

Bacterial Flora of Healthy and Abnormal Chaetognaths

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Freshly captured chaetognath *Sagitta* samples were grouped into apparently healthy and abnormal specimens and the viable heterotrophic bacterial population of both groups were examined. The bacterial population ranged from 1.3×10^2 to 1.0×10^4 per animal, and was one order higher in opaque specimens regardless of whether they were still moving or moribund at the time of capture. Although *Pseudomonas-Alteromonas* was the predominant bacteria in both groups, *Vibrio* comprised 36.3% of the bacterial population in the abnormal *Sagitta*. By the method of numerical taxonomy the total 143 strains were grouped into 14 main phena, of which 4 were exclusively composed of strains from healthy *Sagitta* and 1 from abnormal *Sagitta*. The rest of the strains were unevenly distributed into 9 phena, 4 of which were mostly comprised of strains from abnormal *Sagitta* and 1 of which mostly included strains from healthy *Sagitta*. In general, the isolates of abnormal *Sagitta* grew under a wide range of physiological conditions and showed greater hydrolytic activities of organic compounds.

A proportion of the bacterial population in the aquatic environment is attached to surfaces. Different sections of the naturally occurring bacterial population are the initial colonizers of different types of surfaces.¹⁾ A number of complex adhesive interactions may be involved, and bacterial strains probably differ considerably in their mode of attachment.²⁾ Thus there will be differences in the species composition of attached and free living bacteria. Simidu *et al.*³⁾ observed that the plankton community, in general, carries a microbial flora that is different from the surrounding sea water. It has been shown that *Vibrio* is the predominant group associated with copepods.³⁻⁵⁾ Chitinous skeletons of crustaceans appear to be suitable for bacterial attachment and growth. Exoskeleton surfaces of some isopod and amphipod species are known to support dense bacterial flora in contrast to their digestive tracts which are relatively free of microorganisms.⁶⁾ Data available on the presence of bacteria on *Sagitta* (chaetognaths) are scant, except for the reports by Nagasawa *et al.*^{7,8)} According to their study massive bacterial invasion occurs in *Sagitta*, both in those reared in the laboratory and those which live under natural conditions. Up to 13.3% of two dominant species of chaetognaths in Suruga Bay; *Sagitta nagae* and *S. pacifica* were attacked by bacteria. Nagasawa *et al.*⁹⁾ also

showed that deformed specimens of chaetognaths carry large numbers of bacteria compared to normal specimens. The proportion of abnormal specimens of chaetognaths in natural population were 3.9% to 12.4% in Tokyo Bay. The present study examines the bacterial flora of chaetognath and focuses on the different types of the animal.

Methods

Zooplankton samples were collected from inner Tokyo Bay with a hand net GG-54 (mesh size 0.320 mm, Rigosha, Japan), which had washed with alcohol before towing. *Sagitta* were sorted and transferred to sterilized bottles. Based on appearance (distortion of the body, opaqueness of the muscles) and movement they were grouped as healthy or abnormal (occasionally referred to as weak or unhealthy) *Sagitta* (Fig. 1), and unhealthy and each group was sampled with a sterile forceps. Individual *Sagitta* was washed twice in sterile sea water and homogenized in 1 ml of sterile sea water. An aliquot of 0.1 ml of the homogenate was spread on ORI ager medium (Bacto Proteose peptone (Difco) 0.1 g, Bacto Yeast Extract 0.1 g, Phytone (BBL) 0.05 g, sodium thiosulfate 0.02 g, sodium sulfite 0.005 g, ferric citrate 0.004 g and Bacto Agar (Difco) 15 g), in 90% seawater and incubated for 10 days at 20°C.

