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New techniques for isolation of single prokaryotic cells[1]

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Abstract

Since the 1960s, several new attempts have been made to improve the management of single prokaryotic cells using micromanipulator techniques. In order to facilitate the isolation of pure cultures we have recently developed an improved micromanipulation method for routine work. With the aid of this method single prokaryotic cells can be picked out of a mixed community under direct visual control. The isolated aerobic or anaerobic cells can be grown in pure culture or can be subjected to single cell PCR. Other powerful and completely new approaches are the applications of laser micromanipulation systems, such as optical tweezers or laser microdissection techniques. Of the latter two methods only optical tweezers have been successfully applied to cloning prokaryotic cells.

Keywords: Isolation; Micromanipulator; Optical tweezer; Laser microdissection; Single microbial cell

Contents

1. Introduction

A prerequisite for the biochemical and physiological investigation of microorganisms is the isolation and management of pure cultures. The only absolute criterion of purity for a bacterial culture is that it has been derived from the progeny of a single cell. Failure to apply this criterion may lead to much effort in proving the purity of a culture. All strains upon which research is to be based should therefore be rigorously purified before starting to investigate the properties of individual organisms [1]. Ecologically oriented microbiologists are especially faced with the problem of how to obtain a pure culture of certain microbial strains from their densely populated natural habitats. The methods available range from those requiring only a simple apparatus to those requiring elaborate

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[1] Dedicated to Prof. Dr. Otto Kandler on the occasion of his 80th birthday.

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machines. The principal procedures for obtaining pure cultures of bacterial strains have not been much improved since Robert Koch introduced the agar plating technique more than 100 years ago [2]. Agar plating and the agar shake tube techniques (cf. [3]) do not prevent some colonies arising from clumps of cells. More sophisticated electronic enumeration and sampling systems such as Coulter counter or flow cytometry cannot prevent the formation of cell aggregates.

Two improved methods for isolation of single prokaryotic cells from complex environments have recently been successfully applied. With the aid of a modern micromanipulator single cells can be aspirated by a microcapillary tube (Bactotip method) [4] or they can be separated by using optical tweezers [5].

2. Micromanipulation techniques

2.1. Historical perspective

A survey of the chief methods devised for single organism cultures available up to the end of the 1960s was presented by Johnstone [1,6]. These included (a) the block cut method for the selection of an isolated organism on a lightly inoculated nutrient gel, (b) formation of droplets with micropipettes, which were checked for ones containing single organisms, and (c) isolation by carrying the selected organisms across the sterile gel surface with a microneedle. Because of technical problems and other disadvantages these methods were not adopted for routine isolation.

Several attempts to improve the management of single microbial cells by using micromanipulator techniques have been described in the literature. Either microneedles or microcapillaries were used for the separation of single bacterial cells [6–8]. The techniques suggested more than 25 years ago were based on the state of the art at that time, but had several technical disadvantages which hampered routine usage of the isolation techniques for a broad spectrum of prokaryotes in a microbiological laboratory. The magnification was limited and the transfer of single cells was hardly possible [8]. One instrument [8] was designed for use with low power objectives (e.g. 10×) with a working distance of 7 mm or more, and consisted of a lens collar and magnetic tool carrier. The lens collar was clamped onto the objective and contained two steel slides which permitted the magnet tool carrier to slide freely. Knobs or microloops were the most useful tools for the isolation of cells from colonies on solid agar plates. By several operations cells were floated across the surface of solid medium by lateral movement of the Petri dish and they were well separated from the original population. Attempts to lift single organisms in a loop for transfer were rarely successful. So far this method has been applied for the isolation of large filamentous bacteria [9] and cyanobacteria [10]. Bakoss [7] cloned single cells of leptospires with a micropipette connected to a syringe via a thin polyethylene tube, which was fastened to the holding clip of a micromanipulator. He used a syringe as a simple pneumatic system. The disadvantage of this micromanipulator technique was that it was laborious. A mechanical micromanipulator with a microneedle was also used [11] in order to separate the four spores in a yeast ascus. This technique has been used for this purpose in our institute, and it is also suitable for the separation of larger bacterial cells (>3 μm) by moving them onto an agar surface. Coccoid bacteria from the 'corn cob' of human dental plaques were successfully isolated by Mouton et al. [12] with microneedles designed to be a double angled microhook as described by Johnstone [6]. Single selected spores of *Bacillus cereus* adhering to the glass point of capillary tubes were selectively removed from Petri dishes [13]. Micromanipulation was also successfully applied for the isolation of *Pedomicrobium* cultures from water samples [14].

