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Tests for Detecting Degradation of Gelatin: Comparison of Five Methods

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Five methods for detecting degradation of gelatin by bacteria were compared. These were liquefaction in nutrient broth, hydrolysis in nutrient agar, hydrolysis of charcoal gelatin strips, degradation of the gelatin on strips of photographic film, and alkalinization of gelatin agar. Degradation of photographic film is a rapid and convenient method but, like hydrolysis of gelatin in broth and in agar, may fail to detect weakly positive strains of bacteria. Alkalinization of gelatin in an agar medium is a convenient and sensitive method to detect degradation of gelatin, particularly by *Pseudomonas fluorescens*, but this method may not be applicable to some species.

According to some (2, 5, 19), but not all (13), tabulations, degradation of gelatin is a definitive test for distinguishing *Pseudomonas fluorescens* (all strains are positive) and *P. putida* (all strains are negative). Tests for degradation of gelatin are also useful in the characterization and identification of other bacteria, e.g., species of the genera *Bacillus* and *Flavobacterium* and the Proteus-Providence group in the family *Enterobacteriaceae* and nonpigmented strains of *Serratia marcescens*.

Five methods have been used to detect microbial degradation of gelatin. These are (i) liquefaction in an agar-free basal medium (2–5, 9, 12, 14), (ii) hydrolysis in a nutrient agar basal medium (3, 16, 18, 19), (iii) hydrolysis in a gelatin charcoal disk or strip (8, 10, 12), (iv) hydrolysis on a strip of photographic film (1, 12, 17), and (v) alkalinization in a basal medium containing agar and a pH indicator (6, 15). These five methods have not been compared with respect to sensitivity and applicability, particularly with weakly gelatin-positive bacteria such as some strains of *P. fluorescens*, *P. diminuta*, and *Flavobacterium* (*Sphingobacterium*) *multivorum*. This report presents such comparisons.

MATERIALS AND METHODS

Bacterial strains. All of the bacterial strains used were from our stock culture collection. Most of the strains had been recovered from clinical specimens and were received from local hospitals. Some of the strains, including four of *P. fluorescens* that did not grow at 35°C, had been recovered from environmental specimens. Two strains of *P. putida* were used as negative controls, and five of *F. meningosepticum* served as strongly positive controls.

Media. Gelatin broth was prepared by adding gelatin to heart infusion broth (Difco Laboratories, Detroit, Mich.), dispensing the melted gelatin broth in 3-ml amounts into 13-mm-diameter tubes, and sterilizing this medium by autoclaving it at 121°C for 15 min. Gelatin agar medium was compounded as described by Smith and Goodner (18), namely, with 0.4% neopeptone (Difco), 0.1% yeast extract (Difco), 1.5% agar, and 4% gelatin. This medium was dispensed into tubes (in 20-ml amounts for preparation of petri plates and in 3-ml amounts for use as slants) and sterilized by autoclaving. Gelatin charcoal strips were prepared as described by Kohn (8). The strips, after treatment with formalin, were manipulated aseptically, thus eliminating the need for autoclaving. For each test with gelatin charcoal, a large (ca. 0.5 mm^3 of cell paste) inoculum was suspended in 0.5 ml of sterile distilled water in a 13-mm-diameter tube and a strip was deposited in the suspension. Gelatin film strips, cut to convenient size (e.g., 6 by 30 mm) from Eastman X-ray film type AA, were added, as with the charcoal strips, to heavy bacterial suspensions in distilled water. For some of the tests, the film strips were obtained commercially (Key Scientific Products, Los Angeles, Calif.). Alkalinization of gelatin was determined in an aerobic low-peptone (ALP) basal medium (6). The ALP gelatin medium contained 1.0% gelatin (Difco), 0.05% yeast extract (Difco), 0.05% Casitone (Difco), 0.1% $(NH_4)_2HPO_4$, 0.02% KCl, 0.02% $MgSO_4 \cdot 7H_2O$, 0.002% phenol red, and 1.5% agar. It was adjusted to pH 6.5, dispensed in 3-ml amounts into 13-mm-diameter tubes, autoclaved, and solidified as slants.

