APPLIED AND ENVIRONMENTAL MICROBIOLOGY, July 1996, p. 2673–2675 Vol. 62, No. 7
0099-2240/96/$04.00+0

Identification of *Vibrio proteolyticus* with a Differential Medium and a Specific Probe

M. MUNIESA-PÉREZ, J. JOFRE, AND A. R. BLANCH*

Department of Microbiology, University of Barcelona, 08028-Barcelona, Catalonia, Spain

Received 2 November 1995/Accepted 3 May 1996

A differential medium (VP8) and a specific probe, based on the variable region V3 of the 16S rRNA gene, for the detection of *Vibrio proteolyticus* are defined. The medium contains 8% NaCl, which allows selective growth of moderately halophilic *Vibrio* strains. D-Sorbitol, as the main carbon source, differentiates the species that can ferment it by the pH indicators cresol red and bromothymol blue. *V. proteolyticus* and 8 of 418 strains studied grew on the medium and used the D-sorbitol, forming bright yellow colonies. An oligonucleotide, based on the variable region V3 of the 16S rRNA gene (5′CGCTAACGTCAAATAATGCATCTA3′), was used as the specific probe (V3VPR). Only three strains of *Vibrio* sp. and one strain identified as *V. natriegens* cross-hybridized with the probe. However, unlike *V. proteolyticus*, none of the strains grew on VP8. The combined use of VP8 medium and the probe allowed an unequivocal identification of *V. proteolyticus*.

Vibrio proteolyticus is a gram-negative facultative anaerobic rod which produces an extracellular thermostable aminopeptidase. The gene encoding this protease is expressed at high saline concentrations, which is an outstanding characteristic with respect to other microorganisms that produce proteases (7, 16). An antagonistic effect of *V. proteolyticus* against *V. anguillarum* has also been observed in vitro (10), and it may be worthwhile to explore the interactions of these two species in nature because of the importance of *V. anguillarum* as a fish pathogen (9). However, there are no useful tools to perform these studies, since the identification of *V. proteolyticus* by standard methods requires at least 11 tests (1, 2). This makes it difficult to monitor a large number of samples. Any procedure that improved the identification of *V. proteolyticus* in environmental samples would be very useful. Selective media or highly specific molecular techniques (3, 4, 11, 13, 15, 17, 18) are alternatives to the classical methods. We designed a probe, V3VPR, based on a fraction of the variable region V3 of the 16S rRNA, and developed a differential medium, VP8, which together allow a reliable and specific detection of *V. proteolyticus*.

Forty-five isolates of gram-negative bacteria coming from different collections were used. A total of 373 *Vibrio* strains, which were isolated from different natural environments, were used in the specificity studies (Table 1). These strains are deposited in and available at the Laboratory of Microbiology (Ghent, Belgium) collection. Standard biochemical tests and assays on API 20E strips were performed for each strain. Then, the strains were classified by using a matrix previously described (1, 2) and the software Bacterial Identifier (5a).

The possible components of the medium were defined by considering the metabolic characteristics of *V. proteolyticus* (1, 2, 9). After the different compositions and growth conditions had been assayed, the selective medium chosen had the following composition: yeast extract, 4 g; D-sorbitol, 15 g; NaCl, 80 g; cresol red, 40 mg; bromothymol blue, 40 mg; agar, 15 g; and distilled water, 1,000 ml. The ingredients were dissolved by agitation and boiling. The pH was adjusted to 8.0 with 5 M NaOH after the medium was cooled to 50°C. The VP8 was then poured into petri dishes. This medium does not need to be autoclaved, and it was stored, with the lid down, at 4°C. VP8 is grey-blue, and the identification of *V. proteolyticus* is based on the color change of the colonies when the D-sorbitol is fermented. The colonies are bright yellow with a diffusion halo of the same color. The specificity of the medium was studied by the inoculation, for each strain, of 10 μl of a heavy-cell suspension (McFarland standard 3), prepared as previously described (3), on a plate of VP8. Inoculated plates were incubated at 22°C and examined after 24, 48, and 72 h of incubation. The efficiency of recovery of CFU was determined by comparing the counts of *V. proteolyticus* on VP8 with counts on marine agar (MA) (Pronadisa, Alcobendas, Spain) and on thiosulfate-citrate-bile salt-sucrose agar (TCBS) (Oxoid, Basingstoke, England) following the procedure previously described (3). The detection of *V. proteolyticus* in mixed cultures was studied by spiking a previously calibrated pure suspension of *V. proteolyticus* q113 (10^5 CFU/ml) with different kinds of environmental samples which were also previously determined to be free of *V. proteolyticus*. The suspension of *V. proteolyticus* was calibrated by serial 10-fold dilutions in sterile phosphate-buffered saline (PBS) complemented with 1.5% NaCl at pH 7.2 (marine PBS), inoculation of 100 μl of each dilution on MA, and incubation of the plates at 22°C for 48 h. Twelve samples were taken from a fish farm: two water samples and six, two, and two samples from fish larvae, rotifers, and artemiae, respectively. The whole larvae in 3 ml of marine PBS or the rotifer or artemia samples were homogenized in a Potter-Elvehjem homogenizer. Total counts of bacteria were performed for each sample by serial 10-fold dilutions as described above. The absence of wild strains of *V. proteolyticus* in the samples was determined by using the VP8 medium and standard biochemical methods for the identification of vibrios (1, 2). After the environmental samples were spiked with *V. proteolyticus* q113, serial 10-fold dilutions were performed, and 100 μl of each dilution was inoculated on VP8 and MA. The cell ratios of *V. proteolyticus* to the total bacterial population in the spiked samples were 1:10 for water, $1:10^2$ for fish larvae and rotifers, and $1:10^3$ for artemiae. Plates were incubated at 22°C and examined after 24, 48, and 72 h of incubation.

