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Growth of Silicone-Immobilized Bacteria on Polycarbonate Membrane Filters, a Technique To Study Microcolony Formation under Anaerobic Conditions

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A technique was developed to study microcolony formation by silicone-immobilized bacteria on polycarbonate membrane filters under anaerobic conditions. A sudden shift to anaerobiosis was obtained by submerging the filters in medium which was depleted for oxygen by a pure culture of bacteria. The technique was used to demonstrate that preinduction of nitrate reductase under low-oxygen conditions was necessary for nonfermenting, nitrate-respiring bacteria, e.g., *Pseudomonas* spp., to cope with a sudden lack of oxygen. In contrast, nitrate-respiring, fermenting bacteria, e.g., *Bacillus* and *Escherichia* spp., formed microcolonies under anaerobic conditions with or without the presence of nitrate and irrespective of aerobic or anaerobic preculture conditions.

Growth of bacterial cells on thin agar slides or membrane filters and subsequent visualization of the microcolonies by light or epifluorescence microscopy have been used as an alternative to conventional spread plating (2, 6, 7, 13, 14, 16, 19–21, 24, 25, 27). Advantages of the microcolony growth technique are rapid handling and the possibility of observing both dividing and nondividing cells on the same microscopic slide. It has further been observed that some bacteria (both indigenous and introduced organisms) from environmental samples are able to perform only a few cell divisions before their growth ceases; such organisms would never develop visible macrocolonies by conventional spread plating (3, 7, 25, 26). It has been suggested that the microcolony technique is useful mainly for growth of aerobic organisms (16), and only one report has previously dealt with anaerobic growth of bacteria by the microcolony method (5).

Denitrification enzymes (nitrate, nitrite, and nitrous oxide reductases) are induced at different levels of lowered oxygen conditions (11), and a gradual decrease in oxygen can be necessary for this induction (1, 15). With the anaerobic microcolony technique presented here, we have been able to study the significance of preculture conditions for several denitrifying (*Pseudomonas*) or nitrate-respiring (*Bacillus* and *Escherichia*) species to divide and form microcolonies after a sudden shift to anaerobiosis.

Bacterial strains and growth conditions. Microcolony formation of the following strains was studied: (i) an obligate aerobe, *Pseudomonas fluorescens* DF17, biotype V/VI (23); (ii) three nonfermenting, denitrifying, facultative anaerobes: *P. aeruginosa* PAO303, *P. fluorescens* DF57, biotype III (23), and *P. stutzeri* ZoBell ATCC 14405; and (iii) two fermenting, nitrate-respiring, facultative anaerobes: *Bacillus cereus* DSM31 and *Escherichia coli* K-12.

The bacteria were grown in 1/10-strength tryptic soy broth (TSB) (Bacto; Difco Laboratories, Detroit, Mich.). In one experiment, a defined citrate minimal medium (CMM) (4), was used to obtain a batch culture with low aerobic culturability. Increasing loss of culturability has previously been reported for *P. aeruginosa* PAO303 entering stationary phase in electron acceptor-limited citrate minimal medium (5).

Aerobic precultures were grown at room temperature in baffled 125-ml Erlenmeyer flasks containing 25 ml of medium. To ensure adequate oxygen supply, the cultures were shaken vigorously on a wrist shaker (Flask Shaker SF1; Stuart Scientific Co. Ltd.). Cells from aerobic overnight cultures were shifted to fresh medium (starting optical density at 600 nm [OD_{600}], ~0.01), and cells were harvested in early to mid-log phase (OD_{600}, 0.1 to 0.5) for the subsequent growth experiments.

Microaerobic precultures were established in sealed serum bottles containing 80 ml of TSB (plus 10 mM KNO_3) and 40 ml of air-filled headspace. The bottles were inoculated with cells of *P. aeruginosa* PAO303 (start OD_{600}, ~0.01) obtained from an aerobic mid-log phase preculture (OD_{600}, ~0.8) and incubated on a rotary shaker at 30°C. The oxygen level in the bottles thus decreased gradually due to growth of the incubated cells.

