Preincubation of *Escherichia coli* ATCC 25922 with NaCl Increases Its Attachment to Lettuce Surfaces Compared with Other Chemicals

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The inhibition of microbial attachment to food is important for the prevention of cross-contamination during food processing. The effect of several chemicals that were added in an *Escherichia coli* growth medium on the attachment of the bacterium to lettuce was investigated. *E. coli* ATCC 25922, which is reportedly a useful surrogate for *E. coli* O157:H7 in surface attachment studies, was preincubated in a nutrient broth (NB) containing sodium chloride, potassium chloride, sodium deoxycholate, sodium linear alkylbenzene sulfonate, or sorbic acid. The bacterial cells were placed in contact with cut lettuce in a saline solution at 5°C for 24 hours. Only the addition of NaCl in the NB influenced the attachment of *E. coli*, *Salmonella enterica* subsp. Enteritidis, and *Klebsiella pneumoniae* to the lettuce. The attachment of *E. coli* showed the largest significant increase at 2% NaCl. Changes in the attachment levels were not due to surface hydrophobicity or the motility of *E. coli* cells. Similar results were observed for *S. enterica* although the variation in the degree of attachment of the latter was quite small. These results suggested that the attachment of *E. coli* O157:H7 to food surfaces is influenced by the bacterial growth conditions prior to food exposure and prior to the development of the biofilm; furthermore, the environmental NaCl concentration should be controlled during food processing to prevent the cross-contamination of foods with *E. coli*.

Key words : Sodium chloride / Surface hydrophobicity / Cell motility / Minimally processed vegetables / Biofilm.

1. INTRODUCTION

Outbreaks of food-borne diseases have been increasingly linked to raw and minimally processed fruits and vegetables; the number of reports examining the attachment of bacterial cells to food has also been growing (Kim et al., 2015; Kojima et al., 2015; Kroupitski et al., 2009; Moon et al., 2007; Saggars et al., 2008). Takeuchi et al. (2000) evaluated the attachment of *Escherichia coli* O157:H7, *Listeria monocytogenes*, *Salmonella enterica* subsp. Typhimurium, and *Pseudomonas fluorescens* to iceberg lettuce. The authors described that *L. monocytogenes* preferentially attached to cut edges, while *P. fluorescens* preferentially attached to intact surfaces.

Several studies have detailed the attachment conditions of microorganisms to food (Cerca and Jefferson, 2008; Jahid et al., 2015); however, few studies have determined the influence of growth conditions on the microbial attachment to foods. *E. coli* O157:H7 cells grown in nutrient broth (NB) were found to attach to lettuce and apple surfaces to a lesser extent when compared with cells grown in Tryptic soy broth (TSB) (Hassan and Frank, 2004). The growth temperature has also been shown to significantly (p<0.05) affect the strength of bacterial attachment to food; cells grown at 37°C were more easily removed from leaf surfaces than those grown at 10°C or 22°C (Ells and Hansen, 2006). Gorski et al. (2003) reported that *L. monocytogenes* use different attachment mechanisms at different temperatures.
In a different approach, Islam et al. (2014) showed that the pretreatment of cabbage leaves with a combination of \( \varepsilon \)-polysine and milk serum protein effectively reduced the viable counts of \( S. \) enterica subsp. Enteritidis as a secondary contaminant on cabbage leaves after washing them with water when compared with untreated cabbage leaves. O’Beirne et al. (2015) reported that slicing with a blunt machine blade enhanced the penetration of \( E. \) coli O157:H7 into carrot tissue and its subsequent survival during storage at 8°C.

\( E. \) coli O157:H7 has been found to be particularly adaptable to various conditions (Wang et al., 2011) and to show the ability to attach, colonize, and form biofilms on a variety of surfaces (Uhlich et al., 2006). \( E. \) coli O157:H7 is able to form biofilm not only on a variety of food processing surfaces but also on spinach, lettuce, Chinese cabbage, celery, leeks, basil, and parsley (Morrison et al., 1997; Pawar et al., 2005).

