

Re-Examination of Louis Pasteur's S-Shaped Flask Experiment

Ming-Hua Fu

Abstract— neither negative control nor control to prevent microbes from escaping was set when the S-shaped flask experiments were performed by Pasteur. Microscope was not used to observe the media in the flasks. Louis Pasteur's S-shaped flask experiment was re-examined by using U-shaped flasks, modified S-shaped flasks and microscope. A mixture of microbes was isolated from the room air, from which one rod-shaped *Bacillus* species with proposed name *Bacillus gaso-mobilis* sp nov and one grape-shaped *Staphylococcus* species with proposed name of *Staphylococcus gaso-mobilis* sp nov were identified. Their penicillin and ampicillin resistant strains containing plasmids were isolated. These bacteria could change color, produce odor and automatically move in the air. They did not form colonies on solid media. They had a high suspension capacity in liquid media. Their light absorbance peaked at the wave length of 320nm. It was concluded that there were flaws with Louis Pasteur's S-shaped flask experiments.

Keywords—Bacteria, gaso-mobile, re-examine, S-shaped flasks.

I. INTRODUCTION

IN 1860s, the S-shaped flask experiments were performed by Louis Pasteur [1]. The experiment has been profoundly influencing human life. It has been cited as evidence supporting the theory of biogenesis and against the hypothesis of spontaneous generation [1]. It is also cited by Christian religion society as evidence supporting the creation by God [2], [3]. It has been cited by microbiology textbook as model of scientific experimentation [4]. It has been exhibited in windows by many scientific institutes and galleries as examples for people to follow. It has been the basis of the belief that there is no microbes which can automatically move in the air [4] and influencing the sterilization protocols used in surgery, food preservation, and human daily health. However, there are many flaws with Louis Pasteur's S-shaped flask experiment. First, his experiment missed a flask sterilized and kept sealed as a negative control [1]. Second, no control was set to prevent any microbes from escaping if they would be able to automatically move in the air, that is, a flask with S-shaped pipe opened for a while and then sealed [1]. Third, no microscope was used to examine the media [1]. Neither human naked eyes nor nose has enough resolution or sensitivity to tell if there were or not microbes in the media [5]. Fourth, logically Louis Pasteur's S-shaped flask experiment was based on the assumption that if the medium in the flask kept clear and no odor produced there would be no microbes in the

medium [1]. Indeed, there are many conditions under which there would still be microbes in a clear and odorless medium. There are many microbes in a cup of clear and odorless tap water [6]. If there were microbes which could automatically move in the air they would not only be able to get into the medium through the pipe but also would they get out the flask through the S-shaped pipe. They would not accumulate in the medium, which would leave the medium clear without odor. If there were microbes which were transparent in the medium just like *Amoeba proteus* [7], they would not cause enough contrast for our naked eyes to tell out. If the number of microbes in the medium was so small that they might not cause enough contrast for our naked eyes to tell. Therefore, the concepts generated based on Louis Pasteur's S-shaped flask experiment that there are no microbes automatically moving in the air [1], and that there are no microbes in a clear odorless medium [1], are hypothesis. Louis Pasteur's S-shaped flask experiment needs to be re-examined. In this study, two new species of microbes which could automatically move in the air were isolated using U-shaped flask, S-shaped flask and rDNA identification. These results strongly demonstrated that there were flaws with Louis Pasteur's S-shaped flask experiments.

II. MATERIAL AND METHODS

A. Isolation of Microbes with Sealed U-Shaped Flasks

The sealed U-shaped flasks were designed as in Fig. 1 (a). LB medium was made according to Sambrook et al. [8]. Two 500ml flasks with 200ml of LB medium were connected with a piece of plastic pipe. The empty tube was bent to form a U-shape between the two flasks as shown in Fig. 1 (a). For each experiment, two sets of these flasks were autoclaved at 15 lb/in² for 15 minutes according to Frederick [9]. After the medium in the flasks cooled down to room temperature, flask 3 of experimental set II was opened to the air for 5 minutes and then sealed while flask 1 of control set I was kept sealed. The two sets of flasks were incubated at 37°C overnight without shake. With the forceps of set I untouched and the forceps of set II opened for 5 minutes and then sealed, the two sets of flasks were incubated at 37°C overnight without shake or with vigorous shake for three days. The color of the medium in the flasks was compared with naked eyes and smell was compared with nose. The media in flask 2 of set I and flask 4 of set II were observed under light microscope without treatment or treated with penicillin (100µg/ml), 70% ethanol, eosin (0.5%), neutral red (0.1%) or methylene blue (1%).

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B. Isolation of Microbes with Open U-Shaped Flasks

The open U-shaped flasks were designed as Fig. 1 (c). Each of the two 500ml flasks containing 200ml of LB medium was connected with a piece of plastic pipe. The ends of the plastic pipes were closed with forceps. After autoclavation [9] and when the medium in the flasks cooled down to room temperature, one flask with the pipe sealed was used as control, the plastic pipe on the other flask was opened for 5 minutes and then closed. The two flasks were incubated at 37°C overnight. The medium was treated 70% ethanol, penicillin (100µg/ml), methylene blue (1%) or untreated, and observed under microscope.

