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Comparison of Protein Expression Profiles of Novel *Halomonas smyrnensis* AAD6[T] and *Halomonas salina* DSMZ 5928[T]

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Abstract

In this work, the protein pattern of novel *Halomonas smyrnensis* AAD6[T] was compared to that of *Halomonas salina* DSMZ5928[T], which is the closest species on the basis of 16S rRNA sequence, to understand how AAD6[T] differs from type strains. Using high resolution NEPHEGE technique, the whole cell protein composition patterns of both *Halomonas salina* DSMZ5928[T] and *H. smyrnensis* AAD6[T] were mapped. The expressed proteins of the two microorganisms were mostly located at the acidic side of the gels, at molecular weight values of 60 to 17 kDa, and at isoelectric points 3.8 to 6.0, where they share a significant number of common protein spots. Identification and characterization of protein spots via whole genome sequencing data indicated that these two microorganisms used similar pathways, especially TCA cycle, for their survival; in other words, for their energy requirements. On the other hand, the protein expression differences in AAD6[T] and *H. salina* DSMZ 5928[T] showed that they prefer different metabolic pathways for lipid biosynthesis and in adaptation to extreme environments. Thus, we suggested that phylogenetic dissimilarities between these microorganisms could be related to the protein expression differences; in other words, metabolic flux differences in AAD6[T] and *H. salina* DSMZ 5928[T]. This is the first study to explain the dissimilarities of phenotypic characters and DNA-DNA hybridization between type strain and novel strain AAD6[T] by using protein expression differences.

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*Addresses belong to the Institutions where the study was conducted. #Currently Prof. Wittmann and Prof. Erdmann retired.
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Keywords

1. Introduction

All over the world, an important part of the diversity of life are microorganisms, especially extremophilic microorganisms that have added a new dimension to biodiversity and could represent the direct descending of ancestral forms of life [1]. Due to their ability to adapt very hard environmental conditions like extreme temperature, pH, pressure and salinity, some of the extremophiles may be considered as "living fossils" [2] [3]. According to the requirements of the extreme parameters (temperature, pH, pressure and salinity) for survival; psychrophiles, thermophiles, acidophiles, alkaliphiles, barophiles and halophiles are the more common phenotypes. Within this group, moderately halophilic microorganisms have been in the center of industrial interest in the last decades owing to their growth in a wide range of salt concentrations.

Moderately halophilic novel bacterium, *Halomonas smyrnensis* AAD6[T] from Çamalti saltern, Turkey, produces levan, a fructose homopolymer with many potential uses in various industries [4] [5]. *Halomonas smyrnensis* is Gram-negative, facultatively aerobic, rod-shaped and exopolysaccharide (levan) producing bacterium. It is a moderate halophile that grows optimal at 10% NaCl but even tolerates NaCl concentration up to 25%. It requires magnesium ions for growth, but it doesn't require SO_4^{2-} or K^{1+}. 16S rRNA gene sequence similarities between strain AAD6[T] and type strains were 99.4% for *Halomonas salina* F8-11T, 99.4% for *Halomonas halophila* CCM 3662T, 98.1% for *Halomonas maura* S-31T, 98.0% *Halomonas organivorans* G-16.1T, 97.4% for *Halomonas koreensis* SS20T, 97.2% for *Halomonas elongata* DSM 2581T and 97.1% for *Halomonas EX nitroreducens* 11ST [4] [5]. Phylogenetic analysis of the draft genome of *H. smyrnensis* [6] showed that *Halomonas salina* (99.5%) and *Halomonas halophila* (99.5%) were found to be the closest species on the basis of 16S rRNA sequence comparison. However, according to DNA-DNA hybridization and phenotypic characteristics, strain AAD6[T] is distinguished from the type strains of closely related species as *Halomonas salina*, *Halomonas halophila* and other species of the genus *Halomonas*. For example, strain AAD6[T] differs from *H. salina* in the hydrolysis of tyrosine, in the absence of phenylalanine deaminase activity and for the reduction of nitrate. Additionally, strain AAD6[T] was oxidase-negative and able to hydrolyse starch, casein and Tween 80, while *H. salina*, was not. Also, strain AAD6[T] produced acid from glucose and mannose, while *H. salina* did not.

Proteomics are widely used to analyze the expressional changes of bacterial proteins at defined physiological conditions. Identification of differentially expressed proteins under given physiological conditions by proteomic analysis has gained fundamental importance for functional studies of cellular processes in recent years [7]. High-resolution two-dimensional electrophoresis (2-DE) is still one of the most powerful methods to separate thousands of proteins at once [8]. The availability of genome sequence information for a microorganism is crucial in proteomics studies, since the microbial genome sequence is the starting point for detailed analysis of identifying gene-protein associations.

