Bacterial Contamination of Cucumber Fruit through Adhesion[†,‡]

LAURA D. REINA, HENRY P. FLEMING,* AND FREDERICK BREIDT, JR.

U.S. Department of Agriculture, Agricultural Research Service, and North Carolina Agricultural Research Service, Department of Food Science, North Carolina State University, Raleigh, North Carolina 27695-7624, USA

ABSTRACT

In this study, the adhesion of bacteria to fresh cucumber surfaces in aqueous suspension was shown to be dependent on time of incubation, inoculum species and concentration, and temperature. The adhesion of bacteria to the fruit in wash water was less extensive at lower temperatures and shorter exposure times. Various species of bacteria were adsorbed to cucumber surfaces in the following relative order: *Salmonella* Typhimurium > *Staphylococcus aureus* > *Lactobacillus plantarum* > *Listeria monocytogenes*. Cells were adsorbed at all temperatures tested (5, 15, 25, and 35°C) at levels that depended on incubation time, but the numbers of cells adsorbed were larger at higher incubation temperatures. Levels of adhesion of bacteria to dewaxed fruit were higher for *L. monocytogenes* and lower for *Salmonella* Typhimurium, *L. plantarum,* and *S. aureus* than were levels of adhesion to waxed fruit.

In the past decade, outbreaks of human illness associated with the consumption of raw vegetables and fruits have become more frequent in the United States *(4, 6, 7)*. Fruits and vegetables are often in contact with soil, insects, and animals during growing and harvesting in the field *(26)*. Many different kinds of microorganisms have been found on fresh produce, and the numbers of these microorganisms can range from 10^3 to 10^9 CFU/g *(34)*.

The agricultural practices and hygienic conditions used during harvesting, processing, packaging, transport, and storage influence the initial microbial population *(32, 34)*. Fruits and vegetables are cleaned, cooled, and conveyed in water and are treated with aqueous formulations of chemicals after harvesting to remove foreign matter and microorganisms *(13)*. The degree to which washing reduces the microbial load depends on the produce in question and the procedure used. Recycling of the wash water, a common practice in washing and hydrocooling, can lead to cross-contamination *(5)*. Microorganisms in the water may contaminate fruit through lenticels, stomata, and injuries incurred during harvesting *(28)*. Warmed tomatoes immersed in cool water containing bacteria were found to absorb water and bacteria. When the tomatoes immersed in water were at the same temperature as the water, they did not absorb water, but their surfaces were contaminated with bacteria *(2, 3)*.

Knowledge about microbial contamination of fruits and

vegetables will be helpful in determining ways to prevent or reduce the establishment of pathogens on vegetable tissues, thus reducing the risk of potential illness due to the consumption of contaminated fruits and their products. Such knowledge will also be helpful in determining ways to reduce the risk of cross-contamination during postharvest handling *(18)*. A better understanding of the relative importance of bacteria and of the factors involved in the attachment (adhesion) of bacteria could be useful in designing improved methods for the washing and hydrocooling of produce.

The objectives of this study were to determine the relative levels of adhesion of selected species of bacteria to fresh cucumber surfaces and to determine how adhesion might be related to bacterial hydrophobicity, contact time, and temperature.

MATERIALS AND METHODS

Cucumbers. Size 2B (3.43 to 3.75 cm in diameter) and 3B (4.37 to 5 cm in diameter) Calypso cultivar cucumbers were obtained from Mount Olive Pickle Company. Pickling cucumbers of an unknown cultivar were obtained from a local supermarket. All fruits were in good condition and free of mechanical damage. Fruits were washed with cold water and stored at 6°C for up to 3 days before they were used. None of the cucumbers used in this study were artificially waxed.

Bacterial cultures. Bacteria (Table 1) were stored at −82°C in media containing 15% glycerol (*Staphylococcus aureus, Salmonella* Typhimurium, and *Listeria monocytogenes* were stored in Trypticase soy broth, and *Lactobacillus plantarum* was stored in deMan Rogosa Sharpe [MRS] broth). Chloramphenicol-resistant *S. aureus* and *L. plantarum* were obtained by electroporation with pGk12 as described by Breidt and Fleming *(6)*. *L. monocytogenes* and *Salmonella* Typhimurium were obtained from the culture collection of Dr. P. M. Foegeding (Department of Food Science, North Carolina State University).

