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Native Microflora in Fresh-Cut-Produce Processing Plants and Their Potentials for Biofilm Formation

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ABSTRACT

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Representative food contact and non–food contact surfaces in two mid-sized, fresh-cut processing facilities were sampled for microbiological analyses after routine daily sanitization. Mesophilic and psychrotrophic bacteria on the sampled surfaces were isolated by plating on nonselective bacterial media. Alternatively, bacteria were isolated after an incubation period that allowed the formation of heterogeneous biofilms on stainless steel beads. Of over 1,000 tested isolates, most were capable of forming biofilms, with approximately 30% being strong or moderate biofilm formers. Selected isolates (117) were subjected to species identification by using the Biolog Gen III microbial identification system. They distributed among 23 genera, which included soil bacteria, plant-related bacteria, coliforms, and opportunistic plant- or human-pathogenic bacteria. The most commonly identified bacteria species were *Pseudomonas fluorescens, Rahnella aquatilis,* and *Ralstonia insidiosa*. The high prevalence of *R. insidiosa*, a strong biofilm former, and *P. fluorescens*, a moderate biofilm former, suggests that they were established residents in the sampled plants. These results suggest that native microflora capable of forming biofilms are widely distributed in fresh-produce processing environments.

With a lack of practical kill steps, fresh-cut-produce processors continue to rely on sanitizing washes to maintain the microbiological quality of fresh-cut products and to prevent potential cross-contamination by foodborne pathogens. Rigorous sanitization of the processing environment and equipment is also crucial for preventing transference of pathogenic microorganisms to processed produce *(8, 9)*. Microbial communities in fresh-produce processing facilities comprise diverse microbial species. They can include bacteria proficient at forming biofilms, which can potentially protect food spoilage and pathogenic bacteria and allow them to survive in processing plants *(3, 8)*. Under certain conditions, microorganisms in biofilms can detach and disperse, leading to potential contamination of food products *(16, 20)*.

Several studies were conducted to examine biofilm formation and its association with the contamination of a variety of foods. It was documented that food contaminations because of contact with biofilms can occur in dairy, meat, and fruit-juice processing plants *(17, 18)*. In dairy and meat processing lines, biofilms are often found at locations that are less accessible for routine sanitization *(6)*. These biofilms can increase the likelihood of the presence of pathogenic bacteria in the plant *(6)*. It was suggested as early as 1998 that foodborne outbreaks associated with fresh-cut produce might be related to the existence of biofilms in fresh-cut-produce processing facilities *(4)*. However, no direct evidence of such a link has been established.

The temperature in fresh-cut-produce processing plants must be maintained at 4°C or colder to be in compliance with U.S. Food and Drug Administration regulations *(23)*. In reality, the temperature can fluctuate in the processing environment, and temperature occasionally rises for other reasons. Microenvironments with elevated temperature can also exist in the processing plants. These environments, combined with high humidity, are vulnerable to the establishment of biofilms by native microflora that survive routine sanitization. Such microbial communities can include epiphytic, soil-associated, human-pathogenic, and other bacteria from various environmental sources *(9, 10)*. Some of these bacteria were shown capable of forming biofilms on produce surfaces in laboratory experiments *(19)*, suggesting that they could play a role in forming biofilms on processing surfaces in the plant. Resident strains from various surfaces in a raw-vegetable processing facility were shown to have a higher biofilm-forming capacity than a laboratory *Escherichia coli* strain *(24)*. A large variation in biofilm-forming capacity among the isolates was observed in this study; however, the composition of the bacterial community was not fully determined by species identification *(24)*.

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To better understand the potential of biofilm formation by native microflora in fresh-cut-produce processing plants, samples were obtained from multiple locations in two local facilities. All samplings were conducted after routine daily sanitization. Biofilm-formation potentials of bacteria isolated from the samples were evaluated.

