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SDS-PAGE Heat-Shock Protein Profiles of Environmental *Aeromonas* Strains

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A b s t r a c t

Aeromonas microorganisms normally grow at temperatures between 5°C and 45°C and therefore should have high thermotolerance. Thus it was of interest to find out whether *A. hydrophila*, *A. caviae* and *A. veronii* biovar *sobria* serovars respond to abrupt temperature changes with a heat shock-like response. To this end the present study was undertaken to determine whether *Aeromonas* species exhibits a heat shock response to different temperatures and time factors. The response of *Aeromonas* serovars to 24 h and 48 h of thermal stress at 25°C, 42°C and 50°C involved the synthesis of 12–18 heat shock proteins (HSPs) bands with molecular weights ranging between 83.5–103.9 kDa in the high HSP molecular mass and 14.5–12.0 as low molecular mass HSP. Electrophoretic analysis of the HSPs showed that the serovars do not cluster very tightly and also that they are distinct from each other.

K e y   w o r d s: environmental *Aeromonas* spp., heat-shock proteins (HSP), SDS-PAGE

Introduction

Species of *Aeromonas* are widely distributed in nature, and some of them are of medical and veterinary interest (Albert *et al.*, 2000; Beutin *et al.*, 2004). *Aeromonas* species are now recognized as important enteropathogens. Some strains are able to infect the human gastrointestinal tract and possess virulence properties such as the ability to produce enterotoxins, cytotoxins, haemolysins and/or the ability to invade epithelial cells (Figueras, 2005; Coburn *et al.*, 2007; Janda and Abbott, 2010).

Avian species such as chicken, normally have high body temperatures (40 to 42°C). Therefore, it was noteworthy to hypothesize that microorganisms, such as *Aeromonas* spp., responsible for a variety of diseases in poultry respond to tissue invasion with the production of heat shock proteins. Moreover, in several bacterial species heat shock proteins have been shown to play an important role in pathogenesis (Lathigra *et al.*, 1991; Kaufmann, 1992; Mauchline *et al.*, 1994; Macario, 1995; Schurr and Deretic, 1997). It was therefore proposed that heat shock proteins produced by bacteria affecting avian hosts, may be virulence determinants, since without the ability to produce this unique class of protein, bacteria would be incapable of producing disease (Love and Hirsh, 1994).

Bacterial heat-shock response is a global regulatory system required for effective adaptation to changes (stress) in the environment. The heat-shock response involves the induction of many proteins – called heat-shock proteins, or HSPs in response to rise of temperature (Neidhardt and Van Bogelen, 1987). Induction of this response improves thermotolerance (Kusukawa and Tura, 1988; LaRossa *et al.*, 1991; Volker *et al.*, 1992).

Consequently, as HSP produced by bacteria may be virulence determinants, it was essential to determine the influence of temperature and time of incubation on their production in vitro. This initiated the present endeavor to determine and evaluate whether *Aeromonas* species exhibits a heat shock response to different temperatures and time factors and to indicate any diversity between the HSPs produced by *Aeromonas hydrophila*, *A. caviae* and *A. veronii* biovar sobria through dendrogram analysis.

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Experimental

Materials and Methods

Aeromonas strains and growth conditions. Eighty-two strains of presumptive *Aeromonas* spp. isolated from the sucrose positive strains, which were mainly associated with epizootic outbreaks that occurred in chicken and fish farms from 2006 to 2007, were stored long-term as freeze-dried cultures in 7.5% horse glucose serum as a cryoprotector. They were genetically re-identified although previously biochemically identified as *Aeromonas* spp. isolated from diseased chicken and fish.

