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Phenotypic Characterization of Clinical and Environmental Isolates of *Vibrio cholerae* from Australia

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Abstract. One hundred fifty-seven isolates possessing the biochemical traits associated with *Vibrio cholerae* were submitted to an extensive phenotypic characterization. A numerical analysis of the results suggested that isolates presently assigned to the biotypes *cholerae, eltor,* and *albensis* of *V. cholerae* do not possess consistent phenotypic differences supporting their separation into distinct biotypes. The results further indicated that clinical and environmental isolates of non-O1 serotypes of *V. cholerae* are phenotypically indistinguishable from strains of *V. cholerae* serotype O1. This study also confirmed the persistent presence of *V. cholerae* in the Australian environment.

The concept of the species *Vibrio cholerae* proposed by Shewan and Véron [19] in the most recent edition of *Bergey's Manual of Determinative Bacteriology* was that the species could be divided into four biotypes, namely, *cholerae, eltor, albensis,* and *proteus.* In vitro DNA/DNA hybridization studies [5,18] have supported the inclusion of biotypes *eltor* and *albensis* within the same species as the *cholerae* biotype but clearly excluded the biotype *proteus* from *V. cholerae.* The presently available phenotypic [6] and genotypic [5,18] data do not indicate that the subdivision of *V. cholerae* into the *eltor* and *cholerae* biotypes is necessary; hence, this separation is now based solely on its clinical and epidemiological usefulness. The biotype *albensis* is presently based on a single isolate from the Elbe River which has been maintained in culture collections since 1931 [22]. The growth factor requirements and poor growth yields of this strain have made it difficult to assess whether phenotypic properties other than luminescence exist to support the separation of this strain as a distinct biotype. However, in vitro DNA/DNA hybridization studies and the variable nature of luminescence in *V. harveyi* [18] suggest that the continued use of a separate biotype for luminous strains of *V. cholerae* may not be warranted.

Strains assigned to the *eltor* and *cholerae* biotypes of *V. cholerae* have been further subdivided on the basis of their possession of the cell wall antigen (O antigen) designated group 1 [9], leading to the serotype designations "O1" and "non-O1". Epidemics of cholera have to date been associated with the O1 serotype only [13]. Isolates identifiable as *V. cholerae* by the conventional clinical tests and having cell wall serotypes other than group 1 have been collectively called nonagglutinable (NAG) vibrios. These isolates have exhibited a wide range of pathogenicity, from mild diarrhea to the classical cholera syndrome [1,15,23]; they have also been associated with a variety of nonintestinal diseases [3,8,10,11,12]. Non-O1 serotypes of *V. cholerae* have been far more readily isolated from environmental sources such as rivers and estuaries than the O1 serotype [7,11,14]. In the absence of extensive phenotypic comparisons of large numbers of clinical and environmental isolates of the O1 and non-O1 serotypes of *V. cholerae,* the identity of the environmental isolates of the non-O1 serotypes has remained in doubt [20]; in fact, they are often ambiguously referred to as "non-cholera vibrios" (NCV) [3,8,10,11,12,15,20].

The present study consists of an extensive phenotypic characterization of a total of 157 strains possessing the biochemical traits commonly used to identify *V. cholerae* in the clinic. This collection of strains includes 4 reference cultures, 56 clinical isolates, and 95 environmental isolates of the O1 and non-O1 serotypes. This study also constitutes the first stage in a study of the public health significance of the persistent isolation of *V. cholerae* serotypes O1 and non-O1 from the Australian environment [2,7].

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Table 1. Origin and serotype of *Vibrio cholerae* cultures.

Source of isolation	Serotype		
	O-group 1		
	Ogawa	Inaba	Non-O-group 1
Clinical isolates:			
1. Infections contracted within Australia		B1–B3	VS19–VS21, N33
2. Travelers arriving in Australia	WA	QA, Q6–Q10, Q12, Q13, Q15, Q17–Q19, Q36, Q39, Q43 KLM362, KLM371	VS3–VS7, VS11, VS15–VS17, VS27, N25, N28, N29, KLM361
3. Infections contracted in regions surrounding Australia (Hong Kong, Indonesia, Nauru, New Zealand, Bahrain)	HK757, HK6203, HK82/62	393, HK182, HK772, Q14, Q16, Q42, Q44, Q46–Q48	VS2, VS12–VS14
Nonclinical isolates:			
1. Australian domestic tap water (Beenleigh, Canungra)		B4A, B4B, B5–B10, B19	N6, N7
2. Australian sewerage plants (Beenleigh, Canungra, Bulimba Creek)	B40–B45	B27, B28	N10–N12
3. Australian river water and sediments (Brisbane, Albert and Logan River systems)	B39	B11, B14–B18, B20–B26, B29–B36, B46, B47, B49, B50, B52, B65–B78, B87, B95, B97, B99, B102, B103	VS22–VS25, N1, N5, N14–N16, N18–N21, N23, N24
4. Well-water in Nauru			N30
5. Food imported into Australia			N31, N32, KLM-F
6. Water imported with tropical fish into Australia			FW1, VS8, VS9, VS18
7. Animals (fish, duck) in Australia		B37, B38	VS10