C. Isolation of Antibiotics Resistant Strains

Penicillin and ampicillin resistant strains were isolated using the instrument designed as Fig. 1 (b). Two or more 250ml flasks were connected to a central flask with pieces of plastic pipe. All flasks contained 100ml LB medium. Every piece of pipe connecting the flasks was controlled close or open with a forceps. The whole instrument with media was autoclaved [9]. After the media cooled down to room temperature, the central flask was opened for 5 minutes and then sealed. The whole instrument was incubated at 37°C overnight with the pipes close. Penicillin or ampicillin was injected to the corresponding selective flasks except the central flask to a final concentration of 100µg/ml. The pipes connecting the flasks were opened for 5 minutes and then closed. The whole instrument was incubated at 37°C overnight. The media in the selective flasks were taken, laid on to slides and observed under microscope without treatment or after being mixed with 100% ethanol to a final concentration of 70%. The media were also mixed with methylene blue or eosin and then observed under microscope. Microbes in these flasks were treated with 100 µg/µl penicillin or ampicillin for being tested to be penicillin or ampicillin resistant and used for plasmid preparation.

D. Isolation of Plasmids and Genomic DNA

The microbe plasmid DNA and genomic DNA were isolated with QIAGEN Plasmid Midi Kit (Cat#: 12243, Canada QIAGEN Inc., Mississauga, Ontario) according to the manufacturer's instruction with modifications. Microbes were isolated and incubated at 37°C overnight in 250ml LB medium containing 100 µg/ml of ampicillin in S-shaped flask or instrument designed for isolation of antibiotics resistant strains. Microbes were killed by adding 100% ethanol to a final concentration of 70%. After centrifugation at 6500g at 4°C for 15min, the microbe pellet was suspended in 4 ml of Buffer P1 and stood at room temperature for 1 hour. 4 ml of Buffer P2 was added, mixed and incubated at room temperature for 5min. 4ml of chilled Buffer P3 was added, mixed and incubated on ice for 30min. The mixture was centrifuged at 12000g for 30 min at 4°C. The supernatant was passed a QIAGEN –tip 100 equilibrated with Buffer QBT. The column was washed twice with 10 ml of Buffer QC. The microbe plasmid DNA was eluted with 5 ml of Buffer QF. 3.5

ml of isopropanol was added to the elute and centrifuged at 15000g at 4°C for 30 min. The pellet was washed twice with 0.5 ml of 70% ethanol at room temperature. After being air-dried, the pellet plasmid DNA was dissolved in 50µl of ddH₂O and stored at -20°C for further analysis. To isolate genomic DNA, microbes were isolated and incubated at 37°C overnight in 250 ml LB medium in u-shaped or S-shaped flasks. Microbes were killed by adding 100% ethanol to a final concentration of 70%. After centrifugation at 6500g at 4°C for 15 min, the microbe pellet was suspended in 4 ml of Buffer P1 and stood at room temperature for 1 hour. 4 ml of Buffer P2 was added, mixed and incubated at room temperature for 5 min. 4 ml of chilled Buffer P3 was added, mixed and incubated on ice for 30 min. The mixture was centrifuged at 12000g for 30 min at 4°C. The supernatant was extracted twice with equal volume of Phenol/chloroform. After 0.1 volume of 3 M sodium acetate, pH5.2 was added to the supernatant; two volumes of absolute ethanol were added and incubated at -20°C for two hours. The mixture was centrifuged at 12000g at 4°C for 15 min. The pellet was washed twice with 0.5 ml 70% ethanol, air-dried and dissolved in 50µl of ddH₂O and stored at -20°C for further analysis. Restriction enzyme digestion of the plasmid DNA with EcoRI, EcoRV and ClaI was performed according to the manufacturer's instruction (New England BioLabs Ltd., Ontario, Canada). Both the plasmid DNA and the genomic DNA were displayed through agarose gel electrophoresis [8].

E. Isolation of Microbes with Modified S-Shaped Flasks

Modified S-shaped flask experiments using a set of four s-shaped flasks were designed as Fig. 2 (a). All of the four 250 ml flasks contained 100 ml LB medium. After they were sealed with aluminum film, they were autoclaved according to Frederick [9]. When the medium was cooled down to room temperature, flask 2 was kept sealed as negative control; flask 3 without S-shaped pipe was kept open as a positive control; flask 1 with S-shaped glass pipe was kept open and used as a testing flask; flask 4 with S-shaped pipe was opened for 5 minutes and then sealed to prevent microbes from escaping if they would be able to. The four flasks were incubated at 37°C overnight. Colour of the media was observed and compared with naked eyes. The smell was detected with nose. The viscosity of the media was compared with naked eyes as the flasks were shaken. The media were taken out and observed under microscope directly or after being treated with 70% ethanol, eosin or methylene blue.

