Attachment of and Biofilm Formation by *Enterobacter sakazakii* on Stainless Steel and Enteral Feeding Tubes

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Enterobacter sakazakii has been reported to form biofilms, but environmental conditions affecting attachment to and biofilm formation on abiotic surfaces have not been described. We did a study to determine the effects of temperature and nutrient availability on attachment and biofilm formation by *E. sakazakii* on stainless steel and enteral feeding tubes. Five strains grown to stationary phase in tryptic soy broth (TSB), infant formula broth (IFB), or lettuce juice broth (LJB) at 12 and 25°C were examined for the extent to which they attach to these materials. Higher populations attached at 25°C than at 12°C. Stainless steel coupons and enteral feeding tubes were immersed for 24 h at 4°C in phosphate-buffered saline suspensions (7 log CFU/ml) to facilitate the attachment of 5.33 to 5.51 and 5.03 to 5.12 log CFU/cm^2, respectively, before they were immersed in TSB, IFB, or LJB, followed by incubation at 12 or 25°C for up to 10 days. Biofilms were not produced at 12°C. The number of cells of test strains increased by 1.42 to 1.67 log CFU/cm^2 and 1.16 to 1.31 log CFU/cm^2 in biofilms formed on stainless steel and feeding tubes, respectively, immersed in IFB at 25°C; biofilms were not formed on TSB and LJB at 25°C, indicating that nutrient availability plays a major role in processes leading to biofilm formation on the surfaces of these inert materials. These observations emphasize the importance of temperature control in reconstituted infant formula preparation and storage areas in preventing attachment and biofilm formation by *E. sakazakii*.

Enterobacter sakazakii is a food-borne pathogen capable of causing meningitis (4, 13, 38), sepsis (38), bacteremia (40), and necrotizing enterocolitis (55) in preterm neonates and immunocompromised adults (9, 15, 25, 30, 45). Its genetic and phenotypic diversity has been demonstrated (24, 33), and certain clusters may substantially differ in thermal tolerance (8, 58). Powdered infant formula and milk powder have been implicated as vehicles in outbreaks of *E. sakazakii* infections (3, 18, 37–39, 51, 55). *E. sakazakii* was detected in 20 of 141 (14.2%) powdered infant formulas originating from 13 countries (37). The pathogen has been isolated from various clinical sources, food processing plants, the environment (22, 27), lettuce (52), alfalfa sprouts (5), tomatoes (26), and other vegetables, cheese, minced beef, and sausage (31). Its presence in fresh produce raises the possibility of this food group serving as a vehicle of the pathogen for infections in immunocompromised adults, particularly patients in hospitals and elderly adult assisted-care facilities.

Attachment of bacterial cells to surfaces may be followed by growth, the production of exopolysaccharide, and biofilm formation (29). Biofilms have been defined as sessile communities of cells attached to a surface or to each other, usually embedded in polymeric substances produced by the bacteria (34). The attachment of microorganisms to biotic or abiotic surfaces, followed by biofilm formation, is known to enhance the resistance of cells to environmental stresses and to provide protection against sanitizers (12, 29, 41, 48). Factors affecting attachment and biofilm formation by microorganisms include nutrient availability, the pH of the surrounding medium, and the nature of the cell and abiotic surfaces (11).

E. sakazakii has been reported to be able to attach to and form biofilms on silicon, latex, polycarbonate, stainless steel, glass, and polyvinyl chloride (PVC) (23, 32). Foods such as powdered infant formula and fresh produce represent potential vehicles of *E. sakazakii* infections in infants and immunocompromised adults, respectively. Contact of these and other foods containing the pathogen with abiotic or biotic surfaces could result in attachment and biofilm formation. Attachment or biofilms of *E. sakazakii* on equipment surfaces used in formula preparation and feeding areas or in produce processing plants may increase the risk of infections to infants and immunocompromised adults, respectively. *E. sakazakii* colonization on the surfaces of a spoon, brush, and blender used for infant formula preparation has been documented to occur in a clinical setting where neonatal infections were reported (1, 38, 40, 51). Reuse of enteral feeding tubes and delivery bags after washing may increase the risk of microbial infections (42). Biofilm formation by pathogens on fresh fruits and vegetables during harvesting, transporting, processing, and storage has been conjectured to potentially increase the risk of food-borne diseases in individuals consuming these products (2). We have observed that *E. sakazakii* is able to grow on fresh-cut produce and in produce juice (28).

Removal or inactivation of pathogens on inert surfaces in infant formula preparation areas and produce processing environments by washing with water or treatment with disinfectants or sanitizers is not always achieved, possibly because cells

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are enmeshed in biofilms or otherwise protected against exposure to antimicrobials. Attachment and biofilm formation by *E. sakazakii* as affected by temperature and nutrient availability have been given only meager research attention. The objectives of the present study were to determine the growth characteristics of *E. sakazakii* in a rich microbiological medium, reconstituted infant formula, and lettuce juice as a produce model and characterize subsequent attachment of stationary-phase cells and biofilm formation on the surfaces of stainless steel and enteral feeding tubes as affected by temperature and nutrients provided by these media.

MATERIALS AND METHODS

Strains examined. Five clinical strains (2855, 3231, 3234, 3290, and 3295), four strains (2871, 3270, 3437, and 3439) isolated from foods, and one strain (3396) isolated from an environmental source were examined for colony morphology on tryptic soy agar (TSA; BBL/Difco, Sparks, Md.), infant formula agar (IFA), and lettuce juice agar (LJA) (see the discussion of the preparation of media below). Cultures grown into 10 ml of tryptic soy broth (TSB; BBL/Difco) at 37°C for 24 h were streaked on TSA, IFA, and LJA and incubated at 12, 25, and 37°C for 6, 2, and 1 days, respectively. Three strains (3231, 3234, and 3396) producing colonies distinctly wet and mucoid in appearance and three strains (2855, 3295, and 3439) producing drier nonmucoid colonies were selected for determining growth curves in TSB, infant formula broth (IFB), and lettuce juice broth (LJB). Strains 3231, 3234, 3295, 3439, and 3396 were used for the attachment study; because strain 2855 showed a very different growth pattern compared to the other five strains, it was not included. For the biofilm study, *E. sakazakii* strain 3231, which produces mucoid colonies, and strain 3439, which produces nonmucoid colonies on IFA, were used to determined whether there are significantly different patterns of attachment or biofilm formation based, in part, on these characteristics.

Preparation of media. Similac Neosure Advance infant formula (Ross Pediatrics, Abbott Laboratories, Columbus, Ohio) was selected to make IFB because preliminary studies showed that the development of mucoid colonies by some of the test strains was more evident on IFA prepared using this formula than on IFA prepared using four other formulas. Milk-based infant formulas such as Similac Neosure Advance are consumed more widely than are soy-based formulas, giving additional incentive for its use in attachment and biofilm studies. IFB (pH 6.6) was made by combining Similac Neosure Advance infant formula with distilled water at a 1:10 ratio (wt/vol), dissolving by heating at 50 to 60°C, and autoclaving. Some heat-labile vitamins may have been lost during autoclaving. To prepare LJB (pH 6.3), iceberg lettuce (*Lactuca sativa* L.) was blended in an automatic juice extractor (The Juiceman, Jr.; Trillium Health Products, J. M. Marketing, Inc., Seattle, Wash.), filtered through 16 layers of coarsely woven cheese cloth, brought to a boil, and refiltered through 16 layers of cheese cloth to remove coarse particles. TSB (pH 7.0) and TSA were prepared according to the manufacturer's directions. Powdered infant formula (100 g) and agar (BBL/Difco) (15 g) were combined with 900 ml of distilled water, heated to dissolve the contents, and autoclaved (15 min at 121°C) to make IFA. LJA was made by adding 15 g of agar to 1 liter of LJB, heated to dissolve the contents, and autoclaved. For the growth curve study, 300 ml of each broth in 500-ml capped bottles were used. For attachment and biofilm experiments, 30 ml of each broth in 50-ml centrifuge tubes was used.

