A quasi-universal medium to break the aerobic/anaerobic bacterial culture dichotomy in clinical microbiology

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Abstract

In the mid-19th century, the dichotomy between aerobic and anaerobic bacteria was introduced. Nevertheless, the aerobic growth of strictly anaerobic bacterial species such as *Ruminococcus gnavus* and *Fusobacterium necrophorum,* in a culture medium containing antioxidants, was recently demonstrated. We tested aerobically the culture of 623 bacterial strains from 276 bacterial species including 82 strictly anaerobic, 154 facultative anaerobic, 31 aerobic and nine microaerophilic bacterial species as well as ten fungi. The basic culture medium was based on Schaedler agar supplemented with 1 g/L ascorbic acid and 0.1 g/L glutathione (R-medium). We successively optimized this media, adding 0.4 g/L uric acid, using separate autoclaving of the component, or adding haemin 0.1 g/L or α-ketoglutarate 2 g/L. In the basic medium, 237 bacterial species and ten fungal species grew but with no growth of 36 bacterial species, including 22 strict anaerobes. Adding uric acid allowed the growth of 14 further species including eight strict anaerobes, while separate autoclaving allowed the growth of all tested bacterial strains. To extend its potential use for fastidious bacteria, we added haemin for *Haemophilus influenzae, Haemophilus parainfluenzae* and *Eikenella corrodens* and α-ketoglutarate for *Legionella pneumophila.* This medium allowed the growth of all tested strains with the exception of *Mycobacterium tuberculosis* and *Mycobacterium bovis.* Testing primoculture and more fastidious species will constitute the main work to be done, but R-medium coupled with a rapid identification method (matrix-assisted laser desorption/ionization time-of-flight mass spectrometry) will facilitate the anaerobic culture in clinical microbiology laboratories.

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Introduction

Since the discovery of anaerobic bacteria in the mid-19th century by Louis Pasteur, microbiologists have striven to improve techniques for the growth of anaerobic prokaryotes, notably to reduce oxygen tension, without compromising the anaerobic concept [1]. For example, Hungate [2] introduced roll tubes to prepare an anoxygenic medium for methanogenic archaea cultivation. Finegold *et al.* [3] and Moore *et al.* [4] then comprehensively described the anaerobic human flora in the 1970s. This long, fastidious and costly method was gradually abandoned in favour of metagenomic studies [1]. More recently, by culture-dependent studies using dilution method [5] or microbial culturomics [6,7], designing techniques mimicking the natural environment of the bacteria, the human gut repertoire, composed preferentially of anaerobic bacteria, was dramatically extended [8]. Nevertheless, these techniques remain frequently reserved for specialized laboratories.

Otherwise, the ability to grow strictly anaerobic bacterial species in an atmosphere including a low concentration of oxygen was previously reported [9,10]. We recently reported a preliminary study highlighting the aerobic growth of strictly

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anaerobic bacterial species such as *Ruminococcus gnavus* and *Fusobacterium necrophorum* in petri dishes including a basic culture medium supplemented by ascorbic acid and glutathione [11]. Moreover, we demonstrated that the metronidazole susceptibility of strictly anaerobic bacterial species such as *Bacteroides thetaiotaomicron* and *Parvimonas micra* could be tested aerobically [12]. Nevertheless, several other bacterial species, including strictly anaerobic bacteria, were unable to grow in this medium. Here we report the step-by step optimization of this culture medium, aiming to design a quasi-universal culture medium, incubated aerobically, that is largely usable in clinical microbiology laboratories.

Materials and Methods

Bacterial strains tested

Strain types. In this study, we tested 623 bacterial strains from 276 different species, including 82 strictly anaerobic bacterial species, 154 facultative anaerobic bacterial species, 31 aerobic bacterial species, nine microaerophilic bacterial species and ten fungi.