In order to understand the dissimilarities between AAD6[T] and type strain, we compared the differential expression profiles of *Halomonas smyrnensis* AAD6[T] with closely related species *Halomonas salina* DSMZ 5928[T] on the basis of 16S rRNA sequence comparison. In this work, 2-DE by the NEPHGE technique [9] [10] followed by MALDI-TOF/MS, NANO-LC-ESI-Q TOF MS/MS, N-terminal sequencing and draft genome sequence of *Halomonas smyrnensis* AAD6[T] were used to compare the differential expression profiles of *Halomonas salina* DSMZ 5928[T] and *Halomonas smyrnensis*. This is the first study comparing the protein expression profiles of novel species and type strain to understand how closest species on the basis of 16S rRNA sequence differ from one another according to DNA-DNA hybridization and phenotypic characteristics.

2. Materials and Methods

2.1. Bacterial Strains and Growth Conditions

Halomonas salina (DSMZ No 5928[T]) was supplied from Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Germany. AAD6[T] was isolated from the soil samples of Çamalti saltern area near Izmir, Turkey

[4]. Both microorganisms were cultured by shaking at 180 rpm at 37˚C in 250 ml flasks containing 100 ml of Brown medium containing 0.5% (w/v) yeast extract, 0.3% (w/v) trisodium citrate, 2% (w/v) magnesium sulphate heptahydrate, 0.2% (w/v) potassium chloride, 5% (w/v) sodium chloride at a pH of 7.0.

2.2. Sample Preparation and 2-DE Separation of Bacterial Proteins

Samples were harvested at their late exponential phase by centrifugation at 5000 rpm for 10 minutes at 4˚C and were washed two times with 50 mM Tris buffer (pH 7.8). The whole protein extractions of *H. salina* DSMZ 5928[T] and AAD6[T] were carried out by the SIGMA total protein extraction kit (SIGMA PROT-TOT) according to the manufacturer's instructions.

Protein concentrations of the whole cell extracts were determined by employing the Bradford protein assay [11], using BSA as a protein standard. Prepared samples were stored at −80˚C until their application to 2-DE. Bacterial proteins were separated by large gel 2-DE PAGE (30 × 23 × 0.15 cm) with the NEPHGE technique as described by Klose and Kobalz [12]. In the first dimension, bacterial samples were separated on capillary rods (for analytical gels, capillaries with inner diameter of 0.9 mm, and for preparative gels 1.5 mm inner diameter were used with lengths of 23 cm) using polyacrylamide gels containing 9 M urea, 3.5% acrylamide, 0.3% piperazine diacrylamide and 4% ampholyte mixture providing a pH range of 3 - 7.5. For the analytical and preparative gels, 80 µg and 150 µg of whole cell protein extracts were loaded respectively. The electrophoresis was run starting from 100 V for 60 minutes following 200 V for 60 minutes, 400 V 1050 minutes, 600 V 60 minutes and 1000 V for 45 minutes, respectively. At the end of the run, samples were incubated for 10 minutes at room temperature with gentle shaking in 125 mM Tris buffer containing 40% Glycerin (w/v), 3% SDS (w/v) and 64 mM DTT.

For the second dimensional, sodium dodecyl sulphate-PAGE was performed in 15% separating gels. SDS-PAGE electrophoresis was performed at 65 mA for 15 minutes followed by 100 mA for 400 minutes. The analytical gels were silver stained [13] and preparative gels were stained with coomassie G-250. The gels of each sample were dried between cellophanes under vacuum. Dried samples were scanned and analysed.

2.3. Mass Spectrometer Analyses for Protein Identification

Protein gel spots of interest were excised from the gels with a scalpel and transferred to clean 500 µl Eppendorf tubes. Trypsin solution at a concentration of 0.4 µg/µl was added to the gel pieces and incubated overnight at 37˚C to digest. Trypsin-digested proteins were eluted with a buffer containing 85% MeCN/0.1% TFA. Extracts were dried by a speed-vac concentrator and kept at −20˚C until further analysis by mass spectromety.

Two different MS techniques were applied to various selected protein samples. One of the MS method applied was MALDI-TOF/MS (Waters/Micromass) with a positive ion mode reflection system. The digested and dried protein samples were dissolved in elution buffer containing 85% acetonitrile and 0.1% TFA, mixed 1:1 (v/v) with a freshly prepared matrix solution and spotted onto the MALDI target. Peptides were analysed with MALDI-TOF/MS.

The other MS technique applied was NANO-LC-ESI-Q TOF MS/MS (Micromass). The dried and digested samples were resolved with 0.1% formic acid solution and injected into the sample loop of the MS system. Mass spectra of the samples were recorded in the Nano LC ESI-QTOF MS/MS mass spectrometer. The peptide fingerprint spectra of proteins were identified in the protein database using the MASCOT search engine, http://www.matrixscience.com.

2.4. N-Terminal Sequencing and Genome Sequence of *H. smyrnensis* AAD6[T]

Following the two-dimensional electrophoresis, gels of newly isolated *Halomonas smyrnensis* AAD6[T] were blotted on PVDF membranes with a semi dry blotter (Bio-Rad) using Towbin buffer. The blotting conditions were 300 mA for two hours in the cold room. Blotted membranes were stained using coomassie R-250, dried and kept in a clean environment until N-Terminal sequencing. CBB-R 250 stained protein spots on PVDF membranes were cut out very carefully with a scalpel and placed into the sequencer cartridge. After applying the membrane to the cartridge, Applied Biosystems Model 492A Procise sequencer (WITA GmbH, Teltow, Germany) was run automatically according to the manufacturer's directions.