The risk of cross-contamination is always present when food is processed because of microbial inflow from raw materials and people, the high temperature and humidity in factories, and the presence of food residues that can become a nutrient source for microorganisms. In addition, there are many products and substances that affect microbial growth such as raw meat, drippings from existing on the lettuce did not affect the results when the pretreatment of cabbage leaves with a combi-ning agent, possesses desirable cell surface characteristics (Kim et al., 2009), and is reportedly a useful surrogate for \( E. \) coli O157:H7 in surface attachment studies.

2. MATERIALS AND METHODS

2.1. Preparation of bacterial suspensions

\( E. \) coli ATCC 25922 (=NBRC 15304), \( S. \) enterica subsp. Enteritidis NBRC 105726, and \( K. \) pneumoniae NBRC 3318 were purchased from the National Institute of Technology and Evaluation Biological Resource Center (NBRC). \( S. \) enterica and \( K. \) pneumoniae, which have peritrichous flagella and no flagella, respectively, were selected from among the gram-negative bacteria for comparison with \( E. \) coli. The bacteria were stored in a 10% glycerol solution at -80°C. Cells were thawed and preincubated in brain heart infusion broth (Eiken Chemicals Co., Ltd., Tokyo, Japan) at 37°C for 24 h.

The preincubated culture (0.1 mL) was inoculated into 10 mL of NB containing chemicals at specified concentrations and incubated at 37°C for 24 h (120 strokes/min). Sodium chloride, potassium chloride, sorbic acid, and linear alkylbenzene sulfonate (LAS) were purchased form Wako Pure Chemicals Co., Ltd. (Osaka, Japan). The concentrations of each chemical in the NB were determined to be within the concentration range at which the test bacteria could reach 10⁶ colony-forming units (CFU)/mL by 24 h at 37°C. In a preliminary test, the concentration of each chemical was determined and is shown in Table 1. The culture was washed twice with sterile saline and resuspended in 10 mL of sterile saline. The prepared bacterial suspension was used in all subsequent experiments.

2.2. Quantification of attached bacterial cells

Lettuce was purchased from a city supermarket and several outer leaves were removed. A lettuce leaf was cut into pieces of \( 2 \times 5 \) cm by matching a sterile stainless steel plate with a sterile razor blade. The mass of the lettuce pieces was 0.60 ± 0.17 g. Each lettuce piece was placed into a vial (internal diameter 32 mm) containing 50 mL of sterile saline. Bacterial suspensions (0.1 mL) of the test bacteria that were grown with different concentrations of the test chemicals were added to the vials at an initial concentration of approximately 10⁸ CFU/mL. The vials were incubated at 5°C for 24 h (60 strokes/min). A 0.1-mL aliquot was withdrawn from the suspension, diluted in saline, and plated on nutrient agar (Eiken Chemicals). Colonies were counted following the incubation at 37°C for 48 h.

The number of cells of the test bacteria that were attached to the lettuce pieces was determined by subtracting the number of bacterial cells remaining in the suspension from the starting titer. The initial counts of the original bacteria on the lettuce piece were 3.1 ± 0.4 (log CFU/g). No detectable growth of the original bacteria that was present on the lettuce was observed during the incubation of the lettuce piece at 5°C for 24 h. Therefore, it can be concluded that the original bacteria existing on the lettuce did not affect the results when the initial concentration of each test bacterium was set at 10⁸ CFU/mL. Each test strain was also confirmed not to grow during the incubation at 5°C for 24 h.

2.3. Motility assay

The motility of bacterial cells was determined as described by Wood et al. (2006). The bacterial suspension as prepared in section 2.1 was inoculated at the center of the motility assay plates containing 1% tryptone, 0.25% NaCl, and 0.3% agar. The diameter of the colony formed after the incubation at 37°C for 16 h was...
measured (Sperandio et al., 2002).

