Numerical Taxonomy of Gram-Negative, Oxidase-Positive Rods from Carcharhinid Sharks

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Clustering and ordination were used to analyze the results of a study involving phenotypic characterization (61 tests) of 197 strains of gram-negative, oxidase-positive, rod-shaped bacteria isolated from carcharhinid sharks. Also included in the data set were data for 39 reference strains representing the genera *Aeromonas*, *Plesiomonas*, and *Vibrio*. Unweighted pair group mathematical averaging cluster analysis resolved nine major groups of operational taxonomic units (OTUs) at simple matching coefficient (S_{SM}) values of $\geq 66.5\%$. In turn, each group contained one or more OTU clusters (phena) at S_{SM} values of $\geq 72.6\%$. A total of 27 phena were resolved; 14 phena were identified as *Vibrio* species and contained 182 of the 236 OTUs in the study. Additional phena were identified as members of the genus *Aeromonas* (two phena), the genus *Photobacterium* (three phena), the genus *Alteromonas* (two phena), and *Plesiomonas shigelloides* (one phenon). One phenon resembled descriptions of the genera *Moraxella* and *Neisseria* and *Pasteurella piscicida*. The remaining four phena were not identified. The largest phena were identified as *Vibrio damsela* (52 OTUs) and *Vibrio alginolyticus* (48 OTUs). *Vibrio carchariae* and *Vibrio harveyi* formed subclusters (S_{SM} values, $\geq 82\%$) within the third largest phenon (24 OTUs), which was resolved at an S_{SM} value of $\geq 72.6\%$. Human pathogens (e.g., *Vibrio cholerae*) and fish pathogens (e.g., *Aeromonas salmonicida*, *Pasteurella piscicida*, and *V. damsela*) were isolated from the sharks. Approximately 58% of all of the strains isolated and tested in this study were urease positive.

It has been demonstrated that *Vibrio* spp. can be readily isolated from all tissues, organs, and body cavities of healthy, free-ranging sharks (8). Specifically, *Vibrio* spp. were shown to comprise 59 of 78 pure cultures isolated from 28 neritic sharks that represented five shark species (8). It was hypothesized previously that vibrios act as opportunistic pathogens in sharks, causing disease in compromised individuals (9, 10). Furthermore, regulation of tissue urea concentration was suggested as a possible biological role for shark-associated vibrios (10), and this regulation may be the basis for commensalism between vibrios and sharks. This hypothesis was supported by the results of [¹⁴C]urea studies done to investigate the in situ ureolytic ability of tissue-associated bacteria (15). Therefore, sharks are somewhat unusual among vertebrates, since apparently the tissues and organs of healthy individuals contain bacteria which, on the one hand, are capable of initiating disease but, on the other hand, appear to be either beneficial or fundamental to necessary life processes of the sharks.

In this study we used numerical taxonomy to analyze phenotypic data compiled for 197 strains of bacteria isolated from 10 carcharhinid sharks. Traditionally, numerical taxonomy has been used to cluster operational taxonomic units (OTUs) into groups on the basis of phenotypic or other data recorded for each OTU (20, 26). One of the most popular and useful approaches used in the classification of bacteria has been to calculate measurements of similarity on the basis of bacterial phenotypes (e.g., by using the simple matching coefficient [S_{SM}] and/or the Jaccard coefficient [S_J]), followed by clustering of the OTUs into groups by using unweighted pair group mathematical averaging (UPGMA) (1, 11, 20, 26). We have found that this approach is useful (13, 28), but it has the disadvantage of often accounting for only 50 to 60% of the relatedness among OTUs (notably,

fresh environmental isolates) (1, 26). MacDonell et al. (19) applied ordination analysis to 5S rRNA sequences and concluded that when this method was applied to nucleic acid sequence data, it accounted for close to 90% of the relatedness among OTUs. Principal-component analysis of the data permitted a more informative display of OTUs and inter-group relationships. In this study, both clustering and ordination were used to analyze phenotypic characterization results.

MATERIALS AND METHODS

Sample collection. Most sharks (the exceptions are indicated in Table 1, footnote c) were captured in the vicinity of Bimini, Bahamas, in May 1984 in an area and by using methods that have been described previously (8). In one case, a monogenean trematode, which was identified as *Dermophthirus nigrellii* (6), was aseptically removed from the surface of the skin of a mature lemon shark (Casey tag no. A01510) and was placed into sterile artificial seawater (Instant Ocean; Aquarium Systems, Mentor, Ohio) for transport to the shipboard laboratory for analysis. Water samples were also collected from the capture sites of the blacktip shark, tiger shark A, and lemon shark B. Finally, one of the scientists sustained a shark bite to the thumb during capture of lemon shark A08959. Opportunistically, sterile swabs (Culturette; American Scientific Products, McGaw Park, Ill.) were used to sample the shark's mouth and teeth, and prior to cleaning and suturing, the wound itself was sampled.

Culture methods. Samples were obtained from the sharks in the order described below. (i) Blood samples (50 to 120 ml) were withdrawn from a caudal blood vessel, and 5 ml of each sample was added to a blood bottle (thioglycolate medium lacking an indicator but containing CO₂ [BBL Microbiology Systems, Cockeysville, Md.]). (ii) A tooth was cut aseptically from the upper jaw and placed into broth medium. (iii) The cloaca-anus area was swabbed, and the swab was placed

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into broth. And (iv) the peritoneal cavity was opened, and ca. 1-cm³ tissue sections were removed in the following order: liver, spleen, pancreas, kidney, esophagus and anterior stomach, mid-stomach, posterior stomach, pylorus, duodenum, anterior spiral valve, mid-spiral valve, and posterior spiral valve. Each tissue section was aseptically excised and placed into broth. As in our previous study (8), great care was exercised to prevent sample contamination.

