Relationship Between Bile Tolerance and the Presence of a Ruthenium Red Staining Layer on Strains of *Lactobacillus acidophilus*[1]

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ABSTRACT

Seventeen strains of *Lactobacillus acidophilus* were evaluated to determine the relationship between bile tolerance and the presence of an outer polysaccharide layer exterior to the cell wall when viewed by transmission electron microscopy. Bile tolerance is necessary for survival of lactobacilli in the intestinal tract, and the polysaccharide layer may be responsible for adherence to human intestinal tissue. These two factors may be the basis for use of *L. acidophilus* as a dietary adjunct.

Ten strains exhibited a ruthenium red-stained outer polysaccharide layer. Three of the 10 strains had extremely dense layers, which may indicate stronger adherence properties. Seven strains did not contain a ruthenium red-stained outer layer; however, six strains that did not have the stained layer were resistant to 1.0% bile concentration. Fourteen strains were tolerant to 1% bile, one strain was tolerant to .6% bile, and two strains were sensitive to bile. No relationship between bile tolerance and the presence of the ruthenium red-stained outer polysaccharide layer was apparent.

(Key words: *Lactobacillus acidophilus*, adherence, bile tolerance)

Abbreviation key: EMS = electron microscopy sciences, RR = ruthenium red, TEM = transmission electron microscopy.

INTRODUCTION

Lactobacilli are normal inhabitants of the intestinal tract. Some strains of *Lactobacillus acidophilus* have been shown to inhibit many of the commonly known foodborne pathogens (4). Other reported benefits of *L. acidophilus* include the potential to prevent intestinal infections (5, 11), improve lactose digestion for the lactose-intolerant individual (12), aid in controlling serum cholesterol levels (8), and suppress colon cancer (17).

The metabolic activity of the intestinal microflora may be beneficially altered if these microorganisms are consumed regularly (7). *Lactobacillus acidophilus* may attach and colonize in the intestine and deter pathogens such as *Escherichia coli, Salmonella typhimurium,* and *Staphylococcus aureus* from adhering in the gut. These potentially beneficial properties of lactobacilli contribute to the increased interest in their use as dietary adjuncts (4, 6, 12).

However, to be a successful dietary adjunct, lactobacilli must be able to adapt to the environs of the intestine. They must be able to survive in low pH as well as high levels of bile acids, because the organisms must pass through acidic gastric juices in the stomach before passing into the intestine. They should contain an outer polysaccharide layer exterior to the cell wall and be able to adhere to human intestinal cells. Hood and Zottola (9) reported that an outer polysaccharide layer may be involved in the adherence of the organisms to intestinal tissue. These same researchers (10) found that *L. acidophilus* can survive at low pH values of 3 and 4, and these pH values did not affect the ability of the organism to attach to human intestinal cells.

During digestion, bile flows into the small intestine. Therefore, *L. acidophilus* must be bile-tolerant to survive in the intestinal tract.

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Gilliland et al. (6) reported that when a diet supplemented with a more bile-resistant strain of *L. acidophilus* was fed to newborn dairy calves, greater numbers of facultative lactobacilli were observed in the upper small intestines than when a strain with lower bile resistance was used. Gilliland and Speck (5) and Mayia-Makinen et al. (15) reported that the bile tolerance of *L. acidophilus* varied among strains.

Savage (19) postulated that the mechanism of attachment of *L. acidophilus* to the intestinal wall appeared to be mediated by acidic mucopolysaccharides occurring on the surface of the bacteria. Adherence of *L. acidophilus* to intestinal tissue appeared to involve this polysaccharide layer (9). Detection of the acidic polysaccharide layer is facilitated by ruthenium red (RR) staining (2, 18). Several studies have indicated that various strains of *L. acidophilus* have some host specificity (1, 16, 20). It appears that only strains of *L. acidophilus* isolated from human intestinal tracts will adhere to human intestinal tissue.

The objectives of this study were to determine the presence of an exopolysaccharide layer on 17 strains of *L. acidophilus* by means of transmission electron microscopy (TEM) and to determine the bile tolerance of these strains.

