Appendix A

Siderophore production of the *Aeromonas hydrophila* isolates at 28°C and 37°C before and after treatment with acridine orange under iron-limiting and iron-rich conditions.

Isolates	28°C				37°C			
	Before treatment		After treatment		Before treatment		After treatment	
	-Fe	+Fe	-Fe	+Fe	-Fe	+Fe	-Fe	+Fe
Environmental isolates								
X2	1.27[a]	1.29	1.29	1.27	1.45	1.34	0.23	1.31
X8	1.35	1.34	0.25	1.40	1.31	1.23	0.90	1.34
X13	1.35	1.32	1.31	1.21	1.28	1.20	0.91	1.36
X14	0.28	1.29	1.50	1.30	0.52	1.28	1.23	1.29
X36	0.41	1.31	0.66	1.39	0.47	1.40	0.54	1.29
X38	0.93	1.14	1.08	1.13	1.30	1.29	1.31	1.29
X52	0.97	0.94	0.33	1.30	0.33	1.01	0.68	1.36
X53	1.15	1.29	1.19	1.30	0.78	0.72	0.75	1.11
X54	0.57	1.20	0.85	1.28	1.17	1.22	1.27	1.33
E29	0.40	1.30	0.21	1.09	0.17	1.24	0.51	1.22
H10	0.18	1.03	0.21	1.03	0.16	1.25	0.19	1.24
Clinical isolates								
SL2	0.18	1.25	0.29	0.42	0.37	1.28	0.27	1.26
SL3	0.16	0.61	0.15	0.32	0.17	0.70	0.57	1.29
SL4	0.14	0.85	0.25	1.10	0.18	1.27	0.50	1.22
SL6	0.19	0.92	0.28	0.78	0.24	1.00	0.25	1.39
SL8	0.18	0.31	0.17	0.17	0.18	1.24	0.17	0.34
SL9	0.18	0.42	0.16	0.33	0.17	0.61	0.18	0.35
SL10	0.54	0.98	0.15	0.62	0.94	1.17	0.17	1.00
SL11	1.04	1.24	0.67	0.53	0.80	1.24	1.12	1.25
SL12	0.35	0.74	0.37	0.88	0.31	1.18	0.46	1.26
SL13	1.25	1.25	0.17	1.25	0.97	1.11	0.58	0.56
SL14	0.22	0.67	0.18	1.06	0.67	0.98	0.72	0.85
SL15	0.58	1.23	0.33	1.26	1.12	1.16	0.66	1.32
SL16	1.08	1.29	0.17	1.27	0.47	1.14	0.18	0.36
SL17	0.96	1.24	0.63	1.18	0.48	1.20	0.92	1.11
SL18	0.77	1.27	0.64	1.29	0.19	1.26	0.15	1.24
SL19	0.16	1.16	0.16	1.01	0.19	0.27	0.22	1.23
SL20	0.17	1.20	0.63	1.22	1.09	1.39	0.25	1.27
SL21	0.56	1.00	0.86	1.11	1.28	1.21	1.36	1.26
SL22	0.19	1.18	0.22	0.99	1.02	1.27	0.83	1.03

a. Absorbance value at 630nm of reactants with ferrous ion (Fe^{2+}) present (+Fe) or absent (-Fe). in culture conditions.

Appendix B: Siderophore production of *Vibrio cholerae* O1 El Tor isolates at 28°C and 37°C under iron-limiting and iron-rich conditions.

