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TITLE:

Growing Magnetotactic Bacteria of the Genus *Magnetospirillum*: Strains MSR-1, AMB-1 and MS-1

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SUMMARY:

We present a procedure for growing several strains of *Magnetospirillum* in two different types of growth media. *Magnetospirillum gryphiswaldense* strain MSR-1 is grown in both liquid and O_2 concentration gradient semi-solid media while *M. magneticum* strain AMB-1 and *M. magneto-tacticum* strain MS-1 are grown in liquid medium.

ABSTRACT:

Magnetotactic bacteria are Gram-negative, motile, mainly aquatic prokaryotes ubiquitous in freshwater and marine habitats. They are characterized by their ability to biomineralize magnetosomes, which are magnetic nanometer-sized crystals of magnetite (Fe_3O_4) or greigite (Fe_3S_4) surrounded by a lipid bilayer membrane, within their cytoplasm. For most known magnetotactic bacteria, magnetosomes are assembled in chains inside the cytoplasm, thereby conferring a permanent magnetic dipole moment to the cells and causing them to align passively with external magnetic fields. Because of these specific features, magnetotactic bacteria have a great potential for commercial and medical applications. However, most species are microaerophilic and have specific O_2 concentration requirements, making them more difficult to grow routinely than many other bacteria such as *Escherichia coli*. Here we present detailed protocols for growing three of the most widely studied strains of magnetotactic bacteria, all belonging to the genus *Magnetospirillum*. These methods allow for precise control of the O_2 concentration made available to the bacteria, in order to ensure that they grow normally and synthesize magnetosomes. Growing magnetotactic bacteria for further studies using these procedures does not require the

experimentalist to be an expert in microbiology. The general methods presented in this article may also be used to isolate and culture other magnetotactic bacteria, although it is likely that growth media chemical composition will need to be modified.

INTRODUCTION:

Magnetotactic bacteria (MTB) represent a wide range of Gram-negative prokaryotes ubiquitous in freshwater and marine aquatic habitats[1]. These bacteria share the ability to produce magnetic crystals made of either magnetite (Fe_3O_4) or greigite (Fe_3S_4), which are in most cases assembled into chains inside the cells. This particular structural motif is due to the presence of several specific proteins acting both in the cytoplasm of the bacteria and on the lipid membrane that surrounds each crystal[2]. Each individual crystal and its surrounding membrane vesicle is called a magnetosome and is ranging in size from about 30 to 50 nm in *Magnetospirillum* species[3]. Because of the chain arrangement of magnetosomes, these bacteria possess a permanent magnetic dipole moment that makes them align passively with externally applied magnetic fields. Therefore, these bacteria actively swim along magnetic field lines, acting as self-propelled micro-compasses presumably to more effectively locate the most favorable conditions (*e.g.*, O_2 concentration) for growth.

An interesting property of MTB is their ability to regulate both the chemistry and the crystallography of their magnetosome crystals. Most strains produce relatively high purity crystals of either magnetite or greigite, although some biomineralize both minerals[4]. In all cases, the bacteria are able to precisely control the size and the shape of their single magnetic domain crystals. This explains why a great amount of research is undertaken to develop a better understanding of how MTB perform this biomineralization process. Understanding this process might allow the researchers to tailor-make magnetic nanocrystals for many commercial and medical applications.

A substantial obstacle to extensive research on MTB has been the difficulty of growing them in the laboratory. Most species, including the strains used in this work, are obligately microaerophilic when grown with O_2 as a terminal electron acceptor. This explains why these bacteria are most often found at the transition zone between oxic and anoxic conditions (the oxic-anoxic interface, OAI). This clearly shows that MTB have precise O_2 concentration requirements which obviously needs to be taken into account when devising growth media for these organisms. Moreover, the great existing diversity of MTB implies that different strains will need different types of chemical gradients and nutrients to achieve optimal growth.

In this work, we describe the methods for growing three of the most widely studied MTB: *Magnetospirillum magneticum* (strain AMB-1), *M. magnetotacticum* (MS-1) and *M. gryphiswaldense* (MSR-1). These species phylogenetically belong to the *Alphaproteobacteria* class in the *Proteobacteria* phylum, are helical in morphology and possess a polar flagellum at each end of the cell. We provide the protocols for growing strain MSR-1 in both liquid and O_2 concentration gradient semi-solid media, based on previously published medium recipes[5,6]. We also present a detailed protocol for growing strains AMB-1 and MS-1 in modified Magnetic Spirillum Growth Medium (MGSM)[7].

PROTOCOL:

1. Installation of the N_2 Station

Note: Choose the inner diameter of the tubing so that it can be connected to the gas tank with minimum leakage and so that the cylinder of a 1 mL plastic syringe tightly fits in this tubing. An illustration of the complete N_2 gassing station is provided in **Figure 1**.

1.1. Safely install a N_2 gas tank close to a bench on which there is enough space to set up the N_2 station (a length of approximately 50 cm).

1.2. Connect to the tank a piece of tubing long enough to reach the area where the station will be built. If necessary, apply Teflon tape at the output of the tank to avoid any leakage.

1.3. To build a station capable of bubbling five bottles of medium at the same time, cut four pieces of tubing of approximately 5 cm in length.

1.4. Assemble the pieces of tubing in a line with three three-way T-shaped plastic fittings. Connect one end of this line to the piece of tubing at the output of the N_2 tank through an extra T-shaped fitting. Add a 90° elbow fitting at the other end.

1.5. Use tape to attach the structure to a horizontal metal rod placed approximately 30 cm above the bench.

1.6. Connect five pieces of tubing (approximately 20 cm in length) to the free outputs of the fittings installed in Step 1.4.

1.7. Remove the pistons from five 1.0 mL plastic syringes and cut the larger end of these syringes (*i.e.*, the opposite side to the needle), keeping only the graduated part. Fill these syringes with cotton, not too tightly.

1.8. Insert the syringes in the 20 cm long vertical pieces of tubing and use soapy water to ensure that there is no leakage when N_2 is flowing.

1.9. Remove the caps of five 25G needles (0.5 mm x 25 mm) and insert these needles into the 10 cm pieces of thin tubing. Ensure that the needles tightly fit in the tubing.