Anaerobic precultures were microaerobic precultures grown to stationary phase. Thus, when nitrate was consumed as tested with an analytical paper strip (Nitrate Test, Merckoquant; Merck, Darmstadt, Germany), the batch was considered to be anaerobic and the cells were used for further studies. In experiments where CMM was used as medium, the headspace was initially flushed with nitrogen and cells from a microaerobic preculture were used as inoculum.

Immobilization of cells on membrane filters. Figure 1 shows a flow chart of the experimental protocol. Cells were harvested from the precultures, and serial dilutions were made in phosphate-buffered saline (PBS) (per liter: $Na_2HPO_4 \cdot 2H_2O$, 3.6 g; $NaH_2PO_4 \cdot H_2O$, 1.1 g; NaCl, 7.3 g; pH adjusted to 7.2 to 7.4 with NaOH). Samples were filtered onto black polycarbonate membrane filters (pore size, 0.2 μm; diameter, 25 mm) (Poretics Corp., Livermore, Calif.), which were immediately removed from the filter towers and placed on the surface of a PBS solution to avoid desiccation of the cells. The membrane filters were then mounted with the bacterial side towards a circular glass coverslip (diameter, 25 mm) (Menzel, Braunschweig, Germany) coated with a thin layer (~0.1 mm) of silicone oil

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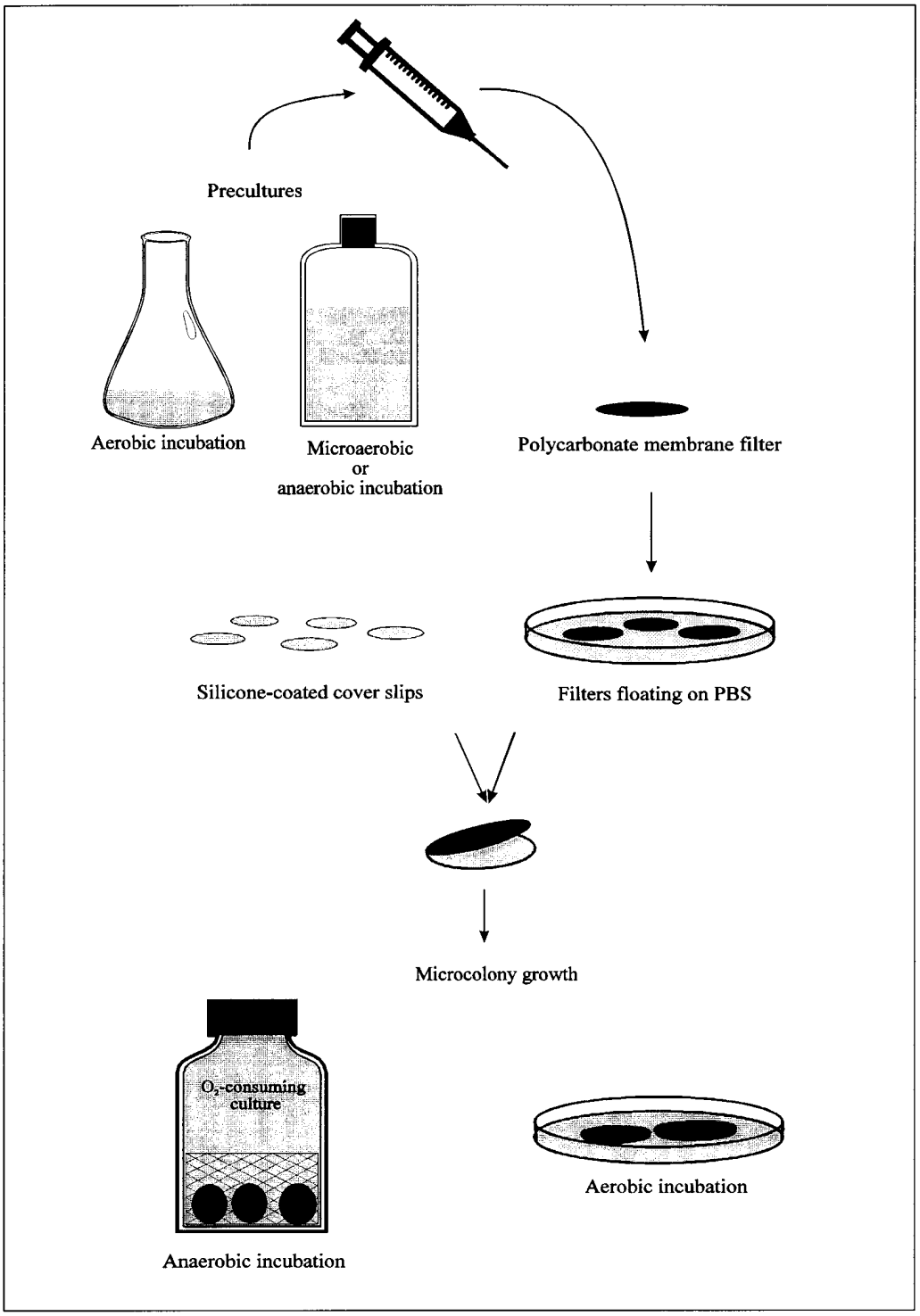