Inocula, incubation temperatures, and readings. The inoculum for each experimental test other than those done with charcoal and film strips was one loop of infusion broth culture. Each inoculum for the ALP gelatin tests was streaked on the surface of the slanted medium; all other gelatin agar tests received one loop of a spot inoculum. Temperatures of incubation for the several experimental tests are given in the tables. Readings for microbial liquefaction of gelatin were made after the tubes had been placed at 5°C for 1 h. Two criteria were used to record hydrolysis of gelatin in agar. One was development of an opacity or halo surrounding the spot-inoculated growth on plates and slants (18), and the other was a transparent zone surrounding spot-inoculated growth upon addition of acidified mercuric chloride (6 g of $HgCl_2$, 8 ml of concentrated hydrochloric acid, 40 ml of distilled water).

RESULTS

We confirmed Lampe's report (9) that slants are more sensitive than stabs for detecting liquefaction of gelatin. Two (of 23) strains of *P. fluorescens* failed to liquefy 12% gelatin

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TABLE 1. Liquefaction of gelatin in broth with gelatin concentration and incubation temperature as variables[a]

Species (no. of isolates)	Gelatin concn (%), incubation temp (°C)	Cumulative % of strains positive after incubation for:						
		1 day	2 days	4 days	7 days	14 days	21 days	28 days
F. meningosepticum (5)	4, 35	100						
	12, 35	60	100					
P. aeruginosa (22)	4, 35	68	82	100				
	12, 35	64	86	91	95	95	100	
P. diminuta (8)	4, 35	75	100					
	12, 35	13	63	100				
P. fluorescens (23)	4, RT[b]	43	78	78	83	100		
	4, 30	13	39	39	39	52	65	70
	12, 30	13	30	39	39	48	48	48
	4, 35	4	4	4	4	13	22	22
	12, 35	4	4	4	4	9	9	9

[a] Two strains of *P. putida* gave negative results in all tests through 28 days of incubation.
[b] RT, room temperature (22 ± 2°C).

stabs after 21 days of incubation at room temperature (22 ± 2°C), but both of these strains liquefied gelatin slants within 14 days. The gelatin stabs were similarly insensitive, relative to slants, with several other species of nonfermenters.

Room temperature incubation is not optimal for detection of degradation of gelatin by some bacteria, but at higher temperatures, 30 and 35°C, gelatin is a fluid rather than a gel and, hence, slants are not applicable. An alternative to slants for increasing sensitivity is to use gelatin at a lower concentration. Four lots of gelatin were examined with respect to gelling. All gelled similarly at several temperatures examined. At a concentration of 4%, gelatin forms a gel at 5°C that is retained at 21°C. This concentration is more sensitive than 12% for detection of bacterial liquefaction of gelatin (Table 1).

Gelatin agar plates of the medium of Smith and Goodner (18), incubated for 2 days, gave negative results with weakly gelatin-positive strains (data not shown). Incubation for more than 2 days was precluded when more than five strains were spot inoculated onto a plate because of the spreading growth of strongly positive strains. When their gelatin agar medium was used as tubed slants, some strains, upon prolonged incubation, gave positive results when negative results had been obtained with plated medium (Table 2). With both plates and slants, readings in terms of halos were less sensitive than those obtained with acidified mercuric chloride. The gelatin agar medium of Pitt and Dey (16), with Fildes' supplement omitted, was examined in both plates and tubes. Early and strong positive results were obtained with strongly gelatin-positive strains, but two strains of *P. putida* also gave positive results.

Two rapid methods for detecting hydrolysis of gelatin, using strips of gelatin charcoal (8) and of photographic film (7), were examined. Both procedures gave early positive results with all strains of *F. meningosepticum* and most strains of *P. aeruginosa*, but neither procedure gave early positive results with weakly gelatin-positive strains of *P. fluorescens* (Table 3). Using gelatin charcoal strips, Lautrop (10) found that better results were obtained with some strains when the bacterial suspensions were prepared in a

TABLE 2. Hydrolysis of 4% gelatin agar in tubed slants[a]