CAUTION: There is a risk of stabbing during this step. Do it slowly and carefully.

1.10. Attach the needles prepared in Step 1.9 to the syringes of the N_2 station. Ensure that N_2 is flowing through all five lines and keep the station on stand-by.

2. Growth Medium Preparation

Note: It is possible to adjust the amount of medium prepared, as explained in Steps 2.1.2, 2.2.2 and 2.3.8. The amount of water and chemicals used just need to be proportionally adjusted. The role of all medium components is described in **Supplementary Table 1**.

2.1. Preparation of liquid growth medium for MSR-1

2.1.1. Prepare a 10 mM ferric citrate solution by adding 0.245 g of ferric citrate to 100 mL of distilled deionized water. Heat and stir to dissolve, until a yellow, clear solution is obtained. Autoclave the solution using a standard cycle (at least 15 min exposure at 121 °C) and then store this stock solution at room temperature in the dark.

Note: Discard the ferric citrate solution when a precipitate becomes obvious.

2.1.2. In a beaker containing 1 L of distilled deionized water, add the following in order while stirring: 1.0 mL of the trace mineral supplement solution, 0.1 g of KH_2PO_4, 0.15 g of $MgSO_4.7\ H_2O$, 2.38 g of HEPES, 0.34 g of $NaNO_3$, 0.1 g of yeast extract, 3.0 g of soy bean peptone, 4.35 mL of potassium lactate (60% w/w solution) and 5 mL of the 10 mM Fe(III) citrate stock solution.

Note: Adjust the quantities proportionally if a smaller amount of growth medium is needed. For a N_2 station capable of bubbling five bottles at the same time, such as the one built in Step 1 of this protocol, prepare 300 mL of medium.

CAUTION: Trace mineral and Fe(III) citrate stock solutions need to be kept sterile. To avoid contamination, use standard sterile technique when using them (flame open tops of bottles using a Bunsen burner) and use sterile pipette tips for dispensing. Store the mineral solution in a refrigerator at 4 °C.

2.1.3. After the addition of all chemicals, adjust the pH to 7.0 with 1 M NaOH solution. Dispense the freshly prepared medium into 125 mL serum bottles. Pour 60 mL of medium in each bottle.

2.1.4. Bubble N_2 into the medium for 30 min to remove the dissolved O_2, using the small tubing connected to the N_2 station described in Step 1. Place a butyl-rubber stopper on top of each bottle, leaving a small opening to allow the excess gas to exit the bottle.

CAUTION: Foam might form while bubbling with N_2. Adjust the gas flow accordingly to avoid foam production.

2.1.5. Crimp seal each bottle with the prepared stopper and an aluminum seal. The aluminum seal ensures that the bottle remains sealed during the rest of the protocol.

2.1.6. Disconnect the needles and the thin tubing from the N_2 station and replace them with clean needles (1 inch, $\leq$ 23G). Adjust the valves of the N_2 tank so that a gentle continuous flow of gas exits the tank (about 50 mL/min).

Note: Needles larger than 23G may leave permanent unsealable holes in the stopper.

2.1.7. Insert one of the needles connected to the N_2 tank into a bottle of medium, through the rubber stopper. Immediately insert another clean needle into the same bottle. Repeat this step for the other bottles and let N_2 flow for about 30 min to replace the air in the bottles by N_2.

2.1.8. Disconnect one bottle from the N_2 station by removing the corresponding needle. Wait for a few seconds until the pressure in the bottle of medium decreases to atmospheric pressure and remove the second needle. Repeat this step for all remaining bottles.

CAUTION: To prevent O_2 from re-entering the bottles of growth medium after Step 2.1.4, perform Steps 2.1.4 – 2.1.8 in quick succession. If all bottles cannot be connected to the N_2 station at the same time, proceed with Steps 2.1.4-2.1.8 for the first set of bottles and then repeat these steps for the remaining bottles.

2.1.9. Autoclave the bottles. Let them cool down to room temperature overnight and store them at room temperature afterwards.

2.2. Preparation of liquid growth medium for AMB-1 and MS-1

2.2.1. Prepare a 10 mM ferric quinate solution. First dissolve 0.19 g of quinic acid in 100 mL of distilled deionized water, then add 0.27 g of $FeCl_3.6H_2O$. Stir to dissolve, until a dark red, clear solution is obtained. Autoclave the solution using a standard cycle (at least 15 min exposure at 121 °C).

Note: Store the ferric quinate solution at room temperature in the dark as a sterile stock solution. Discard the solution when a precipitate becomes obvious.

2.2.2. In a beaker containing 1 L of distilled deionized water, add the following in order while stirring: 10.0 mL of the vitamin supplement solution, 5.0 mL of the trace mineral supplement solution, 0.68 g of KH_2PO_4, 0.848 g of sodium succinate dibasic hexahydrate, 0.575 g of di-Sodium tartrate dihydrate, 0.083g of Sodium acetate trihydrate, 0.45mL of 0.1% aqueous Resazurin, 0.17 g of $NaNO_3$, 0.04 g of ascorbic acid and 3.0 mL of the 10 mM Fe(III) quinate stock solution.

Note: Adjust the quantities proportionally if a smaller amount of growth medium is needed. For a N_2 station capable of bubbling five bottles at the same time, such as the one built in Step 1 of this protocol, prepare 300 mL of medium.

CAUTION: Vitamin, trace mineral and Fe(III) quinate stock solutions need to be kept sterile. To avoid contamination, use standard sterile technique and sterile pipette tips when dispensing. Store the mineral and vitamin solutions in a refrigerator at 4 °C.

2.2.3. After the addition of all chemicals, adjust the pH to 6.75 using 1 M NaOH solution.

2.2.4. Refer to Steps 2.1.3-2.1.9 for the rest of the protocol.

2.3. Preparation of semi-solid growth medium for MSR-1

2.3.1. Prepare a 0.5 M phosphate buffer solution pH 7.0 by dissolving 3.362 g of K_2HPO_4 and 4.178 g of KH_2PO_4 in 100 mL of distilled deionized water. Check if the pH is 7.0 and adjust the pH slightly with KH_2PO_4 or NaOH if needed. Store the solution in a sealed glass bottle (preferable to plastic in order to avoid oxygen exchange).