FIG. 1. Membrane filter design and setups for aerobic and anaerobic incubation. Bacterial cell samples from precultures are filtered onto 0.2-μm-pore-size polycarbonate membrane filters and placed in PBS solution to avoid desiccation. Coverslips are covered with a thin layer of silicone oil. The membrane filters are mounted on the coverslip with the bacteria towards the silicone oil. The filter sandwich can be incubated either floating on a liquid surface or submerged within a liquid growth medium. For aerobic incubation, the filters can also be placed directly on a growth medium without being mounted on the coverslips.

(viscosity index, 10^5 m^2 s^{-1}) (Wacker-Chemie, Munich, Germany). In order to obtain a smooth layer of silicone oil, the silicone-coated coverslips were heated prior to use (~24 h at 105°C). After the filters were mounted on the coverslips, the filter sandwiches were immediately placed (filter side downwards) on the PBS solution again to prevent the hydrophobic silicone oil from imbedding the cells. The silicone-mounted membrane filters could now be incubated on the surface (float-

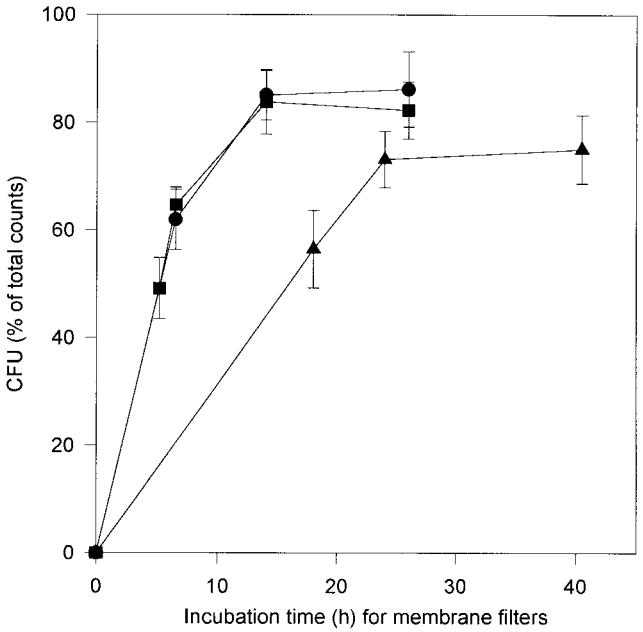

FIG. 2. Microcolony formation (CFU) of *P. aeruginosa* PAO303 on membrane filters as a function of incubation time. The cell samples were taken from an early-stationary-phase anaerobic preculture and incubated anaerobically with nitrate (▲) or aerobically either unmounted (●) or mounted on silicone-coated coverslips (■). Error bars, standard deviations ($n = 3$).

ing) or within (submerged) a liquid medium (see below) without disturbing the position of the individual cells on the filter.

