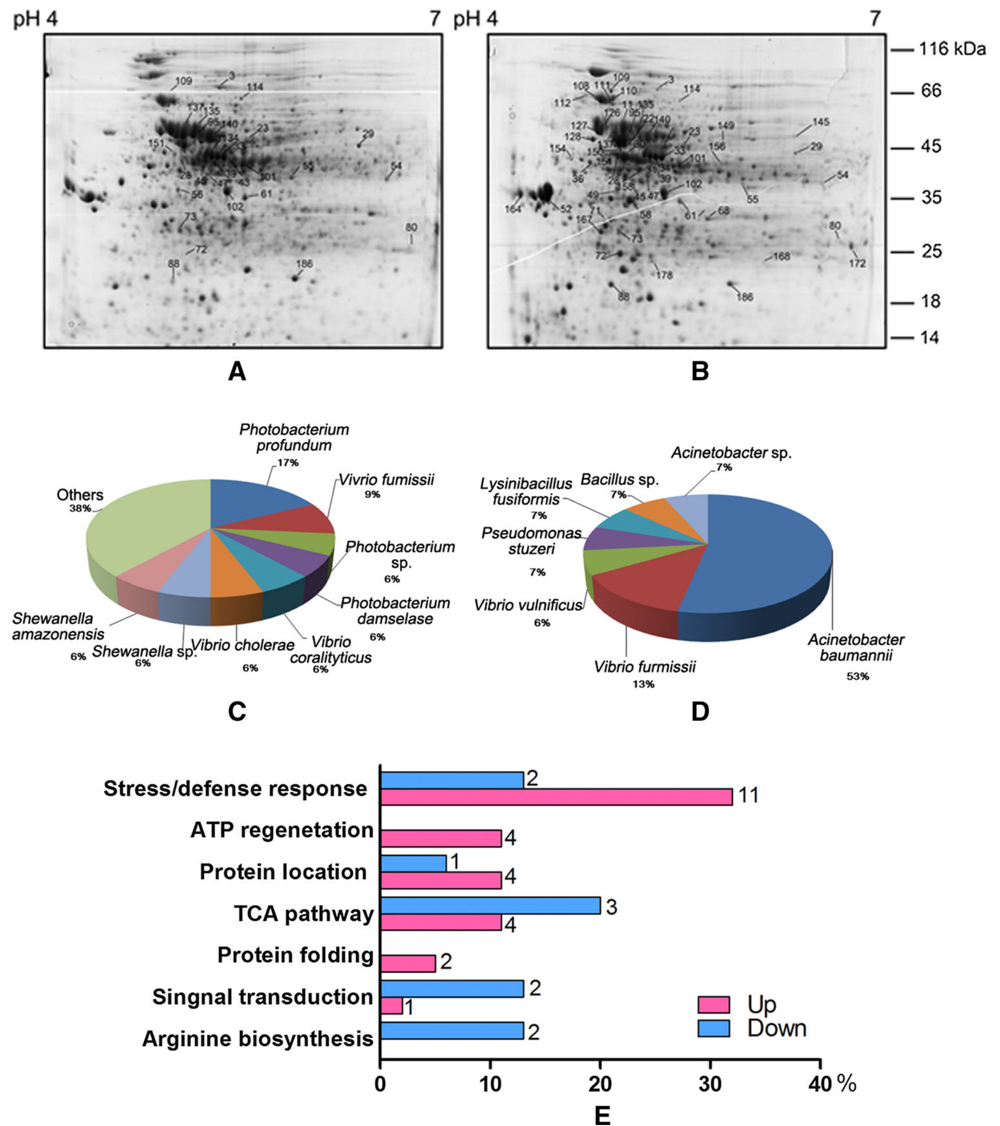

Fig. 3 Proteomics comparison and analysis between aquaculture and natural marine mudflat sediment. **a**, **b** 2-DE map of natural and long-term mudflat aquaculture sediment; **c**, **d** microbial best matching species categories analysis the differentially increasing and decreasing proteins between two comparative groups, respectively. **e** Biological functional categories analysis the percentage of differentially increasing and decreasing proteins between two comparative groups. Each related unique proteins numbers are showed on the *right* of *bars*

analysis on the functional diversity of microbial communities (Maron et al. 2007).

However, due to the physical and chemical complexity of marine sediment and the abundant existence of interfering materials, the development of a direct, efficient, and representative protein extraction from sediment samples has become one of the most daunting tasks for metaproteomic applications (Findlay et al. 1990). In our previous research, using SDS with phenol phase sequential extraction method, we successfully visualized about 1000 separate spots in stained 2-DE gels and identified 189 spots representing 122 proteins in crop soil (Wang et al. 2011a). Since soil in land and sediment in mudflat have partly similar properties due to the fact that they have both complex microbial community habitat and contain similar interfering materials such as humus, we're looking forward to the development of this extraction protocol from soil for application in aquatic sediment protein research. After several optimized steps, a modified protein extraction for marine sediment was established and successfully identified total 151 proteins by mass spectrometry after 2-DE separation.

According to bioinformatics analysis, these identified proteins participated in several important biological process, such as signal transduction, TCA pathway, protein synthesis and stress/defense response. It indicates a complex interrelationship among diverse metabolic pathways in the marine sediment ecosystem. Additionally, there are still substantial protein spots failed to be identified by MS (about 20 %) or have unknown functions (18 %), possibly due to the incomplete environmental metaproteome databases. An improved availability of protein databases derived from environmental samples and *de novo* sequencing strategies will undoubtedly facilitate protein identification, environmental metaproteomic analysis and functional interpretation.