The genome sequence of *H. smyrnensis* AAD6[T] was recently published [6] and deposited at DDBJ/EMBL/

GenBank under the accession AJKS00000000. The sequence data used in the present study is the second version, with accession numbers AJKS02000001 to AJKS02000034. Based on the sequence data, gene prediction and genome annotation were carried out using the RAST auto-annotation server [14]. The gene function and classifications were based on the subsystem annotation of the RAST server. Information on enzyme encoding genes was taken from Kyoto Encyclopedia of Genes and Genomes (KEGG) [15] and Expasy databases [16]. Transport protein coding genes were annotated using the similarity searches against the Transfer Classification Database (TCDB) [17].

3. Results and Discussion

During the last decade, the extensive studies on hypersaline environments carried out in many geographical areas have permitted the isolation and taxonomic characterization of a large number of moderately halophilic species. Although some Gram-negative species were considered members of different genera, phenotypic and phylogenetic data support their close relationship, and they are currently included in the family *Halomonadaceae* as members of two genera: *Halomonas* and *Chromohalobacter* [18] [19]. *Halomonas smyrnensis* strain AAD6[T] represents a novel species within the genus *Halomonas* according to physiological, biochemical and phylogenetic properties. Although, there is 99.4% 16S rRNA gene sequence similarity between strain AAD6[T] and *Halomonas salina* DSMZ 5928[T] [4], there are many dissimilarities between phenotypic characters of type strain and strain AAD6[T]. Proteome analysis, comparative profiling of protein, is one of the powerful tools to analyse and manipulate the microorganisms, since most cellular metabolic activities are directly or indirectly mediated by proteins. So that, in order to elucidate the differences in protein level, we analysed the protein expression patterns of newly isolated moderately halophilic bacteria AAD6[T] and those of *H. salina* DSMZ 5928[T]. Because omics data alone is not enough to understand cellular physiology and regulatory mechanisms [20], we combined proteomics data with genome data of *Halomonas smyrnensis* AAD6[T].

In this work, the NEPHGE technique was used to carry out the first dimension of 2-DE gel electrophoresis. Cho and his group [21] studied 2-DE electrophoresis of *Halobacterium salinarum* by IPG strips and they obtained poor resolution with IPG strips. Shukla [22] studied *Halobacterium* NRC-I also with the 2-DE IPG strip technique and modified the 2-DE procedure in order to improve resolution and to minimize streaking of proteins in the acidic range. He also noted that the common unmodified 2-DE procedure resulted in horizontal and vertical streaking at the high MW range. In this work, by using the high resolution NEPHEGE technique, proteins were well resolved in the acidic range predominant for halophilic organisms and very few streaking effects were observed (**Figure 1** and **Figure 2**).

Protein patterns for *Halomonas salina* DSMZ5928[T] and *H. smyrnensis* AAD6[T] are documented in **Figure 1** and **Figure 2**. It can be seen from the gels that the whole cell protein composition patterns were mostly located at the acidic side of the gels. A number of studies have suggested that the halophilic adaptation correlates with an increase in acidic amino acids in the protein composition [23]. The fraction of acidic residues is extremely large in the surface composition of the halophilic proteins. The large number of acidic residues on the surface of halophilic proteins has been rationalized on the basis of their superior water-binding abilities in the charged forms [24]. An increase in salt concentration from 1 M to 5 M, increases the pKa value of aspartic acid from 4.0 to 4.9, and that of glutamic acid from 4.4 to 5.3 [25] and decreases the population of the charged forms of these acidic residues at the neutral pH.

At the studied conditions, the expressed proteins of the two microorganisms were mostly located at molecular weight values of 60 to 17 kDa and at isoelectric points 3.8 to 6.0 where they share a number of common protein spots on their 2-DE profiles. However there were some proteins that were not expressed by *H. salina* DSMZ 5928[T] while produced by AAD6[T], or visa versa. This is not a surprise that phylogenetically related organisms have many proteins in common, while this number is lower for more distantly related species [23].