Incubation of membrane filters. Aerobic incubation of cells on filters was performed by placing either unmounted filters or filter sandwiches on the surface of 30 ml of TSB in petri dishes (Fig. 1; also, see below). Anaerobic incubation of the membrane-immobilized cells was performed in 100-ml Blue Cap bottles (total volume, 140 ml) with a thin nylon mesh placed along the sides (Fig. 1). The bottles were initially filled with 100 ml of TSB supplemented with 100 μM KNO_3 and inoculated with cells (starting OD_{600}, ~0.01) from an aerobic preculture of *P. fluorescens* 41D7, a Tn*5* mutant of *P. fluorescens* DF57 (12), representing a lower anaerobic growth rate on nitrate compared to the wild type (data not shown). The suspension of strain 41D7 consumed oxygen to establish an anaerobic growth medium, while the bottles were incubated in horizontal position on a rotary shaker at 30°C. When the 100 μM nitrate was consumed by the strain 41D7, the medium was considered to be oxygen free and ready for anaerobic incubation. To start the anaerobic incubation of the filters, the bottles were opened and the filter sandwiches were placed in vertical position towards the sides of the flask supported by the nylon mesh; the filter side pointed towards the interior of the flasks (Fig. 1). Nitrate was added from an oxygen-degassed 1 M KNO_3 stock solution to a final concentration of 10 mM. The bottles were then filled completely with preincubated, and thus oxygen-free, medium and closed so that no air-filled headspace remained in the bottles. The bottles were incubated in the dark at 30°C. Since strain 41D7 is also a denitrifier, it survived as an active oxygen barrier throughout the anaerobic incubation. However, because of its slow anaerobic growth rate the competition for nitrate between cells in the suspension and on the filters was minimal, and incubation for up to 48 h could easily be performed before the medium was depleted for nitrate.

Staining of cells on filters. After incubation, the filter sandwiches were placed on the surface of a 0.04% (wt/vol) solution of acridine orange and stained for 2 min. Surplus stain was removed by gentle shaking of the filter sandwiches in filter-sterilized water in a petri dish for 5 min. The rinsed filters were allowed to dry for 10 to 15 min at 50°C, mounted on microscope slides, and subsequently examined in a Zeiss Axioscope epifluorescence microscope.

Oxygen analysis. Oxygen was analyzed in headspace samples (200 μl) on a gas chromatograph (Perkin-Elmer model 990) equipped with a thermal conductivity detector.

Time course of microcolony formation. *P. aeruginosa* PAO303 cells were harvested from early-stationary-phase anaerobic precultures grown on TSB, and filters were prepared for microcolony growth. Aerobic incubation of the filters whether with or without silicone resulted in microcolony formation for 85% of the cells within 10 h (Fig. 2). Filters incubated anaerobically with nitrate showed microcolony formation for around 75% of the cells within 24 h; prolonged incubation time did not increase these numbers. For the following experiments incubation time of 24 h was thus considered sufficient for all culturable cells in a sample to have initiated microcolony formation on the filters.

Effect of silicone oil on microcolony formation. Aliquots of *P. aeruginosa* PAO303 were sampled from three different batch cultures grown in CMM: (i) aerobic log phase, (ii) anaerobic log phase, and (iii) stationary phase (72 h old). Cells from all three batch cultures had similar culturabilities (given as percent of colony-forming cells relative to total count), whether grown on floating membrane filters or filters mounted on silicone-coated coverslips (Table 1). Silicone oil had no effect on the growth rate of individual cells on the membrane filters as judged from the sizes of the microcolonies (number of cells per colony).

Comparison of bacteria with different types of energy metabolism. The microcolony formation patterns of bacteria with different types of energy metabolism were compared after growth in either aerobic or anaerobic precultures; obligate aerobes were precultured only aerobically. Filters prepared with cells from the precultures were incubated aerobically, anaerobically with nitrate (10 mM KNO_3), or anaerobically without nitrate.