* Author for correspondence. Tel: 919-515-2979; Fax: 919-856-4361; E-mail: hfleming@unity.ncsu.edu.

TABLE 1. *Relative hydrophobicities of bacteria*

Bacterial species	Strain[a]	Relative hydrophobicity (%)[b]	Reference[c]
Bacillus subtilis	B151 (ATCC 19221)	8 (2.1) E	*32*
Listeria monocytogenes[d]	B88 (NCK 157 cm⁺)	23 (5.4) D	22
Lactobacillus plantarum	LA70 (ATCC 14917)	35 (1.3) C	—
L. plantarum[d]	LA219 (MOP 3 cm⁺)	59 (1.6) B	—
Staphylococcus aureus[d]	B195 (153 CPS cm⁺)	96 (1.6) A	27
Salmonella Typhimurium[d]	B38 (ATCC 14028)	63 (3.2) B	25
Enterobacter aerogenes[d]	B146 (ATCC 13048)	63 (0.5) B	27
E. aerogenes	B148 (ATCC 29940)	10 (0.5) E	—

[a] Strain numbers refer to the U.S. Department of Agriculture Food Fermentation Laboratory designations.

[b] Averages for two samples; experiments were repeated four times. Standard deviations are shown in parentheses. Values with different letters are significantly different ($P < 0.05$) by Duncan's multiple-range test.

[c] Studies reporting results similar to ours regarding hydrophobicity.

[d] The time course for adhesion of this species at 4 to 5°C was determined.

Hydrophobicity determination. The microbial-adherence-to-hydrocarbons method was used to determine bacterial cell hydrophobicity *(11)*. Bacteria were grown overnight in the appropriate medium to an optical density (OD) of 1 to 1.2. Then, cells were washed twice with 100 mM sodium phosphate (pH 6.8) by centrifugation at $709 \times g$ for 10 min each time. The cells were diluted to an OD_{440} of 0.8 to 1.0. The resulting turbid, aqueous suspension of washed microbial cells (3 ml) was mixed by vortexing in the presence of 1 ml of hexadecane and was then incubated for 15 min at 35°C in a water bath. After the suspension had been mixed, the two phases were allowed to separate. The percentage of adhering cells was determined by the decrease in absorbance of the original cell suspension. The relative hydrophobicity (%) of the strain was determined as [(initial A_{440} − final A_{440}) $\times$ 100]/initial A_{440}. The integrity of the cells was confirmed by phase-contrast microscopy. Lysis or clumping of the cells due to hexadecane, which might have caused a decrease in absorbance, was ruled out. *Bacillus subtilis* was included in each test as a control. We found the hydrophobicity of this bacterium to be consistent with that reported by Wyncek et al. *(33)*.

Inoculum preparation. *S. aureus* and *L. monocytogenes* were grown overnight in Trypticase soy broth (BBL Microbiology Systems, Cockeysville, Md.) containing 5 μg of chloramphenicol per ml. *L. plantarum* MOP3 (LA 219) was grown in MRS broth (Difco Laboratories, Detroit, Mich.) with 5 μg of chloramphenicol per ml. *Salmonella* Typhimurium was grown in Trypticase soy broth (BBL). Cells were harvested by centrifugation ($709 \times g$ for 10 min) and washed twice with distilled water. Cells were resuspended in water to produce inocula containing approximately 1 $\times$ 10^6 or 1 $\times$ 10^8 CFU/ml. Inoculum levels of 10^3, 10^4, 10^6, and 10^8 CFU/ml were studied.

Adhesion studies. Cucumbers (200 g, three to four fruits) were equilibrated at five different air temperatures (4, 10, 20, 30, and 40°C) and then immersed for 30 or 60 min in water containing approximately 1 $\times$ 10^8 CFU/ml. The cucumbers were held under water with a plastic mesh. The incubation temperatures were 5, 10, 15, 25, and 35°C. After immersion for the designated times, the cucumbers were washed twice with 2$\times$ sterile distilled water (at the temperature of the inoculum) on a platform shaker (gyratory shaker model G2, New Brunswick Scientific Co., Inc., Edison, N.J.) rotating at 100 rpm for 1 min. Bacteria that were not removed by washing were considered to have adsorbed to the surfaces of the cucumbers. Samples of these fruits were obtained for scanning electron microscopy (SEM) and for the determination of bacterial cell counts.