Phosphate-buffered saline (PBS; pH 7.4) used to resuspend *E. sakazakii* cells for use in biofilm experiments was prepared by combining NaCl (8 g), KCl (0.2 g), Na_2HPO_4 (1.44 g), and KH_2PO_4 (0.24 g) with distilled water (1 liter) and autoclaving. Glass beads (425 to 600 μm in diameter; Sigma-Aldrich, St. Louis, Mo.) were added to PBS to facilitate the removal of adherent *E. sakazakii* cells from the surfaces of stainless steel and the feeding tubes. PBS (30 ml) containing 3 g of acid-washed glass beads in 50-ml centrifuge tubes were sterilized by autoclaving. All media and the PBS were stored at 4°C and used within 2 days.

Determination of growth curves. *E. sakazakii* strains 2855, 3231, 3234, 3295, 3439, and 3396 grown in TSB at 37°C for 24 h were serially diluted in sterile distilled water to make suspensions containing ca. 4 log CFU/ml. Three milliliters of each suspension was added to 300 ml of TSB, IFB, and LJB, and the mixture was shaken at 250 rpm by using a Controlled Environment Incubator Shaker (New Brunswick Scientific Co., Edison, N.J.) for 1 min. The inoculated TSB, IFB, and LJB were incubated at 12 or 25°C for up to 10 days. Populations of *E. sakazakii* were determined regular intervals up to 10 days. At each sampling time, 0.1 ml was surface plated on TSA by using an Autoplate 4000 Automated Spiral

Plater (Spiral Biotech, Norwood, Mass.). Colonies formed on plates incubated at 37°C for 24 h were counted.

Preparation of enteral feeding tubes and stainless steel coupons for attachment and biofilm studies. Enteral feeding tubes (PVC, 0.2-cm external diameter, Fr 7; Vygon Co., Ltd., Norriston, Pa.) were used. The feeding tubes were cut into 5-cm long pieces with a sterile blade, and the ends of each piece were sealed by melting with flame, followed by applying pressure. The pieces of feeding tubes were immersed in 70% ethanol for 10 min to disinfect the surface, followed by drying in a laminar flow biosafety hood. Each piece of tube was placed in a sterile 15-ml test tube. The outer surface of each piece was 3.14 cm².

Stainless steel coupons (type 304, 5 by 2 cm, surface area = 20 cm²) with no. 4 finish were also used in attachment and biofilm studies. Coupons were sonicated (model 250D; VWR, Chester, Pa.) in a 15% phosphoric acid solution at 80°C for 20 min, rinsed with distilled water, sonicated in alkali detergent solution (FS Pro-Chlor; Zep, Atlanta, Ga.) at 80°C for 20 min, and rinsed again with distilled water. Washed stainless steel coupons were dried, placed in 50-ml test tubes, and autoclaved.

Preparation of cell suspension for attachment to enteral feeding tubes and stainless steel. *E. sakazakii* strains 3231, 3234, 3295, 3439, and 3396 grown in TSB at 37°C for 24 h were serially diluted in sterile distilled water to give ca. 4 log CFU/ml. TSB, IFB, and LJB (500 ml) were inoculated with each cell suspension (5 ml) to give ca. 2 log CFU/ml and mixed thoroughly. The inoculated TSB, IFB, and LJB were incubated at 25°C for 2 days; TSB was incubated at 12°C for 6 days, and IFB and LJB were incubated at 12°C for 8 days.

Attachment of *E. sakazakii*. Stationary-phase cultures (25 ml) were deposited in sterile 50-ml test tubes, each containing a sterile stainless coupon; 10 ml of each culture were deposited in a sterile 15-ml test tube containing a sterile piece of feeding tube. Stainless steel coupons and feeding tubes immersed in cultures which had been grown at 12 and 25°C were incubated at 12 and 25°C, respectively, for 4 h to facilitate attachment of the cells, aseptically removed with a sterile forceps, immersed in 400 ml of sterile distilled water (22 ± 2°C), and gently agitated for 15 s. Coupons and tubes were rinsed in 200 ml of sterile distilled water with agitation for 5 s and then deposited in 30 ml of sterile PBS containing 3 g of glass beads in 50-ml centrifuge tubes. The PBS containing a stainless steel coupon or a feeding tube with 3 g of glass beads was vortexed (model Vortex Genie-2; Scientific Industries, Inc., Bohemia, N.Y.) at maximum speed for 1 min. Immediately after vortexing, suspensions were serially diluted in 0.1% peptone water and surface plated (0.1 ml, in duplicate) on TSA to determine the populations of *E. sakazakii* attached to the surfaces of stainless steel coupons and feeding tubes. Simultaneously, media in which cells had grown and attached to the surfaces of stainless steel coupons and feeding tubes were serially diluted in 0.1% peptone water and surface plated on TSA to determine the number of planktonic cells in the surrounding media.

Biofilm formation. *E. sakazakii* strains 3231 and 3439 grown to stationary phase in TSB at 37°C for 24 h were centrifuged (4,000 × *g*, 15 min, 4°C), and cells were resuspended in PBS (pH 7.4) to give 7 log CFU/ml. Cell suspensions were kept at 4°C for no longer than 30 min to minimize the changes in cell numbers and physiological state before use in experiments. Suspensions (25 ml) of each strain were deposited in sterile 50-ml test tubes containing a sterile stainless coupon; 10 ml of suspension was deposited in a sterile 15-ml test tube containing a sterile piece of feeding tube. After the coupons and tubes were incubated at 4°C for 24 h, they were removed from the test tubes with a sterile forceps, immersed in 400 ml of sterile distilled water (22 ± 2°C) for 15 s, and rinsed in 200 ml of sterile distilled water with gentle agitation for 5 s. The rinsed stainless steel coupons and feeding tubes were deposited, respectively, in 30 or 10 ml of TSB, IFB, or LJB in 50- or 15-ml centrifuge tubes, respectively, and then incubated at 12 or 25°C for up to 10 days. The numbers of *E. sakazakii* in biofilms formed on coupons and tubes were determined on day 0 (24 h after immersion in PBS cell suspension at 4°C), and after 2, 4, 6, 8, and 10 days at 12°C and 1, 2, 4, 6, 8, and 10 days at 25°C. Coupons and tubes were removed from each medium and rinsed in 400 ml of sterile water (22 ± 2°C) with agitation for 15 s. After another rinse in sterile distilled water (200 ml) for 5 s, the coupons and tubes were placed in 50-ml centrifuge tubes containing 3 g of glass beads and 30 ml of sterile PBS and then vortexed for 1 min. The PBS suspension and each medium in which coupons or tubes had been immersed were serially diluted in 0.1% peptone water, surface plated (0.1 ml, in duplicate) on TSA, and incubated at 37°C. The number of colonies formed on plates within 24 h was counted.