The trematode was washed by shaking it three times serially in fresh 3-ml portions of sterile artificial seawater (Instant Ocean) and was transferred to a sterile petri dish. Trematode tissue was teased apart with a sterile Pasteur pipet and transferred to a broth medium. Water samples were analyzed qualitatively (i.e., by using a sterile swab immersed in seawater and inoculated into broth). All samples except shark blood were inoculated into thioglycolate medium prepared in screw-cap tubes without indicator (Difco Laboratories, Detroit, Mich.) but supplemented with 1.0% NaCl. Broth cultures were incubated overnight at ambient temperature (ca. 24°C), following which blood agar plates (tryptic soy agar [Difco] prepared with 5% defibrinated sheep blood), Levine eosin methylene blue (Difco) agar plates, and thiosulfate-citrate-bile salts-sucrose (Oxoid U.S.A., Inc., Columbia, Md.) agar plates were streaked to isolate organisms from the broth cultures. The plates were incubated at ambient temperature for 4 days. Colonies were picked from the plates after 24 h and 4 days and were streaked onto marine seawater complete agar (10), and the resulting preparations were incubated at ambient temperature. Colonies from the marine seawater complete agar plates were restreaked (if necessary) to achieve purity and were inoculated into semisolid maintenance medium (10). Cultures were also frozen in fresh broth under liquid nitrogen for long-term storage (10).

The isolation and maintenance protocol described above yielded 169 isolates of gram-negative, oxidase-positive bacteria, which are listed in Table 1 along with their sources of isolation. An additional 28 isolates were collected from shark muscle tissue in May 1985, so a total of 197 strains made up the sample set analyzed in this study. Selected reference strains belonging to the genera *Aeromonas*, *Plesiomonas*, and *Vibrio* were also included, and these organisms were maintained as described above (i.e., in semisolid maintenance medium and under liquid N₂). The 39 reference strains included in the study are listed in Table 2.

Characterization of strains. The materials and methods used for phenotypic characterization of each OTU (26) included in the study have been described elsewhere (30, 31). The 66 tests chosen for strain characterization were either tests demonstrated by West et al. (28) to be useful for identification and classification of *Vibrio* spp. (21 of the 66 tests) or tests that are effective in clustering OTUs into taxa (45 tests).

Statistical analysis. The phenotypic tests applied to each OTU were coded (0, negative; 1, positive; 9, not done or no growth), and the codes were entered into a 236-by-66 data matrix. The data matrix was used to calculate the S_{SM} and S_J values for each of the 236 OTUs by using the equations of Pielou (20). A cluster analysis for each of the similarity coefficients was performed by using the UPGMA method. The cophenetic correlation coefficient, a measurement of how well the cluster analysis accounted for OTU relatedness, as determined by the S_{SM} or S_J values (26), was also computed. Once the major groups (i.e., clusters) were determined from the cluster analysis, the phenotypic data were reexamined to determine which test results were unique to

TABLE 1. Sources of strains included in this study

Source ^a	OTU(s)
Tiger shark A	
Water at site.....	5, 7, 8
Tooth.....	17, 19, 20, 21, 90
Cloaca-anus area.....	86, 87, 88, 89, 101
Liver.....	9
Esophagus.....	22, 26, 30, 31, 91
Mid-stomach.....	35, 36
Posterior stomach.....	38, 40, 92
Pylorus.....	45, 49B
Duodenum.....	53, 96
Anterior spiral valve.....	60, 61A, 62, 63, 68
Mid-spiral valve.....	73, 75, 77
Posterior spiral valve.....	79, 82
Blacktip shark	
Water at site.....	103, 106, 108
Blood.....	112, 113, 114, 115, 116, 117
Tooth.....	121, 122, 123
Cloaca-anus area.....	224, 227, 229, 230, 232, 236, 238
Liver.....	133
Spleen.....	125, 126, 129
Pancreas.....	148, 153
Kidney.....	141, 143, 144, 145
Esophagus.....	154, 155, 157, 158, 159, 161, 163, 164, 165, 166, 167, 168, 169, 171, 172
Mid-stomach.....	174, 175, 177, 179
Posterior stomach.....	182, 183, 184, 185, 186, 187, 188, 189
Pylorus.....	191, 193
Duodenum.....	194, 196, 197
Anterior spiral valve.....	201, 202, 208
Mid-spiral valve.....	209, 210, 211, 212
Posterior spiral valve.....	217, 218, 221
Trematode	246
Lemon shark A	
Blood.....	401
Cloaca-anus area.....	425, 427, 429, 431, 432, 433, 434, 435, 436
Spleen.....	423, 424
Esophagus.....	403, 404, 405, 407, 410, 430
Mid-stomach.....	417, 418
Posterior stomach.....	414
Spiral valve.....	421, 422
Lemon shark B	
Water at site.....	464, 466, 467, 468
Cloaca-anus area.....	437, 438, 439, 445, 446, 448, 449
Esophagus.....	451, 452, 454
Spiral valve.....	440, 441, 443
Shark bite^b	
Wound.....	460, 461W, 461T, 462
Shark's mouth.....	469, 470, 471, 472, 473, 475
Tiger shark 95515	
Blood.....	478, 479, 480
Nurse shark A01193	
Blood.....	481, 482
Nurse shark A01191	
Blood.....	484, 485
Lemon shark 499	
Blood.....	488, 490
Lemon sharks C'85^c	
Muscle.....	750, 751, 752, 754, 755, 757, 760, 761, 762, 763, 764, 765, 766, 767, 768, 769, 770, 771, 772, 773, 775, 776, 777, 778, 779, 780, 781, 782

