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Pseudomonas Pellicle in Disinfectant Testing: Electron Microscopy, Pellicle Removal, and Effect on Test Results

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***Pseudomonas aeruginosa* ATCC 15442 is a required organism in the Association of Official Analytical Chemists use-dilution method for disinfectant efficacy testing. When grown in a liquid medium, *P. aeruginosa* produces a dense mat or pellicle at the broth/air interface. The purpose of this investigation was to examine the pellicle by scanning electron microscopy, to evaluate three pellicle removal methods, and to determine the effect of pellicle fragments on disinfectant efficacy test results. The efficacies of three methods of pellicle removal (decanting, vacuum suction, and filtration) were assessed by quantifying cell numbers on penicylinders. The Association of Official Analytical Chemists use-dilution method was used to determine whether pellicle fragments in the tubes used to inoculate penicylinders affected test results. Scanning electron micrographs showed the pellicle to be a dense mass of intact, interlacing cells at least 10 μm thick. No significant differences in pellicle removal methods were observed, and the presence of pellicle fragments usually increased the number of positive tubes in the use-dilution method significantly.**

The use-dilution method (UDM) of the Association of Official Analytical Chemists (AOAC) is the official procedure in the United States for determining bactericidal efficacy of chemical germicides (1). The method specifies the use of *Pseudomonas aeruginosa* ATCC 15442 (American Type Culture Collection, Rockville, Md.). When grown in a nutrient broth, the organism produces a dense mat or pellicle at the broth/air interface. The composition of the pellicle and the effect of pellicle fragments on disinfectant efficacy test results have not been reported. Additionally, the current UDM specifies decanting the *P. aeruginosa* broth culture to another tube to leave the pellicle behind. This method and pellicle removal by a sterile vacuum pipette are currently used by testing laboratories. Broth filtration has been suggested as an alternative pellicle removal method. The purpose of this study was threefold: to examine the composition of the pellicle by scanning electron microscopy, to evaluate the effectiveness of three pellicle removal methods (decanting, vacuum suction, and filtration), and to determine the effect of pellicle fragments on test results.

P. aeruginosa was grown in a 10-ml nutrient broth (AOAC 4.001a) by using Anatone peptone (American Laboratories, Inc., Omaha, Nebr.) and beef extract (Difco Laboratories, Detroit, Mich.) as specified. At least three consecutive 24-h broth transfers were made by using a 4-mm (inside diameter) loop, with incubation at 37°C. Sixteen 10-ml tubes were inoculated and incubated at 37°C for 48 to 54 h.

Previously used penicylinders (type 304 stainless steel [8 ± 1 mm outside diameter, 6 ± 1 mm inside diameter, 10 ± 1 mm long]; S & L Metal Products Corp., Maspeth, N.Y.) were autoclaved (15 min, 121°C), sonically cleaned (≥5 min), and soaked in 1.0 N NaOH overnight. After being thoroughly rinsed in tap water and then in distilled water, the penicylinders were placed in 50-ml polypropylene screw-cap tubes, overlaid with 0.1% asparagine (Difco) solution, and sterilized at 121°C for 15 min. When cooled to room temperature, penicylinders were ready to be used. Before being used, the penicylinders were drained of asparagine.

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Two penicylinders were inoculated with the intact pellicle as follows. A 48- to 54-h broth culture tube was tilted approximately 70° to retract the pellicle. A sterile penicylinder was placed into the culture broth and under the pellicle. As the tube was straightened, the penicylinder was lifted out of the broth, allowing a large portion of the pellicle to adhere. The penicylinders were placed on end on two layers of sterile filter paper in a glass petri dish as described in the AOAC UDM for carrier inoculation (1). The covered dish was placed in a nonhumidified incubator at 37°C for 40 min. After being dried, penicylinders were carefully transferred to tubes of 2% glutaraldehyde–2% paraformaldehyde for scanning electron microscopy processing as previously described (Fig. 1A and B) (2).

