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Cell surface charge and initial attachment characteristics of rough strains of *Listeria monocytogenes*

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J.S. DICKSON AND G.R. SIRAGUSA. 1994. The relative negative surface charge and hydrophobicity of four bacterial strains were evaluated by gravity flow and spin column methods. There was no significant difference between the two methods, indicating that spin column chromatography is an acceptable alternative method of determining cell surface charge or hydrophobicity. Six strains of *Listeria monocytogenes* which exhibited rough colony appearance were evaluated for surface charge and hydrophobicity and their ability to contaminate beef muscle tissue. With one exception, all of the rough strains exhibited greater net negative surface charge and reduced ability to contaminate beef during the initial stages of attachment. Since greater net negative cell surface charge has been positively correlated to attachment to beef tissue surfaces, the reduction in attachment exhibited by the rough strains was interpreted as an indication that attachment to beef tissue cannot be solely explained by cell surface charge.

INTRODUCTION

Hydrophobicity and relative bacterial cell surface charge have been associated with bacterial attachment to a variety of surfaces. Methods of determining hydrophobicity include contact angle, bacterial adherence to hydrocarbons and hydrophobic interaction chromatography (HIC) (Rosenberg and Kjelleberg 1986; Mozes and Rouxhet 1987), while those for relative cell surface charge include electrophoretic mobility and electrostatic interaction chromatography (ESIC) (Pederson 1980; van Loosdrecht *et al.* 1987). While each method has specific advantages and disadvantages, the chromatographic methods provide a reasonable estimate of bacterial surface properties in the context of attachment to a solid substrate.

Hydrophobic interaction chromatography as an estimate of bacterial hydrophobicity was reported by Dahlback *et al.* (1981). The authors examined the surface properties of marine bacteria, using chromatography columns prepared with octyl-Sepharose in Pasteur pipettes. Pedersen (1980) reported the net surface charges of marine bacteria determined by ESIC, using anion and cation exchange resins and methodology similar to that used in HIC. Hydrophobic interaction chromatography and ESIC have previously been performed using gravity flow columns, with the flow rate controlled only by the physical nature of the column and packing material. Spin column chromatography provides a consistent force exerted on the column, which should result in a more uniform flow rate.

Rough strains of *Listeria monocytogenes* have been isolated from clinical samples (Hof *et al.* 1991). While the virulence of these isolates has been evaluated, the ability of these bacterial isolates to contaminate beef tissue has not been documented. Previous research has demonstrated a relationship between cell surface charge and a bacterium's ability to contaminate beef (Dickson and Koohmaraie 1989) and other surfaces (van Loosdrecht *et al.* 1987). The objective of this research was to evaluate the cell surface charge of several rough and smooth *Listeria* isolates and correlate this with the ability to initially contaminate beef tissue. This information will provide a better explanation of the role of cell surface charge in the initial stages of *Listeria* contamination of beef.

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Mention of trade name, proprietary product or specific equipment is necessary to report factually on available data; however, the USDA neither guarantees nor warrants the standard of the product, and the use of the name by USDA implies no approval to the exclusion of others that may be suitable.

MATERIALS AND METHODS

Bacterial cultures and growth conditions

Cultures of *Escherichia coli* O157 : H7 (Food Research Institute, Madison, WI), *Salmonella typhimurium* ATCC 14028 and *Staphylococcus aureus* ATCC 25923 were grown and maintained in tryptic soy broth (TSB) at 37°C. *Listeria monocytogenes* Scott A (U.S. Food and Drug Administration) and rough strains of *L. monocytogenes* (H. Hof, University of Heidelberg, Germany) were grown and maintained in TSB + 0·5% (wt/vol) yeast extract at 37°C. Eighteen hour cultures of each bacterium were grown in a gyrorotary incubator, harvested by centrifugation (1000 *g*, 5°C, 10 min), washed twice in Butterfield's phosphate buffer, pH 6·8 (BPB) (Pertel and Kazanas 1984) and resuspended to the original volume in BPB.

