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Lactobacilli Isolated from the Stomach of Conventional Mice

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Twenty strains of lactobacilli isolated from the stomach of conventional mice were tested for their ability to ferment or hydrolyze substrates that may be present in the stomach habitat. The lactobacilli could be placed in four groups (A to D) depending on their ability to ferment *N*-acetylglucosamine, dextrin, cellobiose, gum arabic, and xylan. The majority of the isolates belonged to groups A and D. Group A strains did not resemble previously described *Lactobacillus* species, but group D strains were identified as *L. leichmannii*. A representative group A isolate colonized the surface of the nonsecretory epithelium of the stomach of gnotobiotic mice; a group D isolate did not.

Microbiological studies of the stomach of monogastric mammals have demonstrated that microbes are present in this region of the host. In some animals (e.g., humans), microbes isolated from the stomach may only be contaminants carried over from the mouth in food and saliva (3). In other animals (e.g., mice, rats, rhesus monkeys, and swine) the stomach provides a habitat for various microbial types (1, 4, 20).

Lactobacilli are considered to be indigenous to the stomach of rodents, rhesus monkeys, and swine because large populations of these bacteria can be consistently isolated from this organ. At least in the case of mice, rats, and swine, lactobacilli colonize the surface of the areas of squamous, keratinized epithelium lining parts of the stomach (4, 20). A similar phenomenon occurs in the crop of chickens, where lactobacilli adhere to the nonkeratinized squamous epithelium (6).

Histological sections reveal a dense layer of gram-positive rod-shaped bacteria in association with the keratinized squamous epithelium of the nonsecretory part of the mouse stomach. This bacterial layer ends abruptly at the cardiac antrum (16). Electron microscopic studies have demonstrated that the bacterial cells are frequently attached to the epithelium by one end (15). Cultural techniques have demonstrated that the bacterial layer associated with the stomach epithelium is composed of lactobacilli (4).

Lactobacilli in the gastrointestinal tract of mice may be involved in host resistance to infection by pathogens. Dramatic reductions in the population levels of lactobacilli in the stomach of mice occur under conditions of dietary and environmental stress (19). Animals subjected to stress are more susceptible to infection with intestinal pathogens than are nonstressed animals (12). *Salmonella typhimurium* population levels are higher in stressed compared with nonstressed animals (21).

Indigenous microbes in the gastrointestinal tract of mice influence the host's physiology. For example, the epithelial cell renewal rate is slower in the small intestine of germfree mice than in conventional mice (10). Alkaline phosphatase activity in duodenal tissue is higher in germfree mice than in conventionalized mice (14). Metabolic products of the lactobacillus population in the stomach could influence the physiology of the adjacent tissues of the host.

In studying the influence of lactobacilli on the host, it is necessary to characterize and identify the types of lactobacilli that can be isolated from the mouse stomach. We have grouped lactobacilli isolated from the stomach of mice according to their ability to utilize or degrade substrates that may be present in the stomach habitat. The strains were then identified to species level where possible.

The ability to adhere to a keratinized epithelium is an important ecological attribute of lactobacilli inhabiting the rodent stomach. Carbohydrate moieties have been suggested to be mediators of lactobacillus adherence in the stomach of mice and chickens (2, 15). Therefore we examined lactobacillus isolates for their ability to adhere to wire surfaces in vitro as has been done with oral microbes (11). The lactobacilli were also tested for agglutination in the presence of concanavalin A (ConA), which specifically binds to carbohydrate moieties with the α-anomeric configuration (5). Some bacteria that produce extracellular polysaccharide are agglutinated by high-molecular-weight

dextrans (7). We tested our lactobacillus isolates in this way also.

Finally, we tested representative lactobacillus isolates for their ability to colonize the nonsecretory epithelium of the stomachs of gnotobiotic mice.

MATERIALS AND METHODS

Mice. Conventional, random-bred male mice (University of Otago Breeding Station), 6 to 8 weeks old, were used. The mice were housed in plastic cages and given commercial food pellets and acidified water (17) ad libitum.

