*2008*年 *8*月

博士學位論文

The Growth of Obligate Anaerobic Bacteria on Brain Heart Infusion Agar by a New Disposable Anaerobic Gas System

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이 論文을 醫學博士學位 論文으로 提出함

*2008*年 *04*月 日

朝鮮大學校 大學院

醫學科

金 振 萬

金振萬의 博士學位論文을 認准함

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*2008*年 *06*月　　日

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新 一回用 無酸素 시스템을 이용한 *BHI agar*에서 절대 無酸素 細菌의 成長에 관한 연구

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임상에서 흔히 분리되는 13가지 절대 무산소 세균 종들이 모두 brain heart infusion (BHI) agar (DIFCO, USA)에서 48시간 내에 배양되었다. 배양에 필요한 엄격한 무산소 환경은 저자가 고안한 일회용 무산소 봉투(DASTech, Gwangju, KOREA), 팔라듐 촉매 10 그람(Heesung Engelhard, Korea) 그리고 역시 저자가 새로 고안한 일회용 무산소 가스팩(H_2 + CO_2, DASTech, Gwangju, KOREA)을 사용하여 조성하였다. 실험에 사용된 한천배지는 환원 혹은 비환원 BHI agar, 5% 사람 적혈구 용해액을 함유한 환원 혹은 비환원 BHI agar, 그리고 5% 사람 적혈구 용해액을 함유한 Wilkins-Chalgren agar 였다. 한천배지의 환원은 사용 전 24시간 동안 무산소 봉투에서 이루어졌다. 절대 무산소 세균 13주 가운데 11주가 비환원 BHI agar에서 성장하였고 그 리스트는 다음과 같다. *Bacteroides fragilis* (ATCC 25285), *Bacteroides vulgatus* (KCTC 2639), *Bifidobacterium bifidum* (KCTC 3281), *Clostridium difficile* (KCTC 5009), *Clostridium perfringens* (KCTC 3269), *Clostridium septicum* (ATCC 12464), *Eubacterium limosum* (KCTC 3266), *Fusobacterium nucleatum subs. polymorphum* (KCTC 2488), *Peptostreptococcus asaccharolyticus* (KCTC 3321), *Propionibacterium acnes* (KCTC 3314), *Veillonella criceti* (ATCC 17747). 그러나 *Mobiluncus mulieris* (ATCC 35239)와 *Porphyromonas gingivalis* (ATCC 33277)는 환원

된 BHI agar에서만 성장하였다. 13 균주 모두 5% 사람 적혈구 용해액이 함유되지 않은 환원된 BHI agar에서 충분한 성장을 보였다. 이 결과는 이제까지 국내외 표준균주 공급처들이 제시하는 까다로운 배지의 조성들을 적용하지 않더라도 BHI agar만으로도 충분히 상기 균주들에 대한 실험을 할 수 있음을 의미한다.

Keywords: Anaerobic bacteria, brain heart infusion agar, disposabe anaerobic gas system

INTRODUCTION

Many anaerobic bacteria researchers have ever felt embarrassed in the choice of media for anaerobic bacteria culture. It is because they often experience difficulty to purchase or make anaerobic culture media recommended by biological resource centers like American Type Culture Collection, Korean Collection for Type Cultures, etc. A number of anaerobic bacteria researchers have generally used disposable anaerobic gas pack in the anaerobic jar or disposable anaerobic pouch (BBL, BECTON DICKINSON, USA) (MERCK, GERMANY) for a simple anaerobic culture. Author had difficulties several times in anaerobic bacteria culture using BBL anaerobic gas pack system. Its preparation time of the absolute anaerobic condition was always over 2 hours, and strict anaerobic bacteria like *Porphyromonas gingivalis* or *Mobiluncus mulieris* did often grow not or very slowly under BBL anaerobic gas system. Author has therefore developed a new disposable anaerobic gas system being able to create the absolute anaerobic condition within 20 minutes. The aim of this study was to evaluate whether well-known obligate anaerobic bacteria will grow on unreduced BHI agar under aerobic manipulation and the new anaerobic gas system or not.

MATERIALS AND METHODS

1. Type strains

Type strains used in this experiment are listed at Table 1.