SEM. Samples of the fruit were obtained for SEM by dissecting sections (5 mm² by 1 mm thick) from three different skin areas (the blossom end, the stem end, and the middle). Samples were prepared for SEM by general procedures described previously *(9)*. The samples were fixed with 3% glutaraldehyde in 0.05 M potassium phosphate buffer, dried by critical point drying with CO_2 (Tousimis Research Corp., Rockville, Md.), and coated with a gold-palladium alloy (80:20) in a Hummer V (Anatech LTD, Springfield, Va.). Samples were observed at various magnification levels at 15 kV with a Philips 505T SEM (Philips, Eindhoven) operating in the secondary electron mode.

Bacterial cell counts. Cucumbers (200 ± 5 g) were blended in a Waring blender for 2 min at high speed with 400 g of sterile saline (0.85%). The slurry was syringe filtered with Whatman no. 4 filter paper to remove large particles. Dilutions were made in sterile saline solution and plated with a spiral plater Model D (Spiral Systems, Inc., Cincinnati, Ohio). Samples were plated on modified MRS agar containing 0.02% sodium azide and 5 μg of chloramphenicol per ml and modified Oxford agar containing 10 mg of colistin sulfate per liter plus 20 mg of moxalactan per liter and 5 μg of chloramphenicol per ml; S110 with chloramphenicol was used for *S. aureus,* and salmonella-shigella agar was used for *Salmonella* Typhimurium.

Dewaxing procedure. Cucumbers were dewaxed by the procedure of Silva Fernandez et al. *(29)*. Cucumbers (150 g) were immersed in beakers containing hexane (300 ml) and placed on a rotary shaker for 1 min at 100 rpm. This procedure was carried out twice. Cucumbers were allowed to dry under the hood and were washed with distilled water. Adhesion studies were carried out as described above.

Statistical analysis. Linear regression analysis and analysis of variance of the data were carried out with SAS software (version 6, SAS Institute, Cary, N.C.).

RESULTS AND DISCUSSION

The relative hydrophobicities of the bacteria studied ranged from 8 to 96%. There were considerable differences
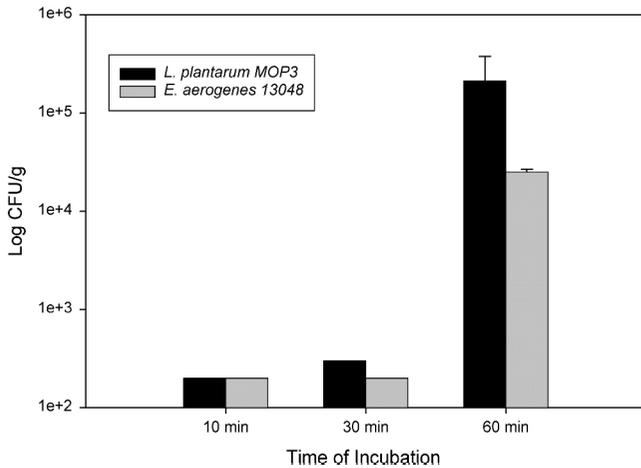
FIGURE 1. *Effect of time of incubation on the adhesion of bacteria to cucumber surfaces in water at 5°C. Numbers are the averages of duplicates obtained by spread plating.*
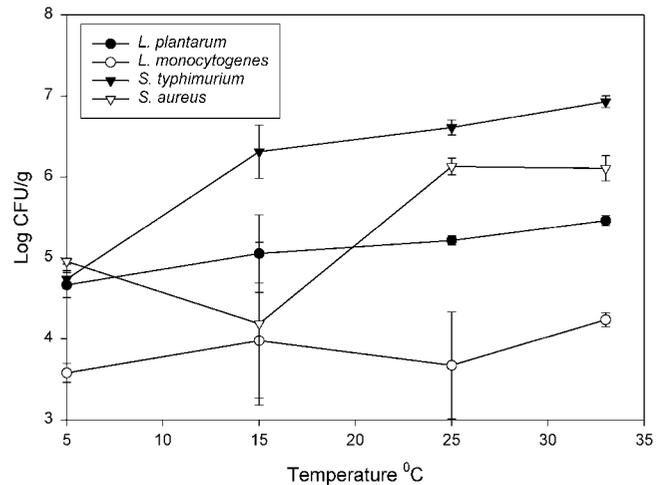
FIGURE 2. *Effect of temperature of incubation on the adhesion of bacteria to cucumber surfaces in water. The incubation time was 60 min. Points represent the means of two replicates.*