Phenotypic identification. All environmental strains were cultured aerobically on trypticase soy agar (TSA) at 25°C for 24 h. The *Aeromonas* isolates were tested for their physiological and biochemical properties as previously described (Abbott *et al.*, 2003; Murray *et al.*, 2003). Before each test, all the cultures were grown on TSA (Oxoid) at 37°C for 18 h. Strains were first identified as *Aeromonas* spp. and growth in 6% sodium chloride was used to discriminate *Aeromonas* from *Vibrio fluvialis*, *V. splendidus*, *V. harvevyi* and *V. alginelyticus* (Castro-Escarpulli *et al.*, 2003). All *Aeromonas* spp. were re-identified biochemically by using 14 tests which were: indole, gas from glucose, cytochrome oxidase, nitrate reduction, arginine dihydrolase, lysine decarboxylase and ornithine decarboxylase by the Moeller's method, esculin hydrolysis, Voges-Proskauer test, acid production from L-arabinose, lactose, sucrose, salicin, m-inositol, D-manitol and the h-hemolysine. To identify the biovars of *Aeromonas veronii*, the following tests were used: arginine dihydrolase, ornithine decarboxylase, acid from salicin and esculin hydrolysis.

Serological testing. All motile *Aeromonas* strains had been previously tested by the O-serogrouping system of the Hydrobiology Department (National Research Centre) according to Eurell *et al.* (1978). Strains were grown on TSA slants overnight at 30°C, harvested with phosphate-buffered saline ($>10^9$ cells/ml), and heated for 1 h at 100°C. After being heated, 20 µl of the boiled cell suspensions (thermostable O antigen of the strains) was mixed with 20 µl of each specific rabbit antiserum (O:1 to O:30) in ceramic rings on agglutination glass sides. The mixtures were rotated for 2 min, and the degree of agglutination (0 to 2+) was recorded. Two negative controls were used, boiled cell suspensions mixed with phosphate-buffered saline and boiled cell suspensions mixed with rabbit serum obtained from nonimmunized animals.

Molecular identification. All strains were re-identified on the basis of the restriction fragment length polymorphism patterns (RFLP) obtained from the 16S rDNA (Ausubel *et al.*, 1994). *Aeromonas* strains ATCC 7966, ATCC 43979, and ATCC 15468, and *Escherichia coli* (ATCC 25922) and *Staphylococcus aureus* (ATCC 25923), were included as quality controls.

Bacterial DNA extraction for PCR. Approximately 100 µl of a TSA culture grown for 16 h at 28°C was used for DNA extraction using the InstaGene matrix (Bio-Rad Laboratories AG, Glattbrugg, Switzerland) in accordance with the manufacturer's instructions. Subsequently, 5 µl of the DNA solution was used as a template for PCR amplification. The sequence, specificities, the primer combination and the size and length of the amplified products are summarized in Table I. The general reaction conditions in a Gene Cycler were 94°C for 1 min, 60°C for 1 min and 72°C for 3 min. This was repeated for 35 cycles. The program also included a preincubation at 94°C for 2 min before the first cycle and an incubation at 72°C for 3 min followed by cooling at 4°C after the last cycle.

Stress induction. Environmental strains of *A. hydrophila*, *A. caviae* and *A. veronii* biovar sobria each was separately grown in 250 ml of Luria-Bertani (LB) broth containing 0.3 M NaCl. The HSP bands were visible if the strains were grown in a broth medium but not after cultivation on an agar medium. Heat shock stress

Table I

Oligonucleotide primer sequences and size of the PCR-targeted products of selected species of *Aeromonas*

