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A Phenotypic and Genetic Study of Sucrose Nonfermenting Strains of *Vibrio mimicus* and *Vibrio cholerae*

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Abstract. Sixty-six strains unable to ferment sucrose and resembling *Vibrio mimicus* and *V. cholerae* were submitted to an extensive phenotypic characterization. DNA–DNA homology among selected strains and the type strain of *V. cholerae* was studied by the S1 endonuclease method. Seven sucrose-negative strains were shown to have the phenotypic properties of and a high percentage DNA relatedness to *V. cholerae* and a low level of homology with *V. mimicus*. Eight luminescent strains phenotypically most closely resembled *V. mimicus;* however, two of these were shown to have a high level of DNA homology with *V. cholerae* and a low level of relatedness to *V. mimicus*. A single strain was found to be phenotypically and genetically unrelated to either *V. cholerae* or *V. mimicus* and may represent a new species. The remaining strains were phenotypically shown to be *V. mimicus,* and selected strains were shown to have a high percentage DNA homology with *V. mimicus* and a low level of homology with *V. cholerae*. Problems associated with the identification of these strains and differential traits are discussed.

There has been increasing interest in the genus *Vibrio* and in particular the species *Vibrio cholerae* in western countries after the occurrence of clinical cases of cholera and the subsequent isolation of the organism from the environment in areas where the disease has not been endemic for many years [2, 4, 5, 18, 21]. An important trait used to identify *V. cholerae* from the increasing number of other potentially pathogenic *Vibrio* species is the ability of the organisms to ferment sucrose.

As early as 1936, Heiberg [13] recognized the existence of non-sucrose-fermenting strains of *V. cholerae*. He proposed a scheme to group *V. cholerae* based on the fermentation of sucrose, mannose, and arabinose. Sucrose-negative *V. cholerae* strains belonged to Heiberg group V. Furniss et al. [12] reported that additional traits including the Voges-Proskauer reaction, amylase production, chick cell agglutination, and polymixin sensitivity, could be used to distinguish the Heiberg group V strains. In a numerical taxonomy study of the family Vibrionaceae, West [22] included two sucrose-negative strains. The strains clustered on the edge of the *V. cholerae* phenon; this suggests that there may be some degree of unrelatedness.

Fanning et al. [11] studied the DNA relatedness among representative strains of the Heiberg groups of *V. cholerae* and showed that the group V strains included in the study were only 20%–50% related to the type strain. Subsequently Davis et al. [7] studied 51 sucrose-negative strains which, on the basis of biochemical traits and DNA relatedness, were found to be a homogeneous group separate from *V. cholerae*. The strains were designated as new species *V. mimicus,* type strain ATCC 33653.

At the same time a phenotypic and genetic analysis of *V. cholerae* conducted by the present authors [8, 9] also showed that the three sucrose-negative strains included belonged to a separate species from *V. cholerae*. This study includes a further phenotypic characterization of 66 sucrose-negative strains and a study of the DNA relatedness among selected sucrose-negative strains and the type strain of *V. cholerae,* NCTC 8021.

Materials and Methods

Bacterial strains. The strains included in the study are listed in Table 1. Thirty strains of *V. cholerae* that ferment sucrose were included in the study. The strains that have computer code numbers 67 to 97 include NCTC 3661 and 8021; Q1; B1–3, 8, 14, 28, 33, 38, 41, 45, 46, 77; N1, 5, 10, 15, 16, 20, 21, 33, 37; VS2, 10,

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Table 1. Origin and details of strains