in hydrophobicity, even between the different strains of *Enterobacter aerogenes* and *L. plantarum* that were tested (Table 1). Hydrophobicity was independent of the Gram stain status or pathogenicity of bacteria. Bacterial surface hydrophobicity has been found to vary depending on a variety of factors, including culture age, growth medium, pH, and subculturing techniques *(15, 25, 27).* In the present study, cultures were of the same age. Differences in hydrophobicity could be species specific. According to our results, the hydrophobicities of *S. aureus,* Salmonella Typhimurium, *E. aerogenes* 13048, and *L. plantarum* MOP3 were higher than those of *L. plantarum* ATCC 14917, *L. monocytogenes,* and *E. aerogenes* 29940. It is not known whether the results obtained here can be considered typical of these particular species. However, the fact that the two organisms showing the highest hydrophobicities were pathogenic indicates the potential for pathogens to adhere well to fruit and vegetable surfaces. Lachica *(20)* reported that 6 of 11 pathogenic bacteria that affect the urinary, digestive, and respiratory tracts were shown to have high cell surface hydrophobicities, which were associated with the binding of bacteria to the host cell.

The time and temperature of exposure of whole cucumbers in water to bacteria significantly influenced the adhesion of the bacterial species tested to cucumber surfaces. In preliminary tests, the six species of bacteria listed in Table 1 adhered to cucumbers at undetectable levels (<20 CFU/g) when incubated in water at 4 to 5°C (data not shown). However, when the levels of inoculum were higher and time of contact with water was extended to 60 min, bacteria could be enumerated (Fig. 1).

The degree of adsorption of bacteria to the fruit surface was somewhat related to the hydrophobicity of the strain. For instance, *L. monocytogenes,* the least hydrophobic bacterium tested, was the least adsorbed (Fig. 2). Similar results were obtained for the adhesion of *L. monocytogenes* to the surfaces of cantaloupes *(31).* However, *S. aureus,* the most hydrophobic bacterium tested (96% hydrophobic), was less adsorbed than *Salmonella* Typhi-

murium, which was 60% hydrophobic. Perhaps other factors, such as the presence of flagella, could have contributed to the adhesion of bacteria *(23).* Flagella have been suggested to play a role in bacterial adhesion and colonization, because flagellated bacteria presumably should reach the surface of attachment faster *(8, 10, 16, 17).* The attachment of bacteria to surfaces has been reported to be a two-phase process. The cells first become associated with the surface through loose, reversible sorption, and then a time-dependent irreversible type of film attachment develops through the formation of viscous polymers *(14).* In this study, we considered only attachment of bacteria to the cucumber surface due to reversible sorption, which could be mediated by hydrophobicity, flagella, London–van der Waals forces, etc.

The effect of incubation temperature on the adhesion of four bacterial species is illustrated in Figure 2. The overall slopes of data shown in the figure indicate that the effect of temperature on the adhesion of *Salmonella* Typhimurium was significantly ($P < 0.05$) stronger than its effects on the adhesion of the other three species. Differences among the other three species were not significant ($P > 0.05$). However, within each species, there was a significant ($P < 0.05$) increase in the degree of adhesion as the temperature was increased from 5 to 35°C.

