Development of a Simple and Rapid Fluorogenic Procedure for Identification of *Vibrionaceae* Family Members

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We describe a simple colony overlay procedure for peptidases (COPP) for the rapid fluorogenic detection and quantification of *Vibrionaceae* from seawater, shellfish, sewage, and clinical samples. The assay detects phosphoglucose isomerase with a lysyl aminopeptidase activity that is produced by *Vibrionaceae* family members. Overnight cultures are overlaid for 10 min with membranes containing a synthetic substrate, and the membranes are examined for fluorescent foci under UV illumination. Fluorescent foci were produced by all the *Vibrionaceae* tested, including *Vibrio* spp., *Aeromonas* spp., and *Plesiomonas* spp. Fluorescence was not produced by non-*Vibrionaceae* pathogens. *Vibrio cholerae* strains O1, O139, O22, and O155 were strongly positive. Seawater and oysters were assayed, and 87 of 93 (93.5%) of the positive isolates were identified biochemically as *Vibrionaceae*, principally *Vibrio vulnificus*, *Vibrio parahaemolyticus*, *Aeromonas hydrophila*, *Photobacterium damsela*, and *Shewanella putrefaciens*. None of 50 nonfluorescent isolates were *Vibrionaceae*. No *Vibrionaceae* were detected in soil, and only *A. hydrophila* was detected in sewage. The COPP technique may be particularly valuable in environmental and food-testing laboratories and for monitoring water quality in the aquaculture industry.

The *Vibrionaceae* family contains a broad group of human and animal pathogens within the genera *Vibrio*, *Aeromonas*, *Photobacterium*, and *Plesiomonas*. Two other genera have been recommended to be placed in the *Vibrionaceae* family: *Shewanella* (9) and *Listonella* (8, 9). In screening for bacterial-virulence-enhancing enzymes, we recently identified and characterized a lysyl aminopeptidase (LysAP) activity associated with phosphoglucose isomerase (PGI) of *Vibrio vulnificus* (12). Subsequently, we demonstrated that PGI with LysAP activity (PGI-LysAP) hydrolyzed the amino-terminal lysyl residue from des-Arg¹⁰-kallidin, converting it to des-Arg⁹-bradykinin (11). Kinin metabolites are known to enhance virulence and mediate inflammatory reactions (10). Kinins and their metabolites influence a host of physiological functions, including inflammation, vasodilation, vascular permeability, and the contraction and relaxation of smooth muscle (2, 14). We also identified PGI-LysAP in chromatographically purified fractions from eight species of *Vibrio* (13). Weak LysAP activity, but no isomerase activity, was detected in purified preparations of *Aeromonas hydrophila*, *Aeromonas veronii*, and *Plesiomonas shigelloides* (13). No LysAP activity could be detected in non-*Vibrionaceae* pathogens (13).

Vibrio PGI-LysAP cleaves the amino-terminal lysyl residue from the synthetic substrate L-lysyl-7-amino-4-methylcoumarin, and enzyme activity can be measured spectrophotometrically (12). To date, the detection of PGI-LysAP has required

substantial efforts to obtain and analyze the enzyme with chromatographic and spectrophotometric techniques (12). We sought to develop a rapid, simple, and inexpensive procedure to identify and enumerate members of the *Vibrionaceae* family without the need for sophisticated instrumentation. This paper reports on the development of a colony overlay procedure for peptidases (COPP) which may be used to identify and quantify a broad range of *Vibrionaceae* in food, environmental, and clinical samples based on the rapid and simple detection of PGI-LysAP.