Isolates	28°C		37°C	
	-Fe	+Fe	-Fe	+Fe
V38	0.67[a]	0.90	0.61	0.86
V57	0.69	0.99	0.57	0.93
V113	0.65	0.94	0.57	0.84
V114	0.70	0.95	0.37	0.87
V115	0.89	1.13	0.20	0.45
V123	0.52	0.82	0.12	0.80
V124	0.51	0.88	0.17	0.90
V125	0.67	0.90	0.33	0.90
V127	0.89	1.26	0.35	1.01
V129	0.62	0.86	0.55	0.88
V130	0.60	0.82	0.47	0.84
V131	0.44	1.06	0.65	0.94
V132	0.45	0.84	0.27	0.96
V133	0.31	0.84	0.18	1.24
V135	0.55	0.93	0.55	0.87
V136	0.77	0.96	0.26	0.80
V137	0.43	0.86	0.36	0.79
V138	0.56	0.91	0.30	0.85
V140	1.12	1.17	0.22	1.13
V141	0.31	0.94	0.25	0.83
V142	0.54	0.92	0.08	0.90
V143	0.46	1.24	0.25	0.97
V144	0.27	0.87	0.49	0.91
V145	0.31	0.90	0.30	0.91
V146	0.72	0.81	0.42	0.92
V147	0.59	0.87	0.47	0.91
V1041	0.50	0.76	0.33	0.89
V1094	0.47	0.93	0.58	1.07
V1100	1.26	1.40	0.40	0.74
V1264	0.65	1.03	0.43	0.91
V1386	0.30	0.95	0.14	1.23
V1387	0.20	1.21	0.45	1.08
V1392	0.53	0.97	0.35	0.92
V1394	0.16	0.68	0.68	0.93
V1398	0.35	0.90	0.51	0.94
V1400	0.67	0.89	0.61	0.94
V1403	0.71	0.90	0.61	0.80
V1482	0.28	0.88	0.66	0.92

a, Absorbance value at 630nm of reactants.

Appendix C: Hemolysin production of the *Aeromonas hydrophila* isolates at 28°C and 37°C under iron-limiting and iron-rich conditions.

ISOLATES	28°C		37°C	
	TSB+EDDA	TSB	TSB+EDDA	TSB
Clinical isolates				
SL2	256[a]	64	128	32
SL3	0	0	0	0
SL4	64	16	512	128
SL6	0	0	0	0
SL8	0	0	0	0
SL9	0	0	0	0
SL10	64	16	64	32
SL11	0	0	0	0
SL12	0	0	0	0
SL13	0	0	0	0
SL14	0	0	0	0
SL15	32	8	32	32
SL16	32	32	128	128
SL17	32	16	128	64
SL18	0	0	0	0
SL20	32	16	128	128
SL21	8	8	4	8
SL22	0	0	0	0
A12	32	32	128	64
A13	32	32	32	16
A14	64	32	16	16
A24	1024	1024	512	1024
Environmental isolates				
X2	0	0	0	0
X8	256	256	256	256
X9	1024	1024	512	512
X13	64	32	128	64
X18	256	256	256	256
X36	0	0	0	0
X38	0	0	0	0
X52	64	64	64	32
X54	1024	512	512	256
H10	16	8	64	32
XF4	128	64	32	32
XF5	128	64	64	64
XF8	0	0	0	0
FC3	0	0	0	0
FC4	32	32	0	0
FP2	0	0	0	0
FP3	0	0	0	0

a, The hemolytic titer (HT) defined as the reciprocal of the highest dilution that gave 50% hemolysis of erythrocytes.

Appendix D: Hemolysin production of the *Vibrio cholerae* O1 El Tor isolates at 28°C and 37°C under iron-limiting and iron-rich conditions.

Isolates	28°C		37°C	
	+EDDA	-EDDA	+EDDA	-EDDA
V38	0[a]	4	0	0
V57	0	0	0	0
V112	0	0	0	0
V113	0	0	0	0
V114	0	0	0	0
V115	0	0	0	0
V122	0	0	0	0
V123	4	2	0	0
V124	0	4	0	0
V125	0	8	0	0
V127	0	0	0	0
V129	0	0	0	0
V130	0	0	0	0
V131	0	0	0	0
V132	0	0	0	0
V133	8	4	0	0
V135	0	0	0	0
V136	1	1	0	0
V137	0	0	0	0
V138	0	0	0	0
V140	0	0	0	0
V141	0	0	0	0
V142	0	0	0	0
V143	0	0	0	0
V144	0	0	0	0
V145	0	0	0	0
V146	0	0	0	0
V147	0	0	0	0
V1041	1	4	0	0
V1094	4	16	0	0
V1100	0	0	0	0
V1264	0	0	0	0
V1386	0	1	0	0
V1387	0	8	0	0
V1392	0	0	0	0
V1394	0	0	0	0
V1398	1	16	0	0
V1400	0	4	0	0
V1403	0	0	0	0
V1482	1	4	0	0

a, The hemolytic titer (HT) defined as the reciprocal of the highest dilution that gave 50% hemolysis of erythrocytes.

Appendix E

Protease production of the *Aeromonas hydrophila* isolates at 28°C and 37°C under iron-limiting and iron-rich conditions.

