APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Sept. 1996, p. 3548–3550 Vol. 62, No. 9
0099-2240/96/$04.00+0

A Selective and Differential Medium for *Vibrio harveyi*

LACHLAN HARRIS,[1]* LEIGH OWENS,[1]* AND SANDRA SMITH[2]

Department of Biomedical and Tropical Veterinary Sciences, Division of Microbiology,[1] and Sir George Fisher Centre for Tropical Marine Studies,[2] James Cook University of North Queensland, Townsville, Australia 4811

Received 18 April 1996/Accepted 9 July 1996

A new medium, termed *Vibrio harveyi* agar, has been developed for the isolation and enumeration of *V. harveyi*. It is possible to differentiate *V. harveyi* colonies from the colonies of strains representing 15 other *Vibrio* species with this medium. This medium has been shown to inhibit the growth of two strains of marine *Pseudomonas* spp. and two strains of marine *Flavobacterium* spp. but to allow the growth of *Photobacterium* strains. Colonies displaying typical *V. harveyi* morphology were isolated from the larval rearing water of a commercial prawn hatchery with *V. harveyi* agar as a primary isolation medium and were positively identified, by conventional tests, as *V. harveyi*. This agar displays great potential as a primary isolation medium and offers significant advantages over thiosulfate-citrate-bile salts-sucrose agar as a medium for differentiating *V. harveyi* from other marine and estuarine *Vibrio* species.

In recent years, *Vibrio harveyi* has become recognized throughout Southeast Asia (3, 5, 17, 18) and northern Australia (9, 12) as a devastating pathogen of penaeid larvae, particularly of the intensively farmed black tiger prawn, *Penaeus monodon* (3, 5, 9). Highly virulent strains of *V. harveyi* result in up to 100% mortality from bath inocula containing as few as 10^2 CFU/ml (5, 9). *V. harveyi* is a ubiquitous bacterium in warm marine waters (5, 10, 12, 13) and a part of the intestinal floras of marine animals (11, 15).

Thiosulfate-citrate-bile salts-sucrose (TCBS) agar is a widely used medium for isolating and enumerating *Vibrio* species from marine and estuarine waters (3, 5, 16). However, the validity of using TCBS agar for isolating many species of *Vibrio* is questionable (6, 16). TCBS agar is inhibitory to many of the *Vibrio* species that are commonly encountered in the marine environment (16). TCBS agar is also not a differential medium in that *Vibrio* species like *V. harveyi*, which are variable in the utilization of sucrose, cannot be distinguished from other sucrose-positive or sucrose-negative species.

A number of selective media have been developed for isolation and enumeration of other *Vibrio* species, in particular *Vibrio cholerae* and *Vibrio vulnificus* (7), *Vibrio anguillarum* (2), and *Vibrio alginolyticus* and *Vibrio parahaemolyticus* (4). An alternative to TCBS for the selective enumeration of marine members of the family *Vibrionaceae* has also been described (16).

The medium described in this paper is termed *V. harveyi* agar (VHA). A high pH, which is tolerated by *Vibrio* species but not by many terrestrial bacteria (10), and an absence of all but contaminating levels of magnesium ions, which are required by other marine bacteria (14), makes VHA inhibitory to most non-*Vibrio* genera. A high NaCl concentration in the medium tends to favor marine *Vibrio* species as does an incubation temperature of 28°C. Lower NaCl concentrations and higher incubation temperatures have been used by other workers when selecting for the human pathogens *V. cholerae* and *V. vulnificus* (7). Utilization by *Vibrio* species which can grow on this medium of cellobiose and ornithine and the resultant pH changes provide the means for the differentiation, based on colonial morphology, of *V. harveyi* from other marine members of the family *Vibrionaceae*. Decarboxylation of ornithine produces a basic pH change in the medium, resulting in colonies appearing blue. Fermentation of cellobiose produces an acid pH change, resulting in colonies appearing green, sometimes with the formation of yellow halos around the colonies. *V. harveyi* can utilize cellobiose as a carbon source and is able to decarboxylate ornithine (8). These properties as well as an optimal NaCl concentration and incubation temperature, promoting a growth rate faster than those of other *Vibrio* spp., probably explain the unique appearance of *V. harveyi* colonies on this medium.