^a Scientific names of the animal sources: blacktip shark, *Carcharhinus limbatus*; lemon shark, *Negaprion brevirostris*; nurse shark, *Ginglymostoma cirratum*; tiger shark, *Galeocerdo curvieri*; trematode, *Dermophthirius nigrellii*.

^b Shark bite sustained to the hand of a scientist attempting to remove a hook fouled in the eye of lemon shark A08959.

^c Muscle samples were collected in May 1985 from two lemon sharks captured in Bimini.

TABLE 2. Reference strains included in this study and their sources

OTU	Strain	Originally isolated from:	Source ^a
735	<i>A. hydrophila</i> ATCC 7966 ^T	Spoiled milk	ATCC
726	<i>A. hydrophila</i> ATCC 15467	Oil emulsion	ATCC
713	<i>Plesiomonas shigelloides</i> ATCC 14029 ^T	Clinical sample	ATCC
731	<i>Plesiomonas shigelloides</i> ATCC 14030	Clinical sample	ATCC
715	<i>V. alginolyticus</i> ATCC 17749 ^T	Spoiled mackerel	ATCC
725	<i>V. anguillarum</i> ATCC 19264 ^T	Cod skin lesion	ATCC
714	<i>V. campbellii</i> ATCC 25920 ^T	Seawater	ATCC
790	<i>V. carchariae</i> ATCC 35084 ^T	Sandbar shark kidney	ATCC
783	<i>V. carchariae</i> ATCC 43515	Lemon shark mouth	UM
784	<i>V. carchariae</i> ATCC 43516	Lemon shark mouth	UM
708	<i>V. cholerae</i> ATCC 14035 ^T	Human feces	ATCC
703	<i>V. cholerae</i> p688		
722	<i>V. cholerae</i> VL 5065		VL
723	<i>V. cholerae</i> VL 3029		VL
730	<i>V. cholerae</i> 569/B	Human feces	
732	<i>V. cholerae</i> VL 1330		VL
719	<i>V. damsela</i> ATCC 33539 ^T	Damselfish lesion	ATCC
712	<i>V. diazotrophicus</i> ATCC 33466 ^T	Sea urchin intestine	ATCC
785	<i>V. fischeri</i> ATCC 7744 ^T	Seawater	ATCC
720	<i>V. fluvialis</i> ATCC 33809 ^T	Human feces	ATCC
736	<i>V. fluvialis</i> DJVP 7225		
709	<i>V. fluvialis</i> DJVP 6957		
789	<i>V. furnissii</i> NCTC 11328 (= ATCC 33812)	River water	VL
728	<i>V. harveyi</i> ATCC 14126 ^T	Luminescent amphipod	ATCC
734	<i>V. mimicus</i> ATCC 33653 ^T	Clinical sample	ATCC
701	<i>V. natriegens</i> ATCC 14048 ^T	Salt marsh mud	ATCC
718	<i>V. nereis</i> ATCC 25917 ^T	Seawater	ATCC
788	<i>V. nigripulchritudo</i> ATCC 27043 ^T	Seawater	ATCC
716	<i>V. nigripulchritudo</i> 1439		
733	<i>V. parahaemolyticus</i> ATCC 17802 ^T	Shirasu	ATCC
710	<i>V. parahaemolyticus</i> SAK 2269	Gastroenteritis	R. Sakazaki
711	<i>V. pelagius</i> ATCC 25916 ^T	Seawater	ATCC
705	<i>V. proteolyticus</i> ATCC 15338 ^T	Wood borer intestine	ATCC
787	<i>V. splendidus</i> ATCC 33125 ^T	Marine fish	ATCC
786	<i>V. splendidus</i> ATCC 25914	Seawater	ATCC
791	<i>V. tubiashii</i> W343	Chesapeake Bay water	UM
792	<i>V. tubiashii</i> W359	Chesapeake Bay water	UM
793	<i>V. tubiashii</i> W428	Seawater	UM
727	<i>V. vulnificus</i> ATCC 27562 ^T	Human blood	ATCC

^a ATCC, American Type Culture Collection, Rockville, Md.; UM, R. R. Colwell, Marine Microbiology Culture Collection, University of Maryland, College Park; VL, J. V. Lee, Vibrio Laboratory, Public Health Laboratory Service, Centre for Applied Microbiology and Research, Porton Down, Salisbury, United Kingdom.

each taxon. The group partitioning index (GPI), a measurement of how effectively a particular phenotypic test divides a group into distinct sets, has a maximum value of 0.5 (16). We used the GPI to rank phenotypic tests in order of importance. Ordination was accomplished by performing a principal-component analysis on the S_{SM} matrix. All of the data analyses were done by using procedures available in SAS, version 5.18 for Conversational Monitor System (SAS Institute Inc., Cary, N.C.).

