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Comparison of Pre-Stain Suspension Liquids in the Contrasting Ability of Neutralized Potassium Phosphotungstate for Negative Staining of Bacteria

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Image contrast of whole bacteria was compared in *Staphylococcus aureus* and *Escherichia coli* depending on pre-stain suspension liquids by energy-filtering transmission electron microscopy. The two bacterial strains were suspended in three most commonly used liquids for negative staining (triple distilled water [DW], phosphate-buffered saline [PBS], and nutrient broth [NB]) and directly observed without staining or stained with neutralized potassium phosphotungstate (PTA), respectively. Even though in low contrast, unstained bacteria were observed owing to their inherent electron density and cell shape in zero-loss (elastic scattering) images. After being suspended in PBS, unstained bacteria appeared to have higher contrast and more refined periphery than DW-suspended ones, and extracellular appendage structures such as fimbriae and flagella could be discerned. The unstained bacteria appeared to be invariably surrounded with electron-lucent precipitates, possibly from PBS. As far as delineation of the structures, the combination of DW or PBS suspension with subsequent staining provided the most satisfactory results, as evidenced by the high contrast of bacterial morphology and appendage structures. However, after being suspended in NB and stained with PTA, bacteria often had too high contrast or poor staining, with electron-dense aggregates around the bacteria. These results suggest that suspension with concentrated organic aliquots including broth media before PTA staining could deteriorate image contrast, and should be used only in dilute form for visualizing bacterial morphology and appendage structures. Moreover the contrast enhancement of unstained bacteria by salt granules would be advantageous in demonstrating bacterial sorption of environmental particles like heavy metals, maintaining minimal contrast for cell imaging.

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There is an increasing demand in disciplines ranging from microbiology to environmental sciences to visualize and document micrometer or submicrometer range organisms, particles, and their related phenomena such as aggregation, adhesion, dissolution, and micropollutant sorption [19]. Negative staining is a simple and rapid method to study the morphology and the structure of particulate specimens such as bacteria, viruses, cell components, and isolated macromolecules [8]. In its simplest form, negative staining involves the deposition of a droplet of dissolved heavy metal salt such as tungsten, uranium, and molybdenum mixed with the specimens onto a thin supporting film [17]. Negative stains do not interact with specimens, but simply form a structureless matrix surrounding all external and accessible internal surfaces of the specimens [18].

Before staining, the concentrated specimens are usually diluted and suspended in liquids for spreading on a supporting plastic film. It is commonly known that any low-molecular weight proteins and organic salts present in the specimen should be minimized or eliminated, because the concentration of almost all components in the specimen occurs on the grid [8]. These extraneous materials often tend to obscure the specimen image. For the dilution of bacteria, diverse pre-stain suspension liquids such as distilled water [11, 23], broth media [3, 20], and salines [12, 14] have been used. Negative staining has a great benefit of simplicity, rapidity, versatile applicability, and high reproducibility [7]. Concomitantly, it has drawbacks like flagella clumping in negatively stained bacteria, and thus much is required to modify and improve for specific purposes [6]. It is worthwhile to ascertain the effects and consequences of pre-stain suspension liquids on contrast formation for

revealing bacterial morphology and extracellular appendage structures.

Uranyl acetate, neutralized potassium phosphotungstate (PTA), and ammonium molybdate are the three most commonly used negative stains. As the first stain specifically used as a negative stain, PTA has the advantage of tolerating high concentrations of nonvolatile salts (*e.g.*, phosphate buffer) without adverse effects [8]. Little information is available on the variations of the contrasting ability of PTA depending on pre-stain suspension liquids. Here, we make comparisons of pre-stain suspension liquids in image formation, and report the precautions and versatility of pre-stain suspension in negative staining of bacteria for routine applications.

MATERIALS AND METHODS

Bacterial Strains

Two bacterial strains were used in this study as follows: (i) *Staphylococcus aureus* ATCC 25923 and (ii) *Escherichia coli* ATCC 25922. Each strain was subcultured at least twice on 5% sheep blood agar (Promed, Seongnam, Korea) to ensure its viability and purity.

