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Note

Growth of various obligate and facultative anaerobic intestinal bacteria in cell culture medium under aerobic and anaerobic culture conditions

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Eight bacterial strains were used in this study to examine the survival of intestinal bacteria in immune cell cultures under aerobic and anaerobic culture conditions. With the addition of penicillin G and streptomycin, viable *Clostridium clostridioforme* and *Fusobacterium varium* cells did not decrease after 6 or 24 hr, even under aerobic conditions. Without antibiotics, eight bacterial strains did not decrease until 4 or 6 hr later, under both aerobic and anaerobic conditions. *Escherichia coli* numbers increased by more than 10 times under both conditions. In order to examine the effects of live gut bacteria on various immune cells, the viability of bacteria should be checked in cell culture media and under different conditions.

Key words: intestinal bacteria, obligate anaerobic bacteria, facultative anaerobic bacteria, cell culture medium, aerobic culture conditions, anaerobic culture conditions

Advances in culture-independent technologies with next-generation metagenomic analyses have shown the enormous diversity, functional capacity, and age-associated dynamics of the human microbiome. Gut microbiota dysbiosis, which is an imbalance in the composition and function of these intestinal microbes, is associated with diseases ranging from localized gastroenterological disorders to neurologic, respiratory, metabolic, hepatic, and cardiovascular illnesses [1].

Mucosal surfaces in the intestinal tract are continuously exposed to both potential pathogens and beneficial commensal bacteria [2]. Commensal bacteria are normally considered tolerant to epithelial and immune cells [3, 4]. However, they can become pathogenic and contribute to irritable bowel syndrome (IBS); inflammatory bowel diseases (IBDs), such as ulcerative colitis (UC) and Crohn's disease (CD); and colorectal cancer [5]. These symptoms depend on an intestinal immune homeostatic balance between tolerance and immunity that represents a unique regulatory challenge to the mucosal immune system [3]. The tolerance and activation of the human immune system are complex processes and depend on the amount and diversity of the microbiota at mucosal sites where epithelial cells and antigen-presenting cells (APCs), such as dendritic cells (DCs) and macrophages, play an important role [4, 6].

Therefore, the effects of commensal bacteria on various immune cells have been actively studied [7–9], but most of the studies have been culture experiments in CO_2 incubators and conducted under aerobic conditions. On the other hand, most intestinal bacteria are anaerobic [10]; aerobic conditions are disadvantageous for their survival, and they are expected to die. Therefore, the effects of dead intestinal bacteria on immune cells have been measured, but those of live bacteria have not been accurately measured in those studies. In addition, antibacterial agents such as penicillin G (PCG) and streptomycin sulfate (SM) are usually added to prevent the growth of bacteria in cell culture, and enteric bacteria susceptible to these antibacterial agents may be killed. Antibiotics have a great influence on the results of studies on cells. To accurately evaluate the effects of live intestinal bacteria on various immune cells, it is necessary to examine the growth of bacteria over time and to determine which cell culture media should be used to cultivate intestinal bacteria with the addition of antibacterial agents under anaerobic conditions. This study was conducted to examine the growth and survival of enteric bacteria over time under aerobic and anaerobic conditions in cell culture medium supplemented with PCG and

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SM. PCG and SM are commonly used antibacterial agents in cell culture, so we chose them as the agents in this study.

Eight strains were used: *Escherichia coli* ATCC1649 and *Lactobacillus bulgaricus* LB- 012001 were used as facultative anaerobes, whereas *Bacteroides vulgatus* ATCC5826, *Clostridium clostridioforme* ATCC1291, *Fusobacterium varium* 113 (clinical isolate), *F. varium* ATCC5801, *Fusobacterium nucleatum* ATCC25586, and *F. nucleatum* 6-8-1 (clinical isolate) were used as obligate anaerobes.

E. coli ATCC1649 was precultured for 24 hr at 37°C in an incubator using a BTB (bromothymol blue) lactose-containing agar medium (Eiken Chemical Co., Ltd., Tokyo, Japan). Other bacteria were precultured on ABCM agar medium (Eiken Chemical Co., Ltd.) under anaerobic conditions in AnaeroPack Kenki (Mitsubishi Gas Chemical Co., Inc., Tokyo, Japan; CO_2 concentration >15%, O_2 concentration <0.1%) at 37°C for 48 hr.