MATERIALS AND METHODS

Cultures

Seventeen different *L. acidophilus* cultures from our collection were transferred daily into 10 ml *Lactobacillus* MRS broth (Difco) for 3 consecutive d and incubated at 37°C for 18 to 24 h prior to preparation for TEM viewing. Identity of the *Lactobacillus* strains was confirmed by carbohydrate fermentation patterns using Rapid CH strips (API; Plainview, NY).

Transmission Electron Microscopy

The 17 cultures were prepared for TEM viewing using the procedure of Hood and Zottola (9). Cultures were centrifuged for 5 min at 4°C (Beckman Microfuge 12; Beckman Instruments, Palo Alto, CA) at 4000 × *g*, supernatants were decanted, and the cell pellets used for TEM fixation.

Fixation

Primary fixation was done with 2.5% glutaraldehyde [Electron Microscopy Sciences (EMS); Port Washington, PA] in .1 *M* sodium cacodylate buffer (pH 7.2) and 500 ppm RR (EMS) for 20 min at room temperature. Cells were washed three times for 10 min in .1 *M* sodium cacodylate buffer.

A secondary fixation was done with 2% osmium tetroxide (EMS) in .1 *M* sodium cacodylate buffer and 500 ppm RR for 20 min at room temperature. Cells were washed three times in distilled water for 10 min.

Dehydration

The cells were subjected to an ethanol dehydration series of 20, 40, 60, 80, and 95% ethanol and three times in 100% ethanol for 10 min in each step. Samples were then infiltrated with a 1:3 resin (Quetol, EMS) to 100% ethanol for 30 min, a 3:1 resin to ethanol for 30 min, and twice in 100% resin for 30 min each. The resulting cell pellets were cured in 100% resin at 70°C for 12 to 16 h.

The samples were sectioned on a microtome with a diamond knife and collected on Formvar-coated (EMS, Fort Washington, PA)

TABLE 1. Bile tolerance and the presence of a ruthenium red (RR) staining outer cell layer for 17 strains of *Lactobacillus acidophilus*.

Strain	Maximum bile tolerance	RR Staining layer
	(%)	
A	1.0	–
B	1.0	–
C	1.0	–
D	1.0	–
E	1.0	–
F	1.0	+
G	0	–
H	0	+
I	1.0	+
J	1.0	+
K	.6	+
L	1.0	+
M	1.0	–
N	1.0	+
P	1.0	+
S	1.0	+
LBZ-101	1.0	+

300-mesh copper grids. Poststaining was done with 4% uranyl acetate for 20 min and triple lead stain (lead nitrate, lead citrate, lead acetate, and sodium citrate) for 3 min. All samples were viewed on a Philips 300 transmission electron microscope (Philips Electronic Instruments, Mahwahy, NJ).

Bile Tolerance Determination

The procedure of Gilliland et al. (6) was used to determine the bile resistance of the 17 strains of *L. acidophilus* cultures used in the TEM study. The cultures were propagated in 10 ml MRS broth for 24 h at 37°C in a CO_2 atmosphere. The cultures were centrifuged at 3000 × *g* at 4°C for 10 min. The supernatant was decanted, and the cell pellet was resuspended in ca. 2 ml of fresh MRS broth. This suspension was used to adjust 10 ml of MRS broth to an optical density of .62 to .64 at 650 nm and was immediately pour plated in MRS agar containing various concentrations (0 to 1%) Oxgall (BBL, Cockeysville, MD). Dilutions were prepared in .1% peptone (Difco Laboratories, Detroit, MI) and .01% Antifoam B (Sigma Chemical Co., St. Louis, MO). All plates were incubated in a CO_2 atmosphere at 37°C for 24 to 72 h.

RESULTS AND DISCUSSION

Maximum bile tolerance and presence or absence of a RR staining layer for the 17 strains of *L. acidophilus* used in this study are shown in Table 1. Ten of the 17 strains contained an outer polysaccharide RR-stained layer. Fourteen of the strains were tolerant to 1.0% bile, one strain was tolerant to .6% bile, and two strains were sensitive to bile. Figure 1 is representative of strains with an exopolysaccharide layer, whereas Figure 2 shows strain C with no apparent RR-stained outer polysaccharide layer.