Chromatography

Initial experiments were conducted to verify that spin and gravity flow chromatography produced comparable results for both ESIC and HIC. *Salmonella typhimurium*, *E. coli* O157 : H7, *Staph aureus* and *L. monocytogenes* Scott A were used to compare the two chromatography methods. Electrostatic interaction chromatography, was conducted using Poly-prep columns for gravity and Bio-spin columns for spin chromatography (Bio-Rad, Richmond, CA). Hydrophobic interaction chromatography was conducted using Bio-spin columns for both gravity and spin chromatography, packed with 1 ml of a 1:1 (vol/vol) octyl-Sepharose CL-4B in BPB (Sigma). Electrostatic interaction chromatography columns were packed with 1 ml of a 1 : 2 (wt/vol) mixture of analytical grade Dowex chloride (form 1 × 8, mesh size 100/200) in BPB (Sigma capacity 1·2 meq ml^{-1}). The exchange capacity of a 100/200 mesh resin, based on a monolayer of cells attached to the bead surfaces, has been determined to be approximately 10^{10} cells g^{-1} of resin (Daniels and Kempe 1966). Electrostatic interaction chromatography columns were packed with 0·5 g of anion exchange resin, which would provide an exchange capacity of approximately 5×10^9 cells column^{-1}. In this report, a maximum of 5×10^8 or 5×10^7 cells was applied to the Poly-Prep and Bio-spin columns, respectively, so that the exchange capacity of the columns was not exceeded. Columns were washed with 10 ml BPB (3 ml for the spin columns), and 100 μl (10 μl for the Bio-spin columns) of the bacterial suspension were adsorbed on to the resin or gel and eluted with 10·0 ml (1·0 ml for the Bio-spin columns) of BPB. Gravity columns were allowed to elute at their natural flow rates, while the spin columns were placed in sterile 12 × 75 mm test tubes and centrifuged at 100 *g* for 2 min in a horizontal rotor. The first 20 s of this 2 min were required to reach 100 *g*, and an additional 90 s were required for the run to complete to return to 0 rev min^{-1}. Populations in the initial and eluted samples were enumerated by plating on tryptic soy agar (TSA) or TSA + 0·5% yeast extract (TSAYE; *L. monocytogenes*) using a Model D Spiral Plater (Spiral Systems Instruments, Inc, Bethesda, MD) and methodology appropriate for spiral plates (Messer *et al.* 1984).

Attachment assays

Post-rigor beef muscle tissue was obtained as boneless trim from the abattoir at the Roman L. Hruska U.S. Meat Animal Research Center. The tissue was sliced (0·5 cm thick), frozen in sterile sealed bags (− 20°C), sterilized with gamma radiation at a minimum dose of 42 kGy, and stored at − 20°C until assay. Prior to use, the slices were cut into 1·0 × 1·0 cm squares (sample size 1·0 × 1·0 × 0·5 cm) and brought to room temperature. Tissue produced in this manner had previously been determined to be representative of pre-rigor tissue, in terms of numbers of bacteria which would attach (Dickson 1992). *Listeria monocytogenes* strains were diluted to approximately $\log_{10}$ 8 colony-forming units ml^{-1} in 50 ml BPB. Beef muscle tissue was inoculated by immersion for 1, 5 and 10 min, transferred to 99 ml bottles of BPB, gently inverted 25 times within 15 s to remove non-adherent, planktonic bacteria, and then removed and homogenized for 2 min in 99 ml BPB in a Stomacher 400 (Tekmar Inc., Cincinnati, OH). Bacteria remaining on the tissue after rinsing were considered to be attached. Homogenized samples and the inoculum samples were enumerated on TSAYE as previously described.

