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A Rapid Test for Chitinase Activity That Uses 4-Methylumbelliferyl-*N*-Acetyl-β-D-Glucosaminide

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A total of 101 strains of bacteria from environmental and clinical sources, most of which were gram negative, were tested for chitobiase activity by using a filter paper spot test with 4-methylumbelliferyl-*N*-acetyl-β-D-glucosaminide as the substrate. The results were compared with those obtained by a conventional plate method for chitinase activity by using colloidal chitin as the substrate. There was excellent agreement in the results for both methods. The filter paper spot test with 4-methylumbelliferyl-*N*-acetyl-β-D-glucosaminide has the advantages of being rapid, simple to perform, and inexpensive. This method should be adaptable to a wider range of microorganisms, particularly those with unusual growth requirements.

Except for cellulose, chitin is probably the most abundant polysaccharide in nature, being a primary constituent of shells in crustaceans and insects (9). Centrate diatoms produce significant amounts of crystalline chitin (3; R. Smucker and R. Dawson, J. Exp. Mar. Biol. Ecol., in press). Chitin also occurs in some protozoa and fungi (16, 23). Chitinase activity has been found in a wide variety of microorganisms (2, 5, 7, 12–15, 19, 22, 23). Chitinase activity plays an important role in the ecology of many marine bacteria (23). For some pathogenic bacteria, such as *Vibrio cholerae*, interactions between the organism and chitinaceous materials have been postulated as playing a role, albeit indirectly, in the epidemiology of disease (6, 10).

Tests for bacterial chitinase activity have relied on conventional plate methods, such as those described by Skerman (17) and West and Colwell (19) in which colloidal chitin is incorporated into a nutrient agar base optimal for the growth of the test organism. Chitinase activity is indicated by zones of clearing around the inoculum growth. While the test itself is straightforward, preparation of the substrate medium is laborious and complicated. Furthermore, the plate test is an insensitive assay for chitinase activity; e.g., some organisms require a long incubation period (up to 7 days or longer). Problems may also be encountered with organisms which have fastidious or unusual growth requirements. Thus, it is understandable why many laboratories are reluctant to include chitinase activity as part of an enzyme profile.

A major end product of chitin degradation by chitinase (or endochitinase) is chitobiose, which is further degraded by the enzyme chitobiase (7, 8, 13). The test described here, which uses 4-methylumbelliferyl-*N*-acetyl-β-D-glucosaminide (4-MUF.GlcNAc), is an assay for chitobiase activity, the inference being that chitobiase activity is produced concomitantly with chitinase activity. The following study was done to find a better method for determining chitinase activity. The test described is an assay for *N*-acetyl-β-D-glucosaminidase activity, the logic being that chitin is essentially a biopolymer of GlcNAc subunits (14, 20, 22).

The strains used in this study are listed in Table 1. No attempt was made to confirm the identity of all of the strains tested. However, whenever possible, American Type Culture Collection type or reference strains or well-characterized isolates received from other laboratories were examined. Many of the strains tested in this study have been used in other studies (11), and they include both environmental and clinical isolates. For the chitinase plate test and culture media, nutrient agar was prepared as described by West and Colwell (19), and brain heart infusion agar and tryptic soy agar were prepared by adding bacteriological agar (Difco Laboratories, Detroit, Mich.) (final concentration, 1.5% [wt/vol]) to brain heart infusion broth (Difco) and tryptic soy broth (Difco), respectively.

The rapid test method was as follows. The 4-MUF.GlcNAc (Sigma Chemical Co., St. Louis, Mo.) stock substrate solution was prepared by dissolving 50 μmol in 2.0 ml of dimethylformamide (Sigma). For the test, 0.6 ml of this solution was diluted in 9.4 ml of phosphate buffer (0.1 M [pH 7.4]) (4). The substrate solutions (stock and buffered) were used immediately, without sterilization, or stored at −20°C. Both solutions were stable for several months at this temperature.

One to five colonies from cultures grown from 24 to 48 h on nutrient agar (*Vibrio* spp.), brain heart infusion agar (*Enterococcus* [*Streptococcus*] *faecalis* and *Enterococcus* [*Streptococcus*] *faecium*), or tryptic soy agar plates were vigorously rubbed onto Whatman no. 1 filter paper, and 20 μl of the 4-MUF.GlcNAc-buffered substrate solution was added. The substrate only and the organism plus the solvent were included as controls.