Statistical analysis. All experiments were performed in triplicate, and two samples representing each combination of test parameters were analyzed at each sampling time. The data were analyzed by using the general linear model of the statistical analysis systems procedure (SAS Institute, Cary, N.C.). The Fisher least-significant-difference test was used to determine whether the attachment and biofilm formation of *E. sakazakii* on stainless steel coupons and enteral
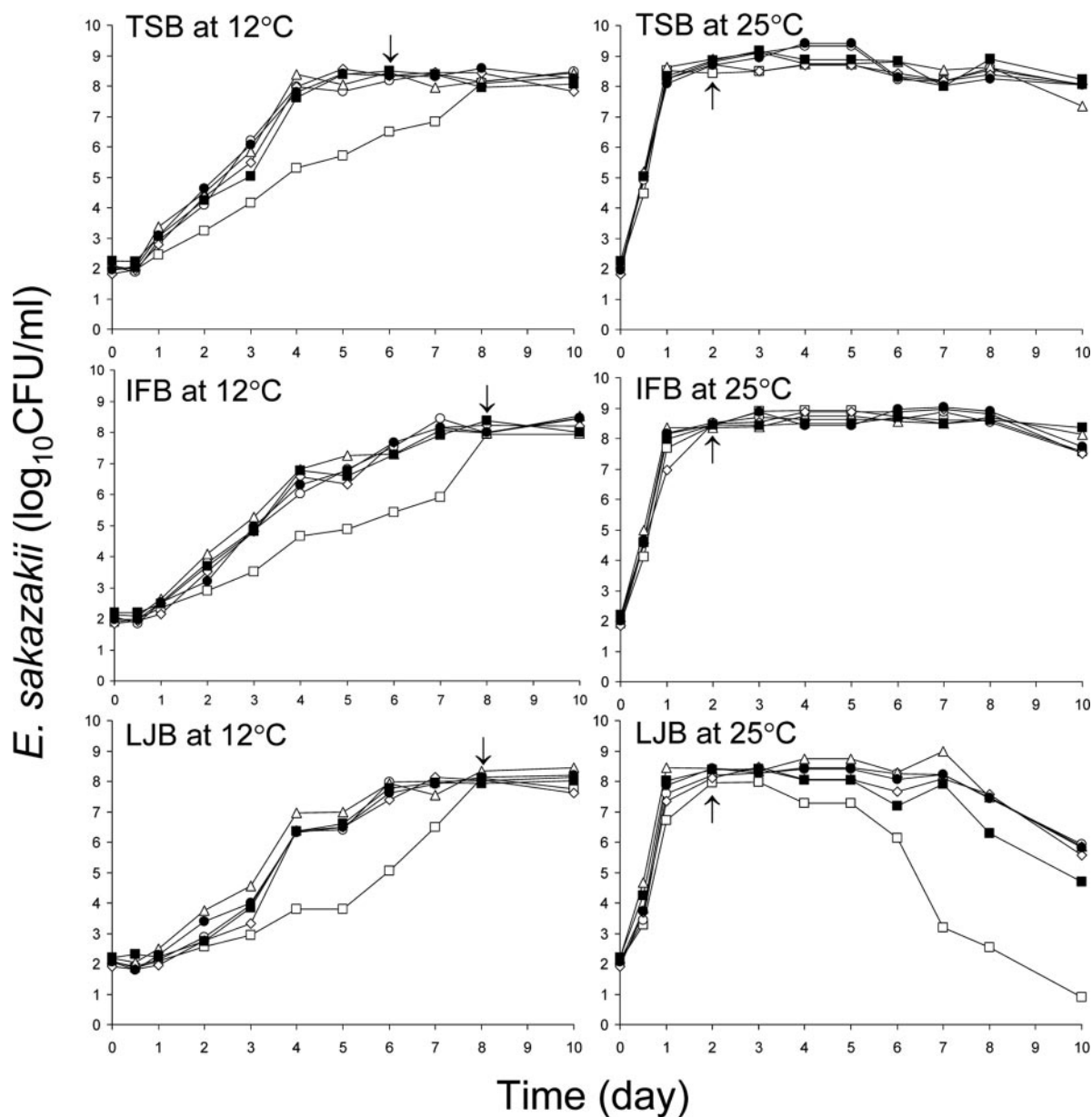
FIG. 1. Populations of *E. sakazakii* strains 2855 (□), 3231 (○), 3234 (△), 3295 (◇), 3439 (■), and 3396 (●) grown in TSB, IFB, or LJB at 12 or 25°C for up to 10 days. Arrows indicate early stationary phase of growth for strains 3231, 3234, 3295, and 3396 at 12°C and all test strains at 25°C.

feeding tubes was significantly affected by temperature and the type of media. Significant differences are presented at a 95% confidence level ($P \leq 0.05$).

RESULTS AND DISCUSSION

Growth curves of *E. sakazakii*. Six strains (2855, 3231, 3234, 3295, 3439, and 3396) of *E. sakazakii* were examined for growth characteristics in TSB, IFB, or LJB at 12 and 25°C. The growth characteristics varied, depending on the strain, medium, and incubation temperature. Figure 1 shows the growth curves of the six test strains in TSB, IFB, or LJB at 12 and 25°C. All strains except strain 2855 showed very similar growth rates in TSB at 12°C. Strains 3231, 3234, 3295, 3439, and 3396 reached the stationary phase in TSB within 6 days at 12°C, but

strain 2855 required about 8 days to reach the stationary phase. Populations of the first five strains increased to 7.63 to 8.38 log CFU/ml within 4 days at 12°C. However, the growth rate of strain 2855 in TSB at 12°C was significantly slower than that of the other five strains. At 25°C, all six strains showed very similar growth patterns in TSB throughout the first 8 days of incubation. Populations of all strains reached 8.09 to 8.64 log CFU/ml within 1 day at 25°C and stationary phase within 1 to 2 days. Growth rates of *E. sakazakii* in TSB at temperatures ranging from 6 to 49°C were determined by Iversen et al. (23). These authors found that the higher the temperature (<40°C), the faster the rate of growth.

E. sakazakii reached early stationary phase of growth within

7 to 8 days in IFB at 12°C (Fig. 1). With the exception of strain 2855, populations in IFB incubated at 12°C increased to 7.30 to 7.68 log CFU/ml within 6 days. As in TSB, compared to the other five strains, strain 2855 grew markedly slower in IFB. All strains reached the early stationary phase of growth in IFB within 1 to 2 days at 25°C. Others have studied the growth rate of *E. sakazakii* in infant formulas and cereals. At temperatures between 6 and 30°C, growth rates in TSB were similar to those in infant formula milk (23). In our study, the growth rate of *E. sakazakii* in IFB at 12°C was slower than that in TSB. Differences in growth rate observed in the two studies are attributed in part to differences in nutrient content of the infant formula. Richards et al. (47) reported that *E. sakazakii* grew to populations of 7.5 and 4.6 log CFU/ml within 72 h at 21 and 12°C, respectively, in infant rice cereal reconstituted with liquid infant formula initially containing ca. 1.0 log CFU/ml.