Strains	28°C			37°C		
	+EDDA	TSB	+$FeSO_4$	+EDDA	TSB	+$FeSO_4$
Clinical isolates						
SL2	0.72[a]	0.96	1.05	0.71	0.79	0.91
SL3	1.47	1.17	1.23	1.05	0.94	0.97
SL4	0.97	1.21	1.20	0.94	0.81	0.90
SL6	0.64	0.72	0.69	0.44	0.41	0.38
SL9	0.88	1.03	0.92	0.66	0.63	0.43
SL12	0.62	0.69	0.68	0.42	0.34	0.41
SL13	0.49	0.63	0.55	0.19	0.12	0.19
SL14	0.50	0.62	0.57	0.34	0.14	0.23
SL15	0.95	1.07	1.21	0.67	0.61	0.56
SL17	1.02	1.19	1.25	0.70	0.75	0.88
SL18	1.07	1.06	1.07	0.80	0.71	0.72
SL19	1.11	1.10	1.20	0.46	0.59	0.64
SL20	0.62	0.73	0.78	0.65	0.60	0.51
SL21	0.77	0.69	0.75	0.79	0.66	0.72
A12	0.83	1.13	0.84	0.83	0.68	0.73
A13	0.83	0.90	0.87	0.50	0.62	0.59
A14	1.00	1.08	1.01	0.78	0.93	0.78
A24	0.61	0.55	0.27	0.44	0.44	0.16
Environmental isolates						
X2	0.83	0.90	1.00	0.89	0.80	0.45
X8	0.12	0.25	0.20	0.21	0.07	0.15
X9	0.77	0.91	0.68	0.64	0.70	0.50
X18	0.77	0.86	0.80	0.63	0.57	0.58
X52	0.21	0.35	0.36	0.42	0.43	0.39
X54	0.65	0.55	0.37	0.44	0.37	0.15
E29	0.40	0.57	0.46	0.38	0.55	0.45
H10	0.69	0.52	0.67	0.52	0.48	0.59
XF4	0.60	0.65	0.50	0.40	0.57	0.37
XF5	0.63	0.68	0.50	0.48	0.40	0.43
XF8	0.65	0.69	0.70	0.64	0.57	0.56
FC3	0.17	0.21	0.10	0.06	0.11	0.04
FP2	0	0	0	0	0	0
FP3	0	0	0	0	0	0

a, The proteolytic activity measured by taking the absorbance value at 450nm of the reactants.

Appendix F

Protease production of the *Vibrio cholerae* O1 El Tor isolates at 28°C and 37°C under iron-limiting and iron-rich conditions.

Strains	28°C			37°C		
	+EDDA	TSB	+$FeSO_4$	+EDDA	TSB	+$FeSO_4$
V38	0.30[a]	0.57	0.73	0.28	0.71	0.71
V57	0.62	0.88	0.87	0.71	0.85	0.82
V113	0.70	0.84	0.88	0.78	0.93	0.82
V114	0.02	0.02	0.05	0.05	0.03	0.01
V115	0.76	0.83	0.92	0.82	0.84	0.91
V122	0.58	0.71	0.89	0.58	0.63	0.76
V123	0.75	0.84	0.85	0.76	0.85	0.81
V127	0.67	0.74	0.80	0.77	0.76	0.77
V130	0.67	0.77	0.89	0.70	0.71	0.84
V131	0.47	0.56	0.57	0.50	0.51	0.55
V132	0.69	0.84	0.85	0.81	0.78	0.80
V133	0.56	0.50	0.95	0.58	0.64	0.67
V135	0.76	0.88	0.88	0.71	0.79	0.85
V137	0.41	0.48	0.69	0.48	0.59	0.56
V138	0.73	0.75	0.91	0.71	0.89	1.05
V140	0.77	0.82	0.84	0.78	0.87	0.82
V142	0.74	0.86	0.97	0.82	0.85	0.90
V143	0.64	0.83	0.87	0.70	0.76	0.87
V145	0.76	0.82	0.89	0.82	0.78	0.91
V146	0.75	0.78	0.90	0.70	0.87	0.77
V147	0.68	0.85	0.88	0.95	0.89	0.98
V1041	0.62	0.82	0.92	0.67	0.80	0.85
V1094	0.56	0.90	0.97	0.63	0.84	0.83
V1264	0.38	0.53	0.61	0.49	0.47	0.62
V1386	0.73	0.68	0.84	0.78	0.74	0.81
V1387	0.60	0.79	0.87	0.72	0.84	0.80
V1394	0.81	0.89	1.03	0.80	0.83	1.00
V1398	0.77	0.83	0.88	0.03	0.05	0.02
V1400	0.03	0.02	0.00	0.04	0.00	0.02

a, The proteolytic activity measured by taking the absorbance value at 450nm of the reactants.