MATERIALS AND METHODS

Bacterial strains and culturing. Sources for the bacterial cultures used in this study are as previously described (13), except for the *Vibrio cholerae* strains, which are listed in Table 1, and *Proteus vulgaris* and *Pseudomonas aeruginosa*, which were cultures 13315 and 10145, respectively, from the American Type Culture Collection, Manassas, VA. Non-*Vibrio* pathogens (*Bacillus cereus*, *Enterobacter aerogenes*, *Escherichia coli* O157:H7, *Klebsiella pneumoniae*, *Listeria monocytogenes*, *Proteus mirabilis*, *P. aeruginosa*, *Salmonella enterica* serovar Typhimurium, *S. enterica* serovar Enteritidis, *Staphylococcus aureus*, and *Yersinia enterocolitica*) were streaked onto plates of tryptic soy agar (TSA; Difco Laboratories, Detroit, MI). *Vibrionaceae*, including eight *Vibrio* spp., two *Aeromonas* spp., and *Plesiomonas shigelloides*, were streaked onto TSA containing 1% NaCl (TSA-N). All isolates were incubated at 37°C for 16 to 18 h.

Preparation of membranes containing synthetic substrate. A stock solution of 20 mM L-lysyl-7-amino-4-trifluoromethylcoumarin (L-Lys-AFC) (catalog no. AFC-08; Enzyme Systems Products, Livermore, CA) was prepared in dimethyl sulfoxide and stored at –20°C. Cellulose acetate membranes (8 by 15 cm) (catalog no. 12200-78-150-K; Sartorius, Goettingen, Germany) were quickly (1 to 2 s) submerged, one at a time, into a solution containing 125 µl of L-Lys-AFC stock per 10 ml of distilled water, soaked for about 30 s, removed, and dried. Each membrane absorbed ~2 ml of the solution. Small metal binder clips taped to the lower side of an overhead cabinet were convenient for hanging the membranes to dry. Once thoroughly dried, the membranes were placed in an opaque envelope and stored at room temperature. The shelf life of the prepared membranes was determined using the COPP procedure, as described below, by

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TABLE 1. *Vibrio cholerae* strains used in this study

Strain	Source ^a	Biotype ^b	Serotype
<i>V. cholerae</i> INDRE-1	INDRE	NA	O139
<i>V. cholerae</i> INDRE-2	INDRE	NA	O139
<i>V. cholerae</i> O1-E	USDA	El Tor, Ogawa	O1
<i>V. cholerae</i> O1-R	CDC	Classical, Ogawa	O1
<i>V. cholerae</i> O139-CDC	CDC	NA	O139
<i>V. cholerae</i> O22-CDC	CDC	NA	O22
<i>V. cholerae</i> O155-CDC	CDC	NA	O155
<i>V. cholerae</i> O139-CA SHD	ICDDR-B	NA	O139
<i>V. cholerae</i> O139-F646	ICDDR-B	NA	O139

^a INDRE, Instituto de Nacional Diagnóstico y Referencia y Epidemiológicos, Mexico City, Mexico; USDA, United States Department of Agriculture, Wyndmoor, PA; CDC, Centers for Disease Control and Prevention, Atlanta, GA; ICDDR-B, International Centre for Diarrhoeal Disease Research—Bangladesh, Dhaka, Bangladesh.

^b NA, not applicable.

overlying colonies of *V. vulnificus* MLT364 with membranes that had been prepared monthly for 6 months and then comparing the fluorescence intensities.

Colony overlay procedure for peptidases. TSA and TSA-N plates were streaked or spread plated with the appropriate bacteria and incubated overnight at 37°C. Previously prepared membranes (≤1 month old) containing L-Lys-AFC were cut to a size appropriate for the area to be overlaid, labeled accordingly, and prewet for 5 s in 20 mM Tris-HCl, pH 9.5. While handling with forceps, excess buffer was dripped from the membranes for 3 to 5 s, and the membranes were carefully placed onto one or more colonies on the agar plates. Care was exercised to prevent bubbles from becoming trapped under the wet membranes. Plates were incubated for 10 min at 37°C without inversion. Each membrane was then removed with forceps and placed on a petri dish, colony side up. The dish was placed on a UV light box, and the membrane was viewed at 364 nm for fluorescent foci at the point of contact between the bacterial colony and the membrane. Membranes were photographed through a deep yellow filter no. 15 (Tiffen Manufacturing Corp., Hauppauge, NY) with a Polaroid camera (Polaroid Corp., Cambridge, MA) on a UV light box with Polaroid 667 film at an F stop setting of 4.5 and exposure for 1/8 to 1/30 s for a permanent record of the results. Results could also be recorded with a digital camera.

