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Capture of bacteria by flexible carbon nanotubes

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1. Introduction

Many carbon materials exhibit excellent molecular adsorption properties. And an activated carbon (AC) [1], which is widely used as an adsorbent, has a high capacity for adsorption owing to their unique porous structures and large surface areas. The occurrence of dental caries is mainly associated with oral pathogens, and *Streptococcus mutans* is one of the primary cariogenic organisms. Therefore, many attempts have been made to eliminate *S. mutans* from the oral cavity. One of these is the use of AC as an effective adsorbent in a wide range of oral care products such as toothpastes and mouthwashes [2,3].

Carbon nanotubes (CNTs) have attracted considerable attention because of their unique physical properties and potential for a variety of applications [4]. In recent investigations for biological applications, CNTs have been utilized as adsorbents to eliminate dyes [5]. In addition, CNTs can adsorb bacteria [6–8]; single-walled carbon nanotubes (SWCNTs) exhibit strong antimicrobial activity toward *Escherichia coli* [9,10]. However, bacterial adhesion, particularly, oral bacterial adhesion, to CNTs has not yet been sufficiently investigated. If bare CNTs are found to have strong adhesive activity and winding CNTs bind oral pathogens, they may be useful as tools at the nano-level for capturing oral pathogens. In this study, we investigated oral bacterial adhesion to CNTs of different diameters and flexibility, and as a control, to the widely used adsorbents AC particles. In general, SWCNTs with diameter of approximately 1 nm are known to be highly flexible, while multi-walled carbon nanotubes (MWCNTs) with diameters > 100 nm are hard. Here we report that CNTs of different diameters exert significantly different effects on the efficiency of *S. mutans* precipitation, and the manner in which they capture bacteria are different and that bundles of SWCNTs and MWCNTs with average diameters of 30 nm can wind around the curved surfaces of bacteria.

2. Experimantal

The SWCNTs employed were synthesized by an arc discharge method; and the MWCNTs used were of two types: 30-MWCNTs (average diameter of 30 nm; produced by NanoLab Inc., Brighton, MA) and 200-MWCNTs (average diameter of 200 nm; produced by MTR Co., Ltd., Ohio, U.S.A.). As a control carbon sample, a commercial activated carbon powder (AC) with an average particle size 20 μm (Kanto Chemical Co. Inc., Tokyo, Japan) was used in this study. *S. mutans* JC2 was grown aerobically in brain heart infusion (BHI) broth at 37°C for several days. The bacteria were harvested by centrifugation at 2500 *g* (Kubota Centrifuge 2700), washed in phosphate-buffered saline (PBS: 20 mM K_2HPO_4/KH_2PO_4, 150 mM NaCl, pH 7.4), and suspended in the same buffer to an optical density (OD) of 1.0 at 700 nm.

For a bacterial precipitation assay, six milliliters of a suspension of CNTs treated by ultrasonication in PBS was added to 3 mL of the bacterial suspension in a glass bottle. As a control, PBS solution was used in substitution for CNTs solutions (initial OD of final volume was 0.34; 1.4×10^8 colony-forming units/mL). The solution was shaken at 200 rpm for 20 min by a universal shaker (Iwaki SHK-U3). After centrifugation (100 *g*, 3 min), the precipitate was at the bottom of the bottle. A 2-mL portion of the upper suspension was transferred to a quartz cell, and OD at 700 nm was measured with a UV-vis spectrometer.

To identify the suspending carbon of the supernatant after centrifugation, aliquots of the supernatant at a carbon sample concentration of 0.66 mg/mL were dried on a slide glass. The dry substances were sputtered using a carbon coater (Meiwa Shoji CC-40F) and then examined by scanning electron microscopy (SEM, Hitachi S4000). For a colony-forming unit (CFU) formation assay of the supernatant after centrifugation, a portion of each supernatant (1 mL) carbon sample at concentration of 0.66 mg/mL was used. Upon serial dilution, the diluted samples (10 μL each in triplicate) were spread

evenly onto solid BHI medium plates for aerobic incubation at 37°C for 3 d, and the colonies were then counted.

For SEM observation of the precipitate after centrifugation, the precipitate was collected on a polycarbonate filter (Advantec, 0.8 μm in pore size) and immersed in a fixative (2% glutaraldehyde in PBS) for 2 h. The samples were dehydrated in graded ethanol and dried with CO_2 in a critical point dryer (Hitachi HCP-1). The cells were sputtered using a carbon coater and then examined by SEM.

