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Factors Affecting Production of Catalase by *Bacteroides*

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Several variables affected the production of catalase by members of the "*Bacteroides fragilis* group" of anaerobic bacteria. Broth media yielded higher catalase levels than the respective agar media. Addition of hemin to media after autoclave sterilization, rather than before, significantly increased production of catalase. Both of these variables could be related to the available hemin concentration present in the medium being tested. Significantly higher amounts of hemin were required for catalase production than were required for growth. For catalase production by *B. fragilis* ATCC 25285, 1 μg of hemin per ml was required. Of the various media tested, the use of chopped meat broth resulted in the highest levels of catalase production (up to 50 to 60 U of catalase per mg of protein). Of the various species and DNA homology groups tested, strains of *B. fragilis* and *Bacteroides distasonis* were catalase positive. Strains of *Bacteroides thetaiotaomicron*, *Bacteroides ovatus*, and *Bacteroides eggerthi* possessed variable catalase activity. *Bacteroides vulgatus*, *Bacteroides uniformis*, and DNA homology groups "3452A" and "subsp. a" were catalase negative. A catalase well test, in which equal volumes of 3% H_2O_2 and chopped meat culture are mixed, is described and recommended for routine catalase tests.

The catalase test has been recommended as a rapid screening procedure to identify members of the "*Bacteroides fragilis* group" of anaerobic, gram-negative, nonsporing bacilli (5a). These investigators tested 650 fresh clinical isolates of anaerobic bacteria for production of catalase with a slide test by using 15% H_2O_2 with Tween 80 and found that members of the *B. fragilis* group and *Bacteroides putredenis* were the only gram-negative anaerobic rods which were catalase positive. Stargel et al. (14) found that catalase production was more consistent when *B. fragilis* was grown without a fermentable carbohydrate. We confirmed this result and found that addition of 0.5% glucose to a culture growing in peptone broth caused an immediate cessation of catalase production (5).

The *B. fragilis* group previously consisted of five subspecies and a phenotypically similar group called "other" (6). These subspecies, subsp. *fragilis*, subsp. *thetaiotaomicron*, subsp. *distasonis*, subsp. *vulgatus*, and subsp. *ovatus*, have all been elevated to species rank on the basis of DNA homology data by Cato and Johnson (3). In additional studies, Johnson (8) has found that strains previously identified as other (6), some strains identified as *B. distasonis* or *B. thetaiotaomicron*, and organisms described by Holdeman and Moore (7) as *B. fragilis* "subsp. a" comprise additional homology groups which warrant species designations.

All of these organisms are of fecal origin, with *B. vulgatus*, *B. thetaiotaomicron*, and *B. uniformis* being the most predominant (11; W. E. C. Moore, personal communication). *B. fragilis*, which occurs at a lower concentration in the intestine (11), is the species of anaerobic bacteria isolated most frequently from clinical specimens (2, 9, 10, 12). *B. thetaiotaomicron* is the other species in this group frequently isolated by clinical laboratories (12). Organisms belonging to the other *Bacteroides* species are isolated much less frequently from clinical specimens (12). Nevertheless, the isolation of any of these organisms from an infection could be cause for concern because, as a group, they are relatively resistant to cephalosporins and penicillins (2, 9). Quick recognition that a member of this group of organisms is present in an infection can therefore be important for a clinical laboratory even though the exact species designation cannot be assigned.

We have noticed considerable variability in the catalase reaction of the same strains of *B. fragilis* grown in different broth and agar media. We report here the extent of this variability, the influence of adding hemin before or after autoclaving, and the catalase reactions of various

species and DNA homology groups of *Bacteroides*.

MATERIALS AND METHODS

Organisms. All bacterial strains used in this study were obtained from the culture collection of the Virginia Polytechnic Institute Anaerobe Laboratory. These strains represented isolates received from clinical laboratories for identification and isolates from the feces of people from several population groups. All strains were separated into species or homology groups on the basis of DNA homology data (8). DNA homology groups that do not have a species designation are listed under the VPI strain number of the DNA reference strain.

Media. Hemin, prepared as an autoclaved stock solution in 0.001 N NaOH (6), was added to the media either before autoclave sterilization or aseptically after autoclaving. Unless otherwise specified, the final concentration of hemin in the medium was 5 μg/ml.

