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A STANDARDIZED PROTOCOL FOR THE RAPID DETECTION OF GELATIN HYDROLYSIS BY *BACILLUS SPHAERICUS*

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ABSTRACT

Seventy out of 75 *Bacillus sphaericus* strains isolated in countries located in four different continents were shown to hydrolyse the incomplete protein gelatin. 61 of these strains are highly toxic to *Culex quinquefasciatus* larvae. Gelatin hydrolysis can be observed in culture media containing bacteriological meat peptone and meat extract alone, and is not dependent on the presence of other additional nutrients. For 93.3% of the strains tested gelatin degradation occurs until day 4 of incubation. However, one bacteriological gelatin failed to be hydrolyzed by many of the tested strains during the same time of incubation, thus indicating the existence of substrates non-degradable by bacteria with a constitutive gelatinase expression. The culture media and conditions employed in this study show that the *B. sphaericus* gelatinases are constitutively expressed.

KEY WORDS: *Bacillus sphaericus,* gelatin hydrolysis, taxonomy, entomopathogenicity, gelatinases.

INTRODUCTION

Gelatin was used for the first time as solidifying agent for bacteriological culture media by Robert Koch in 1881 (Manual de Bacteriologia, 1978; Difco Manual, 1984). However, due to its low melting temperature of 30-36°C (Pelczar, 1957) and the fact that the protein is easily hydrolyzed by many microbial enzymes, gelatin has been replaced later by other solidifiers, such as the polysaccharide agar-agar. The Committee on Bacteriological Techniques of the American Society of Bacteriologists recommended the use of gelatin for the detection of the presence of the enzyme gelatinase in microorganisms (Pelczar, 1957). Levine and Carpenter (1923) studied bacterial degradation of gelatin by formaldehyde titration and viscosity measurement, and Levine and Shaw (1924) showed that a solution of 2% Difco gelatin remained stable for more than 17 days if preheated to 50°C and subsequently stored at 10-20°C. These observations led to taxonomic studies of bacteria, and gelatin hydrolysis became a biochemical test and routine marker in the characterization of many species.

However, the lack of standardized culture media, gelatin quality and test conditions often led to mistakes in the interpretation of results. Furthermore, for a few known *Bacillus* species no information is available regarding the use of this test (Sneath, 1986). In case of *Bacillus sphaericus,* 11-89% of the strains tested were reported to be positive for gelatinase (Gordon et al., 1973; Sneath, 1986).

The present study aims at the standardization of a culture medium and test conditions suited for the detection of gelatin hydrolysis by 75 *B. sphaericus* strains. Dehydrated gelatin preparations from two important manufacturers were evaluated, using formulations described in the literature (Pelczar, 1957; Manual de Bacteriologia, 1978; Manual Merck, 1982; Difco Manual, 1984; Sneath, 1986; Collins et al., 1995).

MATERIALS AND METHODS

Culture media

In this study we used dehydrated Nutrient Broth (NB) reconstituted according to a recommendation of the American Society of Bacteriologists (Pelczar, 1957) and manuals (Manual Merck, 1982; Difco Manual, 1984). NB was prepared according to the manufacturer's suggestions and consisted typically of meat peptone (5 g/L) and meat extract (3 g/L), with a final pH varying between 6.6 and 7.2, depending on NB obtained either from Merck or Difco. These NB preparations were used for all inoculations during this study. Bacteriological gelatins were dissolved at 60°C in distilled water (prefiltered water distilled in a Corning distillator with a Pyrex glass condenser) and subsequently added to the NB medium at a final concentration of 120 g/L, thus yielding medium NBDG [NB plus Difco gelatin] and medium NBMG [NB plus Merck gelatin]. 5 mL aliquots of the media were transferred to 15 x 120 mm test tubes with screw-caps, sterilized for 15 minutes at 121°C and stored in the refrigerator until use. Under these conditions the media remained solidified. A second set of media consisted of the gelatins dissolved in distilled water at a concentration of 120 g/L [DWDG — distilled water plus Difco gelatin; and DWMG — distilled water plus Merck gelatin] (Pelczar 1957).

Bacterial strains

The gelatinase-positive and -negative reference strains used as standards in this study are listed in Table 1. All strains, including the *B. sphaericus* strains tested (Table 2), were maintained in NB agar slants in the refrigerator until use. The agar slants were prepared by supplementing NB with Difco or Merck agar to 15 g/L, with a final pH of 6.6 to 7.0.

Inoculation of gelatin media

The reference strains (Table 1) and 75 *B. sphaericus* strains isolated in 25 different countries in four different continents (see Table 2) were grown in NB medium for 12–18 h at 30°C. 50 µL of these cultures were used for the aseptic inoculation of the four different gelatin media and subsequently incubated at 30°C. In parallel, four sterile tubes containing each of the four media were incubated as control.

Gelatin hydrolysis

Gelatin hydrolysis was determined in 24-h intervals by keeping the test tubes at 3–5°C for 30 minutes and comparing them to the control tubes.