Computer code no.	Strain no.	Laboratory of origin	Source
1	VS21	H. Ghosh, Newcastle	Respiratory tract, Australia
2	VS27	Microbiological Diagnostic Unit, Melbourne University	Diarrhea, traveler returning to Australia
3–7	N47, 52, 123, 124, 127	P.D.[a]	Water, Australia
8–20	S42, 41, 55, 71, 72, 74, 95, 96, 99, 104, 126, 173, 187	P.D.	Water, Australia
21, 22	N57, 130	Australian Government Analytical Laboratory, Sydney	Frozen prawns imported into Australia
23	N62	Commonwealth Health Laboratory, Townsville	Water, Australia
24	N105	H. Ghosh, Newcastle	Wound, Australia
25, 26	611, 612	J.V.L.[b]	Water, Calcutta
27–35	1223, 1229, 1231, 3161, 3311, 3328, 3954, 3955, 8971	J.V.L.	Prawns, Malaysia
36	1357	J.V.L.	Crayfish, United Kingdom
37, 38	2056, 3044	J.V.L.	Feces, Ghana
39–48	3784, 3788, 3789, 3790, 3791, 3793, 3795, 5825, 5872, 5881	J.V.L.	Feces, Bangladesh
49	5961	J.V.L.	Feces, Tunisia
50–53	6579, 7434, 10583, 10586	J.V.L.	Water, UK
54	7150	J.V.L.	Prawns, Thailand
55, 56	7263, 7278	J.V.L.	Water, USA
57	7650	J.V.L.	Water, India
58	8274	J.V.L.	Feces, Bahrain
59	8570	J.V.L.	Feces, Morocco
60–62	8885, 8888, 10127	J.V.L.	Feces, USA
63	9497	J.V.L.	Feces, Iraq
64	9888	J.V.L.	Feces, India
65	10216	J.V.L.	Prawns, Australia
66	*V. mimicus* ATCC 33653	American Type Culture Collection	Designated type strain

[a] P.D., P. Desmarchelier; strains isolated during a study of the distribution of *V. cholerae*.
[b] J.V.L., J. V. Lee, PHLS, Porton Down, England.

16, 18, 22, 28. Details of the sucrose-fermenting strains have been previously described [8]. The strains all possessed the traits necessary for inclusion in the species *V. cholerae* [14]. In particular, they were all oxidase-positive, actively motile, Gram-negative, curved bacilli that fermented D-glucose with the production of acid but no gas, produced lysine and ornithine decarboxylases but no arginine dihydrolase, grew in tryptone water with no added sodium chloride, and were sensitive to the antibiotic substance, 0/129, at 10 μg and 150 μg concentrations. The strains studied included 66 strains unable to ferment sucrose and not agglutinated by *V. cholerae* 01 polyvalent or monovalent antisera (Wellcome Australia). Thirty strains of *V. cholerae* that fermented sucrose were included; 15 were of the 01 serotype. Following isolation or receipt from other laboratories, cultures were maintained in semisolid nutrient agar (0.2% wt/vol) under sterile paraffin oil at room temperature.

Phenotypic analysis. The phenotypic characterization was based largely on a nutritional screening that tested the ability of the strains to utilize 149 different carbon compounds as the sole source of carbon and energy, as previously described [3, 16, 20].

Table 2. Some phenotypic properties of *Vibrio mimicus*, *Vibrio* species N62, and phenotypic subgroups of *Vibrio cholerae*