2.4. Surface hydrophobicity assay

The hydrophobicity of the test bacterial cells was determined as described by Hassan and Frank (2004). The bacterial cells that were grown with different concentrations of the chemicals were resuspended in 0.05 M phosphate-buffered saline (PBS, pH 7.4) at approximately 10^8 CFU/mL. One milliliter of xylene (Wako Pure Chemicals) was added to the test tube containing 4 mL of the bacterial suspension in PBS. The tube was vortexed for 2 min and placed in a 37°C water bath for 30 min for equilibration. The optical density at 600 nm (O) of the control (bacterial suspension in PBS) was also measured. The surface hydrophobicity of the bacterial cells was calculated using the following equation:

\[
\text{Hydrophobicity } (\%) = (1 - A/A_o) \times 100
\]
2.5. Statistical analysis

All experiments were carried out at least in duplicate on three separate occasions. The data points with bars represent the mean ± standard error of the mean. Data were analyzed using on way ANNOVA (Tukey’s method) from the BellCurve for Excel® version 2.0.3 (Social Survey Research Information Co., Ltd., Tokyo, Japan) to determine the significant difference of mean values. The probability level interpreted as statistically significant was p<0.05.

3. RESULTS AND DISCUSSION

3.1. Quantification of attached bacterial cells

Table 1 shows the levels of attachment to the lettuce pieces of the bacteria that were grown with different concentrations of the chemicals in the NB. With increasing concentrations of sorbic acid and LAS in the NB, no significant changes in the attachment levels of E. coli cells to the lettuce pieces were observed. Alternatively, the addition of NaCl at 2% resulted in a significant increase in the attachment level (up to 40-fold) compared with the control. A large reduction could be seen at 4% NaCl.

Since a high concentration of NaCl induces a large osmotic change compared to other chemicals, the growth media were prepared containing the same molar concentrations of KCl as those used for NaCl. However, unlike NaCl, no significant variations in the attachment levels of E. coli were observed with KCl. Therefore, the variations in the attachment levels of E. coli cells observed with NaCl were considered unrelated to the osmotic changes.

For comparison with E. coli, the effect of the NaCl concentration in the NB was investigated using the same procedure with S. enterica, which has peritrichous flagella, and K. pneumoniae, which does not have flagella. Although the peaks of the attachment for both bacteria were observed at 2% NaCl, the variation in the attachment levels of both bacteria were smaller than that of E. coli.

Most E. coli O157:H7 could easily grow at 10°C, with some strains growing at 8°C, and verotoxin production was also detected. Some strains increased 1000-fold in viable counts in 4 to 6 days at 10°C (Paulumbo et al., 1995). Ding et al. (2010) also reported that E. coli O157:H7 inoculated in beef increased 100-fold in viable counts in approximately 10 days at 4°C. Francis and O’beirne (2001) investigated the survival and growth of E. coli O157:H7 during storage (4 and 8°C) in ready-to-use packaged vegetables. In their study, E. coli O157:H7 increased by 1.5 to 2.5 log on shredded lettuce during a 12-day storage period at 8°C. Although no growth of E. coli O157:H7 was observed at 4°C, viable popula-

3.2. Motility of bacterial cells

For flagellar bacteria, it has been reported that their flagella play an important role. Kim et al. (2005) reported that a temperature decrease from 37 to 20°C caused changes in the energy metabolism of E. coli, as well as in flagellar biosynthesis and motility. Larsen et al. (2004) described that the motility and chemotactic responses of Vibrio anguillarum varied with the temperature and that the cell’s speed was higher at a high temperature than at a low temperature. Vatanyoopaisarn et al. (2000) reported that at 22°C, a flagellin mutant of L. monocytogenes was found to attach to stainless steel at levels 10-fold lower than those shown by wild-type cells, and flagella especially facilitated the early stage of attachment. Therefore, we measured the motility of the test bacteria grown at different NaCl concentrations (Tables 2 and 3). The motility changes for E. coli and S. enterica showed a convex upward variation, with the same pattern as that observed for the attachment level due to NaCl concentration. On the other hand, for K. pneumoniae, which has no flagella, there was no correlation between the changes in the attachment level and motility.

Morisaki et al. (1999) described that the attachment of bacterial cells is facilitated by cell motility, and our results suggest that the variation in the motility of E. coli and S. enterica cells, depending on the NaCl concentration in the pretreatment with NB, affected the attachment levels of E. coli and S. enterica to lettuce. Because E. coli that was grown in NB with KCl did not show any correlation between the attachment level and motility, the possibility of specific changes associated with NaCl was also considered.