2.3.2. Prepare 100 mL of a 0.02 M hydrochloric solution. Add 0.2 g of $FeCl_2.4H_2O$ to this solution and stir to dissolve in order to obtain a 10 mM iron chloride solution. Store the solution in the dark in a sealed glass bottle.

2.3.3. Prepare a 0.8 M sodium bicarbonate solution by dissolving 6.72 g of $NaHCO_3$ in 100 mL of distilled deionized water. Store the solution in a sealed glass bottle.

2.3.4. Autoclave the solutions prepared in Steps 2.2.1 – 2.2.3 using a standard cycle (at least 15 min exposure at 121 °C). Store them as sterile stock solutions in the dark.

2.3.5. In a beaker containing 1 L of distilled deionized water, add the following in order while stirring: 5 mL of the trace mineral supplement solution, 0.2 mL of 1% aqueous resazurin solution, 0.4 g of NaCl, 0.3 g of NH_4Cl, 0.1 g of $MgSO_4.7H_2O$, 0.05 g of $CaCl_2.2H_2O$, 1 g of sodium succinate, 0.5 g of sodium acetate, 0.2 g of yeast extract and 1.6 g of agar.

2.3.6. Cover the beaker with aluminum foil and autoclave the solution prepared in Step 2.3.5.

2.3.7. Just before the end of the autoclave cycle, prepare a fresh 4% L-cysteine·HCl·H_2O solution by dissolving 0.8 g of L-cysteine·HCl·H_2O in 20 mL of distilled deionized water. Neutralize the solution to pH 7.0 with 5 M NaOH solution.

CAUTION: it is important that the cysteine solution is prepared fresh to avoid oxidation of the cysteine. Store the mineral solution in a refrigerator at 4 °C.

2.3.8. After autoclaving, let the medium cool down to 50-60 °C and bring the beaker under the flame of a Bunsen burner. Remove the aluminum foil and quickly add the following in order while gently stirring: 0.5 mL of the vitamin solution, 2.8 mL of the sterile phosphate buffer stock solution, 3 mL of the sterile iron chloride stock solution, 1.8 mL of the sterile sodium bicarbonate stock solution and 10 mL of filter-sterilized cysteine solution. Ensure that the solution turns colorless quickly after the cysteine is added.

Note: Adjust the quantities proportionally if a smaller amount of growth medium is needed. It is usually convenient to prepare a batch of 120 mL of medium.

CAUTION: All solutions must remain sterile for future use. Perform Step 2.3.8 using standard

sterile technique and sterile pipette tips when dispensing.

2.3.9. After the addition of all chemicals, transfer the warm medium into 16 mL sterile screw-cap Hungate tubes. Transfer 12 mL of the medium into each tube and seal the tubes.

CAUTION: To avoid contamination, perform Step 2.3.9 under the flame of a Bunsen burner. Perform it before the agar solidifies, while the medium is at 40 °C or above.

2.3.10. Leave the tubes undisturbed for a few hours until the agar solidifies and the OAI is apparent.

3. Inoculation of MTB

Note: The cultures of strains AMB-1, MS-1 and MSR-1 can be obtained commercially (**Table of Materials**).

CAUTION: Perform all the following steps in sterile conditions, under the flame of a Bunsen burner.

3.1. Inoculation of strains MSR-1, AMB-1 and MS-1 in liquid medium

3.1.1. Seal an empty 125 mL serum bottle with a butyl-rubber stopper and an aluminum crimp seal. Insert two needles in the bottle through the stopper, and connect a syringe prepared as in Step 1.7 to one of them. Connect the syringe to a cylinder of O_2 through the same type of tubing as the one used for the N_2 station.

3.1.2. Let O_2 flow through the bottle for about 30 min, to ensure that all air in the bottle is replaced by O_2. Remove both needles, allowing for a slight overpressure in the bottle and then autoclave. Allow the bottle to cool down to room temperature before use.

Note: To save time, perform Steps 3.1.1 and 3.1.2 during the medium preparation and autoclave the bottle along with the medium or the stock solutions.

3.1.3. Sterilize the tops of the stoppers of both the fresh medium bottle and the O_2 bottle by applying a few droplets of 70% ethanol solution on top of them and passing them through the flame of a Bunsen burner.

3.1.4. Using a sterile syringe and a needle, extract 1 mL of O_2 from the O_2 bottle and transfer it into the fresh medium bottle. Make sure that the needle tightly fits on the syringe during this step to avoid any air in the syringe.

3.1.5. If using the inoculum from another culture grown in a glass bottle, sterilize the stoppers of both the fresh medium bottle and the older culture bottle by applying a few droplets of 70% ethanol solution on top of them and passing them through the flame of a Bunsen burner. If using

the inoculum from a tube of frozen culture, just let it warm up to room temperature with the tube sealed under the flame of a Bunsen burner.

3.1.6. If using the inoculum from another culture grown in a glass bottle, inoculate 1 mL of the older culture into the fresh medium. If using the inoculate from a frozen stock, inoculate only 0.1 mL to dilute the glycerol or dimethyl sulfoxide (DMSO) used in the freezing process. In both cases, use a sterile needle and a syringe.

3.1.7. Incubate the culture at 32 °C and inoculate it into fresh medium after 4 to 7 days.

3.2. Inoculation of MSR-1 in O_2 gradient semi-solid medium

3.2.1. Verify that the tube of fresh medium displays a well-defined OAI, materialized by a pink to colorless interface, about 1-3 cm below the surface of the medium.

3.2.2. If the inoculum is coming from another O_2 concentration gradient semi-solid culture, harvest the bacteria by pipetting 50 µL of the culture with a sterile pipette tip placed on the band formed by the bacteria. Slowly inoculate these bacteria at the OAI in the fresh medium (pink/colorless interface), avoiding disturbing the interface. If the inoculum is coming from a frozen culture, proceed in the same way with 100 µL of inoculum instead.

3.2.3. Seal the tube and let the bacteria grow between 25 °C and 30 °C. Transfer into fresh medium using the same procedure when 2/3 of the medium is pink.

4. Observation of the Bacteria

4.1. Sterilize the stopper of the culture bottle by applying 70% ethanol solution on top of it and passing it through the flame of a Bunsen burner. For semi-solid medium, open the tube under the flame of a Bunsen burner. Use a sterile needle and a syringe to extract the bacteria.

