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Biphasic culture of *Arcobacter* spp.

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J.S. DICKSON, T.R. MANKE, I.V. WESLEY AND A.L. BAETZ. 1996. *Arcobacter* spp. have recently been genetically differentiated as a genus distinct from *Campylobacter*. Physiologically, *Arcobacter* spp. are aerotolerant bacteria, while *Campylobacter* spp. are microaerophilic. However, since *Arcobacter* spp. have been difficult to grow to high population densities in broth media, alternative culture techniques were investigated. A biphasic culture system was developed in 25 cm² tissue culture flasks. Biphasic culture, consisting of a solid phase of 10% bovine blood agar and a liquid phase of Brain Heart Infusion broth, was found to increase bacterial population densities by more than 2 log₁₀ cycles for strains of *A. butzleri* and *A. skirrowii*. A strain of *A. cryaerophilus*, which was non-culturable in broth culture, attained population densities of 10⁹ cells ml⁻¹ in biphasic culture. Neither the addition of fetal bovine serum to the liquid nor an increase in the surface area from 25 to 75 cm² resulted in increased cell densities.

INTRODUCTION

*Arcobacter* spp. were first described in the late 1970s, although they were initially identified as *Campylobacter* (Neill et al. 1985). They are microscopically similar to *Campylobacter*, but differ physiologically in their ability to grow aerobically at 30°C, while *Campylobacter* spp. require higher temperatures and microaerophilic atmospheres. However, *Arcobacter* spp. grow poorly on *Campylobacter* isolation media, making cultivation difficult. In 1991, Vandamme proposed the genus *Arcobacter* for these aerotolerant *Campylobacter*, based on immunotyping and DNA hybridization studies (Vandamme et al. 1991).

*Arcobacter* spp. are fastidious bacteria that are difficult to cultivate. Some species grow poorly on blood agar plates (Vandamme et al. 1992), and most are difficult to grow on high cell densities in liquid culture systems. For many bacteriological studies, it is advantageous to have concentrated cultures of the specific bacterium and to able to produce these cultures consistently.

The biphasic culture technique consists of a solid phase of appropriate agar overlayed with a liquid phase of saline, buffer or broth. An early reference to biphasic culture techniques reported the enhanced bacterial production of levansucrase (Hestrin et al. 1943), and this technique was formally documented for routine blood cultures in 1947 (Castaneda 1947). Biphase culture techniques have been used to produce concentrated cultures of many types of bacteria (Tyrrell et al. 1958; Hall et al. 1979). In addition, a biphasic culture technique has been described to enhance the growth and cell numbers of *Campylobacter* spp. (Rollins et al. 1983). The specific objectives of this study were to determine if biphasic culture techniques could enhance the growth of *Arcobacter* spp., and if the inclusion of fetal bovine serum into the liquid phase would augment growth.

MATERIALS AND METHODS

Bacterial cultures

Five strains of *Arcobacter* spp., *A. butzleri*, *A. skirrowii* 3699, *A. skirrowii* 3700, *A. cryaerophilus* 3252 and *A. cryaerophilus* 3256 (ATCC 49615) were obtained from the USDA-ARS National Animal Disease Center, Ames, IA, USA. The lyophilized cultures were revived in brain heart infusion broth (BHI; Accumedia, Baltimore, MD, USA) and maintained in biphasic culture (see following description).

Culture techniques

Liquid media consisted of either BHI broth or BHI + 10% (v/v) inactivated fetal bovine serum (BHI + FBS). Solid
medium consisted of 10% bovine blood in Tryptose Blood Agar Base (BLA; Difco; Detroit, MI, USA). Liquid culture was conducted in screw capped tubes. Biphase culture systems were constructed as described by Krieg and Gerhardt (1994) using 25 or 75 cm$^2$ tissue culture flasks, by adding 7.0 or 15.0 ml of molten BLA, respectively, to the flask and allowing it to solidify on the surface. After the agar solidified, 4.5 ml BHI or 9.0 ml BHI + FBS was added as a liquid phase overlay.

