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Enzymatic Reactions of *Clostridium difficile* in Aerobic and Anaerobic Environments with the RapID-ANA II Identification System

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The RapID-ANA II anaerobic identification system (Innovative Diagnostic Systems, Inc., Atlanta, Ga.) was used to determine whether the incubation environment affects enzyme detection. Twenty strains of *Clostridium difficile* were tested in aerobic, anaerobic, and low-CO_2 anaerobic incubation environments. The percentages of enzymes detected in reactions with the following substrates were noted in the three incubation environments: phenylalanine-β-naphthylamide, aerobic, 0%; anaerobic, 35%; low-CO_2 anaerobic, 35%; arginine-β-naphthylamide, aerobic, 5%; anaerobic, 55%; low-CO_2 anaerobic, 75%; pyrrolidonyl-β-naphthylamide, aerobic, 5%; anaerobic, 65%; low-CO_2 anaerobic, 65%. When the aerobic incubation environment was compared with either the anaerobic or the low-CO_2 anaerobic incubation environments, the results were statistically different with respect to enzyme detection in reactions with the substrates listed above. The results for the anaerobic and low-CO_2 anaerobic environments were not statistically different. The study was repeated twice. Statistical comparisons between the three environments were consistent with the results presented above, with the following exceptions. The aerobic and the anaerobic environments were not different in a reaction with phenylalanine-β-naphthylamide in one of the runs, and there was no significant difference between the three environments in a reaction with arginine-β-naphthylamide in another run. These results suggest that some of the enzymes used in the identification of clinical anaerobes appear to be inactive in an environment containing oxygen.

Rapid anaerobic identification systems based on the detection of preformed enzymes (1, 4, 11, 13) rely on aerobic incubation in their methodologies. When bacterial cells are switched from anaerobic to aerobic environments, rapid inactivation of some enzymes is known to occur (6, 7, 9). The method of inactivation involves degradation of anaerobically specific proteins by reactive oxygen intermediates (2, 7). Intermediates formed by the exposure of cells to oxygen include the superoxide anion radical (O^{2-}), H_2O_2, and the hydroxyl radical (OH ·) (5). This oxidative inactivation of enzymes is presumed to proceed via the Fenton reaction, where H_2O_2 reacts with an iron(II) salt, ultimately forming the hydroxyl radical. Copper(I) salts may also react with H_2O_2 to form OH · . The hydroxyl radical is such an aggressive species that it reacts immediately with any biological molecule located nearby (6). Oxidation of amino acid residues occurs at or near the cation-binding site of an enzyme and generally inactivates the enzyme (2).

The purpose of the present study was to determine, using *Clostridium difficile*, whether a significant difference in enzyme detection exists between aerobic and anaerobic incubation of the RapID-ANA II anaerobic identification system (Innovative Diagnostic Systems, Inc., Atlanta, Ga.).

MATERIALS AND METHODS

Bacterial strains. The study included 20 strains of *C. difficile* that were arbitrarily labeled CD1 through CD20. All strains were received in chopped meat broth from the Clinical Microbiology Laboratory of the Minneapolis Veterans Affairs Hospital and were definitively identified by *Hin*dIII chromosomal restriction endonuclease analysis (8) by the Minneapolis Veterans Affairs Hospital. The 20 strains of *C. difficile* were plated from chopped meat broth onto prereduced anaerobically sterilized brucella blood agar (Anaerobe Systems, San Jose, Calif.) and were incubated anaerobically at 37°C. Colonial growth was harvested at 48 h and was then frozen at −70°C in 0.5 ml of freeze mix (Innovative Diagnostic Systems, Inc.).

RapID-ANA II system. The RapID-ANA II system identifies clinical anaerobic isolates by the reactions of preformed enzymes. The RapID-ANA II panel has 10 reaction wells; 8 of the wells are bifunctional; i.e., two separate tests are contained in each bifunctional well. Well 1 of the panel detects the increase in pH produced by the hydrolysis of urea with the development of a red or purple color. Wells 2 to 9 detect the release of yellow *o*- or *p*-nitrophenol from the enzymatic hydrolysis of the colorless nitrophenyl carbohydrate or phosphoester derivatives. Bacterial aminopeptidases hydrolyze β-naphthylamide derivatives of amino acids to release free β-naphthylamine. These are detected in bifunctional wells 3 to 9 by a modified cinnamaldehyde reaction, which produces a dark pink or purple color. After the addition of Innova spot indole reagent, 1% *p*-dimethylaminocinnamaldehyde in 10% hydrochloric acid, bifunctional well 10 detects the formation of indole by the development of a blue or blue-green color.

