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NOTE

Proteomic characterization of the sulfur-reducing hyperthermophilic archaeon *Thermococcus onnurineus* NA1 by 2-DE/MS–MS

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Abstract *Thermococcus onnurineus* NA1, a sulfur-reducing hyperthermophilic archaeon, was isolated from a deep-sea hydrothermal vent area in Papua New Guinea. The strain requires elemental sulfur as a terminal electron acceptor for heterotrophic growth on peptides, amino acids and sugars. Recently, genome sequencing of *Thermococcus onnurineus* NA1 was completed. In this study, 2-DE/MS–MS analysis of the cytosolic proteome was performed to elucidate the metabolic characterization of *Thermococcus onnurineus* NA1 at the protein level. Among the 1,136 visualized protein spots, 110 proteins were identified. Enzymes related to metabolic pathways of amino acids utilization, glycolysis, pyruvate conversion, ATP synthesis, and protein synthesis were identified as abundant proteins, highlighting the fact that these are major metabolic pathways in *Thermococcus onnurineus* NA1. Interestingly, multiple spots of phosphoenolpyruvate synthetase and elongation factor Tu were found on 2D gels generated by truncation at the N-terminus, implicating the cellular regulatory mechanism of this key enzyme by protease degradation. In addition to the proteins involved in metabolic systems, we also identified various proteases and stress-related proteins. The proteomic characterization of abundantly induced proteins using 2-DE/MS–MS enables a better understanding of *Thermococcus onnurineus* NA1 metabolism.

Keywords *Thermococcus onnurineus* NA1 · Hyperthermophilic archaea · Metabolic pathway analysis · 2-DE/MS–MS

Abbreviations

2-DE/MS–MS	Two-dimensional gel electrophoresis and mass spectrometry analysis

Introduction

Hyperthermophiles are microorganisms that can grow optimally at temperatures between 80 and 110°C. These strains are usually isolated from extreme habitats such as deep sea thermal vents or hot springs (Bae et al. 2006; Holden et al. 2001; Wang et al. 2007). Hyperthermophiles are considered to be valuable sources of thermo-stable enzymes, the study of which allow us to better understand the physiology of hyperthermophiles (Conners et al. 2006; Kelly and Adams 1994). Recently, proteomic techniques were employed to study the proteins of hyperthermophile organisms. Proteomic analysis identified proteins with unique or previously unknown novel properties, and the existence of novel metabolic pathways (Barry et al. 2006). Many hyperthermophiles belong to the Archaea domain. Proteomic studies of several thermophilic Archaea have been reported (Chong and Wright 2005; Trauger et al. 2008; Yamazaki et al. 2006). Among the reported strains, *Aeropyrum pemix* and *Sulfolobus solfataricus* are hyperthermophilic crenarchaea, while *Pyrococcus furiosus* is an anaerobic euryarchaeota. However, proteomic analysis of

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Thermococcus, another major genus of euryarchaeota, has yet to be reported.

Thermococcus onnurineus NA1 was isolated from a deep-sea hydrothermal vent area at the PACMANUS field (3° 44′ S, 151° 40′ E) in Papua New Guinea (Bae et al. 2006). This strain is an obligate heterotrophic, preferentially utilizing yeast extract, beef extract, tryptone, peptone, casein and starch. *T. onnurineus* NA1 requires elemental sulfur as a terminal electron acceptor, with an optimal growth temperature of about 80–85°C. Genome sequencing of *T. onnurineus* was recently completed (Lee et al. 2008), where approximately 1,976 coding DNA sequence (CDS) were predicted from the 1.847-Mb genome. From the analysis of genome data, several hyperthermophilic enzymes (DNA polymerase, aminopeptidase P, and carboxypeptidase) have been characterized (Kim et al. 2007; Lee et al. 2006a, b, 2007). Genomic sequencing has also revealed that *T. onnurineus* NA1 has a close relationship with *T. kodakarensis* KOD1 despite the high frequency of DNA rearrangement, and CDSs unique in *T. kodakaraensis* and *T. onnurineus* NA1 have been proposed to explain the ubiquitous distribution and larger populations of *Thermococcus* spp. compared to *Pyrococcus* (Fukui et al. 2005; Lee et al. 2008).

In this study, we performed 2-DE/MS–MS analysis in order to characterize abundant *T. onnurineus* NA1 proteins expressed in enriched medium.