On each of four days, 80 penicylinders were tested in the UDM. Twenty penicylinders were used for each of the three removal methods, and twenty penicylinders were inoculated in broth containing the pellicle. For each removal method, 3 or 4 tubes of 48- to 54-h broth were pooled after pellicle removal and used for penicylinder inoculation. Decanting involved slowly pouring the broth into another sterile tube (25 by 150 mm), thus leaving behind the intact pellicle as well as any sediment. Suction was accomplished by a sterile Pasteur pipette connected to a vacuum source. After removal of the pellicle by suction, the broth was decanted as described. Filtration required that the broths be carefully decanted through four layers of sterile gauze (4 by 4 in. [ca. 10 by 10 cm]). Tubes with intact pellicles were vigorously mixed on a Vortex-Genie (Fisher Scientific Co., Pittsburgh, Pa.) at setting 4.

After broth preparation, the carriers were inoculated. Tubes containing sterile penicylinders were drained of asparagine, and 22 penicylinders were aseptically transferred to a sterile tube (25 by 150 mm). Prepared broth (22 ml) was added, and the tubes were allowed to remain for 15 min at room temperature. The penicylinders were then removed, placed on end in sterile glass petri dishes (11 per dish), matted with two layers of Whatman no. 2 filter paper, and dried at 37°C for 40 min.

From each batch of 22 penicylinders, 20 were used for

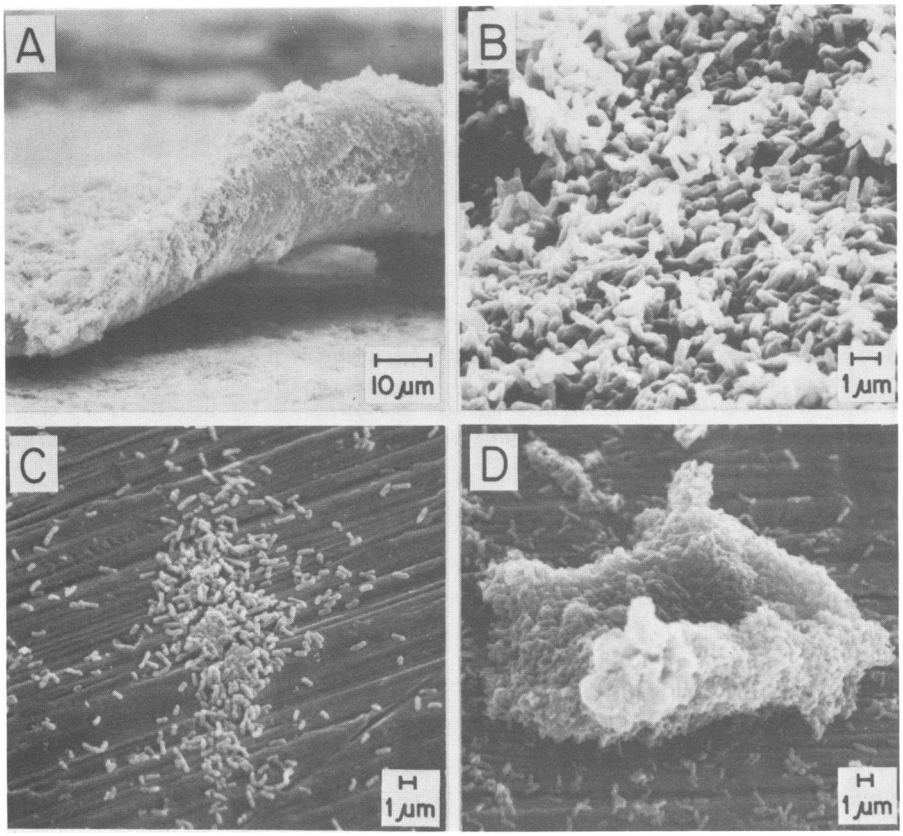

FIG. 1. Scanning electron photomicrographs of *P. aeruginosa* ATCC 15442 showing intact pellicles (A and B) and pellicle fragments on stainless steel penicylinders (C and D).