2.2. Modern equipment

In the last 30 years the technical equipment of micromanipulators has been greatly improved. A long distance objective (Zeiss) with 100× magnification is now available. This allows manipulation at 1000× magnification, and more with an inverse microscope. The capillary tools can be positioned quickly and precisely. The available pneumatic or hydraulic systems are very accurate pressure devices.

For the isolation of microbial cells a commercial micromanipulator (Eppendorf, model 5171) equipped with a pressure device (Eppendorf model 5246 plus or CellTram Oil) and mounted onto an inverse phase contrast microscope (Axiovert 25; objective CP 'Achromat' 100×/1.25 Oil Ph2; Zeiss) can be used (Bactotip method) [4,15–17]. The magnification can be adjusted from 400× to 1000×. The micromanipulator is used according to the manufacturer's instructions (micromanipulator 5171: Operating Manual; CellTram Oil: Operating Manual; Transjector 5246: Operating Manual; Eppendorf, Hamburg). The diameter of the opening of the capillary tip can be adjusted to the size of the bacterial cell of interest. For the isolation of bacteria a sterile capillary tube ('Bactotip'; Fig. 1) is used, which preferably possesses a beveled tip (angle 45°) usually with an opening of about 5–10 μm at the anterior end. The sterile Bactotips are produced by Eppendorf (Hamburg). The posterior end of the Bactotip is sealed with a droplet of sterile oil. If desired, the inner surface of the tip can be siliconized with dichlorodimethylsilane (Fluka Chemie AG, Buchs, Switzerland). This is advisable if the bacteria tend to adhere to glass surfaces. For the isolation of anaerobic microorganisms a glove box (Fig. 2) with a N_2/H_2 (95:5; v/v) atmosphere is used (COY chamber, Toepfer Lab Systems, Göppingen, Germany). The

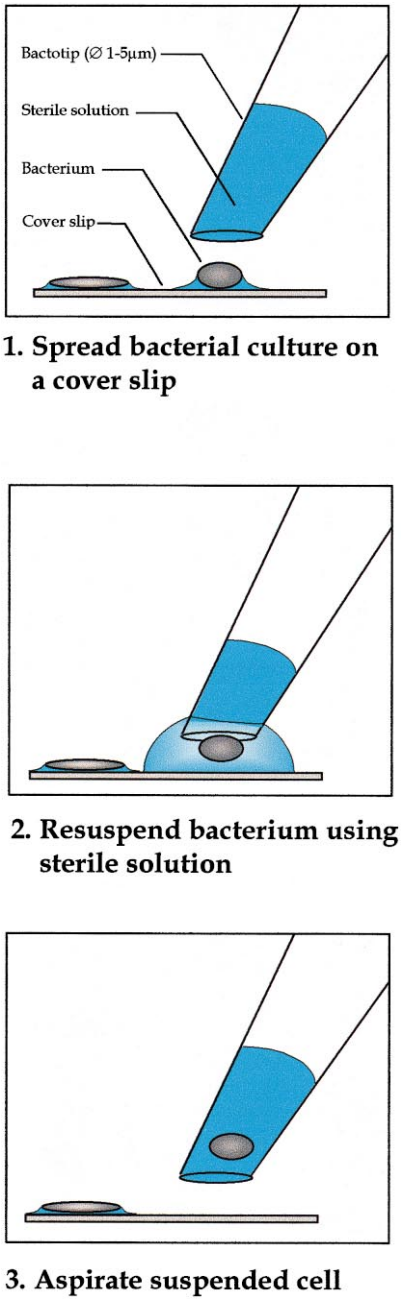

Fig. 1. Schematic drawing of the isolation of a single bacterial cell.

microscope is equipped with a CCD camera (type AVT-BC-12CE Zeiss) and a monitor (type PM 9-5 B, Zeiss). The relative humidity in the anaerobic chamber is adjusted to $>90\%$.