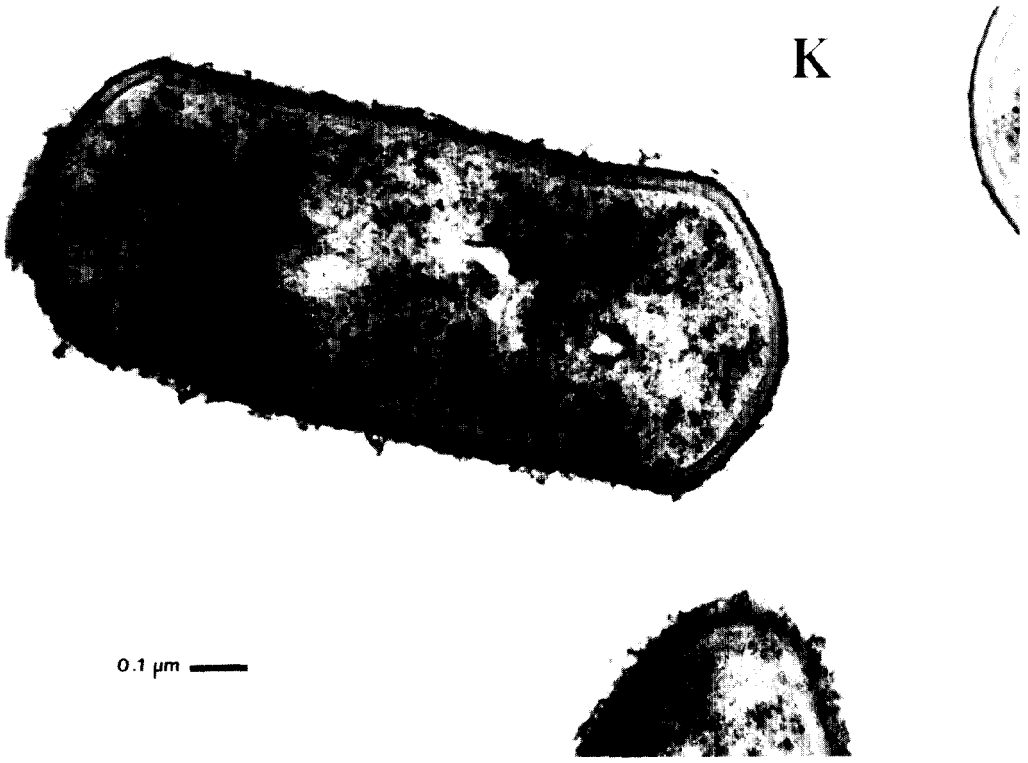

Figure 1. Transmission electron microscopy of *Lactobacillus acidophilus* strain K stained with ruthenium red. An exocellular polysaccharide may be observed.

Brooker and Fuller (2) reported that electron microscopy indicated that lactobacilli may adhere to the gastrointestinal tract by a carbohydrate layer on the bacterial cell. Kleeman and Klaenhammer (14) observed that polysaccharide material may be involved in the adherence of *L. acidophilus* to surfaces. Attachment to human intestinal cells appeared to be dependent on the amount of polysaccharide layer around the cell. The strain with a variable layer showed weak adherence. The strain with no polysaccharide layer did not adhere; however, the strain with a dense layer of polysaccharide material adhered to the intestinal surface. Consequently, in our study the strains that contained a polysaccharide layer may have variable degrees of adherence to human intestinal tissue dependent on the density of the layer. Further study is necessary to determine whether adherence to human intestinal tissue would occur, because Savage (19) and Fuller and Turvey (3) found that *L. acidophilus* strains have specificity for epithelial tissue of a particular animal species.