Histological examination. The whole stomach, with contents intact, was frozen in a 2% solution of methylcellulose in saline on the shelf on a microtome-cryostat (Minotome, Damon/IEC Division). Sections of frozen tissue, cut at 4 μm, were fixed for 60 s in methanol and stained by a tissue Gram stain.

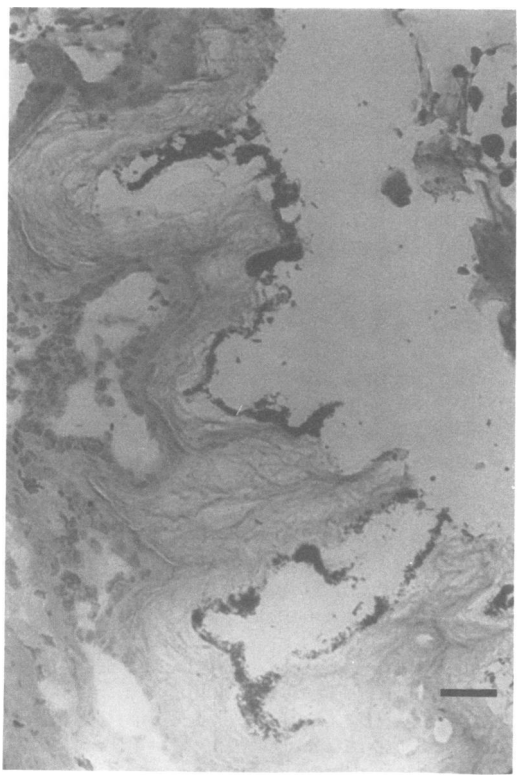


FIG. 1. *Layer of gram-positive bacteria associated with the nonsecretory mucosa of the stomach of a conventional mouse. Gram stain, ×200.*

Isolation of lactobacilli. Twelve mice were killed with chloroform. Approximately 0.5 g of stomach tissue and contents was removed from each mouse and vigorously mixed in 4.5 ml of brain heart infusion broth (Difco) containing glass beads, using a Vortex mixer. The homogenates were diluted in brain heart infusion broth in 10-fold steps. Calibrated loopfuls (0.01 ml) of each dilution were spread on the surface of 10A agar plates (18). The plates were incubated in a candle jar at 37°C for 48 h.

Identification of isolates to genus level. Twenty strains of gram-positive rods were isolated from the 10^{-7} dilution 10A agar plates (one or two strains per mouse). The isolates were identified as lactobacilli on the basis of Gram stain morphology, gas chromatographic analysis of fermentation products of 48-h peptone-yeast extract-glucose (PY-glucose) broth cultures containing 0.1% Tween 80 (PYE series 104 chromatograph; Chromosorb 104 column packing), and the absence of spores (CM agar slant [8], 25°C, 3 weeks) (8).

Characterization of isolates. All tests were carried out by using prereduced media prepared as described in the Virginia Polytechnic Institute anaerobe manual (8) except that an argon (97%)-carbon dioxide (3%) gas mixture (19) was used. Fermentation and other media were those described in the manual, with the addition of 0.1% (vol/vol) Tween 80 (BBL). *N*-acetylglucosamine was used in fermentation tests at a concentration of 0.5%; xylan and gum arabic were each used at a 1% concentration.

The following tests were used to group the isolates: fermentation of cellobiose (BBL), gum arabic (Sigma), xylan (Sigma), *N*-acetylglucosamine (Sigma) and galactose (BDH), glucose (Difco), maltose (BBL), and dextrin (Sigma), sucrose (Difco) and fructose (May and Baker), xylose (BBL), and lactose (Difco). The isolates were tested for their ability to hydrolyze cellulose (Sinar), starch (May and Baker), casein (Koch-Light), keratin (Calbiochem), and tributyrin (Sigma).