As given in **Table 1**, 11 proteins as A1, A2, A3, A4, A5, A6, A7, A8, A9, A10 and A11 are common proteins expressed by both *H. salina* DSMZ 5928[T] and *Halomonas smyrnensis* AAD6[T]. From the common proteins identified, both from *H. salina* DSMZ 5928[T] and strain AAD6[T], protein spot A1 was a pore forming outer membrane protein precursor which was known to take a role in porin formation for solute diffusion. This protein showed high similarities to the major outer membrane protein precursor from various microorganisms. A2, A3, A4, A5 and A7 were identified as aconitate hydratase, succinate semialdehyde dehydrogenase, Acetyl-CoA Acetyltransferase, Malate dehydrogenase and Acetyl CoA synthase. These enzymes are responsible in the energy metabolism. Aconitate hydratase (A2) is an enzyme that catalyses the stereospecific isomerization of citrate to isocitrate
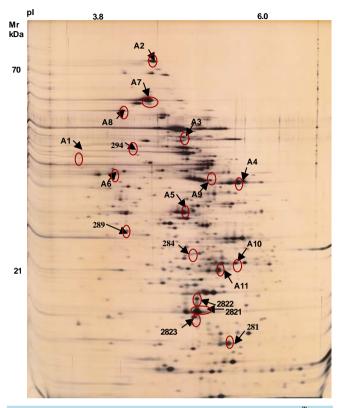
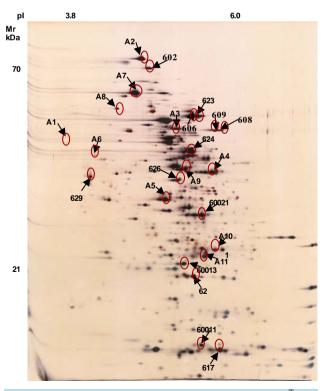**Figure 1.** Silver stained 2-DE gel of *H. Salina* DSMZ5928[T] with the numbered spots showing the identified proteins.

Figure 2. Silver stained 2-DE gel of *Halomonas* sp. AAD6[T] with the numbered spots showing the identified proteins.

Table 1. Overview of *H. salina* DSMZ 5928[T] and *H. smyrnensis* AAD6[T] proteins.

Spot No	Protein	Identification method	Characterization method	pI 2-DE/Pred.	Mr (kDa) 2-DE/Pred.
A1	Gram-negative type porin[*]	N-Termin.seq. **22**ATVYNQDGTKL**32**	WGS[a] (peg.1781)	3.80/3.90	60.00/40.01
	Pore forming outer membrane protein precursor[**]	NanoLC ESI QTOF **65**DLGEGLSALAYAELR**79** **211**LEAGYSQK**218**	Homology search [*Pasteurella multocida*] (gi 32363216, Score: 74[b])	3.50/3.90	58.00/40.01
A2	Aconitate hydratase 2[*] (EC 4.2.1.3)	Nano-LC ESI QTOF (**41**NPPAGEEEAILDLLTNR**57** **58**VPPGVDEAAYVK**69** **79**GEAESPLIDR**88** **324**HGTDEVLTTFELK**336** **364**ESLGLAPSDVFR**375** **413**MTTVGSQDTTGPMTR**427** **491**MLLPDTVGTGGDSHTR**506** **634**WMIANGYGDAR**644** **835**YMSEFDAMAGEIYR**848**	WGS (peg.1297)	5.10/4.83	90.00/99.30
	Aconitate hydratase[**]	Nano-LC ESI QTOF/ **161**WGQQAFDNFR**170** **171**VVPPGTGICHQVNLEYLGK**189** **283** FVEFYGDGLK **292**	Homology search [*C. salexigens* DSM 3043] (gi 92114180, Score:695[b])	4.90/4.96	85.00/94.60
A3	Succinate-semialdehyde dehydrogenase [NADP+] (EC 1.2.1.16)[*]	gi 1500766881 Nano-LC ESI QTOF/ **205**GVFNVVPGR**213** **285**NAGQTCVCTNR**295**	WGS (peg.1111)	5.20/4.98	60.00/54.80
	Succinic semialdehyde dehydrogenase[**]	Nano-LC ESI QTOF/ **112**ETLNADLPLAVDHFR**126** **236**IAFTGSTPVGSHILK**250** **251**CAAENIIPSTVELGGK**266** **340**VGAQASQEQFDK**351** **361**EEGAEFLTGGDK**372** **483**VALEHYQQTK**492**	Homology search [*C. salexigens* DSM 3043] (gi 92114412, Score:96[b])	4.80/4.86	59.00/51.80
A4	Acetyl-CoA acetyltransferase (EC 2.3.1.9)[*]	Maldi-TOF	Homology search [Cellulophage sp. MED134] (gi 86132046, Score:80[b]) + WGS (peg.1731)	5.90/6.86	43.00/42.80
	Acetyl-CoA C-acetyltransferase[**]	Maldi-TOF	Homology search [*C. salexigens*DSM 3043] (gi 92115183, Score: 79[b])	5.90/5.64	42.00/40.20
A5	Malate dehydrogenase (EC 1.1.1.37)[*]	NanoLC ESI QTOF/ **24**IASGDMLGK**32** **234**GASSAASAAIDHMR**250**	WGS (peg.1792)	5.00/5.00	38.00/35.10
	Malate dehydrogenase[**]	NanoLC ESI QTOF/ **99**KDLLEANAAIFSAQGK**114** **100**DDLLEANAA9IFSAQGK**114** **212**DWYENDFIPTVQQR**225**	Homology search [*P. bermudensis* HTCC2503] (gi 84701538, Score: 109[b])	5.20/4.92	36.00/34.40
A6	Phosphate ABC transporter, periplasmic phosphate-binding protein PstS (TC 3.A.1.7.1)[*]	NanoLC ESI QTOF/ **91**MKPSEFER**98** **274**SLFFYVK**280**	WGS (peg.1722)	4.00/4.16	50.00/37.20
	Periplasmic phosphate binding protein[**]	NanoLC ESI QTOF/ **182**DAFEELVMEAASEEMDAYGGEGYTDIR**208**	Homology search [*C. salexigens* DSM 3043] (gi 92113600, Score: 94[b])	4.00/4.16	50.00/37.20
A7	Acetyl-coenzyme A synthetase (EC 6.2.1.1)[*]	NanoLC ESI QTOF/ **71**edgylnvshncldr**84** **362**aimaagdelaqaadsssl**380**	WGS (peg.898)	4.50/4.99	65.00/71.80
	Acetate-CoA ligase[**]	NanoLC ESI QTOF/ **201**DNVDAALTR**209** **506**DEDGYYWITGR**516**	Homology search [*H. chejuensis* KCTC 2396] (gi 83647747, Score: 136[b])	4.50/5.40	63.00/71.80