Negative Staining Regimes

To demonstrate the effects of pre-stain suspension liquids on the contrasting ability of PTA, three types of autoclaved liquids were prepared in this study as follows: (i) triple distilled water (DW), (ii) phosphate-buffered saline (PBS) (pH 7.4), and (iii) nutrient broth (NB) (Difco, Sparks, MD, U.S.A.). One colony of each strain on sheep blood agar was gently suspended in 1 ml of each liquid by finger flicking. A glow-discharged formvar-coated copper grid was floated on a drop of each liquid for 1 min. The excess liquid was drained off with filter paper, and the preparations were either (i) air-dried for 5 min without staining or (ii) stained with 2% (w/v) aqueous PTA (pH 7.0) for 10 s and air-dried. To determine the presence of precipitates in PBS, a formvar-coated grid was floated on a drop of PBS and air-dried without PTA staining. The specimens were then examined with an in-column energy-filtering transmission electron microscope (EF-TEM) (LIBRA 120; Carl Zeiss, Oberkochen, Germany) operated at an accelerating voltage of 120 kV. Zero-loss (elastic scattering) energy-filtered images were recorded with a 4 K×4 K slow-scan charge-coupled device camera (Ultrascan 4000 SP; Gatan, Pleasanton, CA, U.S.A.).

RESULTS

Morphology and Appendage Structures of *S. aureus*

Transmission electron microscopy of the DW-suspended and unstained *S. aureus* revealed gross morphology of the bacterium with low contrast (Figs. 1A and 1B). Because of their considerable inherent electron density, their coccal morphology (approx. 600 nm in diameter) could be discerned. Some *S. aureus* formed chains, and others were arranged in clusters like grapes. After being suspended in PBS, the unstained *S. aureus* appeared to have higher contrast and more refined

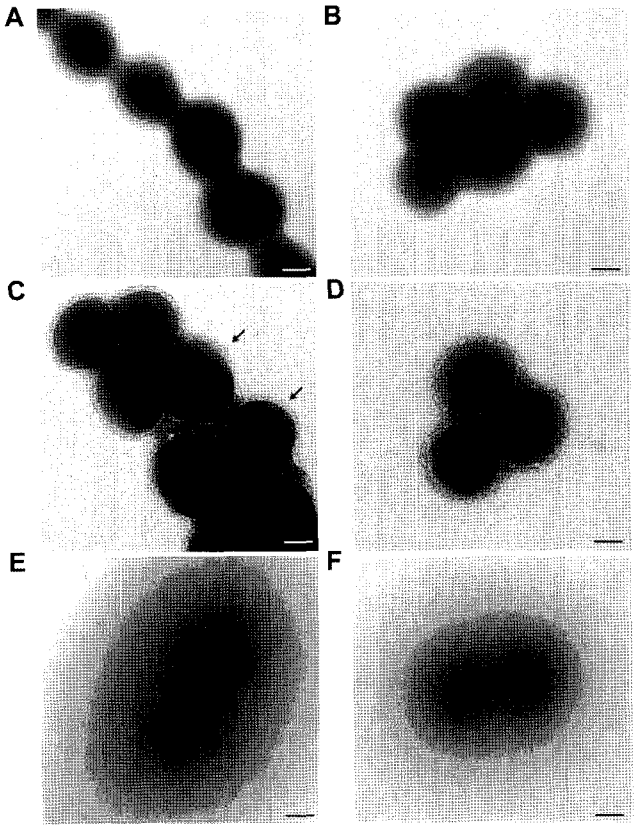

Fig. 1. Transmission electron micrographs of unstained *S. aureus*. **A** and **B**. DW-suspended bacteria. **C** and **D**. PBS-suspended bacteria. Arrows indicate granules surrounding bacteria. **E** and **F**. NB-suspended bacteria. Bars=200 nm.

periphery than DW-suspended and unstained bacteria (Figs. 1C and 1D). Most bacteria were surrounded by small (approx. 20 nm) and irregularly shaped granules. The granules were rarely found after additional suspension in DW (data not shown). Meanwhile, the NB-suspended and unstained *S. aureus* could not be focused properly owing to their lack of contrast (Figs. 1E and 1F).

