NOTE

Effect of strain origin on siderophore production in *Vibrio harveyi* isolates

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ABSTRACT: A total of 92 isolates of *Vibrio harveyi* (as identified by ribotyping) were tested for siderophore activity on a modified chrome azurol sulphate agar. Whilst 4 isolates failed to grow, of the remaining 88 strains, 78.4% were positive for the production of siderophores. There was no correlation between siderophore production and length of time since isolation. There were significant differences between the host groups from which the bacteria were isolated ($F = 3.23$, df = 3,85; $p < 0.05$). The colony halos produced by environmental isolates (halo to colony ratio = 2.7) and fish isolates (halo ratio = 2.4) were significantly larger than the halos from invertebrate isolates (halo ratio = 1.6). Pathogenicity in an Atlantic salmon bioassay was barely significantly related to siderophore production ($t = -1.87$, df = 64; $p < 0.1$). The maximal non-inhibitory levels of 2,2'-dipyridyl showed no association with siderophores ($t = -1.65$, df = 30; $p > 0.05$) nor with pathogenicity ($t = 0.16$, df = 87; $p > 0.05$). Siderophores must be considered a virulence factor in piscine infecting *V. harveyi* but not in strains infecting invertebrates.

KEY WORDS: *Vibrio harveyi* · Siderophore · Virulence

Vibrio harveyi is a major pathogen of aquatic animals including fish (Kraxenberger-Beatty et al. 1990, Anderson & Norton 1991) crustacea (Lavilla-Pitogo et al. 1992, Owens et al. 1992) and bivalves (Pass et al. 1987). It is the main causative agent of luminous vibriosis which plagues tropical prawn hatcheries worldwide and it is considered the most important disease in the Philippines (Lavilla-Pitogo et al. 1990). Despite this, the mechanisms of virulence of this bacterium are unknown. Working with 16 strains including 2 highly virulent strains that killed prawn protozoea at 10^2 and 10^5 cells ml^{-1}, Harris (1993) showed that only 1 strain (and neither of the 2 strains above) had a very small plasmid, and so plasmid-borne pathogenicity was unlikely.

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Siderophores are bacterially secreted molecules with high affinity for iron that scavenge iron from the bacterial cells environment for growth, particularly for replication of the cytochrome systems. Siderophore production is often accompanied by the production of iron repressible outer membrane proteins (IROMP) which act as receptors for the iron-siderophore complex (Crosa 1989). These systems have been found to be major determinates of virulence in *Vibrio anguillarum* (Pybus et al. 1994), *Vibrio cholerae* non-O1 (Amaro et al. 1990) and *Aeromonas salmonicida* (Hirst & Ellis 1994). Harris (1993) failed to demonstrate the importance of siderophores to virulence of *V. harveyi* but his study only included 16 strains. The present study was an attempt to expand on the limited sample size of Harris's study and determine if indeed there might be a role for siderophores as determinates of virulence in *V. harveyi*.

Material and methods. *Bacterial strains:* A total of 93 *Vibrio harveyi* strains identified by ribotyping were taken out of $-70°C$ storage and were grown overnight at 22°C in Oxoid tryptone soya broth supplemented with 1% sodium chloride (w/v). These broth cultures were streaked onto Oxoid tryptone soya agar plates supplemented with 1% sodium chloride (w/v) (NTSA) and checked for purity by colony morphology after overnight growth at 22°C.

Siderophore production: Siderophore production was measured by a modified Chrome Azurol Sulphate (mCAS) agar method of Schwyn & Neilands (1987). The MM9 salts, casamino acids and carbon source were all replaced by the NTSA nutrients. This agar had available iron other than that presented in the CAS-complex. To make iron-limiting conditions, 0.1 mM 2,2'-dipyridyl was added to the agar before autoclaving. The final mCAS plates were a greyish

blue rather than the normal sky blue of CAS. All ingredients were purchased from Sigma Chemical Company.

Eight individual colonies were transferred from the NTSA plates with sterile toothpicks to mCAS. The plates were grown at 22°C and read at 24 and 48 h to ensure the *Vibrio harveyi* had reached stationary phase (Schwyn & Neilands 1987). Only 1 strain, 570, differed between the 24 h (nonproducer) and 48 h (producer) readings. The size of the zone of clearance (orange halo) had to be 1.3 times the size of the colony to be considered a siderophore producer (Amaro et al. 1990) and the mean values of the 8 colonies was used for this calculation. A *Vibrio anguillarum* strain (44) was used on all batches of plates as a known positive siderophore producer (Austin & Austin unpubl. data).