The growth curves of *E. sakazakii* in LJB incubated at 12°C were very similar to those observed in IFB at 12°C (Fig. 1). All test strains reached early stationary phase after 1 to 2 days and after 7 to 8 days of incubation at 12 and 25°C, respectively. *E. coli* O157:H7 grown in LJB reached the stationary phase of growth within 1 to 2 days at 22°C and 4 to 10 days at 12°C (49). In a previous study (28), we observed that *E. sakazakii* reached a maximum population (ca. 5 log CFU/ml) within 24 h in unpasteurized lettuce juice at 25°C, began to decrease at 36 h, and was undetectable (<1 log CFU/ml) after 72 h. The LJB used in the present study was sterilized, so a reduction in pH caused by fermentation by microorganisms naturally present in unpasteurized lettuce juice that may otherwise have an effect on viability of *E. sakazakii* did not play a role. *E. sakazakii* has been known to survive for >4 days at 12°C when inoculated into unpasteurized lettuce juice at a population of ~1.5 log CFU/ml (28). Even though all six test strains reached the stationary phase within 2 days at 25°C in IFB and LJB, the death of strain 2855 and the other five strains in LJB began after 3 and 7 days, respectively. Populations of strain 2855 decreased significantly ($P \leq 0.05$) from 7.98 log CFU/ml at 3 days to 7.29 log CFU/ml after 4 days, 3.20 log CFU/ml after 7 days, and 0.90 log CFU/ml after 10 days in LJB at 25°C. This strain clearly showed growth and death patterns different from those of the other five strains. Lower amounts and different types of nutrients in LJB, compared to TSB and IFB, may have shortened the stationary phase of growth. A lower buffering capacity of LJB, compared to TSB and IFB, may also have contributed to the rapid initiation of the death phase in LJB. Fermentation of sugars in all test media would result in acid production, with concomitant decreases in pH, particularly if the buffer capacity is minimum as would be the case in LJB. Since the growth rate of strain 2855 in TSB, IFB, or LJB was slower than that of the five other strains in those media, particularly at 12°C, it was not included in subsequent studies on attachment and biofilm formation. The arrows in Fig. 1 indicate the incubation times at which cells were harvested for use in an attachment experiment.

Attachment of *E. sakazakii*. Contamination of abiotic surfaces in infant formula preparation and produce processing areas with *E. sakazakii* resulting from attachment and biofilm formation was simulated in the laboratory. Stainless steel and enteral feeding tubes were used as models, and TSB, IFB, and LJB served as nutrient sources. Although the surface charac-

TABLE 1. Populations of *E. sakazakii* in suspension and attached to surfaces of enteral feeding tubes immersed in TSB, IFB, or LJB at 12 and 25°C for 4 h[a]

Strain	Medium	12°C		25°C	
		Attached (log CFU/cm²)	Planktonic (log CFU/ml)	Attached (log CFU/cm²)	Planktonic (log CFU/ml)
3231	TSB	b 5.47 b	b 7.89 a	a 6.09 a	a 8.93 a
	IFB	b 5.86 a	a 8.17 a	a 6.16 a	a 9.10 a
	LJB	b 2.04 c	b 6.09 b	a 4.67 b	a 7.47 b
3234	TSB	b 2.88 a	a 8.05 a	a 4.31 a	a 8.99 a
	IFB	b 2.81 a	b 8.13 a	a 3.55 b	a 8.96 a
	LJB	a 2.34 a	a 7.47 a	a 3.05 c	a 8.11 b
3295	TSB	b 3.14 b	b 7.86 a	a 5.54 a	a 8.92 a
	IFB	a 5.17 a	b 7.82 a	a 5.33 a	a 8.73 a
	LJB	a 3.60 b	a 7.81 a	a 3.76 b	a 7.52 b
3439	TSB	b 3.59 b	b 7.59 a	a 6.03 a	a 8.94 a
	IFB	a 5.28 a	b 7.75 a	a 5.45 b	a 8.91 a
	LJB	a 2.87 b	a 7.20 a	a 3.09 c	a 7.99 b
3396	TSB	b 5.06 b	a 8.05 a	a 6.29 a	a 8.79 a
	IFB	b 5.79 a	b 8.11 a	a 6.28 a	a 9.22 a
	LJB	b 3.35 c	b 6.76 b	a 4.81 b	a 8.12 b

[a] Within attached cell counts, mean values in the same row that are not preceded by the same letter are significantly different ($P \leq 0.05$). Within planktonic cell counts, mean values in the same row that are not preceded by the same letter are significantly different ($P \leq 0.05$). Within the same strain, mean values in the same column that are not followed by the same letter are significantly different ($P \leq 0.05$).

teristics of cells may vary depending on the medium in which the cells are grown, as well as the phase of growth, cells in early stationary phase were selected for investigation with the goal of minimizing the potential influence of different physiological states on attachment and biofilm formation. The effect of temperature on the attachment of strains 3231, 3234, 3295, 3439, and 3396 grown in TSB, IFB, or LJB at 12 and 25°C to stainless steel and enteral feeding tubes immersed for 4 h at the same respective temperatures in the same respective broths was investigated. Populations of *E. sakazakii* in early stationary phase that remained in TSB, IFB, or LJB, i.e., planktonic cells, in which tubes were immersed for 4 h at 12 or 25°C, as well as the number of cells which attached to the tubes, are shown in Table 1. Strains 3231 and 3396 attached in significantly higher ($P \leq 0.05$) numbers to the feeding tubes immersed in a given broth at 25°C than at 12°C. Strains 3295 and 3439 in TSB and strain 3234 in TSB and IFB attached in significantly higher ($P \leq 0.05$) numbers to the feeding tubes at 25°C than at 12°C. Except for strains 3234, 3295, and 3439 at 12°C, significantly higher ($P \leq 0.05$) numbers of planktonic cells were recovered from TSB and IFB than from LJB incubated at 12 or 25°C. Higher numbers of planktonic cells in media did not always correlate with higher numbers of cells attached to the tubes. This could be due in part to differences in the initial number of planktonic cells in the three test broths. Except for strain 3234 at 12°C, the cells of test strains attached in significantly higher ($P \leq 0.05$) numbers to enteral feeding tubes immersed in IFB than to tubes in LJB incubated at 12 and 25°C. At 25°C, significantly higher ($P \leq 0.05$) numbers of all strains attached to the tubes in TSB and IFB than in LJB.

Compared to cells grown in LJB, cells grown in TSB or IFB may have more ability to attach to the hydrophobic surfaces of enteral feeding tubes. A major difference among the three broths is that TSB and IFB are protein-rich media compared to LJB. The TSB and IFB used in our study both contain casein, and IFB also contains whey protein. Other researchers have determined that nutrients and other components in media affect attachment of microorganisms to surfaces of various materials. Hood and Zottola (20) concluded that composition of growth and conditioning media influence the attachment of *Salmonella enterica* serovar Typhimurium and *Listeria monocytogenes* to stainless steel surfaces. These authors observed that *S. enterica* serovar Typhimurium grown in TSB and *L. monocytogenes* grown in 1% reconstituted skim milk supplemented with sucrose attach in the highest numbers to stainless steel chips that were conditioned with TSB and 1% reconstituted skim milk, respectively, when TSB, 1% reconstituted skim milk, and 1% reconstituted skim milk with sucrose were used as growth and conditioning media. Casein has been shown to promote attachment of *E. coli*, *Pseudomonas fluorescens*, and *Aeromonas liquefaciens* to glass surfaces (36) and attachment of milk-associated microorganisms to stainless steel surfaces was enhanced in the presence of whey protein (53). The opposite effects were observed by others. Milk and milk protein were reported to inhibit adhesion of *L. monocytogenes*, serovar Typhimurium, *Staphylococcus aureus*, *Serratia marcescens*, and *Escherichia coli* to abiotic surfaces, including stainless steel or Buna-N rubber (16, 19, 20). These observations were attributed to repulsion between the negative charges of milk protein and bacterial cells (16) and equilibrium between protein in the surrounding medium and proteins that absorb to the contact surfaces (35).