Five percent CO_2 is present in the anaerobic environment, and when dissolved in fluid it yields carbonic acid. Carbonic acid affects tests that are dependent on pH and, thus, can produce false-negative reactions in the urea well. To avoid the production of carbonic acid, 20 ml of 3 N NaOH was injected through a septum into a sealed 3-liter container

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TABLE 1. Comparison of *C. difficile* enzyme detection in reactions with PAL in different incubation environments

Run no. (n = 20)	No. (%) positive in the following incubation environment:			Pairwise comparisons[b]		
	Aerobic[a]	Anaerobic	Low-CO_2	A	B	C
1	0 (0)	7 (35)	7 (35)	$P < 0.02$*	$P < 0.02$*	$P > 0.05$
2	5 (25)	9 (45)	13 (65)	$P > 0.05$	$P < 0.01$**	$P > 0.05$
3	1 (5)	6 (30)	7 (35)	$P < 0.05$*	$P < 0.02$*	$P > 0.05$

[a] Run 1 was incubated at 35°C; the run was repeated at 37°C with a different lot of panels, and identical percent positive results were obtained.
[b] A, aerobic versus anaerobic incubation environment; B, aerobic versus low-CO_2 anaerobic incubation environment; C, anaerobic versus low-CO_2 anaerobic incubation environment. Asterisks indicate significant (*) and highly significant (**).

inside the anaerobic chamber to create a low-CO_2 anaerobic environment.

Test procedure. Each frozen vial of *C. difficile* was thawed anaerobically, and the contents were plated onto brucella blood agar. Plates were incubated for 48 h in a Bactron II anaerobic chamber (Anaerobe Systems) with an anaerobic environment of 5% CO_2, 5% H_2, and 90% N_2. Colonial growth was removed from the agar surface with a cotton swab and was suspended in RapID inoculation fluid at a turbidity equal to or slightly greater than a no. 3 McFarland turbidity standard, as determined by visual comparison. The suspensions were mixed and poured into RapID-ANA II panels and were inoculated as directed by the manufacturer. Inoculum waters and RapID-ANA II panels were inoculated one at a time. Three panels were inoculated for each 1 of the 20 bacterial strains.

The first set of the 20 inoculated panels was incubated anaerobically inside a sealed square plastic container (volume, 3 liter) lined with a paper towel and containing 20 ml of 3 N NaOH. This provided a reduced-CO_2 anaerobic environment. The partial CO_2 pressure was determined by using an IL 1306 pH-blood gas analyzer (Instrumentation Laboratory, Lexington, Mass.), which measures partial CO_2 pressure to 1.14%. The second set of panels was incubated anaerobically, and the third set was incubated aerobically. All panels were incubated at 37°C for 4 h, and reactions were read and interpreted according to the manufacturer's test interpretation guidelines.

The study was repeated twice (runs 2 and 3) as described above by using different lot numbers of RapID-ANA II panels. In addition, one set of panels was reduced for approximately 24 h in the low-CO_2 anaerobic environment, inoculated, and then incubated in the low-CO_2 anaerobic environment as described above for run 1.

Lastly, one randomly chosen *C. difficile* strain (strain CD12) which showed variable positive enzymatic reactions in the aerobic incubation environment in the first two runs was inoculated into two sets of 10 RapID-ANA II panels. One set was incubated aerobically, and one set was incubated anaerobically with decreased CO_2 as described above.

The statistical procedure used was Cochran's Q test (3, 12), and nonparametric post hoc pairwise comparisons were performed (10).

RESULTS

All strains reacted with proline-β-naphthylamide in all test environments. The numbers of *C. difficile* strains that reacted with phenylalanine-β-naphthylamide (PAL), arginine-β-naphthylamide (ARG), and pyrrolidonyl-β-naphthylamide (PYR) in each test environment are listed in Tables 1 to 3. Negative reactions were obtained with all other panel substrates. The results of the post hoc pairwise comparisons of the three test environments are also included in Tables 1 to 3. The three runs were compared by the number of *C. difficile* strains that reacted with PAL, ARG, and PYR in the three test environments (Fig. 1 to 3, respectively).

The strains incubated in anaerobically reduced panels gave positive reactions with the following substrates: PAL, 9 (45%); ARG, 20 (100%); PYR, 20 (100%); and serine-β-naphthylamide (SER), 7 (35%). The numbers of positive reactions of strain CD12 with substrates PAL, ARG, and PYR in aerobic and low-CO_2 anaerobic incubation environments are listed in Table 4.

DISCUSSION

The aminopeptidase which hydrolyzes proline-β-naphthylamide was detected in all strains in each incubation environment tested and appeared to be unaffected by the presence of oxygen. The ability to detect the aminopeptidases which hydrolyze PAL, ARG, and PYR was decreased under aerobic conditions in the present study. In addition, panels incubated aerobically showed a greater variation in within-strain enzymatic reaction than did panels incubated anaerobically (Table 4). The proposed explanation for the difference in enzyme detection between aerobic and anaerobic incubation environments is the inactivation of the aminopeptidases by reactive oxygen intermediates created through reaction with molecular oxygen (6).