3. Results and discussion

The adhesive activities of the CNTs were assessed by a bacterial precipitation assay using *S. mutans*. The results of the precipitation assay are presented in Figs. 1a and 1b. When the bacterial suspensions were mixed with the CNTs, there was loss in supernatant turbidity with an increase in the amount of CNTs. The results clearly show that CNTs have adhesive ability. These results regarding *S. mutans* adhesion to CNTs are in agreement with the previously reported results regarding bacterial adhesion to CNTs in the case of *E. coli* [7–10]. Among the carbon samples, the precipitation efficiency of 30-MWCNTs was the highest; 30-MWCNTs at 0.17 mg/mL concentration had the highest efficiency. SWCNTs were less effective because they were not easily dispersed before mixing with the bacteria; and 200-MWCNTs seemed to be less effective because some of them suspending in the solution and did not precipitate.

Fig. 2 shows that the number of free *S. mutans* and/or *S. mutans* adhered to suspending carbon samples depend on the type of carbon sample employed. A considerable number of free bacteria were observed with PBS and SWCNTs. However, few free bacteria were observed with 30-MWCNTs, 200-MWCNTs, and AC. Moreover, with 200-MWCNTs and AC, a comparatively large amount of suspending carbon was observed. In the AC, the small particles (particle size 1 μm-10 μm) which were included in AC particles were observed. These suspending of carbon would be occurred so that bacteria

or bacterial products act as surfactant. It was found that 200-MWCNTs were strongly bacterial adhesive but had poor precipitation activity in the bacterial suspension.

To remove the influence of the suspending carbon samples in the supernatant on the assessment of bacterial adhesion, the residual amount of bacteria was evaluated by a colony-forming unit (CFU) formation assay. The percentage of CFUs referenced to the control is shown in Fig. 3. The number of free bacteria decreased for all carbon samples, reaching reductions of 65%-96% from the initial numbers of bacteria in the suspension. In particular, 30-MWCNTs and 200-MWCNTs bring about the highest decrement of 92% and 96%, respectively. This result shows that 30-MWCNTs and 200-MWCNTs are highly adhesive to bacteria. However, 200-MWCNTs did not easily precipitate from the suspension, and a large amount of 200-MWCNTs remained suspending in the supernatant (Fig. 2d); and the OD value of its supernatant was comparatively high (Fig. 1).

Kim *et al.* reported that CNTs clusters show high affinity toward and bind *E. coli* cells. No significant difference in the affinity of bacterial adhesion was observed between SWCNTs and MWCNTs [7]. The results of the precipitation assay may be influenced by the aggregation activity of each carbon material, which in turn depends on the differences in their diameters. Bacteria are known attach to AC particles by means of strong van der Waals forces between the bacterial and carbon surfaces [11]. Therefore, all type of carbon materials will have high affinity to bacteria because of the generation of van der Waals forces. However, carbon materials with strong aggregation activity, such as SWCNTs, are not easily dispersed in hydrophilic solution and thus, decrease the area available for bacterial adhesion. In contrast, carbon materials with weak aggregation activity, such as 200-MWCNTs and AC, in the limited size range used in this study are not easily precipitated from hydrophilic solution. Our results show 30-MWCNTs had the highest precipitation efficiency, which was attributable to both their adequate dispersibility and aggregation activity.

Subsequently, bacterial adhesion to the CNTs of different diameters in the precipitates was observed by SEM. Fig. 4 shows SEM image of *S. mutans* adhered to CNTs or AC in the precipitates.

Several *S. mutans* cells adhered to the meshwork comprising bundles of SWCNTs (bundle: rope-like shape, average diameter of about 100 nm). Similar to the fibrous shape of the bundles of SWCNTs, fibrous extracellular polymeric substances (width of about 10 nm) were also observed at right-angles to the bacterial surface (the black arrows in Fig. 4). Furthermore, it is notable that some of the bundles wound around the curved surfaces of the *S. mutans* cells (Figs. 4a and 4b). Thus, the flexibility of the bundles seems to be greater than that of the bacterial cell wall. Sano *et al.* [12] reported that SWCNTs are wormlike polymers in solution and have flexibility. Therefore, SWCNTs can adjust their structure to follow the surface morphology of *S. mutans* (radius of minor axis: 500 nm). Fig. 4a clearly shows that a bundle of SWCNTs captured *S. mutans* passing through a pore of a membrane filter. A flexible net of SWCNTs can be strongly bent without breaking. In a similar manner, 30-MWCNTs (average diameter of 30 nm) captured *S. mutans* and wound around the curved surface of *S. mutans* (Fig. 4c). Poncharal *et al.* [13] reported that the ripple structure in the tube caused the MWCNTs (diameter of ca. 30 nm) to bend uniformly, with a radius of curvature of 400 nm. 30-MWCNTs could wind around the bacteria with the ripple structure. In contrast, though the 200-MWCNTs (average diameter of 200 nm) also adhered to the cells, they did not wind around the surface of *S. mutans* (Fig. 4d). Thus, the flexibility of 200-MWCNTs seems to be less than that of the bacterial cell wall. As for the control material (Fig. 4e), bacterial adhesion occurred at the surface of AC (average particle size 20 μm).