Data analysis

Relative negative charge and hydrophobicity were expressed as r/e and g/e, respectively, where e was the eluted bacterial population and r and g were the populations retained by the resin or gel, calculated as the difference between the initial and eluted populations. Relative negative charges (r/e) were transformed to natural logarithms to minimize variance. The data were analysed using the General Liner Models procedure of SAS (1985), and the reported means are the average of four independent replications per method per bacterium. Natural logarithm transformation of g/e values did not significantly improve the variance, so standard g/e values were used. Estimates of attached bacterial populations were transformed to $\log_{10}$ cfu cm^{-2} values, and statistical analysis was conducted as previously described (SAS 1985). Reported means are the average of three independent replications of each experiment, with the population estimates of the initial inocula used as covariates in the analysis to normalize the results between replications.

RESULTS AND DISCUSSION

The method of chromatography (gravity or spin) was not a significant factor ($P > 0{\cdot}10$) in the g/e or r/e values for any of the bacteria (Table 1). Spin chromatography values were, however, slightly higher than the corresponding

Table 1 Comparison of gravity flow and spin column chromatography methods for determining relative hydrophobicity and relative net negative charge

Bacterium	Surface assay	Chromatography method: Gravity flow	Spin column
Gram-negative			
Escherichia coli O157 : H7	HIC	0·22	0·38
	ESIC	−0·27	−0·11
Salmonella typhimurium	HIC	0·81	0·79
	ESIC	0·35	0·39
Gram-positive			
Staphylococcus aureus	HIC	1·21	1·67
	ESIC	3·60	3·47
Listeria monocytogenes	HIC	3·37	3·47
	ESIC	4·28	4·41

HIC, Hydrophobic interaction chromatography reported as g/e; ESIC, electrostatic interaction chromatography using anion exchange resin, reported as ln (r/e).

Table 2 Relative hydrophobicity and net negative charge of smooth and rough *Listeria monocytogenes* cells determined by spin column chromatography

Bacterial strain	Culture type	Surface charge assay: HIC	ESIC
R1	R	4·22[a]	8·76[a]
R3	R	2·59[a]	10·03[b]
R4	R	7·86[a,b]	7·03[c]
R5	R	4·40[a]	11·12[d]
SV1/2A EGD	R	10·85[b]	5·51[e]
Scott A	S	4·23[a]	4·22[f]
1/2C	R	25·11[c]	10·29[b,d]
1/2C	S	3·38[a]	6·18[e]

R, Rough; S, smooth; HIC, hydrophobic interaction chromatograhy reported as g/e; ESIC, electrostatic interaction chromatography using anion exchange resin, reported as ln(r/e).
Means within columns with different superscripts are significantly different ($P < 0{\cdot}05$).

gravity method. Centrifuging the columns voided all of the BPB in the gel or resin bed, while gravity columns retained a small volume of the buffer used to elute the bacteria. In the case of the HIC columns, approximately 0·1 ml of the buffer was retained in the chromotography media. The additional buffer eluted with the spin columns would have diluted the bacteria in the recovered eluant (e), resulting in a slight decrease in the calculated population. Reducing the population in the eluant would result in an increase in the calculated bacterial population retained by the column (g or r), which would increase the g/e or r/e value. In reality, there was no difference in the numbers of retained or eluted bacteria with the gravity or spin columns, simply a difference in the amount of buffer eluted from the column. The g/e and r/e values determined in these experiments reflect the same general trends in regard to relative surface charge as those previously reported (Dickson and Koohmaraie 1989), although there are some differences in the numerical values, which may be attributable to the type of columns used in each experiment or the fact that phenyl-sepharose was used for HIC.

Two of the rough strains of *L. monocytogenes*, SV1/2A EGD and 1/2C, had relative hydrophobicities which were greater than ($P < 0{\cdot}05$) the two smooth strains used for comparison (Scott A and 1/2C; Table 2). In contrast, all but one of the rough strains (SV1/2A EGD) had relative negative charges greater than ($P < 0{\cdot}05$) the two smooth strains. Since Scott A is a clinical isolate unrelated to the rough strains, the best direct comparison of surface charge can be made between the smooth and rough strains of 1/2C, and the rough strain exhibited both greater hydrophobicity and net negative charge. The difference may be attributable to the lack of specific proteins in the cell-walls (Hof and Chatzipanagiotou 1987; Hof *et al.* 1991). The overall composition of the cell-wall would be altered to fill the physical and chemical deficiencies resulting from the absence of these proteins, although no experiments were conducted in the study presented here to confirm this.