TABLE 2. Comparison of *C. difficile* enzyme detection in reactions with ARG in different incubation environments

Run no. (n = 20)	No. (%) positive in the following incubation environment:			Pairwise comparisons[b]		
	Aerobic[a]	Anaerobic	Low-CO_2	A	B	C
1	1 (5)	11 (55)	15 (75)	$P < 0.01$**	$P < 0.001$**	$P > 0.05$
2	12 (60)	17 (85)	18 (90)	$P < 0.05$*	$P < 0.02$*	$P > 0.05$
3	9 (45)	11 (55)	13 (65)	$P > 0.05$	$P > 0.05$	$P > 0.05$

[a] Run 1 was incubated at 35°C; the run was repeated at 37°C with a different lot of panels, and identical percent positive results were obtained.
[b] A, aerobic versus anaerobic incubation environment; B, aerobic versus low-CO_2 anaerobic incubation environment; C, anaerobic versus low-CO_2 anaerobic incubation environment. Asterisks indicate significant (*) and highly significant (**).

TABLE 3. Comparison of *C. difficile* enzyme detection in reactions with PYR in different incubation environments

Run (n = 20)	No. (%) positive in the following incubation environment:			Pairwise comparisons[b]		
	Aerobic[a]	Anaerobic	Low-CO_2	A	B	C
1	1 (5)	13 (65)	13 (65)	$P < 0.001$**	$P < 0.001$**	$P > 0.05$
2	3 (15)	15 (75)	17 (85)	$P < 0.001$**	$P < 0.001$**	$P > 0.05$
3	2 (10)	12 (60)	14 (70)	$P < 0.01$**	$P < 0.001$**	$P > 0.05$

[a] Run 1 was incubated at 35°C; the run was repeated at 37°C with a different lot of panels, and identical percent positive results were obtained.
[b] A, aerobic versus anaerobic incubation environment; B, aerobic versus low-CO_2 anaerobic incubation environment; C, anaerobic versus low-CO_2 anaerobic incubation environment. Asterisks indicate significant (*) and highly significant (**).

Because enzymatic reactivity with serine-β-naphthylamide occurred only in the prereduced panels, it appears that serine aminopeptidase may be affected by the oxygen dissolved in the panel's plastic molding. It should also be noted that the colors of the positive reactions in the prereduced panels were darker and therefore easier to interpret than those of any other panel runs.

The number of positive enzymatic reactions, particularly those with PAL and ARG, varied greatly between the runs in all incubation environments (Fig. 1 to 3). This variation may partially be explained by the presence of oxygen in the plastic molding of the panels. The oxygen appears to affect mainly the aminopeptidases which hydrolyze ARG and PYR, which were detected at 100% in the prereduced panel run. The number of positive reactions with PAL in the prereduced panel run was not significantly different from the reactions with PAL in runs 1 to 3. This suggests that not all *C. difficile* strains possess the phenylalanine aminopeptidase. Investigation of this possibility was not included in the present study.

The number of strains positive with PAL, ARG, and PYR in all incubation environments was consistently higher in run 2 than in runs 1 or 3 (Fig. 1 to 3). The presentation of this variability, as well as the variability between and within *C. difficile* strains, suggests that the process of enzymatic inactivation by reactive oxygen intermediates is variable in occurrence and in degree. There is a possibility that enzymes, such as catalase or superoxide dismutase, that provide defense to some anaerobic cells against reactive oxygen intermediates may protect anaerobic enzymes against inactivation (7). More studies need to be performed to determine whether the inactivity of these anaerobic enzymes is in fact due to their reaction with oxygen and whether some anaerobes provide enzymatic defense against this inactivation.

As previously determined by Chevalier et al. (2), the extent of oxygen inactivation varies inversely with bacterial cell density; the enzyme studied was not inactivated when cell density exceeded 2.5×10^8 cells per ml. This phenomenon was not observed in the present study. Cell density achieved by inoculation to a turbidity equal to that of a no. 3 McFarland standard is 9×10^8 cells per ml, yet apparent inactivation of phenylalanine, arginine, pyrrolidonyl, and serine aminopeptidase occurred during aerobic incubation. Further study is needed to determine the effect of cell density on the extent of oxygen inactivation.

The results of the present study suggest that some enzymes used to identify anaerobes are detected less frequently and react with greater variability in an environment containing oxygen. Further studies should reveal whether the enzymes of all clinically isolated anaerobes are detected more frequently under anaerobic incubation conditions and whether prereduced panels are required for accurate enzyme detection.