Normal NTSA was supplemented with various amounts of 2, 2'dipyridyl before autoclaving to make up final concentrations of 20, 50, 100, 200, 400 and 1000 μM. All strains were checked for their ability to grow at 20 and 50 μM. A random selection of 30 strains was tested for growth at higher levels of iron binding.

Fish pathogenicity experiments: *Vibrio harveyi* were tested for pathogenicity in Atlantic salmon *Salmo salar* (Austin & Austin unpubl. data) and the conclusions were used herein to determine if there was any association between siderophore production and pathogenicity. Briefly the protocol was as follows. Ten fish (15 to 20 g) were intraperitoneally injected with a 0.1 ml inoculum ranging from 10^4 to 10^6 washed bacterial cells ml^{-1} The fish were monitored for 14 d in aerated, static water at 15°C. Dead and moribund fish were subjected to standard bacteriological examination (Austin & Austin 1989) and, if the mortalities were repeated in a subsequent similar experiment, that categorised the *V. harveyi* strain as pathogenic.

Statistics: Standard statistical tests such as *t*-tests, ANOVA, least-significant difference analysis, regression analysis, and chi-squared tests were performed using Statistix 4.0™ (Analytical Software, St. Paul, MN, USA)

Results. Four isolates failed to grow on the mCAS, 3 isolated from invertebrates and 1 isolated from fish. Of those that grew, 19 of 88 (21.6%) strains were negative for the production of siderophores. Of the negative strains, 9, 6, 2 and 2 respectively were isolated from fish, invertebrates, environment and unknown. Using the chi-squared test, none of the groups were more negative for the production of siderophores than expected by their frequencies in the tested population.

There was no correlation between the year of isolation and siderophore activity ($t = 0.61$, df = 61; $p > 0.05$). This meant that siderophore activity had not been lost or gained by repeated subculturing and maintenance of older isolates. Therefore, conclusions reached regarding siderophore activity should be valid and represent the real population of *Vibrio harveyi*, and do not reflect differences in culturing conditions.

When testing to see if there was any significance difference in siderophore activity between the hosts from which the bacteria were isolated, the variances between the groups were not homogenous ($\chi^2 = 9.3$, df = 2; $p < 0.05$). Therefore, a square root transformation was applied and the variances became homogenous ($\chi^2 = 0.5$, df = 2; $p > 0.05$). This procedure was followed whenever groups had non-homogenous variances. A 1-way ANOVA showed that there were significant differences between the host groups from which the bacteria was isolated ($F = 3.23$, df = 3, 85; $p < 0.05$). Least Significant Difference analysis (LSD) showed that the halos from environmental isolates (ratio of the halo to the colony size = 2.7) and fish isolates (halo ratio = 2.4) were similar. Halos of the bacteria from both these host groups were significantly larger (via LSD) than the halo from invertebrate isolates (halo ratio = 1.6).

Pathogenicity as measured in the fish bioassay was barely significantly related to siderophore production ($t = -1.87$, df = 64; $p < 0.1$).

All strains could grow at 20 and 50 μM concentrations of 2, 2-dipyridyl. The maximal non-inhibitory levels of 2, 2'-dipyridyl showed no association with the levels of siderophore produced ($t = -1.65$, df = 29; $p > 0.05$) nor were they linked to pathogenicity ($t = 0.16$, df = 29; $p > 0.05$). The square-root transformation of the maximal non-inhibitory levels of 2, 2'-dipyridyl showed a limited significant difference between host groups ($t = 2.73$, df = 29; $p < 0.1$). Those strains isolated from fish were most resistant, followed by those from invertebrates, and those from the environment were least resistant.

Discussion. In *Vibrio harveyi*, siderophore activity was linked to the host group from which the bacterium was isolated, with invertebrate isolates having the lowest level of activity. Furthermore, siderophore activity appeared to be linked to pathogenicity in Atlantic salmon although a small doubt must remain. Therefore, siderophore systems should be considered a determinate of pathogenicity in vertebrates, much as in the same way siderophores in *V. anguillarum* are viewed (Pybus et al. 1994).

On the other hand, siderophores in *Vibrio harveyi* from invertebrates appear to be of lesser consequence for maintaining infections. Certainly, invertebrates do not seem to have the extensive iron-binding compounds such as lactoferrin, transferritin and haemoglobin as do vertebrates. The battle for iron between bacteria and invertebrates is not as intense as in vertebrates. The invertebrates seem not to have methods of sequestering iron away from the bacteria and, since

Table 1. *Vibrio harveyi*. Characteristics of the strains tested for siderophore production. Pathogenicity was determined in an Atlantic salmon bioassay. A blank in the siderophore column indicates that the isolate did not grow, probably due to 2, 2'dipyridyl toxicity. A blank in other columns indicates missing data