Cell culture IMDM (Iscove's Modified Dulbecco's Medium, Sigma-Aldrich, MilliporeSigma, Burlington, MA, USA; supplemented with 10% FCS; fetal calf serum) was used for bacterial culture. The bacteria were suspended in IMDM, and the concentration was adjusted to 1×10^8 cfu/mL using a turbidimeter (HACH 2100N, DKK-TOA Corporation, Tokyo, Japan). One milliliter of IMDM suspended with each bacterium was added to a 12-well microplate, and then 1 mL of IMDM was added to the culture. For aerobic culture with the addition of the antibacterial agents, 10,000 units/mL PCG solution and 10,000 µg/mL SM solution (Cosmo Bio Co. Ltd., Tokyo, Japan) were added to the medium at a 100-fold dilution ratio, and cultures were incubated in a CO_2 incubator at 37°C under aerobic conditions (5% CO_2). The viable cell count was measured after 1, 2, 3, 4, 6, 24, and 48 hr. For aerobic culture without the addition of antibacterial agents, culture was performed according to the above procedure, without adding the antibacterial agents, PCG and SM solutions. For anaerobic culture with or without the addition of antibacterial agents, PCG and SM solutions were added to the medium at a 100-fold dilution ratio, and the bacterial suspensions were placed in a square jar with an AnaeroPack Kenki gas generator for anaerobic culture and cultured at 37°C. The viable cell count was measured before incubation and after 1, 2, 3, 4, 6, 24, and 48 hr.

After culturing for the predetermined time, 100 microliter samples were collected, and the samples were serially diluted 10-fold. To measure viable cell counts, *E. coli* was aerobically cultivated using BTB lactose-containing agar, and anaerobic bacteria were anaerobically cultivated with AnaeroPack Kenki using ABCM agar medium. *E. coli* was counted 24 hr later and quantified, while other bacteria were counted 48 hr later and quantified. The experiment was repeated two times, and the averages were taken. The changes in viable cell counts were evaluated logarithmically. That is, increases were evaluated as 10 times or more, 100 times or more, or 1,000 times or more, and decreases were evaluated as 1/10 or less, 1/100 or less, 1/1,000 or less, or 1/10,000 or less. When none of the above logarithmic changes were observed, there was considered to be no change (NC).

During aerobic culture in cell culture medium containing PCG and SM (CO_2 incubator, Table 1), the facultative anaerobic bacterium *E. coli* ATCC1649 decreased to approximately 1/10,000 its original concentration at 1 hr and was below the detection limit (<10^2 cfu/mL) at 3 hr. *L. bulgaricus* LB-012001 decreased to 1/100 its original concentration at 1 hr and was below the detection limit after 4 hr. The obligate anaerobic bacterium *B. vulgatus* ATCC5826 decreased to approximately 1/10, 1/1,000, and 1/10,000 its original concentration at 6, 24, and 48 hr, respectively. *C. clostridioforme* ATCC1291 did not show a decrease in the number of bacteria from 1 to 48 hr. *F. varium* 113 and *F. varium* ATCC5801 decreased to less than 1/10 its original concentration at 48 hr but did not begin decreasing until 24 hr. *F. nucleatum* ATCC25586 decreased to less than 1/100 its original concentration or below the detection limit after 2 hr. *F. nucleatum* 6-8-1 decreased to 1/10 its original concentration at 6 hr and was below the detection limit after 24 hr.

During aerobic culture in cell culture medium without the addition of PCG and SM (Table 1), *E. coli* ATCC1649 grew to 10 times its original concentration at 4 hr and thereafter was 10

Table 1. Changes in viable cell counts during aerobic culture in cell culture medium with or without penicillin G (PCG) and streptomycin sulfate (SM)