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Appendix G

Influence of iron, growth temperature and plasmids on siderophore production in *Aeromonas hydrophila*

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Aeromonas hydrophila strains obtained from diarrhoeal samples of human patients (19 isolates) and freshwater ponds (11 isolates) were analysed for siderophore production. Both clinical and environmental isolates showed significantly increased siderophore production under iron-limiting conditions both at 28°C and at 37°C. Clinical isolates consistently produced higher levels of siderophores than did the environmental isolates. The role of plasmids in moderating siderophore production was studied after curing with acridine orange. Treatment with acridine orange for 24 h removed the larger plasmids but the smaller plasmids (< 5 Mda), more common in the environmental isolates, were resistant to curing. As found in the untreated isolates, the cured clinical isolates produced higher mean levels of siderophores than the cured environmental isolates. Siderophore production in *A. hydrophila* was significantly influenced by iron-limiting cultural conditions and the source of isolates, but plasmid content and growth temperature at 28°C or 37°C had little effect on production. The basis for the greater production of siderophores in clinical isolates than in environmental isolates needs further study.

Introduction

The availability of iron is of crucial importance to the process of infection. The invading microbes often have to overcome the iron-limiting conditions imposed by the presence of high affinity iron-binding proteins in body fluids of vertebrate hosts [1]. Siderophores are low mol.-wt extracellular proteins synthesised by many microbes under iron-limiting conditions to obtain iron essential for the growth of the organism. Siderophores have a high affinity for ferric iron which enters the microbial cell as a ferric–siderophore complex through outer-membrane receptors specific for the siderophore [2–4]. The majority of the siderophores may be broadly classified as hydroxamates or catecholates [2, 5]. The hydroxamate siderophore, aerobactin, and the catecholate siderophore, anguibactin, have been recognised as virulence factors in *Escherichia coli* and *Vibrio anguillarum*, respectively [6], but the role of other siderophores in virulence remains to be established [2].

Aeromonas hydrophila strains are recognised as pathogens of several vertebrate species and in man have been associated with various clinical conditions, including wound infections, septicaemia and acute diarrhoeal disease [7–9]. Most of the *A. hydrophila* strains have been shown to produce the catecholate siderophores, either amonabactin or enterobactin, but not both [10, 11]. Amonabactin has been demonstrated to acquire iron from the vertebrate serum component Fe-transferrin and hence can be considered a virulence factor. Amonabactin-producing strains were also notable for their resistance to lysis by complement [12].

The environmental temperature has been shown to influence the expression of virulence factors, including haemolysins and proteases, in *A. hydrophila* [13–16]. However, as far as we are aware, there is no information on the effect of growth temperature on siderophore production in this organism. In the present study, the effect of iron deficiency and growth temperature on the total production of siderophores in clinical and environmental isolates of *A. hydrophila*

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was investigated. As the siderophore systems of *E. coli* [17–19] and *K. anguillarum* [20, 21] are known to be plasmid-mediated, the relationship between plasmid curing and siderophore production in the *A. hydrophila* isolates was also examined.

Materials and methods

Bacterial strains

A series of 19 clinical isolates of *A. hydrophila* was originally isolated from diarrhoeal samples at the General Hospital of Colombo, Sri Lanka (courtesy of Dr R. P. Subasinghe) and the 11 environmental isolates were obtained from several fresh water fish ponds in University of Malaya campus. These isolates were confirmed as *A. hydrophila* with the API 20E system (API System GA, La Balme Les Grottes, Montalieu-Vercieu, France) and from the metabolic pattern of utilisation of a panel of different carbon sources (Biolog Microstation, USA). Stock cultures were preserved in glycerol 25% in trypticase soy broth (TSB) at −20°C. For routine maintenance the cultures were grown on slants of nutrient agar and were kept at 4°C. All cultures were incubated at either 28°C or 37°C.