F. Treatment with Penicillin, Ethanol, Methylene Blue, Neutral Red and Eosin

For treatment with penicillin, penicillin was injected to the media in the flasks to a final concentration of 100µg/ml. After the flasks stood for one hour and the precipitate settled down to the bottom of the flasks, the medium containing the precipitate was taken out, laid on to slides and observed under microscope. Staining with methylene blue was performed according to Harwood and Cutting [10] with some

modification. Saturated aqueous methylene blue solution was mixed with 0.1% potassium hydroxide at a ratio of 1 to 3 (v/v). The untreated medium was spread on a slide. A drop of the mixture of methylene blue solution was mixed with the medium on the slide. The specimens were observed immediately under light microscope. Eosin staining was performed by adding eosin solution to the media to a final concentration of 0.5% and then observed under microscope. 100% ethanol was added to the medium to a final

concentration of 70%, especially when in some circumstance the microbes were dominantly penicillin resistant and could not be precipitated with penicillin. After the precipitates settled down to the bottom, some medium was taken out, centrifuged at 12000g for 30S and observed under light microscope ($\times 400$). Neutral red solution was added directly to the media to a final concentration of 0.1%, after the media stood for 30 minutes, they were taken out, laid on slides and observed under light microscope.

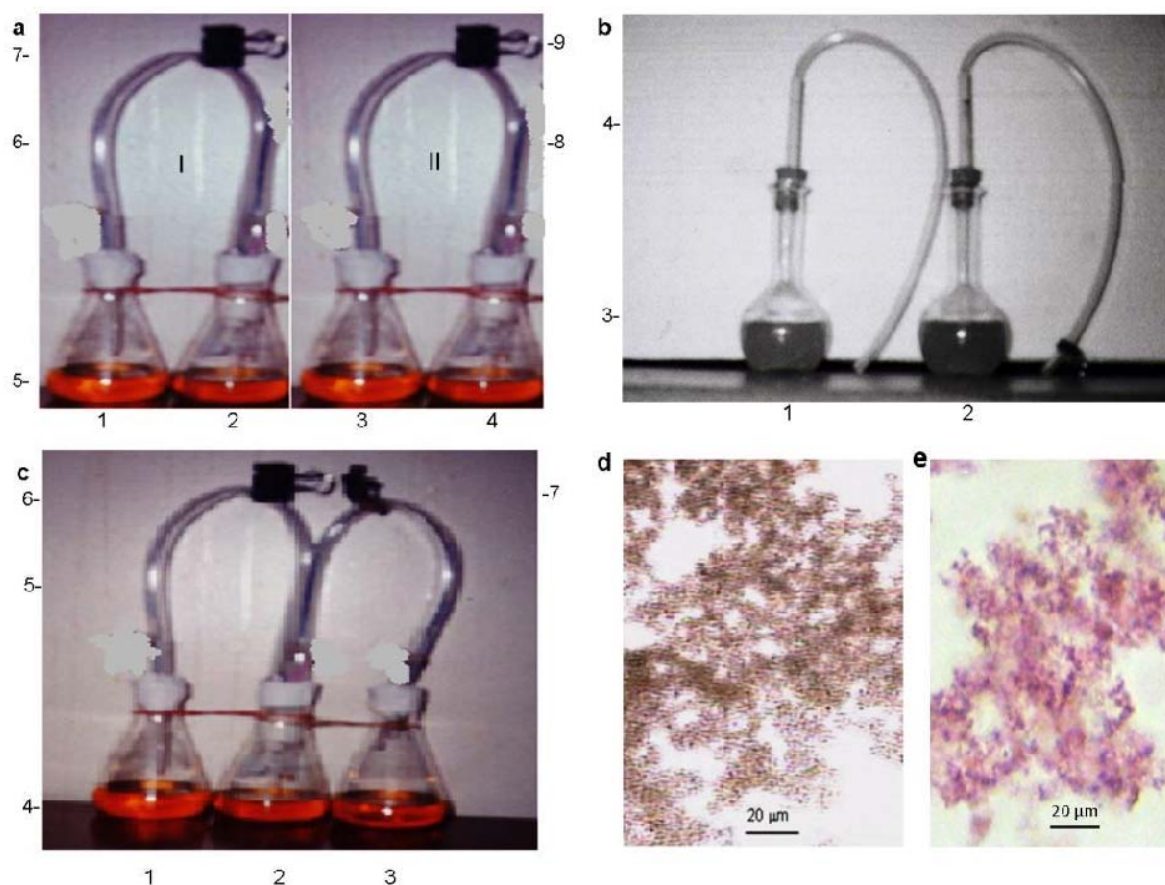


Fig. 1 Isolate of microbes with U-shaped flasks. (a), sealed U-shaped flasks. Two sets of sealed U-shaped flasks were used for the experiment. Each set was made of two 500 ml flasks containing 200 ml of LB medium connected by a piece of plastic pipe and controlled open or close with a forceps. I, control set; II, experimental set; 1, 2, flasks for control set I; 3, 4, flasks for experimental set II; 5, LB medium; 6, 8, U-shaped plastic pipe; 7, 9, forceps. (b), open U-shaped flask experiment. Two flasks containing 200 ml of LB medium were connected to a piece of plastic pipe. The ends of the plastic pipes were controlled with forceps. 1, experimental flask; 2, control flask; 3, LB media; 4, U-shaped plastic pipes; 5, forceps controlling the control flask pipe. (c), instruments used to isolate antibiotics resistant strains. Three 250 ml flasks contained 100 ml of LB medium were connected with pieces of U-shaped plastic pipe. The plastic pipes were controlled open or close with forceps. 1, flask containing penicillin; 2, central flask containing LB medium; 3, flask containing ampicillin; 4, LB media; 5, U-shaped plastic pipe; 6, 7, forceps. (d), microbes in U-shaped flasks ($\times 200$). Media were treated with penicillin, ethanol or eosin and observed under light microscope. (e), microbes in the U-shaped flasks ($\times 400$), which were stained with 0.1% neutral red and observed under light microscope.