Trait	*V. mimicus*	Vibrio species N62	*V. cholerae*		
			Sucrose-negative, nonluminous	Sucrose-negative, luminous	Sucrose-positive[a]
No. of strains	50[b]	1	7	8	157
Voges-Proskauer	0	0	100	0	96
Luminescence	0	0	0	100	1
Growth at 10°C	8(+)[c]2, 13, 28, 41	0	100	0	63
Growth at 43°C	28(+)2, 6, 13, 15, 16, 22, 28, 29, 31, 54, 57, 63, 64, 66	0	100	38(+)8, 9, 11	83
Production of					
Amylase	0	0	43(−)38, 48, 52, 53	0	95
Lipase	90(−)14–16, 42, 64	100	86(−)38	100	100
Utilization of					
D-Ribose	94(−)4, 22, 24	0	100	88(−)5	99
D-Sucrose	0	0	0	0	100
D-Mannose	100(−)	100	71(−)48, 49	63(−)10, 11, 20	73
D-Galactose	94(−)4, 15, 54	0	71(−)48, 50	88(−)10	97
D-Fructose	86(−)26, 29, 39, 40, 43, 45, 51	100	100	100	100
Maltose	90(−)7, 27, 34, 42, 61	100	57(−)48, 50, 53	88(−)20	99
Cellobiose	20(+)2, 3, 7, 14–16, 19, 22, 43, 51	0	0	13(+)8	5
D-Gluconate	98(−)51	100	86(−)56	100	94
D-Glucuronate	92(−)4, 6, 28, 37	0	0	88(−)11	8
L-Malate	100	0	100	75(−)5, 12	94
DL-Lactate	80(−)4, 6, 7, 29, 31, 32, 35, 37, 45, 55	100	86(−)56	75(−)5, 18	99
Citrate	94(−)31, 32, 37	0	100	88(−)12	95
Succinate	98(−)7	100	100	100	96
Fumarate	100	100	86(−)56	100	96
Mannitol	100	0	100	88(−)5	100
Glycerol	96(−)28, 42	0	100	50(−)10–12, 18	97
L-Histidine	90(−)40–43, 45	0	100	75(−)11, 20	44
Proline	98(−)47	100	100	100	100
Glycine	16(+)32, 37, 41, 44, 46, 60, 62, 65	0	29(+)50, 52	13(+)8	9
L-α-Alanine	12(+)28, 31, 37, 40, 41, 44	100	0	13(+)8	1
D-α-Alanine	72(−)15, 21, 26, 30–32, 37, 39–41, 54, 58, 61, 64	0	29(+)38, 52	50(−)8, 12, 18, 20	26
L-Threonine	46(−)4, 15, 16, 19, 21, 22, 30–35, 37, 39–47, 51, 58, 61, 63, 64	100	71(−)48, 56	88(−)18	34
L-Glutamate	90(−)6, 25, 27, 29, 36	100	100	100	90
L-Arginine	74(−)2, 26, 30–32, 37, 39, 40, 47, 51, 58, 59, 66	100	100	75(−)8, 20	100
D-Malate	78(−)2–4, 7, 13, 21, 24, 27, 28, 41, 66	0	43(+)38, 48, 50	13(+)8	0
Tyrosine	8(+)15, 57, 59, 62	0	0	0	0
Leucine	4(+)33, 34	0	0	0	0

[a] Data taken from previous study [8].
[b] Percent positive strains.
[c] Positive or negative strains.

The carbon compounds and the media used were the same as those included in a previous study of *V. cholerae* with the exception of aconitate, caproate, and DL-glycerate [8], which were omitted from the present study. All tests were incubated at 25°C unless otherwise stated. Strains were tested for the production of extracellular amylase by both the method previously described [8], which detected substrate utilization, and by the method described in MacFaddin [15], which detected end-product formation with Benedict's reagent. The Voges-Proskauer test was performed at 25°C and 37°C by use of buffered glucose broth and the method of Barritt [1], and with the medium described by Furniss et al. [12]. Lipase activity on corn oil was determined according to the method of Edwards and Ewing [10]. Sensitivity to polymixin was determined by inoculating 4- to 6-h broth cultures of the strains onto nutrient agar plates containing 15 μg/ml of polymyxin B. The numerical analysis of the phenotypic data was performed by use of the simple matching coefficient and the complete linkage method [19].