The variable region V3 of the 16S rRNA was sequenced for three *V. proteolyticus* strains (CECT 630, q136, and q138). These sequences have been deposited in the GenBank data-

* Corresponding author. Mailing address: Department of Microbiology, University of Barcelona, Diagonal 645, 08028-Barcelona, Catalonia, Spain. Fax: 34 3 411 0592. Electronic mail address: anicet@porthos.bio.ub.es.

TABLE 1. Summary of species used to evaluate VP8 medium and probe V3VPR in this study

Species	No. of strains[a]	No. of strains		
		VP8[b]	V3VPR[c]	VP8-V3VPR[d]
Aeromonas hydrophila	1	0	0	0
Aeromonas salmonicida	1	0	0	0
Aeromonas sobria	1	0	0	0
Edwardsiella tarda	1	0	0	0
Escherichia coli	1	0	0	0
Pasteurella piscicida	1	0	0	0
Plesiomonas shigelloides	1	0	0	0
Pseudomonas aeruginosa	1	0	0	0
Pseudomonas multocida	1	0	0	0
Vibrio adaptus	1	0	0	0
Vibrio aestuarianus	16	1	0	0
Vibrio alginolyticus	25	1	0	0
Vibrio anguillarum	144	1	0	0
Vibrio anguillarum-like	12	0	0	0
Vibrio campbellii	4	0	0	0
Vibrio carchariae	1	0	0	0
Vibrio cincinnatiensis	1	0	0	0
Vibrio costicola	1	0	0	0
Vibrio cholerae	2	0	0	0
Vibrio damsela	14	0	0	0
Vibrio diazotrophicus	4	0	0	0
Vibrio fischeri	1	0	0	0
Vibrio fluvialis	16	0	0	0
Vibrio furnissii	5	0	0	0
Vibrio gazogenes	1	0	0	0
Vibrio harveyi	38	4	0	0
Vibrio logei	5	0	0	0
Vibrio marinus	1	0	0	0
Vibrio mediterranei	10	0	0	0
Vibrio metschnikovii	7	1	0	0
Vibrio mimicus	2	0	0	0
Vibrio natriegens	4	0	1	0
Vibrio navarrensis	3	0	0	0
Vibrio nereis	6	0	0	0
Vibrio ordalii	2	0	0	0
Vibrio orientalis	4	0	0	0
Vibrio parahaemolyticus	14	0	0	0
Vibrio pelagius	6	0	0	0
Vibrio proteolyticus	13	13	13	13
Vibrio salmonicida	3	0	0	0
Vibrio splendidus	15	0	0	0
Vibrio tubiashii	2	0	0	0
Vibrio vulnificus	13	0	0	0
Vibrio sp.	12	0	3	0
Yersinia ruckeri	1	0	0	0

[a] This number includes the collection strains.
[b] Number of strains that grew on VP8 and formed yellow colonies.
[c] Number of strains that hybridized with the probe V3VPR.
[d] Number of strains that grew on VP8, formed yellow colonies, and hybridized with the probe V3VPR.