2-DE and Protein identification

The culture and strain maintenance of *T. onnurineus* NA1 was performed according to standard procedures for Archaea (Robb et al. 1995). To prepare seed cultures of *T. onnurineus* NA1, a 25-ml serum bottle of yeast extract-peptone-sulfur (YPS) medium (Holden et al. 2001) was inoculated with a single colony from a phytagel plate and anaerobically cultured at 85°C for 23 h. Seed cultures were then used to inoculate 700 ml of YPS medium in a 1-L anaerobic jar at 85°C for 23 h. After cultivation, cells were harvested by centrifugation (15,000×*g*, 45 min). Harvested cells were disrupted by a French pressure cell (SLM AMINCO, Urbana, IL, USA) at 20,000 lb/in^2. The supernatants (crude cell extracts) were collected by centrifugation (15,000×*g*, 45 min). 2-DE of cytosolic proteins was performed to analyze the abundant proteins of *T. onnurineus* NA1 expressed in YPS culture medium according to previously modified methods (Kim et al. 2006). However, boiling of the protein sample for complete denaturation in buffer I (SDS 0.3%, DTT 0.2 M, Tris–HCl 50 mM) caused most of the proteins to be degraded to below about 30 kDa (data not shown), suggesting the degradation by unidentified heat-stimulated proteases. As a result, boiling step for protein denaturation was omitted in the 2-DE analysis of *T. onnurineus* NA1. The protein sample was applied to immobiline DryStrip pH3–10, 18 cm (GE healthcare) using IPGphor (Amersham Pharmacia Biotech, Uppsala, Sweden). The second dimension of electrophoresis was a 12% polyacrylamide SDS gel electrophoresis using a Hoeffer Dalt system (Pharmacia Biotech). For staining and image acquisition, silver staining kit and image scanner from Amersham Biosciences were used. Each 2-DE was performed three or more times for the verification of induced proteins. It was expected that major metabolic pathways could be identified by analysis of abundant *T. onnurineus* NA1 proteins. In the pH range of 3–10, 1,136 protein spots were detected and spot intensities were quantified by the 2-D image program Progenesis (version 2005) (Fig. 1). As reported (Lee et al. 2008), 1,976 CDSs were predicted from a single circular chromosome (1,847,607 bp) of *T. onnurineus* NA1. The 1,136 visualized protein spots represent ca. 57% of predicted CDSs, although this percentage could have been overestimated by the multiple appearance of a single protein through posttranslational modification (discussed below). Abundant proteins (about 120 protein spots) were selected for identification by MS/MS analysis, where in-gel digestion was conducted in accordance with previously described methods (Kim et al. 2006).

Protein spots on the 2D gel were excised and digested in 50 mM ammonium bicarbonate with 7–8 µl trypsin (0.1 µg/µl) for 12 to 16 hrs at 37°C after the reduction and alkylation of protein cysteines. The digested peptides were then recovered via two extraction steps, using a solution

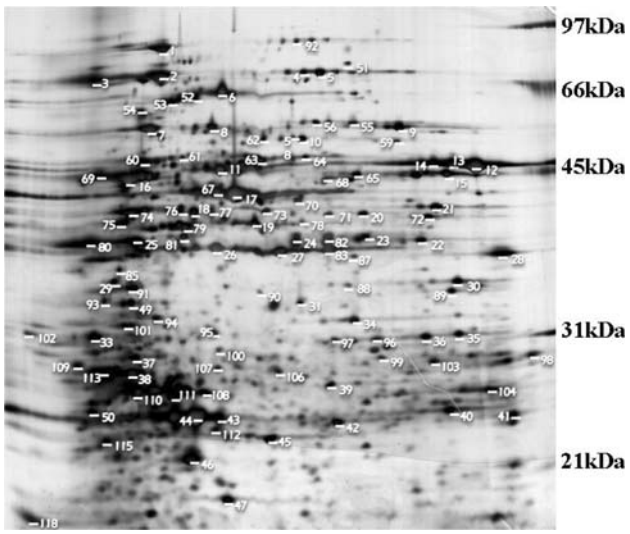

Fig. 1 Representative 2-D gel electrophoresis of soluble *T. onnurineus* NA1 proteome. A measure of 250 µg of soluble proteins were separated on the nonlinear pH 3–10 strip and 12% SDS-PAGE gel electrophoresis. Numbered protein spots were in-gel digested and identified by MS/MS analysis