4.2. Use the hanging drop method[8] to ensure that the bacteria are both magnetic and motile.

Note: Phase contrast microscopy gives great results but is not mandatory. Magnifications ranging from 10X to 60X are suitable.

4.3. Use transmission electron microscopy (TEM) to observe the cell structure and the magnetosomes in detail[8].

REPRESENTATIVE RESULTS:

Successful preparation of the growth media can be assessed as follows. At the end of the process, clear solutions (*i.e.*, free of any precipitate) should be obtained (this is true for both the liquid media and the O_2 gradient semi-solid medium). A picture displaying the expected aspect of MSR-1 liquid medium before inoculation can be seen in **Figure 2a**. A successful O_2 concentration gradient semi-solid medium is signaled by the formation of an OAI after a few hours, indicated by

the presence of 1-3 cm of pink medium at the top of the tube (**Figure 3**, tube A). The rest of the medium should be colorless. An all-pink medium before inoculation is a sign of complete oxidation of the resazurin and therefore of an unsuccessful preparation of the O_2 concentration gradient.

Successful growth in liquid medium can be checked about 48 h after inoculation (or after a few days if the inoculum is coming from a frozen culture) simply by examining the cultures for turbidity using a light source or a spectrophotometer. Successful growth is confirmed by turbidity proving that the bacteria actually grew (**Figure 2b**). If the medium remains clear after 48 h, the bacteria have not grown. For a liquid medium culture growing normally, the presence of bacteria synthesizing magnetosomes can be checked after 48 h by placing the bottle of medium on a magnetic stir plate and by illuminating it with a flashlight. At a low stirring speed, the medium should "flash", looking alternately darker and brighter depending on the orientation of the stirring magnet with respect to the position of the experimentalist. For a non-magnetic culture, the intensity of light scattered by cells towards the experimentalist will remain constant.

Successful growth in semi-solid medium is indicated by the formation of a microaerophilic band of bacteria at the pink/colorless interface. Due to the consumption of O_2 by the bacteria and the changes in the O_2 gradient, the band will slowly migrate to follow the OAI (**Figure 3**, tube B).

The hanging drop method enables the observer to check that the bacteria are both magnetic and motile. After a few minutes, MTB should concentrate at the edge of the hanging drop, proving that they actively swim along the magnetic field lines (**Figure 4a**). To ensure that the cells are magnetic, flip the orientation of the magnet sitting next to the drop. The bacteria should start swimming towards the opposite edge (**Figure 4b**). Finally, the shape of the magnetosomes can be checked with TEM. Cuboctahedral crystals of about 30-45 nm in diameter should be observed[9]. These crystals are normally arranged in one long chain or multiple shorter chains in the species studied here (**Figure 5**).

FIGURE AND TABLE LEGENDS:

Figure 1: Illustration of the N_2 gassing station. (a) Schematic representation. (b) Picture of the setup. A successful station will have the appropriate length of large and thin tubing so that the end of the thin tubing described in Step 1.9 of the protocol remains immersed in the medium during the gas bubbling step. The flow of gas should also be adjustable at all times, to ensure that all O_2 is removed and that the medium does not spill.

Figure 2: Bottles of liquid growth medium before and after inoculation. (a) Clear MSR-1 medium before inoculation. (b) Turbid MSR-1 medium after 3 days of successful growth.

Figure 3: Tubes of O_2 gradient semi-solid medium before (tube A) and 10 days after (tube B) inoculation. The band of bacteria in tube B is indicated by a red arrow.

Figure 4: Typical results of a hanging drop experiment at 20X magnification using phase con-

trast. (a) MTB swim towards the south pole of a magnet and accumulate at the liquid/air interface. (b) 1 min after flipping the magnet, most cells have left the field of view.

Figure 5: TEM observation of an AMB-1 cell. Magnetosome chains are indicated by red arrows. The two flagella are indicated by black arrows.

DISCUSSION:

The specific O_2 concentration requirements of MTB make them non-trivial to grow in the laboratory. A key step of the protocol for liquid medium is the initial removal of all O_2 from the medium in order to control the final concentration by adding a definite volume of O_2, just before inoculation. It has been shown that MSR-1 grows under almost fully aerobic conditions, however, the magnetism of the cells is drastically reduced. The results from the same study showed that strains AMB-1 and MS-1 do not grow under fully aerobic conditions[6]. For the semi-solid culture of MSR-1, all O_2 is first reduced by the cysteine solution and then the O_2 concentration gradient appears, leading to the formation of the OAI. If a culture does not grow well or is not magnetic, the O_2 concentration should be the first thing to check.

Semi-solid growth medium is an interesting choice for isolating unknown MTB or growing the species that are highly sensitive to both O_2 and redox gradients, as it ensures the existence of stable gradients and allows the bacteria to position themselves at the location offering the best growth conditions. Liquid medium is more convenient to work with when higher volumes are needed (*e.g.*, for DNA extraction), or when further analysis needs to be conducted on the cells (*e.g.*, magnetosome studies) as it allows for an easier separation of the cells from the growth medium. The agar in semi-solid medium might indeed interfere with the cell harvesting technique.

In some cultures, the cells sometimes become non-motile, likely due to spontaneous mutations. Non-motile cells survive and grow in the growth medium since they do not need to swim to find nutrients necessary for their survival. The standard race-track method[10] can then be used to recover a motile culture since only motile cells can swim down the race-track. The loss of the magnetic phenotype can also happen in the culture even if the O_2 concentration is optimal, again due to spontaneous mutations[11]. The best solution in that case is to start a new culture by inoculating frozen stocks of the wild-type bacteria into fresh medium. Alternatively, the race-track technique can also allow the recovery of a magnetic culture.

It is essential to avoid contamination of the culture by other organisms and therefore most of the protocol must be performed using sterile techniques. Manipulating under the flame of a Bunsen burner usually gives satisfactory results in keeping aseptic conditions. However, if the culture is grown in a contamination-prone environment, additional precautions should be taken, such as working in a laminar flow hood when preparing the medium and inoculating the bacteria. It should be noted that the risk of contamination is higher in semi-solid medium as the tubes need to be opened every time the cells are removed.