Carbohydrate fermentation media were inoculated with a few drops of 24-h micro-inoculum broth (Difco) culture of each isolate. The cultures were incubated for 48 h at 37°C. The pH of the cultures was determined by using a pH meter. Cultures with

TABLE 1. *Groups of lactobacilli*

Group	No. of isolates	Acid production from:[a]					Adherence to wire
		Gum arabic	Dextrin	*N*-acetylglucosamine	Cellobiose	Xylan	
A	10	–	–	–	–	–	–
B	1	–	–	–	–	A	+
C	1	–	–	–	A	–	–
D	8	A[a]	A	A	A	A	+

[a] –, No acid produced; A, acid produced.

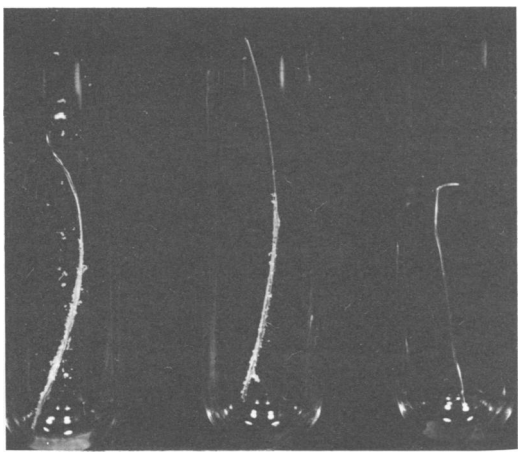


FIG. 2. *Left and middle tubes: Lactobacillus growth adhering to wires. Right tube: No adherence.*

a pH of 6.1 or less were considered to have fermented the substrate. The pH of uninoculated media was 7.0 to 7.2. Cultures that did not ferment the substrate usually had a pH of about 6.8.

Starch hydrolysis was tested as previously described (8) and by inoculating brain heart infusion agar tubes containing 1% (wt/vol) starch with the lactobacillus isolates. *Bacteroides fragilis* and a starch-nonhydrolyzing strain of *Bacteroides vulgatus* were used as positive and negative controls, respectively. After 48 h of incubation at 37°C, iodine solution (8) was allowed to run down the agar tubes, which were examined for zones of starch hydrolysis surrounding the lactobacillus colonies.

Lipid hydrolysis was determined by inoculating tubes of PY-tributyrin (0.1%, vol/vol) with 24-h micro-inoculum broth cultures of lactobacilli. The tubes were incubated for 7 days at 37°C. A *Staphylococcus aureus* strain was used as a positive control in which a clear zone of tributyrin hydrolysis surrounded the bacterial growth.

Casein hydrolysis was determined as previously described (8). Cellulose hydrolysis was tested for by inoculating PY-cellulose (0.1%, wt/vol [9]) broths with lactobacilli and incubating the culture for 3 weeks at 37°C. Keratin hydrolysis was tested by using PY-keratin azure (0.1%, wt/vol) broths which were incubated for 3 weeks at 37°C. A strain of *Trichophyton erinacei* (J. M. B. Smith, Department of Microbiology, University of Otago) was used as a positive control for keratin hydrolysis. The blue-stained keratin fibers were macroscopically observed to be degraded by this keratinolytic fungus.

The ability of the lactobacilli to adhere to a smooth surface in vitro was tested by a modification of the method of McCabe et al. (11). The isolates were compared for their ability to form a plaque-like growth on 5-cm lengths of palladium (20%)-platinum (80%) wire suspended in PY-sucrose (1%, wt/vol) broth. The PY-sucrose medium was inoculated from 24-h cultures of lactobacilli in micro-inoculum broth. The PY-sucrose cultures were incubated for

TABLE 2. *Identification of lactobacillus isolates*

Group	Test[a]																
	Amygdalin	Cellobiose	Fructose	Glucose	Lactose	Maltose	Mannitol	Rhamnose	Ribose	Salicin	Sorbitol	Starch hydrolysis	Sucrose	Trehalose	Xylose	Lipase	Lactic acid isomer
A	–	–	A	A	A	A	–	–	A	–	–	–	A	–	A	–	D
B	A	–	A	A	A	A	–	–	A	A	–	–	A	A	A	–	D
C	–	A	A	A	A	A	–	–	A	–	–	–	A	–	A	–	D
D	A	A	A	A	A	A	–	–	A	A	–	–	A	A	A	–	D

[a] –, No acid produced; A, acid produced.