containing 50 mM ammonium bicarbonate, 50% acetonitrile and 0.1% trifluoroacetic acid (TFA), and lyophilized in a vacuum concentrator. Dried tryptic peptides were dissolved in 0.5% TFA and purified using ZipTipC18. Peptides were eluted onto a MALDI plate after mixing with α-cyano-4-hydroxycinnamic acid (CHCA) matrix solution (10 mg/ml, 0.5% TFA/50% acetonitrile). The digested peptide extracts were then used for MS/MS analysis by a 4700 Proteomic Analyzer (Applied Biosystems, Framingham, MA) according to a previous method (Kim et al. 2006). For protein identification, MS/MS spectra were searched by the MASCOT software (Matrix science, http://www.matrixscience.com, UK) using the genome database of *Thermococcus onnurineus* NA1. Peptide mass tolerance was 50 ppm. Carbamidomethylation of cysteines, oxidation of methionines, and pyroglutamylation of glutamates were considered as protein modification. Proteins harboring at least two significant peptides (≥individual score) were selected from the database search results, from which 110 proteins (more than 90%) were successfully identified. Unique proteins totaled 78 (Table 1), while several protein spots were observed in multiple spots. In particular, phosphoenolpyruvate (PEP) synthetase (no. 1, 2, 7, 25, 26, 47, 53, 81) and elongation factor Tu (no. 12–15, 21, 30, 35, 36, 72, 89) had more than eight multi-spots on the 2-D gel (Fig. 1) and were widely dispersed with a variety of molecular weights. To address the question of why some proteins were present in various forms, Edman sequencing was carried out, whereby protein spots on the 2-D gel were transferred onto a PVDF membrane by the TE77 PWR semi-dry apparatus (Amersham Biosciences) at 0.8 mA/cm^2 for 2 h. Coomassie stained protein spots were excised from the PVDF membrane and installed into the blot cartridge of a model Procise cLC 492 protein sequencer (Perkin-Elmer, Foster City, CA, USA). Interestingly, the Edman sequencing result showed that truncation of the multi-spots happened after the carbonyl side of Arg (R) or Lys (K), similar to the typical cleavage site of trypsin like proteases. Two possibilities for the presence of truncated PEP synthase and elongation factor Tu have to be considered. Firstly, these proteins could be turned over endogenously as part of the metabolic pathway regulation. Two proteins (PEP synthetase and elongation factor Tu) perform the major role of central metabolism and protein synthesis, respectively. In a hydrothermal vent, fluid discharges which sustain the biological communities, can vary by hours, days, and years, as well as by spatial scales. This exposes vent communities to essential nutrient limitations and subsequent ecosystem stress. To thrive within this ecosystem, *T. onnurineus* NA1 may need a rapid regulatory mechanism for central metabolic enzymes. The degradation of proteins involved in central metabolism and translation in this study may be the answer. In 2-DE/MS–MS analysis, several proteases were identified; Proteasome α subunit (no. 101), proteasome β subunit (no. 111), an intracellular protease (no. 6, 52) and deblocking aminopeptidase (no. 19). Since proteasome cleaves the substrate in a random and processive manner, the cleavage seems unlikely to have been made by proteasome activity. Genome analysis showed that *T. onnurineus* NA1 contains more than 20 proteases (Lee et al. 2008). Considering the partial representation of the protein pool in 2-DE/MS–MS analysis, the pinpoint of a protease responsible for cleavage needs further investigation. Secondly, despite the effort to minimize sample modification and damage, we cannot exclude the possibility that the in vitro degradation of these proteins may also take place after cell disruption which has nothing to do with endogenous degradation of proteins. However, the possibility of in vitro digestion after cell disruption seems unlikely because a protease inhibitor was used during the sample preparation and protease digestion was not detected in other abundant proteins.