G. Light Absorption Test

The light absorbance of media in the open S-shaped flask and the negative control flask of the modified S-shaped flask experiment was measured with spectrophotometer at the light wave length of 280nm, 320nm, 360nm, 400nm, 440nm, 480 nm, 520nm, 560nm and 600nm. Autoclaved LB medium was

used as control to set zero reference for each tested light wave length.

H. Suspension Ability Test

Three flasks contained 100ml of LB medium. After autoclaving, one was sealed as control, one with S-shaped pipe was kept opened, and the third one was opened and seeded with *E. coli*. After being incubated at 37°C overnight,

the medium in the S-shaped flask was divided into two portions. One portion was directly used for centrifugation. The other portion was added with ethanol to a final concentration of 70% and stood for 30 minutes. All the media were centrifuged at 6000g and 12000g in SOVA centrifuge at 4°C for 15 minutes to test the suspension ability of the microbes.

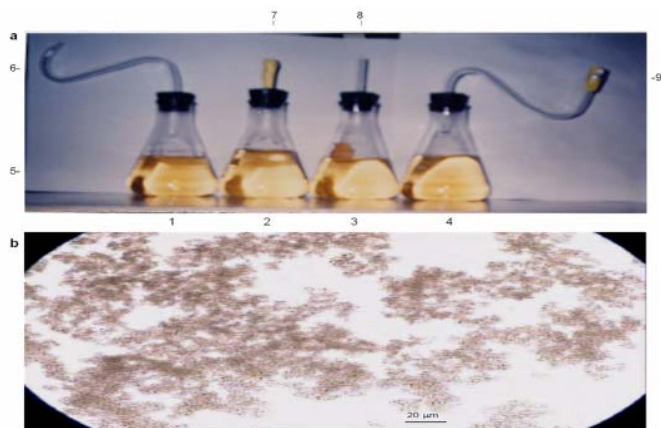


Fig. 2 Modified S-shaped flask experiment. (a), design of the modified S-shaped flasks; a set of four 250 ml flasks were used for the experiment. After they were autoclaved according to Frederick [9] and the medium was cooled down to room temperature, flask 1 with S-shaped glass pipe was kept open and used as testing flask, flask 2 was kept sealed as negative control, flask 3 was kept open as a positive control, and flask 4 with S-shaped pipe was opened for 5 minutes and then sealed to prevent microbes from escaping. (b), microbes in the S-shaped flasks ($\times 400$). The media were taken out and observed under microscope directly or after being treated with 70% ethanol, eosin or methylene blue. Microbes in the S-shaped flasks were filamentous, with a diameter of less than two microns, and similar to the microbes isolated with U-shaped flasks.

I. Protein Content Measurement

Protein content of the microbes was measured with Bio-Rad protein assay (Bio-rad laboratories, Inc., Hercules, USA) according to the manufacture's instruction. Microbes were harvested through precipitation with 70% ethanol. After the weight of the pellet was measured, the pellet was resuspended in 1 volume of TE buffer (pH 8.0) [8], and incubated at room temperature for 1 hour. Then 3 volume of RIPA reagent (SANTA CRUZ Biotechnology, Inc., Santa Cruz, USA, www.scbt.com) was added and homogenized in a glass homogenizer. The homogenate was span at 10000g at 4°C for 10 minutes. The supernatant was collected and used for protein content test with Bio-Rad protein assay (Bio-rad laboratories, Inc., Hercules, USA). Tests were performed in triplicates. Data was expressed as mean \pm SE.

J. Colony Formation Test

Plates containing 10 ml of 2% agarose in LB medium were made according to Sambrook et al. [8]. The microbes in the s-shaped flasks or *E. coli* were laid over the media [8]. After incubation at 37°C overnight, the colonies on the medium were observed.