Further, rounded bacteria adhered to SWCNTs under these conditions. This observation contradicts the previous result that flattened cells are inactivated on SWCNTs, which was reported by Kang *et al*. [9,10] and Brady-Estévez *et al*. [14]. The differences in morphology could be depend on the differences between gram-positive bacteria, which have a thick cell wall (such as *S. mutans*) and gram-negative bacteria, which have a thin cell wall (such as *E. coli*) or on the purity of SWCNTs. In addition, winding CNTs around bacteria have not been clearly observed in previously studies [7,9]. This could be explained by the difference between gram-positive and gram-negative bacteria or by the use of different procedures for mixing bacteria with CNTs.

In our study, data from both the precipitation assay and SEM images prove that CNTs can adhere to *S. mutans*. Although all types of carbon materials show high affinity toward bacteria because of the generation of van der Waals forces, it has been shown that CNTs of different diameters have significantly different effects on the precipitation efficiency and the manners in which they capture the cells are different. We found that 30-MWCNTs had the highest precipitation efficiency, which was attributable to both their adequate dispersibility and aggregation activity. As one of the possibility, this may be attributed to differences in flexibility of the nanotubes. The advantages of capturing pathogens by winding CNTs may be to ensure stronger adhesion and to inhibit the release and budding of captured bacteria. We believe that this could be one of the features of CNTs.

Although the optimum conditions for precipitation are still not clear, we have demonstrated that *S. mutans* can be captured by flexible CNTs. For capturing or eliminating bacteria, the use of CNTs that can adhere to bacteria via physical sorption is not linked to antimicrobial resistance. Bare CNTs having high adhesive ability could be useful as biomaterials, for example, as tools for the elimination of oral pathogens at the nano-level.

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Figure Captions

Fig. 1. (a) OD (at 700 nm) of the supernatant in precipitation assay with carbon samples. Results are presented as means ± SE of three experiments. Upper dotted line is OD value (0.34) of PBS before centrifugation (control). (b) Photographs of *S. mutans* mixed with carbon samples: PBS (b-1); SWCNTs (b-2); 30-MWCNTs (b-3); 200-MWCNTs (b-4); and AC (b-4) after centrifugation.

Fig. 2. SEM images of dried supernatant on a slide glass after mixing with carbon samples at a concentration of 0.66 mg/mL: PBS (a); SWCNTs (b); 30-MWCNTs (c); 200-MWCNTs (d); and AC (e).

Fig. 3. Residual amount of *S. mutans* in supernatant after mixing with carbon samples in 0.66 mg/mL concentration. Results are presented as means ± SE of three experiments.

Fig. 4. SEM images of *S. mutans* adhered to CNTs or AC: bundles of SWCNTs wound around *S. mutans* (a); the bacteria adhered to the meshwork comprising bundles of SWCNTs (b); 30-MWCNTs wound around bacteria (c); 200-MWCNTs adhered but did not wind (d); and AC surface adhered to bacteria (e). The white arrows indicate CNTs. The black arrows indicate fibrous substances produced by bacteria.