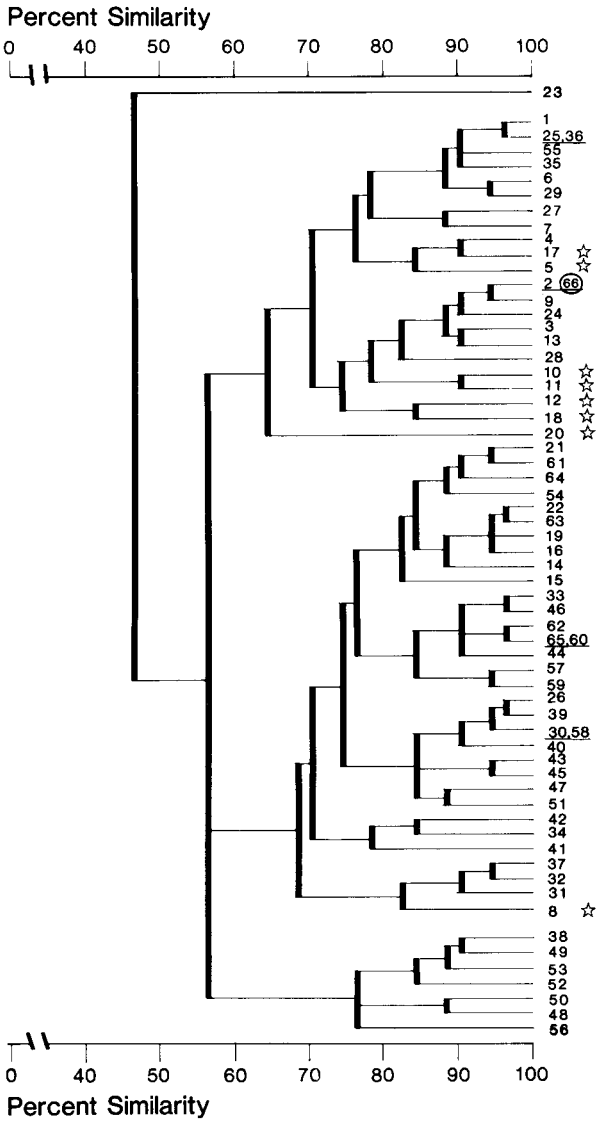

Fig. 1. Numerical analysis of 66 sucrose-negative strains resembling *Vibrio cholerae* and *Vibrio mimicus*. Strains that have identical phenotypic properties have been underlined; ☆ denotes luminous strains; 66 is the type strain of *V. mimicus*.

DNA–DNA hybridization. The method of preparation of both radioactive-labeled and unlabeled DNA was essentially that of Reichelt et al. [17]. The specific activities of the ^{14}C-labeled DNA preparations ranged from 25,000 cpm/μg to 36,000 cpm/μg. Hybrid DNA duplexes were prepared and analyzed with the single-strand, specific endonuclease S1 [6]. Details of the DNA preparation and hybridization have been previously described [9].

Results and Discussion

Phenotypic characterization. The results of the phenotypic characterization of 66 sucrose-nonfermenting strains resembling *V. mimicus* and *V. cholerae* are shown in Table 2. After the exclusion of all the universally positive and negative traits to improve the sensitivity of the analysis, 34 differential traits were submitted to a numerical analysis. The results are shown in the form of a dendrogram in Fig. 1.

A single strain, N62, clustered separately and was phenotypically distinct in that it was much less nutritionally versatile than the other strains. Seven strains—3044, 5881, 5961, 6579, 7278, 10583, and 10586—formed a single cluster before linking with the other strains at 56%. These strains, with the exception of fermentation of sucrose, possessed traits previously described for *V. cholerae* [8]. The remaining strains, including the type strain of *V. mimicus*, formed two clusters linking at the same S value as the former group; however, the strains could not be differentiated by any universally positive or negative traits. Eight strains—N123, S41, S55, S71, S72, S104, S126, and S187—were observed to luminesce, achieving a maximum emission of light of 8×10^{14} quanta/s. Although these strains were linked closely together, they did not form a separate cluster.

A subsequent numerical analysis was performed that included the seven sucrose-negative strains having the traits of *V. cholerae*, eight luminous isolates, and 39 other sucrose-negative isolates chosen from the dendrogram, together with 30 strains of sucrose-positive *V. cholerae* from our previous study [8]. Of the 30 strains, 15 were of the 01 serotype. Thirty-six traits, including the 34 traits used in the former analysis together with sucrose utilization and gelatin hydrolysis, were submitted to a numerical analysis and are presented in the form of a dendrogram (Fig. 2). Strain N62 clustered separately from the other sucrose-negative strains and *V. cholerae*. The seven sucrose-negative strains having the traits of *V. cholerae* were clustered among the sucrose-positive strains before linking together with the other strains at an overall similarity of 46%. The eight sucrose-negative luminous strains showed a tendency to cluster together in a single group, but they did not cluster together with the luminous strain of *V. cholerae*, VS22.