Luttermann et al. [18] described a micromanipulation method for transferring very small objects, such as bacteria, from agar plates using microcapillary tubes. An angled capillary tube (angle 90°) is positioned between the condenser and the objective. The agar plate with the selected bacteria is moved below the opening of the capillary tube using the microscope stage. The aspirated bacterium is placed on the surface of a solid medium or in liquid medium in a microtiter plate.

2.3. Cloning procedure

Cultures or complex mixtures of prokaryotic strains should be diluted in 1–10 ml phosphate buffered saline (1× PBS; 10 mmol sodium phosphate and 130 mmol sodium chloride per liter, pH 7.0). The diluted suspension is spread as a thin film on a sterile microscopic coverslip (24×60 mm) (Fig. 1). A small volume (ca. 0.1–0.2 µl) of PBS is sucked into the Bactotip (Bactotip method). When the opening of the Bactotip (Fig. 1) is brought close to the

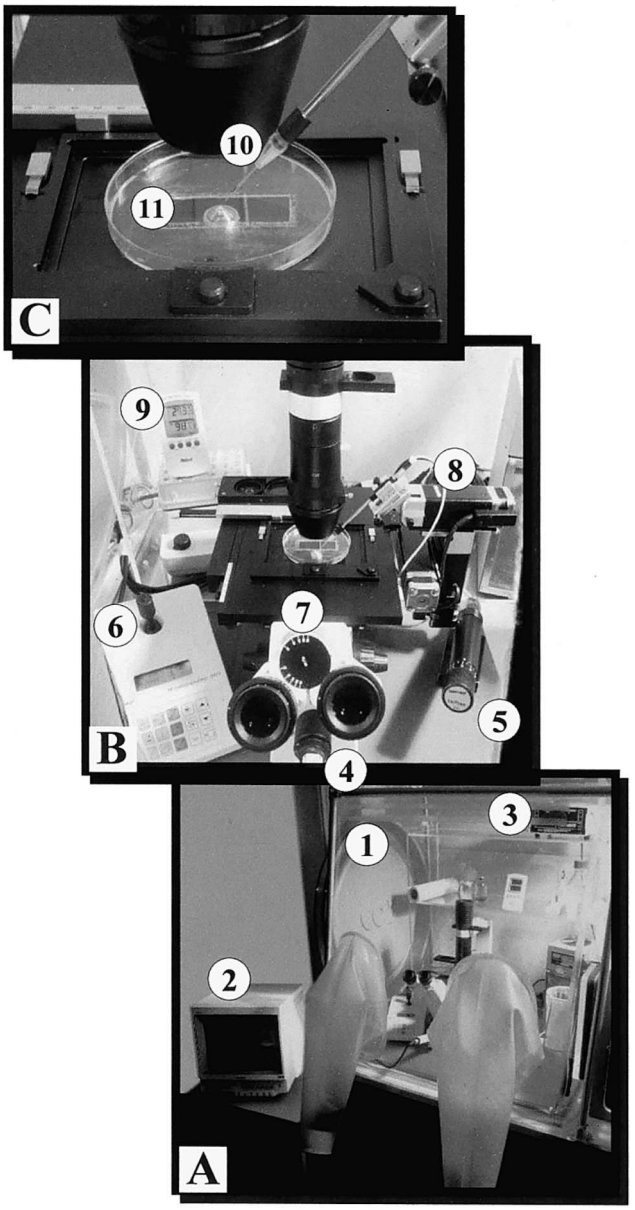

Fig. 2. Working station for the manipulation of single cells with a COY chamber (A) for aerobic and anaerobic isolation. The isolation of single cells is performed using an inverse microscope and a micromanipulator device (B). The spread cells are aspirated by the application of a Bactotip (C). Technical specifications: COY chamber (1), monitor (2), O_2/H_2 electrode (3), camera (4), CellTram Oil (5), joystick (6), inverse microscope (7), micromanipulator (8), thermometer/hygrometer (9), Bactotip (10), coverslip with spread bacteria (11).