Table 2 shows the populations of *E. sakazakii* attached to the surface of stainless steel coupons and the numbers of planktonic cells in the media at the end of the 4-h immersion period. Significantly higher ($P \leq 0.05$) numbers of strains 3234 and 3439 attached to the surfaces of stainless steel coupons immersed in a given broth at 25°C, compared to 12°C. Strains 3231, 3295, and 3396 also showed the same trend, although in some cases the number of cells that attached at 25°C in a particular broth was not statistically different than the number that attached at 12°C. Attachment of *E. sakazakii* to the surface of stainless steel was not correlated with the type of medium in which cells had grown or the number of planktonic cells in the surrounding medium. Except for strain 3439, the number of *E. sakazakii* cells that attached to stainless steel immersed in TSB and IFB was not significantly different than the number that attached to coupons in LJB at 25°C. Under the same attachment conditions, in most cases, higher numbers of *E. sakazakii* adhered to the feeding tubes than to stainless steel. Stainless steel is moderately hydrophilic, whereas PVC (the enteral feeding tube) is highly hydrophobic. Most bacteria adhere more readily to hydrophobic surfaces (46).

The influences of temperature on the attachment of other food-borne pathogens to biotic and abiotic surfaces have been investigated by others. Our observations are in agreement with a study by Gorski et al. (14), who reported that temperature plays a role in the attachment of *L. monocytogenes* to radish tissue. In that study, *L. monocytogenes* cells were found to attach in higher numbers to the radish tissues

TABLE 2. Populations of *E. sakazakii* in suspension and attached to surfaces of stainless steel coupons immersed in TSB, IFB, or LJB at 12 and 25°C for 4 h[a]

Strain	Medium	12°C		25°C	
		Attached (log CFU/cm²)	Planktonic (log CFU/ml)	Attached (log CFU/cm²)	Planktonic (log CFU/ml)
3231	TSB	b 3.55 b	a 7.98 b	a 4.50 a	a 8.83 a
	IFB	b 3.41 b	a 8.57 a	a 4.28 a	a 9.14 a
	LJB	a 4.19 a	a 7.65 b	a 4.45 a	a 7.69 b
3234	TSB	b 3.46 a	a 8.24 a	a 4.17 a	a 8.85 b
	IFB	b 3.01 b	a 7.96 a	a 4.02 a	a 9.25 a
	LJB	b 2.30 c	a 7.55 a	a 3.71 a	a 7.65 c
3295	TSB	b 2.92 b	a 7.30 a	a 4.53 a	a 8.39 a
	IFB	a 4.06 a	b 7.54 a	a 4.10 a	a 8.87 a
	LJB	b 3.31 b	a 7.41 a	a 4.81 a	a 7.22 b
3439	TSB	b 3.64 a	a 8.01 a	a 4.62 a	a 9.03 a
	IFB	b 3.60 a	a 7.82 a	a 4.31 a	a 8.11 ab
	LJB	b 3.01 b	a 7.36 a	a 3.95 b	a 7.54 b
3396	TSB	b 3.54 a	a 7.49 a	a 5.11 a	a 8.56 a
	IFB	a 4.07 a	b 7.79 a	a 4.78 a	a 8.74 a
	LJB	a 4.02 a	a 7.74 a	a 4.36 a	a 7.64 b

[a] Within attached cell counts, mean values in the same row that are not preceded by the same letter are significantly different ($P \leq 0.05$). Within planktonic cell counts, mean values in the same row that are not preceded by the same letter are significantly different ($P \leq 0.05$). Within the same strain, mean values in the same column that are not followed by the same letter are significantly different ($P \leq 0.05$).

at 20°C than at 10°C after ≥ 1 h of exposure. Higher numbers of *E. coli* O157:H7 attached to lettuce leaves at 22°C than at 4 or 10°C (54). Factors affecting attachment at different temperatures, however, may vary, depending on the nature of surfaces to which the cells are exposed. Pompermayer and Gaylarde (43), for example, showed that higher numbers of *S. aureus* attached to polypropylene at 12°C than at 30°C after ≥ 4 h of contact time. They also observed that the numbers of *E. coli* attached to polypropylene within 4 h of exposure at 12°C and 30°C were not significantly different. The adhesion of *Salmonella enterica* serovar Montevideo to tomatoes and tomatillos was not influenced by temperature alone (12, 22, or 30°C) but also by combinations of temperature and relative humidity (21). *E. sakazakii* grown under different conditions in our study could have had different surface charges and hydrophobicity, which are known to affect the ability of bacterial cells to adhere to surfaces at various temperatures (10, 56).

Biofilm formation by *E. sakazakii*. Stainless steel coupons and enteral feeding tubes were immersed in cell suspensions in PBS (ca. 7 log CFU/ml) at 4°C for 24 h to facilitate attachment, followed by rinsing, transfer into TSB, IFB, or LJB, and incubation at 12 or 25°C for up to 10 days. Figure 2 shows the number of *E. sakazakii* cells (strains 3231 and 3439) retrieved from biofilms that formed on feeding tubes. The number of *E. sakazakii* cells initially attached to the surface of the feeding tubes did not change significantly ($P \leq 0.05$) during immersion in TSB at 25°C. Some of the cells would be expected to naturally detach from the surface due to the changes in physiochemical properties (49), starvation (44), and death but, on
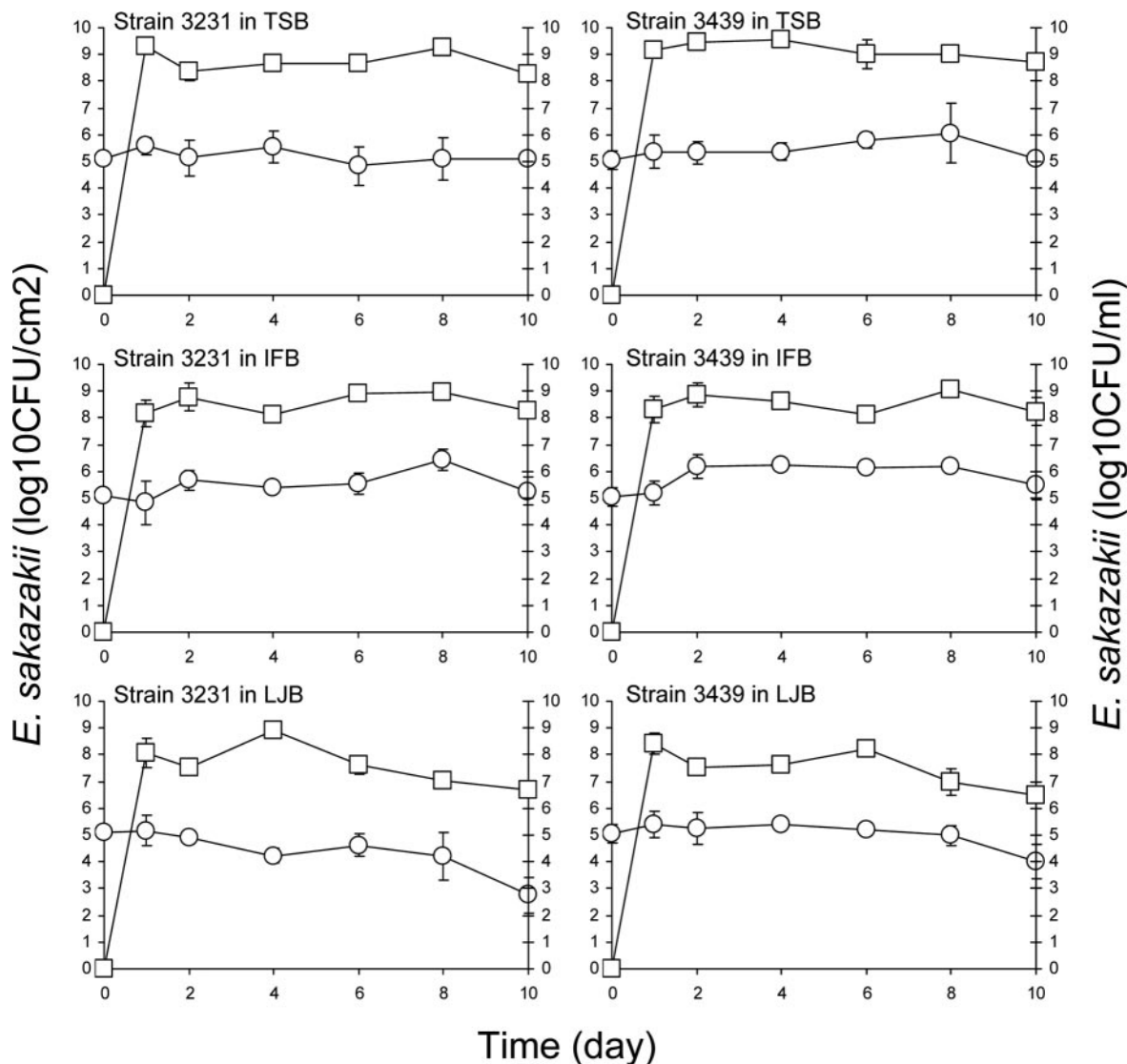
FIG. 2. Populations of *E. sakazakii* strains 3231 and 3396 attached to the surface or in biofilms formed on the surface of enteral feeding tubes (log CFU/cm², ○) immersed in TSB, IFB, or LJB or in broths (log CFU/ml, □) in which tubes were immersed and incubated at 25°C for up to 10 days. Bars indicate the standard deviations.