Prereduced, anaerobically sterilized chopped meat broth (PRAS-CM) was prepared as described in the VPI *Anaerobe Laboratory Manual* (6). Anaerobic Trypticase soy broth, for determining the amount of hemin necessary for catalase production, was prepared in new glassware to eliminate possible hemin contamination. The Trypticase soy broth without dextrose (BBL 11774) plus 0.05% cysteine-HCl was dissolved in distilled water, boiled, dispensed into rubber-stoppered tubes in the anaerobic glove box (85% N_2–10% H_2–5% CO_2), and then autoclaved for 15 min at 15 lb/in^2 and 121°C. Sterile hemin was then added to individual tubes to obtain final concentrations of 0.005, 0.01, 0.05, 0.1, 0.5, 1.0, and 5.0 μg/ml.

For comparing the effect on catalase production of different agar or broth media and of adding hemin before or after autoclave sterilization, the following types of media were used: Columbia (BBL), Wilkins-Chalgren (15), Brucella (Pfizer), Trypticase soy without dextrose (BBL 11774 and 11043), brain heart infusion (Difco) supplemented with 0.5% yeast extract (Difco), peptone-yeast extract, and chopped meat. Hemin was added to the above media either before or after autoclave sterilization to give a final concentration of 5 μg/ml. The above broth media were prepared aerobically, and 10 ml was dispensed into screw-capped tubes and autoclaved 15 min at 15 lb/in^2. For the agar media, dry ingredients were dissolved in distilled water, boiled for 1 min, and autoclaved 15 min at 15 lb/in^2. All plates were dried aerobically and stored in the anaerobic glove box. The peptone-yeast extract broth contained peptone (Bacto, Difco), 0.5%; Trypticase (BBL), 0.5%; yeast extract (Difco), 1.0%; and 0.0005% hemin. The chopped meat agar was prepared by adding Trypticase (BBL), 3%; yeast extract (Difco), 0.5%; K_2HPO_4, 0.5%; and agar, 1.5%, to meat broth (ground beef, 50% [wt/vol] boiled in 0.1 N NaOH, cooled, filtered, the pH neutralized, with the fat removed).

Culture procedures. Overnight anaerobic PRAS-CM cultures were used as inocula for both the broth and agar studies. Broth media, prepared in screw-capped tubes, were boiled for 10 min and then were cooled immediately before use. The tubes were inoculated with 0.1 ml of culture at the bottom of the tube and were incubated overnight at 37°C in an aerobic incubator. The agar plates were streaked with the PRAS-CM inoculum and were incubated in the anaerobic glove box at 37°C.

For determining the amount of hemin required for production of catalase, hemin-depleted cells were used as the inoculum for the anaerobic Trypticase soy broth. These cells were prepared as follows: hemin-free anaerobic Trypticase soy broth was inoculated with 0.05 ml of an overnight PRAS-CM culture and incubated 24 h. When transferred into hemin-free medium, these cells were unable to initiate growth but would grow if hemin was added to the medium.

Catalase assay. Catalase activity was measured by the following methods. For the plate studies, the agar plate was flooded with 3% H_2O_2 and observed for production of bubbles. For the broth studies, equal volumes of culture and 3% H_2O_2 were mixed in either a small test tube or the well of a microtiter plate and observed for gas bubbles.

Previous results in our laboratory indicated that catalase was not induced by exposing the culture to air for 30 min before adding H_2O_2 as currently recommended (6). Therefore, we tested for catalase production immediately after removing a culture from anaerobic conditions.

To determine the specific activity of the catalase that was produced by an individual strain, the broth culture was centrifuged at 16,300 × *g* for 10 min, and the pellet was resuspended in 10 ml of 50 mM sodium phosphate buffer, pH 7.0. The cells were washed with 50 mM sodium phosphate buffer, pH 7.0, and were disrupted by sonic treatment at 60 W for 3 min, followed by 2 min of chilling, and 2 additional min of sonic disruption (model 200, Branson Sonic Power Co.). For agar-grown cultures, the cells were washed off each plate with approximately 3 ml of 50 mM sodium phosphate buffer (pH 7.0). The resulting cell suspension was disrupted by sonic treatment as described above. The disrupted cells were then centrifuged at 34,900 × *g* for 15 min to remove cellular debris. The supernatant was assayed for catalase activity by the method of Beers and Sizer (1). The protein content of the crude lysate was estimated from the optical density at 280 nm, by assuming an extinction of 1 ml·mg^{-1}·cm^{-1}, and the specific activity of the enzyme was calculated.