These protocols enable the growth of enough bacteria to perform many different types of experiments, such as physical studies based on optical microscopy[12,13], electron microscopy imaging[14,15], X-ray spectromicroscopy analyses[16], genomic and protein studies[17,18]. If necessary, higher concentrations of bacteria in suspension can be achieved by centrifugation. In addition, magnetosomes can be extracted and purified for further applications from cell pellets or dense cell suspensions obtained by centrifugation[19].

Growth media formulations are usually based on the same governing principles (see **Supplementary Table 1** for a summary of the list of the role of each component in the growth media described here). A carbon source must be available to the bacteria, via organic acids (*e.g.*, succinate, acetate, tartrate, lactate) or inorganic compounds such as bicarbonate. The choice of a suitable carbon source depends on the metabolism of the bacteria. Nitrogen present in DNA and proteins, and phosphorus (DNA, membranes) are also required and provided by $NaNO_3$, NH_4Cl and KH_2PO_4 in the recipes described here. A buffer (HEPES, phosphate buffer) is necessary to resist pH changes. The trace mineral supplement solution contains cofactors for enzymes, and the vitamins can be used by the bacteria as coenzymes or as functional groups for certain enzymes. Yeast extract and soy bean peptone provide a source of amino acids and salts used for protein production. Since MTB are redox sensitive, one or several reducing agents must often be added to the medium (*e.g.*, cysteine, ascorbic acid, lactate). A major source of iron (*e.g.*, ferric citrate, ferric quinate, iron chloride) is needed in the medium for biomineralization. Finally, a small amount of O_2 is required for microaerophilic bacteria as terminal electron acceptor. Obligate anaerobic MTB use other compounds such as nitrate or nitrous oxide instead.

The principal limitation of these protocols is that there is no guarantee that they will work for other species of MTB. Strains AMB-1, MS-1 and MSR-1 are all freshwater MTB and therefore the recipes described in this article cannot be used to grow marine magnetospirilla such a Magnetospira thiophila strain MMS-120, Magnetospira strain QH-221, or other marine MTB, which require higher salt concentrations. However, to grow other strains of MTB, and for the isolation of new strains, the general methods presented in this article for both liquid and semi-solid media can be used to prepare media with different recipes.

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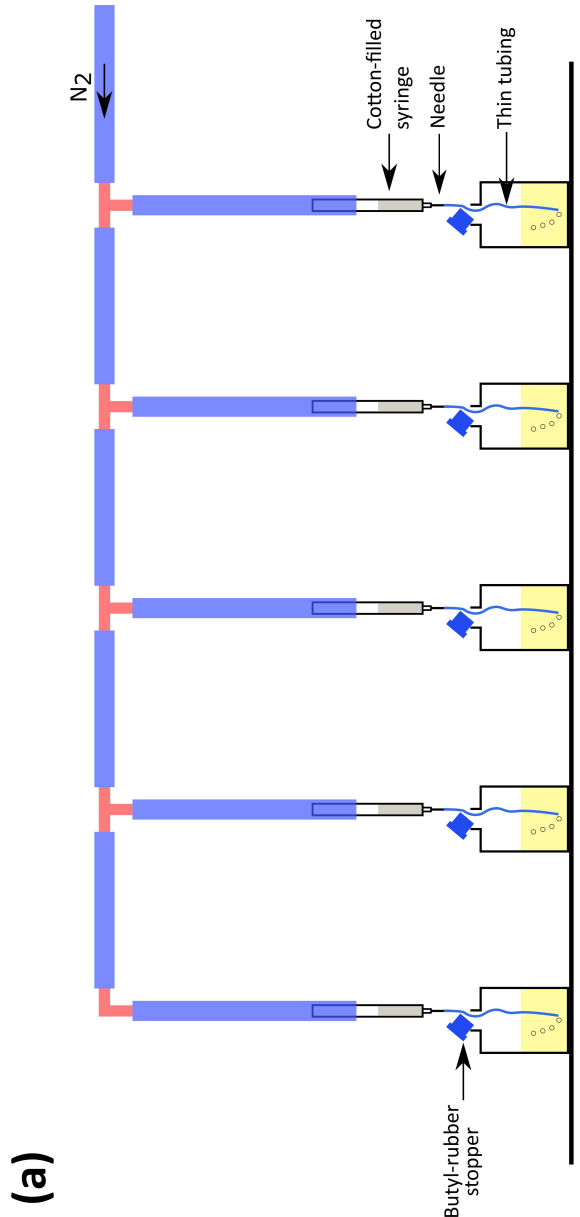