The following ingredients are combined to make VHA: D-cellobiose, 2 g; L-ornithine, 2 g; NaCl, 30 g; Tris[hydroxymethyl]aminomethane, 1.21 g; agar, 20 g; K_2HPO_4, 0.075 g; thymol blue, 0.04 g; bromothymol blue, 0.04 g; Bacto Peptone, 0.1 g; yeast extract, 0.1 g; and distilled water, 1,000 ml. This solution is then boiled for approximately 30 min or until the agar has completely dissolved. After being boiled, the solution is cooled to 56°C and adjusted to pH 9.0 by the addition of 1 M NaOH. The solution is then dispensed into sterile petri dishes. When solidified, VHA is an azure blue color.

Although other workers have used antibiotics to increase the specificities of *Vibrio* species-specific agars (2, 7), the addition of ampicillin, penicillin, colistin, or polymyxin B to VHA was detrimental to the growth of *V. harveyi* on this agar and so these agents were not used. Many marine agars also require additional iron in the form of $FeNH_2$ citrate or $FeCl_3$ (14); however, this was found not to be necessary for the growth of *V. harveyi*. This agar needs to be boiled and not autoclaved to avoid the caramelization of cellobiose. Alternatively, cellobiose can be made into solution separately and filter sterilized before being added to the other components. The addition of NaOH should be performed under sterile conditions by taking an aliquot of the boiled solution and using this as an indicator to judge the amount of NaOH necessary to raise the pH of the larger volume of solution.

In this study, growth of 44 *Vibrio* strains representing 16 species as well as marine isolates from three other bacterial genera on VHA was tested and compared with growth on TCBS agar and on modified luminous agar (LA). LA is a medium suitable for enumerating the total heterotrophic count from marine waters (14). Overnight cultures of bacterial strains on LA were suspended in sterile phosphate-buffered

* Corresponding author. Electronic mail address for Lachlan Harris: Lachlan.Harris@jcu.edu.au. Electronic mail address for Leigh Owens: Leigh.Owens@jcu.edu.au.

TABLE 1. Colony morphologies on VHA

Species (no. of strains)[a]	Colony morphology
Vibrio harveyi (20)	Small (diameter, 2 to 5 mm) colonies; light green with dark green centers; yellow halo; 16 strains with entire margins; 2 strains with crenellated edges
Vibrio natriegens (2)	Small (diameter, 2 to 5 mm) colonies; light green
Vibrio vulnificus (3), *Vibrio anguillarum* (2), and *Vibrio pelagius* (2)	No growth
Vibrio aestuarianus (1) and *Vibrio campbellii* (1)	Very small (diameter, <2 mm) colonies; light blue
Vibrio alginolyticus (3), *Vibrio parahaemolyticus* (3), and *Vibrio carchariae* (1)	Large (diameter, >5 mm) colonies, spreading; light blue large (diameter, 4 to 5 mm) colonies, dark blue, small (diameter, 2 to 5 mm), spreading, dark blue-green
Vibrio cinncinnatiensis (1), *Vibrio furnissi* (1), and *Vibrio orientalis* (1)	Small (diameter, 2 to 5 mm); light blue-green
Vibrio diazotrophicus (1), *Vibrio fluvialis* (1), and *Vibrio gazogenes* (1)	Small (diameter, 2 to 5 mm); dark blue-green
Photobacterium damsela (1) and *Photobacterium fischeri* (1)	Small (diameter, 2 to 5 mm); dark blue-green
Photobacterium leiognathi (1), *Photobacterium angustum* (1), and *Photobacterium phosphoreum* (1)	Very small (diameter, <2 mm); light blue
Pseudomonas spp. (2) and *Flavobacterium* spp. (2)	No growth

[a] *Pseudomonas* spp., *Flavobacterium* spp., and some *V. harveyi* isolates were obtained from Oonoonba Veterinary Laboratories, Queensland Department of Primary Industries, Townsville, Australia. All other isolates were obtained from the Department of Biomedical and Tropical Veterinary Sciences, Australian Collection of Marine Microorganisms, James Cook University, Townsville, Australia.

saline (PBS) to an A_{660} of between 0.1 and 0.5. One hundred microliters of a 10^{-6} dilution of these suspensions was then spread onto VHA, TCBS agar, and LA. Following inoculation, the plates were incubated at 28°C before the number of colonies was counted and colony morphologies were recorded. TCBS agar and LA plates were incubated for 24 h. VHA plates were incubated for 48 h.

As a preliminary field test of VHA, water samples were taken from a commercial *P. monodon* hatchery. Appropriate dilutions were made in sterile PBS, and 100-μl samples were plated on VHA, TCBS agar, and LA. A comparison of colony counts from the different agars was performed. Nine colonies from different plates, displaying typical *V. harveyi* morphology on VHA, were isolated in pure culture and presumptively identified to the species level by the method of Alsina and Blanch (1).