Work flow needs only slight modifications to accommodate anaerobic incubation of RapID-ANA II identification panels. Anaerobic incubation of the panels necessitates an

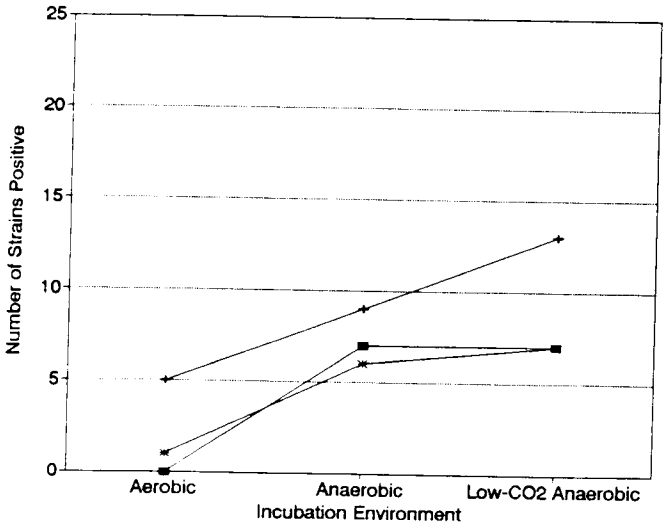

FIG. 1. Comparison of three runs by the enzymatic reaction of 20 *C. difficile* strains with PAL in different incubation environments. ■, run 1; +, run 2; *, run 3.

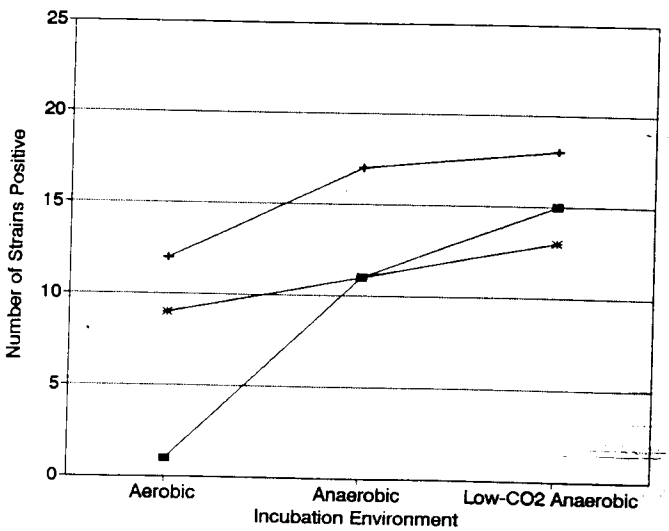

FIG. 2. Comparison of three runs by the enzymatic reaction of 20 *C. difficile* strains with ARG in different incubation environments. ■, run 1; +, run 2; *, run 3.

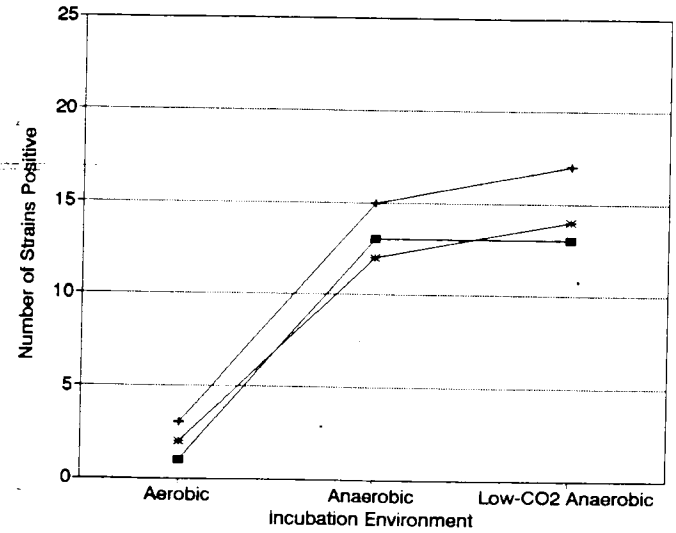

FIG. 3. Comparison of three runs by the enzymatic reaction of 20 *C. difficile* strains with PYR in different incubation environments. ■, run 1; +, run 2; *, run 3.

additional entry into the anaerobic chamber or requires the use of an additional anaerobic biobag. Panels may be reduced on the same day that isolated colonies are subcultured for identification testing. This strategy will avoid additional delay in reporting results.

TABLE 4. Comparison of *C. difficile* CD12 enzyme detection in aerobic and low-CO_2 anaerobic incubation environments at 37°C

Substrate	No. (%) positive in the following incubation environment:	
	Aerobic (n = 10)	Low-CO_2 (n = 10)
PAL	3 (30)	9 (90)
ARG	8 (80)	10 (100)
PYR	0 (0)	5 (50)

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