UDM testing and 2 were used for quantitation of cells on the penicylinder. Cell numbers were determined by placing each carrier in a screw-cap tube (20 by 150 mm) containing 10 ml of phosphate-buffered dilution water (AOAC 4.020f) and vortexing for 1 min at setting 4. Further dilutions in phosphate-buffered dilution water were made, and 1.0-ml samples of the 10^{-4} dilution were plated in duplicate by using a pour plate technique with Plate Count Agar (Difco). Incubation was at 37°C for 48 h, after which CFU were counted.

The UDM test (1) was conducted in a 20°C circulating water bath in which each penicylinder was exposed for 10 min to 10 ml of an Environmental Protection Agency-registered quaternary ammonium disinfectant (7.5% didecyl dimethyl ammonium chloride, 5% *n*-alkyl [C_{14}, 50%; C_{12}, 40%; C_{16}, 10%] dimethylbenzyl ammonium chloride, 1.69% tetrasodium EDTA, 85.81% inert ingredients) prepared with distilled water to its recommended use dilution of 1:256. After exposure to the disinfectant, penicylinders were placed in 10 ml of letheen broth (Difco) for disinfectant neutralization and cell recovery and incubated for 48 h at 37°C. Additionally, two penicylinders inoculated from broth containing the vortexed pellicle were dried and fixed for scanning electron microscopy (Fig. 1C and D) (2).

Scanning electron microscopy (Fig. 1) showed the pellicle to be a dense mat of cells about 10 μm thick. Examination of the pellicle fragments attached to the penicylinders indicated a significant size range. Clumps of cells may have occluded penetration of chemical disinfectants and protected cells from disinfectant exposure, resulting in positive tubes in the qualitative UDM (Table 1; Fig. 1).

UDM testing demonstrated that the pellicle should be removed because it produces more positive tubes in the UDM and therefore biases the results in favor of disinfectant failure. The data also demonstrated that differences in numbers of cells attaching to penicylinders among the three removal methods were not significant ($P > 0.05$, as determined by analysis of variance). Since there were no significant differences in mean numbers of cells remaining on penicylinders after the three removal methods (Table 1), any of the methods may be used satisfactorily in the performance of the AOAC UDM.

Mean log numbers of cells on inoculated penicylinders with no pellicle removal for days 1, 2, 3, and 4 were >7.331,

TABLE 1. AOAC UDM results with three *Pseudomonas* pellicle removal methods versus no removal

Day	No. of positive penicylinders (n = 20) after[a]:			
	Decanting	Suction	Filtering	No removal[b]
1	0	0	1	20
2	0	1	0	17
3	0	0	0	1
4	0	0	0	10

[a] Ranges and mean (n = 8) number ($\log_{10}$) of *P. aeruginosa* attached to penicylinders after pellicle removal methods or no pellicle removal were as follows: decanting, 6.204 to 7.140 (mean, 6.742); suction, 6.653 to 6.954 (mean, 6.813); filtering, 6.544 to 7.049 (mean, 6.853); no removal, 6.140 to >7.477 (mean, >7.106).

[b] Differences in results compared with those obtained with pellicle removal methods were significant ($P < 0.01$; Fisher's exact test).

>7.477, 6.140, and >7.477, respectively. Since the mean number of cells on inoculated penicylinders with no pellicle removal on day 3 was below the range of cell numbers for penicylinders inoculated in broth with pellicles removed, it is not surprising that significantly fewer positive penicylinders were observed on day 3 (Table 1). It is not known why the cell numbers on day 3 were lower.

On the basis of these results, and with the realization that some broth volume is lost by filtration, it is suggested that pipette suctioning followed by decanting be the recommended method of pellicle removal when *P. aeruginosa* is used in disinfectant efficacy testing.

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LITERATURE CITED

1. **Association of Official Analytical Chemists.** 1984. Disinfectants, p. 67–68. *In* Official Methods of Analysis, 14th ed. Association of Official Analytical Chemists, Arlington, Va.
2. **Cole, E. C., W. A. Rutala, and J. L. Carson.** 1987. Evaluation of penicylinders used in disinfectant testing: bacterial attachment and surface texture. J. Assoc. Off. Anal. Chem. **70**:903–906.