Characterization of abundant proteins

Genomic analysis of *T. kodakarensis* KOD1 and *T. onnurineus* NA1 revealed that the genomes encoded proteins for central metabolic pathways, such as glycolysis of various carbon substrates, through a modified EMP pathway, pyruvate conversion to acetic acid or alanine without TCA cycle involvement, versatile amino acid degradation, sulfur reduction as a final electron acceptor, and ATP generation in tandem with hydrogenase and ATPase (Fukui et al. 2005; Lee et al. 2008). In proteomic analysis, we identified key enzymes of major metabolic pathways proposed from genome analysis. As shown in Table 1, enzymes involved in metabolism of pyruvate, glycolysis, amino acids, glyoxylate, ATP synthesis, and proteins synthesis were abundantly present. In particular, enzymes for glycolysis and pyruvate conversion were found as major proteins on the 2D gel. For example, glyceraldehydes-3-phosphate dehydrogenases (no. 23), phosphopyruvate hydratase (no. 16), and phosphoenolpyruvate synthetase (no. 1, 2, 7, 25, 26, 47, 53, 81) for glycolysis were identified. Glutamate dehydrogenase (no. 17, 67), ferrodoxin oxidoreductase (no. 28), and acetyl-CoA synthetase (no. 38, 103) were also identified, which are involved in pyruvate conversion into alanine and acetyl-CoA. In particular, PEP synthetase was highly expressed in our culture condition (YPS medium). PEP synthetase is known to reversibly convert phosphoenolpyruvate (PEP) to pyruvate with the involvement of ATP and AMP. However, in *T. kodakarensis*, this enzyme was reported to perform glycolytic conversion of PEP to pyruvate (Imanaka et al. 2006). Our proteomic result also suggests that PEP synthetase is a major protein in yeast

Table 1 Identification of protein spots on 2D gel by MS/MS analysis using MALDI-TOF/TOF MS

Spot no.[a]	Gene no.[b]	MW/PI	Protein annotation	Related metabolic pathway[c]	Score[d] MS (MS^2)	Match[e] MS (MS^2)	Coverage[f] MS (MS^2)
3	Ton_0987	73577/5.08	Metallophosphoesterase, calcineurin superfamily		125 (179)	20 (4)	41 (8)
6*	Ton_1285	18693/5.36	Intracellular protease I		157 (396)	26 (5)	74 (46)
8	Ton_1001	51536/5.43	Acyl-CoA synthetase (NDP forming), large subunit	Amino acid metabolism	147 (333)	22 (5)	53 (13)
9*	Ton_0755	81872/6.39	Elongation factor EF-2	Protein synthesis	227 (376)	35 (5)	44 (11)
10	Ton_0041	56661/6.02	Methylmalonyl-CoA decarboxylase, alpha subunit	Propanoate metabolism	288 (398)	33 (5)	60 (18)
11	Ton_1234	51060/5.5	Methionine sulfoxide reductase A	Carbon fixation Glyoxylate and dicarboxylate metabolism	181 (282)	24 (5)	64 (12)
16	Ton_1613	46789/4.94	Phosphopyruvate hydratase	Pyruvate metabolism	186 (541)	24 (4)	70 (15)
18*	Ton_0249	42261/5.31	Myo-inositol-1-phosphate synthase	Streptomycin biosynthesis	257 (501)	28 (5)	79 (25)
19	Ton_1032	37917/5.67	Deblocking aminopeptidase	Starch and sucrose metabolism	220 (427)	24 (5)	81 (22)
20*	Ton_0061	42775/6.23	Probable serine-glyoxylate aminotransferase, class V		250 (190)	31 (4)	61 (17)
22	Ton_1301	38019/7.07	Hypothetical protein	Hypothetical protein	90 (245)	14 (4)	47 (22)
23	Ton_0639	37100/6.21	Glyceraldehyde-3-phosphate dehydrogenase	Glycolysis and gluconeogenesis	104 (269)	16 (5)	51 (19)
27*	Ton_1296	36008/5.77	Translation initiation factor IF-2B subunit delta	Glyoxylate and dicarboxylate metabolism One carbon pool by folate	189 (510)	23 (5)	68 (25)
28	Ton_1482	36067/9.04	Ferrodoxin oxidoreductase beta subunit	Reductive carboxylate cycle (CO2 fixation) Butanoate and propanoate and pyruvate metabolism	127 (223)	20 (5)	68 (31)
29	Ton_0105	29543/4.77	DNA-directed RNA polymerase subunit D	RNA polymerase Pyrimidine metabolism Purine metabolism	91 (151)	12 (3)	38 (13)
31	Ton_1067	31203/5.79	Dipeptide transport protein dppa	ABC transporters—general	63 (86)	9 (3)	36 (14)
34	Ton_1463	33193/6.06	D-3-phosphoglycerate dehydrogenase	Glycine, serine and threonine metabolism	193 (194)	21 (5)	54 (24)
35*	Ton_0752	47470/8.42	Elongation factor Tu	Protein synthesis	– (145)	– (4)	– (14)
37	Ton_0620	31446/5.38	Chromosome partitioning protein ParB homolog		85 (110)	13 (3)	46 (14)
38	Ton_0327	25784/4.98	Acetyl-CoA synthetase I (NDP forming), beta subunit	Pyruvate metabolism	103 (479)	13 (5)	67 (28)
40*	Ton_1476	19875/6.17	Indolepyruvate ferredoxin oxidoreductase	Pyruvate and butanoate and propanoate metabolism	69 (94)	8 (2)	46 (17)
41	Ton_0082	20828/9.13	50S ribosomal protein L6	Ribosome	57 (36)	8 (2)	48 (16)
44*	Ton_0829	24711/5.4	Putative peroxiredoxin		220 (427)	21 (5)	91 (23)
45	Ton_1311	21855/5.69	Indolepyruvate ferredoxin oxidoreductase	Pyruvate and butanoate and propanoate metabolism	118 (221)	18 (4)	72 (27)
46	Ton_0866	19450/5.48	Rubrerythrin		122 (288)	16 (5)	73 (26)