Dot blots. For the comparison of fluorescence intensities among the *Vibrionaceae* and the non-*Vibrio* pathogens, templates were made with graph paper by marking a grid to represent columns and rows. The template was taped to the outside bottom of the TSA and TSA-N plates. Isolated colonies were stabbed on the plates according to markings on the template. Each plate was incubated overnight and subjected to the COPP.

Mixed cultures. Sandy loam, raw sewage, oyster homogenates, and seawater were also subjected to the COPP assay. Sandy loam from a local (Dover, Delaware) flower garden and raw sewage from a municipal sewage treatment plant in Harrington, DE, were tested to determine the prevalence of fluorescence from organisms likely to be nonvibrios. The soil was diluted to a ratio of 1:5 with phosphate-buffered saline, vortexed for 15 s, and centrifuged for 5 min at 200 × g. Supernatant and 10-fold dilutions in phosphate-buffered saline were spread onto TSA plates (100 µl/plate), incubated overnight, and overlaid as described above. Oysters obtained from state-approved shellfish harvesting beds in New Jersey were collected and immediately transported to the laboratory, shucked under aseptic conditions, diluted to a ratio of 1:10 with 0.1% peptone, homogenized, and serially diluted in 0.1% peptone, and 100 µl of each dilution was spread plated onto TSA-N. One hundred microliters of seawater and 10-fold dilutions of seawater were also spread plated onto TSA-N. All plates were incubated overnight at 37°C and overlaid according to the COPP technique.

Bacterial identification. Representative bacterial colonies from shellfish, seawater, and sewage were identified biochemically using API 20E and oxidase tests (BioMerieux Industries, Hazelwood, MO), according to the manufacturer's instructions, to determine the relative percentages of fluorescent and nonfluorescent isolates that were in the *Vibrionaceae* family. Colonies on the primary plates were dotted onto duplicate TSA-N plates and incubated overnight. The COPP assay was performed on one set of plates, and colonies producing fluorescent foci were picked from the duplicate plate for API 20E identification. Likewise, nonfluorescent colonies were picked from the duplicate plate and Gram stained, and gram-negative isolates were identified using API 20E. A total of 170 colonies were picked for identification from oysters and seawater cultures. For sewage, all eight isolates producing fluorescent foci were subjected to API 20E identification.

FIG. 1. Colony overlay procedure for peptidases showing PGI-LysAP activity in *Vibrionaceae* and non-*Vibrio* pathogens after bacterial isolates were stabbed onto a TSA-N plate and incubating for 24 h. Overlay was for 10 min, and the membrane was photographed while wet on a UV light box at an F stop setting of 4.5 for 1/30 s. Bacteria are as follows (left to right): (A) *V. vulnificus* strain MLT367, *V. vulnificus* strain MLT1003, *V. cholerae* O1, *V. cholerae* O139, *V. parahaemolyticus* (Kanagawa positive), *V. parahaemolyticus* (Kanagawa negative), *Vibrio fluvialis*; (B) *Vibrio hollisae*, *Vibrio metschnikovii*, *Vibrio mimicus*, *Aeromonas hydrophila*, *Aeromonas veronii*, *Plesiomonas shigelloides*, *E. coli* O157:H7; (C) *Enterobacter aerogenes*, *Salmonella enterica* serovar Typhimurium, *Salmonella enterica* serovar Enteritidis, *Yersinia enterocolitica*, *Staphylococcus aureus*, and *Listeria monocytogenes*. The last space in row C is blank.