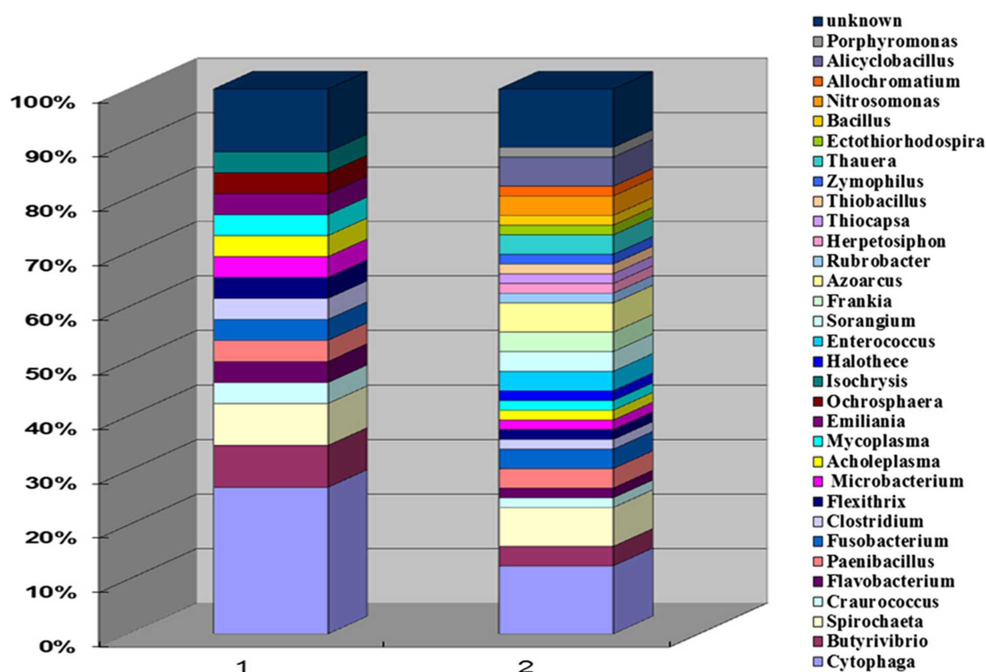

Fig. 4 Terminal restriction fragment length polymorphism (T-RFLP) analysis of (*1*) the relative abundances of bacterial taxa in natural sediment and (*2*) long-term mudflat aquaculture sediment

Furthermore, protein samples from marine long-term mudflat aquaculture sediment and natural mudflat sediment were collected and compared by 2-DE combined with mass spectrometry. A total 53 altered proteins was identified which are involved in various metabolic pathways, including TCA pathway, arginine biosynthesis ATP regeneration, and stress/defense response functions. Of these altered proteins, some heat shock or chaperone proteins which relate to stress conditions (such as Hsp90, GroeL, DnaK) from various microorganisms were found to be abundant in mudflat aquaculture sediment, indicating environmental ecology deterioration after long-term mudflat aquaculture (Jarosz and Lindquist 2010; Selby et al. 2011; Zingaro and Papoutsakis 2012). The detection of these proteins suggests that protein refolding may be integral to pathogens survival in sediment as a response to exposure to environmental stresses (Sowell et al. 2009).

Meanwhile, outer membrane proteins of gram-negative bacteria play important roles on the stress/defense process. OmpU in *Vibrio*, whose homologue mediates the adhesion of *V. mimicus* to host cells and was investigated as a vaccine candidate against *V. harveyi* infection in turbot, was increased abundance in mudflat aquaculture sediment (Liu et al. 2015; Wang et al. 2011b). Furthermore, we found an outer membrane protein, OprD family protein in *Photobacterium* which best matching *P. mendocina* (gi: 146306292) increased after aquaculture cultured. It is interesting that *P. mendocina* is not a common pathogen in fisheries and has been implicated as a candidate for the bioremediation of ecosystems damaged by man-made pollutants (Ramos-Gonzalez et al. 2003). Based on the fact that OprD is a specific porin which facilitates the uptake of basic amino acids and nutrient, this bacteria may play a role as a cleaner on the degradation of organic or toxic compounds in mudflat aquaculture sediment (Tamber and Hancock 2006; Tamber et al. 2006).

Additionally we found several outer membrane proteins which construct the base frame of gram-negative bacterium, fluctuated in different species as well (Kavaliauskas et al. 2012). For example, OmpA was found to be abundant in best matching pathogen *S. amazonensis* but down-regulated in *A. baumannii*. Compared to bacterial community of nature sediment, most of increasing abundant proteins was belong to common pathogens of bivalves, such as *V. furnissii*, *V. cholerae*, *P. profundum* and *S. amazonensis* in clam cultured sediment while down-regulated proteins were found in *A. baumannii*, which presents harmless to most of aquatic livestock (Yue et al. 2010). Thus, the variation tendency of *Vibrio* sp., *Photobacterium* sp. and *Acinetobacter* may be potential indicators for farmed clam ecosystem evaluation. Our findings suggest a complex ecological sediment environment where in various microorganisms compete with one another for nutrition and survival after long-term aquaculture.

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Compliance with Ethical Standards

Conflict of interest The authors have declared that no competing interests exist.

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