Table 1 continued

Spot no.[a]	Gene no.[b]	MW/PI	Protein annotation	Related metabolic pathway[c]	Score[d] MS (MS^2)	Match[e] MS (MS^2)	Coverage[f] MS (MS^2)
47*	Ton_0311	86682/5.17	Phosphoenolpyruvate synthetase	Pyruvate metabolism	– (199)	– (3)	– (3)
50	Ton_0826	28121/4.45	DNA polymerase III subunit beta		42 (98)	7 (3)	37 (22)
51	Ton_0644	8623/6.08	Small nuclear ribonucleoprotein		34 (57)	4 (2)	60 (37)
54	Ton_0395	66000/4.98	Phenylalanyl-tRNA synthetase beta subunit	Aminoacyl-tRNA biosynthesis	144 (59)	22 (2)	43 (4)
				Phenylalanine, tyrosine and tryptophan biosynthesis			
55	Ton_1282	54729/6.2	Sarcosine oxidase, alpha subunit	Glycine, serine and threonine metabolism	279 (172)	35 (4)	77 (10)
56	Ton_0214	55898/5.94	Glycine dehydrogenase subunit 2	Glycine, serine and threonine metabolism	125 (91)	21 (4)	49 (13)
58	Ton_0568	56148/5.75	Prolyl-tRNA synthetase	Aminoacyl-tRNA biosynthesis	128 (38)	21 (3)	40 (7)
				Arginine and proline metabolism			
59	Ton_0129	48628/6.65	NADH:polysulfide oxidoreductase	Oxidative phosphorylation	50 (27)	10 (1)	29 (3)
60	Ton_1785	47555/5.03	Succinyl-diaminopimelate desuccinylase	Lysine biosynthesis	133 (51)	18 (3)	52 (10)
61	Ton_0403	49132/5.35	Glutamyl-tRNA(Gln) amidotransferase subunit D	Glutamate metabolism	49 (103)	12 (4)	27 (10)
62	Ton_0305	48372/5.66	NADH oxidase		179 (147)	24 (4)	53 (11)
63	Ton_1605	49348/5.66	4-Aminobutyrate aminotransferase	Butanoate and propanoate and beta-alanine and alanine and aspartate and glutamate metabolism	226 (368)	29 (5)	65 (17)
64*	Ton_0850	53318/5.43	Hypothetical protein	Hypothetical protein	190 (114)	27 (4)	60 (8)
65	Ton_0704	42764/6.09	Hypothetical protein	Hypothetical protein	67 (63)	13 (2)	36 (8)
67*	Ton_0157	46755/5.48	Glutamate dehydrogenase	Nitrogen metabolism	202 (482)	26 (5)	57 (12)
				Arginine and proline metabolism			
				Glutamate metabolism			
68	Ton_1944	44918/6.13	Translation initiation factor IF-2 gamma subunit	Purine metabolism	189 (227)	26 (5)	53 (17)
				Selenoamino acid metabolism			
				Sulfur metabolism			
69	Ton_0983	54055/5.01	ABC-type iron(III) transport system, periplasmic component		70 (71)	11 (4)	30 (13)
70	Ton_0316	44102/5.79	Geranylgeranyl hydrogenase		261 (277)	29 (5)	61 (13)
73	Ton_1281	43165/5.7	Sarcosine oxidase, beta subunit	Glycine, serine and threonine metabolism	93 (97)	15 (2)	37 (7)
74	Ton_1302	40867/4.88	Acetyl-CoA acetyltransferase	Pyruvate metabolism	109 (243)	17 (5)	51 (19)
75	Ton_1358	40159/4.83	Hypothetical protein	Hypothetical protein	150 (334)	20 (5)	64 (23)
77	Ton_0361	44398/5.51	Hypothetical protein	Hypothetical protein	232 (221)	27 (5)	61 (16)
78	Ton_0481	40844/5.87	x-Pro dipeptidase		172 (113)	23 (4)	61 (10)