Among them, 542 different strains from 252 species were isolated from ongoing culturomics studies [7], and 81 different strains were from 50 species were isolated in our clinical microbiology laboratory. (Supplementary Table 1(A–E)). All these strains are available in Collection de Souches de l'Unité des Rickettsies (CSUR) collection strains except *Campylobacter coli* and *Campylobacter concisus,* which were available in Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ) collection strains (Supplementary Table 2). Among the 276 bacterial species tested here, 161 bacterial species corresponded to 99.8% of the positive samples analyzed over 1 year in our clinical microbiology laboratory (Marseille, France). In addition, all the bacteria that we did not test here were isolated less than once per month in our laboratory.

Culture conditions. A growth baseline of all the strains was obtained on the culture condition reference. All the strains were inoculated by spotting in 120 mm petri dishes of R-medium divided into 25 squares. We inoculated each strain from a fresh culture performed in 5% sheep's blood culture incubated under reference culture conditions. The petri dishes were incubated aerobically without CO_2 at 37°C. To determine the conventional growth atmosphere of each strain, we used the Bacterio.Net website (http://www.bacterio.net/), then studied the seminal article describing the bacterial isolate.

Culture medium. For this large study, we used a culture medium that we named R-medium. To design this, we used a basic medium consisting of Schaedler agar (Sigma-Aldrich, Saint-Quentin Fallavier, France) used routinely to cultivate anaerobic bacteria. Thereafter, we added to this medium compounds with an antioxidant activity. The final R-medium 0 consists of Schaedler agar supplemented by 1 g/L ascorbic acid (VWR, Leuven, Belgium) and 0.1 g/L glutathione (Sigma-Aldrich). The pH was adjusted to 7.5 in both culture media basis and antioxidants mix with 10 M KOH before autoclaving. Antioxidants were dissolved in 10 mL distilled water, filtered using 0.2 µm microfilters and added to the autoclaved culture medium stabilized at 50°C. The final culture medium was then poured into 120 mm square petri dishes.

We optimized R-medium 0 by adding 0.4 mg/L uric acid (Sigma-Aldrich), one of the more powerful antioxidants, as previously described [13].

The preparation R-medium 1bis is essentially based on the principle of independently autoclaving the elements that interact during the period of autoclaving to form free radicals, such as the Maillard reaction (between the sugar and the nitrogen source) [14] and the interaction between the phosphate and agar generating H_2O_2, which is an inhibitor of the growth of anaerobic bacteria [15]. To achieve this, three solutions were prepared and autoclaved or filtered independently. Solution A contained 5.67 g of casein hydrolysate proteose, 5 g of peptone and 5 g of yeast extract (Sigma-Aldrich) dissolved in 400 mL of distilled water. The pH was adjusted to 7.5 with 10 M KOH. It was then autoclaved at 121°C for 15 minutes. Solution B consisted of 0.83 g of dipotassium hydrogen phosphate (Merck Santé, Lyon, France), 1.67 g of sodium chloride (Merck Santé), 5.83 g of glucose (MP Biomedicals, Illkirch, France), 0.40 g of L-cysteine (Sigma-Aldrich), 1 g of ascorbic acid (Sigma-Aldrich), 0.4 g of uric acid (Sigma-Aldrich), 0.1 g of glutathione (Sigma-Aldrich), 0.1 g of haemin (Sigma-Aldrich) and 2 g of α-ketoglutarate (Sigma-Aldrich) dissolved in 200 mL of distilled water and filtered using a 0.2 microfilter. Solution C consisted of 15 g of agar (Oxoid, Dardilly, France) dissolved in 300 mL of distilled water. After adjusting the pH to 7.5, the agar solution was autoclaved for 15 minutes at 121°C. The three solutions were stabilized at 56°C after autoclaving and mixed on a hot plate at 56°C. The final culture medium was then poured into 120 mm square petri dishes and stored at 4°C until use.

To increase the culture of fastidious species, we supplemented haemin (0.1 g/L) (Sigma-Aldrich), Schaedler agar containing only 0.01 g/L haemin. The stock solution of haemin was prepared by dissolving 0.1 g haemin (bovine haemin chloride) (Sigma-Aldrich) in 1 mL of NaOH. This allows better solubilization, as previously described [16].

We added α-ketoglutarate (2 g/L) (Sigma-Aldrich) to allow the growth of *Legionella pneumophila.* Indeed, α-ketoglutarate is

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