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Production of siderophores & effect of iron restriction on the protein profiles of *Aeromonas* species isolated from water & patients suffering from acute diarrhoeal disease

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Among 48 strains of *Aeromonas* species, 21 isolates from patients suffering from acute diarrhoea and 27 from metropolitan water samples grown under iron-restricted conditions, 45 strains produced siderophores. Forty one of the 46 strains tested produced siderophores on chrome azurol S (CAS) agar, while 43 isolates did so when the culture supernatants of the bacterial isolates grown in minimal medium were assayed with chrome azurol S assay solution. The whole cell protein profiles of *A. hydrophila* strains grown under iron restricted conditions expressed new proteins that were not detected in those cultured in iron rich conditions. Five high molecular weight proteins ranging from 70 to 96 kDa were distinctly absent in cultures grown in the presence of iron, indicating their role in iron acquisition by the aeromonads.

Key words *Aeromonas* - protein profile - siderophores

Aeromonas species are ubiquitous water-borne organisms and are recognised as pathogens of humans involved in various clinical conditions such as wound infections, septicaemia and acute diarrhoeal disease[1-4]. Aeromonads produce various cell associated virulence factors such as adhesins, pili, outer membrane proteins *etc.*, and also extra cellular virulence factors such as enterotoxins, haemolysins and siderophores[5]. In order to grow, cause infection and produce disease in the human host, microorganisms must be equipped to acquire essential growth factors and minerals from the host system. Iron, an essential requirement of bacteria, is tightly bound to glycoproteins such as transferrin and lactoferrin in the serum, making it unavailable for the bacteria[6]. Under these iron-restricted conditions, the bacteria develop iron acquisition mechanisms, involving extra cellular factors called siderophores, which selectively dissociate iron complexes such as lactoferrin and transferrin in the host's serum and transfer the iron into the cell[7]. These siderophores are low molecular weight compounds having high affinity for iron. Ability to respond to such iron limitation in the living tissue by production of siderophores along with the mechanism to transfer it in to the cell by the help of cell membrane receptors helps bacteria to initiate the process of infection[6].

Aeromonas species predominantly produce amonabactin, a phenolate siderophore[8] and the

number of clinical isolates producing siderophores is higher compared to environmental isolates[9]. Hence, we undertook to examine the *Aeromonas* isolates recovered from clinical and domestic water samples in Chennai for their ability to elaborate siderophores and to find out if clinical isolates were the predominant siderophore producers. It has been reported that iron-limiting conditions resulted in synthesis of several high molecular weight outer membrane proteins in *Aeromonas salmonicida*[10] and *Acinetobacter baumanni*[11]. Such studies are lacking in the mesophilic *Aeromonas* species. Hence we also attempted to examine the changes if any in the whole cell protein profiles of selected strains of *A. hydrophila* subjected to iron deprivation.

Material & Methods

Bacterial strains : Twenty one *Aeromonas* isolates (17 recovered from children suffering from acute diarrhoea[12] and four standard strains of clinical origin received from Dr Toshio Shimada, Japan) and 27 isolates from drinking water packets, metropolitan water supplies and bore well water samples in Chennai city[13] were used for the present study. Stock cultures of the isolates were maintained in tryptose soya broth (Difco) with 25 per cent glycerol at -20°C. Isolates were sub-cultured on to nutrient agar for further testing.

Detection of siderophore production on blue agar: Production of siderophores by *Aeromonas* isolates was determined using blue agar (chrome azurol S or CAS agar) according to Schwyn and Neilands[14]. The blue agar plates were inoculated with the *Aeromonas* isolates and incubated at 37°C and examined up to 72 h for siderophores, which is indicated by the production of a yellow-orange halo around the bacterial colonies.

Siderophore assay in culture supernatants: Culture supernatants of *Aeromonas* isolates were assayed for siderophores as per the methods described earlier[14] and modified by Naidu and Yadav[9]. *Aeromonas* isolates were grown in iron deficient defined medium M9[15] for 24 h and cell free culture supernatants were obtained by centrifugation of cultures of 12,000 *g* at 4°C for 15 min. Five mililitre of culture supernatant and 0.5 ml of chrome azurol S assay solution prepared according to Schwyn and Neilands[14] were mixed and after an equilibrium was reached, absorbance of this solution at 630 nm was recorded. An uninoculated medium was used as reference. Production of siderophore is inversely proportional to absorbance. Based on the absorbance recorded, siderophore production by the bacterial isolates was categorised as high (A_{630} <0.50), medium or moderate (A_{630}>0.50 to 0.75), low (A_{630}>0.75-1.0) and negative (A_{630}>1.0).