MATERIALS AND METHODS

Sampling at fresh-cut-produce processing plants. Two medium-sized, local fresh-cut-produce processing plants that processed a variety of fresh produce including leafy greens and fruits were sampled for native microflora at multiple food contact and noncontact surfaces. Samples were taken from corresponding locations in both plants during plant visits in the fall of 2010. All samples were taken after routine daily cleaning and disinfection of the facilities, and before the start of the morning shift. An area of 100 cm^2 of the selected surface was defined with a sterile template and sampled by vigorously rubbing five times with a sterile sponge (Whirl-Pak, Nasco, Fort Atkinson, WI) hydrated with 15 ml of sterile phosphate-buffered saline (PBS; pH 7.2; Fisher Scientific, Pittsburg, PA). For surfaces with nonflat contours, the sampled areas were approximated. To neutralize the residual chlorine from facilities surfaces after sanitization, PBS was supplemented with a chlorine-neutralizing reagent, following the manufacturer's instructions (PermaChem, Hach, Düsseldorf, Germany). The materials of sampled surfaces included acrylic-modified cement flooring, high-density polyethylene, nylon, polyvinyl chloride, and stainless steel (SS). Sample sponges were placed in stomacher bags (Whirl-Pak) and transported to the laboratory on ice. Samples were processed within 5 h.

Recovery of bacterial strains. Sample sponges were pummeled for 2 min in a stomacher (Lab Blender, Seward Medical, Ltd., Sussex, UK) and the liquid squeezed out by hand. Each sample was 10-fold serial diluted and then spiral plated on two Tryptic soy agar plates (TSA: BD, Franklin Lakes, NJ), one MacConkey plate (MAC; Neogen, Lansing, MI), and one B-medium *(13)* plate. MAC plates and one set of TSA plates were incubated at 30°C for 2 days. B-medium plates and the other set of TSA plates were incubated at 10°C for 10 days. TSA plates incubated at 30°C were used to determine mesophilic bacteria counts, and MAC plates were used for the recovery of gram-negative bacteria. The population of psychrotrophic microflora was determined with TSA and B-medium plates incubated at 10°C.

Aliquots of all samples collected from processing plants were also incubated in low-nutrient liquid medium with the presence of SS beads to allow formation of heterogeneous biofilms. A 100-μl aliquot of the sample was inoculated into 10 ml of broth supplemented M9 medium (M9 salts supplemented with 0.5% glucose; BD) and 5% Lauria–Bertani broth (BD) *(5)* in a 50-ml tube containing four type 316 SS beads (diameter of 6.3 mm; BioSurface Technology Corp., Bozeman, MT) and incubated at 10°C for 10 days, with moderate shaking. After incubation, the SS beads were rinsed with sterile water to remove unattached and weakly attached bacterial cells. Then the beads were immerged in 10 ml of sterile PBS and vigorously vortexed for 1 min. Recovered bacterial cells were spiral plated, incubated, and enumerated with the same procedures described above.

Screening isolates for biofilm-formation capacity. Single bacterial colonies with distinct morphology (up to three colonies for each type) on the recovery plates were inoculated into 96-well microtiter plates containing 200 μl of a 1:10 dilution of Tryptic soy broth (TSB; BD) in water and simultaneously streaked onto TSA plates. Inoculated microtiter plates were incubated at 30°C for 24 h to examine the biofilm-forming potential of each colony by using a crystal violet staining method described by Stepanović *(21)*. Total biomass formed by each colony was estimated based on an optical density at 590 nm (OD_{590}) measurements, as described by Stepanović *(21)*. Colonies with OD values that exceeded a threshold (OD_C), calculated as the sum of the average OD value for the negative controls plus three times the standard deviation, were considered biofilm formers. Specifically, the potential of biofilm formation for each isolate was categorized as strong ($OD_{590} > 4\ OD_C$), moderate ($2\ OD_C \leq OD_{590} < 4\ OD_C$), weak ($OD_C \leq OD_{590} < 2\ OD_C$), or non–biofilm former ($OD_{590} < OD_C$), based on the total biomass measured as optical density. For each sample, isolates with similar colony morphology and biofilm-formation potentials were considered identical, and one representative was further streaked onto TSA plates for single colonies. All purified isolates were stored at −80°C.

Species identification and verification of biofilm-forming capacity of representative isolates. Purified isolates were subjected to species identification by using the Biolog Gen III microbial identification system (Biolog, Farmingdale, NY), following the manufacturer's instructions. For all identified isolates of a certain species, one isolate was selected to further evaluate biofilm-forming capacity. Each selected isolate was incubated overnight to reach stationary phase in TSB at 30°C, with 150 rpm/min of orbital shaking. The cell cultures were centrifuged at 4,000 × *g* at 10°C for 5 min and the resulting pellet washed three times with sterile PBS. Pellets were resuspended in sterile PBS, and cell density was adjusted to approximately 1.0 OD_{600} unit, which roughly equals 9.0 log CFU/ml. The resuspended cell cultures were 10-fold serially diluted in a 1:10 dilution of TSB in water to achieve a targeted inoculum concentration of 3.0 log CFU/ml. Two hundred microliters of each inoculum was added to each well on a 96-well microtiter plates with six replicates. The plates were incubated at 30°C, with 80 rpm/min of orbital shaking for 24 h to evaluate biofilm formation. The measurement of total biomass was carried out by crystal violet staining method, as described previously.