The bile tolerance of the *L. acidophilus* strains varied slightly. Fourteen of the strains tolerated 1.0% bile, one strain could tolerate up to .6% bile, and two strains were unable to tolerate any of the bile concentrations used in this study. Gilliland and Speck (5) and Klaenhammer and Kleeman (13) also reported a variance of bile tolerance among strains of *L. acidophilus*. A difference in bile sensitivity between rough and smooth colonial variants of *L. acidophilus* RLSK has been reported by Klaenhammer and Kleeman (13). The smooth colony variant was resistant to 1.0% bile, whereas the rough colony variant was sensitive to .6% bile. They concluded that the colonial and cellular morphologies are important consid-

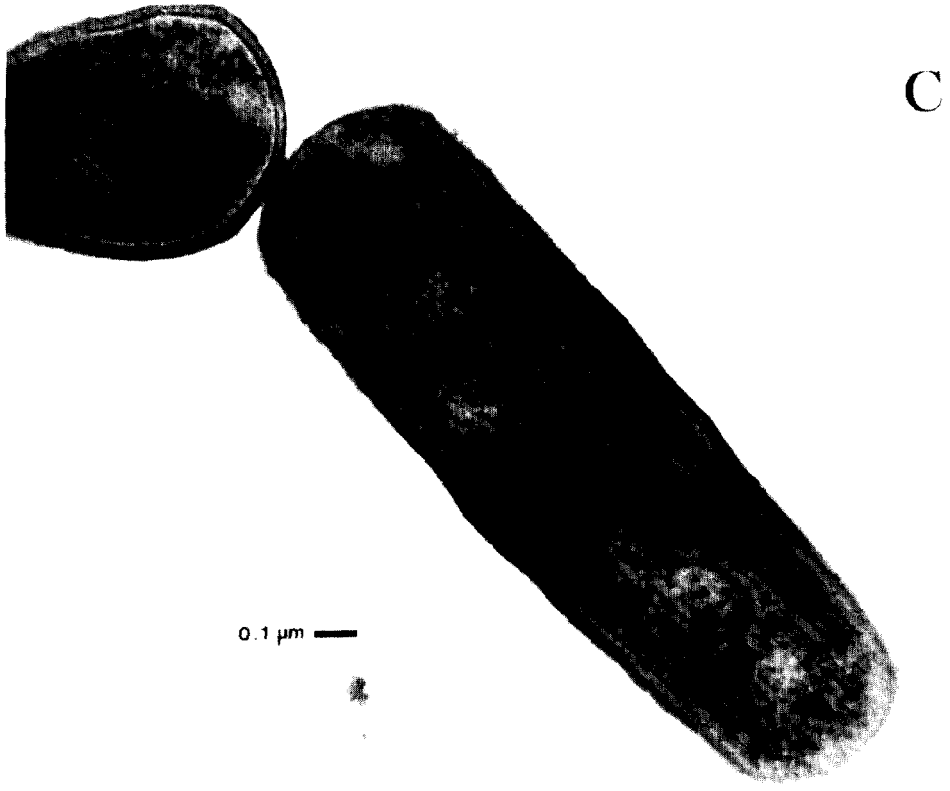

Figure 2. Transmission electron microscopy of *Lactobacillus acidophilus* strain C stained with ruthenium red. No outer cellular polysaccharide layer is apparent.

erations for the selection of *Lactobacillus* strains as dietary adjuncts.

The presence of the outer polysaccharide layer on *L. acidophilus* did not affect the bile resistance of each strain used in this study. Six of the strains that did not have the RR-stained layer were still resistant to 1.0% bile concentration. Strain H, which had an outer polysaccharide layer, was very sensitive to bile and did not grow in the presence of .2% bile. However, strain G, which was also sensitive to .2% bile, did not contain an outer polysaccharide layer.

CONCLUSIONS

Lactobacillus acidophilus must possess several properties to be considered as a possible dietary adjunct. Two of these properties investigated in this study were the presence of an outer RR-stained polysaccharide layer exterior to the cell wall and the ability of the organism to survive in the presence of bile acid. Ten of the strains studied contained an outer polysaccharide layer; however, two of these strains were sensitive to bile and may not be suitable choices as a dietary adjunct. Further study must be conducted to determine whether the strains containing an outer layer would adhere to human intestinal tissue.

Fourteen of the strains survived in 1.0% bile, which is important for their survival under the bile acid conditions in the intestine; however, six of these strains did not have an RR-stained layer. Adhesive strains with a good pH and bile acid tolerance are able to adapt to conditions in the intestine. Therefore, before the eight strains containing an RR-stained layer and tolerance to 1.0% bile concentration can be considered as dietary adjuncts, they must be tested further to determine survival at low pH values and ability to adhere to human intestinal tissue cells.

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