K. rDNA Identification

rDNA identification was performed by Microbial Identification Services of Molzym (<http://www.molzym.com/service.html>). Genomic DNA was isolated from the microbes in the S-shaped flasks and used as template for PCR amplification with the universal rDNA primers (Complete primers, Gram Positive primers and Gram Negative primers) generated by the company. Melting curve analysis of the PCR products was performed. The differentiated PCR products with melting curve peaks were identified and further sequenced. The identified rDNA sequences were used to identify microbes through homology analysis with the rDNA sequences from the known microbes with Sepsitest™ BLAST (www.sepsitest-blast.net).

III. RESULTS

A. Microbes Isolated with U-Shaped Flasks

To test if there were microbes which could pass a piece of U-shaped pipe, an empty U-shaped flask experiment was designed as Fig. 1 (a). It was found that the media in all the four flasks were clear without obvious colour or smell differences among the flasks observed with naked eyes and nose after one day incubation at 37°C without shaking. When the media were taken out and observed directly under light microscope, no microbes were visible in the media from any flasks. After penicillin solution was injected to flask2 and flask 4 to a final concentration of 100μg/ml, precipitates formed in about 1 hour only in flask 4 of the experiment set but not flask 2 of the control set. Microbes (Fig. 1 (d)) were found in the media from the experiment flask 4 but not control flask 2 under light microscope. They were filamentous, and with a diameter of less than 2 microns. After the media were treated with methylene blue, eosin solution or neutral red microbes were stained and visible under microscope in the media from flask 4 but not control flask 2 (Fig. 1 (e)). To determine if microbes could directly come from the air into the media in the flasks with an empty U-shaped pipe, experiments using the instruments designed as Fig. 1 (b) were performed. There were microbes only in the flask with the pipe opened for 5 minutes and then sealed while no microbes were observed in the control flask with the pipe sealed all the time. When the plastic pipe connecting the experimental flask was opened for 5 minutes and then closed, and the flasks were incubated at 37°C for four days with vigorous shaking, the media in the experimental flask turned into black but kept clear in the control flask (Fig. 4 (b)). Surprisingly, the microbes in the experimental flask could be seen jumping up and down, out and into the medium with naked eyes due to being black and long. To isolate penicillin and ampicillin resistant strains of the microbes, instrument as shown in Fig. 1 (c) was designed. After overnight incubation at 37°C, the media in the flasks were clear. The media were taken out and observed under light microscope, they were clear and no microbes visible. When penicillin or ampicillin solution was injected into the flasks to

a final concentration of 100µg/ml, precipitate formed and microbes were visible only in the media from the control flasks without antibiotics added before incubation but not in the media from the flasks with the antibiotics added before incubation. When 100% ethanol was added to the media to a final concentration of 70%, white precipitate formed in all flasks. Microbes were observed in the media from all of the flasks even the ones with the antibiotics added before incubation. From the microbes in the flasks with penicillin or ampicillin added before incubation, plasmid DNA was isolated (Fig. 3 (b)). The plasmid DNA could be digested by EcoRV, EcoRI and ClaI to produce two fragments of ~6 kb and ~4.5 kb (Fig. 3 (b)), indicating that the plasmid is about 10.5 kb. All these results indicated that there were penicillin and ampicillin resistant strains of the microbes, which contained plasmids.

B. Microbes Isolated with Modified S-Shaped Flasks

To test if there were microbes in S-shaped flasks, experiments using the modified S-shaped flasks as shown in Fig. 2 (a) were performed. One flask sealed all the time was set as a negative control; one flask without S-shaped pipe was kept open as a positive control; one flask with a S-shaped pipe opened for 5 minutes and sealed was used to prevent microbes from escaping; one flask with a S-shaped pipe open all the time as an experimental flask. After being incubated at 37°C overnight without shake, the media in all flasks including the positive control, the negative control, the open S-shaped flask and the flask opened for 5 minutes and then sealed, were clear. No difference in the color of the media among the flasks was observed with naked eyes (Fig. 2 (a)). No smell was detectable with nose. When the flasks were shaken, the media in the positive control, the open S-shaped flask and the flask opened and then sealed were found to be more viscous than that in the negative control. When the media were examined under microscope, no microbes were found in the flasks except the positive control. After 100% ethanol was added to the flasks to a final concentration of 70% of ethanol, white precipitate formed in all the flasks except the negative control flask. After the media were span and examined under microscope, many microbes were found in the media from all of the flasks except the negative control (Fig. 2 (b)). These results indicated that living microbes in the S-shaped flasks without shaking were transparent. Microbes in both the opened and the opened and then sealed modified S-shaped flasks were morphologically similar to the microbes isolated with the U-shaped flasks (Fig. 1 (d) and 1 (e), Fig. 2 (b)). Though microbes in the S-shaped flasks without shaking did not cause enough contrast for our naked eyes to detect, they could absorb visible light but not UV light (Fig. 4 (a)). The spectrum of light absorbed by the microbes was from 230nm to 600nm, with an absorbance peak at 560nm (Fig. 4 (a)). The microbes had a high suspension capacity in LB medium. *E. coli* could be precipitated by centrifugation at 6000g at 4°C for 15 minutes while these

microbes could not be precipitated even centrifuged at 12000g at 4°C for 15 minutes (Fig. 4 (c)). Their composition of protein at about 45 µg/mg wet body weight (Fig. 3 (c)) and genomic DNA (Fig. 3 (a)) further demonstrated that they were organisms. Compared with *E. coli*, the microbes did not form visible colonies on solid LB agarose media (Fig. 4 (d)). All these results indicated that some microbes in the S-shaped flasks were overlooked by Louis Pasteur when the S-shaped flask experiments were performed.