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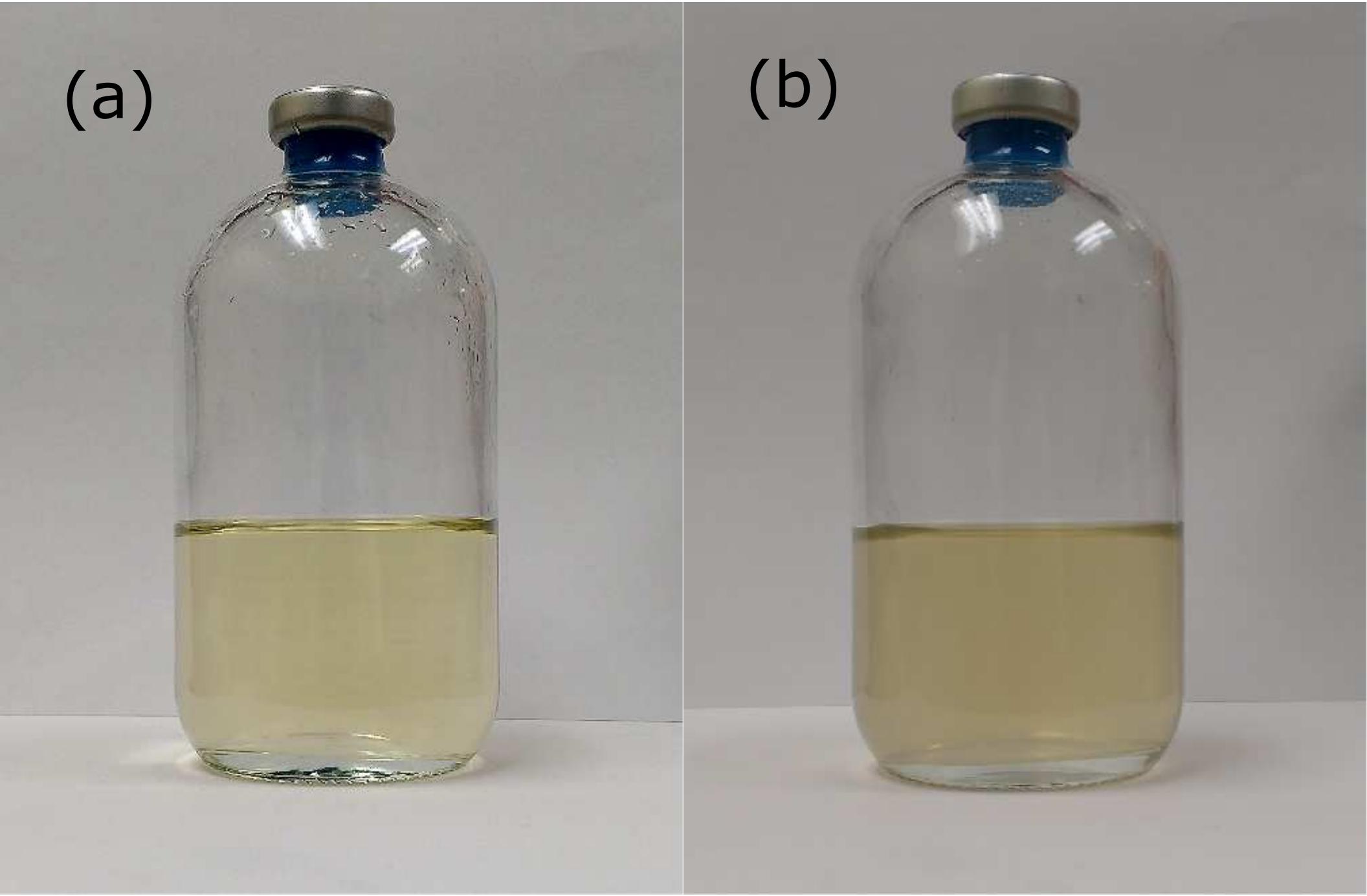

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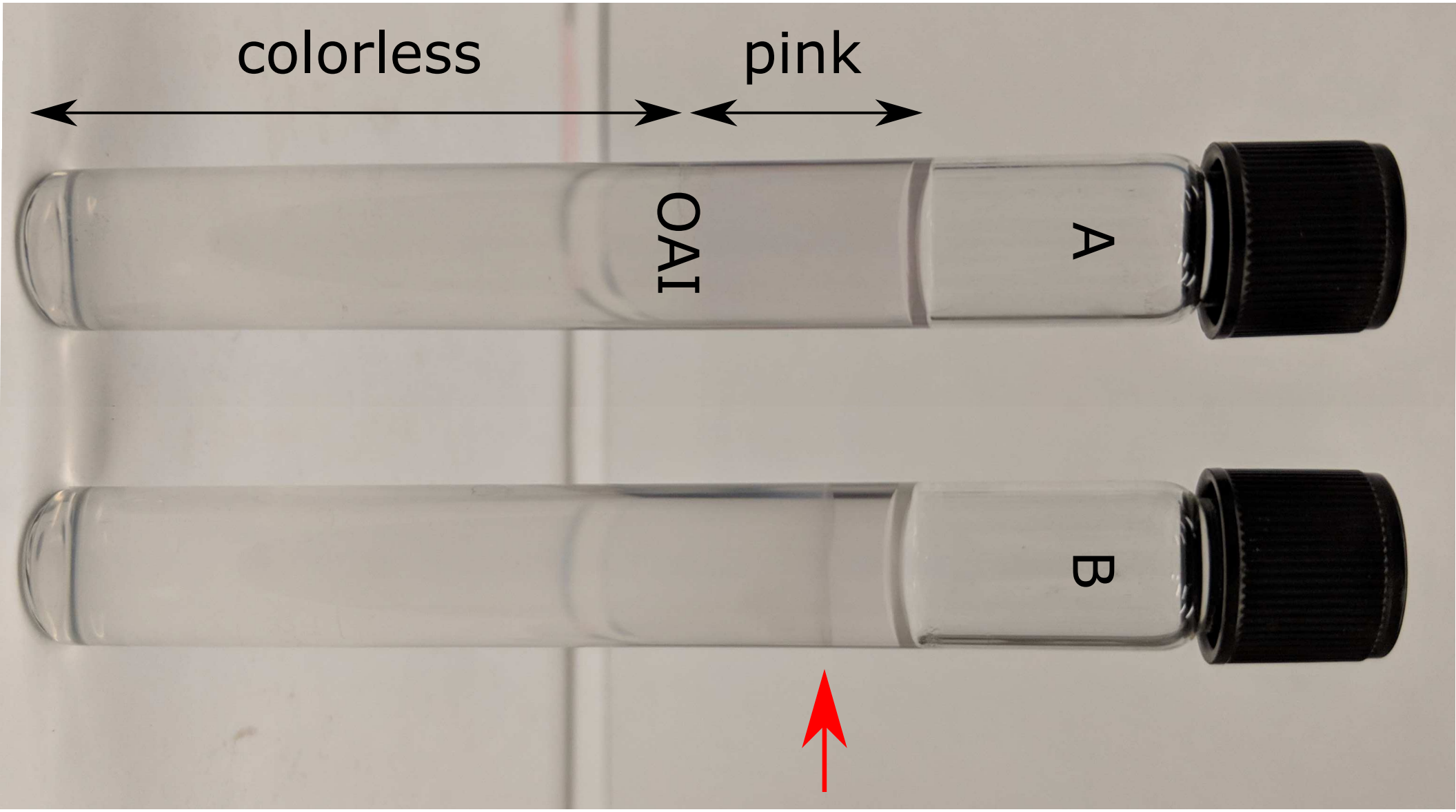