After incubation at 37°C (or 25°C for *Vibrio* strains tested on nutrient agar) for 10 min, each test spot was covered with 1 drop of a saturated sodium bicarbonate solution and exposed to UV light at a wavelength of 366 nm. The sodium bicarbonate solution enhances the fluorescence intensity of the reaction by-product, methylumbelliferone (18).

Depending on the fluorescence intensity, reactions were graded as positive, weakly positive, or negative. In positive reactions, substrate-organism mixtures produced a strong, light-blue fluorescence.

The conventional plate method for determining chitinase activity was a modification of the method of West and Colwell (19) in that tryptic soy agar and brain heart infusion agar were used as alternative preprepared medium bases in addition to nurient agar and a 20% (wt/vol) concentration of colloidal chitin suspension was used. West and Colwell (19) suggest a 15% (wt/vol) concentration. Briefly, a colloidal chitin suspension (20% [wt/vol]) was prepared and added to

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TABLE 1. Results of chitobiase and chitinase assays with 101 bacterial strains

Gram reaction/strain	No. tested	No. (%) positive[a]	
		Chitobiase	Chitinase
Gram positive			
Bacillus subtilis	1	1 (100)	0 (0)
Micrococcus luteus	1	0 (0)	0 (0)
Staphylococcus aureus	1	0 (0)	0 (0)
Enterococcus (Streptococcus) faecalis	1	1 (100)	1 (100)
Enterococcus (Streptococcus) faecium	2	2 (100)	0 (0)
Gram negative			
Aeromonas hydrophila	6	6 (100)	6 (100)
Aeromonas caviae	6	6 (100)	6 (100)
Aeromonas sobria	5	5 (100)	5 (100)
Agrobacterium tumefaciens	1	1 (100)	0 (0)
Enterobacter aerogenes	1	0 (0)	0 (0)
Escherichia coli	7	1 (14)	0 (0)
Klebsiella pneumoniae	1	0 (0)	0 (0)
Plesiomonas shigelloides	10	10 (100)	10 (100)
Proteus vulgaris	1	0 (0)	0 (0)
Pseudomonas aeruginosa	1	0 (0)	0 (0)
Pseudomonas putida	2	0 (0)	0 (0)
Salmonella typhi	7	0 (0)	0 (0)
Shigella dysenteriae 1	4	0 (0)	0 (0)
Shigella sonnei	1	0 (0)	0 (0)
Shigella boydii	1	0 (0)	0 (0)
Vibrio alginolyticus	2	2 (100)	2 (100)
Vibrio campbelli	1	1 (100)	1 (100)
Vibrio carcharae	1	1 (100)	1 (100)
Vibrio cholerae	10	10 (100)	10 (100)
Vibrio cincinnatiensis	1	1 (100)	1 (100)
Vibrio damsela	2	2 (100)	2 (100)
Vibrio diazotrophicus	1	1 (100)	0 (0)
Vibrio fluvialis	3	3 (100)	3 (100)
Vibrio gazogenes	1	0 (0)	0 (0)
Vibrio metschnikovii	1	1 (100)	1 (100)
Vibrio mimicus	2	2 (100)	2 (100)
Vibrio natriegens	1	1 (100)	0 (0)
Vibrio nereis	1	1 (100)	1 (100)
Vibrio nigrapulchritudo	1	1 (100)	1 (100)
Vibrio ordalii	1	0 (0)	0 (0)
Vibrio parahaemolyticus	6	6 (100)	6 (100)
Vibrio pelagius	1	1 (100)	1 (100)
Vibrio proteolyticus	1	1 (100)	1 (100)
Vibrio tubiashii	1	1 (100)	1 (100)
Vibrio vulnificus	3	3 (100)	3 (100)

[a] Includes weakly positive reactions. For chitobiase, the rapid filter paper spot test with 4MUF.GlcNAc as the substrate was used. For chitinase activity, a conventional plate assay with colloidal chitin as the substrate was used.