RESULTS AND DISCUSSION

All 236 strains examined (100%) were gram-negative, oxidase-positive rods. Most strains were capable of glucose oxidation and fermentation (91.6 and 84.5% of the strains, respectively), nitrate reduction (90.4%), growth at 35°C (97.1%), catalase production (98.7%), amylase production (85.4%), growth in the presence of 3.0% NaCl (97.1%), growth at pH 10.0 (86.6%), growth on media containing Tween 20, Tween 40, Tween 60, and Tween 80 (94.6, 94.1, 90.4, and 88.3%, respectively), and DNase production

(95.0%). Only one strain fermented inositol (0.4%), and another strain fermented lactose (0.4%). While these attributes are useful for preparing a general description of OTUs and taxa, some were of little or no value in differentiating the OTUs. A similar conclusion was reached by West et al. (28) for the Gram and oxidase reactions and for growth at pH 10.0. Therefore, we eliminated inositol fermentation, lactose fermentation, oxidase and catalase activities, and the Gram reaction from the data matrix, since essentially all of the strains either lacked the characteristic or possessed it; this left 61 characteristics in the matrix used for cluster and ordination analyses.

Cluster analysis. The results of the cluster analysis of the S_{SM} values calculated for the 236 OTUs are shown in a dendrogram in Fig. 1. The cophenetic correlation coefficient was 0.711. The S_j values were also analyzed, and a similarity matrix was obtained.

Nine major groups were resolved at an S_{SM} value of 66.5% (Fig. 1). Each group in turn contained strain clusters (phena) at S_{SM} values of $\geq 72.6\%$. A total of 27 such phena were identified, and 15 of these phena contained three or more OTUs (Fig. 1).

FIG. 1. Dendrogram, based on UPGMA cluster analysis of the simple match similarity coefficients (S_{sm}), of the 236 OTUs obtained in this study. Nine major groups (designated G1 to G9) were resolved at an S_{sm} value of $\geq 65.5\%$. Phenon (S_{sm} , $\geq 72.6\%$), designated by the numbers listed on the figure, were as follows: 1 and 2, *V. alginolyticus*; 3, *V. carchariae*; 4, *V. harveyi*; 5, *V. cholerae*; 6, *V. campbellii*; 7, *V. vulnificus*; 8, *V. parahaemolyticus*; 9, *V. damsela*; 10, *V. parahaemolyticus*; 11, *V. parahaemolyticus*; 12, *V. parahaemolyticus*; 13, *V. parahaemolyticus*; 14, *V. parahaemolyticus*; 15, *V. parahaemolyticus*; 16, *V. parahaemolyticus*; 17, *V. parahaemolyticus*; 18, *V. parahaemolyticus*; 19, *V. parahaemolyticus*; 20, *V. parahaemolyticus*; 21, *V. parahaemolyticus*; 22, *V. parahaemolyticus*; 23, *V. parahaemolyticus*; 24, *V. parahaemolyticus*; 25, *V. parahaemolyticus*; 26, *V. parahaemolyticus*.