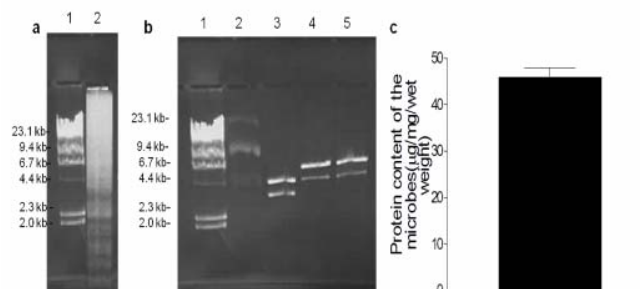


Fig. 3 Biomacromolecules of the microbes; (a), genomic DNA of the microbes; 10 µg genomic DNA was displayed on 1% agarose gel. (b), plasmid DNA from the ampicillin resistant strains; plasmids were isolated from the microbe ampicillin resistant strains using QIAGEN plasmid medi Kit and displayed on 1% agarose gel. The plasmids were designated as pFG. Lane 1, λ /HindIII DNA marker; lane 2, pFG not digested with any enzyme; lane 3, pFG digested with EcoRI; lane 4, pFG digested with EcoRV; lane 5, pFG digested with ClaI. (c), protein content of the microbes in the S-shaped flasks; protein content was measured with Bio-Rad protein assay reagent and expressed as mean \pm SE.

C. Classification of the Microbes

Using the total DNA from the microbes in the S-shaped flask as template, rDNA were amplified with universal primers. Melting curve analysis of the PCR products showed four peaks with different melting temperatures (Fig. 5 (c)). Sequencing analysis generated two different partial 16S rRNA gene sequences (Fugerm1, GenBank accession JN942991 and Fugerm2, GenBank accession JN942992) (Table I), suggesting that there were at least two different species of bacteria in the S-shaped flask. Homology analysis showed the 375 bp sequence JN942991 was 99% homologous with the 16S rRNA gene of *Becillus* bacteria; and the 387 bp sequence JN942992 was 96% homologous with the 16S rRNA gene of *Staphylococcus* sp. and *Staphylococcus cohnii*. High magnitude amplification microscopy indicated there were rod-shaped microbes (Fig. 5 (a)) and grape-shaped microbes (Fig. 5 (b)) in the S-shaped flask. Since these microbes could automatically move in the air and did not form colonies on solid media (Fig. 4 (d)), they were proposed as new species. The rod-shaped ones, Fugerm1 were proposed as *Becillus gasomobilis* sp nov, and the grape-shaped, Fugerm 2 as *Staphylococcus gasomobilis* sp nov.