Specificity	Forward and Reverse sequence	Primer sequence (5'–3')	Length (bp)	Location	Size of amplified product (bp)
Aeromonas species	Aero-F	GGAAACTTCTTGGCGAAAAC	20	IGS	550
	Aero-R	GGTTCTTTTCGCCTTTCCCT	20	23S	
A. hydrophila	A-hydro-F	CCAAACGAGAGAAGCCCTT	19	iGS	700
	A-hydro-R	CCATTCCACTAACTTCCAAGAA		23S	
A. caviae	Acav-F	CGCGCCGTTGCAAACATG	18	IGS	320
	Acav-R	GCGATACCTAGCTTATGCTAA	21	23S	
A. veronii biovar *sobria*	Aver-F	TGGTAGCTAATAACTGCCAG	20	16S	1170
	Aver-R	GGCTTCTCTCGTTTGGCGT	19	IGS	

conditions were imposed according to the following procedures: 1) each strain was cultured at a temperature of 42°C and 50°C, 2) at each temperature, samples were taken at intervals after heat shock, and crude cell extracts were prepared. Culture samples were taken after 3 h, 6 h, 24 h, 48 h and 72 h.

Analysis of *Aeromonas* spp. culture supernatant proteins by SDS-PAGE. Following the procedure of Love and Hirsh (1994), bacterial cells were centrifuged (15,600×g, 5 min, room temperature). Bacteria were lysed in gel sample buffer consisting of 9.5 M urea, 2% Nonidet P-40, 2% ampholytes (Bio-Lyte 5/7 [Bio-Rad Laboratories, Richmond, Calif.] and 2-D Pharmalyte [Pharmacia LKB, Uppsala, Sweden]), and 5% 2-mercaptoethanol. An equal volume of glass beads (212- to 300-[Lm diameter; Sigma Chemical Co., St. Louis, Mo.) was then added to each tube. After the tubes were vortexed for 3 min, they were centrifuged (15,600×g, 5 min, room temperature). The proteins in the supernatants were separated by electrophoresis through a 3% stacking gel and a 10% separating gel. Polyacrylamide gel electrophoresis, Coomassie blue staining analysis of proteins was carried out by standard protocols.

Computer-aided analysis of the gels. Images of the gels were captured using a Sharp JX-330 flat-bed scanner, and image analysis of the protein profiles was performed using Amersham Pharmacia Biotech Image Master 2-D Elite software. The relative amount of each protein spot was calculated and expressed by the software as the percentage of the spot volume and represented the intensity of each individual spot compared to the intensity of the whole gel. The genetic similarity coefficient between two genotypes was estimated according to Dice. The similarity-derived dissimilarity matrix was used in the cluster analysis by using the unweighted pair-group method with arithmetic averages (UPGMA).

Results

Effect of the incubation temperature on for 24 and 48 h on the SDS-PAGE protein pattern. The *Aeromonas* species that were subjected to heat stress in the present investigation were *A. hydrophila*, *A. caviae* and *A. veronii* biovar *sobria.* The SDS-PAGE electrophoresis HSP patterns for cells subjected to temperature downshifts from 42 to 25°C and upshifts to 50°C were examined after 24 and 48 h. The protein profile for isolates of *Aeromonas species* was established out by running eight per cent SDS-PAGE with an objective to find variation in the protein banding pattern of all the isolates. It was found that there was variation in the protein banding pattern. Most proteins were similarly expressed at the three temperatures.

Aeromonas intraspecies generation of heat-shock proteins (HSP). The SDS-PAGE protein profiles of the

A. hydrophila, *A. caviae* and *A. veronii* biovar *sobria* grown in LB- broth revealed that, *Aeromonas* phenospecies produced protein patterns containing several discrete bands in the most important area, with molecular masses in the range of 103.9–83.5 kDa (Table II). Differences between strains were evident in this range of molecular weights in the number of bands that were expressed under different temperatures (25°C, 42°C and 50°C) when incubated for 24 and 48 h. The SDS-PAGE protein pattern revealed that the 3 *Aeromonas* species bands ranged from 12–18 bands (Table II). The *A. veronii* biovar *sobria* serovar which was isolated had a strong band of molecular mass in the range of 103.1–101.0 kDa (Table II) which lacked from all the other serovars. The band with molecular masses of >9 kDa and <100 kDa, in particular, was lacking in all investigated *Aeromonas* serovars (Table II).

The molecular weight of the band of a strain was consistent in repeated SDS-PAGE.

