

Evaluation of the AnaeroPack System for Growth of Anaerobic Bacteria

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Growth of anaerobic bacteria in the AnaeroPack (Mitsubishi Gas Chemical America, Inc., New York, N.Y.) anaerobic atmosphere generation systems, both the AnaeroPack jar and pouch and the AnaeroPack in a GasPak jar were considered equivalent to or better than growth obtained in the corresponding GasPak jar or pouch system (Becton Dickinson Microbiology Systems, Cockeysville, Md.) for 89 (86%) of the 103 anaerobes tested. There were a total of 26 discrepancies after 48 h of incubation, with 16 discrepancies unresolved after 96 h of incubation. The AnaeroPack jar and pouch never failed to reduce the anaerobic indicator. The AnaeroPack systems are easy to use and performed at least as well as or better than the BBL GasPak systems for growth of anaerobic bacteria.

Anaerobic bacteria cause a variety of human infections ranging from mild to severe (3). Methods used to culture anaerobes in the clinical laboratory often include an airtight jar with an anaerobic atmosphere gas generator or with a gas evacuation-replacement system, an anaerobic bag or pouch, and the anaerobic chamber or glove box. Anaerobic jar systems with gas generators and anaerobic pouch systems were reported in one survey to be the most widely used methods for culture of anaerobes in clinical laboratories in the United States (4). The AnaeroPack system is a new anaerobic atmosphere generation system for use in anaerobic jars and pouches. The AnaeroPack system, similar to the Unipath AnaeroGen system, which is also manufactured by Mitsubishi Gas Chemical America, Inc. (New York, N.Y.), is a sachet that is placed directly into the jar or pouch without the need either for a catalyst or for the addition of reagent or water. The AnaeroPack sachet, upon exposure to air, rapidly removes the oxygen to a residual atmosphere of less than 0.1% within 1 h with no hydrogen production and produces an anaerobic atmosphere that contains approximately 20% CO₂ (9).

We compare the AnaeroPack sachet in two jar systems and the AnaeroPouch Anaero sachet (Mitsubishi Gas Chemical America, Inc.) in a resin pouch to the corresponding GasPak jar with GasPak Plus envelope and to the GasPak pouch (Becton Dickinson Microbiology Systems, Cockeysville, Md.) for the ability to grow a variety of clinical isolates of anaerobic bacteria.

(A preliminary report of this work was presented previously [10].)

A total of 103 strains of anaerobic bacteria were tested for growth with three AnaeroPack methods, the AnaeroPack sachet in a 3.2-liter airtight, rectangular AnaeroPack jar, the AnaeroPack sachet in a 2.5-liter GasPak jar, and the smaller AnaeroPouch Anaero sachet in an AnaeroPack resin pouch. Anaerobe growth in the AnaeroPack systems was compared to growth in the 2.5-liter GasPak jar with GasPak Plus envelope and to growth in the GasPak pouch. The test organisms included 96 recent clinical isolates of *Bacteroides* (29 strains, 8

species), *Fusobacterium* (7 strains, 3 species), *Prevotella* (12 strains, 5 species), *Clostridium* (31 strains, 13 species), *Peptostreptococcus* (9 strains, 3 species), *Actinomyces* (2 strains, 2 species), *Bifidobacterium* (2 strains), and one each of *Eubacterium*, *Propionibacterium*, *Veillonella*, and *Streptococcus*. Seven reference strains were also tested; the seven reference strains were *Bacteroides fragilis* ATCC 25285, *Bacteroides uniformis* ATCC 8492, *Porphyromonas gingivalis* ATCC 33277, *Clostridium sordellii* ATCC 9714, *Fusobacterium nucleatum* ATCC 25586, *Peptostreptococcus magnus* ATCC 29328, and *Eubacterium lentum* ATCC 25559. All clinical isolates were identified by the RapID ANA II system (Innovative Diagnostics, Inc., Norcross, Ga.).

A suspension of each freshly grown anaerobe, less than 72 h old, equivalent to the turbidity of a 0.5 McFarland standard was prepared in sterile saline. Each suspension was diluted further in saline to achieve a final organism concentration of approximately 10⁴ CFU/ml. For each anaerobe system tested, the organism was inoculated with a 10- μ l calibrated loop to achieve an approximate final inoculum per plate of 30 to 500 CFU, to two PRAS brucella blood agar plates (BRU; Anaerobe Systems, San Jose, Calif.) and to two Trypticase soy blood agar plates (TSBA; Becton Dickinson Microbiology Systems) reduced at least 24 h prior to inoculation. Four plates for each test organism, 2 BRU and 2 TSBA, were placed into each anaerobe test system. Each anaerobe atmosphere generation system was charged, and a BBL Dry Anaerobic Indicator strip (Becton Dickinson Microbiology Systems) was added to each system. Each system was closed securely, and each system was incubated at 35°C for at least 48 h prior to initial examination. A maximum of one test organism was incubated in each pouch, and two or three organisms were incubated in each jar system.