2. Media

a) Brain heart infusion agar (DIFCO, USA)

b) Human erythrocyte lysate supplement

Citrated-human blood was centrifuged at 2,500 rpm for 15 minutes in a table-top centrifuge with a rotating radius of 14cm, and the supernatant was removed by aspiration. The packed cells were resuspended with phosphate buffered saline (0.01M, pH 7.2) and centrifuged. This wash procedure was repeated twice. Finally, the volumetric conical tubes (50㎖, SUPERCON®, IL-SIN, Seoul, Korea) which contain packed cells were resuspended with distilled water to restore their volumes to whole blood volume. Subsequently, the resuspended erythrocyte solution was poured into flexible plastic bottles (125㎖, REDI-PAK®, WHEATON, MILLVILLE, NJ, USA). Each bottle which contain 50㎖ of erythrocyte suspension was frozen for 20 minutes in a deep freezer (-80℃). After that, bottles were thawed promptly. This freezing and thawing procedures were repeated 5 times for the complete hemolysis of erythrocyte. The hemolyzed erythrocyte solution was diluted two and a half times with distilled water and centrifuged at 15,000 rpm for one hour in a refrigerating centrifuge (VS-21SR, Vision Scientific Co., Seoul, Korea). The supernatant of hemolyzed erythrocyte solution was carefully collected, filter-sterilized by a disposable syringe filter (0.45㎛, CORNING®, Corning Incorporated Corning NY 14831, Germany) and stored in a freezer (-20℃).

c) Wilkins-Chalgren anaerobe agar (OXOID, England)

3. Disposable anaerobic gas system

For the condition of anaerobic environment, author developed the new disposable anaerobic gas system (DASTech, Gwangju, Korea).(1) (2) Its chemical principle of hydrogen generation is as follows. Silica (SiO_2) and sodium borohydride ($NaBH_4$) tablets react with water, and volatile hydride (SiH_4) is generated. Its presence was demonstrated by Inductively Coupled Plasma-Mass Spectrometry (ICP-MS, Thermo Electron Corporation 81 Wyman Street Waltham, MA 02454-9046 United States) (Fig. 1) and hydride generator (SPECTROFLAME, SPECTRO A.I. GmbH Boschstr 10, D-47533 Kleeve, Germany). The chemical reaction formula of volatile hydride generation is deduced as follows; $BH_4^- + 15H^+ + 3SiO_2 \rightarrow H_3BO_4 + 3SiH_4\uparrow + 2H_2O$

Hydrogens of volatile hydride combine easily with oxygen by palladium catalyst under a closed system (DASTech, Gwangju, Korea) and become water vapor. The disposable anaerobic gas pack (Fig. 2) consisted of two parts. Hydrogen generation part contains two sodium borohydride tablets (each tablet weight is 560㎎) and silica (SiO_2, 250㎎). Carbon dioxide generation part contains one citric acid tablet (580㎎) and one sodium bicarbonate tablet (520㎎). Each part has one narrow tipped plastic tube, and each 10㎖ of tap water is poured into the plastic tubes. Catalyst is 10g of 0.5% palladium coating alumina pellets (Heesung Engelhard, Korea).

4. Culture condition

BHI agar, BHI agar supplemented with 5% human erythrocyte lysate and Wilkins-Chalgren anaerobe agar supplemented with 5% human erythrocyte lysate were used in state of reduction or non-reduction. The disposable anaerobic envelope having used in this experiment is shown in Figure 3. All type-strains were handled in the aerobic condition excepting culture condition (strict anaerobic, 5~10% CO_2, 37℃).

RESULTS

Eleven strains have well grown on unreduced BHI agar except *Mobiluncus mulieris* and *Porphyromonas gingivalis* (Table 1). Both strains have grown on reduced BHI agar, but *Porphyromonas gingivalis* has not grown even on reduced Wilkins-Chalgren anaerobe agar supplemented with 5% human erythrocyte lysate (Table 2). *Porphyromonas gingivalis* has grown on reduced BHI agar with supplemented with 5% human erythrocyte lysate better than other media (Table 2).