RESULTS AND DISCUSSION

The COPP procedure successfully discriminated between the *Vibrionaceae* and the non-*Vibrionaceae*. Pure cultures of clinical and environmental strains of the *Vibrionaceae* family dotted onto TSA-N plates all produced strong fluorescent foci on the membrane overlays (Fig. 1). Nine strains of *V. cholerae*, including serogroups O1, O139, O22, and O155 (Table 1), were also strongly positive. Fluorescent foci were not observed for the non-*Vibrionaceae* pathogens, except for a very weak and diffuse fluorescence occasionally associated with *Enterobacter aerogenes* and *E. coli* cultures that had been dotted onto plates (Fig. 1) and with *K. pneumoniae* and *P. vulgaris* cultures which were overlaid directly from a streaked culture (not shown). The diffuse fluorescence was easily discounted and was attributed to other lysyl aminopeptidases present in cell lysates, since activity was not present in chromatographic fractions from these species (13). The overlay of *V. vulnificus* with membranes that were prepared 0 to 6 months previously showed that membranes could be stored up to 2 months with only a slight loss of fluorescence intensity. Older membranes did not produce fluorescence.

The COPP assay was performed on oyster homogenates, seawater, sandy loam, and raw sewage. An overnight culture of diluted oyster homogenate on TSA-N (Fig. 2A) and the corresponding membrane overlay (Fig. 2B) show strongly fluorescent foci (Fig. 2B) associated with nearly all of the bacteria isolated from the oysters. Of 170 natural isolates from seawater and oyster cultures that were picked for biochemical identification over a 2-month period, 120 produced fluorescent foci. Only 93 of these could be identified by API 20E, and 87 (93.5%) were identified as *Vibrionaceae*, including *Shewanella*, whereas 6 (6.5%) were identified as *Chryseobacterium* (*Flavobacterium*) *meningosepticum*, a bacterium which is not presently classified as a *Vibrionaceae* family member but which causes cellulitis and tissue damage (1, 3), much like other *Vibrionaceae* (4, 5, 7, 15). The most prevalent *Vibrionaceae* family member detected was *V. vulnificus*, followed by *Shewanella putrefaciens*, *Aeromonas* spp., *Photobacterium dam-*

FIG. 2. TSA-N plate of oyster homogenate (A) and TSA plate of sandy loam supernatant (C) after incubation for 24 h. Plates were overlaid with membranes containing L-Lys-AFC for 10 min, and the resulting membranes were photographed (B and D) while wet on a UV light box at an F stop setting of 4.5 for 1/8 s. Panel C shows three fungal colonies from the TSA plate (arrows) that produced the fluorescent foci seen in panel D.

sela, and *V. parahaemolyticus*. A lack of discrimination in some assays allowed a portion of the isolates to be identified only to the genus level. Among these, the API 20E listed several isolates as *Vibrio* spp. or *Aeromonas* spp. Only 13 of 50 nonfluorescent isolates could be identified by API 20E, and none were *Vibrionaceae*. They included *Shigella* spp., *Pasteurella multocida*, *Morganella morganii*, *Pseudomonas* spp., and *Stenotrophomonas maltophilia*. Some of the isolates were gram positive and were not identified. The inability to identify some of the isolates by API 20E is commonly experienced for environmental samples, since API 20E was primarily designed for detecting human pathogens in clinical specimens. Bacterial colonies from garden soil (Fig. 2C) did not produce fluorescence when overlaid; however, three fungal colonies (Fig. 2C) produced strong fluorescence (Fig. 2D). Approximately 15% of the isolates obtained from raw municipal sewage produced fluorescent foci and were identified as *A. hydrophila* isolates. No other colonies producing fluorescent foci were detected in sewage. The quantification of *Vibrionaceae* in shellfish and seawater was accomplished with the COPP assay by the overlay of primary spread plates prepared with known amounts of sample. Obviously, quantification is not possible from the overlay of picked or streaked cultures.