RESULTS

Recovery of mesophilic and psychrotrophic bacteria from fresh-cut-produce processing plants. In the present study, a variety of food contact and non–food contact surfaces in two local fresh-cut-produce processing plants were sampled after routine daily sanitization, and the microflora were characterized. Viable bacterial cells were not detected or detected at very low levels on most food contact surfaces. Bacterial populations on the carrot and potato peeler brushes, containers used for washing, and the blades of cutting knives were approximately 2.0 log CFU/cm^2. In contrast, higher bacterial counts (up to 5.8 log CFU/cm^2) were obtained from most non–food contact surfaces including the conveyor frames, knife handles, and the floor (Table 1). In an attempt to increase the recovery of bacteria with strong potential of biofilm-forming capabilities, samples from the processing plants were also incubated with SS beads in a low-nutrient broth. It was hypothesized that bacterial cells with stronger biofilm-forming potentials would be more likely to attach to this solid substrate and form biofilms. After incubation, the SS beads were rinsed,

TABLE 1. *Aerobic plate counts of native microflora from local fresh-cut-produce processing plants by direct spiral plating on TSA, B-medium, and MAC plates*[a]

		Temp (°C)[c]			
		10		30	
Surface	Sample location[b]	TSA plate	B-medium plate	TSA plate	MAC plate
Food contact	Peeler brush (NL)	1.93	2.13	2.31	1.52
	Container (HDPE)	ND[d]	1.86	ND	ND
	Conveyor belt (HDPE)	ND	ND	0.51	ND
	Cutting board (HDPE)	ND	ND	ND	ND
	Knife blade (SS)	1.80	2.05	2.07	ND
	Slicer (SS)	ND	ND	ND	ND
Non–food contact	Conveyor belt (HDPE)	ND	ND	ND	ND
	Conveyor frames (SS)	4.21	3.51	2.73	ND
	Knife handle (PVC)	1.35	1.53	2.16	1.80
	Floor (AMCF)	5.83	5.39	5.11	3.93
	Stainless steel table (SS)	ND	ND	ND	ND

[a] Data are expressed in log CFU per centimeter squared and represent the averages of the two sampled local plants.
[b] Letters in parentheses indicate the material of the surface the sample was taken: NL, nylon; HDPE, high-density polyethylene; SS, stainless steel; PVC, polyvinyl chloride; AMCF, acrylic-modified cement floor.
[c] Incubation temperature used for spiral plated samples.
[d] ND, not detected at a detection limit of 1.0 log CFU/cm^2.

and bacterial cells attached to the beads were then released and plated on nonselective bacterial media and incubated at 10 or 30°C (Table 2). Bacterial cells were recovered from the SS beads for samples collected from multiple locations including both food contact and non–food contact surfaces. The diverse colony morphologies suggested the formation of heterogeneous biofilms on the SS bead during the incubation period at 10°C.

Screening for the potentials of biofilm formation. Selected single colonies obtained by directly plating samples from the processing plants (Table 1) and by plating bacterial cells bound to SS beads after incubation (Table 2) were tested for their biofilm-formation potentials by using crystal violet staining *(21)*. Each isolate was categorized as non–, weak, moderate, or strong biofilm formers, based on the dye retention in comparison with that of the non–biofilm-forming controls. Figure 1 shows the distribution of the isolates across the four categories. A three-way chi-square test (SPSS 17.0, IBM, Armonk, NY) was used to assess the recovery method and sampling surface on the distribution of the isolates relative to their ability of biofilm

TABLE 2. *Aerobic plate counts for samples from biofilms formed on SS beads at 10°C by spiral plating on TSA, B-medium, and MAC plates*[a]