base with the accession numbers U37800, U37801, and U37802, respectively. Sequencing was performed by the amplification of the 16S rDNA. A colony coming from overnight growth on MA at 22°C was harvested with a solution containing 2.5 μl of 10× PCR buffer II (Gene AMP, Perkin Elmer), 2 μl of $MgCl_2$ solution, and 20.5 μl of bidistilled sterile water. The suspension was incubated at 95°C for 15 min and immediately transferred to ice-cold absolute alcohol. After centrifugation at 16,000 × *g* for 5 min, 1 μl of the supernatant was used for the amplification reaction with the universal primers 27f and 1492r (12). PCR was performed with the AmpliTaq DNA polymerase kit (Perkin Elmer) according to the instructions of the manufacturer. The PCR program was 1 cycle at 95°C for 3 min; 35 cycles at 94°C for 1 min, 55°C for 1 min, and 72°C for 2.5 min; and 1 cycle at 94°C for 1 min, 55°C for 1 min, and 72°C for 5 min. The amplified DNA was purified by using the Spin Bind PCR purification system (FMC Bioproducts). The sequencing of the variable region V3 of the amplified 16S rDNA was performed with the T7 sequencing kit (Pharmacia) with the universal primer 519r (12) 5′ labeled with fluorescein and with the Pharmacia LKB Alf-DNA sequencer, used according to the instructions of manufacturer. The sequenced fragments were analyzed and compared with the whole sequences of the 16S rRNA gene of 64 *Vibrio* strains and other gram-negative bacterial species deposited in the EMBL genomic database. Multiple alignment using the software Pileup, based on the method of Needleman and Wunsch (14), was performed, and the software Pretty Box was used to facilitate the visualization of homologies (8). An oligonucleotide 24 nucleotides in length (5′CGCTAACGTCAAATAATGCAT CTA3′) was defined. It is located between positions 467 and 490 of the sequence of the 16S rRNA gene of *Escherichia coli* (5). The oligonucleotide presents neither hairpins nor dimers of more than 4 bp. The specificity of the selected oligonucleotide (V3VPR) was evaluated initially in comparison with the sequences of the EMBL database by using the software FastA (8). Later, the oligonucleotide was synthesized and labeled at the 5′ position with digoxigenin and the specificity was evaluated by colony hybridization using the 418 strains previously described. After different stringencies (temperature and washing conditions) were tested, the hybridization was performed at 58°C under the conditions described previously (13) and a difference of 1 nucleotide among the 24 of the probe was revealed. In order to determine the efficiency and sensitivity of the medium and the probe, sets of 10-fold dilutions of a standardized mixed suspension of *V. proteolyticus* CECT 630 and *V. alginolyticus* ATCC 17749 were prepared in marine PBS at 2% NaCl. Several cell proportions of *V. alginolyticus* to *V. proteolyticus* (10:1, 10^2:1, and 10^3:1) were assayed. Aliquots of 100 μl of each suspension were inoculated in duplicate on VP8 and tryptic soy agar (ADSA, Barcelona, Spain) complemented with 1.5% NaCl (TSA2). Inoculated plates were incubated at 22°C for at least 48 h. The CFU in both media were counted, and yellow colonies (*V. proteolyticus*) were differentiated from blue colonies (*V. alginolyticus*) on VP8. Then, colony hybridization was performed on nylon membranes under the same conditions described above.

VP8 is a medium that is partially selective and differential for *V. proteolyticus*. The 13 strains of *V. proteolyticus* tested presented small, opaque, bright yellow colonies on VP8 because of the fermentation of the D-sorbitol. Other gram-negative bacteria (397 strains; mostly *Vibrio* species) either did not grow or gave blue or green-yellow colonies on VP8. The moderate alkalinity and the high salinity act as inhibitors for bacteria other than halophilic species, especially those belonging to the genus *Vibrio*. In environments colonized mostly by *Vibrio* species, a clear differentiation for strains that ferment D-sorbitol and are able to grow in high levels of salinity is observed. According to taxonomical studies on the genus *Vibrio* (1, 6, 9), a short list of species have strains which can present both metabolic characteristics (percentages in parentheses): *V. proteolyticus* (75), *V. gazogenes* (50), *V. mediterranei* (49.5), *V. aestuarianus* (25), *V. natriegens* (25), *V. metschnikovii* (24.75), *V. fluvialis* (15), a *V. anguillarum*-like species (7.5), and *V. harveyi* (3). However, for the following species we detected the indicated numbers of strains able to ferment sorbitol and grow at 8% NaCl (percentages in parentheses): *V. metschnikovii*, 1 of 7 (14.28); *V. harveyi*, 4 of 39 (10.2); *V. aestuarianus*, 1 of 16 (6.25); *V. alginolyticus*, 1 of 25 (4.0); and *V. anguillarum*, 1 of 144 (0.69). These eight strains formed bright yellow colonies on VP8 (Table 1). According to these results and attending to

the number of strains studied, lower percentages for *V. mediterranei*, *V. aestuarianus*, *V. metschnikovii*, *V. natriegens*, *V. fluvialis*, and the *V. anguillarum*-like species and higher percentages for *V. proteolyticus*, *V. harveyi*, *V. alginolyticus*, and *V. anguillarum* were found.