24 h at 37°C. The wires were transferred into freshly inoculated tubes of media at the end of this period. This process was continued over 9 consecutive days. The wires were then placed in 10% formalin, and the presence or absence of adhering bacterial growth was recorded.

Agglutination of the lactobacilli by ConA (Sigma) was tested by the method described by Fuller (5). Lactobacilli cultured in PY-glucose and PY-sucrose broth media for 48 h at 37°C were used. Similar cultures were used to prepare phosphate buffer (pH 6.7) suspensions of bacteria to test for agglutination by 100 μg of a high-molecular-weight (2×10^6) dextran (type 2000, Sigma). The method described by Gibbons and Fitzgerald (7) was used.

Additional tests used in characterization. The lactobacilli were further characterized by the following additional tests: fermentation of amygdalin (Sigma), mannitol (Difco), rhamnose (Sigma), ribose (L. Light & Co.), salicin (Difco), sorbitol (Difco), trehalose (Sigma); and on the isomer of lactic acid produced (8).

Gnotobiotic mouse experiments. Representative strains of lactobacilli (100-5 [group A], 100-14 [group C], 100-20 [group D]) were cultured in 10 ml of Rogosa broth (Difco) at 37°C for 24 h. Each bacterial culture was placed in a flexible plastic isolator (22) containing five 10-week-old germfree mice (CD-1, Charles River Breeding Laboratories). Two to 3 ml of culture was poured onto sterile food in the bottom of the cages, and the remainder of the culture was added to the drinking water. The next day, the drinking water was replaced with fresh sterile water, and the bedding and food in the cage were replaced with fresh material. Ten days later, the mice were removed from the isolator and killed with chloroform. The stomach was removed from all five mice, frozen, sectioned, and Gram stained as described above.

RESULTS

Histological examination. A layer of gram-positive rod-shaped bacteria was associated with the nonsecretory, keratinized epithelium of the stomachs of all conventional mice examined. The gram-positive rods were closely associated with the epithelial surface of the stomach tissue (Fig. 1).

Identification of isolates to genus level. All 20 isolates of gram-positive rods were nonspore forming and produced lactic acid as their sole major fermentation product. Therefore, all isolates were members of the genus *Lactobacillus* (8).

Characterization of isolates. All the isolates were able to ferment glucose, lactose, maltose, fructose, sucrose, xylose, and galactose. None of the strains hydrolyzed starch, digested casein, hydrolyzed tributyrin, or were keratinolytic or cellulolytic. The isolates differed in their ability to ferment gum arabic, cellobiose, dextrin, *N*-acetylglucosamine, and xylan (Table 1). The lactobacillus isolates also differed in their ability to adhere to wire (Table 1; Fig. 2). The fermentation and adherence properties enabled the isolates to be divided into four groups (A to D; Table 1).

Agglutination by ConA and dextran. A representative strain from each lactobacillus group was tested. The group A strain (100-5) autoagglutinated in buffer. Strains 100-2, 100-14, and 100-20 were agglutinated by ConA. None of the strains were agglutinated by high-molecular-weight dextran.

Identification of isolates to species level. The results of tests used to identify the lactobacillus isolates are given in Table 2. By reference to the Virginia Polytechnic Institute anaerobe manual (8) and Rogosa (13), strains belonging to groups B and D were identified as *Lactobacillus leichmannii*. However, the group B isolate did not ferment cellobiose. Strains belonging to groups A and C did not resemble previously described species.