surface of a distinct bacterial cell a droplet of PBS buffer flows out of the tip and moistens the bacterial cell. The cell is suspended in the droplet after detaching from the glass surface. About 10 single microbial cells can be removed from the microscopic slide within 30 min by aspirating them together with the droplet into the Bactotip. The withdrawn single cells can be transferred to Eppendorf reaction tubes or Hungate tubes (for anaerobes) containing 0.3 ml of the appropriate liquid medium. The tested microbial cells survive the spreading procedure. The tubes are incubated at e.g. 37°C for 10–72 h. Between two and seven growing cultures were obtained out of 10 cloned single cells (*Bacillus cereus*: 4; *Enterobacter cloacae*: 5; *Escherichia coli*: 3; *Staphylococcus aureus*: 7; *Desulfovibrio desulfuricans*: 2). *D. desulfuricans* was isolated anaerobically. The single cells grew up to a visible density or a visible colony in 10–72 h. A single cell could also be transferred to solid medium in Petri dishes as shown with *Bacillus cereus*. The colonies became visible after incubation overnight at 37°C. Furthermore, single cells (e.g. *B. cereus*) were grown directly in the Bactotip.

Spreading the bacterial suspension onto a microscopic slide after an appropriate dilution of the original culture was a prerequisite for the rapid isolation of single cells, while the isolation of a single bacterial cell directly out of a droplet containing a suspension of a mixed microbial population was not very successful. The cells should be transferred to culture medium within 30 min after spreading. The Bactotip method allows the transfer of single prokaryotic cells to different culture vessels, such as Eppendorf reaction tubes or Hungate tubes, or onto the surface of solid media in Petri dishes; it also allows the isolates to be used for single cell PCR or for direct growth in the Bactotip. The advantage of the Bactotip method compared to conventional isolation methods can be seen in the ability to pick out a single prokaryotic cell under direct visual control and to grow pure cultures of distinct aerobic and anaerobic cells directly out of a mixed natural or laboratory population in a relatively short time.

2.4. Applications

The highest number of positive culture tubes with leptospires obtained by Bakoss [7] was 66.6%. Luttermann et al. [18] obtained positive culture tubes in a range of 50–60%. We obtained the best results with laboratory cultures of *Staphylococcus aureus* (70%), which may be due to its relatively high osmotolerance ($a_w = 0.90$–0.86) [19].

Since the cloning procedure described above was successfully applied to laboratory strains we isolated single bacterial cells by the Bactotip method from a complex natural population, the termite gut [4]. The paunch contents (ca. 10 μl) of the termite, *Mastotermes darwiniensis*, were diluted and treated as described above. *Enterococcus* (*Enterococcus* sp. str. JF1; AJ132470) and *Sphingomonas* (*Sphingomonas* sp. str. JF2; AJ1232471) were directly isolated from the termite gut in one step.

If the opening is not larger than 0.5 μm only very small cells can be picked up. With a capillary tube (inner diameter 0.5 μm; Femtotip II, Eppendorf, Germany), a *Mycoplasma alvi* related strain (AJ132469) was isolated from the cytoplasm of the Archaezoon *Koruga bonita* [4].

The trichomonad *Pentatrichomonoides scroa* (length: 30 μm) [20,21], one of the smaller gut flagellates of *M. darwiniensis*, harbors about 50 endosymbiotic methanogens [4,22]. Cloned single cells of the endosymbiotic methanogen were used for the amplification of their SSU rDNA by a single cell semi-nested PCR. The methanogen (*Methanobrevibacter* sp.; AJ132468) isolated from *P. scroa* was related to both clone CD 3 identified in the gut contents of *Cryptotermes domesticus* [23] and *Methanobrevibacter curvatus* [24] isolated from the termite *Reticulitermes flavipes*.