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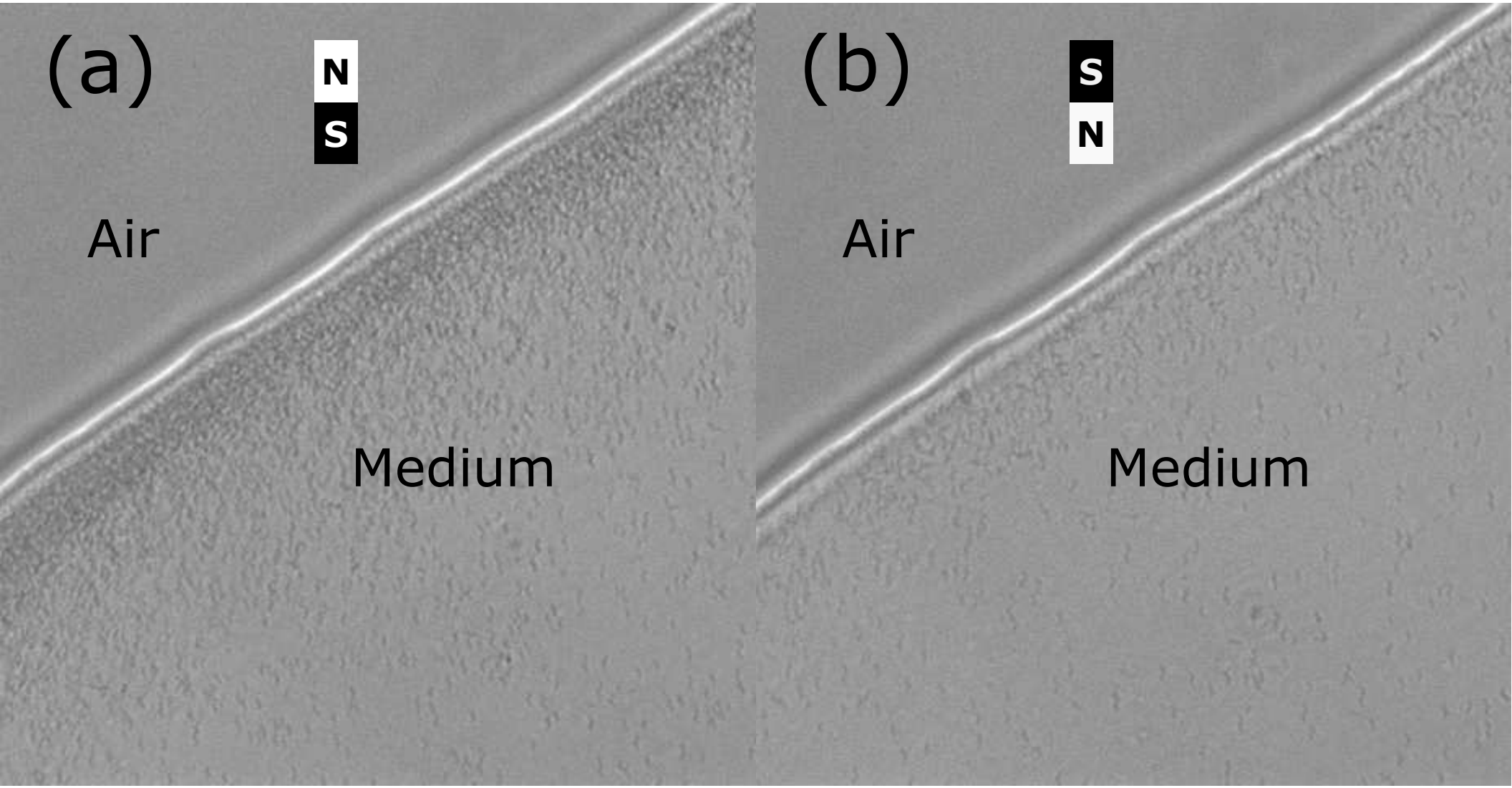

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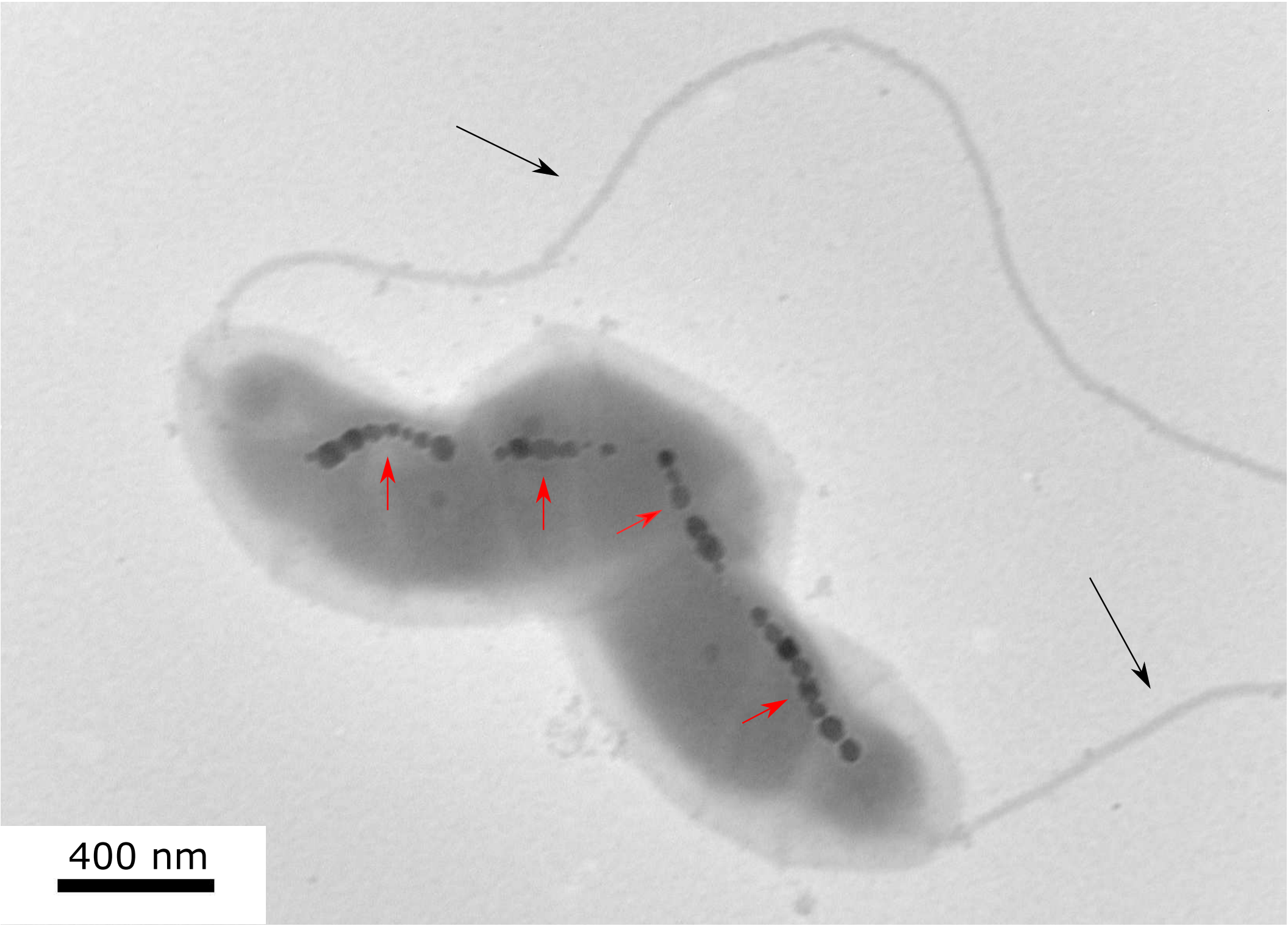