The images of the PTA-stained *S. aureus* were greatly different from those of the unstained ones. After being suspended in DW, the PTA-stained *S. aureus* showed a clearly delineated boundary against the background (Figs. 2A and 2B). Even bacterial septa could be examined in PBS-suspended and PTA-stained *S. aureus* (Fig. 2C). Staining intensity was not homogeneous, depending on the localized surface profiles of bacteria (Fig. 2D). The NB-suspended and PTA-stained *S. aureus* were heavily contrasted with thick stain depth around bacteria (Fig. 2E). Electron-dense aggregates were often found around bacteria on the film (Fig. 2F).

Morphology and Appendage Structures of *E. coli*

Similar results were found in *E. coli* as already shown in *S. aureus*. The DW-suspended and unstained *E. coli* was

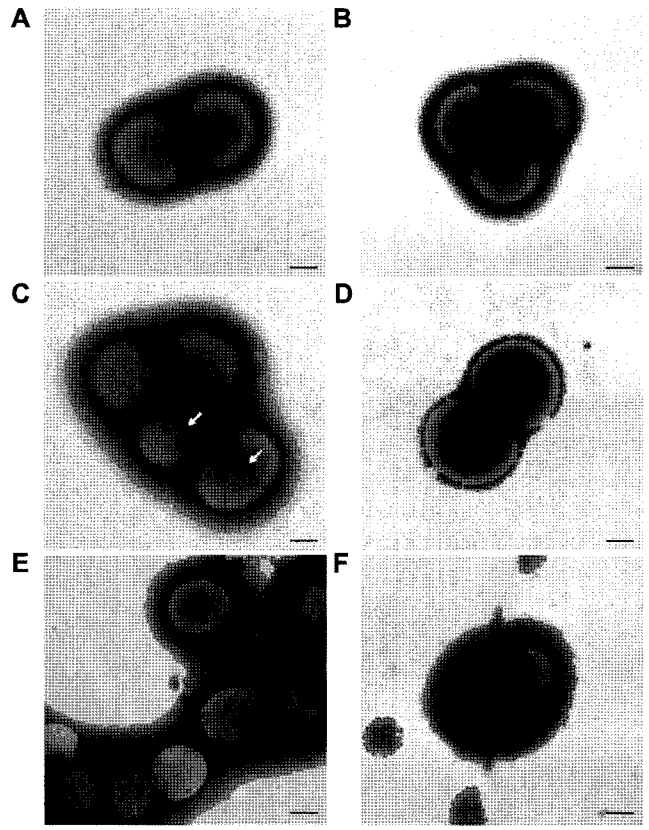

Fig. 2. Transmission electron micrographs of PTA-stained *S. aureus*. **A** and **B**. DW-suspended and PTA-stained bacteria. **C** and **D**. PBS-suspended and PTA-stained bacteria. Arrows indicate bacterial septa. Bars =200 nm. **E** and **F**. NB-suspended and PTA-stained bacteria. Bars=500 nm.