To charge the anaerobic atmosphere generation systems, one AnaeroPack sachet was placed directly into an AnaeroPack 3.2-liter rectangular container and immediately closed. One AnaeroPack sachet was placed directly, without reagent or catalyst, into a 2.5-liter GasPak jar and immediately closed. One AnaeroPouch Anaero sachet was placed directly into an AnaeroPack pouch and immediately closed with a sealing bar. A GasPak Plus anaerobic system envelope with palladium catalyst was placed into a 2.5-liter GasPak jar, and 10 ml of water was added to the envelope immediately prior to jar closure. GasPak Pouch reagent was added to the reagent channel of a

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TABLE 1. Comparison of five anaerobic atmosphere systems for growth of anaerobes

Anaerobe	No. of strains tested	No. of discrepant strains ^a	Percent agreement (no. of discrepancies ^b)				
			AnaeroPack jar	AnaeroPouch	AnaeroPack-GasPak jar	GasPak Plus jar	GasPak Pouch
<i>Bacteroides</i>	31	4	97 (1)	100	97 (1)	87 (6)	97 (1)
<i>Fusobacterium</i>	8	2	100	88 (1)	100	100	100
<i>Prevotella</i> and <i>Porphyromonas</i>	13	2	100	100	92 (1)	100	100
<i>Clostridium</i>	32	5	97 (1)	100	91 (3)	88 (4)	100
Cocci	12	2	92 (1)	92 (1)	92 (1)	83 (2)	92 (1)
NSF GMPOS bacilli ^c	7	1	100	100	86 (1)	100	100
Total	103	14	97 (3)	98 (2)	93 (7)	88 (12)	98 (2)

^a More than one anaerobic system discrepancy may be noted for each discrepant strain.

^b Total number of discrepancies after 48 h of incubation.

^c NSF GMPOS bacilli, nonsporeforming gram-positive bacilli.

GasPak Pouch, and the pouch was immediately closed with a sealing bar. The anaerobic chamber was not tested in this study, even though more consistent results may be obtained in a chamber than in a jar or pouch system (2, 7). The rate and quality of growth of anaerobes with the AnaeroPack have been reported to be similar to the rate and quality of growth of anaerobes when incubated in an anaerobic chamber (1).

All media were marked with a code for the anaerobic test system upon removal from that system and prior to examination and scoring to minimize any examination bias. All plates were examined and interpreted by two microbiologists working independently. Each plate was read initially after at least 48 h of incubation. Plates with sparse or no growth, slow growers, or those with discrepant results were reincubated in the same test systems for an additional 48 h (96 h total). Organism colony counts were obtained for both types of media incubated in each system. Colony counts were considered equivalent if there was less than a 10^1 reduction in growth comparing the CFU in each system to the highest CFU obtained for that anaerobe.

The quality of growth for each organism was based on typical colony morphology characteristics and included colony shape, size, hemolysis, and pigment. A growth characteristic value (GCV) from 0 (no growth) to 5 (the most typical or optimal colony morphology) was obtained based on the observed quality of growth scored by the average of the two microbiologists' readings. At least one plate for each anaerobe with optimal or most typical characteristics was given a GCV of 5, and growth of all others was compared to that GCV. A GCV of 4 was considered slightly less than typical, while a GCV of 3 or below represented a clear decrease in quality of growth as determined by colony morphology characteristics. The quality of growth of the anaerobes tested in each system was considered equivalent to that of the other systems if the average GCV of the four total readings with one medium (two plates with two readings) was within 2 GCV of the highest average GCV for that anaerobe.