Cucumbers were treated with hexane to remove wax and to change the surface characteristics of the fruit (Fig. 3A and 3B). The relative adhesion by *L. monocytogenes* was more extensive for the dewaxed fruit (Table 2). Counts of *L. plantarum, S. aureus,* and *Salmonella* Typhimurium were lower on the dewaxed fruit. *Salmonella* Typhimurium was found to be 60% hydrophobic, and *S. aureus* was found to be 96% hydrophobic. If the attachment of *Salmonella* and *Staphylococcus* to cucumber surfaces is due to hydrophobic interactions *(12),* then the removal of wax from the cucumbers, resulting in a reduction of these hydrophobic interactions, facilitates the removal of the bacteria during washing. The results of an analysis of variance for the data shown in Table
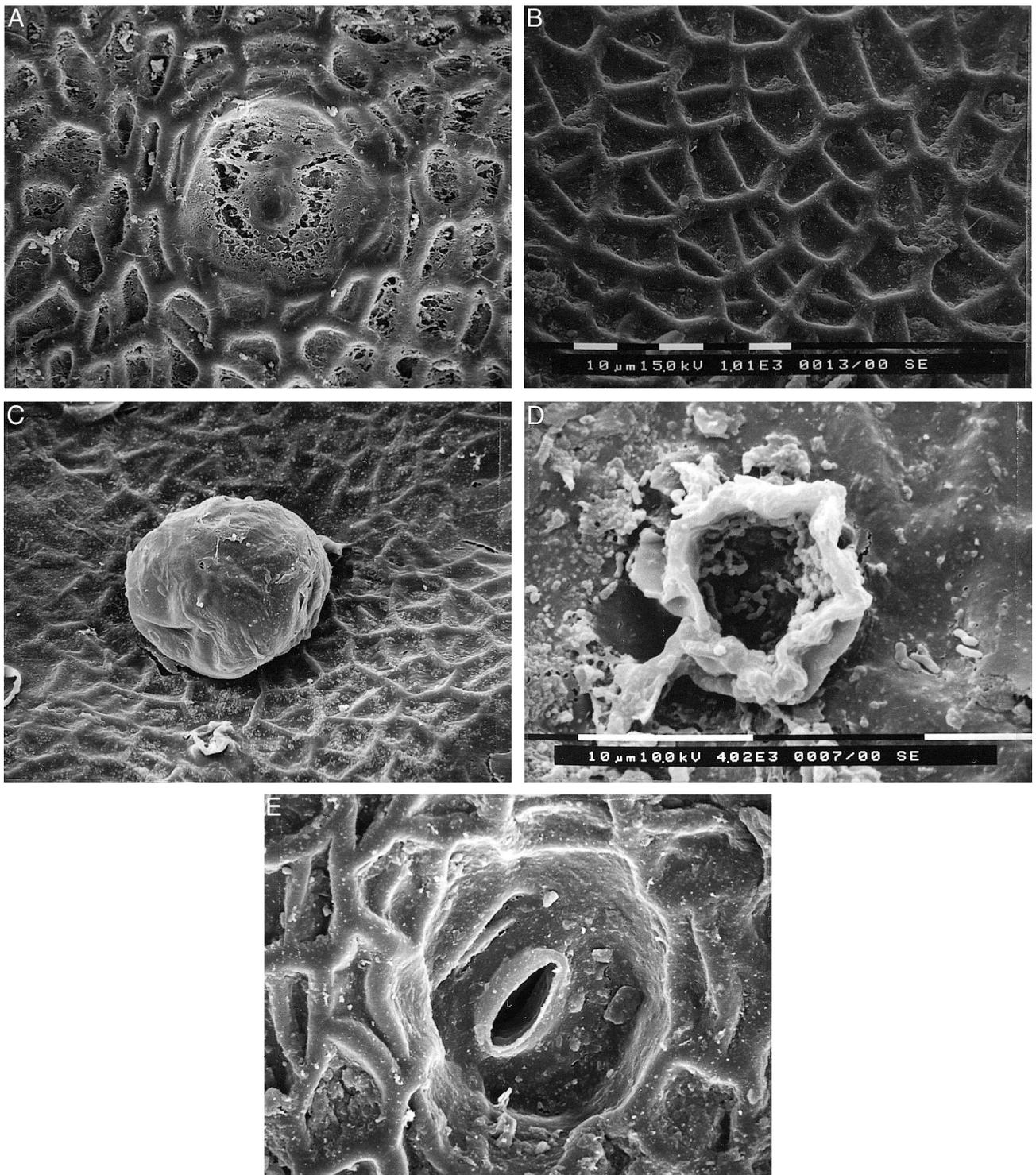
FIGURE 3. *Surfaces of pickling cucumbers. (A) Calypso variety with wax. (B) Calypso variety without wax. (C) Calypso variety with trichome intact. (D) Calypso variety with trichome partially broken. (E) Calypso variety with stomate open. Bar = 10 μm in each of the scanning micrographs. Magnification of pictures without dimensional bars (A, C, and E), ×2,400.*