The colonial morphologies of the strains tested on VHA are described in Table 1. *V. harveyi* colonies displayed distinctive morphologies on VHA. ACMM 656 would be a suitable reference strain for the growth of this species on this agar. However, the 20 strains of *V. harveyi* tested displayed very similar morphologies. After 48 h at 28°C, *V. harveyi* colonies appeared as small (diameter, 2 to 5 mm) light green colonies with dark green centers. Most colonies were circular and had an entire margin. The colonies of two strains showed crenellated edges. When illuminated from below, yellow halos, indicating cellobiose fermentation, could be seen around the colonies.

TABLE 2. Comparison of viable counts obtained on VHA, TCBS agar, and LA

Strain or bacterium	CFU/ml on[a]:		
	VHA (10^8)	TCBS agar	LA (10^8)
Vibrio harveyi ACMM 645	3.0	6×10^7	3.0
Vibrio harveyi ACMM 642	1.5	$<10^7$	1.2
Vibrio harveyi ACMM 656	4.2	2.3×10^8	6.0
Vibrio diazotrophicus ACMM 636	3.1	1.3×10^8	2.6
Vibrio alginolyticus ACMM 102	2.7	3.0×10^8	2.7
Vibrio furnissi ACMM 641	3.1	1.0×10^8	2.7
Photobacterium damsela ACMM 624	3.5	$<10^7$	3.6
Photobacterium phosphoreum	2.3	$<10^7$	2.6

[a] Only a representative sample of the colony counts recorded in this study is shown.

Of the 15 other *Vibrio* species tested on this agar, only the colonies of *Vibrio natriegens* shared some of the characteristics of *V. harveyi* colonies. However, *V. natriegens* colonies did not show a darker green center. *V. vulnificus*, which has very close serological and biochemical homology with *V. harveyi*, did not grow on this agar within 48 h as was intended by using a higher than optimal NaCl concentration and a lower than optimal incubation temperature for this species. *V. alginolyticus* and *V. parahaemolyticus* colonies were easily distinguishable from each other, because of the swarming motility of *V. alginolyticus*, and from *V. harveyi*. These species are very common bacterial floras in *P. monodon* hatcheries, and the easy differentiation of these species from *V. harveyi* with this agar should aid researchers in more accurately assessing *Vibrio* species diversity in this environment.

With the exception of the *Photobacterium* species tested, the other genera of marine bacteria did not grow on VHA. *Pseudomonas* and *Flavobacterium* species are important contributors to the total heterotrophic count in marine waters. The elimination of these genera from growth on VHA is an important selective trait. None of the five *Photobacterium* species which grew on this agar resembled *V. harveyi* in colony morphology.

VHA performed well as a primary isolation medium as shown by the colony counts recorded in Table 2. Colony counts for *V. harveyi* strains and other *Vibrio* species were of the same

TABLE 3. Tests used to identify presumptive *V. harveyi* isolates

Substrate	Growth of isolate[a]:								
	B2	A1	B4	C2	C6	C1	B5	A5	C62
0% NACl	−	−	−	−	−	−	−	−	−
8% NaCl	+	+	+	+	+	+	+	+	+
Citrate	−	+	+	+	−	+	+	+	+
Sucrose acid	−	−	−	−	−	−	−	−	−
VP	−	−	−	−	−	−	−	−	−
L-Arabinose (CS[b])	−	−	−	−	−	−	−	−	−
D-Glucosamine (CS)	−	−	−	−	−	−	−	−	−
Arginine dihydrolase	−	−	−	−	−	−	−	−	−
Lysine decarboxylase	+	+	+	+	+	+	+	+	+
Ornithine decarboxylase	+	+	+	+	+	+	+	+	+

[a] All isolates were gram negative, oxidase positive, and O/F positive and grew on TCBS medium. Isolates A1, B4, B5, A5, and C62 were luminescent on LA.
[b] CS, carbon substrate.

or a slightly higher order of magnitude on VHA compared with those on TCBS agar or LA. This indicates that VHA has the potential to be an excellent medium for enumerating *V. harveyi* in water samples.

The nine isolates that were taken off VHA plates from prawn hatchery water samples were all positively identified as presumptive *V. harveyi*. The results of the tests used to identify these isolates to the species level are shown in Table 3. This confirms the suitability of VHA as a medium for primary isolation of *V. harveyi* from water samples.

The testing of the specificity and differential ability of VHA suggests that the agar is suitable for a full-scale study of *P. monodon* larval rearing water in which *V. harveyi* levels can be closely monitored. Such a study will provide more rigorous testing for this agar, which has already exhibited great potential.