Continued

A8	Dipeptide-binding ABC transporter, periplasmic substrate-binding component (TC 3.A.1.5.2)[*]	NanoLC ESI QTOF/ **181**sfaltnesgtgpyk**194** **104**dvvftidr**111**	WGS (peg.2767)	4.20/5.91	59.00/58.10
	Solute-binding protein[**]	NanoLC ESI QTOF/ **291**VEAFQDPR**298**	Homology search [*C. salexigens* DSM 3043] (gi 92113996, Score: 58[b])	4.00/4.60	62.00/58.90
A9	Precorrin-6A synthase (EC 2.1.1.152)[*]	Maldi-TOF	WGS (peg.681)	5.20/5.58	42.00/36.30
	Putative cobalt-precorrin-6A synthase[**]	Maldi-TOF	Homology search [*M. acetivorans*] (gi 48428102, Score: 59[b])	5.00/6.20	41.80/36.10
A10	Electron transfer flavoprotein beta subunit[*]	NanoLC ESI QTOF/ **83**ailvesndelnslavak**99** **201**vvtpdalgvstastvk**216**	WGS (peg.508)	5.90/9.01	23.00/26.30
	Electron transfer flavoprotein, beta subunit[**]	NanoLC ESI QTOF/ **33**MAMNPFCEIAVEEAVR**48**	Homology search [*C. salexigens* DSM 3043] (gi 92113737, Score: 91[b])	5.80/5.62	21.00/26.80
A11	Single-stranded DNA-binding protein[*]	NanoLC ESI QTOF/ **9**vilignlgk**17** **129**egyaneefnk**138**	WGS (peg.3040)	5.60/5.10	23.00/17.60
	Single-strand binding protein[**]	NanoLC ESI QTOF/ **8**VILIGNLGQDPEVR**21** **97**YSTEIVANDMQMLDSR**112**	Homology search [*C. salexigens* DSM 3043] (gi 92112583, Score: 128[b])	5.40/5.15	21.00/20.60
2821[*]	Sulfate permease	NanoLC ESI QTOF/ **150**KIDWEHTVAGTSGSDDWR**167** **151**IDWEHTVAGTSGSDDWR**167**	Homology search [*H. smyrnensis* AAD6[T]] (Perfect match with peg.2554)	5.10/5.13	16.80/19.10
2822[*]	Hypothetical protein SSO0826	Maldi-TOF	Homology search [*S. solfataricus* P2] (gi 15897726, Score: 91[b])	5.10/10.00	14.80/12.10
2823[*]	Nucleoside diphosphate kinase (EC 2.7.4.6)	NanoLC ESI QTOF/ **6**TLSIIKPDAVAK**17** **88**DLMGATNPK**96**	Homology search [*H. smyrnensis* AAD6[T]] (Perfect match with peg.2161)	5.01/4.72	15.00/15.20
281[*]	Malonate transporter, MadL subunit	Maldi-TOF	Homology search [*H. smyrnensis* AAD6[T]] (Perfect match with peg.110)	5.60/4.12	10.00/11.00
284[*]	Enoyl-CoA hydratase (EC 4.2.1.17)	Maldi-TOF	Homology search [*H. smyrnensis* AAD6[T]] (Perfect match with peg.1521)	5.11/5.59	24.50/29.60
289[*]	Cysteine desulfurase (EC 2.8.1.7)	Maldi-TOF	Homology search [*H. smyrnensis* AAD6[T]] (Perfect match with peg.2159)	4.24/5.36	29.17/40.94
294[*]	Cytidylate kinase (EC 2.7.4.14)	Maldi-TOF	Homology search [*H. smyrnensis* AAD6[T]] (Perfect match with peg.3118)	4.38/4.61	36.75/23.53
62[**]	Alkyl hydroperoxide reductase subunit C-like protein	N-Termin.seq./ **2**SVLVGRQAPDFEA**14**	WGS (peg.693)	5.50/5.03	20.00/22.01
602[**]	Chaperone protein DnaJ	Maldi-TOF	WGS (peg.179)	4.81/6.66	70.00/40.46
606[**]	DNA mismatch repair protein MutS	Maldi-TOF	WGS (peg.765)	5.54/5.58	45.50/104.00