2 indicate a statistically significant difference ($P < 0.05$) between the number of bacteria adsorbed to the waxed fruit and the number adsorbed to the dewaxed fruit. However, with regard to adsorption to dewaxed fruit, only *L. plantarum* was significantly different ($P < 0.05$) among the four species tested. From these results, we can conclude that bacteria will be adsorbed to the surfaces of either waxed or

dewaxed cucumbers, as long as there is enough time to allow surface interactions between bacteria and the cucumber surface. Once bacteria are attached to the surface, it is very difficult to remove them, as demonstrated by Ukuku and Fett *(31)*. Depending on the storage temperature of the fruit, bacteria will grow or will form extracellular polymers, as was the case for *Pseudomonas* attached to meat surfaces at 20°C
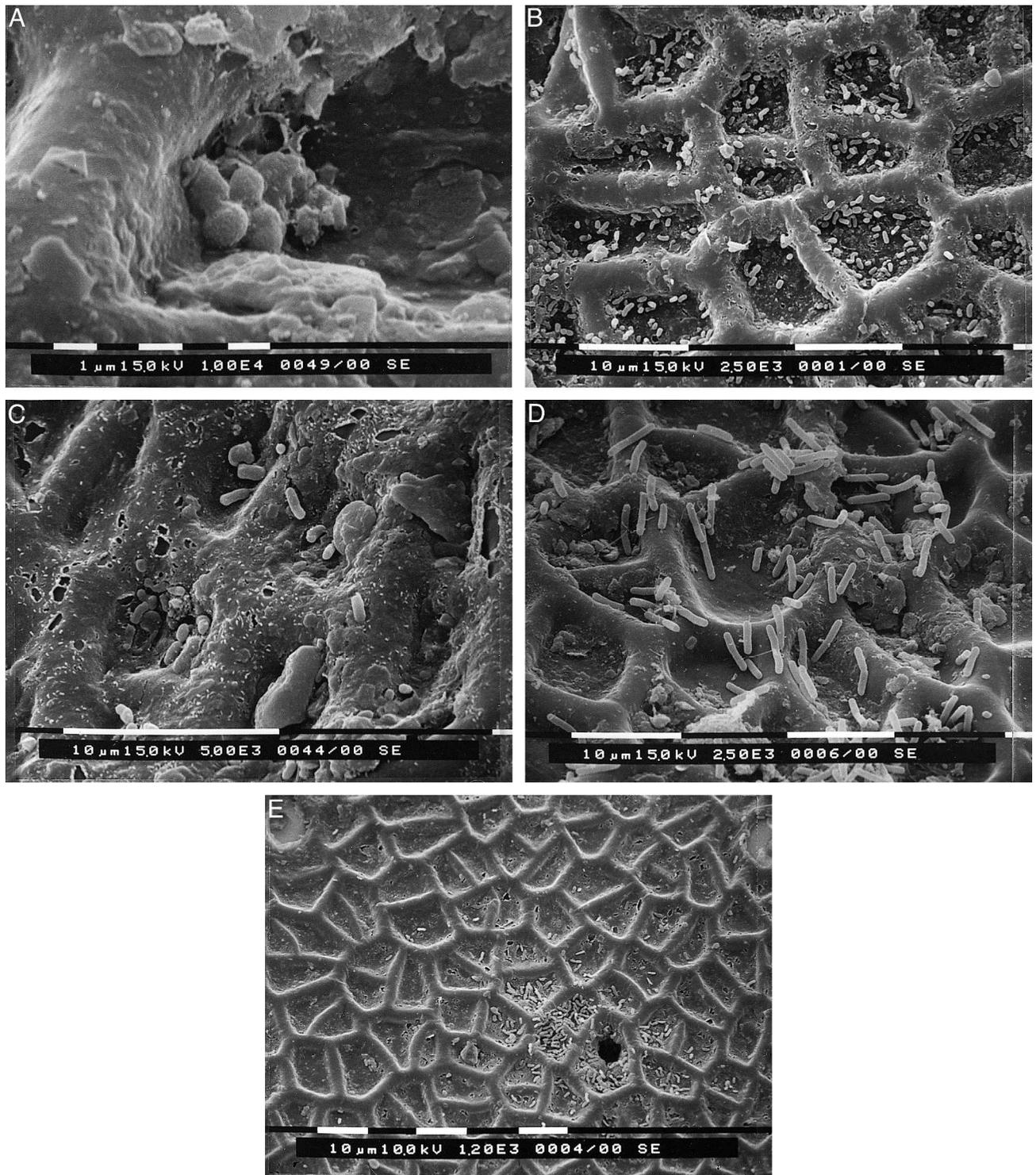
FIGURE 4. *Adhesion of bacteria to the surfaces of pickling cucumbers (Calypso variety) with wax. (A)* S. aureus. *(B)* L. plantarum. *(C)* L. monocytogenes. *(D)* Salmonella *Typhimurium. (E)* E. aerogenes *ATCC 13048. Bar = 10* μm.