3. Laser micromanipulation systems

3.1. Optical tweezers

Ashkin et al. [25] described the use of infrared laser beams (1064 nm) for trapping and manipulating biological specimens, such as the single cells of *E. coli* or *Saccharomyces cerevisiae*. This method was improved and successfully applied to the isolation of hyperthermophilic bacteria and archaea [5,26], and is described in a separate article by R. Huber, H. Huber and K.O. Stetter in this issue. Therefore, only the principles of this procedure will be mentioned here. A neodymium laser is focused by a microscope objective. The movement of the microscope stage is computer-controlled. A rectangular glass capillary with a predetermined breaking point is used as the separation chamber (inside dimensions: 0.1×1 mm, length: 10 cm), which is filled with fresh medium (90%) and the mixed microbial population (10%). A single selected cell is fixed with the laser beam and is separated from the mixed culture by moving the microscope stage. The capillary is broken at the predetermined breaking point and the single cell is transferred to the culture medium. The culture efficiency, after an incubation time of up to 5 days, was 20–100%. The isolation of dead cells could be prevented by application of fluorescent dyes staining viable cells, for example bis-(1,3-dibutylbarbituric acid) trimethine oxonol [27]. Photo damage can be reduced to a background level under anaerobic conditions [28]. This method is a promising tool for the isolation of microorganisms which cannot be obtained in pure culture by conventional methods.

3.2. Laser microdissection

Schütze et al. [29] described a laser pressure catapulting method, which uses a laser (Robot-MicroBeam) for the microdissection and transfer of single cells. This method

has been successfully applied for the isolation of single cells from human tissues. The specimens are spread on a sheet of a 1.35 μm thin polyethylene membrane. With the high photonic energy of a focused nitrogen laser a selected single cell is precisely circumscribed and the selected cell together with a small surrounding strip of the polyethylene membrane is cut out. The round polyethylene slip with the selected cell still adheres to the polyethylene membrane. The laser is then focused below the microdissected target cell and the microdissected sample is catapulted into a common microfuge tube positioned above the sample with a laser shot of increased energy. The cells are subjected to single cell nested PCR. In principle, this method can be applied to cells of any size, but an application for the isolation of viable prokaryotes has not been published so far.

4. Critical outlook

The above mentioned techniques (the Bactotip method, optical tweezers) allow the separation of a single bacterial cell in the presence or absence of oxygen under visual control. Many of the technical problems regarding the separation of single bacterial cells have been solved. The remaining problems are less of a technical nature than, for example, choosing a suitable medium composition for growing bacterial cells of unknown systematic affiliation. Our experiments and previous observations [8] showed that even individual cells of the same species (e.g. *Enterobacter*) differed in the number of resulting grown cultures, depending on the manipulation technique used. Cells aspirated and transferred to fresh solid or liquid media with a capillary tube resulted in a lower number of grown cultures than cells which were separated from a colony and immediately moved over the surface of an agar layer as described by Skerman [8] or Sherman [11]. The reason for this behavior is not clear so far.

It has been known for over 35 years that bacterial populations with cells in a 'sleeping state' could be grown once again after the addition of sterile supernatant of a freshly grown culture [30–32]. In those early studies the specific death rates of high density bacterial populations were lower than those of low density populations. A similar growth stimulating effect could be found with single cells by the addition of fresh culture supernatants. The volume of the medium into which a single bacterium is transferred is also important, because the number of growing cultures decreases with increasing volume.

In recent years it has become clear that bacterial cells can communicate with each other via small signal molecules such as long chain fatty acid esters or *N*-acetylhomoserine lactones [33–35]. Therefore, some of the difficulties in cultivating single isolated bacteria may possibly be due to this quorum sensing effect. Thus, the manipulation technique is suitable for studying the effect of different concentrations of signal molecules on the growth behavior of single cells. These few examples demonstrate that new cultivation approaches have to be applied to grow cloned single bacterial cells with higher efficiency.

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