Curing experiments

Acridine orange was used as the curing agent [22] with the modifications suggested by Borrego *et al.* [23]. After incubation for 24 h in brain-heart infusion broth (BHIB) with shaking, 2-ml samples were added to 1 ml of fresh BHIB and incubated for 3 h at 37°C. The 1 ml of a solution of acridine orange (Sigma) 20 μg/ml was added to the culture and incubated further for 24 h. The cells were centrifuged and resuspended in 2 ml of fresh BHIB. After further growth for 6 h at 37°C, the isolates were tested for their plasmid content and siderophore activity.

Plasmid extraction

Plasmid DNA was extracted by the alkaline lysis method of Sambrook *et al.* [24] with several modifications. After overnight growth in Luria-Bertani (LB) medium the cultures were transferred to Eppendorf tubes, centrifuged and the pellets were resuspended in 150 μl of ice-cold Solution I (50 mM glucose, 25 mM Tris Cl 10 mM EDTA). Then 350 μl of Solution II (SDS 1% in 0.2 N NaOH) were added; the whole was mixed and placed on ice for 5 min before the addition of 250 μl of ice-cold solution III (prepared by adding 30 ml of 5 M potassium acetate to 11.5 ml of glacial acetic acid and the volume made up to 100 ml with distilled H_2O) [24]. The lysate was centrifuged and the supernate was mixed with an equal volume of isopropanol for 5 min and centrifuged again. The supernate was removed and DNA pellet was rinsed with ethanol 70% and left to dry before being dissolved in TE (10 mM Tris.Cl, 1 mM EDTA, pH 8.0) buffer and kept at −20°C.

Agarose gel electrophoresis

Plasmid DNA samples were resolved by electrophoresis in submerged horizontal agarose gels in TBE (Tris-borate) buffer (pH 8.0) at 5 V/cm for 1.5 h. The gels were stained in ethidium bromide solution (0.5 μg/ml) for 30 min and viewed, photographed and analysed with the Vilber Lourmat Bio-1D computer-assisted imaging system. Plasmids from *E. coli* V517 (35.7–1.4 MDa) were used as markers during electrophoresis.

Growth under iron-limiting conditions and production of siderophores

The cultures were grown in the MM9 liquid medium of Schwyn and Neilands [25] which consisted of MM9 salts, Pipes (Piperazine-N,N'-bis[2-ethanesulphonic acid]) buffer (Sigma), and casamino acids (0.3%). Thiamine-HCl (2 ppm) and nicotinic acid (2 ppm) were also added as suggested by Barghouti *et al.* [10]. The medium was treated with Chelex-100 (BioRad) by the batch method according to the manufacturer's instructions to lower the amount of trace metal contamination and then filter sterilised.

Siderophores were obtained by the method of Schwyn and Neilands [25]. A 10-ml volume of MM9 medium in a baffled flask was inoculated and incubated in a shaker at the required temperature. After sufficient growth (18–20 h), 100 μl of this iron-starved culture were transferred to 10 ml of the same medium. Iron-rich conditions were obtained by addition of 2 μM $FeSO_4.7H_2O$ to MM9 medium. After growth for 24 h, the cells were centrifuged and the supernate was assayed for the presence of siderophores.

Siderophore assay

The chrome azurol S (CAS) assay solution of Schwyn and Neilands [25] was used: 0.5 ml of the culture supernate was mixed with 0.5 ml of CAS assay solution and after equilibrium was reached the absorbance was measured at 630 nm with air as the blank. A low absorbance indicates a high production of siderophores and *vice-versa*. As the uninoculated medium gave an absorbance of 1.50, this was taken as the limit of detection. Siderophore production was categorised as follows: $A_{630} \leq 0.50$, high; $0.50 < A_{630} < 1.00$, medium; $A_{630} \geq 1.00$, low. A colour change from blue to orange was obtained within 5 min when catecholate siderophores were present.

Results

Siderophore production under iron-limiting conditions

Under iron-limited conditions at 28°C the mean production of siderophores increased 100% for clinical isolates and 50% for the environmental isolates when compared to normal iron-rich culture conditions (Table 1). Similar increases of 90% for clinical isolates

Table 1. Siderophore production in *A. hydrophila* isolates at 28°C and 37°C before and after treatment with acridine orange under iron-limiting and iron-rich conditions