balance, the number remained the same throughout the 10-day period. Equilibrium was apparently reached between the number of cells that detached from the tube surface, newly attached cells originating from TSB, and newly formed cells originating from cells attached to or in biofilms on the tube surface. Populations of planktonic cells of strains 3231 and 3439 in TSB increased to ≥9.15 log CFU/ml within 1 day, which was followed by slight decreases during after 9 days. *E. sakazakii* initially attached to the feeding tubes grew when the tubes were immersed in IFB at 25°C. The populations of strains 3231 and 3439 increased significantly ($P \leq 0.05$) from initial populations of 5.12 and 5.03 CFU/cm², respectively, to 6.43 and 6.20 log CFU/cm² on the feeding tube surface at 8 days. Planktonic *E. sakazakii* in IFB in which tubes were immersed reached the stationary phase of growth within 2 days. Populations on tubes immersed in LJB at 25°C did not change significantly for the first 8 days but decreased between 8 and 10

days. Planktonic *E. sakazakii* in LJB reached the stationary phase of growth within 2 days. The death phase for strains 3231 and 3439 began at 4 and 6 days, respectively.

Populations of *E. sakazakii* strains 3231 and 3439 recovered from the surface of stainless steel coupons immersed in TSB, IFB, and LJB at 25°C are shown in Fig. 3. The number of *E. sakazakii* strains 3231 and 3439 initially attached to the stainless steel did not change significantly when coupons were immersed in TSB. Initial populations (5.33 to 5.51 log CFU/cm²) on coupons immersed in IFB increased significantly ($P \leq 0.05$) to 6.69 to 6.89 log CFU/cm² within 10 days. The infant formula used to prepare IFB contains nonfat milk, corn syrup, lactose, soy oil, whey protein concentrate, and other nutrients, thus providing protein, fat, carbohydrate, vitamins, and minerals as nutrient sources. As noted above, milk or milk proteins have been shown to either enhance or inhibit bacterial attachment to abiotic surfaces. In our study, *E. sakazakii* attached to sur-
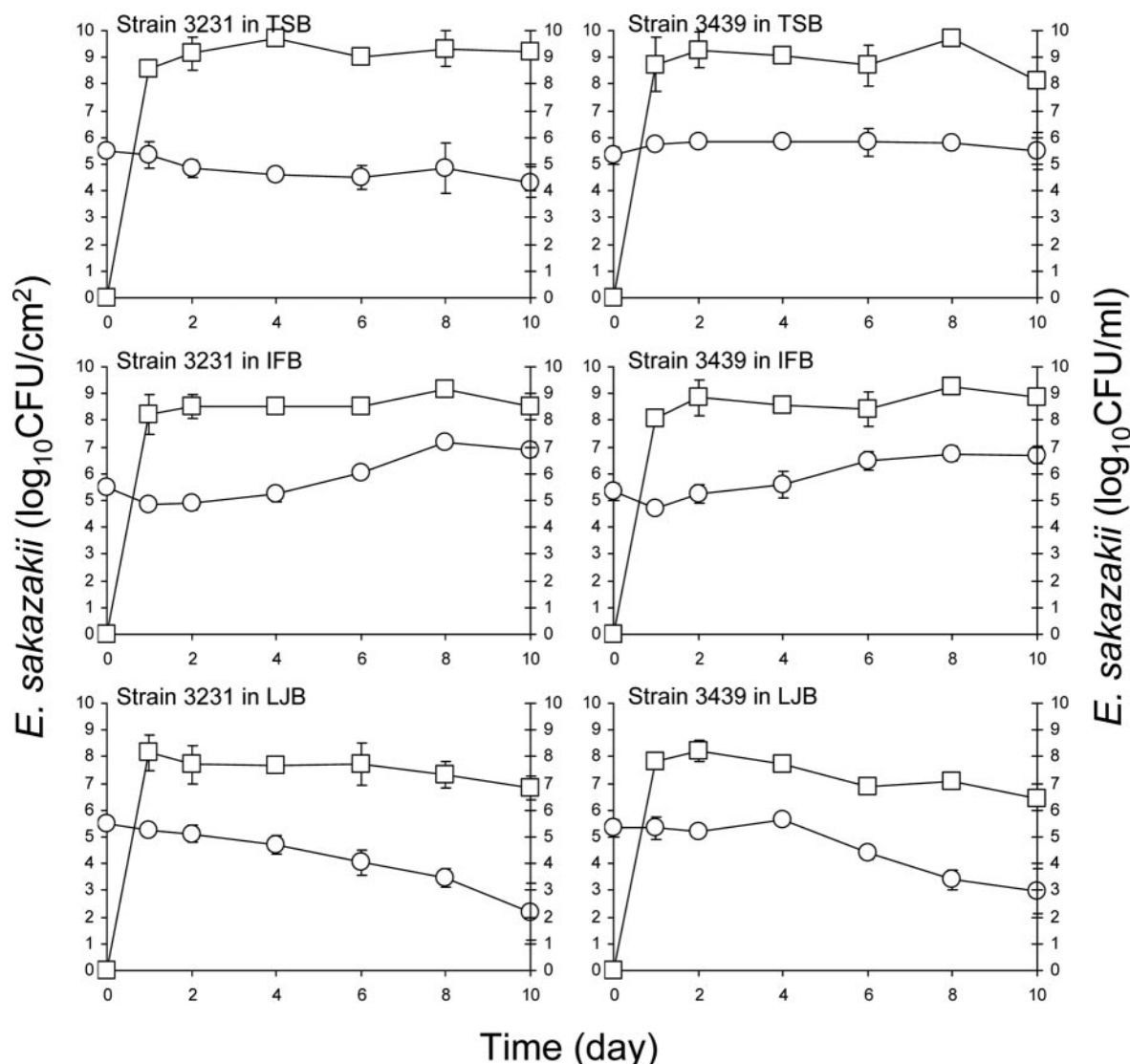
FIG. 3. Populations of *E. sakazakii* strains 3231 and 3396 attached to the surface or in biofilms formed on the surface of stainless steel coupons (log CFU/cm², ○) immersed in TSB, IFB, or LJB or in broths (log CFU/ml, □) in which coupons were immersed and incubated at 25°C for up to 10 days. Bars indicate the standard deviations.

faces of stainless steel in a PBS suspension, followed by immersion in IFB. The IFB may provide the nutrients necessary for *E. sakazakii* cells preattached to the stainless steel or feeding tubes to survive and grow. This observation is in agreement with the study by Helke and Wong (17), who reported that *L. monocytogenes* cells preattached to stainless steel grew in the presence of milk at 25°C. We observed that as incubation time progressed at 25°C, the texture of IFB became markedly viscous. The high viscosity of IFB may facilitate harborage of *E. sakazakii* cells. Biofilm formation by *E. sakazakii* on latex, silicon, and stainless steel immersed in infant formula milk at 37°C has been reported (23). Lehner et al. (32) observed that biofilms were formed by 59% of *E. sakazakii* test strains in PVC microtiter wells and extracellular polysaccharide was produced by 42.8% of the test strains. Extracellular polysaccharide is known to be involved in bacterial adhesion and biofilm formation (6, 7, 11). In the temperature rage of 0 to 30°C, the

highest amount of polysaccharide synthesized by *E. sakazakii* was at 27°C (50).