		Temp (°C)[c]		Surface	
Surface	Sample location[b]	10	*B-medium*	*TSA*	10
Food contact	Peeler brush (NL)	5.62[d]	5.77	5.85	5.53
	Container (HDPE)	4.62	3.57	3.95	4.24
	Conveyor belt (HDPE)	ND[d]	ND	ND	ND
	Cutting broad (HDPE)	ND	ND	ND	ND
	Knife blade (SS)	5.42	5.51	4.92	4.92
	Slicer (SS)	ND	ND	ND	ND
Non–food contact	Conveyor belt (HDPE)	4.04	4.41	ND	ND
	Conveyor frames (SS)	5.06	5.24	4.88	4.84
	Knife handle (PVC)	4.80	4.74	3.50	3.20
	Floor (AMCF)	3.99	3.36	3.48	2.64
	Stainless steel table (SS)	3.91	3.33	ND	ND

[a] Raw samples from all locations in Table 1 were tested, and the results (data are expressed in log CFU per centimeter squared) were the averages of the two local plants.
[b] Letters in parentheses indicate the material of the surface the sample was taken: NL, nylon; HDPE, high-density polyethylene; SS, stainless steel; PVC, polyvinyl chloride; AMCF, acrylic-modified cement floor.
[c] Recovery temperature used for recovery of samples released from SS beads.
[d] All numbers denote the number of colonies (in log CFU per centimeter squared) isolated from SS beads after enrichment. The numbers can be an indication of biofilm formation on SS beads but do not correlate with the numbers of bacterial in the original samples.
[e] ND, not detected at a detection limit of 1.0 log CFU/cm^2.

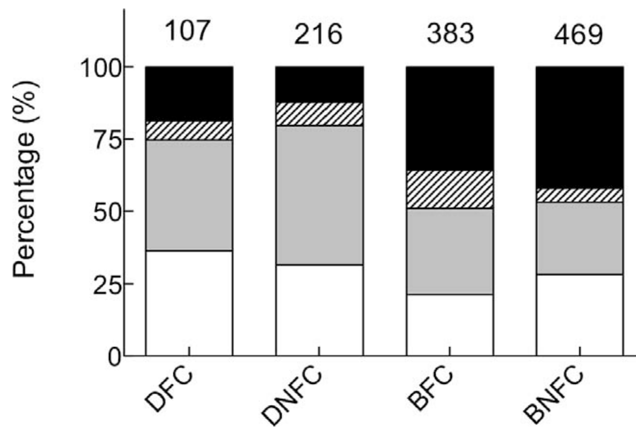

FIGURE 1. *Distribution of bacterial isolates relative to ability of biofilm formation in microplates. Stacked bars represent the percentage of the isolates in each category.* □, *non-biofilm formation;* ■, *weak biofilm formation;* ▨, *moderate biofilm formation; and* ■, *strong biofilm formation. Numbers over the stacked bars indicates total isolates tested. Letters under the stacked bars indicate method of recovery and the type of surfaces for the isolates. DFC, direct plating from food contact surfaces; DNFC, direct plating from non–food contact surfaces; BFC, SS beads from food contact surfaces; BNFC, SS beads from non–food contact surfaces.*

formation on microplates. The methods used to recover the isolates significantly ($P \leq 0.05$) affected this distribution. The distribution notably shifted toward stronger biofilm formation when the isolates where recovered from the SS beads after an incubation period of 10 days at 10°C. When the same methods for recovery were used, the difference in this distribution was statistically significant ($P \leq 0.05$) for the isolates recovered from food contact and non–food contact surfaces. However, the difference relative to the surfaces type on the distribution seemed less pronounced than that of the methods used for obtaining the isolates.

Species identification. Representative isolates from different type of samples and with different colony morphology were selected for species identification by using the Biolog Gen III microbial identification system. The 117 selected isolates were identified as belonging to 23 genera, with 105 belonging to 28 species, and 12 belonging to *Enterobacter, Pseudomonas, Arthrobacter, Mycobacterium,* and *Curtobacterium* genera, without species identification (Table 3). Gram-negative species were abundantly represented among isolates from various surfaces. *P. fluorescens*, *R. aquatilis,* and *R. insidiosa* were the most abundant among the isolated strains, represented by 15, 17, and 14, respectively, among the 117 isolates identified to 28 bacterial species. These three species were isolated from multiple food contact and non–food contact surfaces by using either direct plating or plating after biofilm formation on SS beads.