In vitro DNA/DNA hybridization. Unlabeled DNA was prepared from strains included in the phenotypic analysis. The DNA was hybridized with labeled DNA prepared from strains VS21 and NCTC 8021, the type strain of *V. cholerae*. At the commencement of this work, the type strain of *V. mimicus* was not available, and strain VS21 was chosen for the determination of homology among the sucrose-

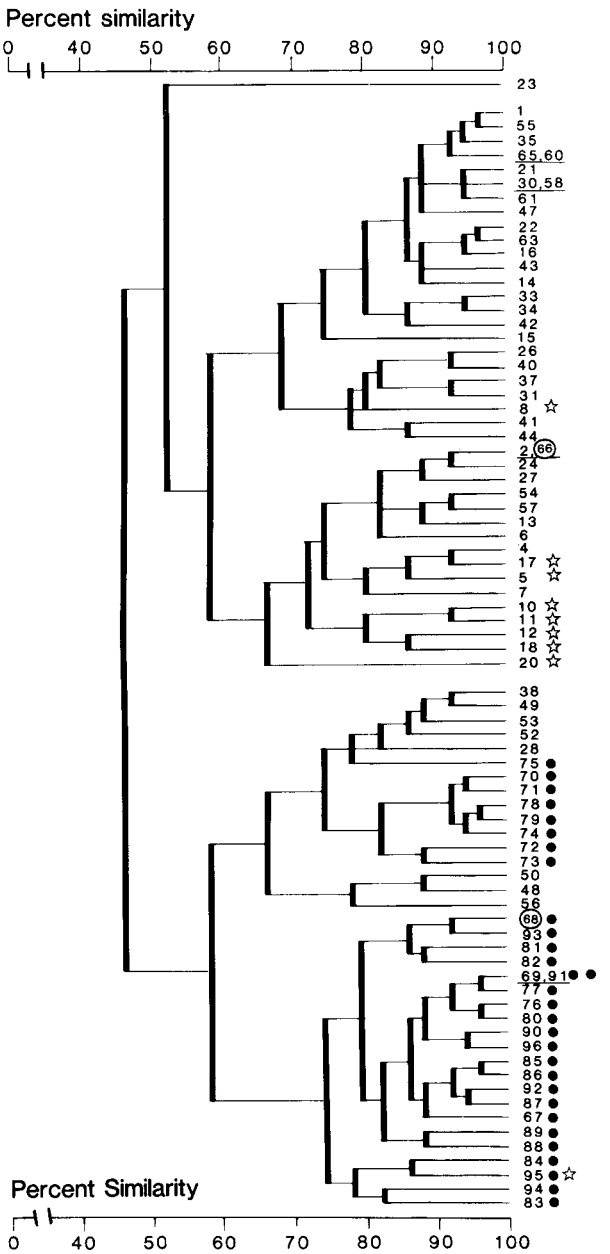

Fig. 2. Numerical analysis of 52 sucrose-negative strains resembling *Vibrio cholerae* and *Vibrio mimicus* and 30 sucrose-positive strains of *Vibrio cholerae*. Strains that have identical phenotypic properties have been underlined; ☆ denotes luminous strains; ● denotes sucrose-positive strains; 66 and 68 are the type strains of *V. mimicus* and *V. cholerae*, respectively.

negative strains. As VS21 has now been shown to be 83% homologous to *V. mimicus* ATCC 33653, it is considered to be a member of this species. The results of the in vitro DNA–DNA hybridizations of the strains are shown in Table 3. Strain N62 was found to have a low percentage homology with both *V. mimicus* and *V. cholerae*. This result supports