***Aeromonas* cluster analysis of heat-shock proteins (HSP).** Pattern storage and comparison were performed with GelCompar version 4.2 (Applied Maths). Of the 400 digitized points in each densitometric trace, only points 11 ± 317 were used for calculation of the similarities between individual pairs of patterns. The dendrogram is derived from the (unweighted pair group arithmetic average-linkage algorithm) clustering of correlation coefficients of the SDS-PAGE protein patterns.

The incubation of A. hydrophila (CR +ve) at 25°C revealed that the HSPs that were produced after 24 h and 48 h of their incubation when analysed dendrogramically varied from each other by a percentage of 26.1%. But, when A. hydrophila (CR +ve) was incubated at 42°C, the HSPs that were produced after 24 h and 48 h were found to exhibit a 42.8% variation in their dendrogram analyses. Dramatically, the dendrogramic variation between the HSPs produced by A. hydrophila (CR +ve) when incubated at 50°C for 24 h and 48 h dropped to an extremely low level of variation (7%) (Fig. 1).

The dendrogramic analysis of *A. caviae* when subjected to incubation at 25°C for 24 h induced a variation in its protein electrophoresis by about 25.9% when compared to the HSPs that were produced during incubation at 25°C for 48 h. The comparative changes in the electrophoretic profile between the two incubation periods increased to 35.5% when the incubation temperature was increased to 42°C. Heat stress became more evident on the A. caviae strain when the incubation was elevated to 50°C and the dendrogramic analysis recorded a difference of 39% between the HSPs produced after 24 h and 48 h incubation (Fig. 2).

The incubation of *A. veronii* biovar *sobria* at 25°C for 24 h disclosed HSPs changes by 38.1% from the HSPs that were produced by the strains that were incubated

Table II

HSPs molecular mass analysis of *A. hydrophila*, *A. caviae* and *A. veronii* biotype *sobria* incubated
at 25°C, 42°C and 50°C for 24 h and 48 h

Aeromonas species	*A. hydrophila* (CR+ve)					
Incubation temperature	25°C		42°C		50°C	
Time of incubation (hours)	24 h	48 h	24 h	48 h	24 h	48 h
Number of bands	15	13	12	14	12	14
Range of MW (kDa)	94.0–13.0	95.0–14.5	96.0–13.5	95.5–13.2	95.3–13.2	94.5–13.0

Aeromonas species	*A. hydrophila* (CR+ve)					
Incubation temperature	25°C		42°C		50°C	
Time of incubation (hours)	24 h	48 h	24 h	48 h	24 h	48 h
Number of bands	16	15	15	16	14	15
Range of MW (kDa)	96.5–12.5	98.7–14.5	98.4–12.1	99.8–12.4	98.4–12.0	97.8–12.4

Aeromonas species	*A. caviae*					
Incubation temperature	25°C		42°C		50°C	
Time of incubation (hours)	24 h	48 h	24 h	48 h	24 h	48 h
Number of bands	15	14	15	13	13	14
Range of MW (kDa)	88.2–14.4	90.7–13.6	95.5–14.1	90.4–13.8	86.2–13.5	83.5–14.5

Aeromonas species	*A. veronii* biotype sobria					
Incubation temperature	25°C		42°C		50°C	
Time of incubation (hours)	24 h	48 h	24 h	48 h	24 h	48 h
Number of bands	17	14	15	18	18	17
Range of MW (kDa)	103.1–13.4	103.5–13.5	103.9–13.8	99.0–13.3	103.1–12.4	101.0–12.2