Colony counts and GCV were considered equivalent after 48 h of anaerobic incubation in all five systems for 89 of the 103 (86%) anaerobes tested (Table 1). The overall quantity and quality of growth of the anaerobes in the two AnaeroPack jar systems (AnaeroPack Jar, 97%; GasPak jar, 93%) appeared to be slightly better than growth in the GasPak Plus jar system (88%) after 48 h of incubation. Growth of the anaerobes in the AnaeroPouch Anaero and GasPak pouch systems was equivalent (Table 1). To our knowledge, this is the first report that evaluates the AnaeroPouch Anaero system; overall growth of the anaerobes tested in the AnaeroPack jar system compares

favorably with results obtained previously (1). Our results are also comparable to results obtained with a similar product, the AnaeroGen system, which is also manufactured by Mitsubishi Gas Chemical Co. (7). Delaney and Onderdonk (1) suggested that the slightly improved growth in the AnaeroPack system may have been due to the increased CO₂ concentrations generated by the AnaeroPack sachet. The improved growth could also be due to the rapidity with which the AnaeroPack achieves anaerobic conditions, reported to be approximately 30 min (9) compared to the approximately 70 to 130 min reported for the GasPak systems (5).

There were a total of 26 system discrepancies detected after 48 h of incubation where the anaerobe grew either with at least a 10^1 CFU reduction or with a GCV of ≥ 2 lower than the value for the best growth in a system. After 96 h of incubation, 10 of the 26 discrepancies were resolved, while 16 remained unresolved (Table 2). In the AnaeroPack jar, one *Peptostreptococcus anaerobius* and one *Bacteroides caccae* failed to grow on TSBA after 96 h, while the quality and quantity of growth on BRU for these two strains was equivalent throughout the other test systems. Quality of growth after 96 h of the same *P. anaerobius* strain noted above was also very poor on TSBA (compared to BRU growth) in the AnaeroPouch, failed to grow on this medium in all other systems, and was considered a discrepancy in each of the five systems. One *F. nucleatum* failed to grow on BRU after 96 h in an AnaeroPouch, while the quantity and quality of BRU growth were equivalent in the other systems (CFU range, 85 to 190; GCV, 4.5 to 4.8). After 96 h of incubation in a GasPak jar with an AnaeroPack sachet added, one *Clostridium butyricum* and one *Clostridium difficile* failed to grow on TSBA, one *Prevotella bivia* failed to grow on BRU, and one *Eubacterium limosum* grew on both media with growth at least 10^1 CFU less (1 CFU on BRU and 22 CFU on TSBA) than growth detected in the other systems (approximately 300 CFU on both media for all other systems). There were six GasPak Plus jar discrepancies and one GasPak pouch discrepancy (Table 2).

A variety of media are available for use by clinical laboratories for the cultivation of anaerobic bacteria. A PRAS brucella agar-based blood agar was used throughout this study and has been reported to be superior to other brucella agar-based media for recovery of anaerobic bacteria (6). A blood agar base (TSBA) often routinely used for isolation of aerobic microorganisms was also used but it was reduced in an anaerobic jar system 24 h prior to use. Growth of the anaerobes tested on BRU was observed to be consistently better than growth on TSBA with 22 of the 26 (85%) discrepancies after 48 h of

TABLE 2. Unresolved anaerobe growth discrepancies in five anaerobe atmosphere systems after 96 h of incubation

System	Organism	Discrepancy
AnaeroPack jar	<i>B. caccae</i>	Failed to grow on TSBA
	<i>P. anaerobius</i>	Failed to grow on TSBA
AnaeroPack-GasPak jar	<i>P. bivia</i>	Failed to grow on BRU
	<i>C. difficile</i>	Failed to grow on TSBA
	<i>C. butyricum</i>	Failed to grow on TSBA
	<i>P. anaerobius</i>	Failed to grow on TSBA
	<i>E. limosum</i>	Decreased CFU on BRU and TSBA ^a
GasPak Plus jar	<i>P. bivia</i>	Failed to grow on TSBA
	<i>C. sporogenes</i>	Failed to grow on TSBA
	<i>P. anaerobius</i>	Failed to grow on TSBA
	<i>B. distasonis</i>	Decreased CFU on BRU (15 vs 300 CFU)
	<i>P. asaccharolyticus</i>	Failed to grow on BRU and TSBA
	<i>C. difficile</i>	Decreased CFU on BRU and TSBA ^b
AnaeroPouch	<i>F. nucleatum</i>	Failed to grow on BRU
	<i>P. anaerobius</i>	Decreased CFU and GCV on TSBA ^c
GasPak Pouch	<i>P. anaerobius</i>	Failed to grow on TSBA

^a Decreased CFU at least 10¹ below the highest system, decreased GCV by ≥2 below the highest graded system (noted system versus the other four systems).