Isolate no.	28°C				37°C			
	Before treatment		After treatment		Before treatment		After treatment	
	−Fe	+Fe	−Fe	+Fe	−Fe	+Fe	−Fe	+Fe
X2	1.27	1.29	1.29	1.27	1.45	1.34	0.23	1.31
X8	1.38	1.31	0.25	1.40	1.31	1.23	0.90	1.34
X13	1.35	1.25	1.31	1.21	1.28	1.20	0.91	1.36
X14	0.78	1.29	1.30	1.30	0.52	1.28	1.23	1.29
X36	0.41	1.31	0.66	1.39	0.47	1.40	0.54	1.29
X38	0.97	1.14	1.08	1.13	1.30	1.29	1.31	1.29
X52	0.97	0.94	0.33	1.30	0.33	1.01	0.68	1.36
X53	1.15	1.29	1.19	1.30	0.78	0.72	0.75	1.11
X54	0.57	1.29	0.85	1.28	1.17	1.22	1.27	1.33
E29	0.40	1.30	0.21	1.09	0.17	1.24	0.51	1.22
H10	0.18	1.02	0.21	1.03	0.16	1.25	0.19	1.24
Mean (SD)	0.81(0.45)	1.22(0.13)	0.81(0.50)	1.25(0.12)	0.81(0.50)	1.20(0.19)	0.77(0.39)	1.29(0.07)
T test	$p < 0.01$		$p < 0.01$		$p < 0.05$		$p < 0.01$	
SL2	0.18	1.25	0.29	0.42	0.37	1.28	0.27	1.26
SL3	0.16	0.61	0.15	0.32	0.17	0.70	0.57	1.29
SL4	0.14	0.85	0.25	1.10	0.18	1.27	0.50	1.22
SL6	0.19	0.92	0.28	0.78	0.24	1.00	0.25	1.39
SL8	0.18	0.31	0.17	0.17	0.18	1.24	0.17	0.34
SL9	0.18	0.42	0.16	0.33	0.17	0.61	0.18	0.35
SL10	0.54	0.99	0.15	0.62	0.94	1.17	0.17	1.00
SL11	1.04	1.24	0.67	0.53	0.80	1.24	1.12	1.25
SL12	0.35	0.74	0.37	0.88	0.31	1.18	0.46	1.26
SL13	1.25	1.25	0.17	1.25	0.97	1.11	0.58	0.56
SL14	0.22	0.67	0.18	1.06	0.67	0.98	0.72	0.85
SL15	0.58	1.23	0.33	1.26	1.12	1.16	0.66	1.32
SL16	1.08	1.29	0.17	1.27	0.47	1.14	0.18	0.36
SL17	0.96	1.24	0.63	1.18	0.48	1.20	0.92	1.11
SL18	0.77	1.27	0.64	1.29	0.19	1.26	0.15	1.24
SL19	0.16	1.16	0.16	1.01	0.19	0.27	0.22	1.23
SL20	0.17	1.20	0.63	1.22	1.09	1.39	0.25	1.27
SL21	0.56	1.05	0.86	1.11	1.28	1.21	1.36	1.26
SL22	0.19	1.18	0.22	0.99	1.02	1.27	0.83	1.03
Mean (SD)	0.47(0.38)	0.99(0.31)	0.34(0.22)	0.88(0.37)	0.57(0.39)	1.09(0.28)	0.50(0.36)	1.03(0.36)
t test	$p < 0.01$		$p < 0.01$		$p < 0.01$		$p < 0.01$	
All isolates								
Mean (SD)	0.63(0.43)	1.08(0.28)	0.51(0.41)	1.02(0.35)	0.66(0.44)	1.13(0.25)	0.60(0.39)	1.12(0.31)
t test	$p < 0.01$		$p < 0.01$		$p < 0.01$		$p < 0.01$	
t test[a]	$p < 0.025$	$p < 0.025$	$p < 0.01$	$p < 0.01$	$p < 0.10$	$p < 0.10$	$p < 0.05$	$p < 0.025$

X, E, H, environmental isolates; SL, clinical isolates

[a]Absorbance value at 410 nm of reactants with ferrous ion (Fe^{2+}) present (+Fe) or absent (−Fe) in culture conditions.

[b]*t* test between the mean values of clinical and environmental isolates in the same column.

and 20% for environmental isolates were noted for siderophore production at 37°C. At 28°C, clinical isolates produced 73% more siderophores under iron-limiting conditions than environmental isolates; while at 37°C, the clinical isolates possessed 44% more siderophore activity than the environmental isolates.