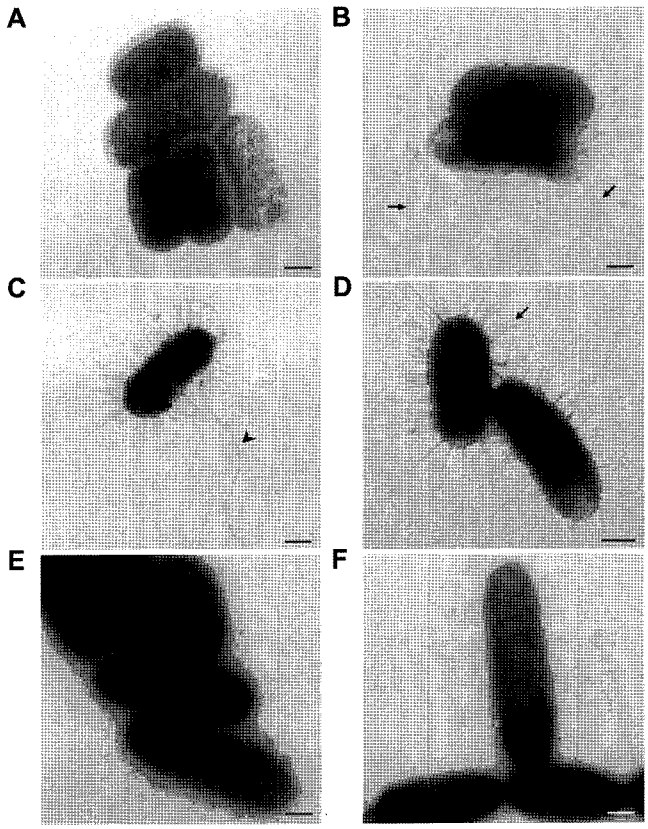

Fig. 3. Transmission electron micrographs of unstained *E. coli*. **A** and **B**. DW-suspended bacteria. Arrows indicate fimbriae. **C** and **D**. PBS-suspended bacteria. An arrow and an arrowhead indicate fimbriae and a flagellum, respectively. **E** and **F**. NB-suspended bacteria. Bars= 500 nm.

characterized by rod cell shape (approx. 2 μm in length) of low contrast throughout the film (Fig. 3A). Fimbriae were slightly visible around the bacterial surface (Fig. 3B). Following suspension in PBS, flagella could be also observed on the film (Fig. 3C). In addition, numerous fimbriae were apparent around the unstained *E. coli* (Fig. 3D). The fimbriae were peritrichous and up to approximately 1 μm long. Small granules were deposited on the bacterial surface. On the other hand, the NB-suspended and unstained *E. coli* were weakly contrasted in that the fine appendage structures could not be readily observed (Figs. 3E and 3F).

The DW-suspended and PTA-stained *E. coli* were contrasted to reveal the fine extracellular appendage structures such as fimbriae and flagella (Fig. 4A). Individual fimbria appeared to be aligned in parallel and often crossed each other (Fig. 4B). The combination of PBS suspension and PTA staining also provided clearly delineated morphology and appendage structures (Figs. 4C and 4D). However, it was common to observe electron-dense aggregates around the NB-suspended and PTA-stained *E. coli* (Fig. 4E). The bacteria were not clearly contrasted against the background. Some round-shaped electron-dense aggregates were in contact with bacteria on the local cell surface (Fig. 4F).

PBS Precipitates

It was common to observe randomly distributed precipitates from PBS on the film (Fig. 5). The precipitates themselves consisted of small amorphous electron-lucent granules that were approximately 20 nm in diameter. These precipitates were strikingly similar to those found around the PBS-suspended and unstained *S. aureus* and *E. coli*.

Discussion

This study provided technical precautions to be considered before conventional negative staining of cultured bacteria. There were significant differences in image contrast depending on suspension liquids before PTA staining. As far as delineation of the structures is concerned, the combination of DW or PBS suspension with subsequent PTA staining provided the most satisfactory results, as evidenced by the high contrast of bacterial morphology and appendage structures (Figs. 2A–2D and 4A–4D). No significant differences in image contrast were observed between negatively stained bacteria suspended in DW and PBS. However, after being suspended in NB and