DISCUSSION

The aim of this study was to evaluate whether well-known obligate anaerobic bacteria will grow on unreduced BHI agar under aerobic manipulation and the new anaerobic gas system[1, 2] or not. Eleven strains of obligate anaerobic bacteria except *Mobiluncus mulieris* (ATCC 35239) and *Porphyromonas gingivalis* (ATCC 33277) showed normal growth on unreduced BHI agar, reduced BHI agar, BHI agar supplemented with 5% human erythrocyte lysate and Wilkins-Chalgren anaerobe agar supplemented with 5% human erythrocyte lysate after 48 hours culture. There was not any distinction in colony sizes between media. *Mobiluncus mulieris* and *Porphyromonas gingivalis* did not grow on unreduced media. *Porphyromonas gingivalis* did not grow even on reduced Wilkins-Chalgren anaerobe agar, but on reduced BHI agar and reduced BHI agar supplemented 5% human erythrocyte lysate. These results were gotten by use of author's new anaerobic gas pack and 10g of sphere type palladium catalyst, but comparative study was not done between the new anaerobic gas system and BBL anaerobic gas system. Many anaerobic microbe researchers are perplexed with difficulty of purchase or making anaerobic culture media recommended by biological resource centers like American Type Culture Collection, Korean Collection for Type Cultures, or several textbooks. Their re-commanding media often contain ingredients like vitamin K1, menadione, haemin, sheep or horse blood, and several extracts. It is, however, easy to purchase brain heart infusion broth or agar, and they are widely used. BHI agar supplemented with yeast extract, haemin and menadione were used by Sutter for *Bacteoides fragilis* culture[8], Markowitz for *Clostridium difficile*[6], Edwards for *Bacteroides* spp.[4], Brazier for *Fusobacterium* spp.[3]. These bacteria were grown on unreduced BHI agar without above ingredients. Silva had used

non-supplemented BHI agar for *Prevotella* spp. culture[7] and Garca-Rodriguez for *Clostridium perfringens*[5]. Author cannot explain the difference of researchers about selection of medium, but can point out that several obligate anaerobes have been able to be cultured on unreduced or reduced BHI agar non-supplemented with above ingredients. It is 'however' suggested that more obligate anaerobes should be tested under the new anaerobic culture system.

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Table 1. The growth result of several obligate anaerobes on the non-reduced media after 48 hours

Type strains	Non-reduced BHI[a] agar	Non-reduced BHI agar supplemented with 5% human erythrocyte lysate	Non-reduced WC[b] agar supplemented with 5% human erythrocyte lysate
Bacteroides fragilis (KCTC 25285)	++	++	++
Bacteroides vulgatus (KCTC 2639)	+	++	++
Bifidobacterium bifidum (KCTC 3282)	++	++	+
Clostridium difficile (KCTC 5009)	++	++	++
Clostridium perfringens (KCTC 3269)	++	++	++
Clostridium septicum (ATCC 12464)	++	++	++
Eubacterium limosum (KCTC 3314)	++	++	++
Fusobacterium nucleatum subsp. polymorphum (KCTC 2488)	++	++	++
Mobiluncus mulieris (ATCC 35239)	-	-	-
Peptostreptococcus asaccharolyticus (KCTC 3321)	++	++	++
Porphyromonas gingivalis (ATCC 33277)	-	-	-
Propionebacterium acnes (KCTC 3314)	++	++	++
Veillonella criceti (ATCC 17747)	++	++	++

a; Brain Heart Infusion

b; Wilkins-Chalgren

Table 2. The growth result on the reduced media after 48 hours

Type strains	Reduced BHI agar	Reduced BHI agar supplemented with 5% human erythrocyte lysate	Reduced WC agar supplemented with 5% human erythrocyte lysate
Mobiluncus mulieris (ATCC 35239)	+	+	++
Porphyromonas gingivalis (ATCC 33277)	++	+++	-