for 48 h. These changes were also close to the changes that were exhibited by the strains that were incubated at 50°C for 24 h and 48 h (35.8%). The HSPs differences dramatically dropped to 29% when the strains of *A. veronii* biovar sobria were incubated at 42°C for 24 h and 48 h (Fig. 3).
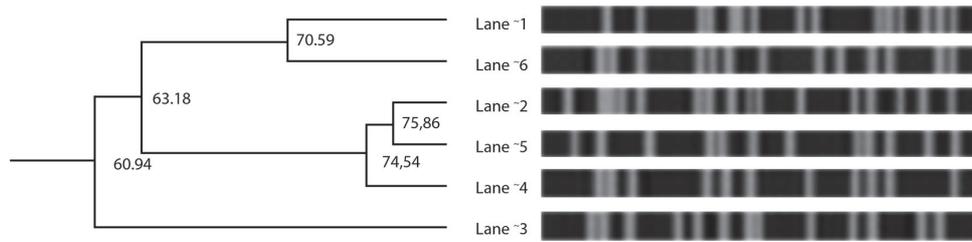

Fig. 1. Dendrogram derived from SDS-PAGE HSP pattern analysis of *Aeromonas hydrophila* (CR +ve) when subjected
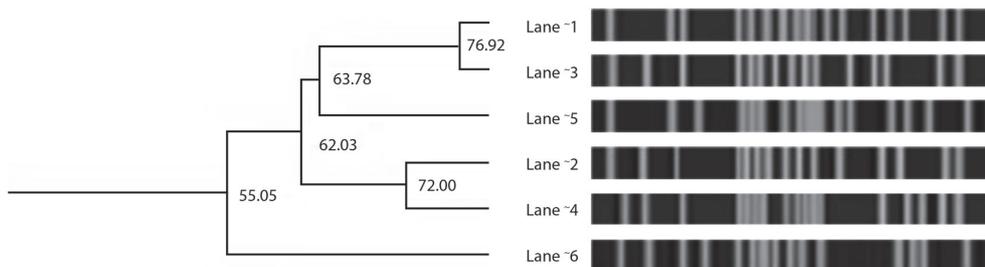to different temperature conditions and time.

Fig. 2. Dendrogram derived from SDS-PAGE HSP pattern analysis of *Aeromonas caviae* when subjected
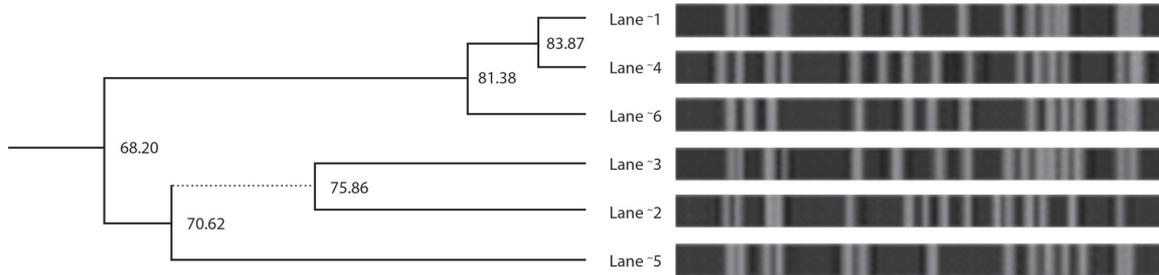to different temperature conditions and time.

Fig. 3. Dendrogram derived from SDS-PAGE HSP pattern analysis of *Aeromonas veronii* biovar sobria when subjected to different temperature conditions and time.

Discussion

Foods are exposed to a wide range of processing treatments intended to kill or control the growth of microorganisms. Thermal treatment of foods is extensively used for the purpose of killing or reducing populations of pathogenic and spoilage microorganisms. The exposure of microorganisms to sublethal heat treatment can, however, occur in food processing plants (Marriott, 1994). Physical stresses can be induced by exposure of microorganisms to elevated or reduced temperatures. The ability of foodborne spoilage and infectious microorganisms in food processing environments to survive under adverse conditions and become cross protected against subsequent stresses depends on intrinsic factors as well as mechanisms induced in the cell upon exposure to sublethal stresses (Abee and Wauters, 1999; Bower and Daeschel, 1999).