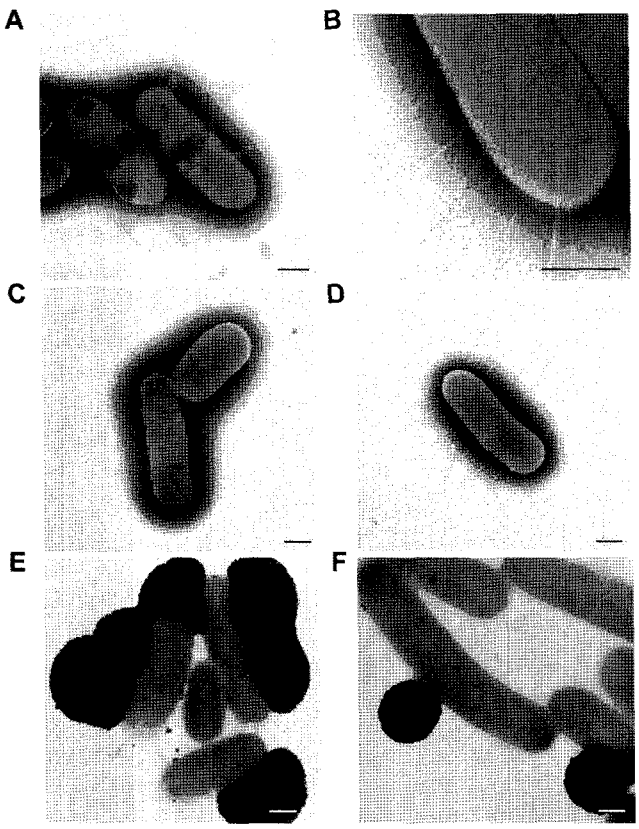

Fig. 4. Transmission electron micrographs of PTA-stained *E. coli*. **A** and **B**. DW-suspended and PTA-stained bacteria. **C** and **D**. PBS-suspended and PTA-stained bacteria. **E** and **F**. NB-suspended and PTA-stained bacteria. Bars=500 nm.

stained with PTA, bacteria often had too high contrast or poor staining, with electron-dense aggregates around bacteria, necessitating additional suspension procedures to achieve homogeneous staining intensity. These results suggest that suspension with concentrated organic aliquots including broth media before PTA staining could deteriorate image contrast, and should be used only in dilute form for visualizing bacterial morphology and appendage structures. The three types of pre-stain suspension liquids assayed in this study are the three most commonly used liquids that are widely available in microbiology laboratories and are easily selected by workers. Therefore, the results in this study might serve as a practical reference in selecting pre-suspension liquids when using PTA for conventional negative staining of bacteria.

It is widely accepted that DW imposes a severe osmotic gradient between the protoplast and the suspension liquid, thereby stressing the bacterial cell wall. The DW-suspended and PTA-stained bacteria appeared to be normal in shape, and did not show any aberrant morphology in this study. Based on the typical morphology of each bacterial type shown in this study, it is reasonable to think that the osmotic gradient exerted by DW is tolerable for the bacterial

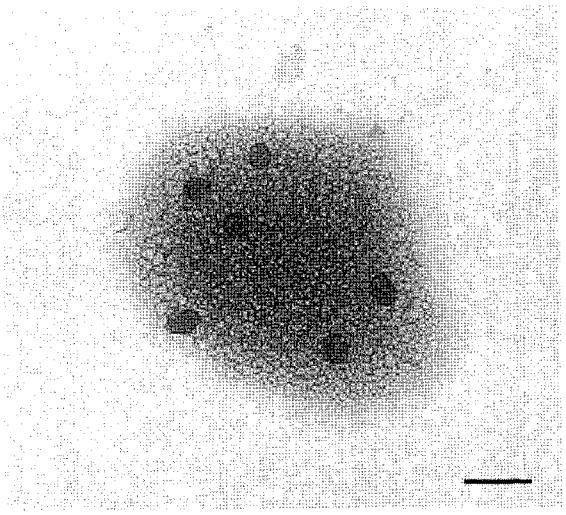

Fig. 5. Transmission electron micrograph of unstained electron-dense aggregates in PBS. Bar=200 nm. The aggregates consisted of small amorphous electron-lucent individual granules.

strains. Instances have been reported where bacteria were fixed with aldehydes before negative staining [22, 24]. The excellent retention of cell shape of unfixed bacteria in this study supports that pre-fixation with aldehydes may not be required for negative staining of bacteria, depending on the research objectives and specimens.

An intriguing finding in this study was the unpredictable staining profiles in the NB-suspended bacteria. The low contrast would be perhaps due to the increase of inelastically scattered electrons, even though zero-loss images of the specimens were observed by EF-TEM. Apart from conventional TEM, EF-TEM is a specialized TEM that can filter the transmitted electrons according to their energy levels *via* an energy filter, leading to the differentiating between elastically scattered electrons (zero-loss) and inelastically scattered electrons [16]. It is considered that the presence of higher concentrations of organic components of NB (beef extracts, peptone, *etc.*) could generate higher amounts of inelastically scattered electrons than the DW- or PBS-suspended treatments, which cause the decrease of elastically scattered electrons and subsequent contrast deterioration in images. However, this study does not exclude the possibility that components of NB act like a surfactant that results in partial or poor staining of bacterial cells and extracellular appendage structures by PTA. In addition, the organic components of NB may react undesirably with PTA to form electron-dense aggregates around the bacteria and interfere with the negative staining procedure, leading to the consequent partial or poor staining of the bacteria. The different staining profiles between the two bacterial strains remain to be investigated in further studies.