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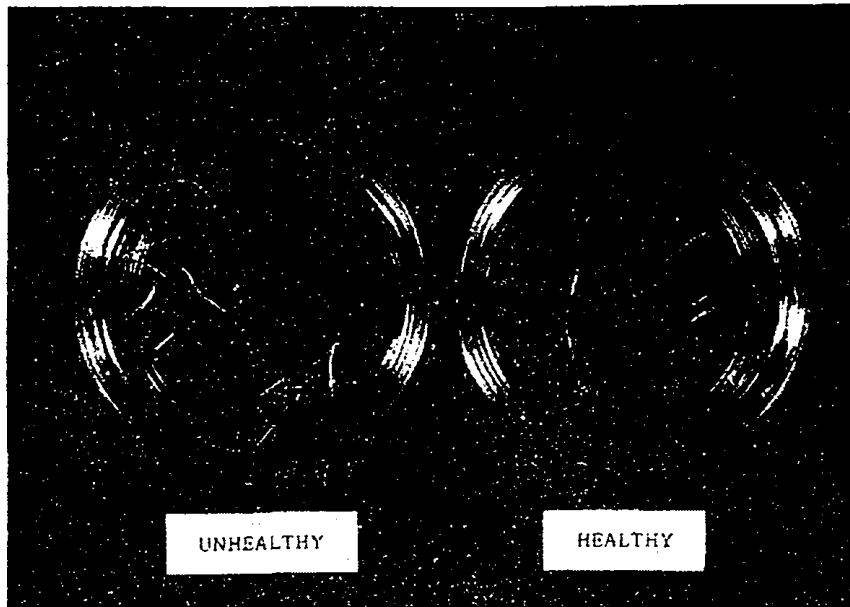


Fig. 1. Photograph of healthy and abnormal *Sagitta*.

The number of colonies were counted and about 10 strains were isolated at random from the plates having 20–200 colonies. Total 143 strains were purified and subjected to various biochemical and physiological tests.

Taxonomical Studies

Twenty four tests (73 features) were performed on the strains, including examination of morphological, physiological and biochemical characteristics. Gram staining was carried out using Hucker's modification.⁶⁾ Motility of the strains was determined microscopically with a phase-contrast microscope. Pigmentation was determined on ORI agar plates. Presence of fluorescent pigment was detected on King's medium.¹⁰⁾ Ability of growth at various temperatures was examined on ORI medium. Requirement of NaCl was determined by the ability of the culture to grow at 0, 0.2, 0.5, 1.0, 2.0, 5.0, 7.0 and 10.0% of salt concentration. Growth at various pH was examined in ORI medium by adjusting the pH from 2.0 to 10.0. Susceptibility to antibiotics was tested using the disk diffusion method. Gelatin hydrolysis was tested in ORI medium containing 25% gelatin. Hydrolysis of Casein was observed using the double-layered agar plate method. Chitin digestion was detected on ORI agar plates containing resuspended chitin particles. DNase activity was tested following the method of Jeffries *et al.*¹²⁾ Hydrolysis of lipid was determined by Rhodes' method.¹³⁾ Bacteriolytic activity was determined by an agar plate method. Lyophilized

cells of *Vibrio parahaemolyticus* were added to ORI agar medium at the concentration of 5 mg/ml. Lytic activity was detected by a clear zone around the area of growth of test organisms. Production of acid from various carbohydrates was also examined. The results were subjected to computer analysis. Similarity among strains was computed with a FACOM M18011 AD (HSH) computer. Using the single matching coefficient of Sokal and Mitcher, clustering was carried out by the single linkage and unweighted average linkage method. Clusters were grouped into phena at 85% similarity using the average linkage method.¹⁵⁾ Strains of healthy and weak *Sagitta* were also treated separately for numerical taxonomy.

Results

Bacterial counts on *Sagitta* groups based on appearance are tabulated in Table 1. The population density was one order of magnitude higher in the opaque *Sagitta* regardless of whether they were living or moribund at the time of capture. It was observed that the *Sagitta* became opaque when large numbers of bacteria were present on them. The percentage generic compositions of bacteria isolated from the healthy and unhealthy *Sagitta* are summarized in Table 2. The dominant flora were *Pseudomonas-Alteromonas*, *Alcaligenes* and *Vibrio* in the healthy *Sagitta*. *Pseudomonas-Alteromonas* followed by *Vibrio* were the predominant genera in the unhealthy *Sagitta*. More diverse flora were observed in

Table 1. Viable counts of heterotrophic bacteria in *Sagitta*

Nature of <i>Sagitta</i>	No. of sample	Viable counts/ <i>Sagitta</i>	SD
Healthy <i>Sagitta</i>			
Transparent & living	15	5.4×10^2	2.2×10^2
Weak <i>Sagitta</i>			
Transparent & living	2	2.0×10^2	1.4×10^2
Transparent & moribund	8	2.0×10^2	3.3×10^2
Opaque & living	3	2.7×10^3	2.7×10^3
Opaque & moribund	7	6.9×10^3	3.2×10^3