TABLE 3—Continued

Growth at:	0	27	33	77	100	0	9	0	33	0	0	14	15	0	0	33	89	33	100	100	71	100	100	62	50	100	0.345	
4°C	100	100	100	100	100	100	100	100	100	100	50	43	100	100	100	100	100	100	100	100	86	100	100	92	100	100	0.081	
35°C	38	96	29	100	100	38	9	0	33	0	50	0	25	50	50	67	100	67	100	100	100	100	62	100	100	0.327		
42°C	13	23	21	77	100	13	100	100	33	0	50	0	31	0	0	0	22	0	100	100	29	100	0	77	0	100	0.385	
pH 4.5	88	94	100	100	100	88	0	0	67	100	50	100	83	100	50	100	100	67	100	100	100	100	100	100	100	100	0.205	
pH 10.0																												
Vibriostatic agent O/129																												
susceptibility																												
10 µg/ml	0	35	29	53	67	50	9	0	33	0	100	71	56	0	50	0	11	0	0	0	0	100	0	0	0	50	0.279	
50 µg/ml	38	69	78	94	100	75	9	50	67	100	100	86	81	0	50	100	22	0	0	50	14	100	100	0	0	50	0.340	
150 µg/ml	100	96	88	94	100	100	91	50	100	100	100	100	87	0	100	100	33	100	0	100	57	100	100	0	0	50	0.308	
Production of:																												
Alginate	75	60	88	6	0	0	0	0	0	0	0	0	14	0	0	0	22	0	0	50	50	0	100	8	50	0	0.248	
Amylase	63	100	100	94	100	63	100	100	33	0	100	100	85	100	50	67	56	100	100	100	100	100	0	54	100	0	50	0.286
Chitinase	63	98	96	88	100	100	9	0	67	0	100	86	79	50	0	33	33	0	0	100	57	0	100	0	50	0	50	0.355
DNase	75	98	100	100	100	100	100	100	100	100	100	50	100	98	100	100	100	100	0	0	100	100	100	85	50	100	50	0.213
Elastase	0	2	0	29	100	0	18	0	0	100	0	14	8	0	0	0	0	67	0	0	0	100	0	0	0	50	0	0.250
Gelatinase	50	100	96	100	67	75	0	0	33	100	0	86	21	50	50	67	100	100	100	86	100	0	39	100	0	100	0.339	
Lecthinase	88	44	75	88	100	50	9	0	33	100	100	86	81	0	50	100	67	67	100	100	43	100	100	100	50	100	50	0.276
Urease	0	40	58	6	33	0	91	100	67	100	50	57	100	50	100	0	100	100	100	50	14	0	100	85	100	0	0	0.355
Degradation of:																												
Tyrosine	88	100	79	88	67	100	55	0	33	100	100	43	31	50	100	67	100	33	0	50	86	100	100	92	100	0	100	0.296
Xanthine	0	0	4	0	33	0	0	0	0	0	100	43	8	0	0	0	0	0	0	0	29	0	0	77	0	0	0	0.168
Hydrolysis of:																												
ONPG ^c	25	2	33	35	33	50	27	100	0	0	100	71	4	100	100	67	0	0	0	0	71	100	100	8	0	0	50	0.349
Tween 20	100	100	100	100	100	100	82	0	100	100	100	100	100	100	100	100	100	100	100	100	100	100	62	0	0	100	0.215	
Tween 40	100	100	100	100	100	100	82	0	100	100	100	100	98	100	100	100	100	100	100	100	100	100	62	0	0	100	0.215	
Tween 60	100	100	100	100	100	100	82	0	100	100	0	100	94	100	100	100	100	33	100	100	100	100	39	0	0	100	0.300	
Tween 80	100	100	100	100	100	100	82	0	100	100	0	100	94	100	50	100	100	33	100	0	100	100	39	0	0	0	0.378	
Utilization of:																												
γ-NH ₄ butyrate	88	92	83	35	100	50	9	0	0	100	0	29	38	0	50	100	67	33	0	100	100	100	0	92	100	0	0.382	
Cellobiose	88	71	100	35	67	20	82	0	0	0	0	86	44	0	0	0	11	67	100	100	100	100	39	100	50	100	0.373	
L-Citrulline	75	79	42	24	0	57	0	0	0	100	0	29	41	50	50	100	33	100	0	100	43	100	100	31	100	50	100	0.325
Ethanol	100	46	50	12	0	50	36	0	0	0	0	0	33	0	50	100	0	0	0	100	86	100	0	62	50	50	0	0.322
D-Gluconate	75	83	88	73	100	13	27	0	0	0	0	43	35	0	50	67	78	100	100	86	100	100	100	100	100	50	0.342	
D-Gluconate	100	61	92	6	67	33	9	0	0	0	0	14	45	0	100	100	33	0	0	50	100	100	100	100	100	0	0.399	
L-Leucine	25	52	33	6	33	25	9	0	0	0	0	32	0	0	0	100	67	44	33	100	50	43	0	92	50	0	100	0.272
Putrescine	88	93	17	18	100	50	0	0	0	100	0	14	36	0	100	33	89	33	0	100	71	0	100	85	100	0	100	0.398
Sucrose	100	70	83	71	67	50	27	50	0	0	0	71	48	0	100	100	78	100	100	50	100	100	ND ^f	46	100	0	0.304	
Urea	25	44	25	0	0	14	18	0	0	0	0	ND	31	0	100	67	0	0	0	100	0	0	0	8	50	0	0	0.217
D-Xylose	38	52	25	6	67	38	0	0	0	0	0	0	41	0	100	67	22	0	0	100	86	100	ND	54	100	0	0	0.330

^a Tests were performed as described by West and Colwell (30) and West et al. (31).^b Values were rounded off to the nearest whole number.^c GPI for characteristics at an S₅₀ value of >72.6%.^d n is the number of OTUs.^e ONPG, o-nitrophenyl-β-D-galactopyranoside.^f ND, test not done.

Table 3 shows the test results for each phenon; Table 3 also shows the GPI for each test at S_{SM} values of $\geq 72.6\%$. The most important test at the level used to distinguish the nine major groups (S_{SM} , 66.5%) was utilization of D-glucuronate (GPI, 0.379), followed by growth in the absence of NaCl, H_2S production, utilization of Tween 80, glucose and trehalose fermentation, utilization of gamma-aminobutyrate, and susceptibility to 150 μ g of vibriostatic agent O/129 per ml (GPIs, 0.362, 0.358, 0.347, 0.346, 0.344, 0.332, and 0.331, respectively).

Group 1 was the largest of the groups observed in the analysis and contained 108 OTUs, including 18 reference strains distributed across five of the six phenon (Fig. 1). The tests that were most useful in distinguishing the six phenon were alginate production, putrescine utilization, growth at 4 and 42°C, growth at pH 4.5, and sorbitol fermentation.

Phenon 1 (S_{SM} , $\geq 75\%$) included eight OTUs, all of which were isolated from lemon shark muscle tissue. In general, the test results for phenon 1 were consistent with the test results recorded previously for *Vibrio alginolyticus* (3, 30).

Phenon 2 (S_{SM} , $\geq 73\%$) included 48 OTUs, making it the second largest phenon, and contained *V. alginolyticus* ATCC 17749^T (T = type strain), *Vibrio parahaemolyticus* SAK 2269, a clinical gastroenteritis isolate, also clustered with this phenon. The test results for phenon 2 were generally consistent with those recorded for *V. alginolyticus*. Phenon 2 joined phenon 1, which was also provisionally identified as *V. alginolyticus*, at an S_{SM} value of $\geq 71\%$. Although all four shark bite wound isolates (OTUs 460, 461W, 461T, and 462) were assigned to this phenon, only one of the five isolates from the mouth of the shark that inflicted the bite was so classified (OTU 469). Also of interest is the observation that 6 of the 10 water isolates (OTUs 5, 8, 464, 466, 467, and 468) were included in this phenon, which is consistent with reports of isolation of *V. alginolyticus* from seawater (3, 8, 30).