^b 1 CFU on BRU and 1 CFU on TSBA compared to 100 and 95 CFU by the other two jar systems.

^c Growth quantity and quality compared to growth on BRU.

incubation noted on TSBA. This high number of discrepancies for one medium but not for the other indicates that the medium may have been responsible for the discrepancies and not the anaerobic atmosphere generation system. Only two strains of *C. difficile* (one in an AnaeroPack-GasPak jar and one in a GasPak Plus jar), and one each of *E. limosum* (in an AnaeroPack-GasPak jar) and *Peptostreptococcus asaccharolyticus* (in a GasPak jar) grew poorly or not at all on both TSBA and BRU, indicating that the discrepancies most likely occurred as a result of the anaerobic atmosphere generation system and were not due to the medium. It is unknown why these discrepancies occurred, since the methylene blue indicator did not reveal any anaerobic atmosphere failure. In contrast, when grown on BRU, only one *F. nucleatum* grown in an AnaeroPouch and one *P. bivia* grown in a GasPak jar with an AnaeroPack sachet failed to grow, while growth on TSBA was equivalent in the systems tested. Trypticase soy-based agar, sometimes used as a blood-containing primary anaerobic isolation medium (4), is not recommended for use as a primary isolation medium for anaerobes (8). These results reaffirm that TSBA is not a reliable medium to obtain consistent growth of anaerobes and should not be used for routine isolation of anaerobes even if reduced prior to use. Although we did not test a selective medium, the AnaeroPack system has been reported to support good growth of anaerobes on a kanamycin-vancomycin laked sheep blood medium (1). Other anaerobic medium formulations must be tested to ensure that the atmosphere generated by the AnaeroPack systems (approximately 20% CO₂) is compatible with these other types of media for growth of anaerobic bacteria.

Each of the 103 test organisms was incubated in a separate

AnaeroPouch and GasPak Pouch with no AnaeroPouch atmosphere failures and only one GasPak Pouch failure (<1% failure rate) as detected with the methylene blue indicator strip. The one GasPak Pouch failure most likely occurred toward the end of incubation, since the test organism grew as well in the GasPak Pouch as in the other systems and was not considered a discrepancy. The AnaeroPouch sealing bar clips were observed to give an apparently tighter and more-secure pouch closure than the GasPak sealing bar clips, even though neither system had any significant anaerobic atmosphere failures. A total of 46 jars were set up for each system with up to three test organisms incubated in each jar system. There were no anaerobic atmosphere failures detected for the AnaeroPack jar, and there were four failures (9% failure rate) detected for the AnaeroPack sachet placed into a GasPak jar and 10 failures (22% failure rate) detected for the GasPak Plus jar, as detected with the methylene blue indicator strip. These jar failure rates are similar to indicator detected failure rates reported by others for these jar systems (1, 5, 7). All test systems were tested again if growth discrepancies occurred for any anaerobe in any system in which an anaerobic atmosphere failure was detected. The cause of these jar failures was not determined. However, each GasPak jar was used for multiple tests with no jar failing on a consistent basis. Also, all GasPak jars used for this study were of good quality and used routinely in this laboratory. Failures with the GasPak jars could have been due to inadequate tightening of the screw-down lid or inadequate lid O-ring sealing. We used the 3.2-liter AnaeroPack jar for this study; however, Mitsubishi is currently marketing a 2.5-liter jar. The 2.5-liter AnaeroPack jar with the AnaeroPack sachet has been reported to support good growth of anaerobes and to also be reliable in producing and maintaining an anaerobic atmosphere (1). The AnaeroPack jar has four clip-down side bars for easy closure and sealing of the lid with no need for tightening a screw. Since there were no AnaeroPack jar atmosphere failures detected, this jar closure system appears to be more efficient than jar systems with screw-down lids.

The AnaeroPack and AnaeroPouch systems are acceptable alternatives to other jar and pouch systems for the cultivation of anaerobic bacteria. The AnaeroPack and AnaeroPouch systems are easy to use, with no requirement for the addition of water, reagent, or catalyst. The production of 20% CO₂ by the AnaeroPack systems does not appear to have an adverse effect on anaerobe growth and may actually enhance growth of some anaerobes. The clip-down lid closure method of the AnaeroPack jar may help to prevent anaerobic atmosphere failures in these jars. Since this study challenged the AnaeroPack systems only with known cultures of anaerobes, future testing of the AnaeroPack systems could include direct testing of clinical specimens.

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