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Distribution of Chitinase and Chitobiase in Bacillus

R.M. Cody

Department of Botany and Microbiology, Auburn University Agriculture Experiment Station, Auburn, Alabama, USA

Abstract. Sixty strains representing 29 taxospecies of *Bacillus* were assayed for their ability to hydrolyze colloidal chitin. A qualitative estimation of chitinolysis was made from the clear zone produced around colonies in the conventional agar plate method and chitobiase activity by use of the fluorescence of 4-methylumbelliferyl-N-acetyl-β-D-glucosaminide.

Strains positive in the chitin–agar plate method were assayed for production of reducing sugar in liquid culture. Seventeen of 52 strains representing 10 species of *Bacillus* were chitinolytic. The most chitinolytic species of *Bacillus* were: *B. chitinosporus*, *B. pulvifaciens*, *B. alvei*, *B. Macerans*, and *B. licheniformis*. Seventy-eight percent of *Bacillus* isolates from chitin-enriched soil (AU Y91B1, AU-X (unidentified), and AU B_2–B_8) were chitinolytic. Twenty-three strains representing 15 species gave a positive test for chitobiase. Many strains negative for endochitinase gave a strong positive reaction (4+) for chitobiase.

Chitin is the second most abundant polysaccharide in nature. It has been estimated that several million tons of chitin come from planktonic crustacea alone [1]. Other major sources of chitin are structural components of other marine forms, fungi, protozoa, nematodes, and insects [3, 8, 14, 23, 27, 28, 30]. Chitin consists of a polymer of β-1,4-linked N-acetylglucosamine (NAG). It is highly resistant to organic solvents and requires strong mineral acids for solubilization [6]. Microbial conversion of chitin to NAG requires the sequential action of two hydrolytic enzymes: (i) chitinase (chitin glycanohydrolase) EC 3.2.2.14 and (ii) chitobiase (acetylaminodexoglucohydrolase) EC 3.2.1.29 [7, 14, 31]. These enzymes have been demonstrated in a wide variety of bacterial and other microbial taxa [1, 2, 4–6, 10, 12, 13, 17, 20, 23–26, 28–30]. Some bacterial species produce both enzymes (chitinoclastic) [32], while others exhibit activity of one or the other enzymes [16].

The best known chitinolytic bacteria are species of *Aeromonas*, *Bacillus*, *Chromobacterium*, *Myxobacter*, *Serratia*, *Vibrio*, and *Streptomyces* (actinomycetes) [2, 6, 12, 13, 17, 18, 20, 30]. *Bacillus* is an industrially important genus capable of producing many different hydrolytic enzymes including cellulase, chitinase, collagenase, protease, as well as a number of antibiotics, amino acids, peptides, and proteins. Unlike its other hydrolytic enzymes, *Bacillus* chitinase has not been studied extensively. The purpose of this study was to determine the distribution of endochitinase and chitobiase in *Bacillus*.

Materials and Methods

Bacterial strains and growth conditions. *Bacillus* strains used are listed in Table 1. Cultures with NRRL designation were obtained from L.K. Nakamura (Northern Regional Research Center, Peoria, Illinois), whereas strains with ATCC numbers were purchased from the American type Culture Collection (Rockville, Maryland). All others were from the Auburn University Culture Collection (AU).

All strains of *Bacillus*, except those with special requirements, were routinely grown and maintained on Tryptone Glucose Extract Agar (TGE) and Bushnell Haas-Chitin Agar (BH-CHA). The BH-CHA contained (μg/l): Bacto Bushnell Haas Agar base (Difco), 3.25; glucose, 1.0; yeast extract, 0.5; and either 0.2 or 1.0 (wt/vol) swollen chitin [13]. Swollen chitin was prepared according to the method of Godoy et al. [8]. *Bacillus larvae* and *B. popilliae* were grown and maintained on J-Agar [9]. Stock cultures were transferred biweekly and maintained at 4°C. *Bacillus coagulans*, *B. circulans*, and *B. polymyxa* were grown at 37°C, *B. stearothermophilus* at 62°C, *B. psychrosaccharolyticus* at 8°C; and all others at 30°C.