Phenon 3 (S_{SM} , $\geq 73\%$) included 24 OTUs, making it the third largest phenon, and contained the three *Vibrio carchariae* reference strains (strains ATCC 35084^T, ATCC 43515, and ATCC 43516) and *Vibrio harveyi* ATCC 14126^T. Within this phenon, two subsets clustered at an S_{SM} value of $\geq 82\%$; one subset contained the three *V. carchariae* reference strains and eight unknown OTUs (OTUs 7, 36, 141, 221, 246, 445, 446, and 752), and the other subset contained the *V. harveyi* reference strain and two unknown OTUs (OTUs 35 and 475). In turn, these two subsets joined each other at a level of ca. 78%. Bochner (4b) compared 28 strains of *V. carchariae* with 35 strains of *V. harveyi* and observed major differences in the results of 5 of 96 carbon source tests (N-acetyl-D-galactosamine, citric acid, D-glucuronate, glycerol, DL-alpha-glycerol phosphate). More recently, Kita-Tsukamoto et al. (14), using 16S rRNA sequence analysis, concluded that *V. carchariae* ATCC 35084^T and *V. harveyi* ATCC 14126^T were distinct species. A similar conclusion had been reached previously by MacDonell et al. (19), who used 5S rRNA sequence analysis. Finally, a whole-cell fatty acid analysis of *V. carchariae* and *V. harveyi* revealed a Sorenson's similarity level of 90.5% among the strains tested, including the type strains of each species, which also indicated that these taxa are two separate species (24a). Clearly, both phenetic and molecular data support the previous classifications of *V. carchariae* and *V. harveyi* as separate species.

Phenon 4 (S_{SM} , $\geq 70.9\%$) contained 17 OTUs, including all six *Vibrio cholerae* reference strains (which clustered separately at an S_{SM} value of 83%), *Vibrio campbellii* ATCC

25920^T, and *Vibrio vulnificus* ATCC 27562^T. With the exception of lactose fermentation, *V. campbellii* and *V. vulnificus* were phenotypically similar (S_{SM} , $\geq 83\%$). In a previous study (28), the type strains of *V. campbellii* and *V. vulnificus* clustered at an S_J value of 88%. In the same study (28), 115 OTUs in a phenon identified as *V. cholerae* clustered with *V. campbellii* and *V. vulnificus* at an S_J value of approximately 73%.

Phenon 5 (S_{SM} , $\geq 75.5\%$) contained three type strains, the type strains of *Vibrio anguillarum*, *Vibrio nereis*, and *Vibrio proteolyticus*. It clustered with phenon 6 at an S_{SM} value of approximately 71.5%. Phenon 6 (S_{SM} , $\geq 74\%$) contained eight OTUs, including *Vibrio furnissii* NCTC 11328.

Group 2 (Fig. 1) comprised 78 OTUs, which were distributed among seven phenon (phenon 7 through 13). This group was distinguished from group 1 by a lack of growth at higher salt concentrations (no growth in the presence of 10% NaCl; 5% growth in the presence of 8% NaCl), an inability to liquefy gelatin, a lack of arabinose fermentation, the results of decarboxylase reactions, and production of urease (Table 3).

Phenon 7 contained 11 OTUs clustering at an S_{SM} of $\geq 74\%$. As in the case of phenon 1, phenon 7 contained only lemon shark muscle tissue isolates. In general, the reactions observed were consistent with those described previously for *Aeromonas salmonicida* subsp. *masoucida* (23).

Phenon 8 also contained only lemon shark muscle tissue isolates and joined phenon 7 at an S_{SM} value of $\geq 72\%$. This small phenon contained two unknown OTUs and was distinguished from phenon 7 by several attributes (Table 3).

Phenon 9 and 10 contained three OTUs and one OTU, respectively. These OTUs were all unknowns, did not share a common habitat, and clustered at an S_{SM} value of $\geq 72\%$. Like phenon 8 strains, both phenon 9 and phenon 10 strains produced gas in glucose broth without showing any evidence of either glucose oxidation or fermentation.

Phenon 11 contained two OTUs (OTUs 422 and 431), both of which were isolated from the intestinal tract of lemon shark A, and joined phenon 12 at an S_{SM} value of $\geq 69.5\%$. Phenon 11 was identified as a *Vibrio* sp. Phenon 12 (S_{SM} , $\geq 74\%$) contained seven OTUs, all of which were *Vibrio* reference strains (*Vibrio fischeri* ATCC 7744^T; *Vibrio nigripulchritudo* ATCC 27043^T; *Vibrio splendidus* ATCC 33125^T and ATCC 25914; *Vibrio tubiashii* W343, W359, and W428). The three strains of *V. tubiashii* (strains W343, W359, and W428) clustered closely at an S_{SM} value of $\geq 92.5\%$.