Five of six rough strains attached to beef in lower numbers than the smooth strain Scott A during the initial stages of attachment (Table 3), with SV1/2A EGD being the exception. Virtually all of the strains exhibited a negative correlation between net negative charge and attachment for the time periods examined. Again, the best direct comparison of attachment ability can be made between the smooth and rough strains of 1/2C, where the smooth strain attached in greater numbers to the tissue ($P < 0{\cdot}05$) than the corresponding rough strain. These data contradict our previous report which indicated that net negative charge and attachment to beef tissue are positively correlated (Dickson and Koohmaraie 1989). However, the cell morphology differs between the smooth and rough strains. Smooth listerias exist as short bacilli, whereas rough strains consist of long filaments (Hof *et al.* 1991), and direct microscopic observation of the cultures used in this research indicated that the filaments typically reached lengths of five to seven smooth cell lengths. This morphological difference may influence the ability of the rough strains to attach to beef tissue during the initial stages of

Table 3 Numbers of *Listeria monocytogenes* attached to beef muscle tissue, expressed as $\log_{10}$ colony-forming units cm^{-2}

Bacterial strain	Culture type	Attachment time (min) 1	5	10	S.E.M.	Different from smooth
R1	R	4·06	4·09	4·18	0·11	L
R3	R	4·00	3·98	4·13	0·11	L
R4	R	3·65	3·82	4·12	0·11	L
R5	R	3·62	3·68	3·72	0·16	L
SV1/2A EGD	R	4·32	4·35	4·79	0·13	E
Scott A	S	4·09	4·68	4·82	0·14	NA
1/2C	R	3·38	3·46	3·71	0·11	L
1/2C	S	3·86	4·01	4·24	0·11	(L)*

* Numbers of attached 1/2C smooth cells less than Scott A, but greater than 1/2C rough. R, Rough; S, smooth; S.E.M., standard error of the means; L, numbers of attached cells less than Scott A; E, equivalent to Scott A; NA, not applicable. Differences determined at the ($P < 0·05$) level.

attachment, although it did not apparently influence the determination of cell surface charge or hydrophobicity by non-specific filtration effects. The sepharose gel used in HIC is a more compact matrix than the ion exchange resin used in ESIC. If the filamentous nature of the rough strains were to produce non-specific filtration effects in the columns, these effects would be most noticeable in HIC. The data (Table 2) indicate that there was generally no significant difference in the hydrophobicity values between smooth and rough strains, indicating that the filamentous nature of the rough strains resulted in no filtration effects.

Hof *et al.* (1991) noted that rough strains of *L. monocytogenes* lacked several proteins in their cell-walls, including an essential virulence factor. Rough strains had an impaired ability to invade the chorio-allantoic membrane of fertilized hen eggs. The virulence factor is a 60 kDa protein, which has been tentatively identified as a murein hydrolase (Wuenscher *et al.* 1991). The lack of this virulence factor may contribute to the reduced ability to attach to tissue. These factors indicate that the attachment of *L. monocytogenes* to beef tissue surfaces cannot be solely explained by bacterial cell surface charge.

In summary, the rough strains of *L. monocytogenes* are generally characterized as having greater net negative cell surface charges than the smooth strains, but a reduced ability to contaminate beef tissue. Since greater net negative cell surface charge has been positively correlated to attachment to beef tissue surfaces, the reduction in attachment exhibited by the rough strains was interpreted as an indication that attachment of *L. monocytogenes* to beef tissue cannot be solely explained by cell surface charge. We speculate that the lack of specific proteins in the cell-walls of the rough strains may account for the reduced ability to attach, and that the subsequent changes in the chemical composition of the cell-wall account for the increases in the surface charge.

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