3.3. Surface hydrophobicity of bacterial cells

Beuchat and Worthington (1976) reported that when Vibrio paraephytus was grown at 0-7.5% NaCl in TSB, the lowest saturated to unsaturated fatty acid ratio was observed in the cells grown at 3% NaCl. In addition, with increasing NaCl concentrations (from 0 to 7.5%), fatty acids with a carbon length of 14-16 increased and
fatty acids with a carbon length of 18 decreased (Beuchat and Worthington, 1976; Tatsuguchi et al., 1989), indicating that the surface hydrophobicity was reduced with an increasing NaCl concentration in the growth medium.

Table 4 shows significant variations in the surface hydrophobicity of the three strains associated with the changes in the NaCl concentrations in NB. The surface hydrophobicity of *E. coli* and *S. enterica* was the lowest at NaCl concentrations of 2-3%. This indicated that the attachment level and surface hydrophobicity were inversely related. There is a possibility that the decrease in the surface hydrophobicity was related to an increase in the attachment level to lettuce. However, no correlation between the attachment level and surface hydrophobicity was observed for *K. pneumoniae*. Goto et al. (2012) investigated the effect of NaCl concentrations in the growth medium on the attachment of *Staphylococcus aureus* to lettuce. In their study, the surface hydrophobicity of *S. aureus* cells decreased with increasing NaCl concentrations in NB, similar to *E. coli*; however, the attachment level decreased, which was the opposite result of what was observed for *E. coli* in this study.

Although effects of NaCl on biofilm formation have been reported in several studies, there are very few studies on the effects of NaCl present in the bacterial growth media prior to the bacterial attachment to food. Jarhid et al. (2015) reported that young cultures (growth time of 24 h) of *Aeromonas hydrophila* that were grown at 0-0.25% salinity showed gradual increases in biofilm formation on stainless steel, glass, and crab shells. There were also increases in motility, exoprotease production, and *N*-acyl homoserine lactone and autoinducer-2 quorum sensing. However, the manifestation of all these phenotypic characteristics was reduced at salinity levels from 0.5 to 3.0%. Cerca and Jefferson (2008) reported a decrease in *E. coli* biofilm formation on polystyrene plates at a 1% concentration of NaCl. On the other hand, Goller et al. (2006) showed that high salt concentrations increased the biofilm formation.

As described above, the effects of growth conditions on bacterial cell attachment to food and surfaces differs among strains. In addition, preculture conditions and biofilm formation conditions were inconsistent across different studies. Di Cicco et al. (2015) investigated the ability of *S. aureus* to form biofilm on food processing surfaces (polystyrene and stainless steel) at different temperatures as related to cellular hydrophobicity. However, their study did not reveal a relationship between the ability to form biofilm and the degree of hydrophobicity. Nowak et al. (2015) also concluded that biofilm formation by *L. monocytogenes* was not determined by one factor but was rather dependent on multiple factors, with the temperature and type of medium having the biggest effects.

Poly-N-acetylglucosamine appears to play an important role in biofilm formation by *E. coli*. Expression of the *pga* gene cluster, which encodes proteins that are neces-

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**TABLE 2.** Motility of bacterial cells grown at different NaCl concentrations in NB.

| NaCl concentration (%) | E. coli | S. enterica | K. pneumoniae |
|------------------------|---------|-------------|---------------|
| Control*               | 70.0 ± 2.4a | 61.7 ± 0.5a | 2.3 ± 0.5a    |
| 1                      | 74.7 ± 4.2a | 65.7 ± 0.5b | 2.7 ± 0.5a    |
| 2                      | 71.7 ± 1.7ab | 69.7 ± 0.9c | 2.7 ± 0.5a    |
| 3                      | 69.7 ± 3.4b | 65.0 ± 1.5ab | 3.3 ± 0.5a    |
| 4                      | 68.3 ± 2.5ab | 64.3 ± 0.5bd | 3.0 ± 0.8a    |

*NB itself (control) contains 0.5% NaCl.