**Experimental design**

The objective of experiment 1 was to determine the effect on bacterial populations of biphase culture in comparison to conventional liquid culture. The liquid medium was also supplemented with fetal bovine serum to determine if this enhanced growth. The bacteria were inoculated independently with a loopful of a 24 h culture into 9.0 ml tubes of BHI broth and thoroughly mixed. After mixing, 4.5 ml of the inoculated broth was transferred to biphase cultures constructed in 25 cm$^2$ tissue culture flasks. This technique assured that the initial populations in both the broth and biphase cultures were equivalent. The experiments were also replicated using comparable liquid and biphase culture systems prepared with BHI + FBS broth. The culture systems were incubated at 30°C in a gyrorotatory incubator at 50 rev min$^{-1}$ for 48 h in ambient air. Biphase culture systems were incubated horizontally, with the liquid phase overlaying the solid phase.

The objective of experiment 2 was to determine if the surface area of the biphase culture system had an effect on bacterial populations. Briefly, biphase culture systems were constructed with 25 or 75 cm$^2$ tissue culture flasks, and inoculated and incubated as previously described.

The objective of experiment 3 was to determine the generation times for *A. skirrowii* 3699 in both conventional liquid and biphase culture systems. Approximately 500 cells of the bacterium were inoculated into either 9 ml tubes or 75 cm$^2$ flasks and incubated at 30°C at 50 rev min$^{-1}$. Samples were evaluated for both optical density (650 nm) and viable cell population by plating.

**Bacterial enumeration**

The populations of *A. butzleri* and *A. skirrowii* 3699 were enumerated by serially diluting in buffered peptone water (Accumedia) and surface plating on BLA. The plates were incubated at 30°C for 24–48 h in ambient air. The populations of *A. skirrowii* 3700, *A. cryaerophilus* 3252 and *A. cryaerophilus* 3256 produced extremely small colonies on BLA plates which could not be enumerated without the aid of a low magnification microscope, and these cultures were subsequently enumerated by direct microscopic count with a Petroff-Hauser counting chamber.

**Statistical analysis**

Each experiment was independently replicated twice, and the populations of bacteria were converted to log$_{10}$ colony-forming units (cfu) ml$^{-1}$. The data were analysed using the general linear models procedure of Statistical Analysis System (Anon. 1985). Generation times were calculated using the formula: $Gt = t/N$; where $t$ equals time in hours, and $N$ is calculated using the formula: $N = 3.3 \log_{10} (x/x_0)$; where $x_0$ equals the initial bacterial population and $x$ equals the population after $t$ hours (Pelczar et al. 1977).

**RESULTS AND DISCUSSION**

Biphase culture systems significantly ($P < 0.05$) increased the populations of all of the *Arcobacter* species studied (Table 1). This increase ranged from approximately 1 log$_{10}$ cycle for *A. cryaerophilus* 3256 to approximately 2 log$_{10}$ cycles for *A. butzleri*. The extreme case was *A. cryaerophilus* 3252, which showed no detectable growth in the liquid culture system after 48 h, but produced a population of approximately 9 log$_{10}$ cells ml$^{-1}$ in the biphase culture system.

*Arcobacter* spp. require a nutrient-rich medium, usually containing blood or albumin, for growth. Fetal bovine serum appeared to be a reasonable component to add to liquid media to enhance the growth of the organisms. However, the addition of 10% FBS resulted in no significant ($P > 0.1$) increase in the populations of *Arcobacter* spp. for a given culture system (Table 1).