Protein profile of A. hydrophila in response to iron deprivation : Log phase cultures of five *A. hydrophila* isolates were inoculated to 10 ml of M9 and simultaneously to another set of M9 medium containing 2 μ*M* iron ($FeCl_3.6H_2O$). Cultures were incubated at 37°C on a shaker (60 rpm) for 24 h and the bacterial cells were harvested by centrifugation at 2200 *g* at 4°C for 10 min. The cell pellets were washed thrice with sterile phosphate buffered saline (PBS, *p*H 7.4) and finally suspended in 1 ml of sterile PBS. These cell suspensions were sonicated for 20 sec with a pulse of 1 sec using a micro tip probe attached to the Vibrasonic ultrasonicator (Germany). The sonicated samples were centrifuged at 12000 *g* for 15 min at 4°C and the supernatants were collected in fresh tubes and preserved at -20°C.

The protein concentration of these extracts was determined by Lowry's method[16]. The samples were appropriately diluted using sample buffer to obtain a μg/μl suspension and 25 μl of each extract was subjected to sodium dodesyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) using 10 per cent gel[17]. Electrophoresis was performed at 20 mA when the samples were in stacking gel at 30 mA in resolving gel. In each run, a protein molecular weight standard (Genei, Bangalore) was included. The electrophoresis was stopped when the bromophenol blue tracking dye was about 1-1.5 cm from the bottom of the gel. The protein bands were stained with coomassie brilliant blue[18] and photographed. The banding pattern in each sample (track) was scanned using a laser densitometer (LKB Broma, Germany) to analyse the differences in the protein fingerprints produced by different isolates.

The molecular weight of protein band of differential value was determined based on its relative mobility (Rf) by referring to the standard graph developed from molecular weight markers and their Rf values plotted on a semi-logarithmic graph paper.

Results

Most of the *Aeromonas* isolates grown under iron stress produced siderophores, which was indicated by development of a yellow-orange halo around the bacterial colonies grown on CAS agar for 48 -72 h at 37°C. Production of siderophores by the *Aeromonas* isolates tested is given in Table I. Though all the 48 isolates were tested for siderophore production, only 44 isolates were tested by both the methods. Of the remaining four, two isolates were tested on CAS agar and two by CAS assay method. Of the 46 isolates tested on CAS agar, 41 were positive, while 43 were positive by CAS assay method. A strain giving positive reaction by either of the methods employed was considered to produce siderophore. Out of 21 clinical isolates and 27 strains of *Aeromonas* from water, siderophore production was observed in 19 and 26 isolates respectively. Eighteen of 20 clinical isolates and 23 of 26 isolates from water showed production of siderophores on CAS agar, while one clinical isolate and two other isolates from water showed positive results when assayed for siderophores using CAS assay solution on their culture supernatants (Table II). Out of 46 *Aeromonas* isolates tested for siderophore production using CAS assay, 14 produced siderophores at moderate level, 29 at low level and 3 were negative (Table III).

The five strains of *A. hydrophila* examined by SDS-PAGE produced a protein fingerprint with 30-40 band (Fig.). The banding pattern of the isolates when digitally processed with the help of laser densitometer, showed several peaks indicating minor protein bands not discernible to the naked eye. The strains grown with iron and those grown without iron in the culture medium showed distinct protein profiles. These differences could be noticed only from the densitometric scan curves and scan data. Although *A. hydrophila* ATCC strain and strain A 13 showed similar protein profiles, many bands were

Table I. Production of siderophores by *Aeromonas* isolates on CAS agar and in culture supernatant