Several factors are critical to the success of the COPP technique. Cultures grown on TSA-N should be ≤ 24 h old and the cell density should be light to moderate, since older or overgrown cultures may acidify the medium, thus preventing PGI-LysAP from cleaving its substrate. Previously, we showed that the optimal pH for PGI-LysAP activity in *V. vulnificus* was 8.0,

with a secondary peak activity at pH 9.5 (12). We also demonstrated that at pH 7.0, enzyme activity was only 20% of the activity obtained at pH 8.0 (12). If the COPP assay is modified to detect other peptidases using L-Lys-AFC or another fluorogenic substrate, it would be necessary to determine the optimal pH for the hydrolysis of that substrate. Membranes should be prewet in buffer approximating the optimal pH for the specific enzyme activity being sought.

The duration of the overlay should be carefully controlled. We chose a 10-min overlay period, which was sufficient to give clearly positive results for the *Vibrionaceae* and essentially negative results for the remaining bacteria. Longer overlays would allow other enzymes present in the bacteria to slowly cleave the substrate, giving various degrees of fluorescence. Once the substrate is cleaved, the fluorescence is nonreversible, leading to a permanent spot on the membrane. Any changes in the time course for the overlay must be determined empirically and should depend on the specificity of the reaction, the quantity of active enzyme, the presence of competing enzymes, the medium used, the pH of the culture, the pH optimum for the enzyme being sought, and the density and age of the culture.

The cellulose acetate membranes employed for the overlays can be readily adapted for use as thin strips of membrane, which can be placed on one or more bacterial colonies for 10 min to quickly detect the presence of a broad array of enzymes, depending on the substrate selected for use. Since the technology is inexpensive and does not require the use of any sophisticated instrumentation, the membranes and the COPP technique could become routinely used in many laboratories, especially if membrane strips were to be commercially marketed. The stability of the membranes at room temperature for up to 2 months provides an added advantage to this method. It should be noted, however, that cellulose acetate membranes must be used in this procedure, because they contain hydrophobic pockets to specifically bind the electronegative fluorine atoms of the amino-trifluoromethylcoumarin (6), thus allowing localization of both the reaction and any subsequently produced fluorescent product.

The only instrument required to perform the COPP assay is a long-wave UV light box capable of irradiating the membranes at 364 nm. Alternatively, an inexpensive "black light" may be used to view fluorescence. There is no need for sample preparation, and the materials used to screen for enzyme activity are inexpensive. Labor costs are substantially reduced by the simplicity and speed of the assay. The entire overlay procedure takes ≤ 20 min from overlay to viewing for fluorescence. Another advantage is the ability to test about a hundred colonies with the overlay of a single petri dish, thus allowing the screening for and enumeration of *Vibrionaceae* on a large scale.

Potential applications for this new technology include the use of COPP as follows: (i) to quantify the levels of *Vibrionaceae* present in food, water, and environmental samples as a potential mechanism to regulate vibrios or to gain a better understanding of their presence, seasonal distribution, and ecology; (ii) to rapidly screen food, water, and environmental samples for *Vibrionaceae* based on the simple presence or absence of fluorescence; (iii) to rapidly screen for *Vibrionaceae* in cultures from tissue, blood, and pus to provide preliminary evidence of *Vibrionaceae* presence or absence; and (iv) to identify the presence of any number of hydrolytic enzymes in bac-

teria after overlaying cultures with membranes containing various substrates. By selecting other fluorogenic substrates, the COPP assay would have expanded use for the detection of a host of bacterial, fungal, and yeast enzymes involved in proteolysis, housekeeping functions, and virulence. Since fish are highly susceptible to infection by *Vibrionaceae*, the COPP technique may be particularly valuable for monitoring water quality in the aquaculture industry.

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