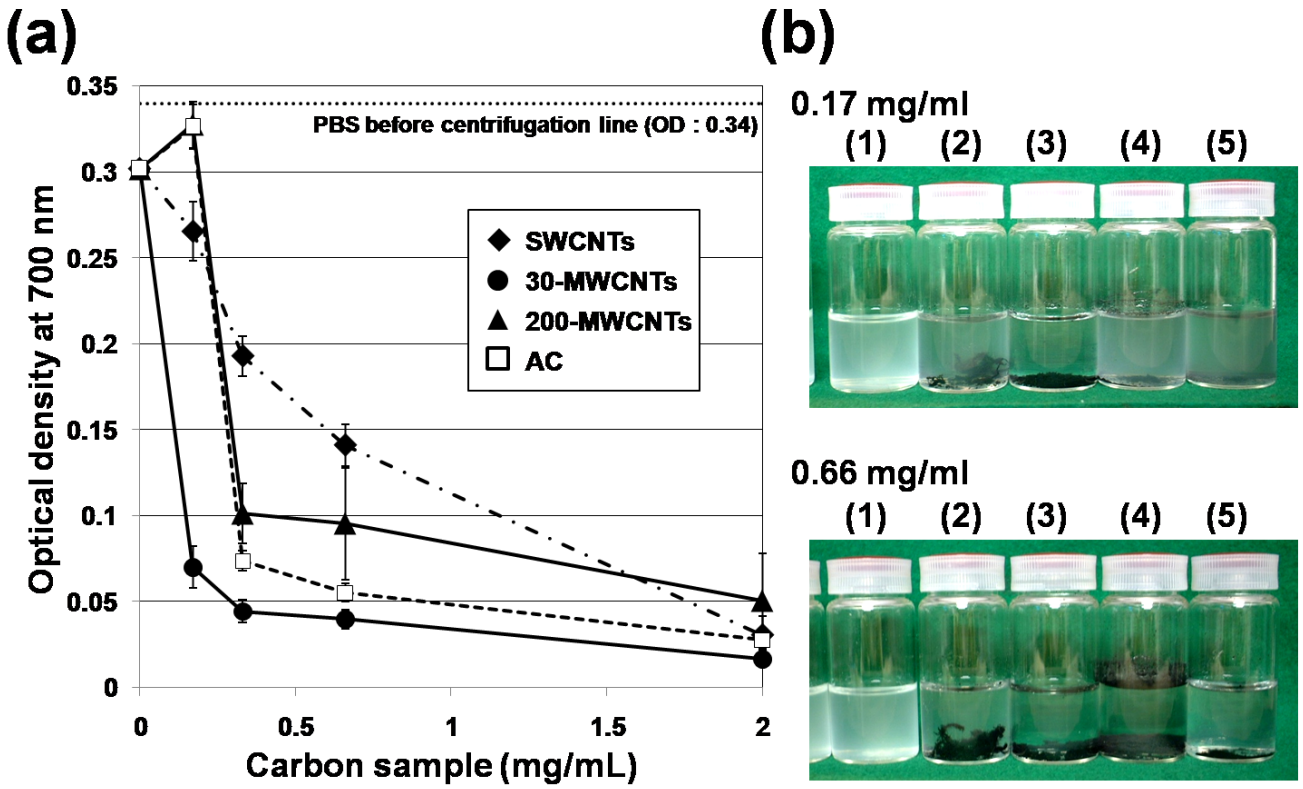
(a)
(b)
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
PBS before centrifugation line (OD : 0.34)
SWCNTs
30-MWCNTs
200-MWCNTs
AC
Optical density at 700 nm
0
0.5
1
1.5
2
Carbon sample (mg/mL)
0.17 mg/ml
(1) (2) (3) (4) (5)
0.66 mg/ml
(1) (2) (3) (4) (5)

Fig. 1.