Sl. no.	Strain	On CAS agar	A_{630}
1.	*A. hydrophila* (ATCC 7966)	+	0.92
2.	*A. hydrophila* (8/143)	+	1
3.	*A. hydrophila* (12)	+	0.96
4.	*A. hydrophila* (13)	+	0.95
5.	*A. hydrophila* (22)	+	0.7
6.	*A. hydrophila* (62)	+	0.85
7.	*A. hydrophila* (71)	+	0.68
8.	*A. hydrophila* (86)	+	0.92
9.	*A. hydrophila* (117)	+	0.74
10.	*A. hydrophila* (121)	–	1.22
11.	*A. sobria* (3/8a)	+	0.66
12.	*A. sobria* (3/74)	+	0.7
13.	*A. sobria* (4/9)	+	ND
14.	*A. sobria* (6/143)	+	0.83
15.	*A. sobria* (8/11)	+	0.69
16.	*A. sobria* (12/62)	+	0.92
17.	*A. sobria* (9)	+	0.6
18.	*A. sobria* (14)	+	0.96
19.	*A. sobria* (16)	+	0.8
20.	*A. sobria* (26)	+	0.6
21.	*A. sobria* (60)	+	0.7
22.	*A. sobria* (66)	–	0.96
23.	*A. sobria* (94)	+	0.88
24.	*A. sobria* (97)	+	0.65
25.	*A. sobria* (110)	+	0.64
26.	*A. caviae* (ATCC 13137)	ND	0.93
27.	*A. caviae* (3/59)	+	0.76
28.	*A. caviae* (5/44)	+	0.87
29.	*A. caviae* (6/49)	+	1.16
30.	*A. caviae* (9/6)	–	1.2
31.	*A. caviae* (11/3)	+	0.64
32.	*A. caviae* (11/54)	–	0.77
33.	*A. caviae* (12/33)	+	0.94
34.	*A. caviae* (12/35)	+	0.79
35.	*A. caviae* (12/64)	+	0.94
36.	*A. caviae* (1/21)	+	0.86
37.	*A. caviae* (7)	+	0.9
38.	*A. caviae* (31)	+	0.88
39.	*A. caviae* (55)	–	0.98
40.	*A. caviae* (59)	+	0.92
41.	*A. caviae* (65)	+	0.79
42.	*A. caviae* (101)	+	0.83
43.	*A. caviae* (109)	+	0.73
44.	*A. veronii* (ATCC 35626)	+	0.76
45.	*A. veronii* (88)	ND	0.7
46.	*A. jandaeii* (307-94)	+	0.94
47.	*A. jandaeii* (18)	+	0.98
48.	*A. jandaeii* (44)	+	ND

ND, no data; CAS agar, chrome azurol S agar

Table II. Siderophore production by *Aeromonas* species from clinical and water samples

Aeromonas species	Number of isolates producing siderophores					
	On CAS agar		In culture supernatant		Total	
	Clinical isolates	Isolates from water	Clinical isolates	Isolates from water	Clinical isolates	Isolates from water
A. hydrophila	2 (2)	7 (8)	1 (2)	7 (8)	2 (2)	7 (8)
A. sobria	6 (6)	8 (9)	5 (5)	9 (9)	6 (6)	9 (9)
A. caviae	8 (10)	6 (7)	9 (11)	7 (7)	9 (11)	7 (7)
A. veronii	1 (1)	–	1 (1)	1 (1)	1 (1)	1 (1)
A. jandaeii	1 (1)	2 (2)	1 (1)	1 (1)	1 (1)	2 (2)
Total	18 (20)	23 (26)	17 (20)	25 (26)	19 (21)	26 (27)

Figures in parentheses are number of isolates tested. Of the 48 isolates, 44 were tested by both methods and of the remaining four, two were tested on CAS agar and two by CAS assay method

Table III. Production of siderophores using CAS assay solution

Aeromonas species	Number of isolates producing siderophores (OD_{630})								
	Moderate (A_{630}>0.50 to 0.75)			Low (A_{630}>0.75 - 1.0)			Negative (A_{630}>1.0)		
	C	W	Total	C	W	Total	C	W	Total
A. hydrophila	0 (2)	3 (8)	3 (10)	2 (2)	4 (8)	6 (10)	–	1	1
A. sobria	3 (5)	5 (9)	8 (14)	2 (5)	4 (9)	6 (14)	–	–	–
A. caviae	1 (11)	1 (7)	2 (18)	8 (11)	6 (7)	14 (18)	2	–	2
A. vernoii	0 (1)	1 (1)	1 (2)	1 (1)	0 (1)	1 (2)			–
A. jandaei	0 (1)	0 (1)	0 (2)	1 (1)	1 (1)	2 (2)			–
Total	4 (20)	10 (26)	14 (46)	14 (20)	15 (26)	29 (46)			3

C, clinical isolates; W, isolates from water; Figures in parentheses are number of isolates tested

Table IV. Effect of iron stress on whole cell protein profile of two strains of *A. hydrophila*

Mol wt	*A. hydrophila* grown in M9 with iron		*A. hydrophila* grown in M9 with no iron	
	A 8/143	A12	A 8/143	A12
70		–		+
74	–	–	+	+
77	–		+	
80		–		+
84	–		+	
88		–		+
96	–	–	+	+

clearly absent in the cultures grown in M9 medium with iron. In the other two strains, A 8/143 and A 12 grown under iron restriction, 4 and 5 high molecular weight bands respectively were present (Table IV), while they lacked several low molecular weight bands.