Table 2. Taxonomic distribution of the 143 heterotrophic bacteria isolated from healthy and weak *Sagitta*

Taxonomic group	Healthy <i>Sagitta</i>		Weak <i>Sagitta</i>	
	No.	%	No.	%
<i>Pseudomonas- Alteromonas</i>	35	55.6	50	62.4
<i>Vibrio</i>	8	12.7	29	36.3
<i>Alcaligenes</i>	13	20.6	1	1.3
<i>Flavobacterium</i>	2	3.2	—	—
Unidentified	5	7.9	—	—
Total	63	100.0	80	100.0

healthy *Sagitta* than in unhealthy *Sagitta*. The percentage of *Vibrio* increased from 12.7% for the healthy *Sagitta* to 36.3% in unhealthy *Sagitta*, and in contrast *Alcaligenes* decreased from 20.6% to 1.3%. Most of the *Vibrio* associated with unhealthy *Sagitta* showed chitinase activity.

Numerical Taxonomy

All the strains were Gram negative, motile except 2 strains and were able to grow at pH 8 and 9 and at temperatures of 20°C and 30°C. They failed to grow at pH 4 and 5 and were resistant to Bacitracin. These 9 characteristics were eliminated from the data matrix. At a similarity coefficient of 85%, the 143 strains were grouped into 20 clusters. Six of these clusters were represented by single strain and hence were omitted from the dendrogram. The remaining 14 were clusters composed of 2 or more strains (Fig. 2). Out of the 14 clusters, 4 each were strains from healthy *Sagitta* (H1, H2, H3 and H4) and one was from unhealthy *Sagitta* (W1). The rest were mixtures of both the groups (HW series) with

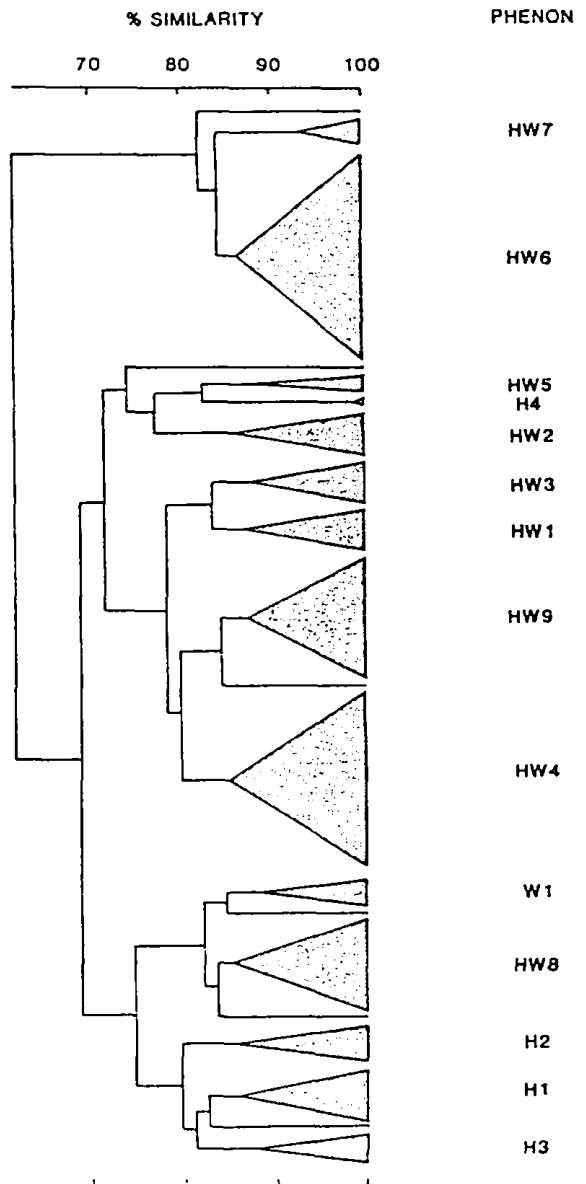


Fig. 2. Dendrogram of strains isolated from *Sagitta*.
 H: strains isolated from healthy *Sagitta*.
 W: strains isolated from abnormal *Sagitta*.
 HW: strains isolated from both groups of *Sagitta*.