Table 1 continued

Spot no.[a]	Gene no.[b]	MW/PI	Protein annotation	Related metabolic pathway[c]	Score[d] MS (MS^2)	Match[e] MS (MS^2)	Coverage[f] MS (MS^2)
79	Ton_0369	38061/5.33	Deblocking aminopeptidase	Starch and sucrose metabolism	200 (465)	27 (5)	78 (24)
80	Ton_0594	44147/4.65	Sugar binding protein		106 (292)	18 (5)	48 (16)
82*	Ton_1603	35790/6.28	Thioredoxin reductase	Pyrimidine metabolism	202 (285)	24 (4)	65 (15)
83	Ton_0435	35058/5.95	Ornithine carbamoyltransferase	Arginine and proline metabolism Urea cycle and metabolism of amino groups	175 (421)	22 (5)	84 (21)
85*	Ton_1014	33143/4.9	Hypothetical protein	Hypothetical protein	95 (99)	14 (3)	42 (12)
88	Ton_1752	65512/5.25	V-type ATP synthase subunit A	ATP synthesis	80 (163)	16 (3)	27 (7)
92	Ton_1816	103946/ 5.98	Ribonucleotide-diphosphate reductase alpha subunit	Pyrimidine metabolism Purine metabolism	230 (190)	34 (4)	42 (5)
93	Ton_1478	44246/5.01	2-Ketovalerate ferredoxin oxidoreductase subunit alpha	Reductive carboxylate cycle (CO2 fixation) Butanoate and propanoate and pyruvate metabolism	130 (245)	21 (5)	46 (11)
94	Ton_0386	29856/5.09	Dihydropteroate synthase	Folate biosynthesis	126 (128)	17 (4)	57 (20)
95	Ton_0332	33020/5.9	Probable transcription regulator, DUF118 helix-turn-helix family		102 (362)	15 (5)	52 (25)
97	Ton_0131	30294/6.16	Uridine phosphorylase	Pyrimidine metabolism	48 (91)	8 (4)	35 (23)
98	Ton_0086	26252/9.51	30S ribosomal protein S5P	Ribosome	72 (88)	12 (4)	49 (21)
99	Ton_1651	29024/6.46	5′-Methylthioadenosine phosphorylase		125 (163)	20 (5)	70 (23)
100	Ton_0117	28861/5.46	Hypothetical protein	Hypothetical protein	104 (229)	13 (5)	54 (26)
101	Ton_0027	28718/4.87	Proteasome subunit alpha		60 (239)	10 (3)	43 (14)
102	Ton_0181	37007/4.69	50S ribosomal protein L10	Ribosome	– (110)	– (3)	– (14)
103	Ton_1097	49879/5.51	Acetyl-CoA synthetase I (NDP forming), alpha subunit	Pyruvate metabolism	– (161)	– (2)	– (7)
104	Ton_0112	23030/8.67	30S ribosomal protein S2	Ribosome	– (148)	– (4)	– (25)
106*	Ton_1312	70727/6.05	Indolepyruvate: ferredoxin oxidoreductase, alpha subunit	Pyruvate metabolism	– (71)	– (2)	– (6)
107	Ton_0281	76996/6.4	Alpha subunit of formate dehydrogenease	Methane metabolism Glyoxylate and dicarboxylate metabolism	44 (150)	13 (4)	22 (8)
108	Ton_1314	24293/5.35	Metal-dependent hydrolase		70 (188)	11 (4)	70 (19)
109	Ton_0168	25110/4.38	Ribose-5-phosphate isomerase A	Carbon fixation Pentose phosphate pathway	– (114)	– (3)	– (21)
110*	Ton_1295	46798/5.44	Multiple substrate aminotransferase		– (493)	– (5)	– (26)
111	Ton_1426	21739/5.35	Proteasome, beta subunit 2		– (142)	– (3)	– (21)
113	Ton_0319	25496/4.67	Protein disulfide oxidoreductase		120 (243)	20 (5)	66 (31)
115	Ton_0852	20318/4.66	Putative ferritin		64 (228)	10 (4)	60 (20)
117*	Ton_1415	43960/5.52	8-Amino-7-oxononanoate synthase	Biotin metabolism	– (101)	– (2)	– (8)