Discussion

In spite of the iron limiting conditions in the culture medium, the *Aeromonas* isolates readily grew, indicating their ability to survive and grow well in the iron deficient conditions prevalent in the host environment. Though initial reports had indicated that *Aeromonas* species produce enterobactin–like siderophore, a study by Barghouthi *et al*[8] has proved that these bacteria produce a siderophore termed as amonabactin, composed of a basic 2, 3-dihydroxybenzoate structure with lysine and glycine; one form containing tryptophane (amonabactin-T) and the other form containing phenyl alanine (amonabactin-P). According to these workers, 44 isolates of *Aeromonas* spp, inclusive of 25 strains of *A. hydrophila*, 16 of *A. sobria* and 3 of *A. caviae* produced siderophores on CAS agar, which

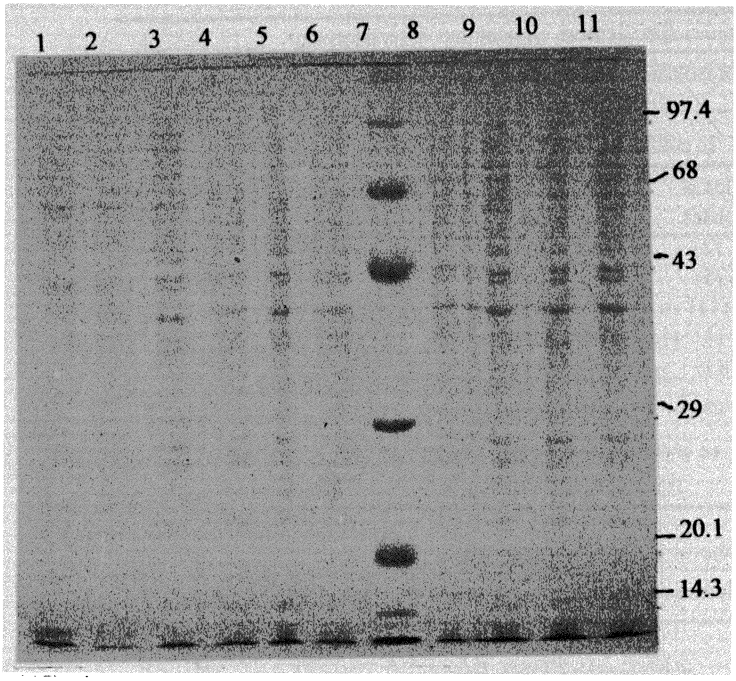

Fig. Protein profiles of *A. hydrophila* strains: Lanes 1, 3, 5, 8 and 10: ATCC 7966, A 8/143, A 12, A 13 and A 62 grown in M9 medium; Lanes 2, 4, 6, 9 and 11: *A. hydrophila* strains grown in M9 medium with 2 μ*M* iron; Lane 7: Molecular weight marker.

predominantly produced amonabactin. The siderophore detection method of Schwyn and Neilands[14] adopted in the present study and also used by Barghouthi and co-workers[8] is a highly sensitive method based on the affinity of siderophores with iron (Fe^{+3}) and is independent of the structure of siderophores, which may be made up of basic structures of either hydroxamates or catechols. The amonabactin producing strains acquire iron from the serum Fe-transferrin complex, whereas isolates synthesising enterobactin cannot utilise iron bound transferrin in the serum[19]. Amonabactin is possibly an important virulence factor of *Aeromonas* species[20]. Earlier studies have indicated that the ability to elaborate siderophores correlated with higher virulence in *Aeromonas* species[21]. Further, iron deficiency in the medium induces microbes to acquire iron acquisition mechanisms and in turn, constitutes an important signal, which regulates expression of a number of virulence factors unrelated to iron metabolism[22]. Presence or absence of a siderophore in *Aeromonas* isolates has also been suggested to be of use in assigning the organism to its DNA-hybridisation group and the DNA-hybridisation groups 7 and 8/10 were considered as siderophore negative groups[23], accordingly, one isolate of *A. hydrophila* and one isolate of *A. caviae* the siderophore negative strains may be presumed to belong to these hybridisation groups.

The protein profiles of the *Aeromonas* strains were altered to a considerable extent when the culture conditions were iron restricted. Similar observations have been reported in coagulase positive staphylococci[24]. In an earlier study, iron limitation resulted in the increased synthesis of several high molecular weight outer membrane proteins of 76.6, 77.7 and 83.2 kDa size in *A. salmonicida*[10]. Another study reported the appearance of four high molecular weight outer membrane proteins of 88, 84, 80 and 77 kDa in *Acinetobacter baumannii* grown under iron-restricted conditions[11]. Similarly, high molecular weight OMPs are expressed by bacteria, which possibly act as receptor for siderophore iron complex[25]. In the present study, of the five strains examined for their protein profiles in response to iron stress, two strains showed presence of 4 - 5 high molecular weight proteins, and these could be involved in iron transport mechanism in the mesophilic aeromonads.

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