Continued

608[**]	Pantothenate kinase type III, CoaX-like (EC 2.7.1.33)	Maldi-TOF	WGS (peg.3084)	5.92/4.60	45.50/48.90
609[**]	DNA polymerase III subunits gamma and tau (EC 2.7.7.7)	Maldi-TOF	WGS (peg.1644)	5.80/5.56	45.70/129.32
617[**]	Universal stress protein family COG0589	N-Termin.seq./ **2**SNEY(R)(H)VLVAV**12**	WGS (peg.120)	5.90/5.11	13.00/16.37
623[**]	Dihydrolipoamide dehydrogenase of 2-oxoglutarate dehydrogenase (EC 1.8.1.4)	NanoLC ESI QTOF/ **2**ADKFDVIVIGAGPGGYVAAIR**22** **112**ANGVTALEGTGK**123**	WGS (peg.2909)	5.60/5.11	64.00/50.40
624[**]	Translation elongation factor Tu	NanoLC ESI QTOF/ **26**TTLTAALTR**34** **60**GITIATAHVEYQSEER**75** **91**NMITGAAQMDGAILVCSAADGPMPQTR**118** **138**ADMVDDEELLELVEMEVR**155** **156**ELLNEYDFPGDDCPIITGSALMALEGK**182** **183**DDNGMGTTAVANLIK**197** **198**ALDAYIPEPER**208** **242**SGEEVEIVGLK**252** **257**TTVTGVEMFR**266** **274**AGENIGALLR**283**	WGS (peg.3072)	5.40/4.89	50.00/43.30
626[**]	Aspartate-semialdehyde dehydrogenase (EC 1.2.1.11)	NanoLC ESI QTOF/ **11**GMVGSVLMQR**20** **274**SHSQAFTIK**282**	WGS (peg.1191)	5.20/5.20	40.00/40.10
629[**]	Type1 restriction-modification system, M subunit	Maldi-TOF	Homology search [*Marinobacter sp.* ELB17] (gi 126665709, Score: 72[b])	4.00/5.10	40.00/56.50
60011[**]	L-ectoine synthase (EC 4.2.1.-)	N-Termin.seq./ **1**MIVRNLEEA**9**	WGS (peg.2772)	5.60/5.44	13.00/13.10
60013[**]	Superoxide dismutase [Fe] (EC 1.15.1.1)	N-Termin.seq/ **2**AFELPALPYE**11**	WGS (peg.2756)	5.30/4.89	22.00/21.64
60021[**]	Succinyl-CoA ligase [ADP-forming] alpha chain (EC 6.2.1.5)	N-Termin.seq./ **2**SILIDKNTKVI**12**	WGS (peg.2907)	5.60/6.32	30.00/29.77

Spot numbers coded with "A" identified from both microorganisms, [*]Protein spots belong to *H. salina* DSMZ5928[T]; [**]Protein spots belong to *H. smyrnensis* AAD6[T]; [a]WGS represent the whole genome sequence of *H. smyrnensis* AAD6[T]. The genome identifiers in the form of peg.X are based on Sogutcu *et al.*, 2012. [6]; [b]Score is −10*Log(P), where P is the probability that the observed match is a random event. Protein scores greater than 50 are significant (p < 0.05) according to NCBI Nucleic acid database.

via cis-aconitate in the tricarboxylic acid (TCA) cycle. It serves as a protective buffer against oxidative stress [26]. Succinate semialdehyde dehydrogenase (A3) participates in the degradation of glutamate, while Acetyl-CoA Acetyltransferase participate in pyruvate metabolism. Acetyl-CoA C-Acetyltransferase belonging to the thiolase family which catalyzes the thiolysis of a linear fatty acid CoA Acetoacetyl-CoA thiolase (also called thiolase II) is specific for the thiolysis of acetoacetyl-CoA and involved in biosynthetic pathways such as poly β-hydroxybutyrate synthesis or steroid biogenesis. Its main function is the synthesis of acetoacyl-CoA from two molecules of acetyl-CoA, which shows its importance in several biosynthetic pathways.

Malate and oxaloacetate of the citric acid cycle were the key metabolites that served as the gateway to gluconeogenesis. Succinic semialdehyde dehydrogenase catalyses the (NAD (P) +)-dependent catabolism reaction of succinic semialdehyde to succinate for metabolism by the TCA cycle. Spot A5 was identified as malate dehydrogenase, which participates in the TCA cycle, and which belongs to the MDH (malate dehydrogenase) type 2 family of LDH/MDH (Lactate dehydrogenase/Malate dehydrogenase) superfamily and catalyzes the reversible oxidation of malate to oxaloacetate utilizing the NAD/NADH cofactor system.

Acetate-CoA ligase belonging to the ATP-dependent AMP-binding enzyme family (also known as acetate-

CoA synthetase and acetyl-activating enzyme) is an ubiquitous enzyme, found in both prokaryotes and eukaryotes, which catalyses the formation of acetyl-CoA from acetate, coenzyme A (CoA) and ATP [27]. The activity of this enzyme is crucial for maintaining the required levels of acetyl-CoA, a key intermediate in many important biosynthetic and catabolic processes (some prokaryotic species can also activate acetate by either acetate kinase/phosphor-transacetylase or by ADP-forming acetyl-CoA synthase).