strains from either group dominating or they were represented in equal numbers. Phenon HW1 was composed of strains mostly isolated from healthy *Sagitta* and HW6, HW7, HW8 and HW9 were mostly from unhealthy *Sagitta*. Thus the taxonomical analysis also clearly revealed the difference in origin of the strains, that is, healthy or abnormal *Sagitta*. The characteristics of each phenon are tabulated in Table 3. The main characteristics of the phenon of the strains from healthy *Sagitta* were very poor hydrolytic activity against various

Table 3. Characteristics of phena

Pehnon name	H1	H2	H3	H4	HW1	HW2	HW3	HW4	HW5	HW6	HW7	HW8	HW9	W1
No. of strains	8	5	4	2	6	6	6	26	2	31	5	14	18	4
% of healthy group	100	100	100	100	83	67	67	57	50	22	20	7	5	0
Catalase	88* ¹	75	50	100	67	100	50	100	50	45	60	50	100	
Growth at:														
2°C			50			33		12						
10°C	25			100	17	100	17	92		26	40	43	100	
37°C	100	20	100		100	67	100	96	50	97	80	100	100	
pH 6				100		83	17	92	100	97	100	21	100	
11	100	100	75	100	100	83	100	100	100	97	100	100	100	25
Growth in:														
0.1% NaCl	13			100		100	17	96	50				94	
0.2% NaCl	63			100	17	100	17	100	50	3	20		100	
7.0% NaCl	13				83	100	100	100		77	40	7	100	
10.0% NaCl					83	100	17	96					100	
Degradation of:														
Casein					50	100	100	92	100	97	60	100	94	
Gelatin	38				100	100	100	81	100	81			89	
Tween 80					100	100	100	100	100	97	100	100	94	75
Starch					100		83	81		97	100	71	72	100
Alginate										3			6	
Urea										6				25
Phosphate			100		67	50	83	77	100	61		93	100	100
Chitin					17	17		4	50	35			6	
Aesculin		40			50		67	92		13	80	7	56	25
Lecithin				100	17			19	100	87	100		11	
DNA	14	20			100	83	100	100	100	100	100	100	94	100
Bacteriolytic activity					100	100	100	96	50	58	40	100	100	75
Acid from:														
Galactose F* ²										100	100			
O	50	60	100	100	33	17	17	92					11	
Glucose F										100	100			
O	63	60	100	100	100	100	17	100	50				22	
Glycerol F										100	100			
O	13		25											
Mannitol F										100	100			
O		60	100		33		4						6	

*¹ Percentage of positive strains.*² F: Fermentative, O: Oxidative.

tested polymers. They also showed little bacteriolytic activity. In the case of strains from unhealthy *Sagittas*, the phena showed a wide range of hydrolytic activity to casein, tween 80, starch and DNA.

(1) Phenon H1 This phenon consisted of 8 isolates from healthy *Sagitta*. 38% hydrolyzed gelatin. About half the strains were able to utilize glucose and galactose oxidatively.

(2) Phenon H2 5 strains from healthy *Sagitta* formed this phenon. Hydrolytic activity was very poor.

(3) Phenon H3 This phenon consisted of 4

strains from healthy *Sagitta*. They had a narrow physiological tolerance and possessed phosphatase activity. The strains oxidatively utilised cellulose, fructose, galactose, glucose, maltose and mannitol. All the strains were *Alcaligenes*.

(4) Phenon H4 The 2 strains from healthy *Sagitta* were only fluorescent with lecithinase activity. They produced acid only from galactose and glucose and were resistant to all antibiotics tested. The strains also grew in media prepared with distilled water.

(5) Phenon HW1 Out of 6 strains, 5 were from healthy *Sagitta* and showed tolerance to

higher temperature, pH and salt concentration and proteolytic, amylolytic and DNA hydrolyzing activity. All the strains oxidatively produced acid from glucose and cellulose.

(6) Phenon HW2 All the 6 strains showed wide NaCl tolerance and proteolytic activity. Unlike HW1, the amylolytic activity and carbohydrate utilization were poor.

(7) Phenon HW3 Out of the 6 strains, 4 were from healthy *Sagitta*. The strains hydrolyzed polymers and utilized only maltose and sucrose oxidatively.

(8) Phenon HW4 This was the second largest phenon with 26 strains. 57% of the strains were represented by healthy *Sagitta*. All the isolates oxidatively utilized only glucose. 92% of the strains possessed aesculin hydrolyzing capacity.

(9) Phenon HW5 This phenon was comprised of 2 strains, one from each group of *Sagitta*. They hydrolyzed most of the polymers. Except for maltose, they were poor utilizers of carbohydrate.