Toxicity test of *B. sphaericus*

B. sphaericus strains were grown in NYSM [nutrient yeast salt medium] (Myers and Yousten, 1980) for 48 h at 30°C. Bacteria were scraped off from the surface of the culture medium and suspended in 0.85% NaCl at an optical density of 0.1 at 600 nm. 1 mL aliquots of the

TABLE 1
Reference bacterial strains utilized as standards for testing hydrolysis of gelatin

Strains	Origin number	Culture medium			
		DWDG	NBDG	DWMG	NBMG
Bacillus cereus[1]	ATCC 11778	+	+	–	+
Bacillus firmus[1]	NCTC 10335	+	+	–	+
Bacillus mascerans[1]	NCTC 6355	+	+	+	+
Bacillus megaterium[1]	ATCC 14581	+	+	–	+
Bacillus polymyxa[2]	20B–15	+	+	–	+
Bacillus subtilis[1]	NCTC 3610	+	+	+	+
Bacillus thuringiensis[1]	LFB 249	+	+	+	+
Bacillus thuringiensis autoagglutinating strain[1]	LFB 859	+	+	+	+
Bacillus thuringiensis subsp. *israelensis*[1]	IPS 82	+	+	+	+
Bacillus thuringiensis subsp. *oswaldocruzi*[1]	LFB 856	+	+	+	+
Bacillus azatofixans[2]	20B–1	–	–	–	–
Escherichia coli[3]	ATCC 6633	–	–	–	–
Escherichia coli[3]	ATCC 11778	–	–	–	–
Proteus mirabilis[3]	342–5	+	+	–	+
Proteus mirabilis[3]	349–K	+	+	–	+
Pseudomonas aeruginosa[3]	ATCC 27853	+	+	–	–
Serratia marcescens[3]		+	+	+	+

[1]Bacteria kindly supplied by the *Bacillus* Culture Collection of the Laboratory of Bacterial Physiology (LFB), Department of Bacteriology, Oswaldo Cruz Institute, Rio de Janeiro.
[2]Strains kindly supplied by Dr. Lucy Seldin from the Institute of Microbiology, Federal University of Rio de Janeiro.
[3]Strains from the Laboratory of Enterobacteria, Department of Bacteriology, Oswaldo Cruz Institute, Rio de Janeiro.
+ = Hydrolizes (– = does not hydrolize) gelatin in the medium tested. Negative results were recorded at least until the 31st day of incubation.

suspensions were added to 50 mL of water (free of chlorine) containing 15 *Culex quinquefasciatus* 4th instar larvae and kept at 25°C for 48 h. As reference for toxicity we used a suspension of *B. sphaericus* strain 2362. All strains were tested in duplicate, and two samples of *C. quinquefasciatus* 4th instar larvae without added bacteria were kept as negative controls. The avarage number of dead larvae was recorded and toxicity classified as follows: 0–20% larval mortality = low toxicity; 21–50% larval mortality = medium toxicity; and >50% larval mortality = high toxicity.

RESULTS AND DISCUSSION

Seventy-five *B. sphaericus* strains, 61 of which highly toxic for mosquito larvae, were grown in four different culture media designed for the gelatinase test. The media tested differed in the overall richness of nutrients and the commercial origin of the bacteriological gelatins employed. Bacterial growth was observed in all four media tested. Fig. 1 shows the percentage of strains hydrolyzing gelatin as a function of the time of cultivation. The results show that 93.3% of the

TABLE 2
Strains of *Bacillus sphaericus* utilized to verify hydrolitic activity on gelatin substrate

LFB Strain No.	Origin	Sero-type	Toxicity*	LFB Strain No.	Origin	Sero-type	Toxicity*
037	Brazil		ND	928	Iraq	5a5b	H
132	Brazil		ND	929	China	5a5b	H
141	Brazil		ND	930	Columbia	5a5b	H
148	Brazil		H	931	Cameroun	5a5b	H
180	Brazil		ND	932	Columbia	5a5b	H
194	Brazil		ND	933	Malaysia	5a5b	H
198	Brazil		H	934	Ghana	6	H
261	Brazil		H	935	Malaysia	25	H
264	Brazil		H	936	Malaysia	25	H
267	Brazil		ND	937	Canada	25	H
305	Brazil		H	938	Singapore	3	H
340	Brazil		ND	939	Scotland	ND	H
344	Brazil		H	940	Czech	5a5b	H
349	Brazil		ND	941	Indonesia	5a5b	H
350	Brazil		H	942	Indonesia	5a5b	H
618	Brazil		ND	943	Egypt	5a5b	H
626	Brazil		ND	944	Guyana	5a5b	H
636	Brazil		H	945	Korea	5a5b	H
664	Brazil		H	946	Malaysia	5a5b	H
711	Brazil		ND	947	Bulgaria	5a5b	H
735	India	5a5b	H	948	Malaysia	5a5b	H
740	El Salvador	5a5b	H	949	URSS	5a5b	H
753	Ghana	6	H	950	Singapore	3	H
832	Nigeria	5a5b	H	951	Singapore	3	H
835	Ghana	6	H	952	Singapore	3	H
836	Ghana	3	H	953	Indonesia	1a	H
838	Scotland	5a5b	H	954	Romania	5a5b	H
841	Thailand	5a5b	H	956	Ghana	48	H
844	Ghana	6	H	957	Israel	25	H
908	Brazil		H	958	Indonesia	1a	H
909	Brazil		H	959	USA	25	H
916	Brazil		H	960	Singapore	3	H
917	Brazil		L	961	Ivory Coast	5a5b	H
922	Brazil		L	962	Thailand	5a5b	H
924	Brazil		L	963	Bulgaria	5a5b	H
925	Brazil		H	964	Ghana	6	H
926	Brazil		H	965	Ghana	6	H
927	Indonesia	1a	H				