Species (no. of strains)	Incubation temp (°C)	Cumulative % of strains positive after incubation for[b]:				
		1 day	2 days	4 days	7 days	14 days
F. meningosepticum (5)	30	100/100				
	35	100/100				
P. aeruginosa (22)	RT[c]	25/45	50/80	80/90	90/95	90/100
	30	80/100	95/100	100/100		
	35	95/95	100/100			
P. diminuta (8)	RT	0/0	0/13	13/25	100/100	
	30	0/0	13/13	13/63	13/100	13/100
	35	0/25	0/25	0/88	0/88	0/88
P. fluorescens (24)	RT	58/58	75/83	88/88	88/96	92/100
	30	50/50	58/71	58/79	58/79	58/83
	35	8/21	33/42	33/42	33/42	33/54

[a] The gelatin agar medium used was that of Smith and Goodner (18) (3 ml per tube). Two strains of *P. putida* gave negative results in all tests through 14 days of incubation.
[b] Shown are cumulative percentages of strains positive with a halo surrounding spot-inoculated growth/with a transparent zone surrounding spot-inoculated growth upon addition of acidified mercuric chloride.
[c] RT, room temperature (22 ± 2°C).

TABLE 3. Degradation of gelatin charcoal and gelatin film strips[a]

Species (no. of strains)	Incubation temp (°C)	Cumulative % of strains positive on gelatin charcoal strips/gelatin film strips after incubation for:					
		4 h	8 h	1 day	2 days	4 days	7 days
F. meningosepticum (5)	35	100/100					
P. aeruginosa (21)	35	62/71	81/71	86/76	86/95	86/100	90/100
P. diminuta (8)	35		0/0	38/25	38/75	100/100	
P. fluorescens (24)	RT[b]	0/4	0/21	38/50	38/67	46/67	54/75
	30	0/25	0/25	17/50	25/54	54/54	63/63
P. putida (2)	RT						0/0
	30						0/0

[a] Preparation and use of the gelatin charcoal strips were done as described by Lautrop (10). Film strips (6 by 30 mm) were prepared from Eastman X-ray film type AA.
[b] RT, room temperature (22 ± 2°C).

menstruum containing calcium chloride than when this salt was omitted. We examined several strains in both sterile distilled water and water supplemented with 0.01 M $CaCl_2$ and found no difference in the time required to obtain positive results.

Except for two strains of *P. fluorescens* incubated at 35°C, all of the strains included in Table 4 alkalinized the gelatin agar medium of Greenwood (6). However, in a sample of other gelatin-positive strains of gram-negative bacteria, neither hemolytic acinetobacters nor *Alcaligenes* species alkalinized this medium (Table 5). After 28 days of incubation, all of the five strains of *Alcaligenes xylosoxidans* subsp. *xylosoxidans* of Table 5 had liquefied 4% gelatin but none had liquefied 12% gelatin (data not shown). Similarly, after 21 days of incubation, all of the six strains of *A. faecalis* of Table 5 had liquefied 4% gelatin but only four had liquefied 12% gelatin.

DISCUSSION

No one procedure was optimal for detecting degradation of gelatin by the species we examined. Tubes of 12% gelatin in a nutrient broth, prepared as stabs and incubated at 20 to 25°C (2, 3, 5, 12, 14), 30°C (5), or 30 to 35°C (2, 3, 12), have been used to detect liquefaction of gelatin. However, at the lower temperature liquefaction may be delayed relative to that obtained with incubation at 35°C. At higher temperatures of incubation, 30 and 35°C, the tubes must be chilled before readings are taken and *P. fluorescens* may fail to liquefy gelatin through 28 days of incubation. With a significant number of the strains examined, 4% gelatin was more sensitive than the conventional 12% gelatin.

Hydrolysis of gelatin in a nutrient agar basal medium dispensed into petri plates has been used as an alternative to testing for liquefaction in a fluid basal medium (3, 16, 18, 19). It has the advantage, relative to stabs of gelatin in broth, that it detects extracellular protease and that it is a solid medium at 30 and 35°C. Additionally, it may give earlier positive results. With our strains of *P. fluorescens*, after 2 days of incubation at room temperature 96% gave positive results with gelatin agar plates but only 57% did so with 12% gelatin stabs and 78% did so with 4% gelatin stabs (data not shown). Hydrolysis of gelatin in gelatin agar plates is commonly detected by depositing a drop of a protein precipitant, such as acidified mercuric chloride, at the margin of spot-inoculated growth. When the precipitant is added after 2 days of incubation, this technique does not distinguish between true-negative and delayed-positive results. This problem can be circumvented in part by using replicate slants rather than plates and by making readings, before addition of the protein precipitant, in terms of halos surrounding the spot-inoculated growth. However, readings in terms of halos are less sensitive with nonfermentative rods than those by a protein precipitant. In brief, hydrolysis of gelatin in agar is a cumbersome and, at least for clinical laboratories, not readily applicable procedure for detection of degradation of gelatin by nonfermenters.