Effect of growth temperature of siderophore production

Under both iron-limiting and iron-rich conditions, clinical isolates produced slightly lower amounts of siderophores at 37°C than at 28°C (Table 1). Siderophore production by the clinical isolates grown under iron-limiting conditions at 37°C was 20% lower than at 28°C. Under iron-rich conditions the production of siderophores at 37°C was 10% lower than at 28°C. However, this difference was not seen with environmental isolates.

Characteristics of plasmid profile and curing

Twenty-four of the 30 isolates of *A. hydrophila* tested contained plasmids (Table 2). Half of the isolates with plasmids harboured more than one plasmid. Four isolates, all environmental, contained only large plasmids (> 20 MDa), 15 isolates contained only small plasmids (< 20 MDa) and five isolates contained both large and small plasmids. The maximum number of plasmids found in a clinical isolate was four and in an environmental isolates was seven. The clinical isolates in all contained 18 plasmids in the range 5–16 MDa, but only one environmental isolate harboured just one plasmid in this range. The environmental isolates in all contained 16 plasmids < 5.0 MDa while the clinical isolates harboured eight plasmids in this range.

The isolates, including those without plasmids, were all subjected to acridine orange treatment to monitor

Table 2. Plasmid profile of *A. hyarophila* isolates compared to the siderophore production before and after curing

Isolate no.	Number of plasmids	Mol. Wt (MDa)	Cultured at 28°C	Cultured at 37°C
SL2	1	11.1	NC	NC
SL3	3	37.0, 12.5, 3.9	NC	−
SL4	3	9.1, 5.5, 2.9	NC	−
SL5	0		NC	NC
SL6	3	35.7, 10.3, 3.9	NC	NC
SL9	4	3.9, 3.4, 1.5, 1.2	NC	NC
SL10	0		+	+
SL11	1	10.6	NC	NC
SL12	1	11.1	NC	NC
SL13	1	11.1	+	+
SL14	1	12.7	NC	NC
SL15	1	12.1	NC	+
SL16	4	9.9, 7.3, 5.2, 4.0	+	NC
SL17	2	11.8, 9.6	NC	−
SL18	1	15.3	NC	NC
SL19	3	15.3, 4.1, 3.6	NC	NC
SL20	0		−	+
SL21	2	39.0, 15.3	NC	NC
SL22	2	3.7, 3.2	NC	NC
X2	0		NC	+
X6	7	33.8, 19.9, 4.3, 4.2, 3.8, 3.3, 3.1	+	NC
X13	1	36.4	NC	NC
X14	1	37.1	−	−
X36	0		NC	NC
X38	0		NC	NC
X52	1	35.7	+	−
X53	5	3.7, 3.6, 3.4, 2.9, 2.2	NC	NC
Y54	1	35.7	NC	NC
E29	6	31.0, 5.0, 4.7, 3.8, 3.7, 3.2	NC	−
H10	1	3.7	NC	NC

Underlining indicates plasmids that were retained after curing; NC, no change in siderophore production after curing; (+) increase and (−) decrease in siderophore production after curing; a difference of at least 0.50 in the absorbance values before and after curing was considered significant.

the possible effect of the agent on chromosomal DNA. Large plasmids and small plasmids of > 5.0 MDa from both clinical and environmental isolates were lost after treatment for 24 h but only nine of 28 plasmids < 5.0 MDa were cured. As regards the smaller plasmids, the environmental isolates were more resistant to curing than the clinical isolates, as eight of the 12 plasmids < 5.0 MDa in clinical isolates were lost by only one of 16 plasmids of same range in environmental isolates was lost.

Effect of plasmid curing on siderophore production

After treatment with acridine orange, the average production of siderophores under iron-limiting conditions at 28°C increased significantly by 150% for the clinical isolates and 55% for the environmental isolates, compared to iron-rich conditions (Table 1). At 37°C the increase was 92% and 68% for the clinical and environmental isolates respectively. Under iron-limiting conditions, the acridine orange-treated clinical isolates produced 140% and 55% more siderophores at 28°C and 37°C, respectively, than environmental isolates. As noted for the untreated clinical isolates, a small non-significant reduction in the siderophore production of the treated clinical isolates was observed at 37°C compared to 28°C, under both iron-limiting and iron-rich conditions. For the environmental isolates, again there was no difference in the siderophore production of the plasmid-cured isolates at 28°C and 37°C under both iron-limited and iron-rich conditions.