RESULTS

We grew the neotype strain of *B. fragilis* (VPI 2553 → ATCC 25285; = NCTC 9343) in 8 types of broth media with 5 μg of hemin per ml added either before or after autoclaving the media. The specific activity of catalase in the various cultures was extremely variable (Table 1). Two factors appear to be responsible for this variability: the type of medium and the manner in which hemin was added. In general, the production of catalase was much lower or absent in the broth media to which hemin had been added before autoclave sterilization. Autoclave sterilization of hemin in the media apparently affected its availability. However, there was sufficient

TABLE 1. *Medium-dependent variability of catalase production by B. fragilis ATCC 25285*

Medium	Broth		Agar	
	Hemin added before autoclaving	Hemin added after autoclaving	Hemin added before autoclaving	Hemin added after autoclaving
Brain heart infusion	1[a]	21	1	13
Brucella	3	10	4	6
Columbia	0	7	1	2
Peptone-yeast	8	24	5	5
Trypticase soy	0	25	2	9
Wilkins-Chalgren	0	10	6	6
Chopped meat	40[b]	NT[c]	0	7

[a] Specific activity of catalase in units per milligram of protein, where 1 U is equivalent to 1 μmol of H_2O_2 decomposed per min.

[b] PRAS-CM (6) was used.

[c] NT, Not tested.

hemin present in all of the media for good growth. Neither the growth rate nor the final turbidity was influenced by the manner in which hemin was added. Only those cultures with a catalase specific activity of 10 or greater gave a positive result when 3% H_2O_2 was added to an equal volume of these cultures.

When we repeated the above experiment with the respective agar media instead of broth media, we obtained very different results (Table 1). In general, much less catalase was produced in agar media than in the corresponding broth media. The manner in which hemin was added to the media had less effect on the amount of catalase produced, but the highest amount of catalase still occurred when hemin was added after autoclave sterilization. This effect was most noticeable in brain heart infusion and chopped meat agar media. Five times more catalase was produced in chopped meat broth than in chopped meat agar. When chopped meat agar cultures were flooded with 3% H_2O_2, a strong bubbling reaction occurred only where cells were growing in close proximity to meat particles. We believe that this was because the hemin adsorbed to the meat particles.

The effect of adding hemin after autoclaving could be shown by placing a paper disk containing 5 μg of hemin on either a brain heart infusion or chopped meat agar plate freshly swabbed with the organism. After incubation, there was not a discernible difference in the amount of growth around the disk, but when the plate was flooded with 3% H_2O_2, the area around the hemin disk was strongly catalase positive. Small catalase-positive areas also occurred in areas where meat particles were close to the surface of the agar. Control plates without bacteria were negative.

The amount of hemin required for production of catalase by *B. fragilis* ATCC 25285 was determined by growing hemin-depleted cells (13) in anaerobic Trypticase soy broth with added hemin and then measuring the specific activity of the catalase produced. Detectable levels of catalase were not produced in media containing 0.5 μg of hemin or less per ml. In media containing 1 μg of hemin per ml, 0.5 U of catalase per mg of protein was produced; in media containing 5 μg of hemin per ml, specific activity was 7. Higher concentrations of hemin (50 μg/ml) did not result in a higher specific activity. These results are similar to those obtained for *B. distasonis* by Gregory et al. (4).

A survey of the various species and DNA homology groups revealed that not all species in the *B. fragilis* group produced catalase. Strains were grown in chopped meat broth and assayed for production of catalase either by determining the specific activity of the catalase produced (Table 2) or checking for a bubbling reaction when equal volumes of culture and 3% H_2O_2 were mixed (Table 3). *B. distasonis* and *B. fragilis* were the only species in which all of the strains tested produced catalase. However, since these experiments were completed, we have found a *B. fragilis* strain that does not produce catalase. No strains of *B. vulgatus*, *B. uniformis*, or of DNA homology groups 3452A and subsp. a produced catalase. The other species and DNA homology groups were variable. Catalase production did not correlate with the original source of each isolate; that is, not all clinical isolates were catalase positive and not all fecal isolates were catalase negative.

DISCUSSION

In this study, we have found several variables, all related to the availability of hemin in the medium, which affect catalase production. The type of medium, presence of agar, and addition of hemin pre- or postautoclaving affected the amount of catalase which was produced. Therefore, these variables should be considered when choosing a medium to grow organisms for the catalase test. Chopped meat, brain heart infusion, peptone-yeast, and Trypticase soy broths yielded the highest level of cellular catalase content, whereas cells grown in brucella, Columbia, and Wilkins-Chalgren (15) broths had lower levels of catalase.