Two rapid tests gave positive results, frequently within 2 h, with strongly proteolytic strains, such as those of *F. meningosepticum* and some strains of *P. aeruginosa*. Photographic strips are easily prepared and more convenient to use than charcoal gelatin strips. They have been favorably evaluated in several reports (1, 7, 11, 17) and can be recommended for general use. However, since this method fails to detect weak proteolysis, for critical tests negative results at 24 to 48 h should be confirmed by a more sensitive procedure, such as liquefaction of 4% gelatin broth or alkalinization of ALP gelatin.

As judged from this study, alkalinization of gelatin in a

TABLE 4. Alkalinization of gelatin agar slants[a]

Species (no. of strains)	Incubation temp (°C)	Cumulative % of strains positive after incubation for:					
		1 day	2 days	4 days	7 days	14 days	21 days
F. meningosepticum (5)	30	100					
	35	100					
P. aeruginosa (22)	30	91	91	100			
	35	91	100				
P. diminuta (8)	30	88	100				
	35	100					
P. fluorescens (24)	RT[b]	67	83	88	92	100	
	30	54	63	63	96	100	
	35	25	50	67	71	79	83

[a] Gelatin (1%) in Greenwood ALP basal medium (6) was used at 3 ml per tube. Two strains of *P. putida* gave negative results in all tests through 28 days of incubation at 22, 30, and 35°C.
[b] RT, room temperature (22 ± 2°C).

TABLE 5. Degradation of gelatin by gram-negative bacteria

Genus or species (no. of strains tested)	Cumulative % of strains that[a]:														
	Liquefied gelatin after:				Hydrolyzed gelatin after:			Alkalinized gelatin after:				Degraded X-ray film after:			
	1 day	2 days	4 days	28 days	1 day	2 days	7 days	1 day	2 days	4 days	21 days	4 h	8 h	1 day	2 days
Acinetobacters (4)	100	100	100	100	100	100	100	0	0	0	0	25	50	100	100
Aeromonas spp. (7)	100	100	100	100	100	100	100	100	100	100	100	71	100	100	100
Alcaligenes faecalis (6)	0	0	0	100	0	0	0	0	0	0	0	0	0	0	0
Alcaligenes xylosoxidans (5)	0	0	0	100	0	0	0	0	0	0	0	0	40	100	100
Flavobacterium indologenes (6)	67	83	100	100	100	100	100	100	100	100	100	83	100	100	100
Flavobacterium multivorum (5)	0	0	0	20	0	20	40	60	100	100	100	0	0	0	20
Flavobacterium odoratum (6)	100	100	100	100	100	100	100	100	100	100	100	83	83	100	100
Proteus mirabilis (5)	100	100	100	100	100	100	100	60	100	100	100	0	60	100	100
Proteus vulgaris (5)	100	100	100	100	100	100	100	20	60	100	100	0	20	100	100
Pseudomonas cepacia (5)	0	20	40	80	60	60	60	80	100	100	100	40	60	80	80
Xanthomonas (*Pseudomonas*) *maltophilia* (4)	50	100	100	100	100	100	100	100	100	100	100	100	100	100	100

[a] Gelatin liquefied, 4% gelatin in infusion broth; gelatin hydrolyzed, 4% gelatin slants in the basal medium of Smith and Goodner; gelatin alkalinized, 1% gelatin in ALP basal medium. All incubations were at 35°C.

low-peptone basal agar medium is the method of choice in clinical bacteriology for detecting degradation of gelatin by fluorescent pseudomonads. This procedure is both convenient and sensitive. In this study, 20 (of 24) strains of *P. fluorescens* gave positive results within 48 h. Two of the four negative strains denitrified, and hence, gelatin was not required for identification. The third strain alkalinized gelatin in 4 days and the fourth strain did so in 14 days. This procedure is not applicable to *Acinetobacter* spp. and *Alcaligenes* spp., but hydrolysis of gelatin is not a significant feature for identification of these taxa.

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