Materials and Methods

The strains used in this study are those listed in Table 1 and four reference cultures of *Vibrio cholerae*: NCTC 8021 (neotype strain; biotype *cholerae*, serotype O1 ogawa), NCTC 7270 (biotype *cholerae*, serotype hikojima), and NCTC 3661 (biotype *eltor*, serotype O1 inaba) from the National Collection of Type Cultures, London, United Kingdom, and strain UNSW (biotype *cholerae*, serotype O1 ogawa) from the University of New South Wales, Sydney, Australia. The criterion for inclusion of cultures in this study was that they possess the biochemical properties currently used to identify *V. cholerae* in clinical practice, namely, actively motile, oxidase-positive rods that ferment D-glucose with the production of acid but no gas, produce lysine and ornithine decarboxylases but not arginine dihydrolase, grow in tryptone water without added sodium chloride, produce indole from tryptophan, and are sensitive to the antibiotic substance called O/129. Isolates found to agglutinate chicken erythrocytes, hemolyze sheep erythrocytes, resist polymyxin (50 units/disk), and give a positive Voges-Proskauer reaction were assigned to the *eltor* biotype. The cultures were serotyped with standard O1 polyvalent antiserum and monovalent ogawa and inaba antisera (Burroughs-Wellcome Corp., Research Triangle Park, North Carolina).

The phenotypic characterization was based largely on a nutritional screening that tested the ability of the isolates to utilize 152 carbon compounds as sole or principal sources of carbon and energy, as previously described [4,16,24]. The carbon compounds included were the same as those used by Baumann, Baumann, and Mandel [4], with the exception of eicosanedioate, D-mandelate, and α-amylamine, which were not used in this study, and *n*-dodecane, D-malate, and L-malate, which were used in this study. The basal medium [16] included 0.3 M sodium chloride, because of the previously observed stimulation of the growth of *V. cholerae* by sodium chloride when minimal media were used [17]. For strains that were unable to grow on the basal medium, Oxoid yeast extract was added to a final concentration of 0.5 g/liter; this amount of yeast extract was insufficient to produce significant growth on media that lacked an added utilizable source of carbon and energy. The tests were incubated at 25°C rather than 37°C because of the use of minimal media and because many of the isolates were from environmental sources rather than from human infections.

Luminescence was measured by means of a photometer capable of detecting light emission down to 10^6 quanta/s and all cultures were tested throughout the exponential and early stationary phases of growth on a complex medium at 23°C.

Numerical analysis of the phenotypic data was performed using the simple matching coefficient and the complete linkage method [21].

Results and Discussion

The results of the serotyping of the isolates are shown in Table 1. All of the Australian isolates with the O1 serotype had the properties of the *eltor* biotype. The results of the phenotypic characterization are shown in Table 2. This table includes only 41 of the carbon compounds tested; the remaining 111 compounds were not utilized by any of the strains tested.

Table 2. Phenotypic properties of *Vibrio cholerae*.

Trait	Positive strains (%)[a]	Positive or negative strains
Motile	100	
Curved rods[b]	3	(+): N20, Q8, B7, B40, B41
Oxidase	100	
$NO_3^- \rightarrow N_2$	0	
$NO_3^- \rightarrow NO_2^-$	100	
Nicotinic acid requirement	15	(+): B1–B3, B4A, B4B, B5–B11, B17, B19–B28
Arginine dihydrolase	0	
Lysine decarboxylase	100	
Ornithine decarboxylase	100	
Luminescence[c]	1	(+): N18, VS22
Gas from D-glucose	0	
Voges-Proskauer	94	(−): B3, VS9, VS18, VS27, UNSW, NCTC 8021, NCTC 7270
Growth at 4°C[c]	0	
Growth at 37°C	100	
Growth at 40°C	95	(−): VS7, VS9, VS10, N5, N28, N33, NCTC 7270
Growth at 45°C	0	
Amylase	94	(−): N18, N28, VS21, VS26, VS27, WA, KLM-F, KLM 361, KLM 362, KLM 371
Lipase	100	
Gelatinase	99	(−): B3
Alginase	0	
Chitinase[c]	91	(−): B17, B28, B31, B57, B75, B76, VS19, VS24, VS28, N1, N12, N16, N20, HK772
D-Ribose	99	(−): Q43
D-Glucose	100	
D-Mannose	72	(−): N10–N12, N15, N18–N20, N23–N25, N28–N33, VS3, VS5, VS7, VS8, VS10–VS13, VS15, VS17, VS19, VS22, VS25, Q6–Q9, Q15–Q19, FW1, KLM 361, KLM 362, KLM 371, KLM-F
D-Galactose	97	(−): N25, VS5, VS6, NCTC 3661
D-Fructose	100	
Sucrose	98	(−): VS21, VS27, NCTC 3661
Trehalose	99	(−): B28
Maltose	99	(−): B28
Cellobiose	4	(+): N5, N10–N12, VS17, VS18, VS20
D-Gluconate	95	(−): B3, B4A, B4B, B5–B10
D-Glucuronate	91	(−): N10–N12, N18, VS6, VS9, VS10, VS17, VS18, VS20–VS22, VS24, VS27
N-Acetyl-glucosamine	100	
Acetate[d]	100	
Propionate	97	(−): N20, N21, VS27, HK82/62, NCTC 7270