Table 1 continued

Spot no.[a]	Gene no.[b]	MW/PI	Protein annotation	Related metabolic pathway[c]	Score[d] MS (MS^2)	Match[e] MS (MS^2)	Coverage[f] MS (MS^2)
118	Ton_0786	25941/9.43	Peroxiredoxin, bacterioferritin comigratory protein homolog, AhpC/TSA family		51 (70)	8 (2)	45 (11)

*These proteins were identified as multi spots on the 2D gel. Other proteins are described as below

9; 9,96/64; 64,90/6; 6,52/85; 49,85/44; 43,44,112/110; 42,110/106; 4,5,106/40; 39,40/117; 33,117/27; 27,87/82; 24,82

20; 20,71/18; 18,76,91/67; 17,67/35; 12,13,14,15,21,30,35,36,72,89/47; 1,2,7,25,26,47,53,81

[a] Spot numbers refer to those in Fig. 1

[b] Accession number of *T. onnurineus* NA1 (Lee et al. 2008)

[c] Databases of *Thermococcus kodakarensis* KOD1, *Pyrococcus abyssi* GE5, *Pyrococcus furiosus* DSM 3638, and *Pyrococcus horikoshii* OT3 were used for KEGG pathway analysis

[d] Mascot Score indicates the confidence of the protein identification. "–" not examined

[e] Number of peptides used for identification. In case of MS^2, maximum score is 5

[f] Amino acid coverage (%) of matched peptides

extract-peptone-sulfur (YPS) culture medium. In addition to glycolysis, several proteins for amino acid metabolism were strongly expressed in our culture condition. Glutamate dehydrogenase (no. 17, 67), indolepyruvate ferredoxin oxidoreductase (no. 4, 5, 106, 39, 40, 45), ferrodoxin oxidoreductase (no. 28), and 2-ketovalerate ferredoxin oxidoreductase (no. 93) appeared involved in the catabolic degradation of amino acids. Succinyl-diaminopimelate desuccinylase (no. 60), sacrosine oxidase (no. 55, 73), and D-3-phosphglycerate dehydrogenase (no. 34) participate in the metabolism of lysine, glycine, and serine, respectively. Dipeptide transport protein dppa (no. 31) and x-pro dipeptidase (no. 78), known to function in peptide uptake and degradation, were identified in our culture condition. It was assumed that the catalytic pathway to utilize amino acids or oligopeptides may be generating energy for growth in the condition of YPS culture medium. The expression of transport and catabolic pathways toward amino acids is consistent with this hypothesis.

The house-keeping proteins related to replication (no. 37, 50, 51), transcription (no. 29), translation (no. 27, 41, 68, 87, 102, 104), tRNA synthase (no. 54, 58, 61), lipid formation (no. 18, 76, 91) and ATP generation (no. 88) were identified as abundant proteins. As reported (Lee et al. 2008), *T. onnurineus* NA1 lost enzymes needed for 5′-phophoribosyl-4-carboxamide-5′-aminoimidazole (AICAR) in purine biosynthetic pathway, implicating that the salvage pathway of purines or histidine biosynthetic pathway is essential to supply the purine building blocks. As a matter of fact, to compensate the loss of de novo biosynthetic pathway, the genome encoded unique enzymes for efficient recycling of deoxynucleotides (manuscript in preparation). As seen in Table 1, we could identify two of the recycling enzymes, uridine phosphorylase (no. 97) and 5′-methylthioadenosine phosphorylase (no. 99), indicating that *T. onnurineus* NA1 uses the recycling pathway for sustainable supply of deoxynucleotides.