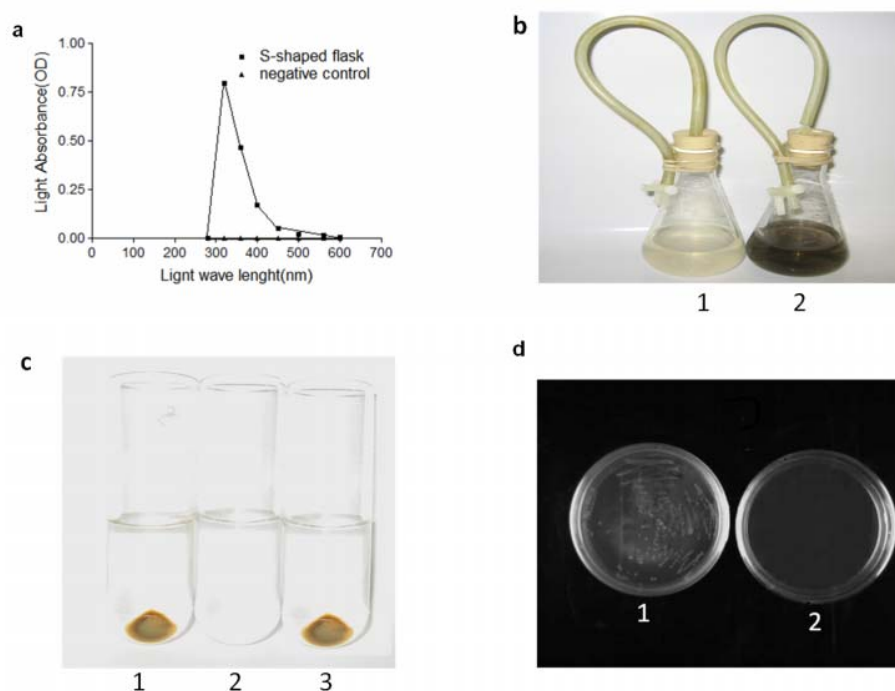


Fig. 4 Characteristics of the microbes in the S-shaped flasks. (a), light absorbance characteristics; the light absorbance of the microbes in the S-shaped flask and negative control flask was measured with spectrophotometer using autoclaved LB medium as control to set zero reference for each test point. Visible light was absorbed by the microbes in the S-shaped flask with a spectrum of 320 nm to 600 nm and peaked at 320 nm. (b), color change of the microbes in sealed u-shaped flasks; 1, control flask without microbes; 2, flask with the microbes. (c), suspension test of the microbes; the suspension capacity of the microbes in the S-shaped flask were compared with *E. coli* through centrifugation at 12000g for 15 minutes at 4°C; 1, living *E. coli*; 2, medium from the S-shaped flask containing the microbes without any treatment; 3, medium from the S-shaped flask treated with 70% ethanol. (d), colony formation test; two plates with the solid agarose media were used, 1, plate seeded with *E. coli*; 2, plate seeded with the microbes in the S-shaped flask. *E. coli* formed many colonies on the surface of solid LB agarose medium in plate 1. No colonies visible on the surface of solid LB agarose medium were formed by the microbes from the S-shaped flasks in plate 2.

IV. DISCUSSION

In this study, two new species of bacteria, *B. gasomobilis* and *S. gasomobilis*, were identified using empty U-shaped flasks, S-shaped flasks and rDNA identification. These microbes could automatically move in the air. They could be transparent or black according to culture conditions. They were filamentous, with a diameter of less than two microns. They could be killed by ethanol while there were penicillin and ampicillin resistant strains, which contained plasmid DNA. These microbes can absorb visible light. They do not form colonies on solid media. They had high suspension ability in liquid media. The existence of the microbes demonstrated that there were flaws with Louis Pasteur's S-shaped flask experiments.

A. Gasomobility of the Microbes

In all the U-shaped flask experiments and modified S-shaped flask experiments, *B. gasomobilis* and *S. gasomobilis* were found only in the experimental flasks not the negative control ones, indicating that the microbes in the experimental and S-shaped flasks suggests that these microbes might automatically pass the U-shaped and S-shaped pipes. This was supported by the observations that they had high suspension ability in LB medium, jumped in the air over the media, swam

in LB media, and did not form colonies on solid media. Louis Pasteur's S-shaped flask experiments [1] and John Tyndall's incubation chamber experiment [11] led people not to try searching for microbes that could automatically move in the air. The gasomobility of the microbes teased them out from other microbes and made them a new group of microbes never found before.

B. Louis Pasteur's S-shaped Flask Experiment Was a Mistake

The existence of the microbes in the U-shaped and S-shaped flasks, which were capable of suspending in liquid against centrifugation, jumping in the air and swimming in the liquid, implicated that these microbes were overlooked in Louis Pasteur's S-shaped flask experiment [4]. The S-shaped flask experiment without proper negative control, a control flask to prevent microbes from escaping, effective visualization methods or using microscope [4] made the experiment less powerful to uncover the microbes. Difference in viscosity of the media caused by these microbes could not be observed due to not setting the negative control. The visible light absorbance by the microbes demonstrated that naked eyes used by Louis Pasteur [4] were not so sensitive to detect the contrast caused by the microbes in S-shaped flasks, and proper instruments,

such as, spectrophotometer, should be used. The existence of microbes in clear media indicated that clarity of media indicated that clarity of media was not sufficient for judging that there were no microbes in any media. Strong odor was smelled in the U-shaped flasks sealed and incubated three days while no odor was smelled by Louis Pasteur [1] in the open S-shaped flask, suggesting that the odorous molecules produced by microbes could diffuse out of the open S-shaped flasks and make the odor not strong enough to be detected by nose. The long time incubation experiments using the S-shaped flasks done by Louis Pasteur [1] were not conclusive since no negative control was set. It should be hard to make the statement that the media in the S-shaped flasks did not change after they stood for weeks or years if longitudinal comparisons of the color, smell and component of the media were not performed [1]. The open S-shaped flasks used by Pasteur [1] not only allowed microbes to escape due to their mobility but also allowed odor molecules to diffuse [12]-[14] and not accumulate in the flask to the extent to be detected by people's nose. The S-shaped flask experiments done by Louis Pasteur at different places, such as, top of mountains, windows, roads and streets should all be re-examined due to the same flaws with the experiment design [1]. The difference in the color of the media between the positive control in the modified S-shaped experiment of this study and the S-shaped flask experiments done by Louis Pasteur [1] might be due to difference in the environments where the experiments were performed and the species of microbes around were different. There might be different species of microbes in the place where Louis Pasteur did his experiments, which got into Pasteur's positive control flasks and turned the media cloudy. There are some flaws with John Tyndall's incubation chamber experiment [11] as Pasteur's S-shaped flask experiments. No negative control was set [11]. No control was set to prevent microbes from escaping [11]. No microscope was used to examine the media [11]. No effective visualization treatments of the media and the space around the media in the chamber were used to detect the microbes, such as, dye staining, killing with antibiotics or ethanol [11]. Transparent and gaso-mobile microbes might be overlooked in John Tyndall's incubation chamber experiment.