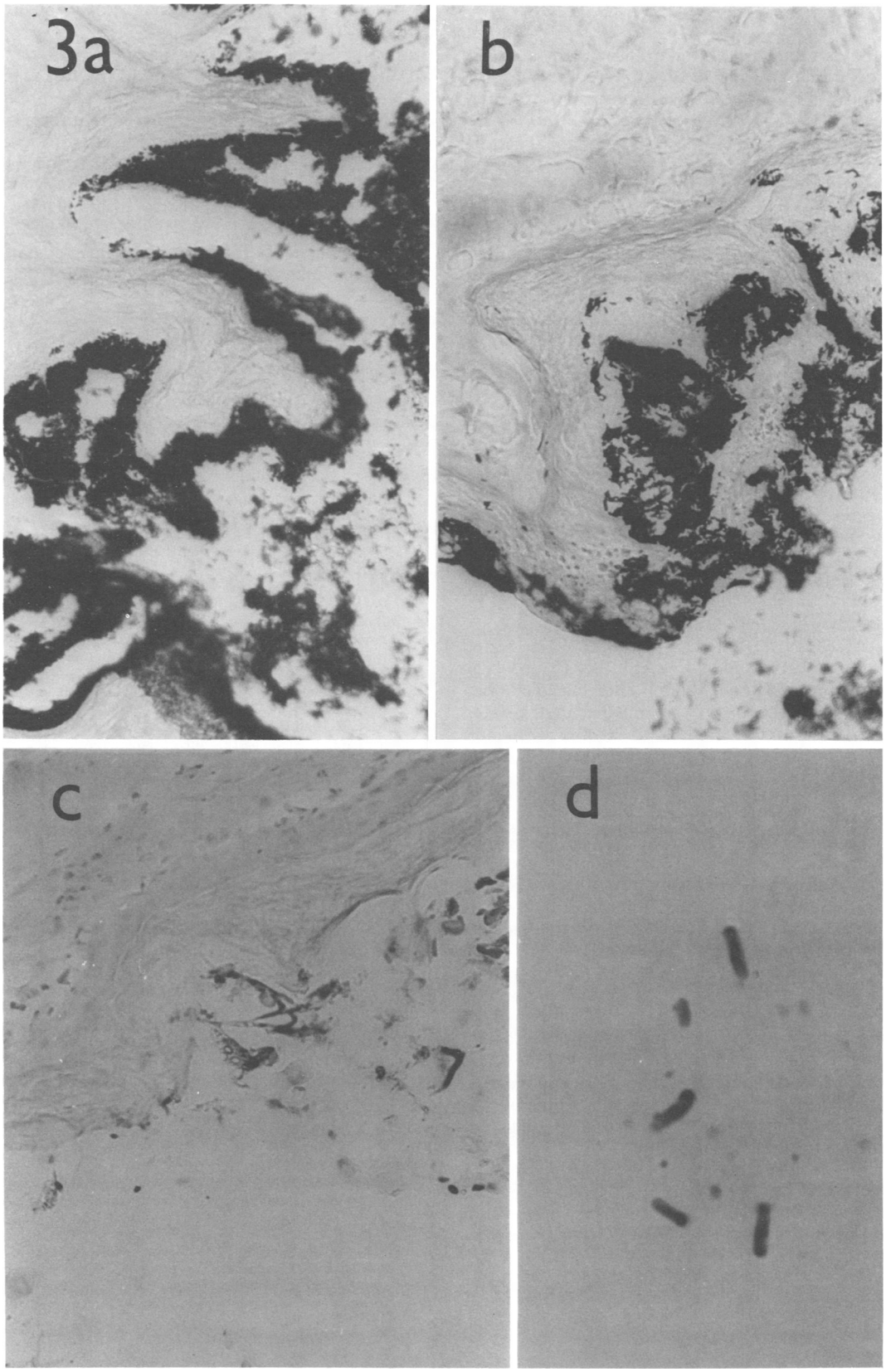
Gnotobiotic mouse experiments. Strains of lactobacilli representing *L. leichmannii* (100-20) and groups A (100-5) and C (100-14) were observed in histological sections of the stomachs of monoassociated mice. However, only the group A (100-5) and group C (100-14) strains colonized the surface of the nonsecretory epithelium (Fig. 3a, b). The *L. leichmannii* strain (100-20) was present only in the lumen of the stomach (Fig. 3c, d).