Stress related proteins

In hyperthermophilic archaea, it has been proposed that various heat-stable proteases and chaperones repress aggregation of unfolded proteins and degrade damaged proteins, regarded as an essential mechanism to live at extreme high temperatures. In line with this proposition, we found several proteases in 2-DE/MS–MS analysis; deblocking aminopeptidase (no. 19), deblocking aminopeptidase (no.79), intracellular protease I (no.6, 52), and proteasome (no.101, 111). The high occurrence of proteases might be also be related to the utilization of oligopeptides in the medium. It is worthwhile to note that two deblocking aminopeptidases (DAP) were abundantly expressed. The physiological roles of DAPs in hyperthermophilic archaea are largely unknown. However, considering that deblocking aminopeptidases (DAPs; EC 3.4.11.-) act at amino-terminals of the polypeptide chain and release amino acids from peptides, it has been suggested that the self-compartmentalizing quaternary structures of DAPs could contribute to maintaining protein quality in concert with ATP-dependent protease system, protein maturation or the metabolism of peptides from proteasomes (Dura et al. 2005; Mori and Ishikawa 2005). In *T. onnurineus* NA1, four copies of DAPs are encoded in the genome displaying different substrate specificities (Lee et al. 2008). The identification of two DAPs indicates that

the enzymes are playing a major role in protein quality control or hydrolyzing peptides in vivo. Several proteins involved in oxidative stress, such as thioredoxin reductase (no. 24, 82), putative peroxiredoxin (no. 43, 44, 112), rubrerythrin (no. 46), NADH oxidase (no. 62), and peroxiredoxin (no. 118) were found. The presence of multiple proteins, assumed to act on oxygen detoxification, indicate that *T. onnurineus* NA1, an obligate anaerobe, constitutively express defensive proteins against oxidative stress. In genomic analysis of *T. onnurineus* NA1, 1,104 CDSs (55.8%) of 1,976 coding DNA sequences (CDSs) were annotatable by homology and domain searches. However, the function of residual 872 CDSs could not be predicted from primary structure. In 2-DE/MS–MS, several hypothetical proteins (no. 49, 64, 65, 75, 77, 85, 100) were abundantly expressed, indicating that they play a role in the physiology of *T. onnurineus* NA1. To understand the functionality of those hypothetical proteins, further analysis of cloning and biochemical characterization is required.

Conclusions

Thermococcus onnurineus NA1 was isolated from a deep-sea hydrothermal vent area. We identified more than 110 abundant proteins from *T. onnurineus* NA1, which was anaerobically cultured in YPS medium at 85°C, using 2-DE/MS–MS. Glycolysis, pyruvate, and amino acid pathways were confirmed to be major energy and carbon metabolism pathways in this strain. In particular, turnover of abundant proteins, such as phosphoenolpyruvate synthetase and elongation factor Tu, was fast. The proteomic characterization of abundantly induced proteins using 2-DE/MS–MS enabled a better understanding of bacterial metabolism. To the best of our knowledge, this study is the first proteomic analysis of a thermophilic marine archaeon (*Thermococcus* sp). Although 110 proteins were identified as abundantly induced, they only represent 10% of visualized protein spots. Ultimately, more proteins should be identified for accurate metabolic characterization of *T. onnurineus* NA1. Recently, shotgun proteomic analysis using ESI-MS spectrometry was carried out for high-throughput identification of marine microorganism proteome (Yamazaki et al. 2006). For further protein identification, we applied this technology to cytosol protein mixtures of *T. onnurineus* NA1 and identified more than 600 proteins (Kim et al. in preparation). Comparative analysis of 2-DE/MS–MS and 1-DE/MS–MS will be performed. However, because 1-DE/MS–MS can only provide information on tryptic peptide mixtures, 2-DE analysis are still useful for characterization of proteome. The 2D reference map can also be useful for screening of *T. onnurineus* NA1 hyperthermophilic proteins by comparing the differently heat-treated proteomes on 2D gels (Prosinecki et al. 2006). Genomic analysis of *T. onnurineus* NA1 revealed that this archaeon induced heterotrophic and carboxydotrophic metabolism according to the different substrates and growth conditions (Lee et al. 2008). This reference map will be used for the study of metabolic shift in *T. onnurineus* NA1. Another application of the reference map is proteomic analysis of other Archaea such as *T. kodakarensis* and *P. furiosus* (Fukui et al. 2005; Trauger et al. 2008). Because *T. kodakarensis* is closely related with *T. onnurineus* NA1, proteomic data of *T. onnurineus* NA1 will be useful for the interpretation of *T. kodakarensis* (Lee et al. 2008). However, a proteomic study of *T. kodakarensis* is yet to be reported. Several identified proteins (phosphoenolpyruvate synthetase, DNA-directed RNA polymerase, indolepyruvate ferredoxin oxidoreductase, etc.) in this study were reported to relate to cold-shock stress in *P. furiosus* (Trauger et al. 2008).

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