Incubation time	1 hr	2 hr	3 hr	4 hr	6 hr	24 hr	48 hr
With addition of PCG and SM							
Escherichia coli 1649	1/10,000	1/10,000	0	1/10,000	0	0	0
Lactobacilus bulgaricus LB-012001	1/100	1/1,000	1/1,000	0	0	0	0
Bacteroides vulgatus 5826	NC	NC	NC	NC	1/10	1/1,000	1/100,000
Clostridium clostridiforme 1291	NC	NC	NC	NC	NC	NC	NC
Fusobacterium varium 113	NC	NC	NC	NC	NC	NC	1/10
Fusobacterium varium ATCC5801	NC	NC	NC	NC	NC	NC	1/10
Fusobacterium nucleatum ATCC 25586	NC	0	0	1/100	1/100	0	0
Fusobacterium nucleatum 6-8-1	NC	NC	NC	NC	1/10	0	0
Without PCG and SM							
Escherichia coli 1649	NC	NC	NC	10	10	10	10
Lactobacilus bulgaricus LB-012001	NC	NC	NC	NC	NC	1/10	1/100
Bacteroides vulgatus 5826	NC	NC	NC	NC	NC	1/10	1/100
Clostridium clostridiforme 1291	NC	NC	NC	NC	NC	NC	1/10
Fusobacterium varium 113	NC	NC	NC	NC	NC	NC	NC
Fusobacterium varium ATCC5801	NC	NC	NC	NC	NC	NC	1/10
Fusobacterium nucleatum ATCC 25586	NC	NC	NC	NC	1/10,000	0	0
Fusobacterium nucleatum 6-8-1	NC	NC	NC	NC	NC	1/10	1/1,000

PCG: penicillin G; SM: streptomycin sulfate; NC: not changed.

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times its original concentration or more until 48 hr. *L. bulgaricus* LB-012001 was unchanged from 1 to 6 hr, decreased to 1/10 its original concentration at 24 hr, and decreased to 1/100 its original concentration or less at 48 hr. *B. vulgatus* ATCC5826 and *C. clostridioforme* ATCC1291 remained unchanged for up to 6 hr, decreased to less than 1/10 its original concentration at 24 hr, and decreased to less than 1/100 its original concentration at 48 hr. *F. varium* 113 was maintained from 1 to 48 hr, with the viable cell count unchanged. *F. varium* ATCC5801 did not decrease until 24 hr, decreased to less than 1/10 its original concentration at 48 hr, and was 1/10 its original concentration or less after 48 hr. *F. nucleatum* ATCC25586 remained unchanged for up to 4 hr, decreased to approximately 1/10,000 its original concentration at 6 hr, and then fell below the detection limit after 24 hr. *F. nucleatum* 6-8-1 decreased to 1/10 and 1/1,000 its original concentration at 24 and 48 hr, respectively.

During anaerobic culture in cell culture medium containing PCG and SM (Table 2), *E. coli* ATCC1649 was 1/1,000 its original concentration or less at 1 hr and below the detection limit after 2 hr. *L. bulgaricus* LB-012001 decreased to 1/1,000 its original concentration or less at 1 hr and below the detection limit after 24 hr. *B. vulgatus* ATCC5826 was 1/10 its original concentration or less at 4 to 6 hr and below the detection limit after 24 hr. *C. clostridioforme* ATCC1291 decreased to less than 1/10 its original concentration at 4 and 6 hr, decreased to less than 1/10,000 its original concentration at 24 hr, and was below the detection limit at 48 hr. *F. varium* 113 decreased to less than 1/10 its original concentration after 4 hr and was below the detection limit after 24 hr. *F. varium* ATCC5801 did not decrease at 4 hr but decreased to less than 1/10 its original concentration at 6 hr and was below the detection limit after 24 hr. *F. nucleatum* ATCC25586 was less than 1/10 its original concentration at 2 hr and below the detection limit after 3 hr. *F. nucleatum* 6-8-1 was not decreased at 4 hr but decreased to 1/10 its original concentration or less at 6 hr and was below the detection limit after 24 hr.

During anaerobic culture in cell culture medium without the addition of PCG and SM (Table 2), *E. coli* ATCC1649 grew to approximately 10 times its original concentration in 3 hr and remained at more than 10 times its original concentration thereafter. *L. bulgaricus* LB-012001 showed no decrease at 24 hr but decreased to approximately 1/10 its original concentration at 48 hr. *B. vulgatus* ATCC5826, *C. clostridioforme* ATCC1291, *F. varium* ATCC5801, and *F. nucleatum* 6-8-1 showed no changes in viable cell counts until 48 hr. *F. varium* 113 did not change until 24 hr but grew to more than 10 times its original concentration at 48 hr. *F. nucleatum* ATCC25586 did not decrease until 24 hr but decreased to less than 1/10 its original concentration at 48 hr.

Cell culture is usually carried out under 5% CO_2 with supplementation of the air in a CO_2 incubator. Since most intestinal bacteria are obligate anaerobes [1], this aerobic condition leads to the death of the bacteria. Intestinal bacteria and immune-related cells are cocultured under aerobic conditions, which is considered unsuitable for studying crosstalk between bacteria and immune cells. Therefore, anaerobic cell culture systems have been developed to study the action of intestinal anaerobic bacteria on intestinal epithelial cells and immune cells [11–14].