Phenon 13 formed at an S_{SM} value of $\geq 73.5\%$ and contained 52 OTUs, making it the largest phenon of the study. *Vibrio damsela* ATCC 33539^T, a recognized fish pathogen (10, 17), was included in this cluster, which was provisionally identified as *V. damsela*. Recently, Smith et al. (25) recommended that *V. damsela* should be reassigned to the genus *Photobacterium* as *Photobacterium damsela* comb. nov. on the basis of their investigation of phenotypic data. Our data do not support this recommendation, since phenon 13 (group 2) joins the *Photobacterium* phenon (group 3) at an S_{SM} value of 65% (Fig. 1). Similarly, the 16S rRNA data of Kita-Tsukamoto et al. (14) do not support this recommendation. In addition, MacDonell and Colwell (18) previously used 5S rRNA sequence data to propose a new genus, *Listonella*, to which *V. damsela* and five additional species (*Vibrio aestuarianus*, *V. anguillarum*, *Vibrio ordalii*, *Vibrio pelagius*, and *V. tubiashii*) were assigned (18, 21, 22). Since Smith et al. (25) did not employ either molecular methods or numerical analysis, the scientific validity of their proposal must be investigated further.

Phenon 14 contained two OTUs (OTUs 122 and 171), which constituted group 8 (Fig. 1) and possessed many of the attributes of the genus *Vibrio*. Unlike most *Vibrio* spp., however, but consistent with phenon 13 (*V. damsela*), this phenon included isolates that produce gas from glucose and joined phenon 13 at an S_{SM} value of $\geq 66.2\%$.

Group 6 contained two phena (phena 15 and 16), which clustered at an S_{SM} value of $\geq 68\%$ and joined group 3 at an S_{SM} value of $\geq 62\%$ (Fig. 1). Phenon 15 contained two OTUs, and phenon 16 contained three OTUs; four of the five OTUs in group 6 were isolated from shark muscle tissue. The strains in phena 15 and 16 neither fermented the sugars tested nor oxidized glucose, but all of these strains produced indole and were susceptible to 150 μg of vibriostatic agent O/129 per ml. In general, phenon 15 resembled the genus *Pasteurella* (5) and was remarkably similar to previous descriptions of *Pasteurella piscicida* (12, 27). By using TAXAN6 (University of Maryland), 65 characteristics determined for five strains of *Pasteurella piscicida* (including strain ATCC 17911) were compared with characteristics simultaneously determined for *V. anguillarum* ATCC 19264^T, *V. ordalii* ATCC 33509^T, *V. parahaemolyticus* ATCC 17802^T, *A. salmonicida* ATCC 33658^T, *Pasteurella multocida* ATCC 29977, and *Photobacterium phosphorum* ATCC 11040^T. It was concluded from the results that *Pasteurella piscicida* is phenotypically closer to the genus *Vibrio* (S_J , $\geq 45\%$) than to *Pasteurella multocida* (S_J , $\geq 25\%$) (4a). The major differences between phenon 15 and *Pasteurella piscicida* were as follows: arginine decarboxylase activity; glucose, mannitol, and sucrose fermentation; indole production; growth in the absence of NaCl; growth at 35°C; and urease activity. Phenon 15 also possessed many of the characteristics reported for the genus *Moraxella*, with the exception of indole production, nitrate reduction, and urease; it also resembled the genus *Neisseria*. Clearly, additional studies, including a comparison with appropriate reference strains, need to be completed before phenon 15 can be properly identified.

Group 3 formed at an S_{SM} value of $\geq 68.5\%$ and comprised 13 OTUs representing three phena (phena 17 through 19), none of which contained reference strains (Fig. 1). Phenon 17 and 18 contained nine and three isolates, respectively. The smallest phenon, phenon 19, contained only one isolate (OTU 485 from a nurse shark). Most of the other test results given by phenon 19 strains were consistent with the description of *Photobacterium leiognathi* (2); therefore, we concluded that this phenon should be identified as a *Photobacterium* sp. In addition, many of the results given by OTUs belonging to phena 17 and 18 were also consistent with the description of the genus *Photobacterium* (2, 30), suggesting that all of group 3 belonged to the genus *Photobacterium*.

Group 5 comprised phena 20 through 23 and joined groups 3 and 6 at an S_{SM} value of $\geq 60\%$ (Fig. 1). Of the 11 OTUs assigned to group 5, 9 were reference strains. Phenon 20 contained two *Vibrio* reference strains, *V. parahaemolyticus* ATCC 17802^T and *Vibrio fluvialis* DJVP 7225. These strains joined at an S_{SM} value of $\geq 74\%$ and, in turn, combined with phena 21 through 23 at an S_{SM} value of $\geq 68\%$. Phenon 21 (S_{SM} , $\geq 73\%$) contained seven OTUs, five of which were *Vibrio* reference strains (*Vibrio diazotrophicus* ATCC 33466^T, *V. fluvialis* ATCC 33809^T and DJVP 6957, *Vibrio pelagius* ATCC 25916^T, and *Vibrio natriegens* ATCC 14048^T), and *Aeromonas hydrophila* ATCC 15467. The single shark isolate in this phenon, OTU 230, was most closely related to *V. natriegens* ATCC 14048^T (S_{SM} , 75.5%). Phenon 22 and 23 each contained one OTU; phenon 22 contained *V.*

nigripulchritudo 1439, and phenon 23 contained OTU 145, a blacktip shark kidney isolate. Phenon 23 was considered a *Vibrio* sp.

Group 4 formed at an S_{SM} value of $\geq 72\%$ and contained two phena, phena 24 and 25 (Fig. 1), which had properties consistent with those reported previously for the genus *Alteromonas* (4). Phenon 24 and 25 contained 13 and 2 shark isolates, respectively. Characteristics that distinguished the phena included growth at pH 4.5, degradation of xanthine, utilization of L-citrulline and cellobiose, and production of gelatinase (GPIs, 0.385, 0.385, 0.346, 0.308, and 0.308, respectively).