**TABLE 3.** Motility of *E. coli* cells grown at different KCl concentrations in NB.

| KCl concentration (%) | Colony diameter (mm) |
|-----------------------|-----------------------|
| Control*              | 64.7 ± 2.6a           |
| 1.3                   | 70.0 ± 2.4ab          |
| 2.6                   | 73.0 ± 2.2ab          |
| 3.8                   | 70.3 ± 1.7b           |
| 5.1                   | 72.3 ± 1.2ab          |

*NB itself (control) contains 0.5% NaCl.

**TABLE 4.** Surface hydrophobicity of bacterial cells grown at different NaCl concentrations in NB.

| NaCl concentration (%) | E. coli | S. enterica | K. pneumoniae |
|------------------------|---------|-------------|---------------|
| Control*               | 46.8 ± 3.9a | 62.1 ± 2.1a | 34.9 ± 6.7a   |
| 1                      | 32.5 ± 1.6b | 58.5 ± 4.3a | 28.7 ± 5.2ab  |
| 2                      | 26.3 ± 1.3c | 38.1 ± 2.8b | 17.0 ± 1.9ac  |
| 3                      | 28.5 ± 2.5bc | 38.3 ± 1.4b | 33.2 ± 3.6abd |
| 4                      | 32.2 ± 6.5abc | 56.0 ± 1.2a | 16.9 ± 10.6a  |

*NB itself (control) contains 0.5% NaCl.

Data are means ± SD of 3 measurements.
sary for the synthesis of polymeric N-acetylglucosamine and polysaccharides, can be induced by NaCl (Cerca and Jefferson, 2008). Colanic acid (or M antigen) is an extracellular polysaccharide that is produced by most E. coli strains, as well as by other species of the Enterobacteriaceae. The genes required for the synthesis of colanic acid are grouped in the wca cluster (Van Houdt and Michiels, 2005). The attachment of E. coli to an abiotic surface induces the expression of the wca operon (Prigent-Combart, 1999). The production of a matrix of extracellular polymeric substances composed of polysaccharides, extracellular DNA, lipids, and proteins, plays an important role during the irreversible attachment in biofilm formation.

However, the contributions of the extracellular polymeric substances to the adherence properties of bacteria during the process of attachment, as well as to biofilm structure and function, have only been examined for a few organisms such as Pseudomonas aeruginosa and Staphylococcus epidermidis (Petrova and Sauer, 2012). Dense et al. (2000) have found that colanic acid production is not required for surface attachment. Hasson and Flank (2004) reported that E. coli O157:H7 that were grown in NB attached to lettuce and apple surfaces to a lesser extent than that grown in TSB, and that TSB but not NB supported capsule formation in E. coli O157:H7. The authors showed that among the factors studied; only capsule formation was associated with attachment ability because there was no difference in the electrokinetic properties of the cells grown in these media. We will further investigate the influence of NaCl concentrations in growth media on the surface hydrophobicity, surface charge, and on capsule and hydrophobic protein production in a future study.

4. CONCLUSIONS

In this study, we investigated the effect of chemicals present in the NB for E. coli ATCC 25922, which is reportedly a useful surrogate for E. coli O157:H7 in surface attachment studies, on the attachment of the bacterium to lettuce. Only increased NaCl concentrations in NB altered the attachment behaviors of E. coli cells to lettuce. The number of the E. coli cells that were attached was significantly increased with 2% NaCl. Although a similar effect was observed for S. enterica, the variation in the attachment levels was smaller. Changes in the surface charge or motility of E. coli cells did not account for the increased attachment ability. Although it is necessary to investigate in detail the capsule and hydrophobic protein production, our results suggest that the attachment of E. coli O157:H7 to a food surface is influenced by the growth conditions prior to bacterial attachment and prior to the development of biofilm. These data also provide an insight into the ecology of E. coli, S. enterica, and K. pneumoniae that grow on fresh vegetables in working environments characterized by high salinity, which is common in food processing. The control of NaCl concentrations in the working environment may be important for the prevention of secondary contamination in food processing processes.

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