Although the identification of *Aeromonas* strains to species level concerns the current number of recognized taxa (n = 24) (Demarta *et al.*, 2008; Beaz-Hidalgo *et al.*, 2009; Alperi *et al.*, 2010), yet certain strains of *Aeromonas* species, in particular *A. hydrophila* and A. sobria, are of potential public health significance (Korbel and Kosters, 1989; Demarta *et al.*, 2008; Beaz-Hidalgo *et al.*, 2009; Alperi *et al.*, 2010). While approximately half of the clinical isolates can grow well at 45°C (Palumbo *et al.*, 1985; Knochel, 1990), most clinical strains grow well at 42°C (Palumbo *et al.*, 1985) and only a few isolates from vegetables stored at 5°C are capable of growing at this temperature (Majeed *et al.*, 1990). Some isolates from cold water may not even grow or grow very slowly at 37°C (Knochel, 1990). Depending on the isolate the maximum growth temperature seems to be from 37°C to 43–44°C (Merino *et al.*, 1995). An observation of growth of several isolates at 55°C (Abou-Shanab, 2007) has not been substantiated by other researchers.

A short exposure of cells to elevated temperatures reduces the synthesis of normal cellular proteins and at the same time induces a transient overproduction of a specific group of proteins, the so-called heat shock proteins (HSPs) (Freeman *et al.*, 1989; Abou-Shanab, 2007). The optimum temperature for the production of HSPs varies from organism to organism. The heat shock temperature range for *E. coli* is 43–47°C, for the yeast 36°C and for the sickle fungus Fusarium oxysporum 40°C or 43°C. In general, a rise of 5°C above the normal physiological temperature will induce the synthesis of HSPs (Freeman *et al.*, 1989; Abou-Shanab, 2007). They are classified according to their respective molecular weights and are divided into six families: HSP100 family, the HSP90 family, the HSP70 family, the HSP60 family, the HSP40 family, and the small HSPs (sHSPs). The small heat shock family members vary in their respective molecular weights; they range in size from 15 kDa to 30 kDa. The HSP70 family represents one of the most widely examined heat shock families. Remarkably, the general features of the HSP70 and HSP60 molecule functional roles are similar. Both types of chaperones are abundant proteins whose rate of synthesis can be enhanced by stress conditions such as heat shock.

The study showed that there are differences in the SDS-PAGE protein profiles between the three *Aeromonas* species grown in LB. The molecular weight of the bands differed between strains. We have shown that at an increased temperature *Aeromonas* species produce heat shock proteins *in vitro* that are typical of the heat shock responses described for other bacteria (Love and Hirsh, 1994). In addition, the heat shock proteins produced in *A. hydrophila*, *A. caviae* or *A. veronii* biovar sobria are of the appropriate size for the previously described families of heat shock proteins (HSP90, HSP70, HSP60, and small heat shock proteins) (Love and Hirsh, 1994). Statner *et al.* (1988) observed temperature-dependent changes in the protein profiles of aeromonads. Kuijper *et al.* (1989) found that A. hydrophila strains had unique patterns in the region of 3 and 45 kDa, and they lacked a protein band of 22–26 kDa, which does not agree with the present study. This could be hypothetically attributed to the fact that Kuijper *et al.* (1989) did not expose their cultures to different temperatures and time of incubation.

Concluding remarks. Foods and food processing environments can impose extreme temperature stress on spoilage and pathogenic bacteria. The behavior of bacterial cells exposed to such a condition is dictated by the duration of exposure, degree, constitutive and induced ability to respond, and ability to synthesize shock proteins. These factors also affect the extent to which cells become cross protected to a secondary stress encountered after exposure to extreme temperature. Investigations of the behavior of bacteria at temperature extremes are made difficult by the fact that cells may act differently upon exposure to simultaneous or sequential stresses

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