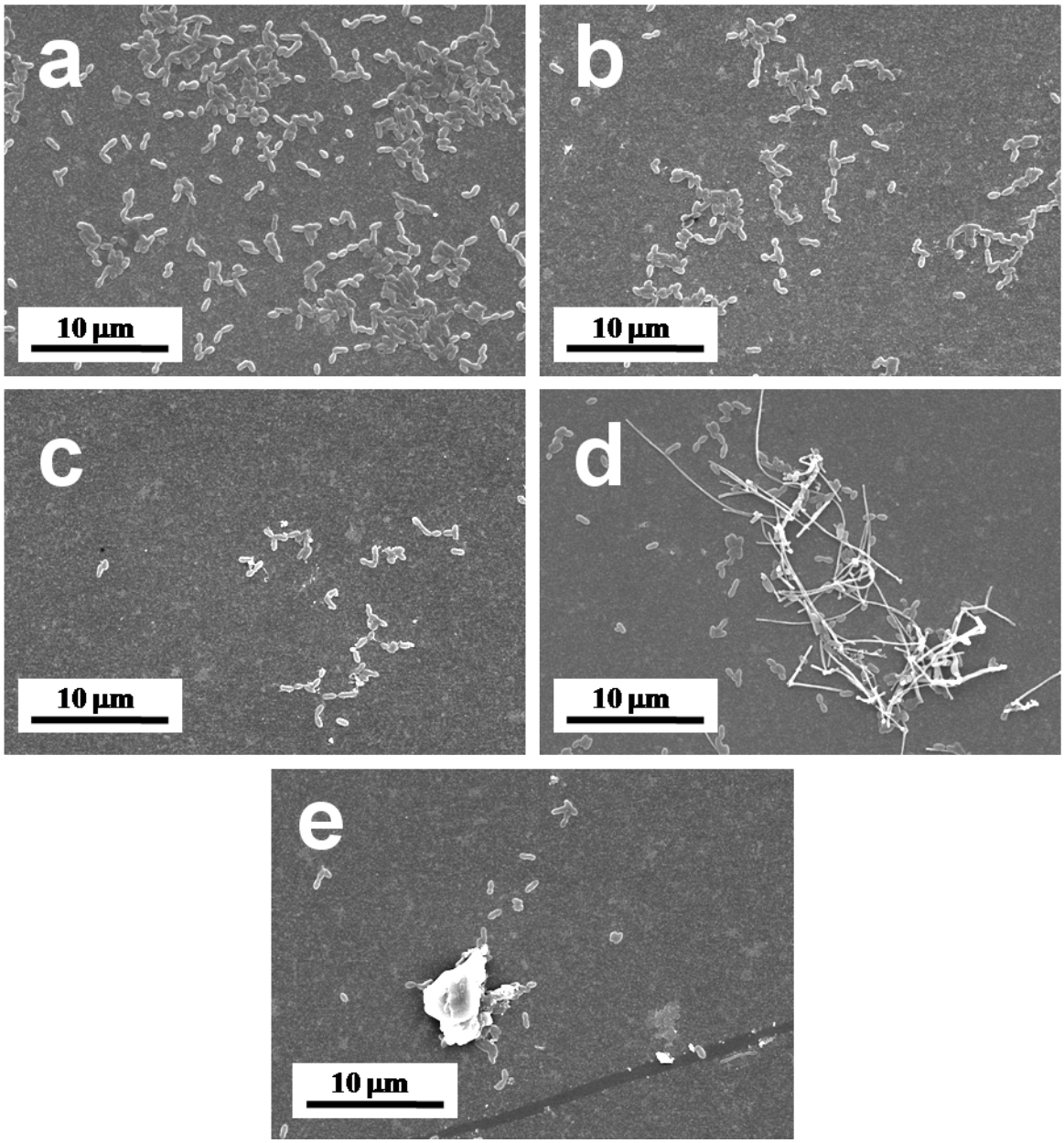

Fig. 2.

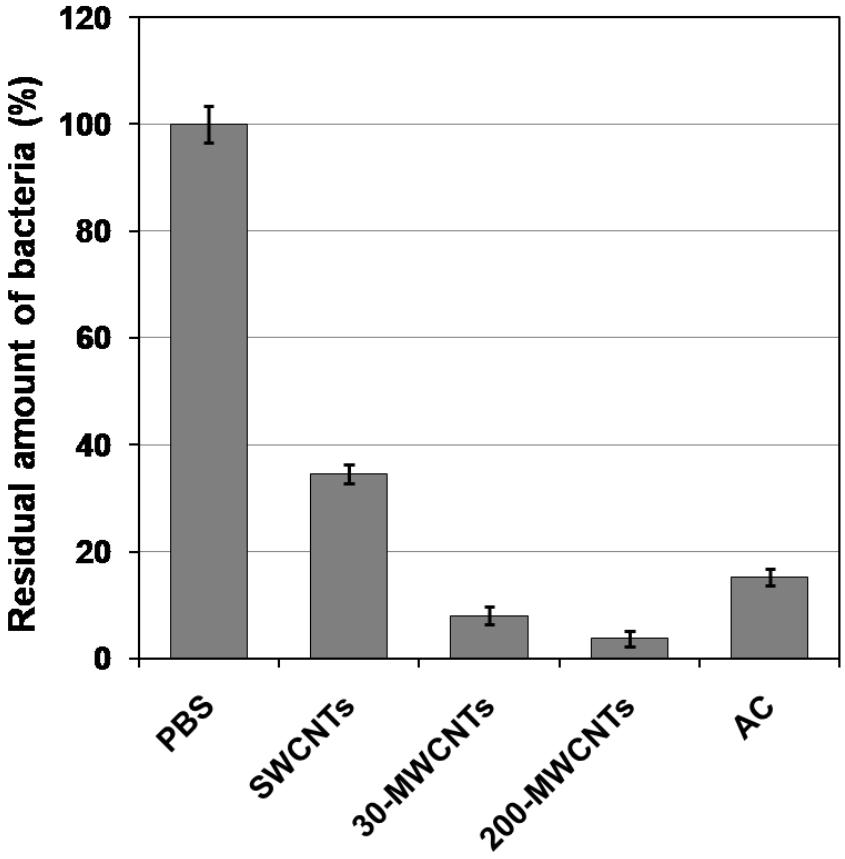
Residual amount of bacteria (%)
120
100
80
60
40
20
0
PBS
SWCNTs
30-MWCNTs
200-MWCNTs
AC

Fig. 3.