The obligate aerobe (*P. fluorescens* DF17) formed microcolonies only when incubated aerobically, whereas anaerobic incubation never resulted in growth. Likewise, cells obtained from aerobic precultures of the three nonfermenting, nitrate-reducing facultative anaerobes showed microcolony formation only when incubated aerobically. However, when precultured anaerobically, they also formed microcolonies when incubated

TABLE 1. Microcolony formation by *P. aeruginosa* PAO303 harvested from aerobic log-phase, anaerobic log-phase, and anaerobic stationary-phase cultures[a]

Mounting	Aerobic log phase		Anaerobic log phase		Anaerobic stationary phase	
	CFU (%)[b]	Size[c]	CFU (%)	Size	CFU (%)	Size
−	74.0 ± 3.0	29.8 ± 4.9	78.1 ± 5.0	17.9 ± 1.6	32.3 ± 3.4	10.6 ± 3.4
+	74.7 ± 3.3	23.3 ± 4.5	77.9 ± 1.7	19.7 ± 4.3	31.6 ± 2.2	8.0 ± 3.0

[a] See the text. Filters were incubated aerobically for 8 h on the surface of liquid medium (CMM), either unmounted or mounted on silicone-coated coverslips.
[b] Mean percent of total counts (single cells plus microcolonies) ± standard deviation. A total of 700 units were counted per filter on four replicate filters.
[c] Number of cells per microcolony (mean ± standard deviation; $n = 100$ colonies).