The colony counts of *V. proteolyticus* in the three media tested showed some differences. The total viable colony counts on MA were higher than on TCBS (8.62×10^8 and 7.0×10^8 CFU/ml, respectively), and these in turn were higher than counts on VP8 (2.21×10^8 CFU/ml). These counts are the averages of duplicate experiments performed with 10 strains of *V. proteolyticus*. No significant differences among the strains were found. The standard deviations for the logarithmic values of the counts on MA, TCBS, and VP8 were 0.13, 0.16, and 0.18, respectively. The counts reflect the stressing effect of the selective medium on the bacteria. On the other hand, though VP8 seems to be more stressing than the selective TCBS, the former allows much clearer and more consistent differentiation of *V. proteolyticus* than it does for other *Vibrio* species.

V. proteolyticus was easily differentiated and recovered from the VP8 plates inoculated with the experimental environmental samples. Only *V. proteolyticus* grew on VP8 and presented the bright yellow colonies in the 12 environmental samples tested. Yellow colonies were confirmed as *V. proteolyticus* by standard biochemical methods. The total counts on VP8 were always lower than those on MA because of the selectivity of the VP8 medium. The recovery of *V. proteolyticus* was in agreement with the ratios described previously for the different spiked environmental samples. The selectivity of the VP8 medium and the yellow coloration of the colonies of *V. proteolyticus* made its detection on VP8 easy, clear, and more consistent than it was on MA.

All 13 strains of *V. proteolyticus* hybridized with the probe V3VPR. Of the 405 strains other than *V. proteolyticus*, only 4 strains presented a positive hybridization with this oligonucleotide (three strains of *Vibrio* spp. and one strain of *V. natriegens*). However, the comparison of the sequences for the V3VPR region with sequences for different species that are on deposit in the EMBL database indicated a sufficient lack of homology to allow cross-hybridization. All the entire 16S rRNA gene sequences of those *Vibrio* species that are available in the EMBL database differ in at least one base from the 24 bp oligonucleotide sequence used to define the V3VPR probe. The sequences of the 16S rRNA gene available for *V. natriegens* in the EMBL database (sequence numbers X74714 and X56581) differ in one base (position 470) from the oligonucleotide V3VPR. Attending to the cross-hybridization we found with one strain of this species with the probe, any additional sequences of this species could be very useful in the future to determine the homology of the 16S rRNA in *V. natriegens*. There is no available information on the sequence of the other three strains presenting cross-hybridization because they do not belong to any of the recognized *Vibrio* species. Additional phenotypic and molecular characterization should be performed before these can be considered as new species for phylogenetic studies or their interest in biodiversity.

The combination of the VP8 medium and the probe V3VPR was highly specific for *V. proteolyticus*. None of the 405 strains tested other than *V. proteolyticus* had yellow colonies on VP8 and hybridized with the probe V3VPR. Only the 13 strains of *V. proteolyticus* studied were positive in both tests. Thus, none of the other strains growing on VP8 was recognized by the probe, and none of those strains recognized by the probe presented yellow colonies on VP8 (Table 1).

In the experiment with mixed cultures, the colonies of *V. proteolyticus* were also easily differentiated from *V. alginolyticus*, which produced round blue colonies without the swarming that this species usually shows in other rich media. We were able to detect 1 colony of *V. proteolyticus* among 10^3 colonies of *V. alginolyticus* when using the selective medium followed by the colony hybridization with the probe V3VPR. This level of detection is difficult to surpass because of the limitations of the visualization of separate colonies growing on the plate.

In summary, VP8 medium allows a partial selection and differentiation of *V. proteolyticus* among other vibrios and even some other gram-negative bacteria. The probe V3VPR is specific, though a limited number of strains of a few *Vibrio* species could be misidentified. The combined use of the medium and probe is highly specific for *V. proteolyticus*, allowing a clear and unequivocal identification. This combined biochemical-molecular technique for detection of bacteria could be practical for environmental and monitoring studies.

This work was supported by the research program AIR2-CT94-1601 of the European Union.