On the other hand, most studies of the effects of commensal bacteria on immune cells [7–9] have employed culture experiments under aerobic conditions. It is unclear whether intestinal bacteria actually die or grow in cell culture medium under aerobic conditions. Moreover, the effect of an antibacterial agent added to the cell culture medium has not been verified.

In general, obligate anaerobes are not affected by aminoglycoside agents, because these agents cannot enter the cells [15]. The antibacterial spectrum of penicillin G is very narrow, and penicillin G has little effect on gram-negative bacteria [16]. Therefore, in this study, *E. coli* (a facultative anaerobic gram-negative bacterium) and *L. bulgaricus* (a facultative anaerobic gram-positive bacterium) were significantly reduced by culture with PCG and SM in the cell culture medium in a CO_2 incubator.

Table 2. Changes in viable cell counts during anaerobic culture in cell culture medium with or without penicillin G (PCG) and streptomycin sulfate (SM)

Incubation time	1 hr	2 hr	3 hr	4 hr	6 hr	24 hr	48 hr
With addition of PCG and SM							
Escherichia coli 1649	1/1,000	0	0	0	0	0	0
Lactobacilus bulgaricus LB-012001	1/1,000	1/1,000	1/1,000	1/1,000	1/1,000	0	0
Bacteroides vulgatus 5826	NC	NC	NC	1/10	1/10	0	0
Clostridium clostridiforme 1291	NC	NC	NC	1/10	1/10	1/10,000	0
Fusobacterium varium 113	NC	NC	NC	1/10	1/10	0	0
Fusobacterium varium ATCC5801	NC	NC	NC	NC	1/10	0	0
Fusobacterium nucleatum ATCC 25586	NC	1/10	0	0	0	0	0
Fusobacterium nucleatum 6-8-1	NC	NC	NC	NC	1/10	0	0
Without PCG and SM							
Escherichia coli 1649	NC	NC	10	10	10	10	10
Lactobacilus bulgaricus LB-012001	NC	NC	NC	NC	NC	NC	1/10
Bacteroides vulgatus 5826	NC	NC	NC	NC	NC	NC	NC
Clostridium clostridiforme 1291	NC	NC	NC	NC	NC	NC	NC
Fusobacterium varium 113	NC	NC	NC	NC	NC	NC	10
Fusobacterium varium ATCC5801	NC	NC	NC	NC	NC	NC	NC
Fusobacterium nucleatum ATCC 25586	NC	NC	NC	NC	NC	NC	1/10
Fusobacterium nucleatum 6-8-1	NC	NC	NC	NC	NC	NC	NC

PCG: penicillin G; SM: streptomycin sulfate; NC: not changed.

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But in aerobic culture without PCG and SM, all the bacteria were viable at 4 hr. Until then, viable cell counts were. maintained. However, *E. coli* ATCC1694 grew to more than 10 times its original concentration. When the antibacterial agents PCG and SM were added to the same cell culture medium for anaerobic culture, the results revealed that *F. varium* ATCC5801 and *F. nucleatum* 6-8-1 maintained their bacterial counts even after 4 hr, but the other bacterial strains were reduced to less than 1/10 their original concentrations or below the detection limit. Without the addition of PCG and SM, all bacteria remained viable for up to 4 hr in anaerobic cultures. In addition, the viable bacterial counts did not change for 6 to 24 hr, except for *F. nucleatum*. However, *E. coli* numbers increased by more than 10 times in the aerobic culture. Surprisingly, even under aerobic conditions, the numbers of viable *C. clostridioforme* and *F. varium* cells did not decrease after 6 or 24 hr. It was found that some obligate anaerobic bacteria are resistant to oxygen.

Based on the above results for the viable bacterial counts under anaerobic and aerobic conditions, it was found that viable bacteria could be maintained for up to 4 hr in both aerobic and anaerobic cultures when the intestinal bacteria were cocultured in cell culture medium, unless PCG and SM were added. Since there was no change in the viable counts for bacteria other than *E. coli* for up to 4 hr and the *E. coli* viable cell count increased by 10 times or more in aerobic culture, it was considered that the number of bacteria to be added should be reduced to 1/10. To the best of our knowledge based on a PubMed search, this is the first research on this topic.

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CONFLICT OF INTEREST

The authors have no conflicts of interest to disclose.

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