The Co-operative Research Centre for Aquaculture provided financial support for Lachlan Harris and the research described in this paper.

REFERENCES

1. **Alsina, M., and A. R. Blanch.** 1994. A set of keys for biochemical identification of environmental *Vibrio* species. J. Appl. Bacteriol. **76:**79–85.
2. **Alsina, M., J. Martinez-Picado, J. Jofre, and A. R. Blanch.** 1994. A medium for presumptive isolation of *Vibrio anguillarum*. Appl. Environ. Microbiol. **60:**1681–1683.
3. **Baticados, M. C. L., C. R. Lavilla-Pitogo, E. R. Cruz-Lacierda, L. D. De La Pena, and N. A. Sunaz.** 1990. Studies on the chemical control of luminous bacteria *Vibrio harveyi* and *Vibrio splendidus* isolated from diseased *Penaeus monodon* larvae and rearing water. Dis. Aquat. Org. **9:**133–139.
4. **Kourany, H.** 1983. Medium for isolation and differentiation of *Vibrio parahaemolyticus* and *Vibrio alginolyticus*. Appl. Environ. Microbiol. **45:**310–312.
5. **Lavilla-Pitogo, C. R., L. J. Albright, M. G. Paner, and N. A. Sunaz.** 1992. Studies on the sources of luminescent *Vibrio harveyi* in *Penaeus monodon* hatcheries, p. 157–164. *In* I. M. Shariff, R. P. Subasinghe, and J. R. Arthur (ed.), Diseases in Asian aquaculture. Fish Health Section, Asian Fisheries Society, Manila, The Philippines.
6. **Makemson, J. C., N. Fulayfil, and P. Basson.** 1992. Association of luminous bacteria with artificial and natural surfaces in Arabian Gulf seawater. Appl. Environ. Microbiol. **58:**2341–2343.
7. **Massad, G., and J. D. Oliver.** 1987. New selective and differential medium for *Vibrio cholerae* and *Vibrio vulnificus*. Appl. Environ. Microbiol. **53:**2262–2264.
8. **Muir, P.** Unpublished data.
9. **Muir, P., D. Sutton, and L. Owens.** Experimental investigation of some bacterial pathogens of *Penaeus monodon* protozoea. Submitted for publication.
10. **Nishibuchi, M., N. C. Roberts, J. R. Bradford, and R. J. Seidler.** 1983. Broth medium for enrichment of *Vibrio fluvialis* from the environment. Appl. Environ. Microbiol. **46:**425–429.
11. **O'Brien, C. H., and R. K. Sizemore.** 1979. Distribution of the luminous bacterium *Beneckea harveyi* in a semitropical environment. Appl. Environ. Microbiol. **38:**928–933.
12. **Owens, L., P. Muir, D. Sutton, and M. Wingfield.** 1992. The pathology of microbial diseases in tropical Australian crustacea, p. 165–172. *In* I. M. Shariff, R. P. Subasinghe, and J. R. Arthur (ed.), Diseases in Asian aquaculture. Fish Health Section, Asian Fisheries Society, Manila, The Philippines.
13. **Ramesh, A., B. G. Loganathan, and V. K. Venugopalan.** 1989. Seasonal distribution of luminous bacteria in the sediments of a tropical estuary. J. Gen. Appl. Microbiol. **35:**363–368.
14. **Reichelt, J. L., and P. Baumann.** 1973. Taxonomy of the marine luminous bacteria. Arch. Microbiol. **94:**283–330.
15. **Ruby, E. G., and J. G. Morin.** 1979. Luminous enteric bacteria of marine fishes: a study of their distribution, densities and dispersion. Appl. Environ. Microbiol. **38:**406–411.
16. **Simidu, U., and K. Tsukamoto.** 1980. A method of the selective isolation and enumeration of marine Vibrionaceae. Microb. Ecol. **6:**181–184.
17. **Sunaryanto, A., and A. Mariam.** 1986. Occurrence of a pathogenic bacteria causing luminescence in penaeid larvae in Indonesian hatcheries. Bull. Brackishwater Aquacult. Dev. Cent. **8:**64–70.
18. **Tansutapanit, D. S. O. A., and L. Ruangpan.** 1987. *Vibrio harveyi*, a causative agent of white shrimp nauplii, *Penaeus merguiensis*, p. 30. *In* Abstract technical paper no. 6/30, Third National Seminar on Marine Science, 6 to 8 August 1986, Bangkok.