Group 7 contained one phenon, phenon 26, which formed at an S_{SM} value of $\geq 82\%$ (Fig. 1) and contained two reference strains, *Plesiomonas shigelloides* ATCC 14029^T and ATCC 14030. This group was most closely related to group 4 (S_{SM} , 64%).

Group 9 (Fig. 1) contained one phenon, phenon 27, which formed at an S_{SM} value of $\geq 79\%$. It contained two type strains, *Vibrio mimicus* ATCC 33653 and *A. hydrophila* ATCC 7966.

An S_{SM} value of $\geq 72.6\%$ was selected as the level at which phena were resolved on the basis of the cophenetic correlation coefficient (0.711) and the number of phena created. This value is low compared with the values used in other studies, in which higher similarity values, usually an S_J value of $\geq 80\%$, have been selected for resolution of phena (13, 24, 28, 29, 31). At an S_{SM} value of $\geq 82\%$ in this study, the six *V. cholerae* strains clustered in a single phenon, as did *V. fluvialis* and *A. hydrophila*, three strains of *V. carchariae*, three strains of *V. tubiashii*, and two strains of *Plesiomonas shigelloides*. In addition, some of the type strains that clustered at an S_{SM} value of $\geq 72.6\%$ did not cluster at an S_{SM} value of $\geq 82\%$. If an S_{SM} value of $\geq 82\%$ had been selected, 93 phena would have resulted from the 236 OTUs examined. Therefore, for expediency of analysis and for the purposes of this study (namely, to assess major groups of strains), the lower value (S_{SM} , $\geq 72.6\%$) was used.

Ordination analysis. The results of the principal-component analysis are shown in Fig. 2. Each OTU in Fig. 2 was coded according to the cluster analysis by using an S_{SM} value of $\geq 66.5\%$. The first three components, components P1, P2, and P3, accounted for 74.2% of the relatedness among OTUs, with components P4 and P5 providing only slight increases in the portrayal of relatedness (4.8 and 3.0%, respectively). Accordingly, principal components P4 and P5 are not shown in Fig. 2.

MacDonell et al. (19) successfully applied ordination analysis to 5S rRNA sequence data and achieved good resolution of strains in clusters. In this study, a principal-component analysis in which phenotypic data were used was approximately as valuable as cluster analysis for differentiating phena. From the data in Fig. 2, the following two observations were made: (i) group 1 occupied a compact, defined, and substantial portion of the three-dimensional array; and (ii) a continuum of OTUs across the hyperspace of the plot occurred. Relationships between and among the groups were such that no clear groupings resulted from the ordination analysis. Unlike the results of the rRNA sequence data analysis obtained by MacDonell et al. (19), which permitted unequivocal identification of three clusters (vibrios, enterics, and *Aeromonas* spp.), there was less clear separation or clustering of OTUs in our study, which was based on phenotypic data. Since this analysis included strains that were freshly isolated from the natural environment and

FIG. 2. (A) The 236 OTUs plotted in three dimensions, on the basis of the three principal components (P1, P2, and P3). Each OTU is represented by its group affiliation at an S_{SM} value of $\geq 65.5\%$, based on UPGMA cluster analysis. Balloons, group 1; pyramids, groups 2 and 8; cubes, groups 3, 5, and 6; cylinders, groups 4, 7, and 9. The 236 OTUs numbered 1 to 9 according to group affiliation, are plotted in two dimensions on the basis of either the first two principal components (P1 and P2) (B) or the first and third principal components (P1 and P3) (C).

phenotypes rather than genotypes, this result was not unexpected.

From the results of the UPGMA cluster analysis of phenotypic data, we concluded that gram-negative, oxidase-positive rods associated with carcharhinid sharks are predominantly members of the genus *Vibrio*; some species were readily identified, and others appeared to be new species. The 14 phena considered to be *Vibrio* spp. accounted for 182 of the 236 OTUs detected in the study. The shark isolates included strains of *V. alginolyticus* (phenon 1 [8 OTUs] and phenon 2 [48 OTUs]); *V. carchariae* and *V. harveyi* (phenon 3 [24 OTUs]); *V. damsela* (phenon 13 [52 OTUs]); the genus *Alteromonas* (group 4, phena 24 and 25 [15 OTUs]); and the genus *Photobacterium* (group 3, phena 17 through 19 [13 OTUs]). A few of the isolates clustered with previously recognized human pathogens (for example, *V. cholerae* in phenon 4 and *V. furnissii* in phenon 6). In previous studies, human pathogens were isolated from sharks (8), and pooled sera from six nurse sharks were found to contain high (titer, ca. 1:1,024) antibody activity against whole cells of *V. cholerae* ATCC 14035^T (7). However, in this study, most of the human pathogens were reference strains and formed clusters separate from the shark isolates. Known fish pathogens (i.e., *V. carchariae*, *V. damsela*, *V. tubiashii*, *Pasteurella piscicida*, and *A. salmonicida*) were also detected among the isolates. However, *V. anguillarum* was not isolated from any of the shark samples collected in this study. In previous studies, shark tissue-associated bacteria were implicated in shark urea homeostasis functions (9, 15). In this study, 58% of the OTUs were found to be urease positive, suggesting that other interactions are also likely.

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