Unless otherwise stated, Brazilian strains were isolated at the Laboratory of Bacterial Physiology (LFB), Department of Bacteriology, Oswaldo Cruz Institute, Rio de Janeiro. The remaining strains were kindly provided by Professor Fergus G. Priest, from the Heriot-Watt University, Edinburgh.
*Toxicity was determined against L_4 *Culex quinquefasciatus* larvae. H, high toxicity. L, low toxicity and ND, not done.

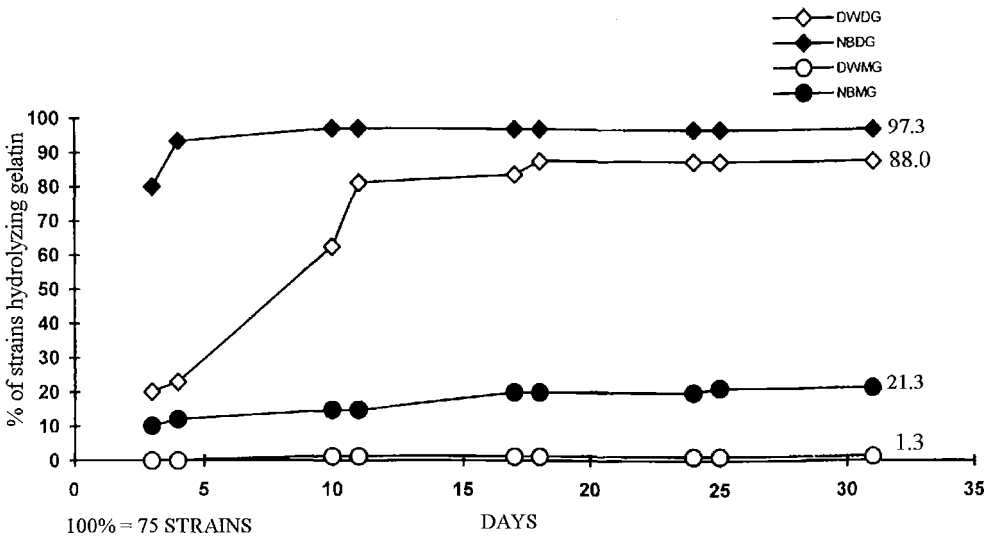

Fig. 1. Gelatin hydrolysis produced by strains of *Bacillus sphaericus* grown in four different gelatin media.

strains hydrolyzed Difco gelatin in the NBDG medium after four days of incubation. At day 9, 97.3% of the strains caused hydrolysis of the NBDG medium, and this value did not change until day 31.

In the case of the DWDG medium, only 18 strains (24%) hydolysed the substrate after four days of incubation. This value increased to 61 strains (81.3%) after day 11, and reached 66 (88%) on day 31. On the other hand, Merck gelatin in the NBMG medium was hydrolyzed only by 16 strains (21.3%), even after 31 days of incubation, and in the DWMG medium, only 1 strain (1.3%) was positive. As demonstrated in Table 1, not all gelatinase positive *Bacillus* strains were able to hydrolyze the substrate in the DWMG medium, indicating that the presence of some additional nutrients is necessary for gelatin hydrolysis. On the other hand, hydrolysis occurred in the DWDG medium, thus suggesting that Merck gelatin is more pure than Difco gelatin but less suited for the test, unless missing nutrients are added separately to the medium. Similar results were obtained with *Proteus mirabilis* (2 strains) and *Pseudomonas aeruginosa*, but not with *Serratia marcescens*, which hydrolyzes effectively the gelatins in all media tested. The same results were obtained with *Bacillus subtilis, B. mascerans* and all *B. thuringiensis* strains tested (see Table 1).

Nevertheless, the NBMG medium gave positive results for the majority of the reference strains, with the exception of *B. azotofixans*, *Escherichia coli* (2 strains) and *P. aeruginosa*. The first two species are known to be gelatinase-negative, and in *P. aeruginosa* the enzyme is expressed only in the presence of certain nutrients (induced expression). However, under the conditions tested, the NBDG medium is better suited for the gelatin hydrolysis test than the DWDG medium (Table 1), since hydrolysis occurs earlier in the former, thus reducing the time required for incubation. In the present study we showed that 93.3% of the investigated *B. sphaericus* strains hydrolyze gelatin until day 4 of incubation. Therefore, gelatin hydrolysis is characteristic for this species.

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