Another interesting finding in this study was the deposition of precipitates from PBS around the bacteria.

Such precipitates have been traditionally regarded as artifacts according to the tenets of high-resolution TEM. Granule-like particles have been often shown in other's TEM images after negative staining of bacteria, but not thoroughly addressed on their significance [1, 12]. Small granules around the PBS-suspended bacteria and PBS precipitates in this study were strikingly similar to granule-like particles in other works in terms of electron density and dimension. Thus, it can be noteworthy to discuss the meaning and applicability of precipitates for contrast tuning or enhancement of unstained bacteria. Unstained bacteria have been specifically used to (i) examine electron-dense bodies [5, 21], (ii) demonstrate peripheral amorphous slime [15], and (iii) perform electron spectroscopy [1]. The low contrast of unstained bacteria can be overcome through the use of Zernike phase contrast [2] or Hilbert differential contrast electron microscopy [10] that require specially crafted apertures in optics designs. It was evident from this study that the contrast of unstained bacteria was enhanced through suspension in PBS before observation. The PBS-suspended and unstained bacteria appeared to be invariably surrounded with precipitates from PBS. The precipitates can be used to unspecifically "decorate" bacteria deposited on a supporting plastic film. The precipitates from PBS are thought to be stable structures, which was evidenced by their presence on the bacterial cell surface after electron beam illumination under the vacuum conditions for TEM. Preliminary energy-dispersive X-ray spectroscopy has revealed that the main elements of the precipitates from PBS included phosphorus, sodium, potassium, and chloride that could possibly form sodium chloride and phosphate microcrystals (data not shown).

Bacteria have surfaces that are reactive with metals largely because of the dissociation of protons from carboxyl and phosphoryl group constituents of cell wall macromolecules [4]. Since the cell surface is in direct contact with the environment, the charged groups within the surface layers are able to interact with ions or charged molecules [13]. The contrast tuning or enhancement by decoration of bacteria using PBS or similar buffered salines would be advantageous in demonstrating bacterial interactions with fine-grained heavy metals, while maintaining minimal contrast for cell imaging. After being stained with conventional negative stains, the PBS-suspended bacterial surface would be covered with metal stains and extraneously treated cell-bound heavy metals, making them difficult to be discriminated based on their electron density, as inferred in this study (Figs. 2C and 2D; 4C and 4D). The discrimination may need significant levels of defocus and consequent contrast transfer function correction. Thus, the ability to simply and rapidly visualize unstained bacteria could be employed in studies of bacterial attachment to solid surfaces, conjugation, and interactions with environmental particles [19], complementing the results of negatively stained specimens. The rationale for unconventional "low contrast" negative stains using chemicals containing light elements for protein crystals [17, 18] can be similar to that from this study. In our recent work [9], PBS-suspended and unstained bacteria were discerned with precipitates around bacteria, and electron-dense particles, probably silver, were contrasted with whole-mount bacteria, so that the sorbed minerals could be attributed to only preformed metals. It was apparent that the PBS-suspended and unstained bacteria did not bear the risk of masking fine minerals around the bacteria, while retaining the low contrast through PBS precipitates deposited on the bacteria.

In summary, we compared a combination of negative staining regimes for bacterial observation, showing that there was significant disparity in image contrast depending on pre-suspension liquids. It was affirmed in this study that the combination of DW or PBS suspension with subsequent PTA staining provided the most satisfactory results. It remains to be validated the effects of pre-stain suspension liquids on diverse bacterial strains and other negative stains such as uranyl acetate and ammonium molybdate in order to confirm their broad-spectrum characteristics and establish optimal staining regimes in further studies. The presently proposed "low contrast" negative stain for bacteria, a simple and rapid decoration procedure for demonstrating bacteria using PBS precipitates, would facilitate characterization of diverse clinical or environmental isolates that are reactive with metal ions present in the external milieu. The knowledge of the implications of the material deposition on bacterial cell surfaces with stainability is likely to enhance our understanding of the specimen-metal stain interactions for visualizing prokaryotes as they are in native environments.

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