C. S-shaped Flask Experiments Should Be Improved to be Evidence for Biogenesis

Though Louis Pasteur's S-shaped flask experiment has been criticized by American historian James Strick [15], it has been cited by Microbiology textbooks as model of scientific experimentation and evidence supporting biogenesis and against spontaneous generation [4]. Louis Pasteur's experiment has been thought to be the foundation of biogenesis. The finding that there were microbes in the S-shaped flasks demonstrated that Pasteur's S-shaped flask experiments were not yet good evidence for the theory of biogenesis. John Tyndall's incubation chamber experiment [11] has many flaws and needs to be re-examined. To support

the theory of biogenesis, both Pasteur's S-shaped flask experiments and John Tyndall's incubation chamber experiment should be improved.

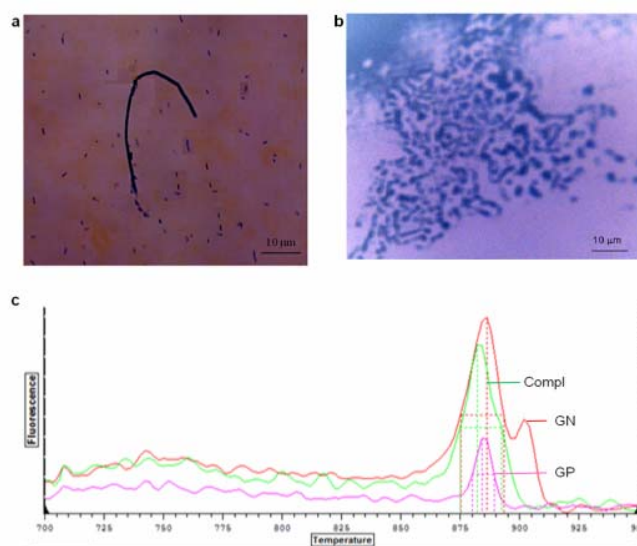


Fig. 5 Identification of the microbes. (a), rod-shaped microbes stained with methylene blue ($\times 800$); (b), an individual microbe with grape-shaped cells stained with methylene blue ($\times 800$). (c), melting curve of the PCR products; rDNAs were amplified with universal rDNA primers and the genomic DNA from the microbes in the S-shaped flask as template; Compl-complete primers; GN-gram negative primers; GP-gram positive primers. Four PCR products with clear melting curve peaks were sequenced and two different sequences were identified

V. CONCLUSION

Identification of two new species of microbes, *B. gasomobilis* and *S. gasomobilis*, which were able to move automatically in the air, has opened a new field to find more microbes mobile in the air since there are so many different microenvironments in the atmosphere. It is of interest to design good methods to further purify the microbes and get single species cultures. It will also be of interest to study the distribution of these microbes in the world, interactions of the microbes with inorganic environment, other microbes, plants, animals and human, and all other life processes of these microbes, especially, their roles in mediating human mental diseases (personal observation).

TABLE I
ALIGNMENT OF THE 16S rDNA SEQUENCES WITH THE SEQUENCES IN GENBANK DATABASE

| sequence identified | GenBank accession | rDNA | match sequence GenBank accession | match microbes | homology | genus | Species |
|---------------------|-------------------|------|-------------------------------------|------------------------------------|----------|-----------------------|-----------------------------|
| Fugerm1 | JN942991 | 16S | HQ285924.1 | <i>Bacillus cereus</i> | 99% | <i>Bacillus</i> | <i>B. gaso- mobilis</i> |
| | | | JF899291.1 | <i>Bacillus sp.</i> | | | |
| | | | JF899291.1 | <i>Bacillus thuringiensis</i> | | | |
| | | | HQ288932.1 | <i>Bacillus weihenstephanensis</i> | | | |
| | | | JF343201.1 | <i>Bacillus subtilis</i> | | | |
| Fugerm2 | JN942992 | 16S | JF343185.1 | <i>Bacillus anthracis</i> | 96% | <i>Staphylococcus</i> | <i>S. gaso- mobilis</i> |
| | | | HM854558.1 | <i>Staphylococcus sp.</i> | | | |
| | | | JN128237.1 | <i>Staphylococcus cohnii</i> | | | |

Note: The identified 16S rDNA sequences were aligned to the known sequences in GenBank database using blast. The Fugerm1 16S rDNA sequence was 99% homologous with those of the *Bacillus* bacteria. The Fugerm2 sequence was 96% homologous with those of the *Staphylococcus* bacteria. The microbes of Fugerm1 were proposed as *Bacillus gaso-mobilis* sp nov. The microbes of Fugerm2 were proposed as *Staphylococcus gaso-mobilis* sp nov.

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