Under iron-limiting conditions, a 40% increase was obtained at 28°C in the siderophore production of the cured clinical isolates compared to the untreated isolates, but only a 14% increase was observed at 37°C. Under iron-rich conditions the cured clinical isolates produce a 11% and 5% more siderophores at 28°C and 37°C, respectively, compared to the untreated isolates. The production of siderophores by the normal and cured isolates at 28°C and 37°C was not significantly altered under both iron-deficient and iron-rich conditions for environmental isolates.

Discussion

Siderophore production, when measured at 28°C and 37°C, increased dramatically in the iron-limiting conditions regardless of the growth temperature or plasmid content. More than 60% of the isolates tested produced high levels of siderophores in iron-limiting

conditions. These results agree with the established view that siderophores are produced in response to iron starvation and the presence of freely available Fe^{2+} acts as a co-repressor for the regulation of biosynthesis of siderophores [5].

Under iron-rich conditions (with 2 μM Fe^{2+} added), most of the clinical isolates continued to synthesise considerable amounts of siderophores ($A_{630} < 1.00$), whereas this effect was not noticed in the environmental isolates, suggesting that clinical isolates have a higher iron requirement than the environmental isolates. Clinical isolates were consistently found to produce higher mean levels of siderophores than did environmental isolates under both iron-deficient and iron-rich conditions at 28°C and also at 37°C. As far as we are aware this is the first attempt to quantify siderophore production in *A. hydrophila*. These results clearly demonstrate a considerable difference in the siderophore producing ability between the isolates from these two different sources. The availability of iron in body fluids is known to be very limited to the invading microbes [26, 27] and the ability of clinical isolates to produce higher levels of siderophores may confer on them a strategy to acquire iron for growth and thus promote their survival in the host. However, three environmental isolates did produce high levels of siderophores that were similar to those of clinical isolates. Possibly these environmental isolates with high siderophore producing ability may have a greater chance of cross-infecting vertebrate species, including man.

The siderophore production of clinical isolates was consistently lower at 37°C than at 28°C under both iron-limited and iron-rich conditions, whereas Mateos *et al.* [16] reported that more haemolysins and cytotoxins were produced at 37°C, but protease activity was lower at this temperature than at 28°C. However, the differential production of siderophores at these two temperatures was not statistically significant suggesting that temperature has little effect on their production.

Some interesting differences were evident in the plasmid profile of the clinical and environmental isolates of *A. hydrophila*. In general, the environmental isolates harboured more plasmids than clinical isolates. Very few plasmids in the 5–20 MDa range were found in environmental isolates, suggesting a difference from the clinical isolates in their ability to acquire plasmids. Acridine orange efficiently cured all plasmids > 5.0 MDa with treatment for 24 h, but smaller (< 5.0 MDa) plasmids were more resistant to treatment. Although environmental isolates harboured more plasmids < 5.0 MDa, the difficulty experienced in curing these plasmids from environmental isolates could not be explained on the basis of size alone, as eight plasmids of the same range were cured from clinical isolates. A prolonged treatment was not attempted to remove the plasmids < 5.0 MDa because of the possible effect of acridine orange on chromosomal DNA.

No definite pattern of increase or decrease was obtained in the normal isolates and their cured derivatives, but there was a small overall non-significant increase in the siderophore activity of the cured isolates compared to the normal isolates under all the various conditions tested. Moreover, there was no correlation between the presence or absence of the plasmids with the production of siderophores. However, Borrego *et al.* [23] found that 60% of the *A. hydrophila* isolates tested lost their siderophore activity after curing. The differences might be due to the methods of siderophore detection employed. The quantitative CAS assay technique in which small differences in the siderophore production were readily detectable was used in this study, but Borrego *et al.* [23] used the CAS agar plate method which only provides a qualitative result. As no plasmid encoded gene(s) for siderophore production in *A. hydrophila* have been identified so far, the results of the present study agree with the current view that genes for catecholate siderophore systems are chromosomal in this species [28].

Siderophore production by the cured clinical and environmental isolates remained unaffected by the curing experiments. Temperature had little effect on siderophore production, although a minor mean decrease was observed at 37°C for both normal and cured clinical isolates. As noted in normal isolates, siderophore production by the cured isolates was significantly increased under iron-limited conditions and the cured isolates also showed a significant increase in the siderophore activity of the clinical isolates compared to the environmental isolates. These observations further support the view that plasmids of *A. hydrophila* have little effect in siderophore biosynthesis.

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