Table 3. DNA–DNA relatedness among strains of *Vibrio mimicus*, *Vibrio* species N62, and phenotypic subgroups of *Vibrio cholerae*

	Source of labeled DNA	
Source of unlabeled DNA	VS21	NCTC 8021
VS21	100	40
VS27	78	41
N57	82	43
1223	85	NT[a]
3784	86	41
V. mimicus ATCC 33653	83	NT
3044	59	NT
7278	58	79
6579	67	NT
5961	54	NT
10586	54	NT
5881	59	78
S126	44	84
S187	50	NT
V. cholerae NCTC 8021	44	100
Vibrio species N62	57	43

[a] NT, not tested.

the phenotypic data and suggests this strain represents a single isolate of a new species. Six sucrose-negative strains possessing the traits of *V. cholerae* and the type strain NCTC 8021 showed a low level of DNA relatedness to *V. mimicus* (40%–67%). Two of these strains were tested for homology with *V. cholerae;* both strains showed species level homology (78% and 79%). Two sucrose-negative luminous strains, S126 and S187, similarly showed a low level of homology with *V. mimicus* (44% and 50%). Strain S126 was found to have 84% homology with *V. cholerae*. These results indicate that the sucrose-negative luminous strains for which DNA homology data are available belong to the species *V. cholerae,* although phenotypically they closely resemble *V. mimicus*. Four nonluminescent strains phenotypically resembling *V. mimicus*—VS27, N57, 1223, and 3784—selected from other clusters of the dendrogram and the type strain, ATCC 33653, were found to have species-level homology (78%–86%) with strain VS21 and a low homology to *V. cholerae*.

Davis et al. [7] published a study of sucrose-negative strains of atypical *V. cholerae* and, on the basis of a phenotypic characterization and DNA–DNA hybridizations, found the strains were a homogeneous group belonging to the species, *V. mimicus*. Properties including sucrose fermentation V.P., corn oil, Jordan tartrate and polymixin sensi-

Table 4. Properties distinguishing sucrose non-fermenting strains of *Vibrio cholerae* and *Vibrio mimicus*

	V. mimicus	*V. cholerae*	
		Nonluminous	Luminous
Growth at 10°C	8[a]	100	0
Growth at 43°C	28	100	38
V.-P.	0	100	0
Growth on D-glucuronate	92	0	88
Lipase (corn oil)	20	100	38
Amylase	0	43	0
Polymixin sensitivity	92	0	63
Luminescence	0	0	100

[a] Percent positive.

tivity were reported to be useful in distinguishing *V. mimicus* from *V. cholerae*. The results of testing 66 strains in the current study, which used some of these tests, are shown in Table 4. The nonluminous, sucrose-negative strains of *V. cholerae* gave reactions consistent with those for that species. The seven luminous sucrose-negative strains of *V. cholerae* gave reactions similar to those of *V. mimicus*. Additional traits which our study has indicated are useful to identify these groups are shown in Table 4.

Conclusions

An extensive phenotypic and genetic characterization of 66 non-sucrose-fermenting strains closely resembling *V. cholerae* has shown that some of these strains belong to the species *V. cholerae*, while others belong to the newly described species, *V. mimicus* [7]. Seven sucrose-negative, nonluminous strains shown to be members of the species *V. cholerae* could be differentiated from *V. mimicus* by the traits of Davis et al. [7] and in addition by their inability to utilize D-glucuronate and their ability to produce extracellular amylase and to grow at both 10°C and 43°C.

Strains that were sucrose-negative and luminesced were shown to belong to the species, *V. cholerae*. These strains could not be distinguished phenotypically from *V. mimicus* other than by their ability to emit light. Since the property of luminescence can be readily lost on subculture in the laboratory, these strains may therefore be distinguished on the basis of in vitro DNA–DNA hybridization only and require further study.

All of the clinical isolates in this study could be identified either with *V. cholerae* or *V. mimicus;* however, during an ecology study, sucrose-negative, luminous strains of *V. cholerae* might be misidentified as *V. mimicus*.

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