Evaluation of biofilm-forming capacities of selected isolates. Among the strains tested for biofilm-forming potential, *Burkholderia caryophylli, Klebsiella pneumoniae,* and *Ralstonia insidiosa* strains exhibited strong capacity of biofilm formation on microtiter plates, as judged by crystal violet staining of attached biomass. *Flavimonas oryzihabitans, Flavobacterium resinovorum, Pseudomonas fluorescens, Sphingomonas terrae, Stenotrophomonas rhizophila,* and *Raoultella planticola* displayed moderate biofilm formation. All other tested strains displayed low or no biofilm formation on the microtiter plates.

DISCUSSION

Microflora in fresh-cut-produce processing plant. In the present study, two local fresh-cut-produce processing plants were sampled after routine daily sanitization, and the native microflora were characterized. Although data from fresh-cut-produce processing plants are limited, previous studies examined the microflora in dairy, meat, and fish facilities *(1, 2, 14).* The aerobic bacterial counts in those studies reached up to 10^4 to 10^5 CFU/cm^2 on sampled surfaces after cleaning and disinfection of the plants. The aerobic bacteria counts in the two fresh-cut-produce processing plants sampled in this study ranged from undetectable to around 10^5 CFU/cm^2 after daily sanitization. In addition to the use of routine sanitization, low operating temperatures are used to inhibit bacteria growth in fresh-cut-produce processing plants, and in meat and dairy plants. The bacterial density determined in this study was in the same range as the microflora in meat processing lines. Kaneko and colleagues reported that the interior surfaces of washing, slicing, dewatering and blending equipments, slicers, and floor surfaces remained high, with aerobic plate counts around 10^5 CFU/cm^2, even after cleaning and disinfection in some environments in which ready-to-eat fresh vegetables are processed *(12).* In contrast, in this study, only floors were found with counts around 10^5 CFU/cm^2 after cleaning and disinfection, indicating the microflora in the fresh-cut-produce processing plants can differ, presumably depending on the practices in the facilities. In the two produce processing plants studied, bacterial counts on several food contact surfaces were below the detection limit. These locations included the conveyor belts, cutting boards, and slicers. Similar results for cutting boards were also found in Kaneko et al.'s study. Among all sampled locations, no particular material was found to significantly differ from the others in terms of remaining bacteria density after sanitization. A similar conclusion was reached in a study by Marouani-Gadri in a beef-processing plant *(14).*

To our knowledge, few studies have characterized the native microflora in fresh-cut-produce processing plants. Van Houdt and colleagues investigated the quorum-sensing-molecule production of isolates collected from the native microflora in vegetable processing plants. However, only a limited number of isolates were subjected to species identification, which included *Vibrio diazotrophicus, Serratia plymuthica,* and *Panthoea agglomerans (24).* Kaneko and colleagues examined the bacterial composition of the microflora in two plants processing ready-to-eat fresh vegetables and reported the presence of *E. coli* and *B. cereus (12).* In this study, we isolated bacterial strains belonging to 28 species including soil bacteria, plant-related

TABLE 3. *Species identification and biofilm-formation potentials of bacterial isolates collected from two local fresh-cut-produce processing plants*