DISCUSSION

A microbial layer associated with the nonsecretory epithelium was observed in histological sections prepared from the stomachs of conventional mice from the University of Otago breeding unit. The microbial layer was composed of gram-positive rod-shaped bacteria closely associated with the stomach epithelium. This is a similar observation to that made in animals from other mouse colonies (4, 19). Cultural results are consistent with the rod-shaped bacteria being lactobacilli.

The lactobacillus isolates from the mouse

FIG. 3. *(a) Layer of gram-positive bacilli on the nonsecretory mucosa of the stomach of a mouse monoassociated with group A lactobacillus strain (100-5). Gram stain, × 200. (b) As in (a), except mouse monoassociated with group C lactobacillus strain (100-14). Gram stain, × 200. (c) Nonsecretory mucosa of a mouse monoassociated with group D lactobacillus strain (100-20). Bacterial layer not present. Gram stain, ×200. (d) Gram-positive bacilli in lumen of stomach of a mouse monoassociated with group D lactobacillus strain (100-20). Gram stain, × 2,000.*


3a
b
c
d

stomach could be placed in four groups (A to D) depending on their ability to ferment *N*-acetylglucosamine, dextrin, cellobiose, gum arabic, and xylan. The majority of the isolates belonged to groups A and D. The group A strains did not resemble previously described species, but group D strains could be identified as *L. leichmannii*. Group A isolates did not ferment *N*-acetylglucosamine, dextrin, cellobiose, gum arabic, or xylan. Group D isolates were able to ferment these substrates. A group A isolate (100-5) was observed to colonize the nonsecretory epithelium of the mouse stomach; a group D isolate (100-20) did not. It is interesting to speculate that group A lactobacilli living in close association with the stomach epithelium may obtain most of their nutrients from the host's secretions rather than from the host's food. The group D lactobacilli, being restricted to the stomach lumen, may obtain their nutrients from the host's food. Hence, the group D bacteria are able to ferment a wider spectrum of substrates than the group A lactobacilli. However, all of the lactobacillus strains could ferment glucose and maltose. The bacteria may rely on the degradation of starch in the diet by host secretions to provide these substrates.

All lactobacillus strains that could be tested were agglutinated by ConA. Fuller (5) observed that lactobacilli capable of adhering to chicken crop epithelial cells in vitro were agglutinated by ConA, but nonadhering strains were not. In our studies, ConA agglutination did not indicate which bacterial strain would colonize the nonsecretory epithelium of the mouse stomach.

Group B and D strains (*L. leichmannii*) formed a plaquelike growth on wire surfaces. The ability to adhere to wire and form plaque similar to *Streptococcus mutans* cultures (11) suggests that these lactobacillus strains produce an extracellular polysaccharide in vitro. We do not know whether such a material is produced in vivo, but if it is, it does not enable the bacteria to colonize the nonsecretory epithelium of the mouse stomach. None of the lactobacilli were agglutinated by high-molecular-weight dextran, suggesting that the mechanism of attachment of lactobacilli to mouse tissue is different from that of oral microbes adhering to tooth surfaces (7).

We believe that it may now be possible to recognize lactobacilli isolated from mice that will associate with the nonsecretory epithelium of the mouse stomach. The adhering types may be recognized on the basis of the results of five fermentation tests: *N*-acetylglucosamine, dextrin, gum arabic, cellobiose, and xylan. However, further in vivo tests are needed to finally establish the relationship between in vitro metabolism of selected substrates and adherence to stomach epithelium. In addition, information obtained in this study can be used to design media suitable for the cultivation of the various types of lactobacilli present in the mouse stomach. The information can also be used in choosing lactobacillus strains for use in gnotobiotic mouse experiments to observe the influence of indigenous microbes on host resistance to *S. typhimurium* infection.

ACKNOWLEDGMENTS

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