We thank L. Grisez (Catholic University of Louvain, Louvain, Belgium), who kindly provided the strains of *V. proteolyticus*.

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., and A. R. Blanch.** 1994. Improvement and update of a set of keys for biochemical identification of *Vibrio* species. J. Appl. Bacteriol. **77:**719–721.
3. **Alsina, M., J. Martínez-Picado, J. Jofre, and A. R. Blanch.** 1994. A medium for presumptive identification of *Vibrio anguillarum*. Appl. Environ. Microbiol. **60:**1681–1683.
4. **Bolinches, J., J. L. Romalde, and A. E. Toranzo.** 1988. Evaluation of selective media for isolation and enumeration of vibrios from estuarine waters. J. Microbiol. Methods **8:**151–160.
5. **Brosius, J., J. L. Palmer, J. P. Kennedy, and H. F. Naler.** 1978. Complete nucleotide sequence of a 16S ribosomal RNA gene from *Escherichia coli*. Proc. Natl. Acad. Sci. USA **75:**4801–4805.

5a. **Bryant, T. N.** 1991. Bacterial identifier. Blackwell Scientific Publications Ltd., Oxford.

6. **Bryant, T. N., J. V. Lee, P. A. West, and R. R. Colwell.** 1986. Numerical classification of species of *Vibrio* and related genera. J. Appl. Bacteriol. **61:**437–467.
7. **David, V. A., A. H. Deutch, A. Sloma, D. Pawlyk, A. Ally, and D. R. Durham.** 1992. Cloning, sequencing and expression of the gene encoding the extracellular neutral protease, vibriolysin, of *Vibrio proteolyticus.* Gene **112:**107–112.
8. **Devereux, J., P. Haeberli, and O. Smithies.** 1984. A comprehensive set of sequence analysis programs for the VAX. Nucleic Acids Res. **12:**387–395.
9. **Farmer, J. J., III, and F. W. Hickman-Brenner.** 1992. The genera *Vibrio* and *Photobacterium*, p. 2952–3011. *In* A. Balows, H. G. Trüper, M. Dworkin, W. Harder, and K.-H. Schleifer (ed.), The prokaryotes: a handbook on the biology of bacteria, 2nd ed., vol. III. Ecophysiology, isolation, identification, applications. Springer-Verlag, New York.
10. **Grisez, L. (Catholic University of Louvain, Louvain, Belgium).** 1994. Personal communication.
11. **Kourany, M.** 1983. Medium for isolation and differentiation of *Vibrio parahaemolyticus* and *Vibrio alginolyticus*. Appl. Environ. Microbiol. **45:**310–312.
12. **Lane, D. J.** 1991. 16S/23S rRNA sequencing, p. 115–175. *In* E. Stackebrant and M. Goodfellow (ed.), Nucleic acid techniques in bacterial systematics. John Wiley and Sons Ltd., Chichester, England.
13. **Martínez-Picado, J., A. R. Blanch, and J. Jofre.** 1994. Rapid detection and identification of *Vibrio anguillarum* by using a specific oligonucleotide probe complementary to 16S rRNA. Appl. Environ. Microbiol. **60:**732–737.
14. **Needleman, S. B., and C. D. Wunsch.** 1970. A general method applicable to the search for similarities in the amino acid sequence of two proteins. J. Mol. Biol. **48:**443–453.
15. **Shimada, T., R. Sakazaki, S. Fujimura, K. Niwano, M. Mishina, and K. Takizawa.** 1990. A new selective, differential, agar medium for isolation of *Vibrio cholerae* O1:PMT (polymyxin-mannose-tellurite) agar. Jpn. J. Med. Sci. Biol. **43:**37–41.
16. **Van Heeke, G., S. Denslow, J. R. Watkins, K. J. Wilson, and F. W. Wagner.** 1992. Cloning and nucleotide sequence of the *V. proteolyticus* aminopeptidase gene. Biochim. Biophys. Acta **1131:**337–340.
17. **Wright, A. C., G. A. Miceli, W. L. Landry, J. B. Christy, W. D. Watkins, and J. G. Morris.** 1993. Rapid identification of *Vibrio vulnificus* on nonselective media with an alkaline phosphatase-labeled oligonucleotide probe. Appl. Environ. Microbiol. **59:**541–546.
18. **Yoh, M., K. Miyagi, Y. Matsumoto, K. Hayashi, Y. Takarada, K. Yamamoto, and T. Honda.** 1993. Development of an enzyme-labeled oligonucleotide probe for the cholera toxin gene. J. Clin. Microbiol. **31:**1312–1314.