Spot A6 is a periplasmic phosphate binding protein, which is found in the periplasmic space of Gram-negative bacteria and serves as an initial high affinity precursor in the uptake of specific nutrient phosphate. Spot A8 is a dipeptide binding ABC transporter. These proteins work with the ABC transport system, in either direction and they can also function in the initiation of sensory transduction pathways.

Another common protein was putative cobalt-precorrin-6A synthase (spot A9) which may catalyze the methylation of C-1 in cobalt-precorrin-5 and the subsequent extrusion of acetic acid from the resulting intermediate to form cobalt-precorrin-6A, taking role mainly in cofactor biosynthesis. Protein A10 was found as electron transfer flavoprotein (ETFs) beta-subunit serving as specific electron acceptors for primary dehydrogenases, transferring the electrons to terminal respiratory systems. Group II ETFs produced by some prokaryotes under specific growth conditions; receive electrons only from the oxidation of specific substrates [28]. ETFs are heterodimeric proteins containing an FAD cofactor and AMP [29]-[31]. FAD is bound in a cleft between domains II and III, while domain III binds the AMP molecule. Interactions between domains I and III stabilize the protein, forming a shallow bowl where domain II resides. Spot A11 which was identified as a single-strand binding protein, is also known as the helix-destabilizing protein. It binds tightly, as a homotetramer, to single-stranded DNA (ss-DNA) and plays an important role in DNA replication, recombination and repair.

Common proteins expressed by *H. salina* DSMZ 5928^T and *H. smyrnensis* AAD6^T show that these two microorganisms use similar pathways, especially the TCA cycle, for their survival, in other words, for their energy requirements.

We also searched for proteins in *Halomonas smyrnensis* AAD6^T, which were not specifically expressed by *H.salina* DSMZ 5928^T. The protein spots 626 and 60011 were identified as enzymes taking a role in ectoine synthesis. Spot 626 was identified as aspartate semialdehyde dehydrogenase, which is found in the cytoplasm, it belongs to the oxidoreductase. Other known names of the enzyme are aspartic semialdehyde dehydrogenase, and L-aspartate-beta-semialdehyde dehydrogenase. This protein takes roles in lysine biosynthesis I, homoserine biosynthesis, and ectoine biosynthesis. The protein has also an oxidoreductase activity, acting on the aldehyde or oxo group of donors. Spot 60011 showed high similarity to L-ectoine synthase (fragment), which is present in various halophilic bacteria and archaea in blast search. This protein belongs to the ectoine synthase family, which plays a role in ectoine synthesis [32] [33] and which is activated by NaCl. It also takes a role in amine and polyamine biosynthesis catalyzing the circularization of gamma-N-actyl-alpha-gamma-diaminobutric acid to ectoine (1,4,5,6-tetrahydro-2-methyl-4-pyrimidine carboxylic acid), which is an excellent osmo-protectant.

The protein spots 62, 617, 629 and 60013 were identified as proteins taking a role in defense mechanisms, which act under stress conditions. Spot 62 showed similarity to alkyl hydroperoxide reductase (AhpC), which is responsible for directly reducing organic hyperoxides in their reduced dithiol form. Thiol specific antioxidant (TSA) is a physiologically important antioxidant, which constitutes an enzymatic defense against sulphur-containing radicals in blast search. It is known that it is induced by heat shock, salt stress, oxidative stress and glucose limitation. Spot 617 showed high similarity to universal stress protein A (USP A), which is a small cytoplasmic bacterial protein whose expression is enhanced when the cell is exposed to stress agents by blast search of N-terminal sequencing. UspA enhances the rate of cell survival during prolonged exposure to such conditions, and may provide a general "stress endurance" activity. Spot 629 was identified as Type I restriction-modification system, M subunit, which protects a bacterial cell against invasion of foreign DNA by endonucleolytic cleavage of DNA that lacks a site-specific modification. The R-M system is a complex containing three polypeptides as M, S and R [34]. The M and S subunits together form a methyltransferase that methylates two adenine residues in complementary strands of a DNA recognition sequence. When the target site is unmodified the DNA is cut, when semimethylated, the complex acts as a maintenance methyltransferase and both strands become methylated. Spot 60013 showed high similarity to superoxide dismutase, which destroys radicals normally produced within the cells and which are toxic to biological systems. SODs (Superoxide dismutases) catalyse the conversion of superoxide radicals to molecular oxygen. Fe/Mn SODs are ubiquitous enzymes that are responsible for the majority of SOD activity in prokaryotes.