(10) Phenon HW6 This was the largest phenon with 31 strains. About 80% were from unhealthy *Sagitta*. They hydrolyzed almost all the polymers and fermentatively utilized all sugars tested. Thirty five per cent showed a chitinase positive reaction. This phenon had the characteristics of the genus *Vibrio*.

(11) Phenon HW7 Out of the 5 strains, only one was from healthy *Sagitta*. This phenon is very similar to HW6 except in negative phosphatase reaction. All the isolates were sensitive to penicillin. This phenon belonged to the genus *Vibrio*.

(12) Phenon HW8 Most (93%) strains were from unhealthy *Sagitta*. All the 14 isolates possessed proteolytic, lipolytic and DNase activities. This phenon showed poor ability to utilize sugars.

(13) Phenon HW9 Except for 1 strain, the remaining 17 came from unhealthy *Sagitta*. They possessed hydrolytic activity for polymers but poor ability to utilize sugars.

(14) Phenon W1 All the 4 isolates were from unhealthy *Sagitta*. This phenon was non proteolytic and do not utilize sugars at all.

Discussion

The surface of animals has been shown to be a suitable substrate for microorganisms.¹³⁾ In general the bacterial population density on healthy

transparent *Sagitta* was one order of magnitude less than on opaque *Sagitta*. Differences were marginal among transparent animals irrespective of their being living, abnormal or moribund. Thus it could be surmised that an increase in the bacterial population led to opaqueness of the animal. Highest densities were observed in *Sagitta* that were opaque and moribund but still living at the time of capture. The opaqueness results from an increase in the bacterial population and is an indication of weakness which eventually leads to death. Nagasawa *et al.*⁷⁾ reported that bacteria colonize on chaetognaths reared in the laboratory and also on specimens collected from Suruga and Tokyo Bays. In their laboratory experiment, the opaqueness of the body appeared either at the head or tail and proceeded toward the centre of the body.

Kogure *et al.*¹⁴⁾ showed that zooplankton provide a better substrate than phytoplankton for colonization of *Vibrio*. Sochard *et al.*¹⁵⁾ reported that *Vibrio* spp. were abundantly associated with copepods. The selective attachment of *Vibrio cholera* on live copepods was also demonstrated by Huq *et al.*¹⁾ *Pseudomonas-Alteromonas* spp. was the major group associated with *Sagitta*. However, the numbers of *Vibrios* were markedly higher in the unhealthy *Sagitta*. Phenon HW6, one of the largest clusters from the *Sagitta* strains, represented *Vibrios* from both types of *Sagitta*, although a majority of the strains were from unhealthy *Sagitta*. About one third of the strains had chitinase activity. It is not yet possible to state if the weakening of the animal occurs as a result of attachment or an invasion of *Vibrio* or other bacteria, or by other causes such as mechanical injury which then lead to invasion and massive growth of bacteria. It is well known that during the rearing of abalone in natural seawater, mass mortality of shellfish occurs as a result of invasion of *Vibrios* including *V. parahaemolyticus*. However, the pathogenicity of *Vibrio* has not been established and the possibility that injury leads to bacterial invasion still exists.

In the present study, based on numerical analysis, the strains from healthy and unhealthy *Sagitta* were grouped into 14 major phenon, in which 5 (H1, H2, H3, H4 and HW1) were composed of, almost exclusively, strains from healthy *Sagittas*. There were also 5 phenon, whose members were mostly from apparently unhealthy *Sagittas*. Thus the numerical taxonomy work revealed that different bacterial groups pre-

dominated in healthy and apparently unhealthy *Sagitta*. The strains from healthy *Sagitta* showed poor hydrolytic activity compared to the strains from unhealthy *Sagitta*. The high and diverse hydrolytic activities of the strains of abnormal *Sagitta* may be accentuated in the degradation of *Sagitta*.

The present study is the first microbiological analysis of bacterial populations of *Sagitta*, although Nagasawa *et al.*^{7,8)} have shown the presence of bacteria by SEM. The three main results of this study are 1) opaqueness of *Sagitta* appears to be associated with high bacterial counts, 2) *Vibrio* may play some role in the decomposition of *Sagitta* and 3) various hydrolyzing activities were observed in the strains from weak *Sagitta*. The results also indicate that the *Sagitta* population may at least be partially controlled by the bacteria that are associated with the animal.

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