Preparation of inocula. The inocula were prepared from cultures grown on BH-CHA plates. Cells were harvested by washing the surface with 10 ml of 0.1 *M* Tris buffer (pH 6.8), packed by centrifugation, and washed a total of three times. The final cell concentration was adjusted to 10^8 cells/ml with Tris buffer. Cells were used either immediately or stored at 4°C.

Address reprint requests to: Dr. R.M. Cody, Department of Botany and Microbiology, Auburn University Agriculture Experiment Station, Auburn, AL 36830, USA.

Table 1. Distribution of chitinase and chitobiase in *Bacillus*

Strain	Source of designation of culture	Chitinase reaction	
		Endochitinase (clear zone–colony size)[a]	Chitobiase (relative fluorescent intensity)
B. acidocaldarius	NRRL-B 1607	0	0
B. alvei	ATCC 6344	4	2
B. alvei	NRRL-B 383[b]	4	2
B. azotoformans	NRRL-B 14310	0	3
B. apiarius	NRRL-B 1438	3	2
B. brevis	ATCC 8264	2	2
B. brevis	NRRL-B 604	1	2
B. cereus	ATCC 14579	0	0
B. cereus	AU-J 617	0	0
B. cereus	AU-J 21	0	0
B. mycoides (*B. cereus* subsp. mycoides)[c]	ATCC 19647	0	0
B. mycoides (*B. cereus* subsp. mycoides)	NRRL-B 4379	0	0
B. mycoides (*B. cereus* subsp. mycoides)	AU J-19	0	0
B. chitinosporus	ATCC 19986[d]	4	3
B. circulans	ATCC 4513	0	2
B. circulans	NRRL-B 378	0	2
B. coagulans	NRRL-NRS 609	0	1
B. coagulans	ATCC 7050	0	3
B. firmus	ATCC 14575	0	0
B. firmus	NRRL-B 14307	0	0
B. insolitus	NRRL-B 3395	0	0
B. larvae	NRRL-B 2605	0	0
B. laterosporus	ATCC 64	1	0
B. laterosporus	NRRL-NRS 314	1	0
B. lentus	NRRL-B 369	4	4
B. licheniformis	ATCC 14580	3	4
B. licheniformis	NRRL-NRS 1264	3	4
B. macerans	NRRL-B 4267	2	4
B. megaterium	ATCC 14581	1	4
B. megaterium	NRRL-B 14308	0	4
B. pantothenticus	NRRL-NRS 1321	0	4
B. pasteurii	ATCC 11859	0	0
B. polymyxa	ATCC 842	0	0
B. popilliae	NRRL-B 2309	IG[g]	IG
B. pulvifaciens	ATCC 13537[e]	4	4
B. pumilus	NRRL-NRS 272	0	2
B. psychrosaccharolyticus	ATCC 23296	0	0
B. sphaericus	ATCC 14577	0	0
B. sphaericus	NRRL-NRS 967	0	0
B. stearothermophilus	ATCC 12016	0	0
B. stearothermophilus	NRRL-B 1172	0	0
B. subtilis	ATCC 23858	0	0
B. subtilis	ATCC 15134	0	0
B. subtilis	AU J-226	0	0
B. subtilis	AU J-227	0	0
B. subtilis	AU H-17	0	0
B. subtilis	AU M-45	0	0
B. subtilis (*B. uniflagellatus*)	ATCC 15134	0	0
B. thuringiensis	ATCC 10792	1	1
B. thuringiensis	NRRL HD-1	1	3

Table 1. Continued

Strain	Source of designation of culture	Chitinase reaction	
		Endochitinase (clear zone–colony size)[a]	Chitobiase (relative fluorescent intensity)
B. thuringiensis (*B. thuringiensis* subsp. israelensis)	BT-1[f]	2	2
Bacillus species (soil isolates):			
	AU Y91B1	4	4
	AU B-2	4	3
	AU B-3	4	4
	AU B-4	4	1
	AU B-5	4	2
	AU B-6	3	2
	AU B-7	0	0
	AU B-8	0	0
	AU X species (unidentified)	3	3

[a] Chitinase reaction: 0 = negative and 1–4 positive (1 = weakest, 4 = strongest).
[b] Same as ATCC 6344.
[c] Correct designation, *B. mycoides* [22].
[d] Not included in 1980 Approved List of Names [21].
[e] *B. pulvifaciens* excluded from the Approved List of Names; revival of *Bacillus pulvifaciens* sp. nov., nom. rev. proposed [15].
[f] Trade name, Bactimos.
[g] Insufficient growth.