Name of Material/ Equipment	Company
AMB-1	American Type Culture Collection (ATCC)
MS-1	ATCC
MSR-1	Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ)
Ferric citrate	Sigma-Aldrich
Trace mineral supplement	ATCC
KH_2PO_4	EMD
$MgSO_4.7\ H_2O$	EMD
HEPES	BioShop Canada Inc
$NaNO_3$	Sigma-Aldrich
Yeast extract	Fischer scientific
Peptone	Fischer scientific
Potassium L-lactate solution (60%)	Sigma-Aldrich
D-(-)-Quinic acid	Sigma-Aldrich
$FeCl_3.6H_2O$	Fischer scientific
Vitamin supplement	ATCC
Sodium succinate hexahydrate	Fischer scientific
Sodium L-tartrate dibasic dihydrate	Sigma-Aldrich
Sodium acetate trihydrate	EMD
Resazurin	Difco
Ascorbic acid	Sigma-Aldrich
K_2HPO_4	Caledon
$FeCl_2\ .4H_2O$	Sigma-Aldrich
Sodium bicarbonate	EMD
NaCl	Caledon
NH_4Cl	EMD
$CaCl_2.2\ H_2O$	EMD
Agar A	Bio Basic Canada Inc
$L\text{-cysteine.HCl.}H_2O$	Sigma-Aldrich
1.0 mL syringes	Fischer scientific

25G x 1 needles	BD
125 mL serum bottles	Wheaton
20 mm aluminum seals	Wheaton
20mm E-Z Crimper	Wheaton
Butyl-rubber stoppers	Bellco Glass, Inc.
Hungate tubes	Chemglass (VWR)
Septum stopper, 13mm, Hungate	Bellco Glass, Inc.
Glass culture Tubes	Corning (VWR)
Hydrochloric acid 36.5-38%, BioReagent	Sigma-Aldrich

Catalog Number	Comments/Description
ATCC 700264	
ATCC 31632	
DSM 6361	
F3388-250G	
MD-TMS	
PX1565-1	
MX0070-1	
HEP001.250	
S5506-250G	
DF210929	
DF0436-17-5	
60389-250ML-F	
138622	
I88-100	
MD-VS	
S413-500	
228729-100G	
SX0255-1	
0704-13	
A4544-25G	
6620-1-65	
44939-250G	
SX0320-1	
7560-1	
1011450500	
1023820500	
FB0010	
C7880-100G	
B309659	

305125	
223748	
224223-01	
W225303	
2048-11800	
CLS-4208-01	
2047-11600	
9826-16X	
H1758-100ML	11.6 - 12 N

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Author(s): Lucas Le Nagard, Viviana Morillo-López, Cécile Fradin, and Dennis A. Bazylinski

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Dear Editor,

please find below our detailed answer to the editorial comments you sent. All changes done to the manuscript have been marked.

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

We have been through the manuscript several times, and we have again made minor changes to improve grammar as indicated in the manuscript (spelling and grammar errors have also been corrected in the Supplementary Table, although those changes have not been marked). We are not aware of any further spelling or grammar issue. If there are specific things you think should be changed, please let us know.

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To the best of our knowledge, all commercial language has now be removed from the manuscript, and the commercial products are instead only referenced in the Table of Materials and Reagents. In particular, we have removed references to ATCC and BD.

3. Please remove trademark (™) and registered (®) symbols from the Table of Equipment and Materials.

Done.

4. Please do not highlight notes for filming.

The note between steps 2.1.1 and 2.1.2 is no longer highlighted (we believe it was the only one that was).

5. Please highlight complete sentences for filming.

Sections 2.1.2 and 2.3.8 have now been highlighted in their entirety, to avoid incomplete sentences. We suggest, however, that, for each of these two steps (which consist in adding different chemicals to a solution), only a fraction of the step is filmed.

6 & 7. Line 147-156 and line 272-280: Please do not use a list. Please write this step in complete sentences.

Done. Steps 2.1.2 and 2.3.8 have been turned into complete sentences.

Note that in addition to the lists found in steps 2.1.2 and 2.3.8, we have also modified the lists previously found in steps 2.2.2 and 2.3.5, to turn them into complete sentences.

Finally, we note that the last highlighted sentence in the manuscript, which refers to the hanging drop method, is further clarified in the section “representative result”. We thought it might help the script writer to know that this step can be further broken down into: Place a droplet of medium on a coverslip. Flip the coverslip and let it sit,

with the droplet facing down, on an O-ring spacer installed on a glass slide. Ensure that the drop is not in contact with the bottom glass slide. Place the hanging drop under a microscope, and focus on one edge of the droplet. Apply a magnetic field by placing the south pole of a magnet close to the observed edge, and watch the bacteria swim towards the edge.

Sincerely, Cécile Fradin.

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