In a previous study we reported on repression of catalase production by fermentable carbohydrates (5). Several of the media used in this

TABLE 2. *Catalase production in chopped meat broth by various species of Bacteroides*

Strain	Catalase[a]	Strain	Catalase
B. fragilis I		*B. thetaiotaomicron*	
2553	38	5482	2
1522	25	0489	0
2362	96	0633	29
2633	56	2579A	3
4509B	70	2302	31
3625	17	3089	0
4147	9	3164A	0
2556	1	3388	4
3390	5	3051	0
B. fragilis II		*B. uniformis*	
2393	45	0061	0
2552	42	3699	0
3392	16	6387	0
4117	42	5444A	0
4225	33	8601	0
C48-32	11	C8-30	0
2360	6	T2-24	0
A3-11B	19	C17-3	0
A10-12B	1	C1-10	0
B. distasonis		*B.* homology group "3452A"	
4243	92	3452A	0
0052	62	8608	0
C14-2	220	B6-11	0
C30-45	30	C7-8	0
S6A-50	43	C14-3	0
S1-35	55	*B.* homology group "subsp. a"	
C2-1	49	B5-21	0
C33-3	65	C1-23	0
B. ovatus		C2-39	0
0038	27	C8-19	0
3524	15	C12-29	0
0435	14	*B. vulgatus*	
3049	0	4245	0
4101	9	0959	0
8605	10	5710	0
C1-45	10	6186	0
1900C	0	2736B	0
B4-11	62	6598B	0
8653	41	4506	0
B. eggerthi		2365	0
B8-51	3		
C18-8	0		
S1A-52	2		
X3-31-1A	0		

[a] Specific activity of catalase in units per milligram of protein, where 1 U is equivalent to 1 μmol of H_2O_2 decomposed per min.

study contained small amounts of glucose (0.09 to 0.25%). This concentration of glucose did not significantly influence catalase production because all the glucose was utilized during the early stages of growth. In media containing 0.5 to 1.0% glucose, very little catalase is produced because the cells continue to utilize the glucose throughout the growth cycle.

B. fragilis cannot synthesize its own hemin and must, therefore, transport preformed hemin for synthesis of heme proteins such as cytochromes and catalase (13). Whereas 1 μg of hemin per ml was required for catalase production, only 0.1 μg/ml was required for optimum growth (13). Because catalase levels were not a prerequisite for growth, hemin-promoted catalase function appeared to be a secondary metabolic process.

TABLE 3. *Number of strains of various Bacteroides species which produce catalase as assayed by the catalase well test*

Species	Catalase	
	Positive	Negative
B. fragilis I	65	0
B. fragilis II	14	0
B. thetaiotaomicron	22	4
B. ovatus	19	14
Bacteroides homology group 3452A	1	5
B. uniformis	0	23
Bacteroides homology group subsp. a	0	13
B. eggerthi	5	1
B. vulgatus	0	38
B. distasonis	9	0

More catalase was produced by cells growing in broth media than in the same type of media prepared with agar (Table 1). Higher catalase levels were observed in the same type of media when the hemin was added after autoclaving. These results indicated that hemin availability and not total hemin concentration was the factor determining catalase production. Catalase produced around a hemin-containing disk placed on the agar medium indicated that, when hemin was available, large quantities of catalase could be produced. The weak catalase-positive areas around meat particles in the chopped meat agar could be due either to intrinsic hemin in the protein or to binding of hemin to the particles during preparation of the medium.

Our study indicates that catalase production is not universal among all organisms in the *B. fragilis* group as reported by Hansen and Stewart (5a). However, almost all strains belonging to the species *B. fragilis*, which is the species of anaerobe most frequently isolated from clinical specimens, were positive by the catalase well test described above.

For a biochemical test to be of value to a clinical microbiologist, it must be reliable, rapid, and easy to perform. We have found that several variables, such as carbohydrate content of the medium and the available hemin concentration, can seriously affect catalase reactions. There-

fore, we recommend using the catalase well test in which equal volumes of a chopped meat culture and 3% H_2O_2 are mixed in either the well of a microtiter plate or in a small test tube. Production of bubbles, within 30 s, indicates a positive catalase reaction. A negative control of uninoculated chopped meat broth should be included in each series of tests. This test avoids the problems caused by carbohydrate repression and low available hemin concentrations. It is quick and reliable and utilizes chopped meat broth, a standard medium for growing anaerobes (6).

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