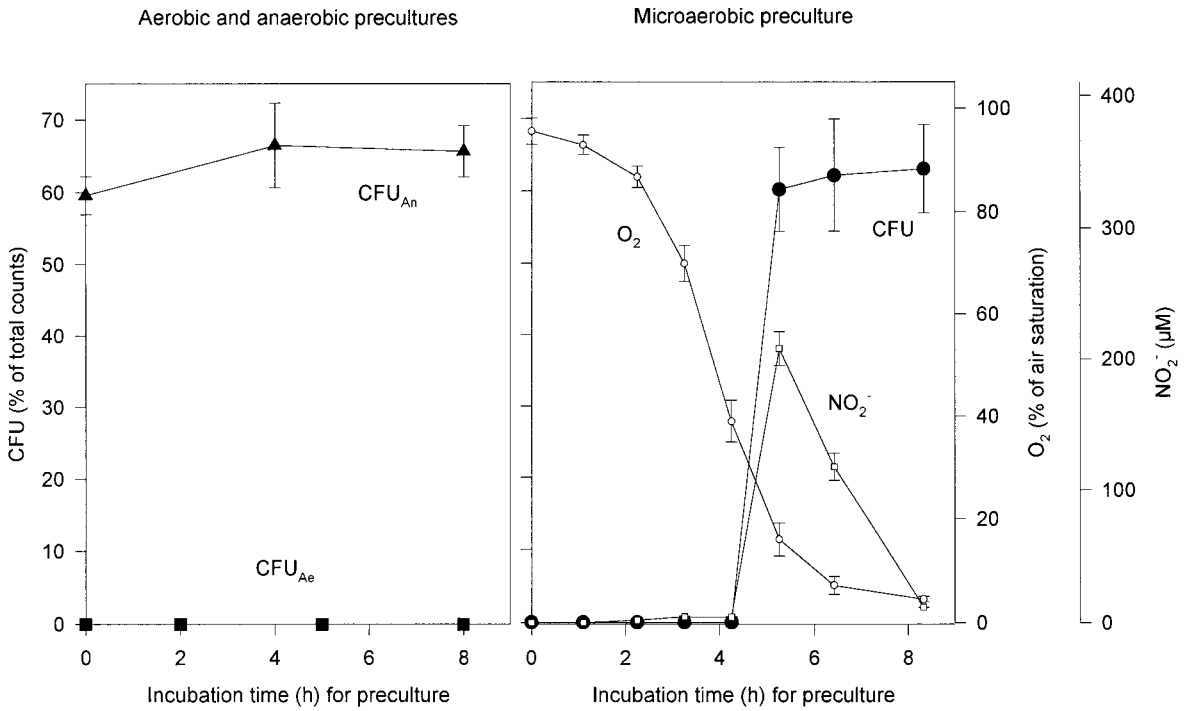

FIG. 3. Microcolony formation (CFU) of *P. aeruginosa* PAO303 on membrane filters as a function of preculture conditions. The cell samples were taken at intervals from aerobic (Ae) (■), anaerobic (An) (▲), or microaerobic (●) precultures and incubated anaerobically with nitrate (20 h at 30°C). The microaerobic preculture was started aerobically in a sealed serum bottle, and oxygen was gradually depleted by growth of the cells. ○, oxygen concentration, measured in headspace of the microaerobic preculture; □, nitrite concentration, measured in the microaerobic preculture. Error bars, standard deviations (n = 3).

aerobically with nitrate; growth was never observed when nitrate was omitted. These results indicate that enzymes needed for anaerobic growth, e.g., nitrate reductase in the respiratory pathway, were not induced in cells of the aerobic precultures. Facultative anaerobic cells originating from anaerobic precultures, however, grew readily on the filters when incubated anaerobically with nitrate since the enzymes needed for nitrate respiration were already expressed in cells of the preculture. Finally, the fermenting, facultative anaerobes (*E. coli* and *B. cereus*) grew on the filters under all examined growth conditions. Thus, as also suggested by Aida et al. (1), the capacity of nitrate-respiring (but nondenitrifying) bacteria to synthesize nitrate reductase after a sudden shift to anaerobiosis may be due to their energy production by fermentative metabolism.

Significance of preculturing under microaerobic conditions. Anaerobic incubation with nitrate for 20 h resulted in microcolony formation by 0 and 60% of the cells originating from aerobic and anaerobic precultures, respectively (Fig. 3). In contrast, cell samples from all three precultures always resulted in microcolony formation by more than 60% of the total number of cells incubated aerobically (data not shown). None of the cells harvested from the microaerobic preculture formed microcolonies under subsequent anaerobic incubation before oxygen level decreased to approximately 15% of air saturation (3 kPa or 40 μM O_2) (Fig. 3). At this point approximately 60% of the cells started to form microcolonies on the anaerobically incubated filters. Induction of nitrate reductase activity in the microaerobic preculture was also indicated by the transient appearance of nitrite as oxygen levels became lower than 15% of air saturation (Fig. 3). Occasionally, nitrite accumulated to higher levels (~700 μM) in the batches and for longer periods than shown in Fig. 3. In these cases the capability of the cells to grow after shift to complete anaerobiosis increased more slowly but was still turned on only when the oxygen level was reduced to approximately 15% of air saturation (data not shown).

Expression of denitrification enzymes in response to changes in oxygen tension has been studied comprehensively as reviewed by Knowles (10). However, only a few studies have distinguished between sudden and transient changes in oxygen conditions for induction of the enzymes (1, 8, 15, 28). While Aida et al. (1) and Payne et al. (15) focused on the oxygen-dependent expression of nitrate, nitrite, and nitrous oxide reductase in whole-batch cultures, we have been able to perform such studies by single-cell observations using the anaerobic microcolony method. Körner and Zumft (11) found that half-maximal expression of nitrate reductase was observed in continuous cultures of *P. stutzeri* only when oxygen level was reduced to between 10 and 20% of air saturation. Combining our observations with these results, it seems that the critical level of nitrate reductase necessary for the cells to cope with the shift to complete anaerobiosis is expressed only at an oxygen level around 15% of air saturation (3 kPa O_2).

The presented anaerobic protocol for microcolony growth allows more comprehensive studies of survival of individual cells to be done, e.g., during lack of terminal electron acceptors (5). Further, it represents a unique opportunity for single-cell studies of general activity and physiological state as determined by 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyltetrazolium chloride (INT) or 5-cyano-2,3-ditolyl tetrazolium chloride (CTC) reduction (9, 22), ribosome content as determined by 16S rRNA probes (17), and specific gene expression as determined by reporter gene technology (18).

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