Populations of *E. sakazakii* initially attached to stainless steel remained constant for 2 to 4 days when immersed in LJB and then significantly decreased ($P \leq 0.05$) by ≥ 2.38 log CFU/cm² after 10 days (Fig. 3). Planktonic *E. sakazakii* in the LJB reached 7.82 to 8.15 log CFU/ml within 1 day, followed by slow decreases during the remainder of the 10-day incubation period. A similar phenomenon was reported by Ryu et al. (49), who showed that *E. coli* O157:H7 attached to stainless steel coupons did not form biofilm when immersed in LJB. The number of *E. sakazakii* and *E. coli* O157:H7 cells that detached from the surface of stainless steel is apparently higher than the number of newly attached cells and preattached cells that grow. A lack of nutrients in LJB, compared to TSB and IFB, as well as a reduction in pH during incubation, may also contribute to significant decreases in the surface populations.
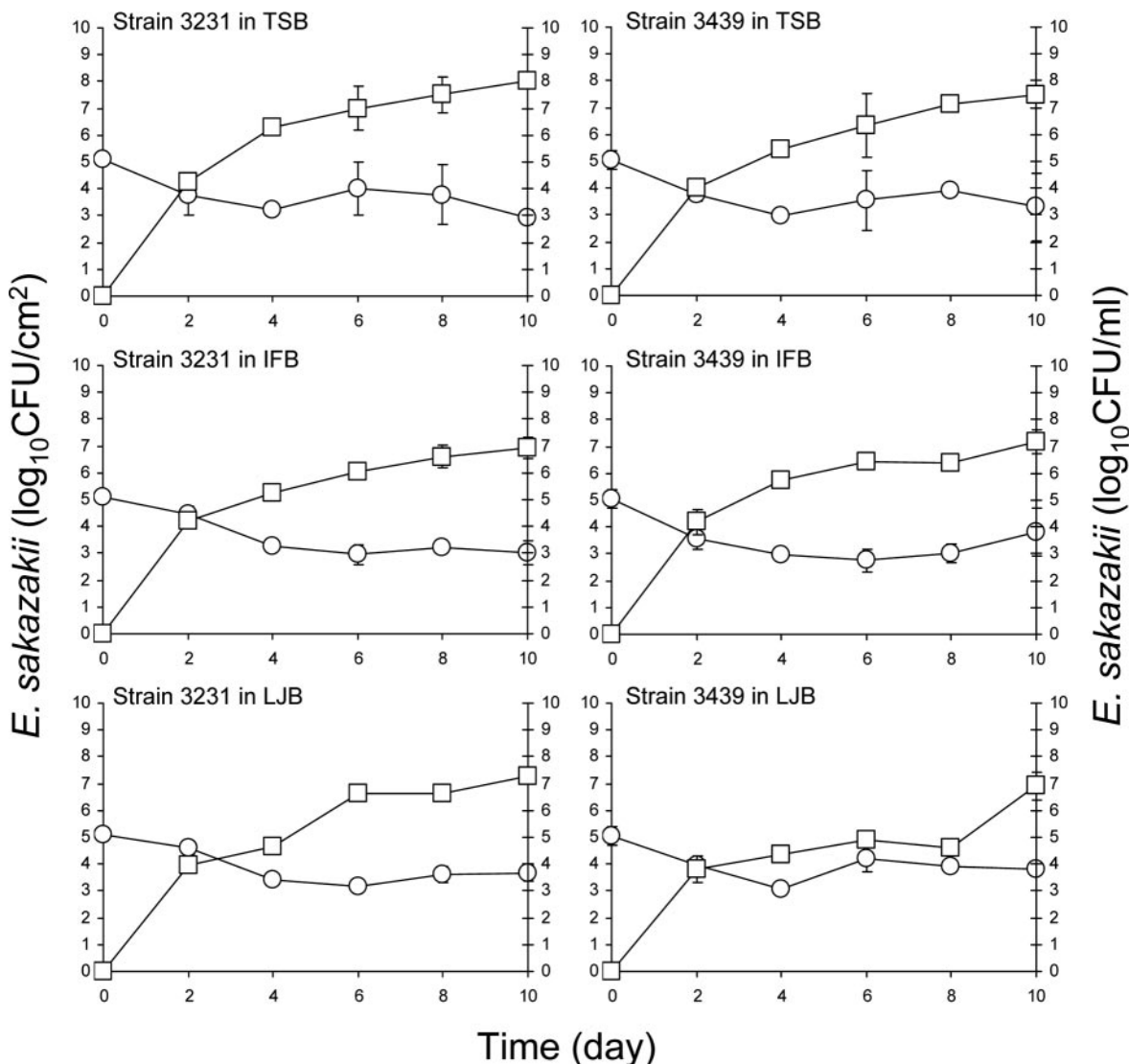
FIG. 4. Populations of *E. sakazakii* strains 3231 and 3396 attached to the surface or in biofilms formed on the surface of enteral feeding tubes (log CFU/cm^2, ○) immersed in TSB, IFB, or LJB or in broths (log CFU/ml, □) in which tubes were immersed and incubated at 12°C for up to 10 days. Bars indicate the standard deviations.

Figure 4 shows the number of *E. sakazakii* strains 3231 and 3439 detected on the surface of enteral feeding tubes and its growth in TSB, IFB, and LJB in which tubes were immersed at 12°C. None of the strains produced biofilms on tubes immersed in TSB. Unlike the behavior of cells in TSB at 25°C, the number of adherent cells gradually decreased at 12°C. Since growth of *E. sakazakii* is very slow at 12°C, biofilm formation in TSB was also restricted. Decreases in the populations of the adherent cells were observed within 2 days. Even though *E. sakazakii* reached a stationary growth phase in TSB in which tubes were immersed within 8 to 10 days, the number of the cells remaining attached to the surfaces of tubes simultaneously decreased. This may be due in part to the presence of fewer planktonic cells at 12°C than at 25°C; to a slower rate of growth of cells preattached to the surfaces at 12°C; and to different physiological states of the cells in media, which has been reported to affect the cell surface characteristics of *E. coli*

(57). *E. sakazakii*, initially attached at 5.03 to 5.12 log CFU/cm^2 of the tube surface, decreased significantly ($P \leq 0.05$) on tubes immersed in IFB at 12°C. This is in contrast to its behavior at 25°C (Fig. 2) and indicates that temperature plays an important role in biofilm formation by *E. sakazakii* in IFB. *E. sakazakii* reached the stationary phase of growth in IFB surrounding the feeding tubes within 8 to 10 days (Fig. 4). Reductions in the numbers of adherent cells on the surface of feeding tubes immersed in LJB at 12°C were 1.23 to 1.47 log CFU/cm^2, which are not substantially different than the 1.05 to 2.37 log CFU/cm^2 reduction at 25°C (Fig. 2).