Protein spot 623 was identified as dihydrolipoamide dehydrogenase, which belongs to the disulfide oxidore-

ductase family and also has several other names such as lipoamide dehydrogenase, lipoamide oxidoreductase, dehydrolipoate dehydrogenase. It is a cytoplasmic enzyme, which is the E3 component of dehydrogenase complexes for pyruvate, 2-oxoglutarate, 2-oxoisovalerate, and acetoine. It can also serve as the L protein of the glycine cleavage system. The protein takes part in isoleucine degradation I, valine degradation I, TCA cycle, glycine cleavage complex, and pyruvate dehydrogenase. Protein spot 624 was identified as translation elongation factor TU Tu which is a member of the G-protein superfamily clan, EF-Tu/EF-1A subfamily. Elongation factors belong to a family of proteins that promote the GTP-dependent binding of aminoacyl tRNA to the A site of ribosomes during protein biosynthesis, and catalyze the translocation of the synthesized protein chain from the A to the P site. This protein promotes the GTP-dependent binding of aminoacyl-tRNA to the A-site of ribosomes during protein biosynthesis. EF1A (or EF-Tu) is responsible for the selection and binding of the cognate aminoacyl-tRNA to the A-site (acceptor site) of the ribosome. EF2 (or EF-G) is responsible for the translocation of the peptidyl-tRNA from the A-site to the P-site (peptidyl-tRNA site) of the ribosome, thereby freeing the A-site so that the next aminoacyl-tRNA can bind to the A-site. Elongation factors are responsible for achieving accuracy of translation and both EF1A and EF2 are remarkably conserved throughout evolution. Spot 60021 showed high similarity to the succinyl-CoA ligase [ADP-forming] subunit alpha, which belongs to the succinate/malate CoA ligase alpha subunit family. This enzyme is a bacterial enzyme that during aerobic metabolism functions in the TCA cycle, coupling the hydrolysis of succinyl-CoA to the synthesis of ATP.

Spots 602 was chaperon protein DnaJ. Chaperone DnaJ, Hsp40 (heat shock protein 40 kD), is a molecular chaperone protein and plays a role in regulating the ATPase activity of Hsp70 heat-shock proteins. Spot 606 was identified as DNA mismatch repair protein, which is involved in the repair of mismatches in DNA and it has also a weak ATPase activity. Sports 608 was determined as pantothenate kinase type III. Pantothenate kinase, an essential enzyme in bacteria and eukaryotes, is involved in catalysing the first step of conversion of pantothenate to coenzyme A (CoA). Three isoforms (type I, II and III) of this enzyme have been reported from various organisms, which can be differentiated from each other on the basis of their biochemical and structural characteristics. Spot 609 was DNA polymerase III subunit gamma and tau. The tau and gamma subunits of DNA polymerase III holoenzyme are both products of the dnaX gene. Since tau and gamma are required as stoichiometric components of the replicative complex, a mechanism must exist for the cell to coordinate their synthesis and ensure that both subunits are present in an adequate quantity and ratio for assembly [35].

Seven more proteins from *Halomonas salina* DSMZ 5928[T] spot 2821, 2822, 2823, 281, 284, 289 and 294 were not expressed by *Halomonas smyrnensis* AAD6[T], although the genes responsible for these proteins are present in *H. smyrnensis* AAD6[T]. Spot 2823 was identified as nucleoside diphosphate kinase, which is required for the synthesis of nucleoside triphosphates (NTP) other than ATP. They provide NTPs for nucleic acid synthesis, CTP for lipid synthesis, UTP for polysaccharide synthesis and GTP for protein elongation, signal transduction and microtubule polymerization. Spot 2821 and 2822 are identified as hypothetical proteins, of which spot 2821 has a probable function in type IV secretion [36], while 2822 has so far an unknown function. 281, 284, 289 and 294 were identified as malonate transporter, Enoyl-CoA hydratase, DNA ligase, cysteine desulfurase and cystdylate kinase. Enoyl-CoA hydratase is essential to metabolizing fatty acids to produce both acetyl CoA and energy. Cysteine desulfurases are applicable to the production of cofactors and the bioconversion of useful compounds. Cystdylate kinase has a role in pyrimidine metabolism.

The protein expression differences in AAD6[T] and *H. salina* DSMZ 5928[T] showed that these microorganisms prefer different metbolic pathways for lipid synthesis and adaptation to extreme environments. Consequently, although, there are 99.4% 16S rRNA gene sequence similarities between strain AAD6[T] and *Halomonas salina* DSMZ 5928[T] [4], dissimilarities between phenotypic characters of both strains is a result of metabolic flux differences.

4. Conclusions

Comparing the protein expression of *Halomonas smyrnensis* AAD6[T] with that of *Halomonas salina* DSMZ 5928[T] and comparing that with the genome sequence of strain AAD6[T] helped us to understand how the microorganism differ from one another, although there were 16S rRNA gene sequence similarities between both strains. Experimental results indicated that as well as common proteins, different proteins were also expressed by AAD6[T] and *H. salina* DSMZ 5928[T].

The protein expression differences in AAD6[T] and *H. salina* DSMZ 5928[T] were related to phylogenetic dissimilarities between these microorganisms. This is not a surprise that specific changes in the metabolism of

the organism cause to change the protein expression pattern and the number of common proteins is lower for more distantly related species. To our knowledge, this is the first study to give an explanation for the dissimilarities of phenotypic characters and DNA-DNA hybridizatios between type strain and novel strain AAD6[T] by using protein expression differences.

Acknowledgements

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