molten bacteriological-grade agar to a final concentration of 1% (vol/vol) and poured as an overlay on a prepared medium base. Thus, the *Vibrio* strains were tested on chitin-nutrient agar, *E. faecalis* and *E. faecium* were tested on chitin-brain heart infusion agar, and chitin-tryptic soy agar was used for all other test organisms. Plates were spot inoculated (four spots per plate) with growth (one to five colonies) from a plate culture of the test organism grown on its optimal medium (without chitin) for 24 to 48 h, as indicated above. Except for the *Vibrio* test strains, which were incubated at 25°C, the chitin plate cultures were incubated at 37°C for at least 7 days to observe clearing zones around growth, indicating chitin hydrolysis. A narrow (1 to 2 mm in width) clearing zone extending from the periphery of the spot growth was considered a positive reaction.

Many of the *Vibrio* test strains were able to grow on tryptic soy agar; therefore, in a separate series of experiments, we tested for chitobiase and chitinase activities on this medium and at 37°C by using the methods described above.

The results for chitobiase (rapid test) and chitinase (conventional plate method) activities are given in Table 1. Overall, there was excellent agreement in the results for both methods. Furthermore, the results obtained here for chitinase activity by the conventional plate method are in very good agreement with the results reported by other workers (2, 7, 19). Certain test organisms, e.g., *Agrobacterium tumefaciens*, *Bacillus subtilis*, *E. faecium*, *Vibrio diazotrophicus* and *Vibrio natriegens*, showed chitobiase activity but no chitinase activity by the conventional plate method. This was most likely a result of the latter being a less sensitive method. Enzyme assays that use 4-methylumbelliferyl-conjugated substrates are notably more sensitive than conventional enzyme test methods (M. O'Brien, Ph.D. thesis, University of Queensland, St. Lucia, Australia, 1985). We never observed chitinase activity in the conventional plate method without observing concomitant chitobiase activity in the rapid test.

The degree of activity for chitobiase and chitinase was not necessarily directly correlated. For example, while many of the strains tested positively with the rapid test, yielding strong reactions within 10 min, corresponding tests with the conventional plate method produced varied results. For some strains, there was strong chitobiase activity and weak chitinase activity; for others, although there was strong chitobiase activity, positive plate tests required incubation for 4 days or longer. Some test organisms, e.g., *Aeromonas* spp., *Vibrio damsela*, *Vibrio nigrapulchritudo*, showed very strong *N*-acetyl-β-D-glucosaminidase activity before the addition of the bicarbonate solution.

Results of tests for enzyme activities in *Escherichia coli* proved interesting, since one of the two strains tested was the recipient of plasmid DNA from chitobiase-positive clones but was otherwise identical to the first strain (21). The chitobiase gene was isolated originally from *Vibrio vulnificus* by cloning chromosomal DNA (21). Very strong chitobiase activity was detected in the transformed strain. The plasmidless strain, as well as five other *E. coli* strains examined, was negative for chitobiase and chitinase activity by both test methods.

Comparable results were obtained with *Vibrio* strains tested for chitobiase activity on tryptic soy agar and for chitinase activity on chitin-tryptic soy agar at 37°C. Some of the strains did show stronger reactivities with both assays, probably more a reflection of the higher incubation temperature than of media differences.

The test for chitobiase activity described in this study has many advantages over the conventional plate method for chitinase activity. It is rapid and simple to perform and uses a substrate that is relatively inexpensive and very stable. The test is specific and sensitive.

Different incubation temperatures may be used for the rapid test, and a variety of growth media may be used as the source of the inoculum. Chitinase is produced in chitinase-positive bacteria even when they are grown in a rich organic medium, since only chitin or glucosamine has been shown to induce chitin hydrolysis (1). Furthermore, the rapid test may be more adaptable to microorganisms whose unusual growth requirements do not allow for determination of chitinase activity by the conventional plate method. For example, we are at present carrying out enzyme profiles of barophilic

bacteria by using chitobiase and other 4-methylumbelliferyl-conjugated substrates, since conventional enzyme assay methods are largely inappropriate for this group of bacteria (W. L. Straube and M. O'Brien, Abstr. Annu. Meet. Am. Soc. Microbiol. 1986, I-37, p. 171). Thus, many useful applications of the test described here can be seen.

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