Figure 5 shows the number of *E. sakazakii* strains 3231 and 3439 on stainless steel coupons immersed in TSB, IFB, or LJB at 12°C and in the immersion broths. As with feeding tubes incubated at 12°C, none of the strains formed biofilms on stainless steel in TSB. Strains 3231 and 3439 attached to stainless steel coupons in PBS cell suspension (ca. 7 log CFU/ml)
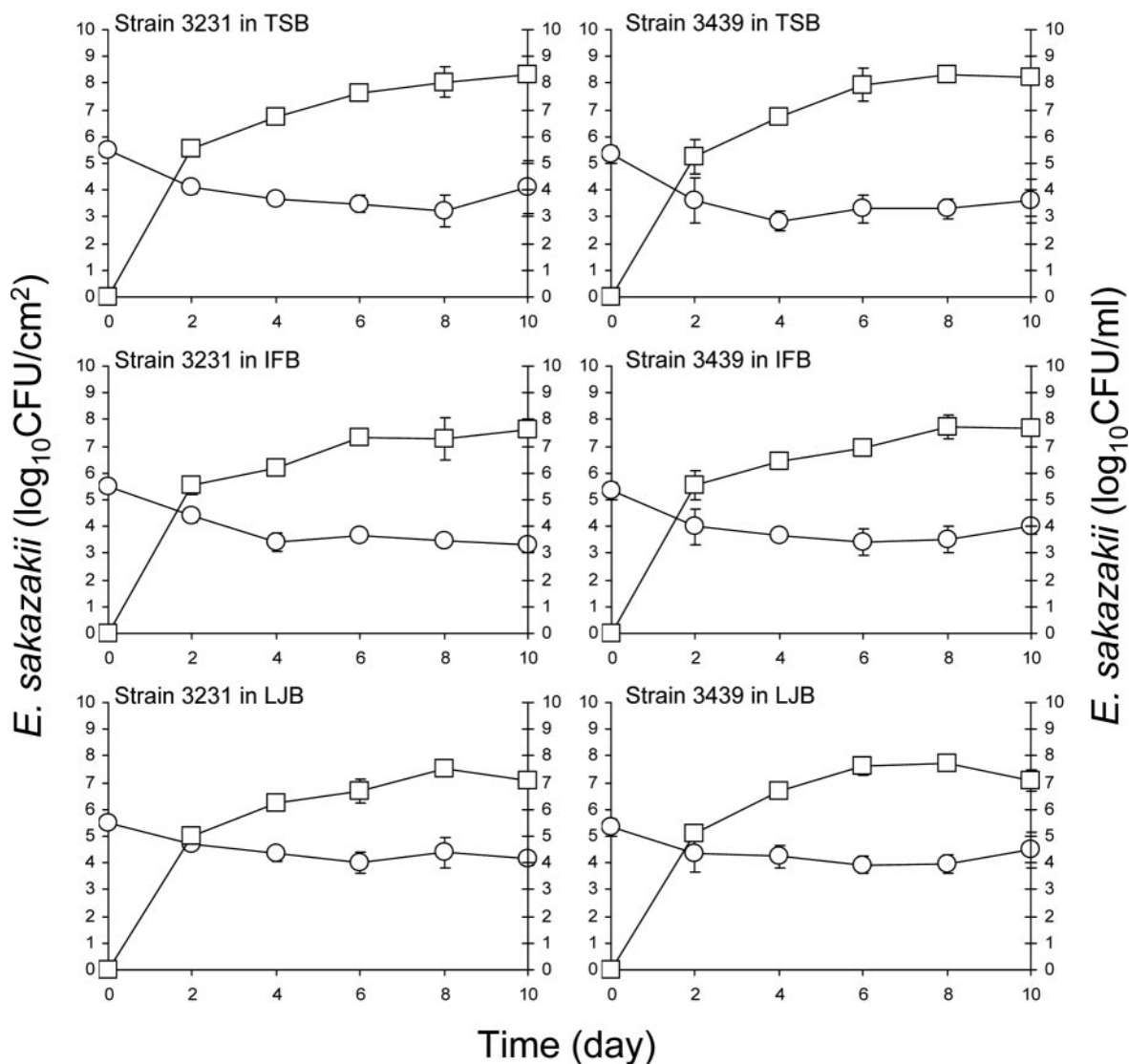
FIG. 5. Populations of *E. sakazakii* strains 3231 and 3396 attached to the surface or in biofilms formed on the surface of stainless steel coupons (log CFU/cm^2, ○) immersed in TSB, IFB, or LJB or in broths (log CFU/ml, □) in which coupons were immersed and incubated at 12°C for up to 10 days. Bars indicate the standard deviations.

during incubation at 4°C for 24 h at populations of 5.51 and 5.33 log CFU/cm^2. Immersion of these coupons in TSB resulted in a reduction of 1.4 log CFU/cm^2 for strain 3231 and 1.7 log CFU/cm^2 for strain 3439 during incubation at 12°C for 10 days. *E. sakazakii* reached the stationary phase of growth in TSB at 25°C within 1 to 2 days (Fig. 3) but required 6 to 8 days when incubated at 12°C (Fig. 5). Numbers of the cells in stationary phase at 12°C reached ≥8.0 log CFU/ml of TSB after 8 days. Unlike the significant growth of *E. sakazakii* observed on stainless steel in IFB at 25°C, the initial numbers of adherent cells decreased significantly ($P \le 0.05$) upon reaching 1.33 to 2.20 log CFU/cm^2 after 10 days at 12°C. *E. sakazakii* did not form biofilm on stainless steel immersed in LJB at 12°C, as was the case at 25°C. However, a slower reduction occurred in the number of cells adhering to stainless steel immersed in LJB at 12°C than at 25°C. Populations of strains 3231 and 3439 attached to stainless steel in LJB

decreased by 3.32 and 2.38 log CFU/cm^2, respectively, at 25°C (Fig. 3) but only 1.37 and 0.84 log CFU/cm^2, respectively, at 12°C (Fig. 5). The larger decreases in populations at 25°C than at 12°C are attributed in part to the death of adherent cells on stainless steel immersed in LJB at 25°C. Like its behavior in TSB and IFB at 12°C, *E. sakazakii* also reached the stationary phase of growth in LJB after 8 to 10 days. *E. sakazakii* behaved similarly in TSB, IFB, and LJB at 12°C. Differences in the composition of three media did not have an influence on the biofilm formation of *E. sakazakii* at 12°C. The results suggest that nutrient depletion did not cause great decreases in the populations of cells attached to feeding tubes or stainless steel coupons at 12°C. It is not the surrounding medium or incubation temperature alone but rather a combination of medium, temperature, or other factors that influence the biofilm formation of *E. sakazakii*.

In summary, *E. sakazakii* attached better to the surfaces of

enteral feeding tubes and stainless steel in TSB, IFB, or LJB at 25°C than at 12°C. *E. sakazakii*, preattached to stainless steel and enteral feeding tubes and immersed in IFB, formed biofilms at 25°C. The importance of preventing the contamination of contact surfaces in areas where powdered infant formulas are reconstituted or fed to infants is reinforced by observations that *E. sakazakii* can attach to and form biofilms on these surfaces. The results emphasize the importance of temperature control in the infant formula and produce processing industries to inhibit attachment and biofilm formation by *E. sakazakii*. Further studies are needed to determine the resistance of *E. sakazakii* in biofilm to various sanitizers and disinfectants used in these industries and in formula reconstitution and feeding areas.

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