An increase in the surface area of a biphase culture system can increase the population of cells in the liquid phase for some bacteria (e.g., *Salmonella*, *Escherichia coli*; Tyrrell et al. 1958; Dickson, unpublished data). Increasing the surface area from 25 to 75 cm$^2$ significantly ($P < 0.05$) increased the populations of *A. butzleri* by approximately 0.75 log$_{10}$ cycles (Table 2). The larger surface area numerically increased the average populations of the other four species, but these results were not significant. Increasing the surface area results in improved oxygen diffusion into the liquid medium. However, since *Arcobacter* spp. are aerotolerant bacteria, increasing the oxygen diffusion into the media would not necessarily enhance their growth.

The growth curves of *A. skirrowii* 3699 confirmed that the maximum population density of this bacterium was approximately 2 log$_{10}$ cycles less in liquid culture than in biphase culture. The generation times were 0.869 h in biphase culture and 1.470 h in liquid culture, based on viable plate counts. The interpretation of the optical density readings for the biphase culture was different, since the bacterium apparently hydrolysed the solid phase, releasing red blood cells into the
Table 1  Effect of biphasic culture and fetal bovine serum on the growth of *Arcobacter* spp.

<table>
<thead>
<tr>
<th>Bacterium</th>
<th>Log$_{10}$ colony forming units ml$^{-1}$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Liquid*</td>
</tr>
<tr>
<td></td>
<td>BHI</td>
</tr>
<tr>
<td><em>A. butzleri</em></td>
<td>8.69§</td>
</tr>
<tr>
<td><em>A. skirrowii</em> 3699</td>
<td>7.74§</td>
</tr>
<tr>
<td><em>A. skirrowii</em> 3700†</td>
<td>3.23§</td>
</tr>
<tr>
<td><em>A. cryaerophilus</em> 3252†</td>
<td>NDG</td>
</tr>
<tr>
<td><em>A. cryaerophilus</em> 3256†</td>
<td>7.93§</td>
</tr>
</tbody>
</table>

*4:5 ml liquid culture.  
†25 cm$^2$ tissue culture flask; solid phase 10% bovine blood agar.  
§Means within rows with different symbols are significantly ($P < 0.05$) different.  
BHI, Brain Heart Infusion Broth;  
BHI + FBS, Brain Heart Infusion Broth + 10% fetal bovine serum;  
NDG, no detectable growth.

liquid phase. As such, the optical density readings could not be directly correlated with an increase in cell numbers.

Although the mechanisms by which biphasic culture systems increase the bacterial populations are unknown, it is speculated that these culture systems enhance bacterial cell numbers by providing the organisms access to nutrients in the solid phase (Rollins et al. 1983). Biphasic culture systems have been shown to have applications to culturing clinically important bacteria (Castaneda 1947; Hall et al. 1979), and have also been useful in culturing other bacteria to high concentrations (Tyrrell et al. 1958). This culture technique appears to have application to producing concentrated liquid cultures of *Arcobacter* spp.

Table 2  Effect of surface area of biphasic culture on the growth of *Arcobacter* spp.

<table>
<thead>
<tr>
<th>Bacterium</th>
<th>Log$_{10}$ colony forming units ml$^{-1}$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>25 cm$^2$ flask*</td>
</tr>
<tr>
<td><em>A. butzleri</em></td>
<td>9.26†</td>
</tr>
<tr>
<td><em>A. skirrowii</em> 3699</td>
<td>9.06†</td>
</tr>
<tr>
<td><em>A. skirrowii</em> 3700†</td>
<td>9.18†</td>
</tr>
<tr>
<td><em>A. cryaerophilus</em> 3252†</td>
<td>9.17†</td>
</tr>
<tr>
<td><em>A. cryaerophilus</em> 3256†</td>
<td>8.86§</td>
</tr>
</tbody>
</table>

*25 or 75 cm$^2$ tissue culture flask; solid phase 10% bovine blood agar, liquid phase Brain Heart Infusion Broth.  
†Bacterial populations enumerated by direct microscopic count.  
§Means within rows with different symbols are significantly ($P < 0.05$) different.

ACKNOWLEDGEMENTS

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