Figure 1. Hydrogen generator

Figure 2. The disposable anaerobic gas pack developed by author

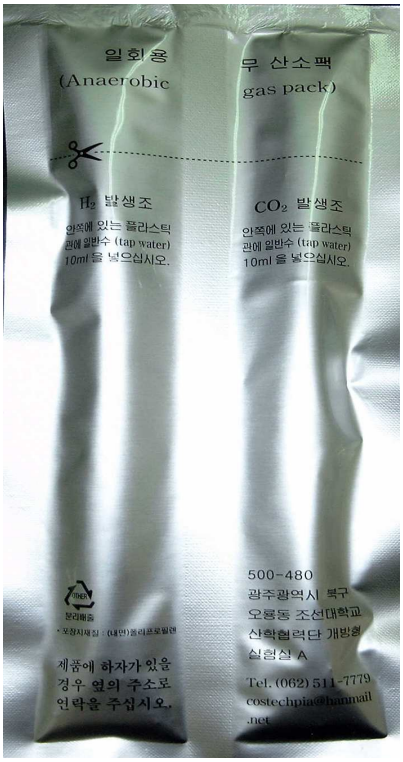
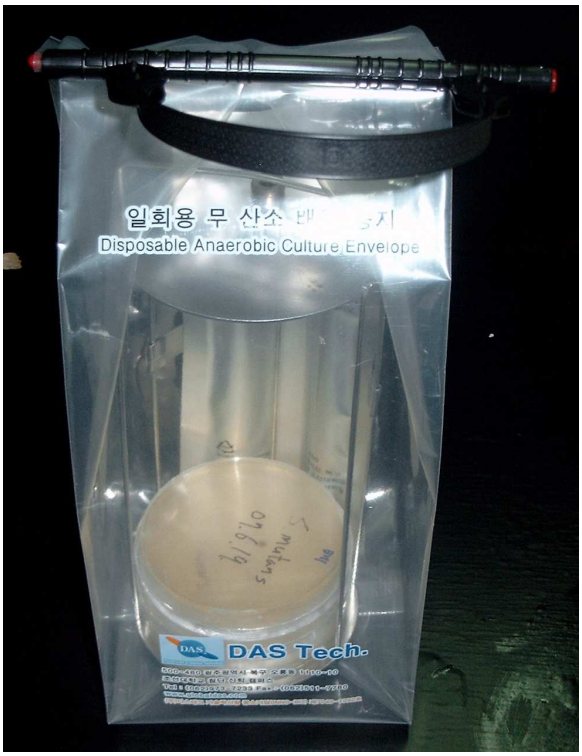


Figure 3. The disposable anaerobic culture system devised by author.



저작물 이용 허락서

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논문제목	한글 : 新 一回用 無酸素 시스템을 이용한 BHI agar에서 절대 無酸素 細菌의 成長에 관한 연구				
	영문 : The Growth of Obligate Anaerobic Bacteria on Brain Heart Infusion Agar by a New Disposable Anaerobic Gas System				

본인이 저작한 위의 저작물에 대하여 다음과 같은 조건 아래 조선대학교가 저작물을 이용할 수 있도록 허락하고 동의합니다.

- 다 음 -

1. 저작물의 DB구축 및 인터넷을 포함한 정보통신망에의 공개를 위한 저작물의 복제, 기억장치에의 저장, 전송 등을 허락함.
2. 위의 목적을 위하여 필요한 범위 내에서의 편집과 형식상의 변경을 허락함. 다만, 저작물의 내용변경은 금지함.
3. 배포·전송된 저작물의 영리적 목적을 위한 복제, 저장, 전송 등은 금지함.
4. 저작물에 대한 이용기간은 5년으로 하고, 기간종료 3개월 이내에 별도의 의사 표시가 없을경우에는 저작물의 이용기간을 계속 연장함.
5. 해당 저작물의 저작권을 타인에게 양도하거나 출판을 허락을 하였을 경우에는 1개월 이내에 대학에 이를 통보함.
6. 조선대학교는 저작물 이용의 허락 이후 해당 저작물로 인하여 발생하는 타인에 의한 권리침해에 대하여 일체의 법적 책임을 지지 않음.
7. 소속 대학의 협정기관에 저작물의 제공 및 인터넷 등 정보통신망을 이용한 저작물의 전송·출력을 허락함.

동의여부 : 동의(0) 반대( )

2008년 월 일

저작자: 김 진만 (인)

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