Chitinase determination. Initially all strains of *Bacillus* listed (Table 1) were screened for hydrolysis of colloidal chitin by the conventional plate method [12, 16]. An actively growing culture of each strain was inoculated on BH-CHA or supplemented BH-CHA plates and incubated at optimal growth temperature. Some of the strains required special growth conditions. *Bacillus larvae* and *B. popilliae* were grown on J-medium with chitin instead of BH-CHA. Other changes made to optimize growth of certain strains were: (i) adjusting the pH of BCH-CHA to 2.0 and 5.0 for *B. acidocaldarius*; (ii) addition of 4% NaCl for *B. pantothenticus*; and (iii) addition of 1% urea (Sigma) to *B. pasteurii*. Strains producing a clear zone around the colony within 8 days were scored positive for chitin hydrolysis [12]. Cultures negative after 8 days were held for an additional 22 days before being discarded as negative for chitinase.

Bacillus strains positive for chitinolysis in the conventional agar plate procedure were assayed qualitatively by measuring the clear zone–colony size ratio. From this value, a comparison of chitin hydrolysis of *Bacillus* strains on an agar medium was determined. This was accomplished by transferring 25 μl of actively growing culture (1.5×10^8 cells/ml) to the center of a BH-CHA plate. Chitinolysis and the time required for clearing to occur was determined at 2-day intervals for a total of 8 days.

Hydrolysis of chitin in broth culture was followed by measuring the reducing sugar concentration in filtrates of Bushnell Haas broth (BH-CHB) [11]. BH-CHB medium was inoculated with a 10% inoculum from a 24-h actively growing culture of each chitinolytic strains. Cultures were incubated at their optimal temperature in a water-bath shaker (Model 406015, American Optical, Buffalo, NY), operated at 126 strokes/min. Immediately after inoculation and at 2-day intervals, a 5-ml sample of the culture was removed and passed through a 0.45 μm membrane filter (Millipore). The reducing sugar concentration of the cell-free filtrate was determined with DNS (3,5-nitrosalicylic acid) reagent [19]. The reducing sugar present in filtrates was extrapolated from a standard curve for N-acetylglucosamine [13].

Chitobiase assay. The rapid qualitative method of O'Brien and Colwell [16] using 4-methylumbelliferyl-N-acetyl-β-D-glucosaminide (4-MUF.GLcNAc) (Sigma Chemical Co., St. Louis, Missouri) was used to determine chitobiase in all the strains except *B. popilliae*. For this assay, 30 μl of 4-MUF.GLcNAc was placed on a 1.25-cm filter paper disc (Schleicher and Schuell No. 740-E). Cells from several colonies were rubbed onto the wet surface of the discs held in a petri dish. Discs were incubated at either 30° or 37°C for 10–15 min or, in the case of *B. psychrosaccharolyticus*, 30 min. After incubation one drop of saturated sodium bicarbonate solution was added to each disc and then exposed to UV light (366 nm). The chitobiase reaction was scored on a numerical basis of 1–4, where 1 was the weakest and 4 the strongest positive, respectively. Cultures giving a weak reaction were retested with a longer incubation period of 30–60 min. All negative reactions are indicated by 0.

Results and Discussion

Biochemical and morphological tests confirmed the authenticity of all the taxospecies of *Bacillus* used. The results of *Bacillus* strains able to hydrolyze colloidal chitin and produce the chitobiase reaction are