Species	Potential source(s)[a]	Surface type(s)[b]	Surface material(s)[c]	Biofilm formation[d]	Isolates Number
Acinetobacter genospecies	Soil	F	NL	+	3
Arthrobacter spp.	Plant*	NF	PVC	0	2
Bacillus pumilus	Rhizosphere	F, NF	HDPE, PVC	+	7
Brevibacterium frigoritolerans	Soil	F	HDPE	0	1
Burkholderia caryophylli	Plant*	NF	AMCF	+++	1
B cepacia	Animal*	NF	AMCF	++	1
Chryseobacterium indoltheticum	Plant	F	NL	+	2
Corynebacterium bovis	Plant*	F	NL	0	1
Curtobacterium spp.	Plant	F, NF	HDPE, AMCF	0	3
Enterobacter cloacae	Animal, soil, plant	NF	AMCF, NL, SS	0	6
E. kobei	Animal, , soil, plant	NF	AMCF	+	1
Enterobacter spp.	Animal, soil, plant	F	NL	+	1
Flavimonas oryzihabitans	Animal*	F	HDPE	++	1
Flavobacterium resinovorum	Soil, water, plant	NF	AMCF	++	1
Klebsiella pneumoniae	Animal, plant	F	NL	+++	3
Microbacterium spp. (CDC.A-5)	Soil	F	NL	0	1
Paenibacillus amylolyticus	Water, rhizosphere	F, NF	HDPE, PVC, SS	0	3
P. pabuli	Water, rhizosphere	F, NF	PVC	0	4
Pectobacterium cypripedii	Plant*	F	SS	0	1
Pseudomonas fluorescens	Plant	F, NF	HDPE, NL, PVC, SS	++	15
P. maculicola	Plant*	F	NL	+	1
P. marginalis	Soil, plant	F	NL	+	1
P. tolaasii	Plant	F	NL	+	1
P. spp.	Plant	F	NL	+	5
Rahnella aquatilis	Rhizosphere, water	F, NF	NL, SS	+	17
Ralstonia insidiosa	Animal	F, NF	HDPE, PVC, SS	+++	14
Raoultella planticola	Animal*	NF	AMCF, SS	++	4
Rhizobium radiobacter	Plant*	F, NF	PVC, SS	+	4
Staphylococcus epidermidis	Animal	F, NF	PVC, SS	+	2
S. kloosii	Animal	NF	PVC	+	2
S. xylosus	Animal	F, NF	SS, PVC	+	4
Sphingomonas terrae	Soil, plant	NF	SS	++	1
Stenotrophomonas rhizophila	Plant	F	NL	++	3

[a] Environment(s) where the bacterial species is most frequently isolated, according to the literature. *, plant or human pathogen, including opportunistic pathogen.
[b] F, food contact surface; NF, non–food contact surface.
[c] Letters in parentheses indicate the material of the surface the sample was taken: NL, nylon; HDPE, high-density polyethylene; SS, stainless steel; PVC, polyvinyl chloride; AMCF, acrylic-modified cement floor.
[d] Biofilm-formation capacity of one representative isolate. +++, ++, +, and 0 represent strong, moderate, weak, and non-biofilm formers, respectively.

bacteria, coliforms, and opportunistic plant or human-pathogenic bacteria. There is considerable overlap in the microbial communities identified in this study compared with findings from studies of meat and dairy processing plants *(1, 7, 14, 15)*. *Pseudomonas* was frequently isolated in both fresh produce and meat and dairy processing environments. Other genera including *Staphylococcus, Bacillus, Corynebacterium, Enterobacter, Flavobacterium,* and *Klebsiella* were commonly present in agricultural product processing facilities. However, several plant- and soil-related bacteria not found in other facilities, including *Rhanella*, *Ralstonia,* and *Rhizobium,* were isolated with high frequencies from produce processing environments.

Raw-produce sources, worker hygiene practices, sanitization practices, and environmental factors all can affect the composition of microflora in particular plants and even areas within plants *(18)*. Therefore, the microbial community composition is highly dynamic. While the presence of many of the species in the plants could be transient, it is likely that some species are adapted to the processing plant environment and have become part of the true native microflora.

Biofilms in fresh-cut-produce processing plant. The presence of biofilms in food processing facilities could be related to foodborne outbreaks *(11)*. Once a biofilm is formed, it can enhance the resistance of microorganisms by creating a protective structure around bacteria, which in turn reduces the efficacy of cleaning and disinfection operations *(8)*. Greater survival rate of *E. coli* O157:H7 was observed in mixed culture biofilms compared with a monoculture biofilm, when biofilms were treated with 5% H_2O_2 *(22)*.

Considering the potential for pathogens to transfer from biofilms to processed food surfaces by direct contact or as a result of physical forces applied during food production *(18),* it is critical to control the presence of biofilms in fresh-cut processing facilities. In a previous study, only two species with relatively strong biofilm-forming capacities, *S. plymuthica* and *P. agglomerans,* were isolated from a raw-vegetable processing line *(24).* In this study, we isolated multiple bacterial species with strong biofilm-forming potentials (Table 3). Several strong and moderate biofilm-forming bacterial species were recovered after allowing biofilm formation on SS beads at 10°C, suggesting good possibility of biofilm formation in produce processing plants by the native microflora at low temperatures.

In particular, the frequent isolation of *P. fluorescens* and *R. insidiosa* from various surfaces suggested they are established residents in the sampled plants. Examination of interactions of these bacteria with foodborne bacterial pathogens could provide insights on the survival of the bacterial pathogens in food processing environments.

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