Table 2 (continued).

Trait	Positive Strains (%)	Positive or negative strains
Caproate[d]	100	
Heptanoate[d]	6	(+): VS21
Caprylate[d]	100	
Pelargonate[d]	25	(+): N10, N20, VS21, B41
Caprate[d]	81	(−): B45, NCTC 3661, NCTC 7270
L-Malate	94	(−): N21, N33, Q6–Q8, Q17, Q19, Q43, Q47, WA
DL-Lactate	99	(−): N5, HK82/62
DL-Glycerate	34	(+): Q13, Q36, Q39, N16, N29, N31, N32, VS2, VS12, VS16, VS25, B1, B2, B4A, B5, B6, B11, B14–B28, B32, B34, B36, B37, B45, B65–B70, B74–B76, HK82/62, HK757, HK6203, UNSW, NCTC 3661, NCTC 7270, NCTC 8021
Citrate	88	(−): B14–B18, B32, HK82/62, NCTC 7270
α-Ketoglutarate	99	(−): VS18, VS24
Pyruvate	97	(−): B16, B19–B21
Aconitate	97	(−): VS11, VS17, UNSW, HK757
Succinate	96	(−): VS18, VS19, VS23, VS24, B77, B78
Fumarate	96	(−): VS18, VS19, VS23, VS24, B77, B78
Mannitol	100	
Glycerol	97	(−): N33, B23, B28, B77, B78
L-Histidine	44	(+): VS9, VS16, N21, N31–N33, Q39, B1–B3, B4A, B4B, B5–B11, B14, B15, B17–B20,B22–B37, B40–B43, B45–B47, B50, B52, B65–B70, B74–B76, WA, HK182, HK757, HK772, KLM-F, KLM 371, UNSW, NCTC 7270, NCTC 8021
L-Proline	100	
Glycine	9	(+): B1–B3, B4A, B4B, B5, B6, B8, B16, B22–B24, B26, HK757
L-α-Alanine	1	(+): Q13, NCTC 8021
D-α-Alanine	27	(+): B2–B4A, B4B, B5–B7, B9, B10, B14, B15, B17–B20, B22–B28, B32, B34, B36, B37, B41, B45, B50, B65–B70, B73–B76, Q13, HK82/62, HK6203
L-Serine	35	(+): VS2, VS5, Q10, Q12, Q13, B1–B3, B4A, B4B, B5–B10, B14, B15, B17–B20, B22–B24, B26, B28, B31, B32, B34–B37, B40–B43, B45, B46, B50, B65–B70,

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Table 2 (continued).

Trait	Positive Strains (%)	Positive or negative strains
		B74–B76, B103, WA, HK182, HK757, HK772, NCTC 7270
L-Threonine	34	(+): VS5, VS8, VS11, N24, N25, N31, N32, Q39, B1–B3, B4A, B4B, B6–B10, B14, B15, B17–B20, B22–B28, B31, B32, B34, B36, B37, B41–B43, B45, B46, B65–B70, B74–B76, WA, HK757, NCTC 7270
L-Aspartate[d]	88	(−): B45, NCTC 8021
L-Glutamate	90	(−): N10–N12, N21, VS12, VS15, VS17, VS19, VS22, Q14, Q42, Q43, Q46–Q48
L-Arginine[d]	100	
L-Ornithine[d]	94	(−): NCTC 3661

[a] Results for 157 strains, except where indicated.
[b] +, Curved rods; −, straight rods.
[c] Tests performed on 155 strains only, omitting strains B4B and B70.
[d] Compounds which gave poor growth on initial testing and were retested in the presence of 0.5 g/liter yeast extract for 16 representative strains, namely, VS18, VS21, N10, N20, N33, B1, B3, B8, B28, B38, B41, B45, B46, NCTC 3661, NCTC 7270, and NCTC 8021.