Strain	Isolated from	Path. to Atl. sal.?	Siderophore (mean ± SD)	Year	Country of origin	Strain	Isolated from	Path. to Atl. sal.?	Siderophore (mean ± SD)	Year –	Country of origin
22	*Dicentrarchus labrax*	Yes	3.1 ± 0.38	91	Greece	609	Unknown	No	5.2 ± 0.53	94	
23	*Dicentrarchus labrax*	Yes	4.0 ± 0.38	91	Greece	618	Penaeid	No	1.6 ± 0.42	94	Venezuela
24	*Sparus aurata*	Yes	3.2 ± 0.38	91	Greece	622	Penaeid	No	1.8 ± 0.53	94	Venezuela
184	*Artemia*	Yes	–	90	Spain	628	*Paguara*	No	1.0 ± 0.18	94	Venezuela
185	*Artemia*	Yes	1.4 ± 0.18	90	Spain	629	Bivalve	No	1.8 ± 0.26	94	Venezuela
214	Seawater	Yes	2.4 ± 0.46	91	Greece	631	Bivalve	No	3.2 ± 1.07	94	Venezuela
286	*Chromis plumbeus*	No	1.4 ± 0.46	82	USA	633	*Paguara*	Yes	3.0 ± 0.74	–	Venezuela
295	*Talorchestia*	No	–	–	USA	641	Shellfish	No	2.8 ± 0.50	–	Japan
326	Eel	No	1.5 ± 0.27	90	Belgium	642	Shellfish	No	0.1 ± 0.18	–	Japan
336	Shrimp	No	1.9 ± 0.37	80	Senegal	643	*Chanos chanos*	No	1.6 ± 0.46	–	Japan
337	Fish	No	0.9 ± 0.18	80	Senegal	644	*Chanos chanos*	No	1.8 ± 0.70	–	Japan
341	Seawater	No	4.5 ± 1.12	81	Senegal	645	Sea bass	No	2.1 ± 0.82	93	Tunesia
343	Caspian seawater	No	1.4 ± 0.32	81	Iran	646	Aquarium water	Yes	3.0 ± 1.31	93	Denmark
350[a]	Unknown	Yes	3.2 ± 1.70	–	–	647	Sea bream	Yes	5.2 ± 1.64	92	Greece
351	Unknown	Yes	2.5 ± 0.46	–	–	648	Shark	Yes	4.2 ± 1.00	93	Denmark
391	Shrimp	Yes	1.2 ± 0.26	90	Thailand	649	Sea bream	Yes	2.9 ± 1.27	93	Malta
392	*Lates calcarifer*	No	1.8 ± 0.26	–	Australia	650	Sea bass	No	2.8 ± 0.18	93	Greece
393	*Lates calcarifer*	No	2.9 ± 0.27	–	Australia	651	Aquarium water	Yes	3.4 ± 0.83	94	Denmark
394	*Lates calcarifer*	No	0.7 ± 0.71	–	Australia	652	Sea bass	Yes	3.1 ± 0.74	93	Italy
395	Prawn	No	2.6 ± 0.26	–	India?	653	Sea bass	Yes	3.2 ± 1.43	93	Turkey
396	Seawater	No	1.1 ± 0.38	–	Red Sea	654	Aquarium water	No	4.7 ± 0.18	94	Denmark
397	Seawater	No	1.1 ± 0.35	–	Hawaii	655	Sea bass	No	1.9 ± 0.42	89	France
398	Seawater	No	2.0 ± 0.35	–	Italy	656	Sea bass	No	1.0 ± 0.18	90	Greece
399	Seawater	Yes	2.2 ± 0.23	–	Hawaii?	657	Sea bass	No	2.7 ± 0.38	90	France
400	Unknown	No	0.7 ± 0.29	–	–	658	Sea bream	Yes	3.1 ± 0.56	90	France
401	Seawater	No	1.4 ± 0.53	–	USA	659	Sea bass	Yes	1.9 ± 0.18	90	Tunesia
402	Seawater	No	6.6 ± 0.96	–	–	660	Sea bass	No	3.9 ± 0.83	90	Greece
403	*Penaeus monodon*	No	0.9 ± 0.23	–	Thailand	661	Sea bass	Yes	1.0 ± 0.23	92	Tunesia
404	*Penaeus monodon*	No	1.8 ± 0.42	–	Thailand	665	Aquarium water	No	2.1 ± 0.91	93	Denmark
405	*Penaeus monodon*	No	1.1 ± 0.18	–	Thailand	666	Aquarium water	No	1.3 ± 0.25	93	Denmark
406	*Penaeus monodon*	No	0.9 ± 0.35	–	Thailand	667	Aquarium water	No	2.1 ± 0.85	94	Denmark
408	*Penaeus monodon*	No	1.7 ± 0.18	–	Thailand	696	Oyster	No	1.7 ± 0.26	89	Spain
409	*Penaeus monodon*	No	–	–	Thailand	697	Oyster	No	1.3 ± 0.18	89	Spain
411	Fish	No	1.0 ± 0.20	86	Sth Africa	771	Blue tang	No	4.9 ± 0.98	–	Australia
413	Seawater	No	4.0 ± 0.89	88	France	775	*Salmo salar*	No	–	–	Australia
538	Shrimp	No	1.1 ± 0.38	92	Thailand	788	*Sparus aurata*	No	1.4 ± 0.18	88	Spain
560	Sea bass	No	1.3 ± 0.60	86	Spain	789	*Sparus aurata*	No	2.6 ± 0.98	88	Spain
561	Sea bass	No	3.2 ± 0.79	88	Spain	796	Unknown	No	1.0 ± 0.14	–	Sri Lanka
568	*Scophthalmus maximus*	Yes	2.1 ± 0.58	90	Spain	803	Shrimp	No	4.8 ± 1.73	92	Thailand
569	*Scophthalmus maximus*	No	2.1 ± 0.42	90	Spain	811	*Dicentrarchus labrax*	No	4.3 ± 1.95	86	Spain
570	*Sparus aurata*	Yes	1.1 ± 0.26	90	Spain	812	*Dicentrarchus labrax*	No	6.3 ± 1.69	88	Spain
571	Sea bass	Yes	1.5 ± 0.82	90	Spain	816	Sediment	No	2.8 ± 0.90	94	Venezuela
572	Sea bream	Yes	2.2 ± 0.27	90	Spain	818	Unknown	No	5.9 ± 1.31	80	France
573	Sea bass	Yes	2.3 ± 0.44	90	Spain	819	Fish	No	1.0 ± 0.24	80	Senegal
574	Sea bass	Yes	2.2 ± 0.59	91	Spain	822	Shrimp	No	1.7 ± 0.26	80	Senegal
584	*Scophthalmus maximus*	No	1.0 ± 0.23	93	Spain	853	*Salmo salar*	No	1.4 ± 0.32	–	Tasmania
585	*Sparus aurata*	No	2.1 ± 0.60	91	Spain						