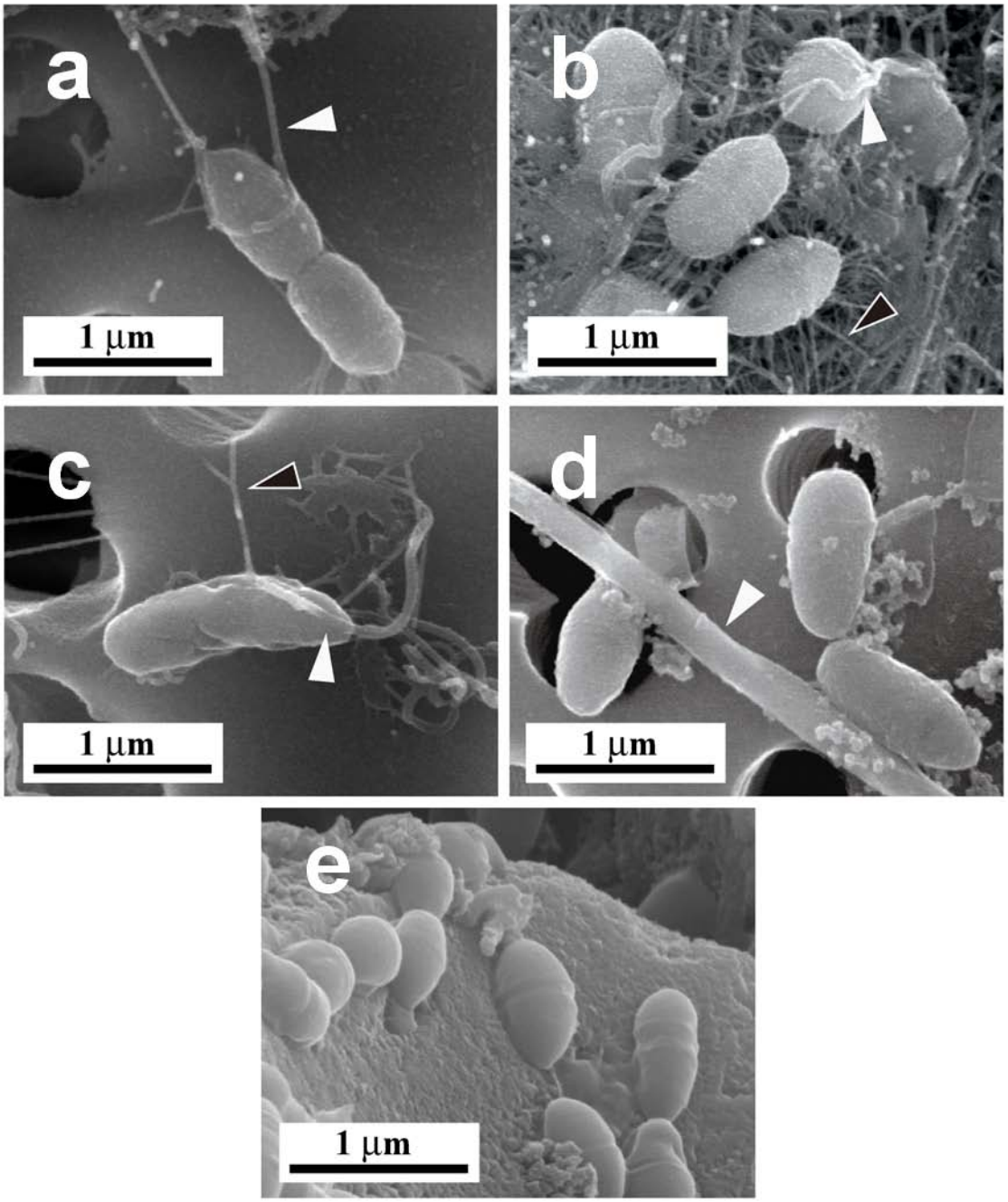

Fig. 4.