Table 2. Ratio of clearing zone to colony size at 2-day intervals

Bacillus strain	Days 2	4	6	8
B. alvei	1.0	1.0	1.9	3.8
B. brevis	0.0	0.0	1.6	1.8
B. chitinosporus	1.3	1.9	2.3	2.5
B. laterosporus	1.4	1.5	1.5	1.5
B. licheniformis	0.0	1.0	1.0	1.0
B. macerans	0.0	1.6	1.7	1.7
B. pulvifaciens	2.0	1.2	1.8	2.1
B. thuringiensis	0.0	1.1	1.1	1.0
Bacillus (sp.) Y-91B	1.8	1.1	1.1	1.2
Bacillus (sp.) AU B-2	1.0	1.1	1.1	1.2
Bacillus (sp.) AU B-3	1.0	1.1	1.6	2.5
Bacillus (sp.) AU B-4	1.0	1.1	1.6	2.5
Bacillus (sp.) AU B-5	1.0	1.0	1.2	1.2
Bacillus (sp.) AU B-6	1.1	1.1	1.2	1.3
Bacillus (sp.) -X (Unidentified)	1.0	2.0	2.5	2.5

Table 3. Reducing sugar concentration in broth cultures of chitinolytic strains

	Reducing sugar (mg/ml)[a] Days 2	4	6	8
B. alvei	0.00	0.31	0.35	0.96
B. brevis	0.00	0.27	0.31	0.71
B. chitinosporus	0.43	0.52	0.19	0.28
B. laterosporus	0.00	0.19	0.14	0.23
B. licheniformis	0.25	0.48	0.49	0.71
B. macerans	0.10	0.22	0.36	1.5
B. pulvifaciens	0.25	0.25	3.5	2.9
B. thuringiensis	0.20	0.29	0.28	0.27
Bacillus (sp.) Y-91B	0.38	1.80	0.23	0.21
Control	0.18	0.15	0.18	0.17

[a] Method of Reissig et al. [19].

given (Table 1). Ten of the 29 species tested exhibited endochitinase activity. These data are not in agreement with results previously reported for chitin hydrolysis by species of *Bacillus* [1]. Species most frequently cited capable of hydrolyzing chitin are *B. cereus*, *B. coagulans*, and *B. megaterium*. Except for one strain of *B. megaterium*, these data show none of these strains to be chitinolytic. *Bacillus laterosporus* was the only species tested that produced only endochitinase; all the other positive strains produced both endochitinase and chitobiase. Many strains negative for endochitinase produced chitobiase. Some endochitinase-negative strains, e.g., *B. azotoformans*, *B. megaterium*, and *B. pantothenticus*, produced a 2+ to 4+ chitobiase reaction. The highest chitinase reaction was observed in (descending order): *B. chitinosporus*, *B. pulvifaciens*, *Bacillus* X (unidentified), *B. alvei*, *B. licheniformis*, and *B. macerans* (Table 2). With *B. macerans*, chitinase activity was slow to appear; however, the size of the clear zone in BH-CHA plates was larger than for most of the other species with the exception of *B. pulvifaciens* and *B. alvei*. The size of the clear zone produced by *B. alvei* was somewhat misleading because of the phenomenon of colony mobility. Colonies of this species growing on BH-CHA plates under optimal conditions were motile and produced a clear zone throughout the entire medium within 3–4 days. *Bacillus circulans* is known to exhibit colony mobility also; however, clearing was not observed with either strain of the species used in this study.

The highest yields of reducing sugar produced in broth cultures were in (descending order): *B. pulvifaciens*, *B. alvei*, *B. macerans*, *B. licheniformis* (ATCC 14580), and *B. brevis* (Table 3). These values correlate well with those obtained for clearing zone–colony size ratios, with the exception of *B. chitinosporus*. This strain was one of three exhibiting the highest (2.5) clear zone–colony ratio; however, it yielded only a third as much reducing sugar. These data indicate that *B. chitinosporus* produced more endochitinase than chitobiase. The exact opposite appears to be true for *B. pulvifaciens*. Reducing sugar concentration for all the strains reached a maximum level after 8 days of incubation, with the exception of *B. pulvifaciens*. This strain not only produced the highest sugar concentration (3.5 mg/ml), but reached a maximum level after 6 days.

In conclusion, this is the first investigation devoted exclusively to assaying the distribution of chitinases in species of *Bacillus*. Data from this study are in disagreement with those previously reported in the literature. This difference may be attributable to poor or inaccurate identification and taxonomy of organisms reported by previous workers. These data may have potential usefulness as a biochemical diagnostic property for separation and identification of *Bacillus* species and may be important industrially in solving pollution and environmental problems.

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