(14). Bacteria are more easily removed when they are not allowed to adsorb to the surface via extracellular polymers. The removal of attached bacteria from the surfaces of cantaloupes upon washing after 12 days of storage at refrigeration temperatures was more difficult *(31).*

Figure 3 shows the surfaces of fresh cucumbers and illustrates features that may be involved in the adhesion and physical entrapment of microorganisms. The presence (Fig.

3A) or absence (Fig. 3B) of wax may influence the types of microorganisms that adhere to the surface. Trichomes (Fig. 3C and 3D) and stomata (Fig. 3E) can serve to physically entrap microorganisms.

SEMs for the attachment of different bacteria are shown in Figure 4. Bacteria were adsorbed to the surface of the cucumber whether or not the fruit was treated with hexane (Fig. 4). Clusters of bacteria were observed with

TABLE 2. *Effects of epicuticular wax on the adhesion of bacteria to the surfaces of cucumbers in water at 20°C for 60 min*[a]

Bacterial species	RA for fruit with wax	RA for dewaxed fruit
Listeria monocytogenes	0.3 (0.3) B	1.85 (1.7) B
Lactobacillus plantarum[b]	0.2 (0.01) B	0.02 (0.01) C
Staphylococcus aureus	13.6 (3.2) A	1.4 (0.03) B
Salmonella Typhimurium	14.6 (3.1) A	1.0 (0.8) B

[a] Relative adhesion (RA) = (CFU/g of fruit/CFU/ml of inoculum) × 100. Values are the means for two replicates. Standard deviations are shown in parentheses. Means with different letters are significantly different ($P \leq 0.05$) by Tukey's multiple-range test.

[b] LA219.

SEM (Fig. 4A, 4D, and 4E). Figure 4B and 4C shows evenly distributed bacteria. This clustering of bacteria could be due to the adhesion of bacteria to cucumber surfaces, as well as interbacterial adhesion *(19)*. When a cell is adsorbed to a surface, other parts of the cell are free to adhere to other surfaces, which may explain why cells tend to appear in clusters under SEM. Figure 4E shows bacteria clustering around a hole of unknown origin (possibly caused by an insect sting or another type of injury). This hole might have allowed the leakage of nutrients from the fruit, thus attracting bacteria. In one study, cells of *Escherichia coli* O157:H7 were found to attach preferentially to cut edges of lettuce over the intact leaf surface *(30)*. In another study, when tomatoes were inoculated with bacteria, the largest numbers of bacteria were recovered from surface scars, blossom scars, and stem scars *(21)*. Annous et al. *(1)* found that *E. coli* attached mainly to the calyx and stem scars of artificially contaminated Golden Delicious apples.

The adhesion of bacteria to cucumber surfaces was found to depend on temperature, species, characteristics of the cucumber surface, and time of exposure. Bacterial adhesion to meat surfaces has been found to be a time-dependent process, and once attached, bacteria have been found to be difficult to remove *(14, 24)*. Our results indicate that low water temperatures and short periods of contact with the fruit will reduce the potential for bacterial adhesion during the postharvest washing and hydrocooling of cucumbers. In addition, treatment of the water with chemicals to keep the microbial load low is fundamental to the avoidance of contamination of the produce.

ACKNOWLEDGMENT

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