[a]This strain was a swarmer and was assumed to be *Vibrio alginolyticus* and therefore not used in any calculations

the bacteria are not so challenged, they do not maximise siderophore expression.

The seawater environment is renowned for the low bioavailability of certain elements. Iron is quickly oxidised to ferric salts and precipitates out, thus becoming biologically unavailable particularly at neutral or alkaline pH. Therefore, it is not surprising that siderophore activity is high in environmental isolates. The fact that the environmental isolates of *Vibrio harveyi* tested here have siderophore activity equivalent to that of the vertebrate isolates tested here suggests that environmental isolates are preadapted for surviving in fish.

The lack of correlation between 2, 2'-dipyridyl tolerance and siderophores was similar to the lack of association between siderophores and EDDA [ethylenediamine-di(o-hydroxyphenylacetic acid] (Amaro et al. 1990), which is another iron chelator similar to 2, 2'-dipyridyl in action. This lack of correlation probably means there are other mechanisms for acquiring iron besides the siderophore system as in *Aeromonas salmonicida* (Hirst et al. 1994). The level of siderophore-

negative strains of *Vibrio harveyi* (22%) was higher than the level of siderophore-negative *V. cholerae* non-O1 (6.5% of 156 strains) found by Amaro et al. (1990). This was probably due to using 2,2'-dipyridyl as an iron chelator in the normal nutrient agar for a bacterium and this method is probably not as sensitive as the original CAS agar.

The demonstration of a role for siderophores in the virulence of *Vibrio harveyi*, particularly in fish, suggests that further studies on IROMP, and maybe a characterisation of the type of siderophores, are warranted. IROMP in particular are good candidate antigens for vaccination (Hirst & Ellis 1994), especially if whole cell preparations are unsuccessful.

Evidence is accumulating that *Vibrio harveyi* has 2 major biotypes. Pizzutto & Hirst (1995) found 2 major DNA and protein profiles for Australian strains of *V. harveyi*. The major pathogenic strains of *V. harveyi* of prawns are sucrose-negative whilst the sucrose-positive strains are benign and even used as probiotics. The BIOLOG™ identification system based on utilisation of 95 carbon sources recognises 2 biotypes, and the production of siderophores tends to suggest 2 biotypes as well. Perhaps, there are 2 separate species now being classified under the a name of *V. harveyi*, and this may be a fruitful area of investigation.

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