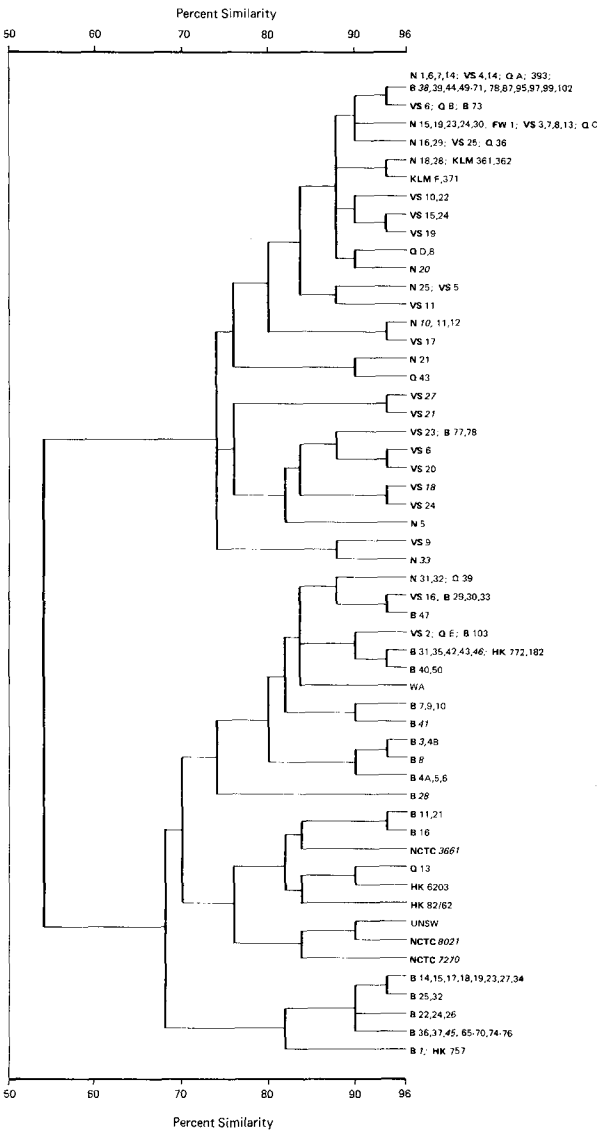

Fig. 1. Numerical analysis of 157 strains of *Vibrio cholerae*. Because they were phenotypically identical, strains Q14, Q42, Q44, and Q46–Q48 have been grouped as QB; Q9, Q15, Q16, and Q18 as QC; Q6, Q7, Q17, and Q19 as QD; and Q10 and Q12 as QE.

Twenty-three of the strains were found to have a specific requirement for nicotinic acid for growth. The strains requiring nicotinic acid were all *V. cholerae* biotype *eltor* serotype inaba from the Albert-Logan River system south of Brisbane; the epidemiological implications of this will be discussed in a later paper. A numerical analysis was performed on the data in Table 2, omitting all of the universally positive and negative traits to improve the sensitivity of the analysis. The results are shown in the form of a dendrogram (Fig. 1).

In general, these data are in agreement with the data of Reichelt, Baumann, and Baumann [18], who list diagnostic tests permitting separation of *V. cholerae* from other species of *Vibrio*. The three strains of the *cholerae* biotype, strains UNSW, NCTC 7270, and NCTC 8021, formed a single cluster in the dendrogram, before linking with strains of the *eltor* biotype. However, no additional consistent phenotypic properties were obtained in this study to suggest a separate biotype status for the hemolytic, polymyxin-resistant, Voges-Proskauer-positive strains of *V. cholerae* that are presently assigned to the *eltor* biotype. The strains, N18 and VS22, were observed to luminesce brightly, achieving a maximum emission of 10^{12} quanta/s at the end of the exponential phase of growth. In comparison, *V. cholerae* biotype *albensis* strain ATCC 14547 achieved a maximum emission of only 10^{8} quanta/s under the conditions used. Strains N18 and VS22 both failed to agglutinate with O1 antiserum; however, they did not cluster together in the dendrogram and did not show any consistent phenotypic differences from the remaining strains of *V. cholerae*, suggesting that a separate biotype for luminescent strains of *V. cholerae* is not justified. Stud-

ies of the genetic homology of these and additional luminous isolates of *V. cholerae* are in progress.

The 25 clinical isolates and 29 environmental isolates of *V. cholerae* serotype non-O1 showed no tendency to cluster separately within the dendrogram. Furthermore, all of these isolates were phenotypically indistinguishable from the 32 clinical isolates and 66 environmental isolates of *V. cholerae* serotype O1, although there was a partial separation of the O1 serotype in the numerical analysis. These data provide further confirmation of the identity of the environmental isolates as *V. cholerae*. Studies of the degree of genetic homology of these isolates are in progress. The public health significance of the persistent isolation of O1 and non-O1 serotypes of *V. cholerae* from environmental sources in Australia is also under study.

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